Experimental myositis inducible with transfer of dendritic cells presenting a skeletal muscle C protein-derived CD8 epitope peptide

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Abstract
It is suggested that polymyositis, an autoimmune inflammatory myopathy, is mediated by autoaggressive CD8 T cells. Skeletal muscle C protein is a self-antigen that induces C protein-induced myositis, a murine model of polymyositis. To establish a new murine model of myositis inducible with a single CD8 T-cell epitope peptide that derives from the C protein, three internet-based prediction systems were employed to identify 24 candidate peptides of the immunogenic fragment of the C protein and bind theoretically to major histocompatibility complex class I molecules of C57BL/6 (B6) mice. RMA-S cell assay revealed that a HILIYSDV peptide, amino acid position 399–406 of the C protein, had the highest affinity to the H2-Kb molecules. Transfer of mature bone marrow-derived dendritic cells pulsed with HILIYSDV induced myositis in naive B6 mice. This myositis was suppressed by anti-CD8-depleting antibodies but not by anti-CD4-depleting antibodies. Because this myositis model is mediated by CD8 T cells independently of CD4 T cells, it should be a useful tool to investigate pathology of polymyositis and develop therapies targeting CD8 T cells.

Keywords: bone marrow-derived dendritic cell, CD4 T cell, RMA-S cell

Introduction
Polymyositis (PM) is an autoimmune inflammatory myopathy of unknown etiology (1). Immunohistochemical studies showed that CD8 T cells infiltrate the pre-necrotic muscle fibers (2–4) and express perforins (5). These findings argue that cytotoxic CD8 T cells (CTLs) drive the pathology of PM.

We established a murine model of PM, C protein-induced myositis (CIM) (6). Biochemical purification studies showed that skeletal muscle fast-type C protein, a myosin-binding protein in the cross-bridge-bearing zone of A bands of myofibrils, appears to be the main immunopathogenic component of the crude skeletal muscle myosin preparation used for the induction of experimental autoimmune myositis in Lewis rats (7, 8). When C57BL/6 (B6) mice wereimmunized with each of four overlapping protein fragments (fragment 1, 2, 3 and 4) from human skeletal muscle C protein in Freund’s complete adjuvant (CFA), histological findings of the proximal muscles showed that mice immunized with fragment 2 (amino acids: 284–580) developed severer myositis than those with other fragments (6). We also found that immunization of a fragment from murine skeletal muscle C protein, corresponding to fragment 2 of the human C protein, induced myositis in B6 mice as well as fragment 2 of the human C protein (9). Since it should contain an epitope with dominant immunogenicity, we have been using the fragment 2 to induce CIM. In CIM, CD8 T cells as well as expression of β2-microglobulin and perforin are essential for myositis induction (10). This fact argues that...
cytotoxicity by CD8 T cells is responsible for CIM as is presumed for PM.

Conventional immunization with protein antigens to evoke CTL responses requires CD4 T cells. Actually, depletion of CD4 T cells before immunization of the C protein fragments inhibited CIM development (6, 10). Here, we identified a CD8 epitope in the murine skeletal muscle C protein fragment to evoke CD4 T-cell-independent peptide-induced myositis. The CD8 T-cell-mediated murine model of myositis will be a tool to develop treatments that address directly CD8 T cells.

Methods

Mice

Female B6 mice at age of 8–10 weeks were purchased from Charles River (Yokohama, Japan). 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.

Candidate peptides

Candidate peptides that bind theoretically to major histocompatibility complex (MHC) class I H2-Db or Kβ were identified with SYFPEITHI (http://www.syfpeithi.de/) based on previous publications on T-cell epitopes and MHC ligands (11, 12). HLA Peptide Binding Predictions (http://www-bimas.cit.nih.gov/molbio/hla_bind/) by Dr Ronald Taylor (BIMAS, CBEL, CIT, NIH, Bethesda, MD, USA) and Dr Kenneth Parker (NIAD, NIH) (13) and NetMHC 3.2 Server (http://www.cbs.dtu.dk/services/NetMHC/) (14, 15). Peptides were synthesized by Custom Peptide Service (Sigma–Aldrich, St Louis, MO, USA).

MHC class I expression assay using RMA-S cells

RMA-S, a mutant lymphoma cell line originating from B6 mice, which lacks a functional MHC-linked transporter with antigen processing-2, expresses reduced levels of MHC class I (16). Addition of exogenous peptides that bind to MHC class I restores its expression. They were analyzed after incubation with each candidate peptide and staining with PE-conjugated anti-MHC class I H2-Dβ antibodies (KH95; BD Biosciences, San Jose, CA, USA) or H2-Kβ antibodies (AF6-88.5).

Immunization of mice with peptides

B6 mice were immunized with 200 μg of synthetic peptides instead of the C protein fragments with the same adjuvants in the method to induce CIM (6, 9). Some mice were intra-peritoneally (i.p.) injected with 50 μg of polyinosinic–polycytidylic acid sodium salt [poly (I:C); Sigma–Aldrich] and 50 μg of agonistic anti-CD40 antibodies (FGK-45).

Transfer of bone marrow-derived dendritic cells presenting immunogenic peptides

Bone marrow-derived dendritic cells (BMDCs), prepared as described previously (17), were incubated with 1 μg ml⁻¹ of lipopolysaccharides (LPS) (Sigma–Aldrich) for 24h to up-regulate MHC class I expression on their surface. Flow cytometry showed that >95% of BMDCs increased expression of MHC class I 24h after the LPS stimulation. Endogenous peptides on MHC class I of BMDCs were replaced with synthetic peptides at 50 μg ml⁻¹ each in the final hour. Two million of the peptide-pulsed or non-pulsed BMDCs were intravenously (i.v.) injected twice with an interval of 3 days to B6 mice that were intra-dermally (i.d.) injected on the footpads with 100 μl of emulsion containing CFA. Seven days after the first transfer, the histological severities of inflammation in the muscles (hamstrings and quadriceps) were graded as in our previous articles (6, 9). Grade 1: involvement of a single muscle fiber or less than five muscle fibers; grade 2: a lesion involving 5–30 muscle fibers; grade 3: a lesion involving a muscle fasciculus; and grade 4: diffuse, extensive lesions. When multiple lesions with the same grade were found in a single block, a 0.5 point was added to the grade.

Immunohistochemistry staining

Cryostat-frozen serial sections (8 μm) of the muscles (hamstrings and quadriceps) from mice 7 days after the first transfer of BMDCs were fixed in 4% paraformaldehyde and stained with 200ng ml⁻¹ anti-CD4 (RM4-5; BD PharMingen) or 400ng ml⁻¹ anti-CD8a (53-6.7; eBioscience, San Diego, CA, USA) monoclonal antibodies or hematoxylin–eosin. Nonspecific staining was blocked with 0.3% hydrogen peroxide in water and 10% rabbit serum (Sigma–Aldrich) in PBS. The sections were incubated with biotin-labeled anti-rat IgG antibodies (Dako, Glostrup, Denmark) and peroxidase-conjugated streptavidin and then visualized with DAB in substrate buffer (R&D systems, Minneapolis, MN, USA). An isotype control, rat IgG2a, κ (eBR2a; eBioscience), was used as a negative control.

In vivo depletion of CD4 or CD8 T cells

To deplete CD4 T cells from B6 mice, the mice were i.p. injected with 500 μg of purified anti-CD4 monoclonal antibodies (GK1.5) (18) every 4 days. To deplete CD8 T cells from B6 mice, the mice were i.p. injected with 1 mg purified anti-CD8 monoclonal antibodies (53.67.2) (19) for three consecutive days. Ten days after, injection of 500 μg of the same antibodies was repeated every other day for 7 days. Rat IgGs were used as controls. Less than 0.5% of peripheral blood cells or splenocytes of the treated mice were positive for CD4 and CD8 staining with FACS analysis 10 or 7 days after the first injection of the antibodies.

Statistical analysis

Histological scores were analyzed with the Mann–Whitney U-test.

Results and Discussion

Identification of an immunogenic peptide derived from murine skeletal muscle C protein

B6 mice develop CIM when immunized with a fragment (amino acids: 279–574) of the murine skeletal muscle C protein (9). Using three internet-based prediction systems, 12 candidate peptides were selected from the fragment for each H2-Dβ and H2-Kβ with high affinity scores in common among
the three systems. Actual binding of the individual peptides to MHC class I molecules was studied with an assay using RMA-S cells. RMA-S cells showed the highest up-regulation of MHC class I expression when pulsed with a peptide (amino acids: 399–406; HILIYSVDV), which binds theoretically to H2-Kb (Table 1).

Peptide-induced experimental myositis

Galea et al. [20] reported that CD8 T-cell cross-competition is governed by peptide–MHC class I stability. To establish a myositis inducible with a single CD8 epitope peptide, B6 mice were immunized with HILIYSVDV. We first i.d. injected the peptide in CFA without myositis induction in five mice. It was reported that coligation of CD40 on DCs by CD4 T cells was critical in priming antigen-specific CD8 T cells (21–23). Agonistic anti-CD40 antibodies and poly (I:C) synergized to prime CTLs without CD4 T cells (24, 25). We thus immunized seven B6 mice with HILIYSVDV with additional i.p. injections of anti-CD40 antibodies and poly (I:C) but found no myositis. The combination adjuvants including CFA, anti-CD40 agonistic antibodies and poly (I:C) might not exert enough efforts to present the muscle antigen to CD8 T cells.

Kawachi et al. [26] established an experimental myositis in BALB/c mice after four injections of BMDCs pulsed with pyruvate kinase M1/M2-derived peptides, which bind theoretically to H-2Kd of BALB/c mice, into the inguinal lymph nodes. We next performed a transfer of peptide-presenting BMDCs to B6 mice. BMDCs were prepared and activated with LPS to up-regulate MHC class I and CD40 and produce IL-2 (27). The MHC class I molecules should present endogenous peptides. HILIYSVDV was or was not pulsed to replace the endogenous peptides on MHC class I of the activated BMDCs. B6 mice were i.v. transferred with the BMDCs twice. Recently, we showed that myositis development requires both autogressive T cells and CFA-induced conditioning of muscle tissues (28). Accordingly, the footpads were treated with i.d. injections of CFA emulsion at the day of the first transfer. The treated mice developed myositis 7 days after the first transfer with histological changes including inflammatory cell infiltration around atrophic or regenerating muscle fibers (Fig. 1A and B). The mice presented higher incidence and severity of myositis than the control mice that were treated with activated BMDC transfer alone (Fig. 1D). Mature BMDCs could substitute the helper effect of CD4 T cells instead of anti-CD40 agonistic antibodies and poly (I:C). When the disease course was followed histologically, we observed that the myositis started to decline 10 days and had disappeared 14 days after the first transfer (Fig. 1E), showing that the myositis is self-limited, as is CIM. This myositis was termed C protein peptide-induced myositis (CPIM). While pyruvate kinase M1/M2 that was used as an autoantigen in the previous report is not expressed specifically by the skeletal muscles (29), HILIYSVDV was a skeletal muscle-specific antigen. CPIM should be appropriate to investigate organ-specific reactions.

We found that a few mice transferred with BMDCs without the peptide pulse developed myositis (the incidence, 23%; the severity, 0.29 ± 0.03 in Fig. 1D). In a separate experiment, some mice developed myositis with a few necrotic muscle fibers 7 days after i.d. injections of CFA alone at the footpads. These results suggested that the myositis might be induced by activation of innate immunity in the muscle tissues. It might be due to CFA-induced up-regulation of MHC class I expression on muscle fibers. It was reported that transgenic mice that expressed MHC class I highly on their muscle fibers naturally developed myositis (30). On the other hand, we have never found myositis histologically in the brachial muscles from 12 mice with CPIM that were injected i.d. with CFA at footpads of hind legs. Since the upper limbs were not treated with CFA, this result agrees with the ‘seed and soil’ model as mentioned above (28). We also found that the histological scores of CPIM mice varied among the experiments, which might depend on the purity and activity of BMDCs.

To determine whether other candidate peptides accelerate CPIM, we selected three candidate peptides (ILTINKCTL, RILTINKCTL and DGGRYQVI) based on the results of the RMA-S cell assay (Table 1). BMDCs pulsed with a mixture of HILIYSVDV and the three candidate peptides were transferred twice to mice treated with CFA at the footpads.

Table 1. Candidate CD8 epitope peptides derived from the C protein fragment

<table>
<thead>
<tr>
<th>MHC class I</th>
<th>Peptide</th>
<th>Fold change of MFI</th>
<th>MHC class I</th>
<th>Peptide</th>
<th>Fold change of MFI</th>
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<tbody>
<tr>
<td>H2-D&lt;sup&gt;b&lt;/sup&gt;</td>
<td>SAKLNFLIEI</td>
<td>2.09</td>
<td>H2-K&lt;sup&gt;c&lt;/sup&gt;</td>
<td>SKYVFENV</td>
<td>1.19</td>
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<tr>
<td></td>
<td>KWFKNQGEI</td>
<td>1.21</td>
<td></td>
<td>HVGRFHKL</td>
<td>0.80</td>
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<tr>
<td></td>
<td>ILTINKCTL</td>
<td>3.00</td>
<td></td>
<td>DEKCFTEL</td>
<td>1.03</td>
</tr>
<tr>
<td></td>
<td>KWYKNGVEV</td>
<td>1.05</td>
<td></td>
<td>ARYRFKRD</td>
<td>1.03</td>
</tr>
<tr>
<td></td>
<td>VVAGNKLRL</td>
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<tr>
<td></td>
<td>KDELEQLODI</td>
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<td>DGGRYQVI</td>
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<tr>
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<tr>
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<td>FVPDGYAL</td>
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<td></td>
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<td>1.81</td>
<td></td>
<td>EDGGRYQVI</td>
<td>1.44</td>
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</tbody>
</table>

The amino acid sequences of 24 candidate peptides are described in single letter codes. Mean fluorescence intensities (MFIs) of the peptide-pulsed RMA-S cells stained with anti-MHC class I H2-D<sup>b</sup> or H2-K<sup>c</sup> antibodies were compared with MFIs of unpulsed RMA-S cells stained with the same antibodies. Fold changes of MFIs show what times candidate peptide-pulsed RMA-S cells up-regulated MHC class I expression compared with unpulsed RMA-S cells.
mice developed myositis with lower incidence and less severity than those treated with transfer of the HILIYSDV-pulsed BMDCs (Fig. 1F). Because mice with transfer of the three candidate peptide-pulsed BMDCs barely developed myositis, the same as those with transfer of unpulsed BMDCs (Fig. 1F), the three peptides are not immunogenic and should have antagonized the effects of HILIYSDV-inducing CPIM. These results argue that HILIYSDV is a peptide with special immunogenicity to induce experimental myositis without promotional effects of C protein-specific CD4 T cells.

**CD4/CD8 T cell dependency of CIM development**

Immunohistochemical analyses of the sections of the CPIM muscles revealed that CD8 positive cells infiltrated into the muscle fibers (Fig. 2A). To investigate whether CD8 effector T cells mediate CPIM and whether the helper effects of CD4 T cells are dispensable for the development, the recipient B6 mice were treated with anti-CD4 or CD8 depleting antibodies before the transfer of the HILIYSDV-pulsed BMDCs. While the development of CPIM was not affected by the CD4-positive cell depletion, it was inhibited by the CD8-positive cell depletion (Fig. 2B and C). The results demonstrate that CD8-positive CTLs are pathogenic in CPIM and that CD4 T cells are not required.

Unexpectedly, HILIYSDV-specific cytotoxic reactions were not assessable in vitro analyses of CD8 T cells isolated from draining lymph nodes of mice with CPIM. The CD8 T cells were stimulated with HILIYSDV-presenting naive splenocytes or BMDCs ex vivo and analyzed with methods including enzyme-linked immunospot assays and flow cytometry assays to detect the interferon-γ production and chromium-51 release assays to measure their cytotoxicities. The difficulties in detecting the in vitro CTL activity might be due to small clonal size or weak cytotoxicity of HILIYSDV-specific CTLs in the mice. In this regard, it has been known that no islet autoantigens can induce detectable in vitro responses to BDC2.5 autoreactive diabetogenic BDC2.5 T cells.

To address whether HILIYSDV is a dominant epitope in CIM, we treated B6 mice with HILIYSDV in incomplete Freund’s adjuvant (IFA) to establish tolerance prior to CIM induction. While injection of C protein fragments in IFA blocked CIM development, that of HILIYSDV did not. Thus, HILIYSDV may not be the dominant CD8 epitope since we screened the peptides based on their affinity to MHC class I molecules. Also, these results do not necessarily argue against the dominant role of the peptide. Tolerance development to C protein by the C protein fragments might depend on tolerance in C protein-specific CD4 T cells because CIM development is mediated by both CD4 and CD8 T cells (6). While systemic or mucosal administration of MHC class II-binding peptides induced tolerance of CD4 T cells in various autoimmune disease models (31–33), peptide-induced tolerance of CD8 T cells has been shown to be limited in antigen-specific T-cell receptor-transgenic mice (34–36) and virus-induced autoimmune diabetes (37, 38). Of most importance is the myositis induction by a CD8 epitope peptide.

CPIM is a new murine model of a CD8 epitope peptide-induced myositis. The peptide, HILIYSDV, derives from the
an unsolved issue, we have established three models of murine skeletal muscle C protein and has a high affinity to MHC class I H2-K\(^d\). Because CPIM is mediated by CD8 T cells independently of CD4 T cells, this new model should be a useful tool to investigate pathology of PM and develop treatments targeting CD8 T cells. We have published research showing that blockade of interleukin (IL)-6, IL-1 or tumor necrosis factor-\(\alpha\) was effective to treat conventional CIM (9, 39). The target cells of these immunosuppressive agents are unclear. Another experimental myositis that we established, which was induced by an adoptive transfer of T cells from CIM mice to recipient B6 mice, was used to clarify if these agents activated innate immunity in the muscle tissues (28). Experiments using CPIM will clarify whether the immunosuppressive agents directly inhibit the activation of CD8 T cells. Although measurement of muscle weakness in mice is still an unsolved issue, we have established three models of myositis as tools to dissect the pathology of the myositis and to develop specific treatments.

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**References**


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**Fig. 2.** Sections of muscle from a mouse with CPIM that include necrotizing muscle fiber with infiltrating inflammatory cells (hematoxylin and eosin staining) were immunohistochemically stained with anti-CD4 or anti-CD8 antibodies or isotype control (A). CD8-positive cells infiltrated into the muscle fiber. Data are representative of three mice. Arrows show positive cells. Development of C protein peptide (HILIYSDV)-induced myositis was inhibited by CD8 T-cell depletion but not by CD4 T-cell depletion. Recipient B6 mice were treated with control rat IgGs, anti-CD4-depleting antibodies (B) or anti-CD8-depleting antibodies (C) before the transfer of BMDCs presenting HILIYSDV. *P < 0.05. The rhombus symbols and the bars represent the histological scores of individual mice and the average scores of individual groups, respectively.


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