MUSCULOSKELETAL PATHOLOGY

Lack of Heme Oxygenase-1 Induces Inflammatory Reaction and Proliferation of Muscle Satellite Cells after Cardiotoxin-Induced Skeletal Muscle Injury

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Heme oxygenase-1 (HO-1, *Hmox1*) regulates viability, proliferation, and differentiation of many cell types; hence, it may affect regeneration of injured skeletal muscle. Here, we injected cardiotoxin into gastrocnemius muscle of *Hmox1*^{+/+} and *Hmox1*^{-/-} animals and analyzed cellular response after muscle injury, focusing on muscle satellite cells (SCs), inflammatory reaction, fibrosis, and formation of new blood vessels. HO-1 is strongly induced after muscle injury, being expressed mostly in the infiltrating leukocytes (CD45⁺ cells), including macrophages (F4/80⁺ cells). Lack of HO-1 augments skeletal muscle injury, evidenced by increased creatinine kinase and lactate dehydrogenase, as well as expression of monocyte chemoattractant protein-1, IL-6, IL-1 β , and insulin-like growth factor-1. This, together with disturbed proportion of M1/M2 macrophages, accompanied by enhanced formation of arterioles, may be responsible for shift of *Hmox1*^{-/-} myofiber size distribution toward larger one. Importantly, HO-1-deficient SCs are prone to activation and have higher proliferation on injury. This effect can be partially mimicked by stimulation of *Hmox1*^{+/+} SCs with monocyte chemoattractant protein-1, IL-6, IL-1 β , and is associated with increased MyoD expression, suggesting that *Hmox1*^{-/-} SCs are shifted toward more differentiated myogenic population. However, multiple rounds of degeneration/regeneration in conditions of HO-1 deficiency may lead to exhaustion of SC pool, and the number of SCs is decreased in old *Hmox1*^{-/-} mice. In summary, HO-1 modulates muscle repair mechanisms preventing its uncontrolled acceleration. (*Am J Pathol* 2018, 188: 491–506; <https://doi.org/10.1016/j.ajpath.2017.10.017>)

Striated muscles consist of highly differentiated muscle fibers and are characterized by a slow cell turnover. Apart from mature myofibers, relatively low number of inflammatory cells (resident macrophages) and progenitor cells [muscle satellite cells (SCs)] are also present in intact muscles, together with fibroblasts, adipocytes, and their progenitors [fibroadipogenic progenitors (FAPs)], endothelial cells, and pericytes.^{1–6} When it comes to myotrauma (eg, induced by cardiotoxin, CTX) the situation changes dramatically. Within a few hours cell debris and activated resident macrophages induce massive infiltration with neutrophils, followed by macrophages, mostly of proinflammatory M1 phenotype.^{6–9} During the first few days, removal of cell debris by macrophages proceeds

concomitantly with activation of key players in skeletal muscle regeneration, namely SCs. In response to growth factors [hepatocyte growth factor, insulin-like growth factor

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(IGF)-1, fibroblast growth factor-2] and proinflammatory cytokines [monocyte chemoattractant protein (MCP)-1/chemokine (C-C motif) ligand 2, IL-1 β , IL-6] SCs enter the cell cycle and proliferate robustly, expanding their population and changing their phenotype.^{2,5,10} SC marker, paired box protein 7 (Pax7) transcription factor, is necessary for cell cycle progression, acting upstream of the group of muscle regulatory factors (Myf5, MyoD, myogenin, and Myf6) and controlling their sequential expression and activity.^{11,12} During successive rounds of proliferation SCs are converted into myoblasts that then differentiate further to fuse into multinucleated myotubes, starting expression of proteins of mature skeletal muscles.^{2,4,5,10,13} This process is accompanied by the change in the macrophage phenotype within injured muscle. Transition from M1 to M2 starts about the third day after injury and modifies the impact of macrophages on SCs—from sustaining activation and proliferation to promotion of differentiation.^{9,14,15} Finally, to fully regenerate skeletal muscle, reconstruction of intramuscular capillaries and arterioles in the processes of angiogenesis and arteriogenesis has to take place, as well as formation of a fibrotic scar. Achieving that can in turn provide a proper niche and supply of nutrients for maturing muscle cells.^{4,6}

The biological effects of heme oxygenase-1 (HO-1, encoded by *Hmox1*) extend far beyond its classic enzymatic function—degradation of heme to carbon monoxide, biliverdin, and iron.¹⁶ Lack of HO-1 strongly induces chronic inflammatory reaction with increased monocyte/macrophage in spleen and elevated proinflammatory cytokines. Reverse effect on the expression of tumor necrosis factor- α , IL-1 β , and IL-6 can be exerted in macrophages by HO-1/carbon monoxide stimulation.^{16–18} HO-1 as immunomodulatory factor promotes macrophage switch from M1 to M2 phenotype.¹⁹ Furthermore, HO-1 is cytoprotective and antiapoptotic in different cell types.^{16,20} It may also promote angiogenesis, and by induction of proangiogenic vascular endothelial growth factor (VEGF) and stromal cell–derived factor-1 α it leads to increased formation of capillaries during wound healing and in ischemic hind limbs.^{16,21} Moreover, HO-1 may reduce fibrosis in kidney, liver, and lungs.^{22,23} Finally, in many cell types HO-1 affects differentiation.²⁴ Recently, it was demonstrated that HO-1 influences *in vitro* differentiation of myoblasts (C2C12 cell line and primary myoblasts).²⁵ Low HO-1 expression increases the differentiation rate but makes myoblasts prone to apoptosis, whereas the opposite effect can be observed in cells overexpressing HO-1.²⁵ Importantly, HO-1 appears to be a potent modulator of the miRNA expression, with especially strong effect exerted on muscle-specific myomirs.^{25,26} However, hypoxia-induced genetic overexpression of HO-1 in muscles during the first days of ischemia enhances cell viability and facilitates recovery on injury.²⁷

Although HO-1 may be considered as a potential target for improving skeletal muscle regeneration, no studies on

its role in this process have been performed so far. We aimed to investigate whether the lack of HO-1 will influence inflammatory reaction, proliferation of SCs, and maturation of new myofibers, as well as if it affects angiogenesis, arteriogenesis, and fibrosis in regenerating skeletal muscle.

Materials and Methods

Animal Models

All animal procedures and experiments were performed after approval by the First Local Ethical Committee on Animal Testing at Jagiellonian University (66/2013). Animals were kept in specific pathogen-free conditions with water and food available *ad libitum*. C57BL/6xFVB *Hmox1*^{+/+} and *Hmox1*^{-/-} breeding pairs were originally kindly provided by Dr. Anupam Agarwal, University of Alabama, Birmingham, AL, in 2004. Female age-matched mice (approximately 12 weeks old) were used for the myoinjury experiment. In addition, for analysis of SC population in intact skeletal muscle 6-, 12-, 15-, 18-, and 24-month-old animals were used.

Gastrocnemius muscles of *Hmox1*^{+/+} and *Hmox1*^{-/-} mice were injected with 25 μ L of 20 μ mol/L CTX (Sigma-Aldrich, St. Louis, MO) in saline. Muscles of control mice were injected intramuscularly with saline and sacrificed immediately. Animals were provided with analgesia (50 μ L, 0.03 mg/mL buprenorphine) after injection and on the next 2 days. To induce multiple injury CTX injections were repeated three times every 2 weeks, and analgesia was applied after each injection. Animals were sacrificed on the 1st, 3rd, 7th, 14th, and 28th day after CTX-induced injury (5 *Hmox1*^{+/+} and 5 *Hmox1*^{-/-} per time point) and 1st, 3rd, and 28th day after triple injury. One day before sacrifice 150 μ L of 5-ethynyl-2'-deoxyuridine (Edu; Thermo Fisher, Waltham, MA) in saline (2 mg/mL) was injected intraperitoneally into CTX-injured or control mice.

After euthanasia injured and control muscles were collected and snap-frozen in liquid nitrogen (for RNA and protein isolation) or preserved in formalin (for histologic analysis). Orbital sinus blood sampling from mice was performed directly to EDTA-coated tubes. Collected blood was analyzed by ABC Vet equipment (Horiba ABX, Kyoto, Japan).

Plasma CPK and LDH Measurement

Plasma was obtained from blood collected from the vena cava just before the terminal procedure, by centrifugation of the clot. Activities of creatine phosphokinase (CPK) and lactate dehydrogenase (LDH) were measured with diagnostic Liquick Cor-CK and Liquick Cor-LDH kit, respectively (Cormay, Warszawa, Poland) according to the vendor's instruction.

Histologic and Immunohistofluorescence Staining

Gastrocnemius muscles, with tightly associated soleus muscles, were placed in 10% formalin for 48 hours. After paraffin embedding, sections were cut, deparaffinized, and subjected to histologic or immunohistofluorescence staining.

Hematoxylin and eosin staining (Sigma-Aldrich) was performed to assess intensity of inflammatory infiltration and muscle degeneration (arbitrary units: 0 = no signs of inflammation; 1 = any sign of leukocyte infiltration and myofiber swelling; 2 = clearly visible inflammation, myofiber swelling, and rhabdomyolysis; 3 = signs of inflammation, myofiber swelling, and rhabdomyolysis take up more than a half of field of view; 4 = all muscle visible in the field of view is infiltrated and degenerated), level of regeneration (percentage of centrally nucleated myofibers in the field of view), and size of myofibers (measurement of the area of at least 50 fibers per mouse; ImageJ software version 1.50i; NIH, Bethesda, MD; <http://imagej.nih.gov/ij>). Trichrome staining was performed by sequential treatment of slides with Biebrich Scarlet-Acid Fuchsin, phosphotungstic acid/phosphomolybdic acid, and Aniline Blue (Sigma-Aldrich, according to the vendor's instructions) to visualize the collagen (arbitrary units: 0 = no signs of collagen deposition; 1 = any sign of collagen deposition; 2 = clearly visible collagen deposition; 3 = collagen deposition takes up more than a half of field of view). All analyses were performed by an observer (M.K.) blinded to the treatment of animals, 10 images of damaged area per mouse were analyzed.

For immunohistofluorescence staining of paraffin sections retrieval of antigens was performed in citric buffer. Then slides were blocked with phosphate-buffered saline (PBS) with 5% bovine serum albumin (BioShop, Burlington, ON, Canada) and 5% goat serum (Sigma-Aldrich), and primary antibodies were applied overnight in 4°C Pax7 (dilution 1:100 and α -smooth muscle actin dilution 1:200; both from Abcam, Cambridge, UK). Secondary anti-rabbit Alexa Fluor 568 or 488 (Thermo Fisher, Waltham, MA) were applied for 2 hours (dilution 1:200–1:500) together with Hoechst 33342 (1 μ g/mL; Sigma-Aldrich). To visualize proliferating cells the slides were then proceeded with Click-iT Edu Alexa Fluor 488 Imaging Kit (Thermo Fisher), according to the vendor's manual. To analyze Pax7⁺Edu⁺ cells at least 10 fields of view per damaged area of muscle were analyzed.

Staining of necrotic fibers was performed on muscle sections after citric buffer-mediated antigen retrieval,²⁸ followed by blocking with 10% goat serum and 2 hours of incubation with goat anti-mouse IgG/IgM/IgA Alexa Fluor 488 (Thermo Fisher). Nuclei were counterstained with Hoechst 33342. To analyze necrosis in the damaged area, percentage of necrotic fibers among total fiber number was counted.

For immunohistofluorescence staining of HO-1 and CD45 or F4/80 colocalization gastrocnemius muscle was

preserved in OCT freezing medium in isopentane, cooled in a bath of liquid nitrogen. Frozen muscle sections of control animals and mice injured with CTX were stained with HO-1 (SPA894; Enzo, Farmingdale, NY) and with CD45 (30-F11; eBioscience, San Diego, CA) or F4/80 (MCA497R; Bio-Rad, Hercules, CA), as described previously.²³ Sections were covered with fluorescence mounting medium (Dako, Carpinteria, CA), visualized under fluorescent confocal microscope (Carl Zeiss, Thornwood, NY) and analyzed in ImageJ (NIH).

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining of DNA fragmentation (Millipore, Billerica, MA) was performed according to the vendor's instruction and followed by overnight incubation at 4°C with primary anti-HO-1 antibody (dilution 1:50; SPA894; Enzo) and 2-hour incubation with anti-rabbit Alexa Fluor 568 secondary antibody (dilution 1:200).

Fluorescence-Activated Cell Sorting

SCs were isolated as previously described.²⁹ Gastrocnemius muscles were minced and digested with 5 mg/mL collagenase IV (Gibco-Thermo Fisher, Waltham, MA) and 1.2 U/mL dispase (Gibco-Thermo Fisher) at 37°C for 45 minutes. Cell suspension was filtered and centrifuged.

For analysis of SCs [CD45⁺CD31⁺ stem cell antigen-1 (Sca1)⁺ α 7i⁺CD34⁺/CD34⁺], a pellet of FAP (CD45⁺CD31⁺ α 7i⁺Sca1⁺CD34⁺), endothelial cells (CD31⁺CD45⁺), and inflammatory cells (CD45⁺CD31⁺) was resuspended in PBS + 2% fetal bovine serum. Thirty-minute incubation on ice was performed with the following antibodies: rat anti-mouse α 7i-phycoerythrin (PE) (dilution 1:15; R&D Systems, Minneapolis, MN), rat anti-mouse CD34-Alexa Fluor 700 (dilution 1:30), rat anti-mouse CD45-allophycocyanin (APC)-eFluor 780 (dilution 1:30), rat anti-mouse CD31-APC (dilution 1:30), rat anti-mouse Sca1-PE-cyanine (Cy)7 (dilution 1:30) (all from BD Biosciences, Franklin Lakes, NJ).

For analysis of macrophages [total macrophages: CD45⁺CD11b⁺F4/80⁺; M1-like macrophages: CD45⁺CD11b⁺F4/80⁺major histocompatibility complex II (MHCII)^{high}CD206^{low}; M2-like macrophages: CD45⁺CD11b⁺F4/80⁺MHCII^{low}CD206^{high}] cells were incubated with rat anti-mouse CD45-APC-eFluor 780 (dilution 1:30), rat anti-mouse F4/80-APC (dilution 1:30), rat anti-mouse MHCII-PE-Cy7 (dilution 1:30), rat anti-mouse 11b-PE (dilution 1:30) (all from BD Biosciences), and rat anti-mouse CD206-peridinin-chlorophyll protein/Cy5.5 (dilution 1:30; BioLegend, San Diego, CA).

For intracellular protein detection in SCs, staining of surface antigens was followed by fixation and permeabilization with BD IntraSure Kit (BD Biosciences) according to the vendor's protocol. Primary rabbit polyclonal anti-Pax7 (dilution 1:200; Abcam) antibodies were applied, and appropriate goat-anti mouse Alexa Fluor 568 secondary antibody (dilution 1:400) was used. Cell cycle phases were

determined from Hoechst 33342 staining. The stained cells were analyzed with LSRFortessa flow cytometer (BD Biosciences).

Isolation of SCs

To isolate SCs (for analysis on RNA level or cell culture) the muscles were prepared similarly as for fluorescence-activated cell sorting (FACS) analysis. Cells were incubated with rat anti-mouse α 7i-PE (dilution 1:15; R&D Systems), rat anti-mouse CD34-fluorescein isothiocyanate (dilution 1:30), rat anti-mouse CD45-APC (dilution 1:30), rat anti-mouse Sca1-PE-Cy7 (dilution 1:30), rat anti-mouse CD31-APC (dilution 1:30) (all from BD Biosciences). After 30-minute incubation cells were sorted with MoFlo XDP cell sorter (Beckman Coulter, Pasadena, CA).

Primary SC Culture and Proliferation Analysis

Five thousand SCs isolated by FACS were cultured on gelatin-coated plates (Sigma-Aldrich) in growth medium that consisted of Dulbecco's modified Eagle's medium 4.5 g/L glucose (Lonza, Basel, Switzerland), 20% fetal bovine serum (Biowest, Nuaille, France), 10% horse serum (PAA Laboratories, Chicago, IL), and 5 ng/mL basic fibroblast growth factor (PeproTech, Rocky Hill, NJ). On the next day, cells were stimulated for 24 hours with a mix of proinflammatory cytokines that included IL-6 (1 ng/mL), IL-1 β (0.1 ng/mL), and MCP-1 (100 ng/mL) (all from Sigma-Aldrich). Afterward (2 days after sort), Edu (10 μ mol/L) was added for 2 hours, and Edu⁺ cells were visualized according to the vendor's instructions.

In addition, cells were immunostained for MyoD. To this purpose blocking was performed with 3% bovine serum albumin in PBS for 1 hour, and primary mouse anti-MyoD antibody (dilution 1:100; BD Biosciences) in blocking buffer was applied overnight at 4°C. Afterward, cells were rinsed and incubated for 2 hours with goat-anti-mouse Alexa Fluor 488 secondary antibody (dilution 1:200 in PBS) and Hoechst 33342 (2 μ g/mL). Percentage of Edu⁺ and MyoD⁺ cells was analyzed under fluorescent microscope Nikon (Melville, NY) Eclipse in at 20 fields of view per mouse.

RNA Isolation and Quantitative RT-PCR

Total RNA was isolated from muscle fragments by homogenization in 1 mL of Quiazol Total RNA Isolation Reagent using Tissue Lyzer (Qiagen, Hilden, Germany), according to the vendor's manual. Concentration and quality of RNA was determined spectrophotometrically (NanoDrop; Thermo Fisher).

cDNA template was synthesized from 1 μ g of total RNA with the use of NCode miRNA First-Strand cDNA Synthesis Kit (Thermo Fisher) according to the manufacturer's protocol. Quantitative PCR (qPCR) was performed with StepOne Plus Real-Time PCR (Applied Biosystems

Thermo Fisher) in a mixture that contained 1 $\times$ concentrated SYBR Green PCR Master Mix (Sigma-Aldrich), 10 μ mol/L primers forward and reverse (Tables 1 and 2) (universal reverse primer for miRNAs was supplied by the vendor), and 2 μ L of cDNA (10 $\times$ diluted). Gene expression was normalized to a constitutive gene (*EF2*) or small RNA (U6). Relative quantification of gene expression was calculated according to the comparative ΔC_T (threshold cycle value) method.

RT-qPCR on SCs

RT-qPCR on SCs was performed with AmpliSpeed system (Beckman Coulter Biomedical, Pasadena, CA). Fifty SCs per field on AmpliGrid slides were sorted and dried overnight at 4°C. RT reaction was performed with NCode VILO miRNA cDNA Synthesis directly on the slide on AmpliSpeed cycler. The obtained cDNA was used for qPCR reaction.

Protein Isolation

Total protein was isolated from snap-frozen muscle fragments by homogenization in 1 mL of lysis buffer (PBS + 1% Triton X-100) that contained inhibitors of proteinases (Roche Diagnostic, Basel, Switzerland), according to the vendor's protocol.

Luminex Cytokine Assay

Concentrations of IL-1 β , IL-6, MCP-1, monokine induced by interferon(IFN)- γ (MIG), IL-12, IFN- γ -induced protein (IP)-10, and VEGF were measured in plasma or in the protein isolated from muscles with the use of Milliplex FlexMap 3D (Millipore, Billerica, MA) according to the vendor's protocol.

Statistical Analysis

Data are presented as means $\pm$ SEM. Differences between groups were tested for statistical significance with the use of the unpaired 2-tailed *t*-test or *U* test for comparison of two groups, and analysis of variance with Bonferroni posttest when changes in multiple time points were analyzed. Grubb's test was used to identify statistically significant outliers. *P* < 0.05 was considered as significant.

Results

Lack of HO-1 Enhances Skeletal Muscle Degeneration and Inflammation after CTX-Induced Injury

In *Hmox1*^{+/+} animals the induction of *Hmox1* expression appeared on the first (mRNA) and third (protein) day after myoinjury, followed by a steady state decrease on subsequent days, concomitantly to decreasing inflammation (Figure 1, A and B and Supplemental Figure S1). HO-1 was

Table 1 Sequence of Primers Used in the Research

Transcript	Sequence of starters	
EF2	Forward	5'-GACATCACCAAGGGTGTGCAG-3'
	Reverse	5'-TCAGCACACTGGCATAGAGGC-3'
HO-1	Forward	5'-CCTCACTGGCAGGAAATCATC-3'
	Reverse	5'-CCTCGTGGAGACGCTTTACATA-3'
SDF-1 α	Forward	5'-CCTTCAGATTGTTGCACGGCTGA-3'
	Reverse	5'-CCCACCACTGCCCTTGCATG-3'
VEGF	Forward	5'-ATGCGGATCAAACCTCACCAAGGC-3'
	Reverse	5'-TTAACTCAAGCTGCCTCGCCTTGC-3'
Myf5	Forward	5'-CCTGTCTGGTCCCGAAAGAAC-3'
	Reverse	5'-GACGTGATCCGATCCACAATG-3'
myoD	Forward	5'-GCTGCCTTCTACGCACCTG-3'
	Reverse	5'-GCCGCTGTAATCCATCATGC-3'
myogenin	Forward	5'-CAGTACATTGAGCGCCTACAG-3'
	Reverse	5'-GGACCGAAGTCCAGTGCAT-3'
Pax7	Forward	5'-CAACCACATGAACCCTGTCA-3'
	Reverse	5'-GAGATGGAGGAAGCCGAGTC-3'
IL-1 β	Forward	5'-CTGGTGTGTGACGTTCCCATTA-3'
	Reverse	5'-CCGACAGCACGAGGCTTT-3'
IGF-1	Forward	5'-CTGGTGGATGCTCTTCAGTT-3'
	Reverse	5'-GCTGCTTTTGTAGCTTCAG-3'

HO, heme oxygenase; IGF, insulin-like growth factor; Pax7, paired box protein 7; SDF, stromal cell-derived factor; VEGF, vascular endothelial growth factor.

expressed in cells infiltrating muscle: CD45⁺ leukocytes, including F4/80⁺ macrophages (Figure 1C), and did not colocalize with apoptotic cells (TUNEL staining) (Supplemental Figure S1). In HO-1-deficient animals plasma activity of muscle degeneration markers (CPK, LDH) were elevated on the first day after CTX injection to a higher level than in *Hmox1*^{+/+} counterparts (Figure 2, A and B). The activity of LDH decreased from day 3 in both groups, but it remained higher in *Hmox1*^{-/-} mice. Moreover, histologic analysis (Figure 2, C and D) and staining of necrotic fibers (Figure 2E) presented more pronounced muscle degeneration and inflammatory infiltration on the third day after injury in *Hmox1*^{-/-} mice.

In skeletal muscle of *Hmox1*^{-/-} mice increased protein level of proinflammatory MCP-1, IL-6 on the first and third day after injury was found (Figure 3A). Although IL-1 β protein was below the threshold of detection, IL-1 β was elevated at the mRNA level (Figure 3B). Similarly, in plasma of injured *Hmox1*^{-/-} animals MCP-1 and IL-1 β were elevated, although no difference was found in the level of IL-6 (Figure 3C). It was accompanied by an increased

Table 2 Sequence of miRNA Primers Used in the Research

miRNA	Sequence of starters
U6	5'-CGCAAGGATGACACGCAAATTC-3'
miR-1	5'-GCTGGAATGTAAAGAAGTATGTAT-3'
miR-206	5'-TGGAATGTAAGGAAGTGTGTGG-3'
miR-133a/b	5'-TTGGTCCCTTCAACCAGCTGT-3'

concentration of MIG (CXCL9), IP-10 (CXCL10), and IL-12 (Figure 3C).

Decreased M1 and M1/M2 Ratio in *Hmox1*^{-/-} Animals after CTX Injury

Increased inflammation in CTX-injected *Hmox1*^{-/-} mice was also evident by increased number of leukocytes and higher percentage of monocytes and granulocytes at the expense of lymphocyte fraction in peripheral blood (Figure 4A). However, enhanced infiltration of injured gastrocnemius was not observed with CD45⁺CD11b⁺F4/80⁺ macrophages either on the first or third day of regeneration (Figure 4, B and C). More detailed FACS analysis of macrophage populations in skeletal muscles (Figure 4, B–F, and Supplemental Figure S2) showed that M1-like macrophages (CD45⁺CD11b⁺F4/80⁺MHCII^{high}CD206^{low}) were decreased in *Hmox1*^{-/-} animals (Figure 4E) in contrast to M2-like macrophages (CD45⁺CD11b⁺F4/80⁺MHCII^{low}CD206^{high}) (Figure 4F). Accordingly, M1/M2 ratio calculated for each mouse was significantly reduced in HO-1-deficient animals (Figure 4D).

Lack of HO-1 Increases Fiber Size Distribution and Arteriogenesis after CTX-Induced Injury

Slight up-regulation of Pax7 expression was observed in the skeletal muscle in *Hmox1*^{-/-} animals on day 28 (Figure 5A), whereas mRNA level of MyoD, myogenin (Figure 5A), and Myf5 (data not shown) was unaffected by *Hmox1* genotype. Similarly, no differences were found in the level of miR-1 (Figure 5A). Accelerated induction of miR-133a/b and miR-206 was visible in skeletal muscles of *Hmox1*^{+/+} animals in comparison with *Hmox1*^{-/-} mice (Figure 5A).

Changes in the percentage of newly formed fibers were not observed between mice of different *Hmox1* genotypes (Figure 5B) and mean cross-sectional area (Figures 5D and 2D). However, more detailed analysis of the size of regenerating myofibers on the 28th day after injury revealed that *Hmox1*^{-/-} muscle fibers of bigger size tend to be more frequent than in animals with normal HO-1 level (Figure 5C). Moreover, *Hmox1*^{-/-} regenerating fibers on the 28th day achieved similar size distribution to the one observed in intact muscles (day 0), whereas in *Hmox1*^{+/+} animals this process was slower (Figure 5C). Accordingly, a tendency to increased level of IGF-1 was observed in skeletal muscles of *Hmox1*^{-/-} animals throughout the process of regeneration after myotrauma (Figure 5E).

Staining of collagen deposition (Supplemental Figure S3A) and FACS analysis of FAP cells (CD45⁻CD31⁻ α 7i⁻Sca1⁺CD34⁺) (Supplemental Figure S3B) did not show differences in the rate of fibrosis between *Hmox1*^{+/+} and *Hmox1*^{-/-} animals. However, increased rate of arteriogenesis occurred in *Hmox1*^{-/-} animals, as reflected by a higher number of positive α -smooth muscle actin arterioles on the 28th day after injury (Supplemental Figure S4A).

Figure 1 Heme oxygenase-1 (HO-1) expression in gastrocnemius muscle of *Hmox1*^{+/+} and *Hmox1*^{-/-} animals after cardiotoxin (CTX)-induced injury in gastrocnemius muscle. **A** and **B**: HO-1 mRNA level; quantitative RT-PCR (**A**) and protein level; immunohistochemistry staining (**B**); representative images. **C**: Cellular expression of HO-1 in CD45⁺ and F4/80⁺ cells; immunohistochemistry staining; representative images. Data are expressed as means $\pm$ SEM. $n = 3$ to 5. $^{\dagger}P < 0.05$, $^{**}P < 0.01$ versus *Hmox1*^{+/+}. Scale bars: 10 μ m (**B**); 100 μ m (**C**).

Percentage of CD45⁻CD31⁺ endothelial cells was similar in skeletal muscles regardless of the level of HO-1 (Supplemental Figure S4B), as well as the expression of mRNA of VEGF and stromal cell-derived factor-1 α in skeletal muscles (Supplemental Figure S4C). *Hmox1*^{-/-} animals had, however, a higher concentration of VEGF in their plasma 1 and 3 days after injury (Supplemental Figure S4D).

Lack of HO-1 Enhances Proliferation of SCs on the First Few Days after CTX Injury

To examine if SCs were affected in *Hmox1*^{-/-} mice, FACS analysis of SCs was performed (Figure 6A). CD45⁻CD31⁻Sca1⁻ α 7i⁺CD34⁺ SCs revealed no differences both in quantity and proliferation between mice of different *Hmox1* genotypes (Figure 6B). However, activated SCs, lacking expression of CD34, (CD45⁻CD31⁻Sca1⁻ α 7i⁺CD34⁻) were decreased in *Hmox1*^{-/-} mice on the third day after CTX injection (Figure 6C). At this time proliferation of

CD45⁻CD31⁻Sca1⁻ α 7i⁺CD34⁻ cells was increased in *Hmox1*^{-/-} mice (Figure 6C), which was also confirmed by higher number of Pax7⁺Edu⁺ cells in muscle sections on the first day after injury (Figure 6D and Supplemental Figure S5A).

Analysis of the expression of Pax7 in SCs showed its raised level in CD45⁻CD31⁻Sca1⁻ α 7i⁺CD34⁻ *Hmox1*^{-/-} cells in intact muscles (Figure 6E), but no differences were found 1 day after CTX injection (Figure 6F). No changes in miR-1 and miR-206 between SCs of different genotypes were visible (Supplemental Figure S5B).

In Vitro Stimulation of *Hmox1*^{+/+} SCs with Proinflammatory Cytokines Partially Mimics the Effect of HO-1 Deficiency on Proliferation

We hypothesized that increased proliferation of SCs may be attributed to elevated concentration of proinflammatory cytokines in *Hmox1*^{-/-} skeletal muscles (Figure 3, A and B). Therefore CD45⁻CD31⁻Sca1⁻ α 7i⁺ cells were

Figure 2 Cardiotoxin (CTX)-induced injury in gastrocnemius muscle of *Hmox1*^{+/+} and *Hmox1*^{-/-} animals. **A** and **B**: Creatine phosphokinase (CPK) (**A**) and lactate dehydrogenase (LDH) (**B**) activity in plasma; activity assay. **C**: Microscopic assessment of gastrocnemius muscle degeneration; semiquantitative assessment. **D**: Representative images of hematoxylin and eosin–stained gastrocnemius muscle. **E**: Microscopic assessment of myofiber necrosis 3 days after CTX, percentage of necrotic fiber among total fiber number in injured area and representative images. Data are expressed as means $\pm$ SEM. $n = 3$ to 5. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus day 1; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, and $^{\dagger\dagger\dagger}P < 0.001$ versus *Hmox1*^{+/+}. Scale bars: 100 μ m (**D**); 50 μ m (**E**).

isolated and stimulated with 100 ng/mL MCP-1, 1 ng/mL IL-6, and 0.1 ng/mL IL-1 β for 24 hours. Slightly but statistically significant increased percentage of SCs was observed in the S phase on cytokine stimulation (Figure 7A). Importantly, HO-1–deficient SCs proliferated more intensively than wild-type counterparts. Simultaneously, increased percentage of MyoD⁺ cells among *Hmox1*^{-/-} SCs and SCs stimulated with cytokines was found (Figure 7B).

Subsequent Phases of Myotrauma and Tissue Regeneration throughout a Life Span Lead to Exhaustion of SCs in *Hmox1*^{-/-} Skeletal Muscle

To examine the effect of HO-1 level in multiple rounds of muscle injury and regeneration, mice of different *Hmox1* genotypes were injected three times with CTX with 2-week intervals, and all analyses were performed after the third injury. Analysis of SCs on the third day after final injection of CTX

Figure 3 Level of proinflammatory cytokines in cardiotoxin (CTX)-injured gastrocnemius muscle and plasma of *Hmox1*^{+/+} and *Hmox1*^{-/-} animals. **A:** Monocyte chemoattractant protein (MCP)-1 and IL-6 protein level in gastrocnemius muscle; Milliplex. **B:** IL-1β mRNA level in gastrocnemius muscle, quantitative RT-PCR. **C:** Protein level of MCP-1, IL-6, IL-1β, monokine induced by interferon (IFN)-γ (MIG; CXCL9), IFN-γ-induced protein (IP)-10 (CXCL10), IL-12 in plasma; Milliplex. Data are expressed as means ± SEM. *n* = 3 to 5. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 versus day 1; †*P* < 0.05, ††*P* < 0.01, and †††*P* < 0.001 versus *Hmox1*^{+/+}.

revealed decreased number of CD45⁺CD31⁺Sca1⁺α7i⁺CD34⁺ with no changes in CD45⁺CD31⁺Sca1⁺α7i⁺CD34⁺ SC quantity (Figure 8, A and B). Proliferation rate of CD45⁺CD31⁺Sca1⁺α7i⁺CD34⁺ *Hmox1*^{-/-} cells was decreased on the first day on the third injury (Figure 8, A and B). Similarly to animals subjected to single injection of CTX, higher level of leukocytes, with increased proportion of granulocytes and monocytes, and decreased lymphocyte percentage were observed in *Hmox1*^{-/-} animals (Supplemental Figure S6A). Increased CPK and LDH activity was visible in plasma of *Hmox1*^{-/-} mice (Supplemental Figure S6B), with no changes in the level of muscle degeneration and regeneration (Supplemental Figure S6C). In addition, on the first day of the final injury M1-like macrophages (CD45⁺CD11b⁺F4/80⁺MHCII^{high}CD206^{low}) were decreased in *Hmox1*^{-/-} mice. Similar tendency was observed on the third day both in M1 and M2 (CD45⁺CD11b⁺F4/80⁺MHCII^{low}CD206^{high}) macrophages, resulting in a decrease of total macrophage quantity at this time point (Supplemental Figure S6D).

Finally, SC quantity was analyzed in *Hmox1*^{+/+} and *Hmox1*^{-/-} mice of different age, demonstrating decreased number of both quiescent and activated SCs in 24-month-old animals lacking HO-1 (Figure 8C).

Discussion

Skeletal muscles are characterized by a high oxygen consumption; thus, enzymes with antioxidant properties were shown to be protective in this tissue.³⁰ High level of oxidative metabolism and reactive oxygen species generation in skeletal muscle is associated with expression of HO-1,^{31,32} that is additionally increased after a series of

contractions^{31,32} and is correlated to myoglobin content.³³ It was suggested that HO-1 can therefore protect against exercise-induced injury,^{32,34} whereas its lack increases apoptosis in skeletal muscles on ischemia.²⁷ Accordingly, in skeletal muscles of *Hmox1*^{+/+} mice in response to CTX an induction of HO-1 expression was observed, which did not colocalize with the presence of apoptotic cells. Accordingly, HO-1 expression was found elevated, particularly in cells infiltrating the muscle—CD45⁺ leukocytes, including F4/80⁺ macrophages. Similar localization of HO-1 is also visible after chronic injury in the murine model of Duchenne muscular dystrophy (*mdx* mice).³⁵ Therefore, HO-1 may be considered as a factor responsible for restricting the intensity of muscle injury. Indeed, a potent elevation of plasma markers of muscle degeneration (CPK, LDH), accompanied by enhanced injury in skeletal muscle tissue, was observed in *Hmox1*^{-/-} animals.

Of note, myogenic cells can be a source of MCP-1, IL-6, and IL-1β,^{36–38} which was found elevated in *Hmox1*^{-/-} skeletal muscle. Those cytokines were down-regulated by HO-1 in other cell types.^{39,40} Persistent HO-1 expression prevents up-regulation of MCP-1 in response to heme and contributes to tissue protection.⁴¹ Therefore, induction of IL-6, IL-1β, and MCP-1 in the injured HO-1-deficient skeletal muscles may be considered as a disturbance of this protective mechanism.

Enhanced inflammatory reaction that is characteristic for mice lacking HO-1^{16,17} was also reflected in the increased number of circulating leukocytes in CTX-injured *Hmox1*^{-/-} animals. Interestingly, in response to local injury, percentage of granulocytes and monocytes in peripheral blood of *Hmox1*^{-/-} mice was elevated at the expense of lymphocytes. Monocytes are considered as

Figure 4 Level of leukocytes in the peripheral blood and macrophages in gastrocnemius muscle of cardiotoxin (CTX)-injected $Hmox1^{+/+}$ and $Hmox1^{-/-}$ animals. **A:** Total blood cell count from peripheral blood. **B:** Gating strategy of CD45⁺F4/80⁺CD11b⁺ cells (macrophages), CD45⁺F4/80⁺CD11b⁺major histocompatibility complex II (MHCII)^{high}CD206^{low} cells (M1-like macrophages), and CD45⁺F4/80⁺CD11b⁺MHCII^{low}CD206^{high} cells (M2-like macrophages); fluorescence-activated cell sorting (FACS). **C–F:** Macrophages (**C**), M1/M2 macrophages ratio (**D**), M1-like macrophages (**E**), M2-like macrophages (**F**) in gastrocnemius muscle; FACS. Data are expressed as means $\pm$ SEM. $n = 5$. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus day 1; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, and $^{\dagger\dagger\dagger}P < 0.001$ versus $Hmox1^{+/+}$. SSC, side scatter.

Figure 5 Skeletal muscle regeneration in cardiotoxin (CTX)-injured gastrocnemius muscle of *Hmox1*^{+/+} and *Hmox1*^{-/-} animals. **A:** Paired box protein 7 (Pax7), MyoD, myogenin, miR-1, miR-133a/b, and miR-206 on mRNA level; quantitative RT-PCR (RT-qPCR). **B:** Percentage of centrally nucleated fibers; semiquantitative assessment. **C:** Percentage of myofibers of different size in intact muscle (day 0) and regenerating myofibers (days 14 and 28). **D:** Mean cross-section area of the fibers. **E:** Insulin-like growth factor (IGF)-1 on mRNA level; RT-qPCR. Data are expressed as means $\pm$ SEM. $n = 4$ to 5. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus day 1; † $P < 0.05$, †† $P < 0.001$ versus *Hmox1*^{+/+}.

major sources of MCP-1, IL-1 β , and IL-12.^{42–44} Accordingly, increased concentration of those proinflammatory cytokines was also observed in the plasma of *Hmox1*^{-/-} animals in response to CTX. Although changes in the level of IFN- γ were not observed between groups (data not shown), IFN- γ -related cytokines MIG

(CXCL9) and IP-10 (CXCL10) were elevated in the plasma of *Hmox1*^{-/-} animals.

Despite increased monocyte percentage in peripheral blood and potent induction of MCP-1 in skeletal muscles, augmented infiltration of CD45⁺CD11b⁺F4/80⁺ macrophages was not observed after injury in *Hmox1*^{-/-} skeletal

Figure 6 Satellite cell (SC) quantity and proliferation in cardiotoxin (CTX)-injured gastrocnemius muscle of *Hmox1*^{+/+} and *Hmox1*^{-/-} animals. **A:** Gating strategy of CD45⁻CD31⁻ stem cell antigen-1 (Sca1)⁻α7i⁺CD34⁺ cells (SCs) and CD45⁻CD31⁻Sca1⁻α7i⁺CD34⁺ cells (activated SCs); fluorescence-activated cell sorting (FACS). **B:** Percentage of SCs and proliferating SCs; FACS. **C:** Percentage of activated SCs and proliferating activated SCs; FACS. **D:** Percentage of proliferating SCs [paired box protein 7 (Pax7)⁺/5-ethynyl-2-deoxyuridine (Edu⁺)] among Pax7⁺ cells; immunohistochemistry staining of paraffin section. **E** and **F:** Level of Pax7 protein in SCs (**E**) and activated SCs (**F**); FACS. Data are expressed as means ± SEM. *n* = 4 to 10. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 versus day 1; †*P* < 0.05, ††*P* < 0.001 versus *Hmox1*^{+/+}. FCS, forward scatter; SSC, side scatter.

muscle. On the contrary, when percentage of proinflammatory macrophages (M1-like phenotype, MHCII⁺C-D206^{low},⁴⁵) was analyzed, there was a tendency to their delayed accumulation. Accordingly, a ratio of M1-like/M2-like macrophages was changed on the first day after injury in *Hmox1*^{-/-} animals. Decreased proportion of M1/M2 macrophages observed in HO-1-deficient mice may influence angiogenesis²¹ and fibrosis⁴⁶ within skeletal muscle. However, differences were not observed either in FAP cells or collagen deposition between *Hmox1*^{+/+} and *Hmox1*^{-/-}

mice. Declined ratio of M1/M2 macrophages, as well as increased concentration of plasma VEGF and MCP-1, can induce blood vessel formation^{21,47} and, hence, may increase arteriole formation in *Hmox1*^{-/-} mice.

Because macrophages are indispensable mediators of myogenic regeneration,⁸ it may be suspected that decreased ratio of M1-like population affects restoration of muscle architecture. However, expression of muscle regulatory factors (MyoD, Myf5, myogenin) was not changed in HO-1-deficient mice during skeletal muscle regeneration.

Figure 7 *In vitro* proliferation and level of MyoD in satellite cell (SC) culture stimulated with monocyte chemoattractant protein (MCP)-1, IL-6, IL-1β. **A:** Percentage of 5-ethynyl-2-deoxyuridine (Edu)⁺ cells in SC culture; immunocytochemical staining. **B:** Percentage of MyoD⁺ cells in SC culture; immunocytochemical staining. Each dot represents cells from 1 animal. *n* = 4. **P* < 0.05 versus unstimulated control; [†]*P* < 0.05 versus *Hmox1*^{+/+}. Scale bars = 100 μm.

No differences were observed in the expression of miR-1; however, miR-206 and miR-133a/b were down-regulated in *Hmox1*^{-/-} animals. Reduction in miR-206 level in *Hmox1*^{-/-} skeletal muscle on the 28th day after CTX injection corresponded with an increased expression of Pax7, which is a target of miR-206 also on the mRNA level.⁴⁸ Although the lack of HO-1 was shown to induce miR-206 expression in primary myoblasts cultured *in vitro*,^{25,26} during regeneration of injured muscle this effect is apparently

overwhelmed by the impact of proinflammatory cytokines, which inhibit myomirs expression⁴⁹ and are elevated in muscles of *Hmox1*^{-/-} mice.

Although no variation in the percentage of newly formed myofibers was found in CTX-injected mice of different *Hmox1* genotype, or mean cross-sectional area, regenerating myofibers of larger size tended to be more frequent in *Hmox1*^{-/-} mice than in *Hmox1*^{+/+} animals. Furthermore, they achieved distribution of size presented by intact muscle

Figure 8 Changes in satellite cell (SC) quantity and proliferation in gastrocnemius muscle of *Hmox1*^{+/+} and *Hmox1*^{-/-} animals after repeated rounds of injury-regeneration and with age. **A** and **B**: Percentage and proliferation of quiescent (**A**) and activated (**B**) SCs in gastrocnemius muscle of *Hmox1*^{+/+} and *Hmox1*^{-/-} control animals after triple injection of cardiotoxin (CTX); fluorescence-activated cell sorting (FACS). **C**: Percentage of SCs and activated SCs in different ages of *Hmox1*^{+/+} and *Hmox1*^{-/-} animals and representative images from FACS analysis of SCs of *Hmox1*^{+/+} and *Hmox1*^{-/-} 24-month-old animals for the same number of nucleated cells. Data are expressed as means ± SEM. *n* = 4 to 9. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 versus day 1; †*P* < 0.05, ††*P* < 0.01 versus *Hmox1*^{+/+}. Sca1, stem cell antigen-1.

earlier than muscles of normal HO-1 level. This moderate effect on myofiber maturation might first be partially ascribed to increased IGF-1 expression, which is a known factor that affects the diameter of muscle fibers.^{2,5} Second, a

potent induction of proinflammatory cytokines observed in *Hmox1*^{-/-} muscles can also result in hypertrophy, because MCP-1⁵⁰ and IL-6^{51,52} were shown to induce myofiber size. Third, miR-206 is expressed in regenerating myofibers,⁵³

acting there as a factor to reduce hypertrophy.⁵⁴ Because its lower expression is found in *Hmox1*^{-/-} animals, it may be an additional factor to increase fiber diameter. Higher expression of miR-206 in *Hmox1*^{+/+} mice may be also a sign that myofibers are immature and are still regenerating. Finally, M2 macrophages were shown to promote fusion and differentiation of SCs⁸; thus, elevated proportion of M2 population in *Hmox1*^{-/-} animals may be responsible for the enlarged myofiber.

SCs in skeletal muscles of *Hmox1*^{+/+} and *Hmox1*^{-/-} mice were analyzed because they might affect the regeneration rate of skeletal muscle. Increased proliferation of both Pax7⁺ cells on the first day of regeneration and activated SCs (CD45⁻CD31⁻Sca1⁻α7i⁺CD34⁻)^{55–57} isolated from skeletal muscle of *Hmox1*^{-/-} animals on the third day after injury was observed. Despite this, no differences were observed in the quantity of SCs (CD45⁻CD31⁻Sca1⁻α7i⁺CD34⁺); however, the number of activated SCs (CD45⁻CD31⁻Sca1⁻α7i⁺CD34⁻) was even decreased in *Hmox1*^{-/-} mice. This effect does not appear to be mediated by the miR-1 or miR-206 because no differences were observed in their expression in SCs. However, activated SCs from HO-1-deficient mice showed higher expression of Pax7. It was shown, that Pax7 is necessary for cell cycle entry because it induces expression of genes that promote proliferation of SCs and therefore allows their population to expand.^{58–60} Hence, elevated expression of Pax7 may make *Hmox1*^{-/-} SCs predisposed to accelerated activation.

Proinflammatory cytokines (MCP-1, IL-1β, IL-6) are known to induce proliferation of SCs.^{51,52,61,62} *In vitro* stimulation of *Hmox1*^{+/+} SCs with these cytokines enhanced proliferation, although not to the level observed in SCs lacking HO-1. In addition, SCs stimulated with proinflammatory cytokines and SCs of *Hmox1*^{-/-} phenotype presented increased expression of MyoD, which is in accordance to previously observed effects of HO-1 deficiency in primary myoblasts.²⁵ Therefore, we suggest that both the lack of HO-1 in SCs and the stimulation of SCs with MCP-1, IL-1β, IL-6 cocktail accelerated their proliferation and shifted SCs toward more differentiated myogenic population. This is in accordance with the accelerated proliferation of *Hmox1*^{-/-} SCs after CTX injury, hypertrophic effect observed in mice of *Hmox1*^{-/-} genotype, and with the previous results that showed the increased differentiation potential of cells with decreased level of HO-1.²⁴

It was further examined whether differences between *Hmox1*^{+/+} and *Hmox1*^{-/-} muscles can be augmented after repeated injury. In *Hmox1*^{-/-} animals subjected to triple CTX injection similar global changes as in animals with single injury were observed: increased leukocytes in peripheral blood with increased proportion of granulocytes and monocytes, increased LDH and CPK plasma activity, decreased M1-like and total macrophage infiltration into skeletal muscle, without significant differences in muscle

damage or regeneration. However, in contrast to single injury, subsequent rounds of muscle damage followed by myogenic restoration seem to depress proliferation of *Hmox1*^{-/-} SCs, what may be a reason for lower number of activated SCs observed on a third day after final CTX injection. The quantity of SC population remains usually stable despite multiple injuries within skeletal muscle.⁶³ Thus, it can be speculated that the proper level of HO-1 during muscle injury may ensure protection against precocious activation of SCs observed in *Hmox1*^{-/-} mice, where repeated rounds of degeneration/regeneration can impair proliferation of SCs and in turn decrease their number.

During a lifetime a reduction in the number of SCs was observed.^{64–66} Therefore, the quantity of SCs in sedentary skeletal muscles of *Hmox1*^{+/+} and *Hmox1*^{-/-} mice was analyzed. In 24-month-old *Hmox1*^{-/-} animals a reduced pool of SCs was found. SCs-depleted mice cannot regenerate muscle at all after CTX injury,^{67–69} and decreased number of SCs is one of the factors to impair the regenerative capacity in aged skeletal muscle,^{70,71} although it does not affect steady state conditions of the tissue.^{64,70} Of note, in Duchenne muscular dystrophy, in which continuous rounds of muscular degeneration and regeneration may lead to exhaustion of the SC pool, hampered regeneration was also shown.⁷² It might be speculated that impaired HO-1 expression may additionally potentiate this effect.

Conclusions

It was shown that the lack of HO-1 augments skeletal muscle injury and expression of proinflammatory MCP-1, IL-6, and IL-1β during the regeneration process in animals subjected to CTX injection. Disturbed proportion of M1/M2 macrophages with a tendency to diminished M1 population, together with increased IL-6, MCP-1, and IGF-1, accompanied by enhanced formation of arterioles may be responsible for hypertrophic effect and increased rate of regeneration observed in injured *Hmox1*^{-/-} skeletal muscle. Finally, HO-1-deficient SCs are prone to activation and have higher proliferation after injury. This effect can be partially mimicked by stimulation of *Hmox1*^{+/+} SCs with MCP-1, IL-6, and IL-1β and is associated with increased MyoD expression, suggesting that SCs from *Hmox1*^{-/-} animals are shifted toward more differentiated myogenic population.

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M.K. and J.D. designed the experiments; M.K., K.B.-S., A.L., A.J., and J.D. analyzed the data; M.K., K.P.-G., M.C., M.S., I.B.-B., P.P., and K.B.-S. performed research; and M.K., A.J., and J.D. wrote the paper.

Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2017.10.017>.

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