Immunopathology of hydatid infection in human liver
PhD Thesis
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1. Introduction

Tissue-dwelling larvae of Platyhelminths belonged to the genus *Echinococcus*, are responsible for serious health problems and a great deal of economical detriment worldwide [1] [4]. *Echinococcus granulosus* is the most prevalent species with global distribution whose larval stage (hydatid cyst) evolves in the internal organs of various mammalian hosts, including humans [10]. The hydatid cyst infection (cystic echinococcosis) in man and animals is characterized by growth and development of one or more spherical, unilocular, fluid-filled cysts mostly in the liver (75% of cases) and with lower frequency in other visceral organs [14]. An evolved cyst is enclosed with a thick membrane which is anatomically composed of two distinguishable layers. The outmost cover of the cyst wall features a thick layer with acellular structure and acts as a protective shield (laminated layer or ectocyst) [14]. The inner layer is actually a germinal epithelium endocyst of which brood capsules and protoscolices are generated [14]. The cyst wall is also a contact border where different compound of either the parasite or the host origin are exchanged [15] [16]. In the liver, hydatid cyst is surrounded by a fibrotic adventitia (pericyst) which is accompanied by massive infiltration of activated fibroblasts and other cellular populations [17] [19] [22]. It may halt the further growth of the cyst, however can also defend the parasite against the host immune agents or administrated chemotherapeutic compounds [37] [16]. Attempt to develop a quite efficient method for treatment of cystic echinococcosis has not been successful yet [37] [38]. Moreover, surgical operation, as the only reliable choice so far, is not practically cost-effective and may sometimes be risky [38]. Meaning to establish non-surgical therapeutic methods, it is obviously important to consider the substantial role of the immune system in pathogenesis of the infection. Therefore, further understanding of the host-parasite immune interplay can be helpful to improve the existing methods and can lead to novel strategies for reducing the pathology and elimination of the parasite.

2. Description of the problem and assigned goals of the present study

A great deal of research has been carried out to elucidate different aspects of the host-parasite immune interplay, although the existing knowledge of the immune functions occur during the course of cystic echinococcosis is not quite sufficient to explain the distinctive pathogenesis of the infection. Constant irritation of the tissue and activation of hepatic stellate cells (HSCs), which cause accumulation of intracellular matrix and formation of fibrosis, are typically
outcomes of parasitic infections of the liver [39] [34] [35] [33]. Evidently, sustained activation of immune components can induce modulation of the responses from T helper (Th)1 to Th2 profile. Th1-related cytokines maintain protective mechanisms and trigger activation of classic macrophages and other inflammatory cells such as neutrophils [35]. On the contrary, Th2 responses are associated with activation of macrophages through an alternative pathway which in turn can induce transformation of HSCs to myofibroblasts and promote fibrosis [35] [11]. The shift of Th1 to Th2 responses is believed to be an evasion mechanism by which such parasites can detract the severity of the host immune reactions, so that they can prolong their life span within the host [17] [37] [7]. As for hydatid infection, the metacestode can manage to survive for several years in the host tissue and often produce no detectable symptom, till the overgrowth of the cyst may physically disrupt the normal function of the organ [39] [6]. Hydatid cyst has been showed to induce concurrent Th1 and Th2 profiles [38] [26]. Study has indicated that the components of the innate immune system are activated primarily against the developing larva which can initiate the other defense mechanisms, particularly antibody-dependent cell-mediated cytotoxicity (ADCC) [38]. The parasite usually escapes these reactions, although remarkable levels of cytokines such as interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin (IL)-2, IL-12, IL-17A as well as IL-4, IL-5 and IL-6 remain detectable in response to the developed cyst [37] [38]. Furthermore, expression of IL-10 is the common trait of chronic hydatid infection which is associated with activation of regulatory T cells (Treg) [38]. Antibodies such as IgG1, IgG4 and IgE can be concomitantly detected in the sera of hydatid patients [37] [38]. Collectively, available data suggest that in response to the chronic hydatid infection both protective and suppressive mechanisms coexist. The current perception of the host immune reactions to cystic echinococcosis can be challenged as it has been principally founded on the basis of achieved results by studies on animal models or by in vitro and ex vivo assays. Reasonably, results of model studies should be doubted due to the crucial differences between host species according to their taxonomical and biological traits. Immune components involved in the defense mechanisms against the parasite can vary in naturally infected hosts in which the host-parasite relationship is reciprocally adjusted throughout the evolution compared to those in unusual hosts (laboratory models) that are experimentally infected. Moreover, in vitro assessment of the immune responses to hydatid cyst may only indicate the activity of peripheral immune system to the circulating parasite antigens, which can change by many intervening factors. It
should be noticed that the metacestode, as a multicellular organism, possesses a great number of antigenic compounds that are potentially able to stimulate the host immune system, however the prompted response is probably specific to a certain antigen. As an instance, somatic protein B (AgB) was showed to skew cytokine responses toward Th2-profile in human PBMC cultures [26] [25], but immunization of BALB/c mice with a plasmid-enclosed gene encoding a subunit of this antigen resulted in predominant Th1 responses [3]. Therefore, it can be hypothesized that the total image of the immune reaction to hydatid cyst is probably the resultant outcome of molecular and cellular events initiated by the parasite antigens which vary according to the specificity of each pathway to the molecular structure of an individual epitope.

Having reviewed the existing data, it can be elicited that there is a lack of information about the tissue-specific immune responses to the established parasite. More importantly, investigation is required to clarify the exact composition of cellular agents which are involved in the inflammatory response at the vicinity of the chronic hydatid lesion and sustain the organ’s hemostasis, particularly in human hosts, as it has been almost neglected so far. Moreover, it can be deduced that the locally provoked inflammatory responses are also regulated by constant exposure of these immune effectors to the parasite antigens. Thus it is worth investigating whether the parasite possesses any antigen with potential ability to induce protective immune responses as well as being essential for its survival. In search of a viability marker, alkaline phosphatase (ALP) has been showed to be a good candidate, as it is retained in all developmental stages of the parasite.

Hence, three major aims were determined for the present study:

1) The composition of cellular infiltrates at the periphery of chronic hydatid lesion was investigated in the liver biopsies of patients with cystic echinococcosis (CE).

2) To elucidate the molecular cross-talk underlying activation of T cells and antigen presenting cells (APCs) around the pericyst, expression of costimulatory molecules (CD28, CTLA4, CD80 and CD86) at the level of mRNA was evaluated in the liver biopsies of CE patients.

3) To investigate whether ALP can be used as viability marker, the enzyme was purified from active (fertile) and inactive (sterile) ovine hydatid cysts and biochemical and molecular characteristics of the enzyme were assessed and results were compared. A well, the cellular and humoral immune reactions to ALP in peripheral blood of human patients were evaluated.
3. Materials and Methods

3.1. Characterization of cellular infiltrates at the periphery of chronic hydatid lesion

Formalin-fixed paraffin-embedded liver biopsies (consisting of total and subtotal cystectomy materials) from 21 patients with cystic echinococcosis (CE) were obtained from the archived samples at the 2nd Department of Pathology, Semmelweis University. Microsections of 2.5µm thickness were prepared and firstly examined by routine hematoxylin-eosin staining method. An array of surface markers including: CD1a, CD3, CD4, CD8, CD20, CD68, myeloperoxidase [6], forkhead box P3 (FOXP3), and smooth muscle actin α (α-SMA) was assayed by immunohistochemistry (IHC), corresponding activation of myeloid dendritic cells (DCs), T cells at all stages of development, proliferated CD4+ T cells, cytotoxic T cells, B cells, macrophages, neutrophils, Treg and HSCs, respectively. The procedure was optimized regarding an indirect IHC protocol which was based on heat-induced epitope retrieval (HIER) method. Biopsy samples from patients with non-parasitic chronic inflammation of the liver: 11 steatohepatitis (SH) samples and 11 chronic hepatitis (CH) samples obtained from the archive at the 2nd Department of Pathology (Semmelweis University) were also examined likewise and results were compared.

3.2. Expression levels of costimulatory molecules

The same biopsy samples (see section 3.1) from CE, SH and CH patients were used to examine the expression levels of CD28, CTLA4, CD80 and CD86 in the inflammatory milieu of the liver. Microsections of 10µm of thickness were deparaffinized and total RNA was isolated. After depletion of DNA contamination, reverse transcription polymerase chain reaction (RT-PCR) technique was used to produce cDNA library of the targeted mRNA molecules. Primer pairs (forward and reverse) were specifically designed for one splicing variant (transcript variants of each molecule mRNA sequence) and oligonucleotides were synthesized by Integrated DNA Technologies (IDT, USA) following the order. To amplify cDNA transcripts of targeted costimulatory mRNAs, a conventional PCR was applied. Amplicons were retrieved from the gel and were sequenced, then all achieved sequences were compared to previously recorded gene alignments in the GeneBank by a BLAST search and identity scores ≥ 98% were considered for evaluation of the designed primers. Ratio of the amplified cDNA to the expression level of a reference gene, β- actin, was measured by quantitative Real-Time polymerase chain reaction (qPCR).
3.3. Molecular characterization and antigenic property of ALP

3.3.1. Purification of ALP from sheep hydatid cysts

Infected sheep livers were obtained from slaughtered animals at an abattoir in Shahryar (Iran). According to the structure of the germinal epithelium and presence of protoscolices or their materials (hooklets), hydatid cysts excised from sheep liver were separated into fertile and sterile cyst groups, in each 30 cysts were included. The content of all cysts (hydatid fluid) was aspirated and ALP was purified as it had been described elsewhere.

3.3.2. Purification of sheep liver-derived ALP

Normal sheep liver was cut into very small pieces while frozen. Then, 1 g of the tissue was homogenized in 9 ml of lysis buffer (15.14 g Tris, 20 g SDS, 100 ml of 100% glycerol, 0.38 g EDTA, pH 6.8) supplied with mercaptoethanol and ammonium sulfate. The extracts were loaded on concanavaline A-sepharose column and active fractions were eluted column-wise with 0.1 ml methylmannoside in equilibration buffer.

3.3.3. Biochemical kinetics of ALP

Optimum reaction pH and the Michaelis-Menten parameters were measured by the method had been previously described. Enzyme inhibitory effects of EDTA, levamisol and L-phenylalanine on activity of ALP were examined. The isoelectric focusing of ALP was determined by using 5% polyacrylamide gel which was enriched with carrier ampholyte, 50% glycerol, 0.1% riboflavin 5'-monophosphate sodium salt hydra, 10% ammonium persulfate and tetramethylethilenediamine (TEMED).

3.3.4. Cellular and humoral responses to hydatid ALP

Blood samples were obtained from 21 CE patients with the liver involvement who had their infection confirmed by ultrasonography and were expected to undergo surgery at cancer Institute, Tehran University of Medical Sciences (Tehran, Iran) in two months. Patients with other parasitic diseases: taeniasis saginata (5 cases) and fascioliasis (2 cases) along with 15 healthy donors were also included in this study. Peripheral blood mononuclear cells (PBMCs) were isolated and cellular responses were examined after incubation with either ALP or hydatid crude antigen (HCF) by using cell proliferation assay and cytokine ELISA assay. The recent method was used to evaluate the production of cytokines including: IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-6 and IL-10 in the supernatants of PBMC cultures after stimulation by applied antigens. Unstimulated cultures and cultures incubated with phytohemagglutinin (PHA) were used as negative and positive
controls, respectively. Humoral immune response to ALP and HCF were tested in the sera from hydatid patients as well as those from patients with fascioliasis, patients with taeniasis and from normal donors using an ELISA which was set up for assessment of total IgG, IgG1, IgG2, IgG3 and IgG4.

3.4. Analysis of the Data

Results of IHC experiment were scored as follows:

N (no stained cell was observed), + (0≤M≤10, very low), ++ (11≤M≤100, low), +++ (101≤M≤300, moderate), ++++ (301≤M≤500, high) and ++++++ (501≤M, very high).

These values along with those measured by costimulatory mRNA experiment were expressed as mean ± SD and Analysis of Variance (ANOVA) test was used for differences between groups. The value of relative frequency (RF) was defined for each cell phenotype in a group and was calculated as follows:

\[
\text{Relative Frequency (RF)} = \frac{\text{Total number of immunostained cells from a certain phenotype}}{\text{Total number of immunostained cells}} \times 100
\]

Due to the low number of individuals in examined groups and to the heterogeneity of the SD between groups, immune parameter for ALP and the other stimuli were compared using one-way ANOVA with post-hoc Tukey test for nonparametric data. Receiver operating characteristic (ROC) curves were used to evaluate the ability of the ELISA to detect specific antibodies against ALP and HCF. The Area under the Curve index was reported with 95% confidence interval (CI) to compare seroreactivity of antigens between hydatid patients and all control groups. The Youden’s index was used to select the best cut-off values for the ELISA. Diagnostic values such as sensitivity, specificity and positive/ negative predictive values for serological tests were also calculated. Spearman’s correlation rank was used to evaluate all the correlations. Differences with \(p \leq 0.05\) were considered statistically significant.
4. Results

4.1. Cellular populations identified in the inflammatory milieu of the liver

Tissue damage and fibrosis were observed in all samples obtained from CE patients. Histopathological examination indicated the presence of hepatocytes with large lipid droplets in their cytoplasm, ductular reaction and mild accumulation of connective tissue fibers in the majority of SH samples, however pericellular scar and septal fibrosis were detected in 2 SH patients (18%). A range of different alterations in the liver from mild cellular infiltrates and slight remodeling in the lobular structures of portal areas to septal fibrosis were observed in CH biopsies, but portal fibrosis was the most frequent pathology among these patients (64%, n=7).

In both SH and CH, MPO\(^+\) neutrophils were detected mostly in sinusoidal spaces with low to moderate aggregation, while these cells were absent or had very low to low accumulation at the periphery of hydatid lesion or in scant numbers within the sinusoidal spaces. Differences were significant between the CE and CH samples in this regard. CD3\(^+\) cells were the most frequent T lymphocytes identified in all examined samples. In the CE samples, these phenotypes were majorly observed as compact clusters of accumulated cells around the pericyst. Quantified numbers of CD8\(^+\) T cells largely varied between CE individuals as ranged from quite absent (in 29% of cases) to moderate (in 9.5% of cases) in these biopsies. In total, aggregation of CD8\(^+\) cells in the inflammatory areas of the liver was significantly higher in the CH samples than that in CE. On the other hand, CD4\(^+\) T cells assemblages contained very low to low scored numbers of immuno-stained cells in the CE biopsies which were not significantly different from the same measurements in the SH and CH samples. Contrary to CD8\(^+\) T cells, there was less disparity according to the number of infiltrated CD4\(^+\) cells between the CE individuals. FOXP3-expressing phenotypes were absent or had very low scores in CE and in SH but were observed in slightly higher numbers in CH biopsies, although the differences between the sample groups were insignificant. Focally aggregated around the scar tissue, CD20\(^+\) B cells were remarkably frequent in all sample biopsies. These cells were the second largest population identified in the inflammatory milieu of the liver in the CE biopsies and their average scores had no significant difference with other sample groups. The number of CD68-expressing cells was highly diverse in the CE livers and was averagely scored as moderate in these samples. The accumulation of CD68\(^+\) macrophages in the inflammatory areas of the liver the in CH biopsies was found significantly higher than that in CE and SH. Myeloid DCs labeled with anti-CD1a antibody were
utterly absent in all CE samples, however these cells had feeble presence in the SH and CH biopsies. The cellular composition of inflammatory infiltrates was illustrated by computing the relative frequency values in the examined samples and indicated B cells and α-SMA⁺ HSCs to be the most abundant populations contributed to the tissue-specific cellular responses in the CE livers. Macrophages composed significantly less proportion of cell assemblages in CE than did they in SH and CH.

**4.2. Expression of costimulatory molecules at the mRNA level**

Antigen presenting-associated costimulatory mRNAs (CD80 and CD86) were highly expressed in all examined samples. The arbitrary ratio of CD80 and CD86 mRNAs to the expression level of house-keeping β-actin gene was almost identical in all individual biopsies, however the quantified level of CD80 mRNA was higher than that of CD86 in the CE and SH samples and the opposite situation was observed in the CH biopsies. The expression level of CTLA4 mRNA in CH livers showed 435-fold and 73-fold increase compared to CE and SH, respectively. Expression level of CTLA4 in SH was also higher (6-fold) than CE, although the average CTLA4 expression level was not significantly different between all groups more likely due to the wide range of inter-sample variations. CD28 mRNA showed far less expression levels in the CE samples compared to the other groups. In both SH and CH samples, the ratios of CD28/CTLA4 according to the expression levels of mRNA were higher than one (CD28/CTLA4=4 in CH and CD28/CTLA4=93 in SH), but showed only a slight increase in the expression of CTLA4 mRNA in the CE samples (CD28/CTLA4=0.3). Correlation between the expression levels of T cell-associated costimulatory molecules and the frequency of immune-labeled cells was only significant in the CH samples where a positive correlation existed between the quantified CTLA4 mRNA and the abundance of CD4⁺ cells. The expression levels of CD80 and CD86 were positively correlated in the CE and CH biopsies, but such a value was insignificant in SH.

**4.3. Immunochemical characterization of hydatid cyst-derived ALP**

**4.3.1. A comparison between the biochemical properties of ALP from fertile and sterile hydatid cysts**

The enzymatic activity of ALP was defined as mean U/ml±SD and indicated stronger affinity of the fertile cyst-derived enzyme to the substrate compared to the other aliquots. No difference was found between the enzyme activity in extracts of sterile cysts and sheep liver. ALP from fertile cysts showed higher activity at pH~9.8, while the maximum enzyme saturation was observed at
pH~11.2 and pH~11.8 for ALP from sterile cysts and from sheep liver, respectively. The value of $V_{\text{max}}$ and the Michaelis-Menten constant [5] were significantly lower when measured for fertile cyst-derived ALP compared to other resources, but the isoelectric point of the enzyme was slightly higher in extracts from fertile cysts. The molecular weight of the enzyme showed no difference between aliquots of each extract either in reduced or in non-reduced condition. L-phenylalanine had no inhibitory effect on the enzyme purified from fertile cysts.

4.3.2. Peripheral immune responses to hydatid ALP in human hosts

Immunoblot assays using a pool of hydatid positive sera showed immunoreactive bands on the nitrocellulose page after sensitization by fertile cyst-derived ALP, however no immune response to ALP from sterile cysts and sheep liver was found. Accordingly, ALP from fertile cysts was chosen as an antigenic resource for further investigation. To study the prognostic value of measured immune parameters, patients involved in this study were grouped based on the existence of fertile active cysts (GI group), semi-calciﬁed cysts (GII group) and completely calcified and inactive cysts (GIII groups) (WHO standardized classiﬁcation of hydatid lesions).

Cell proliferation assay indicated both HCF and ALP to have stimulatory effect on the cultured PBMCs from hydatid patients, however false positive responses were observed in the cultures from taeniasis and fascioliasis patients to HCF.

Cellular reactions to the parasite antigens were also studied by measuring the levels of cytokines in the supernatants of PBMC cultures. Although HCF induced higher IFN-γ response in the control cultures, the levels of this cytokine produced by the hydatid PBMCs against ALP and HCF were not significantly different. The same results were achieved according to the levels of TNF-α in the culture supernatants measured by cytokine ELISA. Production of IL-2, IL-4 and IL-5 in response to both parasite antigens was observed in the supernatants of the hydatid and normal cultures. HCF-induced levels of these cytokines were higher in the hydatid cultures compared to the controls, but PBMCs from the hydatid patients only produced higher levels of IL-2 and IL-4 than did the normal cells in response to ALP. Besides, HCF had more vigorous effect on production of IL-2, IL-4 and IL-5 than did ALP in the hydatid PBMC cultures. Stimulation of the normal PBMCs by HCF yielded the production of IL-2 and IL-4 higher than background by these cells, although ALP only induced IL-2 response in control cultures. PBMCs from both hydatid patients and healthy donors were similarly stimulated by HCF to produce IL-6. The level of this cytokine in the supernatants of hydatid cultures was significantly higher than
that in the normal controls after incubation with ALP. Nonetheless, HCF induced stronger IL-6 response in all sample groups. Measured levels of IL-10 above the background were significantly high in the hydatid cultures compared to the normal controls after stimulation with HCF, but such a difference between two sample groups was not significant when ALP was the stimulator.

Humoral reactions to the parasite antigens measured in the sera showed slightly higher levels of total IgG in response to HCF, however both parasite antigens had good predictive values when used for total IgG ELSA. No difference was found between HCF and ALP according to the peripheral IgG1 and IgG3 responses to these antigens. Antibody ELISA did not confirm any significant IgG2 and IgG4 responses to ALP in the sera of hydatid patients.

Cytokine responses only showed significant prognostic values according to the higher spontaneous levels of IL-6 and IL-10 in GIII and the HCF-induced production of IL-6 in GII and GIII patients. In all stages of the infection, IgG4 response to HCF remained higher than that to ALP, but such a difference was only significant in GI patients for IgG2 due to the decline in the levels of this antibody in GII and GIII sera.

Negative correlation was observed between the serum levels of IgG1 and IgG4 in response to HCF. Concentrations of TNF-α and IL-5 were also negatively correlated in the supernatants of the hydatid cultures stimulated with HCF. On the other hand, ALP-induced levels of IL-2 and IL-6 showed positive correlation in the hydatid PBMC cultures.
5. Discussion

It is alleged that in chronic infections of tissue-dwelling parasites immune modulation toward Th2-profile can suppress the adaptive immune-mediated inflammatory responses and may cause prolonged allergic reactions and fibrosis [29]. In the present study, immunohistochemical analysis could show significant activation of CD3+ and CD4+ T cells along with macrophages, neutrophils and HSCs as major populations composed the structure of cell infiltrates while the ratio of CD4+/CD8+ phenotypes was 1.29 in the CH biopsies. As well, CD4+ T cells and HSCs had significant contribution to the liver inflammation in the SH samples, but aggregation of cytotoxic T cells was expectedly weakened in these biopsies due to the relatively more stable condition of the organ in steatohepatitis. Composition of T lymphocyte assemblages around the hydatid pericyst in CE represented a completely different pattern when compared to SH and CH. The community of T lymphocytes contributed to the inflammatory reactions of the liver in CE dominantly comprised CD3+ T cells with diminished aggregation of CD4+ and CD8+ subtypes. Predominant activation of CD3+ T cells within the pericystic adventitia was reported in hydatid infection of sheep liver, while CD8+ and CD4+ cells were the most frequent populations in cattle with progressive and regressive cysts, respectively [23] [32]. Considering the higher frequency of fertile cysts in humans and sheep [22] [12], results of the present study could also show the impact of the host’s susceptibility on the structure of T cell-mediated immunity in CE. Besides, significant differences between the quantities of CD8+ cells involved the tissue-specific responses to CE and CH could also imply the prognostic value of CD8-dependent activity of T lymphocyte in chronic inflammations of the liver with different etiology. The predominant activation of CD3+ cells may also remark the involvement of CD3+CD56+ natural killer T lymphocytes [9] in the inflammatory reactions of the liver during chronic CE. Although activation of Treg is believed to induce immune tolerance and may be used as an escape mechanism by parasites to impair the protective responses of the host [26] [24] [31], results of the present study showed inefficient participation of FOXP3+ cells in cellular immunity of the liver to CE. As it was mentioned earlier, ADCC is an important defense mechanism primarily activated against the chronic phase of helminthiases [38]. Type 1 cytokines are thought to classically activate macrophages that in turn recruit neutrophils and other leukocytes by expression of various immune mediators [38] [21]. Thus an acute inflammation is initiated which induces overproduction of toxic radicals such as inducible nitric oxide synthase (iNOS) [38]. Th2-like cytokines can be remarkably identified
in the peripheral immune responses to chronic CE and may have downregulatory effects on ADCC mechanisms [18]. Results of the present study did not confirm the significant participation of macrophages and neutrophils in the inflammatory reactions of the liver around the parasite lesions, suggesting the deficient ADCC mechanism. Due to the impaired aggregation of T cell of all identified subtypes at the periphery of hydatid cyst in human liver, it can be assumed that immune suppression within the tissue may be induced by other mechanisms which are rather T cell-independent. It could imply the immune inhibitory properties of the parasite antigens that directly affect the inflammatory cell activation around the pericyst. Furthermore, the activation patterns of T lymphocytes and innate immune cells in hydatid liver seem to be irrelevant to the shift in Th1/Th2 balance but it can be interpreted as generally exhausted T-mediated immunity extended to all major T cell subtypes. Whether such a general anergy is caused by hydatid antigens- induced clonal exhaustion or it is due to the apoptotic elimination of specifically or non-specifically activated T cells are worth elucidating [30]. Such notions could be also supported by the results of molecular experiments through which the expression levels of costimulatory molecules around the hydatid lesion in human infection of the liver was measured for the first time. Negligible expression of CTLA4 and CD28 mRNAs around the hydatid pericyst suggested that the diminished T cell-dependent responses of all sorts (either stimulatory or inhibitory) in the tissue-specific responses to the parasite are more likely due to the existence of mechanisms other than costimulatory signaling pathways.

Abundant numbers of CD20+ B cells with the highest relative frequency among all sample groups were observed at the periphery of hydatid lesions, suggesting significant activity of adaptive humoral immune system in the liver. These results could be the first indication of the humoral functions in locally activated responses to the hydatid infection of the liver, as aspects of the humoral immunity in CE were mostly studied by indirect methods in the peripheral blood of human patients or in animal models. It has become evidence that B cells can contribute to the process of fibrosis by production of IL-6 which has profibrotic effects and promotes transformation of HSCs to myofibroblasts [36]. Therefore, these results can also hallmark the role of B cells in immunopathology of CE in human liver. As well, high frequency of CD20+ B cells along with remarkably expressed CD80 and CD86 mRNAs at the periphery of hydatid lesion can represent efficient antigen presenting functions in tissue-specific cellular immunity against the parasite. It can be supported by IHC results which demonstrated the significant activation of
fibroblasts and aggregation of α-SMA⁺ HSCs in CE livers, implying the existence of sustained irritation in the organ. Another important finding of the present study was the absence of CD1a⁺ cells in the inflammatory reaction to CE in human liver. Collectively, characterization of the inflammatory cell infiltrates around the pericyst evidently denoted the crucial role of APCs, particularly DCs with assumed CD1a⁺ phenotypes, and B cells in immunopathology of CE in human liver. It is worth elucidating whether in chronic hydatid infection APCs or HSCs can directly interact with the parasite antigens and maintain tissue remodeling and fibrosis in absence of other effector (such as T cells and macrophages). Besides, they may be able to induce modal changes in the immune responses through cell-cell cross talk (more likely via costimulatory signals) as it has been showed in other infectious diseases [2].

Biochemical characterization of ALP indicated significant differences between fertile and sterile hydatid cysts in the present study. The potential role of some membranous molecules of the cyst, particularly those of metabolic importance, in susceptibility/resistance of the parasite to chemotherapeutic agents has been implied [8] [13]. Results of the present study also confirmed that in search of a reliable biomarker, ALP can be a good candidate to monitor the anatomy and functional status of the cyst wall. Molecular properties and enzymatic activity of ALP from sterile hydatid cysts and from sheep liver extracts were almost identical. It can be inferred that anatomical damage interferes the selective permeability of the membrane, thus ALP from sterile cysts may be likely of the host origin. Immunoblot test also confirmed that the enzyme from fertile cysts can potentially have immunogenic properties, while sterile cyst-derived ALP does not stimulate the host immune responses.

Immune responses to ALP from fertile hydatid cyst were also characterized and demonstrated the ability of this molecule to activate both cellular and humoral reaction in the host. Cell proliferation test showed higher specificity when ALP was used as the stimulator. PBMCs isolated from hydatid patients proliferated in response to ALP regardless the stage of infection. This finding could indicate the ability of ALP to initiate clonal activation of lymphocytes that later can evolve into memory cells. Cytokine ELISA proved to be a reliable method for screening purposes, especially with regard to significantly higher spontaneous levels of IL-2, IL-4, IL-5, IL-6 and IL-10 in the hydatid cultures compared to normal controls. Results of this study also showed that ALP can induce activation of Th1-like profile, while both type1 and type2 cytokines were produced in response to HCF by the cultured PBMCs. Moreover, ALP was shown to
stimulate hydatid PBMCs to produce significantly higher levels of IL-6 when compared to the cultures from healthy donors; however higher levels of this cytokine were measured in the supernatants of cell cultures from the hydatid patients in response to HCF. Altogether these findings suggest the presence of B cell-activating molecules in the antigenic composition of hydatid metacestode and may introduce ALP as one of these agents which play a role in IL-6 mediated responses to the parasite in human hosts. Negative correlation between the induced levels of TNF-α and IL-5 in response to HCF was in accordance with existing concept of immune responses to the chronic CE during which cross-inhibitory mechanisms are concurrently activated [38]. As well, these findings may underline the role of γδ T cells in inflammatory responses against hydatid cyst [28] [20]. The correlation between IL-2 and IL-6 in response to ALP could also imply the role of this antigen in clonal expansion and proliferation of memory B cells.

The ability of ALP to induce immune memory was also indicated by the antibody responses to the antigen which were independent of the patients’ natural history and the stage of the infection. Detection of anti-ALP antibodies in the sera of hydatid patients also confirmed the existence of protective immune response against the enzyme in the peripheral blood, due to effective total IgG, IgG1 and IgG3 responses to this antigen. Contrary to HCF which had false positive and cross-reactive responses, ALP seemed to be a more specific antigen for serological purposes.

As a conclusion, results of the present study suggest that the inconsistency of immune reactions to hydatid cyst is perhaps correlated with the variations of antigenic components which emerge in a particular stage of the parasite development and manipulate the host immunity. As for the tissue-specific reactions to the parasite, the immune system may be silenced more likely upon phenotypic and functional changes of APCs, particularly DCs, after exposure to specific epitopes that in turn induce anergy in effector cell populations such as T cells. On the other hand, significant infiltration of DCs, B cells, CD3+ T cells, HSCs and accumulation of intracellular matrix indicate constant activation of specific immune responses around the cyst. There are thought to be other antigenic components capable of activating immune responses, however their expression maybe downregulated as an adaptation mechanism or through aging processes. ALP, as an important enzyme for the parasite growth and survival, can potentially induce protective responses and therefore should be considered as a candidate for further investigation of the CE immunopathology.
6. Novel scientific findings of the present study

Tissue-specific responses that maintain the hemostatic changes during the chronic course of hydatid infection were long neglected, particularly in human hosts. The present study for the first time depicted a comprehensive image of the cellular populations involved in locally activated immune reactions in chronic CE of human liver.

The expression of costimulatory CD28, CTLA4, CD80 and CD86 molecules around the hydatid pericyst had not been described before. Results of the present study, for the first time, showed the levels of costimulatory mRNA expression at the vicinity of hydatid lesion in the human liver. It can be the first report to evidence the role of costimulatory pathways in tissue-specific immune responses to the parasite in human hosts.

The present study was the first to indicate differences between fertile and sterile hydatid cysts according to the molecular traits and enzyme activity of ALP. In addition, the immunogenic properties of ALP for the first time were examined by assessment of the cellular and humoral responses to the antigen in human peripheral blood.

Novel findings of this study provided further information about molecular and cellular components which are engaged in immunopathology of CE. Such data can fruitfully improve the existing knowledge of the host-parasite interplay and can be potentially applied to develop new therapeutic methods as well as effective immunization strategies.
7. References


List of Publications:
