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Infiltrating macrophages are broadly activated at the early stage to support acute skeletal muscle injury repair

Xingyu Wang, PhD, Wanming Zhao, MD^{*}, Richard M. Ransohoff, MD⁺, and Lan Zhou, MD, PhD

Department of Neurology & Neurotherapeutics, UT Southwestern Medical Center, Dallas, TX 75390

^{*}Department of Human Oncology, Memorial Sloan Kettering Cancer Center, New York, NY 10065

⁺Neuroimmunology, Biogen, Cambridge, MA 02142

Abstract

Acute skeletal muscle injury repair requires an adequate inflammatory response predominated by macrophage infiltration. We studied the activation of infiltrating macrophages by analyzing the expression of M1/M2 signature genes. Most of the intramuscular macrophages were Ly6C^{hi} at day 1 after BaCl₂ injection, while many were Ly6C^{lo} at day 3. Ly6C^{hi} macrophages at day 1 expressed a high level of both M1 and M2 genes, and the Ly6C^{hi} and Ly6C^{lo} macrophages at day 3 expressed a similar level of many M1/M2 genes. Infiltrating macrophages are broadly activated rather than polarized at the early stage to support acute skeletal muscle injury repair.

Keywords

skeletal muscle injury; macrophage activation; M1/M2; muscle regeneration

1. INTRODUCTION

Adult skeletal muscle possesses remarkable regenerative capacity for injury repair. Satellite cells are muscle stem cells for regeneration. They are located at the basal lamina surrounding myofibers, and are quiescent at the steady state. Upon injury, they are activated, proliferating and differentiating into myoblasts. Myoblasts then undergo fusion and terminal differentiation to become regenerating and mature regenerated myofibers (Charge and Rudnicki, 2004; Tedesco et al; 2010).

Acute skeletal muscle injury repair requires an adequate inflammatory response, which initiates immediately after injury. The first inflammatory cells recruited from blood

Correspondence: Lan Zhou, MD, PhD, Professor, Departments of Neurology & Neurotherapeutics and Pathology, UT Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-8813, Tel: 214-648-3551, Fax: 214-645-6238, lan.zhou@UTSouthwestern.edu.

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circulation are neutrophils with the number peaking at day 1 post injury. This is followed by massive infiltration of monocytes which quickly differentiate into macrophages with the number peaking at day 3 after intramuscular BaCl₂ injections (Lu et al; 2011; Lu et al; 2011). Acutely injured muscle recruits Ly6C^{hi} inflammatory monocytes but not Ly6C^{lo} patrolling monocytes from blood circulation, and Ly6C^{hi} inflammatory monocytes express CC chemokine receptor type 2 (CCR2) to exploit the chemoattraction mediated by CC chemokine ligand 2(CCL2)/CCR2 to enter injured muscle (Arnold et al; 2007; Contreras-Shannon et al; 2007; Sun et al; 2009; Lu et al; 2011; Lu et al; 2011). Within injured muscle, Ly6C^{hi} macrophages phagocytose damaged tissues and switch into Ly6C^{lo} macrophages and produce a high level of myotrophic growth factors, including insulin-like growth factor 1 (IGF-1), to promote muscle regeneration (Arnold et al; 2007; Lu et al; 2011; Lu et al; 2011). Previous studies by our lab and others have demonstrated that infiltrating macrophages are essential to the acute skeletal muscle injury repair. Depleting intramuscular macrophages, or disrupting the recruitment or function of monocytes and macrophages invariably causes poor acute muscle injury repair (Tidball 2005, Summan et al; 2006; Arnold et al; 2007; Contreras-Shannon et al; 2007; Sun et al; 2009; Lu et al; 2011; Lu et al; 2011; Mann et al; 2011; Wang et al; 2014; Zhao et al; 2016).

Macrophages are functionally heterogeneous, which can be pro-inflammatory, anti-inflammatory, and/or pro-regenerative depending on the tissue environment. They have been historically classified into M1 (classically activated) and M2 (alternatively activated) subsets, mainly based on *in vitro* studies and *in vivo* studies of parasite infections (Mosser and Edwards, 2008; Martinez et al; 2009; Martinez and Gordon, 2014). M1 and M2 macrophages are different in their activation stimuli, cell surface markers, arginine metabolism, and cytokine production profiles (Martinez et al; 2009; Martinez and Gordon, 2014). While M1 macrophages, activated by IFN- γ $\pm$ LPS, are pro-inflammatory, M2 macrophages, activated by IL-4 $\pm$ IL-13, can be anti-inflammatory, pro-regenerative, and/or pro-fibrotic. However, there has been increasing evidence demonstrating that the M1/M2 paradigm of the macrophage activation cannot mimic complex *in vivo* settings, in which the macrophage activation status can be influenced by many co-existing other inflammatory cells and tissue resident cells. *In vivo*, M1 and M2 activation stimuli often co-exist, macrophages can display mixed M1/M2 phenotypes, and macrophages may not expand clonally to maintain their phenotypes (Martinez and Gordon, 2014; Murray et al; 2014; Ransohoff, 2016).

In sterile inflammation associated with acute skeletal muscle injury repair, the dynamic temporal activation status of intramuscular macrophages has not been well studied. Based on the available findings, it was hypothesized that the acute muscle injury repair was regulated by the sequential presence of M1 and M2 macrophages, with the M1 macrophages coming first and then switching into M2, and that the M1/M2 phenotype may correlate with the Ly6C^{hi}/Ly6C^{lo} status (Chazaud, 2014; Rigamonti et al; 2014). This hypothesis was challenged by a study of traumatic muscle injury which showed that muscle macrophages upregulated both M1 and M2 genes after muscle laceration (Novak and Koh, 2013; Novak et al; 2014). Given the essential roles of macrophages in acute skeletal muscle injury repair, in the present study, we performed detailed characterization of macrophage activation following acute skeletal muscle injury induced by BaCl₂.

2. MATERIALS AND METHODS

2.1 Animals and acute muscle injury

C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). To induce acute skeletal muscle injury, 100 µl of 1.2% barium chloride (BaCl₂) was injected into the right quadriceps muscle of each mouse (age 10–14 weeks). Our study protocols were approved by the Institutional Animal Care and Use Committee at the Icahn School of Medicine at Mount Sinai (New York, NY) and UT Southwestern Medical Center (Dallas, TX).

2.2 Histopathological analysis

Male mice were sacrificed at days 1, 3, 7, 10, and 14 after BaCl₂ injections. Right quadriceps muscles were collected and frozen in liquid nitrogen-chilled isopentane, sectioned at 8 µm, stained with hematoxylin and eosin, and viewed under a bright-field microscope.

2.3 Isolation of bone marrow-derived macrophages (BMMac) and activation of M1 (M (IFN-γ + LPS)) and M2 (M (IL-4 + IL-13)) phenotypes

To obtain macrophages, bone marrow cells isolated from C57BL/6J mice (age 6–8 weeks) were cultured in 6-well plates (1 million cells per well). Each well was filled with 2 ml of media (DMEM, 20% fetal bovine serum (FBS), 30% supernatant from L929 cells containing M-CSF, 100 µM 2-ME, and antibiotics). At day 6, non-adherent cells were discarded along with the conditioned media. The adherent cells were cultured in fresh DMEM media with 10% fetal bovine serum. The M1 phenotype was induced by treating the BMMac with IFN-γ (10 ng/ml, R&D Systems, Minneapolis, MN, USA) and LPS (100 ng/ml, Sigma-Aldrich, St. Louis, MO, USA) for 6 hours, while the M2 phenotype was induced by the treatment with IL-4 (20 ng/ml, R&D Systems) and IL-13 (10 ng/ml, R&D systems) for 24 hours.

2.4 Preparation of muscle single-cell suspension, flow cytometry analysis, cell sorting, and cell quantification

Male mice were sacrificed at days 0, 1, 3, 7, 10, and 14 after BaCl₂ injections. Right quadriceps muscle single-cell suspension was prepared by collagenase/dispase digestion (Wang et al; 2016; Zhao et al; 2016; Zhao et al; 2017). Briefly, each quadriceps muscle was minced in 2.5 ml digestion solution (1U/ml collagenase B and 1U/ml dispase II (Roche Diagnostics, Indianapolis, IN, USA) in PBS) and incubated at 37°C for 1 hour. The reaction was terminated by adding 10 ml PBS containing 10% FBS. The mixture was then filtered through a 70-µm cell strainer and centrifuged at 250 g for 5 minutes. The pellet was collected and the supernatant was centrifuged again at 250 g for 5 minutes. The pellet was combined with the pellet from the first centrifugation, washed with PBS, and centrifuged at 670 g for 10 minutes. The pellet was re-suspended in 3 ml PBS, filtered through a 40-µm cell strainer, layered on equal volume of the Lympholyte-M solution (Cedarlane, Burlington, NC, USA), and centrifuged at 2,095 g for 45 minutes. Cells at the interface were collected, centrifuged at 670 g for 10 minutes, and re-suspended in FACS staining buffer (PBS

containing 2% normal mouse serum (Invitrogen, Frederick, MA, USA) and 2% BSA (Sigma-Aldrich)).

The following antibodies were used for flow cytometry. FITC-anti-Ly6G, PerCP-Cy5.5- and PE-anti-CD45, PE-anti-TNF- α , PE-anti-TGF- β 1, and PE-anti-IL-10 were purchased from BD Bioscience (San Jose, CA, USA). APC-anti-F4/80, PE-Cy7-anti-Ly-6C, and APC-anti-CD115 were purchased from Biolegend (San Diego, CA, USA). PE-anti-CD31, PE-anti-CD11b, PE-anti-Sca-1, and PE-anti-IL-1 β were purchased from eBiosciences (San Diego, CA, USA). Alexa-700-anti- α 7 integrin was purchased from R&D Systems. To detect cytokine protein expression, intracellular staining was performed with CytoFix/CytoPerm reagents from BD Bioscience following manufacturer's instructions. We used fluorescence-labelled corresponding normal IgG isotypes as negative controls for gating. All flows were done using LSR II (BD Bioscience, San Jose, CA, USA), and data were analyzed using Flowjo 8.2.6 (Tree Star, Inc., Ashland, OR, USA). Cell sorting was performed by the Flow Cytometry Core of the Icahn School of Medicine at Mount Sinai.

For quantifying intramuscular macrophages and satellite cells, we weighed each injured muscle, counted total cell number within single cell suspension of each injured muscle, used flow cytometry to identify CD45⁺/F4/80⁺ macrophages, CD45⁺/F4/80⁺/Ly6C^{hi} and CD45⁺/F4/80⁺/Ly6C^{lo} macrophage subsets, and Sca-1⁻/CD45⁻/CD31⁻/CD11b⁻/ α 7⁺ satellite cells. We measured the percentages of macrophages, macrophage subsets, and satellite cells in each injured muscle by flow cytometry. The numbers of macrophages, macrophage subsets, and satellite cells were then calculated by the total cell number of each muscle $\times$ the percentage of macrophages, macrophage subsets, or satellite cells, and expressed as cell number/muscle weight (mg). The intramuscular CD45⁺/F4/80⁺ cells were macrophages not monocytes, as they did not express CD115, a marker for monocytes (Supplemental Figure).

2.5 RNA preparation, reverse transcription, and real-time PCR

Sorted cells were lysed in the TRIzol reagent (Ambion, Grand Island, NY, USA). Total RNA was then purified and further cleaned up using the RNeasy Micro Kit (Qiagen, Hilden, Germany). Reverse transcription was performed using the SuperScriptTM II kit (Invitrogen, Frederick, MA, USA) following the manufacturer's instructions. The cDNA samples were then subjected to real-time PCR using Sybr-green reagents and an Eppendorf RealPlex 4 cycler. The *gapdh* expression was used as an internal control. The reaction specificity was determined by product melting curves. The PCR products were verified by running 3% agarose gels. Data were analyzed by threshold cycle method and presented as Fold Changes. The primer sequences were listed in Table 1.

2.6 Statistical analyses

GraphPad (GraphPad software, Inc., La Jolla, CA, USA) was used for statistical analyses. All data were presented as mean $\pm$ SEM. Two-tailed Students *t* test was used when comparing two groups, and analysis of variance was performed with Bonferroni correction for multiple comparisons. A *p* value of <0.05 was considered statistically significant.

3. RESULTS

3.1 Infiltrating macrophages and satellite cells showed similar patterns of cell number changes during acute muscle injury repair after BaCl₂ injection

Given the importance of infiltrating macrophages in muscle regeneration following acute muscle injury, we first studied the temporal changes of intramuscular macrophage cell number, and correlated with the changes of satellite cell number at different time points after BaCl₂ injection. The process of normal acute skeletal muscle injury repair was illustrated in Fig. 1A. Massive myofiber necrosis and a few scattered inflammatory cells were seen at day 1. Robust inflammatory cell infiltration was seen at day 3. Many necrotic fibers were replaced by small central-nucleated regenerating myofibers, and inflammatory cells were markedly reduced at day 7. The muscle architecture was largely restored, inflammatory cells were barely detectable, and many regenerated myofibers reached normal size at day 14. During the injury repair, the intramuscular macrophages (CD45⁺/F4/80⁺) and satellite cells (CD11b⁻/CD45⁻/CD31⁻/Sca-1⁻/α7-integrin⁺) showed similar patterns of cell number changes as assessed by flow cytometry (Fig. 1B), and the numbers of both cells peaked at day 3 (Fig. 1C). The findings are consistent with the supporting role for macrophages in the muscle regeneration associated with acute injury repair.

3.2 Only a part of the Ly6C^{lo} intramuscular macrophages were CD206⁺ at day 7

Intramuscular macrophages undergo a phenotype switch from Ly6C^{hi} to Ly6C^{lo} during injury repair (Arnold et al; 2007). We next analyzed the changes of numbers of Ly6C^{hi} and Ly6C^{lo} intramuscular macrophage subsets in our injury model, and addressed whether the Ly6C^{lo} status correlated with the expression of CD206, an important M2 cell marker (Fig. 2). By quantifying the numbers of intramuscular Ly6C^{hi} and Ly6C^{lo} macrophages at different time points using flow cytometry, we found that the numbers of both Ly6C^{hi} and Ly6C^{lo} macrophage subsets peaked at day 3 (Fig. 2A), similar to the total number of intramuscular macrophages. The high numbers of Ly6C^{hi} and Ly6C^{lo} macrophage subsets seen at day 3 as compared to day 1 is due to the continuous recruitment of Ly6C^{hi} monocytes from day 1 to day 3 and the proliferation of Ly6C^{lo} macrophages (Arnold et al; 2007). Consistent with the notion that acutely injured muscle recruits Ly6C^{hi} but not Ly6C^{lo} blood monocytes, and that Ly6C^{hi} macrophages switch into Ly6C^{lo} macrophages within injured muscle (Arnold et al; 2007), most of the intramuscular macrophages at day 1 were Ly6C^{hi}. At day 3, only less than 20% of the intramuscular macrophages were Ly6C^{hi}, and most of the intramuscular macrophages were Ly6C^{lo} at day 7 and day 14 (Fig. 2A). Therefore, the Ly6C^{hi}-to-Ly6C^{lo} switch of macrophages occurs shortly after their infiltration into injured muscle. CD206 is regarded as an important cell surface marker for M2 macrophages, although a combination of markers are needed for identifying M2 macrophages (Martinez and Gordon, 2014). To address whether Ly6C^{lo} macrophages at the early stage represent M2 macrophages, we next used flow cytometry to address the correlation of Ly6C^{lo} status and CD206 expression (Fig. 2B). The percentage of the CD206⁺ macrophages was very small at day 1, which gradually increased at day 3 and day 7, and they were mainly Ly6C^{lo}. However, near 50% of the Ly6C^{lo} macrophages at day 7 were still CD206⁻. Therefore, a significant portion of the Ly6C^{lo} intramuscular macrophages do not express CD206.

3.3 Intramuscular macrophages did not display a clear-cut M1-to-M2 phenotype switch, and they were broadly activated at day 1

M1 and M2 macrophages are activated by different stimuli, and they are different in their cell surface markers, arginine metabolism, and cytokine production profiles (Martinez et al; 2009; Martinez and Gordon, 2014). While M1 cells predominantly express CD68, M2 cells usually express CD206 and CD163. While M1 cells produce inducible nitric oxide synthase (iNOS) which can cause tissue damage via reactive nitrogen species, M2 cells produce arginase to metabolize arginine into polyamines for tissue repair. M1 cells express a high level of pro-inflammatory cytokines, including TNF- α , IL-1 β , IL-6, IL-12, and IL-23. M2 cells express a high level of anti-inflammatory, pro-regenerative, and/or pro-fibrotic cytokines, including IL-10, TGF- β 1, PDGF, IGF-1, IL-4, IL-13, and fibronectin. In addition, M2 cells also express a high level of Ym1 and Fizz1, but M1 cells do not (Raes et al; 2002). It has been shown that macrophages may use different transcription factors, including IRF5 (M1), IRF4 (M2), and PPAR γ (M2), to achieve their polarization status (Lawrence and Natoli, 2011). To precisely determine the activation status of intramuscular macrophages at different stages of the muscle injury repair, we studied the expression of a combination of these M1/M2 genes by qRT-PCR (Fig. 3). We used bone marrow macrophages activated by IFN- γ +LPS (M1) or IL-4+IL-13 (M2) as controls. Among the cell surface markers (Fig. 3A), the gene expression of both CD68 and CD206 gradually increased from day 1 to day 14. The gene expression of CD163 gradually increased from day 3 to day 14, but it surged at day 1. The gene expression of Fizz1 also gradually increased from day 3 to day 14, but it was higher at day 1 than at day 3. The gene expression of Ym1 surged at day 1, and then markedly reduced at day 3. For the enzymes involved in the arginine metabolism, the gene expression of iNOS was low throughout, and the gene expression of arginase 1 showed a pattern similar to Ym1 with a surge at day 1 (Fig. 3B). For the pro-inflammatory cytokines, the gene expression level of TNF- α , IL-1 β , IL-6, and IL-23A was the highest at day 1 (Fig. 3C). The expression of IL-12 was very low throughout (data not shown). For the anti-inflammatory and pro-regenerative cytokines and growth factors (Fig. 3D), the gene expression of TGF- β 1 and IGF-1 gradually increased from day 1 to day 14, and the gene expression of PDGF- α gradually increased from day 1 to day 7. The gene expression of IL-10 was high throughout, with the highest level detected at day 1. The gene expression of fibronectin also surged at day 1 (Fig. 3D). The gene expression of PDGF- β was very low, and the gene expression of IL-4 and IL-13 was undetectable throughout (data not shown). For the transcription factors, the gene expression of IRF4, IRF5, and PPAR γ all gradually increased from day 1 to day 14, with a significant difference detected between day 1 and day 3 for each of these genes (Fig. 3E). Based on the temporal expression patterns of these M1/M2-associated genes, there is no clear-cut M1-to-M2 phenotype switch at the early stage of the injury repair. The expression levels of not only M1 pro-inflammatory cytokine genes but also several M2 genes are the highest at day 1. These M2-associated genes include CD163, arginase 1, Ym1, IL-10, and fibronectin. Therefore, the intramuscular macrophages at day 1 appear broadly activated rather than polarized. The intramuscular macrophages do become less inflammatory and more reparative from day 1 to day 3 with downregulation of the pro-inflammatory cytokine (TNF- α , IL-1 β , IL-6, and IL-23A) genes and upregulation of the regenerative growth factor (IGF-1, TGF- β 1, and PDGF- α) genes.

3.4 The Ly6C^{hi}/Ly6C^{lo} status did not correlate with the M1/M2 phenotype at the early stage of acute muscle injury repair

It is surprising that the day 1 intramuscular macrophages express a high level of several M2 signature genes, while they are mostly Ly6C^{hi} at this stage. The finding suggests that Ly6C^{hi} is not a specific marker for M1, and that the Ly6C^{hi}/Ly6C^{lo} status does not correlate with the M1/M2 phenotype, at least at this early stage. The macrophage function may depend on the requirement by the injury repair at different time points rather than the expression status of Ly6C. To further address these hypotheses, we compared the expression of the M1/M2 genes between day 3 Ly6C^{hi} and Ly6C^{lo} macrophages, and between day 1 and day 3 Ly6C^{hi} macrophages (Fig. 4). Both Ly6C^{hi} and Ly6C^{lo} intramuscular macrophages at day 3 co-expressed many M1 and M2 genes, and the expression of the majority of these genes was not different between the Ly6C^{hi} and Ly6C^{lo} subsets. These genes include CD68, CD206, CD163, arginase 1, TNF- α , IL-23A, IL-10, TGF- β 1, PDGF- α , fibronectin, IRF4, and PPAR γ . The expression of IRF5, a transcription factor promoting M1 polarization (Lawrence and Natoli 2011), was higher in the Ly6C^{hi} macrophages than in the Ly6C^{lo} macrophages. The expression of pro-inflammatory cytokines IL-1 β and IL-6, but not TNF- α or IL-23A, was higher in the Ly6C^{hi} macrophages. The expression of the M2 genes, Fizz1 and Ym1, was also surprisingly higher in the Ly6C^{hi} macrophages. Among the M2 genes, only the IGF-1 gene expression was higher in the Ly6C^{lo} macrophages. Therefore, the Ly6C^{hi} and Ly6C^{lo} macrophage subsets are not fundamentally different at day 3, and the Ly6C^{hi} and Ly6C^{lo} macrophage subsets do not conform to typical M1 or M2 macrophages, although the Ly6C^{lo} macrophages appear less inflammatory and more reparative based on the lower level of IL-1 β and IL-6 gene expression and the higher level of IGF-1 gene expression. For the Ly6C^{hi} macrophages from day 1 to day 3, the expression of the M1/M2 genes was significantly altered, except for the IL-6 and IL-10 genes. The expression of CD68, CD206, TGF- β 1, IGF-1, PDGF- α , IRF4, IRF5, and PPAR γ genes was upregulated, while the expression of Fizz1, Ym1, arginase 1, TNF- α , IL-1 β , IL-23A, and fibronectin genes was downregulated. Therefore, there are considerable differences between day 1 and day 3 Ly6C^{hi} macrophages, with the day 3 Ly6C^{hi} macrophages exhibiting a less inflammatory and more reparative phenotype based on the differences seen. Taken together, the M1/M2 gene expression by intramuscular macrophages appears to correlate with the post-injury time points rather than the Ly6C expression status.

3.5 Intramuscular macrophages at day 1 were broadly activated, co-expressing a high level of both pro-inflammatory and anti-inflammatory cytokines

Intramuscular macrophages at day 1 appear broadly activated based on their M1/M2 gene expression profile. We next addressed whether the co-expression of the M1/M2 cytokines was also detectable at the protein level, and whether the co-expression was contributed by the presence of broadly activated macrophages with mixed M1 and M2 phenotypes or simply by the co-existence of polarized M1 and M2 macrophage subsets. To this end, we performed flow cytometry using muscle single cell suspensions at day 1 and day 3 with intracellular staining of the signature M1 pro-inflammatory cytokines, TNF- α and IL-1 β , and the M2 anti-inflammatory cytokines, IL-10 and TGF- β 1 (Fig. 5). A high percentage of the intramuscular macrophage populations (CD45⁺/F4/80⁺) at day 1 expressed TNF- α (around 60%), IL-1 β (over 85%), and IL-10 (over 85%), and all of the intramuscular

macrophages expressed TGF- β 1 (Fig. 5A). Therefore, the majority of the intramuscular macrophage populations at day 1 are broadly activated, co-expressing both M1 pro-inflammatory and M2 anti-inflammatory cytokines. Similar to the gene expression (Fig. 3C–D), the protein expression of IL-1 β by intramuscular macrophage populations was higher at day 1 than at day 3, and the protein expression of IL-10 and TGF- β 1 was not different between these two time points (Fig. 5B). Although the macrophage gene expression of TNF- α was higher at day 1 than at day 3 (Fig. 3C), the protein expression of TNF- α was similar at these two time points (Fig. 5B). Overall, the pro-inflammatory and anti-inflammatory cytokine gene expression assessed by qRT-PCR was largely consistent with the protein expression evaluated by flow cytometry with intracellular staining.

3.6 Intramuscular neutrophils expressed a high level of TNF- α , IL-1 β , TGF- β 1, and IL-10 cytokines at very early time points after injury

From the flow data shown in Fig. 5, we noticed that a large portion of the CD45⁺/F4/80[−] inflammatory cells also expressed TNF- α , IL-1 β , IL-10, and TGF- β 1 proteins, with the levels of TNF- α and IL-10 being higher than those seen in the CD45⁺/F4/80⁺ macrophages. Since a large portion of the CD45⁺ inflammatory cells at day 1 were neutrophils, we addressed whether these cells were neutrophils and whether intramuscular neutrophils expressed a high level of these cytokines. We first studied the percentages of macrophages and neutrophils in the intramuscular CD45⁺ cell population at day 0 and day 1 by flow cytometry. In uninjured quadriceps muscle, there were very few CD45⁺ cells; over 50% of them were F4/80⁺ resident macrophages, and rare cells were Ly6G⁺ neutrophils (Fig. 6A). At day 1, over 30% of the CD45⁺ cells were macrophages (F4/80⁺), and over 50% were neutrophils (Ly6G⁺) (Fig. 6B). Comparing to the intramuscular macrophages at day 1, the flow cytometry with intracellular staining showed a similar level of IL-1 β and TGF- β 1 but a high level of TNF- α and IL-10 protein expression by the intramuscular neutrophils at this stage (Fig. 6C). Since these cytokines, especially TNF- α and IL-10, could contribute to the macrophage activation, we next addressed whether the intramuscular neutrophils also expressed a high level of these cytokines at earlier time points to contribute to the local tissue environment for the early broad activation of infiltrating macrophages seen at day 1. To this end, we did flow cytometry with intracellular staining of the cytokines at 6 hours, 12 hours, and 24 hours (Fig. 6D). Intramuscular neutrophils expressed a high level of TNF- α , IL-1 β , IL-10, and TGF- β 1 at all these early time points

4. DISCUSSION

Macrophages are essential to acute skeletal muscle injury repair. Infiltrating macrophages exert diverse functions to promote injury repair, including phagocytosing damaged muscle tissue and cell debris, producing pro-inflammatory and anti-inflammatory cytokines to regulate inflammation, and secreting growth factors for myogenic cell regeneration and ECM remodeling (Arnold et al; 2007; Lu et al; 2011; Lu et al; 2011). By studying the intramuscular macrophage expression of a combination of M1 and M2 signature genes and cytokine proteins at different time points after acute muscle injury induced by BaCl₂, we show that there is no clear-cut M1-to-M2 phenotype switch at the early stage of injury repair. The majority of infiltrating macrophage populations are broadly activated at the early

stage, co-expressing a high level of many M1 and M2 genes and displaying mixed pro-inflammatory, anti-inflammatory, and pro-regenerative features for muscle injury repair. The Ly6C^{hi}/Ly6C^{lo} status of intramuscular macrophages does not correlate with the M1/M2 phenotype at the early stage. Our findings demonstrate the phenotypic complexity of *in vivo* macrophages in acutely injured skeletal muscle.

The majority of intramuscular macrophage populations at day 1 are broadly activated rather than polarized, as they not only express the highest level of M1 pro-inflammatory cytokine genes, including TNF- α , IL-1 β , IL-6, and IL-23A, but also express the highest level of several M2 genes, including CD163, arginase 1, Ym1, IL-10, and fibronectin. The co-expression of the M1/M2 cytokines is mainly contributed by the presence of broadly activated macrophage populations with mixed M1 and M2 phenotypes rather than the presence of both M1 and M2 macrophages, as the majority of these cells express a high level of both M1 pro-inflammatory and M2 anti-inflammatory cytokines, including TNF- α , IL-1 β , IL-10 and TGF- β . One alternative explanation is that these macrophages may be at a transitional state, switching from M1 to M2 and expressing both M1 and M2 genes and proteins. One may also speculate that the highest level of the anti-inflammatory IL-10 expression by day 1 macrophages may represent a reactive response to the highest level of pro-inflammatory cytokines at this stage rather than a broad activation status. But the finding that the expression level of several other M2 genes, including the prototypic arginase I gene, is also the highest at day 1 makes these possibilities less likely.

We do see a Ly6C^{hi}-to-Ly6C^{lo} switch of intramuscular macrophages at the early stage in our chemical-induced injury model, as seen in the toxin-induced injury model (Arnold et al; 2007). Our gene expression profile study also reveals a temporal change of macrophages towards a less inflammatory and more reparative phenotype. It has been shown that this change is critical for normal injury repair, and it is regulated by IL-10 (Deng et al; 2012), AMP-activated protein kinase (Mounier et al; 2013), p38/mitogen-activated protein kinase-1 signaling (Perdiguero et al; 2011), CREB-C/EBP β transcription cascade (Ruffell et al; 2009), and regulatory T cells (Burzyn et al; 2013). The findings from our present study suggest that the change of the macrophage phenotype at the early stage appears correlate with the time points after the injury rather than the expression status of Ly6C. First, the day 1 macrophages express a high level of both M1 and M2 genes, while they are mostly Ly6C^{hi}. Second, both Ly6C^{hi} and Ly6C^{lo} macrophages at day 3 co-express many M1 and M2 genes with limited differences between them. Third, there are much more differences seen between the day 1 and day 3 Ly6C^{hi} macrophages than those seen between the day 3 Ly6C^{hi} and Ly6C^{lo} macrophage subsets. The findings suggest that the change of macrophage functions is most likely related to the tissue environment and injury repair requirement at different time points. Although the percentage of Ly6C^{lo} macrophages increases and the macrophages become less inflammatory and more reparative from day 1 to day 3, they do not conform to typical M2 cells based on their M1/M2 gene expression profiles.

The lack of canonical M1 or M2 cells, co-expression of both M1 and M2 markers, high expression of IL-10, and diminished expression of iNOS at the early stage of acute injury repair are not specific to our chemical-induced muscle injury model, as these features have

also been reported in acute muscle injury induced by mechanical laceration (Novak et al; 2014). Moreover, the early broad activation of infiltrating macrophages has also been reported in acute sterile injuries of other tissues, including skin (Daley et al; 2010), brain (Kim et al; 2016), and peritoneum (Loke et al; 2007), which suggests that the early broad activation of infiltrating macrophages may be a common phenomenon in acute sterile tissue injury.

The tissue environmental stimuli driving the early broad activation of intramuscular macrophages remain largely unclear. The early broad activation of macrophages following sterile injury is felt to be an innate immune response, as it is not affected by Rag1 deficiency with absent lymphocytes in the peritoneal injury model (Loke et al; 2007). In our muscle injury model, the expression of IFN- γ and IL-4, the canonical *in vitro* stimuli for the M1 and M2 activation, respectively, are barely detectable in the whole muscle tissue or in the intramuscular macrophages at day 1 (data not shown). Therefore, these two cytokines are unlikely to be the major stimuli inducing the broad activation of macrophages. It has been shown that other stimuli can also activate M1-like and M2-like phenotypes (Mantovani et al; 2004; Martinez and Gordon, 2014). TNF- α can induce M1-like activation, while the toll-like receptor 4 (TLR4) signaling and IL-10 can induce M2-like activation. The high expression of both TNF- α and IL-10 by intramuscular macrophages at day 1 may contribute to their own broad activation. Other inflammatory cells and tissue resident cells may also contribute. Since infiltrating neutrophils express a high level of both TNF- α and IL-10 proteins at very early time points when they are the predominant inflammatory cells in injured muscles, they may contribute to the establishment of a local tissue environment for the broad activation of infiltrating macrophages. BaCl₂ injection induces acute massive muscle necrosis which releases endogenous danger signals in cell debris, including heat shock proteins, nuclear proteins, histones, nucleotides, and ECM components. Macrophages can sense danger signals via TLRs and become activated by TLR4 to display a phenotype with a high expression level of both pro-inflammatory cytokines (TNF- α , IL-1 β , and IL-6) and IL-10, but a very low expression level of IL-12 (Martinez and Gordon, 2014). This phenotype is similar to the one seen in day 1 intramuscular macrophages, which suggests that the TLR4 signaling may contribute to the early broad activation of macrophages during acute muscle injury repair. The change of the macrophage phenotype after day 1 may be due, in part, to the less danger signals (cleared by macrophages) and consequently lesser activation of macrophages by TLR4 at the late time points. Future studies are needed to fully address the macrophage activation signals in acutely injured muscle.

The early broad activation of infiltrating macrophages is likely critical to the initiation of acute muscle injury repair. The high M1-like inflammatory function at early stage is needed to degrade damaged tissues to facilitate phagocytosis, as lack of macrophage infiltration causes delayed clearance of necrotic fibers (Arnold et al; 2007; Lu et al; 2011). The M2-like pro-regenerative and anti-inflammatory functions at early stage are also needed for early initiation of the repairing/regenerative process and inflammation control. Our results show that the day 1 macrophages express not only a high level of M1 cytokine gene, IL-6, but also a high level of M2 genes, IL-10 and fibronectin. IL-6 is not only a pro-inflammatory cytokine but also an important regulator of satellite cell proliferation during muscle injury repair (Serrano et al. 2008; Begue et al; 2013; Zhang et al; 2013). IL-10 is not only an anti-

inflammatory cytokine but also a pro-regenerative cytokine, as it can prevent myogenic cell premature differentiation to allow sufficient proliferation (Deng et al; 2012). Fibronectin, a secreted extracellular matrix protein, can bind Syndecan-4 to activate Wnt7a to induce symmetric expansion of satellite cells, which is important for early muscle regeneration (Bentzinger et al; 2013). Therefore, the highest level expression of these muscle pro-regenerative genes by broadly activated infiltrating macrophages at day 1 is likely essential to the early activation and proliferation of myogenic cells. It has been shown that fibronectin secreted by satellite cells themselves is important for satellite cell proliferation (Bentzinger et al; 2013). The surge of the expression of fibronectin by macrophages at day 1 suggests that infiltrating macrophages may be another important cellular source of fibronectin. We detected a gradual increase of IGF-1 gene expression by intramuscular macrophages. IGF-1 is the key and potent trophic growth factor promoting muscle regeneration following acute injury (Pelosi et al; 2007; Heinemeier et al; 2009; Dumont and Frenette, 2010; Lu et al; 2011). IGF-1 can signal through Akt to promote muscle fiber growth. The macrophage expression of IGF-1 may contribute to the regenerating myofiber size growth at late stages, although other cells, including intramuscular fibroblasts, also express a high level of IGF-1. Infiltrating macrophages are thus pro-regenerative throughout the acute skeletal muscle injury repair process.

In summary, our present study shows that infiltrating macrophage populations are broadly activated rather than polarized at the early stage of acute skeletal muscle injury repair. The high level expression of both M1 and M2 genes, especially IL-6, IL-10, and fibronectin, by broadly activated macrophage populations at day 1 are probably essential to muscle regeneration, as these cytokine proteins are critical for the early activation and proliferation of myogenic cells (Serrano et al; 2008; Deng et al; 2012; Begue et al; 2013; Bentzinger et al; 2013; Zhang et al; 2013). The macrophage phenotype at the early stage does not correlate with the expression status of Ly6C, and it is most likely influenced by the local tissue environment and injury repair requirement.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

BMMac	Bone marrow-derived macrophages
CCL2	CC chemokine ligand 2
CCR2	CC chemokine receptor type 2
ECM	Extracellular matrix
IFN-γ	Interferon gamma

IGF-1	Insulin-like growth factor 1
iNOS	Inducible nitric oxide synthase
PDGF	Platelet derived growth factor
TGF-β1	Transforming growth factor-beta1
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor-alpha

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Highlights

- Infiltrating macrophages are broadly activated rather than polarized at the early stage of acute skeletal muscle injury repair, and they co-express a high level of both M1 and M2 signature genes and cytokine proteins.
- Infiltrating macrophages display a mixed pro-inflammatory, anti-inflammatory, and pro-regenerative functions to support early muscle regeneration for acute skeletal muscle injury repair.
- There is no clear-cut M1 to M2 switch of infiltrating macrophages based on the M1/M2 gene expression profiles.
- The Ly6C^{hi}/Ly6C^{lo} status of infiltrating macrophages does not correlate with the M1/M2 phenotype at the early stage.
- The local tissue environment signals, other than IFN- γ and IL-4, may stimulate the early broad activation of infiltrating macrophages in acutely injured muscle. The danger signals and cytokines produced by neutrophils and other inflammatory and resident cells may contribute.

Figure 1. Infiltrating macrophages and satellite cells showed similar patterns of cell number changes during acute muscle injury repair following BaCl₂ injection

A. H&E staining illustrating the normal acute muscle injury repair process following intramuscular BaCl₂ injection. **B.** Quantification of intramuscular macrophages (CD45⁺/F4/80⁺) and satellite cells (CD11b⁻/CD45⁻/CD31⁻/Sca-1⁻/ $\alpha 7$ -integrin⁺) by flow cytometry. Upper panel: Dot blot samples of macrophages and satellite cells (SCs) at day 3. Lower panel: Quantification results of macrophages and SCs at different time points after acute injury. N = 10 mice/time point. Bar = 50 μ m.

Figure 2. Only a part of the Ly6C^{low} intramuscular macrophages were CD206⁺
A. Flow cytometry assessing Ly6C^{hi}-to-Ly6C^{lo} switch of intramuscular macrophages (left) and quantification of CD45⁺/F4/80⁺/Ly6C^{hi} and CD45⁺/F4/80⁺/Ly6C^{lo} macrophages (right). CD45⁺ cells were gated for analysis of F4/80 and Ly6C expressions. N = 10 mice/time point. **B.** Flow cytometry assessing CD206 expression by intramuscular macrophages. CD45⁺ F4/80⁺ cells were gated for analysis of Ly6C and CD206 expression. N = 5 mice/time point.

Figure 3. Intramuscular macrophages did not display a clear-cut M1-to-M2 phenotype switch, and they were broadly activated at day 1

Quantitative RT-PCR using intramuscular macrophages sorted by FACS ($CD45^+ F4/80^+$) for their gene expression of M1/M2 cell-surface markers (A), arginine metabolism genes (B), pro-inflammatory cytokines (C), anti-inflammatory and pro-regenerative cytokines (D), and transcriptional factors (E). M1 and M2 macrophages, derived from bone marrow and activated *in vitro* by $IFN-\gamma$ +LPS and IL-4+IL-13, respectively, were used as controls. The experiments were performed twice independently. For each experiment at each time point, RNA samples were prepared from 5 to 10 mice. Differences between day 1 and day 3 were evaluated statistically. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 4. Ly6^{Chi}/Ly6^{Lo} status did not correlate with M1/M2 phenotype at the early stage of acute muscle injury repair

Quantitative RT-PCR using Ly6^{Chi} and Ly6^{Lo} intramuscular macrophages (CD45⁺ F4/80⁺) sorted by FACS at day 1 and day 3 for their gene expression of M1/M2 cell-surface markers (A), arginine metabolism gene (B), pro-inflammatory cytokines (C), anti-inflammatory and pro-regenerative cytokines (D), and transcriptional factors (E). M1 (M (IFN- γ +LPS)) and M2 (M (IL-4+IL-13)) macrophages were used as controls. The experiments were performed twice independently. For each experiment at each time point, RNA samples were prepared from 5 to 10 mice. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 5. Intramuscular macrophages at day 1 were broadly activated, co-expressing high levels of both pro-inflammatory and anti-inflammatory cytokine proteins

A. FACS analysis with intracellular staining of intramuscular CD45⁺ cells at day 1 for their surface expression of F4/80 and intracellular expression of TNF- α , IL-1 β , IL-10, and TGF- β 1 proteins. **B.** Quantification of the cytokine protein expression by mean fluorescence intensity (MFI) of intracellular cytokine staining signals. M1 and M2 macrophages were used as controls. N = 5–10 mice/time point. * $p < 0.05$.

Figure 6. Intramuscular neutrophils expressed high levels of TNF-α, IL-1β, TGF-β1, and IL-10 cytokines at very early time points

A. FACS analysis of intramuscular macrophages and neutrophils at day 0. CD45⁺ cells were gated for analysis of F4/80 (macrophages (Mac)) and Ly6G (neutrophils (Neu)) expressions. **B.** FACS analysis of intramuscular macrophages and neutrophils at day 1. CD45⁺ cells were gated for analysis of F4/80 (macrophages (Mac)) and Ly6G (neutrophils (Neu)) expressions. **C.** Quantification of intracellular expression of TNF-α, IL-1β, IL-10, and TGF-β1, based on MFI, by macrophages and neutrophils at day 1. **D.** Quantification of intracellular expression of TNF-α, IL-1β, IL-10, and TGF-β1, based on MFI, by neutrophils at 6 hours, 12 hours, and 24 hours. N = 5–10 mice/time point. *p < 0.05.

Table 1

Primer sequences for qRT-PCR

gene name	primer	sequence
<i>cd68</i>	F	5' - CAATTCAGGGTGGAA GAAAG - 3'
	R	5' - TCTGATGTAGGTCCTGTTG - 3'
<i>cd206</i>	F	5' - GGTGTGGGCTCAGGTAGT - 3'
	R	5' - TGTGGTGAGCTGAAAGGT - 3'
<i>cd163</i>	F	5' - CCTTGGAACA GAGACAGGC - 3'
	R	5' - TCCACACGTCCAGAACAGTC - 3'
<i>fizz1</i>	F	5' - GATGAAGACTACAACCTGTTCC - 3'
	R	5' - AGGGATA GTTAGCTGGATTG - 3'
<i>ym1</i>	F	5' - CTTCTAAGACTGGAATTGGTG - 3'
	R	5' - GTACAAACCTCATAGTAAGCC - 3'
<i>inos</i>	F	5' - TGAAATCCCTCCTGATCTTG - 3'
	R	5' - CCATGTACCAACCATTGAAG - 3'
<i>arginase-1</i>	F	5' - CAATGAAGAGCTGGCTGGTGT - 3'
	R	5' - GTGTGA GCATCCACCCAAATG - 3'
<i>tnfa</i>	F	5' - CTTCTGTCTACTGAACTTCGGG - 3'
	R	5' - CACTTGGTGGTTTGCTACGAC - 3'
<i>il1b</i>	F	5' - GACGGCACACCCACCCT - 3'
	R	5' - AAACCGTTTTTCCATCTTCTCTTT - 3'
<i>il6</i>	F	5'-GAACAACGATGATGCACTTGC - 3'
	R	5'-CTTCATGTACTCCAGGTAGCTATGGT - 3'
<i>il12p40</i>	F	5' - CGCAGCACTTCAGAATCACA
	R	5' - TCTCCCACA GGAGGTTTCTG - 3'
<i>il23a</i>	F	5' - CTTAGTAGATTCATATGTCCTG - 3'
	R	5' - AATAATGCTATGGCTGTTGC - 3'
<i>il10</i>	F	5' - CAGGACTTTAAGGGTACTTG - 3'
	R	5' - ATTTTCACAGGGGAGAAATC - 3'
<i>il4</i>	F	5' - CATCGGCATTTGAACGAGGTCA - 3'
	R	5' - CTTATCGATGAATCAGGCATCG - 3'
<i>il13</i>	F	5' - AGACCAGACTCCCCTGTGCA - 3'
	R	5' - TGGGTCCTGTAGATGGCATTG - 3'
<i>tgfb1</i>	F	5' - GGATACCAACTATTGCTTCAG - 3'
	R	5' - TGTCCAGGCTCCAAATATAG - 3'
<i>igf-1</i>	F	5' - CTACAAAAGCAGCCCGCTCT - 3'
	R	5' - CTTCTGAGTCTTGGGCATGTCA - 3'
<i>pdgfa</i>	F	5' - GTCCAGGTGA GGTAGAGG - 3'
	R	5' - CACGGA GGA GAACAAAGAC - 3'
<i>fibronectin</i>	F	5' - AAACCTGCATCTGGAGGCAAACCC - 3'

gene name	primer	sequence
	R	5' - AGCTCTGATCAGCATGGACCACTT - 3'
<i>irf4</i>	F	5' - GAGTAGGATCTACTGGGATG - 3'
	R	5' - CTTGCAGCTCTGATAGAAAC - 3'
<i>irf5</i>	F	5' - AACTCTTCTATATACCCTGGC - 3'
	R	5' - AAGTCACGGCTTTTGTTAAG - 3'
<i>pparg</i>	F	5' - CAAGAATACCAAAGTGCATCAA - 3'
	R	5' - GAGCAGGGTCTTTTCAGAATAATAAG - 3'
<i>gapdh</i>	F	5' - GTGTTCTACCCCAATGTGTC - 3'
	R	5' - GTAGCCCAAGATGCCCTTCAGT - 3'