The Role of Neutrophil Extracellular Traps and TLR Signaling in Skeletal Muscle Ischemia Reperfusion Injury

Nicole J. Edwards, PhD[1]

Charles Hwang, BS[1]

Simone Marini, PhD[1]

Chase A. Pagani, BA[1]

Philip J. Spreadborough, MD, PhD[2, a]

Cassie J. Rowe, PhD[2]

Pauline Yu, BS[1]

Annie Mei[1]

Noelle Visser, MS[1]

Shuli Li, MD, PhD[1]

Geoffrey E. Hespe, MD[1]

Amanda K. Huber, PhD[1]

Amy L. Strong, MD, PhD[1]

Miriam A. Shelef, MD, PhD[3]

Jason S. Knight, MD, PhD[4]

Thomas A. Davis, PhD[2]

Benjamin Levi, MD[1*]

[1]Department of Surgery, University of Michigan, Ann Arbor MI, United States of America

[2]Department of Surgery, Uniformed Services University of the Health Sciences, Bethesda MD, United States of America

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[3]Division of Rheumatology, University of Wisconsin and William S. Middleton Memorial Veterans Hospital, Madison, WI, United States of America

[4]Department of Internal Medicine, University of Michigan, Ann Arbor, MI, United States of America

[a]Current affiliation: Academic Department of Military Surgery and Trauma, Royal Centre for Defence Medicine, Birmingham, UK

[*]Corresponding Author: Benjamin Levi, MD 1150 W. Medical Center Drive, MSRB 2 Rm A570, Ann Arbor MI, 48109; blevi@umich.edu; telephone: (847-571-6511) fax: 734-763-5354

Running Title: NETs in Ischemia Reperfusion Muscle Fibrosis

Nonstandard Abbreviations

COX: cytochrome oxidase

Ctx: Cardiotoxin

DPI: Days Post Injury

eMHC: Embryonic Myosin Heavy Chain

ERK 1/2: Extracellular signal-regulated kinases 1/2

FAP: Fibroadipogenic progenitor cells

H3Cit: Citrullinated Histone H3

HCQ: Hydroxychloroquine

IL-10: Interleukin 10

IR: Ischemia Reperfusion

Mapk: Mitogen-activated kinase gene

MPO: Myeloperoxidase

NET(s): Neutrophil Extracellular Trap(s)

PAD4: Peptidylarginine deiminase 4

SDH: succinate dehydrogenase

TA: Tibialis Anterior

t-SNE: *t*-distributed stochastic neighbor embedding

TLR: Toll-like receptor

TNFα: Tumor Necrosis Factor alpha

Abstract

Ischemia reperfusion (IR) injury results in devastating skeletal muscle fibrosis. Here, we recapitulate this injury with a mouse model of hindlimb IR injury which leads to skeletal muscle fibrosis. Injury resulted in extensive immune infiltration with robust neutrophil extracellular trap (NET) formation in the skeletal muscle, however direct targeting of NETs via the peptidylarginine deiminase 4 (PAD4) mechanism was insufficient to reduce muscle fibrosis. Circulating levels of IL-10 and TNFα were significantly elevated post injury, indicating toll-like receptor (TLR) signaling may be involved in muscle injury. Administration of hydroxychloroquine (HCQ), a small molecule inhibitor of TLR7/8/9, following injury reduced NET formation, IL-10, and TNFα levels and ultimately mitigated muscle fibrosis and improved myofiber regeneration following IR injury. HCQ treatment decreased fibroadipogenic progenitor cell proliferation and partially inhibited ERK1/2 phosphorylation in the injured tissue, suggesting it may act through a combination of TLR7/8/9 and ERK signaling mechanisms. We demonstrate that treatment with FDA-approved HCQ leads to decreased muscle fibrosis and increased myofiber regeneration following IR injury, suggesting short-term HCQ treatment may be a viable treatment to prevent muscle fibrosis in ischemia reperfusion and traumatic extremity injury.

Key words: muscle fibrosis, inflammation, extracellular signal-regulated kinases (ERK) 1/2, hydroxychloroquine

Introduction

Ischemia reperfusion (IR) tissue injury can occur after any situation in which the blood supply to a tissue is profoundly interrupted resulting in microcirculatory hypo-perfusion and hypoxia. Anaerobic metabolism leads to accumulation of lactic acid, decrease of pH in tissues (metabolic acidosis), mitochondrial dysfunction, vasoactive agents, generation of reactive oxygen species, oxidative stress, endothelial activation, aberrant local inflammatory responses, organelle damage and ultimately cell death (1, 2). Ischemia reperfusion injury occurs clinically both in controlled surgical settings such as organ transplantation and free flap procedures as well as during traumatic events, such as battlefield blast injuries involving emergency tourniquet usage to control hemorrhage. In the skeletal muscle, IR injury translates into inflammation, tissue edema, muscle fibrosis, and/or necrosis, ultimately leading to decreased range of motion or complete loss of function (3). Therefore,

determining the precise mechanism by which IR injury occurs and discover treatments to minimize the long-term consequences is of utmost importance.

The resultant tissue damage occurs secondary to tissue hypoxia and paradoxically from the reperfusion of reoxygenated blood into the ischemic tissue often referred to as hypoxic re-oxygenation injury (2, 4). One cause of IR induced tissue injury is due to an excessive innate local and systemic inflammatory immune response. Perfused leukocytes encounter released intercellular damage-associated molecular patterns (DAMPs), adhesion molecules, chemokines, glycosaminoglycans (GAGs), metalloproteinase, and extracellular calcium and adenosine triphosphate (ATP) released by necrotic cells, which induce the activation of many toll-like receptor (TLR) pathways to promote an inflammatory response (1, 5-7). Neutrophils comprise a substantial portion of the amplifier cell population in the initial immune response, and their involvement in IR injury is hypothesized to be, in part, due to the formation of neutrophil extracellular traps (NETs) containing web-like extrusions of nuclear DNA, citrullinated histones, and antimicrobial peptides released into the tissue by activated neutrophils (8-10). NETs have been implicated in a wide variety of diseases, notably autoimmune diseases such as lupus (11-13) and rheumatoid arthritis (14, 15). Additionally, NETs have also been shown to form following traumatic injury both systemically and at the site of injury (16-21), including in models of ischemic injury in vital organs (5, 6, 18, 22).

NETs have been shown to form through several other independent pathways in addition to the well-characterized peptidylarginine deiminase 4 (PAD4) dependent pathway (23). Recent reports suggest that NET formation also occurs when DNA and RNA stimulate toll-like receptors (TLR) on polymorphonuclear neutrophils in a process termed self-propagation or secondary NETosis (17, 24). TLR 7/8 and 9 are pattern recognition receptors expressed on cells of the innate immune system, which are classically believed to recognize pathogenic RNA and DNA, respectively. However, growing evidence suggests that aberrant activation of these TLRs with self DNA and/or RNA occur when free nucleic material is present in the tissue, such as in autoimmune diseases, viral infections, and following traumatic injuries (17, 25-31).

Small molecule inhibitors of TLR7/8/9, such as hydroxychloroquine (HCQ, Plaquenil®), were developed initially as anti-malarial agents, but have shown to be efficacious against lupus, an autoimmune disease characterized by excess and impaired degradation of NETs (13, 24, 32-34). HCQ has also been shown to be an effective antiviral agent against RNA viruses including dengue virus and retroviruses (35) by de-acidifying endosomes required for endosome-mediated viral entry during TLR7/8/9 activation.

IR injury also leads to abnormal expression of extracellular signal-regulated kinase 1/2 (ERK1/2), however, the precise role of ERK1/2 signaling in IR injury remains controversial. Mitogen-activated protein kinase (*Mapk)* genes, such as *Mapk3* (*Erk1*) and *Mapk1* (*Erk2*), have been found to be dysregulated in multiple pathological states, including fibrotic diseases. Studies have shown inhibition of ERK1/2 signaling has been shown to attenuate fibrosis by minimizing extracellular matrix deposition in several mouse models, including hepatic IR injury (36) and kidney fibrosis (37). Furthermore, inhibition of *Mapk* genes and decreased phosphorylation of ERK1/2 have protective effects in multiple organ systems by decreasing apoptosis and increasing cellular turnover (38-43). In contrast, contradicting reports have shown increased activation of the same ERK pathways provide protection (44-46). These differences suggest ERK signaling may vary depending on pathologic state/condition, thus the precise role in IR injury remains to be elucidated.

Given that excess nuclear material is observed following traumatic injury and may increase NET formation alone and through self-propagation by TLR7/8/9 signaling, we investigated the role of neutrophil signaling and NET formation in the fibrotic response of skeletal muscle in a murine IR injury model. We next hypothesized that modulation of TLR7/8/9 using HCQ, a small molecule inhibitor to TLR7/8/9, may lead to decreased tissue injury and skeletal muscle fibrosis following IR injury by interfering with secondary self-propagated NETosis mediated by TLR7/8/9 activation. More broadly, given the role of ERK1/2 in both cellular apoptosis following IR injury and in fibrotic diseases, we also evaluated the effects of HCQ treatment on *Erk1 and Erk2* gene expression and ERK1/2 signaling following skeletal muscle IR injury. Coupling this with the known importance of neutrophils and the immune response following IR injury, we hypothesized that a combination therapy of TLR7/8/9 inhibition to target NETosis and inhibition of ERK1/2 activation would prevent skeletal muscle fibrosis following IR injury.

Here, we provide evidence that following IR injury in skeletal muscle, the initial immune response drives substantial NET formation in the injured muscle tissue that results in muscle fibrosis. We also show that HCQ treatment both inhibits proliferation of fibroadipogenic progenitor cells (FAPs), the population responsible for fibrotic deposition (47), decreases phosphorylation of ERK1/2, and decreases NET formation. We find that administration of HCQ mitigates muscle fibrosis caused by IR injury through modulation of the initial neutrophil response, FAP proliferation, and ERK1/2 signaling.

Materials and Methods

Animals

Male C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA; stock no. 000664). PAD4-/- mice were crossed 10 generations onto a C57BL/6 background (48). Matching background strains

were used as controls for all transgenic experiments. All mice were 6-9 weeks of age at time of injury. Adult male (11-12 week-old, 350-450g) pathogen free Sprague Dawley rats were obtained from Taconic Biosciences (Germantown, NY, USA). Rats were housed for a minimum of 7 days for acclimatization and quarantine purposes in the vivarium located at the Walter Reed Army Institute of Research (Silver Spring, MD, USA). Animals were housed and maintained under standard conditions: 72 ± 4 ºF, with a 12 hr light/dark cycle and no diet restrictions. All experiments and animal care procedures were approved by the University of Michigan (mouse protocol PRO 7930) and the Naval Medical Research Center and Walter Reed Army Institute of Research (protocol 17-OUMD-31S) institutional animal care and use committees. All activities were conducted in accordance with all applicable regulations.

Hindlimb Ischemia Reperfusion Injury in the Mouse Model

Mice received pre-operative 0.06 mg/kg buprenorphine subcutaneously as an analgesic. Mice were anesthetized with 1-3% isoflurane inhalation. A skin incision was made in the left groin and the groin fat pad was retracted to expose the femoral artery, vein, and nerve. The femoral artery and vein were separated from the nerve and an Ackland clamp was placed immediately distal to the inguinal ligament and proximal to the lateral circumflex femoral artery to induce acute hindlimb ischemia of the left lower limb. Mice remained under 2% isoflurane as left hindlimbs were subjected to 3 hr of ischemia. The clamp was removed and the hindlimb was reperfused until euthanasia at indicated time points. For mice with IR+cardiotoxin (IR+ctx) injury, 10 µL of 3 mg/ml cardiotoxin was injected into the left tibialis anterior muscle immediately after clamp removal. Ctx (*Naja pallida,* Sigma-Aldrich, St. Louis, MO, USA; cat. no. 217503) was dissolved in sterile phosphate buffered saline (PBS). Buprenorphine was administered at 0.06 mg/kg subcutaneously as an analgesic post-operatively and repeated at 12 and 24 hr.

Hindlimb Ischemia Reperfusion Injury in the Rat Model

Rats were administered prophylactic analgesia (Buprenex, 0.05 mg/kg and 1.2 mg/kg buprenorphine SR) via subcutaneous injection prior to injury and repeated at 48-72 hr, with further doses given as indicated based on animal assessment. Rats were anesthetized by intraperitoneal injection (IP; ketamine 80mg/kg and xylazine 10mg/kg). Additional boluses of ketamine and/or xylazine (10-20/1-3mg/kg) were administered to maintain a plane of general anesthesia. A pneumatic tourniquet (UDC1.6™, Hokanson, WA, USA) was used to interrupt blood flow. Limbs were elevated to reduce venous pooling and tourniquet was applied to the proximal aspect of the lower limb. The tourniquet was inflated and maintained at 280-300 mmHg pressure for 150 min.

Test Article Preparation and Dosing

Hydroxychloroquine sulfate (HCQ) (Cayman Chemical Ann Arbor, MI, USA, item no. 17911, CAS 747-36-4), was diluted to 15 mg/ml in sterile PBS and delivered via IP injection at 60 mg/kg daily, starting on the day of injury until euthanasia. A total of 100 µl of sterile PBS was delivered as the vehicle control (vehicle) via IP injection. Cl-amidine (Cayman Chemical, item no. 10599, CAS 1373232-26-8) was diluted to 50 mg/ml in dimethylsulfoxide as a stock solution. The stock solution was diluted to 2 mg/ml in sterile PBS and 10 mg/kg Cl-amidine was delivered via IP injection daily starting on the day of injury until euthanasia. A total of 150 µl of 4% dimethylsulfoxide in sterile PBS was delivered as the vehicle control through IP injection.

Tissue Harvesting and Histology/Immunohistochemistry

Tibialis anterior (TA) muscles were dissected away from the bone and immediately embedded and frozen in optimal cutting temperature (OCT) compound. Contralateral uninjured TA muscles were used as uninjured control samples. Samples were stored in OCT blocks at -80°C until sectioning. Sections were cut at 7-10 µm thick on a Leica 3050S cryostat, mounted on charged slides, and stored at -80°C until analysis. Standard H&E and picrosirius red protocols were followed for histological staining. For studies involving Cl-amidine treatment, 70% ethanol was used as a fixative prior to staining, for all other studies, Bouin's solution (Sigma-Aldrich, St. Louis, MO, USA) was used as a fixative. For immunofluorescent stains, samples were blocked and permeabilized with 2% serum, 1% BSA, 0.1% fish skin gelatin, 0.001% Triton X-100, and 0.0005% Tween 20. Primary antibodies (**Supp. Table 1**) were applied and incubated overnight at 4ºC. Fluorescently conjugated secondary antibodies were added and then counterstained with Hoechst. Fluorescent samples were imaged using confocal fluorescence microscopy using a Leica SPX8 confocal microscope with 20x objective. All exposure times were optimized during initial imagining and kept constant for all subsequent imaging. Standard bright-field microscopy imaging was performed using a 4x, 20x, or 40x air objective. Appropriate primary antibody negative controls were run simultaneously with each tested sample.

NET Quantification

Sections (10 µm) of flash frozen TA muscle were stained with antibodies for citrullinated histone H3 (H3Cit), myeloperoxidase-conjugated with FITC (MPO-FITC), and Hoechst in concentrations described in **Supp. Table 1**. Three high powered representative images (63x resolution) were taken in similar areas of the muscle on one muscle section per animal using a Leica SPX8 confocal microscope. The number of NETs per region of interest (ROI) were quantified by trained personnel and an average number of NETs per ROI was obtained per animal. NETs were considered co-localization of MPO-Hoechst, MPO-H3Cit-Hoechst, and/or classic web-like morphology of Hoechst and/or MPO.

Muscle Fibrosis Analysis

ImageJ (National Institutes of Health, Bethesda, MD, USA) was used to calculate the percentage area of collagen based on staining with picrosirius red. The thresholding tool was used to create a mask of the area of positive staining, and this area was divided by the total area of the sample analyzed. The Huang method for thresholding was used for all images, and thresholding and analysis methods used were constant for every sample analyzed (49). Six non-overlapping images at 40x magnification were taken on one section for each animal. The average positive staining for these images was used as the percent fibrotic area for each animal. Sections from corresponding regions of the muscle were used for every analysis.

Cross Sectional Area Calculations

TA muscle sections (10 µm, flash frozen) were stained with Hoechst 33342, eMHC and laminin fluorescently labeled antibodies. Myovision muscle analysis software (University of Kentucky, Lexington, KY, USA) (50) was used to detect muscle fibers and calculate average myofiber cross-sectional area and minimum Feret diameter in three separate, non-contiguous fields of regenerating muscle at 20x in 4-6 mice per group.

Centralized Nuclei Quantifications

TA muscle sections (10 µm, flash frozen) were stained with hematoxylin and eosin (H&E) using standard protocols. Six non-overlapping images at 40x magnification were taken using bright field microscopy on three sections for each animal. One section was from the proximal, middle, and distal third. Nuclei within the myofiber were identified as either central or peripheral by trained personnel. The number of myofibers in each group was divided by the total number of myofibers per image to obtain the percentage of myofibers with centralized or peripheral nuclei. The average percentage for each section was determined and used to calculate the average per animal, which is reported as % Centralized Nuclei or % Peripheral Nuclei.

Cytochrome C Oxidase/Succinate Dehydrogenase (COX/SDH) Histochemistry and Quantification

COX/SDH staining was performed according to Ross (51). Briefly, slides were incubated in a solution of 1X 3,3'-Diaminobenzidine (Sigma-Aldrich; cat. no. D3939), 100 µM cytochrome c (Sigma-Aldrich; cat. no. C2037) in PBS with 2 µg bovine catalase (Sigma-Aldrich; cat. no. C9322) for 40 minutes at 37°C then washed with PBS. Next, slides were incubated with 1.5 mM nitroblue tetrazolium (Sigma-Aldrich; cat. no. N6876), 130 mM sodium succinate (Sigma-Aldrich; cat. no. 818601), 0.2 mM phenazine methosulfate (Sigma-Aldrich; cat. no. P9625), and 1.0 mM sodium azide (Sigma-Aldrich; cat. no. S8032) in PBS for 40 minutes at 37°C. Slides were washed, mounted and imaged with standard bright field microscopy. Six non-overlapping images at 40x magnification were taken using bright field microscopy on three sections for each animal. One section was from the proximal, middle, and distal third. To quantify, each myofibers was classified as COX positive/negative and SDH positive/negative. The percentage of COX positive/SDH negative myofibers and COX positive/SDH

positive myofibers per total myofibers was determined for each image. The average percentage for each section was determined and used to determine the average per animal, reported as proportion of myofibers COX+/SDH- or COX+/SDH+.

PDGFRα-Ki67 Staining and Quantification

Sections (10 μm) of flash frozen TA muscle were stained with antibodies for platelet derived growth factor alpha (PDGFRα), Ki67, and Hoechst in concentrations described in **Supp. Table 1**. Three high powered representative images (63x magnification) were taken in similar areas of the muscle on three muscle sections per animal (one from each of the proximal, middle, and distal third of the muscle) using a Leica SPX8 confocal microscope. n=4-6 animals per group. The number of cells expressing co-localized PDGFRα and Ki67 were manually identified and counted per region of interest (ROI) by trained personnel. Total PDGFRα and Ki67 quantifications were determined with ImageJ to threshold positive area with the Yen thresholding method to determine total positive staining, which is reported in pixels. A linear regression analysis was used to correlate total PDGFRα and total Ki67 staining. In order to accurately correlate the amount of PDGFRα to the corresponding Ki67 expression in the same ROI, ROIs were evaluated and plotted individually in analyses.

Oil Red O Staining and Quantification

TA muscle sections (10 μm, flash frozen) were fixed in in 4% paraformaldehyde and stained in Oil Red O solution (3 mg/mL Oil Red O; Sigma-Aldrich; CAS 1320-06-5; in 60% isopropyl alcohol) for 15 min. Slides were rinsed in tap water and mounted with an aqueous mountant. Images of the entire muscle section from corresponding locations in the TA were taken at 4x magnification using bright field microscopy. One section from the proximal, middle, and distal third of the TA were analyzed, to total three sections per animal. n=2-5 animals per group. ImageJ was used to calculate the percentage area of positive Oil Red O staining. The image was transformed to YIQ color spectrum, the 'I' spectrum was converted to an 8-bit image, and the MaxEntropy threshold was used to quantify the area stained positively for Oil Red O. The positive area was divided by the total area of the sample to yield the % Oil Red O positive. The mean of three sections per animal was reported as one point per animal.

Western Blots

TA tissue was sonicated and lysed with RIPA buffer lysis system (sc-24948A, Santa Cruz Biotechnology, Dallas, TX, USA) and exposed to three freeze-thaw cycles. Samples were assessed for total protein using Pierce BCA Protein Assay Kit (ThermoFisher Scientific, cat no. 23225). Proteins from lysate were separated in NuPAGE 4-12% bis-tris gel (ThermoFisher Scientific, cat no. NP0335BOX) with NuPAGE SDS running buffer (ThermoFisher Scientific, cat no. NP0001). Protein was transferred to PVDF membrane (ThermoFisher

Scientific, cat no. IPVH00010) in NuPAGE transfer buffer (ThermoFisher Scientific, cat no. NP0006). Membrane was washed in wash buffer (1x Tris buffered saline-0.05% Tween-20) and blocked for 1 hr at room temperature with 5% milk protein in wash buffer. Membranes were incubated with primary antibody (**Supp. Table 1**) diluted in 5% BSA in wash buffer. Appropriate HRP-linked secondary was diluted in block solution. Membranes were developed and ImageJ software was used for quantification.

Cytokine Assays

For mouse cytokine analysis, blood samples were collected at 3 days post injury (DPI) in EDTA tubes from mice treated with vehicle or HCQ (n=3/group). Plasma was stored at -80°C until analysis without additional freeze-thaw cycles. On the day of assay, samples were diluted 1:1 with PBS and assessed using a mouse multi-spot 96-well 10-plex pro-inflammatory assay kit for mice (Mesoscale Diagnostics, Rockville, MD, USA) according to the manufacturer's guidelines. Plates were read and analyzed using the MSD Sector S600 Instrument and MSD Workbench Software version 4.0 (Mesoscale Diagnostics). A standard curve was generated to determine analyte concentration. For rat cytokine analysis, cytokine expression included here was determined in serum samples within a multiplex assay. Bio-Plex Pro™ Rat Cytokine 23-Plex assays (Bio-Rad Laboratories, Inc., Hercules, CA, USA; cat# 12005641,) were performed on a Bio-Plex 200 Luminex system with high throughput fluidics (Bio-Rad Laboratories, Inc., cat# 171000205) which had undergone both validation (Bio-Plex Validation Kit 4.0, Bio-Rad Laboratories, Inc.) and daily calibration prior to use (Bio-Plex Calibration Kit, Bio-Rad Laboratories, Inc.) to standardize signal output and sensitivity between runs. Assay working ranges were determined using Bio-Plex Manager 6.1software (Bio-Rad Laboratories, Inc.) and used for the automatic identification and removal of outliers and optimization of 5-PL standard curves to interpolate the concentrations of analytes in the unknown samples.

RNA Isolation and QRT-PCR

TA muscles were harvested at 7 days post IR injury from mice treated with vehicle or HCQ (n=3/group). Samples were flash frozen and stored at -80°C. Total RNA was isolated using ceramic bead homogenization and the RNeasy mini kit (Qiagen, Venlo, Netherlands). First strand cDNA was generated from 1 µg total RNA using the iScriptAdvanced cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). A custom low-density quantitative reverse transcription polymerase chain reaction array (qRT-PCR; Bio-Rad). The QuantStudio 7 Flex Real-Time PCR system (ThermoFisher Scientific) was used to run the custom arrays. Gene expression levels were calculated as normalized expression relative to housekeeping gene (*Rplp0*; dCt) or fold change gene expression (normalized value relative to vehicle treatment value; ddCt).

Single-cell RNA Sequencing using 10x Genomics

Single-cell tissue preparation and analysis was performed as previously described (52). Briefly, baseline uninjured muscle (day 0) and post-surgery day 3 TA muscles were harvested from six IR+ctx mice and eight uninjured C57BL/6 mice. 3 lanes of IR+ctx (n = 2 pooled mice per lane) and 2 lanes of uninjured (n = 4 pooled mice per lane) was used to maximize cell return. Harvested TAs were digested under agitation in 0.3% Type 1 Collagenase and 0.4% Dispase II (Life Technologies, Carlsbad, CA, USA. cat no 17100017 and 17105041) in RPMI 1640 medium for 45 min at 37°C. Cells were filtered, washed, and resuspended at ~1000 cells/μL. Cell viability was assessed and only samples with 85% or greater viability were used for sequencing. Single-cell 39 library generation was performed on the 10x Genomics Chromium Controller following the manufacturer's protocol for the v2 reagent kit (10x Genomics, Pleasanton, CA, USA). Single-cell gel bead bead-in-emulsions (GEMs) were generated, reverse transcription was performed, and the Post GEM-RT product was cleaned using DynaBeads MyOne Silane beads (ThermoFisher Scientific). cDNA was amplified, quantified and enzymatically fragmented and size selected for optimization using SPRIselect beads (Beckman Coulter, Brea, CA, USA). Double-sided SPRI bead cleanup was performed after end repair and A-tailing, and single-sided cleanup is done after adapter ligation. Indexes were added during PCR amplification, and a final double-sided SPRI cleanup was performed. During PCR amplification, indexes were added and double-sided SPRI was performed for final cleanup. Libraries were quantified by Kapa qPCR for Illumina Adapters (Roche) and size was determined by Agilent tapestation D1000 tapes. Read 1 primer sequence are added to the molecules during GEM incubation. P5, P7, and sample index and read 2 primer sequence are added during library construction via end repair, A-tailing, adaptor ligation, and PCR. Libraries were generated and sequenced on a HiSeq 4000 on a HiSeq 4000, (Illumina, San Diego, CA, USA) using a HiSeq 4000 PE Cluster Kit (PN PE-410-1001) with HiSeq 4000 SBS Kit (100 cycles, PN FC-410-1002) following manufacturer recommended protocols. Cell Ranger Single-Cell Software Suite 1.3 (10x Genomics, Pleasanton, CA, USA) was used to perform sample de-multiplexing, barcode processing, and single-cell gene counting (alignment, barcoding, and unique molecular identifier (UMI) count) at the University of Michigan Biomedical Core Facilities DNA Sequencing Core (52).

Bioinformatics Analysis of Single-cell RNA Sequencing Data

Analysis was performed as previously described (52). Briefly, a total of ~200 million reads were generated from the 10x Genomics sequencing analysis for each of the replicates. The sequencing data were first preprocessed using the 10x Genomics software Cell Ranger and aligned to mm10 genome (GEO accession: GSE144270). Quality control and analysis was performed as previously described (52). Downstream analysis steps were performed using Seurat pipeline (53) and included normalization, scaling, dimensionality reduction (principal component analysis [PCA] and t-distributed stochastic neighbor embedding [t-SNE]), unsupervised clustering, cluster consolidation via centroid rank correlation analysis, discovery of differentially expressed

cluster specific markers, and marker enrichment. Presence of replicate batch effect was excluded by visual inspection of the contribution of each replicate to the PCA and t-SNE projections of the group. Provisional clusters were assigned via unsupervised clustering (Seurat FindClusters, Louvain algorithm, k = 30, resolution = 0.4), resulting in 21 and 17 provisional clusters for the case and control group, respectively. Provisional clusters were aligned according to the rank correlation of their centroids, measured on the gene set derived from the intersection of the genes for each set. Final clusters were obtained by grouping similar clusters based on centroid rank correlation analysis. Clusters appear to be well distinguished in the PCA and t-SNE projections (52). Characteristic gene expression and cluster name abbreviation key is provided in **Supp. Table 2** and previously published data by Stepien, et al. (52)

Statistical Analysis

Statistical tests were performed using GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA, USA) software. Histological quantification of stained images were subjected to Student *t*-test or ANOVA with multiple comparisons post-test. Cross-sectional area and Feret diameter were represented as frequency of distribution graphed in percent frequency and compared using Komolgorov-Smirnov analysis. All data are presented as mean values with standard deviation (SD), and *P* values <0.05 were considered to be statistically significant.

Results

Ischemia reperfusion injury resulted in fibrosis and NET formation in the skeletal muscle

To recapitulate skeletal muscle fibrosis following IR injury, muscle injury and fibrosis were investigated in the injured tibialis anterior muscle and contralateral healthy control of C57BL/6 mice. Skeletal muscle injury was characterized by intense leukocyte infiltration at 3 DPI with lingering necrotic myofibers as indicated by H&E staining (**Supp. Fig. 1***)*. At 3 DPI, picrosirius red staining revealed loose, disorganized collagen deposition (**Supp. Fig 1**) and by 7 DPI, strong collagen deposition was present between regenerating myofibers, a pattern consistent with fibrotic deposition (**Fig 1**, **Supp. Fig. 1***)*. By 7 DPI, necrotic tissue was replaced by regenerating skeletal muscle myofibers with prominent centralized nuclei, cytoplasmic basophilia, and oblong shape with smooth edges (**Fig 1**, **Supp. Fig. 1***)*. IR injury in the skeletal muscle was also characterized by a robust immune system response. To evaluate the formation of NETs in the skeletal muscle following IR injury, muscle sections at 1 and 3 DPI were immunofluorescently labeled with H3Cit, MPO, and Hoechst and NETs were identified. Robust NET formation was demonstrated in the skeletal muscle following IR injury (**Fig 1**, **Supp. Fig. 2**, indicated by white arrowheads). NETs were not present in uninjured skeletal muscle, as shown in **Supp. Fig. 2**.

Decrease of NETosis via PAD4 pathway did not alter muscle regeneration or fibrosis

Protein arginine deiminase 4 (PAD4) is required for NETosis that occurs through citrullination of histone H3 and H4 (48, 54-57). NETs formed in the muscle in the mouse model of IR demonstrate citrullination of histone H3 (**Supp. Fig. 2**), thus PAD4-/- mice and the small molecule PAD inhibitor Cl-amidine were utilized to investigate the role of NETs formed via the PAD4 pathway in fibrotic deposition from IR injury. C57BL/6 mice underwent IR injury and were administered Cl-amidine or vehicle treatment via intraperitoneal injection daily. TA muscles were harvested at 3 and 7 DPI, flash frozen, cryosectioned, and histologically processed to evaluate NET formation, myofiber regeneration, and muscle fibrosis. NET formation was quantified at 3 DPI. Daily administration of Cl-amidine led to a significant decrease in NET formation from 24.1 ± 11.7 to 3.9 ± 2.7 following treatment (NETs per 63x ROI, n=3 per group; $P<0.05$; **Fig 1*A***). Mice treated with Cl-amidine presented with an increased frequency of myofibers with small CSA and Feret diameter ($P<0.0001$, both analyses), indicating that administration of Cl-amidine hindered muscle regeneration at 7 DPI compared to vehicle (**Fig. 1*B***). Similarly, Cl-amidine did not have an effect on muscle fibrosis at 7 DPI compared to the vehicle (17.5 ± 0.41%; vs 50.6% ± 5.5 vs 47.7% ± 11.1 uninjured, Cl-amidine, vehicle, respectively; mean ± SD $P=0.8$, n=3-6/group; ordinary one-way ANOVA plus Tukey's test). (**Fig. 1*C***). As pharmacological intervention may not completely inhibit the PAD4 signaling, we next sought to investigate this phenomenon in a transgenic mouse lacking PAD4. To ensure a robust injury and closely mimic the polytrauma injuries that often accompany IR, in the complimentary transgenic studies IR injury was performed as described and 10 µL of 3 mg/ml ctx was also injected into the injured tibialis anterior immediately following clamp release. Results in the PAD4-/- mice were similar to that of the pharmacological inhibition. Muscle regeneration in the PAD4-/- mouse was decreased compared to the wild type control, as evidenced by an increased relative frequency of myofibers with small Feret diameter ($P<0.0001$; **Fig. 1*D***). Muscle fibrosis was also similar between the PAD4-/- mice and wild type C57BL/6 control mice at 7 DPI (35.0% ± 4.25 vs 39.8% ± 6.7; PAD4-/-, C57BL/6 wild type control respectively, mean ± SD, $P=0.34$, unpaired *t*-test, **Fig. 1*E***). Despite a decrease in NET formation, mice treated with Cl-amidine did not have a significant difference in myofiber regeneration, as assessed by myofiber CSA and Feret diameter. To identify if any treatments or genotypes provided protective effects on the myofibers, we compared the percent of myofibers possessing centralized nuclei (indicating regenerating myofibers) between groups and the percent of myofibers exhibiting nuclei on the periphery of the myofiber (indicating an uninjured/protected myofiber). We found similar composition between vehicle and Cl-amidine treatment (regenerating: 0.05% ± 0.08% vs 99.7% ± 0.24 vs 99.7% ± 0.14; and uninjured/protected: 99.95 ± 0.082 vs 0.35% ± 0.24 vs 0.28% ± 0.14 %; uninjured, Cl-amidine, vehicle, respectively, n=3-6/group, ANOVA followed by Tukey's test), as shown in **Fig 1*F***. Similarly, both C57BL/6 and PAD4-/- muscles were comprised of nearly 100% regenerating fibers without substantial peripherally located nuclei (**Fig 1*G***). This data indicated that PAD4 inhibition both pharmacologically and genetically did not affect the initial injury to the TA muscle or

provide protective benefits to the myofibers. All these findings taken together suggest that NET formation via the PAD4 mechanism was not essential in fibrotic deposition in the skeletal muscle following IR injury.

IR injury induced IL-10 and TNFα production

NETs and TLR7/8/9 have been linked to secondary self-NET propagation and have been shown to augment circulating levels of proinflammatory cytokines IL-10 and tumor necrosis factor-α (TNFα) (32, 58). In our rat model of hind limb tourniquet induced IR injury (59, 60), we show that IL-10 levels were significantly elevated at 6 and 24 hr post injury from 758 ± 244 pg/ml in uninjured animals to 1753 ± 121 pg/ml at 6 hr (P<0.0001) and 1659 ± 213 pg/ml at 24 hr (P<0.0001) (**Fig. 2*A***). We found similar elevations in TNFα at 6 and 24 hr post injury from 2633 ± 1062 pg/ml to 7047 ± 1202 pg/ml (P<0.0001) and 6717 ± 1183 pg/ml (P<0.0001), respectively (**Fig. 2*A***), suggesting that IR injury mimicked other NET-related disease cytokine release, and similar TLR7/8/9 mediated pathways may have also been involved. To understand the cell populations that were involved in TLR7/8/9, IL-10 and TNFα release, we conducted a transcriptome analysis of the cell types at 3 DPI involved in skeletal muscle regeneration using single-cell sequencing (scRNA-seq) in a mouse model of polytrauma (IR+ctx), which has been shown to produce a robust inflammatory reaction that results in ECM deposition, myonecrosis, and ultimately fibrosis (52). Unsupervised clustering identified 11 distinct cellular clusters in uninjured muscle and 8 in IR+ctx (**Fig *2B-C,* Supp. Table 2**). Uninjured muscle was largely composed of endothelial cells/vascular progenitor cells, FAPs, satellite cells, and *scleraxis* expressing mesenchymal stem cells. Injured muscle also contained an FAP population, as well as a greater predominance of immune cells including macrophages, dendritic cells, and granulocytes. Of note, the uninjured muscle was found to contain a *Pax7* positive satellite cell population, indicating quiescent satellite cells, while the injured muscle contained a myoblast population, defined by *Myf5* and *Myod1. Il10* was expressed nearly exclusively by the macrophage population (**Fig 2*D-E*)** and *Tnfα* at highest levels in the granulocyte population (**Fig 2*F-G*).** These results indicate that traumatic injury induced *Il10* and *Tnfα* gene expression at the same time point as robust NET formation and that NETs present in the tissue following injury may stimulate TLR7/8/9 pathways, leading to increased IL10 and TNFα levels observed here.

Treatment with HCQ suppressed IL-10 and TNFα production

We next sought to modulate TLR signaling pathway to mitigate injury caused by IR. HCQ has been shown effective in NET and RNA mediated diseases and viral infections, and is known to lower IL-10 and TNFα levels in lupus patients as well as decrease disease severity (61). Ischemia reperfusion injured C57BL/6 mice treated systemically with 60 mg/kg HCQ daily had a trend towards lower IL-10 and significantly lower TNFα (P<0.05) plasma levels (**Fig. 2*H-I***) at 3 DPI, which correlates with the detected robust NET formation and

presence of *Il10* and *TNFα* expressing immune cells (**Fig 1*A*, Fig. 3*C-G***). Interestingly, no significant differences in other measured pro-inflammatory cytokine were detected (**Supp. Table 3**).

HCQ treatment decreased NET formation

Given that HCQ acts on TLR7/8/9, we evaluated NET formation at 3 DPI in C57BL/6 mice treated with vehicle or HCQ. Mice treated with HCQ showed substantial trend towards decreased NET formation, (NETs per 63x ROI: HCQ: 4.5 ± 1.6, vehicle: 7.4 ± 3.2; n=6/group; P=0.08, unpaired t-test) (**Fig. 3*A***). This potential decrease in NET formation in the HCQ treated mice is unlikely to be due to differences in initial neutrophil recruitment, as treatment with HCQ did not show a difference in neutrophil recruitment to the injured muscle at 3 DPI, as assessed by immunofluorescent staining for Ly6G (**Supp. Fig 3**). This trend of decreased NET formation following administration of TLR7/8/9 inhibitor further indicated that NET formation is likely partially, but not completely, mediated by TLR signaling following traumatic injury.

Treatment with HCQ attenuated fibrosis and improved myofiber regeneration following IR injury

Given the trend towards decreased NET formation observed following HCQ administration, we next sought to investigate the role of HCQ in muscle regeneration and fibrosis after IR injury. C57BL/6 mice underwent IR injury and were treated with vehicle or HCQ. Mice treated with HCQ presented with significantly decreased muscle fibrosis at 7 DPI than those treated with vehicle (18.5% ± 2.3 vs 30.4% ± 5.4 vs 40.5% ± 4.0; uninjured, HCQ, vehicle, respectively; n=6/group, P<0.01, ordinary one-way ANOVA plus Tukey's test) (**Fig. 3*B***). Additionally, treatment with HCQ improved muscle regeneration. HCQ decreased the frequency of myofibers with small CSA and small Feret diameters (both analyses P<0.0001) at 5 DPI (**Fig 3*C***). In order to determine if the differences seen in fibrosis and regeneration in the HCQ treated animals was a result of less severe initial injury, we again compared the percentage of regenerating (centralized nuclei) and uninjured/protected (peripheral nuclei) myofibers in each treatment group to assess if HCQ provided protective effects on the myofibers. The percentage of regenerating myofibers was not significantly different between vehicle and HCQ treated animals (0% vs 98.37% ± 2.08 vs 98.78% ± 0.49; uninjured, HCQ, vehicle, respectively, n=6/group, ANOVA followed by Tukey's test), as shown in **Fig 3*D*.** HCQ did not prevent initial injury to the myofibers either, as the composition of peripherally located myofibers was not significantly different between vehicle and HCQ treated animals (100% vs 1.63% ± 2.08 vs 1.21% ± 0.49; uninjured, HCQ, vehicle, respectively, n=6/group, ANOVA followed by Tukey's test) (**Fig 3*D***). Taken together, this data indicated that HCQ significantly decreased fibrosis and increased regeneration following muscle IR injury. Furthermore, our data indicated that muscle fibrosis was at least in part independent of NET formation in this injury, as the increase in regeneration and reduction in fibrosis was accompanied by a non-significant decrease in NET formation.

HCQ Treatment Did Not Induce Mitochondrial Damage

Antimalarial myopathy and mitochondrial abnormalities have been reported after chronic chloroquine regimens (62). In order to assess the presence of a mitochondrial myopathy in the injured TA following HCQ treatment, COX/SDH double staining was performed. The COX/SDH stain was used to detect fibers that lacked COX activity staining, but in which positive SDH staining was present (COX-/SDH+), which is indicative of mitochondrial myopathies (63, 64), or fibers that are both COX and SDH positive. No myofibers were found to lack COX staining and a significant difference was not found between the proportion of COX+/SDH+ myofibers of muscles treated with HCQ compared to vehicle treated, as shown in **Supp. Fig. 4**, which indicated that 7 days of daily HCQ treatment did not induce significant mitochondrial dysfunction in the injured muscles.

HCQ Treatment Inhibited FAP Proliferation

FAP cells proliferate following injury and differentiate into myofibroblasts, which are responsible for ECM deposition and fibrotic deposition (47). Having shown the HCQ decreased ECM deposition and fibrosis following IR injury, we hypothesized that HCQ may be acting on FAPs. Indeed, staining for FAPs (identified by PDGFRα expression) and the proliferation marker Ki67 at 5 DPI revealed that HCQ treatment significantly decreased the proliferation in FAP cells, as shown by a decreased number of double-positive PDGFRα-Ki67 cells in the HCQ treated muscles compared to vehicle treated (0.1 ± 0.3 vs 2.6 ± 1.6 vs 8.5 ± 4.7; uninjured, HCQ, vehicle, respectively; n=4-6/group, $P<0.0001$, ordinary one-way ANOVA plus Tukey's test) (**Fig 4*A-B***). Furthermore, we used linear regression analysis to correlate total PDGFRα staining with total Ki67 staining and demonstrated that vehicle treated animals showed a significant linear correlation between PDGFRα and Ki67 stains ($P=0.04$, **Fig 4*E***), which suggested that FAPs were the proliferating population. By contrast, there was poor concordance of FAP/proliferation correlation plots of the HCQ treated animals ($P=0.17$ **Fig 4*D***), which suggested that, while Ki67 staining was present, it did not correlate to increased PDGFRα expression. Uninjured TA muscles presented with few FAPs and decreased total staining of both PDGFRα and Ki67 (**Fig 4*A-C***). Following injury, FAPs may also differentiate to adipocytes (47). Oil Red O staining, which identifies lipid deposition, in the vehicle and HCQ treated TAs at 5 DPI identified very little lipid accumulation, indicating that FAPs were not driven towards adipogenesis following IR injury, nor did HCQ lead to adipocyte formation (**Supp. Fig. 5**). Taken together, this indicated that treatment with HCQ decreased proliferation in the FAP population at 5 DPI, which corresponded to a decrease in fibrosis at 7 DPI.

Treatment with HCQ inhibited ERK1/2 phosphorylation after IR injury

The role of ERK signaling following IR injury is not yet completely elucidated, but most studies to date show decreased ERK signaling and/or decreased ERK1/2 phosphorylation in the injured tissue to be beneficial for

tissue survival and regeneration (38-43) and ERK signaling has also been implicated in fibrotic diseases (36, 37, 65-67). In order to investigate the role of ERK signaling in IR injury, we analyzed the IR+ctx single-cell data set to characterize *Erk1* and *Erk2* gene expression in uninjured and injured skeletal muscle without treatment interventions. Given the central role in proliferation, transcription regulation, and differentiation, both genes were widely expressed both in the uninjured and injured muscle at 3 DPI (**Fig. 5*A-J***). Interestingly, *Erk1* and *Erk2* were differentially expressed in the FAP cell population (characteristic genes provided in **Supp. Table 2 and Supp. Fig 6)** after injury (**Fig. 5*C, H***). FAPs expand in response to injury and contribute to fibrotic deposition by myofibroblasts, and an increase in fibrotic gene expression (including *Col3a1, Col1a2, Snai1, Ctgf*) at this time point has previously been shown in this population (52, 68, 69). A full analysis of *Erk1* and *Erk2* expression levels in each cluster of uninjured and injured muscle is included in **Supp. Fig. 7**. Of note, little *Erk1* or *Erk2* expression was present in the granulocyte population of the IR+ctx muscle, the population from which NETs would arise (**Supp. Fig. 7**). Furthermore, low levels *Erk1* and *Erk2* expression was present in the satellite cell population of the uninjured satellite cell population (*Pax7*$^{+}$*, Chodl*$^{+}$*, Sdc4*$^{+}$*)*, however expression of *Erk1* and *Erk2* was present in the myoblast population (*Myod1*$^{+}$*, Myf5*$^{+}$) of the IR+ctx injured muscle at 3 DPI (**Supp. Fig. 7**). We next investigated *Erk1* and *Erk2* expression at 7 DPI in muscles treated with vehicle or HCQ. At 7 DPI, at which point a decrease in fibrotic deposition was seen in HCQ-treated mice, *Erk1* and *Erk2* gene expression was similar in whole muscle tissue of vehicle or HCQ treated mice (**Fig. 5*K-L***), which indicated that treatment with HCQ does not significantly change genetic expression of *Erk1* or *Erk2.* Additional genes of interest are presented in **Supp. Table 4**. Similarly, whole tissue protein analysis by western blot showed similar levels of ERK1/2 protein in tissues treated with vehicle or HCQ at 7 DPI (**Fig. 5*M***). However, animals treated with HCQ showed a significantly lower ratio of pERK1/2 to ERK1/2 than the vehicle treated mice (vehicle: 0.98 ± 0.07; HCQ: 0.61 ± 0.21, n=3/group, $P<0.05$, unpaired *t*-test), which indicated that treatment with HCQ decreased ERK1/2 phosphorylation in the injured muscle tissue (**Fig. 5*M-N***). Concurrently at 7 DPI, mice treated with HCQ also presented with decreased muscle fibrosis, suggesting that inhibition of ERK1/2 phosphorylation contributed to decreased muscle fibrosis. At 7 DPI, *Erk1* and *Erk2* gene expression were similar in bulk muscle treated with vehicle or HCQ, however, HCQ inhibited ERK1/2 phosphorylation in the muscle tissue, which corresponded to a decrease in muscle fibrosis.

Discussion

Following a period of ischemia and subsequent reperfusion, devastating fibrosis is often formed in skeletal muscle (3). Here, we recapitulated this injury in a mouse model of hindlimb IR injury. We showed that IR injury resulted in extensive immune infiltration post injury and led to fibrotic deposition by 7 DPI. As part of the initial immune response, we identified robust NET formation in the skeletal muscle. We found that modulation of TLR7/8/9 signaling by administration of HCQ following injury lowered circulating TNFα levels,

showed a trend to lower IL-10 levels, significantly improved muscle regeneration, and attenuated muscle fibrosis. This effect was at least in part independent of NET formation via TLR7/8/9 signaling as NET formation in the HCQ treated mice was not significantly decreased. Upon further investigation, we identified that HCQ decreased proliferation in FAP cells and modulated ERK1/2 phosphorylation, a pathway known to be involved in the fibrotic response to IR injury. Administration of HCQ may be an effective treatment to mitigate muscle damage and fibrosis following IR injury.

Following IR injury, we identified extensive neutrophil infiltration into the injured muscle and robust NET formation in the injured skeletal muscle. NETs are present in other models of trauma, and are known to exacerbate the injury in many cases (1, 4, 5, 70). For example, following a 30% total body surface area burn in combination with an Achilles tendon tenotomy, mice exhibit robust NET formation at the tenotomy site at three days post injury (17). A mouse model of antibody-mediated transfusion-related acute lung injury also shows NETs in the alveoli of injured mice, and following an experimental model of murine deep vein thrombosis in the inferior vena cava, NETs are present in the thrombus and contribute to thrombosis (18, 19). Therefore, in the current study, we investigated if NET formation following IR injury may also negatively affect tissue regeneration and contribute to fibrosis. Classically, NETs are formed through suicidal NETosis and citrullination of histone H3, a pathway dependent on PAD4. Thus, we inhibited PAD4 pharmacologically as well as deleted PAD4 genetically to investigate the role of suicidal NETosis in muscle fibrosis. While NETs were decreased after Cl-amidine treatment, interestingly, little difference was seen in fibrotic deposition after pharmacological or genetic deletion of PAD4. These results suggested that NET formation via the PAD4 mechanism did not play an essential role in fibrotic deposition in the skeletal muscle following IR injury. However, NETs have also been shown to form through a TLR 7/8/9 mediated pathway that is independent of PAD4 via secondary self-propagation (17, 24). That is, nucleic acid material present in the tissue stimulates the TLR7/8/9 receptors of immune cells, which triggers additional inflammation and a second wave of neutrophils and NETs. We have shown that NETs were abundant in the injured tissue, which may go on to contribute to secondary NETosis. Additionally, while not explicitly investigated here, it is reasonable to hypothesize that free nucleic material was present in the tissue due to mechanical trauma (21, 71). Thus, we investigated TLR7/8/9 signaling further in the context of muscle fibrosis and NET formation.

NETs are perhaps best characterized in autoimmune diseases such as lupus and rheumatoid arthritis, and we saw several similarities between the IR injury and these diseases, despite the vastly different underlying pathologies. First, we showed that the immune response to IR injury led to elevated serum levels of IL-10 and TNFα, which are associated with TLR7/8/9 stimulation and NETs. For example, NETs formed upon activation of neutrophils with *Mycobacterium tuberculosis*, which acts through TLR7/8/9 activation, stimulate macrophages and monocytes to produce IL-10 and TNFα (72). High serum levels of IL-10 and TNFα are also

implicated with rheumatoid arthritis and lupus, both diseases characterized by excessive NET production (11-13, 32). In fact, increased levels of IL-10 in lupus patients has been correlated to increased disease severity (73). We found that in our mouse model of IR injury, high serum levels of IL-10 and TNFα corresponded to the time point at which NETs are present in the injured tissue (3 DPI). Using scRNA-seq in the IR+ctx model, we identified that *Il10* was expressed by the macrophage population, and *Tnfα* largely by the granulocyte population, indicating that the infiltrating immune cells were responsible for increased circulating IL-10 and TNFα levels. Interestingly, pulmonary fibrosis can be seen in lupus, and NETs are also abundant in the airway fluids of cystic fibrosis patients, further suggesting a link between NETs and fibrosis (74). Therefore, we chose to investigate HCQ, a small molecule TLR7/8/9 inhibitor that is prescribed for lupus and rheumatoid arthritis patients and has been shown to decrease TNFα serum levels in NET-associated diseases (75, 76). Indeed, delivery of the TLR7/8/9 inhibitor HCQ to mice following IR injury lowered TNFα levels as well as IL-10. Thus, we hypothesized that administration of HCQ following IR injury may also affect NET formation and muscle injury.

We found that HCQ significantly improved muscle regeneration and decreased muscle fibrosis following IR injury, however, HCQ treatment resulted in a non-significant decrease in NET formation. HCQ is also known to inhibit TLR7/8 signaling as well as have effects on autophagy and metabolic activity, both of which may contribute to the pro-regenerative and anti-fibrotic effect seen here (77, 78). We have also shown that treatment with HCQ decreases proliferation in the FAP cell population, which differentiate to myofibroblasts and are ultimately responsible for fibrotic deposition (47). While further studies are needed to elucidate the cell specific actions of HCQ, we hypothesize that the decrease in FAP proliferation after HCQ treatment is at least partially responsible for the concordant decrease fibrotic deposition.

A growing body of research also ties *Mapk* signaling and ERK1/2 activation to fibrosis, and inhibition of ERK1/2 is shown to attenuate fibrosis (36, 37, 65-67). Here, we showed that differential *Erk1* and *Erk2* signaling in the FAP population of IR+ctx injured skeletal muscle correlated to increased fibrotic gene expression in the FAP population, which suggested a correlation between *Mapk* signaling and fibrosis (52). Thus, we investigated the role of *Erk1* and *Erk2* gene expression and ERK1/2 protein levels and phosphorylation in IR injury. We found that administration of HCQ decreased ERK1/2 phosphorylation at 7 DPI, which corresponded to decreased fibrosis. *Erk1* and *Erk2* expression in the muscle was similar in muscle treated with HCQ or vehicle, as were the levels of ERK1/2 protein, which indicated that the activation and subsequent phosphorylation of ERK1/2 were likely the important step in the ERK signaling process directing fibrosis. Further investigation into the role of ERK1/2 activation and related pathways will be an important next step to characterize the role of HCQ treatment in muscle fibrosis.

We have identified that treatment with FDA-approved HCQ mitigated muscle fibrosis and improved myofiber regeneration following IR injury, suggesting HCQ may be a viable treatment to prevent muscle fibrosis in ischemia reperfusion and traumatic extremity injury. Although chloroquine-induced myopathy has been reported, this side effect is rare and reversible once the dosing regimen is stopped (62, 79, 80). We found that daily dosing of 60 mg/kg HCQ for 7 days did not lead to mitochondrial dysfunction in the injured muscle, as assessed by COX/SDH double staining. Overall, HCQ decreased IL-10, TNFα, FAP proliferation, and ERK1/2 phosphorylation, which contributed to the pro-regenerative and anti-fibrotic effects observed. Administration of HCQ resulted in a modest decrease of NETs in the skeletal muscle, which suggested that the muscle fibrosis following IR injury was at least partially independent of NET formation. Similarly, mice with PAD4 pharmacologically inhibited or genetically deleted and therefore lacking NETs formed via citrullination of histone H3 did not present with differences in muscle fibrosis, which suggested fibrosis is independent of NET formation via PAD4. These insights into the role of NETs in fibrotic injuries and mechanisms of HCQ may offer important therapies in addition to HCQ and contribute to understanding the role of NETs in traumatic and fibrotic injuries. Further investigation will focus on the mechanism of action of HCQ in muscle fibrosis.

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Figure 1. Muscle fibrosis is independent of PAD4 mediated NETosis. *A)* Immunofluorescent staining showing NETs (examples indicated by white arrowheads) in the TA at 3 DPI in C57BL/6 mice treated with Cl-amidine or vehicle. Treatment with Cl-amidine significantly decreased NET formation. *B)* Representative images of C57BL/6 mice uninjured TA, and treated with Cl-amidine or vehicle stained with eMHC and laminin at 7 DPI. Histogram of frequencies of distribution of myofiber cross sectional area (CSA) and Feret diameter at 7 DPI, indicating muscles with Cl-amidine treatment have significantly increased frequency of smaller myofibers as indicated by CSA or diameter on x-axis. *C)* Representative histological sections of picrosirius red staining at 7 DPI of C57BL/6 mice uninjured TA, and treated with Cl-amidine or vehicle. ImageJ quantification of positive picrosirius red staining in the same groups. D) Representative sections of PAD4-/- mice or C57BL/6 wild type control injured with IR+ctx stained with eMHC and laminin at 7 DPI. Histogram of frequencies of distribution of myofiber CSA and Feret diameter at 7 DPI, indicating PAD4-/- muscles have significantly increased frequency of smaller diameter myofibers. *E)* Representative histological sections of picrosirius red staining at 7 DPI of PAD4 -/- or C57BL/6 wild type control injured with IR+ctx. ImageJ quantification of positive picrosirius red staining in the same groups. *F)* Representative images of H&E stained TA 7 DPI of uninjured and treated with Cl-amidine or vehicle. Quantification of percentage of total myofibers with centralized or peripheral nuclei in each group. *G)* Representative images of H&E stained muscle 7 DPI of PAD4-/- and C57BL/6 mice. Quantification of percentage of total myofibers with centralized or peripheral nuclei in each group. + indicates difference compared to uninjured group. All scale bars indicate 100 μm. * indicates $P<0.05$; ++ indicates $P<0.005$; **** and ++++ indicate $P<0.0001$. ns, not significant. Frequency distributions analyzed with Kolmogorov-Smirnov test. Bars represent means ± standard deviation. Each point represents one animal.

Figure 2. HCQ decreases IL-10 and TNFα in an IR injury model. *A)* In a rat model of tourniquet-induced hind limb IR injury, serum levels of IL-10 and TNFα are significantly elevated at 6 and 24 hr post injury compared to uninjured levels (n=6-8/group, one-way ANOVA with Tukey test). *B)* Clusters resulting from unsupervised clustering in mouse uninjured TA muscle, overlaid letters correspond to adjacent table to indicate cluster identification. *C)* Clusters resulting from unsupervised clustering in mouse IR+ctx injured muscle at 3 DPI, overlaid letters correspond to adjacent table to indicate cluster identification. Violin plots indicate distribution of expression between clusters of cells at 3 DPI of *D-E) Il10* and *F-G) Tnfα.* HCQ lowers circulating *H)* IL-10 and *I)* TNFα levels at 3 DPI in the C57BL/6 mouse model of IR injury (n=3 per group, unpaired *t*-test). * indicates $P<0.05$, ****indicates $P<0.0001$. Each point represents one animal.

Figure 3. HCQ decreases muscle fibrosis following IR injury. *A)* Immunofluorescent staining showing NETs (examples indicated by white arrowheads) in the TA at 3 DPI in C57BL/6 mice treated with HCQ or vehicle. Quantification shows HCQ treatment modestly decreased NET formation. *B)* Representative histological sections of picrosirius red staining of TA at 7 DPI of C57BL/6 mice uninjured and treated with HCQ or vehicle. ImageJ quantification of positive picrosirius red staining in the same groups shows HCQ treatment decreased collagen deposition. *C)* Representative images of C57BL/6 mice uninjured TA and treated with HCQ or vehicle stained with eMHC and laminin at 5 DPI. Histogram of frequencies of distribution of myofiber CSA and Feret diameter, indicating muscles with vehicle treatment have significantly increased frequency of small CSA and feret diameter. *D)* Representative images of H&E stained TA 7 DPI in uninjured and treated with HCQ or vehicle. Quantification of percentage of total myofibers with centralized or peripheral nuclei in each group. All scale bars indicate 100 μm. + indicates difference compared to uninjured group. ** indicates $P<0.01$.; ++ indicates $P<0.005$; **** and ++++ indicate $P<0.0001$. ns, not significant. Frequency distributions analyzed with Kolmogorov-Smirnov test. Bars represent means ± standard deviation. Each point represents one animal.

Figure 4. HCQ treatment decreases FAP proliferation. *A)* Immunofluorescent staining of FAPs co-stained with Ki67 (examples indicated by white arrowheads) in the TA at 5 DPI in C57BL/6 mice uninjured and treated with HCQ or vehicle. *B)* Quantification of number of FAPs expressing Ki67. HCQ treatment significantly reduces the number of FAPs expressing Ki67. Correlation curves of total PDGFRα+ and Ki67+ area in *C)* uninjured, *D)* HCQ treated, *E)* vehicle treated TA muscles. All scale bars indicate 100 μm *indicates $P<0.05$; ****indicates $P<0.0001$. Each point represents one ROI.

Figure 5. HCQ treatment decreases ERK1/2 phosphorylation in IR injury model. *A)* Single-cell RNAseq expression of *Erk1* in all cells of uninjured TA. *B)* Expression of *Erk1* in FAP population of uninjured TA. *C)* Comparison of *Erk1* expression in FAP population of uninjured and IR+ctx FAP populations. *D)* Single-cell RNAseq expression of *Erk1* in all cells of IR+ctx injured TA without treatment at 3 DPI. *E)* Expression of *Erk1*

in FAP population of IR+ctx injured TA without treatment at 3 DPI. *F)* Single-cell RNAseq expression of *Erk2* in all cells of uninjured TA. *G)* Expression of *Erk2* in FAP population of uninjured TA. *H)* Comparison of *Erk2* expression in FAP population of uninjured and IR+ctx FAP populations. *I)* Single-cell RNAseq expression of *Erk2* in all cells of IR+ctx injured TA without treatment at 3 DPI. *J)* Expression of *Erk2* in FAP population of IR+ctx injured TA without treatment at 3 DPI. Gene expression of *K) Erk1* and *L) Erk2* in the TA at 7 days post IR injury in mice treated with vehicle or HCQ. HCQ treatment does not significantly change *Erk2* or *Erk1* gene expression. Bars indicate fold change in gene expression relative to vehicle treatment. *M)* Western blot indicating levels of ERK1/2, phosphorylated ERK1/2 (pERK1/2), and alpha tubulin at 7 DPI in TA treated with vehicle or HCQ. *N)* Corresponding quantification of pERK1/2 relative to ERK1/2 shows significant decrease in ERK1/2 phosphorylation after HCQ treatment. * indicates $P<0.05$. Bars represent means ± standard deviation. Each point in *K) –N)* represents one animal.

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Author Contributions

N. Edwards, B. Levi designed research; N. Edwards, C. Hwang, P. Spreadborough, C. Pagani, S. Marini, C. Rowe, P. Yu, A. Mei, N. Visser performed experiments; N. Edwards, S. Marini, C. Hwang analyzed data; A. Huber, A. Strong, G. Hespe, S. Li, J. Knight, M. Shelef, T. Davis, B. Levi provided intellectual contributions and critical review; N. Edwards and B. Levi wrote the paper with input and final approval from all authors.

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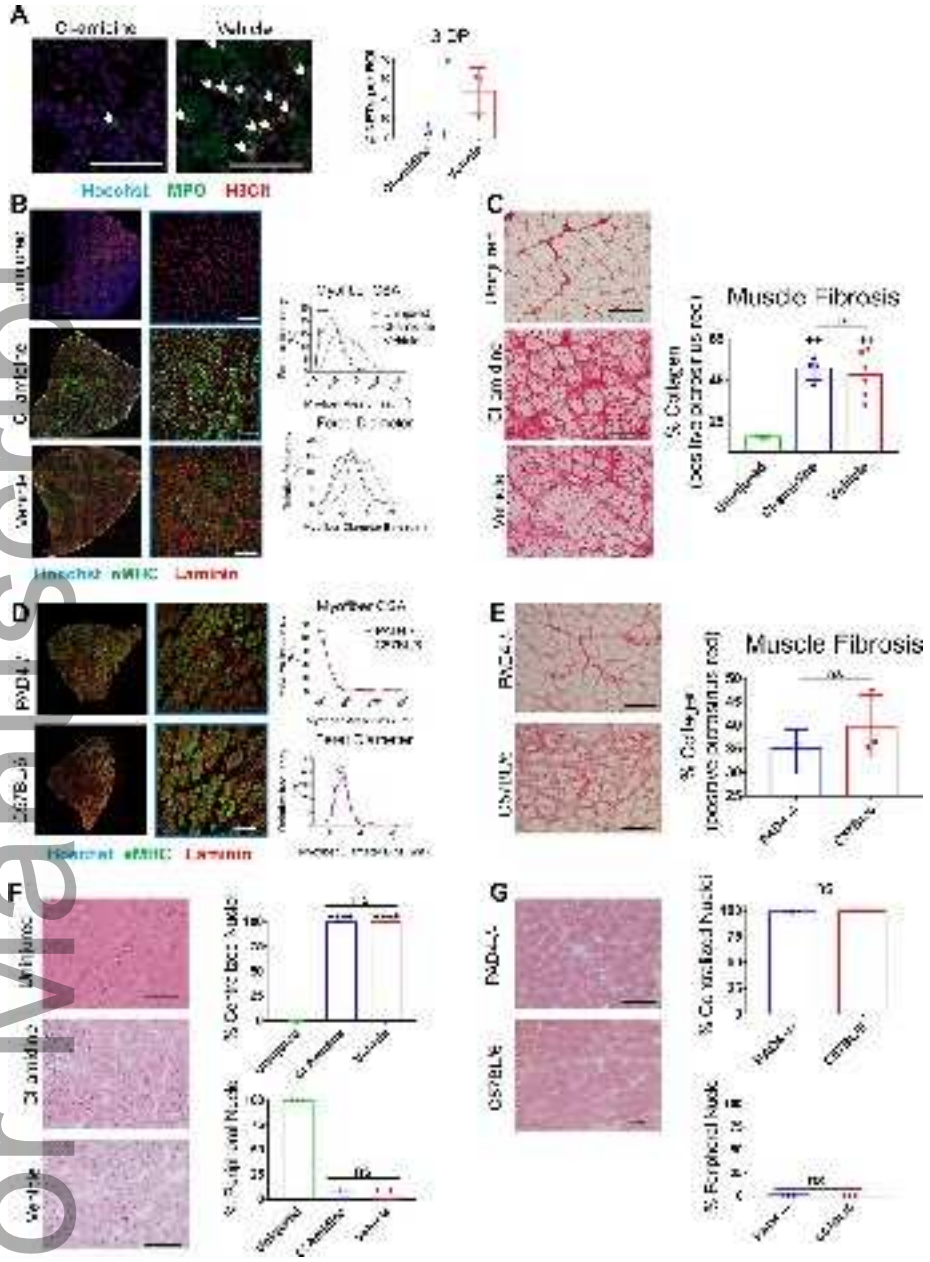

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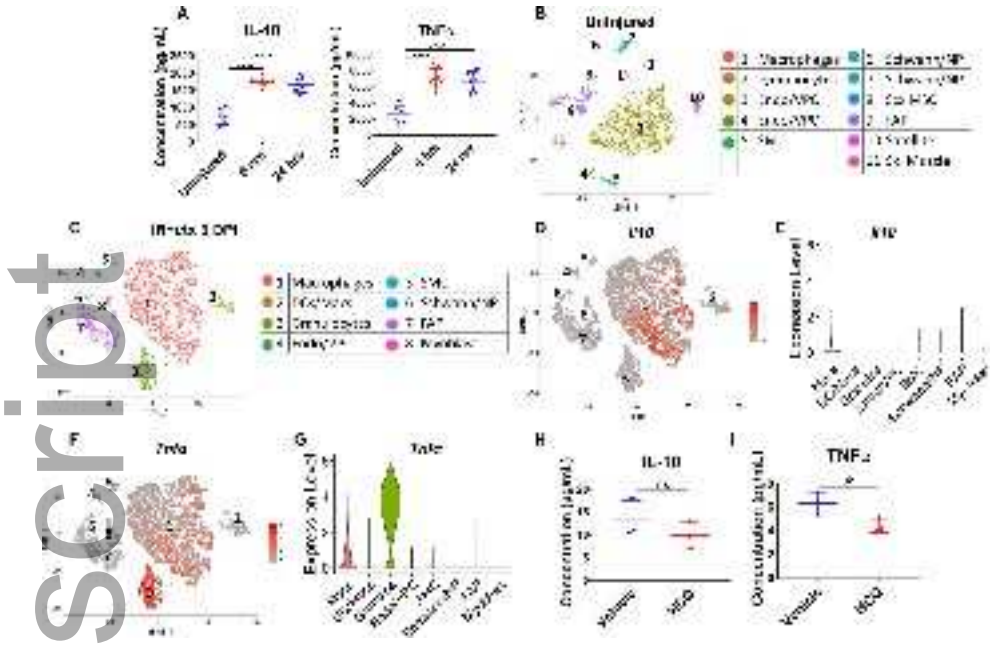

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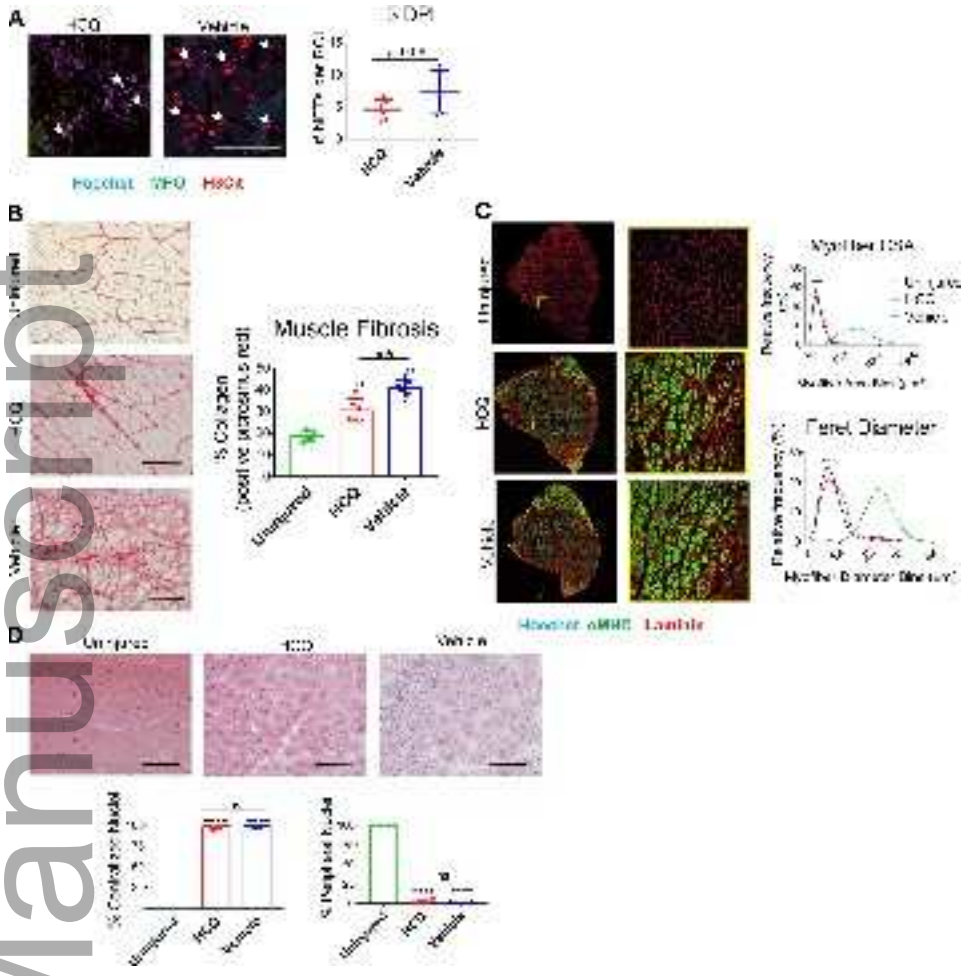

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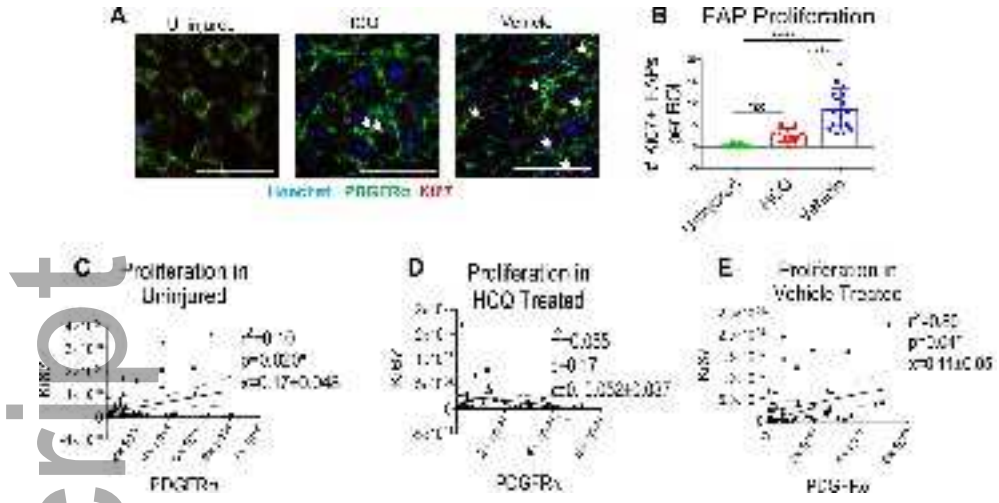

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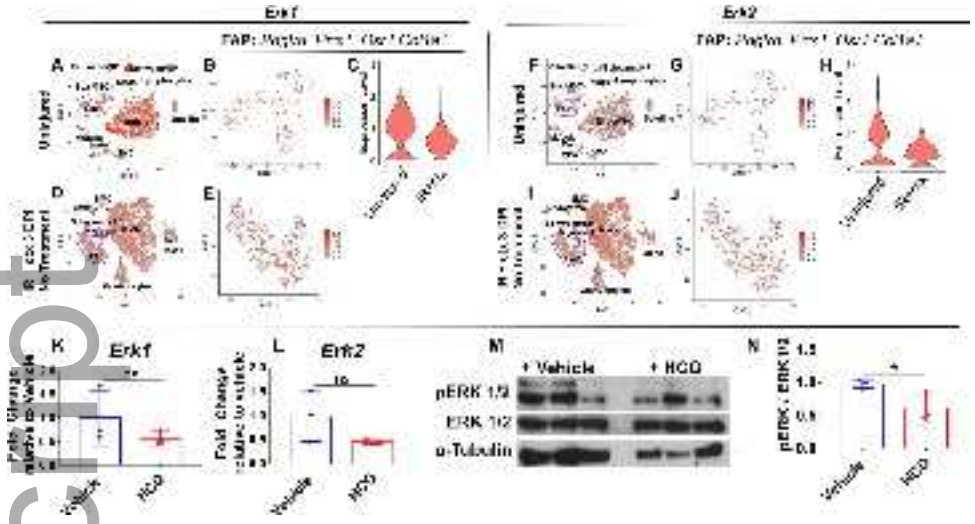

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