RESEARCH ARTICLE

Involvement of IL-10 and IL-4 in evasion strategies of *Echinococcus granulosus* to host immune response

Manel Amri, Dalila Mezioug, Chafia Touil-Boukoffa

Team « Cytokines et NOSynthases », Laboratory of Cellular and Molecular Biology, FSB-USTHB, PB 32, 16111, Algiers, Algeria

Correspondence: Pr C. Touil-Boukoffa, Laboratoire de Biologie Cellulaire et Moléculaire, FSB-USTHB, Université Bab-Ezzouar, PB 32, 16111, Algiers, Algeria
<touilboukoffa@yahoo.fr>

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ABSTRACT. Human echinococcosis is one of the world's major zoonotic infections. In this study, our aim is to clarify one of the strategies used by the parasite for evasion and prolonged infestation in the host. We wished to provide further immunological evidence for the involvement of IL-10/IL-4 in these mechanisms. In this regard, we investigated the effects of IL-4 and IL-10 on protoscoleces (PSC: the larval form of the parasite), co-cultured with patient PBMC. Furthermore, we used IL-4 and IL-10 antibodies to confirm this effect. Our results showed that IL-4 and IL-10 reduced PSC killing. This reduction correlates with an decrease in NO production by PBMC. In conclusion, the results reported here suggest that IL-4/IL-10 impairs the Th1 protective response and allows the parasite to survive in hydatid patients.

Keywords: IL-10, IL-4, human hydatidosis, evasion

Echinococcus granulosus infections are among the most common infections worldwide. There has been significant work characterizing the immune response against this macroparasite [1-5], but little is known regarding the precise mechanisms responsible for the parasite evasion of the host's defense mechanisms. It is important to try to understand the parasite's evasion strategies that allow the its persistence and survival, for several years, despite specific cellular and humoral immune responses being present.

Many later studies have suggested that parasite evasion involves a variety of adaptive strategies that exploit their host's immune response. According to the current view, in parasitic helminth infections, a strong Th2 response correlates with susceptibility to the disease, whereas a Thl response correlates with protective immunity [6]. Th2 cells express IL-4, IL-5, IL- 6 and IL-10, whereas Th1 cells produce IL-2 and IFN-γ. Furthermore, it is widely accepted that Th2 cytokines downregulate Th1-derived cytokines and *vice versa*.

In human hydatid disease, there is ample evidence that shows that Thl- and Th2-type cytokine patterns coexist: the Th2 response benefits the parasite, whereas the Th1 response benefits the host [7, 8]. These have been implicated in the active and inactive stages of hydatid disease [9].

More recently, we have investigated *in vitro* effects of IFN-γ on protoscoleces using PBMC-PSC co-culture. The results of our experiments highlight an obvious role for IFN-γ in protoscoleces killing by iNOS induction [10]. Our aim in this study was to provide further immunological evidence for the involvement of IL-10 and IL-4 in evasion mechanisms adopted by the parasite. We wished to confirm this hypothesis by investigating whether these two cytokines inhibit PSC killing, elicited by iNOS induction in PBMC-PSC co-culture.

PATIENTS, METHODS AND MATERIALS

Patients and normal donors

Twenty blood samples were obtained from Algerian patients with *E. granulosus* infections (30 ± 13 years old, 60% men), before and after surgery (1 week before and 24-72 h after surgical removal). Clinical diagnosis was confirmed surgically in each patient by the presence of cysts in the lung (Department of Surgery, Mustapha Bacha Hospital, Algiers, Algeria). Subjects having secondary infection and other acute or chronic diseases were not included in this study. Ten healthy donors (mean age 25 ± 2 years, 60% women) (from the same region of Algeria) were included in this study. They did not have inflammatory disease or show any sign of infection at the time of blood collection. In addition, none of the subjects had ever received a blood transfusion. No pharmacological treatment was given before blood sampling. All participants gave informed consent for the study, which was carried out in accordance with the guidelines of the local Ethics Working Group. Blood samples collected from healthy donors and patients were used immediately for PBMC isolation.

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PBMC preparation

PBMCs were separated by density gradient centrifugation as previously described by Touil-boukoffa *et al.* [2]. Briefly, after centrifugation, the mononuclear fraction was collected and washed. Cell viability was checked using 0.2% trypan blue dye exclusion and was always > 98%. Freshly isolated PBMCs were resuspended at a final concentration of 10^6 viable cells/mL in RPMI 1640 culture medium supplemented with antibiotics and 10% heat-inactivated foetal bovine serum (FBS) (Sigma). Cells were used immediately for co-culture.

Protoscoleces collection

E. granulosus protoscoleces (PSC) were obtained by aseptic puncture of fertile, human, pulmonary hydatid cysts. PSC were stored at 4°C in complete culture medium.

PBMC and PSC co-culture activation

PBMCs from healthy donors or hydatid patients were co-cultured with intact *E. granulosus* PSC in supplemented RPMI 1640. Co-cultures were stimulated with human recombinant IL-10 or IL-4 (20 ng/mL) alone or with neutralizing antibodies anti-HuIL-10 or anti-HuIL-4 (20 µg/mL) (Peprotech, EC, Ltd., London, UK). In all cases, cultures without PSC and co-cultures without cytokines (or anti-cytokines) were included as negative controls. Cultures were incubated for 20 h at 37°C in a humidified atmosphere with 5% CO_2. At the end of the culture period, the medium of each culture was collected for NO determination and PSC viability test.

NO production measurement in co-culture supernatants

iNOS activity was assessed by measuring the total nitrite (NOx) (nitrite: NO_2^- + nitrate: NO_3) concentration in each cell culture supernatant as described by Touil-Boukoffa *et al.* [2]. Briefly, after nitrate reduction using nitrate reductase from *Pseudomonas oleovorans* (ATCC 8062), the NOx concentration was measured by spectrophotometry at 543 nm after reaction with the Griess reagent.

PSC viability assay

Before and after each co-culture, PSC viability was assayed microscopically using 0.1% eosin staining. It was based on PSC staining, motility, and morphological criteria as described by Amri *et al.* [10]. Live PSCs remained unstained, with conserved membrane integrity, order of hooks, and homogeneity of calcareous corpuscles. There were contractile movements of the membrane and suckers. Dead PSCs took on a reddish color, and the morphological appearance was disturbed. To perform this study, all samples had a viability > 97% at the time of their use.

Statistical analysis

All results were expressed as mean ± standard deviation (SD). Data analysis was performed using the SYSTAT 12. Student's *t*-test was used for comparison between different groups (stimulated/unstimulated cells). Differences were considered to be statistically significant at $p < 0.05$.

RESULTS

Effects of IL-10 and IL-4 on protoscoleces viability - correlation with NO production

In an attempt to determine the effect of IL-10 and IL-4 on PSC viability, we stimulated PBMC-PSC co-cultures with HuIL-10 or HuIL-4. Our results indicate that the percentage of PSC viability is higher when PBMC (before surgery) had been incubated with IL-10 (69.58 ± 5.05%) or IL-4 (67.07 ± 7.35%) than in unstimulated PBMC (without cytokines, 56.33 ± 6.26%) *(figure 1A)*. Interestingly, NOx levels evaluated in the same supernatants, show a marked decrease from 132.8 ± 8.31 µM to 80.22 ± 5.19 µM and 85.85 ± 10.63 µM in the presence of IL-10 and IL-4 respectively *(figure 2A)*.

In co-cultures performed with PBMC from post-surgical patients, similar findings are observed after stimulation with IL-10 (78.53 ± 7.11% *versus* 67 ± 6.59%, and 49.33 ± 11.58 µM *versus* 118 ± 10.95 µM) or IL-4 (76.4 ± 5.97% *versus* 67 ± 6.59%, and 40.22 ± 11.4 µM *versus* 118 ± 10.95 µM) *(figures 1B, 2B)*.

The same results are also observed for PBMC prepared from healthy donors and stimulated with IL-10 (86.83 ± 4.71% *versus* 76.49 ± 0.82%, and 26.75 ± 5.09 µM *versus* 68 ± 13.04 µM) or IL-4 (83.59 ± 5.64% *versus* 76.49 ± 0.82%, and 28.25 ± 7.81 µM *versus* 68 ± 13.04 µM) *(figures 1C, 2C)*.

Effects of antibodies anti-IL-10 and anti-IL-4 on protoscoleces viability - correlation with NO production

After addition of anti-IL-10 or anti-IL-4, we observed with interest a decrease in the percentage of live PSC (from 56.33 ± 6.26% to 31.38 ± 4.68% and 39.94 ± 4.5% respectively) in co-culture performed with patient PBMC before surgery *(figure 1A)*. In comparison with control co-cultures (without cytokines or corresponding antibodies) *(figures 3A, B)*, we show that the addition of neutralizing antibodies anti-IL-10 induces a marked morphological change and degeneration *(figures 3C, D, E)*. This effect correlates with an increase in NO production by the same cells after neutralization by anti-IL-10 (from 132.8 ± 8.31 µM to 148 ± 10.59 µM) or anti-IL-4 (from 132.8 ± 8.31 µM to 147 ± 18.28 µM) *(figure 2A)*.

The same results are also observed in co-cultures performed with PBMC from post-surgical patients *(figures 1B, 2B)* and healthy donors *(figures 1C, 2C)*.

DISCUSSION

Implication of IL-10 and IL-4 in parasite protection

The results reported here suggest that IL-4 and IL-10 impair the Th1 protective response and allow the parasite to survive in hydatid patients. Based on our results, inhibition of NO production by PBMC is involved in PSC protection. These findings are in agreement with our previous studies related to a relevant role of NO in the host defense against hydatid infection [2, 11, 10]. This

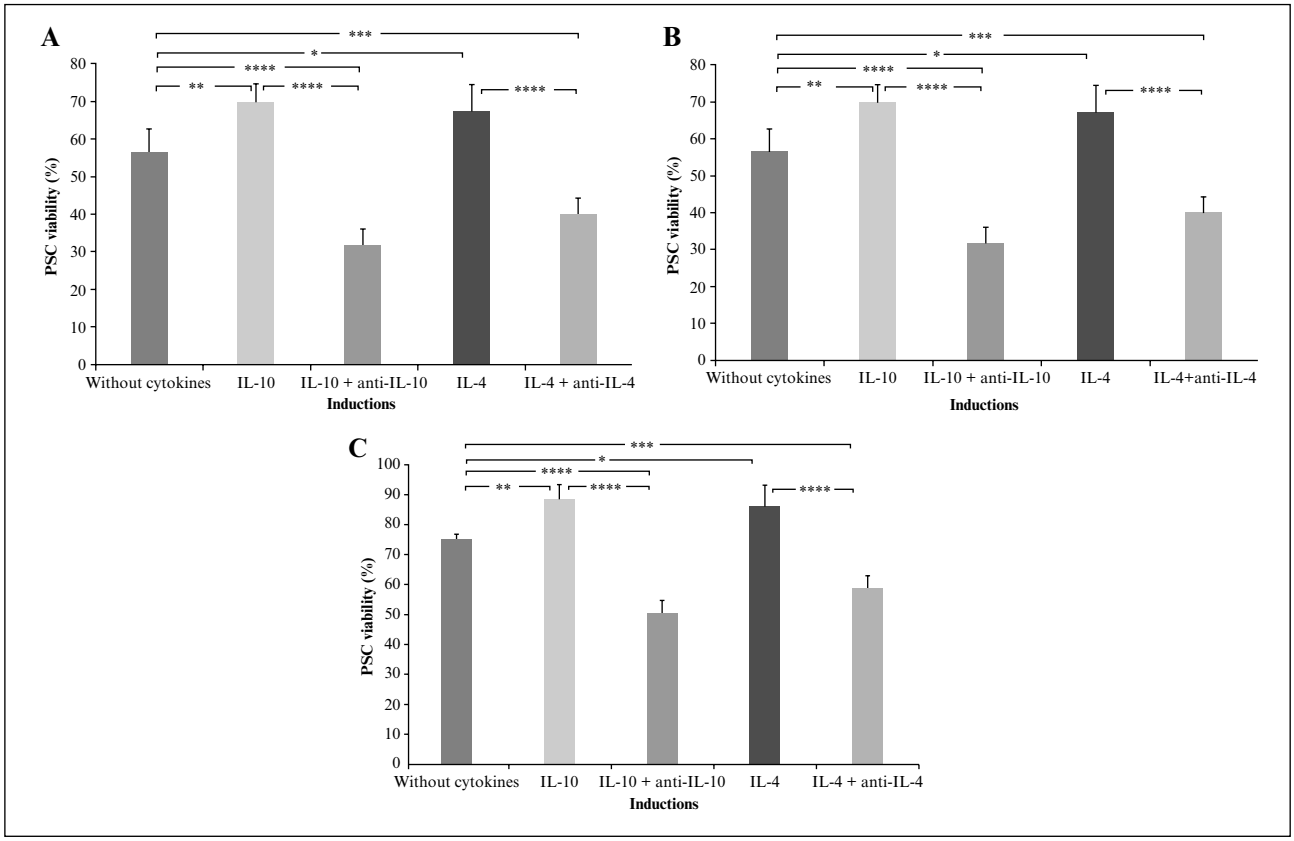

Figure 1

Effect of IL-10, IL-4 and their antibodies on protoscoleces (PSC) viability (%) in PSC-PBMC cocultures. PBMC from hydatid patients before (**A**) and after (**B**) surgery, and healthy donors (**C**) were cocultured with intact *E. granulosus* PSC. Cocultures were stimulated by HuIL-10 or HuIL-4 alone or with neutralizing antibodies Anti-HuIL-10 or Anti-HuIL-4. Cocultures without cytokines are included as negative controls. After 20 h, percentage of PSC viability was evaluated in each coculture. All results are expressed as mean ± SD. Significant differences between two groups (*e.g.* without cytokines/with IL-10) are indicated (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; and **** $p < 0.0001$).

conclusion is supported by our previous experiments using IFN-γ and NOS inhibitor in PBMC-PSC co-cultures. We have shown that IFN-γ-mediated iNOS induction is one of the host defense mechanism against human *E. granulosus* infection [10]. Of note, it is widely accepted that IL-4/IL-10 cytokines downregulate Th1-derived cytokines [12].

The results presented here reinforce previous notions that disease progression is associated with IL-4/IL10 production, whereas the disease control is associated with Th1 responses. In previous *in vivo* studies, IL-4 and IL-10 were found to be dominant in serum samples from *Echinococcus granulosus*-infected individuals before treatment. After treatment with albendazole, those patients who responded to therapy showed a decrease in IL-4 and IL-10 levels, whereas those patients who did not respond continued to have high IL-4 and IL-10 levels [7, 8]. Furthermore, in relapsing hydatid cases, IFN-γ, as well as nitric oxide, were absent [2, 11]. Moreover, the *in vitro* experiments showed that in the patients who responded to pharmacological treatment, high IFN-γ production is associated with a lack of IL-4 and low IL-10 production by PBMC. Conversely, high IL-4 and IL-10 production are associated with lack of or low IFN-γ production in the non-responders [7, 8]. Our results are in the line with these studies. These findings suggest that in human hydatidosis, high IL-4/IL-10 production is related to the failure of therapy.

More recently, investigations have showed that the cell lines from patient with an inactive cyst had a predominantly Th1 profile. Conversely, the T cell lines derived from patients with active and transitional hydatid cysts had mixed Th1/Th2 and Th0 clones, thus providing evidence that Th1/Th2 cytokines are implicated in the inactive and active stages of hydatid disease [9].

In a murine model, previous reports have demonstrated that BALB/c mice are susceptible to *E. granulosus* infection [13]. Cyst development was characterized by the dominance of a Th2 response, whereas a dominant Th1 response was found to be associated with the death and clearance of injected PSC in mice [14]. Furthermore, the pattern of cytokines secreted *in vitro* by splenocytes from infected mice indicated that, as early as week 1, a Th2-type response was stimulated. These results suggest that a Th2-type response would not be able to impair the establishment of *E. granulosus* infection [15].

Many studies have investigated the role of IL-4 and IL-10 in the regulation of immune responses induced by a variety of important human parasite. The results from these studies have clearly identified IL-10 and/or IL-4 as important regulatory cytokines in infections by *Schistosoma mansoni* in mice [16] and humans [17, 18], *Schistosoma haematobium* [19], *Trichuris muris* [20], and *Trichinella spiralis* [21].

Taken together, our results suggest that IL-10/IL-4 production is implicated in parasite evasion of the host

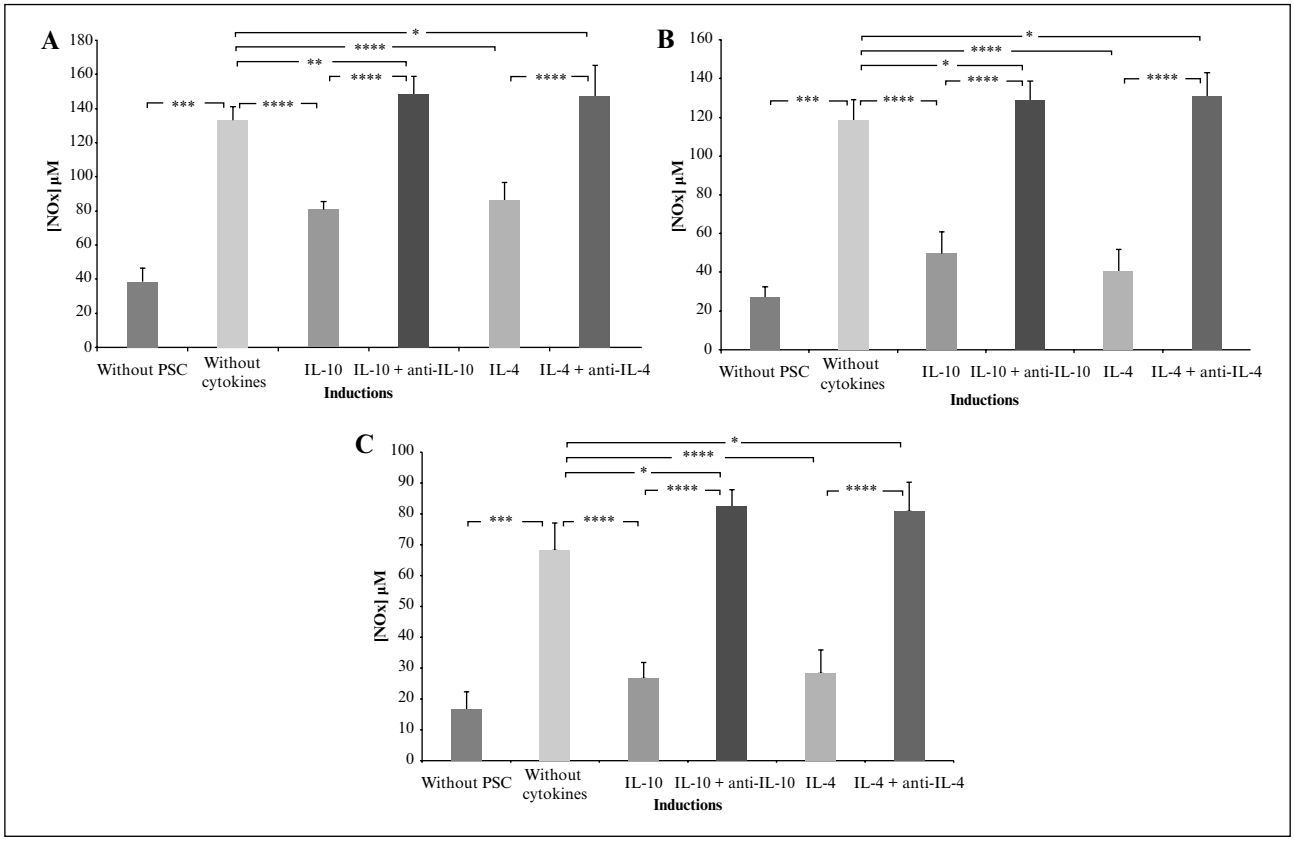

Figure 2

Effect of IL-10, IL-4 and their antibodies on NOx production in PSC-PBMC cocultures. PBMC from hydatid patients before (**A**) and after (**B**) surgery, and healthy donors (**C**) were cocultured with intact *E. granulosus* PSC. Cocultures were stimulated by HuIL-10 or HuIL-4 alone or with neutralizing antibodies Anti-HuIL-10 or Anti-HuIL-4. Cultures without PSC and cocultures without cytokines are included as negative controls. After 20 h, NOx levels were evaluated in each coculture. All results are expressed as mean ± SD. Significant differences between two groups (e.g. without cytokines/with IL-10) are indicated (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; and **** $p < 0.0001$).

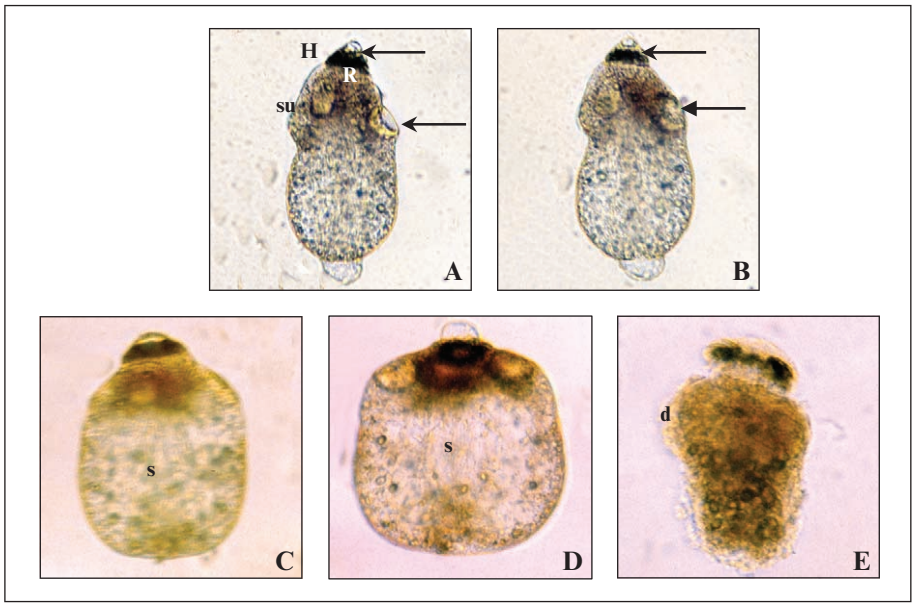

Figure 3

Light microscopy of progressive change in PSC morphology after treatment of coculture with Anti-IL-10. Coculture without cytokines or antibodies (**A-B**), Coculture stimulated with Anti-IL-10 after 10 h (**C**), 15 h (**D**), 20 h (**E**).
(**d**): degeneration; (**H**): hooks; (**s**): swelling; (su): suckers; (R): rostellum; (→): shift. x 269.

immune response. However, more work is required to identify the factors present in the parasite cyst that affect the protective Th1 responses in human, *Echinococcus granulosus* infections. Antigen B, one of the two major antigens present in cyst fluid, seems to be implicated in this effect. Shepherd *et al.* first suggested the role of anti-

gen B in the reduction of the early inflammatory response against parasite [22]. Recently, Rigano *et al.* have confirmed this finding. They showed that antigen B impairs the Th1 protective response by up-regulating IL-4 and IL-13 production in patients with cystic echinococcosis [23]. IL-10/IL4 is thought to act via its downregulatory effects on APC function by inhibition of MHCII and B7 molecules expression [24, 25]. In addition to depressing APC function, IL-10 affects dendritic cell maturation, thereby providing a potential, bimodal feedback inhibition of Th1 cells [26].

Implication of antibodies anti-IL-10 and anti- IL-4 in parasite killing

In addition, our results provide evidence that inhibition of the development of IL-4/IL-10 responses, reduced protoscoleces viability. These findings are similar to those reported by Al-Qaoud *et al.* who stated that injection of mice with the IL-4 gene exacerbates the infection after four weeks. In contrast, transfection of mice with IFN-γ or IL-12 genes yielded up to a 60% protection rate from secondary hydatidosis [27]. These results and our findings suggest that antibodies directed against IL-10 and IL-4 might be useful tools in human hydatidosis treatment. Indeed, more research is required to evaluate the physiological effect of IL-10 and IL-4 antibodies during experimental hydatidosis.

Implication of parasite in NO production

We report here a significant increase in NOx production by human PBMC (from patients before and after surgery, and healthy donors) in response to parasite (PSC) stimulation. These findings are related to our previous studies and confirm that PSC may provide an activation signal, triggering NO induction in PBMC from patients and healthy donors [10].

Moreover, our results provide evidence that levels of NO are higher in co-cultures performed with PBMC from hydatid patients. This increase is in agreement with previous data on the role of NO in host anti-hydatic defense, and underscores the ability of the larval stage of *Echinococcus granulosus* to trigger NO production [2, 11, 10]. Furthermore, the higher NO levels produced by PBMC of pre-surgical patients, support the notion of a relationship between clinical stage and NO induction. This may be explained by the fact that surgical cyst removal reduces the availability of soluble, parasitic antigens. Our findings confirm the involvement of parasitic burden in NOS2 induction [2, 11, 10]. These observations are in line with those reported by Touil-Boukoffa *et al.*, showing that the reduction in NO levels after surgery is associated with the absence of IFN-γ production, which is related to decreased CD4+ T-cells [2].

CONCLUSION

This study highlights the role of IL-10/IL-4 in regulating the host immune responses against *E. granulosus*. These cytokines may play a pivotal role early in the regulation of iNOS-mediated protoscoleces killing by modulating the Th1-type cytokines. This inhibition may reflect a biological adaptation of parasite to limit killing mechanisms during larval development within the human. However, it remains a challenge for us to establish the exact mechanism by which IL-10/IL-4 are produced and act during human *E. granulosus* infection. Interestingly, inhibition of these mechanisms seems to be an important consideration in the design of anti-hydatic treatment.

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