BMSC Transplantation Aggravates Inflammation, Oxidative Stress, and Fibrosis and Impairs Skeletal Muscle Regeneration

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Skeletal muscle contusion is one of the most common muscle injuries in sports medicine and traumatology. Bone marrow mesenchymal stem cell (BMSC) transplantation has been proposed as a promising strategy to promote skeletal muscle regeneration. However, the roles and underlying mechanisms of BMSCs in the regulation of skeletal muscle regeneration are still not completely clear. Here, we investigated the role of BMSC transplantation after muscle contusion. BMSCs were immediately transplanted into gastrocnemius muscles (GMs) following direct contusion. Comprehensive morphological and genetic analyses were performed after BMSC transplantation. BMSC transplantation exacerbated muscle fibrosis and inflammation, as evidenced by increased leukocyte and macrophage infiltration, increased inflammatory cytokines and chemokines, and increased matrix metalloproteinases. BMSC transplantation also increased muscle oxidative stress. Overall, BMSC transplantation aggravated inflammation, oxidative stress and fibrosis and impaired skeletal muscle regeneration. These results, shed new light on the role of BMSCs in regenerative medicine and indicate that caution is needed in the application of BMSCs for muscle injury.

Keywords: bone marrow mesenchymal stem cells, skeletal muscle, regeneration, inflammation, oxidative stress, fibrosis

INTRODUCTION

Severe skeletal muscle injuries are commonly observed inclinics of sports medicine and traumatology (Liu et al., 2018). However, there are no effective strategies for treating skeletal muscle injuries. Conservative treatment, such as "rest, ice, compression and elevation" are insufficient for muscle injury repair (Natsu et al., 2004), and muscle fibrosis and dysfunction are commonly

Abbreviations: BMSCs, Bone marrow mesenchymal stem cells; CCL2, Chemokine (C-C motif) ligand 2; CCL5, Chemokine (C-C motif) ligand 5; CCR2, C-C chemokine receptor type 2; CXCR4, C-X-C chemokine receptor type 4; Fn14, fibroblast growth factor-inducible protein 14; GMs, gastrocnemius muscles; H&E, Hematoxylin and Eosin; IFN-γ, Interferon gamma; IL-1β, Interleukin 1 beta; IL-6, Interleukin 6; MMP-1, Metalloproteinase-1; MMP-2, Metalloproteinase-2; MMP-9, Metalloproteinase-9; MMP-10, Metalloproteinase-10; MMP-14, Metalloproteinase-14; PBS, phosphate buffer solution; RT-PCR, Real-Time Polymerase Chain Reaction; TGF-β, Transforming growth factor beta; TWEAK, TNF-related weak inducer of apoptosis.

observed after severe muscle injury, such as contusion, in patients following a conservative treatment protocol (Xiao et al., 2016b).

Recently, stem cell transplantation has been proposed as a promising treatment for various muscle diseases, such as skeletal muscle injury (Aldahmash et al., 2012; Farjah et al., 2018). Muscle satellite cells are generally used as a source of skeletal muscle stem cells to treat muscle injury or muscle dystrophy (Bashir et al., 2014). However, skeletal satellite cells are relatively rare in skeletal muscle tissue, and they often lose their myogenic potential after *in vitro* expansion (Sassoli et al., 2012). BMSCs have higher proliferative potential and pluripotency and lower rates of donor site morbidity than common satellite cells (Winkler et al., 2009).

Bone marrow mesenchymal stem cells can also effectively differentiate into skeletal muscle cells both *in vivo* and *in vitro* (Galli et al., 2014). Several studies have demonstrated that transplantation of mesenchymal stem cells derived from bone marrow promotes muscle regeneration and accelerates the functional recovery of injured skeletal muscle (Winkler et al., 2008; von Roth et al., 2012b, 2013). However, the mechanism responsible for the beneficial effects on in skeletal muscle regeneration after transplantation of BMSCs remains to be investigated. Moreover, BMSCs have been used to treat muscle atrophy (Geng et al., 2009), toxicant injection-induced muscle injury (Dezawa et al., 2005; de la Garza-Rodea et al., 2011), traumatic muscle injury (Merritt et al., 2010), crush trauma (Winkler et al., 2012), and laceration (Natsu et al., 2004). Here, we investigated the role of BMSCs in regulating skeletal muscle regeneration after contusion.

MATERIALS AND METHODS

Animals

Eighty-eight male C57BL/6J mice weighing 18.1–21.3 g at 7 weeks of age were obtained from Shanghai Jiesijie Laboratory Animal Co., Ltd. After acclimatization to the local environment for 1 week, the mice were divided into the following three groups: normal control mice without muscle injury (group 1), muscle contusion mice treated with vehicle (group 2), and muscle contusion mice treated with BMSCs (group 3). The animals were housed at a constant temperature of 25°C with free access to pellet food and water. The study was approved by the Ethics Review Committee for Animal Experimentation of the Shanghai University of Sport, Shanghai, China (reference number 2016006).

Isolation and Culture of BMSCs

Tibia and femur bones were harvested from male C57BL/6J male mice. Bone marrow was flushed from the tibia and femur bones with DMEM complete medium. Cells were cultured without disturbance for 24 h, were washed to remove non-adherent cells, and were supplied with fresh DMEM complete medium, with medium renewal every 3 days (Leroux et al., 2010; Su et al., 2014).

Generation of Mouse Hind Limb Injury

The mice were anesthetized with 400 mg/kg chloral hydrate administered intraperitoneally. The hind limb contusion was operatively induced as previously described with a simple pendulum device. Briefly, the hind limb was positioned by extending the knee and plantarflexing the ankle to 90°. A 16.8 g (diameter, 15.9 mm) stainless steel ball was dropped from a height of 125 cm through a tube (interior diameter of the tube, 16 mm) onto an impactor with a surface of 28.26 mm^2, resting on the middle of the gastrocnemius muscle (GM) of the mice. The muscle contusion created by this method was a high-energy blunt injury that created a large hematoma, which was followed by muscle regeneration, a healing process that is very similar to that observed in humans (Liu et al., 2016, 2018; Xiao et al., 2016a).

BMSCs Intramuscular Injection

Bone marrow mesenchymal stem cells were collected, washed twice in PBS, and resuspended in PBS. Either 1×10^6 BMSCs or PBS was injected into the injured muscle. Cell injections were performed with a 27-gauge needle immediately after muscle injury by direct intramuscular injection into the middle point of the gastrocnemius muscle. The GMs were harvested from the mice 3, 6, 12, and 24 days after the treatment for further analyses (Leroux et al., 2010).

Flow Cytometry

Flow cytometry was performed on a Cytomics™ FC 500 System (Beckman Coulter) using a blue laser (488 nm). The culture medium was removed, and BMSCs were washed twice resuspended in PBS at a concentration of $1x10^5$ cells/mL, and stained with the following monoclonal antibodies: CD29-phycoerythrin (PE), CD44 (PE), at a concentration of 0.2 mg/mL, CD11b (FITC) and CD45 (FITC), at a concentration of 0.5 mg/mL, and isotype controls for FITC and PE (both from Biolegend, San Diego, CA, United States). Cells were incubated in the dark for 30 min at room temperature. The cells were washed with 2 mL of PBS and resuspended in 300 μL of PBS for image acquisition. A minimum of 10,000 events were counted for each analysis.

Hematoxylin and Eosin (H&E) Staining

Skeletal muscle sections (8 μm) were cut from the mid-belly region of the gastrocnemius muscle and stained with H&E to evaluate the general morphology of the skeletal muscle regeneration.

Masson's Trichrome Staining

Masson's trichrome staining was used to measure the area of fibrotic tissue in the injured skeletal muscle. The collagen fibers were stained blue, the nuclei were stained black, and the background was stained red. After this staining procedure, the ratio of the fibrotic area to the total cross-sectional area was calculated to estimate fibrosis formation using ImageJ 1.44 (NIH, Bethesda, MD, United States).

Real-Time Polymerase Chain Reaction

The total RNA of the skeletal muscle was isolated using a modified guanidinium isothiocyanate-CsCl method (Liu et al., 2018). RNA was reverse transcribed into cDNA using a

commercially available kit (RevertaidTM First Strand cDNA Synthesis Kit, Thermo Scientific). Quantitative PCR was carried out in triplicate via reactions utilizing 10 µL of $2 \times$ Maxima SYBR Green/ROX qPCR Master mix (Vazyme), 1 µL of cDNA, nuclease-free water and 300 nM of each primer (**Table 1**).

Statistical Analysis

The data were presented as the mean $\pm$ SD and analyzed using SPSS 20.0. The mean values of the genetic data were compared using repeated-measure analysis. *Post hoc* multiple comparisons were performed using the Bonferroni test. The data of the scar tissue area were compared using an independent samples *t*-test. Differences between values were considered statistically significant when P-values were less than 0.05.

RESULTS

The Characterization and Cell Surface Antigens of BMSCs

The cultured BMSCs were fibroblast-like cells and the BMSCs formed homogenous colonies. Most of the BMSCs were had clear cellular boundaries (**Figure 1A**). FC analysis showed that BMSCs had high expression levels of CD29, CD44, Sca-1 and low expression of CD11b and CD45 (**Figures 1B–I**). The general morphological characteristics and the expression of relevant cell surface markers were consistent with the criteria used to define mesenchymal stem cells by the International

Society for Cellular Therapy (ISCT) (Dominici et al., 2006; Carvalho et al., 2008).

Transplantation of BMSCs Impaired Skeletal Muscle Regeneration

We found that the BMSC treated mice showed irregular lumps in their GMs, while irregular lumps were absent in the vehicle-treated group. In addition, the gastrocnemius muscle mass of the BMSCs treated mice was significantly larger than that of the gastrocnemius muscle collected from the vehicle-treated mice (**Figures 2A–F**).

Hematoxylin and eosin staining was performed to evaluate whether BMSC transplantation improved skeletal muscle regeneration after injury. H&E staining showed that the GMs from the BMSCs treated mice 3 and 6 days after injury had significantly less central-nucleated regenerating muscle fibers and significantly more infiltrated leukocytes compared with the GMs collected from the corresponding vehicle treated mice. In addition, H&E staining showed that the GMs from BMSC-treated muscle does not exhibit improved morphology of the injured skeletal muscle. Rather, the BMSC treatment may have impaired muscle regeneration after contusion, as there were fewer central-nucleated regenerating myofibers and more inflammatory cells in the BMSC-treated group compared with the vehicle group 3 and 6 days after muscle injury (**Figures 3A–D**). In addition, at 12 and 24 days post-injury, the damaged area in the vehicle group had been replaced mostly by intact skeletal muscle fibers, whereas numerous necrotic myofibers dominated the injured muscle regions of the BMSC-treated mice (**Figures 3E–H**).

TABLE 1 | Primers used for qRT-PCR.

Target gene	Forward primer sequences	Reverse primer sequences
CD68	5′-CAAAGCTTCTGCTGTGGAAAT-3′	5′-GACTGGTCACGGTTGCAAG-3′
F4/80	5′-AACATGCAACCTGCCACAAC-3′	5′-TTCACAGGATTCGTCCAGGC-3′
TNF-α	5′-CTTCTGTCTACTGAACTTCGGG-3′	5′-CACTTGGTGGTTTGCTACGAC-3′
INF-γ	5′-GCTTTGCAGCTCTTCCTCAT -3′	5′-GTC ACC ATCCTTTTGCCAGT -3′
IL-1β	5′-TGACGTTCCCATTAGACAACTG -3′	5′-CCGTCTTTCATTACACAGGACA-3′
TGF-β	5′-TGCGCTTGCAGAGATTAAAA-3′	5′-CGTCAAAAGACAGCCACTCA-3′
IL-6	5′-GAACAACGATGATGCACTTGC-3′	5′-CTTCATGTACTCCAGGTAGCTATGGT-3′
Col1a1	5′-GAGCGGAGAGTACTGGATCG-3′	5′-GCTTCTTTTCCTTGGGGTTC-3′
Col3a1	5′-GTCCACGAGGTGACAAAGGT-3′	5′-GATGCCCACTTGTTCCATCT-3′
MMP-1	5′-AGTTGACAGGCTCCGAGAAA-3′	5′-CACATCAGGCACTCCACATC-3′
MMP-2	5′-ACCCTGGGAGAAGGACAAGT-3′	5′-ATCACTGCGACCAGTGTCTG-3′
MMP-9	5′-CGTCGTGATCCCCACTTACT-3′	5′-AACACACAGGGTTTGCCTTC-3′
MMP-10	5′-GAGTGTGGATTCTGCCATTGA-3′	5′-TCTCCGTGTTCTCCAACTGC-3′
MMP-14	5′-CCTGGCTCATGCCTACTTCC-3′	5′-GCACAGCCACCAAGAAGATG-3′
CCL2	5′-GCTCAGCCAGATGCAGTTAAC-3′	5′- CTCTCTCTTGAGCTTGGTGAC-3′
CCR2	5′-GAAAAGCCAACTCCTTCATCAG-3′	5′-TCTAAGCACACCACTTCCTCTG-3′
CCL5	5′-CATATGGCTCGGACACCA-3′	5′-ACACACTTGGCGGTTCCT-3′
CXCR4	5′- CAAGGCCCTCAAGACGACAG-3′	5′- CCCCCAAAAGGATGAAGGAG-3′
TWEAK	5′-GCTCCCAAAGCCCCTACTTAT-3′	5′-AGGTCCAGCCCATCTCAGT-3′
Fn14	5′-GGCGCTCTTAGTCTGGTCCT-3′	5′-TGGATCAGTGCCACACCTG-3′
gp91phox	5′-CCAGTGAAGATGTGTTCAGCT-3′	5′-GCACAGCCAGTAGAAGTAGAT-3′
GAPDH	5′-ACTCCACTCACGGCAAATTC-3′	5′-TCTCCATGGTGGTGAAGACA-3′

Transplantation of BMSCs Aggravated the Fibrosis of Contused Muscles

The BMSC-treated skeletal muscle showed significantly more fibrosis than the vehicle group 24 days after the contusion injury (7.23 ± 2.26 vs. 50.73 ± 15.5, $p < 0.01$) (**Figure 4**). Moreover, we used RT-PCR to test the expression of collagen I and III. As expected, the examination revealed that BMSC transplantation significantly increased the expression of collagen I mRNA levels at 3, 6, 12, and 24 days post-injury ($p < 0.01$) compared with the collagen I mRNA levels of the vehicle group (**Figure 5A**). The expression of collagen III mRNA in the BMSCs-treated group also increased significantly 3, 12, and 24 days after the contusion injury compared with the collagen III mRNA levels of the vehicle group (**Figure 5B**).
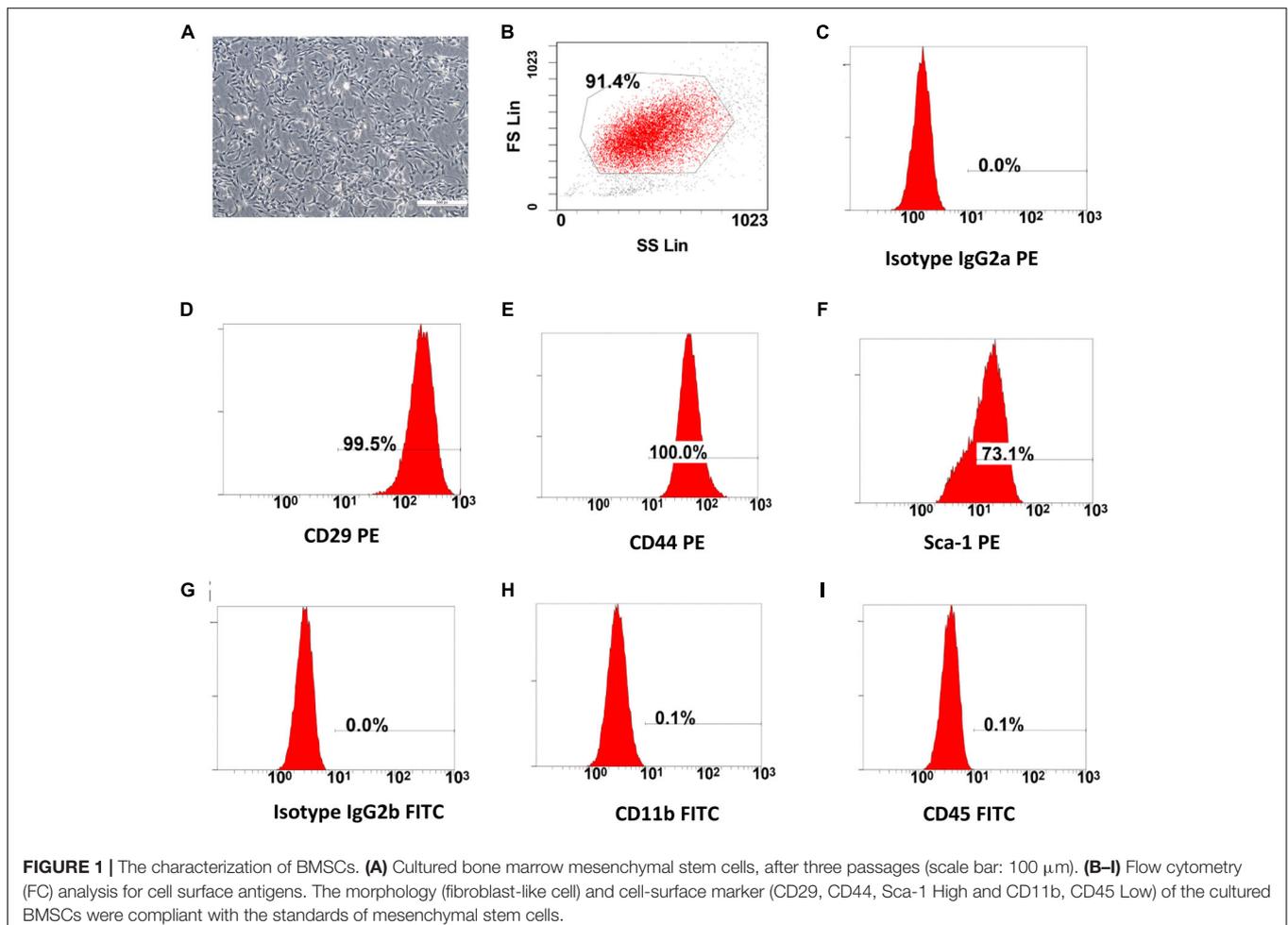
Transplantation of BMSCs Increased the Expression of Specific Markers of Macrophages in Contused Muscles

F4/80 a mouse macrophage-specific membrane marker (Starkey et al., 1987). The RT-PCR data showed that F4/80 mRNA increased significantly at the early stage of regeneration, especially in the first 6 days (**Figure 6A**). In addition, the expression of F4/80 mRNA increased in the BMSC-treated group at 3, 6, 12, and 24 days post-injury ($P < 0.05$). BMSC transplantation increased the expression of CD68, a macrophage-specific endosomal protein (da Silva and Gordon, 1999), at 12 days ($p < 0.01$) and 24 days ($p < 0.01$) after muscle contusion compared with the CD68 expression of the vehicle group (**Figure 6B**).

Transplantation of BMSCs Increased the Expression of Inflammatory Cytokines in Contused Muscles

Real-time polymerase chain reaction demonstrated that the expression levels pro-inflammatory cytokines (such as TNF-α, IL-1β, IFN-γ, IL-6, and TGF-β) significantly increased in the early stage of regeneration. With the exception of IL-6, the mRNA levels of these pro-inflammatory cytokines returned to normal 24 days post-injury. BMSC treatment significantly exacerbated the increases in these pro-inflammatory cytokines (such as TNF-α, IL-1β, IFN-γ, IL-6, and TGF-β), indicating that BMSC transplantation enhanced the inflammatory response in skeletal muscle regeneration (**Figure 7**).

FIGURE 1 | The characterization of BMSCs. **(A)** Cultured bone marrow mesenchymal stem cells, after three passages (scale bar: 100 μm). **(B–I)** Flow cytometry (FC) analysis for cell surface antigens. The morphology (fibroblast-like cell) and cell-surface marker (CD29, CD44, Sca-1 High and CD11b, CD45 Low) of the cultured BMSCs were compliant with the standards of mesenchymal stem cells.
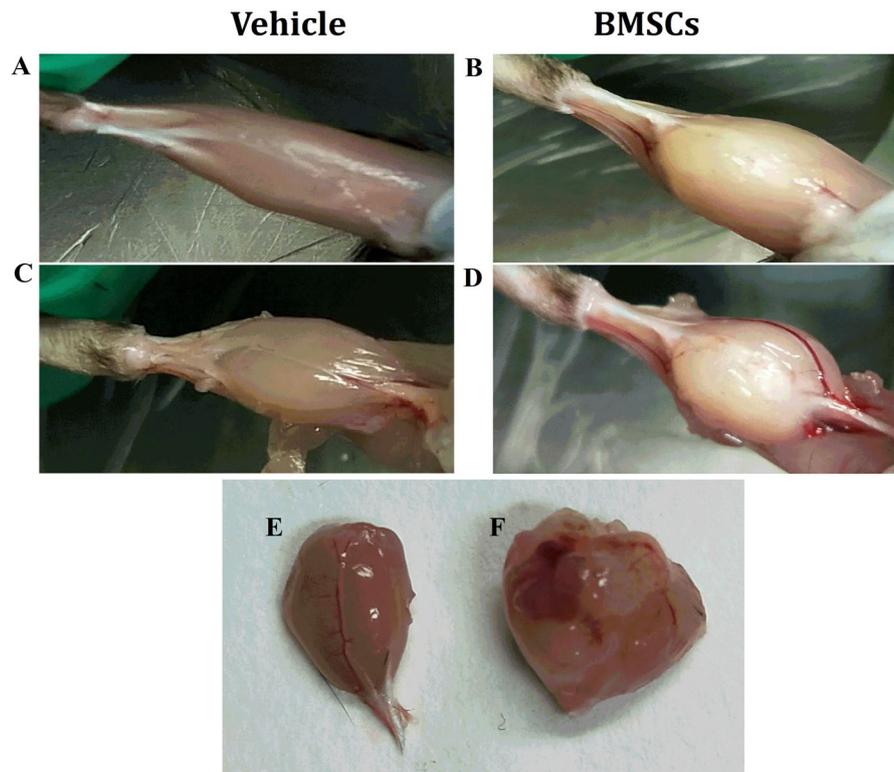
FIGURE 2 | Macroscopic appearances of GMs after BMSCs were treated. **(A)** Vehicle treated GMs at 24 days post-injury; **(B)** BMSCs treated GMs at 24 days post-injury; **(C)** Vehicle treated and fascia stripped GMs at 24 days post-injury; **(D)** BMSCs treated and fascia stripped GMs at 24 days post-injury; **(E)** harvested GMs with vehicle treated at 24 days post-injury; **(F)** Harvested GMs with BMSCs treated at 24 days post-injury; Irregular lumps were found in the BMSCs treated mice other than the vehicle treated mice.

Transplantation of BMSCs Increased the Expression of Chemokines in Contused Muscles

To understand the mechanism underlying the increased leukocyte infiltration after BMSC transplantation, we further analyzed skeletal muscle chemokines. Compared with the control group, the injured skeletal muscle showed increased content of CCL2, CCR2, CCL5, CXCR4, TWEAK, and Fn14 at the early stage of regeneration. With the exception of CCL5, the above chemokines returned to normal at 24 days post-injury. Compared with vehicle treated groups, the BMSC-treated group exhibited significantly enhanced expressions of CCL2 (297.85-fold, $p = 0.001$), CCR2 (20.51-fold, $p = 0.001$), CCL5 (76.16-fold, $p = 0.001$), CXCR4 (14.14-fold, $p = 0.001$), TWEAK (2.39-fold, $p = 0.001$) and Fn14 (38.85-fold, $p = 0.001$) in injured gastrocnemius muscle at 24 days post-injury (**Figures 8A–F**).

Transplantation of BMSCs Increased the Expression of Matrix Metalloproteinase in Injured Skeletal Muscles

The skeletal muscle injury caused significant increases in muscle MMP-1 (31.43-fold), MMP-2 (5.03-fold), MMP-9 (21.62-fold), MMP-10 (23.87-fold), and MMP-14 (20.66-fold) 6 days
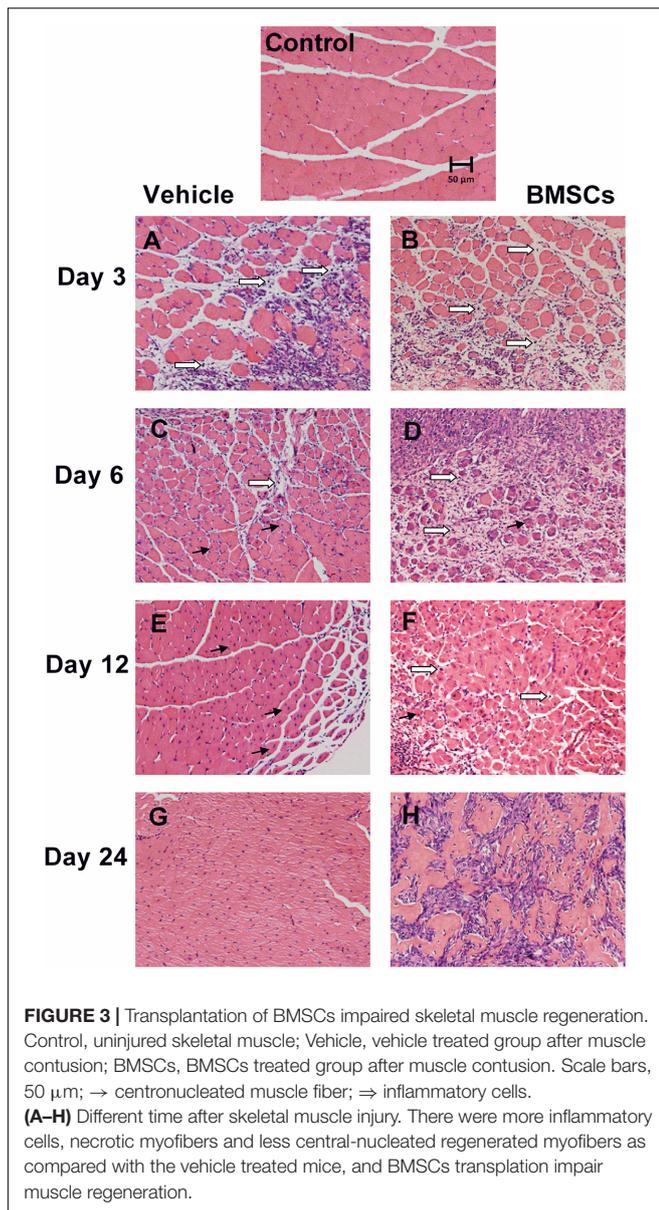
after injury, which returned to normal at 24 days post-injury. The transplantation of BMSCs resulted in significantly greater increases in MMP-1, MMP-2, MMP-9, MMP-10, and MMP-14 at 12 and 24 days ($p < 0.01$) post-injury (**Figures 9A–E**).

Transplantation of BMSCs Increased the Expression of NADPH Oxidases in Injured Skeletal Muscles

Gp91phox is a key subunit of NADPH oxidases and often used as a marker of NADPH oxidases (Xiao et al., 2013). RT-PCR data showed that gp91phox mRNA levels were significantly increased at 3, 6, and 12 days post-injury. Compared with the vehicle treated groups, transplantation of BMSCs resulted in a significant increase in gp91phox at 3 days (1.98-fold, $p = 0.001$), 6 days (2.35-fold, $p = 0.001$), 12 days (4.98-fold, $p = 0.001$) and 24 days (34.17-fold, $p = 0.001$) post-injury (**Figure 8**).

DISCUSSION

Skeletal muscle contusion is a common muscle injury in humans, which is particularly common in sport activities and high speed vehicle accidents. It is important to develop effective methods to

FIGURE 3 | Transplantation of BMSCs impaired skeletal muscle regeneration. Control, uninjured skeletal muscle; Vehicle, vehicle treated group after muscle contusion; BMSCs, BMSCs treated group after muscle contusion. Scale bars, 50 μm; → centronucleated muscle fiber; ⇒ inflammatory cells.
(A–H) Different time after skeletal muscle injury. There were more inflammatory cells, necrotic myofibers and less central-nucleated regenerated myofibers as compared with the vehicle treated mice, and BMSCs transplation impair muscle regeneration.

treat muscle injuries (Liu et al., 2016). Although muscles have a strong regenerative ability, the severity of the injury (such as contusion) might prevent complete regeneration. BMSCs have been intensively studied in the past decade as a promising therapy for many diseases. BMSCs transplantation has been proposed as a treatment for skeletal muscle injury. Although no agreement has been reached on the clinical applications of BMSCs, some reports have generated high expectations for this kind of therapeutic approach (Carvalho et al., 2008). Several studies have found the application of BMSCs to treat muscle injury, although the exact mechanisms leading to this process remain to be elucidated.

The original aim of this study was to investigate the role of BMSC transplantation in muscle regeneration after contusion. We found that BMSC transplantation impaired skeletal muscle regeneration.

Macroscopic appearances and H&E staining revealed that BMSCs treated mice showed irregular lumps, and numerous necrotic myofibers dominated the injured muscle regions after contusion. Furthermore, the Masson's trichrome staining results showed that the BMSC-treated mice exhibited significantly more fibrosis than the vehicle treated mice 24 days after the contusion injury (**Figure 4**). As is the case in most tissues, the major ECM muscle protein was collagen, of which the type I and type III isoforms dominated (Lieber and Ward, 2013). Fibrosis was demonstrated by large increases in collagen I and III in the muscle ECM (Huebner et al., 2008). Consistent with the Masson's trichrome staining results, levels of collagen I and III increased significantly in the BMSC-treated group compared with the vehicle group. Taken together, these findings suggest that transplantation of BMSCs impairs skeletal muscle regeneration and aggravates muscle fibrosis after contusion. Our results suggest that BMSC transplantation is a double-edged sword in injured skeletal muscle and that inappropriate transplantation impairs skeletal muscle regeneration. This viewpoint has been tested in other disease models, such as lung injury (Yao et al., 2018), and various cancers (Norozi et al., 2016; Lee and Hong, 2017) including leukemic (Low et al., 2015) and hepatocarcinoma (Zong et al., 2018).

To explore the mechanism related to BMSC transplantation that impairs skeletal muscle regeneration, we tested the expression of specific markers of macrophages. Macrophages play complex roles in injured skeletal muscle and are involved in muscle fibrosis (Kharraz et al., 2013; Novak et al., 2014; Wang et al., 2014; Tonkin et al., 2015; Xiao et al., 2016a). Our previous studies and other studies have suggested that increased macrophage recruitment or macrophage depletion impairs skeletal muscle regeneration (Shen et al., 2008; Wang et al., 2014; Liu et al., 2016; Xiao et al., 2016a). Interestingly, we found that F4/80 and CD68, markers of macrophages, increased significantly 12 days ($p < 0.01$) and 24 days ($p < 0.01$) after contusion in the BMSCs treated group as compared with the vehicle treated group. Secondly, we found that BMSCs transplantation increased the expression of inflammatory cytokines (such as TNF-α, IL-1β, IFN-γ, IL-6 and TGF-β) in the later stage of skeletal muscle regeneration (**Figure 7**). Indeed, disease microenvironments have profound impacts on transplanted MSC sinmediating and modulating their therapeutic effects (Sui et al., 2017). Inflammatory cytokines (especially TGF-β) modulate MSC proliferation and myofibroblast differentiation. For example, in a study by Desai et al. (2014), adipose-derived mesenchymal stem cells (ADSCs) treated with TGF-β developed a myofibroblastic phenotype with increases in a-smooth muscle actin (a-SMA), a myofibroblast marker, and the ECM proteins type I collagen and fibronectin. di Bonzo et al. (2008) found that the differentiation of transplanted BMSCs, particularly under conditions of chronic injury, into pro-fibrogenic potential cells significantly contributed to liver fibrosis. In addition, Kim et al. (2014) found that normal human prostate-derived mesenchymal stem cells (MSCs) exposed to TGF-β1 can differentiate into myofibroblasts. Myofibroblasts are the primary extracellular matrix (ECM)-secreting cells during wound healing and fibrosis, and are largely responsible
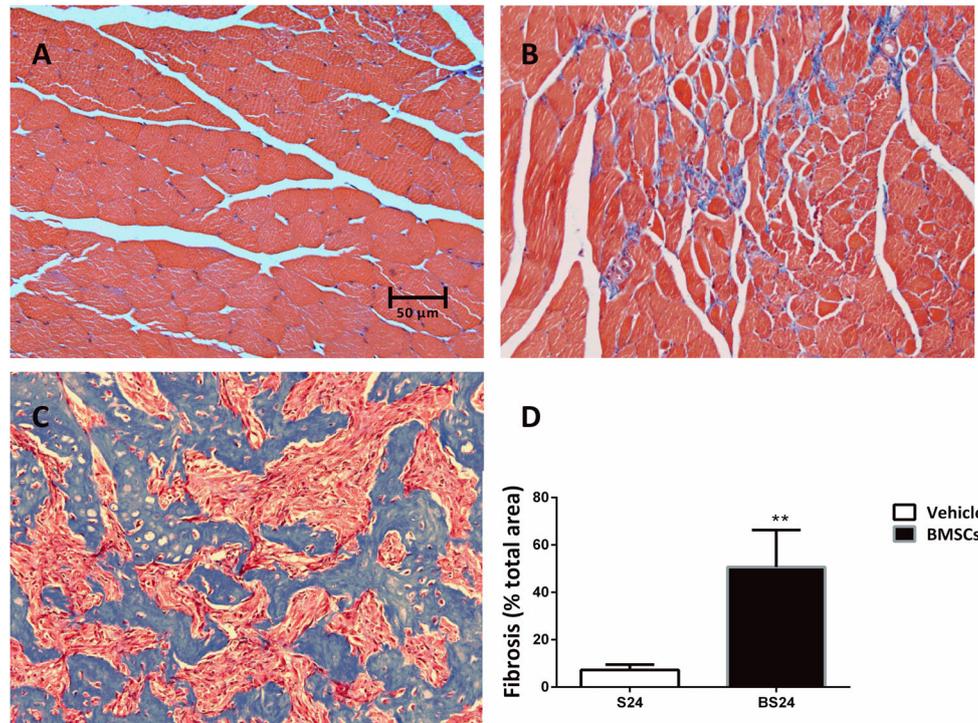
FIGURE 4 | Representative images of fibrosis formation in injured GMs. **(A)** Control muscle; **(B)** Vehicle treated group (24 days post-injury); **(C)** BMSCs treated group (24 days post-injury); **(D)** Quantification of the scar tissue area in injured GMs. Scale bars = 50 μm. Data are means ± S.D., $n = 6$. ** Significant difference from S24, **$P < 0.01$. The BMSCs treated mice showed more fibrosis than the vehicle treated mice at 24 days post-injury.
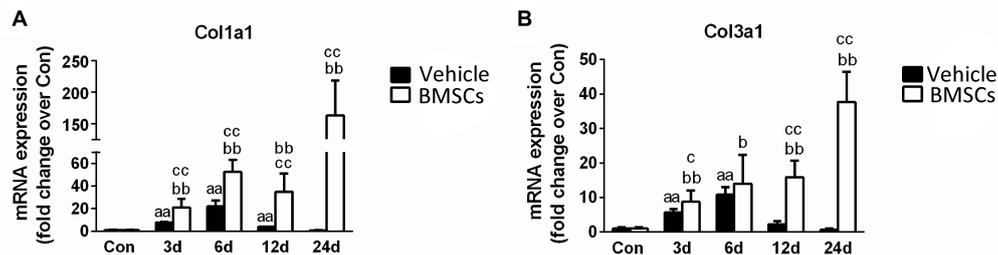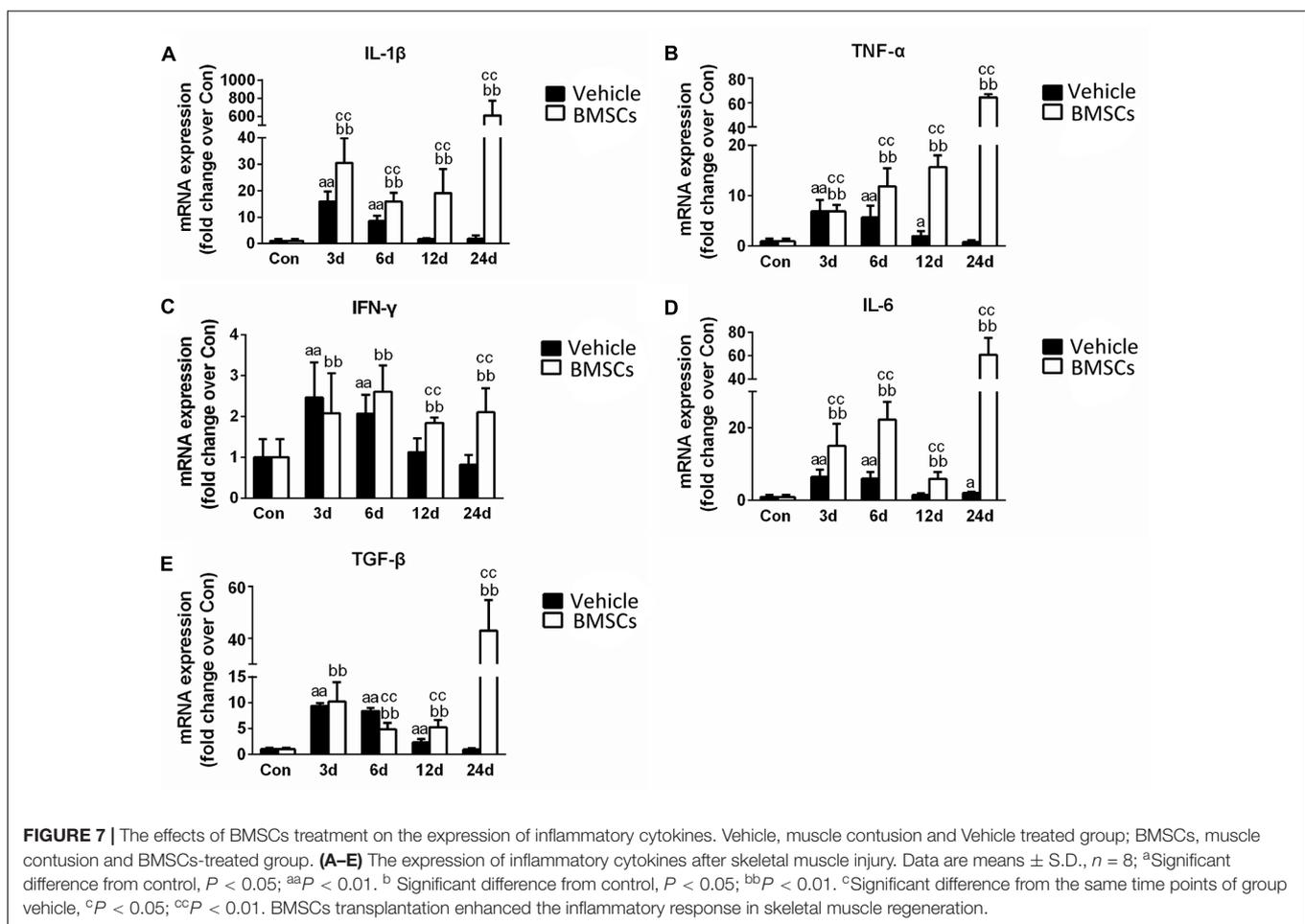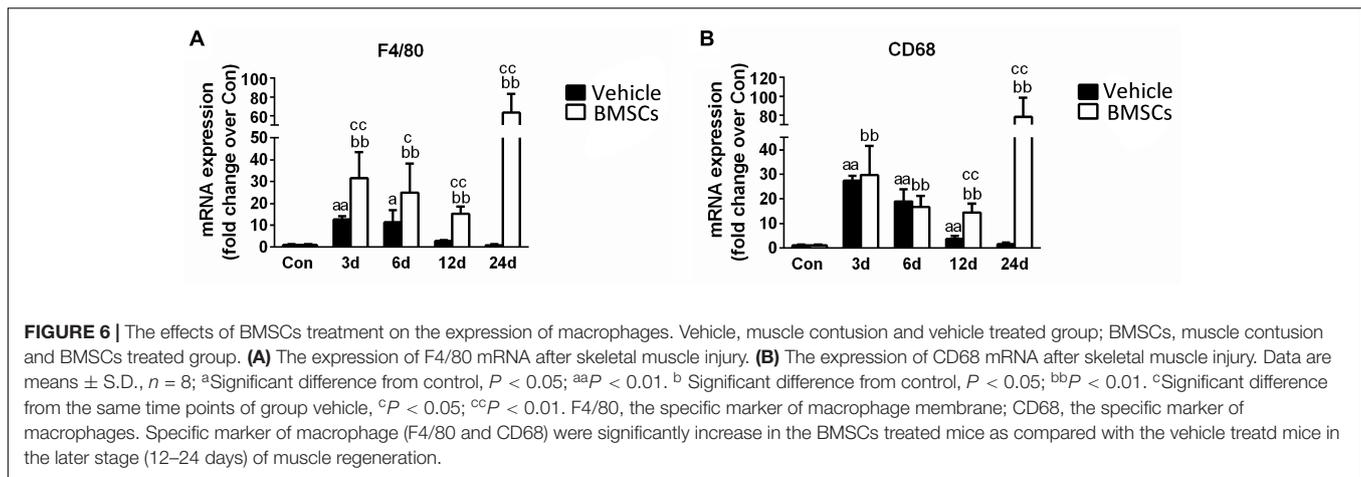

FIGURE 5 | The effects of BMSCs treatment on the expression of collagen. **(A)** The expression of mRNA of Col1a1. **(B)** The expression of mRNA of Col3a1. Vehicle, muscle contusion and vehicle treated group; BMSCs, muscle contusion and BMSCs treated group. Data are means ± S.D., $n = 8$; [a] Significant difference from Control, $P < 0.05$; [aa]$P < 0.01$. [b]Significant difference from control, $P < 0.05$; [bb]$P < 0.01$. [c]Significant difference from the same time points of group vehicle, [c]$P < 0.05$; [cc]$P < 0.01$. The transplantation of BMSCs significantly increased the expression of collagen I and III mRNA as compared with the vehicle treated mice after muscle contusion.

for scar tissue formation as the wound matures (Klingberg et al., 2013). Furthermore, in our previous study, we found that levels of pro-inflammatory cytokines (such as IL-1β, Il-6 and MCP-1) and MPO (a specific markers of neutrophils) were significantly increased at 6 h post-injury (Xiao et al., 2016b). Levels of the pro-inflammatory cytokines TGF-β, TNF-α and myostain were significantly increased 12 h post-injury (Xiao et al., 2016a). Taken together, these findings may suggest that pro-inflammatory microenvironments may induce BMSCs differentiation into myofibroblasts and impair skeletal muscle regeneration.

Moreover, we investigated muscle chemokines. The data showed that the expression pattern of chemokines, like inflammatory cytokines, increased significantly in the BMSCs treated mice in the later stage of muscle regeneration (**Figure 8**). CC chemokines are mainly involved in the recruitment of monocytes/macrophages, eosinophils, basophils, and lymphocytes, whereas CXC chemokines attract neutrophils to sites of injury (Boyd et al., 2006; Contreras-Shannon et al., 2007). In addition to their chemotactic effects on leukocytes, multiple chemokines have broader functions, such as influencing collagen production and proliferation of hematopoietic precursor

FIGURE 6 | The effects of BMSCs treatment on the expression of macrophages. Vehicle, muscle contusion and vehicle treated group; BMSCs, muscle contusion and BMSCs treated group. **(A)** The expression of F4/80 mRNA after skeletal muscle injury. **(B)** The expression of CD68 mRNA after skeletal muscle injury. Data are means ± S.D., $n = 8$; [a]Significant difference from control, $P < 0.05$; [aa]$P < 0.01$. [b] Significant difference from control, $P < 0.05$; [bb]$P < 0.01$. [c]Significant difference from the same time points of group vehicle, [c]$P < 0.05$; [cc]$P < 0.01$. F4/80, the specific marker of macrophage membrane; CD68, the specific marker of macrophages. Specific marker of macrophage (F4/80 and CD68) were significantly increase in the BMSCs treated mice as compared with the vehicle treatd mice in the later stage (12–24 days) of muscle regeneration.

FIGURE 7 | The effects of BMSCs treatment on the expression of inflammatory cytokines. Vehicle, muscle contusion and Vehicle treated group; BMSCs, muscle contusion and BMSCs-treated group. **(A–E)** The expression of inflammatory cytokines after skeletal muscle injury. Data are means ± S.D., $n = 8$; [a]Significant difference from control, $P < 0.05$; [aa]$P < 0.01$. [b] Significant difference from control, $P < 0.05$; [bb]$P < 0.01$. [c]Significant difference from the same time points of group vehicle, [c]$P < 0.05$; [cc]$P < 0.01$. BMSCs transplantation enhanced the inflammatory response in skeletal muscle regeneration.
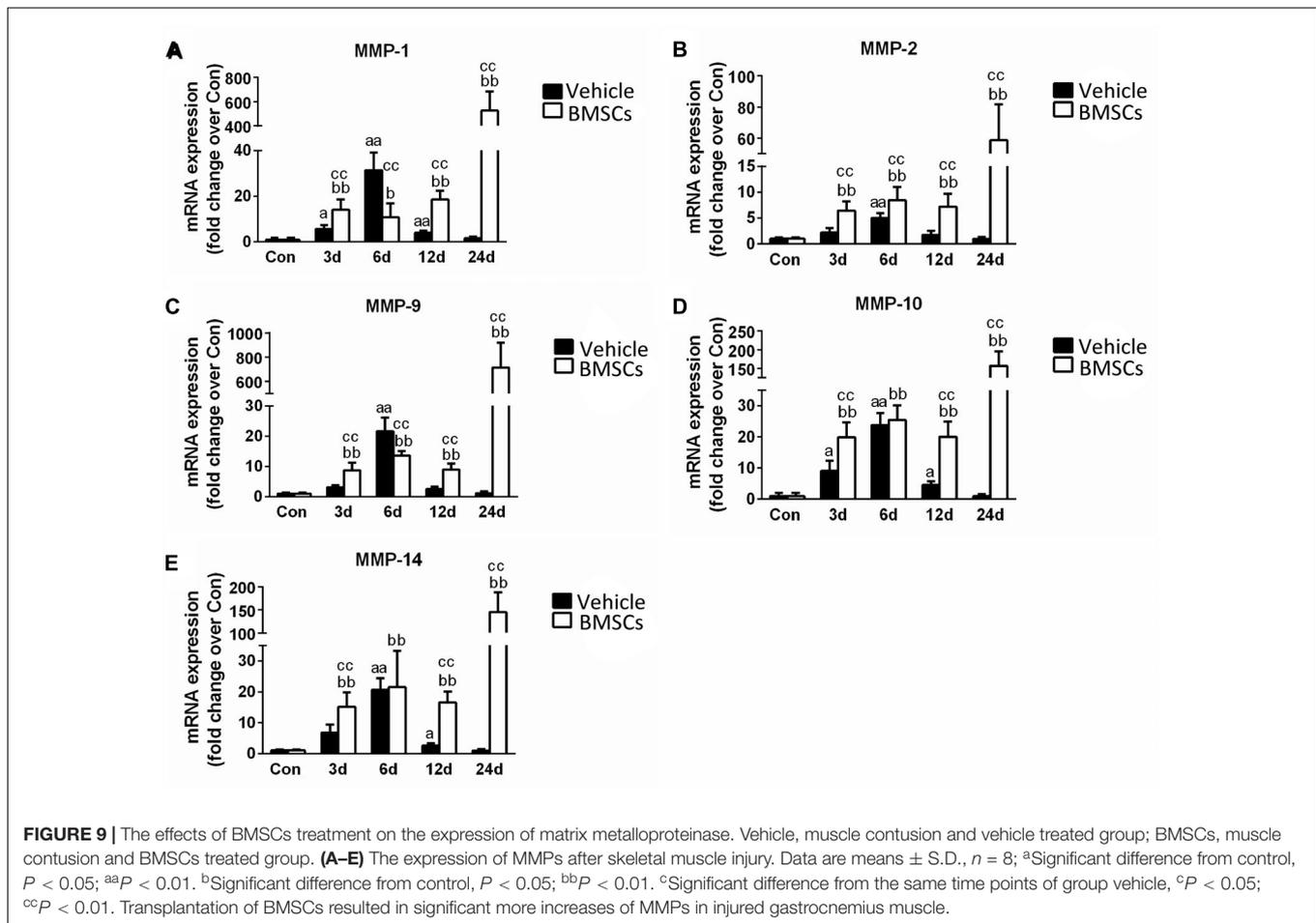
cells (Warren et al., 2004). In previous studies, we showed that there was a high expression of chemokines in injured skeletal muscle with severe fibrosis (Xiao et al., 2016a). This result was similar to the outcome observed in the contused skeletal muscle of the BMSC treatment group. It has been suggested that BMSC transplantation impairs muscle regeneration and multiple chemokines may be involved.

In addition, we tested the expression of matrix metalloproteinases (MMPs), which are zinc-dependent endopeptidases that play an important role in the digestion of the ECM, inflammation and fibrosis in pathophysiological conditions (Kumar et al., 2010; Davis et al., 2013). Compared with the vehicle group, the transcription levels of MMPs were significantly upregulated in the BMSC-treated group after
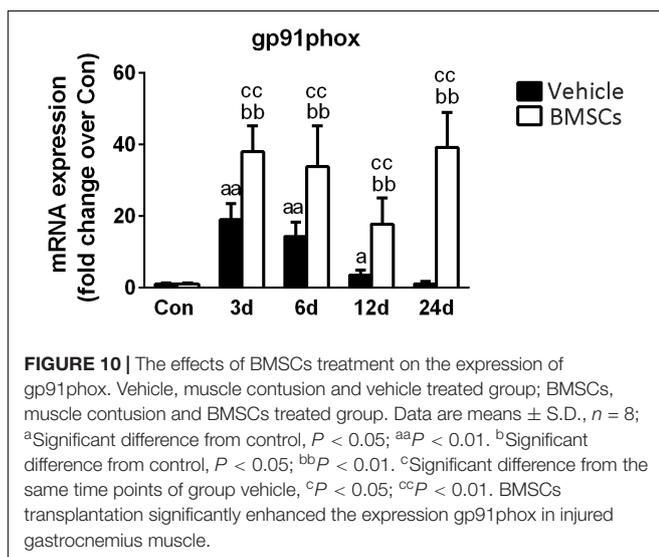
FIGURE 8 | The effects of BMSCs treatment on the expression of chemokines. Vehicle, muscle contusion and vehicle treated group; BMSCs, muscle contusion and BMSCs treated group. **(A–F)** The expression of chemokines after skeletal muscle injury. Data are means ± S.D., $n = 8$; [a]Significant difference from control, $P < 0.05$; [aa]$P < 0.01$. [b]Significant difference from control, $P < 0.05$; [bb]$P < 0.01$. [c]Significant difference from the same time points of group vehicle, $P < 0.05$; [cc]$P < 0.01$. BMSCs transplantation significantly enhanced the expression of CCL2, CCR2, CCL5, CXCR4, TWEAK, and Fn14 in injured gastrocnemius muscle.

contusion (**Figure 9**). Interestingly, this phenomenon is similar to the process involved in other muscle disease models, such as dystrophic muscle. In dystrophic muscle of mdx mice, MMPs are significantly upregulated, whereas tissue inhibitors of MMPs are down regulated (Kumar et al., 2010). Deletion or inhibition of MMPs was found to dramatically improve muscle structure and function, as well as reduce muscle injury, inflammation and fiber necrosis in the muscle of mdx mice (Li et al., 2009; Hindi et al., 2013). In addition, BMSCs cocultured with C2C12 cells or their conditioned medium (MSC-CM) upregulated MMP-2 and MMP-9 expression (Sassoli et al., 2014). Moreover, we found that the increase in MMP (MMP-1, MMP-2, MMP-9, MMP-10 and MMP-14) levels for the BMSCs treated injury group is delayed compared to the MMP increase in the vehicle treated group. High MMPs expression impair muscle regeneration, and inhibition of MMPs using batimastat contributes muscle regeneration (Kumar et al., 2010; Ogura et al., 2014). These results suggest that MMPs may be involved in delayed skeletal muscle regeneration after BMSC transplantation. However, further studies need to explore the interaction between MMPs and MSCs and the mechanism involved in skeletal muscle regeneration.

Next, we investigated gp91phox (formerly known as Nox2), which is a key membrane-bound subunit of NADPH oxidase and is used as a marker of NADPH oxidase (Ghaly and Marsh, 2010; Xiao et al., 2012; Liu et al., 2018). It has been acknowledged that NADPH oxidase is a primary source of ROS generation, and the consequential of oxidative stress in various tissues (Chan et al., 2009). Recently, Rabani et al. (2018) found that when BMSCs cocultured with macrophages, BMSC-induced ROS production in macrophages is dependent on the activation of gp91phox. In this study, the expression of gp91phox increased significantly after muscle contusion and returned to normal at 24 days post-injury. However, BMSC transplantation significantly upregulated the expression of gp91phox after injury compared with the gp91phox expression in the vehicle group ($p < 0.01$) (**Figure 10**). Similar results were seen in other disease models. Compared to wild mice, gp91phox levels were significantly increased in the tibialis anterior muscles of mdx mice (Whitehead et al., 2010). Likewise, pharmacologically induced liver fibrosis was attenuated in gp91phox-deficient mice (Novitskiy et al., 2006). Moreover, administration of apocynin (the NADPH oxidase inhibitor) suppressed the development of renal fibrosis hypertensive rats (Zhao et al., 2008). These

FIGURE 9 | The effects of BMSCs treatment on the expression of matrix metalloproteinase. Vehicle, muscle contusion and vehicle treated group; BMSCs, muscle contusion and BMSCs treated group. **(A–E)** The expression of MMPs after skeletal muscle injury. Data are means ± S.D., $n = 8$; [a]Significant difference from control, $P < 0.05$; [aa]$P < 0.01$. [b]Significant difference from control, $P < 0.05$; [bb]$P < 0.01$. [c]Significant difference from the same time points of group vehicle, $P < 0.05$; [cc]$P < 0.01$. Transplantation of BMSCs resulted in significant more increases of MMPs in injured gastrocnemius muscle.

results indicate that BMSC transplantation impairs muscle regeneration and that NADPH oxidase may play an important role in this process.

FIGURE 10 | The effects of BMSCs treatment on the expression of gp91phox. Vehicle, muscle contusion and vehicle treated group; BMSCs, muscle contusion and BMSCs treated group. Data are means ± S.D., $n = 8$; [a]Significant difference from control, $P < 0.05$; [aa]$P < 0.01$. [b]Significant difference from control, $P < 0.05$; [bb]$P < 0.01$. [c]Significant difference from the same time points of group vehicle, $P < 0.05$; [cc]$P < 0.01$. BMSCs transplantation significantly enhanced the expression gp91phox in injured gastrocnemius muscle.

These results may be different from the results of other studies, that found that mesenchymal stem cells [BMSCs (von Roth et al., 2012a; Winkler et al., 2012), adipose MSCs, embryonic stem cells (Ninagawa et al., 2013), umbilical cord MSCs (Grabowska et al., 2013) and skeletal muscle MSCs (Meligy et al., 2012)] contribute to muscle regeneration and improve muscle force after injury. However, in other disease models [such as lung injury (Yao et al., 2018) and various cancers (Norozi et al., 2016; Lee and Hong, 2017), including leukemia (Low et al., 2015), and hepatocarcinoma (Zong et al., 2018)], BMSC transplantation aggravated the disease condition that was regulated by disease microenvironments. In this study, we first found BMSC transplantation impaired muscle regeneration after muscle contusion.

CONCLUSION

Our results suggest that BMSC transplantation induced impaired skeletal muscle regeneration and that macrophages, inflammatory cytokines, chemokines, matrix metalloproteinases and oxidative stress-related enzymes may be involved in the process. These findings shed new light on the role of BMSC in regenerative medicine and indicate

that caution is needed in the application of BMSCs for muscle injury.

AUTHOR CONTRIBUTIONS

WX and PC designed this study and helped to draft the manuscript. XL carried out data analysis and drafted the manuscript. LZ and YZ performed the isolation and cultured BMSCs. XL, LZ, and YZ performed the histological staining and carried out the real time PCR. All authors have read and approved the final version of the manuscript, and agreed with the order of presentation of the authors.

FUNDING

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