Immunopathology of hydatid infection in human liver

PhD Thesis

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List of abbreviations

Ab: Antibody
ADCC: Antibody-dependent cell-mediated cytotoxicity
Ag5: Antigen 5
AgB: Antigen B
ALP: Alkaline phosphatase
APC: Antigen presenting cell
AUC: Area under curve
CD: Cluster of differentiation
CE: Cystic echinococcosis
CH: Chronic hepatitis
CI: Confidence interval
CTLA4: Cytotoxic T Lymphocyte-Associated protein 4
CT-(Scanning): (X ray) Computed tomography
DAB: Diaminobenzidine
DC: Dendritic cell
dNTP: Deoxynucleoside 5-triphosphates
EDTA: Ethylene deamine tetra-acetic acid
ELISA: Enzyme-linked immunosorbent assay
FBS: Fetal bovine serum
FFPE: Formalin-fixed paraffin-embedded (tissue sample)
FN: False negative
FOXP3: Forkhead box protein 3
FP: False positive
HCF: Hydatid cyst fluid
HEPES: 4-2-Hydroxyethyl-1-piperazineehtane sulfonic acid
HIER: Heat-induced epitope retrieval
HRP: Horse-radish peroxidase
HSC: Hepatic stellate cell(s)
IB: Immunoblotting test
IEP: Immunoelectrophoresis test
IFA: Indirect immunofluorescence antibody test
IFN-γ: Interferon γ
Ig: Immunoglobulin
IHC: Immunohistochemistry
IL: Interleukin
iNOS: Inducible nitric oxide synthase
LA: Latex agglutination
MCP-1: Monocyte chemotactic protein 1
MHC: Major histocompatibility complex
MIP-2: Macrophage inflammatory protein 2
M-MLV: Moloney murine leukemia virus
MPO: Myeloperoxydase
MRI: Magnetic resonance imaging technique
mRNA: Messenger ribonucleic acid
NK: Natural killer (cell)

PBMC: Peripheral blood mononuclear cell(s)
PCR: Polymerase chain reaction
PHA: Phytohemagglutinin
POPOP: 1,4-bis-5-Phenyloxazole-2-yl-benzene
PPO: 2,5-Diphenyloxazole
qPCR: Quantitative Real-Time polymerase chain reaction
RF: Relative frequency
ROC: Receiver operating characteristic curve
RPMI: Roswell Park Memorial Institute
RT-PCR: Reverse transcription polymerase chain reaction
SDS: Sodium dodecyl sulfate
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH: Steatohepatitis
TAE: Tris base+acetic acid+EDTA buffer
TCR: T cell receptor
TE: Tris base+EDTA buffer
TEMED: Tetramethylethylene diamine
TGF-β: Transformation growth factor β
Th: Helper T cell
TN: True negative
TNF-α: Tumor necrosis factor α
TP: True positive
Treg: Regulatory T cell
α-**SMA**: Smooth muscle actin α

1. Introduction

The pathogenesis of an infection thoroughly depends on how the immune system interacts with released antigens. Triggered immune functions are normally meant to sustain the homeostatic balance of the organ upon being interfered by the infection as well as depletion of infective agents and compensation of probable tissue damage through hiring various mechanisms. Considering the forceful abidance of the infection to survive, immune system is not always the ultimate winner of this combat and may fail to manage its defensive task. Ironically, immune reaction appears to be harmful if production of immune mediators is drown out and left uncontrolled. Indeed, investigating the pathogen-immune system interplay can help to decode underlying mechanisms by which the resultant pathology is induced and may lead to employ more efficient therapeutic strategies against infections. Apparently, such an assumptive reasoning appears to be so disputable when the pathogenesis of parasitic helminthes is the issue.

Despite being as one of the major health problems in the human history, the importance of helminthiases in public health was distinctively brought into consideration when Stoll uttered his epic presentation titled "The Wormy World" in Rockefeller Institute for Medical Research, later published in 1947 [251]. Since then, a huge body of research has been endured to expound various aspects of these infections with a particular emphasis on the host-parasite interaction and its association with the immunopathology of parasitic diseases.

Echinococcus granulosus is a Platyhelminth with a multistage life cycle that causes cystic echinococcosis in a wide range of mammalian hosts. The infecting larva usually lives in the liver and other visceral organs of the intermediate host. In an infected organ, the larva evolves to a fluid-filled cyst (hydatid cyst) surrounded by periparasitic granuloma and fibrosis [158]. Human is an incidental intermediate host whose infection is expectedly associated with drastic tissue damage and turns into a serious disease with clinical manifestations range from asymptomatic infection to severe, potentially fatal disease [38]. In most cases, the infection tends to be chronic and clinical signs are detectable only when the organ destruction and failure have already occurred [158]. *Echinococcus* species are found all around the globe, although some species have restrictive distributions. This infection is considered as one the major public health problems worldwide, particularly in developing regions with limited economic resources. Furthermore, there are indications of an increasing number of cases in certain areas implying emergence or re-emergence of the disease [231] [266]. So far, seven species of *Echinococcus* have been identified,

among which four species are of more importance: *Echinococcus granulosus* (which causes cystic echinococcosis), *Echinococcus multilocularis* (which causes alveolar echinococcosis), and *Echinococcus vogeli* and *Echinococcus oligarthrus* (which cause polycystic echinococcosis). Two new species have recently been identified: *Echinococcus shiquicus* in small mammals from the Tibetan plateau [27], *Echinococcus felidis* in African lions [117] and *Echinococcus ortleppi* (also known as *Echinococcus granulosus* cattle strain G5) [69], but their zoonotic transmission potential is not fully understood yet.

Hepatic alveolar and cystic echinococcoses are both life-threatening diseases with medical and economic impact and wide geographical distribution [144]. Polycystic echinococcosis is, on the contrary, restricted to Central and South America and only few cases have been reported in human [65].

1.1. Cystic echinococcosis

In both human and animal, cystic echinococcosis is characterized by growth and development of the *Echinococcus granulosus* metacestode mostly in the liver (almost 75% of cases) and less frequently in other internal organs [197]. The evolved metacestode in the harboring tissue features a fluid- filled, spherical, unilocular vesicle which is called hydatid cyst. Thus the infection in intermediate hosts sometimes is termed as hydatidosis.

1.1.1. Biology and the life cycle of *Echinococcus granulosus*

Echinococcus granulosus is a small tapeworm (rarely exceeding 7 mm in length) and adults are morphologically recognizable by a flattish body containing three segments. The adult worm lives firmly attached to the mucosa of the small intestine in definitive hosts, usually dogs and other canines (coyotes, dingoes, red foxes), where the adult-stage reaches sexual maturity within 4 to 5 weeks. This is followed by the shedding of gravid proglottids (each containing several hundred eggs) and/or of released eggs in the feces of definitive hosts [158]. These eggs remain alive and keep their capability to produce infection for a long time after defecation [260]. If eggs are ingested by the intermediate hosts, majorly ungulates such as sheep, pigs, goats, horses, every single egg releases an embryo (onchosphere) which possesses six hooklets. Onchospheres can penetrate the intestinal lamina propria and travel via blood or lymph, thus are trapped in the liver, lungs, and other sites where cystic development begins. This process involves transformation of

the onchospheral stage to the metacestode larva. *Echinococcus* larvae can inhabit a broad range of other mammals, such as marsupials, hares, rabbits, rodents, carnivores and primates. Humans can accidentally become "aberrant" intermediate hosts, after ingestion of *Echinococcus* eggs excreted by infected carnivores [4] [86].

About 5 days after ingestion of eggs, the metacestode evolves a large unilocular cyst, so-called hydatid cyst which is usually surrounded by a host-derived collagen capsule (adventitial layer or the pericyst), but can be also enclosed by host inflammatory cells [158] [156]. The cyst wall comprises an inner cellular layer with 20–25 micron of thickness (germinal epithelium or the endocyst) and an outer protective acellular layer (laminated membrane or the ectocyst) [158].

Microvilli-like extensions (microtriches) are extended from the germinal layer towards the apical membrane of its syncytial tegument and protrude into the matrix of the laminated layer. These microvilli increase the resorbing surface of the cyst [170] [135]. In addition, the germinal layer contains highly differentiated cell types including connective tissue, muscle cells, and glycogen storage cells, as well as many undifferentiated cells, e.g. "stem" cells. The brood capsules (daughter cysts) are small vesicular structures that are originated from the germinal layer toward the cyst cavity [140] [158]. These vesicles are primarily attached to the germinal layer by pedicles and may resemble a cluster shape in microscopic images. A large number of spherical larval heads (protoscolices) that typically possess a set of hooklets and visible suckers are asexually generated from the germinal epithelium and/or in the daughter cysts and later every one of them can evolve to an adult worm. The sucker discs are sometimes pulled inside (invaginated) or are extended out (evaginated) [4] [140]. When surrounding membrane of brood capsules rupture, the protoscolices and other larval particles such as hooks and proboscis are released inside the cyst lumen and because of their microscopic appearance are usually known as hydatid sand. Protoscolices are only produced in fertile cysts (contain germinal layer) cysts of 5 to 20 mm in diameter. Due to structural defect or absence of the germinal epithelium, some cysts do not produce protoscolices and remain sterile [170] [135] [140]. The outer laminated layer is a hyalinated carbohydrate-rich structure which is synthesized by the parasite and is secreted into peripheral entities of the cyst [170] [4] [81]. As there is no junction between cells toward the cyst cavity, the intercellular fluid of the germinal layer is apparently linked with the cyst/vesicle fluid [81].

The size of cysts is highly variable and usually ranges between 1 and 15 cm, but much larger cysts (>20 cm in diameter) may also occur. The exact time required for the development of protoscolices within cysts in the human host is not known, but it thought to be more than 10 months post- infection. The cyst cavity is filled by a clean, clear, aseptic liquid (hydatid fluid) containing secretions from either the parasite or the host [4] [140]. It may consist of some elements identical to those of the host's serum (Na, K, Cl, CO2, a density between 1.008 and 1.015, alkaline pH) and some proteins with antigenic properties such as Ag5 and AgB [124] [155] [64].

If fertile metacestodes in the cyst-containing organs of the infected intermediate host are ingested by a suitable definitive host, the *Echinococcus granulosus* life cycle is completed, in that the protoscolices evaginate, attach to the intestinal mucosa and develop into adult stages and start to produce parasite eggs in 32 to 80 days [170] [158].

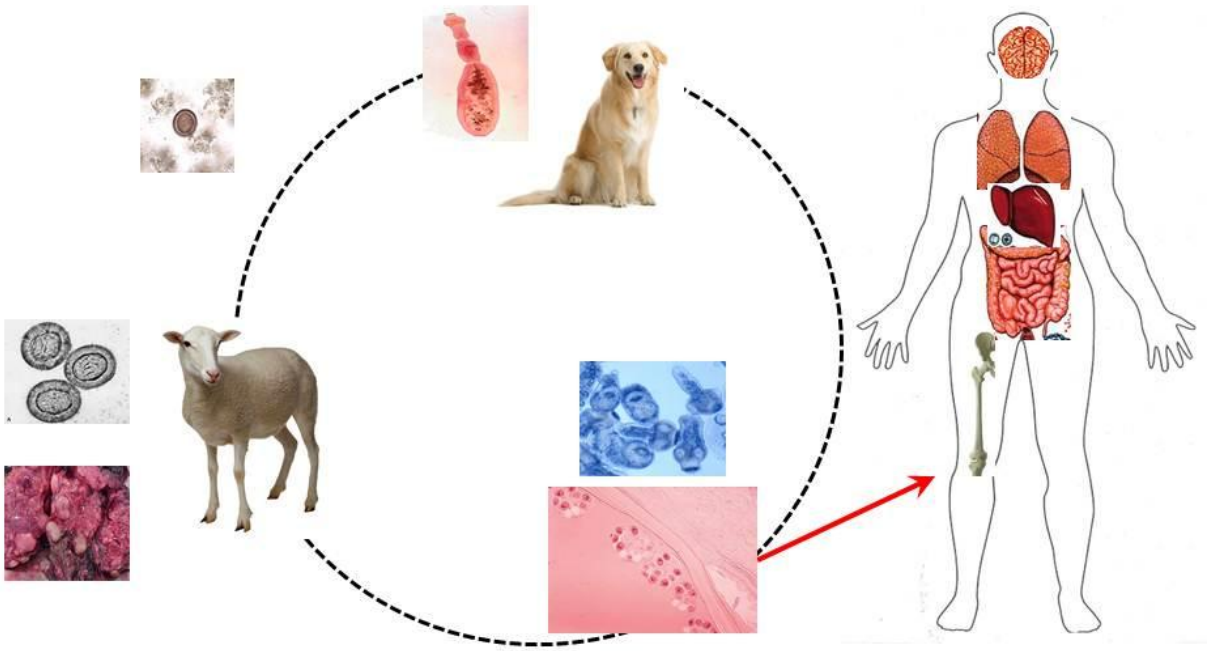

Figure 1: The domestic life cycle of *Echinococcus granulosus* has been shown in this picture. Adults normally live adjacent to the intestinal mucosa of dogs where they become sexually mature and produce fertile eggs. Gravid proglottids are detached from the body of adult worms and eggs are released into the lumen of the intestine. These eggs are passed through feces and infect the intermediate host (in domestic cycle are sheep, goats, cattle, etc.). The embryo is hatched in the digestive system of the intermediate hosts and migrates via blood circulation into various organs. Since the embryo is settled in the targeted organ, evolves into the larva (hydatid cyst) and continues growing to produce protoscolices. Each protoscolex can evolve to an adult worm if the infected visceral organs of the intermediate host are ingested by dogs. In the sylvatic life cycle of the parasite, other canines such as foxes, wolves, bears, etc., can be involved as the definitive hosts while a wide range of ungulates bear larvae as the intermediate hosts. Humans can be accidentally infected by ingesting fertile eggs and the metacestode can be formed in different organs, as it has been shown in the picture [158].

1.1.2. Epidemiology

The life cycles of E. granulosus can be classified as domestic, involving the domestic dog as the main definitive host and various species of domestic ungulates as intermediate hosts, or as sylvatic, involving wild carnivores (Foxes, wolves) and Cervidae (elk; *Alces alces*, reindeer; *Rangifer tarandus*, and red deer; *Cervus elaphus*) as intermediate hosts. The sylvatic cycle is reported in confined regions of the world such as North America and Eurasia [261]. In many areas where the infection is endemic, both forms of the parasite life cycle coexist or overlap [86]. Molecular studies using mitochondrial DNA sequences have identified 10 distinct genetic types (G1–10) within *Echinococcus granulosus* [262]. These include two sheep strains (G1 and G2), two bovid strains (G3 and G5), a horse strain (G4), a camelid strain (G6), a pig strain (G7), and a cervid strain (G8). A ninth genotype (G9) has been described in swine in Poland [222] and a tenth strain (G10) in reindeer in Eurasia [201]. The sheep strain (G1) composes the largest global population of *Echinococcus granulosus* and is that most common strain associated with human infections [262]. The 'cervid', or northern sylvatic genotype (G8), is upheld in cycles involving wolves and dogs and moose and reindeer in northern North America and Eurasia. Human infection with this strain is rare and is predominantly characterized by pulmonary cysts which grow slowly and the infection may remain benign or manifests less frequent clinical complications than reported for other forms [262].

Recently, the World Health Organization [37] included echinococcosis as part of a Neglected Zoonosis subgroup for its 2008–2015 strategic plans for the control of neglected tropical diseases [66]. The distribution of E. granulosus is worldwide, with only a few areas such as Iceland, Ireland, and Greenland that are seemed to be free of endemic human cystic echinococcosis [38]. The infection is mostly prevalent in the temperate zones, including South America, the entire Mediterranean region, Russia, central Asia, China, Australia, and north and east Africa [293] [46] [68]. In the USA, most cases are diagnosed in immigrants from countries in which echinococcosis disease is endemic. Sporadic autochthonous transmission is currently recognized in Alaska, California, Utah, Arizona, and New Mexico [169]. This is more likely due to the trade of live livestock between endemic and non-endemic regions. Moreover, diagnosed human cases in sporadic regions are more often among immigrants from countries where the infection is prevalent [188].

Current status of the infection in Europe shows the highest prevalence in the Mediterranean regions. It is suggested that the human infection rates from 0.28 to 10.8 per 100,000 inhabitants in the continent [23] [16] [46] [104]. The prevalence of infection obviously corresponds to the distribution of livestock farming and fluctuates according to the presence of other risk factors such as semi-industrial or non-industrial breeding of domestic animals, illegal slaughtering and improper extermination of infected viscera that are available to domestic and wild canids [44]. Human infection is usually considered as a 'dead-end' in the normal development of the parasite, whereas some cultural issues in endemic areas (such as Turkana in Kenya where human dead bodies are traditionally left unburied) may change the human involvement to an active intermediate host in the parasite life cycle [28].

1.2. Pathology of cystic echinococcosis

Despite carrying a massive parasite burden, definitive hosts do not normally show any symptom while being infected by adult worms. On contrary, the larval stage of *Echinococcus granulosus* induces significant pathology in the intermediate host [266]. Indeed, the pathogenicity of hydatid cyst differs from host to host and depends on many factors such as age, sex, genetic traits, physiological condition and species of the host [266]. Besides, severity of clinical symptoms is closely correlated to the size, number and localization of evolved cysts [4] [266].

Almost in all intermediate hosts, hydatid cyst is principally located in the liver with a frequency of about 70%, although can be found in other organs such as the lungs (20%), kidneys, spleen, brain, heart and bones with less frequency [302]. About 20–40% of human patients have multiple cysts or multiple organ involvement. After an undefined incubation period which may last months or years, the exerted pressure on adjacent tissue by a grown cyst may cause symptoms and can be followed by other pathologic events. As hydatid cysts grow slowly, the host often tolerates it remarkably well and therefore hydatid patients may come to clinical attention only when the normal function of the infected organs is interfered by the mechanical pressure of the cyst. Other clinical signs such as allergic reactions, eosinophilia or accidental cyst rupture which triggers acute hypersensitivity responses can also indicate the existence of the infection. Cysts or a cystic mass may also be discovered by chance during body scanning or surgery for other clinical complications [180].

1.2.1. Cystic echinococcosis of the liver

Once the onchosphere passes through the intestinal wall, it is carried by the portal venous or lymphatic system to the the liver as the first line of defense. That is why the liver is the most frequently involved organ. Most cysts tend to be harbored in the right lobe [125]. Natural history of the hydatid cyst can be divided into two phases [125] [250]:

1- During the first phase, continuous growth and the enlargement of the cyst can cause increased compression on the surrounding parenchyma and may result in upper abdominal pain and other non-specific signs. While hydatid cyst is growing, the cyst wall may lose its resistance against the pressure of the hydatid fluid, thus cyst rupture occurs. As well, this condition can happen due to a trauma or even surgical intervention. In general, symptoms such as acute allergic reactions, obstructive jaundice and emesis can be detectable as consequences of the cyst rupture.

2- If protoscolices and daughter cysts are overproduced during the first phase, the hydatid fluid will be replaced by these components which results in stiffness of the cyst cavity and is followed by the calcification of the cyst wall. In this phase, cyst growth usually halts and the ectocyst is detached from the fibrous capsule. Partial calcification of the cyst does not always indicate the death of the parasite; nevertheless, densely calcified cysts may be assumed to be inactive.

Hepatic cysts can cause complications in about 40% of cases. In order of frequency, these can be commonly observed as secondary infections, the cyst rupture to the biliary tree and rupture to the peritoneal or the pleural cavities [125] [75].

Secondary infection (i.e. bacterial, fungal) of the hydatid lesion is the most common complication and can be somewhat symptomatic. Infection occurs only after communicating and direct rupture when both the pericyst and endocyst fracture, which allows pathogens to pass easily into the cyst, in 5%–8% of cases [141]. The evolvement of an infected hydatid cyst is usually dormant, sub-acute and is clinically identified by pain in the right hypochondrium, hepatic abscess, and fever [168] [235].

Biliary rupture may occur through a small fissure or bile duct fistula [125] [75] [295]. A wide perforation allows the access of hydatid membranes to the main biliary ducts, which can cause symptoms simulating choledocholithiasis [125] [33]. Intrabiliary rupture of a hepatic cyst can be indicated as an occult drainage of hydatid fluid into the biliary tree and is observed in 10-37% of patients mainly in centrally localized cysts [125] [200] [270]. The increased pressure of the hydatid fluid can be also a prompting factor of the rupture usually in the right hepatic ducts (55-

60 cases), although the left hepatic ducts are sometimes involved [125] [200] [270]. More severe complication can be detected due to an overt passage of intra-cystic material to the biliary tract in 3-17% patients [247]. Perforation into the gallbladder can be detected in 5-6% of cases. The hydatid cyst rupture to the biliary ducts and the dissemination of the hydatid material in the biliary tree lead to the occurrence of other biliary complications such as biliary obstruction, ascending cholangitis and hydatid-induced biliary lithiasis [200] [33].

Hepatic cyst rupture to the gastrointestinal tract is very rare [125], however involvement of the diaphragm and thoracic cavity can be detected in 0.6-16% of patients [101]. Perforation of the hydatid cyst into the peritoneal area and the thorax may cause serious consequences such as anaphylactic shock due to the discharge of highly antigenic compounds exist in the cyst content [235] [40] [77]. On the other hand, released protoscolices settle other visceral organs and every one of them can potentially evolve to a hydatid cyst. This condition is termed as secondary cystic echinococcosis and may occur either spontaneously or after trauma. Formation of secondary cysts has been also observed as a complication after inattentive surgery [235] [40] [77]. Involvement of the pulmonary parenchyma or peritoneum is usually the most frequent trait of secondary cystic echinococcosis. Nonetheless, primary infection of the peritoneum has been also reported [244] [286] [132].

1.2.2. Cystic echinococcosis of other organs

In human hosts, the lungs are the second most frequent sites of infection in adults, while the involvement of the lungs is the most common feature of cystic echinococcosis in children [19]. In organs such as the lungs and the brain, hydatid cysts may grow faster and achieve larger size more likely due to the softness of the tissues which is easy to compress. Calcification in pulmonary cysts is very rare (0.7% of cases) [122], although it may be seen in pericardial, pleural, and mediastinal cysts [140]. Sudden coughing attacks, hemoptysis, and chest pain are the most common clinical symptoms [83] [122]. Expectoration of the fluid or other materials of the cyst and its rupture into the pleural cavity may also occur. Bacterial infection of the cyst is the most serious complication commonly seen after rupture [83] [122].

The prevalence of renal infection is 3% and the involvement of the kidneys usually remains asymptomatic for many years, although symptoms such as flank mass, pain and dysuria can be commonly seen [280] [182]. Renal hydatidosis can be characterized more frequently by solitary

cysts located in the cortex, and they may reach 10 cm before any clinical symptoms are noted [182]. Uncomplicated cysts may produce a lump of rounded mass in the outline of the kidney that extends the infundibula and calices. In up to 18% of cases, the cyst may rupture into the collecting system, a situation which may induce acute renal colic and hydatiduria. Several round masses may be seen in the excretory system due to daughter cysts [182].

The splenic involvement in human hydatid disease has been reported in 8% to 9% of cases [96]. The metacestode can be harbored in the spleen majorly after systemic or inter-peritoneal dissemination of protoscolices due to the rupture of hepatic cysts. Consequently, the spleen is usually considered as the third most frequent site of infection in humans. Clinical symptoms such as abdominal pain, splenomegaly and fever are often observed in patients with splenic hydatid infection [96] [2].

The osseous involvement in hydatid disease is most commonly seen in the spine and pelvis, followed by the femur, tibia, humerus, skull, and ribs with a frequency ranged between 0.5-4% [19] [267] [118]. The absence of the cellular infiltrate and fibrosis (pericyst) around the cysts in the skeletal system allows them to grow vastly in an irregular manner and they may produce subsidiary branches that penetrate through less resistant compartments of the tissue, especially in the bone canals [267] [183]. The growing metacestode in the bone can also possess a vesicular shape which fills the space between trabecular structures and destroys the osseous tissue. If the tissue damage exceeds to the cortex, the subsequent diffusion of the parasite materials into the surrounding tissues may occur [267] [12].

Hydatid disease affects the central nervous system in 1% of cases [182] and is usually diagnosed during childhood. The hemispheres are the most common locations of the cerebral cysts, particularly around the middle cerebral artery, although the metacestode may be harbored anywhere in the brain [131].

The infection may involve almost any anatomic site due to hematogenous dissemination. Unusual locations include the heart, pericardium, orbit, retrocrural space, mediastinum, subcutaneous space, muscle, and adrenal glands [19] [14] [129] [21] [128].

1.3. Host- parasite immune interaction in cystic echinococcosis

Existing data about host immune reactions to cystic echinococcosis is still insufficient. Many studies have been carried out to unveil the association of immune responses to the host- parasite

relationship in different stages of the infection, although a definitive picture to show the clear-cut traits of hydatid- induced immune mechanisms cannot be concluded by the achieved results. It probably reflects the impact of the intermediate host phenotype on immune reactions, as a wide range of mammalian species is involved in the parasite life cycle. Furthermore, genotypic variability within a certain species is likely determinative to the immunity against cystic echinococcosis [292] [236]. The main problem is raised by taking into account that these results are primarily derived from studies on laboratory animals, so cannot be a precise indication of immunity against the parasite in naturally infected intermediate hosts. Immunoserological assays have also provided indirect evidence to characterize parasite- induced immune reactions in human patients and other mammalian intermediate hosts. Nevertheless, such findings seem inadequate to thoroughly illustrate the real feature of host- parasite immune interaction, particularly in human.

It is assumed that immune responses are initiated at the very beginning of infection course when the onchosphere invades the host tissues. As well, evolvement of the embryo to hydatid cyst more likely gives rise to numerous hemostatic changes within the harboring organ. Therefore, immunopathology of the infection can be studied during two distinctive phases: the establishment (pre-encystment) phase and established (encystment) phase [207] [31], however this concept has appeared to be controversial as the Echinococcus species are multicellular organisms shown to bear a large number of antigenic compounds that may frequently change during their life span [242].

1.3.1. Immune responses during the pre-encystment phase

Little is known about innate immune mechanisms are primed against early stages of hydatid infection. Study has suggested the innate immune responses have a crucial role in host susceptibility/resistance to the infection [281]. Evidently, various effector mechanisms corresponding to innate and adaptive immune functions are induced against experimentally produced hydatid infection either with eggs (primary infection) or with active onchospheres (secondary infection) in laboratory animals. Perhaps activation of complement system, particularly through its alternative pathway, associates with the host resistance against the infection during the pre-encystment phase. It has been implied that C5- mediated complement reaction followed by activation of inflammatory cells may cause pathologic changes when the

embryo is trapped in the harboring organ [207] [91]. Infiltration of leukocytes such as eosinophils, neutrophils and macrophages is a hallmark of cellular reaction against invasive larvae of tissue-dwelling helminthes and is detectable after 3-5 days post-infection in mice [218]. Eosinophils seem not to have participation in the host defense against adult Echinococcus granulosus [160]. Reuben and Tanner in 1983 showed that the induction of non-specific immunity in rats by bacillus Calmette- Guérin (BCG) or phytohemagglutinin can enhance the resistance of rats against hydatid infection [204]. There is no evidence to show the role of natural killer (NK) cells in elimination of hydatid cyst however elevated number of NKs in the peripheral blood has been indicated in patients with active cysts [110]. Allegedly, IL-12 has an important role in innate defense against the parasite as the increased resistance to hydatid infection was indicated by inducing IL-12 expression in mice [88] [3].

Both humoral and cellular schemes of adaptive immune system are shown to contribute to the host resistance against developing hydatid cyst. Detectable levels of immunoglobulin (Ig) G are produced against primary infection after 2 and 11 days in mice and sheep, respectively [296] [303]. Activation of neutrophils and macrophages upon presence of IgM and IgG suggests that the antibody-dependent cell-mediated cytotoxicity (ADCC) has likely a pivotal role in depletion of the parasite, as well conducts the pathologic changes during early stages of the infection [94]. There is a lack of information about early cytokine production in primary infection of hydatid cyst is insufficient, although it is believed that T lymphocyte profile activated against developing cyst is initially polarized toward Th1 cytokines which is capable to deplete the parasite and provoke inflammatory changes in the tissue [281]. In contrast, experimentally induced secondary infection in mice resulted in immediate infiltration of macrophages, neutrophils and eosinophils, but cytokines such as interleukin (IL)-4, IL-5, and IL-10 were measurable after one week suggesting the probable domination of Th2- mediated profile in early response to protoscolex antigens [73]. Activation of Th2- mediated immunity is thought to be associated with less inflammation and may facilitate the parasite survival [215].

The effectiveness of defense mechanisms against cystic echinococcosis is normally evaluated by the number vital protoscolices that can evolve to hydatid cyst or by the capability of larvae to form germinal epithelium after encystment. About 50% of oncospheres remain alive and develop to hydatid cysts in experimentally infected sheep [296].

1.3.2. Immune response during the encystment phase

Host immune reaction to the established cyst has received more attention for investigation about immunopathology of cystic echinococcosis. As soon as the embryo is lodged in a suitable organ, it starts to develop into hydatid cyst with complete endocyst and ectocyst [303]. In both animal and human host, humoral immune reaction against the established cyst is characterized by presence of circulating IgM, IgG1 and IgG4, and IgE [194] [60] [67]. Animal models challenged with either parasite eggs or active protoscolices initially show lower levels of expressed IgG subclasses, however gradual increase in antibody response is observed along with the cyst growth [74] [190] [303]. Expression of IgM, IgG1, and IgG3 may induce complement activation [24] [229] and suggests the role of innate immune mechanisms during the early stage of encystment phase. Although complement factors are not clearly shown to have contribution to the host defense against established cysts, their activation has been indirectly confirmed. Knock- out mice with deficiency of C5- mediated reaction show significantly higher susceptibility to the cyst establishment during the early stage of the secondary infection [91]. Complement effector molecules such as C3 and C4 were also measured in sera obtained from hydatid patient and remained detectable after surgery [265] [39]. Perhaps it can be inferred that the activation of complement system may enhance host resistance and sustain inflammatory responses to the newly formed hydatid cyst, although it seems to have no effect on the parasite survival in chronic infection [79] [92]. Deposition of complement agents around the cyst wall with no defect in biological functions of the parasite has been observed in secondary infection of animals [78]. Altogether these findings can show the restricted contribution of innate immune mechanisms (particularly complement agents) to the host defense and unreliable diagnostic value of these proteins during the chronic infection.

In patients with primary or relapsed infection IgE can be detected in serum and may decrease due to successful cyst extraction [265] [258]. Specific humoral reaction to the established hydatid cyst is dominated by expression of IgG1 and IgG4 in the intermediate host [73] [249] [258]. Serum levels of IgG1, IgG4, and IgE recline in patients with calcified cysts or after surgery [76] [67]. Moreover, relapsing disease in an individual induces elevated levels of IgG4 and IgE upon seeding of protoscolices [214] [67]. Consequently, circulating levels of IgG1, IgG2, and IgG4 antibodies are potentially valuable for the assessment of the infection stage [214] [136] [258].

The established cyst is thought to induce activation of different immune effector cells in the intermediate host. Cytokine profiling assays have demonstrated the presence of IL-2, interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), IL-4, IL-5, IL-6, and IL-12 in the sera obtained from animals with the primary or secondary infection and from human patients [107] [268] [73] [213] [17]. Collectively, available data suggests that in response to chronic hydatid infection both Th1 and Th2- mediated profiles are induced [211] [242]. Furthermore, chronic infection of *Echinococcus granulosus* is evidently characterized with remarkable expression of IL-10 [211] [213] [239] [185]. This cytokine may associate with the parasite survival and its production reclines after successful treatment but remains high in patients who does not respond to chemotherapy [213] [176]. Expression of IL-10 is considered in association with down-regulation of inflammatory responses and suppression of cell- mediated immunity principally by inducing the differentiation and proliferation of T lymphocytes to Treg subtype [271] [85] [298]. It is not clear yet whether how Treg cells participate in host immune reactions against cystic echinococcosis.

Activation of other cell populations involved in hydatid- induced immunity is not thoroughly explored yet. Existing concept of host cellular responses mediated by different cells from lymphcytic and monocytic background is largely based on results of experiments on animal models or is conferred from studies on other tissue- dwelling helminthes. Early studies suggested that the local granulomatous reaction around the pericyst comprises activated eosinophils, fibroblasts and mesothelial cells [243] [206]. In 1986, Riley *et al*., reported that primary exposure of Balb/c mice to protoscolex antigen induces cell proliferation in lymph nodes and production of specific IgM and IgE, although the inflammatory reaction dwindles after 3-8 weeks and protoscolices survive. They indicated that the second exposure to protoscolices initiates more intensive cell infiltration in infected tissues consisting of neutrophils, eosinophils, mast cells and macrophages which may associate with more efficient cellular response in challenged animals [218]. Immunohistochemical examination of hydatid lesion in cattle showed predominant activation of $CD8^+$ T lymphocytes at the periphery of progressive cysts [224]. Study on cattle infection also suggested that the composition of cell infiltrates around hydatid lesions of the liver and lungs originates from cells in the draining lymph nodes of these organs [224]. Peritoneal immunization of mice with glycoconjugate antigens derived from *Echinococcus granulosus* was reported to trigger differentiation of peritoneal B lymphocytes into IgM, IgG2, and IgG3-

secreting cells and initiates expression of Th2- like cytokines such as IL-4, IL-5, and IL-6 by these cells [173]. Such a phenomenon seems to be independent of T cell function; however an intensive response of NK cells and late Th2 response which was substituted for primary activation of Treg and Th1 subtypes was observed in experimentally induced secondary infection of mice [172].

The precise involvement of macrophages in protective immune responses to hydatid cyst is not investigated yet. It is assumed that the laminated layer can reduce macrophage- mediated functions and secretion of nitric oxide in murine models [248]. Presence of monocyte- derived inflammatory proteins such as S100 family along with infiltration of epithelioid and multinuclear giant cells have been also indicated in granulomatous reaction around the hydatid lesion of cattle [15]. Experimental infection of mice was shown to induce serum levels of monocyte chemotactic protein-1 (MCP-1) and macrophage inflammatory protein-2 (MIP-2) after 20 days which declined after 4 months post- infection [259]. Moreover, local cell infiltration in response to tissue- dwelling parasites is normally characterized by eosinophilia and remarkable deposition of neutrophil granulocytes [57], although eosinophil activation is usually impaired or is absent in chronic response to cystic echinococcosis [59] [242]. Activation of polymorphonuclear cells has been thought to associate with effective immune reactions and depletion of pathogens in the host tissue. Therefore down- regulation of their function is possibly an important mechanism parasites employ to evade the host immune system. Evidently, Echinococcus granulosus possesses antigenic molecules by which can diminish activation of neutrophils in vitro [217] [155] [277].

1.4. Diagnosis and Treatment of cystic echinococcosis

Due to the bad prognosis of developed infection, early diagnosis is an essential part of the treatment and control procedure in cystic echinococcosis. Considerably long incubation period of the infection during which clinical manifestations are usually absent, is an important challenge to plan an efficient strategy for early diagnosis of the infection [103]. Parasite larvae show the highest susceptibility to chemotherapeutic compounds during the pre-encystment phase or before maturation of the established cyst [87] [35] [103].

Because of the distinctive natural history of the infection, patients are usually found accidentally or when physical damage to the harboring organ and cyst rupture has occurred. A reliable diagnosis requires combination of physical examination, imaging techniques and

serology methods. Upper abdominal discomfort, loss of appetite and pain are of the major complaint that along with results of physical examinations such as hepatomegaly, presence of abdominal palpable mass and abdominal distention may lead clinicians to consider potential occurrence of cystic echinococcosis [219]. Manifested signs and symptoms resemble those of many other disorders, thus differential diagnosis is necessary to distinguish cystic echinococcosis from various health problems such as jaundice, colicky pain, portal hypertension, ascites, compression of the inferior vena cava and Budd-Chiari syndrome and can be misdiagnosed as non-parasitic cysts, single or multiple hemangiomas, pyogenic or amebic liver abscess, hematoma, adenoma, adenocarcinoma, hepatocellular carcinoma, metastases, focal or diffuse lymphoma, alveolar echinococcosis, and textiloma [36] [49]. Consequently, it is very difficult, if not impossible; to diagnose cystic echinococcosis by clinical signs, and therefore further examination (i.e. imaging techniques, serology) is necessary.

1.4.1. Imaging techniques

Generally used imaging techniques include ultrasonography, CT- scanning and magnetic resonance (MR) imaging. Ultrasonography is the most common imaging method to identify hydatid lesion and is useful to determine the number and size of hydatid cysts in almost all anatomical sites. This technique can be used in field surveys by applying portable machines [150]. Ultrasonography can only visualize cysts with at least partial calcification where echogenic regions (either hyperechogenic or hypoechogenic) are scattered throughout the lesion. Regarding this trait, it has been tried to introduce an internationally accepted system to standardize the hydatid cyst diagnosis based on classification of its ultrasonographic features. Gharbi *et al*., were the first to propose a world-wide accepted classification system [99]. In 2003, the World Health Organization [37] published a new standard system which was actually an amended version of Gharbi's classification method introducing six categories: cystic lesion (a unilocular cyst with unknown origin which is to undergo more investigation), CE1 (unilocular fertile cysts with visible wall and 'snowflake' sign), CE2 (multivesicular septated fertile cysts), CE3 (laminated layer is detached from the cyst wall which makes 'water lily' sign), CE4 (cysts with scattered hypo- and hyperechoic degenerative contents and no visible daughter cysts), and CE5 (cysts with thick wall showing partial or complete calcification) [105].

1.4.2. Immunodiagnosis

Although imaging techniques provide valuable information about physical appearance, dimension and anatomical site of hydatid cysts, diagnosis can be sometimes presumptive [180]. A visualized lesion can be reliably confirmed by percutaneous aspiration or biopsy sections and microscopic examination of the cyst content, but every intervention of the cyst is anticipated to bear serious risks because of the capability of protoscolices to produce invasive infection in both clinical practitioners and patients are involved in this procedure [180]. Besides, release of hydatid fluid and protoscolices can have life- threatening consequences such as anaphylaxis [162].

Serology of hydatid cyst is normally based on tracing specific antibodies, parasite circulating antigens and circulating immune complexes in serum samples [241] [45] [119]. So far, various serologic methods have been tested for diagnosis of the infection. Due to the lack of sensitivity and presence of non- specific results, early methods such as Cassoni intradermal test and complement- fixation test were replaced by more sensitive examinations. Routine laboratory tests include indirect hemagglutination [55], enzyme- linked immunosorbent assay (ELISA), latex agglutination (LA), indirect immunofluorescence antibody test (IFA), immune- electrophoresis (IEP), and immunoblotting (IB) [185]. Immunoassays have appeared to be expedient not only for detection of the infection and follow- up analyses but also for screening studies in endemic areas.

Recent advances of molecular genetics have suggested application of more accurate methods such as molecular chain reaction (PCR) for detection of hydatid antigens or expression of specific antibodies, although performing these methods requires high- tech equipment and well- trained laboratory staff and other conditions which are often difficult to provide particularly in endemic areas of the Third- world or developing countries.

1.4.3. Therapeutic methods

Cystic echinococcosis treatment is based on surgery, chemotherapy or both. Cyst removal followed by anthelminthic medication is often proposed as the best choice for the disease management [35] [159]. Surgical operation is normally associated with many risk factors and may not be very cost- effective, although seems to be the best option for precise depletion of the parasite [106] [58]. Furthermore, clinical status of the patient and presence of other health complications as well as cyst indications (such as size, number and location which may make it

surgically inaccessible) and presence of well- experienced medical team can restrict surgery [13] [159] [180]. The most common techniques are used for hydatid cyst surgery include open procedure, laparoscopy and percutaneous treatment. Cyst removal by surgery is divided into radical (when pericyst and parasitic content of the lesion are entirely excised or cystectomy) and conservative (when only parasite cyst is removed or hydatidectomy) methods [123] [66].

Chemotherapy has recently received more attention for treatment of cystic echinococcosis. Benzimidazole and albendazole are current chemotherapeutic choices for the disease, although albendazole is more recommended because of its better absorption [283] [9]. A widely accepted protocol advocates oral administration of 10-15 mg/kg per day which must continuously last 3-6 months [37]. Although some degrees of success have been achieved by using anti- hydatid agents, adverse drug reactions, non-responsiveness to chemotherapy and relapse of the infection, parasite resistance to the administrated drug and in some cases aggravated conditions have been observed in a note- worthy proportion of patients [264] [263] [257] [95] [142].

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

Literature review shows that there are still many unanswered questions about host-parasite immune interaction in cystic echinococcosis. The lack of knowledge is particularly conspicuous about exact cellular and molecular mechanisms involved in immunopathology of the infection in human hosts.

1.5.1. Immunopathological aspects of cystic echinococcosis

So far, immunopathology of cystic echinococcosis has been majorly studied by model experiments and/or determination of peripheral immune responses. Results of these studies generally show concurrent activation of Th1 and Th2 profiles in hydatid patients. As an instance, immunization by *Echinococcus granulosus* tetraspanin induced expression of serum IFN-γ and IL-12 in mice [114]. Naik *at al*., showed that using anthelminthic drugs may change the serum cytokine profile in responders to the chemotherapy [176]. Expression of IL-6 and IL-17A was reported to increase in serum samples and supernatants from cultures of peripheral blood mononuclear cells (PBMCs) obtained from hydatid patients compared to healthy controls [161]. Patients with relapsed disease did not display IL-6 and IL-17A activity. These results implied the contribution of IL-17A in the host defense against hydatid cyst [161] although no significant

difference in expression of IL-17A had been previously noted between hydatid patients and healthy controls [233]. Chandrasekhar and Parija in 2009 showed the predominance of Th2 cytokine profile with presence of specific serum IgE and IgG in hydatid patients [52]. Immune response to *Echinococcus* larvae can be dynamically altered in correlation with various influential factors dependent on either the parasite or the host conditions. Secondary infection with 500 protoscolices induced early Th0 and late Th2 reaction in Balb/c mice whereas inoculation with 2000 protoscolices elicited early Th2 and late Th0 cytokine profile in this host [72]. Few studies have indicated the local immune reaction to the hydatid cyst in naturally infected ungulates. The most recent investigation by Vismarra *et al*., showed the predominance of $CD3^+$ T cells at the periphery of the hydatid lesion in sheep along with infiltration of forkhead box P3 (FOXP3)- expressing cells in immunohistochemistry assay [278]. Another experiment performed by Sakamoto and Cabrera in 2003 showed the presence of $CD8^+$ cells around the pericyst in the liver and lungs of cattle with significant likelihood of infiltrating inflammatory cells to be originated from draining lymph nodes of these organs [224]. however In 2012, Tuxun *et al*., suggested the role of Treg cells in impairment of effective immune response and the cyst survival by measuring the Th17 and Treg surface activation markers in the PMBCs of hydatid patients [271]. Classification of lymphocytes in the peripheral blood of hydatid patients showed significantly lower activation of $CD3^+$ $CD8^+$ cells in individuals with clinical symptoms and relative increase in $CD56^+$ $CD8^-$ cell populations in patients with active cysts [110]. Some investigators have also tried to examine the immunomodulatory effects of parasite specific antigens. In between, hydatid cyst- derived AgB has received more attention. Exposure of human PBMCs to AgB prompted Th2 activation profile and reduced production of IFN-γ and IL-12. Such results suggested the capability of this antigen to modulate host immune response in favor of the parasite survival [217], although recombinant subunits of this antigen were shown later to induce Th1 cytokine response [29]. Assessment of the peripheral immunity may reflect systemic immune reaction to the circulating antigens but cannot precisely explain cellular and molecular events occur in response to the cyst establishment [212] [17] [184]. It can be assumed that the systemic immune reaction in the host is provoked when peripheral inflammatory cells encounter circulating antigens or immune complexes and may change due to the localization of the infection even in different parts of a tissue compartment [265] [93] and temporal factors [187] [299]. Results of model studies should also be also considered watchfully. Indeed, evolutionary

changes have made reciprocal adaptation between hosts and parasites. Thus, immune components involved in the responses to natural infections can differ from those induced by experimental challenge. Moreover, taxonomy and biological variations of hosts have pivotal impact on the parasite immunopathology [242].

1.5.2. Costimulatory molecular cross- talk in cystic echinococcosis

The expression level of costimulatory molecules in tissue- specific immune reaction to hydatid cyst is not studied yet. Apparently, murine bone marrow- derived dendritic cells (DCs) achieve specific morphology with upregulated CD86 marker when stimulated by laminated layer extract [48]. Recombinant AgB also enhanced expression of CD80 and CD86 in the same cell lines [234]. Monocyte precursors isolated from peripheral blood of healthy human donors gave rise to semimature DCs with $CD1a^{-}$ $CD86^{+}$ phenotype in response to either hydatid fluid fraction or purified AgB [126] [216]. These cells were later cultured with human peripheral blood- derived lymphocytes and induced activation of Th2 cytokines in these cells [216]. Macintyre *et al*., reported the ability of hydatid fluid crude antigen to reduce proliferation of $CD28^{+}$ cells in the culture of human peripheral blood lymphoblasts [149]. Expression of CTLA4 in response to hydatid antigens has not been measured yet.

1.5.3. *Echinococcus granulosus* alkaline phosphatase, characterization of the parasite proteome

Proteomic analyses have appeared to be a precious tool for identification of Echinococcus granulosus antigens. Over the past decades, many proteins with immunogenic properties have been characterized in adult forms or the larval stage of the parasite; however the larva (hydatid cyst) has appealed more interest for proteomics studies. Protoscolex and hydatid fluid are the most frequently used resources for purification of parasite antigens [226] [53] [45] [166]. As the main protective barrier around the cyst which is in direct contact with the host- derived components, the cyst wall has been recently appreciated for its immune- reactive elements [166] [301]. Structure of hydatid cyst membrane reflects the cyst viability [47] [164]. Ectocyst maintains the chemical composition of hydatid fluid in active cysts by importing host- originated substances as well as providing a passage through which either somatic or excretory- secretory antigens leak out of the cyst cavity [152] [294]. Defect in the structure of laminated layer impairs

transportation of molecules through this membrane which may lead to the cyst calcification [221] [151]. Completely calcified cysts are normally inactive; however partial calcification does not necessarily indicate the cyst death [134] [246]. It has been documented that host immune components or chemotherapeutic agents can deteriorate the organization of ectocyst [116] [275] [246] [32]. Whether effective immune reactions and successful chemotherapy can truly cause calcification in naturally occurred hydatid lesions or this phenomenon is just a normal process of the parasite aging, merit further investigation [108] [80]. On the other hand, endocyst gives rise to protoscolices and therefore its structure is directly correlated with the cyst fertility [189]. Higher frequency of sterile cysts in some intermediate hosts or in particular tissue compartments [143] [11] [177] suggests that immune responses may damage cellular and molecular structure of the germinal epithelium in a host- dependent and/or tissue- dependent manner. Defect in the endocyst has been also reported after chemical treatment [116] [274] [164]. Even with lack of protoscolex germination, hydatid cysts can keep alive but are often associated with efficient defense mechanisms [42] [11] [208]. Perhaps biological functions of protoscolices are determinative to the biochemical content and concentration of the parasite metabolites [186]. Furthermore, hydatid cysts unable to produce active protoscolices cannot evolve to adult worms when ingested by the definitive host, thus disrupt the normal life cycle of the parasite. Altogether this information shows the importance of proteomic studies in finding specific substances by which viability and fertility of the parasite can be assessed. Novel antigens can be also applied for development of immunodiagnosis as well as vaccination methods.

In addition to somatic antigens such as Arc5 and AgB, hydatid- derived metabolic enzymes have been introduced as the parasite viability markers and their immunogenic traits have been characterized. Alkaline phosphatase (ALP) activity was firstly indicated by Reissenweber *et al*., in protoscolex and brood capsules of *Echinococcus granulosus* cysts [203]. These scientists assumed that ALP is responsible for basic phosphorylation in cells within the calcareous corpuscle- associated cells of mature protoscolices [203]. Fractionation of microtriche from a horse strain of *Echinococcus granulosus* showed the presence of ALP in tegumental surface of mature protoscolices [157]. Inhibition of ALP activity in hydatid cyst of experimentally infected mice after administration of 50 mg/kg^{-1} isatin for 18 days suggested the enzyme role in sustaining metabolic balance of the parasite microenvironment [192]. Presence of ALP in the cyst wall and antagonistic effect of albendazole, mebendazole and praziquantel on the enzyme activity was

demonstrated by Feng *et al*, in 1992 [89]. ALP was later purified and partially characterized from the ovine hydatid cyst membrane [137] and it was shown to have specific biochemical properties in compare with the enzyme purified from *Echinococcus multilocularis* and with that of mammalian liver origin [138]. Cytochemical analysis of the germinal epithelium suggested high ALP activity of the stem cells located in the innermost layer of endocyst that could be an indicator for the parasite development or degenerative changes in the cyst wall [70]. Presence of anti- ALP specific antibodies in human patients was confirmed by using ELISA and immunoblotting (IB) and showed significantly higher sensitivity and specificity than hydatid crude antigen in immunoassays [153]. Recent application of molecular techniques has also elucidated that the hydatid cyst enzymes play an essential role in the host- parasite interaction and are potentially valuable targets for diagnosis, chemotherapy and vaccination strategies [223] [62]. According to the above mentioned data, it can be hypothesized that metabolic enzymes, such as ALP, probably possess different immunochemical properties in hydatid cysts with complete (fertile) and incomplete (sterile) membranes. Such a difference is to be investigated for better understanding of causative factors by which hydatid cyst fertility is impaired, an approach to further explanation of the parasite immunology.

Evidently, immune reactions are initiated against the parasite antigens at the very beginning when the egg is ingested and oncosphere is released, albeit immune components are not always measurable and the infection becomes persistent in almost all individual hosts. Moreover, defense mechanisms are seemingly baffled while encountering the hydatid antigens in immunologically responder hosts.

Current understanding of host-hydatid cyst immune interactions is so poor. Allegedly, T helper1 (Th1) and T helper2 (Th2) profiles are simultaneously activated in the chronic inflammatory response to hydatid cyst. The involvement of regulatory T cells (Treg) in the hydatid-induced immunity has been also reported. Yet, it cannot be explained how different immune profiles with cross-inhibitory effects are concomitantly activated in the host and how these responses conduct tissue-specific cellular functions associated with distinctive pathology such as fibrogenesis.

In fact, the role of host immunity in body defense and pathogenesis of hydatid infection has remained unexplained, particularly in humans. Therefore, attempts to clarify different aspects of the host- parasite relationship can be pivotally helpful for further understanding of the infection

immunopathology and may provide valuable information about other chronic inflammatory diseases. In addition, characterization of parasite- derived excretory- secretory molecules is important not only as an approach toward proteomics study, also improves existing knowledge about the host- parasite immune interaction and achieved data can be used for development of specific immunodiagnostic methods and vaccination strategies.

Accordingly, the present study was designed and carried out by considering three major objectives as followed:

1. Characterization of the inflammatory infiltrate at the periphery of hydatid cyst in chronic cystic echinococcosis of the human liver with an emphasis on activation profile of T lymphocytes.

2. Evaluation of costimulatory CD80, CD86, CTLA4, and CD28 molecules mRNA expression within the inflammatory infiltrate around the periparasitic areas of the liver for assessment of the cross- talk between T cells and antigen presenting cells (APCs) at the periphery of hydatid lesion.

3. Determination of biochemical and immunogenic traits of hydatid cyst- derived alkaline phosphatase as a candidate for further proteomics approach.

2. Materials and Methods

To achieve the defined objectives, a multidisciplinary study was designed and was carried out using different methods to identify a panel of cell surface markers and to measure mRNA expression levels of costimulatory molecules for characterization of inflammatory infiltrate and evaluation of tissue- specific cellular immunity in chronic cystic echinococcosis of the human liver. Immune response to parasite- derived ALP was also assessed to provide more information of the hydatid cyst immunopathology in human host.

2.1. Cellular characterization of hepatic infiltrates at the periphery of human hydatid cyst

Orchestration of intrahepatic immune cells in granulomatous reactions to cystic echinococcosis (CE) of the human liver was studied by application of immunohistochemistry (IHC). All laboratory experiments were carried out at the 2nd Department of Pathology, Semmelweis University. Formalin- fixed paraffin- embedded (FFPE) liver biopsies and clinical examination data of patients attending the university's hospitals were obtained through an archive at the 2nd Department of Pathology. As a comparison, FFPE samples from patients with steatohepatitis (SH) and chronic hepatitis (CH) were also included in this study. According to the institutional review board of the Semmelweis University no license was needed for the study being retrospective in manner.

2.1.1. Patients and sample preparation

Twenty one patients with surgically confirmed chronic hydatid infection of the liver were selected. Post- surgically prepared FFPE biopsy samples from these patients consisting of total or subtotal cystectomy materials (hydatid cyst lumen, fibrous capsule and the adjacent liver parenchyma) were used for the present study. Likewise, samples from 11 patients with SH and 11 patients with CH were included. Microsections of 2.5μm thickness were prepared using Thermo Scientific Finesse E^+ microtome (Thermo Scientific, Waltham, MA, USA). For histological observations, microsections were primarily stained by hematoxylin-eosin method [148]. In brief, sections were deparaffinized by 95% alcohol solution in deionized water and were stained by Lillie Mayer's alum hematoxylin (ready-to-use solution, Dako) for 1 min. After rinsing in water, 0.3% acid alcohol solution (commercial grade ethanol 2800 ml, conc. hydrochloric acid 12 ml,

and distilled water 1200 ml) followed by 0.2% ammonia water solution (concentrated ammonium hydroxide 2 ml, and distilled water 1000 ml) (Sigma- Aldrich) were used for differentiation (30 seconds) and blueing (1 min) procedures, respectively. Sections were washed again, rinsed in Scott's tab water substitute (sodium hydrogen carbonate 10 g, magnesium sulfate 100 g, and distilled water 5 L) and were placed in eosin counterstain including 1% eosin Y 400 ml, 1% aqueous phloxine 40 ml, 95% alcohol 3100 ml, and glacial acetic acid 16 ml (Sigma- Aldrich) for 1 min. The procedure was followed by dehydration of stained samples by two changes each of reagent alcohol, 95%, absolute ethanol and xylene for 1 to 2 min each and sections were mounted on slides for microscopic examinations.

2.1.2. Immunohistochemistry

Immunohistochemistry (IHC) was performed by using BenchMark XT automated slide preparation system (Ventana Medical Systems, Tucson, AZ, USA) and assays were run by applying UltraView DAB detection kit (Ventana Medical Systems). The kit was optimized regarding an indirect IHC protocol which was based on heat-induced epitope retrieval (HIER) method. Citrate-EDTA buffer was used for antigen retrieval and removing calcium ion residues from the tissue [193]. The buffer was made by adding 1.92 g of 10mM anhydrous citric acid and 0.74 g of 2mM EDTA to 1000 ml of deionized water, then the pH was adjusted to 6.2 with 1N sodium hydroxide and 0.5 ml of 0.05% Tween20 (all reagents from Sigma- Aldrich) was added to the solution. To abolish the endogenous peroxidase activity, all slides were immersed in a mixture of methanol and 0.1% hydrogen peroxidase (H_2O_2) (Sigma- Aldrich) for 10 minutes and were washed twice each for 5 minutes with PBS. Unspecific and background staining was reduced by applying avidin- biotin peroxidase complex (Vector Laboratories, USA) according to the manufacturer's instruction [113]. A panel of primary mono and poly clonal antibodies was used for in situ detection of the targeted clusters of differentiation that are listed in Table 1. Appropriate anti-mouse immunoglobulin- horse radish peroxidase (HRP) conjugates (Dako) at 1:50 to 1:120 dilutions were used as secondary antibody probes. 2,3-diamino-benzidine (DAB) (Liquid DAB^+ Substrate, Chromogen System, Dako) was diluted to the final concentration of 0.5 mg/ml in DAB buffer provided in the kit and was used as chromogenic substrate. To enhance the chromogenic reaction, 0.4% cupric sulfate in 0.9% NaCl (sigma- Aldrich) was applied and slides were counterstained by hematoxylin solution (1 g/L, Sigma- Aldrich).

2.2. Expression of costimulatory molecules in hydatid- induced inflammatory infiltrates of the human liver

To provide more evidence about T lymphocytes and APCs molecular cross- talk at the periphery of chronic hydatid lesion, expression of CD28, CTLA4, CD80 and CD86 was assessed by using reverse transcription polymerase chain reaction (RT- PCR) to produce cDNA library from mRNA template of the target molecules. The 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). Liver biopsies from all patient groups (see section 2.1of the Materials and Methods) were subjected for this analysis and 15 microsections from each individual FFPE sample were collected in three tubes (each tube contained 5 microsections) then extracts with high concentration of RNA were pooled.

Table 1- List of primary antibodies which were used for immunophenotyping of the hepatic infiltrates in biopsies from patients with cystic echinococcosis, steatohepatitis and chronic hepatitis.

Cluster of Differentiation	Cell	Type of Antibody	Positive Control	Working Dilution	Source
CD1a	Monocyte-derived dendritic cells	Mouse anti-human moAb, clone MTB1	Thymus	1/100	Cell Marque
CD3	Mature T lymphocytes	Mouse anti-human moAb, clone UCHT1	Tonsil	1/100	Dako
CD4	T helper cells	Mouse anti-human moAb, clone 1F6	Tonsil	1/50	Novocastra labs LTD
CD8	Cytotoxic T cells	Rabbit anti-human moAb, clone SP 57	Tonsil	1/150	Dako
CD20	B lymphocytes	Mouse anti-human moAb, clone L26	Tonsil	1/250	Dako
CD68	Macrophages	Mouse anti-human moAb, clone KP1	Tonsil	1/200	Dako
MPO*	Neutrophil granulocytes	Rabbit anti-human polyclonal	Tonsil	1/100	Cell Marque
FOXP3	Regulatory T cells	Mouse anti-mouse/rat/human moAb, clone 150D	Tonsil	1/100	BioLegend
α-SMA**	Activated HSCs	Mouse anti-human moAb, clone 1A4	Bowel	1/200	Dako

**Myeloperoxidase*

***Smooth muscle actin α*

2.2.1. Extraction of total RNA from FFPE samples

High Pure RNA Paraffin Kit (Roche) was used for extraction of total RNA from the liver biopsies. Microsections of 10μm thickness were collected into 1.5 ml eppendorf tubes and were deparaffinized by adding 800 μl xylene to each tube and incubation at room temperature for 5 minutes. Then samples were washed by adding 400 μl and 1000 μl of absolute ethanol for the first and second time, respectively, each time the supernatant was discarded after centrifugation at 13000Xg for 2 minutes. To dry out the tissue pellet, all tubes were incubated at 55C° for 10 minutes, then 100 μl of tissue lysis buffer, 16 μl of 10% SDS and 40 μl of proteinase K (High Pure RNA Paraffin Kit, Roche, lyophilized, prior to work was diluted in 3.5 ml of dilution buffer according to the manufacturer instruction) were added to each tissue pellet and samples were incubated overnight at 55C°. The content of each tube was mixed with 325 μl binding buffer and 325 μl of absolute ethanol, and then was transferred into a High Pure Filter tube (Roche) placed in an eppendorf tube. The undesired residue on the filter fleece was removed by using wash buffers (provided in the kit pack) and the flowthrough was discarded. This stage was repeated three times and 90 μl of elution buffer was added into each High Pure Filter tube placed in a collection tube. After centrifugation, the collected extracts were treated by 0.1 μl of DNase (diluted 1:800 in elution buffer) and were incubated for 45 minutes at 37°C. Added enzymes and other peptide moieties where depleted by incubation all samples for 1 hour at 55°C with 10% SDS (18 μl), tissue lysis buffer (20 μl) and proteinase K (40 μl) complex. The procedure was continued by binding the nucleic acid molecules to the microfilter in High Pure Filter and washing the residues and collecting the tissue extracts containing total RNA in eppendorf tubes, as it was explained above. The level of RNA molecule was measured by pipetting 2 μl of each extract on optical eye of Nanodrop ND 1000 spectrophotometer (RIC Facility, BYU, Utah, USA) and the optical density was read at 260nm. Elutes were stored at -80°C for further examination.

2.2.2. Polymerase chain reaction (PCR) and agarose- gel electrophoresis

In a microtube, 1 μl of deoxynucleoside 5'- triphosphates (dNTPs) (10 mM of each nucleotide) (Life Technologies, Thermo Fisher Scientific, USA), 0.5 μl of 2.5 Unit/μl taq DNA polymerase (Life Technologies, Thermo Fisher Scientific, USA), 11 μl of DNA polymerase buffer 10x containing 200 mM Tris HCl (pH 8.4) + 500 mM KCl + 50mM $MgCl_2$, and 2 μl of a sample

extract were added and mixed. Primer sets (forward and reverse) for a housekeeping gene, β-actin, provided by Dr. Erzsébet Rásó (2nd Department of Pathology, Semmelweis University) and 2.5 µl of each was added to the PCR mixture and the total volume of each microtube was adjusted to 23 µl by nuclease- free water. Swift Max Pro Version 2009-1.2 (ESCO, USA) thermal cycler was used for running PCR with following parameters: 94°C for 3 minutes, 30 cycles comprising 94°C for 1 minute, 59°C for 1:10 minutes and 72°C for 1:20 minutes, and 72°C for 5 minutes. Nuclease- free water was used as the negative control.

Amplified fragments were visualized by agarose- gel electrophoresis. Briefly, 2.5g of agarose powder (Seakem LE, Lenza Rockland, ME, USA) was dissolved in 100 ml of TAE buffer (0.04 ml triacetate + 0.0001 ml EDTA) (Merk, Germany) and the gel was loaded with 10 µl of each specimen mixed with 2 µl of loading dye and was put into the tank connected to a power supply at 100V for 45 minutes. Electrophoretic bands were illuminated by rinsing the gel in ethidium bromide (BIO- RAD) for 30 minutes and were observed by a trans- illuminator (ultraviolet lightbox, G-Box SYNGENE UV, Synoptics Group, UK).

2.2.3. Reverse transcription of cDNA from mRNA templates

After isolation of RNA from FFPE samples, all extracts were treated with 1:10 v/v of Turbo buffer and 2µl DNase I (Turbo DNA- Free Kit, Life Technologies, USA) and were incubated for 1 hour at 37°C. Reaction was stopped by adding 1:10 v/v DNase inhibitor solution (Turbo DNA-Free Kit, Life Technologies, USA) and samples were centrifuged at 14000g for 1 min. The efficacy of DNA depletion was examined by β- actin PCR and agarose- gel electrophoresis (see section 3.2.2 of Materials and Methods). This procedure was repeated till no DNA contamination was detected, thus a pure RNA extract was obtained from each FFPE sample.

Reverse transcription PCR (RT-PCR) was performed to produce cDNA from total RNA template. In a microtube, 1 µl of 100 µM random primer- oligo dT (containing 30 µg oligo dT, Life Technologies, Thermo Fisher Scientific, USA), 1 µl of 100 µM dNTPs (Life Technologies, Thermo Fisher Scientific, USA) were added to 8 µl of each RNA isolate and all specimens were incubated at 70°C for 10 minutes in a micro-processor controlled thermal cycler (Crocodile III, Appligene Oncor, Durham, UK). After cooling down in ice for 2 minutes, following reagents:

- Moloney Murine Leukemia virus reverse-transcriptase buffer (M-MLV Reverse-Transcriptase Kit, SIGMA), 2μl,

- M-MLV reverse-transcriptase enzyme (recombinant, expressed in E.coli, SIGMA), 1μl,

- RNase inhibitor (M-MLV Reverse-Transcriptase Kit, SIGMA), 0.5μl,

- nuclease- free water, 6.5μl,

were added to each tube and then all tubes were incubated at 37°C for 60 minutes in the same thermal cycler (Crocodile III). RT-PCR products were checked due to the presence of cDNA transcribed from the RNA template by applying β-actin PCR and agarose gel electrophoresis as it has been described above.

To amplify cDNA transcripts of targeted costimulatory mRNAs, a conventional PCR (see section 2.2.2 of the Materials and Methods) was applied by using primer sets (forward and reverse) for CTLA4, CD28, CD80, and CD86 molecules. Primer pairs 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 (Table 2). Lyophilised primers were reconstituted in 1xTE buffer to make a stock solution of 100 μM/μl.

Table 2- Primer pairs were used for amplification of costimulatory molecules.

Gene	Primer Pairs	Annealing Parameter
CD28	Sense:5′- AAGCATTACCAGCCCTATG-3′ Antisense: 5′- GGAATCATCTAGGAAGGTCAACTC-3′	56.3°C 54.6°C
CTLA4	Sense: 5′- TGGCTTGCCTTGGATTTCAG-3′ Antisense: 5′- GGAATCATCTAGGAAGGTCAACTC-3′	55.9°C 54.6°C
CD80	Sense: 5′- CCTGGTTGGAAAATGGAGAA -3′ Antisense: 5′- AGGAAAATGCTCTTGCTTGG -3′	52.1°C 53.4°C
CD86	Sense: 5′- TGTCAGTGCTTGCTAACTTCAG-3′ Antisense: 5′- TGGTCATATTGCTCGTAACATCAG-3′	55.2°C 54.8°C

To evaluate the specificity of primer pairs, RNA isolates from normal human lymph nodes microsections (FFPE samples provided by Dr. Erzsébet Rásó, 2nd Department of Pathology, and Semmelweis University) were reverse transcribed by RT-PCR and cDNA molecules from the template of costimulatory mRNAs were amplified by PCR using designated primers. After electrophoresis, each amplicon was retrieved by excision the band from agarose gel and isolation the polynucleotides using EZ-10 Speed Column DNA Kit (Bio Basic, Canada). The kit was optimized for recovering 40 bp-40 kb DNA fragments from agarose gel. Briefly, the excised bands were collected into microtubes and 500 µl of binding buffer (Bio Basic, Canada) was added to each tube. After incubation within water bath at 55°C for 10 minutes, 10 µl of isopropanol was added to each tube and then the mixture was transferred into a filter tube combined with a collection tube. The flowthrough was discarded after centrifugation at 14000g for 1 min and 100 µl of washing buffer (Bio Basic, Canada) was added up to the filter membrane. Filter was dried by two times centrifugation at 14000g each for 1 minute. Filter tube was placed into a fresh reaction tube and amplicon was retrieved by adding 30 µl of elution buffer (Bio Basic, Canada) onto the filter followed by incubation for 5 minutes at room temperature. After

centrifugation at 14000g for 1 min, the supernatant was collected and DNA sequencing was performed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, Canada). All achieved sequences were compared to previously recorded gene alignments in the GeneBank by a BLAST search and identity score ≥ 98% was considered for evaluation of the designed primers.

2.2.4. Quantitative Real- Time polymerase chain reaction (qPCR)

Expression level of costimulatory mRNAs was measured by qPCR based on SYBR Green method. The assays were designed, validated and optimized according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [41]. As for the control template, total RNA extracts of intestinal crypts stem cells (human colorectal cancer) was provided by Dr. Erzsébet Rásó (2nd Department of Pathology, Semmelweis University). After reverse transcription of cDNA, the same primers (see Table 2.2) were used to amplify costimulatory molecules mRNAs and products were evaluated on agarose gel. Aliquots containing cDNA of each molecule were pooled and used as qPCR control library. Fractions (unknown and control libraries) were collected into microtubes and the content of each tube was diluted with nuclease- free water to the total volume of 150 µl. Five 5x serial dilutions (by using nuclease- free water) were prepared from all aliquots to generate a titration curve.

MicroAMP Optical 96- Well Reaction plates (Life Technologies, Thermo Fisher Scientific, USA) were used for qPCR assays. On a plate, each well was loaded by 3 µl of cDNA isolate, 6.3 µl of Absolute qPCR SYBR Green ROX Mix (Thermo Scientific, USA) containing following components:

- Thermo scientific Thermo- Start DNA polymerase,
- Proprietary reaction buffer including an inert blue dye for further visualization of SYBR Green mix after aliquoting into the wells,
- dNTPs including dTTP in order to increase the sensitivity and efficiency of the reaction.
- SYBR Green dye,
- ROX passive reference dye (500 nM),
- 1M $MgCl_2$,

0.4 µl each primer (primer sets designed for each molecule of interest), and nuclease- free water to the total volume of 12.5 µl. To seal the samples into the wells, loaded plates were covered by MicroAMP Optical Adhesive Film (Life Technologies, Thermo Fisher Scientific, USA) and then were centrifuged at 2500g for 2 minutes by a Z300 Universal Mid- Capacity centrifuge (Labnet Hermle Centrifuge Rotors, BioExpress, USA). qPCR was performed on a Light Cycler 480II Real- Time PCR System (Roche, Swizerland) and reactions were carried out in technical quadruplicate using the following cycle parameters: 95°C for 15 minutes, 50 cycles (95°C for 15 seconds, 56°C for 30 seconds and 72°C for 40 seconds), followed by a melting curve rising from 56°C to 95°C and a cooling cycle at 37°C for 10 seconds. Standard error in technical quadruplicate ranged from 0.9% to 3.0% (average: 2.2%). Nuclease- free water was used as the no-template control. To evaluate the relative mRNA expression, data for each target were normalized against β- actin mRNA level (reference gene) and were defined as the target mRNA/β- actin mRNA ratio in every isolate.

2.2.5. Data analysis

Results of IHC test were evaluated by applying a semi- quantitative assessment. Briefly, each slide was observed three times by a light microscope at X400 magnification and about 10 fields were randomly selected in each round. Consequently, the number of immunostained cells was counted in 30 objective fields under a fixed focus for each slide and the value of total count median for each cell phenotype was scored and assigned to groups as follows: N (no stained cell was observed), + ($0 \leq M \leq 10$, very low), ++ ($11 \leq M \leq 100$, low), +++ ($101 \leq M \leq 300$, moderate), ++++ ($301 \leq M \leq 500$, high) and +++++ ($501 \leq M$, very high). Assessment was performed independently by two observers and the mean ± standard deviation (SD) of their counts was used for data analysis. Where a dense accumulation of stained cells was observed, the area was divided into four squares and the number of stained cells in a square was multiplied to four. Moreover, all results were also expressed as mean ± SD and Analysis of Variance (ANOVA) test was used for differences between groups. Spearman's rank correlation coefficient [205] was computed to show the correspondence of costimulatory molecules expression (at the mRNA level) with T cells activation phenotype identified by IHC. To illustrate the composition of inflammatory infiltrates of the liver in examined biopsies, a value of relative frequency (RF) was defined for each cell phenotype in a group and was calculated as follows:

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

Value of $p \leq 0.05$ was regarded as statistically significant.

2.3. Molecular characterization and antigenic property of ALP from fertile and sterile hydatid cysts

Impact of the parasite viability on host immune reactions was examined by purification and characterization of ALP from fertile and sterile hydatid cysts and results were compared. The sampling parameters of this phase such as selection of patient groups and sampling strategies were independent of the first two phases of this study.

2.3.1. Patients and healthy donors

Hydatid patients (21 cases) participated to this study were randomly selected among individuals who had their infection confirmed by clinical examinations and ultrasonography. All selected patients were expected to undergo surgery at Cancer Institute (Tehran University of Medical Sciences, Tehran, Iran) in two months. This group included 9 males (mean age: 51 years, range: 34-65 years) and 12 non-pregnant, non-lactating females (mean age: 45 years, range: 26-69 years) and the natural history of each patient was obtained by using the clinical database cross-checked with laboratory records. All patients suffered from cysts in the liver and none had any other infectious diseases or was under anthelminthic medication at the time of sampling. After surgical treatment, FFPE liver biopsies from each patient containing total or subtotal cystectomy materials were prepared and were preserved for histopathological examinations. Moreover, 7 patients with other parasitic infections: 2 cases with fascioliasis, both males at the age of 23 and 31 years, and 5 cases with Taeniasis (*Taenia saginata*), 2 males at the age of 9 and 17 years old, 3 females at the age of 6, 10 and 14 years old were selected among those who referred the Department of parasitology, Pasteur Institute of Tehran (Tehran, Iran). Uninfected (control) samples were collected from 15 healthy volunteers. The study protocol was approved by the local

Ethical Committee of and the written informed consent for the sampling procedure was obtained from all adult individuals or from the parents of those who were under the legal age.

2.3.2. Human blood samples

Fresh blood specimens (10 ml from each individual) were obtained by venipuncture. Five ml of each specimen was removed, was heparinized at 20 U/ml for cultures and was used within 6 hours. The remainder was allowed to clot, thus the serum was collected, and aliquots were made and were stored at -20°C.

2.3.3. Purification of ALP from hydatid cyst fluid (HCF)

Hydatid cysts were obtained from infected livers of slaughtered sheep at a local abattoir (Sharyar, Iran). Livers were carried to the laboratory in ice packs and the content of each cyst was aspirated aseptically and was collected and labeled in Corning Centrifuge tubes (50 ml, Sigma-Aldrich). After centrifugation at 10,000 g for 10 minutes, the sediment was checked by a light microscope for presence of protoscolices [18]. As well, the membrane of each cyst was fixed in 10% neutral buffer formalin and was checked according to the presence of germinal layer. Absence of protoscolices or hooklets and impaired structure of germinal epithelium was considered as the cyst sterility. A total number of 30 fertile and 30 sterile cysts were collected and ALP concentration of their pure fluid (without hydatid sand or other parasitic particles) was measured by using Alkaline Phosphatase Detection kit, Fluorescence (Sigma- Aldrich) in a Technicon RA-1000 autoanalyzer (Bayer, Germany). These samples were separately aliquoted into clean vials (fluid of fertile and sterile cysts were marked separately) and were frozen at 20°C until used.

The purification of ALP from HCF was performed according to Lowton *et al*., 1994, with some modifications [137]. Briefly, ALP containing extracts from fertile and sterile HCF (2 ml, protein concentration: 10 mg/ml) was thawed and loaded on a 5 ml concanavaline A-sepharose column previously equilibrated in equilibration buffer (100 mM/l Tris/ HCl, pH 7.6, 100 mM/l NaCl, 1 mM/l $CaCl_2$ and 1 mM/l $MgCl_2$) at a flow rate of 1 ml/min at 4°C. After washing with at least six column volumes of the same buffer, the retained glycoproteins were eluted with five column volumes of 0.5 mol/l methylmannoside (Sigma- Aldrich) in equilibration buffer. The solid phase

was removed by washing with at least ten column volumes of the same buffer and ALP recovered by two batch-wise treatments at 37°C for 30 minutes with four column volumes of 0.1 mol/l methylmannoside in equilibration buffer containing 1% (w/v) Triton X-100 (Sigma- Aldrich). The eluted fraction was subsequently applied to a sephacryl S-300 (Sigma-Aldrich) column, equilibrated with buffer A (100 mM/l ammonium bicarbonate, pH 7.5 containing 20 mM/l NaCl, 0.5 mM/l $MgCl_2$) at a flow rate of 1 ml/min. The collected fractions were assayed for ALP activity by making serial dilutions (1:10, 1:100 and 1:1000) and mixing 20 µl of each dilute which was equilibrated at 37°C with the following reagents: 100 µl of activity buffer, 100 µl of p-nitrophenyl phosphate solution (one tablet in 1.4 ml distilled water, equilibrated at 37°C) and 2 ml of stop solution (all reagents used in this procedure were provided from Alkaline Phosphatase Activity Colorimetric Assay Kit, BioVision, USA). Absorption density was read at 405 nm and the most active fractions were concentrated by dialyzing against buffer B (100 mM/l Tris/HCl, pH 7.6 containing 100 mM/l NaCl, 1 mM/l $CaCl_2$, 1 mM/l $MgCl_2$, 2 mM/l ethyleneglycol 5%). Sephadex G-200 (Sigma- Aldrich) was added to increase the speed of the process [100].

2.3.4. Purification of sheep liver- derived ALP

Normal sheep liver was cut into small pieces, washed several times in cold saline to remove blood and stored at 20°C before use. This tissue 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) using an homogenizer (Kinematica Polytron PT10-35, Switzerland). While being homogenized, 5% (v/v) 2- mercaptoethanol was added to the suspension. The homogenized suspension was collected into Eppendorf tubes and placed into boiling water for 2 minutes to denature the proteins. After centrifugation at 10,000 g for 3 minutes, the supernatant was transferred to Eppendorf tubes and stored at 20°C. To concentrate the tissue extract, 56.8 g of ammonium sulfate was added slowly to 100 ml of extract while stirring at 4°C. The stirring was continued for 10-30 minutes to complete the incorporation of the ammonium sulfate into the solute. Samples were centrifuged at 3,000 g for 10 minutes and the sediment diluted in phosphate buffer, pH 7.2, 1:2 v/v, and retained. The sheep liver extract was loaded onto the concanavaline A-sepharose column as already described (see section 3.3.1 of Materials and Methods), except that the active fraction was eluted column-wise with 0.1 ml

methylmannoside in equilibration buffer. The most active fractions were pooled, concentrated and processed as above.

2.3.5. Kinetic study of ALP

Determination of the optimum reaction pH was performed using 100 mM/l sodium acetate buffer, pH 4.5-5.5, 100 mM/l Tris/HCl pH 6.8 and 1 mM/l diethanolamine pH 9. The kinetic parameters were determined according to the method of Sarciron *et al*., 1991 [228].

For inhibition experiments, the following inhibitors were employed: EDTA, levamisol and L-phenylalanine (Sigma- Aldrich). Each had been incubated for 10 minutes with the purified ALP in phosphate buffer before addition of the substrate [174].

To determine the isoelectric focusing of the purified ALP, samples at a concentration of 1.5 mg/ml protein were loaded on to a 2 mm-thick 5% polyacrylamide gel (2 ml) combined with 40% carrier ampholyte (0.6 ml), pH range 3.5-10, 50% glycerol (1.2 ml), 0.1% riboflavin 5'-monophosphate sodium salt hydrate (60 μl), 10% ammonium persulfate (18 μl), tetramethylethilenediamine (TEMED) (4 μl) and 8.2 ml of deionized water (all reagents from Sigma- Aldrich). Procedure was carried out on a horizontal gel casting apparatus (Multiphor II, GE Healthcare Life Sciences, USA). The gel was silver stained, and the isoelectric point of the protein was determined with markers (IEF Mix 3.6-9.3, Sigma- Aldrich) [7]. The pH of the gel strip was measured as it had been formerly described.

2.3.6. SDS-PAGE and immunoblot assay

To determine the molecular weight of ALP, the protein extracts were resolved by SDS- PAGE in 5% gradient gel and loaded after heating with 1:5 (v/v) sample buffer (0.6 ml Tris/HCl 1M, pH 6.8, 5 ml glycerol 50%, 2 ml SDS 10%, 0.5 ml 2- mercaptoethanol, 1% bromophenol blue 1 ml at 100°C for 210 minutes. Electrophoresis was carried out at 100-110 mA, 220 V, for 3040 minutes. The bands were stained by silver staining and the molecular weight was determined using Sigma-Aldrich standard marker [287].

Proteins were transferred on nitrocellulose membranes (Sigma- Aldrich). Transfer buffer (1.93 g Tris 15.6 mmol, 9 g glycine 120 mmol, 1 l distilled water, pH 8.1) was used and the system was

set on 100 V, 0.2 A. Sera from hydatid patients (n=21) as well as patients with fascioliasis (n=2), taeniasis (*Taenia saginata*, n=5) and uninfected controls (15 samples) were examined in this assay. Nitrocellulose strips were incubated overnight at 4°C in 1:64 dilution of each serum sample in Tris buffer, and immunoreactive bands were visualized after incubation in horseradish peroxidase (HRP) conjugated anti- human IgG (1:500 Sigma- Aldrich), diluted with Tris buffer containing 0.5% bovine serum albumin. Next, the strips were washed for 310 minutes with Tris buffer and then incubated with substrate solution (1ml chloronephtol dissolved in 30 mg/ml of methanol, 10 ml methanol, 30 μmol H2O2 30%, with the addition of Tris buffer to give a total volume of 50 ml at 37°C for 30 minutes. The reaction was stopped by rinsing three times in distilled water for 10 minutes [198].

2.3.7. Culture conditions and lymphocyte proliferation assay

Peripheral blood mononuclear cells (PBMCs) were isolated from blood by density centrifugation through Ficoll- Hypaque (GE Healthcare Life Sciences). A total of $2x10^5$ cells/well were cultivated in Roswell Park Memorial Institute (RPMI)- 1640 medium (Sigma-Aldrich) supplemented with 2mM L- glutamine (Sigma- Aldrich), 100 IU/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, 50 μM 2- mercaptoethanol, 23 mM HEPES (Sigma- Aldrich), 1 mM sodium pyrovate and 10% heat- inactivated fetal bovine serum (FBS) (Gibco, ThermoFisher Scientific) and 1X amino acid non-essential (ThermoFisher Scientific). Cells were plated in 96- well flat bottom TPP tissue culture plates (Sigma- Aldrich) and were incubated with medium alone, or stimulated with 5 μg/ml phytohemagglutinin (PHA) (Sigma-Aldrich), or with saline extracts of hydatid ALP (10 μg/ml), or with saline extracts of fertile HCF (10 μg/ml). All experiments were carried out in triplicate and plates were kept in a 5% CO_2 humidified atmosphere at 37°C. Supernatants were collected after 48 hours from mitogen-stimulated and after 120 hours from ALP/HCF- stimulated cultures and were stored at -80°C until use.

All wells were pulsed with 1 μCi [^{3}H]- thymidine (Sigma- Aldrich) for the final 18 hours of culture and the cells were then harvested onto glass fiber filters on a cell harvester (INOTECH, USA) and were allowed to dry overnight. Two ml of scintillation fluid (0.05mg/ml POPOP [1,4-bis-5-phenyloxazole-2-yl-benzene], and 4 mg/ml PPO [2,5-diphenyloxazole] in 1 liter of toluene)

was added to each tube containing the dried filter discs and the incorporated radioactivity in the cells was evaluated by using a liquid scintillation beta counter (Beckman, USA). The [^{3}H] - thymidine incorporation of stimulated cells was described as mean cell per minute (cpm) above the background for triplicate cultures which was calculated as below:

$$\text{Mean } \Delta\text{cpm} = \frac{\sum [\text{cpm of a culture (stimulated)} - \text{cpm of the same culture (unstimulated)}]}{3}$$

2.3.8. Cytokine assays

Concentrations of IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-10 and TNF-α in supernatants were measured by double sandwich ELISA. The assays were carried out using commercially available ELISA kits for quantification of human cytokines (ThermoFisher Scientific) upon the manufacturer's instructions. Quantification of cytokines was determined by reference to recombinant human standards provided in each kit and the assays limit of detection for IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-10 and TNF-α ranged as 15.6-1000 pg/ml (sensitivity: 4 pg/ml), 20-700 pg/ml (sensitivity: 6 pg/ml), 7.8-300 pg/ml (sensitivity: 2 pg/ml), 11.7-750 pg/ml (sensitivity: 4 pg/ml), 10.2-400 pg/ml (sensitivity: 1 pg/ml), 7.8-500 pg/ml (sensitivity: 1 pg/ml) and 15.6-700 pg/ml (sensitivity: 1,7 pg/ml), respectively. All tests were performed in duplicates and the mean concentration of the test samples were adjusted for background and spontaneous cytokine production by subtracting the mean levels of cytokines recorded in the unstimulated cultures.

2.3.9. Humoral response to hydatid ALP

Antibody response to hydatid ALP was detected by enzyme- linked immunosorbat assays (ELISA). All tests were optimized by checkboard titration using 1:100 diluted pools of sera from hydatid patients with positive immunoblot reactions and from negative healthy donors. Microtiter 96-well plates (Nunc- Immuno MicroWell, Sigma- Aldrich) were sensitized by placing 50 µl of 10 µl/ml ALP or 5 µl/ml HCF diluted in 0.05 M carbonate/bicarbonate buffer, pH 9.6, and were incubated for 2 hours at 37°C then overnight at 4°C. Plates were washed trice with PBS containing 0.05% Tween 20 and uncoated sites were blocked with 200 µl of PBS/Tween 20

containing 5% bovine serum albumin (BSA) for 2 hours at room temperature. To detect antigen-specific total IgG and IgG subclasses, sera were diluted in PBS/Tween 20 (1:500 for total IgG, 1:200 for IgG1, 1:100 for IgG2 and 1:50 for IgG3/4), then 50 µl per well of each sample was added in duplicate and the plates were incubated for 2 hours at room temperature. The plates were washed three times with PBS/Tween 20 and 50 µl/well of murine anti- human IgG, IgG1, IgG2, IgG3 and IgG4 secondary antibodies peroxidase conjugated (ThermoFisher Scientific), were added in optimal dilutions in PBS. After incubation for 1 hour at 37°C, the plates were washed with PBS/Tween 20 and 50 µl of the substrate solution containing 1 mg/ml o-phenylenediamine dihydrochloride and 15 µl of 30% H_2O_2 in citrate- phosphate buffer, pH 5.0, was added. The procedure continued by incubation for 10- 15 minutes at room temperature in the dark, then the reaction was stopped with 50 µl/well of H_2SO_4 and the plates were read at 450 nm. Results were expressed as the mean optical density (OD) for duplicate test samples. The cut-off values were calculated as mean OD of negative controls + 3SD. Individuals with OD values > cut-off were considered as positive responders. To assess the reproducibility of the ELISA results, coefficients of variance were calculated for reference hydatid and normal control sera. Intra- and inter-assay variability for each experiment was 10% or lower.

2.3.10. Statistical analysis

Due to the low number of individuals in examined groups and to the heterogeneity of the SD between groups, one-way ANOVA with post-hoc Tukey test for nonparametric data were employed to determine differences in measured immune parameters between different stimuli. 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 (J= max {sensitivity+specificity-1}) was used to select the best cut-off values for the ELISA. Diagnostic values for serological tests were calculated as follow:

$$\text{Sensitivity} = \frac{TP}{TP+FN} \times 100$$

$$\text{Specificity} = \frac{TN}{TN+FP} \times 100$$

$$\text{Positive predictive value} = \frac{TP}{TP+FP} \times 100$$

$$\text{Negative predictive value} = \frac{TN}{TN+FN} \times 100$$

These parameters were defined as: true positive (TP), true negative (TN), false positive (FP) and false negative (FN). Spearman's correlation rank was used to evaluate all the correlations. Differences with $p \leq 0.05$ were considered statistically significant.

3. Results

To assess the objectives of the present study, cellular arrangements of the inflammatory infiltrates in the vicinity of chronic hydatid lesion of human liver were characterized by immunohistochemistry. Moreover, quantitative analysis of costimulatory mRNAs demonstrated the in situ expression level of these molecules in response to chronic hydatid infection of the liver. To determine the impact of the cyst viability on immunopathology of the infection, ALP from hydatid cyst was purified and its immunochemical properties were compared between fertile and sterile cysts. Humoral and cellular responses to hydatid ALP were also examined in human peripheral blood.

3.1. Characterization of cellular infiltrates in the inflammatory milieu of the liver

3.1.1. Histopathological observation

Tissue damage and fibrosis were observed in all samples obtained from CE patients. Inflammatory cell infiltrates were detected in all CE samples (100%) around the fibrous capsule (Figure 2) mainly consisting of fibroblast-like cells along with infiltrating lymphocytes, occasional polymorphonuclear cells and monocytes. Hydatid cysts with total or partial calcification were observed in 23.8% (5 of 21) patients (Figure 3 and Figure 4). Due to the presence of protoscolices or hooklets in histology sections, cysts were identified as fertile in all patients (Figure 4 and Figure 5). Ductular proliferation and hydatid cyst rupture into biliary tracts was observed in 4 patients (19%). Hepatocytes with large lipid droplets in their cytoplasm were detected in all SH samples. Ductular reaction (n=8, 73%) and slight tissue remodeling with mild accumulation of connective tissue fibers (n=5, 45%) were also observed in some SH biopsies (Table 3). Pericellular scar and septal fibrosis along with macrovesicular steatosis and inflammatory nodules were detected in 2 (18%) SH patients. Iron staining showed the present of hemosiderin in ~30% of Kupffer cells in these biopsies. Besides, apoptotic cells were observed among normal cells in the parenchyma. Histopathological examination of CH biopsies showed a range of different alterations in the liver from mild infiltration of inflammatory cells and slight remodeling in the lobular structures of portal areas to septal fibrosis. Portal fibrosis was observed in 64% (n=7) of these CH biopsies (Table 3).

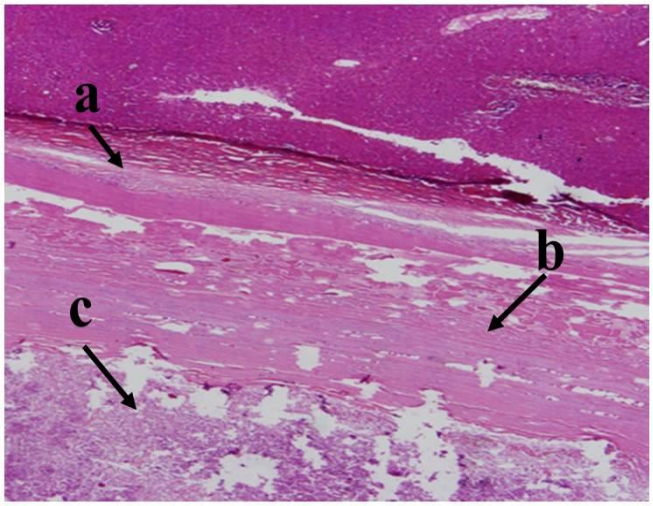

Figure 2- Histological image of chronic hydatid cyst infection in human liver typically represents: a) derangement of hepatocytes, accumulation of fibroblasts and subsequent fibrosis (pericyst) which separates the normal parenchyma from the cyst wall, b) the acellular laminated membrane (ectocyst) which is the outermost layer of the cyst wall, and c) the cyst lumen that indicates progressive internal calcification. (Hematoxylin-eosin, 40x)

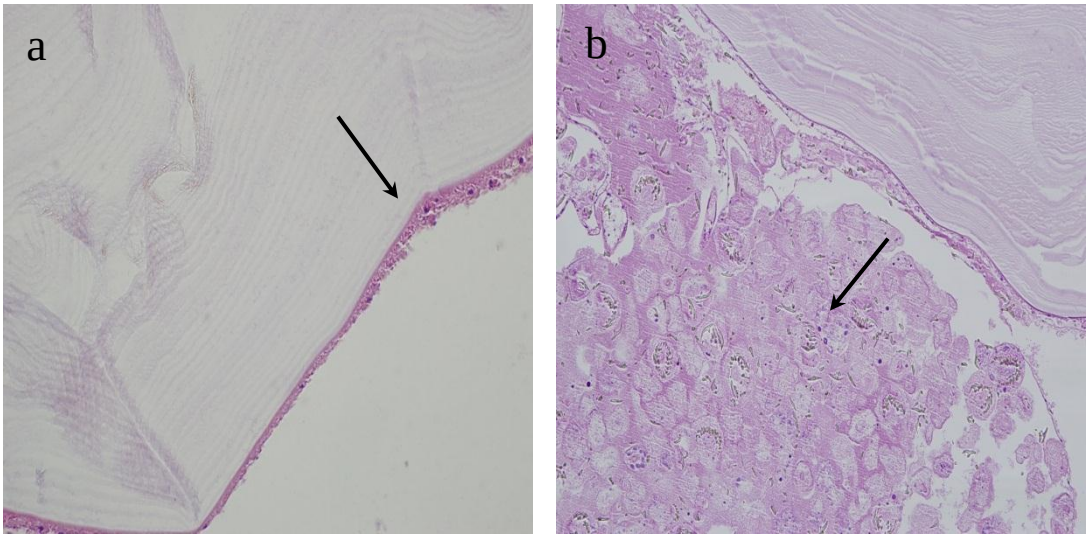

Figure 3- These slides show the primary stages of aging in human hydatid cyst of the liver: a) a young cyst with folded laminated layer and cellular nucleated germinal epithelium (arrow), and b) pre-calcification stage which is featured by deformation of protoscoleces (arrow) in daughter cysts caused by failure in membranous transportation of molecules. (Hematoxilin-eosin, a 400x and b 200x)

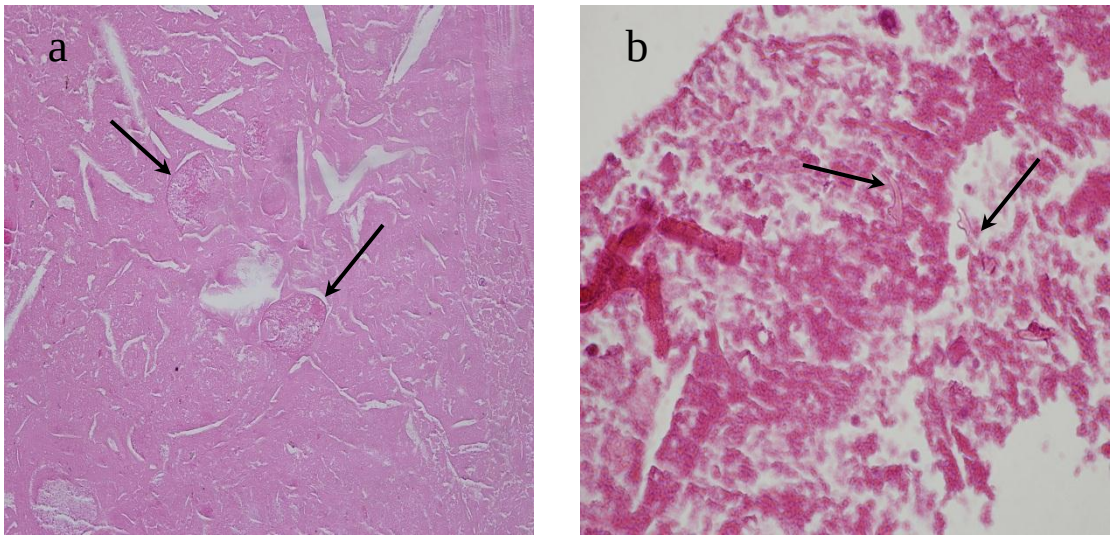

Figure 4- These slides show final stages of aging in established hydatid cysts of human liver: picture (a) shows deformed protoscoleces (arrows) in a calcified cyst lumen, and picture (b) indicates a completely calcified cyst. Hooklets (arrows) may remain visible after cyst death and should be considered as markers for post-calcification diagnosis. (Hematoxilin-eosin, a 200x and b 600x).

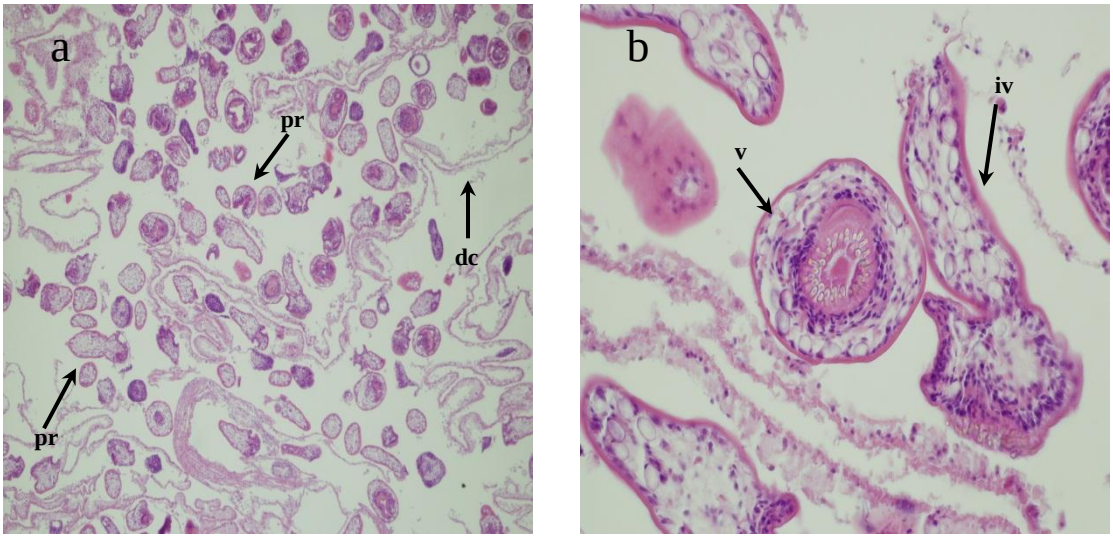

Figure 5- Content of a fertile hydatid cyst in human liver has been showed in these slides: a) hydatid sand including protoscolices (pr) and daughter cysts (dc), b) image of vaginated (v) and invaginated (iv) protoscolices with larger magnification that shows hooklets and calcareus corpuscles. (Hematoxilin-eosin, a 200x and b 600x)

Table 3- Demographic data and histopathology of patients with cystic echinococcosis (CE, n=21), steatosis (SH, n=11) and chronic hepatitis (CH, n=11).

Sample Groups	Range of Age (Year)	Sex		Pathology				
		Male (n)	Female (n)	Intrabiliary Rupture (n)	Portal Fibrosis (n)	Periportal Fibrosis (n)	Septal Fibrosis (n)	Ductular Reaction (n)
CE	41±19.31	7	14	4	10	2	1	-
SH	26±22.31	7	4	-	5	-	2	8
CH	26±19.31	7	4	-	7	-	1	7

3.1.2. In situ identification of cell phenotypes in the inflammatory infiltrates of the liver

In both SH and CH, neutrophils detected by expression of MPO had low to moderate aggregation mostly in sinusoidal spaces of the liver. In CE liver, these cells were absent or had very low to low accumulation and were mostly localized at the periphery of hydatid lesion or had scant number within the sinusoidal spaces. The density of MPO^+ cells scattered within the inflammatory areas of the parenchyma in SH and CH was higher than that in CE, although the statistical analysis only confirmed the significant difference between CE and CH in this regard, p=0.01, (Table 4 and Figure 6).

T lymphocytes predominantly consisted of $CD3^+$ cells in all liver biopsies. In CE samples, these phenotypes were majorly observed as compact clusters of accumulated cells around the pericyst with an averagely moderate quantity (265±138.66 cells/field). Immunostained $CD3^+$ cells showed almost the same aggregation pattern in SH and CH biopsies with moderate quantities in peri-fibrotic areas of the liver (232±198.11 cells/field and 241±143.89 cells/field in SH and CH, respectively). There was no significant difference between the patient groups according to the

number of labeled $CD3^+$ T lymphocytes. In CE livers, $CD8^+$ cells were absent or had very low to low counts at the site of injury. Aggregation of this subtype in CE- induced inflammatory infiltrates was characterized by small clusters of adhered cells to the pericystic adventitia as well as sparsely scattered cells throughout the parenchyma. CE liver biopsies from 6 patients (29%) lacked $CD8^+$ cells and the amount of these cells was relatively moderate at the periphery of hydatid lesion in two patients (9.5%). There was no significant difference according to the density $CD8^+$ cells between CE and SH (quantified as 20±50.61 cells/field and 17±26.62 cells/field, respectively), whereas such a value was found significantly higher in CH biopsies (36±23.27), p=0.02, (Table 4 and Figure 7). Aggregation of $CD4^+$ T cells around the pericyst was scored as very low to low (from 0 to max 67 cells/filed, mean: 29±18.94 cells/field) with less disparity regarding the number of infiltrated cells between individual samples and was not significantly different than that in SH (averagely 24±22.86 cells/field) and in CH (57±46.16 cells/field). Cells with $FOXP3^+$ phenotypes were absent or had very low scores in CE (4±4.53 cells/field) and in SH (10±8.64 cells/field) but were observed in slightly higher numbers (20±29.87 cells/field) in CH biopsies, although the differences between all sample groups were insignificant (Table 4).

B lymphocytes comprised the second largest cellular population and were identified by expression of CD20 surrounding the hydatid lesion. The amount of $CD20^+$ cells quantified in CE livers (230±174.86 cells/field) was higher than in SH (50±53.87 cells/field) and in CH (140±128.50 cells/field), however this difference was not statistically significant. In all samples, these cells formed focally aggregated clusters adjacent to the scar tissue (Table 4).

The number of $CD68^+$ cells was found highly variable in CE livers, ranged from 0 (in one sample) to ≥500 (in two samples) and it was averagely scored as moderate in this group (101±147.13 cells/field). The density of $CD68^+$ macrophages in the inflammatory infiltrate of CH (258±107.82 cells/field) livers was significantly higher than that in CE and SH samples, p<0.001, (Table 4). In all biopsies, $CD68^+$ cells were observed diffusely scattered between hepatocytes particularly around the scar tissue. In CE livers, these cells had more condensed aggregation at the periphery of the pericyst mostly in areas with tissue derangement and accumulation of fibroblasts and also were observed in adjacent to the cyst wall (Figure 8).

$CD1a^+$ cells were observed scantly scattered throughout the parenchyma SH (13±11.85 cells/field) and CH (8±9.63 cells/field) livers, whereas were completely absent in CE biopsies. No difference was found between SH and CH regarding the number of $CD1a^+$ cells in the inflammatory milieu of the liver (Table 4 and Figure 9).

Among the perihydatid cells, α-SMA^+ myofibroblasts were the most frequent cell type constituted the non-parenchymal cell population in all biopsies. Relative frequency of these cells was higher in CE (RF=32%) and SH (RF=36%) than that in CH (RF=23%), $p \leq 0.05$. There was no significant difference between CE (RF=6%) and SH (RF=10%) groups according to the relative frequency of $CD68^+$ cells in Inflammatory infiltrates of the liver, but this value was significantly higher in CH samples (RF=16%), $p<0.05$. On the contrary, $CD20^+$ cells showed the highest frequency in CE (RF=13%) compared to SH (RF=4%) and CH (RF=9%), $p<0.05$. Altogether, $CD3^+$ cells were the most frequent T lymphocyte subtypes and showed relatively the same pattern in all biopsies. Other activation markers of T cells were detected on much smaller proportion of infiltrated cells within inflammatory regions of the liver in all groups (Figure 10).

Table 4- Density of inflammatory cells of the liver in cystic echinococcosis (CE), steatosis (SH) and chronic hepatitis (CH) biopsy samples.

	CE	SH	CH	p Value		
Marker	Cells/field (mean±SD)	Cells/field (mean±SD)	Cells/field (mean±SD)	CE vs. SH	CE vs. CH	SH vs. CH
CD1a	**0±1.1**	**13±11.85**	**8±9.63**	**0.00001**	**0.006**	**0.5**
CD3	265±138.66	232±198.11	241±143.89	0.9	0.9	0.9
CD4	29±18.94	24±22.86	57±46.16	0.1	0.1	0.1
CD8	**20±50.61**	**17±26.62**	**36±23.27**	**1**	**0.02**	**0.1**
FOXP3	4±4.53	10±8.64	20±29.87	0.1	0.1	0.1
CD20	230±174.86	50±53.78	140±128.50	0.06	0.06	0.06
CD68	**101±147.13**	**108±77.47**	**258±107.82**	**1**	**0.0006**	**0.05**
MPO	**35±30.89**	**65±28.82**	**133±118.14**	**0.06**	**0.01**	**1**
α-SMA	**537±75.63**	**394±104.19**	**364±130.97**	**0.001**	**0.001**	**1**

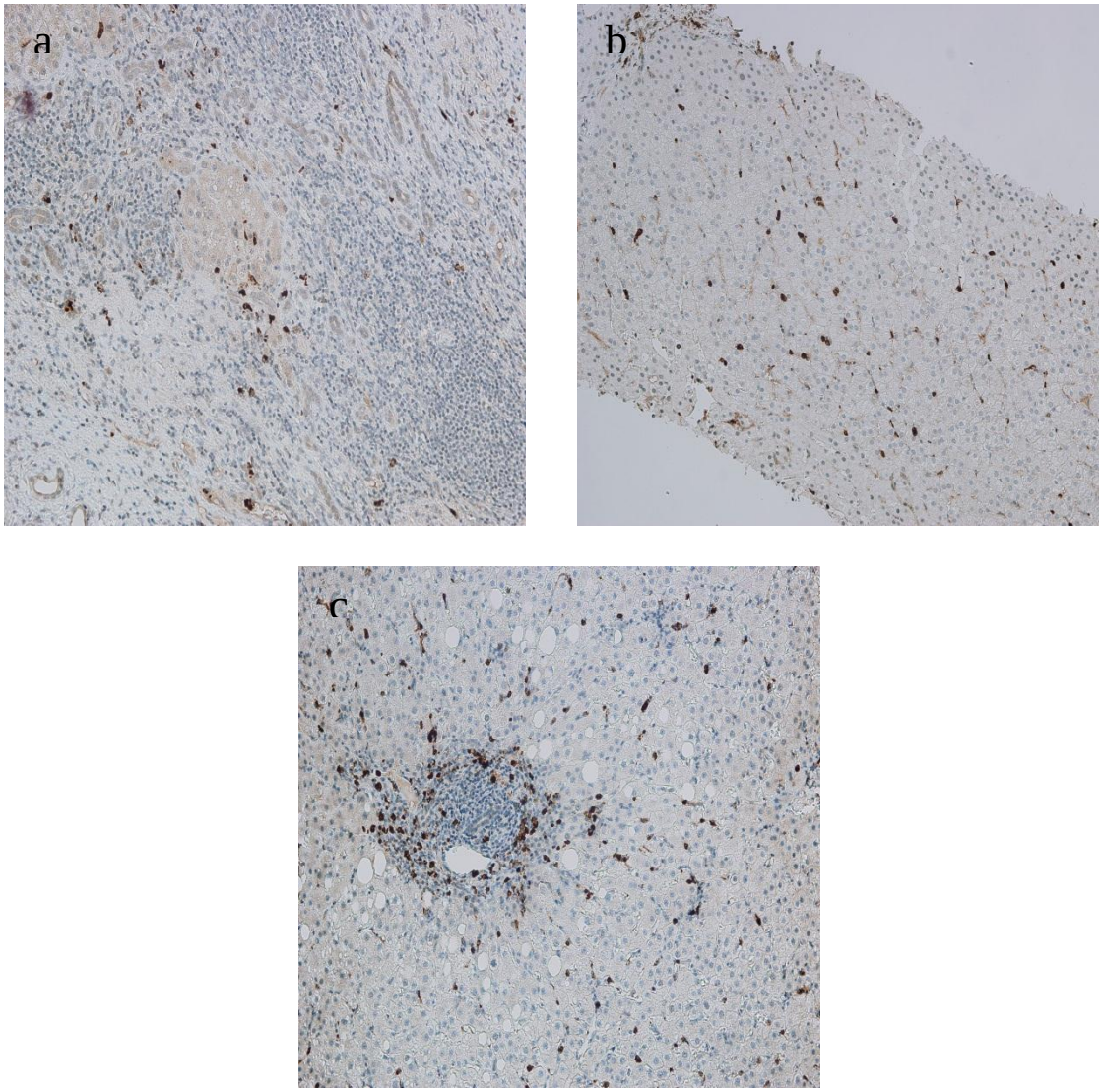

Figure 6- Distribution of MPO^+ cells within the inflammatory infiltrates of the liver in cystic echinococcosis (a), in steatosis (b) and in chronic hepatitis (c). (200x)

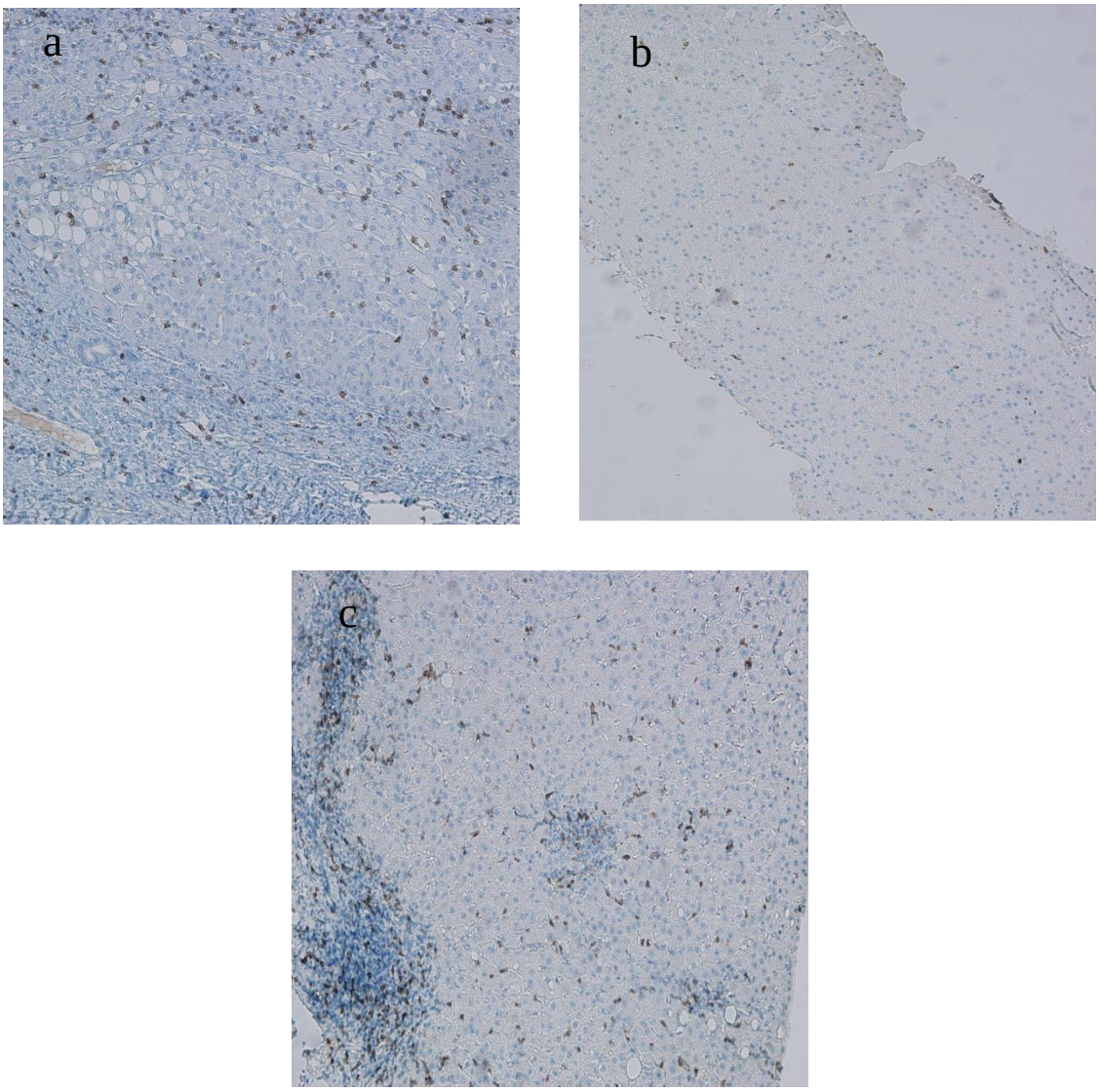

Figure 7- Distribution of $CD8^+$ cells within the inflammatory infiltrates of the liver in cystic echinococcosis (a), in steatosis (b) and in chronic hepatitis (c). (200x)

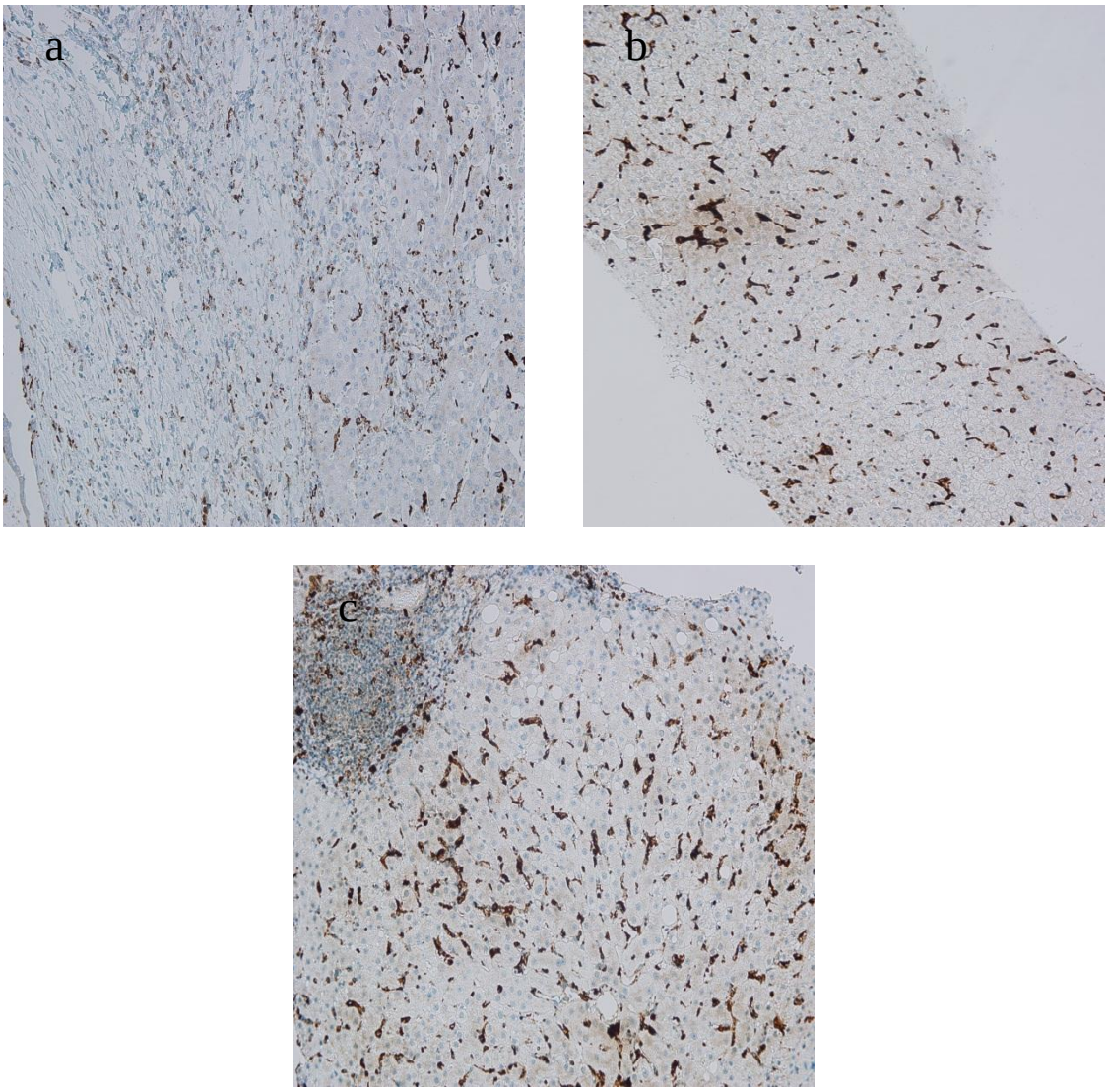

Figure 8- Distribution of $CD68^{+}$ cells within the inflammatory infiltrates of the liver in cystic echinococcosis (a), in steatosis (b) and in chronic hepatitis (c). (200x)

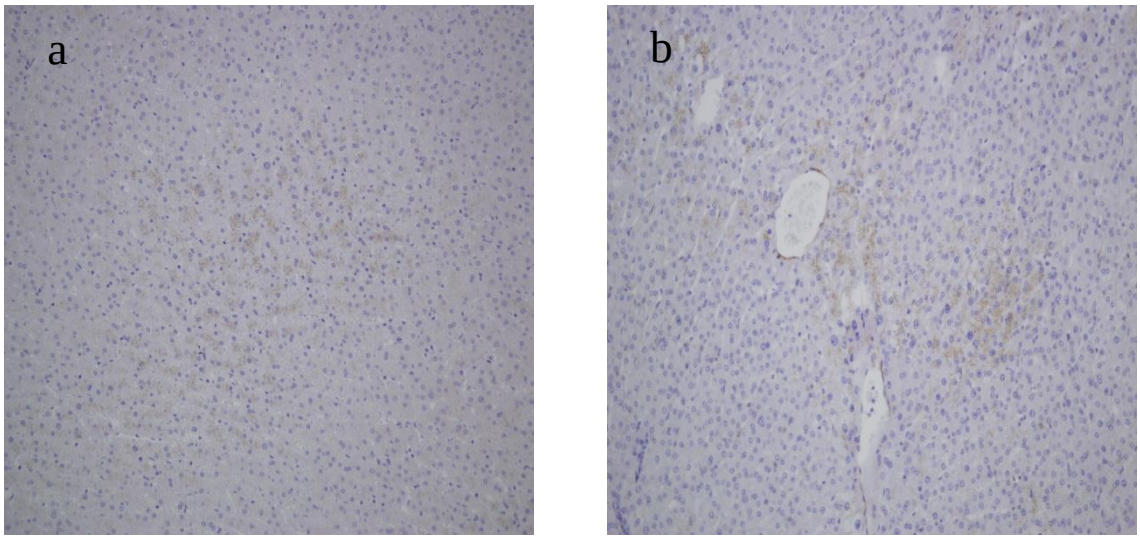

Figure 9- Distribution of CD1a^{+} cells within the inflammatory infiltrates of the liver in steatosis (a) and in chronic hepatitis (b). These cells were not observed in cystic echinococcosis (CE) samples. (200x)

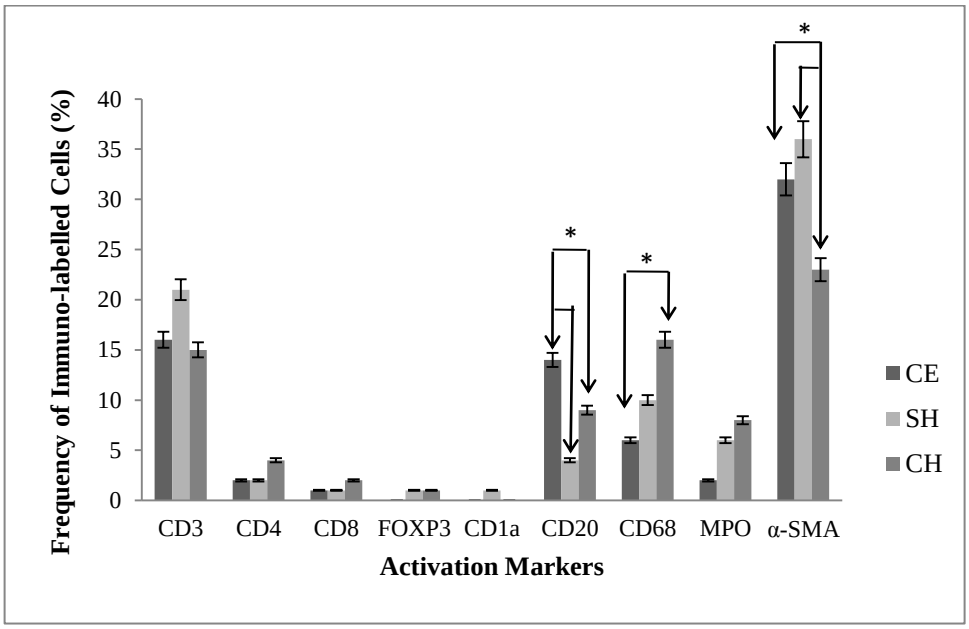

Figure 10- Relative frequency (RF) of identified cellular populations in the inflammatory milieu of cystic echinococcosis (CE), steatosis (SH), and chronic hepatitis (CH) livers. Bars show the standard error of each value. Significantly different values ($p \leq 0.05$) are pointed by the arrows.

3.2. Expression of costimulatory molecules at the mRNA level

The qPCR results were represented as arbitrary ratios of costimulatory mRNAs to the expression level of β-actin gene for each individual specimen. Intra- and inter-group variations between relatively quantified mRNAs (median) have been demonstrated in Figure 3.10. Despite comparatively constant levels of CD80 and CD86 mRNAs in all livers (Figure 13), expression of T cell- derived costimulatory molecules was found highly deviant between individuals in each group (Figure 12).

Antigen presenting- associated costimulatory CD80 and CD86 had high expression levels in all biopsies. Both CD80 and CD86 were highly expressed at the mRNA levels in CH biopsies which were numerically greater than other two groups but differences were not significant (Table 5 and Figures 11 and 13). The expression level of CTLA4 mRNA in CH livers showed 435-fold and 73-fold increase compared to CE and SH, respectively. Expression of CTLA4 in SH was also higher (6-fold) than CE, however the average CTLA4 expression level was not significantly different between all groups more likely due to the wide range of inter-sample variations (Table 5 and Figures 11 and 12). Expression of CD28 had a remarkable decrease in CE livers compared to other sample groups and such a difference was statistically significant (SH/CE=2171 fold, $p \leq 0.00001$), (CH/CE=6191 fold, $p \leq 0.00001$) (Table 5 and Figures 11 and 12).

The ratio of CD28/CTLA4 in CE samples was 0.3 showing a slightly higher prevalence of CTLA4 expression around the hydatid lesion. Such a ratio in CH samples was found to be 4, and was the highest (CD28/CTLA4=93) in SH biopsies. On the other hand, the quantified level of CD80 mRNA was higher than CD86 at CD80/CD86 ratios of 1 and 2 in SH and CE, respectively, but such a ratio was reversed in CH biopsies (CD80/CD86=0.7).

Expression of CTLA4 and CD28 had no significant correlation with the density of various T cell phenotypes ($CD3^+$, $CD4^+$, $CD8^+$, and $FOXP3^+$) identified by immunohistochemistry in CE and SH samples. In CH livers (Figure 14), expression of CTLA4 was positively correlated with the quantity of $CD4^+$ cells in each individuals, Rho=0.664, p=0.03.

Expression levels of CD80 and CD86 measured in CE and CH livers were significantly correlated (Rho=0.525, *p*=0.01 and Rho=0.755, *p*=0.007, respectively), but this correlation was not significant in SH (Figure 15).

Table 5- Expression of costimulatory molecules at the mRNA level in inflammatory infiltrates of cystic echinococcosis (CE), steatosis (SH) and chronic hepatitis (CH) livers.

	CE	**SH**	**CH**	**P Value**		
Costimulatory Molecules	Mean±SD	Mean±SD	Mean±SD	CE vs SH	CE vs CH	SH vs CH
CTLA4	0.073±0.099	0.182±0.233	5.293±16.949	0.1	0.07	0.1
CD28	**0.087±0.018**	**24.361±22.860**	**71.128±60.365**	**≤0.0001**	**≤0.0001**	**0.1**
CD80	50.779±67.682	54.731±74.778	67.712±98.575	0.5	0.1	0.5
CD86	61.528±117.973	27.526±33.573	62.451±89.896	0.2	0.5	0.5

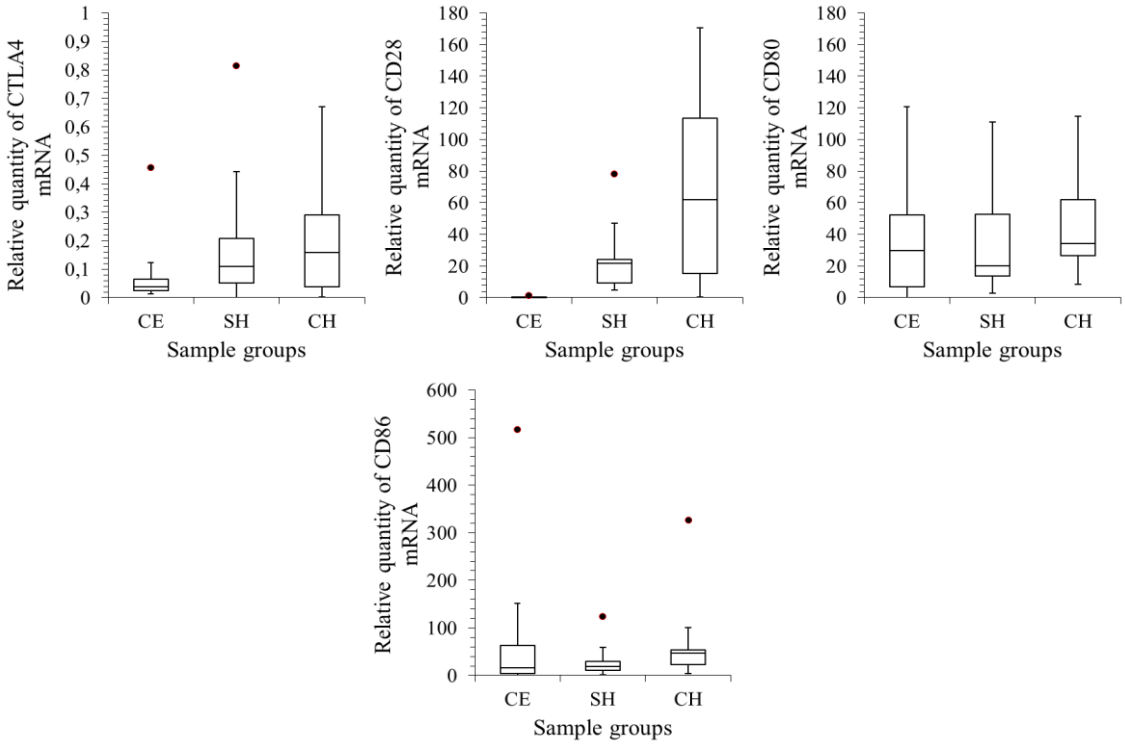

Figure 11- Box- plot graphs represent the variations of molecular measurement datasets (medians) in cystic echinococcosis (CE), steatosis (SH) and chronic hepatitis (CH) livers. The boxes show interquartile ranges (differences between the first and third quartiles) of values. Bars within each box and whiskers show the median and min/max points of data, respectively.

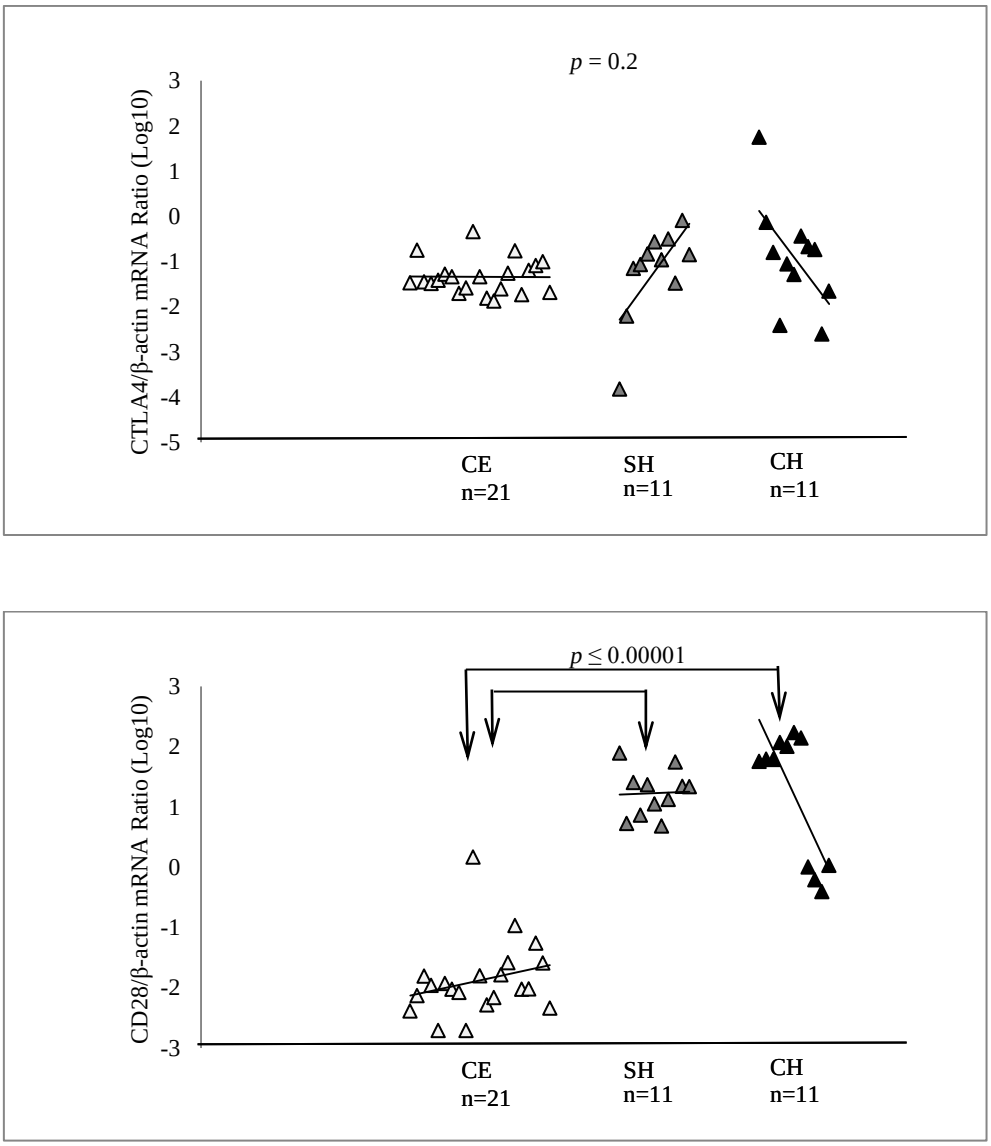

Figure 12- Expression levels of T cell-associated costimulatory molecules in inflammatory infiltrates of cystic echinococcosis (CE), steatosis (SH) and chronic hepatitis (CH) livers have been demonstrated in this figure. The number of individuals in each group (n) and the value of significance (*p*) are noted on graphs. Trend lines show the linear deviation of values from the standard ratio in each group.

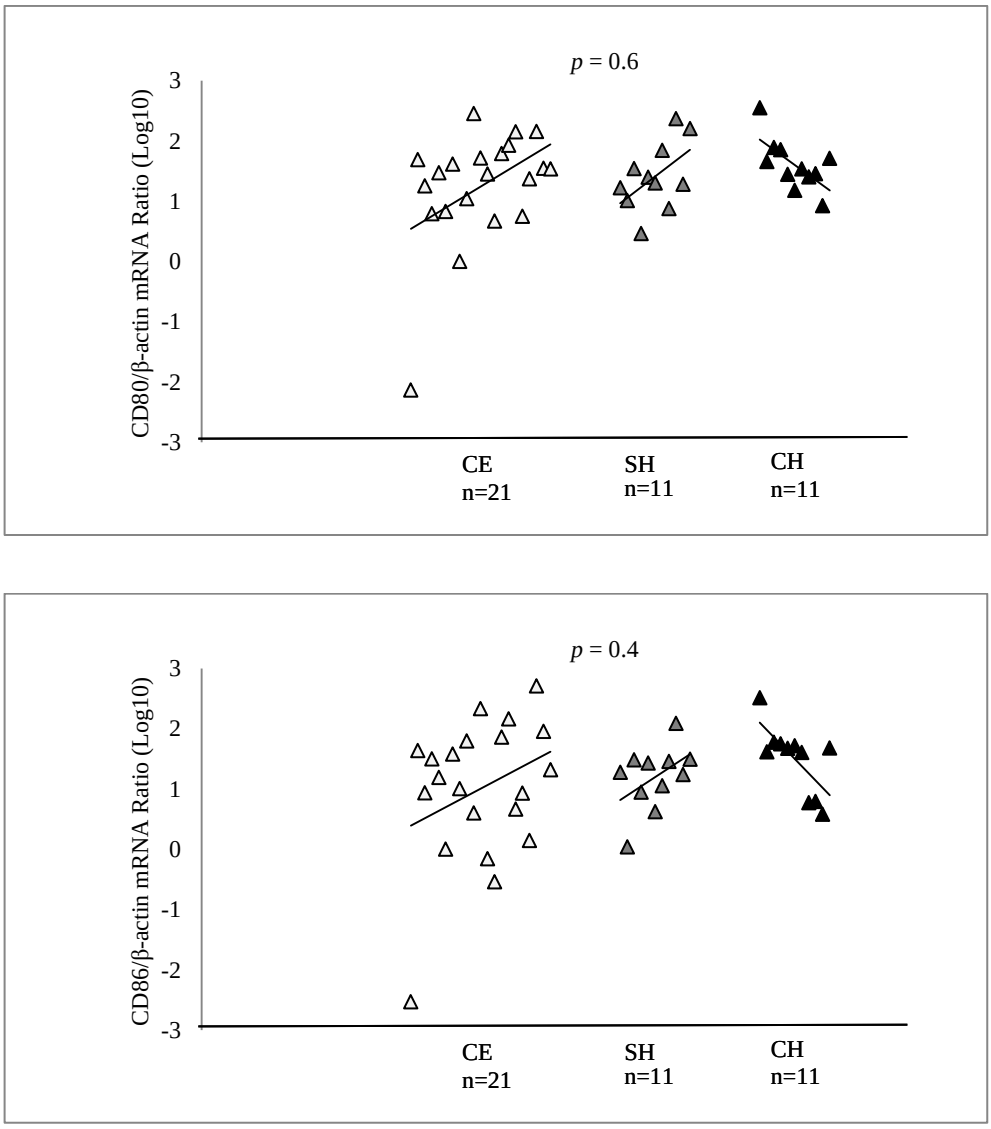

Figure 13- Expression levels of antigen presenting-associated costimulatory molecules in inflammatory infiltrates of cystic echinococcosis (CE), steatosis (SH) and chronic hepatitis (CH) livers have been demonstrated in this figure. The number of individuals in each group (n) and the value of significance (*p*) are noted on graphs. Trend lines show the linear deviation of values from the standard ratio in each group.

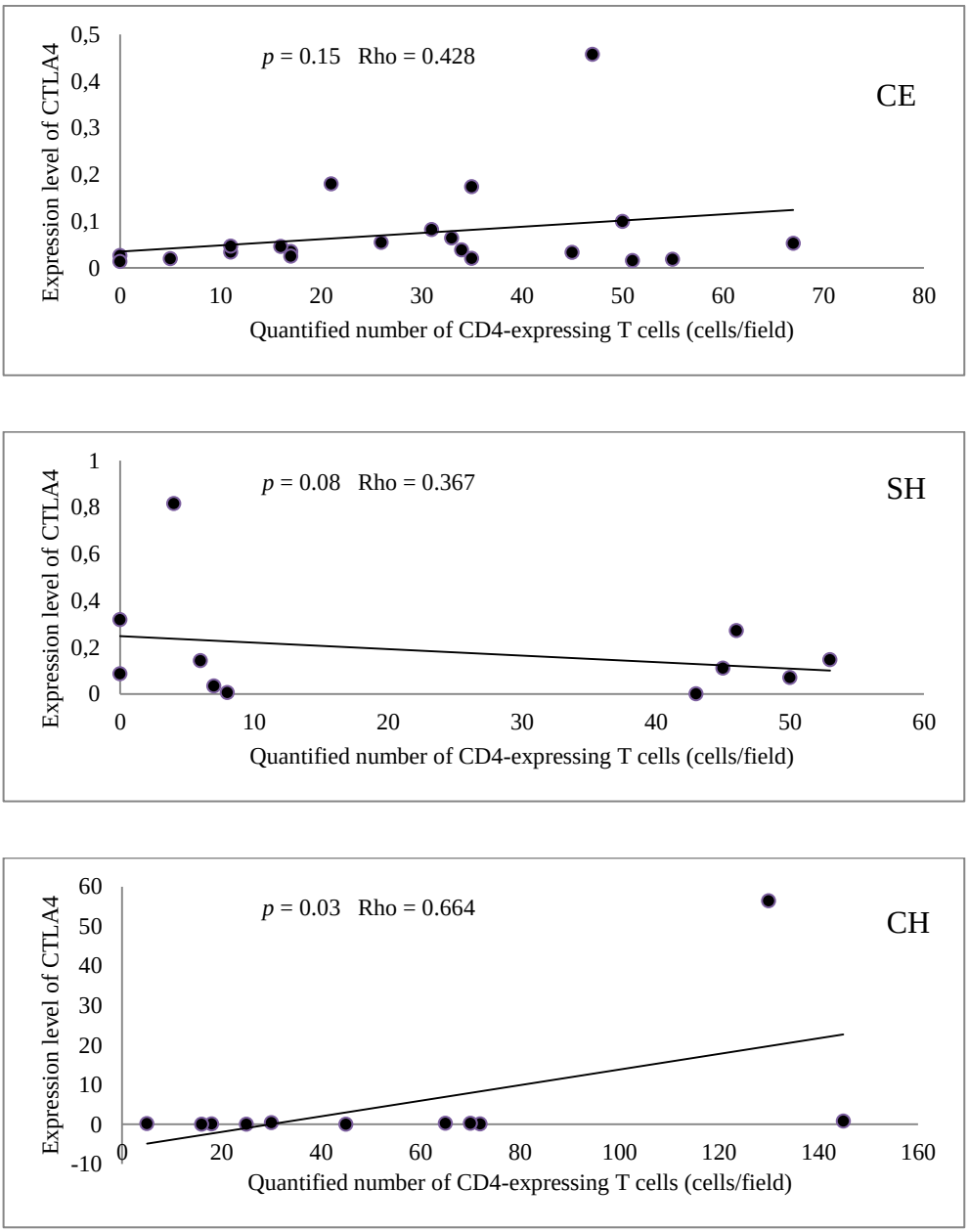

Figure 14- Linear correlation between the expression level of CTLA4 and activation of $CD4^+$ T cells in liver inflammatory infiltrates was assessed by the Spearman's correlation coefficient and was found significantly positive in chronic hepatitis ©, but was insignificant in cystic echinococcosis (a) and steatosis (b). The corresponding values of *p* and Rho are shown on each graph.

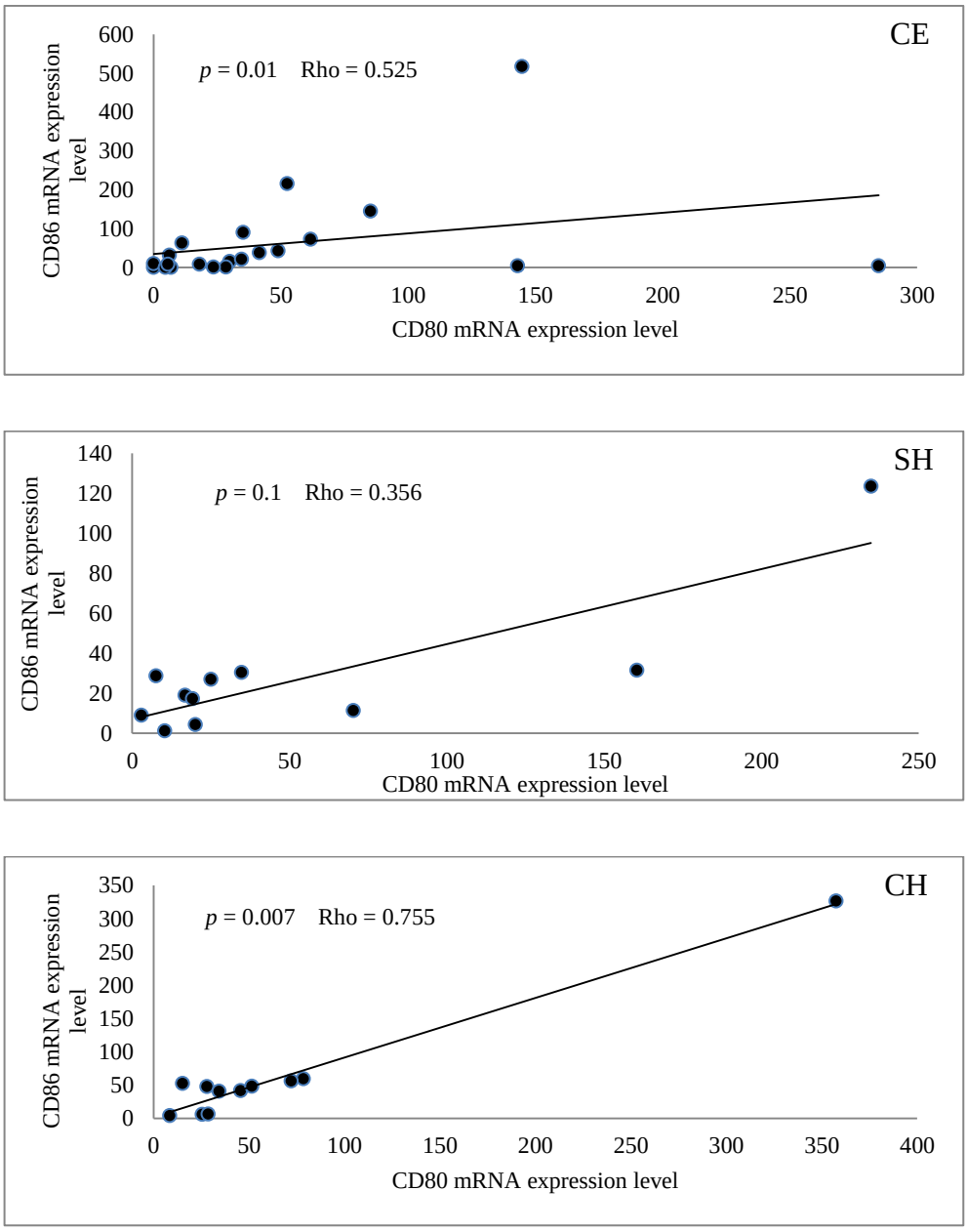

Figure 15- Linear correlation between the expression levels of antigen presenting- associated costimulatory molecules (CD80/CD86) in inflammatory miliue of the liver was computed by the Spearman's correlation coeficinet and was found significantly positive in cystic echinococcosis (CE) and in chronic hepatitis (CH), but was insignificant in steatosis (SH) samples. The corresponding values of *p* and Rho are shown on each graph.

3.3. Biochemical characterization of ALP from fertile and sterile hydatid cysts and assessment of its immunogenicity in human peripheral blood

3.3.1. Kinetic parameters and molecular properties of ALP from different extracts

Biochemical examinations revealed significant differences between fertile and sterile cysts due to the enzymatic activity and molecular traits of ALP. Moreover, the present study indicated the immunogenic properties of ALP purified from fertile hydatid cysts, although the enzyme from sterile cysts seemed not to be capable of inducing immune reactions in the host.

The results of enzymatic activity measurements were expressed as mean values (U/ml±SD) in 30 aliquots of each ALP extract obtained from hydatid cyst (fertile and sterile cysts) or from sheep liver. The assay showed higher activity of ALP in fertile hydatid cysts (10.75 U/ml, SD=3.78) when compared to that from sterile cysts (6.25 U/ml, SD=2.43) and sheep liver (7.03 U/ml, SD=1.83), $p \leq 0.01$. No significant difference was found between sterile cyst extracts and those from sheep liver concerning the activity of ALP.

The pH optima of maximum ALP activity to catalyze p-nitrophenyl phosphate were measured as about 9.8, 11.2 and 11.8 for fertile cysts, sterile cysts and sheep liver- derived fractions, respectively (Figure 16).

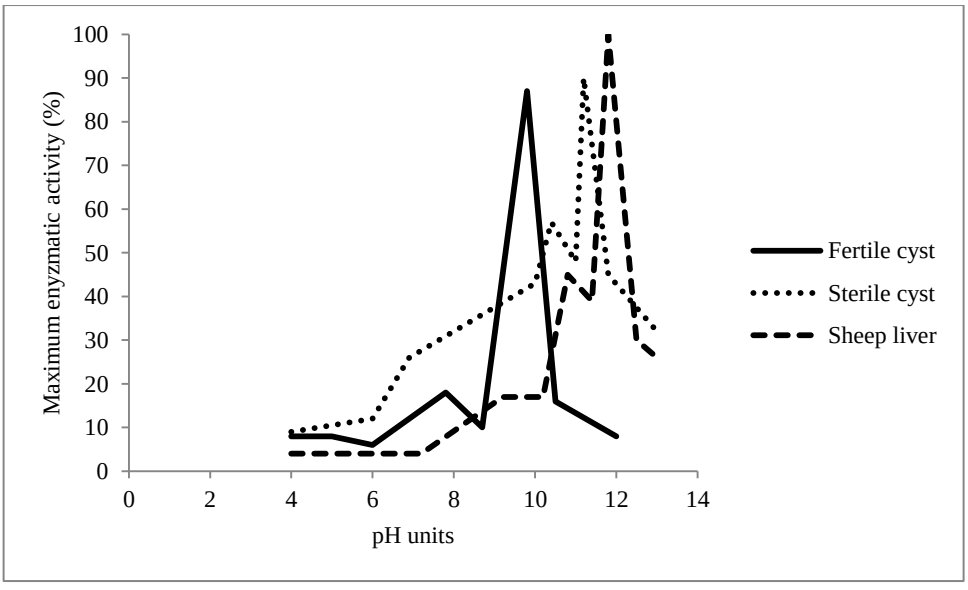

Figure 16- Enzymatic activity of ALP from different extracts according to changes in pH.

Michaelis-Menten kinetics also showed comparative variations in ALP activity between different enzyme resources. Both V_{max} and K_m values showed lower saturation rate of the enzyme from fertile hydatid cysts compared to that from sterile cysts and sheep liver. Isoelectric point of fertile cysts- derived ALP was slightly higher than the enzyme from the other resources, however the difference was not significant (Table 6). Enzyme inhibition assays using three different inhibitors also indicated that relatively lower concentration of levamizole or EDTA can yield 50% inhibition in the activity of ALP purified from sterile cysts or sheep liver compared to that from fertile cysts. L- phenylalanine had rather no inhibitory effect on ALP activity from fertile hydatid cysts (Table 7). In spite of significant differences due to the kinetics of ALP, SDS-PAGE examination showed almost the same molecular weight of the purified enzyme in either reduced or non-reduced conditions in all extracts. The non-reduced fraction of ALP formed electrophoretic band at the range of 180-190 kilo Dalton (kDa) while the molecular weight of reduced enzyme was measured as 50-60 kDa on SDS according to the standard (Figure 17).

Table 6- Biochemical parameters of ALP derived from hydatid cysts and sheep liver extracts.

	V_{max} (nmol/min/mg)	**K_m (µmol/l)**	**Thermal stability (C°)**	**Isoelectric point (C° in 30 min)**
Fertile cysts	137±17	0.27±0.02	37-66	5.3
Sterile cysts	708±85	0.65±0.2	37-57	3.9
Sheep liver	723±110	0.68±0.17	37-60	4.2

Table 7- Concentration yielding 50% inhibition [IC_{50} (mM)] of different enzyme inhibitors examined through ALP inhibition assays.

Extract	Fertile cysts	Sterile cysts	Sheep liver
Inhibitor			
L-phenylalanine	Not inhibitory	47	21
Levamizole	0.35	0.23	0.12
EDTA	0.17	0.10	0.03

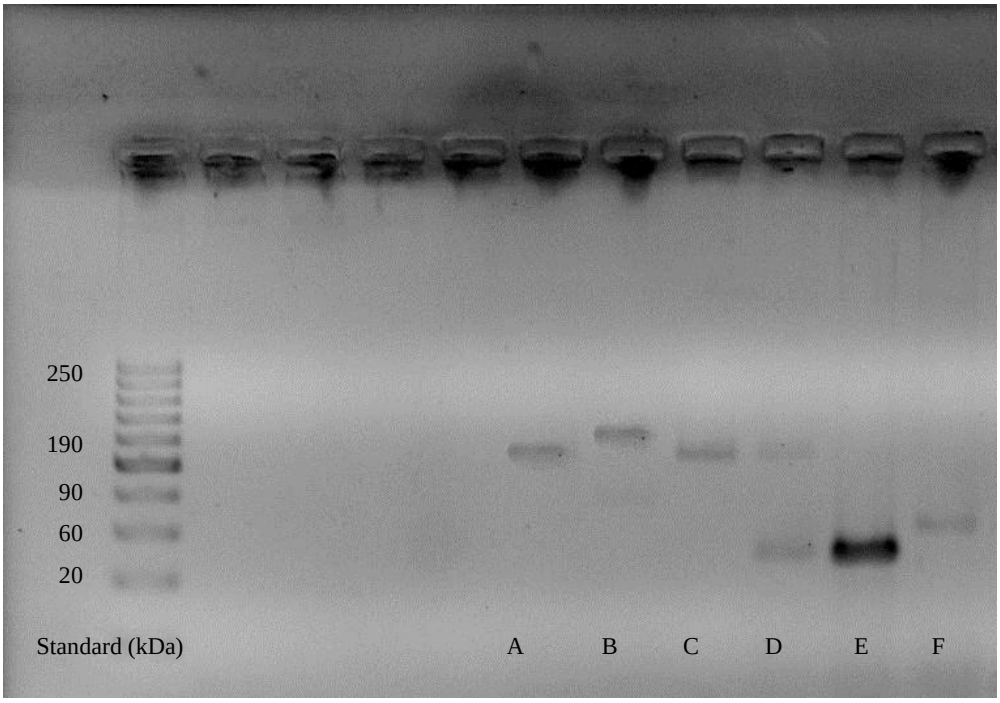

Figure 17- Gel electrophoresis (SDS-PAGE) of ALP extracts purified from fertile hydatid cysts (A: non-reduced, D: reduced), sterile hydatid cysts (B: non-reduced, E: reduced) and sheep liver (C: non-reduced, F: reduced).

3.3.2. Humoral and cellular immune reactions to hydatid cyst-derived ALP in human hosts

The ability of hydatid cyst ALP to induce immune responses in humans was studied by *in vivo* and *ex vivo* assays. To evaluate the feature of humoral and cellular functions which are specifically activated against ALP, all experiments were also carried out using HCF extracts as an alternative antigenic source of the parasite.

Post-surgical examinations

Microsections of FFPE liver biopsies obtained from hydatid patients (n=21) were stained by hematoxilin-eosin (see section 2.1.1 of the Material and Methods) and microscopic appearance of excised cysts was observed. According to the post-operation records, 19 patients (90.5%) had a single cyst and multiple cysts (more than 2 cysts) were removed from the other two cases. Hydatid patients were mostly asymptomatic or had mild abdominal pain complaint. Health complications such as obstructive jaundice were observed in 4 patients whose surgery results showed ductular proliferation and intrabiliary rupture of the cyst. One patients among the recent group had fissured cyst with a large fistula (>5 mm in diameter) into bile ducts. Hydatid cysts were located in the hepatic dome (n=11 out of 21, 52%), in the anterior right lobe (n=7 out of 21, ~34%) and in the left lobe (n=3 out of 21, 14%) of the liver. Hydatid cysts in 7 patients were characterized by the presence of a soft ectocyst with hyaline appearance directly attached to the distal tegument of germinal membrane and a clear lumen (with or without internal septa) with no visible solidification (GI group). The viability rate of protoscolices in this group was ≥20%. Relatively stiff ectocyst, disconnected in some parts from degenerating germinal epithelium and partial solidification with dense contents of the inner cavity (0-20% viable protoscolices) were observed in hydatid cysts removed from 9 patients (GII group). Completely calcified cysts with degenerated germinal layer and 0% viable protoscolices were detected in 5 patients (GIII group). Such grading criteria correspond to the WHO standardized classification of hydatid lesions [105], however could be more reliable as they were directly based on morphological and histological observations. The viability rate was determined by light microscopy considering the presence of ovoid form protoscolices showing motility as well as eosin exclusion by dropping 0.1% aqueous eosin solution on slides. Protoscolices with intact anatomical shape (mostly in GI cysts) or their

disintegrated body parts such as rostellar hooklets were observed within the cyst contents of all hydatid lesions.

Ex vivo cell proliferation and cytokine responses induced by parasite antigens

The threshold of positive proliferation response was defined as mean cpm+2SD of unstimulated cell cultures from healthy individuals (namely 318±152.35 cpm). Therefore, in this study an average cpm ≥622.69 was considered spontaneous cell proliferation.

A significant lymphocyte proliferation was observed in PHA-stimulated cell cultures from all groups compared to non-stimulated controls, indicating the vivid cellular function in the PBMC cultures. There was no significant difference between examined groups according to the mitogen-induced cell proliferation. In general, PBMCs from hydatid patients showed relatively higher response to HCF than to ALP (p=0.03), whereas both antigens had significantly stronger stimulatory effect on cells from hydatid patients when compared to the patients with other parasitic infections or to the healthy donors ($p\leq0.001$) (Figure 18). Due to the insufficient number of individual samples with fascioliasis (n=2), all statistical indices were computed by considering patients with fascioliasis and taeniasis as one group. Collectively, PBMCs isolated from the recent groups showed significantly higher proliferative responses to HCF than did the identical cultures to ALP (p=0.002). As well, HCF-stimulated cell proliferation in these cultures was greater than those from healthy donors ($p\leq0.001$). No difference was found between patients with non-hydatid infections and the normal individuals concerning the ex vivo proliferation responses of PBMCs to ALP (Figure 18).

Levels of cytokines in supernatants were compared between the normal and hydatid cultures under different conditions. Although spontaneous production of IFN-γ was similarly observed in the supernatants of unstimulated cultures from both groups (Table 8), mitogen-induced levels of this cytokine were significantly higher in the cultures from hydatid patients compared to those from healthy donors ($p\leq0.0002$) (Table 9). Both parasite antigens induced IFN-γ production in the supernatants from hydatid cultures and at significantly lower levels in the cultures from healthy donors ($p\leq0.0001$) (Table 9). Besides, HCF induced higher levels of IFN-γ in the control supernatants compared to the identical cultures stimulated with ALP (p=0.0008), but such a difference was not significant in the hydatid samples (Figures 19 and 20).

Despite statistically similar levels of TNF-α in supernatants of unstimulated cultures from both groups, production of this cytokine in hydatid samples was higher than controls in response to either the parasite antigens or to the mitogen ($p \leq 0.0001$ for the parasite antigens and $p=0.0006$ for the mitogen) (Tables 8 and 9). No significant difference was found between HCF and ALP according to their ability to induce secretion of TNF-α in samples from hydatid patients whereas those from healthy donors produced higher levels of this cytokine when stimulated by HCF ($p=0.008$) (Figures 19 and 20).

In compare with the controls, significantly higher spontaneous levels of IL-2, IL-4 and IL-5 were produced by the hydatid PBMCs. Although mitogen-induced levels of IL-2 and IL-4 were indifferent between two groups, IL-5 production was higher in the hydatid supernatants ($p=0.005$) after stimulation with PHA (Table 9). In response to HCF, PBMCs from the hydatid patients produced significantly higher amounts of IL-2, IL-4 and IL-5 than did those from healthy donors ($p \leq 0.0001$), while such a difference between two groups was only observed according to the levels of IL-2 and IL-4 (but not IL-5) in the ALP-stimulated cultures ($p=0.003$ for IL-2 and $p \leq 0.0001$ for IL-4) (Table 9). In all examined samples from the hydatid patients, concentration of IL-2, IL-4 and IL-5 had more increase in response to HCF than to ALP ($p \leq 0.0002$). Among these cytokines, levels of IL-4 were significantly above background in the ALP-stimulated cultures from hydatid patients ($p=0.008$) (Figure 19). Detectable IL-2 and IL-4 responses to HCF were observed in the control supernatants, whilst ALP only induced slightly elevated levels of IL-2 ($p=0.01$) and had no effect on IL-4 production in this sample group. Concentration of IL-5 in the normal controls was not significantly above background in response to both parasite antigens; although these cultures produced measurable amounts of this cytokine after stimulation with the mitogen (Figure 20).

In absence of antigen stimulation, hydatid PBMCs produced higher levels of IL-6 than did normal controls ($p \leq 0.0001$) (Table 8), although PHA-induced levels of IL-6 showed insignificant differences between the examined cultures (Table 9). IL-6 production in response to HCF was not significantly different between the sample groups, while levels of this cytokine were higher in the hydatid cultures compared to the normal controls when ALP was the stimulant ($p=0.02$) (Table 9). A comparison between the applied parasite antigens according to their ability to induce IL-6

response indicated that HCF had stronger effect than did ALP in both hydatid and control cultures (Figures 19 and 20).

Spontaneous IL-10 production measured in the supernatants of hydatid cultures was greater than that in the normal samples (p=0.002) (Table 8). Accordingly, stimulation with PHA induced more increase in IL-10 levels above the background in the control cultures than in the hydatid samples (p=0.008) (Table 9). In response to HCF, the hydatid PBMCs produced higher levels of IL-10 than did cells from healthy donors (p=0.05), although such a difference between the sample groups was not significant when ALP was used as the stimulator (Table 9). Overall IL-10 reactions of both normal and hydatid PBMCs were the lowest in the cultures stimulated with ALP compared to HCF and PHA (Figures 19 and 20). Cultures from the healthy controls showed slightly stronger IL-10 responses to PHA than did to HCF (p=0.05) (Figure 20).

Table 8- Levels of cytokines measured in supernatants of unstimulated cultures from the hydatid patients and normal donors.

	Cytokines (pg/ml) **Median (SD)** **Range** **n/N**						
	TNF-α	IFN-γ	IL-2	IL-4	IL-5	IL-6	IL-10
Culture							
Hydatid	14.41 (24.71) ≤8-87 15/21	5.35 (9.90) ≤29 19/21	12.69 (11.09) ≤41 17/21	2.51 (2.27) ≤10 17/21	10.63 (6.88) ≤26 20/21	11.39 (9.30) ≤30 21	20.97 (28.15) ≤2-129 19/21
Normal	8.52 (21.67) ≤8-54 9/15	10.69 (10.98) ≤5-34 11/15	1.01 (3.98) ≤38 4/15	2.44 (1.46) ≤3 8/15	4.12 (5.38) ≤4-17 8/15	1.52 (1.01) ≤2 9/15	8.38 (5.80) ≤5-18 9/15
***p* value**	-	-	**≤0.0001**	**0.03**	**0.0002**	**≤0.0001**	**0.002**

- The value of median (pg/ml) and the standard deviation (SD) have been demonstrated for each group. The number of responders out of the total number of individuals (n/N) has been also indicated for each measured cytokine. Values of significance (p) show the differences between the hydatid and normal samples.

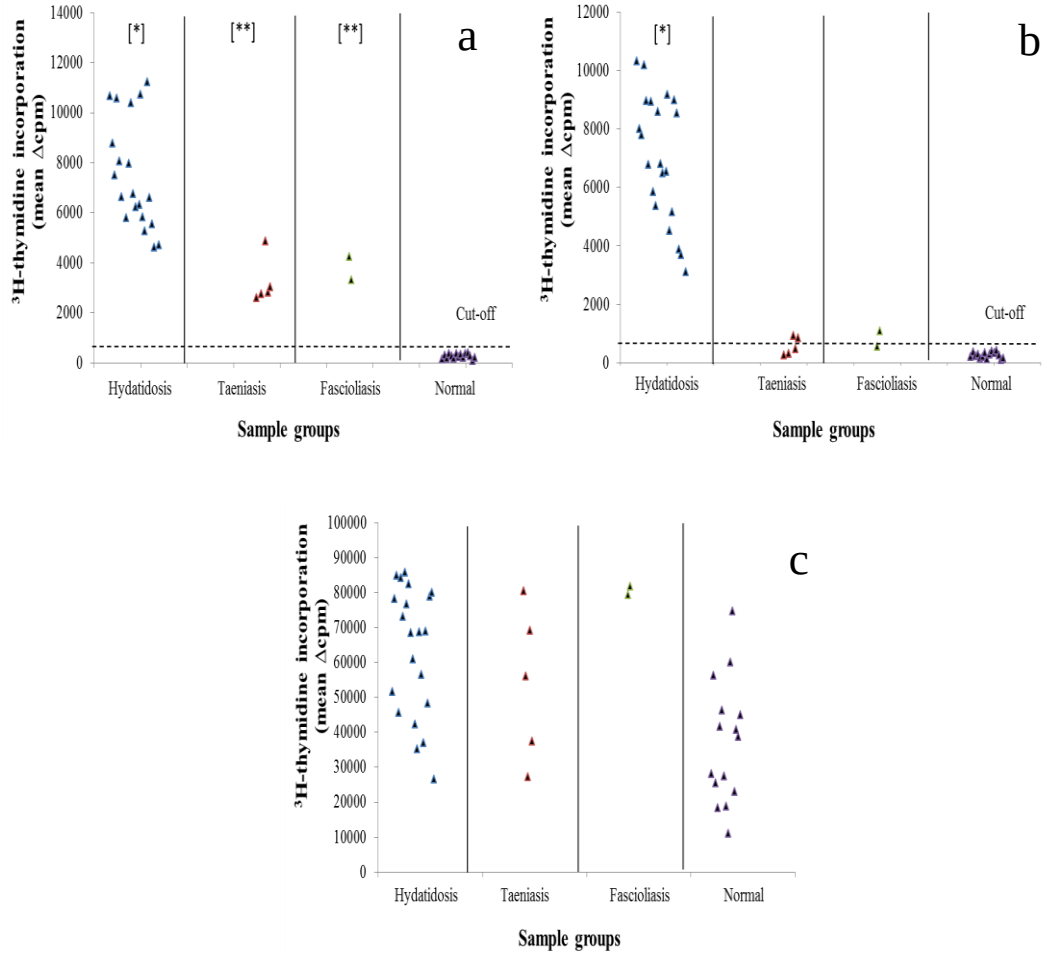

Figure 18- Cell proliferation (mean Δcpm of triplicate assays) in PBMC cultures from the hydatid patients (n=21), patients with taeniasis (n=5) and fascioliasis (n=2) as well as from normal donors (n=15) in response to parasite antigens and the mitogen. Dashed lines indicate the cut-off point of proliferative responses to HCF and ALP. a) Δcpm of HCF-stimulated cultures shows a significant difference between the hydatid samples ([*], $p \leq 0.001$) and other groups as well as between patients with other parasitic infections and normal donors ([**], $p \leq 0.001$). b) Δcpm of ALP-stimulated cultures shows only a significant difference between hydatid samples and other groups ([*], $p \leq 0.001$). c) All samples showed vigorous cell proliferation after stimulation with PHA.

Table 9- Levels of cytokines measured in the supernatants of cultures from hydatid patients and normal donors after stimulation with parasite antigens and the mitogen.

		Cytokines (pg/ml) **Median** **(SD)** **Range** **n/N**						
		TNF-α	IFN-γ	IL-2	IL-4	IL-5	IL-6	IL-10
Stimulant	**Cultures**							
HCF	Hydatid	263.97 (94.41) 77-352 21/21	452.08 (102.32) 175-536 21/21	110.56 (49.81) 48-303 20/21	27.11 (23.31) 3-87 21/21	35.02 (21.04) 11-74 21/21	79.59 (26.80) 17-113 21/21	123.21 (52.15) ≤20-178 19/21
	Normal	137.00 (73.09) ≤72-280 14/15	309.06 (147.04) ≤32-452 14/15	9.30 (14.42) ≤5-52 13/15	12.43 (5.91) ≤1-15 13/15	11.29 (9.37) ≤1-35 13/15	63.79 (37.23) ≤26-124 14/15	74.72 (59.34) ≤3-164 14/15
	***p* value**	**≤0.0001**	**≤0.0001**	**≤0.0001**	**≤0.0001**	**≤0.0001**	**-**	**0.05**
ALP	Hydatid	212.44 (97.38) 88-379 21/21	391.36 (101.04) 204-542 21/21	86.35 (13.42) 70-128 20/21	4.46 (8.57) ≤1-42 20/21	6.59 (7.28) ≤1-25 20/21	12.25 (24.62) ≤4-79 20/21	23.70 (26.21) ≤5-88 18/21
	Normal	90.92 (72.84) ≤20-215 13/15	78.75 (89.25) ≤5-247 14/15	7.18 (8.04) ≤2-27 11/15	0.32 (1.36) ≤1-4 5/15	3.72 (9.25) ≤4-25 9/15	8.60 (11.85) ≤1-43 13/15	16.58 (12.53) ≤1-40 14/15
	***p* value**	**≤0.0001**	**≤0.0001**	**0.003**	**≤0.0001**	**-**	**0.02**	**-**
PHA	Hydatid	390.91 (83.45) 167-487 21/21	582.14 (113.33) 286-755 21/21	98.18 (25.76) 39-137 21/21	3.25 (3.93) ≤2-12 17/21	38.77 (18.81) 8-68 21/21	115.77 (56.86) 43-275 21/21	134.90 (69.54) ≤50-266 19/21
	Normal	237.10 (115.50) ≤121-408 14/15	409.38 (113.43) (251-611) 15/15	68.70 (32.13) 30-117 15/15	8.24 (4.57) ≤2-15 14/15	17.92 (8.88) 2-34 15/15	135.41 (66.10) ≤97-215 13/15	205.49 (67.03) ≤125-262 14/15
	***p* value**	**0.0006**	**≤0.0002**	**-**	**-**	**0.005**	**-**	**0.008**

- For each individual sample, mean pg/ml of a desired cytokine in unstimulated cultures was deducted from mean pg/ml of that cytokine in stimulated cultures. Due to the disparity of achieved data, the value of median (as it is more indicative for highly variant data) and the standard deviation (SD) have been showed for each group. The number of responders out of total number of individuals in each group (n/N) has been also demonstrated for the assayed cytokines. Values of significance (p) show the differences between the hydatid samples and the controls.

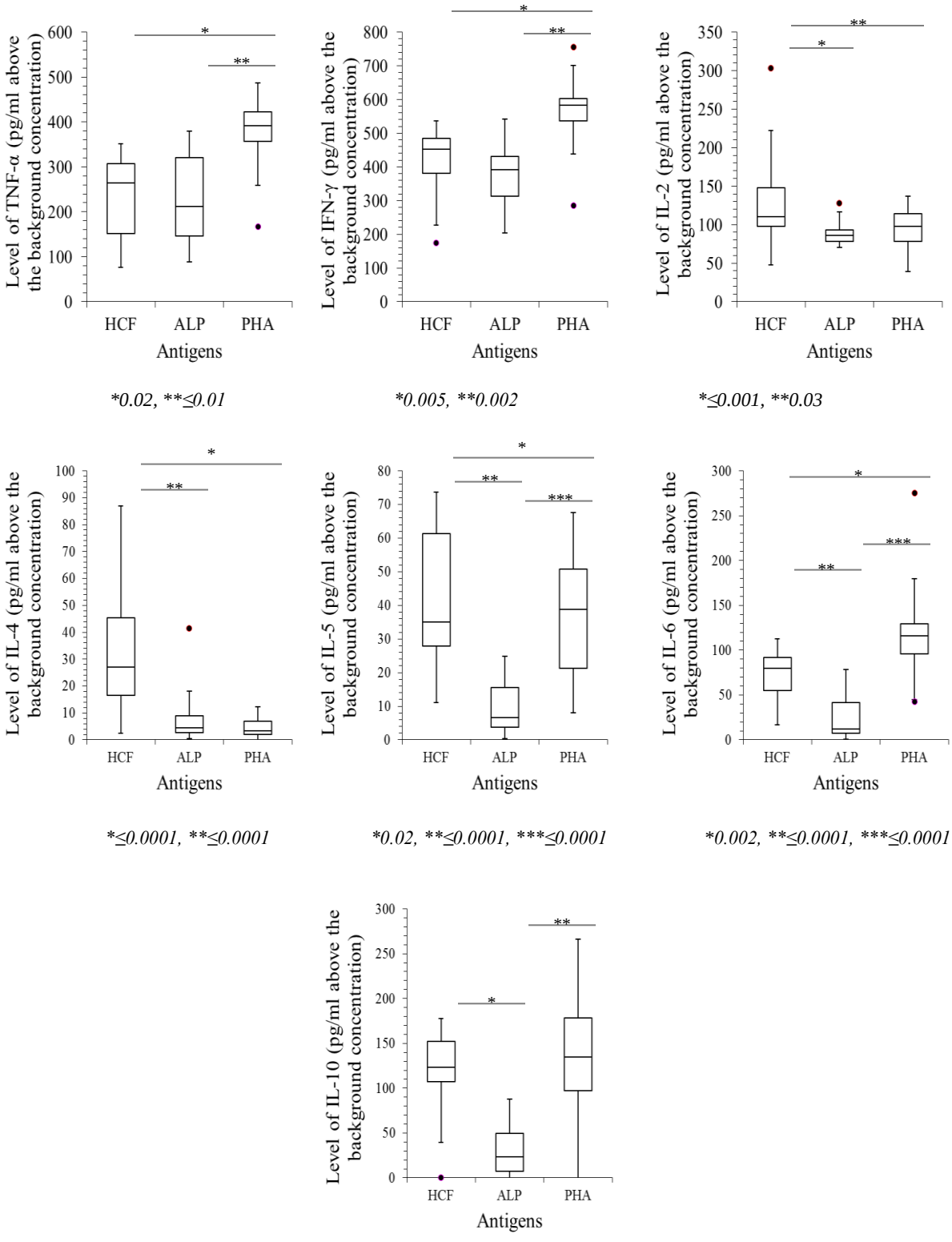

Figure 19- Box-plots show levels of cytokines (pg/ml above the background) in hydatid samples after stimulation with the antigens.

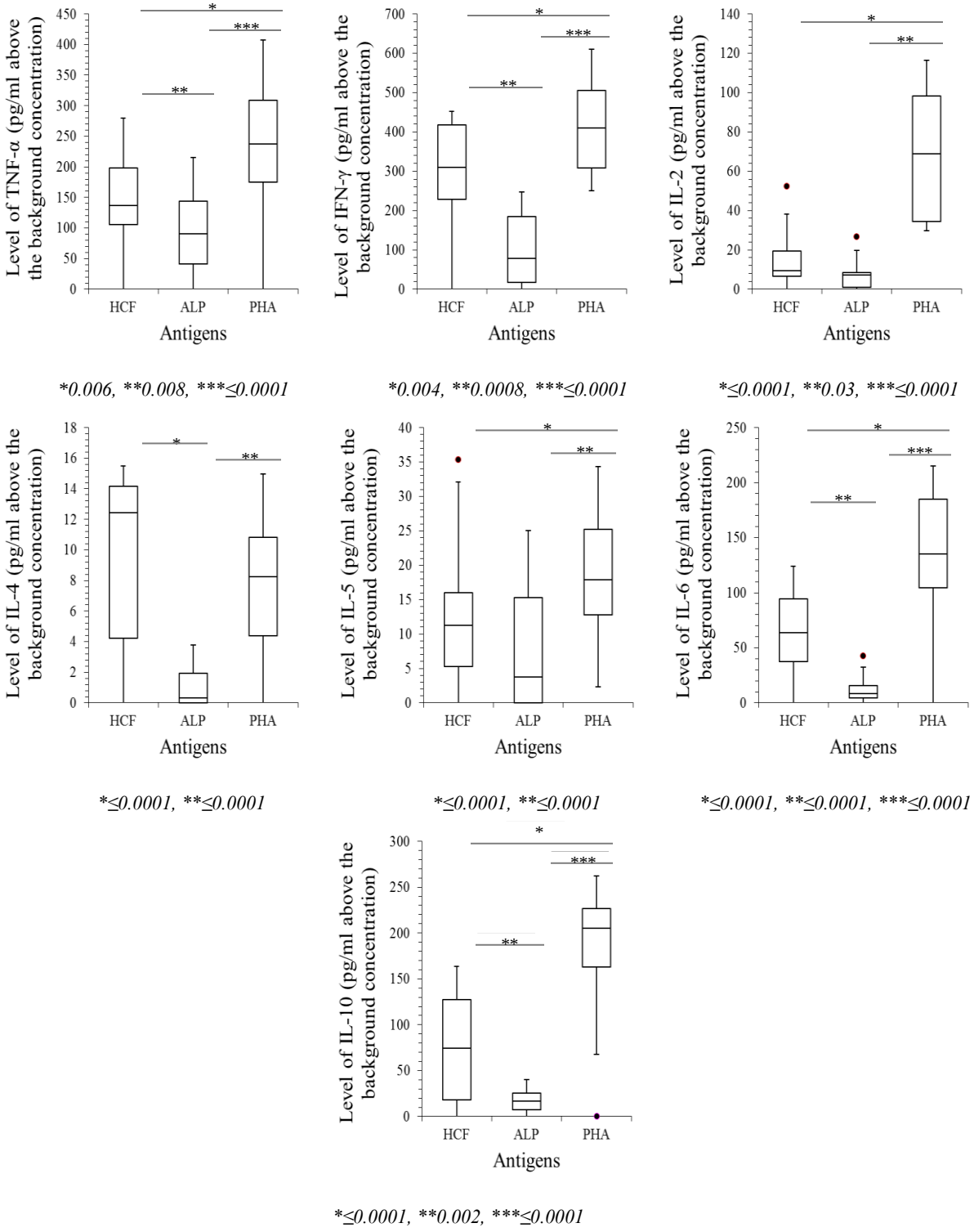

Figure 20- Box-plots show levels of cytokines (pg/ml above the background) in normal control samples after stimulation with the antigens.

In the last two figures (Figures 19 and 20), stimulatory effects of examined antigens on the PBMC cultures have been demonstrated in the hydatid and control samples, respectively. Lines within boxes indicate values of median corresponding concentration of cytokine (pg/ml) in response to a particular antigen. Whiskers show maximum and minimum values in each data group. Solid marks represent outliers in each data set; however they only remark maximum and minimum outliers to simplify the graph plots. Asterisks show significant differences and corresponding values of *p* are noted under each graph.

Humoral responses to ALP and HCF in serum samples

As it has been demonstrated in Figure 21, sera from hydatid patients showed immunoreactive bands on the nitrocellulose page after sensitization with fertile cyst- derived ALP. Both reduced and non-reduced antigen extracts showed affinity to anti-hydatid IgG at the molecular weight of 65-75 kDa and 185-190 kDa, respectively.

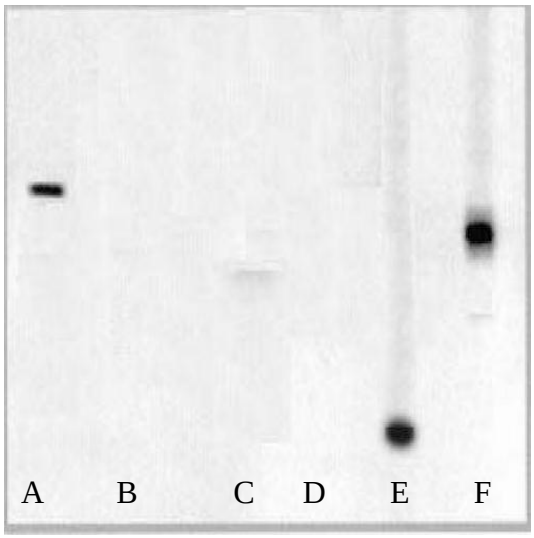

Figure 21- Immunoblot reactions of ALP from each extract have been showed in this picture. In compare with the control (A), no immunoreactive band was observed between IgG-positive serum and sterile cyst-derived ALP (B). The band was also absent when IgG-positive serum was incubated with ALP from host liver tissue (C). Besides, ALP from fertile cysts had no reaction to normal serum (D), however the immunoblot results were positive with either reduced or non-reduced extracts of ALP from fertile hydatid cysts when sensitized nitrocellulose page was incubated with anti-hydatid positive serum (E and F).

Specific IgG responses to the parasite antigens in sera of hydatid patient were also examined using ELISA and were compared to those in taeniasis/fascioliasis and normal samples. The overall results of ELISA assays have been summarized in Table 10.

Table 10- Total IgG and IgG subclasses against HCF and ALP measured in examined serum samples by ELISA.

	HCF				**ALP**				***p* value**
	Mean OD±SD	Hydatid patients (n/N)	Normal controls (n/N)	Cross reactivity (n)	Mean OD±SD	Hydatid patients (n/N)	Normal controls (n/N)	Cross reactivity	
Total IgG	0.623±0.129	21/21	2/15	Taeniasis (2)	0.435±0.185	15/21	0/15	-	≤0.01
IgG1	0.412±0.165	15/21	2/15	Taeniasis (1) Fascioliasis (1)	0.404±0.137	15/21	0/15	-	0.12
IgG2[1]	0.365±0.158	13/21	0/15	Taeniasis (1)	0.204±0.091	4/21	0/15	-	≤0.01
IgG3	0.238±0.107	14/21	0/15	Taeniasis (1)	0.316±0.072	12/21	0/15	-	0.94
IgG4	0.386±0.167	16/21	0/15	Taeniasis (3) Fascioliasis (1)	0.111±0.028	0/21	0/15	-	≤0.001

- ELISA results have been showed as mean OD±SD of duplicate assays in this table. The numbers of individuals with mean OD above the threshold (n) in total number of hydatid patients or normal controls (N) have been indicated according to the adjusted cut-off point using ROC curves for each antibody assay. To optimize the evaluations, the same cut-off point was assigned for both antigens by considering anti-HCF responses as the reference.

1: IgG2 responses to ALP were only measurable in 4 hydatid samples when the threshold was determined as the mean OD+3SD of normal controls in this test, however ELISA did not show any IgG2 activity against ALP when the cut-point was adjusted (mean OD >0.3).

To evaluate the diagnostic accuracy of ELISA tests, critical indices were calculated by measuring the area under ROC curves plotted for each antibody in response to HCF and ALP separately, as they have been demonstrated in Tables 11.

Table 11- The diagnostic performance of ELISA in accordance with serum antibody reactions to the parasite antigens.

		Indices							
		Cut-off	Sensitivity (%)	Specificity (%)	PP value (%)	NP value (%)	Accuracy (%)	Area under the ROC curve	J
Antigens									
	Total IgG	>0.3	100	81	84	100	92.3	0.994	0.9
	IgG1	>0.3	61.9	81.8	79.0	73.9	72.1	0.723	0.4
HCF	IgG2	>0.3	52.4	100	76.5	72.4	76.2	0.911	0.5
	IgG3	>0.2	66.7	95.5	93.3	74.1	76.2	0.851	0.6
	IgG4	>0.2	66.7	85.7	80.0	77.3	76.2	0.886	0,5
	Total IgG	>0.3	90.5	100	100	75.0	95.2	0.977	0.9
	IgG1	>0.3	71.4	100	100	78.0	86.0	0.940	0.7
ALP	IgG2	>0.3	33.3	86.4	57.1	51.4	60.5	0.608	0.2
	IgG3	>0.2	51.1	100	100	70.0	79.1	0.981	0.6
	IgG4	>0.2	N/A	N/A	N/A	N/A	N/A	N/A	N/A

- All cut-off values were pre-assigned according to the mean OD of normal sera + 3SD for each assay and then were adjusted by the highest distance between the ROC curve and the diagonal (chance) line of that assay to maximize the number of correctly classified individuals. Positive predictive, PP, and negative predictive, NP, values as well as the Youden indices (J) are also shown for each test. These analyses were not applicable for IgG4 responses to ALP as the measured ODs in hydatid samples were at the same levels or lower than the threshold (mean OD+3SD of normal sera for this assay). Antibody responses to HCF were considered as the reference for evaluating the cut-off points.

Prognostic value of the ex vivo immune responses to the parasite antigens

According to the results, both parasite antigens somewhat induced measurable proliferation responses in cultures from normal donors. Moreover, the proliferation response to both antigens was found irrelevant to the viability of hydatid cyst as it was compared between GI, GII and GIII patients.

Correlations between cytokine responses in the supernatants of peripheral blood cultures and stage of parasitic lesions were also analyzed in hydatid patients. Under the same condition, the pattern of cytokine production was almost the same in supernatants from all patient groups (GI, GII and GIII). Differences were only significant according to higher levels of the spontaneous IL-6 and IL-10 production in GIII cultures compared to the cultures from the other two groups. As well, HCF significantly increased the IL-6 levels in the GII and GIII supernatants (p=0.05). Regardless the grade of hydatid lesions, IFN-γ was predominant among the examined cytokines produced in response to either parasite antigens or to the mitogen in all hydatid samples. Besides, all antigens induced remarkable production of TNF-α, however levels of this cytokine were significantly lower than IFN-γ (p=0.01) and there were no differences between hydatid groups in this regard. On the contrary, concentrations of IL-4, IL-5, IL-6 and IL-10 were significantly lower in all GI, GII and GIII culture supernatants after stimulation with ALP (Figure 22).

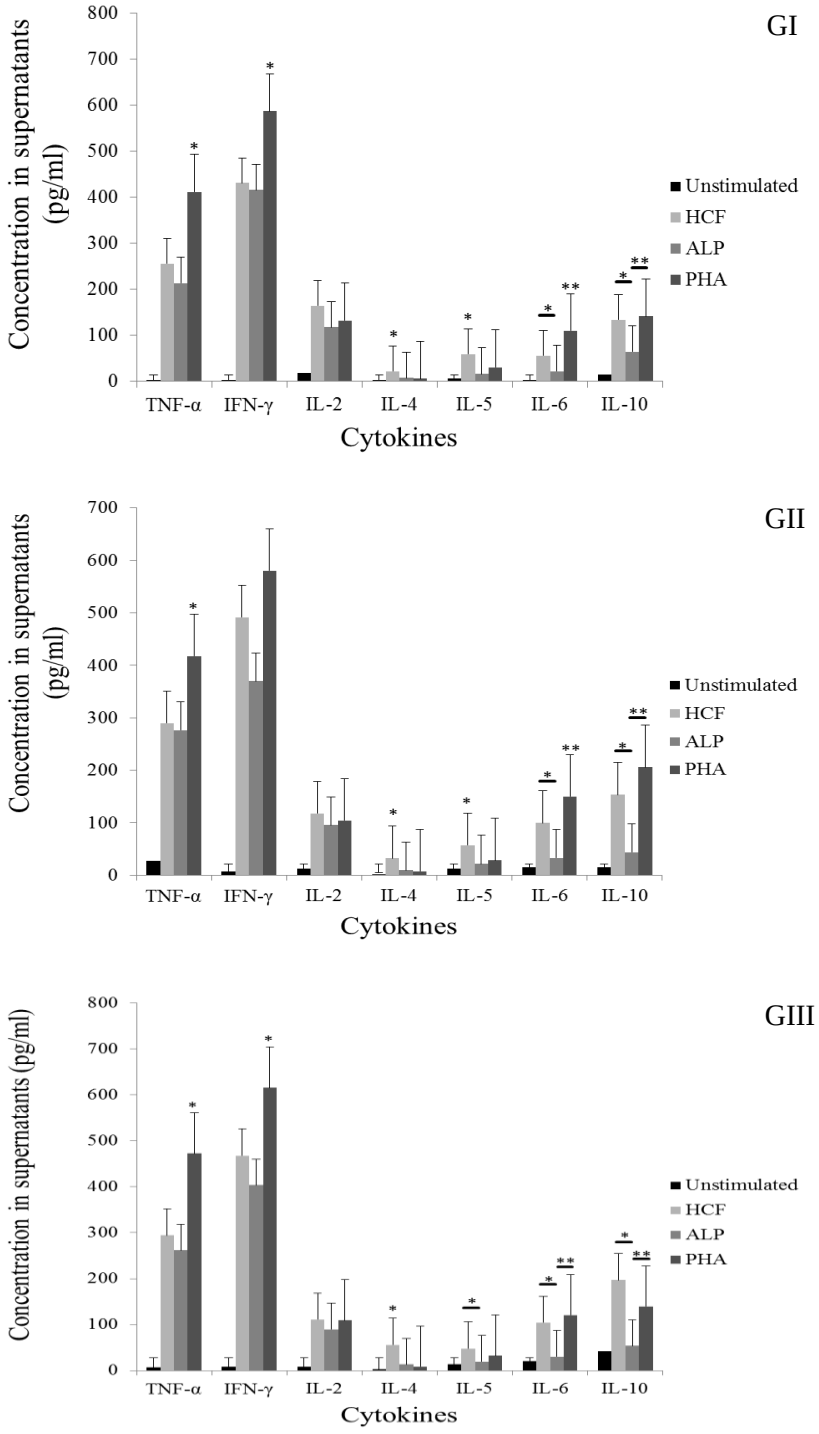

Figure 22- Ex vivo cytokine profiles in response to the examined antigens for each group of hydatid patients (GI, GII and GIII) have been summarized in this figure. Asterisks above bars show the significant differences between datasets. The values of significance (*p*) were ≤0.05 for all comparisons.

Comparative analyses of serum antibody responses to the parasite antigens showed significant differences in all hydatid groups (GI, GII, GIII) according to the detection of higher levels of IgG4 against HCF. In GI patients, IgG2 responses to HCF were higher than those to ALP (p=0.02), although such differences were not found significant in the other two groups (Figure 23).

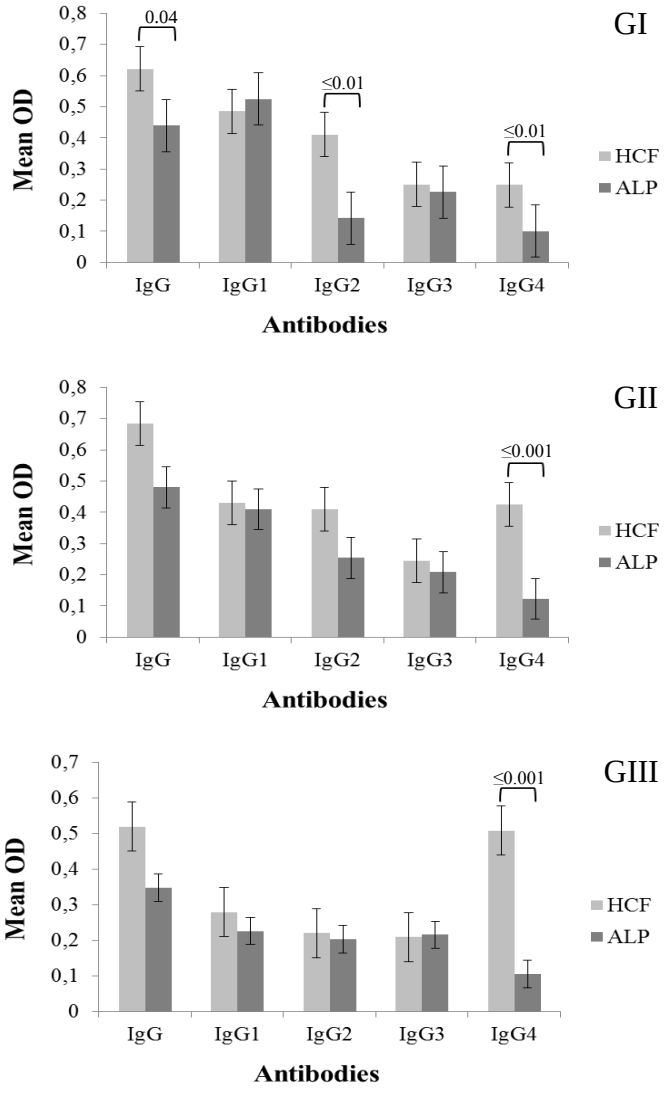

Figure 23- Levels of total IgG and IgG subclasses in response to the parasite antigens were compared between the sera of the hydatid patient groups (GI, GII and GIII). Values of significance have been indicated for different pairs. The standard error for each measurement has been showed by bars.

Correlation between cellular and humoral responses to the parasite antigens measured in the peripheral blood of the hydatid patients

The Spearman coefficient showed a negative correlation between IgG1 and IgG4 responses to HCF in hydatid patients (p=0.002) (Figure 24). There were no correlations between antibodies detected against ALP in these patients. As well, no relationship was found between HCF and ALP according to the humoral responses to these antigens. Assessment of correlations between the examined cytokines also revealed that the levels of TNF-α were negatively correlated with production of IL-5 in HCF-stimulated supernatants from hydatid patients ($p \leq 0.05$) (Figure 25). Furthermore, IL-2 production was found significantly correlated with IL-6 in response to ALP ($p \leq 0.05$) (Figure 26). Cytokine responses to HCF and ALP were only correlated according to the measured amounts of IL-10 in the hydatid supernatants (p=0.005). Correlations between antibody responses (IgG and IgG subclasses) assessed in the sera and the levels of cytokines in the culture supernatants of the hydatid patients were insignificant when assessed for either HCF or ALP.

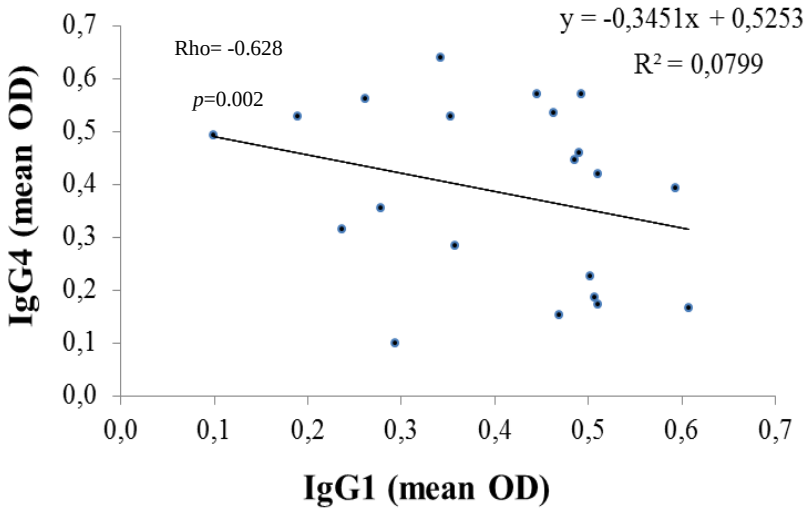

Figure 24- ELISA assays indicated a negative correlation between IgG1 and IgG4 responses to HCF in the sera from the hydatid patients. The Spearman correlation coefficient [205], the linear equation with R square the value of p are noted on the plot.

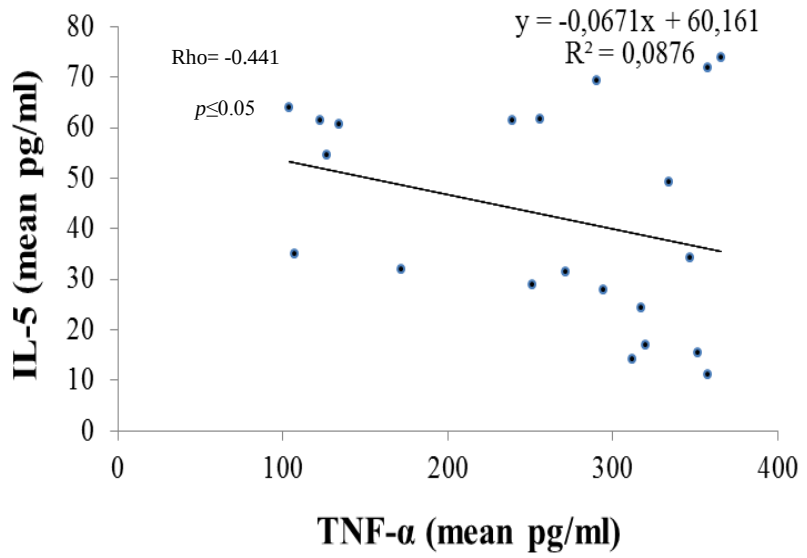

Figure 25- A negative correlation was found between IL-5 and TNF-α in responses to HCF in the supernatants of the hydatid PBMC cultures. The Spearman correlation coefficient [205], the linear equation with R square and the value of *p* are noted on the plot.

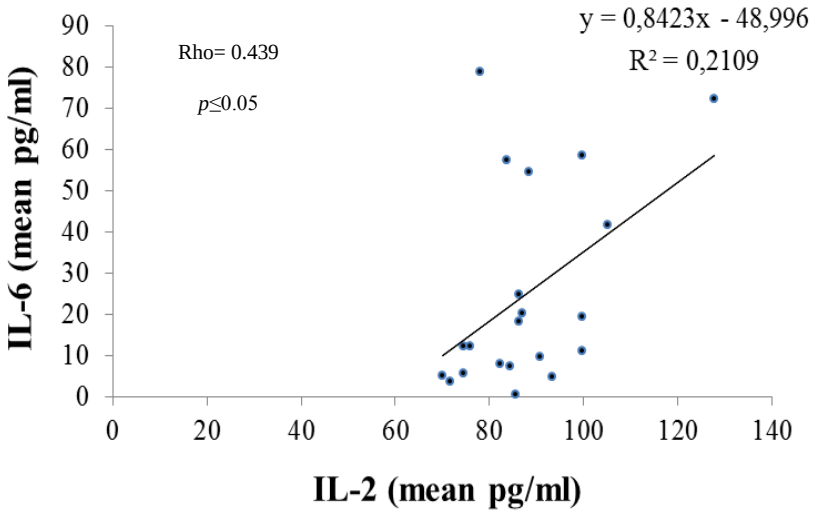

Figure 26- A positive correlation was found between IL-2 and IL-6 in responses to ALP in the supernatants of the hydatid PBMC cultures. The Spearman correlation coefficient [205], the linear equation with R square and the value of *p* are noted on the plot.

4. Discussion

Results of the present study illustrated different features of immune responses to cystic echinococcosis that may lead to pathogenesis of the infection in human hosts. These findings for the first time showed the overall tissue-specific reactions during the chronic infection in human liver. Moreover, in vitro assays also indicated the potential role of ALP as a biomarker of the cyst viability in immunogenicity of the parasite.

4.1. Cellular composition of inflammatory infiltrates around the pericyst

Tissue scarring and formation of fibrosis in chronic injuries of the liver is generally associated with phenotypic changes of T lymphocytes that are involved in perpetuation of the normal tissue homeostasis. Evidently, long-lasting pathogens induce differentiation of $CD4^+$ Th2 cells whereby macrophages are alternatively activated and fibrosis is promoted [191]. It has been also implied that $CD8^+$ T cells are recruited to the site of injury and although can effectively activate defense mechanisms, but may enhance tissue damage upon cytotoxicity functions [288]. Th2-profile cytokines are indicated to suppress these adaptive immune-mediated inflammatory responses that in turn may cause prolonged allergic reactions and fibrosis [145]. In the present study, immunohistochemical analysis could show significant activation of $CD3^+$ and $CD4^+$ T lymphocytes along with $CD68^+$ 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 chronic hepatitis biopsies (Figure 27). These findings could be in accordance with existing concept of immune functions that regulate chronic immune responses of the liver and maintain fibrogenesis. Cellular responses to steatohepatitis manifested weakened activation of $CD8^+$ T cells and macrophages; however aggregation of $CD4^+$ cells had no significant difference than that in chronic hepatitis at the $CD4^+/CD8^+$ ratio of 0.85. According to the significant activation of α-SMA expressing cells in these biopsies, a relatively typical chronic inflammation was characterized in steatohepatitis (Figure 27).

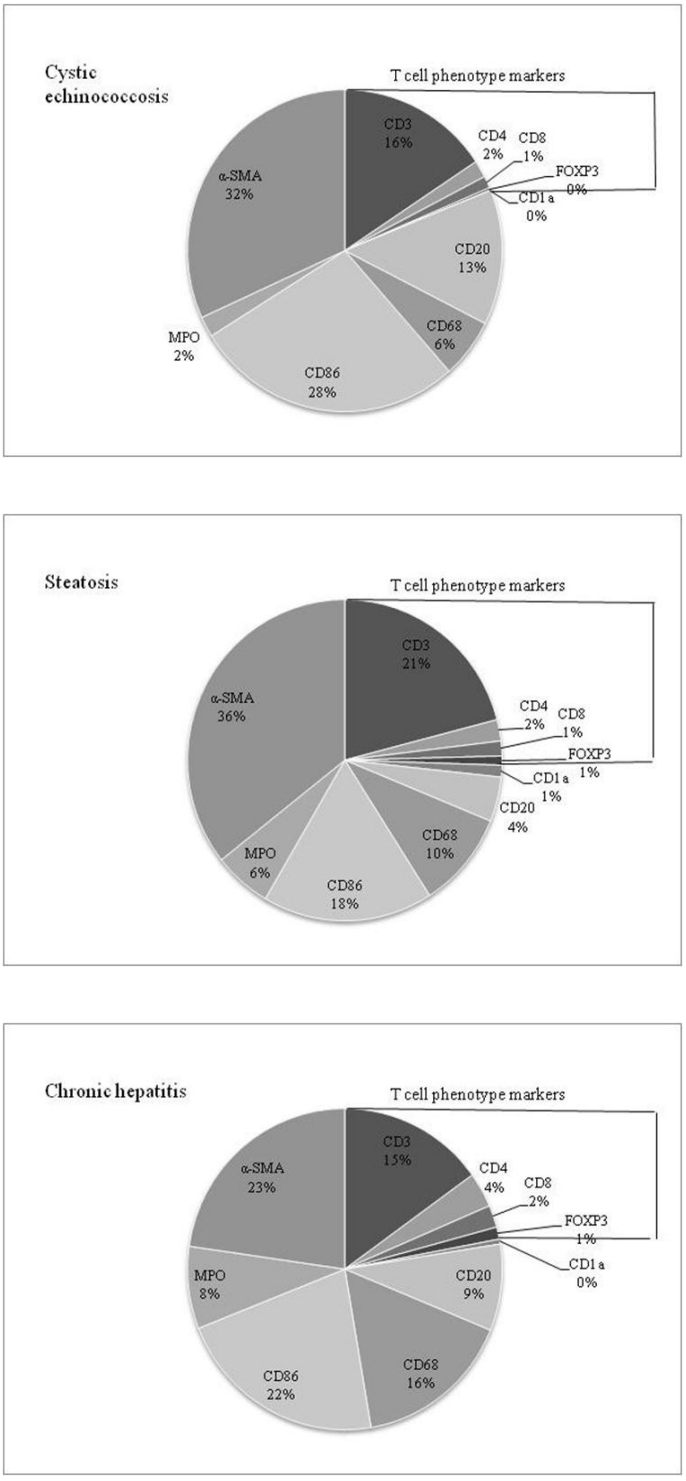

Figure 27- Cellular structure of the inflammatory infiltrates of the liver in cystic echinococcosis, steatosis and chronic hepatitis have been demonstrated in this figure. The percentage of immune-stained cells has been indicated for each identified cell marker.

Local activation of T cells in response to cystic echinococcosis had completely different feature as it was assessed by immunohistochemistry. The assemblages of T lymphocytes at the periphery of hydatid cyst were dominantly composed of $CD3^+$ phenotypes (Figure 27). Although the activation of CD4-labeled cells showed no difference when compared to the other two sample groups, both $CD4^+$ and $CD8^+$ cells had feeble presence around the hydatid pericyst at the $CD4^+/CD8^+$ ratio of 10.3. Assemblages of T cells dominated by $CD3^+$ subtypes within the pericystic adventitia were 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 [278] [224]. Intriguingly, up to 70% of cysts in humans and sheep are fertile, while 70%-100% of cysts in cattle have degenerated or undeveloped structures [220] [133] [225] [163] [177]. Altogether these data along with the outcome of the present study suggest the impact of the host's susceptibility on the composition of T lymphocytes which are locally activated in response to hydatid infection of the liver. Besides, significant differences between the quantities of activated $CD8^+$ cells in the tissue-spacific immune responses to cystic echinococcosis and chronic hepatitis could also imply the prognostic value of CD8-dependent activitiy of T lymphocyte in chronic inflammations of the liver with different etiology.

Presumably, the predominance of $CD3^+$ immunostained cells with weaker presence of $CD4^+$ and $CD8^+$ subtypes in inflammatory responses to hydatid cyst of the liver can highlight the role of $CD3^+CD56^+$ natural killer T cells in the organ defense against cystic echinococcosis. It has been implied that T cells compartment of the normal liver consists of both normal $CD3^+CD56^-$ and natural $CD3^+CD56^+$ T subsets, while natural killer T cells can prompt T cell receptor-mediated cytotoxicity and are capable of simultaneously producing Th1 and Th2 cytokines upon activation [84].

Results of the present study also showed negleagible accumulation of $FOXP3^+$ cells adjacent to the hydatid pericyst in human liver. However no significant difference was found between the sample groups in this regard, the numbers of these cells in chronic hepatitis and steatohepatitis were comparably higher than those in cystic echinococcosis. $FOXP3^+$ $CD25^+$ $CD4^+$ Treg cells are thought to be among resident cell population in the normal liver which may play a key role in maintainance of the homeostatic status of the organ [63]. It has been demonstrated that these cells are activated through T cell receptor signaling pathway in an antigen-idependent manner and

have immune inhibitory effect on all T cell subsets [238]. The contribution of Treg cells in the liver pathology has not been well-defined yet. Nonetheless, study has revealed that the enhanced activation of these cells can promote the oragn tolerance and is probably an efficient tool for treatment of autoimuune diseases and transplants rejection [238]. On the other hand, chronic inflammations are thought to induce differentiation of Treg subsets which may cause hyporesponsiveness of protective immune functions as it has been showed in viral infections of the liver and cancers [273] [252]. A body of research has also indicated that $FOXP3^+$ $CD4^+$ regulatory cells are capable of repressing tissue injuries via immunosuppressive cytokines such as IL-10 and transformation growth factor (TGF)-β. Perhaps such a mechanism can alter hyperimmune responses to pathogens and facilitates the establishment of chronic inflammation, whereas the contribution of Treg subsets to liver fibrosis has not been fully understood yet and different studies have implied either profibrotic or antifibrotic functions of these cells [90] [181] [254]. Parasitic helminths that chronically infect the liver can likely evade the host protective responses due to the augmented Treg functions during the course of infection as levels of TGF-β and IL-10 have been measured in response to their antigens [242] [271] [165]. Using murine models has suggested that the *Echinococcus* metacestode can locally supress defense mechanisms of the liver by inducing TGF-β and IL-10 responses [165]. In contrast to these data, results of the present study showed that Treg cells may have no participation in cellular inflammatory responses to chronic hydatid infection of human liver. Therefore, it is assumed that the role of $FOXP3^+$ T lymphocytes in cell-mediated immunity against cystic echinococcosis needs further investigation.

Abundant numbers of B cells expressing CD20 with the highest relative frequency amidst all sample groups were observed at the periphery of hydatid lesions, suggesting significant activity of adaptive humoral immune system in the liver. In a normal condition, B cells compose a very low proportion of the liver resident cells, but they infiltrate the parenchyma during the inflammatory response [1]. Aspect of the humoral immune response to hydatid cyst in human has been mostly studied indirectly in patients sera or by using animal models [300]. Therefore, achieved data by the present study could denote the importance of B cells in immune functions which are locally activated against the parasite in the liver. Besides, these results are in agreement with the recent findings which imply the role of B lymphocytes in chronic inflammations associated with liver fibrosis. A model study has demonstrated decreased collagen deposition and

faster depletion of apoptotic hepatocytes in absence of B cells [179]. There are two plausible hypotheses to explain the role of B cells in formation of fibrosis. First, B cells may contribute to the process of fibrosis by production of IL-6 which has profibrotic effects and promotes transformation of HSCs to myofibroblasts [291]. Second, B cells that express major histocompatibility complex (MHC) II can potentially act as professional antigen presenting cells, thus may have an impact on modal changes of Th1 to Th2 when contact with T cells through TCR signaling pathways. Th2 skewed cytokine profile (e.g. IL-4) has been showed to have a pivotal role in activation of HSCs and formation of fibrosis [290]. Expression of IL-6 elevates in both human and animal hosts during the chronic cystic echinococcosis [281] [300]. Moreover, results of the present study showed inadequate differentiation of $CD4^+$ T cells at the periphery of the metacestode. Accordingly, the first hypothesis is assumed to be more explicable in context of illustrated features of the immune response to chronic hydatid infection.

Immunohistochemistry also showed attenuated aggregation of either polymorphonuclear or macrophages in cellular inflammatory responses of the liver to cystic echinococcosis. Evidently, the antibody-dependent cell mediated cytotoxicity (ADCC) is an important defense mechanism primarily activated against the chronic phase of helminth infections [300] [302]. During the early phase of infection, type 1 cytokines classically activate macrophages that in turn recruit neutrophils and other leukocytes by expression of various immune mediators [218] [300]. As a consequence, an acute inflammation is initiated which induces overproduction of toxic radicals [300]. As such, expression of inducible nitric oxide synthase (iNOS) is enhanced via activation of proinflammatory cytokines (e.g. IFN-γ, IL-1β and TNF-α) which stimulate release of nitrite free radicals, however may result in apoptotic damage of the parenchymal cells [26]. A specific hydatid antigen (Arc 5) has been shown to induce production of nitric oxide and IFN-γ in PBMC cultures derived from hydatid patients [5]. Immunohistochemical detection of iNOS in liver biopsies and its significant levels in the sera as well as in hydatid fluid have been also reported in CE patients [5]. Th2-like cytokines (such as IL-4, IL-5 and IL-13) predominantly produced in immune reactions against chronic phases of the infection can induce alternative activation of macrophages and downregulate the ADCC response. Therefore, tissue-dwelling helminths may employ escape mechanisms whereby they suppress ADCC-mediated responses to prolong their survival within the host tissue [167]. As an instance, Study showed that some antigenic compounds of the parasite reduce neutrophil activation in vitro [155] [217]. Results of the current

study could also confirm that macrophages and neutrophils are weakly activated in local immune responses to cystic echinococcosis of human liver. Nonetheless, generally impaired T lymphocytes activities could suggest that in human liver hydatid cyst likely induces immune suppression 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 [256].

Strikingly, results of the present study showed the absence of $CD1a^+$ dendritic cells (DCs) in all liver biopsies obtained from hydatid patients. Perhaps, the absence of $CD1a^+$ cells in the pericystic adventitia represents the participation of specific DC phenotypes in hydatid-induced inflammation of the liver. In human, heterogeneous lineages of DCs comprising $CD1a^+$ and $CD1a^-$ subsets have been identified in different compartment such as the peripheral blood [120]. Myeloid DCs express CD1a in response to type 1 cytokines, but evolve into $CD1a^-$ subtypes in absence of exogenous mediators in vitro [30]. As well, $CD1a^+$ DCs produce significant amount of IL-12 and can polarize naïve T cell to Th1 [50]. The involvement of DCs in immunopathology of chronic CE has not been thoroughly studied yet. The only available data show the ability of parasite antigens to induce alternative differentiation of murine and human DCs in vitro, which results in overproduction of CD86 and downregulation of CD1a and their enhanced ability to activate Th2 or Treg responses [126] [216] [284] [285]. Altogether, these findings suggest that in response to chronic hydatid infection DCs are more probably differentiate to $CD1a^-$ subtypes which can be a new field for further approach toward identification of host immune reactions against cystic echinococcosis.

Application of molecular methods also confirmed the results of immunohistochemistry according to the impaired activation of T cell-dependent immune responses at the periphery of chronic hydatid lesions. Molecular assays for the first time showed the expression pattern of costimulatory molecules in the inflammatory cellular infiltration surrounding chronic hydatid

lesions in human. According to the results, the expression of CTLA4 mRNA was very low in all sample groups. As well, CD28 mRNA had a significantly lower level in CE than that in SH and CH. Signal transduction through CD28, which is constitutively expressed on both $CD4^+$ and $CD8^+$ subtypes, is essential for T cell activities [175]. To reduce the magnitude of immune reactions, some $CD4^+$ T cells express CTLA4 which has downregulatory effect on CD28. The function of CTLA4 is associated with the Treg cells activity and its presence is dependent on expression of FOXP3 [112]. The expression of CTLA4 mRNA was significantly correlated with the presence of $CD4^+$ cells only in CH biopsies; however this correlation between CTLA4 and FOXP3 was insignificant in all sample groups. The exacerbated expression of CD28 in the inflammatory milieu of hydatid liver could be referred to phenotype change of T cells to $CD28^-$ subtypes as it has been reported in infections, autoimmune diseases and cancer [154] [195] [253] [34]. As well, insignificant quantities of CD28 and CTLA4 mRNA could suggest the poor activity of T cell-dependent mechanisms at the periphery of hydatid cyst in human chronic infection, probably due to the clonal deletion of specifically activated T cells [289]. The role of costimulatory molecules in CE has been barely defined, but hydatid fluid antigen seems to induce diminished expression of CD28 in T cell cultures [149]. These results appear to be more important when it is taken into consideration that signal transduction through costimulatory pathways may have a key role in modulation of T cell responses [6].

B7 molecules, including CD80 (B7-1) and CD86 (B7-2), are expressed on professional antigen presenting cells and are up-regulated after activation [6]. Following the engagement of TCR by antigen which induces the first activation signal, B7 molecules bind to their ligands on the surface of T cells (CD28 and/or CTLA4) and generate the second (costimulatory) signal. This pathway is determinant in functional outcomes and activation threshold of antigen-specific T cell response. CD28 is constitutively expressed by up to 95% of $CD4^+$ T cells and conducts costimulatory signals after binding to CD80 and CD86 [25] [6]. In contrast, activated $CD4^+$ T cells up-regulate surface expression of CTLA4 which binds to the same ligands with higher affinity and delivers a negative signal to impair the T cell function. B7-CD28/CTLA4 costimulatory pathway has an essential role in activation of naïve T cells and is seemingly responsible for influencing the subsequent differentiation of $CD4^+$ T lymphocytes regarding the activation of Th1, Th2 or Treg subsets [6] [253]. As for antigen presenting- associated costimulation, CD86 is constitutively expressed on dendritic cells and monocytes and is rapidly

up-regulated after activation. CD80, however, is only detectable on activated antigen presenting cells with gradually increasing levels and evidently has an opposite function [195] [196]. Research has shown that the signals through CD86 may enhance immune response while CD80 induces immune suppression via interaction with CTLA4 [196]. These molecules are constitutively expressed on the surface of DCs, as professional antigen presenting cells, but can be also seen on macrophages and B cells upon stimulation [196]. Therefore, the imbalance between CD86 and CD80 can be an indicator for pathogenesis of inflammatory diseases [232]. In contrast with T cell costimulatory proteins, molecular assay indicated the remarkable levels of CD80 and CD86 mRNA in cystic echinococcosis biopsies as well as in the other sample groups. The quantities of CD80 and CD86 at mRNA levels were positively correlated so that could indicate continuous activation of antigen presenting functions that are normally attributed to chronic inflammations. Significant levels of CD80 and CD86 RNAs with slightly higher proportion of CD80 in hydatid samples also suggest the essential roles of these molecules in normal liver tolerance which is essential for sustaining the liver immunological balance [146].

Finally, an excessive accumulation of α-SMA^{+} cells and consequent fibrosis in the immediate vicinity of hydatid cyst in all CE samples, showed the presence of unremitting wound-healing processes. It could be challenged by the inefficient activity of inflammatory cells around the pericyst, which was detected in this study. Additionally, the decisive contribution of DCs and B cells in the liver inflammation may underline their critical role in the organ pathology and formation of fibrosis [22] [199]. It still remains unanswered whether antigen presenting DCs or HSCs can directly interact with hydatid antigens and mediate liver fibrosis, as it has been implied in other helminth infections such as schistosomiasis [8].

4.2. ALP as a potential marker for hydatid cyst viability

Immunochemical characterization of ALP indicated significant differences between fertile and sterile hydatid cysts in the present study. The assessment of molecular characteristics of this enzyme showed higher thermal stability of ALP from fertile cyst compared to the enzyme purified from sterile cysts or host liver. Kinetics of enzymatic reactions also demonstrated higher affinity to substrate when measured for fertile cysts- derived ALP compared to the isolated enzyme from the other resources. Interestingly, the molecular weight of all three extracts was the

same in both reduced and non-reduced conditions. In 1994, Lawton *et al*., characterized the molecular traits of ALP isolated from hydatid cyst membrane and showed that this molecule varies from the host derived enzyme. They associated these variations to the structural heterogeneities between the enzymes from different species. Besides, these researchers proposed that ALP can be potentially an activity maker for the cyst viability tests and may be of diagnostic value [137]. To examine viability biomarkers of the parasite, recent study has also demonstrated the potential role of enzymes from the cyst membrane in susceptibility/resistance of the parasite to chemotherapeutic agents [62].

ALP isoenzymes are hydrolase enzymes that are found in almost all tissue compartments. The levels of these enzymes are routinely measures as a diagnostic marker in different medical conditions. The structure of these enzymes differs in accordance with the type of the organ where the isoenzyme is produced [171]. Human liver is an important site of ALP production and the elevated levels of the enzyme in this organ can be associated with various health complications such as cirrhosis, toxic irritations of the liver and malignancies [171]. Interestingly, *Echinococcus* metacestode possesses this enzyme which has an essential role in the parasite metabolism presumably as a regulator of phosphate turnover via phosphorylation of various phosphoesters [70]. The association of this enzyme with calcareous corpuscles in protoscolices also indicates its possible role in calcium metabolisms [70].

Although a few studies have focused on immunochemical traits of ALP in *Echinococcus multilocularis* (a close relative of *E. granulosus* which causes alveolar echinococcosis), the potential role of this enzyme in immunopathology of hydatid infection has not been fully investigated yet [227]. Moreover, there has been no data to show the differences between fertile and sterile hydatid cysts according to the enzymatic activities of ALP.

Results of the present study confirmed that in sterile cyst the structural damage of the membrane can be associated with metabolically inactivated metacestode and/or with the parasite death due to defects in selective permeability of the cyst barriers [230]. ALP isolated from sheep liver showed almost the same molecular characteristics as that from sterile cysts. Knowing the structural aberration of the membrane in inactive cysts [81], these results may indicate the existence of an uncontrolled passage between the cyst cavity and the parasite microenvironment

which allows host-originated compounds to pass through. Therefore, it can be inferred that the detected enzyme in extracts from sterile cysts is perhaps from the host origin. This point can be also supported by negative response of anti-hydatid sera to ALP from sterile cysts in immunoblot assays, whereas all sera from hydatid patients contained antibodies with specific affinity to ALP from fertile cysts. Indeed, the cyst membrane in *Echinococcus* species is a protective shield against agents that are involved in the host defense, and may selectively permit the transportation of parasite metabolites and excretory- secretory components. As a consequence, this barrier plays an important role in the cyst viability as well as in pathogenesis of the infection [82] [282]. It has been also indicated that the apoptotic cell death can be reciprocally induced in the basal layer of germinal epithelium by the host immune agents or in host-derived lymphocytes by antigenic metabolites of the parasite [245].

Results of this study could be biased concerning the fact that ALP isoenzymes are of the largely unchanged molecules between different species [97]. Of course, it is should be considered that somatic proteins which are structurally conserved between prokaryotic and eukaryotic species are showed to have different functions in every individual organism. As well, functional similarities have been also observed between proteins which have completely different molecular compositions [171]. These variations are mostly due to only a few replacements in their amino acid sequences have occurred throughout the evolution [56]. It could be hypothesized that in parasitic organisms (such as *E. granulosus*) metabolically important molecules have undergone changes in order to maximize the capability of organisms to conform to life conditions within a host. Hence, these molecules can potentially be immunogenic and therefore are good targets for vaccination or chemotherapy strategies.

Altogether these findings can confirm that the host immune responses are probably induced when either adaptive or innate immune agents are in contact with fertile cysts, while an inactive parasite metacestode (sterile cyst) may have no impact on the host immunity. Such an assumption can explain the existence of immunologically non responder individuals in the course of chronic infection.

4.3. Cellular and humoral responses to ALP

Peripheral immune responses to hydatid cyst-derived ALP were investigated in the present study. To evaluate the significance of the achieved data, all examinations were performed by using hydatid crude antigen and results were compared.

Lymphoproliferative responses to the mitogen were observed in all samples, showing no impairment in ability of cultured PBMCs to react against antigen stimulation. Cell proliferation assays were able to distinguish between hydatid patients and normal controls as PBMCs in 100% of the hydatid cultures noticeably responded to both parasite antigens. The presence of peripheral cell-mediated immune responses to HCF has been indicated by other researchers, although there are only a few studies specifically focused on this subject. Siracusano *et al.*, reported the enhancement of mitotic cell division in PBMCs isolated from hydatid patients after stimulation with hydatid fluid and a fraction of hydatid specific antigen contained two major parasite proteins, Arc 5 and antigen B [240]. It was also shown that circulating lymphocytes can perpetuate their responsiveness during the course of chronic hydatid infection in human patients, as they significantly responded to PHA stimulation [10]. This notion was also supported by other studies demonstrating the ability of isolated PBMCs from patients with primary infection to respond to the recall of the parasite antigens [109] [217]. The present study also confirmed that ex vivo proliferation assay can be considered as an indicator to show the presence of cellular immune responses to circulating antigens, however the results should be trusted carefully when crude extracts of the parasite is used as a stimulant. HCF was observed to stimulate cultures from patients with taeniasis and fascioliasis above the threshold, which could highlight the presence of cross-reactive epitopes in these parasite species. It has been previously indicated that molecules with the same epitopic residues are shared not only between helminths belonging to the same class [130] [147] but can be detected in those of different taxonomical classes [237] [54] [139]. Hydatid cyst- derived ALP, on the other hand, showed no stimulatory effect on PBMCs from the patients with other parasitic diseases, suggesting the significance of specific immune responses to this antigen. So far, attempt for characterization and localization of ALP in *Echinococcus* metacestode has suggested that as a membrane-bound enzyme of metabolic importance and has associated its activity with the cyst viability [157] [228] [137] [70]. Thus it can be inferred that ALP likely participates in the host-parasite immune interaction, although there is no evidence to

show whether levels of this antigen alone or involved in immune complexes are detectable in the peripheral blood. Furthermore, the specific responses to ALP can be presumably influenced by clinical heterogeneity, stage of the infection and individual traits of the host as well as by the parasite strain. The hydatid patients participated to this study showed different lymphoproliferative responses to ALP, but there was no correlation found between ex vivo PBMCs responses and the stage of the disease when these patients were divided into three distinctive groups according to the histological markers of the metacestode viability. Taking into account that all patients carried the infection in the liver, these results can underline the presence of locally activated adaptive immune agents against the parasite antigens in the circulating blood which undergo somatic hypermutation. This phenomenon exerts irreversible mutations in genes encoding antigen receptors on lymphocytic clones that further are inherited by the progeny of these clones including memory T cells and memory B cells [297]. Underlying mechanisms are closely controlled by the magnitude of the inflammatory response, cytokine production and are also affected by either paracrine or endocrine regulators upon antigen presenting functions [297]. Somatic hypermutation causes avidity maturation in naive lymphocytic clones which in antigen-specific B cells can be characterized by enhanced affinity of activated antigen receptors, while it is normally observed as an accelerated functional responsiveness in antigen-primed T cells. Exposure to the antigen elicits stronger responses in the selected clones compared to those after the first antigen encounter [279]. Parasitic helminths are known to constantly stimulate the host immune system either by their somatic antigens or by excretory-secretory compounds [127] [45]. These immune reactions are seemingly inadequate to eliminate the infection and instead, repeatedly induced responses by a vast quantity of antigenic compounds and subsequent suppression of effector mechanisms (such as apoptotic deletion of activated lymphocytes) intervene the generation of memory responses [98] [51] [272]. Therefore, results of this study can probably indicate the importance of hydatid ALP in provoking the clonal expansion in specifically responder lymphocytic populations and in induction of immunological memory.

Levels of cytokines measured in the supernatants of PBMC cultures also signified distinctive differences between HCF and ALP according to the T cell-mediated immune profiles stimulated by these antigens. Cytokine production at the basal level was assessed in unstimulated cultures and showed distinguishably different spontaneous cytokine production between hydatid patients and normal donors. In brief, all hydatid cultures produced measurable amounts of IL-2, IL-4, IL-

5, IL-6 and IL-10 in higher quantities than those in the normal cultures. Basal levels of IFN-γ and TNF-α showed no differences between the hydatid and normal cultures. Hydatid patient PBMCs have been formerly shown to produce higher amounts of IL-6 in unstimulated cultures when compared to the same samples from healthy donors, although the levels of simultaneous IFN-γ in cultures from the patients were more than those in the controls [161]. Other researchers, however, showed no difference between IL-4 and IFN-γ productions by PBMCs from hydatid patients and healthy donors [217]. It was also shown that the levels of IL-5 measured in the supernatants of hydatid and normal cell cultures were not significantly different in the absence of stimulant but IL-6 had higher concentration in unstimulated hydatid cultures than in normal controls [210]. Besides, an earlier study indicated insignificant differences between patients and normal controls PBMCs according to their abilities to produce IL-4, IL-10 and IFN-γ in unstimulated cultures [211]. As there is an inconsistency in the available data about spontaneous levels of cytokines in ex vivo assays, they may not exclusively reflect the cellular responses to the parasite in the peripheral blood. Perhaps these results majorly correlate with biological factors such as age, sex, and immune responsiveness of individuals as well as with the culture conditions which can induce different levels of stress to the cells [111] [121].

Concentrations of cytokines were measured above the background in the supernatants of PBMC cultures from the hydatid patients and healthy donors after stimulation with the applied antigens. Patients with other parasitic infections were not included in this assay due to the irrelevant value of possible results to the aim of this study. HCF induced production of all examined cytokines above the background in PBMC cultures. Significantly increased levels of IL-2, IL-4, IL-5, IL-10, IFN-γ and TNF-α were measured in samples from hydatid patients compared to normal controls in response to HCF, while concentration of IL-6 was not different between the hydatid and normal PBMC cultures after incubation with this antigenic fraction for five days. The existing data about the impact of IL-6 on human immune reactions against cystic echinococcosis is so rare. In 1996, Rigano *et al*., reported that PBMCs from hydatid patients and normal donors significantly showed IL-6 responses to a fraction of hydatid fluid [210]. Serum levels of IL-6 along with IFN-γ and TNF-α measured in hydatid patients suggested the correlation between nitric oxide production and responses of these cytokines in human immune mechanisms against the parasite [269]. Patients with cystic echinococcosis also showed strong IL-6 responses positively related to activation of C-reactive proteins which declined after successful surgical

treatment [202]. Significant levels of IL-6, IFN-γ and IL-17A in the sera and supernatants of PBMC cultures from hydatid patients and their lower concentrations in samples from healthy donors in response to a specific parasite antigen, Arc 5, suggested the role of these cytokines in immune protection against the parasite in human hosts [161]. On the other hand, hepatic expression of IL-6 and TNF-α mRNAs decreased after eight weeks post-infection in mice while IL-10 and TGF-β had elevated expression levels [165]. IL-6 has complicated functions and is involved in both acute and chronic inflammatory reactions. It is considered as a progenitor of the early T cells and natural killer cells development and helps B cells to proliferate and facilitates their differentiation to plasma cells [115]. Inflammation-induced IL-6 can be also secreted by cells other than T lymphocytes such as B cells and monocyte/macrophages [115]. In the present study 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 antigens which play a role in IL-6 mediated responses to the parasite in human hosts.

Except for IL-6, unstimulated cultures from the hydatid patients produced significantly higher levels of the other cytokines when compared to the normal samples. In the absence of antigen stimulation, levels of TNF-α and IFN-γ were comparatively higher than the other cytokines in the supernatants of normal PBMC cultures, although concentrations of cytokines were generally low in these samples. Besides, the hydatid PBMCs spontaneously produced higher levels of TNF-α, IL-2 and IL-10 than the other cytokines. Differences between levels of IFN-γ, IL-5 and IL-6 were insignificant and IL-4 had the lowest concentration among the other cytokines in these cultures (Figure 28). Comparison between the background levels of the cytokines indicated significantly enhanced responses of IL-2, IL-4, IL-5, IL-6 and IL-10 in cells isolated from the peripheral blood of hydatid patients. These results are consistent with former studies that implied the concurrent activation of type 1 and type 2 cytokines in response to hydatid antigens [281] [302], however may represent the predominance of Th2 profiles in peripheral immune reactions to chronic hydatid infection in human hosts [207] [211] [300]. Such an assumption can be also confirmed by noticeable amounts of proinflammatory cytokines such as IFN-γ, TNF-γ and IL-2 with a significant increase in levels of IL-4, IL-5, and IL-10 measured in HCF-stimulated cultures from

hydatid patients. Interestingly, TNF-α and IL-5 were negatively correlated in these samples Moreover, HCF-driven cytokine profile of the normal PBMC cultures showed the activation of either type 1 or type 2 mediators above the background, which suggests the involvement of both immune profiles in the innate mechanisms activated by the parasite crude antigen in the peripheral blood. It can be explained by the presence of cellular sources of cytokines other than T lymphocytes [102] [178]. As well, these findings may underline the role of γδ T cells in inflammatory responses against hydatid cyst. The recent cell types are shown to compose around 2-5% of lymphocyte populations in the peripheral blood and contribute to innate and adaptive immunity by production of different cytokines such as IL-17A, IL-10, IL-21, IL-22, IFN-γ and TNF-α [205] [255] [61]. Unlike αβ T cells, their prompt reaction against pathogens is independent of TCR/MHC-antigen signaling pathways [255] [61]. On the other hand, ALP induced significantly lower levels of IL-4, IL-6, and IL-10 in both sample groups compared to those stimulated with HCF. In the supernatants of hydatid PBMCs, IL-5 responses to ALP were weaker than to HCF. Such differences were insignificant in normal samples, although numbers of the healthy individuals who had detectable levels of IL-5 in their PBMC cultures were 9 out of 15 and 13 out of 15 in responses to ALP and HCF, respectively. Besides, levels of IL-5 and IL-10 had no difference between hydatid patients and normal donors when measured in ALP-stimulated cultures from both groups. Results of the present study also showed that the hydatid PBMCs produced higher levels of IL-2 in response to HCF than to ALP, albeit both fractions had the same stimulatory effect on normal cells in this regard and a correlation was observed between IL-2 and IL-6 in the ALP-stimulated hydatid samples. Strikingly, levels of TNF-α and IFN-γ were invariably detected in supernatants from hydatid patients when stimulated with either parasite antigens, while HCF had somewhat more influence on production of TNF-α and IFN-γ by the normal PBMCs. Along with significant increase of HCF-induced IL-6 in both sample groups, these results may specify higher amplitude of immunogenic molecules which can induce cell proliferation and cell differentiation in the structure of parasite crude antigen compared to ALP. The patterns of cytokine response to both parasite antigens showed no difference according to the stage of the disease when assessed in three patient groups, more likely due to the existence of immune memory in lymphocytic populations. Serum antibody ELISA also showed that IgG responses to ALP mainly comprised IgG1 and IgG3 subclasses in all hydatid patients. The diagnostic performance of the ELISA was acceptably high for both fractions according to the

total IgG measurements in the sera, although 7 patients had total IgG responses lower than threshold to ALP. Due to the presence of chronic lesions with partial or general calcification in some patients, it is presumed that the magnitude of serum antibody responses to ALP is closely dependent on the stage of the infection and may reflect the existence of metabolically active cysts. Identification of IgG subclasses demonstrated the predominance of IgG1 and IgG4 in peripheral immune responses to HCF, while IgG3 showed very low affinity to this antigen in ELISA assays (Figure 29). A negative correlation was also observed between serum levels of IgG1 and IgG4 in hydatid samples. These findings are supported by available information about humoral responses to the metacestode in human hosts which generally suggests the highest frequency of IgG1 and IgG4 levels in sera of patients with the chronic infection [302].

A growing body of evidence has also proved that the serum levels of different antibodies can be essentially correlated with biological factors of patient individuals and change after chemotherapy or surgical treatments [209] [302] [258]. As well, the pattern of antibody reactions varies between patients with primary infection and those who have relapsed disease [20]. Thus antibody detection has been widely used in post-treatment follow-up of hydatid patients; however it seems not to be adequately indicative of the infection outcomes which are correlated with the metacestode development [103]. Using crude antigen preparations for ELISA may result in cross reactive responses and besides a considerable proportion of patients with parasitic burden have been reported to be seronegative or had low antibody responses perhaps due to entrapment of specific antibodies in the circulating immune complexes [302]. Therefore it is necessary to identify novel antigens with higher diagnostic values to make more reasonable balance between the serology and the parasite activity.

Results of ELISA showed that IgG1 and IgG3 were predominant subclasses in the sera of hydatid patients against ALP. Analysis of the AUC of the ROC curve also showed that these two subclasses had acceptable diagnostic performances. Moreover, ALP-ELISA was able to distinguish between the hydatid patients and the other groups, indicating a highly specific antigen. On the other hand, IgG2 and IgG4 responses to ALP were almost at the cut-off levels. The encounter of polysaccharide/carbohydrate antigens triggers class switching to IgG2 in a T cell-independent manner [276] which can explain the lack of ALP specific levels of this antibody in patients' sera. Furthermore, the role of IgG4 in pathogenesis of chronic inflammations

associated with fibrogenesis has been already implied [71]. Activity of this antibody has been also reported in relation with hyporesponsiveness and immune suppression during parasitic infections [43] [242].

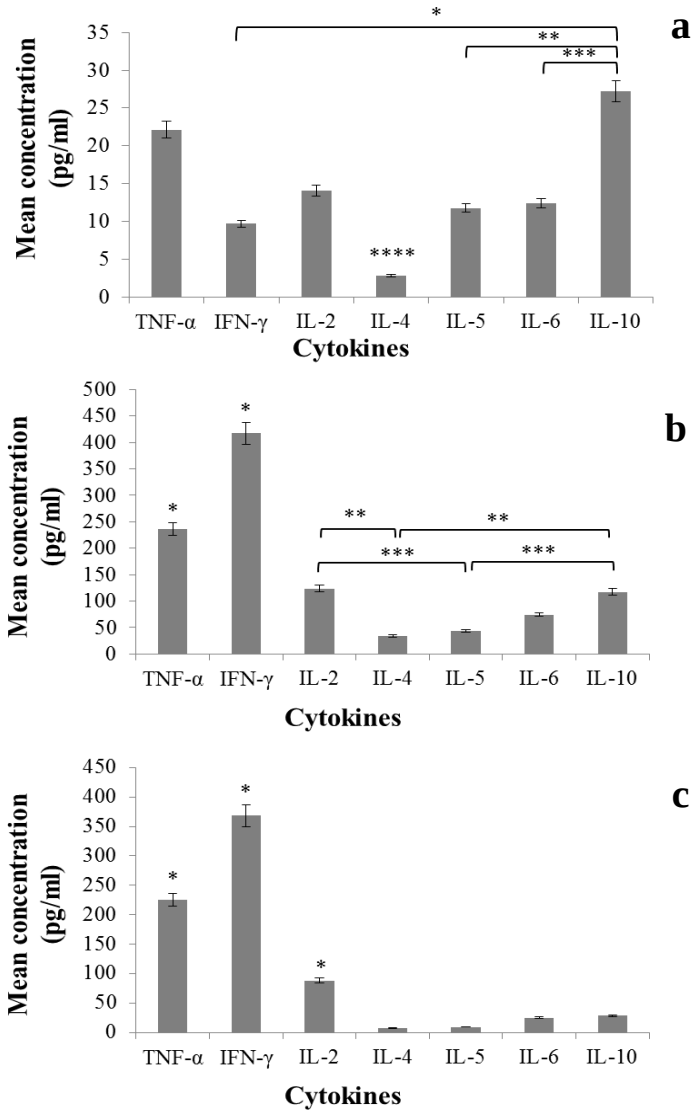

Figure 28- Arrays of examined cytokines produced by unstimulated (a), HCF-stimulated (b) and ALP-stimulated PBMCs from hydatid patients have been demonstrated in this figure. Bars show 5% of the total value (mean pg/ml) measured in 21 patients. Significant differences between the mean concentration values of cytokines are indicated by asterisks and interconnection bars ($p \leq 0.05$).

These data may show the impact of ALP as a tegumental protein on locally activated immune responses at the site of the infection and consequent fibrosis. It can be hypothesized that ALP is involved in activation of early inflammatory responses to the metacestode which decline upon degenerative changes and impairment in metabolic functions of the parasite. To analyze the results, it should be also considered that the diagnostic performance of the ELISA was acceptable for detection of total IgG and IgG3 specific antibodies against HCF, whereas the Youden index was higher than 0.6 for all antibodies (total IgG, IgG1 and IgG3) which showed affinity to ALP.

As a conclusion, overall results indicated the important role of ALP in immunopathology of cystic echinococcosis and suggested that human immune reactions to this antigen more likely comprise Th1 cytokine profile with predominance of IgG1 and IgG3 subclasses in IgG-mediated inflammatory responses, as they were measured in the peripheral blood of hydatid patients. The rationale is that antigens with ability to elicit protective immune responses (e.g. significant IFN-γ production in previously exposed individuals) perhaps is a good candidate for further investigation in order to improve therapeutic and/or vaccination strategies. Moreover, ALP is proposed as a useful marker for cyst viability that can be applied for the stage-related diagnosis of the infection.

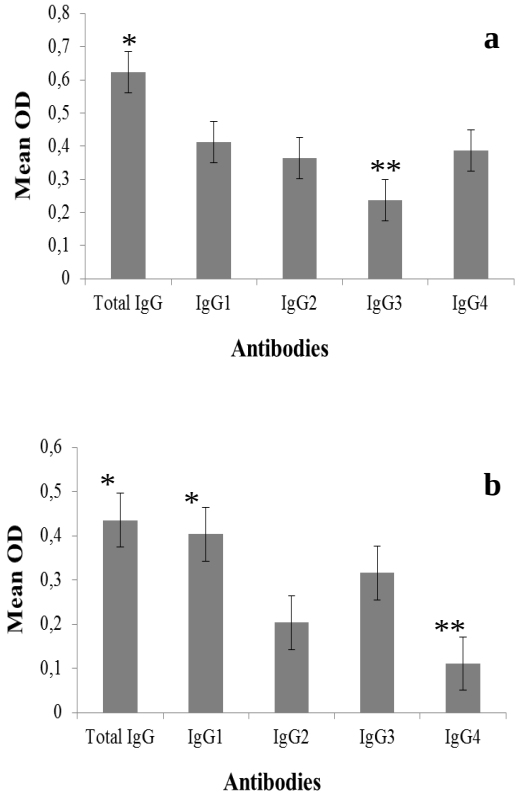

Figure 29- Panel of antibodies detected in the sera from hydatid patients by HCF-ELISA (a) and ALP-ELISA (b). Asterisks show the significantly highest (*) and lowest (**) mean OD values for each assay.

5. Novel scientific findings of the present study

The present study provided new information about molecular and cellular components that are engaged in immunopathology of chronic CE in human hosts. Results of this study can be also used for further approaches toward developing chemotherapeutic methods or immunization strategies.

1- The present study was the first to depict a comprehensive image of the cellular structure of inflammatory infiltrates around chronic hydatid lesions in human liver. Using a panel of antibodies against different cell surface antigens provided an opportunity to accurately determine the phenotypic traits of various cell components participating tissue-specific responses which are somewhat distinguishable from systemic reactions to the circulating antigens. These results suggested the existence of a general anergy in the activity of all major subtypes of T lymphocytes at the periphery of hydatid cyst in human liver. It was demonstrated that the CD8-mediated cytotoxicity is broadly silenced and may have no role in the organ immunity against the chronic infection while inadequate presence of both $CD4^+$ and $FOXP3^+$ cells also indicated exhaustion in either helper or regulatory functions of T lymphocytes in response to the chronic CE of human liver. $CD3^+$ T cells, however, were showed to have predominant role in the liver immunity during the course of the chronic CE. Such a finding remarks the participation of natural T cells and natural killer T cells in maintaining the immunological balance of the liver in human CE. Although the ADCC is generally considered as the main defense mechanism against tissue-dwelling helminths, results of the present study for the first time indicated a modest contribution of inflammatory cells such as macrophages and neutrophils to the immune responses against CE in human liver. Contrary to the cell-mediated functions, humoral immunity was showed to have a prominent role in the tissue-specific defense mechanisms against the chronic CE. Characterization of cell infiltrates around the pericyst demonstrated the abundant presence of B cells and DCs and a perpetuated fibrotic reaction due to the significant activation of HSCs expressing α-SMA. Altogether, these findings for the first time directly evidenced that in CE-induced chronic inflammation of the liver, professional APCs such as DCs and B cells are major role players in conducting the activation of resident and/or non-resident cells and may be responsible for phenotypic changes of HSCs and formation of fibrosis.

2- Expression of costimulatory molecules at the periphery of the hydatid lesion in human liver for the first time was measured in the present study. By application of quantitative PCR methods, mRNA expression levels of CTLA4, CD28, CD80 and CD86 were evaluated and the results of molecular experiments in parallel with immunohistochemistry showed the attenuated T cells function due to negligible expression of CTLA4 and CD28 while CD80 and CD86 molecules were abundantly expressed at the level of mRNA in the vicinity of chronic hydatid lesions. These findings also provided further evidence of the leading role of APCs (particularly DCs) in immunopathology of the infection.

3- Hypothetically, hydatid cyst contains an enormous amount of different epitopes with the ability to activate various immune mechanisms, thus the overall response to the parasite is likely the subsequent outcome of all these functions which vary according to the host-dependent (such as species, physiological status, the harboring tissue) as well as the parasite-dependent factors (such as strain, adaptation, aging). Therefore, the viability of the hydatid cyst is of the essential importance in pathogenesis of the infection. In search for a potential marker of the cyst viability which could be probably involved in immunopathology of CE, the present study for the first time showed the significant differences between fertile and sterile cysts in molecular characteristics, functional kinetics and immunogenicity of ALP as an important enzyme in parasite metabolism. Moreover, cellular and humoral responses to ALP were measured in the peripheral blood obtained from hydatid patients and healthy donors which indicated the significant proliferation of PBMCs and activity of proinflammatory cytokines as well as the existence of peripheral IgG1 and IgG3 responses to ALP. Therefore, hydatid cyst-derived ALP for the first time was shown to be a viability marker of the metacestode with potential immunogenic traits and was proposed as a good candidate for further studies on serology and immunology of CE.

6. Summary

In the present study, cellular composition of inflammatory infiltrates around the hydatid lesion in CE liver biopsies (n=21) was assessed by immunohistochemistry using a panel of antibodies against cell surface markers. Expression levels of costimulatory CD80, CD86, CTLA4 and CD28 mRNAs in inflammatory milieu of CE liver were measured by quantitative RT-PCR. Results were compared to those of steatosis (SH), n=11, and chronic hepatitis (CH), n=11, liver biopsies. Immunochemical characteristics and molecular kinetics of ALP were studied and differences between fertile and sterile cysts were observed. Peripheral blood mononuclear cells (PBMCs) and sera from hydatid patients undergoing surgery (n=21) and from taeniasis (n=5), fascioliasis (n=2) and from healthy donors (n=15) were obtained. Ex vivo proliferative reactions and cytokine profile were examined in PBMC cultures stimulated with either ALP or hydatid cyst fluid extract (HCF). Serum levels of total IgG and IgG subclasses in response to ALP and HCF were measured and results were compared.

Excessively aggregated α-SMA$^+$ and CD20$^+$ cells indicated the presence of a chronic inflammatory response, however T CD8$^+$ cells, macrophages and neutrophils were meagerly scattered within pericystic areas. CD4$^+$ and FOXP3$^+$ T cells had inadequate participation in hepatic infiltrates and T cell assemblages were predominantly composed of CD3$^+$ cells. CD1a$^+$ cells were absent in CE samples. Negligible expression of CTLA4 and CD28 mRNAs with high quantities of CD80 and CD86 mRNAs were observed in CE livers. ALP derived from fertile cysts showed significant molecular characteristics with ability to elicit immune responses when compared to ALP isolated from sterile cysts. Specific lympho-proliferative responses and the dominance of type 1 cytokines were observed in ALP-stimulated cultures. Total IgG, IgG1 and IgG3 showed high affinity while IgG2 and IgG4 responses were at the threshold levels in ALP-ELISA.

These results suggested that chronic inflammatory response of the liver to CE may comprise weakened cellular functions mediated by either helper or regulatory T cells, whereas B lymphocytes and dendritic cells (more probably with distinctive phenotypes) are actively involved in pathogenesis of the infection. As well, ALP was found to be potentially a cyst viability marker which can induce immune responses in human hosts more likely toward Th1-mediated profile. Therefore, hydatid cyst may possess various epitopes that provoke either types of immunity but type2 profiles dominate due to the downregulation of type1 inducing antigens.

Összefoglalás

Jelen tanulmány szerint, a gyulladást okozó beszűrődések sejtösszetétele a hydatid károsodások körül a "CE" máj mintákban (n=21) immunhisztokémia segítségével lettek megbecsülve, ellenanyag panelek felhasználásával a sejtfelület jelölők ellen. A kostimulációs CD80, CD86, CTLA4 és CD28 mRNS-ek megjelenési szintje a CE máj gyulladást okozó környezetében kvantitativ RT-PCR-el lett megmérve. Az eredmények ezekkel a steatosisok-kal, (SH), krónikus hepatitisz (CH), (n=11) májmintákkal lettek összehasonlítva.

Az immunkémiai jellemzők és az ALP molekuláris mozgások lettek tanulmányozva, illetve a különbségek a termékeny és steril ciszták között lettek megfigyelve. Perifériku mononukleáris vérsejtek (PBMCs) és a szérum a műtéten áteső hydatid páciensektől, taeniasis (n=5), fascioliasis (n=2)-tól valamint egészséges donoroktól lett levéve.

Ex vivo proliferatív reakciók és *citokin profil volt megvizsgálva,* PBMC kultúrákban lettek stimulálva vagy ALP vagy pedig hydatid ciszta folyadék kivonattal (HCF). Az IgG és IgG teljes szérum szintjének alosztályai válaszként az ALP és HCF volt megmérve és az eredmény összehasonlítva. Túlzottan aggregált α-SMA$^+$ és CD20$^+$ sejtek mutatták a krónikus gyulladásos válaszok jelenlétét, habár a T CD8$^+$ sejtek, makrofágok és neutrofilek szűkösen voltak szétszóródva a *pericisztikus* területen belül. A CD4$^+$ és FOXP3$^+$ T sejteknek elégtelen volt a részvétele a hepatic beszűrődésekben és a T sejtek gyülekezése túlnyomó részt CD3$^+$ sejtekből állt. CD1a$^+$ sejtek hiányoztak a CE mintákból. Elhanyagolható a CTLA4 és CD28 mRNS-ek kimutathatósága a magas CD80 és CD86 mRNS-ek mennyiségével volt megfigyelhető a CE májakban. A termékeny cisztákból származó ALP, jelentős molekuláris jellemzőket mutatott immunreakciókat ellenben a steril cisztából származó ALP nem mutatott immunreakciókat. Jellegzetes lymphoproliferativ válaszok és a 1 típusú citokinek dominanciája volt megfigyelhető az ALP-stimulált tenyészetekben. A teljes IgG, IgG1 és IgG3 magas affinitást mutatott miközben IgG2 és IgG4 válaszok voltak a küszöbértékű szinteknél az ALP-ELISA-ban. Ezek az eredmények felvetik, hogy a máj krónikus gyulladását okozó válaszai a CE tartalmazhat legyengült sejtfunkciókat vagy a segítő vagy pedig a szabályozó T sejtek általi közbejárással, mivel a B limfociták és dendritikus sejteknek (valószínűbben különböző

fenotípusokkal) aktív szerepe van a fertőzés kórfejlődésében. Ugyanúgy az ALP potenciálisan egy ciszta életképesség jelzőként volt megtalálva, mely előidézhet immunis válaszokat az emberi hordozókban inkább a Th1 közvetített profil felé.

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8. List of Publications

1. Vatankhah, A., Halász J., Piurkó V., Barbai T., Rásó E., Tímár J., (2015), *Characterization of the inflammatory cell infiltrate and expression of costimulatory molecules in chronic echinococcus granulosus infection of the human liver.* BMC Infect Dis. **15**: p. 530.

2. Vatankhah, A., Assmar M., Vatankhah GR., Shokrgozar MA., (2003), *Immunochemical characterization of alkaline phosphatase from the fluid of sterile and fertile Echinococcus granulosus cysts.* Parasitol Res. **90**(5): p. 372-6.

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