Spleen and liver as effectors against blood stages of *Plasmodium chabaudi* malaria in lymphotoxin β receptor-deficient mice

Inaugural-Dissertation
zur
Erlangung des Doktorgrades der
Mathematisch-Naturwissenschaftlichen Fakultät
der Heinrich-Heine-Universität Düsseldorf

vorgelegt von

Mohamed Abdel-Monem Mohamed Dkhil Hamad

aus Giza, Helwan University, Ägypten

Düsseldorf
2004

Gedruckt mit Genehmigung der
Mathematisch-Naturwissenschaftlichen Fakultät
der Heinrich-Heine-Universität Düsseldorf.

Referent: Prof. Dr. F. Wunderlich
Koreferent: Prof. Dr. H. Mehlhorn

Tag der prüfung: 13.05.2004

TO MY FAMILY

CONTENTS

1 INTRODUCTION

1.1 History of malaria

Malaria was recognized as a human disease more than 5000 years ago. Enlarged spleens, presumably due to malaria, have been found in Egyptian mummies more than 3000 years old, and the Ebers Papyrus (*c.* 1570 bc) mentions splenomegaly, fevers and a variety of cures for such ailments (Sherman, 1999).

The identification of malaria parasites in the blood was made by Charles Louis Laveran (1845–1922). In 1880, while serving as a physician in the army of Napoleon in North Africa, Laveran examined microscopically the blood of soldiers suffering from intermittent fevers and noticed crescent-shaped bodies that were clear except for some pigment granules (gametocytes); he also observed transparent, mobile filaments emerging from clear spherical bodies (exflagellation of microgametes) (Sherman, 1999). Some 6 years later, Camillo Golgi (1843–1926), using thin smears of fresh blood, discovered the asexual development and multiplication by schizogony for *Plasmodium malaria* and *P. vivax* and he showed that the beginning of fever in malaria coincided with the rupture of the erythrocyte and liberation of the merozoites (Kean *et al*., 1978).

Ronald Ross (1857–1932), a Surgeon-Major in the British Indian Medical Service, definitively demonstrated the mosquito transmission of *Plasmodium*. That day, while examining the stomach of an *Anopheles* mosquito that had fed 4 days earlier on human subjects with crescents (gametocytes) in their blood, he saw a clear and almost circular outline and within it a cluster of malaria pigment. Ross recognized he had discovered the oocyst. By June of 1898 he was able to see sporozoites developing in the oocysts, and later he found the sporozoites in the mosquito salivary glands (Bynum, 1999).

In 1948, H. E. Shortt, P. C. C. Garnham and their collaborators in England found malaria parasites developing in the livers of monkeys that had been infected with sporozoites from a primate malaria, and later similar stages were described in biopsy samples taken from the livers of human volunteers infected either by the bite of infected mosquitoes or after intravenous injection of sporozoites dissected from the salivary glands of mosquitoes infected

with *Plasmodium vivax* or *Plasmodium falciparum*. Primate and human malarias have a single pre-erythrocytic cycle; however, among those species that cause relapsing malaria (*Plasmodium vivax* and *Plasmodium ovale*), persistent 'resting parasites' called hypnozoites have been shown to be present in the liver, and when 'reactivated' they are presumed to result in relapse (Sherman, 1999).

1.2 Geographical distribution of malaria and populations at risk

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 areas where malaria occurs, 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 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.

1.3 The life cycle of the malaria parasite in mammals

Briefly, the life cycle is as follows (Fig. 1, P. 8). Sporozoites are released from the female mosquito's salivary glands, in her saliva, into the circulating blood of the host and within 3 to 45 min they have entered hepatocytes (Rosenberg *et al*., 1990). It is neither known, how sporozoites squeeze through the sinusoid lining into the space of Disse or, as might be the case, pass through Kupffer cells on the walls of the sinusoids (Vreden, 1994) to reach the

hepatocytes nor what the precise nature of the sporozoite-hepatocyte ligand-receptor interactions which enables the parasite to recognize the host cell. Peptides forming part of the major surface protein on the sporozoite, the circumsporozoite protein (CSP), have been suggested to interact with receptors on the hepatocyte (Cerami *et al*., 1992; Sinnis and Sim, 1997). Growth and division in the liver for the human malaria parasites take approximately 6 to 15 days depending on the species: approximately 6, 10, and 15 days for *P. falciparum*, *P. vivax*, and *P. ovale* and *P. malariae*, respectively. At the end of the pre-erythrocytic cycle, thousands of merozoites are released into the blood flowing through the sinusoids and, within 15 to 20 s, attach to and invade erythrocytes. Recognition and attachment are via a receptor-ligand interaction, and at least for *P. vivax* and *P. falciparum*, the host and parasite molecules involved are different (Galinski and Barnwell, 1996). In *P. vivax* and *P. ovale*, some of the sporozoites appear to develop for about 24 h before becoming dormant as a hypnozoite stage; this form can remain as such for months and even years until reactivated to complete the liver cycle, releasing merozoites into the blood to initiate a relapse infection. The asexual erythrocytic cycle produces more merozoites that are released with the destruction of the red blood cell after 48 or 72 h for the human malaria parasites, depending on the species. These merozoites immediately invade new erythrocytes. The asexual cycle usually continues until controlled by the immune response or chemotherapy or until the patient dies (in the case of *P. falciparum*). Most malaria parasites developing in the host's red blood cells grow in synchrony with one another, for at least some animal species apparently tuning into the host's circadian rhythms (Hawking *et al*. 1968). Consequently, they complete schizogony together at the end of the asexual cycle, releasing pyrogenic materials which induce the characteristic fever spike and clinical symptoms.

After invading red blood cells, eventually some merozoites differentiate into sexual forms (gametocytes) and, following ingestion by another female mosquito, will mature to male and female gametes in the blood meal. After fertilization, the resulting zygote matures within 24 h to the motile ookinete, which burrows through the midgut wall to encyst on the basal lamina, the extracellular matrix layer separating the hemocoel from the midgut. Within the developing oocysts, there are many mitotic divisions resulting in oocysts full of sporozoites. Rupture of the oocysts releases the sporozoites, which migrate through the hemocoel to the salivary glands to complete the cycle approximately 7 to 18 days after gametocyte ingestion, depending on host-parasite combination and external environmental conditions. All stages in

the life cycle are haploid, apart from the diploid zygote, which immediately after fertilization undergoes a two-step meiotic division, the resulting cell containing a nucleus with four haploid genomes (Billingsley and Sinden, 1997). The sexual process and meiotic division following fertilization allow genetic recombination to occur, which is reflected in the genetic makeup of the sporozoites and together with mutations provides the raw material upon which selective pressures such as antimalarial drugs can work.

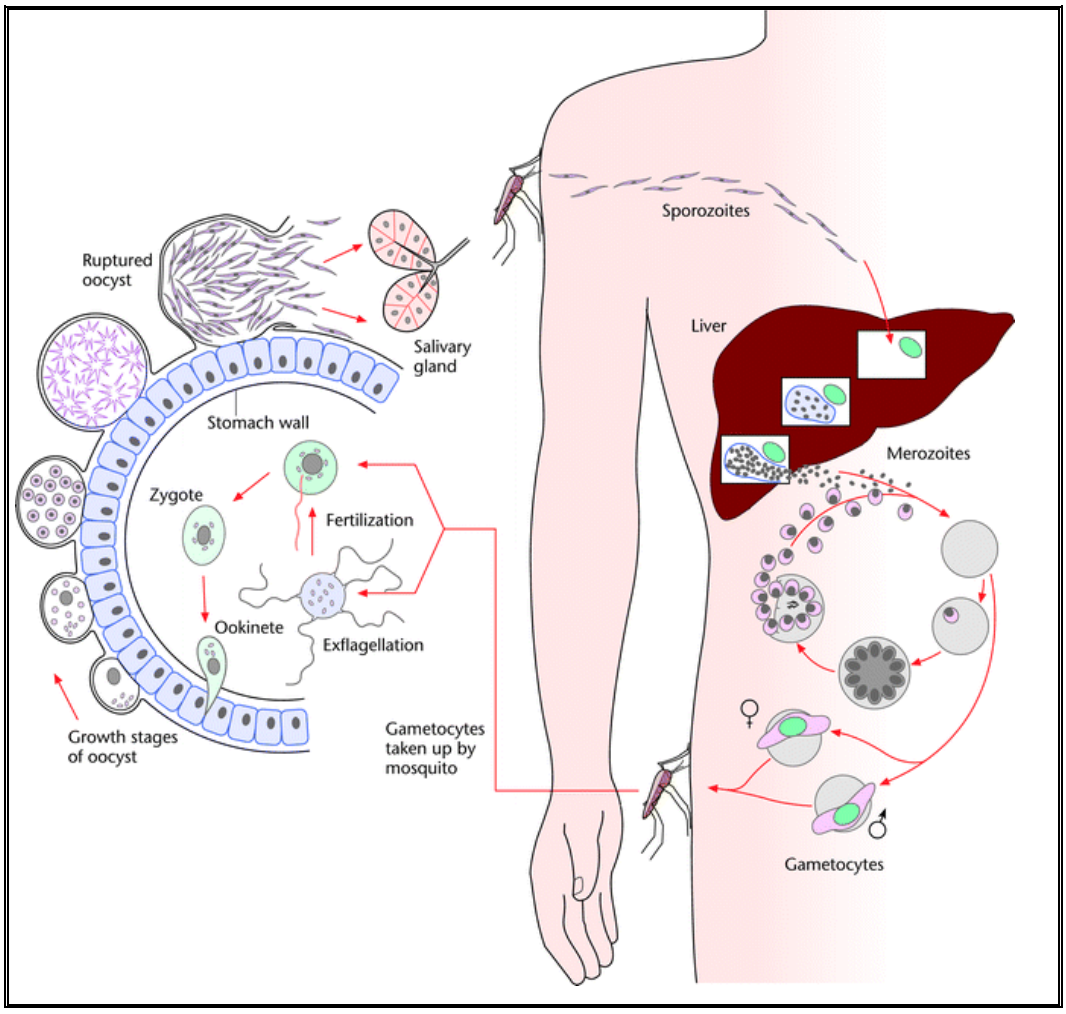


Figure 1. The life cycle of *Plasmodium* sp. (Sherman, 1999)

1.4 Malaria pathology

Malaria, an infectious disease frequently associated with fever and anaemia, is caused by various species of *Plasmodium* that ultimately destroy the red blood cells in which they grow

and reproduce. *Plasmodium* species infect mammals, birds and lizards, and are transmitted by the bite of female *Anopheles* (mammals) or *Culex* (birds, lizards) mosquitoes (Paul and Brey, 2003). Mammalian malarias are confined to antelopes, lemurs, bats, rodents and primates, including humans. There are four species of *Plasmodium* (*P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*) pathogenic for humans. Most disease and death is due to the malignant tertian malaria, *P. falciparum*, although the benign tertian malaria, *P. vivax*, is an important pathogen in parts of Asia and Latin America. The World Health Organization (WHO) estimates that there are more than 4 billion people at risk of developing malaria each year, there are up to 500 million cases, and 1.5–2.7 million deaths annually (WHO, 2000). This averages to one person dying of malaria every 12 seconds. At present over 90% of the deaths occur within the continent of Africa (Sherman, 1999).

All the pathology of malaria is due to the asexual blood stage parasites; the hepatic (pre-erythrocytic) and sexual stages (gametocytes) cause no organ dysfunction (Mehlhorn, 2001). Pathology results from the destruction of erythrocytes, the liberation of parasite and red blood cell material into the circulation, and the host's reaction to these. *P. falciparum*-infected erythrocytes sequester: they attach to the walls of the capillaries and postcapillary venules of vital organs. Attachment is via knobby protuberances, visible by electron microscopy, and the presence of knobs on the outer surface of erythrocytes containing the more mature and haemozoin-containing stages of *P. falciparum* is correlated with sequestration. Knobs contain proteins that both promote infected red cell adhesion and contribute to antigenic variation. Sequestration of infected cells interferes with blood flow and impairs tissue function (Mehlhorn, 2001).

Chills, fever, anaemia, renal failure, liver and placental dysfunction, hypoglycaemia and lactic acidosis are consistent features of severe malaria. Although no specific malaria toxin has been found to be associated with disease, parasite substances that induce proinflammatory cytokines have been identified. The rapid and characteristic enlargement of the spleen have been postulated to be associated with an increased capacity to remove parasitized erythrocytes from the circulation (Sherman, 1999; Shah *et al*., 2003). Blackwater fever (the excretion of dark brown–black urine) may occur in patients with severe malaria after being treated with quinine or artemisinin derivatives; how quinine causes this condition is not known. The severity of infection depends on both host and parasite factors. Among the host factors are

genotype, age and previous exposure. Parasite virulence factors associated with severe malaria are multiplication capacity, red cell preference, ability to induce cytokines, adhesivity, antigenicity and antimalarial drug resistance (Kareier 1980; Sherman, 1999).

1.5 Immune response to malaria

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; Kwiatkowski, 1992).

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. 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).

1.5.1 Role of antibodies

In both humans and mice, passive transfer of antibodies from immune individuals to those suffering from acute malaria results in quick and marked reduction of parasitaemia. In addition, infection with *Plasmodium berghei* and *P. yoelii* cannot be controlled in mice from which B cells have been removed by neonatal anti-μ treatment (Grun and Weidanz, 1981). The elimination of parasites is also impaired in mice with targeted gene deletions resulting in B-cell deficiency (Van der Heyde *et al*., 1994; Von der Weid *et al*., 1996). While immunoglobulin (Ig) G2a is essential in the mouse model (White *et al*., 1991), IgG1 and IgG3 (Groux and Gysin, 1990) appear to be most effective in humans.

IgG1 and IgG3 in humans and IgG2a in mice are cytophilic immunoglobulin isotypes that are able to promote activation of monocytes and macrophages via Fc receptors. The antibody-

dependent killing of parasites *in vitro* is dependent on the presence of either mouse neutrophils or human monocytes. One mechanism possibly involved in this antibody activity is the induction of TNF-α secretion by monocytes, leading to growth inhibition of intracellular parasites in neighbouring cells (Röllinghoff et *al*., 1999)

1.5.2 Role of T cells

Two stages of the malaria parasite are truly intracellular: 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 (Hoffman *et al*., 1996).

The central role of CD4+ T cells in the protective immunity against the 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.

In *Plasmodium chabaudi*-infected mice both T_H1-type and T_H2-type CD4 T cells are involved in protective immunity (Taylor-Robinson *et al*., 1993). However, the relative contribution of these subsets changes during the course of infection: T_H1 cells predominate during the acute phase, and T_H2 cells are found primarily during later phases of infection. The protective effect of transferred T_H1 cells in mice infected with *P. chabaudi chabaudi* can be blocked by inhibitors of inducible nitric oxide synthase (iNOS), whereas resistance conferred by T_H2 cells is not affected (Taylor-Robinson *et al*., 1993). Even in the case of T_H1 cells, there appear also to be nitric oxide-independent mechanisms of protection, as shown in *P. yoelii*-infected mice (Amante and Good, 1997). Protective T_H2 cell clones specific for *P.*

chabaudi chabaudi drive a strong protective malaria-specific IgG1 response *in vivo* which is promoted by interleukin (IL) 4 (Amante and Good, 1997).

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

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 periarteriolar lymphoid sheath or PALS) and the B cells distributed around the T zone in tightly packed follicles (Fig. 2). The spleen contains an additional population of B cells in a compartment that surrounds the follicles, known as the marginal zone (Kraal, 1992). Antigen-presenting dendritic cells (DCs) are prevalent in marginal zones, T cell zones, and in the bridging channels between these two compartments (Steinman *et al*., 1997). The T zones, follicles, and marginal zones 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), 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).

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).

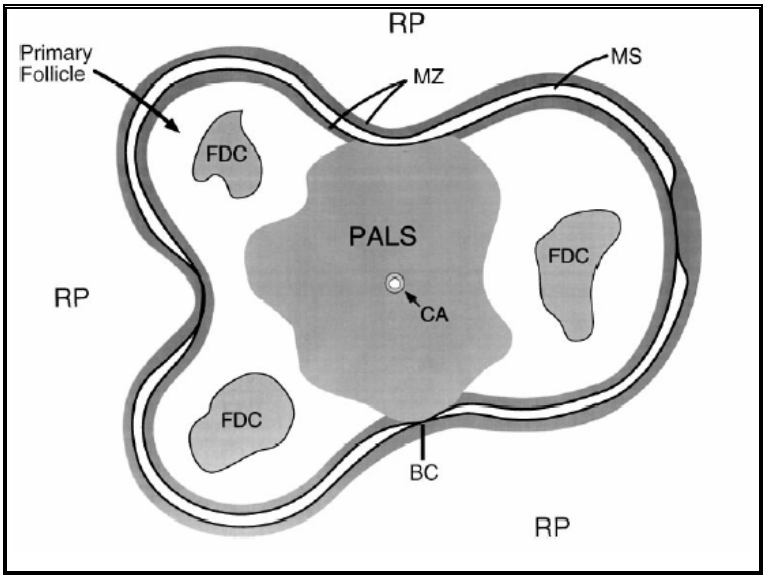


Figure 2. Structure of the spleen white pulp nodule. The white pulp nodule is separated into a central T cell–rich zone [periarteriolar lymphoid sheath (PALS)] surrounded by B cell–rich primary follicles. Within each primary follicle is a cluster of follicular dendritic cells (FDC). The white pulp nodule is separated from the red pulp (RP) by the marginal sinus (MS). The MS is embedded in a layer of marginal zone (MZ) lymphocytes. Also adjacent to the MS near to the red pulp a layer of marginal zone macrophage and near to the white pulp is a layer of marginal metallophilic macrophages that are thought to be important to regulate antigen trafficking into the red and white pulp spaces. The bridging channels (BC) are thought to represent areas by which lymphocytes enter and leave the white pulp. CA, central arteriole (Yang-Xin and Chaplin, 1999).

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). 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 and Weiss, 1989). However, recent studies suggest that alterations in the cellular composition of the spleen during infection may be of prime importance (Yadava *et al*., 1996). 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 a 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). The role of the spleen in the effector phase of parasite killing was demonstrated by the finding that splenectomy of *P. chabaudi adami* mice during crisis, when the parasitemia was rapidly falling, resulted in increased parasitemia, which was eventually cleared much later than in normal or sham-splenectomized animals (Yadava *et al*., 1996). The role of the spleen in resistance to infection with *P. yoelii* 17XNL appears to be dependent upon the genotype of the host (Sayles *et al*., 1991). A compensatory increase in IFN-γ production by lymph node cells of splenectomized resistant mice may be responsible for the lack of effect of splenectomy on the outcome of *P. yoelii* infection in these hosts (Hauda *et al*., 1993). Solid protection against P. *berghei* infection was achieved in naive rats following transplantation of spleens from immune rats (Favila-Castillo *et al*., 1996). Interestingly, IFN-γ was detected in the sera of rats receiving immune spleens but not in rats receiving spleens from naive animals (Smith *et al*., 1997).

Grun and others showed that acute infection with *P. chabaudi adami* in splenectomized mice persisted with significant parasitemia for a period lasting for several months post infection before resolution occured, whereas acute infections in sham-operated mice were cleared within three weeks (Grun *et a*l., 1985). Splenectomy of immune mice results in loss of immunity (Kuramar and Miller, 1990).

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*., 2000). 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).

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 (Fig. 3), 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 (Fig. 3).

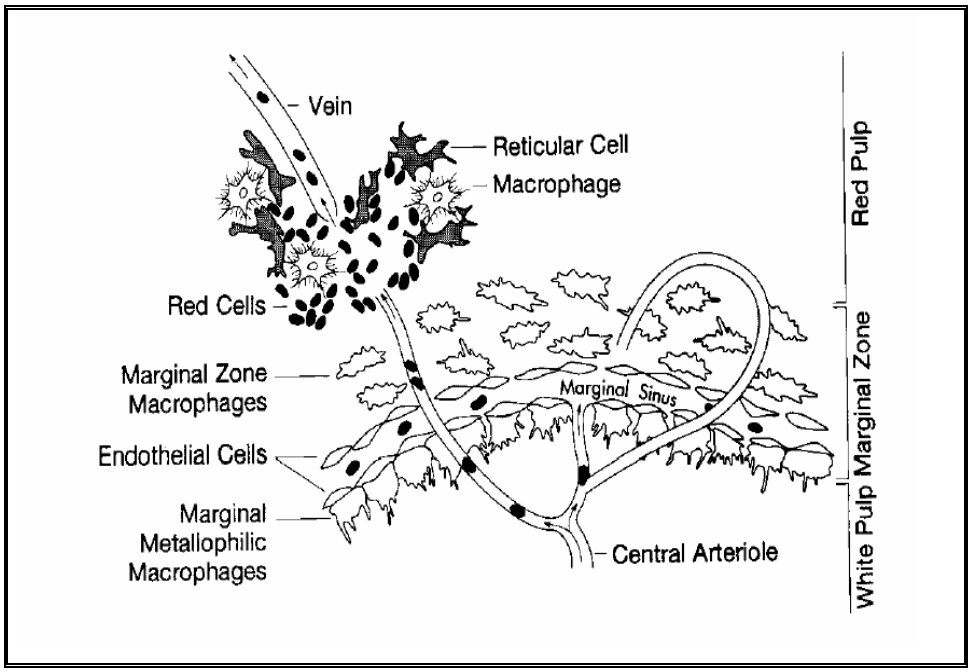


Figure 3. Schematic representation of the mouse spleen showing the marginal zone and red pulp areas. Blood flows from the central arteriole into the marginal sinus, marginal zone, or red pulp, where it comes into contact with macrophages and other immune effector cells before flowing into the veins (Yadava *et al*., 1996).

1.8 The role of apoptosis in malaria

Apoptosis occurs in various processes in mammalian life (Jacobson *et al*., 1997). It is a natural mechanism of cell death, which involves cell membrane blebbing, cell shrinkage, chromatin condensation, nuclear fragmentation and DNA degradation (Kerr *et al*., 1972). This mechanism, which has been also called programmed cell death, is essential for the homeostasis of the whole organism and has a central role from development to the maintenance of body shape and function. There have been major advances in the understanding of the biochemical events that underlie the apoptotic process (Hengartner,

2000). Apoptosis is triggered by a variety of both internal and external signals. Most of the morphological changes observed in a cell that is undergoing apoptosis are caused by a family of cysteine proteases (Salvesen and Dixit 1997) which are activated in a cascade and are known as caspases. These enzymes have restricted protein targets which are normally inactivated after cleavage by caspases. In some cases, the target of a caspase is an enzyme inhibitor so the final action of the caspases is the activation of the enzyme (Nicholson and Thornberry 1997).

During the course of an immune response, T cell clones responding to the antigen undergo extensive proliferation. When the antigen is eliminated, the number of T cells must be down regulated and this is achieved by inducing apoptosis in the responding T cells, a process termed activation-induced cell death. The apoptotic signal is received by the T cell through a membrane receptor called CD95 or Fas, which is a type I transmembrane receptor. The death signal is provided by the ligand of CD95 (CD95L or FasL), which is expressed on lymphoid cells in the late phases of the immune response. The elimination of responding cells is considered a normal mechanism to turn off an ongoing immune response and to avoid self damage (Van Parijs and Abbas, 1998). Helmby *et al*. (2000) investigated apoptosis in spleen cells of mice infected with *P. chabaudi chabaudi* AS. It was found that the frequency and absolute numbers of apoptotic cells in the spleen increased during primary infection, reached a peak around 4 days following peak parasitemia and then descended to almost normal levels after the parasite was cleared. The findings also demonstrated that apoptosis involves T cells ($CD4^+$ and $CD8^+$), B cells and macrophages and that the majority of apoptotic cells are B cells.

It seems that an increase in apoptosis, in some or most of the lymphoid cells, is a general phenomenon in a variety of infections. In principle, this can be explained as a normal homeostatic response involved in the termination of the immune response induced by the microorganism (Van Parijs and Abbas, 1998). Apoptosis is an important mechanism regulating the development, mutation and activation of lymphocytes. In addition, apoptosis may also prevent and terminate lymphocyte responses. (Krammer *et al*., 1994; Van Parijs and Abbas, 1998).

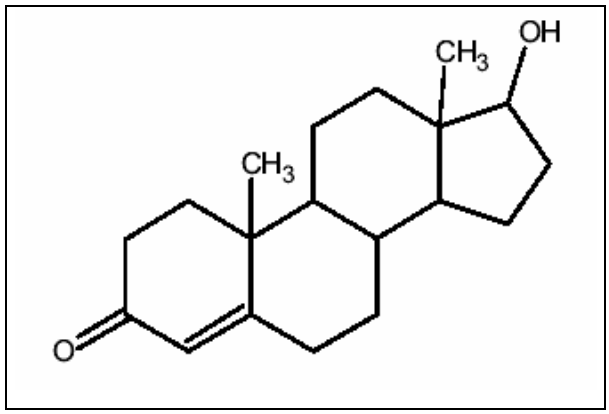
1.9 Lymphotoxin beta receptor deficient mice ($LT\beta R^{-/-}$)

The lymphotoxin-beta receptor (LTßR) is a member of the tumor necrosis factor receptor (TNFR) superfamily and is ubiquitously expressed on the surface of most of cell types, except T and B lymphocytes (Chen *et al*., 2003). It is essential for the genesis of lymph nodes (LN), normal structural development of peripheral lymphoid tissues and the differentiation of natural killer (NK) cells. LTßR binds to the heterotrimeric cytokines $LT\alpha_1\beta_2$ and LIGHT (Kather *et al*., 2003).

$LT\beta R^{-/-}$ mice lack lymph nodes (LN) and Peyer's patches (PP) and show a severe disorganization of splenic architecture, characterized by the absence of T/B cell segregation, marginal zones, follicular dendritic cell (FDC) networks, and germinal centers (GC) (Fütterer *et al*., 1998; Fu and Chaplin, 1999; Ehlers *et al*., 2003).

1.10 Testosterone

The structure of testosterone contains the notable features of a 17-β-OH (hydroxyl) and a 3-keto group which are necessary for its specific activity. Oxidation of the 17-β-OH to a 17-keto group, or conversion to a 17 α-OH, results in steroids with significantly decreased androgen activity. Likewise, a 3-OH instead of a 3-keto group is much less active, partly due to increased metabolism.



Structural formula of testosterone

Gonadal steroids can have a pronounced influence on immune function and disease. One indirect line of evidence for an effect of a gonadal steroid on immune function comes from studies demonstrating a pronounced sexual dimorphism in immune function. In general, females have a stronger immune response and higher immunoglobulin (IgG) concentrations than males (Alexander and Stimson, 1988; Brabin and Brabin, 1992). Likewise, in skin graft rejection studies, female animals reject grafts faster than males, suggesting a more robust immune response. This sex difference in immune function is apparently modulated by gonadal steroids: oestrogens generally enhance, while androgens typically suppress immune function (Henderson *et al*., 1982). In accordance, females are more resistant than males towards a wide variety of viral, bacterial and parasitic infections (Alexander and Stimson, 1988; Brabin and Brabin, 1992).

The primary site of testosterone synthesis is the testes (Leydig cells) with low level synthesis occurring in the adrenal cortex. In the periphery steroids equilibrate rapidly between various organs and blood. This fact can be derived from identical levels of free testosterone in saliva and blood (Wang *et al*., 1981). The total concentration of steroids in the target tissues and body fluids is mainly dependent on the presence of binding proteins such as sex hormone binding globulin (SHBG), and albumin. Binding proteins in the body fluids can act as a storage form of steroids which have a high rate of metabolism during passage of blood through the liver (Mendel, 1989).

The male sex hormone testosterone is an internal environmental factor which is known to enhance immunosupressive activities (Stimson, 1987), thereby promoting different forms of cancer such as hepato and prostate-carcinomas (Henderson *et al*. 1982; Kemp *et al*., 1989), and increasing susceptibilities of vertebrate hosts to a wide variety of parasitic infections (Bundy, 1988). Testosterone makes mice more susceptible to infections with *Plasmodium chabaudi* (Wunderlich *et al*., 1988) and *P. berghei* (Kamis and Ibrahim, 1989). Moreover, there is information available that the testosterone-induced immune suppression is regulated by H-2 linked gene(s) (Ahmed *et al*., 1987; Wunderlich *et al*., 1988), that genes encoding androgen–metabolizing enzymes are linked to H-2 complex (Klein, 1975) and that testosterone levels vary among different mouse strains including H-2 congenic strains (Wunderlich *et al*., 1991).

1.11 Aim of the work

The present study aimed at investigating the outcome of *Plasmodium chabaudi* blood stage malaria in LTβR-deficient mice. In contrast to males, female mice exhibit a robust resistance to blood stages of *Plasmodium chabaudi* malaria with both spleen and liver acting as anti-malaria effectors. This resistance is completely suppressible by testosterone, and the lethal outcome of malaria coincides with distinct, but different changes in gene expression of both spleen and liver, indicating their presumable dysfunction as anti-malaria effectors.

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
Diethyl-pyrocarbonat (DEPC)	Fulka, Neu-Ulm, Germany
Bovine serum albumin (BSA)	Serva, Heidelberg, Germany
Calf serum	Boehringer, Mannheim, Germany
Chloramphenicol spray (colourless)	A. Albrecht, Aulendorf, 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 Suspention, 3 μm in diameter (Green)	Ducke Scientific Corporation, Palo Alto, CA, USA
Fluorescent Polymer Microsphere Suspention, 2.9 μm in diameter (Red)	Ducke Scientific Corporation, Palo Alto, CA, USA
Giemsa	Merck, Darmstadt, Germany
Ketanest®	Parke Davis GmbH, Berlin, Germany
MaxiSorp F96 microtiter plates	Nunc, Wiesbaden, Germany
May-Gruenwald`s Eosine-methylene blue	Merck, Darmstadt, Germany
OCT compound	Sakura, Zouterwede, The Netherlands
P 23-Cellulose	Serva, Heidelberg, Germany
Percoll	Amersham, Pharmacia, Uppsala, Sweden
Rompun®	Bayer, Leverkusen, Germany
RPMI medium	GIBCO-BRL, Eggenstein, Germany
Sea sand	Merck, Darmstadt, Germany

Sephadex G 25 and P 23 cellulose	Servacel, Heidelberg, Germany
Serum separator tubes	BD Bioscience, Heidelberg, Germany
Streptavidin-bionylated horseradish peroxidase complex	Amersham-Buchler, Braunschweig, Germany
Superfrost® plus slides	Menzel-Glaser, Braunschweig, Germany
Testosterone	Schering, Berlin, Germany

2.1.2 Kits

PIQOR^{TM} cDNA array system	Memorec, Cologne, Germany
PolyA^{+}-RNA purification kit	Qiagen GmbH, Hilden, Germany
Qiagen PCR Purification Kit	Qiagen GmbH, Hilden, Germany
Total RNA isolation kit	Qiagen GmbH, Hilden, Germany

2.1.3 Solutions and buffers

Annexin V labelling buffer	10 mM Hepes, 8 g/l NaCl, 0.278 g/l $CaCl_2$
Blocking Buffer	8 g NaCl, 0.2 g KCl, 1 g $Na_2HPO_4 \times 2\ H_2O$, 0.5 g $NaH_2PO_4 \times H_2O$, 0.2 g KH_2PO_4, 20 g Milk Powder, adjust pH to 7.2, add H_2O up to 1 l
Buffered formalin (10%)	100 ml 40% formaldehyde, 900 ml dist. H_2O, 4 g $NaH_2PO_4 \times H_2O$, 6.5 g $Na_2HPO_4 \times 2\ H_2O$
CPD-Puffer	34.7 g Sodium citrat, 0.7 g Citric acid, 22,5 g D-Glucose, 1.8 g $Na_2HPO_4 \times 2\ H_2O$, pH 7.2 add H_2O up to 1 l
Citrate Buffer	1.43 g Citric acid, 2.37 g $Na_2HPO_4 \times 2\ H_2O$, adjust pH to 5, add H_2O up to 200 ml

Citrate phosphate glucose (CPG)	34.7 g Na-citrate × 2 H_2O, 0.7 g Citric acid, 1.8 g Na_2HPO_4 × 2 H_2O, 22.5 g Glucose × H_2O, adjust pH to 7.2, add H_2O up to 1 l
Coating Buffer	0.8 g Na_2CO_3, 1.46 g $NaHCO_3$, adjust pH to 9.6, add H_2O up to 1 L
ELISA Buffer.	12.114 g Tris, 21.915 g NaCl, 0.2% Tween-20, adjust pH to 7.5, add 500 ml distilled H_2O
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, $MgCl_2$ × 6 H_2O, 1.8 g Glucose, 1.3 g Hepes, 2 g $NaHCO_3$, add H_2O 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 H_2O up to 1 L, Sterilize by autoclaving.
Phosphate buffer (5mM)	1.42 g/2L Na_2HPO_4, 138 mg/200 ml NaH_2PO_4, adjust pH to 8, add the second to the first

Phosphate buffer saline glucose monohydrate (PBS-HG)	900 ml PBS, 20.25 glucosemonohydrate, add H_2O up to 1 l
PBS^-	8 g NaCl, 0.2 g KCl, 1.15 g $Na_2HPO_4 \times 2\ H_2O$, 0.15 g $NaH_2PO_4 \times H_2O$, 0.2 g KH_2PO_4, adjust pH to 7.2, add H_2O up to 1 l
PBS^+	8 g NaCl, 0.2 g KCl, 1.15 g $Na_2HPO_4 \times 2\ H_2O$, 0.2 g KH_2PO_4, 0.1 g $MgCl_2 \times 6\ H_2O$, 0.1g $CaCl_2 \times 2\ H_2O$, adjust pH to 7.2, add H_2O up to 1 l
PFA (1%)	Paraformaldehyde in PBS^+, adjust to pH 7.2
Solubilization solution	0.5% SDS, 1 mM EDTA, 50 mM Tris, (Soerensen buffer), 0.49 g KH_2PO_4, 1.14 g Na_2HPO_4 adjust pH to 6.8, add H_2O up to 1 l
Soerensen buffer	0.49 g $KH_2PO_4 \times H_2O$, 1.14 g Na_2HPO_4, adjust pH to 8.6, add H_2O up to 1 l
TBE (5×)	54 g Tris-base, 70 g boracic acid, 3.4 g EDTA, add H_2O up to 1 l
TE buffer	10 mM Tris, 1 mM EDTA, pH 7.5
Triton X-100 (1%)	10 g Triton X-100, add up to 1l H_2O
Washing buffer for ELISA	5 g Bovine serum albumin (BSA), 500 µl Tween-20, add PBS^+ up to 1 l

2.1.4 Antibodies

Table 1. ***Antibodies and specific reagents used in ELISA***

IgG and specific reagents	*Supplier*	*Usage*	*Working dilution*
Rabbit antiserum to mouse kappa light chain	Cappel, Durham, N.C, USA	First antibody for total IgG & IgG1	1:1000
HRP-Rabit antimouse IgG (Gamma)	Zymed, San Francisco, CA, USA	Second antibody	1:200 (ELISA titer) 1:2000 for total IgG
Mouse immunoglobulin Reference serum	Bethyl, Montgomery, AL, USA	Standard	Serially diluted from 1:2000 to 1:15.63
Biotin–Rabbit-Anti-Mouse IgG1	Zymed, San Francisco, CA, USA	Second antibody	1:1000
Mouse Myeloma IgG1	Zymed, San Francisco, CA, USA	Standard	Serially diluted from 1:2000 to 1:15.63
Goat Anti-Mouse Kappa UNLB	Southern Biotechnology Associates, Inc., Birmingham, AL, USA	First antibody for IgG2a, IgG2b & IgG3	1:1000 for IgG2a & IgG3, 1:500 for IgG2b
Goat Anti-Mouse IgG2a biotin	Southern Biotechnology Associates, Inc., Birmingham, AL, USA	Second antibody	1:2000
Purified Mouse IgG2a	Pharmingen, BD Biosciences, Heidelberg, Germany	Standard	Serially diluted from 1:2000 to 1:15.63
Goat Ant-Mouse IgG2b biotin	Southern Biotechnology Associates, Inc., Birmingham, AL, USA	Second antibody	1:1000
Purified Mouse Myeloma IgG2b	Zymed, San Francisco, CA, USA	Standard	diluted from 1:2000 to 1:15.63
Goat Anti-Mouse IgG3 biotin	Southern Biotechnology Associates, Inc., Birmingham, AL, USA	Second antibody	1:2000
Mouse IgG3 standard	Bethyl, Montgomery. AL, USA	Standard	Serially diluted from 1:2000 to 1:15.63

Table 2. ***Antibodies and specific reagents for FACS***

Antibodies and specific reagents	*Supplier*	*Working dilution*	*Incubation time*
FITC labelled anti-mouse CD8a (Ly-2)(53-6.7)	BD PharMingen, Heidelberg, Germany	1:200	1 h
FITC labelled anti-mouse F4/80 (macrophages) (C1:A5-1)	ImmunoKontact, Wiesbaden, Germany	1:150	1 h
FITC labelled anti-mouse CD45/B220 (clone RA3-6B2)	BD PharMingen, Heidelberg, Germany	1:200	1 h
FITC labelled anti-mouse Ly-6G (Gr-1)(RB6-8C5)	BD PharMingen, Heidelberg, Germany	1:200	1 h
FITC labelled anti-mouse NK cells/2B4 (CD244.2)	BD PharMingen, Heidelberg, Germany	1:200	1 h
CD16/CD32(FcIII/ II receptor) FC Block	BD PharMingen, Heidelberg, Germany	1:200	15 min
PE labelled anti-mouse CD4 (L3T4)(H129.19)	BD PharMingen, Heidelberg, Germany	1:200	1 h
Annexin V, alexa fluor ® 568 conjugate	Molecular Probes, Leiden, The Netherlands		10 min
7-AAD staining solution	BD PharMingen, Heidelberg, Germany		10 min

2.2 Animals

Mice were housed in plastic cages, and standard diet (Nohrlin, Bad Salzuflen, Germany) and water were available *ad labitum*. The experiments were approved by the state authorities and followed German law on animal protection.

Breeding pairs of LTβR-deficient mice generated by gene disruption and backcrossed on a C57BL/6 background (Fütterer *et al*., 1998) were provided by Charles River (Sulzfeld,

Germany). C57BL/6 and NMRI mice were bred under specific pathogen-free conditions in the central animal facilities of the Heinrich-Heine-University. Balb/c mice and Hox11-deficient mice were bred at the Max-Plank-Institute for Immunobiology (Freiburg, Germany). Experiments were performed only with 9-14 weeks old female and male mice.

2.3 Parasite infection

2.3.1 *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). The parasitemia was evaluated in Giemsa stained smears prepared from tail blood. Both NMRI and experimental mice were challenged with 1×10^6 *P. chabaudi*-infected erythrocytes.

2.3.2 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 centrifuging at 1500 × g for 5 min., the pellet containing both infected and non-infected erythrocytes was suspended in PBSG-buffer. Aliquots of 2-3 ml were layered over a 16 ml percoll-step (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).

2.3.3 Isolation of parasites

P. chabaudi infected erythrocytes were subjected to a glycerol-enhanced osmotic shock (Wunderlich, *et al*., 1985). Infected erythrocytes were concentrated in PBSG-buffer supplemented with 3% FCS and incubated in an equal volume of glycerol buffer at room temperature for 1.5 min. Then, a 4-fold volume of IMV-buffer supplemented with 5% FCS

was added, which induces the release of parasites from the infected erythrocytes and the emergence of infected erythrocyte ghosts.

Parasites and ghosts were separated in a continuous Percoll gradient (Wunderlich, *et al.,* 1985). About 3 ml of the suspension of the osmotically shocked erythrocytes were layered on top of a 20 ml continuous Percoll gradient (1.02-1.10 g cm^{-3}) in IM-buffer and centrifuged at 5000 × g for 20 min. Parasites banding at density of about 1.05 g cm^{-3} were carefully removed by a Pasteur pipette, suspended in IM-buffer supplemented with 10% FCS, and centrifuged at 5000 × g for 20 min. Parasites were resuspended, centrifuged again and store at –20°C. Parasites were resuspended and centrifuged once.

2.3.4 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). 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 Perccoll 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 infected ghosts.

2.4 Treatment of mice

2.4.1 Treatment of mice with testosterone

Mice were subcutaneously injected twice a week for 3 weeks with 0.9 mg testosterone (Testoviron-Depot-50, Schering, Berlin Germany) in 100 µl sesame oil as a vehicle (Wunderlich *et al*., 1988). Controls received only the vehicle. Mice were kept under standard conditions.

2.4.2 Vaccination of mice with ghosts from *Plasmodium chabaudi*-infected erythrocytes

Mice were vaccinated once or twice with 5×10^5 ghosts which were suspended in 100 µl PBS, mixed with an equal volume of Freund's complete adjuvant, and administrated

subcutaneously at the base of the tail (Wunderlich *et al*., 1993). After one week the vaccinated mice were challenged with 1×10^6 *P. chabaudi* infected erythrocytes.

2.5 Castration

Male mice were anaesthetized with Ketavet (Park-Davis, Berlin, Germany) in combination with Rompun (Bayer, Leverkusen, Germany). The testes and epididymis were pulled off through a scrotal incision and removed by electrocautery. After operation, the mice were kept under an IR-lamp for 1 h and then under standard conditions for 3 weeks before further experimental use (Wunderlich *et al*., 1991).

2.6 Splenectomy

Age-matched C57BL/6 and LTβR$^{-/-}$ adult males were infected with 10^6 *Plasmodium chabaudi*-infected erythrocytes. After 55 days they were anesthetized with Avertin (2,2,2-tribromoethanol; Sigma-Aldrich, St Louis, MO, USA). After surgical skin preparation, the spleen was exteriorized through a 1-cm left subcostal incision. The splenic artery and vein were double ligated, and the spleen was removed. The peritoneum and skin were closed in separate layers, using 4.0 absorbable suture (Roubinian, 1980). Mice were rested for 2 weeks before reinfection again with 10^6 *Plasmodium chabaudi*-infected erythrocytes. Parasitemia was calculated.

2.7 Enzyme Linked Immunosorbent Assay (ELISA)

2.7.1 Preparation of serum fractions for ELISA

Blood from female LtβR$^{-/-}$ mice was taken from the heart (5 weeks after first infection), kept at 37°C for 30 min and, subsequently, at 4°C overnight. The blood was then centrifuged at 3000 rpm. for 5 minutes, the supernatant was recentrifuged, and then inactivated at 56 °C for 45 min. The inactivated serum was centrifuged at 13000 r.p.m. The supernatant was recentrifuged and stored at –20°C until use (Wunderlich *et al*.,1992). Hyperimmune serum was taken from 3 times reinfected female C57BL/6 mice with 10^7 *Plasmodium chabaudi* infected erythrocytes and used as a standard in the determination of *Plasmodium chabaudi* antibody titers by ELISA.

2.7.2 Preparation of soluble *P. chabaudi* antigens

Parasitic erythrocytes isolated before were centrifuged with 5000 × g for 10 minutes at 4 °C, the pellet was removed, the supernatant resuspended in 1 ml solubilization solution, centrifuged again as above. The supernatant was transferred to a new tube and passed through a syringe using a 26 G needle. The OD_{280} was measured; the used parasites for coating should have an OD_{280} of 0.05.

2.7.3 Determination of IgG isotypes

MaxiSorp F96 microtiter plate (Nunc, Wiesbaden, Germany) were coated with 50 µl of first antibody for specific IgG overnight at 4°C with different dilutions (table 1). Wells were washed three times with 100 µl washing buffer and subsequently blocked with 250 µl blocking buffer for 2 h at room temperature. After three additional washing steps individual serum samples of $LT\beta R^{-/-}$ mice diluted in Elisa buffer (1:2000; 1:4000; 1:8000; 1:16000; 1:32000; 1:64000; 1:128000; 1:256000) and the standards (table 1) were added in 50 µl to the wells, then incubated for 1 h at 37°C, then followed by three washing steps.

After washing, 50 µl of the secondary antibody diluted in ELISA buffer (table 1) were added to each wel for 45 min at 37°C. After incubation the plates were washed three times with washing buffer and one time with ELISA buffer. The next step was the addition of 50 µl 1:2000 diluted Streptavidin-biotinylated horseradish peroxidase complex (Amersham-Buchler, Braunschweig, Germany) in each well then, incubated 45 min at 37°C. This was followed by washing two times with washing buffer, one time with ELISA buffer and finally two times with citrate buffer. The last step was the addition of 50 µl of 0.53 mg/ml 1,2 phenylenediamine (DAKO, Copenhagen, Germany) diluted with citrate buffer and added to this solution 1.6 µl/ml 30% H_2O_2 in each well, after waiting about 3 min the reaction was stopped by adding 25 µl 3 M H_2SO_4 in each well. OD_{492} values were determined in Anthos-Reader 2001 (Anthos, Cologne, Germany).

2.7.4 Determination of *Plasmodium chabaudi* antibody titers by ELISA

Levels *of Plasmodium chabaudi*–specific total IgG antibodies in serum were determined by ELISA. *Plasmodium chabaudi* antigen was prepared as described previously. MaxiSorp F96 microtiter plates (Nunc, Wiesbaden, Germany) were coated with 50 µl parasite antigen diluted in coating buffer (OD_{280} = 0.05) in each well overnight at 4°C. The wells were washed 3 times with 100 µl washing buffer then dried and subsequently blocked with 250 µl blocking buffer for 2 h at room temperature, followed by three additional washing steps. Individual diluted serum samples (1:100 & 1:200) and hyperimmune serum from C57BL/6 mice as a standard (1:500; 1:250; 1:125; 1:62.5; 1: 31.25) were serially diluted and 50 µl of each dilution were added to each plate and incubated for 1 h at 37°C. The OD_{492} values were measured as described above in details. Antibody levels of total IgG in serum are expressed as ELISA titers.

2.8 Liver pathology

2.8.1 Biochemical measurements

Female LTβR-deficient mice treated with testosterone and vehicle treated controls were infected with 10^6 *P. chabaudi*-parasitized erythrocytes. At maximum parasitemia blood was collected from mice and centrifugally depleted of cells in serum separator tubes (BD Bioscience, Heidelberg, Germany). Sera were assayed for total bilirubin, lactate dehydrogenase, aspartate aminotransferase, alanine aminotransferase, γ-glutamyltransferase, albumin, and alkaline phosphatase using commercially available kits according to the instructions of the manufacturer (Roche Diagnostics, Mannheim, Germany). All measurements were carried out on a Hitachi 917 analyzer (Roche Diagnostics, Mannheim, Germany). Statistical analysis was performed using an unpaired Student's t test.

2.8.2 Liver histology

Small pieces of the liver were quickly removed from dissected mice and fixed in PBS buffered 10% formalin overnight. Specimens were then routinely dehydrated with ethanol and chloroform and embedded in paraffin. Sections of 6 µm were stained with haematoxylin-eosin, picrosirius red, Perl's Prussian blue, and Giemsa standard methods for determination of

liver morphology, fibrosis, iron overload and parasites, respectively. Using a modified quantitative Ishak scoring system for inflammatory activity (Ishak *et a*l., 1995), scores of 1 to 3 were assigned to cases of minimal liver damage, scores of 4 to 8 to mild, scores of 9 to 12 to moderate and scores of 13 to 18 to severe cases, respectively.

2.9 Flow cytometry

2.9.1 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, Austeria). After erythrocytes were removed by NH_4Cl lysis (Benten *et al*., 1991), total leukocytes were counted in a Neubauer chamber.

2.9.2 Quantification of splenic cell populations

Splenic leukocytes were labeled with antibodies and analyzed by flow cytometry (FACS Calibur, BD Bioscience, Heidelberg, Germany) as detailed previously (Benten *et al*., 1991). In brief, cells were preincubated with anti-CD16/CD32 (FcIII/II receptor) FC block (BD PharMingen, Heidelberg, Germany) for 15 min and then labeled 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 Gr1 (RB6-8C5) (all BD PharMingen, Heidelberg, Germany), and anti-mouse F4/80 (C1:A3-1) (ImmunoKontact, Wiesbaden, Germany). 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, Heidelberg, Germany).

2.9.3 Determination of apoptotic Cells

Splenic leukocytes were preincubated with anti-CD16/CD32 (FcIII/II receptor) FC block (BD PharMingen, Heidelberg, Germany) for 15 min and then labelled 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 Gr1 (RB6-8C5) (all BD PharMingen, Heidelberg, Germany), and anti-mouse F4/80

(C1:A3-1) (Immunokontact, Wiesbaden, Germany). The cells were incubated with AnnexinV conjugated to alexa fluor 568 (Molecular Probes, Leiden, The Netherland) and with 7-AAD (BD PharMingen, Heidelberg, Germany) at room temperature for 10 min and then directly measured in the FACSCalibur as described above.

2.10 Filtration capacity of spleen and liver

2.10.1 Determination of the filtration capacity

Each mouse was anesthetized with diethylether and weighed, before 200 µl PBS containing 1.3×10^8 green fluorescent beads (Duke Scientific, Palo Alto, USA) with a diameter of 3 µm were injected into the retroorbital plexus according to Pinkerton and Webber (1964). After 5 min the mouse was killed by cervical dislocation and the spleen and a part of liver were removed, weighed and squeezed with a glass rod in 8 ml 2.3 M KOH, 0.5% Tween 80 in ethanol. After addition of 5×10^5 red beads (2.9 µm diameter, Duke Scientific, Palo Alto, USA) to control for losses during extraction, the tissues were dissolved by shaking for 48 h at 50°C and interrupted several times by vigorous vortexing. Samples were centrifuged at 2000 × g 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) at excitation/emission wavelengths (450/480) and (520/590) for green and red beads, respectively. The number of fluorescent green beads in the spleen and liver were calculated from the standard curve made from the serial dilution measurements of red and green beads at the same excitation/emission wavelengths.

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

The spleens of female LTβR-deficient 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. 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 using a FITC filter set.

2.11 cDNA microarrays

2.11.1 RNA-Isolation

Three testosterone-treated and three vehicle-treated LTβR-deficient mice were infected with 10^6 *P. chabaudi*-parasitized erythrocytes. At peak parasitemia, spleens and livers were removed from dissected mice, rapidly frozen in liquid nitrogen and stored at -80°C. Spleens and livers were pooled, respectively, and total RNA was isolated by standard procedures using a commercially available RNA isolation Kit (Qiagen, Hilden, Germany). PolyA$^+$-RNA was purified using Oligotex (Qiagen, Hilden, Germany). 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, Palo Alto, USA.) and a spectrophotometer.

2.11.2 cDNA arrays

The used PIQOR^{TM} immuno/onco cDNA arrays commercially available at Memorec (Cologne, Germany) contain, on treated glass slides, quadruplicate samples of defined 200 bp to 400 bp cDNA-fragments selected from 642 different genes of the mouse (Bosio *et al*., 2002; Gerstmayer *et al*., 2003). The arrays contain buffer and herring sperm DNA as negative controls, 4 control RNAs of *E. coli* and the 6 positive control cDNAs GAPDH, actin α, tubulin α, tubulin β, cyclophilin and HPRT.

2.11.3 Labelling and hybridization

Labelling of cDNAs with Cy3/Cy5 and hybridization on the arrays were performed as detailed previously (Bosio *et al*., 2002). In brief, 100 µg of total RNA were combined with a control RNA of an *in vitro* transcribed *E. coli* genomic DNA fragment with a polyA$^+$ tail, before isolating mRNA. Isolated mRNA was combined with 3 different control transcripts and then labeled during reverse transcription with FluoroLink^{TM} Cy3/Cy5-dCTP (Amersham

Bioscience, Freiburg, Germany). The labeled samples were cleaned up using QIAquick™ spin columns (Qiagen, Hilden, Germany) and hybridized using a Gene TAC™ hybridization station (Perkin Elmer, Langen, Germany) according to the guidelines of the manufacturer (Memorec, Colone, Germany).

2.11.4 Fluorescence readout and data analysis

The hybridized arrays were scanned and analyzed as detailed elsewhere (Bosio *et al.*, 2002). Briefly, image capture and signal quantification were done with the ScanArrayLite 4000 (Perkin Elmer, Langen, Germany) and ImaGene software 4.1 (Bio-Discovery, Los Angles, USA.).

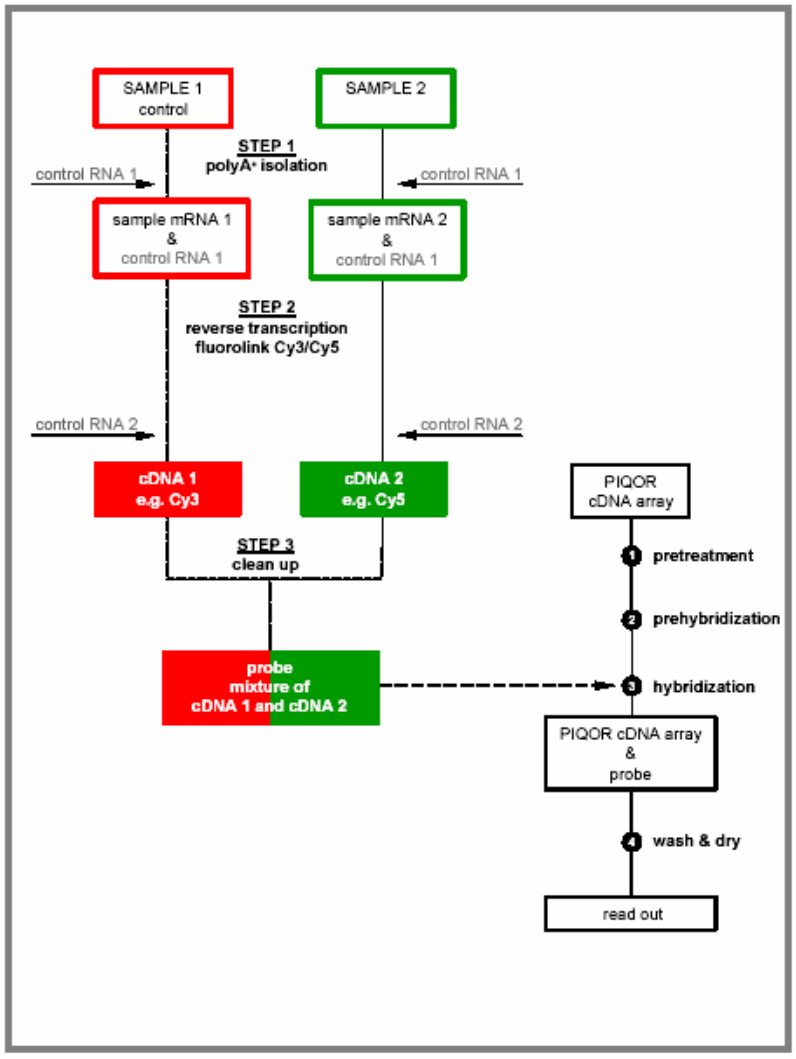


Figure 4. The PIQOR™ cDNA array hybridization procedure

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 obtained and the ratio of Cy5/Cy3 was calculated. The ratios were normalized to the median of all ratios by using only those spots with fluorescence intensity two times higher than that of the negative controls consisting of four spots of herring sperm DNA and four spots of spotting buffer alone. Values represent means of 4 individual spots and standard deviations (Fig. 4).

2.12 Statistical Analysis

Unless otherwise mentioned, each data was normalized to the control value, the mean and standard deviation or standard error of the mean (SEM) from at least three experiments were determined. The data were analyzed by using Excel 2000 (Microsoft, USA), and SigmaPlot 2001 (SPSS, USA).

3 RESULTS

3.1. Resistance of LTßR-deficient mice to *P. chabaudi* malaria

3.1.1 Course of blood stage malaria

Mice with disrupted LTβR on a C57BL/6 genetic background do not only exhibit severe defects in the architecture of the spleen, but also lack major immune organs such as lymph nodes, Peyer's patches, and colon-associated lymphoid tissues (Fütterer *et al*., 1998) making these mice highly vulnerable to pathogens such as *Mycobacterium tuberculosis* and *Listeria monocytogenes* (Ehlers *et al*., 2003). It is surprising, however, that LTβR-deficient mice are highly resistant to blood stage infections with *P. chabaudi*. Indeed, challenge with 10^6 *P. chabaudi*-parasitized erythrocytes results in a self-healing course of the infection. Precrisis culminates in a fulminant parasitemia of about 35% on day 7-10 p.i., but mice are able to clear parasitemia during crisis and to survive the infection (Fig. 5*A*). On day 21 p.i., parasitemia has declined to less than 0.5% in the peripheral blood. By contrast, wild type C57BL/6 mice exhibit a survival rate of only about 80% and the same parasite strain of *Plasmodium chabaudi* induces a lethal outcome in Balb/c mice, where all mice were dead on day 13 postinfection (Fig. 5*B*).

3.1.2 Spleen and liver as effectors against *P. chabaudi* malaria

3.1.2.1 Effect of splenectomy on the course of malaria infection

Self-healing of blood stage *Plasmodium chabaudi* malaria coincides with development of long-lasting immunity. When immune LTβR-deficient mice, which have survived a primary infection with *P. chabaudi* for 70 days, are rechallenged with homologous *P. chabaudi*, the parasitemia is kept below 0.5% for more than 6 weeks (cf. Fig. 6*A*). The successful mounting of protective immunity requires the spleen as an effector against malaria blood stages. First, female Hox11-deficient mice on B6-background which lack a spleen (Roberts *et al*., 1994) are not able to survive blood stage infections with *P. chabaudi* (Fig. 6*B*). Second, when immune LTβR-deficient mice are splenectomized before homologous reinfection, periodic malarial resurgences are observed (Fig. 6*A*).

However, these resurgences do normally not exceed 6% parasitemia, which is rather low in comparison to the fulminant parasitemias of non-immune LTβR-deficient mice.

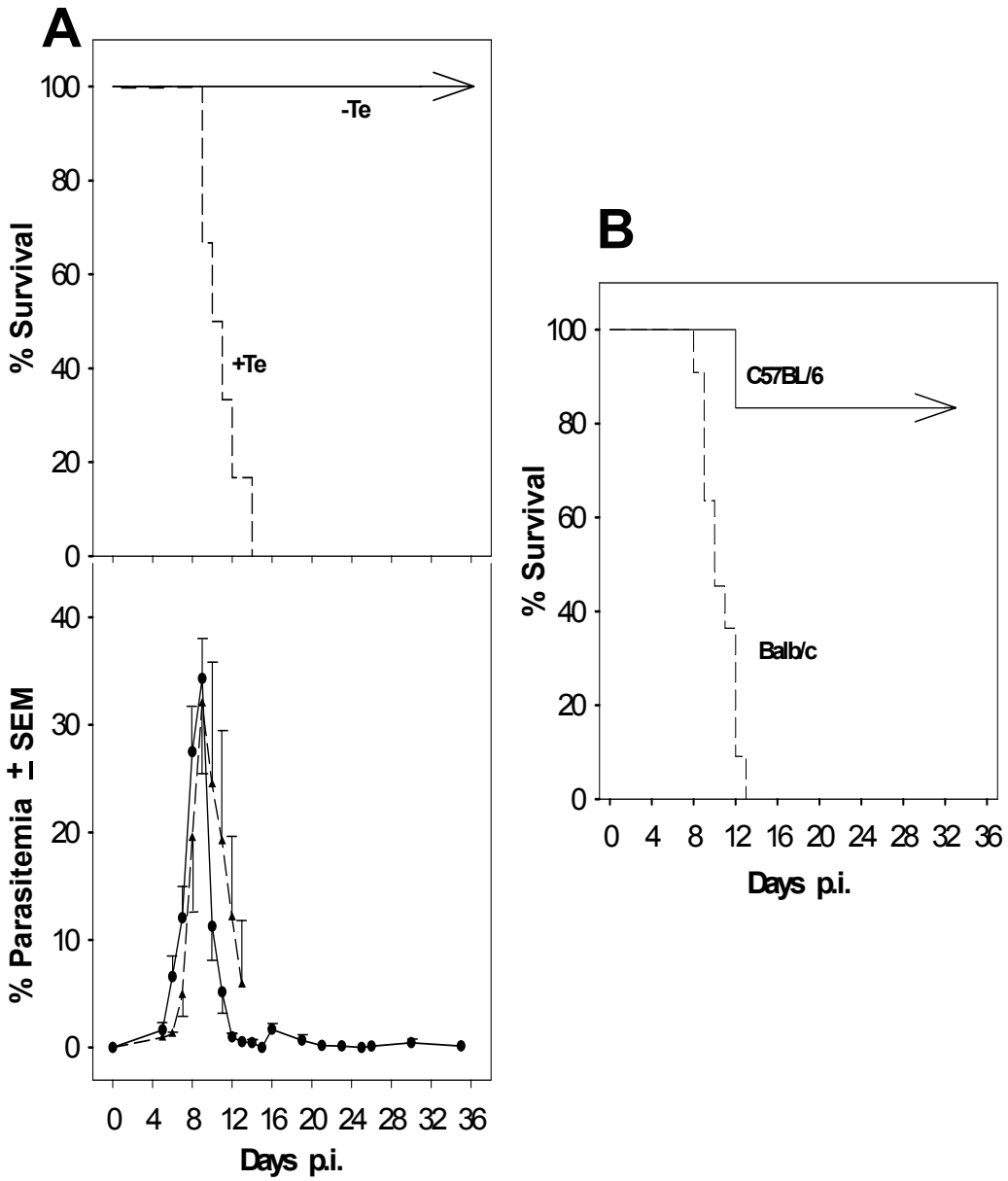


Figure 5. Survival and parasitemia of female C57BL/6 mice treated with testosterone (+Te; triangles) (n=9) or vehicle (-Te; circles) (n=23) and infected with 1×10^6 *Plasmodium chabaudi*-parasitized erythrocytes. All values are means ± SD.

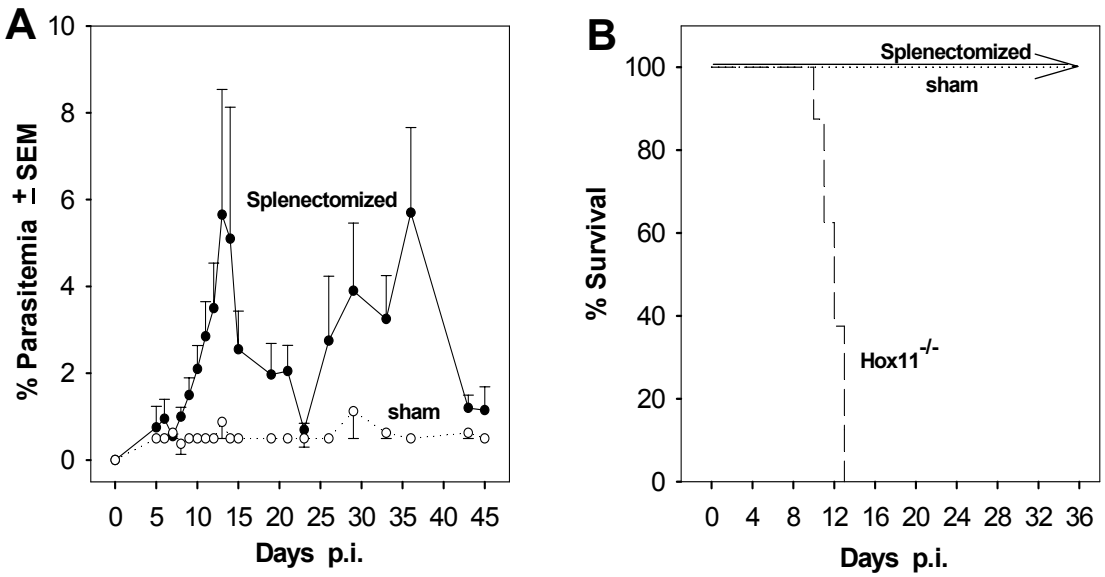


Figure 6. Effects of the spleen on *P. chabaudi* infections. Splenectomized (n = 6) and sham-operated (n = 6) female LTβR-deficient (*A*) or Hox11-deficient (n = 8) (*B*) mice were challenged with 10^6 *P. chabaudi*-infected erythrocytes.

3.1.2.2. Changes in total and apoptotic spleen cell number

The differentiation of the spleen to an anti-parasite effector is associated with reorganizations of the spleen at maximal parasitemia. There is an apparent enlargement of white pulp areas preceding the disappearance of white and red pulp segregation. This coincides with an increase in numbers of leukocytes. Flow cytometric analysis of the total and apoptotic splenic leucocytes were determined and it was found that, the numbers of $CD4^+$ T cells, $CD8^+$ T cells, $F4/80^+$ macrophages, $Gr1^+$ granulocytes and $2B4^+$ NK cells have more than doubled and, in parallel, also the number of apoptotic cells in all cell populations. Only the number of $B220^+$ cells remains about the same during precrisis, but percentage of apoptotic cells has also almost doubled at peak parasitemia (Fig. 7).

3.1.2.3. Differential gating of the spleen

There are two distinct components of the spleen, the red pulp and the white pulp. 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.

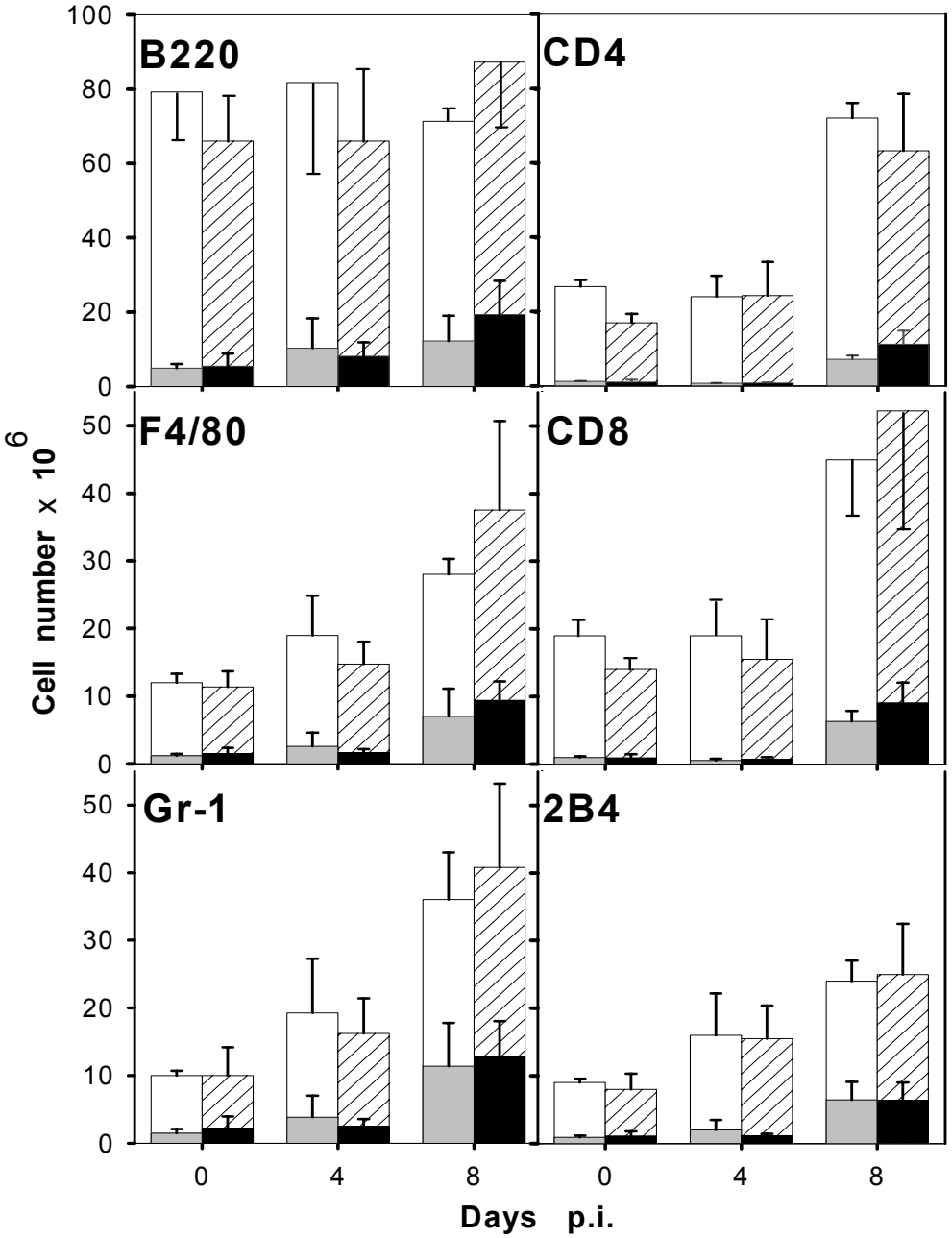


Figure 7. Effects of testosterone on total and apoptotic cell numbers in the spleen of female LTβR-deficient mice. Cell numbers are given for total cells of vehicle- (open columns) and testosterone-treated (hatched) mice and for apoptotic cells of vehicle- (grey) and testosterone-treated (black) mice. Data represents mean ± SEM.

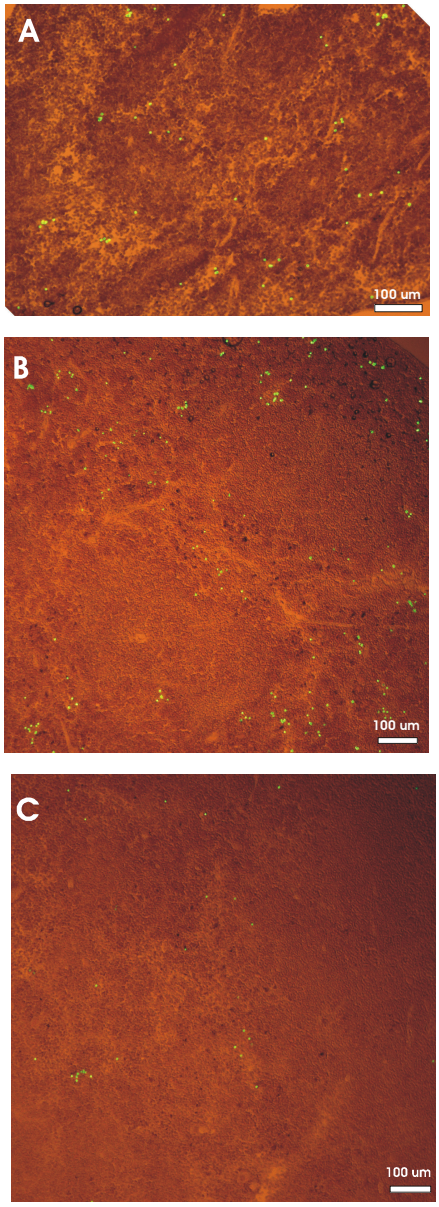


Figure 8. Particle distribution in spleen of female LTßR-deficient mice. Localization of the green beads in red and white pulp on day 0 (A), 4 (B) and 8 (C) p.i. with *P. chabaudi malaria*. Sections counterstained with Hematoxylin.

P. chabaudi infections induce a dramatic enlargement of the spleen (Fig. 8 and 9). This coincides with a dramatic decrease in filtration capacity of the spleen as measured by injecting 3 µm fluorescent polystyrol particles for 5 min and quantifying the particle uptake by isolation of particles from the dissoluted spleen. The number of beads per total spleen were increased on day 4 p.i., then significantly decreased on day 8 p.i. The specific uptake, i.e. number of beads per 100 mg spleen weight, has not significantly changed on day 4 p.i., but then dramatically drops with almost no particle uptake on day 8 p.i. (Fig. 9). This almost complete 'closing' of the spleen is retained during crisis.

Since LTβR-deficient mice have no marginal zone, the cryosections of spleens showed that more particles are observed in the red pulp and only occasional particles over white pulp areas (Fig. 8). At the peak of parasitemia, the separation of red and white pulp becomes increasingly disentangled and the beads are then predominantly found within the red pulp. The number of particles has decreased in comparison to control mice.

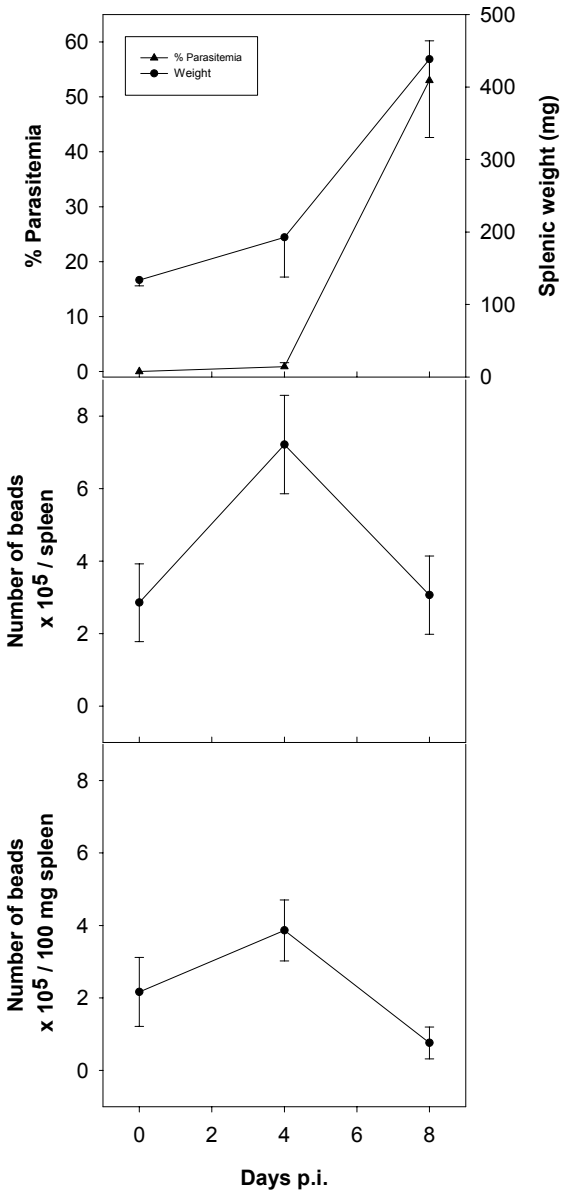
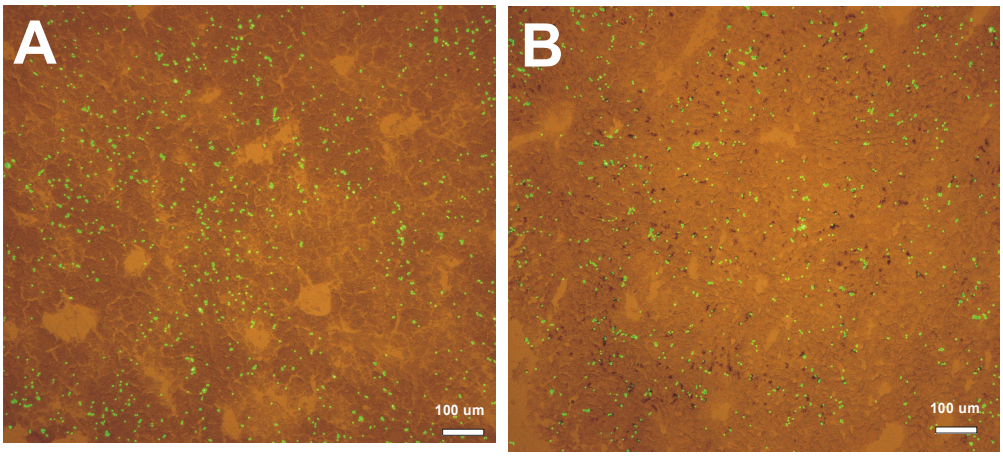


Figure 9. Filtration capacity of the spleen during *P. chabaudi* infection of female LTßR-deficient mice. Animals were infected with 1×10^6 *P. chabaudi*-parasitized erythrocytes. Parasitemia, spleen weight, total uptake of particles and uptake per 100 mg spleen weight are shown. All values are means ± SD.

3.1.2.4 Filtration capacity of liver

Fluorescence microscopy of liver sections reveals that the particles taken up by the liver are more pronounced in pericentral areas of the liver acinus and decreased towards the periportal areas (Fig. 10*A* and *B*).

The specific filtration capacity of the liver of female LTßR$^{-/-}$ mice on day 4 p.i. with *P. chabaudi* is about the same as that on day 8 p.i. (Fig. 10*C*). The number of green beads / 100 mg liver in non infected mice is a little bit lower than that of the infected mice.



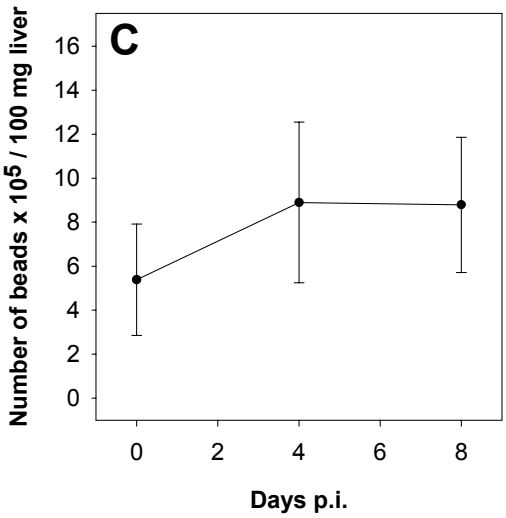


Figure 10. Filtration capacity of the liver during *P. chabaudi* infection of female LTßR$^{-/-}$ mice. (A and B are cryosections on day 0 and 8, respectively, C shows specific uptake of the liver)

3.1.2.5 Pathology of the LTβR$^{-/-}$ mice due to P. chabaudi infection

3.1.2.5.1. Histological changes

For the non-infected mice, the liver shows normal structure where the hepatic lobule (the unit structure of the liver) is made up of radiating plates, cords or strands of cells forming a network around the central vein. Outside the hepatic lobule at certain angle lies the portal area of connective tissue, each enclosing a branch of bile ductile, a hepatic portal vein and a branch of the hepatic artery. The hepatic cells are polyhedral in shape with a noticeable granular cytoplasm. Each cell has a centrally located nucleus or two nuclei (Fig. 11)

The liver strands are alternating with narrow blood sinusoids and also radially extending along the liver lobules. The boundaries of the sinusoids are composed of a single layer of endothelial cells, in addition to the phagocytic cells called Kupffer cells (Fig. 11). These cells are characterized by their flattened condensed nuclei and attenuated poorly stained cytoplasm.

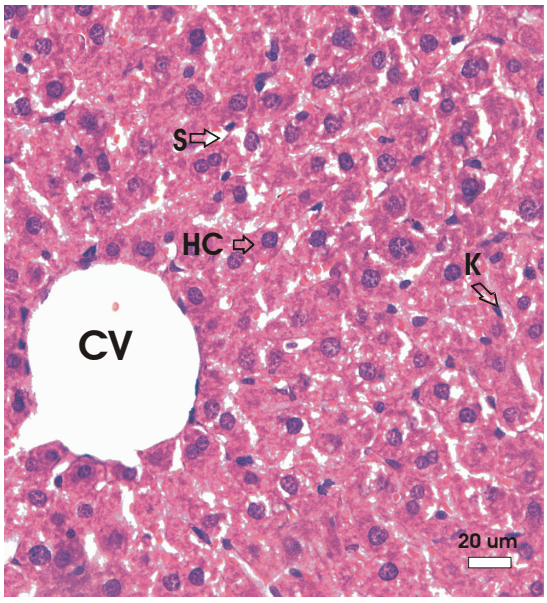


Figure 11. Section of control liver showing, central vein (CV) and surrounding hepatocytes (HC) with nuclei and blood sinusoids (S) lined with Kupffer cells (K). The sections were stained with haematoxylin-eosin.

The liver also undergoes reorganizations in response to blood stage *P. chabaudi* malaria. At maximum parasitemia, the liver has become dark-brown and extremely friable, and it is highly edematous with largely dilated, in part peliosis-like sinusoid cords, enriched with

macrophages and parasite-containing erythrocytes (Fig. 12, table 3). The Kupffer cells are enlarged, contain hemozoin and occasionally parasites (Fig. 12*B*). Moreover, there is always a strong inflammation in the liver. The lobular inflammation is characterized by predominant infiltrations of lymphocytes, plasma cells, and histiocytes, which are localized in perivascular and parenchymal areas (Fig. 12*A*). Furthermore, the liver reveals proliferation of bile ducts presumably with activation of oval cells, an increase in extracellular matrix (ECM) and collagen fibrils, iron-containining hemozoin pigment deposits in the Kupffer cells, rare apoptotic bodies and portocentral (bridging) necrosis (Fig. 12). All these alterations are considered in the histological liver activity index according to Ishak, which can be categorized as 11-13 for the liver at maximal parasitemia in comparison to 4 for non-infected controls (table 3).

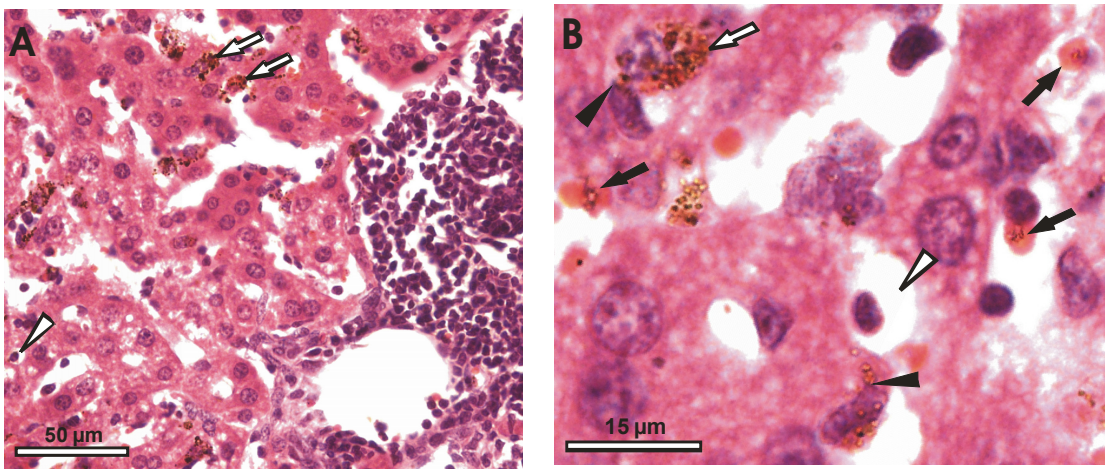


Figure 12. *P. chabaudi*-induced changes in liver histology of female LTβR-deficient mice. *A*, Lobular necroinflammatory changes in the liver. The inflammatory infiltrate on the right is dominated by lymphocytes, the Kupffer cells contain the Malaria-pigment hemozoin. The liver displays cholangiohepatitis, destruction of the periportal limiting plate and dissolution of parenchyma. *B*, *Plasmodium*-infected erythrocytes, apoptotic bodies, hemozoin in Kupffer cells and *P. chabaudi*-parasitized erythrocytes attached to Kupffer cell. The sections were stained with haematoxylin-eosin. White arrow: hemozoin granules; white arrowheads: apoptotic bodies; black arrow: *P. chabaudi*-parasitized erythrocyte; black arrowhead: *P. chabaudi*-parasitized erythrocyte attached to Kupffer cell.

Table 3: ***Histochemical and biochemical parameters of the liver of testosterone-treated and control female LTβR-deficient mice and those infected with Plasmodium chabaudi at peak parasitemia.***

Liver Parameters	Non-infected mice		Acute phase malaria	
	- testosterone	+ testosterone	- testosterone	+ testosterone
Histological activity index [a]	4	2	11-13	11
Inflammatory infiltrates	+	+	+++	++
Sinusoid dilatation	+	+	++	++
Cytoplasmic vacuolization	no	no	+	+
Binucleated cells	+	+	++	+++
Cell swelling	no	no	no	+
Apoptosis	no	no	+	++
Malaria pigment (Hemozoin)	no	no	++	+++
Hyperplasia of Kupffer cells	+	+	+++	++++
Total Bilirubin (mg/dl)	0.14 ± 0.08	0.15 ± 0.13	0.28 ± 0.18	0.27 ± 0.13
Lactate dehydrogenase (U/l)	606± 326[b]	545 ± 113[c]	3855 ± 1470[b]	4147 ± 757[c]
Aspartate aminotransferase (U/l)	72 ± 43	55 ± 14[d]	134 ± 76	125 ± 43[d]
Alanine aminotransferase (U/l)	26 ± 4	24 ± 5.3	38 ± 21	40 ± 14
γ-Glutamyl transferase (U/l)	< 3	< 3	< 3	< 3
Albumin (g/dl)	2.9 ± 1.4	3.4 ± 0.07[e]	2.1 ± 1.6	2.9 ± 0.15[e]
Alkaline phosphatase (U/l)	233 ± 60[f,g]	141 ± 13[f,h]	153 ± 3.6[g,i]	104 ± 11[h,i]

[a] Modified according to Ishak *et al*. (1995). Score: 1-3, minimal; 4-8, mild; 9-12, moderate; 13-18, severe.

[b,c] $p < 0.01$ between indicated groups (Student's t test)

[d] $0.05 < p < 0.06$

[e,f,g] $p < 0.05$

[i] $p < 0.01$

3.1.2.5.2 Biochemical changes

The liver injuries at maximal parasitemia can be further substantiated by measuring

diverse liver functional parameters in blood serum (table 3). There is an increase in total bilirubin originally derived from haem degradation, in aspartate aminotransferase and in alanine aminotransferase. However, the activity of alkaline phosphatase is decreased at peak parasitemia in comparison to non-infected mice (table 3), indicating immunologically based liver injuries (Xu *et al*., 2002). Also, albumin is slightly decreased which indicates reduced protein synthesis of the liver at peak parasitemia (table 3).

3.1.2.5.3 Determination of IgG isotypes

In order to examine the possible effect of *Plasmodium chabaudi* infection on antibody levels, IgG isotypes were determined in the sera of non-infected and infected female LTβR$^{-/-}$ mice 5 weeks p.i. Infection with *Plasmodium chabaudi* malaria produce an increase in total IgG by more than 60% in LTβR$^{-/-}$ mice when compared to the non-infected mice (Fig. 13*A*).

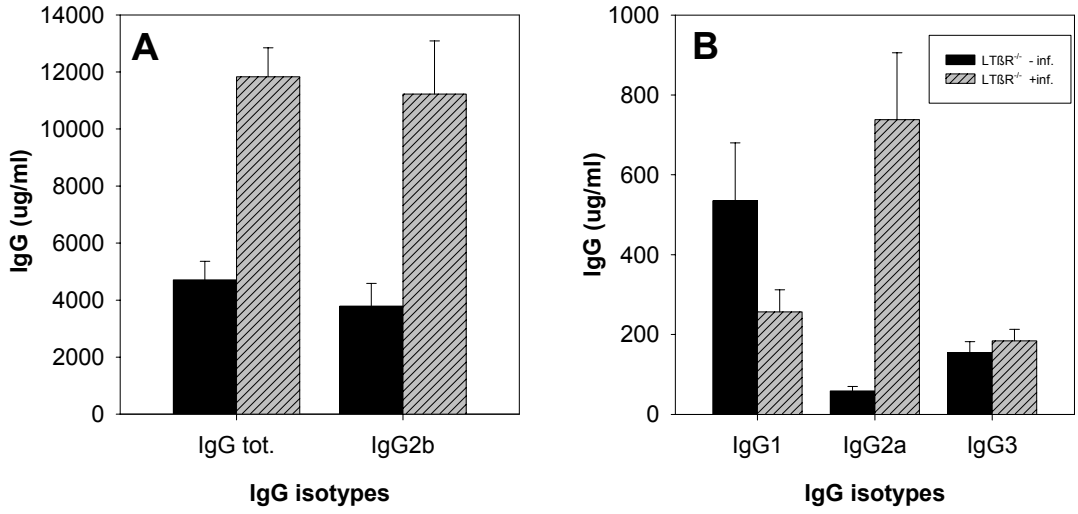


Figure 13. IgG isotype concentrations in sera of female LTβR$^{-/-}$ mice, non-infected and infected with 10^6 *P. chabaudi* parasitized erythrocytes for 5 weeks. Sera concentrations of IgG isotypes were determined by ELISA. Data represents means ± SEM of at least 3 separate duplicate determinations.

Also, the infection was associated with changes in the isotype pattern, in particular IgG2a which have a more significant increase in infected mice (< 0.02) than in non-infected mice (Fig. 13*B*). A double increase in IgG2b (Fig. 13*A*) and a little increase of IgG3 were noticed

due to infection with *Plasmodium chabaudi*. However, a reduction of IgG1 concentration in sera were observed after infection (Fig. 13*B*).

3.1.2.5.4. Determination of Plasmodium chabaudi antibody titers by ELISA

Level of *Plasmodium chabaudi* total IgG were determined using ELISA in sera of both female C57BL/6 and LTβR$^{-/-}$ mice after 5 weeks p.i. using a hyperimmune serum from female C57BL/6 mice as a standard. It was found that there is no significant difference in the total *Plasmodium*-specific IgG between the two mouse strains (Fig. 14).

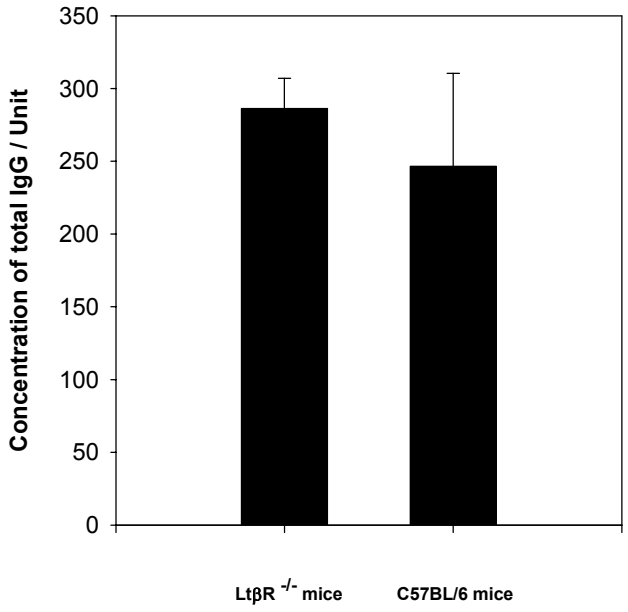


Figure 14. Total IgG concentrations in sera of C57BL/6 and LTβR$^{-/-}$ mice infected with 10^6 *P. chabaudi* parasitized erythrocytes for 5 weeks. Sera concentrations of IgG isotypes were determined by ELISA. Data represent means ± SD of at least 3 separate duplicate determinations.

3.2. Testosterone-induced lethal outcome of malaria

3.2.1. Course of blood stage malaria

The highly resistant phenotype of LTβR-deficient mice is completely converted to susceptibility by testosterone. When female mice were pretreated with 0.9 mg testosterone

twice a week for three weeks, subsequent blood stage infections take a lethal course, i.e. all mice succumb to infection which normally takes place during or shortly after fulminant parasitemia (Fig. 5, page 38). The peak of parasitemia was about 35 % between day 7-10 p.i.

3.2.2. Changes in total and apoptotic spleen cell number

Testosterone-treatment does not affect the cellularity of the spleen of LTβR-deficient mice when compared to non-treated mice (Fig. 7, page 40). Moreover, testosterone does not impair the *P. chabaudi*-induced increase in cell numbers of the spleen during precrisis. At maximal parasitemia, the number of $F4/80^+$ macrophages, $Gr1^+$granulocytes, $CD8^+$ T cells, $B220^+$ B cells, and $2B4^+$ NK cells have not too much changed in comparison to vehicle-treated mice, while $CD4^+$ T cells appear to be slightly decreased. Also, the number of apoptotic cells of the different cell types in the spleen remains to be largely unaffected by testosterone.

3.2.3. Gene profiling of the spleen

In order to evaluate possible effects of testosterone on malaria-induced alterations in gene expression of the spleen, we have compared expression of 642 different genes using the PIQOR immuno/onco arrays. The arrays were hybridized with Cy3 and Cy5 labeled cDNA isolated from spleens of testosterone-treated and vehicle-treated female LTßR-deficient mice at maximal parasitemia. The scatter plot of normalized signals in figure 15 indicates that a few genes are regulated negatively by testosterone and some more genes positively. Table 4 summarizes these testosterone-regulated genes at maximal parasitemia. Expression of IFNγ is slightly increased just as the IFNγ-regulatable genes CCL2, CLTA4, CXCL10, CCL22, IL-1R2. Some genes associated with the extracellular matrix such as thrombospondin and fibulin are slightly downregulated, whereas TIMP3 and CTGF are upregulated.

3.2.4. Liver pathology

In contrast to the spleen, the liver apparently responds to testosterone in non-infected mice which becomes evident as a decreased alkaline phosphatase activity (table 3, page 44). An additional drop in this activity is induced by *P. chabaudi* infections. The latter induce several additional differences in the liver between testosterone- and vehicle-treated mice. Thus, the

liver of testosterone-treated mice reveals increases in binucleated cells, apoptosis, hyperplasia of Kupffer cells, hemozoin deposition, and cell swelling at peak parasitemia. Conversely, there is a slight decline in inflammatory cell infiltrates (table 3). Bilirubin, lactate dehydrogenease, aspartate aminotransferase, and alanine aminotransferase reveal the same increases at maximum parasitemia in sera of testosterone-treated mice and vehicle-treated mice (table 3).

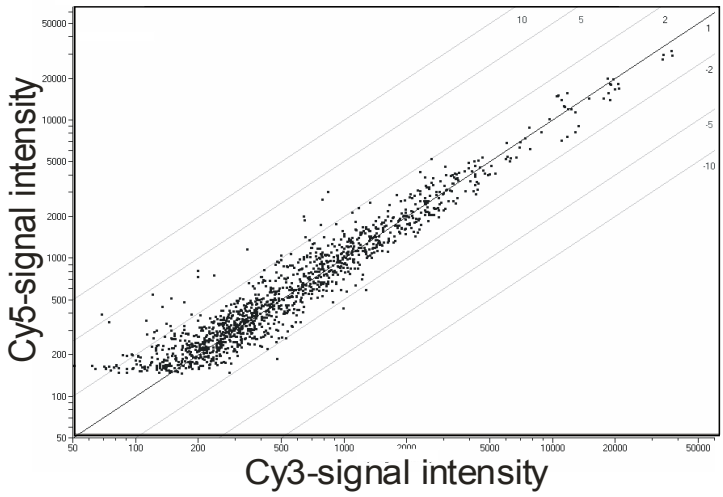


Figure 15. Effects of testosterone-treatment on gene expression of spleen of female LTβR-deficient mice at maximal parasitemia after infection with *P. chabaudi*. Scatterplots represent normalized fluorescence intensities. Differential regulation of genes increases with their distance to the diagonal of the plot.

3.2.5. Gene profiling of liver

Gene expression in the liver has been comparatively analyzed between testosterone-treated and vehicle-treated mice at peak parasitemia (Fig. 16, table 5). Conspicuously, there are again alterations in a number of distinct genes related to the extracellular matrix ECM. This does not only comprise ECM-constituents such as the downregulated syndecan 3 and collagen α1 and the upregulated matrix Gla precursor, but also factors involved in the control of ECM such as inhibitors of matrix metalloproteases (TIMP3) and serine proteases (PAI). Moreover, genes associated with signaling of chemokines, cytokines and growth factors are differentially regulated. In particular, there is a down-regulation of STAT2 and some suppressors of cytokine signaling (SOCS3, SOCS2) whereas SOCS1 is upregulated. Also, the chemokines CXCL10, CXCL9, and CCL2 are upregulated, while CCL5 is downregulated. Finally, testosterone stimulates the expression of the inhibitory transcription factors ATF3 and

NF-IL3A, whereas it suppresses the activating transcription factor junB, which all bind to AP-1/CRE promoter elements.

Table 4. ***Testosterone-induced changes in splenic gene expression of female LTβR-deficient mice infected with P. chabaudi at maximal parasitemia***

Gene name	*Swiss Prot acc.-no.*	*Ratio Te/contr.*	*SD*
Thrombospondin1 precursor	P35441	0.44	21%
Transferrin receptor (CD71)	Q62351	0.48	14%
Activating transcription factor 5 (ATF5)	O70191	0.52	5%
Growth differentiation factor 3 (GDF3)	Q07104	0.55	6%
Fibulin-2 precursor	P37889	0.56	14%
Aortic smooth muscle α-actin2 (ACTA2)	P03996	1.8	13%
Tumor necrosis factor receptor family member 4 (OX40L)	P47741	1.80	8 %
c-rel protooncogene	P15307	1.81	10 %
Macrophage derived chemokine A22 (CCL22)	O88430	1.88	35 %
IFNγ induced monokine precursor (CXCL9, MIG)	P18340	1.92	8 %
Interleukin-1 receptor type II precursor (IL1R2, CD121B)	P27931	1.93	7 %
cAMP-dependent transcription factor 3 (ATF3)	Q60765	1.95	14 %
Metalloprotease inhibitor 3 precursor (TIMP3)	P39876	2.02	12 %
Connective tissue growth factor (CTGF)	P29268	2.16	5 %
ADP-ribosylcyclase 2 precursor (BST1)	Q64277	2.68	42 %
Interferon-γ (IFNγ)	P01580	2.77	13 %
IFNγ induced protein 10 (CXCL10; IP10)	P17515	3.08	9 %
Cytotoxic T lymphocyte protein 4 (CTLA4; CD152)	P09793	3.39	11 %
Monocyte chemotactic protein 1 (MCP1; CCL2)	P10148	4.33	20 %

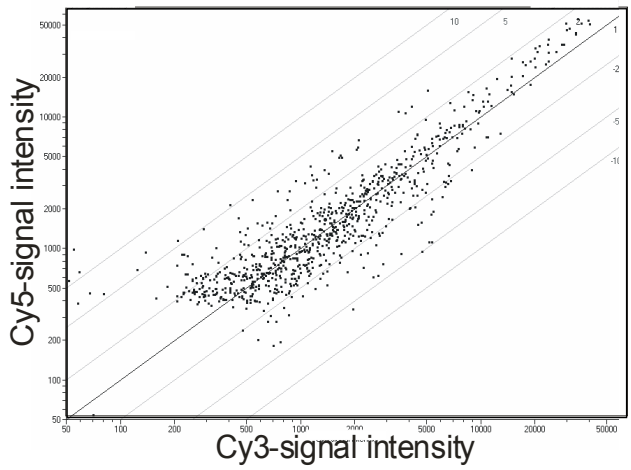


Figure 16. Effects of testosterone-treatment on gene expression of liver of LTβR-deficient mice at maximal parasitemia after infection with *P. chabaudi*. Scatterplots represents normalized fluorescence intensities. Differential regulation of genes increases with their distance to the diagonal of the plot.

Table 5. ***Testosterone-induced changes in liver gene expression of female LTβR-deficient mice infected with P. chabaudi at maximal parasitemia***

Gene name	Swiss Prot. acc. No.	Ratio Te/contr.	SD
Syndecan-3	Q64519	0.2	13 %
Transcription factor junB	P09450	0.21	13%
Suppressor of cytokine signaling 3 (SOCS3)	O35718	0.31	2%
Kinesin-like motor protein 1C (KIF1C)	O35071	0.35	7 %
Cyklin-dependent kinase inhibitor 1B (CDKN1B)	P46414	0.36	5 %
Interleukin-6 receptor-α (IL-6R-α)	P22272	0.37	21 %
Collagen-α1 XVIII containing endostatin	Q62001	0.41	7 %
CSF-1 receptor (CSF-1R)	P09581	0.43	5 %
5'-nucleotidase precursor (NT5)	Q61503	0.44	18 %
Cytokine inducible SH2 containing protein (G18)	Q62225	0.44	3 %
G1/S specific cyclin D1 (CCND1)	P25322	0.44	5 %
Small inducible cytokine A5 precursor (CCL5)	P30882	0.46	4 %
Suppressor of cytokine signaling 2 (SOCS2)	O35717	0.47	16 %
CREB-related protein 1 (CREB-RP)	O35451	0.48	16 %
Signal tranducer and activator of transcription (STAT2)	Q64189	0.5	3 %
Matrix GLA-protein precursor (MGP)	P19788	2.13	24 %
Growth arrest and DNA damage inducible protein (GADD45αCD152)	P48316	2.25	7 %
Insulin-like growth factor binding protein10 (ILGFBP10)	P18406	2.39	19 %
Small inducible cytokine B10 precusor (CXCL10, IP-10, CRG-2)	P17515	2.46	7 %
Serine protease HTRA1 precursor	Q9QZK6	2.57	29 %
Suppressor of cytokine signaling 1 (SOCS1)	O35716	2.61	8 %
IFNγ induced monokine precursor (CXCL9, MIG)	P18340	2.74	6 %
Cyclin-dependent kinase inhibitor 1A (CDKI1A)	P39689	2.75	5 %
Small inducible cytokine A2 precursor (CCL2, MCP1)	P10148	2.76	13 %
Lysosome membrane associated glycoprotein 3 (LAMP-3, CD63)	P41731	2.93	10 %
Plasminogen activator inhibitor 1 (PAI1)	P22777	3.48	9 %
cAMP-dependent transcription factor 3 (ATF3)	Q60765	3.94	20 %
Nuclear factor IL-3A (NF-IL3A, E4BP4)	O08750	5.18	18 %
Tissue inhibitor of metalloproteinases 3 (TIMP-3)	Q91WL9	9.24	59 %

3.3 *Plasmodium chabaudi* infection in male LTβR-deficient mice

3.3.1. Outcome of *Plasmodium chabaudi* infections

Male C57BL/6 mice are susceptible to blood stage infections with *Plasmodium chabaudi* malaria. Challenge with 10^6 *P. chabaudi*-parasitized erythrocytes results in a lethal outcome of the infection (Fig. 17). The course of infection is characterized by rising parasitemias during precrisis culminating at maximal parasitemia of about 55% on about day 8 p.i. Though parasitemias decrease during the following crisis, it is that phase in which the majority of male mice succumb to infection. However, male mice have the potency to survive *P. chabaudi* malaria. This becomes evident after surgical castration or (Fig. 18) vaccination with ghosts of *P. chabaudi*-infected erythrocytes before challenging. Blood stage infections take then a self-healing course in the majority of mice (Fig. 18).

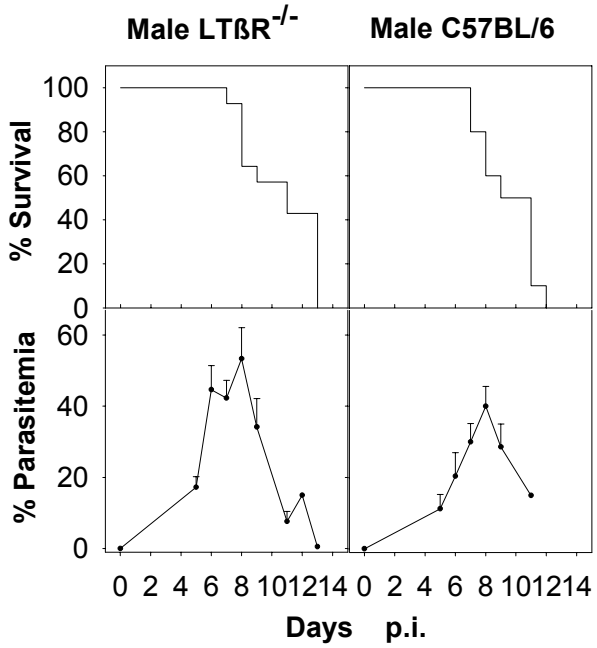


Figure 17. Survival and parasitemia of male LtßR$^{-/-}$ mice and control C57BL/6 mice infected with 1×10^6 *P. chabaudi*-parasitized erythrocytes. All values are means ± SD.

Male mice with a disrupted LTßR on a C57BL/6 genetic background are also susceptible to blood stages of *P. chabaudi* malaria (Fig. 17). By contrast to wild-type controls, these mice remain susceptible after castration or vaccination, i.e. both castrated and vaccinated mice

succumb to infections (Fig. 18). However, when mice are vaccinated two times and then rechallenged, about 20% of the mice are able to survive the infection. About 50% of the mice survive an infection, when castrated LTβR-deficient mice are two times vaccinated (Fig. 18). This indicates that LTßR$^{-/-}$-deficient mice also have the potency of surviving blood stage *P. chabaudi*- infections.

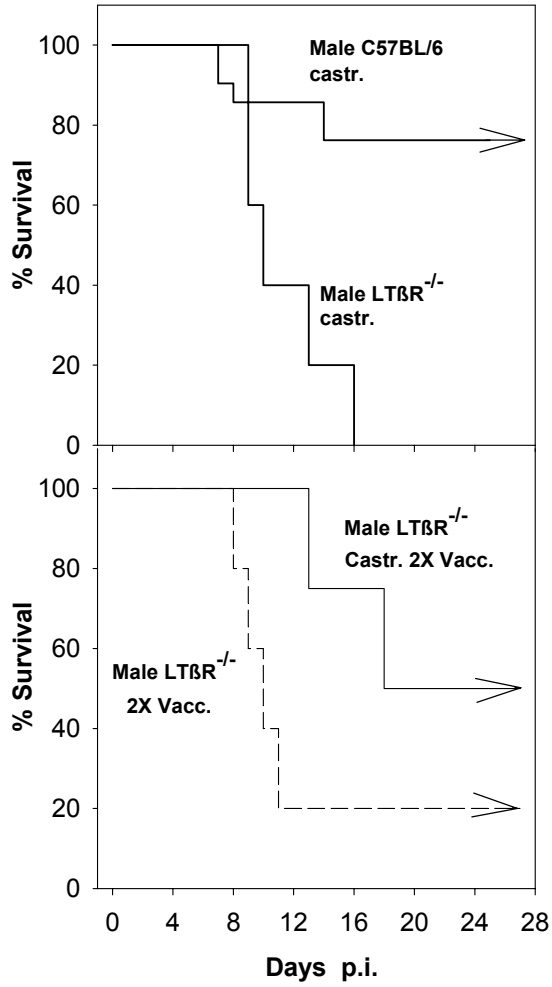


Figure 18. Survival of castrated and vaccinated male LTßR$^{-/-}$ mice and control C57BL/6 mice infected with 1×10^6 *P. chabaudi*-parasitized erythrocytes.

3.3.2. Differential gating in the spleen

As in case of female, the spleen also shows a dramatic enlargement where the weight increased about 5 folds in day 8 p.i. with *Plasmodium chabaudi* malaria and it was noticed that there is no difference in spleen weight between castrated or vaccinated male LTßR-

deficient mice and its C57BL/6 control as well.

The filtration capacity on day 0 p.i., is about 3 fold higher in both male and castrated male C57BL/6 mice when it is compared to male and castrated male LTßR$^{-/-}$ mice. On day 4 p.i. both splenic uptake and specific uptake were increased while on day 7 p.i. there was a dramatic decrease in the filtration capacity (Fig. 19). On day 8 p.i. the spleen reopens slightly and the uptake increased more than that on day 7 p.i.

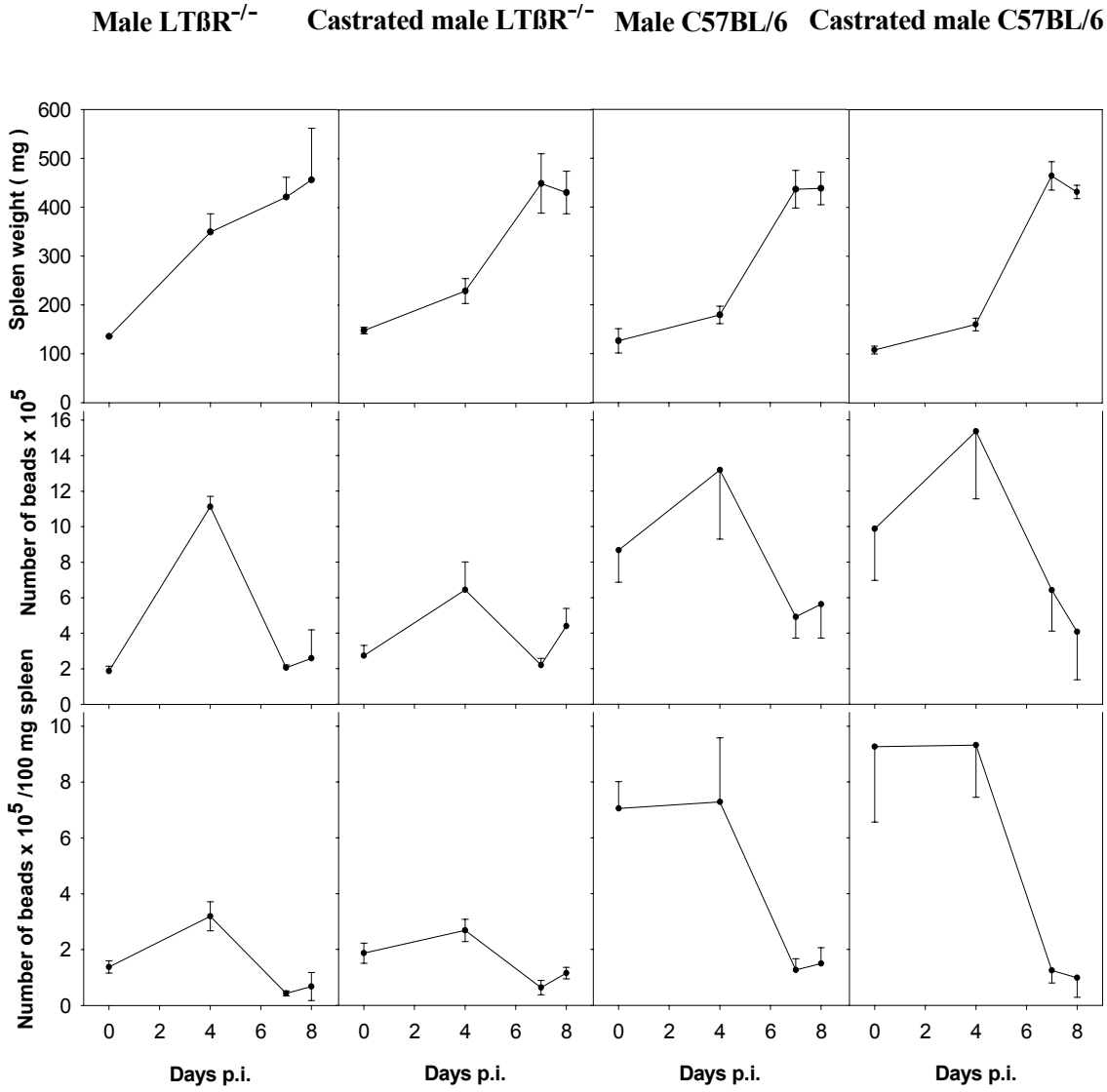


Figure 19. Filtration capacity of the spleen during *P. chabaudi* infection. Mice were infected with 1×10^6 *P. chabaudi*-parasitized erythrocytes. Parasitemia, spleen weight, total uptake of particles and uptake per 100 mg spleen weight are shown. All values are means ± SEM.

3.3.3. Gene expression of the spleen

The transcriptional response to malaria infection in spleens of female and castrated male LTßR$^{-/-}$ mice was evaluated on day 7 p.i. We have compared expression of 642 different genes using the PIQOR immuno/onco arrays. The arrays were hybridized with Cy3 and Cy5 labeled cDNA isolated from spleens of *P. chabaudi*-resistant female and *P. chabaudi*-suscebtible castrated male LTßR-deficient mice at maximal parasitemia. The scatter plots presented in fig. 20 gives an impression of the overall transcriptional responses to *P. chabaudi* infection. In the spleen, there is a strong upregulation of several genes. Downregulation of genes also occurs, but it is less often observed. Table 6 summarizes relative differences for those genes. In the spleen, malaria infection induced strong upregulation of the B cell receptor associated α chain (CD79A), B lymphocyte antigen CD19, B cell differentiation antigen (CD72), B lymphocyte antigen CD20 (MS4A2), and B cell receptor associated β chain (CD79B). In addition, NF-κB p50, tyrosine-proteine kinase BLK, cyclin D1, lymphotoxin-β (LTβ), and G protein coupled receptor EDG1 are induced by infection. In the spleen, downregulation of growth differentiation factor 3 (GDF3), cytokine inducible SH2-containing protein (G18) and the ECM protein fibulin-2 (FBLN2) were observed.

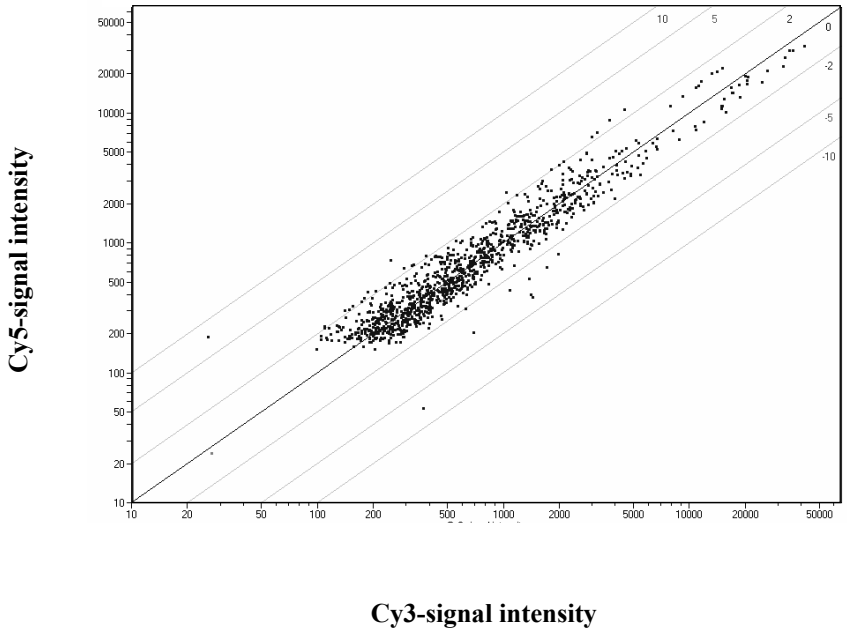


Figure 20. Gene expression of spleens of female and castrated male LTβR-deficient mice at maximal parasitemia after infection with *P. chabaudi*. Scatterplots represents normalized fluorescence intensities. Differential regulation of genes increases with their distance to the diagonal of the plot.

Table 6. ***Gene expression in the spleen of female and castrated male LTβR-deficient mice infected with P. chabaudi at maximal parasitemia***

Gene name	SWall	day 7 ♂ / day 7 ♀	SD
Growth differentiation factor 3 (GDF3)	Q07104	0.28	5%
Cytokine inducible SH2-containg protein (G18)	Q62225	0.39	4%
fibulin-2 (FBLN2)	Q9WUI2	0.53	6%
G protein coupled receptor EDG1	O08530	1.78	18%
B cell receptor associated β chain (CD79B)	P15530	1.79	8%
Lymphotoxin-β (LTβ)	P41155	1.79	8%
B lymphocyte antigen CD20 (MS4A2)	P19437	1.84	11%
Cyclin D1	P25322	1.85	16%
Tyrosine-proteine kinase BLK	P16277	1.86	9%
B cell differentiation antigen CD72	P21855	2.04	7%
NF-κB p50	P25799	2.07	59%
B lymphocyte antigen CD19	P25918	2.08	8%
B cell receptor associated α chain (CD79A)	P11911	2.28	4%

3.3.4. Gene expression of the liver

Gene expression in the liver has also been comparatively analyzed between female and castrated male LTßR$^{-/-}$ mice at peak parasitemia (Fig. 21, table 7). The data shows that some genes are upregulated while others are downregulated. Conspicuously, there are alterations in a number of distinct genes related to the ECM as collagen-α1 XV (COL15A1) and collagen-α1 V (COL5A1). Moreover, genes associated with B cells are upregulated. In particular, B cell receptor associated β chain (CD79B) and CD83 (B cell activation protein). Downregulation was noticed on the chemokine interleukin-6 receptor (IL6R) and the cytokine inducible SH2-containg protein (G18).

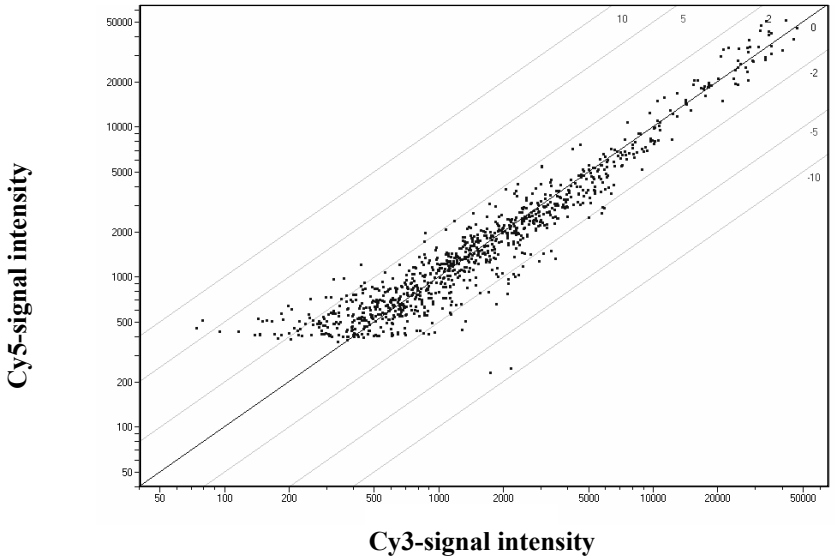


Figure 21. Gene expression of livers of female and castrated male LTβR-deficient mice at maximal parasitemia after infection with *P. chabaudi*. Scatterplots represents normalized fluorescence intensities. Differential regulation of genes increases with their distance to the diagonal of the plot.

Table 6. ***Gene expression in the liver of female and castrated male LTβR-deficient mice infected with P. chabaudi at maximal parasitemia***

Gene name	SWall	day 7 ♂ / day 7 ♀	SD
Plasminogen activator inhibitor-1 (PAI-1; serpine1)	P22777	0.12	11%
Interleukin-6 receptor (IL6R)	P21183	0.41	12%
Bone morphogenetic protein 6 (BMP-6)	P20722	0.42	11%
Cyclin-dependent kinase inhibitor 1 (CDKN1A)	P39689	0.44	10%
Heat shock protein 70 (HSP70)	P17879	0.50	14%
Cytokine inducible SH2-containg protein (G18)	Q62225	0.50	4%
Integrin-α1 (ITGA1, CD49A)	P56199	2.02	88%
Proteinkinase Cβ (PKCβ)	P04411	2.06	15%
Collagen-α1 V (COL5A1)	O88207	2.08	38%
CD83 (B cell activation protein)	O88324	2.26	69%
B cell receptor associated β chain (CD79B)	P15530	2.34	24%
Collagen-α1 XV (COL15A1)	O35206	2.91	3%
Nuclear factor IL-3A (NF-IL3A, E4BP1)	O08750	5.35	112%

4 DISCUSSION

This study provides evidence that protective immunity against blood stage malaria can successfully be established without an intact immune system. Indeed, intact secondary lymphoid tissues require, for organogenesis and maintenance, permanent signaling through LTβR (Fütterer *et al*., 1988; Mackay *et al*., 1997). Disrupting LTβR by gene targeting results in loss of lymph nodes, Peyer's patches, and colon-associated lymphoid tissue, in profound defects of the spleen, in impaired affinity maturation of antibodies, and in a reduced number of NK cells (Fütterer *et al*., 1988; Wu *et al*., 2001).

The sex steroid hormone testosterone is another factor that is critically involved in the control of blood stage malaria. Indeed, testosterone is known for its immunosuppressive activity (Schuurs and Verheul, 1990; Roberts *et al*., 2001) and this is presumably the major reason, why testosterone causes self-healing malaria to take a lethal course (Wunderlich *et al*., 1988; Wunderlich *et al*., 1991). Both spleen and liver are targets for testosterone. For instance, testosterone has been reported to induce alterations in spleen cellularity in C57BL/10 mice (Benten *et al*., 1991) and even to cause hepatocarcinomas (d'Arville and Johnson, 1990; Chen *et al*., 1997). Such actions of testosterone are achieved via changes in gene expression of cells, which can be mediated directly through intracellular androgen receptors (Benten *et al*., 1999; Guo *et al*., 2002) and indirectly through membrane-associated androgen receptors (Heinlein and Chang, 2002).

Gender is critical for the outcome of diseases, especially infectious diseases. In general, males are more susceptible than females to numerous infectious diseases caused by viruses, bacteria, and parasites including protozoa, helminths and even arthropods (Alexander and Stimson, 1988; Brabin and Brabin 1992). This male preponderance of infectious diseases is often ascribed to the sex steroid hormone testosterone which is known to have immunosuppressive activity. Gender and testosterone have been demonstrated to control the outcome of malaria, particularly in the experimental murine blood stage malaria caused by *Plasmodium chabaudi*. For instance, female mice of the inbred strain C57BL/10 are much more resistant than males to blood stages of *Plasmodium chabaudi*. Resistance manifests itself as a self-healing course of *Plasmodium chabaudi* infections and, hence, the successful mounting of protective immune mechanisms, which is controlled by genes of the H-2

complex and the non H-2 background (Wunderlich *et al*., 1988). By contrast, male mice are susceptible, i.e. they succumb to blood stage infections with *Plasmodium chabaudi*. Nevertheless, males also possess the potency to self-heal malaria and to develop long-lasting protective immunity. This becomes evident after lowering the circulating testosterone levels by surgical castration (Wunderlich *et al*., 1991; Benten *et al*., 1993).

4.1 Susceptibility of LTßR-deficient mice to *P. chabaudi* malaria infection

Although female LTßR$^{-/-}$ mice have defects in secondary lymphoid tissues, they are able to self-heal blood stage infections with *P. chabaudi* and to develop long-lasting immunity against homologous rechallenge. Male LTβR-deficient mice are susceptible to blood stage malaria where they succumb to infection just as wildtype B6 mice. However, wildtype B6 mice become resistant after castration or vaccination whereas LTβR-deficient mice remain fully susceptible. Nevertheless, even in the absence of LTβR-signaling it is possible to establish at least partially protective immune responses against *P. chabaudi*. Infection after two rounds of vaccination results in a survival rate of 20% and castration followed by two vaccinations elevated survival to 50%.

Though development of protective immunity to blood stage malaria is rather robust in LTR-deficient mice, it can be completely suppressed by testosterone, since all female mice succumb to infection after pretreatment with testosterone. This is consistent with a series of other studies showing that testosterone has immunosuppressive activity (Schuurs and Verheul, 1990; Roberts *et al*., 2001) and that this activity even superimposes those genes of the H-2 complex and the non H-2 background which control development of protective immunity to blood stage malaria in mice (Wunderlich *et al*., 1988; Sayles and Wassom, 1988; Foote *et al*., 1997).

4.2 The spleen as an effector against *P. chabaudi* malaria infection

The spleen is currently regarded to be the predominant effector of the immune defense against blood stage malaria (Weiss, 1990; Chotivanich *et al*., 2000). It eliminates *Plasmodium*-infected erythrocytes via phagocytosis and initiates adaptive protective immune responses. Splenectomized mice are normally not able to survive a primary infection with blood stage

malaria (Oster *et al*., 1980). The function as an anti-malaria effector is thought to require the intactness of the spleen (Grun *et al*., 1985; Yap *et al*., 1994). Critical for maintaining spleen architecture is permanent lymphotoxin β (LTβ)-signaling through its receptor LṬR, which integrates signals not only from LṬ and LTβ, but also from LIGHT (Zhai *et al*., 1998), another member of the TNF family. Disruption of LTβR gene by homologous recombination has been described to result in profound defects of the spleen (Fütterer *et al*., 1998). Such defects in turn let suspect a severe dysfunction of the spleen in its combat against blood stage malaria.

The profound defects of the spleen in LTβR-deficient mice manifest themselves as absence of the marginal zone, T/B cell segregation, follicular dendritic cell networks, and peanut agglutinin positive cells around central arterioles (Fütterer *et al*., 1998). Nevertheless, the spleen of LTβR-deficient mice is able to differentiate to a powerful anti-parasite effector against blood stages of *P. chabaudi* malaria, as demonstrated by our finding that splenectomy of immune mice results in partial loss of the ability to control parasitemia (cf. also Favila-Castillo *et al*., 1999). Moreover, the role of the spleen as an anti-parasite effector is further substantiated by our finding that the spleen displays hemozoin granules during the acute phase of blood stage malaria. Hemozoin is generated by intraerythrocytic parasites to detoxify the lytic host ferriprotoporphyrin IX (Pagola *et al*., 2000) released during hemoglobin digestion by parasites (Zhang *et al*., 1999). The hemozoin granules are almost exclusively localized in the red pulp of the spleen, thus indicating that the red pulp predominantly harbors the anti-malaria effectors of the spleen. The absence of the marginal zone in spleens of LTβR-deficient mice (Fütterer *et al*., 1998) demonstrates that this zone does not essentially contribute to splenic activity as an anti-parasite effector, confirming previous reports describing the red pulp to be the most important site for phagocytosis of *Plasmodium*-infected erythrocytes (Yadava *et al*., 1996).

Our finding that splenectomy of immune LTβR-deficient mice results only in a partial loss of the ability to control parasitemia upon homologous reinfection indicates that anti-parasite effectors have developed not only in the spleen, but also at other sites in LTβR-deficient mice. In the absence of other secondary lymphoid tissues, one of these sites is apparently the liver. This view is supported by our finding that the liver of LTßR-deficient mice contains numerous hemozoin granules, in particular in the Kupffer cells in accordance

with previous data (Sullivan *et al*., 1996; Levesque *et al*., 1999). Presumably, the anti-malaria activity of Kupffer cells is not only confined to removal of hemozoin from circulation, but also directly contributes to the destruction of *Plasmodium*-parasitized erythrocytes. In this context, it should be emphasized that neither Kupffer cells nor spleen macrophages require LTβR-mediated signaling for their optimal activation to execute effector functions against malaria. This contrasts to the situation found in infections with *Mycobacterium bovis*, *Mycobacterium tuberculosis* and *Listeria monocytogenes*, where macrophages have to be activated through LTβR to acquire the competence to destroy these obligate intracellular pathogens (Ehlers *et al*., 2003; Sayles *et al*., 1988).

Throughout the course of self-healing of blood stage infections with *Plasmodium chabaudi* the spleen increases in weight i.e. splenomegaly resulting in enlargement of both the red and the white pulp. This comes in agreement with Alves *et al*. (1996). The gating process in the spleen is associated with changes in the size and portion of several splenic cell populations. These changes as well as migration of cells in the spleen might allow the organ to control entrance of *Plasmodium*-infected erythrocytes, probably by remodeling the microarchitecture of the spleen.

The malaria-induced gating in the spleen becomes evident as a dramatic drop in uptake of polystyrol particles during late precrisis and crisis. This contrasts to the situation found in infections with *P. yoelii* and *P. chabaudi adami* malaria, where a fast reopening of extravasular routes with much higher efficiency has been described at the beginning of crisis (Alves *et al*., 1996; Weiss, 1989 and Weiss *et al*., 1989). The strong and only slow reversible drop in filtration capacity might be due to the higher parasitemia evoked by *P. chabaudi* in comparison with *P. yoelii* 17XNL (Weiss 1989; Weiss *et al*., 1989) and *P. chabaudi adami* (Alves *et al*., 1996; Yadava *et al*., 1996).

The capacity of this splenic pathway for filtration of blood-borne particles undergoes dramatic alterations during a malaria infection. At the initial phase of precrisis, the spleen is open, but at a later stages of precrisis, this pathway becomes closed presumably by malaria activated barrier cells (Weiss, 1990). It also remains closed during crisis and becomes reopened only at the end of crisis, but with a still higher efficiency as before, this is in contrast to Alves *et al*. (1996).

The testosterone-induced immunosuppression in LTβR-deficient mice is associated with transcriptional changes in spleen and liver. In the spleen, these changes are not large, though the outcome of the infection differs dramatically. For instance, INF-γ and some INF-γ-inducible genes are only slightly upregulated, but these slight upregulations may cause several subtle changes in the spleen, which together may contribute to an impairment of the spleen to differentiate to an anti-parasite effector. First, INF-γ is known to activate intracellular cytotoxicity of macrophages and granulocytes which are then anticipated to degrade more *Plasmodium*-infected erythrocytes. The higher accumulation of hemozoin inside cells may cause a decline of phagocytosis after a while, as hemozoin has been shown to exhibit an inhibitory effect on activity of macrophages (Prada *et al*., 1996; Schwarzer *et al*., 2003a; Schwarzer *et al*., 2003b). Second, the initially higher phagocytic activity should result in a higher release of toxic oxygen radicals, NO, and lysosomal enzymes causing more damages in the spleen, in particular in the red pulp. Third, the increased expression of INF-γ obviously induces a shift in the local Th1/Th2 cell responses in favor of Th1. Even if only transiently, such a shift could severely contribute to a lethal outcome of blood stage malaria, since development of protective immunity is known to require a delicately balanced sequential Th1/Th2 activation in the spleen (Langhorne and Simon-Haarhaus, 1991; Phillips *et al*., 1994). Fourth, the distinct changes in ECM-related genes suggest some specific reorganizations in the splenic ECM, involved for example in the control of extravascular traffic of *Plasmodium*-parasitized erythrocytes (Weiss, 1990; Yadava *et al*., 1996; Alves *et al*., 1996) which may be also impairing anti-parasite effector activity of the red pulp in the spleen.

Gene expression in spleens of susceptible castrated LTßR-deficient males respond differently to blood stage malaria in comparison to resistant female LTßR-deficient mice, where some genes are upregulated that are necessary for the activation and maturation of B cell as B lymphocyte antigen CD19 (Carter and Fearon, 1992), B cell differentiation antigen CD72 (Adachi *et al*., 2000) and B lymphocyte antigen CD20 (MS4A2) (Marti *et al*., 1992). Also G protein coupled receptor EDG1 which is important for the vascular maturation in spleen (Liu *et al*., 2000) is upregulated.

4.3 Liver as an effector against *P. chabaudi* malaria infection

The liver plays a central role in malaria. It is that site where the pre-erythrocytic stages of the malaria parasites asexually multiply and where host immune mechanisms develop to fight these pre-erythrocytic stages (Cohen and Lambert, 1982; Miller *et al*., 2002). However, it has been neglected for a long time that the liver is also able to actively phagocytose constituents of *Plasmodium*-parasitized erythrocytes (Sullivan *et al*., 1996; Levesque *et al*., 1999) and that it may therefore also function as an effector against blood stage malaria. 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 (Balmer *et al*., 2000; Mannoor *et al*., 2002). Deletion of LTβR does not result in structural defects of the liver as found in spleen, though there is a massive infiltration of lymphocytes in perivascular areas (Fütterer *et al*., 1998).

The present study supports findings by Rajesh *et al*., (2003), showing that liver injury due to malaria is accompanied by increased serum bilirubin, hepatomegaly, elevated liver enzymes like aspartate and alanine transferases. Alkaline phosphatase (AP), a membrane-bound ectoenzyme, is used as an indicator to reflect hepatobiliary or bone diseases (Oss, 1987). However, excluding obstructive jaundice or cholestasis, the role of AP activity remains unknown in a variety of liver diseases such as viral hepatitis and cirrhosis. There is a relationship between AP and Wilson's disease, an autosomal recessive disease. Its clinical and pathological manifestations are the consequence of an excessive accumulation of copper in tissues, particularly in the liver, brain, cornea, and kidneys (Sternlieb 1984; Nagai *et al*., 1988; Bellary *et al*., 1995; Wang *et al*., 2003). Although many reports have indicated the relationship between low serum AP level and fulminant hepatic failure caused by Wilson's disease and indicated the usefulness of AP as a marker for diagnosing the disease (Hoshino *et al*.,1995; Nomiyama *et al*., 1999).

AP might be an important factor involved in the mechanisms against liver injury. The consumption of AP might be considered as one of the possible reasons for the decrease in serum level. Such a decrease was consequently observed in both serum and liver tissue of mice with liver injury that proceeded for one week and may be linked to abnormal liver

function (Xu *et al*., 2002).

At the histological level, the tissue sections reflect the severe hepatic disfunction during maximum parasitemia where the liver is enlarged with more sinusoidal dilatation and hyperplasia of Kupffer cells (Dharmeshkumer *et al*., 2003). The increase in Kupffer cell numbers with more lymphocytic infiltration in the portal spaces are a prominent response of body tissue facing any injurious impacts (Miura *et al*., 1991). The intensity of fluorescence was most pronounced in pericentral areas of the liver acinus and decreased from the periportal to pericentral areas where the unidirectional blood flow in the liver acinus is characterized by a continuous decrease in blood from the pericentral areas to the periportal areas (Jungermann and Kietzmann 1996; Paxian *et al*., 2004).

Still more than the spleen, the liver is dysregulated by testosterone which may impair its differentiation to an anti-malaria effector. This can be deduced from the following findings: First, testosterone induces liver injuries in non-infected mice as indicated by the decreased activity of alkaline phosphatase. Second, several additional testosterone-induced liver changes become evident in response to infection at peak parasitemia as increased numbers of binucleated and apoptotic cells, as increased hyperplasia of Kupffer cells, as increased hemozoin depositions, as cell swelling, and as slightly reduced infiltration of lymphocytes. Third, there are testosterone-induced expression changes in distinct liver genes related to ECM and to cytokine and chemokine signaling. However, testosterone does not affect several other liver parameters such as serum bilirubin, lactate dehydrogenease, apartate aminotransferase, and alanine aminotransferase as well as the liver activity index according to Ishak are not significantly changed. This indicates that testosterone-pretreatment does not lead to a general liver failure in malarial blood stage infections, though distinct liver functions appear to be pertubed.

Castration affects gene expression in the liver of male LTßR-deficient mice where some genes are upregulated and others are downregulated. Collagen-α1 XV (COL15A1) and collagen-α1 V (COL5A1) genes are upregulated genes in the liver which are important for the antiangiogenic activity (Ramchandran *et al*., 1999; Sasaki *et al*., 2000). B cell activation protein CD83 and B cell receptor associated β chain (CD79B) are also upregulated where they are needed for the B cell activation and increased amounts of interferon-γ and

interleukin-2 (Wolenski *et al*., 2003).

This study shows changes in the production of IgG during *Plasmodium chabaudi* malaria infection. As regulation of these isotypes is influenced by prevailing Th1/Th2 cytokine balance (Abbas *et al*., 1996). IgG antibodies are primarily important for mediating protective immunity against *P. chabaudi* malaria (Langhorne *et al*., 1984; Falanga *et al*., 1987; Wunderlich *et al*., 1992) as well as other *Plasmodium* species including *P. falciparum* (Groux and Gysin, 1990; Bouharoun-Tayoun and Druilhe, 1992).

Production of IgG2a is common to all infections but its rapid upregulation is a feature of self-resolving infections only, i.e. *Plasmodium chabaudi* (Smith and Taylor-Robinson 2003). Mice that suffer low parasitemia might have higher amount of memory Th1 cells, which would produce high amounts of INF-γ which promotes IgG2a responses (Waki *et al*. 1995; D'Imperio Lima *et al*., 1996). Nevertheless, it might explain why mice that suffered low parasitemia have good immunity. The experiment show that IgG1 is reduced in primary infections with *Plasmodium chabaudi* malaria in LTßR$^{-/-}$ mice while it is reported to be increased after secondary challenge (Burns *et al*., 1997).

Collectively, our data indicate that both spleen and liver differentiate to anti-parasite effectors in response to blood stages of *P. chabaudi* malaria and they are important for survival of a primary malaria infection as well as for maintenance of acquired immunity against challenge infections. This immunity is associated with distinct changes in gene expression and splenic and liver microarchitecture. These data imply that vaccine design will have to consider target molecules that are able to provoke such changes in spleen and liver in the absence of a virulent infection. Testosterone causes a dysregulation of these effectors and this may contribute to the testosterone-induced lethal outcome of blood stage malaria.

5 SUMMARY

This study was designed to analyze the role of spleen and liver as effectors against *Plasmodium chabaudi* malaria using the model of lymphotoxin β receptor (LTβR)-deficient mice. Disrupted signaling through LTβR results in severe defects of the spleen and even loss of all other secondary lymphoid tissues, making mice susceptible to diverse infectious agents. Here we show that female LTβR$^{-/-}$ mice are highly resistant to blood stage infections with *P. chabaudi* while males are particularly susceptible to infections. Even after castration or vaccination male LTβR$^{-/-}$ mice succumb to infection in contrast to wild type male C57BL/6 mice.

Mice develop protective immunity with both spleen and liver as anti-malarial effectors. Splenectomy of immune LTβR$^{-/-}$ mice results only in a partial loss of the ability to control parasitemia. Flow cytometric analysis of total and apoptotic splenic leucocytes showed that, the numbers of CD4^{+} T cells, CD8^{+} T cells, F4/80^{+} macrophages, Gr1^{+} granulocytes and CD244^{+} NK cells were increased during *P. chabaudi* infections. In addition, malaria induced dramatic changes in splenic filtration capacity as determined by retention of fluorescent microbeads after intravenous injection. At the initial phase of infection (early precrises) the spleen is open and takes up large numbers of beads. However, at late precrisis filtration beds in the spleen are closed and microbeads have no longer access as indicated by a 2 fold lower uptake. Histological examination of liver sections and clinical biochemistry of serum real severe liver damage of LTβR $^{-/-}$ mice at maximum parasitemia.

Testosterone suppresses resistance of female LTβR $^{-/-}$ mice to *P. chabaudi* malaria and infections take a lethal outcome during crisis. Comparative analysis of spleen and liver reveal differences between self-healing and lethal malaria at peak parasitemia. Hybridization of cDNA arrays detects that testosterone induces a slight increase in splenic expression of INFγ and INFγ-regulated genes and a decrease in transcript levels of extracellular matrix (ECM)-associated genes. In liver, testosterone also induces changes in expression of genes related to ECM, to chemokine and cytokine signaling, and to cell cycle control. These data suggest that testosterone dysregulates the inflammatory response in both liver and spleen. Since male LTβR $^{-/-}$ mice are susceptible to *P. chabaudi* even after castration, at least one additional determinant of male susceptibility remains to be identified. Indeed, comparison of gene expression in spleen and liver of susceptible castrated male with resistant female LTβR $^{-/-}$ mice demonstrates differences in the inflammatory response as indicated by e.g. changes in expression of ECM-related and cytokine/growth factor signaling-related genes especially in the liver.

Collectively, this study indicates that both spleen and liver differentiate to anti-parasite effectors in response to blood stages of *P. chabaudi* malaria and that they are both important for survival of a primary malaria infection as well as for maintenance of acquired immunity against challenge infections. This immunity is associated with distinct changes in gene expression and in microarchitecture of spleen and liver. These data imply that vaccine design will have to consider target molecules that are able to provoke such changes in spleen and liver in the absence of a virulent infection. Testosterone causes a dysregulation of these effectors and this may contribute to the testosterone-induced lethal outcome of blood stage malaria.

6 ZUSAMMENFASSUNG

Ziel der vorliegenden Arbeit war, die Rolle von Milz und Leber als Effektoren gegen *Plasmodium chabaudi* Malaria am Modell Lymphotoxin β Rezeptor (LTβR)-defizienter Mäuse zu analysieren. Das Ausschalten des LTβR-Signalwegs führt zu schweren Defekten in der Milz und zum völligen Verlust aller anderen sekundären lymphatischen Organe, so daß Mäuse suszeptibel für eine Reihe von Erregern werden. Hier wird gezeigt, daß weibliche LTβR$^{-/-}$-Mäuse hochresistent gegen Infektionen mit *P. chabaudi* Blutstadien sind, während männliche Mäuse für diese Infektion besonders empfindlich sind. Selbst nach Kastration oder Vakzinierung sterben männliche LTβR$^{-/-}$-Mäuse im Gegensatz zu wt C57BL/6 Männchen.

Mäuse entwickeln eine schützende Immunität, wobei Milz und Leber als anti-Malaria Effektoren fungieren. Splenektomie führt bei immunen LTβR$^{-/-}$-Mäusen nur zu einem partiellen Verlust der Fähigkeit, die Parasitämie zu kontrollieren. Analysen der Milzzellen und der apoptotischen Milzzellen mittels Durchflußcytometrie zeigten, daß die Anzahl der CD4$^+$ T-Zellen, der CD8$^+$ T-Zellen, der F4/80$^+$ Makrophagen, der Gr1$^+$ Granulocyten und der CD244$^+$ NK-Zellen während einer *P. chabaudi*-Infektion zunimmt. Außerdem verursacht Malaria dramatische Veränderungen in der Filtrationskapazität der Milz, die durch Quantifizierung der Retention fluoreszierender, intravenös injizierter Mikropartikel gemessen wurde. In der ersten Phase der Infektion (frühe Präkrise) ist die Milz geöffnet und nimmt eine große Anzahl von Partikeln auf. In der späten Präkrise werden die filtrierenden Bereiche der Milz verschlossen, so daß praktisch keine Mikropartikel mehr aufgenommen werden. Untersuchungen an Leberschnitten und an Serumproben ergaben, daß im Maximum der Parasitämie bei LTβR$^{-/-}$-Mäusen eine schwere Schädigung der Leber vorliegt.

Testosteron supprimiert die Resistenz weiblicher LTβR$^{-/-}$-Mäuse, so daß die Infektionen mit dem Tod in der Krise-Phase der Infektion enden. Vergleichende Analysen von Milz und Leber zeigen Unterschiede zwischen selbstheilender und letaler Malaria im Maximum der Parasitämie. Durch Hybridisierung von cDNA-'arrays' konnte gezeigt werden, daß Testosteron eine leichte Zunahme der Expression von IFNγ und IFNγ-regulierten Genen in der Milz verursacht, während eine Abnahme der Transkriptmenge von Genen beobachtet wurde, die mit der Extrazellulären Matrix (ECM) assoziiert sind. In der Leber kommt es ebenfalls zu Änderungen in der Expression von Genen, die mit der ECM, mit Chemokin- und Cytokin-Signalwegen sowie mit der Kontrolle des Zellzyklus in Zusammenhang stehen. Diese Daten legen nahe, daß Testosteron die Entzündungsantwort sowohl in der Milz als auch in der Leber dysreguliert. Da männliche LTβR$^{-/-}$-Mäuse auch nach Kastration suszeptibel für *P. chabaudi*-Infektionen sind, bleibt zumindest eine weitere Determinante für die männliche Suszeptibilität zu identifizieren. In der Tat zeigen Vergleiche der Genexpression von Milz und Leber zwischen resistenten, weiblichen und suszeptiblen, männlichen LTβR$^{-/-}$-Mäusen, daß es Unterschiede in der Entzündungsantwort – besonders in der Leber – gibt, z.B. in der Expression ECM-relevanter bzw. mit Cytokin- und Wachstumskator-Signalwegen zusammenhängender Gene.

Diese Arbeit zeigt, daß sowohl Milz als auch Leber sich als Antwort auf Blutstadieninfektionen mit dem Malaria-Erreger *P. chabaudi* zu anti-parasitischen Effektoren differenzieren, und daß beide nicht nur für das Überleben einer primären Malaria-Infektion, sondern auch für die Aufrecherhaltung bestehender Immunität gegen Reinfektionen wichtig sind. Diese Immunität ist mit distinkten Veränderungen in der Genexpression und der Mikroarchitektur von Milz und Leber assoziiert. Testosteron verursacht eine Deregulation dieser Effektoren und könnte so zum letalen Ausgang von Blutstadien Malaria beitragen. Aus den Daten ergibt sich, daß die Suche nach einer Vakzine Zielmoleküle in Betracht ziehen muß, die in der Lage sind, solche Veränderungen ohne eine virulente Infektion hervorzurufen.

7 REFERENCES

Abbas, A. K., Murphy, K. M., and Sher, A. (1996). Functional diversity of helper T lymphocytes. *Nature* **383**, 787-793.

Adachi, T., Wakabayashi, C., Nakayama, T., Yakura, H., and Tsubata, T. (2000). CD72 negatively regulates signaling through the antigen receptor of B cells. *Journal of Immunology* **164**,1223-1229.

Ahmed, S. A., Talal, N., and Christadoss, P. (1987). Genetic-Regulation of Testosterone-Induced Immune Suppression. *Cellular Immunology* **104**, 91-98.

Alexander, J., and Stimson, W. H. (1988). Sex-Hormones and the Course of Parasitic Infection. *Parasitology Today* **4**, 189-193.

Alves, H. J., Weidanz, W., and Weiss, L. (1996). The spleen in murine *Plasmodium chabaudi adami* malaria: Stromal cells, T lymphocytes, and hematopoiesis. *American Journal of Tropical Medicine and Hygiene* **55**, 370-378.

Amante, F. H. and Good, M. F. (1997). Prolonged Th1-like response generated by a *Plasmodium yoelii*-specific T cell clone allows complete clearance of infection in reconstituted mice. *Parasite Immunology* **19**, 111-126.

Angus, B. J., Chotivanich, K., Udomsangpetch, R., and White, N. J. (1997). *In vivo* removal of malaria parasites from red blood cells without their destruction in acute falciparum malaria. *Blood* **90**, 2037-2040.

Balmer, P., Alexander, J., and Phillips, R. S. (2000). Protective-immunity to erythrocytic *Plasmodium chabaudi* AS infection involves IFN gamma-mediated responses and a cellular infiltrate to the liver. *Parasitology* **121**, 473-482.

Bellary, S., Hassanein, T., and Van Thiel, D. H. (1995). Liver transplantation for Wilson's disease. *Journal of Heptology* **23**,373-381

Benten, W. P. M., Bettenhaeuser, U., Wunderlich, F., Van vliet, E., and Mossmann, H. (1991). Testosterone-Induced Abrogation of Self-Healing of *Plasmodium chabaudi* Malaria in B10 Mice - Mediation by Spleen Cells. *Infection and Immunity* **59**, 4486-4490.

Benten, W. P. M., Wunderlich, F., Herrmann, R., Kuhn-Velten, W. N. (1993). Testosterone-induced compared with oestradiol-induced immunosuppression against *Plasmodium chabaudi* malaria. *Journal of Endocrinology* **139**, 487-494.

Benten, W. P. M., Lieberherr, M., Giese, G., Wrehlke, C., Stamm, O., Sekeris, C. E., Mossmann, H., and Wunderlich, F. (1999). Functional testosterone receptors in plasma membranes of T cells. *FASEB Journal* **13**, 123-133.

Billingsley, P. F. and Sinden, R. E. (1997). Determinants of malaria-mosquito specificity. *Parasitology Today* **13**, 297-301.

Bosio, A., Knorr, C., Janssen, U., Gebel, S., Haussmann, H. J., and Muller, T. (2002). Kinetics of gene expression profiling in Swiss 3T3 cells exposed to aqueous extracts of cigarette smoke. *Carcinogenesis* **23**, 741-748.

Bouharoun-Tayoun, H. and Druilhe, P. (1992). *Plasmodium falciparum* Malaria - Evidence for an isotype imbalance which may be responsible for delayed acquisition of protective immunity. *Infection and Immunity* **60**, 1473-1481.

Bowdler, A. J. (1990). The Spleen. Structure, Function and Clinical Significance. Chapman and Hall Medical, London. 515 pp.

Brabin, L., and Brabin, B. J. (1992). Parasitic infections in women and their consequences. *Advances in Parasitology* **31**, 1-81.

Bundy, D. A. P. (1988). Sexual Effects on Parasite Infection - Gender-Dependent Patterns of Infection and Disease. *Parasitology Today* **4**, 186-189.

Burns, J. M., Dunn, P. D., and Russo, D. M. (1997). Protective immunity against *Plasmodium yoelii* malaria induced by immunization with particulate blood-stage antigens. *Infection and Immunity* **65**, 3138-3145.

Bynum, W. F. (1999). Ross, Ronald. In: *Nature Encyclopedia of Life Sciences*. London: Nature Publishing Group. http://www.els.net/

Carter, R. H, and Fearon, D. T. (1992). CD19: lowering the threshold for antigen receptor stimulation of B lymphocytes. *Science* **256**, 105-107.

Cerami, C., Frevert, U., Sinnis, P., Takacs, B., Clavijo, P., Santos, M. J., and Nussenzweig, V. (1992). The Basolateral Domain of the Hepatocyte Plasma-Membrane Bears Receptors for the Circumsporozoite Protein of *Plasmodium falciparum* Sporozoites. *Cell* **70**, 1021-1033.

Chen, C. J., Yu, M. W., and Liaw, Y. F. (1997). Epidemiological characteristics and risk factors of hepatocellular carcinoma. *Journal of Gastroenterology and Hepatology* **12**, S294-S308.

Chen, M. C., Hwang, M. J., Chou, Y. C., Chen, W. H., Cheng, G. H., Nakano, H., Luh, T. Y., Mai, S. C., and Hsieh, S. L. (2003). The role of apoptosis signal-regulating kinase 1 in lymphotoxin-beta receptor-mediated cell death. *Journal of Biological Chemistry* **278**, 16073-16081.

Chotivanich, K., Udomsangpetch, R., Dondorp, A., Williams, T., Angus, B. J., Simpson, J. A., Pukrittayakamee, S., Looareesuwan, S., Newbold, C. I., and White, N. J. (2000). The mechanisms of parasite clearance after antimalarial treatment of *Plasmodium falciparum* malaria. *Journal of Infectious Diseases* **182**, 629-633.

Cohen, S., and Lambert, P. H. (1982). Malaria In: Cohen S, Warren KS eds. Immunology of parasitic infections. 2nd ed., Oxford, UK: Blackwell Scientific Publications, 422-74.

Crane, G. G. (1977). Pathogenesis of Tropical Splenomegaly Syndrome - Role of Immune-Complexes. *Papua New Guinea Medical Journal* **20**, 6-14.

d'Arville, C. N., and Johnson, P. J. (1990). Growth factors, endocrine aspects and hormonal treatment in hepatocellular carcinoma-an overview. *Journal of Steroid Biochemistry and Molecular Biology* **37**, 1007-1012.

Demoy, M., Gibaud, S., Andreux, J. P., Weingarten, C., Gouritin, B., and Couvreur, P. (1997). Splenic trapping of nanoparticles: Complementary approaches for in situ studies. *Pharmaceutical Research* **14**, 463-468.

D'Imperio Lima, M. R., Alvarez, J. M., Furtado, G. C., Kipnis, T. L., Coutinho, A., and Minoprio P. (1996). Ig-isotype patterns of primary and secondary B cell responses to *Plasmodium chabaudi chabaudi* correlate with IFN-gamma and IL-4 cytokine production with CD45RB expression by CD4+ spleen cells. *Scandinavian Journal of Immunology* **43**, 263-270.

Dharmeshkumar, N. P., Pradeep, P., Surti, M.M., and Agarwal S. B. (2003). Clinical manifestation of complicated malaria an overview. *Journal of Indian Academy of clinical medicine* **4**, 323-331

Dockrell, H. M., Desouza, J. B., and Playfair, J. H. L. (1980). The Role of the Liver in Immunity to Blood-Stage Murine Malaria. *Immunology* **41**, 421-430.

Ehlers, S., Holscher, C., Scheu, S., Tertilt, C., Hehlgans, T., Suwinski, J., Endres, R., and Pfeffer, K. (2003). The lymphotoxin beta receptor is critically involved in controlling infections with the intracellular pathogens *Mycobacterium tuberculosis* and *Listeria monocytogenes*. *Journal of Immunology* **170**, 5210-5218.

Falanga, P. B., Lima, M. R. D., Coutinho, A., and Dasilva, L. P. (1987). Isotypic Pattern of the Polyclonal B-Cell Response During Primary Infection by *Plasmodium-Chabaudi* and in Immune-Protected Mice. *European Journal of Immunology* **17**, 599-603.

Favila-Castillo, L., Monroy-Ostria, A., Kobayashi, E., Hirunpetcharat, C., Kamada, N., Good, M. F. (1996). Protection of rats against malaria by a transplanted immune spleen. *Parasite Immunology* **18**, 325-331.

Favila-Castillo, L., Monroy-Ostria, A., and Garcia-Tapia, D. (1999). *Plasmodium chabaudi chabaudi*: Effect of low parasitemias on immunity in CB6F1 mice. *Experimental Parasitology* **92**, 73-80.

Foote, S. J., Burt, R. A., Baldwin, T. M., Presente, A., Roberts, A. W., Laural, Y. L., Lew, A. M., and Marshall, V. M. (1997). Mouse loci for malaria-induced mortality and the control of parasitaemia. *Nature Genetics* **17**, 380-381.

Foster, S., and Phillips, M. (1998). Economics and its contribution to the fight against malaria. *Annals of Tropical Medicine and Parasitology* **92**, 391-398.

Fu, Y. X., and Chaplin, D. D. (1999). Development and maturation of secondary lymphoid tissues. *Annual Review of Immunology* **17**, 399-433.

Fütterer, A., Mink, K., Luz, A., Kosco-Vilbois, M. H., and Pfeffer, K. (1998). The lymphotoxin beta receptor controls organogenesis and affinity maturation in peripheral lymphoid tissues. *Immunity* **9**, 59-70.

Galinski, M. R., and Barnwell, J. W. (1996). *Plasmodium vivax*: Merozoites, invasion of reticulocytes and considerations for malaria vaccine development. *Parasitology Today* **12**, 20-29.

Gerstmayer, B., Kusters, D., Gebel, S., Muller, T., Van Miert, E., Hofmann, K., and Bosio, A. (2003). Identification of RELM gamma, a novel resistin-like molecule with a distinct expression pattern. *Genomics* **81**, 588-595.

Groux, H. and Gysin, J. (1990). Opsonization as an effector mechanism in human protection against asexual blood stages of *Plasmodium falciparum* - functional-role of IgG subclasses. *Research in Immunology* **141**, 529-542.

Grun, J. L., and Weidanz, W. P. (1981). Immunity to *Plasmodium chabaudi-adami* in the B-Cell-Deficient Mouse. *Nature* **290**, 143-145.

Grun, J. L., Long, C. A., and Weidanz, W. P. (1985). Effects of Splenectomy on Antibody-Independent Immunity to *Plasmodium chabaudi-adami* malaria. *Infection and Immunity* **48**, 853-858.

Guo, Z., Benten, W. P. M., Krücken, J., Wunderlich, F. (2002). Nongenomic testosterone calcium signaling. Genotropic actions in androgen receptor-free macrophages. *Journal of Biological Chemistry* **277**, 29600-29607.

Hauda, K. M., Sayles, P. C., and Wassom, D. L. (1993). *Plasmodium yoelii* - Cellular Immune-Responses in Splenectomized and Normal Mice. *Experimental Parasitology* **76**, 385-393.

Hawking, F., Worms, M. J., and Gammage, K. (1968). 24- and 48-Hour Cycles of Malaria Parasites in Blood - Their Purpose Production and Control. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **62**, 731-765.

Heinlein, C. A., and Chang, C. (2002). The roles of androgen receptors and androgen-binding proteins in nongenomic androgen actions. *Molecular Endocrinology* **16**, 2181-2187.

Helmby, H., Jonsson, G., and Troye-Blomberg, M. (2000). Cellular changes and apoptosis in the spleens and peripheral blood of mice infected with blood-stage *Plasmodium chabaudi chabaudi* AS. *Infection and Immunity* **68**, 1485-1490.

Henderson, B. E., Ross, R. K., Pike, M. C., and Casagrande, J. T. (1982). Endogenous Hormones as a Major Factor in Human Cancer. *Cancer Research* **42**, 3232-3239.

Hengartner, M. O. (2000). The biochemistry of apoptosis. *Nature* **407**, 770-776.

Ho, M., White, N. J., Looareesuwan, S., Wattanagoon, Y., Lee, S. H., Walport, M. J., Bunnag, D., and Harinasuta, T. (1990). Splenic Fc Receptor Function in Host Defense and Anaemia in Acute *Plasmodium falciparum* Malaria. *Journal of Infectious Diseases* **161**, 555-561.

Hoffman, S. L., Franke, E. D., Hollingdale, M. R, and Druihle, P. (1996). Attacking the infected hepatocyte. In: Hoffman SL (ed.) *Malaria Vaccine Development: A Multi-immune Response Approach*, pp. 105-143. Washington DC: ASM Press.

Hoshino, T., Kumasaka, K., Kawano, K., Yamagishi, F., Koyama, I., Fujimori-Arai, Y., Nakajima, T., and Komoda, T. (1995). Low serum alkaline phosphatase activity associated with severe Wilson's disease. Is the breakdown of alkaline phosphatase molecules caused by reactive oxygen species? *Clinica Chimica Acta.* **238**, 91-100.

Ishak, K., Baptista, A., Bianchi, L., Callea, F., Degroote, J., Gudat, F., Denk, H., Desmet, V., Korb, G., Macsween, R. N. M., Phillips, M. J., Portmann, B. G., Poulsen, H., Scheuer, P. J., Schmid, M., and Thaler, H. (1995). Histological Grading and Staging of Chronic Hepatitis. *Journal of Hepatology* **22**, 696-699.

Jacobs, P., Radzioch, D., and Stevenson, M. M. (1996). A Th1-associated increase in tumor necrosis factor alpha expression in the spleen correlates with resistance to blood-stage malaria in mice. *Infection and Immunity* **64**, 535-541.

Jacobson, M. D., Weil, M., and Raff, M. C. (1997). Programmed cell death in animal development. *Cell* **88**, 347-354.

Jungermann, K., and Kietzmann, T. (1996). Zonation of parenchymal and nonparenchymal metabolism in liver. *Annual Review of Nutrition* **16**,179–203

Kamis, A. B. and Ibrahim, J. B. (1989). Effects of Testosterone on Blood Leukocytes in *Plasmodium berghei*-Infected Mice. *Parasitology Research* **75**, 611-613.

Kareier, J. P. (1980). Malaria., Volume 1 Epidemology, Chemotherapy, Morphology, and Metabolism. P. 2-4. Academic press, INC, New York.

Kather, A., Chantakru, S., He, H., Minhas, K., Foster, R., Markert, U. R., Pfeffer K., Croy, B. A. (2003). Neither lymphotoxin alpha nor lymphotoxin beta receptor expression is required for biogenesis of lymphoid aggregates or differentiation of natural killer cells in the pregnant mouse uterus. *Immunology* **108,** 338-345.

Kean, B., Mott, K., and Russell, A. (1978). Tropical Medicine and Parasitology. Vol. 1: *Classical Investigations*. Ithaca: Cornell University Press.

Kemp, C. J., Leary, C. N., and Drinkwater, N. R. (1989). Promotion of Murine Hepatocarcinogenesis by Testosterone is Androgen Receptor-Dependent but not Cell Autonomous. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 7505-7509.

Kerr, J. F. R., Wyllie, A. H., and Currie, A. R. (1972). Apoptosis - Basic Biological Phenomenon with Wide-Ranging Implications in Tissue Kinetics. *British Journal of Cancer* **26**, 239-257.

Klein, J. (1975). In: Biology of the mouse histocompatibility-2 complex. P. 504, Springer-Verlang, Berlin.

Kraal, G. (1992). Cells in the marginal zone of the spleen. *International Review of Cytology* **132**,31-73.

Knudsen, A. B, and Slooff, R. (1992). Vector-borne disease problems in rapid urbanization: new approaches to vector control. *Bulletin of the World Health Organization* **70**, 1-6.

Krammer, P. H., Behrmann, I., Daniel, P., Dhein, J., and Debatin, K. M. (1994). Regulation of Apoptosis in the Immune-System. *Current Opinion in Immunology* **6**, 279-289.

Kumar, S., Good, M. F., Dontfraid, F., Vinetz, J. M., and Miller, L. H. (1989). Interdependence of $CD4^+$ T-Cells and Malarial Spleen in Immunity to *Plasmodium vinckei-vinckei* - Relevance to Vaccine Development. *Journal of Immunology* **143**, 2017-2023.

Kuramar S, Miller L. H. (1990). Cellular mechanisms in immunity to blood stage infection. *Immunological Letters* **25**, 109-114.

Kwiatkowski, D. (1992). Malaria - Becoming More Specific About Nonspecific Immunity. *Current Opinion in Immunology* **4**, 425-431.

Langhorne, J., Evans, C. B., Asofsky, R., and Taylor, D. W. (1984). Immunoglobulin Isotype Distribution of Malaria-Specific Antibodies Produced During Infection with *Plasmodium chabaudi adami* and *Plasmodium yoelii*. *Cellular Immunology* **87**, 452-461.

Langhorne, J. and Simon-Haarhaus, B. (1991). Differential T-cell Responses to *Plasmodium-chabaudi chabaudi* in Peripheral-Blood and Spleens of C57BL/6 Mice During Infection. *Journal of Immunology* **146**, 2771-2775.

Langhorne, J., Mombaerts, P., and Tonegawa, S. (1995). αβ and γδ T cells in the immune response to the erythrocytic stages of malaria in mice. *International Immunology* **7**: 1005–1011.

Lee, S. H., Looareesuwan, S., Wattanagoon, Y., Ho, M., Wuthiekanun, V., Vilaiwanna, N., Weatherall, D. J., and White, N. J. (1989). Antibody-Dependent Red-Cell Removal During *P-falciparum* Malaria - the Clearance of Red-Cells Sensitized with an IgG Anti-D. *British Journal of Haematology* **73**, 396-402.

Levesque, M. A., Sullivan, A. D., and Meshnick, S. R. (1999). Splenic and hepatic hemozoin in mice after malaria parasite clearance. *Journal of Parasitology* **85**, 570-573.

Liu, Y., Wada, R., Yamashita, T., Mi, Y., Deng, C. X., Hobson, J. P., Rosenfeldt, H. M., Nava, V. E., Chae, S. S., Lee, M. J., Liu, C. H., Hla, T., Spiegel, S., and Proia, R. L. (2000). Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. *Journal of Clinical Investigat*ion **106**, 951-961.

Mackay, F., Majeau, G. R., Lawton, P., Hochman, P. S., and Browning, J. L. (1997). Lymphotoxin but not tumor necrosis factor functions to maintain splenic architecture and humoral responsiveness in adult mice. *European Journal of Immunology* **27**, 2033-2042.

Mannoor, M. K., Halder, R. C., Morshed, S. R. M., Ariyasinghe, A., Bakir, H. Y., Kawamura, H., Watanabe, H., Sekikawa, H., and Abo, T. (2002). Essential role of extrathymic T cells in protection against malaria. *Journal of Immunology* **169**, 301-306.

Marti, G. E., Faguet, G., Bertin, P., Agee, J., Washington, G., Ruiz, S., Carter, P., Zenger, V., Vogt, R., and Noguchi, P. (1992). CD20 and CD5 expression in B-chronic lymphocytic leukemia. *Annual of the New Year Academy of Scence.* **651**, 480-483.

Mehlhorn, H. (2001). Encyclopedic Reference of Parasitology. 2nd edition, Berlin,Vol. 1 pp. 291. springer press.

Mendel, C. M. (1989). The Free Hormone Hypothesis - A Physiologically Based Mathematical-Model. *Endocrine Reviews* **10**, 232-274.

Miller, L. H., Baruch, D. I., Marsh, K., and Doumbo, O. K. (2002). The pathogenic basis of malaria. *Nature* **415**, 673-679.

Miura, S., Suematsu, M., Tanaka, S., Nagata, H., Houzawa, S., Suzuki, M., Kurose, I., Serizawa, H., and Tsuchiya, M. (1991). Microcirculatory disturbance in indomethacin-induced intestinal ulcer. *American Journal of Physiol*ogy **261**, 213-219.

Nagai, H., Yakuo, I., Yamada, H., Shimazawa, T., Koda, A., Niu, K., Asano, K., Shimizu, T., and Kasahara, M. (1988). Liver injury model in mice for immunopharmacological study. *Japanese Journal of Pharmacology* **46**, 247-54.

Nakato, M., Nakayama, T., and Kankura, T. (1973). A new method for separation of human blood components. *Nature New Biol*ogy **246**, 94.

Nicholson, D. W. and Thornberry, N. A. (1997). Caspases: killer proteases. *Trends in Biochemical Sciences* **22**, 299-306.

Nomiyama, K., Nomiyama, H., Kameda, N., Tsuji, A., and Sakurai, H. (1999). Mechanism of hepatorenal syndrome in rats of Long-Evans Cinnamon strain, an animal model of fulminant Wilson's disease. *Toxicology* **132**, 201-214.

Oss, D. W. (1987). Diagnostic aspects of alkaline phosphatase and its isoenzymes. *Clinical Biochemistry* **20**, 225-230.

Oster, C. N., Koontz, L. C., and Wyler, D. J. (1980). Malaria in asplenic mice: effects of splenectomy, congenital asplenia, and splenic reconstitution on the course of infection. *American Journal of Tropical Medicine and Hygiene* 29:1138-1142.

Pagola, S., Stephens, P. W., Bohle, D. S., Kosar, A. D., and Madsen, S. K. (2000). The structure of malaria pigment beta-haematin. *Nature* **404**, 307-310.

Paul, R. E. L. and Brey, P. T. (2003). Malaria parasites and red blood cells: from anaemia to transmission. *Molecules and Cells* **15**, 139-149.

Paxian, M., Keller, S. A., Cross, B., Huynh, T. T., and Clemens, M. G. (2004). High-resolution visualization of oxygen distribution in the liver in vivo. *Americam Journal of Physiology: Gastrointestinal and Liver Physiology* 286, 37-44.

Phillips R. S., Mathers, K. E., and Taylor-Robinson, A. W. (1994). T cells in immunity to *Plasmodium chabaudi chabaudi*: operation and regulation of different pathways of protection. *Research in Immunol*ogy **145**, 406-12.

Phillips, R. S., Brannan, L. R., Balmer, P., and Neuville, P. (1997). Antigenic variation during malaria infection - the contribution from the murine parasite *Plasmodium chabaudi*. *Parasite Immunology* **19**, 427-434.

Pinkerton, W. and Webber, M. (1964). A method of injecting small laboratory animals by opthalmic plexus route. *Proceedings of the Society for Experimental Biology and Medicine* **116**, 959-961.

Playfair, J. H. L., Taverne, J., Bate, C. A. W., and Desouza, J. B. (1990). The Malaria Vaccine - Antiparasite or Antidisease. *Immunology Today* **11**, 25-27.

Prada, J., Malinowski, J., Muller, S., Bienzle, U., and Kremsner, P. G. (1996). Effects of *Plasmodium vinckei* hemozoin on the production of oxygen radicals and nitrogen oxides in murine macrophages. *American Journal of Tropical Medicine and Hygiene* **54**, 620-624.

Prinzen, F. W., and Glenny, R. W. (1994). Development in non radioactive microsphere technique for blood flow measurement. *Cardiovascular Research* **28**, 1467-1475.

Quinn, T. C., and Wyler, D. J. (1980). Resolution of Acute Malaria (Plasmodium berghei in the Rat) - Reversibility and Spleen Dependence. *American Journal of Tropical Medicine and Hygiene* **29**, 1-4.

Rajesh, B. Purnima, L., R. S. Gehlot (2003). Liver Involvement in Falciparum Malaria :A Histo-pathological Analysis *Journal, Indian Academy of Clinical Medicine* **4**, 34-38.

Ramchandran, R., Dhanabal, M., Volk, R., Waterman, M. J. F., Segal, M., Lu, H., Knebelmann, B., and Sukhatme, V. P. (1999). Antiangiogenic activity of restin, NC10 domain of human collagen XV: comparison to endostatin. *Biochemical and Biophysical Research Communication* **255**, 735-739.

Roberts, C. W. M., Shutter, J. R., and Korsmeyer, S. J. (1994). Hox11 Controls the Genesis of the Spleen. *Nature* **368**, 747-749.

Roberts, C. W., Walker, W., and Alexander, J. (2001). Sex-associated hormones and immunity to protozoan parasites. *Clinical Microbiology Reviews* **14**, 476-488.

Röllinghoff, M., Bogdan, C., Gessner, A., and Lohoff, M. (1999). Immunity to Protozoa. In: *Nature Encyclopedia of Life Sciences*. London: Nature Publishing Group. http://www.els.net

Rosenberg, R., Wirtz, R. A., Schneider, I., and Burge, R. (1990). An Estimation of the Number of Malaria Sporozoites Ejected by a Feeding Mosquito. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **84**, 209-212.

Roubinian, J. (1980). Selected surgical procedures. In: Mishel BB, Shiigi SM, eds. Selected methods in cellular immunology. San Francisco: W. H. Freeman and Company, 325-342.

Rudolph, A. M. and Heymann, M. A. (1967). Circulation of Fetus in Utero - Methods for Studying Distribution of Blood Flow Cardiac Output and Organ Blood Flow. *Circulation Research* **21**, 163-184.

Salvesen, G. S., and Dixit, V. M. (1997). Caspases: intracellular signaling by proteolysis. *Cell* **91**, 443-446.

Sasaki, T., Larsson, H., Tisi, D., Claesson-Welsh, L., Hohenester, E., and Timpl, R. (2000) Endostatins derived from collagens XV and XVIII differ in structural and binding properties, tissue distribution and anti-angiogenic activity. *Journal of Molecular Biology* **301**, 1179-1190.

Sayles, P. C. and Wassom, D. L. (1988). Immunoregulation in Murine Malaria - Susceptibility of Inbred Mice to Infection with *Plasmodium yoelii* Depends on the Dynamic Interplay of Host and Parasite Genes. *Journal of Immunology* **141**, 241-248.

Sayles, P. C., Cooley, A. J., and Wassom, D. L. (1991). A Spleen Is Not Necessary to Resolve Infections with *Plasmodium yoelii*. *American Journal of Tropical Medicine and Hygiene* **44**, 42-48.

Sayles, P. C., and Rakhmilevich, L. (1996). Exacerbation of *Plasmodium chabaudi* malaria in mice by depletion of TCR alpha beta(+) T cells, but not TCR gamma delta(+) T cells. *Immunology* **87**, 29-33.

Schuurs, A. H. W. M., and Verheul, H. A. M. (1990). Effects of Gender and Sex Steroids on the Immune Response. *Journal of Steroid Biochemistry and Molecular Biology* **35**, 157-172.

Schwarzer, E., Kuehn, H., Valente, E., and Arese, P. (2003a). Malaria-parasitized erythrocytes and hemozoin nonenzymatically generate large amounts of hydroxy fatty acids that inhibit monocyte functions. *Blood* **101**, 722-728.

Schwarzer, E., Kuehn, H., and Arese, P. (2003b). The hidden faces of hemozoin and its dangerous midwives. *Trends in Parasitology* **19**, 197-198.

Shah, N., Johnson, K., and Ghaly, S. (2003). Fever of unknown origin. *Journal of National Medical Association* **95**, 1099-1102.

Shear, H. L., Srinivasan, R., Nolan, T., and Ng, C. (1989). Role of IFN-Gamma in Lethal and Nonlethal Malaria in Susceptible and Resistant Murine Hosts. *Journal of Immunology* **143**, 2038-2044.

Sherman, I. W. (1999). Plasmodium. In: *Nature Encyclopedia of Life Sciences*. London: Nature Publishing Grouphttp://www.els.net/

Silverman, P. H., Schooley, J. C., and Mahlmann, L. J. (1987). Murine Malaria Decreases Hematopoietic Stem-Cells. *Blood* **69**, 408-413.

Sinnis, P., and Sim, B. K. L. (1997). Cell invasion by the vertebrate stages of Plasmodium. *Trends in Microbiology* **5**, 52-58.

Smith, E. C., and Taylor-Robinson, A. W. (2003). Parasite-specific immunoglobulin isotypes during lethal and non-lethal murine malaria infections. *Parasitology Research* **89**, 26-33.

Smith, N. C., Favila-Castillo, L., Monroy-Ostria, A., Hirunpetcharat, C., and Good, M. F. (1997). The spleen, IgG antibody subsets and immunity to *Plasmodium berghei* in rats. *Immunology and Cell Biology* **75**, 318-323.

Steinman, R. M., Pack, M., and K., Inaba. (1997). Dendritic cells in the T-cell areas of lymphoid organs. *Immunology Review* **156**, 25-37.

Sternlieb, I. (1984). Wilson's disease: indications for liver transplants. *Hepatology* **4**, 15s-17s

Stimson, W. H. (1987). Sex steroids, steroid receptors and immunity. In Hormones and immunity, eds. I. Berczi & K. Kovacs, p. 43-53, MTP Press, Lancaster.

Stevenson, M. M., Tarn, M.F., and Rae, D. (1990). Appearance of crisis forms during *Plasmodium chabaudi* infection: dependence on cell mediated mechanisms. *Microbial Pathogenesis* **9**, 303-314.

Sullivan, A. D., Ittarat, I., and Meshnick, S. R. (1996). Patterns of haemozoin accumulation in tissue. *Parasitology* **112**, 285-294.

Taylor-Robinson, A. W., Phillips, R. S., Severn, A., Moncada, S., and Liew, F. Y. (1993). The Role of T(H)1 and T(H)2 Cells in a Rodent Malaria Infection. *Science* **260**, 1931-1934.

Van der Heyde, H. C., Pepper, B., Batchelder, J., Cigel, F., and Weidanz, W. P. (1994). The time course of selected malarial infections in cytokine-deficient mice. *Experimental Parasitology* **85**, 206-213

Van Parijs, L. and Abbas, A. K. (1998). Homeostasis and self-tolerance in the immune system: Turning lymphocytes off. *Science* **280**, 243-248.

Villeval, J. L., Gearing, A., and Metcalf, D. (1990). Changes in Hematopoietic and Regulator Levels in Mice During Fatal or Nonfatal Malarial Infections. 2. Nonerythroid Populations. *Experimental Parasitology* **71**, 375-385.

Von der Weid, T., Honarvar, N., and Langhorne, J. (1996). Gene-targeted mice lacking B cells are unable to eliminate a blood stage malaria infection. *Journal of Immunology* **156**, 2510-2516.

Vreden, S. G. S. (1994). The Role of Kupffer Cells in the Clearance of Malaria Sporozoites from the Circulation. *Parasitology Today* **10**, 304-308.

Waki, S., Uehara, S., Kanbe, K., Nariuch, H., and Suzuki, M. (1995). Interferon-Gamma and the Induction of Protective IgG2a Antibodies in Nonlethal Plasmodium-Berghei Infections of Mice. *Parasite Immunology* **17**, 503-508.

Wang, C., Playmate, S., Nieschlag, E., and Paulsen, C. A. (1981). Salivary Testosterone in Men - Further Evidence of a Direct Correlation with Free Serum Testosterone. *Journal of Clinical Endocrinology and Metabolism* **53**, 1021-1024.

Wang, X. H., Cheng, F., Zhang, F., Li, X. C., Qian, J. M., Kong, L. B., Zhang, H., Li, G. Q. (2003). Copper metabolism after living related liver transplantation for Wilson's disease. *World Journal of Gastroenterol*ogy **9**, 2836-2838.

Weiss, L., Geduldig, U., and Weidanz, W. (1986). Mechanisms of splenic control of murine malaria: reticular cell activation and the development of a blood-spleen barrier. *American Journal of Anatomy* **176,** 251-285.

Weiss, L. (1989). Mechanisms of Splenic Control of Murine Malaria - Cellular Reactions of the Spleen in Lethal (Strain-17Xl) *Plasmodium yoelii* Malaria in Balb/C Mice, and the Consequences of Pre-Infective Splenectomy. *American Journal of Tropical Medicine and Hygiene* **41**, 144-160.

Weiss, L., Johnson, J.,and Weidanz, W. (1989). Mechanisms of splenic control of murine malaria: tissue culture studies of the erythropoietic interplay of spleen, bone marrow, and blood in lethal (strain 17XL) *Plasmodium yoelii* malaria in BALB/c mice. *American Journal of Tropical Medicine and Hygiene* **41***,* 135-143

Weiss, L. (1990). The Spleen in Malaria - the Role of Barrier Cells. *Immunology Letters* **25**, 165-172.

White, W. I., Evans, C. B., and Taylor, D. W. (1991). Antimalarial Antibodies of the Immunoglobulin G2a Isotype Modulate Parasitemias in Mice Infected with *Plasmodium-Yoelii*. *Infection and Immunity* **59**, 3547-3554.

Wolenski, M., Cramer, S. O., Ehrlich, S., Steeg, C., Fleischer, B., and von Bonin, A. (2003) Enhanced Activation of CD83-Positive T Cells. *Scandinavian Journal of Immunol*ogy **58**, 306-11.

World Health Organization (WHO) (1996). World malaria situation in (1993), part I. *Weekly Epidemiological Record* **71**, 17-22.

WHO, (2000). Expert Committee on Malaria - Twentieth Report**.** 2000, World Health Organization: Geneva.

Wu, Q., Sun, Y. L., Wang, J., Lin, X. Q., Wang, Y., Pegg, L. E., Futterer, A., Pfeffer, K., and Fu, Y. X. (2001). Signal via Lymphotoxin-beta R on bone marrow stromal cells is required for an early checkpoint of NK cell development. *Journal of Immunology* **166**, 1684-1689.

Wunderlich, F., Stuebig, H., and Koenigk, E. (1982). Development of *Plasmodium chabaudi* in Mouse Red Blood Cells - Structural-Properties of the Host and Parasite Membranes. *Journal of Protozoology* **29**, 60-66.

Wunderlich, F., Schillinger, G., and Helwig, M. (1985). Fractionation of *Plasmodium-chabaudi*-Infected Erythrocytes into Parasites and Ghosts. *Zeitschrift fuer Parasitenkunde-Parasitology Research* **71**, 545-551.

Wunderlich, F., Mossmann, H., Helwig, M., and Schillinger, G. (1988). Resistance to *Plasmodium chabaudi* in B10 Mice - Influence of the H-2-Complex and Testosterone. *Infection and Immunity* **56**, 2400-2406.

Wunderlich, F., Marinovski, P., Benten, W. P. M., Schmitt Wrede, H-P., and Mossmann, H. (1991). Testosterone and other Gonadal Factor(S) Restrict the Efficacy of Genes Controlling Resistance to *Plasmodium chabaudi* Malaria. *Parasite Immunology* **13**, 357-367.

Wunderlich, F., Benten, W. P. M., Bettenhaeuser, U., Schmitt Wrede, H-P., and Mossmann, H. (1992). Testosterone-Unresponsiveness of Existing Immunity Against *Plasmodium chabaudi* Malaria. *Parasite Immunology* **14**, 307-320.

Wunderlich, F., Maurin, W., Benten, W. P. M., and Schmitt Wrede, H-P. (1993). Testosterone Impairs Efficacy of Protective Vaccination Against *Plasmodium chabaudi* Malaria. *Vaccine* **11**, 1097-1099.

Wyler, D. J., Oster, C. N., and Quinn, T. C. (1979). Role of the Spleen in the Immunology of Parasitic Diseases. Basel: Schwabe; *Tropical Diseases Research Series No. 1.* 183-204.

Wyler, D. J., Quinn, T. C., and Chen, L. T. (1981). Relationship of alterations in splenic clearance function and microcirculation to host defense in acute rodent malaria. *Journal of Clinical Investigation* **67,** 1400-1404.

Wyler, D. J. (1983). Splenic Functions in Malaria. *Lymphology* **16**, 121-127.

Xu, Q., Lu, Z. H., and Zhang, X. M. (2002). A novel role of alkaline phosphatase in protection from immunological liver injury in mice. *Liver* **22**, 8-14.

Yadava, A., Kumar, S., Dvorak, J. A., Milon, G., and Miller, L. H. (1996). Trafficking of *Plasmodium chabaudi adami*-infected erythrocytes within the mouse spleen. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 4595-4599.

Yang-Xin, F. U. and Chaplin, D. D. (1999). Development and maturation of secondary lymphoid, *Annual Review of Immunol*ogy **17**, 399-433.

Yap, G. S. and Stevenson, M. M. (1992). *Plasmodium chabaudi* AS - Erythropoietic Responses During Infection in Resistant and Susceptible Mice. *Experimental Parasitology* **75**, 340-352.

Yap, G. S. and Stevenson, M. M. (1994). Differential Requirements for an Intact Spleen in Induction and Expression of B-cell-Dependent Immunity to *Plasmodium chabaudi* As. *Infection and Immunity* **62**, 4219-4225.

Zhai, Y. F., Guo, R. B., Hsu, T. L., Yu, G. L., Ni, J., Kwon, B. S., Jiang, G. W., Lu, J. M., Tan, J., Ugustus, M., Carter, K., Rojas, L., Zhu, F., Lincoln, C., Endress, G., Xing, L. L., Wang, S., Oh, K. O., Gentz, R., Ruben, S., Lippman, M. E., Hsieh, S. L., and Yang, D. J. (1998). LIGHT, a novel ligand for lymphotoxin beta receptor and TR2/HVEM induces apoptosis and suppresses in vivo tumor formation via gene transfer. *Journal of Clinical Investigation* **102**, 1142-1151.

Zhang, J. M., Krugliak, M., and Ginsburg, H. (1999). The fate of ferriprotoporhyrin IX in malaria infected erythrocytes in conjunction with the mode of action of antimalarial drugs. *Molecular and Biochemical Parasitology* **99**, 129-141.

8 ABBREVIATIONS

7-AAD	7 amino-actinomycin D
AP	Alkaline phosphatase
ATF	activating transcription factor
B6	C57BL/6
Bp	base pair (s)
BSA	bovine serum albumin
cAMP	cyclic adenosine 3', 5', -monophosphate
cpm	counts per minute
CSP	Circumsporozoite protein
DEPC	diethyl pyrocarbonate
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DUSP	dual specificity protein phosphatase
EDTA	ethylene diaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ECM	Extracellular matrix
FACS	fluorescent activated cell scanner
FCS	fetal calf serum
FDC	Follicular dendritic cell
FITC	fluorescein isothiocyanate
FRA	fos-related antigen
GC	Germinal center
HEPES	N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid

IFN-γ	Interferon-γ
IGF	insulin-like growth factor
IGFBP	IGF-binding protein
IL2RB	interleukin 2 receptor B
LTßR$^{-/-}$	Lymphotoxin beta receptor deficient mice
MMP	matrix metalloprotease
MZ	Marginal zone
iNOS	Inducible nitric oxide synthase
OD	Optical density
PALS	Periarteriolar lymphoid sheath
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
p.i.	post infection
PIQOR^{TM}	Parallel identification and quantification of RNAs
pfEMP1	*Plasmodium falcibarum* erythrocyte membrane protein 1
RNA	ribonucleic acid
RNase	ribonuclease
rpm	rounds per minute
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SAA	serum amyloid A
SHBG	Sex hormone binding globulin
SOCS	suppressor of cytokine signaling

STAT	signal transducer and activator of transcription
T	testosterone
TCR	T cell receptor
TEMED	*N,N,N',N'*-tetramethylethylenediamine
TIMP	tissue inhibitor of MMP
TNF	Tumour necrosis factor
Tris	Tris-(hydroxylmethyl) aminomethane
U	unit (s)

9 ACKNOWLEDGMENT

First and foremost thanks are to **Allah** who helped me to accomplish this work.

Words will never be able to express my deepest gratitude to all those who helped me to make this work possible.

I would like to express my deep gratitude and sincere thanks to **Prof. Dr. Frank Wunderlich,** Head of the Division of Molecular Parasitology, Faculty of Natural Science and Mathematics, Heinrich-Heine-University, Düsseldorf, Germany, for his good supervision, kind advice and support during the work.

My thanks also extends to **Prof. Dr. Mehlhorn,** Head of the Institute of Zoomorphology, Cell biology and Parasitology, Faculty of Natural Science and Mathematics, Heinrich-Heine-University, Düsseldorf, Germany for acting as my referee.

I am deeply indebted to **Dr. Jürgen Krücken,** Division of Molecular Parasitology, Faculty of Natural Science and Mathematics, Heinrich-Heine-University, Düsseldorf, Germany, for giving much of his supervision and for giving much of his precious experience.

I do feel greatly indebted to **Dr. W. Peter M. Benten,** Division of Molecular Parasitology, Faculty of Natural Science and Mathematics, Heinrich-Heine-University, Düsseldorf, Germany, for his generous help during this work.

I am appreciated and grateful to **Dr. Hans-Peter Schmitt-Werde,** Division of Molecular Parasitology, Faculty of Natural Science and Mathematics, Heinrich-Heine-University, Düsseldorf, Germany, for his intimate encouragement and continuous advice.

I would like to thank all the stuff members of the Division of Molecular Parasitology, Faculty of Natural Science and Mathematics, Heinrich-Heine-University, Düsseldorf, Germany, specially Mrs. **Andrea Grunwald** and Mr. **Predrag Marinovski** for their great help.

My thanks are also extended to my Master supervisors in Egypt and the Egyptian government which allowed me to establish this work in Germany.

My foremost and greatest grateful thanks go to my father, mother and brothers for their patience and interest that stimulated the production of this thesis.

Special cordial acknowledgement and warm grateful thanks go to my wife for her support and valuable help during the present work.

10 LEBENSLAUF

Persönalisches

Name	Mohamed Abdel-Monem Mohamed Dkhil Hamad
Geburtsdatum	22. Juni 1972
Geburtsort	Giza/Ägypten
Wohnort	Giza
Nationalität	ägyptisch
Familienstand	Verheiratet, 2 kinder

Schulische Ausbildung

Sept. 1978 - Jul. 1984	Grundschule/Giza (Ägypten)
Sept. 1984 - Jul. 1987	Mittelschule/Giza (Ägypten)
Sept. 1987 - Jul. 1990	Gymnasium/Giza (Ägypten)

Hochschulausbildung

Sept. 1990 - Jul. 1994	Studium der Zoologie an der Wissenschaftlichen Fakultät, Kairo Universiät/Ägypten
Jul. 1994	Bachelor of Science (B.Sc. Zoology)

Studium

Sept. 1995 - Juli 1999	Master degree in Biology
Seit September 2000	Anfertigung der Dissertation in der Abteilung für Molekulare Parasitologie an der Heinrich-Heine-Universität, Düsseldorf