2014

Ischemia-induced inflammation is increased and satellite-cell activation is decreased in TNFR2/P75 knockout hindlimb ischemia model

BOSTON UNIVERSITY

SCHOOL OF MEDICINE

Thesis

ISCHEMIA-INDUCED INFLAMMATION IS INCREASED AND SATELLITE-CELL ACTIVATION IS DECREASED IN TNFR2/P75 KNOCKOUT HINDLIMB ISCHEMIA MODEL

by

LAYLA M. RAHIMI

B.A., Boston University, 2010

Submitted in partial fulfillment of the

requirements for the degree of

Master of Science

2014

Approved by

First Reader

Gwynneth D. Offner, Ph.D.
Director, M.A. in Medical Sciences Program
Assistant Dean for Admissions
Associate Professor of Medicine

Second Reader

David Goukassian, M.D., Ph.D.
Director, CardioVascular Research Center
Associate Professor of Medicine
Tufts University School of Medicine

ACKNOWLEDGMENTS

I would first like to thank my readers, Dr. David Goukassian, Sharath Sasi, and Dr. Gwynneth Offner, for your mentorship and all that you've taught me while working on this project. I have learned so much during this past year and it has been such a wonderful experience.

I would also like to thank my parents for their endless encouragement and for inspiring me to always work extremely hard to achieve my goals. To my younger brother and sister, thank you for being a constant source of support.

ISCHEMIA-INDUCED INFLAMMATION IS INCREASED AND SATELLITE-CELL ACTIVATION IS DECREASED IN TNFR2/P75 KNOCKOUT HINDLIMB ISCHEMIA MODEL

LAYLA M. RAHIMI

ABSTRACT

Objective: Tumor necrosis factor-alpha (TNF-α) is a multifunctional proinflammatory cytokine that plays a critical role in mediating inflammatory and immunological responses. TNF-α has been shown to elicit both beneficial and detrimental biological effects by acting through its two receptors, TNFR1/p55 and TNFR2/p75. Previous studies from this laboratory have shown that TNF-TNFR2/p75 signaling plays a critical role in ischemia-induced neovascularization in muscle and heart tissues. However, the role of TNF-TNFR2/p75 signaling in ischemia induced inflammation and muscle regeneration remains to be characterized.

Methods: To evaluate ischemia induced inflammation responses, young wild type (WT) and young TNFR2/p75 knockout (p75KO) mice were subjected to unilateral hind limb ischemia (HLI) surgery. Operated hind limb tissue samples were collected at 1, 3, 7, and 10 days post-HLI surgery and studied for neutrophil (myeloperoxidase-1 positive cells) and macrophage (F4/80 positive cells) infiltration as well as satellite-cell activation (neural cell adhesion molecule positive cells) at each time point. To determine possible synergistically negative roles of tissue aging and the absence of TNFR2/p75 in either the tissue or bone marrow (BM), two chimeric BM transplantation (BMT) models were generated where young Green Fluorescent Protein (GFP) positive (+) p75KO and WT

BM-derived cells were transplanted into adult p75KO mice. HLI surgery was performed one month post-BMT, after confirming complete engraftment of the recipient BM with GFP donor cells. Operated hind limb tissue samples were evaluated up to 28 days post-surgery to examine proliferation and apoptosis of BM-derived cells in ischemic tissue.

Results: Ischemia induced significant and long-lasting inflammation associated with a considerable decrease in satellite-cell activation in p75KO muscle tissue 1-10 days post-HLI surgery. For the BMT studies, in adult p75KO with the WT-BMT, proliferative (Ki67+) cells were detected only by day 28 and were exclusively GFP (+), suggesting delayed contribution of young WT-BM cell to adult p75KO ischemic tissue recovery. No GFP (+) young p75KO BM cells survived in adult p75KO tissue.

Conclusion: The data demonstrate that: (1) ischemia-induced recovery in skeletal muscle tissue is impaired in young p75KO mice; (2) inflammatory responses are significantly increased and long-lasting in p75KO mice; (3) in the absence of TNFR2/p75 signaling, satellite-cell activation is affected in p75KO mice; (4) during post-ischemic recovery, tissue aging combined with decreased/absent TNFR2/p75 signaling may have synergistically negative roles on survival and proliferation in the damaged tissue.

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BM	Bone Marrow
BMT	Bone Marrow Transplantation
NCAM	Neural Cell Adhesion Molecule
FACS	Fluorescent Activated Cell Sorting
GFP	Green Fluorescent Protein
H&E	Hematoxylin and Eosin
HLI	Hind Limb Ischemia
MAPK	Mitogen-Activated Protein Kinase
MNCs	Mononuclear Cells
NF-κB	Nuclear Factor-kappa-B
MPO-1	Myeloperoxidase-1
p75KO	p75 Knockout
PB-MNC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
TNF-α	Tumor Necrosis Factor-alpha
TNFR1/p55	Tumor Necrosis Factor Receptor 1/p55
TNFR2/p75	Tumor Necrosis Factor Receptor 2/p75
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling
WT	Wild Type

INTRODUCTION

Tumor Necrosis Factor-Alpha

Tumor Necrosis Factor-alpha (TNF-α) is a multifunctional pleiotropic cytokine produced by activated macrophages and other mononuclear leukocytes. It elicits a wide variety of both beneficial and detrimental biological effects, with its primary role involving mediation of inflammatory and immunologic responses.[1,2] TNF-α induces cell adhesion molecules, elicits hemorrhagic necrosis of some tumor types, activates macrophages, T and B cells, and can serve an antiviral role in vivo by inhibiting cells with a number of viruses.[3,4] It is also constitutively produced in tumors, augmenting their development and spread by inducing angiogenesis. [5–10]

Angiogenesis and TNF-α

Angiogenesis is the development of new collateral blood vessels and is a physiological response to ischemia.[11] Following extensive arterial perfusion impairment, the extent of damage and ischemic recovery are largely dependent upon this process of forming new blood vessels.[12] TNF-α is a proinflammatory cytokine expressed in ischemic tissue[13] and plays a critical role in ischemia-induced neovascularization in muscle and heart tissues.[12] TNF-α can be both proangiogenic and antiangiogenic by acting through its two receptors, Tumor Necrosis Factor Receptor 1 (TNFR1)/p55 and Tumor Necrosis Factor Receptor 2 (TNFR2)/p75, which are present on a wide variety of cell types.[14–17] Because p55 signaling mediates cytotoxic effects while p75 facilitates

protective effects of TNF-α,[1,18] TNF signaling through its two receptors may have opposing actions regarding neovascularization and recovery following an ischemic event. Although angiogenesis is critical to recovery in cases of arterial obliteration, this process can also be considered pathological, as angiogenesis is required for tumor growth and metastasis.[11]

Angiogenesis and Aging

Many studies have previously documented age-related impairment of angiogenesis, [19–22] including decreased expression of angiogenic growth factors[19,23–25] and inhibition of endothelial cell proliferation and function with age. Since aging has also been shown to be associated with increased expression of p55 and decreased expression of p75 in human lymphocytes,[2] prior studies from this laboratory have examined ischemia-induced neovascularization and aging in p75-knockout (p75KO) mice.[12] Through this model, it was demonstrated that signaling through the p75 receptor plays a critical role in ischemia-induced neovascularization with advanced age via modulation of several angiogenic growth factors.[12] The role of TNFR2/p75 receptor in ischemia-induced inflammation and muscle regeneration remains to be characterized.

TNF-α and Inflammation

Monocyte/macrophage accumulation, which produces a variety of cytokines including TNF-α, is critical to the development of new collateral blood vessels.[13] TNF-α is a potent paracrine and endocrine mediator of inflammatory and immune functions[13,26]

and can induce the expression of many angiogenesis related and immunologically relevant genes through its two receptors.[27–31] While p75 facilitates the protective effects of TNF-α, p55 has been shown to modulate the cytotoxic effects, including apoptotic signaling.[26,32]

TNF-α binding to its TNFR1/p55 receptor is thought to be the signaling pathway by which most of the prototypical TNF-α functions occur, including induction of adhesion molecules, antiviral activity, and cytotoxicity. It is the receptor believed to be required by various cells to induce the expression of inflammatory response proteins by activating the pro-inflammatory nuclear factor-kappa-B (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways.[33] The molecular mechanisms underlying p55 signaling have been well characterized and many of the relevant proteins involved in this process have been identified.[34] Upon stimulation with TNF-α, adaptor proteins are recruited to the cytosolic death domain of TNFR1/p55.[33,34] Additional cytosolic adaptor proteins are recruited and mediate the activation of inflammatory signaling and the expression of TNF-α target genes as part of the pro-inflammatory response.[33,34]

Although the underlying mechanisms of TNFR2/p75 signaling are still unknown, previous studies have suggested that this receptor facilitates neuro-, cardio-, and osteo-protective effects, and promotes survival following myocardial infarction in mice.[33] Prior studies have shown that p75 signaling mediates several angiogenic growth factors in mice, promoting ischemia-induced angiogenesis. In addition, it has been demonstrated that blocking p75 in cultured Lewis Lung Carcinoma cells led to increases in TNF mediated apoptosis in the cancer cells.

Because aging is associated with a steady decline in immune functions[35,36] as well as decreased p75 expression[2], the present report examines the specific role of TNFR2/p75 signaling in ischemia-induced inflammation .

The Present Study

In the current study, it is hypothesized that ischemia induced inflammation responses are impaired in TNFR2/p75KO mice after hind limb surgery and TNFR2/p75 deficiency affects satellite-cell activation at the time of ischemic recovery. To test these hypotheses, neutrophil and macrophage infiltration were studied as well as satellite-cell activation following hind limb ischemia induction in young p75KO and age-matched WT mice. The possible synergistically negative roles of tissue aging combined with absence of TNFR2/p75 receptor in either tissue or bone marrow (BM) was examined by using BM transplant (BMT) models. In the present report, it is shown that ischemia induced inflammation responses are increased and longer lasting, and ischemia-induced satellite-cell activation is impaired in p75KO mice.

Specific Aims (hypotheses)

(1) Decrease in TNFR2/p75 expression contributes to impairment in post-ischemic skeletal muscle tissue recovery

(2) Ischemia induced inflammatory responses are impaired in TNFR2/p75 after hind limb surgery

(3) TNFR2/p75 deficiency affects satellite-cell activation at the time of ischemic recovery

(4) Tissue aging combined with absent TNFR2/p75 signaling may have synergistically negative roles on survival, proliferation and regeneration of the damaged tissue during post-ischemic recovery

METHODS

Animal Model

All animals were handled in accordance with and housed under protocols approved by GeneSys Research Institute Institutional Animal Care and Use Committee (Boston, MA). The experimental animal models used in this study were male WT (C57BL/6J) and p75KO (B6. 129S2-Tnfrsf1btm1Mwm/J) mice purchased from the Jackson Laboratory (Bar Harbor, ME). Young (4-6 weeks) and adult (10-12 months) mice were used for hind limb ischemia (HLI) and BMT studies.

For BMT studies, young homozygous p75KO/Green Fluorescent Protein (GFP) positive (+) mice were created. In order to obtain these mice, young WT/GFP (+) mice were crossed with p75KO/GFP negative (-) mice for generations until GFP (+) homozygous breeders were acquired. Any GFP (-), WT, or heterozygous littermates were excluded and all homozygous TNFR2/p75KO and GFP (+) mice were genotyped to respective controls as per guidelines and protocols set by Jackson Laboratory.

Hind Limb Surgery and Tissue Collection

Unilateral hind limb ischemia was surgically induced in young WT, young p75KO, and adult p75KO BMT mouse models. For each mouse, an incision was made in the middle of the hind limb in order to expose the femoral artery. This artery was then ligated at the proximal end, followed by the saphenous end. Next, side-branches of the femoral artery were dissected free, the femoral artery along with its side branches were

excised, and the incision was closed.[11,12] All animals were monitored carefully post-surgery and those found to exhibit severe infection or pain with limited mobility, reduced food and water consumption, weight loss of 15% or more were euthanized immediately.

All mice were euthanized at designated time points using a pentobarbital based euthanasia solution (200 mg/kg intra-peritoneal). Operated HL muscle samples for both young WT and p75KO genotypes were collected at 1, 3, 7 and 10-days post-HLI surgery (Figure 1) and at 28 days post-HLI surgery for adult p75KO BMT model studies along with pre-surgery controls. Muscle samples were bisected completely and divided into two parts. First, for paraffin embedding after buffered formalin fixation overnight. Second, for optimal cutting temperature embedding (Tissue-Tek, Torrance, CA) after 4% paraformaldehyde (PFA) fixation overnight followed by subsequent 15% and 30% sucrose incubation overnight at 4°C. All animals had free access to food and water pre- and post-operatively.

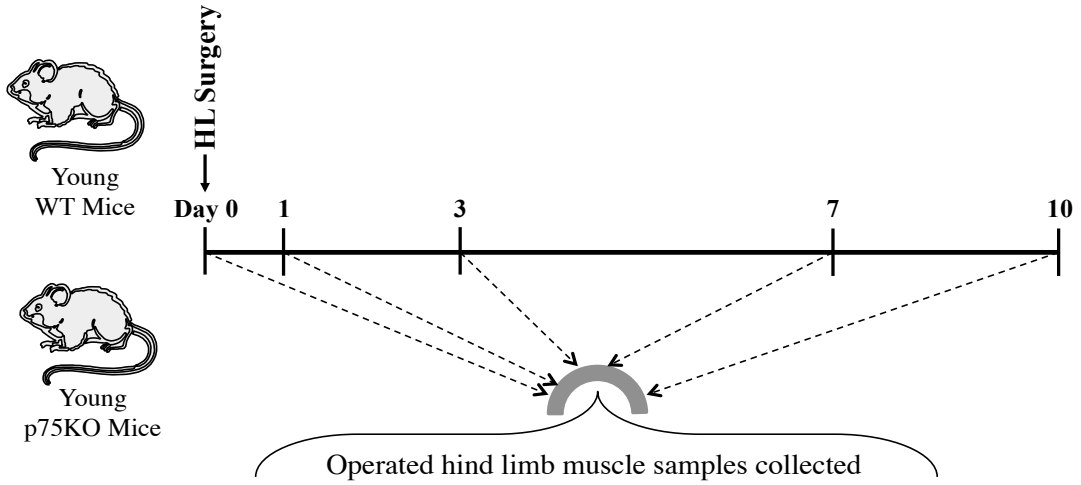

Figure 1. Timeline of hind limb ischemia surgery and tissue collection. Following ischemia induction, tissue samples were obtained on days 1, 2, 7, and 10 post-surgery in both wild type (WT) and p75 knockout (p75KO) mice.

Histology Staining and Immunohistochemistry

Paraffin embedded sections of HL muscle post-HLI surgery were processed for Hematoxylin and Eosin (H&E) using manufacturer's protocol (Electron Microscopy Sciences, Hatfield, PA). Macrophage infiltration in ischemic tissue was assessed by enzyme pre-treatment using proteinase K solution (20 ug/mL), followed by staining paraffin sections with rat anti-mouse F4/80 monoclonal antibody (AbD Serotec, Raleigh, NC), as per manufacturer data sheet. Tissue sections were then incubated with biotinylated secondary antibody, ABC Vectastain (Vector Laboratories, Burlingame, CA, USA) followed by 3, 3´-diaminobenzidine (Vector Laboratories) for visualization of F4/80 staining[37,38] and counterstained with hematoxylin for nuclei.

Fixed frozen muscle tissue sections obtained at 6-8 μm thickness were fixed in cold acetone (4°C) for 10 minutes[12,39] and then processed for immunofluorescent stainings. Neutrophil infiltration in ischemic tissue post-HLI surgery was evaluated by staining with myeloperoxidase-1 (MPO-1, Biocare Medical, Concord, CA) antibody and Alexa Fluor 488 goat anti-rabbit secondary antibody (Life Technologies).

To evaluate post-HLI surgery satellite-cell activation in ischemic tissue, frozen sections were stained with monoclonal rat anti-mouse embryonic neural cell adhesion molecule (NCAM, BD Bioscience, San Jose, CA) and Alexa Fluor 555 goat anti-rat secondary antibody (Life Technologies).

Imaging and analysis

Post-HLI surgery, muscle samples stained for H&E and F4/80 were imaged using light microscopy at x20 magnification. All immunofluorescent stained muscle slides were imaged using a Laser Scanning Confocal Microscope (Zeiss 510, Carl Zeiss, Oberkochen, Germany). Number of MPO-1 (+) cells and NCAM (+) cells were evaluated in at least 4-5 animals/group using Image J program (v1.40, NIH) in 7-8 separate visual fields of 176,400 μm^2 per mouse per time point for each genotype (x20 images). Results were plotted as a graph between mean number of cells and treatment time point post-HLI surgery.

Quantification of Central Nuclei/Muscle Fiber

Muscle tissue post-HLI surgery from young WT and p75KO mouse HL were carefully bisected, cross-sectioned through the middle, and fixed in formalin. H&E stained tissue sections (6 μm) were visualized using a light microscope (Leica Microsystems GmbH, Wetzlar, Germany). Images of the full circumference of the muscle cross sections were taken at x20 magnification and the average number of muscle fibers per visual field was 85 individual fibers (Nikon Instruments Inc., Melville, NY, USA). Muscle tissue samples from 4-5 animals of WT and p75KO mice were coded and analyzed by two laboratory members independently of each other, who were blinded to the treatment conditions. Results were represented as mean number of central nuclei/muscle fiber.

Murine Bone Marrow Transplantation Studies

Two chimeric BMT models were generated in order to investigate the roles of both tissue aging and p75KO. In model A, BM mononuclear cells (BMCs) from young (4-6 weeks old) WT/GFP (+) were transplanted into adult (10-12 months old) p75KO mice. In model B, BMCs from young p75KO/GFP (+) were transplanted into adult p75KO mice.

BM cells from young GFP (+) donor mice were obtained by flushing the tibias and femur and then subjecting to density centrifugation using Histopaque-1083 (Sigma, St. Louis, MO).[40,41] Isolated BM-MNCs at a concentration of 3 x 10^6 cells per mouse were administered by tail-vein injection into 9-11 Gy lethally gamma-irradiated, adult p75KO recipient mice (Figure 2).[42]

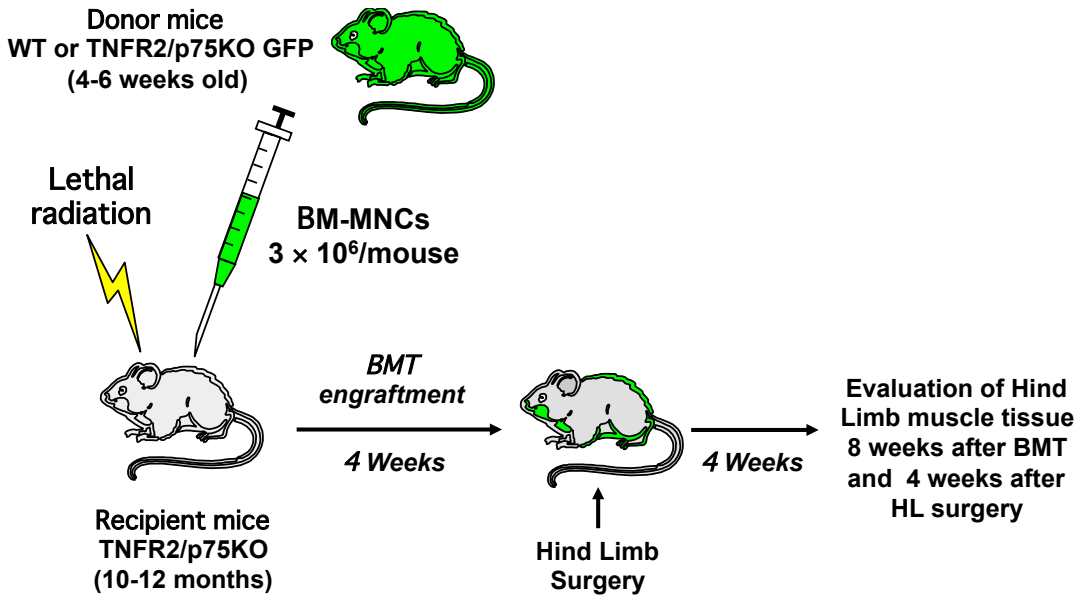

Figure 2. Experimental design for Bone Marrow Transplantation experiments. Chimeric BMT models were created using young GFP (+) WT and p75KO donor mice and adult p75KO recipient mice. After full engraftment (4 weeks after BMT), HLI surgery was performed on adult p75KO mice transplanted with either WT or P75KO young bone marrow and HL muscle tissue samples were evaluated 8 weeks post-surgery.

To evaluate the engraftment of recipient BM with donor GFP (+) cells, BM-derived cells as well as peripheral blood mononuclear cells (PB-MNCs) from these recipient mice at 1 and 2 months post-BMT were processed for fluorescent activated cell sorting (FACS) analysis. HLI surgery was performed on these recipient mice 4 weeks after BMT, by which time the BM of recipient mice usually regenerates by donor GFP (+) BM cells.[12]

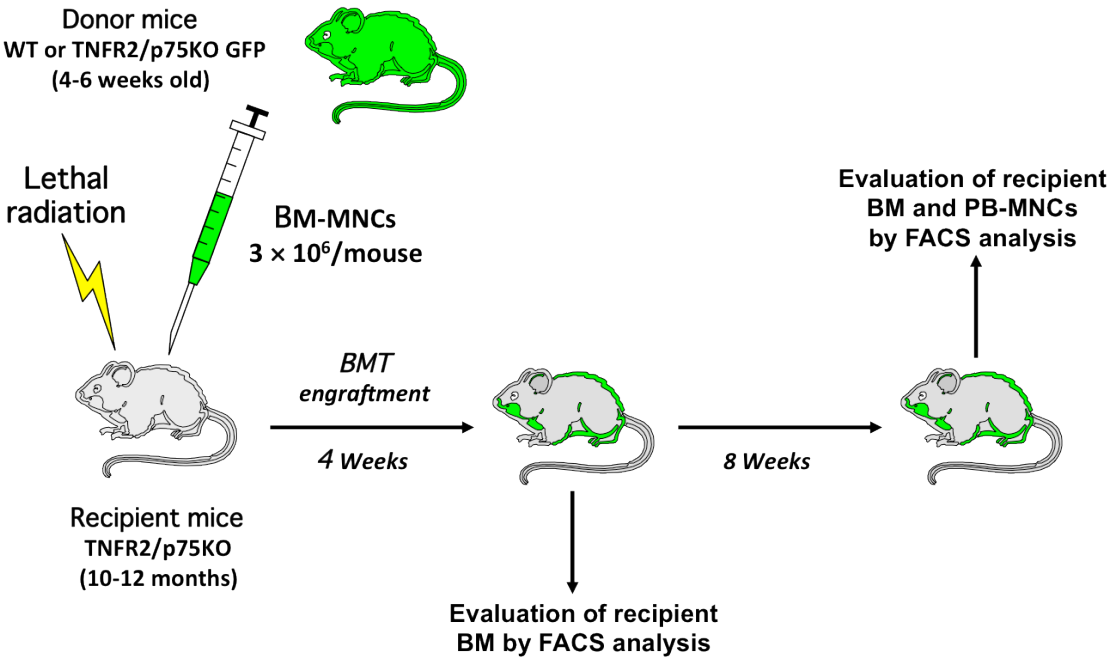

Figure 3. Experimental design for Bone Marrow Transplantation study evaluating complete engraftment of recipient bone marrow with donor cells. Adult p75KO chimeric BMT models were created using young GFP (+) WT and p75KO mice as donors for young BM cells. FACS analysis of cells derived from BM and peripheral blood (PB) mononuclear cells (MNCs) was used to evaluate engraftment of BM-derived GFP (+) cells in to recipient p75KO adult mice at 4 weeks and 8 weeks after BMT.

Proliferation and apoptosis of BM-derived cells in ischemic tissue for BMT studies were evaluated by separately staining for rabbit polyclonal Ki67 antibody (Vector Labs) along with Alexa Fluor 555 goat anti-rabbit secondary antibody (Life Technologies) and ApopTag Red In Situ Apoptosis Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling Kit (TUNEL, Millipore, Billerica, MA). TO-PRO3 nuclear staining (Life Technologies) was used in conjunction with all immunofluorescent staining to visualize cell nuclei.

Statistical Analysis

All results were expressed as mean $\pm$ SEM and plots obtained from at least 4-5 mice per group for each time point. Statistical analysis was performed on the data by one-way ANOVA (Stat View Software, SAS Institute Inc., Middleton, MA). Differences were considered significant at $P < 0.05$.

RESULTS

Diminished Post-Ischemic Recovery in Young p75KO Mice

Histological analysis of WT and p75KO ischemic skeletal muscle tissue demonstrated contrasting morphologic changes over 3-10 days post-HLI surgery when compared to each other and respective normal tissue pre-surgery controls. Compared to pre-surgery normal muscle tissue (Figure 4A), H&E staining of young WT ischemic tissue over 10-days showed decreased muscle fiber density and increased non-specific, cellular infiltrates and conglomerates, perhaps immature inflammatory or progenitor cells, on days 3 (Figure 4C). By day 7, there was a mild inflammatory response, prominent myofibroblastic proliferation and early immature fibrosis (Figure 4D), as well as nuclear fibers with central nuclei, an indication of muscle regeneration. There was a substantial increase in regenerative activity of muscular tissue by day 10 post-HLI surgery, which is evident by a large number of muscle fibers with one or more centrally located nuclei, accompanied by significant capillary hyperplasia and intermysial cellular expansion (Figure 4E). This can be attributed to early and mild inflammatory response followed by substantial muscle regeneration in WT tissue.

Compared to pre-surgery normal p75KO tissue (Figure 4F), young p75KO ischemic tissue revealed significant loss of normal skeletal muscle morphology by day 3 (Figure 4H). In p75KO muscle tissue on days 7 and 10, there were features of degeneration such as prominent apoptosis, cytoplasmic vacuolization, kariorexis, and kariopyknosis. These pathological changes were accompanied by a significant loss of

muscle fibers that were replaced by cellular infiltration and central location of residual nuclei, mostly represented by chronic inflammatory cells (Figure 4I and J).

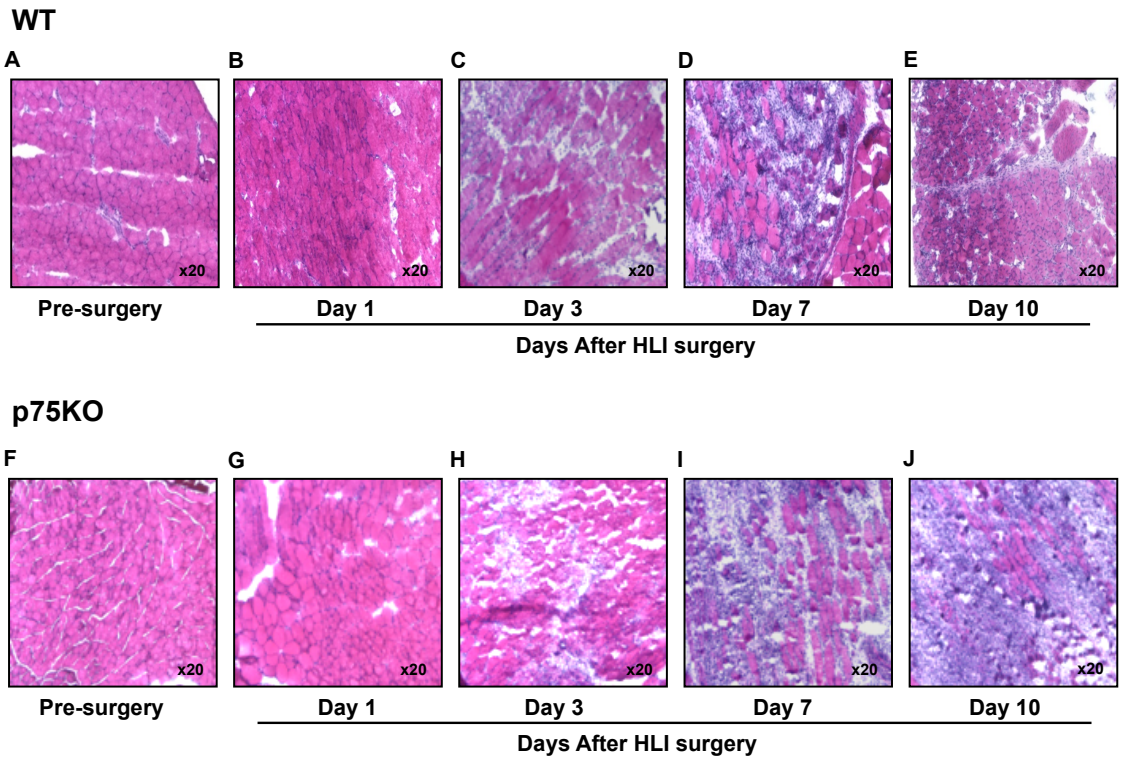

Figure 4. Diminished post-ischemic recovery in young p75KO mice. Representative x20 brightfield microscopy images for H&E staining in HL muscle tissue from WT and p75KO mice respectively at pre-surgery control (A and F), Day 1 post-HLI surgery (B and G), Day 3 post-HLI surgery (C and H), Day 7 post-HLI surgery (D and I) and Day 10 post-HLI surgery (E and J).

Increased and Prolonged Neutrophil and Macrophage Infiltration in Young p75KO Mice Post-HLI Surgery

To evaluate post-ischemic inflammatory responses in WT and p75KO mice, skeletal muscle sections of operated hind limbs were stained separately for MPO-1, a neutrophil marker, and F4/80, a macrophage marker. TO-PRO3 staining was used to visualize nuclei. Pre-surgery control muscle tissue did not reveal any MPO-1 positive (+) cells (Figure 5A, F) in WT or in p75KO tissue. WT ischemic tissue had a gradual increase in MPO-1 (+) cells from 1 to 3 days, with a peak at day 3 (Figure 5B, C and K). Compared to WT mice, there was a significant increase (4.4-fold, ~340%) of MPO-1 (+) cells in p75KO ischemic tissue on day 1 (Figure 5B, G and K). Although there was a ~47% decrease in MPO-1 (+) cells in p75KO muscle tissue by day 3, it was still ~2-fold higher when compared to WT (Figure 5C, H and K). By day 7, neutrophil infiltration returned to pre-surgery control levels in WT ischemic tissue (Figure 5D-E and K), while p75KO ischemic tissue still demonstrated significantly higher MPO-1 (+) cells during days 7 to 10 (~100% and ~25% higher respectively) post-HLI surgery, when compared to WT tissue (Figure 5I, J and K).

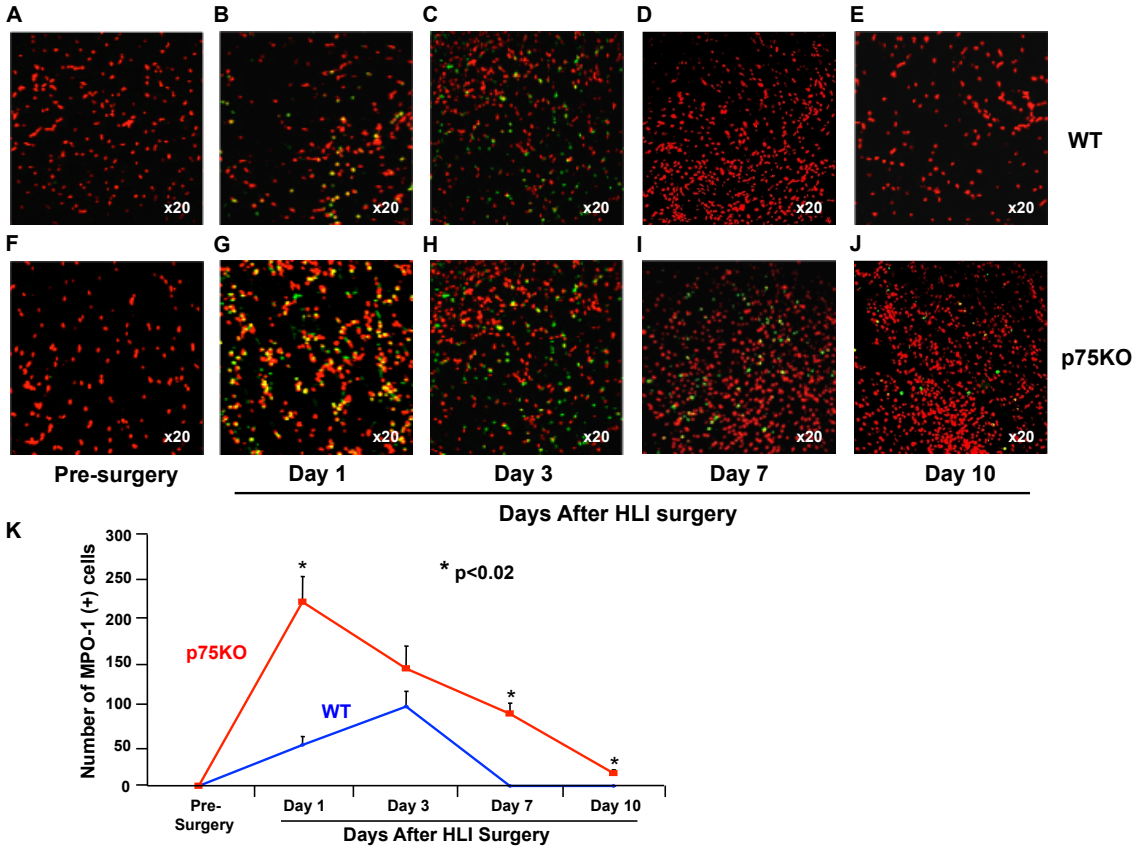

Figure 5. Ischemia induced neutrophil infiltration is increased and long-lasting in young p75KO mice. (A-J) Representative x20 confocal microscopy images for immunostaining with neutrophil marker, MPO-1 (green), and Topro-3 stained nuclei (red) in HL muscle tissue. (A and F) pre-surgery control, (B and G) Day 1 post-HLI surgery, (C and H) Day 3 post-HLI surgery, (D and I) Day 7 post-HLI surgery, and (E and J) Day 10 post-HLI surgery demonstrates neutrophil infiltration from WT and p75KO mice respectively. (K) Graphic representation of number of MPO-1 (+) cells in the HL muscle tissue isolated from WT and p75KO mice pre-surgery and up to 10 days post-HLI surgery. All results are presented as mean ± SEM; *N = 3-5* animals per time point/group for WT (solid blue line) and p75KO (solid red line) groups. Statistical significance was assigned when $P < 0.05$.

Quantification of F4/80 (+) cells showed no macrophage infiltration in both p75KO and WT ischemic tissue by day 1 post-HLI surgery, which was comparable to respective pre-surgery control tissues (Figure 6A-B, F-G and K). By 3-days post-HLI surgery, both WT and p75KO ischemic tissue revealed significantly increased macrophage infiltration with a substantial 5-fold (~400%) increase in F4/80 (+) cells in p75KO tissue, when compared to WT (Figure 6C, H and K). From 7-10 days post-surgery, both WT and p75KO ischemic tissues had decreasing trends in F4/80 (+) cells with p75KO tissue still showing a significantly higher macrophage infiltration: ~400% and ~500% higher at 7 and 10-days respectively, when compared to WT (Figure 6D-E, I-J and K). Taken together this data suggests that in the absence of TNFR2/p75 signaling post-HLI surgery, inflammatory responses are increased and longer-lasting in p75KO muscle tissue.

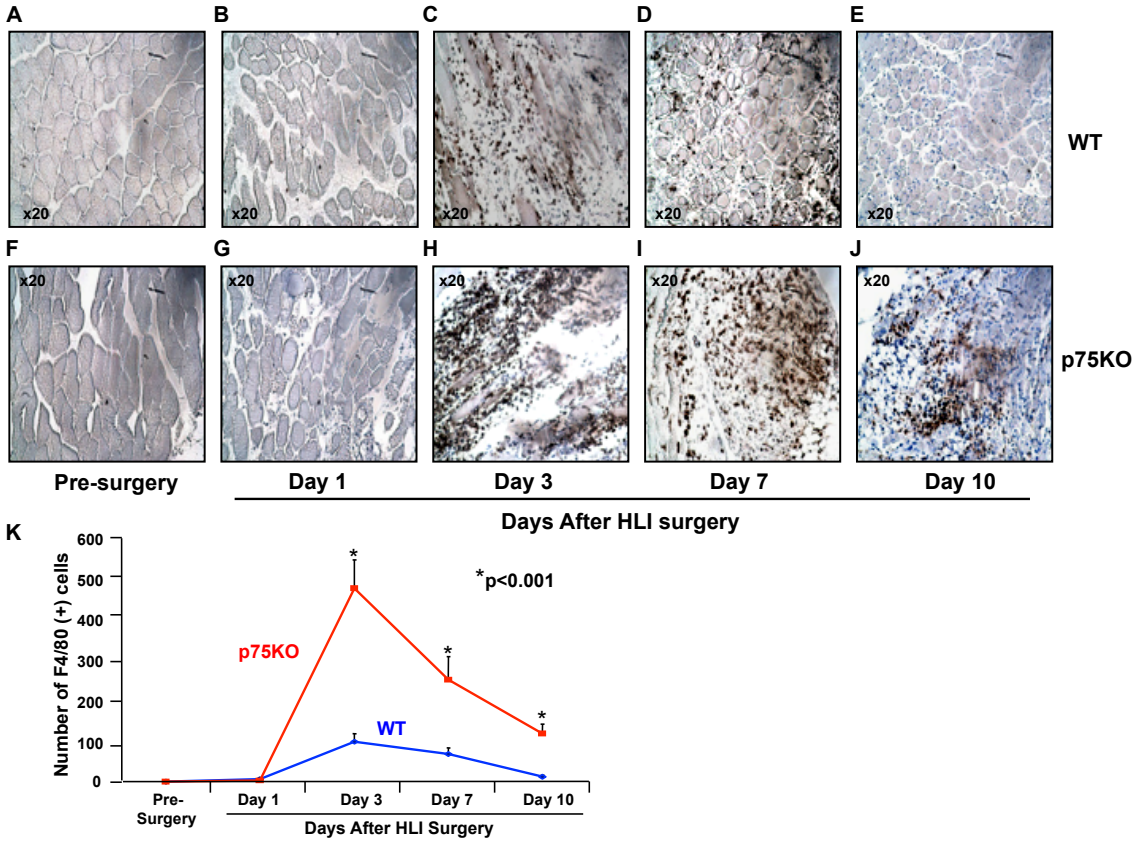

Figure 6. Ischemia induced macrophage infiltration is increased and long-lasting in young p75KO mice. Representative x20 brightfield microscopy images for immunostaining with macrophage marker, F4/80 (brown cells), and hematoxylin stained nuclei (blue) in HL muscle tissue from WT and p75KO mice respectively at (A and F) pre-surgery control, (B and G) Day 1 post-HLI surgery, (C and H) Day 3 post-HLI surgery, (D and I) Day 7 post-HLI surgery and (E and J) Day 10 post-HLI surgery. (K) Graphic representation of number of F4/80 (+) cells in the HL muscle tissue isolated from WT and p75KO mice pre-surgery and up to 10 days post-HLI surgery. All results are presented as mean ± SEM; *N = 3-5* animals per time point/group for WT (solid blue line) and p75KO (solid red line) groups. Statistical significance was assigned when $P < 0.05$.

Impaired Activation of Satellite-Cells in Young p75KO Mice After HLI Surgery

To evaluate if the absence of TNFR2/p75 signaling affects satellite-cell activation during post-ischemic recovery, p75KO and WT muscle sections post-surgery were double stained with NCAM, a satellite-cell marker[43,44], and TO-PRO3 to visualize nuclei. Compared to pre-surgery controls, on days 1, 3 and 7 post-HLI surgery there was a ~5-6-fold increase in the number of satellite-cells in WT ischemic tissue (Figure 7B-D and K), whereas satellite-cells were not detectable in p75KO ischemic tissue after day 1 and through day 7 (Figure 7G-I and K). Satellite-cells became detectable on day 10 in p75KO ischemic tissue (Figure 7J and K), but the number of NCAM (+) cells were still twice as low as in WT ischemic tissue on day 10 (Figure 7E, J and K). Post-HLI muscle samples at day 10 from WT showed a significant increase (7-fold) in the mean number of central nuclei per muscle fiber when compared to p75KO at the same time point (Figure 7L-N). Taken together these findings indicate impaired ischemia-induced activation of satellite-cells and substantially decreased muscle regeneration in p75KO mice that further signifies the role played by TNFR2/p75 signaling in post-ischemic recovery.

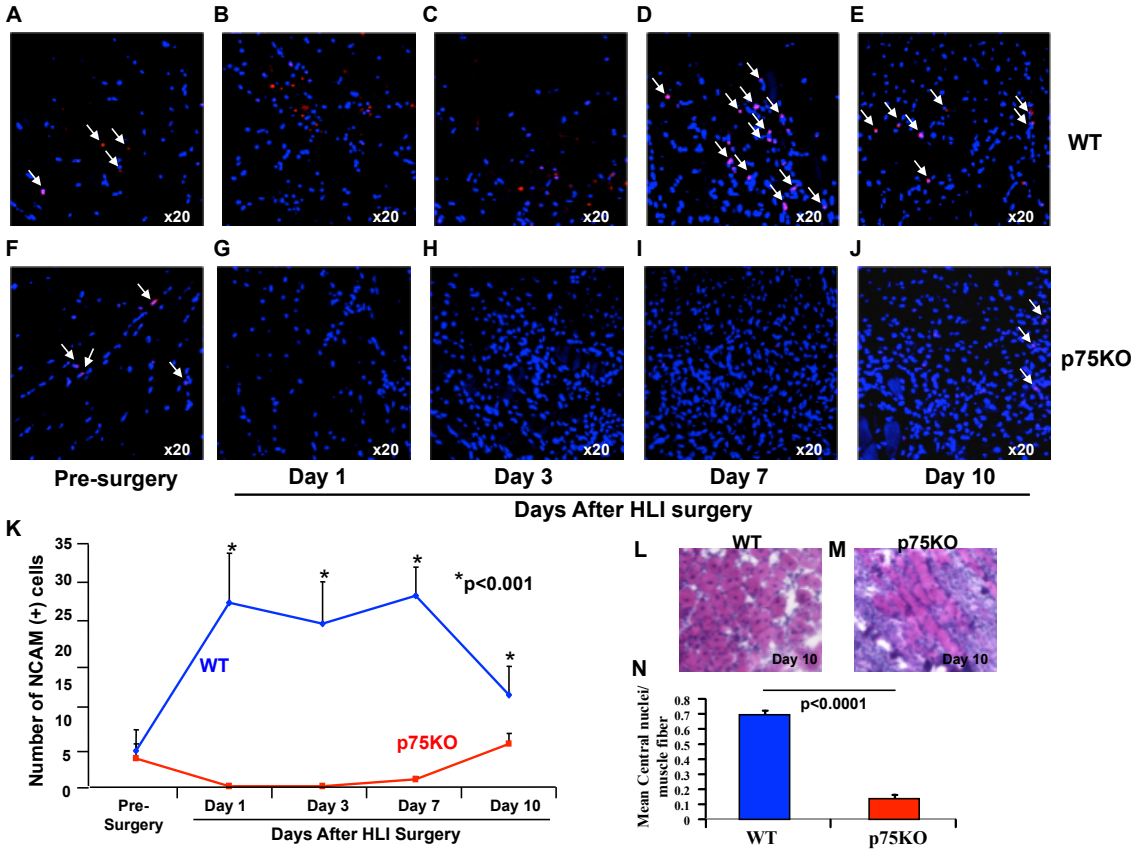

Figure 7. Ischemia induced satellite-cell activation is impaired in young p75KO mice. Representative x20 confocal microscopy images for immunostaining with satellite-cell marker, NCAM (red), and Topro-3 stained nuclei (blue) in HL muscle tissue from WT and p75KO mice respectively at (A and F) pre-surgery control, (B and G) Day 1 post-HLI surgery, (C and H) Day 3 post-HLI surgery, (D and I) Day 7 post-HLI surgery and (E and J) Day 10 post-HLI surgery. White arrows indicate NCAM (+) satellite-cells in muscle tissue. (K) Graphic representation of number of NCAM (+) cells in the HL muscle tissue isolated from WT and p75KO mice pre-surgery and up to 10 days post-HLI surgery. Representative x40 brightfield microscopy images for H&E staining in HL muscle tissue from (L) WT and (M) p75KO mice 10-days post-HLI surgery. (N) Graphic representation of mean number of central nuclei/muscle fiber in the HL muscle tissue isolated from WT and p75KO mice 10-days post-HLI surgery. All results are presented as mean ± SEM; *N = 3-5* animals per time point/group for WT (solid blue line) and p75KO (solid red line) groups. Statistical significance was assigned when $P < 0.05$.

Ischemia Decreases Proliferation and Increases Apoptosis in Adult p75KO Tissue Transplanted with Young p75KO But Not Young WT BM Cells

The engraftment of the recipient BM with GFP (+) donor cells was confirmed using BMT model where young WT/GFP (+) and young p75/GFP (+) BM were transplanted into lethally gamma-irradiated adult p75KO mice (Figure 3). FACS analysis of BM engraftment from recipient adult p75KO mice at 4 and 8 weeks post-BMT showed similar percentage of GFP (+) cells when compared to the number of BM GFP (+) cells in the marrow of positive control GFP mice (Figure 8B-C and E-F). Eight weeks post-BMT, we also tested the number of circulating PB MNCs in GFP mice versus circulating PB MNCs in GFP BM cell transplanted mice. There was a similar percentage of GFP (+) cells in PB-MNCs for both positive control (actual GFP mice) and GFP/BMT recipient mice (Figure 8H-I). Negative control BM-derived cells and PB-MNCs showed no GFP (+) cells, as expected (Figure 8A, D and G).

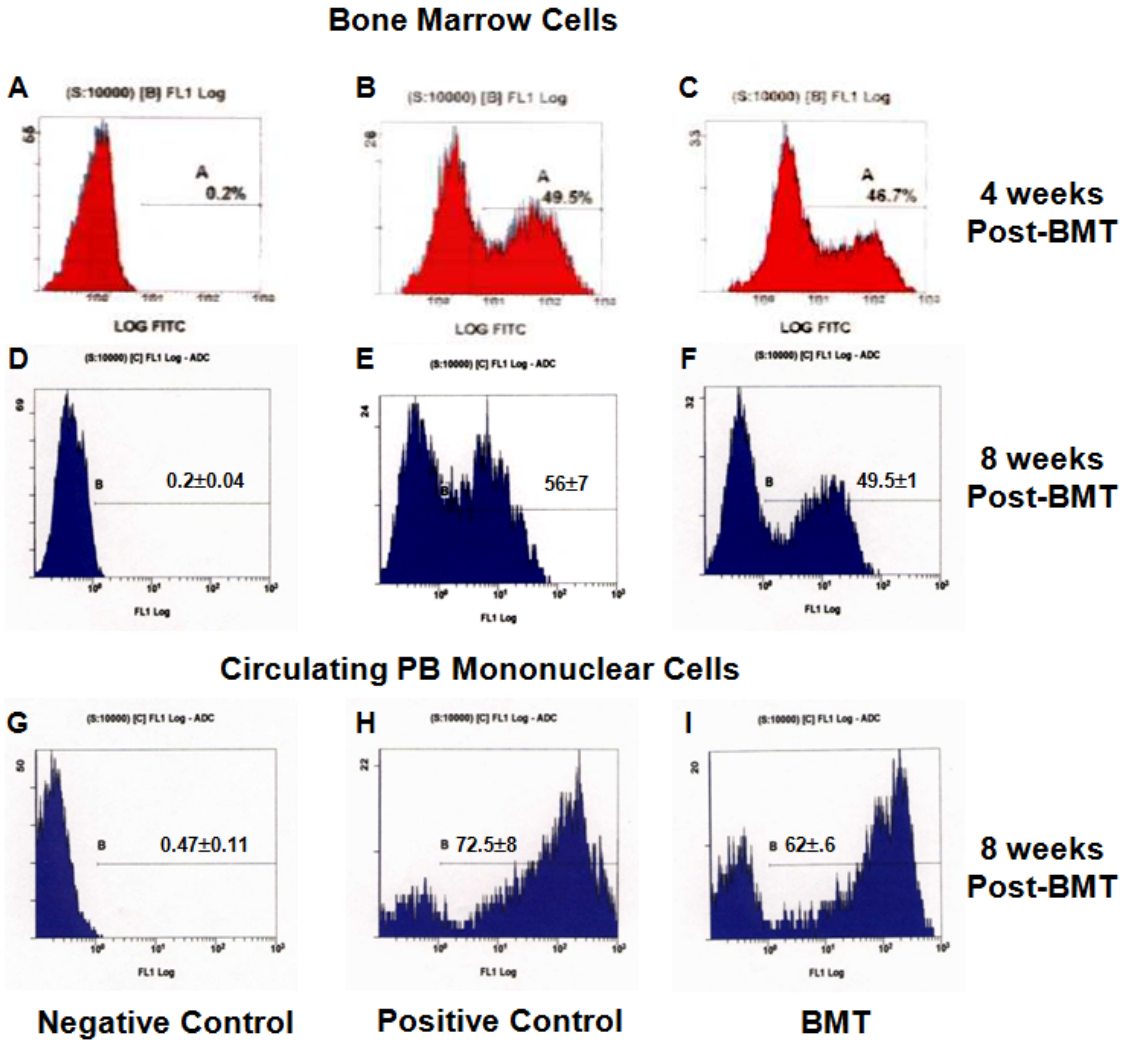

Figure 8. Representative histograms of FACS analysis showing the percentage of GFP (+) BM-derived cells at 4 and 8 weeks in adult p75KO mice after bone marrow transplantation: (A and D) negative control non-GFP mice, (B and E) positive control GFP mice and (C and F) BMT recipient mice. Representative histograms of FACS analysis showing the percentage of GFP (+) PB-MNCs at 8 weeks time point from: (G) negative control non-GFP mice, (H) positive control GFP mice and (I) BMT recipient mice.

The possible synergistically negative roles of tissue aging and the absence of TNFR2/p75 either in the tissue or in the BM cells were evaluated using two chimeric BMT models (Figure 2). By 28 days post-HLI surgery in the ischemic tissue of adult p75KO recipients that were transplanted with young WT GFP (+) BM cells, there was a significant difference in the number of Ki67 (+), presumably proliferative cells, in GFP (+) BM-derived (Figure 9A-C, arrow heads) versus GFP (-) resident cells (Figure 9B and C, circles). By 28-days after HLI surgery, the number of Ki67 (+) cells (Figure 9E, arrow heads) in the muscle tissue of adult p75KO mice transplanted with young p75KO GFP (+) BM were exclusively GFP (+) (Figure 9D, E, F, arrow heads). No Ki67 (+) resident cell in adult p75KO ischemic tissue transplanted with young p75KO donor BM-derived cells were detected (Figure 9F), indicating the synergistic negative roles of aging and the absence of TNFR2/p75 in BM-derived cells and the muscle tissue resulting in impaired proliferation in adult p75KO ischemic skeletal muscle tissue.

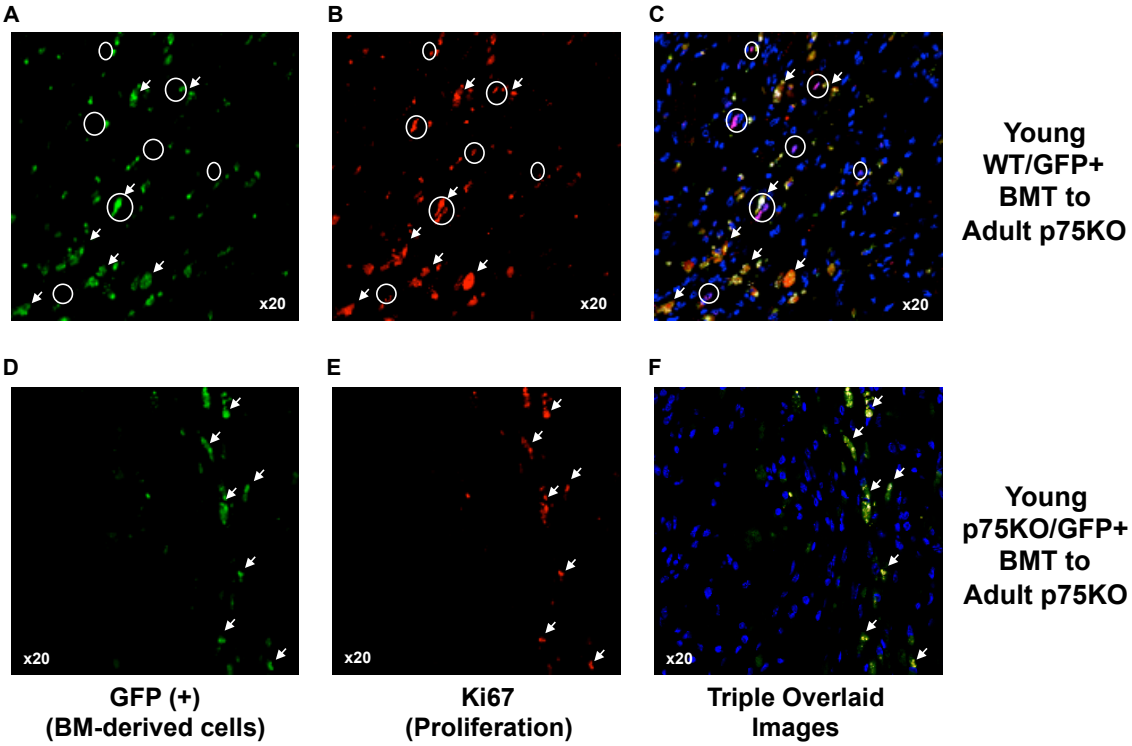

Figure 9. Proliferation of BM-derived and resident muscle cells is decreased in adult p75KO tissue transplanted with young p75KO but not young WT BM cells. Representative x20 confocal microscopy images of HL muscle tissue from two chimeric BMT models: (A-C) young WT/GFP (+) BMT to adult p75KO and (D-F) young p75KO/GFP (+) BMT to adult p75KO mice, 28-days post-HLI surgery. Representative images of young WT (A) and young p75KO (D) BMT GFP (+) cells (green) identified in adult p75KO tissue; Ki67 positive (red) cells in adult p75 mice transplanted with young WT (B) and p75KO (E) bone marrow cells. (C and F) Triple overlaid images of GFP(+)/Ki67(+)/TopRo-3(+) immunostained cells. White arrowheads represent BM-derived cells transplanted from GFP (+) young WT and p75KO donor mice that are also proliferative in adult p75KO HLI tissue shown as double positive yellow colored cells in the overlaid images. White circles represent GFP (-) and Ki67 (+), proliferative, resident adult p75KO cells (magenta colored) in HLI tissue.

Analysis of apoptosis in the adult p75KO ischemic tissue transplanted with young WT donor BM cells (Figure 10A, arrow heads) showed equal number of GFP (+) BM-derived cells that were both apoptotic, TUNEL (+) (Figure 10A-C, arrow heads) and non-apoptotic, TUNEL (-) (Figure 10A-C, circles). In contrast, in adult p75KO mice transplanted with young p75KO BM cells, none of the GFP (+) young p75KO donor BM cells (Figure 10D, arrow heads) survived in adult p75KO tissue (Figure 10E and F, arrow heads). Taken together, the Ki67 and TUNEL data in the ischemic tissue of two chimeric models of adult p75KO mice transplanted with young WT or p75KO BM cells showed decreased proliferative activity and increased apoptosis in adult p75KO mice transplanted with young p75KO BM cells that strongly suggests synergistically negative roles of tissue aging combined with decreased/absent TNFR2/p75 signaling in post-ischemic recovery.

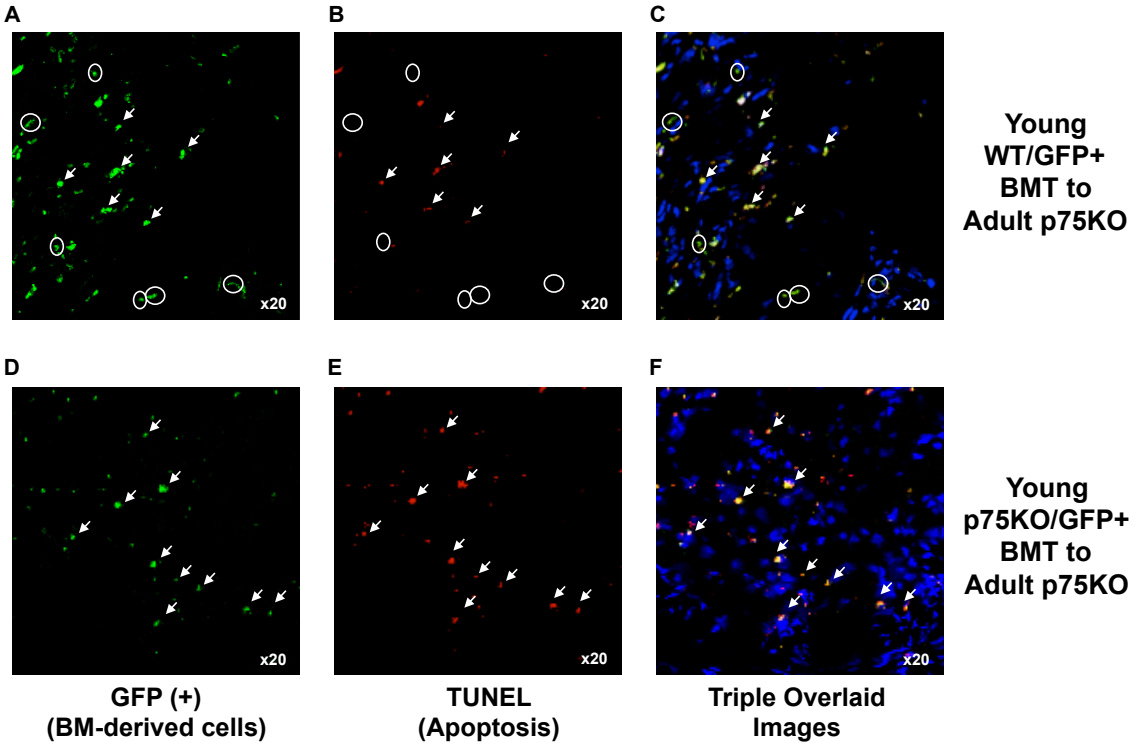

Figure 10. Young WT but not young p75 BM cells survive in adult p75KO tissue. Representative x20 confocal microscopy images of HL muscle tissue from two chimeric BMT models: (A-C) young WT/GFP (+) BMT to adult p75KO and (D-F) young p75KO/GFP (+) BMT to adult p75KO mice, 28-days post-HLI surgery. Representative images of young WT (A) and p75KO (D) BMT GFP (+) cells (green) identified in adult p75KO tissue; TUNEL positive (red) cells in adult p75 mice transplanted with young WT (B) and young p75KO (E) bone marrow cells. (C and F) Triple overlaid images of GFP(+)/TUNEL(+)/TopRo-3(+) immunostained cells. White arrowheads represent BM-derived cells transplanted from GFP (+) young WT and p75KO donor mice that are also apoptotic in adult p75KO HLI tissue shown as double positive yellow colored cells in the overlaid images. (A-C) white circles represent GFP (+) and TUNEL (-), non-apoptotic, BM-derived young p75KO cells (yellow colored) surviving in adult p75KO HLI tissue.

DISCUSSION AND CONCLUSIONS

This study demonstrated that ischemia induced significant and long-lasting inflammation associated with a considerable decrease in satellite-cell activation in p75KO muscle tissue 1-10 days post hind limb ischemia surgery. In the absence of TNFR2/p75 receptor, the major findings of this study were: (i) ischemia-induced skeletal muscle tissue recovery was impaired, (ii) inflammatory responses, including neutrophil and macrophage infiltration, following HLI surgery were increased and longer lasting, (iii) ischemia induced satellite-cell activation was affected, and (iv) tissue aging combined with decreased/absent TNFR2/p75 signaling may have a synergistically negative role on survival, proliferation and regeneration of damaged tissue.

Previous studies in this laboratory have shown that TNF-TNFR2/p75 signaling plays a critical role in ischemia-induced neovascularization in muscle and heart tissues.[12] To determine the role of TNF-TNFR2/p75 signaling in ischemia-induced inflammation and muscle regeneration, WT and TNFR2/p75KO mice were subjected to HLI surgery. Post HLI surgery, tissues were harvested from WT and p75KO mice hind limb and were evaluated for inflammatory responses. This murine HLI model represents a manifestation of peripheral artery disease and a means for studying inflammatory responses *in vivo*. This animal model also allows observation of the natural compensatory mechanisms following the development of limb ischemia.[11]

In the first seven days following HLI surgery, histological analysis indicated that inflammatory cascades were triggered for both young WT and p75KO mice. While

young WT muscle tissue showed recovery over the 10 day period post-surgery, young p75KO mice continued to show prominent apoptosis and significant loss of muscle tissue. The inflammation response was therefore longer lasting in p75KO mice and these mice failed to show signs of muscle regeneration, unlike their young WT counterparts. These histological findings suggest prolonged inflammatory and apoptotic response possibly due to the absence of pro-survival p75 signaling, and perhaps enhanced anti-survival p55 signaling. These muscle tissue recovery observations further demonstrate previous findings from our laboratory,[12] where a significant increase in apoptosis of endothelial cells was observed by 10-days in p75KO ischemic tissue, which is indicative of continued impaired recovery in p75KO mice due to the absence of TNF-TNFR2/p75 signaling. Without the TNFR2/p75 receptor, TNF action on its p55 receptor may be intensified, hindering the ability of muscle tissue to recover following ischemia.

Evaluation of post-ischemic neutrophil and macrophage infiltration also revealed increased and longer lasting inflammatory responses in p75KO tissue compared to WT. Studies have shown that once inflammation and repair signals are activated, the associated actions and sequence of this process are very consistent.[45] White blood cells are activated and recruited to injured tissue by specific cellular signals. Once these cascades are stimulated, neutrophils are the first to arrive, whose defensive mechanisms can cause cell death and necrosis.[45–48] Macrophages are then recruited to sites of tissue stress, where they produce and release proinflammatory cytokines and growth factors that contribute to tissue repair and regeneration.[45,49,50] However, studies have also shown extended neutrophil accumulation to be associated with impaired tissue regeneration.[51]

The present results show that while neutrophil infiltration gradually increased, peaked, and then returned to pre-surgery levels in WT ischemic tissue, levels in p75KO tissue were consistently higher during the 10 days post-surgery. Moreover, during peak neutrophil infiltration for the p75KO group, levels were 4.4 times those of young WT tissue on the same day. Quantification of macrophage levels demonstrated similar patterns over the 10 days post-HLI surgery. Both WT and p75KO ischemic tissue showed no infiltration on day 1, followed by peak macrophage infiltration levels on day 3, indicating that macrophage levels increased after neutrophil infiltration, which corresponds with the temporal inflammatory response patterns specified previously. However, macrophage tissue levels were found to be significantly higher on days 3, 7, and 10 post-surgery in p75KO mice relative to WT. These findings suggest that considerably different ischemia-induced inflammatory responses occurred in WT and p75KO tissue and the processes of muscle repair and regeneration was negatively affected in p75KO mice.

Following HLI surgery, the above data show that ischemia induced significant and long-lasting inflammation in TNFR2/p75KO mice group compared to their WT counterparts. Although the precise roles of the two TNF receptors, TNFR1/p55 and TNFR2/p75, have not been completely elucidated, our data support the involvement of these receptors in the post-ischemic recovery process. Following an ischemic event, previous reports have shown these two receptors to have divergent actions. While TNFR2/p75 facilitates the protective effects of TNF-α, ubiquitously expressed TNFR1/p55 has been shown to modulate the cytotoxic effects, including apoptotic

signaling.[1,18] Therefore, increased muscle damage and impaired inflammation responses in the p75KO mice compared to WT are conceivably due to up-regulated signaling through the p55 receptor, increasing the pro-apoptotic cascade. In the absence of TNFR2/p75 receptor, increased and prolonged inflammation may be the result of a microenvironment with increased cytotoxic TNF signaling through unopposed p55 receptor signaling.

Prior studies have supported the TNFR1/p55 receptor's role in inducing inflammatory response proteins via activation of the NF-κB and MAPK pro-inflammatory signaling pathways.[33,52] It is believed that many different types of cells require the TNFR1/p55 receptor in order to signal the expression of pro-inflammatory proteins. Upon TNF-α binding to TNFR1/p55, adaptor proteins are recruited to its cytosolic death domain, followed by the recruitment of additional cytosolic adaptor proteins.[33,34] The proteins in this cascade serve as critical mediators of inflammatory signaling and the activation of gene expression in response to TNF-α.

Contrary to the role of TNFR1/p55, previous reports have demonstrated TNFR2/p75 signaling induces anti-inflammatory effects in mice.[33] Although the underlying mechanism of TNFR2/p75 signaling is not well known compared to TNFR1/p55, studies suggest this receptor mediates many protective actions, including promoting survival following myocardial infarction in mice.[33] The protective effects of TNF-α through TNFR2/p75 has been well documented in endothelial and hematopoietic lineage cells.[53,54] Because TNFR2/p75 has been shown to be involved in processes with beneficial and protective functions, Ruspi et al. predicted that its blockade could have

harmful consequences.[33] A prior study of p75KO mice in this laboratory supported this hypothesis by demonstrating that deficiency in the TNFR2/p75 receptor expression led to impaired post-ischemic recovery following unilateral HLI-surgery.[12] All p75KO mice were found to have significantly fewer capillaries per muscle fiber compared to WT post surgery, and old p75KO exhibited 100% limb loss.[12] Examination of muscle tissue recovery as well as neutrophil and macrophage infiltration in ischemic tissue in our current study supports the potential negative effects decreased or absent TNFR2/p75 signaling may have on post-ischemic recovery.

In order to investigate the role of TNFR2/p75 in satellite-cell activation during post-ischemic recovery, the number of satellite-cells in ischemic tissue was quantified in p75KO and WT mice following HLI-surgery. Satellite-cells are progenitor precursors to skeletal muscle cells and are responsible for muscle growth and repair.[55] Under normal physiological conditions in adult muscle, satellite-cells are mitotically quiescent and are attached to muscle fibers between the basal lamina and sarcolemma. Once activated by injury or exercise, these cells proliferate and differentiate into myocytes.[56] A small portion of activated satellite-cells undergo self-renewal and return to the quiescent state for future maintenance and repair. Neural cell adhesion molecule (NCAM) has been vastly identified as a developmental marker that is expressed by activated satellite-cells during muscle regeneration in both humans and mouse models.[44,57–61]

In the present study, evaluation of satellite-cell activation on days 1-10 post-HLI surgery revealed 5-6 fold higher levels in WT tissue when compared to pre-surgery control levels. In contrast, satellite-cells were not detectable in p75KO ischemic tissue

within the 10 days post-surgery compared to pre-surgery controls. The lack of satellite-cells in p75KO mice following HLI-ischemia surgery provides insight into the significant loss of muscle tissue experienced by this group, as described above. In response to muscle injury, the capacity of skeletal muscle to regenerate is dependent upon satellite-cell activation, proliferation, movement to site of injury, and fusion into existing or new muscle fibers.[62–64] Satellite-cells provide damaged muscle fibers with the extra nuclei that are needed for initiation of rapid repair processes to prevent cell death and muscle loss.[65] Therefore, the undetectable levels of NCAM positive satellite-cells in young p75KO compared to WT mice likely contributed to the failed muscle recovery in these mice over 10 days post-HLI surgery.

Previous studies have demonstrated contradictory roles of pro-inflammatory cytokines in either inducing or inhibiting the skeletal muscle differentiation process.[64] An investigation of TNF-KO mice revealed that TNF deficiency significantly reduced muscle mass and contributed to impaired muscle regeneration.[66] Reports have shown TNF-α to be a requirement for myogenesis; however, the concentration of this cytokine appears to play an important role in the process, and high versus low levels of TNF-α results in conflicting actions regarding differentiation.[67]

In addition to TNF-α being predominantly produced by activated macrophages, muscle fibers have been shown to constitutively produce this cytokine as well.[4,67,68] During differentiation, myoblast synthesis of TNF-α increases, causing levels to increase substantially. In cases of muscle injury, TNF-α expression is upregulated by both increased myoblast differentiation and macrophage infiltration to injured tissue.[69]

Although muscle regeneration is generally successful in a high TNF-α environment,[67] studies have also shown exogenously added TNF-α to have an inhibitory effect on myoblast differentiation.[70–72] Chen et al. concluded that while temporary increases in TNF-α during differentiation may stimulate myogenesis, prolonged pathological increases in TNF-α would have an inhibitory effect on muscle regeneration.[67] Perhaps the lack of p75KO signaling in our current study combined with perceived upregulation in muscle fiber TNF synthesis in injured tissue with prolonged inflammation led to the decreased satellite-cell activation and muscle regeneration observed. Ultimately, our results indeed suggest that the absence of TNFR2/p75 signaling affects satellite-cell activation during post-ischemic recovery.

Chimeric BMT models were used to evaluate the possible synergistically negative roles of tissue aging combined with the absence of TNFR2/p75 receptor in either the tissue or BM. Previous studies using animals models of ischemia[40,73,74] have demonstrated that BMT cells are recruited to areas of ischemia and significantly improve neovascularization and collateral vessel development, helping to restore blood flow in ischemic regions.[40,73–75] Because BM MNCs contribute to neovascularization and because postnatal recovery is impaired in p75KO mice, we examined whether restoration of p75 receptor expression in BM of lethally irradiated adult p75KO mice would help post-ischemic recovery. To further explore contribution of BM-derived EPCs, we transplanted adult p75KO mice with young GFP(+) p75KO BM. Following complete engraftment of the recipient BM with GFP donor cells, HLI surgery was performed one month post-BMT.

Analysis of resident versus BMT cell proliferation (Ki67+) in ischemic tissue 28-days post-HLI surgery revealed differing patterns in each model. In model A, where adult p75KO mice were transplanted with GFP (+) BMCs from young WT mice, there was an equal number of Ki67(+)/GFP(+) cells and Ki67(+)GFP(-) cells by day 28 post-HLI surgery, meaning there were an equal number of proliferative cells from both the donor and recipient. This model, representing the presence of p75 receptor in BM and absence in the tissue, indicated that transplanted BM cells were indeed recruited to the ischemic tissue. Apoptosis of BM-derived cells in ischemic tissue was also evaluated for both BMT models in order to examine survival of donor GFP (+) cells in recipient ischemic tissue. In model A, there were more non-apoptotic (surviving) cells that were BM-derived (TUNEL-/GFP+), by 28-days post-HLI surgery.

In model B, where adult p75KO mice were transplanted with BMCs from young p75KO mice, ischemic tissue of recipients consisted of much fewer proliferating cells and, those that were proliferative, were exclusively BM derived GFP (+) transplants. This model, representing absence of p75 from tissue and BM, showed the inability of resident adult p75KO cells to proliferate even after receiving BMT from young p75KO mice, which supports the synergistically negative role of aging combined with the absence of TNFR2/p75 receptor. The non-proliferative resident cells signify impaired recovery and regeneration of muscle tissue in this model. Analysis of apoptosis showed that a large number of young p75KO GFP (+) BM-derived cells did not survive in adult p75KO ischemic muscle tissue. The exaggerated levels of apoptosis in mice lacking the TNFR2/p75 receptor in both the tissue and BM supports our hypothesis that absence of

signaling via p75 receptor in the microenvironment affects survival processes within the tissue. These histological findings underscore earlier findings by this laboratory,[12] where a significant increase in apoptosis was observed in endothelial cells by 10 days post HLI surgery in p75KO ischemic tissue, which is indicative of continued impaired recovery due to the absence of TNF-TNFR2/p75 signaling. In this study, BM cells from young WT mice also helped rescue limbs of old (16-18 months) p75KO mice from ischemia-induced auto amputation and functional TNFR2/p75 was required in old WT ischemic tissue for efficient post-ischemic recovery.[12] Taken together, the results from the current and prior studies from this laboratory signify the importance of signaling through TNFR2/p75 receptor in cell survival and recovery of muscle tissue post-ischemia.

In summary, the present results demonstrate that deficiency of TNFR2/p75 expression led to the failure of post-ischemic recovery in adult mice over 10 days following HLI surgery. These data suggest that age related dysfunction in ischemia recovery is in part a result of impaired signaling through TNFR2/p75. Ischemia induced inflammatory responses were increased and longer lasting in p75KO after HLI surgery. Ischemia-induced satellite-cell activation was impaired in TNFR2/p75KO mice. Tissue aging combined with absent TNFR2/p75 signaling appeared to have a synergistically negative role on survival, proliferation and regeneration of the damaged tissue.

LIST OF JOURNAL ABBREVIATIONS

Acta Neuropathol	Acta Neuropathologica
Aging Cell	Aging Cell
Am J Nephrol	American Journal of Nephrology
Am J Pathol	The American Journal of Pathology
Am J Physiol Cell Physiol	American Journal of Physiology – Cell Physiology
Ann Surg Oncol	Annals of Surgical Oncology
Arch Ophthalmol	Archives of Ophthalmology
Bone Marrow Transplant	Bone Marrow Transplantation
Br J Sports Med	British Journal of Sports Medicine
Cancer Res	Cancer Res
Cell	Cell
Cell Signal	Cellular Signaling
Circulation	Circulation
Cytokine	Cytokine
EMBO J	European Molecular Biology Organization Journal
Exp Cell Res	Experimental Cell Research
Exp Gerontol	Experimental Gerontology
FASEB	Federation of American Societies for Experimental Biology
Folia Neuropathol	Folia Neuropathologica

Glia	Glia
Hypertension	Hypertension
Immunol Rev	Immunological Reviews
Immunol Ser	Immunology Series
J Appl Physiol	Journal of Applied Physiology
J Anat	Journal of Anatomy
J Athl Train	Journal of Athletic Training
J Cell Biochem	Journal of Cellular Biochemistry
J Cell Biol	The Journal of Cellular Biology
J Cell Physiol	Journal of Cellular Physiology
J Cell Sci	Journal of Cell Science
J Appl Physiol	Journal of Applied Physiology
J Clin Invest	Journal of Clinical Investigation
J Immunol	The Journal of Immunology
J Natl Cancer Inst	Journal of the National Cancer Institute
J Orthop Res	Journal of Orthopedic Research
J Physiol	The Journal of Physiology
Leuk Lymphoma	Leukemia & Lymphoma
Life Sci	Life Sciences
Med Sci Sports Exerc.	Medicine and Science in Sports and Exercise
Mol Cell Biochem	Molecular and Cellular Biochemistry
Mol Cell Biol	Molecular and Cellular Biology

Mol Med	Molecular Medicine
Muscle Nerve	Muscle & Nerve
Nat Med	Nature Medicine
Nature	Nature
Neuromuscul Disord	Neuromuscular Disorders
Oncogene	Oncogene
Physiol Genomics	Physiological Genomics
PloS One	Public Library of Science One
Proc Natl Acad Sci	Proceedings of the National Academy of Sciences
Sci Signal	Science Signaling
Science	Science
Skelet Muscle	Skeletal Muscle
Stem Cells	Stem Cells
Trends Cell Biol	Trends in Cell Biology
Trends Mol Med	Trends in Molecular Medicine

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