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Attenuation of Experimental Autoimmune Myositis by Blocking ICOS-ICOS Ligand Interaction¹

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Polymyositis (PM) is an acquired, systemic, connective tissue disease characterized by the proximal muscle weakness and infiltration of mononuclear cells into the affected muscles. To understand its etiology and immunopathogenesis, appropriate animal model is required. It has been demonstrated that immunization with native human skeletal C protein induces severe and reproducible experimental autoimmune myositis (EAM) in Lewis rats, and that the muscle inflammatory lesions in the EAM mimic those of human PM. In the present study, we prepared recombinant skeletal C protein fragment and succeeded in inducing as severe EAM as that by native C protein. We found ICOS expression on muscle fiber-infiltrating T cells in the EAM rats, but not in normal rats. Treatment with anti-ICOS mAb reduced incidence and severity of myositis; decreased the number of muscle-infiltrating CD11b/c⁺, TCR⁺, and CD8α⁺ cells; and inhibited the expression of IL-1α and CCL2 in the hamstring muscles of the EAM rats. However, the treatment neither inhibited serum anti-C protein IgG level, C protein-induced proliferation of lymph node (LN) cells, or LN T cells, nor production of IFN-γ by C protein-stimulated LN cells in EAM rats. These data indicate that analysis of C protein-induced EAM provides not only insights into pathogenesis of PM, but also useful information regarding development of effective immunotherapy against the disease. ICOS-ICOS ligand interaction would be a novel therapeutic target for PM. The Journal of Immunology, 2007, 179: 3772–3779.

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Abbreviations used in this paper: PM, polymyositis; EAM, experimental autoimmune myositis; FCM, flow cytometry; ICOSL, ICOS ligand; LN, lymph node; PLP, proteolipid protein; rh, recombinant human.

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To study effects of anti-ICOS mAb on this animal model, we administered JmA5b0 (3 mg/kg) or control mAb (JmA2b16) (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 5–50 muscle fibers in one muscle block; grade 3, a lesion involving a muscle fascicle; 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 cross-avidation between the rat specific mAbs and the anti-rat mAb, was used. 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 (1:1000; anti-ICOS; Serotec), 10E6 (1:200; anti-CD4; Cedarlane Laboratories), and CD8a (1:400; Serotec). W3/25 (1:400; anti-CD8; Serotec) and OX-8 (1:100; anti-CD8; Oxford Biotechnology), OX42 (1:1000; anti-CD8; Oxford Biotechnology), JmA5b9 (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 CD1a+ 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 pieces of muscles was minced into 2- to 4-mm pieces with sterile scissors. The tissue pieces were minced with forceps, using scissors. The tissue pieces were added to cold DMEM (Nikken) supplemented with 10% heat-inactivated FBS, 1% penicillin, 1% streptomycin, and 1% heat-inactivated PBS. The solution was incubated in a 37°C incubator for 2 to 3 hr. Particles suspended in collagenase type II (1 mg/ml; Worthington Biochemical) dissolved in DMEM at 37°C for 2 h, mixing intermittently. The cells were gently dispersed by pipetting. The cell suspension was filtered through fine nylon mesh and washed two to three times. Cells were resuspended in DMEM, and cell yield and viability were measured. Single-cell suspensions of LN cells and splenocytes were prepared by passing through fine nylon mesh in DMEM supplemented with 10% heat-inactivated PBS, 1% penicillin, 1% streptomycin, and 10X 10^3 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 (JmA5b9). All FITC-, PE-, or PerCP-conjugated mAbs were used. The concentration of conjugation was estimated to be 10 μg/ml/mAb. JmA5b9 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 FCMBuffer (PBS-0.1% BSA-0.1% sodium azide). Cells were then incubated at 4°C with saturating amounts of the fluorochrome (anti-rHc-FITC or PE-) or rHc-conjugated mAb for 30 min. Cells were washed twice in ice-cold FCMBuffer and incubated at 4°C with streptavidin-PerCP for 30 min when necessary. After incubation, cells were washed three times in ice-cold FCMBuffer and fixed in PBS containing 1% paraformaldehyde.
paraformaldehyde. The expression of cell surface markers was evaluated using an EPICS ALTRA (Beckman Coulter) cell sorter and EXPO32 analysis software (Beckman Coulter).

**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 × 10⁴) from EAM rats and 2 × 10⁴ 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 Dunn’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 representatively 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 CD8a+ 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). The vascular endothelial cells also expressed MHC class I Ag, but the muscle fibers did not. MHC class II Ag cells were mainly found in the perimysium (Fig. 2F). 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 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).
Effect of ICOS inhibition on cellular immunity against C protein

To evaluate the effect of the treatment with anti-ICOS mAb (JmAb50) on cellular immunity, we examined Ag-induced proliferation of LN cells and LN T cells in EAM rats. LN cells as well as LN T cells from untreated EAM rats proliferated in response to rhC protein fragment 2 in a dose-dependent manner, whereas LN cells from naive rats did not proliferate even at a high concentration of Ag (Fig. 5, A and B). Unexpectedly, the [3H]thymidine incorporation of both LN cells and LN T cells from the anti-ICOS mAb-treated EAM rats was higher than that of control IgG-treated EAM rats, and the difference reached statistical significance at all the concentrations of rhC protein fragment 2 examined in LN cells (Fig. 5A) and at 0 and 1.56 μg/ml in LN T cells (Fig. 5B). Similar results were obtained in another independent set of experiments.

We also measured IFN-γ production of LN cells stimulated with rhC protein fragment 2. LN cells from naive rats did not produce IFN-γ in both the presence and absence of C protein (<8 pg/ml, data not shown). IFN-γ was produced at low levels by unstimulated LN cells of both control mAb-treated EAM rats and anti-ICOS mAb-treated rats, and the treatment with anti-ICOS mAb increased the mean levels of IFN-γ production without stimulation (n = 7 in each group) (Fig. 5C). 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 vitro. 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 JmAb50 and streptavidin-PerCP) and flow cytometry. Both CD4+ 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 (fascioscapulohumeral; 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 is activated by the treatment with IFN-γ, and it plays an important role in the pathogenesis of anti-CD28 autoantibody-induced myocarditis and subsequent dilated cardiomyopathy. Although ICOS-ICOSL interaction would be a novel therapeutic target of PM, anti-ICOS mAbs (JmAb49 and JmAb50) would be a novel therapeutic agent for 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.

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**Disclosures**

The authors have no financial conflict of interest.

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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 effferent 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.


