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Single cell analysis reveals satellite cell heterogeneity for proinflammatory chemokine expression

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Background: The expression of proinflammatory signals at the site of muscle injury are essential for efficient tissue repair and their dysregulation can lead to inflammatory myopathies. Macrophages, neutrophils, and fibroadipogenic progenitor cells residing in the muscle are significant sources of proinflammatory cytokines and chemokines. However, the inducibility of the myogenic satellite cell population and their contribution to proinflammatory signaling is less understood.

Methods: Mouse satellite cells were isolated and exposed to lipopolysaccharide (LPS) to mimic sterile skeletal muscle injury and changes in the expression of proinflammatory genes was examined by RT-qPCR and single cell RNA sequencing. Expression patterns were validated in skeletal muscle injured with cardiotoxin by RT-qPCR and immunofluorescence.

Results: Satellite cells in culture were able to express *Tnfa*, *Ccl2*, and *Il6*, within 2 h of treatment with LPS. Single cell RNA-Seq revealed seven cell clusters representing the continuum from activation to differentiation. LPS treatment led to a heterogeneous pattern of induction of C-C and C-X-C chemokines (e.g., *Ccl2*, *Ccl5*, and *Cxcl0*) and cytokines (e.g., *Tgfb1*, *Bmp2*, *Il18*, and *Il33*) associated with innate immune cell recruitment and satellite cell proliferation. One cell cluster was enriched for expression of the antiviral interferon pathway genes under control conditions and LPS treatment. Activation of this pathway in satellite cells was also detectable at the site of cardiotoxin induced muscle injury.

Conclusion: These data demonstrate that satellite cells respond to inflammatory signals and secrete chemokines and cytokines. Further, we identified a previously unrecognized subset of satellite cells that may act as sensors for muscle infection or injury using the antiviral interferon pathway.

KEYWORDS

satellite cells, chemokines, scRNA-seq, interferon, inflammation, muscle repair

1 Introduction

Skeletal muscle has the intrinsic capacity to repair itself in response to acute injury through crosstalk between the muscle and the innate immune system (reviewed in Tidball, 2017; Howard et al., 2020). Inflammatory signals, such as cytokines and chemokines, expressed at the site of injury, serve to activate and recruit monocytes and neutrophils responsible for phagocytosis of debris and necrotic fibers. These signals also promote a promyogenic signaling cascade directing the activation, proliferation, and differentiation of satellite cells (MuSCs), which are necessary to regenerate muscle fibers. Inhibition, or prolonged expression, of these factors diminishes muscle regeneration and promotes fibrosis (Dogra et al., 2006; Dogra et al., 2007; Munoz-Canoves et al., 2013). Despite the importance of cytokines and chemokines to this process, the contribution of muscle cells to immune cell recruitment and the proinflammatory environment remains poorly understood.

In response to acute injury, a proinflammatory environment is established at the site of injury that is necessary for the clearance of necrotic muscle fibers and cell debris (Yang and Hu, 2018). This is initiated immediately after tissue damage by resident neutrophils and degranulating mast cells releasing proinflammatory cytokines, including tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and interleukin-1β (IL-1β) (Gordon and Galli, 1990; Fielding et al., 1993). Subsequently, tissue resident macrophages responding to damage-associated molecular patterns (DAMPs) secrete TNF-α, IFN-γ, IL-1β, and IL-6, as well as chemoattractants, such as C-X-C-chemokine ligand 1 (CXCL1) and C-C-chemokine ligand 2 (CCL2) (Brigitte et al., 2010; Venereau et al., 2012; Yang and Hu, 2018). Collectively, these signals promote the recruitment of additional neutrophils and proinflammatory (M1) macrophages that produce reactive oxygen species necessary for the degradation of the damaged muscle fibers (Collins and Grounds, 2001; Teixeira et al., 2003). Single-cell transcriptomic analyses of regenerating muscle have demonstrated that macrophages, neutrophils, and fibroadipogenic progenitor cells (FAPs) residing in the endomysial space of muscle are the primary source of proinflammatory signals (Oprescu et al., 2020; McKellar et al., 2021). The importance of these signals to successful muscle repair has been demonstrated in mice that were deficient for *Tnfa*, *Ccl2*, or their respective receptors (Collins and Grounds, 2001; Warren et al., 2002; Chen et al., 2005; Lu et al., 2011). These mutations resulted in reduced muscle inflammation, as measured by decreased infiltration of neutrophils and M1 macrophages after injury, concomitant with a decrease in the clearance of necrotic fibers and diminished muscle fiber regeneration.

Proinflammatory cytokines also promote muscle repair by direct regulation of the myogenic progenitor cell population. Quiescent satellite cells are positioned between the basement and plasma membrane of muscle (Chargé and Rudnicki, 2004). They are distinguished by *Pax7* expression, but not the myogenic transcription factors, *Myod1*, *Myf5*, or myogenin (*Myog*). Upon injury, MuSCs start expressing *Myod1* and *Myf5* and pass through a period of rapid cell population expansion. Daughter cells undergoing differentiation to contribute to muscle fiber repair decrease *Pax7* expression and increase expression of a third myogenic transcription factor, *Myog*. IL-6 and IFN-γ secreted at the site of injury contribute to the activation, proliferation, and migration of the MuSC population (Munoz-Canoves et al., 2013; Fu et al., 2015). TNF-α differentially

regulates MuSCs based on its level of expression, at high levels it promotes proliferation and migration and inhibits differentiation. As the inflammation is resolved, TNF-α levels decrease, and MuSCs begin to differentiate and form new muscle fibers (Li, 2003; Torrente et al., 2003; Langen et al., 2004; Dogra et al., 2006; Fu et al., 2015). C-C and C-X-C chemokines have been implicated in the migration, proliferation, and differentiation of satellite cells during muscle repair (Griffin et al., 2010; Ge et al., 2013; Zhu et al., 2016; Hogan et al., 2018). Thus, proinflammatory cytokines tightly coordinate the activity of both myeloid and myogenic cell lineages during muscle repair.

Proinflammatory factors also contribute to idiopathic inflammatory myopathies, including dermatomyositis (DM) and polymyositis (PM) (Kuru et al., 2003). The affected muscle expresses proteins associated with the activation of the type I interferon pathway (Boehm et al., 1997; Greenberg et al., 2005; Salajegheh et al., 2010; Suárez-Calvet et al., 2014). These observations and several *in vitro* studies raise the possibility that skeletal muscle can also contribute to activation of the inflammatory response associated with healthy muscle repair in response to injury. For example, the well-studied inflammatory activator, bacterial lipopolysaccharide (LPS), induced C2C12 and L6 myoblast cell lines to express cytokines (Frost et al., 2003). LPS treatment is used to stimulate sterile inflammation in culture (Frost et al., 2002) by acting on Toll Like Receptor 4 (TLR4), a cellular membrane sensor of damage and infection that also triggers the caspase-11 non-canonical inflammasome (Yang et al., 2015; Cieslelska et al., 2021). LPS treatment induced *Tnfα, Il6, Ccl2,* and *Cxcl1* expression within 2 h in these cell lines (Frost et al., 2002; Frost et al., 2003; Bivona et al., 2021). Primary human MuSCs have also been shown to express a broad set of cytokines and chemokines associated with muscle repair, including *Tnfa*, *Il6*, *Ccl2*, and *Ccl5* when treated with TNF-α or IFN-γ (De Rossi et al., 2000).

In this study, we wanted to explore whether MuSCs contributed to the proinflammatory response during healthy muscle repair post-injury. We observed that primary mouse MuSC cultures were comprised of cells along the continuum from activation through differentiation and they upregulated cytokine expression in response to LPS treatment. Single cell RNA sequencing (scRNA-Seq) revealed that a broad set of cytokines and chemokines were differentially expressed in response to LPS. For example, *Ccl2* and *Cxcl1* were broadly expressed throughout the culture. In contrast, a small subset of MuSCs expressed *Ccl5* and genes belonging to the antiviral IFN pathway. These cells were identifiable in PBS treated control cells, suggesting this is a stable cell population. Further, CCL5 positive MuSCs were detectable in cardiotoxin (CTX) injured muscle suggesting that chemokine and cytokine expression by these cells has a distinct function in muscle repair.

2 Materials and methods

2.1 Animals

B6129SF1/J mice were purchased from Jax Labs (Bar Harbor, ME) and bred and housed in a vivarium at Arizona State University (ASU). These mice were kept on a 10 h light: 14 h dark schedule with *ad libitum* access to food and water. ASU is accredited by the

2.2 Cell culture and satellite cell isolation

Primary muscle satellite cell cultures (MuSCs) were established from cells isolated from five 12 week old B6129SF1/J mice as previously described (Palade et al., 2019). Briefly, hind limb quadriceps femoris muscles were excised, trimmed of fat and connective tissue, and finely minced. The muscle tissue was digested with 1.25 mg of protease XIV (Sigma-Aldrich, St. Louis, MO) for 1 h at 37°C. The cell suspension was filtered, differentially centrifuged and pre-plated in DMEM (Corning, Corning NY), containing 2% donor horse serum (HS) (Atlanta Biologicals, Flowery Branch, GA), and 100 µg/mL Primocin (Invivogen, San Diego, CA). Fibroblasts were removed by three pre-plating rounds of 3 h. Satellite cells were grown in a humidified chamber at 37°C with 5% CO_2, on Matrigel Matrix Basement Membrane (BD Biosciences, Bedford, MA) coated plates, in growth medium, Hams F-10 (Corning), containing 20% FBS (Atlanta Biologicals), 10 ng/mL bFGF (BD Biosciences) and 100 µg/mL Primocin (Invivogen).

2.3 LPS induction and quantitative RT-PCR (RT-QPCR)

MuSCs were seeded onto Matrigel (BD Biosciences) coated 6 well plates at a density of 1×10^5 cells/well in growth medium. Cells were then treated with LPS (1 mg/mL; Sigma-Aldrich) or phosphate buffered saline (PBS) for 1, 2, 4, 6, or 8 h. Cells were lysed in TRIzol (ThermoFisher Scientific, Waltham, MA) for RNA isolation, per the manufacturer's protocol, and 2 µg of total RNA was used to synthesize cDNA using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) and random hexamer primers.

Quantitative Real Time PCR (RT-qPCR) was carried out using SYBRgreen (Eurogentec, Fremont, CA) on an ABI 7900 HT thermocycler using a 384 well format in 10 µL reactions. All samples were normalized to the *Gapdh* transcript and relative gene expression was calculated using ΔΔCt analysis (Haimes and Kelley, 2010). Primer sequences are listed in Supplementary Table S1. All data are the result of triplicates from 3 biological samples.

2.4 Single cell RNA sequencing

MuSCs were treated with LPS as above for 2 h. MuSCs were then trypsinized (0.05% trypsin 0.53 M EDTA, Mediatech, Manassas VA) to create a single cell suspension. Cells were then counted on an automated cell counter and RNA library preparation was performed by the ASU Genomics Core facility. Samples were processed using a 10X Chromium single cell 3′ GEM, Library and Gel Bead kit V3 with a Chromium single cell B chip. The quality of each library was determined using Agilent TapeStation automated electrophoresis. Samples were sequenced

at a read depth of ~31,000 reads per cell (BioFrontiers Sequencing Core, University of Colorado, Boulder). The 10X Genomics CellRanger v6.0.1 was used to align to the *Mus musculus* reference genome mm10-2020-A (GRCm39), quantify, and provide basic quality control metrics for the scRNA-seq data. Data was then analyzed utilizing the Seurat R package. Cells with < 2000 reads or >80,000 reads were discarded as well as cells with < 0.9% or >20% mitochondrial genes. Clusters of cells with similar expression patters were identified using the 'FindClusters' function which used a shared nearest neighbor modularity optimization-based clustering approach.
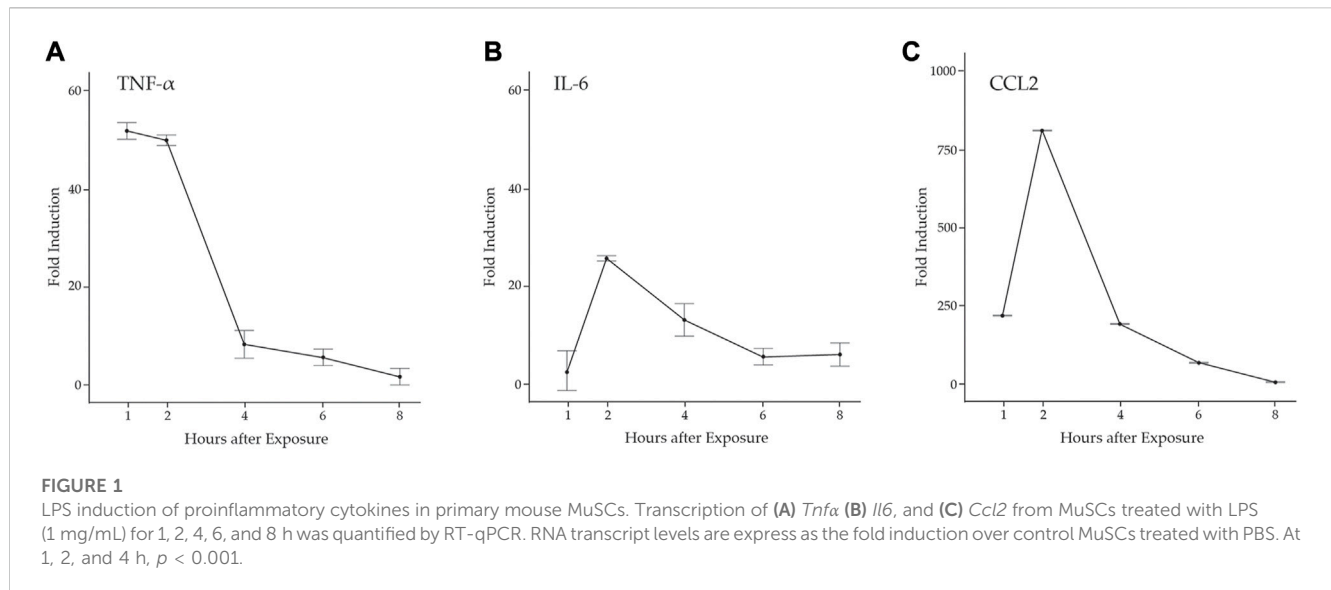
2.5 Cardiotoxin injury of skeletal muscle

Acute muscle injury was generated in the quadriceps of 3 month old mice by intramuscular injection of 50 µL of 10 µM cardiotoxin (CTX) (Sigma-Aldrich) solution in DMSO (Garry et al., 2000). At 24 h post-injection, Evan's blue dye (Sigma-Aldrich) was injected intraperitoneally at 1 mg/10 g body weight to allow for visualization of damaged cellular membranes. Mice were sacrificed at designated time points and tissue was harvested for analysis. The uninjured quadriceps muscle from the contralateral leg was also harvested for use as control tissue. Total RNA was isolated from muscle biopsies using TRIzol (ThermoFisher Scientific) as described in Majumdar et al. (2012).

2.6 Immunofluorescence

Mice (n = 3) were injected with CTX, quadriceps were harvested, and fixed in 4% paraformaldehyde in PBS overnight at 4°C. Tissue was cryopreserved in sucrose prior to embedding in Tissue-Tek OCT. Sections were then permeabilized and blocked in a buffer containing 0.1% Triton-X 100% and 5% normal donkey serum (NDS, Sigma-Aldrich) in PBS. Samples were then incubated in primary antibody solution containing antibodies recognizing a muscle marker, M-cadherin (Santa Cruz #sc-81471 1:250) and CCL5 or CCL2 (CCL5, R&D Systems AF478 1:300, CCL2 Novus Biologicals NBP 1-07035 1:500). Sections were then rinsed and incubated in secondary antibody solutions containing Alexa-Fluor conjugated antibodies against mouse (Thermofisher, A-21202, 1:1000), rabbit (Thermofisher, A-31573, 1:1000) or goat IgG (Thermofisher, A-21447, 1:1000) and DAPI (Sigma Aldrich 10,236,276,001, 1:1000) diluted in blocking solution. Tissue was rinsed in PBS and cover-slipped for microscopic analysis. Images were collected on a Zeiss LSM800 laser scanning confocal microscope and optimized for brightness and contrast. Blinded evaluators quantified M-cadherin positive (MCAD[+ve]) and MCAD[+ve]/CCL5[+ve] cells in random 20X fields. Data were averaged for both CTX injured (n = 418 MCAD[+ve] cells) and contralateral control quadriceps (n = 416 MCAD[+ve] cells). Student's t-test was used to determine statistical significance.

MuSCs treated with LPS (1 mg/mL) or PBS control for 2 h, were prepared for immunofluorescence (IF) by washing twice with cold PBS followed by fixation in 4% PFA for 20 min. Cells were then washed 3 times with PBS before antibody staining. Monoclonal mouse anti-MYOD (NB100-56511, Bio-techne Minneapolis, MN, 1:

FIGURE 1
LPS induction of proinflammatory cytokines in primary mouse MuSCs. Transcription of **(A)** *Tnfα* **(B)** *Il6*, and **(C)** *Ccl2* from MuSCs treated with LPS (1 mg/mL) for 1, 2, 4, 6, and 8 h was quantified by RT-qPCR. RNA transcript levels are express as the fold induction over control MuSCs treated with PBS. At 1, 2, and 4 h, $p < 0.001$.

200) and polyclonal goat anti-CCL5 (AF478-SP, R&D Systems, 1: 200) were used to detect cellular expression in culture. Anti-mouse-Alexa Fluor 488 (1:1000), anti-goat Alexa Fluor 647 (1:1000) and DAPI (1:1000) were then used to visualize MYOD, CCL5, and nuclei, respectively. Images were collected on a Zeiss LSM800 laser scanning confocal microscope at 20X magnification. Cells from 50 fields were then counted to determine the percent of total cells positive for these markers.

2.7 Statistical analysis

To assess the significance of the expression of cultured MuSCs post-treatment a one-way ANOVA was used. The 'FindMarkers' function in the Seurat package was used to determine the significance of the differential expression of marker genes from each cluster in the scRNA-Seq data. Marker genes were determined as having an adjusted *p*-value less than 0.05 and an average log 2 fold-change greater than 0.25.

3 Results

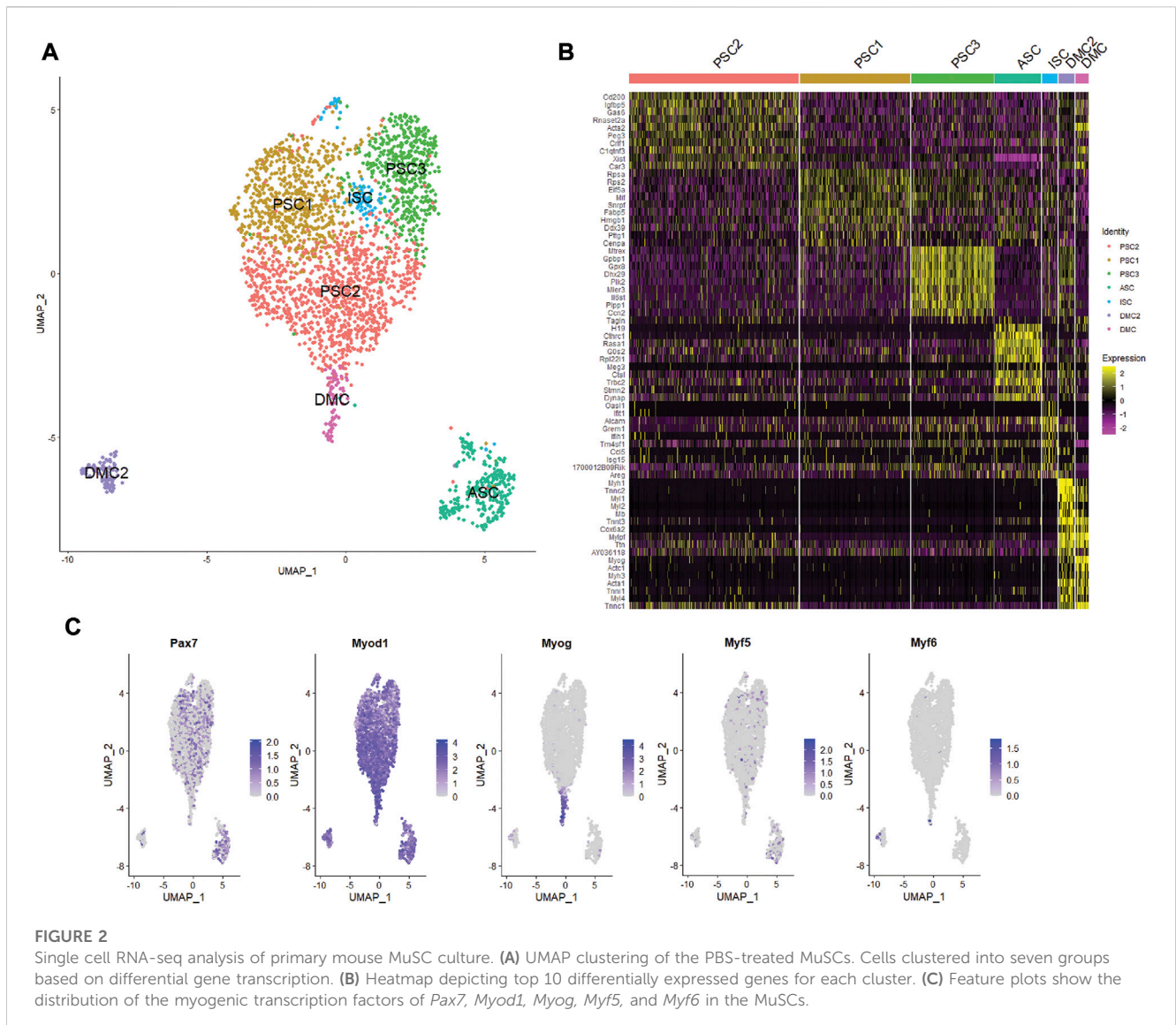### 3.1 Treatment of MuSCs with LPS induces inflammatory cytokine expression

Proinflammatory cytokines promote neutrophil and M1 macrophage infiltration to the damaged muscle *via* diapedesis and promote the activation and proliferation of MuSCs necessary for muscle regeneration (Howard et al., 2020). There are several studies that indicate the potential for MuSCs to contribute to the proinflammatory environment. For example, the pathogen-associated molecular pattern molecule (PAMP) LPS induced the expression of *Tnfa*, *Il6*, *Ccl2, and Ccl5* in C2C12 and L6 muscle cell lines and human MuSCs (De Rossi et al.,

2000; Frost et al., 2002; Frost et al., 2003). Further, expression of genes in the antiviral IFN pathway was elevated in the muscle of DM patients, predicting that muscle plays a role in the onset of inflammation (Moneta et al., 2019; Bivona et al., 2021). To examine the potential induction of an inflammatory response by MuSCs, we examined primary cell cultures derived from MuSCs isolated from the quadriceps of 3 month old B6129SF1/J mice treated with LPS (1 mg/mL) or a PBS control for 1, 2, 4, 6, or 8 h. Total RNA was isolated and transcription of *Tnfa, Il6*, and *Ccl2* was assessed by RT-qPCR. We found that *Tnfa* was rapidly induced and reached its maximum at 1 h post-treatment ($p < 0.0001$) with a significant drop off between 2 and 4 h. In contrast, *Il-6* and *Ccl2* transcription were induced to maximal levels at 2 h ($p < 0.0001$) (Figure 1).

3.2 Single cell RNA-SEQ analysis of primary MuSCs

It is becoming increasingly clear that MuSCs are heterogeneous based on the expression of specific markers, cell function, and their ability to contribute to muscle regeneration (Biressi and Rando, 2010; Rodriguez-Outeiriño et al., 2021). Thus, it is likely that the chemokine and cytokine profiles of individual MuSCs will vary across different subpopulations. To examine this, scRNA-Seq was performed on actively proliferating MuSCs treated with LPS or PBS. Based on the results in Figure 1, cells were exposed to LPS for 2 h for maximal induction of chemokines and cytokines. Single cells were isolated and profiled by scRNA-Seq. Following quality control for mitochondrial RNA content and reads per cell, this resulted in a PBS-treated library of 2,751 cells with 20,235 mean reads/cell and an LPS-treated library of 2,802 cells with 19,330 mean reads/cell.

Unsupervised clustering and UMAP embedding was performed using the Seurat R package and identified seven unique clusters in PBS-treated MuSCs based on differentially expressed genes (Figures

FIGURE 2
Single cell RNA-seq analysis of primary mouse MuSC culture. **(A)** UMAP clustering of the PBS-treated MuSCs. Cells clustered into seven groups based on differential gene transcription. **(B)** Heatmap depicting top 10 differentially expressed genes for each cluster. **(C)** Feature plots show the distribution of the myogenic transcription factors of *Pax7, Myod1, Myog, Myf5,* and *Myf6* in the MuSCs.

2A,B; Supplementary Table S2). All clusters were determined to consist of myogenic lineage cells based on the expression of one or more of the muscle-specific transcription factors, *Pax7*, *Myod1*, *Myog*, and *Myf5* (Figure 2C), known to have dynamic expression in satellite cells as they progress along the continuum from quiescent satellite cell through activation, proliferation, and differentiation (Figure 3A). The pattern of transcription of these four factors and other differentially expressed genes was used to identify the cell clusters within the PBS-treated MuSC culture (Figure 3B). Activated satellite cells (ASC) were identified based on transcription of *Pax7* and the satellite cell activation and proliferation markers *H19*, *EIF2S3Y*, and *HGF* (Sheehan et al., 2000; Li et al., 2016; Martinet et al., 2016). The vast majority of the cells sorted into 3 cell clusters, PSC1—PSC3, with a similar pattern of reduced *Pax7,* and increased *Myod1,* transcription. PSC1 had a marked increase in ribosomal protein expression (*Rspa, Rps2, Rps26,* and *Rps29*), necessary for ribosome biogenesis and post-transcriptional regulation. Expression of these genes is associated with the increase in protein production needed for the transition of ASCs to proliferating myoblasts

(Chaillou et al., 2017; Gayraud-Morel et al., 2018). The second and largest cell cluster, PSC2, was enriched for markers of proliferating myoblasts, including *Peg3*, *Gas6*, and *C1qtnf3* (Correra et al., 2018; Mervis et al., 2020; Otani et al., 2020). The third PSC cluster (PSC3) had a higher expression level of glutathione peroxidase 3 (*Gpx3*) and the RNA helicase *Mtrex,* implying that these cells were responding to local environmental stressors that may not be related to the normal progression of satellite cells (El Haddad et al., 2012). There was a small cell population related to the PSC cell clusters, except for reduced transcription of *Myod1* and *Myog* and enrichment for genes in the antiviral IFN pathway and downstream interferon stimulated genes (ISG), including *Oasl1, Ifit1*, *Ifit3*, and *Rsad2* (Figure 3B). Thus, we have designated this cluster, IFN stimulated cells or ISCs. Finally, two clusters of differentiating myocytes (DMC1 and 2) were present in the culture, based on the expression of *Myog* and *Myod1,* as well as sarcomeric proteins including *Myl1*, *Acta1*, *Tnnc2*, and *Myh1*. Thus, the PBS treated primary satellite cell culture was comprised of cells along the continuum from activated satellite cells to differentiating

FIGURE 3
Myogenic transcription factors and differentially expressed genes in MuSCs. **(A)** Schematic of satellite cell progression to differentiated myotube, including the myogenic transcription factor profile for each stage. **(B)** Dotplot showing average and percent expression of genes that differentiate the 7 cell clusters in PBS-treated MuSCs.
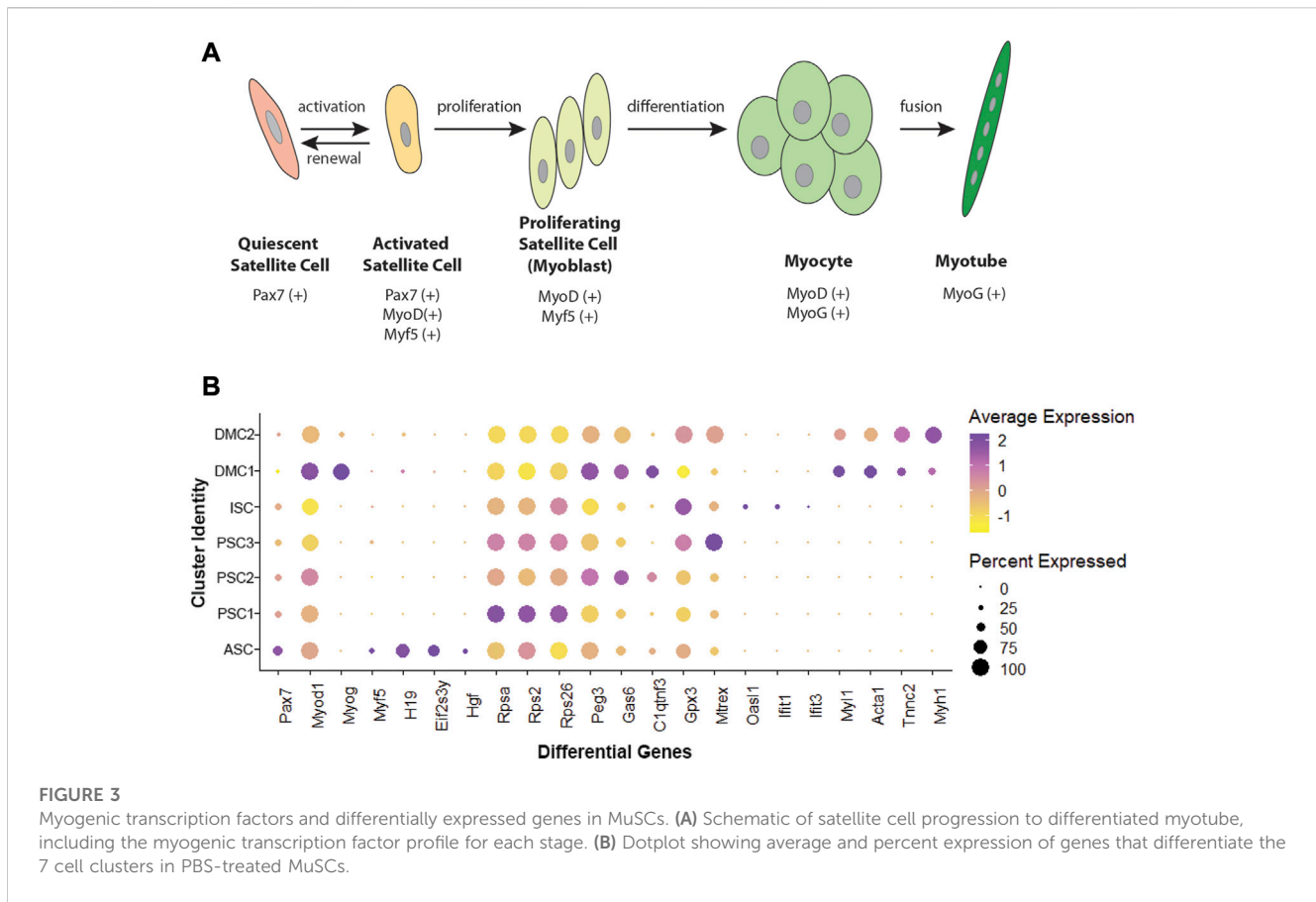
myocytes with several subsets of myoblasts with distinct patterns of gene transcription.
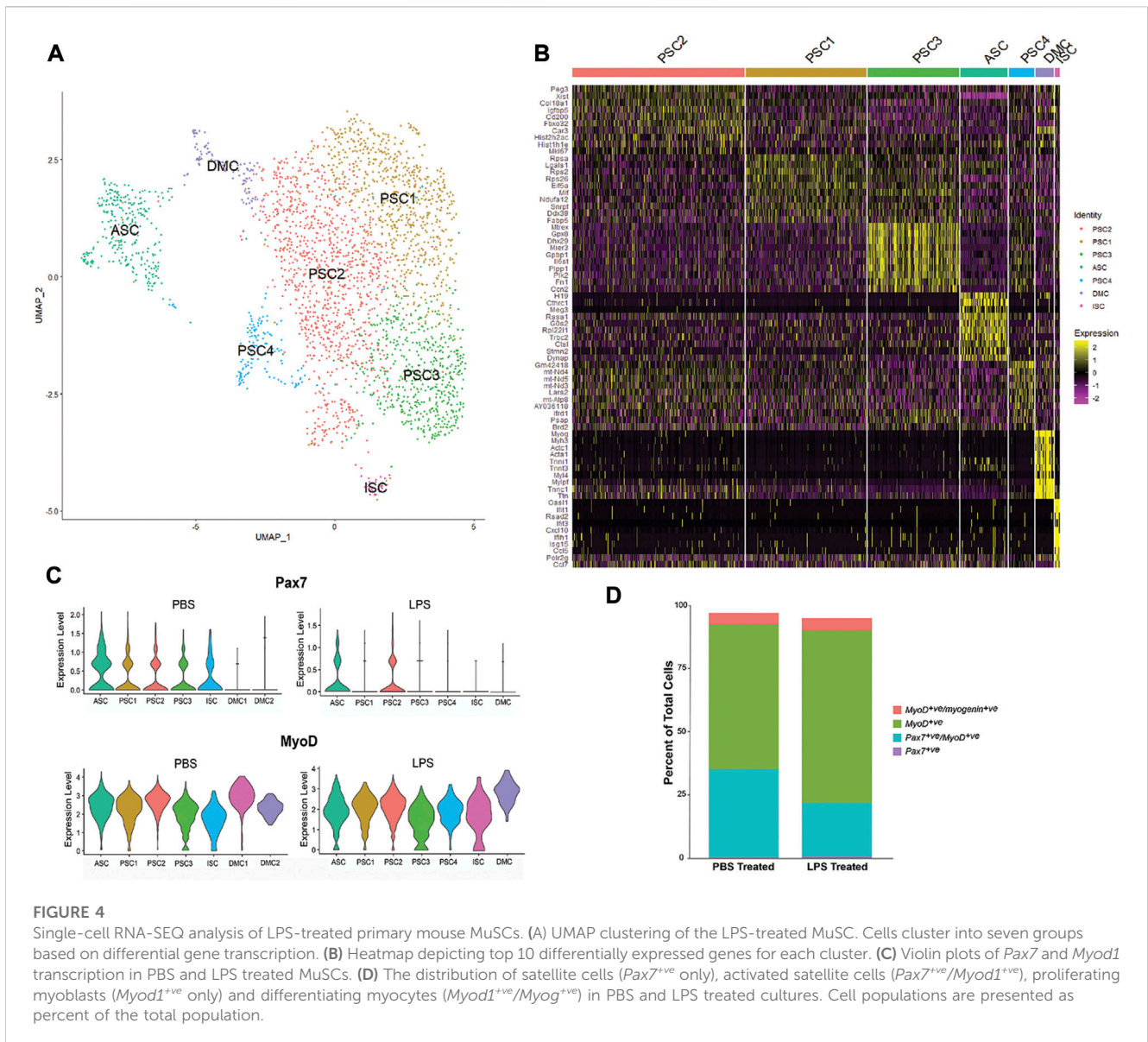
3.3 LPS induces chemokines in primary MuSCs

To extend our evaluation of LPS induction of cytokines and chemokines in muscle (Figure 1), MuSC cultures were treated with LPS (1 mg/mL) for 2 h and profiled by scRNA-Seq. Unsupervised clustering and UMAP embedding was performed using the Seurat R package under the same conditions as the control culture (Figure 4, Supplementary Table S3). A similar set of cell clusters, as described above, were identified based on differential gene expression. However, there were exceptions, including a fourth PSC cluster (PSC4) and the absence of the second DMC cluster. The PSC4 cluster was distinguished by decreased expression of more than 20 ribosomal proteins. The significance of this reduction of the translational machinery is not clear, though there is evidence of LPS reducing protein synthesis in muscle tissue (Gordon et al., 2013).

The level of transcription of the myogenic markers, *Pax7* and *Myod1*, was examined in the cell clusters of PBS and LPS treated cells (Figure 4C). *Pax7* transcription was reduced in PSC1, PSC3 and ISC clusters and reduced in the ASC cluster. In contrast, *Myod1* transcription levels are unaffected by LPS treatment. Alternatively, the distribution of cells along the satellite cell continuum was evaluated independent of cell clusters by comparing the number of cells co-expressing the myogenic markers, *Pax7*, *Myod1*, and *Myog* (Figure 4D). While the number of *Pax7*$^{+ve}$-only satellite cells and *Myod1*$^{+ve}$/*Myog*$^{+ve}$ differentiating myocytes remained relatively constant, there was an expansion of the *Myod1*$^{+ve}$ myoblasts cells at the cost of the *Pax7*$^{+ve}$/*Myod1*$^{+ve}$ activated satellite cells. This is consistent with the reduction of *Pax7* transcription after LPS treatment. It remains to be determined if this is due to direct regulation by LPS signaling, or the indirect result of promoting proliferation of myoblasts through cytokine production.

An evaluation of chemokine production in response to LPS by scRNA-Seq showed that 13 members of the C-C and C-X-C subfamilies (*Ccl2, Ccl5, Ccl7, Ccl17, Ccl20, Ccl27a, Cxcl1, Cxcl2, Cxcl4, Cxcl10, Cxcl12, Cxcl16,* and *Cx3cl1*) were expressed in MuSCs. A comparison of transcription of these factors across the cell clusters in the LPS and PBS control conditions revealed two distinct patterns (Figure 5). The first pattern included chemokines not expressed in the control cells and broadly transcribed after treatment with LPS (e.g., *Ccl2, Cxcl1, Ccl7,* and *Cx3cl1*). This is in contrast to chemokines that were induced in a single cell cluster. This was observed for *Ccl5* and *Cxcl10*, which were induced in the ISC cluster, and *Ccl17* and *Cxcl16* in the ASC cluster (Figure 5B). *Ccl5* and *Cxcl10* are induced by type I IFNs (Kelly-Scumpia et al., 2010; Nakano et al., 2012) consistent with our designation of this cluster as IFN stimulated.

LPS induction of chemokines was validated in independently isolated primary MuSCs by RT-qPCR of total RNA using gene specific primers. Transcripts for the chemokines *Ccl2, Ccl5, Ccl7, Cxcl1,* and *Cxcl10* were significantly higher in LPS-treated cells as

FIGURE 4
Single-cell RNA-SEQ analysis of LPS-treated primary mouse MuSCs. **(A)** UMAP clustering of the LPS-treated MuSC. Cells cluster into seven groups based on differential gene transcription. **(B)** Heatmap depicting top 10 differentially expressed genes for each cluster. **(C)** Violin plots of *Pax7* and *Myod1* transcription in PBS and LPS treated MuSCs. **(D)** The distribution of satellite cells (*Pax7^{+ve}* only), activated satellite cells (*Pax7^{+ve}/Myod1^{+ve}*), proliferating myoblasts (*Myod1^{+ve}* only) and differentiating myocytes (*Myod1^{+ve}/Myog^{+ve}*) in PBS and LPS treated cultures. Cell populations are presented as percent of the total population.
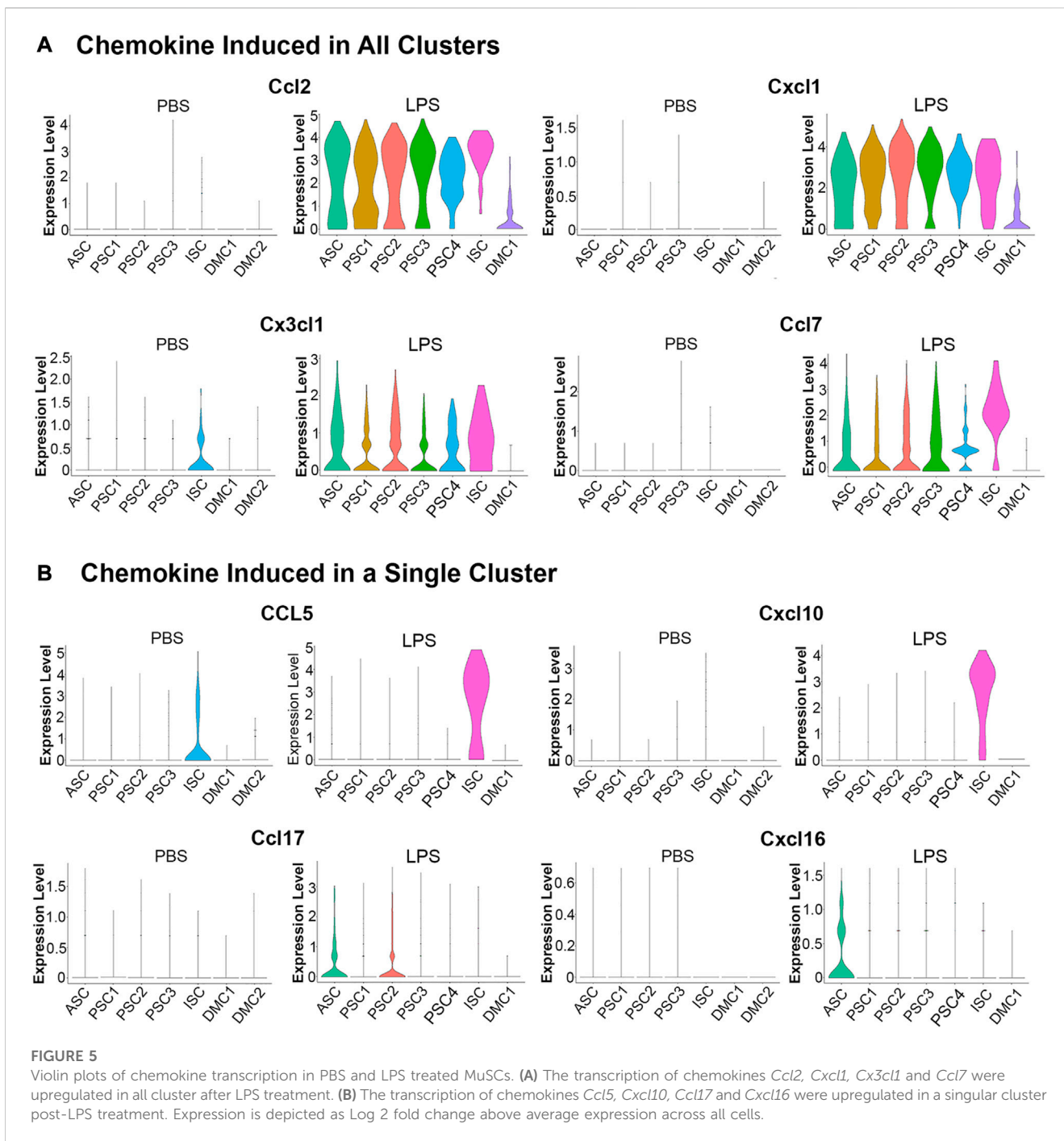
compared to the PBS-treated controls (Supplementary Table S4). Overall, these studies revealed that MuSCs can be induced to express many C-C and C-X-C chemokines with LPS and they are differentially expressed across the cell clusters. ASC and ISC cell clusters express separate sets of chemokines, raising the possibility that they serve unique functions during muscle inflammation response.

An examination of proinflammatory cytokine transcription in response to LPS treatment revealed a modest induction of a limited number of factors when compared to the robust induction of chemokines (Figure 5). Among the TGFβ superfamily, known to positively regulate cells of the innate immune system (Chen and ten Dijke, 2016), *Tgfb1* was constitutively transcribed at low levels in the ASC, PSC2, PSC3, and ISC clusters. With LPS treatment, *Tgfb1* was induced in PSC1 cells (Figure 6A). Similarly, *Tgfb2* was induced in PSC1 and PSC2 cells, but constitutively expressed in DMCs (data not shown). In contrast, *Bmp2*, a known antiviral ISG in bone marrow macrophages (Liu et al., 2012), was induced selectively in

the ISC cluster (Figure 6A). Interestingly, *Il18* and *Il33*, members of the IL-1 family of proinflammatory cytokines (Chan et al., 2019; Martinon et al., 2009), were weakly induced in ISCs (Figure 6B). Overall, the MuSCs expressed cytokines and chemokines in a heterogeneous pattern in response to inflammatory signals.

3.4 LPS induces the interferon signaling pathway in the ISC cell cluster
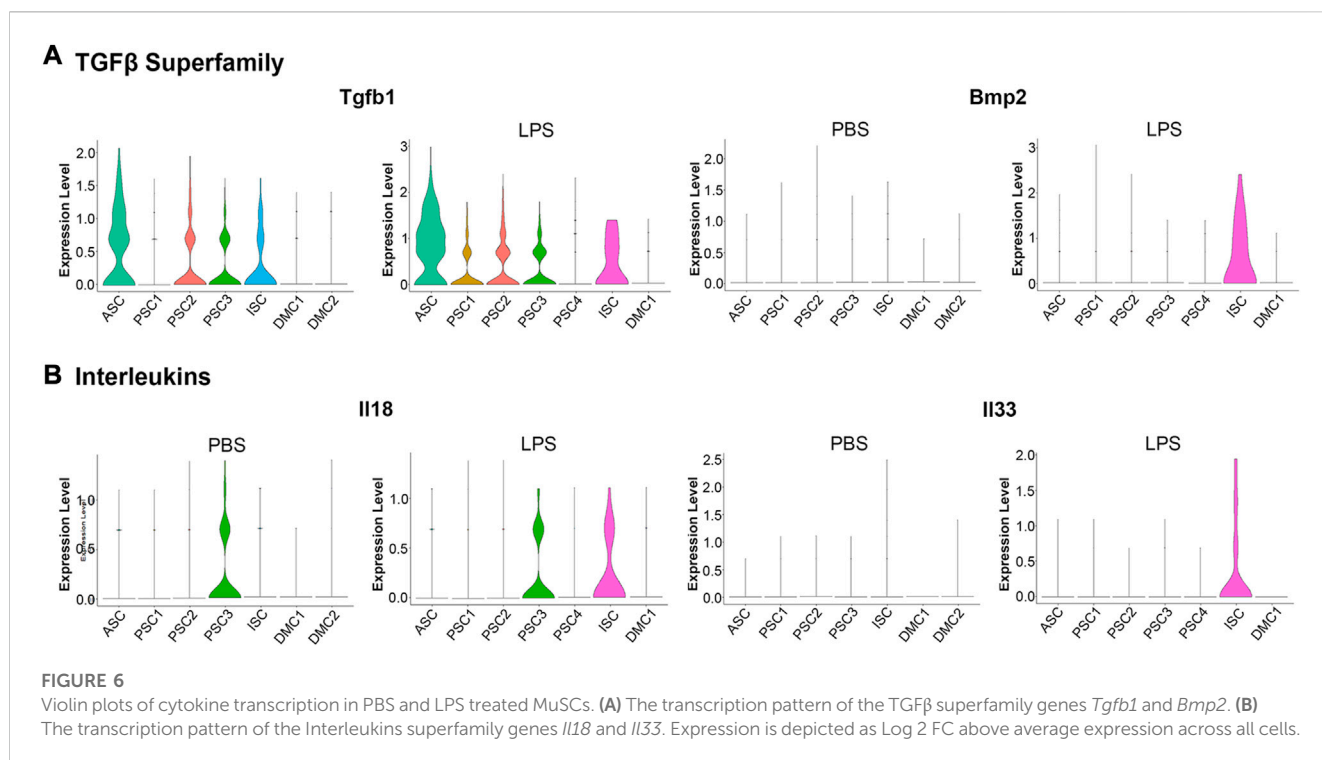
Single cell sequencing of the PBS control cells revealed a low level expression of components of the IFN signaling pathway in the ISC cluster (Figure 2). LPS induced the expression of *Ccl5, Cxcl10, Il18,* and *Il33* in these cells (Figures 5, 6), supporting a proinflammatory role for this cluster. Since LPS acts through the TLR4 receptor to induce IFN α and γ expression (Richez et al., 2010; Wang et al., 2017), transcription of members of the antiviral IFN pathway were examined (Table 1). Of note, two cytosolic sensors of

FIGURE 5
Violin plots of chemokine transcription in PBS and LPS treated MuSCs. **(A)** The transcription of chemokines *Ccl2, Cxcl1, Cx3cl1* and *Ccl7* were upregulated in all cluster after LPS treatment. **(B)** The transcription of chemokines *Ccl5, Cxcl10, Ccl17* and *Cxcl16* were upregulated in a singular cluster post-LPS treatment. Expression is depicted as Log 2 fold change above average expression across all cells.

viral DNA and RNA, *Ifih1/MDA-5* and *Ddx58/Rig-1* (Loo and Gale, 2011), as well as their downstream transcription factor, *Irf7*, were selectively induced in the ISC cells. Further, twelve other IFN induced signaling factors associated with antiviral activity were also induced (Table 1). It should be noted that IFN-α, -β, or -γ transcription was not detected, raising the possibility that the antiviral IFN pathway was activated through the cytosolic receptors independent of the IFNs.

The expression of the IFN pathway marker, CCL5, in a small subset of MuSCs was confirmed by IF done using antibodies specific to both MYOD and CCL5. CCL5[+ve]/MYOD[+ve] cells were detected in newly isolated MuSCs from the *quadriceps femoris* of 3 month old mice treated with LPS or PBS (Figure 7). Double positive cells represented 2.3% of the LPS treated MuSCs, which was comparable to the distribution (2.08%) of *Myod1* positive cells expressing ISG genes identified in the scRNA-Seq analysis (Table 2). These genes were also detectable in PBS treated control cultures (Figure 7), indicating that activation of the ISG gene expression was independent of LPS activation, but the expression level was increased in response to this inflammatory signal.

FIGURE 6
Violin plots of cytokine transcription in PBS and LPS treated MuSCs. **(A)** The transcription pattern of the TGFβ superfamily genes *Tgfb1* and *Bmp2*. **(B)** The transcription pattern of the Interleukins superfamily genes *Il18* and *Il33*. Expression is depicted as Log 2 FC above average expression across all cells.

3.5 Induction of IFN pathway in injured muscle

Activation of the antiviral IFN pathway is associated with the chronic muscle inflammation noted in DM patients and IFN-γ has been linked to muscle regeneration (Greenberg et al., 2005; Cheng et al., 2008; Suárez-Calvet et al., 2014). Since we observed that the IFN pathway was activated in a small percentage of MuSCs isolated from healthy mice, it was possible that it has a role in skeletal muscle repair as well. We further examined if we could detect activation of a subset of the ISG in response to acute injury. CTX was injected into the *quadriceps femoris* of Bl10 mice and total RNA isolated from the site of injury at 1, 2, 3, and 5 days post-injury (DPI) and from the uninjured contralateral leg. RT-qPCR was done using gene specific primers. When compared to the uninjured muscle, both *Ifih1* and *Isg15* transcription were rapidly induced at 1 DPI followed by a gradual decline. In contrast, expression of the IFN-regulated chemokines, *Ccl5* and *Cxcl10*, peaked at 3 DPI before sharply declining (Figure 8). This is distinctly different from the broadly transcribed chemokines, *Ccl2* and *Cxcl1*, that peaked earlier at 1 DPI. Thus, these data demonstrate that components of the antiviral IFN pathway originally observed with LPS treatment are expressed *in vivo* in response to injury also.

The distribution of satellite cells that had the antiviral IFN pathway activated in muscle was evaluated by IF with antibodies specific for CCL5 and M-Cadherin (MCAD), a cell adhesion protein expressed on satellite cells in their niche and proliferating myoblasts. MCAD expression is used to distinguish the mononuclear satellite cells from the adjacent muscle fiber (Yin et al., 2013). IF was performed on sections of CTX injured and contralateral control *quadriceps*. Analysis demonstrated that CCL5[+ve]/MCAD[+ve] cells represented 10.18% of total MCAD[+ve] satellite cells of uninjured muscle and 10.52% of the total MCAD[+ve] cells in CTX injured muscle at 3 DPI (Figure 9A). In comparison, we found CCL2, which was transcribed at high levels in LPS-induced MuSCs, was broadly expressed in both satellite cells and intact muscle fibers at 1 DPI (Figure 9B).

4 Discussion

In this study, we re-evaluated the muscle-immune paradigm during muscle damage and repair. The transcription of immune effector genes has been reported in muscle through a MYF6-dependent pathway (Barruet et al., 2020; Lazure et al., 2020). However, their capacity to be induced to express pro-inflammatory genes in response to infection or injury remains less clear. Here we leveraged primary satellite cell cultures and single cell RNA sequencing technology to examine the capacity of satellite cells and myoblasts to express cytokines and chemokines. Here, we demonstrated that non-sterile PAMPs (LPS) induced a heterogeneous pattern of expression of proinflammatory cytokines and chemokines (Figures 3–6). Single-cell RNA-SEQ revealed that proliferating MuSCs in culture were comprised of distinct clusters that ranged from activated satellite cells, proliferating myoblasts, to differentiating myocytes (Figures 2, 3). Though many of the chemokines and cytokines were broadly expressed across these clusters, there were examples of selective transcription in ISC, ASC, and PSC1. Thus, the responsiveness of the MuSCs to

inflammatory signals can vary dependent on a cell's myogenic status on the pathway towards differentiation. Of particular interest are the ISCs that appear to have primed the antiviral IFN pathway which then can be activated by LPS to selectively increase the expression of *Ccl5, Cxcl10, Il18,* and *Il33*. There are descriptions of satellite cell subsets predicted to have an adaptive response for injury and stress (G$_{Alert}$) (Rodgers et al., 2014; Scaramozza et al., 2019; De Micheli et al., 2020). Our data is the first indication that a subgroup of satellite cells that could be acting as sensors for infection and injury in the muscle.

An evaluation of the chemokines and cytokines expressed in response to LPS predicts that MuSCs can promote the proinflammatory environment in muscle. Many of these factors have been reported to recruit neutrophils, monocytes, and T cells to the site of muscle injury, including CCL2, CCL5, CCL7, CXCL16, and CX3CL3 (Figure 5; Zhang et al., 2009; Martinez et al., 2010; Crescioli et al., 2012; Kohno et al., 2011; Strömberg et al., 2016). Further, our finding that CCL2 and CCL5 were expressed in satellite cells and muscle fibers at the site of CTX-induced injury, indicated that induction occurs *in vivo* and this response is not restricted to LPS. Several of the chemokines expressed in LPS-treated MuSC are predicted to participate in the expansion of the satellite cell population during skeletal muscle repair. CCL2 and CXCL16 promote satellite cell proliferation (Yahiaoui et al., 2008; Zhang et al., 2009), while CXCL1, CXCL10, and CCL17, negatively regulate myoblast differentiation, preventing premature exit from the cell cycle (Ge et al., 2013; Hogan et al., 2018). Thus, the pattern of expression is consistent with a dual role for MuSCs during regeneration, recruiting the innate immune cells and promoting myoblast expansion.

An unexpected outcome of the scRNA-Seq analysis was the identification of a small population of MuSCs with the antiviral IFN pathway activated in the absence of LPS. Approximately, 2% of the proliferating MuSCs in culture and 10% of satellite cells in muscle tissue expressed genes associated with the IFN-independent early antiviral components of the pathway that sense cytosolic viral DNA and double stranded RNA. Among the IFN genes expressed were the

TABLE 1 Induction of genes in the antiviral IFN pathway in the ISC cluster of PBS and LPS treated MuSCs. Transcription of genes reported to participate in the antiviral IFN pathway or be downstream induced genes were quantified by scRNA-Seq and expressed as Log2 fold change above average expression across all cells in the PBS- or LPS-treated MuSC library.

IFN stimulated genes	PBS treated MuSC	LPS treated MuSC
	avg log2 FC	avg Log2 FC
Intracellular Sensors		
Ifih1	1.3457	2.6062
Ddx60	0.2366	0.8932
Ddx58	0.2047	0.4750
IFN Regulatory Transcription Factors		
Irf7	0.1605	0.3152
IFN Inducible Chemokines		
Ccl5	2.3052	4.3164
Cxcl10	1.5864	4.1414
IFN Inducible Host Defense Genes		
Ifit1	3.0999	4.7017
Isg15	2.5953	4.4797
Rsad2	1.2518	3.4335
Oasl1	1.5689	2.9600
Oasl2	0.5788	1.5041
Bmp2	0.2401	1.0713
Apol9a	0.3192	0.9115
Ifit3b	0.2866	0.8729
Apol9b	0.2094	0.8477
Ifit2	0.5599	0.8431
Gbp3	0.1478	0.6641
Rtp4	0.1878	0.4956

cytosolic sensors, *Ddx58* and *Ifih1*, which bind the triphosphate group at the 5' end of viral RNA (Wu and Chen, 2014). This interaction induces expression of type I IFNs and a subset ISG
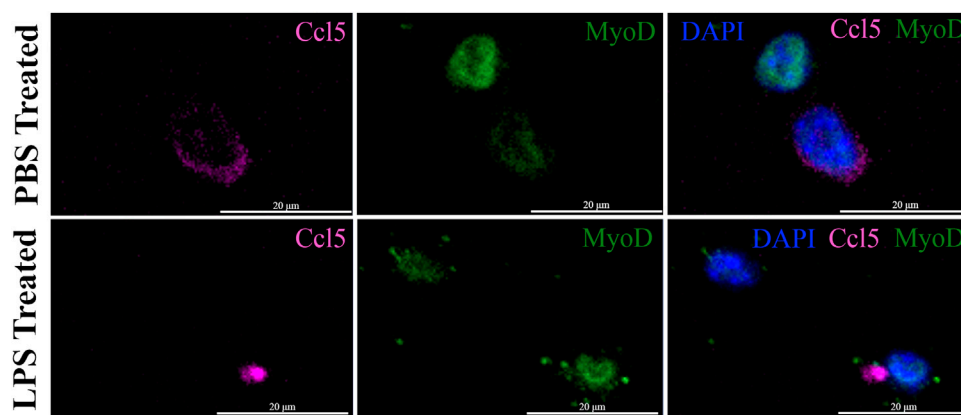

FIGURE 7
Co-expression of CCL5 and MYOD in proliferating MuSCs treated with LPS and PBS. The expression of CCL5 (magenta) and MYOD (green) was detected by indirect IF in MuSCs treated with LPS (1 mg/mL) or control PBS, for 2 h or control PBS treatment. CCL5 can be detected in the cytoplasm and MYOD in the nucleus. The nuclei were visualized with DAPI (blue). Images were taken at ×20 magnification. Scalebar = 20 μm.

TABLE 2 Distribution of MuSCs with the activated antiviral IFN pathway in PBS- and LPS-treated cells. Representation of the ISC cluster within the control and LPS treated MuSCs was evaluated by co-expression of *Isg15*, *Ifih1*, Oasl, *Ifit1*, and *Myod1* as measured by scRNA-Seq. ISC cells were independently assess in culture by co-expression of CCL5 and MYOD by IF.

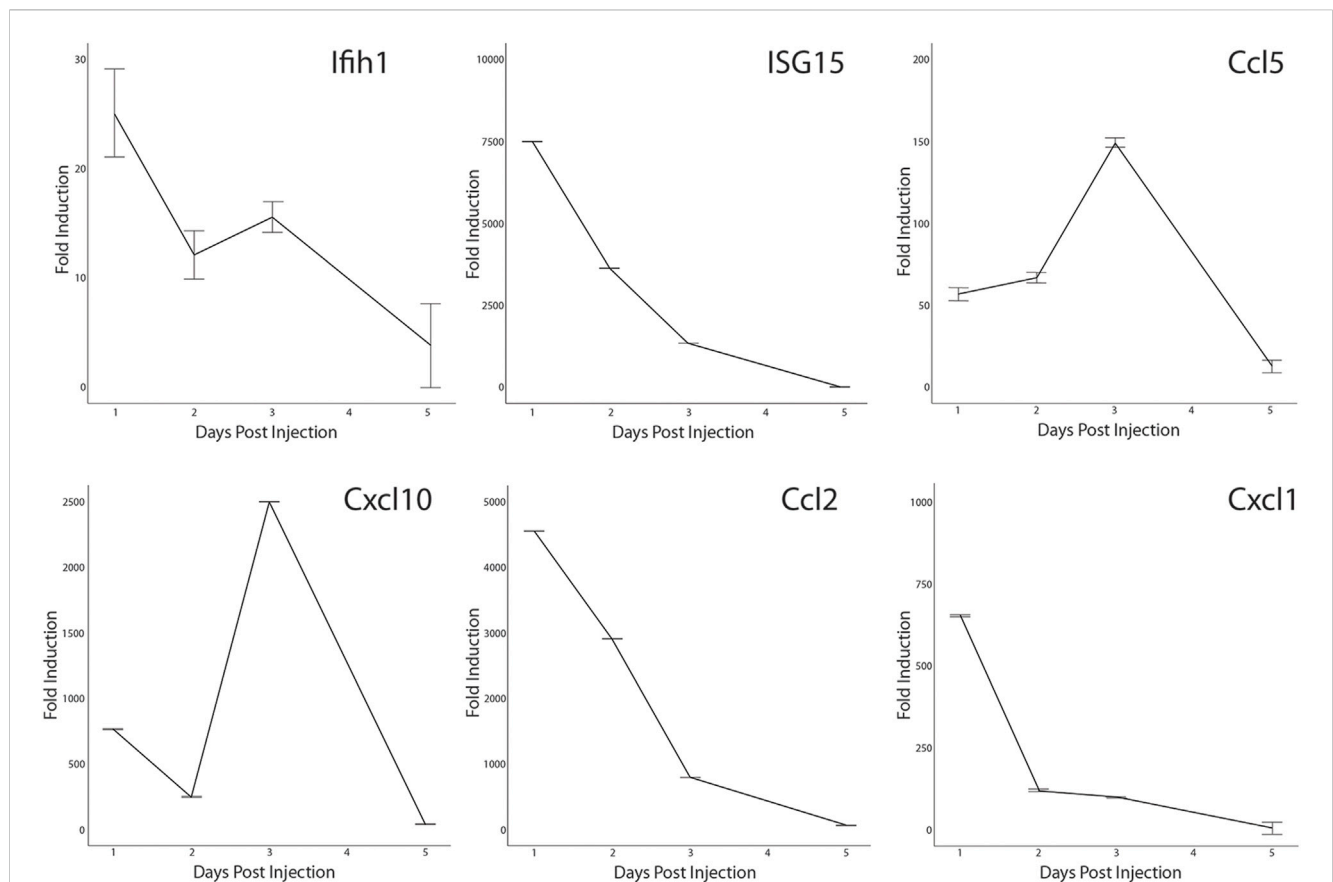| | Single cell RNA-Seq | | | Immunostaining | | |
| | Transcription of antiviral IFN pathway | | | CCL5/MYOD positive cells | | |
Treatment	+^{ve} Cells	Total Cells	%	+^{ve} Cells	Total Cells	%
PBS	72	2,751	2.61	49	3,600	1.36
LPS	58	2,801	2.08	97	4,200	2.3

FIGURE 8
Transcription of antiviral interferon pathway genes in CTX-injured *quadriceps*. CTX-injured *quadriceps* had total RNA isolated at 1, 2, 3, and 5 DPI. Transcripts of *Ifih1, Isg15, Ccl5, Cxcl10, Ccl2*, and *Cxcl1* were quantified by RT-qPCR. RNA transcript levels are expressed as the fold induction over RNA isolated from the uninjured contralateral leg. Significance was determined by Student's t-test, all timepoints $p < 0.0001$ except for *Cxcl1, Ifih1*, and *Ccl5* at 5 DPI.

proteins mediated by the transcription factors nuclear factor κ-light-chain enhancer of activated B cells (NF-κB), and the IFN regulatory factors IRF3 and IRF7 (Grandvaux et al., 2002). Among the antiviral pathway genes detected at high levels in uninduced MuSC ISCs, *Oasl, OasII, Isg15, Rsad2,* and *Irf7,* are positive regulators of IFIH1/DDX58-dependent signaling (Kim et al., 2008; MacMicking et al., 2012; Hee and Cresswell, 2017; Li et al., 2017; Liu et al., 2021). Transcription of these genes in the PBS treated controls suggests these cells are maintained in a primed state. How the antiviral

pathway is activated at low levels in the absence of a viral infection is unclear. Further, the presence of satellite cells with activated antiviral IFN pathway 3 days after a sterile muscle injury reinforces the presence of a mechanism of activation that is virus independent. One possible explanation comes from the observation that the endoribonuclease, RNase L, and the $2',5'$-oligoadenylate synthetase, OAS, can produce small self-RNAs from cellular mRNA that is recognizable by IFIH1 and DDX58 (Malathi et al., 2007; Stok et al., 2020). This suggests that ISCs are capable of self-priming or
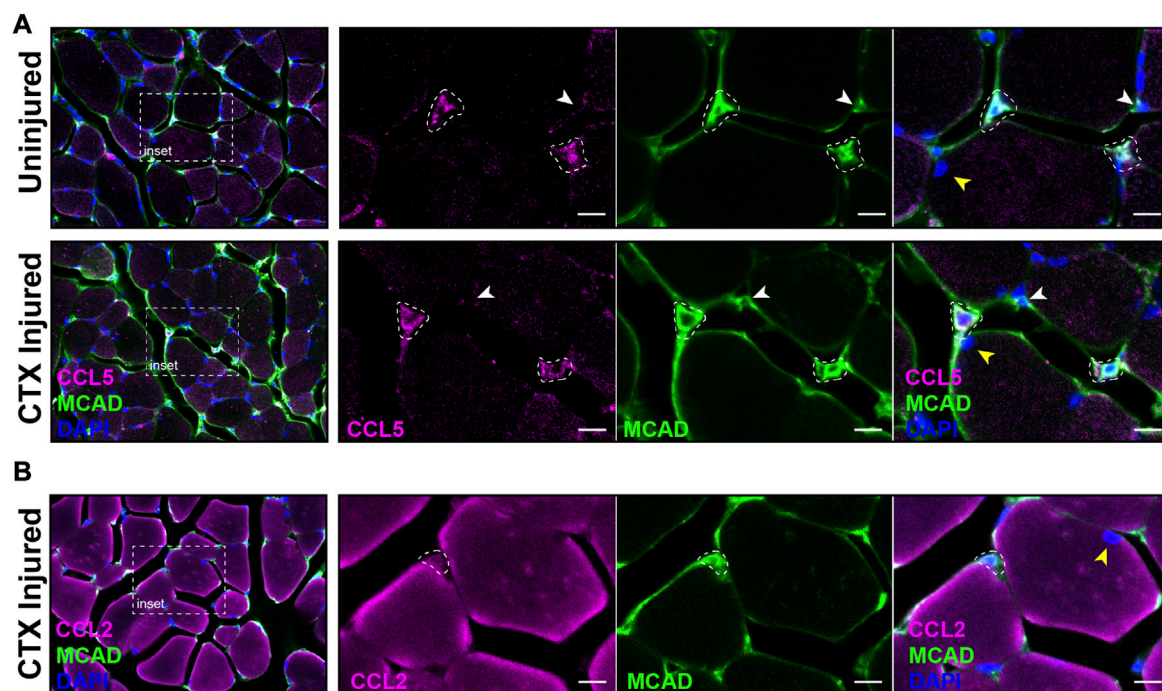
FIGURE 9
Co-expression of CCL2, CCL5 and MCAD in CTX-injured skeletal muscle. **(A)** The expression of CCL5 (magenta) and MCAD (green) were visualized in DAPI-labeled (blue) CTX-injured and uninjured *quadriceps* at 3 DPI by IF. White dotted outlines mark CCL5/MCAD co-expressing satellite cells, the white arrowhead indicates a MCAD-only expressing satellite cell, and the yellow arrowhead marks a muscle fiber nucleus. **(B)** CCL2 (magenta) and MCAD (green) co-expressing satellite cells (white dotted outline) were detected in CTX-injured *quadriceps* at 1 DPI. The nuclei were labeled with DAPI (blue). In contrast to CCL5, significant expression of CCL2 was detected in muscle fibers. The yellow arrowhead denotes a muscle fiber nucleus. Scalebar = 10 µm.

possibly detecting RNA from adjacent damaged cells post-injury in the absence of infection.

Expression of the IFN signaling pathway in satellite cells has been previously described in patients with dermatomyositis (DM) and polymyositis, demonstrating the capacity of muscle to regulate this pathway (Greenberg et al., 2005; Salajegheh et al., 2010; Suárez-Calvet et al., 2014). This suggests a mechanism for the autoimmune pathology of DM patients through overstimulation of IFIH1/DDX58 sensors by self-RNA. Consistent with this, muscle from these DM patients express type I IFN and upregulate *Isg15* and other ISG genes at levels approaching 100 fold higher than unaffected muscle (Baechler et al., 2011). Interestingly, activation of the IFN pathway also was detected in uninjured human *vastus lateralis* satellite cells by scRNA-Seq (Barruet et al., 2020). They identified a small population of *Pax7*[+ve] cells that expressed many of the IFN genes described in our study. It should be noted that the chemokines, CCL5 and CXCL10, were not observed in the human satellite cells, whereas our data demonstrated expression of *Ccl5* and *Cxcl10* transcripts in cultured satellite cells, and CCL5 protein in muscle. As our current studies demonstrate that IFN pathway and chemokine expressing MuSCs are present and expression of these genes can be upregulated by LPS, we have an opportunity for further study of the role of *Ccl5* and IFN pathway genes in muscle inflammation.

Heterogeneity of the adult satellite cell population has been well studied as it relates to tissue origin, cell cycle, and function in skeletal muscle repair (Biressi and Rando, 2010). In this study, we expand our view of heterogeneity to include the capacity of MuSCs to produce chemokines and cytokines that act on the myeloid and myogenic cells present at the site of injury. *Pax7*[+ve] satellite cells (ASC) selectively expressed *Ccl17* and *Cxcl16* that promoted cell proliferation in myogenic cells, as well recruiting neutrophils and macrophages. Perhaps more surprising, was the identification of ISC population, present in both treated and control MuSC cultures and in the satellite cells of injured muscle. Their presence in the absence of injury or inflammatory signals suggests that they serve as sentinel cells. The cells are specifically predisposed to activate the IFIH1/DDX58-dependent pathway. This is underscored by the results of our LPS treatment of MuSCs. LPS induces a broad set of proinflammatory cytokines and chemokines in immune and non-immune cells through a TLR4-dependent manner, including the IFIH1/DDX58-dependent pathway (Lu et al., 2008; Imaizumi et al., 2013). While LPS induced transcription of *Ccl2, Ccl7,* and *Cxcl1* in all cell clusters, induction of *Ifih1, Ddx58, Ccl5* and *Cxcl10* transcription was restricted to the ISC cluster. The nature of the signals regulating the heterogeneity of MuSC response to LPS or other immunogens remains to determined.

Overall, our studies demonstrate that satellite cells and proliferating myoblasts are competent to respond to PAMPs and DAMPs by expressing of chemokines and cytokines. Functionally, this is likely a combination of amplifying the signals at the site of injury that recruit and activate

proinflammatory immune cells and autocrine signaling promoting the rapid expansion of the satellite cells prior to differentiation into muscle fibers. These studies suggest that the antiviral IFN pathway plays a greater role in sterile inflammation in response to muscle damage than previously considered. Additional studies are needed to understand its contribution to the regulation of the innate immune response and satellite cell proliferation and differentiation.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/, GSE216848.

Ethics statement

The animal study was reviewed and approved by Arizona State University Institutional Animal Care and Use Committee.

Author contributions

AA as the first author to lead on the execution of all aspects of the research. KR and JN provided expertise with indirect immunofluorescence and confocal imaging of chemokine

expression in culture and injured muscle. SO'C and CP provide expertise on the scRNA-Seq analysis of chemokine and cytokine induction in cell culture. GS contributed to the validation of chemokine transcription by RT-qPCR. AR and JW-R contributed to the design and interpretation of the experiments.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2023.1084068/full#supplementary-material

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