

Activation and Recruitment of Regulatory T Cells via Chemokine Receptor Activation in *Trichinella spiralis*-Infected Mice

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Abstract: As most infections by the helminth parasite elicit the recruitment of CD4⁺CD25⁺Foxp3⁺ T (T_{reg}) cells, many scientists have suggested that these cells could be used for the treatment of immune-mediated inflammation and associated diseases. In order to investigate the distribution and alteration of activated T_{reg} cells, we compared the expression levels of T_{reg} cell activation markers in the ileum and gastrocnemius tissues 1, 2, and 4 weeks after infection. The number of T_{reg} cells was monitored using GFP-coded Foxp3 transgenic mice. In mice at 1 week after *Trichinella spiralis* infection, the number of activated T_{reg} cells was higher than in the control group. In mice at 2 weeks after infection, there was a significant increase in the number of cells expressing Foxp3 and CTLA-4 when compared to the control group and mice at 1 week after infection. At 4 weeks after infection, *T. spiralis* was easily identifiable in nurse cells in mouse muscles. In the intestine, the expression of *Gzmb* and *Klrg1* decreased over time and that of *Capg* remained unchanged for the first and second week, then decreased in the 4th week. However, in the muscles, the expression of most chemokine genes was increased due to *T. spiralis* infection, in particular the expression levels of *Gzmb*, *OX40*, and *CTLA-4* increased until week 4. In addition, increased gene expression of all chemokine receptors in muscle, *CXCR3*, *CCR4*, *CCR5*, *CCR9*, and *CCR10*, was observed up until the 4th week. In conclusion, various chemokine receptors showed increased expressions combined with recruitment of T_{reg} cells in the muscle tissue.

Key words: *Trichinella spiralis*, CD4⁺CD25⁺Foxp3⁺T cell, muscle phase, recruitment

INTRODUCTION

For 2 decades, the hygiene hypothesis - which proposes that the lack of early childhood exposure to infectious agents increases susceptibility to allergic diseases in later life - provided the conceptual framework for unravelling the mechanisms that could account for the increased incidence of allergic diseases [1]. Based on the “hygiene hypothesis”, as people migrate to a more hygienic environment, there are less opportunities to encounter the sources of infection [2]. The difference in the occurrence of allergic and immune-mediated disorders between developing and developed countries may be attributed to the prevalence of helminth infections [3,4]. Childhood

microbial and helminth infection have been shown to decrease the risk of developing the inflammatory bowel disease (IBD) [5]. Infection may result in changes in the gut microbiota, which could lead to the changes in the complex immune response mechanisms, involving regulatory T (CD4⁺CD25⁺Foxp3⁺T, T_{reg}) cells, toll-like receptor stimulation, etc. [6]. Almost all helminth infections elicit the recruitment of T_{reg} cells [7]. T_{reg} cells can inhibit the immune responses by directly killing cytotoxic cells through cell-to-cell contact, by inhibiting cytokine production of cytotoxic cells through IL-2 secretion, or by direct secretion of TGF-β and IL-10 as immunomodulatory cytokines [8]. It has been proven that T_{reg} cells are useful in the treatment of immune-mediated inflammation, autoimmune and autoinflammatory disorders, allergies, acute and chronic infections, cancers, and metabolic inflammations [9].

Trichinella spiralis infection activates the Th2 response until the nurse cell formation but after the formation of nurse cells, the Th2 response to the infection declines. At the same time, T_{reg} cell numbers increase significantly in the spleen and in sev-

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eral lymph nodes [10]. This parasite infection or the products can ameliorate several immune disorders, allergic airway inflammation, intestinal inflammation, and experimental autoimmune encephalomyelitis in a mouse model by T_{reg} cell recruitment and IL-10⁺ T cell activation [11-14]. Previous reports have suggested that T_{reg} cells are the most important cells in the amelioration of the allergic airway inflammation and intestinal inflammation [11-14]. Kang et al. [13] found that *T. spiralis* induced T_{reg} cells to migrate to the inflammation site and suppress the immune responses using adoptive transfer of T_{reg} cells.

However, the distribution of activated T_{reg} cells in specific tissues particularly the intestine and muscle tissues during the parasite life cycle is yet to be studied. In this study, we investigated the distribution and alteration of activated T_{reg} cell population by real-time PCR and immunohistochemistry analysis at each time point after *T. spiralis* infection.

MATERIALS AND METHODS

Parasites

T. spiralis strains (isolate code ISS623) were maintained in our laboratory through serial infection of rats. The parasites were isolated from the infected mice using dissection. Skin, fat, and internal organs were removed, and only the muscle and bones were used. The muscles and bones were cut into pieces, mixed with a digestive solution of 1% pepsin and 1% hydrochloride, and stirred continuously for 1 hr at 37°C. Then pepsin and hydrochloride were separated from the solution using a separating funnel, and the remains were washed with sterile PBS. After the wash, the muscle larvae of *T. spiralis* were collected.

Preparation of samples

GFP-tagged Foxp3 mice was purchased at Jackson Laboratory (Bar Harbor, Maine, USA) and kept in a pathogen-free facility at the Institute for Laboratory Animals of Pusan National University. Five-week-old mice were used. We designed 4 groups with 3 mice in each group; uninfected mice, 1-week-infected mice, 2-week-infected mice, and 4-week-infected mice. Each mouse was infected with 250 larvae of *T. spiralis* and was sacrificed after the designated infection time.

Total RNA extraction and real-time PCR

Total RNA was extracted from muscles and the intestine using 1 ml of RiboEx (GeneAll, Seoul, Korea), 200 µl chloroform

Table 1. Primer sequences for real-time PCR

Primer	Sequence
GAPDH-for	5'-TAC CCC CAA TGT GTC CGT C-3'
GAPDH-rev	5'-AAG AGT GGG AGT TGC TGT TGA AG-3'
CTLA-4-for	5'-GGA CGC AGA TTT ATG TCA TTG ATC-3'
CTLA-4-rev	5'-CCA AGC TAA CTG CGA CAA GGA-3'
Klrg1- for	5'-GGC TTG AGG AAC ATT GAT GG-3'
Klrg1-rev	5'-TCA AGC TGT TGG TAA GAA TCC TC-3'
Capg- for	5'-GCT GTG TGG CAA AAT CTA CAT C-3'
Capg- rev	5'-GAT GAA GCC ATC AGC CAC TT-3'
Gzmb- for	5'-GCT GCT CAC TGT GAA GGA AGT-3'
Gzmb- rev	5'-TGG GGA ATG CAT TTT ACC AT-3'
GARP-for	5'-TGA ATT CAT GAG CCA CCA GAT-3'
GARP-rev	5'-AGC GGC CGC TCA GGC TTT GTA-3'
OX40-for	5'-TAT GGT GAG CCG CTG TGA TC-3'
OX40-rev	5'-ACA GTC AAG GGA GCC AGC AG-3'
CD62L-for	5'-CAT TCC TGT AGC CGT CAT GG-3'
CD62L-rev	5'-AGG AGG AGC TGT TGG TCA TG-3'
CXCR3-for	5'-AGA ATC ATC CTG GTC TGA GAC AA-3'
CXCR3-rev	5'-AAG ATA GGG CAT GGC AGC TA-3'
CCR4-for	5'-ATC GTG CAC GCG GTA TTC TCC-3'
CCR4-rev	5'-GAC GGG GTT AAG GCA GCA GTG A-3'
CCR5- for	5'-GGA TTT TCA AGG GTC AGT TC-3'
CCR5-rev	5'-AAC CTT CTT TCT GAG ATC TGG-3'
CCR9- for	5'-ATT GCA CAA GAG TGA AGA CC-3'
CCR9-rev	5'-GTC AAC AGC CTG CAC TAC AA-3'
CCR10- for	5'-CGG AGA AAC CCT TGT AGC CAG-3'
CCR10-rev	5'-GGC CAA GAC TAG GCC ATT GCC-3'

for, forward; rev, reverse

and 500 µl isopropanol to remove the protein, DNA, and polysaccharides and to stabilize RNA. Extraction was carried out based on the protocol of the manufacturer. The cDNA was synthesized from the extracted RNA with MMLV reverse transcriptase (Promega, Madison, Wisconsin, USA) and oligo dT primer. Then, the cDNA of interest was amplified using gene specific primers (Table 1). The target genes were chemokine (C-X-C motif) receptor3 (CXCR3), chemokine (C-C motif) receptor4 (CCR), CCR5, CCR9, CCR10, killer cell lectin-like receptor subfamily G member 1(Klrg1), capping protein gelsolin-like (Capg), granzyme (Gzmb), glycoprotein A repetitions predominant (GARP), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), CD62 ligand (CD62L), and OX40 (CD134). The genes were amplified and measured by real-time PCR (Applied Biosystems 7500, Foster City, California, USA), and the relative gene transcription level was calculated based on the transcription level of the housekeeping gene GAPDH.

Immunohistochemistry and confocal microscopy

The muscles and intestines were dissected from each mouse,

fixed with paraffin, sliced into thin slices, and mounted onto slides. Paraffin was then removed and the tissues were treated with an antigen retrieval solution of 0.1 M citric acid, 0.1 M sodium citrate, and pH 6.0 for 20 min. The slide was rinsed and treated with methanol (0.3% H₂O₂) for 15 min in order to inhibit endogenous peroxidase activity. Before the incubation, the slides were placed in 1% BSA for 1 hr at room temperature. It was then incubated with hamster anti-mouse CTLA-4 (1:500; Santa Cruz Biotechnology, Santa Cruz, California, USA) for 1 hr at 4°C, was then washed several times with PBS before Alexa Fluor 594 goat anti-hamster IgG secondary antibody (1:500; Jackson Immuno Research Laboratories, West Grove, Pennsylvania, USA) was applied for 1 hr at room temperature. After the application of CTLA-4 antibody and DAPI, the slides were washed and incubated for 2 min. The prepared slides with their fluorescent signals were then observed under an inverted fluorescence microscope.

Statistical analysis

Means ± SD were calculated, and significant differences were determined using the Student's *t*-test comparing all groups

with the control group.

RESULTS

Alteration of T_{reg} cell population in intestinal tissue during *T. spiralis* infection

In order to know how many T_{reg} cell were activated and where they migrate after infection, the T_{reg} cells were monitored in the intestine and muscle tissues after infection using GFP-tagged Foxp3 expressed mice. In the intestine, we easily located T_{reg} cells, not only in *T. spiralis*-infected mice, but also in uninfected mice (Fig. 1). In the uninfected mice (control), there was only a small number of activated T_{reg} cells (expressed both Foxp3 and CTLA-4). In mice at 1 week after *T. spiralis* infection, the number of activated T_{reg} cells increased in comparison to the control group. In the 2-week infected mice, the number of cells expressing Foxp3 and CTLA-4 was much greater than in the control and 1-week infected mice. However, 4-week infected mice showed a reduction in T_{reg} cell numbers to levels lower than that seen in 2-week infected mice (Fig. 1).

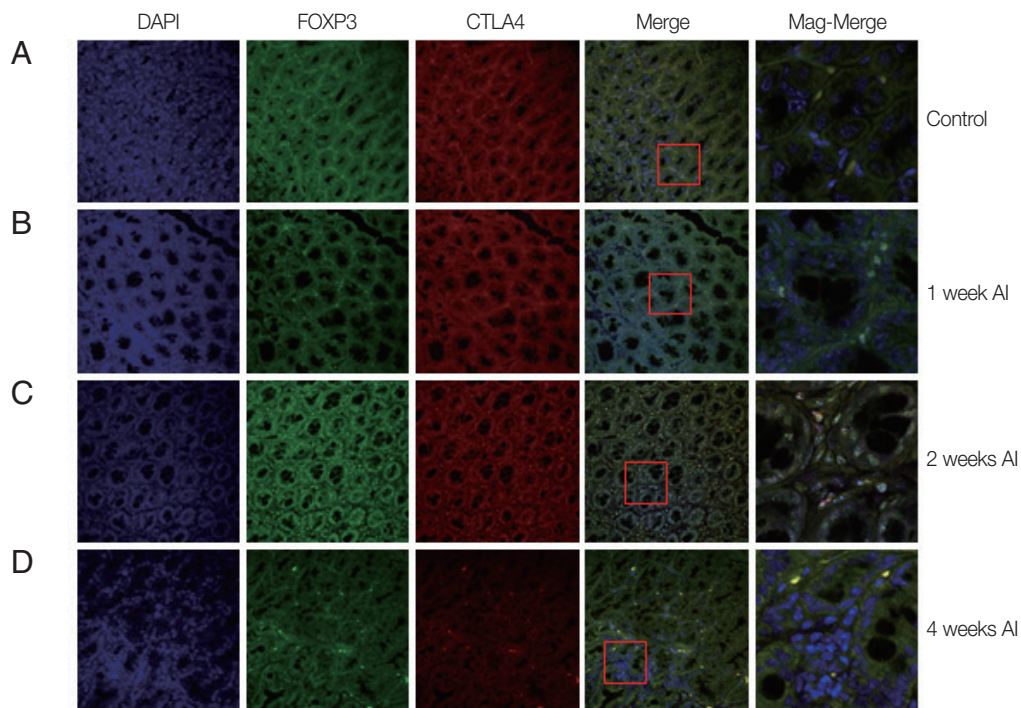


Fig. 1. Recruitment of T_{reg} cells in the intestine after *T. spiralis* infection. Mice were infected with *T. spiralis* and sacrificed at 0, 1, 2, and 4 weeks after infection. A fraction of the ileum tissue was dissected, and the sections were fixed and soaked in paraffin block. The thin section of the tissue was stained with DAPI and anti-CTLA-4 monoclonal antibody. The stained tissue was analyzed for cells expressing Foxp3, CTLA-4 using a confocal microscope. (A) Non-infected tissue. (B) 1 week after infection. (C) 2 weeks after infection. (D) 4 weeks after infection.

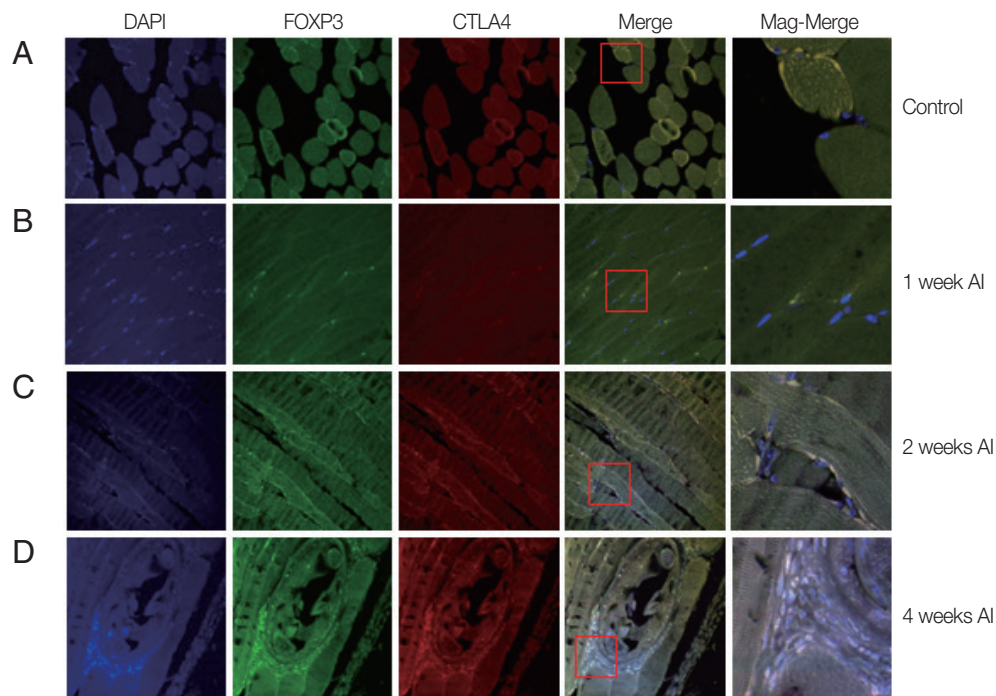


Fig. 2. Recruitment of T_{reg} cells in muscles after *T. spiralis* infection. Mice were infected with *T. spiralis* and sacrificed at 0, 1, 2, and 4 weeks after infection. The gastrocnemius tissue was dissected, and the tissues were fixed and soaked in paraffin block. The thin section of the tissue was stained with DAPI and anti-CTLA-4 monoclonal antibody. The stained tissue was analyzed for cells expressing Foxp3, CTLA-4 using a confocal microscope. (A) Non-infected tissue. (B) 1 week after infection. (C) 2 weeks after infection. (D) 4 weeks after infection.

Recruitment of T_{reg} cells around nurse cells

Auto-fluorescence was observed for muscle tissues infected mice or un-infected mice. At 1 week after infection, the muscle tissue was quite similar to that of the control group. However, 2 weeks after infection, small-sized *T. spiralis* larvae were sparsely found in the muscle tissues (Fig. 2). The CTLA-4 expressed T_{reg} cells were recruited around *T. spiralis* larvae. At 4 weeks after infection, nurse cells containing *T. spiralis* were easily located in the muscle. A number of immune cells were recruited to the nurse cells and many expressed Foxp3 and CTLA-4 (Fig. 2).

Activation markers of T_{reg} cells and gene expression levels of homing receptors in the intestine and muscle tissues during *T. spiralis* infection

The gene expression of activation marker of T_{reg} cell was evaluated to understand the functional features of T_{reg} cells recruited to the tissue. *CTLA-4*, *Klrg1*, *Capg*, *GARP*, *Gzmb*, *OX40*, and *CD62L* were measured. In the intestine, the expression of *Gzmb* and *Klrg1* was decreased over time, and *Capg* expression was unchanged for first and second week but decreased in the

4th week. *GARP* showed a significant and gradual increase until week 4. *OX40* expression decreased 1 week after infection, then gradually increased until week 4 (Fig. 3A). However, in muscles, the gene expression levels of most chemokines were elevated due to *T. spiralis* infection, especially *Gzmb*, *OX40*, and *CTLA-4* whose expression increased until week 4. In addition, *GARP*, *Klrg1* expression increased in first and second week but decreased 4 weeks after infection (Fig. 3B). The expression levels of chemokine receptors, *CXCR3*, *CCR4*, *CCR5*, *CCR9*, and *CCR10*, were measured to understand how the T_{reg} cells were recruited to the intestine and muscle after *T. spiralis* infection (Fig. 4). T_{reg} cell homing receptors guide T_{reg} cells to sites of inflammation quickly and accurately. The chemokine receptor expressed changes depending on the immunological setting and a change in chemokine receptor leads to a change in the distribution of T_{reg} cells in the tissue. In the intestine, expression of *CXCR3*, *CCR4*, *CCR5*, *CCR9* did not change significantly until week 4 but that of *CCR10* had increased significantly at week 4 (Fig. 4A). However in muscle, all chemokine receptors in this study, *CXCR3*, *CCR4*, *CCR5*, *CCR9*, and *CCR10*, showed an increase in their expression levels until week 4 (Fig. 4B).

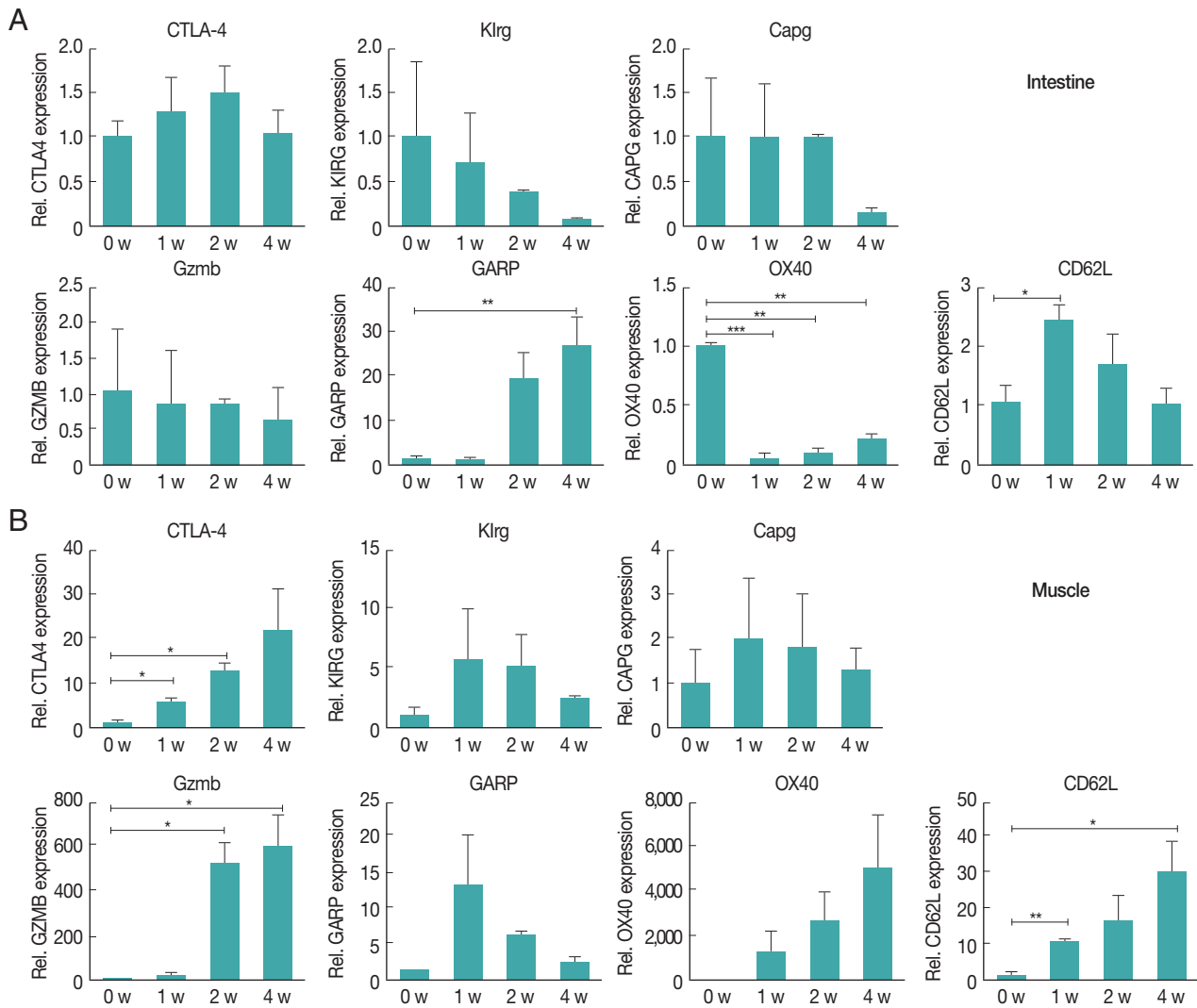


Fig. 3. Increased gene expression levels in *T*_{reg} activation in the intestinal (A) and muscle (B) tissues after infection. Activation and functional marker gene expression levels of *T*_{reg} cells were measured using real-time PCR. At 1, 2, and 4 weeks after infection, the ileum and gastrocnemius tissues were isolated from mice. Total RNA was isolated from 1 g of the tissue. To determine activation and functional marker expression, the gene expression levels of *CTLA-4*, *Klrg1*, *Capg*, *GARP*, *Gzmb*, *OX40*, and *CD62L* were measured.

DISCUSSION

In this study, we observed the dynamic recruitment of *T*_{reg} cells during *T. spiralis* infection, especially around muscles and the small intestine, by confocal microscopy (Figs. 1, 2). In addition, although most *T*_{reg} cells were activated in both the intestine and muscle tissue, the patterns were quite different (Fig. 3). This might be caused by *T. spiralis* larva migration according to their life cycle. In the small intestine, *T. spiralis* invade the mucosal layer at 10–28 hr after infection and remain for 2–3 weeks [15]. The number of *T*_{reg} cells increased 1 and 2 weeks after infection, coinciding with the development stage

of *T. spiralis*. At 4 weeks after infection, as *T. spiralis* reached the end of its life cycle, the number of *T*_{reg} cells decreased [10]. In muscles, the parasites started to invade at around 10 days and its nurse cells remained fully developed for 6 weeks after infection [15]. Thus, the number of the activated *T*_{reg} cells did not change in muscle tissue for 0–1 weeks after infection, after that it rapidly increased around the parasite until the 4th week of infection when the nurse cells were fully developed (Fig. 3).

Why do *T*_{reg} cells increase during invasion and development of *T. spiralis*? When infected with *T. spiralis*, the immune system activates the Th2 pathway which induces an inflammatory response against the intruder [10]. However, too strong an im-

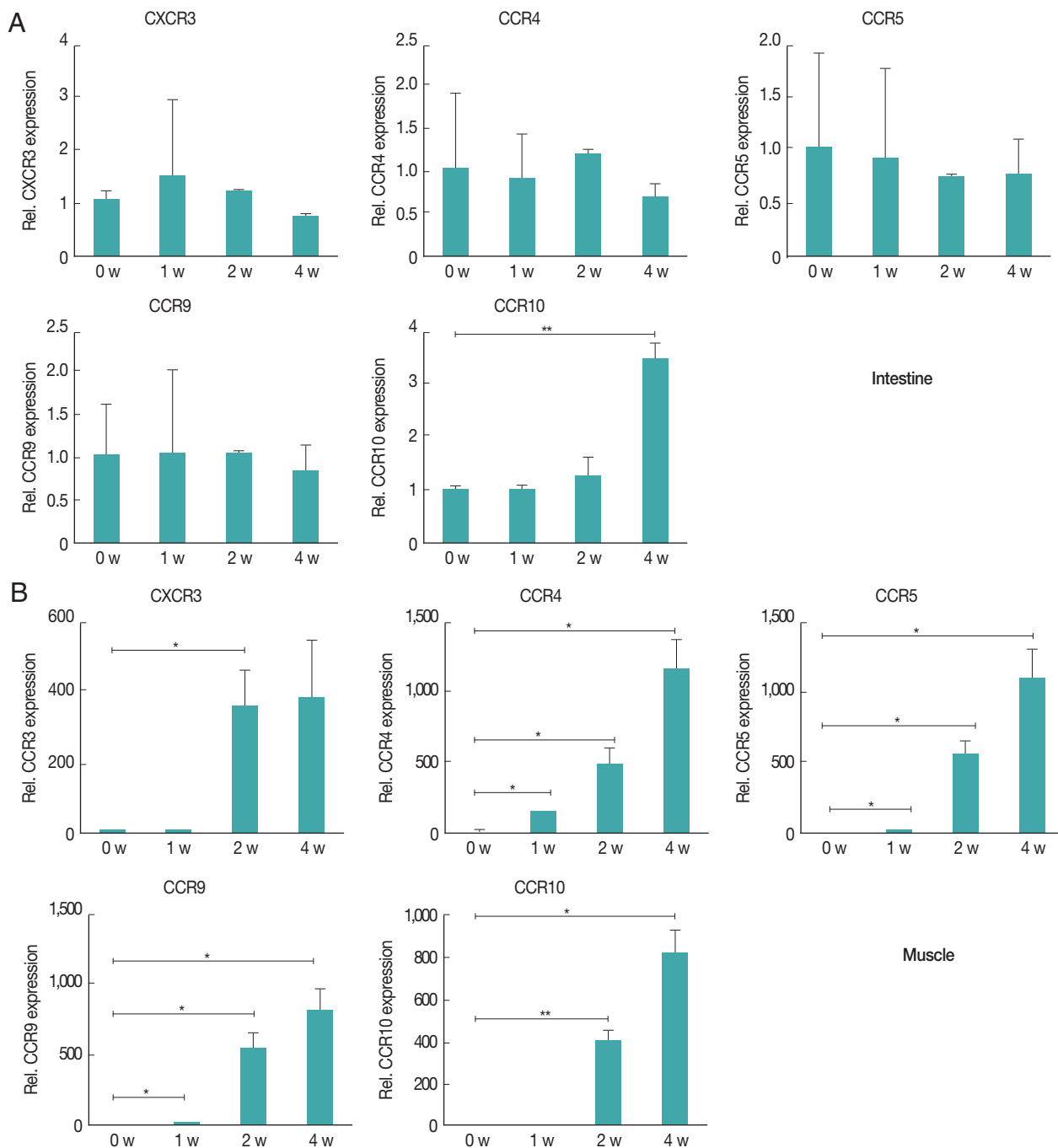


Fig. 4. Chemokine receptors in the muscle tissue showed higher levels of increased gene expression compared to receptors in the intestine after infection. Gene expression levels of chemokine receptors were measured using real-time PCR. At 1, 2, and 4 weeks after infection, the intestinal (A) and gastrocnemius (B) tissues were isolated from mice. Total RNA was isolated from 1 g of the tissue. *CXCR3*, *CCR4*, *CCR5*, *CCR9*, and *CCR10* gene levels were measured.

mune reaction cannot only kill the foreign substance but also the host's own tissues. Thus, the immune response is regulated by T_{reg} cells that are activated when there is an excessively large or long immune response. Some parasite infections induce T_{reg} cells, and these T_{reg} cells activate specific genes, such as those

encoding Foxp3, CTLA-4, CD103, GITR, OX40, Gzmb, Klr1, secretory leukocyte peptidase inhibitor (Slpi), fatty acid-binding protein 5 (Fabp5), nuclear factor, IL-3 regulated (Nfil3), suppressor of cytokine signaling 2 (Socs2) and G protein-coupled receptor 177 (Gpr177) [16-20]. In this study, we also ob-

served highly expression of several genes for T_{reg} cell activations [GARP and CD62L in intestine; CTLA-4, Gzmb, and CD62L in muscle tissue] (Fig. 3). The CTLA-4 activates the transmission of immunosuppressive signals on T effector cells by interacting the T effector ligands CD80 and CD86 [21,22]. GARP, which are expressed on the surface of activated T_{reg} cells, suppresses IL-2 and IFN- γ production and the differentiation and proliferation of naïve T cells into T effector cells, leading to the differentiation of naive T cells into induced T_{reg} cells [23,24]. Blocking OX40 on T_{reg} cells inhibits the cells' ability to suppress and restores effector T-cell proliferation [25]. Gzmb is released by activated T_{reg} cells, and it functionally induces apoptosis of naive B cells [26]. CD62L high-expressed T_{reg} cells have a higher immunosuppressive capacity than CD62 low expressed T_{reg} cells [27]. Therefore, *T. spiralis* could activate T_{reg} cell to suppress host Th2 immune response to them.

Activation of T_{reg} cell as well as How T_{reg} cells reach the target organ is also important. Studies about the localization of T_{reg} cells into specific sites reveal that the migratory behavior of T_{reg} cells is crucial for its regulatory role [28]. Chemokine receptors that are expressed on the surface of the cells play a major role in T_{reg} migration. Both the expression level and pattern of chemokine receptors determine the destination of a T_{reg} cell. The result from the intestines show that only *CCR10* expression increased significantly during the 4 weeks of infection (Fig. 4). The expression levels of other chemokine receptors, *CXCR3*, *CCR4*, *CCR5*, and *CCR9* remained mostly unchanged (Fig. 4). *CCR10* is a chemokine receptor that homes to epithelial cells and interacts with *CCL27* and *CCL28* ligands [6]. Although *CCL27* is mainly expressed in keratinocytes [29], *CCL28* is widely expressed in various mucosal tissues [30-32]. Eksteen et al. [30] isolated a subset of *CCR10*-expressing T_{reg} cells from a chronically inflamed human liver. Exposure of *CCR10*⁺ T_{reg} cells to *CCL28* in vitro stimulated migration and adhesion to mucosal addressin cell adhesion molecule-1 (MAdCAM-1) and vascular cell adhesion protein-1 (VCAM-1) [30]. During the 4 weeks of infection, *T. spiralis* stayed in the mucosa layer for the entirety of its 4-week life span (Fig. 1). The increased expression of *CCR10* in T_{reg} cells leads to the localization of T_{reg} cells into mucosal tissue of small intestine and thus, the regulation of the inflammatory response caused by *T. spiralis*. The result from muscle tissues show a significant increase in the level of all the chemokine receptors, *CXCR3*, *CCR4*, *CCR5*, *CCR9*, and *CCR10* (about 400 fold - about 1,500 fold than control) during the 4 weeks of infection (Fig. 4). The increase

in the expression of the *CCR4* gene was the most remarkable (> 1,500 fold than control). *CCR4* is expressed on the majority of T_{reg} cells, especially on effector T_{reg} cells [33]. In vivo administration of anti-*CCR4* mAb markedly reduced the effect T_{reg} cells population in an adult T-cell leukemia-lymphoma patient [34]. In addition, *CCR4* interacts with *CCL22* expressed on the surface of matured dendritic cells (DCs) [35]. Therefore, *CCR4* expression might be closely related to T_{reg} cell activation. *CCR4* upregulation in T_{reg} cells can home the cell to the lymph nodes for efficient activation [34,35].

Along with *CCR4*, *CCR5*, *CXCR3*, and *CCR9* have T_{reg} cell related immunological effects on inflammation. *CCR5* prevents uncontrolled post-infarction inflammation and protects from adverse remodeling by recruiting suppressive mononuclear cells [36]. Moreover, enhanced inflammation in the absence of *CCR5* was associated with impaired recruitment of T_{reg} cells [36]. The *CCR5*⁺ T_{reg} cell subset exhibited increased IL-10 expression, reflecting potent anti-inflammatory activity [36]. The chemokine *CXCR3* is expressed in the liver, lung and intestine and it reacts reacted with Th1-associated chemokines *CXCL9*, *CXCL10*, and *CXCL11* [37]. Therefore, when Th1-associated chemokine-expression is high in an organ, it might suppress *CXCR3*⁺ T_{reg} cells. In trichinellosis, Th1 cytokine expression was highly increased temporarily, but this elevation was suppressed by T_{reg} cell activation and recruitment [10]. Interestingly, *CCR9* (gut homing receptor) expression did not increase in the intestine tissue but increased in the muscle tissue during *T. spiralis* infection. *CCR9*, a gut-trophic chemokine receptor expressed by lymphocytes and dendritic cells, has been implicated in the regulation of IBD as it mediates the recruitment of T cells to site of inflammation [38,39]. Further studies are need to determine the *CCR9*-related mechanisms in trichinellosis.

In conclusion, we found higher levels of T_{reg} cell activation markers and recruitment in muscle tissue than in intestinal tissue. The expression of various chemokine receptors (most of them were closely related with T_{reg} cell recruitment) increased in the muscle tissue. These chemokine receptors might help in the migration and activation of T_{reg} cells and thus might be one of host's immunomodulation strategies in response to *T. spiralis* infection.

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CONFLICT OF INTEREST

We have no conflict of interest related to this work.

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