specifically target the critical molecules involved in PM/DM pathology.

The present review attempts to show where we stand in PM/DM research and clinical practice, and hopefully will facilitate development of more specific and effective treatments for PM/DM.

Muscle pathology

Muscle histopathology of PM/DM is characterized by mononuclear cell infiltration among non-necrotic skeletal muscle fibers, and degeneration/regeneration of the fibers. The cellular infiltrates in the affected muscle tissues include T and B lymphocytes, NK cells, macrophages and dendritic cells. Thus, effector T cells, immunoglobulins, and muscle fibers appear to be critical components for final damage of the muscles (Fig. 1).

Immunohistochemical studies of the PM/DM muscles by Engel and Arahata showed that CD8 T cells were more abundant in the endomysium of the PM muscles (82.5%) than in that of the DM muscles (68.2%).[2–6] Later, deposition of complement 5b-9 membrane attack complexes (MAC) was found in the intramuscular microvasculature of the DM muscles.[9] It was suggested that high-dose intravenous infusion of immunoglobulin (IVIg) exerts its effects by removing the complement deposition.[10] Based on these observations, it was proposed that DM is a humorally mediated vasculopathy triggered by the activation of CD4 T cells and B cells, followed by complement deposition on intramuscular microvessels. Whereas PM, in contrast, is a cell-mediated disease characterized by activated cytotoxic CD8 T cells directly damaging muscle fibers.

These assumptions have hardly been supported by the knowledge base of modern immunology. There is no evidence that subtle differences in CD4/8 T lymphocyte counts dictate dominance of humoral and cellular immune reactions. Primary sites for CD4 T cells to provide help to B cells are lymphoid tissues, including the lymph nodes and the spleen, but not peripheral inflammatory sites. Furthermore, there has been no evidence that shows *in vivo* MAC-dependent injury of eukaryotic cells. Clinical features and treatment responses of the PM and DM patients are also similar, except for skin manifestations. IVIg exerts its therapeutic effects both on PM and DM. A molecular attempt to discriminate between genes expressed intramuscularly has failed to distinguish the two diseases.[11] Based on these facts, it has also been proposed that the two diseases – including amyopathic DM – represent a spectrum of illnesses in which some patients suffer only from a muscle disease or from a skin disease.[12]

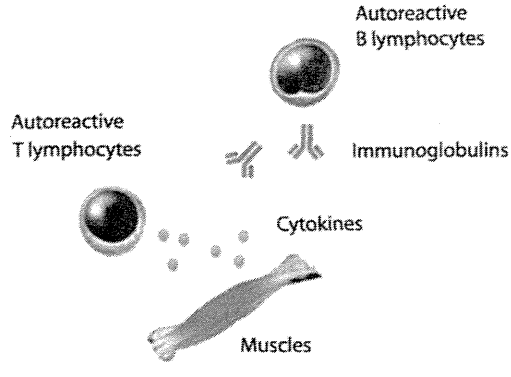

Figure 1 Components that could determine the fate of the muscles affected by polymyositis and dermatomyositis.

Of particular interest is perifascicular atrophy (PFA) seen in the affected muscle tissues. It denotes small, abnormal in appearance myofibers at the periphery of muscle fascicles and has been considered to be a hallmark of DM-related muscle pathology by quite a few muscle pathologists. It was hypothesized that autoantibodies against endothelial cells attach to the endothelium, activate complements and cause vascular injury leading to ischemic myofiber damage at a watershed region in the periphery of the fascicles. However, no myositis-related autoantibodies that react specifically to endothelial cells have been identified, although great efforts to search for disease-specific autoantibodies succeeded in discovering several myositis-related autoantibodies. An experimental model suggested that the perifascicular fibers are less vulnerable to ischemia than central fibers.[13] Microscopic polyangitis occasionally affects perimysial microvessels in the muscles and causes muscle weakness and increased signals in MRI of the muscle tissues. This typical ischemic microvascular damage of the muscles does not induce PFA.[14] Some myositis patients without skin manifestations have lesions indistinguishable from PFA in the biopsy muscles.

Does PFA characterize pathology of DM? Is it caused by ischemia? To reconcile conflicting observations (Table 1) by establishing a new model, these questions have to be revisited with modern cellular and molecular biology. In this regard, Greenberg et al. proposed that type I interferon (IFN) should be responsible for PFA. They found that the periphery of the muscle fascicles affected by myositis is rich in plasmacytoid dendritic cells (pDC), which produce a large quantity of type I IFN.[15]

Table 1 The facts that do not agree with the classical model of dermatomyositis pathology

1. There is no instance of CD4/8 ratio in the peripheral tissues dictating humoral versus cellular immunity
2. No myositis-related antibodies react to endothelial antigens
3. There is no instance of membrane attack complexes damaging eukaryotic cells *in vivo*
4. Microscopic angitis in the muscles or ischemic peripheral arterial diseases does not induce perifascicular atrophy
5. Therapeutic responses to treatments, including IVIg, do not differ in polymyositis and dermatomyositis

T lymphocytes in PM/DM

Antigens recognized by autoreactive T cells responsible for PM/DM have not been identified as is in many other human autoimmune diseases. However, the ideal treatment should rely on the induction of immune tolerance specific to the autoantigens targeted in the disease. A reverse genetic approach taken to identify the autoantigens was the characterization of T cell clones activated in myositis patients. We and another group found an expansion of peripheral blood CD8 T cell clones in PM patients more frequent than in healthy age-matched controls.[16,17] Of note, the CD8 clonal expansion was also found in DM patients.[18] Some of those clones infiltrated into the endomysium of the affected muscles and expressed cytotoxic molecules. Clonal tracking showed a persistent expansion of these clones in the peripheral blood of patients, even after recovery from active myositis.[17,19] Interestingly, the infiltrating cells expressed anti-apoptotic genes that could contribute to their extended survival,[20] which might possibly account for the disease relapse when tapering immunosuppressive treatment.

The T cell receptors (TCR) utilized by the expanded clones have been characterized with molecular biological techniques. The cDNA of those TCR (Vγ1.3 and Vδ2) was transferred into the murine T cell hybridoma, BW58 α-β-, which after the transfer acquired reactivity against a cytosolic protein expressed in human myoblasts.[21] However, the target molecule recognized by these T cells remains elusive.

Finally, analyses of genetic polymorphisms showed the association of PM/DM with specific alleles of IFN-γ and interleukin (IL)-4 genes,[22] which encode the two prototypical Th1/2 cytokines. This aspect is interesting because the preferential induction of Th1/2 cytokines might dictate T cell commitment towards possibly pathogenic or protective immune responses. Th17 cells belong to a newly identified subset of helper T lymphocytes that produces IL17A and other cytokines as effector molecules. Studies of mice lacking the IL17A gene have shown that Th17 cells are involved primarily in many autoimmune models of mice, although their role in humans is still controversial. It as been shown that IL17A is dispensable for the induction of a murine model of PM, arguing that autoimmune diseases depend differentially on IL17A.[23]

In experimental animals, some antigens are immunogenic enough to induce myositis. Classically, experimental autoimmune myositis (EAM) can be induced by immunizing Lewis rats with crude skeletal muscle myosin. Myosin itself, as well as the minor component skeletal muscle C-protein, can induce experimental myositis in rats as suggested by studies using biochemically purified proteins.[24,25] The immunization of SJL mice with crude myosin also induces classical EAM. However, there is a possible confounding complication in studying myositis in SJL mice, which have mutated dysferlin genes. It is well known that the muscles with human dysferlinopathy occasionally have T lymphocyte infiltration. This is associated with an elevated expression of human leukocyte antigen (HLA) class I molecules on the muscle fibers.[26,27] Thus, the impaired function of dysferlin (and related immune dysfunction) could be associated with the pathology of EAM.[28] Additionally, murine EAM is histologically characterized by dominant CD4 T cell infiltration, whereas human PM is likely to be mediated by cytotoxic CD8 T cells. Myositis can be evoked in naïve animals by the transfer of serum from EAM mice.[29] Given those considerations, it is an open question whether classical murine EAM might reproduce human PM even if the early phase of reactivity might seem to be driven by immunological responses to an immunizing antigen.[30] By contrast, immunization with recombinant C-protein also induced experimental myositis in C57BL6 mice.[31] In analogy with human PM, the C-protein-induced myositis (CIM) is mediated by CD8 cytotoxic T cells. Availability of many genetic mutant mice on the C57BL6 background has clearly facilitated genetic studies of this experimental myositis (Fig. 2).[23,31] Other immunogens that can induce experimental myositis include laminin, which is a major non-collagenous component of the basal lamina,[32] and pyruvate kinase M1/M2 (expressed by muscle fibers), peptides of which incubated with dendritic cells results in myositis in BALB/c mice.[33]

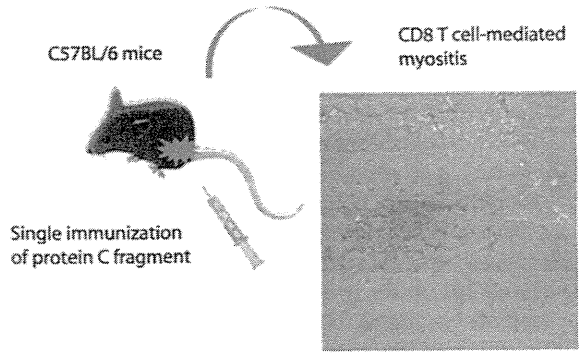

Figure 2 C-protein-induced myositis.

B cells in PM/DM

Although B cells might not be involved directly in the muscle injury of PM/DM, sera from patients react against many autoantigens, including aminoacyl transfer (t)RNA synthetases – a group of enzymes that catalyze the esterification of a specific amino acid to tRNA to form aminoacyl tRNA. Until an antiphenylalanyl tRNA synthetase (anti-Zo) autoantibody was found in the serum of a PM patient, eight aminoacyl tRNA synthetases have been found to be targeted by antoantibodies in the sera of PM/DM patients.[34] In particular, the antihistydyl-tRNA synthetase (anti-Jo-1) antibody was detected in approximately 20% of PM/DM patients. The antithreonyl-tRNA (anti-PL-7) antibody was found to be present more in Japanese PM/DM patients (17%), and associated with milder myositis and interstitial lung disease (ILD).[35,36] Also the anti-isoleucyl-tRNA (anti-OJ), antiasparaginyl-tRNA (anti-KS), and anti-alanyl (anti-PL-12) antibodies associate more with ILD than myositis.[37–39] These facts show that the presence of antisynthetase antibodies might be a hallmark of ILD rather than myositis.

It has been known that some aminoacyl tRNA synthetases can act as chemoattractants. Hystidyl-tRNA synthetase activates CCR5-transfected cells, which can be blocked by anti-CCR5 antibodies.[40] Aspraginyl-tRNA synthetase activates monocytes, immature dendritic cells and CCR3-transfected cells, whereas seryl-tRNA synthetase activates CCR3-transfected cells but not immature dendritic cells. How aminoacyl tRNA synthetases exert chemotactic effects remains to be elucidated. Some aminoacyl-tRNA synthetases, such as asparatyl- and lysyl-tRNA synthetases, are neither recognized by autoantibodies nor chemoattractants, whereas retinal S-antigen (an immunizing antigen to induce experimental autoimmune uveitis) is also a chemoattractant.[41] This, it was proposed that the release of autoantigens might represent a danger signal to facilitate the repair of damaged tissues and induce, at the same time, autoimmunity in those subjects with impaired immune regulation. Another factor that might account for the autoantibody and disease development is preferential expression of autoantigens. Histydyl-tRNA synthetase is expressed in two different conformations; one is cleavable with granzyme B, whereas the other is not. The granzyme-cleavable form is expressed preferentially in the lungs and localized to the alveolar epithelium, which are the major targets of PM/DM-associated ILD.[42]

Some other myositis-related autoantibodies are also associated with specific clinical features, and are thus of clinical value. Autoantibodies against RNA helicase encoded by melanoma differentiation-associated gene 5 (MDA5) can be found in half of sera from patients with DM accompanying mild or no myositis, and were previously named anti-CADM (clinically amyopathic dermatomyositis) autoantibodies.[43] Most of the patients positive for the anti-MDA-5 antibodies develop acute ILD, which often leads to a fatal outcome. Antibodies against unidentified proteins with molecular weight 155 and 140 (p155 and p140) were found in 13–21% of DM patients.[44,45] The p140 protein is perhaps a degraded p155 protein fragment. These antibodies are commonly detected (71–75%) in DM patients accompanying malignancy. Heliotrope rash, Gottron's papules and signs, and other DM-associated skin rashes were more frequent, whereas ILD is less frequent in patients with anti-p155 antibodies. Patients with antibodies against signal recognition particles often have severe symmetric proximal muscle weakness. Most of their muscle biopsy samples show necrotic muscle fibers with few inflammatory infiltrates.[46] Although the presence of these antibodies was originally thought to be confined to PM patients who are resistant to conventional treatment, a larger scale study did not support this notion.[47] Altogether, it is now possible to roughly define subsets of myositis patients by the newly defined autoantibodies.

Cytokines in PM/DM

A body of evidence accumulated in recent years has implicated the increased expression of IFN-regulated genes in the pathogensis of systemic lupus erythematosus.[48] This seems to be the case in DM and possibly PM. Microarray analyses of DM muscles showed increased expression of type I IFN-inducible genes.[15] MxA protein, an IFN-inducible gene

product, was highly expressed by muscle fibers and capillaries. Peripheral blood cells from DM patients also expressed type I IFN-inducible genes,[49] and the IFN signature was present both in the muscles and in the peripheral blood. The same results were observed in muscles and peripheral blood from juvenile DM patients.[50] Immunohistochemical analyses of skin lesions of DM patients showed that the MxA protein is highly expressed in the epidermis and infiltrating cells, showing that the IFN signature is also present in the skin.[51] MxA expression was accompanied by the expression of CXCL10, a chemokine that should help recruit a subset of T cells into the dermis. Although the source of type I IFN is unknown, pDC becomes a major source of type I IFN when their surface toll-like receptors (TLR) are triggered. Anti-Jo-1 and anti-SS-A/Ro antibodies mixed with necrotic cell materials stimulated peripheral blood mononuclear cells to produce IFN-α,[52], which was abrogated by RNAse treatment. It is possible that RNA in immune complexes stimulates pDC through TLR. Because pDC are abundant in PM/DM muscles, this pathway might be involved in DM and PM. Finally, anecdotal case reports have described DM and PM during therapeutic administration of INF-α for other diseases.[53–55]

Because mononuclear cell infiltration characterizes the PM/DM muscle tissue, involvement of chemokines and their receptors have been investigated. It was reported that CCL2 (MCP-1) is expressed in the endothelial cells and infiltrating cells in PM/DM muscle tissues, and in mononuclear cells invading non-necrotic muscle fibers – especially in PM muscles – and in perifascicular and perimysial endothelial cells in the DM muscles.[56,57] The importance of CCL2 is further confirmed by the expression of its receptors, CCR2A and 2B, in the inflamed muscle tissues.[58,59] In injured muscles, CCR2 co-localized with myogenin, which is a marker of activated muscle precursor cells. Muscle regeneration processes are delayed in CCR2 deficient mice, implying that signaling through CCR2 is involved in the muscle precursor cell activities that are necessary for the regeneration of injured muscle tissue.[60] Although it has been suggested that CCL2/CCR2B interaction might be the most attractive target in the treatment of PM/DM, other chemokines and receptors are reportedly involved in PM/DM.[61,62] It is difficult to predict which chemokine is the best to inhibit *in vivo* amelioration of muscular damage. This issue can possibly be addressed using animal models of PM. In myosin-induced EAM, CX3CL1 (fraktalkine) was expressed on mononuclear cell infiltrates and endothelial cells.[63] Its receptor, CX3XR1, was expressed on mononuclear cell infiltrates. *In vivo* inhibition of the interaction between fraktalkine and CX3XR1 ameliorated myositis, suggests that CX3XL1 is involved in the destruction of the muscles in EAM.

TNF-α blockers are used successfully in the treatment of rheumatoid arthritis. Like in the rheumatoid synovial tissues, TNF-α was detected in intramuscular macrophages, in myonuclei of regenerating muscle fibers and in endomysial or perimysial tissue of PM/DM muscles.[64] Its receptors were found expressed on endothelial cells in inflammatory areas. A corresponding increase in mRNA levels of the ligand and receptor combination was also noted.[65,66] The genotyping of TNF- α genes in juvenile DM patients showed that the 308A allele is associated with the disease.[67] Patients with this allele tend to have peripheral mononuclear cells that produce more TNF-α *in vitro*, and have elevated muscle immunohistochemical reactivity to anti-TNF- α staining antibody.[68] The clinical use of TNF-blocking reagents in inflammatory myopathies has been limited to case reports and small case series.[69–77] Some reports showed that PM/DM developed during anti-TNFα monoclonal antibody treatment of rheumatoid arthritis.[78–80] At present, it needs to be determined which of the PM/DM patients might respond to TNF-blocking reagents if TNF blockade has any therapeutic effects.

Classical major histocompatibility complex in PM/DM

Muscle biopsy specimens from PM/DM patients show variable T cell and macrophage infiltration. Notably, HLA class I molecules are overexpressed in muscle fibers from PM/DM patients.[81] These molecules serve as platforms for antigenic peptides when the peptides are recognized by cytotoxic T cells. Overexpression of major histocompatibility complex (MHC) class I molecules should facilitate the recognition of muscle fibers by cytotoxic T cells. In addition, conditional upregulation of the MHC class I gene in the skeletal muscles in mice led to self-sustaining myositis.[82] This form of myositis was characterized by the degeneration of muscle fibers and massive infiltration of macrophages, but no lymphocytes. Injection of a proinflammatory cytokine, IFN-γ, induced the expression of MHC class I on regenerating, but not mature, myofibers.[83] Introduction of the MHC class I gene into myoblasts impaired the differentiation of the muscle *in vitro* and *in vivo*. Subsequent studies showed that the overexpression of MHC class I molecules induced the responses of

endoplasmic reticulum (ER), suggesting that ER stress can be a major non-immune pathway in the muscle damage and the impaired muscle regeneration of autoimmune myositis.[84] Nevertheless, MHC class I overexpression might not be enough to induce human myopathies. Indeed, MHC class I is overexpressed on muscle fibers together with IL-1α on capillary endothelial cells, even in the muscles of inactive PM/DM patients.[81] From a diagnostic viewpoint, two reports indicated that immunohistochemical staining of MHC class I could be a useful tool for the diagnosis of inflammatory myopathies,[85,86] and the staining remains elevated after initiation of immunosuppressive treatment. The same holds true in the diagnosis of juvenile DM.[87] However, one report showed that the detection of MHC class II rather than MHC class I might be more specific for diagnosis of PM/DM.[88]

Muscle fibers in PM/DM

Although cytotoxic T cells expressing perforins and granzymes invade the affected muscles, especially PM muscles, there is no evidence of apoptotic myocyte death in the affected muscle. Protection of muscle fibers from classic apoptosis seems to be mediated in several ways. The non-classical HLA class I molecule, HLA-G molecule, is expressed in the inflamed muscles.[89] Human myoblasts can escape from alloreactive cell lysis by T lymphocytes and NK cells when HLA-G genes are transduced.[90] The death-inducing receptor, Fas, is expressed on most muscle fibers, particularly in regenerating muscle fibers, showing that they could be vulnerable to apoptosis.[91,92] Although the expression of its ligand, Fas-L, in the inflamed muscle tissues is controversial, this molecule can be expressed on infiltrating lymphocytes. However, muscle fibers express a high level of the Fas-associated death domain-like IL-1 converting enzyme-inhibitory protein, which confers resistance to Fas-induced apoptosis.[92] The inhibitor of apoptosis protein (IAP)-like protein is another molecule that inhibits apoptotic cell death and is highly expressed on muscle fibers.[93] Also, myoblasts produce immunosuppressive molecules, transforming growth factor-beta and IL-10, which can serve as inhibitors of inflammation.[94] Thus, inflamed muscle tissues seem to resist immunological insults.

Diagnosis and treatment of PM/DM

The Bohan and Peter criteria[7,8] for diagnosis are imprecise in the terminology, which led to wide acceptance of the criteria over three decades. Several researchers attempted to establish defined criteria.[95–97] Dalakas and Hohlfeld placed emphasis on histopathological findings of the biopsy muscles.[95] The argument was raised that their criteria force highly specialized histopathological investigations to be carried out in daily practice and are so low in sensitivity that many myositis patients might be left diagnostically adrift and excluded from receiving potentially effective treatments.[98] Criteria proposed by Japanese investigators are clinically practical, but do not incorporate modern diagnostic modalities, such as MRI and the detection of myositis-associated autoantibodies other than anti-Jo-1 antibodies.[97]

Conventional treatment of PM/DM is the administration of high-dose glucocorticoids. About 70% of patients respond initially and move on to the tapering of the glucocorticoids. Primary resistance to glucocortioid therapy, intolerance or glucocorticoid dependency requires treatment with immunosuppressants. Methotrexate, azathioprine, cyclophosphamide and calcineurin inhibitors (ciclosporine A and tacrolimus) have been commonly used successfully. Recently, myocophenolate mofetil has been brought into clinics. It seems effective for patients who are refractory to other immunosuppressants, although infection is often a serious side-effect.[99,100] An anecdotal report described the successful use of leflunomide in the treatment of PM.[101] Based on the assumption that humoral immunity drives inflammation in DM, the B cell-depleting monoclonal antibody, rituximab, has been trialed in therapy. Although no randomized studies have yet been carried out, rituximab appeared to be effective in both DM and PM.[102–104]

Although IVIg might be used in patients who are resistant and/or intolerant to other drugs, the mechanisms of how IVIg exerts its effects are largely unknown. Potentially, they include FcγR blockade, anti-idiotypic antibodies to inhibit pathogenic autoantibodies, increased catabolism of IgG mediated by FcRn blockade, inactivation of complement activation or effector function, inactivation of B cells and monocytes, and neutralization of cytokines and toxins. In the case of idiopathic thrombocytopenic purpura, FcγRIIb modulation has been proposed.[105] Molecular studies to address this issue have been carried out, but showed limitations.[106–108] In this regard, the mouse PM model, CIM, can be treated successfully with IVIg. Because CIM is a B cell-independent myositis, the result suggested that the suppression of B cells and/or the immunoglobulin-mediated process is dispensable for the therapeutic effect.[31]

Acute progression of ILD is one of the most challenging manifestations in PM/DM, and often accounts for disease-associated death. Analyses of T lymphocytes in the bronchoalveolar lavage fluid suggested that pulmonary lesions are driven by autoreactive T cells.[109] Genotype identification of the patients showed preferential association with selected HLA class II haplotypes.[110] In agreement with these observations, suppressants of the T cell function, such as ciclosporine A and tacrolimus, seem to improve the clinical outcome of ILD.[111,112] As such, it has been proposed that calcineurin inhibitors should be started simultaneously with glucocorticoids in the treatment of myositis-associated acute ILD.[111,113]

Conclusion

The classic view on the ethiopathology of PM/DM has been established on the basis of microscopic findings and is now being revisited. Further studies on a molecular basis should be carried out to elucidate the real picture of PM/DM. The results will help to update the diagnostic criteria and to develop new treatment strategies.

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T Lymphocytes and Muscle Condition Act Like Seeds and Soil in a Murine Polymyositis Model

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Objective. **It has been reported that polymyositis (PM) is driven by CD8+ cytotoxic T lymphocytes. The C protein–induced myositis (CIM) model we have established is similar to PM in pathology except that it undergoes spontaneous remission. We undertook the present study to delineate the roles of innate and acquired immunity in myositis.**

Methods. **C57BL/6 mice were immunized with recombinant C protein fragments together with Freund's complete adjuvant (CFA) and Toll-like receptor (TLR) ligands at hind leg footpads and tail bases. CIM mediated by adoptive transfer of T cells to naive mice was treated with cytokine antagonists.**

Results. **Second immunization with C protein fragments revealed no induction of tolerance. Injection of CFA and TLR ligands at the hind leg footpads reinduced myositis in the same legs. Interestingly, initial myositis was observed only in the CFA-treated forelegs. Transfer of C protein fragment–specific T cells from mice with CIM induced myositis in CFA- and TLR ligand–treated legs of recipient mice. CFA treatment resulted in the recruitment of macrophages producing inflammatory cytokines. Induction of myositis was inhibited by blocking interleukin-1 receptor or tumor necrosis factor α.**

Conclusion. **Myositis development requires activation of autoaggressive T cells and conditioning of muscle tissue. CIM regression is due to attenuation of local CFA-induced immune activation. These results are in accordance with a "seed and soil" model of disease development and might offer clues to decipher clinical aspects of PM.**

Polymyositis (PM) is a chronic inflammatory myopathy of unknown etiology. It affects striated muscles and induces varying degrees of muscle weakness, especially in the proximal muscles (1). Dysphagia and respiratory muscle weakness with choking episodes and/or recurrent aspiration pneumonia can lead to premature death of patients. Current standard treatment is administration of high-dose glucocorticoids and/or immunosuppressants, which do not address the specific pathology of PM. Some patients have unwanted side effects, while other patients' disease is refractory to these drugs.

Immunohistochemical analysis of biopsy specimens of muscle tissue in PM suggested that muscle injury is driven primarily by CD8+ cytotoxic T lymphocytes (CTLs) (2). Thus, the disease process derives from systemic autoimmune reactions to muscle autoantigen(s), which could incite systemic muscle inflammation. However, the entire muscle system is not necessarily

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subject to inflammation in PM. This is evident especially when the muscles of the whole body are scanned with magnetic resonance imaging (MRI). The affected muscles are often separated clearly from unaffected muscles (3). Moreover, peripheral blood of PM patients in clinical remission still contained expanding CD8+ cytotoxic T cell clones (4,5). These clones appeared autoreactive since they were found in the inflamed muscles prior to the remission induction (5).

In order to gain mechanistic insights into the pathology of PM, we have established a murine PM model, skeletal muscle C protein–induced myositis (CIM) (6). CIM can be induced by single immunization with recombinant mouse or human skeletal muscle C protein fragments in Freund's complete adjuvant (CFA). Our previous studies demonstrated that CIM is primarily mediated by CD8+ CTLs (6,7). This is in sharp contrast with the classic myosin-induced experimental autoimmune myositis model, which is driven by CD4+ T cells and humoral immunity (8,9). While experimental autoimmune myositis is induced in SJL/J mice carrying a mutated dysferlin gene that causes spontaneous muscle necrosis and secondary inflammation (10), CIM can be induced in C57BL/6 (B6) and other strains of mice.

As with most other models of autoimmune disease that are inducible by immunization with autoantigens, CIM resolves spontaneously. The myositis peaks 2 to 3 weeks after the immunization with C protein fragments and regresses in 10 weeks (6,11). Since muscle fibers regenerate rapidly, no histologic abnormality is observed after the resolution of disease.

Spontaneous regression of inducible autoimmune disease models has been intensively investigated because the results might grant insight into the development of new treatment modalities. For example, disease regression in experimental autoimmune encephalomyelitis (EAE; a model of multiple sclerosis) in rats induced by immunization with myelin basic protein is associated with the appearance of immunosuppressive cytokines including transforming growth factor β and interleukin-4 (IL-4) (12). Recently, accumulating evidence suggests that naturally arising CD25+CD4+ Treg cells actively act to maintain peripheral self tolerance (13). CD25+ cell–depleted mice had significantly more severe diseases in murine models of multiple sclerosis, rheumatoid arthritis (RA), myasthenia gravis, and Hashimoto thyroiditis (14–18).

In the present studies, extensive investigation was directed at addressing the mechanistic processes involved in spontaneous regression of CIM. The results show that attenuated activity of autoaggressive T cells was not exclusively responsible for the spontaneous regression of CIM. Instead, attenuation of innate immunity in the muscles also contributed to disease regression. These facts led us to propose a "seed and soil" theory of autoimmune tissue damage, in which autoaggressive T cells and activation of innate immunity act as seed and soil, respectively. Combination therapies that address pathomechanisms of both aspects involved in autoimmune disease damage may be an effective approach to explore in the treatment of disease.

MATERIALS AND METHODS

Mice. B6 mice were purchased from Charles River 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.

CIM induction and recurrence. To induce CIM, female B6 mice ages 8–10 weeks were immunized intradermally with 200 μl of an emulsion consisting of 200 μg of murine C protein fragments emulsified in CFA containing 100 μg of heat-killed *Mycobacterium butyricum* (Difco) (11). The immunogens were injected at the hind leg footpads and tail bases. At the same time, 0.2–2 μg of pertussis toxin (Seikagaku Kogyo) in phosphate buffered saline (PBS) was injected intraperitoneally. Some mice were subjected to additional intradermal injections at the front paws of 100 μl of an emulsion consisting of PBS/CFA or PBS/Freund's incomplete adjuvant (IFA). In a modified protocol, mice were immunized intradermally with 200 μl of the C protein fragment/CFA emulsion only at the left hind leg footpads and tail bases together with intraperitoneal injection of 0.2–2 μg of pertussis toxin. These mice were treated after 42 days with an intradermal injection of 100 μl of a C protein fragment/CFA emulsion, a CFA emulsion containing PBS vehicle, a C protein fragment/CFA emulsion containing 1 mg of polymyxin B (Sigma-Aldrich), an IFA emulsion containing PBS vehicle, an IFA emulsion containing 100 μg of poly(I-C) sodium salt (Sigma-Aldrich), or an IFA emulsion containing 100 μg of lipopolysaccharide (LPS; Sigma-Aldrich) at the contralateral hind leg foot pads and tail bases.

Hematoxylin and eosin–stained 10-μm sections of the hamstrings and quadriceps and the brachial triceps were examined histologically for the presence of mononuclear cell infiltration and degeneration of muscle fibers. Histologic severity of myositis in each muscle block was graded as follows (6): grade 1 = involvement of a single muscle fiber or <5 muscle fibers; grade 2 = a lesion involving 5–30 muscle fibers; grade 3 = a lesion involving a muscle fasciculus; grade 4 = diffuse, extensive lesions. When multiple lesions with the same grade were found in a single block, 0.5 points was added to the grade. The stained sections were evaluated by 2 independent observers (NO and TS), who reported comparable results.

Cell proliferation assay and interferon-γ (IFNγ) detection in culture supernatants. Bone marrow cells from B6 mice were treated with granulocyte–macrophage colony-

stimulating factor to prepare bone marrow–derived dendritic cells (BMDCs) (19). More than 70% of the treated cells were positive for CD11c staining. Lymph node (LN) cells were prepared from the inguinal and popliteal LNs from the mice with CIM 21 days after the immunization. One hundred thousand LN cells and 1×10^4 C protein fragment–pulsed or untreated mature BMDCs were cultured for 3 days. Proliferation was evaluated with incorporation of ^{3}H-thymidine during the last 8 hours of the incubation. The culture supernatants were examined for the concentration of IFNγ with an enzyme-linked immunosorbent assay (ELISA) kit (Mouse IFN-gamma DuoSet; R&D Systems).

Adoptive transfer of CIM. LN cells were prepared from the inguinal and popliteal LNs of the mice with CIM 21 days after the immunization. Three million LN cells and 1.5×10^6 C protein fragment–pulsed mature BMDCs were cultured with 100 IU/ml of recombinant human IL-2 (Shionogi Pharmaceuticals) for 3 days. Eight million LN cells were adoptively transferred to naive mice that were treated simultaneously with subcutaneous hind leg footpad injection of 50 μl of CFA emulsion, IFA emulsion, or IFA emulsion containing 100 μg of poly(I-C), LPS, or CpG-containing oligonucleotide (CpG ODN) 1826 (InvivoGen). Their muscles were examined histologically after 14 days.

Immunohistochemical analysis. Cryostat frozen sections (6 μm) fixed in cold acetone were stained with anti-CD68 monoclonal antibodies (mAb) (FA-11; AbD Serotec). Nonspecific staining was blocked with 4% Blockace (DS Pharma Biomedical). Bound antibodies were visualized with peroxidase-labeled anti-rat IgG antibodies and associated substrates (Histofine Simple Stain Max PO; Nichirei Biosciences). Isotype controls were used as a negative control.

Antiinflammatory cytokine treatment. Hamster/mouse chimeric anti-murine IL-1 receptor (IL-1R) IgG1 mAb (M147) (20), rat/mouse chimeric anti–tumor necrosis factor α (anti-TNFα) IgG2a mAb (cV1q) (21), and rat anti–IL-6R IgG1 mAb (MR16-1) (22) were provided by Amgen, Centocor, and Chugai Pharmaceutical, respectively. Bovine serum albumin (Sigma-Aldrich) or rat antidinitrophenol IgG1 mAb (KH-5; Chugai Pharmaceutical) was used as a control.

Statistical analysis. Histologic scores were analyzed statistically using the Mann-Whitney U test.

RESULTS

No C protein–specific tolerance induction after spontaneous regression of CIM. As is the case in most inducible models of autoimmune disease, our model of CIM regresses spontaneously (6,11). To study whether immunologic tolerance to C protein fragments had been established after the regression, we rechallenged mice with CIM with C protein fragments emulsified in CFA. First, B6 mice were immunized with a C protein fragment/CFA emulsion at only the left hind leg footpads and tail bases with intraperitoneal injections of pertussis toxin. This treatment resulted in myositis development in the muscles of the ipsilateral hind legs 3 weeks after initial immunization. Then, after disease regression, these mice were reimmunized with the same antigen/CFA emulsion in the footpads and tail bases. Contralateral legs were used for repeat immunization due to skin damage in previously immunized ipsilateral legs. IFA alone was injected as a vehicle control. Histologic evaluation of the muscles of the contralateral hind legs 14 days after the repeat C protein fragment/CFA immunization revealed that repeat immunization with C protein fragment/CFA had reinduced the myositis, while control IFA treatment had not (Figure 1A). These results showed that tolerance to the C protein fragment was not established following disease regression.

Notably, CFA injection without C protein fragments, but not IFA, at hind leg footpads and tail bases induced myositis after disease regression. Myositis reinduced with CFA alone was less severe than that reinduced with C protein fragment/CFA reimmunization, suggesting that attenuated activity of the autoaggressive T cells may also partly account for disease regression. As a control, CFA alone was used in the first and second treatments, and we found no inflammation that damaged muscles (Figure 1A).

To study the mechanism of CFA-induced recurrence more carefully, we tried to inhibit the recurrence by adding polymyxin B, an inhibitor of LPS (a Toll-like receptor 4 [TLR-4] ligand), in CFA emulsion. Polymyxin B partially inhibited myositis recurrence (Figure 1B). The partial inhibition was likely attributable to activators of innate immunity other than LPS contained within CFA (23). The effect of TLR ligands was examined directly with injection of LPS- and poly(I-C) (a TLR-3 ligand)–containing emulsions without C protein fragments as the second treatment. Injection of these TLR ligands induced recurrence of myositis (Figure 1B). Thus, redevelopment of myositis requires activation of local innate immunity, but not necessarily T cell reactivation.

Requirement of local CFA treatment for the development of myositis. Previously, we immunized mice at their hind leg footpads and tail bases and examined their femoral muscles for histologic changes. The results of the reimmunization experiments prompted us to examine the brachial muscles of the immunized animals. We found that they did not develop myositis at the brachial muscles (Table 1). We then injected CFA at the right front paws and IFA at the left front paws in mice at the same time that they were immunized in the conventional way to induce CIM. These mice developed myositis only in the CFA-treated forelegs and hind legs. The brachial muscles of the IFA-treated forelegs had no myositis (Table 1). No myositis was observed in the

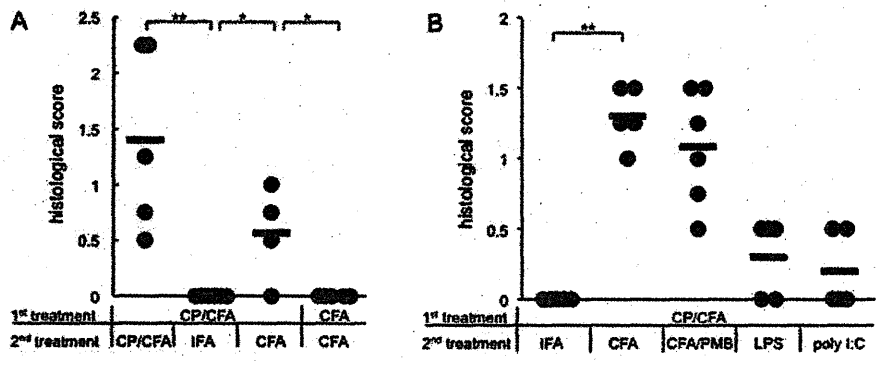

Figure 1. No establishment of tolerance to C protein fragments (CP) after disease regression. **A**, C57BL/6 mice were immunized with C protein fragments/Freund's complete adjuvant (CFA) emulsion only at the footpads of left hind legs and tail bases (first treatment: CP/CFA). Treatment with C protein fragments/CFA resulted in myositis development in the muscles of the ipsilateral legs followed by spontaneous regression that was confirmed 42 days after the initial immunization. After the myositis regression, these mice were reimmunized with C protein fragments/CFA or treated with Freund's incomplete adjuvant (IFA) or CFA emulsion (second treatment). Another group of mice was treated with CFA alone twice (first and second treatment: CFA). **B**, In a separate experiment, the same C protein fragment/CFA–immunized mice were treated afterward with CFA, CFA together with polymyxin B (PMB), lipopolysaccharide (LPS), or poly(I-C) emulsions at the footpads of the contralateral hind legs. IFA was injected as a vehicle control. Histologic evaluation of the muscles of the right legs was performed 14 days after the second treatment. Horizontal bars indicate the mean. * = $P < 0.05$; ** = $P < 0.01$.

forelegs of naive mice treated in the same way. These findings reinforced the fact that effector T cell development and local activation of innate immunity are both critical for myositis development.

Requirement of local activation of innate immunity for adoptive transfer of CIM. To explore the mechanism of effector T cell activation (a component of acquired immunity) in muscle cells engaged in local innate immunity, we used an adoptive transfer CIM model (7). Prior to the transfer to naive mice, the LN cells from mice with CIM were cocultured for 5 days with C protein fragment–pulsed BMDCs together with recombinant IL-2. Upon coculture with C protein fragment–pulsed BMDCs, LN cells showed enhanced proliferation and IFNγ induction compared with LN cells cocultured with untreated BMDCs (Figure 2A). Thus, transferred LN cells contained T cells reactive specifically to C protein fragments. Because we had confirmed that intradermal injection of CFA at leg footpads did not cause myositis (Figure 2B), we injected CFA intradermally at the right hind leg footpads and IFA intradermally at the left hind leg footpads simultaneously upon transfer of LN cells. Two weeks later, we examined the muscles of the hind legs histologically and found myositis only in the hind legs that were injected with CFA, but not in the hind legs that were injected with IFA (Figure 2B).

As in the aforementioned studies on CFA-induced recurrence, TLR ligands, including poly(I-C), LPS, and CpG ODN, successfully primed muscle tissues as well as CFA and facilitated adoptive transfer of myositis (Figure 2C). Thus, it was confirmed that not only effector T cells, but also conditioning of the muscle tissues via activation of innate immunity, is essential for the development of myositis.

Local activation of innate immunity as a therapeutic target in CIM. Activation of local innate immunity with CFA or other TLR ligands was crucial to

Table 1. Requirement of local CFA treatment for myositis induction*

Foreleg status, muscle, footpad treatment	Histologic score, mean ± SD
Untreated	
Femoral	
CP/CFA	1.88 ± 0.75
Brachial	
None	0
Treated	
Femoral	
CP/CFA	1.00 ± 0.42
Brachial	
CFA	0.83 ± 1.03
IFA	0

* Four mice that were treated to induce C protein–induced myositis (CIM) at the hind leg footpads and tail bases (foreleg untreated) did not develop myositis of the brachial muscles. In 6 mice, at the same time as treatment to induce CIM, Freund's complete adjuvant (CFA) and Freund's incomplete adjuvant (IFA) emulsions were injected additionally into the right and left footpads, respectively, of forelegs (foreleg treated), and myositis developed in the brachial muscles of the CFA-treated legs. Myositis of the femoral and the right/left brachial muscles was histologically assessed 21 days after the immunization. CP = C protein fragments.

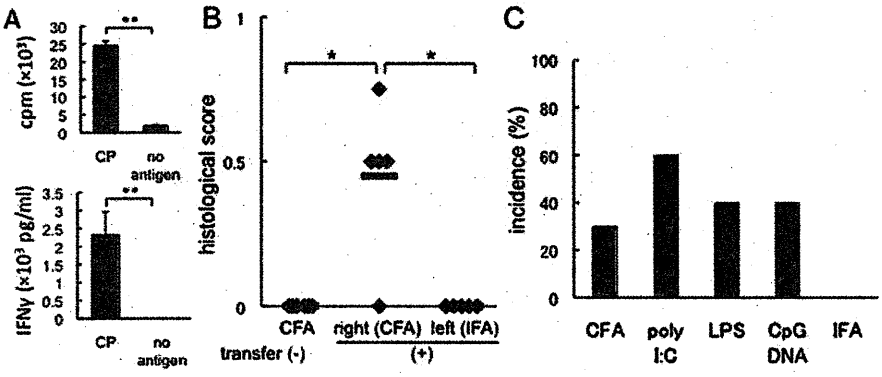

Figure 2. Selective transfer of myositis to CFA/Toll-like receptor (TLR) ligand–treated legs. **A,** Lymph node (LN) cells from mice with C protein–induced myositis (CIM) were stimulated with C protein fragment–pulsed mature bone marrow–derived dendritic cells (BMDCs) or untreated mature BMDCs (no antigen) for 3 days. Their proliferation was determined by ^{3}H-thymidine incorporation (top). Interferon-γ (IFNγ) in the culture supernatants was quantified by enzyme-linked immunosorbent assay (bottom). Values are the mean ± SD of 3 independent experiments. ** = $P < 0.01$. **B,** LN cells from mice with CIM stimulated with interleukin-2 (IL-2) and C protein fragment–pulsed mature BMDCs were transferred to 5 naive mice with their right hind leg footpads treated with CFA and their left hind leg footpads treated with IFA. Six naive mice were treated with CFA on their hind leg footpads without adoptive transfer. Myositis of the bilateral femoral muscles was histologically assessed 14 days after the transfer. Horizontal bars indicate the mean. * = $P < 0.05$. **C,** Upon adoptive transfer of LN cells from mice with CIM stimulated with IL-2 and C protein fragment–pulsed mature BMDCs, the legs of the recipient mice were treated with the TLR ligands poly(I-C), LPS, and CpG-containing oligonucleotide (CpG ODN; CpG DNA). CFA and IFA were included as positive and negative controls, respectively. The incidence of myositis resulting from the transfer is shown. Each group included 5 mice. See Figure 1 for other definitions.

facilitate autoaggressive T cell attack of muscle fibers. To elucidate the distal effects of CFA treatment, muscles were histologically examined 14 days after CFA treatment. In comparison with normal muscles, muscle tissue 14 days after CFA treatment contained more mononuclear cells but showed no signs of damage. Pathologic scores remained grade 0 since no muscle damage was observed. Mononuclear cells were positive for CD68 (Figure 3A), but not for CD4, CD8, or B220 (data not shown). Thus, macrophages were recruited into the muscles of the CFA-treated limbs without tissue damage.

Triggering of macrophage TLRs induces production of type I IFNs and inflammatory cytokines including IL-1, TNFα, and IL-6 (24). Since a separate experiment showed that IFNα/β/ω receptor 1–null B6 mice, lacking type I IFN receptors, were as susceptible to CIM induction as wild-type mice (data not shown), it is unlikely that macrophage type I IFN contributed to autoaggressive T cell infiltration into muscle tissue. We thus assumed that alternative cytokines, including IL-1, TNFα, and IL-6, were crucial to recruit autoaggressive T cells.

To study the role of IL-1, TNFα, and IL-6, anti–IL-1R, anti-TNFα, and anti–IL-6R mAb were employed to block individual cytokines in the CIM adoptive transfer model. Intraperitoneal administration of these reagents was initiated when activated LN cells from mice with CIM were transferred to naive mice that were treated simultaneously with CFA at the hind leg footpads. Anti–IL-1R, anti-TNFα, and anti–IL-6R mAb were administered until 14 days following transfer of LN cells from mice with CIM, when muscles of the recipient mice were histologically evaluated. Blockade of both IL-1R and TNFα suppressed the transfer, while anti–IL-6R mAb, a dose of which suppressed development of conventional CIM (11), failed to suppress induction of myositis (Figure 3B). Thus, IL-1 and TNFα from macrophages may be responsible for the conditioning of muscle tissues that mediates attack by autoaggressive T cells.

DISCUSSION

By investigating spontaneous regression of mononuclear cell infiltration and muscle injury in CIM, we found that activation of autoaggressive effector T cells and that of innate immunity at the target tissues are required for autoimmune tissue injury. TNFα and IL-1 are essential in the activation of innate immunity. Presumably, local macrophages are the source of TNFα and IL-1 in muscles that are activated by CFA. CFA may have dual roles since it should provoke systemic T cell responses specific to C protein fragments by activating

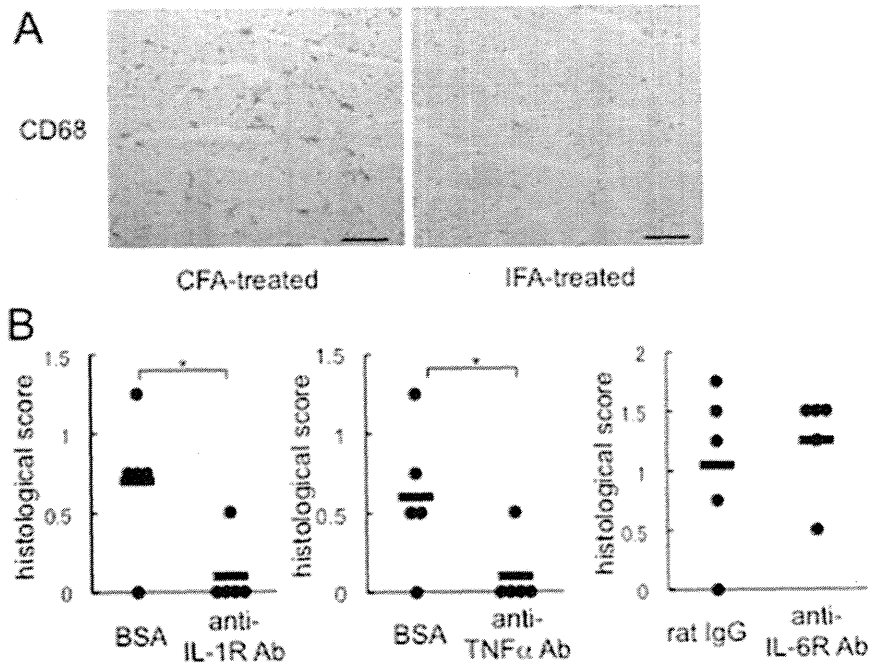

Figure 3. Effect of blockade of inflammatory cytokines on adoptive transfer of C protein–induced myositis (CIM). **A,** Femoral muscles from mice treated with intradermal CFA injection at the footpads and from mice treated in the same way with IFA were stained immunohistochemically with anti-CD68 antibodies. Although CD68+ macrophages were increased in CFA-treated mice, no muscle damage was observed. Bars = 50 μm. **B,** Activated lymph node cells from mice with CIM were transferred to naive recipient mice that were treated with CFA at the hind leg footpads to induce myositis. At the same time, the recipient mice were subjected to intraperitoneal injection of 100 μg of anti–interleukin-1 receptor (anti–IL-1R) antibodies (Ab), which was followed by repeat administration every 3 days; 100 μg of anti–tumor necrosis factor α (anti-TNFα) antibodies, which was followed by repeat administration 3 times a week; or 4 mg of anti–IL-6R antibodies, which was followed by 0.3-mg injections twice a week. Bovine serum albumin (BSA) or rat IgG was used as a control. The proximal muscles were examined for histologic scoring 14 days after transfer. Each group included 5 mice. Horizontal bars indicate the mean. * = $P < 0.05$. See Figure 1 for other definitions.

DCs. Once T cell responses against muscles are established, local injection of CFA and TLR ligands at the footpads can cause myositis to recur in legs otherwise free from myositis. The effect of CFA in activating local innate immunity appeared more potent than individual TLR ligands and could not be inhibited fully by a TLR-4 inhibitor. This is most likely because CFA contains activators of innate immunity other than TLR-4 ligands (23,25–27). Thus, CIM depends on systemic T cell autoimmunity as well as activation of innate immunity, especially that of TLR signaling in the muscles.

In CIM, TNFα and IL-1 production from macrophages stimulated with CFA or TLR ligands is a key event to activate innate immunity. Activation of innate immunity per se is insufficient for development of myositis, because single and repeat CFA injections increased local macrophages in number while not inducing muscle damage. The other essential event is activation of C protein fragment–reactive T cells. In contrast to LN cells from mice with CIM cocultured with C protein fragment–pulsed BMDCs, LN cells cocultured with untreated BMDCs did not proliferate or produce IFNγ. Moreover, they failed to transfer myositis to a naive recipient mouse with footpad CFA injection (data not shown). Histologic scores were set at zero to indicate no muscle damage. Involvement of activated T cells is crucial for muscle damage; therefore, histologic scores greater than zero suggested that CIM and its recurrence were mediated by C protein fragment–reactive T cells inducing muscle damage.

These observations echo the "seed and soil" model, which had been proposed in metastatic processes of tumor cells. Metastasis depends on tumor cells liberated from tumor mass as seeds and also depends on target tissues as soil that accepts the tumor cells readily for their local growth. Analogously, autoreactive CD8+

T cells act as seeds while muscle tissues act as soil. Requirement of the 2 factors was demonstrated for the first time in animal models of autoimmunity.

Molecular events of the "soil" activation in muscle tissues in CIM may include up-regulation of adhesion molecules on endothelial cells, chemokine release from macrophages, and costimulatory molecule expression by myofibers, which can be promoted by TNFα and/or IL-1 (28). To address this point, we used immunohistochemistry to study the expression of intercellular adhesion molecule 1 and class I major histocompatibility complex in the muscles from the CFA- and IFA-treated legs, and we found no difference in their expression levels (data not shown). Although more intensive studies will be required to draw definitive conclusions, other molecules should be of importance for recruitment of autoaggressive T cells.

It was reported previously that collagen-induced arthritis, a murine model of RA, could be reactivated by oral administration of LPS (29). Since the reactivation accompanied increased levels of antibodies against the immunizing antigen (type II collagen), the authors concluded that B cell activation by LPS might be responsible for the reactivation. EAE was also reactivated by intravenous injection of LPS (30). It appeared to be mediated by proliferation and cytokine production in a fraction of effector/memory CD4+ T cells. While these studies implied that systemic delivery of LPS stimulated pathogenic lymphocytes in the systemic pool, our studies revealed the contribution of local innate immunity activated by LPS.

It is still an open question whether the same "seed and soil" model applies to human PM. Apparently, CFA is not involved in PM. Since the recurrent myositis and the T cell adoptive transfer in the present studies showed that TLR ligands were sufficient to prime the muscles, stimulation by endogenous TLR ligands might account for production of IL-1 and TNFα, both of which were reportedly expressed by infiltrating cells in muscles in PM/dermatomyositis (DM) and CIM (6,11,31). Alternatively, aberrant production of inflammatory cytokines and chemokines might be responsible for attraction of autoaggressive T cells. It is known that muscle fibers can produce these cytokines under physiologic conditions, especially during exercise (32–35). Etiologic studies revealed heavy muscular exertion as a risk factor for development of PM/DM (36). As was reviewed earlier, MRI scans typically show that some muscle fascicles are affected and that others remain unaffected (3). Cytotoxic CD8+ T cells, which appeared specific to the muscles, were present for a long time after successful treatment of the disease (4). These facts could be explained by the 2 requirements: activation of effector T cells and that of the muscle tissues.

Experiments of repeat antigen/CFA immunization and CFA treatment have demonstrated that tolerance was not induced during spontaneous regression of disease and that activation of local innate immunity alone can cause recurrence of myositis. We also noted that reimmunization of repeat antigen/CFA provoked more severe myositis than CFA treatment alone. Thus, the T cell activation level following regression must have waned, which could also contribute to the disease regression. In this regard, contribution of Treg cells was studied with antibody-mediated CD25+ T cell depletion, which did not alter the disease course of CIM (data not shown). Genetic absence of IL-10, which is one of the key effector molecules of Treg cells, did not interfere with the regression (data not shown). It was thus suggested that no active suppression of autoreactive T cells operates in the regression phase.

Waning T cell activation might partially account for the fact that myositis was modest in the transfer model, which was assessed histologically 2 weeks after the T cell transfer. Incidence of myositis transfer varied among experiments (25–80%), which might depend on the frequency and activation levels of C protein fragment–specific T cells. We had to compare the severity and incidence of transferred myositis in the same set of experiments. Nonetheless, the adoptive transfer model has enhanced the value of CIM since it allows us to evaluate the effector phase of myositis. Together with conventional CIM, we can dissect the pathologic processes involved in the induction and effector phases of myositis. In this regard, IL-6 inhibition was effective for treating conventional CIM (11), while IL-6 inhibition in the recipient animals did not prevent adoptive transfer of CIM. It is likely that IL-6 plays a more prominent role in the induction phase of myositis. Of special interest are interventions targeting the myositis effector phase, since we always have to treat patients after disease has been established.

At the moment, histologic evaluation is the only reliable way to assess severity of rodent models of PM, a problem shared by all murine myositis models. Serum levels of creatinine kinase or other muscle-derived proteins are unreliable. They are often high in normal mice, presumably because of their physical activity. The rotarod test is difficult because some mice become accustomed to it and avoid falling off. Other mice are willing to drop off from the device. We believe that we need

devices that directly quantify muscle strength of rodents to follow clinical disease course.

Glucocorticoids, which are a first-line medication in the current therapeutic approaches to PM, should suppress activation of T cells as well as innate immune cells. They are effective, but have quite a few side effects. On the other hand, most small-molecule immunosuppressants target primarily lymphocyte activation. The results of the present study suggest that combinatorial approaches that address activation of T cells and innate immunity could be optimal for treating autoimmune myositis and suggest that glucocorticoids could become dispensable if activation of innate immunity in the muscles is suppressed by alternative treatment. This implication appears interesting for designing future clinical trials to treat 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. Okiyama, Yokozeki, Miyasaka, Kohsaka.

Acquisition of data. Okiyama, Sugihara, Oida, Ohata.

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

ADDITIONAL DISCLOSURE

Author Ohata is an employee of Chugai Pharmaceutical.

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ORIGINAL ARTICLE

TLR3-mediated apoptosis and activation of phosphorylated Akt in the salivary gland epithelial cells of primary Sjögren's syndrome patients

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Abstract This study aimed at ascertain whether innate immunity is involved in the apoptosis of primary cultured salivary gland epithelial cells (SGECs) in primary Sjögren's syndrome (pSS). Induction of apoptosis of SGECs was performed using a TLR3 ligand, poly (I:C). Activation of phosphorylated-Akt (pAkt) and cleaved-caspase 3 was determined by Western blotting or immunofluorescence. Expression of TLR2 and TLR3 with pAkt was observed in cultured SGECs after 24-h stimulation with each ligand. Compared with stimulation with the peptidoglycan or lipopolysaccharide, that with poly (I:C) induced significant nuclear fragmentation, as determined by Hoechst staining ($p = 0.0098$). Apoptosis was confirmed by terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) staining of SGECs from pSS patients and a normal subject. A significant increase in TUNEL-positive cells was observed by the addition of a PI3K inhibitor, LY294002. Poly (I:C) phosphorylated stress-activated protein kinase/Jun-terminal kinase and p44/42 MAP kinase as well as Akt. Furthermore, poly (I:C)-induced caspase 3 cleavage in SGECs was also inhibited by LY294002. Similar results were obtained using SGECs obtained from a normal subject. The results demonstrated for the first time that TLR3 induces the apoptotic cell death of SGECs via the PI3K-Akt signaling pathway.

Keywords Sjögren's syndrome · TLR3 · Akt · MAP kinase · Caspase3

Abbreviations

IFN	Interferon
IRF3	Interferon (IFN) regulatory factor 3
FITC	Fluorescein isothiocyanate
LPS	Lipopolysaccharide
LSG	Labial salivary gland
MAP	Mitogen-activated protein
PBS	Phosphate-buffered saline
PGN	Peptidoglycan
PI3K	Phosphatidylinositol 3-kinase
pSS	Primary Sjögren's syndrome
SGECs	Salivary gland epithelial cells
TRIF	TIR domain-containing adaptor-inducing IFNβ
TRITC	Tetramethyl rhodamine isothiocyanate
TUNEL	Terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling

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Introduction

Toll-like receptors (TLRs) are known as intermediation receptors involved in innate immunity [1]. Some TLRs are signaling adaptor molecules that are stimulated by bacterial or viral nucleic acid sequences [2, 3]. We previously reported the expression of TLR2, 3, and 4 in labial salivary glands (LSGs) obtained from patients with primary Sjögren's syndrome (pSS) [4] showing sicca symptoms due to salivary gland destruction [5, 6]. These three types of TLRs