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# Role of Toll-like receptors and retinoic acid inducible gene I in endogenous production of type I interferon in dermatomyositis

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ABSTRACT

To explore the possible mechanisms implicated in the endogenous production of type I interferons within the muscle tissue of dermatomyositis (DM) patients. We detected the co-localization of plasmacytoid dendritic cells (pDCs) with Toll-like receptors (TLRs) and retinoic acid inducible gene (RIG)-I by immunohistochemistry and immunofluorescence. Western blotting confirmed the expression of TLRs and RIG-I. TLR-3 and RIG-I was preferentially expressed in the perifascicular atrophy fibers of DM. TLR-7 was only in inflammatory infiltrates of a few DM patients. TLR-4 and TLR-9 was expressed mainly in inflammatory infiltrates. Immunofluorescence showed extensive co-localization of BDCA-2 with TLR-9 and little co-localization with TLR-7. Western blotting showed upregulation of expression of TLRs and RIG-I in DM compared with the controls. Our findings indicate that endogenous production of type I IFN in DM is generated by pDCs, mainly through the TLR-9 pathway and in part by TLR-7. TLR-3 and RIG-I are implicated in the formation of perifascicular atrophy in DM.



## 1. Introduction

Dermatomyositis (DM) belongs to the idiopathic inflammatory myopathies (IIMs), which are characterized by subacute onset of symmetric proximal muscle weakness, specific skin involvement, inflammatory infiltrates within the muscle, and pathologically perifascicular atrophy. To date, the immunopathogenesis of DM has not been fully clarified.

More recently, much attention has been focused on type I interferon (IFN), because of its potential role in IIM, as well as in other autoimmune diseases, such as systemic lupus erythematosus (SLE) and Sjogren's syndrome (Lovgren et al., 2004; Gaipl et al., 2005; Vakaloglou and Mavragani, 2011). An IFN signature has been identified in the muscle tissues of DM patients from both gene expression and protein expression (Greenberg et al., 2005), and expression of type I IFN in peripheral blood cells is involved in DM (Walsh et al., 2007; Baechler et al., 2007). Plasmacytoid dendritic cells (pDCs), a type of natural IFN-α-producing cell, are also detected in the perimysium and endomysium of DM muscle (Greenberg, 2007). Greenberg et al. further documented the presence of an IFN-inducible protein, myovirus resistance A (MxA), in muscle fibers and capillaries of DM patients (Greenberg et al., 2005). Activation of the type I IFN pathway plays a major role in DM, but not in other types of IIM (Lundberg and Helmers, 2010; Baechler et al., 2011).

The predominant forms of type I IFN are the IFN-α and IFN-β families, which are crucial in innate antiviral immunity in mammals, and all cells carry specialized receptors that recognize viral nucleic acids and induce IFN production (Sozzani et al., 2010). Molecular mechanisms of IFN induction depend on the activation of specific receptors, so-called pattern-recognition receptors, which recognize pathogen-associated molecular patterns (PAMPs) (Akira and Hemmi, 2003).

Toll-like receptors (TLRs) and retinoic acid inducible gene (RIG)-I-like receptors (RLRs) constitute distinct families of pattern-recognition receptors that can detect nucleic acids derived from viruses, and trigger antiviral innate immune responses (Yoneyama et al., 2004; Theofilopoulos et al., 2005). TLR-3, TLR-4, TLR-7 and TLR-9 are membrane proteins localized on the endosomes that can recognize viral double-stranded RNA, lipopolysaccharide, single-stranded RNA, and DNA, respectively (Hemmi et al., 2000; Alexopoulou et al., 2001; Diebold et al., 2004). RIG-I is one of the major type of RLRs, which are cytoplasmic proteins that recognize viral RNA (Loo and Gale, 2011). Upon recognition of these nucleic acid species, TLRs and RLRs recruit specific intracellular adaptor proteins to initiate signaling pathways culminating in the activation of nuclear factor (NF)-κB, mitogen-activated protein kinases, and IFN-regulatory factors, which control the transcription of genes encoding type I IFN and other inflammatory cytokines (Alexopoulou et al., 2001; Yoneyama et al., 2004).

It is postulated that autoantibodies bound to DNA or RNA in the muscle of DM patients stimulate pDCs to secrete IFN-α/β locally, as occurs in SLE (Lovgren et al., 2004). In this study, we investigated the possible

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mechanism involved in the endogenous production of type I IFN by detecting co-localization of pDCs with TLR-3, TLR-4, TLR-7, TLR-9 and RIG-I within the muscle tissue of DM patients.

## 2. Materials and methods

### 2.1. Patients

Muscle specimens were obtained retrospectively from 20 DM patients and 11 controls; of whom, 4 had polymyositis (PM), 4 had facioscapulohumeral muscular dystrophy (FSHD), and 3 were initially suspected of having muscle disorders. In the latter, 3 biopsies showed normal histological findings, and these non-disease controls did not have any neuromuscular disease with longer follow-up times. Patients with DM and PM fulfilled Bohan and Peter's criteria (Bohan and Peter, 1975a, 1975b). The diagnosis of FSHD was based on the characteristic muscle weakness distribution and positive family history. The profiles of DM patients are summarized in Table 1.

No patients were receiving any immunosuppressive agents at the time of biopsy or had discontinued immunosuppressants for ≥3 months. Patients who were complicated with other autoimmune and/or infectious diseases were excluded. Open biopsies were performed for clinical indications with written informed consent. The Institutional Review Board of Qilu Hospital Affiliated to Shandong University approved the study.

### 2.2. Immunohistochemistry

Six-micrometer cryostat sections of muscle tissue were air-dried at room temperature and fixed in cool acetone (4 °C) for 10 min. The PV-9000 polymer detection system (ZsBio Ltd., China) was used. After rehydrating in phosphate-buffered saline (PBS), all sections were incubated overnight at 4 °C with primary antibodies (BDCA-2, RIG-I, TLR-3, TLR-4, TLR-7 and TLR-9; Table 2). After washing with PBS, the sections were incubated with polymer helper for 20 min followed by polymerized-horseradish-peroxidase-conjugated anti-mouse/rabbit immunoglobulin G (IgG) for 30 min at 37 °C. Peroxidase activity was determined using diaminobenzidine. The slides were counterstained with hematoxylin and eosin, dehydrated and mounted in neutral balsam. In the negative control tissue sections, the primary antibody was replaced by isotype-specific non-immune mouse IgG.

**Table 2**
Major antibodies used for IM, IF and WB.

| Primary Ab | Clone | Species | Dilution (IM/IF) | Dilution (WB) | Company |
|---|---|---|---|---|---|
| Anti-BDCA-2 | AC144 | Mouse | 1:50 | – | Miltenyi Biotec |
| Anti-BDCA-2 | Polyclonal | Rabbit | 1:100 | – | PL Laboratory |
| Anti-TLR-3 | 40C1285 | Mouse | 1:50 | 1:250 | Abcam |
| Anti-TLR-4 | 76B357.1 | Mouse | 1:50 | 1:250 | Abcam |
| Anti-TLR-7 | Polyclonal | Rabbit | 1:100 | 1:250 | Abcam |
| Anti-TLR-9 | 26C593 | Mouse | 1:50 | 1:250 | Abcam |
| Anti-RIG-I | Polyclonal | Rabbit | 1:50 | 1:250 | Santa Cruz |

IM = immunohistochemistry; IF = immunofluorescence; WB = western blotting.

### 2.3. Immunofluorescence double staining

Double immunofluorescence labeling was used to detect the possible co-localization of BDCA-2 with TLRs or with RIG-I in the DM muscle biopsies. Frozen muscle tissue sections (6 μm thick) were fixed in methanol, blocked in 10% normal goat serum for nonspecific site binding. Anti-BDCA-2 (mouse monoclonal or rabbit polyclonal; Table 2) was applied first to the slides for 30 min at 37 °C, followed by application of goat anti-mouse or anti-rabbit fluorescein-isothiocyanate-conjugated IgG as the secondary antibody (1:100 dilution; Zhongshan Bio, China). Anti-TLR-7/RIG-I (rabbit polyclonal) or anti-TLR-3/4/9 (mouse clonal) and the secondary antibody, goat anti-rabbit or anti-mouse tetramethylrhodamine-isothiocyanate-conjugated IgG (1:100 dilution; Southern Biotech Associates, Birmingham, AL, USA), were sequentially applied to the slides. All slides were counterstained with DAPI for nuclear staining. As a negative control, isotype-specific nonimmune IgG was applied at the same working dilution as the primary antibodies. Images were acquired using DP manager version 3.1.1 2008 (Olympus, Tokyo, Japan) and merged using Image-Pro Plus version 5.1.0.20 (Media Cybernetics, Bethesda, MD, USA).

### 2.4. Western blotting

Western blotting was performed on muscle homogenates. Specific primary antibodies against RIG-I, TLR-3, TLR-4, TLR-7 and TLR-9 are listed in Table 2. GAPDH (dilution 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as internal standard control. The protein

**Table 1**
Clinical and pathological profile and the expressions TLRs and RIG-I in DM patients.

| Patient no. | Sex (M:F) | Age | Disease duration | CK (U/L) | Muscle weakness | Skin rash | Perifascicular atrophy | Inflammatory infiltrates | Semi-quantitative immunohistochemistry results | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | TLR-3 | TLR-4 | TLR-7 | TLR-9 | RIG-I |
| 1 | M | 32 | 2 years | 5000 | Prox, N, Dys | + | − | + | − | + | + | ++ | − |
| 2 | F | 6 | 4 months | 499 | Prox | + | + | + | +++ | + | ++ | +++ | +++ |
| 3 | F | 42 | 3 months | 8090 | Prox, Dys | + | − | + | − | + | − | + | − |
| 4 | F | 44 | 1 year | 5277 | Prox, Dys | + | − | + | − | − | + | ++ | − |
| 5 | F | 23 | 5 months | 80 | Prox, N, Dys | + | + | + | ++ | ++ | + | + | ++ |
| 6 | F | 9 | 1 year | 1700 | Prox | + | + | + | ++ | + | − | + | + |
| 7 | F | 31 | 6 months | 1827 | Prox, N, Dys | + | + | + | +++ | + | ++ | +++ | ++ |
| 8 | F | 50 | 9 months | 2348 | Prox, N | + | + | + | ++ | + | − | + | ++ |
| 9 | M | 57 | 10 months | 2682 | Prox, Dys | + | + | + | + | ++ | − | ++ | ++ |
| 10 | M | 13 | 1 year | 50 | Prox | + | + | + | + | + | − | + | + |
| 11 | M | 62 | 3 years | 330 | Prox, Dys | + | + | + | ++ | + | − | + | + |
| 12 | F | 48 | 6 months | 306 | Prox, N, | + | + | + | ++ | + | ++ | +++ | ++ |
| 13 | F | 15 | 2 months | 5138 | Prox, N, Dys | + | + | + | ++ | + | + | ++ | ++ |
| 14 | F | 70 | 1 year | NA | Prox, N | + | + | + | + | + | − | ++ | + |
| 15 | M | 27 | 6 months | 2700 | Prox, N | + | + | + | ++ | + | + | + | + |
| 16 | F | 74 | 2 months | 2044 | Prox, | + | − | + | − | + | − | ++ | − |
| 17 | F | 19 | 8 months | 89 | Prox, N | + | + | − | ++ | − | − | ++ | ++ |
| 18 | F | 37 | 4 months | 160 | Prox, N | + | + | + | + | + | − | ++ | ++ |
| 19 | M | 18 | 3.5 months | 516 | Prox, | + | + | − | ++ | − | − | + | + |
| 20 | F | 42 | 2 months | 111,40 | Prox, N, Dys | + | + | + | + | + | + | ++ | + |
| | (6:14) | (6–70) | (2 months–3 years) | | | | | | | | | | |