



Published in final edited form as:

J Vasc Surg. 2018 December ; 68(6 Suppl): 22S–29S. doi:10.1016/j.jvs.2017.10.068.

Osteopontin May be a Driver of Abdominal Aortic Aneurysm Formation

S. Keisin Wang, MD,

Linden A. Green, PhD,

Ashley R. Gutwein, MD,

Alok K. Gupta, MD,

Clifford M. Babbey, BS,

Raghu L. Motaganahalli, MD,

Andres Fajardo, MD,

Michael P. Murphy, MD

Division of Vascular Surgery, Department of Surgery, Indiana University School of Medicine, Richard Roudebush VA Medical Center, Indianapolis, IN

Abstract

Objective: Previous *in vitro* and animal studies have suggested that Osteopontin (OPN), an inflammatory extracellular matrix (ECM) protein, is involved in the formation and growth of Abdominal Aortic Aneurysms (AAA). However, the mechanism in which this occurs continues to be nebulous. The relationship between OPN and inflammation suppressing lymphocytes present in the human AAA condition was investigated and presented herein.

Methods: Serum OPN concentrations were measured in healthy, risk-factor matched (RFM) non-AAA, and AAA patients by Enzyme Linked Immunosorbent Assay (ELISA). Immunohistochemistry (IHC) was used to determine the source of OPN secretion using aortic tissue collected from multi-organ donors and AAA patients undergoing open surgical repair. Vascular smooth muscle cells (VSMCs) were exposed to various inflammatory mediators and OPN expression was evaluated by Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR) and ELISA. The inflammatory nature of OPN and the aortic wall was determined using a TR1 suppressor cell induction assay as a surrogate and characterized by ELISA and Fluorescence Activated Cell Sorting (FACS).

Corresponding author: Michael P. Murphy M.D., Associate Professor, Department of Surgery - Division of Vascular Surgery, Indiana University School of Medicine, 1801 Senate Blvd MPC# 2-3500, Indianapolis, IN 46202, Phone: (317) 962-0282, Fax: (317) 962-0289, mipmurph@iupui.edu.

Presentation: Midwest Vascular Surgery Society Annual Meeting, Chicago 2017, Plenary Session (Charles C. Guthrie Basic Science Award)

Disclosures: The authors have nothing to disclose pertinent to the experiments described in this manuscript.

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Results: OPN was found to be elevated in both the plasma and aortic homogenate of AAA compared to controls. On IHC, OPN localized to the tunica media of the diseased aorta but was minimally expressed in healthy aorta. *In vitro*, cigarette smoke extract (CSE) was the most potent stimulator of OPN secretion by VSMCs and increased both mRNA and supernatant concentrations. OPN demonstrated an ability to inhibit the induction of IL-10 secreting TR1 lymphocytes, a depleted population in the AAA patient, from naïve precursors. Lastly, neutralizing receptor targets of OPN in the setting of AAA homogenate coincubation abrogated the inhibition of TR1 induction.

Conclusions: OPN, secreted by the VSMCs of the tunica media, is elevated in the circulating plasma and aortic wall of patients with AAA. It can inhibit the induction of the TR1 suppressor cell leading to an overall proinflammatory state contributing to progressive aortic wall breakdown and dilation.

Background

AAA is a significant cause of morbidity and mortality in the United States.¹ Currently, treatment consists of watchful waiting and regimented surveillance until cross-sectional diameter reaches 5.5 cm. At this size, surgical intervention is offered to negate the risk of rupture and death.² However, aortic surgery is associated with immense postoperative morbidity and mortality.³ Therefore, the discovery of a pharmaceutical to slow diameter expansion and decrease the need for surgical intervention has been the subject of intense investigation.

OPN, a proinflammatory ECM protein, has been previously implicated in cardiac dysfunction after ischemic injury⁴, pathological neutrophil recruitment⁴, anti-apoptosis⁵, amplification of inflammation through mast cell degranulation, and immunoglobulin production.⁵ Additionally, OPN is intimately involved in atherosclerosis⁶ and proteolysis⁷, processes crucial in aortic degeneration.⁸

Methods

All results described in this manuscript were obtained while adherent to the principles outlined by the latest Declaration of Helsinki.⁹ Additionally, all experiments were reviewed and approved by the Indiana University Institutional Review Board (IRB #1408881234).

Collection and Banking of Blood

After informed consent was obtained, peripheral blood was collected from patients presenting for their United States Preventative Task Force (USPTF) recommended AAA screening at Indiana University School of Medicine affiliated hospitals (Table I). Patients screening negative were deemed RFM and used as a control. Samples from healthy patients (no comorbidities) were sourced from surgical resident volunteers. Peripheral blood mononuclear cells (PBMCs) and plasma were isolated using Ficoll-Paque (Sigma, St Louis, MO) assisted density centrifugation within 24 hours of collection and stored at -80°C. All assays performed were from randomly selected banked PBMC and plasma samples.

Generation of Cigarette Smoke Extract (CSE)

1R6F research cigarettes were obtained from the University of Kentucky Center for Tobacco Reference Products. The chemical composition of these cigarettes are available on the vendor website.¹⁰ The steps in the generation of CSE is described in detail elsewhere.¹¹ In short, the smoke from 10 1R6F research cigarettes were vacuum filtered through 500 mL of Phosphate Buffered Saline (PBS) in a negative pressure fume hood. The resulting solution was sterilized via passage through a 22 µm filter.

Generation of Aortic Homogenate

1 g of aneurysmal or normal aortic tissue was immediately flash frozen and ground in liquid nitrogen on the day of sample collection. The resulting blend was dissolved in 50 mL of PBS and vigorously vortexed to a suspension. This solution was sterilized through a 22 µm filter and used immediately; any remaining aortic homogenate was stored at -20°C.

Tissue Culture and VSMC OPN mRNA

Healthy human aortic VSMCs were obtained from ATCC (Manassas, VA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma) supplemented with 10% Fetal Bovine Serum (FBS, Sigma) and 1× Penicillin/Streptomycin (Sigma) at 37°C (5% CO₂). Tissue culture flasks were passaged when cells reached 90% confluency. To assay OPN expression, VSMCs were plated on a 12-well plate at 90% confluency before exposure to suspected OPN stimulants such as CSE (1%), angiotensin-II (AT2, 50 µM, Sigma), IFN-γ (10 ng/mL, R&D), and TNF-α (10 ng/mL, R&D). Supernatant was harvested at 48 hours for ELISA. To determine OPN mRNA, VSMCs were harvested at 24 hours and lysed using a Qiagen RNeasy kit (Germantown, MD) to collect total mRNA; resultant mRNA yields were determined using a NanoDrop 8000. 10 ng of total mRNA was used for qRT-PCR with the SYBR Green RT-PCR Master Mix Kit (Agilent, Santa Clara, CA). The primers to OPN and GADPH (Origene, Rockville, MD) were employed per manufacturer's instructions and the PCR product confirmed on a 0.8% agarose gel.

Immunohistochemistry

AAA tissue samples were obtained intraoperatively at the time of elective open aneurysm repairs. Normal healthy aortic tissue was collected at the time of multi-organ donor harvests. All samples were immediately stored in 10% neutral buffered formalin (NBF). After 48 hours, samples were transitioned to 70% EtOH for long-term storage. Preserved tissues were eventually embedded in paraffin blocks and sectioned. Antigen retrieval was performed if staining was suboptimal as deemed by the performing histologist. Alkaline phosphatase and horseradish peroxidase reporting systems (Sigma) were used to label actin and OPN in the aortic walls, respectively. Sections were counterstained with hematoxylin or methyl green at the discretion of the performing histologist.

Induction of TR1 Lymphocytes

The induction of TR1 lymphocytes from naïve T-cells has been previously described using plate-bound αCD3 and high molecular weight hyaluronan (HMW-HA) in the presence of αCD28.¹² Non-memory CD4⁺CD45RO⁻ naïve T-cells were isolated from healthy donors

using Magnetic Activated Cell Sorting (MACS, Miltenyi, Auburn, CA) per manufacturer's instructions. The purity of the isolated product has previously been demonstrated to be >90%.¹³ Isolated naïve T-cells were plated in Optimem (Sigma) supplemented with α CD28 (0.5 μ g/mL, Sigma) at a concentration of roughly 2.5×10^5 cells/mL. These plates were previously coated with α CD3 (0.5 μ g/mL, Sigma) and blocked with 100 μ g/mL of HMW-HA (Genzyme, Boston, MA) to activate and induce TR1 suppressor cells. After 96 hours, lymphocytes were harvested and analyzed by FACS (BD Accuri C6) using a triple stain for CD4-FITC (Miltenyi), CD49b-PE (Miltenyi), and LAG3-APC (Biolegend, San Diego, CA). TR1% was expressed as a ratio of total CD4⁺ cells. Results were confirmed by IL-10 supernatant concentration assayed by ELISA. The neutralization of OPN in aortic homogenate was performed with a combination of α CD44 (Abcam, Cambridge, MA), α OPN (Abcam), and RGD peptide targeting the α V β 3 integrin, a target of OPN activity, per manufacturer's instructions.

Results

Osteopontin is Increased in the AAA condition

We quantified OPN concentrations in cohorts of healthy, RFM, and AAA patients. Isolated plasma was subject to ELISA demonstrating an increase in serum OPN (Figure 1A) in the AAA patients compared to healthy and RFM controls. Next, aortic tissue homogenate of both healthy and aneurysmal samples were assayed for OPN to determine local aortic OPN levels. AAA patients demonstrated a 4-fold increase in tissue OPN as compared to healthy multi-organ donor controls (Figure 1B).

VSMCs of the Tunica Media Secrete OPN in Response to CSE

Aortic VSMCs were cultured with and without the presence of suspected stimulators of OPN expression. Supernatant and VSMCs were harvested after 48 and 24 hours, respectively. OPN mRNA harvested from VSMCs were amplified using qRT-PCR and normalized to constitutively expressed GADPH. A 4-fold increase in OPN mRNA was observed in the VSMCs stimulated with CSE as compared to controls (Figure 2A). Additionally, supernatant OPN from VSMCs exposed to CSE measured by ELISA demonstrated a 2-fold increase compared to controls (Figure 2B).

AAA specimens collected from patients undergoing open surgical repair were immediately preserved in formalin before paraffinization and sectioning. Antibodies specific to OPN and α -actin confirmed localization of OPN to the tunica media of the aneurysmal wall (Figure 3). In comparison, little to no OPN expression was noted in the healthy aortic specimens harvested from multi-organ donors (Figure 4).

IL10 is Decreased in AAA Corresponding to a Loss of TR1 Lymphocytes

Our previous preliminary studies demonstrated a depletion of IL-10, a potent anti-inflammatory cytokine in the AAA population. In fact, A greater than 10-fold decrease in circulating IL-10 was noted in the AAA population compared to controls (Figure 5A). We suspected this decrease was related to a loss of the TR1 lymphocyte, a potent secretor of IL-10. Therefore, PBMCs from both AAA and control cohorts were stained

for the CD4⁺CD49b⁺LAG-3⁺ TR1 population, quantified via FACS, and normalized as a percentage of total CD4⁺ cells. In the AAA cohort, we found a greater than 4-fold decrease in total circulating TR1 cells (Figure 5B).

OPN Inhibits Induction of TR1 Lymphocytes from Naïve T-Cells

To determine the reason for TR1 depletion in the AAA condition, TR1 lymphocytes were induced from naïve CD4⁺CD45RO⁻ T-cells in tissue culture wells saturated with plate bound aCD3 and blocked with HMW-HA. Soluble aCD28 was added at the same time as the naïve CD4 cells isolated via MACS sorting. Supernatant and lymphocytes were harvested after 96 hours and assayed using FACS and ELISA to determine induction efficiency. OPN proved to be a potent inhibitor of IL-10 expression from these lymphocytes suggesting an inhibition of TR1 induction (Figure 6A). FACS analysis confirmed the changes in IL-10 expression correlated with changes in TR1 induction. Additionally, CSE and AAA homogenate proved to inhibit TR1 induction to a similar extent as compared to OPN (Figure 6B). Next, we attempted to block the effect of OPN in AAA homogenate on TR1 induction via introduction of aCD44, OPN neutralizing antibodies, and RGD peptide. With this OPN neutralizing cocktail, the inhibitory effect of AAA homogenate on TR1 induction was abrogated (Figure 6C).

Discussion

OPN is a glycoprotein that was first identified in osteoblasts and was first implicated in bony growth and calcification.¹⁴ Additionally, OPN signaling seems to be crucial in tissue remodeling via post-translational modification of matrix metalloproteinases (MMP) and binding to ECM glycoproteins such as heparan, fibronectin, and collagen.¹⁵ Importantly to AAA, OPN is highly expressed by M1 macrophages, the first inflammatory cells observed in the newly aneurysmal aorta. These inflammatory cells further upregulate monocyte migration via OPN secretion as it also serves as a strong chemotactic cytokine.¹⁶ Not surprisingly, macrophages from OPN^{-/-} mice are more susceptible to apoptosis, are less chemotactic, and has a lower cytotoxic ability.^{15,17}

OPN has a myriad of functions which leads to progression of atherosclerosis. For example, foamy macrophages in animal models express significantly higher concentrations of OPN.¹⁸ In fact, serum OPN levels seems to drop with the initiation of atherosclerosis modifying treatments such as angiotensin converting enzyme inhibitors and statins.¹⁹ Secretion of OPN within the vasculature has been demonstrated in VSMCs secondary to high glucose exposure and endothelial cells secondary to cigarette smoke exposure affecting disease severity via NFAT inhibition and PPAR γ signaling.^{20,21} After coronary revascularization, restenosis is significantly decreased with α OPN antibody graft pretreatment suggesting a role in neointimal hyperplasia as well.²²

The connection between AAA and OPN was first described by Gollege *et al.* In his initial publication, he established an association even stronger than that of CRP and AAA. Furthermore, increased OPN levels seemed to predict AAA growth over time after adjusting for other known risk factors.²³ We confirm his initial report in both the peripheral blood and local aortic wall of AAA patients. It appears that the source of this increased OPN

originates from the VSMCs as a response to inflammatory mediators such as TNF- α , IFN- γ , and cigarette smoke.¹⁷ Interestingly, we found the strongest stimulator of *in vitro* VSMC OPN expression was CSE compared to nonsignificant increases in OPN mRNA by IFN- γ , TNF- α , and AT2 (not presented).

We do not know the initiator of AAA formation; however, risk factors identified include advancing age, male gender, smoking, COPD, CAD, HTN, HLD, and a positive family history.²⁴ Diabetes seems to be protective of both formation and growth for unknown reasons.²⁵ After initiation, the tunica adventitia of the abdominal aorta is infiltrated with macrophages, neutrophils, and cytotoxic T-cells which release a gamut of elastases, collagenases, and MMPs directly causing structural degradation and vessel dilation.^{26,27} Theories of pathogenesis linking inflammation and autoimmunity to AAA formation have steadily become more accepted over time.²⁸

The CD4⁺CD49b⁺LAG3⁺ TR1 suppressor lymphocyte is a primary secretor of IL-10, a potent anti-inflammatory cytokine.²⁹ This unique T-cell is antigen-specific and becomes activated when recognition occurs.³⁰ IL-10 and the corresponding suppressor lymphocyte were found to be severely depleted in the AAA condition compared to RFM controls and is the target of our current investigations. Of interest, OPN, CSE, and AAA homogenate all demonstrated a strong ability to inhibit the induction of TR1 lymphocytes from naïve T-cells using the action of HMW-HA on CD44 and the α V β 3 integrin. However, when the OPN activity of AAA homogenate was inhibited by a neutralizing cocktail, a loss of induction inhibition was observed suggesting OPN as the effector molecule in the aneurysmal aortic wall.

Based on our results, we argue that OPN plays an important role in AAA propagation and growth through inhibition of the immune suppressor response. We believe that initiation occurs secondary to an insult from cigarette smoke exposure which causes an increase in circulating elastin and collagen degradation products causing autosensitization.³¹ We have recently submitted a manuscript highlighting the discovery of a higher concentration of antibodies to elastin fragments in the AAA plasma compared to RFM controls. As a result, the infrarenal aortic segment, under the highest mechanical stress, becomes a target of sensitized inflammatory cells leading to ECM degradation.^{29,32} It is presently unclear to us why some patients never develop aortic wall sensitization despite cigarette smoke exposure and elastin fragment generation. However, predisposed patients are victim to local OPN accumulation through expression by M1 inflammatory macrophages of the innate immune system and stimulated VSMCs of the tunica media. This upregulation of OPN in the aortic wall inhibits the ability of TR1 suppressor cell function and further propagates local inflammation and eventual vessel ectasia.

Conclusion

In this series of experiments, we suggest that AAA propagation may result from OPN overexpression, possibly through prolonged cigarette smoke exposure, causing a deficit in TR1 suppression of the runaway local inflammatory response.

Acknowledgments

The work described in this article was in part supported by the National Institutes of Health grants 1UM1HL113457 and R01HL128827.

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Little is known about the initiating event of AAA formation. However, the early histology of the ectatic aorta is characterized by massive infiltration of mononuclear cells and elaboration of matrix metalloproteinases (MMPs), collagenases, and elastases. This runaway inflammatory response directly leads to extra-cellular matrix (ECM) degradation and loss of aortic integrity. In this study, we report elevations of osteopontin (OPN), an ECM glycoprotein associated with inflammation in other pathologies. Additionally, OPN demonstrated an ability to inhibit the expression of the TR1 lymphocyte, a potent suppressor of inflammation. Therefore, we argue that accumulation of OPN in the aortic wall may be a potent driver for AAA propagation.

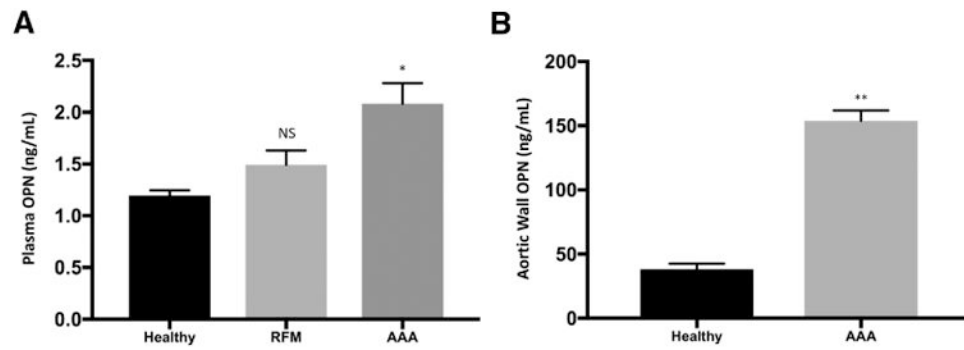


Figure 1.

(A) Osteopontin (OPN) plasma concentrations were quantified in healthy, RFM, and AAA patients demonstrating a 2-fold increase in the AAA as compared to the healthy condition ($*p<0.05$; $n=32$ /group). There was no difference (NS) between RFM and healthy controls, but a difference was observed between RFM and AAA cohorts ($p<0.05$). (B) Healthy aortic tissue was harvested from multi-organ donors and compared to AAA samples collected at the time of open surgical repair. A 4-fold increase in the local OPN concentration was observed in the AAA condition ($**p<0.01$; $n=6$ /group).

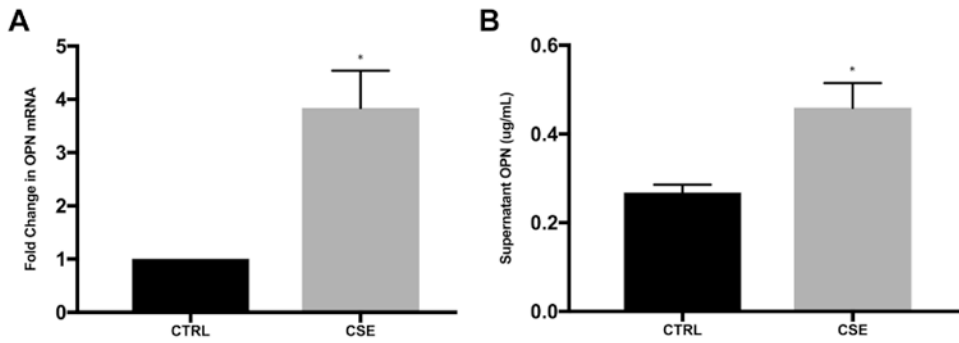


Figure 2.

(A) Human vascular smooth muscle cells (VSMC) were plated into 12-well plated and exposed to cigarette smoke extract (CSE) at 90% confluency for 24 hours. Cells were harvested and OPN mRNA concentration was assayed with qRT-PCR. CSE mRNA was normalized to the mean of the control (* $p < 0.05$; $n = 4$ /group). (B) After 48 hours, the supernatant was collected and assayed using ELISA. A 2-fold OPN increase was observed (* $p < 0.05$; $n = 6$ /group).

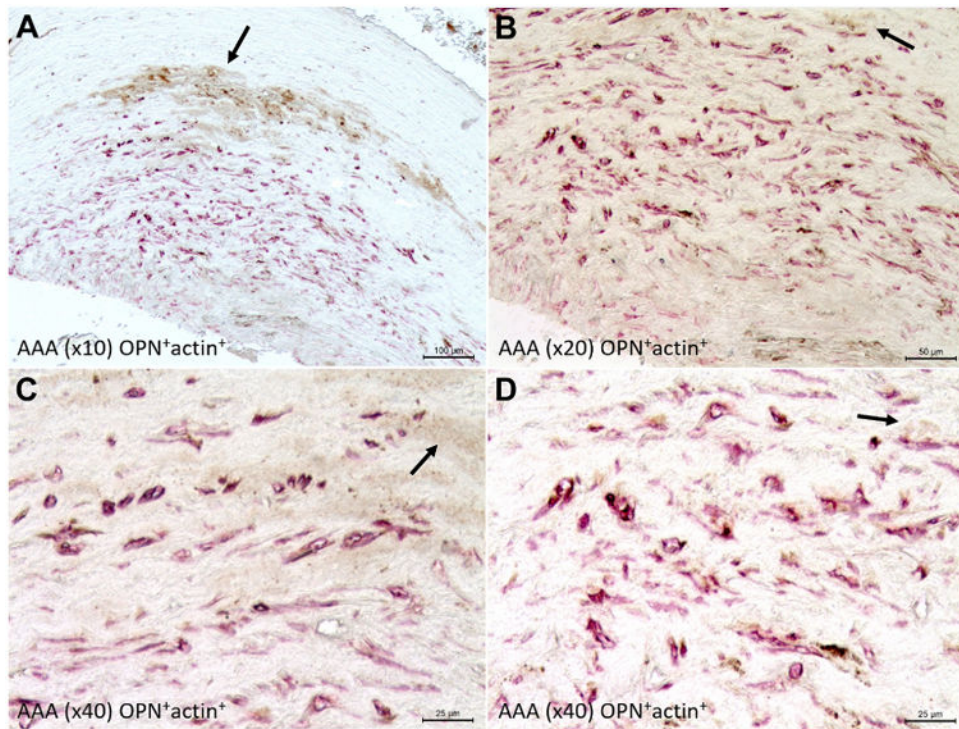


Figure 3. Representative IHC images of the tunica media AAA samples stained for OPN (brown/black, arrow) and actin (red) through a hematoxylin (blue, nucleus) counterstain under various magnifications. High concentrations of OPN was observed in the tunica media throughout.

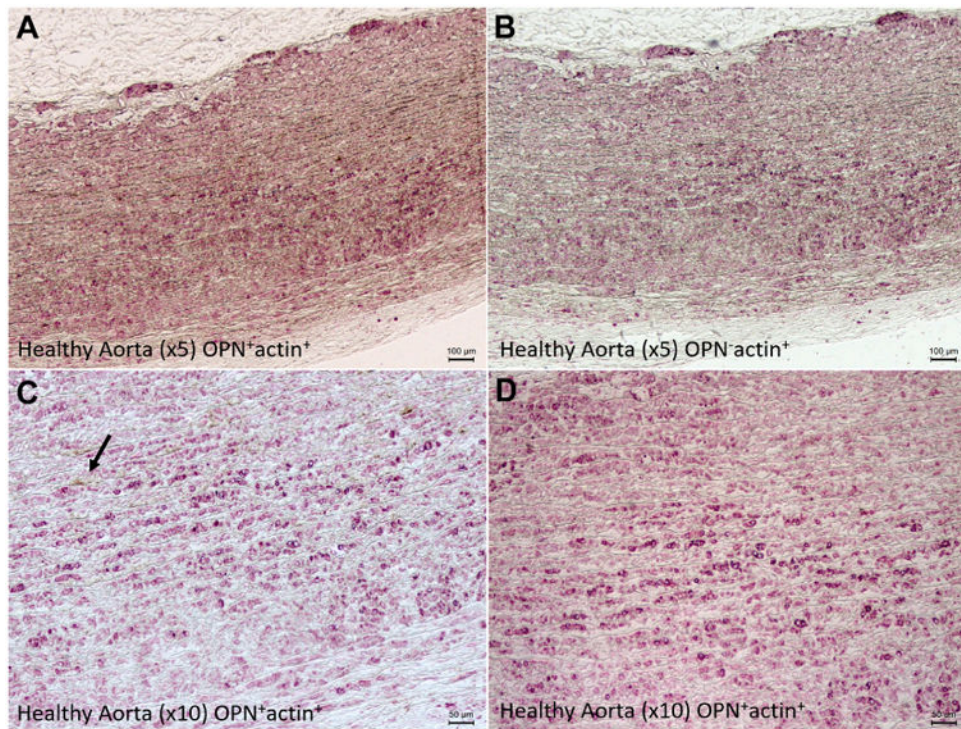


Figure 4. Representative IHC images of the tunica media of healthy aortic samples stained for OPN (brown/black, arrow) and actin (red) through a hematoxylin (blue, nucleus) counterstain under various magnifications. Little to no OPN was observed in the tunica media of healthy aorta.

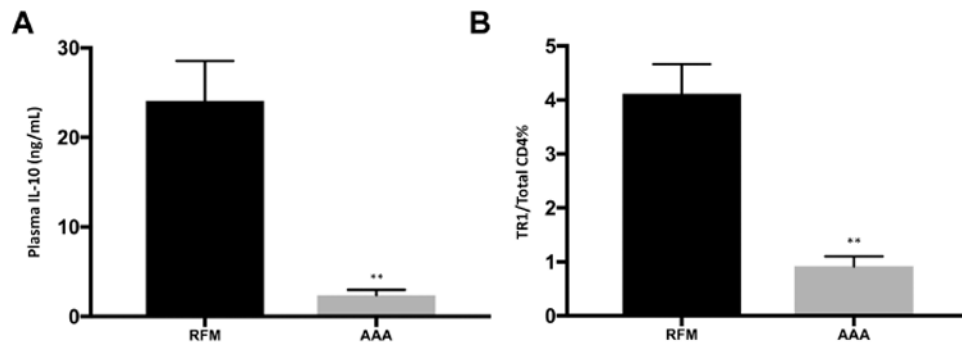


Figure 5. (A) IL-10, a potent anti-inflammatory cytokine, is severely depressed in the plasma of AAA patients compared to RFM controls. (B) TR1 lymphocytes are one of the more effective secretors of IL-10 and is severely depressed in the AAA population compared to RFM controls. (* $p < 0.05$, ** $p < 0.01$; $n = 20-26$ /group)

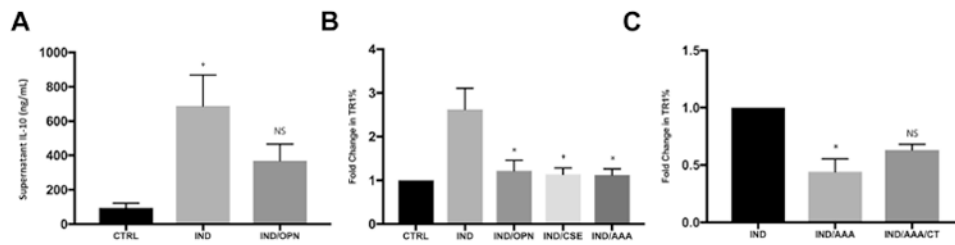


Figure 6.

(A) TR1 lymphocytes were induced (IND) *in vitro* in the presence of α CD3, α CD28, and high molecular weight hyaluronan (HMW-HA) from naïve CD4⁺CD45RO⁻ T-cells and compared to non-induction conditions. Efficacy of induction was assayed by supernatant IL-10 at 96 hours. The presence of OPN in the media significantly reduced the effect of IND conditions (* p <0.05 and NS compared to CTRL; n=14/group). (B) *in vitro* IL-10 changes of the previous panel were confirmed to be secondary to the induction of TR1 lymphocytes by FACS triple staining for CD4⁺CD49b⁺LAG-3⁺. Data was normalized to the mean of the controls and depicted as a TR1/CD4⁺ percentage (* p <0.05 compared to IND; n=10/group). (C) The inhibitory effect of AAA homogenate on TR1 induction was then determined via FACS in the presence of an α OPN cocktail (CT) consisting of neutralizing antibodies to OPN/CD44 and RGD peptide inhibition of the α V β 3 integrin. Data was normalized to the mean of the control (* p <0.05 and NS compared to IND; n=8/group).

Table 1

All blood analyzed was sampled from a prospectively maintained blood bank isolated from patients undergoing USPTF AAA screenings or from subjects with known AAA previous to operative repair. Patients screening negative were designated “risk-factor matched” (RFM) non-AAA and used as a control. All blood samples assayed were randomly selected from the blood bank. Demographics from the blood bank participants are depicted here. Categorical variables were compared with Fisher's Exact testing while continuous variables were compared with Student's T-Test.

	RFM Non-AAA (N=116)	AAA (N=112)	P-Value
COPD	20.2%	35.7%	0.01
DM	36.8%	26.8%	0.15
HTN	68.4%	90.2%	<0.01
PAD	7.9%	11.6%	0.37
HLD	77.2%	87.5%	0.03
CAD	23.7%	39.2%	0.01
CKD	7.9%	10.7%	0.50
FHx	4.4%	3.6%	1.0
BMI>30	50.0%	40.2%	0.19
Smoking Hx	87.0%	95.5%	0.03
Framingham Risk Score	35.0%	40.6%	0.01
	RFM Non-AAA (N=116)	AAA (N=112)	P-Value
Metformin	23.7%	14.3%	0.09
ACEi	30.7%	46.4%	0.01
Steroids	12.3%	18.8%	0.20
ARB	16.7%	11.6%	0.34
Nitrates	1.8%	8.0%	0.03
BB	34.2%	53.8%	<0.01
ASA	43.9%	55.4%	0.08
Clopidogrel	6.1%	5.4%	1.0
Statin	67.5%	72.3%	0.39