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Effect of Garlic Extract Treatment on the Expression of CD8$^+$ and/or γδ T Cells Receptors in the Intraepithelial Lymphocytes in *Eimeria Vermiformis*-Infected Mice

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Abstract

Background: garlic has been used for food and medicine purposes and this recorded in many cultures for thousands of years. Coccidiosis is serious health problem causing destruction of intestinal mucosa.

Objective: In this study, we investigated the immunomodulatory and parasiticidal effects of garlic on the coccidiosis caused by *Eimeria vermiformis* in male ICR/c mice. The immunomodulation and CD8$^+$ and γδ$^+$ T cells proliferative induced by garlic extract in *Eimeria vermiformis* infected male ICR mice were detected.

Methods: Thirty mice divided into two groups, group one was received garlic extract (500 mg/kg body weight) daily by oral route. Garlic was given 10 days before parasitic infection (*E. vermiformis*; given once as 300 sporulated oocysts orally) and continued up to the end of the experiment (infected-garlic$^+$). The second group served as control positive with *E. vermiformis* infection alone (infected-garlic$^-$) and both groups injected with Bromodioxyurinidin (BRDU) intraperitoneally 24 hours before scarification.

Results: In the infected-garlic$^+$ group, garlic extract treatment impaired the intracellular development of the parasites this confirmed by lowering of the oocyst output number. Significant increases in the expression of CD8$^+$ and γδ$^+$ T cells also observed by flowcytometric analysis. Immunohistochemically examination of the ileum tissue showed that garlic extract treatment increased the expressed CD8$^+$ number in the intraepithelial lymphocytes (IELs). On the other hand, no double positive CD8$^+$ T cells were expressed

Conclusion: garlic extract treatment increased the over expression of the immune cells (CD8$^+$ and γδ$^+$) T cells. The overexpressed cell not originated from the site of infection. In addition, further studies needed for exploring the origin of activated CD8$^+$ T cells during *Eimeria* infection.

Keywords: *Eimeria vermiformis*; Garlic; Intraepithelial Lymphocytes; Immunohistochemistry; CD8$^+$, BRDU

Introduction

Garlic (*Allium sativum*) is an herbal plant has been used both for culinary and medical purposes by many cultures [1]. Several extensive studies done on laboratory and animal models have demonstrated that garlic has wide range of biological activities including high free radical scavenging activities [2] as well as anticancer activity as it inhibits the growth of several different cancer cells *in vitro*, as well as cancer growth *in vivo* [3]. Furthermore, it has anti-inflammatory activity by suppressing inflammatory mediators [4] and immunostimulatory activities [5].

Several studies and reports were done on laboratory and animal models described the wide range of biological activities of garlic including high free radical scavenging activities [2] and anticancer effects as it inhibits the growth of many different cancer cells in vitro and in vivo [3]**.** Furthermore, it has anti-inflammatory role as it suppresses some inflammatory mediators **[4]** as well as the immunostimulatory activities **[5]**. Garlic and most of its constituents have anti-parasitic activities against to some animal and human parasites [6] fore examples, Leishmania infection [7], Schistosoma parasite [8,9], Trypanosoma[10], Giardia[11], Entamoebasis [12] and the Plasmodium species **[13].** Garlic extract contains compounds have immuno-modulating activity [14-15]**.** Garlic also enhances the shifting of the polarization of CD4+ T cells towards Th1 cells [16]**.** Moreover, garlic extract activates the function of natural killer T cells**, [17].** In accordance, it stimulates the frequency and proliferation of lymphocytes **[18].** Garlic extract also improve the CD4+/CD8+ T cells ratio in vitro. Increase also the production of IFN-γ in the splenocytes. **[19].** In other study, supplementation of the diet with garlic extract intensify the immune cells performance through γδ-T cells and NK cells proliferation [20]**.** Garlic extract treatment in mice stimulates the expression of miR-142-5p and miR-29b in the jejunum that they are involved in the transition of $CD4^{-}CD8^{-}$ T cells to $CD4^{+}CD8^{+}$ T cells [21]**.**

Coccidiosis is a widespread disease affecting animals and birds. Coccidial infection caused by the unicellular eukaryote *Eimeria* spp., **[22].** The disease causes well-known signs of mortality, morbidity, diarrhea or bloody feces. Coccidial infection may be sub-clinical and evidenced mainly by poor weight gain, reduced efficiency of feed conversion and leading to highest percentage of the total economic losses [23]**.** *E. vermiformis* is a pathogenic coccidian parasite known to infect several laboratory strains of mice [24]**.** Several studies have denoted that *E. vermiformis* is an effective model for the study of the developmental, pathogenetic, immunological, ultrastructural, and genetic aspects of coccidiosis **[25].** Recently the *E. vermiformis* parasite has used as a model to examine the title role of the cellular mitochondria in managing the activation state of the intra-epithelial T lymphocytes **[26]**. Gamma delta (γδ) T- cells demonstrated that enhance the rapid activation of other lymphoid cells and the transferable role as anti -pathogen effect in αβ T-cell-deficient mice infected by *Eimeria vermiformis* oocysts [**25].** Regardless, these results CD T cells are in some way involved in the response to Eimeria infection by increases in intra epithelial γδ+ in either the mice or chickens **[27].** challange by Eimeria encourage antibody and cell-mediated immune responses. However, cellular immunity mediated by various cell populations, including T lymphocytes, NK cells, and macrophages, plays a major role in disease resistance [28].

Depending on the own's author study which indicated that garlic activate the intra epithelial lymphocytes $CD8^{+}$ [29]. This study was done to investigate the effect of garlic extracts treatment on the population of $CD8^{+}$ T cells and γδ T cells. In addition, to where is the activated T cells proliferated? in the site of infection or come from other sources? during E. *vermiformis* infection.

Material and methods

Experimental animals

Male ICR mice 7-8 weeks' age and weighing 39-42 g were purchased from Charles River Japan, Inc., Yokohama, Japan. Animals were given healthy standard diet and clean water *ad libitum* and were housed in an air-conditioned room (23 ± 3º C) with 12:12 hour light: dark cycle. Animals were kept for 10 days before starting of the experiment for acclimatization. All protocols for animal care and dealing were approved by the institutional review board for animal experiments of the University of Miyazaki (UOM 2011-007-5), Japan.

Parasite and infection

E. vermiformis parasite was maintained in veterinary parasitic diseases laboratory by passaging the oocysts in mice and were purified then sporulated [30]. After examination of the stock microscopically for sporulation, the mice were given 300 sporulated oocysts/mouse in diluted in 100 µl of distilled water by oral gavage.

Garlic extract preparation

Garlic extract was prepared daily by mixing dried garlic powder mixed in distilled water in concentration of 80 mg/ml then incubated overnight [31]. The mixture was centrifuged at 3500 rpm for 10 minutes and the supernatant was kept at 4º C. The extract was given orally to the mice at a dose of 500 mg/kg by stomach tube.

BRDU and Anti-BRDU detection kit

Bromodeoxyuridine (BRDU) 25 mg (BD pharmingen, U.S.A), was used as a structural analog of thymidine base and it can be incorporated into DNA during the synthesis-phase of the cell cycle. BrdU In-Situ detection kits (BD pharmingen, U.S.A) used for BRDU proliferation assay in ileum tissues.

Monoclonal antibodies (mAbs):

The monoclonal antibodies were used, 1) Rat anti mouse CD8, clone YTs105.18 (mab) specific for mouse, recognizes the surface epitope on the mouse CD8 alpha chain. It was obtained from (Bio-Rad laboratories. Inc, U.S.A), the monoclonal antibody was used in 1:200 dilutions for immunohistochemical staining.
2) Hamster anti mouse gamma delta (γδ) T cell (mab) clone GL3 ((Bio-Rad laboratories. Inc, U.S.A) used with CD8 (mab) for Flocytometry analysis.

Experimental design

Thirty male ICR mice were randomly divided into two groups (n=15/group). The groups were as follows: (1) infected-garlic- group; infected with 300 sporulated oocysts of *E. vermiformis*

orally without garlic treatment (2) infected-garlic$^+$ group; infected with 300 sporulated oocysts of *E. vermiformis* orally and given garlic extract (500 mg/kg body weight) orally for 10 days before infection and continue to the end of the experiment. Ileum samples were collected on days 0, 3 and 6 p.i.

BRDU injection

Ten mg/ml solution of Bromodeoxyuridine (BRDU) in sterile 1X PBS was used. BRDU solution dose was 200 µl (1-2 mg) per mouse and was prepared before injection. In brief, 200 µl (1-2 mg) of BRDU solution was mixed with the same volume of sterile saline to increase the volume, the mixed solution was injected intra peritoneal (I.P) 24 hours before scarifying.

Intraepithelial Lymphocytes (IELs) isolation

IEL isolation method was based on the previous report by Godman et al., [32]. In brief, ileum was removed and placed in petri dish and flushed with ice cold PBS to remove intestinal contents. Peyer's patches were cut out and discarded. The ileum was cut longitudinally, scraped lightly to remove mucus, and cut into 1-cm pieces that were rinsed several times in cold PBS (mM phosphate buffered saline) to remove debris and placed into a clean 15-ml conical tube containing 10 ml of 1mM of Dichlorodiphenyltrichloroethane (DTT) and 0.5mM of EDTA-PBS working solution (Roche, Penzberg, Germany) to remove the epithelial layer. Then the ileum sections were incubated at 37º C for 40 minutes after incubation the tube undergoes well pipetting then sieved with mesh 40 nm (BD Bioscience, U.S.A). The remain tissues discarded and the viability of cells confirmed by trypan blue dye (Sigma Chemical Co. St. Louis, MO, United States).

Flow cytometry analysis

The different cell populations analyzed by flowcytometry. CD8$^+$ and γδ$^+$ T cells were stained at 37°C for 60 min with the following fluoro-chrome conjugated antibodies: FITC-anti-CD8 (YTs105.18) and PE-anti-γδ T (GL3) (all reagents, BD-Pharmingen U.S.A). Multiple-color flow analyses performed using a FACScan flow cytometer up-regulated by Cytec Development (Fremont, CA, United States) to allow for 2-color analysis. Facs analysis was done as the following: Isolated intraepithelial lymphocytes transferred to 96 wells plate and incubated with the antibodies for 60 minutes. Then cells were passed on Facs for sorting. Optained results analyzed with CELLQUEST Software (BD Biosciences CA, United States).

BRDU/ CD8 double immunohistochemistry.

For double immunohistochemical analysis, ileum tissues were collected from infected-garlic$^-$ and infected-garlic$^+$ at days 0, 3 and 6 p.i., were frozen in Optimal Cutting Temperature compound (OCT) at -80°C. Tissues were sectioned using a Cryostat (Leica Biosystem) and stained with CD8α monoclonal antibody (Bio-Rad Laboratories Inc., USA) using an indirect immunoperoxidase technique according to Yasuda et al., [33]. CD8$^+$ cytotoxic T cells counted in ten random Villous Crypt Units (VCU). Tissue staining continued for BRDU detection. In brief, after the CD8α (mab) staining slides placed in retrieval solution for 10 minutes then incubated with 10 times diluted biotinylated anti-BrdU antibody for 60 min at room temperature. Next, the tissues section incubated with ready-to use Streptavidin-HRP secondary antibody for 30 minutes at room temperature. Finally, the reactions were visualized with Vector VIP substrate solution (Vector Laboratories, Inc, U.S.A) [34].

Statistical analysis

Statistical analysis performed with the statistical software package SPSS for Windows (version 20.0; SPSS Inc., Chicago, Ill.). Student's t test used to compare data from the infected-garlic$^-$ and the infected-garlic$^+$ groups. Results were expressed as M ± SE of the mean (S.E.M.). The probability (P) value which less than 0.05 was considered as significant result.

Results

Effects of garlic treatment on expression of CD8 and γδ in IELs

Ileal tissue samples infected with *E. vermiformis* collected from infected-garlic$^-$ and infected-garlic$^+$ groups and examined by Flowcytometry for the expression of CD8 and/or γδ surface markers of IELs. Flowcytometry analysis of IELs was performed on 0, 3 and 6 days post infection (P.i). On day 0 p.i., garlic extract treatment induced significant increase in the percentage of CD8$^+$γδ$^-$ and CD8$^+$γδ$^+$ double positive T cells as shown in (Figure 1A) in association with significant increase in the total CD8$^+$γδ$^-$ and CD8$^-$γδ$^-$ (Figure 1B) in comparison to infected-garlic$^-$ group. Furthermore, garlic extract treatment induced only significance increase in the percentage of double positive CD8$^+$γδ$^+$ T cells on day 3 and 6 p.i., when compared with infected-garlic$^-$ group (Figure 2 & 3A). On the other hand, treatment of the E. vermiformis infected mice with garlic extracts caused significant increase in the percentage of total CD8$^+$γδ$^-$ and Cd8$^-$γδ$^+$ on day 3 and 6 p.i., respectively as shown in (Figure 2 & 3B) in comparison to infected-garlic$^-$ group.

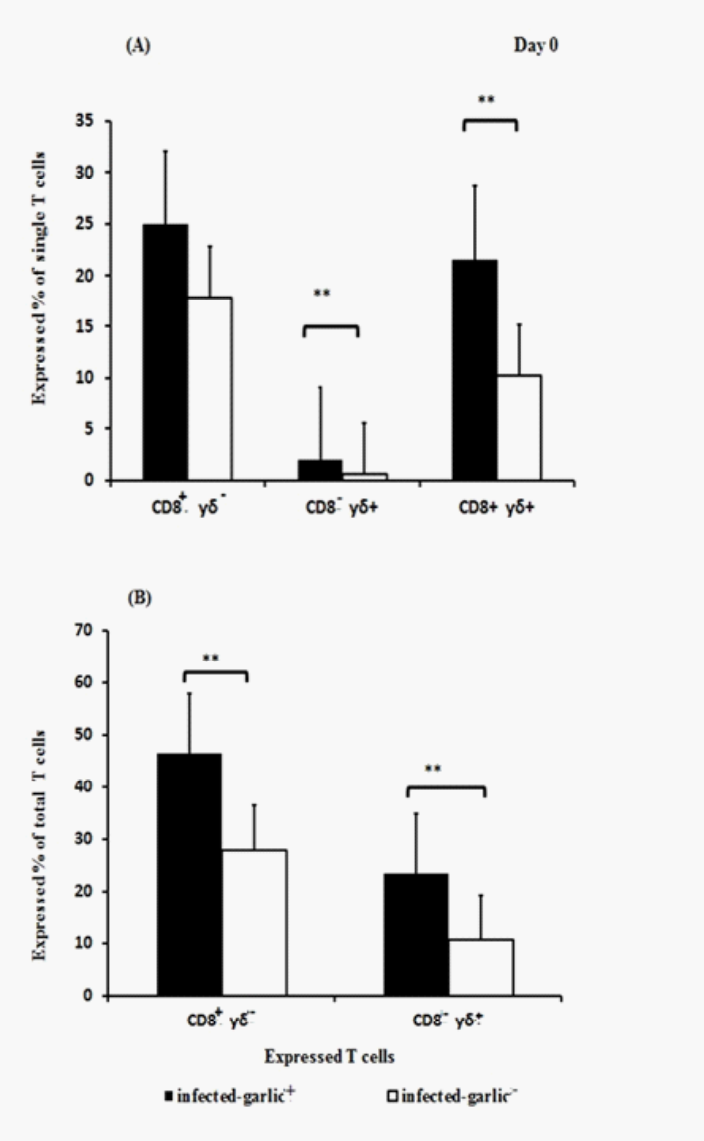

Figure 1: Effect of garlic extract treatment on the expressed % of CD8$^+$ and γδ T cells in *E. vermiformis*-infected male ICR mice on day 0 p.i., (infective dose: 300 sporulated oocysts). Garlic treatment (500 mg/ kg; orally) induced a significant increase in the percentage of expressed CD8$^+$ and γδ T cells in the infected treated-mice (infected-garlic$^+$) in comparison to infected mice (infected-garlic$^-$). **P < 0.01, compared with infected-garlic$^-$ values. Bars represent means ± S.E.M. (n = 5).

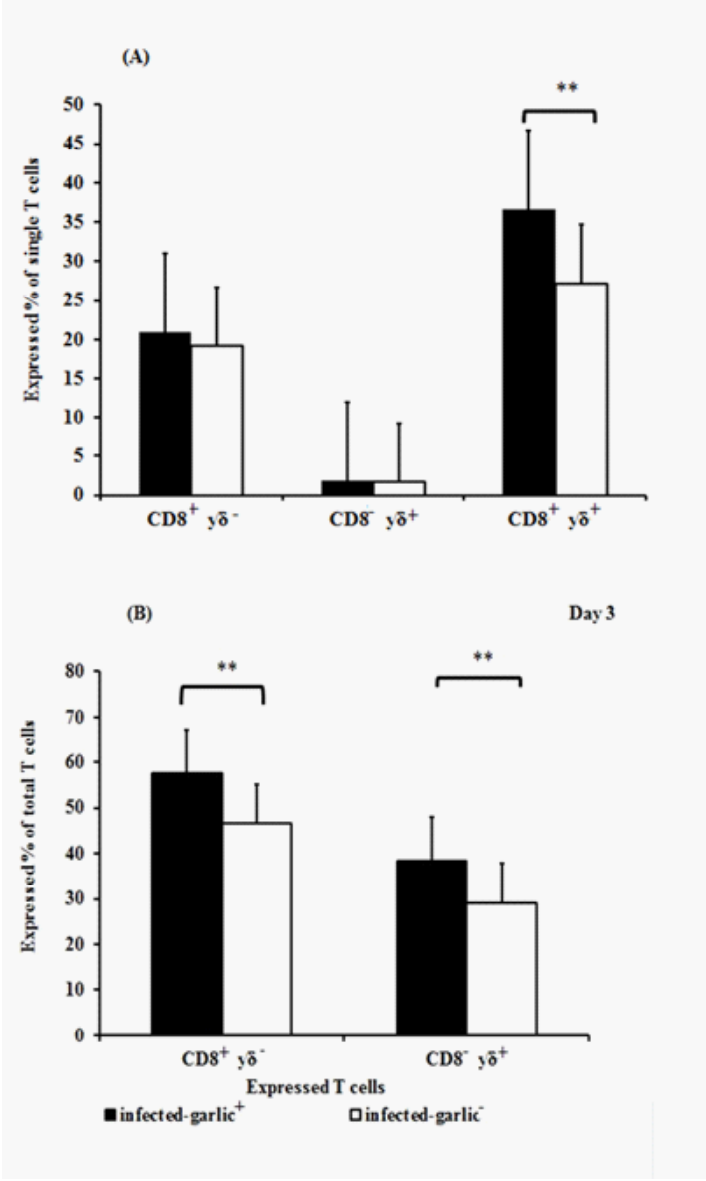

Figure 2: Effect of garlic extract treatment the expressed % of CD8$^+$ and γδ T cells in *E. vermiformis*-infected male ICR mice on day 3 p.i., (infective dose: 300 sporulated oocysts). Garlic treatment (500 mg/ kg; orally) induced a significant increase in the percentage of expressed CD8$^+$ and γδ T cells in the infected treated-mice (infected-garlic$^+$) in comparison to infected mice (infected-garlic$^-$). **P < 0.01, compared with infected-garlic$^-$ values. Bars represent means ± S.E.M. (n = 5).

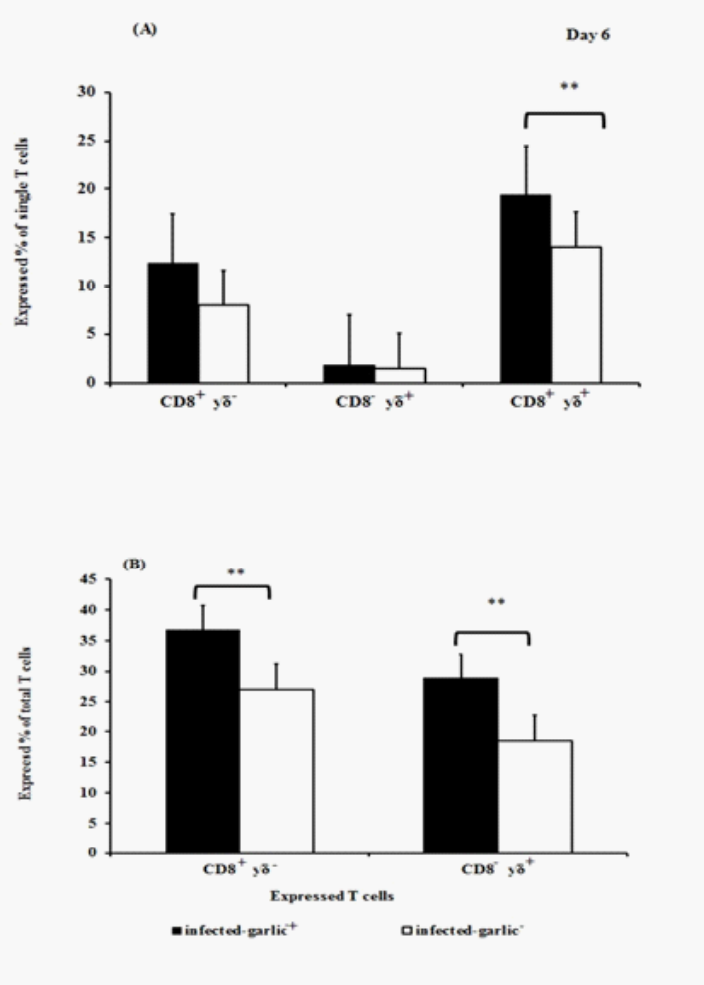

Figure 3: Effect of garlic extract treatment the expressed % of $CD8^+$ and γδ T cells in *E. vermiformis*-infected male ICR mice on day 6 p.i., (infective dose: 300 sporulated oocysts). Garlic treatment (500 mg/ kg; orally) induced a significant increase in the percentage of expressed $CD8^+$ and γδ T cells in the infected treated-mice (infected-garlic$^+$) in comparison to infected mice (infected-garlic$^-$). **P < 0.01, compared with infected-garlic$^-$ values. Bars represent means ± S.E.M. (n = 5).

Effect of garlic extract on $CD8^+$ T cells expression and proliferation in the intraepithelial layer

Eimeria vermiformis infection induced increase in the $CD8^+$ T cells expression, which confirmed by Immunohistochemical (IHC) examinations. IHC revealed that treatment of the infected mice with garlic extracts induced significant increase in the cytotoxic T cells ($CD8^+$) population that highly distributed in the intraepithelial tissues of the ileum in comparison to infected-garlic$^-$ group as shown in (Figure 4). The $CD8^+$ T cells number began to increase from the day 0 which becomes significantly higher at day 3 as shown in (Figure 5), then decreased to low expression at day 6 p.i.

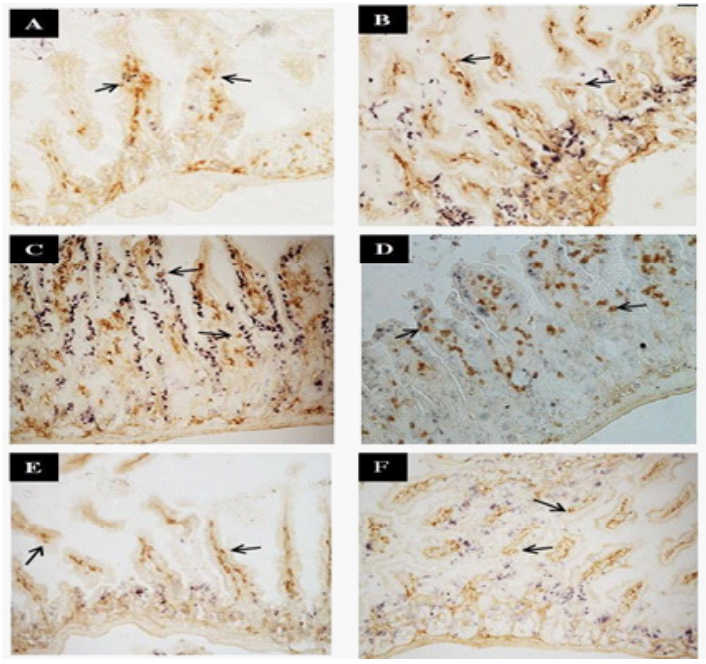

Figure 4: Immunohistochemical examination showed $CD8^+$ T cells population (arrow) in random ten VCUs of ileum on day 0, 3 and 6 p.i., infected-garlic$^-$ (A, C and E) and infected-garlic$^+$ (B, D and F); respectively. Garlic extract treatment induced increased number of $CD8^+$ cells which started to increase from day 0 (B) and reached the peak at day 3 (D) and then declined by day 6 (F) p.i., in comparison to infected-garlic- group (A, C and G). HPF means high power field, VCUs means villous crypt units.

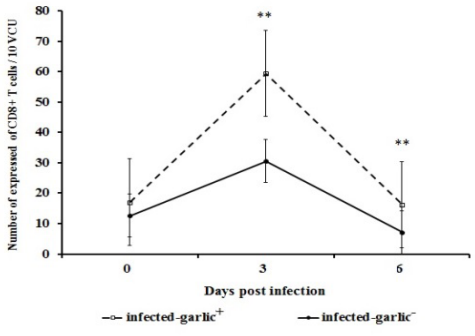

Figure 5: Kinetics of $CD8^+$T cells expression in 10 random VCUs of ileum infected with *E. vermiformis* oocysts. After garlic extract (infected-garlic$^+$) treatment, at day 3 and 6 p.i in comparison to infected-garlic$^-$ group. **P<0.01, infected-garlic$^+$ compared with infected-garlic$^-$ value. Bars represent means ± SEM (n = 5). VCUs means villous crypt units.

Investigation of the ($CD8^+$) T cells proliferation in the site of infection

Ileal tissues stained with BRDU stain. Although BRDU incorporated cells were observed clearly at the site of infection in infected-garlic$^-$ and infected-garlic$^+$ at days 0, 3 and 6 p.i., while on the other hand, no double positive ($CD8^+$- BRDU incorporated) T cells were seen in site of infection of the ileal tissues.

Kinetics of $CD8^+$ T cells expression in 10 random VCUs of infected ileum

After garlic extract (infected-garlic$^+$) treatment, $CD8^+$ T cells population began to increased gradually from day 0, and become significantly higher at day 3 and 6 p.i in comparison to infected-garlic$^-$ group.

Discussion

This study focuses on the effect of garlic extract treatment on the expression and activation of the intra epithelial lymphocytes (IELs) γδ and $CD8^+$ T cells in the intestinal epithelium of male ICR mice model infected with Eimeria vermivormis. The study clearly demonstrated that garlic extract treatment enhanced immunity against E. vermiformis infection to protect the host by increasing the expression of intraepithelial $CD8^+$ &γδ T cells.

It well known that lymphocytes population in the mouse intestinal epithelial are Predominantly $CD8^+$ T cells [35]. Totally more than 70% of IELs in the small intestine are $CD8\alpha^+$ and about 5 - 10 % are $CD4^+$ [36-37]. The result of these study found that garlic extract treatment increases the expression of IELs $CD8^+$ T cells in the site of infection on day 0, 3, 6 post infection which observed clearly by immunohistochemistry analysis as well as flocytometry exploration. The increase in the number of cytotoxic $CD8^+$ T lymphocytes explains the enhancement of specific effector-cell-mediated immunity against the different parasitic stages in intestinal tissue. On the other hand, cytotoxic contents such as perforin and granzymes that produced by these cells cause direct or indirect cytotoxic effect on the infected cells [38]. The cellular characterization of intestinal immune response during parasitic infections could not be interpreted far from that of intestinal epithelial cells (IECs). Increasing evidence suggests an active role for IECs as non-professional antigen-presenting cells in sampling luminal antigens, regulating associated T-cell responses and preferentially activating $CD8^+$ T cells [39]. This in part, would explain the activation in the function of $CD8^+$ T cell and their role during *E. vermiformis* infection in our experiment.

Infact, within the $CD8\alpha^+$ subset population of the IELs in the murine small intestine roughly equal residence of TCRαβ and TCRγδ expressing cells were evinced [40]. Moreover, most of the present γδT cells in intestinal epithelium express $CD8^+$ surface molecules composed of α homodimeric chain [41].

γδT cells induced activate IgA responses in the mucosal immunity [42]. The immunoglobulin A (IgA) is one of the humoral responses to coccidial infection,. This immunogloulin, play role against mucosal pathogens and has a fast effectiveness [43].

In our study, within the intra epithelial lymphocytes garlic extract treatment increases the expression of $CD8^+$ T cells & γδT cells %, as well as increases the double $CD8^+$ /γδT cells % in the site of infection. Increase in the expression of total $CD8^+$ and γδT cells suggesting the immune enhancement role induced by garlic extract treatment. Garlic extract treatment played an immunoregulatory role through increase in the γδT cells % in agreement with other previous reports [44], which mentioned that following infection of the intestinal epithelium with the coccidian parasite, *Eimeria vermiformis*, γδT cells support the rapid activation of other lymphoid cells.

An objective of this study was to investigate the origin of the expressed cytotoxic $CD8^+$ T cells in the site of infection in the presence and absence of garlic extract treatment. Although BRDU incorporated epithelial cells observed clearly at the site of infection in both groups at different days post infection, no double positive ($CD8^+$- BRDU incorporated) T cells seen in site of infection of the ileal tissues. These results may explain that the expressed cytotoxic T cells migrate from other immune places to the site of infection. Same data were described in previous studies in years of 2003 and 2005 respectively [45-46], which described that IELs are thought to be activated in the gut-associated lymphoid tissues, such as Peyer's patches (PP) or mesenteric lymph nodes (MLNs) and gain entry into intestinal epithelium through lamina propria (LP).

Conclusion

Garlic extract treatment enhanced the host immunity against *Eimeria vermiformis* infection in ICR mice through activation of the intraepithelial lymphocytes. $CD8^+$ T cell populations play an important role in the host cellular immunity against infection, in addition to, increase the expressed % of $CD8^+$ and γδ T cells in the ileum tissues. All expressed lymphocytes reach to the site of infection from immune organs and no proliferation was done in the ideal tissues.

Collective findings from this study suggest that garlic extract partially protects host against *E. vermiformis* infection through enhancement of the host's cellular immune responses.

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