OPN-a induces muscle inflammation by increasing recruitment and activation of pro-inflammatory macrophages

Running Title: OPN-a induces muscle inflammation

Gina M. Many[1,2], Yasuyuki Yokosaki[3], Kitipong Uaesoontrachoon[1], Peter P. Nghiem[1,2,6], Luca Bello[1], Sherry Dadgar[1], Ying Yin[7], Jesse M. Damsker[1,4], Heather B. Cohen[5], Joe N. Kornegay[6], Marcas M. Bamman[8], David M. Mosser[5], Kanneboyina Nagaraju[1,2], Eric P. Hoffman[1,2*]

[1]Research Center for Genetic Medicine, Children's National Medical Center, Washington, DC, USA

[2]Department of Integrative Systems Biology, George Washington University School of Medicine & Health Sciences, Washington, DC, USA

[3]Hiroshima University, Minamiku, Hiroshima, Japan

[4]ReveraGen BioPharma, Rockville, MD, USA

[5]Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, MD, USA

[6]Department of Veterinary Integrative Biosciences, Texas A&M University, College Station, TX

[7]National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD, USA

[8]Department of Cell, Developmental and Integrative Biology, University of Alabama Birmingham, Birmingham, AL, USA

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***Correspondence to:**

Eric Hoffman

Center for Genetic Medicine Research

111 Michigan Ave. NW

Washington DC 20010

Email: ericphoffman@childrensnational.org

Phone: 202 476 6011

Fax: 202 476 6014

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New Findings:

- Human OPN spliced isoform variants are upregulated in human and dog dystrophic muscle—we thus sought to explore differences in the inflammatory properties of each isoform.
- The full-length human OPN-a isoform is the most pro-inflammatory, acting on macrophages and myoblasts in an RGD-integrin dependent manner.
- OPN-a upregulates tenascin-c (TNC) expression, a known TLR4 agonist.
- Blocking TLR4 signaling inhibits the pro-inflammatory effects of OPN-a, suggesting a potential mechanism of OPN action is by promoting TNC-TLR4 signaling.

Abstract

While OPN is an important mediator of muscle remodeling in health and disease, functional differences in human spliced OPN variants in the muscle microenvironment have not been characterized. We thus sought to define the pro-inflammatory activities of human OPN isoforms (OPN-a, OPN-b, and OPN-c) on cells present in regenerating muscle. OPN transcripts were quantified in normal and dystrophic human and dog muscle. Human macrophages and myoblasts were stimulated with recombinant human OPN protein isoforms and cytokine mRNA and protein induction was assayed. OPN isoforms were greatly increased in dystrophic human (OPN-a> OPN-b> OPN-c) and dog muscle (OPN-a = OPN-c). In healthy human muscle, mechanical loading also upregulated OPN-a expression (8-fold; $p < 0.01$), but did not significantly upregulate OPN-c expression (2-fold; $p > 0.05$). *In vitro,* OPN-a displayed the most pronounced pro-inflammatory activity among isoforms, acting on both macrophages and myoblasts. *In vitro* and *in vivo* data revealed OPN-a upregulated tenascin-c (TNC), a known TLR4 agonist. Inhibition of TLR4 signaling attenuated OPN-mediated macrophage cytokine production. In summary, OPN-a is the most abundant and functionally active human spliced isoform the skeletal muscle microenvironment. Here, OPN-a promotes pro-inflammatory signaling in both macrophages and myoblasts possibly through induction of TNC-TLR4 signaling. Together, our findings suggest that specific targeting of OPN-a and/or TNC signaling in the damaged muscle microenvironment may be of therapeutic relevance.

Introduction

Osteopontin (OPN), or secreted phosphoprotein 1 (SPP1), is a member of the small integrin-binding ligand N-linked glycoprotein (SIBLING) protein family. SIBLING family proteins are structurally characterized by their small flexible structure and the presence of an Arg-Gly-Asp (RGD) tripeptide, an authentic integrin-binding sequence (Fisher *et al.*, 2001). OPN is a secreted protein with diverse physiological functions influenced by multiple pre- and post-translational modifications. Increasing importance has been attributed to OPN as an immunoregulatory protein in inflammation and tissue remodeling (acute and chronic) and cancer (Shin, 2012; Bandopadhyay *et al.*, 2014). However, the amino acid sequence is relatively poorly conserved between mouse and human (~63% homology), with the greatest degree of conservation occurring around receptor binding sites (e.g. RGD domain) and proteolytic cleavage sites. The human but not mouse gene shows three alternatively spliced isoforms (Gimba & Tilli, 2013). Full-length OPN protein is designated OPN-a, with a molecular weight of ~54kDa prior to extensive post-translational modifications. OPN-b lacks exon 5 (~50kDa), and OPN-c lacks exon 4 (~47kDa) of the seven exons.

All human OPN isoforms contain the RGD and SVVYGLR integrin binding peptide sequences (Yokosaki *et al.*, 1999), a CD44 binding domain, and thrombin and MMP cleavage sites. Unlike the RGD integrin binding peptide sequence, the SVVYGLR integrin binding site is only exposed upon thrombin cleavage of OPN (Yokosaki *et al.*, 1999). The three spliced isoforms differ in protein cross-linking sites (Gln residues) that undergo covalent polymerization by transglutaminase 2 (TG2), a difference that significantly alters OPN function (Higashikawa *et al.*, 2007; Nishimichi *et al.*, 2011). TG2 catalyzes OPN polymerization at multiple Gln residues coded by exons 2-5 (Christensen *et al.*, 2014). The deleted exons 5 (OPN-b) and 4 (OPN-c) contain one and three Gln residues, respectively, making the degree of isoform polymerization proportional to the number of transglutamination sites, with OPN-a>OPN-b>OPN-c (Nishimichi *et al.*, 2011). OPN

transglutamination is thought to modify protein function by altering the conformational state of OPN and thereby increasing its cell binding and chemotactic abilities (Kaartinen *et al.*, 1999; Nishimichi *et al.*, 2011).

Despite the widely characterized role of OPN as a pro-inflammatory protein, little research has investigated the function or expression patterns of OPN spliced isoforms outside of the field of cancer biology. In tumor cells, differential OPN isoform expression patterns are related to cancer cell behaviors and pathological outcomes such as proliferation, migration and metastasis (Gimba & Tilli, 2013). In breast cancer cells OPN-a but not OPN-c transfected cells display STAT DNA binding (Shi *et al.*, 2014). STAT activation occurs in response to cytokines, such as IL-6, suggesting that OPN isoforms may differentially modulate inflammatory cytokine induction. However, the effects of OPN isoforms on inflammatory signaling in other cellular microenvironments, such as skeletal muscle, remain unclear.

The role of OPN in muscle remodeling has received increased interest due to three recent observations. First, ablation of OPN expression in the mouse model of Duchenne muscular dystrophy (dystrophin-deficient *mdx* mice) improves disease pathology (Vetrone *et al.*, 2009). Second, a genetic polymorphism in the human OPN gene altering gene transcription is associated with disease severity in Duchenne muscular dystrophy (DMD) (Pegoraro *et al.*, 2011; Bello *et al.*, 2015). Finally, the same genetic polymorphism is associated with increased muscle size (Hoffman *et al.*, 2013) and increased damage in response to eccentric contraction in young adults (Barfield *et al.*, 2014). In normal skeletal muscle under homeostatic conditions OPN expression is very low or undetectable. OPN expression is rapidly induced in acute and chronic muscle injury with expression primarily localized to infiltrating immune cells and proliferating myogenic cells (Uaesoontrachoon *et al.*, 2008; Zanotti *et al.*, 2011; Paliwal *et al.*, 2012; Pagel *et al.*, 2014). In myogenic cells, OPN is expression is upregulated with inflammatory stimuli and degeneration/remodeling (Uaesoontrachoon *et al.*, 2008; Paliwal *et al.*,

2012). Despite increasing importance attributed to OPN in muscle remodeling, functional differences between spliced isoforms in the regenerating muscle microenvironment have not been characterized.

In healthy and diseased muscle (e.g. DMD), macrophages are the predominant cellular infiltrate and express high levels of OPN (Hirata *et al.*, 2003). Despite increasing importance attributed to OPN in muscle remodeling, the relative contribution of spliced isoforms to muscle remodeling has not been studied in either health or disease. We thus sought to better define the role of OPN spliced isoforms in the skeletal muscle microenvironment by studying macrophage and myoblast/muscle inflammatory signaling. We observed that OPN-a is the most abundantly expressed isoform in human muscle. In both macrophages and myoblasts OPN-a was the most pro-inflammatory spliced isoform.

Methods

Ethical Approval:

All tissue samples were obtained by protocols approved by the Institutional Animal Care and Use Committee (IUCAC) or Institutional Review Board (IRB), for respective animal and human studies at respective institutions. For human studies, all participants consented to study participation at the enrolling institution. All study procedures were conducted according to standards set forth by the Declaration of Helsinki.

Human and Canine Skeletal Muscle OPN Expression:

Human. All human muscle biopsies were obtained by fine needle biopsy of *vastus lateralis* muscle and flash-frozen for processing. Briefly, samples were collected using local anesthetic (1% lidocaine). DMD, BMD and control human muscle biopsies were archival specimens from a tissue

bank at Children's National Medical Center. These samples were obtained from consenting subjects and/or their legal guardian, if under the age of 18 years. All subjects were male due to the X-linked nature of DMD. For OPN isoform detection, the human forward primer (ATTGCAGTGATTTGCTTTTGC) was designed against human exon 2 and the reverse primer (GCAACCGAAGTTTTCACTCC) was designed against exon 6.

For loading stress studies, biopsies were obtained from healthy male (n = 11) and female (n = 11) human subjects (age: 57 ± 3 yrs) immediately before and 24 hr after loading induced stress. Here, subjects performed unaccustomed resistance exercise, by performing 9-10 repetitions at ~65% 1-RM (repetition maximum). All subjects were > 18 yrs and consented to study participation at the University of Alabama, Birmingham (Stec *et al.*, 2015). qPCR was performed by Taqman gene array on a StepOnePlus Real-Time PCR System (Applied Biosystems; Foster City, CA). Here, GAPDH was used as an endogenous control.

Canine. Muscle samples were taken at either biopsy or necropsy from the cranial sartorius (CS), *vastus lateralis* (VL), and long digital extensor from the opposite limbs of golden retriever muscular dystrophy (GRMD) dystrophin deficient dogs (n = 8) and wild-type littermates (n = 4) at 4-9 weeks (early stage disease) and 6 months (symptomatic disease), as previously described (Nghiem *et al.*, 2013). All dogs were used and cared for according to principles outlined in the National Research Council Guide for the Care and Use of Laboratory Animals. Dogs were housed at the Kornegay laboratory either at the University of Missouri or University of North Carolina at Chapel Hill. All animals were given *ad libitum* access to food and water. GRMD dogs were identified at one day of age based on dramatic elevation of serum creatine kinase. Genotype was confirmed by PCR when creatine kinase results were ambiguous. Characteristic clinical signs subsequently developed. For muscle biopsy, dogs were given a general anaesthetic comprising of acepromazine maleate (0.02

mg/kg), butorphanol (0.4 mg/kg), and atropine sulfate (0.04 mg/kg). On average, 400 mg of tissue was sampled per dog.

For dystrophic human and dog samples, HPRT1 was used as an endogenous control for OPN isoforms expression. RNA was isolated by TRIzol extraction and cDNA synthesis was performed using an Applied Biosystems' High Capacity cDNA Reverse Transcription Kit (Life Technologies) according to methods described previously (Nghiem *et al.*, 2013). Briefly for PCR analyses, cDNA was incubated at 90°C for one minute; a multiplex reaction was then carried out with OPN and HPRT1 probes according to the manufacturer's instructions using AccuPrime SuperMix II (Invitrogen). Reactions were performed in triplicate for samples from both normal (n = 4; all male) and GRMD (n = 8; n = 4 male; n = 4 female) cDNA (CS, LDE, VL) at 4-9 weeks and 6 months. PCR conditions for human samples were 30 cycles at: 94°C for 5 min, 94°C for 30 sec, 60°C for 1 min, 72°C for 30 sec, and 72°C for 7 min. OPN mRNA isoform bands were detected with GeneSnap software and quantified and normalized to HPRT1 in GeneTools software (Syngene, Frederick, MD).

Human primary monocyte-derived macrophages

Consenting healthy adult males not taking anti-inflammatory or immunoregulating medications underwent venipuncture for the isolation of primary human monocytes by negative selection. All subjects consented to participate in this study conducted at Children's National Medical Center. To obtain monocytes, venous blood was collected in sodium heparin vacutainers and immediately processed for isolation of peripheral blood derived mononuclear cells (PBMCs) via density centrifugation using Ficoll Paque Plus (GE Lifesciences; Marlborough, MA). Monocytes were then isolated from the PBMC layer via negative magnetic selection for $CD14^+$ cells, without $CD16^+$ cell depletion (StemCell Technologies; Vancouver, Canada). Monocyte cell identity was

confirmed via staining for human CD14 and CD16 (BD Biosciences). Negative selection for monocytes resulted in a cell population of ~94% purity as defined as: $CD14^{hi}$ $CD16^{lo}$, $CD14^{lo}$ $CD16^{hi}$, and/or $CD14^{hi}$ $CD16^{hi}$ as determined by FACS. Monocytes were plated at a density of ~100,000 cells/well in 96-well plates for culture in DMEM-F12 media containing 1% Penicillin-Streptomycin and 10% fetal bovine serum, and cultured with 50 ng/mL human recombinant M-CSF (R&D; Minneapolis, MN). Cells were cultured for five days at 37°C with 5% CO_2, in order to differentiate cells into primary human macrophages. Human monocyte-derived macrophages were phenotypically characterized by an increase in size and the formation of cytoplasmic projections.

Recombinant Protein Derivation

Human OPN isoforms (OPN-a, -b, -c) were expressed as glutathione S-transferase (GST) fusion proteins in *E. coli* with pGEX6P plasmid, affinity purified and cleaved from GST according to previously described methods (Nishimichi *et al.*, 2011). Recombinant human OPN-a proteins containing: 1) RGD (arginine-glycine-aspartic) acid residues; and 2) OPN-a with the RGD domain mutated to KAE (lysine-alanine-glutamic) (ΔRGD→KAE [OPN-a-KAE]) were a kind gift from Dr. Larry Fisher at the NIH. Briefly, recombinant proteins were generated by subcloning OPN-a-RGD and OPN-a-KAE cDNA into pAd/CMV/V5-DEST adenovirus vectors in HEK293 cells. Viral transfection of human stromal fibroblasts was then used to generate recombinant proteins, which were purified via column chromatography (Fedarko *et al.*, 2000). Briefly, the proteins were purified using Sartobind Q strong basic anion exchanger (Sartorius; Göttingen, Germany). Here, the Sartobind Q 15 membrane was equilibrated with 50 mL PBS prior to sample filtration. The membrane was then washed with 8 M urea, 0.2% beta mercaptoethanol/PBS, and 100 mL PBS. A linear salt gradient to 2.0 M NaCl/PBS was employed to purify proteins to ~95% purity as measured by Stains-All (Sigma; St. Louis, MO).

Human Macrophage Stimulation

Human recombinant OPN proteins showed evidence of endotoxin contamination (Pierce LAL assay; Thermo Scientific; Waltham, MA). To inhibit endotoxin effects on assays, we pre-treated all samples with polymixin-B (PMB) (InvivoGen; San Diego, CA). This was shown to inhibit the effects of *E. coli* derived LPS (tested range: 10-100 ng/mL) on human macrophages. Macrophages were pre-treated with 100 µg/mL filtered PMB sulfate in PBS 30 min prior to stimulation with OPN (InvivoGen; San Diego, CA). PMB is a positively charged cyclic polypeptide that binds to and inhibits the actions of the negatively charged and bioactive lipid A tail of endotoxin (Cavaillon & Haeffner-Cavaillon, 1986). The pre-treatment of cells with PMB was optimized through testing of IL-6 and TNFα secretion from human macrophages assayed by ELISA and FACS bead array. Endotoxin depleted recombinant human and murine OPN-a was also obtained for testing and optimization of inflammatory responses (R&D Systems; Minneapolis, MN; not shown).

For mRNA assays, macrophages were treated with OPN proteins for 4 hrs. For protein assays, macrophages were treated with OPN proteins for 24 hrs. For protein assay by ELISA, R&D Duo Set kits were used (R&D Systems; Minneapolis, MN).

Nanostring Expression Profiling

After culturing for 5 days with 50 ng/mL human M-CSF, fresh human monocyte media was added and primary human monocyte-derived macrophages were stimulated for 4 hrs with human recombinant OPN proteins for expression profiling by Nanostring according to methods described previously (Dillingham *et al.*, 2015). Briefly, cells were harvested in cell stripper (Cellgro; Manassas, VA), washed and resuspended in RLT Buffer (Qiagen; Hilden, Germany). mRNA molecule counting

was performed on 10,000 macrophages per treatment condition using a custom Nanostring probe set and a NanoString nCounter analytics system (Nanostring Technologies; Seattle, WA). Eight negative control probes (targeting RNA sequences not expressed in humans) were used to adjust for background noise. Six positive probes (targeting housekeeping gene transcripts) were included in each probe set for initial content normalization, using geometric means of control transcript counts as a normalization factor. Additional normalization was performed against reference genes that were abundantly expressed (> 2 SD over negative control probes) and did not display variability between treatment conditions and donors ($p>0.05$). Based on these normalization procedures, GIGYF2, RMBX2, STK35, and TOX4 were used as normalization controls for macrophage studies, and HDAC3, RBMX2, STK35, TOX4 and USP4 for muscle studies.

Human myogenic cell culture

Immortalized human myoblasts were a gift from Dr. Vincent Mouly at the Centre for Research in Myology in Paris, France. These primary human myoblasts were derived from a healthy 25 year-old female subject, and immortalized according to previously described methods (Di Donna *et al.*, 2003). Muscle was obtained via fine needle biopsy of the *vastus lateralis* for cell isolation. Once immortalized, cells were grown in skeletal muscle cell media (Promocell; Heidelberg, Germany) supplemented with 20% FBS at 37°C with 5% CO_2. Cells were plated overnight prior to stimulation with OPN.

Phagocytosis assay

Freshly isolated primary human monocytes were cultured in human macrophage media without added M-CSF and left to adhere overnight at 37°C with 5% CO_2. Cells were then washed and

fresh media was added 1hr prior to 30 min pre-treatment with PMB. Cells were then stimulated for 2 hrs with recombinant OPN proteins. Following stimulation with OPN, fluorescently conjugated *E. coli* particles (K-12 strain) were suspended in Hanks balanced salt solution and added to the solution, according to the manufacturer's instructions (Vybrant Phagocytosis Assay Kit; Molecular Probes; Eugene, OR). After an additional 2 hr incubation period, cells were washed to remove non-phagocytosed *E.* coli particles and cellular fluorescence of non-viable cells was quenched with trypan blue for 1 min. The trypan blue solution was then aspirated. To determine cellular uptake of fluorescent *E. coli* particles, fluorescence was measured first on fluorescence plate reader using wavelengths of 485 nm for excitation and 525 nm for emission, and a repeat experiment was read on a flow cytometer using the FITC channel (FL1) a repeat experiment was performed measuring fluorescence by flourometer. All experiments were ran in technical replicates of n = 5, according to the manufacturer's instructions. For FACS reading, cells were harvested with cell stripper, centrifuged, and resuspended in FACS buffer. All fluorescent (FACS: FITC) measures were normalized for background fluorescence (*E. coli* particles only) prior to normalization to vehicle control cells (PMB treated).

Intramuscular OPN transfection and expression profiling

Use of mice for research was approved and conducted according to the Children's National Medical Center Institutional Animal Care and Use Committee guidelines. Mice were given *ad libitum* access to food and water. Animals were sacrificed by CO_2 overdose. For transfection experiments, murine OPN-a plasmid (GeneCopoeia; 50 μg total in 100 μL PBS) was injected into the right tibialis anterior (TA) muscle of 3-month male BLA/J mice (n = 3). The TA muscle was chosen as it is accessible by injection without surgery, and is relatively homogeneous in fiber type composition (predominantly fast twitch). As surgery is well known to induce inflammation, we wished to avoid

this added confounding variable as inflammation was a key endpoint. The contralateral left TA muscle was injected with 50 μg of scrambled (empty) plasmid (GeneCopoeia; Rockville, MD) and used as a vehicle and injection control. TA muscles were harvested 7 days post-injection for microarray expression profiling. The belly of the TA muscles were sectioned and total RNA was extracted from muscle ~50 sections using TRI reagent (Sigma-Alrich; St. Louis, MO). Illumina beadchip microarray was performed on the extracted mRNA according to previously described methods (Uaesoontrachoon *et al.*, 2013). Data was analyzed through use of Ingenuity Pathway Analysis software. A p-value of ≤ 0.05 and fold change of ≥ 1.5.between vector and OPN-a injected groups was used as a cutpoint for pathway analysis.

Statistical Analyses

Statistical significance between treatment conditions was assessed via student's t-tests, unless otherwise indicated. Statistical significance was set at $p < 0.05$. For Nanostring expression profiling, ANOVA was performed and differences between groups were assessed using Bonferonni correction for multiple testing (cytokines and doses) through use of Partek Genomics Suite software, Version 6.6. Additionally, paired t-tests were used to examine single dose responsiveness between donors, where paired comparisons between treatment per donor was performed. For transient transfection experiments, statistical powering for microarray was based on initial measures of inflammation (inflammatory foci per tissue cross section). For these analyses, a sample size of n = 3 was considered sufficient for statistical powering (Cohen's d = 2.9).

Results

Prominent expression of OPN isoforms in dystrophic muscle

Spliced OPN isoform (Figure 1, Panel A) expression patterns in dystrophic muscle were determined by RT-PCR in dystrophin-deficient human and dog muscle. Consistent with previous reports, OPN was scarcely expressed in dystrophin sufficient but highly expressed in dystrophic muscle both in human and dog (Porter *et al.*, 2004; Zanotti *et al.*, 2011; Kornegay *et al.*, 2014) (Figure 1, Panels B-C). In patients with DMD, all three OPN RNA isoforms were observed; OPN-a (full length), OPN-b (minus exon 5), and OPN-c (minus exon 4), with relative levels of OPN-a>OPN-b>OPN-c (Figure 1, Panel B). In GRMD dog muscle, OPN-a and OPN-c were expressed at similar levels and OPN-b was not recognized (Figure 1, Panel C).

To determine if the relative levels of these isoforms changed as a function of age or muscle histological severity, we studied the GRMD dog model of DMD, a spontaneously occurring large animal model that shares many clinical and histological features of the human disease (Kornegay *et al.*, 2012). OPN expression in the severely affected *vastus lateralis* (VL) and *long digital extensor* (LDE), and mildly affected *cranial sartorius* (CS) of GRMD dogs (Nghiem *et al.*, 2013) was assessed by qRT-PCR. This differential involvement of muscles in GRMD is shared with human DMD patients (Li *et al.*, 2015). OPN transcripts increased as a function of both age and muscle histological severity (Figure 1, Panel D). Here, OPN-c expression closely paralleled OPN-a expression (not shown).

Human OPN-a and OPN-c isoform ratios change as with DMD severity and mechanical loading

To confirm relevance of our PCR findings, we performed Nanostring expression profiling on *vastus lateralis* biopsies from a larger cohort of human DMD (dystrophin deficient) and Becker's muscular dystrophy patients (BMD; partially dystrophin deficient). Total OPN (all isoforms) and OPN-c levels were quantified as the sequences of OPN-a and OPN-b displayed too high overlap for

accurate sequence coverage by Nanostring expressing profiling. Histology (H&E) was performed on muscle biopsy sections prior to analyses. Histology was used to group by histological severity as determined by the number of degenerating and regenerating fibers and inflammatory foci per image field. No significant differences in total OPN (sequence specific to OPN exon 7) or OPN-c (sequence specific to exons 3-5, with exon 4 deleted) were observed between patients based on diagnosis (DMD vs. BMD) or histological severity (mild vs. severe) (not shown). However, when comparing the ratio of OPN-c expression relative to all forms of OPN, DMD patients displayed a higher ratio of total OPN relative to OPN-c expression. Additionally, when grouping DMD and BMD patients based on histological severity, increased severity was associated with a higher ratio of total OPN relative to OPN-c expression (Figure 2, Panel A). This suggests that the expression of OPN-a and OPN-b isoforms, not OPN-c, is more closely associated with dystrophic pathological severity.

Since OPN is expressed during muscle degeneration and remodeling, which is a prominent feature of DMD, we sought to characterize OPN isoform expression in healthy human muscle in response to a mild inflammatory stimulus. Subjects performed one bout of unaccustomed mechanical loading (knee extension) and muscle biopsies of the *vastus lateralis* muscle were taken before and 24 hr after loading. This stimulus was previously observed to induce mild muscle damage and inflammation (Merritt *et al.*, 2013). Here, we observed, similar to DMD patient muscle biopsies that OPN-a, not OPN-c, was the most prominent isoform upregulated in response to mechanical loading. OPN-a expression increased ~8-fold ($p < 0.05$) with mechanical loading. OPN-c expression increased ~2-fold, but did not reach statistical significance ($p > 0.05$). OPN-a expression levels also correlated to the induction of the pro-inflammatory proteins TNC, a TLR4 agonist (Midwood *et al.*, 2009), and IL-1β. OPN-c levels did not correlate to expression of either of these pro-inflammatory proteins (Figure 2, Panel B).

To understand the role of elevated OPN isoform expression in dystrophic muscle, we next observed the effects of each human OPN isoform on macrophage activation, since macrophages extensively infiltrate dystrophic muscle and play critical roles in muscle remodeling. Primary human macrophages derived from circulating monocytes from normal volunteers (n = 4 healthy male donors) were incubated with recombinant human OPN protein isoforms (Yokosaki *et al.*, 1999). Cells were pre-treated with polymixin-B (PMB) to inhibit the effects of any endotoxin contamination within the recombinant proteins. Cells were then stimulated with recombinant OPN isoforms (1.0 μg/mL) or vehicle (PMB) for 4 hrs. mRNA molecule counting (Nanostring) was performed to quantify expression of pro-inflammatory and pro-fibrotic transcripts: CCL5, IL-1β, IL-10, IL-15, IFITM1, TNC, TGFβ isoforms 1-3. The transcripts included in the custom Nanostring panel were chosen based on previous microarray data demonstrating the acute upregulation of these transcripts in response to pro-inflammatory and pro-fibrotic stimuli and have been published elsewhere (Dillingham *et al.*, 2015). OPN-a induced the greatest amount of pro-inflammatory transcript induction (Figure 3, Panel A). Significant main and between treatment effects were observed when comparing differences in cytokine production induced by OPN-a, OPN-b and OPN-c as determined by ANOVA testing, with Bonferroni correction (Main effects: CCL5: $p < 0.001$; IL-1β: $p < 0.001$; IL-10: $p = 0.009$; TNC: $p = 0.015$; IFITM1: $p = 0.010$; between group effects for all proteins: OPN-a vs. OPN-b, $p<0.01$; OPN-a vs. OPN-c $p<0.001$). TGFβ-1 expression did not change in response to OPN treatment and TGFβ-2/3 isoform expression was not detectable (not shown). Together this data suggests that OPN-a promotes strong cytokine induction from human macrophages, while OPN-b and OPN-c induce less inflammatory activity.

OPN contains an RGD integrin-binding domain on exon 6, which is conserved in all three spliced isoforms. To determine if the pro-inflammatory activity of OPN was mediated by integrins

that bind to the RGD site, we used mutant OPN in which RGD was changed to KAE (OPN-a-KAE) (Young *et al.*, 1990). RGD sufficient OPN-a significantly increased the expression of inflammatory cytokines, while OPN-a-KAE displayed attenuated effects (Figure 3, Panel B).

So far, OPN was upregulated in dystrophic muscle and exerted pro-inflammatory effects on macrophages. To further understand the roles of OPN in muscle remodeling, we examined the pro-inflammatory effects of OPN on myoblasts. After incubating myoblasts in the presence of OPN isoforms secreted cytokine protein was assessed by IL-6 ELISA. Here, OPN-a was the most potent inducer of IL-6 production (Figure 4, Panel A). This also appeared to be mediated by RGD-integrin binding because like on macrophages the OPN-a RGD→ KAE mutant induced less IL-6 production (Figure 4, Panel B).

Human OPN-a cytokine induction is attenuated in macrophages upon inhibition of TLR4 signaling

Since we observed OPN induced TNC expression from macrophages and TNC is a known TLR4 agonist (Midwood *et al.*, 2009), we sought to determine whether OPN promoted macrophage cytokine production in a TLR4-dependent manner. To determine if OPN-RGD pro-inflammatory activity occurs via TLR4, a small molecule intracellular inhibitor of TLR4 signaling, TAK-242, was used. TAK-242 decreased ~98% of IL-6 expression induced by 100 ng/mL (not shown) and 10 ng/mL LPS (Figure 5) in primary human macrophages. TAK-242 did not affect cell viability (not shown). IL-6 induction by human OPN-a-RGD was similarly blocked by TAK-242 (~93% decrease in IL-6 production), whereas OPN-a-KAE did not significantly increase IL-6 production from macrophages. With OPN-a-KAE treatment the IL-6 levels were: 5.3 pg/mL (vehicle) and 4.0 pg/mL (TAK-242) (Figure 5).

Enhancement of monocyte phagocytosis equally by OPN-a, OPN-b or OPN-c

Extensive macrophage infiltration is a feature of dystrophic muscle, where macrophages phagocytose cellular debris from necrotic myofibers. We tested the effects of OPN isoforms on macrophage phagocytosis (Figure 6). All OPN spliced isoforms increased phagocytosis of fluorescently conjugated bacterial particles by ~6-8-fold (Figure 6, Panel A). OPN-a-RGD and OPN-a-KAE equally induced phagocytosis with 8-9-fold increase in macrophage phagocytosis relative to vehicle (PMB) (Figure 6, Panel B). These results suggest, in contrast to cytokine induction, stimulation of phagocytosis is not mediated by RGD-integrins, and another OPN domain is required for the phagocytosis that is shared and equally accessible between the three isoforms.

Intra-muscle OPN-a expression induces upregulates pathways indicative of macrophage recruitment and TNC-TLR4 signaling

In response to acute muscle injury by cardiotoxin, pro-inflammatory macrophages express OPN (Paliwal *et al.*, 2012). To provide insight into the role of OPN-a expression on skeletal muscle transcriptomics, we performed transcriptional profiling on muscle tissue sections following intramuscular OPN-a overexpression in 3-month old BLA/J mice. We observed OPN-a overexpression upregulated transcripts indicative of pro-inflammatory macrophage recruitment and activation. When comparing the expression of genes in TA muscles of mice injected with either OPN-a or empty-plasmid, 432 genes were differentially expressed at the $p < 0.01$ level at 7 days post-injection. Over-expression of OPN-a led to a significant increase in its own transcript (SPP1; 3.8-fold; $p = 0.008$; Figure 7). OPN-a induction also increased expression of its RGD binding receptors: integrin α_V (1.6-fold; $p = 0.005$) and integrin α_5 (1.5-fold; $p = 0.003$) (not shown). At 7 days post-injection, the most highly upregulated pro-inflammatory transcripts by OPN-a included: TNC (3.9-

fold, p = 0.004), IL-18 (1.8-fold; p = 0.010), IL-17 receptor A (1.5-fold; p = 0.007), and CD40 (1.9-fold; p = 0.008) (Figure 7). CX3CR1 expression was also upregulated 1.9-fold (p = 0.005), suggesting increased macrophage infiltration post-acute injury in response to OPN-a relative to the empty vector control. The marker of alternatively activated (less pro-inflammatory) macrophages, CD163, was also downregulated 2.3-fold (p = 0.008). Through network analysis, CSF2 appeared to be a major upstream regulator of OPN-a inducible transcripts within the skeletal muscle (z-score: 4.95; p-value of overlap: 1.04E-09). Together, data from these two experiments suggest that: 1) OPN-a inducible proteins are upregulated in acute muscle injury and; 2) intramuscular overexpression of OPN-a promotes macrophage chemotaxis and pro-inflammatory activation within the skeletal muscle.

Discussion

OPN is increasingly recognized as an important mediator of muscle inflammation after damage. A genetic study showed that gene polymorphisms in *OPN* modulate the response of adult volunteer muscle to inflammation detected by MRI after damaging contractions (Barfield *et al.*, 2014), followed by another study showing these same polymorphisms alter the clinical severity of Duchenne muscular dystrophy (Bello *et al.*, 2015). In this experiment, we found the prominent upregulation of OPN isoforms in dystrophic muscles and with mechanical loading, a mild inflammatory stimulus. This led us to define the pro-inflammatory effects of OPN spliced isoforms on the skeletal muscle microenvironment. Macrophages, an extensively infiltrating immune cell type in dystrophic muscle, were found to be highly responsive to OPN-a as indicated by increased pro-inflammatory cytokine production. OPN-a also exerted some pro-inflammatory effects on myoblasts. Among the three human OPN isoforms, OPN-a, OPN-b and OPN-c, cytokine-induction was most prominent in response to OPN-a in macrophages and myoblasts. On the other hand, each of the three isoforms stimulated monocyte phagocytosis equally well. Through expression profiling we observed

that intramuscular overexpression of OPN-a induced TNC expression, an endogenous activator of TLR4 signaling. Further, OPN-a upregulated TNC expression in human macrophages *in vitro*. Upon examining the effects of TLR4 signaling ablation on OPN-mediated cytokine production, we observed blockade of TLR4 signaling attenuated OPN-a-mediated cytokine production in human macrophages. Together, our findings suggest OPN-a acts as the most pro-inflammatory isoform in the muscle microenvironment, possibly through induction of TNC-TLR4 signaling.

In the present study, we compared OPN splice variants to each other because of distinct properties of the variants in tumor cells and increased isoform expression in dystrophic human muscle. Mutating the RGD sequence of OPN-a revealed that the pro-inflammatory effects of OPN-a, both on macrophages or myoblasts, is mediated by the RGD sequence that binds to integrins including $\alpha v\beta 3$, $\alpha v\beta 5$ and $\alpha 5\beta 1$ (Figure 1, Panel A). Structural differences between these isoforms include the presence or absence of exon 4 or exon 5, while the RGD and encompassing sequence, TYDGRGDSVVYGLR, in exon 6 is consistently present in the isoforms. The distinct pro-inflammatory effects of the OPN isoforms observed in this study would not likely occur, unless exon 4 or exon 5 influenced the interaction between OPN and integrins. The ability of exon 4 or exon 5 to affect OPN-integrin interactions is supported by several observations. First, OPN displays enhanced integrin-mediated function upon polymerization by a cross-linking enzyme, TG2 that covalently links Gln and Lys residues. OPN-b and OPN-c are less polymerized than OPN-a (Nishimichi *et al.*, 2011) due to loss of Gln residues at exons 4 and 5, which serve as crosslinking sites. Since TG2 is abundantly expressed by macrophages (Zakrzewicz *et al.*, 2015) and skeletal muscle (Park *et al.*, 1994), it is possible that the OPN isoforms were polymerized during incubation with macrophages and myoblasts. OPN-a might sufficiently polymerize for cytokine induction due to its enhanced ability to undergo polymerization. Further, polymeric OPN forms a new binding site for another

integrin α9β1, whose signals might also be less intensive in OPN-b and OPN-c than OPN-a. Second, OPN is thought to bind with many proteins through its naturally disordered domain including the regions encompassed at exons 4 and 5. If some proteins in the culture supernatant from cells or FBS bind differentially to the isoforms, the interactions between OPN and integrins would not be the same between isoforms. On the other hand, observations of RGD independent and equally enhanced monocyte phagocytosis among isoforms strongly suggests another OPN receptor whose binding site is equally accessible among isoforms promotes monocyte phagocytosis. The αxβ2 integrin might serves as such function as it is abundantly expressed on macrophages and documented to promote murine macrophage phagocytosis (Schack *et al.*, 2009).

From expression profiling, our data suggests that OPN-a promotes TNC and TLR4 responsive cytokines (IL-1β and IL-18) in humans and mice. This was evident as OPN-a induced TNC and IL-1β expression from human macrophages. Further, OPN-a expression correlated to TNC and IL-1β expression levels in human muscle following mechanical loading. In mice, overexpression of OPN-a also increased expression of TNC and IL-18, another TLR4-responsive cytokine (Rhee *et al.*, 2013). This led us to examine whether OPN-a induced cytokine production via TLR4 signaling. We observed that the pro-inflammatory activity of OPN-a on human macrophages was attenuated by pre-treatment with a small molecule inhibitor of TLR4 signaling, TAK-242. In murine RAW264.7 macrophages TAK-242 inhibits TLR4 signaling by binding to the TIR domain (Cys747) of TLR4 to inhibit downstream signaling activation (Matsunaga *et al.*, 2011). Our *in vitro* and *in vivo* profiling data suggest OPN upregulates TNC expression. TNC is an extracellular matrix protein upregulated in response to acute injury that promotes TLR4 signaling (Midwood *et al.*, 2009). Thus, a possible mechanism of OPN-mediated cytokine production may be induction of TNC-TLR4 signaling. Previous reports have also observed that OPN ablation attenuates TNC and IL-1β expression levels in

a model of lung injury (Sabo-Attwood *et al.*, 2011), which further supports this hypothesis. It is additionally possible that OPN could be acting as a TLR4 agonist itself, as numerous endogenous activators of TLR4 signaling have been reported.

In contrast to our findings, OPN isoforms derived from tumor (MCF-7) cells do not display pro-inflammatory activity (Sun *et al.*, 2013). Here, Sun *et al.* observed no effect of OPN isoforms on LPS-mediated IL-8, IL-6 or IL-12 production from primary human monocytes, while all isoforms decreased TNFα and increased IL-10 production. Only OPN-c was observed to increase the expression of CD163 on monocytes, a marker of alternative macrophage activation (Kowal *et al.*, 2011), suggesting potential anti-inflammatory activity of OPN-c. We did not observe an anti-inflammatory effect of OPN-c *per se.* However, we observed that OPN-c displayed the least pro-inflammatory activity of all of the OPN-a isoforms. The differential activities of OPN isoforms may vary widely among tissues and cell types due to multiple post-translational modifications of OPN and differential OPN receptor expression patterns (i.e. CD44 vs. integrins). Such differences may explain varying outcomes between studies and highlight a need to investigate isoform specific post-translational effects of OPN in different tissues.

In dystrophic human muscle, we observed higher OPN-c expression levels, relative to total OPN, were associated with reduced histological severity. This suggests that within the skeletal muscle, OPN-a is likely the primary contributor to the pathological effects of OPN. Targeting of OPN-a may thus be a useful approach to reducing OPN-mediated muscle inflammation. Previously, inhibition of OPN has been shown to reduce skeletal muscle inflammation in dystrophic mice (Vetrone *et al.*, 2009) and promote regeneration following acute injury in aged mice (Paliwal *et al.*,

2012). Targeting OPN-a, but not OPN-c, signaling in human skeletal muscle may be a useful therapeutic strategy as OPN does exert some positive effects on skeletal muscle regeneration. Substratum OPN promotes myoblast attachment and myotube fusion in murine cells (Uaesoontrachoon *et al.*, 2008). Conversely, soluble OPN-a inhibits myotube fusion. These differential effects of OPN on myoblast proliferation and myotube fusion are congruent with the pro-inflammatory effects of OPN as inflammatory stimuli typically promote myoblast proliferation and inhibit myotube fusion (Arnold *et al.*, 2007). As the beneficial effects of OPN ablation in dystrophic and aging muscle are presumably due to inhibition of its inflammatory activities (OPN-a is the only isoform expressed in mice), inhibiting the inflammatory action of OPN-a while maintaining some activity of OPN-c may serve as a useful therapy in humans. Future studies examining the effects of OPN isoforms on myogenesis (proliferation and fusion) may further identify the therapeutic utility of OPN isoform targeting in muscle disease.

In *mdx* mice, ablation of OPN attenuates muscle fibrosis and TGFβ expression (Vetrone *et al.*, 2009). However, we did not observe a direct effect of OPN on TGFβ production in macrophages or myoblasts (not shown). Based on our findings, the ability of OPN to promote fibrosis is likely due to its pro-inflammatory, chemotactic and phagocytic activities, rather than directly inducing TGFβ production from myoblasts or macrophages. This is supported as genetic ablation of TLR4 reduces both inflammation and fibrosis in *mdx* mice, suggesting that inflammation, particularly innate immunity, contributes to muscle fibrosis in the context of dystrophin deficiency (Giordano *et al.*, 2015). However, it is possible that OPN expression in the muscle microenvironment promotes TGFβ secretion by another cell type.

Limitations

In dystrophic human muscle, this study was limited by a conservative sample size. Future profiling of OPN-a and OPN-c expression levels and their correlation to muscle pathology may elucidate the utility of OPN isoform ratios as a biomarker of disease severity. We did not specifically examine the effects of inhibiting TNC on OPN-mediated inflammatory cytokine production. Further studies investigating OPN-TNC signaling are thus warranted. Additionally, it is unknown whether OPN isoform expression and function is fiber type specific. Future studies examining the effects of OPN overexpression in murine muscle groups with varying fiber type composition may thus be of interest.

Together our data suggests OPN is an important mediator of muscle inflammation, whereby its expression in response to mechanical loading and in dystrophin deficiency contributes to both macrophage and muscle cytokine production. Further, our data support a body of literature that localized myofiber OPN-a expression increases inflammation by promoting pro-inflammatory monocyte recruitment and macrophage activation to damaged myofibers. The pro-inflammatory activity of OPN-a may be mediated, in part, by induction of TNC-TLR4 signaling. Our data further suggests differential activities of human spliced OPN isoforms and suggests that targeting RGD-mediated integrin signaling and/or transglutamination may attenuate the pro-inflammatory activity of OPN-a within the skeletal muscle microenvironment.

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Author Contributions

GMM, KU, PPN, LB, SD, and Y.Yin, contributed to data acquisition, analysis and/or interpretation. GMM, YY, JMD, HBC, JNK, MMB, DMM, KN, and EPH contributed to study design and data analysis and interpretation.

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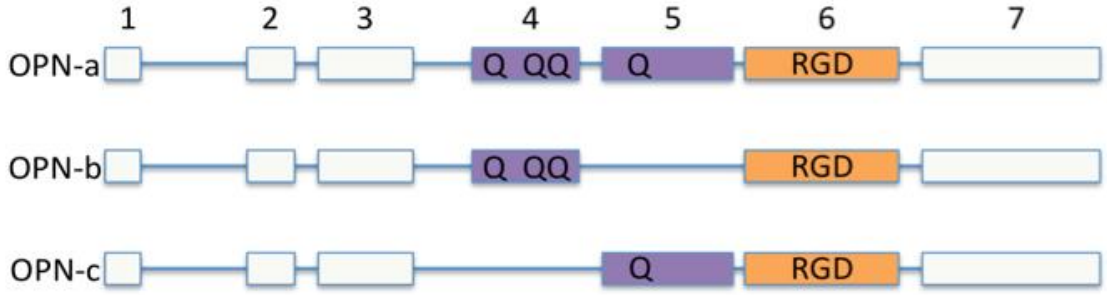
Figure Legends:

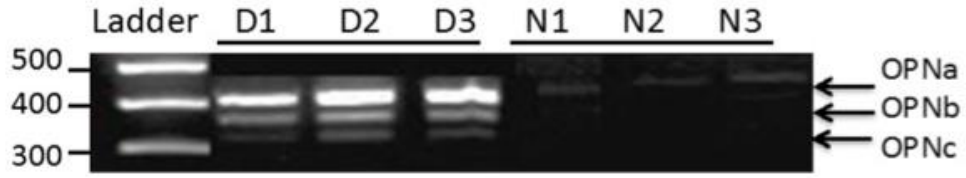
Figure 1. Alternatively spliced osteopontin isoforms are expressed in dystrophic muscle. (Panel A) OPN spliced isoform transcript structure. The blocks in this figure represent exons and the lines represent introns. Here, OPN-a is full length (top), OPN-b is missing exon 5 (middle), and OPN-c is missing exon 4 (bottom). (Panel B) Osteopontin isoform transcript expression in muscle biopsies of three dystrophin deficient Duchenne muscular dystrophy patients (DMD) [D 1-3], and three dystrophin sufficient controls [N 1-3] as determined by RT-PCR. Minimal expression is detected in dystrophin sufficient muscle, whereas all three isoforms are expressed in DMD muscle (full length OPN-a, OPN-b lacking exon 5, and OPN-c lacking exon 4). (Panel C) Dog dystrophin deficient muscle similarly shows no detectable expression in dystrophin sufficient littermates (N1), but high expression of OPN-a and OPN-c in dystrophin deficient GRMD *vastus lateralis* muscle (D1). (Panel D) RT-PCR analysis of muscle biopsies from three muscles and two age points in dystrophin sufficient (N) and deficient (D) dogs shows elevated OPN mRNA in all dystrophic muscles at all age points. An increase in OPN-a mRNA levels with age is seen in the three muscle groups tested (CS – *cranial sartorius*; LDE – long digital extensor; VL – *vastus lateralis*). Here, OPN-a mRNA levels correlated with both age and severity of muscle involvement (CS: mildly affected; LDE and VL: severely affected). Triplicates are shown per sample. Significant difference between dystrophic and non-dystrophic littermates: ** $p < 0.01$; Significant differences with age per muscle group: # $p \leq 0.05$; ## $p \leq 0.01$ [GRMD: n = 8; dystrophin sufficient littermates: n = 4]).

A.



RGD: Integrin binding domain
Q: Glutamine residue; transglutamination site

B.



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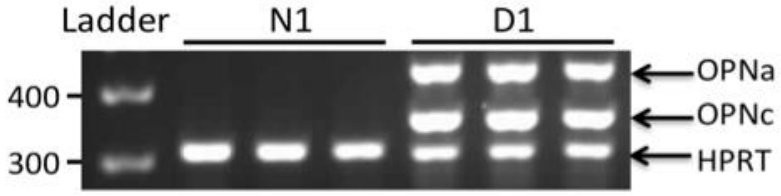

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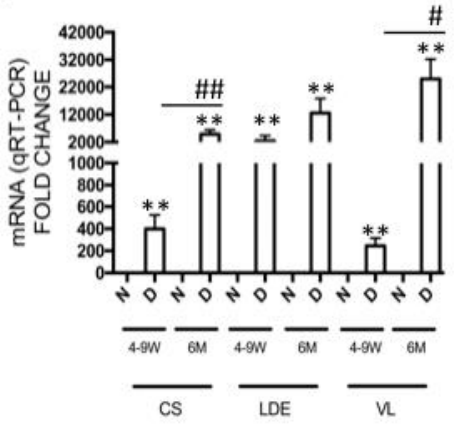
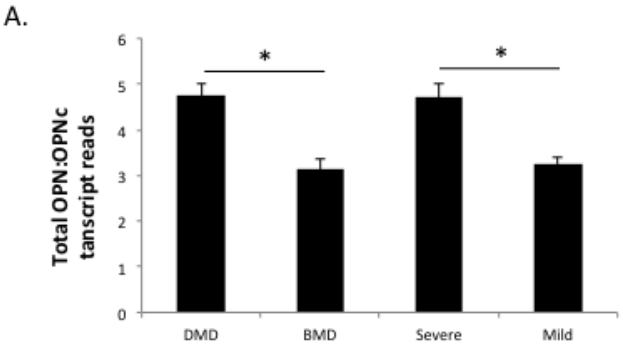
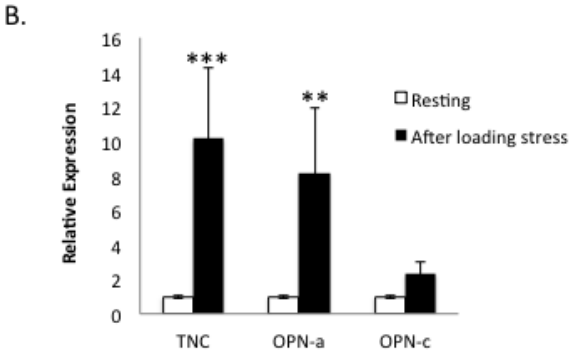


Figure 2. Human OPN-a and OPN-c isoform ratios change as with dystrophic severity and mechanical loading. (Panel A) The expression ratio of total OPN (using primers specific to exon 7) to OPN-c (specific to mRNA sequence spanning from exons 3-5, with exon 4 deleted) in DMD and BMD *vastus lateralis* human muscle biopsies (n=18; all male). Patients were grouped by histological severity prior to Nanostring mRNA molecule counting, as indicated by total fiber de- and re-generation and inflammatory foci as assessed via H&E staining. (Panel B) Transcript fold-changes (ΔΔCT) were quantified before and 24 hrs after mechanical loading (n = 22, matched for sex) by qRT-PCR. IL-1β and OPN-c were undetectable in some subjects (n = 3 and n = 4, respectively) at either baseline or at 24 hrs and were excluded from analysis. The tables below displays Pearson Product-Moment Correlation Coefficients between fold changes in transcripts (ΔΔCT values) in all subjects. * $p < 0.05$; ** $p < 0.01$ between groups.

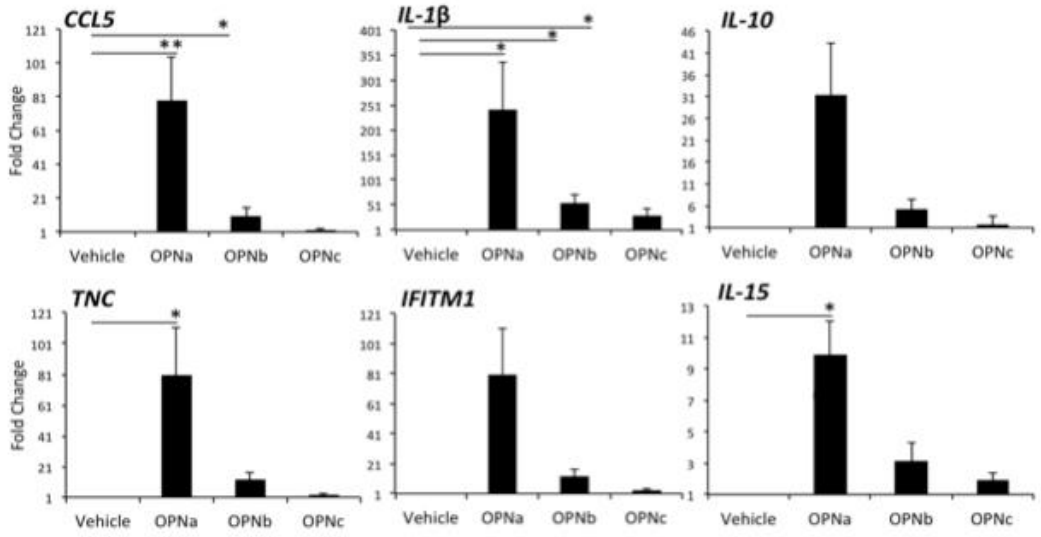




	ΔΔTNC	ΔΔIL-1β
ΔΔOPN-a	0.82**	0.71**
ΔΔOPN-c	0.12	-0.05

Figure 3. Human primary macrophages show a pro-inflammatory response to recombinant OPN-a in an RGD dependent manner. Shown are fold changes of immunoregulatory transcripts from primary human macrophages treated with 1 μg/mL OPN isoforms (Panel A), or RGD sufficient (OPN-a-RGD) and deficient (ΔRGD→KAE [OPN-a-KAE]) OPN (Panel B). (Panel A) OPN-a increased pro-inflammatory transcripts, whereas OPN-b and OPN-c show less activity. Statistical significance is presented relative to vehicle control. (Panel B) Macrophages were stimulated with 2 μg/mL OPN-a-RGD or OPN-a-KAE show that the pro-inflammatory activity of OPN-a requires RGD-mediated binding to integrins on macrophages. Statistical significance is compared between treatment conditions. Expression values after treatment with OPN isoforms are presented average fold-change relative to vehicle (PMB) treatment for n = 4 healthy male donors. *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$ as assessed via paired t-tests (average donor response).

A.



B.

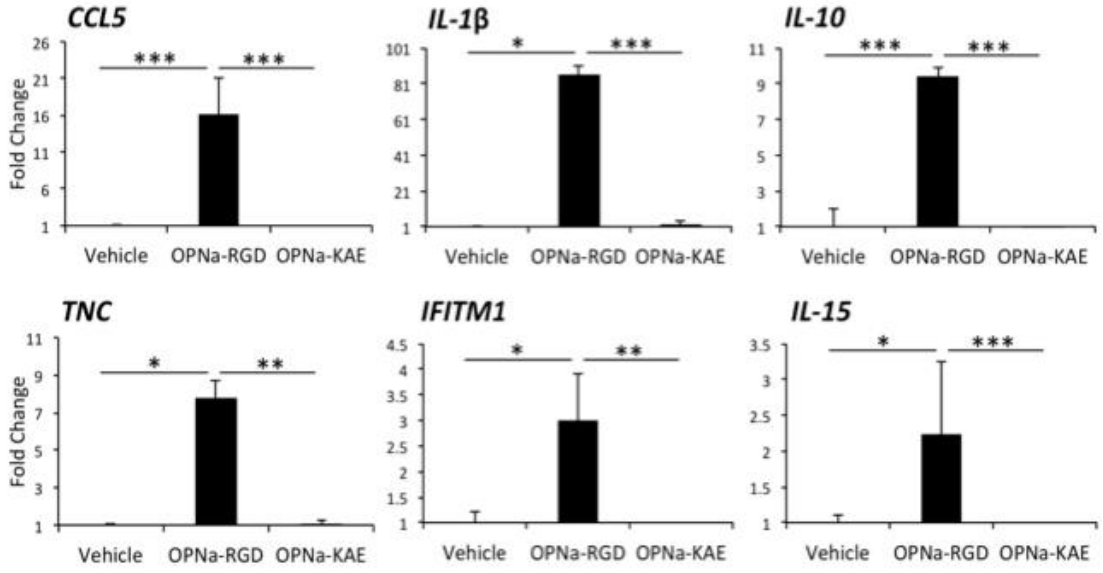


Figure 4. OPN isoforms differentially induce pro-inflammatory cytokine production from human myoblasts in an RGD-dependent manner. Immortalized human myoblasts were cultured for 48 hr with recombinant human OPN proteins treated with: (Panel A) 0.25 or 1.0 μg/mL human OPN -a, -b, or -c, or (Panel B) 1.0 μg/mL recombinant human OPNa-RGD or OPNa-KAE. IL-6 levels in the secreted media were quantified after 48 hr by ELISA. Samples were ran in replicates of n = 5.

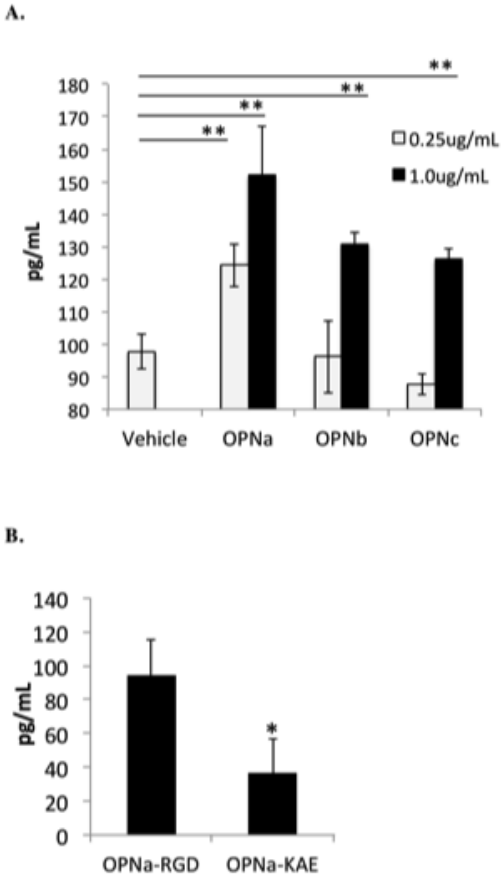


Figure 5. Pretreatment of human macrophages with TAK-242, an inhibitor of TLR4 signaling, inhibits human OPN-a mediated IL-6 production. Primary human macrophages were treated with TAK-242 (100 nM) or vehicle (DMSO) for 1 hr prior to stimulation with 1 μg/mL OPN-a-RGD and OPN-a-KAE or 10 ng/mL LPS (positive control). IL-6 levels in the secreted media were measured at 24 hr by ELISA. The pro-inflammatory activity of OPN-a was largely blocked by TAK-242. TAK-242 had little effect on OPN-a-KAE activity. Samples were ran in replicates of n = 4.

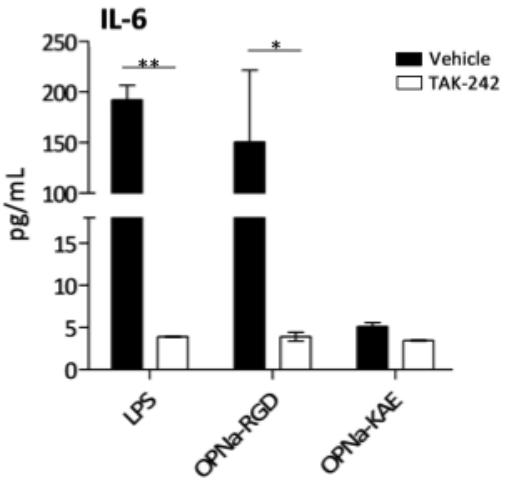


Figure 6. Human OPN mediated monocyte phagocytosis is isoform and RGD independent. After 30 min pretreatment with PMB, primary human monocytes were treated for 2 hr with recombinant OPN proteins or vehicle (PMB) and in the presence of fluorescently conjugated *E. coli* particles. Cells were washed and cellular fluorescence of non-viable cells was quenched with trypan blue. Phagocytosis is presented as fold increase in fluorescence from viable cells, relative to vehicle control cells (PMB). All conditions were normalized for background fluorescence (*E. coli* particles only) prior to calculation of fold-change relative to vehicle control. (Panel A) Phagocytosis induced by 0.5 µg/mL OPN spliced isoforms relative to vehicle (PMB). (Panel B) Phagocytosis induced by 1.0 µg/mL OPN-a-RGD and OPN-a-KAE. All samples were ran in replicates of n = 5, according to the manufacturer's instructions (Vybrant Phagocytosis Assay Kit; Life Technologies). Significance is presented relative to vehicle control.

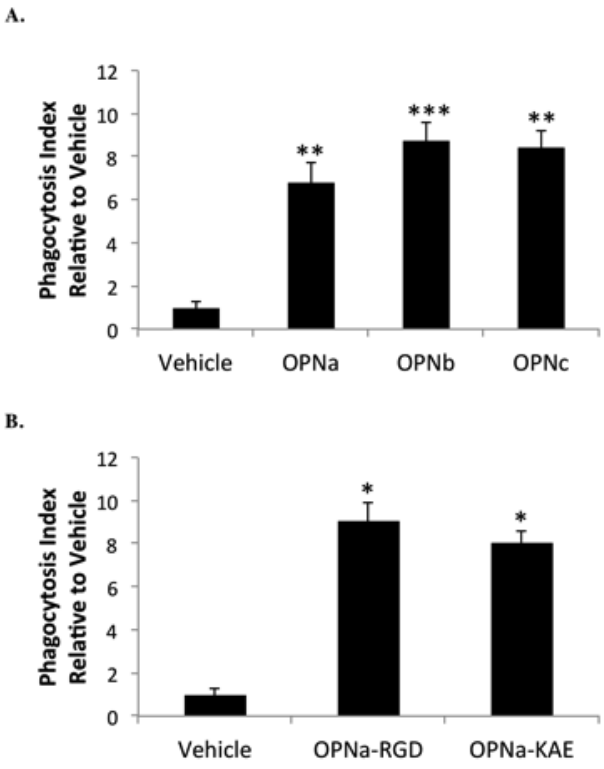


Figure 7. OPN-a promotes pro-inflammatory macrophage infiltration *in vivo*. BLA/J mice (n = 3) were injected with OPN-a and an empty vector in contralateral TA muscles. TA muscles were collected 7 days post-injection and Illumina mRNA expression profiling was performed. Data is represented as average transcript fold-change by OPN-a relative to empty vector control. Changes in all transcripts shown were statistically significant ($p < 0.05$). Spp1= OPN; TG2= transglutaminase 2; TNC= tenascin-C; CSF2= GM-CSF.