Journal of Helminthology

cambridge.org/jhl

Immunohistochemical observation of local inflammatory cell infiltration in the host-tissue reaction site of human hydatid cysts

R. Jafari[1], B. Sanei[2], A. Baradaran[3], M. Kolahdouzan[2], B. Bagherpour[4] and H. Yousofi Darani[1,*]

[1]Department of Parasitology and Mycology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran; [2]Department of Surgery, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran; [3]Department of Pathology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran and [4]Acquired Immunodeficiency Research Centre, Isfahan University of Medical Sciences, Isfahan, Iran

Research Paper

*Current address: Infectious Disease and Tropical Medicine Research Center, Isfahan University of Medical Sciences, Isfahan, Iran

Cite this article: Jafari R, Sanei B, Baradaran A, Kolahdouzan M, Bagherpour B, Yousofi Darani H (2019). Immunohistochemical observation of local inflammatory cell infiltration in the host-tissue reaction site of human hydatid cysts. *Journal of Helminthology* **93**, 277–285. https://doi.org/10.1017/S0022149X1800024X

Received: 20 September 2017
Accepted: 13 February 2018
First published online: 30 April 2018

Author for correspondence:
H. Yousofi Darani, E-mail: yousofidarani@gmail.com

Abstract

The aim of this study was to evaluate the pattern of local immune cell infiltration in human cystic echinococcosis (CE) by identifying the subtypes of immune cells using immunohistochemistry (IHC). Fifty surgically removed hydatid cyst samples and surrounding tissues were collected from patients referred to Al-Zahra Hospital, Isfahan, Iran. IHC was performed on the surrounding host tissue of hydatid cysts using anti-human CD3, CD19, CD8, CD4, CD68, CD56, Ki-67 and Foxp3 (forkhead box P3) antibodies. The results were then compared to hepatocellular carcinoma and chronic hepatitis. In the host-tissue reaction site of liver hydatid cysts, a distinct pattern of local immune cell response, which outwardly consisted of a pack of the fibrous elements, a layer of palisading macrophages, an eosinophil-containing layer and a layer of accumulated lymphocytes, was observed. However, in some cases there were no positive cells for $CD56^+$ natural killer cells and $Foxp3^+$ regulatory T cells. The $CD3^+$ T cells were the predominant inflammatory cells in all groups, followed by $CD19^+$ B cells. It can be concluded that different immune cells are involved in the local response to human hydatid cysts.

Introduction

Cystic echinococcosis (CE) is a parasitic disease of humans and some animals, caused by the metacestode of the tapeworm *Echinococcus granulosus*. Humans may be infected accidentally through ingestion of vegetables or any other foodstuffs contaminated by the ova of the parasite. The infection can also be acquired by direct contact with the definitive hosts, carnivorous animals, having adult *E. granulosus* in their gut (Pakala *et al.*, 2016; Thompson, 2017). CE has a worldwide distribution and is a major public health problem in some regions (Craig *et al.*, 2007).

The phenotypic variations in *E. granulosus* were described in the early 1970s. The development and maturation of *E. granulosus* from sheep origin in culture media have been demonstrated, whereas isolates of the parasite from horses failed to develop or mature (McManus, 2013). Ten genotypes of *E. granulosus* have been described by mitochondrial DNA sequence. Based on phenotypic and genetic characteristics, that which was formerly known as a single species *E. granulosus sensu lato* with ten genotypes is now a cluster of species: *E. granulosus sensu stricto* G1–G3 (G1, sheep strain; G2, Tasmanian sheep strain; G3, buffalo strain) (Romig *et al.*, 2015), *E. equinus* (G4, horse strain), *E. ortleppi* (G5, cattle strain), *E. intermedius* (G6, camel strain; G7, pig strain), *E. canadensis* (G8 and G10, cervid strains) and *E. felidis* (Thompson, 2017).

The metacestode of *E. granulosus sensu lato*, which forms in intermediate hosts, is a complex structure covered outwardly by a laminated layer and a germinal layer. A host-derived layer, the fibrous layer, encircles the hydatid cyst (Tamarozzi *et al.*, 2016; Thompson, 2017). The laminated layer is a non-cellular multi-laminated structure, which is synthesized initially by the developing hexacanth larva and then by the germinal layer. It is made up of glycosylated glycoproteins and is crucial in the metacestode's immune evasion in the intermediate host (Díaz *et al.*, 2011a, b; Tamarozzi *et al.*, 2016).

Untreated hydatid cysts generally pass through several stages, active to inactive (Tamarozzi *et al.*, 2016), or tend to be stable over time (Frider *et al.*, 1999). Two essential immunological mechanisms – immune evasion and immune modulation – are highlighted for the often long-lasting and asymptomatic CE. There are also some degrees of protective immunity to reinfection (Lightowlers, 2010; Zhang *et al.*, 2012; Tamarozzi *et al.*, 2016). Both humoral and cellular immune responses are involved against hydatid cysts in intermediate hosts (Kharebov *et al.*,

CAMBRIDGE UNIVERSITY PRESS

1997), yet 30–40% of patients with CE are antibody-negative. This suggests that the proliferation and activity of B cells may be inhibited and/or regulated by *E. granulosus* antigens. It is not known how these antigens target and regulate the immune mechanisms of the host (Zhang *et al.*, 2012).

There are variations in the interaction of intermediate hosts and hydatid cysts, and the causes of these variations are not completely known. However, the parasite genotype and host-related factors are suggested to be involved. Furthermore, it has been demonstrated that in naturally infected sheep, antibody response may be accompanied by a cellular inflammatory response involving neutrophils, lymphocytes, eosinophils and macrophages (Rickard & Williams, 1982). The same cellular immune response has also been reported for experimental secondary infections in mice (Rogan *et al.*, 2015).

Most of our knowledge about the immunology of CE is based on experimental studies and observations in animals, yet we know little about the local cellular immune response in human infections. Studies on human subjects usually represent only single-point observations and we do not really know much about how immune response varies within humans (Riganò *et al.*, 2004; Rogan *et al.*, 2015). We have even less information about the local immune response to hydatid cysts, especially regarding the lymphocyte subtypes involved in local responses. There have been three studies on the local proliferation of different subtypes of lymphocytes in hydatid cysts: one on ovine CE (Vismarra *et al.*, 2015), one on bovine cases (Sakamoto & Cabrera, 2003) and one on hydatid cysts of the liver in humans (Vatankhah *et al.*, 2015). Thus in the present study we aimed to evaluate and explain the local immune cell infiltration pattern using CD3 as pan T cell, CD19 as pan B cell, CD4 as T helper cell, CD8 as cytotoxic T cell (CTL), CD56/NCAM as natural killer (NK) cell, CD68 as macrophage, Foxp3 (forkhead box P3) as regulatory T cell (Treg) and Ki-67 as proliferation biomarkers in human CE. The similarities in antigens of hydatid cysts and some cancers have been reported (Darani & Yousefi, 2012), and thus we also investigated the pattern of cellular immune response to hydatid cysts using the above-mentioned biomarkers, and compared it with samples of hepatocellular carcinoma (HC) and chronic hepatitis (CH).

Materials and methods

Sample collection and fixation

We collected 44 tissue samples from livers, five from lungs and one from a kidney, from surgically removed human hydatid cysts (containing the fibrous layer and the host-tissue reaction site) from patients referred to Al-Zahra Hospital, Isfahan, Iran, during April 2015–March 2017. The isolated hydatid cysts belonged to *E. granulosus* G1 (43 samples) and G3 (3 samples), and *E. intermedius* G6 (4 samples). The genotypes of the collected samples have been defined previously. The ultrasound features of the samples were also defined by the method described by the World Health Organization Informal Working Group on Echinococcosis (WHO-IGWE) (Wuestenberg *et al.*, 2014). Samples from paraffin-embedded blocks of four hepatocellular carcinoma and four non-autoimmune chronic hepatitis tissues were included in the study for comparison. The tissue samples were cut into small pieces and kept in 10% natural buffered formalin for 48–72 hours. The paraffin-embedded blocks were prepared from the collected tissues and stored in a cool, dry place until examination. The fertility of the hydatid cysts was also determined.

Slide preparation

Using a microtome, 4 µm sections were prepared, placed on Superfrost Plus positively charged slides (Thermo Scientific, Waltham, Massachusetts, USA) and incubated at 37°C overnight. Routine eosin and haematoxylin staining was also performed for histopathological analysis of the eosinophils.

Immunohistochemistry (IHC)

1. De-waxing and hydrating

The tissue sections were deparaffinized in three changes of xylene for 5 minutes each and then hydrated through a graded series of ethanol (100, 96, 70, 50%) and deionized water, each for 5 minutes. The hydrated tissue sections were incubated in phosphate-buffered saline (PBS), pH 7.0 (0.02 mol/l sodium phosphate buffer, 0.15 mol/l NaCl; Dako, Denmark), for 5 minutes.

2. Antigen retrieval

A target retrieval solution, Tris/EDTA buffer, pH 9 (Dako, Denmark), was used for heat-mediated antigen retrieval. The slides were then immersed in the 1X solution, heated to the boiling threshold (95°C) and kept at this temperature for 30 minutes (the buffer was boiled very slowly), and then cooled for 15 minutes at room temperature (RT).

3. Endogenous peroxidase blocking

After the antigen retrieval, the slides were incubated in wash buffer for 5 minutes (0.05 mol/l Tris/HCl, 0.15 mol/l NaCl, 0.05% Tween 20, pH 7.6; Dako, Denmark). To block endogenous peroxidase the slides were incubated in 3% hydrogen peroxide (H_2O_2 30%; Merck, Germany) for 10 minutes, washed for 1 minute in two changes of deionized water to remove excess H_2O_2, and then incubated in PBS for 5 minutes.

4. Incubation with the primary antibody

Water-repelling circles were drawn around the tissue sections using a Dako Pen (Dako, Denmark), and primary antibodies were added to the sections. The following biomarkers were used: polyclonal rabbit anti-human CD3 (Dako, Denmark), monoclonal mouse anti-human CD4 (clone 4B12; Dako, Denmark), monoclonal mouse anti-human CD8 (clone C8/144B; Dako, Denmark), monoclonal mouse anti-human CD19 (clone LE-CD19; Dako, Denmark), mouse monoclonal anti-human CD56/NCAM-1 (clone 123C3.D5; Diagnostic Biosystems, USA), monoclonal mouse anti-human CD68 (clone KP1; Dako, Denmark), monoclonal mouse anti-human Foxp3 (clone 236A/E7; eBioscience, USA) and monoclonal mouse anti-human Ki-67 (clone MIB-1; Dako, Denmark). Except for anti-human Foxp3, all antibodies were prediluted (ready to use) and incubated for 20 minutes in a humidity chamber. Anti-human Foxp3 was diluted in TTBS (Tris-buffered saline or TBS and Tween 20), optimized in 5 µg/ml concentration and incubated for 120 minutes in a humidity chamber. After incubation with the primary antibody, the slides were washed in two changes of wash buffer for 7 minutes each and then washed once in PBS for 7 minutes.

5. Secondary antibody and visualization

EnVision Detection System, Mouse/Rabbit, Peroxidase/DAB (3,3'-diaminobenzidine) (Dako, Denmark) was used for visualization (in the ready-to-use/prediluted format). The slides were incubated with the secondary antibody for 30 minutes. The washing step was repeated as previously described. DAB was diluted

https://doi.org/10.1017/S0022149X1800024X Published online by Cambridge University Press

(25 µl DAB in 1000 µl of DAB buffer) and used fresh. Slides were incubated with the diluted DAB for 5 minutes at RT to develop the enzymatic reaction. Then slides were washed with water, stained with haematoxylin for 1–2 minutes, dehydrated, cleared in xylene and mounted in Entellan (Merck Millipore, USA).

Negative and positive controls

Negative and positive control tissues were stained with each IHC run. Normal human tonsil tissue was used as the positive control for all the biomarkers studied. Biliary epithelial layer and Kupffer cells in the liver were also used as additional positive controls for CD56 and CD68, respectively (see supplementary fig. S5). The same slides incubated with PBS instead of the primary antibody were used as the negative controls.

Data analysis

Slides were studied under a microscope, and micrographs were taken from the stained tissue sections using 40X objective, considering all the microscopic fields with immune cell infiltration. Then the positive and negative inflammatory cells for CD4, CD8, CD3, CD19, CD56, CD68, Ki-67 and Foxp3 were counted using ImageJ software (https://imagej.nih.gov/ij/). The mean numbers of cells counted were then reported as a ratio of the inflammatory cells, using the following formula: positive cells/total cells × 100. The observatory data were reported as seen, and the counted cells were compared in groups and analysed by one-way analysis of variance (ANOVA), regression and Student's *t*-tests using SPSS v. 22.0 (IBM Corp., Armonk, USA).

Results

In this investigation we analysed 50 tissue samples of isolates of human hydatid cysts (44 from livers, 5 from lungs and one from kidney). To compare the inflammatory cell distribution in the host-tissue reaction site of liver hydatid cysts with other diseases, four samples from patients with hepatocellular carcinoma and four from patients with chronic hepatitis were also studied. The mean age of patients with hydatid cysts, hepatocellular carcinoma and chronic hepatitis was 40 ± 14, 65 ± 13 and 26 ± 12 years, respectively. All hydatid cysts belonged to the CE2 category and were also fertile.

Considering the biomarkers, there were positive cells for CD3, CD19, CD8, CD4, CD68 and Ki-67 in all cases, yet in some there were no positive cells for CD56 (seven CE patients) and Foxp3 (one CE and one chronic hepatitis), and there were no eosinophils in non-CE samples. The studied cells were arranged in distinct ways in the various organs, as described below.

Pattern of inflammatory cell distribution in the host-tissue reaction site of liver hydatid cysts

In the host-tissue reaction site of liver hydatid cysts, a general pattern of inflammatory cell distribution was observed. This pattern consisted outwardly of a pack of the fibrous element (adjacent to the laminated layer of the parasite), a layer of palisading macrophages, an eosinophil-containing layer, a layer of aggregated lymphocytes, and normal host tissue (fig. 1; supplementary figs S1 and S2). In the aggregated lymphocyte layer, $CD3^+$ T cells, $CD4^+$ T helper cells, and $CD19^+$ B cells were dominant (table 1). However, $Foxp3^+$ regulatory T cells (Tregs) were not aggregated and there were few $CD56^+$ natural killer (NK) cells. The $CD8^+$ CTL population was less dense than the $CD4^+$ Th cell population and was scattered all around the inflammation site. $CD68^+$ macrophages were mostly around the aggregation site, with a few located inside the site, and were cut into the fibrous layer as palisading macrophages connecting fibrous tissue to the inflammatory site (fig. 1). The fibrous layer was thick, separating the inflammatory site from the parasite (mean thickness 551 ± 224 µm). The Ki-67^+ proliferating cells tended to cluster in the core of lymphocyte aggregation where $CD19^+$ B cells were localized (fig. 1, micrographs CD19 and Ki-67), and there were very few in the palisading macrophage location. This pattern of immune cells around the liver hydatid cyst was consistent among nearly all individuals.

Pattern of inflammatory cell distribution in the host-tissue reaction site of lung hydatid cysts

No host-derived fibrous capsule was observed in lung hydatid cysts. Inflammatory cells were observed in most areas of the lung samples, and mostly encompassed all the cells mentioned above in relation to the liver. $CD19^+$ B cells mostly clustered at the core of the immune cell aggregation (fig. 2; supplementary fig. S3), and $CD3^+$ T cells were around and inside the aggregated and non-aggregated sites. As in the liver, a significant number of $CD4^+$ Th cells were observed quite close together, whereas the $CD8^+$ CTLs were less populous and were scattered all around the inflammation site. The $CD68^+$ macrophage arrangement in lungs differed from that in the liver. They were scattered all around the immune cell aggregated sites, with a few inside the sites, and were present in a considerable number inside the alveoli and small airways. $CD56^+$ NK cells were rare, and present in low numbers in the host-tissue reaction site of four out of five samples of lung hydatid cyst. Eosinophils were predominantly outside the lymphocyte aggregation sites and were present in considerable number in all the lung tissues. Similar to the liver samples, the Ki-67^+ proliferating cells were observed in most of the same locations as the $CD19^+$ cells. The $Foxp3^+$ Tregs were sparsely scattered in the inflammatory sites (supplementary fig. S3). The aggregation of immune cells around the lung hydatid cyst was almost the same in nearly all individuals.

Pattern of inflammatory cell distribution in the host-tissue reaction site of a kidney hydatid cyst

Only one hydatid cyst of the kidney was studied, and the results showed similarity between hydatid cysts of the liver and kidney. As observed in the liver cysts, the kidney cyst had a fibrous layer. Unlike in the liver, the palisading macrophages in the kidney cyst were lined closer to the cyst (fig. 3). The inflammatory cell infiltration was similar to the liver.

Pattern of inflammatory cell distribution in patients with hepatocellular carcinoma and chronic hepatitis

HC and CH tissue samples were studied and compared to the inflammatory site of liver hydatid cysts only. The immune cell infiltration was not as dense or intense as in the host-tissue reaction site of hydatid cysts. Considering the studied cells, the arrangement in HC and CH was visually similar to that in CE except that in non-CE samples there was no eosinophil, or clustering of Ki-67^+ cells as in liver and lung hydatid cysts. The distinct pattern of cell layers in liver hydatid cysts was limited

https://doi.org/10.1017/S0022149X1800024X Published online by Cambridge University Press

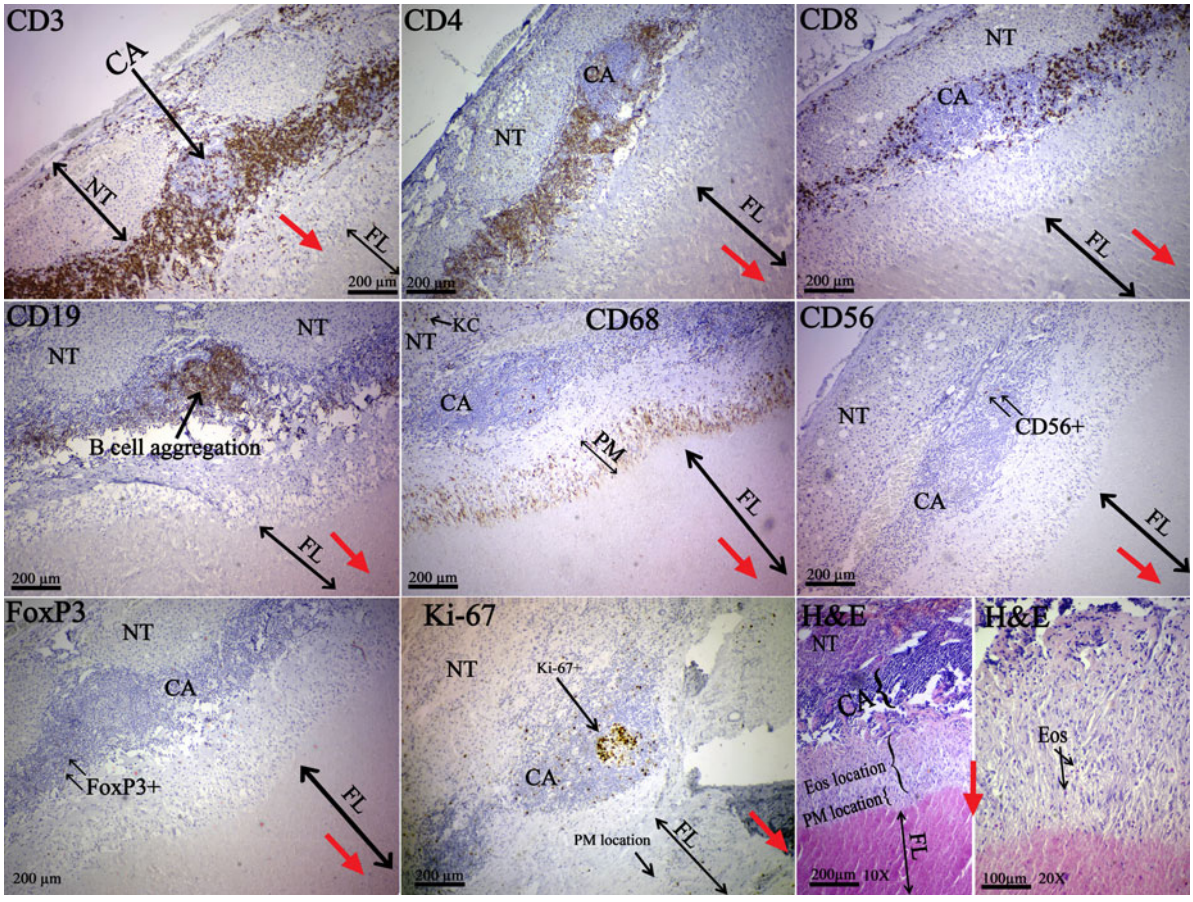

Fig. 1. The studied cell types in liver hydatid cysts. The red arrows point towards the location of the parasite. A thick fibrous layer separates the parasite from the host parenchyma, and a considerable number of eosinophils and macrophages are visible at the border of the fibrous layer and the inflammatory site (H&E and CD68). B-cell clusters are mostly present within the granulomas. Ki-67^{+} cells tend to cluster at the centre of the granuloma (micrograph Ki-67), where the B-cell follicle is present. The observed B cells inside the granuloma may indicate germinal centres. There are few proliferating cells in the area where palisading macrophages are present (CD68 and Ki-67). All of the micrographs are of the same individual. NT, normal tissue; CA, immune cell aggregation; FL, fibrous layer; PM, palisading macrophages; Eos, eosinophil; KC, Kupffer cell. CD3 (10X), CD4 (10X), CD8 (10X), CD19 (10X), CD68 (10X), CD56 (10X), Foxp3 (10X), Ki-67 (10X), and H&E (10X and 20X).

to the host-tissue reaction site of patients with CE and was not seen in HC and CH cases. CD56^{+} NK cells were rare, and present in low numbers in all the CH and HC cases (supplementary fig. S4).

Quantitative analysis

The mean positive cells for CD3, CD4, CD8, CD19, CD56, Foxp3 and Ki67 are presented as a ratio of the inflammatory cells, and CD68^{+} macrophages and eosinophils are presented as number per high-power microscopic field. The mean numbers of cells are available in table 1.

Multiple comparisons of the mean positive cells showed no significant difference between CH, HC and liver hydatid cyst inflammatory sites (supplementary table S1). There was no significant difference between the number of eosinophils, CD3^{+} T cells, CD4^{+} Th cells, CD8^{+} CTLs, CD19^{+} B cells, CD56^{+} NK cells, CD68^{+} macrophages and Foxp3^{+} Tregs with the genotype of the parasite in CE cases (supplementary table S2). Considering the cell numbers in different organs of the patients with CE, the CD8^{+} CTL population was larger in the lungs compared to the liver ($P = 0.003$) (table 2). There was no significant difference in the mean cell count between males and females.

In CE patients, the numbers of CD19+ B cells were significantly and positively correlated with the numbers of Ki-67^{+} proliferating cells ($R^2 = 0.183$, $P = 0.032$). This phenomenon is also true for CD4^{+} Th cells with CD19^{+} B cells ($R^2 = 0.153$, $P = 0.005$), and CD8^{+} CTLs with Foxp3^{+} Tregs ($R^2 = 0.81$, $P = 0.045$). However, the numbers of Ki-67^{+} proliferating cells were significantly and negatively correlated with the age of the patient ($R^2 = 0.165$, $P = 0.044$) (supplementary fig. S5 and table S3). No other significant correlation was observed in the other studied diseases.

Discussion

In this investigation a pattern of immune cell aggregation in the host-tissue reaction sites of human isolates of the hepatic hydatid cysts was observed (summarized in fig. 4). The local immune response against hydatid cysts in humans is barely known. The inflammatory cell aggregation and composition around the hydatid lesion in humans may be driven by specific mechanisms that are likely to be specific to the affected organ and may be activated directly in response to parasite antigens; however, this has not been completely described (Vatankhah *et al.*, 2015). In this study, localization of the immune cell infiltration was described and analysed using IHC. Given the inadequate numbers of G3

https://doi.org/10.1017/S0022149X1800024X Published online by Cambridge University Press

Table 1. Mean numbers of cells in liver tissue samples infected with cystic echinococcosis, hepatocellular carcinoma or chronic hepatitis; numbers for CD3, CD4, CD8, CD19, CD56, Foxp3 and Ki67 are relative numbers (0–100) of positive cells per all immune cells, and numbers for CD68 and eosinophils are numbers per high-power microscopic field. The ratios of Foxp3/CD4, CD4/CD8 and CD3/CD19 are also shown.

		Eos	Ki67	CD3	CD4	CD8	CD19	Foxp3	CD68	Foxp3/CD4	CD4/CD8	CD3/CD19
Cystic echinococcosis	Mean ± SD	57 ± 63	12.5 ± 10.4	56.2 ± 29	51.7 ± 16	28.1 ± 9	37.9 ± 19	5.05 ± 4	79.8 ± 69	0.08 ± 0.06	2.3 ± 0.9	2.1 ± 2.1
	CV (%)	111.92	86.75	52.49	32.24	33.8	50	80	86.9	–	–	–
Hepatocellular carcinoma	Mean ± SD	–	3.5 ± 0.7	79.2 ± 9.6	68.3 ± 8.2	37.1 ± 7.8	23.3 ± 5.4	7.1 ± 5.4	53.2 ± 3.7	0.1 ± 0.08	1.9 ± 0.6	3.5 ± 0.8
	CV (%)	–	20	12.12	12.1	21	23.25	76.37	7.1	–	–	–
Chronic hepatitis	Mean ± SD	–	25 ± 18	53.5 ± 21	47.3 ± 17	36.3 ± 9	25.6 ± 6.6	4.1 ± 4.7	50.5 ± 12.2	0.07 ± 0.08	1.3 ± 0.4	2 ± 0.5
	CV (%)	–	73.36	39.4	35.94	25	26	98.75	24.33	–	–	–

No significant numbers of CD56+ cells were detected.

and G6 genotypes, they were excluded from further analysis and discussion. Further investigations with a higher number of genotypes other than G1 are recommended.

The results showed a predominant $CD3^+$ T cell population, followed by $CD19^+$ B cells in the local host reaction sites in hydatid cysts. The $CD4^+$ Th cell population was 2.3 times larger than that of $CD8^+$ CTL. In general the $CD3^+$ T cell population was observed to be 2.1 times larger than that of $CD19^+$ B cells in CE cases. $Foxp3^+$ cells comprised *c.* 8% of $CD4^+$ T helper cells. In agreement with our work, a higher T cell population in the host-tissue reaction site of hydatid cysts was previously reported for humans and sheep infected with CE (Vatankhah *et al.*, 2015; Vismarra *et al.*, 2015). However, Vatankhah *et al.* (2015) reported different $CD8^+$ CTL populations in patients with CE, CH and steatosis (SH), and proposed it as a prognostic value in CD8-dependent T cell activity in some chronic liver inflammations with different etiology (Vatankhah *et al.*, 2015), whereas such difference was not observed in this study.

B cells are important immune cells that produce immunoglobulin; they are also regarded as antigen-presenting cells (APCs) and are important in humoral immune response (LeBien & Tedder, 2008). In this study a considerable proportion of inflammatory cells were $CD19^+$ B cells, and among these, plasma cells (not counted) were also observed in histopathological observation, which shows the importance of $CD19^+$ B cells in the local immune response in CE patients. Only a fraction of people with CE (10–91.7%.) show positive levels of antibodies against hydatid cysts, using commercially available immunoassay kits (Zhang *et al.*, 2012). Moreover, most of the proliferating cells tend to cluster in the B cell area in CE patients, and the numbers of $CD19^+$ B cells were significantly and positively correlated with the numbers of $Ki\text{-}67^+$ cells, indicating that B cell proliferation is active in the area. A similar phenomenon was reported in the granulomas of *Mycobacterium tuberculosis* infection in non-human primates (Phuah *et al.*, 2012). It has been reported that the B cells at the centre of mycobacterial granuloma are probably suggestive of germinal centres (Ulrichs *et al.*, 2004; Zhang *et al.*, 2011; Phuah *et al.*, 2012). Considering the clustering and proliferation of B cells, our results show similarities between mycobacterial granuloma and hydatid cyst host reaction. However, there is no similar study indicating the mentioned results in human hydatid cysts. Furthermore, the localization of B and $Ki\text{-}67^+$ cells is reminiscent of their clustering in normal human tonsil tissue (supplementary fig. S6). The presence of $CD19^+$ B cells in great numbers, and considering their proliferation in the host-tissue reaction site of human hydatid cysts, shows a probable strong humoral response against the parasite, but the reason that the immunological diagnosis of CE is not sensitive and specific is not completely clear.

Eosinophils were observed in considerable numbers only in CE patients. Eosinophils have been considered as the end-stage cells involved in host protection against multicellular parasites. These leukocytes are now known to be involved in the initiation and even propagation of various inflammatory responses, as well as innate and adaptive immunity modulation (Rothenberg & Hogan, 2006).

A large number of macrophages were observed in all of the studied CE patients, and notably the macrophages were seen as the first-line combat against hydatid cysts. $CD68^+$ macrophages were also observed inside and around the lymphocyte aggregation site, and in almost all cases they lined like a guard just behind the fibrous capsule (the palisading macrophages). Considering these cells as important APCs, they may have a significant role in

https://doi.org/10.1017/S0022149X1800024X Published online by Cambridge University Press

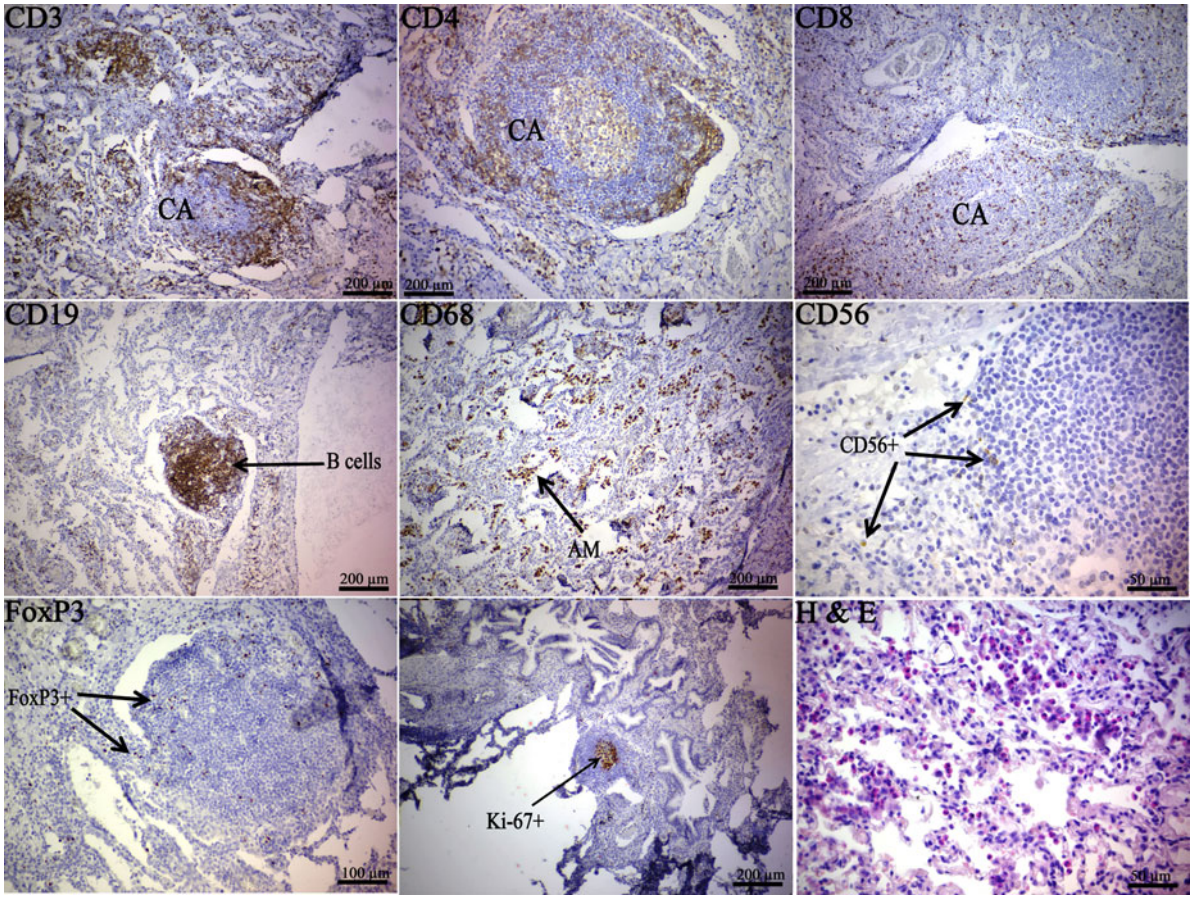

Fig. 2. The studied cell types in a human subject with a lung hydatid cyst. There was no apparent fibrous layer, and massive inflammatory cell aggregation all over the tissue, thus the location of the hydatid cyst could not be determined. No palisading macrophages are visible. Considerable numbers of eosinophils are visible around the immune cell aggregation sites (H&E). All of the micrographs are of the same individual. CA, immune cell aggregation; Eos, eosinophil; AM, alveolar macrophages. CD3 (10X), CD4 (10X), CD8 (10X), CD19 (10X), CD68 (20X), CD56 (20X), Foxp3 (20X), Ki-67 (10X), and H&E (40X).

processing and presenting the *E. granulosus* antigens to the other immune cells, such as T cells, which are also present in great number in the lesion site.

In this study, $CD56^+$ NK cells were the least populous. In fact, NK cells were defined as effector lymphocytes of innate immunity with cytolytic functions. NK cells are now known to express activating and inhibitory receptors that are calibrated for self-tolerance while having efficacy against infections such as viral infections and tumours. It is suggested that NK cells can also be a form of immunologic memory for antigens, thus exerting complex biological functions that are properties of innate and also adaptive immunity (Vivier *et al.*, 2011). It was previously recommended that $CD56^+$ NK cells be investigated in patients with CE (Vatankhah *et al.*, 2015) and we have done so comprehensively in this study. The results showed that in the local inflammatory cell aggregation sites of hydatid cysts the $CD56^+$ NK cells comprised the smallest proportion of the inflammatory cells (0.6%). The highest quantity of these cells was observed in hepatic tumours (1.2%). Moreover, $CD56^+$ NK cells were absent in seven CE patients. The role of $CD3^+CD56^+$ NK T cells in the hydatid-induced local immune response is presumed (Vatankhah *et al.*, 2015). Our findings do not support this hypothesis; however, the function of these cells was not studied in the present work and we recommend further investigation of the function of NK cells in the immune response against hydatid cysts.

APCs are a group of cells of the immune system that mediate the immune response by processing and presenting antigens for identification by T cells. Macrophages, B cells, dendritic cells and Langerhans cells are examples of APCs (Hamilos, 1989). In this study, macrophages were lined behind the fibrous capsule of hydatid cysts and were in a considerable quantity. They may be the most important APCs in the immune response to established human hydatid cysts.

Foxp3 protein is an accepted marker for Tregs. These cells are a population of lymphocytes that regulate and suppress overresponses from other immune cells. Tregs are able to control a variety of other immune cell subsets, including activated effector cells, and inhibit APCs, B cells, NK cells and innate immunity (Gliwiński *et al.*, 2017). $Foxp3^+$ Tregs were absent in one CE sample, yet 5.05% of inflammatory cells of 49 CE patients were $Foxp3^+$ Tregs, which is a considerable number. Studies have shown that *E. granulosus* modulates anti-parasite immune responses in human hosts and this may be the reason the parasite can persist for a long time in intermediate hosts. Furthermore, the probable immune-suppressing role of TGF-β and Tregs has been shown in *E. multilocularis* infection in animal models (Siracusano *et al.*, 2012). Based on the results of the present study, Tregs may play an immunomodulatory role in human CE. Vatankhah *et al.* (2015) reported almost similar results regarding Foxp3 expressing Tregs in CE (Vatankhah *et al.*, 2015). It is accepted in the

https://doi.org/10.1017/S0022149X1800024X Published online by Cambridge University Press

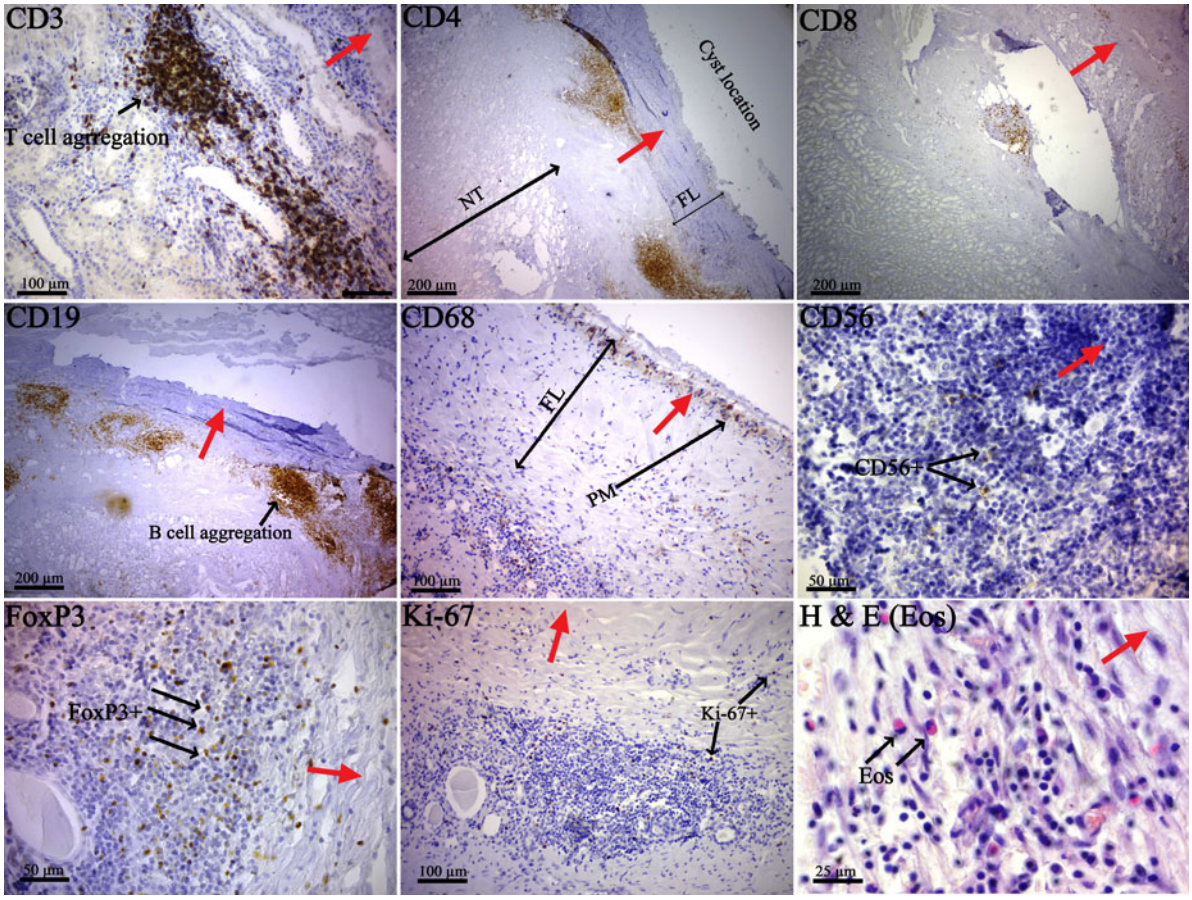

Fig. 3. The studied cell types in a human subject with a kidney hydatid cyst. The red arrows point towards the location of the parasite. There is a fibrous layer between the cyst and the host-tissue reaction site, separating the cyst from normal tissue. As in the liver cysts, the palisading macrophages (PM) and eosinophils are located between the immune cell aggregation site and the cyst. Lymphocyte proliferation (Ki-67) is not as pronounced as in the lung and liver samples; however, there are few Ki-67+ cells in the location where palisading macrophages are present (micrographs Ki-67 and CD68). The micrographs are all from the single individual studied. NT, normal tissue; FL, fibrous layer; PM, palisading macrophages; Eos, eosinophil. CD3 (20X), CD4 (10X), CD8 (10X), CD19 (10X), CD68 (20X), CD56 (40X), Foxp3 (40X), Ki-67 (20X), and H&E (40X).

literature that the Th2 response probably favours the establishment of the parasite in an intermediate host, as opposed to a parasite-killing Th1 response. However, often a mixed Th1/Th2 response is present in infected intermediate hosts, and regulatory mechanisms may control the Th1 and Th2 parasite-killing effector mechanisms. For example, Tregs have been reported to increase locally in the first 5 days post protoscolex injection in an animal model (Mourglia-Ettlin *et al.*, 2011; Tamarozzi *et al.*, 2016). *Echinococcus multilocularis*-infected mice with Treg depletion have been shown to exhibit a lower parasite load and an enhanced Th1/Th17 polarization (Wang *et al.*, 2017).

Vatankhah *et al.* (2015) observed a significant difference in the distribution of CD1a expressing dendritic cells (between CE and CH, and CE and SH), α-SMA expressing myofibroblasts (between CE and CH, and CE and SH) and CD68 expressing macrophages (between CE and CH, and SH and CH). Despite the larger sample size in the present study, a significant difference was not observed in the studied cells (CD3, CD4, CD8, CD19, CD56, CD68, Foxp3 expressing cells) in the three diseases studied. The CD1a and α-SMA markers were not used in this study.

Vismarra *et al.* (2015) studied the lymphocyte populations surrounding established ovine hydatid cysts using the IHC method. They reported a higher number of $CD3^+$ T cells compared to $CD79^+$ B cells, in agreement with the findings of this study on human CE. Similarly, they also reported a fraction of infiltrating lymphocytes as $Foxp3^+$ Tregs and suggested that established ovine cysts may be protected from aggressive immune response through the suppressive effect of $Foxp3^+$ Tregs (Vismarra *et al.*, 2015).

Similar to our findings, Sakamoto & Cabrera (2003) and Díaz *et al.* (2000) reported a kind of macrophage epithelioid cell layer in bovine hydatid cysts (Rickard & Williams, 1982; Sakamoto & Cabrera, 2003). Sakamoto & Cabrera (2003) also showed predominant $CD8^+$ CTLs in most cases of progressive hydatid cysts. In these cases they observed a relatively small number of $CD4^+$ Th cells. The eosinophil-mediated destruction of the laminated layer was also reported in regressive and involuted hydatid cysts (Sakamoto & Cabrera, 2003). Considering human CE, the infiltrating immune cell populations, especially T helpers and CTLs, are different from those in cattle. In humans, hydatid cysts persist for a long time, whereas in cattle, hydatid cysts are mostly reported to be sterile or degenerated and calcified (Rinaldi *et al.*, 2008). In the present study, in CE patients the numbers of $Foxp3^+$ Tregs were significantly and positively correlated with the numbers of $CD8^+$ CTL.

In conclusion, we have shown a distinct pattern of inflammatory cells localized in the host-tissue reaction site of human hydatid cysts. The suppressive effects of regulatory T cells and a considerable number of proliferating B cells may be the cause of the persistence of hydatid cysts in human cases for a long period.

https://doi.org/10.1017/S0022149X1800024X Published online by Cambridge University Press

Table 2. Mean numbers of cells studied in liver and lung tissue from humans with cystic echinococcosis.

Cell	Organ	Mean ± SD	Mean difference	*P*	95% CI of difference	
					Lower	Upper
Eosinophil	Liver	51 ± 52.5	−56.5	.386	−216.3	103.2
	Lung	107 ± 129				
$CD3^+$ T cell	Liver	53.8 ± 29.5	−19.7	.161	−47.6	8.1
	Lung	73.5 ± 27.4				
$CD4^+$ Th	Liver	60.1 ± 16.3	−8.5	.265	−23.8	6.7
	Lung	68.7 ± 14.2				
$CD8^+$ CTL	Liver	26.5 ± 8	−12.5	**.003***	−20.7	−4.3
	Lung	39 ± 13.6				
$CD19^+$ B cell	Liver	35.1 ± 19	−16.1	.075	−33.8	1.6
	Lung	52 ± 15.6				
$Foxp3^+$ Treg	Liver	4.7 ± 3.8	−2.1	.266	−5.8	1.6
	Lung	6.8 ± 4.9				
$CD68^+$ MΦ	Liver	74.8 ± 61	−51.5	.418	−207.7	104.6
	Lung	126.4 ± 126.6				
$Ki67^+$	Liver	11.9 ± 10.2	−5.05	.394	−17.1	6.9
	Lung	17 ± 12.8				

***Significant at $P < 0.05$**
No significant numbers of CD56+ cells were detected.
MΦ, macrophage

Moreover, NK cells are present in low numbers in the host-tissue reaction site of human hydatid cysts. Further studies on the functions of these immune cells in human CE are recommended, using a greater number of different genotypes and other organs than the liver to figure out the possible role of each of these cells in the durability of the disease in humans, and possible immunotherapeutic strategies.

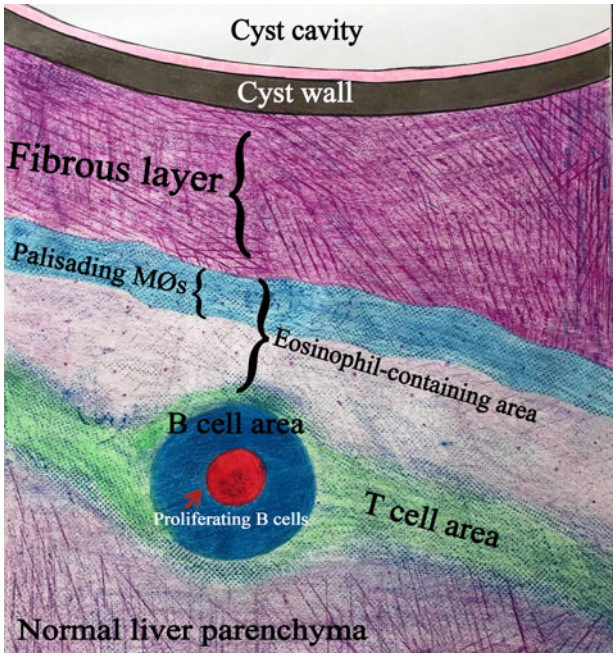

Fig. 4. Diagram of the observed immune cells and their approximate location, based on the findings of this study.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/S0022149X1800024X

Acknowledgments. The authors would like to acknowledge the Vice-chancellor of Research and Technology, Isfahan University of Medical Sciences, Isfahan, Iran, for the approval of this study. Fig. 4 was kindly produced by Maryam Sajadian. This paper is derived from a PhD thesis in parasitology (No. 394952).

Financial support. This study was supported financially by the Vice-chancellor of Research and Technology, Isfahan University of Medical Sciences, Isfahan, Iran.

Conflict of interest. None.

Ethical standards. This study was approved by the ethical committee of Isfahan University of Medical Sciences (No. 394952). No tissue sample was taken deliberately from patients for this study, and the samples used were derived from those sent to the histopathology lab.

References

Craig PS, McManus DP, Lightowlers MW *et al.* (2007) Prevention and control of cystic echinococcosis. *The Lancet Infectious Diseases* **7**, 385–394.

Darani HY and Yousefi M (2012) Parasites and cancers: parasite antigens as possible targets for cancer immunotherapy. *Future Oncology* **8**, 1529–1535.

Diaz A, Willis AC and Sim RB (2000) Expression of the proteinase specialized in bone resorption, cathepsin K, in granulomatous inflammation. *Molecular Medicine* **6**, 648–659.

Díaz A, Casaravilla C, Allen JE, Sim RB and Ferreira AM (2011a) Understanding the laminated layer of larval *Echinococcus* II: immunology. *Trends in Parasitology* **27**, 264–273.

Díaz A, Casaravilla C, Irigoín F, Lin G, Previato JO and Ferreira F (2011b) Understanding the laminated layer of larval *Echinococcus* I: structure. *Trends in Parasitology* **27**, 204–213.

Frider B, Larrieu E and Odriozola M (1999) Long-term outcome of asymptomatic liver hydatidosis. *Journal of Hepatology* **30**, 228–231.

Gliwiński M, Iwaszkiewicz-Grześ D and Trzonkowski P (2017) Cell-based therapies with T regulatory cells. *BioDrugs* **31**, 335–347.

Hamilos DL (1989) Antigen presenting cells. *Immunologic Research* **8**, 98–117.

Kharebov A, Nahmias J and El-On J (1997) Cellular and humoral immune responses of hydatidosis patients to *Echinococcus granulosus* purified antigens. *The American Journal of Tropical Medicine and Hygiene* **57**, 619–624.

LeBien TW and Tedder TF (2008) B lymphocytes: how they develop and function. *Blood* **112**, 1570–1580.

Lightowlers MW (2010) Fact or hypothesis: concomitant immunity in taeniid cestode infections. *Parasite Immunology* **32**, 582–589.

McManus DP (2013) Current status of the genetics and molecular taxonomy of *Echinococcus* species. *Parasitology* **140**, 1617–1623.

Mourglia-Ettlin G, Marqués JM, Chabalgoity JA and Dematteis S (2011) Early peritoneal immune response during *Echinococcus granulosus* establishment displays a biphasic behavior. *PLoS Neglected Tropical Diseases* **5** (8), e1293. doi: 10.1371/journal.pntd.0001293.

Pakala T, Molina M and Wu GY (2016) Hepatic echinococcal cysts: a review. *Journal of Clinical and Translational Hepatology* **4**, 39–46.

Phuah JY, Mattila JT, Lin PL and Flynn JL (2012) Activated B cells in the granulomas of non-human primates infected with *Mycobacterium tuberculosis*. *The American Journal of Pathology* **181**, 508–514.

Rickard MD and Williams JF (1982) Hydatidosis/cysticercosis: immune mechanisms and immunization against infection. *Advances in Parasitology* **21**, 229–296.

Riganò R, Profumo E, Buttari B, Delunardo F, Ortona E, Margutti P, Teggi A and Siracusano A (2004) Cytokine expression in the follow-up of patients with cystic echinococcosis. *Parassitologia* **46**, 441–442.

Rinaldi L, Maurelli MP, Veneziano V, Capuano F, Perugini AG and Cringoli S (2008) The role of cattle in the epidemiology of *Echinococcus granulosus* in an endemic area of southern Italy. *Parasitology Research* **103**, 175–179.

Rogan MT, Bodell AJ and Craig PS (2015) Post-encystment/established immunity in cystic echinococcosis: is it really that simple? *Parasite Immunology* **37**, 1–9.

Romig T, Ebi D and Wassermann M (2015) Taxonomy and molecular epidemiology of *Echinococcus granulosus* sensu lato. *Veterinary Parasitology* **213**, 76–84.

Rothenberg ME and Hogan SP (2006) The eosinophil. *Annual Review of Immunology* **24**, 147–174.

Sakamoto T and Cabrera PA (2003) Immunohistochemical observations on cellular response in unilocular hydatid lesions and lymph nodes of cattle. *Acta Tropica* **85**, 271–279.

Siracusano A, Delunardo F, Teggi A and Ortona E (2012) Host–parasite relationship in cystic echinococcosis: an evolving story. *Clinical and Developmental Immunology* **2012**, 639362. doi: 10.1155/2012/639362.

Tamarozzi F, Mariconti M, Neumayr A and Brunetti E (2016) The intermediate host immune response in cystic echinococcosis. *Parasite Immunology* **38**, 170–181.

Thompson RC (2017) Biology and systematics of *Echinococcus*. *Advances in Parasitology* **95**, 65–109.

Ulrichs T, Kosmiadi GA, Trusov V, Jörg S, Pradl L, Titukhina M, Mishenko V, Gushina N and Kaufmann SH (2004) Human tuberculous granulomas induce peripheral lymphoid follicle-like structures to orchestrate local host defence in the lung. *The Journal of Pathology* **204**, 217–228.

Vatankhah A, Halász J, Piurkó V, Barbai T, Rásó E and Tímár J (2015) Characterization of the inflammatory cell infiltrate and expression of costimulatory molecules in chronic echinococcus granulosus infection of the human liver. *BMC Infectious Diseases* **15**, 530. doi: 10.1186/s12879-015-1252-x.

Vismarra A, Mangia C, Passeri B *et al.* (2015) Immuno-histochemical study of ovine cystic echinococcosis (*Echinococcus granulosus*) shows predominant T cell infiltration in established cysts. *Veterinary Parasitology* **209**, 285–288.

Vivier E, Raulet DH, Moretta A, Caligiuri MA, Zitvogel L, Lanier LL, Yokoyama WM and Ugolini S (2011) Innate or adaptive immunity? The example of natural killer cells. *Science* **331**, 44–49.

Wang J, Müller S, Lin R, Siffert M, Vuitton DA, Wen H and Gottstein B (2017) Depletion of FoxP3^{+} Tregs improves control of larval *Echinococcus multilocularis* infection by promoting co-stimulation and Th1/17 immunity. *Immunity, Inflammation and Disease* **5**, 435–447.

Wuestenberg J, Gruener B, Oeztuerk S, Mason RA, Haenle MM, Graeter T, Akinli AS, Kern P and Kratzer W (2014) Diagnostics in cystic echinococcosis: serology versus ultrasonography. *The Turkish Journal of Gastroenterology* **25**, 398–404.

Zhang M, Wang Z, Graner MW *et al.* (2011) B cell infiltration is associated with the increased IL-17 and IL-22 expression in the lungs of patients with tuberculosis. *Cellular Immunology* **270**, 217–223.

Zhang W, Wen H, Li J, Lin R and McManus DP (2012) Immunology and immunodiagnosis of cystic echinococcosis: an update. *Clinical and Developmental Immunology* **2012**, 101895. doi: 10.1155/2012/101895.