VARIATION IN PD-1/PD-L1 PROTEIN EXPRESSION IN LIVER AND SPLEEN AFTER TREATMENT OF CYSTIC ECHINOCOCOSIS: AN EXPERIMENTAL STUDY

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ABSTRACT

The stimulatory signaling molecules programmed death-1 and its ligand (PD-L1) is associated with immunoregulatory and suppressive immune effector mechanisms. The aim of this study is to evaluate the liver and spleen tissue expression of PD-1 and PD-L1 after treatment with Oxfendazole (OXF), Oxfendazole + Praziquantel (OXF+PZQ), Oxfendazole + Albendazole (OXF+ABZ) and Albendazole + Praziquantel (ABZ+PZQ) of experimentally mice model of cystic echinococcosis. After mice model induction of hydatid disease, four months of treatment were initiated and both liver and spleen were processed for immunohistochemical staining of PD-1 and PD-L1 proteins. The results showed that higher expression of PD-1 and PD-L1 proteins in positive control group and statistically reduced expression in treated groups suggesting an involvement of PD-1/PD-L1 signaling pathway in the pathogenesis of cystic echinococcosis.

Keywords: PD-1/PD-L1, Immunohistochemistry and cystic echinococcosis


INTRODUCTION

Cystic echinococcosis (CE) is one of the most chronic helminthic wide spread zoonotic disease in many countries caused by metacestode of Echinococcus granulosus⁴. CE can persist chronically for years unless treated successfully. The treatment strategies lied on surgical removal followed by treatment with broad spectrum anthelmintic “albendazole” or Mebendazole which act as parasitostatic rather than parasiticidal²,³. Studies have been characterize the immunological profile in the lesion between cyst and host tissue like: liver and spleen of mice ⁴, or human ⁵. Indicating the chronic behavior of local reaction emphasizing
immunoregulatory capabilities of helminthic infections, it has been suggested that Th2 responses play a crucial role in chronic helminthiasis\(^6\). However, a remarkable feature of chronic CE infection is the coexistence of IFN-gamma, IL-4 and IL-10 at high levels in human echinococcosis\(^7\). It is unclear why hydatid infection can induce high levels of both Th1 and Th2 cytokines\(^8\) since they usually downregulate each other. Antigen and the amount of antigens released may play key roles. For instance, *E. granulosus* antigen B skewed Th1/Th2 cytokine ratios towards a preferentially immunopathology-associated Th2 polarization, predominantly in patients with progressive disease\(^9\). Limited studies have been described the profile of inhibitory signals including Programed cell death 1 (PD-1) and programmed death ligand (PD-L1). The interaction act as a negative regulator of immune responses\(^10\). Studies have shown its important to maintain a balance between peripheral tolerance and autoimmunity, it also impairs viral\(^11\) and tumor immunity\(^12\), promoting chronic infection and tumor progression\(^13\). However, serum level of soluble PD-1 and soluble PD-L1 have been evaluated in patients with cystic echinococcosis\(^14\). They act as regulatory molecules for PD-1/PD-L1 interaction suggesting they create a dynamic balance in immune response to parasite. A recent study showed that PD-1/PD-L1 pathway blockade could possibly promote a curative immune response against AE through tipping the Th1/Th2 balance and through inhibition of T-reg cells\(^15\). The current study aimed to investigate the variation in PD-1 and PD-L1 protein expression in liver and spleen of mouse model of cystic echinococcosis under treatment with different modalities of anthelminthic drugs.

**MATERIAL AND METHODS**

**Experimental design**

This in vivo mouse model experiment for cystic echinococcosis was approved by College of Pure Sciences (Ibn AL-Haitham) Baghdad University. This experiment was done in Animal housing of the college. Balb/C male mice were housed inside cages according to the instructions. Collection of hydatid cysts samples and isolation of protoscolices: Hydatid cysts from lungs and livers of infected sheep carcasses collected in AL-Sadir slaughter in Baghdad. These cysts were stored and processed to separate the protoscoleses from the fluid\(^16\).

**Preparation and administrations of drugs**

Three drugs were used in this study, Albendazole (ABZ) 10mg/kg of body weight (BW), equivalent to 0.01 mg/ml, Praziquantel (PZQ) 40mg/kg of BW, equivalent to 0.06 mg/ml and Oxfendazole (OXF) 30 mg/kg of BW, equivalent to 0.04mg/ml was obtained on the property locally. These drugs were dissolved in distilled water and stored at 4°C for no more than 24 hours. Drugs were used in single form or combination with others as the following:

- OXF treated group,
- OXF+PZQ treated group,
- OXF+ABZ treated group and
- ABZ+PZQ treated group.

Additionally, not infected group served as negative control (NC) group while none treated group served as positive control group (PC). All drugs were given after four months of experimental infection with available protoscolices (2000±5 / mice) accordingly to the group of study as single dose orally at early morning for 60 days.
Immunohistochemistry staining of PD-1 / PD-L1 protein in liver and spleen sections

Animals were sacrificed by cervical dislocation, then examined their internal organs (such as Liver, spleen, lung, stomach intestine and etc.) of mice were removed under sterile conditions by abdominal incision. Both liver and spleen were washed in distilled water and stored in 70% ethanol and processed as paraffin embedded tissue blocks.

Sections of 5 micrometer were obtained and mounted on positive charge glass slides. Before labeling, sections were deparaffinized in xylene and rehydration by graded series of ethanol baths. Endogenous peroxidase activity was blocked with peroxidase block. After washing, sections were then incubated in protein block for 20 minutes at room temperature.

About 100 microliters of diluted primary antibody against PD-1 (orb13641) or PD-L1 (orb228661) was added on tissue section for 60 minutes. After washing, visualization steps achieved by Rabbit IgG SABC Kit (orb90444) using DAB Chromogen Kit (orb219876) and counterstained by hematoxylin.

Data analysis

Results of IHC test were evaluated by applying a semi-quantitative assessment: each slide was counted under light microscope for three times at x400 magnification and about 5 fields were randomly selected in each round. Thus, the number of immunolabeled cells was counted in 5 fields under a fixed focus for each slide and value of mean for positive count in total number of all counted cells (each tissue section) for each sample group. Data were expressed as mean ± standard deviation (SD) and Analysis of Variance (ANOVA) test was used for differences between groups. Values p≤ 0.05 was regarded as statistically significant.

RESULTS

The current work involved an evaluation of PD-1/PD-L1 protein expression in liver and spleen tissue sections in mice experimentally infected with *E. granulosus* then treated with different drugs.

Liver and spleen PD-1 and PD-L1 were down regulated after treatment:

The results in table 1 showed that, PD-1 protein expression in negative control (normal liver tissue) was 5.8% while highly statistical increased expression in infected group (PC) 36.7%. However, significant reduction was seen in OXF treated group (12.4%) than PC group, similarly in OXF+PZQ treated group 22.8%, OXF+ABZ treated group 16.8% and ABZ+PZQ treated group 19.64%. PD-L1 protein was 34.33% in NC (none treated group) and 45.38 % found in (PC) infected group, a highly statistical significant reduction in the mean value of PD-L1 after treatment with OXF (13.2%), OXF+PZQ treated group (30.2%), OXF+ABZ treated group (34.3%) and ABZ+PZQ treated group (28.12%).
These results strongly suggesting that restoration of inflammatory processes after reduction of PD-1/PD-L1 axis after treatment.

Table 1 showed that there were statistical significant reduction of PD-1 expression in spleen treated groups than those of none treated group (PC) (29.4%), OXF treated group was 8.9%, OXF+PZQ treated group was 17.92%, OXF+ABZ treated group was 17.32% and ABZ+PZQ treated group was 16.91%. Also, PD-L1 have the same pattern of expression; the none treated group (PC) was 37.3%, significant reduction in the mean of expression in OXF treated group was 17.2%, OXF+PZQ treated group was 21.29%, OXF+ABZ treated group was (28.4%) and ABZ+PZQ treated group was 29.44%.

Table 1: Comparison of PD-1/PD-L1 in liver and spleen among treated groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Liver</th>
<th>Spleen</th>
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<tbody>
<tr>
<td></td>
<td>PD-1</td>
<td>PD-L1 CD274</td>
</tr>
<tr>
<td>NC</td>
<td>5.8±2.93</td>
<td>34.3±20.9</td>
</tr>
<tr>
<td>PC</td>
<td>36.7±12.8A</td>
<td>45.3±14.74A</td>
</tr>
<tr>
<td>OXF</td>
<td>12.4±5.3AB</td>
<td>13.2±10.7AB</td>
</tr>
<tr>
<td>OXF+PZQ</td>
<td>22.8±8.4AB</td>
<td>30.2±17.4B</td>
</tr>
<tr>
<td>OXF+ABZ</td>
<td>16.8±4.44AB</td>
<td>34.3±21.4B</td>
</tr>
<tr>
<td>ABZ+PZQ</td>
<td>19.64±7.83AB</td>
<td>28.12±13.28B</td>
</tr>
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</table>

A: statistically significant difference from NC, B: statistically significant difference from PC.
Figure 1: Bar chart illustration of PD-1/PDL1 protein expression in liver and spleen after treatment. (A-D): PD-1 expression in liver of mice treated (A) with OXF, (B) with OXF+PZQ, (C) with OXF+ABZ and (D) with ABZ+PZQ. (E-H): PD-1 expression in spleen of mice treated (E) with OXF, (F) with OXF+PZQ, (G) with OXF+ABZ and (H) with ABZ+PZQ. (I-L): PD-L1 expression in liver of mice treated (I) with OXF, (J) with OXF+PZQ, (K) with OXF+ABZ and (L) with ABZ+PZQ. (M-P): PD-L1 expression in spleen of mice treated (M) with OXF, (N) with OXF+PZQ, (O) with OXF+ABZ and (P) with ABZ+PZQ. (OXF) Oxfendazole, (PZQ) Praziquantel and (ABZ) Albendazole.
Figure 2: Immunoperoxidase staining of PD-1 protein expression in liver and spleen in study groups using polyclonal rabbit anti-PD-1 (5pg/ml). (A-F): PD-1 expression in spleen tissue, (A) control negative group with low expression, (B) positive control group without treatment showing high expression of PD-1 protein, (C) OXF treated group, (D) OXF+PZQ treated group, (E) OXF+ABZ treated group and (F) ABZ+PZQ treated group. (G-L): PD-1 expression in liver tissue, (G) control negative group with low expression, (H) positive control group without treatment showing high expression of PD-1 protein, (I) OXF treated group, (J) OXF+PZQ treated group, (K) OXF+ABZ treated group and (L) ABZ+PZQ treated group.
Figure 3: Immunoperoxidase staining of PD-L1 protein expression in liver and spleen in study groups using polyclonal rabbit anti-PD-L1 (5pg/ml). (A-F): PD-L1 expression in spleen tissue, (A) control negative group with low expression, (B) positive control group without treatment showing high expression of PD-L1 protein, (C) OXF treated group, (D) OXF+PZQ treated group, (E) OXF+ABZ treated group and (F) ABZ+PZQ treated group. (G-L): PD-L1 expression in liver tissue, (G) control negative group with low expression, (H) positive control group without treatment showing high expression of PD-L1 protein, (I) OXF treated group, (J) OXF+PZQ treated group, (K) OXF+ABZ treated group and (L) ABZ+PZQ treated group.

DISCUSSION

The negative co-stimulatory molecule like: programmed death-1 (PD-1) and its ligands, PD-ligand-1 (PD-L1; also known as B7-H1) negatively regulate anti-infection pathways. The PD-1/PD-L1 pathway could down regulate T cell activation, cytokine production and pathogen elimination, and can also enhance host resistance to pathogens, thereby promoting the suppression of the immune system. The PD-1 molecule is an inhibitory receptor that is induced and activated by CD28 and CTLA-4 cell surface receptors, which are predominantly expressed in activated T cells.
The slow growing lesion of hydatid cyst is highly suggestive for induction of local immune suppression in the infected organ. The current study demonstrated the higher expression of inhibitory molecules of PD-1 and PD-L1 in both liver and spleen of mice experimentally infected with hydatid cyst compared with normal very low expression in normal liver or spleen. This result highly argued the importance of PD-1/PD-L1 signaling pathway in the induction of local immune suppression mediated by *Echinococcus granulosus* antigen. La et al. (2015) study found that the upregulation of PD-1 expression is essential for induction of tolerance that stimulate CD4+CD25+ regulatory cell functions (inhibition of T-cell function, cytokine secretion and T-cell proliferation). Human liver tissue showed impairment of cell mediated immunity, phagocyte function and their numbers were significantly decreased due to *E. granulosus* antigen. In the current study, the PD-1 and PD-L1 protein expression in liver and spleen were investigated under treatment with anthelmintic drugs. Statistically significant reduction in the mean of PD-1 and PD-L1 protein were documented after using Oxfendazole, Oxfendazole + Praziquantel, Oxfendazole + Albendazole and Albendazole + Praziquantel. The reduction of PD-1 and PD-L1 upon treatment may be due to clearance of parasite and reduction of cyst antigenic stimulation of immune system cells as compared with positive and negative control groups. After 4 months of treatment, Oxfendazole + Praziquantel showed highest percentage of efficacy of treatment (91.08%) followed by using Oxfendazole (88.62%), Oxfendazole + Albendazole (82.97%) and Albendazole + Praziquantel (79.6%). A recent study done by Wang et al. (2018) used anti-PD-L1 blocking agent to treat echinococcosis is associated with fewer liver lesions, improves IL-2, IL-12 and interferon gamma mRNA level and decreased IL-4 and Foxp3 mRNA level. Thus, the reduction of PD-1 and PD-L1 tissue expression in both liver and spleen might govern the upregulation of pro-inflammatory cytokines such as: Tumor necrosis factor – alpha (TNF-α), IL-12 and IL-2 as restoration of immunosuppressive state mediated by parasitic antigens.

**CONCLUSION**

Liver and spleen tissue expression PD-1/PD-L1 signaling pathway is up regulated during infection with *E. granulosus* and these proteins were downregulated or reduced after effective treatment suggesting an involvement of PD-1/PD-L1 signaling pathway in the pathogenesis of Hydatid disease.

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**ETHICAL CLEARANCE**

The Research Ethical Committee at scientific research by ethical approval of both environmental and health and higher education and scientific research ministries in Iraq

**CONFLICT OF INTEREST**

The authors declare that they have no conflict of interest.

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REFERENCES


