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Testosterone-induced persistent susceptibility to *Plasmodium chabaudi* malaria: Long-term changes of lincRNA and mRNA expression in the spleen

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ABSTRACT

Testosterone (T) is known to induce persistent susceptibility to blood-stage malaria of *Plasmodium chabaudi* in otherwise resistant female C57BL/6 mice, which is associated with permanent changes in mRNA expression of the liver. Here, we investigate the spleen as the major effector against blood-stage malaria for any possible T-induced long-term effects on lincRNA and mRNA expression. Female C57BL/6 mice were treated with T for 3 weeks, then T was withdrawn for 12 weeks before challenging with *P. chabaudi*. LincRNA and mRNA expression was examined after 12 weeks of T-withdrawal and after subsequent infections using Agilent whole mouse genome oligo microarrays. Our data show for the first time long-term effects of T on lincRNA expression evidenced directly as persistent changes after T-withdrawal for 12 weeks and indirectly as altered responsiveness of expression to *P. chabaudi* infections. There are 3 lincRNA-species upregulated and 10 lincRNAs downregulated by more than 2-fold ($p < 0.01$). In addition, 11 and 10 mRNAs are persistently up- and downregulated by T, respectively. These changes remain not sustained during infections at peak parasitemia, when 15 other lincRNAs and 9 other mRNAs exhibit an altered expression. The only exception is the Tnk1-mRNA encoding the non-receptor tyrosine kinase 1 that is persistently downregulated by 0.34-fold after T-withdrawal and that becomes upregulated by 5.9-fold upon infection at peak parasitemia, suggesting an involvement of tyrosine phosphorylation by Tnk1 in mediating long-term effects of T in the spleen. The T-induced changes in splenic mRNA expression are totally different to those previously observed in the liver. Collectively, our data support the view that T induces long-term organ-specific changes in both lincRNA and mRNA expression, that presumably contribute to organ-specific dysfunctions upon infection with blood-stage malaria of *P. chabaudi*.

1. Introduction

Testosterone (T) is a critical player in the outcome of different diseases: T is known to exhibit immunosuppressive activity [1–3] and to increase susceptibility towards a wide variety of infectious diseases [4,5]. A convenient model to investigate this suppressive effect of T is *Plasmodium chabaudi* malaria in the mouse [6,7]. Indeed, T is able to induce a lethal outcome of otherwise self-healing blood-stage infections in female mice [8,9]. This T-induced susceptibility is persistent for rather a long time. When mice are treated with T for 3 weeks, and thereafter T-treatment is discontinued for 12 weeks, the circulating T levels decline again to those characteristic for untreated female mice, yet the mice still succumb to *P. chabaudi* infections [10]. Obviously, T is able to´imprinť changes in mice which continue to exist even at normal T levels.

To trace out such presumably complex T-induced long-term changes it is reasonable to begin with investigations at the level of gene expression in spleen and liver as the major effector organs against malaria. The liver is known to raise specific immune responses to blood-stage malaria [11–16]. Recently, we have shown that the T-induced persistent susceptibility of female mice to *P. chabaudi* malaria is associated with permanent deregulations of hepatic expression of numerous genes including genes involved in the immune response and hepatic metabolism [17]. Currently, however, the spleen is regarded as to be the predominant effector site against blood-stage malaria [18]. Indeed, macrophages in the red pulp of the spleen eliminate *Plasmodium*-parasitized

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erythrocytes, and the number of T- and B-cells in the spleen increase during acute malaria [19–21]. However, the spleen has not yet been examined with respect to T-induced persistent changes in gene expression.

Evidence is increasing that also long integrated non-coding (linc) RNAs are emerging new players as regulators of gene expression [22]. There is some information available that lincRNAs are associated with pathogenesis of diseases, which has been particularly investigated in diverse cancer diseases [23,24]. Also, lincRNAs have been recently characterized in human malaria parasites [25,26]. However, changes in expression of host lincRNAs in response to infectious diseases including malaria have not yet been reported to date. Here, we show that the T-induced persistent susceptibility to blood-stage malaria is associated with persistent changes in expression of distinct species of both lincRNAs and mRNAs in the spleen.

2. Experimental

2.1. Mice

C57BL/6 mice were inbred under specified pathogen-free conditions at central facilities of the University Duesseldorf. Only female mice were used throughout the experiments. They were housed in plastic cages and they received a standard diet (Wohrlin, Bad Salzuflen, Germany) and water *ad libitum*. Experiments were approved by the state authorities and followed the German law on animal protection.

2.2. Treatment with testosterone

Approximately 10–12 weeks old mice were subcutaneously injected with 0.9 mg Testosterone-Depot-50 (Schering, Berlin, Germany) suspended in 100 µl sesame oil twice a week for 3 weeks [8,10,17]. Control mice received only the vehicle. After treatment, mice were kept under standard conditions for 12 weeks, before challenging with *P. chabaudi*.

2.3. Plasmodium chabaudi malaria

Blood-stage infections of *P. chabaudi* were routinely kept in outbred NMRI mice at weekly passages [27,28]. The experimental C57BL/6 mice were intraperitoneally injected with 10^6 *P. chabaudi*-parasitized erythrocytes. Parasitemia was determined in Giemsa-stained smears from tail blood. Cell number was counted in a Neubauer chamber.

2.4. RNA isolation

Spleens were aseptically removed from mice killed by cervical dislocation and rapidly frozen in melting nitrogen and stored at -80°C until use. RNA was isolated from the individually frozen spleens using the standard RNA extraction protocol with Trizol (Peqlab Biotechnology, Erlangen, Germany) [17]. Quality and integrity were checked using the Agilent RNA 6000 NanoKit on the Agilent 2100 Bioanalyzer (Agilent Technologies, Böblingen, Germany). RNA was measured at 260 nm on the ND-1000 spectrophotometer (NanoDrop Technologies).

2.5. Amplification and labeling of RNA

This was performed as detailed in the ´one-colórmicroarray-based gene expression analysis protocol (version 6.5, part. No. G4140-90040). In brief, amplification and labeling was done with 100 ng of each RNA sample using the Agilent Low Input Quick Amp Labeling Kit (Agilent Technologies). Yields of cRNA and dye-incorporation, respectively, were determined with the ND-1000 spectrophotometer.

2.6. Hybridization of Agilent whole mouse genome oligo microarrays

The procedure of hybridization followed the protocol of the one-colór Microarray-Based Gene Expression Analysis (version 6.5, part No. G4140-90040) using the Agilent Gene Expression Hybridization Kit (Agilent Technologies). To this end, 0.6 µg Cy3-labeled fragmented cRNA was hybridized in hybridization buffer at 65 °C for 17 h to Agilent Whole Mouse Genome Oligo Microaarays 8 × 60 K using Agilent́s recommended hybridization chamber and oven. After hybridization, the microarrays were washed once with the Agilent Gene Expression wash buffer (AGEWB 1) for 1 min at 23 °C, followed by a second wash with preheated AGEWB 2 at 37 °C for 1 min and the last washing step with acetonitrile.

2.7. Scanning and data analyses

Agilent́s Microarray Scanner System (Agilent Technologies) was used to detect fluorescence signals of the hybridized and washed microarrays. The Agilent Feature Extraction Software (FES) 10.7.3.1 was applied to readout and process the microarray image files. Differential gene expression was determined with the FES derived output data files subjected to further analyses using the Rosetta Resolver gene expression data analysis system (Rosetta Biosoftware, Rosetta error model) [29]. Only those data were evaluated which were more than 2-fold deregulated at high significance ($p < 0.01$) as determined by Student́s *t*-test.

3. Results

Approximately 70–80% of female C57BL/6 mice, when challenged with 10^6 *P. chabaudi*-parasitized erythrocytes, survive the infections that take a self-healing course with approximately 40–60% peak parasitemia on day 8 p.i., as described previously [17,30]. T-pretreatment, however, causes a lethal outcome of infections in all mice [10,17]. To detect possible long-term effects of T on splenic mRNA expression, we have removed spleens from 3 vehicle-treated control mice (C_{d0}) and from 3 T-treated mice after T-withdrawal for 12 weeks before infections (T_{d0}) as well as during infections at peak parasitemia on day 8 p.i. (C_{d8}, T_{d8}). Individual spleens are subjected to RNA-isolation and subsequent hybridizations to 1-color Agilent whole mouse genome oligo microarrays. The overall expression of RNA are similar among the three spleens

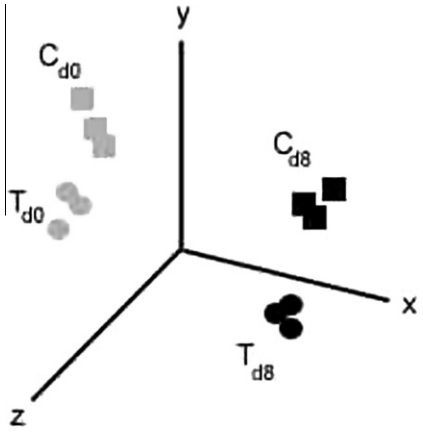

Fig. 1. Principal component analysis representing the three major vectors contributing to variance between expression profiles.