Disruption of CD40-CD40 Ligand Pathway Inhibits the Development of Intestinal Muscle Hypercontractility and Protective Immunity in Nematode Infection

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\textbf{Running title:} CD40-CD40 ligand interaction in muscle function and worm expulsion

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ABSTRACT

In our previous studies, we have demonstrated that during *Trichinella spiralis* infection, T helper (Th) 2 cells contribute to the development of intestinal muscle hypercontractility and worm expulsion from the gut via signal transducer and activator of transcription factor 6 (Stat6). In addition, we have linked the altered muscle contractility to the eviction of the parasite, and thereby to host defense. However, the initial events linking infection to the development of muscle hypercontractility are poorly understood. In this study, we examined the contribution of CD40-CD40 ligand interaction in the development of intestinal muscle hypercontractility, monocyte chemoattractant protein-1 (MCP-1) production and in the Th2 response in CD40 ligand deficient (CD40L -/-) mice infected with *T. spiralis*. Expulsion of intestinal worms was substantially delayed in CD40L -/- mice as compared to the wild-type mice following *T. spiralis* infection. Consistent with delayed worm expulsion, there was a significant attenuation of intestinal muscle contractility in CD40L -/- mice. Infected CD40L -/- mice also exhibited marked impairment in production of MCP-1, IL-4, IL-13, IgG1, IgE, mouse mucosal mast cell protease 1 (MMCP-1), and in goblet cell response. These results demonstrate that CD40-CD40 ligand interaction plays an important role in MCP-1 production, Th2 response, intestinal muscle hypercontractility, and worm expulsion in nematode infection. The present data suggest that the early events leading to the generation of Th2 response include CD40-CD40 ligand interaction, which subsequently influences the production of Th2 cytokines most likely via up-regulation of MCP-1.

**Key words:** CD40 ligand, MCP-1, Th2, muscle, *Trichinella spiralis*, host defense
INTRODUCTION

T helper (Th) 2 cells play an essential role in the development of host protective immunity in many intestinal nematodes infections including *Trichinella spiralis* (12, 14, 17). Signal transducer and activator of transcription factor 6 (Stat6) is critical in the differentiation of Th cells towards Th2 phenotype (43) and in a previous study by using Stat6 deficient (Stat6 -/-) mice we have shown a significant attenuation in infection-induced intestinal muscle contractility in Stat6 -/- mice as compared to the wild type mice and this was accompanied by delayed worm expulsion from the intestine (28). We were also able to demonstrate the presence of IL-4 and IL-13 in the muscularis externa layer during the primary infection with *T. spiralis*. These observations clearly indicated that Th2 cytokines, IL-4 and IL-13, acting via Stat6, mediate the development of nematode infection-induced intestinal muscle hypercontractility and that this, in turn contributes to the host defense by effective expulsion of worms. Nevertheless, the initial events linking infection to the development of the changes in intestinal muscle function are poorly understood. Moreover, in contrast to an increasing understanding of the mechanisms by which bacteria, virus or protozoa generates Th1 type responses, little is known about the initiation of Th2 type responses activated by nematode infection and this remains as an exciting area to explore.

The differentiation of naïve T cells into effector cells requires antigen recognition and signals provided by costimulatory molecules expressed on antigen presenting cells (APCs). Among the costimulatory pathways, the CD40-CD40 ligand (CD40L) pathway plays an important role in T cell responses (19). Although the mechanisms by which this
pathway effects T cell responses are not clear, up-regulation of other costimulatory pathways or enhancement of chemokines and cytokines production are likely relevant. CD40 is a cell surface receptor that belongs to the tumor necrosis factor-R (TNF-R) family, is constitutively expressed on APCs, whereas CD40L is a member of TNF family, and is induced on T cell activation (47). The interaction between CD40 and CD40L has been shown to regulate both humoral and cellular immune responses (19, 47). The demonstration of the critical role of CD40-CD40L interaction in T cell dependent B cell responses was revealed by the findings that the hyper-IgM syndrome, an X-linked immunodeficiency disorder is due to the mutation of CD40L gene. This disease is characterized by a severe impairment in T cell dependent antibody responses with no B cell memory, defective germinal center formation and a markedly reduced antibody repertoire (4, 15). Mice deficient in CD40 and in CD40L genes demonstrate a phenotype comparable to that of the individuals suffering from the hyper-IgM syndrome (25, 40, 49). A contribution of CD40-CD40L interactions to the process of T cell priming, differentiation and effector functions has been also described (20, 36).

Manipulation of CD40-CD40L pathway in animal models by the use of mice deficient in CD40 or CD40L as well as the use of neutralizing antibodies enables study on the role of CD40-CD40L interaction in several disease models, including transplantation, autoimmunity, and infectious diseases (47). Most of the studies on the CD40-CD40L interactions during infection have focused on the infective agents that generate Th1 responses (18) and have convincingly demonstrated the importance of this interaction in the development of Th1 immune responses during infection. In contrast, there are few
studies that have examined CD40-CD40L interactions in the development of Th2 based immune responses and host defense. Recently a role for CD40-CD40L interaction in the development of Th2 mediated host protective immunity has been described in mice infected with *Schistosoma mansoni* (35).

Monocyte chemoattractant protein-1 (MCP-1) is a member of the C-C chemokine family and acts on T cells, monocytes, and natural killer cells. Studies in MCP-1 deficient mice suggest that this chemokine is involved in the development of Th2 type immune response and in intestinal worm expulsion during nematode infection (11).

In the present study we examined the contribution of CD40 and CD40L interaction in the development of intestinal muscle hypercontractility, in MCP-1 production and in the Th2 response in *T. spiralis* infection using CD40L deficient (CD40L -/-) mice. We reasoned that if CD40-CD40L interaction and MCP-1 are required for the development of Th2 response, then interruption of CD40-CD40L interaction should not only prolong the infection but should also attenuate infection-induced muscle hypercontractility and inhibit MCP-1 production. For the first time we demonstrate that interaction between CD40 and CD40L is required for the generation of infection-induced intestinal muscle hypercontractility during the primary infection with *T. spiralis*. Our results also confirm the importance of CD40-CD40L interaction in the development of Th2 response and host defense. The results also suggest an important role of CD40-CD40L interaction in the production of MCP-1, which subsequently may influence the generation of Th2 response during nematode infection.
MATERIALS AND METHODS

Animals

CD40L deficient (CD40L -/-) mice on C57BL/6 background and C57BL/6 (CD40L +/+) were purchased from the Jackson Laboratories (Bar Harbor, Maine). Mice were kept in sterilized, filter-topped cages, and fed autoclaved food in the animal facilities of McMaster University. Only the male mice were used at the age of 8-10 weeks. The protocols employed were in direct accordance with guidelines drafted by the McMaster University Animal Care Committee and the Canadian Council on the Use of Laboratory Animals.

Parasitological Techniques

The *T. spiralis* parasites used in the study originated in the Department of Zoology at the University of Toronto and the colony was maintained through serial infections alternating between male Sprague-Dawley rats and male CD1 mice. The larvae were obtained from infected rodents 60-90 days post-infection (p.i.), using a modification (48) of the technique described by Castro and Fairbairn (6). Mice were killed at various time points after infection. Adult worms were recovered from mice after the intestine had been opened longitudinally, rinsed and placed in Hank’s balanced salt solution for 3 hours at 37°C. Worms were counted under a dissecting microscope.

Measurement of Muscle Contraction in Intestinal Muscle Strips

Preparation of intestinal muscle strips for muscle contractility experiments, and analysis of carbachol-induced contraction have been described previously (45). Briefly, the
jejunum was removed and placed in oxygenated (95% O2 – 5% CO2) Kreb’s solution and 1 cm sections of whole gut were cut from the jejunum. The lumen of each segment was flushed with Kreb’s buffer, prior to the insertion of short (2-3 mm) lengths of Silastic tubing (0.065 in. OD, 0.030 in. ID; Dow Corning, Midland, MI) into the open ends of the gut segments. Tubing was then tied in place with surgical silk. The insertion of the tubing was found to maintain patency of the gut segments, over the course of experiments. Segments were then hung in the longitudinal axis and attached at one end to a grass FT03C force transducer (Grass, Quincy, MA) and responses were recorded on a Grass 7D polygraph. Tissues were equilibrated for 30 minutes at 37 °C in Kreb’s buffer, oxygenated with 95% O2 –5% CO2 before starting the experiment. The previously identified optimal tension was then applied in carbachol dose-response experiments before the addition of the first dose of carbachol (45). Previous experiments indicated that this was optimal tension to determine the maximal responsiveness of both control and inflamed tissues. After the application of tension, gut segments were exposed to different concentrations of carbachol. After the maximal response to each dose was obtained, tissues were rinsed twice and equilibrated in fresh Krebs solution for 15 min before addition of the next dose. Contractile responses to carbachol were expressed as milligrams of tension per cross sectional area as described previously (45). For each mouse, the mean tension was calculated from at least three segments.

**Mouse Mast Cell Protease-1 (MMCP-1) ELISA**

Levels of MMCP-1 in serum were measured with an MMCP-1 ELISA kit purchased from Moredun Animal Health (Penicuik, UK) as previously described (23). Briefly,
rabbit anti-MMCP was used as capture antibody. Tenfold serial dilutions of serum were made from 1/10 to 1/10,000. Horseradish peroxidase-conjugated rabbit anti-mouse MMCP-1 was then added, quantification was made by reference to purified MMCP-1.

Evaluation of in vitro IL-4, IL-13, MCP-1 and IFN-γ production from spleen cells

Single-cell suspensions of spleen were prepared in RPMI 1640 containing 10% FCS, 5 mM L-glutamine, 100 U/ml penicillin, 100μg/ml streptomycin, 25 mM Hepes, 0.05 mM 2-ME (all Gibco-BRL). Cells (1x10^7) were incubated in the presence of 5μg/ml Concanavalin A (Con A) or 50 μg/ml T. spiralis antigen.

Culture supernatants were harvested after 24 hours and were stored at -20 °C. IL-4, IL-13, MCP-1 and IFN-γ levels in the supernatants were measured by enzyme immunoassay technique using commercially available kits purchased from R & D Systems (Minneapolis, MN).

Investigation of intestinal tissue IL-4 level

Frozen intestinal tissues were homogenized in lysis buffer containing protease inhibitor cocktail (Sigma). The homogenates were freeze-thawed three times, centrifuged and supernatant collected and stored at -20°C until analyzed.

IL-4 level in the supernatant was measured by enzyme immunoassay technique using commercially available kit purchased from R & D Systems (Minneapolis, MN). Concentration of protein in the intestinal tissue was determined by commercially
available DC Protein Assay Kit (BIORAD) and the amount of IL-4 in the tissues were expressed per mg of tissue protein.

**Determination of serum IgG1, IgG2a and IgE levels**

Levels of parasite specific IgG1, IgG2a and total IgE responses were determined as previously described (16) with slight modification. Briefly, wells of 96 wells microtiter plate was coated with 5 µg *T. speralis* antigen in 0.05M carbonate buffer (pH 9.6) and incubated over night at 4°C. After washing with saline containing 0.1% Tween-20, free binding sites of each well were blocked with blocking buffer [100 ml of washing buffer and 2 gm of Bovine Serum Albumin (BSA)]. Following washing, serum samples diluted with blocking buffer were applied. After incubation at 37°C for 1 h, the wells were washed and then peroxidase conjugated anti-mouse IgG1 or IgG2a were added to the wells. The plate was incubated at 37°C for 1 h. After washing, a mixture of H₂O₂ and substrate, 2,2-azino-di-(3-methylbenzthiazoline sulphonic acid) (ABTS, Sigma, St. Louis, Mo, USA) in substrate buffer was added to each well, incubated for 30 minutes and then the absorbance was read at the wave length of 405 nm.

For determination of total IgE, wells were coated with monoclonal anti-mouse IgE antibody (5µg/ml) and incubated overnight at 4°C. Following washing and blocking serum samples and IgE standard diluted in the blocking agent were added to the wells. Following incubation, wells were washed and peroxidase-conjugated anti-mouse IgE were applied to the wells. After washing, a mixture of H₂O₂ and substrate, ABTS, was
added to each well, incubated for 30 minutes and then the absorbance was read at the wave length of 405 nm.

Histology
A segment (1 cm in length) of small intestine or cecum tip was fixed in 10% neutral buffered formalin (NBF), and processed using standard histological techniques. The sections from NBF were stained with periodic acid-Schiff reaction for counting intestinal goblet cells. The numbers of goblet cells were expressed per 10 villus crypt units.

Statistical analysis
Data were analyzed using Student’s t-test with \( P < 0.05 \) considered significant. All results are expressed as the mean ± SEM.

RESULTS

Expulsion of worms is inhibited in CD40L -/- mice infected with \textit{T. spiralis}
To elucidate the role of CD40-CD40L interaction in \textit{T. spiralis} expulsion, CD40L +/+ and CD40L -/- mice were infected with \textit{T. spiralis} larvae and sacrificed on different days after infection. Almost all worms were expelled from CD40L +/+ mice by day 21 p.i. However, worm expulsion was significantly delayed in CD40L -/- mice. We recovered significantly higher number of worms from CD40L -/- mice as compared to that from CD40L +/+ mice on day 21 p.i. (Figure 1), indicating prolonged infection in CD40L -/- mice.
CD40-CD40L interaction is required in the development of infection-induced intestinal muscle hypercontractility during *T. spiralis* infection

The contribution of CD40-CD40L interaction to intestinal muscle contraction during *T. spiralis* infection was investigated using CD40L -/- mice. There was no significant difference in intestinal muscle contractility between non-infected CD40L +/+ and CD40L -/- mice. Infection in CD40L +/+ mice was accompanied by intestinal muscle hypercontractility evident on days 7 and 14 p.i. This infection-induced hypercontractility was not evident in infected CD40L -/- mice. We observed significantly lower intestinal muscle contraction in CD40L -/- mice on days 7 and 14 p.i. as compared to that in CD40L +/- mice (Figure 2). This was not a reflection of the carbachol dose used in these experiments as differences between infected CD40L -/- and infected CD40L +/- were observed over several doses, as shown in Figure 3.

Absence of CD40-CD40L interaction reduced Th2 type cytokine production during *T. spiralis* infection

Measurement of in vitro cytokine production from spleen cells by stimulation with Con A and *T. spiralis* antigen, which stimulates T lymphocytes non-specifically and specifically, respectively, revealed severe impairment of IL-4 and IL-13 production in CD40L -/- mice as compared to CD40L +/- mice after *T. spiralis* infection (Table 1). As expected we observed high amounts of both IL-4 and IL-13 in CD40L +/- infected mice. However, production of IL-4 and IL-13 was severely impaired in CD40L -/- infected mice. We observed significantly lower amount of IL-4 and IL-13 following stimulation with both Con A and *T. spiralis* antigen in CD40L -/- mice. We also observed markedly lower
amount of IFN-γ in infected CD40L+/+ mice as compared to IL-4 and IL-13 production and this was further reduced in infected CD40L -/- mice.

To evaluate local Th2 cytokine production we also measured intestinal tissue IL-4 levels. As shown in Figure 4 there was significantly lower amount of intestinal tissue IL-4 in infected CD40L -/- mice as compared to that in infected CD40L +/+ mice both on days 7 and 14 p.i.

**Production MCP-1 impaired in the absence of CD40-CD40L interaction**

Investigation of MCP-1 production in the supernatant of *in vitro* Con A stimulated spleen cells demonstrated significantly reduced level of MCP-1 in CD40L -/- mice as compared to CD40L +/+ mice after *T. spiralis* infection on days 7 and 14 p.i. (Figure 5), indicating an important role for CD40-CD40L interaction in the production of this chemokine following the infection.

**Absence of CD40-CD40L interaction reduced IgG1 and IgE responses during *T. spiralis* infection**

We next investigated IgG1, IgG2a and IgE responses in the serum of CD40L +/+ and -/- mice. We observed significantly higher *T. spiralis* antigen specific IgG1 response in CD40L +/+ mice following infection as compared to that in CD40L -/- mice. Indeed, the level of IgG1 in CD40L -/- infected mice was almost similar to that in non-infected CD40L +/+ and non-infected CD40 -/- mice (Figure 6a). There was no significant
increase in antigen specific IgG2a response in both CD40L +/+ and CD40L -/- mice following infection.

Total IgE level in the serum was not detected on day 7 p.i in either CD40L +/+ and CD40L -/- mice. We observed significantly elevated IgE level on days 14 and 21 p.i in CD40L +/+ mice as compared to that in control non-infected mice. However, CD40L -/- mice failed to exhibit IgE until day 21 p.i. (Figure 6b).

Absence of CD40-CD40L interaction attenuated MMCP-1 production and development of intestinal goblet cell hyperplasia during *T. spiralis* infection

We next investigated serum MMCP-1 and intestinal goblet cell hyperplasia, which are considered to be a Th2 mediated characteristic components of intestinal nematode infection. Serum MMCP-1 levels were undetectable in non-infected mice. Infection with *T. spiralis* caused a significant increase in serum MMCP-1 concentration in CD40L +/+ mice as compared to that in non-infected control. However, the serum MMCP-1 level was significantly lower in CD40L -/- mice following infection on both days 7 and 14 p.i (Figure 7).

Marked increased numbers of goblet cells were observed in the intestine of CD40L +/+ mice on days 7 and 14 p.i. following *T. spiralis* infection as compared to that in non-infected control. However, we observed significantly lower number of goblet cells in CD40L -/- mice as compared to that in CD40L +/+ mice on day 14 following the infection (Figure 8).
DISCUSSION

The present study demonstrates a critical role for CD40-CD40L interaction in the development of intestinal muscle hypercontractility and in the generation of effective Th2 immune response during infection of mice with *T. spiralis*. As we have shown previously that intestinal muscle hypercontractility is mediated largely via Stat6 and Th2 cytokines (28), we interpret the attenuation of muscle contractility as a consequence of an impaired Th2 response due to the absence of CD40-CD40L interaction. It seems likely that CD40-CD40L interaction following infection activates Th cells, and subsequently Th cells differentiate towards Th2 cells via Stat6 pathway. Disruption of CD40-CD40L pathway inhibited this process. The results of the present study also show marked attenuation of MCP-1 production in the absence of CD40-CD40L interaction; the absence of MCP-1 may be a reason for the attenuated Th2 response in infected CD40L -/- mice. Taken together, these data suggest that the initial events regulating differentiation of Th cells toward Th2 pathway in nematode infection include CD40-CD40L interaction and the generation of MCP-1.

Altered intestinal muscle function has been described in rodents following infection by several gastrointestinal nematodes including *Nippostrongylus brasiliensis* (13), *Heligosphoides polygyrus* (50), *T. muris* (27) and *T. spiralis* (28, 45, 48). We consider this increase in muscle contractility as a critical component of host protection during *T. spiralis* infection. As the expelled worms in *T. spiralis* infection retain their viability and fecundity, the worms are actually evicted from the gut and this draws attention to the role of the motor system in this process. Previous studies from our laboratory showed
regional differences in the intestine in response to inflammation, with muscle from the jejunum exhibited increased contractility whilst that from the ileum and colon exhibits reduced contractility (21, 37). We assumed that the distribution of changes in contractility would create an aboral gradient during infection, thereby enhancing aboral propulsion of luminal contents. If such forces contribute to the host defense against parasite, then one might expect to see a relationship between the magnitude of intestinal muscle hypercontractility and the ability of the host to evict the parasites from the gut. This is indeed the case, with strong responder to *T. spiralis* infection such as NIH Swiss mice, which rapidly expel the parasite exhibiting the greater degree of muscle contractility and slow responders such as B10.BR mice, which expel the parasite slowly, exhibiting a lesser degree of hypercontractility (45).

There is infiltration of intestinal muscle layers by T lymphocytes during *T. spiralis* infection and ultrastructural analysis revealed close communication between lymphocytes and muscle cells indicating a direct interaction between these cells (8). Recent work has revealed the presence of IL-4 and IL-13 in the intestinal muscle layer and attenuation of muscle hypercontractility in mice deficient in Stat6, suggesting an important role for Th2 cytokines (28). It has been also shown that IL-4 and IL-13 enhances carbachol induces contraction in dispersed smooth muscle cells isolated from the jejunum of mice (1). Very recently, it has been also reported that IL-4 and IL-13 play important role in the generation of intestinal muscle contractility in mice infected with *N. brasiliensis* and *H. polygyrus* (50) further supporting the hypothesis that Th2 response mediate intestinal muscle contractility in nematode infection. Previously we have shown that transfer and
overexpression of IL-12 gene during Th2-based *T. spiralis* infection shifts the immune response towards Th1 and inhibits worm expulsion. In addition, this shift in immune response attenuated infection induced muscle hypercontractility (26). Although considering the influence of enteric nerves, interstitial cells of Cajal and endocrine factors, changes in contractility observed *in vitro* cannot be extrapolated directly into the *in vivo* situation, muscle contractility is an acceptable and convenient method of assessing how inflammatory and immune cells alter this component of intestinal motility. Taken together, these results clearly indicate that the Th2 type immune response is critical for the development of intestinal muscle hypercontractility in this infection and also that the muscle hypercontractility may be an important component in parasite eviction from the intestine.

Th2 cells are not pre-committed phenotypes but rather, denote the outcome of a multi-step differentiation process by which a common precursor population acquires a distinct cytokine secretion profile. The observation that the deficiency in CD40L in mice is associated with attenuated muscle contractility, and prolonged worm expulsion suggest that the CD40-CD40L interaction is an important event in the early stages of effective protective immunity development following this nematode infection. This requirement is evident in the markedly impaired ability of CD40L -/- mice to produce Th2 cytokines, IL-4 and IL-13 from both *in vitro* stimulated spleen cells and intestinal tissues, and in their reduced ability to produce antibodies, IgG1 and IgE, which are characteristic of Th2 based infection. This observation clearly emphasized the importance of CD40-CD40L interaction in the development of a Th2 type immune response. Recently an important
role of CD40-CD40L interaction in the development of Th2 immune response has been also shown in another helminth infection, *Taenia crassiceps* (41). The reduction in Th2 cytokines production in the absence of CD40-CD40L interaction was not associated with a shift towards Th1 response as there was no up-regulation in IFNγ production in CD40L -/- mice. Indeed, we observed a reduction in IFN-γ production in infected CD40L +/- mice suggesting a difference in activation of Th2 response between infected CD40L +/- mice CD40L -/- mice rather than a difference due to a shifting of immune response.

Chemokines play well important roles in directing cell movements necessary for the initiation of T cell immune responses. They are required to attract APCs to the sites of inflammation, direct APCs to lymphatic vessels, and bring APCs and lymphocytes together within lymphoid organ (9). Chemokines are also considered important in recruiting appropriate effector cells to the sites of inflammation, including the differential recruitment of Th1 and Th2 cells (42). Recent studies indicate that they also play substantial roles in regulation of T cell differentiation (34). We observed a marked reduction in MCP-1 production in the CD40L -/- mice following *T. spiralis* infection. MCP-1 is produced by a variety of cells including dendritic cells, macrophages, endothelial cells and fibroblasts, and expression is up-regulated after exposure to inflammatory stimuli such as IL-1, TNF-α and IL-4 (46). Expression of MCP-1 is up-regulated in serum and intestinal muscle layer following *T. spiralis* infection (10, 39). MCP-1 originally identified as a monocyte-specific chemoattractant but later shown to act on T cells, mast cells, basophils and natural killer cells (2, 3, 5, 31). In relation to the polarization of Th cells, MCP-1 has been reported to cause a decrease in IL-12
production from activated macrophages (7), and an increase in IL-4 production from CD4+T cells (24, 33), suggesting a role of this chemokine in the regulation of Th2 response. MCP-1 deficient mice are resistant to *Leishmania major* infection, the model that requires Th1 immune response for clearing the infection (22). Recently it has been reported that in the absence of MCP-1, worm expulsion is inhibited during infection with intestinal nematode, *T. muris* and this was associated with a shift of immune response toward Th1 type from Th2 type (11). We also observed an enhancement of Th2 response in *T. spiralis* mice following transfer and overexpression of MCP-1 gene (29). Thus the reduced level of Th2 response following *T. spiralis* infection in CD40L-/- mice we observed in this study may be associated with the impairment in MCP-1 production resulting from the absence of CD40-CD40L interaction.

Characteristic components of the Th2-based immune response to nematode infections include mucosal mastocytosis and goblet cell hyperplasia (12, 17). Here, we also investigated the role of CD40-CD40L interaction in the development of mast and goblet cells. Mast cells are generally considered important in host protective immunity in *T. spiralis* infection, although some controversy exists (30, 44). To investigate the mast cell response we have quantified the serum levels of MMCP-1, a protease known to be produced by mucosal mast cells *in vivo* (38). Although the level of MMCP-1 was significantly elevated during *T. spiralis* infection in CD40L +/- mice, the infection-induced MMCP-1 response was significantly affected in CD40L -/- mice. Similarly, we also observed an inhibition in the development of hyperplasia of mucin secreting intestinal goblet cells. These observations further support the notion that CD40-CD40L
interaction is a major component in the development of effective Th2 mediated host responses. Related to this it has been also reported that treatment of mice with anti-CD40L antibody blocked the *H. polygyrus* induced elevation of IgG1, and inhibited the development of mucosal mastocytosis and blood eosinophilia (32), further suggesting that CD40-CD40L interaction may be required for the proliferation/differentiation of cells other than B cells.

Taken together, our data provide substantial evidence that the initial events in the generation of Th2 mediated muscle hypercontractility and host protective immunity during nematode infection include the interaction between CD40 and CD40L, which subsequently influence the production of Th2 cytokines most likely via up-regulation of MCP-1. These results are also consistent with our hypothesis that intestinal physiological changes such as muscle function and the worm expulsion in nematode infection share a common immunological basis and may be causally linked.

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### Table 1. Cytokine produced by *in vitro* stimulated spleen cells from CD40L +/- and CD40L -/- mice infected and non-infected mice.

<table>
<thead>
<tr>
<th></th>
<th>CD40L +/-</th>
<th>CD40L -/-</th>
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<tbody>
<tr>
<td></td>
<td>ConA</td>
<td>Antigen</td>
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<tr>
<td><strong>Non-Infected</strong></td>
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<tr>
<td>IL-4</td>
<td>20.38±3.19</td>
<td>10.02±4.07</td>
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<tr>
<td>IL-13</td>
<td>105.40±25.31</td>
<td>35.32±8.82</td>
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<tr>
<td>IFN-γ</td>
<td>20.20±3.73</td>
<td>7.99±2.62</td>
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<td><strong>Day 7 post-infection</strong></td>
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<tr>
<td>IL-4</td>
<td>611.13±73.16</td>
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<tr>
<td>IFN-γ</td>
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<tr>
<td><strong>Day 14 post-infection</strong></td>
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<tr>
<td>IL-4</td>
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<td>110.27±10.83</td>
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<tr>
<td>IL-13</td>
<td>1066.15±99.48</td>
<td>817.25±59.29</td>
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<tr>
<td>IFN-γ</td>
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Spleen cells from non-infected and infected CD40L +/- and CD40L -/- mice were stimulated with Con A or *T. spiralis* antigen for 24 hours and the levels of IL-4, IL-13 and IFN-γ present in the supernatant was investigated by ELISA. Each value (pg/ml) represents mean±SE from five mice. *Significantly different between Con A stimulated spleen cells from CD40L +/- and spleen cells from CD40L -/- mice. **Significantly different between antigen stimulated spleen cells from CD40L +/- and spleen cells from CD40L -/- mice.
LEGEND FOR FIGURES

Figure 1. Worm recovery from CD40L +/+ and CD40L -/- mice after *T. spiralis* infection. Mice were infected with 375 *T. spiralis* larvae orally and killed on the days indicated to investigate the worm recovery from intestine. Each bar represents mean±SEM from five animals. *Significantly different between CD40L +/+ and CD40L -/- mice.

Figure 2. Contraction of intestinal muscle from CD40L +/+ and CD40L -/- mice. Maximum tension generated by intestinal muscle taken from non-infected and *T. spiralis* infected CD40L +/+ mice and CD40L -/- mice in response to carbachol. CD40L +/+ and CD40L -/- mice were infected with 375 *T. spiralis* larvae orally and killed on the time points indicated. *Significantly different between CD40L +/+ and CD40L -/- mice.

Figure 3. Dose-response relationships for carbachol induced contraction of muscle from non-infected and *T. spiralis* infected CD40L +/+ mice and CD40L -/- mice. CD40L +/+ and CD40L -/- mice were infected with 375 *T. spiralis* larvae orally and killed on day 14 p.i. Each value represents mean±SEM from five animals. *Significantly different between infected CD40L +/+ and infected CD40L -/- mice on day 14 p.i.

Figure 4. Intestinal tissues IL-4 levels. CD40L +/+ and CD40L -/- mice were infected with 375 larvae *T. spiralis* orally and killed on days 7 and 14 p.i to investigate intestinal tissue IL-4. Each value (pg/ml) represents mean±SE from five mice. *Significantly different between CD40L +/+ and CD40L -/- mice.
Figure 5. MCP-1 production from in vitro ConA stimulated spleen cells. CD40L +/- and CD40L -/- mice were infected with *T. spiralis* orally and killed on days 7 and 14 p.i. Spleen cells were stimulated with Con A for 24 hours and the levels of MCP-1 present in the supernatant was investigated by ELISA. Each value (pg/ml) represents mean±SE from five mice. *Significantly different between CD40L +/- and CD40L -/- mice.

Figure 6. Levels of serum immunoglobulins in CD40L +/- and CD40L -/- mice following *T. spiralis* infection. Mice were infected with *T. spiralis* orally and killed a) on day 14 p.i to assess serum IgG1 and IgG2a responses and b) on days 7, 14 and 21 to investigate IgE. Each value represents mean±SEM from four animals.

Figure 7. Serum MMCP-1 response in CD40L +/- and CD40L -/- mice following *T. spiralis* infection. Mice were infected with *T. spiralis* orally and were killed on the time points indicated to investigate serum MMCP-1. Each value represents mean±SEM from four animals.

Figure 8. Intestinal goblet cell response in CD40L +/- and CD40L -/- mice following *T. spiralis* infection. Mice were infected with *T. spiralis* and were killed on days 7 and 14 p.i to investigate PAS stained intestinal goblet cell number. Each bar represents mean±SEM from five animals. *Significantly different between CD40L +/- and CD40L -/- mice.*
Figure 1

Worm burden

CD40L +/+  CD40L -/-

Day 14  Day 21

Time After Infection

*
Figure 2

After *T. spiralis* Infection

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<tr>
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<th>Non-infected</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
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Figure 3

Non-infected CD40L +/+  
Non-infected CD40L -/-  

T. spiralis + CD40L +/+  
T. spiralis + CD40L -/-  

Carbachol conc. (log M)  

Tension (mg/mm²)
Figure 4

After T. spiralis infection

IL-4 (pg/mg protein)

CD40L +/+  
CD40L -/-
Figure 5

After *T. spiralis* infection
**Figure 6**

(a) Abs 405 nm

- CD40L +/+ and CD40L -/
- No Infection and T. spiralis

(b) IgE (µg/ml)

- CD40L +/+ and CD40L -/
- Day 0, Day 7, Day 14, Day 21
Figure 7

MMCP-1 (ng/ml)

- CD40L +/+  
- CD40L -/-

Non-infected Day 7 Day 14

After * T. spiralis* infection
Figure 8

After *T. spiralis* infection

<table>
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<th>CD40L -/-</th>
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Goblet cells/10 VCU