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 *Ifn γ* 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,

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 Salzufflen, 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 10⁶ *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 ($P < 0.01$). 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. Hierarchical 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 (*Ifn γ*), peroxisome proliferator-activated receptor alpha (*Ppar α*), liver X receptor (*Lxr*, listed as *Nr1h3* in the MGI database), retinoid X receptor alpha (*Rxr α*), pregnane X receptor (*Pxr*, *Nr1i2*), farnesoid X receptor (*Fxr*, *Nr1h4*), *Ar*, estrogen receptor alpha (*ER α* , *Esr1*), estrogen receptor beta (*ER β* , *Esr2*), aryl hydrocarbon receptor (*Ahr*), hepatic nuclear factor 4 alpha (*Hnf4 α*), 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 ($P < 0.05$).

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 lose 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 IFN γ

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

Gene symbol	Gene name	T ₄₀ /C ₄₀	T ₄₀ /C ₄₈	Affymetrix probe ID	Entrez gene	Representative public ID (A)	Functions	PMID
Phase I metabolism	<i>MGC225972</i>	390-20	42-70	1424352_at	277 753	BC025936	Decreased expression in HGF-knockout mice	17241389
	<i>Cyp2d9</i>	22-58	4-38	1419349_a_at	13 105	BC010593	Male-specific; regulated by GH; sterol 16 α -hydroxylase	16547391, 4074718
	<i>Cyp7b1</i>	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
	<i>Cyp7b1</i>	4-45	3-55	1421074_at	13 123	NM_007825		
	<i>Fmo2</i>	0-36	0-42	1422904_at	55 990	NM_018881	Typically expressed at high levels in lung	16872995
	<i>Cyp3a44</i>	0-10	0-20	1426064_at	337 924	AB039380	Female-specific expression depends on GH	12147261
	<i>Cyp2b9</i>	0-06	0-13	1419590_at	13 094	NM_010000	Female-specific expression depends on GH	15381067
	<i>Cyp3a41</i>	0-02	0-01	1419704_at	53 973	NM_017396	Female-specific isoenzyme	10775455
	<i>Fmo3</i>	0-01	0-02	1449525_at	14 262	NM_008030	Female-specific expression in the liver	7473608
Phase II metabolism	<i>Cyp2b13</i>	<0-01	0-01	1449479_at	13 089	NM_007813	Female-specific expression	15155787
	<i>Cml5</i>	9-51	24-01	1424811_at	69 049	BC024605	Putative N-acetyltransferase	
	<i>Ugt2b38</i>	9-47	3-06	1424934_at	71 773	BC027200	Male-predominant expression in the liver; inductive effects by testosterone	19131521
	<i>Cml4</i>	6-30	8-91	1419520_at	68 396	NM_023455	Putative N-acetyltransferase	
	<i>Ugt2b1</i>	1-76	2-08	1424934_at	71 773	BC027200	Male-predominant expression in the liver; inductive effects by testosterone	19131521
	<i>Sult3a1</i>	0-01	0-04	1421669_at	57 430	NM_020565	Female-specific expression	16807285
	<i>Sult2a2</i>	<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	<i>Elovl3</i>	40-51	6-61	1420722_at	12 686	BC016468	Circadian expression in the liver; cold induction in brown adipose tissue is controlled by PPAR α and crucial for accumulation of very long chain	17003504, 15855229
	<i>Vldlr</i>	0-21	0-30	1434465_x_at	22 359	AV333363	Involved in uptake of triglycerides	11453330
	<i>Vldlr</i>	0-17	0-22	1417900_a_at	22 359	NM_013703		
	1810022C23Rik	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 ₄₀ /C ₄₀	T ₄₀ /C ₄₈	Affymetrix probe ID	Entrez gene	Representative public ID (A)	Functions	PMID
<i>Hao3</i>	Hydroxyacid oxidase (glycolate oxidase) 3	0.02	0.05	1418654_at	56 185	NM_019545	Peroxisomal-2-hydroxy acid oxidase	10777549
General metabolism								
<i>Hsd3b5</i>	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
<i>Ela3b</i>	Elastase 3B, pancreatic	4.19	3.82	1415884_at	67 868	NM_026419	Protease of the exocrine pancreas	2826474
<i>Serpine2</i>	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 <i>Pat1</i> gene results in partial loss of the ability to control the course of <i>P. chabaudi</i> infections	15618182
4833442J19Rik	RIKEN cDNA 4833442J19 gene	2.80	2.29	1427202_at	320 204	AV002340	Highly conserved protein containing putative methyltransferase domain	
<i>Csad</i>	Cysteine sulfinic acid decarboxylase	2.37	2.74	1427981_a_at	246 277	AY033912	Rate-limiting enzyme in taurine biosynthesis	11997111
<i>Mcm10</i>	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
<i>Serpipb1a</i>	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
<i>Serpipb1a</i>	Serine (or cysteine) proteinase inhibitor, clade B, member 1a	0.36	0.41	1448301_s_at	66 222	AF426024		
<i>Nt5e</i>	5' nucleotidase, ecto	0.18	0.22	1422974_at	23 959	NM_011851	Reduced activity by ischemia	9306922
Carcinogenesis								
<i>Lama3</i>	Laminin, alpha 3	3.93	2.64	1427512_a_at	16 774	X84014	Expression correlated with dedifferentiation of hepatocellular tumors	12079511
<i>Nox4</i>	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
<i>Nox4</i>	NADPH oxidase 4	3.29	2.44	1451827_a_at	50 490	BC021378		
<i>Rad51l1</i>	RAD51-like 1 (S. cerevisiae)	0.24	0.55	1421430_at	19 363	NM_009014		
<i>Prom1</i>	Prominin 1	0.06	0.51	1419700_a_at	19 126	NM_008935	Involved in genetic instability	15723711
Immune response								
<i>Il1r1</i>	Interferon gamma	11.72	0.58	1425947_at	15 978	K00083	Marker for hematopoietic and endothelial progenitor cells	1569483
<i>C6</i>	Complement component 6	8.66	6.37	1449308_at	12 274	NM_016704	Plays a protective role during cerebral malaria	16088835
							Complement component produced in the liver; higher expression in males than in females	4007963
<i>Ck1f</i>	Chemokine-like factor	0.43	0.49	1436242_a_at	75 458	BE852312	Chemoattractant for leukocytes	11415443
<i>Tox</i>	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
<i>Lag3</i>	Lymphocyte activation gene 3	0.36	0.54	1449911_at	16 768	NM_008479	Negatively regulates T-cell proliferation	12421911
<i>Igk-C</i>	Immunoglobulin kappa chain variable 8 (V8)	0.22	0.52	1426200_at	16 071	AY058910		

(continued)

Table 1 Continued

Gene symbol	Gene name	T _{ad} /C _{ad}	T _{ad} /C _{as}	Affymetrix probe ID	Entrez gene	Representative public ID (A)	Functions	PMID
Signal transduction <i>Prlr</i>	Prolactin receptor	0.50	0.48	1425853_s_at	19 116	M22958	Treatment of female mice with testosterone reduces <i>Prlr</i> levels	6329976
<i>Prlr</i>	Prolactin receptor	0.45	0.52	1448556_at	19 116	BC005555		
<i>Dscr11l</i>	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 endothelial cells and inhibits angiogenesis calcineurin signaling pathways in	10756093
<i>Mmd2</i>	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
<i>Mmd2</i>	Monocyte to macrophage differentiation-associated 2	0.23	0.47	1438654_x_at	75 104	AV269411		
Transport <i>Slc01a1</i>	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
<i>Slc01a1</i>	Solute carrier organic anion transporter family, member 1a1	22.05	41.28	1449844_at	28 248	AB031813		
<i>Slc1a4</i>	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
D630002G06l C730048C13Rik	Hypothetical protein D630002G06l/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 BC014805	cDNA sequence AB056442 cDNA sequence BC014805	0.05 0.05	0.11 0.15	1419751_x_at 1425751_at	171 405 236 149	NM_134256 AJ132857	Organic anion transporter 6 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 <i>Omd</i>	Osteomodulin	17.26	2.55	1418745_at	27 047	NM_012050	Bone-specific extracellular matrix protein	10607915
<i>Susd4</i>	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	
<i>Clec2h</i>	C-type lectin domain family 2, member h	2.88	3.16	1451438_s_at	94 071	AF350410	Unknown	
<i>Artdc4</i>	Arrestin domain-containing protein 4	0.42	0.54	1426818_at	66 412	BC025091	Unknown	
<i>Pcp4l1</i>	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 SUSL4 remained upregulated, but at significantly lower levels after infection on day 8 p.i. By contrast, only the expression of *Ifn γ* was increased by testosterone on day 0 p.i. ($T_{d0}/C_{d0}=11.78$), but its upregulation appeared relatively suppressed on day 8 p.i. ($T_{d8}/C_{d8}=0.58$), 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 *Ifn γ* 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 RXR α , LXR α , and PPAR α 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 $\pm$ 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 ($P < 0.05$).

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 *Igh-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}
<i>Pparα</i>	Peroxisome proliferator-activated receptor alpha	0.76 ± 0.07	1.25 ± 0.18
<i>Lxrα</i> (<i>Nr1h3</i>)	Liver X receptor alpha	0.76 ± 0.06	0.72 ± 0.19
<i>Rxrα</i>	Retinoid X receptor alpha	0.53 ± 0.33	0.92 ± 0.33
<i>Pxr</i> (<i>Nr1i2</i>)	Pregnane X receptor	1.13 ± 0.12	1.01 ± 0.57
<i>Fxr</i> (<i>Nr1h4</i>)	Farnesoid X receptor	0.56 ± 0.42	0.98 ± 0.09
<i>Ar</i>	Androgen receptor	0.91 ± 0.22	1.15 ± 0.27
<i>ERα</i> (<i>Esr1</i>)	Estrogen receptor alpha	0.88 ± 0.22	1.19 ± 0.21
<i>ERβ</i> (<i>Esr2</i>)	Estrogen receptor beta	1.46 ± 0.33	0.95 ± 0.14
<i>Ahr</i>	Aryl hydrocarbon receptor	1.16 ± 0.16	1.09 ± 0.17
<i>Hnf4α</i>	Hepatic nuclear factor 4 alpha	1.28 ± 0.33	1.13 ± 0.38
<i>Car</i> (<i>Nr1i3</i>)	Constitutive androstane receptor	0.31 ± 0.06	0.99 ± 0.26
<i>Vdr</i>	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 *Ifn γ* which is upregulated by the end of the testosterone withdrawal period, but the subsequent malaria infection induces expression of *Ifn γ* 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 reprogramming 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 HNF4 α , 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 *Hnf4 α* in the female mouse liver. Also, any other transcription factors including common nuclear factors are apparently not affected by testosterone. Moreover, testosterone-induced reprogramming 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 IFN γ 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 IFN γ -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 *Ifn γ* 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 IFN γ 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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