Attenuation of Experimental Autoimmune Myositis by Blocking ICOS-ICOS Ligand Interaction

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*J Immunol* 2007; 179:3772-3779; doi: 10.4049/jimmunol.179.6.3772

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Polymyositis (PM) is an acquired, systemic, connective tissue disease characterized by the clinical and pathologic effects of chronic muscle inflammation (1). Although the etiology and much about the pathogenesis of PM remain elusive, it has increasingly been appreciated that in the muscles of patients with PM, clonally expanded CD8+ CTLs invade muscle fibers that express MHC class I Ags (2, 3), which leads to fiber necrosis via the perforin pathway (4). However, no specific target Ags have been identified, and an agent initiating self-sensitization remains unknown (1). Although corticosteroid is the first-line drug for PM, many patients become steroid resistant and the addition of an immunosuppressive drug may become necessary (1).

Experimental autoimmune myositis (EAM) is an animal model for human idiopathic inflammatory myopathies and has served for elucidation of the pathomechanism of the disease and the development of immunotherapy. Kohyama and Matsumoto (5) previously induced severe and reproducible EAM in Lewis rats by immunization with human skeletal C protein, and demonstrated that muscle inflammatory lesions mimicked those of human PM. C protein is a single polypeptide chain of 140 kDa present in thick filaments of skeletal and cardiac muscles (6). Its physiological function remains obscure, but thick filament structural support and conformational change during muscle activation have been suggested (6). However, the low purity of native C protein could result in the unstable myositis-inducing ability, and it is practically difficult to obtain highly purified native C protein at a sufficient amount for the induction of EAM in a large number of rats. The similar technical problem has been pointed out, and Matsumoto et al. (7) have recently prepared recombinant cardiac C protein and succeeded in inducing severe experimental autoimmune myocarditis in Lewis rats by immunization with the protein.

ICOS is a member of CD28 family costimulatory receptor expressed on activated T cells, and its ligand, ICOS ligand (ICOSL)/B7 homologous protein, belongs to B7 family. The human ICOS is 62–67% identical with rat ICOS, and 68% to mouse ICOS in amino acid sequence. The expression level of rat ICOS on muscle fibers constitutively expresses low levels of ICOSL, whereas
muscle fibers in patients with inflammatory myopathies express markedly increased ICOS-L expression (14). These observations led us to investigate effects of ICOS blockade in C protein-induced EAM in Lewis rats.

In the present study, we prepared recombinant skeletal C protein and induced EAM with the Ag to overcome the above problems. In addition, we analyzed the expression of ICOS in the muscles to clarify the pathomechanisms of the C protein–induced EAM in more detail and administered anti-ICOS mAb to the EAM rats to evaluate possible therapeutic value of ICOS blockade. Analysis of C protein–induced EAM provides not only insights into pathogenesis of human PM, but also useful information regarding development of effective immunotherapies against the disease.

Materials and Methods

Animals and proteins

Lewis rats were purchased from Charles River Laboratories and bred in our animal facility. Rats used in the present study were 6 wk old. Partially purified and purified skeletal myosin and native C protein were prepared, as described previously (5). The experimental protocol was approved by the Institutional Animal Care and Use Committee of Tokyo Women’s Medical University.

Preparation of recombinant C protein fragments

Because C protein is too large to prepare recombinant protein as a whole protein, we planned to produce four protein fragments designated as fragments 1, 2, 3, and 4, corresponding to the 1–290, 284–580, 567–877, and 864–1142 aa sequences, respectively. Total RNA was isolated from human skeletal muscle using RNAzol B (Biotecx Laboratories) and then reverse transcribed into cDNA using ReverTra Ace-α (Toyobo). Then cDNA was PCR amplified with KOD DNA polymerase (Toyobo) and fragment-specific primers. Each primer was designed to contain a restriction enzyme site at both ends. PCR products were inserted into a cloning vector, pCR4-Blunt-TOPO in the Zero Blunt TOPO kit (Invitrogen Life Technologies), and clones with right sequences were obtained by the standard method. Several clones were subcloned into an expression vector, pQE30 (Qiagen), and used for large-scale preparation of C protein fragments. Recombinant C protein fragments produced in transformed Escherichia coli were isolated after denaturing conditions and purified using Ni-NTA agarose (Qiagen). Then purified protein fragments were diluted and refolded in 100 mM Tris-HCl (pH 8.0) containing 500 mM l-arginine, 2 mM glutathione (reduced form), and 2 mM EDTA, after which they were concentrated and dialyzed in PBS. As a final purification step, protein fragments were incubated with DETox-Gel (Pierce) overnight to remove endotoxins. Obtained protein fragments contained endotoxins (<10 EU/1 mg protein, as determined with Toxinometer ET-2000 (WAKO)). Because preliminary study demonstrated that fragment 2 had the highest water solubility among the four fragments and showed same ability to induce EAM as whole human C protein (5) (data not shown), we used fragment 2 in the following experiments.

Preparation of anti-ICOS Ab

Anti-rat ICOS mAbs (JmAb50 and JmAb50) and isotype-matched control IgG1 mAb against keyhole limpet hemocyanin (JmAb216) were prepared at JT Frontier Research Laboratory, as described previously (15). JmAb49 was used for immunohistochemistry, and JmAb50 was used for in vivo experiments.

EAM induction and tissue sampling

Lewis rats were immunized by s.c. injection of the recombinant human (rh) C protein fragments with CFA (2.5 mg/ml Mycobacterium tuberculosis) in hind footpads, tail base, and neck one time each on a weekly basis, and were sacrificed 2 wk after the last immunization. For controls, PBS/CFA was injected according to the same protocol. To prevent degeneration of the Ags, only freshly prepared or short-term preserved (1 mo or less at −80°C) preparations were used. At the time of immunization, each rat received an i.p. injection of 2 μg of pertussis toxin (Seikagaku Kogyo). Rat muscles were removed from proximal portion of lower extremities and frozen in chilled isopentane precooled in liquid nitrogen on day 28. Rat inguinal lymph nodes (LNs) and spleen were also removed from each rat on day 28.

Treatment by anti-ICOS mAb

To study effects of anti-ICOS mAb on this animal model, we administered JmAb50 (3 mg/kg) or control mAb (JmAb216) (3 mg/kg) i.v. twice per week for 3 consecutive wk (days 7, 10, 14, 17, 21, and 24) to the rats along with the immunization by rhC protein fragment 2. All of the rats were sacrificed on day 28, and muscles, inguinal LNs, and spleen were removed from each rat, as described above.

Histological grading of inflammatory lesions and immunohistochemistry

For histological and immunohistological study, frozen sections (5 μm) were cut in a cryostat and air dried. For histological study, they were fixed in ether for 10 min and stained with H&E. Using H&E-stained sections, histological severity of inflammation was graded into the following four categories: grade 1, single or <5 muscle fiber involvement in one muscle block; grade 2, a lesion involving 5–30 muscle fibers in one muscle block; grade 3, a lesion involving a muscle fasciculus; grade 4, a diffuse extensive lesion. When multiple lesions were found in one muscle block, 0.5 was added to the score (5).

For immunohistochemical analysis, the biotin-free immunohistochemical staining through use of the HRP-labeled polymer system, without crosslinking, was performed in accordance with the manufacturer’s instructions (Histofine Simple Stain Rat MAX-PO (mouse; rabbit); Nichirei) (16). In brief, frozen sections were air dried and fixed in ether (at room temperature) or acetone (at −20°C) for 10 min. After a wash with PBS and incubation with normal goat or rabbit serum for 30 min, serial sections were incubated for 60 min at room temperature with primary mouse anti-rat mAbs, followed by the polymer-conjugated anti-mouse or rabbit IgG (Nichirei), and washed with PBS. For the substrate-chromogen reaction, diaminobenzidine tetrahydrochloride (Nichirei) was used according to the manufacturer’s protocol. Control sections were subjected to the isotype-matched normal mouse IgG. Sections were counterstained with hematoxylin for 1 min and washed in tap water for 10 min. The mouse mAbs used in the present study for immunohistochemistry were R73 (1:500 dilution; anti-TCRα/β; Serotec), W3/25 (1:400–2.5 μg/ml; anti-CD4; Cedarlane Laboratories), 1H10 (OX-8; 2.5 μg/ml; anti-CD8; Oxford Biotechnology), OX42 (1:1000 = 1 μg/ml; anti-CD11b/c used as anti-macrophage; Oxford Biotechnology), JmAb49 (50 μg/ml; anti ICOS), OX18 (1 μg/ml; anti-MHC class I; Abcam), 3D6 (1 μg/ml; anti-MHC class II; Abcam), and goat anti-rat IL-1α (1/200 dilution; Santa Cruz Biotechnology). To evaluate the severity of inflammation in addition to the above mentioned histological grading, we counted the number of CD11b/c+ TCRα+ and CD8α+ cells in serial sections according to the method of Suzuki et al. (17). The evaluation of histological inflammatory changes was performed in a blind fashion for the experimental group identity.

Cell isolation protocol

Isolated piece of muscles was minced into 2- to 4-mm pieces with sterile scissors. The tissue pieces were added to cold DMEM (Nikkon) supplemented with 10% heat-inactivated FBS (Pigma-Aldrich), 1% penicillin, 1% streptomycin, and 10 mM heat-inactivated PBS, 1% penicillin, 1% streptomycin, and 2.5 × 10^2 M 2-ME. RBC were depleted by incubating in hypotonic solution (0.83% NH4Cl, 20 mM Tris-HCl (pH 7.6)) at 37°C for 30 min. After this, cells were washed twice in ice-cold PBS supplemented with 10% heat-inactivated PBS and 1% penicillin, 1% streptomycin, and 2.5 × 10^2 M 2-ME. RBC were depleted by incubating in hypotonic solution (0.83% NH4Cl, 20 mM Tris-HCl (pH 7.6)) at 37°C to prepare splenocytes.

Flow cytometric analysis of muscle cells and LN cells

mAbs against the following Ags were used for flow cytometry analysis: CD3 (G4.18, mouse IgG3), CD4 (OX-38, mouse IgG2a), CD8a (OX-8, mouse IgG1), and ICOS (JmAb49). All FITC-, PE-, or PerCP-conjugated mAbs were prepared in our laboratory and were used at a concentration of 10 μg/ml. mAbs JmAb49 was biotinylated in our laboratory and was detected by streptavidin-PerCP (BD Biosciences). Multicolor analysis was performed using flow cytometry (FCM). Rat muscle cells or LN cells were washed three times in ice-cold FCMM buffer (PBS-0.1% BSA-0.1% sodium azide). Cells were then incubated at 4°C with saturating amounts of the fluorochrome 1H10 (OX-8 or PE)– or OX42–conjugated mAb for 30 min. Cells were washed twice in ice-cold FCMM buffer and incubated at 4°C with streptavidin-PerCP for 30 min when necessary. After incubation, cells were washed three times in ice-cold FCMM buffer and fixed in PBS containing 1%
In the adjuvant-treated rats (B) and normal rats (C) showed normal appearance with neither inflammatory foci nor necrosis. A–C. H&E staining. Original magnification, ×40 (A–C) and ×400 (inset of A–C). D. Evaluation of myositis by the histological grades among rhC protein-immunized rats, adjuvant-treated rats, and normal rats. The histological grades of the rhC protein fragment 2-immunized rats were significantly higher than those of adjuvant-treated rats and normal rats. The histological grade of one muscle (hamstrings) of each rat. Data represent the mean ± SEM values of eight muscles of four animals.

FIGURE 1. Histopathological features of EAM rats. Lewis rats were immunized on days 0, 7, and 14 with rhC protein fragment 2 and sacrificed on day 28. A. Immunization with rhC protein fragment 2 elicited obvious EAM. B and C. Most muscle specimens from adjuvant-treated rats (B) and normal rats (C) showed normal appearance with neither inflammatory foci nor necrosis. A–C. H&E staining. Original magnification, ×40 (A–C) and ×400 (inset of A–C).

Quantitative PCR

Total RNA was extracted from freshly isolated muscle using TRIzol (Invitrogen Life Technologies) and was reverse transcribed to cDNA using SuperScript II RNase H (Invitrogen Life Technologies). One microliter of the cDNA was used as templates for quantitative PCR, which was performed using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) with ΔΔCt method, according to the instructions of the manufacturer. Primers and probes for quantitative RT-PCR of rat GAPDH, IL-1α, and CCL2/MCP-1 were also purchased from Applied Biosystems.

Proliferation assay by rhC protein-stimulated LN cells and LN T cells

LN cells and splenocytes were prepared, as described above. OX-52 (pan T cell)-positive LN T cells of EAM rats were purified using mAb-conjugated microbeads and magnetic cell separation columns (Miltenyi Biotec). An APC-enriched population was prepared from normal rat splenocytes by depleting OX-52-positive T cells and HIS24 (CD45R)-positive B cells using MACS. Purified LN T cells (8 × 10^4) from EAM rats and 2 × 10^4 APC-enriched normal splenocytes were cocultured in 96-well culture plates in DMEM supplemented with 10% heat-inactivated FBS, 1% penicillin, 1% streptomycin, and 5 × 10^-3 M 2-ME with various concentrations of denatured (60°C, 30 min) rhC protein fragment 2 for 72 h, and pulsed with [3H]thymidine (1 μCi/well; Amersham Biosciences) for the last 8 h. Con A was added to some wells as a positive control for stimulation. [3H]Thymidine incorporation was measured using Matrix96 (Packard Instrument).

ELISA for IFN-γ

Concentration of IFN-γ in the culture supernatant was measured using an ELISA kit (Amersham Biosciences), according to the protocol provided by the manufacturer.

ELISA for serum anti-C protein Ab

IgG anti-rhC protein fragment 2 Ab levels in the sera of EAM rats and normal rats were measured using ELISA, according to the protocol described below. A 96-well microtiter plate was coated with the rhC protein fragment 2 used for the induction of EAM and incubated at 4°C overnight. After incubation with normal calf serum at room temperature for 2 h, appropriately diluted rat serum samples were added. The plate was allowed to react with biotinylated goat anti-rat Ig F(ab′)2 (BioSource International) at room temperature for 2 h, followed by HRP-labeled streptavidin (Sigma-Aldrich) at room temperature for 60 min. The reaction was visualized using ABTS Microwell Peroxidase Substrate System (Kirkegaard & Perry Laboratories), and color development was measured at 415 nm by a Benchmark microplate reader (Bio-Rad).

Statistical analysis

Data are expressed as the mean ± SEM. Significant differences between the experimental groups were evaluated using Fisher’s exact test (for the incidence of myositis), Mann-Whitney’s U test (for the histological grades, the number of muscle-infiltrating CD11b/c^+, TCR^+^, or CD8a^+^ cells and quantitative RT-PCR), Student’s unpaired t test (for the [3H]thymidine incorporation and the concentration of IFN-γ), or Dunnett’s test (for the serum anti-C protein Ab). All statistical analyses were performed using JMP statistical software (version 5.1.2; SAS Institute). Values of p < 0.05 were considered statistically significant.

Results

Histological grading of inflammatory lesions and immunohistochemistry

As representative shown in Fig. 1A, immunization with fragment 2 of rhC protein elicited obvious EAM, the pathology of which is essentially the same as that induced with purified whole human native C protein, as previously reported (5). In the inflammatory foci of hamstrings and quadriceps muscles of rhC protein-immunized rats, numerous mononuclear cells were present in the endomysium and perimysium, and around the blood vessels in some fasciculuses. These cells often surrounded, invaded, and replaced the muscle fibers. Some muscle fibers were atrophic or necrotic and had internal nuclei. In moderate to severe inflammatory lesions, several muscle fibers were infiltrated with a large number of mononuclear cells. On the contrary, most muscle specimens from the control rats showed normal appearance with neither inflammatory foci nor necrosis, but some adjuvant-treated rats had a scatter of mononuclear cell infiltrates in the endomysium (Fig. 1, B and C). We also compared the histological grades among rhC protein-immunized rats, adjuvant-treated rats, and normal rats. The histological grades of the rhC protein-immunized rats were significantly higher than those of adjuvant-treated rats and normal rats (Fig. 1D).
To characterize the inflammatory cells in rhC protein-induced EAM, an immunohistochemical study was performed using a panel of mAbs (Fig. 2). As shown in Fig. 2A, anti-TCR mAb-positive T cells were mainly located in the endomysium and some infiltrated muscle fibers. Interestingly, very few CD4\(^+\) cells (T cells and macrophages) infiltrated muscle fibers, although there were many CD4\(^+\) cells in the endomysium (Fig. 2B). The total number of CD8\(^+\) T cells was small in comparison with that of CD4\(^+\) cells. However, the numbers of CD8a\(^+\) cells infiltrating muscle fibers were larger than those of CD4\(^+\) cells (Fig. 2C). A huge number of OX42\(^+\) cells (macrophages) was recognized in both muscle fibers and the endomysium (Fig. 2D). These results were also very similar to those with whole native C protein, as previously reported (5). MHC class I Ag\(^+\) mononuclear cells were located in the perivascular area as well as in the endomysium (Fig. 2E). The vascular endothelial cells also expressed MHC class I Ag (Fig. 2F). MHC class II Ag\(^+\) cells were mainly found in the perimysium (Fig. 2G). Expression of ICOS on T cells in the muscles of EAM rats was analyzed by flow cytometry. ICOS was expressed on 18.7–68.2% of CD3-positive T cells in the muscle of an EAM rat (Fig. 3C). Because few mononuclear cells infiltrated into muscles of normal rats, we could not perform flow cytometry analysis of these rats.

**ICOS expression in the inflammatory lesions of EAM rats**

We next examined the expression of ICOS in muscles of EAM rats by immunohistochemistry. ICOS was expressed on some of muscle fiber-infiltrating mononuclear cells in EAM rats (Fig. 3, A and B), but not in normal rats; those cells were considered to be T cells by staining of serial sections (data not shown).

Expression of ICOS on T cells in the muscles of EAM rats was analyzed by flow cytometry. ICOS was expressed on 18.7–68.2% of CD3-positive T cells in the muscle of an EAM rat (Fig. 3C). Because few mononuclear cells infiltrated into muscles of normal rats, we could not perform flow cytometry analysis of these rats.

**Anti-ICOS Ab therapy attenuated the disease progression of EAM**

Because immunohistological examination suggested that ICOS might play an important role in the immunopathogenesis of EAM and treatment with anti-ICOS mAb ameliorated several animal models of autoimmune diseases, we explored the therapeutic effects of anti-ICOS mAb in our EAM rats. In these experiments, we used anti-ICOS mAb, JmAb50, which has been shown to inhibit binding of ICOS to ICOSL in vitro (15). EAM were induced by the immunization with rhC protein fragment 2, and anti-ICOS mAbs
(JmAb50) or control mAb (JmAb216) (3 mg/kg) were administered i.v., as described in Materials and Methods. The muscles of control mAb-treated rats (Fig. 4A) showed severe inflammation, whereas the muscles of anti-ICOS mAb-treated rats showed only marginal inflammation (Fig. 4B). In the control group, the histological grade was 2.6 ± 0.23 and incidence of myositis was 100% (n = 14 muscles of 7 rats). The histological grade of anti-ICOS mAb-treated rats (0.9 ± 0.25) and incidence of myositis (n = 14 muscles of 7 rats, 57%) were significantly lower than those of controls (Fig. 4C) (p = 0.0001 and 0.0158, respectively). Similar results were obtained in two other independent sets of experiments to treat EAM rats with anti-ICOS mAb (data not shown). To confirm the therapeutic effects of the treatment with anti-ICOS mAb, we performed an immunohistochemical analysis. Numbers of TCRαβ+, CD8α+, and CD11b/c+ cells in the muscles of the anti-ICOS mAb-treated rats were significantly lower than those of the control mAb-treated rats (Fig. 4D). These findings clearly indicate that treatment with anti-ICOS mAb ameliorated myositis in EAM rats. We also examined the effects of anti-ICOS mAb treatment on the expression of IL-1α and CCL2 in the hamstring muscles of the EAM rats using quantitative RT-PCR. The expression of IL-1α and CCL2 mRNA in the anti-ICOS mAb-treated EAM rats was significantly lower than that in the control mAb-treated rats (Fig. 4E). IL-1α and CCL2 were not detected in the hamstring muscle of normal mice (data not shown). Taken together, these results strongly indicate that the treatment with anti-ICOS mAb ameliorated the C protein-induced EAM.

FIGURE 3. Expression of ICOS in muscles of EAM rats. Lewis rats were immunized on days 0, 7, and 14 with rhC protein fragment 2 and sacrificed on day 28. A and B, Expression of ICOS was examined by immunohistochemistry (original magnification, ×200 and ×400, A and B, respectively). ICOS was expressed on some of muscle fiber-infiltrating mononuclear cells in EAM rats. C, Single-cell suspension of muscles was obtained, as described in Materials and Methods, and the cells were stained with FITC-conjugated anti-CD3 mAb and biotinylated anti-ICOS mAb (JmAb49, solid line) or biotinylated control mAb (JmAb216, dotted line) with streptavidin (SA)-PerCP. CD3-positive cells were gated and analyzed. ICOS was expressed on 68.2% of CD3-positive T cells in the muscle of this EAM rat by flow cytometry analysis.

FIGURE 4. Effect of treatment with anti-ICOS mAb on EAM. Rats were immunized with rhC protein fragment 2, as described in Materials and Methods; given 3 mg/kg anti-ICOS mAb (JmAb50) or isotype-matched control mAb i.v. 7, 11, 14, 18, 21, and 25 days after the first immunization; and sacrificed on day 28. A and B, Representative findings of the muscles taken from a control IgG-treated C protein-induced EAM rat (A) and an anti-ICOS mAb-treated EAM rat (B). The muscles of control rats showed as severe inflammation as untreated EAM rats, whereas the muscles of anti-ICOS mAb-treated rats showed only marginal inflammation at most. A and B, H&E staining. Original magnification, ×40 (A and B) and ×400 (inset of A and B). C, The histological grade of one muscle (hamstrings) of each rat (n = 14 for each treatment group). The data shown are representative of three independent experiments with similar results. D, Decreased numbers of infiltrating cells of each subset by anti-ICOS mAb treatment. Numbers of CD11b/c+, TCRαβ+, and CD8α+ cells were counted in the hamstring muscles from the control mAb-treated and anti-ICOS mAb-treated EAM rats. E, Reduction of IL-1α and CCL2 expression by anti-ICOS mAb treatment in the hamstring muscles of the EAM rats. IL-1α and CCL2 mRNA was measured using quantitative RT-PCR. Data represent the mean ± SEM (D and E).
and streptavidin (SA)-PerCP) and flow cytometry. CD3 (JmAb49, solid line) or biotinylated control mAb (JmAb216, dotted line), or PE anti-CD8a mAb (H9253), and biotinylated anti-ICOS mAb (H9255), prepared on day 28. LN cells were also prepared from untreated naive rats (A) or LN T cells (B) were prepared on day 28. LN cells were also prepared from untreated naive rats (A). LN cells (1 x 10^6 cells/well) (A) or LN T cells (8 x 10^5 cells/well) and APC-enriched splenocytes (2 x 10^6 cells/well) from untreated naive rats (B) were cultured in the presence or absence of the indicated concentration of rhC protein fragment 2 for 72 h and pulsed with [3H]thymidine for the last 8 h, and the incorporated radioactivity was measured. C. The supernatants of LN cells after a 72-h culture were assessed for IFN-γ production by ELISA. All data were shown as the mean ± SEM of seven mice in each group (the number of naive rats is 3) (A and C) or three mice (B). Data are representative of three independent experiments with similar results. *p<0.05; **p<0.01 (using Student’s t test). D and E, LN cells from the control mAb-treated EAM rats were analyzed for the expression of ICOS using triple-color staining (FITC anti-CD3 mAb, PE anti-CD4 mAb (D), or PE anti-CD8a mAb (E), and biotinylated anti-ICOS mAb (JmAb49, solid line) or biotinylated control mAb (JmAb216, dotted line) and streptavidin (SA)-PerCP) and flow cytometry. CD3+CD4+ cells (D) or CD3+CD8a+ cells (E) were gated and analyzed for the expression of ICOS. Both CD3+CD4+ (D) and CD3+CD8a+ (E) T cells of control mAb-treated EAM rats expressed ICOS (CD4+ T cells, 22.9–39.9%; CD8a+ T cells, 23.5–26.0%). Stimulation with rhC protein fragment 2 up-regulated the production of IFN-γ by the LN T cells from both treatment groups. The mean levels of IFN-γ production of anti-ICOS mAb-treated rats were higher than those of control mAb-treated rats, but the difference did not reach statistical significance (n = 7 in each group). Similar results were obtained in another independent set of experiments (data not shown). We also analyzed the inhibitory effect of the anti-ICOS mAb in vivo. C protein-specific proliferation of LN cells or LN T cells was not inhibited by the addition of JmAb50 (data not shown).

We prepared LN cells from both treatment groups and analyzed the expression of ICOS using triple-color staining (FITC anti-CD3 mAb, PE anti-CD4 mAb, or PE anti-CD8a mAb, and biotinylated JmAb49 and streptavidin-PerCP) and flow cytometry. Both CD3+ and CD8a+ T cells of control mAb-treated EAM rats expressed ICOS (CD4+ T cells, 22.9–39.9%; CD8a+ T cells, 23.5–26.0%) (Fig. 5, D and E), whereas those of anti-ICOS mAb-treated rats were very low (CD4+ T cells, 2.8–5.9%; CD8a+ T cells, 2.5–3.3%).

**Effect of ICOS inhibition on humoral immunity against C protein**

We next examined effect of anti-ICOS mAb on EAM by studying the humoral immune response. To evaluate the effect of the treatment with anti-ICOS mAb (JmAb50) on humoral immunity, sera taken at the end of the treatment were subjected to ELISA using rhC protein fragment 2. Anti-C protein Ab were markedly elevated in control IgG-treated EAM rats (0.984 ± 0.0403) than unimmunized normal rats (0.286 ± 0.0376) (p < 0.05) or adjuvant-treated rats (0.257 ± 0.0262) (p < 0.05), whereas levels of anti-C protein Ab in anti-ICOS mAb-treated EAM rats (0.839 ± 0.0734) were not significantly different from those of control IgG-treated EAM rats (p = 0.12).

These results suggest that treatment with anti-ICOS mAb did not affect the above aspects of humoral and cellular immunity against rhC protein 2 in EAM rats.

**Discussion**

The major findings of the present study were as follows: 1) skeletal rhC protein fragment 2 induced as severe myositis as native skeletal whole C protein in Lewis rats; 2) treatment with anti-ICOS mAb reduced incidence of myositis, with amelioration of histopathological features of myositis and reduction of IL-1α and CCL2 expression in EAM rats; and 3) treatment with anti-ICOS mAb did not affect serum anti-C protein Ab levels, and it unexpectedly enhanced the proliferative responses of LN cells and LN T cells to the Ag. Because ICOS blockade provided only histological improvement and the reduction of the expression of cytokine and chemokine, the clinical significance of the treatment remains unclear and requires further investigation.
We induced severe and reproducible EAM in Lewis rats by immunization with skeletal rhC protein fragment 2 and demonstrated that muscle fiber-infiltrating cells in the EAM were mainly CD8a⁺ T cells and macrophages. Muscle inflammatory region of patients with PM is characterized by CD8⁺ CTLs invading muscle fibers (1–3). The histopathological features of muscle inflammatory regions and type of muscle fiber-infiltrating cells in the EAM are similar to those of human PM, and thus the C protein-induced EAM could be served as a model for human PM. However, some of the pathological features of the EAM in rats are distinct from PM. Not only CD8a⁺ T cells, but also many OX42⁺ macrophages infiltrated muscle fibers in this model, whereas in humans, macrophages infiltrate muscle fibers less frequently in PM in comparison with toxic, necrotizing, or dystrophic myopathies (facioscapulohumeral; due to deficiency of dystrophin or dysferlin) in which macrophages predominate (1). Recently, Sugihara et al. (18) reported C protein-induced myositis in murine, which also shared several common histological features with human PM, including abundant infiltration of CD8 and perforin-expressing cells in the affected muscle.

For optimal stimulation, naive T cell requires the following two signals: the first one is provided by MHC-TCR interaction, and the second one is provided by costimulatory ligand and receptor pairs (19, 20). Muscle fibers do not express the classic costimulatory molecules B7-1 (CD80) or B7-2 (CD86) (21); instead, they express at least three costimulatory molecules, CD40 (22), BB-1 (21), and ICOSL (14). We previously demonstrated that CD40 was expressed on muscle cells, whereas CD154, the ligand of CD40, was expressed on muscle-infiltrating T cells in patients with PM/dermatomyositis, and that the expression of CD40 could be induced by the treatment with IFN-γ in vitro (22). Stimulation of human muscle cells with rCD154 enhanced production of IL-6, IL-8, and MCP-1 (22). BB-1 is one of the receptors for CD28 and human muscle cells with rCD154 enhanced production of IL-6, diminished by the treatment with IFN-γ (14). We also demonstrated that CD40 was expressed on muscle cells, whereas CD154, the ligand of CD40, was expressed on muscle-infiltrating T cells in patients with PM/dermatomyositis, and that the expression of CD40 could be induced by the treatment with IFN-γ in vitro (22). Stimulation of human muscle cells with rCD154 enhanced production of IL-6, IL-8, and MCP-1 (22). BB-1 is one of the receptors for CD28 and muscle fibers make direct cell-to-cell contact with the infiltrating CD8⁺ cells via CD28 or CTLA-4 on their surface (21, 23). The results of the present study and the expression of ICOSL in patients with PM and other inflammatory myopathies (14) indicate that the ICOS-ICOSL interaction may also serve as costimulatory pairs for muscle-infiltrating T cells and may be involved in the pathogenesis of inflammatory myopathies.

To investigate immunological mechanisms of the treatment with anti-ICOS mAb (JmAb50), we measured rhC protein fragment 2-induced LN cell and LN T cell proliferation and IFN-γ production and serum anti-rhC protein fragment 2 IgG levels. Unexpectedly, none of them was inhibited by the treatment with JmAb50. In other animal models of autoimmune diseases, blockade of ICOS-ICOSL interaction inhibited proliferation of T cells, production of cytokines, and Ag-specific Ab levels, which would explain the beneficial effects of the treatment (10–13). However, we found that biotinylated anti-ICOS mAb (JmAb49) bound to LN T cells from the control mAb-treated EAM rats (Fig. 5, D and E), but did not bind to those from anti-ICOS mAb-treated EAM rats. Because vascular endothelial cells (24) and muscle cells (14) were reported to express ICOSL, these observations supported the possibility that the treatment may hamper migration of activated T cells into muscles or proliferation and/or survival of effector T cells in muscles by inhibiting interaction between ICOS and ICOSL. Unfortunately, rat ICOSL cDNA has not been cloned and we could not perform further analysis in our animal model.

Although ICOS-ICOSL blockade has been shown to inhibit T cell responses in many animal models of autoimmune diseases (10–13), it enhanced T cell responses under some experimental conditions. Rottman et al. (11) induced experimental allergic encephalomyelitis in SJL mice with PLP, and found that ICOS blockade during the efferent immune response (9–20 days after immunization) abrogated the disease, but blockade during Ag priming (1–10 days after immunization) exacerbated it. Upon culture with PLP and compared with immunized controls, proliferation and production of IFN-γ of splenocytes were decreased by efferent blockade, but increased by priming blockade. We administered anti-ICOS mAb 7–24 days after the first immunization to the rats along with the immunization (7 and 14 days after the first immunization) because mild inflammation was already observed in the EAM muscles on day 7 (data not shown) and we considered that day 7 would be already in efferent immune response phase. Administration of anti-ICOS mAb from day 7 might be too early to decrease cellular immune response against the Ag, but it requires further investigation to assess this possibility.

In conclusion, skeletal rhC protein fragment 2 induced as severe EAM as whole native C protein in Lewis rats, and the treatment with anti-ICOS mAb ameliorated EAM. ICOS-ICOSL interaction would be a novel therapeutic target of PM. Analysis of C protein-induced EAM provides not only insights into pathogenesis of human PM, but also useful information regarding development of effective immunotherapies against the intractable disease.

Acknowledgments
We thank Dr. Katsunari Tezuka and Dr. Masashi Sasabuchi (JT Pharmaceutical Frontier Research Laboratories, Kanagawa, Japan) for providing anti-ICOS mAbs (JmAb49 and JmAb50). We also thank Drs. Hitoshi Kohsaka and Toshihiro Nanki (Tokyo Medical and Dental University, Tokyo, Japan) for their critical advice.

Disclosures
The authors have no financial conflict of interest.

References


