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Original article

A new *in vitro* model of polymyositis reveals CD8$^+$ T cell invasion into muscle cells and its cytotoxic role

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Abstract

Objectives. The hallmark histopathology of PM is the presence of CD8$^+$ T cells in the non-necrotic muscle cells. The aim of this study was to clarify the pathological significance of CD8$^+$ T cells in muscle cells.

Methods. C2C12 cells were transduced retrovirally with the genes encoding MHC class I (H2K^b) and SIINFEKL peptide derived from ovalbumin (OVA), and then differentiated to myotubes (H2K^{b}OVA-myotubes). H2K^{b}OVA-myotubes were co-cultured with OT-I CD8$^+$ T cells derived from OVA-specific class I restricted T cell receptor transgenic mice as an *in vitro* model of PM to examine whether the CD8$^+$ T cells invade into the myotubes and if the myotubes with the invasion are more prone to die than those without. Muscle biopsy samples from patients with PM were examined for the presence of CD8$^+$ T cells in muscle cells. The clinical profiles were compared between the patients with and without CD8$^+$ T cells in muscle cells.

Results. Analysis of the *in vitro* model of PM with confocal microscopy demonstrated the invasion of OT-I CD8$^+$ T cells into H2K^{b}OVA-myotubes. Transmission electron microscopic analysis revealed an electron-lucent area between the invaded CD8$^+$ T cell and the cytoplasm of H2K^{b}OVA-myotubes. The myotubes invaded with OT-I CD8$^+$ T cells died earlier than the uninvaded myotubes. The level of serum creatinine kinase was higher in patients with CD8$^+$ T cells in muscle cells than those without these cells.

Conclusion. CD8$^+$ T cells invade into muscle cells and contribute to muscle injury in PM. Our *in vitro* model of PM is useful to examine the mechanisms underlying muscle injury induced by CD8$^+$ T cells.

Key words: idiopathic inflammatory myopathy, polymyositis, *in vitro* model, cytotoxic T lymphocyte

BASIC SCIENCE

Rheumatology key messages

- CD8$^+$ T cells invade into muscle cells and contribute to muscle injury in PM.
- Our new *in vitro* model can dissect the mechanisms underlying muscle injury in PM.

Introduction

PM is a subacute inflammatory myopathy, its primary clinical feature being manifested as proximal muscle weakness. The histopathological features in PM include necrotic and regenerating muscle cells and mononuclear inflammatory cell infiltrates, which are predominantly CD8$^+$ T cells in the endomysial area of the skeletal muscles [1, 2]. Some CD8$^+$ T cells are observed in non-necrotic muscle cells as a characteristic finding of PM [3, 4]. CD8$^+$ T cells in non-necrotic muscle cells are also observed in other forms of idiopathic inflammatory myopathy, including IBM and DM, and thus are not specific to PM. Nevertheless, it is useful to distinguish inflammatory myopathies from other types of myopathies such as dystrophic, metabolic, mitochondrial and toxic myopathies [5].

While histological analyses have been widely conducted for the diagnosis and classification of inflammatory myopathies in clinic, the mechanisms underlying muscle injury in PM are poorly understood. Histological analysis of muscle biopsy samples has revealed an increase in the expression of MHC class I molecules in the muscle cells and cytotoxic molecules such as perforin and granzyme in the CD8$^+$ T cells in patients with PM [6]. In addition, analysis of the peripheral blood from patients with PM revealed clonally expanded CD8$^+$ T cells, some of which infiltrate in the affected muscles [7–9]. These observations suggest the crucial role of CD8$^+$ T cells in muscle injury.

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The presence of $CD8^+$ T cells in non-necrotic muscle cells suggests that $CD8^+$ T cells may invade into the muscle cells to injure muscles in PM [5, 10, 11]. The expression level of inducible nitric oxidase synthase, a stress protein, increases in the muscle cells with $CD8^+$ T cells in the cytoplasm [12, 13]. However, the pathological significance of the presence of $CD8^+$ T cells in muscle cells has been yet to be determined. The correlation between the presence of $CD8^+$ T cells in non-necrotic muscle cells and clinical characteristics has never been investigated. In addition, the histological analysis cannot disclose whether the $CD8^+$ T cells invade into the muscle cells and if the invaded $CD8^+$ T cells are involved in the muscle injury. It is even possible to hypothesize that the muscle cells may engulf $CD8^+$ T cells to survive the attack by $CD8^+$ T cells, as in the case of cancer cells that escape from tumour immunity via entosis of immune cells after the engulfment process [14–16].

Understanding of the pathological significance of $CD8^+$ T cells in muscle cells would reveal the pathogenesis of PM, eventually leading to the development of new therapeutic strategies. To investigate the significance of $CD8^+$ T cells in muscle cells, we developed an *in vitro* model of the antigen-dependent muscle injury by $CD8^+$ cytotoxic T lymphocytes (CTLs). The *in vitro* model enabled us to study whether $CD8^+$ T cells invade into muscle cells and to clarify the fate of the muscle cells and $CD8^+$ T cells after the invasion process. In addition, we investigated the differences in the severity of myositis between PM patients with and without $CD8^+$ T cells in muscle cells. These approaches using both the *in vitro* model and biopsy specimens disclosed that the $CD8^+$ T cells in myotubes contribute to muscle injury.

Methods

Plasmid construction and generation of myotubes expressing $H2K^b$ and SIINFEKL peptide derived from ovalbumin

Plasmid containing the sequence of mouse β2-microglobulin, SIINFEKL and $H2K^b$ fused with a linker ($H2K^b$-SIINFEKL) was provided by Dr Satoru Senju (Kumamoto University, Kumamoto, Japan). C2C12 ($H2\text{-}K^k$) cells were provided by Dr Shin-ichi Takeda (National Center of Neurology and Psychiatry, Tokyo, Japan). To construct a retroviral vector expressing $H2K^b$-SIINFEKL, the coding region was amplified with PCR primers, 5′-CCGCTCGAG CGGCCACCATGGCTCGCTCGGTGACCCTGGTCTTT-3′ and 5′-ATAAGAATGCGGCCGCTAAACTATTCACGCTAG AGAATGAGGGTC-3′. The PCR product was digested with XhoI and NotI and cloned into a retroviral vector, pMXsIP, to generate pMXsIP-$H2K^b$-SIINFEKL. pMXsIP-$H2K^b$-SIINFEKL was transfected into Platinum-E cells with FuGENE HD (Promega, Madison, WI, USA) to produce the virus. C2C12 cells were infected with the virus for 48 h and cultured in the presence of 2.5 μg/ml of puromycin for 12 days. The puromycin-resistant cells were stained with allophycocyanin-conjugated anti-mouse H2-Kb bound to SIIFEKL antibody (25-D1.16, eBioSciences, Waltham, MA, USA) and sorted to purify $H2K^b$-SIINFEKL-expressing C2C12 cells ($H2K^b$OVA-C2C12) with FACSAria (BD Biosciences, Franklin Lakes, NJ, USA). The purity of $H2K^b$OVA-C2C12 was >99% after sorting. The cells were maintained with high-glucose DMEM (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum, penicillin and streptomycin. Upon reaching confluency, cells were cultured in DMEM supplemented with 2% horse serum to induce differentiation into myotubes ($H2K^b$OVA-myotubes). For additional methods see supplementary material, section Methods, available at *Rheumatology* online.

Patients and muscle biopsy

Muscle specimens were obtained using a percutaneous conchotome muscle biopsy technique from the anterior tibial muscles of 10 untreated patients with PM, who were hospitalized in our department between August 2016 and June 2018, and met the Bohan and Peter criteria and Tanimoto's criteria for classification of PM [17, 18]. Patients with IBM, DM or immune-mediated necrotizing myopathy (IMNM) were not included in the study. None of the patients had finger flexor weakness, arthritis, DM-associated skin manifestations, mechanic's hands or RP. Rimmed vacuoles were not observed in all of the muscle specimens.

Histological evaluation of the presence of $CD8^+$ T cells in muscle cells

Two pieces of muscle biopsy specimens per patient were used for the histological analysis. Sections were made in 10 μm thickness from the frozen biopsy specimens. Three sections per piece were randomly taken for the analysis. The serial sections were subjected to haematoxylin and eosin staining and the immunohistological staining against CD8. For the immunohistological staining, the sections were fixed with 4% paraformaldehyde, blocked with 5% goat serum in PBS, incubated with primary antibodies against CD8 (C8/144B; Nichirei, Tokyo, Japan) and then treated with a peroxidase-labelled amino acid polymer-conjugated goat anti-mouse IgG (K4000, Dako Cytomation, Glostrup, Denmark), followed by treatment with 3, 3′-diaminobenzidine substrate (K3468, Dako Cytomation, Glostrup, Denmark) for visualization. Forty high-power fields representing a total area of 7.5 mm^2 in each section were evaluated for the presence of $CD8^+$ T cell in muscle cells.

Ethics approval and consent to participate

The study protocols were approved by the institutional review board at Tokyo Medical and Dental University (TMDU) and are in accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained from all participants. All animal experiments were approved by the Institutional Animal Care and Use Committee of TMDU and were performed in accordance with the institutional and national guidelines.

Statistical analyses

Statistical significance was determined using Student's *t*-test for comparison between two groups. In time course analysis, the statistical significance was determined using Log-rank test. The correlation between the two variables was examined with linear regression analysis. In multiple comparison between two categorical variables, χ^2 test was performed and the statistical significance was determined based on Benjamini and Hochberg method.

Results

An *in vitro* model of CTL-mediated muscle injury was developed using SIINFEKL peptide and OT-I CTLs

The presence of $CD8^+$ T cells in muscle cells can be attributable to invasion of $CD8^+$ T cells to kill muscle cells, engulfment of $CD8^+$ T cells by muscle cells to survive the attack or behaviour of the dying muscle cells. To clarify the pathological significance of $CD8^+$ T cells in non-necrotic muscle cells, we developed an *in vitro* model of CTL-mediated muscle injury. To induce an antigen-specific muscle injury by CTLs, we utilized the SIINFEKL peptide, which is an antigenic peptide recognized by T cell antigen receptor of CTLs derived from OVA-specific class I restricted T cell receptor transgenic mice (OT-I) in the context of MHC class I K^b ($H2K^b$). C2C12 cells were retrovirally transduced with genes encoding the OVA peptide and $H2K^b$, and subsequently differentiated into myotubes ($H2K^b$OVA-myotubes).

We tested if the $H2K^b$OVA-myotubes were killed upon co-culture with OT-I CTLs, using a ^{51}Cr release cytotoxic assay. While the cytotoxic effect of OT-I CTLs was observed against $H2K^b$OVA-myotubes in the co-culture system, no effect was observed against C2C12-derived myotubes without $H2K^b$OVA expression (Fig. 1A). The cytotoxic effect was next studied with time-lapse confocal microscopy. Death of $H2K^b$OVA-myotubes was observed over hours until all the myotubes died. An increase in the rate of $H2K^b$OVA-myotube death was observed as the number of OT-I CTLs increased. In contrast, no cell death was detected for C2C12-derived myotubes without $H2K^b$OVA expression in the presence of OT-I CTLs (Fig. 1B). These results indicate that CTLs killed the myotubes in an antigen-dependent manner.

CTLs were internalized in myotubes

We examined the non-necrotic muscle cells for the presence of OT-I CTLs. Confocal microscopic analysis revealed the presence of OT-I CTLs in the $H2K^b$OVA-myotubes aside from CTLs on the myotubes (Fig. 1C). CTLs were not observed in the myotubes when wild-type CTLs and the $H2K^b$OVA-myotubes were co-cultured or when OT-I CTLs and the myotubes without $H2K^b$OVA expression were co-cultured (Table 1). These results indicate that the presence of CTLs in the myotubes was antigen dependent.

To see if CTLs were internalized thoroughly in the myotubes, we first stained the cells extracellularly with an Alexa Fluor 647-conjugated anti-CD8a antibody (eCD8 staining), fixed with 4% paraformaldehyde, permeabilized with Triton X-100 and stained intracellularly with a phycoerythrin-conjugated anti-CD8a antibody (iCD8 staining). The CTLs on the surface of the myotubes were positive for both eCD8 and iCD8 staining. In contrast, the CTLs in the myotubes were positive only for iCD8 staining and were protected from eCD8 staining, indicative of the complete internalization in the myotubes (Fig. 1D). Transmission electron microscopy analysis revealed the electron-lucent area between the internalized CTL and the cytoplasm of the myotube, indicating that CTL should be surrounded by the plasma membrane of the myotube (Fig. 1E).

CTLs invaded into myotubes and contributed to muscle injury

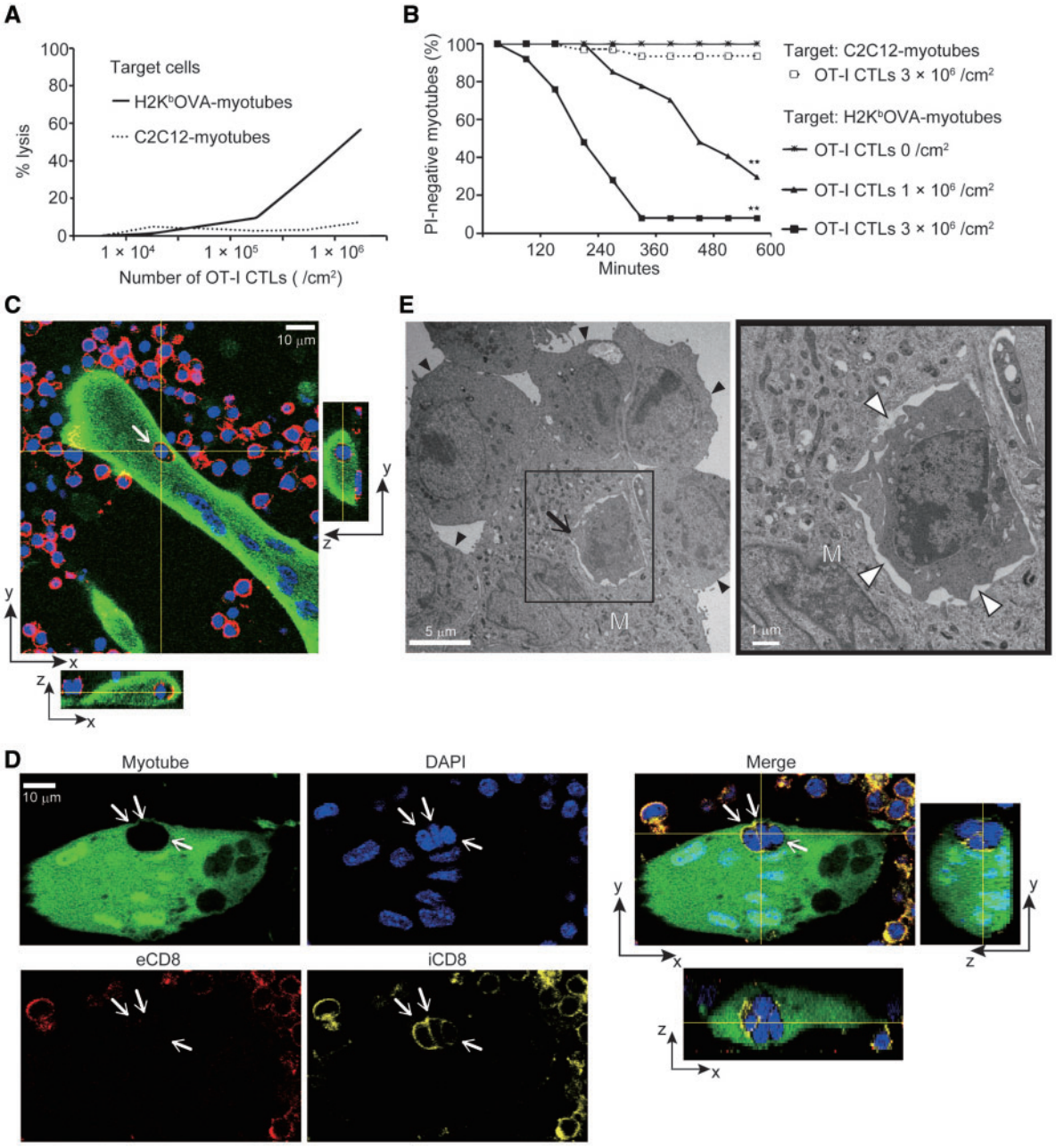
To discern if the CTLs invade into the myotubes and induce the myotube death, we evaluated the morphological changes of the myotubes and CTLs during the co-culture using time-lapse confocal microscopy (Fig. 2A). The CTLs started to invade into myotubes as early as 1 h after initiation of the co-culture [150 (108) min, mean (S.D.)]. Subsequent to the invasion, CTLs stayed in the myotubes for several hours [193 (139) min, mean (S.D.)], after which the myotubes shrank and detached from the dish. The myotubes were stained positive for propidium iodide (PI), indicative of cell death (Fig. 2B). Forty percent of the CTLs in myotubes evaded before the myotube death, while the others stayed in the myotubes during the myotube death (Table 2). We observed no PI-positive CTLs in the myotubes, while some CTLs on the myotubes were positive for PI staining.

To study if the myotubes with the CTL invasion are more prone to death than those without the CTL invasion, we compared the time from the initiation of the co-culture to the myotube death. The myotubes with the CTL invasion died earlier than those without the CTL invasion by about 20% ($P < 0.01$, Fig. 2C). The result did not change irrespective of the evasion of CTLs from the myotubes before their death (Table 2). The early cell death of myotubes could be associated with the increase in the number of CTLs attached on the surface of myotubes, as the increase in the number of CTLs on the surface of a myotube would result in faster cell death ($r = 0.48$, $P < 0.01$, Fig. 2D). However, the number of CTLs on the myotubes was comparable between the myotubes with and without CTL invasion (Fig. 2E). These results indicate that CTLs in the myotubes should be involved in the myotube death.

CTL invasion did not result from the behaviour of dying myotubes

The myotube death after the CTL invasion could be associated with the increase in susceptibility of the dying myotubes to the CTL invasion. To discern whether the dying myotubes are susceptible to the CTL invasion, myotubes were subjected to starvation in a serum-free medium to induce cell death prior to their co-culture with CTLs [19, 20]. The proportions of PI positive myotubes with or

Fig. 1 Cytotoxicicy of OT-I CTLs against H2K^{b}OVA-myotubes and internalization of OT-I CTLs in H2K^{b}OVA-myotubes



(**A**) The cytotoxicity of OT-I CTLs on myotubes assessed with the ^{51}Cr release assay. (**B**) The cytotoxicity of OT-I CTLs on myotubes assessed with time-lapse confocal microscopy. $^{**}P < 0.01$, compared with H2K^{b}OVA-myotubes without OT-I CTLs. (**C**) The co-cultured OT-I CTLs (red) and H2K^{b}OVA-myotubes (green). Arrow, OT-I CTL in myotube. (**D**) The co-cultured cells analysed for eCD8 and iCD8 expression. Arrows, CD8$^+$ cells with iCD8 but without eCD8 expression. (**E**) The co-cultured cells analysed with TEM. Arrow, a CTL in a myotube. Black arrowheads, CTLs on the myotube. White arrowheads, electron-lucent area between CTL and myotube. CTL: cytotoxic T lymphocyte; OT-I: OVA-specific class I restricted T cell receptor transgenic mice; OVA: SIINFEKL peptide derived from ovalbumin; M: myotube; TEM: transmission electron microscopy; eCD8: staining extracellularly with an Alexa Fluor 647-conjugated anti-CD8a antibody; iCD8: fixed with 4% paraformaldehyde, permeabilized with Triton X-100 and stained intracellularly with a phycoerythrin-conjugated anti-CD8a antibody.

without starvation were 13.4 (3.4)% and 5.0 (2.6)% [mean (S.D.)] respectively ($P < 0.01$). When the number of myotubes showing CTL invasion was enumerated, we observed no difference between the starved and non-starved cells (Fig. 2F). These results suggest that the death of myotubes after CTL invasion would not be attributable to the increased susceptibility of the dying myotubes to CTL invasion, but result from the cytotoxic effect of CTLs on the myotubes.

TABLE 1 Percentage of myotubes with CTL in the cytoplasm

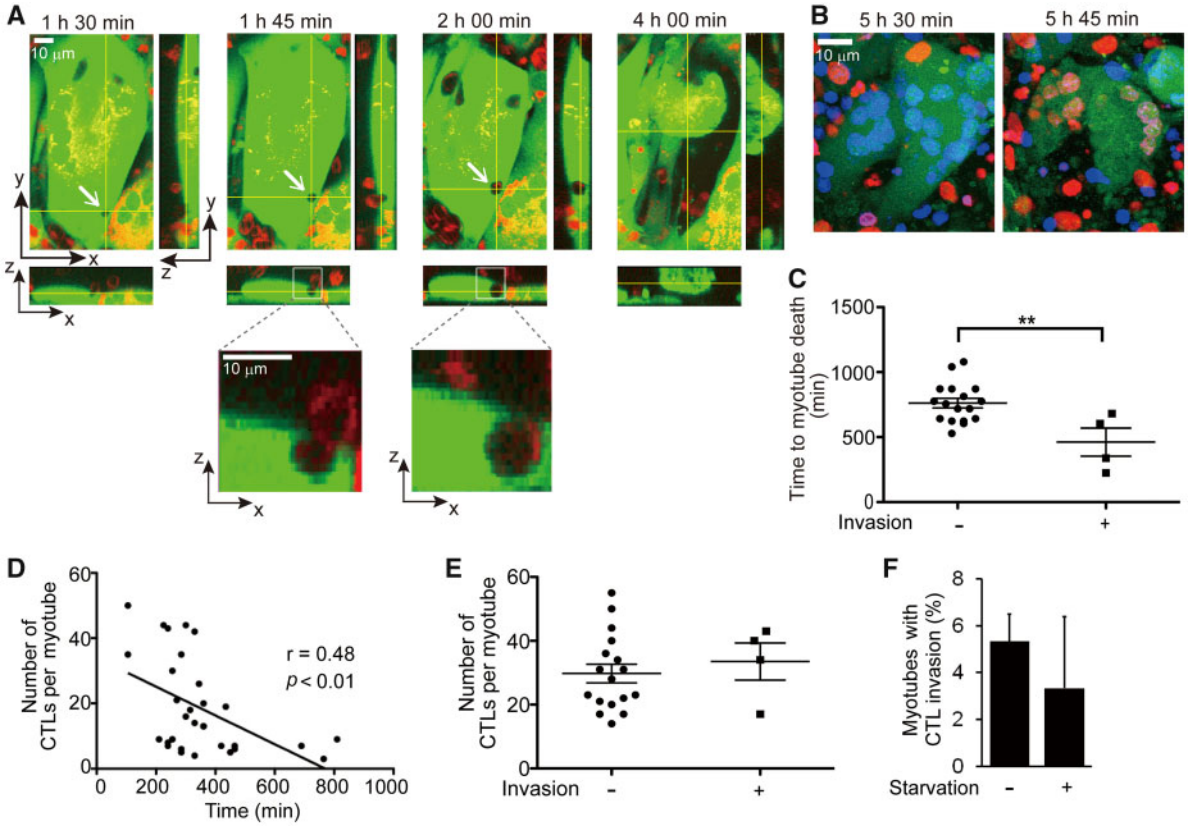
	H2K^{b}OVA-myotubes	C2C12-myotubes
OT-I CTLs	13.4 (2.9)	0.0
Wild-type CTLs	0.0	NA

H2K^{b}OVA-myotubes or C2C12-myotubes were co-cultured with either OT-I CTLs or wild-type CTLs. Values are the percentage of myotubes with CTL in the cytoplasm. Results represent the mean (S.D.) of three independent experiments. CTL: cytotoxic T lymphocyte; NA: not analysed; OT-I: OVA-specific class I restricted T cell receptor transgenic mice; OVA; ovalbumin.

Serum creatinine kinase level was high in patients with the presence of CTLs in non-necrotic muscle cells

Considering the cytotoxic role of CTLs in the myotubes in the *in vitro* model, we reasoned that the myositis should be more severe in patients with the presence of CD8$^+$ T cells in non-necrotic muscle cells than those without. We collected muscle biopsy samples from 10 PM patients and investigated the differences in the severity of myositis between the patients with and without CD8$^+$ T cells in muscle cells. CD8$^+$ cells in the non-necrotic muscle cells were observed in 5 out of the 10 patients. The inflammatory infiltrate was observed in the endomysium area and perivascular or perimysium areas in all subjects. Myofiber

FIG. 2 CTL invasion into myotubes and its cytotoxic role



(**A**) The co-cultured CellTracker-labelled OT-I CTLs (red) and H2K^{b}OVA-myotubes (green). Arrow, invading CTL. (**B**) The co-cultured CellTracker-labelled H2K^{b}OVA-myotubes (green) and unlabelled OT-I CTLs in the presence of PI (red). Blue, nuclei. (**C**) The time to death for myotubes with and without CTL invasion. Mean (S.D.). $^{**}P < 0.01$. (**D**) Correlation between the time of myotube death and numbers of CTLs on myotubes at 4 h of co-culture. (**E**) Numbers of CTLs on myotubes with and without CTL invasion. Mean (S.D.). (**F**) Percentage of myotubes with CTL invasion with or without starvation. Mean (S.D.). CTL: cytotoxic T lymphocyte; OT-I: OVA-specific class I restricted T cell receptor transgenic mice; OVA: SIINFEKL peptide derived from ovalbumin PI: propidium iodide.

Table 2 Number of myotubes that died earlier or later than median time

CTL invasion	–	+	+
CTL evasion before myotube death		–	+
Died earlier than median	41	23	16
Died later than median	59	11	7
P-value		0.028	0.029

The median time of myotube death was calculated in each experiment, and the number of myotubes that died earlier or later than the median was shown. Values are the sum of 10 independent experiments. *P*-values were calculated in the comparison with the myotubes without CTL invasion. CTL: cytotoxic T lymphocyte.

necrosis was not the predominant feature in all specimens. Immunohistological analysis revealed the presence of dystrophin-positive membrane around $CD8^+$ T cells in the non-necrotic muscle cells, indicating that the $CD8^+$ T cells were surrounded by the plasma membrane of the muscle cells (Fig. 3).

Next, we looked for differences in the clinical characteristics between patients with and without $CD8^+$ cells in non-necrotic muscle cells (Table 3) and found that the level of creatinine kinase (CK) was higher in patients with $CD8^+$ cells than in those without $CD8^+$ cells in non-necrotic muscle cells ($P < 0.05$). No statistical difference was observed in other clinical manifestations including interstitial pneumonia and arthralgia. The disease duration was comparable between the two groups. These results suggest that the presence of $CD8^+$ cells in the non-necrotic muscle cells should be associated with muscle injury.

Discussion

Our *in vitro* model of muscle damage in PM revealed the invasion of $CD8^+$ CTLs in myotubes and their cytotoxic role in muscle injury. We observed no signs of death of the invaded CTLs, indicating that presence of CTLs in muscle cells should not be attributed to the efforts of the muscle cells to escape from the attack by $CD8^+$ CTLs. In addition, we observed that the level of serum CK was higher in PM patients with $CD8^+$ cells in muscle cells than in patients without those cells.

We found that some myotubes died after the evasion of CTLs from the invaded myotubes. The time from the start of the co-culture to the death of these myotubes was similar to the time of the myotubes with CTLs in the cytoplasm, but less than the time of the myotubes without the CTL invasion. In addition, transmission electron microscopy analysis revealed that the invaded CTL was surrounded with an intact plasma membrane of the myotube with no signs of the myotube death, consistent with the results of previous studies; it was shown that most lymphoid cells in the muscle cells were surrounded

Table 3 Patient characteristics

	$CD8^+$ T cells in non-necrotic muscle cells	
	Absent ($n = 5$)	Present ($n = 5$)
Age (years)	50.4 (13.7)	64.4 (21.1)
Gender (female), (*n*)	2	3
Disease duration (months)	4.0 (0.7)	7.4 (4.1)
Total MMT score of 8 muscles (% of max)	94.4 (2.9)	92.2 (0.9)
Arthralgia	1	0
Interstitial pneumonia	3	0
Serum CK* (U/l)	1577 (543)	4117 (900)
Autoantibodies (*n*)		
ANA ($>\times 80$)	4	2
ARS		
Jo-1	0	2
Others	1	1
TIF1γ	0	1
RNP	1	0

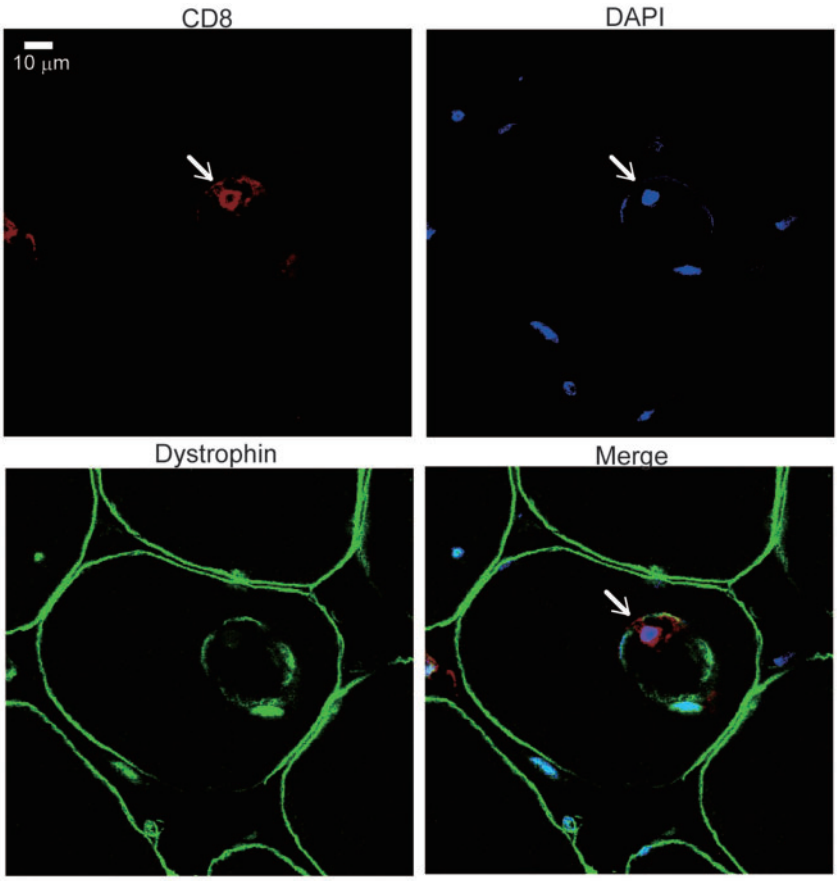
Values are presented as mean (S.D.). *$P < 0.05$. ARS: anti-aminoacyl tRNA synthetase antibodies; CK: creatinine kinase (reference interval: male 62–287 U/l, female 45–163 U/l); Jo-1: anti-Jo-1 antibodies; MMT: manual muscle testing; *n*: number of patients; RNP: anti-RNP antibodies; TIF1γ: anti-transcription intermediary factor 1-gamma antibodies.

with cell membrane in electron microscopic analysis of muscles from patients with PM [10, 11]. In PM, CTLs in myotubes would not necessarily kill the invaded myotubes immediately, but damage the cells or increase the vulnerability of the myotubes to cytotoxicity of other CTLs on the myotubes.

In our *in vitro* model of muscle damage in PM, a large number of OT-I CTLs was required to induce cell death of target myotubes. In addition, the time required by OT-I CTLs to kill the myotubes was longer than that required to kill other cell types such as cancer cells [21, 22]. These results suggest that myotubes are relatively resistant to CTL-induced cell death, which may be the reason for the invasion of CTLs in the muscle cells observed in PM. To the best of our knowledge, the cytotoxic role of CTL invasion has not been reported in other diseases including cancers, graft-versus-host disease and viral infections. CTL invasion may be the mechanism underlying the induction of cell death in resistant cells.

To examine the cytotoxic effect of T cells on muscle cells, co-culture systems using the myotubes differentiated from patient biopsy samples and T cells from the peripheral blood or muscle tissues of the same donors were employed in past studies [20, 23]. While the system offers an advantage of the use of autologous cells in the co-culture, the requirement to obtain samples from patients with PM and the difficulty of obtaining samples from the control donors posed challenges. In addition, it is hard to ascertain if muscle injury results from antigen-specific effects of CTLs in the autologous co-

Fig. 3 CTL invasion observed in muscle specimens of PM



Immunofluorescence staining of muscle specimen of PM. Arrow, a $CD8^+$ cell in a muscle cell. The $CD8^+$ cell was surrounded by plasma membrane of the muscle cell. CD8, red; DAPI, blue; Dystrophin, green. CTL: cytotoxic T lymphocyte.

culture system. To overcome these limitations, we used the SIINFEKL peptide and OT-I CTLs as a model autoantigen and antigen-specific $CD8^+$ CTLs. The analyses using the SIINFEKL peptide and OT-I CTLs have revealed the pathogenesis of a variety of CTL-mediated human diseases [24–27]. For example, a crucial role of Fas and Fas ligand, and the involvement of perforin and granzyme B, were demonstrated in human HCV infection and graft-versus-host skin disease, respectively, using OT-I CTLs [25, 26, 28–31]. This model should be useful to elucidate the mechanisms of antigen-dependent muscle injury by CTLs.

A caveat of our *in vitro* model is that we used C2C12-myotubes for the assays. C2C12-myotubes have been widely used in studies of muscle development and diseases. However, they are incompletely differentiated into muscle cells; thus, the observations reported with this model may not always mimic the pathogenesis of PM, wherein the muscle cells are damaged by CTLs. In addition, the differences between mice and human may lead to translational limitation. To employ this model for the analysis of PM, it is important to confirm that the findings of the *in vitro* model are consistent with those reported with the muscle samples from patients with PM.

While we observed differences in CK level between the patients with and without $CD8^+$ T cells in muscle cells, no differences were observed in muscle strength, as evaluated with a manual muscle testing in the two groups. This discrepancy may be related to the fact that muscle weakness was not severe in our cohort, which makes it difficult to find the differences between the groups. The average total manual muscle testing scores of eight muscles were $>90\%$ of the maximal strength, whereas the average CK level was about 14 times the upper limit of the normal range in our cohort.

In this study population, four patients had anti-aminoacyl tRNA synthetase antibodies, three of whom have $CD8^+$ cells in non-necrotic muscle cells. These four patients could be sub-classified as suffering from anti-synthetase syndrome (ASS). Indeed, one patient with $CD8^+$ T cells in non-necrotic muscle cells met the diagnostic criteria for ASS proposed by Solomon *et al.* [32]. Recently, unsupervised multivariate analyses based on various characteristics including clinical features, histological findings and

autoantibodies were performed using a database of the French myositis network cohort to develop a new classification scheme for idiopathic inflammatory myopathies. In this study, 67.7% of the patients with clinical diagnosis of PM were classified into the same category with IMNM in the four identified groups; IBM, IMNM(+PM), DM and ASS [33]. However, the proportion of patients with the presence of $CD8^+$ cells in muscle cells were similar between IMNM(+PM), DM and ASS categories, implying that the presence of $CD8^+$ cells in muscle cells might not necessarily be a characteristic of PM.

Conclusions

In conclusion, we disclosed the cytotoxic role of $CD8^+$ CTLs in muscle cells in PM using two approaches, namely, the newly developed *in vitro* PM model and the analysis of muscle samples of patients with PM. In PM, $CD8^+$ CTLs in the muscle cells would play the cytotoxic role in cooperation with the CTLs on the muscle cells. Targeting CTLs in myotubes as well as those on the myotubes may serve as an important strategy for the treatment of PM. Clarifying the mechanisms of CTL invasion and muscle injury by invaded CTLs may lead to the development of new therapies for PM.

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Supplementary data

Supplementary data are available at *Rheumatology* online.

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