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Original Article

Development of an improved animal model of experimental autoimmune myositis

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Abstract: Multiple animal models of experimental autoimmune myositis (EAM) have been developed. However, these models vary greatly in the severity of disease and reproducibility. The goal of this study was to test whether vaccination twice with increased dose of rat myosin and pertussis toxin (PT) could induce EAM with severer disease in mice. BALB/c mice were injected with 1 mg rat myosin in 50% complete Freund's adjuvant (CFA) weekly for four times and one time of PT (EAM) or twice with 1.5 mg myosin in CFA and PT (M-EAM). In comparison with that in the CFA and PT injected controls, vaccination with rat myosin and injection PT significantly reduced the muscle strength and EMG duration, elevated serum creatine kinase levels, promoted inflammatory infiltration in the muscle tissues, leading to pathological changes in the muscle tissues, demonstrating to induce EAM. Interestingly, we found that vaccination twice with the high dose of myosin and PT prevented EAM-related gain in body weights and caused significantly less muscle strength in mice. More importantly, all of the mice receiving high dose of myosin and PT survived while 3 out of 16 mice with four times of low dose of myosin died. Finally, vaccination with high dose of myosin promoted $CD4^+$ and $CD8^+$ T cell infiltration in the muscle tissues and up-regulated MHC-I expression in the muscle tissues of mice. Hence, the new model of EAM is a time-saving, efficient and easily replicable tool for studying autoimmune myositis.

Keywords: Experimental autoimmune myositis (EAM), animal models, idiopathic inflammatory myopathies (IIM), immunosuppressive

Introduction

Idiopathic inflammatory myopathies (IIM) are a heterogeneous group of skeletal muscle diseases characterized by the presence of muscle weakness, inflammation and elevated levels of serum creatine kinase [1-4]. While previous studies have shown that IIMs result from an autoimmune disorder, the etiology and pathogenesis of the disease remain unclear [5]. Immunosuppressive drugs and corticosteroids can help to alleviate symptoms, however, these types of treatments have many side effects [6-8]. The development of new therapeutic strategies has been hindered at least in part by the lack of suitable animal models for experimental autoimmune myositis (EAM).

Multiple rodent models of EAM have been created to mimic various types of IIM. For example, several studies have shown that injection of muscle homogenates induces mild disease in a mouse model of EAM [9, 10]. Furthermore, many rat models of EAM have also been generated by injection of partially purified skeletal myosin [11], native C-protein [12], and recombinant C-protein fragment 2 (SC2) [13]. These models of animals take at least four weeks to develop EAM. The C-protein comprises only 2% of muscular proteins, which is difficult to perform experiments using a large amount of the protein. Furthermore, generation of purified SC2 is technically complex. Other EAM mouse models have been developed by manipulating immune system. Nagaraju et al. [14] generated mouse model of EAM by conditionally up-regulating MHC-I expression, but this strain of transgenic mice did not distribute worldwide. In addition, Kimura et al. [15] generated mouse model of EAM by inducing muscle injury and regeneration, but this model has poor reproducibility. Hence, the available animal models of EAM have limitations, including methodological complexity, time-consuming, mild inflammation, poor reproducibility, and low success rate. Accordingly, a new reproducible EAM animal model is urgently needed.

In this study, we modified an existing method of generating mouse model of EAM, and evaluated its pathogenesis. Our approach resulted in a much more efficient and consistent model of EAM.

Materials and methods

Animals

Six week-old female BALB/c mice weighing 15-17 g and a guinea pig weighing 480 g were purchased from Experimental Animal Center of the Fourth Military Medical University, Xian, China, and housed in a specific pathogen free facility in our University. All animals were free access to normal chow and water. The experiments were performed according to the National Institute of Health Guide for the Care and Use of Laboratory Animals (revised 1996) and the protocol was approved by the Animal Research Care Committee of the Fourth Military Medical University.

Preparation of myosin

Skeletal muscle myosin was partially purified, as described previously with several modifications [11, 16]. Guinea pig skeletal muscle was dissected, weighed, frozen at -70°C, thawed and minced. The minced muscle tissues (10 g) were homogenized in 30 ml of cold 0.3 M KCl, 0.15 M sodium phosphate buffer (pH 6.5) on ice for 60 minutes. The homogenates were centrifuged at 12,000 g for 30 minutes at 4°C and the supernatants were collected and filtered. The filtrates were diluted with 5 volumes of cold Milli-Q-filtered water to extract myosin. After being centrifuged at 7000 g at 4°C for 30 minutes, the aggregated myosin was dissolved in 0.5 M KCl and stored at -70°C.

Mouse immunization protocol

Forty-eight BALB/c mice were randomly divided into modified model (M-EAM), traditional model (EAM) and control groups (CON). The mice in the EAM group were immunized subcutaneously with 100 μl of 50% complete Freund's adjuvant (CFA, Sigma) containing 1 mg myosin and 5 mg/ml *M. tuberculosis* at the left hindlimb and boosted at the tail base and flanks weekly for 4 times. The mice were injected intraperitoneally with pertussis toxin (PT, Sigma, 500 ng in 200 μl saline) immediately after the first immunization [16]. To prevent antigen degeneration, only freshly prepared or short-term stored (one month or less at -70°C) antigen preparations were used for experiments. The mice in the M-EAM group were immunized with 1.5 mg myosin in CFA weekly for two times and injected with 500 ng PT twice immediately after each immunization. The CON group received saline/CFA and PT twice, similar to that in the M-EAM group. Ten days after the last immunization, their body weights and skeletal muscle strengths were measured. Furthermore, their left and right hindlimb were randomly selected for electromyogram (EMG) examination. Their blood samples were collected and the levels of serum creatine kinase in individual mice were measured using an automatic biochemical analyzer. The mice were sacrificed and their bilateral gastrocnemius, quadriceps, and triceps muscle tissue samples were dissected (avoiding injection and acupuncture sites), immediately frozen in liquid nitrogen, and blocked for histology and immunohistochemistry.

Evaluation of muscle strength in mice

The strength of skeletal muscles of individual mice was evaluated by an inverted screen test, as described previously [16, 17]. Briefly, each mouse was placed in the center of the wire mesh screen (50 cm^2) consisting of 12 mm^2 of 1 mm diameter wire. The screen was smoothly inverted and held steadily 20 cm above a padded surface. The time at which the mouse fell off was recorded to a maximum of 1 minute. Each mouse was assessed 5 times, and then averaged. The mice were evaluated by one investigator in a blinded manner.

Histological grading of inflammation

The muscle tissue sections (10 μm) from each block were randomly selected and stained with hematoxylin and eosin (H&E). The degrees of inflammatory infiltrates in six muscle sections were graded by two evaluators into four categories in a blinded manner and expressed as a mean score [12, 13, 18]. Grade 1: <5 muscle fibers involved; 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 were found in one section of muscle, 0.5 was added to the score. A disagreement for evaluating a sample between these two observers was resolved by discussion.

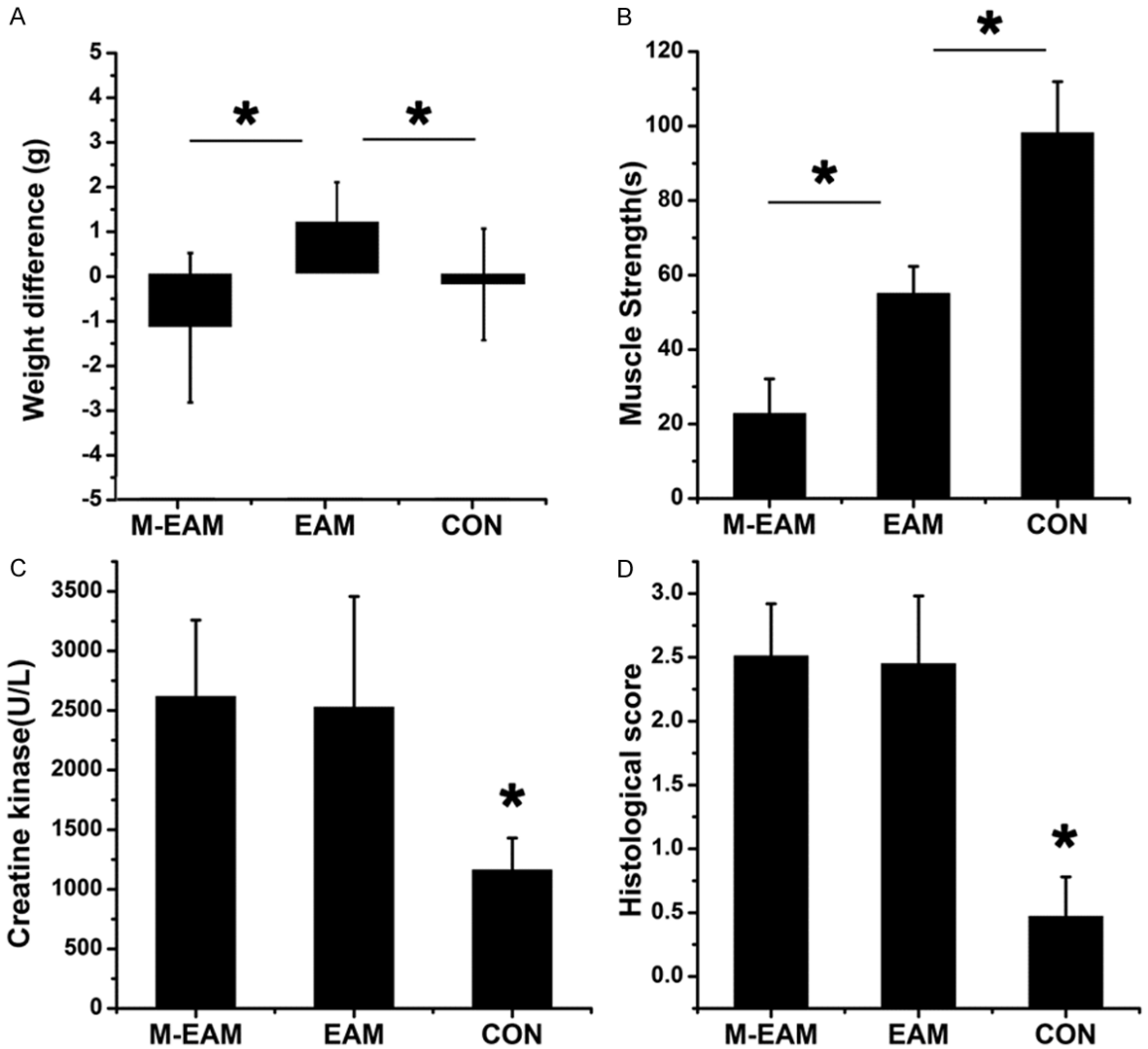

Figure 1. Vaccination twice with a higher dose of myosin and PT induces severer EAM in mice. BALB/c mice were induced with EAM using the modified protocol (M-EAM), or the traditional method (EAM), and were compared to controls (CON). The mice were assessed for their body weights (A); muscle strength (B); serum creatine kinase concentrations (C). Data are expressed as the mean ± SD of each group (n=16) of mice from at least two separate experiments. (D) And histological score of muscle tissue sections (*P≤0.05).

Immunohistochemistry

The specific type of immunocompetent infiltrates in the muscle tissues was characterized by immunohistochemistry. Briefly, the serial muscle cryosections (10 μm) were air-dried and washed with 0.01 mM PBS. After being blocked with normal sheep serum, the sections were incubated with rat monoclonal anti-CD4 (clone GK1.5, 1:300, Abcam China), rabbit anti-CD8 (clone EP 1150Y, 1:200), and rabbit anti-mouse MHC-I (clone EP 1395Y, 1:200, Epitomics. USA) overnight at 4°C. The sections were incubated with biotinylated goat anti-rat or goat anti-rabbit IgG (1:200) (Vector, Burlingame, CA) and horseradish peroxidase (HRP)-labeled VECTASTAIN® Elite ABC Kit (Vector), followed by visualizing with DAB (Sigma). The sections were examined using an inverted microscope (Leica, Germany).

Statistical analysis

Data are present as mean ± SD unless indicated. The difference between groups was analyzed by LSD-t test using SPSS 16.0 software. A *P* value less than 0.05 was considered statistically significant.

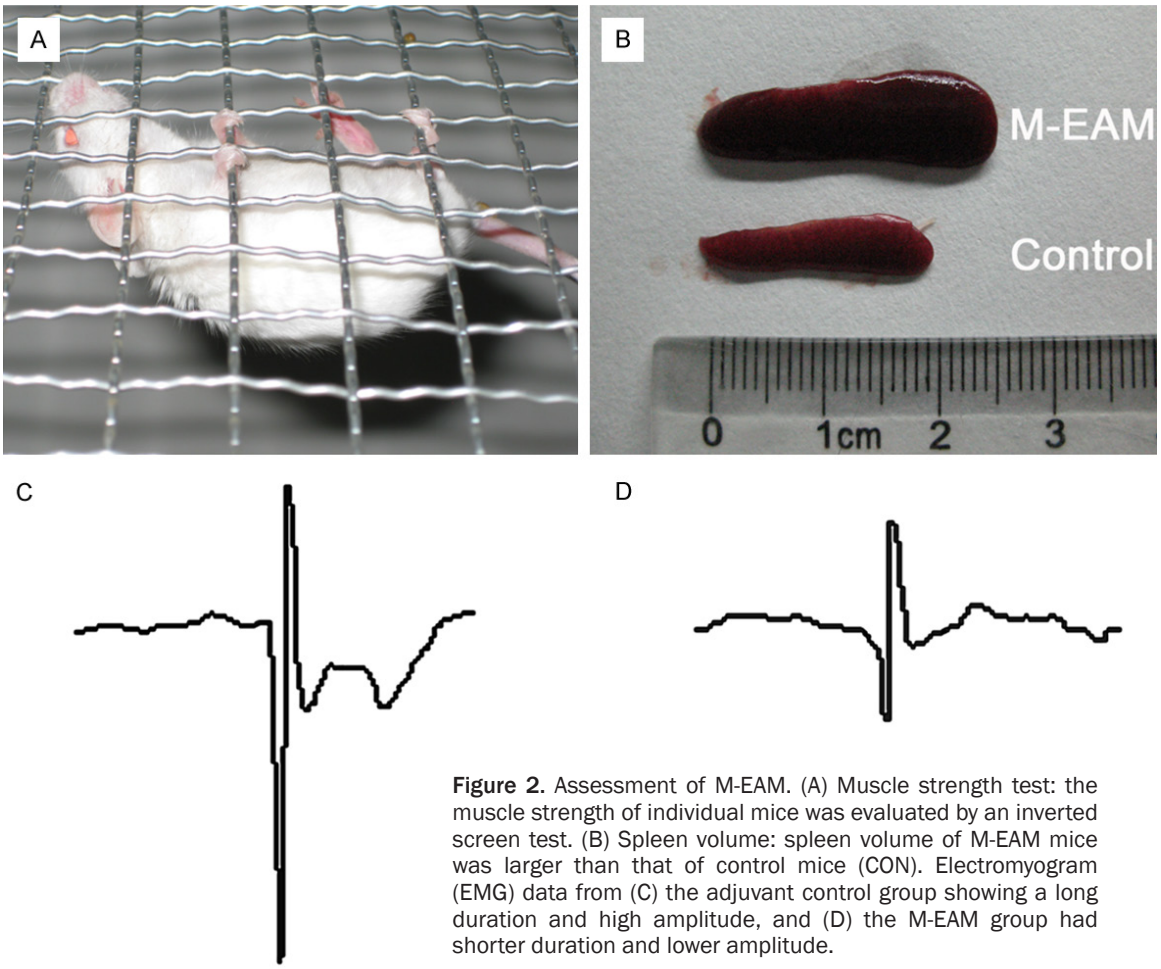

Figure 2. Assessment of M-EAM. (A) Muscle strength test: the muscle strength of individual mice was evaluated by an inverted screen test. (B) Spleen volume: spleen volume of M-EAM mice was larger than that of control mice (CON). Electromyogram (EMG) data from (C) the adjuvant control group showing a long duration and high amplitude, and (D) the M-EAM group had shorter duration and lower amplitude.

Results

During the observation period, while three out of 16 mice died in the EAM group all mice in the M-EAM and CON group survived at 10 days post the last immunization. Furthermore, measurement of body weights indicated that the body weights in the EAM group increased and were significantly higher than that in the M-EAM and CON groups of mice (**Figure 1A**, $P<0.05$). There was no significant difference in the body weights between the M-EAM and CON groups of mice although the body weights in the M-EAM group (17.6 g) was slightly decreased (1.46±2.03 g).

Analysis of the muscle strength by the inverted screen test indicated that the muscle strength in the EAM group of mice was significantly higher than that in the M-EAM group of mice, but less than that in the CON group ($P<0.05$ for both, **Figure 1B**). Furthermore, serum creatine kinase levels in both the EAM and M-EAM groups were significantly higher than that in the CON group of mice (2618.19±640.35 U/L; 2526.77±930.22 U/L vs. 1161.69±267.98 U/L, $P<0.05$, respectively, **Figure 1C**), but there was no significant difference in the levels of serum creatine kinase between the EAM and M-EAM groups of mice. In addition, EMG revealed that both the EAM and M-EAM groups of mice displayed significantly shorter duration and lower amplitude than the CON group (**Figure 2A**).

Using H&E staining, we found that there were two types of muscle fibers and a few inflammatory infiltrates in the muscle tissues in the CON group of mice (**Figure 3A** and **3B**). In contrast, endomysial inflammation, scattered fibers undergoing necrosis and phagocytosis, or invaded and regenerated fibers, the hallmarks of myositis were observed in the muscles of both the EAM and M-EAM groups of mice (**Figure 3C-F**). There were obvious inflammatory infiltrates and muscle fiber atrophy in the mus-

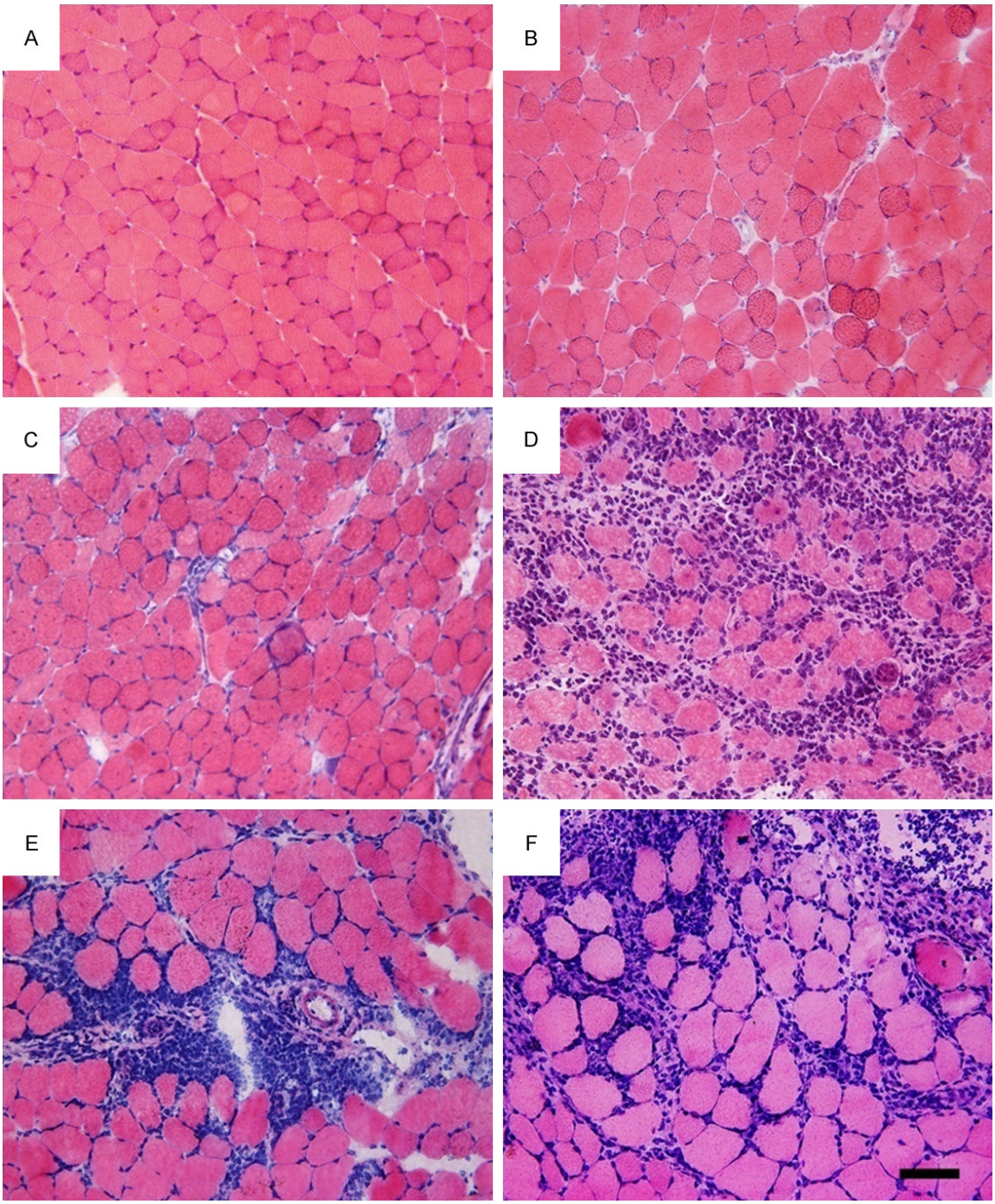

Figure 3. Histological analysis of the muscle tissues. The muscle tissue sections from individual groups of mice were stained by H&E and the inflammation degrees in the muscle tissues were evaluated. Data are representative images (magnification ×200, Bar =50 μm) or expressed as the mean ± SD of inflammation scores of each group (n=16) of mice. A. Two types of muscle cells in the CON group of mice. B. A few inflammatory infiltrates in the muscle tissue of the CON group. C. Inflammatory infiltrates and muscle fiber atrophy in the muscle tissues of the EAM group of mice two weeks after the last immunization. D. Endomysial inflammation, scattered fibers undergoing necrosis and phagocytosis, as well as invading and regenerating fibers in the muscle tissues of the M-EAM group of mice at two weeks post the last immunization. E. Severe inflammation in the muscle tissues of the EAM group of mice at four weeks post the last immunization. F. Inflammation in the muscle tissues of the M-EAM group of mice at four weeks post the last immunization. Inflammation scores. $^{*}P<0.05$ vs. the CON group.

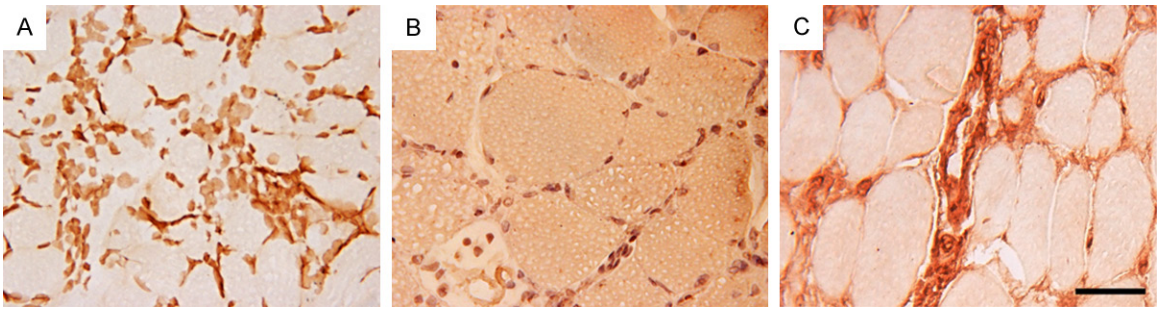

Figure 4. Immunohistochemical analysis of immune cells in the muscle tissues from the M-EAM group of mice. A. A few $CD4^+$ T cells infiltrated into muscle fibers. Specifically, many $CD4^+$ T cells were present in the endomysium, perimysial and perivascular areas. B. $CD8^+$ T cells were found in the endomysium and infiltrated muscle fibers. C. MHC-I expression was up-regulated in various muscle fibers (400×, Bar =50 μm).

cle tissues of the EAM group of mice (**Figure 3C**). Similar levels of inflammatory infiltrates in the muscle tissues in the M-EAM group of mice (**Figure 3D** and **3E**). Except for three dead mice in the EAM group, all other immunized mice, but not a single control, developed myositis. Quantitative analyses revealed that the inflammatory scores in the EAM (2.45±0.73) and M-EAM (2.51±0.61) groups of mice were significantly higher than that in the CON group (0.47±0.31, $P<0.05$) and there was no significant difference in the inflammatory scores between the EAM And M-EAM groups of mice (**Figure 3F**).

Finally, immunohistochemical analysis of the muscle tissue sections revealed that there were many $CD4^+$ T cells in the endomysium, perimysial and perivascular areas and some infiltrated in the muscle fibers in the M-EAM group of mice (**Figure 4A**), which was similar to that in the EAM group (data not shown). Furthermore, anti-CD8 staining was mainly located in the endomysium and less in the muscle fibers (**Figure 4B**). In addition, strong anti-MHC I staining was observed in some fibers of the EAM and M-EAM groups of mice, but not in the CON group of mice (**Figure 4C**).

Discussion

In the present study, we modified the traditional protocol to induce severer EAM in mice by injecting twice of a higher dose of myosin and PT. This new model was a quicker, more efficient, and repeatable method to induce EAM that resembled human IIM. Therefore, our findings may help in studying the pathogenesis and developing new therapies for IIM.

The clinical diagnosis of IIM is dependent on elevated serum creatine kinase, abnormal EMG and pathologic muscle inflammation [19]. We found that in comparison with that in the control, vaccination with myosin and PT induced EAM in mice. Evidently, vaccination with myosin and PT significantly reduced the muscle strength and EMG duration and amplitude, increased the levels of serum creatine kinase, promoted inflammatory cell infiltration in the muscle tissues and muscle damages in mice. Interestingly, the EAM group of mice, but not the M-EAM and control groups of mice, gained body weights. The muscle strength in the M-EAM groups of mice was significantly less than that in the EAM group of mice. Furthermore, while some mice died in the EAM groups while all of the mice in the M-EAM group survived through the observation period. Our data clearly indicated that new model of EAM was severer and safer to mice. The mouse death in the EAM group may be associated with multiple vaccination-induced systemic inflammations, which may cause multiple organ failure. However, it is unclear why most mice in the same group gained their body weights. We are interested in further investigating the potential mechanisms underlying these findings.

Previous studies have shown that autoreactive T cell responses may participate in the pathogenesis of IIM and EAM [5, 13, 16]. We found that CD4 and $CD8^+$ T cell infiltrates in the perimysial and perivascular areas of the muscle tissues, consistent with previous observations [1, 13, 20, 21]. However, some of the pathological features of EAM are different from that in polymyositis. We found that MHC-I expression was upregulated in some fibers, but not diffused in

the muscle tissues of the M-EAM group of mice. Anti-CD8 staining was mainly located in the endomysium and some infiltrated muscle fibers. Many CD4-positive T cells infiltrated in the endomysium, perimysial and perivascular areas, but only a few in the muscle fibers, consistent with previous reports [16, 22]. Therefore, the inflammatory pathogenesis in the muscle tissues of the M-EAM group of mice was more like dermatomyositis and polymyositis rather than pure polymyositis. Our findings suggest that an increased dose of myosin can induce severe EAM and the partially purified skeletal myosin may have more myositis-inducing properties than protein fragments [13] and higher immunogenicity. The severer disease induced by high dose of myosin and PT may be associated with down-regulation of regulatory T cell responses by PT [16, 23, 24].

In summary, we have successfully induced severe EAM by vaccination twice with a higher dose of myosin and PT. This modified protocol saved time and was easily performed. This animal model may provide a useful tool to analyze the pathogenesis of these diseases, and to develop specific therapies for human IIMs.

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Disclosure of conflict of interest

None.

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