Resistance/Susceptibility to *Plasmodium chabaudi* malaria

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Vorwort

Herr Denis Delić hat seine experimentelle kumulative Dissertation mit dem Thema "Resistance/Susceptibility to *Plasmodium chabaudi* malaria" angefertigt. Grundlage für die kumulative Dissertation sind folgende Publikationen:

1. Publikation:

Krücken J, **Delić D**, Pauen H, Wojtalla A, El-Khadragy M, Dkhil MA, Mossmann H & Wunderlich F 2009 Augmented particle trapping and attenuated inflammation in the liver by protective vaccination against *Plasmodium chabaudi* malaria. *Malaria Journal* **8** 54. PMID:19341445

2. Publikation:

Delić D, Warskulat U, Borsch E, Al-Qahtani S, Al-Quraishi S, Häussinger D & Wunderlich F 2010 Loss of ability to self-heal malaria upon taurine transporter deletion. *Infection and Immunity* **78** 1642-1649. PMID: 20100858

3. Publikation:

Delić D, Grosser C, Dkhil M, Al-Quraishy S & Wunderlich F 2010 Testosterone-induced upregulation of miRNAs in the female mouse liver. *Steroids* **75** 998-1004. PMID: 20600201

4. Publikation:

Delić D, Gailus N, Vohr HW, Dkhil M, Al-Quraishi S & Wunderlich F 2010 Testosterone-induced permanent changes of hepatic gene expression in female mice sustained during *Plasmodium chabaudi* malaria infection. *Journal of Molecular Endocrinology* **45** 379-390. PMID: 20844152

5. Publikation:

Delić D, Dkhil M, Al-Quraishi S & Wunderlich F 2010 Hepatic miRNA expression reprogrammed by *Plasmodium chabaudi* malaria. *Parasitology Research* [Epub ahead of print]. PMID: 21085987

6. Publikation:

Delić D, Dkhil M, Al-Quraishi S & Wunderlich F 2010 Attenuated responiveness to testosterone of hepatic gene expression in female mice immune *Plasmodium chabaudi* malaria. *Journal of Molecular Endocrinology* [submitted]

7. Publikation:

Wunderlich CM, **Delić D**, Behnke K, Stroehle P, Wunderlich F, Brüning J & Wunderlich FT 2010 Disrupted IL-6 trans-signaling augments ability to self-heal malaria [to be submitted]

weitere Publikationen:

8. Publikation:

Dkhil M, Abdel-Baki AA, **Delić D**, Wunderlich F, Sies H & Al-Quraishi S 2010 *Eimeria papillata*: Upregulation of specific miRNA-species in the mouse jejunum. *Experimental Parasitology* [Epub ahead of print] PMID: 21093440

9. Publikation:

Dkhil M, **Delić D**, Abdel-Baki AA, Wunderlich F & Al-Quraishi S 2010 Differential miRNA expression in the mouse jejunum during garlic treatment of *Eimeria papillata* infections. *Parasitology Research* [submitted]

1. Introduction

1.1 Malaria – key facts

Malaria is the most important parasitic disease of man which is caused by a eukaryotic protist of the genus *Plasmodium*, a member of the phylum Apicomplexa, and which is naturally transmitted *via* the bite of a female *Anopheles* mosquito. Malaria occurs in 108 countries worldwide, i. e. approximately 40% of the global population live in high risk areas. In 2008, there were 243 million cases of malaria worldwide, whereas the vast majority cases (85%) were in sub-Saharan Africa, followed by the South-East Asia (10%) and Eastern Mediterranean Region (4%) (Fig. 1). Malaria accounted for 863,000 deaths in 2008, the majority of whom are children under 5 years of age (WHO, 2009). In sub-Saharan Africa a child dies every 45 seconds of malaria so that the disease accounts for 20% of all childhood deaths. Moreover, pregnant women are at higher risk to suffer from malaria.

During the last six years, the commitments for malaria control are fivefold increased, from approximately US$ 0.3 billion per year in 2003 to US$ 1.7 billion in 2009. Interventions to control malaria are mainly based on diagnosis and treatment of malaria, including preventive medications. A wide variety of anti-malarial drugs are available to date, in particular based on a combination of drugs containing an arteminisine-derivate. Furthermore, malaria prevention is achieved by mosquito control using insecticide-treated nets and indoor residual spraying. Indeed, the number of cases and death per capita is reduced by 50% or more between 2000 and 2010 and expectedly by 75% or more between 2000 and 2015 (WHO, 2009).

Four species of *Plasmodium* are pathogenic for humans: *P. falciparum*, *P. vivax*, *P. ovale,* and *P. malariae. P. falciparum* is responsible for the majority of malaria-induced deaths associated with malaria. Moreover, two or more human plasmodial species can infect an individual and the parasites can coexist, interact with each other, and exert cross-species regulatory effects (Snounou 2004).

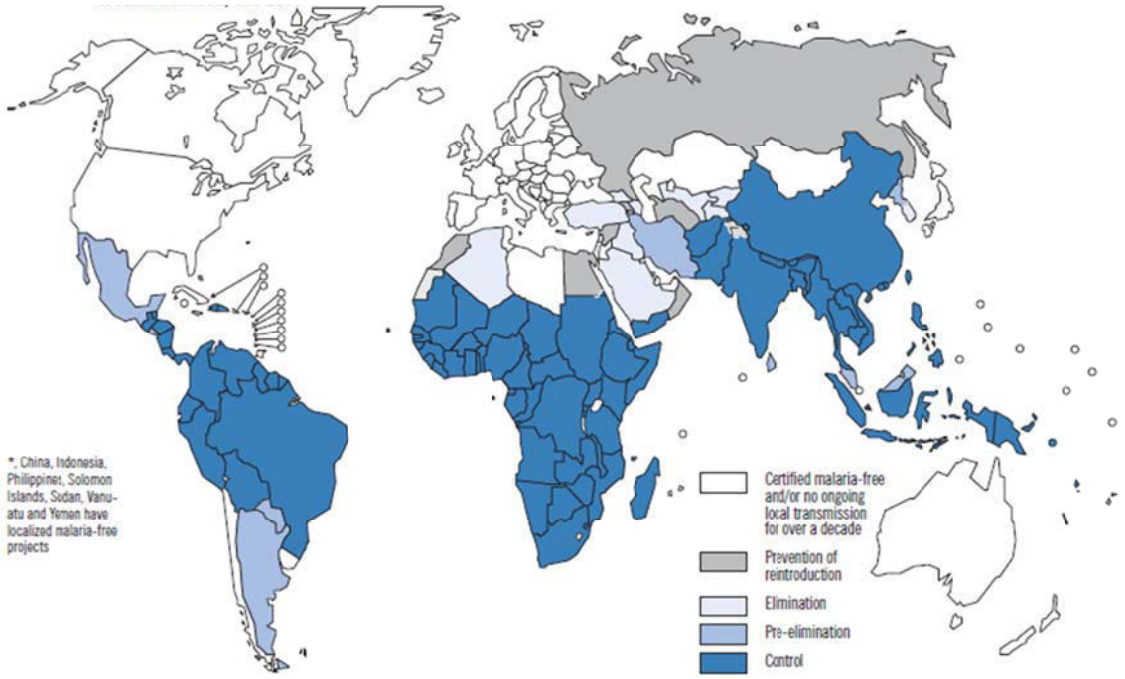

Figure 1. Malaria-free countries and malaria-endemic countries in phases of control*, pre-elimination, elimination and prevention of reintroduction, end 2008 (from WHO, 2009).

The malaria parasite exhibits a complex life cycle involving an insect vector (*Anopheles*) and a vertebrate host (human). The sporozoites are inoculated from the saliva of the biting insects into the human during blood feeding. After reaching the liver through the bloodstream, parasites invade hepatocytes, which may even endure less than 30 min. The intracellular parasite undergoes an asexual replication termed pre-erythrocytic schizogony. Each hepatic schizont ruptures releasing a great number of merozoites which then invade erythrocytes. After intra-erythrocyte multiplication, schizonts then also rupture and merozoites are released, which infect other erythrocytes (erythrocyte schizogony), so that the parasite burden expands logarithmically approximately ten-fold per cycle (Simpson *et al*. 2002, White 2004). These asexual blood stages are responsible for the severe pathology, morbidity and mortality associated with malaria.

After several asexual cycles, some merozoites invade red blood cells and develop into gametocytes, either male flagellated microgametocytes or female macrogametocytes (gamogony). Only these sexual stages can develop in *Anopheles*. After fertilization, the resulting zygote develops into a motile ookinete, which penetrates the midgut and encyst on the basal lamina. The oocyst undergoes multiple rounds of asexual replication resulting in the

production of sporozoites, which finally migrate to the salivary glands of the female *Anopheles* mosquito (sporogony).

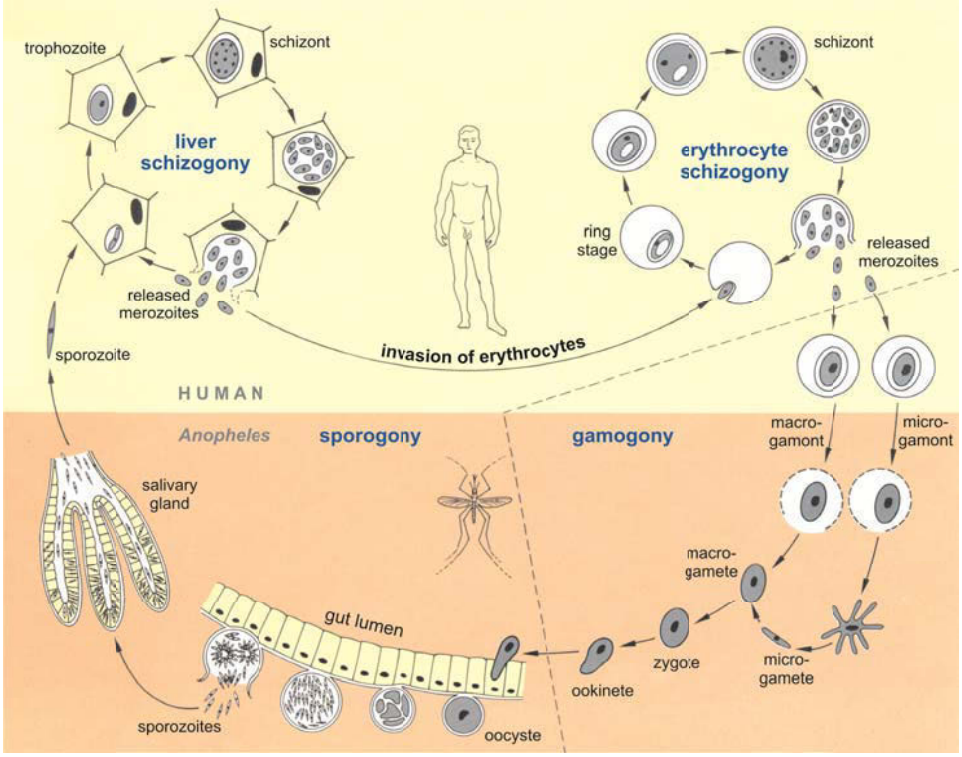

Figure 2. Life cycle of *Plasmodium sp.* The life cycle can be subdivided into three phases: schizogony, gamogony and sporogony (from Wunderlich & Schmitt 1988c).

An infection with malaria is associated with fever and anemia caused by hemolysis. Further symptoms can be hemoglobinuria, hypoglycemia, renal failure, splenomegaly, hepatomegaly, headache and cerebral ischemia (Trampuz *et al.* 2003). The classic symptoms of malaria are cyclical occurrence of sudden coldness followed by rigor and then fever and sweating lasting four to six hours, taking place every two days in *P. vivax* and *P. ovale* infections, while every three days for *P. malariae*. The most dangerous form malaria tropica caused by *P. falciparum* is characterized by recurrent fever every 36-48 hours or even a less pronounced and almost continuous fever. The pathology is due to the asexual blood stages, whereas the pre-erythrocytic and sexual stages cause no organ dysfunction (Mehlhorn 2008). Pathology results from the destruction of erythrocytes, the liberation of parasite and the release of red blood cell material into the circulation, and the dysregulations of the host responses directed against the blood-stages. The occurrence of sequestration of infected erythrocytes impairs blood flow and tissue functions. The severity of infection depends on

both host and parasite factors. Host factors, which are critical for the course of malaria are genotype, age and previous exposure (Kareier 1980, Sherman 1999).

Natural immunity to malaria underlies rather complex control. It is directed against blood-stages of *Plasmodium*, it can completely abolish disease symptoms, but it cannot prevent parasitemia during malaria seasons (Playfair *et al*. 1990, Kwiatkowski 1992). *Plasmodium* infections cause a series of antibody- and cell mediated immune responses. Both T cell- and antibody-mediated responses play a role in protective immunity to malaria. In liver-stage immunity, for example, $CD8^+$ T cells cells are important (Overstreet *et al*. 2008, Tsuji 2010), whereas $CD4^+$ T cells and B cells are essential for blood-stage immunity (Stevenson & Riley 2004, Langhorne *et al*. 2008, Pierce 2009). 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 and class II molecules. T_H1-related cytokines are predominantly produced during the acute phase of infection and are sufficient for antibody-independent parasite control early in infection *via* macrophage-associated inflammatory responses (Langhorne *et al.* 2008). Further progression of the infection is associated with a switch towards a T_H2-related response with antibody production by mature B cells, so that the later stages of infection are predominantly antibody-dependent. Immunoglobulin (Ig) G1 and IgG3 appear to be most effective in humans (Groux & Cysin 1990), while IgG2a is essential in the mouse model (White *et al*. 1991).

Despite enormous efforts during the last 30 years, all attempts to develop a safe and effective vaccine against malaria have failed to date: no effective vaccine is yet available! Several types of malaria vaccines have been developed which can be classified according to the target parasite stage. (i) Pre-erythocytic vaccines are directed against the infective sporozoites, in particular vaccines based on the surface protein circumsporozoite protein (CSP), so that this anti-sporozoite vaccine is expected to prevent infection. (ii) Anti-merozoite vaccines would not prevent infection, but would reduce malaria morbidity and mortality. (iii) Transmission-blocking vaccines (TBV) are directed against sexual blood-stages and would prevent the development of parasites in the mosquito, thus reducing transmission in endemic areas. However, there are also three major difficulties hindering the development of a vaccine for malaria. Firstly, for all three stages of the parasite life cycle, there is still an incomplete understanding of the precise type of immune response. Secondly, only a few of the many hundreds of parasite-derived antigens have been explored in animal models to date. Thirdly,

there is strong evidence that the parasite can evade host immunity, for example by antigenic variation (Playfair *et al*. 1990, Good & Doolan 2010).

1.2 Resistance/Susceptibility to *Plasmodium chabaudi* malaria

Since the first human vaccination trials have failed in the middle of the 80ies of the last century, basic research about malaria is urgently required more than ever. A convenient model to study the role of malaria is the murine malaria *P. chabaudi*, which shares several common characteristics with the most dangerous human parasite *P. falciparum*. Both *P. falciparum* and *P. chabaudi* prefer normocytes as host cells (Pasvol *et al*. 1980, Jarra & Brown 1989, Clough *et al*. 1998). Both species exhibit cytoadherence and sequestration of the schizont stages from peripheral circulation to internal microvasculature, which are considered as a major virulence factor (Roberts *et al*. 2000, Pain *et al*. 2001). Moreover, both malaria species induce strain-specific immunity (Snounou *et al*. 1989, Mota *et al*. 1998) and display clonal antigen variation (Biggs *et al*. 1991, Roberts *et al*. 1992).

Using this mouse malaria model, it has been revealed that mice are able to self-heal blood-stage infections, which is controlled by both genes of the *H-2* complex and the *non-H-2* background (Wunderlich *et al*. 1988b, 1991). Female mice with the *H-2*^{d} haplotype on BALB- or DBA-background were susceptible, while strains with the same *H-2*^{d} haplotype on B6- or B10-background were resistant (Wunderlich *et al*. 1988b). Further investigations using mouse strains with different *H2*-complex on the same B10-background showed different levels of resistance to *P. chabaudi* in female mice. Resistant strains were those with b, d and o2 *H-2* haplotype, in which mice survived the infection, and mice with the *H2*-haplotypes k, a and h4 were susceptible (Wunderlich *et al*. 1988b). Furthermore, several genes and/or loci of the non-*H-2* background have been already reported to be critical for a fatal outcome of malaria (Foote *et al*. 1997, Fortin *et al*. 2001, Hernandez-Valladares *et al*. 2004, Kwiatkowki *et al*. 2000, Min-Oo *et al*., 2007, Roberts *et al*. 2001).

Remarkably, ′gene-independent′ soluble ′environmental factors′ such as testosterone (Wunderlich *et al*. 1988, 1991) and estrogen (Benten *et al.* 1992b, 1993) have been also found to be critical for the outcome of *P. chabaudi* malaria. In contrast to female mice, the gene-controlled mechanisms of resistance to *P. chabaudi* did not become evident in male mice, which always succumbed to malarial infections. This gender-dependence of the outcome of

malaria is presumably modulated by sex hormones, especially testosterone (Krücken *et al*. 2005). Indeed, castration of male B10 mice entailed a dramatic increase in survival. Castrated males, however, became susceptible again, when pre-treated with testosterone (Wunderlich *et al*. 1988b, 1991). Also, testosterone pre-treatment of female mice caused susceptibility (Wunderlich *et al*. 1988b, 1991). By contrast immune mice, which have survived a *P. chabaudi* infection, are not affected by testosterone at all (Wunderlich *et al*. 1992).

1.3 Project objectives

The present work is aimed at investigating several ′non-genic′ factors of the host response which cause resistance and/or susceptibility to *Plasmodium chabaudi* blood-stage malaria in female mice, with particular emphasis on the liver as an anti-malaria effector site.

Specific aims are:

- to evaluate those protein-encoding genes in the liver which become deregulated by testosterone and which remain persistently deregulated after testosterone withdrawal, even upon malaria infection;

- to investigate the effect of testosterone on the miRNA signature in the liver;
- to identify possible changes of the miRNA signature in the liver of mice upon acquiring protective immunity;
- to detect the responsiveness to testosterone of hepatic gene expression in mice having acquired protective immunity to blood-stage malaria;

- to characterize the role of taurine and IL-6 trans-signaling on the outcome of blood-stage malaria;

- to identify the effect of protective vaccination against blood-stages of *P. chabaudi* on the trapping capacity and gene expression of the liver.

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2. Results

2.1 Loss of ability to self-heal malaria upon taurine transporter deletion

Background: Taurine is the most abundant free amino acid in mammalian tissues, which is not incorporated into proteins, and the uptake of dietary taurine *via* the taurine transporter TAUT is the major route for taurine provision. Taurine is an important modulator of immune responses and its role for the outcome of blood-stage malaria was investigated in this study by comparing surviving *taut*$^{+/+}$ mice and *taut*$^{-/-}$ mice, which succumbed to infection with *Plasmodium chabaudi*.

Methods: Female *taut*$^{+/+}$ and *taut*$^{-/-}$ mice were infected with 10^6 *P. chabaudi*-paratisized erythrocytes. Determination of hepatic particle trapping was done using green fluorescent beads. Hepatic inflammatory and metabolism markers were detected by analyzing the mRNA levels by qRT-PCR and the protein levels in blood by enzyme-linked immunosorbent assay (ELISA). Histopathology of different tissues was performed in *taut*$^{-/-}$ mice that succumbed to *P. chabaudi* malaria.

Results: Deletion of the taurine transporter (TAUT) resulted in lethal outcome of otherwise self-healing *P. chabaudi* malaria, whereas peak parasitemia is increased from approximately 40% in *taut*$^{+/+}$ mice to approximately 65% in *taut*$^{-/-}$ mice. The circulating taurine levels are lowered in *taut*$^{-/-}$ from 540 to 264 µmol/liter. Deletion of *taut*$^{-/-}$ is characterized by increased systemic ammonia levels, a perturbed particle trapping capacity, and more increased mRNA expression of TNF-α, IL-1β, IL-6, iNOS, NF-κB, and VDR upon infection on day 8 *p.i.*. In blood, the levels of TNF-α, IL-1β, and ammonia were higher increased in *taut*$^{-/-}$ mice at peak parasitemia than in *taut*$^{+/+}$ mice. Autopsy of different tissues of succumbed *taut*$^{-/-}$ mice revealed multi-organ failure.

Conclusion: These data show for the first time that the *taut* gene and taurine homeostasis are essential for self-healing of *P. chabaudi* blood-stage malaria.

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Loss of Ability To Self-Heal Malaria upon Taurine Transporter Deletion▽†

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Deletion of the taurine transporter gene (*taut*) results in lowered levels of taurine, the most abundant amino acid in mammals. Here, we show that *taut*$^{-/-}$ mice have lost their ability to self-heal blood-stage infections with *Plasmodium chabaudi* malaria. All *taut*$^{-/-}$ mice succumb to infections during crisis, while about 90% of the control *taut*$^{+/+}$ mice survive. The latter retain unchanged taurine levels even at peak parasitemia. Deletion of *taut*, however, results in the lowering of circulating taurine levels from 540 to 264 μmol/liter, and infections cause additional lowering to 192 μmol/liter. Peak parasitemia levels in *taut*$^{-/-}$ mice are approximately 60% higher than those in *taut*$^{+/+}$ mice, an elevation that is associated with increased systemic tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β) levels, as well as with liver injuries. The latter manifest as increased systemic ammonia levels, a perturbed capacity to entrap injected particles, and increased expression of genes encoding TNF-α, IL-1β, IL-6, inducible nitric oxide synthase (iNOS), NF-κB, and vitamin D receptor (VDR). Autopsy reveals multiorgan failure as the cause of death for malaria-infected *taut*$^{-/-}$ mice. Our data indicate that *taut*-controlled taurine homeostasis is essential for resistance to *P. chabaudi* malaria. Taurine deficiency due to *taut* deletion, however, impairs the eryptosis of *P. chabaudi*-parasitized erythrocytes and expedites increases in systemic TNF-α, IL-1β, and ammonia levels, presumably contributing to multiorgan failure in *P. chabaudi*-infected *taut*$^{-/-}$ mice.

Taurine, a nonprotein sulfur-containing amino acid, is the most abundant amino acid in mammals, occurring both in cells and in blood plasma (7, 22). It plays an important role in diverse biological processes, such as cell volume regulation, neuromodulation, antioxidant defense, protein stabilization, and stress responses (11, 16, 46, 48, 54). Taurine may protect cells against various types of injury (11, 16, 29, 38, 45, 48, 54, 56). In particular, taurine is considered a basic regulator of cell homeostasis, presumably as an osmolyte and chaperone (19). According to the current view, taurine is synthesized from cysteine primarily—if not exclusively—in hepatocytes, and it is then exported to the plasma and imported into other cells via the taurine transporter (22).

The disruption of the taurine transporter gene (*taut*) by homologous recombination causes reductions of 80 to 98% in taurine levels in plasma and blood cells, as well as in tissues such as the brain, the kidney, and skeletal and heart muscles (20, 50, 51). Moreover, *taut*$^{-/-}$ mice exhibit decreased resistance to osmotic shock and oxidation stress (31). Taurine levels in the liver are decreased by about 70% in adult *taut*$^{-/-}$ mice; in particular, they are decreased by more than 80% in Kupffer and sinusoidal endothelial cells and by only approximately 30% in liver parenchymal cells (50). Taurine availability is an important modulator of Kupffer cell functions, such as phagocytosis and eicosanoid synthesis (52). *taut*-deficient mice develop moderate unspecific hepatitis and liver fibrosis at older than 1 year (50, 51). Moreover, though our knowledge is still poor, some information is available on how the immune system is affected by *taut* depletion (17). For instance, taurine modulates basic functions of leukocytes, such as phagocytosis, prostanoid formation, and cytokine formation (22, 39, 43, 45, 52, 53). However, the consequences of lowered taurine levels for the outcomes of infectious diseases have not been investigated to date.

Malaria is one of the major infectious diseases worldwide, with about 1 million to 3 millions deaths per year (13). The liver plays a central role in malaria: it is the site where the preerythrocytic stages of the malaria parasites have to develop and multiply (in hepatocytes), but it is also the site where the intraerythrocytic stages of the parasites, which are responsible for disease and death, can be trapped and even destroyed (2, 3, 28). Predominantly the Kupffer cells, which constitute approximately 80 to 90% of total macrophages, contribute to the trapping capacity of the liver. Recent evidence obtained in experimental *Plasmodium chabaudi* malaria indicates that during the crisis phase of infection, when parasitemia drops from about 50% to below 1% within 3 to 4 days, the liver improves its trapping capacity, whereas the spleen is largely closed (27).

In the present study, we show that the lowering of taurine levels due to *taut* deletion results in a lethal outcome of oth-

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† Supplemental material for this article may be found at http://iai.asm.org/.

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erwise self-healing blood-stage malaria caused by *P. chabaudi* in mice.

MATERIALS AND METHODS

Animals. Mice with disrupted taurine transporters (*taut*$^{-/-}$) have been generated previously by homologous recombination using embryonic stem (ES) cells of 129/SvJ origin in C57BL/6 mice (20). The same mixed genetic background is present in the *taut*$^{+/+}$ mice. Mice were bred under specific-pathogen-free conditions in our central animal facilities. Experiments were performed with 10- to 14-week-old female mice. They were housed in plastic cages and received a standard diet (Wohrlin, Bad Salzuflen, Germany) and water *ad libitum*. The experiments were approved by the State authorities and followed German law on animal protection.

Blood-stage malaria. We used a nonclonal line of *P. chabaudi* (59) exhibiting a restriction length polymorphism pattern very similar, but not identical, to that of *Plasmodium chabaudi chabaudi* AS (28). Erythrocytic stages of *P. chabaudi* were passaged weekly in NMRI mice. Blood was taken from these mice, and 10^6 *P. chabaudi*-infected erythrocytes were injected intraperitoneally (i.p.) into the *taut*$^{+/+}$ and *taut*$^{-/-}$ mice. Parasitemia was evaluated in Giemsa-stained blood smears. The total number of erythrocytes was determined in a Neubauer chamber.

Liver histology. Five *taut*$^{+/+}$ mice and 5 *taut*$^{-/-}$ mice, all infected with *P. chabaudi*, were killed at peak parasitemia on day 8 postinfection (p.i.) by cervical dislocation. Livers were removed, cut into small pieces, fixed first with 2.5% glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 7.2) at room temperature for 1 h and then with 2% OsO_4 at 4°C for 2 to 3 h, dehydrated in graded solutions of ethanol, and embedded in Spurr's resin. Semithin sections were stained with toluidine blue-borax.

Determination of particle trapping. Mice were injected with 200 μl phosphate-buffered saline (PBS) containing 1.3×10^8 green fluorescent beads (diameter, 3 μm) by the method of Pinkerton and Webber (40), and uptake by the liver after 5 min was measured by a procedure detailed recently (27). In brief, mice were killed by cervical dislocation, and parts of liver lobes were removed and weighed. Then the tissue was dissolved in KOH and 0.5% Tween 80 in ethanol. Red beads were added as an internal control. The samples were then subjected to several extractions, and the purified beads were resuspended in distilled H_2O. Their fluorescence intensity was measured at excitation and emission wavelengths of 450 and 480 nm for green beads and 520 and 590 nm for red beads, respectively (27).

Histopathology. *taut*$^{+/+}$ mice killed by cervical dislocation at peak parasitemia on day 8 p.i. and *taut*$^{-/-}$ mice that had succumbed to infection during crisis were ventrally opened by longitudinal cuts and were then immersed in 4% neutral formaldehyde. Organs were then prepared and embedded in paraffin. Five-micrometer-thick sections were cut, dewaxed with xylene, rehydrated, and stained with hematoxylin and eosin as well as with Giemsa stain for routine morphology. In addition, the sections were washed three times, for 5 min each time, in Tris buffer (pH 6.8), incubated at 4°C for 24 h with 3 μg/ml of a fluorescein isothiocyanate (FITC)-labeled isolectin IB_4 antibody (Sigma, Deisenhofen, Germany), and examined by fluorescence/phase-contrast microscopy for brain microglial cells and stimulated murine macrophages and monocytes. Control sections were stained without IB_4.

RNA isolation. Approximately 250 mg frozen liver was homogenized with an Ultra-Turrax homogenizer in 5 ml Trizol (Peqlab Biotechnologie, Erlangen, Germany) for 1 min. After being mixed with 1 ml chloroform for 15 s, the suspension was incubated for 15 min at room temperature and was centrifuged at $3{,}000 \times g$ for 45 min. After isopropanol precipitation of the supernatant, the pellet was washed twice with 80% ethanol, air dried, and dissolved in 200 μl RNase-free water. RNA concentrations were determined at 260 nm.

qRT-PCR. All RNA samples were treated with DNase (Applied Biosystems, Darmstadt, Germany) for at least 1 h and were then converted into cDNA by following the manufacturer's protocol using the reverse transcription kit (Qiagen, Hilden, Germany). Quantitative real-time PCR (qRT-PCR) was performed using the ABI Prism 7500HT sequence detection system (Applied Biosystems, Darmstadt, Germany) with SYBR green PCR master mix from Qiagen (Hilden, Germany). We investigated the genes encoding the mRNAs for the following proteins: interleukin-1β (IL-1β), tumor necrosis factor alpha (TNF-α), IL-6, nuclear factor κB (NF-κB), inducible nitric oxide synthase (iNOS), vitamin D receptor (VDR), CYP3A11 (cytochrome P450, family 3, subfamily a, polypeptide 11), CYP7A1, SULT2B1 (sulfotransferase family 2B, member 1), UGT1A1 (UDP-glucuronosyltransferase family 1, polypeptide A1), multidrug resistance protein 4 (MRP4), and beta-actin (Actb). All primers used for qRT-PCR were obtained commercially from Qiagen. PCRs were conducted as follows: 2 min at 50°C to activate uracil-*N*-glycosylase (UNG); 95°C for 10 min to deactivate UNG; and 40 cycles at 94°C for 15 s, 60°C for 35 s, and 72°C for 30 s. Reaction specificity was checked by performing dissociation curves after PCR. For quantification, mRNA levels were normalized to those of 18S rRNA. The threshold cycle (C_T) value is the cycle number, selected from the logarithmic phase of the PCR curve, in which an increase in fluorescence above background can be detected. ΔC_T is determined by subtracting the C_T of 18S rRNA from the C_T of the target. The relative mRNA levels in noninfected mice are described as the ratio of the target mRNA copy number to the 18S rRNA copy number ($2^{-\Delta CT}$). The fold induction of mRNA expression on day 8 p.i. was determined using the $2^{-\Delta\Delta C_T}$ method ($-\Delta\Delta C_T = \Delta C_T$ at day 0 p.i. $-\Delta C_T$ at day 8 p.i.).

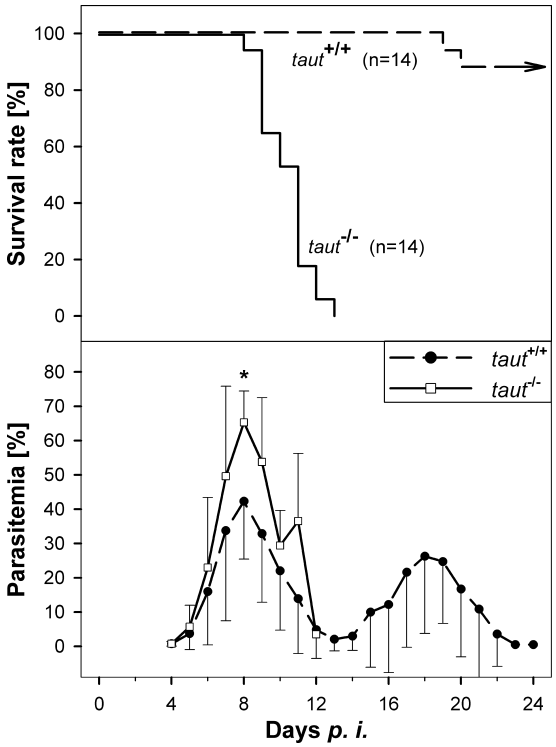

FIG. 1. Course of *P. chabaudi* infections in female *taut*$^{-/-}$ and *taut*$^{+/+}$ mice. Mice were infected with 10^6 *P. chabaudi*-parasitized erythrocytes. *n* stands for the number of mice infected. All values are given as means ± standard deviations. *taut*$^{-/-}$ mice differed significantly from *taut*$^{+/+}$ mice with respect to survival ($P < 0.001$ by Fisher's exact test) and peak parasitemia ($P < 0.01$ by the *t* test).

Blood analysis. Plasma and serum were prepared from blood and were then analyzed as follows. Plasma taurine levels were detected as described previously (20). Levels of ammonia, bilirubin, and bile acids (3α-hydroxysteroid dehydrogenase assay) in plasma, as well as activities of aspartate aminotransferase and alanine aminotransferase, were determined using the standard methods of the International Federation of Clinical Chemistry. In sera, IL-1β, IL-6, and TNF-α levels were measured using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN) according to the manufacturer's protocols. Total NO was analyzed using a commercially available kit (R&D Systems).

Statistical analysis. A two-tailed Student *t* test and a Fisher exact test were used for statistical analysis. Only *P* values of <0.01 were considered to be highly significant.

RESULTS

Course of infections. Blood-stage infections with *P. chabaudi* take a self-healing course in female *taut*$^{+/+}$ mice, i.e., approximately 90% of the challenged *taut*$^{+/+}$ mice survive the infections (Fig. 1). The self-healing course is characterized by a precrisis phase with increasing parasitemia, culminating in

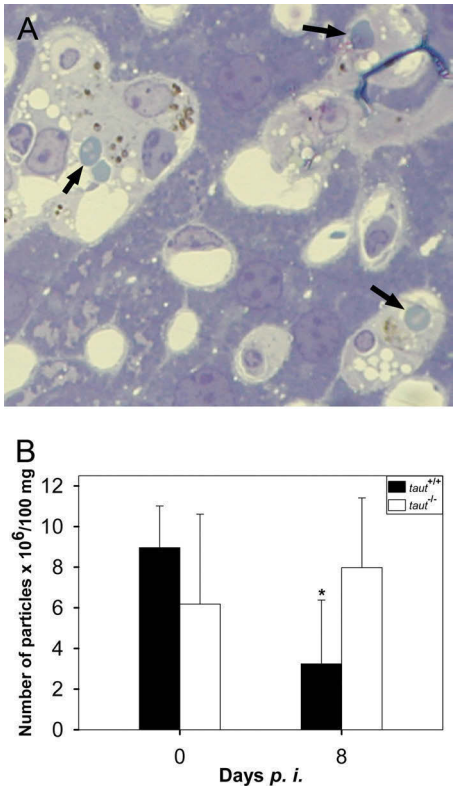

FIG. 2. Liver structure and particle trapping. (A) Light microscopy of liver from a *taut*$^{-/-}$ mouse infected with *P. chabaudi* for 8 days. Arrows indicate parasitized erythrocytes in tight association with Kupffer cells. (B) The trapping capacity of the liver was determined on days 0 and 8 after infection with *P. chabaudi* as described in Materials and Methods. Data are means ± standard deviations for at least 5 different mice.

peak parasitemia of approximately 40% on day 8 p.i., followed by the crisis phase, with parasitemia falling dramatically, below about 2%, within 5 days. Thereafter, a second peak with a parasitemia of approximately 25% occurs on day 18 p.i., followed by the chronic phase of persistent low-grade parasitemia, presumably controlled by protective immune mechanisms.

In contrast to *taut*$^{+/+}$ mice, however, all *taut*$^{-/-}$ mice succumb to infection. Peak parasitemia occurs on day 8 p.i. also but is significantly increased, to approximately 65% (Fig. 1), i.e., there are about 60% more parasitized erythrocytes in *taut*$^{-/-}$ mice than in *taut*$^{+/+}$ mice at peak parasitemia. All *taut*$^{-/-}$ mice die during crisis, between days 8 and 13 p.i. Figure 1 indicates the days of death of the individual mice.

Liver structure and particle trapping. Deletion of the *taut* gene does not result in any visible changes in the structures of the livers of 2-month-old mice at the light microscopic level (50). Upon infection, however, the liver progressively experiences alterations. At peak parasitemia, the tissue reveals some necrotic areas and changes typical of inflammation. Kupffer cells of *taut*$^{-/-}$ mice are apparently more hypertrophic and more overloaded with hemozoin (Fig. 2A) than those of *taut*$^{+/+}$ mice (see Fig. S1 in the supplemental material). It appears that Kupffer cells of *taut*$^{-/-}$ mice are more engaged in phagocytosis than those of *taut*$^{+/+}$ mice, as suggested by an increased content of hemozoin and more-frequent occurrence of tightly associated *Plasmodium*-infected erythrocytes (Fig. 2A; see also Fig. S1 in the supplemental material).

In order to semiquantify possible differences in phagocytic activity between *taut*$^{-/-}$ and *taut*$^{+/+}$ mice *in vivo*, we have determined the trapping capacity of the liver. Figure 2B shows that the livers of noninfected *taut*$^{+/+}$ mice are able to entrap approximately 9×10^6 fluorescent, 3-μm-diameter polystyrene beads per 100 μg of liver. At peak parasitemia, however, this trapping capacity is significantly reduced, by about 60%. In contrast, there is no significant reduction in the specific trapping capacity of the liver in *taut*$^{-/-}$ mice at peak parasitemia (Fig. 2B).

Hepatic gene expression. Quantitative real-time PCR was used to detect changes in the mRNA levels of different genes in the liver. Deletion of the *taut* gene did not affect the expression of the genes tested in noninfected mice. Upon infection, however, there was a significant increase in the mRNA expression of genes in *taut*$^{-/-}$ mice in comparison to that in *taut*$^{+/+}$ mice: IL-1β, TNF-α, IL-6, and iNOS were more highly expressed in *taut*$^{-/-}$ mice. Also, expression of the genes encoding NF-κB and VDR was increased. In contrast, the mRNA expression patterns of CYP3A11, SULT2B1, UGT1A1, MRP4, and Actb in response to malaria did not differ significantly between *taut*$^{-/-}$ and *taut*$^{+/+}$ mice (Fig. 3).

Blood parameters. Deletion of *taut* results in a significant reduction (about 50%) in taurine levels in the plasma (Table 1), in accordance with previous data (20). Also, aspartate transaminase (AST) levels are apparently diminished by about the same percentage (Table 1). However, the concentrations of alanine transaminase (ALT), bile acids, and bilirubin in plasma are not affected by the deletion of *taut*. Also, there is no influence on the levels of IL-1β, IL-6, TNF-α, and total NO in serum (Table 2).

Upon infection, the taurine levels remained unaffected at peak parasitemia in *taut*$^{+/+}$ mice, whereas *taut*$^{-/-}$ mice exhibited a further dropping of the circulating taurine level in comparison to that in noninfected *taut*$^{-/-}$ mice on day 8 p.i. (Table 1). Infection induced strong increases in AST, ALT, bile acid, and bilirubin levels on day 8 p.i., but these parameters were not significantly different between *taut*$^{+/+}$ and *taut*$^{-/-}$ mice. The major significant difference between *taut*$^{+/+}$ and *taut*$^{-/-}$ mice at peak parasitemia was the content of ammonia in plasma, which was increased by about 270% in *taut*$^{+/+}$ mice and by about 400% in *taut*$^{-/-}$ mice (Table 1).

As expected, infections caused increases in the levels of the three cytokines IL-1β, IL-6, and TNF-α, and in total NO levels, in both *taut*$^{+/+}$ and *taut*$^{-/-}$ mice at peak parasitemia (Table 2). Although IL-6 levels increased dramatically at peak parasitemia, the difference between *taut*$^{+/+}$ and *taut*$^{-/-}$ mice was not significant (Table 2). The only significant differences at peak parasitemia were the higher levels of TNF-α and IL-1β in *taut*$^{-/-}$ mice (Table 2).

Histopathology. *P. chabaudi* infections caused increased levels of ammonia in the blood of *taut*$^{-/-}$ mice at peak parasitemia (Table 1), suggesting undesired adverse effects on other organs. The brain is especially sensitive to ammonia intoxication, which eventually leads to hepatic encephalopathy (8, 9, 18, 19, 36). When we examined slices of cerebrum and cerebellum from *taut*$^{-/-}$ mice, which had succumbed to *P.*

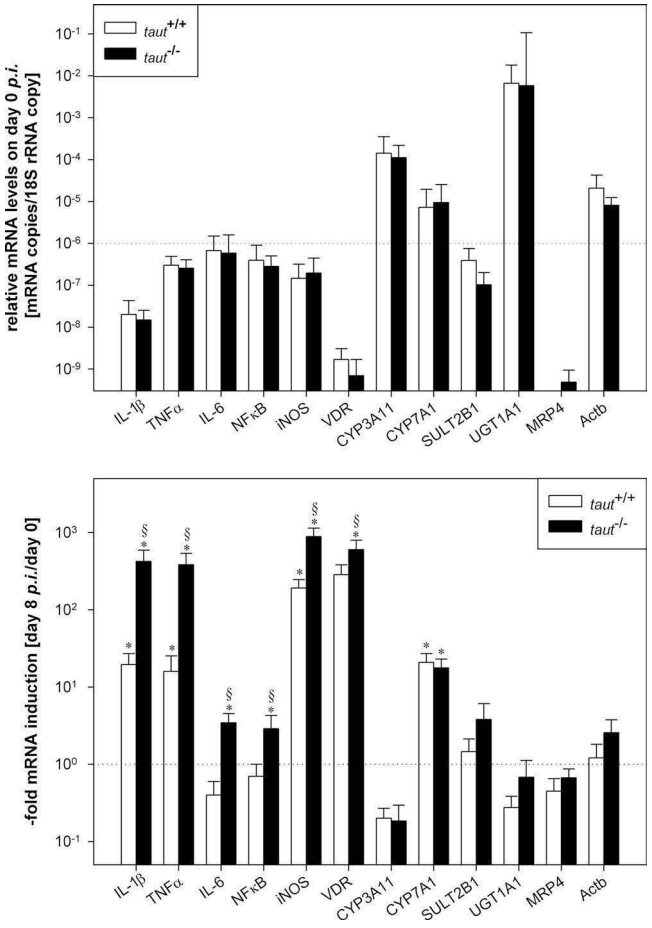

FIG. 3. Hepatic gene expression. The mRNA levels and fold mRNA induction of malaria-relevant genes were determined by quantitative real-time PCR as described in Materials and Methods. Data are means ± standard deviations for 5 different mice. Symbols: *, significant difference between infected and noninfected mice; §, significant difference between *taut*$^{+/+}$ and *taut*$^{-/-}$ mice.

chabaudi infections during the crisis phase, destruction of the microvasculature was widespread (Fig. 4A). In particular, the vessels contained parasitized erythrocytes, as well as monocytes and macrophages with hemozoin deposits (Fig. 4A and B). The vessel walls were partially destroyed, allowing blood cells to invade adjacent brain tissue (Fig. 4C). Damage of the microvasculature also manifested as intravascular monocyte aggregation, vasodilatation, edema, endothelial cell activation, swelling of endothelial cell nuclei, increased numbers of activated microglial cells in perivascular species, plugging of small vessels with erythrocytes and mononuclear cells, and occasional rupture of vessel walls (37). In particular, small vessels were plugged with both parasitized and nonparasitized erythrocytes, causing focal microhemorrhages. Moreover, the lumens of vessels were occasionally occluded by monocytes that adhered tightly to the endothelium of the microvasculature.

Moreover, the hearts of these dead *taut*$^{-/-}$ mice revealed a series of morphological alterations. There was interstitial edema with moderate mononuclear cell infiltration; the sizes of cardiomyocytes differed; and myofibers often appeared thinner than normal and wavy (Fig. 4D). Furthermore, severe changes were also detected in the lungs. These changes became evident as thickened alveolar septa, interstitial and/or intra-alveolar edema, increased numbers of macrophages, intensified adhesiveness of malaria pigment-containing monocytes, septal pneumonitis with monocyte infiltrates, and occasional hyperplasia of type II pneumocytes (Fig. 4E to G). Three out of 16 mice revealed hemorrhagic infarcts of the lung, and 1 mouse showed alveolar macrophages with hemosiderin pigment (Fig. 4G).

The deceased mice also suffered from acute renal failure, as indicated by acute tubular necrosis and chromoprotein cylinders (Fig. 4H). In acute tubular necrosis, necrosis occurs mainly in the proximal tubular epithelium and is characterized by cells with no nuclei and homogenous, intensely eosinophilic cytoplasm. Necrotic cells penetrate the lumens of tubules, which become obliterated, eventually resulting in acute renal failure (Fig. 4I). Finally, the liver revealed large necrotic areas (Fig. 4K).

In this context, however, it should also be mentioned that *taut*$^{+/+}$ mice killed at peak parasitemia on day 8 p.i. did not reveal such dramatic pathological changes in the brain, lung, heart, kidney, and liver as *taut*$^{-/-}$ mice that succumbed to infection during crisis (see Fig. S1 in the supplemental material).

DISCUSSION

Previous studies of different mouse malaria models have revealed that blood-stage malaria is under complex control,

TABLE 1. Plasma parameters of *P. chabaudi*-infected *taut*$^{+/+}$ and *taut*$^{-/-}$ mice on days 0 and 8 p.i.[a]

Parameter	Day 0 p.i.			Day 8 p.i.			*P* (day 8 p.i. vs day 0 p.i.)	
	Mean value ± SEM (no. of mice)		*P* (*taut*$^{-/-}$ vs *taut*$^{+/+}$ mice)	Mean value ± SEM (no. of mice)		*P* (*taut*$^{-/-}$ vs *taut*$^{+/+}$ mice)		
	taut$^{+/+}$	*taut*$^{-/-}$		*taut*$^{+/+}$	*taut*$^{-/-}$		*taut*$^{+/+}$ mice	*taut*$^{-/-}$ mice
Taurine (μmol/liter)	540 ± 47 (3)	264 ± 25 (3)	<0.01	569 ± 16 (6)	192 ± 19 (4)	<0.01	0.48	0.05
AST (U/liter)	93 ± 28 (6)	42 ± 4 (6)	0.14	574 ± 117 (9)	589 ± 142 (7)	0.88	<0.01	<0.01
ALT (U/liter)	19 + 2 (6)	14 + 3 (6)	0.41	150 + 32 (9)	131 + 30 (7)	0.68	<0.01	<0.01
Bile acids (μmol/liter)	20 ± 2 (3)	22 ± 5 (5)	0.79	40 ± 8 (9)	51 ± 9 (6)	0.39	0.2	0.02
Bilirubin (ng/dl)	0.13 ± 0.02 (6)	0.17 ± 0.03 (6)	0.19	1.18 ± 0.03 (6)	0.80 ± 0.23 (6)	0.33	<0.01	<0.01
Ammonia (μg/dl)	61 ± 6 (4)	74 ± 9 (4)	0.31	165 ± 16 (8)	291 ± 23 (8)	<0.01	<0.01	0.02

[a] Blood was taken from mice, and plasma was prepared and analyzed, as described in Materials and Methods. Significance was evaluated with Student's *t* test; *P* values of <0.01 were considered significant. γ-Glutamyltransferase activity was lower than 3 U/liter in all samples.

TABLE 2. Serum parameters of *P. chabaudi*-infected *taut*$^{+/+}$ and *taut*$^{-/-}$ mice on days 0 and 8 p.i.[a]

Parameter	Day 0 p.i.			Day 8 p.i.			*P* (day 8 p.i. vs day 0 p.i.)	
	Mean value ± SEM (no. of mice)		*P* (*taut*$^{-/-}$ vs *taut*$^{+/+}$ mice)	Mean value ± SEM (no. of mice)		*P* (*taut*$^{-/-}$ vs. *taut*$^{+/+}$ mice)	*taut*$^{+/+}$ mice	*taut*$^{-/-}$ mice
	taut$^{+/+}$	*taut*$^{-/-}$		*taut*$^{+/+}$	*taut*$^{-/-}$			
IL-1β (ng/liter)	1.27 ± 0.1 (3)	2.1 ± 0.2 (3)	0.15	4.7 ± 1.1 (3)	14.5 ± 1.9 (3)	<0.01	0.05	0.02
IL-6 (ng/liter)	1.2 ± 0.1 (3)	2.1 ± 0.5 (3)	0.37	39.1 ± 12.3 (5)	69.4 ± 22.6 (5)	0.25	<0.01	<0.01
TNF-α (ng/liter)	23.8 ± 0.6 (4)	27.3 ± 2.1 (4)	0.22	29.4 ± 6.2 (5)	97.2 ± 23.6 (5)	<0.01	0.46	0.02
Total NO (μmol/liter)	25 ± 2 (3)	74 ± 41 (3)	0.35	118 ± 9 (4)	198 ± 35 (3)	0.08	<0.01	0.02

[a] Blood was taken from mice, and serum was prepared and analyzed, as described in Materials and Methods. Significance was evaluated with Student's *t* test; *P* values of <0.01 were considered significant. γ-Glutamyltransferase activity was lower than 3 U/liter in all samples.

involving both genes of the mouse major histocompatibility complex (MHC), i.e., the *H-2* complex, and genes of the non-*H-2* background (42, 57, 58), as well as soluble factors, such as testosterone (57, 58) and estrogen (5, 6). Different genes and/or loci of the non-*H-2* background have already been reported to be critical for a fatal outcome of malaria (14, 15, 21, 30, 35, 41). Here we report another critical gene of the non-*H-2* background, i.e., the *taut* gene, encoding the taurine transporter TAUT. Indeed, deletion of the *taut* gene causes loss of the mice's ability to self-heal blood-stage infections with *P. chabaudi*.

Our data, however, also indicate that it is not the loss of the *taut* gene or the gene product TAUT *per se* that causes the fatal outcome of malaria. Rather, it is the physiological consequence, namely, the breakdown of taurine homeostasis, evident as lowered levels of taurine in the cells and blood, since taurine is the major factor transported by TAUT. Indeed, deletion of *taut* results in a reduction of about 50% in taurine levels in the blood plasma, which, in turn, is associated with a lowering of intracellular taurine levels, as shown previously (20, 50, 51). Moreover, the taurine levels in *taut*$^{-/-}$ mice are lowered another 25% at peak parasitemia. This suggests that the fatal outcome of *P. chabaudi* infections during crisis is causally related to taurine deficiency. However, the taurine deficiency *per se* is not lethal; rather, it becomes lethal only when mice have to respond to *P. chabaudi* infection. This becomes evident at peak parasitemia, when *taut*$^{-/-}$ mice exhibit significant increases in parasitemia, systemic cytokine levels, and liver damage relative to those for *taut*$^{+/+}$ mice, which exhibit normal taurine homeostasis even at peak parasitemia.

There is ample evidence that lowered taurine levels also critically affect the stability of cells (18). In accordance, our findings suggest altered stability of, e.g., macrophages and parasitized erythrocytes. Indeed, *P. chabaudi* infections in taurine-deficient nonhealer *taut*$^{-/-}$ mice result in a peak parasitemia that is approximately 60% higher, on average, than that for self-healer *taut*$^{+/+}$ mice. But even noninfected erythrocytes exhibit a different level of stability, as indicated by previous data showing that erythrocytes of *taut*$^{-/-}$ mice are characterized by impaired eryptosis (apoptosis of erythrocytes) (31). Such impaired eryptosis may be even further delayed by intraerythrocytic *P. chabaudi*, as is known to occur for host cells infected by other intracellular parasites (1, 32, 49). Recent evidence indeed has shown impaired eryptosis of erythrocytes infected with *Plasmodium berghei* (26) or *Plasmodium falciparum*, presumably due to a parasite-maintained low Ca^{2+} concentration in the cytosol of the host erythrocyte (23). Moreover, the macrophages of *taut*$^{-/-}$ mice appear to change their stability in response to malaria, as circumstantially indicated by the increased systemic levels of TNF-α and IL-1β, which are produced primarily by macrophages. This accords with other data showing, conversely, that taurine is able to dampen the effect of proinflammatory cytokines, including IL-1β (12) and TNF-α (10, 24, 33, 34, 44, 47, 60).

About 80% to 90% of all macrophages reside as Kupffer cells in the liver, in particular in the periportal area, supervising the invasion of pathogens (18). It is therefore reasonable to assume that taurine deficiency also affects the stability of malaria-activated Kupffer cells in *taut*$^{-/-}$ mice, which presumably release more proinflammatory cytokines than *taut*$^{+/+}$ mice, thus contributing to the increased levels of circulating TNF-α and IL-1β. The increased release of TNF-α and IL-1β may, in turn, induce local inflammatory responses, which may be associated with more-pronounced liver injuries in *taut*$^{-/-}$ mice than in *taut*$^{+/+}$ mice (4). This view, that increased liver injuries are due predominantly to overwhelming host responses to infection, is also supported by our data showing increased ammonia levels in malaria-infected *taut*$^{-/-}$ mice and perturbed particle-entrapping activity of the liver at peak parasitemia. However, our analyses of hepatic gene expression indicate that the liver is obviously not uniformly damaged in response to malaria, since only some genes, such as those encoding IL-1β, TNF-α, IL-6, iNOS, NF-κB, and VDR, show increased expression; the expression of others, such as the CYP3A11, CYP7A1, SULT2B1, UGT1A1, and MRP4 genes, is the same in *taut*$^{-/-}$ mice as in *taut*$^{+/+}$ mice.

The malaria-induced increase in the systemic ammonia levels of *taut*$^{-/-}$ mice ultimately contributes to injuries in other organs, which also suffer from the lowered taurine levels due to the breakdown of taurine homeostasis. In particular, it is known that increased ammonia levels lead to hepatic encephalopathy, characterized by astrocyte swelling and low-grade cerebral edema (19, 36, 55). Our data reveal that the brains of the *taut*$^{-/-}$ mice that succumbed to malaria were massively damaged. Incidentally, human *P. falciparum* malaria patients suffer from hepatic encephalopathy (25). Although brain damage, such as that which occurs in *taut*$^{-/-}$ mice, could be a sufficient and exclusive cause for death, our autopsy analysis also revealed massive injuries in other organs, such as the lung, kidney, and heart. Incidentally, *taut*$^{-/-}$ are prone to pressure overload and cardiac hypertrophy (50, 51). All these data indicate multiple organ failure as the cause of death for malaria-

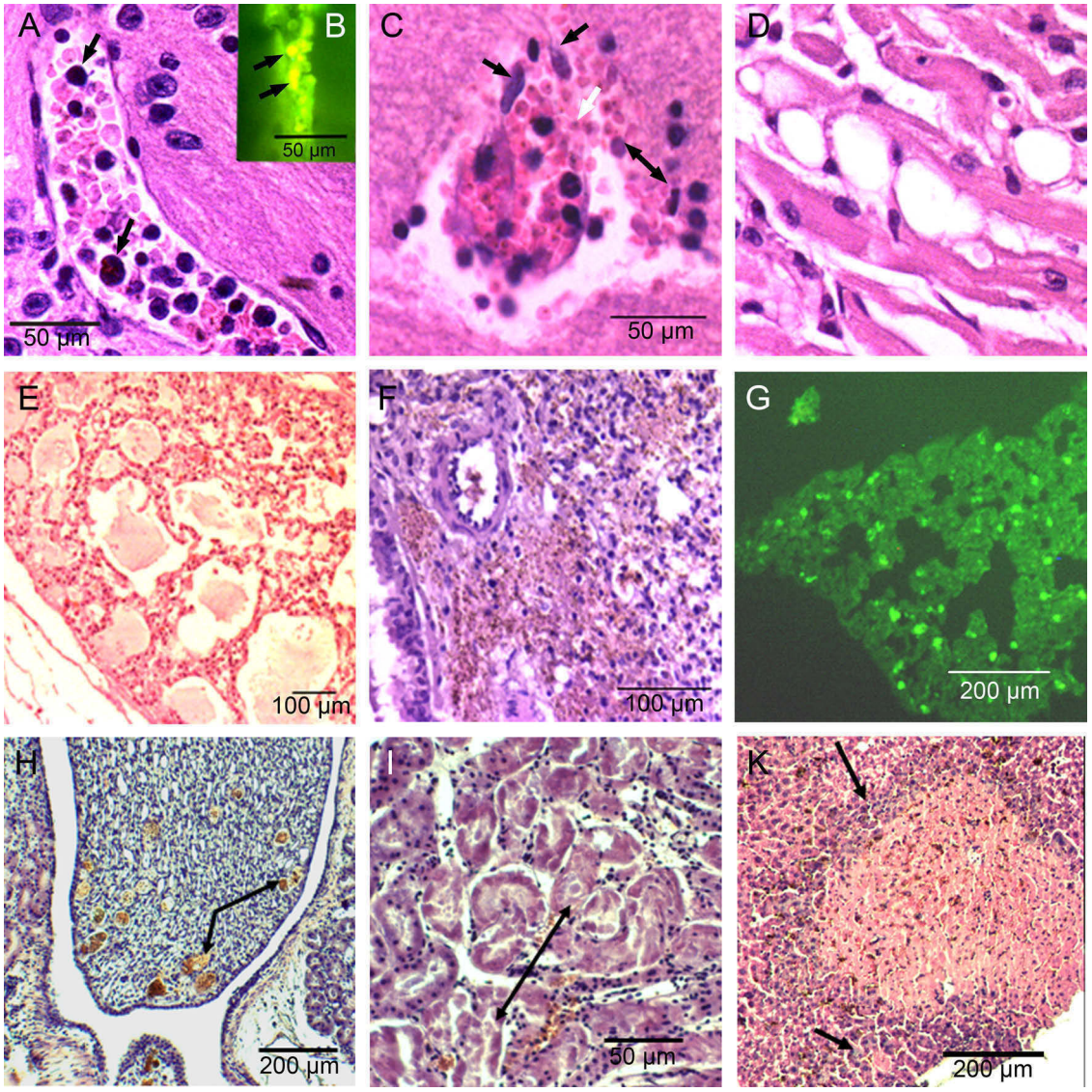

FIG. 4. Histopathology of tissues of *taut*$^{-/-}$ mice that succumbed to *P. chabaudi* malaria. (A, B, and C) Slices of the brain including the cortex cerebri. Histological changes of the brain (A and C) include the presence of several parasitized erythrocytes in the cerebral blood vessels, infiltration of malarial-pigment-containing hypertrophic monocytes (A, arrow), endothelial cell activation with enlarged nuclei (C, black arrows), adherence of mononuclear cells to endothelial cells of small cerebral vessels, and focal vessel disruption (C, white arrow) with perivascular hemorrhage (C, double-headed arrow). Arrows in panel B show isolectin IB_4 antibody staining of stimulated murine macrophages in a blood vessel. (D) Slices of heart muscle show interstitial edema, differences in the sizes of cardiomyocytes, and abnormal, thinner myofibers with a wavy appearance. (E) Pulmonary edema of the lung. (F) Hemorrhagic infarct of the lung. (G) Isolectin staining showing interstitial stimulated murine pigment-containing macrophages/monocytes in the lung. (H) Arrows indicate chromoprotein cylinders in the kidney. (I) Double-headed arrow shows tubular necrosis in the kidney. (K) Small and large arrows indicate small and large necrotic areas in the liver. Some tissues were stained with hematoxylin and eosin (A, C, D, E, and F) or were subjected to immune staining with the FITC-labeled isolectin IB_4 antibody (B and G). Original magnifications, ×400 (A, B, C, and D), ×100 (E, G, H, and K), and ×200 (F and I).

infected *taut*$^{-/-}$ mice. Obviously, *taut*$^{-/-}$ mice, due to their taurine deficiency, are not able to robustly activate those mechanisms that mediate self-healing from *P. chabaudi* malaria, as *taut*$^{+/+}$ mice do.

Collectively, our data show that the breakdown of taurine homeostasis, manifesting as lowered taurine levels as a consequence of *taut* deletion, is not lethal *per se* but becomes lethal in response to blood-stage malaria. Obviously, the *taut* gene and taurine homeostasis are essential for self-healing of *P. chabaudi* blood-stage malaria. It remains to be seen whether taurine deficiency also affects the outcome of human malarial infections.

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2.2 Testosterone-induced upregulation of miRNAs in the female mouse liver

Background: Evidence is increasing for a new player in gene expression, that is the miRNA. MicroRNAs are small (18-25 nts long) non-coding RNAs, which post-transcriptionally regulate gene expression in a sequence-specific manner by binding to the 3'UTR of target genes resulting in either inhibition of translation or degradation of mRNA. Most recent information indicates that there is a sexually dimorphic miRNA expression. Testosterone (T) is known to affect protein-encoding gene expression directly through androgen receptor which binds to specific androgen response elements in promoters of target genes or indirect through non-genotropic mechanisms. To date, there is no information available about T effects on hepatic miRNA expression *in vivo*.

Methods: Female C57BL/6 mice were treated with T or vehicle for 3 weeks twice a week. Hepatic miRNA expression was determined using miRXplore microarrays and verified by qRT-PCR. Moreover, qRT-PCR analysis is used to detect effects of T during different time-points of T treatment, after 12 weeks of T withdrawal, and after infection with *P. chabaudi* malaria. Affymetrix microarrays and qRT-PCR analysis are used to identify target genes of miRNAs and *in silico* analysis to find out AREs in the promoter regions.

Results: T caused a transient upregulation of the 6 miRNAs miR-22, miR-690, miR-122, let-7A, miR-30D, and let-7D in the female mouse liver, reaching maximal expression on different time-points, but disappeared after 12 weeks of T withdrawal. Moreover, this upregulated miRNA expression is robust because it was not affected by *Plasmodium chabaudi* blood-stage malaria. The majority of the 6 upregulated miRNA and their protein-encoding target genes did not contain any ARE in their promoters.

Conclusion: These data provide first indications that T affects hepatic miRNA expression *in vivo* through both genotropic and non-genotropic mechanisms.

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Testosterone-induced upregulation of miRNAs in the female mouse liver

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ABSTRACT

Testosterone (T) regulates expression of protein-encoding genes directly through androgen receptor (AR) targeting androgen response element (ARE) in gene promoters or indirectly through non-genotropic mechanisms, but only limited information is available about T effects on expression of gene-regulatory non-coding miRNAs. Here, we investigate the effect of T on miRNA expression profiles in the female mouse liver using miRXplore microarrays and quantitative RT-PCR. T treatment for 3 weeks induced upregulation of the 6 miRNAs miR-22, miR-690, miR-122, let-7A, miR-30D and let-7D, reaching maximal expression at different time-points during T treatment. This upregulation was transient, i.e. it disappeared after T withdrawal for 12 weeks, and it was rather robust since it was not essentially affected by blood-stage infections with *Plasmodium chabaudi* malaria. *In silico* analysis revealed an ARE in the miR-122 promoter, while the other 5 miRNAs did not contain any ARE in their 2000 bp promoters. The T-induced upregulation of the 6 miRNAs coincided with a downregulation of some of their target protein-encoding genes, the majority of which did incidentally not contain any ARE in their promoters. T treatment did not affect expression of AR and estrogen receptor β (ERβ), but significantly downregulated the miR-22 target genes ERα and aromatase. This downregulation is presumably not caused by T after its aromatase-mediated conversion to E_2 through ER, but rather by the T-induced upregulation of miR-22. Collectively, our data suggest that T can regulate expression of distinct miRNAs *in vivo* by both genotropic and non-genotropic mechanisms.

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1. Introduction

Testosterone (T) exerts a wide spectrum of physiological and pathological activities: it does not only contribute to sculpturing the male phenotype [1,2], but it is also known, for example, to exhibit immunosuppressive activity [3,4] and to increase susceptibility towards a wide variety of infectious diseases [5,6]. T is known to regulate gene expression through both genotropic and non-genotropic mechanisms. The genotropic T effects manifest themselves as direct action on gene expression through the intracellular androgen receptor (AR) [7]. More specifically, the 110 kDa protein AR binds T and the AR–T-complex is translocated into the nucleus, where it binds to androgen response element (ARE) in promoters of target genes thus inducing or repressing expression of protein-encoding genes [1,8,9]. Non-genotropic mechanisms are regarded as those which are either only indirectly mediated through the AR, i.e. by cross-talk of the T–AR-complex with other signaling pathways [7,10–13], or as those which are initiated at specific membrane androgen receptors belonging to the G-protein coupled receptors [12–16]. The latter non-genotropic mechanisms are associated with an increase in intracellular free Ca^{2+} concentration [14,17–19], which in turn can also modulate gene expression by cross-talk with other signaling pathways [20].

Recently, microRNAs (miRNAs) are getting more and more attention as regulators of gene expression. These miRNAs are one of the non-coding RNA species which are approximately 20 bp in size and which regulate gene expression by inhibiting translation of RNA into proteins and/or by reducing stability of mRNAs [21–23]. In mice, more than 600 miRNA have been discovered to date [24] and each miRNA is supposed to target hundreds of mRNAs of protein-coding genes, respectively [25]. The miRNAs affect outcome of diseases and they may even function as oncogenes or tumor suppressors [26–32]. However, there is available only very limited information about T action on miRNAs. Only prostate cancer cells and other cancerous cells have been investigated showing T-dependent changes in miRNA expression [33–35]. To our knowledge, however, T effects on miRNAs in organs *in vivo* have not been described to date.

Recent studies have revealed profound and unexpected roles of miRNAs in the control of diverse aspects of the liver, includ-

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ing hepatocyte growth, stress response, metabolism, infection, proliferation, gene expression, and maintenance of hepatic phenotype [27,36–38]. The liver is also known as a T target organ. For instance, T affects liver metabolism [41], depresses hepatic immune responses [39,40] and even induces hepatocellular carcinoma [42–44]. Here, we provide first indications that T induces transient expression of 6 different miRNA species in the female mouse liver, at least 5 of them are presumably activated by non-genotropic mechanisms.

2. Experimental

2.1. Mice

Female mice of the inbred strain C57BL/6, bred under specified pathogen-free conditions in the animal facilities of the Heinrich-Heine University Düsseldorf, were used in all experiments. They were housed in plastic cages, and they received a standard diet (Wohrlin, Bad Salzuflen, Germany) and water ad libitum. The experiments were approved by the state authorities and followed the German law on animal protection.

2.2. Testosterone treatment

Mice at an age of 10–12 weeks received subcutaneous injections of 100 μl sesame oil containing 0.9 mg testosterone (Testosterone-Depot-50; Schering, Berlin, Germany) twice a week for 3 weeks; controls were treated only with the vehicle [45]. This T treatment has been previously shown to increase the circulating T concentrations in female mice from 0.18 ng/ml to 3.79 ng/ml [46], which is in that range normally found in male mice amounting to about 3–5 nM [47]. Discontinuation of T treatment for 12 weeks resulted in a decline of the T levels to 0.21 ng/ml as shown previously [46].

2.3. Malarial infections

Blood-stage infections of *Plasmodium chabaudi* [48,49] were passaged weekly in NMRI mice. C57BL/6 mice were challenged *i. p.* with 10^6 *P. chabaudi*-parasitized erythrocytes. Parasitemia was determined in Giemsa-stained tail blood, and cell number was measured in a Neubauer chamber.

2.4. RNA-isolation

Mice were killed by cervical dislocation, livers aseptically removed and pieces of the liver were immediately frozen in nitrogen and stored at −80 °C until use. For isolating RNA, approximately 250 mg frozen liver was homogenized with an ultraturrax in 5 ml Trizol (Peqlab Biotechnology, Erlangen, Germany), mixed with 1 ml chloroform for 15 s, kept at room temperature for 15 min and centrifugated at 3000 × *g* for 45 min. After isopropanol precipitation of the supernatant, the pellet was washed twice with 80% ethanol, air dried and dissolved in 200 μl RNase-free water. RNA concentrations were determined at 260 nm.

2.5. RNA labeling and hybridization

Quality of RNA samples was controlled using the Agilent 2100 Bioanalyzer platform (Agilent Technologies, Böblingen, Germany). Labeling and hybridization was done according to user manuals of the miRXplore™ manufacturer (Miltenyi Biotec, Bergisch-Gladbach, Germany) [24,50]. In brief, the experimental samples were always labeled with the red fluorescent Hy5 and the control miRXplore Universal Reference (UR), representing a defined pool of synthetic microRNAs, was labeled with green fluorescent Hy3. Subsequently, the labeled material was hybridized overnight to miRXplore™ Microarrays using the a-Hyb™ Hybridization Station (Miltenyi Biotec, Bergisch-Gladbach, Germany). Fluorescence signals of the hybridized miRXplore™ Microarrays were detected using a laserscanner (Agilent Technologies, Böblingen, Germany).

2.6. Data analysis

The miRXplore™ Microarrays were scanned and quantified as detailed previously [24,50]. Mean signal and mean local background intensities were obtained for each spot of the microarray images using the ImaGene software (Biodiscovery, El Segundo, U.S.A.). Low-quality spots were flagged and excluded from data analysis. Unflagged spots were analyzed with the PIQOR™ Analyzer software (Miltenyi, Biotec). The PIQOR Analyzer allows automated data processing of the raw data text files derived from the ImaGene software. This includes background subtraction to obtain the net signal intensity, data normalization, and calculation of the Hy5/Hy3 ratios for the species of interest. As an additional quality filtering step, only spots were taken into account for the calculation of the Hy5/Hy3 ratio with a signal that was equal or higher than the 50% percentile of the background signal intensities. A “sample versus universal reference” modus was chosen allowing absolute quantification and indirect comparisons of several experimental samples, i.e. we calculated the ratio of signals from experimental sample versus UR over the ratio of vehicle control versus UR and the resulting re-ratio indirectly reflected the ratio of experimental sample versus control [24].

2.7. Quantitative real-time PCR

RNA was treated with DNase (Applied Biosystems, Darmstadt, Germany) at 37 °C for at least 1 h prior to reverse transcription and then converted into cDNA following the manufacturer's protocol using the miScript™ Reverse Transcription Kit (Qiagen, Hilden, Germany). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the ABI Prism® 7500HT Sequence Detection System (Applied Biosystems, Darmstadt, Germany) with miScript™ SYBR Green PCR Kit (Qiagen, Hilden, Germany). Primers used for qRT-PCR were commercially provided as miScript™ primer assays by Qiagen (Hilden, Germany). PCR reactions were conducted as follows: 94 °C for 15 min as initial activation step to activate HotStarTaq DNA polymerase followed by 40 cycles at 94 °C for 15 s, at 55 °C for 35 s and at 70 °C for 30 s. Reaction specificity was checked by analyzing melting curves. Relative quantitative evaluation of amplification data was analyzed using the Taqman7500 system software v.1.2.3f2 (Applied Biosystems, Darmstadt, Germany) and the $2^{-\Delta\Delta ct}$-method [51]. For quantification, the U6 small nuclear RNA was used as an internal standard.

2.8. Identification of miRNA target genes

Recently, Affymetrix microarrays containing 22,690 probe sets representing 14,000 genes were recently posted at NCBI/GEO DataSets/GSE13388, which were generated from livers obtained from the same female C57BL/6 mice as described here. To determine T-deregulated genes after 3 weeks of T treatment we removed probe sets with an expression intensity <20 in each sample. Only those probe sets were selected, which were at least 2-fold deregulated by T (two-tailed Student's *t*-test with p-value < 0.01). Genes were categorized according to their biological pathways using the Databank for Annotation, Visualization, and Integrated Discovery (DAVID) [52]. To identify and to allocate miRNAs to their corresponding T-deregulated target genes we used the web tool for bioinformatics miRanda algorithms [http://www.microrna.org] [53,54].

Table 1
T-upregulated miRNA expression in the female mouse liver as revealed by miRXplore microarrays and qRT-PCR.

miRNA name	GeneID	Gene accession	T/C (microarray)	T/C (qRT-PCR)	miRNA functions described to date	PMID
MIR-22	35886	MI0000570	6.49	6.62 ± 1.22	miR-22 inhibits estrogen signaling by directly targeting the ERα mRNA, highly expressed in mammary progenitor cells	19414598, 18079172
MIR-690	38880	MI0004658	5.34	4.43 ± 1.1	miR-690 downregulated by high glucose treatment	19096044
MIR-122	35826	MI0000256	3.70	3.2 ± 1.24	Regulation of lipid metabolism, downregulates the high affinity cationic amino acid transporter CAT-1, downregulated in HCC, targets hepatitis C virus	16459310, 17179747, 19296470, 19021529
LET-7A	38314	MI0000061	2.51	3.9 ± 1.51	Suppresses therapeutics-induced cancer cell death by targeting caspase-3, functions as a potential tumor suppressor in human laryngeal cancer	18758960, 19787239
MIR-30D	35901	MI0000549	2.50	5.2 ± 1.92	Upregulated by glucose treatment, downregulated in human thyroid anaplastic carcinomas	19096044, 17563749
LET-7D	39153	MI0000405	2.28	4.49 ± 1.44	n-3 polyunsaturated fatty acids modulate carcinogen-directed non-coding miRNA signatures in rat colon, low-level expression as prognostic marker of head and neck squamous cell carcinoma	19825969, 19179615

2.9. Statistical analysis

Significance was evaluated by two-way ANOVA with Dunnett and Bonferoni post hoc tests using the statistical package program SPSS version 17.0.

3. Results

In order to identify possible effects of T on miRNA expression profiles in the female mouse liver, female mice of the inbred strain C57BL/6 were treated with T or vehicle for 3 weeks and, then, RNA was extracted from individual livers of vehicle- and T-treated mice. Equal amounts of RNA were pooled from 3 vehicle-treated mice and 3 T-treated mice, respectively, and these experimental samples were subjected first to a screening using miRXplore™ microarrays, each containing 634 mouse miRNAs according to miRBase version 12.0. The microarrays obtained from the T-treated sample (T) and that of the vehicle-treated sample (C), represented as false-color images in supplementary material, were evaluated in terms of calculating the Hy5/Hy3 ratios of the experimental samples versus the universal references (T/UR and C/UR). Then, calculation of the re-ratios allowed the direct comparison of T versus C. Only 6 miRNAs were detected which were at least 2-fold upregulated by T in the female mouse liver. These miRNAs were miR-22, miR-690, miR-122, let-7A, miR-30D and let-7D summarized in Table 1, which also includes their functions ascribed to these miRNAs to date. Then, the T-upregulated expression of the 6 miRNAs was re-investigated by qRT-PCR analysis in the three individual livers of the two pooled samples, respectively, which essentially verified the microarray data (Table 1).

Moreover, about the same expression levels of the 6 miRNAs could be found in a second, completely independent cohort of T-treated mice and vehicle-treated mice as represented in Fig. 1. In this approach, we also tracked the rate of expression of the 6 miRNAs during the T treatment. Conspicuously, the expression of the 6 different miRNAs is already significantly increased after 1 week of the T treatment (Fig. 1). This is also maximal expression of miR-122 and let-7A, whereas the expression of miR-22, miR-690, miR-30D and let-7D is further increased during the following 2 weeks.

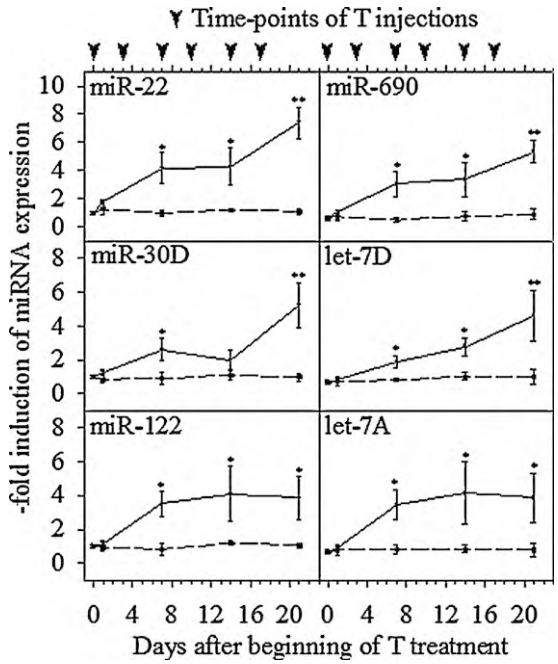

Fig. 1. Upregulation of miRNA expression during treatment with testosterone. Mice were treated with T and vehicle twice a week for 3 weeks. The vertical arrowheads indicate the time-points of the injections. Quantitative RT-PCR was performed with livers of 3 T-treated and 3 vehicle-treated mice. Means are given ± SD. Expression of all miRNAs is significantly increased already after 1 week of T treatment as indicated by one asterisk ($p < 0.01$). Two asterisks indicate significant upregulation ($p < 0.01$) after 3 weeks in relation to 1 week T treatment. ■ – – – ■ T-treated mice and ■ - - - ■ vehicle-treated control mice.

The T-induced upregulation of the 6 miRNAs was not persistent, but only transient. Indeed, when qRT-PCR was used to examine the expression of the 6 T-induced miRNAs after 12 weeks of T withdrawal, none of the 6 miRNAs was still upregulated, i.e. the T-induced expression of the 6 miRNAs was reversible (Fig. 2a).

The T treatment for 3 weeks was previously shown to induce a suppression of protective responses of the liver to blood-stage infections with *P. chabaudi* [39]. In particular, the malaria-induced

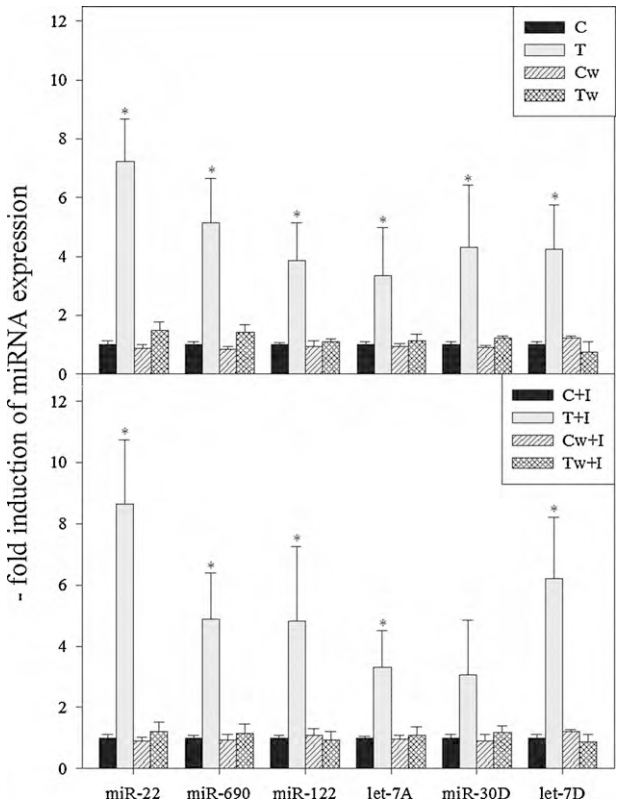

Fig. 2. T-induced transient upregulation of hepatic miRNA expression and insensitivity to *P. chabaudi*. (A) The fold induction of miRNAs was determined by qRT-PCR after 3 weeks of T treatment (T) or vehicle treatment (C) and after withdrawal of T (Tw) or vehicle (Cw), respectively. (B) Quantitative RT-PCR was used to determine expression of miRNAs on days 8 *p. i.* after T treatment (T+I) and after T withdrawal for 12 weeks (Tw+I). Relative miRNA expression was normalized to the mean expression of the corresponding control mice (C+I, Cw+I). Values indicate means ± SD from 3 different mice. Asterisks indicate significant differences ($p < 0.01$).

hepatic alterations differed between T-treated and vehicle-treated mice [39,40]. We therefore wondered if *P. chabaudi* malaria possibly affected the expression of the 6 miRNAs. However, the T-induced expression of the 6 miRNAs is rather stable: it is not affected by malaria at all (Fig. 2b). Indeed, when mice after the 3 weeks treatments with T or vehicle and after T withdrawal for 12 weeks were infected with 10^6 *P. chabaudi*-infected erythrocytes culminating at peak parasitemias of about 45% on days 8 *p. i.*, respectively, qRT-PCR analysis did not reveal any significant effect of *P. chabaudi* infections on the expression levels of the 6 miRNAs in both T-treated and control samples, neither after the 3 weeks T treatment nor after 12 weeks of T withdrawal (Fig. 2b).

The T-induced upregulation of the 6 miRNAs coincided with an at least 2-fold downregulation of some of their known target genes. This was evaluated from the MOE430A Affymetrix chips recently posted at NCBI/GEO DataSets GSE 13388, which were generated from the livers of the same mice investigated here, i.e. 3 chips for 3 T-treated and 3 chips for vehicle-treated mice were evaluated after the end of 3 weeks T treatment and after T withdrawal for 12 weeks. After the T treatment we found a downregulation of 5 target genes for the miR-22, 1 target gene for the miR-690, 2 target genes for the liver-specific miR-122, 6 target genes for the let-7A/let-7D, and 3 target genes for the miR-30D, as summarized in Table 2. Remarkably, the downregulation of the protein-encoding target genes of the 6 different miRNAs was largely abolished after 12 weeks of T withdrawal (Table 2). There was only one exception: the miR-22 targeted mRNA of Cyp17a1 (Table 2). These data could be verified for at least 6 genes arbitrarily selected from Table 2 by qRT-PCR (Fig. 3).

Genotropic effects of T are known to be exerted directly through the androgen receptor (AR) and the androgen response element (ARE) in the target gene promoters, respectively [1]. In order to identify possible ARE-mediated expression of the T-upregulated miRNAs, the data bank ALGGEN with the research tool PROMO [55,56] was used to analyze the 2000 bp DNA upstream regions of the transcription start of the 6 T-sensitive miRNAs. Only 1 of the T-upregulated miRNAs, i.e. the liver-specific miR-122, contained an ARE at position −47 bp to −53 bp. The other 5 miRNAs did not exhibit any ARE in their promoters. Incidentally, the downregulated target genes of the 6 miRNAs did also not contain any ARE. There were only two exceptions: the mRNA-encoding gene for growth hormone receptor targeted by let-7A/let-7D and the mRNA of 5730420B22Rik targeted by miR-22 (Table 2). In accordance, qRT-PCR did not reveal any significant change in the mRNA expression of AR after 3 weeks of T treatment and after 12 weeks of T withdrawal, respectively (Fig. 4).

T is also known to act, after its conversion to estradiol (E_2) by the aromatase Cyp19a1, through the estrogen receptor (ER). However, Fig. 4 shows data obtained with qRT-PCR that T downregulated the mRNA expression of ERα, but did not significantly affect ERβ. This downregulation of ERα was transient, i.e. it could be observed only after the 3 weeks of T treatment, but not after 12

Table 2
The 6 T-upregulated miRNAs and their downregulated target genes.

miRNA	Target gene	Target gene title	Affymetrix ID	Entrez gene	T/C	Tw/Cw
miR-22	Fmo1	Flavin containing monooxygenase 1	1417429_at	14261	0.49	0.7
	5730420B22Rik	RIKEN cDNA 5730420B22 gene	1427050_at	70561	0.46	0.71
	Nr3c1	Nuclear receptor subfamily 3, group C, member 1	1421866_at	14815	0.34	0.84
	Slc39a14	Solute carrier family 39 (zinc transporter), member 14	1425649_at	213053	0.33	0.96
	Cyp17a1	Cytochrome P450, family 17, subfamily a, polypeptide 1	1417017_at	13074	0.11	0.26
miR-690	Mccc2	Methylcrotonoyl-Coenzyme A carboxylase 2 (beta)	1428021_at	78038	0.43	1.09
miR-122	Nedd4	Neural precursor cell expressed, developmentally downregulated gene 4	1421955_a_at	17999	0.46	0.89
	Ceacam1	CEA-related cell adhesion molecule 1	1452532_x_at	26365	0.43	1.13
let-7A/let-/D	Ghr	Growth hormone receptor	1451871_a_at	14600	0.48	1.24
	Dusp6	Dual specificity phosphatase 6	1415834_at	67603	0.46	0.79
	Rtn4	Reticulon 4	1421116_a_at	68585	0.45	0.57
	Sdccag3	Serologically defined colon cancer antigen 3	1431760_a_at	68112	0.45	1.86
	Ceacam1	CEA-related cell adhesion molecule 1	1452532_x_at	26365	0.43	1.13
	Mmp19	Matrix metalloproteinase 19	1421976_at	58223	0.4	0.96
miR-30D	Eif4e3	Eukaryotic translation initiation factor 4E member 3	1417977_at	66892	0.47	0.79
	Nedd4	Neural precursor cell expressed, developmentally downregulated gene 4	1421955_a_at	17999	0.46	0.89
	Ahcyl1	S-adenosylhomocysteine hydrolase-like 1	1425576_at	229709	0.38	1.24

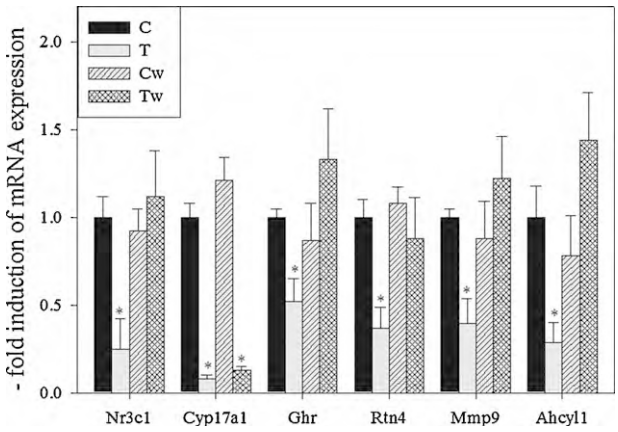

Fig. 3. Verification of gene expression levels evaluated from Affymetrix microarrays. The fold induction of mRNA expression of selected miRNA-regulated target genes *Nr3c1*, *Cyp17a1*, *Ghr*, *Rtn4*, *Mmp9* and *Ahcyl1* was determined by qRT-PCR [49] after T treatment (T) and after T withdrawal for 12 weeks (Tw). Relative mRNA expression was normalized to the mean expression of the corresponding control mice (C, Cw). Values represent means ± SD from 3 different mice. Asterisks indicate significant differences ($p < 0.01$).

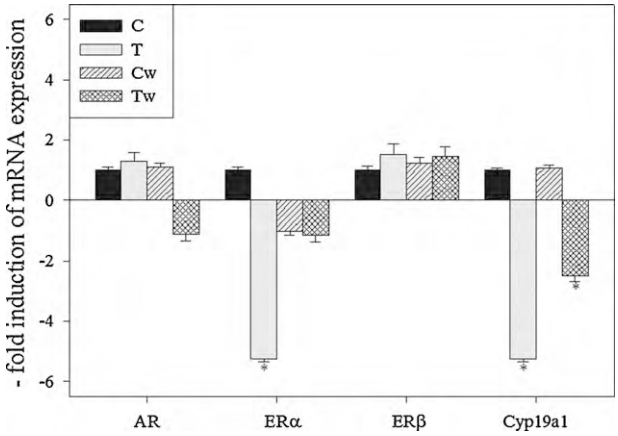

Fig. 4. Gene expression of AR, ERα, ERβ and aromatase CYP19A1. Quantitative RT-PCR [49] detects the mRNA expression profiles in the different groups of mice described in legend to Fig. 2. Relative mRNA expression was normalized to the mean expression of the corresponding control mice. Values represent means ± SD from 3 different mice. Asterisks indicate significant differences ($p < 0.01$).

weeks of T withdrawal (Fig. 4). Moreover, the mRNA of aromatase was also downregulated after 3 weeks of T treatment, which even remained downregulated after T withdrawal for 12 weeks (Fig. 4). The downregulation of aromatase could be also confirmed by data obtained from Affymetrix microarray analysis (Table 2). Remarkably, however, the transient downregulation of ERα by T could not be confirmed by evaluating the Affymetrix microarrays. The reason is that the signals of ERα were removed from analysis due to the applied criteria with highly significant *p*-values ($p < 0.01$) used for evaluation.

4. Discussion

This study has revealed a T-induced upregulation of the 6 miRNAs miR-22, miR-690, miR-122, let-7A, miR-30D and let-7D in the female mouse liver. None of these miRNAs has been yet reported to be T-responsive in other cells and/or tissues. The upregulation of the 6 hepatic miRNAs is transient, i.e. it has disappeared after 12 weeks of T withdrawal. Moreover, the transient upregulation induced by T is specific since the majority of the miRNA species in the liver is not affected by T at all. Specificity is further supported by the course of upregulation. Indeed, the 2 miRNAs miR-122 and let-7A reach its maximal expression after 1 week, however the 4 miRNAs miR-22, miR-690, miR-30D and let-7D only after 3 weeks of T treatment. Furthermore, the T-induced transient upregulation of the 6 miRNAs is rather robust, since it is not affectable by blood-stage infections with *P. chabaudi* malaria. This is remarkable since these infections seriously injure the liver, which becomes evident as dramatic changes in diverse structural and biochemical parameters of the liver [39,40]. For instance, there is an increase in inflammatory infiltrates, in cytoplasmic vacuolization, in the number of binucleated cells and apoptotic bodies, in cell swelling, in the occurrence of malaria pigment, in hyperplasia of Kupffer cells, in increase in total bilirubin, in increased activities of lactate dehydrogenase, aspartate aminotransferase, and alanine aminotransferase, and in a decline of alkaline phosphatase [40].

A direct effect of T on gene expression is mediated through AR by binding to ARE in promoters of target genes [1]. However, the role of AR in the transcription and regulation of miRNA expression has been recently investigated only in different prostate cell lines to date. Thus, ectopic expression of AR in the AR-null prostate cancer cell line PC3 induces differential expression of 11 miRNAs [34,57]. In particular, there is an upregulation of miR-18h, miR-361, and miR-424 and a downregulation of miR-19b miR-29b, miR-128b, miR-146a, miR-221, miR-22 and miR-663 [57]. These miRNAs differ from those 6 miRNAs we have found here in the female mouse liver to be upregulated by T. Additionally, it has been reported that treatment with androgen increases the abundance of miR-125b in AR-negative prostate epithelial cells after transfection with AR [34]. This increase is due to binding of AR to ARE in the promoter of the miR-125b-2 locus. Our study indicates that abundance of AR in the female liver is not affected by T at all. Moreover, only one miRNA, namely miR-122, contains an ARE in the promoter region adjacent to the transcription start. Such a close adjacency is currently considered as to predict an active functional ARE [58]. It is therefore reasonable to assume that the T-induced upregulation of miR-122 in the female mouse liver is mediated through direct ARE-mediated AR signaling. The majority of T-responsive miRNAs in the female mouse liver, however, do not contain any ARE in their promoter regions. A direct ARE-mediated genotropic action of T through AR on these miRNAs appears therefore rather unlikely. In this context, however, it is worthwhile mentioning that, meantime, information has recently become available that genotropic effects are not only mediated through ARE within a 2 kb 5′ region of target genes, but also in AR-occupied regions within 10 kb 5′-flanking regulatory sequences, some of which were intragenic and even in gene deserts as revealed in prostate cancer by chromatin immune precipitation analysis [59].

Nonetheless, the T-induced upregulation of only the distinct 5 miRNAs in the liver indicates that the T effect on miRNA expression is mediated through specific mechanisms. One mechanism could be that T is converted by aromatase to E_2 which then induces miRNA expression through ER. However, our data demonstrate that ERβ-mRNA is not affected by T treatment, and that the ERα-mRNA just as the mRNA of aromatase are even downregulated by T. In this context, it is noteworthy that both ERα [60,61] and aromatase (see Table 2) have been described to be target genes of miR-22. It is therefore conceivable that the T-induced downregulation of ERα and aromatase is not a direct T action on these two genes, but rather an indirect T action through miR-22, i.e. T induces an upregulation of miR-22, which in turn downregulates ERα and aromatase. This view is supported by recent findings in cultivated Hep-G2- and MCF7-cells that miR-22 targets the 3′-UTR of the ERα-mRNA, downregulates ERα, and represses estrogen signaling [60,61]. It is therefore reasonable that the upregulation of miR-22 as the other 4 miRNAs miR-690, let-7A, miR-30D and let-7D reflects indirect effects of T mediated through spe-

cific, but still unknown non-genotropic mechanisms, which may even operate following a primarily direct genotropic T action and which are possibly parts of larger regulatory circuits mediating T effects. Such circuits may involve intracellular transcriptional networks [62] but also supracellular regulatory programs, as e.g. the hypothalamus–pituitary gland–liver axis, which is neonatally imprinted by androgen, which operates through pulsatile versus continuous patterns of growth hormone secretion in males versus females, respectively, and which is affectable by T [63,64].

Collectively, our data provide first indications that T affects expression of miRNAs *in vivo* through both genotropic mechanisms and still unknown non-genotropic mechanisms, which increases complexity of T signaling in general.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.steroids.2010.06.010.

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2.3 Testosterone-induced permanent changes of hepatic gene expression in female mice sustained during *Plasmodium chabaudi* malaria infection

Background: Gender is critical for the outcome of numerous diseases, which particularly holds true for a wide variety of infectious diseases, including human malaria. A central role in this gender-dependence is played by the male sex hormone testosterone, which exhibits immunosuppressive activity and increases susceptibility towards infectious diseases. Indeed, male mice succumb to blood stage malaria, while female mice and castrated mice are able to survive and self-heal the same infections. However, T-treatment of the self-healer mice induces a lethal outcome of malaria. Most remarkably, the suppressive lethal T-effect is not reversible, but persists after discontinuation of T-treatment for rather a long time. However, it is not yet known to date as to whether this persistence is associated with any changes in gene expression.

Methods: Female C57BL/6 mice were treated with T for 3 weeks; then, T treatment was discontinued for 12 weeks before infecting with *P. chabaudi* malaria for 8 days. Hepatic gene expression was examined after 12 weeks of T withdrawal and subsequent infection on day 8 *p.i.* using Affymetrix microarrays and qRT-PCR analysis.

Results: T treatment resulted in a persistent masculinized hepatic gene expression characterized by an upregulation of male-specific genes *Cyp2d9*, *Cyp7b1*, *Cyp4a10*, *Ugt2b1*, *Ugt2b38*, *Hsd3b5*, and *Slco1a1* and a downregulation of female-specific genes *Cyp2b9*, *Cyp2b13*, *Cyp3a41*, *Cyp3a44*, *Fmo3*, *Sult2a2*, *Sult3a1*, and *BC014805*. The expression of genes encoding nuclear receptors was not affected. T induced persistent upregulation of markers for hepatocellular carcinoma, as e. g. *Lama3* and *Nox4*, and a persistent suppression of genes involved in immune responses such as *Ifnγ* and *Igk-C*.

Conclusion: This study provides new detailed insights of persistent T effects in the female mouse liver characterized by a masculinized liver metabolism and impaired immune response, which may be critical for the T-induced persistent susceptibility to *P. chabaudi* malaria.

Testosterone-induced permanent changes of hepatic gene expression in female mice sustained during *Plasmodium chabaudi* malaria infection

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Abstract

Testosterone has been previously shown to induce persistent susceptibility to *Plasmodium chabaudi* malaria in otherwise resistant female C57BL/6 mice. Here, we investigate as to whether this conversion coincides with permanent changes of hepatic gene expression profiles. Female mice aged 10–12 weeks were treated with testosterone for 3 weeks; then, testosterone treatment was discontinued for 12 weeks before challenging with 10^6 *P. chabaudi*-infected erythrocytes. Hepatic gene expression was examined after 12 weeks of testosterone withdrawal and after subsequent infection with *P. chabaudi* at peak parasitemia, using Affymetrix microarrays with 22 690 probe sets representing 14 000 genes. The expression of 54 genes was found to be permanently changed by testosterone, which remained changed during malaria infection. Most genes were involved in liver metabolism: the female-prevalent genes *Cyp2b9*, *Cyp2b13*, *Cyp3a41*, *Cyp3a44*, *Fmo3*, *Sult2a2*, *Sult3a1*, and *BC014805* were repressed, while the male-prevalent genes *Cyp2d9*, *Cyp7b1*, *Cyp4a10*, *Ugt2b1*, *Ugt2b38*, *Hsd3b5*, and *Slco1a1* were upregulated. Genes encoding different nuclear receptors were not persistently changed. Moreover, testosterone induced persistent upregulation of genes involved in hepatocellular carcinoma such as *Lama3* and *Nox4*, whereas genes involved in immune response such as *Ifnγ* and *Igk-C* were significantly decreased. Our data provide evidence that testosterone is able to induce specific and robust long-term changes of gene expression profiles in the female mouse liver. In particular, those changes, which presumably indicate masculinized liver metabolism and impaired immune response, may be critical for the testosterone-induced persistent susceptibility of mice to *P. chabaudi* malaria.

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Introduction

Testosterone is known to increase susceptibility to a wide variety of infectious diseases (Müller 1992, Klein 2000, Roberts *et al.* 2001, Marriott & Huet-Hudson 2006), which also concerns human malaria (Müller 1992, Kurtis *et al.* 2001, Muehlenbein *et al.* 2005). In the experimental mouse malaria *Plasmodium chabaudi*, testosterone has been shown to induce a lethal outcome of otherwise self-healing infections (Wunderlich *et al.* 1988, 1991). Remarkably, this testosterone-induced conversion from resistance to susceptibility becomes somehow imprinted in female mice, i.e. it persists for rather a long time. Thus, when mice are pretreated with testosterone for 3 weeks, and then testosterone treatment is discontinued for 12 weeks, thereafter the mice are still susceptible to *P. chabaudi* infections (Benten *et al.* 1997). Obviously, this testosterone-induced susceptibility persists, even though the circulating testosterone levels have declined to those levels characteristic for female mice after withdrawal for 12 weeks (Benten *et al.* 1997). This indicates that testosterone is able to induce changes in mice, which continue to exist at low testosterone levels.

The liver is known to be a target organ for testosterone to mediate intrahepatic immune responses (Häussinger *et al.* 2004) and to play a central role in malaria. Indeed, the liver is not only that site in which the pre-erythrocytic development of malaria parasites takes place, but also it is an important effector against malarial blood stages (Balmer *et al.* 2000, Krücken *et al.* 2005), though largely neglected by current research. Moreover, specific populations of lymphocytes have been described to be generated in the liver, which mediate novel protective immune mechanisms against malaria blood stages in the mouse (Mannoor *et al.* 2001,

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2002). Also, Kupffer cells are able to eliminate, via phagocytosis, parasite-derived material such as hemozoin and even *Plasmodium*-infected erythrocytes (Aikawa *et al.* 1980). Moreover, the liver is known for its sexual dimorphism, in particular for its sex- and testosterone-dependent pattern of phase I and phase II metabolism (Waxman & Holloway 2009). All this information led us to suppose that the liver may be one of those sites which is critically involved in mediating the suppressive testosterone effects on *P. chabaudi* malaria.

Testosterone acts on gene expression either directly through the androgen receptor (AR; Zhou *et al.* 1994, Quigley *et al.* 1995, Bennett *et al.* 2009) or indirectly by crosstalk with other signaling pathways (Guo *et al.* 2002, Rahman & Christian 2007, Wendler & Wehling 2009). Moreover, testosterone has been described to induce changes in gene expression, including those genes involved in liver metabolism (Kato & Onada 1970, Krücken *et al.* 2005, Waxman & Holloway 2009). However, there is no information available that testosterone is able to induce permanent changes in gene expression of the liver, i.e. changes that persist even after withdrawal of testosterone. This view prompted us to investigate possible permanent testosterone effects on hepatic gene expression using the Affymetrix microarray technology. At least, the present study provides evidence that testosterone is able to induce permanent changes in the expression of distinct genes in the liver, which, when once induced by treatment with testosterone for 3 weeks, remain existing even for at least 12 weeks after discontinuation of the testosterone treatment. In addition, the expression of such genes is rather robust upon infecting with *P. chabaudi* malaria, which is discussed with respect to relevance for the testosterone-induced persistent susceptibility of mice to *P. chabaudi* malaria.

Materials and methods

Mice

C57BL/6 mice were bred under specific pathogen-free conditions at the central animal facilities of our university. Experiments were performed with female mice. They were housed in plastic cages, and they received a standard diet (Wohrlin, Bad Salzuflen, Germany) and water *ad libitum*. The experiments were approved by the state authorities and followed German law on animal protection.

Testosterone treatment

Mice aged 10–12 weeks received s.c. injections of 100 µl sesame oil containing 0·9 mg testosterone (Testosterone-Depot-50, Schering, Berlin, Germany) twice a week for 3 weeks (Wunderlich *et al.* 1988, Benten *et al.* 1997). Controls were treated only with vehicle, i.e. sesame oil. Thereafter, they were kept under standard conditions for 12 weeks.

Infections

Blood stages of *P. chabaudi* were passaged weekly in NMRI mice (Wunderlich *et al.* 1982, Krücken *et al.* 2009). C57BL/6 mice were challenged with 10^6 *P. chabaudi*-parasitized erythrocytes. Parasitemia was determined in Giemsa-stained tail blood, and cell number was measured in a Neubauer chamber.

RNA isolation

Three mice per time point were killed by cervical dislocation, and livers were aseptically removed. Liver pieces were rapidly frozen in melting nitrogen and stored at −80 °C. For isolation of RNA, ∼250 mg frozen liver were homogenized with an ultra turrax in 5 ml Trizol (Peqlab Biotechnology, Erlangen, Germany) for 1 min, mixed with 1 ml chloroform for 15 s, incubated for 15 min at room temperature, and centrifugated at 3000 ***g*** for 45 min. The supernatant was treated with isopropanol and centrifugated, and the pellet was washed twice with 80% ethanol, air-dried, and dissolved in 200 µl RNase-free water. RNA concentrations were determined at 260 nm, and the quality of the RNA was examined with agarose gel electrophoresis.

Hybridization of microarrays

Quality control of RNA was performed with a Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany) on an RNA 6000 Nano chip. RNA was quantified using the RiboGreen RNA Quantitation kit (Molecular Probes, Leiden, The Netherlands). Biotin-labeled cRNA was synthesized from 5 µg total RNA using the One cycle kit (Affymetrix, Inc., Santa Clara, CA, USA) according to the manufacturer's protocol. Biotin-labeled cRNA (15 µg) was then hybridized to Affymetrix MOE430A Gene Chips for 16 h at 45 °C. The chips were stained and washed on an Affymetrix Fluidics Station 400, and the fluorescence of the hybridized cRNA was read with an Affymetrix 300 Scanner. The chips were quality controlled with the software 'Expressionist Refiner' (GeneData AG, Basel, Switzerland) detecting and correcting gradients, spots, and distortions. Each probe set is represented by 11 pairs of 25 mer perfect match and mismatch oligonucleotides. Using the MAS 5.0 statistical algorithms implemented in the Expressionist software, the intensities of all 11 probe pairs per probe were condensed to one intensity value. For comparability, the microarrays were scaled after condensing to an average signal intensity of 100.

Data analysis

Gene expression analysis was done using the software 'Expression Analyst' (GeneData AG). Gene expression profiles between individual mice were overall compared by principal component analysis using Genesis 1.7.2. (Sturn *et al.* 2002). To select testosterone-deregulated genes, we removed probe sets with an expression intensity <20 in each sample. In the next step, only those probe sets were selected, which were deregulated twofold by testosterone on day 0 post infectionem (p.i.) and on day 8 p.i., and these were subjected to two-way ANOVA ($P<0{\cdot}01$). Genes were analyzed by the Database for Annotation, Visualization, and Integrated Discovery (Dennis *et al.* 2003) and categorized according to their major biological pathways involved. Genes with similar expression patterns were identified using Gene Cluster 3.0 (Eisen *et al.* 1998, de Hoon *et al.* 2004). Data were $\log_2$-transformed and normalized to the mean expression value for control mice. Hierachical clustering was done using uncentered correlation and average linkage mode.

Quantitative real-time PCR

All RNA samples were treated with DNase of the DNA-free kit (Applied Biosystems, Darmstadt, Germany) for 1 h and then converted into cDNA following the manufacturer's protocol using the QuantiTect Reverse Transcription (RT) kit (Qiagen). Amplifications were performed in the ABI Prism 7500HT Sequence Detection System (Applied Biosystems) using QuantiTect SYBR Green PCR kit (Qiagen) according to the manufacturer's instructions using gene-specific QuantiTect primer assays (Qiagen) for prominin 1 (*Prom1*), 5′-nucleotidase, ecto (*Nt5e*), sulfotransferase family 2A, dehydroepiandrosterone-preferring, member 2 (*Sult2a2*), 3 beta-hydroxysteroid dehydrogenase 5 (*Hsd3b5*), elongation of very long chain fatty acid-like 3 (*Elovl3*), interferon gamma (*Ifnγ*), peroxisome proliferator-activated receptor alpha (*Pparα*), liver X receptor (*Lxr*, listed as *Nr1h3* in the MGI database), retinoid X receptor alpha (*Rxrα*), pregnane X receptor (*Pxr*, *Nr1i2*), farnesoid X receptor (*Fxr*, *Nr1h4*), *Ar*, estrogen receptor alpha (*ERα*, *Esr1*), estrogen receptor beta (*ERβ*, *Esr2*), aryl hydrocarbon receptor (*Ahr*), hepatic nuclear factor 4 alpha (*Hnf4α*), constitutive androstane receptor (*Car*, *Nr1i3*), and vitamin D receptor (VDR). PCRs were conducted as follows: 2 min at 50 °C to activate uracil-*N*-glycosylase (UNG), 95 °C for 10 min to deactivate UNG and to activate the PCR, 45 cycles at 94 °C for 15 s, at 60 °C for 30 s, and at 72 °C for 30 s. Reaction specificity was checked by performing dissociation curves after PCR. Relative quantitative evaluation of amplification data was done using Taqman7500 system software v.1.2.3f2 (Applied Biosystems), and the mRNA was normalized to 18S rRNA. The relative expression was measured using the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen 2001).

Statistical analysis

Student's *t*-test was used for determination of significant differences ($P<0{\cdot}05$).

Results

Experimental design

This study is aimed at identifying genes in the female mouse liver, i) the expression of which is persistently deregulated by testosterone and ii) which even remains deregulated during malaria infection. To this end, we followed the experimental outline given in Fig. 1. Female C57BL/6 mice were treated with testosterone or vehicle twice a week for 3 weeks. The testosterone-treated mice were previously shown to loose their capability of self-healing malaria infections when challenged with 10^6 *P. chabaudi*-infected erythrocytes (Wunderlich *et al.* 1988, 1991). The treatment was discontinued for 12 weeks, before the mice were challenged with *P. chabaudi* malaria reaching peak parasitemia of ~50% on day 8 p.i. in both testosterone- and vehicle-pretreated mice. Previously, we have shown that, though the circulating testosterone levels have declined to values characteristic for untreated females, from 3·79 to 0·21 ng/ml, by the end of the 12-week period of testosterone withdrawal, the mice still succumb to malaria (Benten *et al.* 1997). Livers were aseptically removed from three animals at each of the four time points on days 0 and 8 p.i., i.e. at C_{d0}, C_{d8}, T_{d0}, and T_{d8} as outlined in Fig. 1. RNA was extracted from individual livers before subjecting to Affymetrix chip analysis. The used MOE430A array contains 22 690 oligo probe sets representing 14 000 different genes. Persistent testosterone effects were identified by two-way ANOVA test evaluating only those genes with at least twofold expression changes at a highly significant *P* value <0·01. Functional annotations of the genes were searched in several databases including SwissProt, Proteome, PubMed, and NetAffx from Affymetrix and

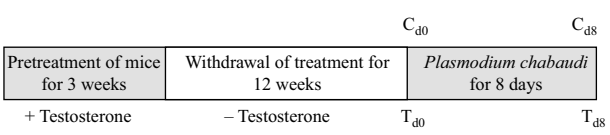

Figure 1 Experimental outline. Mice were treated with testosterone (T) or vehicle (C) twice a week for 3 weeks. The treatment was then discontinued for 12 weeks, before mice were challenged with 10^6 *P. chabaudi*-parasitized erythrocytes. Livers were removed from three mice just prior to infection at C_{d0} and T_{d0} as well as at peak parasitemia on day 8 p.i. at C_{d8} and T_{d8}.

were categorized into nine different groups. Quantitative RT-PCR (qRT-PCR) was used to verify expression profiles of several, arbitrarily selected genes from chips.

Overall expression

Principal component analysis revealed that the overall expression profiles were relatively similar among the three different biological replicates per time point, but differed among the four different time points (Fig. 2). The numbers of testosterone-deregulated genes identified on the microarrays of the four different groups are summarized in a Venn diagram (Fig. 3). *In toto*, the expression of 143 genes was still deregulated by testosterone after 12 weeks of testosterone withdrawal, especially 48 genes were upregulated and 95 genes were downregulated by testosterone (horizontal ellipses in Fig. 3). Upon infection with *P. chabaudi* malaria, 63 genes were deregulated, with 30 genes upregulated and 33 genes downregulated (vertical ellipses in Fig. 3). In particular, 24 genes out of the 48 upregulated genes on day 0 p.i. were still upregulated, and 29 genes out of the 95 genes downregulated by testosterone remained suppressed on day 8 p.i. (Fig. 3). Only one gene was upregulated by testosterone on day 0 p.i. and became relatively downregulated by malaria on day 8 p.i. (Fig. 3).

Gene expression profiles

Out of the 143 genes persistently deregulated by testosterone at the end of the 12-week period of testosterone withdrawal, 54 genes remained significantly deregulated during infection with *P. chabaudi* malaria on day 8 p.i. (Table 1; cf. overlaps in Venn diagram of Fig. 3). The expression profile clustering of these genes is presented in Fig. 4.

Testosterone persistently upregulated the gene expression of well-known male-prevalent enzymes such as CYP2D9, CYP7B1, CYP4A10, UGT2B1, UGT2B38, and

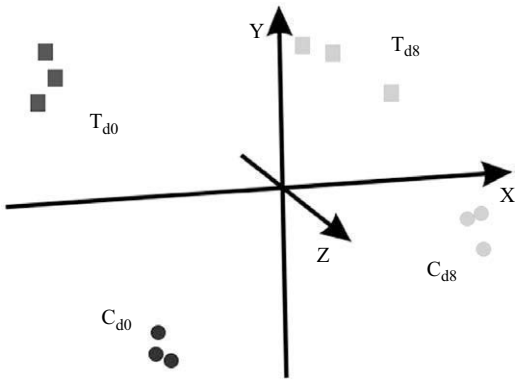

Figure 2 Principal component analysis represents the three major vectors contributing to variance between arrays. Variations exist in expression profiles within and between triplicates.

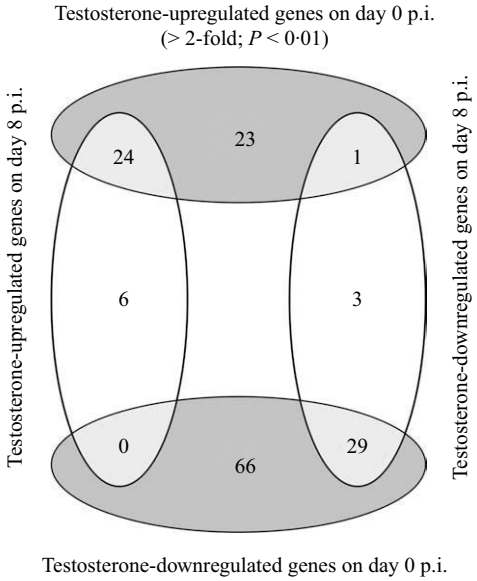

Figure 3 Venn diagram summarizing the numbers of testosterone-deregulated genes on day 0 p.i. (horizontal ellipses) and testosterone-deregulated genes on day 8 p.i. (vertical ellipses). The overlap of the ellipses represents the numbers of those genes persistently deregulated by testosterone.

HSD3B5 as well as the male-prevalent transporter SLCO1A1 (Table 1). By contrast, there also remained a persistent downregulation of gene expression of female-prevalent enzymes such as CYP2B9, CYP2B13, CYP3A41, CYP3A44, FMO3, SULT2A2, and SULT3A1 as well as the female-prevalent transporter BC014805. Remarkably, 27 genes, i.e. 50% of the genes, which were deregulated by testosterone, belonged to the categories phase I and phase II metabolism, lipid, and general metabolism. Phase I expression of genes encoding CYP2B13, CYP3A41, and FMO3 and phase II expression of genes encoding SULT2A2 and SULT3A1 were persistently suppressed by testosterone (>100-fold), whereas HSD3B5 showed the highest persistent testosterone-upregulated expression (>100-fold; Table 1).

Remarkably, the expression of four genes, which were involved in cancerogenic processes, was persistently deregulated by testosterone. These genes encode LAMA3, NOX4, RAD51L1, and PROM1 (Table 1). LAMA3 is an indicator of hepatocellular carcinoma dedifferentiation, NOX4 is normally expressed in cancerous tissues, RAD51L1 is involved in genetic instability, and PROM1 is a marker for hematopoietic and endothelial progenitor cells. Furthermore, persistent deregulation also occurred with four genes involved in signal transduction and genes encoding diverse transporters and seven genes of miscellaneous function.

Long-term testosterone-induced deregulation also occurred in expression of genes involved in the immune response, in particular those encoding IFNγ

Table 1 Expression of genes persistently deregulated by testosterone and their functions annotated to date

Gene symbol	Gene name	T_{d0}/C_{d0}	T_{d8}/C_{d8}	Affymetrix probe ID	Entrez gene	Representative public ID (A)	Functions	PMID
Phase I metabolism								
MGC25972	Similar to cytochrome P450, 4a10	390·20	42·70	1424352_at	277 753	BC025936	Decreased expression in HGF-knockout mice	17241389
Cyp2d9	Cytochrome P450, family 2, subfamily d, polypeptide 9	22·58	4·38	1419349_a_at	13 105	BC010593	Male-specific; regulated by GH; sterol 16α-hydroxylase	16547391, 4074718
Cyp7b1	Cytochrome P450, family 7, subfamily b, polypeptide 1	5·37	3·59	1421075_s_at	13 123	NM_007825	Oxysterol-7-α-hydroxylase involved in synthesis of 7-α-hydroxylated bile acids; male-specific expression in the liver	9295351, 8530364, 11284740
Cyp7b1	Cytochrome P450, family 7, subfamily b, polypeptide 1	4·45	3·55	1421074_at	13 123	NM_007825		
Fmo2	Flavin-containing monooxygenase 2	0·36	0·42	1422904_at	55 990	NM_018881	Typically expressed at high levels in lung	16872995
Cyp3a44	Cytochrome P450, family 3, subfamily a, polypeptide 44	0·10	0·20	1426064_at	337 924	AB039380	Female-specific expression depends on GH	12147261
Cyp2b9	Cytochrome P450, family 2, subfamily b, polypeptide 9	0·06	0·13	1419590_at	13 094	NM_010000	Female-specific expression depends on GH	15381067
Cyp3a41	Cytochrome P450, family 3, subfamily a, polypeptide 41	0·02	0·01	1419704_at	53 973	NM_017396	Female-specific isoenzyme	10775455
Fmo3	Flavin-containing monooxygenase 3	0·01	0·02	1449525_at	14 262	NM_008030	Female-specific expression in the liver	7473608
Cyp2b13	Cytochrome P450, family 2, subfamily b, polypeptide 13	<0·01	0·01	1449479_at	13 089	NM_007813	Female-specific expression	15155787
Phase II metabolism								
Cml5	Camello-like 5	9·51	24·01	1424811_at	69 049	BC024605	Putative *N*-acetyltransferase	
Ugt2b38	UDP glucuronosyltransferase 2 family, polypeptide B38	9·47	3·06	1424934_at	71 773	BC027200	Male-predominant expression in the liver; inductive effects by testosterone	19131521
Cml4	Camello-like 4	6·30	8·91	1419520_at	68 396	NM_023455	Putative *N*-acetyltransferase	
Ugt2b1	UDP glucuronosyltransferase 2 family, polypeptide B1	1·76	2·08	1424934_at	71 773	BC027200	Male-predominant expression in the liver; inductive effects by testosterone	19131521
Sult3a1	Sulfotransferase family 3A, member 1	0·01	0·04	1421669_at	57 430	NM_020565	Female-specific expression	16807285
Sult2a2	Sulfotransferase family 2A, dehydroepiandrosterone (DHEA)-preferring, member 2	<0·01	<0·01	1419528_at	20 865	NM_009286	Female-specific expression in the liver; involved in control of the amounts of active androgens in cells; protects against the toxic effects of lithocholic acid	8570624, 9566751, 16864508
Lipid metabolism								
Elovl3	Elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 3	40·51	6·61	1420722_at	12 686	BC016468	Circadian expression in the liver; cold induction in brown adipose tissue is controlled by PPARα and crucial for accumulation of very long chain	17003504, 15855229
Vldlr	Very low-density lipoprotein receptor	0·21	0·30	1434465_x_at	22 359	AV333363	Involved in uptake of triglycerides	11453330
Vldlr	Very low-density lipoprotein receptor	0·17	0·22	1417900_a_at	22 359	NM_013703		
1810022C23Rik	RIKEN cDNA 1810022C23 gene	0·06	0·19	1451588_at	69 123	BC014724	78% identical and 85% similar to murine peroxisomal delta 3, delta 2-enoyl-coenzymeA isomerase involved in β-oxidation of fatty acids	11781327

(continued)

Table 1 *Continued*

Gene symbol	Gene name	T_{d0}/C_{d0}	T_{d8}/C_{d8}	Affymetrix probe ID	Entrez gene	Representative public ID (A)	Functions	PMID
Hao3	Hydroxyacid oxidase (glycolate oxidase) 3	0·02	0·05	1418654_at	56 185	NM_019545	Peroxisomal-2-hydroxy acid oxidase	10777549
General metabolism								
Hsd3b5	Hydroxysteroid dehydrogenase-5, delta<5>-3-beta	1203·67	119·72	1420531_at	15 496	NM_008295	Involved in steroid synthesis with both dehydrogenase and isomerase activity; male-specific	8319586, 8477648, 8647315
Ela3b	Elastase 3B, pancreatic	4·19	3·82	1415884_at	67 868	NM_026419	Protease of the exocrine pancreas	2826474
Serpine2	Serine (or cysteine) proteinase inhibitor, clade E, member 2	2·91	2·78	1416666_at	20 720	NM_009255	Plasminogen activator inhibitor (PAI1); protective against malaria, since disruption of the *Pai1* gene results in partial loss of the ability to control the course of *P. chabaudi* infections	15618182
4833442J19Rik	RIKEN cDNA 4833442J19 gene	2·80	2·29	1427202_at	320 204	AV002340	Highly conserved protein containing putative methyltransferase domain	
Csad	Cysteine sulfinic acid decarboxylase	2·37	2·74	1427981_a_at	246 277	AY033912	Rate-limiting enzyme in taurine biosynthesis	11997111
Mcm10	Minichromosome maintenance deficient 10 (*S. cerevisiae*)	2·20	2·16	1433408_a_at	70 024	AK010648	DNA replication protein required for assembly and progression of the replication fork	19081065
Serpinb1a	Serine (or cysteine) proteinase inhibitor, clade B, member 1a	0·42	0·46	1416318_at	66 222	AF426024	Inhibitor of neutrophil serine proteases (elastase, cathepsin G and proteinase 3)	17664292
Serpinb1a	Serine (or cysteine) proteinase inhibitor, clade B, member 1a	0·36	0·41	1448301_s_at	66 222	AF426024		
Nt5e	5′ nucleotidase, ecto	0·18	0·22	1422974_at	23 959	NM_011851	Reduced activity by ischemia	9306922
Carcinogenesis								
Lama3	Laminin, alpha 3	3·93	2·64	1427512_a_at	16 774	X84014	Expression correlated with dedifferentiation of hepatocellular tumors	12079511
Nox4	NADPH oxidase 4	3·88	2·52	1419161_a_at	50 490	AB041034	NADPH oxidase generating ROS; *Nox4* is most frequently expressed in tumor cells	11376945, 15155719
Nox4	NADPH oxidase 4	3·29	2·44	1451827_a_at	50 490	BC021378		
Rad51l1	RAD51-like 1 (*S. cerevisiae*)	0·24	0·55	1421430_at	19 363	NM_009014	Involved in genetic instability	15723711
Prom1	Prominin 1	0·06	0·51	1419700_a_at	19 126	NM_008935	Marker for hematopoietic and endothelial progenitor cells	1569483
Immune response								
Ifnγ	Interferon gamma	11·72	0·58	1425947_at	15 978	K00083	Plays a protective role during cerebral malaria	16088835
C6	Complement component 6	8·66	6·37	1449308_at	12 274	NM_016704	Complement component produced in the liver; higher expression in males than in females	4007963
Cklf	Chemokine-like factor	0·43	0·49	1436242_a_at	75 458	BE852312	Chemoattractant for leukocytes	11415443
Tox	Thymocyte selection-associated HMG box gene	0·38	0·55	1425484_at	252 838	BB547854	Upregulated in immature thymocytes but not in matured naive T-cells upon stimulation of the T-cell receptors	11850626
Lag3	Lymphocyte activation gene 3	0·36	0·54	1449911_at	16 768	NM_008479	Negatively regulates T-cell proliferation	12421911
Igk-C	Immunoglobulin kappa chain variable 8 (V8)	0·22	0·52	1426200_at	16 071	AY058910		

(continued)

Table 1 *Continued*

Gene symbol	Gene name	T_{d0}/C_{d0}	T_{d8}/C_{d8}	Affymetrix probe ID	Entrez gene	Representative public ID (A)	Functions	PMID
Signal transduction								
Prlr	Prolactin receptor	0·50	0·48	1425853_s_at	19 116	M22958	Treatment of female mice with testosterone reduces *Prlr* levels	6329976
Prlr	Prolactin receptor	0·45	0·52	1448556_at	19 116	BC005555		
Dscr1l1	Down syndrome critical region gene 1-like 1	0·25	0·31	1421425_a_at	53 901	NM_030598	Mimics the inhibitory effects of DSCR1 on calcineurin signaling pathways in endothelial cells and inhibits angiogenesis	10756093
Mmd2	Monocyte to macrophage differentiation-associated 2	0·23	0·47	1424534_at	75 104	BC025064	Member of the PAQR seven transmembrane protein family that also encompasses non-genomic progesterone receptors	16044242, 12617826
Mmd2	Monocyte to macrophage differentiation-associated 2	0·23	0·47	1438654_x_at	75 104	AV269411		
Transport								
Slco1a1	Solute carrier organic anion transporter family, member 1a1	25·65	22·65	1420379_at	28 248	AB031813	Organic anion transporter with male preponderance in the liver; regulated by androgens and GH secretion pattern	16807376, 12399219
Slco1a1	Solute carrier organic anion transporter family, member 1a1	22·05	41·28	1449844_at	28 248	AB031813		
Slc1a4	Solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	0·37	0·44	1423549_at	55 963	BB277461	Neutral amino acid transporter	12533615
D630002G06|C730048C13Rik	Hypothetical protein D630002G06|RIKEN cDNA C730048C13 gene	0·17	0·31	1451635_at	236 293-319 800	AB056443	Cation transport protein	
D630002G06	Hypothetical protein D630002G06	0·15	0·31	1425222_x_at	236 293	AB056443	Cation transport protein	
AB056442	cDNA sequence AB056442	0·05	0·11	1419751_x_at	171 405	NM_134256	Organic anion transporter 6	
BC014805	cDNA sequence BC014805	0·05	0·15	1425751_at	236 149	AJ132857	64% identical and 79% similar to Slc22a9, an organic anion transporter in transepithelial transport of steroid sulfates	16150593
BC014805	cDNA sequence BC014805	0·04	0·13	1425752_at	236 149	AJ132857		
Miscellaneous								
Omd	Osteomodulin	17·26	2·55	1418745_at	27 047	NM_012050	Bone-specific extracellular matrix protein	10607915
Susd4	Sushi domain-containing protein 4	8·14	2·49	1424221_at	96 935	BC021842	Unknown	
2810439F02Rik	RIKEN cDNA 2810439F02 gene	3·49	3·04	1426223_at	72 747	BC020021	Unknown	
Clec2h	C-type lectin domain family 2, member h	2·88	3·16	1451438_s_at	94 071	AF350410	Unknown	
Arrdc4	Arrestin domain-containing protein 4	0·42	0·54	1426818_at	66 412	BC025091	Unknown	
Pcp4l1	Purkinje cell protein 4-like 1	0·41	0·36	1452913_at	66 425	AV337888	Reported to be expressed only in the central nervous system	15053978
2610528H13Rik	Coiled-coil domain containing 25	0·37	0·55	1451799_at	67 179	BC025545	Unknown	

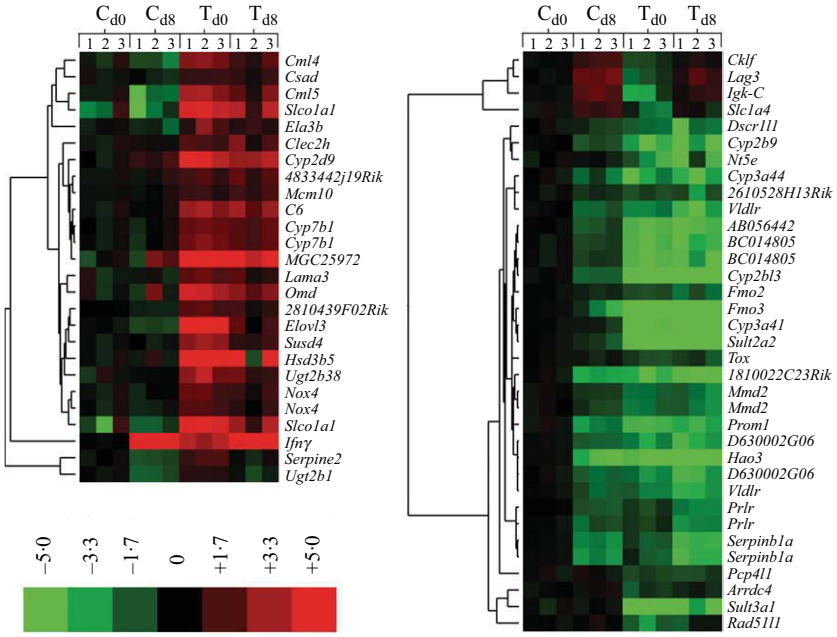

Figure 4 Hierarchical cluster analysis of expression levels of persistently deregulated genes by testosterone, with the upregulated genes on the left and the downregulated genes on the right. Analysis was performed using Gene Cluster 3. All expression levels were normalized to the mean signal intensity of control mice (C_{d0}; cf. outline in Fig. 1), and data are $\log_2$-transformed. Green and red colors represent down- and upregulation respectively as indicated by the logarithmic color scale bar.

and IGK-C (Table 1). Out of the 24 upregulated genes, 3 genes encoding CYP2D9, ELOVL3, and SUSD4 remained upregulated, but at significantly lower levels after infection on day 8 p.i. By contrast, only the expression of *Ifnγ* was increased by testosterone on day 0 p.i. ($T_{d0}/C_{d0}=11{\cdot}78$), but its upregulation appeared relatively suppressed on day 8 p.i. ($T_{d8}/C_{d8}=0{\cdot}58$), i.e. it was strongly induced by infection at C_{d8} than at T_{d8} (Table 1). Finally, Fig. 5 shows that the expression profiles of arbitrarily selected genes from microarrays, i.e. testosterone-upregulated *Hsd3b5*, *Elovl3*, and *Ifnγ* and testosterone-downregulated *Sult2a2*, *Nt5e*, and *Prom1*, could be verified by real-time PCR analysis.

Nuclear receptors

Nuclear receptors play a central role in liver metabolism of endo- and xenobiotics (Tirona & Kim 2005, Plant & Aouabdi 2009). However, some nuclear receptors such as RXRα, LXRα, and PPARα are not represented on the Affymetrix chip, and others may be removed according to the criteria we applied for analysis. We therefore decided to re-examine a possible persistent testosterone effect on expression of genes encoding some known nuclear receptors using qRT-PCR. Table 2 summarizes data obtained for 12 different receptors. It is conspicuous that after testosterone withdrawal for 12 weeks, only the expression of *Car* was more than twofold downregulated. However, expression of *Car* did not remain deregulated by testosterone after infection with *P. chabaudi* on day 8 p.i.

Discussion

Using Affymetrix microarray technology, the present study has revealed that, among 14 000 genes examined, only 54 genes were persistently deregulated by testosterone in livers of female mice and remained deregulated after subsequent infection with *P. chabaudi* for 8 days. The fact that only 54 genes out of 14 000 are persistently deregulated by testosterone indicates that this deregulation reflects specific testosterone effects rather than long-term toxic testosterone effects. The genes permanently changed by testosterone can be summarized into three groups: 1) genes involved in liver metabolism, 2) genes involved in hepatocellular carcinogenesis, and 3) genes involved in immune response.

Most of the genes permanently changed by testosterone belong to the first group, i.e. genes encoding enzymes involved in phase I–III liver metabolism. Conspicuously, there is a persistent downregulation of some female-prevalent genes such as *Cyp2b9*, *Cyp2b13*, *Cyp3a41*, *Cyp3a44*, *Fmo3*, *Sult2a2*, *Sult3a1*, and

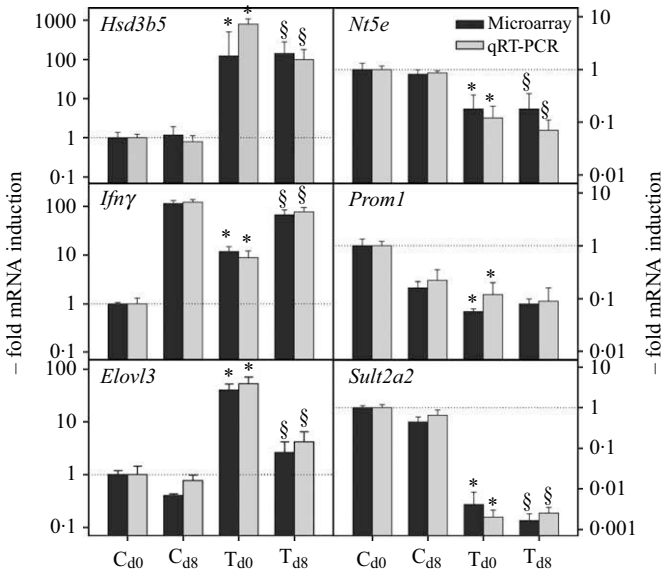

Figure 5 Expression of testosterone-deregulated genes analyzed by quantitative real-time PCR. Gene expression profiles of testosterone-upregulated *Hsd3b5*, *Ifnγ*, and *Elovl3* on the left and testosterone-downregulated *Nt5e*, *Prom1*, and *Sult2a2* on the right, as revealed by microarrays, can be verified by real-time PCR analysis. Relative mRNA expression was normalized to the mean expression of control mice (C_{d0}). For definition of C_{d0}, C_{d8}, T_{d0}, and T_{d8}, see outline in Fig. 1. Means ± S.D. from three different mice are indicated for each group. Significant differences between T_{d0} and C_{d0} are indicated by *, and between T_{d8} and C_{d8} by § using *t*-test ($P<0{\cdot}05$).

BC014805, and an upregulation of some male-prevalent genes such as *Cyp2d9*, *Cyp7b1*, *Hsd3b5*, *Ugt2b1*, *Ugt2b38*, and *Slco1a1* (cf. Table 1). The testosterone-induced permanent changes of these genes contribute to an at least partial masculinization of the metabolism of the female mouse liver. Such a masculinization is ultimately also in accordance with the testosterone-induced permanent changes of those genes listed in the second group, which are involved in and contribute to hepatocellular carcinogenesis, such as *Lama3* and *Nox4*. Indeed, hepatocellular carcinoma is known for a long time to be much more prevalent in male mice than in female mice (Drinkwater *et al.* 1990, Kemp & Drinkwater 1990, Nagasue & Kohno 1992). The changes of *Igk-C* and *Ifnγ* in the third group of testosterone-deregulated genes indicate that testosterone also affects genes involved in the immune response, though our data cannot discriminate as to whether testosterone affects only genes of intrahepatic T- and B-cells and/or also circulatory B- and T-cells. At least,

Table 2 Effects of testosterone pretreatment and *Plasmodium chabaudi* malaria on gene expression of different nuclear receptors in the female mouse liver as revealed by quantitative reverse transcription-PCR

Gene symbol	Gene name	T_{d0}/C_{d0}	T_{d8}/C_{d8}
Pparα	Peroxisome proliferator-activated receptor alpha	0·76±0·07	1·25±0·18
Lxrα (*Nr1h3*)	Liver X receptor alpha	0·76±0·06	0·72±0·19
Rxrα	Retinoid X receptor alpha	0·53±0·33	0·92±0·33
Pxr (*Nr1i2*)	Pregnane X receptor	1·13±0·12	1·01±0·57
Fxr (*Nr1h4*)	Farnesoid X receptor	0·56±0·42	0·98±0·09
Ar	Androgen receptor	0·91±0·22	1·15±0·27
ERα (*Esr1*)	Estrogen receptor alpha	0·88±0·22	1·19±0·21
ERβ (*Esr2*)	Estrogen receptor beta	1·46±0·33	0·95±0·14
Ahr	Aryl hydrocarbon receptor	1·16±0·16	1·09±0·17
Hnf4α	Hepatic nuclear factor 4 alpha	1·28±0·33	1·13±0·38
Car (*Nr1i3*)	Constitutive androstane receptor	0·31±0·06	0·99±0·26
Vdr	Vitamin D receptor	ND	0·31±0·17

the persistent downregulation of *Igk-C* by testosterone indicates that testosterone ultimately leads to a partially reduced production of antibodies. Indeed, this confirms previous studies that testosterone lowers the production of antibodies (Fujii *et al.* 1975, Hirota *et al.* 1980, Morton *et al.* 1981, Kincade *et al.* 1994, Benten *et al.* 1997). More complicated is the situation with *Ifnγ* which is upregulated by the end of the testosterone withdrawal period, but the subsequent malaria infection induces expression of *Ifnγ* in both the vehicle- and the testosterone-pretreated mice, however, at a higher extent in control than in testosterone-pretreated mice.

The mechanisms by which testosterone exerts reprograming of gene expression, evidenced as permanent changes in the expression of distinct genes in the female mouse liver, are unknown, but are expected to be rather complex and, possibly, even to be different for different genes, involving both direct and indirect actions of testosterone on gene expression (Wunderlich *et al.* 2002, Centenera *et al.* 2008, Bennett *et al.* 2009). An indirect action of testosterone may also take place through the hypothalamus–pituitary gland–liver axis, which is imprinted neonatally by androgens and which operates through pulsatile versus continuous patterns of GH secretion in males versus females respectively (Colby *et al.* 1973, Einarson *et al.* 1973, Sakuma *et al.* 2002, Gustafsson 2005, Waxman & Holloway 2009). Indeed, this axis is currently envisaged as to maintain the sexual dimorphism of hepatic gene expression, especially expression of cytochrome P450 genes involved in liver metabolism. GH in turn activates the GH-responsive transcription factor STAT5b, which is, besides HNF4α, essential for establishment and maintenance of sexually dimorphic gene expression in the liver of male mice (Clodfelter *et al.* 2007, Holloway *et al.* 2007, 2008). In female mice, however, STAT5b plays only – if at all – a minor role in sexually dimorphic pattern of hepatic gene expression (Holloway *et al.* 2007). Remarkably, our microarray and qRT-PCR data indicate that testosterone affects expression of neither *Stat5b* nor *Hnf4α* in the female mouse liver. Also, any other transcription factors including common nuclear factors are apparently not affected by testosterone. Moreover, testosterone-induced reprograming of liver gene expression may involve changes in the epigenome. Indeed, steroid hormones are known to induce long-lasting chromatin remodeling through epigenetic mechanisms, as e.g. DNA methylation and/or covalent modifications of histones (Grunstein 1997, Goldberg *et al.* 2007, Murray *et al.* 2009, Waxman & Holloway 2009).

Some of those genes that may be important for the testosterone-induced persistent susceptibility to *P. chabaudi* malaria have been possibly not detected due to masking since our experiments have been conducted with livers *in toto*. Also, it is not unlikely that genes may be critical in other organs, as e.g. in the spleen representing the other major effector site against blood stage malaria (Wunderlich *et al.* 2005). Nevertheless, it appears as if the testosterone-induced permanent changes we have found in hepatic expression of those genes involved in liver metabolism and in particular those involved in immune response are presumably relevant for the testosterone-induced persistent susceptibility to *P. chabaudi* malaria. For instance, masculinization of liver metabolism possibly reflects a decreased hepatic capacity to detoxify substances, which are derived in abundance from destroyed parasites and host cells during malaria infection. At least, the testosterone-induced decrease in production of antibodies and IFNγ indicates an association with the testosterone-induced persistent susceptibility to *P. chabaudi* malaria. Indeed, the current view predominates that protective immunity to *P. chabaudi* malaria is eventually mediated through antibodies (Achtman *et al.* 2005, 2007) requiring activation by the T_H2 response. However, this activation has to be preceded by an IFNγ-dependent activation of the T_H1 response (Taylor-Robinson & Phillips 1998, Balmer *et al.* 2000, Su & Stevenson 2000, Batchelder *et al.* 2003, Cernetich *et al.* 2006). The specific upregulation of *Ifnγ* we observe after testosterone withdrawal could reflect an overactivation of the T_H1 response; the delicately balanced switching to the T_H2 response may be then perturbed (Zhang *et al.* 2000), thus delaying the maturation and secretion of the antibodies from plasma B cells. In addition, the testosterone-increased production of IFNγ may perturb the proper activation of those genes, which encode, e.g. other effectors directed against infections such as diverse GTPases (Boehm *et al.* 1998, Klamp *et al.* 2003, Degrandi *et al.* 2007).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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2.4 Hepatic miRNA expression reprogrammed by *Plasmodium chabaudi* malaria

Background: MiRNAs exhibit a wide spectrum of biological functions and are increasingly reported to be associated with the outcome of several human malignancies and even infectious diseases. Moreover, miRNAs control diverse aspects of the liver, including hepatocyte growth, stress response, metabolism, infection, proliferation, gene expression, and maintenance of hepatic phenotype. miRNAs play a key role in both innate and adaptive immunity, but there is only little information available about their role in the response of the anti-malaria effector-site liver in female mice.

Methods: Female C57BL/6 mice were challenged with 10^6 *P. chabaudi*-infected erythrocytes and homologuously re-infected after 56 days *p.i.*. The miRNA expression patterns were examined on days 0 and 8 *p.i.* after primary infection, and on days 0 and 8 *p.i.* after secondary infection using miRXplore microarrays and qRT-PCR. ELISA was used to determine IgG levels on the different time-points and light microscopy and PCR-analysis of a *P. chabaudi* specific protein-encoding mRNA, Pc90, was used to detect the presence of *P. chabaudi*-infected parasites. Inflammatory and liver metabolism marker genes were analyzed by qRT-PCR.

Results: Primary infections resulted in approximately 50% peak parasitemia on day 8 and approximately 80% survival, whereas secondary infections were characterized by 1.5% peak parasitemia and 100% survival. In immune mouse liver, there were still *P. chabaudi*-infected erythrocytes. Moreover, immune mice contained increased levels of IgG2a- and IgG2b-isotypes. Primary infections were described by a transcriptional upregulation of IL-1β, TNFα, IFNγ, NFκB, and iNOS, and a transcriptional downregulation of CYP7A1 and SULT2A2. The hepatic miRNA signature is designated by an upregulation of the 3 miRNA-species miR-26b, MCMV-miR-M23-1-5p, and miR-1274a, and a downregulation of the 16 miRNA-species miR-101b, let-7a, let-7g, miR-193a-3p, miR-192, miR-142-5p, miR-465d, miR-677, miR-98, miR-694, miR-374*, miR-450b-5p, miR-464, miR-377, miR-20a*, and miR-466d-3p.

Conclusion: This study describes persistently deregulated miRNAs as potential new players in acquired protective immunity against *P. chabaudi* malaria.

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Hepatic miRNA expression reprogrammed by *Plasmodium chabaudi* malaria

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Abstract Evidence is accumulating that miRNAs are critically implicated in the outcome of diseases, but little information is available for infectious diseases. This study investigates the hepatic miRNA signature in female C57BL/6 mice infected with self-healing *Plasmodium chabaudi* malaria. Primary infections result in approximately 50% peak parasitemia on day 8 p.i., approximately 80% survival, and development of protective immunity. The latter is evidenced as 100% survival and 1.5% peak parasitemia upon homolog re-infections of those mice which are still alive on day 56 after primary infection. Such immune mice exhibit increased levels of IgG2a and IgG2b isotypes and still contain *P. chabaudi*-infected erythrocytes in their livers as revealed by light microscopy and PCR analysis. Primary infections, but not secondary infections, induce an upregulation of hepatic mRNAs encoding IL-1β, TNFα, IFNγ, NF-κB, and iNOS, and a downregulation of mRNAs for CYP7A1 and SULT2A2, respectively. Using miRXplore microarrays containing 634 mouse miRNAs in combination with quantitative RT-PCR, the liver is found to respond to primary infections with an upregulation of the three miRNA species miR-26b, MCMV-miR-M23-1-5p, and miR-1274a, and a downregulation of the 16 miRNA species miR-101b, let-7a, let-7g, miR-193a-3p, miR-192, miR-142-5p, miR-465d, miR-677, miR-98, miR-694, miR-374*, miR-450b-5p, miR-464, miR-377, miR-20a*, and miR-466d-3p, respectively. Surprisingly, about the same pattern of miRNA expression is revealed in immune mice, and this pattern is even sustained upon homolog re-infections of immune mice. These data suggest that development of protective immunity against malarial blood stages of *P. chabaudi* is associated with a reprogramming of the expression of distinct miRNA species in the female mouse liver.

Introduction

Natural immunity against malaria is directed against the blood stages of the parasitic protozoon *Plasmodium* and can be acquired after repeated infections (Cohen et al. 1982; Miller et al. 2002; Doolan et al. 2009). A general feature of this immunity is that it can suppress disease symptoms, but it is not able to prevent parasitemia during malaria season (Gomase and Tagore 2008; Pierce and Miller 2009; Goodman and Draper 2010). The rodent malaria *Plasmodium chabaudi* is a convenient model to investigate blood-stage malaria: it shares several characteristics with *Plasmodium falciparum*, the most virulent humanpathogenic malaria species (Mehlhorn 2008; Mackinnon and Read 2003). Both *P. falciparum* and *P. chabaudi* prefer normocytes as host cells (Pasvol et al. 1980; Jarra and Brown 1989; Clough et al. 1998). Both species exhibit cytoadherence and sequestration of the schizont stages from peripheral circulation to internal microvasculature, which are considered as major virulence factors of human malaria (Roberts et al. 2000; Pain et al.

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2001). Moreover, both malaria species induce strain-specific immunity (Snounou et al. 1989; Mota et al. 1998) and display clonal antigenic variation (Biggs et al. 1991; Roberts et al. 1992).

Primary blood-stage infections with *P. chabaudi* malaria induce innate and adaptive responses, which help to self-heal and to survive the infections. This self-healing is associated with the development of immune mechanisms protecting against secondary homolog re-infections (Wunderlich and Helwig 1987; Jarra and Brown 1989; Langhorne et al. 2002). Remarkably, self-healing and acquisition of immunity against *P. chabaudi* malaria can even take place in the absence of secondary lymphoid organs as we have previously shown in lymphotoxin β receptor-deficient mice (Krücken et al. 2005; Wunderlich et al. 2005). Even the spleen of these mice exhibits severe defects such as absence of the marginal zone, T/B cell segregation, and absence of the follicular dendritic cell network (Fütterer et al. 1998; Fu and Chaplin 1999), which suggests at least an impairment of the widely accepted function of the spleen as the major effector site against blood-stage malaria.

The liver plays a central role in malaria. It is not only that site in which pre-erythrocytic development of *Plasmodium* takes place but also this lymphoid organ (Häussinger et al. 2004; Crispe 2009) apparently differentiate to an essential effector against blood-stage malaria (Balmer et al. 2000; Krücken et al. 2005). Indeed, blood-stage malaria activates Kupffer cells, which constitute 80–90% of all macrophages, which are then able to phagocytose *Plasmodium*-infected erythrocytes (Dockrell et al. 1980; Taverne et al. 1987; Delić et al. 2010). Moreover, specific extrathymic lymphocyte populations are generated in the liver, which mediate protective immunity to blood-stage malaria (Mannoor et al. 2001, 2002). Even the liver metabolism, especially its detoxifying capacity, appears to be critical for the outcome to blood-stage malaria (Delić et al. 2010).

During the last years, evidence is accumulating that miRNAs are involved in the control of diseases. MicroRNAs are small non-coding RNAs, approximately 20 bp in size, that are involved in post-transcriptional regulation of gene expression by affecting both the stability and translation of mRNAs (Bartel 2004; Ruvkun 2008; Pawlicki and Steitz 2010). In mice, more than 600 miRNA have been discovered to date (Bissels et al. 2009) and each miRNA is supposed to target hundreds of mRNAs of protein-coding genes, respectively (Griffiths-Jones et al. 2008). There is also evidence that characterizes miRNAs as key regulators of immune reactions (Baltimore et al. 2008; Bi et al. 2009; Davidson-Moncada et al. 2010). Thus, a number of miRNAs are implicated in both innate and adaptive immune responses, including the release of inflammatory mediators, the proliferation of monocytes and neutrophils, the development and differentiation of B and T cells and antibody switching (Li et al. 2007; O'Connell et al. 2007; Johnnidis et al. 2008; Lindsay 2008). Moreover, miRNAs are known to control diverse features of the liver, including hepatocyte growth, stress response, metabolism, infection, proliferation, gene expression, and maintenance of hepatic phenotype (Bala et al. 2009; Chen 2009; Lu and Liston 2009; Wang et al. 2009; Kerr and Davidson 2010). However, only little information is available about the role of miRNAs in infectious diseases and the miRNA signature of anti-malaria effector organs has not yet been investigated in response to malaria.

This prompted us to investigate possible effects of blood-stage malaria on the miRNA signature of the liver of female mice. Here, we show that self-healing infections with *P. chabaudi* blood-stage malaria induce changes in the hepatic miRNA, which remain sustained in immune mice, even after homolog re-infections.

Material and methods

Mice

C57BL/6 mice were bred under specified pathogen-free conditions in the animal facilities of the Heinrich-Heine University Düsseldorf. They were housed in plastic cages, and they received a standard diet (Wohrlin, Bad Salzuflen, Germany) and water ad libitum. The experiments were approved by the state authorities and followed the German law on animal protection.

Malaria infections

Blood-stage infections of *P. chabaudi* (Wunderlich et al. 1982; Krücken et al. 2009) were passaged weekly in NMRI mice. Female C57BL/6 mice were challenged *i. p.* with 10^6 *P. chabaudi*-parasitized erythrocytes. Parasitemia was determined in Giemsa-stained tail blood. Erythrocytes were counted in a Neubauer chamber.

Preparation of sera and livers

Mice were anesthetized and blood was taken from the retroorbital plexus. After clotting and centrifugation, supernatants were used as sera and stored at −20°C until use. Immediately after taking blood, mice were killed by cervical dislocation. Livers were aseptically removed, cut up in sterile PBS, and frozen at −80°C until use—if not otherwise stated.

Histopathology

Pieces of livers, freshly prepared, were fixed with 10% neutral buffered formalin at room temperature before

embedding in paraffin. Sections were cut and stained with hematoxylin/eosin.

Elisa of IgG isotypes

Sera were assayed for IgG antibodies as described previously (Benten et al. 1997). In brief, maxiSorp F96 microtiter plates (Nunc, Wiesbaden, Germany) were coated first with capture antibody specific for mouse IgG isotypes overnight at 4°C. Then, serially diluted sera were added at 37°C for 1 h before incubation with biotinylated secondary antibodies at 37°C for 45 min. Thereafter, streptavidin-biotinylated horseradish peroxidase complex (Amersham-Buchler, Braunschweig, Germany) was added at 37°C for 45 min before loading with 0.53 mg/ml 1,2-phenylendiamine (DAKO, Copenhagen, Germany) and 1.6 μl/ml 30% H_2O_2 in each well. Absorbance was measured at 492 nm.

RNA isolation

Approximately 250 mg frozen liver was homogenized in 5 ml Trizol (Peqlab Biotechnology, Erlangen, Germany), before mixing with 1 ml chloroform and centrifugating at 3,000×*g* for 45 min. The supernatant was precipitated with isopropanol and subsequently the pellet was washed twice with 80% ethanol, air-dried, and dissolved in 200 μl RNase-free water. RNA concentrations were determined at 260 nm and quality control was generally determined by agarose gelelectrophoresis.

RNA labeling and hybridization of microarrays

The Agilent 2100 Bioanalyzer platform (Agilent Technologies, Böblingen, Germany) was used to control the quality of RNA samples, before the RNA was labeled and hybridized according to protocols of the miRXplore™ manufacturer (Miltenyi Biotec, Bergisch-Gladbach, Germany) (Landgraf et al. 2007; Bissels et al. 2009). The control miRXplore Universal Reference (UR), representing a defined pool of synthetic microRNAs, was labeled with green fluorescent Hy3. The experimental samples were labeled with the red fluorescent Hy5. Subsequent hybridization was done overnight to miRXplore™Microarrays using the a-Hyb™ Hybridization Station (Miltenyi Biotec, Bergisch-Gladbach, Germany). The hybridized miRXplore™ Microarrays were monitored for fluorescence signals using a laser scanner (Agilent Technologies, Böblingen, Germany).

Data analysis

The miRXplore™ Microarrays were scanned and quantified as detailed previously (Landgraf et al. 2007; Bissels et al. 2009). In brief, the ImaGene software (Biodiscovery, El Segundo, USA) was used to determine mean signal and mean local background intensities for each spot of the microarray images. Low-quality spots were flagged and excluded from data analysis, and unflagged spots were evaluated using the PIQOR™ Analyzer software (Miltenyi, Biotec), which allows automated data processing of the raw data text files derived from the ImaGene software. After background subtraction, the net signal intensity was subjected to data normalization and calculation of the Hy5/Hy3 ratios for the species of interest. Only those spots were taken into account for the calculation of the Hy5/Hy3 ratio, which are characterized by a signal that was equal or higher than the 50% percentile of the background signal intensities. The "sample versus universal reference" modus was chosen for absolute quantification and indirect comparisons of multiple experimental samples. Accordingly, the ratio of signals from experimental sample versus UR over the ratio of vehicle control versus UR was calculated and the resulting re-ratio yielded the ratio of experimental sample versus control (Bissels et al. 2009).

Quantification of miRNA

Quantitative real-time polymerase chain reaction (qRT-PCR) were performed with miScript™ SYBR Green PCR Kit (Qiagen, Hilden, Germany) using the ABI Prism® 7500HT Sequence Detection System (Applied Biosystems, Darmstadt, Germany). RNA was treated with DNase (Applied Biosystems, Darmstadt, Germany) at 37°C for at least 1 h prior to conversion into cDNA following the manufacturer's protocol using the miScript™ Reverse Transcription Kit (Qiagen, Hilden, Germany). Primers for miR-1274A, miR-26B, miR-101B, let-7A, miR-694, let-7B, and miR-122 were commercially provided as miScript™ primer assays by Qiagen (Hilden, Germany). PCRs were as follows: 94°C for 15 min as initial activation step to activate HotStarTaq DNA polymerase followed by 40 cycles at 94°C for 15 s, at 55°C for 35 s, and at 70°C for 30 s. Reaction specificity was analyzed by melting curves, and the Taqman7500 system software v.1.2.3f2 (Applied Biosystems, Darmstadt, Germany) and the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) were applied for relative quantitative evaluation of amplification data. The U6 small nuclear RNA was used as an internal quantification standard.

Quantification of mRNA

RNA samples were first treated with DNase as described above and then converted into cDNA following the manufacturer's protocol using the QuantiTect™ Reverse Transcription Kit (Qiagen, Hilden, Germany). The QuantiTect™ SYBR® Green PCR kit (Qiagen) was used for

amplifications according to the manufacturer's instructions using gene-specific QuantiTect™ primer assay (Qiagen) for 18S rRNA, interleukin-1β (IL-1β), tumor necrosis factor alpha, IL-6, interferon gamma (IFNγ), nuclear factor κB (NF-κB), inducible nitric oxide synthase (iNOS), cytochrome P450, family 7, subfamily a, polypeptide 1 (CYP7A1), sulfotransferase family 2A, member 2 (SULT2A2), and the pair for the *P. chabaudi*-specific Pc90 primer was synthesized using the sense (5′-AAT GCT CCT GAA GAA ACT CAA GCC GAA GAA AT-3′) and antisense (5′-TTG CAA TCT CGA CAT TTT CAT CAA CCG TAA AG-3′) primers. PCRs were conducted as follows: 2 min at 50°C to activate uracil-*N*-glycosylase (UNG), 95°C for 10 min to deactivate UNG and to activate the PCR, 45 cycles at 94°C for 15 s, at 60°C for 30 s, and at 72°C for 30 s. Reaction specificity was checked by performing dissociation curves after PCR and agarose gelelectrophoresis.

Statistical analysis

Significance was evaluated by two-way ANOVA ($p<0.01$) with Dunnett and Bonferoni post hoc tests using the statistical package program SPSS version 17.0.

Results

Female mice of the inbred strain C57BL/6 are able to survive blood-stage infections with *P. chabaudi* malaria. When mice were challenged with 10^6 *P. chabaudi*-infected erythrocytes, the infections took a self-healing course in more than 80% of the mice (Fig. 1). The pre-crisis phase of infection culminates at a peak parasitemia of approximately 50% on day 8 p.i. The subsequent crisis phase is characterized by a dramatical decline of parasitemia to approximately 1% to 2% on day 13 p.i., before a second smaller peak of approximately 15% followed on day 18 p.i. Thereafter, parasitemia declined to below 0.1% and *P. chabaudi*-infected erythrocytes largely disappeared from the peripheral blood (Wunderlich and Helwig 1987; Jarra and Brown 1989).

Self-healing is associated with acquisition of protective immune mechanisms against *P. chabaudi* malaria. Indeed, homolog re-infections of mice after 8 weeks of primary infections resulted in survival of all mice with a remarkable low peak parasitemia of only approximately 1.5% on day 8 after re-infection (Fig. 1). Such immune mice exhibited a strong increase in circulating total IgG-antibodies, mainly due to increased levels of IgG2a and IgG2b isotypes (Table 1), which is consistent with previous studies (Langhorne et al. 2002; Smith and Taylor-Robinson 2003; Wipasa et al. 2009). Remarkably, *P. chabaudi*-infected

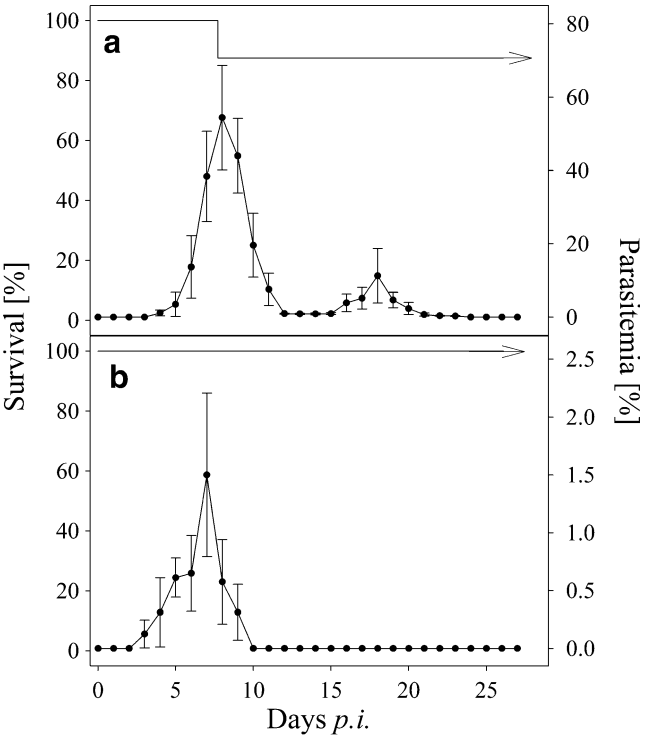

Fig. 1 Primary (**a**) and secondary (**b**) *P. chabaudi* infections in female C57BL/6 mice. Mice were infected with 10^6 *P. chabaudi*-parasitized erythrocytes. All values are given as means ± SD ($n=8$)

erythrocytes, which had disappeared from peripheral blood, were still apparent in liver sections of immune mice before re-infection on day 56 after primary infection (Fig. 2). In accordance, analysis with quantitative RT-PCR clearly revealed *P. chabaudi*-specific mRNA encoding the parasite protein Pc90 in the liver (Fig. 3). This protein is the major immunogenic parasite protein localized at the host erythrocyte membrane: it is synthesized by trophozoite stages of *P. chabaudi*, it is exported to the host cell cytoplasm, and it finally associates with the inner surface of the plasma membrane of host erythrocytes (Wunderlich et al. 1985; 1987; 1988a, b, c; Lanners et al. 1999). The cDNA sequence of Pc90 has been largely characterized (Schmitt-Wrede et al. 1993; also Hartz et al. 1993; Giraldo et al. 1999).

The liver strongly responded to primary infections. At peak parasitemia on day 8 p.i., there was a dramatic increase in the mRNAs of the cytokines IL-1β, TNFα, and

Table 1 IgG-isotypes in naive (N_{d0}) and immune (I_{d0}) mice

IgG-isotype	N_{d0}	I_{d0}
IgGtot	1,706.34±107.5	12,757.71±1,048.6
IgG1	223.165±24.9	195.52±39
IgG2a	9.21±2.7	518.08±122.8
IgG2b	3,497.12±492.5	12,567.31±1,846.5
IgG3	469.87±87	604.32±77.9

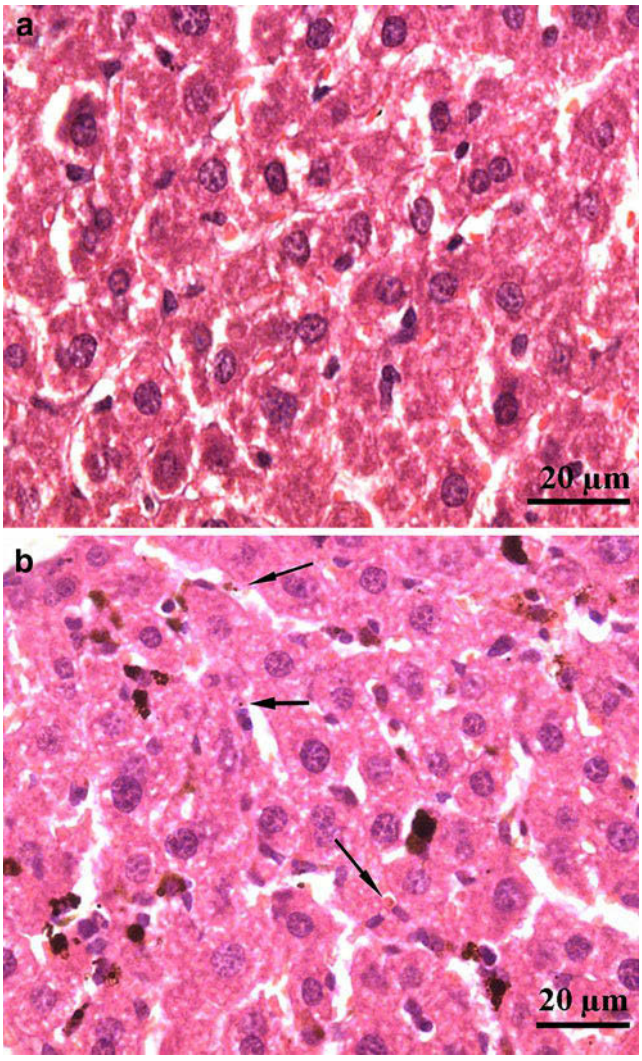

Fig. 2 Light microscopy of liver sections of non-infected naïve (**a**) and immune female C57BL/6 mice (**b**). Arrows indicate *P. chabaudi*-infected erythrocytes in immune mice after 56 days of primary infection

IFNγ, respectively (Fig. 4). Also, mRNA expression of NF-κB and iNOS was upregulated, while the mRNA expression of CYP7A1 and SULT2A2 involved in phases I and II liver metabolism were downregulated at peak

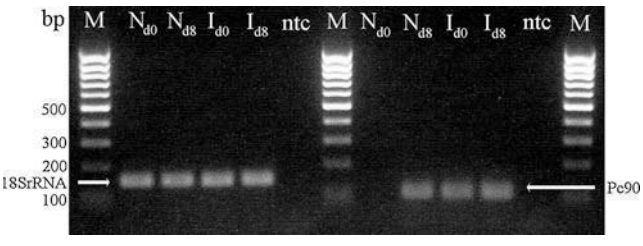

Fig. 3 Quantitative RT-PCR analysis of 18S rRNA and Pc90 mRNA. The PCR products of the mouse 18S rRNA (146 bp) and the *P. chabaudi* specific Pc90 mRNA (90 bp) were seperated on a 1% agarose gel. *M* marker; *ntc* non-template control; N_{d0} and I_{d0} indicate non-infected naïve and immune mice, N_{d8} and I_{d8} after infection and homolog re-infection on day 8 p.i., respectively

parasitemia (Fig. 5). Surprisingly, immune mice exhibited about the same level of TNFα- and iNOS-mRNA in comparison with naïve mice, whereas the mRNA expression of IL-1β, IFNγ, IL-6, NF-κB, CYP7A1, and SULT2A2 were even significantly downregulated. Also, it is remarkable that none of these examined mRNAs in immune mice did significantly respond to homolog re-infections, in contrast to primary infections in naïve mice (Figs. 4, 5).

The miRNA signature of the liver was investigated using the miRXplore microarray technology. Pieces of frozen liver were taken from three naïve and immune mice of the same age of 19 weeks as well as three naïve and immune mice after infection with 10^6 *P. chabaudi*-infected erythrocytes on day 8 p.i, respectively. Equal amounts of RNA of the individual mice were pooled from the four groups of mice, and these RNAs were subjected to a screening using miRXplore™ microarrays containing 634 mouse miRNAs according to miRBase version 12.0. Hy5/Hy3 false-color images of the scanned microarrays are shown in Fig. S1 (supplemental material), and the signal intensities of those miRNAs, which passed the quality filtering are contained in the double-log scatter plots in Fig. 6 (Material and methods). Calculation of the re-ratios detected 19 miRNAs being at least 2-fold up- or downregulated in mice with primary infections of *P. chabaudi* at peak parasitemia on day 8 p.i. (Table 2).

Surprisingly, about the same deregulation of miRNA expression was also detected in immune mice on day 56 after the primary infections and was even sustained upon homolog re-infection on day 8 p.i. In toto, the expression of the three miRNAs miR-26b, MCMV-miR-M23-1-5p, and miR-1274a was upregulated, whereas the expression of 16 miRNAs was downregulated as summarized in Table 2. These downregulated miRNAs comprised miR-101b, let-7a, let-7g, miR-193a-3p, miR-192, miR-142-5p, miR-465d, miR-677, miR-98, miR-694, miR-374*, miR-450b-5p, miR-464, miR-377, miR-20a*, and miR-466d-3p. Table 2 also contain the annotated functions of these deregulated miRNAs, as far as known. The *P. chabaudi*-induced deregulation of the miRNAs was also verified for six arbitrarily selected miRNAs by quantitative RT-PCR in six individual livers obtained from each of the four groups of mice, respectively, which essentially confirmed the microarray data (Fig. 7).

Discussion

This is the first study that reports malaria-induced changes in the miRNA-signature of an anti-malaria effector site. Indeed, the female mouse liver responds to self-healing infections with blood stages of *P. chabaudi* with an altered expression of 19 different miRNA species. Surprisingly, these changes remain sustained in those mice which have acquired immunity, even after homolog re-infection.

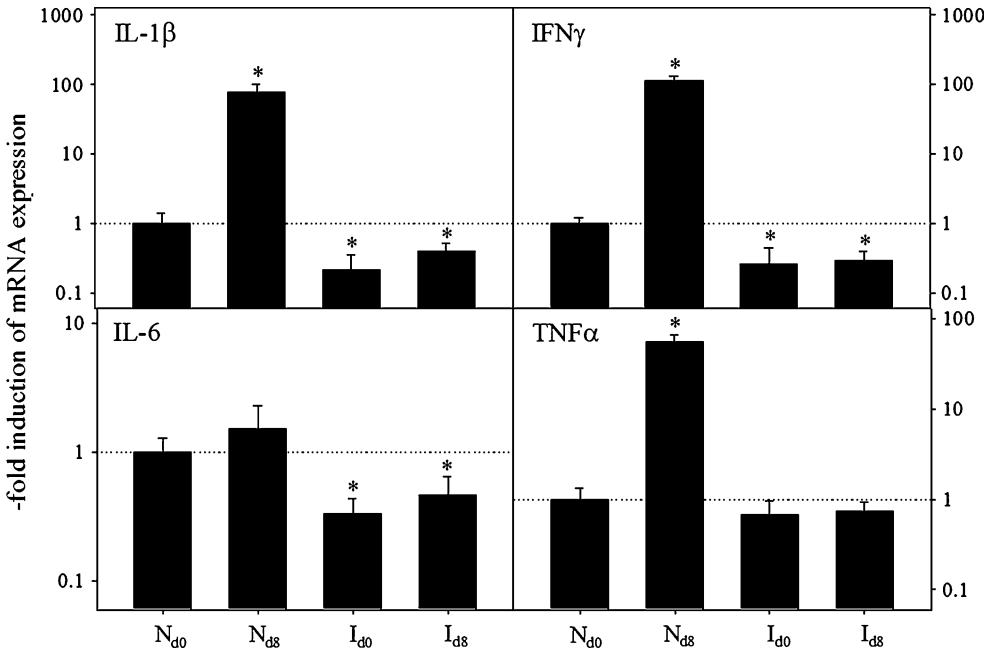

Fig. 4 Quantitative RT-PCR of hepatic cytokines in naïve (N_{d0}) and immune mice (I_{d0}) as well as after infection with 10^6 *P. chabaudi*-infected erythrocytes at peak parasitemia on day 8 p.i. (N_{d8}, I_{d8}). Values represent means ± SD (n=6). *Asterisks* indicate significant differences (p<0.01) with respect to N_{d0}

Actually, primary infections of *P. chabaudi* at peak parasitemia have induced a 2- to 3-fold upregulation of the three miRNA species miRNAs miR-26b, MCMV-miR-M23-1–5p, and miR-1274a, and a 1.5–15-fold downregulation of the 16 miRNA species miR-101b, let-7a, let-7 g, miR-193a-3p, miR-192, miR-142-5p, miR-465d, miR-677, miR-98, miR-694, miR-374*, miR-450b-5p, miR-464, miR-377, miR-20a*, and miR-466d-3p. The upregulated miRNA miR-1274a is relatively abundant in the liver, but its functions are completely unknown to date, just as those of the seven downregulated miRNAs miR-465d, miR-677, miR-694, miR-374*, miR-450b-5p, miR-464, and miR-466d-3p. The other 11 miRNAs appear to be involved in processes normally associated with apoptosis and cancer (Table 2). According to our databank researches, however, the functions of none of these 11 miRNA species are known in context with malaria at all.

The liver is known as a lymphoid organ and is able to generate intrahepatic innate and adaptive immune responses (Häussinger et al. 2004; Crispe 2009). Here, the liver has been found to respond to primary infections at peak parasitemia with dramatic increases in mRNA expression

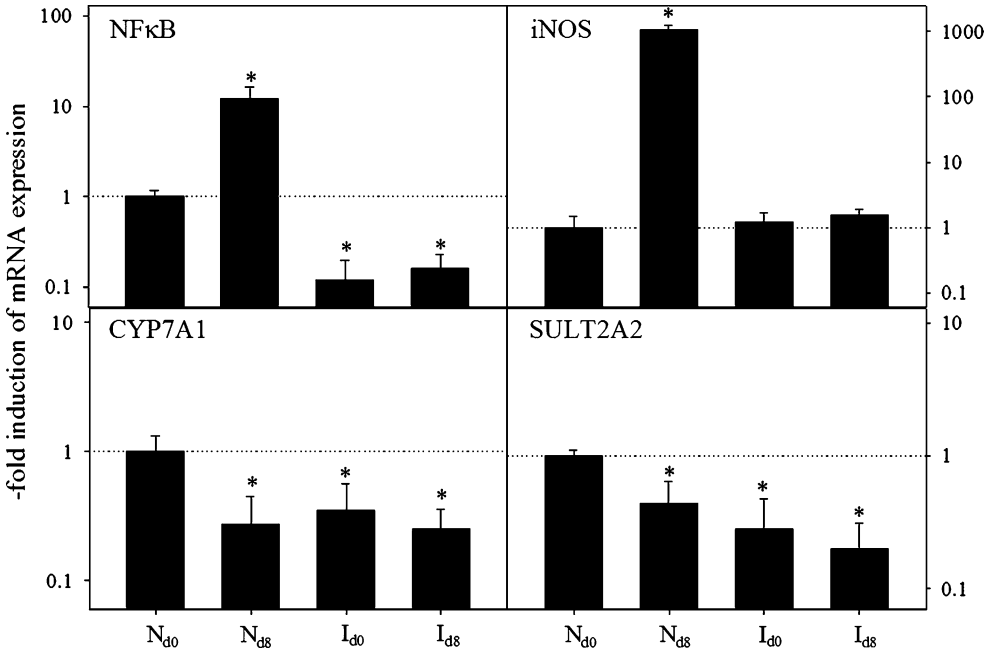

Fig. 5 Quantitative RT-PCR of mRNAs of NF-κB, iNOS, CYP7A1, and SULT2A2 in naïve and immune and upon infection with *P. chabaudi* malaria on day 8 p.i. Further eyplanations, see legend to Fig. 4

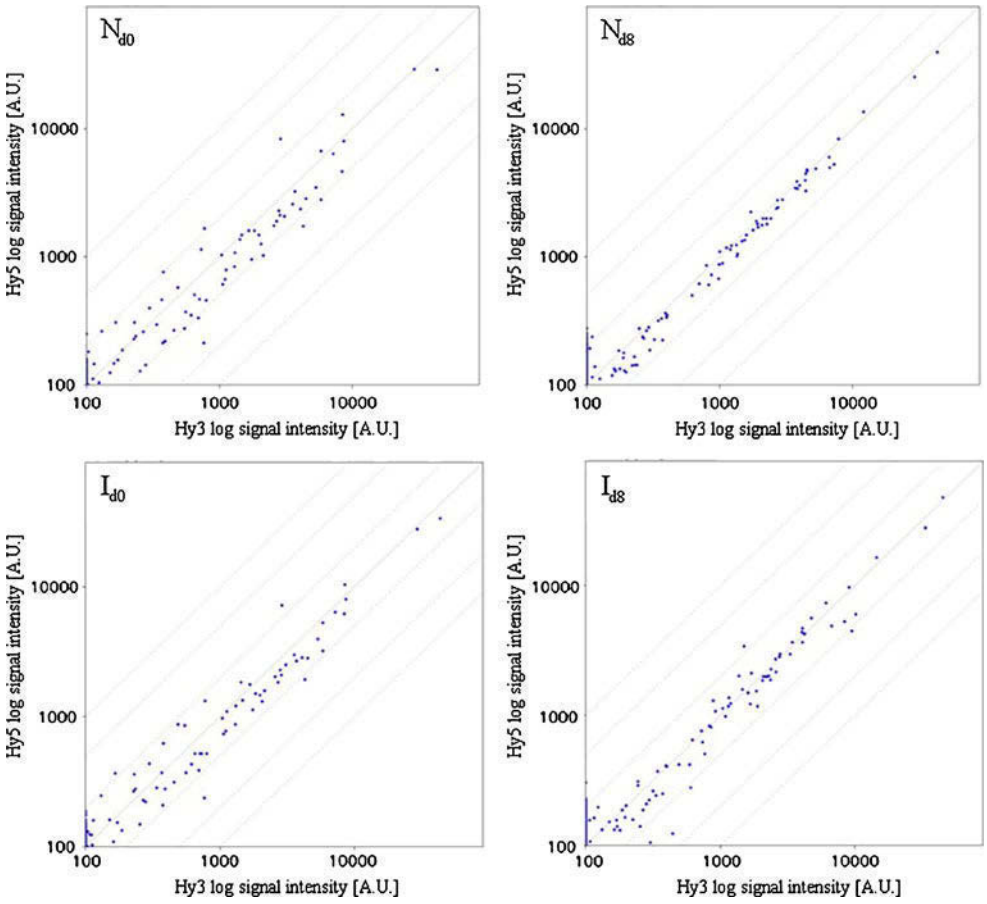

Fig. 6 Double-log scatter plots of non-infected naïve (N_{d0}) and immune (I_{d0}) female C57BL/6 mice as well as naïve (N_{d8}) and immune (I_{d8}) mice upon infections with *P. chabaudi* on day 8 p.i. The signal intensities of each miRNA that passed the quality filtering are represented by a *dot*. *X*-axis: Hy3 signal intensity (experimental sample), *y*-axis: Hy5 signal intensity (universal reference)

of the cytokines IL-1β, TNFα, and IFNγ as well as in NF-κB and iNOS mRNA expression. These factors are basically involved in the regulation of immune responses and host defense against infections in general and against blood-stage malaria in particular (McCall and Sauerwein 2010). Conspicuously, however, primary infections of *P. chabaudi* do not induce any changes in the expression of those miRNA species which have been reported to be associated with diverse aspects ascribed to innate immune responses, though corresponding hybridization signals can be observed on our microarrays (Fig. S1). For instance, the *P. chabaudi* infections do not affect those miRNA species, which have been described to be involved in TLR-induced signaling such as miR-21, miR-25, miR-27b, miR-100, miR-140, miR-142-3p, miR-181c, miR-187, miR-194, miR-214, miR-223, and miR-224 (Moschos et al. 2007; Sonkoly et al. 2008); in proliferation and activation of neutrophils, as e. g., miR-223 (Johnnidis et al. 2008); and in regulation of the inflammatory response of macrophages, as e.g. miR-132, miR-146, and miR-155 (Taganov et al. 2007). Moreover, it is known that IL-1β and TNFα downregulate liver metabolism (Kim et al. 2004; Krücken et al. 2005). In accordance, our data show that primary blood-stage infections with *P. chabaudi* have induced a downregulation of the mRNA expression of CYP7A1 and SULT2A2, which are typical enzymes of phases I and II liver metabolism, respectively. Again, however, it is conspicuous that *P. chabaudi* malaria does not change those miRNA species which are known to be associated with liver metabolism, as e.g. miR-21, being majorly involved in hepatocyte proliferation (Marquez et al. 2010).

The changes of the hepatic miRNA signature induced by *P. chabaudi* malaria are rather robust: they persist even after parasitemias of primary infections have largely resolved. This resolution coincides with acquisition of stable protective immune mechanisms. This becomes evident in secondary infections with *P. chabaudi*. Homolog re-infections result only at very low peak parasitemia of approximately 1.5% on day 8 p.i., in contrast to approximately 50% in primary infections. Protective immunity is known to be mediated by antibodies, in particular by IgG2a and IgG2b isotypes, which are dramatically increased in immune mice, in accordance with previous studies (Su and Stevenson 2000; Langhorne et al. 2002; Smith and Taylor-Robinson 2003; Wipasa et al. 2009). Once again, however, it is conspicuous that we have not detected any changes of those miRNA species which are currently known to be critically involved in adaptive immune responses such as antibody formation, development and maturation of B cells, and formation of memory B cells, $CD4^+$ T cells, and $CD8^+$ T

Table 2 *P.chabaudi* reprogrammed miRNA expression in the female mouse liver as revealed by miRXplore microarrays

miRNA name	GeneID	Gene accession	Nd0	Nd8	Id0	Id8	miRNA functions described to date	PMID
miR-1274A	40350	MI0009969	28.85	83.38	72.19	52.66	unknown	
MCMV-miR-M23-1-5P	40249	MI0006252	7.69	16.62	13.20	12.21	mouse cytomegalovirus-regulated miRNA	
miR-26B	35892	MI0000575	1.30	2.60	2.44	2.63	the miR-26 status of patients is with hepatocellular carcinoma is associated with survival and response to adjuvant therapy with interferone alpha	19812400
miR-142-5P	39219	MI0000167	0.15	0.11	0.06	0.05	repressed in murine and human lung cancers	19228723, 19618089
miR-192	35876	MI0000551	1.15	0.56	0.64	0.64	p53-responsive miR-192 is capable of inducing cell cycle arrest, regulates dihydrofolate reductase and cellular proliferation through the p53-miRNA circuit, potential biomarker for drug-induced liver injury	19074875, 19088023, 19246379
miR-193A-3P	39256	MI0000235	0.67	0.32	0.49	0.47	downregulation of expression through tumor-specific hypermethylation	18381414
let-7G	39156	MI0000137	0.35	0.17	0.19	0.18	decreased expression in heptocelluler carcinoma cell lines	19912688
let-7A	38314	MI0000061	0.92	0.37	0.42	0.44	suppresses therapeutics-induced cancer cell death by targeting caspase-3, functions as a potential tumor suppressor in human laryngeal cancer	18758960, 19787230
miR-466D-3P	40131	MI0005546	0.46	0.10	0.14	0.22	unknown	
miR-101B	38961	MI0000648	3.30	0.92	1.02	1.35	decreased in Hmga1-knockout mouse embryonic fibroblasts	19169275
miR-20A*	39973	MI0000568	0.26	0.05	0.06	0.11	upregulated in colonic cancer tissues	20132431
miR-377	35914	MI0000794	0.13	0.02	0.02	0.04	overexpression of miR-377 in diabetic nephropathy indirectly leads to increased fibronectin protein production	18716028
miR-374*	39863	MI0004125	0.42	0.05	0.04	0.09	unknown	
miR-694	38884	MI0004664	0.37	0.04	0.04	0.08	unknown	
miR-450B-5P	38803	MI0004705	0.60	0.06	0.05	0.12	unknown	
miR-465D	38812		0.58	0.06	0.06	0.12	unknown	
miR-677	38867	MI0004634	0.51	0.05	0.04	0.09	unknown	
miR-98	38497	MI0000586	0.49	0.05	0.05	0.09	regulates expression of tumor suppressor gene FUS1, estradiol induces expression of miR-98	19671678, 19528081
miR-464	38808	MI0002399	0.53	0.04	0.04	0.09	unknown	

cells, though their hybridization signals are available on the microarrays. For instance, miR-155 regulates the generation of Ig-class-switched plasma cells and targets the transcription factor Pu.1, which is important for the production of antibodies (Vigorito et al. 2007). Also, miR-155 mutant mice display defective B- and T-cell immunity and abnormal function of antigen-presenting cells (Rodriguez et al. 2007; Thai et al. 2007). The miRNA species miR-16, miR-21, miR-142-3p, miR-150, miR-15b, and let-7f have been found to control T-cell differentiation and activation (Neilson et al. 2007; Bi et al. 2009). The undetectability of any changes in these hepatic miRNA species that are normally associated with adaptive immune responses is the more astonishing since the liver contains both intra-hepatic B and T cells and also migratory B and T cells, in particular during *P. chabaudi* infections (Krücken et al. 2005). A

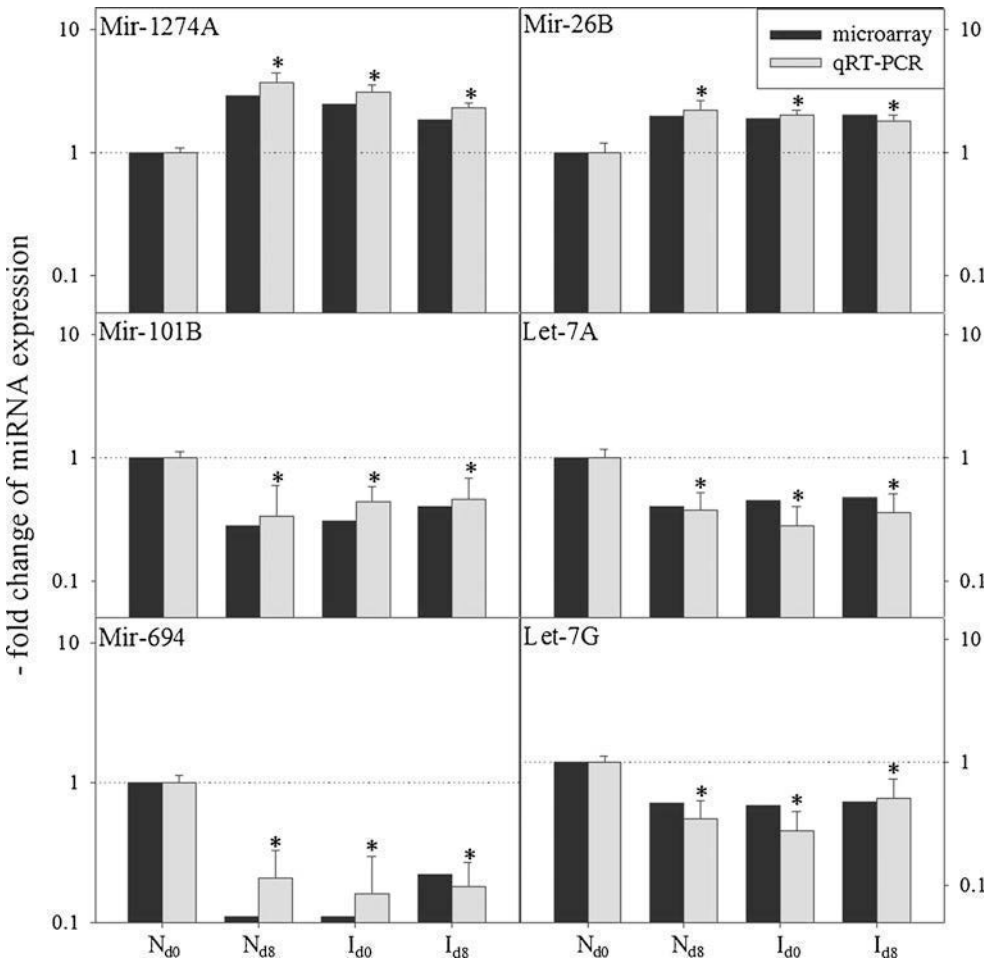

Fig. 7 Expression of selected miRNA species evaluated by miRXplore microarrays. The fold change of miRNA expression of miR-1274A, miR-26B, miR-101B, let-7A, miR-694, and let-7G was determined by qRT-PCR for naïve mice on days 0 (*Nd0*) and 8 p.i. (*Nd8*) and for immune mice on days 0 (*Id0*) and 8 p.i. (*Id8*). Values represent means±SD from six different mice. *Asterisks* indicate significant differences ($p<0.01$)

plausible explanation is, therefore, that there occur changes in expression of miRNAs of B and T cells, but these changes cannot be detected due to masking effects since our microarray analysis has been conducted with livers in toto. On the other hand, this observation is not valid for all miRNAs. Indeed, we observe a slight downregulation of miR-142-5p in immune mice, and this miRNA species is known to be downregulated during T-cell activation and differentiation, especially during differentiation from double-negative to double-positive T cells (Sonkoly et al. 2008).

The mechanisms, whereby *P. chabaudi* infections induce changes in the hepatic miRNA signature, are completely unknown to date. However, the persistence of these changes in immune mice and upon re-infections suggests that these changes in the liver induced by the malarial infections are rather robust. The most straight forward explanation is that epigenetic mechanisms are involved, which may reprogram hepatic miRNA expression. Consistent with this view is our finding that the miRNA-species miR-193a-3p and miR-101b are persistently downregulated by primary infections. Indeed, the expression of miR-193a-3p is known to be downregulated through tumor-specific hypermethylation (Kozaki et al. 2008) and miR-101b to be decreased in high mobility group a 1 (*hmga1*)-knockout mice (De Martino et al. 2009). Moreover, very recent information even indicates that there exist an intricate network between the epigenetic machinery and miRNAs (Iorio et al. 2010). The expression of miRNA genes cannot be only epigenetically regulated but also miRNAs per se can induce epigenetic changes in expression of other genes. Future work is required to unravel these reprogramming mechanisms and their relevance for the strongly decreased pathogenecity of malarial infections in mice which have acquired immunity to malaria. At least, our data suggest that miRNAs in the liver, during its differentiation to an effector site against malarial blood stages, are new players in acquiring protective immunity against *P. chabaudi* malaria.

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2.5 Attenuated responsiveness to testosterone of hepatic gene expression in female mice having acquired testosterone-unresponsive immunity to *Plasmodium chabaudi* malaria

Background: Protective immunity against *P. chabaudi* malaria, when once established, has become unresponsive to T. Those mice, which acquire protective immunity, concomitantly acquire T-unresponsiveness. However, it is completely unknown to date as to whether this acquired T-unresponsiveness of immunity encompasses the lymphoid anti-malaria effector site liver.

Methods: Naïve and immune female C57BL/6 mice, which have acquired immunity to *P. chabaudi* malaria, were treated with T for 3 weeks. Then, immune mice were challenged with 10^6 *P.chabaudi*-infected erythrocytes for one day. T-induced effects on the hepatic gene expression were examined in immune and naïve mice using Affymetrix microarrays and qRT-PCR analysis. These methods are also used to identify *P. chabaudi*-induced effects on hepatic gene expression in T-treated and vehicle-treated immune mice.

Results: A significantly decreased number of genes, i. e. 39 genes vs. 156 genes, were identified, whose mRNA expression is T-responsive in immune vs. naïve mice. In both groups of mice, T treatment resulted in a strong masculinized hepatic gene expression characterized by an upregulation of male-specific genes *Cyp2d9*, *Cyp7b1*, *Ugt2b1*, *Hsd3b2* and *Hsd3b5*, and a downregulation of female-specific genes *Cyp2b9*, *Cyp2b13*, *Cyp3a41*, *Cyp3a44*, *Cyp7a1, Fmo3*, and *Sult2a2*. HSD3B5, encoding an important T-metabolizing enzyme, is 5-fold higher expressed in immune mice than in naïve mice. Moreover, Stom, which is involved in erythropiesis, and Ehmt2, which is responsible for the T_H1/T_H2-balance, were T-downregulated in naïve mice, but T-unresponsive in immune mice. T-unresponsive genes encompass those which encode diverse regions of heavy and light chains of IgG-antibodies, infection-inducible acute phase proteins SAA1, SAA2, and ORM2, and cytokines IL-1β, IL-6, TNFα, and IFNγ as well as iNOS, which are even not inducible by infection in immune vs. naïve mice.

Conclusion: This study shows that the occurrence of adaptive persistent changes in the liver of female mice resulting in an attenuated T-unresponsiveness of gene expression, possibly due to diminished effective T, upon acquiring T-unresponsive, IgG-antibody mediated protective immunity to blood-stage malaria of *P. chabaudi*.

Attenuated responsiveness to testosterone of hepatic gene expression in female mice having acquired testosterone-unresponsive immunity to *Plasmodium chabaudi* malaria

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Abstract

Acquisition of protective immunity against *P. chabaudi* malaria coincides with an acquired unresponsiveness to testosterone (T). Here, we have investigated as to whether a T-unresponsiveness becomes also evident in the liver as an important T-target and anti-malaria effector site. Using Affymetrix microarray technology, in combination with quantitative RT-PCR, we have identified a significantly decreased number of genes, i. e. 39 genes vs. 156 genes among 14,000 genes, whose hepatic mRNA expression is T-responsive in immune vs. naïve mice. Among the T-responsive genes, there are those encoding the T-downregulated female-prevalent enzymes CYP2B9, CYP2B13, CYP3A41, CYP3A44, CYP7A1, FMO3 and SULT2A2, and the T-upregulated male-prevalent enzymes CYP2D9, CYP7B1, UGT2B1, HSD3B2 and HSD3B5. Remarkably, the T-metabolizing enzyme exhibits even a 5-fold T-induced increase in its mRNA expression in immune mice. The T-unresponsive genes encompass those which encode (i) diverse regions of heavy and light chains of IgG-

antibodies, (ii) the T_H2-response promoting EHMT2, (iii) the erythrocyte membrane protein band 7.2 STOM , and (iv) the infection-inducible acute phase proteins SAA1, SAA2, and ORM2. The cytokines IL-1β, IL-6, TNFα, and IFNγ, as well as iNOS, are even not inducible by infection in immune vs. naïve mice. Collectively, our data indicate the occurrence of adaptive persistent changes in the liver of female mice resulting in an attenuated T-unresponsiveness of gene expression, possibly due to diminished effective T, upon acquiring T-unresponsive, IgG-antibody mediated protective immunity to blood-stage malaria of *P. chabaudi*.

Introduction

Testosterone (T) is known to promote a wide variety of diseases incl. different forms of cancer such as prostate cancer (Wirén & Stattin, 2008, Rhoden & Averbeck 2009, Wu *et al*. 2010) and hepatocarcinoma (Drinkwater *et al*. 1990, Kemp & Drinkwater 1990, Nagasue & Kohno 1992). T also contributes to the higher preponderance of infectious diseases in males than in females (Müller 1992, Klein 2000, Roberts *et al*. 2001, Verthelyi 2001, Marriott & Huet-Hudson, 2006), including the four different human pathogenic malaria species *P. falciparum*, *P. ovale*, *P. malariae*, and *P. vivax* (Müller 1992, Kurtis *et al*. 2001, Muehlenbein *et al*. 2005). An extreme sex-dependence due to testosterone has been also described for the experimental malaria *Plasmodium chabaudi* (Wunderlich *et al*. 1988, 1991, Mossmann *et al*. 1997). Indeed, male mice succumb to blood-stage infections, whereas the same infections take a self-healing course after lowering the circulating T-levels after castration, as it is also typical for female mice. Conversely, T-treatment of females and castrated males results in a lethal outcome (Wunderlich *et al*. 1988, 1991). Obviously, T suppresses the development of protective immunity against malarial blood-stages. This immunosuppressive T-effect is not transient, but rather persistent, i. e. it is sustained even after withdrawal of T for 12 weeks (Benten *et al*. 1997).

The liver plays a central role both in malaria and as a target for testosterone. Indeed, the liver as an important lymphoid organ (Häussinger *et al*. 2004, Crispe 2009) is not only that site in which the pre-erythrocytic development of the blood-stages of malaria takes place (Mehlhorn 2001), but also the liver apparently differentiates to an effector site against the malarial blood stages (Krücken *et al*. 2005, Delić *et al*. 2010). The importance of the liver as a prominent anti-malaria effector site has been recently also demonstrated in lymphotoxin β

receptor-deficient mice (Wunderlich *et al*. 2005). These mice are devoid of secondary lymphoid tissues and their spleen is defect (Fütterer *et al*. 1998, Fu & Chaplin 1999), but they are rather resistant to malaria, i. e. they are able to self-heal blood-stage infections with *P. chabaudi* (Wunderlich *et al*. 2005). The liver is also known to generate extrathymic T cells conferring protective immunity to blood stage malaria (Balmer *et al*. 2000, Mannoor *et al*. 2001, 2002). Moreover, the Kupffer cells, which contribute 80-90% of all macrophages, are able to eliminate *P. chabaudi*-infected erythrocytes (Dockrell *et al*. 1980, Taverne *et al*., 1987, Delić *et al*. 2010). Even the metabolism of the liver apparently contributes to its anti-malaria effector function, since it is required for detoxification of endogenous substances originated during malaria infections (Delić *et al*. 2010). Moreover, the liver is characterized by a sexual dimorphism, especially by a sex- and testosterone-dependent pattern of phase I- and phase II metabolism (Kato & Onada 1970, Waxman and Holloway 2009). It is therefore not astonishing that T apparently impairs the differentiation of the liver to an anti-malaria effector during a malaria infection (Krücken *et al*. 2005).

There is information available that T suppresses only the development of protective immunity against *P. chabaudi* malaria. When once acquired, however, protective immunity has become unresponsive to T (Wunderlich *et al*. 1992). Obviously, mice which have acquired protective immunity, have concomitantly acquired a T-unresponsiveness. However, it is completely unknown to date as to whether this acquired T-unresponsiveness of immunity also encompasses the liver. This prompted us to investigate the effect of T on the liver transcriptome in immune mice, i. e. those mice which have already acquired T-unresponsive protective immunity to *P. chabaudi*.

Materials and methods

Mice

Mice of the inbred strain C57BL/6 were bred under specified pathogen-free conditions at the central animal facilities of our university. They were housed in plastic cages and received a standard diet (Wohrlin, Bad Salzuflen, Germany) and water *ad libitum*. The experiments were performed exclusively with female mice and were approved by the State authorities.

Testosterone treatment

Naïve mice aged 16-18 weeks and immune mice received subcutaneous injections of 100 µl sesame oil containing 0.9 mg T (Testosterone-Depot-50, Schering, Berlin, Germany) twice a week for 3 weeks (Wunderlich *et al*. 1988, Delić *et al*. 2010). Controls were treated only with the vehicle.

Infections

P. chabaudi was passaged weekly in NMRI mice (Wunderlich *et al*. 1982, Krücken *et al*. 2009). Female C57BL/6 mice were challenged with 10^6 *P. chabaudi*-parasitized erythrocytes. Parasitemia was determined in Giemsa-stained blood from the tail, and cell number was measured in a Neubauer chamber.

Mice immune to *P. chabaudi*

Approximately 10-12 weeks old C57BL/6 mice were challenged with *P. chabaudi*. Those mice which survived these infections for at least 6 weeks were taken as immune mice for T-treatment.

RNA-Isolation

Mice were killed by cervical dislocation, livers aseptically removed, and liver pieces rapidly frozen in nitrogen and stored at -80 °C until use. RNA was isolated as described recently (Delić *et al*. 2010) using approximately 250 mg frozen liver homogenized in 5 ml Trizol (Peqlab biotechnology, Erlangen, Germany). RNA concentrations were determined at 260 nm and RNA-quality was examined by agarose gel electrophoresis.

Hybridization of microarrays

Quality of RNA was re-controlled with a Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany) on a RNA 6000 Nano chip. The One cycle kit (Affymetrix, Inc. Santa Clara, USA) was then used to synthesize biotin-labeled cRNA from 5µg total RNA according to the manufacturer′s protocol. Approximately 15µg biotin-labeled cRNA was then hybridized to Affymetrix MOE430A Gene Chips® for 16 h at 45 °C, before staining and washing on an Affymetrix Fluidics Station 400. The fluorescence of the hybridized cRNA was read with an Affymetrix 300 Scanner. The chips were quality-controlled with the software "Expressionist Refiner" (GeneDataAG, Basel, Switzerland) which corrected gradients, spots and distortions. Each probe set is represented by 11 pairs of 25mer perfect match and mismatch oligonucleotides. The intensities of all 11 probe pairs per probe were

condensed to one intensity value using the MAS 5.0 statistical algorithms implemented in the Expressionist software. For reasons of comparability, the microarrays were scaled after condensing to an average signal intensity of 100.

Data analysis

The software ′Expression Analyst′ (GeneDataAG, Basel, Switzerland) was applied for gene expression analysis. Overall expression between individual mice was compared by principal component analysis using Genesis 1. 7. 2. (Sturn, 2002). Only those probe sets were evaluated which revealed an expression intensity > 20 in each sample and were more than 2-fold deregulated. These probe sets were then subjected to two-way ANOVA ($p < 0.01$). Genes were analyzed using the Database for Annotation, Visualization and Integrated Database (DAVID) (Dennis *et al*. 2003) and categorized according to their major biological pathways involved. Gene Cluster 3.0 (Eisen *et al*. 1998, de Hoon *et al*. 2004) was used for principal component analysis. Data were log_2-transformed and normalized to the mean expression value for control mice. Hierachical clustering was done using uncentered correlation and average linkage mode.

Quantitative real-time PCR

All RNA samples were treated with DNase of the DNA-free™ Kit (Applied Biosystems, Darmstadt, Germany) for 1 h, before cDNA was synthesized using the QuantiTect™ Reverse Transcription Kit (Qiagen, Hilden, Germany) following the manufacturer′s protocol. The QuantiTect™ SYBR® Green PCR kit (Qiagen) was applied for amplifications in the ABI Prism® 7500HT Sequence Detection System (AppliedBiosystems, Darmstadt, Germany) according to the manufacturer's instructions using gene-specific QuantiTect™ primer assays (Qiagen) for cytochrome P450, family 7, subfamily a, polypeptide 1 (Cyp7a1), 5′-nucleotidase,ecto (5Nte), sulfotransferase family 2A, dehydroepiandrosterone (DHEA)-preferring, member 2 (Sult2a2), 3 beta-hydroxysteroid dehydrogenase 5 (Hsd3b5), complement component 6 (C6), interferone gamma (Ifnγ), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), tumor necrosis factor alpha (TNFα) and inducible nitric oxide synthase (iNOS). PCR reactions were as follows: 2 min at 50 °C to activate uracil-N-glycosylase (UNG), 95 °C for 10 min to deactivate UNG and to activate the PCR reaction, 45 cycles at 94 °C for 15 sec, at 60 °C for 30 sec and at 72 °C for 30 seconds. Reaction specifity was checked by performing dissociation curves after PCR. The Taqman75000 system software v.1.2.3f2 (AppliedBiosystems) was used for relative quantitative evaluation. The mRNA was

normalized to 18S rRNA and its relative expression was determined with the $2^{-\Delta\Delta}$ct method (Livak & Schmittgen, 2001).

Statistical analysis

Significance was evaluated by two-way ANOVA with Dunnett and Bonferoni post-hoc-tests using the statistical package program SPSS version 17.0.

Results

T-unresponsiveness of acquired immunity

Female mice of the inbred strain C57BL/6 are able to self-heal blood-stage infections with *Plasmodium chabaudi* malaria. When mice are challenged with 10^6 *P. chabaudi*-infected erythrocytes, approximately 80% of the mice survive the infections (Fig. 1). A fulminant peak parasitemia of approximately 48% occurred on day 8 *p.i.*, which is followed by a smaller peak of approximately 12% on day 16 *p.i.*. Testosterone (T) prevents self-healing and all mice succumb to infection, mainly during the crisis phase between day 8 and day 13 of infection, and peak parasitemia is raised to approximately 70%.

Mice which have self-healed the infections have acquired protective immunity. This becomes evident upon homolog re-infections of those mice, which have survived primary infections, as survival of all mice without any visible symptoms of sickness and as a very low peak parasitemia of approximately 3% on day 8 *p.i.* (Fig. 1). T-pretreatment of immune mice for 3 weeks affects neither survival rate nor causes any significant rise in parasitemia (Fig. 1).

T-responsive genes in liver of immune vs. naïve mice

In order to identify possible effects of T on hepatic gene expression in immune mice, we have used Affymetrix microarray analyses for comparing immune mice vs. naïve mice. Both naïve and immune mice were treated with T or vehicle for three weeks, before individual livers were subjected to Affymetrix microarray analysis.

The principal component analysis in Fig. 2 reveals that the overall expression profiles were relatively similar among the three different biological replicates in the four different mouse groups, but differ between the four groups, i. e. (i) control naïve mice (C), (ii) naïve mice

treated with T (T), (iii) control immune mice (C_{imm}), and immune mice treated with T (T_{imm}), respectively.

The Venn-Diagram in Fig. 3 summarizes the numbers of T-upregulated and T-downregulated genes in immune vs. naïve mice identified by Affymetrix microarrays. *In toto*, the expression of 156 genes was affected by T in naïve mice with 51 T-upregulated and 105 T-downregulated genes (horizontal ellipses in Fig. 3). By contrast, significantly less genes respond to T in immune mice: the expression of only 39 genes is affected, with 17 T-upregulated genes and 22 T-downregulated genes (vertical ellipses in Fig. 3). The overlap of the ellipses represents the same genes deregulated by T in both immune and naïve mice (Fig. 3).

T-responsive hepatic gene expression in immune vs. naïve mice

Fig. 4 shows the expression profile clustering of T-responsive genes in livers of immune and naïve mice. The evaluated data are summarized in Table 1, which also provides information about functional annotations of the genes as searched in several databases including NettAffx (Affymetrix), SwissProt, Proteome, and Pubmed. We have categorized the genes in 10 different groups.

In the categories of liver metabolism, i. e. phase I and phase II metabolism and general metabolism of carbohydrates, lipids and proteins (Table 1), the gene expression of 22 genes is significantly affected by T in immune mice, in contrast to 50 genes in naïve mice. There are only 4 genes among the 50 T-responsive genes in naïve mice, which have significantly become T-unresponsive in immune mice, namely *Pfkfb3, C1galt1, Habp2*, and *Usp12*, respectively (Table 1). Most conspicuously, T induces a strong masculinization of expression of those genes. In particular, T upregulates the expression of genes encoding male-prevalent enzymes such as CYP2D9, CYP7B1, UGT2B1, HSD3B5, and HSD3B2, whereas T downregulates that of female-prevalent enzymes such as CYP2B9, CYP2B13, CYP3A41, CYP3A44, CYP7A1, FMO3 and SULT2A2, respectively. The T-induced response of these genes is about the same in both immune and naïve mice. Only the male-prevalent HSD3B5 exhibits a T-induced 5-fold higher expression in immune than naïve mice.

In the categories of cell cycle/apoptosis/carcinogenesis and cytoskeleton, the expression of the 2 genes *Lama3* and *Nox4* is significantly changed by T in immune mice, whereas 12 genes are T-responsive in naïve mice. Among the latter, the four genes *Gadd45g*, *Phlda1*, *Lasp1*, and *Stom* have become unaffectable by T in immune mice (Table 1).

The category of genes involved in immunity contains only 2 genes, which are T-responsive in immune mice, whereas 12 genes are deregulated by T in naïve mice (Table1). The complement component C6 exhibits an approximately 2-fold higher expression in T-treated immune mice than in T-treated naïve mice. The expression of the cytokine inducible SH2-containing protein Cish is significantly upregulated by T in naïve mice, while the same gene is T-unresponsive in immune mice (Table 1).

The expression of only 7 genes, summarized in the categories of gene regulation and signal transduction (Table 1), were significantly deregulated by T in immune mice, while the expression of 39 genes responded to T in naïve mice. The following genes, whose expression was significantly deregulated by T in naïve mice, became T-unresponsive in immune mice: *Foxq1, Foxa1, Fbxw11, Ehmt1, Egfr, Plk3, Sorbs1, Rad23a, Spred1, Hnf4a,* and *Sdc1*, respectively (Table 1).

Furthermore, the expression of only 8 genes, which belong to the categories transporter or miscellaneous functions, was T-responsive in immune mice, whereas 43 genes were T-affectable in naïve mice. The genes *Chacl1, Slc9a8, Zfml, Mrpl15, Sel1h*, *D4Wsu53e*, and *Rnf14* became T-unresponsive in immune mice (Table 1).

Finally, Fig. 5 shows that the mRNA expression profiles of arbitrarily selected genes from microarrays, i. e. the T-upregulated *Hsd3b5* and *C6* as well as the T-downregulated *Cyp7a1*, *Sult2a2*, *IL-1β* and *Nt5e,* could be essentially verified by quantitative RT-PCR analysis.

T-unresponsive genes encoding antibody chains

Protective immunity to *P. chabaudi* malaria is mediated by antibodies (Wunderlich *et al*. 1992, Su & Stevenson 2000, Langhorne *et al*. 2002, Achtman *et al*. 2005, 2007). We have therefore tried to identify T-induced changes in expression of genes encoding immunoglobulins in the liver of immune mice. To this end, we have compared the groups C, C_{imm}, T, and T_{imm} using two-way ANOVA analysis. Expectedly, the liver of immune mice expresses a number of genes encoding diverse regions of IgG such as the IgG heavy chains IgH-1A, IgH-4, and IgH-6, the kappa and lamda light chains IgK-V21, IgK-V8, IgK-V28, IGK-V32, and IGL-V1 (Table 2). Also, we have identified those genes encoding the joining chain IgJ and heavy chains of IgM in immune mice. None of these genes were significantly affected by pre-treatment with T (Table 2).

T-unresponsiveness of genes affected by *P. chabaudi*

In order to explore possible T-effects on expression of *P. chabaudi*–affectable genes, immune mice (C_{imm}) and T-treated immune mice (T_{imm}) were challenged with 10^6 *P. chabaudi*-infected erythrocytes for 1 day (C_{inf}, T_{inf}), before individual livers were subjected to Affymetrix microarray analysis.

Principal component analysis revealed that the overall expression profiles were relatively similar among the three different biological replicates within the four described mouse groups. Only marginal differences exist in the overall gene expression between C_{imm} and C_{inf} as well as T_{imm} and T_{inf}, but somewhat larger differences can be obseverd between C_{imm} and T_{imm}, as well as C_{inf} and T_{inf} (Fig. 2).

Two-way ANOVA analysis over the four groups C_{imm}, C_{inf}, T_{imm}, and T_{inf} surprisingly revealed that the *P. chabaudi* infections affected hepatic expression of only 4 genes in immune mice. The expression of the genes encoding the acute phase proteins SAA2, SAA1, and ORM2 were upregulated and that of TXNL1 was significantly downregulated. In T-pretreated immune mice, the expression of *Saa2*, *Saa1*, *Orm2*, and *Acnat2* was upregulated by infection, whereas *Tenc1*, *Adamts5*, *Nt5e*, *Atoh8*, and *Lipg* were significantly downregulated. Remarkably, there was only one gene, namely *Acnat2*, whose response to *P. chabaudi* was significantly affectable by T (Table 3).

To our surprise, none of the genes encoding the cytokines IL-1β, IL-6, TNFα, ans INFγ, as well as iNOS were identified to be affected by infection in immune mice as identified by the Affymetrix microarrays. However, this result could be confirmed using quantitative RT-PCR analysis. The expression of these genes was completely unresponsive to *P.chabaudi* infection in immune mice on day 1 *p.i.* and even on day 8 *p.i.* (Fig. 6). Moreover, this unresponsiveness is not changed by pre-treatment with T (Fig. 6). By contrast, hepatic expression of these genes resulted in a massive induction of mRNA in naïve mice on day 1 *p.i.* and day 8 *p.i.* Pre-treatment with T did not significantly influence this infection-induced upregulation in naïve mice. IL-6 mRNA expression was not detected in naïve mice on day 8 *p.i.*, whereas T-treatment induced an upregulation of IL-6 mRNA expression on day 8 *p.i.*. In immune mice, the mRNA expression of genes encoding IL-1β, IL-6, TNFα, and INFγ, and iNOS are obviously affected neither by infection nor T-pre-treatment.

Discussion

Mice which have acquired immunity to *P. chabaudi* malaria have concomitantly acquired an unresponsiveness to testosterone (T), evidenced here as a T-induced lethal outcome of blood-

stage malaria in only naïve mice, but not in immune mice (Fig. 1). Our data indicate that this acquisition of T-unresponsive immunity is associated with an attenuated T-responsiveness of gene expression in the liver, which is both a T-target organ (Holloway *et al*. 2008) and an immune organ (Häussinger *et al*. 2004, Crispe 2009) involved in host defense against blood-stage malaria (Krücken *et al*. 2005, Wunderlich *et al*. 2005, Delić *et al*. 2010). This attenuated T-responsiveness manifests itself as (i) a decrease in T-responsive genes, and (ii) an increased T-responsiveness of 2 genes, when immune mice are compared with naïve mice.
. Moreover, there is a decrease in malaria-inducible genes in immune mice.

Overall, we have identified only 39 T-responsive genes in immune mice vs. 156 genes in naïve mice among 14,000 genes as analyzed by Affymetrix microarray technology. Among the 39 T-responsive genes, a number of gender-specific genes have retained their T-responsiveness during acquisition of T-unresponsive immunity. Indeed, the expression of genes encoding the male-prevalent enzymes CYP2D9, CYP7B1, UGT2B1, HSD3B5 and HSD3B2 is upregulated by T, whereas it is downregulated in genes encoding the female-prevalent enzymes such as CYP2B9, CYP2B13, CYP3A41, CYP3A44, CYP7A1, FMO3 and SULT2A2, respectively, in both immune and naïve mice. Incidentally, the gene encoding HNF4α, which has been previously described to be involved in the control of sexually dimorphic gene expression (Clodfelter *et al*. 2007, Holloway *et al*. 2008) has lost its T-responsiveness in immune mice. This supports our previous view that HNF4α is not directly involved in the control of gender-specific gene expression in female mice (Delić *et al*. 2010). Remarkably, the expression of gender-specific genes reveals approximately the same T-responsiveness in immune and naïve mice, with one spectacular exception: this is the gene encoding HSD3B5 which was even 5-fold more upregulated by T in immune than naïve mice. This enzyme is involved in the metabolism of steroid hormones (Wang *et al*. 2005). It is therefore reasonable to assume that the increased expression of this enzyme contributes to lower concentrations of effective T in the liver of immune mice in comparison to naïve mice.

Among those genes, whose hepatic expression has lost their T-responsiveness with acquiring immunity, we have identified Stomatin (*Stom*). This encodes the erythrocyte membrane protein band 7.2b. The deregulation of band 7.2b is known to be associated with stomatocytosis, in which erythrocyte membrane permeability is changed leading to final hemolytic anemia (Fricke *et al*. 2003). The non-responsiveness of Stom to T is consistent with previous findings suggesting that erythrocytes of immune mice are more robust and less penetrable by malaria parasites in immune than naïve mice (Wunderlich *et al*. 1987). Moreover, it is noteworthy that C1galt1 is another gene which has lost its T-responsiveness in

immune mice. The protein-encoding gene C1galt1 is known to be involved in thrombopoiesis. Conceivably, T induces an increased thrombopoiesis in naïve mice vs. immune mice. Furthermore, genes encoding the transcription factors FOXQ1 and FBXW11 are not T-responsive in immune mice. FOXQ1 is an important regulator of NK cell effector function (Jonsson & Peng 2005) and FBXW11 is described to be involved in the ubiquitination-dependent destruction of IκBα (Suzuki *et al*. 2000), which is the inhibitor of NF-κB (Baldwin 1996). The latter is a multifunctional transcription factor that regulates the expression of a number of genes involved in immune and inflammatory responses. Also, Cish has lost its T-responsiveness, which is an important negative regulator of cytokine signaling after infection (Hanada *et al*. 2003).

Genes encoding diverse regions of heavy and light chains of IgG antibodies have been identified to be T-unresponsive in immune mice. Though our data cannot discriminate between intrahepatic and/or immigrant B cells, they are compatible with other findings showing that production of protective antibodies by B cells is T-insensitive and adoptive transfer of such antibodies can protect T-treated naïve mice from a lethal outcome (Wunderlich *et al*. 1992). Also, we have found that IgM-encoding genes are T-unresponsive. This supports the view that T suppresses development of the antibody response of protective immunity at a rather early stage in naïve mice. For instance, the formation of protective antibodies requires a T_H2-response preceded by a T_H1-response (von der Weid & Langhorne 1993). Previously, we have found that T persistently upregulates the T_H1 promoting IFNγ (Delić *et al*. 2010), and the production of biologically active IL-10, an inhibitor of T_H2-response, is almost completely suppressed by T in naïve mice (Mossmann *et al*. 1997). Obviously, T shifts the delicate balance between T_H1- and T_H2- response in direction to the T_H1-response, thus ultimately impairing the formation of protective antibodies. This view is further corroborated by our present finding that the euchromatic histone lysine N-methyltransferase 2 (Ehmt2) is identified to be T-unresponsive in immune mice, whereas its expression is decreased by T in naïve mice. This gene is thought of mediating epigenetic changes in genes which are involved in the switch of T_H1-response to T_H2-response. Indeed, mice with a T-cell specific deletion of this gene fail to develop a protective T_H2 cell response after infection with the helminth parasite *Trichuris muris* (Lehnertz *et al*. 2010).

Collectively, a series of changes have been here identified, in response to T, in the liver transcriptome of immune mice as compared with naïve mice. Overall, immune mice are characterized by an attenuated T-responsiveness of hepatic gene expression. Moreover, our finding is noteworthy, that important inflammatory cytokines such as IL-1β, IL-6, TNFα and

IFNγ, as well as iNOS are not inducible by *P. chabaudi* infections in the liver transcriptome of immune mice, in contrast to naïve mice. This is also consistent with our very recent finding that there occurs a reprogramming of the hepatic miRNA-signature in mice during acquisition of immunity (Delić *et al*. 2010). It is rather obvious that the liver undergoes adaptive persistent changes upon acquiring protective immunity to blood-stage malaria of *P. chabaudi*. All these data emphasize the role of the liver as an important effector site against blood-stage malaria.

Declaration of interest

The authors have no conflict of interest.

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Figure legends

Figure 1 Course of blood-stage infections with *P. chabaudi* in naïve and immune C57BL/6 mice. Mice were pre-treated with T or vehicle for 3 weeks before infecting with 10^6 *P. chabaudi*-parasitized erythrocytes. Values represent means ± SD (n=8). Only half of the SD bar is given for clarity reasons.

Figure 2 Principal component analysis represents the three major vectors contributing to variance between arrays. Variations exist in expression profiles between triplicates of different mouse groups, whereas similarity occurs within triplicates of a given mouse group. C, control naïve mice; T, T-treated mice; C_{imm}, immune mice; T_{imm}, T-treated immune mice; C_{inf}, immune mice infected with *P. chabaudi* for 1 day; T_{imm}, T-treated immune mice infected with *P. chabaudi* for 1 day.

Figure 3 Venn-diagram summarizing the numbers of T-affected genes in naïve mice (horizontal ellipses) and T-affected genes in immune mice (vertical ellipses). The overlap of the ellipses represents numbers of those genes deregulated by T in both groups of mice.

Figure 4 Hierarchical cluster analysis of hepatic expression levels of T-deregulated genes in immune and naïve mice. The T-upregulated genes are shown on the left and the T-downregulated genes on the right. Analysis was performed using Gene Cluster 3. The expression levels of immune mice (C_{imm}), T-pre-treated naïve mice (T), and immune mice (T_{imm}) were normalized to the mean signal intensity of control naïve mice (C) and data are log_2-transformed. Yellow and blue colors represent up- and downregulation, respectively, as indicated by the logarithmic color scale bar.

Figure 5 Verification of microarray data by quantitative real-time PCR. Gene expression microarray profiles of T-upregulated *Hsd3b5* and *C6* as well as T-downregulated *Nt5e, Cyp7a1, IL-1β* and *Sult2a2* reveal about the same T-induced deregulation by real-time PCR analysis. Relative mRNA expression was normalized to the mean expression of control mice (C). Values represent means ± SD from 3 different mice in each group. * indicates significant differences ($p < 0.01$).

Figure 6 Quantitative RT-PCR of cytokines IL-1β, IL-6, TNFα, IFNγ and iNOS in vehicle-treated immune (C_{imm}) and T-treated immune mice (T_{imm}), infected with 10^6 *P. chabaudi*-parasitized erythrocytes. Values represent means ± SD (n=3). Asterisks indicate significant differences ($p<0.01$) with respect to C_{imm}.

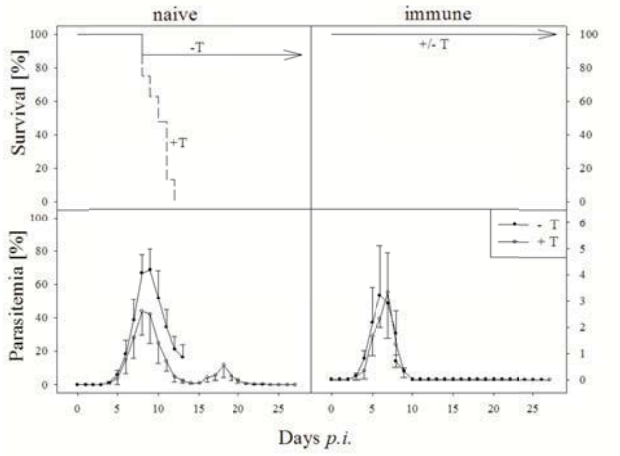

Figure 1

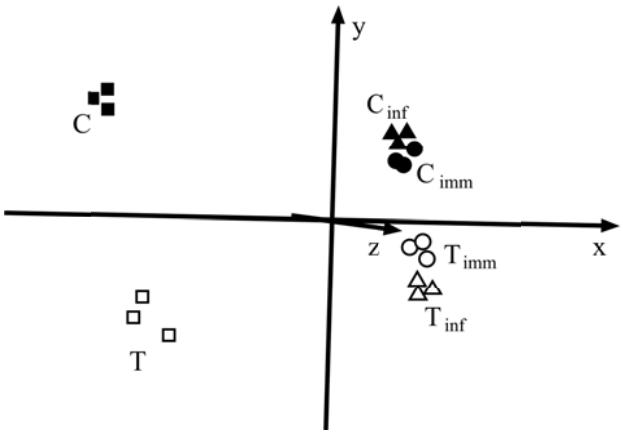

Figure 2

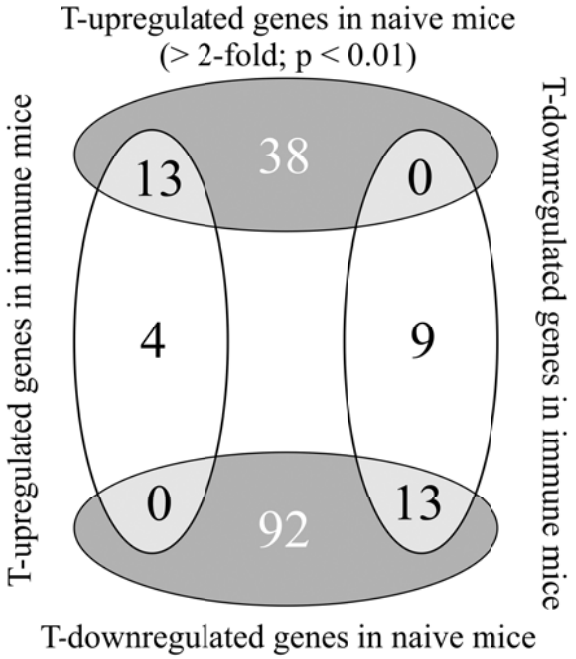
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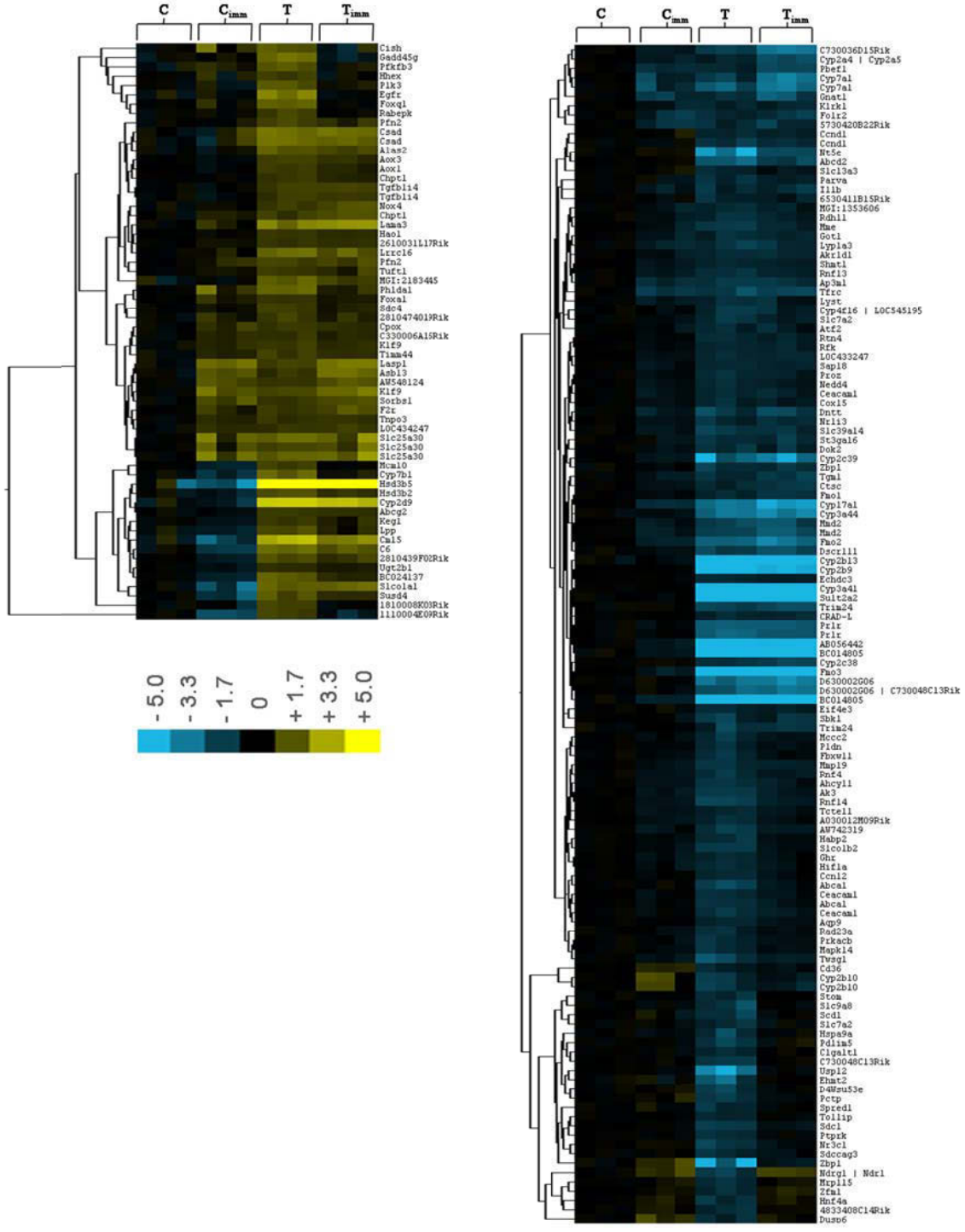
Figure 3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Alas2
Aox3
Aoxi
Chpt1
Tgfbi14
Tgfbi14
Nox4
Chpt1
Lama3
Hao1
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1110004E09Rik
- 5.0
- 3.3
- 1.7
0
+ 1.7
+ 3.3
+ 5.0
C730036D15Rik
Cyp2a4 | Cyp2a5
Pbef1
Cyp7a1
Cyp7a1
Gnat1
Klrk1
Foxr2
5730420B22Rik
Ccnd1
Ccnd1
Nt5e
Abcd2
Slc13a3
Parva
Il1b
6530411B15Rik
MGI:1353606
Rdh11
Mme
Gotl
Lypla3
Akr1d1
Shmt1
Rnf13
Ap3m1
Tfrc
Lyst
Cyp4f16 | LOC545195
Slc7a2
Atf2
Rtn4
Rfk
LOC433247
Sap18
Proz
Medd4
Ceacam1
Cox15
Dntt
Nr1i3
Slc39a14
St3gal6
Dok2
Cyp2c39
Zbp1
Tgm1
Ctsc
Fmo1
Cyp17a1
Cyp3a44
Mad2
Mad2
Fmo2
Dscr111
Cyp2b13
Cyp2b9
Echdc3
Cyp3a41
Suit2a2
Trim24
CRAD-L
Prlr
Prlr
AB056442
BC014805
Cyp2c38
Fmo3
D630002G06
D630002G06 | C730048C13Rik
BC014805
Eif4e3
Sbk1
Trim24
Mccc2
Pldn
Fbxw11
Map19
Rnf4
Ahcy11
Ak3
Rnf14
Tctel1
A030012M09Rik
AW742319
Mabp2
Slco1b2
Ghr
Hif1a
Ccnl2
Abca1
Ceacam1
Abca1
Ceacam1
Aqp9
Pad23a
Prkacb
Mapk14
Twsg1
Cd36
Cyp2b10
Cyp2b10
Stom
Slc9a8
Scd1
Slc7a2
Hspa9a
Pdlim5
Clgalt1
C730048C13Rik
Usp12
Ehmt2
D4Wsu53e
Pctp
Spred1
Tollip
Sdc1
Ptpxk
Nr3c1
Sdccag3
Zbp1
Ndrg1 | Ndr1
Mrpl15
Zfm1
Hnf4a
4833408C14Rik
Dusp6

Figure 4

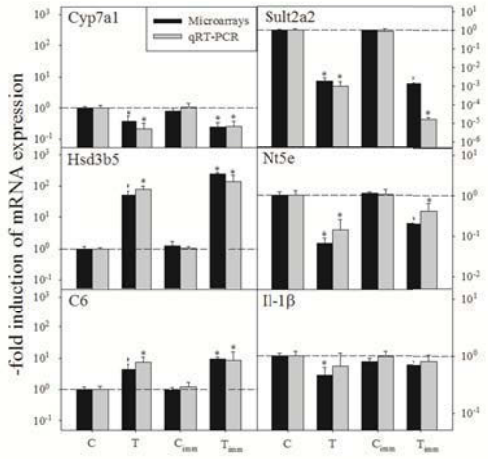

Figure 5

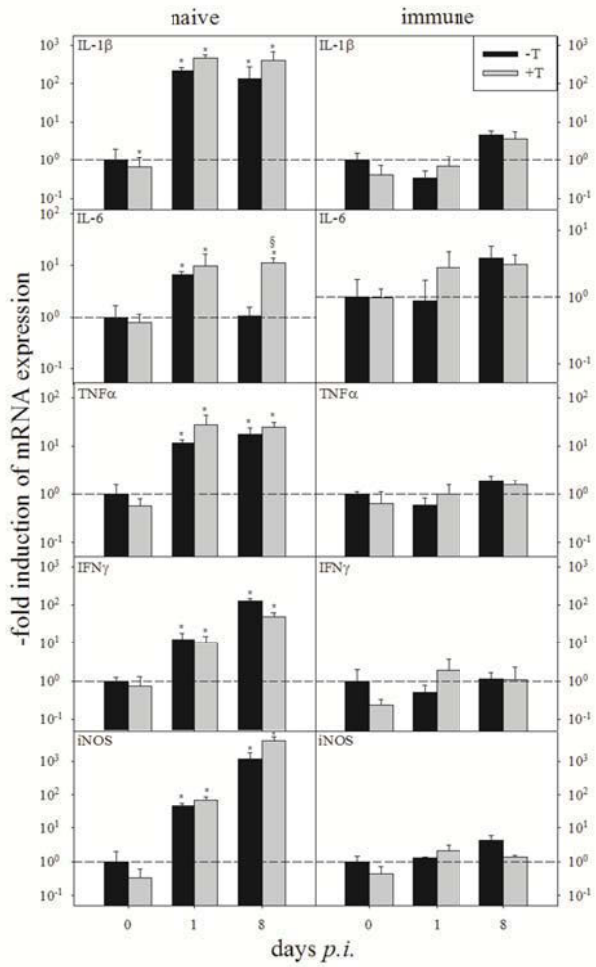

Figure 6

Table 1. T-responsive gene expression in the liver of female mice immune to *P. chabaudi* malaria vs. naïve mice.

	Gene symbol	Gene name	T_{imm}/C_{imm}	p-value	T/C	p-value	$T_{imm}:C_{imm}$/ T:C	p-value	Affymetrix probe ID	Entrez Gene	Representative Public ID	Functions	PMID
Phase I metabolism	*Cyp2d9*	cytochrome P450. family 2. subfamily d. polypeptide 9	23.738	0.004	16.544	4.291E-06	1.435	0.048	1419349_a_at	13105	BC010593	male-specific; regulate by growth hormone; sterol 16α-hydroxylase	16547391, 4074718
	Cyp7b1	cytochrome P450. family 7. subfamily b. polypeptide 1	3.188	0.008	3.961	0.008	0.805	0.011	1421075_s_at	13123	NM_007825	oxysterol-7-α-hydroxylase involved in synthesis of 7-α-hydroxylated bile acids; male specific expression in the liver	9295351, 8530364, 11284740
	Cyp2a4/Cyp2a5	cytochrome P450. family 2. subfamily a. polypeptide 4/5	0.404	0.001	0.529	0.007	0.764	0.014	1422230_s_at	13086 | 13087	NM_007812	Circadian expression of the steroid 15 alpha-hydroxylase (Cyp2a4) and coumarin 7-hydroxylase (Cyp2a5) genes in mouse liver is regulated by the PAR leucine zipper transcription factor DBP	10490589
	Fmo1	flavin containing monooxygenase 1	0.447	0.0013	0.494	0.0027	0.905	0.048	1417429_at	14261	BC011229	expression of Fmo1 is suppressed by overproduced nitric oxide	10462538
	LOC433247	similar to Cyp2c40 protein	0.635	0.002	0.459	0.0016	1.385	0.579	1423244_at	433247	AI265721		
	Cyp2c38	cytochrome P450. family 2. subfamily c. polypeptide 38	0.250	1.059E-05	0.392	0.003	0.638	0.02	1452501_at	13097	AF047725	91,8% sequence identity to Cyp2c39	19358898
	Cyp4f16	cytochrome P450. family 4. subfamily f. polypeptide 16	0.600	0.03	0.389	0.0069	1.541	0.254	1430172_a_at	70101	AK009445	Catalyzes the hydroxylation of eicosanoids; LPS-induced downregulation in the liver is in part mediated by peroxisome proliferator-activated receptor alpha	11160642, 12384299
	Cyp2b10	cytochrome P450. family 2. subfamily b. polypeptide 10	0.309	0.129	0.387	0.0039	0.799	0.039	1422257_s_at	13088	NM_009998		
	Cyp2b10	cytochrome P450. family 2. subfamily b. polypeptide 10	0.284	0.101	0.387	0.0073	0.736	0.18	1425645_s_at	13088	AF128849		
	Cyp7a1	cytochrome P450. family 7. subfamily a. polypeptide 1	0.238	0.08	0.370	0.007	0.644	0.071	1438743_at	13122	BB667338	Cholesterol-7alpha-hydroxylase that is expressed in a female-specific manner	1036899
	Cyp7a1	cytochrome P450. family 7. subfamily a. polypeptide 1	0.285	0.11	0.207	0.0014	1.376	0.194	1422100_at	13122	NM_007824		
	Fmo2	flavin containing monooxygenase 2	0.211	0.0003	0.154	0.0012	1.367	0.224	1422904_at	55990	NM_018881	typically expressed at high levels in lung	16872995
	Cyp2c39	cytochrome P450. family 2. subfamily c. polypeptide 39	0.135	0.0013	0.133	0.0011	1.019	0.565	1421363_at	13098	NM_010003	Acts as a retinoic acid 4-hydroxylase.Metabolizes arachidonic acid to 14, 15-cis-epoxyeicosatrienoic acid	14623888, 9721182
	Cyp3a44	cytochrome P450. family 3. subfamily a. polypeptide 44	0.108	0.021	0.120	0.001	0.896	0.206	1426064_at	337924	AB039380	female-specific expression of this expression depends on growth hormone	12147261
	Cyp17a1	cytochrome P450. family 17. subfamily a. polypeptide 1	0.111	0.0001	0.111	0.00063	0.998	0.0163	1417017_at	13074	NM_007809	Exhibits 17alpha-hydroxylase/17,20-lyase activity, a crucial enzyme for the synthesis of all steroid hormenes, and squalene monooxygenase (epoxidase) activity involved in cholesterol biosynthesis.	15761033
	Cyp3a41	cytochrome P450. family 3. subfamily a. polypeptide 41	0.013	0.0004	0.020	0.004	0.674	0.148	1419704_at	53973	NM_017396	female specific isoenzyme	10775455
	Cyp2b9	cytochrome P450. family 2. subfamily b. polypeptide 9	0.039	0.0008	0.015	3.799E-05	2.570	0.027	1419590_at	13094	NM_010000	female specific expression depends on growth hormone	15381067
	Cyp2b13	cytochrome P450. family 2. subfamily b. polypeptide 13	0.005	0.007	0.001	0.029	3.578	0.159	1449479_at	13089	NM_007813	female-specific expression	15155787
	Fmo3	flavin containing monooxygenase 3	0.001	0.0106	< 0.001	0.0003	1.11	0.846	1449525_at	14262	NM_008030	female specific expression in the liver	7473608
Phase II metabolism	*Cml5*	camello-like 5	20.406	0.014	12.398	0.007	1.646	0.0181	1424811_at	69049	BC024605	putative N-acetyltransferase	
	Ugt2b1	UDP glucuronosyltransferase 2 family. polypeptide B1	2.036	0.0004	1.868	0.0017	1.090	0.0016	1424934_at	71773	BC027200	male-predominant expression in the liver; inductive effects by testosterone	19131521
	Sult2a2	sulfotransferase family 2A. dehydroepiandrosterone (DHEA)-preferring. member 2	0.001	0.002	0.002	0.008	0.726	0.541	1419528_at	20865	NM_009286	female specific expression in the liver; involved in control of the amounts of active androgens in cells; protects against the toxic effects of lithocholic acid	8570624, 9566751, 16864508
General metabolism	*Hsd3b5*	hydroxysteroid dehydrogenase-5. delta<5>-3-beta	248.626	0.0034	49.878	0.005	4.985	0.009	1420531_at	15496	NM_008295	involved in steroid synthesis with both dehydrogenase and isomerase activity; male-specific	8319586, 8477648, 8647315
	Csad	cysteine sulfinic acid decarboxylase	2.900	0.036	4.634	0.002	0.626	0.992	1448986_x_at	246277	AY033912	rate-limiting enzyme in taurine biosynthesis	11997111
	Csad	cysteine sulfinic acid decarboxylase	2.578	0.053	4.629	0.0004	0.557	0.067	1427981_a_at	246277	AY033912	Rate limiting enzyme in taurine biosynthesis, predominantly expressed in the liver	11997111
	Alas2	aminolevulinic acid synthase 2. erythroid	2.547	0.004	3.328	0.004	0.765	0.097	1451675_a_at	11656	M63244	Catalyzes the first step in heme synthesis in erythroblasts and is induced by erythroblast differentiation	10773455
	Pfkfb3	6-phosphofructo-2-kinase/fructose-2.6-biphosphatase 3	0.776	0.329	3.189	0.005	0.243	0.0035	1416432_at	170768	NM_133232	Important activator of anaerobic glycolysis and essential for embryonic survival	15845370
	Hsd3b2	hydroxysteroid dehydrogenase-2. delta<5>-3-beta	3.284	0.00052	3.009	0.003	1.091	0.0023	1425127_at	15493	BC026757	Important enzyme for steroid synthesis with both dehydrogenease and isomerase activity; minor, male-preponderant isoform	8647315
	Aox3	aldehyde oxidase 3	2.311	0.0051	2.432	8.077E-05	0.950	0.0194	1418858_at	71724	NM_023617	Maturation of secretory cells was associated with progressive increases in the expression of Fmo3, Pon1, Aox3, and Cyp2f2 between late embryonic and postnatal periods	18757308
	Mcm10	minichromosome maintenance deficient 10 (S. cerevisiae)	1.885	0.018	2.381	0.0031	0.792	0.00065	1433408_a_at	70024	AK010648	Knockdown of human MCM10 exhibits delayed and incomplete chromosome replication	17997977
	Chpt1	choline phosphotransferase 1	2.003	0.0005	2.294	0.0077	0.873	0.04503	1455901_at	212862	AI642069	involved in phospholipid metabolism	12097155
	Chpt1	choline phosphotransferase 1	1.648	0.0023	2.118	0.003	0.778	0.0227	1435870_at	20962	BM899344		
	Aox1	aldehyde oxidase 1	1.859	0.003	2.260	0.0009	0.822	0.0075	1419435_at	11761	NM_009676	AOX1 catalyzes the oxidation of a wide range of endogenous and exogenous aldehydes and N-heterocyclic aromatic compounds	16702728
	Keg1	kidney expressed gene 1	2.243	0.072	2.157	0.0012	1.040	0.148	1416833_at	64697	NM_029550	Expression is upregulated in regenarating liver and in hepatocellular carcinomas	10194414
	Cpox	coproporphyrinogen oxidase	0.914	0.615	2.131	0.0014	0.429	0.047	1422493_at	12892	BG067254	involved in heme biosynthesis and upregulated by PPARα	19289130
	Hao1	hydroxyacid oxidase 1. liver	1.634	0.001	2.131	1.329E-05	0.767	0.0036	1420420_at	15112	NM_010403	liver-specific peroxisomal enzyme that oxidizes glycolate to glyoxylate with concomitant production of H_2O_2	14578854
	Shmt1	serine hydroxymethyl transferase 1 (soluble)	0.826	0.079	0.489	9.407E-05	1.687	0.101	1422198_a_at	20425	NM_009171	both Shmt1 and Shmt2 are shown to contribute to nuclear de novo thymidylate biosynthesis	19513116
	CRAD-L	cis-retinol/3alpha hydroxysterol short-chain dehydrogenase-like	0.474	0.0001	0.481	0.0016	0.985	0.871	1451681_at	216454	BC018263	catalyze the dehydrogenation of retinols, including 9-*cis* retinol, and to exhibit 3α- and 17β-hydroxysteroid dehydrogenase activities	10876094

	C1galt1	core 1 UDP-galactose:N-acetylgalactosamine-alpha-R beta 1.3-galactosyltransferase	1.112	0.452	0.481	0.0014	2.311	0.0054	1422772_at	94192	NM_052993	Targeted disruption of the gene causes embryonic lethality and defective angiogenesis in mice	17113876
	Got1	glutamate oxaloacetate transaminase 1. soluble	0.776	0.21	0.466	0.004	1.665	0.769	1450970_at	14718	AA792094	greatest diagnostic utility for manifestations of hepatocellular necrosis and biliary injury	20466777
	Akr1d1	aldo-keto reductase family 1. member D1	0.644	0.081	0.458	0.0035	1.408	0.969	1425771_at	208665	BC018333	AKR1D1 (steroid 5beta-reductase) reduces all Delta(4)-3-ketosteroids to form 5beta-dihydrosteroids, a first step in the clearance of steroid hormones and an essential step in the synthesis of all bile acids	18407998
	Tgm1	transglutaminase 1. K polypeptide	0.526	0.028	0.454	0.0075	1.159	0.109	1451416_a_at	21816	BC026422	retinoic acid treatment that was reported to increase transglutaminase activity, wheraes CCl4 decreases ist activity	14758042
	Proz	protein Z. vitamin K-dependent plasma glycoprotein	0.703	0.031	0.437	0.002	1.609	0.124	1431721_a_at	66901	BC024104	HNF-4alpha plays a crucial role in human PZ gene expression in hepatocytic cells	17958743
	Mccc2	methylcrotonoyl-Coenzyme A carboxylase 2 (beta)	0.859	0.218	0.431	0.002	1.993	0.0392	1428021_at	78038	BI155184	biotin-dependent carboxylase that catalyzes the fourth step in the leucine catabolic pathway	11181649
	Rdh11	retinol dehydrogenase 11	0.765	0.148	0.429	0.006	1.781	0.168	1449209_a_at	17252	AB030503	abundantly expressed in the liver of mice	15790565
	St3gal6	ST3 beta-galactoside alpha-2.3-sialyltransferase 6	0.512	0.122	0.407	0.0051	1.256	0.918	1449078_at	54613	NM_018784	IL-6 and IL-8 increase the expression of St3gal6	17944600
	Mmp19	matrix metalloproteinase 19	0.741	0.056	0.403	0.0004	1.838	0.07	1421976_at	58223	AF153199	MMP19 is essential for T cell development and T cell-mediated cutaneous immune responses	18523579
	Habp2	hyaluronic acid binding protein 2	0.945	0.454	0.402	0.0048	2.351	0.0022	1443696_s_at	226243	AI035669	HABP2 negatively regulates vascular integrity	20042707
	Lypla3	lysophospholipase 3	0.860	0.48	0.384	0.008	2.241	0.498	1422341_s_at	192654	NM_133792	involved in lipid metabolism and of Lypla3 increases artherosclerosis in ApoE-deficient mice	15781238
	Ahcyl1	S-adenosylhomocysteine hydrolase-like 1	0.910	0.55	0.382	0.008	2.384	0.096	1425576_at	229709	BC018218	possible role in inositol phospholipid signaling	16754674
	Hspa9a	heat shock protein 9A	1.222	0.27	0.377	0.006	3.244	0.018	1431274_a_at	15526	AA543265	involved in protein export from nucleus	11900485
	Scd1	stearoyl-Coenzyme A desaturase 1	0.922	0.78	0.365	0.002	2.524	0.014	1415965_at	20249	NM_009127	a delta-9 fatty acid desaturase that catalyzes the synthesis of monounsaturated fatty acids and has emerged as a key regulator of metabolism	18460915
	Dntt	deoxynucleotidyltransferase. terminal	0.475	0.027	0.337	0.003	1.410	0.59	1450545_a_at	21673	AF316014	involved in generating immunglobulins and T cell antigen receptors	19596089
	Ak3	adenylate kinase 3	0.922	0.493	0.336	0.0029	2.746	0.03	1432436_a_at	56248	AK007618	involved in erythropoiesis	
	Mme	membrane metallo endopeptidase	0.724	0.185	0.332	0.0001	2.178	0.051	1422975_at	17380	NM_008604	present in epithelial cells of the liver, inactivates several peptide hormones including glucagon, enkephalins, substance P, neurotensin, oxytocin bradykinin, natriuretic peptides	20692264
	C730036D15Rik	acyl-coenzyme A amino acid N-acyltransferase 2	0.169	0.0005	0.315	0.012	0.537	0.003	1425150_at	209186	BC010829	72% similar to bile acid-Coenzyme A: amino acid N-acyltransferase	
	Usp12	ubiquitin specific protease 12	0.952	0.72	0.077	6.905E-05	12.343	0.009	1425805_a_at	22217	AF441835	WDR20 regulates activity of the USP12/UAF1 deubiquitinating enzyme complex	20147737
	Nt5e	5' nucleotidase. ecto	0.206	0.0007	0.068	0.004	3.040	0.0182	1422974_at	23959	NM_011851	reduced activity by ischemia	9306922
Cell cycle, apoptosis, carcinogenesis	*Lama3*	laminin. alpha 3	4.872	0.0003	5.559	0.028	0.876	0.1183	1427512_a_at	16774	X84014	expression correlated with dedifferentiation of hepatocellular tumors	12079511
	Gadd45g	growth arrest and DNA-damage-inducible 45 gamma	0.515	0.008	4.465	0.0013	0.115	0.0045	1453851_a_at	23882	AK007410	Cdc2/cyclinB1 kinase inhibitor with role in S and G2/M cell cycle checkpoints	12124778
	Phlda1	pleckstrin homology-like domain. family A. member 1	0.586	0.42	4.057	0.0008	0.144	0.0031	1418835_at				
	Nox4	NADPH oxidase 4	2.666	0.009	2.176	0.006	1.225	0.0566	1451827_a_at	50490	AB041034	NAPDH oxidase generating ROS; Nox4 is most frequently expressed in tumor cells	11376945, 15155719
	Ccnd1	cyclin D1	0.443	0.063	0.483	0.0008	0.918	0.1709	1417419_at	12443	NM_007631	Cyclin D1/cdk4 complexes can phosphorylate Rb and E2F factors to overcome a late G1 restriction point in the cell cycle	10728791
	Ccnd1	cyclin D1	0.470	0.059	0.369	0.008	1.275	0.281	1448698_at	12443	NM_007631		
	MGI:1353606	Cd27 binding protein (Hindu God of destruction)	0.874	0.53	0.464	0.007	1.883	0.246	1418377_a_at	30954	NM_013929	apoptosis-inducing factor	
	Ccnl2	cyclin L2	0.876	0.39	0.459	0.0019	1.910	0.065	1453740_a_at	56036	BI112766	induces G0/G1 cell cycle arrest and apoptosis	17543181
	Rtn4	reticulon 4	0.630	0.003	0.448	7.152E-05	1.408	0.1102	1421116_a_at	68585	NM_024226	induces apoptosis	
Cytoskeleton	*Pfn2*	profilin 2	1.737	0.066	2.530	0.002	0.687	0.108	1418209_a_at	20338	AK005544	G-actin-binding protein regulating endocytosis and exocytosis	16319076; 17541406
	Pfn2	profilin 2	1.509	0.079	2.493	0.004	0.605	0.772	1418210_at	18645	NM_019410		
	Lasp1	LIM and SH3 protein 1	1.187	0.102	2.425	0.0043	0.489	0.0034	1455470_x_at	16796	AV027151	regulates HCC cell growth	19155088
	Stom	stomatin	1.066	0.031	0.430	0.008	2.477	0.0034	1419098_at	13830	AF093620	erythrocyte membrane protein 7.2b	10090952
	Parva	parvin. alpha	0.642	0.24	0.420	0.002	1.529	0.139	1416818_at	57342	NM_020606	regulates actin cytoskeletal dynamics and cell survival	16314921
	Pdlim5	PDZ and LIM domain 5	1.141	0.58	0.341	0.005	3.345	0.061	1421413_a_at	56376	NM_022554	involved in actin organization	
Immune response	*C6*	complement component 6	9.456	0.009	4.348	0.0003	2.175	0.0033	1449308_at	12274	NM_016704	complement component produced in the liver; higher expression in males than in females	4007963
	Cish	cytokine inducible SH2-containing protein	0.389	0.318	3.214	0.0032	0.121	0.0068	1448724_at	12700	NM_009895	Negative regulator of cytokine signaling involved in hematopoiesis, immune regulation, and inflammation	12687406
	Cd36	CD36 antigen	0.468	0.009	0.545	0.0003	0.860	0.0467	1450884_at	12491	BB534670	CD36, a scavenger receptor involved in immunity, metabolism, angiogenesis, and behavior	19471024
	Klrk1	killer cell lectin-like receptor subfamily K. member 1	0.795	0.315	0.498	0.0013	1.596	0.8402	1450495_a_at	27007	AF039026	expressed on NK cells and subsets of T and NKT cells, and recognizes a group of such inducible "stress" ligands and important regulatory component of the inflammatory response	20488792
	Pbef1	pre-B-cell colony-enhancing factor 1	0.598	0.016	0.494	0.0067	1.210	0.022	1448607_at	59027	AW989410	PBEF is upregulated in neutrophils by IL-1beta and functions as a inhibitor of apoptosis in response to a variety of inflammatory stimuli	15124023
	Tollip	toll interacting protein	0.969	0.738	0.488	0.007	1.987	0.0231	1423048_a_at	54473	BB400304	overexpression of Tollip inhibits NF-kappaB activation in response to TLR2 and TLR4 signaling, role of Toll-interacting protein and IL-1 receptor signaling molecules in Toll-like receptor 2 signaling	11441107
	Ctsc	cathepsin C	0.501	0.0002	0.479	0.002	1.046	0.106	1416382_at	13032	NM_009982	Cathepsin C null mice show deficiencies in disease-relevant proteases including neutrophil elastase, cathepsin G, chymases and granzymes and the Cat C mice are protected in a number of disease models	20337582
	Pldn	pallidin	0.858	0.074	0.471	0.0027	1.821	0.021	1417350_at	18457	NM_019788	involved in the biogenesis of lysosome-related organelles	12191018
	Il1b	interleukin 1 beta	0.685	0.118	0.451	0.0073	1.520	0.873	1449399_a_at	16176	BC011437	Interleukin-1beta (IL-1beta) and IL-18 are important proinflammatory cytokines that on the one hand activate monocytes, macropages, and	20195505

											neutrophils, and on the other hand induce Th1 and Th17 adaptive cellular responses	
Sdccag3	serologically defined colon cancer antigen 3	0.769	0.116	0.449	0.005	1.714	0.0485	1431760_a_at	68112	AK021273	necessary for the presentation of TNF receptor 1 on cell surface	16332174
Lyst	lysosomal trafficking regulator	0.988	0.95	0.444	0.0035	2.225	0.3101	1421384_at	17101	NM_010748	mutations in the *lyst* gene result in defective membrane targeting of the proteins present in secretory lysosomes, and it is associated with a variety of features, including a lymphoproliferative syndrome with hemophagocytosis	10411929
Ndrg1/Ndrl	N-myc downstream regulated gene 1/N-myc downstream regulated-like	1.118	0.579	0.346	0.003	3.228	0.0008	1450977_s_at	17988 | 17990	AI987929	ubiquitously expressed in tissues in response to cellular stress signals; involved in cellular differentiation, proliferation and growth arrest, neoplasia, tumor progression and metastasis, heavy metal response, the hypoxia response and DNA damage response	17916902
Mmd2	monocyte to macrophage differentiation-associated 2	0.334	0.017	0.291	0.0036	1.148	0.086	1438654_x_at	75104	BC025064	member of the PAQR seven transmembrane protein family that also encompasses non-genomic progesterone receptors	16044242, 12617826
Mmd2	monocyte to macrophage differentiation-associated 2	0.375	0.0158	0.284	9.924E-05	1.319	0.629	1424534_at	75104	AV269411		
Gene regulation												
Foxq1	forkhead box Q1	0.743	0.392	3.801	0.0002	0.196	0.0001	1422735_at	15220	NM_008239	Important for NK cell effector function	15719160
Lpp	LIM domain containing preferred translocation partner in lipoma	2.754	0.057	2.840	0.0058	0.970	0.019	1438597_x_at	210126	AU015158	Histone H4-Acetyltransferase	
Klf9	Kruppel-like factor 9	1.249	0.45	2.646	0.00017	0.472	0.058	1422264_s_at	16601	NM_010638	thyroid hormone-inducible	18426912
Klf9	Kruppel-like factor 9	1.062	0.52	2.217	0.003	0.479	0.056	1428289_at	16601	AW488885		
Tgfb1i4	transforming growth factor beta 1 induced transcript 4	2.617	0.002	2.331	0.002	1.122	0.395	1454758_a_at	21807	AU016382	contributes to the regulation of haematopoietic precurser cells	19329776
Tgfb1i4	transforming growth factor beta 1 induced transcript 4	2.556	0.004	2.208	0.0056	1.158	0.797	1425742_a_at	21807	AF201285		
Foxa1	forkhead box A1	0.870	0.499	2.066	6.235E-05	0.421	0.0022	1418496_at	15375	NM_008259	required for normal bile duct development through prevention of excess cholangiocyte proliferation	20347503
Snrpd3	small nuclear ribonucleoprotein D3	1.075	0.596	2.034	0.009	0.528	0.755	1449629_s_at	---	AW046420	target of autoantibodies associated with systemic lupus erythematosus	11823543
Asb13	ankyrin repeat and SOCS box-containing protein 13	1.446	0.017	2.016	0.009	0.717	0.0036	1419401_at	142688	AF403041	involved in proteolysis	
Nedd4	neural precursor cell expressed. developmentally down-regulated gene 4	0.846	0.178	0.463	0.001	1.825	0.0581	1421955_a_at	17999	NM_010890	E3-ubiquitin ligase; Nedd4 knockout leads to perinatal lethality	19345204
Mrpl15	mitochondrial ribosomal protein L15	0.975	0.85	0.435	0.0012	2.239	0.0074	1451623_at	27395	BC021304	regulator of embryonetic stem cell pluripotency and self-renewal	19619308
Fbxw11	F-box and WD-40 domain protein 11	0.990	0.94	0.425	0.0094	2.330	0.0064	1425461_at	103583	AY038079		
Rnf4	ring finger protein 4	0.887	0.064	0.410	0.0066	2.163	0.0262	1451072_a_at	19822	AV045658	a SUMO-dependent ubiquitin E3-ligase which plays a key role in active DNA-methylation	20696907
Dok2	docking protein 2	0.674	0.091	0.399	0.002	1.691	0.385	1416333_at	13449	NM_010071	play an essential role in the negative regulation of TCR signaling	17329234
Rnf14	ring finger protein 14	0.836	0.049	0.277	0.001	3.017	0.0029	1431030_a_at	56736	AK010162	ubiquitin E3-ligase; might function as coactivator for AR-mediated transactivation in prostate cancer DU145 cells	10085091
Ehmt2	euchromatic histone lysine N-methyltransferase 2	1.002	0.99	0.242	0.008	4.141	0.0066	1426888_at	110147	BI412952	arginine methylation on chromatin	17898715
Signal tranduction												
Egfr	epidermal growth factor receptor	0.838	0.49	5.769	0.0086	0.145	0.008	1432647_at	13649	AK014017	involved in liver cell proliferation, as well as to apoptotic liver cell death, and represents an important regulator of hepatic regeneration	19558330
Spsb4	SPRY domain-containing SOCS box 4	2.383	0.0029	3.643	0.0059	0.654	0.028	1451418_a_at	211949	BC023083	unknown	
Plk3	polo-like kinase 3 (Drosophila)	1.404	0.333	3.209	0.005	0.438	0.0077	1434496_at	12795	BM947855	Involved in G1/S phase transition and expression peaks in G1 phase	17264206
Chacl1	cation transport regulator-like	1.225	0.487	2.481	0.0009	0.494	0.0003	1451382_at	69065	BC025169	Presumably involved in cation transport (based on domain architecture)	
Hhex	hematopoietically expressed homeobox	0.714	0.458	2.330	0.0009	0.306	0.01001	1423319_at	15242	AK014111	Hhex is a critical regulator of hematopoietic development and is necessary for the maturation and proliferation of the earliest definitive hematopoietic progenitors	20472829
Sdc4	syndecan 4	1.232	0.141	2.300	0.0007	0.536	0.0087	1417654_at	20971	BC005679	a transmembrane heparan sulftate proteoglycan involved in defense mechanisms	12975610
F2r	coagulation factor II (thrombin) receptor	1.629	0.071	2.155	0.0025	0.756	0.123	1450852_s_at	14062	BQ173958	essential role in coagulation	9695019
Sorbs1	sorbin and SH3 domain containing 1	0.997	0.987	2.064	0.0024	0.483	0.008	1436737_a_at	20411	BB737680	key factor modulating actin polymerization in migrating cells	16483316
Atf2	activating transcription factor 2	0.752	0.279	0.492	0.0031	1.528	0.499	1427559_a_at	11909	M31629	transcription factor important for normal cellular development and survival	18677098
Rfk	riboflavin kinase	0.640	0.008	0.479	0.0016	1.334	0.421	1416229_at	54391	NM_019437	Riboflavin kinase couples TNF receptor 1 to NADPH oxidase	19641494
Folr2	folate receptor 2 (fetal)	0.814	0.550	0.477	0.0073	1.706	0.144	1451648_a_at	14276	BC022108	results in profound developmental abnormalities in the fetus, ranging from neural tube defects to neurocristopathies such as cleft-lip and cleft-palate, cardiac septal defects, and eye defects	17284762
Ghr	growth hormone receptor	1.109	0.568	0.477	0.0009	2.326	0.073	1451871_a_at	14600	M33324	important in the regulation of lipid and carbohydrate metabolism, particularly by liver and adipose tissue, and for the sexual dimorphism of hepatic drug, xenobiotic and steroid metabolism	18063438
Ceacam1	CEA-related cell adhesion molecule 1	0.763	0.169	0.477	0.0047	1.602	0.527	1450494_x_at	26365	NM_011926	plays a role in intercellular adhesion of hepatocytes; involved in liver histogenesis; belongs to a class of androgen-repressed genes associated with enriched stem/amplifying cell population after prolonged castration	6339528, 8106416
Ceacam1	CEA-related cell adhesion molecule 1	0.829	0.303	0.428	0.0002	1.938	0.045	1452532_x_at	26365	M61907		
Ceacam1	CEA-related cell adhesion molecule 1	0.686	0.084	0.389	0.004	1.761	0.0306	1427711_a_at	26365	X15351		
Nr1i3	nuclear receptor subfamily 1. group I. member 3	0.607	0.0014	0.470	0.005	1.291	0.653	1425392_a_at	12355	AF009328		
Sap18	Sin3-associated polypeptide 18	0.705	0.093	0.469	0.0041	1.502	0.0471	1419444_at	20220	NM_009119	in a complex with the transcriptional corepressor mSin3 and histone deacetylase	9511770
Eif4e3	eukaryotic translation initiation factor 4E member 3	0.552	0.058	0.469	0.0069	1.177	0.092	1417977_at	66892	BC027014	binds to mRNA cap-structure	15153109
Zfml	zinc finger. matrin-like	1.223	0.143	0.468	0.00604	2.612	0.0082	1417792_at	18139	BM238431	unknown	
Dusp6	dual specificity phosphatase 6	0.585	0.149	0.461	0.0042	1.268	0.026	1415834_at	67603	NM_026268	negative regulator of MAPK activity; involved in initiation and development of cancer	18330678
Rad23a	RAD23a homolog (S. cerevisiae)	0.969	0.813	0.459	0.0059	2.109	0.0082	1453623_a_at	19358	AK009733	plays a role in nucleotide excision repair, cell cycle regulation and the ubiquitination pathways	11788722
Hif1a	hypoxia inducible factor 1. alpha subunit	0.991	0.968	0.447	0.0004	2.219	0.0912	1448183_a_at	15251	BB269715	essential for myeloid-cell-mediated inflammation; involved in apoptosis, cell differentiation and tumor angiogenesis	12628185; 9697772
Spred1	sprouty protein with EVH-1 domain 1. related sequence	0.898	0.648	0.441	0.0048	2.038	0.0089	1423161_s_at	114715	BQ044290	bone marrow-derived mast cells and eosinophils from Spred-1–/– mice were more sensitive to IL-3 and IL-5	19597303
Prkacb	protein kinase. cAMP dependent. catalytic. beta	0.885	0.361	0.439	0.0083	2.013	0.0192	1420610_at	18749	AV024339	involved in carbohydrate and lipid metabolism	
Gnat1	guanine nucleotide binding protein. alpha transducing 1	0.631	0.235	0.433	0.0063	1.457	0.1016	1460212_at	14685	BC022793	a transducin normally involved in photoreceptor modulation	

Mapk14	mitogen activated protein kinase 14	0.908	0.252	0.432	0.0077	2.104	0.0115	1451927_a_at	26416	AF128892	involved in the IL-2 induction of TNF-β gene	11741287
Ptprk	protein tyrosine phosphatase. receptor type. K	1.015	0.903	0.424	0.0016	2.393	0.0263	1431680_a_at	19272	AK019870	regulates CD4+ T cell development through ERK1/2-mediated signaling	19800317
Trim24	tripartite motif protein 24	0.391	0.0018	0.418	0.03	0.934	0.744	1427259_at	21848	BB611004	Trim24 ubiquitylates and negatively regulates p53 levels	19556538
Trim24	tripartite motif protein 24	0.220	0.0038	0.290	0.026	0.758	0.1388	1427258_at	21848	BB611004		
Hnf4a	hepatic nuclear factor 4. alpha	0.978	0.873	0.405	0.0022	2.412	0.0052	1450447_at	15378	NM_008261	a direct transactivator of numerous xenobiotic-metabolizing cytochrome P450 (CYP) genes	19689247
Rnf13	ring finger protein 13	0.842	0.055	0.398	0.0055	2.114	0.0138	1420620_a_at	24017	NM_011883	an E3 ubiquitin ligase embedded in endosome membranes	20230530
Zbp1	Z-DNA binding protein 1	0.458	0.0072	0.384	0.027	1.191	0.7167	1429947_a_at	58203	AK008179	upregulated in macrophages; cytosolic DNA sensor involved in type I interferon response	10564822, 17618271
Sbk1	SH3-binding kinase 1	0.510	0.044	0.377	0.009	1.355	0.5801	1423978_at	104175	BC025837	involved in diverse signal transduction processes	
Nr3c1	nuclear receptor subfamily 3. group C. member 1	0.678	0.0265	0.342	0.0004	1.984	0.0098	1421866_at	14815	NM_008173	glucocorticoid receptor directly regulates expression of various genes involved in bile acid transport and detoxification; involved in the transcriptional regulation of various functions including repression of inflammatory signals	20422498
D4Wsu53e	DNA segment. Chr 4. Wayne State University 53. expressed	0.803	0.159	0.337	0.0039	2.383	0.0097	1448538_a_at	27981	BE447520	unknown	
Sdc1	syndecan 1	0.706	0.038	0.328	3.092E-06	2.152	0.00549	1448158_at	20969	BI788645	heparan sulfate proteoglycan mediates the clearance of both hepatic and intestinally derived triglyceride -rich lipoproteins	
Rcan2	regulator of calcineurin 2	0.313	0.0004	0.310	0.0219	1.009	0.211	1421425_a_at	53901	NM_030598	functions as calcineurin fascilitator	16648267
Tfrc	transferrin receptor	0.796	0.109	0.307	0.0029	2.593	0.628	1422966_a_at	22042	BB810450	necessary for development of erythrocytes (erythropoiesis)	10192390
Twsg1	twisted gastrulation homolog 1 (Drosophila)	0.804	0.1385	0.276	0.0018	2.913	0.0478	1426179_a_at	65960	AF295097	unknown	
Prlr	prolactin receptor	0.382	0.0033	0.219	0.0156	1.747	0.394	1451844_at	19116	M22958	treatment of female mice with testosterone reduces prlr levels	6329976
Prlr	prolactin receptor	0.218	0.0079	0.170	0.0129	1.285	0.322	1450226_at	19116	NM_008932		
Prlr	prolactin receptor	0.209	0.0057	0.152	0.0113	1.372	0.079	1421382_at	19116	NM_008932		
Transport												
Slco1a1	solute carrier organic anion transporter family. member 1a1	14.590	0.0002	4.375	0.0012	0.301	0.091	1420379_at	28248	AB031813	organic anion transporter with male predonderance in the liver; regulated by androgens and growth hormone secretion pattern	16807376, 12399219
Slco1a1	solute carrier organic anion transporter family. member 1a1	16.227	0.0025	3.712	0.0024	4.372	0.3109	1449844_at	28248	AB031813		
Slc25a30	solute carrier family 25. member 30	1.221	0.717	4.181	0.008	0.292	0.785	1450018_s_at	67554	BB032012	Transporter in the inner mitochondrial membrane; expression is elevated when mitochondrial metabolism is increased, in particular und a pro-oxidant cellular redox state	15809292
Slc25a30	solute carrier family 25. member 30	1.217	0.678	3.693	0.0051	0.330	0.439	1420836_at	67554	BB032012	mitochondrial carrier protein, upregulated in the kidney during tubular cell regeneration	15809292
Slc25a30	solute carrier family 25. member 30	1.214	0.671	2.533	0.0001	0.479	0.503	1420835_at	67554	BB032012		
Timm44	translocator of inner mitochondrial membrane 44	1.166	0.031	2.311	0.006	0.505	0.151	1439371_x_at	21856	AV102008	involved in mitochondrial import	11450972
Abcg2	ATP-binding cassette. sub-family G (WHITE). member 2	2.346	0.0004	2.215	0.0059	1.059	0.0169	1422906_at	26357	NM_011920	breast cancer resistance protein which mediates biliary elimination	19084913
Tnpo3	transportin 3	1.189	0.0472	2.005	0.0031	0.593	0.986	1453124_at	320938	AV306751	involved in retroviral preintegration complex nuclear import in humans	19846519
Aqp9	aquaporin 9	0.754	0.024	0.495	0.0054	1.523	0.011	1421605_a_at	64008	NM_022026	crucial to determine glucose metabolism	12351427
Slc13a3	solute carrier family 13 (sodium-dependent dicarboxylate transporter). member 3	0.595	0.058	0.481	0.0055	1.235	0.062	1416560_at	114644	NM_054055	Na(+)-dependent dicarboxylate transporter	12915942
Slc7a2	solute carrier family 7 (cationic amino acid transporter. y+ system). member 2	0.948	0.811	0.452	0.0056	2.095	0.087	1450703_at	11988	BF533509	cationic amino acid transporter abundant expressed in the liver	9736269
Slc7a2	solute carrier family 7 (cationic amino acid transporter. y+ system). member 2	0.707	0.022	0.392	0.0051	1.802	0.06	1426008_a_at	11988	M62838		
Abca1	ATP-binding cassette. sub-family A (ABC1). member 1	0.836	0.237	0.401	0.0015	2.083	0.058	1421839_at	11303	BB144704		
Abca1	ATP-binding cassette. sub-family A (ABC1). member 1	0.722	0.099	0.275	0.0012	2.630	0.033	1450392_at	11303	BB144704	controls cholesterol and phospholipid efflux	11950702
Pctp	phosphatidylcholine transfer protein	0.635	0.171	0.358	0.00073	1.774	0.0009	1420983_at	18559	AF114437	involved in cholesterol and phospholipid transport	16099870
Ap3m1	adaptor-related protein complex 3. mu 1 subunit	0.876	0.418	0.352	0.0044	2.485	0.536	1448309_at	55946	AF242857	unknown	
Slc9a8	solute carrier family 9 (sodium/hydrogen exchanger). member 8	1.008	0.96	0.342	0.0032	2.944	0.0066	1426274_at	77031	AF482993	Na+/H+-antiporter/exchanger	12845533
Slco1b2	solute carrier organic anion transporter family. member 1b2	0.970	0.794	0.329	0.0096	2.945	0.011	1427826_a_at	28253	AB037192	organis anion transporter which plays an essential role in hepatic reuptake of conjugated bilirubin and uptake of unconjugated bile acids and drugs	20644253
Slc39a14	solute carrier family 39 (zinc transporter). member 14	0.629	0.022	0.328	0.0081	1.914	0.233	1425649_at	213053	BC021530	Interleukin-1beta contributes via nitric oxide to the upregulation and functional activity of the zinc transporter Zip14 (Slc39a14) in murine hepatocytes	19179618
Abcd2	ATP-binding cassette. sub-family D (ALD). member 2	0.277	0.0001	0.170	0.00011	1.629	0.072	1419748_at	26874	NM_011994	ABC transporter in the peroxisome membrane, LXR prevents expression by interfering with binding of SREB1c	8577752, 16249184
BC014805	cDNA sequence BC014805	0.027	0.0002	0.017	0.0185	1.567	0.884	1425752_at	236149	AJ132857	64% identical and 79% similar to Slc22a9, anorganic anion transporter in transepithelial transport of steroid sulfates	16150593
BC014805	cDNA sequence BC014805	0.026	5.669E-05	0.012	0.0127	2.112	0.8401	1425751_at	236149	AJ132857		
Miscellaneous												
Lrrc16	leucine rich repeat containing 16	2.605	0.048	4.394	0.0038	0.593	0.168	1451804_a_at	68732	BC012229	Inhibits actin capping; concentrates in lamellipodia and increases the fraction of cells with large lamellipodia	16054028
BC024137	cDNA sequence BC024137	3.243	0.0079	3.568	0.0024	0.909	0.0149	1427513_at	234757	BI144810	unknown	
Susd4	sushi domain containing 4	5.449	0.0031	3.079	0.0279	1.770	0.084	1424221_at	96935	BC021842	unknown	
2810439F02Rik	RIKEN cDNA 2810439F02 gene	3.712	0.023	2.878	0.0081	1.290	0.0997	1426223_at	72747	BC020021	unknown	
Rabepk	Rab9 effector protein with kelch motifs	1.003	0.982	2.399	0.0041	0.418	0.0159	1448046_at	227746	AA217054	locates inside a QTL region known to modulate prion disease incubation time	17303092
1110004E09Rik	RIKEN cDNA 1110004E09 gene	2.626	0.084	2.389	0.0019	1.099	0.00073	1435301_at	68001	BF460829	unknown	
2810474O19Rik	RIKEN cDNA 2810474O19 gene	1.037	0.885	2.263	0.007	0.458	0.124	1427334_s_at	67246	BE196832	unknown	
Tuft1	tuftelin 1	1.522	0.238	2.156	0.0006	0.706	0.653	1416689_at	22156	NM_011656	involved in mouse tooth development	
AW548124	protein kinase domain containing, cytoplasmic	1.087	0.514	2.047	0.0098	0.531	0.024	1454838_s_at	106522	BB323985	unknown	
Prpf6	PRP6 pre-mRNA splicing factor 6 homolog (yeast)	1.527	0.0001	2.043	0.0001	0.748	0.186	1454789_x_at	68879	BB085604	involved in the formation of the spliceosome	
Armcx3	armadillo repeat containing, X-linked 3	1.193	0.0724	2.018	0.0041	0.591	0.278	1460359_at	434247	AK004598	unknown	
C330006A16Rik	RIKEN cDNA C330006A16 gene	0.990	0.914	2.013	0.0052	0.492	0.129	1433618_at	109299	BB397899	unknown	
Tcte1l	t-complex-associated-testis-expressed 1-like	0.989	0.899	0.495	0.0005	1.998	0.0381	1449928_at	67117	NM_025975	unknown	
Tmem98	transmembrane protein 98	0.636	0.093	0.488	0.0041	1.303	0.323	1424133_at	103743	BC011208	unknown	
Cox15	COX15 homolog. cytochrome c oxidase assembly protein (yeast)	0.794	0.073	0.485	0.003	1.637	0.274	1460376_a_at	226139	BC011509	unknown	

Txndc16	thioredoxin domain containing 16	1.004	0.987	0.457	0.0024	2.196	0.72	1427050_at	70561	BC027108	unknown
Echdc3	enoyl Coenzyme A hydratase domain containing 3	0.574	0.014	0.457	0.00013	1.255	0.018	1418862_at	67856	NM_024208	unknown
Pcmtd1	protein-L-isoaspartate (D-aspartate)-O-methyltransferase domain containing 1	1.080	0.471	0.454	0.0018	2.380	0.0174	1435635_at	319263	BB549335	unknown
Ptpl1	protein tyrosine phosphatase-like A domain containing 1	0.826	0.307	0.347	0.0025	2.380	0.024	1452427_s_at	57874	BM950003	involved in different signal cascades (e. g. Ras, Rho)
Sel1h	Sel1 (suppressor of lin-12) 1 homolog (C. elegans)	1.033	0.731	0.344	0.0014	3.001	0.0051	1453559_a_at	20338	AK005544	involved in Notch signaling pathway, functions as a tumor suppressor
C730048C13Rik	hypothetical protein D630002G06/RIKEN cDNA C730048C13 gene	0.125	0.026	0.196	6.776E-05	0.639	0.147	1451635_at	236293	AB056443	cation transport protein
D630002G06	hypothetical protein D630002G06	0.080	0.0102	0.124	0.0044	0.647	0.216	1425222_x_at	236293	AB056443	cation transport protein
AB056442	cDNA sequence AB056442	0.042	0.0025	0.023	0.0049	1.869	0.395	1419751_x_at	171405	NM_134256	organic anion transporter 6

Table 2 Permanent change of immunglobulin expression in vehicle- and T-treated female immune mouse liver

Gene Symbol	Gene name	Affymetrix probe ID	Entrez Gene	Representative public ID	T_{imm}/T	C_{imm}/C	T_{imm}/C_{imm}
Igh-1a	Immunoglobulin heavy chain 1a (serum IgG2a)	1425385_a_at	380793	BC018365	63.357	81.407	0.778
Ighg	Immunoglobulin heavy chain (gamma polypeptide)	1424631_a_at	380794	BC025447	15.352	26.282	0.584
Ighg	Immunoglobulin heavy chain (gamma polypeptide)	1426174_s_at	380794	S69212	22.000	15.166	1.451
Igh-4	Immunoglobulin heavy chain 4 (serum IgG1)	1427756_x_at	16017	M60430	6.288	10.917	0.576
Igk-V21	Immunoglobulin kappa chain variable 21 (V21)-12	1425738_at	243469	M35669	35.847	36.946	0.970
Igk-V8	Immunoglobulin kappa chain variable 8 (V8)	1452463_x_at	16071	BG966217	42.483	22.036	1.928
Igk-V28	Immunoglobulin kappa chain variable 28 (V28)	1452417_x_at	16114	AV057155	20.766	12.164	1.707
Igk-V28	Immunoglobulin kappa chain variable 28 (V28)	1427455_x_at	16114	BI107286	19.485	11.834	1.647
Igk-V28	Immunoglobulin kappa chain variable 28 (V28)	1427660_x_at	16114	BC013496	16.433	10.359	1.586
Igk-V32	Immunoglobulin kappa chain variable 32 (V32)	1427837_at	16116	U25103	6.322	8.255	0.766
Igl-V1	Immunoglobulin lambda chain, variable 1	1424931_s_at	16142	M94350	7.978	5.303	1.504
Igj	Immunoglobulin joining chain	1424305_at	16069	BC006026	11.266	10.306	1.093
IgM	Immunoglobulin heavy chain (V7183 family)	1460423_x_at	381774	U29768	15.316	8.979	1.706
Igh-6	Immunoglobulin heavy chain 6 (heavy chain of IgM)	1427870_x_at	16017	K00686	3.928	7.456	0.527
Igh-6	Immunoglobulin heavy chain 6 (heavy chain of IgM)	1425247_a_at	16017	BC008237	2.639	3.650	0.723

Table 3 Effect of *P. chabaudi* infection on hepatic gene expression and vehicle- and T-treated immune mice

Gene symbol	Gene name	Entrez gene	Affymetrix probe ID	T_{inf}/T_{imm}	C_{inf}/C_{imm}
Saa2	serum amyloid A 2	20209	1449326_x_at	8.794	7.639
Saa1	serum amyloid A 1	20208	1419075_s_at	4.282	4.478
Saa1	serum amyloid A 1	20208	1450788_at	3.827	4.365
Orm2	orosomucoid 2	18406	1420438_at	2.893	4.233
Acnat2	acyl-coenzyme A amino acid N-acyltransferase 2	209186	1425150_at	2.688	0.884
Tenc1	tensin like C1 domain-containing phosphatase	209039	1452264_at	0.458	0.916
Adamts5	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 5	100048332	1422561_at	0.488	0.912
Nt5e	5' nucleotidase, ecto	23959	1422974_at	0.485	0.889
Atoh8	atonal homolog 8 (Drosophila)	71093	1426418_at	0.480	0.839
Lipg	lipase, endothelial	16891	1421262_at	0.380	0.564
Lipg	lipase, endothelial	16891	1450188_s_at	0.395	0.517
Txnl1	thioredoxin-like 1	53382	1459817_at	1.031	0.410

2.6 Disrupted IL-6 trans-signaling augments ability to self-heal-malaria

Background: The pleiotropic cytokine IL-6 is critically involved in the response to malaria. IL-6 signals through a protein complex, which includes the membrane-bound, non-signaling receptor α (IL-6Rα) and two signal transducing sgp130 subunits. IL-6Rα is mainly expressed on hepatocytes, neutrophils, monocytes/macrophages and lymphocytes. On the other hand, IL-6 can also act *via* a soluble IL-6Rα (sIL-6Rα), which is termed trans-signaling, leading also to an activation of a signaling cascade. IL-6 trans-signaling has been investigated in the murine arthritis model resulting in an improvement in systemic arthritis, when IL-6 trans-signaling was blocked by using soluble sgp130. In this study, the murine malaria model is used to examine the role of IL-6 trans-signaling in lethal outcome of *P. chabaudi* infection.

Methods: Here, we used IL-6Rα knockout-mice (IL-6Rα$^{-/-}$) and corresponding control IL-6Rα$^{FL/FL}$ mice. Female IL-6Rα$^{-/-}$ and IL-6Rα$^{FL/FL}$ mice were challenged with 10^6 parasitized erythrocytes derived from a highly virulent strain of *P. chabaudi*. ELISA was used to determine circulating levels of IL-6 and sIL-6Rα. To induce IL-6 trans-signaling, IL-6Rα$^{-/-}$ mice were injected with 8µg, 4µg, and 4µg sIL-6Rα on days 1, 4, and 7 after infection, respectively. To reduce IL-6 trans-signaling, IL-6Rα$^{FL/FL}$ mice were injected with 16µg , 8µg, and 8µg sgp130 on day 1, 4, and 7 after infection. STAT3 phosphorylation was used as an indicator of hepatic IL-6 trans-signaling analyzed by Western-Blots.

Results: Deletion of the IL-6Rα gene resulted in approximately 50% self-healing of otherwise lethal *P. chabaudi* malaria. Injection with sIL-6Rα during infection in IL-6Rα$^{-/-}$ caused lethal outcome of *P. chabaudi* malaria, whereas 40% of IL-6Rα$^{FL/FL}$ mice injected with sgp130 are able to self-heal these infections. In sIL-6Rα-injected IL-6Rα$^{-/-}$ mice, the circulating levels of sIL-6Rα were raised from non-detectable levels to approximately 70 ng/ml at peak parasitemia on day 8 *p.i.*, whereas the circulating IL-6 is decreased from approximately 0.60 ng/ml to approximately 0.11 ng/ml. In sgp130-injected IL-6Rα$^{FL/FL}$ mice sIL-6Rα levels decline from approximately 200 ng/ml to 120 ng/ml and the IL-6 levels from approximately 0.35 ng/ml to 0.1 ng/ml on day 8 *p.i.*

Conclusion: This study provides first evidence that disruption of IL-6 trans-signaling augments ability of mice to self-heal otherwise lethal malaria.

Disrupted IL-6 trans-signaling augments ability to self-heal malaria

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ABSTRACT

Increasing levels of interleukin 6 (IL-6) are associated with increasing morbidity and even lethal outcome of malaria. IL-6 is known to act through both the ′classic-signaling′ pathway, i. e. the specificity-determining membrane IL-6 receptor alpha (IL-6Rα) and two recruited membrane gp130 to activate the JAK/STAT pathway, and the ′trans-signaling′ pathway mediated by the soluble form of IL-6Rα (s IL-6Rα). To disrupt IL-6 signaling pathways, we created an IL-6Rα-deficient mouse by floxing exons 2 and 3 of the IL-6Rα gene and its deletion by crossing with Cre-deleter mice. Approximately 50% of these IL-6Rα$^{-/-}$ mice are able to self-heal an infection of otherwise lethal blood-stage malaria of *P. chabaudi*. To induce IL-6 trans-signaling, IL-6Rα$^{-/-}$ were injected with sIL-6Rα during infection, which causes a lethal outcome. By contrast, when IL-6 trans-signaling in IL-6Rα carrying mice is reduced by injecting soluble form of gp130 (sgp130), which binds and inhibits circulating sIL-6Rα/IL-6 complexes, approximately 40% of these mice are able to self-heal the infections. Our data demonstrate that IL-6 trans-signaling contributes to a lethal outcome of blood-stage malaria. Possibly, the malaria-induced increase in IL-6 is inactivated by sIL-6Rα,

and protective response *per se* is superposed by increasing levels of sIL-6Rα/IL-6 complexes, which can interact and the increasing levels of sIL-6Rα/IL-6 complexes then activate other cells through their gp130.

Malaria is still a major health problem: about 1.3-2.7 billion people live in malaria-endemic regions, about 300-500 million people suffer from malaria, and mortality is high with about 1-3 million deaths per year, mainly among children below 5 years in sub-Saharan areas (Garcia, 2010; O´Meara *et al*., 2010). The blood stages of the infectious agent, protozoan parasites of the genus *Plasmodium*, induce both protective and pathologic host responses not yet really understood, however the dysregulations of which are assumed to mainly contribute to malaria morbidity and mortality (Pierce and Miller, 2009).

The pleiotropic cytokine IL-6 is essential for both health and disease in general (Heinrich *et al*., 2003), and it is also critically involved in the response to malaria. Indeed, evidence has accumulated that circulating levels of IL-6 are increased in patients suffering from mild and severe malaria caused by *P. falciparum* and *P. vivax* (Kern *et al*., 1989; Mshana *et al*., 1991; Jakobsen *et al*., 1994; Thuma *et al*., 1996; Jason *et al*., 2001; Gourly *et al*., 2002; Lyke *et al.,* 2004), which can be associated with polyclonal B cell activation (Donati *et al*., 2004) and hypergammoglobulinemia (Grau *et al*., 1990). Conversely, decreased IL-6 levels are reported to prevail after anti-malarial treatment (Hugosson *et al*., 2006), with decreasing hyperpyrexia (Seoh *et al*., 2003), with decreasing parasitemia (Tabone *et al*., 1992), and after survival (Sarthou *et al*., 1997).

The mode of IL-6 action is rather complex (Heinrich *et al.,* 2003; Drucker *et al*., 2010). IL-6 signals through the membrane IL-6 receptor α (IL-6Rα), that requires the recruitment of two chains of the membrane receptor glycoprotein 130 (gp130) for signal transduction to activate the JAK/STAT pathway. This IL-6 ´classic-signaling` is restricted to those cells which express IL-6Rα on their surface, i. e. hepatocytes and immune cells such as neutrophils, monocytes/macrophages, and lymphocytes. Nevertheless, IL-6 is also able to communicate with all other cells through a process termed IL-6 ´trans-signaling`, i. e IL-6 can to signal through a naturally occurring soluble IL-6Rα (sIL-6Rα), which is derived mainly by shedding of the ectodomain of the membrane IL-6Rα, and which can bind to membrane gp130 expressed by all cells. Incidentally, this gp130 is also used for signaling by other members of the IL-6 family such as IL-11, IL-27, ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), leukemia inhibitory factor (LIF), oncostatin M (OSM), and neurotrophin-1 (NT-1) (Heinrich et al., 2003; Häussinger, 2009). Since the role of IL-6 trans-signaling has not yet

been investigated in context with malaria, we have tried to approach this in the mouse malaria model *Plasmodium chabaudi*. First, we have explore a possible role of IL-6Rα in malaria by generating an IL-6Rα-deficient mouse strain on a C57BL/6 background (Fig. 1). The gene encoding the 80 kDa large IL-6Rα consists of 10 exons. IL-6Rα$^{FL/FL}$ mouse were created (Wunderlich *et al*., 2010), which still carry a functional IL-6Rα gene, but with exons 2 and 3 flanked by loxP sites (Fig. 1A). These mice were then crossed with deleter Cre-mice, and further F1 intercrosses yielded the IL-6Rα$^{-/-}$ mice (Fig. 1A). The disruption of IL-6Rα was verified at the DNA-level by Southern Blot analysis and PCR (Fig. 1B, C). As anticipated, no expression of IL-6Rα was observed at the protein-level in different tissues of the IL-6Rα$^{-/-}$ mice (Fig. 1D). The IL-6Rα$^{-/-}$ mice do not exhibit any apparent phenotype. In particular, the immune system does not appear to be grossly affected. For instance, the mutant mice exhibit similar numbers of B and T cells in lymph nodes, mesenterial lymph nodes, and the spleen (Fig. S1). Surprisingly, however, the disruption of IL-6Rα becomes evident as an altered responsiveness to malaria. Upon challenge with blood-stage malaria of *P. chabaudi* (Delić et al., 2010), approximately 50% of the IL-6Rα$^{-/-}$ mice are able to self-heal the infections, while the same infections take a lethal outcome in all IL-6Rα$^{FL/FL}$ control mice (Fig. 2A). In both types of mice, the course of infections was the same, i. e. there was an increasing parasitemia during the precrisis phase culminating at peak parasitemia of approximately 50-55% on around day 8 *p.i.*. Mice then succumb mainly during the subsequent crisis phase, which was characterized by dramatically falling parasitemias (Fig. 2A). The IL-6Rα$^{FL/FL}$ mice exhibit a continuous elevation of circulating levels of sIL-6Rα during the precrisis phase from approximately 12 ng/ml to 190 ng/ml at peak parasitemia on day 8 *p.i.* in IL-6Rα$^{FL/FL}$ mice (Fig. 2B), whereas sIL-6Rα was not detectable in the IL-6Rα$^{-/-}$ mice (Fig. 2B). Concomitantly, there was a higher increase increase in circulating IL-6 the IL-6Rα$^{-/-}$ mice than in IL-6Rα$^{FL/FL}$ mice (Fig. 2B).

Approximately 70% of the circulating IL-6 has been estimated to bind to the sIL-6Rα (Gaillard *et al*., 1999), and the IL-6/sIL-6Rα-complex is known to induce IL-6 trans-signaling in all cells via the membrane-bound gp130 (Heinrich et al., 2003; Drucker et al., 2010). It is therefore possible that the *P. chabaudi*-induced increase in circulating endogenous sIL-6Rα of the IL-6Rα$^{FL/FL}$ mice indicates an increased IL-6 trans-signaling which contributes to the lethal outcome of malaria. In order to support this view, we have next examined the occurrence of IL-6 trans-signaling. Hepatic STAT3 is a downstream target of IL-6 signaling. At peak parasitemia, STAT3 is phosphorylated in IL-6Rα$^{FL/FL}$ mice, which is higher than that in IL-6Rα$^{-/-}$ mice (Fig. 3A). The latter indicates that STAT3-phosphorylation is apparently not

exclusively activated in response to IL-6 signaling during malaria infection. However, a much stronger STAT3 phosphorylation occurs when 1µg mouse recombinant sIL-6Rα is injected *i.p.* in IL-6Rα$^{-/-}$ mice during infection on days 1, 4, and 7 *p.i.*, respectively (Fig. 3A). The injections apparently induced IL-6 trans-signaling. In accordance, the circulating levels of sIL-6Rα were raised from non-detectable levels to approximately 70 ng/ml at peak parasitemia on day 8 *p.i.*(Fig. 3B), and, coincidently, the circulating IL-6 is decreased from approximately 0.6 ng/ml to approximately 0.11 ng/ml. Importantly, however, all IL-6Rα$^{-/-}$ mice injected with sIL-6Rα succumb to malaria during the crisis phase (Fig. 3C). Though parasitized erythrocytes appear earlier in the sIL-6Rα-injected IL-6Rα$^{-/-}$ mice (Fig. 3C) than in the non-treated mice (Fig. 2A), parasitemia in both group of mice was maximal on around day 8 *p.i.* and its percentage was not significantly altered (Fig. 2C).

Besides the sIL-6Rα, there exists also a soluble gp130 (sgp130), which is derived by shedding of the ectodomain from the membrane gp130 (Heinrich et al., 2003; Knüpfer & Preiss, 2008). This sgp130 is able to bind the IL-6/sIL-6Rα-complex and, thus, to inhibit IL-6 trans-signaling (Knüpfer & Preiss, 2008). In order to further substantiate a critical role of IL-6 trans-signaling in the outcome of malaria, we have also attempted to reduce IL-6 trans-signaling in the IL-6Rα expressing mice. Thus, we have *i.p.* injected IL-6Rα$^{FL/FL}$ mice with mouse recombinant sgp130 (R&D Systems, Minneapolis, United States) in doses of 16 µg, 8 µg, and 8 µg during of infection on days 1, 4, and 7 *p.i.*, respectively. This schedule caused the endogenous sIL-6Rα to decline from approximately 200 ng/ml to 120 ng/ml and the IL-6 from approximately 0.35 ng/ml to 0.1 ng/ml on day 8 *p.i.* (Fig. 3D). Also, this schedule lowers STAT3 phosphorylation in the liver (Fig. 3A). Obviously, the injected sgp130 binds at least some of the endogenous sIL-6Rα and lowers IL-6 trans-signaling. Under these conditions, approximately 40% of the *P. chabaudi* challenged mice survived the infection (Fig. 3E). The injected sgp130 slightly affected the course of infections: peak parasitemia of approximately 30 % occurred one day earlier than in non-treated IL-6Rα$^{FL/FL}$ (Fig. 3E vs. Fig. 2A). This report is the first that provides unequivocal evidence for IL-6 trans-signaling as to promote a lethal outcome of *P. chabaudi* malaria in mice. Indeed, IL-6 trans-signaling is critically responsible for about 40-50% of the mortality induced by *P. chabaudi* malaria and its disruption augments the ability of mice to self-heal the malarial infections. This self-healing ability has been previously shown to be under control of both genes of the *H2*-complex and genes of the *non-H2* background (Wunderlich *et al*., 1988; 1991; Foote et al., 1997; Roberts et al., 2001). The efficacy of these genes-mediated protective host responses is apparently superposed by the counteracting IL-6 trans-signaling. Currently, we consider the

following explanation as to be plausible. The sIL-6Rα levels continuously increase during malaria, which appears to be induced by the preceding malaria-induced increase in the circulating IL-6. This represents *per se* a ´beneficial´response of the host, since it is to inactivate overshooting of IL-6. However, this protective host response becomes harmful with the continuous elevation of IL-6/sIL-6Rα-complexes causing increased IL-6 trans-signaling, which in turn increasingly activates those mechanisms and cells, respectively, which promote harmful pathologic conditions thus raising morbidity and mortality to malaria.

Finally, our data appear to be also relevant for human malaria. Indeed, the few data currently available indicate that sIL-6Rα levels directly correlate with severity of malaria caused by *P. falciparum* and *P. vivax* (Wenisch et al., 1999). This also emphasizes that the development of an efficient anti-malaria vaccine will become more complicated than hitherto assumed. For, the vaccine has to be designed such that it intervenes in the complex process of IL-6 trans-signaling, the extent of which presumably varies among individuals, for example due to prevailing inflammatory and/or infectious diseases.

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Figure legends

Fig. 1 Generation of IL-6Rα$^{-/-}$ mice. (A) The IL-6Rα gene comprises 10 exons. The exons 2 and 3 were flanked by loxP-sites to create the IL-6Rα^{FL} allele, as detailed recently (Wunderlich *et al*., 2010). Mice carrying the IL-6Rα^{FL} allele were crossed with deleter-Cre mice to yield knock out mice termed IL-6Rα$^{-/-}$ on a C57BL/6 background. (B) Southern

blot analysis of Bg1I-digested DNA isolated from tails reveals the 6.3 kb FL allele using the radioactively labeled probe indicated in (A), while the Cre-mediated recombination of the loxP-flanked exons 2 and 3 resulted in the 4.3 kb allele (cf. A); us = unspecific band. (C) Confirmation of mouse genotypes of DNA isolated from tail biopsies from IL-6Rα$^{FL/FL}$, IL-6Rα$^{-/-}$ and corresponding heterozygous genotypes using PCR with 5′-CCG-CGG-GCG-ATC-GCC-TAG-G-3',5′-CCA-GAG-GAG-CCC-AAG-CTC-TC-3' and 5′-TAG-GGC-CCA-GTT-CCT-TTA-T-3' primers. (D) Western blot analysis of different tissues of IL-6Rα$^{FL/FL}$ mice and IL-6Rα$^{-/-}$ mice. We used the polyclonal rabbit anti-serum sc-660 from Santa Cruz biotechnologies for IL-6Rα as primary antibodies, anti-rabbit antibody linked with HRP as secondary antibody and the anti-rabbit AKT polyclonal antibody for the loading control AKT. ECL solutions (Amersham) were used for development of the HRP-coupled antibodies.

Fig. S1 Flow cytometric analysis of B- and T-cells in different lymphoid organs of IL-6Rα$^{FL/FL}$ mice and IL-6Rα$^{-/-}$ mice. Spleens, mesenterial lymph nodes (LN) and axial lymph nodes (LN) were aseptically removed from mice. Single cell suspensions were labeled with anti-CD19 and anti-TCR-β monoclonal antibodies and measured in a FACS calibur. FACS dotblots are representative for three different mice and numbers indicate percentage of total cells.

Fig. 2 Response of IL-6Rα$^{FL/FL}$ and IL-6Rα$^{-/-}$ mice to *P. chabaudi* malaria. (A) Outcome and course of blood-stage infections after challenge with 10^6 *Plasmodium chabaudi*-infected erythrocytes. The IL-6Rα$^{FL/FL}$ mice succumb to infection, mainly during the crisis phase after reaching peak parasitemia. However, approximately 50% of the IL-6Rα$^{-/-}$ mice are able to self-heal the same infections. (B) Circulating levels of sIL-6Rα and IL-6. At different time points during infection, sIL-6Rα and IL-6 in blood plasma were determined using the commercially available mouse IL-6 and mouse sIL-6Rα ELISA kits (R&D Systems, Minneapolis, USA). Stars indicate significant differences ($p < 0.01$) as evaluated using two-way ANOVA with Dunnett and Bonferoni post-hoc-tests using the statistical package program SPSS version 17.0.

Fig. 3 Effects of sIL-6Rα and sgp130 on *P. chabaudi*-infected mice. IL-6Rα$^{FL/FL}$ and IL-6Rα$^{-/-}$ mice were challenged with 10^6 *P. chabaudi*-infected erythrocytes. (A) To activate IL-6 trans-signaling, IL-6Rα$^{-/-}$ mice were *i.p.* injected with commercially available mouse recombinant 1 µg sIL-6Rα (R&D Systems, Europe, UK) during infection on days 1, 4, and 7 *p.i.*, respectively. To reduce IL-6 trans-signaling IL-6Rα$^{FL/FL}$ mice were treated with 16, 8, and 8µg sgp130 during infection on days 1, 4, and 7, respectively. Liver tissue of mice

was subjected to Western blotting using anti-phosphotyrosine STAT3 antibody (Cell Signaling #9145), anti-STAT3 antibody (Cell Signaling #4904) as primary antibodies and anti-rabbit HRP-coupled antibody as detecting secondary antibody.

(B) Effect of sIL-6Rα in IL-6Rα$^{-/-}$ mice. The mice were *i.p.* injected with 1µg sIL-6Rα (R&D Systems, Europe, UK) three times during infection as in (A) and sIL-6Rα and IL-6 levels in blood plasma of at least three different mice were determined on day 0 and day 8 *p.i.*, respectively, by commercially available ELISA kits (R&D systems, Minneapolis, USA). Significance was evaluated using Student´s t-test ($p < 0.01$). (C) Outcome and parasitemia of *P. chabaudi* in IL-6Rα$^{-/-}$ mice injected with sIL-6Rα as in (A).

(D) Effect of sgp130 in IL-6Rα$^{FL/FL}$ mice. Mice infected with *P. chabaudi* were *i.p.* injected sgp130 (R&D Systems Europe, UK) as in (A). On day 0 and 8 *p.i*., sIL-6Rα and IL-6 were determined in blood plasma by ELISA (R&D systems, Minneapolis, USA). Significance was evaluated using Student´s t-test ($p < 0.01$). (E) Outcome and parasitemia of infections in IL-6Rα$^{FL/FL}$ mice injected with sgp130 at different time-points during infection (arrows).

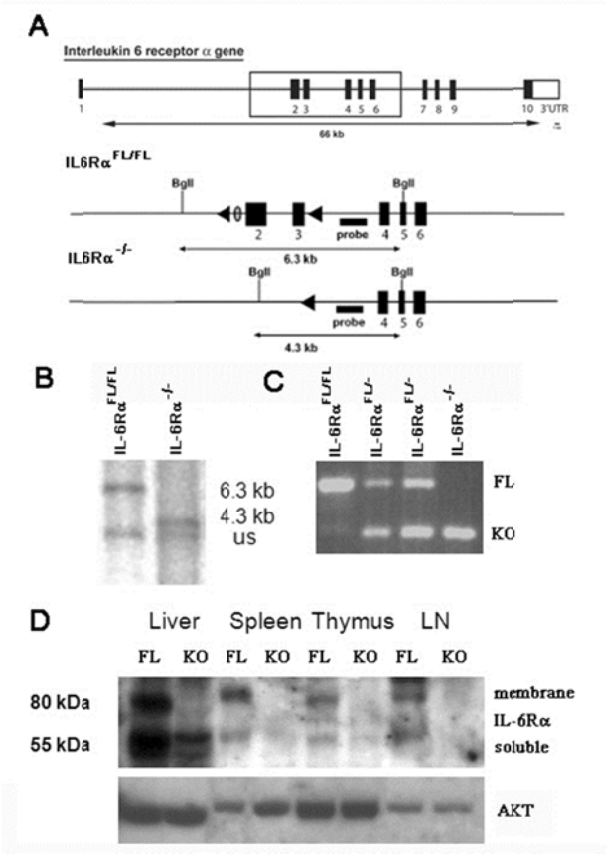
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Figure 1

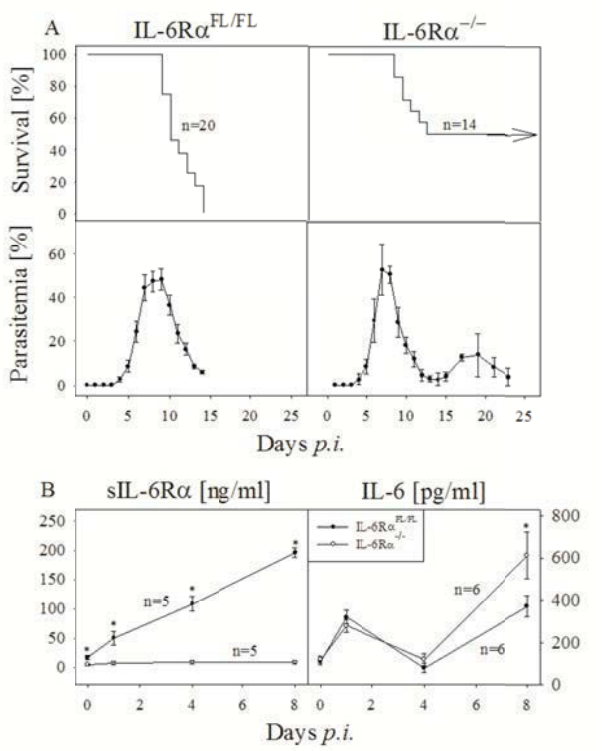


Figure 2

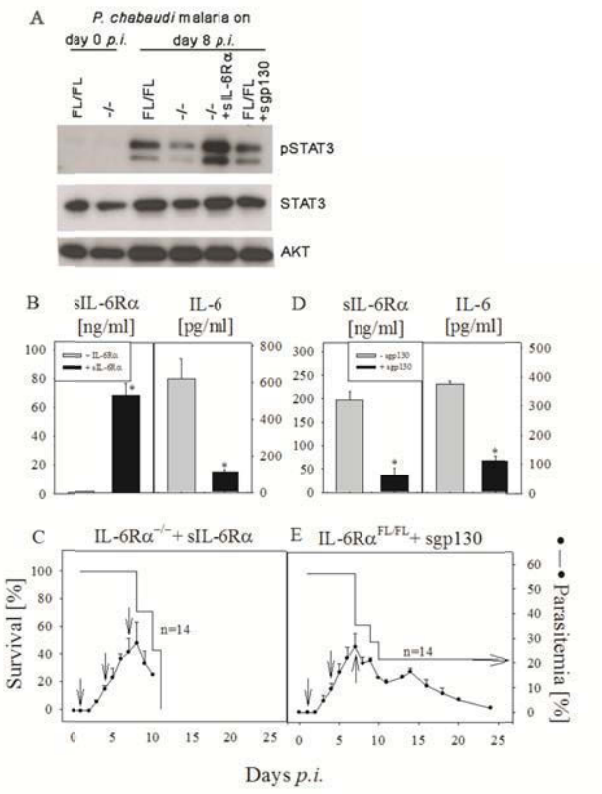
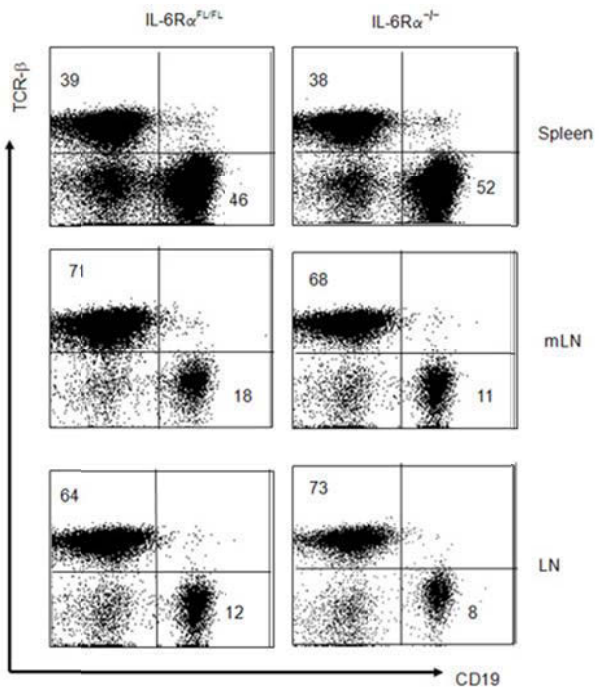


Figure 3


IL-6Rα^FL/FL
IL-6Rα^-/-
TCR-β
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46
38
52
Spleen
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11
mLN
64
12
73
8
LN
CD19

Figure S1

2.7 Protective vaccination against *Plasmodium chabaudi* malaria: augmented particle trapping and attenuated inflammation in the liver

Background: To date all efforts to develop a malaria vaccine have failed, reflecting the still fragmentary knowledge about protective mechanisms against malaria. For instance, it is not yet really understood which changes have to be activated by a protective vaccination in the anti-malaria effectors spleen and liver. In order to find such changes, we have compared Balb/c mice succumbing to infection with *Plasmodium chabaudi* with those surviving after vaccination.

Methods: Mice were vaccinated with host cell plasma membranes isolated from *P. chabaudi*-injected erythrocytes. Hepatic and splenic capacity to trap particulate material was determined after injection of fluorescent polystyrol beads. Hepatic gene expression was measured using quantitative real-time PCR and Northern blotting.

Results: Survival of Balb/c mice was raised from 0% to 80% and peak parasitemia was decreased by about 30% by vaccination. Vaccination boosted particle trapping capacity of the liver during crisis when splenic trapping is minimal due to spleen ′closing′. Also, malaria-induced inflammation is attenuated, thus diminishing severe damages and hence liver failure. Vaccination increased hepatic IFNγ production but mitigated acute phase response. Vaccination has a complex influence on infection-induced changes in expression of hepatic nuclear receptors (CAR, FXR, RXR, and PXR) and of the metabolic enzymes Sult2a and Cyp7a1. Although vaccination decreased CAR mRNA levels and prevented Cyp7a1 suppression by the CAR ligand 1,2-bis [2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) on day 8 *p.i.*, Sult2a-induction by TCPOBOP was restored.

Conclusion: These data support the view that the liver is an essential effector site for a vaccine against blood stage malaria: vaccination attenuates malaria-induced inflammation thus improving hepatic metabolic activity and particle trapping activity of the liver.

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Augmented particle trapping and attenuated inflammation in the liver by protective vaccination against *Plasmodium chabaudi* malaria

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Abstract

Background: To date all efforts to develop a malaria vaccine have failed, reflecting the still fragmentary knowledge about protective mechanisms against malaria. In order to evaluate if vaccination changes responses of the anti-malaria effectors spleen and liver to blood stage malaria, BALB/c mice succumbing to infection with *Plasmodium chabaudi* were compared to those surviving after vaccination.

Methods: Mice were vaccinated with host cell plasma membranes isolated from *P. chabaudi*-infected erythrocytes. Hepatic and splenic capacity to trap particulate material was determined after injection of fluorescent polystyrol beads. Hepatic gene expression was measured using real-time RT-PCR and Northern blotting.

Results: Survival of BALB/c mice was raised from 0% to 80% and peak parasitaemia was decreased by about 30% by vaccination. Vaccination boosted particle trapping capacity of the liver during crisis when splenic trapping is minimal due to spleen 'closing'. It also attenuated malaria-induced inflammation, thus diminishing severe damages and hence liver failure. Vaccination increased hepatic IFN-γ production but mitigated acute phase response. Vaccination has a complex influence on infection-induced changes in expression of hepatic nuclear receptors (CAR, FXR, RXR, and PXR) and of the metabolic enzymes Sult2a and Cyp7a1. Although vaccination decreased CAR mRNA levels and prevented Cyp7a1 suppression by the CAR ligand 1,2-bis [2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) on day 8 p.i., Sult2a-induction by TCPOBOP was restored.

Conclusion: These data support the view that the liver is an essential effector site for a vaccine against blood stage malaria: vaccination attenuates malaria-induced inflammation thus improving hepatic metabolic activity and particle trapping activity of the liver.

Background

Despite intense efforts to develop a vaccine against malaria during the last 30 years, a safe and effective vaccine candidate is not yet available [1]. One reason for this failure may be that knowledge about the effector sites and mechanisms that have to be activated for successful protection is still rather incomplete. Moreover, natural immunity to malaria underlies rather complex control. It is directed against the blood stages of *Plasmodium* parasites, but it is never solid, i.e. it mitigates and can even completely abolish disease symptoms, but it does not prevent re-infections during malaria seasons [2,3]. In an experimental malaria model, *Plasmodium chabaudi* in rodents, a vaccination model has been previously developed that resembles natural immunity in so far that it helps susceptible mice to survive an otherwise lethal blood stage infection without preventing parasitaemia [4]. This vaccination model uses host cell plasma membranes of *P. chabaudi*-parasitized red blood cells (pRBC) as an immunogen. These erythrocyte membranes contain several parasite proteins [5,6] the functions of which have remained unknown to date, including a major immunogenic *P. chabaudi* protein *Pc*90 against which most of the antibodies induced by this type of vaccination are directed [4]. This vaccination model is used here to further study the effector sites and mechanisms, which have to be activated to survive blood stage infections.

The spleen is widely considered to be the central effector site of the host defence against blood stage malaria [7,8], and it is thought to destroy pRBC by the same mechanisms which normally remove senescent and other aberrant erythrocytes from circulation [9]. Basically, pRBC are eliminated by macrophages in the red pulp areas of the spleen, specifically in extravascular beds through which blood is percolated before reaching the collecting veins. This open circulation and, hence, the direct contact between pRBC and macrophages, has been described to become 'closed' during acute *P. chabaudi* malaria or at least transiently closed during acute *Plasmodium yoelii* 17XNL [10,11] and *P. chabaudi adami* malaria [12]. However, it is possible that vaccination prevents – at least partially – this 'closing' mechanism, thus enabling the spleen to destroy pRBC during crisis when parasitaemia is dramatically falling.

The liver is another important effector site against blood stage malaria, though research in this field is largely neglected to date [13,14]. Indeed, research concentrates on the role of the liver in the pre-erythrocytic development of parasites. However, the liver, although not exhibiting any extravascular beds as the spleen, is also able to phagocytose senescent erythrocytes [15,16] and pRBC [17,18]. In particular, the intravascular Kupffer cells, which constitute about 80–90% of all resident macrophages of the reticuloendothelial system [19], are competent for erythrophagocytosis. In *P. chabaudi* malaria it has been recently shown that the liver improves its trapping capacity, especially during crisis of self-healing infections, i.e. that phase, when the spleen is 'closed' [13,20]. However, the effect of protective vaccination on liver trapping capacity has never been investigated to date.

Here, protective vaccination against blood stages of *P. chabaudi* is shown to convert non-healer BALB/c mice to self-healer mice. This vaccination-induced self-healing coincides with an augmented trapping capacity of the liver – but not of the spleen – especially during crisis, when parasitaemia drops from more than 50% to about 1% or even below. Furthermore, vaccination boosts production of IFN-γ and strongly attenuates inflammation and promotes recovery of liver metabolism from infection-induced dysregulation during crisis.

Methods

Mice

BALB/c mice were bred under specific pathogen-free conditions in the central animal facilities at the Max-Planck-Institute for Immunobiology in Freiburg and at the University of Düsseldorf. Experiments were performed only with female mice at an age of 10–14 weeks. They were housed in plastic cages and received standard diet (Wohrlin, Bad Salzufeln, Germany) and water *ad libitum*. In some experiments, mice received 60 μg of the synthetic CAR ligand 1,2-bis [2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP) in 100 μl DMSO by intraperitoneal injection 24 h before they were killed for isolation of RNA. All experiments were approved by the state authorities and followed German law on animal protection.

Infections

A non-clonal line of *P. chabaudi* was used which behaves very similarly to *P. chabaudi chabaudi* AS in terms of restriction fragment length polymorphism analysis [14]. Also, the AS clone and the line used here reveal sequence identity for dihydrofolate reductase and for a cysteine protease [21] with only a single nucleotide exchange in the latter (Krücken and Wunderlich, unpublished data). Blood stages of *P. chabaudi* were weekly passaged in NMRI mice [14,22]. BALB/c mice were challenged with 10^6 *P. chabaudi*-parasitized erythrocytes by intraperitoneal injection. Parasitaemia was evaluated in Giemsa-stained blood smears. Total erythrocytes were counted in a Neubauer chamber.

Vaccination

Mice were vaccinated with a modified procedure that has been developed previously [4]. As an immunogen, host cell plasma membranes were used which were isolated in the form of ghosts from *P. chabaudi*-pRBC as described

elsewhere [23]. About 10^6 ghosts in 100 μl FCA were subcutaneously injected twice, three weeks and one week before challenging with *P. chabaudi* pRBC. Controls received plasma membranes in the form of ghosts from non-infected erythrocytes in FCA or only FCA.

Flow cytometry

Spleens were processed for flow cytometry in a FACScan (BD Biosience) as detailed previously [14]. After aseptic removal, spleens were gently dissociated through a stainless steel sieve into RPMI medium (Invitrogen) supplemented with 5% foetal calf serum (PAA Laboratories). After lysis of erythrocytes by NH_4Cl, total leukocytes were counted in a Neubauer chamber. Leukocytes were preincubated with anti-CD16/CD32 (FcIII/II receptor) FC block (BD Bioscience) for 15 min and then labelled with one of the following FITC-labelled 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 Br1 (RB6-8C5) (all BD Bioscience), and anti-mouse F4/80 (C1:A3-1) (Immunokontact). FACS analyses were done with a sample size of 10.000 cells gated on the basis of forward and sideward scatter [24]. Data were stored and processed using Cell Quest Pro software (BD Bioscience).

Trapping capacity

Trapping of 3 μm green fluorescent polystyrol beads (Ducke Scientific corporation, Palo Alto, California) was measured according to the procedure described recently [13,20]. Particles were intravenously injected, mice were killed 5 min later, spleens and parts of livers were removed, weighed and then incubated in ethanolic KOH, to which 5×10^5 red fluorescent beads (diameter 2.9 μm) were added as an internal standard. The samples were incubated until complete tissue resolution before fluorescence of beads was measured in a spectrometer (Perkin Elmer LS 55, Germany) at excitation/emission wavelengths (450/480) and (520/590) for green and red beads, respectively. For localization of beads [20], cryosections of spleen and liver were stained with haematoxilin and eosin. Fluorescence and bright field pictures of the same field were take separately and superimposed electronically.

RNA-Isolation

Spleens and liver pieces were aseptically removed, rapidly frozen, and stored in liquid N_2. Total RNA was isolated using Trizol (Invitrogen).

Northern blot analysis

Total RNA (20 μg) was glyoxylated, separated in agarose gels, and transferred to positively charged Biodyne7PLUS nylon membrane (PALL Corporation, Pensacola, FI) as described previously [25]. Hybridiziation was carried out with cDNA fragments labelled with [α^{32}P]dCTP using the DecaLabel™ DNA Labeling Kit (Fermentas) in ExpressHyb solution (Clontech) at 65°C overnight [26]. Blots were washed at 65°C twice for 15 min in 2 × SSC, 0.1% SDS and twice for 30 min in 0.1 × SSC, 0.1% SDS, and subjected to autoradiography at -80°C using Biomax MS film and screen (Kodak). For densitometric analyses, autoradiographs were scanned and evaluated using QuantiScan 3.0 software (Biosoft, Cambridge, UK).

Quantitative real-time RT-PCR

Contaminating genomic DNA was removed from total RNA by digestion with DNase using the DNA-free™ kit (Ambion). Then, cDNA was synthesized using the QuantiTect® Reverse Transcription kit (Qiagen) which includes an additional step to remove contaminating genomic DNA. Amplifications were performed in a TaqMan7500 (AppliedBiosystems) using QuantiTect™ SYBR® Green PCR kit (Qiagen) and gene-specific QuantiTect™ primer assays (Qiagen) according to the manufacturer's instructions. Following an initial incubation at 50°C for 2 min, Taq polymerase was activated by incubation at 95°C for 10 min. During the following 55 cycles made up of 15 s at 95°C, 35 s at 60°C, and 30 s at 72°C, the amount of double stranded PCR product was measured as SYBR green fluorescence at the end of the extension phase. All PCR reactions yielded only a single product species of the expected size as revealed by melting point analysis and gel electrophoresis. Relative quantitative evaluation of amplification data was done using Taqman7500 system software v.1.2.3f2 (AppliedBiosystems) and the $2^{-\Delta\Delta c}{}_T$ method [27]. Expression of the genes of interest was compared to 18S rRNA.

Statistical analyses

Differences between vaccinated and non-vaccinated mice were analysed using Student's t tests.

Results

Protective vaccination

The vaccination procedure used here converts mice from a non-healer to a self-healer phenotype, i.e. it prevents mortality, but it does not prevent parasitaemia [4]. Indeed, female BALB/c mice are highly susceptible to *P. chabaudi* malaria. Challenge with 10^6 *P. chabaudi*-parasitized erythrocytes resulted in a peak parasitaemia of approximately 58% on day 7 *p. i.* (Figure 1). Though the following crisis was characterized by falling parasitaemia, all mice succumbed to infection during this period. Vaccination, however, protected BALB/c mice against otherwise fatal *P. chabaudi* malaria. Indeed, more than 80% of these mice survived the infection (Figure 1). Vaccination did not prevent parasitaemia, but peak parasitaemia was significantly decreased to 42%, which dropped during crisis to about 1% and in individual mice to even below 1% on

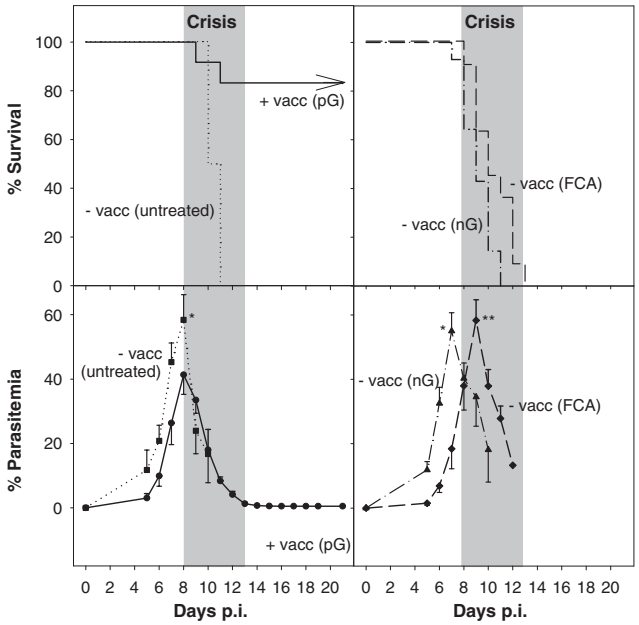

Figure 1
Vaccination-induced protection against *P. chabaudi* malaria. BALB/c mice were vaccinated with ghosts from pRBC (+ vacc (pG); n = 18) or were not vaccinated (- vacc (untreated); n = 8) or were treated with ghosts from non-infected erythrocytes in FCA (nG; n = 8), or treated only with FCA (FCA; n = 17), before challenging with 10^6 pRBC. All values are means and half standard errors of the mean (S.E.M.). **, $p < 0.01$, *, $p < 0.05$ vs. peak parasitaemia in vaccinated mice. The crisis phase (days 8–13 p.i.) is accentuated in grey.

day 13 p.i. (Figure 1). Mice vaccinated with erythrocyte ghosts from non-infected mice were not protected, i.e. all mice succumbed to infection during crisis. The same was observed in control mice, which received only FCA. Remarkably, blood glucose level, anaemia, and percentage of reticulocytes in the blood did not significantly differ between vaccinated and non-vaccinated mice (Additional file 1).

Particle trapping by liver and spleen

Though vaccination did not directly affect spleen size in terms of weight, there was an indirect effect on size, which became evident upon infection. Thus, increase in splenic weight of vaccinated mice was about twice as much as in non-vaccinated mice (Figure 2A). In the latter, average spleen weight rose from about 130 mg on day 0 to about 660 mg on day 8 p.i. Infection of vaccinated mice resulted in a dramatic increase to more than 1,200 mg per average spleen on day 8 p.i. (Figure 2B). On day 0 p.i., vaccination already induced a significantly increased proportion (Figure 3A) and absolute number (Figure 3B) of phagocytes in the spleen, i.e. frequency of $Gr1^+$ granulocytes increased by about 75% and that of $F4/80^+$ macrophages increased by about 40% (Figure 3). This vaccination-induced increase in phagocytes was retained during infection. However, the frequency of different lymphoid cell populations was not significantly altered by vaccination with the exception of $B220^+$ B cells showing a significant but temporary increase in absolute number on day 4 p.i. (Figure 3B).

In preliminary experiments, particle trapping by spleen and liver was analysed by fluorescence microscopy after injection of 3 μm fluorescent beads. Splenic cryosections, taken on day 0 p.i., clearly show trapping of beads predominantly in the marginal zone of the spleen that was lost around peak parasitaemia (Figure 2A) as described previously for C57BL/6 mice [20]. Hepatic sections reveal a more or less uniform distribution of trapped beads in the liver on days 0 and 8 p.i., however, on day 11 p.i. beads appear to be restricted predominantly to regions with large distance to the central veins (Figure 2A). The cryosections were insufficient to resolve whether trapped beads have been directly phagocytosed by macrophages, adhere to the surface of phagocytic cells or are passively trapped in the organs. Incidentally, paraffin sectioning was not possible since all solvents used for dewaxing the sections did also dissolve the beads.

Since number of beads varied considerably between slides a more quantitative method employing dissolution of tissues by ethanolic KOH followed by fluorescence spectroscopy was used to look for differences in particle trapping between vaccinated and non-vaccinated mice. Despite the increase in the frequency of phagocytic cells in the spleen, vaccination did not significantly affect the specific capacity of the spleen to trap particles – neither before nor during infection. After injection of 3 μm fluorescent polystyrol beads, approximately 2.2×10^5 particles/100 mg spleen were trapped in non-vaccinated mice and about the same number in vaccinated mice before infection (Figure 2B). On day 4 p.i., trapping capacity remained at about the same level as before, but at peak parasitaemia on day 8 p.i., there was a dramatic reduction in particle trapping both in vaccinated and non-vaccinated mice (Figure 2B).

Obviously, the entry of particles into the spleen was largely prevented at peak parasitaemia. This so-called 'closing' of the spleen lasted during the major part of crisis and, only at the end of crisis, a slight 'reopening' began in vaccinated mice, but the spleen re-gained its initial trapping capacity only on day 36 *p.i.* – if at all (Figure 2B). Despite the enormous malaria-induced splenomegaly in vaccinated mice, total splenic uptake of fluorescent beads

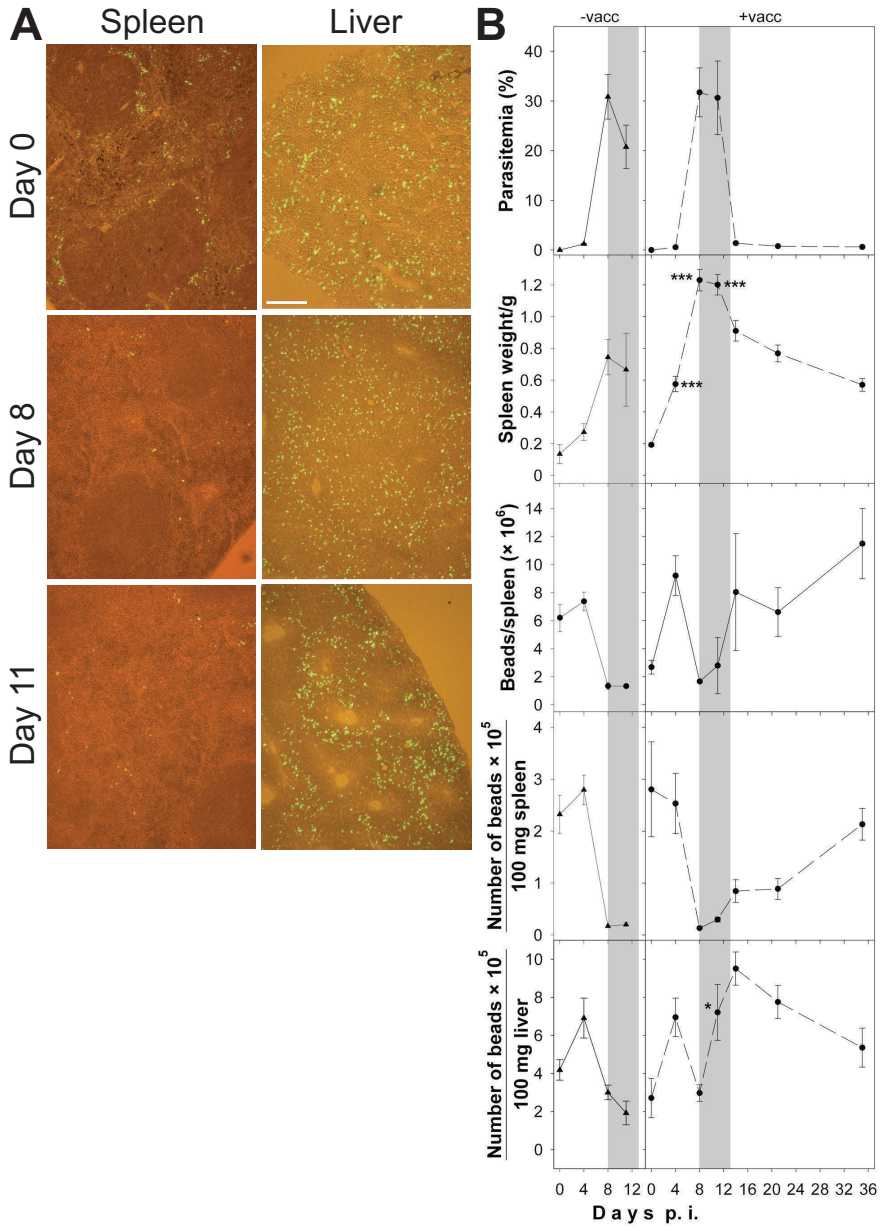

Figure 2
Splenic and hepatic particle trapping during *P. chabaudi* malaria. (A) Localization of 3 μm green fluorescent beads in splenic and hepatic cryosections of non-vaccinated BALB/c mice. The scale bar represents 200 μm. (B) Quantification of splenic and hepatic trapping. BALB/c mice were vaccinated (+vacc) or not vaccinated (-vacc) and then were challenged with 10^6 pRBC. Parasitaemia, spleen weight, and total number of beads per spleen, and numbers of beads per 100 mg spleen or liver are given as means ± S.E.M. The crisis phase (days 8–13 p.i.) is accentuated in grey. ***, $p < 0.001$ vs. non-vaccinated control on the same day p.i. *, $p < 0.05$ vs. non-vaccinated control on the same day p.i.

was not significantly higher during crisis. However, splenomegaly resulted in rapid recovery of total splenic trapping capacity after crisis.

Since preliminary tests had shown that there were no significant effects of vaccination or *P. chabaudi* infection on liver weight, trapping capacity was only determined for pieces of liver and no total liver trapping capacity was determined. In contrast to spleen, specific trapping capacity per 100 mg liver was significantly affected by vaccination, which became evident upon infection during crisis (Figure 2B). Indeed, vaccination did neither affect the size of the liver nor modify the capacity of the liver to trap particles before infection. On day 4 p.i., there was an increase

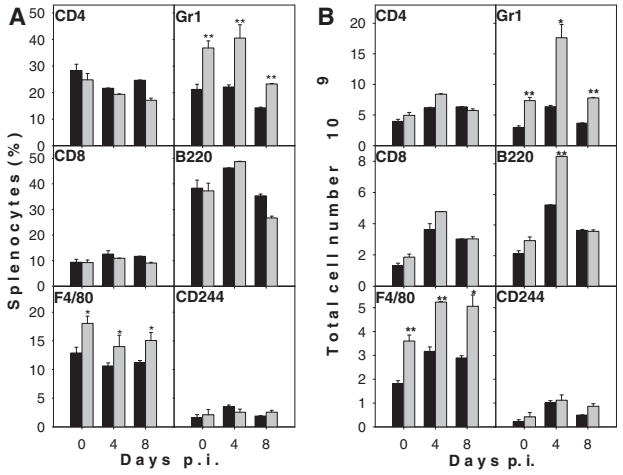

Figure 3
Cellular composition of the spleen during *P. chabaudi* malaria. Vaccinated (black columns) and non-vaccinated (gray columns) mice were infected with 10^6 *P. chabaudi* pRBC and spleen cells were isolated on the days indicated. The frequency (A) and total number (B) of $CD4^+$ and $CD8^+$ T cells, $F4/80^+$ macrophages, $Gr1^+$ granulocytes, $B220^+$ B cells, and $CD244^+$ NK cells among nucleated spleen cells are given as means + half S.E.M. **, $p < 0.01$ vs. non-vaccinated control on the same day p.i. *, $p < 0.05$ vs. non-vaccinated control on the same day p.i.

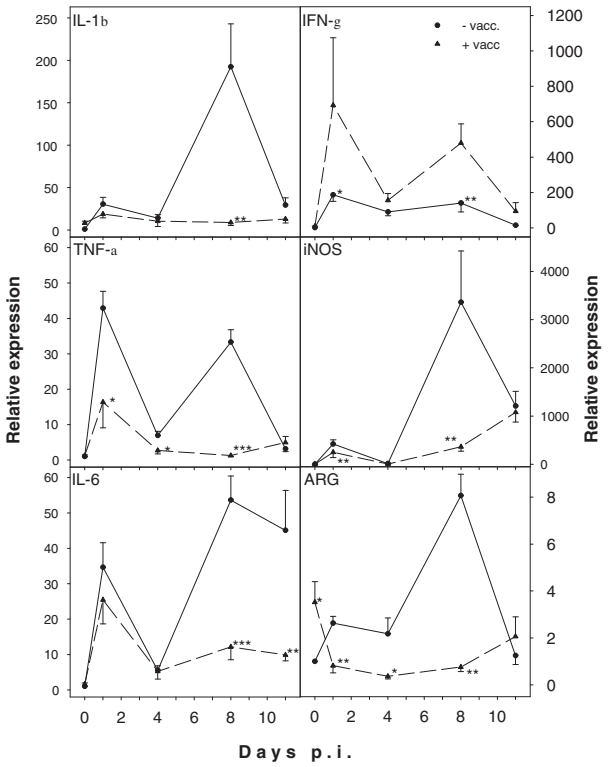

Figure 4
Real-time RT-PCR analysis of cytokines, iNOS, andarginase (ARG) in the liver. Expression was analysed in hepatic RNA from vaccinated (circles) and non-vaccinated (triangles) mice on the indicated days after challenging with *P. chabaudi*. Signals for genes of interest were normalized to 18S rRNA signals and relative expression is given as fold increase compared to non-vaccinated mice on day 0 p.i. All values are mean and half S.E.M. ***, $p < 0.001$ vs. non-vaccinated control on the same day p.i. **, $p < 0.01$ vs. non-vaccinated control on the same day p.i. *, $p < 0.05$ vs. non-vaccinated control on the same day p.i.

in the trapping capacity of the liver, but on day 8 p.i., there was a decline to about the initial trapping capacity with no difference between vaccinated and non-vaccinated mice (Figure 2B). During crisis, particle trapping further dropped in non-vaccinated control mice, whereas vaccinated mice exhibited a sharp increase in their capacity to trap particles in the liver and this increase further progressed reaching a maximum on day 14 p.i., i.e. shortly after crisis (Figure 2B).

Liver inflammation

In order to detect possible effects of vaccination on inflammatory and immune responses in the liver, real-time RT-PCR was used to measure the proinflammatory cytokines IL-1β, TNF, and IL-6 and the T_H1 cytokine IFN-γ, which is protective against *P. chabaudi* malaria [28-30]. The mRNA levels of these cytokines followed a biphasic pattern in non-vaccinated mice during infection, with a first peak on day 1 p.i. and a second peak on day 8 p.i. (Figure 4). After vaccination, infection induced a large increase in expression of IFN-γ especially on days 1 and 8 p.i., whereas vaccination dampened the infection-induced increases in mRNA levels for the proinflammatory cytokines IL1-β, TNF, and IL-6 – in particular around peak parasitaemia. Kupffer cells are a well-known source of these cytokines in the liver suggesting strong Kupffer cell activation in non-vaccinated mice during malaria. In accordance, mRNA levels of iNOS, marker for M1 macrophages, also exhibited a biphasic expression pattern in response to infection, whereas induction of iNOS by *P. chabaudi* malaria is largely depressed in vaccinated mice (Figure 4). Arginase, a marker for alternatively activated M2 macrophages, followed about the same expression profile as iNOS although at much lower levels.

In the liver, the above proinflammatory cytokines are especially known to induce acute phase and other innate immune responses. Expression profile of two acute phase proteins and one proinflammatory chemoattractant were, therefore, compared between vaccinated and non-vaccinated mice. Similar to the cytokines, total serum amyloid

A (SAA) followed a biphasic kinetic during infection in non-vaccinated mice (Figure 5). Vaccination, however, abolished this biphasic pattern due to decreased expression, especially on days 8 and 11 p.i. By contrast, C-reactive protein (CRP) and the CXC chemokine ligand CXCL10 were activated only in the early phase of precrisis, i.e. on days 1 and 4 p.i., and their induction by infection was largely prevented by vaccination.

Liver metabolism

IL-1β, TNF and IL-6 are also well known to depress liver metabolism by interfering with expression of key nuclear receptors regulating expression of hepatocyte-specific metabolic enzymes [31-35]. Therefore, mRNA levels of five members of the nuclear receptor family were examined by real-time RT-PCR. Infection caused a transiently increased expression of RXR, FXR, CAR, PXR, and VDR in non-vaccinated mice, however, expression dramatically dropped during crisis on day 11 p.i (Figure 6). In contrast, vaccinated mice initially showed increased mRNA levels of these receptors before infection (day 0 p.i.), whereas infection down-regulated their expression on days 1, 4 and 8 p.i. (Figure 6). Surprisingly, however, these receptors were significantly up-regulated again during crisis. By the end of crisis, there was an almost complete recovery of RXR, FXR, and CAR to those levels induced by vaccination before infection, whereas PXR and VDR were significantly higher expressed (Figure 6).

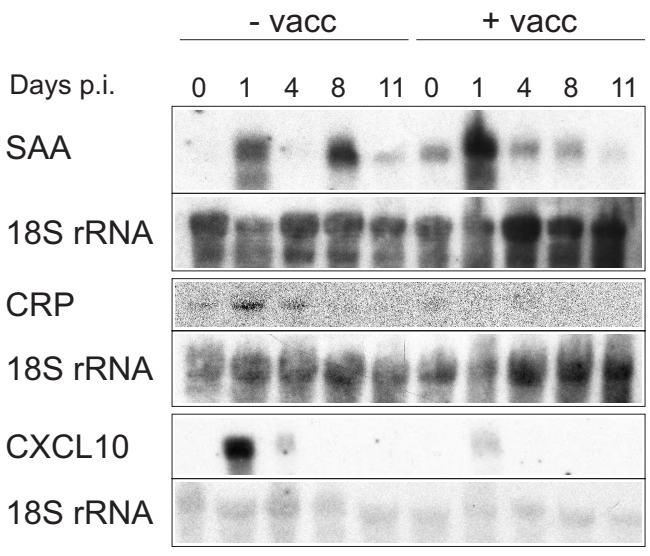

Figure 5
Northern blot analyses of inflammatory markers in the liver. Hepatic RNA isolated from *P. chabaudi*-infected vaccinated (+ vacc) or non-vaccinated (-vacc) mice on the days indicated was hybridized to probes recognizing all isoforms of SAA, CRP, or CXCLI10 before reprobing with an 18S rRNA-specific probe. Blots are representative of at least three independent experiments.

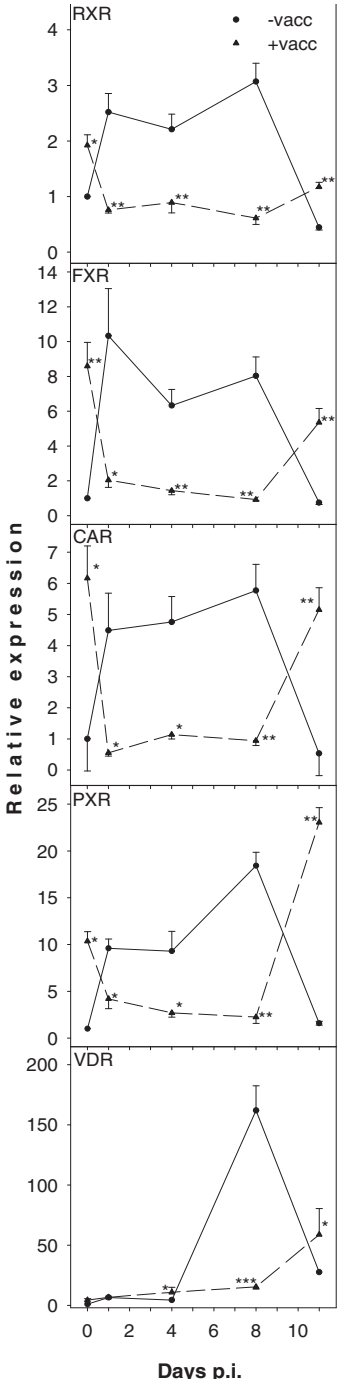

Figure 6
Real-time RT-PCR analyses of nuclear receptor expression in the liver. Levels of RXR, FXR, CAR, PXR, and VDR mRNA were analysed in hepatic RNA of vaccinated (circles) and non-vaccinated (triangles) mice on the indicated days after *P. chabaudi* infection. Signals were normalized to 18S rRNA and relative expression is given as fold increase compared to non-vaccinated mice on day 0 p.i. All values are mean and half S.E.M. **, $p < 0.01$ vs. non-vaccinated control on the same day p.i. *, $p < 0.05$ vs. non-vaccinated control on the same day p.i.

Rapid destruction of pRBC is expected to lead to highly elevated levels of cholesterol-derived toxic bile acids and haem-derived bilirubin. Since CAR is involved in regulation of both bile acid detoxification [36] and bilirubin elimination [37], CAR activity during malaria is of particular interest. Expression of the positive and negative CAR response genes Sult2a and Cyp7a1 involved in bile acid detoxification and cholesterol degradation to bile acids, respectively, were measured by real-time RT-PCR. These analyses confirmed suppression of Sult2a mRNA levels by blood stage malaria in BALB/c mice on days 8 and 11 p.i. (Figure 7A) as previously shown in C57BL/6 and LTβR$^{-/-}$ mice [13,38]. Vaccination, however, increased Sult2a1 mRNA levels by approximately two- and tenfold on days 0 and 8 p.i., respectively (Figure 7A). Cyp7a1 was severely down-regulated on days 8 and 11 p.i., whereas vaccination already induced expression on day 0 p.i. In contrast to Sult2a1, however, infection strongly intensified Cyp7a1 down-regulation in vaccinated mice (Figure 7A).

In order to test directly, whether elevated CAR mRNA levels on day 8 p.i. correspond to higher hepatic receptor activity, mice were challenged with TCPOBOP, a nuclear receptor ligand known to up-regulate Sult2a [39] and to down-regulate Cyp7a1 [40] via activation of CAR. Figure 7B clearly shows that treatment with TCPOBOP approximately doubles Sult2a1 mRNA levels in both vaccinated and non-vaccinated mice on day 0 p.i. Surprisingly, non-vaccinated mice on day 8 p.i. did not show any TCPOBOP-inducibility, but rather displayed TCPOBOP-induced suppression of Sult2a1 levels. This suppression is not observable in vaccinated mice, which maintained inducibility of Sult2a1 by TCPOBOP (Figure 7B), although they exhibited lower expression of the TCPOBOP receptor CAR (Figure 6). In order to substantiate these very unusual results, these experiments were not only confirmed three times with new groups of mice but also using Northern blotting (Figure 7C) to exclude any errors due to quantification by PCR.

TCPOBOP-regulation of Cyp7a1 expression revealed an exactly reciprocal effect. As expected, TCPOBOP suppressed expression of Cyp7a1 in non-vaccinated mice on days 0 and 8 p.i. and in vaccinated mice on day 0 p.i. (Figure 7B). In contrast, however, Cyp7a1 mRNA levels were elevated in vaccinated mice on day 8 p.i. after treatment with TCPOBOP. Therefore, negative regulation of Cyp7a1 was restricted to non-vaccinated mice with high CAR mRNA levels, while positive regulation of Sult2a by TCPOBOP was only observable in vaccinated mice with low CAR mRNA levels.

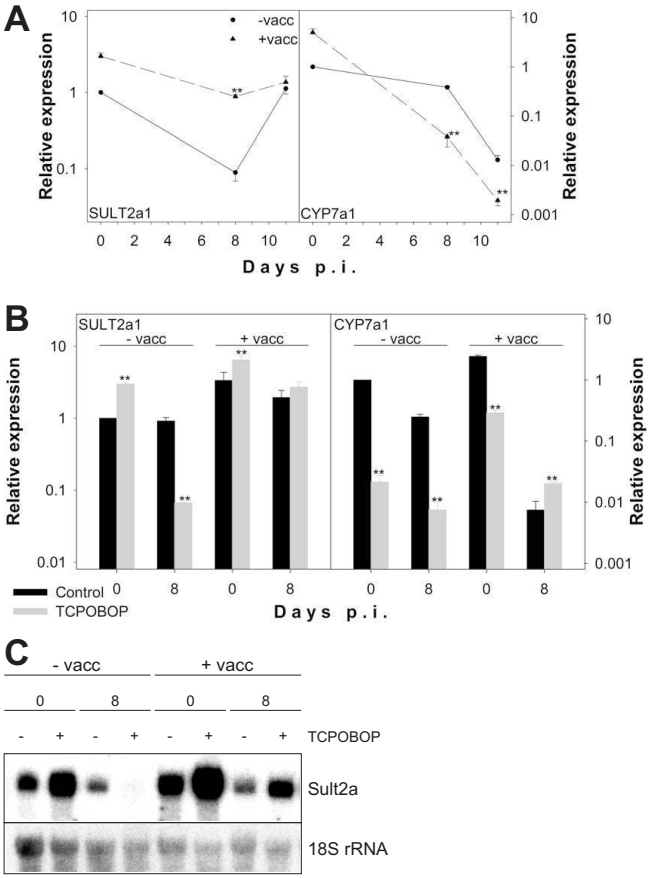

Figure 7
Effects of vaccination on malaria-induced suppression of SULT2a and CYP7a1. (A) Analysis of hepatic SULT2a1 and CYP7a1 expression on the indicated days of *P. chabaudi* malaria in vaccinated (triangles) and non-vaccinated (circles) mice by real-time RT-PCR. Signals were normalized to 18S rRNA signals and relative expression is given as fold increase compared to non-vaccinated mice on day 0 p.i. All values are means and half S.E.M. (B) Analysis of TCPOBOP effects on expression of SULT2a1 and CYP7a1. Vaccinated (+ vacc) or non-vaccinated (- vacc) mice were subjected to injection of TCPOBOP (grey columns) or vehicle (black columns) before analysis of SULT2a1 and CYP7a1 expression on the indicated days of *P. chabaudi* infection using real-time RT-PCR as in (A). (C) TCPOBOP effects on expression of SULT2a were confirmed by Northern blotting. Blot is a representative of three independent experiments. Equal loading of the blot was confirmed by rehybridization with a probe specific for 18S rRNA.

Discussion

The vaccination model under investigation here protects mice against blood stages of *P. chabaudi* malaria. It does not prevent parasitaemia, though peak parasitaemia is significantly decreased, but rather helps mice to overcome the disease. Indeed, vaccination converts non-healer to self-healer mice [4]. Self-healing of *P. chabaudi* malaria in turn is known to be associated with acquiring long-lasting protective immunity against homologous re-challenge [41]. This capability is normally controlled by genes of the

H-2 complex and genes of the non-*H-2* background [42-44]. The susceptible BALB/c mice exhibit the malaria-'resistant' *H-2*^{d} haplotype, but a malaria-'susceptible' non-*H-2* background [44]. Hence, the vaccination procedure used here abolishes/overcomes those genetic restrictions which cause susceptibility and which are controlled by mouse non-*H-2* genes.

Vaccination of BALB/c mice appears to improve the potential phagocytic capacity of the spleen as indicated by a significant increase in the percent proportion of both G1$^+$ granulocytes and F4/80$^+$ macrophages as well as the concomitant increase in spleen size. Nevertheless, this did not contribute to an increased trapping capacity since the spleen became almost 'closed' during peak parasitaemia and subsequent crisis, when masses of pRBC were destroyed as evidenced by dramatically falling parasitaemias [25]. In contrast to spleen, however, trapping capacity of the liver is augmented after vaccination, especially during crisis. This augmentation is preceded by a remarkable increase in the *P. chabaudi*-induced production of IFN-γ-mRNA. In accordance, IFN-γ is known to activate the host defence in other malaria vaccination models too [45,46]. This supports the view that the liver can function as an active effector against malarial blood stages, in particular during crisis, when the spleen is 'closed' [20], i.e. when the spleen excludes the uptake of pRBC and, thus, cannot be mechanically involved in the drop of peripheral parasitaemia observed during crisis.

In the liver, the Kupffer cells are important sites for phagocytosis of damaged and senescent erythrocytes [15,16], and phagocytic activity of Kupffer cells during *Plasmodium berghei* malaria has been described to be increased both in vitro [18] and in perfused livers [17]. The vaccination procedure used here is shown to modulate hepatic inflammation as indicated by altered responses of typical macrophage activation parameters towards to *P. chabaudi* malaria. Indeed, *P. chabaudi* infections induce dramatic up-regulations of IL-1β, TNF, IL-6, and iNOS in the non-vaccinated susceptible BALB/c mice. High levels of TNF have been shown to result in lethal hepatic damage during blood stage *P. chabaudi* malaria [47]. Indeed, the increases in TNF and IL-6 are even about 10-times higher than during lethal endotoxic shock [48]. Also, iNOS expression observed at peak parasitaemia is comparable to that expression occurring during LPS-induced lethal shock [49]. These high levels of proinflammatory cytokines and especially iNOS appear to contribute to severe liver failure, which entails lower particle trapping during crisis. In the vaccination model used here, however, there is a significant dampening of the infection-induced increase in IL-1β, TNF, IL-6 and iNOS, especially observable at peak parasitaemia. This indicates that vaccination does not prevent activation of Kupffer cells and other inflammatory cells by malaria infection but rather prevents their over-activation and, hence, severe liver failure. In this context, it is also noteworthy that high levels of IL-1β and TNF prevent efficient phagocytosis by Kupffer cells, reasonably due to impaired hepatic microvascular blood flow by promoting leukocyte adhesion to the walls of sinusoids [50]. Thus, vaccination-induced decreases in the expression of these cytokines, together with the decreased production of the chemoattractant CXCL10, presumably allow improved blood circulation in the liver and enhance contact between Kupffer cells and circulating material thus favouring increased particle trapping in the liver during crisis.

Conspicuously, mRNA levels of iNOS correlate well with those of VDR in both non-vaccinated and vaccinated mice. The mRNAs of iNOS and VDR were strongly up-regulated by *P. chabaudi* infection during crisis on days 8 and 11 p.i., and vaccination largely prevented this effect. In this context, recent findings are noteworthy that VDR signalling is able to inhibit IFN-γ-induced expression of macrophage activation markers including CXCL10 [51] and mitigates production of toxic nitric oxide by inducing arginase expression which lowers the intracellular pool of the iNOS substrate arginine [52]. In non-vaccinated mice, induction of arginase is presumably only of minor importance since iNOS induction is more than 100-fold stronger than induction of arginase. The up-regulation of VDR by infection in non-vaccinated mice may thus represent an insufficient counter-regulatory response to protect liver tissue from excessive damages due to extreme up-regulation of iNOS producing high NO levels. In vaccinated mice, VDR up-regulation appears to be dispensable since there is only minor induction of iNOS. Although vaccination strongly down-regulates markers for classically activated M1 macrophages such as TNF and iNOS, it does not apparently result in differentiation of macrophages to an alternatively activated M2 phenotype, since arginase as marker for alternatively activated macrophages [53] is even down-regulated by vaccination.

The vaccination-induced attenuation in the inflammatory response of the Kupffer cells appears to have modulatory effects on hepatocyte-based reactions involved in the defence against blood stages of malaria. Indeed, data indicate that vaccination diminishes the infection-induced acute phase response with respect to the CRP- and SAA3-proteins. Also, vaccination affects the infection-induced metabolic response with respect to SULT2A1 and CYP7A1, respectively. The phase I enzyme CYP7A1 is involved in cholesterol degradation/bile acid biosynthesis and the phase II enzyme SULT2A1 in the detoxification of bile acids, the latter majorly derived from cholesterol released during destruction of pRBC. At peak parasitaemia, the vaccination-induced elevated levels of SULT2A

and decreased levels of CYP7A1 may be, therefore, part of a protective response, which diminishes liver damages due to high levels of toxic bile acids.

Liver metabolism including phase I and II detoxifications are not only regulated by CAR, but also by other nuclear receptors such as FXR, PXR, and RXR. Here, it is shown that these nuclear receptors are also responding to both challenge-infection and vaccination due to cytokines released by Kupffer cells. Conspicuously, the nuclear receptors PXR, FXR, RXR, CAR, and VDR are significantly up-regulated during crisis after vaccination, while non-vaccinated mice exhibit a down-regulation. However, the results concerning CAR activation with TCPOBOP show that mRNA levels of nuclear receptors are not predictive for hepatic metabolic capacity during blood stage malaria. The expression of SULT2A and CYP7A1 is known to respond positively and negatively to CAR activation, respectively. Direct testing of CAR receptor function by treating non-vaccinated mice with TCPOBOP revealed that both *P. chabaudi* infection and vaccination altered CAR functionality on day 8 p.i. In non-vaccinated mice, Sult2a was no longer up- but even down-regulated by TCPOBOP on day 8 p.i. Vaccination prevented Sult2a down-regulation and retained its inducibility by TCPOBOP. Surprisingly, however, vaccination also abolished TCPOBOP-mediated down-regulation of Cyp7a1 on day 8 p.i. and even caused induction of Cyp7a1 by TCPOBOP. At peak parasitaemia, vaccination apparently promotes up-regulation of gene expression by CAR and, concomitantly, interferes with down-regulation. The simplest explanation for this complex response pattern is that CAR interacts with different cofactors, which determine whether it acts as a transcriptional activator or repressor. Characterization of such cofactors in the future will help to identify pathways leading to liver dysfunction in malaria and thus provide important information for prophylactic and therapeutic interventions.

Conclusion

The improvement of hepatic trapping and metabolic capacity in protectively vaccinated mice support the previous view that the liver has an effector function against blood stage malaria – at least during acute infection. This effector function can be strengthened by vaccination due to dampening the production of the proinflammatory cytokines IL-1β, TNF, and IL-6 by Kupffer cells. Moreover, the data presented here suggest that the efficacy of a human anti-malaria vaccine can be improved by including anti-inflammatory components protecting the liver from overwhelming inflammatory responses.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JK, HM, and FW designed the study. JK and FW planned and supervised all experiments and drafted the manuscript. DD and HP performed real-time RT-PCR analyses, AW did Northern blot experiments, MEK and MD did particle trapping experiments. All authors were involved in revising the manuscript and approved the final version.

Additional material

Additional file 1

Additional figure. *Diagram showing blood glucose level, anaemia, and percentage of reticulocytes in the blood between vaccinated and non-vaccinated mice.*

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3. General discussion

3.1 Relevance of ′environmental factors′ for the outcome of *Plasmodium chabaudi* malaria

Female mice are able to self-heal blood-stage malaria infections with *P. chabaudi*, which is under control by genes of the *H-2* complex and genes of the non-*H-2* background (Wunderlich *et al*. 1988b, 1991). These gene-controlled mechanisms of resistance do not become evident in male mice, which are highly susceptible and always succumb to malarial infections presumably due to the immunosuppressive activity of testosterone (T) (Seli & Arici 2002). Obviously, T superposes the efficacy of the resistance-mediating genes and ultimately leads to a suppression of the development of protective immunity.

T is known to exert its functions on gene expression through the classical intracellular androgen receptor (AR), a member of the nuclear receptor superfamily (Evans 1988, Chen 2008). However, the suppressive effect of T on the development of protective immunity in *P. chabaudi*-infected mice is mediated neither through the classical AR (Benten *et al.* 1992a) nor – after conversion of T to estradiol – through the intracellular estrogen receptor (Benten *et al*. 1993). Remarkably, T is also able to act through non-genomic mechanisms (Benten *et al*. 2004, Rahman & Christian 2007), as other steroids too. Such ′non-genomic′ T signaling has been shown in different immune cells such as splenic T cells and macrophages of mice. This becomes evident as increases in intracellular free Ca^{2+} concentrations (Benten *et al*. 1997, 1999 a, b, Wunderlich *et al.* 2002), which cross-talk with gene expression induced by other signaling pathways (Guo *et al*. 2002).

This study also emphasizes that T acts through AR-independent non-genotropic mechanisms because the majority of protein-encoding target genes and miRNA-target genes, we have identified in the liver after T-treatment, do not contain androgen-response elements in their promoter regions. Moreover, it is possible that still unknown non-genotropic mechanisms are responsible for the T effect, which may even operate following a primarily direct genotropic T action and which may be parts of larger regulatory circuits, as e. g. the hypothalamus-pituitary-gland-liver axis (Gustafsson 2005, Waxman & Holloway 2009). Possibly, T induces changes in the epigenome because steroid hormones are known to induce

long-lasting chromatin remodeling through epigenetic mechanisms, as e. g. DNA methylation and/or covalent modifications of histones (Grunstein 1997, Goldberg *et al*. 2007, Waxman & Holloway 2009). Such epigenetic changes associated with a reprogramming of gene expression can reasonably explain that the suppressive action of T causing lethal malaria is not transient, but rather persists for a rather long time, even after withdrawal of T for 12 weeks (Delić *et al*. 2010a).

Moreover, this study describes two other new endogenous ′environmental factors′, namely taurine and IL-6 trans-signaling, which are involved in the outcome of *Plasmodium chabaudi* malaria. Taurine has been previously identified as an important organic osmolyte in liver parenchymal, endothelial Kupffer and stellate cells with impact for immune functions such as phagocytosis and cytokine production (Warskulat *et al*. 1997, Weik *et al*. 1998, Peters-Regehr *et al*. 1999). A reduction of about 50% of the circulating taurine is found after deletion of the *taut* gene, which encodes the taurine transporter (TAUT), and these decreased levels are even lowered another 25% at peak parasitemia suggesting that *P. chabaudi*-induced lethal outcome in *taut*$^{-/-}$ mice is related to taurine deficiency. It has to be emphasized that the deletion of *taut* is not lethal *per se*, but rather its consequences, i. e. lowered taurine levels and a breakdown of taurine homeostasis.

The cytokine IL-6 has been demonstrated to correlate with severity of human malaria infections (Kern *et al*. 1989, Jason *et al*. 2001, Lyke *et al*. 2004), but its action is not yet really understood. Here, we show that the lethal effect of IL-6 is exerted through uncontrolled IL-6 trans-signaling. Disruption of IL-6 trans-signaling protects from malaria-induced lethality as evidenced by an approximately 50% survival of IL-6Rα$^{-/-}$ mice of otherwise self-healing malaria. In corresponding control mice, which still carry the IL-6Rα gene (IL-6Rα$^{FL/FL}$), sIL-6Rα levels are increased during an infection with *P. chabaudi*. Injection of sIL-6Rα in IL-6Rα$^{-/-}$ mice induces IL-6 trans-signaling, which results in a lethal outcome to malaria. On the other hand, when IL-6 trans-signaling is reduced in IL-6Rα$^{FL/FL}$ mice by injecting sGP130, 40% of mice survive the infection. These results are consistent with reports indicating that sIL-6Rα levels directly correlate with severity of human malaria caused by *P. falciparum* and *P. vivax* (Wenisch *et al*. 1999). On the base of these findings, novel interventions may become feasible for treatment of human malaria in future.

3.2 The liver as a tastosterone-target and anti-malaria effector site

The liver is known as a lymphoid organ generating both innate and adaptive immune reactions (Häussinger *et al*. 2004, Crispe 2009) and as to play a central role in malaria. Indeed, the liver is not only that site in which the pre-erythrocytic development of malaria parasites takes place (Mehlhorn 2008), but also appears to be an important effector against malarial blood-stages (Balmer *et al*. 2000, Krücken *et al*. 2005). For instance, specific populations of lymphocytes have been described to be generated in the liver, which mediate novel protective immune mechanisms against malaria blood-stages in the mouse (Mannoor *et al*. 2001, 2002). Also, activated Kupffer cells are apparently able to eliminate, *via* phagocytosis, parasite-derived residual material and even *Plasmodium*-infected erythrocytes (Taverne *et al*. 1987, Delić *et al.* 2010b) as already recognized by Aikawa *et al*. as early as (1980). Moreover, the importance of the liver as a prominent effector site against blood-stage malaria has been recently also demonstrated in lymphotoxin β receptor-deficient mice (Wunderlich *et al*. 2005). These mice are devoid of secondary lymphoid tissues and their spleen is defect (Fütterer *et al*. 1998, Fu & Chaplin 1999), but they are rather resistant to malaria, i. e. they are able to self-heal blood-stage infections with *P. chabaudi* (Wunderlich *et al*. 2005).

The liver is also known as a T-target organ, in particular its sexual dimorphism with its sex- and T-dependent pattern of gene expression involved in liver metabolism (Gustafsson 2005, Waxman & Holloway 2009). Thus, male and female mice express genes differently including those which are involved in phase I and phase II metabolism. Also, the male pattern of gene expression of the liver can be altered by lowering T levels, as e. g. by castration, and this pattern can be restored by T replacement (Kato & Onada 1970). First hints for short-term T effects on hepatic gene expression have been previously shown for 9 genes out of 299 genes tested, as e. g. for genes encoding plasminogen activator inhibitor (PAI1) and sulfotransferase 2A2 (SULT2A2) are deregulated by T (Krücken *et al*. 2005).

The data presented in this thesis suggest that T dysregulates the liver function which may impair its differentiation to an anti-malaria effector. Indeed, this view is supported by the following findings:

Firstly, T-induced conversion of mice from resistance to susceptibility is persistent even after 12 weeks of T treatment, which is associated with T-induced reprogramming of hepatic gene expression. Using Affymerix microarray technology, 54 genes have been identified to be

persistently deregulated by T. The majority of these genes show a strong persistent pattern of ′masculinization′ of the phase I-III liver metabolism evidenced as a persistent T-induced down-regulation of some female-prevalent genes such as *Cyp2b9, Cyp2b13, Cyp3a41, Cyp3a44, Fmo3, Sult2a2, Sult3a1*, and *BC014805*, and an up-regulation of some male-prevalent genes such as *Cyp2d9, Cyp7b1, Hsd3b5, Ugt2b1, Ugt2b38,* and *Slco1a1,* respectively (Delić *et al*. 2010a). This T-induced reprogramming of genes involved in liver metabolism is presumably androgen receptor (AR) - and nuclear receptor-independent, since the promoters of the reprogrammed genes do not contain any androgen response elements (ARE). The expression of genes involved in immune responses such as *Ifnγ* and *Igk-C* is significantly increased and decreased, respectively, by T. IFNγ is required for the activation of the T_H1-response. Currently, the view predominates that protective immunity to *P. chabaudi* malaria is mediated by antibodies (Achtman *et al*. 2005, 2007) requiring the activation by the T_H2-response preceded an IFN-γ-dependent activation of the T_H1-response. The observed T-induced persistent upregulation of IFN-γ may therefore reflect an over-activation of the T_H1-response thus impairing the switch to the T_H2-response. This scenario is further corroborated by previous findings shown that T suppresses the production of biologically active IL-10 (Mossmann *et al*. 1997), which normally promotes the T_H2-response.

Secondly, acquisition of protective immunity against *P. chabaudi* coincides with an acquired unresponsiveness to T. The investigation of the liver transcriptome of immune mice compared to that of naïve mice after 3 weeks of T treatment shows an attenuated responsiveness to T of hepatic gene expression. It is important to note that the T_H2-promoting *Ehmt2* is downregulated by T only in naïve mice, but not in immune mice. In particular, it is remarkable that the attenuated responsiveness to T of hepatic gene expression coincides with a 5-fold higher expression of that gene encoding the T-metabolizing HSD3B5 in immune mice than in naïve mice, thus possibly leading to lower concentrations of effective T in the liver of immune mice.

Thirdly, recent reports describe profound and unexpected roles of miRNAs in the control of diverse aspects of the liver, including hepatocyte growth, stress response, metabolism, infection, proliferation, gene expression, and maintenance of hepatic phenotype (Bala *et al*. 2009, Chen 2009, Kerr & Davidson 2009, Wang *et al*. 2009). The present study describes first hints suggesting that hepatic miRNAs play a central role for the T-induced lethal outcome to malaria. T induces the expression of the 6 miRNA-species miR-22, miR-690,

miR-122, let-7A, miR-30D, and let-7D, respectively. This T-induced deregulation is widely independent of the AR because *in silico* analyses have revealed an ARE only for miR-122. Since the main action of miRNAs occurs at the post-transcriptional level during the translation process, it is possible that there may be even more effects at the proteome level. Furthermore, there is circumstantial evidence that miRNAs are implicated in an intricate network with the epigenetic machinery. Although we have not detected any persistently deregulated miRNAs after 12 weeks of T withdrawal, it cannot be excluded at the present state of knowledge that the T-deregulated miRNAs are involved in the persistence of T-induced susceptibility to malaria: Once induced, they are able to influence the epigenome, thus inducing persistently deregulated expression of protein-encoding genes. Future work is required to unravel the regulatory circuits of miRNAs described here to be involved in the outcome of *P. chabaudi* malaria.

Fourthly, the liver is found to respond to primary *P. chabaudi* infections with an upregulation of the miRNA-species miR-26b, MCMV-miR-M23-1-5p, and miR-1274a, and a downregulation of the 16 miRNA-species miR-101b, let-7a, let-7g, miR-193a-3p, miR-192, miR-142-5p, miR-465d, miR-677, miR-98, miR-694, miR-374*, miR-450b-5p, miR-464, miR-377, miR-20a*, and miR466d-3p, respectively. Surprisingly, about the same pattern is even sustained in mice having acquired protective immunity and even upon homolog re-infections of such immune mice. The development of protective immunity against malarial blood-stages of *P. chabaudi* correlates with a robust reprogramming of distinct miRNA-species in the female mouse liver. Collectively, these data exhibit a novel regulatory role of hepatic miRNAs in the development of protective immunity to malaria.

3.3 Vaccination against malaria

The concept of a malaria vaccine has sparked great interest for decades, but all efforts to develop an effective malaria vaccine have failed to date. This thesis has also conducted vaccination studies with the *P. chabaudi* model using a previously developed procedure in mice of the inbred strain B10.A (Wunderlich *et al*. 1988b). This procedure uses surface membranes isolated in forms of ghosts from *P. chabaudi*-infected erythrocytes, which contain several parasite-encoded proteins, with Pc90 as the most prominent parasite protein (Wunderlich *et al*. 1988a). This vaccination technique protects Balb/c mice from a lethal outcome to *P. chabaudi* malaria. In the non-vaccinated susceptible Balb/c mice, *P. chabaudi*

infections induce extreme upregulations of inflammatory markers such as IL-1β, IL-6, TNFα, and iNOS. In accordance, high levels of TNFα have been recently found to be associated with lethal hepatic damage during blood-stage malaria (Seixas *et al*. 2008). Upon vaccination, these inflammatory parameters become downregulated and are not as strongly inducible by *P. chabaudi* infections as in non-vaccinated mice. Conspicuously, the protective vaccination is associated with a dampening of the hepatic inflammatory response and an attenuated uptake of injected particles by the liver.

Obviously, the vaccination procedure used here overcomes those restrictions which cause susceptibility. These restrictions encompass both genetic predispositions as well as the described ′environmental factors′ such as T, taurine, and IL-6 trans-signaling. Our data reveal that the vaccination leads to an activation and development of protective mechanisms, which apparently correlate with diminished pathological consequences. In particular, vaccination protects the liver from pathological alterations induced by malarial infections. Collectively, the data emphasize that it should be feasible to develop an effective anti-malaria vaccine for humans, possibly by including liver-protecting substances.

4. Summary

Malaria is still a major health problem with 243 million cases of malaria and 863,000 deaths alone in 2008. Malaria morbidity and mortality are presumably due to not yet really understood dysregulations of the host responses induced by the malaria parasites. Using the mouse malaria model *Plasmodium chabaudi*, this thesis provides novel results, which contribute to our still poor understanding of the control of resistance/susceptibility to blood-stage malaria.

Firstly, decreased levels of circulating taurine, achieved by deletion of the taurine transporter gene, result in perturbed taurine homeostasis, which causes lethal outcome of otherwise self-healing blood-stage malaria. Secondly, testosterone, which suppresses self-healing of malaria, upregulates miRNA expression in the female mouse liver. Thirdly, the testosterone-induced persistent lethal effect on malaria is associated with changes in hepatic gene expression such as a masculinization of liver metabolism and an impaired antibody response. Fourthly, the hepatic miRNA-signature becomes apparently reprogrammed upon acquisition of protective immunity against malaria. Fifthly, acquiring testosterone-unresponsive, IgG-antibody-mediated protective immunity to blood-stage malaria is associated with adaptive persistent changes in the liver resulting in an attenuated testosterone responsiveness of gene expression. Sixthly, disruption of IL-6 trans-signaling augments ability of mice to self-heal otherwise lethal malaria. Seventhly, protective vaccination against blood-stage malaria overcomes restrictions imposed to self-healing such as genetic predispositions and endogenous environmental factors. Indeed, vaccination attenuates malaria-induced inflammatory responses thus improving metabolic activity and particle trapping capacity of the liver.

Collectively, the present data indicate (i) the importance of endogenous environmental factors such as testosterone, taurine and IL-6 trans-signaling for the outcome of malaria, and (ii) the importance of the liver as an anti-malaria effector site, which is more important than hitherto assumed.

5. Zusammenfassung

Malaria ist immer noch eines der größten Gesundheitsprobleme mit 243 Millionen gezählten Fällen und 863.000 Toten allein im Jahre 2008. Die Malaria-Morbidität und -Mortalität basiert wahrscheinlich auf noch nicht wirklich verstandenen Dysregulationen der Wirtsantworten, welche durch die Malaria-Erreger induziert werden. Die vorgelegte Arbeit hat an dem murinen Malaria-Modell *Plasmodium chabaudi* neue Ergebnisse erzielt, die zum bislang noch dürftigen Verständnis von Resistenz bzw. Suszeptibilität gegenüber Infektionen mit Blutstadien der Malaria-Erreger beitragen.

Erstens, erniedrigte Taurin-Level, die durch eine Deletion des Taurintransporter-Gens entstanden sind, resultieren in einer gestörten Taurin-Homöostase, welche wiederum zu einem letalen Ausgang einer andernfalls selbst-heilenden Infektion führt. Zweitens, Testosteron, welches eine Selbst-heilung der Malaria supprimiert, induziert die Expression bestimmter miRNA-Spezies in der Leber weiblicher Mäuse. Drittens, der Testosteron-induzierte letale Effekt auf eine Malariainfektion ist assoziiert mit Veränderungen in der hepatischen Genexpression, die sich u. a. in einem ′maskulinisierten′ Lebermetabolismus und einer verzögerten Immunantwort widerspiegeln. Viertens, die hepatische miRNA-Signatur wird offensichtlich nach Erwerb protektiver Immunität gegen Malaria reprogrammiert. Fünftens, die durch Testosteron nicht beeinflussbare erworbene IgG-Antikörper-vermittelte protektive Immunität gegen Malaria ist mit adaptiven, persistenten Veränderungen in der Leber assoziiert, die in einer abgeschwächten Testosteron-empfindlichen Genexpression resultiert. Sechstens, eine Unterdrückung des IL-6 ′trans-signaling′ erhöht die Fähigkeit der Mäuse eine andernfalls letale Infektion mit dem Malariaerreger zu selbst-heilen. Siebtens, protektive Vakzinierung gegen Malaria überkommt Restriktionen, die normalerweise eine Selbst-heilung unterdrücken, und die durch genetische Faktoren, aber auch durch endogene Faktoren wie Testosteron, Taurin und IL-6 ′trans-signaling′ verursacht werden. Eine Vakzinierung schwächt die Malaria-induzierte inflammatorische Antwort der Leber ab, so dass die Stoffwechselaktivität und die ′Trapping′-Kapazität der Leber verbessert werden.

Zusammenfassend zeigen die hier präsentierten Daten (i) die Bedeutung von endogenen Faktoren Testosteron, Taurin und IL-6 ′trans-Signaling′ für den Ausgang einer Malariainfektion, und (ii) die Bedeutung der Leber als Anti-Malaria-Effektor, welche offenbar wichtiger ist als bisher angenommen.

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7. Anteilserklärung

1. Publikation:

Delić D, Warskulat U, Borsch E, Al-Qahtani S, Al-Quraishi S, Häussinger D & Wunderlich F 2010 Loss of ability to self-heal malaria upon taurine transporter deletion. *Infection and Immunity* **78** 1642-1649.

Herr Denis Delić war für einen großen Teil der praktischen Experimente verantwortlich und trug maßgeblich zur Manuskriptherstellung bei. Beitrag: 40%

2. Publikation:

Delić D, Grosser C, Dkhil M, Al-Quraishy S & Wunderlich F 2010 Testosterone-induced upregulation of miRNAs in the female mouse liver. *Steroids* **75** 998-1004.

Herr Denis Delić war für das experimentelle Design und für die Durchführung der praktischen Experimente verantwortlich. Die Auswertung der Versuche führte er vollständig unabhängig durch und war entscheidend an der Manuskriptherstellung beteiligt. Beitrag: 80%

3. Publikation:

Delić D, Gailus N, Vohr HW, Dkhil M, Al-Quraishi S & Wunderlich F 2010 Testosterone-induced permanent changes of hepatic gene expression in female mice sustained during *Plasmodium chabaudi* malaria infection. *Journal of Molecular Endocrinology* **45** 379-390.

Herr Denis Delić war für das experimentelle Design, für die Auswertung sämtlicher Analysen und bei der Entstehung der Publikation entscheidend beteiligt. Beitrag: 75%

4. Publikation:

Delić D, Dkhil M, Al-Quraishi S & Wunderlich F 2010 Hepatic miRNA expression reprogrammed by *Plasmodium chabaudi* malaria. *Parasitology Research* [Epub ahead of print].

Herr Denis Delić führte den größten Teil der praktischen Experimente und deren Auswertung durch. Ferner trug er entscheidend bei der Manuskriptherstellung bei. Beitrag: 80%

5. Publikation:

Delić D, Dkhil M, Al-Quraishi S & Wunderlich F 2010 Attenuated responiveness to testosterone of hepatic gene expression in female mice immune *Plasmodium chabaudi* malaria. *Journal of Molecular Endocrinology* [submitted]

Herr Denis Delić war für das experimentelle Design und der damit verbundenen Durchführung der Experimente und Analysen verantwortlich. Desweiteren war er bei der Entstehung der Publikation entscheidend beteiligt. Beitrag: 80%

6. Publikation:

Wunderlich CM, **Delić D**, Behnke K, Stroehle P, Wunderlich F, Brüning J & Wunderlich FT 2010 Disrupted IL-6 trans-signaling augments ability to self-heal malaria [prepared to submit]

Herr Denis Delić führte die Arbeiten mit Malaria (Infektionsversuche und ELISA) durch und war an der Manuskriptherstellung beteiligt. Beitrag: 40%

7. Publikation:

Krücken J, **Delić D**, Pauen H, Wojtalla A, El-Khadragy M, Dkhil MA, Mossmann H & Wunderlich F 2009 Augmented particle trapping and attenuated inflammation in the liver by protective vaccination against *Plasmodium chabaudi* malaria. *Malaria Journal* **8** 54.

Herr Denis Delić führte einen großen Teil der qRT-PCR- und Northern blot-Analysen durch und beteiligte sich an der Diskussion bei der Entstehung der Publikation. Beitrag: 25%

_____ _____

Denis Delić Prof. Dr. Frank Wunderlich

8. Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertation mit dem Titel ′Resistance/Susceptibility to *Plasmodium chabaudi* malaria′ selbstständig verfasst und keine anderen als die angegebenen Hilfsmittel und Quellen verwendet habe.

Tag der Abgabe: 30.11.2010 Denis Delić

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10. Lebenslauf

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Academic Education

2001-2006	Study of Biology at the Heinrich-Heine-Universität Düsseldorf Main subject*: Molecular biology* Degree: Diplom-Biologist (M. Sc.), predicate ′excellent′ Diploma thesis: ′The influence of the *E. coli* protein DksA on the synthesis of ribosomal RNAs under conditions of the stringent control′
2006-2010	PhD Thesis at the division of *Molecular Parasitology* at the Heinrich-Heine-Universität Düsseldorf; Supervisor: Prof. Dr. Frank Wunderlich Theme of the PhD Thesis: "Resistance/Susceptibility to *Plasmodium chabaudi* malaria"
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