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ARTICLE TYPE

PNIPAM hydrogel induces skeletal muscle inflammation response

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Although researchers believe hydrogels have favorable biocompatibility *in vitro* and *in vivo*, these kinds of synthetic polymers still have the different immunogenic behavior *in vivo*, and bring forth a long-lasting inflammatory response. In this study, we attempt to explore the possibility of the intramuscular implantation of the specific PNIPAM hydrogel to induce a chronic inflammatory-myoinjury model in mice. We injected temperature-sensitive PNIPAM hydrogel mixed with or without fast-type skeletal muscle C protein, into tibialis anterior muscle (TA) of WT B6 mice, and analyzed mononuclear cell infiltration, muscle autoantigens and TLRs expression, and the intramuscular $CD8^+$ T cells infiltration and MHC-I expression on the surface of new myofiber in TA muscle on day 15, 30 and 60 post-injection. We found that, PNIPAM hydrogel induces a skeletal muscle inflammation during 2 months. The inflammation response in hydrogel injecting area reached the peak on day 30, and then gradually decreased. Interestingly, the mixture of PNIPAM hydrogel/C protein initiated a more severe and persistent muscle inflammatory response, than hydrogel, or C protein injection alone. Immunofluorecence analysis demonstrated that the hydrogel-induced the chronic muscle inflammation featured by the $CD8^+$ T cell infiltration and the increased class I MHC–positive regenerating myofibers. Our work holds a new promise for preparing animal model of muscle inflammation using hydrogel as a bio-driver. Hydrogel induced-myoinjury model maybe useful for investigating pathologic mechanisms of muscle injury, inflammation and regeneration.

Introduction

Multiple cellular and molecular processes are rapidly activated in response to various types of muscle damage. The local response to muscle tissue damage typically involves an inflammatory process, and the beneficial outcome of muscle repair relies largely on the coordination between muscle inflammation and repair or regeneration[1]. Although the degenerative phase and the regenerative phase of the muscle regeneration process are similar among different muscle types and after varying causes of injuries, the kinetics and amplitude of each phase may vary depending on the extent of the injury, the muscle injured, or the animal model[2].

To establish the animal model of chronic muscle injury is important to explore the pathologic mechanisms of inflammatory myoisits, e.g. autoimmune polymyositis. Researchers developed different skills to prepare myositis model, including muscle homogenate or myosin administration[3, 4], C protein induction[5], and so on. However, the myositis phenotypes of those model animals are unstable, and difficult to repeat at the different experimental conditions. It is necessary to explore the new way to induce chronic muscle injury *in vivo*. Until now, no teams report the chronic inflammatory-myoinjury model prepared by the intramuscular implantation of the specific biomaterial.

Hydrogel is synthetic biomimetic extracellular matrices (ECM), and usually is taken as a vehicle of therapeutic cells and regulators, presenting customized tissue like properties for cell in-growth and exchange of metabolites[6-8]. However, biocompatibility of different kinds of hydrogel with host tissues is variable, because of their tailored physical and chemical properties, biodegradability, stability, and so on. A considerable body of evidence has showing that synthetic polymer hydrogels have the different immunogenic behavior *in vivo,* and their degradation can produce a local acidic environment, bringing forth a long-lasting inflammatory response[9, 10]. Therefore, it is possible to stimulate intramuscular inflammatory response by implanting synthetic hydrogel into skeletal muscle. Recently, Malcolm M. Q. Xing's group reported a novel cell-compatible hydrogel based on a multifunctional crosslinker, and demonstrated its favorable biocompatibility *in vitro* and *in vivo* [11]. In this study, we injected this new hydrogel (PNIPAM hydrogel, temperature-sensitive, a gift from Pro. Xing) mixed with or without muscle C-protein, into TA muscle of WT B6 mice, and explored the hydrogel-induced intramuscular inflammation response. We analyzed mononuclear cell infiltration, and muscle autoantigens and Toll like receptors (TLRs) expression, in mice TA muscle on day 15, 30 and 60 days post-injection. For evaluating the immune features of hydrogel induced muscle inflammation, the intramuscular $CD8^+$ T cells infiltration and MHC-I expression on the surface of new myofibers was detected.

Materials and Methods

Preparation of PNIPAM hydrogel

Temperature-sensitive PNIPAM hydrogel was made by N-isopropyl acrylamide (NIPAM, Sigma Aldrich) and multifunctional crosslinking agent poly amidoamines (PAAs, provided by Pro. Xing) [11]: 444ul 50mg/ml NIPAM and 6ul 50mg/ml PAA were all prepared in PBS solution (pH=7.4) initiated by ammonium persulfate (APS, Sigma Aldrich) (5.7 mg ml^{-1}) and catalyzed by addition of N,N,N',N'-tetramethylethanediamine (TEMED, Sigma Aldrich) (2.9 mg ml^{-}

[1]) at 37^{0}C for 5 min. The synthesized hydrogel was stored at 4^{0}C for next use.

Preparation of recombinant human skeletal muscle C protein

Complementary DNA (cDNA) fragments encoding overlapping cDNA fragments of human fast-type skeletal muscle C protein were amplified from human skeletal muscle cDNA using polymerase chain reaction. Primers used were 5' cgc ggatcc GACCTGACCCTCAAGTGGTTCAAG 3' and 5' ccg ctcgag CTTCAGCCAGGTAGCGACGGG 3'. Primers were reacted with pET-28a plasmid (DongSheng，China), and then introduced into the DH5α bacterial host (DongSheng，China) and were used to prepare recombinant C protein fragments. Soluble recombinant C proteins were dialyzed using SnakeSkinTM Dialysis Tubing (Cat. 68035, Thermo). Endotoxin was removed using Detoxi-Gel^{TM} Endotoxin Removal Gel (Cat. 20339, Thermo).

Animals and induction of myoinjury

6-to 8-wk-old female wild C57BL/6 mice were used. All experiments were performed in accordance with the guidelines of the Animal Experimentation Ethics Committee of the Southern Medical University. The animals were randomly divided into four groups of 6 animals each: control, C-protein, hydrogel, and the mixture of C-protein and hydrogel. Animals of the C-protein and hydrogel groups were respectively injected with 30ul of C-protein or hydrogel into tibialis anterior (TA) muscles, while animals of the last group received the mixture of hydrogel (30ul) and C-protein (30ul). Mice were sacrificed at day 7 (only for animals injected with C protein), 15, 30 and 60 after injection. TA muscles were collected and snap frozen for RNA analyses. For histology, muscles were collected and directly frozen in liquid nitrogen-cooled isopentane.

RNA extraction and quantitative real-time PCR analysis

Total RNA was extracted from muscle tissue using RNA extraction kit (DongSheng，China), following manufacturer's recommendations. Total RNA (1ug) was then used for reverse transcription (RT) with commercially available kit (Revert Aid First Strand cDNA Synthesis Kit, Fermentas). Real-time polymerase chain reaction (PCR) was performed in triplicate with an ABI Step One Plus system (Applied Biosystems, USA) and a fluorescence-labeled SYBR Green/ROX qPCR Master Mix kit (Fermentas) using specific primers. Muscle autoantigens (Mi-2, HARS, Ku-70), TLR3, MHC-1(H-2k^b) and with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) taken as an endogenous control (primer sequences and sizes of product are listed in table 1), were detected. The results were analyzed with SOS2.1 software (Applied Biosystems). Expression of the genes was calculated from the accurate threshold cycle (Ct), which is the PCR cycle at which an increase in fluorescence from SYBR Green probes above the baseline signal can first be detected. The Ct values for GAPDH were compared with these from Mi-2, HARS, Ku-70, TLR3 and H-2k^b and in each well to calculate ΔCt. Data of the treated conditions were expressed relative to the signal obtained for the average of the untreated controls by the ΔΔCt calculation. The triplicate ΔΔCt values for each sample were averaged.

Histological and immunofluorescence detection

Snap-frozen whole TA muscle was transversely cryosectioned (8μm), and either stained with hematoxylin and eosin（H&E） or prepared for immunofluorescence. For immunofluorescence staining, muscle sections were fixed with cold acetone and incubated with rat anti-mouse F4/80 (1:100， eBioscience， USA); rat anti-mouse CD11b (1:100，eBioscience, USA); rat anti-mouse CD8α (1:100，eBioscience, USA); rat anti-mouse H-2K^b (1:100, BD, USA); rabbit polyclonal anti-Dystrophin (1:200, Santa Cruz, USA); and rabbit polyclonal anti-CD3ε (1:100, Abcam, USA). Rhodamine-conjugated goat anti-rat IgG (1:200, Santa Cruz, USA), FITC-conjugated goat anti-rabbit IgG (1:400, Santa Cruz, USA) were used as secondary antibodies. Nuclei were counterstained with DAPI. Slides were viewed with an Olympus BX51 fluorescence microscope (Olympus, Japan). For results analysis, the Image-Pro-Plus software was performed to quantify the intensity of staining. The integrated optical density (IOD) and area of interest (AOI) of all the positive staining (intense red staining) were measured, respectively. The mean density (IOD/AOI) was then calculated.

Table 1 Primer sequences used for qRT-PCR

Gene name	Forward (F) and Reverse (R) Primer sequence (5'-3')	Product size
Mi-2	F:CCCGAGGAGTGTGGAACTA	62bp
	R:CCCTACCACCCTAGCCAAG	
HARS	F:GATGGGATGTTTGCTGTCTG	114bp
	R:TCCCACCATCTCATTCTTCA	
Ku-70	F:GACAGCAGGAAGTGGAGAC	91bp
	R:GCCACGAACAGAGTCTTGAA	
TLR3	F:TCTGTTTGCGAAGAGGAATG	114bp
	R:AATTCCGAGATCCAAGTGCT	
H-2K^b	F:TGCTCGCTGTATTCTTGGTG	169bp
	R:GGCTCCTTCTGTCGAGTGAC	
GAPDH	F:CAATGTGTCCGTCGTGGATCT	124bp
	R:GTCCTCAGTGTAGCCCAAGAT	

Statistical analysis

All data were expressed as mean ± standard deviation (SD). One-way ANOVA was used for multiple comparisons (Duncan's multiple range test) using SPSS ver.13.0 software. P values<0.05 were considered as statistically significant.

Results

Histological features of muscle inflammation induced by intramuscular hydrogel/C protein injection

Recombinant human fast-type skeletal muscle C protein fragments were prepared using a prokaryotic expression system according to Kohsaka's method[5] (Fig.1). C protein was mixed with hydrogel before the injection. Histological analysis showed that, C protein injection i.m. induced a conspicuous mononuclear cell infiltration in TA muscle of WT mice at the day 7, and a small number of myofiber necrosis. However, C protein induced inflammation response disappeared quickly. At the day 15, no inflammatory infiltration and muscle degeneration, was observed (Fig.2). In contrast, the persistent inflammatory response can be observed in TA muscle injected with hydrogel or the mixture of hydrogel and C-protein during two months, and inflammation of

the muscle tissue peaked on day 30 after the injection. We observed that, the injection of the mixture of hydrogel and C protein induced more severe inflammatory infiltration and myofiber necrosis than hydrogel injection alone at the day 15 and 30. Moreover, at the day 60, mononuclear cell infiltration was absent from muscle treated with hydrogel alone, but still can be observed in perimysial site of TA muscle treated with the mixture of hydrogel and C protein (Fig.2).

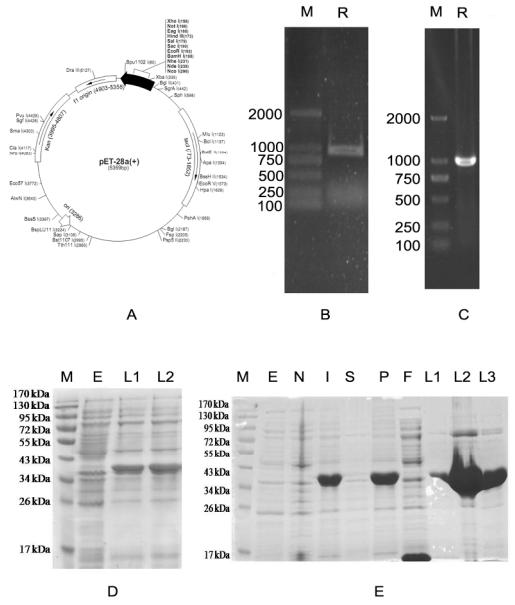

Fig.1 Preparation of recombinant human skeletal muscle C protein. (A)The pET-28a(+) vectors. It carries an N-terminal His•Tag®/thrombin/T7•Tag® configuration plus an optional C-terminal His•Tag sequence. Unique sites are shown on the circle map. (B)PCR amplification of cDNA fragments of muscle C protein. M, DL2000 DNA Marker; R, PCR result (891 bp). (C)PCR identification of positive clone. M, DL2000 DNA Marker; R, PCR product. (D)Expression of Cloned Gene [BL21(DE3)-pET28a-C-protein]. M, prestained protein molecular weight marker; E, BL21(DE3)-pET28a; L1 and L2, BL21(DE3)-pET28a-C-proteins; (E)Purification of C protein. M, protein marker; E, BL21(DE3)-pET28a; N, with no IPTG induction; I, IPTG induction; S, supernant; P, precipitate; F, flowthrough; L1, L2, and L3, eluted proteins.

Investigation of the expression levels of muscle autoantigens and TLRs in TA muscle treated with hydrogel/C protein

Toll-like receptors (TLRs) or of potential muscle autoantigens represent the important immune/inflammatory mediators in damaged muscle. Since the concomitant up-regulation of TLRs or of autoantigens could potentially aggravate muscle inflammation and damage in immune-mediated muscle disease by stimulating cytokines and chemokines production, as well as the activation of autoimmune T cells[12-14], in this study, we explored whether intramuscular injection of the mixture of hydrogel and C protein, or of hygdrogel alone, may influence on the expression of muscle autoantigens and TLRs.

Although DNA-PKcs and TLR7 expression has no change (data not shown), we found most of autoantigens (Mi-2, HRS and Ku-70) and TLR3 up-regulated in the inflammatory TA induced by hydrogel injection i.m., compared to that of normal muscle. As shown in Figure 3, mRNA levels of Mi-2, HRS, Ku-70 and TLR3

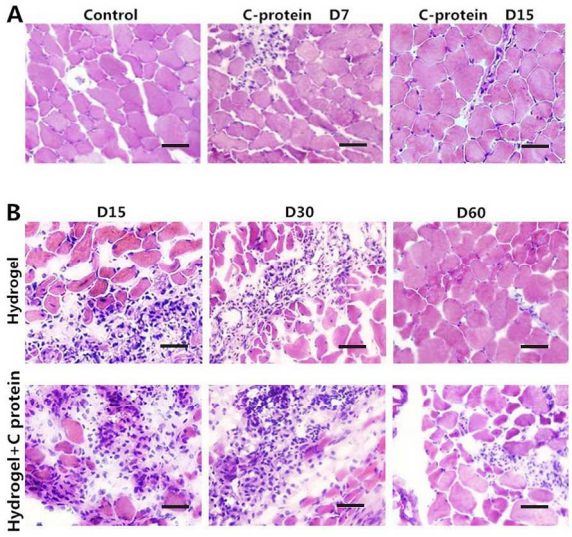

Fig.2 Representative H&E images of tibialis anterior (TA) muscle treated with hydrogel/C protein. (A)The normal muscle, and C protein injected-muscle were detected at day 7 and 15; (B) TA muscle injected with the mixture of hydrogel and C protein, or with hydrogel alone at day 15, 30, and 60. Bar=50μm.

significantly up-regulated following the injection of the mixture of hydrogel and C protein, or of hygdrogel alone, compared to normal muscle, and reached their expression peak around day 30. It's worth noting that the expression levels of Mi-2, HRS, Ku-70 and TLR3 in TA muscle treated with the mixture of hydrogel and C-protein conspicuously higher than that of muscle treated with hydrogel alone, at the day 15 and 30. At the day 60, the mRNA levels of autoantigens and TLR3 still higher significantly in hydrogel treated muscle than the normal, however, at this detecting time point, we did not find the difference between hydrogel mixture treated and hydrogel treated muscle. This data suggested that, the injection of hydrogel alone is enough to induced chronic inflammation of TA muscle, moreover, the sustained release effects of C protein in muscle received the injection of the mixture of hydrogel and C-protein help to induce the more severe intramuscular inflammation.

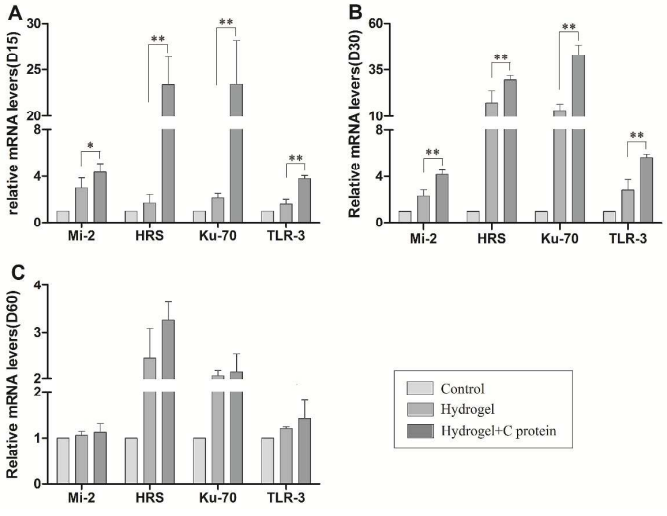

Fig.3 The mRNA expression of muscle autoantigens and TLR3 in TA muscle treated with hydrogel/C protein. mRNA levels of Mi-2, HRS, Ku-70 and TLR3 were quantified by qRT-PCR analysis in TA muscle

treated with the mixture of hydrogel and C protein, or with hydrogel alone at day 15 (A), 30 (B), and 60(C). One-way ANOVA was used for multiple comparisons. All data are presented as mean ± SD (n=3). (**p<0.01, *p<0.05).

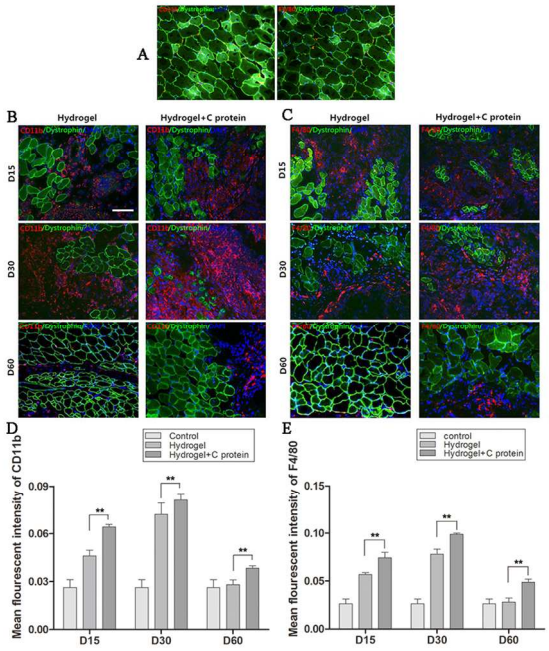

Fig.4 Hydrogel/C protein injection i.m. induces TA intramuscular infiltration of monocytes/macrophages. Representative immunofluorescence-stained muscle section (CD11b and F4/80) obtained from normal muscle (A), and hydrogel/C-protein injected muscle (B, C). Quantification of the mean fluorescent intensity values of CD11b (D) and F4/80 (E) in muscle cross sections was shown. One-way ANOVA was used for multiple comparisons. All data are presented as mean ± SD (n=10). (**p<0.01, *p<0.05). Bar = 50μm.

Hydrogel/C protein treatment induces intramuscular infiltration of monocytes/macrophages

Monocytes and macrophages dominate a basic inflammatory response in muscle that follows particular stimulus or injury[1]. Our H&E staining had evidenced the persistent inflammatory infiltration in TA muscle injected with hydrogel or the mixture of hydrogel and C protein during two months (Fig.2). We next investigated the details of hydrogel/C protein injection-induced intramuscular infiltration of monocytes and macrophages. We conducted immunostaining to assess the infiltration of $CD11b^+$ and $F4/80^+$ cells in treated and intact muscles of B6 mice on day 15, 30 and 60 post-injection. Dystrophin staining was used to identify myofibers from infiltrated mononuclear cells. The extent of infiltration was evaluated by fluorescent staining intensity analysis [The integrated optical density (IOD) and area of interest (AOI) of all the positive staining (intense red staining) were measured, respectively. The mean density (IOD/AOI) was then calculated. For every TA sample, we selected 10 slices from the serial sectioned TA muscle, and measured 3 AOI for every slice]. As shown in figure 4, the number of $CD11b^+$ and $F4/80^+$ cells increased significantly in TA muscles following the injection of the mixture of hydrogel and C-protein, or of hygdrogel alone, compared to normal muscle, at the day 15 and 30, and the number of positive cells reached the peak at day 30. The mean value of fluorescence intensity (red color of Rhodamine for CD11b or F4/80) of infiltrated monocytes/macrophages in TA muscle treated with the mixture of hydrogel and C protein conspicuously higher than that of muscle treated with hydrogel alone, on day 15 and 30 post-injection. Consistent with our histologic analysis, at the day 60, $CD11b^+$ and $F4/80^+$ cells can still be observed in perimysium of TA muscle treated with the mixture of hydrogel and C protein, but absent in hydrogel treated muscle.

Intramuscular infiltration of $CD8^+$ T cell and MHC-I expression on new myofibers after hydrogel/C protein injection

$CD8^+$ T cell-mediated and class I major histocompatibility complex (MHC) restricted autoimmune attack of myofibers is the distinct histopathologic feature of chronic inflammatory myopathies[15]. Muscle autoantigens have been shown to induce migration of T lymphocytes to the endomysium or perimysium after myoinjury, and MHC-I gene expression on regenerated myofibers is a possible reason of intramuscular $CD8^+$ T cells infiltration[16]. We have found the injection i.m. of hydrogel/C protein up-regulated mRNA levels of muscle autoantigens, we next wish to find out whether hydrogel material is involved in the myofiber MHC-I expression and intramuscular $CD8^+$ T cell infiltration.

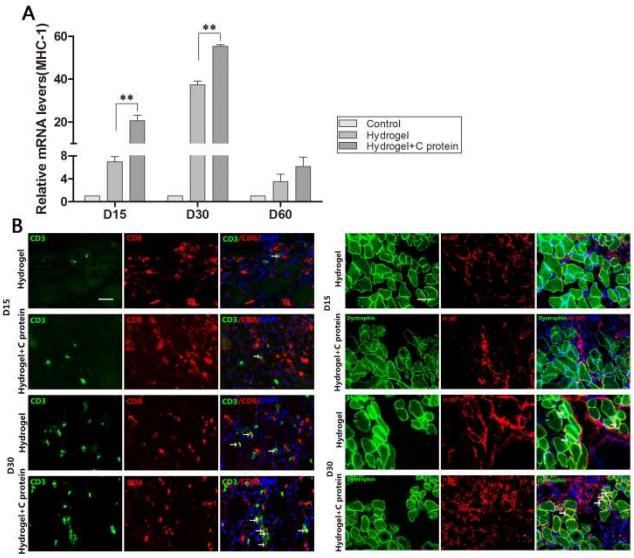

Fig.5 Intramuscular infiltration of $CD8^+$ T cell and MHC-I expression on new myofibers after hydrogel/C protein injection. (A) mRNA levels of MHC-I in TA muscle treated with hydrogel/C protein at day 15, 30 and 60. (B) The number of $CD3\varepsilon^+CD8\alpha^+$ T cells and of $H\text{-}2K^{b+}$ myofiber increased after hydrogel/C protein treating on day 30. Arrows indicate $CD3\varepsilon^+CD8\alpha^+$ T cells and $H\text{-}2K^b$-positive myofibers. Bar= 50μm.

Firstly, we detected MHC-I expression in hydrogel/C protein treated muscle tissue, and found mRNA levels of MHC-I significantly up-regulated following the injection of the mixture of hydrogel/C protein, or of hygdrogel alone, with their expression peak around day 30. As expectedly, MHC-I expression level in TA muscle treated with hydrogel/C protein mixture conspicuously higher than that of muscle treated with hydrogel alone, at the day 15 and 30 (Fig.5A). Next, we conducted double immunostaining to assess the presence of $CD3\varepsilon^+CD8\alpha^+$ T cells, and MHC-I molecule $H\text{-}2K^b$-expressing myofibers on TA muscles on day 15 and 30 post-injection.

Sarcolemmal H-2K^b expression was assessed by comparison to basement membrane staining with Dystrophin. As shown in figure 5B, the number of CD3ε$^+$CD8α$^+$ T cells and H-2Kb$^+$ myofiber were rather less in hydrogel injected TA muscle (0-1/per slide), no matter adding C protein or not, on day 15 post-injection. Interestingly, on day 30, hydrogel treatment resulted in an increased number of H-2K^b positive myofibers (3-5/per slide), and of the infiltrated CD3ε$^+$CD8α$^+$ T cells in TA muscle. However, we did not found the difference of the number of CD8$^+$ T cell and H-2K^{b+} myofibers between muscle injected with hydrogel/C protein mixture, and with hydrogel alone. All together, these results suggest the induction of TA muscle-injected hydrogel on CD8$^+$ T cell intramuscular infiltration, and imply that hydrogel treatment induces chronic skeletal muscle inflammation.

Discussion

Various pathological conditions, such as primary or acquired myopathies, can lead to considerable intramuscular inflammation and myofiber degeneration. It is necessary to develop animal models of muscle injury to study the process of muscle inflammation in a controlled and reproducible way. The use of myotoxins such as bupivacaine (Marcaine) [17], cardiotoxin (CTX) [18], and notexin (NTX)[19] is perhaps the easiest and most reproducible way to induce muscle injury， however, the potentially unknown effects of this toxin on various muscle cell types including satellite cells is a potential "caveat" to this protocol. The other ways developed for triggering the process of muscle inflammation and regeneration, involve in the repeated bouts of intensive exercise, the direct infliction of a wound by crushing and/or freezing the muscle, or the denervation-devascularization by transplantation of a single muscle[2,20,21]. Experimental chronic myoinjury model of mouse involved in: i) experimental autoimmune myositis (EAM), established as an model of polymyositis (PM), is inducible specifically in SJL/J mice by repeated administration of muscle homogenate or partially purified myosin. Immunohistochemical studies have shown that infiltrating T cells in the muscle are dominated by CD4$^+$ T cells[3]; ii) C protein–induced myositis (CIM) that can be induced with a single injection of a recombinant skeletal muscle fast-type C protein. Functional studies for CIM indicate that cytotoxic CD8$^+$ T cells are primarily responsible for the pathologic mechanisms of this disease [5].

Hydrogels are a class of hydrophilic biomaterials made of water-soluble components exhibiting high porosity and high water uptake. Their structure is basically a network of macromolecules that can be stabilized either by chemical or physical cross-linking. By mimicking native ECM, synthetic hydrogel polymers hold enormous potential in biological applications[7,8,22]. As well, cell- or bio-compatibility of polymers have been promoted by chemical modifications of a synthetic surface with biomimetic moieties like peptides, receptor–ligand fragments, or signalling molecules[23,24]. In the field of skeletal muscle regeneration, synthetic hydrogel polymers are taken as scaffolds to embed myoblasts to promote the regeneration and reconstruction of a partially ablated skeletal-muscle injury[25-27]. Using poly amidoamines (PAAs) with disulfide linkages as the backbone of the crosslinker to improve bio-compatibility and biodegradability, the team of Malcolm M. Q. Xing fabricates a 3D hydrogel system by copolymerizing it with N-isopropyl acrylamide (NIPAM). This new PNIPAM hydrogel is temperature-responsive, injectable and biocompatible[11]. In this study, we focused on this new injectable hydrogel, and attempt to explore whether the implantation of PNIPAM hydrogel into TA muscle of B6 mice will trigger muscle injury and inflammation. We investigated the features of inflammation response induced by PNIPAM hydrogel, and demonstrated the effects of the sustained-release capability of PNIPAM hydrogel to C protein on muscle inflammation.

Adopting histological and immunofluorecent evaluation of intramuscular infiltration of monocytes/macrophages, and qRT-PCR analysis of muscle autoantigens (Mi-2, HRS and Ku-70) and TLR3 mRNA levels, we found that PNIPAM hydrogel induces a skeletal muscle inflammation during 2 months. The most severe inflammation response in hydrogel injecting area appeared on day 30, and then gradually decreased. As well, at this time point, the severe muscle degeneration can be observed (Fig.2). Our work therefore indicates that PNIPAM hydrogel has immunogenic behaviour *in vivo*, and this material provides a possibility to induce a persistent muscle inflammation. Because of acidification and the formation of proinflammatory fragments upon degradation *in vivo*, hydrogel-based ECM system has been used to yield potent immune responses in mice (e.g., hydrogel vaccine adjuvant enhancing antigen-specific immune responses)[28,29]. We presume that, the muscle injury and inflammation were initiated by the degradation elements of the PNIPAM hydrogel, or the local proinflammatory environment produced by the hydrogel.

Giving the extended-release capacity, currently, hydrogels have been used to delivery of therapeutic molecules, such as hepatocyte growth factor (VEGF)[30], hepatocyte growth factor (HGF)[31], stromal cell-derived factor-1 (SDF-1)[32], and so on. We next studied the extended-release capacity of hydrogel as a carrier for C protein on the effects of muscle-inflammation response. C protein is the myosin-binding protein in the cross-bridge–bearing zone of A bands of myofibrils. Biochemical purification studies showed that C protein appears to be the main immuno-pathogenic component of the crude skeletal-muscle myosin preparation used for the induction of experimental myositis[33]. C protein induced muscle inflammation maintained about 50 days after treatment, even though C protein was emulsified in Freund's complete adjuvant (CFA) containing mycobacterium butyricum and pertussis toxin (PT), for augmenting and extending its immune response[34]. In our work, C protein was firstly injected i.m. alone, and we detected a severe but temporal mononuclear cells infiltration in TA muscle (2 weeks). However, hydrogel injection induced-muscle inflammation sustained about one month. Interestingly, the injection i.m. of the mixture of PNIPAM hydrogel/C protein initiated a more severe and persistent muscle inflammatory response, because on the day 60 after the injection, we still observed cellular infiltration in perimysial site of TA muscle (Fig.2,4). Thus, we assume that, in treated TA muscle, C protein was slowly released from PNIPAM hydrogel, and may has synergistic effect with hydrogel, to stimulate a long-lasting muscle inflammation.

For chronic inflammatory myopathy (such as polymyositis and inclusion body myositis), CD8$^+$ T cells are most abundant in the

endomysial site and invade non-necrotic muscle fibers, as well, surface expression of class I major histocompatibility complex (MHC-I) molecules on muscle fibers is up-regulated[15,16]. Using fluorescent staining to detect MHC-I (H2-K^b) expression in damaged muscle of B6 mice treating with PNIPAM hydrogel/C protein, we observed an obviously increased number of H-$2K^b$ positive myofibers on day 30 post-injection. In accord with this result, we found the increased number of $CD3\varepsilon^+CD8\alpha^+$ T cells in the injecting site at the same detecting time point. We therefore confirm that the PNIPAM hydrogel induced muscle inflammation featured by the $CD8^+$ T cell infiltration and the increased class I MHC–positive regenerating myofibers. We assume the inflammatory T cell infiltration is initiated by hydrogel itself, or by its degradation products, not by C protein, because we did not find the significant differences for the number of T cell and H-$2K^b$ positive myofibers between hydrogel alone and the mixture of hydrogel/C protein treatment.

Conclusions

In a summary, in this study, we used the injectable PNIPAM hydrogel as a new bio-driver to induce chronic skeletal muscle inflammation response. Our work holds a new promise for preparing animal model of muscle injury, which maybe a useful tool for investigating pathologic mechanisms of muscle inflammation and regeneration.

Acknowledgements

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Notes and references

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1 James G.Tidbal, *Am J Physiol Regul Integr Comp Physiol*,2005, **288**, 345-353.

2 J. P. Lefaucheur and A. Sebille, *Neuromuscul Disord*, 1995, **5**, 501-509.

3 N. L. Rosenberg, S. P. Ringel and B. L. Kotzin, *Clin Exp Immunol*, 1987, **68**, 117-129.

4 S. Matsubara, T. Shima and M. Takamori, *Acta Neuropathol*, 1993, **85**, 138-144.

5 T. Sugihara, C. Sekine, T. Nakae, K. Kohyama, M. Harigai, Y. Iwakura, Y. Matsumoto, N. Miyasaka and H. Kohsaka, *Arthritis Rheum*, 2007, **56**, 1304-1314.

6 P. Ferruti, S. Bianchi, E. Ranucci, F. Chiellini and A. M. Piras, *Biomacromolecules*, 2005, **6**, 2229-2235.

7 A. M. Kloxin, C. J. Kloxin, C. N. Bowman and K. S. Anseth, *Adv Mater*, 2010, **22**, 3484-3494.

8 L. S. Wang, J. E. Chung, P. P. Chan and M. Kurisawa, *Biomaterials*, 2010, **31**, 1148-1157.

9 A. K. Lynn, I. V. Yannas and W. Bonfield, *J Biomed Mater Res B Appl Biomater*, 2004, **71**, 343-354.

10 M. P. Lutolf, F. E. Weber, H. G. Schmoekel, J. C. Schense, T. Kohler, R. Muller and J. A. Hubbell, *Nat Biotechnol*, 2003, **21**, 513-518.

11 J. B. Shi, J. Ouyang, Q. T. Li, L. Y. Wang, J. Wu, W. Zhong and M. Q. Xing, *J. Mater. Chem.*, 2012, **22**, 23952–23962

12 L. Casciola-Rosen, K. Nagaraju, P. Plotz, K. Wang, S. Levine, E. Gabrielson, A. Corse and A. Rosen, *J Exp Med*, 2005, **201**, 591-601.

13 A. Tournadre, V. Lenief and P. Miossec, *Arthritis Rheum*, 2010, **62**, 2144-2151.

14 Z. E. Betteridge, H. Gunawardena and N. J. McHugh, *Curr Opin Rheumatol*, 2009, **21**, 604-609.

15 M. C. Dalakas and R. Hohlfeld, *Lancet*, 2003, **362**, 971-982.

16 A. M. Emslie-Smith, K. Arahata and A. G. Engel, *Hum Pathol*, 1989, **20**, 224-231.

17 E. C. Hall-Craggs, *Exp Neurol*, 1974, **43**, 349-358.

18 A. D'Albis, R. Couteaux, C. Janmot, A. Roulet and J. C. Mira, *Eur J Biochem*, 1988, **174**, 103-110.

19 J. B. Harris and M. A. Johnson, *Clin Exp Pharmacol Physiol*, 1978, **5**, 587-600.

20 E. Schultz, D. L. Jaryszak and C. R. Valliere, *Muscle Nerve*, 1985, **8**, 217-222.

21 A. Irintchev and A. Wernig, *Cell Tissue Res*, 1987, **249**, 509-521.

22 J. P. Vacanti, M. A. Morse, W. M. Saltzman, A. J. Domb, A. Perez-Atayde and R. Langer, *J Pediatr Surg*, 1988, **23**, 3-9.

23 M. Chanana, A. Gliozzi, A. Diaspro, I. Chodnevskaja, S. Huewel, V. Moskalenko, K. Ulrichs, H. J. Galla and S. Krol, *Nano Lett*, 2005, **5**, 2605-2612.

24 J. Chen, C. Wu and D. Oupicky, *Biomacromolecules*, 2009, **10**, 2921-2927.

25 J. P. Beier, J. Stern-Straeter, V. T. Foerster, U. Kneser, G. B. Stark and A. D. Bach, *Plast Reconstr Surg*, 2006, **118**, 1113-1121, 1122-1124.

26 L. Boldrin, A. Malerba, L. Vitiello, E. Cimetta, M. Piccoli, C. Messina, P. G. Gamba, N. Elvassore and P. De Coppi, *Cell Transplant*, 2008, **17**, 577-584.

27 C. A. Rossi, M. Flaibani, B. Blaauw, M. Pozzobon, E. Figallo, C. Reggiani, L. Vitiello, N. Elvassore and P. De Coppi, *Faseb J*, 2011, **25**, 2296-2304.

28 A. K. Lynn, I. V. Yannas and W. Bonfield, *J. Biomed. Mater. Res., Part B*, 2004, **71**, 343–354.

29 Noh KH, Park YM, Kim HS, Kang TH, Song KH, Lee YH, Byeon Y, Jeon HN, Jung ID, Shin BC, Lee KM, Seong SY, Han HD, Kim TW, *BMC Immunol.* 2014,**15**,48.

30 J. Lee, S. H. Bhang, H. Park, B. S. Kim and K. Y. Lee, *Pharm Res*, 2010, **27**, 767-774.

31 E. Ruvinov, J. Leor and S. Cohen, *Biomaterials*, 2010, **31**, 4573-4582.

32 D. Kuraitis, P. Zhang, Y. Zhang, D. T. Padavan, K. McEwan, T. Sofrenovic, D. McKee, J. Zhang, M. Griffith, X. Cao, A. Musaro, M. Ruel and E. J. Suuronen, *Eur Cell Mater*, 2011, **22**, 109-123.

33 Kohyama K and Matsumoto Y, *J Neuroimmunol*, 1999, **98,** 130–135.

34 Sugihara T, Okiyama N, Suzuki M, Kohyama K, Matsumoto Y, Miyasaka N, Kohsaka H, *rthritis Rheum.* 2010 ,**62**,3088-3092.

In this work, we used the injectable PNIPAM hydrogel as a new bio-driver to induce chronic skeletal muscle inflammation response.