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ORIGINAL ARTICLE

Expression of TLR2, TLR4, and TLR9 in dermatomyositis and polymyositis

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Abstract The aim of this study was to investigate the expressions of Toll-like receptor (TLR) 2, TLR4, TLR9, and their correlations with the expression of cytokines that are associated with activation of $CD4^+$ T cells and inflammation including interferon γ (IFNγ), interleukin 4 (IL4), interleukin 17 (IL17), and tumor necrosis factor α (TNFα) in muscle tissues of patients with dermatomyositis (DM) and polymyositis (PM). The expressions of TLR2, TLR4, TLR9, IFNγ, IL4, IL17, and TNFα were measured by real-time reverse transcription–polymerase chain reaction in muscle tissues from 14 patients with DM and PM (nine patients with DM, five patients with PM) and three controls. The expressions of TLR2, TLR4, and TLR9 were also localized with immunohistochemistry. The expression levels of TLR2, TLR4, TLR9, IFNγ, IL4, IL17, and TNFα were significantly high in patients with DM and PM compared with those in the controls, and the expression levels of TLR4 and TLR9 had significant positive correlations with the expressions of IFNγ, IL4, IL17, and TNFα. Immunohistochemistry showed that TLR2, TLR4, and TLR9 were expressed by infiltrating cells of perimysium in DM, whereas they were expressed by infiltrating cells of endomysium in PM. These results suggest that the involvement of TLR4 and TLR9 in immunopathogenesis of DM and PM might be connected with activation of $CD4^+$ T cells.

Keywords Dermatomyositis · Polymyositis · Toll-like receptors

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Introduction

Dermatomyositis (DM) and polymyositis (PM) are chronic muscle disorders characterized by inflammatory infiltrate in the muscle tissue. DM and PM are clinically characterized by features of symmetric proximal muscle weakness associated with muscle cell destruction [1]. Previous studies have identified different proportions of $CD4^+$, $CD8^+$ T cells and macrophages with various localizations in DM and PM. In PM, cytotoxic $CD8^+$ T cells and macrophages were detected surrounding and invading non-necrotic muscle fibers expressing major histocompatibility complex (MHC) class I [2]. In DM, perivascular infiltrates composed mainly of $CD4^+$ T cells, B cells, and macrophages were observed [3].

The cytokine-driven differentiation of distinct lineages of effector and regulatory T cells (Tregs) from naive $CD4^+$ T cell precursors is a hallmark of the adaptive immune system. T helper (Th) 1 and Th2 are the best understood effector $CD4^+$ T cells generated during immune responses, with each subset characterized by distinct transcription factor activity and cytokine-secreting phenotype. Classically, Th1 cells produce interferon (IFN) γ and mediate immune responses against intracellular bacteria, viruses, and tumor cells through the activation of macrophages and cytotoxic T cells. Th2 cells make mostly interleukin 4 (IL4), which stimulate humoral responses and are thought to have evolved to enhance resistance against extracellular parasites [4, 5]. In recent years, a distinct T-cell subset, termed Th17 cells, has also been identified and seems to play key roles in the activation of neutrophils and immunity to bacteria, particularly at mucosal surfaces. IL17, also termed IL17A, is the signature cytokine of Th17 cells [6].

Recently reported data suggest that $CD4^+$ T cells play roles in immunopathogenesis of DM and PM [7]. Identification of Th1 and Th17 cytokine (IFNγ and IL17) producing cells from muscle tissue of DM and PM contributes to the roles of $CD4^+$ T cells in DM and PM [8].

Cytokines are potent mediators of a number of cell functions and are essential in coordinating inflammatory responses. They can be produced by a large variety of cells and exhibit pro-inflammatory as well as anti-inflammatory effects. Their key role in chronic inflammatory diseases has been well documented by the often strikingly good response to therapies targeting proinflammatory cytokines, one of the best examples being tumor necrosis factor (TNF) α blockade in patients with rheumatoid arthritis and Crohn's disease. Recent findings suggest cytokines as important key molecules in the pathogenic mechanisms of idiopathic inflammatory myopathies (IIMs), myositis [9].

Toll-like receptors (TLRs) are primarily involved in the innate immune response to microbial pathogens through the recognition of conserved pathogen-associated molecular patterns. However, they also contribute to sterile inflammation by sensing "danger signals", the endogenous molecules that are generated during tissue damage or inflammation [10–13]. The activation of TLRs is an important bridge between innate and adaptive immunity by regulating the expression of co-stimulatory molecules on antigen-presenting cells that drive T-cell activation and by creating a cytokine milieu in which the differentiation of T cells into the desired subsets occurs [14]. It was known that activation of TLR4 and TLR9 is generally to induce a Th1 response in dendritic cells (DCs), and TLR2 might induce Th2-based immune response in experimental asthma, and activation of TLR4 also induces Th17 response through IL17 production [14–21].

The aim of this study was to investigate the expression of TLR2, TLR4, TLR9, and cytokines that are related to activation of $CD4^+$ T cells (Th1, Th2, and Th17 cells) and inflammations including IFNγ, IL4, IL17, and TNFα, and to examine the connection of TLRs to these cytokines in muscle tissues of patients with DM and PM.

Here, we demonstrate that the TLR4 and TLR9 expressions are significantly increased, and they are connected with the expressions of those cytokines in muscle tissues of patients with DM and PM.

Patients and methods

Patients

Muscle biopsy samples from patients newly diagnosed with active DM (n=9) or PM (n=5), according to the classification system of Bohan and Peter [22, 23], were evaluated in this study. Seven patients were women and seven were men. The time from symptom onset to diagnosis was 1–12 months, and they had not been treated. The mean age at diagnosis was 41 years (41±14 years). Patient data are presented in Table 1. Muscle tissue sections from three patients with non-specific muscle manifestations but normal histological findings were used as the controls. All patients and controls gave their informed consent, and the local ethics committee at Pusan National University Hospital approved the study.

Laboratory assessments

Serum levels of C-reactive protein (CRP), the erythrocyte sedimentation rate (ESR), and serum aldolase and creatinine kinase (CK) levels were analyzed at the Department of Clinical Chemistry, Pusan National University Hospital.

Muscle biopsies

Biopsy samples were obtained from the vastus lateralis or deltoid muscle with a "semi-open" muscle biopsy technique, with the patient under local anesthesia. The biopsy specimens were immediately frozen in dry ice and isopentane and stored at −70°C until analysis.

Real-time RT–PCR

After biopsy of the muscle tissues from patients with PM or DM, these were immersed immediately in liquid nitrogen. Total RNA was extracted from the frozen muscle biopsies with TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). Each solution containing 1 μg of RNA was heated at 65°C for 15 min, and a mixture containing

Table 1 Clinical and laboratory characteristics and the expression of TLR2, TLR4, TLR9, IFNγ, IL4, IL17, and TNFα in 14 patients with DM and PM

Patient/ diagnosis	Sex	Age	Symptom duration, months	CK (U/l)	Aldolase (U/l)	ESR (mm/h)	CRP (mg/dl)	Relative mRNA expression levels compared to controls[a]						
								TLR2	TLR4	TLR9	IFNγ	IL4	IL17	TNFα
1/DM	F	66	1	502	10.1	85	3.41	16.4	48.4	11.2	587	9.4	40	9.5
2/DM	M	36	3	2,815	46.1	75	0.9	18.6	140	15.1	1,430	23.3	68	27
3/DM	M	39	2	1,163	7.1	85	0.5	25.1	164	5.6	278	4.6	18	3.7
4/DM	M	37	12	70	5.7	78	0.5	15.1	106	11.6	484	6.7	27	13.9
5/DM	M	58	1	3,006	8.4	13	0.5	48.1	60.8	4.6	273	1.8	9	4.8
6/DM	M	37	2	14,139	36.7	73	2.8	57.1	81	6.0	462	5.3	19	21.7
7/DM	M	55	1	3,034	21.9	24	2.3	13.1	153	37.2	885	15.3	62	17.1
8/DM	F	30	6	6,000	54.3	14	0.1	69.8	232	26.3	2,760	23.7	100	20.2
9/DM	F	18	3	668	16.5	52	0.1	28.7	297	34.4	3,140	71.0	122	82.5
10/PM	F	47	2	10,994	109.5	37	0.4	18.6	169	14.8	674	15.9	75	31.3
11/PM	M	22	3	32	6.9	26	1.3	16.1	149	10.0	382	4.7	22	8.2
12/PM	F	59	1	2,207	44	108	1.8	50.3	228	35.2	1,830	22.6	139	23.5
13/PM	F	33	7	2,743	64	50	1.0	39.5	444	44.0	2,180	47.1	218	50.5
14/PM	F	48	1	1,820	44.8	19	0.6	35.9	108	5.2	334	3.4	11	3.2
DM/PM (9/5)	M/F (7/7)	41± 14	3.2±3.1	3,510± 4,180	34±30	53± 31	1.2± 1.0	32.3± 18.1	170.0± 104.8	18.6± 13.8	1,121.3± 978.9	18.1± 19.5	66.4± 60.6	22.7± 21.5

[a] The mRNA expressions were showed by relative levels compared to controls. Muscle tissue sections from three patients with non-specific muscle manifestations but normal histological findings were used as the controls

reverse transcriptase was added to the solution. cDNA was transcribed with reaction cycles of 25°C for 10 min, 42°C for 60 min, 99°C for 5 min, and 4°C for 5 min. A First Strand cDNA Synthesis Kit for reverse transcription–polymerase chain reaction (RT–PCR; AMV, Roche Applied Science, Indianapolis, IN, USA) was used for the reactions described above, and real-time PCR was performed in a LightCycler System Instrument (Roche Applied Science). LightCycler-DNA Master SYBR Green I (Roche Applied Science), the cDNA template, each primer, and 25 mM of $MgCl_2$ were added to microcapillary tubes to a final volume of 20 μl. The PCR cycling parameters were 50 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 5 s, and primer extension at 72°C for 20 s.

The primers 5′-GCCACCATTTCCACGGACT-3′ (sense) and 5′-GGCTTCCTCTTGGCCTGG-3′ (antisense) were used to amplify TLR2, 5′-CTCTGCCTTCACTACA GAGACTTT-3′ (sense) and 5′-TGTGGAAGC CTTCCTGGATG-3′ (antisense) for TLR4, 5′-ACTGAGCACCCCTGCTTCTA-3′ (sense) and 5′-AGATTAGTCACGG CAGGAA-3′ (antisense) for TLR9, 5′-TCCCATGGGTTGTGTGTTTA-3′ (sense) and 5′-AAGCA CCAGGCATGAAATCT-3′ (antisense) for IFNγ, 5′-ACCCAAACTAGGCCTCACCT-3′ (sense) and 5′-ACAGGTGGCATCTTGGAAAC-3′ (antisense) for IL4, 5′-TCTCAT CCAGCAAGAGATCC-3′ (sense) and 5′-AGTTTGGGACCCCTTTACAC-3′ (antisense) for IL17, and 5′-CAAACCACCAAGTGGAGGAG-3′ (sense) and 5′-AGATAGCAAATC GGCTGACG-3′ (antisense) for TNFα, all purchased from Bioneer (Daejun, Korea).

The threshold cycle (C_T) was determined by monitoring the fluorescent signal for each cycle, and the amounts of mRNA in the experimental groups were determined relative to those of the control group.

Immunohistochemistry for TLR2, TLR4, and TLR9

The cryotissue was fixed in 4% paraformaldehyde, and endogenous peroxidase activity was quenched with 3% H_2O_2 in methanol. Immunohistochemistry was performed using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). The tissues were incubated with the primary antibody directed against TLR2 (sc-8689), TLR4 (sc-10741), or TLR9 (sc16247) overnight at 4°C. All the primary antibodies were purchased from Santa Cruz Biotechnology, Inc. The slides were incubated with biotinylated secondary antibody solution and then treated with the avidin/biotinylated enzyme complex. The final color product was developed using diaminobenzidine chromogen (Dako, Carpinteria, CA, USA).

Statistical analysis

Differences between experimental groups were tested using the Mann–Whitney *U* test and Student's *t* test, depending

on the parameter. The data are expressed as means ± standard deviations (SD).

Results

The expression levels of TLR2, TLR4, TLR9, IFNγ, IL4, IL17, and TNFα were significantly high in patients with DM and PM compared to controls

The mRNA expression levels of TLR2 (32.3±18.1), TLR4 (170.0±104.8), TLR9 (18.6±13.8), IFNγ (1121.3±978.9), IL4 (18.1±19.5), IL17 (66.4±60.6), and TNFα (22.7±21.5) were significantly high in patients compared to those of the controls, and the levels of TLR4 and IFNγ were prominently high (Table 1). These expression levels were not significantly different between PM and DM, and any of these levels did not significantly correlate with disease duration, age, serum level of ESR, CRP, CK, and aldolase.

The expression levels of TLR4 and TLR9 had significant positive correlations with those of IFNγ, IL4, IL17, and TNFα

The expression levels of TLR2 had no significant correlations with those of IFNγ, IL4, IL17, and TNFα, whereas the levels of TLR4 and TLR9 had positive correlations with those of IFNγ (r=0.76 in TLR4 and 0.73 in TLR9, p<0.01), IL4 (r=0.8 in TLR4 and 0.76 in TLR9, p<0.01), IL17 (r=0.92 in TLR4 and 0.9 in TLR9, p<0.01), and TNFα (r=0.71 in TLR4, p<0.01; 0.66 in TLR9, p=0.01; Fig. 1).

Immunohistochemistry of TLR2, TLR4, and TLR9

TLR2, TLR4, and TLR9 were expressed by perimysial infiltrating cells in DM, whereas they were expressed by endomysial infiltrating cells in PM (Figs. 2, 3, and 4).

Discussion

Data originating predominantly from animal models of autoimmune disease and circumstantial data from human patients suggest that the inappropriate activation of the TLR pathway by endogenous or exogenous ligands may lead to the initiation and/or perpetuation of autoimmune responses and tissue injury [24]. The expression of TLRs by both immune cells and the resident cells in the involved tissues supports their important roles in tissue injury, destruction, and repair. We showed that the expression levels of TLR2, TLR4, and TLR9 were elevated in the muscle tissues of patients with DM and PM compared to controls, and as far as we know, this is the first report of this phenomenon in DM and PM.

We reported that inflammatory cytokines such as IL17, TNFα, IL1, and IL6 increase the production of TLR2, TLR4, and TLR9 from mice synoviocytes in autoimmune arthritis [25]. The possible mechanisms of TLR over-expressions are as follows: (1) various microbial pathogens considered as etiologic agents of IIMs contribute to over-expressions; (2) endogenous molecules and cytokines that are generated during muscle damage or immune response induce the overexpressions.

DM is a microangiopathy affecting the skin and muscle, in which the early activation and deposition of complement causes the lysis of endomysial capillaries and muscle ischemia. In PM, clonally expanded $CD8^+$ cytotoxic T cells invade muscle fibers that express MHC class I antigens, leading to fiber necrosis via the perforin pathway. Perimysial and perivascular inflammation with $CD4^+$ T cell, B cells, and macrophage infiltrations are histological features of DM, whereas endomysial inflammation with $CD8^+$ T-cell infiltration is a histological feature of PM [1]. We have demonstrated that TLR2, TLR4, and TLR9 are expressed by perimysial infiltrating cells in DM and by endomysial infiltrating cells in PM. These expression patterns of TLR2, TLR4, and TLR9 were consistent with the histopathological features of DM and PM.

$CD4^+$ T cells play roles in immunopathogenesis of DM and PM [7]. Detection of IFNγ and IL17 producing cells in muscle tissues of DM and PM suggests the involvement of activated $CD4^+$ T cells in the pathophysiology of both DM and PM. These data showed that non-specific amplification of muscle inflammation by T lymphocytes may result from the local production of cytokines and chemokines [8]. We have demonstrated that the expressions of cytokines that are associated with $CD4^+$ T-cell activation including IFNγ (Th1 cytokine), IL4 (Th2 cytokine), and IL17 (Th17 cytokine) were increased in patients with DM and PM compared to controls. These results imply that activation of $CD4^+$ T cells is involved in immunopathogenesis of DM and PM.

TLR signaling may promote autoimmunity by several mechanisms. TLR signaling of B lymphocytes enhance their antigen-presenting capacity towards $CD4^+$ T cells through up-regulation of HLA-DR. TLR signaling in plasmacytoid DCs results in maturation of DCs, and mature DCs promote induced activation of $CD4^+$ T cells [24]. $CD4^+$ T cells are categorized as Th1, Th2, or Th17 cells, and the function of these cells is to produce reciprocal sets of cytokines: IFNγ by Th1 cells, IL4 by Th2 cells, and IL17 by Th17 cells [4–6]. Specific TLRs might be associated with $CD4^+$ T-cell activation. Activation of TLR2 might induce Th2 immune response, TLR4 induced Th1 and Th17 immune response, and TLR9 induced Th1

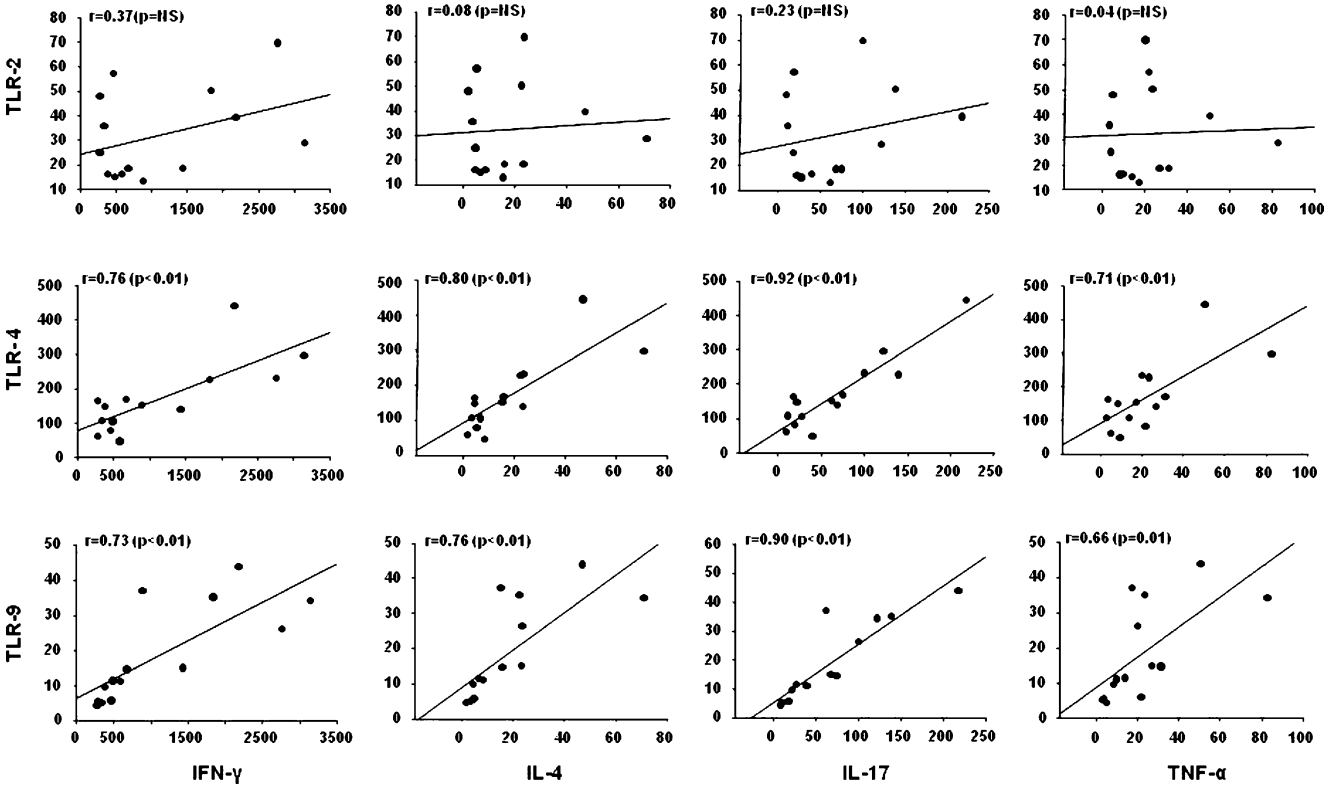

Fig. 1 Correlations of TLR2, TLR4, TLR9, IFNγ, IL4, L17, and TNFα expressions in patients with DM and PM. The expression levels of TLR4 and TLR9 had significant positive correlations with those of IFNγ, IL4, IL17, and TNFα. The mRNA expressions were measured by real-time RT–PCR from muscle tissues of patients with DM and PM

immune response [14–21]. We showed that the expression levels of TLR4 and TLR9 were increased compared to controls and had significant positive correlations with those of IFNγ, IL4, and IL17. These results suggest that the overexpression of TLR4 and TLR9 might be involved in the immunopathogenesis of DM and PM through activation of Th1, Th2, and Th17 cells. And the data showed by our study that the expression levels of TLR4 and IFNγ were

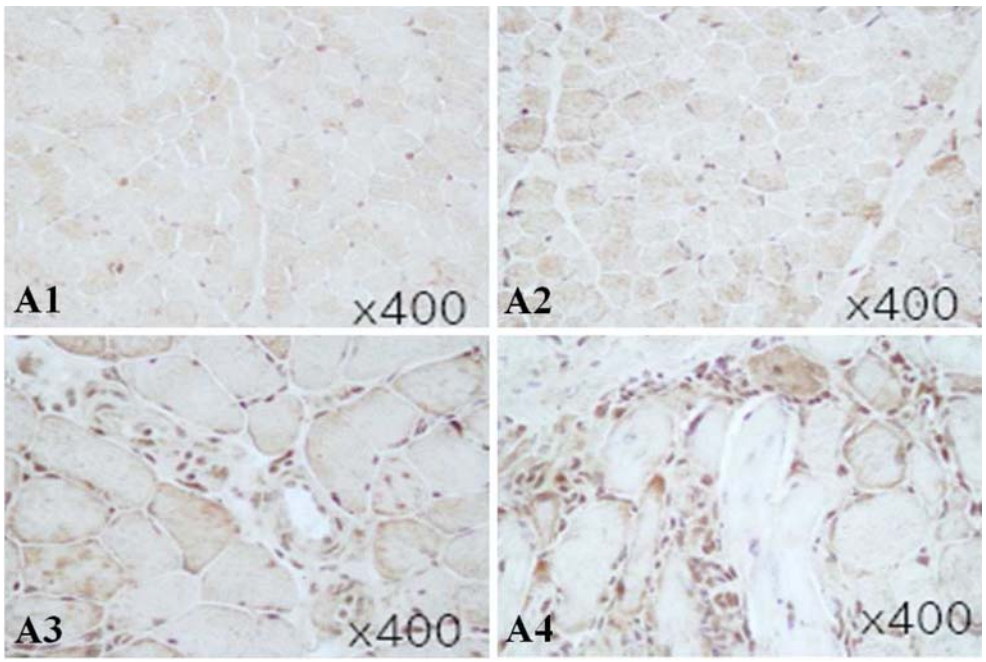

Fig. 2 Immunohistochemistry of TLR2 in muscle tissues of patients with DM, PM, and controls. In the muscle tissues of DM, TLR2 was expressed by infiltrating cells in the perimysial area (*A3*), whereas in PM, TLR2 was expressed by infiltrating cells in the endomysium (*A4*). Control muscle tissues were obtained from patients with non-specific muscle manifestations but with normal histological findings (*A1*, *2*). Magnification was ×400

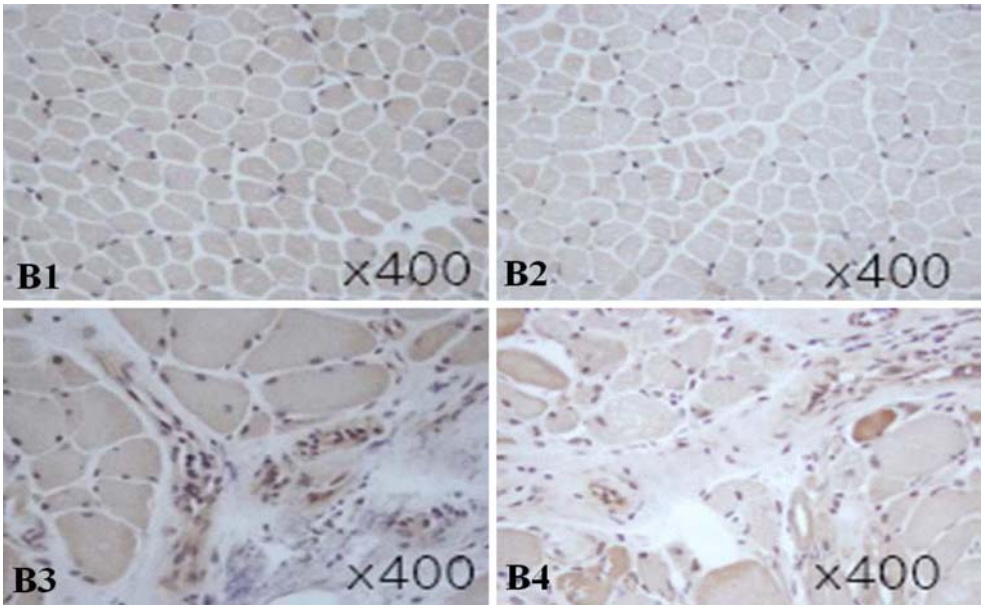

Fig. 3 Immunohistochemistry of TLR4 in muscle tissues of patients with DM, PM, and controls. In the muscle tissues of DM, TLR4 was expressed by infiltrating cells in the perimysial area (*B3*), whereas in PM, TLR4 was expressed by infiltrating cells in the endomysium (*B4*). Control muscle tissues were obtained from patients with non-specific muscle manifestations but with normal histological findings (*B1*, *2*). Magnification was ×400

prominently high and had significantly positive correlations with each other suggest that TLR4-induced Th1 response might play a more important role in immunopathogenesis of DM and PM.

TNFα gene expression can be detected in the muscle tissues of most patients with IIMs [26–28]. TNFα acts via several possible mechanisms, including the positive feedback loop in which increased numbers of TNFα-producing cells in infiltrating cell clusters enhance the inflammatory reaction by releasing more TNFα. This mediates the upregulation of adhesion molecules on endothelial cells and hence increases transendothelial cell trafficking as well as enhancing the T-cell response and thereby also T-cell-mediated muscle injury [29, 30]. We have showed that the expression level of TNFα was elevated and had significant positive correlations with those of TLR4 and TLR9 in the muscle tissues of patients with DM and PM.

According to recent studies, including our previous reported study and our present data, the possible immunopathogenic involvement of TLRs and $CD4^+$ T cells are as follows: (1) overexpression of TLR4 and TLR9 induced by microbial pathogen, endogenous molecules, and inflammatory cytokines contributed to activation of $CD4^+$ T cells resulting in increased expression of IFNγ, IL4, and IL17; (2) overexpressed $CD4^+$ T-cell cytokines induce the inflammatory response, resulting in an increase of various inflammatory cytokines and endogenous molecules; (3) overexpressed $CD4^+$ T-cell and inflammatory cytokines,

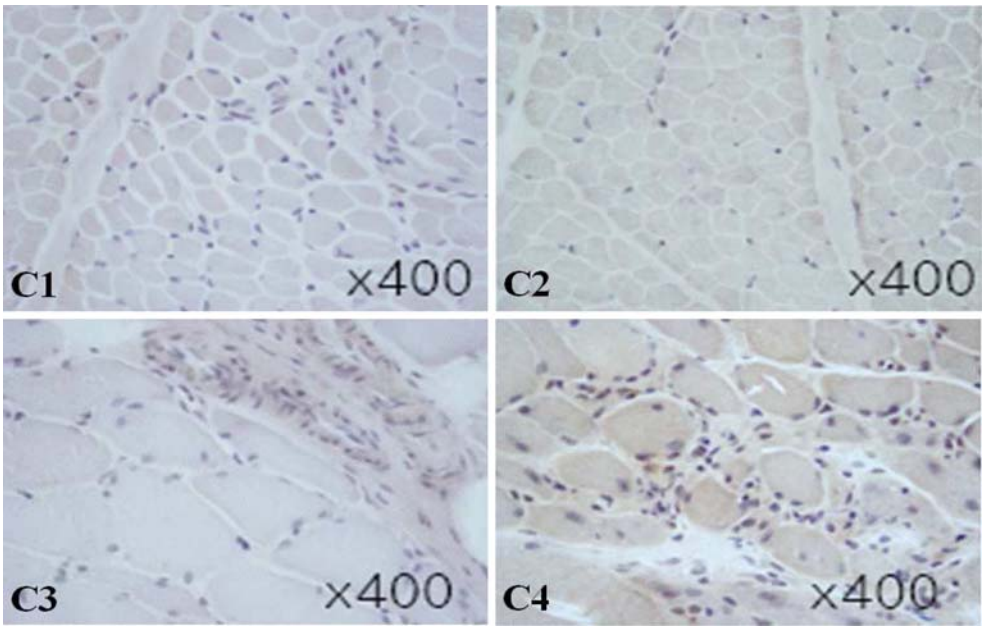

Fig. 4 Immunohistochemistry of TLR9 in muscle tissues of patients with DM, PM, and controls. In the muscle tissues of DM, TLR9 was weakly expressed by infiltrating cells in the perimysial area (*C3*), whereas in PM, TLR9 was expressed by infiltrating cells in the endomysium (*C4*). Control muscle tissues were obtained from patients with non-specific muscle manifestations but with normal histological findings (*C1*, *2*). Magnification was ×400

including IFNγ, IL4, IL17, and TNFα, and endogenous molecules induce the overexpression of TLRs, causing the vicious cycle of amplification of chronic inflammation in muscle tissues of DM and PM.

In conclusion, we have demonstrated that the expressions of TLR2, TLR4, and TLR9 were elevated in muscle tissue of patients with DM and PM, and their expression patterns were consistent with pathophysiology of DM and PM. We also showed that the expression levels of TLR4 and TLR9 had significant positive correlations with those of Th1, Th2, and Th17 cell cytokines. These results suggest that the involvement of TLR4 and 9 in the immunopathogenesis of DM and PM may be connected with activation of $CD4^+$ T cells.

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Disclosures None

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