Protective vaccination against blood stages of

*Plasmodium chabaudi* malaria: Role of spleen and liver
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TO MY FAMILY
CONTENTS

1 INTRODUCTION ........................................................................................................... 5

1.1 Geographical distribution of malaria ................................................................. 5
1.2 The parasite and the disease ............................................................................. 6
1.3 Life cycle of malarial parasite ......................................................................... 8
1.4 Immune responses in malaria ........................................................................ 11
1.5 Vaccine development ..................................................................................... 13
1.6 Role of the spleen in immunity to blood stage malaria parasites ............... 15
1.7 Filtration capacity of the spleen ................................................................... 18
1.8 Aim of the work .............................................................................................. 19

2 MATERIALS AND METHODS ............................................................................. 20

2.1 Chemical reagents, kits, solutions and buffers, and antibodies ....................... 20
2.1.1 Chemical reagents ..................................................................................... 20
2.1.2 Kits ............................................................................................................ 21
2.1.3 Solutions and buffers ............................................................................. 22
2.1.4 Antibodies ............................................................................................... 24

2.2 Animals ............................................................................................................. 25

2.3 Plasmodium chabaudi infection in mice ........................................................... 25

2.4 Vaccination of mice ........................................................................................ 25
2.4.1 Isolation of parasitized erythrocytes ....................................................... 25
2.4.2 Isolation of ghosts from infected erythrocytes ......................................... 26
2.4.3 Vaccination of mice with ghosts from P. chabaudi- infected erythrocytes .... 26

2.5 Flow cytometry ................................................................................................ 26
2.5.1 Isolation of spleen cells ........................................................................... 26
2.5.2 Isolation of liver cells .............................................................. 26
2.5.3 Quantification of splenic cell populations ...................................... 27
2.5.4 Quantification of hepatic mononuclear cell populations ................. 27

2.6 Filtration capacity of spleen and liver ........................................ 28
2.6.1 Determination of the filtration capacity ........................................ 28
2.6.2 Distribution of the fluorescent beads in spleen and liver using fluorescence microscopy ............................................................ 28
2.6.3 Localization of Gr-1⁺ granulocytes in the spleen using fluorescence microscopy ............................................................ 29

2.7 Adoptive transfer experiments .................................................... 29
2.7.1 Isolation of liver cells .............................................................. 29
2.7.2 Isolation of spleen cells ............................................................ 29

2.8 cDNA microarrays ................................................................. 30
2.8.1 RNA isolation ........................................................................ 30
2.8.2 cDNA arrays ........................................................................... 30
2.8.3 Labeling and hybridization ......................................................... 30
2.8.4 Readout and data analysis .......................................................... 31

2.9 Statistical analysis ....................................................................... 32

3 RESULTS .................................................................................. 33

3.1 Course of blood stage malaria after protective vaccination ........... 33

3.2 Flow cytometry ................................................................. 34
3.2.1 FACS analysis of spleens of mice vaccinated with iG .................. 34
3.2.2 FACS analysis of livers of mice vaccinated with iG .................... 37

3.3 Gating of spleen and liver .......................................................... 38
3.3.1 Differential gating of the spleen .................................................. 38
### Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.3.2</td>
<td>Differential gating of the liver</td>
<td>41</td>
</tr>
<tr>
<td>3.4</td>
<td>Adoptive transfer experiments</td>
<td>43</td>
</tr>
<tr>
<td>3.4.1</td>
<td>Transfer of liver cells from vaccinated mice to infected non-immune mice</td>
<td>43</td>
</tr>
<tr>
<td>3.4.2</td>
<td>Transfer of liver cells from immune mice to infected non-immune mice</td>
<td>46</td>
</tr>
<tr>
<td>3.4.3</td>
<td>Transfer of spleen cells from immune mice to infected non-immune mice</td>
<td>47</td>
</tr>
<tr>
<td>3.5</td>
<td>Effect of vaccination on gene expression in spleen</td>
<td>49</td>
</tr>
<tr>
<td>3.6</td>
<td>Effect of vaccination on gene expression in liver</td>
<td>51</td>
</tr>
<tr>
<td>4</td>
<td>DISCUSSION</td>
<td>53</td>
</tr>
<tr>
<td>4.1</td>
<td>The spleen as an effector against <em>P. chabaudi</em> malaria infections</td>
<td>53</td>
</tr>
<tr>
<td>4.2</td>
<td>Liver as an effector against <em>P. chabaudi</em> malaria infections</td>
<td>54</td>
</tr>
<tr>
<td>4.3</td>
<td>Effects of protective vaccination on the spleen and liver</td>
<td>55</td>
</tr>
<tr>
<td>4.4</td>
<td>Effects of adoptive transfer of liver and spleen cells on recipient mice</td>
<td>56</td>
</tr>
<tr>
<td>4.5</td>
<td>Impact on human vaccine development</td>
<td>57</td>
</tr>
<tr>
<td>5</td>
<td>SUMMARY</td>
<td>58</td>
</tr>
<tr>
<td>6</td>
<td>ZUSAMMENFASSUNG</td>
<td>59</td>
</tr>
<tr>
<td>7</td>
<td>REFERENCES</td>
<td>60</td>
</tr>
<tr>
<td>8</td>
<td>ABBREVIATIONS</td>
<td>78</td>
</tr>
<tr>
<td>Chapter</td>
<td>Title</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>-------------------</td>
<td>------</td>
</tr>
<tr>
<td>9</td>
<td>ACKNOLEDGEMENTS</td>
<td>82</td>
</tr>
<tr>
<td>10</td>
<td>LEBENSLAUF</td>
<td>84</td>
</tr>
</tbody>
</table>
1 INTRODUCTION

1.1 Geographical distribution of malaria

Malaria is one of the top three killers among communicable diseases. There are 300 to 500 million clinical cases every year and between one and three million deaths, mostly of children, are attributable to this disease (Sachs and Malaney, 2002). Malaria occurs in over 90 countries worldwide. According to figures provided by the World Health Organization (WHO, 1996), 36% of the global population live in areas where there is risk of malaria transmission, 7% reside in areas where malaria has never been under meaningful control, and 29% live in areas where malaria was once transmitted at low levels or not at all, but where significant transmission has been re-established (WHO, 1996). Malaria transmission occurs primarily in tropical and subtropical regions in sub-Saharan Africa, Central and South America, the Caribbean island of Hispaniola, the Middle East, the Indian subcontinent, South-East Asia, and Oceania. In Africa, childhood deaths from malaria are continually increasing. In some regions, as for example in parts of eastern and southern Africa, malaria has spread into previously non-endemic or low-transmission areas (WHO, 1999a). In Kenya, an increase in epidemics was recorded in the 1980s. In malaria endemic areas, however, there is considerable variation in the intensity of transmission and risk of malaria infection (Knudsen and Slooff, 1992). The economic effects of malaria infection can be tremendous. These include direct costs for treatment and prevention, as well as indirect costs such as lost productivity from morbidity and mortality, time spent in seeking treatment, and diversion of household resources. The annual economic burden of malaria infection in 1995 was estimated at US$ 0.8 billion for Africa alone (Foster and Phillips, 1998). This heavy toll can hinder economic and community development activities throughout the region.

The scope of the malaria problem is broad. With the continuing rapid spread of parasite and vector resistance against long-established but also new drugs and insecticides, no striking improvement of the situation is foreseen in the near future. Moreover, the resurgence of malaria that is linked to civil conflict and human migrations, climatic and environmental changes, and inadequate or deteriorating health systems (Trigg and Kondrachine, 1998; WHO, 1999a).
Introduction

1.2 The parasite and the disease

Malaria is caused by apicomplexan protozoa of the genus plasmodium. It is the most important parasitic disease in humans, with 300 to 500 million cases resulting in the death of 1.5 to 2.7 million people annually (Sherman, 1999; WHO, 2000; Smith et al., 2002). Malaria is transmitted to vertebrates by female Anopheles mosquitoes. In the vertebrate host, the asexual blood forms of the parasite are the life cycle stages that are exclusively responsible for morbidity and mortality of plasmodial infections. Four species of malaria parasites cause disease in humans, *Plasmodium falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. Whereas three species give rise to considerable malaria morbidity, only *P. falciparum* results in high mortality (WHO, 1997; Sherman, 1999).

Malaria parasites cause several pathophysiological effects. The disease is characterized by fever associated with symptoms, including headaches, chills, myalgia, malaise and joint pain that can be resolved into life threatening complications, such as cerebral malaria or severe malaria anaemia. Chronic anaemia may be a result of continued haemolysis due to chronic malaria infection (Kean et al., 1978; Akiwa et al., 1980; Deitsch and Wellems, 1996; White, 1999a). Malarial anaemia is induced by the direct destruction of parasitised red blood cells, but also by immune mechanisms leading to a destruction of non-parasitised red blood cells and abnormalities in erythrocyte production in the bone marrow (dyserythropoiesis). Malaria induced iron deficiency further contributes to anaemia (Mockenhaupt et al., 2003). Kurtzhals et al. (1997) showed that *P. falciparum* infection causes a rapidly reversible suppression of the bone marrow response to erythropoietin, while the severity of anaemia depends on the peripheral destruction of parasitized erythrocytes. The secondary hypersplenism may also contribute to increased red blood cells (RBCs) destruction (Sen et al., 1994). Cytokine levels, especially low plasma levels of the anti-inflammatory cytokine interleukin-10 (IL-10), were also shown to be associated with severe anaemia (Kurtzhals et al., 1998; Mockenhaupt et al., 2003).

The main cause of cerebral malaria is a mechanical obstruction of capillaries in the central nervous system due to adherence of sticky parasitised red blood cells to capillary endothelium (sequestration). The resulting ischaemia, hypoxia and anoxia may provoke a variety of
encephalopathic signs and symptoms such as stiff neck, headache, increased muscle tone, ataxia, aphasia, convulsions, local paralysis, and delirium (Modiano et al., 1993). Cerebral malaria is associated with the production of excessive levels of TNF-α, IL-1 and IL-6. They are released by monocytes and macrophages in response to the glycosylphosphatidyl-inositol (GPI) anchors of parasite proteins that are released during rupture of parasitized erythrocytes (PEs) (Berendt et al., 1989; Molyneux et al., 1989; Oquendo et al., 1989). TNF-α has been shown to upregulate the levels of intercellular adhesion molecule-1 (ICAM-1) and other receptors in brain endothelium, which may increase sequestration of PEs to cerebral microvasculature, resulting in cerebral malaria (Molyneux et al., 1989; Day et al., 1999; Dobbie et al., 1999; Ricke et al., 2000; Smith et al., 2002).

A typical symptom of \textit{P. falciparum} malaria is splenomegaly. Clearance of infected and altered uninfected red blood cells from the circulation may provoke enlargement of the spleen (White, 1999a; Sherman, 1999; Shah et al., 2003). A chronic complication of malaria is the hyperreactive malarial splenomegaly (HMS), with its clinical presentation of persistent and progressive splenic enlargement (Färnert et al., 1999).

Hypoglycaemia is a common complication during severe malaria that may lead to irreversible brain damage or a lethal outcome if not immediately treated (White et al., 1987; Taylor et al., 1988; Molyneux et al., 1989). Further severe and fatal complications include dehydration as a result of decreased fluid intake and loss of fluids due to high fever, sweating, vomiting and diarrhoea, metabolic acidosis, impaired liver function, jaundice, spontaneous bleeding and others (McDonald and Phillips, 1978; WHO, 1990; 1999a; 1999b; Oaks et al., 1991).

The distinction between infection and disease is particularly important in malaria, since infection with the parasite does not necessarily result in disease. In areas where malaria transmission from mosquitoes to human is intense, such as in many parts of sub-Saharan Africa, almost all of the children will have parasites in their blood constantly, without appreciable disease effectes (Smith et al., 1993). These children have developed an anti-disease immunity (Playfair et al., 1991), while their anti-parasite immunity has not reached
levels high enough to clear the infection (Greenwood et al., 1987; Miller et al., 2002). This suggests that asymptomatic, especially multiclonal, *P. falciparum* infections protect against clinical disease and provide with a status of primunition (Perignon and Druilhe, 1994; al-Yaman et al., 1997; Smith et al., 1999). The premunition is characterized by a decrease in the frequency and severity of disease episodes over several years, despite almost continuous infection, suggesting that immunity may develop through the acquisition of a repertoire of specific protective antibodies directed against polymorphic target antigens. In such infections the phenotype of the parasites sometimes may remain stable over extended periods of times (Greenwood et al., 1987; Sen et al., 1994; Contamin et al., 1996; Snow et al., 1999).

The resistance of malaria parasites to drugs and the resistance of mosquitoes to insecticides have resulted in a resurgence of malaria in many parts of the world and a pressing need for vaccines and new drugs (Kareier, 1980; Sherman, 1999).

### 1.3 Life cycle of malaria parasite

Malaria is caused by protozoan parasites from the genus *Plasmodium*. The analysis of small subunit ribosomal RNA gene sequences, have shown that *P. falciparum* is more closely related to *Plasmodium* species that infect chimpanzees, also rodents (*P. chabaudi*) and birds than to other *Plasmodium* species that infect humans and non-human primates (Bynum, 1999). The genome of *P. falciparum* was recently sequenced and consists of 23 million base pairs of DNA split over 14 chromosomes (Bynum, 1999). The life cycle of *Plasmodium* is complex. It includes a sexual phase developing in an invertebrate host (definitive host) and an asexual phase occurring in a vertebrate host (intermediate host). All mammalian *Plasmodium* species have similar life cycles. In human malaria the female mosquito of the genus *Anopheles* is the definitive host where the sexual reproduction takes place. Only the female mosquitoes have haematophagous habits, necessary as a protein source for the development of a batch of eggs (Bynum, 1999).

Infection in humans begins with the bite of an infected female *Anopheles* mosquito (Sherman, 1999). Sporozoites released from the salivary glands of the mosquito enter the bloodstream. Within minutes, inoculated sporozoites reach the liver and invade the
hepatocytes. In the hepatocyte the parasite undergoes differentiation and asexual reproduction (schizogony) to produce a large pre-erythrocytic schizont that contains a few thousand merozoites (Sherman, 1999). This stage is completely asymptomatic and in humans can last approximately 5-15 days, depending on the parasite species. In the liver stage of *P. vivax* and *P. ovale*, some sporozoites may transform into hypnozoites, and remain latent for months or even years, being responsible for subsequent relapses. With the rupture of the infected hepatocytes, the merozoites are released into the bloodstream and are able to invade erythrocytes (Sherman, 1999).

Each species of parasites shows specific capacity to infect immature or mature erythrocytes. The merozoite, after erythrocyte invasion, differentiates into a round trophozoite that grows and develops the erythrocytic schizont, which contains a few merozoites. During this process the parasite induces an increase in the permeability of the erythrocyte membrane which enables the uptake of nutrients and discharge of waste products; the parasite also ingests and digests 70% of the haemoglobin in the erythrocyte (Metseelaar and Van Thiel, 1959; Sherman, 1999). In humans, the time of the intra-erythrocytic development can be either 48 or 72 hours and the number of produced merozoites varies from 8 to 32, depending on the *Plasmodium* species. The erythrocyte rupture releases merozoites that are able to invade more erythrocytes, starting a new cycle. The clinical manifestations of the disease are associated with the rupture of the infected red blood cells. A small percentage of merozoites, upon invading a new red blood cell, differentiate into male or female gametocytes (Sherman, 1999; Ricke *et al.*, 2000).
During the mosquito meal the gametocytes are taken up and after some minutes they transform into gametes in the acidic, low-temperature environment of the midgut. The male microgametes fertilize the female macrogametes to form a zygote that develops into an ookinete. This is a mobile form able to cross the ciliated wall of the stomach and situate itself in the outer surface where it develops into an oocyst that grows and matures. The oocyst undergoes repeated division (sporogony) and a large number of sporozoites are produced. Sporogony can take from 7 days to 8 weeks depending on the parasite species, nature of the vector and environmental factors (Sherman, 1999). After oocyst rupture the infective sporozoites, are released to the hemolymph of the mosquito and migrate to the salivary glands where they wait to be injected during a blood meal into a new host, continuing the transmission cycle.
1.4 Immune responses in malaria

Immune systems have developed to protect multicellular organisms from foreign non-self substances. During evolution, two general immune systems have developed to detect foreign substances namely innate (natural) immunity and adoptive (acquired) immunity. Malaria infection gives rise to host responses which are regulated by both the innate and adaptive immune system (Janeway, 1989; Facer and Tanner, 1997).

Natural immunity to malaria is known to be predominantly directed against the blood stages of Plasmodium. Remarkably, however, this immunity is unable to prevent parasitemia during malaria season, but it can completely suppress disease symptoms (Playfair et al., 1990; Fearon and Locksley, 1996). The adaptive immune system is further divided into two systems: humoral and cell-mediated immunity, together mobilizing different specific molecular and cellular components to eliminate different types of pathogens. The key-players are the two distinct types of lymphocytes: B cells are lymphocytes that mature in the bone marrow (BM), which exert their function in humoral immunity by producing antibodies, and T cells that are responsible for cell-mediated immunity. Both B cells and T cells recognize specific antigens through membrane-bound receptors (Janeway, 1992; Fearon and Locksley, 1996; Shi et al., 2001). Experiments performed in B cell deficient mice have demonstrated that infection with P. yoelii parasites was lethal, while it was nonlethal in normal mice (Molineaux, 1988). This indicates that the humoral immune system appears to play a major role in protecting mice against this parasite. However, mice depleted of B cells were able to control their infections with P. chabaudi, although they could not completely clear parasitemia. The involvement of T cells was demonstrated in mice depleted of both T and B cells, where transfer of normal or immune T cells protected mice from the lethal effect of P. chabaudi, whereas transfer of immune B cells led to complete clearance of parasitemia. Plasmodium species with complex life cycles often stimulate both antibody and cell-mediated defence mechanisms, whose effectiveness depends on the particular parasite and the stage of infection (Janeway, 1992; Fearon and Locksley, 1996; Shi et al., 2001). The different stages of the parasite occur inside cells that either express (e.g. hepatocytes) or lack (e.g. erythrocytes) major histocompatibility complex (MHC) class I or class II molecules. Since T cells are able to recognize parasite antigens only as processed peptides presented by appropriate MHC molecules, extracellular forms of the parasite or parasite stages in
erythrocytes devoid of the presentation machinery are controlled mainly by antibody-dependent acquired immune effector mechanisms. However, the immunological control is only partial (Röllinghoff et al., 1999). Two stages of the malaria parasite are truly extracellular: the sporozoites that infect the hepatocytes and the asexual merozoite stage that resides in red blood cells. Infection with *Plasmodium* species stimulates both CD4+ and CD8+ T cells expressing an αβ or a γδ T-cell receptor (TCR). While mice genetically deficient for αβ TCR T cells are very susceptible to *P. chabaudi chabaudi* infection and die rapidly after infection, there is no difference between γδ TCR-deficient mice and control mice (Langhorne et al., 1995; Sayles and Rakhmilevich, 1996). CD8+ T cells mediate killing of the liver stage of plasmodia, possibly by producing cytokines (interferon γ (IFN-γ), TNF) which induce the production of nitric oxide by infected hepatocytes (Nussenzweig and Nardin, 1993; Hoffman et al., 1996; Mannorr et al., 2000).

CD4+ T cells play an essential role in regulating the human immune responses to asexual blood stages of *P. falciparum* both with regard to production of cytokines and with providing help to the humoral component. CD4+ T cells also have important functions in anti-plasmodial immunity, including release of cytokines such as IFN-α involved in the activation of mononuclear and polymorphonuclear leukocytes which phagocytose or lyse infected erythrocytes. While, CD8+ T cells with a cytotoxic potential play an important role in immunity to the preerythrocytic stages of malaria parasites. The Production of TNF-α may be essential for protection against *P. falciparum* malaria (Hoffman et al., 1996; Mannorr et al., 2000).

The central role of CD4+ T cells in protective immunity against asexual blood stages of experimental malaria has been shown by in vivo cell depletion analysis and by cell transfer studies. Since transfer of purified CD4+ T cells or malaria-specific CD4+ T cell clones to severe combined immunodeficient or lethally irradiated mice clears the infection only in the presence of B cells, T–B cell interaction is thought to be required for the establishment of a fully protective immune response to malaria parasites (Taylor-Robinson et al., 1993). In *Plasmodium chabaudi*-infected mice both Th1-type and Th2-type CD4+ T cells are involved in protective immunity (Abbas et al., 1996). However, the relative contribution of these subsets changes during the course of infection: Th1 cells predominate during the acute phase,
and TH2 cells are found primarily during later phases of infection. The protective effect of transferred TH1 cells in mice infected with *P. chabaudi chabaudi* can be blocked by inhibitors of inducible nitric oxide synthase (iNOS), whereas resistance conferred by TH2 cells is not affected (Taylor-Robinson *et al.*, 1993). Even in the case of TH1 cells, there appear also to be nitric oxide-independent mechanisms of protection, as shown in *P. yoelii*-infected mice (Amante and Good, 1997). Protective TH2 cell clones specific for *P. chabaudi chabaudi* drive a strong protective malaria-specific IgG1 response *in vivo* which is promoted by IL-4 (Amante and Good, 1997).

### 1.5 Vaccine development

Vaccination has been an important tool to combat infectious diseases over the past 200 years. The first human vaccine was developed in 1798 when Edward Jenner successfully prevented smallpox infection in milkmaids. Today, prevention of bacterial and viral infections through vaccination is very beneficial in reducing disease mortality and healthcare costs. Successful development of vaccines has resulted in control of many major diseases, such as diphtheria, poliomyelitis and measles nowadays, and smallpox are even totally eradicated (Mäkelä, 2000). The first vaccines were mainly based of either live but attenuated or inactivated, killed microbes. Also today, most of the vaccines used routinely in childhood vaccination programs are whole-organism vaccines consisting of attenuated or killed bacteria or viruses (Plotkin, 1993).

With the panorama of newly emerging and spreading drug resistance, the development of an effective vaccine against malaria would be a valuable and cost-effective intervention instrument to complement other control tools. Several types of malaria vaccines are being developed which can be classified according to the parasite stage that is their target. (i) Preerythrocytic vaccines are directed against infective sporozoites injected by the mosquito vector, and liver stages. An anti-sporozoite vaccine would prevent infection, thus avoiding clinical manifestations of malaria. (ii) Merozoite vaccines are vaccines against asexual erythrocytic stages. Merozoites cause most of the clinical symptoms of malaria. An anti-merozoite vaccine would therefore, not prevent infection, but it would reduce malaria morbidity and mortality. (iii) Transmission-blocking vaccines (TBVs) are directed against sexual blood stages and aim to prevent the development of parasites in the mosquito vector. A
TBV would not prevent disease in people who were infected, but would reduce transmission in endemic areas. Several potential candidate vaccines of all three types are currently in clinical trials (Tanner, 2000). The complexity of the parasite and the immune response of the human host, as well as the enormous costs, hinder rapid progress in the development of a malaria vaccine. Up to now, the only candidate to have progressed to phase III of clinical trials is the multi-stage (sporozoite and merozoite) vaccine SPf66. The synthetic peptide vaccine SPf66, developed in Colombia (Patarroyo et al., 1988), has shown mixed results in different trials in South America, South-East Asia and Africa. The overall efficiency, calculated from 6 Phase III clinical trials, has been estimated at 23% (Graves, 1997; Tanner, 2000). In a recent trial in infants under 7 months old, SPf66 was not found to reduce the risk of clinical malaria (Acosta et al., 1999). The efficacy of SPf66 vaccine in its present form is thus much below the standard of other vaccines currently in use against other diseases, which can reach efficiency levels of 90% or more. It is also below the goal set by the Vaccine Discovery Research group of TDR, to find a vaccine which reduces the incidence of clinical attacks in children under the age of five by at least 30% (WHO, 2000b).

Vaccination seems to be the best way to prevent malaria (Miller, 2002). Malaria vaccines are feasible. Immunisation with irradiated sporozoites protects or partially protects humans from being infected by sporozoites (Rieckmann, 1979; Clyde, 1990; Egan et al., 1993; Elodie et al., 2004). Immunisation studies performed over the past 15 years show that vaccines already in hand can protect against malaria infection in animal models and in humans, but efficiency of these vaccines is still too low and the duration of protection still too short to be of practical value (Richie, 2002). First vaccination trials with subunit malaria vaccines in male human volunteers have not been successful (Siddiqui, 1991). There is increasing information available describing genetic restrictions in malaria vaccination. In particular, genes of the HLA complex have been reported to restrict the immune response to subunit vaccines against malaria (Pink and Sinigaglia, 1989; Quakyi et al., 1989; Patarroyo et al., 1991). Besides genetic restrictions, however, environmental restrictions may also exist. Indeed, it is known that endogenous environmental factors such as hormones play an important role in the modulation of the immune response (Schuurs and Verheul, 1990). Testosterone has been found to suppress the development of protective immunity in mice against infections with the malaria parasites *Plasmodium chabaudi* (Wunderlich et al., 1988a) and *Plasmodium berghei* (Kamis and Ibrahim, 1989). Vaccination experiments were conducted with the *P. chabaudi*
model previously developed in mice of the inbred strain B10.A (Wunderlich et al., 1988b). This model uses surface membranes of *P. chabaudi*-infected erythrocytes as an anti-disease rather than an anti-parasite vaccine (Playfair et al., 1991). The surface membranes were isolated in the form of ghosts from *P. chabaudi*-infected erythrocytes (Wunderlich et al., 1988c). In the present study, vaccination with surface membranes isolated from *Plasmodium chabaudi*-infected erythrocytes can protect female Balb/c mice from the lethal outcome of *P. chabaudi* malaria.

1.6 Role of the spleen in immunity to blood stage malaria parasites

Efficient control of pathogens by the immune system is promoted by a highly organized microarchitecture of secondary lymphoid organs. These structures form the basis for trapping, transport, processing, and presentation of antigens Ags, a prerequisite for initial constraint of pathogens and successful induction of specific immunity. The spleen is responsible for filtering/clearing of blood borne particles. The spleen is divided into white pulp and red pulp separated by the marginal zone. The marginal zone consists of sinus-lining reticular cells, marginal zone B cells, dendritic cells (DC) (Kraal and Janse, 1986; Weiss, 1990), marginal metallophilic macrophages (MM), and marginal zone macrophages (MZM). In the marginal zone, the blood leaves the terminal arterioles into open sinuses, the blood flow is slowed down, and bloodborne particles are trapped with high efficiency (Weiss, 1990; Kraal, 1992; Moghimi, 1995; Avles et al., 1996).

The spleen participates in immune responses against many types of pathogens and it is also involved in autoimmune diseases and lymphoid malignancies (Bowdler, 1990). Within the spleen, lymphocytes are organized as sheathes around arterioles, with the T zone located centrally (also called the perierteriolar lymphoid sheath or PALS) and the B cells distributed around the T zone in tightly packed follicles (Dijkstra et al., 1985; Kraal, 1992).

Antigen-presenting dendritic cells (DCs) are prevalent in the marginal zone, the T cell zone, and in the bridging channels between these two compartments (Snook, 1964; Steinman et al., 1997). The T zone, follicles, and marginal zone of the spleen are commonly referred to as the white pulp cords and they account for approximately half of the splenic tissue
(Bowdler, 1990). The remainder of the spleen, termed red pulp, contains large numbers of macrophages, vascular cells, and transiting blood cells. This compartment functions in red cell and immune complex clearance and leukocyte exit. The spleen plays a central role in immunity against blood stage malaria (Wyler, 1983; Yap and Stevenson, 1994; Alves et al., 1996; Balmer et al., 2001), and a significant role in mediating resistance to malaria infection. It is the major site of (a) elimination of parasite-infected erythrocytes via erythrophagocytosis, (b) elaboration of protective immune mechanisms, and (c) hypersensitivity reactions manifesting themselves as spleen enlargement (Crane, 1977; Chotivanich et al., 2002).

**Figure 2.** Schematic representation of the mouse spleen showing the marginal zone and red pulp areas. Blood flows from the central arteriole into marginal sinus, marginal zone, or red pulp, where it comes into contact with macrophages and other immune effector cells before flowing into the veins (Yadava et al., 1996).
The spleen is greatly enlarged during malaria in experimental animals and humans, and splenomegaly is used as a measure of malaria endemicity. The precise mechanisms by which the spleen exerts its protective functions are not well understood but are likely to be complex, given the unique anatomical features and cellular composition of this organ. The primary induction of immunity to blood stage parasites may occur in the spleen, although the liver may assume this function in the absence of the spleen (Dockrell et al., 1980).

The spleen may also serve as a mechanical filter for the removal of rheologically or immunologically modified erythrocytes, including parasitized as well as uninfected erythrocytes (Wyler et al., 1979; Humphrey and Grennan, 1981). During malaria due to blood stage parasites in mice, the spleen serves as the primary site of erythropoiesis to replace erythrocytes lost as a result of the stress-induced anaemia caused by malaria infection (Silverman et al., 1987; Weiss et al., 1989; Villeval et al., 1990; Yap and Stevenson, 1992). Earlier studies suggested that dramatic changes in the microcirculation in the spleen during malaria due to blood stage parasites in rodents were critical for the killing of parasites (Wyler et al., 1981; Weiss, 1986). However, recent studies suggest that alterations in the cellular composition of the spleen during infection may be of prime importance (Yadava et al., 1996; Chotivanich et al., 2002). During the course of infection, the numbers of parasite specific antibody-forming cells in the spleen increased (Sayles and Wassom, 1988) and increased activation and homing of T cells to the spleen occurred (Langhorne and Simon-Haarhaus, 1991). There are also marked increases in the number and function of splenic macrophages, responses mediated by chemotactic factors, and cytokines produced by CD4+ T cells and other activated lymphocytes (Shear, 1989). Studies demonstrated high levels of TNF-α and NOS2 mRNA expression in the spleens of P. chabaudi AS-resistant mice, while there was up-regulation of expression of these genes in the livers of susceptible mice just before death occurred (Jacobs et al., 1996). A convenient model to study the role of the spleen in malaria is the murine malaria Plasmodium chabaudi, which shares several common characteristics with the most dangerous human parasite Plasmodium falciparum, causing malaria tropica (Phillips et al., 1997). Studies with Plasmodium chabaudi adami, P. chabaudi AS, and P. vinckei have demonstrated the absolute requirement for an intact spleen in the resolution of both primary and reinfection immunity (Grun and Weidanz, 1981; Kumar et al., 1989; Stevenson et al., 1990; Weiss, 1991).
1.7 Filtration capacity of the spleen

The normal function of the spleen is to remove abnormal erythrocytes and intraerythrocytic inclusions. Malaria-infected red blood cells (RBCs) with *P. falciparum* contain an increasingly large and rigid parasite. Starting at about 13-16 h and peaking toward the middle of the asexual life cycle (24 h), the parasitized RBCs adhere to vascular endothelium and thereby avoid splenic removal. The younger ring-stage parasite is small and flexible and does not perturb the membrane configuration of RBCs or express parasite antigens externally. The more mature stages (the mature trophozoites and schizonts), which are larger, change the discoid shape of the infected RBCs and alter the host RBC membrane by the insertion of neoantigens, such as the ring-infected erythrocyte surface antigen (RESA) and *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1). The antigenic parasite adhesion protein PfEMP1 is expressed on the exterior of these RBCs. These various changes result in a loss of RBC deformability and an increase in antigenicity (Angus *et al*., 1997; Chotivanich *et al*., 2002). The spleen plays a pivotal role in policing the circulating RBC population, removing RBCs that are coated with antibodies or have reduced deformability and extracting intracytoplasmic particulate materials such as nuclear remnants (Howell Jolly bodies) or oxidized haemoglobin (Heinz bodies). Splenic clearance function has been reported to be increased in malaria (Lee *et al*., 1989; Ho, 1990; Prinzen *et al*., 1994). Microspheres have been used for the measurement of regional blood flow for more than 30 years (Rudolph and Heymann, 1967) and their use eliminates the problems associated with radioactivity (Prinzen and Glenny, 1994). The use of polysterene microspheres made it possible to reliably, safely, and inexpensively demonstrate quantitative changes in splenic clearance in *P. chabaudi* infection (Alves *et al*., 1996). The massive spleen marginal zone capture of nanoparticles might be attributed to the high blood flow passing through this area, leading to a sieve effect (Demoy *et al*., 1997). Quinn and Wyler (1980), from their studies of *P. berghei* infection in the rat, showed that crisis was associated with a sudden rise in clearance of heat-treated erythrocytes and Heinz body-containing erythrocytes (Wyler *et al*., 1981). They suggested that circulation through the spleen converts from an open circulation of the normal, uninfected animal to a closed circulation in precrisis. Crisis may be initiated by a reopening of the circulation in the red pulp. Weiss *et al*., (1986), studying *P. yoelii* in the mouse, proposed an ultrastructural basis for this shift in circulation during precrisis. During this period, the reticular cells in the red pulp become highly activated, and their processes form a tightly knit meshwork that channels blood directly from the artery into the vein. Thus,
a blood-spleen barrier is formed, causing limited access of blood into the filtration beds. These barrier cells disappeared during crisis, and normal blood circulation was restored (Alves et al., 1996).

1.8 Aim of the work

The present study was aimed at investigating the mechanisms by which vaccination with surface membranes isolated from Plasmodium chabaudi-infected erythrocytes can protect female Balb/c mice from the lethal outcome of P. chabaudi malaria, i.e. how-vaccination converts non-healer mice to self-healers. Normally, self-healing is characterized not only by overcoming the infection, but also by developing protective immunity controlled by genes of both H-2 complex and non-H-2 background. The susceptible Balb/c mice exhibit the resistant H-2 haplotype, but the susceptible non-H-2 background. Therefore, functional changes in spleen and liver of vaccinated Balb/c mice were analyzed in comparison with non-vaccinated mice.
2 MATERIALS AND METHODS

2.1 Chemical reagents, kits, solutions and buffers, and antibodies

2.1.1 Chemical reagents

1,2 phenylenediamine | DAKO, Copenhagen, Denmark
Avertin (2,2,2-tribromoethanol) | Sigma-Aldrich, St Louis, MO, USA
Bovine serum albumin (BSA) | Serva, Heidelberg, Germany
Diethyl-pyrocarbonat (DEPC) | Fulka, Neu-Ulm, Germany
DNA EcoR I/Hind III Marker, PNK A, PUC Mix Marker 8 | MBI Fermentas, St. Leon Rot, Germany
FluoroLink™ Cy3-dCTP | Amersham, Pharmacia, Uppsala, Sweden
FluoroLink™ Cy5-dCTP | Amersham, Pharmacia, Uppsala, Sweden
Fetal calf serum (FCS) | PAA, Linz, Austria
Fluorescent Polymer Microsphere Suspension, 3 µm in diameter (Green) | Ducke Scientific Corporation, Palo Alto, CA, USA
Fluorescent Polymer Microsphere Suspension, 2.9 µm in diameter (Red) | Ducke Scientific Corporation, Palo Alto, CA, USA
Giemsa solution | Merck, Darmstadt, Germany
**Material and methods**

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ketanest®</td>
<td>Parke Davis GmbH, Berlin, Germany</td>
</tr>
<tr>
<td>MaxiSorp F96 microtiter plates</td>
<td>Nunc, Wiesbaden, Germany</td>
</tr>
<tr>
<td>May-Gruenwald’s Eosine-methylene blue solution</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>OCT compound</td>
<td>Sakura, Zouterwede, The Netherlands</td>
</tr>
<tr>
<td>P 23-Cellulose</td>
<td>Serva, Heidelberg, Germany</td>
</tr>
<tr>
<td>Percoll</td>
<td>Amersham, Pharmacia, Uppsala, Sweden</td>
</tr>
<tr>
<td>Rompun®</td>
<td>Bayer, Leverkusen, Germany</td>
</tr>
<tr>
<td>RPMI medium</td>
<td>GIBCO-BRL, Eggenstein, Germany</td>
</tr>
<tr>
<td>Sea sand</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Sephadex G 25 and P 23 cellulose</td>
<td>Servacel, Heidelberg, Germany</td>
</tr>
<tr>
<td>Serum separator tubes</td>
<td>BD Bioscience, Heidelberg, Germany</td>
</tr>
<tr>
<td>Streptavidin-bionylated horseradish peroxidase complex</td>
<td>Amersham-Buchler, Braunschweig, Germany</td>
</tr>
<tr>
<td>Superfrost® plus slides</td>
<td>Menzel-Glaser, Braunschweig, Germany</td>
</tr>
<tr>
<td>Testosterone</td>
<td>Schering, Berlin, Germany</td>
</tr>
</tbody>
</table>
Material and methods

2.1.2 Kits

<table>
<thead>
<tr>
<th>Kit Description</th>
<th>Supplier Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIQOR™ cDNA array system</td>
<td>Memorec, Cologne, Germany</td>
</tr>
<tr>
<td>PolyA⁺-RNA purification kit</td>
<td>Qiagen GmbH, Hilden, Germany</td>
</tr>
<tr>
<td>Qiagen PCR Purification Kit</td>
<td>Qiagen GmbH, Hilden, Germany</td>
</tr>
<tr>
<td>Total RNA isolation kit</td>
<td>Qiagen GmbH, Hilden, Germany</td>
</tr>
</tbody>
</table>

2.1.3 Solutions and buffers

- **Blocking Buffer**: 8 g NaCl, 0.2 g KCl, 1 g Na₂HPO₄ × 2 H₂O, 0.5 g NaH₂PO₄ × H₂O, 0.2 g KH₂PO₄, 20 g Milk powder, adjust pH to 7.2, add H₂O up to 1 l
- **Buffered formalin (10%)**: 100 ml 40% formaldehyde, 900 ml dist. H₂O, 4 g NaH₂PO₄ × H₂O, 6.5 g Na₂HPO₄ × 2 H₂O
- **CPD-Puffer**: 34.7 g Sodium citrat, 0.7 g Citric acid, 22.5 g D-Glucose, 1.8 g Na₂HPO₄ × 2 H₂O, pH 7.2 add H₂O up to 1 l
- **Citrate Buffer**: 1.43 g Citric acid, 2.37 g Na₂HPO₄ × 2 H₂O adjust pH to 5, add H₂O up to 200 ml
- **Citrate phosphate glucose (CPG)**: 34.7 g Na-citrate × 2 H₂O, 0.7 g Citric acid, 1.8 g Na₂HPO₄ × 2 H₂O, 22.5 g Glucose × H₂O, adjust pH to 7.2, add H₂O up to 1 l
<table>
<thead>
<tr>
<th>Material and methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coating Buffer</td>
</tr>
</tbody>
</table>
| 0.8 g Na₂CO₃ 1.46 g NaHCO₃ adjust pH to 9.6, add H₂O up to 1 l  
| Ethanolic KOH solution                |  
| 3 g KOH, 0.5 g Tween 80, add ethanol up to 100 ml  
| Glycerine buffer (10%)                |  
| 1 ml Glycerin (100%), 500 µl FCS, 8.5 ml PBS  
| Intracellular medium (IM)             |  
| 1.17 g NaCl, 8.95 g KCl, MgCl₂ × 6 H₂O, 1.8 g Glucose, 1.3 g Hepes, 2 g NaHCO₃, add H₂O up to 1 l  
| IM shocking buffer                    |  
| 1 ml FCS, add 9 ml IM-buffer          |  
| (IMV)-buffer                          |  
| IM supplemented with 1 mM adenosine triphosphate (ATP) and vitamins as in RPMI 1640  
| LB-agar                              |  
| LB-medium with 1.5% agar              |  
| LB-medium (Luria-Bertani Medium)      |  
| 10 g NaCl, 10 g Trypton, 5 g Yeast extract, adjust pH to 7.5, add H₂O up to 1 l, Sterilize by autoclaving.  
| Phosphate buffer saline glucose monohydrate (PBS-HG) |  
| 900 ml PBS, 20.25 glucosemonohydrate, add H₂O up to 1 l  
| PBS⁺                                 |  
| 8 g NaCl, 0.2 g KCl, 1.15 g Na₂HPO₄ × 2 H₂O, 0.15 g NaH₂PO₄ × H₂O, 0.2 g KH₂PO₄, adjust pH to 7.2, add H₂O up to 1 l  

- 23 -
### Material and methods

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Composition and Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS⁺</td>
<td>8 g NaCl, 0.2 g KCl, 1.15 g Na₂HPO₄ × 2 H₂O, 0.2 g KH₂PO₄, 0.1 g MgCl₂ × 6 H₂O, 0.1 g CaCl₂ × 2 H₂O, adjust pH to 7.2, add H₂O up to 1 l</td>
</tr>
<tr>
<td>PFA (1%)</td>
<td>Paraformaldehyde in PBS⁺, adjust to pH 7.2</td>
</tr>
<tr>
<td>Soerensen buffer</td>
<td>0.49 g KH₂PO₄ × H₂O, 1.14 g Na₂HPO₄, adjust pH to 8.6, add H₂O up to 1 l</td>
</tr>
<tr>
<td>TBE (5×)</td>
<td>54 g Tris-base, 70 g boracic acid, 3.4 g EDTA, add H₂O up to 1 l</td>
</tr>
<tr>
<td>TE buffer</td>
<td>10 mM Tris, 1 mM EDTA, pH 7.5</td>
</tr>
<tr>
<td>Triton X-100 (1%)</td>
<td>10 g Triton X-100, add up to 11 H₂O</td>
</tr>
<tr>
<td>Washing buffer</td>
<td>5 g Bovine serum albumin (BSA), 500 µl Tween-20, add PBS⁺ up to 1 l</td>
</tr>
</tbody>
</table>
2.1.4 Antibodies

Table 1. Antibodies and specific reagents for FACS

<table>
<thead>
<tr>
<th>Antibodies and specific reagents</th>
<th>Supplier</th>
<th>Working dilution</th>
<th>Incubation time</th>
</tr>
</thead>
<tbody>
<tr>
<td>FITC labelled anti-mouse CD8a (Ly-2)(53-6.7)</td>
<td>BD PharMingen, Heidelberg, Germany</td>
<td>1:200</td>
<td>1 h</td>
</tr>
<tr>
<td>FITC labelled anti-mouse CD4 (L3T4)(H129.19)</td>
<td>BD PharMingen, Heidelberg, Germany</td>
<td>1:200</td>
<td>1 h</td>
</tr>
<tr>
<td>FITC labelled anti-mouse F4/80 (macrophages) (C1:A5-1)</td>
<td>ImmunoKontact, Wiesbaden, Germany</td>
<td>1:150</td>
<td>1 h</td>
</tr>
<tr>
<td>FITC labelled anti-mouse CD45/B220 (clone RA3-6B2)</td>
<td>BD PharMingen, Heidelberg, Germany</td>
<td>1:200</td>
<td>1 h</td>
</tr>
<tr>
<td>FITC labelled anti-mouse Ly-6G (Gr-1)(RB6-8C5)</td>
<td>BD PharMingen, Heidelberg, Germany</td>
<td>1:200</td>
<td>1 h</td>
</tr>
<tr>
<td>FITC labelled anti-mouse NK cells/2B4 (CD244.2)</td>
<td>BD PharMingen, Heidelberg, Germany</td>
<td>1:200</td>
<td>1 h</td>
</tr>
<tr>
<td>CD16/CD32(FcIII/II receptor) FC Block</td>
<td>BD PharMingen, Heidelberg, Germany</td>
<td>1:200</td>
<td>15 min</td>
</tr>
</tbody>
</table>

2.2 Animals

Balb/c mice were bred under specific pathogen-free conditions in the central animal facilities at the Max-Plank-Institute for Immunobiology (Freiburg, Germany). Experiments were performed only with female mice at an age of 10-14 weeks. They were housed in plastic
Material and methods

cages and received standard diet (Wohrlin, Bad Salzuflen, Germany) and water *ad libitum*. The experiments were approved by the state authorities and followed German law on animal protection.

2.3 *Plasmodium chabaudi* infection in mice

Blood stages of *Plasmodium chabaudi* (originally provided by the National Institute of Medical Research, Mill Hill, London) were maintained in 9-14 weeks old NMRI-mice (28-35 g) by weekly passages of infected blood (Wunderlich *et al*., 1982). Balb/c mice were challenged with $10^6$ *P. chabaudi*-parasitized erythrocytes. Parasitaemia was evaluated in Giemsa-stained blood smears. Total erythrocytes were counted in a Neubauer chamber.

2.4 Vaccination of mice

2.4.1 Isolation of parasitized erythrocytes

*P. chabaudi*-infected NMRI-mice were anaesthetized with ether at a parasitemia of 20-45%. Blood was drawn from the retroorbital plexus into Pasteur-pipettes, diluted with CPG-buffer at a ratio of about 1:1, and then passed through a column containing about 25 ml sea sand (Merck, Darmstadt, Germany) overlaid by 25 ml of a 1:1 mixture of sephadex G 25 and P 23 cellulose (Servacel, Heidelberg, Germany) to remove leukocytes and platelets (Nakato *et al*., 1973). After centrifugation at 1500 × g for 5 min, the pellet containing both infected and non-infected erythrocytes was suspended in PBS-HG-buffer. Aliquots of 2-3 ml were layered on top of a 16 ml percoll-cussion (p: 1.10 g cm$^{-3}$) and centrifuged at 5000 × g for 20 min. The parasitized erythrocytes were enriched on top of the percoll-step with an average yield of 90-98% (Wunderlich, *et al*., 1985).

2.4.2 Isolation of ghosts from infected erythrocytes

In the continuous Percoll gradient, the ghosts from infected erythrocytes remained on top of the gradient in a red coloured zone (Wunderlich *et al*., 1985; 1987). After careful removal, these ghosts were diluted with about 30 ml IM-buffer, centrifuged at 500 × g for 30 min, and the pellet was resuspended in IM-buffer. Aliquots (1-2 ml) were layered on an about 8 ml two-step Percoll gradient (p: 1.01 + 1.02 g cm$^{-3}$) and centrifuged at 5000 × g for 30 min. Ghosts were recovered from the top of the gradient, diluted with about 30 ml phosphate buffer and centrifuged at 5000 × g for 30 min. The pellet was resuspended in phosphate-buffer and recentrifuged as above. The pellet contains the hemoglobin-free, purified ghosts from infected erythrocytes (Wunderlich *et al*., 1985; 1987).
2.4.3 Vaccination of mice with ghosts from *Plasmodium chabaudi*-infected erythrocytes

Mice were vaccinated once or twice with \( 5 \times 10^5 \) ghosts which were suspended in 100 µl PBS, mixed with an equal volume of Freund’s complete adjuvant (FCA) (Sigma, USA), and administrated subcutaneously at the base of the tail (Wunderlich *et al*., 1993). After one week the vaccinated mice were challenged with \( 1 \times 10^6 \) *P. chabaudi* infected erythrocytes.

2.5 Flow cytometry

2.5.1 Isolation of spleen cells

Mice were killed by cervical dislocation, spleens were aseptically removed and gently dissociated through a stainless steel sieve into RPMI medium (GIBCO-BRL, Eggenstein, Germany) supplemented with 5% fetal calf serum (PAA Laboratories, Linz, Austria). After erythrocytes were removed by NH\(_4\)Cl lysis (Benten *et al*., 1991), total leukocytes were counted in a Neubauer chamber.

2.5.2 Isolation of liver cells

Hepatic mononuclear cells were prepared as previously described (Watanabe *et al*., 1992). Briefly, the liver was removed from mice and the hepatic mononuclear cells were prepared by pressing the liver through a stainless steel and suspending them in RPMI medium containing 5% fetal calf serum. After washing once, cells were resuspended in 35% percoll solution and centrifuged for 15 min at 2000 rpm and 4°C. After washing once, the pellet was resuspended in NH\(_4\)CL lysis solution and washed once with medium. The number of cells was determined in a Neubauer chamber.

2.5.3 Quantification of splenic cell populations

Flow cytometry was performed as described previously (Van Vliet *et al*., 1986). In brief, spleen cells prefixed with 1% paraformaldehyde were diluted to \( 5 \times 10^6 \) per ml. Aliquots of 75 µl were centrifuged, splenic leukocytes were labeled with antibodies and analyzed by flow cytometry (FACS Calibur, BD Bioscience) as detailed previously (Benten *et al*., 1991). In brief, cells were preincubated with anti-CD16/CD32 (FcIII/II receptor) FC block (BD PharMingen) for 15 min and then labeled with one of the following FITC-labeled monoclonal antibodies for 1 h: anti-mouse CD45R/B220 (clone RA3-6B2), anti-mouse CD4 (H129.19), anti-mouse CD8a (53-6.7), anti-mouse CD244.2 (2B4), anti-mouse Gr1 (RB6-8C5) (all BD PharMingen), and anti-mouse F4/80 (C1:A3-1) (ImmunoKontact). Finally cells were suspended in 1% paraformaldehyde and FACS analysis was done with a sample size of
Material and methods

10,000 cells gated on the basis of forward and sideward scatter. Data were stored and processed using Cell Quest Pro software (BD Bioscience). Leukocytes were counted in a Neubauer chamber.

2.5.4 Quantification of hepatic mononuclear cell populations

Hepatic mononuclear cells prefixed with 1% paraformaldehyde were diluted to 5x10⁵ cells per ml. Aliquots of 37.5 ul were centrifuged, and the cell pellets were incubated with 12.5 µl of monoclonal antibodies. After labeling the hepatic mononuclear cells were analysed by flow cytometry (FACS Calibur, BD Bioscience) as detailed previously (Benten et al., 1991). In brief, cells were preincubated with anti-CD16/CD32 (FcIII/II receptor) FC block (BD PharMingen) for 15 min and then labeled for 1h with one of the following FITC-labeled monoclonal antibodies: anti-mouse CD45R/B220 (clone RA3-6B2), anti-mouse CD4 (H129.19), anti-mouse CD8a (53-6.7), anti-mouse CD244.2 (2B4), anti-mouse Gr-1⁺ (RB6-8C5) (all BD PharMingen), and anti-mouse F4/80 (C1:A3-1) (ImmunoKontact). Finally, cells were suspended in 1% paraformaldehyde. FACS analysis was done with a sample size of 10,000 cells gated on the basis of forward and sideward scatter. Data were stored and processed using Cell Quest Pro software (BD Bioscience). Cells were counted in a Neubauer chamber.

2.6 Filtration capacity of spleen and liver

2.6.1 Determination of the filtration capacity

Each mouse was anesthetized with chloroform and weighed, before 200 µl PBS containing 1.3x10⁸ green fluorescent beads (Duke Scientific, Palo Alto, USA) with a diameter of 3 µm were injected into the retroorbital plexus according to W. Pinkerton and M. Webber (1964). After 5 minutes the mouse was killed by cervical dislocation and the spleen, kidney and the total or a part of the liver were removed, weighed and squeezed with a glass rod into 8 ml 2.3 M KOH, 0.5% Tween 80 in ethanol. After addition of 5x10⁵ red beads (2.9 µm diameter, Duke Scientific) to control for losses during extraction, the tissues were dissolved by shaking for 48 h at 50°C interrupted several times by vigorous vortexing. Samples were centrifuged at 2000xg for 20 minutes at 20°C. The pellet with the beads was washed once with 8 ml 1% Triton X-100 and once with 8 ml phosphate buffer and finally resuspended in 1 ml distilled water. Fluorescence intensity was measured in a luminescence spectrometer (Perkin Elmer LS 55, Langen, Germany) using a 10×2 mm Quartz Precision cell (Hellma, Mühlheim, Germany)
Material and methods

at excitation/emission wavelengths (450/480) and (520/590) for green and red beads, respectively.

2.6.2 Distribution of the fluorescent beads in spleen and liver using fluorescence microscopy

Spleens and livers of female Balb/c mice were embedded in OCT compound (Sakura, Zouterwede, The Netherlands), and serial 7-10 μm cryosections were mounted on superfrost® plus slides (Menzel-Glaser, Braunschweig, Germany). Slides were allowed to air dry at room temperature for at least 1 h. They were fixed in 10% PFA for 10-20 min, washed with PBS+ then counter stained with Haematoxylin (Merk, Darmstadt, Germany). Sections were mounted with a special mounting medium (Sigma Diagnostic, St. Louis, USA). Localization of fluorescent beads was analyzed with a Leica DM LB microscope (Leica Bensheim, Germany) using a FITC filter set.

2.6.3 Localization of Gr-1+ granulocytes in the spleen using fluorescence microscopy

For localization of Gr-1+ granulocytes in the spleen, sections were air dried, fixed for 20 min in 1% paraformaldehyde in PBS and blocked for 20 min with FC block (1:200 in PBS+). Then cells were incubated for 1 h with the FITC-coupled primary antibody GR-1 in PBS+. Signal amplification was performed using a biotinylated secondary mouse anti-rat antibody (Dianova, Hamburg) and alexa fluor 488-coupled streptavidin. After counterstaining with haematoxilin sections were embedded in Mowiol with DABCO and analyzed with a Leica DM LB microscope using a FITC filter set.

2.7 Adoptive transfer experiments

The cells were suspended 200 µl of PBS+ at the desired cell concentration and injected intravenously into the tails of the recipient syngeneic female Balb/c mice, which were 10 to 12 weeks old.

2.7.1 Isolation of liver cells

Hepatic mononuclear cells were prepared as previously described (Watanabe et al., 1992). Briefly, the liver was aseptically removed from mice and the hepatic mononuclear cells were prepared by pressing the liver through a stainless steel sieve and suspending them in RPMI medium containing 5% fetal calf serum. After washing once, cells were resuspended in 35% percoll solution and centrifuged for 15 min at 2000 rpm and 4°C. Cells were washed again,
the pellet was resuspended in NH₄CL lysis solution to remove erythrocytes and washed once with medium. The number was determined in a Neubauer chamber.

2.7.2 Isolation of spleen cells
Spleens were aseptically removed from mice and gently dissociated through a stainless steel sieve into RPMI medium (GIBCO-BRL) supplemented with 5% fetal calf serum (PAA Laboratories) and collected by pelleting at 1,200 rpm in a Beckman GPKR centrifuge. Erythrocytes were lysed for 1 min with NH₄Cl, the suspension was then diluted 10-fold with RPMI medium containing 5% fetal calf serum and centrifuged, and the lysis was repeated once. Total leukocytes were counted in a Neubauer chamber.

2.8 cDNA microarrays

2.8.1 RNA-Isolation
Spleens and liver pieces were aseptically removed, rapidly frozen, and stored at -80°C. Total RNA was isolated by standard procedures using a commercially available RNA isolation Kit (Qiagen, Hilden, Germany) and PolyA⁺-RNA was purified using Oligotex (Qiagen). The quality of RNA was routinely tested for genomic DNA impurity, 28s/18s ratio, OD 280/260 ratio, and integrity by capillary electrophoresis using a Bioanalyzer 2100 (Agilent Technologies, Parlo Alto USA) and spectrophotometer.

2.8.2 cDNA arrays
PIQORTM immuno cDNA arrays (Memorec, Cologne, Germany) were used, which were applied with quadruplicate samples of defined 200 bp to 400 bp cDNA-fragments selected from 1076 immune relevant genes of the mouse (Bosio et al., 2002; Gerstmayer et al., 2003). All genes including their accession numbers are available in the internet (www.memorec.com). The arrays contained buffer and herring DNA as negative controls, 4 control RNAs of E. coli and the 6 positive control cDNAs GAPDH, actin, tubulin, cyclophilin and HPRT.

2.8.3 Labeling and hybridization
Probes were labelled with Cy3/Cy5 and hybridized as detailed previously (Bosio et al., 2002). In brief, 100 µg of total RNA was combined with a control RNA of an in vitro transcribed E. coli genomic DNA with a polyA⁺ tail, before isolating mRNA. To the latter, 3 different control transcripts were added before reverse-transcribing. The samples were
Material and methods

labelled with FluoroLink™ Cy3/Cy5-dCTP (Amersham Pharmacia Biotech, Freiburg, Germany), cleaned up using QIA quick™ (Qiagen), and hybridized using an Gene TAC™ hybridization station (Perkin Elmar, Langen, Germany) according to the guidelines of the manufacturer (Memorec, Cologne, Germany).

2.8.4 Readout and data analysis

The hybridized arrays were scanned and analyzed as detailed elsewhere (Bosio et al., 2002). Image capture and signal quantification were performed using the ScanArray 3000 (GSI Lumonics, Watertown, USA) and ImaGene software 4.1 (Bio-Siscorery, Los Angles, USA). The local signal of each spot was measured inside a 300 µm diameter circle, and, outside of this circle, the background was determined within a 40 µm wide ring approximately 40 µm distant to the signal. After subtraction of local background, the net signal intensity was used for calculation of the ratio of Cy5/Cy3. The ratios were normalized to the median of all ratios using only those spots with a fluorescence intensity of which was three times larger than that of the negative controls consisting of two spots of herring sperm DNA and two spots of only spotting buffer. Values represent means of 4 individual spots and standard deviations.
Figure 3. The PIQORTM cDNA array hybridization procedure

2.9 Statistical analysis

Unless otherwise mentioned, each data was normalized to the control value, the mean and standard deviation (SD) or standard error of the mean (SEM) from at least three experiments were determined. The data were analysed by using Excel 2000 (Microsoft, USA), and Sigma Plot 2001 (SPSS, USA). Curves analysed according to Kaplan and Meier using a log rank significance test and the SPSS software.
3 RESULTS

3.1 Course of blood stage malaria after protective vaccination

Blood stage infection of *P. chabaudi* malaria take a lethal outcome in female Balb/c mice. Challenge with $10^6$ *P. chabaudi*-parasitized erythrocytes results in a parasitemia of about 30-40% on approximately days 8-10 p.i. All mice succumb to infection. Vaccination, however, converts these non-healers to self-healers. Mice were vaccinated by injecting subcutaneously $10^6$ ghosts isolated from *P. chabaudi*-infected erythrocytes in FCA and boostering once with the same dose after 14 days. On day 7 after boostering, mice were challenged and more than 80% of these mice survived the infection. The precrisis of infection results in approximately 35% parasitemia on day 8 p.i. The subsequent crisis is characterized by falling parasitemias reaching parasitemias below 1% on approximately day 14 p.i. (Fig. 4).

![Graph of survival and parasitemia](image)

**Figure 4.** Survival and parasitemia of female Balb/c mice (n=18) (triangles) and vaccinated female Balb/c mice (n=20) (circles), infected with $1\times10^6$ *Plasmodium chabaudi*-parasitized erythrocytes. All values are means ± SEM.
3.2 Flow cytometry

3.2.1 FACS analysis of spleens of mice vaccinated with iG

Flow cytometric analysis of total nucleated spleen cells were determined and it was found that vaccination changes cellularity of the spleen. The total number of cells increases by about 50%. The spleen of non-vaccinated control mice contains $2.34 \times 10^8$ leukocytes and that vaccinated mice $3.28 \times 10^8$. The percentage of Gr-1$^+$ granulocytes has approximately doubled after vaccination (Fig. 5A). There is a significant increase of Gr-1$^+$ cells on day 0 ($p<0.01$), day 4 ($p<0.01$) and day 8 ($p<0.01$), and also a significant increase in F4/80 macrophages on day 0 ($p<0.01$), day 4 ($p<0.05$) and day 8 ($p<0.01$) p.i. after vaccination (Fig. 5A). No significant effect of vaccination was found on 2B4$^+$ NK cells (CD244), CD4$^+$ T cells and CD8$^+$ T cells. Moreover, a significant increase in the total number of F4/80 macrophages on day 0 ($p<0.001$), day 4 ($(p<0.01)$ and day 8 ($(p<0.05)$ p.i., and a significant increase in the total number of Gr-1$^+$ granulocytes on day 0 ($p<0.001$), day 4 ($p<0.05$) and day 8 ($p<0.001$) after infection was observed in vaccinated mice (Fig. 5B), while the number of B cells (B220) was only elevated on day 4 p.i. ($p<0.001$) (Fig. 5B). The described increase in Gr-1$^+$ cells of iG vaccinated mice was observed in the marginal zone and red pulp of the spleen (Figs. 6, 7).
Figure 5. FACS analysis of spleens of *P. chabaudi*-infected female Balb/c mice vaccinated with iG/FCA (grey) or treated with FCA (black) alone. Data represent mean ± SEM. (A) Percentage and (B) total number of CD4⁺ T cells, CD8⁺ T cells, F4/80 macrophages, Gr-1⁺ granulocytes, B220⁺ B cells and CD244 NK cells.
Results

Figure 6. Localization of Gr-1\(^+\) granulocytes in cryosections of spleens of *P. chabaudi* infected female Balb/c mice on day 0 and day 4 p.i.. Bars indicate 100 µm.

(A) Balb/c, day 0 p.i.  
(B) Balb/c, day 4 p.i.

Figure 7. Localization of Gr-1\(^+\) granulocytes in cryosections of spleens of iG vaccinated female Balb/c mice infected with *P. chabaudi*, on day 0 and day 4 p.i.. Bars indicate 100 µm.

(A) Vacc. Bab/c, day 0 p.i.  
(B) Vacc. Balb/c, day 4 p.i.
3.2.2 FACS analysis of livers of mice vaccinated with iG

Flow cytometric analysis of purified liver leukocytes were determined and it was found that in vaccinated female Balb/c mice there is a significant increase in 2B4$^+$ NK cells, CD4$^+$ T cells, B220$^+$ B cells, and F4/80 macrophages compared to mice treated with FCA alone. No significant difference was found for CD8$^+$ T cells. In contrast Gr-1$^+$ granulocytes decreased after vaccination with iG (Fig. 8).

**Figure 8.** FACS analysis of livers of uninfected female Balb/c mice vaccinated with iG/FCA (black) or treated with FCA (white).
3.3 Gating of spleen and liver

3.3.1 Differential gating of the spleen

The spleen is accepted as the major effector of the immune defense against blood stage malaria (Chotivanich et al., 2002). Splenic macrophages eliminate *Plasmodium*-infected erythrocytes via phagocytosis, which occurs predominantly in unique extravascular pathways (Weiss, 1991; Yadava et al., 1996). Arterioles open directly into the reticular meshwork through which blood cells are squeezed before reaching the collecting veins. This open circulation of the spleen switches to a closed state during precrisis of acute malaria, presumably controlled by malaria-activated barrier cells (Weiss, 1991). After reopening during crisis, the spleen has even gained a much higher effectivity to remove particulate material from the circulation (Alves et al., 1996).

There are two distinct components of the spleen, the red pulp and the white pulp separated by the marginal zone. The red pulp consists of large numbers of sinuses and sinusoids filled with blood and is responsible for the filtration function of the spleen. The white pulp consists of aggregates of lymphoid tissue and is responsible for the immunological function of the spleen. Vaccination alone has no effect on spleen size. However, an obvious effect of vaccination on spleen size during infection can be observed. Control mice display a twofold increase in spleen size at peak parasitemia, but there is a fourfold increase after vaccination.

Vaccination significantly decreases particles/100 mg in spleen on day 0 and day 4 p.i. (p<0.05) (Fig. 9). After injection of 3 µm fluorescent polystyrol beads, there are trapped approximately 2×10^5 particles/100 mg spleen in control mice (Fig. 9B) and 1.2×10^5 particles/100 mg spleen in vaccinated mice (Fig. 9A). Trapping occurs predominantly at the marginal zone of the spleen as detected in cryosections by fluorescence microscopy (Figs. 10, 11). On day 4 p.i., trapping capacity has slightly increased, but there is a dramatic reduction in particle trapping on day 8 p.i. This closing of the spleen lasts during whole crisis. There after, a slight reopening begins to occur, but only on day 36 p.i., the initial trapping capacity is restored. Moreover localization of Gr-1^+ granulocytes in the spleen is approximately increased in vaccinated mice than non-vaccinated mice (Figs. 6, 7).
Figure 9. Filtration capacity of the spleen during *P. chabaudi* infection. (A) Vaccinated female Balb/c mice and (B) non-vaccinated female Balb/c mice. Animals were infected with $1 \times 10^6$ *P. chabaudi*-parasitized erythrocytes. Parasitemia, spleen weight, total uptake of particles and uptake per 100 mg spleen weight are shown. All values are means ± SEM.
Figure 10. Particle distribution in cryosections of spleens of female Balb/c mice. Localization of the green beads at the marginal zone on day 0 and day 4 of *P. chabaudi* malaria infection. Sections were counterstained with Hematoxylin. Bars indicate 100 µm.

Figure 11. Particle distribution in cryosections of spleens of iG vaccinated female Balb/c mice. Localization of the green beads at the marginal zone on day 0 and day 4 p.i. Sections were counterstained with Hematoxylin. Bars indicate 100 µm.
Results

3.3.2 Differential gating of the liver

Vaccination does not visibly affect the size of the liver. Also, the specific capacity of liver to trap particles remains unaffected. However, there is a dramatic difference in trapping capacity during crisis between vaccinated (Fig. 12A) and non-vaccinated control mice (Fig. 12B). Whereas controls reveal a further drop in particle trapping between day 8 and day 11 p.i., there is a sharp increase in particle trapping in vaccinated mice, and this increase further progresses reaching a maximum on day 14 p.i., i.e. at the end of crisis. On day 11 p.i. particles trapping is 4.7 fold higher in the liver of vaccinated mice compared to controls (p<0.005).

Fluorescence microscopy of liver sections reveals that the injected beads are predominantly found in periportal areas of the liver acinus and the density decreases rapidly from the periportal to pericentral areas indicating that beads are rapidly trapped after entering the sinusoids from the portal vein or hepatic artery (Figs. 13, 14).

(A)                                                        (B)

Figure 12. Filtration capacity of the liver during *P. chabaudi* infection. (A) Vaccinated female Balb/c mice and (B) non-vaccinated female Balb/c mice. Animals were infected with $1 \times 10^6$ *P. chabaudi*-parasitized erythrocytes. All values are means ± SEM.
Results

Figure 13. Filtration capacity of the liver during *P. chabaudi* infection of female Balb/c mice. (A and B are cryosections on day 0 and 4 p.i.). Bars indicate 100 µm.

Figure 14. Filtration capacity of the liver during *P. chabaudi* infection of vaccinated female Balb/c mice. (A and B are cryosections on day 0 and day 4 p.i.). Bars indicate 100 µm.
3.4 Adoptive transfer experiments

3.4.1 Transfer of liver cells from vaccinated mice to infected non-immune mice.

Adoptive transfer of $5 \times 10^5$ liver cells from iG vaccinated female Balb/c mice into recipient non-vaccinated *P. chabaudi* infected female Balb/c mice on day 4 and day 8 p.i. caused a significant increase in survival time ($p<0.001$). No significant increase was observed when adoptive transfer was performed with $5 \times 10^5$ liver cells on day 4 p.i. and $5 \times 10^7$ spleen cells on day 8 p.i. (Table 1 and Fig. 15). Also no significant increase in survival occurred after adoptive transfer of $5 \times 10^5$ liver cells from iG vaccinated and infected mice into recipient non-vaccinated infected mice on day 4 and day 8 (Table 2 and Fig. 15).

### Table 1: Adoptive transfer (A.T.) of liver cells from iG vaccinated female Balb/c mice to recipient non-vaccinated *P. chabaudi* infected female Balb/c mice.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Vacc. controls</th>
<th>A.T. of liver cells ($5 \times 10^5$) from FCA treated control mice to recipient mice on day 0</th>
<th>A.T. of liver cells ($5 \times 10^5$) from vaccinated mice to recipient mice on day 4 of infection</th>
<th>A.T. of liver cells ($5 \times 10^5$) from vaccinated mice to recipient mice on day 0</th>
<th>A.T. of liver cells ($1 \times 10^6$) from vaccinated mice to recipient mice on day 4 of infection</th>
<th>A.T. of liver cells ($5 \times 10^5$) from vaccinated mice to recipient mice on day 4 and spleen cells ($5 \times 10^7$) on day 8 of infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of mice</td>
<td>18</td>
<td>20</td>
<td>5</td>
<td>6</td>
<td>5</td>
<td>8</td>
<td>17</td>
</tr>
<tr>
<td>Time until death of succumbing mice (Mean±SD)</td>
<td>10.05±1.2</td>
<td>10.0±1.41</td>
<td>9.6±0.89</td>
<td>10.17±0.98</td>
<td>10.4±1.52</td>
<td>10.5±1.31</td>
<td>9.8±0.84</td>
</tr>
<tr>
<td>% Survival</td>
<td>0</td>
<td>85</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>16.7</td>
</tr>
<tr>
<td>% Mice surviving longer than day 10</td>
<td>33.3</td>
<td>95</td>
<td>20</td>
<td>33</td>
<td>40</td>
<td>62.5</td>
<td>33</td>
</tr>
</tbody>
</table>
**Table 2:** Adoptive transfer (A.T.) of liver cells from iG vaccinated female Balb/c mice to recipient non-vaccinated *P. chabaudi* infected female Balb/c mice.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Vacc. controls</th>
<th>Controls treated with FCA alone</th>
<th>A.T. of liver cells (5×10^5) from vaccinated and infected mice to recipient mice on day 4 and 8 of infection.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of mice</td>
<td>18</td>
<td>20</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>Time until death of succumbing mice (Mean±SD)</td>
<td>10.05±1.20</td>
<td>10.0±1.41</td>
<td>10±0.93</td>
<td>11.2±0.45</td>
</tr>
<tr>
<td>% Survival</td>
<td>0</td>
<td>85</td>
<td>0</td>
<td>16.7</td>
</tr>
<tr>
<td>% Mice surviving longer than day 10</td>
<td>33.3</td>
<td>95</td>
<td>12.5</td>
<td>100</td>
</tr>
</tbody>
</table>
Results

Figure 15. Adoptive transfer of cells from iG vaccinated female Balb/c mice to recipient non-vaccinated *P. chabaudi* infected female Balb/c mice.
3.4.2 Transfer of liver cells from immune mice to infected non-immune mice

Blood stage infections with *Plasmodium chabaudi* malaria take a self-healing course in iG vaccinated female Balb/c mice. The present data show that adoptive transfer of $5 \times 10^5$ liver cells from immune mice to recipient non-immune *P. chabaudi* infected female Balb/c mice on day 4 of infection and $5 \times 10^7$ spleen cells on day 8 of infection caused a significant increase in survival time ($p<0.001$) and induced a higher survival rate of the recipient mice, and adoptive transfer of $5 \times 10^5$ liver cells from immune mice into recipient non-immune infected mice on day 4 of infection induced a significant increase in survival time ($p<0.06$) but survival rate is only slightly elevated (Table 3 and Fig. 16).

Table 3: Adoptive transfer (A.T.) of liver cells isolated from immune female Balb/c mice to *P. chabaudi* infected non-immune female Balb/c mice.

| Controls | Vacc. controls | A.T. of liver cells ($5 \times 10^5$) from immune to recipient mice on day 4 and 8 of infection | A.T. of liver cells ($1 \times 10^6$) from immune to recipient mice on day 0 | A.T. of liver cells ($3,3 \times 10^6$) from immune to recipient mice on day 4 of infection | A.T. of liver cell ($1 \times 10^6$) from immune to recipient mice on day 4 of infection | A.T. of liver cell ($5 \times 10^5$) from immune to recipient mice on day 4 of infection | A.T. of liver cell ($5 \times 10^5$) from immune to recipient mice on day 4 and spleen cells ($5 \times 10^7$) on day 8 of infection |
|----------|----------------|-----------------------------------------------------------------------------------------------|---------------------------------------------------------------------------|-----------------------------------------------------------------------------|---------------------------------------------------------------------------|-----------------------------------------------------------------------------|-----------------------------------------------------------------------------|-----------------------------------------------------------------------------|
| Number of mice | 18 | 20 | 5 | 6 | 2 | 8 | 15 | 5 |
| Time until death of succumbing mice (Mean±SD) | 10.05±1.2 | 10.3±1.15 | 10±0.71 | 10.5±1.378 | 10.7±0.58 | 12±1.69 | 9.83±2.08 | 9±0.0 |
| %Survival | 0 | 85 | 0 | 0 | 0 | 0 | 20 | 60 |
| % Mice surviving longer than day 10 | 33.3 | 95 | 20 | 33.3 | 50 | 87.5 | 60 | 60 |
3.4.3 Transfer of spleen cells from immune mice to infected non-immune mice

Adoptive transfer of $5 \times 10^7$ spleen cells from immune female Balb/c mice into recipient *P. chabaudi* infected non-immune female Balb/c mice on day 4 of infection induced a significant increase in survival time ($p < 0.03$) and a slightly increase in survival rate. However adoptive transfer of $5 \times 10^7$ spleen cells from immune mice into recipient non-immune infected mice on day 0 did not prevent a fatal outcome of the infection for all recipient mice (Table 4 and Fig. 16).

**Table 4:** Adoptive transfer (A.T.) of spleen cells isolated from immune female Balb/c mice to *P. chabaudi* infected non-immune female Balb/c mice.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Vacc. controls</th>
<th>A.T. of spleen cells ($5 \times 10^7$) from immune to recipient mice on day 0</th>
<th>A.T. of spleen cells ($5 \times 10^7$) from immune to recipient mice on day 4 of infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of mice</td>
<td>18</td>
<td>20</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Time until death of succumbing mice (Mean±SD)</td>
<td>10.05±1.20</td>
<td>10.3±1.15</td>
<td>11 ± 1.26</td>
<td>10.75±1.58</td>
</tr>
<tr>
<td>% Survival</td>
<td>0</td>
<td>85</td>
<td>0</td>
<td>11,1</td>
</tr>
<tr>
<td>% Mice surviving longer than day 10</td>
<td>33.3</td>
<td>95</td>
<td>50</td>
<td>55.5</td>
</tr>
</tbody>
</table>
Figure 16. Adoptive transfer (A.T.) of cells from immune female Balb/c mice to recipient non-immune *P. chabaudi* infected female Balb/c mice.
3.5 Effect of vaccination on gene expression in spleen

Vaccination-induced changes in gene expression of spleens were analysed using cDNA-microarrays to compare the RNA levels of 1076 immunorelevant genes in the system on day 11 p.i. The scatter plot in Fig. 17 shows that 49 genes are upregulated more than 2-fold, and 29 genes down-regulated in spleens of iG vaccinated female Balb/c mice. More than 3-fold changes occurred in only 11 genes with 6 genes down-regulated and 5 genes upregulated as summarized in Table 5. The down-regulated genes are LOX, INHBB, BF, SOCS3, IL1R2, and IP3, whereas the upregulated genes are FY, MGST3, IRF7, MMPI2, and MAP17.

Figure 17. Effects of vaccine-treatment on gene expression of spleens of female Balb/c mice on day 11 after infection with *P. chabaudi*. Scatterplots represent normalized fluorescence intensities. Differential regulation of genes increases with their distance to the diagonal of the plot.
Table 5. Vaccine-induced changes in splenic gene expression of *P. chabaudi* infected female Balb/c mice on day 11 p.i.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Swiss Prot.</th>
<th>Ratio/CD (Vaccinated/controls)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysyloxidase (LOX) protein-lysine 6-oxidase precursor</td>
<td>P28301</td>
<td>0.23/8%</td>
</tr>
<tr>
<td>Inhibin beta B chain precursor (INHBB)</td>
<td>Q04999</td>
<td>0.25/24%</td>
</tr>
<tr>
<td>Complement factor B precursor BF: (BF)</td>
<td>P04186</td>
<td>0.31/6%</td>
</tr>
<tr>
<td>Stat induced stat inhibitor-3 (SOCS3)</td>
<td>P97803</td>
<td>0.32/14%</td>
</tr>
<tr>
<td>Interleukin-1 receptor, type II precursor (IL1R2 or IL1RB)</td>
<td>P27931</td>
<td>0.33/28%</td>
</tr>
<tr>
<td>BCL2/Adenovirus E1B 19-KDA Protein-interacting protein 3 (BNIP3 or IP3)</td>
<td>O55003</td>
<td>0.33/8%</td>
</tr>
<tr>
<td>Duffy antigen/chemokine receptor (FY or GPD or DARC)</td>
<td>O35970</td>
<td>3.51/4%</td>
</tr>
<tr>
<td>Microsomal glutathione s-transferase 3 (MGST3)</td>
<td>Q9CPU4</td>
<td>3.54/6%</td>
</tr>
<tr>
<td>Interferon regulatory factor 7 (IRF-7)</td>
<td>P70434</td>
<td>4.25/12%</td>
</tr>
<tr>
<td>Macrophage metalloelastase precursor (MMP12 OR HME)</td>
<td>P34960</td>
<td>6.65/63%</td>
</tr>
<tr>
<td>Membrane associated protein 17 KDA (MAP17)</td>
<td>Q9CQH0</td>
<td>6.72/3%</td>
</tr>
</tbody>
</table>
3.6 Effect of vaccination on gene expression in liver

Vaccination induced also some changes in the expression of immunorelevant genes of the liver as evidenced by cDNA-microarrays. Only 4 genes out of 1200 genes are down-regulated more than 3-fold, whereas 5 genes are upregulated 3-fold or more in iG vaccinated female Balb/c mice in comparison to non-vaccinated mice. The down-regulated genes are C-FOS, ETS2, IL1R1, IL1R2, and upregulated are MGST3, HSP105, MAP17, FOXA2 and ELA2 (Table 6 and Fig. 18).

Figure 18. Effects of vaccine-treatment on gene expression of livers of female Balb/c mice on day 11 after infection with *P. chabaudi*. Scatterplots represents normalized fluorescence intensities. Differential regulation of genes increases with their distance to the diagonal of the plot.
Table 6. Vaccine-induced changes in liver gene expression of *P. chabaudi* infected female Balb/c mice on day 11.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Swiss Prot.</th>
<th>Ratio/CD (Vaccinated/controls)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proto-oncogene protein (FOS) P55-C-FOS</td>
<td>P01101</td>
<td>0.49 / 13%</td>
</tr>
<tr>
<td>ETS2 : C-ETS-2 protein (ETS2)</td>
<td>P15037</td>
<td>0.65 / 13%</td>
</tr>
<tr>
<td>Interleukin-1 receptor, type I precursor (IL-1R-1) (IL-1R-ALPHA) (P80)</td>
<td>P13504</td>
<td>0.74 / 14%</td>
</tr>
<tr>
<td>Interleukin-1 receptor, type II precursor (IL-1R-2)</td>
<td>P27931</td>
<td>0.33 / 28%</td>
</tr>
<tr>
<td>Interleukin-1 alpha receptor (IL-1 ALPHA)</td>
<td>P01582</td>
<td>0.77 / 11%</td>
</tr>
<tr>
<td>Microsomal glutathione s-transferase 3 (MGST3)</td>
<td>Q9CPU4</td>
<td>3.03 / 5%</td>
</tr>
<tr>
<td>Microsomal glutathione s-transferase 3 (MGST3)</td>
<td>Q9D834</td>
<td></td>
</tr>
<tr>
<td>Heat-shock protein 105 KDA (HSP105 or HSP110 or KIAA0201)</td>
<td>Q61699</td>
<td>3.03 / 7%</td>
</tr>
<tr>
<td>Heat-shock protein 105 KDA (HSP105 or HSP110 or KIAA0201)</td>
<td>Q62578</td>
<td></td>
</tr>
<tr>
<td>Heat-shock protein 105 KDA (HSP105 or HSP110 or KIAA0201)</td>
<td>Q62579</td>
<td></td>
</tr>
<tr>
<td>Membrane associated protein (MAP17) 17 KDA</td>
<td>Q9CQH0</td>
<td>3.70 / 5%</td>
</tr>
<tr>
<td>Hepatocyte nuclear factor 3-Beta (FOXA2 or HNF-3B)</td>
<td>Q60602</td>
<td>3.70 / 9%</td>
</tr>
<tr>
<td>Hepatocyte nuclear factor 3-Beta (FOXA2 or HNF-3B)</td>
<td>P35583</td>
<td></td>
</tr>
<tr>
<td>NELASTASE: (ELA2) Leukocyte elastase precursor (NEUTROPHIL ELASTASE) (PMN ELASTASE) (BONE MARROW SERINE PROTEASE) (MEDULLASIN).</td>
<td>Q61515</td>
<td>5.00 / 5%</td>
</tr>
</tbody>
</table>
4 DISCUSSION

For more than 20 years intense efforts have been undertaken to develop a vaccine against malaria (Norman et al., 2001), but a safe and effective vaccine is not yet available. One reason is that we still know too little about those immune mechanisms which ultimately confer immunity to malaria and which have to be activated by a vaccine to mediate long-term protection. Indeed, natural immunity to malaria underlies rather complex control: It is directed against the blood stages of the *Plasmodium* parasites and it is never solid, it mitigates and can even completely abolish disease symptoms, but it does not prevent blood stage infections during malaria seasons (Doolan et al., 2003). One of the major aims of an anti-malaria vaccine should be therefore to active and strengthen those effector arms of the host defense which target and destroy the blood stages (Sedegah et al., 1994).

This study demonstrates that self-healing infections with blood stages of *P. chabaudi* malaria induce specific gating mechanisms in both spleen and liver, which coincides with a differentiation of both organs to anti-malaria effectors.

4.1 The spleen as an effector against *P. chabaudi* malaria infections

The spleen is accepted as the major effector of the immune defense against blood stage malaria (Chotivanich et al., 2002). Spleen macrophages eliminate *Plasmodium*-infected erythrocytes via phagocytosis and initiate adaptive protective immune responses, which occur predominantly in unique extravascular pathways (Bernard et al., 1985; Weiss, 1990; Weiss, 1991; Yadava et al., 1996; Chotivanich et al., 2000). There are two phagocytically active regions in the spleen: the marginal zone and the reticular meshwork of the red pulp (Groom et al., 1991; Zhang et al., 1999; Pagola et al., 2000). The latter is responsible for erythrophagocytosis and contains extravascular beds, i.e. arterioles open directly into the reticular meshwork through which blood cells are squeezed before reaching the collecting veins. This open circulation switches to a closed state during acute malaria, presumably controlled by malaria-activated barrier cells. At the beginning crisis, however, this extravascular pathway is described to reopen with much higher effectivity to remove particulate material from the circulation (Weiss, 1991; Alves et al., 1996).
The malaria-induced gating in the spleen becomes evident in *P. yoelii* and *P. chabaudi adami* infected mice as a dramatic drop in the uptake of polystyrol particles during precrisis, and a rapid reopening at the beginning of crisis (Weiss, 1989; Weiss *et al*., 1989; Alves *et al*., 1996). Yadava *et al.* (1996) could show that *Plasmodium chabaudi adami*-infected erythrocytes accumulate in the red pulp, however these authors were unable to measure reduced uptake during precrisis. Here reduced uptake was shown to begin in precrisis in *P. chabaudi*-infected mice and to become maximal at peak parasitemia. However, in contrast to previous studies, no rapid reopening could be observed during crisis. Reopening was rather slow and even on day 21 only 64% of the trapping capacity on day 0 was measured. These differences might reflect differences in parasite virulence. The strong and only slowly reversible drop in filtration capacity might be due to the higher parasitemia evoked by *P. chabaudi* in comparison with *P. yoelii* 17XNL (Weiss *et al*., 1989; Weiss, 1989) and *P. chabaudi adami* (Yadava *et al*., 1996; Alves *et al*., 1996).

### 4.2 The liver as an effector against *P. chabaudi* malaria infections

The liver plays a central role in malaria and it is another important site of the reticular endothelial system (RES). Furthermore, it plays a key role in the pre-erythrocytic development of parasites (Cohen and Lambert, 1982; Miller *et al*., 2002). There is increasing information available that the liver is not only the site of preerythrocytic development of *Plasmodium* parasites but it is also an important effector of immunity against malarial blood stages. The reticular endothelial system (RES) of the liver is able to eliminate parasite-derived hemozoin and even *Plasmodium*-infected erythrocytes through active phagocytosis (Akiwa *et al*., 1980). However, the RES of the liver, though not exhibiting any extravascular beds like those in the spleen, may be also able to phagocytose pRBC, in particular the intravascular Kupffer cells, which constitute about 80%-90% of all resident macrophages of the body (Sullivan *et al*., 1996; Levesque *et al*., 1999). Only recently, there is awakening an increased attention to the liver as an effector against erythrocytic malaria. Several authors have shown that the liver is capable of generating specific populations of lymphocytes mediating novel protective immune mechanisms against blood stage malaria (Miura *et al*., 1991; Blamer *et al*., 2000; Mannoor *et al*., 2002; Dharmeshkumer *et al*., 2003).
Discussion

Fluorescence microscopy of liver sections reveals that the injected beads are predominantly found in periportal areas of the liver acinus and the density decreases rapidly from the periportal to pericentral areas indicating that beads are rapidly trapped after entering the sinusoids from the portal vein or hepatic artery. Presumably, only a few beads entering the liver will reach the central vein (Jungermann and Kietzmann 1996; Paxian et al., 2004). There are differences between periportal and pericentral Kupffer cells, and periportal Kupffer cells are the main phagocytic cells in the liver. Though the RES of both liver and spleen may be critical for the outcome of blood stage malaria, these sites have never been investigated as to whether protective vaccination increases, for example, their trapping capacity.

4.3 Effects of protective vaccination on the spleen and liver

A vaccination procedure of high efficacy has been developed for the murine malaria Plasmodium chabaudi. This method uses erythrocyte ghosts isolated from pRBC as an immunogen and converts mice from a non-healer to a self-healer phenotype, i.e. it prevents mortality, but it does not prevent propagation of parasitemia (Wunderlich et al., 1988b; Wunderlich et al., 1993). This situation basically resembles that of natural immunity in humans and it is therefore appropriate for investigations about mechanisms conferring vaccination-induced immunity. Here, this vaccination procedure was analysed for its possible effects on particle trapping capacity of the RES of the spleen and liver.

Mice were vaccinated against blood stages of P. chabaudi malaria, thus making susceptible Balb/c mice resistant to homologous challenge. Normally, self-healing is characterized not only by overcoming the infection, but also by developing solid protective immunity, a process controlled by genes of both H-2 complex and non-H-2 background. The susceptible Balb/c mice exhibit a resistant H-2 haplotype, but a non-H-2 background that confers susceptibility (Wunderlich et al., 1988b; 1993). The vaccination procedure overcomes genetic restrictions of susceptibility. The mechanisms underlying this model vaccination should therefore also be of significant relevance for the design of a human anti-malaria vaccine (Pink and Singaglia, 1989; Quakyi et al., 1989; Patarroyo et al., 1991).
A major effect of vaccination occurs on spleen size. Spleen weight doubles by infection in comparison to non-infected mice. However, vaccination induces a stronger splenomegaly leading to a spleen weight twice as high as that of non-vaccinated controls at peak parasitemia. This is reminiscent to hypersplenomegaly in humans. Furthermore, the percent portion of Gr-1$^{+}$ granulocytes doubles compared with non-vaccinated controls. However, this increase in spleen size and number of splenic granulocytes does not result in destruction of infected erythrocytes as can be deduced from the following findings: First, peak parasitemia is not reduced in vaccinated mice, indicating that parasite growth is not impaired. Second, the phagocytic activity of the RES in the spleen is shutdown at peak parasitemia. The spleen remains closed during the subsequent crisis, when *Plasmodium chabaudi* infected erythrocytes are destroyed as evidenced by extremely falling parasitemias.

During crisis expression of only a few genes is changed out of 1076 immuno-relevant genes analysed. These genes may have therefore something to do with increase in size rather than spleen closing since closing also occurs in succumbing mice. For instance, the 5-fold overexpression of N-elastase in vaccinated mice directly relates to increased numbers of granulocytes since N-elastase is specifically transcribed in neutrophiles.

The liver is also affected by vaccination. There is a dramatic increase in specific trapping capacity during crisis. This suggests that the liver can function as highly effective parasite-eliminator when the spleen is closed thus causing the drop in peripheral parasitemia. In contrast to spleen, gene expression changes observed in liver have something to do with host defense directly and/or indirectly. Changes observed in gene expression during crisis might therefore signal changes responsible for increased effector function of liver.

### 4.4 Effect of adoptive transfer of liver and spleen cells on recipient mice.

Adoptive transfer of $5 \times 10^5$ liver cells from vaccinated mice into recipient mice on day 4 and day 8 of infection induced a significant increase in survival time ($p<0.001$), and conferred resistance to some of the recipient non-vaccinated mice against challenge with *P. chabaudi* malaria. Moreover, adoptive transfer of $5 \times 10^5$ liver cells from immune mice to
recipient mice on day 4 of infection and $5\times10^7$ spleen cells on day 8 of infection induced a significant increase ($p<0.001$) in survival time and survival rate of the recipient mice. Adoptive transfer of $5\times10^5$ liver cells from immune mice into recipient mice on day 4 of infection caused an increase ($p<0.06$) in survival time. Adoptive transfer of $5\times10^7$ spleen cells from immune mice into recipient non-immune infected mice on day 0 does not help recipient mice to develop immunity against *P. chabaudi* under certain experimental conditions (Favilia-Castillo *et al.*, 1990), and did not prevent a fatal outcome of the infection for all recipient mice.

Moreover, the adoptive transfer experiments demonstrate that the liver has higher relevance for immunity against *Plasmodium chabaudi* malaria than the spleen. Similar to spleen cells, liver cells can confer immunity to *P. chabaudi* infections but do not lead to a dramatic reduction in parasitaemias as observed in immune mice. Whatever the reason may be, this indicates at least that the protective immunity transferred by spleen cells is not mainly directed against the parasites (Playfair *et al.*, 1990).

### 4.5 Impact on human vaccine development

The results of this study may also be relevant for design of a human anti-malaria vaccine. It is rather likely that the liver has higher relevance for elimination of *Plasmodium*-infected erythrocytes than the spleen. The vaccine should therefore contain such components which increase liver capacity to trap and destroy *Plasmodium*-infected erythrocytes (Cohen and Lambert, 1982). It is therefore necessary to further explore those factors and mechanisms which cause increase in trapping capacity of the liver in order to include components in a vaccine which activate these liver mechanisms.

Collectively, the presented data demonstrate that both spleen and liver are important for survival of a primary malaria infection as well as for maintenance of acquired immunity against rechallenge infections. This immunity involves distinct changes in gene expression and microarchitecture of spleen and liver.
Resistance to *Plasmodium chabaudi* blood stage malaria is controlled by genes of the *H-2* complex and the non-*H-2* background. Balb/c mice exhibit the resistant *H-2*<sup>d</sup> haplotype, but they are highly susceptible due to the susceptible non-*H-2* background. However, vaccination of female Balb/c mice with plasma membranes isolated from infected erythrocytes leads to conversion of the phenotype, and the mice gain the ability to self-heal the infection. Vaccination has no effect on peak parasitemia and non-vaccinated mice usually die in the crisis phase when parasitemia is falling again. The present study was aimed at identification of protective responses in spleen and liver that are activated by vaccination.

Vaccination alone has no effect on spleen size. However, an obvious effect of vaccination on spleen size during infection can be observed. Control mice display a twofold increase in spleen size at peak parasitemia, but there is a fourfold increase after vaccination. Furthermore, the number of Gr1<sup>+</sup> granulocytes is twice as high in vaccinated mice even before challenge, and this increased number of granulocytes is maintained throughout infection.

Conspicuously, the increase in spleen size and in the number of phagocytic cells which can be observed during infection does not lead to increased phagocytic activity in the spleen. By contrast, at peak parasitemia and throughout crisis trapping capacity of the spleen for 3 µm fluorescent polystyrol particles is severely impaired. Vaccination does not influence this closing of the spleen. Similar to the spleen, the liver displays partial closing around peak parasitemia. However, in vaccinated mice a rapid reopening of the liver occurs leading to a much higher trapping capacity than in non-vaccinated controls on day 11 post infection.

cDNA arrays containing 1076 different genes were hybridized to cDNAs of vaccinated and non-vaccinated mice on day 11 post infection. Surprisingly, only a few differentially expressed genes could be identified in spleen and liver. Furthermore, increased expression of at least some of these genes might be the result of differences in spleen cell populations. For instance, the fivefold overexpression of N-elastase in the spleen in vaccinated mice directly correlates with the higher proportion of granulocytes in the spleen of vaccinated mice since N-elastase is specifically transcribed in neutrophils. Therefore, only a minor impact of vaccination on the transcriptional response in spleen and liver could be found.

A very important role of the liver in the development of protective immunity against *P. chabaudi* malaria was demonstrated in adoptive transfer experiments. Total spleen cells and mononuclear cells isolated from the liver of vaccinated or immune mice were transferred to naive mice before challenge. Although 100 times lower amounts of liver cells than spleen cells were used in these experiments, liver cells were more effective in increasing both survival rate and survival time. Furthermore, transfer of cells from vaccinated mice was more effective than transfer of cells from immune mice.

The importance of the liver for the development of immunity against blood stage malaria is mostly underestimated. However, the results of this study emphasize that the phagocytic system as well as lymphoid cells in the liver might be very important effectors against blood stage malaria. Protective vaccination against malaria preferentially activated immune mechanisms in the liver rather than in the spleen indicating that such unconventional immunological mechanisms should also be considered for design of a vaccine for use in humans against *Plasmodium falciparum*.
ZUSAMMENFASSUNG


7 REFERENCES


References


ABBREVIATIONS

7-AAD 7 amino-actinomycin D
AP  Alkaline phosphatase
A.T.  Adoptive transfer
ATF  activating transcription factor
ATP  Adenosine triphosphate
Bp  base pair (s)
BM  bone marrow
BSA  bovine serum albumin
cAMP  cyclic adenosine 3’, 5’,-monophosphate
CPG  citrate phosphate glucose
cpm  counts per minute
CSP  Circumsporozoite protein
DC  dendritic cells
DEPC  diethyl pyrocarbonate
DMSO  dimethylsulphoxide
DNA  deoxyribonucleic acid
DUSP  dual specificity protein phosphatase
ECM  Extracellular matrix
EDTA  ethylene diaminetetraacetic acid
FACS  fluorescence activated cell scanner
FCA  Freund’s complete adjuvant
FCS  fetal calf serum
FDC  Follicular dendritic cell
FITC  fluorescein isothiocyanate
<table>
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<th>Description</th>
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<tbody>
<tr>
<td>FRA</td>
<td>fos-related antigen</td>
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<tr>
<td>GPI</td>
<td>glycosylphosphatidyl-inositol</td>
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<tr>
<td>GC</td>
<td>Germinal center</td>
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<tr>
<td>Gr-1⁺</td>
<td>Granulocytes</td>
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<tr>
<td>HEPES</td>
<td>N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid</td>
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<tr>
<td>HMS</td>
<td>hyperreactive malarial splenomegaly</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IM</td>
<td>Intracellular medium</td>
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<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
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<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
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<tr>
<td>iG</td>
<td>Infected ghosts</td>
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<tr>
<td>IgG</td>
<td>immunoglobuline G</td>
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<tr>
<td>ICAM-1</td>
<td>intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>IGFBP</td>
<td>IGF-binding protein</td>
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<tr>
<td>IL2RB</td>
<td>interleukin 2 receptor B</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MM</td>
<td>metallophilic macrophages</td>
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<tr>
<td>MMP</td>
<td>matrix metalloprotease</td>
</tr>
<tr>
<td>MZ</td>
<td>Marginal zone</td>
</tr>
<tr>
<td>MZM</td>
<td>marginal zone macrophages</td>
</tr>
<tr>
<td>INOS2</td>
<td>Inducible nitric oxide synthase 2</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PALS</td>
<td>Periarteriolar lymphoid sheath</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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### Abbreviations

<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>PBS-HG</td>
<td>Phosphate buffer saline glucose monohydrate</td>
</tr>
<tr>
<td>PEs</td>
<td>Parasitized erythrocytes</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>p.i.</td>
<td>post infection</td>
</tr>
<tr>
<td>PIQOR™</td>
<td>Parallel identification and quantification of RNAs</td>
</tr>
<tr>
<td>pfEMP1</td>
<td><em>Plasmodium falciparum</em> erythrocyte membrane protein 1</td>
</tr>
<tr>
<td>RBCs</td>
<td>red blood cells</td>
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<tr>
<td>RESA</td>
<td>ring-infected erythrocyte surface antigen</td>
</tr>
<tr>
<td>RES</td>
<td>reticular endothelial system</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>RNase</td>
<td>ribonuclease</td>
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<tr>
<td>rpm</td>
<td>rounds per minute</td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
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<tr>
<td>T</td>
<td>testosterone</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-base EDTA</td>
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<tr>
<td>T&lt;sub&gt;H&lt;/sub&gt; cells</td>
<td>T helper cells</td>
</tr>
<tr>
<td>TBVs</td>
<td>Transmission-blocking vaccines</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TEMED</td>
<td>(N,N,N',N'-)tetramethylethylenediamine</td>
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<tr>
<td>TIMP</td>
<td>tissue inhibitor of MMP</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>Tris</td>
<td>Tris-(hydroxylmethyl) aminomethane</td>
</tr>
<tr>
<td>U</td>
<td>unit(s)</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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