Impairment of Macrophage Presenting Ability and Viability by *Echinococcus granulosus* Antigens

Naceur Mejri[1]*, Imed Eddine Hassen[2], Jenny Knapp[3], Mouldi Saidi[1]

[1]Radiopharmaceutical Unit, National Centre of Sciences and Nuclear Technology, [2]Laboratory of Methods and Techniques of Analysis, National Institute for Research and Physical and Chemical Analysis, Ariana, Tunisia, [3]Laboratory of Chrono-environnement, Faculty of Medicine and Pharmacy, University of Bourgogne Franche-Comté, Besançon, France

ABSTRACT

Background: Despite advances toward an improved understanding of the evasive mechanisms leading to the establishment of cystic echinococcosis, the discovery of specific immunosuppressive mechanisms and related factors are of great interest in the development of an immunotherapeutic approach. **Objective**: To elucidate immunosuppressive effects of bioactive factors contained in chromatographic fractions from hydatid cystic fluid (HCF) of *Echinococcus granulosus*. **Methods:** Hydatid cystic fluid was fractionated by reverse phase chromatography. Non-specific Concanavalin A-driven proliferation of spleen cells was used to determine specific inhibitory fractions. Trypan blue exclusion test and flowcytometry analysis were performed to check whether highly inhibitory fractions of HCF have apoptotic effect on peritoneal macrophages. Western blot analysis was used to determine proteolytic effects of parasitic antigens on major histocompatibility complex (MHC) class II (I-a) contained in membrane proteins extract from macrophages. **Results**: High concentrations of HCF and few of chromatographic fractions suppressed spleen cells proliferation. Fractions 7 and 35 were the highest inhibitory fractions. Specifically fraction 35 and to a lesser extent HCF induced apoptosis in peritoneal naive macrophages. However, HCF and the fraction 7 proteolytically altered the expression of MHC class II molecules on peritoneal macrophages. The proteolytic molecule was identified to be a serine protease. Macrophages taken at the chronic and end phase from cystic echinococcosis-infected mice were able to uptake and process C-Ovalbumine-FITC. These cells expressed a drastically reduced level of (I-a) molecules. **Conclusion**: Our study present new aspects of immune suppression function of *E. granulosus*. Further molecular characterization of apoptotic and proteolytic factors might be useful to develop immunotherapeutic procedure to break down their inhibitory effects.

Mejri N, et al. Iran J Immunol. 2017; 14(1):35-50.

Keywords: Cystic Antigens, *Echinococcusgranulosus*, Immunosuppression, Reverse Phase Chromatography

---

*Corresopnding author: Dr. Naceur Mejri, National Centre for Nuclear Sciences and Technology, Sidi Thabet 2020, Ariana, Tunisia, Tel: (+) 216 24 542 780, Fax: (+) 216 71 537 555, E-mail: naceur.mejri@cnstn.rnrt.tn

INTRODUCTION

Cystic echinococcosis (CE) or hydatidosis is a cosmopolitan zoonosis caused by the taeniid tapeworm *Echinococcus granulosus* (1). Adult cestode lives mainly in the small intestine of dogs (definitive host), while the larval stage or metacestode (hydatid cyst) can infect intermediate hosts in a wide range of mammalian species, mainly sheep, cattle, camel and accidentally humans (2). The disease is characterized by the growth in the internal organs, mostly liver and lungs of unilocular cysts surrounded by an inner germinal layer and an outer laminated layer (3). Hydatidosis has a worldwide distribution. Tunisia is considered as an echinococcosishyperendemic region (4). Experimentally the intraperitoneal infection of mice with the larval stage of *echinococcusgranulosus* is referred to clinic as secondary infections. The intraperitoneal murine infection with cystic echinococcosis offers the opportunity to study the direct regulatory effects of metacestodes on periparasitic peritoneal cells, including dendritic cells (DCs) T cells, granulocytes such as eosinophils and especially macrophages (MØs) as predominant type of peritoneal cells (5). In the host–parasite interplay, metacestode surface molecules as well as excretory⁄secretory (E⁄S) products are considered as important key players (1). In the peritoneal cavity of CE-infected mice, inter-visceral growth of the hydatid cyst (metacestode) overcomes the immune system and subsequently establishes a chronic phase of infection. As in many helminthiases, surface-associated molecules and metabolic (E/S) products of the parasite tissue (metacestodes) exhibit immunoregulatory effects, favorable for the parasite survival and for the host by limiting immunopathology (6). Thus parasitic products are implicated in immune evasive strategies of hydatid cyst which have evolved an outstanding arsenal of mechanisms to modulate and/or suppress the host's immune system (7). Infected mice are characterized by a deeply impaired immune response. Increasing our knowledge on how cystic antigens modulate the immune response of the host is crucial for identifying and controlling parasitic factors involved in metacestodes growth and proliferation.
In the present work HCF (as crude antigen) including secreted/excreted (E/S) metabolic products and chromatographic fraction(s) of (HCF) were used as antigens. The purpose was to determine a part of numerous facets of immunosuppressive pathways which occur during CE-infection. To this end it was proposed first to determine whether HCF as crude antigen containing molecules with non-specific inhibitory effect on splenic T cells proliferation. Similarly inhibitory chromatographic fractions were subject of identification. Subsequently we aimed to determine accurate and specific effects of highly suppressive fractions on especially peritoneal-macrophages (pe-MØs). We propose to first tackle the effect of parasitic antigens on the viability of pe-MØs. Considering the role of pe-MØs as antigen presenting cells we checked a priori the ability of pe-MØs to uptake and process antigens. Subsequently we assessed the expression of MHC class II (I-a) molecules necessary to provide the first signal during leucocytes reactions. The influence of suppressive fractions on MHC class II (I-a) molecules expression on naive pe-MØs was studied.

MATERIALS AND METHODS

Mice, Parasites and Infection. Female 6-10 week-old C57BL/6 mice were obtained as a kind gift from the Pasteur Institute, Tunisia. Animals were housed for three days before the onset of the experiments in our laboratory. All mice were handled according

to the principles of the guide for animal experimentation. To obtain viable protoscoleces (PSC) fertile hydatid cysts were aseptically punctured and cystic fluid aspirated. The infectious protoscoleces belonged to genotypes G1 (sheep strain) of *E. granulosus*. The G1 strain is the most widespread genotype in Tunisia and especially in the north area of the country (8). Protoscolices and membrane debris contained in HCF were allowed to decant for 20 min at room temperature and the fluid was carefully discarded (9). PSC were washed with PBS to separate non-viable PSC and membrane debris. PSC exhibited more than 95% viability. A group of five C57BL/6 mice were inoculated intraperitoneally with 200 µL of a suspension containing approximately 1000 live protoscoleces in PBS. Five mice were injected with 200 µl PBS and used as controls.

Analysis of Hydatid Cystic Fluid by Reverse Phase Chromatography. The analysis by reverse phase chromatography (RPC) of HCF as crude antigen was done with a C8 column at a flow rate of 6 ml/min. Antigens in 300 µl of sheep HCF were loaded and separated through the C8 column. The gradient used for elution was developed using Buffer A (0.05% trifluoroacetic acid in water) and Buffer B (0.05% trifluoroacetic acid in 90% acetonitrile, 10% water). The column that was initially equilibrated with 90% Buffer A and 10% Buffer B, reached a composition of 10% Buffer A and 90% Buffer B in 45 min. A total of 44 fractions of 6 ml each were collected at the end of this fractionation.

Hydatid Cysts Antigens. Fertile hydatid cysts developed in liver of "barbarin" sheep breed naturally infected with *E. granulosus*, were obtained from the slaughterhouse of Mateur, Tunisia. The hydatid cystic fluid was aspirated aseptically by a syringe containing 25x protease inhibitor cocktail (Roche, Basel, Switzerland) diluted in 100 mM phosphate buffer, pH 7.0. HCF was centrifuged at 3000×g for 30 min at 4°C and the supernatant was collected. Chromatographic fractions collected from reverse phase chromatography and the supernatant of HCF were dialyzed, using cellulose ester membrane tube with a molecular weight cut-off of 100 Da (Spectrum, Socochim, Switzerland) against two changes of PBS 10 mM, pH 7.4 per 24 h over two successive days. Echinococcal antigens of different fractions were lyophilized for about 24 h and dissolved in 50 µl of PBS (10 mM, pH 7.4) to obtain suitable concentrations of antigens. Protein concentration was estimated by the Bio-Rad Bradford protein assay kit (Bio-Rad AG, Basel, Switzerland). Chromatographic fractions and the supernatant of HCF were stored at 4 °C until use.

Cell Culture. Spleen cell suspension was obtained by teasing the spleen from naive BALB/c mice with the large striated end of forceps. Red blood cells contained were removed by incubation of spleen cell suspension in hypotonic lysis buffer (0.15 MNH4Cl, 1 mM KHCO3, 0.1 mM EDTA). Splenic leucocytes were plated into 96-well culture plates (Falcon, Corning, NY) at 10^6 cells per well in RPMI-1640 supplemented with 10% fetal calf serum (v/v), 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM non-essential aminoacids, 0.05 mMmercaptoethanol, 100 U/ml penicillin/streptomycin and 25 µg/ml Fungizone. Splenic leukocytes were then stimulated with different doses of HCF: 6.25, 12.5, 25 and 50 µg/ml.

10^5 of splenic leukocytes per well in RPMI-1640 complete medium were stimulated separately with 20 µl from different HCF fractions derived from reversed phase chromatography. In different cultures, cells were cultivated for 24 h at 37°C and 5% CO_2 before being stimulated with 10 µl of concanavalin A (ConA), (Sigma, Lezennes, France) (0.2 µg/10 µl) for additional 24 h in a total volume of 200 µl (10). Cells cultivated without antigens were considered as a negative control, while cells treated

with only Con A was used as positive control. Cells were then incubated for additional 24 h in the presence of 10 μM 5-bromo-2'-deoxyuridine (BrdU). The incorporation of BrdU into the newly synthesized DNA of proliferating cells was determined by BrdU Cell Proliferation ELISA Kit (Roche, Mannheim, Germany), according to the manufacturer's instructions. The absorbance or optical density (OD) was measured by spectrophotometer at 450 nm and corrected to the absorbance measured with cultures containing only the medium.

Macrophages Preparation and Stimulation. Peritoneal exudate cells (PECs) from groups of five naive mice and five CE-infected mice, sacrificed separately after 6 weeks (early stage) and 12 weeks (late stage) of infection, were collected by peritoneal rinsing with 10 ml RPMI-1640. Cells were subsequently washed twice with Hank's Balanced Salt Solution (HBSS) and resuspended in RPMI-1640 (Gibco, Basel, Switzerland). To separate peritoneal macrophages, PECs were incubated in 5 ml RPMI-1640 +20% fetal calf serum in a Petri dish for 2 h at 37°C, in an atmosphere containing 5% CO_2. T cell-enriched non-adherent cells were discarded and pe-MØ-enriched adherent cells washed twice with 5 ml RPMI-1640 before being dislodged by treatment with non-enzymatic cell dissociation solution (Sigma, Lezennes, France). The purified pe-MØs were 90% Mac-1+ as assessed by fluorescence-activated cell sorting (FACS) analysis. Pe-MØ-enriched cells were resuspended in RPMI-1640 supplemented with heated and inactivated 10% fetal calf serum (v/v) (Gibco), 2 mM l-glutamine, 1 mM sodium pyruvate, 1 mM non-essential amino acids, 0.05 mM 2-mercaptoethanol, 100 U/ml penicillin and 100 μg/mL streptomycin.

$5x10^5$ cells/well of harvested pe-MØs were plated into 96-well tissue culture plate and incubated during 48 h separately with only the complete RPMI-1640 medium (as control), 20 μl of Fr 19, Fr 7, Fr 35 and 50 μl of HCF. Upon completion pe-MØs, viability was determined by trypan blue exclusion (Biochrom, Germany) on a Neubauer counting chamber. The trypan blue was filtered with 0.2 μm filter and used at the final concentration of 0.2%.

Preparation of Quenched C-Ova-FITC. The chicken ovalbumin (C-Ova), used as protein antigen, was highly labelled with FITC (isomer I) (Sigma, Lezennes, France). C-Ova-FITC-labelled antigen was prepared in a way that fluorescein moieties were quenched at 98% in the native globular C-Ova structure; this was according to a procedure carried out for bovine serum albumin (BSA) labelling by others (11). Briefly 10 mg C-Ova per milliliter were dissolved in sterile water with an equal weight of K_2CO_3, 2 mg/ml of fluorescein isothiocyanate isomer I (FITC) (Sigma, Switzerland) was added and reacted at 37°C with mild stirring for 24 h in an amber tube. The derived product was dialyzed against sterile distilled water to remove free fluorescein.

Fluorescent Microscopy. To investigate whether uptake and processing activities of CE-MØs were affected, $5x10^5$ofnaivepe-MØs, pe-CE-MØs and naive pe-MØs treated with chloroquine (for the negative control) were separately incubated overnight with 50 μg/ml of quenched C-Ova-FITC incomplete RPMI-1640 medium. On the following day, the medium was replaced with fresh complete RPMI-1640 containing 1μM lyso Tracker Red DND-99 molecular probes (Life Technologies, Carlsbad, CA) as a dye to stain lysosome compartments, with a further incubation for 1.5 h (12). The cells were subsequently fixed for 30 min in 3% buffered paraformaldehyde, washed twice with PBS and resuspended in 20 μl of PBS, before being loaded onto poly-L-lysine-coated glass slides. To visualize the nucleus, cells were reacted for 2 min with 5 μL of 4',6-diamidino-2-phenylindole (DAPI) (Reactolab, Servion, Switzerland) and covered with a

cover slip. Cells were viewed with a Nikon Eclipse E 800 digital confocal fluorescence microscope (Nikon Instruments Inc., Amsterdam, Netherlands). Processing of images was performed using the Open-lab 2.0.7 software (Improvision, Heidelberg, Germany).

Assessment of MØs Processing Activity. An increasing number of naïve pe-MØs and pe-CE-MØs ranging from $1x10^4$ to $1x10^6$ cells were treated or not with chloroquine and plated into 96-well tissue culture plates (Corning Costar, NY). Cells were incubated with a constant concentration (50 µg/ml) of quenched C-Ova-FITC as antigen incomplete RPMI-1640. To assess cells' processing activity, the levels of fluorescence were determined after 24 h of culture by spectrofluorometry (Bio-Tek Instruments, VT), using excitation at 485 nm and emission at 528 nm. Results are expressed as the mean value of fluorescence.

Flowcytometric Analysis. To analyze whether the expression of MHC class II molecules on pe-MØs of naive and CE-infected mice taken at the early and late stages of infection was modified, pe-MØs were washed and resuspended in the following staining buffer: PBS, 0.05% NaN_3 and 0.5% BSA. Aliquots of $5x10^5$ cells/50 µl per well were incubated each with 1 µg of Fc receptor-blocking reagent FcγIII/IIR (CD16/CD32) for 20 min in the dark at 4°C. Blocked cells were incubated for 30 min with 1µg of FITC-anti-I-aβ. The corresponding primary labelledisotype control antibodies were used for staining controls.

To show the level of naïve pe-MØs apoptosis, $5x10^5$ cells were treated separately with 20 µl of Fraction (Fr 35) and 50 µg of HCF in final volume of 200 µl complete RPMI-1640, non-treated cells determine the basic level of apoptosis. For the staining treated or non-treated naïve pe-MØs were directly resuspended in 50 µl of 1X annexin-V binding buffer (BD Pharmingen, Heidelberg, Germany). Subsequently cells were then incubated for 30 min with 1.5 µg of annexin-V–FITC and 1.5 µg of 7-AAD. All antibodies were purchased from (BD Pharmingen, Heidelberg, Germany). Thereafter, cells were washed twice with the staining buffer and resuspended in 500 µL of FACS buffer (0.15 M NaCl, 1 mM NaH2PO4 H2O, 10 mM Na2HPO4 2H2O and 3 mM NaN3). Cells were analyzed by flowcytometer (BD, Heidelberg, Germany) using the corresponding CELL QUEST software (version).

Determination of ProteolyticActivity. Pe-MØs expressing MHC class II (Ia) molecules were isolated from mice injected with 1.5 ml of 3% Brewer thioglycolate medium. After 4 days injected mice developed an intraperitoneal inflammatory response that allowed the harvesting of 10 time pe-MØs (1×10^7) than non-injected mice. These pe-MØs were used to investigate whether HCF and chromatographic fraction (Fr 7) have proteolytic effect. Aliquots of 10^6pe-MØswere incubated separately in PBS plus CaCl2 (1 mM) with 0.1 µg of Fr 7 and (25 and 50µg) of HCF for 2 h at 37°C.

To determine the class of protease(s) we used phenyl-methyl-sulfonyl fluoride (PMSF), E-64, pepstatin, and phenanthroline (Sigma, Switzerland) as inhibitors of the four classes of proteases which are serine, cysteine, aspartic, and metallo-protease, respectively. Aliquots of 10^6 pe-MØs were preincubated with different protease inhibitors at working concentrations for PMSF (1 mM), E-64 (0.02 mM), pepstatin (0.1 mM), and phenanthroline (0.5 mM), during 1 h before adding 25 µg of HCF. The enzymatic reactions were maintained for 2 h at 37°C.

Extraction of Membrane Proteins from Macrophages. To extract membrane proteins, pe-MØs from different cultures were resuspended separately in 330 µl of PBS containing 3 µl of a protease inhibitor cocktail (Thermo Scientific, FL) and 33 µl of Triton-X-114. The mixture was alternatively vortexed and chilled for 5 min before

being sedimented (10,000 xg) for 10 min at 4°C to remove the insoluble residue. The extraction was repeated twice. Pooled supernatants were incubated for 5 min at 37°C to allow the formation of micelles. Water phase and Triton phase containing the membrane proteins were separated after sedimentation at 10,000 xg for 5 min at room temperature as previously reported (13). The water phase was discarded. Membrane proteins in the Triton phase were washed with 1 ml of PBS supplemented with protease inhibitor cocktail (1%) and precipitated using a rapid method based on a defined methanol-chloroform-water mixture as previously described (14). The pellets were dried, resuspended in sample buffer for SDS–PAGE according to the literature (15). Membrane proteins suspension was used to examine whether HCF and chromatographic fraction (Fr 7) have proteolytic effect on MHC class II (I-a) molecules.

Western Blots. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used with a 12% separation gel and a 6% stacking gel under denaturing conditions. Membrane protein fractions from different pe-MØ cultures were heated at 65°C for 20 min before loading on a 12% gel. Proteins were transferred by electrophoresis onto a nitrocellulose sheet. Blots were rinsed in PBS and blocked in PBS 10mM with (0.3% Tween 20 + 5% fat-free milk powder), during 2 h at room temperature. The nitrocellulose membrane was then washed three times and incubated with the first antibody, rat anti-mouse MHC class II (I-A ⁄ I-E) (eBioscience, Paris, France), diluted (1:500) in PBS-Tween 20(0.3%) during 1 h. The membrane was subsequently washed three times and incubated with the secondary antibody, anti-rat-IgG-alkaline phosphatase-conjugate (Sigma-Aldrich, Lezennes, France) for 1 h at a dilution 1:1000 in PBS-Tween 20 (0.3%). To visualize bands, the membrane was incubated in10 mL of substrate buffer [$MgCl_2$ (10 mM) + NaCl (100 mM) + Tris (100 mM)] adjusted to pH 9.5 and supplemented with 66 µl of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 66 µl nitro blue tetrazolium chloride (NBT), prepared, respectively, in 100% and 70% of dimethyl formamide (DMF). To stop the enzymatic reactions, the membrane was transferred into water and then dried on absorbent paper at room temperature.

Statistical Analysis. Results are presented as the mean of triplicate values ± standard deviation (SD).

RESULTS

Hydatid Fluid Inhibited Splenic T Cell Proliferation to Concanavalin A. To elucidate the existence and the activity of suppressor substances contained in HCF of *Echinococcus granulosus* metacestodes, naïve spleen cells were preincubated *in-vitro* with different protein concentrations of HCF as crude antigens ranging between 6.25 and 50 µg/ml. Thereafter cell cultures were stimulated *in-vitro* with Con A. Cell proliferation was determined by BrdU incorporation assay. Results (Figure 1) showed that HCF crude antigens reduced splenic T cells proliferation in response to Con Astimulation. This non-specific immunosuppressive activity was proportional to antigenic doses. The higher reduction (>80%) of cells proliferation was observed with 50 µg/ml of HCF proteins.

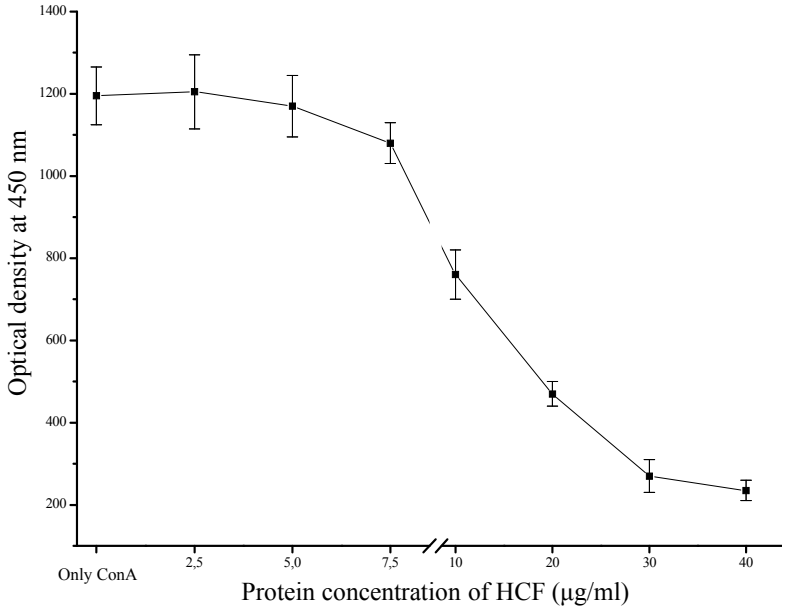

Figure 1. High concentrations of hydatid cystic fluid of *Echinococcus granulosus* metacestodes inhibit naive splenic T cell proliferation in response to Con A. 10^6 of naive spleen cells/well were preincubated in medium containing increasing protein concentrations of HCF and then stimulated *in vitro* with Con A. The optical density registered in different cultures was corrected with the measured absorbance of buffer containing only splenic leucocytes (as negative control). Results are presented as the mean of triplicate cultures of three independent experiments. Error bars refer to standard deviations.

Non-Specific Inhibition of Spleen Cells Proliferation with Chromatographically Defined Fractions of HCF. Spleen cells prepared from naive BALB/c mice responded to a wide range of HCF fractions resulting from a reverse phase chromatography (RPC). Among them, few fractions displayed an inhibitory effect on splenic T cells proliferation. Results (Figure 2B) showed that especially Fr 7, 34 and 35 obtained from RPC triggered the highest suppressive effect on splenic T cells proliferation, while Fr 19 appeared to stimulate the proliferation of splenic cells in response to Con A. From HCF mixture, molecules with stimulative and inhibitory effects might be eluted separately by reverse phase chromatography.

Molecules from HCF of *E. granulosus* Specifically Induce Apoptosis in pe-MØs.The influence of HCF as crude antigen, (Fr 19) and immunosuppressive (Fr 7; Fr 35) fractions on pe-MØs viability was tested. For this, pe-MØs were incubated with different antigens for 48 h and the number of viable cells was assessed by trypan blue exclusion. As shown in Figure 3A, a high reduction in viability of pe-MØs was registered when cells were incubated with HCF and Fr 35 with 40-50% and 30-40% of surviving cells compared to the control, respectively.

To confirm whether apoptotic mechanism induced pe-MØs death, Annexin-V/7-AAD dual staining was performed using necrotic (7AAD+) from apoptotic (Annexin-V+) markers. Pe-MØs were separately treated with HCF as crude antigen and the Fr 35 for 24 h, harvested and processed for staining. As shown in Figure 3B, 1.5 times more pe-MØs underwent apoptosis following incubation with Fr 35 than HCF as crude antigen.

These results indicated apoptosis as the primary mechanism by which HCF and Fr 35 induce pe-MØs death.

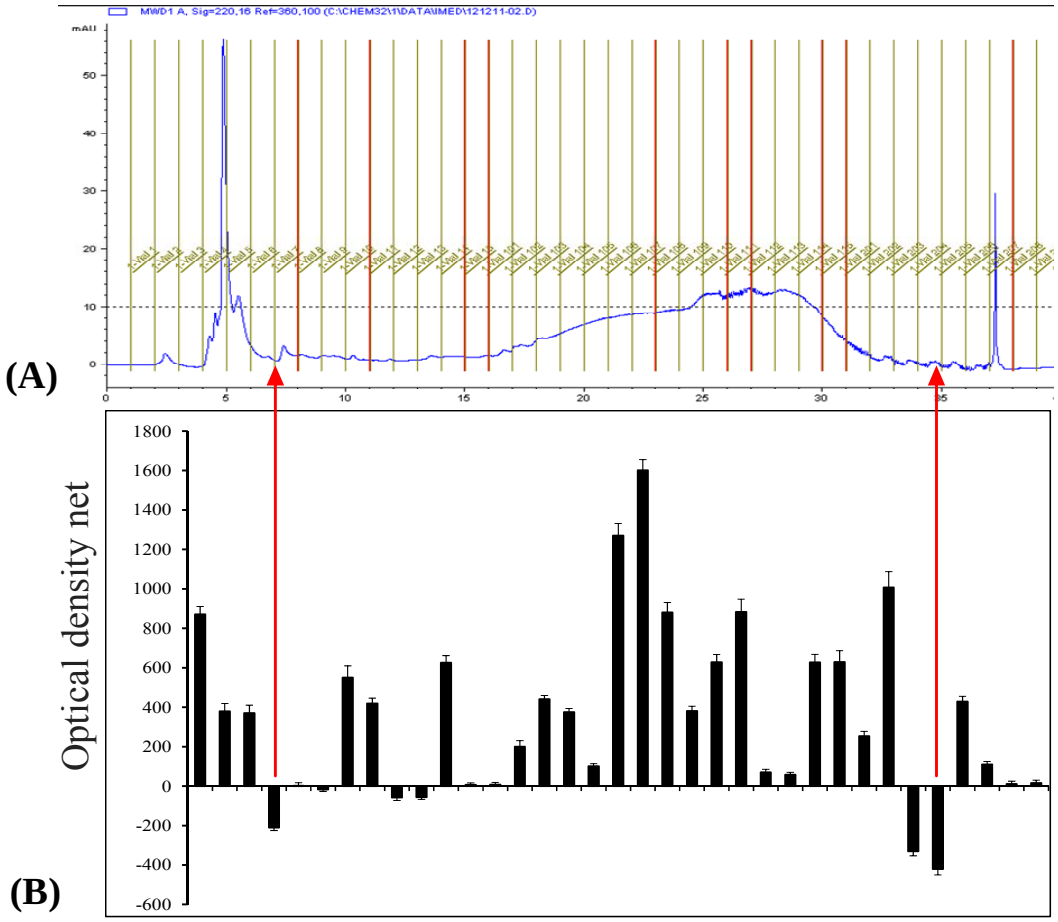

Figure 2. (A) Chromatogram illustrating the elution profile of chromatographic fractions of hydatid cystic fluid from reverse phase chromatography. Molecules of hydatid cystic fluid are bound to the hydrophobic matrix in an aqueous buffer (polar) and eluted in different fractions using a gradient of organic solvent (non-polar). **(B)** Few chromatographic fractions of hydatid cystic fluid (mainly fraction 7 and 35 indicated by arrows) triggered a nonspecific suppressive effect on the proliferation of naive splenic T cells following the stimulation with Con A. The proliferation was quantified by measuring the optical density, (OD) of each culture in the microplate at a wavelength of 450 nm. This OD is proportional to the quantity of bromodeoxyuridine incorporated into proliferating cells. The OD was corrected by the measured absorbance of buffer control. Each OD net value represents the difference between the absorbance registered in each culture with fractions + Con A minus the absorbance in the culture with only Con A (positive control). All results are means of duplicate values ± the difference between these values.

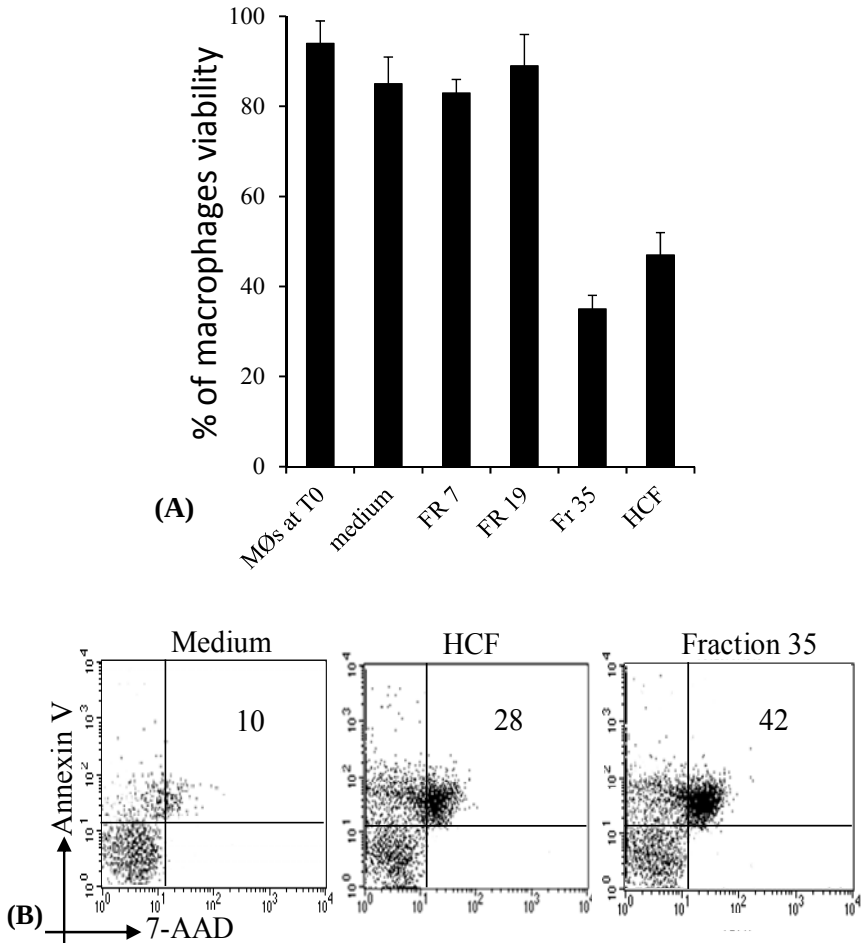

Figure 3. (A) Peritoneal macrophages incubated with chromatographic fractions (Fr 7; Fr 35) (Fr 19) and hydatid cystic fluid (HCF) for 48 h. Cells viability was then assessed using the Trypan Blue exclusion test. The number of surviving peritoneal-macrophages (pe-MØs) is expressed as a percentage of the initial number of pe-MØs plated. As a control, the percentage of viable pe-MØs at the beginning of the experiment is given (t0). Results are the means ± SD of 3 independent experiments. **(B)** Fraction 35 and products contained in HCF of *E. granulosus*induce naive pe-MØs death via apoptosis. Pe-MØs were treated with Fr 35 and HCF and analyzed by flowcytometry for the expression of Annexin-V and 7-AAD. The proportion of apoptotic pe-MØs (AnnexinV+, 7-AAD+) was then determined. Representative plots of the proportion of Annexin V+ 7-AAD+ cells, gated were considered.

CE-MØ Uptake and Processing Potential. To check the processing potential of naive MØs, CE-MØs and naive MØs treated with the chloroquine, respective cells were incubated overnight with quenched C-Ova-FITC. Photos of fluorescence microscopy showed (Figure 4A) the presence of numerous green brilliant endosomes within the cytoplasm of naive MØs and CE-MØs. The emitted green fluorescence is explained by the striped fluorescein moieties linked to the generated peptides following quenched C-Ova-FITC breakdown by naive and CE-MØs processing activity. The overlay of the red fluorescence emitted by lysosome compartments with the green ones emitted by peptides into endosomes revealed a small area of orange color representing a stack consisting of a few endosomes and some of the lysosome compartments. As a control naive MØ treated with the chloroquine, as inhibitor of the processing activity, did not

show any green fluorescence. This finding showed the efficacy of quenching C-Ova with FITC as well.

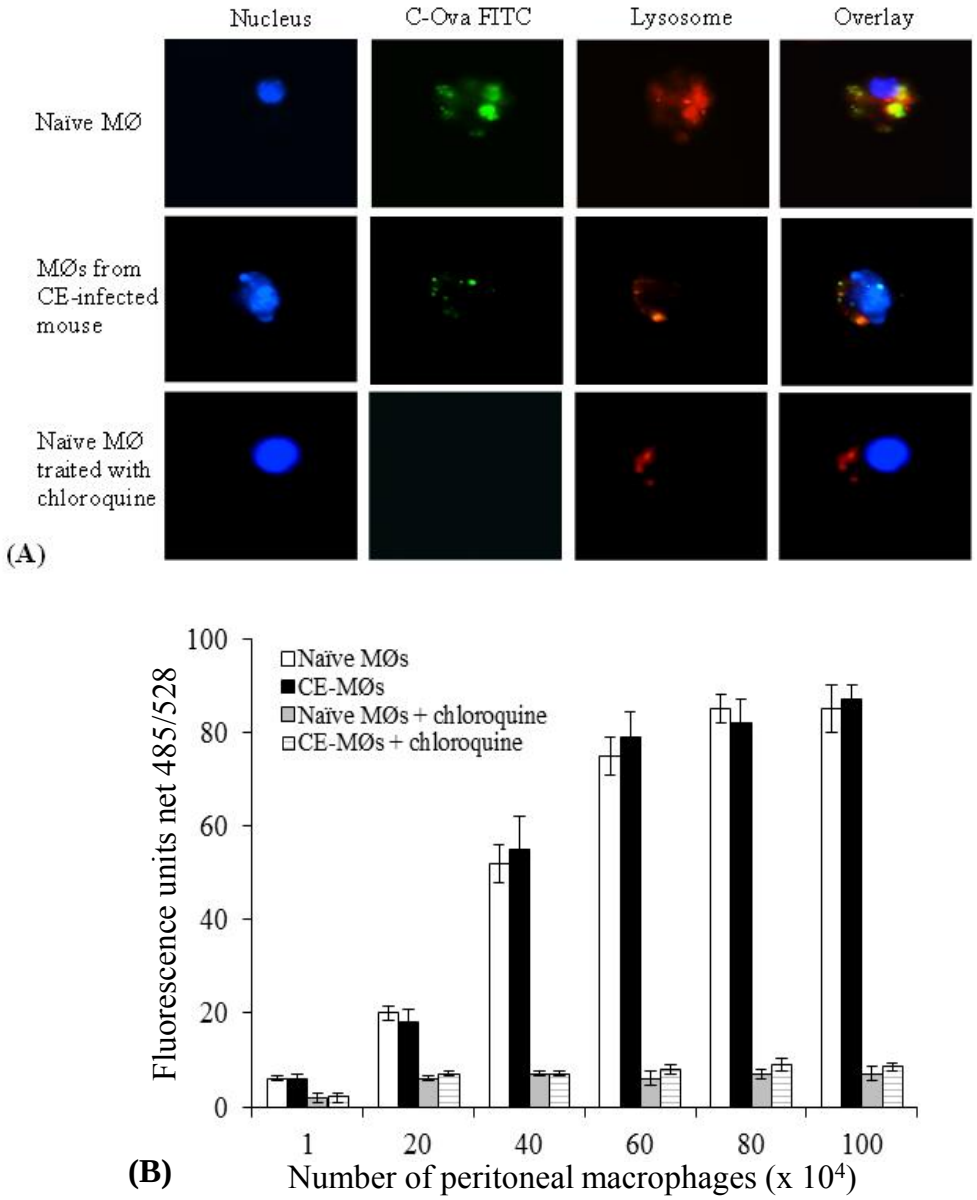

Figure 4. (A) Fluorescence microscopy revealed that peritoneal-macrophages (pe-MØs) isolated from naive and CE-infected mice were able to uptake and process quenched C-Ova-FITC after 24 h of incubation. Processed C-Ova-FITC antigens are represented by the green brilliant endosomes that partially overlaid lysosomes. Naive MØs treated with chloroquine considered as control didn't show green fluorescent antigen. **(B)** The quantification of the processing potential of CE-MØs and naive MØs (positive control) was determined by assessment of emitted fluorescence derived from processed quenched C-Ova-FITC. Both MØ populations enhanced the fluorescence after 24 h of incubation with C-Ova-FITC. Chloroquine inhibited the processing activity of the two populations of pe-MØs. Each value represents the mean of triplicate cultures ± SDs. Similar results were obtained in three independent experiments.

To determine whether the processing activity is due to the quality than to the quantity of MØs, an increasing number of MØs were incubated for 24 h with quenched C-Ova-FITC and assessed for their ability to process antigens. Results (Figure 4B) showed that fluorescence emitted by fluorescent-generated peptides increased with increasing number of naive MØs as well as CE-MØs.

However, the increasing fluorescence reached practically constant level with high numbers of macrophages (more than 7 x 10^5 cells). This finding indicates that rather the quality of MØs had influence on the processing activity. When chloroquine was added at the beginning of cultures, the fluorescence induced by naive MØs as well as CE-MØs remained very low at different doses of cells which provide evidenced the inhibition of cells processing activity.

MHC Class II (I-a) Expression by CE-MØs. In comparison with naive pe-MØs, the flowcytometry analysis revealed an increase in the surface expression of MHC class II (I-a) on CE-pe-MØs from CE-infected mice taken at early phase of infection and a pronounced reduction on CE-pe-MØs isolated from CE-infected mice taken at the chronic phase (Figure 5).

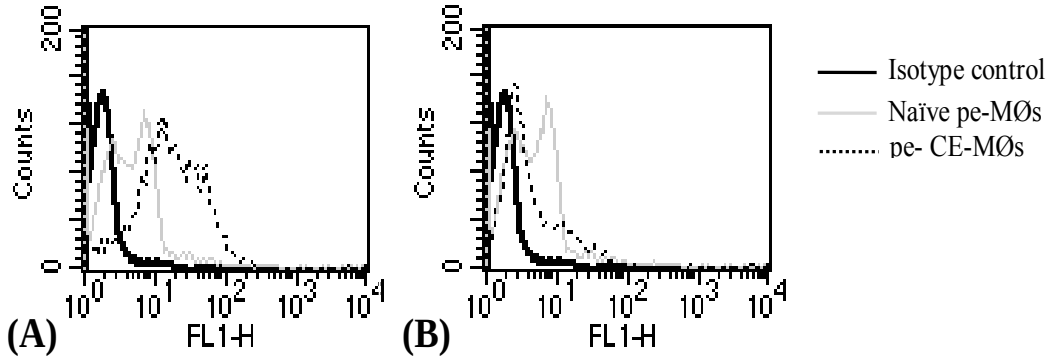

Figure 5. Flowcytometry analysis of MHC class II (I-a) molecules on the surface of peritoneal-macrophages (pe-MØs) isolated from naive mice as a control and pe-CE-MØs from CE-infected mice taken at **(A)** the early stage **(B)** the late (chronic) stage of infection. This experiment was repeated three times with similar results.

Alteration of MHC Class II (I-a) Molecules Expressed on the Surface of pe-MØs. HCF components Fr 7 were investigated for their putative involvement in the reduction of functional MHC class II (I-a) in vitro. Pe-MØs were separately treated with HCF and Fr 7 for 2 h. Isolated membrane-associated proteins were investigated by Western blotting with anti-MHC class II antibodies. Figure 6 showed that banding signal was suppressed with HCF and highly reduced with Fr 7 in comparison with that of non-treated control pe-MØs. These findings suggested that HCF and to a lesser extent Fr 7 modified intact MHC class II (I-a) molecule expressed on the surface of pe-MØs.

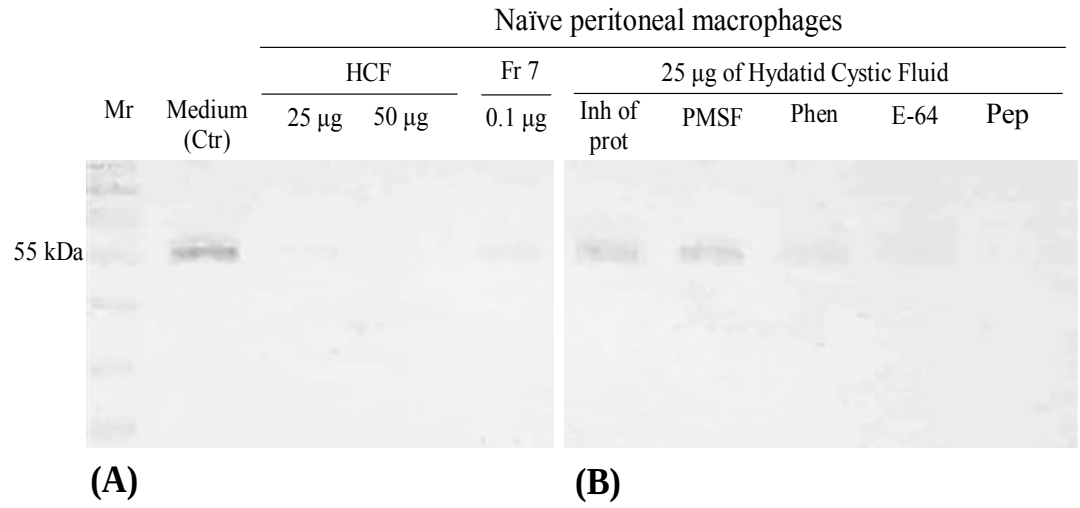

Figure 6. (A) Proteolytic effect of Fr 7 and HCF of *Echinococcus granulosus*on MHC class II (I-a) molecules expressed by naive (pe-MØs). MHC class II (I-a) molecules contained in membrane protein extracts of antigen-treated cells were analysis by Western blotting. Pe-MØs incubated with medium are considered as negative control. Westernblot revealed weak signals of the remaining MHC class II (I-a) molecules following the treatment of naive pe-MØs with either hydatid cystic fluid (HCF) or Fr 7. **(B)** The nature of proteases was determined using selective inhibitor of the four classes of proteases. Reduced signals of I-a molecules were observed following incubation of naive pe-MØs with HCF antigens, pretreated with phenanthroline, E-64 and pepstatin, separately, while signals close to that of the control when the I-a molecules were blotted alone and detected in the presence of PMSF(an inhibitor of serine protease) or mixture of protease inhibitors. Thus the MHC class II (I-a) molecule proteolysis was triggered by a serine protease. Data from one representative Western blotting analysis are shown.

To determinate whether parasitic antigens have a proteolytic effect on MHC class II (I-a) molecule, pe-MØs were preincubated with a mixture of protease inhibitors before adding HCF. Results showed that a practically similar intense signal was detected in comparison the control when inhibitors were omitted in the enzymatic control reaction. In comparison to the control, a similarly intense signal of MHC class II (I-a) molecule was detected when pe-MØs preincubated with PMSF (a serine protease inhibitor) were treated with HCF. The other protease inhibitors (E-64, phenanthroline and pepstatin) did not interfere in the proteolysis of the I-a molecule as illustrated by the faint signals of I-a molecule. Thus, the protease containing HCF and eluted in the chromatographic Fr 7was revealed to belong to the class of serine proteases.

DISCUSSION

For the helminthic parasite *E. granulosus*, the use of a laboratory mouse model to study the host-parasite interplay is highly appropriate, as small rodents act as a natural intermediate host in the life cycle of this pathogen. In the peritoneal cavity of secondary infected BALB/c mice (i.p. inoculation of protoscoleces), the growth of the parasite and the evolution of the disease into its chronic phase strongly evoke the presence of immunomodulatory events orchestrated by the parasite to reduce periparasitic host

effector mechanisms and, thus, to increase the survival potential of the parasite (7). Most likely, these immunomodulatory activities involve metabolically active molecules expressed by the germinal layer of the metacestode and secreted through the laminated layer into the periparasitic environment (16). In our study HCF of *E. granulosus* used as source of antigens contains excreted/secreted (E/S) molecules that may regulate the immune system by modifying their function. In a preliminary experiment we found that high concentrations of HCF provoke non-specific inhibitory effects on Con A-driven proliferation of splenic T cells. So far a total of 32 proteins included in HCF and coming from the E/S products of the germinal layer or protoscoleces of *E. granulosus*have been identified by the proteomic analysis (17,1,18). Among them few molecules have been described for their immunoregulatory activity, thus contributing to the establishment of the chronic infection. In this study chromatographic fractionation was designed to separate HCF bioactive molecules. We have a special interest for fractions with inhibitory effects on leucocytes proliferation. In term of immunology the proliferation is one of the most important features of the immune response. Results (Figure 2) showed that there are three categories of fractions. Fr 21 and 22 were able to enhance Con A-driven proliferation of splenic cells while Fr 7, 34 and 35 depressed profoundly the proliferative response of cells to Con A stimulation. However the major part of fractions did not interfere with the non-specific proliferation of splenic cells. Our finding revealed the presence of stimulative and suppressive antagonist molecules in HCF mixture of antigens. We found that the low concentrations of HCF did not influence on the proliferation of splenic cells stimulated with Con A while higher concentrations (more than 10 µg/ml) decreased progressively cells proliferation (Figure 1). This finding displayed the resulting effect of competing antigens in HCF taken as crude antigen, moreover, at high concentrations inhibitory molecules prevail over those that arestimulative. *In vivo*, the high concentrations of parasitic antigens reached at the end of the chronic phase of infection explained the deep suppression of the immune system, especially, the activation of T cells. In accordance with this statement a general immunosuppression affecting both mitogenic and parasite-specific T-cell responses at the end of the infection was observed in a close model (mice/*E. multilocularis*) (19). This issue has been demonstrated in many models of parasitosis too far from each other (10). Macrophages from secondary infected mice with *E.multilocularis* produce nitric oxide (NO) that mediates immunosuppression at early and late stages of infection (20). It had been indicated that *E.multilocularis*-492 (Em492) an antigen with protein/carbohydrate ratio of 0.25 activated macrophages to produce increased levels of nitric oxide (NO). This antigen markedly depressed proliferative responses of splenocyte upon mitogenic (ConA) or antigenic (Em crude antigen) stimulation. Thus, elevated levels of NO could be responsible for the cellular immune suppression (21)**.** To deeply investigate the regulatory mechanisms triggered by the most inhibitory chromatographic fractions Fr 7 and 35, we used peritoneal macrophages to examine whether these fractions affect their viability and activity as antigen presenting cells. Pe-MØs represent the major population of cells that may interact with parasite in the peritoneal cavity of intraperitoneally infected mice. Results (Figure 3) showed that Fr 35 and to a lesser extent HCF reduce the viability of pe-MØs. This finding was confirmed by the increasing expression of Annexin-V (as a marker of apoptosis) into pe-MØs treated separately by HCF and Fr 35 in comparison with untreated pe-MØs. Inline with these results treatment of human lymphocytes with HCF from fertile cysts leads to the activation of caspace-3 and Bax (proapoptotic protein) pathways (22,23). It

has also been demonstrated that E/S-products of *E. multilocularis* larvae induce apoptosis in DC (24).

Concerning the antigen presenting activity of viable macrophages, one has to first evaluate the ability of pe-MØs isolated from CE-infected mice to uptake and break antigens as a first step in the process of antigen presenting activity. We found that CE-MØs incubated overnight with quenched C-Ova labelled with FITC, were able as naive pe-MØs (positive control) to uptake and process antigens (Figure 4). Green brilliant color of the endosomes within the CE-MØs revealed the proteolytic digestion of quenched C-Ova-FITC within the phagolysosomal compartment. Pe-MØs treated with the chloroquine as inhibitor of processing did not show any green fluorescence which indicates the failure of processing activity and the accuracy of trails. The decomposition of quenched C-Ova-FITC into fluorescent peptides by CE-MØs was not only evidenced by imaging procedures, but also by the quantification of fluorescent moieties assessed by spectrofluorimetry as a function of varying number of antigen presenting cells (APCs). The highest values of fluorescence were registered after 24 h of incubation with CE-MØs. The treatment of cultures with the chloroquine (as negative control) maintained the fluorescence at low and basic values. Similarly it had been showed that alveolar echinococcosis (AE)-MØs as well as naive MØs (positive control) were able to uptake and process C-Ova-FITC (10). Subsequent question concerned the capacity of peritoneal CE-MØs to present processed antigens in the context of MHC-peptide complex. Flowcytometry analysis showed in (Figure 5) an upregulation in the expression of MHC class II (I-a) molecules on CE-MØs taken from the early stage of infection and a down-regulation of these molecules in the level of CE-MØs isolated from the late (chronic) stage of infection. In the early stage of infection immunogenic peptides, generated by peritoneal CE-MØs, are expected to be presented in association with MHC class II molecules (I-a) to responding lymphocytes. However late at the chronic and end phase the drastic reduction of MHC class II (I-a) molecules expression makes CE-MØs unable to provide the first signal which hampered their function as APCs. In accordance with this finding alveolar echinococcosis-pe-DCs had a weaker surface expression of MHC class II (I-a) molecules as compared to naive pe-DCs. The MHC class II (I-a) molecules synthesis and formation of MHC class II (I-a)-peptide complexes were down-regulated (12). Herein CE-MØs may play other roles. AgB and particularly its predominant subunit Eg-AgB8/1induce an anti-inflammatory phenotype in macrophages (25). It has been demonstrated that AgB, hydatid fluid antigens and protoscoleces could increase serum level of TGF-ß in mice. However, protoscoleces could only induce IL-10 (26). Previously we demonstrated that DCs isolated from *E. multilocularis*infected mice express a high level of TGF-ß (13). It has been described that TGF-ß attenuates CIITA gene expression and consequently inhibits HLA-DRA expression (27). We speculate here that the suppression of MHC class II (I-a) molecules expression is due, at least partially, to the high amount of TGF-ß secreted at the end phase of the CE-infection. Results (Figure 6) revealed another way used by the parasite to affect the expression of MHC class II (I-a) molecules. Western blot analysis of (I-a) molecules contained in membrane protein extracts of naive pe-MØs treated with parasitic antigens (HCF and Fr 7) displayed a weak signal (band) in both cases. Signals with a close intensity to that obtained from untreated naive pe-MØs considered as control were detected when pe-MØs were preincubated separately with a mixture of protease inhibitors and PMSF before adding HCF. Thus, HCF may contain proteases that would alter the structure of MHC class II molecules (I-a) expressed by pe-MØs.

The proteolytic molecule was determined as serine protease. This additional proteolytic effect may explain the practical absence of (I-a) molecules on pe-MØs isolate at the late stage of CE-infection as showed by flowcytometry analysis.
In conclusion, at the chronic phase the high level of compounds released by the large parasitic mass *in vivo* conditioned the surrounding environment which affects the viability of pe-MØs and their capacity of antigen presenting. Such suppressive events represent additional evasive mechanisms ensuring the survival of the parasite. Better molecular characterization of HCF factors may be very useful in the design of new treatment strategies, not only for echinococcosis but also for organ transplantations.

ACKNOWLEDGEMENTS

This project was financially supported by the budget (Ref N° LR16CNSTN01) of Radiopharmaceutical Unit, National Centre of Sciences and Nuclear Technology, SidiThabet, Tunisia. We would like to thank the veterinary in the slaughterhouse of Mateur, and the staff of the laboratory of radiopharmacy, Sidi Thabet, Tunisia for their assistance.

REFERENCES

1. Aziz A, Zhang W, Li J, Loukas A, McManus DP, Mulvenna J. Proteomic characterization of *Echinococcusgranulosus*hydatid cyst fluid from sheep, cattle and humans. J Proteomics. 2011; 74:1560-1572.
2. Eckert J, Deplazes P. Biological, Epidemiological, and Clinical Aspects of Echinococcosis, a Zoonosis of Increasing Concern. ClinMicrobiol Rev. 2004; 17:107-135.
3. Carmena D, Cardona GA. Canine echinococcosis: Global epidemiology and genotypic diversity. Acta Trop. 2013; 128:441-460.
4. Chaâbane-Banaoues R, Oudni-M'rad M, Cabaret J, M'rad S, Mezhoud H, Babba H. Infection of dogs with *Echinococcusgranulosus*: causes and consequences in an hyperendemic area. Parasit Vectors. 2015; 8:231-240.
5. Gottstein B, Hemphill A. *Echinococcusmultilocularis*: the parasite-host interplay. ExpParasitol. 2008; 119:447-52.
6. Riganò R, Profumo E, Bruschi F,Carulli G, Azzarà A, Ioppolo S, et al. Modulation of Human Immune Response by *Echinococcusgranulosus* Antigen B and Its Possible Role in Evading Host Defenses. Infect Immun. 2001; 69:288-296.
7. Mejri N, Hemphill A, Gottstein B. Triggering and modulation of the host-parasite interplay by *Echinococcusmultilocularis*: a review. Parasitology.2010; 137:557-568.
8. Oudni-M'rad M,Cabaret J,M'rad S,Bouzid W,Mekki M,Belguith M,et al.Genetic differences between Tunisian camel and sheep strains of the cestode*Echinococcusgranulosus* revealed by SSCP. Parasite.2006; 13:131-136.
9. Baz A, Hernández A, Dematteis S, Carol H, Nieto A. Idiotypic modulation of the antibody response to *Echinococcusgranulosus* antigens. Immunology.1995; 84:350-354.
10. Mejri N, Rutti B, Brossard M. Immunosuppressive effects of *Ixodesricinus* tick saliva or salivary gland extracts on innate and acquired immune response of BALB/c mice. Parasitol Res. 2002; 88:192-197.
11. Mejri N, Gottstein B. Intraperitoneal*Echinococcusmultilocularis* infection in C57BL/6 mice affects CD40 and B7 costimulator expression on peritoneal macrophages and impairs peritoneal T cell activation. Parasite Immunol.2006; 28:373-385.
12. Belkhiri A, Lytvyn V, Guilbault C, Bourget L, Massie B, Nägler DK, et al. A noninvasive cell-based assay for monitoring proteolytic activity within a specific subcellular compartment. Anal Biochem. 2002; 306:237-246.

13. Mejri N, Müller J, Gottstein B. Intraperitoneal murine *Echinococcusmultilocularis* infection induces differentiation of TGF-β-expressing DCs that remain immature. Parasite Immunol.2011; 33:471-482.
14. Wessel D, Flügge UI. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal Biochem.1984; 138:141-143.
15. Laemmli U K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature.1970; 227:680-685.
16. Gottstein B, Hemphill A. Immunopathology of *echinococcosis*. ChemImmunol. 1997; 66:177-208.
17. Monteiro KM, de Carvalho MO, Zaha A, Ferreira HB. Proteomic analysis of the *Echinococcusgranulosus*metacestode during infection of its intermediate host. Proteomics. 2010; 10:1985-1999.
18. Virginio VG, Monteiro KM, Drumond F, de Carvalho MO, Vargas DM, Zaha A, et al. Excretory/secretory products from in vitro-cultured *Echinococcusgranulosus*protoscoleces. MolBiochemParasitol. 2012; 183:15-22.
19. Emery I, Liance M, Deriaud E, Vuitton DA, Houin R, Leclerc C. Characterization of T-cell immune responses of *Echinococcusmultilocularis*-infected C57BL/6J mice. Parasite Immunol. 1996; 18:463-72.
20. Dai WJ,Waldvogel A,Jungi T,Stettler M,Gottstein B. Inducible nitric oxide synthasedeficiencyinmiceincreasesresistancetochronicinfectionwith*Echinococcusmultilocularis*. Immunology.2003; 108:238-244.
21. Mirjam Walker,Adriana Baz,Sylvia Dematteis, Marianne Stettler,Bruno Gottstein,Johann Schaller, et al. Isolation and Characterization of a Secretory Component of*Echinococcusmultilocularis*Metacestodes Potentially Involved in Modulating the Host-Parasite Interface. Infect Immun. 2004; 72:527-536.
22. MokhtariAmirmajdi M, Sankian M, EftekharzadehMashhadi I, Varasteh A, Vahedi F, Sadrizadeh A, et al. Apoptosis of human lymphocytes after exposure to hydatid fluid. Iran J Parasitol. 2011; 6:9-16.
23. Spotin A, Majdi MM, Sankian M, Varasteh A. The study of apoptotic bifunctional effects in relationship between host and parasite in cystic *echinococcosis*: a new approach to suppression and survival of hydatid cyst. Parasitol Res. 2012; 110:1979-1984.
24. Nono JK, Pletinckx K, Lutz MB, Brehm K. Excretory/secretory-products of *Echinococcusmultilocularis* larvae induce apoptosis and tolerogenic properties in dendritic cells in vitro. PLoSNegl Trop Dis. 2012; 6:e1516.
25. Silva-Álvarez V, Folle AM, Ramos AL, Kitano ES, Iwai LK, Corraliza I, et al. *Echinococcusgranulosus* Antigen B binds to monocytes and macrophages modulating cell response to inflammation. Parasit Vectors. 2016; 9:69-86.
26. Haniloo A, Ghasemi F, Shikhi A, Ghavami MB. Immunoregulatory cytokine (TGF-ß And IL-10) responses in mice inoculated with protoscoleces and major hydatid fluid antigens of cystic *echinococcosis*. Iranian J Parasitol. 2008; 3:18-23.
27. Nandan D, Reiner NE. TGF-beta attenuates the class II transactivator and reveals an accessory pathway of IFN-gamma action. J Immunol. 1997; 158:1095-1101.