



## RESEARCH ARTICLE

### Early Antibody, Cytokine and Chemokine Responses during *Echinococcus Granulosus* Infection in Kazakh Sheep with Cystic Echinococcosis Resistance Haplotype

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#### ABSTRACT

Our previous study showed that the haplotype of MHC *Mvalbc-SacIIab-HinI1ab*, was associated with cystic echinococcosis (CE) resistance in Kazakh sheep. The present study described here was designed to analyze the system immune response in the very early stage of *Echinococcus granulosus* (*E. granulosus*) infection in sheep with CE resistant haplotype. Fourteen healthy Kazakh sheep were divided into two groups: seven sheep with CE resistant haplotype referred to group A, while the others without this haplotype constituted group B. These sheep were all experimentally infected with *E. granulosus* eggs. Blood samples were collected on day 0 (prior to infection), hours 2, 3, 4, and 9 post-infection as well as days 1, 2, 3, and 7 post-infection, respectively. ELISA assay was used to measure serum levels of antibodies (IgE and IgM), cytokines (Th1:TNF- $\alpha$  and IFN- $\gamma$ ; Th2: IL-4 and IL-10) and chemokines (Th1: CXCL-9; Th2: CCL17) at different time points of *E. granulosus* infection in the two groups. Results showed that, in the early stage of *E. granulosus* infection, antibodies like IgM and IgE, Th1 cytokines such as IFN- $\gamma$  and TNF- $\alpha$ , as well as Th1 chemokines CXCL-9 were predominant in group A, especially for IgE and Th1 cytokines, which were significantly higher, most were at or began from 4h post-infection, as compared with group B. Our findings revealed that the influence of the host's genetic background on the immunopathology of *E. granulosus* infection in the early stage could be partially mediated by Th1-type cytokines and IgE.

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#### INTRODUCTION

Genetic factors, especially the major histocompatibility (MHC) systems of the immune response, have been demonstrated to be related to the resistance or susceptibility of CE infection (Azab *et al.*, 2004; Abdul-Basit *et al.*, 2010; Li *et al.*, 2010), since MHC molecules, encoded by MHC genes, play an important role in the immune response by presenting antigens (peptides from bacteria, viruses, and parasites) to T lymphocytes. The individual variation of MHC molecules, caused by the highly polymorphic genes, may influence effective presentation of antigens, thereby triggering different outcome of the disease in hosts (Aydinli *et al.*, 2007).

Genetic markers, MHC *Mvalbc-SacIIab-HinI1ab* haplotype, on CE resistance and susceptibility in Kazakh sheep have been divergently selected by us. The rates of *E.*

*granulosus* infection in the internal organs of Kazakh sheep with this haplotype, is confirmed to be significantly lower than sheep without this haplotype, when exposure to the same level of parasites (Li *et al.*, 2010). CE resistant sheep therefore have an increased genetic capability to respond to and subsequently reject parasites when challenged. Carrying out study of the immune response in these sheep was, therefore, worth doing, since it could produce some interesting clues on the mechanism of immune resistance to *E. granulosus* infection.

At the time of initial encounter with *E. granulosus*, the host might modulate the immune response. The changes that it induces are dynamic and depend on the stage of development, for example, ranging from oncosphere, to early stage vesicles up to a fully matured and fertile metacystode (Vuitton and Gottstein 2010; Paredes *et al.*, 2011). However, very little is known of immune response in

the early phase of a primary (oral challenge with eggs) *E. granulosus* infection in vivo (Zhang *et al.*, 2008), although it is important to evaluate cell-mediated immunity, the generation of associated cytokines/chemokines in the early stage of infection (Zhang *et al.*, 2008, 2012), which might be able to stop the larval growth at the very first stages or during early development in liver (Pater *et al.*, 1998), since *in vitro* experiment has shown that killing of *E. granulosus* oncosphere was caused by antibody dependent, cell-mediated cytotoxicity reactions (Rogan *et al.*, 1992).

The present work, therefore, was carried out to analyze immune responses in the very early stage of primary *E. granulosus* infection in Kazakh sheep with CE resistant MHC haplotype, the experiment period was focused within the first week post-infection, since we previously found that the ability to eliminate *E. granulosus* infection in CE resistance sheep depended on the MHC system in the first week post-infection (Hui *et al.*, 2012).

## MATERIALS AND METHODS

**Preparation of *E. granulosus* eggs for oral infection:** Two dogs purchased locally were ingested sheep offal containing protoscoleces and housed in an isolation facility. 45 days later, the dog was euthanized. The gut contents were washed several times with physiological saline at 37°C. The undissolved material was allowed to sediment by gravity. Mature proglottids and eggs were obtained.

**Experimental animals and infection:** Fourteen healthy two-year-old Kazakh sheep with different haplotype were selected and maintained during the course of the experiments. They were negative for antibodies to hydatid cyst fluid (HCF) antigen, assayed by a commercial ovine hydatidosis ELISA kit (Shenzhen Combined Biotech Co., Shenzhen, China), and no hydatid cysts presented in internal organs detected by ultrasonography (50S Tringa Vet Pie Medical, Netherland), prior to the experiment. The seven sheep with the MHC *MvaIbc-SacIIab-HinIIab* haplotype (CE resistant haplotype) referred to group A, while the other seven sheep without this haplotype constitute group B. Each sheep in the two groups was infected per orally via a medical syringe with 5000 *E. granulosus* eggs suspended in 1000ul physiological saline.

**Samples collection and storage:** Following infection, 5ml of blood sample in sodium-heparin solution was collected from the jugular vein of each sheep, on day 0 (prior to infection), hours 2, 3, 4, and 9 post-infection as well as days 1, 2, 3, and 7 post-infection, respectively. After centrifugation (3000g for 10 minutes, at 4°C), the serum was aliquoted and stored at -20°C until analysis.

Measurement of antibodies, cytokines and chemokines in serum levels of antibodies (IgE, IgM), cytokines (Th1:TNF- $\alpha$ , IFN- $\gamma$  and IL-2; Th2: IL-4, IL-10) and chemokines (Th1: CXCL-9; Th2: CCL17) were measured using commercial kits for ELISA (Shanghai BlueGene Biotech Co Ltd, Shanghai, China) following the manufacturer's instructions. Results were expressed in pg/ml.

**Statistical analysis:** The statistical analysis was carried out by SPSS 17.0. Independent T-test, One-way ANOVA test,

and repeated measure of ANOVA were used for statistical analysis of changes of antibodies, cytokines and chemokines concentration in serum, with a P-value of 0.05 considered indicative of a statistically significant difference.

## RESULTS AND DISCUSSION

**Changes in antibodies:** Infection of sheep with *E. granulosus* eggs resulted in significant alterations in the statistical analysis of both IgE and IgM. Graphical representations of the sequential changes in the concentration of IgE and IgM are shown in Table 1.

IgE: After initial decrease in all animals (from 0 to 2h post-infection), the concentration of IgE underwent different changes, which still decreased in group B while increased in group A. A significant difference between the two groups (P=0.044) was observed at 4h post-infection. It is inferred that, at this time, systemic non-specific IgE in group A, probably plays a certain role in regulation the *E. granulosus* oncosphere hatched by eggs through the gut wall, thus inhibiting the parasite further development, thereby, partially leading to the fact that a significantly lower infection rates occurred in these animals (Li *et al.*, 2010). It was noteworthy that after fluctuation from 4h post-infection to day 2 post-infection both in group A and group B, IgE in group A rose rapidly and reached a third peak on day 3 post-infection, which thereafter sustained this level till the end of the experiment. On the contrary, IgE in group B showed slight decline during this period (Table 1). During this phase, the remaining living oncosphere may be transported passively through blood or lymph to the internal organs where the subsequent larval proliferates. Increasing IgE level in group A may play an important role in killing the parasite or inhibiting its growth at this stage, to some extent, because anti-oncosphere antibodies play a major role in parasite killing and are central to the protective immune response against *E. granulosus* (Siracusano *et al.*, 2012). Also, this hypothesis can be evidenced by the previous study that the main function of mast cells, which rely on IgE, is to enhance the ability of eosinophils to kill the parasite (Sterla *et al.*, 1999).

IgM: Compared with group B, although IgM in group A decreased after infection with *E. granulosus*, it rose to the pre-infection level at 4h post-infection and sustained this level for a long period. No significant difference was observed during the early stage of infection, even though a dramatic drop occurred from day 3 post-infection (Table1). In group B, Systemic non-specific IgM concentration in serum decreased significantly within the first week post-infection (day 7 post-infection VS day 0, P=0.044), although it once increased slightly and leveled with pre-infection on day 1 post-infection (Table1). Similar results were found in susceptible Balb/C mouse oral infected with *Echinococcus multilocularis* eggs by Pater *et al.* (1998), who reported that systemic non-specific IgM did not increase significantly until 8 weeks post-infection.

**Changes in cytokines:** Cytokine profiles, due to the secretion of characteristic cytokines by T "helper" (Th) cells give an insight into immune mechanisms involved in host-infectious organism relationship and in the types of immune responses that are developed after the early stage of antigen recognition (Vuitton and Gottstein, 2010).

**Table 1:** Early antibody, cytokine and chemokine responses during *Echinococcus granulosus* (*E. granulosus*) infection in Kazakh sheep with different haplotype

Index	Group	Antibody	Post infection									
			0d (pre-infection)	2h	3h	4h	9h	1d	2d	3d	7d	
Antibody	IgE	A	852.9±9.5	770.5±1.6	945.9±2.8	967.9±1.7*	680.2±3.1	963.2±5.9	716.8±2.4	963.9±1.8	965.8±18.2	
		B	632.1±10.7	579.5±2.1	523.4±1.6	469.0±1.7	541.3±2.3	801.6±1.9	538.5±2.3	522.3±2.4	521.3±22.5	
Antibody	IgM	A	973.3±6.1	777.4±2.0	673.8±3.1	973.8±10.1	970.4±1.3	977.2±8.8	977.4±1.3	979.4±1.4	673.5±3.1	
		B	965.1±5.9	782.4±2.0	710.1±2.7	773.7±18.0	730.2±2.6	986.5±5.5	751.8±2.2	725.4±2.5	484.8±2.5	
Cytokine	IFN- $\gamma$	A	564.9±1.7	578.6±1.9	762.0±21.3	968.1±1.6*	968.1±1.6*	974.7±10.4*	969.2±17.6*	967.0±18.5	974.0±3.6*	
		B	685.0±1.7	269.9±7.3	433.6±18.1	465.1±1.7	252.9±6.8	248.8±3.6	274.3±6.7	457.4±24.9	274.1±7.7	
	TNF- $\alpha$	A	119.4±22.3	172.1±7.5	163.0±22.9	238.9±4.1*	245.0±9.1	197.2±36.1	197.2±5.9	186.5±22.6	165.0±4.2	
		B	137.2±7.5	46.9±2.5	60.5±10.2	64.3±8.9	132.1±6.1	61.2±8.7	64.2±3.8	56.7±5.9	72.2±3.7	
	IL-4	A	615.4±13.1	591.6±1.6	487.2±12.7	496.4±6.5	639.1±3.6	674.9±18.1	550.8±21.3	505.0±5.7	621.3±16.0	
		B	686.2±13.5	684.6±1.8	581.5±17.6	603.1±5.8	639.9±4.8	622.6±20.5	623.7±20.4	499.2±4.5	451.1±20.0	
	IL-10	A	49.6±8.2	70.8±6.5	79.5±8.2	70.6±7.1	63.6±10.0	71.0±20.0	70.8±21.0	67.1±18.6	70.8±30.2	
		B	97.6±5.4	133.0±9.9	131.2±10.2	154.5±8.7	133.8±15.4	201.5±40.4	153.9±42.2	196.1±35.3	201.5±41.5	
	Chemokine	CXCL-9	A	2.8±0.2	3.5±0.3	3.5±0.3	3.4±0.3	4.3±0.3	4.3±0.3	4.1±0.3	4.2±0.3	4.2±0.3
			B	2.3±0.2	2.3±0.2	2.2±0.2	2.8±0.2	3.3±0.3	3.5±0.3	2.8±0.2	2.8±0.2	2.7±0.2
CCL17		A	1363.3±86.2	1199.4±18.5	1322.0±100.0	1236.0±93.2	1186.5±37.6	1157.6±96.6	1141.6±77.0	1155.5±42.1	905.3±13.6	
		B	1267.0±86.9	1318.1±10.2	1344.9±142.4	1272.0±79.3	1240.5±56.6	1290.5±110.9	1241.2±62.6	1177.1±61.5	918.6±14.2	

Table 1 illustrates serum levels of antibodies (IgE and IgM), cytokines (Th1: TNF- $\alpha$  and IFN- $\gamma$ ; Th2: IL-4 and IL-10) and chemokines (Th1: CXCL-9; Th2: CCL17) at different time points of *E. granulosus* infection in Kazakh sheep with different haplotype. Group A: sheep with CE resistant haplotype: MHC-DRB1 *Mvalbc-SacIab-Hin1lab* (n=7), group B: sheep without this haplotype (n=7). Each value is the mean  $\pm$  SE (pg/ml). \*:  $P < 0.05$  (Group A vs. Group B).

**Th1 cytokines:** After remaining the same level as pre-infection, IFN- $\gamma$  in group A showed a marked increase from 2h post-infection to 4h post-infection, which thereafter sustained the high level till day 7 post-infection. In contrast, IFN- $\gamma$  in group B displayed a declining trend during the experiment, although a slight increase occurred between hours 2-4 and days 2-3 post-infection (Table 1). It is reported that early IFN- $\gamma$  production has a crucial role in the activation of peritoneal macrophages for the induction of protective immune responses against parasite, as well as the control of parasite invasion into organs and tissues, which is found in mice infected with *Neospora caninum* (Nishikawa *et al.*, 2001). The elevated IFN- $\gamma$  production in group A occurred from 2h post-infection, at this time, the oncosphere hatched by eggs might be transported to intestine, so higher IFN- $\gamma$  in CE resistant sheep (group A) might induce the activation of macrophages, thereby triggering the immune response of killing the oncosphere in the blood or inhibiting its invasion into liver, further preventing from the larval growth in the liver. On the other hand, Compared with group B, IFN- $\gamma$  production in group A is significantly higher from 4h post-infection to the end of the experiment (day 7 post-infection), except on day 3 post-infection ( $P > 0.05$ ) (Table 1). These significant differences may be associated with different MHC haplotype, thus resulted in differences of the susceptibility of *E. granulosus* infection between the two groups.

TNF- $\alpha$ , which is mainly produced by monocytes and activated T cells, plays an important immunoregulatory role. It was proved that TNF- $\alpha$  could inhibit echinococcosis growth in experimental mice (Amiot *et al.*, 1999). In this study, TNF- $\alpha$  level in group A rose significantly from initial infection to 9h post-infection, while in group B, it dropped largely within the two hours post-infection, despite a slight increase occurred subsequently. Generally, TNF- $\alpha$  level is much higher in group A than that in group B (Table 1). The difference for TNF- $\alpha$  level in the two groups seemed to be associated with different MHC haplotype, which was also observed previously by Godot *et al.* (2000), who found that AE patients with different MHC haplotype secreted different level of TNF- $\alpha$ . Additionally, significant difference of TNF- $\alpha$  level in the two groups occurred on 4h post-infection ( $P = 0.037$ ) in our experiment (Table 1). It is

found that TNF- $\alpha$  play a protective role at the initial stages of larval development, which could be beneficial to the host (Godot *et al.*, 2000), so, in our study, the early elevated level of TNF- $\alpha$  in group A might have a certain function in killing the parasite or preventing from its development.

In the present study, Th1-type systemic cytokine (IFN- $\gamma$ , TNF- $\alpha$ ) prevailed significantly in sheep with resistant haplotype, compared with others without this haplotype, at the early stage of *E. granulosus* infection. Immune responses in human CE as well as in animal models indicated that the development of Th1 cytokines was associated with disease control (Hernandez-Pomi *et al.*, 1997; Zhang *et al.*, 2003), while Th2 cytokines (IL-4 and IL-10) were found dominant in the sera of chronic CE patients (Rigano *et al.*, 1999), which were beneficial for parasite growth (Zhang *et al.*, 2008).

**Th2 cytokines:** In this study, we found that both IL-4 and IL-10 were lower in group A than that in group B, but no significant difference was observed between the two groups during the whole experiment (Table 1), which indicated that in the early stage of infection, Th2 type cytokines might not be dominant in CE resistance in Kazakh sheep with CE resistant haplotype. Whether Th2 cytokines were associated with CE susceptibility in Kazakh sheep without this haplotype, the mechanism need to be further determined.

**Changes in chemokines:** Chemokines account for monocyte, granulocyte and lymphocyte dissemination, directed cellular migration, cell adhesion, tissue infiltration and cell activation, and such, these chemotactic cytokines may promote host resistance by activating immune functions that kill, expel, or sequester *E. multilocularis* (Kocherscheidt *et al.*, 2008). In the present study, we have investigated the cellular Th1 type chemokine (CXCL-9) and Th2 type chemokine (CCL17) responses in CE resistant sheep and other random sheep. CXCL-9, the monokine, which is induced by IFN- $\gamma$ , was higher in group A than that in group B in this study (Table 1), such higher release of CXCL9 in group A was consistent with the high level of IFN- $\gamma$ , suggesting that Th1 chemokine might play a certain role in CE resistance. The chemokine CCL17 play a critical role for the

recruitment of Th2 type cells into target tissues of allergic inflammation (Romagnani, 2001). In this study, CCL17 was slightly lower in group A than that in group B, although there was no significant difference (Table1), suggesting that CCL17 was not associated with CE resistance.

The current study revealed that in the early stage of *E. granulosus* infection, antibodies like IgM and IgE, Th1 cytokines such as IFN- $\gamma$  and TNF- $\alpha$ , as well as Th1 chemokines CXCL-9 were predominant in sheep with CE resistant characters, especially for IgE and Th1 cytokines, which were more significant higher, most were at or began from 4h post-infection, when compared with sheep without this haplotype.

**Conclusion:** Our findings seem thus to confirm that the role of Th1-type in the controlling of the disease and suggest that the influence of the host's genetic background on the immunopathology of *E. granulosus* infection in the early stage could be partially mediated by Th1-type cytokines and Ig E.

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