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Characterising granuloma regression and liver recovery in a murine model of schistosomiasis japonica

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ABSTRACT

For hepatic schistosomiasis the egg-induced granulomatous response and the development of extensive fibrosis are the main pathologies. We used a *Schistosoma japonicum*-infected mouse model to characterise the multi-cellular pathways associated with the recovery from hepatic fibrosis following clearance of the infection with the anti-schistosomal drug, praziquantel. In the recovering liver splenomegaly, granuloma density and liver fibrosis were all reduced. Inflammatory cell infiltration into the liver was evident, and the numbers of neutrophils, eosinophils and macrophages were significantly decreased. Transcriptomic analysis revealed the up-regulation of fatty acid metabolism genes and the identification of Peroxisome proliferator activated receptor alpha as the upstream regulator of liver recovery. The aryl hydrocarbon receptor signalling pathway which regulates xenobiotic metabolism was also differentially up-regulated. These findings provide a better understanding of the mechanisms associated with the regression of hepatic schistosomiasis.

1. Introduction

Schistosomiasis is a chronic helminth disease of humans caused by digenean trematodes of the genus *Schistosoma*. Although this disease has been successfully controlled in many countries, it is still a major threat to public health as approximately 260 million people in over 78 countries are infected (World Health Organization, 2015). Chronic infection with the Asiatic *Schistosoma japonicum* ranges from mild hypersensitivity reactions to granuloma formation, periportal fibrosis, portal hypertension, portocaval shunting, and bleeding from gastrointestinal varices, which may be fatal (Gryseels et al., 2006; Burke et al., 2009). Pathology is associated with the host CD4+ Th2 response, with IL-4 and IL-13 the dominant cytokines responsible (Wynn et al., 2004). Chemokines and their receptors have also been shown to play an important role in the development of schistosome egg-induced granuloma formation (Chuah et al., 2014a). The transcriptional regulation of hepatic schistosomiasis japonica in mouse models has been investigated during the acute phase of granuloma formation (Burke et al., 2010a,b; Perry et al., 2011; Chuah et al., 2013); however less is known regarding the more chronic phases and the events that occur in the liver during recovery after parasite clearance.

Current efforts to control schistosomiasis rely solely on the drug praziquantel (PZQ) (Xiao et al., 2009). The pharmacological actions of PZQ on adult schistosomes are considered to be calcium-dependent (Doenhoff et al., 2008), and the participation of host complement in vivo is also an important co-factor for parasite elimination (La Flamme et al., 2003). PZQ, in addition to its anti-helminth effect, is reported to have anti-inflammatory properties (Ribeiro-dos-Santos et al., 2006). PZQ administration significantly reduces granuloma area, the number of inflammatory cells within the granulomas (Yang et al., 1984; Huang et al., 2011), and the levels of inflammatory cytokines in the blood (Silveira-Lemos et al., 2013). PZQ treatment successfully decelerated hepatic fibrosis in patients suffering from schistosomiasis mansoni (Zwingenberger et al., 1990), and can also reverse pulmonary hypertension and vascular remodelling in a murine model of *Schistosoma mansoni* infection (Crosby et al., 2011). The transcriptional response of the parasite to PZQ has advanced the understanding of its mode of action (Gobert, 2010). While some insights have been

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made in regards to the anti-parasite response of PZQ for both *S. japonicum* (You et al., 2013) and *S. mansoni* (Aragon et al., 2009; Hines-Kay et al., 2012; Kasinathan et al., 2014), less is understood regarding the impact of the drug on host tissues, particularly in the main organ of pathology, the liver.

Schistosomiasis is a disease caused predominantly by the host immune response to schistosome eggs (ova) and the granulomatous reaction they evoke (Pearce and MacDonald, 2002). Granulomas are formed to destroy eggs and sequester or neutralise otherwise pathogenic egg antigens but this process also leads to host tissue fibrosis (Wilson et al., 2007). Transforming growth factor beta (TGF-β) is involved in the process of liver regeneration after partial hepatectomy (Braun et al., 1988), and it plays a role in hepatocyte proliferation in the regenerating liver (Thenappan et al., 2010). In schistosomiasis, the regression of hepatic granuloma and the down-modulation of the Th2 response are thought to be mediated by IL-10 secreting T regulatory cells (Hesse et al., 2004), and the role of IL-13Rα2 in the resolution of fibrosis has been reported (Chiaramonte et al., 2003). However, the specific mechanisms leading to liver recovery are still not clear. In this study, we describe the application of a mouse model of *S. japonicum* treated with PZQ to allow liver recovery, and the characterisation of these events using histology and by whole genome microarray analysis. These results provide novel insights on hepatic cellular events during recovery after hepatic schistosomiasis.

2. Materials and methods

2.1. Ethics statement

All animal studies were conducted with the approval of the Animal Ethics Committee of QIMR Berghofer Medical Research Institute, Brisbane, Australia.

2.2. Parasites and mouse infections

A total of 42, 4–6 week old female C57BL/6 mice ($n = 6$ per group) were percutaneously infected with 14 ± 1 *S. japonicum* cercariae (Chinese mainland, Anhui population). An additional 14 C57BL/6 mice were used as uninfected controls.

2.3. Drug administration

The mice were divided into groups (Fig. 1) designated as: PZQ-treated infected mice (PI; $n = 18$, six mice per time point); untreated infected mice (NI; $n = 24$, six mice per time point); PZQ-treated uninfected mice (PU; $n = 12$, four mice per time point); and untreated uninfected mice (NU; $n = 2$). A group of six infected mice (from group NI) was euthanised at 6 weeks p.i. before the commencement of PZQ treatment, to provide baseline data. Mice receiving PZQ treatment (Groups PI and PU) were orally administered 150, 200, 250, 300 and 350 mg/kg PZQ prepared in 2.5% (v/v) Cremophor EL (Sigma, USA) for five consecutive days at 7 weeks p.i. Groups NI, PI and PU were euthanised 3, 6 and 7 weeks post PZQ treatment (10, 13 or 14 weeks post cercarial challenge, respectively) and the absence of worms was confirmed at sacrifice (see Section 2.4).

2.4. Parasitological, pathological and histological assessment

Adult worms were obtained and counted following perfusion from the intestinal mesenteric veins, and mouse livers and spleens were collected for assessment of hepatosplenomegaly. The small lobe of the liver was used for histology (see below), then the remaining tissue was divided for RNA isolation and egg counts. Hepatic egg burden was calculated as the number of eggs per gram of liver (EPG) as previously described (Burke et al., 2010a). Faeces were collected 1 week before PZQ administration to microscopically confirm the presence of parasite eggs and again 1 week after PZQ treatment to ensure that treatment was effective, as described (You et al., 2012).

The small lobe of liver from each mouse was formalin-fixed 10% (v/v) and paraffin-embedded, and liver sections were then stained with H&E to assess granuloma density, and PicroSirius Red for collagen to measure hepatic fibrosis (Perry et al., 2011). Leder and Giemsa staining measured neutrophil and eosinophil infiltration, while quantitation was carried out by determining the average number of positive-stained cells over 20 fields at high magnification as previously described (Burke et al., 2010a). Macrophage recruitment was evaluated by F4/80 immunostaining and quantitated using Aperio's Spectrum Plus software Positive Pixel Count Algorithm (Burke et al., 2010b). All slides were digitised using the Aperio Slide Scanner (Aperio Technologies, USA).

2.5. Total RNA isolation

Total RNAs from all liver samples were isolated using Trizol and a RNeasy Mini Kit (Qiagen, Germany) following the manufacturer's instructions. Total RNA quantity was measured using a Nanodrop-1000 spectrophotometer (Nanodrop Technologies, USA) and RNA quality was assessed using an Agilent Bioanalyzer (Agilent Technologies, USA).

2.6. Microarray analysis

Microarray analyses were performed using Illumina Mouse Ref-8 Version 2 whole genome expression arrays as previously reported (Chuah et al., 2013). Details of cRNA synthesis and Whole Genome Microarray Hybridisation, Feature Extraction and data analysis, and Ingenuity Pathway Analysis (IPA), are outlined in Supplementary Data S1. All gene expression data have been submitted to Gene Expression Omnibus (National Center for Biotechnology Information (NCBI), USA) and are publicly available (Series Accession Number, GSE59276).

2.7. Quantitative real-time PCR (qRT-PCR)

cDNA was synthesized using a Quantitect Reverse Transcription kit (Qiagen) and cDNA concentration quantified using a Nanodrop-1000 spectrophotometer (Nanodrop Technologies). qRT-PCR was performed using SYBR Green master mix (Applied Biosystems, USA) on a Corbett Rotor Gene 6000 (Corbett Life Sciences, Australia). Hypoxanthine phosphoribosyltranferase (HPRT) was used as a housekeeping gene as described (Dheda et al., 2004). Quantitative real-time PCR (qRT-PCR) was used to validate a subset of microarray data representing those transcripts that were significantly expressed (up- or down-regulated) by microarray analysis. Primers for qRT-PCR were designed using Primer 3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) and are listed in Supplementary Table S1.

2.8. Immunohistochemistry

The Ki67 (a marker for cellular proliferation) and tissue inhibitor of metalloproteinase-1 (TIMP1) proteins were immunolocalised using paraffin-embedded section described in Section 2.4. Full details of the processing procedures are presented in Supplementary Data S1. Two analysis strategies were used for quantitation of staining as described below.