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TNF α induces inflammatory stress response in microvascular endothelial cells via Akt- and P38 MAP kinase-mediated thrombospondin-1 expression

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Abstract

Tumor necrosis factor- α (TNF α) and thrombospondin-1 (TSP-1) are well-known mediators of inflammation. However, a causal relationship between TNF α stimuli and TSP-1 expression in endothelial cell stress, and the underlying mechanisms has not yet been investigated. In our study, human microvascular endothelial cells (hMEC) were treated with TNF α and analyzed for endothelial dysfunction, TSP-1 expression and associated mechanisms. TNF α treatment induced a dose-dependent increase in TSP-1 expression in hMEC associated with increased endothelial permeability, apoptosis and reduced proliferation. Whereas TNF α activated Akt, ERK and P38 MAPK simultaneously in hMEC, inhibitors of Akt and P38 MAPK, but not ERK blunted TNF α -induced TSP-1 expression. Silencing of NF κ B gene had no significant effect on TNF α -induced TSP-1 expression. Our study demonstrates the novel role of TNF α in inducing inflammatory stress response in hMEC through Akt- and P38 MAPK-mediated expression of TSP-1, independent of NF κ B signaling.

Keywords

TNF α ; TSP-1; inflammation; endothelium; P38 MAPK; Akt

Introduction

Tumor necrosis factor alpha (TNF α), a mediator of inflammation acts on the endothelium, the first line of action during inflammatory stimuli [1]. Activated macrophages, the major source of TNF α , have the ability to lyse many cell types including tumor cells [2]. As a result of the cellular stress inflicted by TNF α , endothelial cells undergo apoptosis [3] leading to barrier breakdown and increased vascular permeability aiding migration of

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inflammatory cells into the tissue environment [2, 4]. TNF α deregulation has been reported in many human diseases such as Alzheimer's disease [5], cancer [6], depression [7] and inflammatory bowel disease [8]. Although TNF α signaling pathway is considered as a new therapeutic strategy for inflammatory diseases [9], lack of knowledge on the precise molecular and cellular events in TNF α -induced stress response limits its application in anti-inflammation therapy.

Thrombospondin-1 (TSP-1) is a multifunctional 450 kDa glycoprotein, which is secreted by many cell types including endothelial cells, and are also stored in the platelet α -granules [10]. Whereas macrophages, fibroblasts and endothelial cells are known to lodge cell surface TSP-1 during tissue injury or inflammation [11-15], TSP-1 is also essential for the extracellular matrix remodeling in skin and other tissues [16], thus suggesting a key role for TSP-1 in the inflammatory response and post-inflammatory tissue remodeling. TSP-1 acts as a chemo-attractant and increases the adherence of monocyte and neutrophils to the endothelium [17-20]. Due to its affinity towards multiple cell surface receptors, TSP-1 elicits pleiotropic effects in many cell types. Whether or not TSP-1 is required for TNF α -induced inflammation is not clear.

In the current study, we report that treatment with TNF α results in cellular stress in human microvascular endothelial cells (hMEC) via activation of P38 MAPK in a dose-dependent manner, without compromising the Akt and ERK survival pathways. Interestingly, activation of P38 MAPK by TNF α leads to hMEC apoptosis despite co-activation of Akt and ERK. Increased hMEC stress by TNF α was associated with increased synthesis and secretion of TSP-1 by the hMEC, which is blunted by pharmacological inhibition of Akt or P38 MAPK, but not ERK. However, gene silencing of NF κ B, a transcription factor regulated by Akt and p38 MAPK, did not show any effect of TSP-1 expression. Our study provides novel insight into the role of TNF α /TSP-1 axis in hMEC inflammatory stress response via Akt and P38 MAPK activation independent of NF κ B, and renders reasonable optimism for utilizing this pathway for the treatment of inflammatory diseases.

Materials and Methods

Reagents, cell lines and antibodies

Telomerase immortalized hMEC were purchased from ATCC (Manassas, VA). The primary antibodies for TSP-1, phospho-Akt^{S473}, panAkt, phospho-Erk1/2, phospho-P38 MAPK, NF κ B p65, cleaved caspase-3, cleaved caspase-9 and GADPH were from Cell Signaling Technology (Danvers, MA). NF κ B p65 SiRNA was purchased from SA Biosciences (Frederick, MD). Piceatannol and TSP-1 ELISA kit was purchased from R&D systems (Minneapolis, MN). TNF α was obtained from Fisher Scientific (Pittsburgh, PA). Thiazolyl blue tetrazolium bromide (MTT) was purchased from Amresco (Solon, OH).

Electric cell-substrate impedance sensing (ECIS) method

ECIS assay was performed as previously described [21, 22]. Briefly, 8-well chamber arrays covered with gold plated electrodes were filled with 400 μ l of electrode-stabilizing solution and incubated for 30 minutes. Next, arrays were washed with 400 μ l complete

media and 5×10^5 hMEC in total 400 μ l of media was added directly into the each array well. Then, cells were cultured until a monolayer is formed (~48 hours), followed by treatment with 0, 1, 5, 10, 25, and 50 ng/ml TNF α and real-time analysis using ECIS instrument up to 12 h. ECIS equipment and arrays were purchased from Applied Biophysics (Troy, NY).

Immunocytochemistry and fluorescent imaging

Immunofluorescence staining was performed as described previously [23]. Briefly, hMEC monolayer plated on 8-well chamber slides was treated with various doses of TNF α for 12 hours. Next, cells were fixed with 2% paraformaldehyde in $1 \times$ PBS followed by permeabilization with 0.1% Triton X-100 in $1 \times$ PBS. Nonspecific staining was blocked with 2% BSA for 1 h at room temperature. Monolayers were then incubated with Alexa Fluor 488-labeled phalloidin (dilution 1:1000) (Life technologies, Carlsbad, CA) at room temperature for 1 hour. The slides were mounted with Vectashield (Vector Laboratories, PA), and imaged by a Zeiss fluorescent microscope.

Western blot analysis

Western blot analysis was performed as described previously [21, 22]. In the first set of experiments, western blot analysis was carried out to measure the phosphorylation and/or expression level of TSP-1, pAkt, pERK, pP38 MAPK, cleaved caspase-3 and 8 and NF κ B p65 in the conditioned media and/or cell lysates after dose-dependent treatment with TNF α . In order to do that, hMEC were plated in 6-well plates using EMB complete media (2ml/well) and allowed to attach for 48 hours prior to treatment. Next, cells were washed with PBS and incubated in serum free media supplemented with different concentrations of TNF α (0, 1, 5, 10, 25 and 50 ng/ml) for 12 hours. Next, conditioned media were collected and cell lysates were prepared. Protein estimation was performed using D_c protein assay (Bio-Rad, Hercules, CA). Samples (30 μ g of protein/well) were separated on 8 % SDS-PAGE gel and then transferred to PVDF membranes. Membranes were blocked with 5% milk blocking buffer for 1 hour, and incubated with primary antibodies against TSP-1 (1:1000 dilution), GADPH (1:5000 dilution), pAkt (1:1000 dilution), panAkt (1:1000 dilution), pErk (1:1000 dilution), cleaved caspase-3 (1:1000 dilution), cleaved caspase-8 (1:1000 dilution), NF κ B p65 (1:1000 dilution) and pP38MAPK (1:500 dilution) overnight at 4°C. Membranes were washed with 1X TBST and incubated with secondary antibodies at room temperature for 1 hour. Membranes were then washed again with 1 X TBST and enhanced chemiluminescence was used to detect the signal. The data are presented as mean $\pm$ SD (n=3). In the second set of experiments, western blot analysis was performed to measure the phosphorylation and/or expression level of TSP-1 in cell lysates and the conditioned media after treatment with 10 μ M SH-5 (Akt inhibitor), 10 μ M U0126 (Mek1/2 inhibitor) and 5 μ M SB239063 (P38 MAPK inhibitor). To do this, hMEC cells were plated in 6-well plates using EMB complete media (2ml /well) and allowed to attach for 48 hours prior to treatment, and the samples were processed as above.

ELISA assay for TSP-1

For ELISA assay, hMEC control SiRNA and NF κ B SiRNA (2 pM or 5 μ l of each SiRNA was transfected using lipofectamine and allowed to express for 72 hours) were plated in 6-well plates using EMB complete media (2ml/well) and allowed to attach for 48 hours prior

to treatment (a total time of 72 hours after SiRNA transfection). Next, cells were washed with PBS and incubated in serum free media supplemented with 10 ng/ml of TNF α for 12 hours. Next, conditioned media were collected and subjected for ELISA assay for the secreted TSP-1. ELISA analysis was performed using the Quantikine Human TSP-1 solid-phase Immunoassay kit (R&D systems, Minneapolis, MN) according to the manufacturer's protocol.

3-(4,3-Dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay

MTT assay was performed as previously described [21]. Briefly, hMEC cells were plated in 96-well plates using EMB complete media (100 μ l/well). After the formation of monolayer (~48 hours), cells were treated with various doses of TNF α for 12 hours in serum free media. Media were removed and cells were treated with 150 μ l (0.5 mg/ml) of MTT/well for 3 hours. Absorbance was measured at a wavelength of 570 nm.

Trypan blue assay

Trypan blue assay was performed as previously described [24]. Briefly, hMEC were plated in 96 well plates using EMB complete media (100 μ l/well). After the formation of monolayer (48 hours), cells were treated with various doses of TNF α for 12 hours in serum free media. Then, serum free media were removed and cells were detached by 25 μ l/well of trypsin followed by adding 25 μ l of Trypan blue solution. Total and dead cells were counted using hemocytometer under light microscope.

Apoptosis assay

Apoptosis assay was performed as previously described [21, 22] and determined based on cytoplasmic histone-associated DNA fragments detection, quantified by the Cell Death Detection ELISA^{PLUS} kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer's protocol. Briefly, hMEC were plated in 96-well plates at a density of 10⁴ cells/well. After 48h, cells were treated with treatment with various doses of TNF α for 12 hours. Next, cells were lysed and centrifuged at 200g for 10 min, and the collected supernatant was subjected to ELISA apoptosis detection plate. The absorbance was measured at 405 nm (reference wavelength, 492 nm).

Statistical analysis

Results were expressed as mean $\pm$ SD for either n=3 experiments (western blots and ECIS) or n=6 (MTT, viability and apoptosis assays). Paired two-tailed student t-test was used to determine the level of significance. Two-way ANOVA was used to determine the statistical analysis of data with 3 or more groups. A 'p value' less than 0.05 were considered statistically significant.

Results

TNF α induces endothelial-barrier permeability

Since endothelial cells are the first to respond to any inflammatory stimuli, we determined whether TNF α can directly modulate the endothelial-barrier. Our results indicated that

TNF α induces endothelial-barrier permeability in a dose dependent manner in a highly sensitive ECIS method that measures endothelial-monolayer resistance (Figure 1A and B). Whereas as little as 1 ng/ml TNF α was able to significantly induce breakdown of the hMEC-barrier, maximum effect was seen at 25 ng/ml concentrations (Figure 1B). Interestingly, at 50 ng/ml dose of TNF α , a minimum level of recovery in endothelial-barrier resistance was noticed as compared to 25 ng/ml dose. Our immunocytochemistry analysis with phalloidin revealed that TNF α induces cytoskeletal changes leading endothelial-barrier junction gap formation and vascular permeability (Figure 1C).

TNF α induces synthesis and secretion of TSP-1 by hMEC and increases activating phosphorylation of Akt, P38 MAPK and ERK

Many clinical studies have suggested a correlation between activation of TNF α pathway and increased expression of TSP-1, another pro-inflammatory protein. Hence we determined whether TNF α is able to directly regulate the expression of TSP-1 in hMEC. Our data indicated that treatment with TNF α results in a dose-dependent increase in the total expression of TSP-1 both in the cell lysates and the conditioned media (Figure 2A and B). Interestingly, as observed in the endothelial-monolayer permeability study, although treatment with 50 ng/ml TNF α resulted in increased expression of TSP-1 in cell lysates as compared to 25 ng/ml dose, levels of secreted TSP-1 in the conditioned media was lesser in 50 ng/ml dose as compared to 25 ng/ml treated hMEC monolayers (Figure 2A and B). Surprisingly, both the survival pathways, Akt and ERK signaling as well as stress activated P38 MAPK pathway was observed to be significantly activated together by TNF α in hMEC in a dose-dependent manner (Figure 2C and D).

TNF α -mediated cell stress induces apoptosis and inhibits survival and proliferation of hMEC *in vitro*

Whereas both Akt and ERK pathways activate cell survival and proliferation in mammalian cells, respectively, P38 MAPK is involved in the regulation of cell stress and apoptosis. Since TNF α was able to activate Akt, ERK and P38 MAPK simultaneously, we determined what the net effect of these signaling pathways will be on hMEC survival, apoptosis and proliferation. Our results indicated that TNF α treatment results in a significant inhibition of proliferation (Figure 3A), cell survival (Figure 3B) and induces apoptosis (Figure 3C) in hMEC in a dose-dependent manner. Interestingly, even though significant reduction in cleaved caspase-3 expression in hMEC was noticed upon treatment with P38 MAPK inhibitor SB239063, neither P38 MAPK inhibition nor Akt inhibition using SH-5 exhibited any effect on TNF α -induced expression of cleaved caspase 3 (Figure 4A and B) and cleaved caspase 8 (Figure 4A and C) in hMEC. These results indicated that although Akt, P38 MAPK and ERK pathways were activated in hMEC in response to TNF α treatment, TNF α was able to overcome the cell survival and proliferation effects of these kinases in hMEC in induction of apoptosis.

Pharmacological inhibition of Akt and P38 MAPK, but not ERK blunted TNF α – induced TSP-1 synthesis by hMEC

Since TNF α was able to activate Akt, ERK and P38 MAPK simultaneously, we next sought to determine which of these pathways are responsible for the regulation of synthesis and

secretion of TSP-1 by the hMEC. To do this, we treated hMEC monolayers with 5 ng/ml TNF α in the absence and presence of pharmacological inhibitors Akt (10 μ M SH-5), Mek1/2 (10 μ M U0126) or P38 MAPK (5 μ M SB239063) for 12 hours in serum free media. Our results indicated that while low dose of TNF α (5 ng/ml) was able to significantly increase TSP-1 expression both in hMEC lysates and the conditioned media, inhibition of either P38 MAPK or Akt blunted the TNF α -induced synthesis and secretion of TSP-1 by hMEC (Figure 5A-C). Treatment with either Akt or P38 MAPK inhibitors reduced TSP-1 expression in the cell lysates and the conditioned media to below the basal level of expression by the control, vehicle treated hMEC monolayers (Figure 5A-C). Our data thus indicated that both Akt and P38 MAPK activities is necessary for the expression of TSP-1 by hMEC in response to TNF α .

Pharmacological inhibition or gene silencing of NF κ B had no significant effect on TNF α – induced TSP-1 synthesis by hMEC

We next determined if TNF α -induced TSP-1 expression involved activation of pro-inflammatory transcription factor NF κ B. In our results, piceatannol an NF κ B inhibitor although did not exhibit any effect on TSP-1 expression in hMEC lysates, a high dose of 100 μ M induced TSP-1 expression (Figure 6A). In contrast, although lower doses on piceatannol had no effect on TNF α -induced TSP-1 synthesis in hMEC, 100 μ M piceatannol demonstrated a significant inhibitory effect (Figure 6A). However, SiRNA-mediated knockdown of NF κ B p65 did not show any effect on the TNF α -induced TSP-1 expression (Figure 6B). Our analysis of the hMEC conditioned medium for TSP-1 through an ELISA assay further confirmed that silencing of NF κ B in hMEC had no significant effect on TNF α -induced TSP-1 secretion (Figure 6C and D). Interestingly, we observed changes in TSP-1 expression at higher doses of piceatannol, which was once again opposite in control vs. TNF α treated hMECs (Figure 6A). This, we think is due to the toxicity and/or off-target effects of supra-optimal doses of piceatannol. Our data thus indicated that Akt and P38 MAPK-mediated expression of TSP-1 by hMEC in response to TNF α was independent of NF κ B activity.

Discussion

Integral role of TNF α in mediating inflammation has been indisputably demonstrated [25]. However, the mechanism regulating TNF α -mediated inflammatory response is only partly characterized. Among the various steps involved in an inflammatory response to stimuli, first and the foremost is activation of endothelium to express recognition receptors for inflammatory cells, barrier breakdown and sustenance of vascular leakage for a longer period of time so as to assist in the exudation of plasma components and infiltration of inflammatory cells across endothelial-barrier [26]. In the current study, we investigated the specific effects of TNF α on hMEC-barrier and the mechanisms how TNF α mediate endothelial-stress response that prepares the endothelium for plasma exudation and infiltration of inflammatory cells.

Our study revealed several novel details on the mechanisms regulating TNF α -induced endothelial stress and how TNF α sustains and/or amplifies its effects. First, our study

showed that treatment with TNF α results in a dose- and time-dependent breakdown of endothelial-barrier as evidenced by the real-time changes in endothelial-barrier resistance measured by a highly sensitive ECIS technology, corroborated with phalloidin immunocytochemistry data showing endothelial junction gap formations. Second, TNF α induces activation of two major survival pathways Akt and ERK, and stress-activated p38 MAPK in hMEC simultaneously. Third, we show for the first time that TNF α treatment results in a dose-dependent increase in the expression of another pro-inflammatory cytokine TSP-1 by the hMEC. Fourth, TNF α treatment results in decreased hMEC proliferation and viability, and increased apoptosis. Inhibitors of Akt and p38MAPK, but not Mek/ERK blunted TNF α -induced TSP-1 expression in hMEC. Finally, genetic or pharmacological inhibition of NF κ B did not exhibit any effect on TNF α -induced TSP-1 expression. Together, these results demonstrated that TNF α -mediated inflammatory stress in hMEC is mediated via activation of Akt and P38 MAPK, which is sustained through the expression of TSP-1 (Figure 7).

P38MAPK, ERK and Akt are 3 major signaling pathways activated in cells in response to many growth factors and cytokines [27]. However, there are conflicting reports on cross-talk between these 3 signaling pathways in various cell types and tissues [28-33]. It is extremely rare to have P38MAPK activated in conjunction with either Akt or ERK. In the current study, we report a highly rare scenario where TNF α is able to activate all the three serine-threonine kinases simultaneously in a dose-dependent manner. While TNF α is known to induce cell death via activation of death-receptor signaling-mediated extrinsic apoptosis pathway [34], Akt pathway protects apoptosis via activation of Bcl2 expression and inhibition of intrinsic apoptosis pathway [35, 36]. Potential role of co-activation of Akt and ERK along with stress-induced P38MAPK activation by TNF α is to reduce detrimental effects of P38MAPK on hMEC apoptosis and proliferation during the inflammatory stress.

Increased expression of TSP-1 and its binding to CD47 has been reported to enhance monocyte binding to endothelial monolayer through enhanced expression of intracellular adhesion molecule-1 (ICAM1) and E-selectin [17]. Furthermore, upon binding to CD36, TSP-1 induces receptor dimerization and CD36 activation [37], thus eliciting an adhesive, anti-angiogenic and apoptotic response in endothelial cells [38]. Both these are important endothelial cell-specific steps essential for an inflammatory response. Although many clinical studies have suggested a correlation between TNF α activation and TSP-1 expression in inflammation, to our knowledge there are no existing reports demonstrating a causal relationship between TNF α -mediated activation and TSP-1 production in any cell types. A TNF α -308 G/A promoter polymorphism that activates TNF α gene expression, is an underlying cause of many inflammatory diseases such as juvenile dermatomyositis [39, 40] and Crohn's disease [41]. Interestingly, the former studies have reported a correlation between TNF α activation and increased TSP-1 expression in juvenile dermatomyositis patients. However, these studies did not reveal whether there is a causative connection between TNF α and TSP-1 expression with regard to disease progression. Our current study revealed for the first time that TNF α is able to induce expression of TSP-1 in hMEC.

Our previous studies have demonstrated that Akt is necessary for the expression of TSP-1 in endothelial cells [42]. In the current study, we show that both Akt and P38MAPK cooperate

in mediating TNF α -induced TSP-1 expression in hMEC. Since TNF α , Akt and P38MAPK have been implicated in the NF κ B activation [34, 43-45], a signaling mediator of inflammation, we presumed that NF κ B activation downstream of Akt and P38MAPK activation may be responsible for TNF α -induced TSP-1 expression in hMEC. However, our further analysis using piceatannol, a pharmacological NF κ B inhibitor or NF κ B SiRNA exhibited no significant difference in the expression of TSP-1 in response to TNF α . Nevertheless, our results indicate the potential benefits of targeting TNF α and/or TSP-1 for inflammatory diseases.

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Abbreviations used

TNFα	Tumor necrosis factor- α
p38 MAPK	p38 mitogen activated protein kinase
TSP-1	Thrombospondin-1
MAPK	Mitogen activated protein kinase
ERK	Extracellular regulated kinase
hMEC	Human microvascular endothelial cells
ECIS	Electric cell-substrate impedance sensing

References

1. Zhang H, et al. Role of TNF- α in vascular dysfunction. Clin Sci (Lond). 2009; 116(3):219–30. [PubMed: 19118493]
2. MacEwan DJ. TNF ligands and receptors—a matter of life and death. British journal of pharmacology. 2002; 135(4):855–875. [PubMed: 11861313]
3. Espino J, Rodríguez AB, Pariente JA. The inhibition of TNF- α -induced leucocyte apoptosis by melatonin involves membrane receptor MT1/MT2 interaction. Journal of pineal research. 2013; 54(4):442–452. [PubMed: 23397952]
4. Flier JS, et al. The tumor necrosis factor ligand and receptor families. New England Journal of Medicine. 1996; 334(26):1717–1725. [PubMed: 8637518]
5. Swardfager W, et al. A meta-analysis of cytokines in Alzheimer's disease. Biological psychiatry. 2010; 68(10):930–941. [PubMed: 20692646]
6. Locksley RM, Killeen N, Lenardo MJ. The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell. 2001; 104(4):487–501. [PubMed: 11239407]
7. Dowlati Y, et al. A meta-analysis of cytokines in major depression. Biol Psychiatry. 2010; 67(5): 446–57. [PubMed: 20015486]
8. Brynskov J, et al. Tumour necrosis factor alpha converting enzyme (TACE) activity in the colonic mucosa of patients with inflammatory bowel disease. Gut. 2002; 51(1):37–43. [PubMed: 12077089]

9. Feldmann M. Development of anti-TNF therapy for rheumatoid arthritis. *Nature Reviews Immunology*. 2002; 2(5):364–371.
10. Dixit VM. Thrombospondin and tumor necrosis factor. *Kidney Int*. 1992; 41:679–682. [PubMed: 1573846]
11. Wight TN, et al. Light microscopic immunolocalization of thrombospondin in human tissues. *Journal of Histochemistry & Cytochemistry*. 1985; 33(4):295–302. [PubMed: 3884704]
12. Naganuma H, et al. Quantification of thrombospondin-1 secretion and expression of $\alpha v\beta 3$ and $\alpha 3\beta 1$ integrins and syndecan-1 as cell-surface receptors for thrombospondin-1 in malignant glioma cells. *Journal of neuro-oncology*. 2004; 70(3):309–317. [PubMed: 15662972]
13. Raugi GJ, Olerud JE, Gown AM. Thrombospondin in early human wound tissue. *Journal of investigative dermatology*. 1987; 89(6)
14. DiPietro LA, et al. Thrombospondin 1 synthesis and function in wound repair. *The American journal of pathology*. 1996; 148(6):1851. [PubMed: 8669471]
15. Gotis-Graham I, Hogg PJ, McNeil HP. Significant correlation between thrombospondin 1 and serine proteinase expression in rheumatoid synovium. *Arthritis & Rheumatism*. 1997; 40(10): 1780–1787. [PubMed: 9336411]
16. Resovi A, et al. Current understanding of the thrombospondin-1 interactome. *Matrix Biol*. 2014
17. Narizhneva NV, et al. Thrombospondin-1 up-regulates expression of cell adhesion molecules and promotes monocyte binding to endothelium. *The FASEB journal*. 2005; 19(9):1158–1160. [PubMed: 15833768]
18. Mansfield PJ, Suchard SJ. Thrombospondin promotes chemotaxis and haptotaxis of human peripheral blood monocytes. *The Journal of Immunology*. 1994; 153(9):4219–4229. [PubMed: 7930624]
19. Suchard SJ. Interaction of human neutrophils and HL-60 cells with the extracellular matrix. *Blood cells*. 1993; 19(2):197. [PubMed: 7508769]
20. Mansfield PJ, Boxer LA, Suchard SJ. Thrombospondin stimulates motility of human neutrophils. *The Journal of cell biology*. 1990; 111(6):3077–3086. [PubMed: 2269666]
21. Goc A, et al. TGF β - and bleomycin-induced extracellular matrix synthesis is mediated through Akt and mammalian target of rapamycin (mTOR). *Journal of cellular physiology*. 2011; 226(11):3004–3013. [PubMed: 21302298]
22. Goc A, et al. P21 activated kinase-1 (Pak1) promotes prostate tumor growth and microinvasion via inhibition of transforming growth factor β expression and enhanced matrix metalloproteinase 9 secretion. *Journal of Biological Chemistry*. 2013; 288(5):3025–3035. [PubMed: 23258534]
23. AL-Husein B, Goc A, Somanath PR. Suppression of interactions between prostate tumor cell-surface integrin and endothelial ICAM-1 by simvastatin inhibits micrometastasis. *Journal of cellular physiology*. 2013; 228(11):2139–2148. [PubMed: 23559257]
24. Kochuparambil ST, et al. Anticancer efficacy of simvastatin on prostate cancer cells and tumor xenografts is associated with inhibition of Akt and reduced prostate-specific antigen expression. *Journal of Pharmacology and Experimental Therapeutics*. 2011; 336(2):496–505. [PubMed: 21059805]
25. Luan Y, Yao Y, Sheng Z. The tumor necrosis factor-alpha-induced protein 8 family in immune homeostasis and inflammatory cancer diseases. *J. Biol. Regul. Homeost. Agents*. 2013; 27:611–19. [PubMed: 24152830]
26. Cromer WE, et al. The effects of inflammatory cytokines on lymphatic endothelial barrier function. *Angiogenesis*. 2013:1–12.
27. Cross TG, et al. Serine/threonine protein kinases and apoptosis. *Experimental cell research*. 2000; 256(1):34–41. [PubMed: 10739649]
28. Goc A, et al. PI3 kinase integrates Akt and MAP kinase signaling pathways in the regulation of prostate cancer. *International journal of oncology*. 2011; 38(1):267–277. [PubMed: 21109949]
29. Mathur RK, et al. Reciprocal CD40 signals through p38MAPK and ERK-1/2 induce counteracting immune responses. *Nat Med*. 2004; 10(5):540–4. [PubMed: 15107845]
30. Rommel C, et al. Differentiation stage-specific inhibition of the Raf-MEK-ERK pathway by Akt. *Science*. 1999; 286(5445):1738–41. [PubMed: 10576741]

31. Tzivion G, Luo Z, Avruch J. A dimeric 14-3-3 protein is an essential cofactor for Raf kinase activity. *Nature*. 1998; 394(6688):88–92. [PubMed: 9665134]
32. Blanc A, Pandey NR, Srivastava AK. Synchronous activation of ERK 1/2, p38mapk and PKB/Akt signaling by H₂O₂ in vascular smooth muscle cells: potential involvement in vascular disease (review). *Int J Mol Med*. 2003; 11(2):229–34. [PubMed: 12525883]
33. Somanath P, et al. The role of PAK-1 in activation of MAP kinase cascade and oncogenic transformation by Akt. *Oncogene*. 2009; 28(25):2365–2369. [PubMed: 19421139]
34. Li J, Yin Q, Wu H. Structural basis of signal transduction in the TNF receptor superfamily. *Advances in immunology*. 2013; 119:135. [PubMed: 23886067]
35. Kandel ES, Hay N. The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB. *Exp Cell Res*. 1999; 253(1):210–29. [PubMed: 10579924]
36. Somanath PR, et al. Akt1 in endothelial cell and angiogenesis. *Cell cycle (Georgetown, Tex.)*. 2006; 5(5):512.
37. Daviet L, et al. Thrombospondin induces dimerization of membrane-bound, but not soluble CD36. *Thrombosis and haemostasis*. 1997; 78(2):897–901. [PubMed: 9268192]
38. Jiménez B, et al. Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1. *Nature medicine*. 2000; 6(1):41–48.
39. Pachman LM, et al. Juvenile dermatomyositis: The association of the TNF α - 308A Allele and disease chronicity. *Current rheumatology reports*. 2001; 3(5):379–386. [PubMed: 11564368]
40. Lutz J, et al. Increased plasma thrombospondin-1 (TSP-1) levels are associated with the TNF α -308A allele in children with juvenile dermatomyositis. *Clinical Immunology*. 2002; 103(3): 260–263. [PubMed: 12173300]
41. Segundo González M, et al. TNF-alpha-308A promoter polymorphism is associated with enhanced TNF-alpha production and inflammatory activity in Crohn's patients with fistulizing disease. *The American journal of gastroenterology*. 2003; 98:1101–1106. [PubMed: 12809834]
42. Chen J, et al. Akt1 regulates pathological angiogenesis, vascular maturation and permeability in vivo. *Nature medicine*. 2005; 11(11):1188–1196.
43. Aslam M, et al. TNF-alpha induced NF κ B signaling and p65 (RelA) overexpression repress Cldn5 promoter in mouse brain endothelial cells. *Cytokine*. 2012; 57(2):269–275. [PubMed: 22138107]
44. Li W, et al. An essential role for the Id1/PI3K/Akt/NF κ B/survivin signalling pathway in promoting the proliferation of endothelial progenitor cells in vitro. *Molecular and cellular biochemistry*. 2012; 363(1-2):135–145. [PubMed: 22139302]
45. Klettner A, et al. Regulation of constitutive vascular endothelial growth factor secretion in retinal pigment epithelium/choroid organ cultures: p38, nuclear factor kappaB, and the vascular endothelial growth factor receptor- 2/phosphatidylinositol 3 kinase pathway. *Molecular vision*. 2012; 19:281–291. [PubMed: 23401656]

Figure 1. Treatment with TNFα induces endothelial-barrier permeability

hMEC were treated with 0, 1.0, 5, 10, 25 and 50 ng/ml of TNFα for 12 hours then subjected to ECIS analysis. (A) Real-time measurements on the hMEC endothelial-barrier resistance upon treatment with different concentrations of TNFα recorded up to 12 hours. (B) Bar graph representation of hMEC endothelial-barrier resistance upon treatment with different concentrations of TNFα recorded by ECIS equipment at 8 hour post TNFα treatment. (C) Fluorescent images of endothelial cells treated with various doses of TNFα for 12 hours and stained with phalloidin indicating cytoskeletal changes and gap formations between endothelial cells. Data presented as mean $\pm$ SD (n=3), *p < 0.05, # p < 0.001.

Figure 2. TNFα induces synthesis and secretion of TSP-1 by hMEC associated with activating phosphorylation of Akt, P38 MAPK and ERK

A) Representative Western blot images of hMEC after treatment with TNFα in serum free media probed with antibodies against TSP-1, p^{S473}Akt, panAkt, pErk, pP38 MAPK and GAPDH in lysates, and TSP-1 in the conditioned media. B) Bar graphs showing densitometry analysis of the hMEC lysates and conditioned media for the cellular and secreted TSP-1, as well as phosphorylation of Akt, ERK and P38 MAPK. Data presented as mean ± SD (n=3), *p < 0.05.

Figure 3. TNFα-mediated cell stress modulates survival and proliferation of hMEC

(A) hMEC treated with 0, 1.0, 5, 10, 25 and 50 ng/ml of TNFα for 12 hours was subjected to 3-(4,3-Dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay for proliferation (B) Trypan blue staining for cell viability and (C) cytoplasmic histone-associated DNA fragments detection assay for apoptosis. Data presented as mean $\pm$ SD (n=6), *p < 0.05.

Figure 4. TNF α -induced cleaved caspase 3 and cleaved caspase 8 expression in hMEC was not reversed with Akt or p38 MAPK inhibitors

A) Representative Western blot images of hMEC lysates and conditioned media treated with 1 ng/ml and 5 ng/ml TNF α alone or in combinations with one of 10 μ M Akt inhibitor (SH-5) or 5 μ M of P38 MAPK inhibitor (SB239063) for 12 hours in serum free media, probed with antibodies against cleaved caspase 3 and cleaved caspase 9. B) Bar graphs showing densitometry analysis of amount of cleaved caspase 3 and 9 in cell lysates in response to TNF α in the presence and absence of Akt or P38 MAPK inhibitors. Data presented as mean $\pm$ SD (n=3), *p < 0.05.

Figure 5. Pharmacological inhibition of Akt and P38 MAPK, but not ERK blunts TNF α – induced TSP-1 synthesis by hMEC

A) Representative Western blot images of hMEC lysates and conditioned media treated with 5 ng/ml TNF α alone or in combinations with one of 10 μ M Akt inhibitor (SH-5), 10 μ M of Mek1/2 inhibitor (U0126) or 5 μ M of P38 MAPK inhibitor (SB239063) for 12 hours in serum free media, probed with antibodies against TSP-1. B) Bar graphs showing densitometry analysis of amount of TSP-1 in cell lysates and the conditioned media in response to TNF α in the presence and absence of Akt, P38 MAPK and Mek1/2 inhibitors. Data presented as mean $\pm$ SD (n=3), *p <0.05.

Figure 6. TNF α -induced TSP-1 expression in hMEC was reversed upon pharmacological or genetic inhibition of NF κ B

A) Representative Western blot images of hMEC lysates treated with 0, 25, 50 and 100 μ M doses of NF κ B inhibitor Piceatannol in the presence and absence of 10 ng/ml TNF α incubated for 12 hours in serum free media, probed with antibodies against TSP-1. Bar graph showing densitometry analysis of amount of TSP-1 in cell lysates in response to 0, 25, 50 and 100 μ M doses of NF κ B inhibitor Piceatannol in the presence and absence of 10 ng/ml TNF α incubated for 12 hours in serum free media. B) Representative Western blot images of control and SiNF κ B expressing hMEC lysates treated with 10 ng/ml TNF α , incubated for 12 hours in serum free media, probed with antibodies against TSP-1. Bar graph showing densitometry analysis of amount of TSP-1 in control and SiNF κ B expressing hMEC lysates in response to 10 ng/ml TNF α , incubated for 12 hours in serum free media. C) Bar graph showing the amount of TSP-1 in control and SiNF κ B expressing hMEC conditioned media in response to 10 ng/ml TNF α , incubated for 12 hours in serum free media measured using ELISA. D) Bar graph showing relative fold changes in TSP-1 levels in control and SiNF κ B expressing hMEC conditioned media in response to 10 ng/ml TNF α , incubated for 12 hours in serum free media measured using ELISA. Data presented as mean $\pm$ SD (n=3), *p < 0.05, # p < 0.001.

Figure 7. Schematic representation of the working hypothesis

Figure shows the overall conclusions from the current study supported by currently available information from the literature.