

Cytokine mRNA profiles in pigs exposed prenatally and postnatally to *Schistosoma japonicum*

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Abstract – The pig is a natural host for *Schistosoma japonicum* and a useful animal model of human infection. The aim of the present study was to assess the differences between the cytokine profiles in prenatally or postnatally *S. japonicum* exposed pigs. Seven prenatally exposed pigs, 7 postnatally exposed pigs and 4 uninfected control pigs were compared 27 weeks post exposure. Variables included worm burdens, tissue egg counts, liver pathology and mRNA levels of IL-2, IL-4, IL-10, IL-12, TNF- α , TGF- β 1 and IFN- γ in the liver and the caecum, assessed by RT-PCR. Infection intensity and level of septal fibrosis were significantly higher in the postnatal group compared to the prenatal group ($P < 0.05$). A significant increase of IL-4 ($P < 0.01$), IL-10 ($P < 0.01$), IL-12 ($P < 0.01$) and TNF- α ($P < 0.05$) mRNA level was also observed in the caecum of prenatally infected animals compared to the control group ($P < 0.01$). The prenatal group showed higher levels of TGF- β 1 in the liver compared with the postnatally infected group ($P < 0.05$) and the control group ($P < 0.01$). This suppressive immune response correlated with previously reported low hepatic pathogenesis in prenatally exposed pigs.

swine / *Schistosoma japonicum* / cytokine / prenatal infection / postnatal infection

1. INTRODUCTION

Schistosomiasis remains one of the most prevalent infections with more than 200 million people infected worldwide. The disease constitutes a major public health problem in sub-Saharan Africa, re-

gions of South America, the People's Republic of China, the Philippines and in minor endemic foci in other Southeast Asian countries [27, 37]. Thus, there is still a critical need for a further understanding of the immune responses elicited during infection, in order to generate insight into plausible mechanisms of immune regulation and treatment strategies [42].

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In chronic infections with *Schistosoma mansoni* and *S. japonicum*, parasite development and granuloma formation is believed to be a T-helper (Th) lymphocyte dependent process [11, 24]. In experimental murine schistosome infections, it was originally thought that a Th2 response, involved in the development of chronic infection, was the main cause of pathology, whereas a Th1 response participated in protection against the infection [9]. However, more recent studies suggest that the situation is more complicated, showing that schistosome infections in mice with reduced type 2 responsiveness lead to a more severe pathological outcome than in mice capable of mounting type 2 responses [14]. In agreement with the more recent studies, immuno-epidemiological studies of human populations in endemic areas have revealed a positive correlation between high, specific immunoglobulin (Ig) E levels, eosinophilia, interleukin (IL)-5 levels and resistance to reinfection after treatment [30,48]. However, high levels of gamma interferon (IFN- γ) and tumor necrosis factor (TNF) together with lower levels of IL-5 seem to be associated with hepatosplenic disease [33]. These results support a beneficial role of Th2 responses in both experimental murine infections and human schistosomiasis.

The current understanding of the immunological basis of schistosomiasis is based mainly on results obtained using murine models [7,39]. There are however, problems in extrapolating results from this unnatural host to humans, especially with regards to granuloma induced fibrosis formation in schistosomiasis patients [2, 13]. The pig is a natural host for *S. japonicum* [8] and experimental *S. japonicum* infection has been readily established in Danish Landrace/Yorkshire/Duroc cross-bred sows, causing pathogenesis similar to that described in humans, with the most severely affected organs being the intestine and the liver [17]. Willingham et al. [45]

showed that *S. japonicum* readily establishes in the pig fetus if the sow is infected during mid to late pregnancy. Interestingly, prenatally exposed pigs were later shown to develop less severe hepatic pathology, compared to challenge control animals, when given a challenge infection [22]. Johansen and Ørnbjerg [23] further showed that treatment with praziquantel, previously reported to give a 100% cure rate [20], did not cure the prenatal infection and that a challenge infection was readily established in these pigs. These results indicate that pigs might develop partial tolerance towards *S. japonicum* after infection in utero.

The prenatal *S. japonicum* model is well established in terms of parasitological and pathological effects of the infection [45], but knowledge on induced production of immune mediators is very scarce [41]. A deeper understanding of the cytokine responses induced in prenatally and postnatally exposed pigs, might reveal important differences, reflecting which factors are likely to be involved in immunopathology, given that the pathological outcome of disease is less severe in prenatal infections. Here the mRNA expression levels for 7 different cytokines were measured, in order to investigate the cytokine profiles in prenatally versus postnatally *S. japonicum* exposed pigs.

2. MATERIALS AND METHODS

2.1. Parasites

Schistosoma japonicum of the Anhui strain [21], were maintained by laboratory passage in *Oncomelania hupensis* snails and female NMRI mice housed at DBL – Institute for Health Research and Development. Cercariae were harvested as previously described [31].

2.2. Animals and experimental design

Six pathogen-free and helminth-naïve Danish Landrace/Yorkshire/Duroc cross-breed sows were used in this experiment. Ten weeks post-insemination, 3 of the sows were infected by intramuscular injection of 10 000 *S. japonicum* cercariae [44]. Seven piglets born from the infected sows were removed immediately after delivery, and received colostrum and milk from an uninfected sow, to ensure that the immunity of the sows had no influence on the immune responses mounted in the prenatally exposed piglets. Among piglets born from uninfected control sows, seven were infected intramuscularly with 1000 cercariae 9 weeks post delivery, while 4 piglets remained as uninfected controls and received an injection of suspension medium as previously described [41]. All animals were housed at Sjaelland III and treated in accordance with protocols approved by the animal ethics committee of Denmark.

2.3. Parasitological techniques

At 27 weeks post-infection (i.e. at 21 and 36 weeks of age for the prenatally and postnatally exposed animals respectively), all the pigs were perfused as previously described [4]. Briefly, pigs were killed by an intravenously injected overdose of pentobarbital (30 mg/kg). Heparin sulfate (5000 IU) was also injected intravenously. One central longitudinal cut was performed from the sternum to the lower abdomen and selective perfusion of the portal hepatic system and the intestinal mesenteric system was achieved by clamping the vessels supplying blood to other organs and hind legs. The perfusion tube was inserted into the aorta and sodium citrate containing saline was pumped through the portal and mesenteric vessels and collected at the punctured portal vein. The perfusate was sieved and worms were collected [4]. Adult *S. japonicum* worms were

counted and classified according to sex and maturity. After perfusion, the intestinal tract of each pig was examined for residual worms, and the caecal mucosae were scraped for tissue egg counts. The number of eggs in the faeces, liver and caecum was determined as previously described [41]. Liver fibrosis was examined and scored as none = 0, mild = 1, moderate = 2, or severe = 3 depending on the number of worm nodules, lesions and levels of interlobular connective tissue related to fibrosis [21].

2.4. Determination of cytokine mRNA expression by semi-quantitative RT-PCR

Tissue samples were taken at the autopsy of the animals from lesion sites in the liver and the caecum. They were immediately preserved in Trizol Reagent (GibcoBRL, Life Technologies, Germany) and homogenized on ice using a Cat homogenizer. Total RNA was extracted as recommended by the manufacturer and quantitated by spectrophotometry. Semi-quantitative determination of IL-2, IL-4, IL-10, IL-12, TNF- α , TGF- β 1 and IFN- γ levels was carried out using RT-PCR as previously described, using cyclophilin as the housekeeping gene control [34]. Briefly, mRNA was reverse transcribed with Moloney leukaemia virus reverse transcriptase (Promega, Charbonnières, France) and amplified with DNA Taq polymerase enzyme (Invitrogen, Life Technology, Cergy-Pontoise, France). The primer sequences, number of cycles and the annealing temperature used for PCR amplification of each cytokine and cyclophilin are summarized in Table I. Semi-quantitative analysis of PCR products was done by hybridization of 32 P-labelled specific oligonucleotide probes to the PCR products, which were immobilized on nitrocellulose membranes by dot blotting as previously described [10, 35]. The

Table 1. Oligonucleotide sequences and DNA probes designed for the detection of different porcine cytokines, with the respective extension temperatures and number of PCR cycles used in samples from the caecum and liver.

Cytokine	Primer sequences ^a (5'-3')		Annealing t° Caecum/ Liver		Number of cycle Caecum/ Liver		DNA probe sequence (5'-3')
	S	AS	Caecum	Liver	Caecum	Liver	
IL-2 ^b	S	GATTACAGTTGCTTTTGAA	54/54	54/54	45/46	45/46	AGGAATCAATGAACAATATCAACGT
	AS	GTTGAGTAGATGCTTTTGACA					
IL-4 ^b	S	TACCAGCAACTTCGTCCAC	54/54	54/54	45/45	45/45	ACAAGAGATCATCAAAAACCTTGAAC
	AS	ATCGTCTTTTAGCCTTTTCCAA					
IL-10 ^b	S	GCATCCACTTCCCAACCA	54/54	54/54	45/45	45/45	TAGGCTTCTAIGTAGTGTGATGAAGAATGT
	AS	CTTCCCTCATCTTCATCGTCAT					
IL-12p40 ^b	S	GATGCTGGCCAGTACACC	54/54	54/54	35/35	35/35	ACTCCGGACGTTTACACCT
	AS	TCCAGCACGACCTCAATG					
IFN- γ ^b	S	GAAGAAAGGTCAGCCAAGGGC	54/60	54/60	33/33	33/33	CCAAAATTGTCTCCTTCTACTTCAAA
	AS	GCTTGTATCACATCCATGCTCC					
TNF- α ^b	S	ATCGGCCCCAGAAAGGAAGAG	56/56	56/56	30/30	30/30	ATCATCGTCTCAAACCTCAGATAAG
	AS	GATGGCAGAGAGGAGGTTGAC					
TGF- β 1 ^c	S	CTACTACGCCAAGGAGGTC	54/54	54/54	30/30	30/30	ACGTGGAGCTATACCAGAAATACAG
	AS	TGACCCCGCAGAGAGGCTAT					
Cyclophilin ^b	S	TAAACCCACCGTCTTCTT	50/50	50/50	22/22	22/22	TGGTGACTTCACACGCCATA
	AS	TGCCATCCAACCACCTCAG					

^a S, sense primer; AS, antisense primer.

^b Oligonucleotide sequences previously described [10, 34, 35].

^c Newly designed primer and probe sequences.

sequence of the DNA probes used for hybridization to the different cytokines is listed in Table I. The relative amount of each product was determined by measuring radioactivity with a Phosphor Imager (Molecular Dynamics, Sunnyvale, CA, USA). For each cytokine, the amount of RT-PCR product was normalized to the values obtained for cyclophilin, as an internal standard for each sample.

2.5. Statistical Analysis

Data from individual pigs are presented as the group mean \pm standard deviation. Statistical differences between group means for worm burdens, fecal and liver egg counts and cytokine expression levels were determined using One-way analysis of variance, and post hoc comparisons were done with the Scheffe range test. Prior to analysis, caecum and liver egg counts were transformed to \log_{10} . Correlations between parasitological variables were assessed by using the Pearson correlation test. The ranked degree of liver gross pathology was compared between the two groups using the Kruskal-Wallis test and Mann-Whitney U-test. All analyses were done using the program Statistical Package for Social Sciences¹ (SPSS) version 11.0. Values of $P < 0.05$ were considered significant.

3. RESULTS

3.1. Level of parasitic infection

Piglets were exposed prenatally or postnatally to *S. japonicum* infection. By examination of faecal egg excretion, infection was demonstrated in 5 of the 7 prenatally exposed piglets and in 6 of the 7 postnatally exposed piglets. The total number

of worms was significantly higher in the postnatally exposed group in comparison to the prenatally exposed group ($P < 0.05$) (Tab. II).

Faecal egg excretion remained low throughout the experiment, and due to high variation within each group, there was no significant difference at any time between the two groups (data not shown). At autopsy, the number of eggs in the liver was higher in the postnatally exposed group ($P < 0.01$). There was no difference in the level of portal fibrosis between the two groups, but a significantly higher degree of septal fibrosis was seen in the postnatally exposed group compared to the prenatally exposed group ($P < 0.05$) (Tab. II).

3.2. Cytokine mRNA expression levels in liver and caecum samples

Twenty-seven weeks post infection, liver and caecum samples were collected for investigation of IL-2, IL-4, IL-10, IL-12, IFN- γ , TNF- α and TGF- β 1 mRNA expression levels (Figs. 1 and 2). In the liver, the TGF- β 1 mRNA expression level was significantly higher in the prenatally exposed group compared to the levels observed in both the control group ($P < 0.01$) and the postnatally exposed group ($P < 0.05$). Furthermore, significantly higher levels of TNF- α and IL-2 mRNA were seen in the postnatally exposed group compared to the control ($P < 0.05$), and a tendency towards higher IFN- γ levels was also observed in the postnatally exposed group.

In the caecum IL-4, IL-10, IL-12 and TNF- α levels were significantly increased in the prenatally exposed group compared to the control group (for IL-4, IL-10, IL-12 $P < 0.01$, and for TNF- α , $P < 0.05$), while a tendency to higher TGF- β 1 levels was observed. The levels of IFN- γ and TNF- α in the postnatally exposed group were

¹ www.SPSS.com.

Table II. Characteristic of the parasitic infection in prenatally and postnatally exposed pigs.

	Exposition to the parasite ^a			Statistical effect of the type of exposure (pre versus post)
	Prenatally (<i>n</i> = 7)	Postnatally (<i>n</i> = 7)		
		Low infection level (<i>n</i> = 3)	High infection level (<i>n</i> = 4)	
Total worms (mean number per animal)	0.7 ± 0.8 ^b	7.3 ± 7.0	20.0 ± 17.5	<i>P</i> < 0.01
Egg count (number per gram of tissue)				
Liver	2.9 ± 7.6	19.7 ± 20.0	233.3 ± 139.3	<i>P</i> < 0.01
Caecum	0	6.7 ± 11.5	28.0 ± 32.2	NS
Liver pathology (arbitrary unit)				
Septal fibrosis	0.3	1	1	<i>P</i> < 0.05
Portal fibrosis	0.4	0.5	0.5	NS

^a Pigs were prenatally exposed to *S. japonicum* at 10 weeks gestation or postnatally exposed 9 weeks post delivery.

^b Results are expressed as mean ± SD.

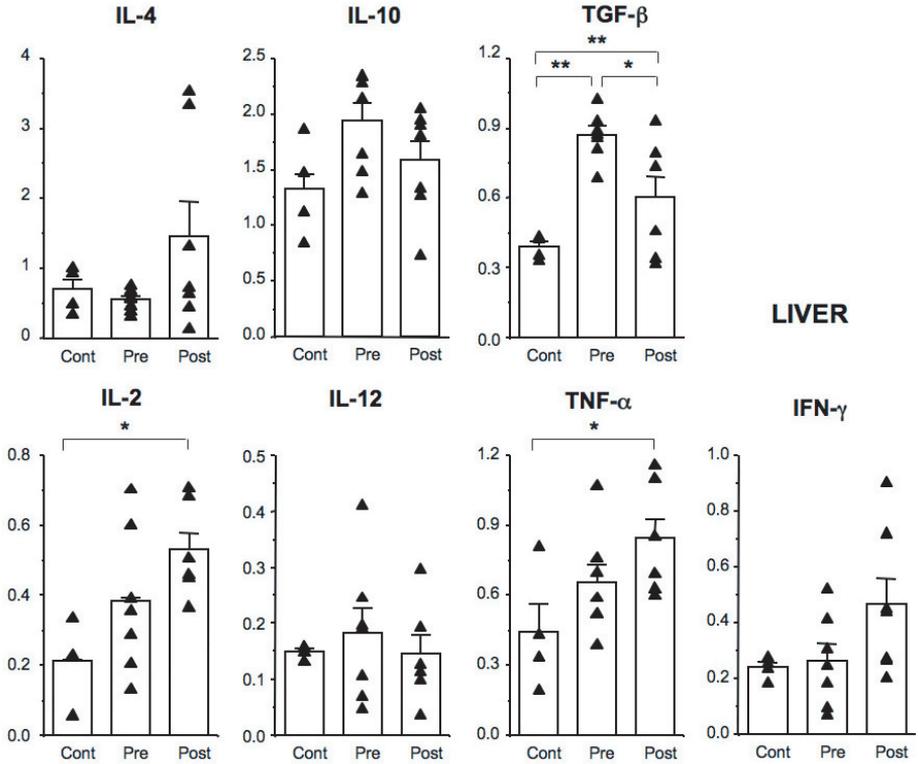


Figure 1. Cytokine mRNA expression levels in liver tissue from pigs prenatally or postnatally exposed to *Schistosoma japonicum*. Closed triangles represent the individual values and vertical bars the mean (\pm SD) of these results for each group. Comparison of the cytokine levels observed in control, prenatally and postnatally exposed animals, * $P < 0.05$ and ** $P < 0.01$.

significantly higher than the levels in the control group ($P < 0.05$).

The results obtained could be reflecting differences in infection levels between the prenatally and the postnatally exposed group. In order to investigate this hypothesis, the postnatally exposed group was divided into two subgroups consisting of pigs with lower (3 pigs) or higher infection intensities (4 pigs), and data was re-analysed with respect to these groups. The postnatal subgroup with low infection levels was comparable to the prenatally exposed group regarding worm and egg counts. Levels of cytokine mRNA for

each of the postnatally exposed subgroups were then compared to the prenatally exposed group and to the control group. As before, the prenatally exposed group had significantly higher levels of mRNA encoding TGF- β 1 in the liver compared to the control and both postnatally exposed subgroups ($P < 0.05$). No significant differences were found between the groups regarding TGF- β 1 levels in the caecum. The above mentioned differences between the postnatal group (7 pigs) and the control group were also observed when comparing each of the postnatal subgroups with the control group.

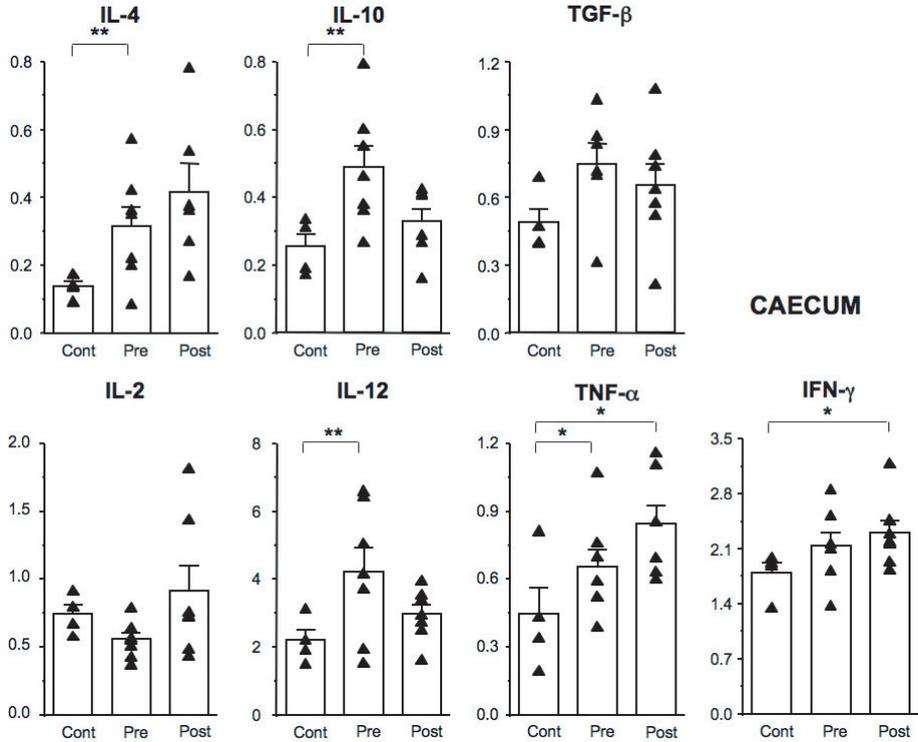


Figure 2. Cytokine mRNA expression levels in caecum tissue from pigs prenatally or postnatally exposed to *Schistosoma japonicum*. Closed triangles represent the individual values and vertical bars the mean (\pm SD) of these results for each group. Comparison of the cytokine levels observed in control, prenatally and postnatally exposed animals, * $P < 0.05$ and ** $P < 0.01$.

4. DISCUSSION

In line with previous studies on prenatal schistosomiasis in pigs, significantly higher establishment of worms and higher tissue egg counts were seen in the postnatally exposed group compared to the prenatally exposed group, emphasizing that infections obtained through vertical transmission are generally characterised by low infection intensities [18]. Limited fibrosis formation was observed in the prenatal group, and the level of septal fibrosis was significantly higher in the postnatal group.

We employed a semiquantitative RT-PCR procedure, in order to investigate the cytokine expression in granulomatous liver

and caecum tissue in prenatally and postnatally exposed pigs. Our data indicate that there are differences in the cytokine profiles between prenatally and postnatally exposed pigs, independent of infection intensity when compared to the control group 27 weeks post infection. IL-10 is an anti-inflammatory agent, which has been shown to dampen a number of responses, including macrophage cytokine secretion, Th2 cell proliferation, IL-4 induced IgE synthesis and IL-5 production [3, 36, 50]. Our observation that prenatally exposed pigs show high IL-10 levels and low hepatic fibrosis suggests a similar role for IL-10 in pigs. In a number of human schistosomiasis studies, high levels of IL-10 have been

shown to be associated with reduced susceptibility to infection, and to play a role in antigen-specific suppression [26,29]. TGF- β 1 is also regarded as a potent immunoregulatory cytokine, regulating the differentiation, proliferation and state of activation of a large number of immune cells, thereby modulating both cellular and humoral mediated responses [28]. The prenatally exposed group had significantly higher levels of mRNA encoding TGF- β 1 in the liver compared to the control and the postnatally exposed group ($P < 0.05$), whereas no significant differences were found between caecum TGF- β 1 levels within the three groups. Our observation, that TGF- β 1 mRNA levels were higher in prenatally exposed pigs, showing less septal fibrosis, suggests that TGF- β 1 may mediate down-regulatory responses beneficial to the prenatally exposed pigs.

In the postnatally exposed pigs, significantly higher levels of TNF- α , together with a tendency to higher levels of IL-2 and IL-4, indicate the existence of a dynamic and not yet polarized Th2 response [40]. Apart from being the principal mediator of acute inflammatory responses, TNF- α is important for the induction of fibroblast mitosis [19]. Interestingly, TNF- α treatment has been shown to restore granuloma formation in *S. mansoni* infected SCID mice, and furthermore to lead to a dose-dependent increase in the total number of parasite eggs [1]. Our results suggest that TNF- α may also play a role in hepatic fibrosis development in pigs postnatally infected with *S. japonicum*.

Blockade of IL-2 responses in *S. japonicum* infected mice, leads to reduced hepatic fibrosis, an effect thought to be caused by the down-regulation of Th2 responses [5]. Moreover, anti-IL-4 treatment of *S. mansoni* or *S. japonicum* infected mice has been shown to result in decreased fibrosis formation and reduced granuloma size [6, 9]. These results emphasise the importance of a Th2 response in murine

schistosome fibrosis and granuloma formation [38, 39]. Our observations are in line with these results, indicating that high IL-2 and IL-4 responses in postnatally exposed pigs, may mediate fibrosis formation. IFN- γ responses have been shown to be prominent in early disease, while down-regulated in chronic human schistosome infections [15, 32]. In our experiment, levels of IFN- γ mRNA did not differ between infected and control pigs, but some variation in the expression level of this cytokine was observed.

The cytokine profiles in the caecum displayed similar differences between prenatally or postnatally exposed pigs, as observed for the liver. A high IL-10/IL-4 ratio in prenatally exposed pigs together with high TGF- β 1 levels seem to dominate, while postnatally exposed pigs show higher expression of IL-2 and IL-4. Intestinal tissue damage, due to toxic products released from developing schistosome eggs, is often combined with secondary bacterial infection [37], which might explain the higher IL-12 and TNF- α levels seen in the caecum of prenatally exposed pigs.

Interleukin (IL)-13 is a key inducer of several type-2 cytokine-dependent pathologies [46]. Recently, this cytokine has been described to regulate tissue remodelling and fibrosis formation in the murine schistosomiasis model and is regarded as a central mediator of chronic infection-induced liver pathology [46, 47]. At the time of this study, antibody towards porcine IL-13 and porcine IL-13 sequence were not available and hence IL-13 levels could not be determined. In future experiments, however, it will be of interest to characterize the expression pattern of IL-13 in the porcine schistosomiasis model.

In line with a previous study [26], we show that postnatally exposed pigs seem to mount a Th2 like response, with high IL-2 and IL-4 levels locally in lesion sites of the liver and caecum. Th2 responses,

although related to hepatic fibrosis, seem to be associated with reduced susceptibility and host protection against overt human hepatosplenic disease due to inflammatory responses [25, 30, 33]. Indeed, also porcine IL-4 has been shown to play an important role in the regulation of macrophage mediated inflammatory responses [49]. In prenatally exposed pigs, a high IL-10/IL-4 ratio, together with high levels of TGF- β seem to dominate, suggesting that these pigs mount a more Th3 like response [16, 43]. Such IL-10 and TGF- β 1 dominated responses have been shown to dominate and mediate antigen-specific cellular hyporesponsiveness in other chronic human helminth infections [12, 26]. This indicates that the partial tolerance induced in prenatally exposed pigs [22] might be mediated by antigen-specific hyporesponsiveness, resulting in lower levels of hepatic fibrosis.

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