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# Interleukin-1 Is Overexpressed in Injured Muscles Following Spinal Cord Injury and Promotes Neurogenic Heterotopic Ossification

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## ABSTRACT

Neurogenic heterotopic ossifications (NHOs) form in periarticular muscles after severe spinal cord (SCI) and traumatic brain injuries. The pathogenesis of NHO is poorly understood with no effective preventive treatment. The only curative treatment remains surgical resection of pathological NHOs. In a mouse model of SCI-induced NHO that involves a transection of the spinal cord combined with a muscle injury, a differential gene expression analysis revealed that genes involved in inflammation such as interleukin-1 $\beta$  (IL-1 $\beta$ ) were overexpressed in muscles developing NHO. Using mice knocked-out for the gene encoding IL-1 receptor (IL1R1) and neutralizing antibodies for IL-1 $\alpha$  and IL-1 $\beta$ , we show that IL-1 signaling contributes to NHO development after SCI in mice. Interestingly, other proteins involved in inflammation that were also overexpressed in muscles developing NHO, such as colony-stimulating factor-1, tumor necrosis factor, or C-C chemokine ligand-2, did not promote NHO development. Finally, using NHO biopsies from SCI and TBI patients, we show that IL-1 $\beta$  is expressed by CD68<sup>+</sup> macrophages. IL-1 $\alpha$  and IL-1 $\beta$  produced by activated human monocytes promote calcium mineralization and RUNX2 expression in fibro-adipogenic progenitors isolated from muscles surrounding NHOs. Altogether, these data suggest that interleukin-1 promotes NHO development in both humans and mice. © 2021 American Society for Bone and Mineral Research (ASBMR).

**KEY WORDS:** OSTEOIMMUNOLOGY; CYTOKINES; DISEASES AND DISORDERS OF/RELATED TO BONE (OTHER)

## Introduction

Neurogenic heterotopic ossifications (NHOs) are pathological bone formations in soft tissues after central nervous system (CNS) injuries such as traumatic brain injuries (TBI), spinal cord injuries (SCI), strokes, and cerebral anoxia. About 10% to

23% patients with TBI and 10% to 53% with SCI develop NHO, and the prevalence increases to 68% in victims suffering from severe combat blast injuries.<sup>(1-3)</sup> NHOs develop in periarticular muscles and can lead to partial or complete ankylosis of affected joints, compression of blood vessels and nerves and, hence, consequently compromises life quality of patients.<sup>(4)</sup> Because our

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Additional Supporting Information may be found in the online version of this article.

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understanding of NHO pathogenesis is limited, the only effective curative treatment for NHO is surgical excision; however, even after surgical resection, NHO reoccur in 6% to 7% of patients.<sup>(5)</sup> Preventive treatments such as radiation therapy and bisphosphonates have been proposed but may negatively impact other complications associated with SCI, such as wound and bone fracture healing.<sup>(6)</sup> The only preventive treatments that show some efficacy at reducing the incidence and size of NHO in retrospective studies are non-steroid anti-inflammatory drugs (NSAIDs) such as indomethacin and celecoxib.<sup>(7)</sup>

To better understand the cellular and molecular mechanisms driving NHO and discover potential therapeutic targets, we established the first SCI-induced NHO mouse model in which NHO development requires a combination of SCI and muscle injury without the artifice of genetic manipulation.<sup>(8)</sup> In this mouse model, NHO development is induced by the combination of spinal cord transection between T<sub>11</sub> and T<sub>13</sub> combined with a muscle injury induced by injection of cardiotoxin.<sup>(8)</sup> In this model, NHOs are featured by mineralized calcium deposition detected by micro-computed tomography ( $\mu$ CT) from day 4 post-SCI and muscle injury. NHO volumes peak around day 7 to 14. As NHOs gradually mature, osterix-positive osteoblasts lining the NHOs lay down collagen I and osteocalcin-containing bone matrix and form small heterotopic bone nodules that are remodeled by multinucleated osteoclasts.<sup>(8)</sup>

There are striking differences between normal muscle repair and NHO development. In the absence of SCI, muscle repair is completed within 3 weeks by satellite cells, which are myogenic stem cells that differentiate into myoblasts and fuse into myocytes to replace damaged myofibers. Effective muscle repair begins with acute inflammation in response to injury. CC chemokine ligand 2 (CCL2), also called macrophage chemoattractant protein 1 (MCP-1), and its receptor CC chemokine receptor 2 (CCR2), known to mediate monocyte recruitment in various inflammatory conditions, recruit inflammatory monocytes to the site of muscle injury.<sup>(9-12)</sup> Inhibition of macrophage infiltration by depleting intramuscular CD11b<sup>+</sup> myeloid cells or deletion of CCL2 or CCR2 encoding genes significantly reduces infiltration of monocyte/macrophages and subsequently delays clearance of necrotic muscle and muscle regeneration.<sup>(9,10,12,13)</sup> Recruited monocytes and muscle-resident macrophages produce cytokines that enhance myogenesis such as interleukin-6 (IL6), insulin-like growth factor 1 (IGF1), and interleukin-1 $\beta$  (IL1- $\beta$ ).<sup>(9,14)</sup> Another muscle-resident progenitor population, called fibro-adipogenic progenitors (FAPs), expands rapidly after muscle injury and provides a supportive environment for satellite cell expansion and myogenic differentiation.<sup>(15)</sup> However, unlike satellite cells, dividing FAPs undergo apoptosis in a tumor necrosis factor (TNF)-dependent manner between 4 and 7 days after injury and are eliminated by macrophages to prevent scar tissue formation and fibrosis.<sup>(16)</sup> Therefore, muscle resident and infiltrating macrophages are pivotal to orchestrate several steps of muscle regeneration.

Interestingly, we and others have demonstrated that macrophages are also essential in HO formation in various mouse models.<sup>(8,17,18)</sup> In our SCI-NHO model, we discovered abnormally increased inflammatory monocyte/macrophage infiltration in injured muscle 4 days after SCI and muscle injury.<sup>(19)</sup> Depletion of macrophages significantly reduced the size of NHO, establishing the critical roles of macrophages in NHO.<sup>(8)</sup> We showed that in the context of a SCI, macrophages infiltrating the injured muscle secrete abnormally high levels of oncostatin M (OSM), which promotes NHO development.<sup>(20)</sup> OSM exerts its effect in part by

binding to a specific cell surface receptor made of GP130 complexed with the OSM receptor  $\alpha$  chain (OSMR), which signals via JAK1 and JAK2 tyrosine kinases, which in turn tyrosine phosphorylate and activate the transcription factor STAT3 by promoting its translocation from the cytoplasm to the nucleus. JAK-STAT3 signaling is persistently activated at abnormally high levels in muscles developing NHO after SCI.<sup>(19)</sup> Deletion of the *Osmr* gene or blocking OSM receptor downstream signaling with the selective JAK1-2 tyrosine kinase inhibitor ruxolitinib significantly reduces NHO formation,<sup>(19,21)</sup> demonstrating the important role of OSM/OSMR/JAK1-2 signaling in NHO development.

Because numerous macrophage-mediated inflammatory responses can promote muscle repair or fibrosis or ossification, our working hypothesis was that CNS injury skews these inflammatory responses toward fibrosis and ossification rather than muscle repair.<sup>(22)</sup> To better understand which inflammatory pathways are activated after a SCI and how these promote NHO instead of muscle repair, we undertook gene expression profiling of muscles in the early phase after muscle injury. Gene set enrichment analysis (GSEA) showed that inflammatory responses, IL6-JAK-STAT3 and TNF-NF $\kappa$ B signaling gene sets, were significantly enriched in muscles developing NHO with enhanced expression of OSM, colony-stimulating factor (CSF)-1, TNF, CCL2, and IL-1 $\beta$ . Functional studies with selective inhibitors, neutralizing antibodies, and knockout mice revealed that among these pro-inflammatory cytokines, only OSM and IL-1 signaling positively contributed to NHO development after SCI. Finally, expression of IL-1 $\beta$  was confirmed in the muscular tissue surrounding NHO from patients with SCI or TBI, and in vitro osteogenic assays of human FAPs isolated from muscles surrounding NHOs confirmed that both IL-1 $\alpha$  and IL-1 $\beta$  produced by activated human monocytes promote calcium mineralization and *RUNX2* expression.

## Materials and Methods

### Animals

All experimental procedures were approved by the Health Sciences Animal Ethics Committee of The University of Queensland (# MRI-UQ/TRI/050/17) and followed the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. C57BL/6 mice were purchased from the Australia Resources Centre. B6.129S-*Tnfrsf1a*<sup>tm1mx</sup> *Tnfrsf1b*<sup>tm1mx</sup>/J (abbreviated as *Tnfrsf1a/1b*<sup>-/-</sup>), B6.129S4-*Ccr2*<sup>tm1lc</sup>/J (abbreviated as *Ccr2*<sup>-/-</sup>), B6.129S7-*Il1r1*<sup>tm1j</sup> (*Il1r1*<sup>-/-</sup>) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). *Il1a*<sup>tm1Yim</sup> (*Il1a*<sup>-/-</sup>) and *Il1b*<sup>tm1Yim</sup> (*Il1b*<sup>-/-</sup>) were kindly provided by Prof Yoichiro Iwakura<sup>(23)</sup> and backcrossed into C57BL/6 background over 10 times. Experimental mice were maintained in a specific pathogen-free animal facility 12-hour light/dark cycle with free access to standard chow pellets and water. Animals were randomly allocated to treatment groups and different genotypes were co-housed in the same cages. Mice were identified with ear notches.

### NHO mouse model

For microarray analysis, 6-week-old female C57BL/6 mice underwent either spinal cord transection injury (SCI) at level T<sub>11</sub> to T<sub>13</sub> or sham surgeries followed by intramuscular (i.m.) injection of cardiotoxin (CDTX) purified from the venom of *Naja mosseimbica* snake (Sigma-Aldrich, St. Louis, MO, USA) at 0.32 mg/kg or saline

into the hamstring muscle as previously described.<sup>(8)</sup> Combinations of surgery and i.m. injections in each group (4 mice per group) were (i) SCI + CDTX, (ii) SCI + saline, (iii) Sham+CDTX, and (iv) Sham+saline. Two days post-injury, mice were euthanized by cervical dislocation and hamstring muscles were immediately harvested to extract total RNA for gene expression microarray analyses.

Samples for quantitative real-time reversed transcribed polymerase chain reaction (qRT-PCR) validation were collected from another cohort of mice 2 and 4 days post-injuries following identical surgical procedures with the following minor modifications<sup>(19)</sup>: (i) purified CDTX from *Naja pallida* venom was from Latoxan (Portes-lès-Valence, France; catalog #L8102) due to discontinuation of *N. mossembica* CDTX production by Sigma-Aldrich, and (ii) saline was replaced by PBS, which does not cause muscle injury. All muscle samples were snap-frozen and stored at  $-80^{\circ}\text{C}$  until RNA extraction.

For in vivo analysis of gene deletion and drug effects on NHO formation, mice underwent SCI surgery with an injection of CDTX from *Naja pallida* as described above.

### RNA extraction

Whole muscle samples were homogenized in 4 mL of Trizol (Invitrogen, Carlsbad, CA, USA) with TissueRuptorII (Qiagen, Hilden, Germany) or IKA T10 basic homogenizer on ice for 30 seconds for 3 times with 10-second intervals followed by incubation at room temperature (RT) for 30 minutes. Tissue debris were removed by centrifugation at 12,000g and supernatants were mixed with 1/5 volume of chloroform. After centrifugation at 12,000g,  $4^{\circ}\text{C}$  for 15 minutes, aqueous phases containing RNA were transferred to fresh tubes for RNA precipitation with isopropanol. The RNA pellets were further washed in 75% ethanol, air-dried, and dissolved in RNase/DNase free water.

### Gene expression microarray analysis

RNA samples for microarray analysis were cleaned up using the RNeasy MinElute Cleanup Kit (Qiagen) and the quantity and quality was verified using a Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). Total RNA samples were amplified and purified using the Ambion Illumina RNA amplification kit (Ambion, Austin, TX, USA) to yield biotinylated cRNA following the Illumina protocol, and further hybridized to MouseRef-8 v. 2.0 Gene Expression BeadChips and read on an IScan System using the IScan control software version 3.5.31 (Illumina). Microarray data were analyzed by Illumina Genomestudio software (v. 2011.1), Gene Expression module (v. 1.9.0) using normalization algorithms with background subtraction followed by Illumina custom differential analysis algorithms. Normalized data are accessible on NCBI database with the assigned GEO accession number GSE165062. Genes with signal intensity higher than background (detection  $p$  value  $<0.01$ ) in at least one sample were included for further analyses. Principal component analysis (PCA) was performed using R studio (v. 1.1.456) prcomp function, while a heat map was generated using complexHeatmap packages.<sup>(24)</sup>

### Gene set enrichment analysis (GSEA)

Gene set enrichment analysis (GSEA software v. 4.0.3) was performed using microarray signal intensity.<sup>(25,26)</sup> In the present study, we selected the Hallmark gene set in the MSigDB molecular signature database v. 7.0. The Hallmark gene sets were

generated by a computational methodology based on identifying overlaps between gene sets in other MSigDB collections including 50 well-defined biological processes that summarize and represent specific well-defined biological states or processes and display coherent expression.<sup>(27)</sup>

### qRT-PCR validation

For qRT-PCR validation, cDNA were synthesized from RNA samples using iScript cDNA kit (Bio-Rad, Hercules, CA, USA) per manufacturer's instructions. mRNA expression was quantified using single-step qRT-PCR Taqman system. Reactions were prepared following instruction of TaqMan Universal PCR Master Mix using mouse *Il1b*, *Tnf*, *Osm*, *Ccl2*, *Csf1*, *Il1a*, and ribosomal protein S20 (*Rps20*) (Supplemental Table S1). Ct values were normalized by the expression of the housekeeping gene *Rps20*. The amplifications were performed using Applied Biosystems Viiia7 real-time PCR system.

### Micro-computed tomography ( $\mu\text{CT}$ ) analyses

NHO volumes were measured in live mice in the Inveon PET-CT multimodality system (Siemens Medical Solutions Inc., Malvern, PA, USA) with the following parameters:  $360^{\circ}$  rotation, 180 projections, 80 kV voltage, 500  $\mu\text{A}$  current, and effective pixel size 36  $\mu\text{m}$ . Mice were anesthetized with 2% isoflurane oxygen mixture during imaging. After 3D image reconstruction, NHO volume was quantified in the Inveon Research Workplace (Siemens Medical Solutions Inc.) as previously described.<sup>(19)</sup> To calculate NHO volumes, the region of interest (ROI) was drawn around the injured muscle containing calcification (excluding any normal skeletons). The quantification was not performed with any blinding, but after defining the ROI, the calcified regions were defined by setting the threshold Hounsfield units (HU) to 450 HU; therefore, only radiodensity  $>450$  HU was quantified as NHO. NHO development in *Ccr2*<sup>-/-</sup> mice was repeated in three separate experiments. Due to instrument upgrade between experiments, the NHO formation in the first two experiments (wild type [WT]  $n = 10$ ; *Ccr2*<sup>-/-</sup>  $n = 10$ ) were measured in  $\mu\text{CT}$  scanner ( $\mu\text{CT}40$ , SCANCO Medical AG, Brüttisellen, Switzerland) at a resolution of 30  $\mu\text{m}$  and three-dimensional (3D) images of the lower parts of the mouse bodies were reconstructed from the scans using the  $\mu\text{CT}$  system software package. Quantitative assessments of bone volumes in the muscular mass by a subtraction technique of orthotopic mouse skeleton (hip, femur, tibia, fibula) were measured to detect and quantify NHO. The remaining *Ccr2*<sup>-/-</sup> and wild-type mice (WT  $n = 5$ ; *Ccr2*<sup>-/-</sup>  $n = 3$ ) were measured in the Inveon PET-CT scanner. All other mice were analyzed in the Inveon PET-CT scanner.

### In vivo treatment with GW2580 and anti-IL1 $\alpha$ /anti-IL1 $\beta$ neutralizing antibodies

C57BL/6 mice were administered with GW2580 (Biorbyt, Cambridge, UK, catalog# orb61037) at 80 mg/kg in 0.5% hydroxypropyl methylcellulose in water, twice daily oral gavage<sup>(28)</sup> or vehicle from day 0 to day 10 post-surgery. To confirm the effects of GW2580 on myeloid cell development, bone marrow (BM) and blood samples were analyzed by flow cytometry.

C57BL/6 mice were also injected intraperitoneally with 200  $\mu\text{g}$  daily of neutralizing Armenian hamster anti-mouse IL-1 $\alpha$  (Bio X Cell, Lebanon, NH, USA, catalogue #BE0243, clone ALF-16) together with 200  $\mu\text{g}$  neutralizing Armenian hamster anti-mouse IL-1 $\beta$  (clone B122) monoclonal antibodies or 200  $\mu\text{g}$  control

Armenian hamster IgG monoclonal antibody (Bio X Cell, catalogue #BE0091, clone PIP) from day 0 to day 7 post-surgery.

### Phenotypic characterization of mouse leukocytes by flow cytometry

Muscle leukocytes were isolated from hamstring muscles 4 days post-surgery using a skeletal muscle dissociation kit (Miltenyi Biotec, Bergisch Gladbach, catalog #130-098-305) and GentleMACS Dissociator tissue homogenizer (Miltenyi Biotec) as previously described.<sup>(19)</sup> To characterize inflammatory infiltrating injured muscle in C57BL/6 and *Ccr2*<sup>-/-</sup> mice, leukocytes were stained with the following cell surface markers: anti-lineage (B220, Ter119, CD3 $\epsilon$ )-FITC, anti-Ly6C-Pacific blue, anti-F4/80-APC, anti-LY6G-PE-Cy7, CD48-APC-CY7, CD11b-BV510, CD45-BV785, and anti-CCR2-PE (Supplemental Table S2). Stained cells were analyzed with a CytoFlex flow cytometer (Beckman Coulter, Brea, CA, USA) equipped with 405 nm, 488 nm, 561 nm, and 635 nm lasers.

To confirm the effect of GW2580 on myelopoiesis, separate cohorts of mice were injected subcutaneously with either saline control or 1 mg/kg/d long-lived porcine CSF-1-IgG Fc fusion protein that cross-reacts with mouse as previously described.<sup>(29)</sup> These two cohorts were both subdivided into two groups and gavaged with either vehicle control or 80 mg/kg GW2580 twice daily. Mice were treated for 2 consecutive days and femoral bone marrow (BM) harvested the next morning. BM from one femur was flushed with 1 mL PBS containing 2% newborn calf serum (NCS) in a 1 mL syringe mounted with a 25G needle and washed in PBS/2% NCS before staining. BM cells were subsequently stained with CD11b-BV605, anti-F4/80-AF647, anti-Ly6G-PE-CY7, CD115-PE, anti-Ly6C-Pacific Blue, and CD48-PerCP-CY5.5 monoclonal antibodies (Supplemental Table S2). Cells were analyzed using a CyAn flow cytometer (Beckman Coulter) equipped with 405 nm, 488 nm, and 635 nm lasers. All FCS data files were exported without compensation and analyzed on FlowJo v. 10.7.1 software following compensation with single color controls.

### Human ethics statement and samples collection

Blood, NHO, and muscle samples were obtained with the informed consent of the patients and under the approval of an independent ethics committee (CPP approval no. 09025, study BANKHO). Blood samples from healthy donors (HD) and NHO patients were collected on citrate solution from Percy Hospital (Clamart, France) and Raymond Poincaré Hospital (Garches, France). NHOs and muscle surrounding NHOs were collected from surgical waste after their excision from patients with brain injuries (TBI) or spinal cord injuries (SCI) at Raymond Poincaré Hospital.

### Human CD68 and IL-1 $\beta$ immunohistochemistry

Human NHO biopsies were fixed in 4% formaldehyde, demineralized in 10% nitric acid, and embedded in paraffin. Sections (4  $\mu$ m) were deparaffined in xylene and rehydrated. For IHC staining, sections were permeabilized 15 minutes at room temperature (RT) in PBS-0.1% Triton X-100 followed by a 15-minute treatment at 95°C in a pH = 6.0 antigen retrieval citrate-based solution (Vector Laboratories, Burlingame, CA, USA), then cooled down for 15 minutes. Sections were then incubated in Bloxall blocking solution (Vector Laboratories) for 10 minutes at RT to inactivate endogenous peroxidase followed by a 30-minute incubation in PBS-goat serum 10% for 30 minutes at RT. Anti-human CD68 (mouse monoclonal IgG3, clone PG-M1, Dako

[Glostrup, Denmark], dilution 1/100) and anti-human IL-1 $\beta$  (rabbit polyclonal antibody, NB600-633, Novus Biologicals [Littleton, CO, USA], dilution 1/200) primary antibodies were diluted in PBS containing 5% goat serum and 1.5% bovine serum albumin (BSA) and incubated overnight at 4°C. Mouse IgG3 isotype (MAB007, R&D Systems, Minneapolis, MN, USA) and rabbit polyclonal isotype (ab37415, Abcam, Cambridge, MA, USA) were used as controls. After washing in PBS-Triton 0.1%, ready-to-use ImPRESS FLEX REAGENT goat anti-rabbit IgG and goat anti-mouse IgG (Vector Laboratories) were incubated for 30 minutes at RT. After washing in PBS-Triton 0.1%, FLEX DAB substrate solution (Vector Laboratories) was added on sections for 5 minutes at RT. Counter coloration was performed using hematoxylin. All sections were analyzed using a Panoramic Midi II slide scanner and Case Viewer software (3D HISTECH Ltd., Budapest, Hungary).

### Human plasma IL-1 $\beta$ quantification by ELISA

Blood samples were centrifuged at 820 g for 10 minutes at 4°C and plasma fractions were stored at -80°C. IL-1 $\beta$  plasma level was evaluated using Human IL-1 $\beta$  ELISA Kit (DLB50, R&D Systems) according to the manufacturer's procedure.

### Isolation of PDGFR $\alpha$ <sup>+</sup>CD56<sup>-</sup> fibro-adipoprogenitors (FAPs) from human muscles in surgically resected NHO

Muscle fragments associated with NHO biopsies were minced using scalpel and small scissors, placed in a 50 mL Falcon tube, and incubated in 1.5 mg/mL pronase (catalog #10165921001, Sigma-Aldrich) in  $\alpha$ -minimal essential medium ( $\alpha$ -MEM), 45 minutes in a 37°C water bath. After addition of  $\alpha$ -MEM supplemented with 15% FBS and 1% P/S antibiotics, the cell suspension was filtered through a 100  $\mu$ m cell strainer followed by a 40  $\mu$ m cell strainer (BD Falcon). Isolated muscle progenitor cells (MPCs) were maintained 10 days in  $\alpha$ -MEM supplemented with 15% FBS, 1% penicillin/streptomycin (P/S) antibiotics, and 10 ng/mL basic fibroblast growth factor (bFGF) (R&D Systems). Human MPCs were trypsinized and incubated 30 minutes with biotinylated anti-human PDGFR $\alpha$  (BAF322, R&D Systems) goat polyclonal antibody and CD56-PE (clone B159, BD Pharmingen, San Diego, CA, USA) monoclonal antibody in PBS 2% fetal bovine serum (FBS), 2 mM EDTA or with control isotypes IgG1 PE (A07796, Beckman Coulter), and biotinylated goat IgG (BAF108, R&D Systems). Cells were washed and incubated 30 minutes with Streptavidin-APC/Cy7 and the viability dye 7-aminoactinomycin D (7-AAD) (Sony). Cells were washed and filtered through a 30  $\mu$ m cell strainer (Sysmex, Kobe, Japan) and sorted using a FACSAria III SORP sorter (BD Biosciences, San Jose, CA, USA). PDGFR $\alpha$ <sup>+</sup>CD56<sup>-</sup> FAPs were seeded at 3000 cells per cm<sup>2</sup> in  $\alpha$ -MEM supplemented with 10% FBS and 1% P/S antibiotics.

### Human CD14<sup>+</sup> monocytes isolation and preparation of conditioned medium (CM)

Human CD14<sup>+</sup> blood monocytes were isolated from whole blood donation buffy coat from the Centre de Transfusion Sanguine des Armées (Clamart, France) or NHO bone marrow. PBMC and NHO-bone marrow mononuclear cells were recovered from using Ficoll gradient (Pancoll, Pan-Biotech, Aidenbach, Germany). CD14<sup>+</sup> monocytes were then isolated after magnetic separation using CD14 microbeads (130-050-201, Miltenyi Biotec). CD14<sup>+</sup> cells were seeded at  $1.25 \times 10^6$  cells per cm<sup>2</sup> and stimulated with 100 ng/mL lipopolysaccharide (LPS) (L6529,

Sigma-Aldrich) or without LPS for 72 hours. Conditioned media with (CM<sup>+</sup>) or without (CM<sup>-</sup>) LPS stimulation were collected and centrifuged 5 minutes at 500g. Pooled CM<sup>+</sup> were used in a 1 to 10 ratio in cell culture medium during osteogenic differentiation assays. Due to a limited number of CD14<sup>+</sup> monocytes retrieved from NHO bone marrows, only CM<sup>+</sup> derived from CD14<sup>+</sup> blood monocytes were used. IL-1 $\beta$  and IL-1 $\alpha$  concentrations in conditioned media were evaluated using Human IL-1 $\beta$  and IL-1 $\alpha$  ELISA assays (DY201 and DY200, R&D Systems) according to the manufacturer's instructions.

### In vitro osteogenic differentiation assay and mineralization quantification

Human PDGFR $\alpha$ <sup>+</sup> CD56<sup>-</sup> FAPs were seeded at 3000 cells per cm<sup>2</sup> in  $\alpha$ -MEM 10% FBS with standard P/S. After 3 days, culture medium was replaced by control medium ( $\alpha$ -MEM 10% FBS 1% P/S antibiotics) or osteogenic medium ( $\alpha$ -MEM 10% FBS 1% P/S antibiotics, 0.052 mg/mL dexamethasone, 12.8  $\mu$ g/mL ascorbic acid, and 2.15 mg/mL  $\beta$ -glycerophosphate (D-1159, A-8960, G-9422, Sigma-Aldrich) for 13 days. Human recombinant IL-1 $\alpha$ , IL-1 $\beta$ , and Interleukin 1 receptor antagonist (IL-1RA) were purchased from PeproTech (Rocky Hill, NJ, USA; 200-01A, 200-01B, and 200-01RA). FAPs were incubated 2 hours with IL-1RA before conditioned medium (CM) addition. Neutralizing mouse anti-human IL-1 $\alpha$  and human IL-1 $\beta$  monoclonal IgG1 antibodies and mouse IgG1 isotype control (mabg-hil1a, mabg-hil1b, mabg1-ctrlm) were purchased from InvivoGen and used at the indicated concentration with a 2-hour pre-incubation in CM<sup>+</sup> before addition to FAPs. At the end of the differentiation process, cells were washed in PBS, fixed in 70% ethanol, washed in water, and stained in 20 g/L Alizarin red S (A5533, Sigma-Aldrich). Excess stain was removed during three washing steps. Alizarin red S dye was extracted with 0.5 N hydrochloric acid and 5% sodium dodecyl sulfate (SDS) and quantified by spectrophotometry at 405 nm.

### Western blot

Cultured human PDGFR $\alpha$ <sup>+</sup>CD56<sup>-</sup> FAPs were washed in PBS before incubation in cell lysis buffer composed of PBS, 1% NP-40, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 0.5% deoxycholic acid, and protease inhibitors (11697498001, Roche, Mannheim, Germany). Cells were then scrapped, incubated 20 minutes on ice, and supernatant was collected after centrifugation (13,000g, 15 minutes at 4°C). Protein concentrations were quantified using Pierce BCA protein assay kit following the manufacturer's instructions (23252, Thermo Fisher Scientific, Waltham, MA, USA). Twenty micrograms of protein were loaded for separation in 10% acrylamide SDS-PAGE gels (161-0182, Bio-Rad) before transfer on PVDF membrane (Trans-Blot Turbo, Bio-Rad). After saturation in 5% non-fat dry milk, membranes were incubated with specific RUNX2 antibody (dilution 1/1000, ab192256, Abcam) overnight at 4°C. Then, they were probed with HRP secondary antibody (dilution 1/200,000, ab205718, Abcam) 2 hours at RT. After enhanced chemoluminescence incubation (170-5061, Bio-Rad), imaging and densitometric analysis were performed on a ChemiDoc XRS+ (Bio-Rad) using ImageLab for StainFree normalization.

### Statistical analyses

Statistical analyses were performed using non-parametric two-sided Mann-Whitney test for two comparisons or repeated

measures Friedman test with Dunn's correction or ANOVA with Tukey's post hoc test for multiple comparisons depending on normality of data distribution as specified in figures.

## Results

### SCI exacerbates inflammation in injured muscles as determined by expression microarray analyses

We first performed differential gene expression microarray analyses in mouse muscles 2 days after SCI and muscle injury to identify potential triggers of NHO development in response to SCI. To this end, a gene expression microarray analysis was performed using total RNA according to the following four treatment groups: (i) SCI + CDTX: SCI surgery plus intramuscular (i.m.) CDTX injection, the only condition in which NHOs form in the injured muscle; (ii) SCI + Saline: SCI surgery plus i.m. injection of saline; (iii) Sham + CDTX: Sham surgery plus i.m. injection of CDTX; or (iv) Sham + Saline: Sham surgery plus i.m. injection of saline. Normalized data are accessible on NCBI database with the assigned GEO accession number GSE165062.

We noticed that one SCI + CDTX sample showed similar gene expression signatures to that of the Sham + CDTX group (not shown). Incomplete spinal cord transection can occur during surgery; however, it takes more than 2 days to observe motor recovery. Therefore, we were unable to confirm if the spinal cord transection injury was complete or not in this particular mouse by day 2. As a result, this sample was excluded from further analysis.

Principal component analysis (PCA) of the remaining samples demonstrated that the dominant principal component 1 (PC1) segregated samples according to CDTX-mediated muscle injury, while PC2 segregated samples according to SCI (Fig. 1A). As a result, all muscle samples specifically clustered according to treatment groups.

Expression heat map confirmed that the combination of SCI plus CDTX muscle injury regulates unique sets of genes in the injured muscle that can be distinguished from genes activated in response to the SCI or CDTX injury alone (Fig. 1B).

GSEA were subsequently performed to identify which pathways and genes may be important in triggering NHO development by comparing differentially expressed gene sets between pairs of treatment groups using MSigDB Hallmark gene set collection.<sup>(25-27)</sup> We performed the following comparisons:

Comparison 1: SCI + CDTX group versus the other three experimental groups (other) (Fig. 2A).

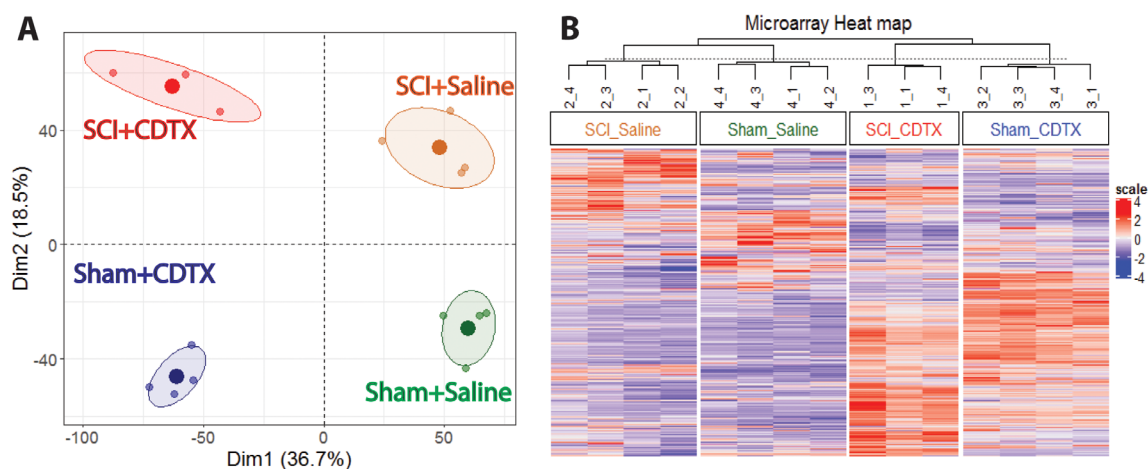
Comparison 2: SCI + CDTX group versus Sham + CDTX group (Fig. 2B).

Comparison 3: SCI + saline group versus Sham + Saline group (Fig. 2C).

Comparison 4: SCI + CDTX group versus SCI + Saline group (Fig. 2D).

Comparison 5: Sham + CDTX group versus Sham + Saline group (Fig. 2E).

Analysis of normalized enrichment scores (NES) with significant false discovery rates (FDR  $q < 0.05$ ) revealed that seven of the eight top gene sets enriched in the SCI + CDTX group developing NHO relative to the three other treatment groups that do not develop NHO (Fig. 2A) were gene sets related to inflammation such as "IL6-JAK-STAT3", "allograft rejection", "TNFA signaling via NFKB", "inflammatory response", "complement", "coagulation" and "interferon gamma response" further demonstrating that SCI exacerbates the inflammatory responses in injured muscles as early as day 2 post-injury. Conversely, the three gene sets that were most



**Fig 1.** Spinal cord injury (SCI) causes expression of different sets of genes in injured skeletal muscles. RNA was isolated from injured hamstring muscles 2 days post-injury from four experimental groups: (i) SCI + CDTX (red): SCI plus intramuscular (i.m.) CDTX injection, the only condition in which NHOs form in the injured muscle; (ii) SCI + saline (orange): SCI surgery plus i.m. injection of saline; (iii) Sham + CDTX (blue): Sham surgery plus i.m. injection of CDTX; or (iv) Sham + saline (green): Sham surgery plus i.m. saline injection of saline. (A) Principal component analysis (PCA) analysis. Each dot represents an individual mouse. (B) Clustering analysis with dendrogram and heat map of differentially expressed genes in the four groups.

significantly reduced in the SCI + CDTX group were “oxidative phosphorylation”, “myogenesis” and “fatty acid metabolism” suggesting that muscle repair and muscle energy usage are reduced subsequent to a SCI. Enriched gene sets in both comparisons 2 and 3 were more likely to be the consequence of the SCI. Enriched gene sets in both comparisons 4 and 5 revealed gene sets enriched as a consequence of the CDTX-induced muscle injury. Remarkably, “IL6-JAK-STAT3 signaling” and “TNFA signaling via NF- $\kappa$ B” Hallmark gene sets were enriched in all possible pairwise comparisons, including in the comparison SCI + CDTX versus all other three groups (Fig. 2, black arrows). The broader “inflammatory response” gene set was enriched in all comparisons that included at least one group with CDTX muscular injury. Heat maps of these three gene sets also showed distinct expression patterns between SCI + CDTX and the other three groups (Fig. 3). For initial validation, we confirmed that the *Osm* gene (encoding oncostatin M) in the “inflammatory response” gene set (which we have previously shown to be overexpressed in muscles after SCI + CDTX injury and playing a major role in SCI-NHO development<sup>(20)</sup>) was overexpressed in the SCI + CDTX group in the microarray analysis. We next selected candidate genes that were found upregulated in the SCI + CDTX group and known to play important roles in macrophage-mediated inflammatory responses such as *Csf1*, *Tnf*, *Il1b*, and *Ccl2*. Comparison 2 between the SCI + CDTX group and Sham + CDTX group confirmed that “TNF/NF- $\kappa$ B signaling” and “IL6/JAK/STAT3” gene sets were significantly enriched in the SCI + CDTX group. The “inflammatory response” gene set was also enriched ( $p = 0.043$ ), but the false discovery rate was 0.2 (Supplemental Fig. S1). Nevertheless, *Csf1*, *Tnf*, *Il1b*, *Ccl2*, and *Osm* mRNA were still among the leading-edge subsets (Supplemental Fig. S1C).

Next, we validated these gene targets in another cohort of mice by quantitative real-time RT-PCR (qRT-PCR) (Fig. 4). CDTX injection increased the expression of *Csf1*, *Tnf*, *IL1b*, and *Ccl2* 2 days post-procedures regardless of SCI, whereas *Il1b* expression was significantly higher in SCI + CDTX compared with

Sham + CDTX similar to *Osm*.<sup>(20)</sup> To further test the functional roles of CSF-1, TNF, CCL2, and IL-1 $\beta$  signaling pathways in SCI-NHO development in vivo, the four signaling pathways were blocked by pharmacological inhibitors, neutralizing antibodies, or using gene knockout mouse models.

### CSF-1 receptor, TNF, and CCR2 signaling do not promote NHO development

We first inhibited CSF-1 receptor signaling by administering GW2580, an orally bioavailable small inhibitor of CSF-1 receptor (CSF1R) tyrosine kinase.<sup>(28,30)</sup> We first assessed the effect of GW2580 at inhibiting medullary monopoiesis stimulation induced by administration of a long-lived recombinant pig CSF-1-IgG Fc (pCSF1-Fc) fusion protein in vivo<sup>(29)</sup> (Supplemental Fig. S2). Flow cytometry analyses of BM cells showed that GW2580 abrogated increases in the numbers of CD11b<sup>+</sup> F480<sup>+</sup> Ly6G<sup>-</sup> monocytes/macrophages in response to pCSF-1-Fc treatment, and within this population, GW2580 was particularly effective at reducing the numbers of Ly6C<sup>bright</sup> CSF1R<sup>+</sup> and Ly6C<sup>+</sup> CSF1R<sup>low</sup> monocytes, whereas these treatments had no effect on the number of CD11b<sup>+</sup> Ly6G<sup>+</sup> granulocytes or CSF1R<sup>-</sup> monocytes. Next, mice were administered daily GW2580 or vehicle from the day of SCI surgery and i.m. injection of CDTX (day 0) until day 10 and NHO volume were measured on day 10 by  $\mu$ CT ex vivo. Unexpectedly, GW2580 treatment significantly increased NHO volumes 1.6-fold after SCI (Fig. 5A). Thus CSF-1 is not a cytokine that promotes NHO development in the inflamed muscle after SCI.

Next, to disrupt the TNF signaling pathway, we used mice with germinal deletion of the two genes encoding the two TNF receptors (*Tnfrsf1a/1b*<sup>-/-</sup> mice). Unexpectedly, NHO volume were twofold higher in *Tnfrsf1a/1b*<sup>-/-</sup> double KO mice compared with C57BL/6 wild-type mice (Fig. 5B). Therefore, similar to CSF-1, TNF signaling does not trigger NHO development after SCI but rather dampens it.

We have previously reported that infiltration of Ly6C<sup>hi</sup> inflammatory monocyte/macrophages in the CDTX-injured muscles is

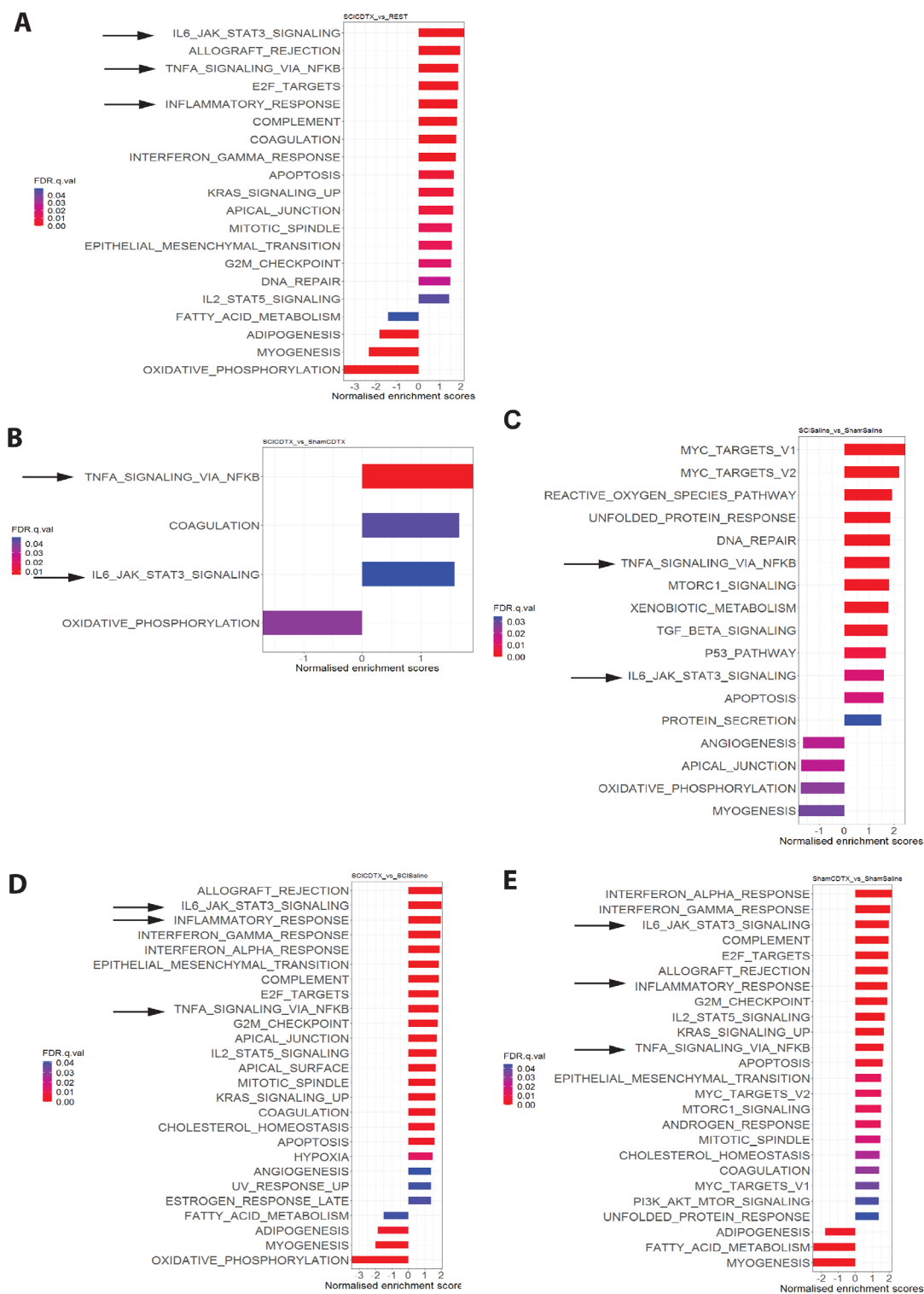


Figure 2

**Fig 2.** Gene set enrichment analyses of muscles from mice with spinal cord injury (SCI) and/or muscle injury. Gene set enrichment analysis was performed to investigate genes enriched in injured muscles from the following experimental groups: (A) SCI + CDTX group, which is the only developing NHO versus the other three experimental groups; (B) SCI + CDTX group versus Sham + CDTX group; (C) SCI + saline group versus Sham + saline group; (D) SCI + CDTX group versus SCI + saline group; (E) Sham + CDTX group versus sham + saline group. Gene sets with false discovery rate (FDR) below 0.05 are shown in the figures. Normalized enrichment scores (NES) were presented in x-axes and FDR are represented on a color scale from high (blue) to low (red). Black arrows highlight the “inflammatory response,” “IL6-JAK-STAT3 signaling,” and “TNF signaling via NFκB” gene sets across the different comparisons.



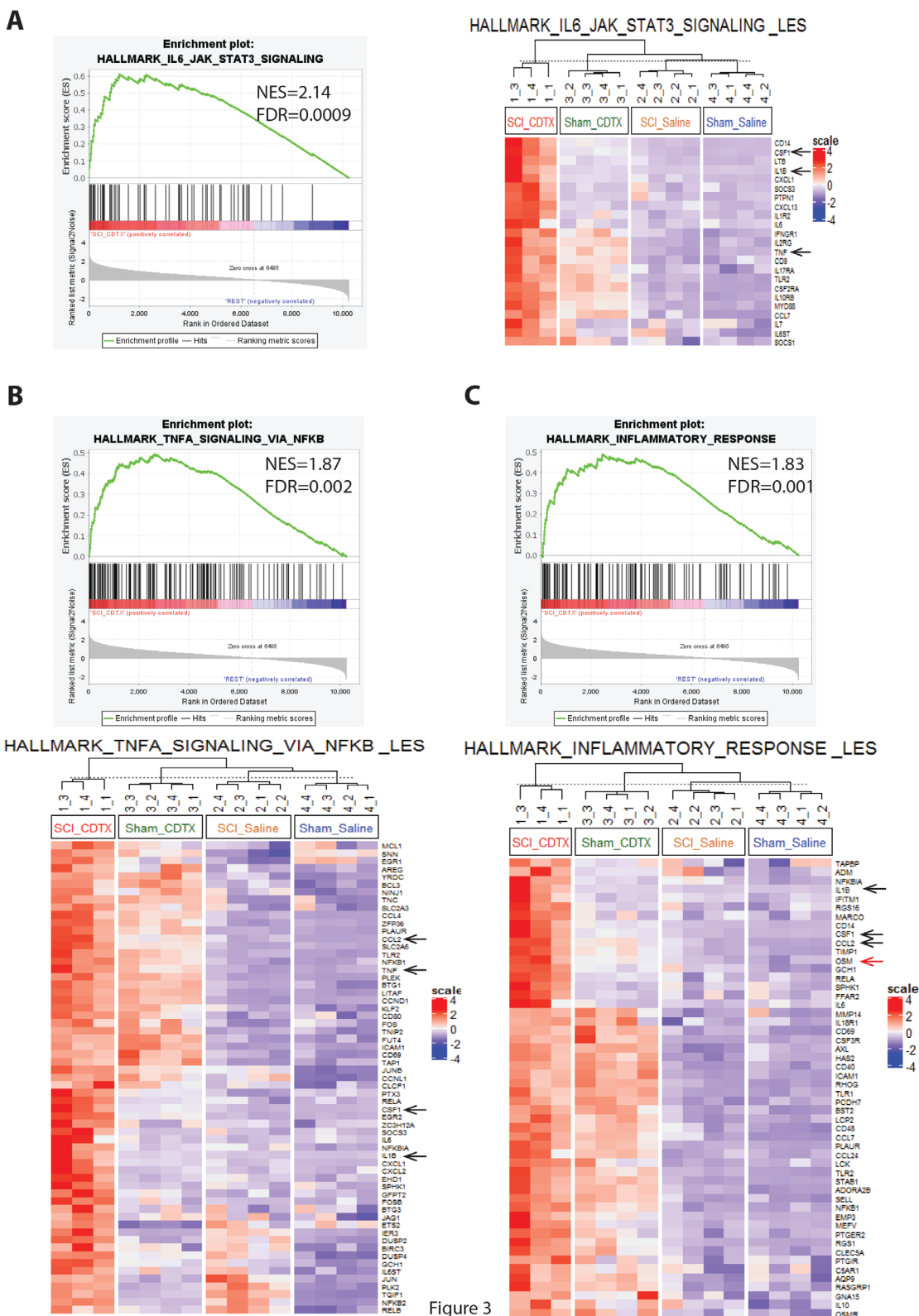
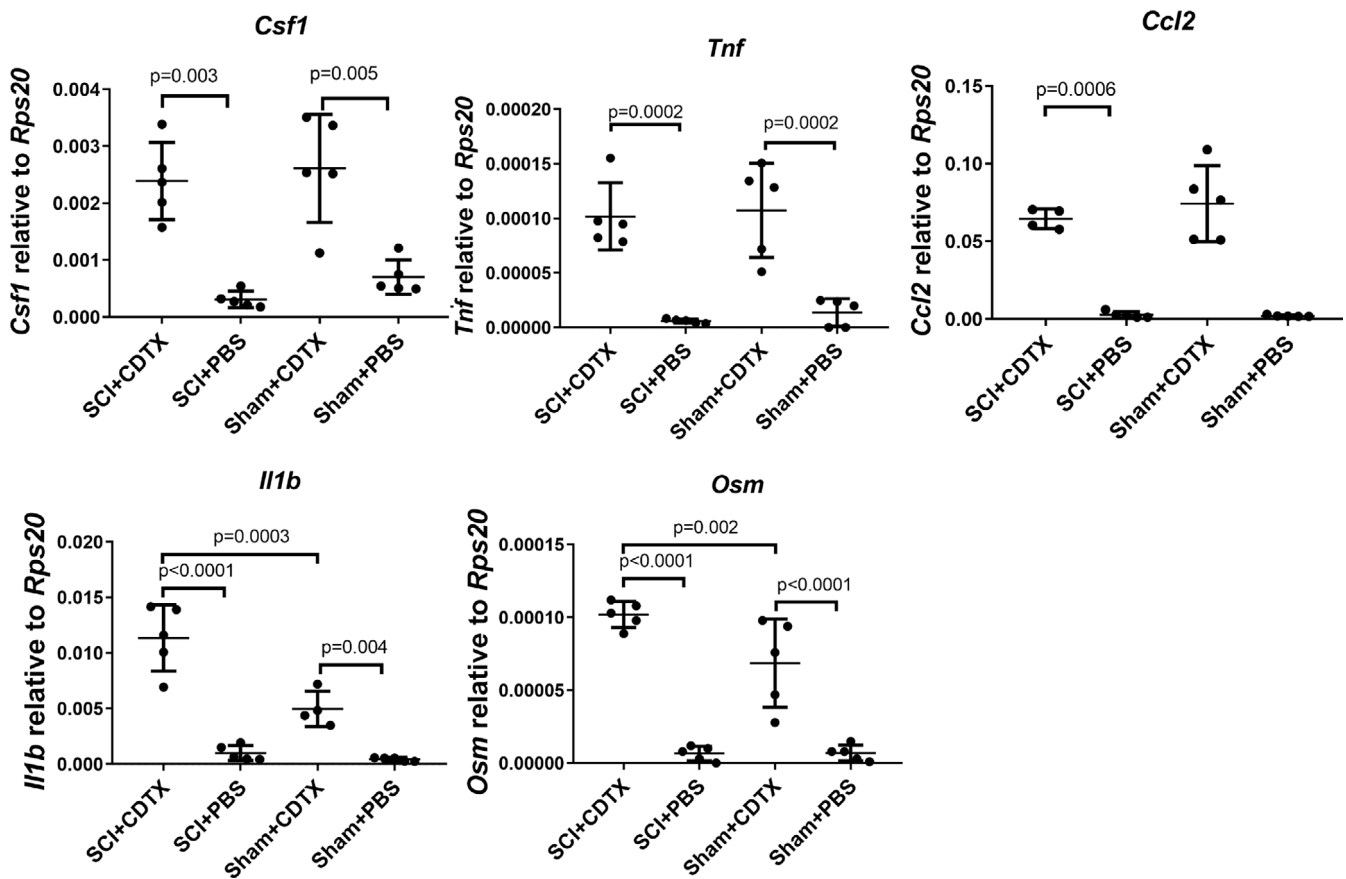


Figure 3

**Fig 3.** Heat maps of differentially expressed gene sets from injured muscles from mice that develop neurogenic heterotopic ossifications (NHO) after spinal cord injury (SCI) and muscle injury. Enrichment score plots for (A) “IL6-JAK-STAT3 signaling,” (B) “TNF signaling via NFκB,” and (C) “inflammatory response signaling” gene sets comparing injured muscles from the SCI + CDTX group, which develops NHO, to the other three experimental groups that do not develop NHO. Heat maps of leading-edge subsets for each of these gene sets are shown. Genes studied in subsequent experiments are indicated by black arrows. *Osm* previously published by Torossian and colleagues<sup>(20)</sup> is indicated by a red arrow.



**Fig 4.** Validation of differentially expressed in injured muscles by qRT-PCR. *Csf1*, *Tnf*, *Ccl2*, *Osm*, and *Il1b* mRNA expression in injured muscles from mice that underwent either SCI + CDTX muscle injury, SCI + PBS, Sham + CDTX, or Sham + PBS. Muscles and total RNA were isolated 2 days post-surgery. Specified mRNAs were quantified relative to *Rps20* housekeeping gene. Each dot represents a separate mouse and bars are means  $\pm$  SD ( $n = 4$ –5/group). Statistical differences were calculated by ANOVA with Tukey's multiple comparison test.

significantly higher in mice with SCI compared with injured muscles from mice without SCI.<sup>(19)</sup> Furthermore, *in vivo* depletion of macrophages with clodronate-loaded liposomes reduced NHO formation dramatically.<sup>(8)</sup> As CCL2/CCR2-mediated macrophage chemotaxis and recruitment largely contribute to inflammatory monocyte/macrophage infiltration in injured muscles,<sup>(9,16)</sup> we further investigated whether defective CCR2-mediated signaling reduced NHO formation. Both CCR2-deficient (*Ccr2*<sup>-/-</sup>) and C57BL/6 wild-type mice underwent SCI surgery and i.m. CDTX injection and muscle leukocyte infiltrates were analyzed 4 days post-surgery. CCR2 deficiency led to a significant reduction in the infiltration of overall F4/80<sup>+</sup> monocytes/macrophages as well as the Ly6C<sup>hi</sup> subset. This was compensated in part by an increased recruitment of CD11b<sup>+</sup> F4/80<sup>-</sup> Ly6G<sup>+</sup> neutrophils (Supplemental Fig. S3). Surprisingly, however, NHO volumes were significantly increased 1.6-fold in *Ccr2*<sup>-/-</sup> mice (Fig. 5C) despite reduced infiltration of inflammatory macrophages.

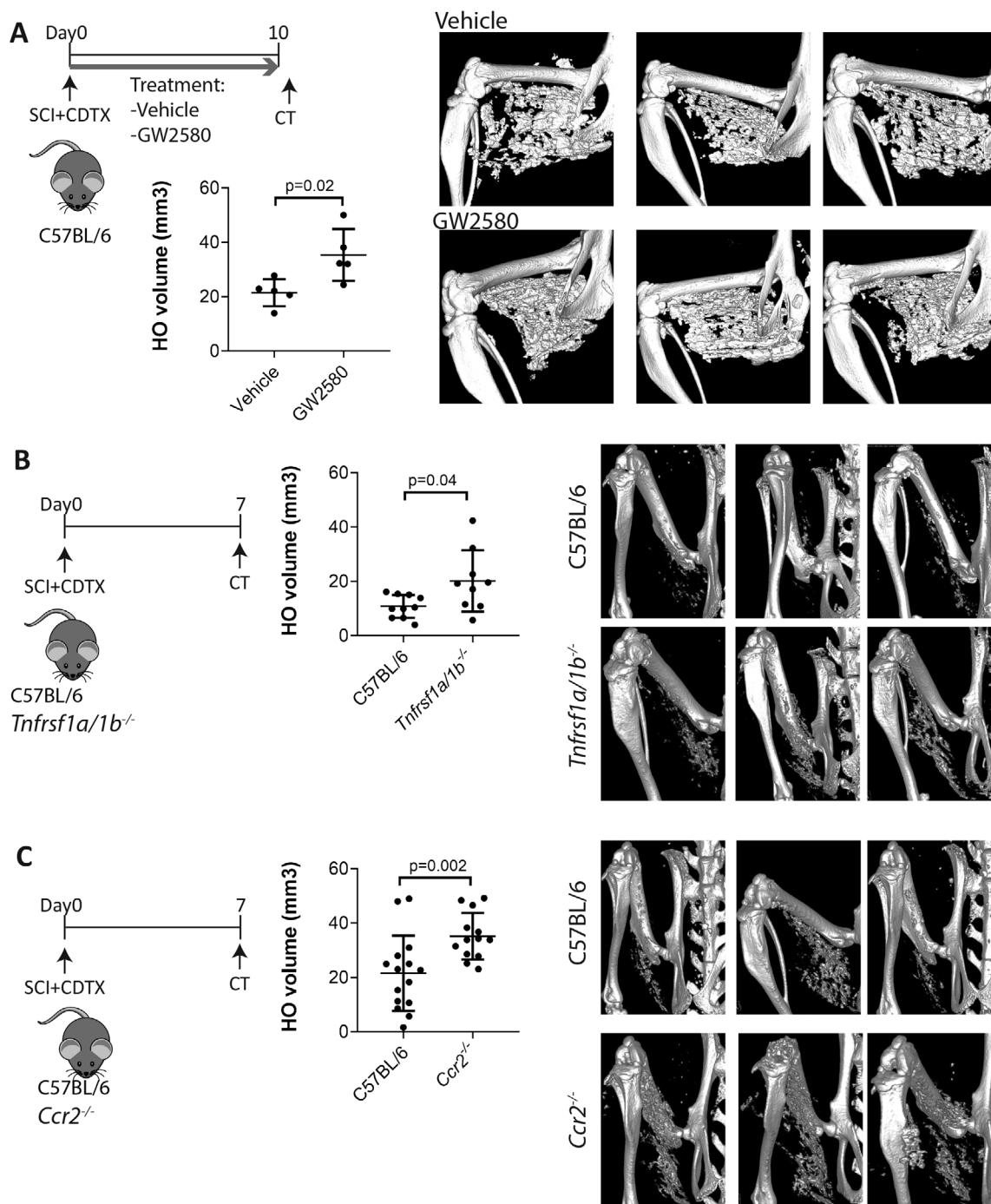
#### IL-1-mediated signaling contributes to SCI-NHO development in mice

Because IL-1 comprises two distinct but functionally redundant proteins, namely IL-1 $\alpha$  and IL-1 $\beta$  encoded by two distinct genes, which both bind to their common IL-1 receptor IL1R1,<sup>(31,32)</sup> we tested the involvement of IL1-mediated signaling in *Il1r1*<sup>-/-</sup> mice defective

for IL1R1. *Il1r1*<sup>-/-</sup> mice exhibited a significant 32.2% reduction in NHO volume compared with wild-type C57BL/6 control mice, indicating IL-1 signaling plays some role in NHO development (Fig. 6A). However, when we conducted this experiment in mice defective for either IL-1 $\alpha$  (*Il1a*<sup>-/-</sup> mice) or IL-1 $\beta$  (*Il1b*<sup>-/-</sup> mice), deficiency of neither IL-1 $\beta$  nor IL-1 $\alpha$  alone affected NHO volumes (Supplemental Fig. S4). Interestingly, by qRT-PCR, we found that *Il1b* and *Il1a* mRNA expression changed over time. On day 2 post-injury, *Il1b* expression was significantly higher in SCI + CDTX compared with Sham + CDTX groups, whereas it was significantly decreased from day 2 to day 4 (Figs. 4 and 6B). On the other hand, *Il1a* expression was significantly increased from day 2 to day 4 in SCI + CDTX muscles (Fig. 6B). These results suggest different kinetics of induction of IL-1 $\alpha$  and IL-1 $\beta$  in the injured muscle after SCI with potential functional redundancy. Therefore, we measured the effects of daily injections of combined neutralizing anti-mouse IL-1 $\alpha$  and anti-mouse IL-1 $\beta$  monoclonal antibodies on NHO volumes. Antibody-mediated neutralization of both IL-1 $\alpha$  and IL-1 $\beta$  caused a trend toward reduced NHO volumes, although this did not reach statistical significance (Fig. 6C).

IL-1 $\beta$  protein is expressed in human NHO biopsies and increases NHO-derived FAP mineralization *in vitro*

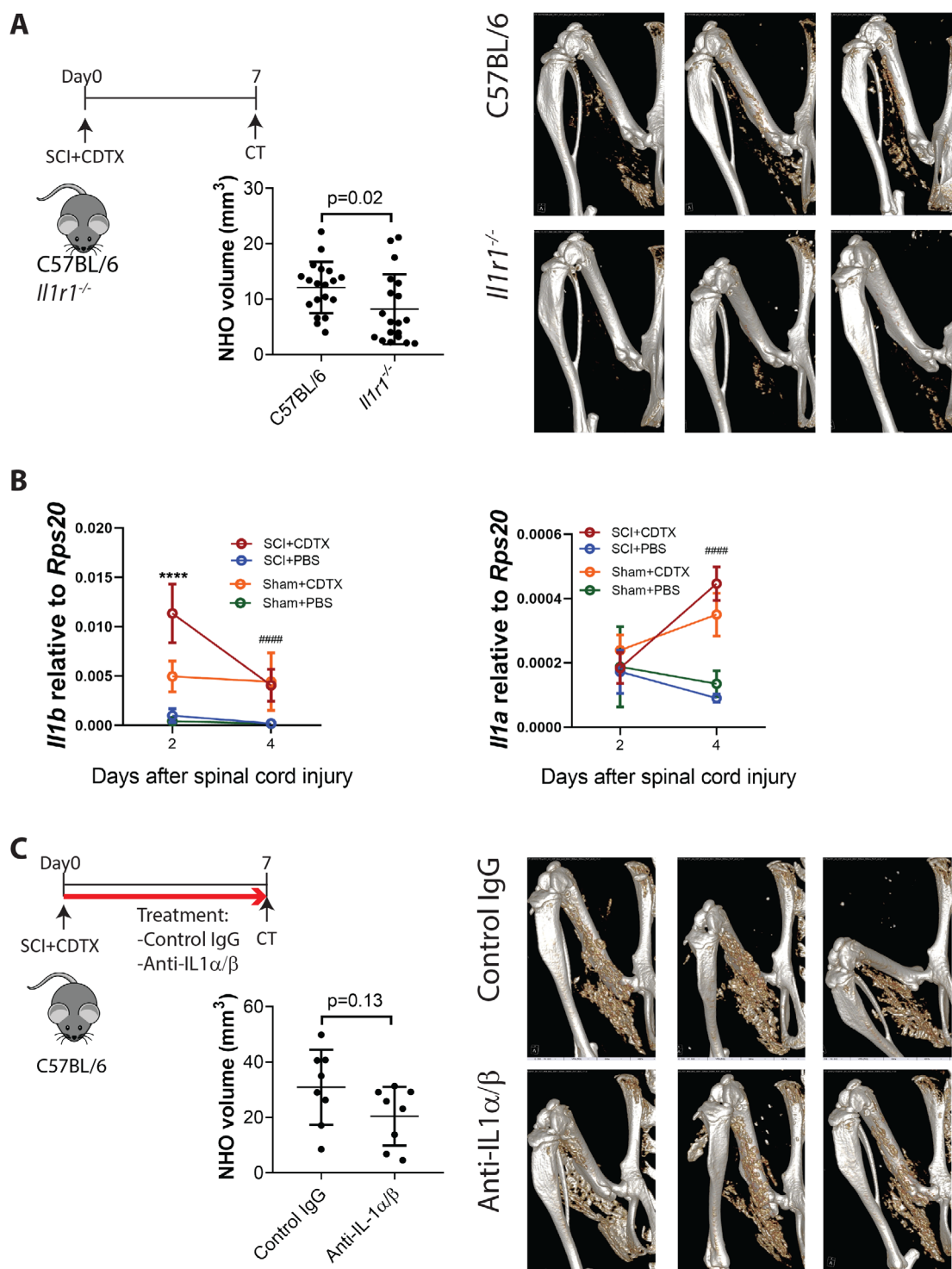
To assess relevance to the human pathology, we studied IL-1 $\beta$  protein expression in human NHO biopsies taken from



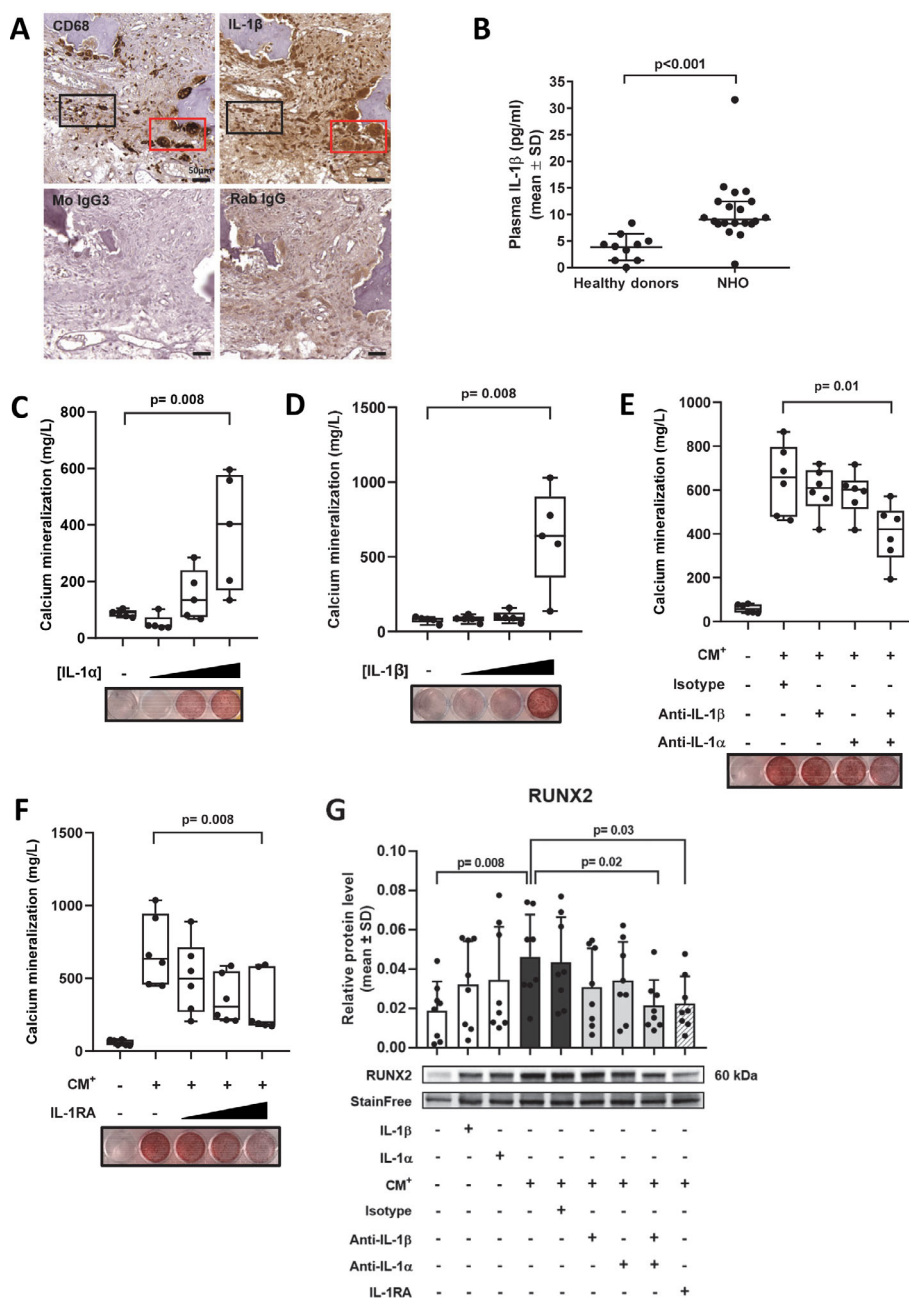
**Fig 5.** Blocking CSF1, TNF, or CCR2 receptor signaling exacerbates neurogenic heterotopic ossification (NHO) formation after spinal cord injury (SCI) in mice. (A) C57BL/6 mice underwent SCI surgery and CDTX i.m. injection. Mice were then treated with vehicle or GW2580 (80 mg/kg) twice daily from day 0 to 10. NHO formation was measured ex vivo by  $\mu$ CT on day 10 ( $n = 5$  mice/group). (B) *Tnfrsf1a/1b*<sup>-/-</sup> double knockout and C57BL/6 mice ( $n = 10$ /group from two separate experiments) and (C) *Ccr2*<sup>-/-</sup> and C57BL/6 mice ( $n = 14$ –15 mice/group from three separate experiments) underwent SCI surgery and CDTX i.m. injection. NHO formation was measured in  $\mu$ CT on day 7. Representative  $\mu$ CT 3D reconstruction images from each group and quantifications of NHO volumes for each mouse are shown. Each dot represents a separate mouse, and bars are means  $\pm$  SD. The *p* values were calculated using two-sided Mann–Whitney test.

13 patients by immunohistochemistry. Five patients had NHO after SCI and 8 after TBI. There was a clear presence of CD68<sup>+</sup> macrophages and multinucleated osteoclasts within NHO

(Fig. 7A and Supplemental Fig. S5) as we previously described.<sup>(8,20)</sup> We noted the accumulation of IL-1 $\beta$ <sup>+</sup> cells adjacent to the compact bone of NHOs and also distributed among



**Fig 6.** IL1 receptor deficiency reduces neurogenic heterotopic ossification (NHO) formation after spinal cord injury (SCI) in mice. (A) *Il1r1<sup>-/-</sup>* and C57BL/6 mice underwent SCI surgery and i.m. injection of CDTX. NHO volumes were quantified by  $\mu$ CT 7 days post-injury. (B) qRT-PCR quantification of *Il1b* and *Il1a* mRNA in whole muscle from mice that underwent either SCI + CDTX, SCI + PBS, Sham + CDTX, or Sham + PBS on day 2 and day 4 post-injury. The mRNA samples on day 2 are the same as in Fig. 4. Expression of *Il1b* and *Il1a* were normalized to *Rps20* housekeeping gene. Data are presented as means  $\pm$  SD ( $n = 4-5$ /group). Statistical differences were calculated by two-way ANOVA with Sidak's multiple comparison test. \*\*\*\* $p < 0.0001$  SCI + CDTX versus Sham + CDTX on day2. #### $p < 0.0001$  SCI + CDTX day 2 versus day 4. (C) C57BL/6 mice underwent SCI surgery plus i.m. injection of CDTX and injected daily with a combination of anti-mouse IL1 $\alpha$  and anti-mouse IL1 $\beta$  neutralizing Armenian hamster monoclonal antibodies or control Armenian hamster IgG. NHO volumes were quantified by  $\mu$ CT 7 days post-injury. Each dot represents a separate mouse, and bars are means  $\pm$  SD. The  $p$  values were calculated using two-sided Mann-Whitney test.  $\mu$ CT scans of NHO formation are shown with representative images from each group.



**Fig 7.** IL-1 $\beta$  is expressed in human neurogenic heterotopic ossification (NHO) biopsies and stimulates calcium mineralization of NHO-derived fibroadipogenic progenitors (FAPs) in vitro. (A) Representative immunohistochemistry staining of serial sections of a human NHO biopsy (spinal cord injury [SCI] patient) demonstrating the colocalization of the macrophage marker CD68 (top panel left) and IL-1 $\beta$  protein expression (top panel right). The black rectangle shows IL-1 $\beta$  expressing CD68<sup>+</sup> macrophages in the fibrotic tissue surrounding NHO, whereas the red rectangle shows IL-1 $\beta$  expressing multinucleated CD68<sup>+</sup> osteoclasts. Mouse IgG3 (bottom left panel) and rabbit IgG (bottom right panel) confirm the specificity of CD68 and IL-1 $\beta$  antibodies. 20 $\times$  magnification and scale bar = 50  $\mu$ m. (B) IL-1 $\beta$  concentration in plasma of healthy volunteers and NHO patients. Each dot represents a different individual. Bars are means  $\pm$  SD. Statistical difference was calculated using two-sided non-parametric Mann-Whitney test. (C) Dose-response of recombinant human IL-1 $\alpha$  (0.01; 1 and 100 ng/mL) and (D) IL-1 $\beta$  (0.01; 0.5 and 10 ng/mL) on calcium mineralization of PDGFR $\alpha$ <sup>+</sup> CD56<sup>-</sup> FAPs derived from 5 different NHO biopsies/patients cultured in osteogenic medium. (E) Osteogenic differentiation assay of FAPs cultured with LPS-stimulated CD14<sup>+</sup> blood monocyte-conditioned medium (CM<sup>+</sup>) and incubated with anti-IL-1 $\alpha$ , anti-IL-1 $\beta$  neutralizing antibodies, or isotype control (100 ng/mL). (F) Osteogenic differentiation assay of FAPs derived from 6 different biopsies/patients cultured with CM<sup>+</sup> and incubated with 10, 100, or 500 ng/mL of recombinant human IL-1RA. In C–E, each dot represents a result from a different NHO biopsy/patient. Boxes extend from the 25th to 75th percentiles; middle line and whiskers represent median and minimum and maximum value, respectively. Statistical differences were analyzed using non-parametric repeated measure Friedman test with Dunn’s correction for multiple comparisons. (G) RUNX2 protein expression level of FAPs evaluated by Western blot after 7 days of osteogenic differentiation with IL-1 $\beta$  (10 ng/mL), IL-1 $\alpha$  (100 ng/mL), NHOmac CM<sup>+</sup>, control isotype antibody, anti-IL-1 $\alpha$ , and anti-IL-1 $\beta$  antibody (100 ng/mL) or IL-1RA (500 ng/mL). Statistical differences were analyzed using non-parametric repeated measure Friedman test with Dunn’s correction for multiple comparisons.

the inflamed fibrotic tissue surrounding NHOs (Fig. 7A and Supplemental Fig. S5). The distribution of these IL-1 $\beta$ -producing cells overlapped with that of CD68, suggesting that for the most part, IL-1 $\beta$  is expressed by CD68<sup>+</sup> cells. Interestingly, for four biopsies, we observed the presence of large IL-1 $\beta$ <sup>+</sup> multinucleated cells lining the mineralized bone of NHO nodules consistent with the morphology and location of osteoclasts (Fig. 7A and Supplemental Fig. S5). Although we were unable to perform TRAP staining on these NHO biopsies decalcified with nitric acid, these results suggest that IL-1 $\beta$  is likely secreted by CD68<sup>+</sup> macrophages and osteoclasts in these NHO biopsies. This is consistent with the known expression profile of *IL1B* mRNA expression in myeloid cells in the BioGPS database (<http://biogps.org/#goto=genereport&id=3553>) and the Human Protein Atlas database (<https://www.proteinatlas.org/ENSG00000125538-IL1B/celltype>) and IL-1 $\beta$  immunoreactivity of osteoclasts on sections of growth plate, fracture callus, osteomyelitis, and Paget's disease.<sup>(33)</sup> We also confirmed that patients with NHOs had significantly elevated plasma IL-1 $\beta$  concentration compared with healthy donors (Fig. 7B), suggesting that IL-1 $\beta$  may play a role in NHO development.

To further investigate the involvement IL-1 on NHO pathology, we collected surgical residues of muscle tissue surrounding NHO after resection surgery. Muscle progenitor cells were expanded in vitro and PDGFR $\alpha$ <sup>+</sup> CD56<sup>-</sup> FAPs were isolated by FACS (Supplemental Fig. S6). Osteogenic differentiation assays showed that FAPs exhibited higher calcium mineralization activity when cultured with increasing doses of either recombinant IL-1 $\alpha$  (Fig. 7C) or IL-1 $\beta$  (Fig. 7D), suggesting that IL-1 stimulates FAP osteoblastic differentiation. The addition of anti-IL-1 $\beta$  neutralizing antibody significantly inhibited FAP calcium mineralization activity when cultured with IL-1 $\beta$  (Supplemental Fig. S7A). Considering the pivotal role of macrophages in NHO formation, we isolated CD14<sup>+</sup> blood monocytes from healthy donors and NHO-bone marrow-derived CD14<sup>+</sup> monocytes. CD14<sup>+</sup> cells were cultured with or without LPS to generate CM<sup>-</sup> and LPS-stimulated CM<sup>+</sup> conditioned media. CM<sup>+</sup> displayed significantly higher IL-1 $\alpha$  and IL-1 $\beta$  concentrations compared with CM<sup>-</sup> (Supplemental Fig. S7B, C) and, as expected, pooled CM<sup>+</sup> stimulated FAPs calcium mineralization activity in osteogenic differentiation assays (Fig. 7E, F). When added individually, anti-IL-1 $\alpha$  and anti-IL-1 $\beta$  neutralizing antibodies did not significantly reduce calcium mineralization of CM<sup>+</sup>-stimulated FAPs; however, the combination of both antibodies significantly reduced FAP mineralization activity associated with significant decrease in RUNX2 protein expression (Fig. 7E, G), suggesting a synergistic effect of IL-1 $\alpha$  and IL-1 $\beta$ . This was further confirmed by the dose-dependent inhibitory effect of IL-1RA, the endogenous protein antagonist of IL1R1 common receptor for IL-1 $\alpha$  and IL-1 $\beta$ , on FAP mineralization associated with reduced RUNX2 protein expression (Fig. 7F, G). Overall, these results confirm the involvement of IL-1 as an inflammatory mediator of NHO formation in the human pathology.

## Discussion

Using non-supervised unbiased GSEA of whole muscle RNA in mice, we demonstrate herein that superimposition of a SCI to a muscle injury exacerbates the local inflammatory response to muscle injury and most particularly expression of genes associated with IL6 cytokine family/JAK/STAT3 signaling and TNF signaling via NF- $\kappa$ B. The proinflammatory effect of the SCI is rapid

as we measured gene expression 2 days after SCI and/or muscle injury. This is consistent with our previous studies showing that SCI causes persistent and abnormally high expression of the IL6 family cytokine OSM, together with persistently high levels of active tyrosine phosphorylated STAT3 via JAK1/2 tyrosine kinase activation in the injured muscle.<sup>(19)</sup> In addition to OSM that we have already shown to promote NHO in response to SCI,<sup>(20)</sup> we also found that SCI increased the expression of other inflammatory cytokines in injured muscles, including TNF, CSF-1, and IL-1 $\beta$ , as well as inflammatory chemokines such as CCL2, CXCL1, or CXCL13 (Fig. 3). As we have previously shown that macrophages play a key role in NHO development,<sup>(8)</sup> we focused on TNF, CSF-1, and CCL2, which are well known to regulate macrophage polarization and recruitment,<sup>(34-37)</sup> as well as IL-1 $\beta$ , which is specifically produced by phagocytes after activation of inflammasomes and mediates inflammatory responses in many tissues.<sup>(38)</sup> Using mice lacking the cognate cytokine receptors for TNF, CCL2, and IL-1, neutralizing antibodies (IL-1), or small receptor antagonists (CSF-1), we found that of these four potential mediators, only IL-1 promoted NHO development after SCI. Germinal deletion of the *Il1r1* gene that encodes the common receptor for IL-1 $\alpha$  and IL-1 $\beta$  significantly reduced NHO volumes after SCI, although this decrease was not as pronounced as observed in *Osmr*<sup>-/-</sup> mice lacking the OSM receptor.<sup>(20)</sup> Active IL-1 $\beta$  release by macrophages is a two-step process, first requiring activation of NF- $\kappa$ B-dependent transcription of the *Il1b* gene followed by inflammasome-dependent activation of non-active IL-1 $\beta$  pro-peptide by caspase-1-mediated proteolytic cleavage. In contrast, the IL-1 $\alpha$  pro-peptide is biologically active and preformed intracellularly in many cell types, particularly endothelial cells and fibroblasts, and its activity can be further enhanced by calpain and several caspases upon cell death.<sup>(39)</sup> Therefore, although IL-1 $\beta$  needs two distinct signals to be produced in active form by macrophages, preformed IL-1 $\alpha$  is a "preloaded gun" that is released upon death of many cell types.<sup>(38,39)</sup> Although we could not detect enhanced IL-1 $\alpha$  transcription in our gene expression microarray analysis at day 2 post-injury, *Il1a* was upregulated later at day 4, and it is likely that muscle destruction caused by CDTX injection would release IL-1 $\alpha$ . To assess the respective role of IL-1 $\alpha$  and IL-1 $\beta$  in NHO pathogenesis, we used mice targeted deletion of either the *Il1a* gene or *Il1b* gene. Deletion of either IL-1 gene alone did not alter NHO development, suggesting that both IL-1 proteins act in concert to promote NHO development. This is further suggested by reduced NHO development in mice treated simultaneously with two neutralizing antibodies specific for IL-1 $\alpha$  and IL-1 $\beta$ , although this decrease did not reach significance ( $p = 0.13$ , Fig. 6B).

To validate our results in the human pathology, we show that IL-1 $\beta$  protein is present in the NHO itself and the non-ossified and peripheral part of the NHO, particularly by CD68<sup>+</sup> macrophages. It indicates that IL-1 $\beta$  is produced locally by activated macrophages and could contribute to the development of NHO. Furthermore, IL-1 $\beta$  protein concentration was doubled in the plasma of SCI patients developing NHO compared with healthy subjects. Polynucleated osteoclasts were also identified for several NHO biopsies, suggesting active bone remodeling. Functionally, we find that purified recombinant IL-1 $\alpha$  and IL-1 $\beta$  both potentially promote calcium deposition and mineralization of human FAPs purified from the muscles surrounding patient NHOs. Furthermore, we had previously shown that LPS-stimulated monocyte-conditioned medium (CM<sup>+</sup>) promotes human FAP osteogenic differentiation and calcium

mineralization in part via OSM secretion.<sup>(20)</sup> Herein, we show that IL-1 $\alpha$  and IL-1 $\beta$ , which are both secreted by human monocytes in response to LPS, both contribute to the pro-osteogenic effect of CM<sup>+</sup> on human FAPs. Similar to our results suggesting that antibody neutralization of both IL-1 $\alpha$  and IL-1 $\beta$  is necessary to reduce NHO development in mice in vivo, neutralization of both IL-1 $\alpha$  and IL-1 $\beta$  was necessary to reduce the pro-osteogenic effect of CM<sup>+</sup> on human FAPs in vitro, an effect recapitulated by addition of recombinant IL-1RA, the physiological endogenous antagonist of the IL-1 receptor. As we have shown that muscle FAPs are the cells of origin of NHO in both humans and mice (Tseng and colleagues, data not shown), this suggests that both IL-1 $\alpha$  and IL-1 $\beta$  are potential mediators of NHO development in patients.

In respect to the other inflammatory cytokines and chemokines that we found to be highly expressed in muscles developing NHOs in mice, namely TNF, CCL2, and CSF-1, we unexpectedly found that they did not promote but instead dampened NHO development after SCI. In respect to TNF and CCL2, both have been shown to play a major role in normal muscle repair by promoting apoptosis of FAPs proliferating in the first few days in injured skeletal muscles and are therefore essential to prevent muscle fibrosis.<sup>(16)</sup> As FAPs are the cells of origin of NHO in both humans and mice (Tseng and colleagues, data not shown), we speculate that *Ccr2*<sup>-/-</sup> and *Tnfrsf1a/1b*<sup>-/-</sup> mice may have reduced apoptosis and prolonged proliferation of FAPs,<sup>(16)</sup> which could be responsible for increased muscle fibrosis and NHO volumes. The enhancing effect of CSF1R tyrosine kinase inhibitor GW2580 on NHO development was also surprising because CSF1R signaling is necessary to maintain many tissue-resident macrophages and osteoclasts but dispensable to monocyte production in the bone marrow,<sup>(40,41)</sup> although extra-physiological treatment with stable CSF-1 protein considerably enhances monopoiesis.<sup>(29,41)</sup> As macrophages are necessary to the development of SCI-induced NHO<sup>(8)</sup> as well as genetically driven HO development in fibrodysplasia ossificans progressiva,<sup>(17,42)</sup> we anticipated that inhibition of CSF1R signaling would reduce NHO formation. The opposite effect that we observed on NHO formation in vivo may be due to the complex function of CSF-1 on macrophage biology. CSF-1 is not only necessary to the maintenance of many types of tissue-resident macrophages, but it also induces and modulates macrophage pro-inflammatory or anti-inflammatory polarization in a context-dependent manner.<sup>(36,43)</sup> Our results herein suggest that CSF-1 polarizes macrophages in an inflammatory manner in injured muscles in the context of a SCI similar to the effect of GW2580 treatment or CSF-1-deficiency, which both inhibit functional recovery and increase tubulointerstitial fibrosis in a mouse model of acute kidney injury.<sup>(44,45)</sup>

Although our mouse model of SCI-NHO recapitulates many features of NHO observed in patients with SCI,<sup>(8)</sup> a limitation of our mouse model is that it does not recapitulate the endochondral phase observed within NHO from patients. Indeed, safranin-O staining showed a clear chondrogenic phase with different stages of cartilage and chondroblast maturation in NHO biopsies resected from patients with SCI, suggesting some endochondral bone formation. However, we could not detect any chondrogenic phase in the NHO nodules forming in the injured muscles in mice that underwent SCI (Supplemental Fig. S8), suggesting intramembranous bone formation. Despite this limitation, functional in vivo experiments in mice, immunohistology, and functional in vitro experiments with biopsies from patients

suggest a commonality of triggering mechanisms involving exacerbated inflammation in muscles developing NHO<sup>(8)</sup> with a role of OSM<sup>(20)</sup> and IL-1 reported herein.

In conclusion, we report herein that SCI exacerbates the transcription of several inflammatory cytokines and chemokines such as IL-1 $\beta$ , IL-1 $\alpha$ , TNF, CSF-1, and CCL2 in injured muscles. We established that TNF, CSF-1, and CCL2 had a dampening effect on NHO development. In sharp contrast, IL-1 signaling promoted NHO development in injured muscles in our mouse model of SCI-induced NHO. The expression of IL-1 $\beta$  in NHO biopsies from SCI and TBI patients and the osteogenic effect of IL-1 $\alpha$  and IL-1 $\beta$  secreted by activated monocytes was confirmed in cultures of FAPs isolated from the muscles surrounding NHO biopsies from SCI and TBI patients. These results are consistent with the observation that 80% of infants with the extremely rare genetic deficiency of IL-1 receptor antagonist (DIRA) syndrome, in which IL-1 activity cannot be inhibited by endogenous IL-1RA, develop periarticular HO.<sup>(46)</sup> Considering that both OSM<sup>(20)</sup> and IL-1 (herein) promote NHO development after SCI, prophylactic combinations of drugs antagonizing IL-1 signaling (such as anakinra or IL-1RA) and JAK1/2 tyrosine kinases (such as ruxolitinib) activated by the OSM receptor<sup>(19)</sup> may be studied in more detail to assess their potential synergism in inhibiting NHO development. How SCI causes this peculiar exacerbation of inflammation in injured muscles that leads to NHO formation remains incompletely understood. In our SCI-NHO mouse model, transection of the spinal cord between T<sub>11</sub> and T<sub>13</sub> leads to a significant increase of epinephrine blood plasma concentration 24 hours post-surgery, and we have shown chemical sympathectomy or inhibition of  $\beta$ 1/2-adrenergic receptors with propranolol significantly reduced NHO formation, suggesting that sympathetic adrenergic signaling plays an important role in NHO pathogenesis.<sup>(47)</sup> By the same token, the increased adrenergic signaling subsequent to an SCI may exacerbate the inflammatory response in injured muscle. In support of this, binding of epinephrine to macrophages via  $\beta$ 2 adrenergic receptors has been shown to modulate inflammatory responses by, for instance, upregulating OSM and IL-1 $\beta$  expression in macrophages and microglial cells.<sup>(48-50)</sup> A similar mechanism may occur via systemic release of epinephrine from adrenal glands in response to low level SCI.<sup>(47)</sup>

## Disclosures

KS is a co-inventor on patent applications for NLRP3 inhibitors, which have been licensed to Inflazome Ltd, a company headquartered in Dublin, Ireland. Inflazome is developing drugs that target the NLRP3 inflammasome to address unmet clinical needs in inflammatory disease. KS served on the Scientific Advisory Board of Inflazome in 2016–2017 and serves as a consultant to Quench Bio, USA, and Novartis, Switzerland. All other authors have no conflicts of interest to declare.

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## PEER REVIEW

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## DATA AVAILABILITY STATEMENT

Normalized gene expression microarray data are accessible on NCBI database with the GEO accession number GSE165062. All other data that support the findings of this study are available from the corresponding author upon reasonable request.

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