의학박사 학위논문

Therapeutic effects of anti-CXCL10 antibody on C protein-induced myositis (CIM) mouse

C단백유발근육염(C protein-induced myositis, CIM) 생쥐에서 항CXCL10항체의 치료효과에 관한 연구

2013년 2월

서울대학교 대학원

의학과 면역학 전공

김 진 현

C단백유발근육염(C protein-induced myositis, CIM)

생쥐에서 항CXCL10항체의 치료효과에 관한 연구

지도교수 송 영 욱

이 논문을 의학박사 학위논문으로 제출함

2012년 10월

서울대학교 대학원

의학과 면역학 전공

김 진 현

김진현의 의학박사 학위논문을 인준함

2012년 12월

위 원 장 _____

부위원장 _____

위 원 _____

위 원 _____

위 원 _____

Therapeutic effects of anti-CXCL10 antibody

on C protein-induced myositis (CIM) mouse

by

Jinhyun Kim, M.D.

Submitted to the Department of Immunology

and the Faculty of the Seoul National University

Graduate School of Medicine

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

December, 2012

Doctoral Committee:

Professor _____, Chairman

Professor _____, Vice Chairman

Professor _____

Professor _____

Professor _____

Abstract

Therapeutic effects of anti-CXCL10 antibody on C protein-induced myositis (CIM) mouse

Jinhyun Kim

Department of Medicine (Immunology)

The Graduate School

Seoul National University

Introduction. CXCL10 (also called interferon-γ-inducible protein 10; IP-10) is a chemokine that plays a critical role in the infiltration of T cell in autoimmune diseases. CXCL10 is reported to be expressed in muscle tissue of polymyositis. Thus, the role of CXCL10 and the effect of CXCL10 blockade were investigated in C protein-induced myositis (CIM), an animal model of polymyositis.

Materials and Methods. CIM was induced with human skeletal muscle C

protein fragment in 8-week-old female C57BL/6 mice. Immunohistochemistry was performed to detect CXCL10 and CXCR3, the receptor of CXCL10, in muscle tissue. Serum levels of CXCL10 were measured by enzyme-linked immunosorbent assay. Surface markers including CD3, CD4, F4/80, and B220 and other effector molcules in mouse lymph node cells was investigated by flow cytometry. Migration assay of CIM lymph node cells was performed with 5-μm pore transwell system. Mice with CIM were treated with anti-CXCL10 antibody or control antibody from day 8 after the induction of myositis until day 20 every other day and the inflammation in muscle tissue was assessed 21 days after the induction.

Results. Immunohistochemistry showed increased expression of CXCL10 and CXCR3 in the inflammatory lesion of muscle in CIM. Especially, CD8+ T cells invading myofiber expressed CXCR3. Serum level of CXCL10 was increased in CIM compared to normal mice (mean ± SD, normal mouse, 14.3 ± 5.3 pg/ml vs. CIM, 368.5 ± 135.6 pg/ml, $p < 0.001$). Flow cytometry demonstrated localization of T cells in the lymph node of CIM and increased CXCR3 positivity in CD8+ T cells compared to CD4+ T cells in

the lymph node cells of CIM (CXCR3+/CD4+ T cell, 23.5 ± 4.7 vs. CXCR3+/CD8+ T cell, 65.9 ± 2.1, n = 6, $p < 0.001$). Moreover, IFN-γ+ cells were increased among CXCR3+CD8+ T cells compared to CXCR3−CD8+ T cells (CXCR3+CD8+ T cell, 28.0 ± 4.2% vs. CXCR3−CD8+ T cell, 9.5 ± 1.5%, $p = 0.016$). Migration of lymph node cells was increased in response to CXCL10 (chemotactic index = 1.91 ± 0.45, $p = 0.011$). After inducing CIM, mice treated with anti-CXCL10 antibody (n = 10) showed less inflammation score in muscles than those with control antibody (anti-RVG1) (n = 10; median [range], anti-CXCL10, 0.75 [0.25-2.00] vs. anti-RVG1, 1.43 [1.125-4.25], $p = 0.045$).

Conclusion. CXCL10 / CXCR3 expression was increased in the inflammation of CIM model and its blockade suppressed inflammation in muscle.

Keywords: polymyositis, CXCL10, CXCR3

Student number: 2007-30971

Contents

List of Tables

List of Figures

Introduction

Chemokines are 8- to 10-kDa proteins with 20 to 70% amino acid sequence homology and produce chemotactic activity in various cells, especially immune cells (Zlotinik and Yoshie, 2000). To date, approximately 50 different chemokines and at least 20 different receptors have been identified (Murphy et al., 2000). Chemokines are subclassified into four subfamilies based on the relative position of conserved cysteine residues (Baggiolini et al., 1997; Rollins, 1997). Among them, CXCL10 (also known as interferon-γ inducible protein-10, IP-10) is a chemokine that potentially plays a role in the immunopathogenesis of autoimmune disease such as rheumatoid arthritis, systemic lupus erythematosus, systemic sclerosis, and idiopathic inflammatory myopathy (IIM) (Patel et al., 2001; Narumi et al., 2000; Fujii et al., 2004; De Paepa, 2007). It is secreted by various cell types, such as, monocytes, neutrophils, endothelial cells, keratinocyte, fibroblasts, mesenchymal cells, dendritic cells, and astrocytes (Luster et al., 1987). CXCL10 binds to its receptor CXCR3, and regulates immune responses by

activation and recruitment of immune cells.

CXCR3 is a seven–transmembrane, G protein–coupled cell surface chemotactic receptors for CXCL9/MIG, CXCL10/IP-10, and CXCL11/I-TAC, and predicted to play an important role in lymphocyte trafficking, preferentially that of activated T cells (Liu et al., 2005). Originally it was cloned from T cells (Loetscher et al., 1996) but, now it is clear that CXCR3 is expressed on activated T cells, NK cells, monocytes, dendritic cells, endothelial cells, and microglia (Qin et al., 1998; Janatpour et al., 2001; Cella et al., 1999). The differential activation of CXCR3 by CXCL10, CXCL9, and CXCL11 may be related to their distinct biological function in vivo (Khan et al., 2000; Hancock et al., 2001; Zhang et al., 2004). Previous reports suggested that chemokines not only play an important role in lymphocyte recruitment to inflammatory sites but also participate in T-cell activation (Taub et al., 1996).

IIM are rare autoimmune diseases characterized by proximal muscle weakness, elevated muscle enzymes, abnormal electromyographic findings, and inflammation or vasculopathy in muscle tissue. IIM are subdivided into

dermatomyositis, polymyositis, and sporadic inclusion body myositis according to the immunopathological features (Mammen, 2010). Dermatomyositis is a humoral endotheliopathy initiated by complement deposition in intramuscular blood vessels, and it is characterized by perimysial inflammation and muscle fiber atrophy in perifascicular regions. In polymyositis and inclusion body myositis, non-necrotic muscle fibers are actively invaded by autoaggressive macrophages and cytotoxic T cells (Dalakas et al. 2012; Mammen, 2010). Despite recent advance in immunosuppression, the treatment of IIM is not sufficient. Although many kind of treatment including high-dose corticosteroid, intravenous immunoglobulin, azathioprine, cyclophosphamide or other immunosuppressant is available, some patients do not respond to these treatments, especially, when they have lung involvement (Dalakas et al. 2012; Kang et al., 2005).

Chemokines are also known to play an essential role in sustaining the inflammation associated with IIM. In previous reports on IIM, CXCL10 was abundantly expressed on macrophages and T cells in polymyositis, inclusion

body myositis and dermatomyositis whereas CXCL9 or CXCL11 was not altered compared to control (De Paepe et al,. 2005, 2007). Strong CXCR3 expression has also been observed on the majority of T cells in both polymyositis and dermatomyositis (De Paepe et al., 2005, 2007). In other studies, CXCL10 is abundantly expressed on infiltrates of muscle in inclusion body myositis and of muscle and skin in dermatomyositis (Raju et al., 2003; Fall et al., 2005; Wenzel et al., 2006). In addition, CXCR3 expression on the majority of T cells in dermatomyositis was reported (Fall et al., 2005). The above data suggest that T cell-mediated immunity is involved and the CXCL10/CXCR3 interaction in particular may be a potential therapeutic target in IIM.

Recently, animal model of human polymyositis, C protein-induced myositis (CIM), was established by Sugihara, et al (Sugihara et al., 2008). CIM can be induced easily by injection of human skeletal muscle C protein on C57BL/6 mice and its main mechanism of muscle damage by CD8+ T cell is similar to human polymyositis (Sugihara et al., 2010). The purpose of this study was to determine the therapeutic efficacy of anti-CXCL10 antibody in the CIM

model. First, the expression of CXCL10 and CXCR3 in C-protein induced myositis mice was investigated. Second, the functional aspect of CXCR3 positive cell was studied and last, the change of muscle inflammation was evaluated after administration of anti-CXCL10 antibody.

Materials and Methods

1. C-protein induced myositis model

Complementary DNA (cDNA) encoding fragments 2 of human fast-type skeletal muscle C protein were given by Prof. Kohsaka in Tyoko Medical & Dental University, Japan. The establishment of CIM model was described in the previous study (Sugihara et al., 2008). cDNA fragments were introduced into the TOP10F' bacterial host (Invitrogen, Carlsbad, CA, USA) and were used to prepare recombinant C protein fragments according to the manufacturer's protocol. Soluble recombinant C proteins were dialyzed against 0.5M arginine, 2 mM reduced glutathione, 0.2 mM oxidized glutathione in phosphate buffered saline (PBS), pH 7.4. Endotoxin was removed using Detoxi-Gel Endotoxin Removal Gel (Pierce, Rockford, IL, USA).

C57BL/6 mice were purchased from OrientBio (Sungnam, Korea). Female mice, ages 8–10 weeks, were immunized intradermally with 200 μg of the C

protein fragments emulsified in Freund's complete adjuvant (CFA) containing 100 μg of heat-killed *Mycobacterium butyricum* (Difco, Franklin Lakes, NJ, USA). The immunogens were injected at multiple sites of the back and foot pads, and 250 ng of pertussis toxin (PT) (Sigma-Aldrich, St. Louis, MO, USA; Cat No. P7208) diluted with 0.03 % Triton X was injected intraperitoneally at the same time. Hematoxylin and eosin–stained 10-μm sections of the proximal muscles (hamstrings and quadriceps) were examined histologically for the presence of mononuclear cell infiltration and necrosis of muscle fibers. The histologic severity of inflammation in each muscle block was graded as follows: grade 1 = involvement of a single muscle fiber; grade 2 = a lesion involving 2-5 muscle fibers; grade 3 = a lesion involving 6-15 muscle fiber; grade 4 = a lesion involving 16-30 muscle fibers; grade 5 = a lesion involving 31-100 muscle fibers; and grade 6 = a lesion involving >100 muscle fibers. When multiple lesions with the same grade were found in a single muscle block, 0.5 point was added to the grade. All experiments were done under specific pathogen–free conditions. The experiment was approved by Institutional Animal Care and Use Committee in Seoul National

University Hospital.

2. Immunohistochemistry

Immunohistochemical staining for the presence of CXCL10 or CXCR3 was performed according to the manufacturer's protocol based on the conventional streptavidin-biotin-peroxidase method. Representative sections of 3-μm thickness of paraffin-embedded muscle tissue were rehydrated after deparaffinization by xylene. Antigen retrieval was performed and the sections were washed with citrate buffer. Then, the sections were immersed in 3% H_2O_2 for 10 minutes to inhibit endogenous peroxidase activity and washed three times by phosphate-buffered saline (PBS) over the course of 5 minutes. Then, the sections were incubated with various primary antibodies. The primary antibodies were as follows: anti-CXCL10 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:200) or anti-CXCR3 (Invitrogen, Carlsbad, CA, USA; 1:200). Antigen retrieval was performed by boiling in citrate buffer. 3,3-Diaminobenzidine tetrahydrochloride (DAB) was used as a

chromogen. Counterstaining with Meyer's hematoxylin stain followed. As a positive control, synovial tissue of collagen-induced arthritis was used and as a negative control, primary antibody was omitted.

Cryostat-frozen sections (8 μm) were also used for detection of CXCR3. The section fixed in cold acetone were stained overnight at 4ºC with mouse anti-mouse CD4 (Abcam, Cambridge, UK), rat anti-mouse CD8a (Santa Cruz Biotechnology), rabbit anti-mouse CXCR3 (Invitrogen) in blocking reagents. A second layer of Alexa Fluor 555-conjugated anti-rabbit, Alexa Fluor 488-conjugated anti-mouse, and Alexa Fluoro 647-conjugated anti-rat antibody (all antibodies were purchased from Molecular Probes, Eugene, OR, USA) were used as secondary antibodies, respectively. All sections were washed and incubated for an additional 5 minutes with 4'-6-diamidino-2-phenylindole (DAPI, Molecular Probes) for counterstaining. For negative control, primary antibodies were omitted. The bound antibodies were visualized using LSM510 META confocal laser microscopy (Carl Zeiss, Jena, Germany).

3. Flow cytometry

Splenocytes of normal mice and CIM mice, inguinal lymph node cell of CIM mice was harvested. The splenocytes were purified by Ficoll-gradient methods. The cells were enumerated, and 5×10^5 cells were incubated with Fc Block™ (1 μg/mL; BD Bioscience, San Jose, CA, USA). Staining of the cells was performed with the following antibodies: PerCP-labeled anti-mouse CD3 (BD Bioscience), PE-labeled anti-mouse CXCR3, APC-labeled anti-mouse CXCR3, FITC-labeled anti-mouse CD4, FITC-labeled anti-mouse CD8, PE-Cy5- labeled anti-mouse F4/80, APC-labeled anti-mouse B220, PE-labeled anti-mouse IFN-γ, PE-labeled anti-mouse TNF-α, PE-labeled anti-mouse perforin, PE-labeled anti-mouse granzyme or PE-labeled anti-mouse FasL (all antibodies were purchased from eBioscience, San Diego, CA, USA).

For intracellular cytokine staining, 5×10^5 lymph node cells were plated in RPMI 1640 supplemented with 10% fetal calf serum, 100U penicillin/mL and 100 μg/mL streptomycin (Gibco, Carlsbad, CA, USA). Cultures were incubated with phorbol 12-myristate 13-acetate (PMA; 100 ng/mL; Sigma-

Aldrich) plus ionomycin (500 ng/mL; Sigma-Aldrich) in the presence of brefeldin A (10 μg/mL; BD Pharmingen, San Diego, CA, USA) for 4 hours at 37°C. After stimulation, cells were permeabilized with BD Cytofix/Cytoperm™ solution according to the manufacturer's instructions (BD Pharmingen), stained with antibodies, and fixed with 1% paraformaldehyde. Flow cytometry was performed using a FACSCanto (Becton Dickinson), and results were analyzed using FlowJo software (TreeStar Inc. Ashland, OR, USA).

4. Enzyme-linked immunosorbent assay (ELISA) of CXCL10

Concentrations of CXCL10 in mouse sera were measured with sandwich ELISA kits (MCX100, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

5. Migration assay

Inguinal lymph node cells of CIM were harvested and the migration of lymph node cell was evaluated by using a Costar Transwell system (24-well, 5-μm pore size membrane; Corning Costar, Cambridge, MA). Briefly, a total of 5×10^5 cells were added to the top chamber with 0.1 ml serum-free RPMI 1640 media. The bottom chamber was filled with 0.6 ml serum-free RPMI 1640 media with or without 200 ng/ml recombinant mouse CXCL10 from R&D systems (Campanella et al. 2008). The chambers were incubated for 3 hours at 37ºC. Then, the transmigrating cells in the bottom well were counted. Each experiment was performed in triplicate.

6. Anti-CXCL10 antibody treatment in CIM

Mouse ascites containing monoclonal anti-CXCL10 antibody and monoclonal anti-RVG1 antibody (anti-rotavirus antibody used as a control treatment) were generously given by Prof. Kawachi at Niigata University Graduate School of Medical and Dental Sciences, Japan. After filtering ascites with cell strainer, acetate buffer (4 times the volume of ascites) was

added. After the mixture was adjusted to pH 2.2~ 4.5 using NaOH, caprylic acid (28 μl for 1 ml of the mixture) was added and the mixture was agitated at 4ºC for 30 minutes. Then, the mixture was centrifuged at 4ºC, 12000g for 35 minutes and the supernatant was collected and 1M HEPES buffer was added to final concentration of 20 mM. Then, saturated ammonium sulfate was mixed to a final concentration of 45% and incubated at 4ºC for 3 minutes. Centrifuging the mixture at 4ºC, 10,000g for 30 minutes, supernatant was removed and the pellet was dissolved by phosphate-buffered saline (PBS) and the mixture were dialyzed 2 times in PBS.

CIM mice were treated with anti-CXCL10 antibody (n = 10) or anti-RVG1 (n = 10) as purified by the above methods. Nine CIM mice were observed without any treatment. Mice were immunized with C protein at day 0 and treated by injecting monoclonal antibody 200 μg in 100 μL PBS intraperitoneally every other day from day 8 till day 20.

7. Statistical analysis

All values are expressed as mean ± standard deviation (SD) or median (minimum, maximum). Mann-Whitney U-test, Kruskal-Wallis test, t-test or paired t-test (SPSS software; SPSS Inc) was used to compare groups. A p-value < 0.05 was considered statistically significant.

Results

1. C protein purification and induction of C protein-induced myositis

C protein was purified as described above method. The purified protein was loaded to 12% acrylamide gel and Coumassie staining showed the same band at about 40 kDa with positive control C protein that was given by Prof. Kohsaka (Figure 1). CIM was induced with purified C protein and the muscle tissue section was evaluated 3 weeks after induction. The incidence rate of myositis was 90% (9/10 mice, Figure 2).

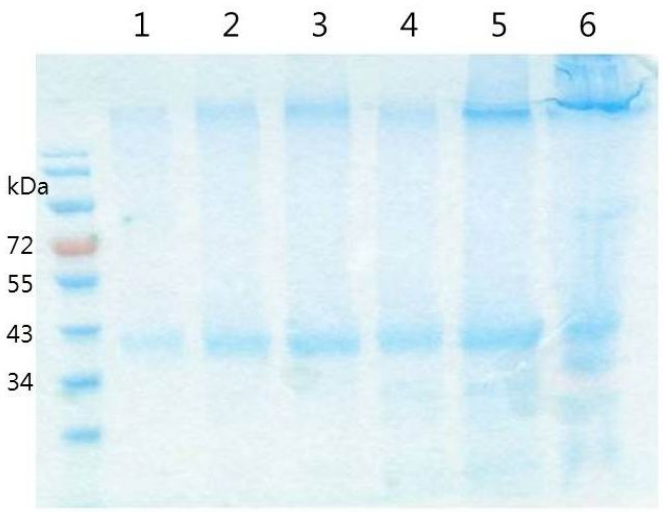

Figure 1. Gel electrophoresis of C protein

Acrylamide gel electrophoresis of C protein and Coomassie staining

Lane 1-3. C protein (positive control, from Prof. Kohsaka): lane 1, 2 μg; lane 2, 4 μg; lane 3, 6 μg.

Lane 4-6. Purified C protein: lane 4, 1/10 dilution; lane 5, 1/4 dilution; lane 6, 1/2 dilution

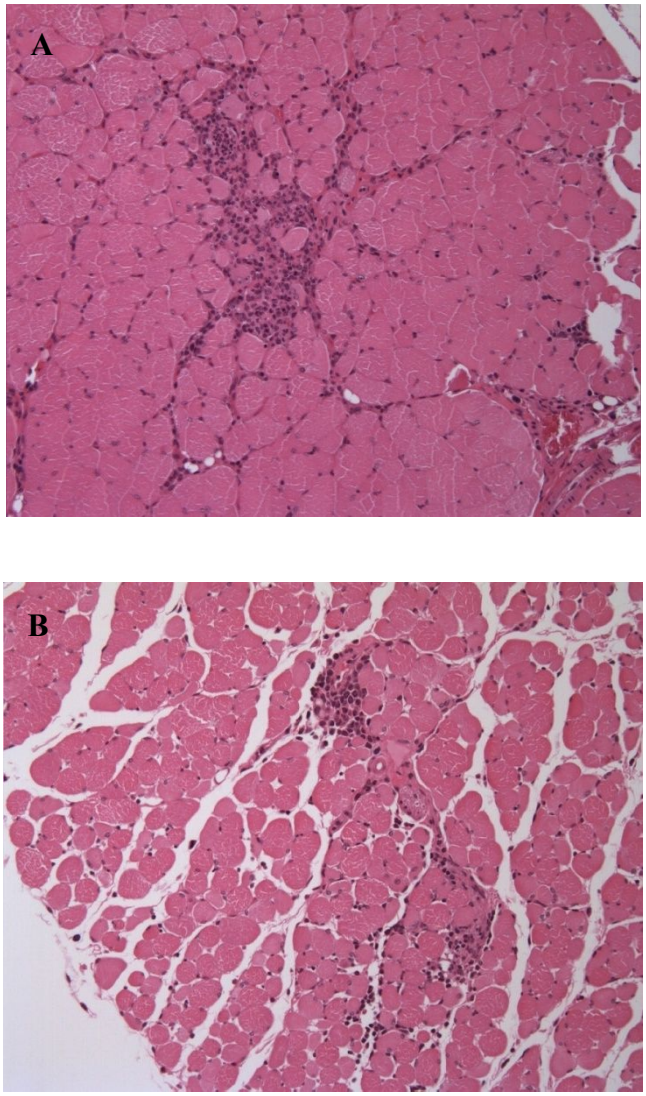

Figure 2. Histology of C protein-induced myositis

Leg muscle tissue of CIM in the study showed inflammation (H&E stain, x 200). (A) Hamstring muscle showed histologic grade 4. (B) Quadriceps muscle showed two inflammatory lesions of grade 2.

2. The presence of CXCL10 in the muscle tissue and serum of CIM and CXCR3 positive cell infiltration in the muscle of CIM

Immunohistochemistry showed the positive staining of CXCL10 and CXCR3 in the muscle tissue of CIM. CXCL10 was stained strongly in the inflammatory lesion (Figure 3). Serum levels of CXCL10 increased in CIM compared to normal mice (normal mouse, 14.3 ± 5.3 pg/ml vs. CIM, 368.5 ± 135.6 pg/ml, $p < 0.001$, Figure 4). CXCR3 positive cells also scattered in the lymph node and inflammatory lesion of muscle tissue (Figure 5). Moreover, CD8+ T cells invading myofiber expressed CXCR3 (Figure 6).

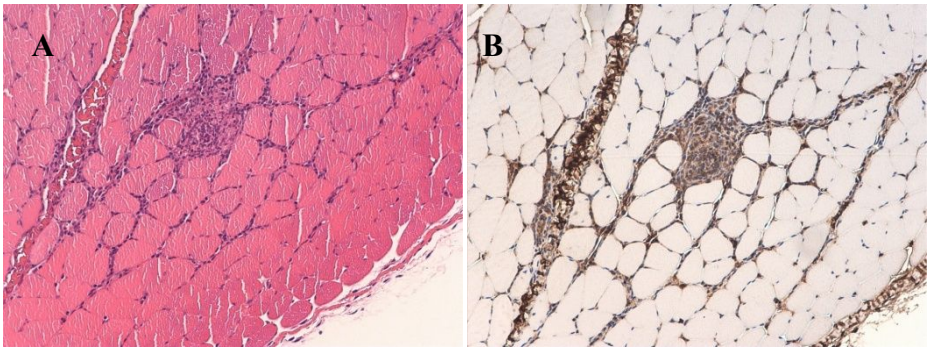

Figure 3. Immunohistochemistry of CXCL10 in the muscle of CIM

CXCL10 was stained in the inflammatory lesion of CIM. Two pictures were the same section of muscle tissue. H&E stain (A, x 200) and CXCL10 immunohistochemistry (B, x 200).

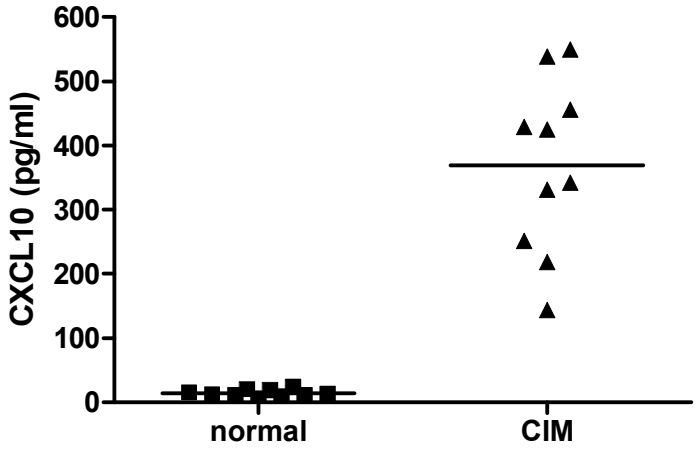

Figure 4. Serum level of CXCL10 in normal mice and CIM mice

The level of CXCL10 was measure by Enzyme-linked immunosorbent assay in the sera of normal mice (n = 10) and CIM mice (n = 10) at 3 weeks after induction. The serum level of CIM was more elevated in CIM than in normal (normal mouse, 14.3 ± 5.3 pg/ml vs. CIM, 368.5 ± 135.6 pg/ml, $p<0.001$). The horizontal lines means mean.

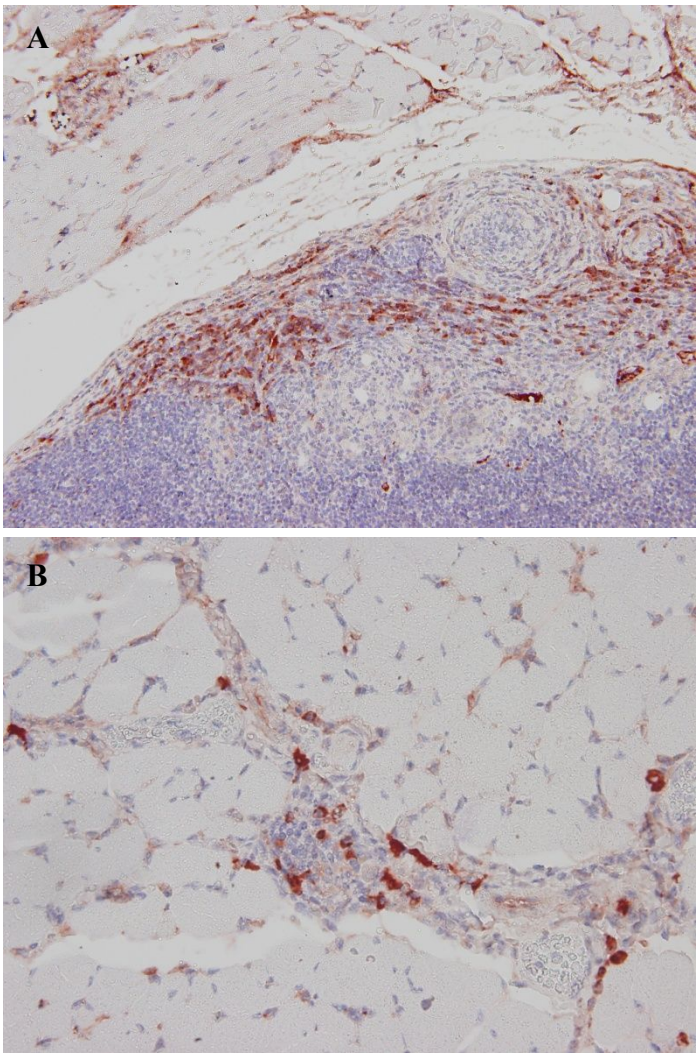

Figure 5. Immunohistochemistry of CXCR3 in the lymph node and muscle of CIM

CXCR3 was expressed in some cells of the lymph node cells (A, x 200) and inflammatory lesion in the muscle of CIM (B, x 400).

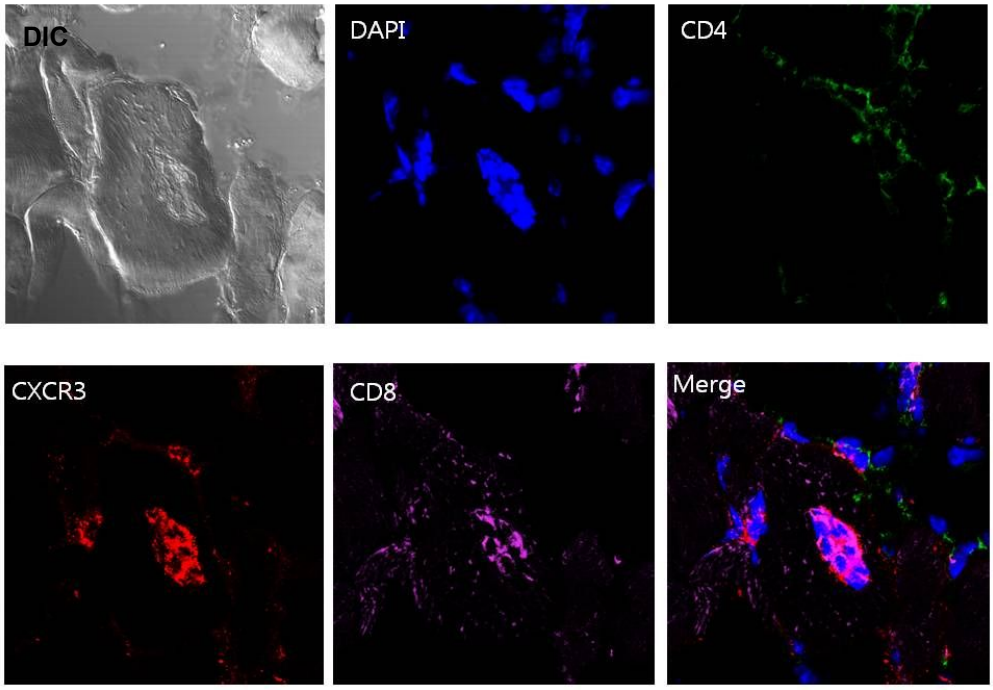

Figure 6. Immunostaining of cells invading myofiber in muscle of CIM.

Differential interference contrast (DIC) showed myofiber and invasive cells in the middle of myofiber. The CD8 was co-stained with CXCR3 whereas CD4 was not.

3. Abundance of CXCR3 expression in CD8+ T cells in regional lymph node of CIM mouse

Localization of CXCR3+ cells in the regional lymph node

The proportion of T cell in the spleen and lymph node was measured by flow cytometry. The normal splenocytes contained CD4 + T cells and CD8+ T cells while splenocyte of CIM contained few CD4+ T cells and CD8+ T cells. However, the proportion of CD4+ and CD8+ T cells was increased in the inguinal lymph node of CIM (Figure 7, Table 1). Normal mice did not show discrete lymphadenopathy, thus, lymph node cells could not be obtained.

CXCR3+CD8+ T cells are abundant in regional lymph nodes

The CXCR3 positivity in the lymph node cells of CIM was analyzed using flow cytometry. CXCR3+ cells of CIM lymph node were 15.7 ± 3.7% and composed of various immune cells (Figure 8) and CXCR3+ T cells were abundant in CD3+CD8+ T cells (51.5 ± 3.0%, Table 2).

The proportion of CXCR3+ T cells among CD4+ T cells was 23.5 ± 4.7% while the proportion of CXCR3+ T cell among CD8+ T cells was 65.9 ± 2.1% (n=6, $p<0.001$, Figure 9).

IFN-γ+ cell are more abundant in CXCR3+CD8+ T cells compared to CXCR3−CD8+ T cells

The effector molecule of CD8+ T cells was analyzed by flow cytometry. CXCR3 positivity was associated with IFN-γ positivity ($p=0.016$). However, TNF-α, perforin, granzyme, or FasL was not associated with CXCR3 positivity in the lymph node cells of CIM (Figure 10, Table 3).

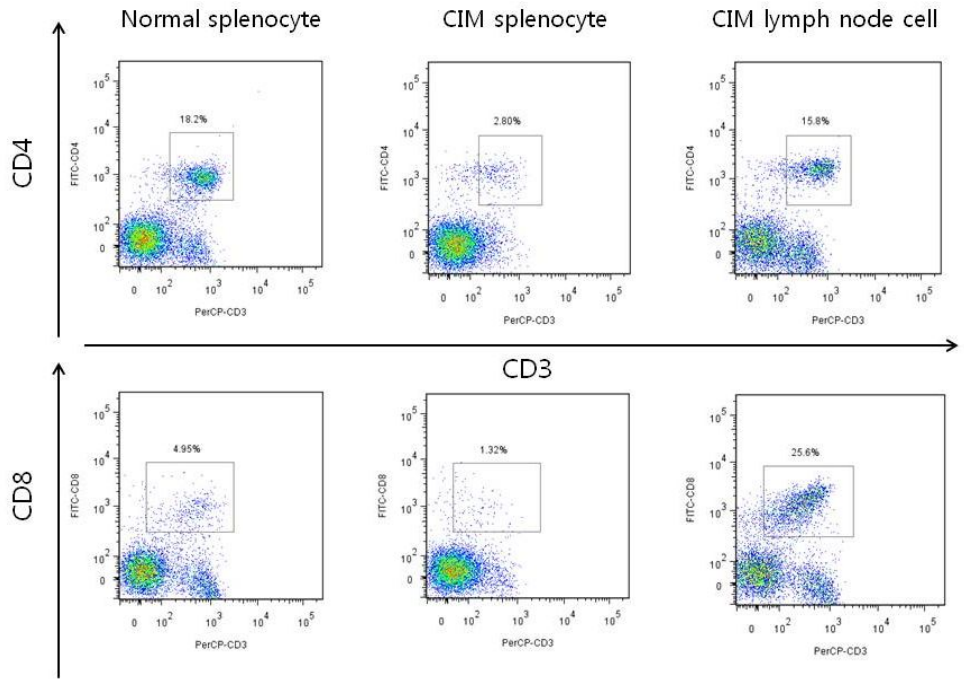

Figure 7. Distribution of CD4+ T cells and CD8+ T cells in normal mice and CIM mice

Representative result of normal splenocytes (left), CIM splenocytes (middle), CIM lymph node cells (right). There were few CD4+ T cells and CD8+ T cells in CIM splenocytes compared to normal splenocytes. However, there were many CD4+ T cells and CD8+ T cells in the inguinal lymph node cells in CIM.

Table 1. The proportion of CD4+ T cell and CD8+ T cell in normal splenocytes, CIM splenocytes and lymph node cells

	Normal splenocytes (n=4)	CIM splenocytes (n=3)	CIM lymph node cells (n=3)	*P**
CD4+ T cell	16.1 ± 1.7	6.9 ± 2.8	10.5 ± 1.5	0.024
CD8+ T cell	6.4 ± 0.7	1.0 ± 0.3	9.8 ± 3.7	0.018

*by Krushkal-Wallis test (comparing normal splenocytes, CIM splenocytes and CIM lymph node cell)

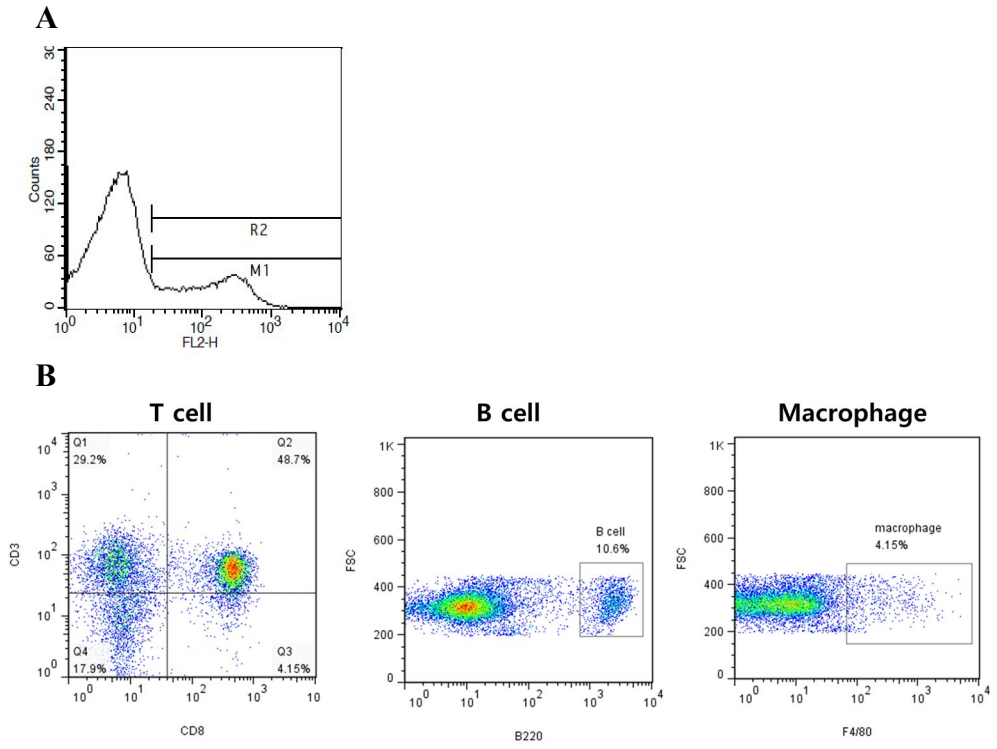

Figure 8. CXCR3 positive immune cells in CIM lymph node

(A) CXCR3 positive cell was gated from lymph node cells. (B) CXCR3 positive cells were analyzed by cell-specific markers (CD3, CD8 for T cells; B220 for B cells; F4/80 for macrophages).

Table 2. The distribution of immune cells among CXCR3 positive cell in CIM lymph node (n=3)

Cell marker	The proportion (%) among CXCR3+ cells
CD3+CD8−	31.4 ± 2.9
CD3+CD8+	51.5 ± 3.0
B220+	12.1 ± 6.0
F4/80+	4.3 ± 2.6

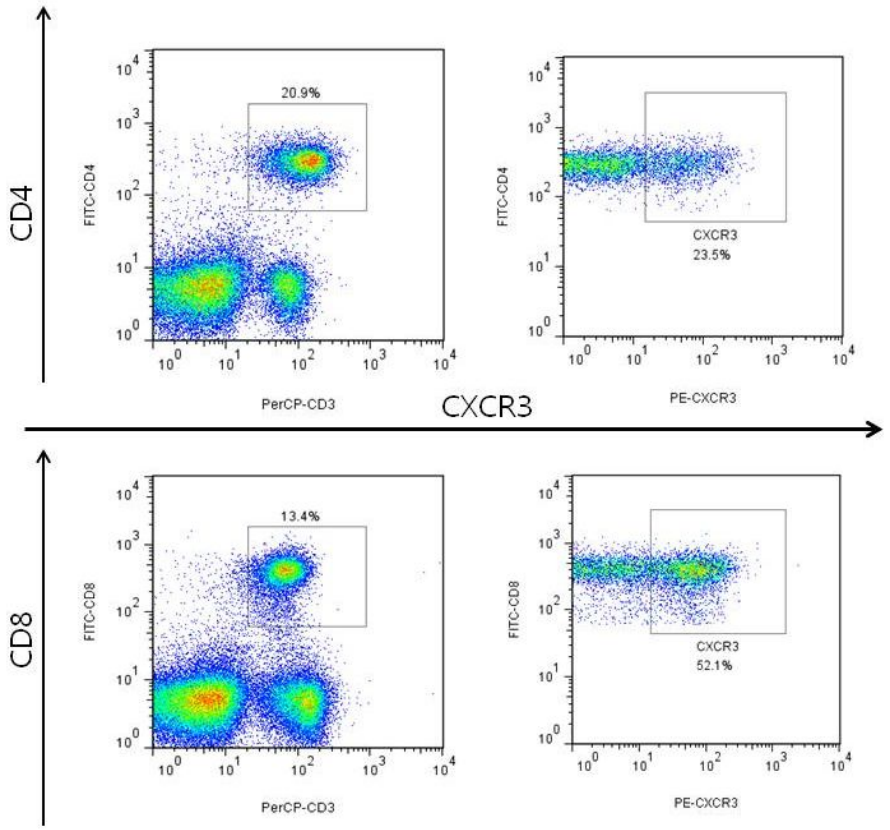

Figure 9. The proportion of CXCR3 positive cells among CD4+ T cells and CD8+ T cells

Representative result of CIM lymph node cells: CD4+ T cells (upper row) and CD8 + T cells (lower row). CD4+ T cells and CD8+ T cells were gated (left column) and CXCR3 positivity was analyzed in the gated cells (right column). Compared to CD4+ T cells, CD8+ T cells showed more CXCR3 positivity.

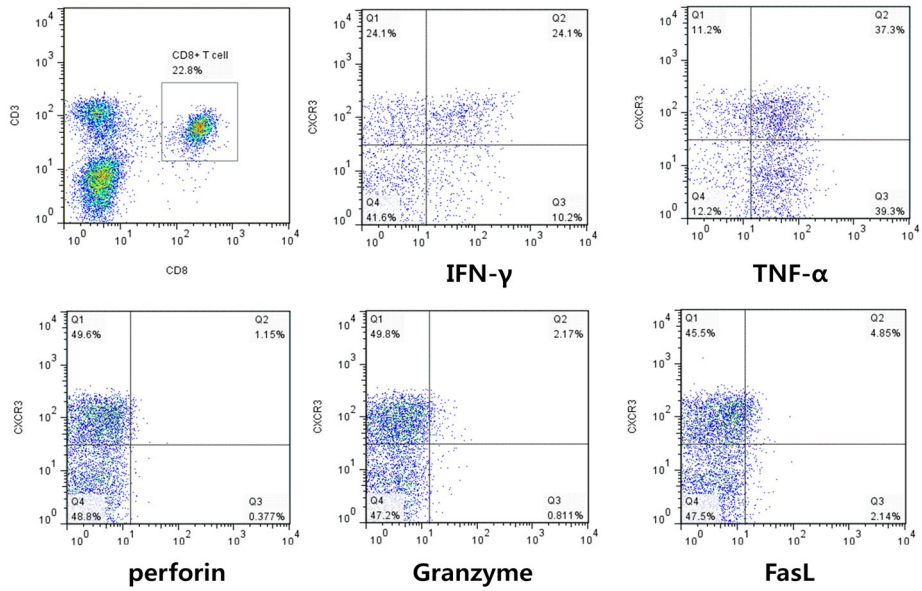

Figure 10. The effector molecules of CD8+ T cells

Lymph node cells of CIM were stimulated with phorbol 12-myristate 13-acetate, ionomycin and brefeldin A for 5 hours. CD3+CD8+ cells were gated (left upper figure) and analyzed according to presence of CXCR3 and effector molecules.

Table 3. The comparison of effector molecules positive cells between CXCR3+CD8+ T cells and CXCR3-CD8+ T cells (n=3)

Effector molecule	positive cell /CXCR3+ CD8+ T cell	positive cell /CXCR3- CD8+ T cell	p*
IFN-γ	28.0 ± 4.2%	9.5 ± 1.5%	0.016
TNF-α	34.7 ± 4.3%	38.0 ± 1.5%	0.362
Perforin	0.90 ± 0.22%	0.43 ± 0.16%	0.127
Granzyme	1.22 ± 0.82%	0.47 ± 0.29%	0.133
FasL	2.74 ± 1.85%	1.60 ± 0.79%	0.295

*Paired t-test

4. The migration of CIM lymph node cells was increased by CXCL10

Inguinal lymph node cells of CIM was stimulated with CXCL10 (200 ng/ml) or without CXCL10 in the migration assay. The degree of migration was calculated as chemotactic index (number of migrated cell in the presence of CXCL10 / number of migrated cells in the absence of CXCL10). The increased migration of the cells in the presence of CXCL10 was observed (chemotactic index was 1.91 ± 0.45, n=5, p = 0.011, by Kolmogorov-Smirnov test, Figure 11)

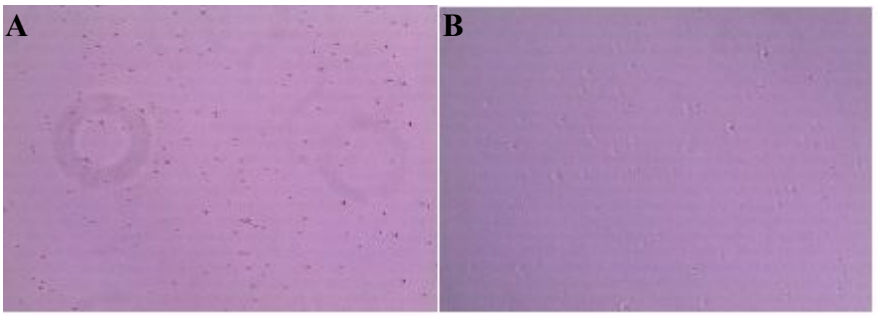

Figure 11. Picture of migrated cells in the migration assay of inguinal lymph node cells

Representative pictures of migrated cells were taken from the bottom well of Transwell system. The cells from inguinal lymph node of CIM were stimulated with CXCL10 (200 ng/ml, A) versus control (B).

5. The therapeutic effect of CXCL10 blockade in CIM

The CIM mice were treated with the intraperitoneal injection of monoclonal anit-CXCL10 (200 μg / 100 μl) or anti-RVG1 antibody (as control, 200 μg / 100 μl) every other day from day 8 to day 20. Three weeks after induction, muscle inflammation was compared between treatment groups by histologic score. The group treated with monoclonal anti-CXCL10 antibody showed significant improvement of muscle inflammation compared to the group treated with anti-RVG1 ($p=0.045$) or the group which did not receive any treatment ($p=0.002$). The results were summarized in Table 4 and Figure 12.

Table 4. The results of anti-CXCL10 or control antibody treatment in CIM

Group	No treatment (n=9)	Anti-CXCL10 (n=10)	Anti-RVG1 (n=10)	p*
Histologic score	2.23 (1.125, 3.875)	0.75 (0.25, 2.00)	1.875 (1.125, 4.25)	0.005

Data are expressed as median (min, max).

*by Kruskal-Wallis test

p = 0.002, no treatment vs. anti-CXCL10, by Mann-Whitney test

p = 0.045, anti CXCL10 vs. anti-RVG1, by Mann-Whitney test

p = 0.120, no treatment vs. anti RVG1, by Mann-Whitney test

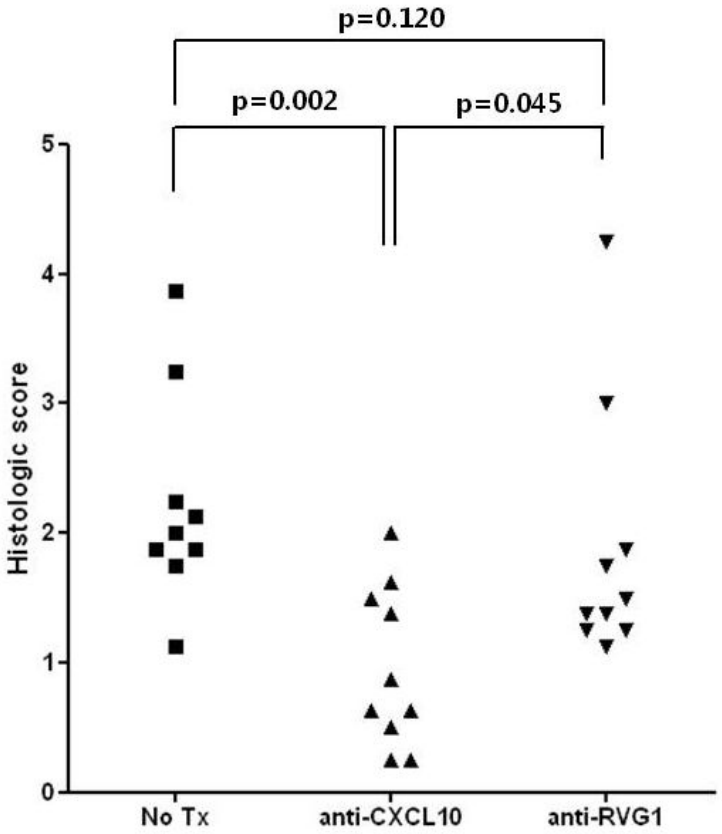

Figure 12. The results of anti-CXCL10 or control antibody treatment in CIM

No Tx: no treatment group, anti-CXCL10: anti-CXCL10 treatment group, anti-RVG1: anti-RVG1 treatment group (control antibody)

Discussion

We investigated the role of CXCL10/CXCR3 axis using murine model of polymyositis based on the previous study on chemokine profile of human IIM (De Paepe et al., 2007). The major findings of the present study were the following: CXCL10 and CXCR3 were expressed in the inflammatory lesion in the CIM muscle tissue. The level of CXCL10 was increased in the serum of CIM as well. In this study, most of the CXCR3 positive cells were CD8+ T cell (51.5 ± 3.0%). CXCR3 was expressed in more than half of CD8+ T cells in regional lymph node (65.9 ± 2.1%). CD8+ T cells had been known as principal pathogenic cells in CIM (Sugihara et al., 2010). Moreover, CXCR3+CD8+ T cells infiltrated in myofiber. CXCR3+CD8+ T cells showed more IFN-γ positivity compared to CXCR3−CD8+ T cells. Regional lymph node cells migrated with stimulation of CXCL10 more than without stimulation of CXCL10. Treatment with anti-CXCL10 ameliorated muscle inflammation in CIM mice. These results suggest that CXCL10/CXCR3 interaction seems to play a crucial role in inflammatory cell migration into

muscle in CIM.

Several animal models of myositis have been introduced (Rosenberg et al., 1987; Nagaraju et al., 2000; Katsumata et al., 2007; Sugihara et al. 2008). Among them, transgenic mouse and special diet model resembles inclusion body myositis (Rosenberg et al., 1987). Antigen-induced models include laminin, myosin, or C protein-induced rats or mice models. Most commonly used animal model of polymyositis, experimental autoimmune myositis (EAM), is inducible specifically in SJL/J mice by repeated administration of muscle homogenate or partially purified myosin (Rosenberg et al., 1987; Matsubara et al., 1993; Suzuki et al. 2005). This model is a complex representation of disease, because SJL/J mice have a dysferlin gene mutation that causes spontaneous muscle necrosis and secondary muscle inflammation (Bittner et al., 1999). Immunohistochemical studies have shown that infiltrating T cells in the muscle are dominated by CD4+ T cells, suggesting that the EAM disease model is mediated by CD4+ T cells (Rosenberg et al., 1989). Conditional upregulation of MHC class I molecule in muscle of mouse can mimic autoimmune myopathies (Nagaraju et al., 2000).

Nonetheless, that model requires special transgenic technology and showed endoplasmic reticulum stress response due to MHC class I overexpression and disturbance of muscle metabolism before inflammation (Nagaraju et al., 2005; Coley et al., 2012). Thus, it is possible that pathogenesis could be attributed to non-immunologic mechanism. Newly developed histidyl-tRNA synthetase immunization model uniquely shows extramuscular manifestation like lung inflammation and autoantibody to histidyl-tRNA synthetase (Katsumata et al., 2007; Soejima et al., 2011). However, this model usually needs intramuscular injection which itself can induce inflammatory lesion.

CIM used in this study was established as a simple murine model of polymyositis. A single injection of mice with recombinant human muscle protein induced severe and clinically significant inflammation of the skeletal muscles. Previous studies on the CIM demonstrated that several types of immune cells could be involved. Macrophages and CD4+ T cells are also abundant in the muscle inflammation. CD4+ T cell depletion exerted the same effect as CD8+ T cell depletion (Sugihara et al., 2008). Thus, CD4+ T cells as well as CD8+ T cells might participate in pathogenesis. However,

CD8+ T cells were enriched in the endomysial site, the site of muscle injury, and expressed perforins preferentially at the endomysial site. Class I MHC expression was up-regulated in muscles with severe inflammation in mice with CIM (Sugihara et al., 2008). Moreover, removal of class I MHC significantly suppressed myositis and adoptive transfer model featured that the CD8 T cell-induced muscle injuries were significantly more severe than the CD4 T cell-induced muscle injuries (Sugihara et al., 2010) Especially when evaluating the necrotic muscle area representing direct muscle injury, CD8+ T cells were dominant. In this regard, the new CIM model provides a clear contrast to the previous EAM model, which appears to be driven by CD4+ T cells. The finding that CD8+ cytotoxic T lymphocytes primarily damage the muscle fibers in CIM confirmed that CIM is the mouse myositis model most analogous to human polymyositis. The present study showed that CXCR3 was expressed in CD8+ T cells more than CD4+ T cells in regional lymph node and moreover, muscle-invasive CD8+ T cells expressed CXCR3. In addition, CXCR3+CD8+ T cells showed more frequent IFN-γ positivity in the inguinal lymph node. Perforin or granzyme can be the cytotoxic

mechanism for CD8+ T cells to injure muscle fiber in this model (Sugihara et al., 2010). However, this study could not detect perforin / granzyme or FasL in CD8+ T cells. This result could be attributed by using lymph node cells rather than directly infiltrated cells.

The source of CXCL10 is not clear based on this study. The previous study of immunolocalization of CXCL10 showed expression in the inflammatory lesion and vessel, but not in the muscle fiber (De Paepa et al., 2007) Immunohistochemistry of CIM muscle in this study showed similar pattern of staining on infiltrating cell. However, it might be expressed in the muscle tissue of inflammatory state. Recently, primary human muscle cell was known to secret CXCL10 after stimulation with TNF-α or IFN-γ (Crescioli et al., 2012). Those results suggested the active role of muscle cells in immune response. Further study can be focused in the interaction between muscle cell and immune cells such as CD8+ T cells, CD4+ T cells, or macrophage.

IIMs are Th1-driven autoimmune processes characterized by significant inflammatory cell infiltrates in muscle and other tissue resulting in muscle injury (Grundtman et al., 2007). About 25% of patients cannot tolerate or are

refractory to the conventional therapies (Wiendl et al., 2005) and there are no defined guidelines for myositis treatment (Tournadre et al., 2010). Therefore, the developments of new therapeutic agents are necessary. In addition to polymyositis, CXCL10/CXCR3 axis was also reported to be involved in inclusion body myositis and dermatomyositis. CXCL10 is abundantly expressed on macrophages and T cells surrounding and invading nonnecrotic muscle fibers in inclusion body myositis (Raju et al., 2003). CXCL10 expression on T cells in the perimysial infiltrates of dermatomyositis and CXCR3 expression on the majority of T cells in dermatomyositis was also known. Juvenile type dermatomyositis also showed high expression of CXCL10 in muscle tissue (Fall et al., 2005) and the expression of CXCL10 expression and recruitment of CXCR3+ T cells was detected in the skin lesions of dermatomyositis (Wenzel et al., 2006).

In clinical aspects, the development of therapeutic agent against CXCL10/CXCR3 in IIM is plausible. As mentioned in introduction, the studies demonstrated that the expression of CXCL10 and CXCR3 was increased in collagen-induced arthritis model, and neutralizing anti-CXCL10

antibodies ameliorated disease manifestations in these models (Salomon et al., 2002; Kwak et al. 2008). Moreover, CXCL10 and CXCR3 expression is also increased in the synovial membrane of rheumatoid arthritis patients (Qin et al., 1998; Patel et al., 2001). Thus, clinical trial of blocking antibody against CXCL10 showed promising outcome (Yellin et al., 2012). Taken the results of this study into consideration in parallel with the studies on rheumatoid arthritis, CXCL10/CXCR3 pathway can be candidate as a therapeutic target in human IIMs.

References

Baggiolini M, Dewald B, and Moser B. Human chemokines: An update. Annu Rev Immunol 1997;15: 675–705.

Bittner RE, Anderson LV, Burkhardt E, Bashir R, Vafiadaki E, Ivanova S, et al. Dysferlin deletion in SJL mice (SJL-Dysf) defines a natural model for limb girdle muscular dystrophy 2B. Nat Genet 1999;23:141–142

Campanella GS, Medoff BD, Manice LA, Colvin RA, Luster AD. Development of a novel chemokine-mediated in vivo T cell recruitment assay. J Immunol Methods 2008;331(1-2):127–39

Cella M, Jarrossay D, Facchetti F, Alebardi O, Nakajima H, Lanzavecchia A, Colonna M. Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. Nat Med 1999; 5: 919–923

Coley W, Rayavarapu S, Pandey GS, Sabina RL, Van der Meulen JH, Ampong B, et al. The molecular basis of skeletal muscle weakness in a

mouse model of inflammatory myopathy. Arthritis Rheum 2012;64(11):3750-3759

Crescioli C, Sottili M, Bonini P, Cosmi L, Chiarugi P, Romagnani P, et al. Inflammatory response in human skeletal muscle cells: CXCL10 as a potential therapeutic target. Eur J Cell Biol 2012;91(2):139–149

Dalakas MC. Pathogenesis and therapies of immune-mediated myopathies. Autoimmun Rev 2012;11(3):203–206

De Paepe B, De Keyzer K, Martin JJ, De Bleecker JL. Alpha-chemokine receptors CXCR1-3 and their ligands in idiopathic inflammatory myopathies. Acta Neuropathol 2005;109:576–582

De Paepe B, Creus KK, De Bleecker JL. Chemokine profile of different inflammatory myopathies reflects humoral versus cytotoxic immune responses. Ann N Y Acad Sci 2007;1109:441–453

Fall N, Bove KE, Stringer K, Lovell DJ, Brunner HI, Weiss J, et al. Association between lack of angiogenic response in muscle tissue and high

expression of angiostatic ELR-negative CXC chemokines in patients with juvenile dermatomyositis: possible link to vasculopathy. Arthritis Rheum 2005;52:3175–3180

Fujii H, Shimada Y, Hasegawa M, Takehara K, Kamatain N. Serum levels of a Th1 chemoattractant IP-10 and Th2 chemoattractants, TARC and MDC, are elevated in patients with systemic sclerosis. J Dermatol Sci 2004;35(1):43–51

Grundtman C, Malmstrom V, Lundberg IE. Immune mechanisms in the pathogenesis of idiopathic inflammatory myopathies. Arthritis Res Ther 2007;9:208–220

Hancock WW, Gao W, Csizmadia V, Faia KL, Shemmeri N, Luster AD. Donor-derived IP-10 initiates development of acute allograft rejection. J Exp Med 2001;193: 975–980.

Janatpour MJ, Hudak S, Sathe M, Sedgwick JD, and McEvoy LM. Tumor necrosis factor-dependent segmental control of MIG expression by high endothelial venules in inflamed lymph nodes regulates monocyte recruitment. J Exp Med 2001;194:1375–1384

Jinquan T, Jing C, Jacobi HH, Reimert CM, Millner A, Quan S, et al. CXCR3 expression and activation of eosinophils: role of IFN-γ-inducible protein-10 and monokine induced by IFN-γ. J Immunol 2000;165:1548–1556

Kang EH, Lee EB, Shin KC, Im CH, Chung DH, Han SK, Song YW. Interstitial lung disease in patients with polymyositis, dermatomyositis and amyopathic dermatomyositis. Rheumatol (Oxford) 2005;44:1282–1286

Katsumata Y, Ridgway WM, Oriss T, Gu X, Chin D, Wu Y, et al. Species-specific immune responses generated by histidyl-tRNA synthetase immunization are associated with muscle and lung inflammation. J Autoimmun 2007;29(2-3):174–186

Khan IA, MacLean JA, Lee FS, Casciotti L, DeHaan E, Schwartzman JD, Luster AD. IP-10 is critical for effector T cell trafficking and host survival in Toxoplasma gondii infection. Immunity 2000;12: 483–494.

Kwak HB, Ha H, Kim HN, Lee JH, Kim HS, Lee S, et al. Reciprocal cross-talk between RANKL and interferon-gamma-inducible protein 10 is responsible for bone-erosive experimental arthritis. Arthritis Rheum

2008 ;58(5):1332-42

Liu L, Callahan MK, Huang DR, Ransohoff RM. Chemokine Receptor CXCR3: an unexpected enigma. Current Topics in Developmental Biology, 2005; 68:149–181

Loetscher M, Gerber B, Loetscher P, Jones SA, Piali L, Clark-Lewis I, et al. Chemokine receptor specific for IP10 and Mig: Structure, function, and expression in activated T-lymphocytes. J Exp Med 1996;184: 963–969

Luster AD, Ravetch JV. Biochemical characterization of a γ interferon-inducible cytokine (CXCL10). J Exp Med 1987;166:1084–1097

Mammen AL. Dermatomyositis and polymyositis: Clinical presentation, autoantibodies, and pathogenesis. Ann N Y Acad Sci 2010;1184:134–153

Matsubara S, Shima T, Takamori M. Experimental allergic myositis in SJL/J mice immunized with rabbit myosin B fraction: immunohistochemical analysis and transfer. Acta Neuropathol (Berl) 1993;85:138–144

Murphy PM, Baggiolini M, Charo IF, Hébert CA, Horuk R, Matsushima K,

et al. International union of pharmacology. XXII. Nomenclature for chemokine receptors. Pharmacol Rev 2000;52(1):145–176.

Nagaraju K, Raben N, Loeffler L, Parker T, Rochon PJ, Lee E, et al. Conditional up-regulation of MHC class I in skeletal muscle leads to self-sustaining autoimmune myositis and myositis-specific autoantibodies. Proc Natl Acad Sci U S A 2000;97:9209–9214

Nagaraju K, Casciola-Rosen L, Lundberg I, Rawat R, Cutting S, Thapliyal R, et al. Activation of the endoplasmic reticulum stress response in autoimmune myositis: potential role in muscle fiber damage and dysfunction. Arthritis Rheum. 2005;52(6):1824-1835

Narumi S, Takeuchi T, Kobayashi Y, Konish K. Serum levels of IFN-inducible protein-10 relating to the activity of systemic lupus erythematosus. Cytokine 2000;12(10):1561–5.

Patel DD, Zachariah JP, Whichard LP. CXCR3 and CCR5 ligands in rheumatoid arthritis synovium. Clin Immunol 2001;98:39–45

Qin S, Rottman JB, Myers P, Kassam N, Weinblatt M, Loetscher M, et al. The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. J Clin Invest. 1998;101(4):746–754

Raju R, Vasconcelos O, Granger R, Dalakas MC. Expression of IFN-gamma-inducible chemokines in inclusion body myositis. J Neuroimmunol 2003 ;141(1-2):125–131

Rollins BJ. Chemokines. Blood 1997; 90:909–928

Rosenberg NL, Ringel SP, Kotzin BL. Experimental autoimmune myositis in SJL/J mice. Clin Exp Immunol 1987;68:117–129

Rosenberg NL, Kotzin BL. Aberrant expression of class II MHC antigens by skeletal muscle endothelial cells in experimental autoimmune myositis. J Immunol 1989;142:4289–4294

Salomon I, Netzer N, Wildbaum G, Schif-Zuck S, Maor G, Karin N. Targeting the function of IFN-γ-inducible protein 10 suppresses ongoing

adjuvant arthritis. J Immunol 2002;169:2685–2693

Soejima M, Kang EH, Gu X, Katsumata Y, Clemens PR, Ascherman DP. Role of innate immunity in a murine model of histidyl-transfer RNA synthetase (Jo-1)-mediated myositis. Arthritis Rheum 2011;63(2):479–487

Sugihara T, Sekine C, Nakae T, Kohyama K, Harigai M, Iwakura Y, et al. A new murine model to define the critical pathologic and therapeutic mediators of polymyositis. Arthritis Rheum 2007;56(4):1304–1314

Sugihara T, Okiyama N, Suzuki M, Kohyama K, Matsumoto Y, Miyasaka N, Kohsaka H. Definitive engagement of cytotoxic CD8 T cells in C protein-induced myositis, a murine model of polymyositis. Arthritis Rheum 2010;62(10):3088–3092

Suzuki F, Nanki T, Imai T, Kikuchi H, Hirohata S, Kohsaka H, Miyasaka N. Inhibition of CX3CL1 (Fractalkine) improves experimental autoimmune myositis in SJL/J mice. J Immunol 2005;175:6987–6996

Taub DD, Lioyd AR, Conlon K, Wang JM, Ortaldo JR, Harada A, et al. Recombinant human interferon-inducible protein 10 is a chemoattractant for human monocytes and T lymphocytes and promotes T cell adhesion to endothelial cells. J Exp Med 1993;177:1809–1814

Tournadre A, Dubost JJ, Soubrier M. Treatment of inflammatory muscle disease in adults. Joint Bone Spine 2010;77: 390–394

Wenzel J, Schmidt R, Proelss J, Zahn S, Bieber T, Tüting T. Type I interferon-associated skin recruitment of CXCR3+ lymphocytes in dermatomyositis. Clin Exp Dermatol 2006;31(4):576–582

Wiendl H, Hohlfeld R, Kieseier BC. Immunobiology of muscle: advances in understanding an immunological microenvironment. Trends Immunol 2005;26:373–380

Yellin M, Paliienko I, Balanescu A, Ter-Vartanian S, Tseluyko V, Xu LA, et al. A phase II, randomized, double-blind, placebo-controlled study evaluating the efficacy and safety of MDX-1100, a fully human anti-CXCL10

monoclonal antibody, in combination with methotrexate in patients with rheumatoid arthritis. Arthritis Rheum 2012;64(6):1730-1739

Zhang Z, Kaptanoglu L, Tang Y, Ivancic D, Rao SM, Luster A, et al. IP-10-induced recruitment of CXCR3 host T cells is required for small bowel allograft rejection. Gastroenterology 2004;126(3):809-818

Zlotnik A, Yoshie O. Chemokines: A new classification system and their role in immunity. Immunity 2000;12:121–127

요 약 (국문초록)

서론. CXCL10 (interferon-ɣ-inducible protein 10; IP-10)는 자가면역질환에서 T세포의 침윤에 중요한 역할을 하는 케모카인이다. CXCL10은 다발근육염의 근육조직에서 발현되는 것으로 보고되고 있다. 따라서 다발근육염의 동물모델인 C단백유발근육염 생쥐에서 CXCL10의 역할과 CXCL10 차단의 효과를 연구하였다.

재료 및 방법. C단백유발근육염을 인간골격근의 C단백절편을 이용하여 8주령 암컷 C57BL/6 생쥐에서 유도하였다. CXCL10과 그 수용체인 CXCR3를 근육조직에서 발견하기 위해서 면역화학염색을 실시하였다. 또한 생쥐의 혈청에서 CXCL10의 농도를 효소결합면역흡착측정법으로 측정하였다. 생쥐의 림프절세포와 비장세포에서 유세포 분석법으로 표면표지자 및 효과분자(effector molecule)를 분석하였다. 5-μm pore 트랜스웰 시스템 (transwell system)을 이용하여 쥐의 림프절세포의 이동분석을 하였다. 마지막으로 C단백유발근육염이 유도된 쥐를

항CXCL10항체 또는 대조항체로 근육염 유도 후 8일째부터 20일째까지 격일로 치료를 하였고 유도 후 21일째 근육조직에서 염증을 평가하였다.

결과. 면역화학염색으로 C단백유발근육염의 근육에서 CXCL10과 CXCR3가 발현됨을 확인하였으며, 특히, 근육을 침범하는 CD8+ T세포에서 CXCR3가 발현됨을 확인하였다. CXCL10의 혈청농도는 정상 생쥐와 비교하여 C단백유발근육염 생쥐에서 증가되었다 (정상생쥐, 14.3 ± 5.3 pg/ml vs. C단백유발근육염 생쥐, 368.5 ± 135.6 pg/ml, $p < 0.001$). 유세포 분석에서 T세포는 C단백유발 근육염의 비장보다 림프절에 주로 분포하였으며, C단백유발근육염 림프절세포에서 CXCR3의 양성율은 CD4+ T세포보다 CD8+ T세포에서 증가되어 있었다 (CXCR3+/CD4+ T cell, 23.5 ± 4.7 vs. CXCR3+/CD8+ T cell, 65.9 ± 2.1, $n = 6$, $p < 0.001$). 더구나 IFN-γ 양성세포는 CXCR3+CD8+ T세포에서 CXCR3-CD8+ T세포보다 증가되어 있었다 (CXCR3+CD8+ T cell, 28.0 ± 4.2% vs. CXCR3-CD8+ T cell, 9.5 ± 1.5%, $p = 0.016$). 림프절세포의 이동은 CXCL10에 반응하여 증가되어 있었다 (chemotactic index

= 1.91 ± 0.45, p = 0.011). C단백유발근육염을 유도후 항CXCL10항체로 치료하였을 때 (n = 10), 대조치료(anti-RVG1, n = 10)에 비해서 낮은 염증수치를 보였다 (median [range], anti-CXCL10, 0.75 [0.25-2.00] vs. anti-RVG1, 1.43 [1.125-4.25], p = 0.045).

결론. C단백유발근육염모델에서 CXCL10/CXCR3이 염증에서 발현이 증가되어 있으며, 이를 차단하는 것은 근육의 염증을 억제하였다.

Keywords: 다발근육염, CXCL10, CXCR3

학번: 2007-30971