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

Xingyu Wang[a], Wanming Zhao[b], Richard M. Ransohoff[c], Lan Zhou[a,*]

[a] Department of Neurology & Neurotherapeutics, UT Southwestern Medical Center, Dallas, TX 75390, United States
[b] Department of Human Oncology, Memorial Sloan Kattering Cancer Center, New York, NY 10065, United States
[c] Neuroimmunology, Biogen, Cambridge, MA 02142, United States

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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 $BaCl_2$ 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.

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 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 $BaCl_2$ injections (Lu et al., 2011a, 2011b). 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., 2011a, 2011b). 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., 2011a, 2011b). 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., 2011a, 2011b; 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 ± LPS, are pro-inflammatory, M2 macrophages, activated by Il-4 ± IL-13, can be anti-inflammatory, pro-regenerative, and/or pro-fibrotic. However, there

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

* Corresponding author at: Departments of Neurology & Neurotherapeutics and Pathology, UT Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-8813, United States.
E-mail address: lan.zhou@UTSouthwestern.edu (L. Zhou).

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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-exit, 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 $BaCl_2$.

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 ($BaCl_2$) 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 $BaCl_2$ 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 h, 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 h.

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 $BaCl_2$ 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 (1 U/ml collagenase B and 1 U/ml dispase II (Roche Diagnostics, Indianapolis, IN, USA) in PBS) and incubated at 37 °C for 1 h. 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 min. The pellet was collected and the supernatant was centrifuged again at 250*g* for 5 min. The pellet was combined with the pellet from the first centrifugation, washed with PBS, and centrifuged at 670*g* for 10 min. 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 2095*g* for 45 min. Cells at the interface were collected, centrifuged at 670*g* for 10 min, 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-β1, 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-1β were purchased from eBiosciences (San Diego, CA, USA). Alexa-700-anti-α7 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 gaiting. 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 $Sca\text{-}1^-/CD45^-/CD31^-/CD11b^-/\alpha7^+$ 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 × 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 Fig. 1).

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 ΔΔ 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 ± 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.