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Tumor necrosis factor-α-induces aortic intima-media thickening via perivascular adipose tissue inflammation

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Running Title
TNF-α induces aortic IMT

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Abstract

Background/Aims: Neointimal thickening results from inflammation in association with vascular smooth muscle cell (VSMC) proliferation. We studied the role of perivascular adipose tissue (PVAT) on VSMC proliferation and intima-media thickening (IMT) in a rodent model of chronic inflammation.

Methods: The abdominal aorta and surrounding PVAT of TNF-α-injected mice were examined 28 days after administration. Plasma and PVAT cytokines were measured with Milliplex assays. Inflammatory cells were examined with immunofluorescence. TGF-β1, MMP-2, -9, and -12 expressions were examined with immunohistochemistry, immunoblotting and zymography. IMT was determined. Cell proliferation and TGF-β1 mRNA levels were examined after treating VSMC with PVAT homogenates -/+ MMP-2 inhibitors (batimastat, ARP 100 or TIMP-2) and SB431542, a selective inhibitor of the TGF-β-type1 receptor.

Results: A significant increase of CD3, CD68, neutrophils, VCAM-1 and MMP-2 in PVAT, and TGF-β1 and IMT of the aorta of TNF-α-injected mice was observed. PVAT of TNF-α-injected mice significantly upregulated TGF-β1 and increased cell proliferation in a dose-dependent manner and was attenuated by SB431542, batimastat, ARP 100 andTIMP-2.

Conclusions: Our study shows that chronic PVAT inflammation leads to MMP-mediated increase of TGF-β1 and hence VSMC proliferation.

Key words: perivascular adipose tissue, vascular smooth muscle cell, chronic inflammation, TNF-α, MMP-2

Introduction

Neointimal thickening and vascular smooth muscle cells (VSMCs) proliferation are hallmarks of many pathological vascular conditions including atherosclerosis as well as restenosis following angioplasty and stent placement [1]. Conventionally, these pathological conditions progress from disturbances within the lumen, such as pathologically high levels of LDL causing endothelial dysfunction, transmigration of inflammatory cells including monocytes which differentiate into macrophages, transcytosis of atherogenic lipoproteins from plasma to the intima, foam-cell formation, and VSMC pleiocytosis [2].

Chronic inflammation plays a major part in obesity and obesity-related cardiovascular diseases. Increased levels of tumor necrosis factor (TNF)-α, monocyte chemoattractant protein (MCP)-1, interleukin (IL)-1 and IL-6 in the circulation and adipose tissue are associated with obesity-related insulin resistance and cardiac dysfunction in obese subjects [3]. We recently demonstrated that single administration of TNF-α could induce ventricular dysfunction in a mouse model 7 days and 28 days post-injection [4]. Nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase)-mediated oxidative stress and increased levels of IL-1β, IL-2, IL-6 and TNF-α in the ventricles were observed in these mice, suggesting chronic inflammation following single dose of TNF-α.

Perivascular adipose tissue (PVAT) has recently been recognized to be a distinct form of adipose tissue in which adipocytes are heterogeneous in shape, smaller in size, and exhibit a reduced state of adipogenic differentiation [5]. PVAT adipocytes are not separated from the walls of blood vessels but instead encroach into the outer adventitial region. Furthermore, the vasa vasorum is interspersed within PVAT and proliferates during inflammation and injury

[6]. Inflammatory cells and expression of inflammatory genes have been reported to be upregulated in PVAT surrounding the atherosclerotic aorta and coronary arteries of obese subjects [7]. This is substantiated by reports of increased accumulation of inflammatory cells in the adventitia and PVAT of obese mice [8].

Recent studies have demonstrated that PVAT regulates VSMC proliferation by secreting growth promoters such as fibroblast growth factor, insulin-like growth factor, transforming growth factor (TGF)-β and heparin-binding epidermal growth factor and PVAT-secreted visfatin stimulates VSMC proliferation in culture [9], In this study, we explored the effect of chronic inflammation on intima-media thickening (IMT) and VSMC proliferation and the role of PVAT in this disease process.

Materials and Methods

Materials

Anti-mouse antibodies CD3, NIMP-R14 (neutrophil marker), vascular cell adhesion molecule (VCAM)-1, alpha-smooth muscle actin (α-SMA), TGF-β1, matrix metalloproteinase (MMP)-2, MMP-9, and MMP-12 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Ki-67 antibody was purchased from eBioscience (San Diego, CA, USA). Anti-mouse CD68 antibody was purchased from AbD Serotec (Oxford, UK). Alexa Fluor 555 goat anti-mouse antibody was purchased from Cell Signaling Technology (Beverly, MA, USA). SB-431542, batimastat and ARP 100 were obtained from Tocris Bioscience (Ellisville, MO, USA) and human recombinant TIMP-2 was purchased from Millipore (Bedford, MA, USA). Oil Red O stain and Verhoeff–van Gieson stain were

purchased from Sigma–Aldrich (St. Louis, MO, USA) and Electron Microscopy Sciences (Hatfield, PA, USA) respectively.

Animal model

The details of animal experiments have been described previously [4]. Briefly, male Swiss Albino mice (7–8 weeks; 25–30 g) were administered intravenous injection of a single dose of murine TNF-α (8 μg/kg body weight, n=8; R&D Systems, Minneapolis, MN, USA) slowly, without anaesthesia. An equivalent volume of physiological (0.9%) saline was administered to control mice (n=9). The mice were sacrificed 28 days after injection. The abdominal aorta (4-6 mm below diaphragm) which was defined previously [8] and plasma were collected. All protocols and experimental procedures were undertaken in accordance with the Guidelines for the Care and Use of Research Animals as approved by the Animal Care and Use Committee of SingHealth, Singapore.

Histology, immunofluorescence and immunohistochemical (IHC) analyses

Cryostat sections (6 μm) of the abdominal aorta were prepared. Hematoxylin and eosin (H&E) staining for morphological analyses, Oil Red O staining for adipocyte measurements, and Verhoeff–van Gieson staining for elastin examination were undertaken following previously described methods [10]. Measurements for IMT and adipocyte areas were carried out on ten randomly chosen fields on at least three sections for each animal. Adipocyte area was traced manually with Image Pro Plus software 6.0 (Media Cybernetics, Silver Spring, MD, USA).

Expression of CD3, CD68, NIMP-R14 and VCAM-1 in the PVAT and aorta was examined using immunofluorescence. Sections were incubated with anti-mouse CD3 (dilution, 1:50), CD68 (1:50), neutrophils (1:50) and VCAM-1 (1:50) overnight at 4°C. Sections were then washed with phosphate-buffered saline (PBS)-Tween-20 thrice and then incubated with the secondary antibody, Alexa Fluor 555 goat anti-mouse (1:1000), for 2 h at room temperature. The immunofluorescence of the each section was visualized with a Zeiss LSM 510 confocal microscope (Carl Zeiss Microscopy, Jena, Germany). The fluorescence intensity of each section was quantified in a blinded fashion at 5–7 randomly chosen fields on ≥3 sections for each animal using Image Pro Plus software 6.0 (Media Cybernetics).

Expression of TGF-β1, MMP-2 α-SMA and Ki-67 in PVAT and the aorta was examined using anti-mouse TGF-β1 (1:50), MMP-2 (1:50), α-SMA (1:6400), and Ki-67 antibodies following previously described IHC methods [11-13]. Images were analyzed using Image Pro Plus software 6.0 (Media Cybernetics) and signal intensities quantified in a blinded fashion at 5–7 randomly chosen fields on ≥3 sections for each animal. Negative controls for immunofluorescent and IHC staining were carried out by substitution of immunoglobulin (Ig) G controls for the primary antibodies.

Immunoblotting

PVAT homogenates from TNF-α-injected and control mice were prepared following previously described methods [14]. Protein concentrations were quantified with a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, USA). Levels of MMP-2,

MMP-9 and MMP-12 proteins in PVAT homogenates were examined by immunoblotting following previously described methods [15]. Signals were analyzed using AlphaEase® FC software and FluorChem HD2 (Alpha Innotech, San Leandro, CA, USA).

Zymography

Gelatinase activity was detected by zymography usingmethods described previously with some modifications [16]. PVAT homogenates (50μg/lane) were resolved under non reducing conditions in an 8% polyacrylamide gel containing 0.5 mg/ml gelatin (Merck, Darmstadt, Germany). After electrophoresis, the gels were washed twice at room temperature for 30 min in 2.5% Triton X-100, subsequently washed in buffer containing 100 mMTris/HCl, 30 mM CaCl2, 0.01% NaN3 and incubated in this buffer at 37°C for 24 h. Staining of protein was performed with Coomassie Blue solution brilliant blue R-250 (Sigma, St. Louis, MO, USA) for 1 h and lightly destained in methanol:acetic acid:water (3:1:6). After staining, white bands on blue gels indicated enzyme species. We used human recombinant MMP-2 and MMP-9 as standards (Sigma, St. Louis, MO, USA).

Milliplex Immunoassays

Quantification of levels of IL-1β, IL-2, IL-6, TNF-α and C-reactive protein (CRP) in plasma as well as levels of IL-6, TNF-α, MCP-1, plasminogen activator inhibitor (PAI)-1 and resistin in PVAT homogenates was undertaken using Milliplex™ MAP Mouse Cytokine/Chemokine Immunoassays (Millipore, Billerica, MA, USA) following previously described methods[17].

VSMC culture and cell proliferation assays

Human aortic VSMC were obtained from Clonetics (Cambrex Bio Science Walkersville, Inc. Walkersville, MD, USA) and maintained in SmGM-2 (reconstituted according to manufacturer's instruction). Cells were used between passage 3 to 5 and at 85–90% confluence. Cells were distributed in 96-well plates at an initial density of 2×10^3 cells per well and allowed to attach overnight. Cells were rendered quiescent in serum-free media with 0.2% bovine serum albumin (BSA) for 24 h. They were then incubated with PVAT homogenate (10 μg/mL) of TNF-α-injected mice and control mice for 48 h following previously described methods [18]. Cell proliferation was measured with a Celltiter 96 Aqueous One Solution Proliferation Assay kit (Promega, Madison, WI, USA) according to manufacturer instructions. In a separate experiment, cells were incubated with or without SB-431542 (10 μM; a selective inhibitor of the TGF-type 1 receptor) [19], batimastat (10 μmol/L; an inhibitor of MMP-1, 2, 3, 7, and 9), ARP-100 (10 μmol/L; a specific inhibitor of MMP-2)[20] or TIMP-2 (50 ng/ml, tissue inhibitor of MMP-2) [21]. Cells were incubated with different doses (1, 5, 10, and 20 μg/ml) of PVAT homogenates of TNF-α-injected mice and assayed for cell proliferation. Each measurement was carried out in triplicate.

Real-time reverse-transcription-polymerase chain reaction (RT-PCR)

Human VSMCs were treated with PVAT homogenate (10 μg/mL) of TNF-α-injected mice and control mice for 48 h. TGF-β1 mRNA levels were determined by real-time RT-

PCR using a Taqman® Gene Expression Assay following previously described methods [4]. Cells were incubated with PVAT homogenates of TNF-α-injected mice for 48 h with or without batimastat (10 μmol/L), ARP 100 (10 μmol/L) or TIMP-2 (50 ng/ml). Separately, cells were incubated with different doses (1, 5, 10, and 20 μg/ml) of PVAT homogenates of TNF-α-injected mice and TGF-β1 mRNA levels were examined

Statistical analyses

Data are expressed as means ± SD. Statistically significant differences in means were tested using Student's *t*-test. $P<0.05$ was considered significant. Data were analyzed using GraphPad Prism, version 4.02 (GraphPad, San Diego, CA, USA). The data obtained from TGF-β upregulation and VSMC proliferation induced by PVAT of TNF-α-injected mice were analysed by one-way ANOVA with post hoc Dunnett test for comparisons.

Results

Measurement of plasma cytokines and PVAT adipokines

We previously reported a significant increase in IL-1β, IL-2, IL-6 and TNF-α protein concentrations in ventricular homogenates of TNF-α-injected mice 28 days after TNF-α administration [4]. In the present study, circulating levels of the pro-inflammatory cytokines IL-1β, IL-2, IL-6, TNF-α and CRP of these mice were measured in the plasma 28 days after

TNF-α administration using Milliplex™ MAP Mouse Cytokine/Chemokine Immunoassays to determine if there was systemic inflammation.

IL-1β and CRP levels of TNF-α-injected mice were significantly elevated compared to controls (Table 1). Analyses of PVAT adipokines showed that levels of IL-6, MCP-1, PAI-1, and resistin of TNF-α-injected mice were significantly higher than in controls (Table 2). Of note, PAI-1 was undetectable (minimum detectable concentration, 19.4 pg/mL) in the PVAT of controls. However, significantly elevated PAI-1 levels were observed in the PVAT of TNF-α-injected mice (78.33 ± 14.85 pg/mL).

Chronic inflammation of PVAT

Increased accumulation of macrophages and T lymphocytes in adipose tissue has been reported in obesity-associated chronic inflammation. In the present study, a significant increase in expression of CD3 (fig. 1A–D), CD68 (fig. 1E–H) and NIMP-R14 (fig. 1I–L) in the PVAT of TNF-α-injected mice compared with controls was observed. The fluorescence intensities of CD3, CD68 and NIMP-R14 of TNF-α-injected mice were approximately 10-, 20- and 8-times higher than those of controls, respectively. Moreover, VCAM-1 expression in the PVAT and aorta of TNF-α-injected mice was significantly higher than controls (fig. 1M–P). VCAM-1 expression was confined not only to the aorta but also around adipocytes, suggesting enhancement of the microcirculation in the PVAT of TNF-α-injected mice.

Phenotypic changes in VSMCs and PVAT

The IMT of TNF-α-injected mice was significantly increased compared with controls (73.77 ± 8.73 μm *vs* 56.75 ± 3.88 μm, fig. 2A-C).

Moreover, elastin fibres in the aorta were degraded in areas where VSMC proliferation was evident in TNF-α-injected mice but not in controls (fig. 2A & B, arrowheads). Furthermore, adventitia degradation in TNF-α-injected mice was also noted (fig. 2B, arrows). Our findings were consistent with the observations with Verhoeff–van Gieson stain (fig. 2D & E). The elastin fibres (fig. 2E, arrowheads) and adventitia (arrows) of TNF-α-injected mice were degraded.

Hypertrophy of PVAT adipocytes that surround the abdominal and thoracic aorta have been reported in *ob/ob* mice [8]. Hyperplasia and hypertrophy of adipocytes have been observed in diet-induced obese mice [22]. In the present study, cryostat sections were stained with Oil Red O and adipocytes were examined (fig. 2F–H). There was no significant difference in the surface area of adipocyte cells between TNF-α-injected mice and control mice (697.54 ± 193.24 *vs* 688.23 ± 118.4 μm^2). In contrast, elastin and adventitia degradation were detected in TNF-α-injected mice (fig. 2G, arrowheads) but not in controls.

α-SMA expression has been reported to reflect the proliferation and differentiation of VSMCs [23]. Hence, we investigated α-SMA expression in the aorta using IHC staining. α-SMA expression in the aorta of TNF-α-injected mice was significantly increased compared with that seen in controls (fig. 2I-L). Moreover, Ki-67, a marker for cell proliferation [9, 13]

was also significantly increased in the aorta of TNF-α-injected mice compared to controls (fig. 2M-P).

Increased expression of TGF-β1 and activated MMP-2

Proliferation of VSMCs is regulated by several growth factors and cytokines. A recent study reported that TGF-β1 promoted VSMC proliferation via Smad3 signaling [24]. We examined TGF-β1 expression in the PVAT and aorta using IHC (fig. 3A–D). Slight expression of TGF-β1 was observed in the aorta, but not in the PVAT of control mice (fig. 3B). However, significantly increased expression of TGF-β1 was observed in the aorta and PVAT of TNF-α-injected mice compared with controls.

TGF-β1 can be activated by several mechanisms, including those involving MMPs [25]. We examined the expression of MMP-2, MMP-9 and MMP-12. IHC analyses of MMP-2 in PVAT and the aorta revealed significantly increased expression of MMP-2 in the PVAT but not in the aorta of TNF-α-injected mice (fig. 3E-H). However, MMP-2 was not detectable in the PVAT and aorta of control mice. Our IHC findings were consistent with immunoblotting, which also revealed a significant level of MMP-2 in the PVAT of TNF-α-injected mice, but not in controls (fig. 3I & K). However, immunoblotting analysis showed that MMP-9 expression was similar in PVAT of both TNF-α-injected and control mice (fig. 3J & L). Moreover, MMP-12 expression was not detectable in PVAT of both groups (fig. not shown). Zymography of PVAT homogenates obtained from TNF-α-injected and control mice were performed. By gelatin zymography, MMP-9 was detected in PVAT of both groups whilst MMP-2 was detected only in TNF-α-injected-mice but not in controls (fig. 3M).

In vitro VSMC proliferation and TGF-β1 upregulation

We next examined the effect of PVAT on TGF-β1 mRNA levels in human VSMCs using real-time RT-PCR. PVAT homogenates of TNF-α-injected mice significantly upregulated TGF-β1 gene expression with a 30-fold increase after 48 h incubation (fig. 4A). Moreover, PVAT treatment of cells together with batimastat, TIMP-2 or ARP 100significantly attenuated PVAT-induced TGF-β1 expression by 24%, 34% and 90% respectively, suggesting that activated MMP-2 or MMP-9 in the PVAT of TNF-α-injected mice upregulated TGF-β1 expression. Furthermore, incubation with different doses (1, 5, 10 μg/ml) of PVAT homogenates of TNF-α-injected mice showed dose dependent increased upregulation of TGF-β1 gene expression in VSMCs (fig. 4B). However, 20 μg/ml of PVAT homogenates did not show further increased upregulation.

Finally, we examined the effect of PVAT homogenates on VSMC proliferation *in vitro*. Human aortic VSMCs were treated with PVAT homogenates obtained from TNF-α-injected mice and control mice. PVAT homogenates of TNF-α-injected mice significantly increased VSMC proliferation at 10 μg/mL compared with controls (121 ± 5%, fig. 4C). In contrast, PVAT homogenates of control mice did not induce VSMC proliferation. We then investigated the role of TGF-β1 in PVAT-induced VSMC proliferation. Treating the cells with PVAT homogenates of TNF-α-injected mice together with SB-431542 significantly attenuated VSMC proliferation (82 ± 9%). To ascertain if MMPs mediate TGF-β1-induced VSMC proliferation, cells were treated together with PVAT and batimastat, ARP 100 or TIMP-2. Batimastat, ARP 100 and TIMP-2 significantly decreased PVAT-induced VSMC proliferation (75 ± 10%, 78 ± 6% and 80.9 ± 5% respectively). Furthermore, dose dependent

VSMC proliferation was also observed when VSMCs were incubated with different doses (1, 5, 10 and 20 μg/ml) of PVAT homogenates of TNF-α-injected mice (fig. 4D).

Discussion

We show that a single bolus i.v injection of TNF-α (8 μg/kg body weight) induces chronic inflammation in mice and increased vascular intima-media thickening. We demonstrate for the first time that this is due to significantly increased expression of activated MMP-2 in PVAT leading to increased TGF-β1 in VSMCs. This in turn results in degradation of adventitia and elastin as well as VSMC proliferation, evidenced by increased expression of α-SMA and Ki-67 in VSMCs, culminating in increased IMT. Our *in vitro* data confirmed that PVAT homogenates of TNF-α-injected mice but not control mice, upregulated TGF-β1 expression in VSMC and PVAT-induced TGF-β1 upregulation was blocked by MMP-2 inhibition, suggesting that activated MMP-2 in PVAT upregulates TGF-β1 in VSMCs. We also demonstrated that PVAT homogenates of TNF-α-injected mice but not PVAT homogenates of saline-injected mice increased VSMC proliferation, and PVAT-induced VSMC proliferation was attenuated by TGF-β R1 inhibition as well as MMP inhibition. Our study shows that PVAT chronic inflammation leads to VSMC proliferation via mediation of MMP activity and TGF-β1.

A growing body of evidence shows that chronic inflammation plays a critical role in obesity. Elevated levels of pro-inflammatory cytokines in the plasma and increased infiltration of inflammatory cells in PVAT are observed in obese subjects [26]. We recently

reported that TNF-α administration induces ventricular dysfunction and elevated levels of IL-1β, IL-2, IL-6 and TNF-α in ventricular homogenates 7 days and 28 days after injection [4]. The inflammatory status of our mouse model was further examined in the present study. We showed significant elevation of levels of IL-1β and CRP in the plasma as well as IL-6, MCP-1, PAI-1 and resistin concentrations in the PVAT of TNF-α-injected mice. These findings demonstrate that a single bolus injection of TNF-α can induce systemic inflammation and inflammation in PVAT, 28 days after injection. However, contrary to other findings [27], our study did not detect elevated level of circulating TNF-α. This warrants further investigation into regulatory mechanism of TNF-α in TNF-α-induced chronic inflammation.

The present study also demonstrated significantly increased levels of PAI-1 in the PVAT of TNF-α-injected mice compared with controls, suggesting that adipose tissue can produce PAI-1 not only in obesity but as a consequence of chronic inflammation [28]. Moreover, a significant increase in resistin level in the PVAT of TNF-α-injected mice compared with controls also suggests that resistin might function as a signaling protein of chronic inflammation in PVAT as well as mediating the production of pro-inflammatory cytokines such as IL-6 [29]. The present study also demonstrated a significant increase of MCP-1 levels in the PVAT of TNF-α-injected mice compared with controls. Increased levels of MCP-1 might lead to recruitment of $CD3^+$ cells, $CD68^+$ cells and neutrophils in the PVAT of TNF-α-injected mice. However, a twofold increase (14.53 ± 1.13 *vs* 7.38 ± 1.19 pg/mL, P=0.007) in MCP-1 level does not in itself explain the several-fold increase in the number of $CD3^+$ cells, $CD68^+$ cells and neutrophils in the PVAT of TNF-α-injected mice. It can be conjectured that several other mechanisms (including those involving adhesion molecules such as VCAM-1) contribute to the recruitment and activation of inflammatory cells. Interestingly, a ~100% increase in expression of VCAM-1 in the PVAT and aorta of TNF-α-

injected mice was observed in the present study. In the PVAT of these mice, adipocytes were surrounded by VCAM-1 expression, suggesting activation of the microcirculation in PVAT and which might support recruitment of inflammatory cells into PVAT.

Several studies have reported the influential role of MMPs in the development of VSMC proliferation and vascular remodelling [30]. MMP-2 and MMP-9 have been reported to cause VSMC proliferation in organ culture and in animal models [31]. Some studies suggest that MMP-induced VSMC proliferation could be mediated by degradation of type-IV collagen and proteoglycans [32], whereas other studies show mediation through non-matrix substrate N-cadherin [33] and mobilization of growth factors such as fibroblast growth factor (FGF)-1 and FGF-2 [34]. Recent studies also report that VSMC proliferation is mediated by PVAT-derived visfatin [9], and the synergistic combination of adipocyte-conditioned media and oleic acid [35]. Moreover, several MMPs were identified in gonadal and subcutaneous adipose tissue, and their important role in adipocyte differentiation recognized [36]. However, the types of MMPs present in PVAT and whether they have a role in VSMC proliferation are not known. The present study reveals that PVAT chronic inflammation leads to VSMC proliferation via mediation of MMP activity and TGF-β1.

TGF-β is a pluripotent growth factor implicated in various aspects of vascular pathology (including VSMC proliferation). Evidence that TGF-β can be activated by many factors, including MMPs, integrins, pH and reactive oxygen species has been reported as well as MMP-2 activates TGF-β1 in aged arterial wall via mediation of the TGF-β type-II receptor and Smad2/3 and Smad4 signaling [37]. Conversely, TGF-β has been shown to induce MMP-2 transcriptional activation via mediation of activation of transcription factors for malignant progression of human breast epithelial cells [38]. The present study shows that activated

MMP-2 was localized in PVAT and that TGF-β1 was identified in PVAT and VSMCs. *In vitro* experiments confirmed that PVAT homogenates of TNF-α-injected mice upregulate TGF-β1 in VSMCs and that this was attenuated by a specific inhibitor of MMP-2, showing MMP-2-induced upregulation of TGF-β1. However, the molecular mechanisms of TGF-β1 upregulation in VSMCs (e.g., mediation of early growth response factor-1 and/or the Smad2/3 signaling pathway) remain to be elucidated.

Intimal thickening consistently features in many vascular pathological conditions, including pulmonary hypertension and luminal stenosis after percutaneous coronary interventions. A recent study showed that neointimal hyperplasia as characterized by an increased neointima–media ratio in mouse femoral arteries and rat iliac arteries after endovascular injury was mediated by TNF-α. Takaoka et al. suggested that phenotypic changes in PVAT were crucial for the development of neointimal hyperplasia [39]. The present study also shows that VSMC proliferation *in vivo* as characterized by increased aortic IMT was the result of chronic inflammation in PVAT. Our findings are similar to reports demonstrating increased aortic-wall thickness in high fat diet-induced mast cell-deficient SI/SI^{d} mice, suggesting chronic inflammation plays a crucial role in these models.

In conclusion, the present study reports a novel mechanism of VSMC proliferation *in vivo* and *in vitro*. TNF-α-induced chronic inflammation in the PVAT of the abdominal aorta results in VSMC proliferation via a crosstalk mechanism between PVAT and VSMCs in which MMPs in PVAT increase TGF-β1 in VSMCs.

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Table 1. Plasma protein concentrations

	Control (n=5)	**TNF-α (n=5)**	**P value**
IL-1β	12.29 ± 1.88	19.25 ± 1.81	0.01
IL-2	4.97 ± 0.48	5.34 ± 0.72	0.68
IL-6	9.95 ± 1.19	9.02 ± 1.19	0.61
IL-10	23.87 ± 7.68	29.68 ± 10.94	0.66
TNF-α	6.41 ± 1.56	3.17 ± 0.38	0.06
CRP	45.14 ± 3.59	54.87 ± 2.27	0.04

All plasma values are given in pg/ml except CRP that is given in ng/ml. Mean ± SD.

Table 2. Protein concentrations in PVAT

	Control (n=5)	**TNF-α (n=5)**	**P value**
IL-6	5.06 ± 0.72	9.65 ± 1.49	0.032
TNF-α	2.22 ± 0.34	2.18 ± 0.33	0.942
MCP-1	7.38 ± 1.19	14.53 ± 1.13	0.007
PAI-1	0.00 ± 0.00	78.33 ± 14.85	0.001
Resistin	324.5 ± 46.37	704.60 ± 130.55	0.042

All PVAT protein values are given in pg/ml. Mean ± SD.

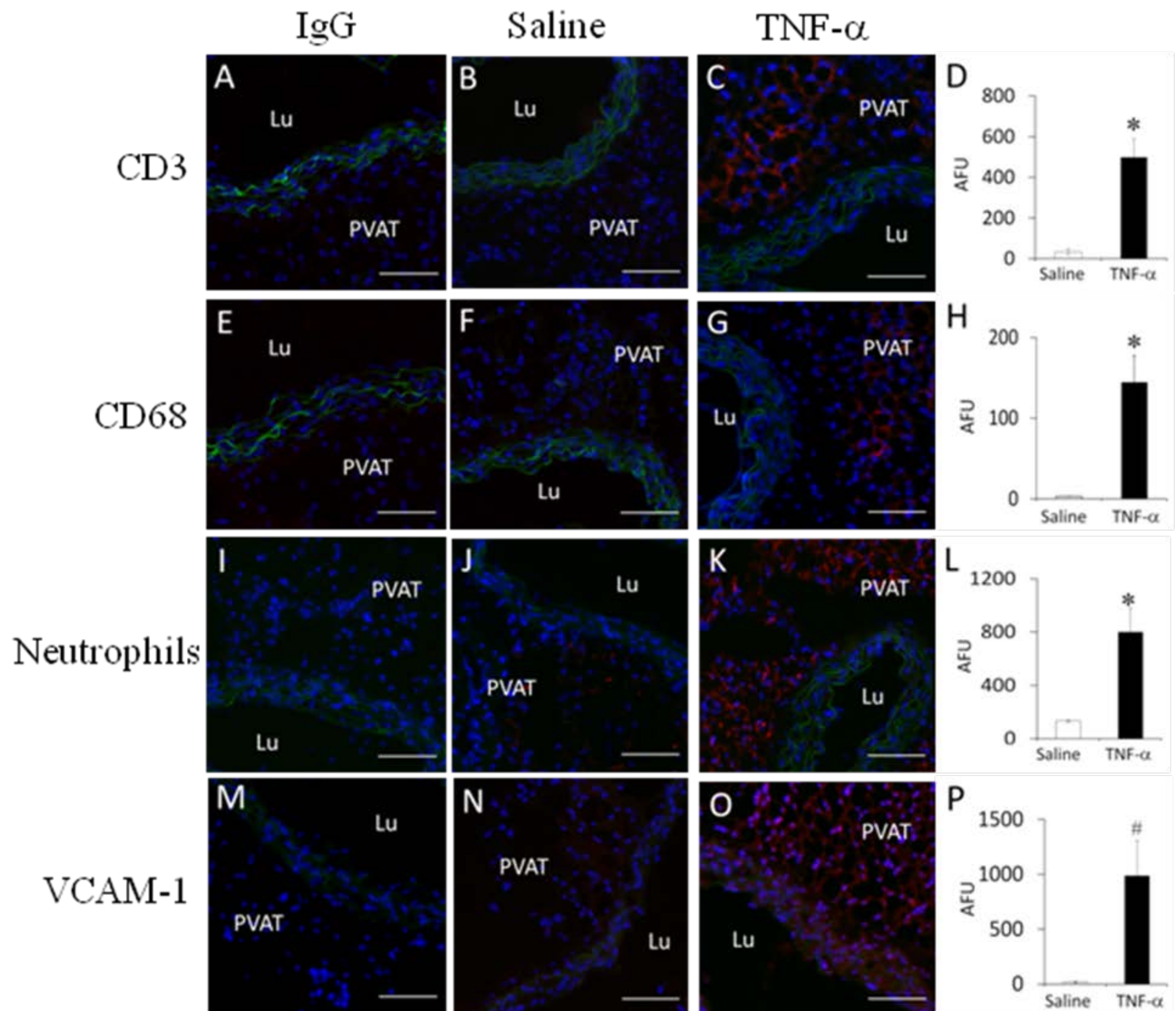
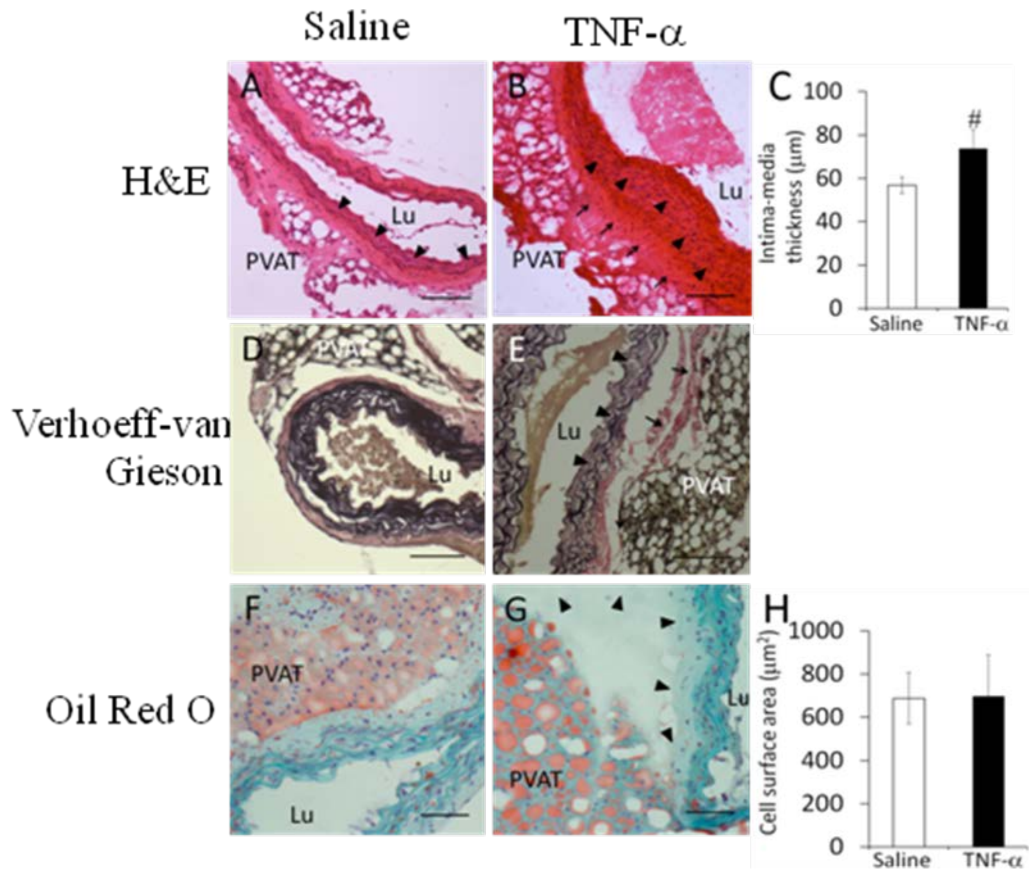


Fig. 1. TNF-α-induced chronic inflammation in PVAT. $CD3^+$ T lymphocytes, $CD68^+$ monocyte/macrophages, NIMP-R14-positive neutrophils and VCAM-1 expressions were examined in the cryostat sections of the abdominal aorta and PVAT using immunofluorescence staining methods. Representative immunofluorescence staining of CD3 (A-D), CD68 (E-H), NIMP-R14 (I-L) and VCAM-1 (M-P)expression were shown. Data were expressed as arbitrary fluorescence unit (AFU) ± S.D of 5–7 randomly chosen fields on ≥3 serial sections for each animal (n= 5 in each group). Lu indicates lumen. Scale bar = 50μm. $^{*}P$=0.001 and $^{\#}P$=0.009 versus controls.


Saline
TNF-α
H&E
Verhoeff-van Gieson
Oil Red O
A
B
C
D
E
F
G
H
Lu
PVAT
Intima-media thickness (μm)
Cell surface area (μm²)
Saline
TNF-α
#

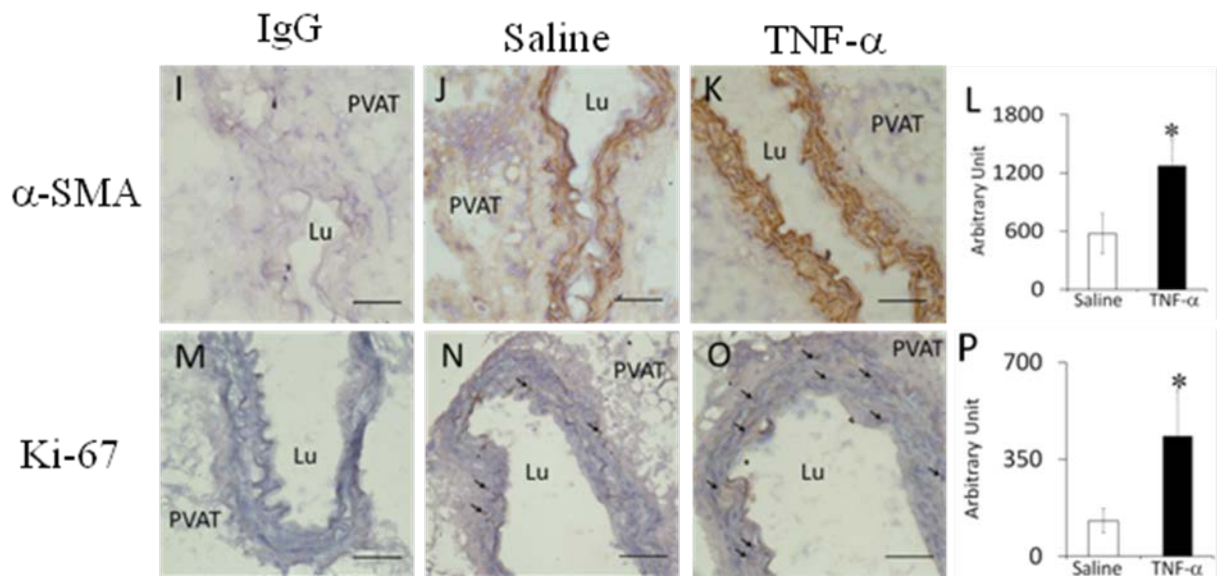

IgG
Saline
TNF-α
α-SMA
Ki-67
I
J
K
L
M
N
O
P
Lu
PVAT
Arbitrary Unit
Saline
TNF-α
*

Fig. 2. Phenotypic changes of abdominal aorta and surrounding PVAT. The cryostat sections of the abdominal aorta and PVAT of TNF-α-injected and saline-injected control mice were stained with Hematoxylin and eosin (H&E), Verhoeff–van Gieson and Oil Red O. Representative H&E stain of the cryostat sections showed a significant increase of IMT in abdominal aorta of TNF-α-injected mice compared to controls (A-C).. Elastin fibres degradation (arrowheads) and adventitia degradation (arrows) were observed in TNF-α-injected mice (B). Representative Verhoeff–van Gieson stain of the sections for elastin showed degradation of elastin fibres (arrowheads) and adventitia (arrows) in TNF-α-injected mice (E). Representative Oil Red O stain of the sections showed adipocyte area between TNF-α-injected mice and controls (F-H). Representative IHC analysis for α-SMA (I-L) and Ki-67 (M-P) expressions was shown in VSMC. Data were expressed as arbitrary unit ± S.D of 5–7 randomly chosen fields on ≥3 serial sections for each animal (n= 5 in each group). Lu indicates lumen. Scale bar = 50μm. $^{\#}P$=0.004 and $^{*}P$=0.001 versus controls.

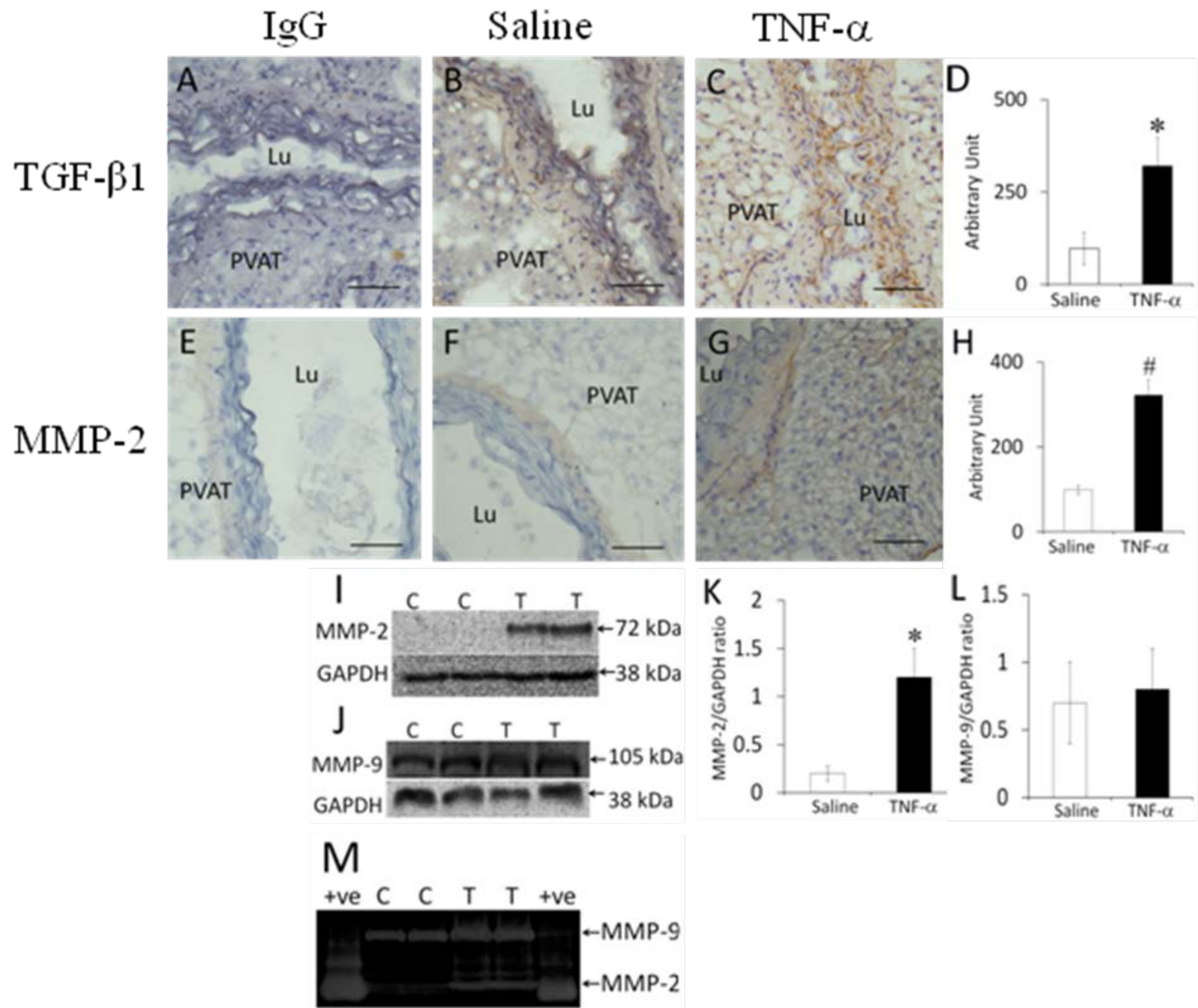


Fig. 3. Increased expression of TGF-β1 and activated MMP-2 in PVAT and VSMC. Representative IHC analysis of TGF-β1 (A-D) and activated MMP-2 (E-H) in the cryostat sections of the abdominal aorta and PVAT of TNF-α-injected and saline-injected control mice were shown. Representative immunoblot analysis of activated MMP-2 (I) and MMP-9 (J) in PVAT of TNF-α-injected mice and controls were shown with the densitometric analysis (K & L respectively). Representative zymogram of PVAT of control and TNF-α-injected mice are shown (M). A mixture of human recombinant MMP-2 and MMP-9 are used as standards. Data were expressed as arbitrary unit ± S.D of 5–7 randomly chosen fields on ≥3 serial sections for each animal (n= 5 in each group). Lu indicates lumen. C, control; T, TNF-α. Scale bar = 50μm. $^{*}P$=0.01 and $^{\#}P$=0.001 versus controls.

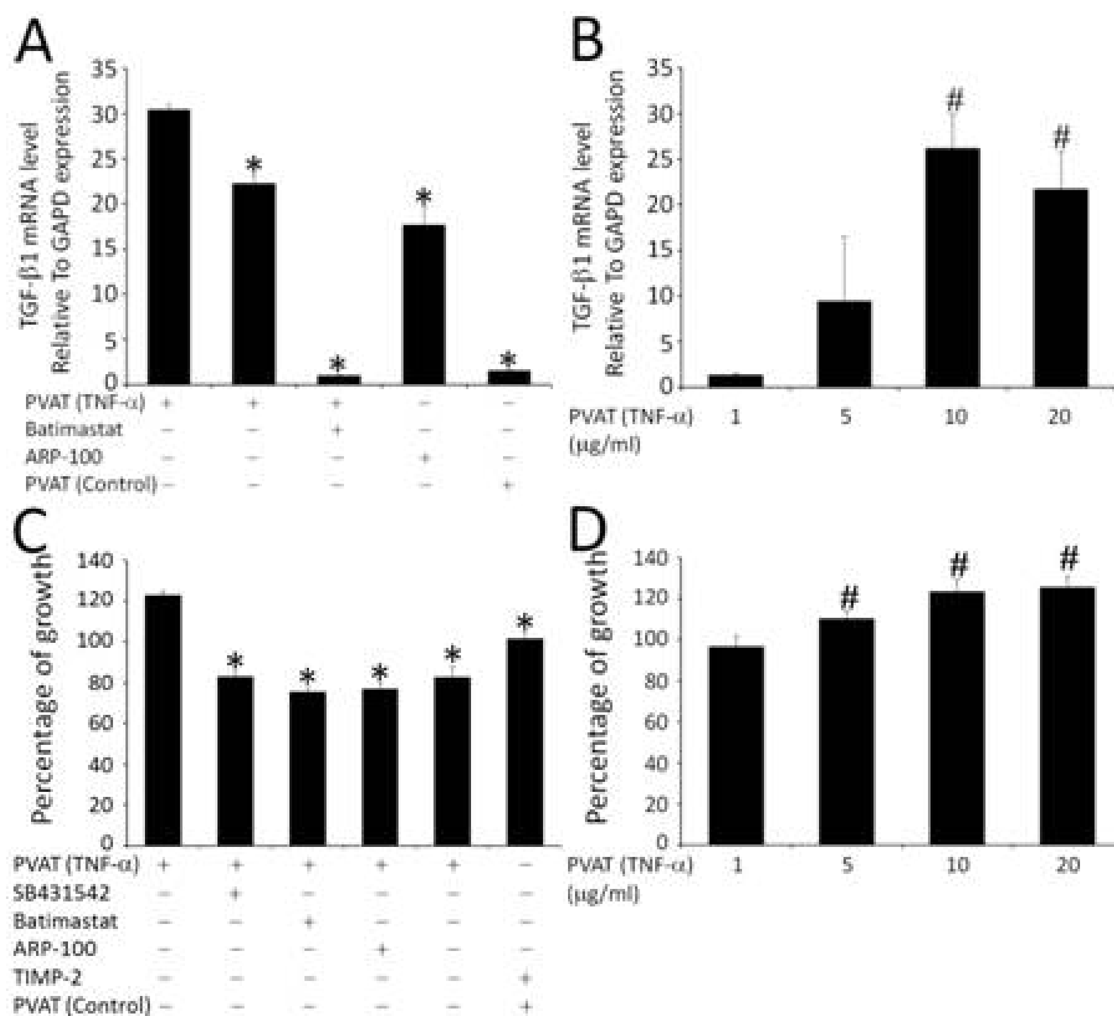


Fig. 4. *In vitro* TGF-β1 upregulation and VSMC proliferation. Human aortic smooth muscle cells were treated with PVAT homogenates (10 μg/mL) of TNF-α-injected and saline-injected control mice for 48h and TGF-β1 mRNA level was determined using real-time RT-PCR (A). Cells were treated with PVAT homogenates of TNF-α-injected mice with or without batimastat (10 μmol/L), ARP 100 (10 μmol/L), or TIMP-2 (50ng/ml). In some experiments, cells were treated with different doses (1, 5, 10, and 20 μg/mL) of PVAT homogenates of TNF-α-injected mice and TGF-β1 mRNA level was determined (B). Cells were treated with PVAT homogenates (10 μg/mL) of TNF-α-injected and saline-injected control mice for 48h and cell proliferation was examined using Celltiter 96® Aqueous One

Solution Proliferation Assay (C). Cells were treated with PVAT homogenates of TNF-α-injected mice with or without SB-431542 (10 μmol/L), batimastat (10 μmol/L), ARP 100 (10 μmol/L) or TIMP-2 (50ng/ml). In some experiments, cells were treated with different doses (1, 5, 10, and 20 μg/mL) of PVAT homogenates of TNF-α-injected mice and cell proliferation assay was performed. $^{*}P<0.001$ versus no-treatment control and $^{\#}P<0.01$ versus PVAT treatment.