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Original Article

Dysregulation of Ovine Toll-Like Receptors 2 and 4 Expression by Hydatid Cyst-Derived Antigens

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Abstract

Background: Cystic echinococcosis (CE) is a zoonotic disease caused by infection with *Echinococcus granulosus*. Toll-like receptors (TLRs) as the first line of defense against various parasites play a critical role in sensing and triggering anti-parasite responses.

Methods: The study was conducted at the Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Iran in 2019. Ovine peripheral blood mononuclear cells (PBMCs) were stimulated with hydatid cyst-derived antigens including hydatid cyst fluid (HCF), germinal layer antigens (GL), somatic and excretory/secretory (ES) products of protoscoleces (PSC). To investigate whether the expression of TLR2 and TLR4 was altered during exposure to these antigens, PBMCs were stimulated with two different concentrations at different time points.

Results: After exposure of PBMCs to ES and somatic antigens of protoscoleces (PSC) the expression of TLR2 and TLR4 was down-regulated in comparison with control group. Similarly, HCF markedly down-regulated TLR2 and TLR4 transcripts independent of dose and time. GL antigens significantly down-regulated TLR2, while TLR4 expression was up-regulated as compared with control group.

Conclusion: Hydatid cyst-derived antigens could dysregulate the expression of TLR2 and TLR4 in ovine PBMCs, suggesting a possible mechanism to suppress host immunity to establish chronic infection.

Introduction

Cystic echinococcosis (CE) is a major public health concern caused by the larval stage of tapeworm, *Echinococcus granulosus* (1). The massive size and high proliferation of echinococcal cysts in the animal body, no significant inflammatory responses are induced against them (2). Although, CE in the early stages induces neutrophils and macrophages infiltration along with leukocytosis, in the "established metacestode" phase, the parasite successfully sets up the chronic infection (2,3). Innate immunity as the first line of host defense plays a critical role in the preservation and early diagnosis of *E. granulosus* infection (3,4). Excretory secretory (ES) products of *E. granulosus* through inhibition of dendritic cells (DCs) maturation and suppression of their functions strictly confine innate immunity (5). In addition, up-regulation of the $CD4^+CD25^+Foxp3^+$ T cells is another important mechanism exploited by *E. granulosus* to prevent antiparasite responses (5). Altogether, the exact method through which *E. granulosus* manipulates the innate immune system is not fully understood; however there are growing proofs suggesting that toll-like receptors (TLRs), as the most important part of pattern recognition receptors (PRRs) family, may well be relevant in echinoccocal infections (6,7). TLRs are one of the strongest arms of innate immunity which sense and respond to helminth-derived antigens (8). CE infection possibly through TLRs expression up-regulate anti-inflammatory cytokines and suppresses host immunity (9,10).

Up to now, very limited studies have been done concerning the effects of CE infection on TLRs expression in susceptible animals such as sheep and cows. This study aimed to assess the effects of hydatid cyst-derived antigens, including somatic and ES products of *E. granulosus* protoscoleces, germinal layer (GL), and hydatid cyst fluid (HCF) on the expression of TLR2 and TLR4 in ovine PBMCs.

Materials and Methods

Extraction of hydatid cyst fluid antigens (HCF)

Hydatid cysts were obtained from the lung tissues of infected sheep slaughtered at abattoirs in Mashhad, Khorasan-Razavi Province, Iran in 2019. Lung tissues were transferred to the Parasitology Lab of Faculty of Veterinary Medicine. Then, the surface of the cysts was disinfected by iodine alcohol and the cyst fluid was aspirated from fertile cysts under sterile conditions. To remove the protoscoleces and large particles, hydatid cyst fluid was centrifuged at 1500 g for 15 min and the supernatant was collected as hydatid cyst fluid antigens (HCF). Then, HCF was concentrated using polyethylene glycol 6000 (Merck, Darmstadt, Germany) and dialysis membrane (cut off 12000). The concentrated HCF antigens were kept at -20 °C until used.

Extraction of germinal layer antigens (GL)

Upon aspiration of cyst fluid, the germinal layer was removed The LL was separated from the GL by peeling and scraping with a scalpel under light microscope. The separated tissues were washed several times in phosphate-buffered saline (PBS) and examined by light microscope to confirm the absence of protoscoleces. The freeze/thaw process was performed on GL extracts and then homogenized in a glass homogenizer with an equal volume of PBS. The preparation was then disrupted by sonication in a 150 W ultrasonic disintegrator (10s on 5 sec off) on ice for 25 minutes. The main part of the sonicated material (milky suspension) was kept at -20 °C until used (11).

Extraction of somatic and excretory/secretory (ES) antigens of protoscoleces (PSC)

To obtain the ES antigens of protoscoleces, at first, PSC was aseptically collected from fertile cysts and maintained at 4 °C in sterile PBS containing 1µg/ml gentamicin. PSC viability was tested by eosin exclusion (12), and those batches with over 95% viability were used. Opsonized PSC was cultured in PBS complemented with 10% glucose, 100 unit/ml penicillin, and 100µg/ml streptomycin at 37 °C in 5% CO_2. The medium was renewed every 8h and after 50h of culture, the ES products were collected for concentrating and storing at -20 °C. Moreover, for obtaining the somatic antigens, PSC was thawed and sonicated (10 cycles of 12 sec at 60 Hz frequency) (UP 200s Dr. Hielscher GmbH, Germany) on ice for 25 minutes. freeze-thawed once more and centrifuged for 35 min at 2300×g. Supernatants were stored at -20 °C.

Somatic antigens were obtained from protoscoleces removed by aseptic cyst puncture as described by Smyth and Davies (13). Briefly, the protoscoleces were washed three times with PBS; pH 7.2 and subjected to three cycles of freezing and thawing and re-suspended in 10 volumes of PBS 7.2 containing 0.5M phenylmethylsulfonyl fluoride (PMSF) (at a ratio of 1 in 100). Samples were sonicated in the vicinity of ice (1 min, 0.5 amplitude) until no intact protoscoleces were visible microscopically. Sonicate was left at 4 °C overnight and then centrifuged at 10,000rpm for 30 minutes. The supernatant was collected, dispensed in small aliquots and stored as protoscolex somatic antigens at -20 °C.

The concentration of all extracted antigens was measured by standard Bradford method and the integrity of their protein contents was analyzed by SDS-PAGE (data not shown).

Isolation of PBMCs and stimulation with hydatid cyst derived antigens

Only lambs aged between 1 and 2 months, which received 5 mL blood from each lamb, were included in the study. Peripheral Blood Mononuclear Cells (PBMCs) were collected intravenously from five healthy lambs in sterile venipuncture tubes containing EDTA and transferred to Immunology lab of Faculty of Veterinary Medicine. PBMCs were isolated with the standard Ficoll-hypaque method and the cell viability was tested by Tripan-blue staining. Then, the PBMCs were placed in 6-well cell culture plates (12 x10^6 cells/well) and cultured in RPMI1640 (Biosera, East Sussex, UK,) containing Hepes (25mM), L-Glutamine (2mM), 10%FBS, Penicillin (100 unit/ml) and Streptomycin (100 µg/ml). The PBMCs were immediately exposed to different concentrations of hydatid cyst-derived antigens (50 µg/ml and 100 µg/ml) for 2 h and 18 hours. Moreover, in each plate, the cells of one well are considered as control without Ag stimulation. During the culture period, the plates were kept at 37 °C with 95% humidity and 5% CO_2.

RNA isolation & cDNA synthesis

Total RNA was extracted from the cell pellets using high pure RNA isolation kit (Roche Applied Science, Penzberg, Germany) based on the manufacturer's instructions. Then RNA integrity was tested by running the RNA samples on the denaturing agarose gel and observing the intensity and sharpness of 18 sec and 28 sec rRNA bands. The quantity and purity of the extracted RNA were determined by measuring the A260/A280 ratio by a nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). For cDNA synthesis, 2µg of total RNA was mixed with 1µl of oligo $(dT)_{12-18}$ primer (Fermentas, Vilnius, Lithuania) and diluted to 13µl with DEPC treated water, heated to 70 °C for 5 min, and then rapidly chilled on ice. Then 2µl of 10mM dNTP mix (Cinagen, Karaj, Iran) plus 4µl of 5X buffer were added and the mixture was incubated for 5 min at 37 °C. Finally, to each reaction 1µl (200 units) M-MuLV reverse transcriptase (Fermentas, Vilnius, Lithuania) was added and the mixture was incubated for 60 min at 42 °C and at 72 °C for 10

min to stop the reaction. All incubations were carried out using Thermal Cycler apparatus (Primus 25, Germany). A non-RT control was also prepared for each sample. The cDNA product was kept at -20 °C until used.

Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR)

Expression of TLR2 and TLR4 was analyzed by qRT-PCR using the Corbett 6 apparatus (Rotor-Gene, Australia). The exon-specific primer sequences to amplify transcripts of ovine TLR2, TLR4 and GAPDH were designed by primer premier software (Primer Premier 5 PREMIER Biosoft Int. Palo Alto, CA) and synthesized by Bioneer (Daejeon, South Korea) (Table 1).

Table 1: Gene-specific primers used for quantitative real-time RT-PCR assays

Specific primer	*Forward*	*Reverse*	*Product Size (Amplicon)*
TLR2	5'-GGTTTTAAGGCAGAATCGTTTG-3'	5'- AAGGCAC-TGGGTTAAACTGTGT-3'	190 Kb
TLR4	5'- CTTGCGTACAGGTT-GTTCCTAA-3'	5'- CTGGGAAGCTGGA-GAAGTTATG-3'	153 Kb
GAPDH	5'- TCAAGAAGGTGGTGAA-GCAG-3'	5'- TGTCGTACCAGG AAATGAGC-3'	174 Kb

TLR: Toll-like receptor, GAPDH: glyceraldehyde-3-phosphate dehydrogenase

The specificity of the amplification reaction was determined by a melting curve analysis, including fluorescence measurement every 15 sec, 0.3 degree of temperature rise from 65 °C to 95 °C. We performed relative quantification through normalizing the TLR2 and TLR4 genes signals with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) signal. To provide uniformity across all plates reactions were performed in triplicate in a 20 µl volume, including 400 ng cDNA, 10 µl **S**YBRGreen PCR mastermix (QIAGEN, Germany), nuclease-free water, 2 pmol forward, and reverse primers. Parallel to each cDNA sample, a non-RT control was also run. qPCR products were visualized by 2% agarose gel electrophoresis stained with ethidium bromide. Relative fold change expression of genes was calculated using the Pfaffl method (17). To quantify the results, a 10-fold serial dilution standard curve of a pooled of five cDNA samples obtained from PBMCs of one-month-old healthy young lambs as the calibrator for any primer (TLR2, TLR4, and GAPDH) was performed. The same cDNA sample was used for all the standard-curve runs. A threshold of detection was set based on the duplicate control samples lacking a template.

Statistical analysis

SPSS software, version 19 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 6 (GraphPad Software, Inc, San Diego, CA, USA) were used to conduct the statistical analysis. One samples t-test was used to analyze experimental data. Expressions of TLR2 and TLR4 in all groups were screened for normality using Shapiro-Wilk test. All data was expressed as mean ± standard error of mean (SEM). Overall P values were given in the figure legends ($P<0.05$ was considered as significant) and significant differences between treatments/groups were shown in the figures.

Results

Excretory/secretory (ES) antigens of protoscoleces (PSC) can down-regulate the expression of ovine TLR2 and TLR4 in PBMCs

We performed qRT-PCR to investigate whether the expression of TLR2 and TLR4 is altered by ES exposure in a dose and time-point-dependent manner. Based on qRT-PCR results the expression level of TLR2 and TLR4 genes in the PBMCs exposed to ES was determined on the mRNA level and compared with ES-unexposed PBMCs as a control group. Totally, after treatment with ES the expression of both TLR2 and TLR4 mRNA was reduced relative to the control group. Expression analysis of TLR2 showed that ES down-regulate the expression of this receptor on the ovine PBMCs (Fig.1A). Differences between treatment groups and control groups in the expression of TLR2 were not significant at different doses and time points tested following exposure to ES antigens (P>0.05).

Expression of TLR4 was also measured in all groups of PBMCs exposed to ES. Except PBMCs treated with 50 µg/ml of ES for 2h, expression of TLR4 in other groups was down-regulated (Fig. 1B). Analysis of mRNA expression showed a marked reduction of TLR4 expression in the groups (ES 100 2 h and ES 50 18 h) as compared to control (P<0.05). Quantification of TLR4 transcripts in PBMCs exposed to 50 µg/ml of ES for 2 h (ES 50 2 h) showed a highly significant up-regulation (P=0.008) (Fig. 1B). The expression of TLR4 showed transient up-regulation after 2 h exposure to 50 µg/ml of ES. Interestingly, this up-regulation was shown to be specific only to this group, while in other PBMCs treated with ES, there was no increase in expression of TLR2 and TLR4.

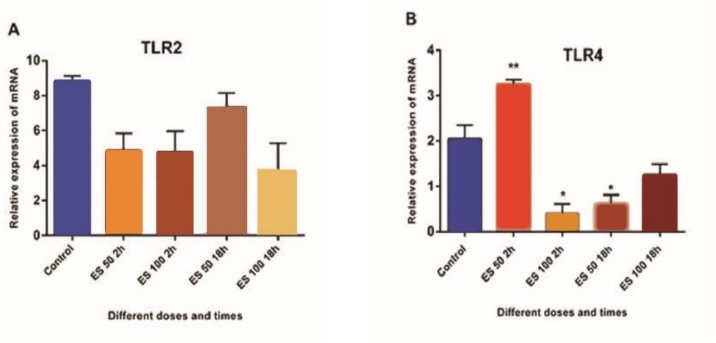

Fig. 1: Expression pattern of TLR2 and TLR4 in ovine PBMCs stimulated with ES antigens. Baseline expression of TLR2 and TLR4 corresponds to control group. Values were mean ± SEM in this figure. Significant difference in expression between ES-treated PBMCs and control group is indicated by asterisk (*) (P < 0.05). All fold changes is normalized to the housekeeping gene GAPDH

Exposure to germinal layer (GL) antigens significantly down-regulates TLR2, while up-regulates TLR4 in ovine PBMCs

The expression level of TLR2 and TLR4 genes in the PBMCs treated with GL antigens was measured by qRT-PCR and compared with GL-unexposed PBMCs as a control group. As shown in Fig. 2A, GL antigens at different doses and time-points significantly suppressed TLR2 expression in PBMCs (P<0.004) (Fig. 2A). By contrast, TLR4 expression was found to be increased after exposure to GL antigens, but this up-regulation was not statistically substantial. There was no significant increase in expression of TLR4 in PBMCs treated with GL antigens (P>0.07) (Fig. 2B).

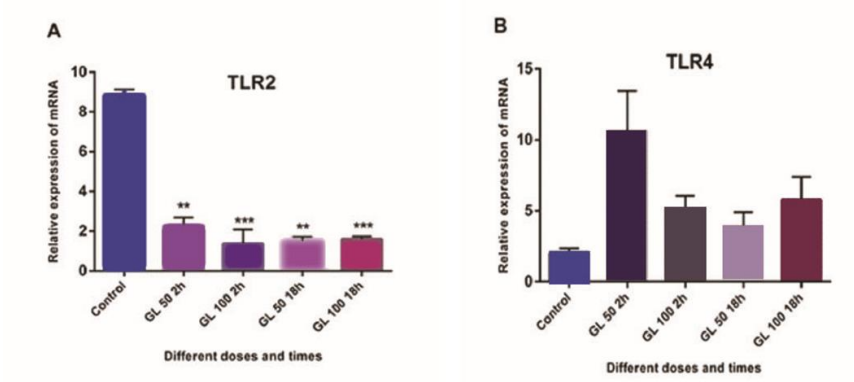

Fig. 2: The effect of GL antigens on the expression pattern of TLR2 and TLR4 in ovine PBMCs. Baseline expression of TLR2 and TLR4 corresponds to control group. Values were mean ± SEM in this figure. Significant difference in expression between GL-treated PBMCs and control group is indicated by asterisk (*) ($P < 0.05$). All fold changes is normalized to the housekeeping gene GAPDH

Expression of TLR2 and TLR4 in PBMCs is markedly down-regulated in response to hydatid cyst fluid antigens (HCF)

The expression of TLR2 and TLR4 transcript was quantified using qRT- PCR in PBMCs cultured with HCF antigens. As demonstrated in Fig. 3A, HCF antigens were found to extremely down-regulate the expression of TLR2 in all groups of PBMCs treated with these antigens. The expression of TLR2 transcript at different doses and time-points of HCF exposure was highly significant in comparison with un-exposed PBMCs ($P<0.001$).

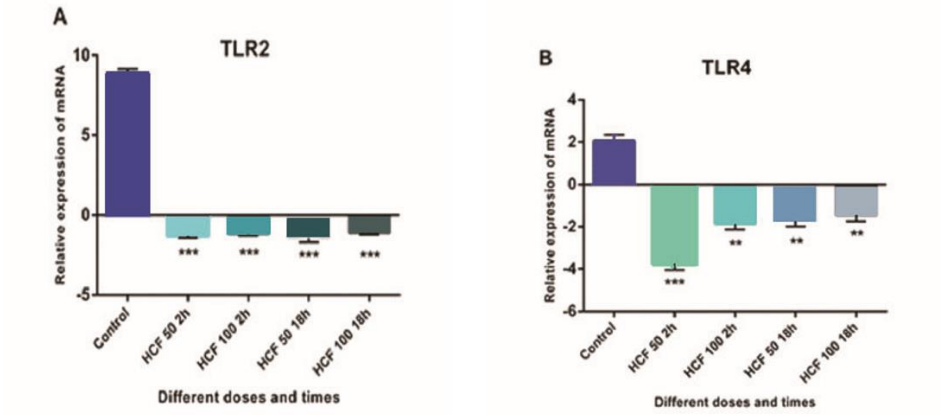

Fig. 3: The expression of TLR2 and TLR4 in ovine PBMCs stimulated with HCF in different doses and time points. Baseline expression of TLR2 and TLR4 corresponds to control group. Values were mean ± SEM in this figure. Significant difference in expression between HCF-treated PBMCs and control group is indicated by asterisk (*) ($P < 0.05$). All fold changes is normalized to the housekeeping gene GAPDH

Just like TLR2, after analysis of the TLR4 expression in PBMCs treated with HCF antigens, a significant reduction was observed in comparison with un-exposed PBMCs (Fig. 3B). Among treated groups, the most significant reduction of TLR4 expression belonged to PBMCs exposed to 50 μg/ml of HCF for 2h (HCF 50 2h) ($P<0.001$). Whereas, the level of TLR4 expression in other treated groups were equally down-regulated ($P=0.001$).

Somatic antigens of protoscoleces (PSC) decrease the expression level of ovine TLR2 and TLR4 in PBMCs

The expression level of ovine TLR2 and TLR4 after culturing the PBMCs with 50 μg/ml and 100 μg/ml of somatic antigens was measured by qRT-PCR. As shown in Fig. 4A, the expression of TLR2 in all treatment groups was significantly reduced (P<0.01), except for the PBMCs treated with 50 μg/ml of PSC (P>0.05).

The level of TLR4 expression was also determined by qRT-PCR and treatment groups were compared with the PSC-unexposed group. Apart from the groups treated with 50 μg/ml and 100 μg/ml of somatic antigens of PSC for 2h (PSC 50/100 2h), other two treatment groups showed a significant reduction of TLR4 expression (P<0.04) (Fig. 4b). The expression of TLR4 in PBMCs exposed to 50 μg/ml of PSC for 2h showed transient up-regulation, but not significant (P=0.17). Moreover, the level of TLR4 transcript in PBMCs was not markedly changed after exposure to 100 μg/ml of somatic antigens for 2h (P=0.24) (Fig. 4B).

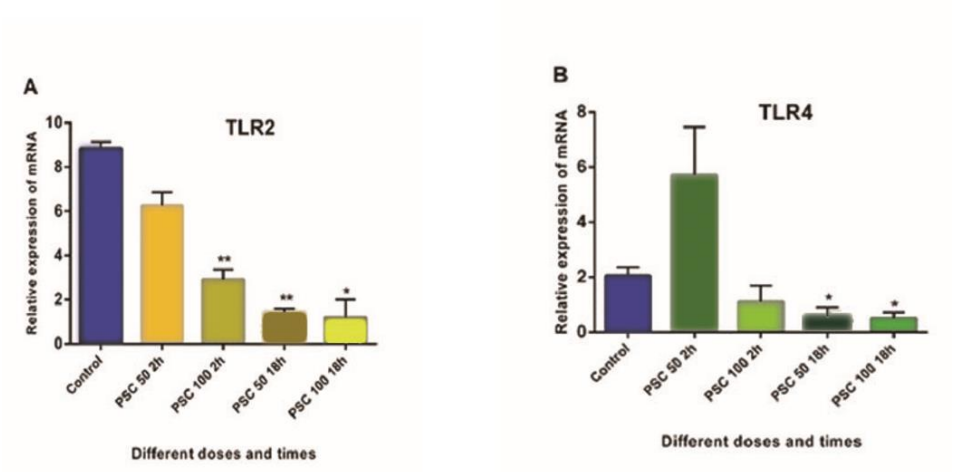

Fig. 4: Gene expression of TLR2 and TLR4 in ovine PBMCs exposed to somatic antigens of protoscoleces. Baseline expression of TLR2 and TLR4 corresponds to control group. Values were mean ± SEM in this figure. Significant difference in expression between PSC-treated PBMCs and control group is indicated by asterisk (*) (P < 0.05). All fold changes is normalized to the housekeeping gene GAPDH

Discussion

Many helminth-derived antigens can engage TLR2 and TLR4 and even manipulate their downstream signaling, (9). However, limited data are available concerning the molecular interactions between *E. granulosus* and host innate immunity (14,15). An in vivo study measured the expression of a cluster of innate immunity genes in the intestine tissue of sheep infected with *E. granulosus* eggs (3). Based on the microarray results most of the up-regulated genes were related to innate immune responses. Undoubtedly, focusing on the possible interplays occurred between TLRs and *E. granulosus* antigens might provide us invaluable data concerning the immunoevasion strategies. In this study, exposure to two different doses of hydatid cyst-derived antigens differently regulates the expression of ovine TLR2 and TLR4 transcription patterns. To determine whether TLRs expression was differently regulated by hydatid cyst-derived antigens, we examined the mRNA expression of TLR2 and TLR4 in control and treated PBMCs. Hydatid cyst-derived antigens were found to be able to

alter the expression of TLR2 and TLR4 on ovine PBMCs, suggesting the possible role of these receptors in recognition and initiation of immune responses against hydatidosis. Nearly all antigens we studied here were able to down-regulate the TLR2 and TLR4 expression. Induction of immunoregulatory responses during infection with cystic echinococcosis may be mediated through TLR2 and TLR4 (13). In addition, stimulation of TLR2 and TLR4 on the Tregs results in IL-10 secretion, suggesting there is a strong correlation between stimulation of these TLRs and immunosuppression (10, 16).

Hydatid cyst -derived antigens can shift Th1 responses toward Th2 in the host (3,4). In fact, in the chronic stage of *E. granulosus* infection, up-regulation of Th2-related cytokines suppresses inflammatory responses raised against parasite (17, 18). TLRs play an essential role in deviation of adaptive immune responses, their manipulation during infection with *E. granulosus* may be an efficacious strategy to suppress Th1 responses for increasing life span.

TLR2 and TLR4 in immune cells recognize lipopeptide and lipopolysaccharide structures from pathogens, respectively (19). It is yet unknown whether hydatid cyst antigens contain biomolecules structurally similar with these putative ligands. Exploiting proteomics analysis technique has, in part, addressed the potential of hydatid cyst antigens in activation of TLRs. AgB is the main immunosuppressive antigen of hydatid cyst and it has only been detected in HCF and ES products of PSC (20). We showed that only the expression of TLR4 is significantly up-regulated in PBMCs exposed to 50 ug/ml of ES for 2h, whereas the expression of TLR2 was not markedly altered (Fig.1A, B). Our observation of reduced TLR4 and TLR2 expression in PBMCs cultured with HCF antigens in two different concentrations and time-points (Fig. 3A, B) supports the immunosuppressive effects of HCF antigens, suggesting it likely occurs through the TLR2 and TLR4 down-regulation. Helminthes exploit complex mechanisms beyond TLRs manipulation to suppress and evade host immunity, thus more investigations need to be done in order to understand complex interactions happened during helminthic infections.

The GL is an inner syncytial tissue containing the parasite's tegument and able to generate PSC, capsules, and HCF. GL damage, like fissures or rupture, results in antigenic stimulation taking for an indefinite period (21). Since, most molecules derived from helminthes can stimulate surface TLRs (6), it is not strange to suppose GL antigens can involve TLR2 and TLR4 during interaction with PBMCs. With regards to the possible immunostimulatory role of GL, we observed a significant down-regulation of TLR2 in all treatment groups, while TLR4 expression showed up-regulation in treated PBMCs but this observation was not significant (Fig. 2A, B).

HCF, secreted by germinal membrane and PSC (22), represents another important component of hydatid cyst, as it can interact with and modulate the host immune system (9, 23). HCF markedly down-regulated both TLR2 and TLR4 expression in treated PBMCs as compared to control group (Fig.3A, B), suggesting the possible anti-inflammatory effects of HCF on immune cells. Many HCF-derived molecules were found to down-regulate both Th1 responses and pro-inflammatory cytokines production (24). HCF contains a complex mixture of immunosuppressive, immunostimulatory, and immunomodulatory components. For instance, AgB as a major antigen of HCF possesses immunosuppressive properties which enable it to induce and maintain a Th2-polarized microenvironment, facilitating the establishment of an *E. granulosus* chronic infection (9). AgB by manipulation of TLRs interferes with monocyte differentiation and DC maturation. We used HCF derived from sheep in which AgB is more abundant compared with HCF originated from cattle and human (25,26). This could also highlight-

ed the suppressive properties of HCF on TLRs expression as reported in this study.

Conclusion

We assessed the effects of four different hydatid cyst antigens on the expression of ovine TLR2 and TLR4 in two different doses and time points. The most obvious finding to emerge from this study was that except ES antigens, other hydatid cyst antigens significantly suppress the expression of TLR2 and partially TLR4, in ovine PBMCs. Since stimulation of TLR2 and TLR4 can significantly promote Th1 response and associated cytokines against CE infection suppression of TLRs expression is one of the mechanisms by which hydatid cyst antigens prevent Th1 and anti-parasite immune responses.

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Conflict of interest

The authors declare that there is no conflict of interests.

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