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Original paper

Recombinant C-type lectin protein of *Toxocara canis* increases the population of spleen Foxp3+ regulatory T cells in BALB/c mice

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ABSTRACT. *Toxocara canis* (*T. canis*) is a common parasitic nematode in dogs and cats. Parasitic worms can cause chronic and long-term infections in their host, due to their ability to neutralize the host's defense mechanisms. They can stimulate immune response-mediated regulatory T (T-reg) cells. The aim of this study is to evaluate the effect of recombinant *T. canis* C-type lectin protein (TCTL-1) on cell infiltration in the brains of BALB /c mice as well as the number of regulatory T cells. Six-week-old female BALB/c mice received the recombinant C-type lectin protein of *T. canis* six times intravenously and intraperitoneally. Twenty-eight days after the first injection, the spleen and brain of mice were removed under sterile conditions. The brains of mice were examined by histopathological staining methods. The FOXP3+ regulatory T cell population was determined by flow cytometry. The cell populations of regulatory T cells in spleen mononuclear cell culture of 3 female BALB/c mice injected with recombinant TCTL-1 (group I) were 2.59%, 1.64%, and 1.78 and in spleen mononuclear cell culture of three female BALB/c mice injected with sterile PBS (group II) as a control group were 1.14%, 1.13%, and 1.15%. Also, no cell infiltration was seen around the cerebral arteries of mice receiving this protein. This recombinant protein would increase the population of FOXP3+ regulatory T cells. These results suggest that recombinant C-type lectin protein of *T. canis* can modulate immune responses, reduce severe inflammatory responses, and induce FOXP3+ regulatory T cells.

Keywords: *Toxocara canis*, C-type lectin, FOXP3+ regulatory T cells, mouse

Introduction

Toxocara canis (*T. canis*) is a common parasitic nematode in dogs and cats. In humans, toxocarosis is a global zoonotic disease [1]. Fertilized eggs are infectious to a wide range of mammals. Larvae can migrate in the tissues of the host body for several months to several years and prevent the host's immune attack.

The second stage larvae of this nematode can survive for a long time in the host tissue. This survival strategy is due to the excretory-secretory (ES) products [2,3]. Infective larvae release significant amounts of *T. canis* excretory-secretory (TES) antigens, about 1% of the host body weight per day [4]. Large excretory-secretory macromolecules are glycoproteins that are structurally slightly different [5].

Parasitic worms can cause chronic and long-term infections in their host, due to their ability to neutralize the host's defense mechanisms [6]. *T. canis* can cause a long-live infection even in the presence of humoral and cellular immune responses, since this nematode has effective strategies to escape immune system damage. Studies have shown that the parasite ES products, such as TES-32 and TES-70, play an important role in modulating the immune responses against *T.canis* infection [2,7,8].

Toxocara infection elevates the level of

cytokines such as IL-4, IL-5, IL-10, and IL-13 through induction of Th2 cells, where these cytokines induce responses to the parasite such as elevated level of circulating IgE antibodies, differentiation of B cells (IL-4) and eosinophils (IL-5), hyperleukocytosis, as well as eosinophilia with infiltration to the site of larval persistence [9]. The interaction between worm-derived molecules and host cells can lead to changes in immune responses from one inflammatory reaction to an anti-inflammatory reaction. Helminth-derived molecules can alter the function of dendritic cells and downregulate adaptive immune responses by inducing a regulatory network which include regulatory T (Treg) cells, alternatively activated macrophages (AAMs), and regulatory B (B-reg) cells [9].

Previous studies have found that the experimental model of mice infected with *Toxocara* induced a strong response similar to the response of T-helper (Th2) cells as well as elevated interleukin-10 (IL-10) and tumor growth factor-β (TGF-β) levels [10–12].

Cytokine production by mouse spleen cells can be induced by recombinant protein of *T. canis* excretory-secretory (TES), but complete TES has a greater effect on the secretion of cytokines by these cells. It is still unknown whether this effect is related to one component of TES or a set of effects of all TES proteins [13].

Some constituents of TES include 32-kilodalton (kDa) (TES-32), 55-kDa (TES-55), 70-kDa (TES-70), 120-kDa (TES-120), and 400-kDa (TES-400) proteins. All of these proteins are glycosylated, antigenic, and are found in various species of *Toxocara* as well as other ascarid nematodes. TES is one of the most important of these proteins and shows serious closeness with C-type lectins found in mammalian immune cells that are involved in the response to the pathogen [14].

Most of the *T. canis* ES products are C-type lectins [8]. Many C-type lectins are cell surface receptors that play a key role in activation of the vertebrate immune system. Their principal examples are collectins, selectins, macrophage mannose receptor, natural killer cell receptors, and CD23 (the low-affinity IgE receptor) [8].

C-type lectins or calcium-dependent lectins are a family of available lectins that are bonded with carbohydrates, including simple monosaccharides and complex glycoconjugates [15]. C-type lectins play a role in various processes of cells including signaling, plus traffic and activation of innate immunity in vertebrate and invertebrate organisms [8]. These proteins are particularly involved in immune system evasion. An important characteristic of C-type lectins and proteins similar to C-type lectins is that they are cellular surface receptors that play important roles in activation of vertebrate immune system [16].

The study of BALB/c mice infected with *T. canis* provided new insights into anti-inflammatory responses innate immune cell activation, as well as Th2 dependent immune responses during primary infection in these mice. This protein can block cell infiltration in the brain through T cell-dependent responses [17].

The aim of the present study was to evaluate the effect of *T. canis* recombinant C-type lectin on the population of Foxp3+ T cells as well as to demonstrate the immune cells infiltration in the brain tissue of BALB/c mice.

Materials and Methods

Expression and purification of T. canis C-type lectin

The original reading frame (ORF) encoding the C-type lectin protein of *T. canis* was extracted from the Genbank (AF041023). This sequence includes 657 nucleic acid and 219 amino acids. The recombinant plasmid pET32a containing this sequence was then synthesized (Generay Co., China). The recombinant plasmid was transferred to the competent cells of *Escherichia coli* BL21 (DE3) (Bon Yakhteh Co., Iran) using $CaCl_2$-$MgCl_2$ solution and heat shock method [18]. Induction of recombinant protein expression was performed using isopropyl β-d-1-thiogalactopyranoside (IPTG) (Sigma Co., USA) at a concentration of 1 mM at 37°C for 4 hours [19].

The protein was purified by the QIAGEN Ni-NTA spin kit (Qiagen, USA) under denaturing conditions according to the kit instructions [20].

The purity of the recombinant proteins was assessed by SDS-polyacrylamide gel 12% electrophoresis (SDS-PAGE) [21]. Recombinant proteins separated by SDS-PAGE were transblotted in Tris-glycine buffer at 45 V for 5 h onto a 0.45 μm nitrocellulose membrane (Bio-Rad, Germany). Western blot analysis was carried out by applying anti-six-histidine antibody at a dilution of 1:1000 and DAB (3-3’ diamino banzidine([20].

Animals

Six female BALB/c mice (6 weeks of age) were selected from laboratory animals' breeding and keeping center in Rastgar Central Laboratory at the Faculty of Veterinary Medicine, University of Tehran. Ethical approval for this study was granted by the Multiple Sclerosis Research Center in Tehran University of Medical Sciences (approval number: IR.TUMS.VCR.REC.1396.4800).

Six injections of 10 µg recombinant C-type lectin per 100 µl PBS (the contents prepared for injection were sterilized using 0.45 µm filters) were given both intravenously and intraperitoneally (50 µl in each site) into three female BALB/c mice (group I) every other day. Six injections of 100 µl of sterile PBS were also administered into the next three mice (group II) as a control group both intravenously and intraperitoneally exactly like the previous group.

Cell culture

Mice were anesthetized with ether with the spleen of mice isolated 28 days after the first injection under sterile conditions. The spleen cells were isolated under perfectly sterile conditions and washed with RPMI-1640 culture medium (Sigma, Germany). Ficoll Solution (Inno-train, Germany) was added at a 1: 2 ratio to the culture containing the cells. The cells were centrifuged at 20°C for 30 minutes at 2300 rpm. The formed clear ring was isolated and washed with RPMI-1640 medium containing 1% penicillin-streptomycin with the cells stained using trypan blue (Merck, USA) and counted by hemocytometer under light microscope ×40 magnification [22]. Trypan blue is a vital dye that discriminates living and dead cells.

One and a half million cells, counted using hemocytometer, were cultured in each well of a 24-well plate containing RPMI-1640 culture medium, 10% fetal bovine serum, and 1% penicillin-streptomycin [23]. The cells obtained from the spleen of each mouse were cultured in three wells. Fifteen micrograms of recombinant antigen were added to the first well and 10 micrograms of phytohemagglutinin to the second well. The third well was considered as a control. The cells were incubated at 37°C with 5% CO_2 in humidified atmosphere. After 72 hours, the cells were collected and examined by flow cytometry.

Initially, frequencies of T cell subpopulations were calculated as percentages of positive cells in the total lymphocyte gate using CD4-FITC antibodies (eBioscience, Inc., San Diego, USA). Also, the CD25 positives were counted sequentially by CD25-APC antibodies (eBioscience, Inc., San Diego, USA). CD4+CD25+ T cells express FoxP3 were accepted as Treg. Total cells were stained with the following monoclonal antibodies and were incubated for 45 min at dark and 4°C: CD4-FITC, CD25-APC (eBioscience, Inc., San Diego, USA).

Intracellular detection of FOXP3 (eBioscience, Inc., San Diego, USA) was performed on fixed and permeabilized cells using appropriate buffers (eBioscience, Inc., San Diego, USA), then incubated at 4°C for 20 min. The cells were acquired on a flow cytometer (Becton Dickinson, USA) and analyzed using FlowJo software version 10.6.1 (Tree Star, Inc., USA). Dead cells were excluded through forward/side scatter gating.

Histology

For histological assessment of the central nervous system (CNS) tissue, the brains were removed from mice under sterile conditions. Brains were fixed in 10% formalin and embedded in paraffin, where 5 µm sections were cut and stained with hematoxylin and eosin (H and E). These sections were examined with a light microscope to demonstrate cell infiltration.

Results

Preparation of recombinant C-type lectin of T. canis

Recombinant C-type lectin of *T. canis* was expressed at 1 mM IPTG, 37°C. The purity of the recombinant proteins was assessed by SDS-polyacrylamide gel 12% electrophoresis (SDS-PAGE). The results revealed a band with a molecular mass of approximately 41 kDa. Western blot results demonstrated the presence a band with a molecular mass of approximately 41 kDa (Fig. 1).

Flow cytometry results

The cell populations of FOXP3+ regulatory T cells in spleen mononuclear cell culture of three female BALB/c mice injected with recombinant C-type lectin protein (group I) were 2.59%, 1.64%, and 1.78 and in spleen mononuclear cell culture of three female BALB/c mice injected with sterile PBS (group II) as a control group were 1.14%, 1.13%, and 1.15% (Fig. 2).

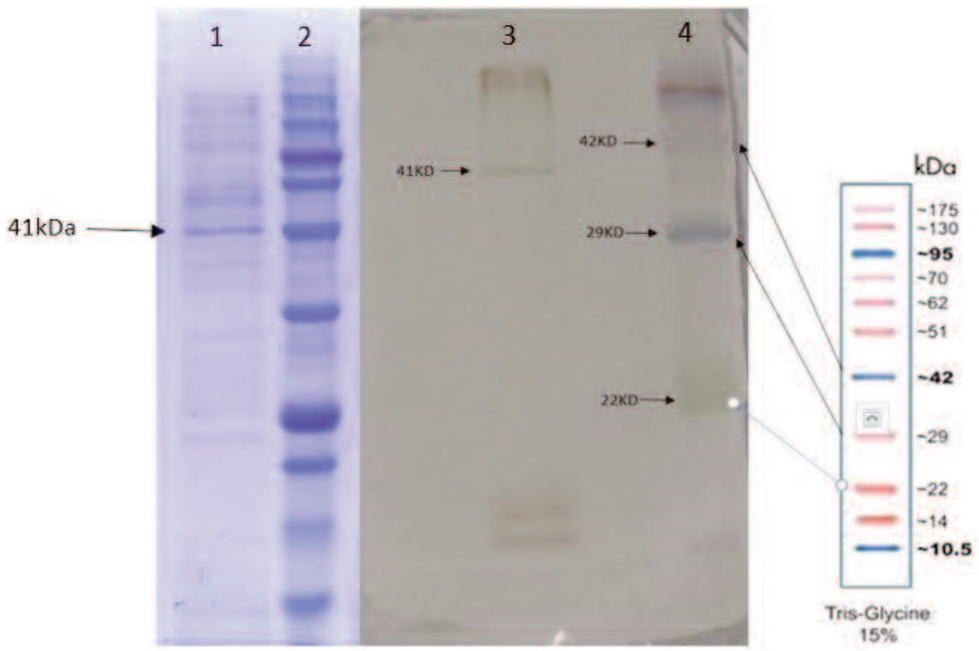

Figure 1. SDS-PAGE and Westerb blot analysis of the recombinant C-type lectin protein of *T. canis*. A SDS-PAGE gel of recombinant C-type lectin protein stained with Coomassie Blue (12% gel). 1: purified recombinant C-type lectin protein by the QIAGEN Ni-NTA spin kit (QIAGEN, USA) under denaturing conditions. 2: protein marker (Sinacolon, Iran). Western blot analysis using anti-six-histidine antibody at a dilution of 1:1000 and DAB (3-3' Diamino banzidine) confirmed expression of the recombinant C-type lectin protein of *T. canis*. 3: purified recombinant C-type lectin protein by the QIAGEN Ni-NTA spin kit (QIAGEN, USA) under denaturing conditions (41 KDa). 4: protein marker.

Histological results

Hematoxylin-eosin-stained slides were examined using a light microscope to analyze cell infiltration around cerebral arteries. No immune cell infiltration was observed around the cerebral arteries in group I and group II (Fig. 3).

Discussion

The results of the present study indicated that the cell populations of regulatory T cells in spleen mononuclear cell culture of three female BALB/c mice injected with recombinant C-type lectin

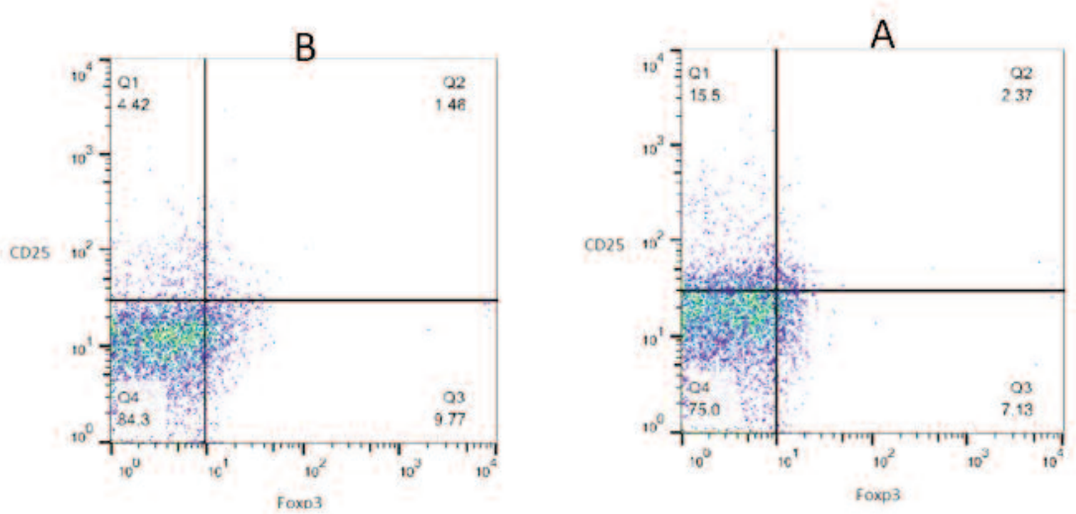

Figure 2. Flow cytometry analysis of FOXP3+ regulatory T cells in spleen cell culture of mice receiving recombinant C-type lectin protein of *T. canis* as a mitogen (A), with the control group (B)

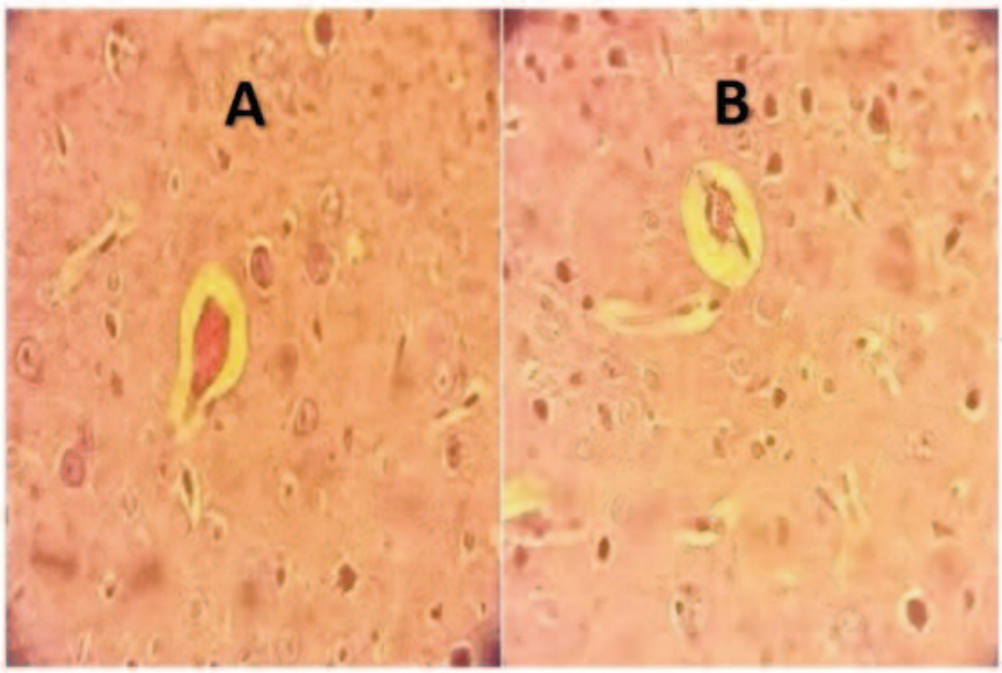

Figure 3. Brain parenchyma of BALB/c mice (H and E 40×). A. Control group: normal brain parenchyma; B. Absence of inflammatory mononuclear cells and polynuclear cells around the cerebral arteries of brain tissue as well as hemorrhagic areas in brain parenchyma of mouse from group I injected with recombinant C-type lectin of *T. canis*

protein (group I) were 2.59%, 1.64%, and 1.78, while in spleen mononuclear cell culture of 3 female BALB/c mice injected with sterile PBS (group II) as a control group they were 1.14%, 1.13%, and 1.15%. According to these results, this study should be performed on more mice in the future. T-reg cells have been identified as the main cells in many infections, including worm infections. Also, these cells help in regulation of parasitic-induced immunopathology in many parasitic infections [24]. Experimental infection of *T. canis* increases the population of T-reg cells of spleen of mice. Studies have also found that T-reg cells a key role in evolution of immunopathology during *T. canis* infection [24]. The spleen plays a substantial role in several chronic infectious diseases because of its lymphoreticular tissue [25].

TCTL-1 is secreted from the larval stage causing chronic infection, and study of the effect of this protein on cell population is of particular importance. These findings may confirm that *T. canis* C-type lectin can modulate dendritic cells (DCs) function and host immunity [26].

It seems that nematode tissue C-type lectins can interfere with cell infiltration by inhibiting inflammation-mediated selectin [8].

In experimental infection of gal3+/+ mice with *Toxoplasma gondii*, the expression of galectin3 increased in infiltrated leukocytes in the intestine, liver, lungs, and brain, Lack of galectin in gal3-/- mice would promote the production of IL-12 by dendritic cells. This indicates the role of host galectin3 in controlling parasitic infections. The protective role of galectin-3 and galectin-7 in the neuropathology of brain parasite infections has recently been investigated in a preclinical model of neurocysticercosis. The results showed high level of galectin-7 in the brain endothelial cells of mice infected with *Mesocestoides corti* [27].

So far, not many studies have been published on the role of lectins in modulating host immunity during parasitic worm infections migrating to the brain. However, the role of galectins, a family of lectins, has been studied in mice infected with protozoa such as *Toxoplasma gondii*. The results of these studies revealed that in *Toxoplasma gondii* infection, galectin-3 modulates the production of IL-10 and IL-6 dependent on regulatory T cells [27]

The present study showed that lectin did not induce neutrophil, eosinophil, and mononuclear cell infiltration in the brain. However, it is necessary to examine the expression of lectin in the brain tissue following the challenge with this protein in future studies.

Histopathological results did not show any cellular infiltration around the cerebral arteries,

suggesting that this recombinant protein did not cause a cellular immune response in the brain. On the other hand, larvae of *T. canis* can migrate to various organs of human body including central nervous system and cause neurotoxocariosis [27]. The present study indicated that this recombinant protein can increase the number of regulatory T cells of spleen. These results may indicate the effect of recombinant C-type lectin protein on modulating immune responses.

Thus, this protein can be used as a candidate to control unwanted immune responses to autoimmune diseases such as autoimmune colitis and multiple sclerosis [23]. Note that further studies are required to investigate the immune responses induced by this protein.

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