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TSG-6 is Highly Expressed in Human Abdominal Aortic Aneurysms

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Abstract

Background—The formation of abdominal aortic aneurysms (AAA) is characterized by a dominance of pro-inflammatory forces that result in smooth muscle cell apoptosis, extra-cellular matrix degradation, and progressive diameter expansion. Additional defects in the anti-inflammatory response may also play a role, but have yet to be fully characterized. TSG-6 (TNF-stimulated gene-6) is a potent anti-inflammatory protein involved in extracellular matrix stabilization and cell migration active in many pathological conditions. Here, we describe its role in AAA formation.

Methods—Blood and/or aortic tissue samples were collected from organ donors, subjects undergoing elective AAA screening, and open surgical AAA repair. Aortic specimens collected were preserved for IHC or immediately assayed after tissue homogenization. Protein concentrations in tissue and plasma were assayed by ELISA. All immune cell populations were assayed using FACS. *In vitro*, macrophage polarization from monocytes were performed with young, healthy donor PBMCs.

Results—TSG-6 was found to be abnormally elevated in both the plasma and aortic wall of patients with AAA compared to healthy and risk-factor matched non-AAA donors. We observed the highest tissue concentration of TSG-6 in the less diseased proximal and distal shoulders compared to the central aspect of the aneurysm. IHC localized most TSG-6 to the tunica media with minor expression in the tunica adventitia of the aortic wall. Higher concentrations of both M1 and M2 macrophages where also observed, however M1/M2 ratios were unchanged from healthy controls. We observed no difference in M1/M2 ratios in the peripheral blood of risk-factor

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matched non-AAA and AAA patients. Interesting, TSG-6 inhibited the polarization of the anti-inflammatory M2 phenotype *in vitro*.

Conclusions—AAA formation results from an imbalance of inflammatory forces causing aortic wall infiltration of mononuclear cells leading to vessel breakdown. In the AAA condition, we report an elevation of TSG-6 expression in both the aortic wall and the peripheral circulation.

Introduction

An abdominal aortic aneurysm (AAA) is characterized by pathologic, progressive vessel dilation which, if left untreated, results in eventual rupture, massive uncontrolled hemorrhage, and death. This anomaly usually occurs in the infrarenal aortic segment (80%), an area relatively deplete of vascular smooth muscle cells (VSMCs) and subjected to high wall tension.[1]

AAA may affect up to 10% of men older than 60 and is currently the 11th leading cause of mortality in the United States.[2] For this serious condition, no pharmaceutical therapy is currently available.[3] Treatment consists of surgical reconstruction after cross-sectional diameter exceeds 5.5 cm but exposes the patient to high risk of perioperative morbidity and mortality.[4] Although the initial insult causing AAA formation is not known, the pathogenesis is histologically characterized by an infiltration of activated macrophages and inflammatory mononuclear cells to the aortic wall, increased matrix metalloproteinases (MMPs), degradation of the extracellular matrix (ECM), and loss of VSMCs.[5]

TSG-6 (TNF-stimulated gene-6) is a member of the hyaluronate binding protein family and was first isolated from fibroblasts stimulated with TNF-α.[6,7] TSG-6, through the CD44 receptor of resident macrophages, has the ability to decrease zymosan/TLR-2 mediated nuclear translocation of NF-κB to attenuate inflammation.[8] Previous studies have also demonstrated the ability of TSG-6 to inhibit neutrophil protease release and chemotaxis demonstrating its potent anti-inflammatory properties.[9,10] TSG-6 is additionally able to directly affect ECM mediated cellular signaling via interaction with a host of protein and glycosaminoglycan (GAGs) ligands such as HA (hyaluronan), heparin, aggrecan, TSP1, and PTX3.[11]

AAA formation and initial growth occurs because of a runaway pro-inflammatory response within the aortic wall. In this paper, we report an increased expression of the anti-inflammatory cytokine TSG-6 in the aortic wall and peripheral blood of patients with AAA.

Methods

Ethics Statement

All experiments with human tissues and blood were approved by the Indiana University Institutional Review Board. Informed consent was obtained for all tissue and blood samples before collection. The work presented here was performed in concordance with the 7th edition of the Declaration of Helsinki.[12]

Collection of Whole Blood and Tissues

Human AAA specimens were collected from patients undergoing open aortic repair at IU Health hospitals (Indianapolis, IN). All abdominal aneurysm tissue was obtained on the longitudinal axis spanning the length of the aneurysmal segment from infrarenal or juxtarenal AAAs opposite the origin of the inferior mesenteric artery (IMA). Disease-free human aorta was obtained from multi-organ tissue donors (Figure 1) from the aortic cuff at the origin of the renal arteries. All adherent clot, calcium, and debris, if present, was stripped at the time of collection by the operating team. Tissue samples were immediately flash frozen in liquid nitrogen or preserved in 10% normal buffered formalin (NBF) depending on downstream application.

Blood specimens were collected from healthy donors, preoperative AAA patients, or from subjects presenting to the vascular lab for their U.S. Preventative Task Force (USPTF) recommended AAA screenings. Subjects who screened AAA negative by ultrasound were thusly classified as a risk-factor matched non-AAA control population. PBMCs (peripheral blood mononuclear cells) and plasma were isolated by Ficoll-Paque (GE Healthcare, Princeton NJ) density centrifugation and stored at −80°C.[13]

Analysis of Aortic and Serum TSG-6

Aortic wall homogenates were created within 24 hours of tissue collection by grinding weighed samples under liquid nitrogen as previously described.[14] TSG-6 concentrations in the tissue homogenates and plasma were assayed using prefabricated TSG-6 ELISA kits per manufacturer's recommendations (Sigma, St Louis MO).

Immunohistochemistry and Fluorescence Microscopy

AAA tissue preserved in NBF was embedded into paraffin blocks and sectioned as needed. Primary monoclonal antibodies used were against human TSG-6 (R&D Systems, Minneapolis MN), CD14 (R&D), and alpha-actin (R&D). Epitope retrieval was performed if needed. All reactions were performed using the avidin-biotin complex immunoperoxidase method (Vector Labs, Burlingame CA).[15] Fluorescence microscopy was performed using previously described primary antibodies and Alexa Fluor secondary antibodies per manufacturer's instructions (Thermofisher, Waltham MA).

Induction of M1 Phenotype

A PBMC monocyte isolation kit (Miltenyi Biotec, GER) was used to harvest circulating monocytes from healthy donor PBMCs. Monocytes were subsequently plated in a 48-well plate for 7 days at a concentration of 2.5×10^6 cells/mL in complete RPMI media (RPMI, 10% FBS, 100 ug/mL penicillin/streptomycin) with supplementation of IFN-γ (50 ng/mL) and M-CSF (50 ng/mL). TSG-6 (50 ng/mL) and/or osteopontin (OPN) (10ug/mL), when used, was added at the time of media supplementation of IFN-γ and M-CSF. All media and supplements were refreshed on day 4.[16]

Analysis of M1/M2 Aortic Phenotypes

Fresh aortic tissue was cut into mm^3 cubes in a tissue culture plate and incubated in the presence of collagenase (0.5 mg/mL) and DNase (150 u/mL) at 37°C for one hour. The resulting tissue suspension was passed through a 30 μm cell strainer. Remaining cellular clumps were further broken down by addition for 15 minutes of 0.05% trypsin/EDTA. Mononuclear cells were isolated with Ficoll-Paque density centrifugation.

Identification of M1/M2 phenotypes were performed using Fluorescence-Activated Cell Sorting (FACS) analysis. Single cell suspensions from aortic samples, peripheral blood, or tissue culture were stained for $CD14^+CD206^-$ (M1) and $CD14^+CD206^+$ (M2) macrophages/monocytes per manufacturer's instructions (Miltenyi) and analyzed using an Accuri C6 Plus (BD Biosciences, San Jose CA) flow cytometer.

Statistical Analysis

Continuous variables were compared using Student's T-Test while categorical variables were compared using χ^2 analysis where appropriate. All testing was two sided with p-values less than 0.05 considered to be statistically significant.

Results

Serum TSG-6 is Increased in AAA Patients

Baseline characteristics between the AAA and non-AAA groups are depicted in table 1. A combination of cytokines previously determined to be relevant to the inflammatory response were assayed from the banked plasma of patients with AAA and non-AAA controls. We found a 60% increase in concentration of the anti-inflammatory cytokine TSG-6 ($p<0.05$) (Figure 2). The anti-inflammatory cytokine PGE-2 was also elevated in AAA patients but did not reach statistical significance. IL-10 was significantly diminished ($p<0.05$) in the AAA cohort. The pro-inflammatory cytokines IL-4, IL-17, IFN-γ, and TNF-α were all significantly elevated ($P<0.05$) in the AAA condition.

Aortic TSG-6 is Increased in AAA Patients

We next sought to determine if TSG-6 levels were elevated in aortic aneurysm tissue. In fact, a 10-fold increase ($p<0.01$) in TSG-6 was detected in the aortic homogenates of AAA as compared to healthy controls (Figure 3A). To determine if TSG-6 levels were equivocally distributed in the aneurysm, we obtained aortic tissue from the proximal aorta just below the renal arteries, tissue from the middle of the aneurysm sac midway between the renal arteries and the aortic bifurcation, and distally at the aortic bifurcation. Subsequent measurements of TSG-6 concentrations in the proximal, middle, and distal segments of the aorta demonstrated more pronounced elevation in the less diseased proximal and distal sections of the aneurysm as compared to the more structurally attenuated middle section (Figure 3B).

TSG-6 Localizes to the Tunica Media and VSMCs

We next sought to determine the source of TSG-6 in the aortic wall. Using immunohistochemical staining, the highest expression of TSG-6 in aneurysms localized to the tunica media (Figure 4A). No TSG-6 was observed in the tunica intima in any samples.

In contrast to the AAA condition, the healthy aorta did not express TSG-6 in any of the arterial layers (Figure 4B). Next, fluorescence microscopy confirmed colocalization of TSG-6 to SMA^+ VSMCs in the tunica media and the vascular pericytes of the tunica adventitia (Figure 5).

M1/M2 Macrophages Infiltrate the AAA Tissue

Initially, paraffinized tissue sections were stained with Masson Trichrome (Figure 6). Subsequently, M1 ($CD14^+CD206^-$) and M2 macrophages ($CD14^+CD206^+$) were stained to determine macrophage phenotype within the aortic wall. Infiltrates of both macrophage phenotypes were observed in the tunica media of the aneurysmal tissue but not in the healthy aorta. Next, fresh aneurysmal tissue was homogenized to obtain a single cell suspension for FACS analysis (Figure 7B). A large, greater than 100×, increase in both the M1 and M2 macrophage phenotypes were observed in the AAA condition which reached statistical significance ($p<0.01$) when compared to the healthy aortic controls. However, there was no difference in M1/M2 ratio between the healthy control and the AAA condition in the aortic wall or peripheral blood (Figure 7B).

TSG-6 Inhibits Polarization of M1 macrophages

Monocytes from healthy donors were isolated and plated in media supplemented with IFN-γ and M-CSF to induce M1 macrophages with and without presence of TSG-6. When exposed to polarizing conditions, the M2 fraction of all macrophages decreased by half ($p<0.01$) (Figure 8). However, if TSG-6 was added to this polarizing media, no difference was found between the baseline and the supplemented groups. This effect of TSG-6 on M2 polarization was significantly diminished when OPN was added to culture supplementation.

Discussion

We found TSG-6 to be elevated in the peripheral blood of patients diagnosed with AAA. This finding was also maintained in the aortic walls of specimens harvested during resection. Interestingly, the highest TSG-6 concentrations localized to the less diseased and dilated proximal and distal thirds of the aortic samples. Using IHC, we found TSG-6 concentrated to the tunica media of the AAA wall and to a lesser extent the tunica adventitia; however, no TSG-6 was visualized in the healthy aortic controls. Furthermore, fluorescence microscopy localized the source of TSG-6 to the VSMCs and perivascular cells. Although total macrophage infiltration was greater in the AAA samples, the M1/M2 phenotype ratio was unchanged. *In vitro*, we observed M1 polarization inhibition by TSG-6 addition.

TSG-6, which maps to human chromosome 2q23.3, is highly conserved across mammalian species and has been implicated as a modulator of the inflammatory cascade.[17] This 35kDa secreted protein is comprised of two triple-stranded antiparallel β–sheets and two α–helices arranged around a hydrophobic core.[18] TSG-6 binds with avidity to GAGs including heparin, chondroitin-4-sulfate, aggrecan, and has the extra ability to crosslink hyaluronan. TSG-6 has also been shown to regulate CD44 activity, affect cellular proliferation, and inhibit neutrophil migration.[19] There is no constitutive elaboration of TSG-6 in unstimulated tissues or cells; rather, it is secreted in response to pro-inflammatory cytokines such as IL-1,

IL-6, and TNF-α. The rate of synthesis can further be augmented by introduction of EGF, FGF-1, and TGF-β.[7] High serum and tissue levels have been detected in pro-inflammatory conditions such as lupus, inflammatory bowel disease, and rheumatoid arthritis.[11]

Relevant to the pathogenesis of AAA, VSMC TSG-6 mRNA was found to be highly expressed in response to TNF-α stimulation.[20] TSG-6's affinity for CD44, also a receptor for immunomodulatory ECM proteins such as OPN and HA, was subsequently shown to be of particular importance as activation of both T-cells and antigen presenting cells were inhibited by TSG-6 in a CD44-dependent manner.[21] Furthermore, rodent atherosclerosis models suggests TSG-6 elaboration, in response to injury to the intima, is key in stimulation of VSMC proliferation and arterial healing.[22]

The catalyst for this aortic investigation arose from the unresolved question of the initiating step of AAA formation. Assays of peripheral blood from AAA patients for mediators of inflammation demonstrated anticipated increased pro-inflammatory chemokines IL-17, IFN-γ, and TNF-α correlating to histologic appearance of aortic wall macrophage infiltration followed by elaboration of inflammatory chemokines, recruitment of mononuclear cells, and degradation of the ECM.[23] This reflects a previous observation in which almost all of the top 80 upregulated genes in the murine AngII/ApoE$^{-/-}$ AAA model were associated with inflammation.[24] In our initial experiments, we observed a paradoxical increase in TSG-6 concentration in the serum of AAA patients. We hypothesized increased systemic concentrations may be functioning as a feedback mechanism to reign in the AAA inflammatory response.

The role of TSG-6 in AAA formation and growth has never been described in the literature. However, limited descriptions in the context of atherosclerosis and arterial injury have been made. Wang *et al* described a rabbit carotid artery atherosclerosis model in which animals with vulnerable plaques were treated with mesenchymal stem cells. Animals exposed to these stem cells had significantly lower serum concentrations of hs-CRP, TNF-α, and IL-6. In contrast, TSG-6 expression was found to be markedly increased correlating closely to a histological appearance of a higher fibrous cap to lipid core ratio suggesting TSG-6 as a potent plaque stabilizer.[25] The arterial benefits of TSG-6 extend to trauma as well. Following balloon catheter injury of rat blood vessels, TSG-6 was found to be significantly elevated in the neointima and VSMCs correlating with cellular proliferation, inhibition of neutrophil infiltration, decreased plasmin activity, and inflammation inhibition through NF-κB suppression.[8]

In addition to elevated TSG-6 in the plasma, our tissue samples from organ donors and patients undergoing vascular surgery demonstrated a significant increase in aortic wall TSG-6. TSG-6 was found to be localized to the tunica media and concentrated to the less dilated portions of the proximal and distal aneurysm consistent with small aneurysm formation. Previous studies in humans using MRI and PET/CT localized macrophage activity, *in vivo*, to the shoulders of the aneurysm, suggesting a correlation between TSG-6 and macrophage activity.[26,27]

Monocytes/macrophages are the first mononuclear cell type observed in the AAA wall and are responsible for the recruitment of further inflammatory cell chemotaxis.[28] As monocytes migrate from blood into peripheral tissues, they mature into macrophages and are polarizable into the M1 or M2 phenotypes depending on local cytokine signaling. M1 macrophages have microbicidal and tumoricidal activity while elaborating pro-inflammatory cytokines, MMPs, nitric oxide, and reactive oxygen species. In contrast, M2 macrophages are intricately involved with parasite clearance, regulation of tumorigenesis, and tissue healing while modulating the M1 response via elaboration of TGF-β and IL-10.[29] *In vitro*, these two phenotypes can be polarizable with the addition of IFN-γ/LPS or IL-4/IL-10.[30]

We found no significant difference in the ratio of M1/M2 macrophages in tissue or blood of non-AAA and AAA patients. However, the absolute number of $CD14^+$ monocytes/macrophages were significantly increased in the AAA condition consistent with previous reports.[28,31] It is worth noting, however, that one would anticipate an elevated ratio of M1 macrophages in AAA given the local inflammatory cytokine soup which was not observed in our experiments. Interestingly, we detected the ability of TSG-6 to abrogate the effect of a M1 polarizing inflammatory media *in vitro*. Therefore, perhaps a regulatory feedback mechanism causing an increase in TSG-6 keeps this M1/M2 ratio at basal levels despite the inflammatory cytokine signals. Specifically, perhaps TSG-6 works to counteract the effects of OPN, through competitive inhibition of CD44 activity as demonstrated by our *in vitro* experiment. OPN, an ECM component which is found in excess in the aortic wall and serum of AAA patients,[32] has previously shown to inhibit M2 polarization and while promoting M1 activity through CD44 signaling.[16,33]

Conclusion

TSG-6 is a potent anti-inflammatory mediator which is elevated in the aortic wall of AAAs and to a lesser extent the systemic circulation compared to risk-factor matched controls. It may represent a potential therapeutic target for modulation of AAA initiation and growth and should be the target of further investigation.

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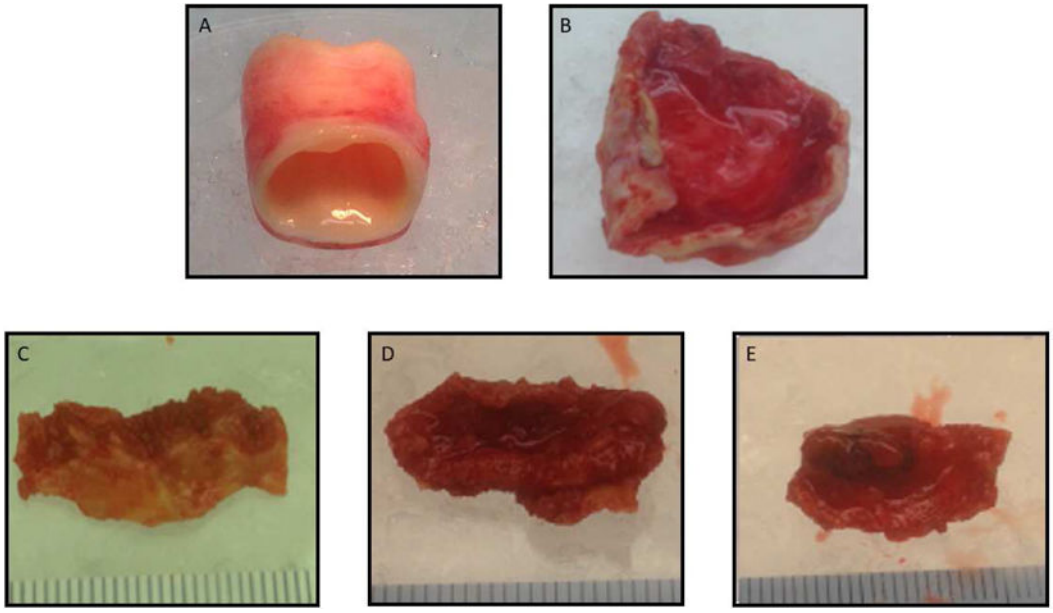

Figure 1. Representative depiction of the tissues used for downstream analysis

(A) Healthy aorta harvested from organ donors compared to aneurysmal aorta harvested during open AAA repair (B). Diseased aorta was divided into proximal (C), middle (D), and distal segments (E) for further analysis of aortic wall protein and cell phenotype expression.

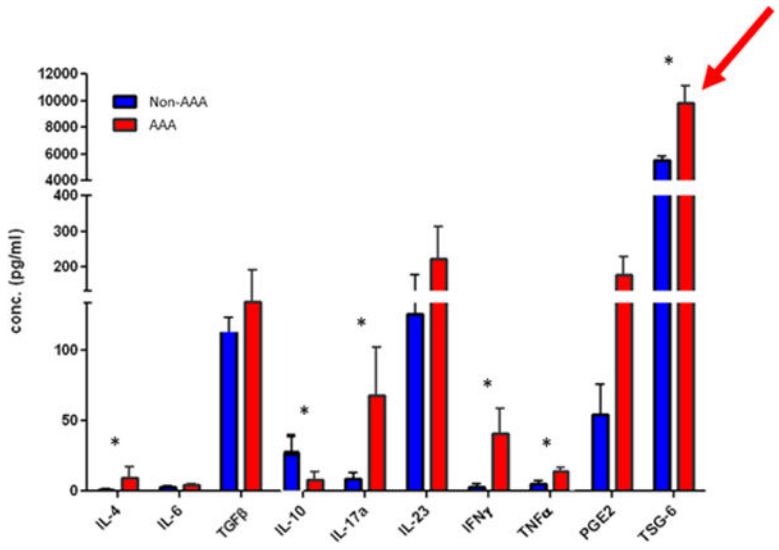

Figure 2. An environment of inflammation exists in the plasma of the AAA patient

Plasma proteins were assayed by ELISA in both patients with AAA and risk-factor matched non-AAA subjects. Mediators of inflammation were generally increased while anti-inflammatory cytokines were decreased in the AAA condition. A paradoxical increase in plasma TSG-6 was observed in the AAA population (red arrow). Mean ± SEM; (n=8/group); *p<0.05.

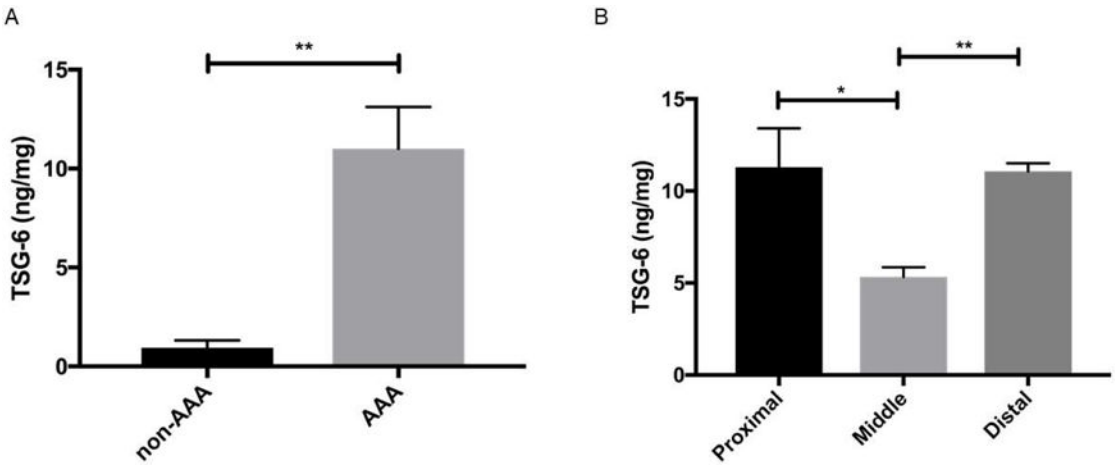

Figure 3. Increased tissue expression of TSG-6 is present in AAA patients compared to non-AAA controls

Normal aorta and infrarenal aneurysms were flash frozen and ground under liquid nitrogen to create a tissue homogenate (A), TSG-6 concentration was then assayed via ELISA; Mean ± SEM; (n=4/group). (B) Aneurysms were then divided into proximal, middle, and distal thirds on the longitudinal axis and TSG-6 expression was assayed. Mean ± SEM; (n=3/group); *p<0.05, **p<0.01.

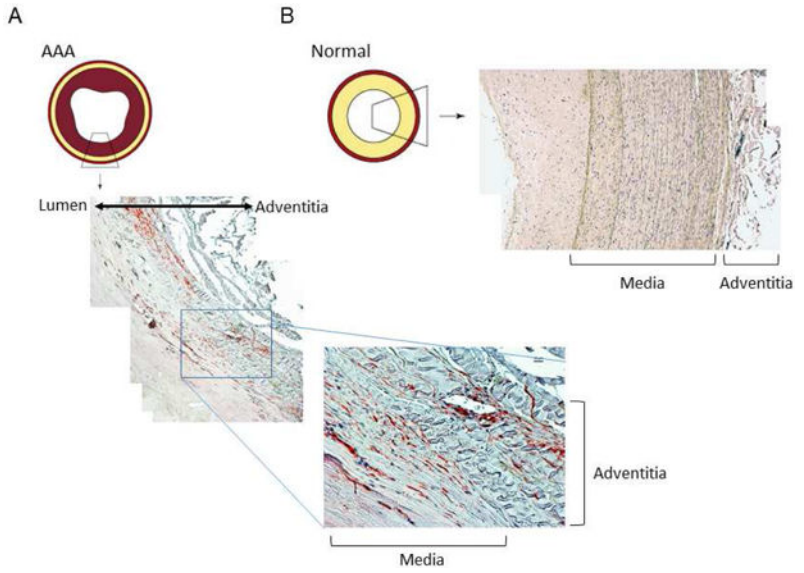

Figure 4. TSG-6 is localized to the tunica media of aortic aneurysms

Normal and aneurysmal aorta was preserved in NBF and encased in paraffin. IHC on both healthy and diseased tissue was performed with anti-human TSG-6 primary antibodies. Secondary antibodies were conjugated to alkaline phosphatase. Counterstaining was performed using hematoxylin. Significant deposition of TSG-6 (red) was observed in the AAA samples in the tunica media as compared to the other arterial layers. No TSG-6 staining was observed in the healthy aortic specimens.

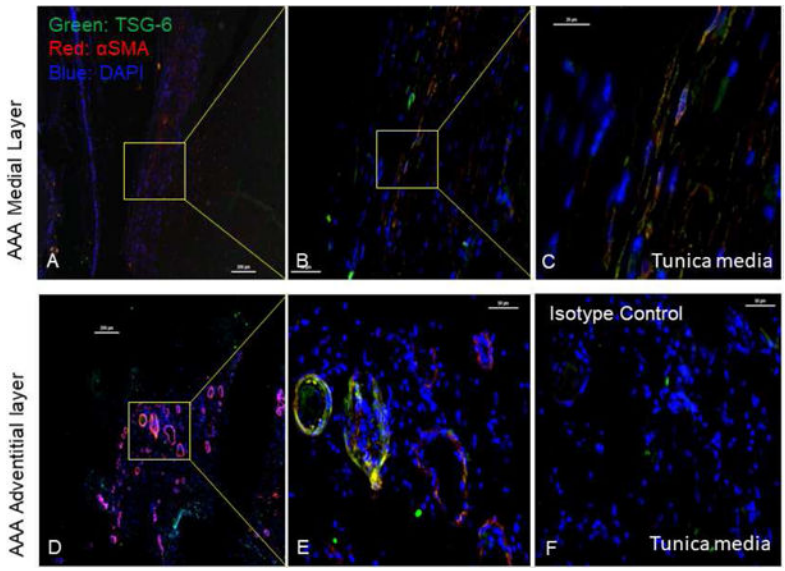

Figure 5. TSG-6 protein in AAA localizes to aortic SMA$^+$ VSMCs and perivascular cells

(A–C) TSG-6 protein in AAA tunica media smooth muscle cells. (D–E) TSG-6 protein in SMA$^+$ cells of the adventitial perivascular niche.

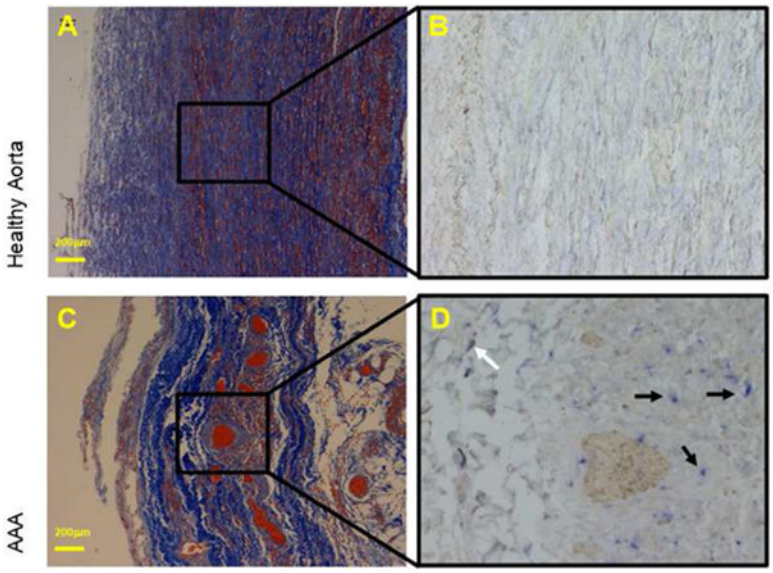

Figure 6. Histologic appearance of M1 macrophage infiltration into AAA tissue

Masson.s trichrome stain of normal (A) and aneurysmal aorta (C). M1 macrophages ($CD14^{+}CD206^{-}$, black arrow) and M2 macrophages ($CD14^{+}CD206^{+}$, white arrow) were found in aneurysmal tissue (D) but not normal aorta (B).

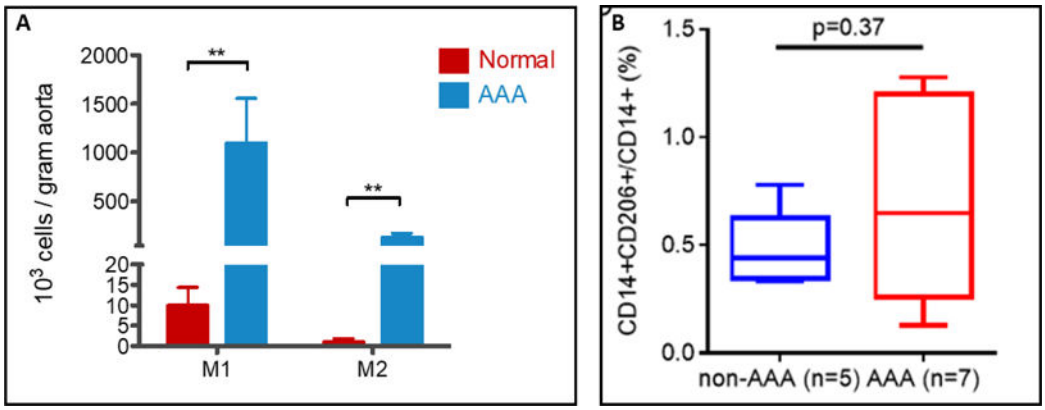

Figure 7. Increased distribution of M1 and M2 macrophages in AAA

Tissue samples were dissociated within 24 hours into a single cell suspension. The abundance of M1 and M2 macrophages in aortic tissue was measured by flow cytometry (A). Relative ratios of M2 macrophages in the healthy patient and normal condition are depicted in (B). Mean ± SEM (n=5–7/group); **p<0.01.

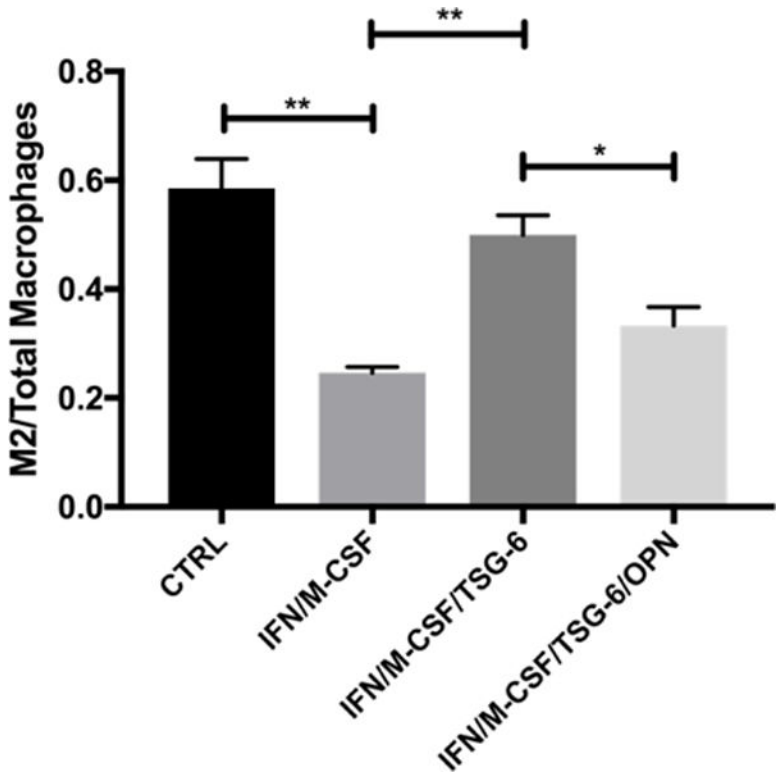

Figure 8. TSG-6 inhibits M1 polarization in vitro

Monocytes harvested from the peripheral blood of healthy donors were exposed to IFN-γ and M-CSF for 7 days for M1 polarization. TSG-6 and OPN, if used, was added concurrently. Cells were harvested, stained for CD14/CD206, and evaluated for M1/M2 phenotypes by FACS analysis. Mean ± SEM; (n=4/group); *p<0.05, **p<0.01.

Table 1

Representative demographics of risk-factor matched (non-AAA) and AAA patients

Baseline characteristics between the AAA and non-AAA cohorts. Plasma was obtained from risk-factor matched and AAA patients and cytokine levels were assayed by ELISA. P values less than 0.1 are illustrated. COPD, Chronic Obstructive Pulmonary Disease. DM, Diabetes Mellitus. HTN, Hypertension. HLD, Hyperlipidemia. CAD, Coronary Artery Disease. CHF, Congestive Heart Failure.

	Non-AAA (n=11)	AAA (n=9)	P value
Age	68.1	67.5	NS
Male	100%	100%	NS
COPD	45%	44%	NS
DM	36%	22%	NS
HTN	73%	89%	NS
HLD	55%	100%	0.02
CAD	27%	67%	0.08
CHF	9%	0%	NS
Active Smoker	36%	78%	0.06
Smoker (Former)	50% (of non-active)	100% (of non-active)	0.04