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Initial Studies on the Role of Hydatid Fluid in the Immune Evasion Strategies of *Echinococcus granulosus*

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Abstract- Modulation of the immune response is an important strategy in hosts chronically infected with *Echinococcus granulosus*, which modulates the response of the host immune system for long periods of time. The induction of NK cell function might be a considerable component of this infection. This study explores the modulation of NK cells and T lymphocytes by hydatid cyst fluid *in vitro*. Splenocytes from BALB/c mice were treated with hydatid fluid. After 72 h of exposure to the fluid, NK cell cytotoxicity and the expression of NKG2D on NK cells were reduced. In the co-culture system, hydatid cyst fluid also modulated $CD4^+CD25^+$ T cell differentiation and enhanced splenocyte secretion of TGF-β. In conclusion, hydatid fluid (HF) can stimulate the differentiation of T lymphocytes into Treg cells and induce the secretion of TGF-β, which could be involved in the suppression of NK cell-mediated cytotoxicity and the reduction of NKG2D receptor expression. The potential role of hydatid fluid in the regulation of the innate immune response of the host to hydatid cysts is discussed.

Keywords: Cystic echinococcosis, hydatid fluid, NK cell, Treg cell, TGF-β.

INTRODUCTION

Cystic echinococcus (CE) is a chronic endemic helminthic disease caused by infection with metacestodes (*i.e.*, the larval stage) of the tapeworm *Echinococcus granulosus* (Singh *et al*., 2014). Secondary infection in humans is an important medical problem that occurs when protoscoleces disseminate after the accidental rupture of cysts and develop into new cysts, evading the host immune response (Mourglia-Ettlin *et al*., 2011). Parasites can survive in the host for long periods of time, and this survival requires effective immune evasion mechanisms. In addition to the physical barrier of the fibrous cyst, antigenic components of the cyst fluid are also involved in the escape from the host immune response (Siracusano *et al*., 2008a). In recent years, a number of studies on the mechanisms involved in the establishment of chronic *E. granulosus* infection have been reported. These mechanisms enhance immunoregulatory molecules that directly suppress the function of certain immune cell subsets and stimulate other cell populations to evade the host immune system (Haniloo *et al*., 2008). Hydatid fluid is a complex mixture of components derived from the host and components derived from the metabolic activity of the metacestode, which include the lipoproteins antigen B and antigen 5. These lipoproteins are considered the main antigenic source for the immunoregulatory effects of CE (Carmena *et al*., 2006).

Extensive efforts have been made in recent years to determine the mechanisms by which the parasite modulates the host immune response (Mourglia-Ettlin *et al*., 2011; Siracusano *et al*., 2008a). Several suppressive molecules have been reported to modulate the innate immune and adaptive immune responses of the host, with which the parasite must actively interact to decrease the effect of a host response (Siracusano *et al*., 2008a). Naceur Mejri reported that the expression of TGF-beta and IL-4 mRNA was significantly increased in the peripheral blood in echinococcosis infection models compared to the uninfected group. The infected group exhibited higher numbers of $CD4^+CD25^+$ and $CD8^+CD25^+$ T cells than the

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uninfected group. In addition, high mRNA levels of Foxp3, which is a specific marker of T regulatory cells, were observed in the infected group. These results suggest that Treg cells play an important role in development and differentiation during hydatid infection (Mejri *et al*., 2011).

Experimental studies of echinococcosis immune evasion are limited to studies of whether hydatid cyst contents and protoscoleces inhibit or modulate host immune responses, particularly with respect to changes in the balance of Th1 and Th2 responses. During echinococcosis, Th1-type responses have been shown to be protective, while Th2-type responses allow the parasite to survive for long periods of time in the host (Dematteis *et al*., 1999, 2003). A small number of studies have focused on innate immune cells in hosts with hydatid disease. Nicod found that NK cell activity was significantly reduced, and the observed decline in the proportion of NK cells in the peripheral blood mononuclear cells (PBMCs) of patients with alveolar echinococcosis (AE), which is another hydatid disease caused by *Echinococcus multiocularis*, indicated that the NK cell activity was related to the low proportion of NK cells in the PBMCs of AE patients (Nicod *et al*., 2002). One prominent interaction between the infected tissue and the effector cells involves NKG2D and its ligands. High expression of TGF-β leads to the modulation of NKG2D, with subsequent inhibition of NKG2D-dependent cytotoxicity in AE (Zhang *et al*., 2008). However, the role of this interaction in CE infection remains unclear; to date, there have been no reports concerning NK cell function and the NKG2D system in hosts with CE.

Experimental studies of the interaction between *E. granulosus* and the host are not scarce. Because NK cells are a first line of defence essential for identifying pathogen invasion, their failure to activate the innate immune may be an important cause of hydatid disease. Thus, it is important to determine whether hydatid fluid plays a role in the induction of NK cells. The aim of this work was to determine whether HF influences splenocytes *in vitro* and whether Treg cells, NK cells or the suppressive immune molecule TGF-β are altered in the co-culture system.

MATERIALS AND METHODS

Hydatid fluid collection

Sheep liver hydatid cysts containing protoscoleces (PSCs) were acquired from a slaughterhouse in the city of Shihezi, Xinjiang province, China. To isolate the PSCs from a fertile or infertile unilocular hydatid cyst, the cyst was sprayed with 75% (v/v) ethanol and the membrane was punctured with a 21-gauge needle. The hydatid fluid was obtained from the hydatid cysts by aseptic aspiration and clarified by centrifugation at 1000 g at 4°C for 10 min. For all experiments, the HF was filtered through a sterile 0.22 µm membrane. The protein concentration of the HF was 1.86 mg/ml, measured using the Bradford assay.

Mice and cell preparation

Female 6- to 10-week-old BALB/c mice were purchased from the First Affiliated Hospital of Xinjiang Medical University Experimental Animal Center. Suspensions of mixed splenocytes were obtained from the spleens of the BALB/c mice by mechanically squeezing the tissue between glass slides in cold PBS and subsequently separated using the Ficoll-Hypaque gradient centrifugation method. Briefly, after centrifugation, the mononuclear fraction was collected, and the splenocytes were washed with PBS three times, removed, stained with Trypan blue dye and counted in a haemocytometer. The cells were cultured in plastic flasks in RPMI-1640 medium supplemented with 10% foetal bovine serum.

Co-culture of hydatid fluid with splenocytes

The effect of HF on the differentiation of T lymphocytes into Treg cells was tested by adding different concentrations of hydatid fluid to complete medium supplemented with ConA.

After verification of the viability of the cells, 1 ml of the cell suspensions was added to the wells of a 24-well culture plate. Different volumes of hydatid fluid, including 100 µl, 200 µl, 300 µl and 500 µl, were subsequently added to each well. Each well contained 1.8 ml of medium. PBS was included as a control group. ConA (5 µg/ml) was added to each well, and the plates were incubated at 37°C in a humidified atmosphere of 5% CO_2.

TGF-β cytokine assays

After 72 h, the concentration of TGF-β was determined in the co-culture supernatants from each group using capture ELISA kits from EXCELL (Shanghai, China). ELISAs were performed according to per the manufacturer's instructions.

Flow cytometry analysis

After exposure to hydatid fluid for 72 h, lymphocytes were collected by centrifugation at 200 g for 5 min. The HF-treated cells were then analysed using flow cytometry. The cells were stained with antibodies directly labelled with different colours prior to analysis. The following antibodies were used: anti-mouse CD3-FITC, CD4-PE, CD25-APC, DX5-FITC, and NKG2D-PE. All antibodies were purchased from EBioscience (USA).

NK cell cytotoxicity assay measuring LDH release from Yac-1 cells

After co-culturing with HF for 72 h, the cytotoxicity of the NK cells in the splenocytes was detected by LDH release using the *In Vitro* Toxicology Assay Kit. The cytolytic activity of splenocytes against Yac-1 cells was determined in an LDH release assay at 1:100 target:effector ratio. Spontaneous release was assessed from wells that contained Yac-1 cells (i.e., target cells) alone, and maximum LDH release was assessed after the addition of 1% NP40 (Solarbio, China). Specific cytotoxicity was calculated as follows: percent LDH release = 100(cpm experimental – cpm spontaneous release) / (cpm maximum release – cpm spontaneous release).

RT-PCR

The expression levels of Foxp3 mRNA were analysed using semi-quantitative reverse transcriptase PCR (RT-PCR) after 72 h of lymphocyte exposure to hydatid fluid. Lymphocytes from HF-treated wells and control wells were separated by centrifugation at 13,000x g for 1 min, and the pellets were subjected to RNA extraction using Tri-Pure Isolation Reagent (Roche, Germany) according to the manufacturer's instructions.

The RNA was reverse transcribed into cDNA using MBI Revert Aid (Fermentas, Germany). RT-PCR was performed to determine the expression levels of the Foxp3 mRNA. All of the primers used in the RT-PCR reaction were designed using Primer Premier Software (Table I). β-actin was used as an internal control to normalise the amount of mRNA in each sample. The samples were denatured for 5 min at 95°C, and Foxp3 cDNA was amplified using 35 cycles each of 95°C for 30 s, 60°C for 80 s, and 72°C for 45 s, followed by a final extension at 72°C for 3 min on a TAKARA thermocycler (Tokyo, Japan). A total of 5 μL of the amplification product was analysed by electrophoresis on an ethidium bromide-stained 2% agarose gel and documented using a gel documentation system. Quantification of the PCR band intensities was accomplished using Bio-Rad Quantity One analysis software. The relative Foxp3 mRNA expression levels were given by normalization with β-actin mRNA expression levels.

Table I.- Primers used for RT-PCR of Foxp3 and β-actin.

Primer name	Nucleotide sequence	Length	Product length
β-actin			
F	5'-AATTCCATCATGAAGTGTGA-3'	20	248
Foxp3			
R	5'-ACTCCTGCTTGCTGATCCAC-3'	20	248
F	5'-GAGAGGCAGAGGACACTCAATG-3'	22	108
R	5'-GCTCAGGTTGTGGCGGATG-3'	19	108

Statistical analysis

Data were analysed using SPSS 18.0 Software. For comparisons with unequal variances, the logarithms of the cytokine concentrations and the percentages were used. Statistical significance was determined using Student's t-test. *P*-values less than or equal to 0.01 were considered significant.

RESULTS

Cytokine production after co-culture of splenocytes with hydatid fluid

The addition of HF to splenocytes cultured with ConA (*i.e.*, standard T lymphocyte differentiation assays) induced a dose-dependent release of TGF-β. The results of the supernatant TGF-β analysis for all experimental groups are presented in Figure 1, including the mean and the

range of TGF-β concentrations observed for cells co-cultured with hydatid fluid. Compared to the control groups (32.4±8.6 pg/ml), the mean concentration of TGF-β in all of the hydatid fluid-treated splenocyte groups was significantly higher ($P < 0.01$). As the amount of hydatid fluid increased, the amount of TGF-β present in the supernatants from the hydatid fluid-treated groups slowly increased. The group treated with 500 µl displayed the highest concentration of TGF-β (382.5±23.6 pg/ml). The differences observed in the co-cultures after hydatid fluid treatment suggest that the hydatid fluid induced the splenocytes to release the suppressive cytokine TGF-β.

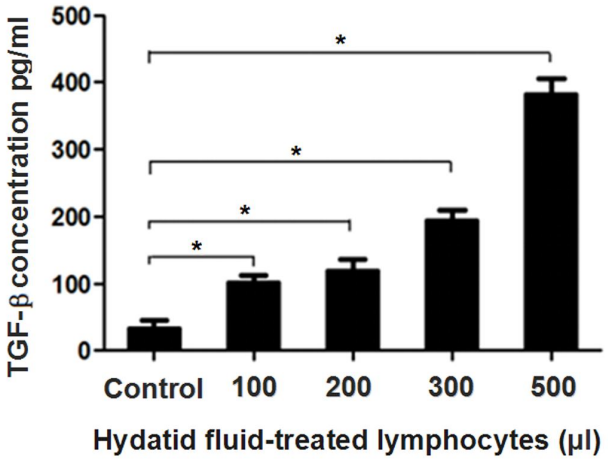

Fig. 1. HF induces the release of TGF-β from splenocytes from BALB/c mice. The splenocytes were cultured in the presence or absence of Con A (*i.e.*, control group) of HF, as shown. The supernatants were collected after 3 days of co-culture and analysed for TGF-β by ELISA. The concentration (mean ± S.D) of supernatant TGF-β was significantly higher ($P < 0.01$) in the presence of HF than in the control group. Asterisks indicate $P < 0.01$ compared to control.

Effect of hydatid fluid on $CD4^+CD25^+$ T cells in the co-culture system

The data presented in Fig. 3 illustrate the effects of exposure to HF on T lymphocyte differentiation into Treg cells. The percentages and numbers of $CD4^+CD25^+$ T cells in the co-culture samples, which contained spleen cells that were incubated with different concentrations of hydatid fluid antigens for 72 h, were measured using a flow cytometer (Fig. 2A). The mean percentages of Treg cells in all of the experimental groups were higher than that of the control group (Fig. 2B), but only the groups treated with a low concentration of HF (*i.e.*, 100 µl and 200 µl) were significantly increased ($P<0.05$). As the amount of fluid antigens in the system increased, the ratio of $CD4^+CD25^+$ T cells in the co-culture system did not increase. It is worth noting that the antigens from hydatid fluid can induce the differentiation of T lymphocytes into $CD4^+CD25^+$ Treg cells, which may play a key role in the immunosuppression that occurs during hydatid infection. However, in the presence of high concentrations of HF, some factors inhibit $CD4^+CD25^+$ T cell differentiation.

Foxp3 is one of the principal markers of Treg cells. To assess the influence of hydatid fluid on the expression of Foxp3 mRNA in splenocytes, lymphocytes that were exposed to different concentrations of hydatid fluid for 72 h were harvested, and the expression of Foxp3 mRNA was analysed using RT-PCR. Fig. 2C depicts the density of the labelled bands for the amplified cDNA. Compared to the control group, all HF-treated groups displayed higher levels of Foxp3, but not all of these differences were significant (Fig. 2D). The Foxp3 mRNA levels were only significantly increased in the groups treated with 100 µl and 200 µl of HF ($P<0.05$). Overall, these results indicate that HF induces an increase in the proportion of cells expressing Foxp3.

Hydatid fluid inhibits NK cell function in vitro

To directly assess the capacity of hydatid fluid antigens to interfere with NK cell activity, we co-cultured NK cell-containing splenocytes from BALB/c mice with hydatid fluid. After exposure to hydatid fluid for 72 h, the NK cell cytotoxicity of all of the experimental groups and the control group was measured using a standard LDH release assay. The results of these independent experiments are shown in Figure 3C. In these assays, NK cell-mediated cytolysis of Yac-1 cells decreased after treatment with the HF antigens in all groups except the 100 µl treatment group. All of the remaining treatment groups exhibited a significant reduction in

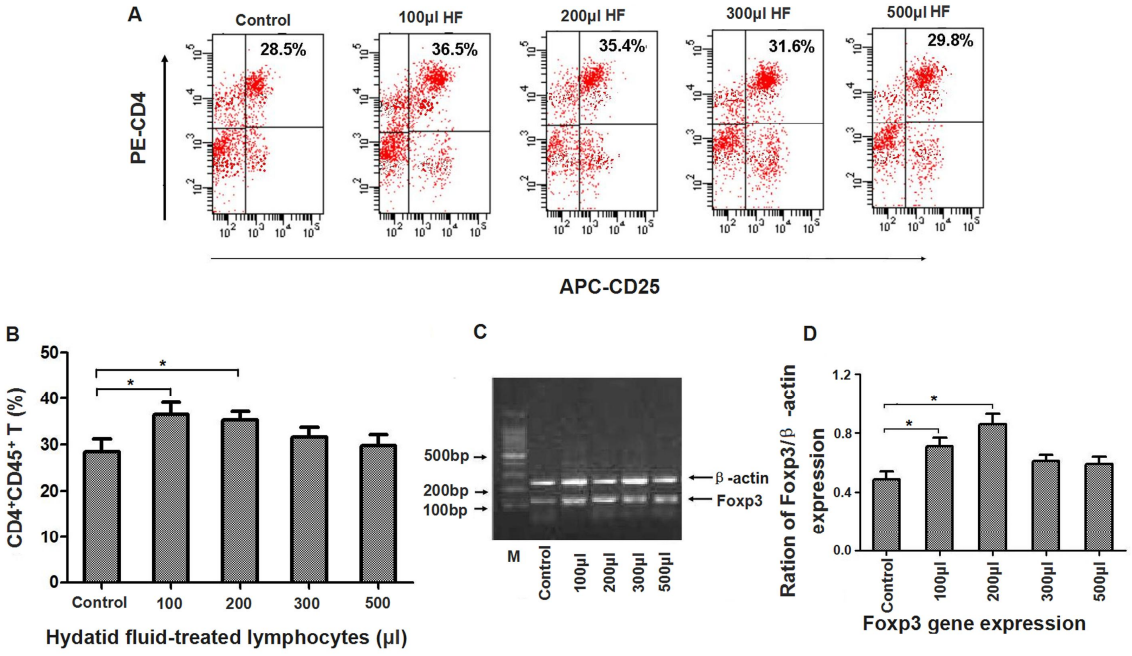

Fig. 2. HF enhanced the differentiation of Treg cells. Splenocytes were cultured with Con A in the presence or absence of HF for 3 days, as described in the Methods. The cells were then harvested, and the percentages of $CD4^+CD25^+$ T cells were analysed using flow cytometry. A shows the flow cytometry scatter plot from one representative experiment, and B shows the mean percentages of $CD4^+CD25^+$ positive cells in different groups, Mean±SD, n=5; C: Semi-quantitative RT-PCR analysis of Foxp3 expression in HF-treated lymphocytes and in the control group. β-actin was used as an internal control. The densities of the labelled bands, representing the amplified products of the Foxp3 gene (128 bp) and the β-actin gene (246 bp), are shown for each group. D: The Foxp3/β-actin expression ratio in HF-treated lymphocytes compared to the control group. The bar graph indicates the mean ± S.D. Asterisks indicate $P < 0.05$.

NK cell-mediated cytolysis ($P<0.05$) compared to the control group. In these assays, when high concentrations of antigens were present in the co-culture system, the cytolysis was significantly reduced ($P<0.05$). NK cell-mediated cytolysis might be blocked by the antigens via an unknown mechanism, potentially eliminating the natural cytotoxicity of splenocytes against the Yac-1 target cell line. These results suggest that the hydatid fluid antigens indirectly down-regulate Yac-1 cell lysis by HF-treated splenocytes.

To further investigate the involvement of hydatid fluid in NKG2D-mediated NK cell cytotoxicity, we examined the expression of NKG2D on $DX5^+$ NK cells from HF-treated splenocytes using a flow cytometer. As shown in Figure 3A and B, this co-culture system demonstrated that in all of the experimental groups, the expression of NKG2D was lower than that observed in the control group. However, only the 300 μl and 500 μl treatment groups displayed significant down-regulation ($P<0.05$). It has been demonstrated, however, that altered NKG2D expression is induced by chronic exposure to hydatid fluid antigens in NK cells. Overall, our data demonstrated that weak cytolysis of NK cells was accompanied by unusually low expression of NKG2D on immune effector cells within the hydatid fluid-treated group. This finding could contribute to changes in the destruction of effector NK cells during co-culture with hydatid fluid antigens.

DISCUSSION

Despite being under constant barrage by the

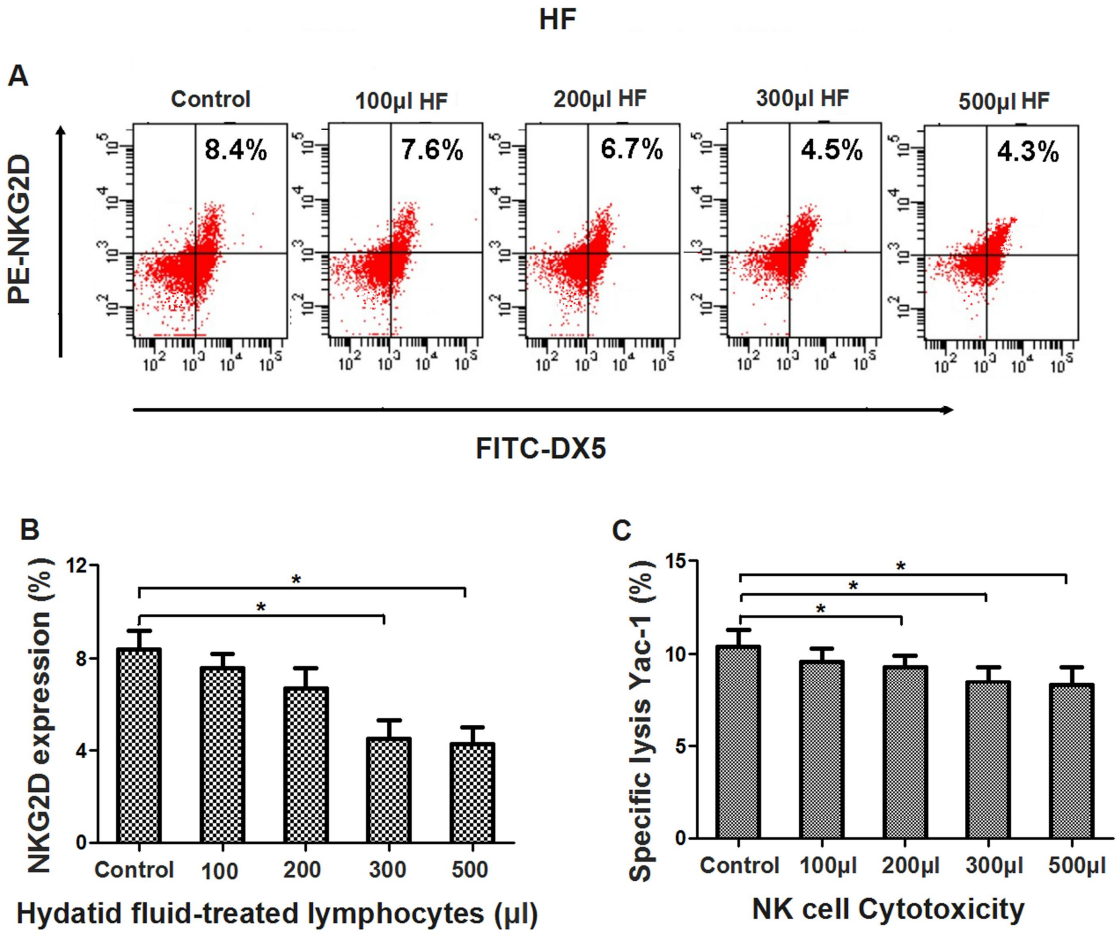

Fig. 3. *In vitro* inhibition of NK cell function by hydatid fluid. A, After co-culture with hydatid fluid, splenocytes were collected and analysed for expression of cell surface molecules, including NKG2D, using flow cytometry as described. The flow cytometry scatter plots from all groups and the corresponding percentages of NKG2D cells in the Dx5 cell population from a representative experiment are shown. B shows the mean percentages of NKG2D-positive cells in the $DX5^+$ NK cell population. C, After co-culture, the cells were collected and incubated with Yac-1 target cells, and NK-mediated cytolysis was measured in all groups using the LDH release assay. The results of the LDH release assay are represented as the Mean±SD of triplicate wells from one of three independent experiments. Asterisks indicate $P < 0.05$.

host, *E. granulosus* has complex defence mechanisms that protect it from the immune responses and modulate anti-parasite immune responses (Siracusano *et al*., 2012), facilitating long-term parasite survival in the host. Little is known concerning the innate immune mechanisms that affect susceptibility to primary or secondary *E .granulosus* infection. In past decades, several studies have described immune evasion mechanisms and demonstrated that hydatid fluid antigens play an important role in the parasite's immune evasion (Janssen *et al*., 1992).

In this work, we document the effect of hydatid fluid on both cytokine production and immune splenocytes (*i.e*., T cells and NK cells) from healthy BALB/c mice. We studied the number of Treg cells and the function of NK cells in splenocytes treated with hydatid fluid *in vitro* to analyse the effects of hydatid fluid on host defence mechanisms. Hydatid fluid was initially observed to stimulate robust production of TGF-β by splenocyte co-cultures *in vitro*. The results also demonstrate

that hydatid fluid antigens can increase the number of immunosuppressive $CD4^+CD25^+$ T cells and the mRNA expression of Foxp3, which is a key molecule required for the stimulation and differentiation of Treg cells, in splenocytes. The data presented here suggest that hydatid fluid affects T cells by promoting the differentiation and maturation of T lymphocytes into Treg cells, which can counteract the host immune response during parasite infection. *In vitro*, the ability of $CD4^+CD25^+$ Treg cells to suppress responder T cell proliferation and cytokine production requires activation, is cell contact dependent, and is antigen non-specific (Chen and Wahl, 2003). The induction of immunosuppressive cytokine production and suppressor T cells by host cells is observed during infection with a variety of parasites and modulates the host inflammatory responses (Maizels *et al*., 2004). The data presented in this study demonstrate that hydatid fluid has effects on splenocytes under these culture conditions, including the induction of TGF-β expression and the increase of $CD4^+CD25^+$ T cells.

The inhibitory effects of HF on NK cell function were investigated by measuring LDH release after Yac-1 cell lysis and by assessing NKG2D expression using flow cytometry analysis of the resulting population. We demonstrated, as expected based on previous studies, that NK cell cytotoxicity was reduced and that NKG2D expression was down-regulated after exposure to hydatid fluid. It has been demonstrated that hydatid fluid restricts NK cell effector function *in vitro*. In AE, TGF-β is strongly expressed by most of the T cells, but there are low numbers of NK cells and a lack of expression of NKG2D on the $CD8^+$ T cells in the periparasitic infiltrate. In our study, the NK cells cultured in the presence of HF had a significantly impaired ability to express NKG2D, a key cytokine required for the stimulation of NK cell cytoxicity (Zhang *et al*., 2008). This down-regulation of NK cell effector function and NKG2D receptor expression could not be explained by the robust secretion of TGF-β by lymphocytes. However, in previous tumour studies designed to identify the relationship between Treg cells and NK cells, TGF-β was responsible for the Treg cell-mediated down-regulation of NKG2D on NK cells, which was detectable *in vitro* in a Treg cell and NK cell co-culture system (Ghiringhelli *et al*., 2005).

In conclusion, the results of this study suggest that HF contains factors that can affect T cell and NK cell functions, but these effects may be different after acute and chronic exposure. HF contains soluble factors that are normally sequestered from the host immune response but may escape into the lymphatic system, where parasite antigens can activate Treg cells to produce IL-10 and TGF-β, prevent the stimulation of a mixed Th1/Th2 response and regulate NK cell effector functions.

Once the hydatid cyst is present 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 TGF-β and subsequently down-regulating NKG2D expression and impairing NK cell effector function.

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Conflict of interest

All authors have no conflict of interests.

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