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Definitive Engagement of Cytotoxic CD8 T Cells in C Protein–Induced Myositis, a Murine Model of Polymyositis

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Objective. **To substantiate a pathogenic role of cytotoxic CD8 T cells in the development of a murine polymyositis model, C protein–induced myositis (CIM).**

Methods. **Beta$_2$-microglobulin–null mutant, perforin-null mutant, and wild-type (WT) C57BL/6 mice were immunized with skeletal muscle C protein fragments to provoke CIM. Regional lymph node CD8 or CD4 T cells stimulated with C protein–pulsed dendritic cells were transferred adoptively to naive mice. Inflammation and damage of the muscle tissues were evaluated histologically.**

Results. **The incidence of myositis development was significantly lower in β_2-microglobulin–null and perforin-null mutant mice compared with WT mice. Inflammation was less severe in mutant mice, and the incidence of muscle injury was reduced significantly. Adoptive transfer of lymph node T cells from mice with CIM induced myositis in naive recipient mice. The CD8 T cell–induced muscle injuries were significantly more severe than the CD4 T cell–induced muscle injuries.**

Conclusion. **Perforin-mediated cytotoxicity by CD8 T cells is definitively responsible for muscle injury in CIM.**

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Polymyositis (PM) is a chronic autoimmune inflammatory myopathy that affects the systemic striated muscles. An accumulated body of evidence shows that muscle injury in PM is driven by cytotoxic CD8 T cells (1). We recently established C protein–induced myositis (CIM) in mice as an animal model of PM (2). This murine myositis is readily induced by a single immunization of C57BL/6 (B6) mice with recombinant skeletal muscle C protein fragments. In mice with CIM, CD8 T cells are enriched at the site of the muscle injury. Cytotoxic molecules (perforins) were expressed by T cells surrounding the muscle fibers. Also, class I major histocompatibility complex (MHC) expression was upregulated on the muscle fibers (2). Inducibility of CIM in the B6 strain of mice, which is a background strain for most genetically mutated mice, prompted us to show that B cells are not required for CIM induction. These findings suggested that the muscle injury in CIM is also driven by cytotoxic CD8 T cells. This is in sharp contrast to a classic model, experimental autoimmune myositis, that is induced by repeat immunization with crude myosin. Experimental autoimmune myositis has dominant CD4 T cell infiltration in the muscles and can be transferred adoptively to naive animals by serum transfer (3).

Previously, we showed that pretreatment with anti-CD8α monoclonal antibodies (mAb) to deplete CD8 T cells conferred resistance to CIM. However, the anti-CD8α antibody treatment could deplete a subset of dendritic cells (DCs) expressing CD8α (4). In the inflamed muscles, macrophages and CD4 T cells are also abundant. CD4 T cell depletion exerted the same effect as CD8 T cell depletion (2). Thus, CD4 T cells might play some roles not only as helper cells of CD8 T cell

differentiation but also as effector cells in the injured tissue.

Based on our study of mutant mice and adoptive T cell transfer, we report here definitively that CIM is driven primarily by cytotoxic CD8 T cells. The analogous pathology of this model and that of PM should facilitate further studies of inflammatory myopathies.

MATERIALS AND METHODS

Induction of murine CIM. B6 mice were purchased from Charles River. Beta$_2$-microglobulin–null mutant and perforin-null mutant B6 mice were purchased from The Jackson Laboratory. Female mice ages 8–10 weeks were immunized intradermally with recombinant C protein fragments for CIM induction (2). All experiments were carried out under specific pathogen–free conditions in accordance with the ethics and safety guidelines for animal experiments of Tokyo Medical and Dental University and RIKEN.

Generation of antigen-pulsed mature DCs. DCs were prepared from the bone marrow of B6 mice (5) and were incubated with 50 μg/ml of the recombinant C protein fragments and 1 μg/ml lipopolysaccharide (Sigma-Aldrich). Purity of DCs (>70%) was confirmed with a FACSCalibur (Becton Dickinson) using allophycocyanin-conjugated anti-CD11c mAb (eBioscience).

Adoptive T cell transfer. Draining lymph nodes were removed from wild-type (WT) mice 21 days after the C protein immunization. Three million lymph node cells were cultured with 1.5×10^5 antigen-pulsed DCs and 100 units/ml recombinant human interleukin-2 (Shionogi Pharmaceuticals) for 72 hours. CD8- or CD4-positive T cells were sorted with MACS magnetic beads (Miltenyi Biotech). Purity of these cells was confirmed using a flow cytometer. Either 4 million CD8-positive T cells or 4 million CD4-positive T cells were injected intraperitoneally into naive B6 mice whose foot pads had been pretreated with Freund's complete adjuvant. Prior to the transfer, the CD8 T cells of some mice were depleted by administration of anti-CD8 mAb (53.67.2) (2). The muscles of the hind legs were evaluated histologically 14 days after the transfer.

Histologic analysis of myositis. Hamstring and quadriceps muscles were examined for histopathologic changes. Two 10-μm sections derived from proximal and distal sites of individual muscles were stained with hematoxylin and eosin (H&E). All fields of the 2 cross sections from each muscle piece were examined for histologic changes. Histologic severity of inflammation in each block was graded as previously described (2). We defined necrotic muscle fibers as muscle fibers that showed decreased H&E stainability and those replaced by mononuclear cell infiltration (6). Microscopic views of the entire muscle sections were captured digitally, and the lesions of necrotic muscle fibers in a total of 4 sections were then marked with ImageJ software (NIH Image, National Institutes of Health; online at: http://rsbweb.nih.gov/ij/) for calculation of total necrotic area.

Statistical analysis. The histologic scores were statistically analyzed using the Mann-Whitney U test. The necrotic muscle areas were statistically analyzed using Student's t-test.

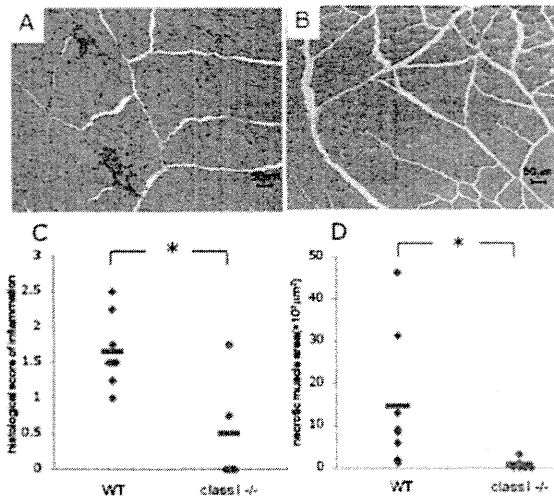

Figure 1. C protein–induced myositis in β_2-microglobulin–null mutant (class I$^{-/-}$) mice. Eight wild-type (WT) female C57BL/6 (B6) mice and 6 β_2-microglobulin–null mutant female B6 mice were immunized. Muscle fibers invaded by inflammatory mononuclear cells were abundant in the WT mice (**A**) but not in the β_2-microglobulin–null mutant mice (**B**). The histologic scores of inflammatory mononuclear cell infiltration (**C**) and the areas of muscle fiber necrosis (**D**) are shown. Horizontal lines indicate the mean. $* = P < 0.05$.

RESULTS

The role of CD8 T cells in mice with CIM. As was stated earlier, pretreatment with anti-CD8α mAb made mice resistant to CIM (2). To demonstrate the requirement of CD8 T cells directly, β_2-microglobulin–null B6 mice, which are devoid of mature CD8 T cells, were immunized with recombinant skeletal muscle C protein fragments for induction of CIM. All experiments were carried out using female mice. In WT mice with CIM, mononuclear cells infiltrated predominantly at the endomysial site, and many necrotic muscle fibers and muscle fibers were invaded by inflammatory mononuclear cells (2) (Figure 1A). The incidence of CIM was reduced in mutant mice compared with WT mice (33% versus 100%). Inflammatory mononuclear cell infiltration and muscle fiber necrosis were sparse in the mutant mice (Figure 1B). The histologic scores, which represent inflammatory mononuclear cell infiltration, were significantly lower in the mutant mice (Figure 1C). The difference was more apparent when necrotic muscle areas were compared (Figure 1D). These results demonstrated the critical role of CD8 T cells in the muscle injury of CIM.

Adoptive transfer of CD4 or CD8 T cells. The relative importance of CD4 and CD8 T cells in myositis was investigated using adoptive transfer of lymph node T cells. Cells were isolated from the draining lymph nodes of mice with CIM 21 days after immunization. These lymph node cells were stimulated in vitro with DCs that were prepared from the bone marrow of naive mice and preincubated with the C protein fragments. The stimulated cells were separated into CD4 and CD8 T cell populations. Flow cytometry analyses showed that the purity of the sorted CD4 and CD8 T cells was >95%, and that CD11c-positive cells were absent. Intraperitoneal transfer of the CD8 T cells into naive mice induced myositis with an incidence of 100%, which accompanied necrosis of muscle fibers. As in CIM, inflammatory mononuclear cells infiltrated predominantly around non-necrotic and necrotic muscle fibers at the endomysial site (Figure 2A). Transfer of the CD4 T cells induced much milder muscle damage, with a lower incidence (41%) (Figure 2B). Necrotic muscle areas induced by the 2 T cell subsets were significantly different (Figure 2C). Next, CD4 T cells were transferred to mice pretreated with depleting anti-CD8 mAb or control mAb. Necrotic muscle areas were comparable between the CD8-depleted and control mice (Figure 2D), showing that CD4-mediated injury was independent of endogenous CD8 T cells. Lymph node T cells derived from naive mice stimulated with the same antigen did not induce myositis (data not shown).

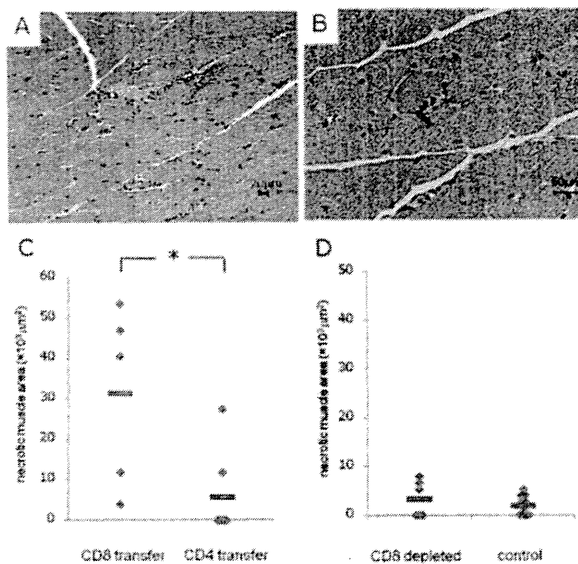

Figure 2. Adoptive transfer of CD8 or CD4 T cells. Lymph node cells from mice with C protein–induced myositis were stimulated with C protein fragment–pulsed dendritic cells, and CD8-positive and CD4-positive T cells were separated and transferred into recipient C57BL/6 mice. **A** and **B**, Adoptive transfer of CD8 T cells (**A**) or CD4 T cells (**B**) induced muscle fiber necrosis. **C**, The areas of muscle fiber necrosis in myositis induced by CD8-positive cells and by CD4-positive cells are shown. **D**, Mice were pretreated with depleting anti-CD8 monoclonal antibodies or control monoclonal antibodies before transfer of the purified CD4 T cells, and purified CD4 T cells were transferred. Necrotic muscle areas of 6 CD8-depleted mice and 9 control mice are shown. Horizontal lines indicate the mean. * = $P < 0.05$. Results shown are representative of those from 2 independent experiments.

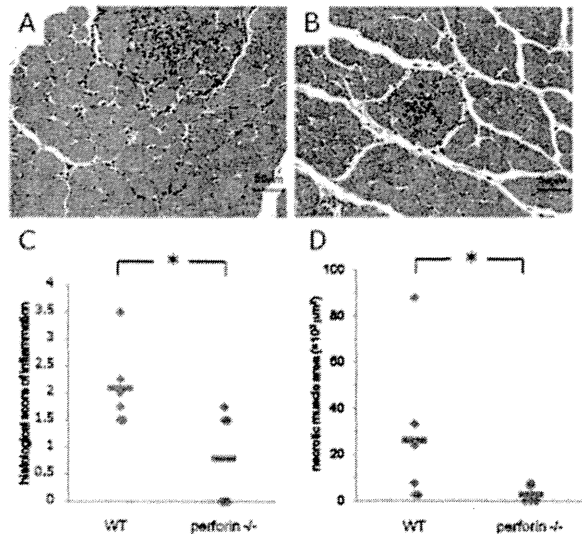

Figure 3. C protein–induced myositis in perforin-null (perforin$^{-/-}$) mutant mice. Six wild-type (WT) C57BL/6 (B6) mice and 6 perforin-null mutant B6 mice were immunized. Necrosis of muscle fibers with mononuclear cell infiltration was observed in WT mice (**A**), and scattered necrosis of single muscle fibers was observed in perforin-null mice (**B**). Histologic scores for inflammatory mononuclear cell infiltration (**C**) and necrotic muscle area (**D**) are shown. Horizontal lines indicate the mean. * = $P < 0.05$.

The role of perforin in mice with CIM. Immunohistochemical studies of the muscles with PM implied that perforin-mediated cytotoxicity should operate in the muscle injury. We next immunized perforin-null B6 mice to induce CIM. It has been reported that these mice have no defect in T cell maturation, and that their CD8 T cells expand normally but lack cytotoxity (7). The incidence of myositis development in mutant mice (50%) was reduced compared with that in WT mice, and mononuclear cell infiltration was milder (Figures 3A–C). Whenever necrosis was found in the perforin-null mice, it was limited to single muscle fibers. Thus, the

areas of muscle injury were significantly reduced in the mutant mice (Figure 3D). This supports the idea that perforin-dependent cytotoxicity is critical for muscle damage in CIM.

DISCUSSION

Resistance of class I MHC mutant mice to CIM demonstrated the crucial role of CD8 T cells in muscle injury. The perforin-null mice showed that the injury should be mediated by effector molecules secreted from the CD8 T cells. Adoptive transfer experiments with CD4 and CD8 T cells from mice with CIM have confirmed the critical importance of CD8 T cells.

Since macrophages and CD4 T cells were abundant in the muscles of mice with CIM (2), histologic grading (which simply reflects the numbers of infiltrating cells) and actual muscle injury might be discordant in mice lacking CD8 effector molecules. To circumvent this problem, we evaluated the necrotic muscle area, which should represent muscle injury directly. Indeed, the muscles from 1 class I MHC mutant mouse with CIM had a histologic score comparable with that of muscles from WT mice (Figure 1C). However, these muscles had a small necrotic area, suggesting that the tissue injury should depend on CD8 cytotoxity.

In a set of experiments, donor T cells derived from green fluorescent protein (GFP)–transgenic mice with CIM were injected intraperitoneally into naive B6 mice. However, GFP-positive cells were not seen in the muscles of the recipients at the peak of myositis (data not shown). Thus, the majority of inflammatory mononuclear cells in adoptive myositis seem to be recruited by the transferred CD8 T cells after the primary muscle injury. We believe that induction of adoptively transferred myositis is antigen dependent, because donor cells stimulated with concanavalin A instead of concanavalin C protein failed to transfer the myositis (data not shown). Also, 3-day stimulation with antigen-pulsed DCs is not long enough for induction of nonspecific lymphokine-activated killer cells (8).

Class I MHC expression was up-regulated in muscles with severe inflammation in mice with CIM (2). We tried to evaluate class I MHC expression in the muscles in the adoptive transfer model and in perforin-null mice with CIM. However, no obvious up-regulation was observed. Based on our observation and on a report of a decrease in class I MHC expression in muscle fibers after corticosteroid treatment (9), we assume that class I MHC up-regulation is associated with the severity of myositis.

To measure muscle function as the clinical outcome, we previously used a Rotarod test (2). However, this technique turned out to be less reliable than histologic analyses, because mice learn how to avoid falling off and do not necessarily run consistently. Although magnetic resonance imaging appears better in detecting myositis, its use still needs to be optimized.

Perforin-dependent cytotoxicity mediated by CD8 T cells is also crucial in pancreatic tissue injury in NOD mice, which is an animal model of type 1 diabetes mellitus. Spontaneous development of diabetes was suppressed in perforin-deficient NOD mice (7), and CD8 T cell clones isolated from pancreatic islets of prediabetic NOD mice are able to induce diabetes in nondiabetic NOD mice (10). Interestingly, the islets of the perforin-deficient NOD mice had numbers of CD4 and CD8 T cells comparable with those of control mice. In contrast, muscle fiber necrosis and mononuclear cell infiltration were both suppressed in perforin-deficient mice immunized with C protein fragments. This result implies again that inflammatory mononuclear cells in the muscles of mice with CIM should be recruited as a secondary event.

Scattered necrotic muscle fibers were found in the perforin-null and β_2-microglobulin–null mice. Transfer of activated CD4-positive T cells induced mild muscle fiber necrosis, which did not depend on endogenous CD8 T cells. In this regard, beta cell loss and diabetes are observed in 16% of perforin-deficient NOD mice (7), and some islet-specific CD4 T cell clones can induce diabetes when transferred to neonatal NOD mice (11). Also, it was reported that T cell–independent muscle injury was observed in muscle tissues overexpressing class I MHC molecules (12). Thus, the muscle damage could be partly attributable to these CD8 T cell–independent pathways.

CD4 T cells mediate cytotoxicity via Fas ligand or, in a certain instance, perforins. Indeed, Fas antigen is expressed on muscle fibers, while Fas ligand is expressed on T cells in the inflamed muscles (13). However, it was shown that muscle fibers were resistant to apoptosis, possibly because the inflammatory environment induced endogenous antiapoptotic molecules (14).

The present study demonstrated that CD8 cytotoxic T lymphocytes primarily damage the muscle fibers in CIM and confirmed that CIM is the mouse myositis model most analogous to human PM. Recently, a randomized, double-blind, placebo-controlled trial failed to show efficacy of the anti–tumor necrosis factor α (anti-TNFα) mAb infliximab in treating patients with PM and dermatomyositis (15). This might be consistent with the fact that CIM does not require TNFα for its develop-

ment (2). We expect that further analyses of CIM will shed more light on the pathology of PM.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Kohsaka had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
Study conception and design. Sugihara, Miyasaka, Kohsaka.
Acquisition of data. Sugihara, Okiyama, Suzuki, Kohyama, Matsumoto.
Analysis and interpretation of data. Sugihara, Okiyama, Kohsaka.

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Interleukin-1 and Tumor Necrosis Factor α Blockade Treatment of Experimental Polymyositis in Mice

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Objective. **Histologic studies of the muscles suggest that cytokines are involved in inflammatory myopathy. The therapeutic effects of cytokine blockade are controversial, with anecdotal reports of clinical efficacy. The aim of this study was to discern the significance of interleukin-1 (IL-1) and tumor necrosis factor α (TNFα) as therapeutic targets in polymyositis (PM) by studying their involvement and the effects of their blockade in C protein–induced myositis (CIM), a murine model of PM.**

Methods. **C57BL/6 mice were immunized with recombinant skeletal C protein fragments to induce CIM. The expression of IL-1 and TNFα in the muscles of mice with CIM was detected using immunohistochemical and real-time polymerase chain reaction analyses. After the onset of myositis, the mice with CIM were treated with recombinant IL-1 receptor antagonist (IL-1Ra), anti–IL-1R monoclonal antibody, recombinant TNF receptor (p75)–fusion protein (TNFR-Fc), or anti-TNFα monoclonal antibody. The muscles were examined histologically for the severity of myositis.**

Results. **IL-1α– and TNFα-positive macrophages were observed in the muscle tissue of mice with CIM as early as 7 days after immunization. IL-1α, IL-1β, and TNFα expression in the muscles increased as the severity of myositis peaked, at both the messenger RNA and protein levels. Continuous subcutaneous delivery of IL-1Ra resulted in suppression of established CIM. Intermittent delivery (1-day intervals) of anti–IL-1R monoclonal antibody suppressed myositis, while intermittent delivery of IL-1Ra did not suppress myositis. Treatment with anti-TNFα monoclonal antibody and with TNFR-Fc also reduced the severity of CIM.**

Conclusion. **IL-1 and TNF blockade ameliorated CIM after disease onset and should potentially be a new strategy for the treatment of inflammatory myopathy. As IL-1 blockade, treatment with anti–IL-1R monoclonal antibody appeared more feasible than the other approaches.**

Polymyositis (PM) is a chronic autoimmune inflammatory myopathy affecting striated muscles (1). An accumulated body of evidence supports the notion that the pathology of PM is driven by cytotoxic CD8 T cells (2–7), but the event that initiates the inflammatory processes has not been identified. Currently, patients with PM are treated primarily with nonspecific immunosuppressants, including high-dose corticosteroids, methotrexate, and/or other small-molecule immunosuppressants. Because the administration of therapeutic agents can elicit a wide variety of adverse reactions, treatments that address the specific pathology of PM need to be developed.

In the development of new therapeutic approaches to human diseases, animal models have served as a means with which to identify therapeutic targets and to test the effect of new treatments (8–11). Despite the known limitations, experiments in animals with collagen-induced arthritis (CIA) have facilitated development of new treatments for rheumatoid arthritis (RA). Treatment approaches such as blockade of interleukin-1 (IL-1), tumor necrosis factor α (TNFα), and IL-6 have had an enormous effect in modulating the disease course of RA (12–15).

However, in myositis research, no appropriate

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animal model of PM had been available until the murine model of C protein–induced myositis (CIM) was developed (16). Unlike the classic model of experimental autoimmune myositis (EAM), which is induced by repeated immunizations to desferlin gene–mutated mice (SJL/J strain) with crude myosin, CIM can be induced in C57BL/6 (B6) mice by a single immunization with recombinant human fast-type skeletal muscle C protein fragments. Although serum can be used to transfer EAM to naive mice (17), CIM is primarily mediated by cytotoxic CD8 T cells (18). Among the available models, CIM mimics human PM best in terms of pathology (16,18).

Because of the availability of biologic anticytokine reagents for clinical use, these reagents have been anecdotally tested for the treatment of patients with PM and patients with dermatomyositis (DM) who did not respond to conventional treatment (19–21). In this regard, results of animal experiments using anticytokine reagents will represent a rationale for conducting controlled clinical studies in humans. We recently observed that anti–IL-6 receptor (anti–IL-6R) antibodies were effective for the treatment of CIM (22).

In mice with CIM and in the muscles of patients with PM (23,24), both IL-1 and TNFα are expressed by infiltrating mononuclear cells. Previously, we observed that the development of CIM was suppressed in IL-1α/β–null mutant mice but not in TNFα-null mutant mice (16). This observation suggested the differential requirement of inflammatory cytokines for CIM induction. However, it is unclear whether blockade of IL-1 or TNFα after disease onset can suppress CIM. Also, genetically mutated mice may undergo skewed development of the immune system and respond aberrantly to immunogens. In the present study, we examined the therapeutic effects of IL-1 blockade and TNFα blockade in mice with established CIM. In addition, antibodies and soluble decoy molecules were compared for the in vivo effects of IL-1 blockade.

MATERIALS AND METHODS

Induction of CIM. Female B6 mice (Charles River), ages 8–10 weeks, were immunized by intradermal injection of recombinant human fast-type skeletal muscle C protein fragments emulsified in Freund's complete adjuvant (CFA) together with intraperitoneal injection of pertussis toxin (16). All experiments were carried out under specific pathogen–free conditions in accordance with the ethics and safety guidelines for animal experiments of Tokyo Medical and Dental University and RIKEN.

Anticytokine treatment. Murine IL-1R antagonist (IL-1Ra), hamster anti-mouse IL-1R chimeric IgG1 monoclonal antibody (clone M147), and murine recombinant TNF receptor (p75)–fusion protein (TNFR-Fc) were provided by Amgen. Anti-TNFα chimeric (rat and murine) IgG2a monoclonal antibody (clone cV1q) and control chimeric IgG2a monoclonal antibody with unknown antigen specificity (clone cVaM) were provided by Centocor. IL-1Ra was administered continuously with subcutaneously implanted osmotic minipumps (Durect), while the other treatments were administered intraperitoneally 3 times weekly.

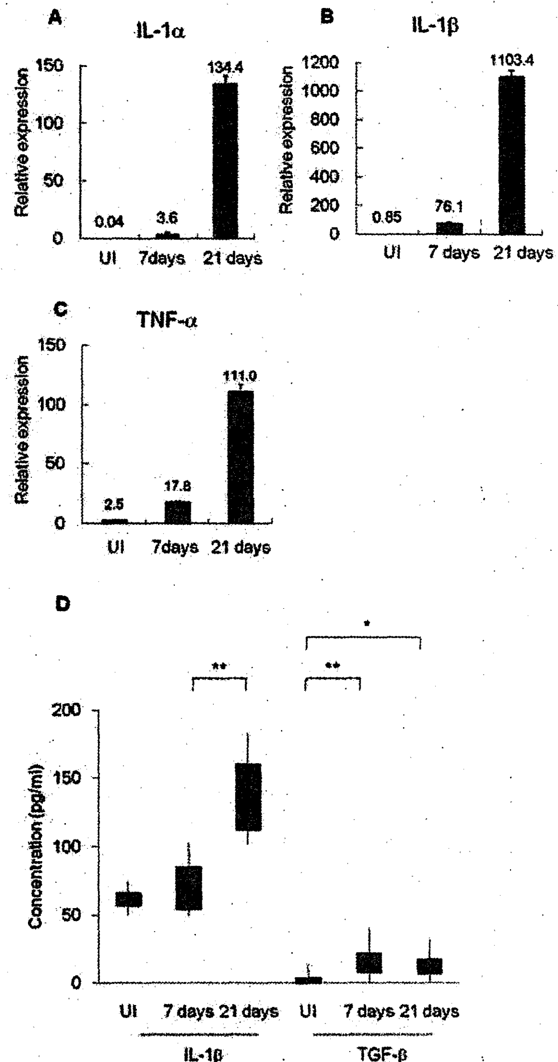

Figure 1. Interleukin-1 (IL-1), tumor necrosis factor α (TNFα), and transforming growth factor β1 (TGFβ1) expression in the muscles of mice with C protein–induced myositis (CIM). **A–C,** Real-time polymerase chain reaction analysis was performed to quantify the expression of IL-1α (**A**), IL-1β (**B**), and TNFα (**C**) mRNA in the muscle tissue of unimmunized (UI) mice and mice with CIM, 7 days and 21 days after immunization. Expression levels are normalized to expression of GAPDH. Bars show the mean ± SD (n = 3 mice). **D,** Enzyme-linked immunosorbent assay of IL-1β and active TGFβ1 was carried out in the muscles of unimmunized mice (n = 6) and mice with CIM (n = 8–10), 7 days and 21 days after immunization. Data are presented as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the lines outside the boxes represent 0–100%. * = $P < 0.05$; ** = $P < 0.01$.

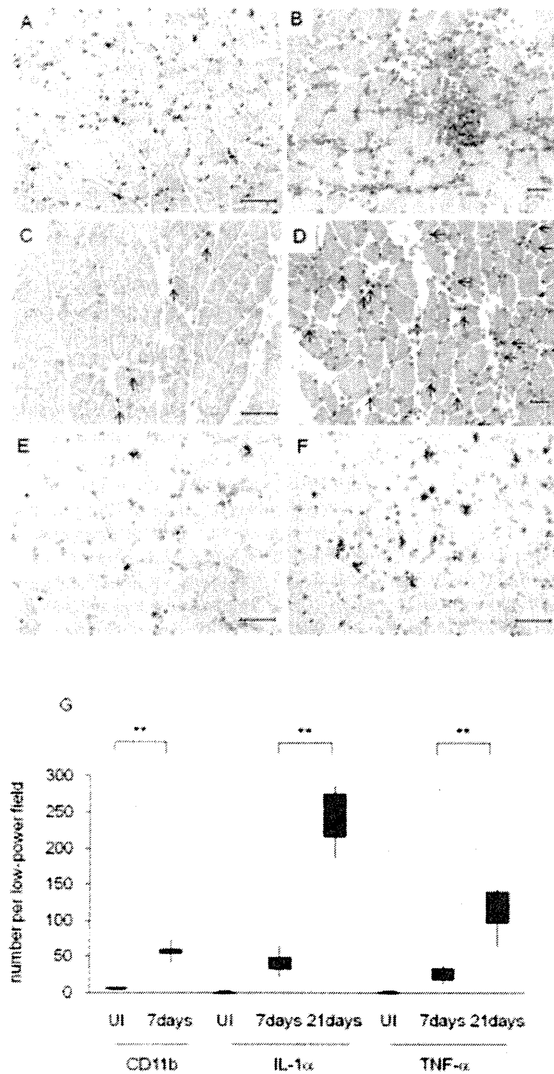

Figure 2. Immunohistochemical analysis of muscle tissue. IL-1α (**A** and **B**), TNFα (**C** and **D**; **arrows indicate positively stained cells**), and CD11b (**E** and **F**) were detected immunohistochemically in muscle sections from unimmunized mice (**E**) and mice with CIM 7 days after immunization (**A**, **C**, and **F**) and 21 days after immunization (**B** and **D**). In these sections, 10 low-power (200×) fields were selected to include myositis lesions, where CD11b-, IL-1α–, and TNFα-positive cells were enumerated (**G**). IL-1– and TNFα-positive cells appeared in the muscles 7 days after immunization with C protein/Freund's complete adjuvant and become more abundant 21 days after immunization. CD11b-positive cells were present in the muscle tissue of unimmunized mice and expressed no detectable inflammatory cytokines. Seven days after immunization, the number of CD11b-positive cells in the muscles increased significantly. The distribution pattern of CD68-positive cells in muscles was the same as the distribution pattern of CD11b-positive cells (data not shown). Bars in **A**, **C**, **E**, and **F** = 50 μm; bars in **B** and **D** = 30 μm. Data in **G** are presented as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the lines outside the boxes represent 0–100%. ** = $P < 0.01$. See Figure 1 for definitions.

Histologic severity of inflammation. The system for scoring the histologic severity of inflammation in muscles affected by experimental myositis was originally established using a Lewis rat model of myositis (25,26). This system was applied successfully to the evaluation of several treatments of CIM (16,22).

For each mouse, 2 sections of bilateral muscles (hamstrings or quadriceps) were evaluated. Myositis was defined as mononuclear cell infiltration spreading around muscle fibers, including at least 1 necrotic muscle fiber. The histologic score for myositis severity was determined according to the number of muscle fibers associated with cellular infiltration (grade 1 = lesions with <5 muscle fibers involved, grade 2 = lesions involving 5–30 muscle fibers, grade 3 = lesions involving an entire muscle fasciculus, and grade 4 = extensive lesions across muscle fasciculi). When multiple lesions with the same grade were observed in the 2 sections of a muscle block, 0.5 point was added to the grade. The mean score of bilateral muscles was calculated and used as the score for each mouse. The muscle sections were evaluated in a blinded manner by at least 2 independent observers, who reported comparable results.

Quantification of cytokine messenger RNA (mRNA) expression. Total RNA was isolated from hindleg muscle tissue with Isogen reagent (Nippon Gene). Quantitative polymerase chain reaction (PCR) was performed on a PerkinElmer 7700 sequence detector using sets of primers and FAM-labeled TaqMan probes specific for IL-1α, IL-1β, TNFα, and GAPDH complementary DNA (Assays-on-Demand; Applied Biosystems).

Enzyme-linked immunsorbent assay (ELISA) of muscle homogenate. The muscles were homogenized in phosphate buffered saline containing protease inhibitors (cOmplete Mini tablets; Roche Diagnostics) and radioimmunoprecipitation assay buffer (Millipore), 3 times for 20 seconds with homogenizer (Mini-BeadBeater; BioSpec) at 2500 revolutions per minute. The supernatants were collected, and the concentrations of IL-1β and active transforming growth factor β1 (TGFβ1) were measured with Quantikine ELISA kits (R&D Systems) according to the manufacturer's directions.

Immunohistochemical analysis. The expression of CD11b, CD68, IL-1α, and TNFα in muscle sections was examined immunohistochemically, as previously described (16). To quantify the stained cells, 10 low-power (200×) fields in each section were selected to include myositis lesions. The numbers of CD11b-, IL-1α–, and TNFα-positive cells in these fields were then determined.

Statistical analysis. Histology scores and quantitative analysis of immunohistochemical studies were analyzed using the Mann-Whitney U test.

RESULTS

IL-1 and TNFα expression in mice with CIM. In the murine model of CIM, which was induced by immunizing B6 mice with recombinant C protein fragments, necrotic muscle fibers with surrounding mononuclear cell infiltration appeared as early as 7 days and peaked 14–21 days after immunization (16). Real-time quantitative PCR analysis of mRNA in the muscles revealed that IL-1α, IL-1β, and TNFα mRNA were up-regulated 7 days after immunization, with increased expression observed at the peak of inflammation (Figures 1A–C).

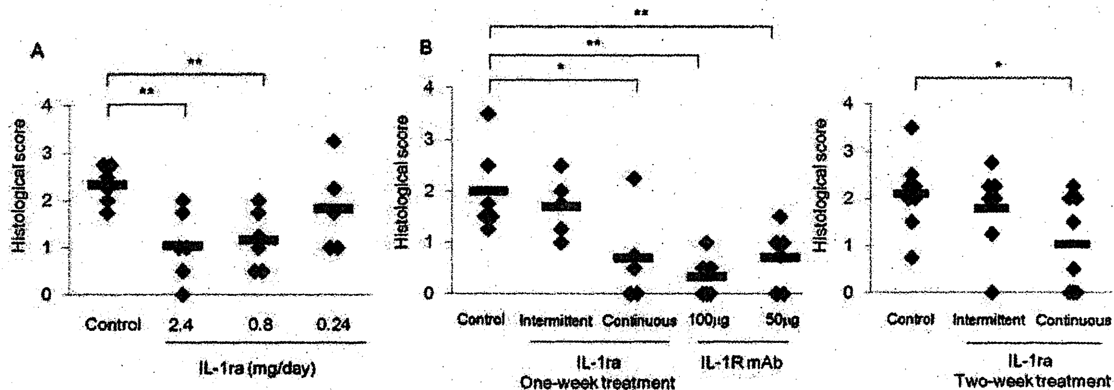

Figure 3. Effect of IL-1 blockade in mice with established CIM. **A,** IL-1 receptor antagonist (IL-1Ra) was administered to mice with CIM by continuous subcutaneous infusion beginning 7 days after immunization with C protein. Bovine serum albumin (2.4 mg/day) was used as a control. The severity of myositis was assessed histologically 14 days after immunization. Each experimental group consisted of 5 or 6 mice. **B,** IL-1Ra (total 7.2 mg) was intraperitoneally injected 7 days, 9 days, and 11 days after immunization (intermittent 1-week treatment). IL-1Ra was injected continuously (0.8 mg/day, total 5.6 mg) with a subcutaneously implanted minipump (continuous 1-week treatment). Anti–IL-1R monoclonal antibody (mAb) was administered intraperitoneally 7 days, 9 days, and 11 days after immunization. Saline was used as a treatment control. The severity of myositis was assessed histologically 14 days after immunization. Each experimental group consisted of 5 or 6 mice. For the 2-week treatment protocol for IL-1Ra, intermittent treatment (total 14.4 mg) and continuous treatment (total 11.2 mg) were started 7 days after immunization, and myositis was assessed histologically 21 days after immunization. Bovine serum albumin (0.8 mg/day) was used as a control. Each experimental group consisted of 7 or 8 mice. Bars represent the mean scores of individual groups. * = $P < 0.05$; ** = $P < 0.01$ versus control. See Figure 1 for other definitions.

Similar results were observed at the protein level. ELISA showed that IL-1β expression was significantly increased in the muscles of mice with CIM (Figure 1D). Immunohistochemical studies showed that IL-1– and TNFα-positive cells appeared in the muscles 7 days after the C protein immunization and become more abundant 21 days after immunization (Figures 2A–D and G). In contrast, active TGFβ1 was barely detectable 7 days after immunization and stayed at a constant level even at the peak of inflammation. (Figure 1D).

Resident macrophages were present around nonnecrotic muscle fibers, since CD11b-positive cells were present in the muscle tissue of unimmunized mice (Figures 2E and G). Immunohistochemical studies showed that the resident macrophages expressed no detectable inflammatory cytokines (Figure 2G). Seven days after the C protein/CFA immunization, the number of macrophages in the muscles increased significantly (Figures 2F). Thus, macrophages were recruited and activated to produce inflammatory cytokines in the muscles beginning in the early stage of myositis.

Therapeutic effects of IL-1 blockade on established CIM. IL-1–null mutant mice have been shown to be resistant to CIM induction. In the present study, IL-1 was blocked to discern whether this approach can alleviate the severity of CIM after disease onset. Blockade was carried out using IL-1Ra or anti–IL-1R monoclonal antibodies. Treatment was started 7 days after immunization. IL-1Ra was administered continually (0.24, 0.8, and 2.4 mg/day/mouse) for 7 days with minipumps implanted under the back dermis. The same amount of bovine serum albumin acted as a control. Histologic scoring of the muscles from the treated mice showed that IL-1Ra successfully suppressed CIM, in a dose-dependent manner (Figure 3A).

Although continuous administration of IL-1Ra was effective, this mode of infusion would not be practical for the treatment of patients. Thus, we next treated the mice with CIM intermittently (7, 9, and 11 days after immunization) and examined the muscles 3 days after treatment completion. Although a total of 7.2 mg of IL-1Ra was administered, intermittent administration of IL-1Ra failed to ameliorate the disease (Figure 3B). Continuous administration of IL-1Ra (0.8 mg/day), starting on the same day as the intermittent injections, was effective. The advantage of continuous treatment over intermittent treatment was maintained in a 2-week treatment protocol (Figure 3B).

Generally, monoclonal antibodies bind more stably to cell surface receptors than do soluble forms of the corresponding ligands. Indeed, when recombinant TNF receptor (p75)–fusion protein (TNFR-Fc) (100 μg/day and 50 μg/day) was injected intraperitoneally according to the same 1-week intermittent administration

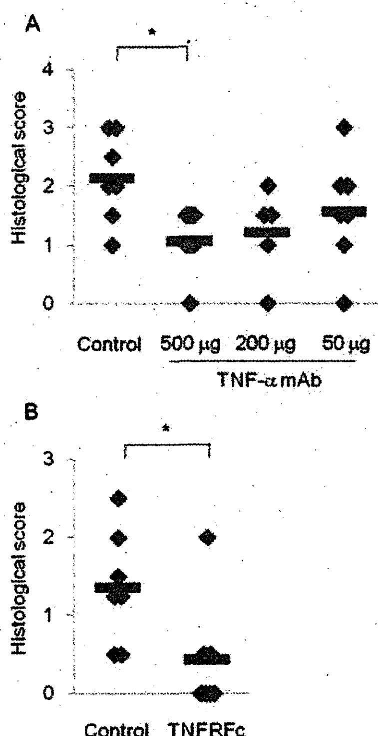

Figure 4. Effect of TNFα blockade on established CIM. **A,** Anti-TNFα monoclonal antibodies (50 μg, 200 μg, and 500 μg) and control antibodies (500 μg) were administered intraperitoneally 3 times weekly for 2 weeks, starting 7 days after immunization with C protein. **B,** Murine recombinant TNF receptor (p75)–fusion protein (TNFR-Fc) (100 μg) and bovine serum albumin (100 μg) were administered as described in **A.** Myositis was assessed histologically 21 days after immunization. Each experimental group consisted of 7 mice. Bars represent the mean scores of each group. * = $P < 0.05$ versus control. See Figure 1 for other definitions.

protocol, treatment with both doses ameliorated CIM (Figure 3B).

Therapeutic effects of TNFα blockade on ongoing CIM. Mice with CIM were also treated with TNFα-blocking reagents, including anti-TNFα monoclonal antibody and TNFR-Fc. Different doses of the anti-TNFα monoclonal antibody (50 μg, 200 μg, and 500 μg) and TNFR-Fc (100 μg) were administered 3 times weekly for 2 weeks, starting 7 days after immunization. The muscles of the treated mice were assessed 3 days after treatment completion. The histologic scores of the mice treated with either type TNFα (500 μg) or TNFR-Fc were significantly lower than those of the control mice (Figures 4A and B).

DISCUSSION

The expression of IL-1 and TNFα in the muscles of mice with CIM was observed beginning in the early phase of disease (day 7) and increased as the severity of inflammation peaked. Blockade of either cytokine after disease onset suppressed CIM. These results suggest that both IL-1 and TNFα are potential therapeutic targets in the treatment of PM.

Both IL-1 blockade and TNFα blockade reduced the severity of CIM. Previous histologic studies of the muscles of patients with PM showed that IL-1 expression by mononuclear cells accompanied up-regulation of class I major histocompatibility complex (MHC) molecules on the muscle fibers (27), and that IL-1R expression on muscle fibers was most pronounced in the vicinity of IL-1–expressing cells (28). TNFα-positive mononuclear cells have also been observed in the muscles of patients with PM (24). Like IL-1, TNFα increased class I MHC expression on human myoblasts in vitro (29,30). Also, TNF can damage muscle fiber directly (31). These findings suggest that IL-1 and TNFα expression of activated macrophages in muscles may contribute to both up-regulation of class I MHC molecules on muscle fibers and direct muscle damage.

IL-1 is involved in antigen-specific T cell differentiation. T cell proliferative responses to type II collagen were impaired in IL-1α/β–double-null mutant mice immunized with type II collagen for induction of CIA, which is also a model of induced autoimmune disease. In vitro experiments suggested that dendritic cells cannot activate T cells fully if they are not activated by IL-1 (32–34). Recently, it was shown that IL-1, together with TGFβ and IL-6, is involved in the differentiation of Th17 cells (35). In addition, IL-1 promoted Th17 cell differentiation in mice with experimental autoimmune encephalomyelitis (EAE) (36) and also in IL-1Ra–deficient mice with destructive arthritis (37). However, IL-17A was dispensable in the development of CIM (22). Actually, CD3 cells from the inguinal lymph nodes of mice with CIM that had or had not received IL-1Ra treatment proliferated equally in response to C protein–pulsed dendritic cells (data not shown). Thus, we did not see attenuation of pathogenic T cell responses in the IL-1Ra–treated mice.

The therapeutic effects of cytokine blockade could not be tested until CIM had developed. The classic EAM model not only is mediated by CD4 humoral immune responses (17) but also requires continual administration of CFA throughout the disease course. This makes it difficult to discern whether any treatment blocked the adjuvant effects of CFA or the pathologic

processes of myositis per se. In contrast, CIM can be induced with a single immunization and can be treated after disease onset. However, it is still difficult to initiate treatment at the very peak of the disease, because regression occurs spontaneously.

We performed ELISA of active TGFβ as an antiinflammatory cytokine. Previous studies showed that TGFβ was expressed in the muscles of patients with PM (23) and suggested that TGFβ from macrophages may contribute to muscle regeneration after muscle injury (38). This is consistent with the marginal elevation of TGFβ expression observed in the muscles of mice with CIM.

Because a major clinical manifestation of myositis is muscle weakness, rotarod testing was used to measure muscle function as the clinical outcome in our previous study (16). We actually used the same rotarod test in the anti-TNFα antibody treatment experiment and observed that the mean running time of the treated mice was longer than that of control mice. However, the difference did not reach statistical significance. According to our experience, the rotarod test is not as sensitive as histologic analyses, because mice become accustomed to the device and thus are able to avoid falling off, and the efforts of mice are inconsistent. We have used different techniques to measure muscle weakness in mice with CIM, including measurement of walking time, walking distance, and rearing time in open-field tests but have not yet identified an appropriate technique for use in mice with CIM.

In a clinical trial of the treatment of multiple sclerosis, TNF inhibitors occasionally exacerbated the disease (39). In accordance with this finding, TNF-null mice developed severe EAE (40). TNF inhibitors sometimes induce a lupus-like syndrome in the clinical setting (41). In an animal model, TNF-null (NZB × NZW)F_1 mice developed lupus nephritis (42), and injection of high doses of TNF delayed disease onset (43). In contrast, TNF inhibitors were proven to be useful in the treatment of both RA and CIA (8,12). Nevertheless, TNFα-null mice are fully susceptible to CIA (44). The current study showed that TNFα inhibitors are effective in treating a murine model of PM, although TNFα-null mice are fully susceptible to CIM induction. Thus, this is the second instance in which the inducibility of autoimmune diseases is different between TNFα-null mice and mice treated with TNFα inhibitors. It has been proposed that increased numbers of memory CD4 T cells and augmented interferon-γ production from CD4 T cells are responsible for exacerbated disease activity of CIA in TNFα-null mice (44). Although CD4 T cells and IL-17–producing lymphocytes play a critical role in CIA (45), both are dispensable for muscle injury in CIM. The genetic absence of TNFα appears to have extensive effects on the effector function of lymphocytes. The efficacy of TNF inhibitors in the treatment of PM and DM is controversial (19–21). According to anecdotal case reports, myositis occurred in patients with RA even after initiation of treatment with TNF inhibitors (46,47). It would be of particular interest to know the clinical effects of TNF inhibitors in patients with myositis.

In the US, IL-1Ra (anakinra) has been approved for the treatment of RA. Because its terminal half-life ranges from 4 hours to 6 hours, IL-1Ra should be injected subcutaneously every day. The discrepant efficacy of IL-1Ra and anti–IL-1R monoclonal antibody in the present studies may be explained by differences in the half-life as well as the affinity to IL-1R.

DM is another inflammatory myopathy that is driven by autoimmunity. It has been proposed that PM is mediated by cytotoxic CD8 T cells, while DM is mediated by humoral responses. However, the accumulated body of evidence suggests that PM and DM are similar in terms of muscle pathology as well as responses to various treatments (48). Sontheimer proposed that both PM and DM are within a single disease spectrum (49). With the CIM model, we believe that by using the CIM model in this study, we provide support for clinical trials of IL-1 and TNF blockade to treat PM and DM.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Kohsaka had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Sugihara, Okiyama, Miyasaka, Kohsaka.
Acquisition of data. Sugihara, Okiyama, Watanabe, Miyasaka, Kohsaka.
Analysis and interpretation of data. Sugihara, Okiyama, Watanabe, Miyasaka, Kohsaka.

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Definitive Engagement of Cytotoxic CD8 T Cells in C Protein–Induced Myositis, a Murine Model of Polymyositis

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Objective. **To substantiate a pathogenic role of cytotoxic CD8 T cells in the development of a murine polymyositis model, C protein–induced myositis (CIM).**

Methods. **Beta$_2$-microglobulin–null mutant, perforin-null mutant, and wild-type (WT) C57BL/6 mice were immunized with skeletal muscle C protein fragments to provoke CIM. Regional lymph node CD8 or CD4 T cells stimulated with C protein–pulsed dendritic cells were transferred adoptively to naive mice. Inflammation and damage of the muscle tissues were evaluated histologically.**

Results. **The incidence of myositis development was significantly lower in β_2-microglobulin–null and perforin-null mutant mice compared with WT mice. Inflammation was less severe in mutant mice, and the incidence of muscle injury was reduced significantly. Adoptive transfer of lymph node T cells from mice with CIM induced myositis in naive recipient mice. The CD8 T cell–induced muscle injuries were significantly more severe than the CD4 T cell–induced muscle injuries.**

Conclusion. **Perforin-mediated cytotoxicity by CD8 T cells is definitively responsible for muscle injury in CIM.**

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Polymyositis (PM) is a chronic autoimmune inflammatory myopathy that affects the systemic striated muscles. An accumulated body of evidence shows that muscle injury in PM is driven by cytotoxic CD8 T cells (1). We recently established C protein–induced myositis (CIM) in mice as an animal model of PM (2). This murine myositis is readily induced by a single immunization of C57BL/6 (B6) mice with recombinant skeletal muscle C protein fragments. In mice with CIM, CD8 T cells are enriched at the site of the muscle injury. Cytotoxic molecules (perforins) were expressed by T cells surrounding the muscle fibers. Also, class I major histocompatibility complex (MHC) expression was up-regulated on the muscle fibers (2). Inducibility of CIM in the B6 strain of mice, which is a background strain for most genetically mutated mice, prompted us to show that B cells are not required for CIM induction. These findings suggested that the muscle injury in CIM is also driven by cytotoxic CD8 T cells. This is in sharp contrast to a classic model, experimental autoimmune myositis, that is induced by repeat immunization with crude myosin. Experimental autoimmune myositis has dominant CD4 T cell infiltration in the muscles and can be transferred adoptively to naive animals by serum transfer (3).

Previously, we showed that pretreatment with anti-CD8α monoclonal antibodies (mAb) to deplete CD8 T cells conferred resistance to CIM. However, the anti-CD8α antibody treatment could deplete a subset of dendritic cells (DCs) expressing CD8α (4). In the inflamed muscles, macrophages and CD4 T cells are also abundant. CD4 T cell depletion exerted the same effect as CD8 T cell depletion (2). Thus, CD4 T cells might play some roles not only as helper cells of CD8 T cell

differentiation but also as effector cells in the injured tissue.

Based on our study of mutant mice and adoptive T cell transfer, we report here definitively that CIM is driven primarily by cytotoxic CD8 T cells. The analogous pathology of this model and that of PM should facilitate further studies of inflammatory myopathies.

MATERIALS AND METHODS

Induction of murine CIM. B6 mice were purchased from Charles River. Beta$_2$-microglobulin–null mutant and perforin-null mutant B6 mice were purchased from The Jackson Laboratory. Female mice ages 8–10 weeks were immunized intradermally with recombinant C protein fragments for CIM induction (2). All experiments were carried out under specific pathogen–free conditions in accordance with the ethics and safety guidelines for animal experiments of Tokyo Medical and Dental University and RIKEN.

Generation of antigen-pulsed mature DCs. DCs were prepared from the bone marrow of B6 mice (5) and were incubated with 50 μg/ml of the recombinant C protein fragments and 1 μg/ml lipopolysaccharide (Sigma-Aldrich). Purity of DCs (>70%) was confirmed with a FACSCalibur (Becton Dickinson) using allophycocyanin-conjugated anti-CD11c mAb (eBioscience).

Adoptive T cell transfer. Draining lymph nodes were removed from wild-type (WT) mice 21 days after the C protein immunization. Three million lymph node cells were cultured with 1.5×10^5 antigen-pulsed DCs and 100 units/ml recombinant human interleukin-2 (Shionogi Pharmaceuticals) for 72 hours. CD8- or CD4-positive T cells were sorted with MACS magnetic beads (Miltenyi Biotech). Purity of these cells was confirmed using a flow cytometer. Either 4 million CD8-positive T cells or 4 million CD4-positive T cells were injected intraperitoneally into naive B6 mice whose foot pads had been pretreated with Freund's complete adjuvant. Prior to the transfer, the CD8 T cells of some mice were depleted by administration of anti-CD8 mAb (53.67.2) (2). The muscles of the hind legs were evaluated histologically 14 days after the transfer.

Histologic analysis of myositis. Hamstring and quadriceps muscles were examined for histopathologic changes. Two 10-μm sections derived from proximal and distal sites of individual muscles were stained with hematoxylin and eosin (H&E). All fields of the 2 cross sections from each muscle piece were examined for histologic changes. Histologic severity of inflammation in each block was graded as previously described (2). We defined necrotic muscle fibers as muscle fibers that showed decreased H&E stainability and those replaced by mononuclear cell infiltration (6). Microscopic views of the entire muscle sections were captured digitally, and the lesions of necrotic muscle fibers in a total of 4 sections were then marked with ImageJ software (NIH Image, National Institutes of Health; online at: http://rsbweb.nih.gov/ij/) for calculation of total necrotic area.

Statistical analysis. The histologic scores were statistically analyzed using the Mann-Whitney U test. The necrotic muscle areas were statistically analyzed using Student's t-test.

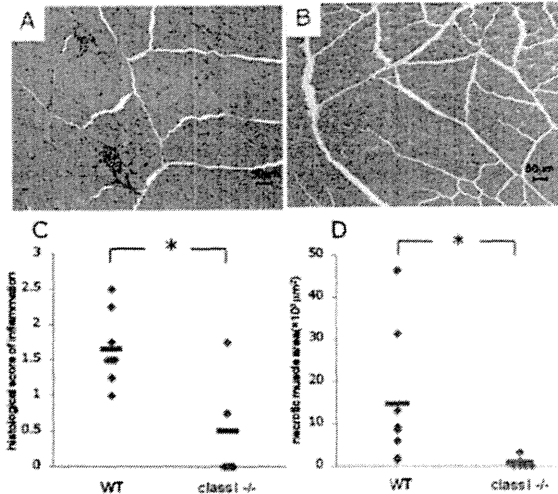

Figure 1. C protein–induced myositis in β_2-microglobulin–null mutant (class I$^{-/-}$) mice. Eight wild-type (WT) female C57BL/6 (B6) mice and 6 β_2-microglobulin–null mutant female B6 mice were immunized. Muscle fibers invaded by inflammatory mononuclear cells were abundant in the WT mice (A) but not in the β_2-microglobulin–null mutant mice (B). The histologic scores of inflammatory mononuclear cell infiltration (C) and the areas of muscle fiber necrosis (D) are shown. Horizontal lines indicate the mean. $* = P < 0.05$.

RESULTS

The role of CD8 T cells in mice with CIM. As was stated earlier, pretreatment with anti-CD8α mAb made mice resistant to CIM (2). To demonstrate the requirement of CD8 T cells directly, β_2-microglobulin–null B6 mice, which are devoid of mature CD8 T cells, were immunized with recombinant skeletal muscle C protein fragments for induction of CIM. All experiments were carried out using female mice. In WT mice with CIM, mononuclear cells infiltrated predominantly at the endomysial site, and many necrotic muscle fibers and muscle fibers were invaded by inflammatory mononuclear cells (2) (Figure 1A). The incidence of CIM was reduced in mutant mice compared with WT mice (33% versus 100%). Inflammatory mononuclear cell infiltration and muscle fiber necrosis were sparse in the mutant mice (Figure 1B). The histologic scores, which represent inflammatory mononuclear cell infiltration, were significantly lower in the mutant mice (Figure 1C). The difference was more apparent when necrotic muscle areas were compared (Figure 1D). These results demonstrated the critical role of CD8 T cells in the muscle injury of CIM.

Adoptive transfer of CD4 or CD8 T cells. The relative importance of CD4 and CD8 T cells in myositis was investigated using adoptive transfer of lymph node T cells. Cells were isolated from the draining lymph nodes of mice with CIM 21 days after immunization. These lymph node cells were stimulated in vitro with DCs that were prepared from the bone marrow of naive mice and preincubated with the C protein fragments. The stimulated cells were separated into CD4 and CD8 T cell populations. Flow cytometry analyses showed that the purity of the sorted CD4 and CD8 T cells was >95%, and that CD11c-positive cells were absent. Intraperitoneal transfer of the CD8 T cells into naive mice induced myositis with an incidence of 100%, which accompanied necrosis of muscle fibers. As in CIM, inflammatory mononuclear cells infiltrated predominantly around non-necrotic and necrotic muscle fibers at the endomysial site (Figure 2A). Transfer of the CD4 T cells induced much milder muscle damage, with a lower incidence (41%) (Figure 2B). Necrotic muscle areas induced by the 2 T cell subsets were significantly different (Figure 2C). Next, CD4 T cells were transferred to mice pretreated with depleting anti-CD8 mAb or control mAb. Necrotic muscle areas were comparable between the CD8-depleted and control mice (Figure 2D), showing that CD4-mediated injury was independent of endogenous CD8 T cells. Lymph node T cells derived from naive mice stimulated with the same antigen did not induce myositis (data not shown).

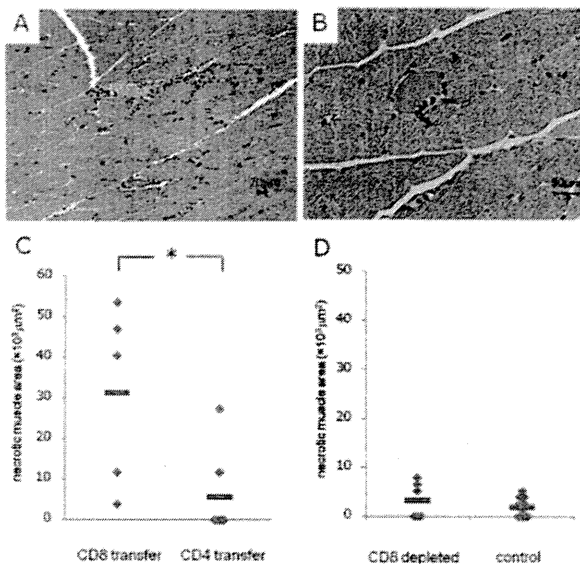

Figure 2. Adoptive transfer of CD8 or CD4 T cells. Lymph node cells from mice with C protein–induced myositis were stimulated with C protein fragment–pulsed dendritic cells, and CD8-positive and CD4-positive T cells were separated and transferred into recipient C57BL/6 mice. **A** and **B,** Adoptive transfer of CD8 T cells (**A**) or CD4 T cells (**B**) induced muscle fiber necrosis. **C,** The areas of muscle fiber necrosis in myositis induced by CD8-positive cells and by CD4-positive cells are shown. **D,** Mice were pretreated with depleting anti-CD8 monoclonal antibodies or control monoclonal antibodies before transfer of the purified CD4 T cells, and purified CD4 T cells were transferred. Necrotic muscle areas of 6 CD8-depleted mice and 9 control mice are shown. Horizontal lines indicate the mean. * = $P < 0.05$. Results shown are representative of those from 2 independent experiments.

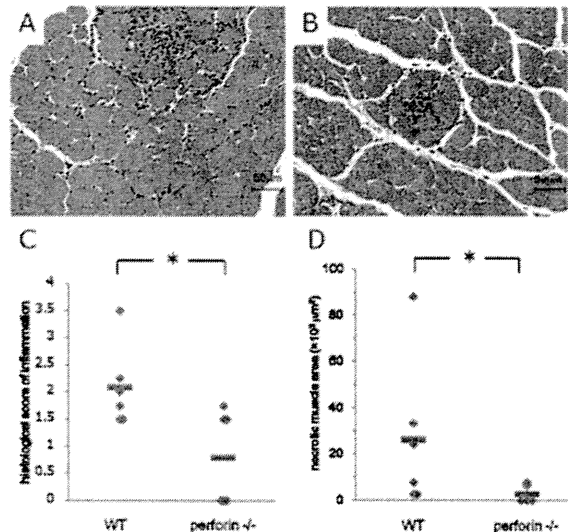

Figure 3. C protein–induced myositis in perforin-null (perforin$^{-/-}$) mutant mice. Six wild-type (WT) C57BL/6 (B6) mice and 6 perforin-null mutant B6 mice were immunized. Necrosis of muscle fibers with mononuclear cell infiltration was observed in WT mice (**A**), and scattered necrosis of single muscle fibers was observed in perforin-null mice (**B**). Histologic scores for inflammatory mononuclear cell infiltration (**C**) and necrotic muscle area (**D**) are shown. Horizontal lines indicate the mean. * = $P < 0.05$.

The role of perforin in mice with CIM. Immunohistochemical studies of the muscles with PM implied that perforin-mediated cytotoxicity should operate in the muscle injury. We next immunized perforin-null B6 mice to induce CIM. It has been reported that these mice have no defect in T cell maturation, and that their CD8 T cells expand normally but lack cytotoxity (7). The incidence of myositis development in mutant mice (50%) was reduced compared with that in WT mice, and mononuclear cell infiltration was milder (Figures 3A–C). Whenever necrosis was found in the perforin-null mice, it was limited to single muscle fibers. Thus, the

areas of muscle injury were significantly reduced in the mutant mice (Figure 3D). This supports the idea that perforin-dependent cytotoxicity is critical for muscle damage in CIM.

DISCUSSION

Resistance of class I MHC mutant mice to CIM demonstrated the crucial role of CD8 T cells in muscle injury. The perforin-null mice showed that the injury should be mediated by effector molecules secreted from the CD8 T cells. Adoptive transfer experiments with CD4 and CD8 T cells from mice with CIM have confirmed the critical importance of CD8 T cells.

Since macrophages and CD4 T cells were abundant in the muscles of mice with CIM (2), histologic grading (which simply reflects the numbers of infiltrating cells) and actual muscle injury might be discordant in mice lacking CD8 effector molecules. To circumvent this problem, we evaluated the necrotic muscle area, which should represent muscle injury directly. Indeed, the muscles from 1 class I MHC mutant mouse with CIM had a histologic score comparable with that of muscles from WT mice (Figure 1C). However, these muscles had a small necrotic area, suggesting that the tissue injury should depend on CD8 cytotoxity.

In a set of experiments, donor T cells derived from green fluorescent protein (GFP)–transgenic mice with CIM were injected intraperitoneally into naive B6 mice. However, GFP-positive cells were not seen in the muscles of the recipients at the peak of myositis (data not shown). Thus, the majority of inflammatory mononuclear cells in adoptive myositis seem to be recruited by the transferred CD8 T cells after the primary muscle injury. We believe that induction of adoptively transferred myositis is antigen dependent, because donor cells stimulated with concanavalin A instead of concanavalin C protein failed to transfer the myositis (data not shown). Also, 3-day stimulation with antigen-pulsed DCs is not long enough for induction of nonspecific lymphokine-activated killer cells (8).

Class I MHC expression was up-regulated in muscles with severe inflammation in mice with CIM (2). We tried to evaluate class I MHC expression in the muscles in the adoptive transfer model and in perforin-null mice with CIM. However, no obvious up-regulation was observed. Based on our observation and on a report of a decrease in class I MHC expression in muscle fibers after corticosteroid treatment (9), we assume that class I MHC up-regulation is associated with the severity of myositis.

To measure muscle function as the clinical outcome, we previously used a Rotarod test (2). However, this technique turned out to be less reliable than histologic analyses, because mice learn how to avoid falling off and do not necessarily run consistently. Although magnetic resonance imaging appears better in detecting myositis, its use still needs to be optimized.

Perforin-dependent cytotoxicity mediated by CD8 T cells is also crucial in pancreatic tissue injury in NOD mice, which is an animal model of type 1 diabetes mellitus. Spontaneous development of diabetes was suppressed in perforin-deficient NOD mice (7), and CD8 T cell clones isolated from pancreatic islets of prediabetic NOD mice are able to induce diabetes in nondiabetic NOD mice (10). Interestingly, the islets of the perforin-deficient NOD mice had numbers of CD4 and CD8 T cells comparable with those of control mice. In contrast, muscle fiber necrosis and mononuclear cell infiltration were both suppressed in perforin-deficient mice immunized with C protein fragments. This result implies again that inflammatory mononuclear cells in the muscles of mice with CIM should be recruited as a secondary event.

Scattered necrotic muscle fibers were found in the perforin-null and β_2-microglobulin–null mice. Transfer of activated CD4-positive T cells induced mild muscle fiber necrosis, which did not depend on endogenous CD8 T cells. In this regard, beta cell loss and diabetes are observed in 16% of perforin-deficient NOD mice (7), and some islet-specific CD4 T cell clones can induce diabetes when transferred to neonatal NOD mice (11). Also, it was reported that T cell–independent muscle injury was observed in muscle tissues overexpressing class I MHC molecules (12). Thus, the muscle damage could be partly attributable to these CD8 T cell–independent pathways.

CD4 T cells mediate cytotoxicity via Fas ligand or, in a certain instance, perforins. Indeed, Fas antigen is expressed on muscle fibers, while Fas ligand is expressed on T cells in the inflamed muscles (13). However, it was shown that muscle fibers were resistant to apoptosis, possibly because the inflammatory environment induced endogenous antiapoptotic molecules (14).

The present study demonstrated that CD8 cytotoxic T lymphocytes primarily damage the muscle fibers in CIM and confirmed that CIM is the mouse myositis model most analogous to human PM. Recently, a randomized, double-blind, placebo-controlled trial failed to show efficacy of the anti–tumor necrosis factor α (anti-TNFα) mAb infliximab in treating patients with PM and dermatomyositis (15). This might be consistent with the fact that CIM does not require TNFα for its develop-

ment (2). We expect that further analyses of CIM will shed more light on the pathology of PM.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Kohsaka had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Sugihara, Miyasaka, Kohsaka.

Acquisition of data. Sugihara, Okiyama, Suzuki, Kohyama, Matsumoto.

Analysis and interpretation of data. Sugihara, Okiyama, Kohsaka.

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REVIEW ARTICLE

Current insights in polymyositis and dermatomyositis

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Keywords
autoantibody; dermatomyositis; disease model; lymphocyte; polymyositis

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Abstract

Polymyositis (PM) and dermatomyositis (DM) are characterized by immune-mediated muscle damage. Early immunohistochemical studies of PM and DM muscle biopsy samples suggested that PM is mediated by cytotoxic CD8 T lymphocytes, whereas DM is a vasculopathy mediated by antibodies and complement deposition to the intramuscular microvessels. This classic view is now challenged by recent advances in immunology and molecular biology, as well as by clinical observation of the two diseases. Clonal expansion of cytotoxic CD8 T cells was found in the affected muscles and in peripheral blood from PM and DM patients. Autoantigens recognized by these clones are yet to be identified. Patient sera contain various autoantibodies. Although they are not directed to endothelial antigens, detection of specific antibodies helps when classifying the patients. Infiltrating cells, as well as muscle cells, produce cytokines and chemokines that could potentiate inflammation. Myofibers overexpress major histocompatibility complex class I and class II molecules that render themselves vulnerable to immune attacks while they also express molecules that protect themselves from apoptotic cell death. Corticosteroid administration is still a gold standard for PM/DM treatment. A major life-threatening complication is an interstitial lung disease, the acute form of which should not be treated with corticosteroids alone. Future studies should be carried out to further elucidate their ethiopathology and to develop more effective and specific treatment. (Clin. Exp. Neuroimmunol. doi: 10.1111/j.1759-1961.2009.00002.x, January 2010)

Introduction

Polymyositis (PM) and dermatomyositis (DM) are idiopathic inflammatory myopathies and are considered to be relatively benign diseases. However, disease-related death occurs in at least 10% of patients during a median 5-year follow up,[1] and is typically associated with malignancy and pulmonary complications. Furthermore, after the follow up period, 80% of patients still require continuous medical treatment, indicating the intractability of these diseases.

Efforts to study pathological processes were initiated by Engel and Arahata a quarter century ago. They carried out a series of precise immunohistochemical studies of PM/DM muscle biopsy samples.[2–6] Since then, molecular biological techniques have been applied to PM/DM research, but have failed to confirm the hypothesis established by their immunohistochemical analyses. The Bohan and Peter diagnostic criteria were established for the purpose of clinical research 34 years ago.[7,8] New diagnostic modalities, including the detection of serum autoantibodies and magnetic resonance imaging (MRI), have been brought into clinical use but have failed to revise the criteria. For treatment, administration of corticosteroids was proven to be effective and soon became the gold standard, although their long-term use is detrimental to many organs including the muscles. Many small-molecule immunosuppressants and biological agents have been developed, but failed to offer treatments that

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