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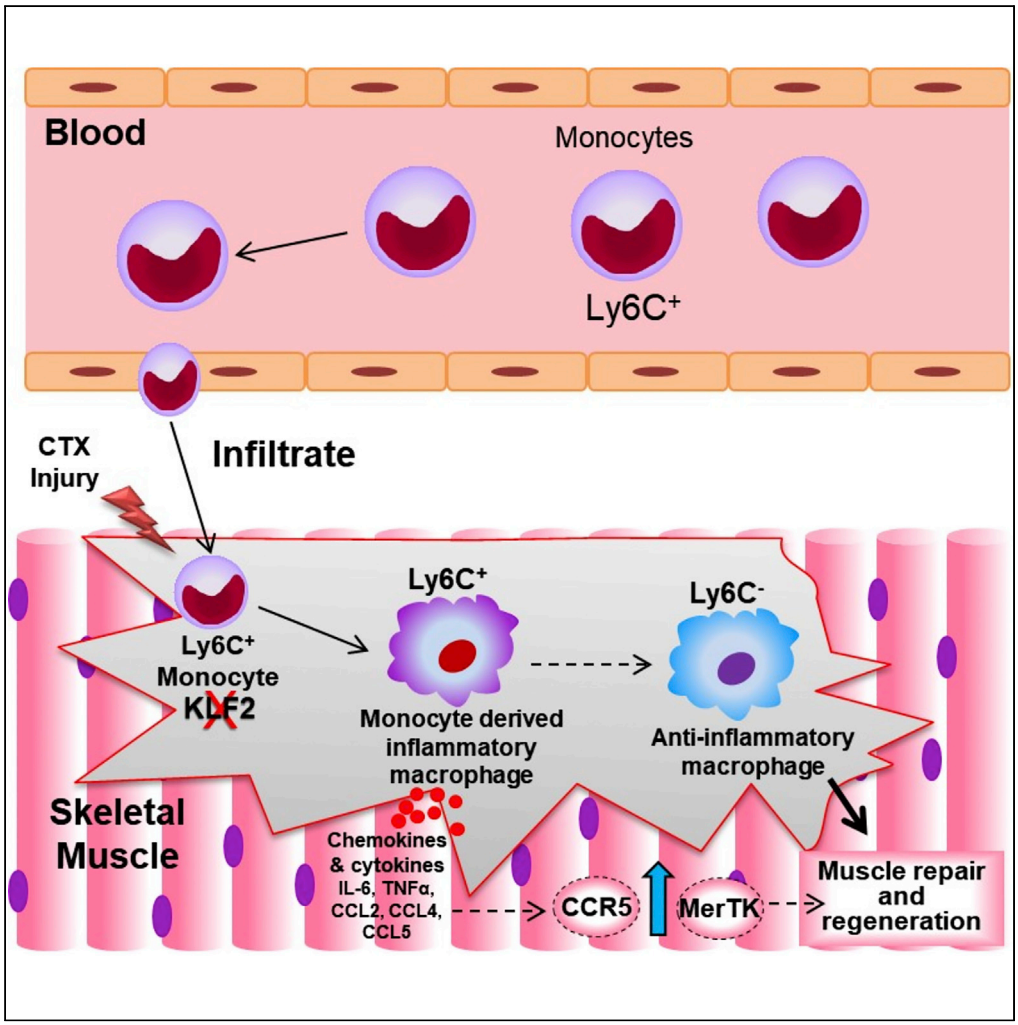

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HIGHLIGHTS

Loss of KLF2 in myeloid cells accelerates skeletal muscle regeneration after injury

Myeloid KLF2 determines key muscle-immune cell interactions during muscle regeneration

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KLF2 in Myeloid Lineage Cells Regulates the Innate Immune Response during Skeletal Muscle Injury and Regeneration

Palanikumar Manoharan,[1,4,*] Taejeong Song,[2] Tatiana L. Radzyukevich,[3] Sakthivel Sadayappan,[2] Jerry B Lingrel,[1] and Judith A. Heiny[3,*]

SUMMARY

Skeletal muscle repair and regeneration after injury requires coordinated interactions between the innate immune system and the injured muscle. Myeloid cells predominate in these interactions. This study examined the role of KLF2, a zinc-finger transcription factor that regulates immune cell activation, in specifying myeloid cell functions during muscle regeneration. Loss of KLF2 in myeloid lineage cells (*myeKlf2*$^{-/-}$ mice) dramatically enhanced the initial inflammatory response to acute muscle injury (cardiotoxin). Injured muscles showed dramatically elevated expression of inflammatory mediators and greater numbers of infiltrating, pro-inflammatory monocytes that matured earlier into activated macrophages. Notably, the inflammatory phase resolved earlier and regeneration progressed to myogenesis, marked by elevated expression of factors that promote the formation of new fibers from satellite cells. Regeneration was completed earlier, with phenotypically normal adult fibers integrated into the muscle syncytium. These findings identify myeloid KLF2 as a key regulator of myeloid cell functions in adult skeletal muscle regeneration.

INTRODUCTION

Adult skeletal muscles retain a remarkable capacity to regenerate lost or damaged tissue. Skeletal muscle regeneration after acute injury progresses through a continuum of overlapping phases, broadly defined as an initial pro-inflammatory phase followed by an anti-inflammatory, myogenic phase (Arnold et al., 2007; Karalaki et al., 2009; Tidball, 2005; Villalta et al., 2011; Yin et al., 2013). The regenerative phase recapitulates, in many ways, the process of embryonic myogenesis, whereas the innate immune response to muscle injury is unique to adult skeletal muscle. It is now recognized that coordinated interactions between the injured muscle and the innate immune system mediate every phase of this process and are essential for its proper completion (Smith et al., 2008). In this respect, the innate immune system functions as the maestro in orchestrating the program of adult muscle regeneration.

Myeloid-lineage immune cells predominate in this process (Arnold et al., 2007; McLennan, 1996; Mounier et al., 2013; Tidball and Wehling-Henricks, 2007; Villalta et al., 2011). Depletion of circulating monocytes slows regeneration, and reconstitution of myeloid lineage cells restores regeneration (Summan et al., 2006). Myeloid-lineage monocytes dominate the initial inflammatory infiltrate and are present at up to 100,000 cells/mm^3 of muscle (Wehling et al., 2001). As regeneration progresses, myeloid cells, largely macrophages, assume a continuum of distinct phenotypes that support each phase of repair (Saclier et al., 2013; Tidball and Wehling-Henricks, 2007). Macrophages secrete the cytokines, chemokines, and growth factors that drive the removal or phagocytosis of necrotic tissue, produce the fibrotic scaffold and blood supply needed for new fibers to attach and grow, and help resolve the inflammation. Macrophages also support myogenesis of new fibers from muscle-resident progenitor cells (satellite cells) by producing factors that promote satellite cell activation, proliferation, and differentiation into adult fiber types (Arnold et al., 2007; Chazaud et al., 2009). Macrophages eventually disappear as regeneration is completed and the newly formed adult fibers are integrated into the muscle syncytium. Despite the crucial roles played by myeloid-lineage cells in adult muscle regeneration, the molecular mechanisms that determine their phenotypes and functions in this process remain poorly understood.

The zinc-finger transcription factor, KLF2, plays a central role in the activation and phenotypic determination of a variety of immune cell types, both lymphoid and myeloid (Carlson et al., 2006; Das et al., 2012;

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Sebzda et al., 2008). KLF2 is a central, negative regulator of gene clusters that mediate the pro-inflammatory activation of monocytes and macrophages *in vitro* (Das et al., 2006), and its loss promotes an inflammatory monocyte or macrophage profile *in vitro* and *in vivo* (Das et al., 2012; Lingrel et al., 2012; Manoharan et al., 2014). Macrophages of mice that lack KLF2 in all myeloid cells assume an enhanced inflammatory profile with elevated expression of inflammatory mediators and greater immune cell infiltration into atherosclerotic plaques (Lingrel et al., 2012; Manoharan et al., 2014).

These considerations led us to propose that KLF2 may play an important role in directing the immune response to acute muscle injury. To test this hypothesis, we compared regeneration in skeletal muscles of wild-type (WT) mice with that in mice that lack KLF2 in all myeloid lineage cells (*myeKlf2*$^{-/-}$). We subjected the mice to cardiotoxin (CTX) or to sham injury to the lateral gastrocnemius muscle and followed regeneration for 21 days post injury using established indices—the expression levels of inflammatory mediators and myogenic markers, macrophage phenotypes, histological changes, and functional restoration of muscle strength.

Our results show that loss of KLF2 in myeloid-lineage cells significantly enhances the inflammatory immune response to muscle injury; notably, an enhanced inflammatory response accelerates regeneration. These findings demonstrate that KLF2 plays a central role in determining myeloid cell phenotypes and functions during skeletal muscle regeneration. In addition, they suggest that KLF2 in myeloid-lineage cells, which are accessible from the circulation, may be a useful therapeutic target for improving outcomes after muscle injury.

RESULTS

Loss of KLF2 in Myeloid-Derived Cells Enhances the Inflammatory Immune Response to Muscle Injury

CTX muscle injury induces a highly stereotypical regeneration process. Rapid necrosis and inflammation, characterized by neutrophil and macrophage infiltration, is followed by resolution of inflammation and myogenesis from satellite cells. The initial, pro-inflammatory phase is marked by significantly elevated levels of inflammatory mediators, many of which are produced within the muscle by myeloid-derived monocytes and macrophages. To investigate the role of myeloid KLF2 in their production, we compared the expression of *Cox2*, *IL-6*, *Ccl2*, *Ccl4*, *Ccl5*, and *Tnfα* in regenerating muscles of WT and *myeKlf2*$^{-/-}$ mice at days 3 and 5 post CTX or vehicle treatment (Figure 1). This was found to be the period during which the greatest differences between WT and *myeKlf2*$^{-/-}$ were seen.

Each of these cytokines or chemokines plays a known role in adult muscle regeneration. Interleukin (IL)-6 is one of the earliest cytokines detected after acute muscle injury, and the initial pool of IL-6 is produced by monocytes recruited from the circulation (Zhang et al., 2013). The signaling cytokines, *Ccl2*, *Ccl4*, and *Ccl5*, are released from the injured tissue and are also secreted by infiltrating monocytes. *Ccl2 is* regulated by KLF2 via miR124a and miR150 (Manoharan et al., 2014). The cytokines tumor necrosis factor (TNF)-α and cyclooxygenase (COX)-2, which mediate inflammation through prostaglandins, originate from activated macrophages that matured *in situ* from infiltrating monocytes. *Cox2* signaling is essential during the early stages of skeletal muscle regeneration (Bondesen et al., 2004). In injured muscle, TNF-α and COX-2 originate from both inflammatory macrophages and injured muscle fibers and participate in both inflammatory and regenerative functions (Bondesen et al., 2004; Chen et al., 2005; Warren et al., 2002).

In injured muscles of WT mice, *IL-6*, *Ccl2*, *Ccl4*, *Ccl5*, and *Tnfα* are present at day 3 post injury (Figure 1A) and decline by day 5 (Figure 1C). Their appearance indicates that recruitment and infiltration of circulating monocytes is underway. Their decline by day 5 indicates that inflammation is resolving. Expression of *Tnfα* indicates that some of the infiltrating monocytes have matured into activated, pro-inflammatory macrophages. Injured muscles of *myeKlf2*$^{-/-}$ mice express these markers at 15- to 30-fold greater levels than WT at day 3 post injury (Figure 1B); all but *Ccl5* remain significantly elevated at day 5 (Figure 1D), a time at which their expression has returned toward baseline in WT. None of these markers are significantly expressed in muscles of either genotype after sham injury (Figures 1A–1D, solid bars). The dramatically increased expression of inflammatory mediators and their earlier decline in muscles of *myeKlf2*$^{-/-}$ mice suggest that loss of KLF2 in myeloid cells enhances and accelerates the pro-inflammatory phase of skeletal muscle regeneration. Next, we measured the expression of these pro-inflammatory mediators in CD11b^{+}

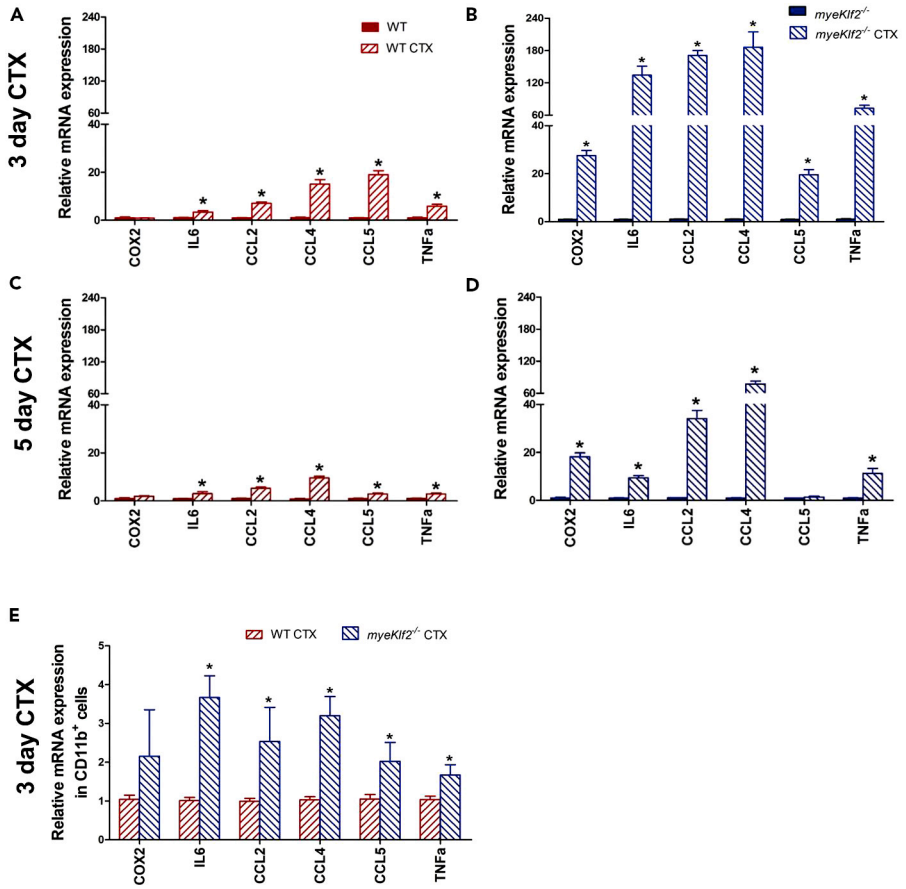

Figure 1. Loss of KLF2 in Myeloid Lineage Cells Enhances the Expression of Pro-inflammatory Mediators in Regenerating Skeletal Muscles

(A–D) Lateral gastrocnemius muscles of WT (red) and *myeKlf2*$^{-/-}$ (blue) mice were taken at day 3 (A and B) and day 5 (C and D) following vehicle (solid fill) or CTX (striped) injury and probed by RT-qPCR.

(E) CD11b^{+} macrophages isolated from the site of injury on day 3 showed enhanced expression of inflammatory markers in *myeKlf2*$^{-/-}$ mice compared with WT. * Indicates significant difference at $p < 0.05$; $n = 4$.

monocytes or macrophages isolated from the injured muscles (Figure 1E). Except for *Cox2*, expression of these markers is significantly increased in *myeKlf2*$^{-/-}$ mice compared with the WT, indicating that they originate from myeloid-lineage cells that infiltrated from the circulation.

Injured Muscles of *myeKlf2*$^{-/-}$ Mice Recruit Greater Numbers of Circulating Ly6C^{+} Monocytes than WT Mice

The enhanced expression of inflammatory markers in injured muscles of *myeKlf2*$^{-/-}$ mice suggests that these muscles recruit greater numbers of inflammatory Ly6C^{+} monocytes from the circulation. Circulating Ly6C^{+} monocytes provide the pool that matures *in situ* into monocyte-derived macrophages (Arnold et al., 2007). To examine this possibility, we compared the number of Ly6C^{+} cells present in regenerating skeletal muscles of *myeKlf2*$^{-/-}$ and WT mice at day 3 (Figures 2A and 2B) and day 5 (Figures 2D and 2E) post injury. Ly6C^{+} monocytes and macrophages are present in both genotypes, as expected. However, by day 3, injured muscles of *myeKlf2*$^{-/-}$ mice have recruited a significantly greater number of Ly6C^{+} monocytes than WT (Figures 2A and 2B, 55.7% versus 35.7%, $n = 6$; $p < 0.05$). The number of Ly6C^{+} monocytes subsequently declines, and their decline starts earlier in *myeKlf2*$^{-/-}$ muscles. Their decline is apparent by day 5, a time at which their number is still increasing in WT (Figures 2D and 2E, 20.4% versus 24.6%, $n = 6$; $p < 0.05$). Overall, injured muscles of *myeKlf2*$^{-/-}$ mice recruit more circulating Ly6C^{+} cells at day 3 (Figure 2C, rightmost panels, blue versus red counts), and this population declines earlier than in WT mice (Figure 2F).

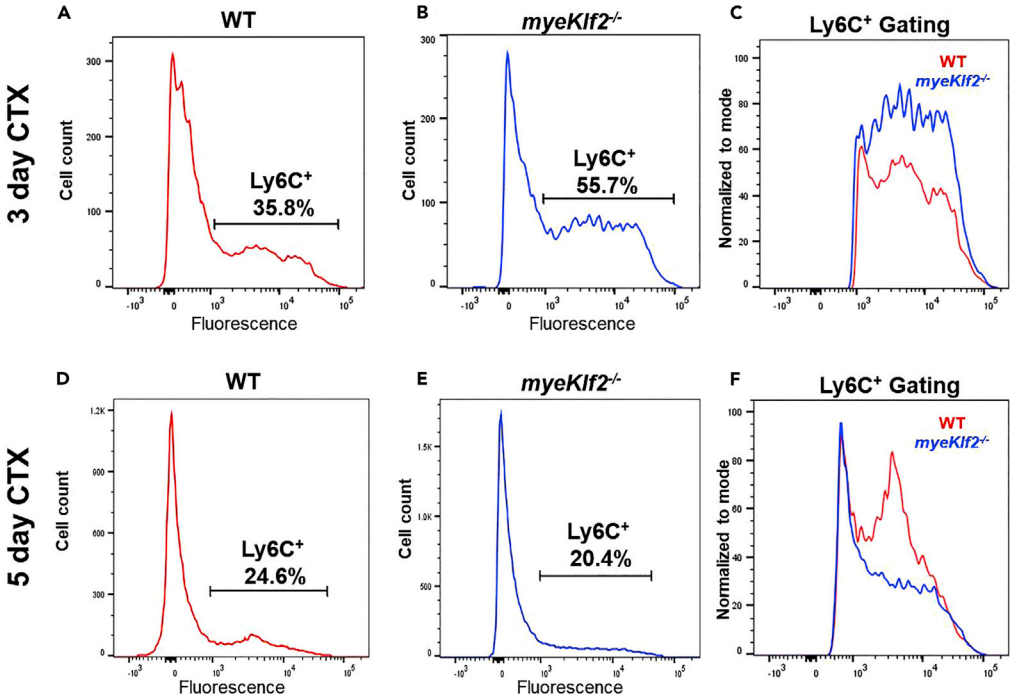

Figure 2. Injured Muscles of *myeKlf2*$^{-/-}$ Mice Recruit a Greater Number of Ly6C^{+} Monocytes

(A, B, D, and E) Representative flow cytometric analysis showing Ly6C^{+} cells present in injured muscles of WT (red) and *myeKlf2*$^{-/-}$ (blue) mice at day 3 (A and B) and day 5 (D and E) following CTX injury. Vertical axis: Ly6C count. Horizontal axis: cell count. Homogenates were prepared from CTX-treated lateral gastrocnemius muscles of WT and *myeKlf2*$^{-/-}$ mice and sorted by fluorescence-activated cell sorting using an antibody against Ly6C. Results are expressed as the percentage of mononuclear cells isolated from muscle and represent the means ± SEM of measurements on six mice of each genotype.

(C and F) Normalized difference in Ly6C^{+} cell counts between WT (red) and *myeKlf2*$^{-/-}$ (blue) at days 3 and 5 ($n = 6$; $p < 0.05$).

Infiltrating Ly6C^{+} Monocytes Mature Earlier into Inflammatory Macrophages that Are Phenotypically Ly6C^{+}, CD11b^{+}, F4/80^{+}

Pro-inflammatory macrophages within the injured tissue derive from circulating Ly6C^{+} monocytes (Arnold et al., 2007). Macrophages in regenerating skeletal muscles do not assume the canonical M1/M2 phenotypes defined for other inflammatory conditions (Novak et al., 2014; Wang et al., 2014). To identify the relevant populations, we further sorted immune cells by CD11b and F4/80 markers (Figure 3). Myeloid-lineage monocytes and macrophages express the surface marker CD11b, which is upregulated upon activation. F4/80 is a macrophage-specific marker of monocyte differentiation that identifies intramuscular, mature macrophages. It is not expressed by circulating monocytes. Cells that are positive for all three markers—Ly6C^{+}, CD11b^{+}, and F4/80^{+}—represent the population of intramuscular, pro-inflammatory macrophages that derived from circulating Ly6C^{+} monocytes. Differences between WT and *myeKlf2*$^{-/-}$ reveal populations that are regulated, directly or indirectly, by KLF2.

By day 3, pro-inflammatory, monocyte-derived macrophages are evident in both genotypes. This population is significantly greater in *myeKlf2*$^{-/-}$ compared with WT (Figures 3E versus 3B; 60.7% in *myeKlf2*$^{-/-}$ versus 37.4% in WT; $n = 6$; $p < 0.05$). Only trace numbers of anti-inflammatory, Ly6C^{-} cells are detected in both genotypes at day 3 (Figures 3C and 3F, 9.75% and 6.4%, $n = 6$; $p > 0.05$). The near absence of Ly6C^{-} cells is consistent with a previous report that Ly6C^{-} cells are not present in uninjured skeletal muscles, and injured muscles do not recruit Ly6C^{-} monocytes (Arnold et al., 2007).

By day 5, the Ly6C^{+} population declines in both genotypes (Figures 3H and 3K; see also Figure 2), and the decline is associated with an increase in anti-inflammatory Ly6C^{-} populations (Figures 3I and 3L). The majority of these cells are phenotypically Ly6C^{-}, CD11b^{+}, F4/80^{+}. This cell population is significantly greater in

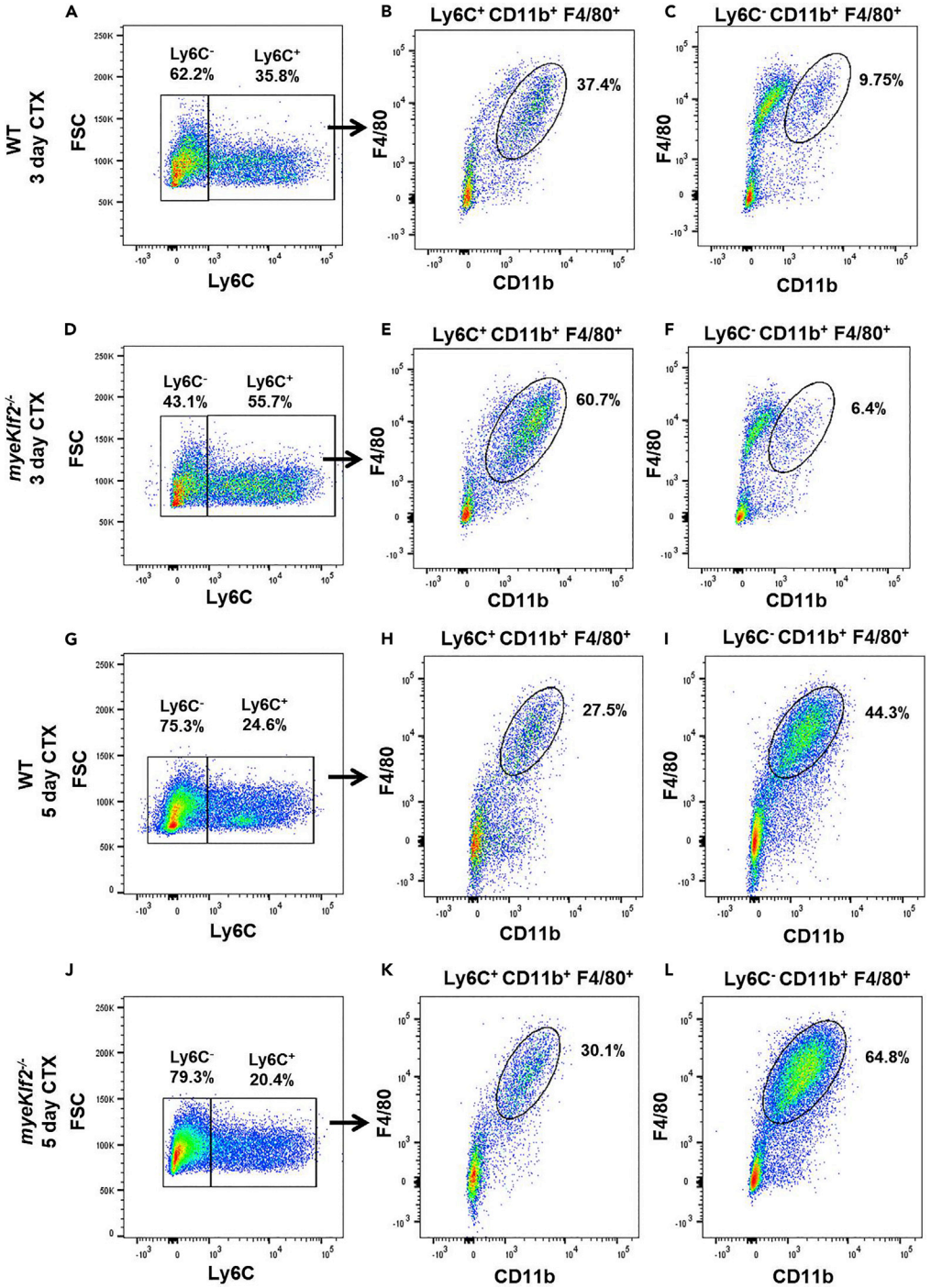

Figure 3. Monocyte-Derived Macrophage Populations Present in Regenerating Skeletal Muscles of WT and myeKlf2$^{-/-}$ Mice at Days 3 and 5 Post Injury

(A–L) Days 3 and 5 post injury: Ly6C^{+} and Ly6C^{-} mononuclear cells were isolated on days 3 and 5 post injury from regenerating muscles of WT (A–C) and *myeKlf2*$^{-/-}$ (D–F) mice, gated by Ly6C, and further sorted by flow cytometry using antibodies against CD11b (horizontal) and F4/80 (vertical). (B and E) Macrophages that are triple positive for Ly6C^{+}, CD11b^{+}, and F4/80^{+} (circled). (C and F) Macrophages that are Ly6C^{-}, CD11b^{+}, and F4/80^{+}. Circles indicate the triple-labeled cells used for counting ($n = 6$; $p < 0.05$).

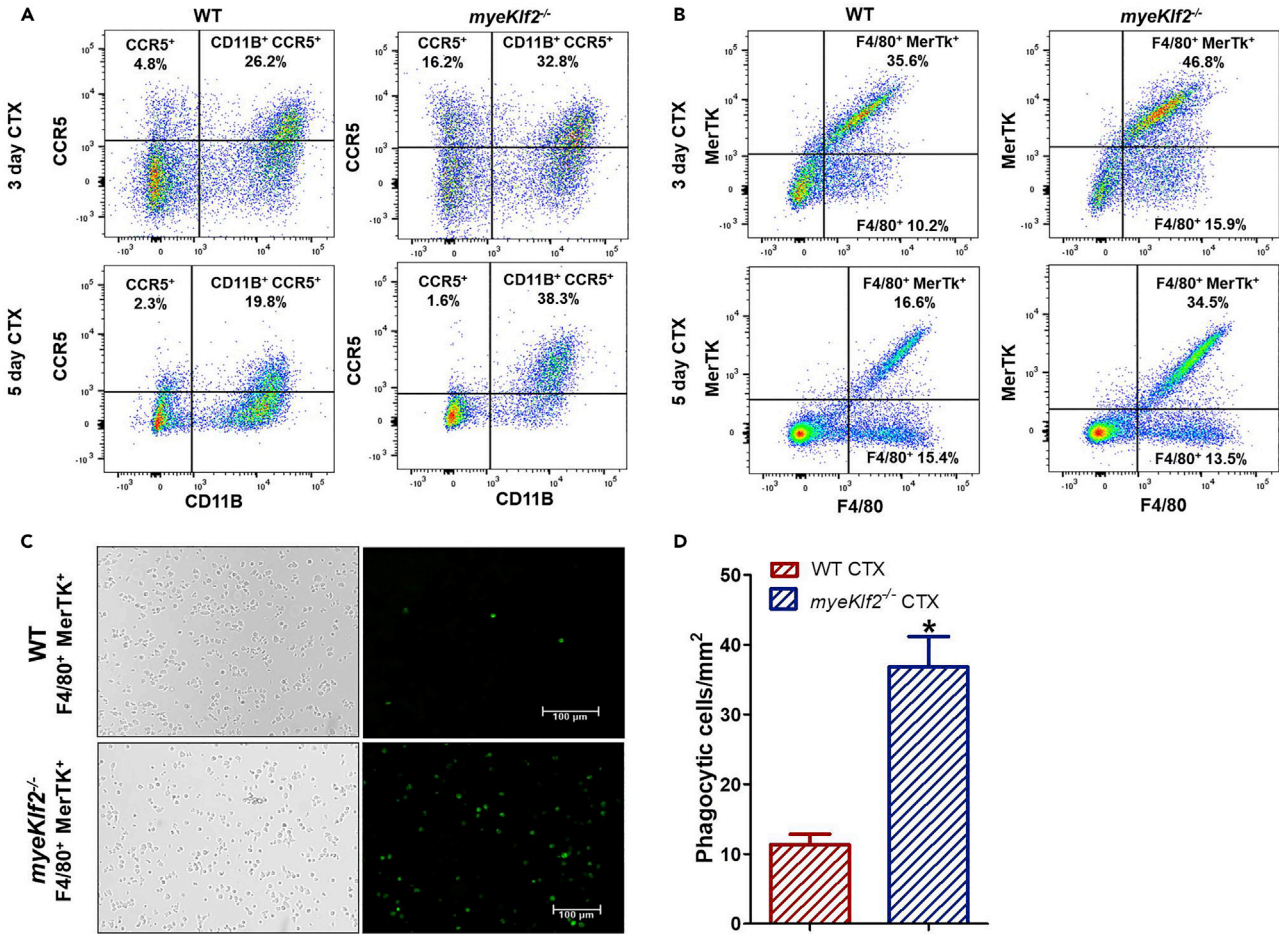

Figure 4. Loss of KLF2 Increases the Number of Intramuscular CCR5$^+$ and MerTK$^+$ Monocytes and Increases the Phagocytic Capacity of Inflammatory Macrophages at Days 3 and 5 Post Injury

(A and B) (A) Representative flow cytometry figure of CCR5$^+$ cells gated on CD11b$^+$ population and (B) representative flow cytometry figure of MerTK-expressing F4/80$^+$ macrophages obtained from muscles of WT and *myeKlf2*$^{-/-}$ mice at days 3 and 5 post injury. Each graph is representative of results obtained from five mice of each genotype.

(C) Phagocytic activity of inflammatory macrophages from each genotype. Intramuscular MerTK$^+$F4/80$^+$ macrophages were obtained by cell sorting with MoFlo XDP. The isolated macrophages were plated and assayed for phagocytic activity using pHrodoTM Green Bio Particle. Green fluorescence identifies actively phagocytosing cells. Images show positive phagocytic events at the endpoint after 4 h of live recording.

(D) Number of phagocytic cells counted in *myeKlf2*$^{-/-}$and WT samples. * Indicates statistically significant difference $p < 0.001$ for n = 4 samples of each genotype.

myeKlf2$^{-/-}$ compared with WT (Figures 3L versus 3I: 64.8% in *myeKlf2*$^{-/-}$ versus 44.4% in WT, n = 6; $p < 0.05$). The decline in Ly6C$^+$ macrophages and associated increase in Ly6C$^-$ macrophages marks the resolution of inflammation and transition to regeneration. The greater abundance of anti-inflammatory Ly6C$^-$ macrophages in *myeKlf2*$^{-/-}$ at day 5 suggests that resolution of inflammation is advanced in *myeKlf2*$^{-/-}$ compared with WT.

Expression of CCR5 and MerTK Is Enhanced in Injured Muscles of *myeKlf2*$^{-/-}$ Mice: Macrophages Obtained from Injured Muscle Show Enhanced Phagocytic Activity, Reflecting Elevated Expression of MerTK

Macrophage-mediated inflammatory processes must end when the damaged tissue has been cleared so as to not destroy adjacent, undamaged tissue. To further examine the role of KLF2 in resolving inflammation, we compared the expression of the chemokine receptors CCR5 and MerTK in intramuscular monocytes of regenerating *myeKlf2*$^{-/-}$ and WT muscles (Figure 4A). KLF2 controls the expression of chemokine

receptors in other immune cells (Sebzda et al., 2008). For example, only activated inflammatory T cells, which downregulate KLF2, express CCR5. Deletion of KLF2 in T cells induces expression of the CCR5 gene, which has consensus KLF2-binding sequences (Sebzda et al., 2008). Interestingly, CCR5 is the cytokine receptor for CCL4 (MIP1β) and CCL5 (RANTES), both of which are dramatically elevated in injured muscles of *myeKlf2*$^{-/-}$ mice (Figure 1). Upregulation of CCR5 is implicated in a range of inflammatory conditions (Schroder et al., 2007; Warren et al., 2004) and plays a key role in resolving inflammation in arthritis (Doodes et al., 2009).

CCR5-expressing cells are more abundant in regenerating muscles of *myeKlf2*$^{-/-}$ compared with WT on days 3 and 5 post injury. Interestingly, two distinct populations of CCR5-expressing cells appear in the injured muscle—CD11b^{+}CCR5^{+} and CD11b^{-}CCR5^{+}. CD11b^{+}CCR5^{+} cells are present in both genotypes at comparable abundance (32.8% in *myeKlf2*$^{-/-}$ versus 26.2% in WT, $n = 5$, $p > .05$), whereas the CD11b^{-}CCR5^{+} population is significantly increased in *myeKlf2*$^{-/-}$ samples (16.2% in *myeKlf2*$^{-/-}$ versus 4.8% in WT, $n = 5$; $p < 0.05$). This population remains elevated on day 5 in *myeKlf2*$^{-/-}$ muscles (38.3% in *myeKlf2*$^{-/-}$ versus 19.8% in WT, $n = 5$; $p < 0.05$), whereas the CD11b^{-}CCR5^{+} population declines sharply in both genotypes after day 3. The elevated expression of CD11b^{+}CCR5^{+} populations and their complimentary ligands (*Ccl4* and *Ccl5*) in injured *myeKlf2*$^{-/-}$ muscles suggests that CCR5 receptors contribute to monocyte recruitment after injury.

MerTK is a member of the Tyro3/Axl/Mer (TAM) receptor tyrosine kinase family that plays a role in resolving inflammation and dampening inflammatory responses after acute injury in other conditions (Choi et al., 2013; Crane et al., 2014; DeBerge et al., 2017; Eken et al., 2010; Triantafyllou et al., 2018; Wan et al., 2013). MerTK expression in macrophages defines a late stage of phagocytosis. Macrophages must induce MerTK expression to engulf apoptotic cells, and only actively phagocytosing macrophages elevate MerTK (Scott et al., 2001). When intramuscular macrophages (i.e., F4/80^{+}) are sorted by MerTK^{+}, the MerTK^{+} F4/80^{+} population is elevated at day 3 (46.8% versus 35.6%, $n = 5$, $p < 0.01$) and day 5 (34.5% versus 16.6%, $n = 5$, $p < 0.01$) in muscles of *myeKlf2*$^{-/-}$ compared with WT (Figure 4B). The MerTK^{+}F4/80^{+} cell population shows significantly greater phagocytic activity in *myeKlf2*$^{-/-}$ compared with WT (36.83 ± 4.34 versus 11.33 ± 1.52 phagocytic cells/mm^{2}, $n = 4$, $p < 0.001$) (Figures 4C and 4D), confirming the relationship between MerTK expression and phagocytosis. In other words, the clearance of dead cells, which prepares the environment for myogenesis, is advanced in *myeKlf2*$^{-/-}$ compared with WT muscles.

KLF2 deficiency did not change the quantity of intramuscular CCR2-expressing cells on days 3 and 5 post injury (data not shown). CCR2 is a receptor for the ligand CCL2, which is upregulated in *myeKlf2*$^{-/-}$ muscles at early times after injury (Figure 1). CCR5 and MerTK expressions in these samples originate largely in myeloid-lineage monocytes or macrophages. CCR5 and MerTK expressions do not arise from T cells (data not shown), which also express these markers but comprise less than 1% of the immune cell population in these samples. Collectively, the results in Figure 4 demonstrate that suppressing KLF2 expression in myeloid-lineage cells promotes phagocytosis and accelerates the resolution of inflammation in regenerating skeletal muscles.

Markers of Satellite Cell Activation and Myogenesis Appear Earlier and Are Enhanced in Regenerating Muscles of *myeKlf2*$^{-/-}$ Mice

To examine whether or not the earlier resolution of inflammation in *myeKlf2*$^{-/-}$ leads to accelerated myogenesis from satellite cells, we measured the expression of key transcription factors or myogenic regulatory factors (MRFs) that determine satellite cell activation, proliferation, and differentiation into adult fiber types (Figure 5). Pax7 is a canonical satellite cell-specific marker in muscle (Yin et al., 2013). Upon activation, satellite cells co-express Pax7 with MyoD (also known as Mrf3) (Zammit et al., 2006). MyoD, Mrf5, and Mrf6 (also called herculin) are expressed only by activated satellite cells (Francetic and Li, 2011; Zammit, 2017) and are among the earliest markers of myogenic commitment. Increasing expression of this cluster of markers and decreasing expression of inflammatory markers marks a critical transition in the regeneration program, during which myogenesis predominates over inflammation.

Regenerating muscles of *myeKlf2*$^{-/-}$ mice express these markers earlier and at higher levels than WT. Pax7, MyoD, and MyoG are already expressed at day 3 in *myeKlf2*$^{-/-}$ (Figure 5B), are elevated 3- to 5-fold at day 5 (Figure 5D), and remain elevated through day 8 (data not shown). By day 5, Myf5 expression is significantly

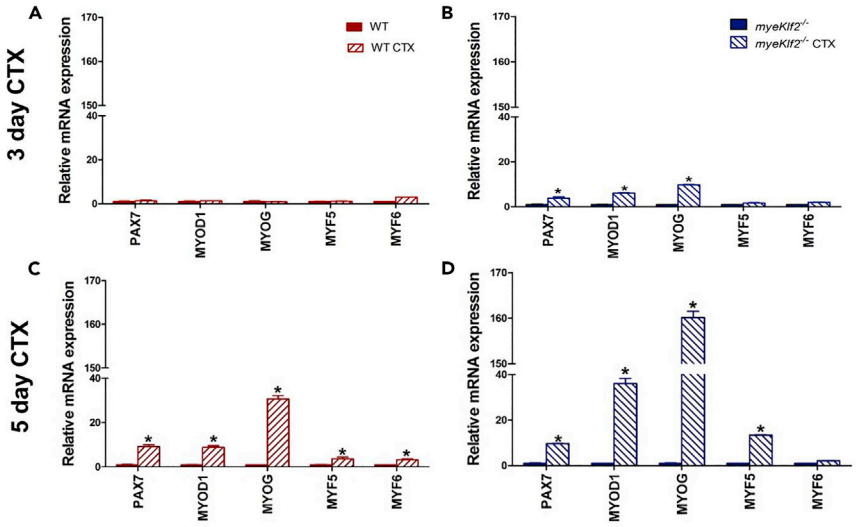

Figure 5. Markers of Muscle Satellite Cell Activation and Differentiation in Regenerating Muscles of WT and *myeKlf2*$^{-/-}$ Mice

(A–D) Lateral gastrocnemius muscles of WT (red) and *myeKlf2*$^{-/-}$ (blue) mice were taken at day 3 (A and B) and day 5 (C and D), following vehicle (filled bars) or CTX (striped bars) treatment, and probed for the indicated markers by RT-qPCR. * Indicates significant difference at $p < 0.05$. n = 4.

elevated in *myeKlf2*$^{-/-}$ compared with WT. The earlier appearance of these markers in *myeKlf2*$^{-/-}$ compared with WT indicates an advanced stage of myogenesis.

Regeneration Is Advanced and Completed Normally in Injured Muscles of *myeKlf2*$^{-/-}$ Mice

The progression of muscle regeneration in WT and *myeKlf2*$^{-/-}$ mice was further investigated using histological and functional indices (Figure 6). Uninjured H&E-stained muscle sections (Figure 6A) of both genotypes show phenotypically normal histology. Injured muscles of WT mice show the typical progression from inflammation through myogenesis from satellite cells (Garry et al., 2016; Hardy et al., 2016). By days 3, 5, and 8 post injury, muscles show extensive necrosis. By day 14, the inflammation has largely resolved and myogenesis from satellite cells is underway. Myogenesis is evident from the presence of cells with the canonical features of newly forming fibers—small diameters and a central nucleus. By day 21, many new fibers have differentiated into adult fibers with larger cross-sectional areas and multiple, peripherally localized nuclei. Injured muscles of *myeKlf2*$^{-/-}$ mice show an accelerated pattern of repair. Smaller necrotic regions with fewer monocytes are evident at day 5, consistent with an earlier resolution of inflammation. By day 8, necrotic regions have largely disappeared and newly forming fibers are already present. These muscles are transitioning to the myogenic phase of regeneration. By day 14, many new fibers have differentiated into larger diameter, multi-nucleated adult phenotypes in which nuclei have moved to the fiber periphery. By day 21, muscles of *myeKlf2*$^{-/-}$ mice show a regenerated adult architecture that is nearly indistinguishable from that of uninjured muscle.

The accelerated progress of regeneration in *myeKlf2*$^{-/-}$ was further confirmed by immunolabeling using morphometric and myogenic markers of new fiber formation. At day 8, regenerating fibers of *myeKlf2*$^{-/-}$ show more fibers with larger cross-sectional areas (Figures 6B and 6Ei), indicating advanced myogenesis. These muscles have a greater number of fibers that have central nuclei and express eMHC (Figures 6C, 6Eii, and 6Eiii). As the new fibers mature into adult fiber types, eMHC expression and central nuclei both decrease, and this decrease occurs earlier in *myeKlf2*$^{-/-}$ than in WT muscles (day 14, Figures 6B and 6C). The transcription factor Pax7 is uniformly expressed in proliferative-state satellite cells and is subsequently downregulated as myogenic differentiation advances. At day 8, regenerating muscles of *myeKlf2*$^{-/-}$ mice show fewer fibers expressing Pax7 (Figures 6D and 6Eiv). Taken together, these multiple indices independently demonstrate that myogenesis from satellite cells is advanced in *myeKlf2*$^{-/-}$ compared with WT regenerating muscles.

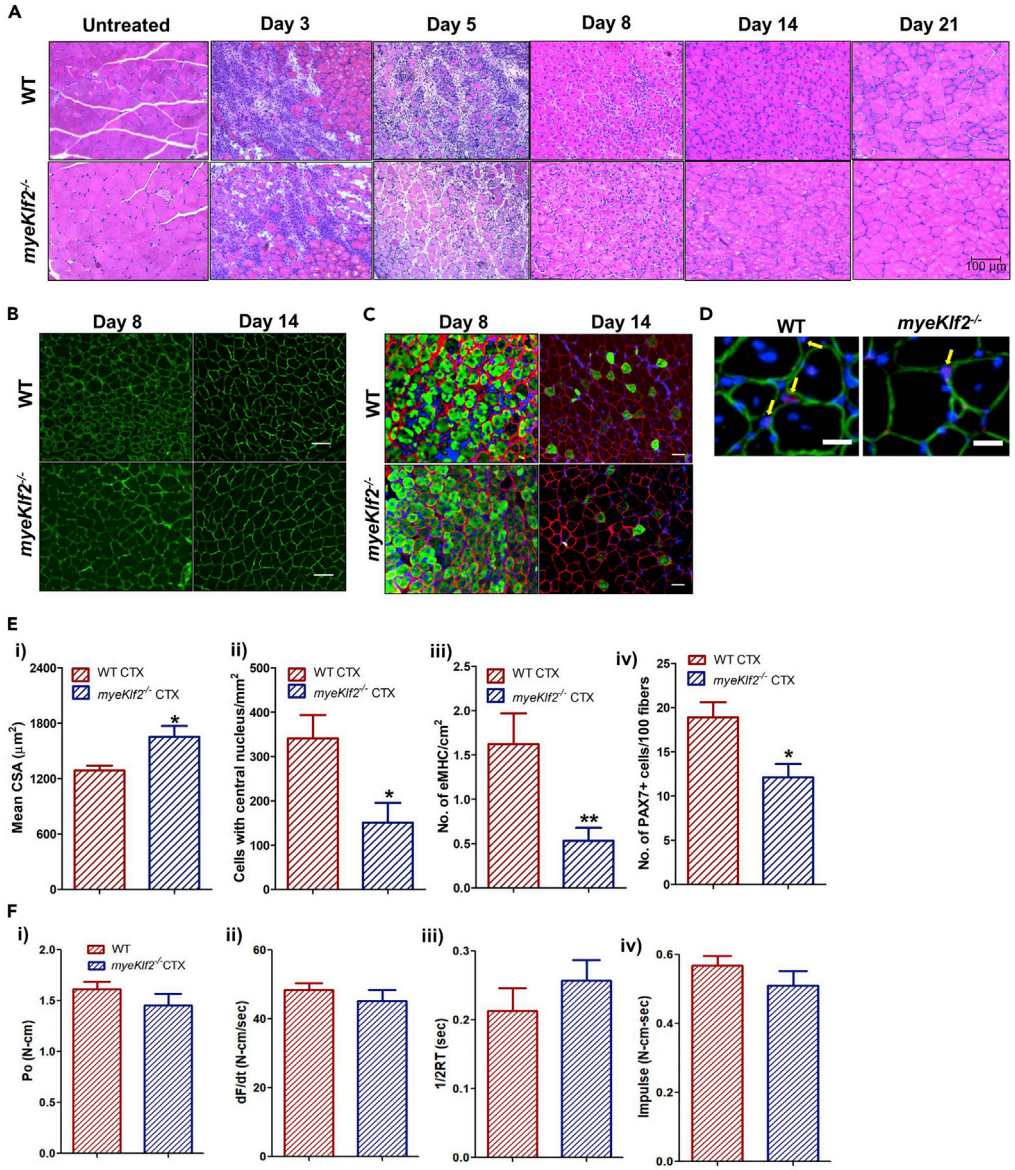

Figure 6. Regeneration Is Accelerated and Completed Normally in Injured Muscles of *myeKlf2*$^{-/-}$ Mice

(A) Representative H&E-stained cross sections of lateral gastrocnemius muscles from male WT and *myeKlf2*$^{-/-}$ mice. Sections were obtained from uninjured and CTX-injured muscles at days 3, 5, 8, 14, and 21 post treatment. Scale bar, 100 µm.

(B) Laminin-labeled sections at days 8 and 14 post treatment. Laminin (green) was used to visualize fiber perimeters for measuring cross-sectional areas. n = 3–5 mice of each genotype at each time point. Scale bar, 100 µm.

(C) Embryonic myosin heavy chain (eMHC)-labeled (green) sections at days 8 and 14 post treatment. Dystrophin (red) and DAPI (blue) were used to mark skeletal muscle fibers and nuclei, respectively. Dystrophin is expressed only by skeletal muscle fibers and is used to exclude non-muscle cells from the quantification. Fibers that express eMHC and dystrophin and have a central nucleus represent newly forming fibers. Scale bar, 50 µm.

(D and E) (D) Pax7-labeled (red) sections along with dystrophin (green) and DAPI (blue) at day 8 post treatment. Small, newly forming fibers at an early stage of myogenesis (arrows) are positive for all three markers. Scale bar, 10 µm; n = 6 per genotype. (E) Mean cross-sectional areas (i), density of fibers with central nuclei (ii), and density of fibers expressing eMHC (iii) on day 14 after CTX injury; Pax7-positive cells (iv) on day 8 after CTX injury. CSAs were measured from laminin-labeled sections; number of fibers with central nuclei was counted using H&E-stained sections. * Indicates statistically significant difference for n = 5 mice of each genotype at *$p < 0.05$ or **$p < 0.001$. For each genotype, three labeled sections per muscle were used to count Pax7-expressing cells. Approximately 300 fibers per labeled section of each sample were examined to obtain the number of Pax7-positive cells per 100 fibers.

Figure 6. *Continued*

(F) Functional recovery of plantar flexion force in *myeKlf2*$^{-/-}$ and WT mice at day 21 post injury. Isometric plantar flexion force was measured *in vivo* using conditions in which the gastrocnemius muscle makes the major contribution (Methods). Po, peak isometric tetanic force. dF/dt, rate of force development. $^{1}/_{2}$ RT, half relaxation time. Impulse, area under strength curve. n = 4 mice of each genotype. Po, dF/dt, $^{1}/_{2}$RT, and impulse in uninjured gastrocnemius muscles were comparable in both genotypes (n = 4, $p > 0.05$ for each parameter; data not shown). A pilot study at day 28 showed no further differences between genotypes (data not shown).

Finally, we compared functional recovery of muscle force in *myeKlf2*$^{-/-}$ and WT mice at day 21 post injury (Figure 6F). The ability to generate force is a fundamental benchmark of muscle function. *myeKlf2*$^{-/-}$ mice recovered plantar flexion strength comparable with WT, indicating full functional recovery.

DISCUSSION

Skeletal muscle regeneration after injury follows a specific program of coordinated interactions between the innate immune system and the injured muscle. The immune system's contribution is essential for the proper completion of repair (Smith et al., 2008) and is dominated by myeloid-lineage cells. In this study, we found that the transcription factor KLF2 determines important myeloid cell fates and functions during skeletal muscle repair and regeneration. Loss of KLF2 enhances the initial inflammatory response to injury and, notably, promotes an earlier resolution of inflammation and an earlier completion of myogenesis.

During the initial inflammatory phase of repair, injured muscles and isolated macrophages of *myeKlf2*$^{-/-}$ mice express dramatically increased levels of known inflammatory mediators in association with greater numbers of infiltrating, pro-inflammatory Ly6C^{+} monocytes/macrophages. In addition, the critical transition from inflammation to myogenesis, marked by a decline in Ly6C^{+} macrophages and their replacement by anti-inflammatory Ly6C^{+} macrophages, begins earlier and is enhanced. During the myogenic phase of repair, muscles of *myeKlf2*$^{-/-}$ show elevated levels of factors that promote the activation, growth, and differentiation of satellite cells into new adult fibers. In all phases of repair, the expected histological indices coincide with the appearance of pro- and anti-inflammatory mediators and cell populations.

A more robust, inflammatory response to muscle injury in myeKlf2$^{-/-}$ mice is consistent with known functions of myeloid KLF2 as a negative regulator of inflammatory genes in other conditions (Carlson et al., 2006; Das et al., 2012; Sebzda et al., 2008). Overexpression of KLF2 in leukocytes induces quiescence (Buckley et al., 2001) and represses inflammatory genes (Das et al., 2012). Peritoneal macrophages of *myeKlf2*$^{-/-}$ mice show a chronic inflammatory profile, produce elevated levels of cytokines, and show enhanced adhesion and infiltration into atherosclerotic lesions (Lingrel et al., 2012; Manoharan et al., 2014). KLF2 directly regulates *Ccl2*/MCP-1, *Tnfα*, and *Cox2* expression in various activated monocytes *in vitro* and *in vivo* (Das et al., 2006); KLF2 deficiency in myeloid cells induces expression of *Ccl2*/MCP-1, *Ccl5*, *IL-6*, and *Cox2* in inflammatory, M1-type peritoneal macrophages (Manoharan et al., 2014).

On the other hand, the finding that enhanced inflammation progresses to an accelerated and normal completion of regeneration was not expected. In other inflammatory conditions, enhanced inflammation amplifies tissue damage and delays repair; in addition, a change in KLF2 levels is required to both mount and terminate the inflammation. For example, in atherosclerosis, the absence of KLF2 in macrophages promotes inflammatory gene expression, and a subsequent *rise* in KLF2 is required to terminate their expression (Lingrel et al., 2012; Manoharan et al., 2014). A different transcriptional program must operate in myeloid cells during skeletal muscle repair, in which inflammation resolves in the complete absence of KLF2.

The critical switch of macrophage phenotypes from Ly6C^{+} to Ly6C^{-} populations is required for skeletal muscle regeneration to progress to myogenesis (Varga et al., 2013; Wang et al., 2014). The mechanisms by which inflammation resolves and this transition proceeds without KLF2 may involve local signals related to phagocytosis, as suggested for muscle (Arnold et al., 2007) and other cell types (Fadok et al., 2001; Huynh et al., 2002; Savill and Fadok, 2000; Xu et al., 2006). The unique MerTK^{+} population of macrophages, which increases in *myeKlf2*$^{-/-}$ muscle at this transition, may also contribute to resolving inflammation.

Myeloid-derived monocytes and macrophages produce the chemokines, cytokines, and growth factors that drive each stage of repair (Warren et al., 2004). The dramatically elevated expression of these factors in regenerating muscles of mye*Klf2*$^{-/-}$ mice suggests that KLF2, either directly or indirectly, negatively regulates their expression. In particular, IL-6 plays important roles in all stages of muscle regeneration. The initial IL-6 pool originates in monocytes recruited from the circulation (Zhang et al., 2013). Subsequently, activated satellite cells also produce IL-6, which promotes the formation and differentiation of new fibers (Munoz-Canoves et al., 2013). Our finding that KLF2 regulates Il-6 expression early in muscle regeneration may explain, in part, the ability of an enhanced inflammatory phase to positively drive the myogenic phases of repair. IL-6 produced by pro-inflammatory macrophages (together with other chemokines and factors) may promote satellite cell activation and thereby further raise IL-6 levels in the satellite cell niche, as proposed by other researchers (Munoz-Canoves et al., 2013).

Ly6C$^+$ monocytes recruited from the circulation are non-dividing, but mature within the injured muscle into macrophages capable of proliferation and differentiation (Arnold et al., 2007). Loss of KLF2 may promote their *in situ* maturation, consistent with the greater abundance of Ly6C$^+$, CD11b$^+$, F4/80$^+$ macrophages found in injured *myeKlf2*$^{-/-}$ muscles. Interestingly, a population of inflammatory Ly6C$^+$, CD11b$^+$, F4/80$^+$ monocytes is significantly increased at sites of inflammation in rheumatoid arthritis when KLF2 is reduced systemically (Das et al., 2012).

The pattern of cytokine receptor expression determines the response of monocytes and macrophages to chemokines. Myeloid cells *of myeKlf2*$^{-/-}$ mice cannot downregulate CCR5 by upregulating KLF2, as occurs in T cells, monocytes, and macrophages in other immune conditions (Das et al., 2006; Sebzda et al., 2008). The enhanced and sustained expression of CCR5 in regenerating muscles of *myeKlf2*$^{-/-}$ mice may contribute to the larger population of Ly6C$^+$ monocytes and macrophages. In other immune conditions, CCR5 receptors stimulate migration of inflammatory monocytes to the site of injury (Sebzda et al., 2008). CCR5 expression increases during differentiation of human monocytes and monocyte-derived macrophages (Naif et al., 1998; Tuttle et al., 1998). In atherosclerosis, CCR5 and CX3CR1 expression is required for Ly6C$^+$ monocytes to accumulate in plaques (Tacke et al., 2007).

Our finding that enhancing the innate immune response to muscle injury *accelerates* regeneration has important clinical implications. It challenges the common strategy of treating skeletal muscle injuries with anti-inflammatory agents and is consistent with reports that some anti-inflammatory drugs, in fact, delay muscle regeneration after injury (Shen et al., 2005). In addition, it raises the possibility that drugs that elevate myeloid KLF2, for example, some statins (Bu et al., 2010), may delay muscle regeneration.

In conclusion, loss of the zinc-finger transcription factor, KLF2, in myeloid cells enhances the initial inflammatory phase of repair and positively drives subsequent phases of regeneration to a normal and earlier completion. KLF2 controls important macrophage phenotypes and effector functions during skeletal muscle regeneration after injury.

Limitations of the Study

The CTX model of muscle injury is well validated but does not perfectly recapitulate regeneration after other types of mechanical or exercise-induced injury. The gating strategy used to identify monocytes (CD11b+, Ly6C+, F4/80+) may include nonmonocytes such as eosinophils because they share common surface markers. This study focused on intramuscular myeloid cell populations from day 3 post injury through completion of regeneration. It is possible that loss of KLF2 may produce upstream effects on myeloid progenitor cells in the bone marrow or circulating monocytes and thereby bias the pool recruited to the injured muscle. Additional mechanistic studies are needed to address these questions.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2019.07.009.

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AUTHORS CONTRIBUTIONS

P.M. generated the hypothesis, designed experiments, performed experiments, analyzed data, and wrote and edited the manuscript. T.S. performed experiments and assisted in data analysis. T.L.R. assisted with functional measurements. S.S. analyzed the data, provided funding, and edited the manuscript. J.B L. and J.A.H. guided the project, analyzed the data, provided funding and infrastructure, and wrote and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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ISCI, Volume *17*

Supplemental Information

KLF2 in Myeloid Lineage Cells Regulates the Innate Immune Response during Skeletal Muscle Injury and Regeneration

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S1. Supplemental Information

TRANSPARENT METHODS

Animals – *Klf2*$^{fl/fl}$ mice were generated and backcrossed to a C57BL/6 background as described (Lingrel et al., 2012). *Klf2*$^{fl/fl}$ mice were crossed with transgenic mice expressing *Cre* recombinase under the control of the LysM promoter (LysM-*cre*, Jackson Laboratories; (Abram et al., 2014) to produce offspring with a targeted deletion of KLF2 in all myeloid-derived cells. *Klf2*$^{fl/fl}$ mice without the *Cre* recombinase transgene were used as controls. All mice were housed in pathogen-free conditions at the University of Cincinnati. In all experiments we used male mice at the age between 8 weeks to 12 weeks, except in flow cytometry experiments (Fig 2, 3 and 4) were we used both genders at the same age. Tissue removal was performed under anesthesia (Avertin). Muscle strength was measured in mice anesthetized with isoflurane (1.5 - 2%). All procedures involving animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council of the National Academies, USA) and were approved by the Institutional Animal Care and Use Committee of the University of Cincinnati.

Muscle Injury – Acute injury was produced by injecting Cardiotoxin (CTX; EMD Millipore, 100 µl of 10 mM stock dissolved in PBS) into the lateral gastrocnemius muscle of anesthetized mice (Avertin). This dose of CTX produces a maximal immune response that does not increase with higher doses (Garry et al., 2016). CTX injury induces a stereotypical and reproducible sequence of changes that reflect the physiological repair process after traumatic injury (Garry et al., 2016; Guardiola et al., 2017; Hardy et al., 2016). The muscles were surgically removed for analysis at time points up to 21 days post injury and the animal was sacrificed. Macrophages from the injured tissue were captured by CD11b microbeads (Cat. No. 130-049-601, Miltenyi Biotec) and subjected for RT-qPCR analysis.

RNA isolation and real-time quantitative PCR (RT-qPCR) – Total RNA was isolated from skeletal muscle and macrophages using mirVana miRNA isolation kit (Invitrogen). cDNA was synthesized using the Superscript IV reverse-transcription system (Invitrogen, # 18091050) and genomic DNA was eliminated using EZDNASE (Invitrogen, # 11766051). mRNA expression was quantified using gene-specific Taqman primers and probes (Applied Biosystems) following the manufacturer's protocol, and normalized to 18sRNA levels. RT-qPCR was performed on an ABI7300 real˙time PCR system (Applied Biosystems) using Taqman universal master mix (Applied Biosystems, #4440044). Relative gene expression was calculated using the $\Delta\Delta C_t$ method following the manufacturer's instructions. All reactions were carried out in triplicate using RNA isolated from muscles of 4 - 8 mice at each time point.

Histological analysis – The lateral gastrocnemius muscle was removed, snap frozen in nitrogen-chilled isopentane, embedded in O.C.T. (Fisher Scientific) and kept at -80°C until use. Cryosections of 10 µm thickness were prepared for haematoxylin/eosin (H&E) and laminin (Sigma, L9393) staining. To avoid bias in selecting representative sections, the entire length of muscle was sectioned. The regions of greatest damage were identified and analyzed quantitatively for the percentage of necrotic and regenerating myofibers, fiber cross-sectional areas, and the number of fibers with central nuclei. Sections obtained from at least three mice per time point were analyzed. For immuno-labeling with dystrophin, eMHC and PAX7, cross-sections (10 µm) of lateral gastrocnemius muscle were fixed with cold acetone for 5 min and permeabilized with 0.5% Triton X-100 in PBS for 20 min. Antigen retrieval utilizing cottage buffer for PAX7 staining was performed as described by Shi *et al.* (Shi et al., 1993). After blocking in blocking buffer (0.1% BSA, 0.1% gelatin, 0.1% tween-20, 0.0001% NaN_3 in PBS) for 1hr at RT, the sections were incubated overnight at 4°C with primary antibodies against embryonic MHC (Sigma, HPA021808, 1:5,000), dystrophin (Invitrogen, MA1-26837) and PAX7 (Proteintech,

20570-1-AP). Sections were incubated with secondary antibodies (Alexa Fluor 488 and 568, Invitrogen) for 1 h in at room temperature and mounted with anti-fade mounting media and DAPI stain (Invitrogen, S36942). Images were captured at 10x using a Leica DMi8 inverted microscope and analyzed with Las X (Leica) and Image J software.

Cell isolation and flow cytometry – Flow cytometry was performed using a suspension of single cells obtained from the muscle. Briefly, for cell isolation from tissue, injured muscles were placed in warmed DMEM (Life Technologies). The muscles were chopped into small pieces and enzymatic disaggregation was performed using a skeletal muscle dissociation kit (Miltenyi Biotec, #130-098-305). The dissociated sample was filtered through a 70 µm cell strainer (Fisher Brand). Red blood cells (RBCs) were lysed with RBC Lysing buffer (eBioscience, #00-4300-54) at room temperature for 10 min. After lysis, cells were centrifuged and the pellet was re-suspended in flow cytometry buffer (eBioscience, # 00-4222-26). Following the lysis of RBC cells the samples were blocked with CD16/32 for 10 min on ice. For flow cytometry analysis of monocytes and macrophages, the cells were stained with Ly6C-APC (eBioscience), CD11b-FITC (Biolegend) and Super Bright 436 (eBioscience), F4/80-PECy7 (Biolegend), MerTK-PerCP-eflour 710 (eBioscience), CCR5-APC Vio770 (Miltenyi Biotech), CCR2-PE (Biolegend). Sytox blue (Invitrogen) was used to exclude dead cells and debris, and unstained controls were used to set gates. These fluorescent antibodies were selected to avoid spectral overlap. In addition, all experiments included single-stain controls with compensation heads to validate that no spectral spillover occurred. Samples of 30,000 cells per run were analyzed in the flow cytometer (LSR Fortessa, BD Bioscience) and the resulting data was analyzed using FlowJo software (FlowJo, LLC). Only samples that passed the appropriate filters and quality checks were analyzed. Cell counts were expressed as the percentage of positive cells per sample. We used both male and female mice at the age between 8 to 12 weeks in this analysis. Cell suspensions were obtained from 4 — 8 mice for each time point studied.

Isolation of MerTK^{+} macrophages by cell sorting and In vitro phagocytosis assay – CTX injured gastrocnemius muscles of WT and *myeKlf2 $^{-/-}$* mice were processed and labeled for MerTK and F4/80 as described above. MerTK^{+}F4/80^{+} macrophages were sorted by FACS (MoFlo XDP cell sorter, Beckman Coulter) and collected in DMEM media containing 1% fetal bovine serum. The sorted cells were plated into an 8 well plate (IBIDI, Cat.No. 80826) at a density of 100,000 cells per well in a final volume of 400 µL DMEM, and incubated for 3 h at 37°C. 100 µl of pHrodo™ Green Bio Particles Conjugates (P35367, Thermofisher Scientific) was added to each well to assay phagocytic activity. Control wells contained no macrophages or no BioParticles. Live cell recording was performed for 4h. Phagocytosis events were analyzed using an inverted, incubated microscopy chamber (Leica DMi8 live cell microscope), and the number of phagocytic positive cells were counted using ImageJ software.

Measurement of muscle strength – Isometric plantar flexion force was measured *in vivo* in anesthetized mice (1.5 – 2% of isoflurane) at days 21 and 28 post injury using a force transducer (Aurora Scientific, model 300C-LR). The mouse was placed in a supine position on a heated platform. One knee was fixed and the foot was secured to the pedal of the force transducer with the ankle joint at a 90^{o} angle. A radiant lamp above the animal helped maintain body temperature. The plantar flexor muscles were stimulated using a pair of needle electrodes inserted percutaneously into the posterior side of the knee close to the tibial nerve. Under these conditions, gastrocnemius muscle force makes the major contribution to plantar flexor strength. Stimulus amplitude and optimal leg/muscle length were determined from measurements of peak isometric twitch strength (Pt) using 0.2 ms pulses at 50 mA (701C stimulator, Aurora Scientific). Peak isometric tetanic strength (Po) was measured at the optimal length and stimulus amplitude using pulse trains of 350 ms duration and 50 – 150 Hz applied every 2 min.

Statistics – Statistical analysis was performed with GraphPad Instat program and values were expressed as mean ± SD. Multiple comparisons were tested by ANOVA, with Student Newman-Keuls post hoc analysis. A difference of $p \leq 0.05$ was considered statistically significant.

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