

Original Article

Impact of intestine mucosal immune barrier in sheep naturally infected with *Echinococcus granulosus*

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Abstract: Cystic echinococcosis, caused by larval stage of *Echinococcus granulosus*, is a globally distributed food-borne infectious disease continues to be a major public health issue in developing countries. The pathogenesis process has been considered as “cross-talk” between the parasite and host’s immune system. Although efforts have been made to illustrate circulatory and local immunological response, however, few studies have been reported on the possible role of intestinal mucosal barrier as it might constitute magnificent role. In this study, interleukin (IL)-6, IL-10, and interferon (IFN) γ levels in serum and intestinal mucosa tissues were respectively detected in 10 naturally infected CE sheep and 10 controls to assess Th1/Th2 cell activation in vivo. Significantly higher serum levels (measured with an ELISA) of IL-10 were found in CE sheep ($P<0.1$) than in controls. In contrast, significantly higher levels (measured with Immunohistochemistry) of IFN- γ were found in CE sheep intestinal mucosa ($P=0.005$) than in controls. In conclusion, our data showed Th2 dominated immune response in serological immune, however, it maybe Th1 dominated immune response in intestinal mucosa immune barrier in CE sheep.

Keywords: Parasite immunology, echinococcosis, cytokine, intestinal mucosa immune barrier

Introduction

Cystic *Echinococcosis* (CE) is a widespread chronic endemic helminthic disease caused by the larval stage of *Echinococcus granulosus* (*E.granulosus*) [1]. It is well known as a public health hazard to the intermediate hosts include sheep, goats, pigs, horses, cattle, and as well as humanbeings [2]. The disease is prevalent in many areas, such as South America, the Mediterranean littoral, Eastern Europe, the Near and Middle East, East Africa, Central Asia, and China [3]. Dogs and other canids, as the definitive hosts, harbor the intestinal stage of tapeworm which produces infective eggs. The intermediate hosts are infected by ingesting the eggs in contaminated food or water [4]. After ingestion by a suitable intermediate host (herbivore: sheep, humans), the egg attaches to the intestinal mucosa, and hatches in the small intestine and releases a hooked larva called

oncosphere. The embryo, by means of its six hooks, penetrates the intestinal barrier and migrates via the blood stream into major filtering organs, such as liver and lungs [5]. *E.granulosus* stimulates both humoral and cellular immune responses. It could use two mechanisms to subvert the host immune response: passive escape, in which the parasite, by developing into a hydatid cyst, avoids the damaging effects of an immune response, and immunomodulation, through which the parasite actively interacts with the host immune system to reduce the impact of a host response [6].

As the first line of defense, intestinal mucosa forms a dynamic defensive barrier that is responsive to the external environment [7]. The barrier has four major components: mechanical barrier that consists of the intestinal mucosa epithelial cells and intercellular tight junctions with biofilm; micro-ecological barrier that con-

sists of the intestinal tract in fungi and the host micro space structure form of interdependence and interaction; chemical barrier that consists of hydrochloric acid in gastric juice, bile, digestive enzymes, lysozyme, sticky polysaccharides, glycoproteins and glycolipids secreted by intestinal mucosa epithelial cells, and gut associated lymphoid tissue and immune activity of products constitution the immune barrier [8]. The mucosal surface of the intestinal tract is the largest body surface in contact with the external environment (200 to 300 m²). The host is protected from attack by potentially harmful enteric microorganisms by the four kinds of intestinal mucosa barriers [9]. The intestinal mucosa is provided with an important branch of the immune system, which has the difficult task of protecting the intestinal tract while maintaining a non-inflammatory status despite the presence of massive amounts of bacteria and other microbes. Changes in intestinal immune barrier function have been extensively studied in relation to parasitic infections, particularly intestinal dwelling nematodes [10] and *Trichuris muris* (*T. muris*) [11]. It is clear that intestinal immune systems play a key role in resistance and susceptibility to chronic parasitic infections [12]. Few studies, however, have been published on the study of the impact of intestine mucosal immune barrier in sheep naturally infected *Echinococcus*. In this study, we, therefore, investigated serum and in intestine levels of interleukin(IL)-6, IL-10 and interferon (IFN) γ in sheep naturally infected with *E.granulosus* in comparison to healthy controls aiming to obtain further insight into the possible role of intestine mucosal immune function in sheep with CE.

Materials and methods

Chemicals

Sheep cytokines including IL-6, IL-10 and IFN- γ were purchased from Shanghai Kejian Chemical Ltd (Shanghai, China). Anti-rabbit antibody (Shanghai Zhongshan Jingqiao Chemical Ltd, China) was used as a secondary antibody. Immunohistochemical primary antibodies IL-6, IL-10 were purchased from Biorbyt (USA) and IFN- γ were purchased from Abdsertec (USA).

Experimental animals and controls

The study animals consisted of 10 sheep, with hydatid cyst placed in the livers (Type CE1 or

CE2 according to the WHO classification, determined by ultrasonography), which were obtained from the Bayinbuluke of Xinjiang. This region has a high prevalence of CE in sheep (36.9%) as previously reported [13]. Ultrasonography was performed using an OptiGo equipment (LOGIQ BOOK, American GE Company) with a 3-5 MHz transducer. Sheep were selected and assigned to groups in two steps. First, a total of 10 experimental according to the requirements of experiment, and 10 controls ewes with eight-tooth erupted (24 to 36 months of age) were randomly selected by applying systematic random sampling procedure in a corral from a flock of 986 sheep already determined for culling by the cooperative. Animals that were clinically sick, unable to move and feed by them were excluded before applying the systematic randomization. Each selected sheep was identified using numbered ear tags at the beginning of the study. Next, having a list with those selected and numbered sheep, these animals were maintained in the same corral with the rest of the flock under identical food and water availability. Animal conditions were monitored prior to and throughout the experimental period. Animal procedures and management protocols were approved by the ethical committee of 1st affiliated hospital of Xinjiang Medical University (IACUC20141021001). Blood samples were collected at similar day-time in all sheep. Sheep were fasting for at least 12 h. Blood samples (5 ml) were taken from the jugular vein using 10 ml heparinised Vacutainers tubes. Plasma was separated by centrifugation at 2000 $\times$ g for 15 min, placed into plastic tubes and kept frozen at -20°C until analysis. A portion of approximately 2.0 cm of small intestine was removed and immediately fixed in 10% formaldehyde solution at least 48 h when the animals were slaughtered. Intestinal samples fixed in 10% formaldehyde solution, were stored at 4°C until quantification of parameters.

ELISA and Immunohistochemistry

Peripheral serum concentrations of total IL-6, IL-10 and IFN- γ were quantified by enzyme-linked immune sorbent assay (ELISA), with standard curves from 5 to 60 ng/ml (IL-6, first standard 0.39 ng/ml), 6 to 72 pg/ml (IL-10, first standard 0.78 pg/ml), and 75 to 900 pg/ml (IFN- γ , first standard 0.39 pg/ml). Intra-assay and inter-assay variations, as determined by the manufacturer, were 9%/15% for IL-6, IL-10,

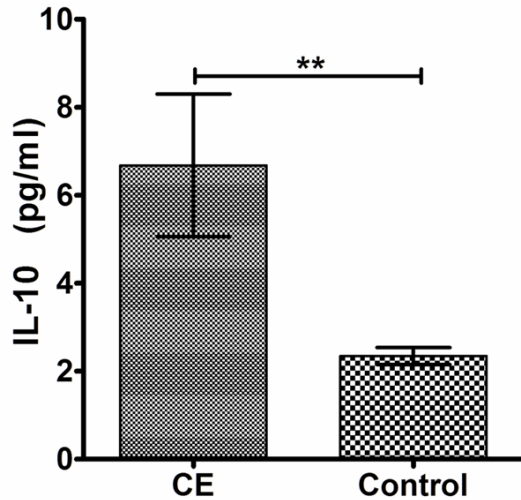


Figure 1. Serum level of IL-10 (pg/ml) in the CE sheep (n/10) and healthy controls (n/10). The box plots display the median. Significance of difference was analyzed by Mann-Whitney U test, (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.00$ respectively; n. s.: not significant).

and IFN- γ . Cytokine levels of samples were determined using commercial ELISA kit closely according to the manufacturer's instruction.

After the procurement of intestinal tissue of both control and experimental sheep, the intestinal tissues were formalin-fixed and paraffin-embedded for subsequent immunohistochemical analysis. Serial sections (4 μ m) were mounted on charged glass slides and deparaffinized using xylene and a decreasing series of ethanol. After washing with TBS/Tween (pH 7.4), antigen retrieval was performed by microwaving in 10 mmol/l citrate buffer (pH=6.0). The sections were blocked in normal goat serum for 30 min (ZSGB-BIO). Next, the sections were incubated for either one night with the corresponding primary antibodies against IL-6 in 4°C (1:200, sheep monoclonal, Biorbyt), IL-10 (1:100, sheep monoclonal, Biorbyt) and IFN- γ (1:100, sheep monoclonal, Abdsertec). Afterwards, the sections were incubated with secondary antibodies for 30 min in 37°C. The specific staining reaction was completed by incubating the slides in the presence of 3, 3'-diaminobenzidine (DAB) in buffer reaction solution (Dako) and observed as a brown staining. The sections were counterstained with hematoxylin. The negative controls were carried out by substitution of the primary antibodies with

non-immunized serum; resulting in no immunostaining signal detection. The cells were initially observed at a low magnification ($\times 100$) to assess the general distribution of the primary antibody. The samples were subsequently examined at a higher magnification ($\times 400$). The evaluation of cell staining was performed in intestinal mucosa tissue. The intestinal mucosa cells (exhibiting gross and evident nucleoli, and irregular chromatin) were identified and counted at the higher magnification. Immunohistochemical staining was evaluated in the cytoplasm of intestinal mucosa cells. The intensity of staining of each section was interpreted by a specialized pathologist (who was blinded to the experiment) using the following designations: 0-10% of cells stained, score 0; 11-25% of cells stained, score 1; 26-50% of cells stained, score 2; 51-100% of cells stained, score 3. Those scoring 0-1 were considered to be negative, and those scoring 2-3 were considered to be positive.

Statistical analysis of the data

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS); version 17.0. All continuous variables were expressed as median (interquartile range, IQR) in text. Mann-Whitney U test was performed to detect the difference between the two groups. Spearman correlation was used as a test of correlation and determined by Spearman correlation coefficients. A probable value of $P \leq 0.05$ was considered to be statistically significant.

For immunohistochemical staining, the concordance between staining intensity scores for each sample was calculated according to Cohen's κ coefficient: $\kappa < 0.4$, slight concordance; $\kappa \geq 0.4$ and < 0.8 , moderate concordance; $\kappa \geq 0.8$ and < 1 , strong concordance; and $\kappa = 1$, perfect concordance. The first κ inter-rater was between 0.8 and 1.0 of 1% (between strong and perfect concordance). All discordant cases were reevaluated and the result was determined by consensus. The association between staining intensity and tumor classification was evaluated using Fisher's exact test. $P < 0.05$ was considered to indicate a statistically significant difference.

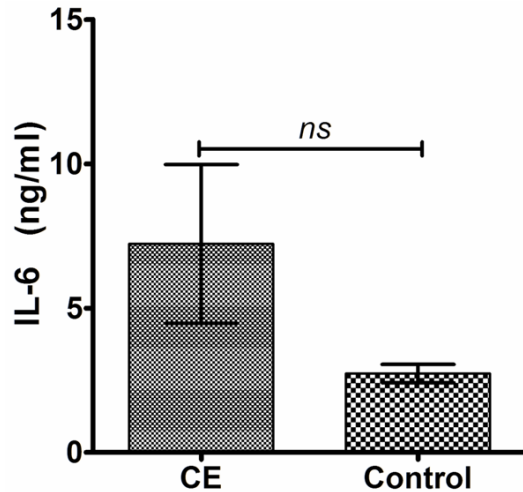


Figure 2. Serum level of IL-6 (ng/ml) in the CE sheep (n/10) and healthy controls (n/10). The box plots display the median. Significance of difference was analyzed by Mann-Whitney U test, * $P < 0.5$; ** $P < 0.1$; *** $P < 0.00$ respectively; n. s.: not significant.

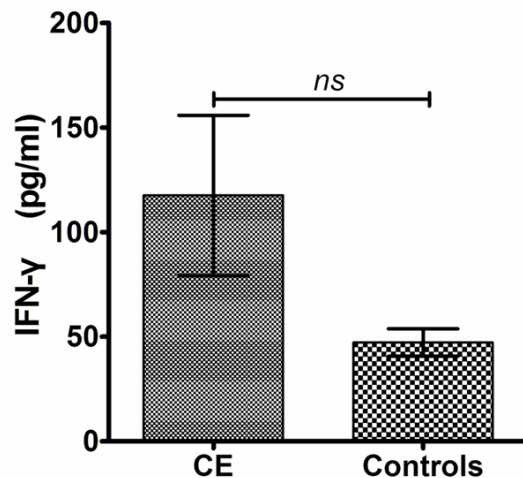


Figure 3. Serum level of IFN-γ (pg/ml) in the CE sheep (n/10) and healthy controls (n/10). The box plots display the median. Significance of difference was analyzed by Mann-Whitney U test, * $P < 0.5$; ** $P < 0.1$; *** $P < 0.00$ respectively; n. s.: not significant.

Results

Cytokine serum levels of CE sheep compared to healthy controls

As showed in **Figure 1**, IL-10 concentration levels were significantly increased in CE group (4.65; IQR, 2.94-11.06) when comparing to control group (2.30; IQR, 2.08-2.68; ** $P < 0.1$). IL-6 and IFN-γ levels in CE group (3.05; IQR, 1.76-12.93, 58.61; IQR, 34.23-219.06) were

elevated compared to those in control group (2.68; IQR, 2.03-3.22, 45.59; IQR, 33.29-54.71), however, with no statistical difference ($P > 0.5$, **Figures 2 and 3**).

Cytokine intestinal mucosal levels of CE sheep compared to healthy controls

The immunohistochemical analysis of control and experimental (*E.granulosus* infected) histological sections for IL-6 showed absent or weak (0-1) staining in three (30.0%) control group and in six (60.0%) *E.granulosus* infected group (tissues), and moderate or strong (2-3) staining in seven (70.0%) control group and in four (40.0%) *E.granulosus* infected group (tissues). Regarding IL-10, there was absent or weak (0-1) staining in six (60.0%) control group and five (50.0%) *E.granulosus* infected group (tissues), and moderate or strong (2-3) staining in four (40.0%) control group and five (50.0%) *E.granulosus* infected group (tissues). IFN-γ staining analysis showed absent or weak (0-1) staining in six (60.0%) and moderate or strong (2-3) staining in four (40%) control group (tissues). All the tissues in *E.granulosus* infected group were moderate or strong (2-3) stained (**Figure 4**).

Immunohistochemical staining for IL-6 was more intense in control group compared with *E.granulosus* infected group, but this difference was not statistically significant ($P = 0.185$). As for IL-10, it was more intense in *E.granulosus* infected group when comparing to control group, however with no statistically differences ($P = 0.500$). Immunohistochemical staining of IFN-γ was obviously more intense in *E.granulosus* infected group (tissues) compared with control group ($P = 0.005$), as seen in **Table 1**.

Discussion

The intermediate host (such as sheep, and humans) produces a significant immune response against *E.granulosus* infection [14]. Several studies have focused on the mechanisms of host-parasite interplay in CE. The immune response to *E.granulosus* infection has been investigated through both clinical studies on patients with hydatidosis sheep and murine experimental models [4]. *E.granulosus* stimulates both TH1 and TH2 response: elevated levels of Th1 cytokines, especially IFN-γ [15], but

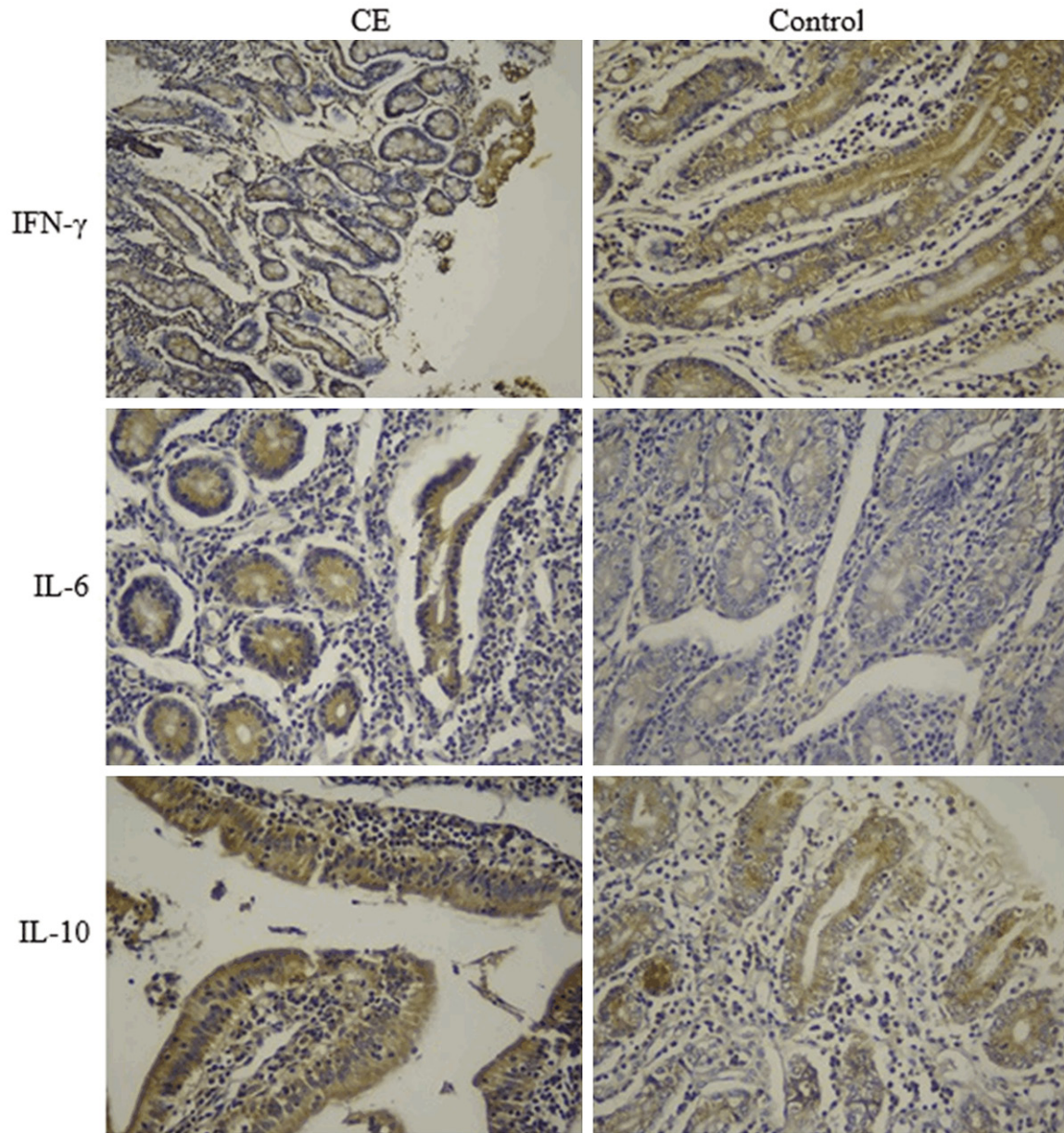


Figure 4. One representative picture from each group of at least four is shown. Magnification, $\times 400$.

also Th2 cytokines, such as IL-5, IL-6 [16] and IL-10 [17], have been recorded in patients with hydatidosis. The reason for this ambiguous cytokine secretion pattern is not known: Th1 and Th2 responses usually down-regulate each other, with a cross-inhibitory mechanism; it is assumed that the complex antigenic organization of *Echinococcus* may induce both T-cell subsets [18].

Intestine is not only one of the oldest tissues and exists for digestion and adsorption of nutri-

ents even in lower class animals, but also is the largest organ of immunity in the body [19]. After the opportunistic ingestion, the parasite has to undergo a close interaction with intestinal mucosa, since it is mandatory for hatching process and dwelling in the liver. More important is that the intestinal mucosal immune system provides the first line of defense against most parasites entering into the body. In fact, 60% of the immune cells in the body are proven to be present in the intestine. We believe that a complex immune response mechanism occurs

Table 1. Immunohistochemical staining of IL-6, IL-10 and IFN- γ in *E.granulosus* infected and control group

	IL-6				IL-10				IFN- γ			
	Score 2-3		Score 0-1		Score 2-3		Score 0-1		Score 2-3		Score 0-1	
	n	%	n	%	n	%	n	%	n	%	n	%
Control Group (n=10)	7	70	3	30	4	40	6	60	4	40	6	60
Experimental Group (n=10)	4	40	6	60	5	50	5	50	10	100	0	0

$P=0.185$ (IL-6), $P=0.500$ (IL-10) and $P=0.005$ (IFN- γ). IL-6 (10), Interleukin-6 (10); IFN- γ , Interferon- γ .

when the parasite enters the gut. The intestine is an important immune interface, and polarized cytokines profiles produced by Th1 and Th2 cells are integral to the coordination of immune and nonimmune cell interactions that mediate protection against parasites. Miller [20] and Nawa [21] have already reported that *Nippo strongylus brasiliensis* and *Strongyloides ratti* expulsion are mediated by T-cell-dependent intestinal immune response of the host. It was found that IFN- γ produced by intestinal macrophages can lead to the loss of tryptophan and iron in cells, and the proliferation of *Toxoplasma gondii* in macrophages and epithelial cells is inhibited [22]. However, there is plenty evidence show Th2 immune response play a critical role in the intestine. Murine Infection with intestinal nematode parasites, such as *N. brasiliensis*, induces a strong Th2 cytokine response required for worm expulsion [23].

In line with this, human and murine experimental study also displayed Th1/Th2 imbalance during the infection. Enhanced Th1 is correlated with resistance, while Th2 is correlated with susceptibility. In the present study, we aimed to characterize the changes within the intestinal mucosal immune barrier, in particular in its immune cytokines including IL-6, IL-10 and IFN- γ that may play a role in parasite rejection, by using naturally infected CE sheep models.

At our knowledge, this is the first report on the immune profile in circulatory and intestinal mucosa. The study displayed Significantly higher intestinal mucosal levels of IFN- γ in CE sheep compared to controls (moderate or strong (2-3) staining in four (40.0%) control group and ten (100%) *E.granulosus* infected group (tissues), $P=0.005$). Cytokine levels of IL-10 in the intestinal mucosal of CE sheep was increased (moderate or strong (2-3) staining in four (40.0%) control group and five (50%) *E.granulosus* in-

fectured group), then, IL-6 was decreased (moderate or strong (2-3) staining in seven (70%) control group and in four (40%) CE group), however both of them with no statistically differences (IL-10: $P=0.500$, IL-6: $P=0.185$). On the other hand, concentration of IL-6, IL-10, and IFN- γ in peripheral serum from CE infected and control group were assessed using ELISA technique. As it was showed, IL-10 levels were significantly increased in CE group, when comparing to control group (** $P<0.1$). IL-6 and IFN- γ levels in CE group were elevated compared to those in control group, however, with no statistical difference ($P>0.5$). Given the recent advances in understanding the immunoregulatory capabilities of helminthic infections, it has been suggested that Th2 responses play a crucial role in chronic helminthiasis [24]. Ample evidence shows that the coexistence of significantly high IL-10 concentrations in the peripheral serum observed in most of hydatid patients supports Th2 cell activation in human hydatidosis [25]. Our team's previous research results also show that plasma concentration levels of IL-5, IL-6, and IL-10 were slightly increased in AE and CE groups compared with those in healthy controls group with no statistical differences ($P>0.05$). Concentration levels of IFN- γ in CE subjects were markedly higher than those in healthy controls subjects ($P<0.01$) [26]. IL-10 is an anti-inflammatory cytokine and actively involved in the immune tolerance process of parasite. Opposite to TH1 related cytokines, IL-10 is reported to increase the viability of the parasite and exaggerate the parasitic infection [27]. Both human and experimental study showed imbalanced TH response during the infection highlighted with increased number of Treg cells in circulation and infected liver [28]. The interaction of the *E.granulosus* organisms with their mammalian hosts may provide a highly suitable model to address some of the fundamental questions remaining such as the

molecular basis underpinning the different effects of IL-10 on different celltypes, the mechanisms of regulation of IL-10 production, the inhibitory role of IL-10 on monocyte/macrophage and CD4 T cell function, its involvement in stimulating the development of B cells and CD8 T cells, and its role in the differentiation and function of T regulatory cells [29].

IL-6 is produced by the T cells and macrophage and acts as pro-inflammatory cytokine. During the infection, the parasite secretory and/or excretory antigens can attract effective T cells and macrophages to surround infective tissue. Kanan et al reported that that hydatid fluid (HF) contains factors that can affect dendritic cell function, but that the effects may vary for acute and chronic exposure. Soluble factors from the HF may escape into the lymphatic system, and activate DC within draining lymph nodes to produce IL-12, IL-6 and PGE₂, and stimulate a mixed Th1/Th2 response to the parasite antigens. Once the hydatid cyst is fixed in a suitable host tissue, however, components of its fluid are likely to be released chronically into the pericystic microenvironment and stimulate a host inflammatory response, producing at least PGE₂ and IL-6 [30]. A human CE subsets showed significantly elevated levels of IL-6 [26, 31], in line with human study, experimental study also displayed the increased level of IL-6 [32].

IFN-γ mainly secreted by activated Th1 cell, with a variety of biological activity. It induced by antigen, inhibits the secretion of Th2 cytokines, mainly through enhanced the phagocytosis of macrophage proliferation and development of the capabilities and limitations of the intracellular parasite infection control. Current finding that most patients' PBMC produced abundant IL-4, IL-5, IL-6, IL-10 and IFN-γ demonstrates that the human immune response to *E.granulosus* infection is predominantly regulated by Th2 cell activation but also by Th1 cell subset [33]. It is unclear why hydatid infection can induce high levels of both Th1 and Th2 cytokines, since they usually downregulate each other [34]. Antigen and the amount of antigens released may play key roles. For instance, *E.granulosus* antigen B skewed Th1/Th2 cytokine ratios towards a preferentially immunopathology-associated Th2 polarization, predominantly in patients with progressive disease [35].

Few studies related to changes in intestinal immune factor of hydatid infection. In this study, we have focussed on the variety of intestinal mucosa immune cytokine IL-6, IL-10 and IFN-γ. Our new data highlighted the significance of late events in infection, which TH1 responses may be more active in intestinal mucosa. Immunohistochemical staining of IFN-γ was obviously more intense in *E.granulosus* infected group (tissues) compared with control group ($P=0.005$). Immunohistochemical staining for IL-6 was more intense in control group compared with *E.g* infected group, but this difference was not statistically significant ($P=0.185$). As for IL-10, it was more intense in *E.granulosus* infected group when comparing to control group, however with no statistically differences ($P=0.500$). Previous studies showed that not all parasites cause Th2-based intestinal immune responses. Oral infection with *Toxoplasma gondii* is controlled by a strong intestinal Th1 response, which is impaired in vitamin-A deficient mice [36]. Perhaps, the oncosphere hatch and become activated in the small intestine when a suitable intermediate host ingests Echinococcal eggs, but activate Th1 type intestinal immune response, causing it to be cleared or "evade" to elsewhere, and being not continue to grow in the intestinal wall. In fact, lytic secretions of the oncosphere then facilitate its passage through the intestinal mucosa and into the host circulatory system (venous and lymphatic) through which they are distributed to the liver, lungs, and other sites where post-oncospherical development continues, however, rarely reported about Intestinal hydatid.

In this study, naturally infected sheep was selected as a large animal model and related T helper cell related cytokines were detected. Despite of the preliminary results which may indicate possible correlation of intestinal immune profile and infection, some limitations exist in the current study. Firstly, only Th1 and Th2 immune profile in this study was considered, while more and more increasing data showed possible role of Th17 cells in mucosa immunity; second, only two groups were considered in this study, in the future study, it would be more informative if we analyze the role of anti-parasitic drugs on the intestinal immune barrier. At last but not least, human study could be taken into plan list due to ample number of patients in our center.

In conclusion, the present study is the first to demonstrate intestinal mucosa immune regulation of IL-6, IL-10 and IFN- γ activity and expression. The major findings to emerge from this study are that *E.granulosus* infection induced intestinal mucosa Th1 response up-regulation, and Th2 function down regulation. Further studies are, however, definitely necessary not only to investigate regulation of specific intestinal immune response in CE but also to determine the role of intestinal mucosa cytokine measurements in different stages of infection, in order to prevention of disease and therapeutically success.

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Disclosure of conflict of interest

None.

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