

Original Article

Pro-inflammatory angiogenesis is mediated by p38 MAP kinase

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SUMMARY

Chronic inflammation is tightly linked to diseases associated with endothelial dysfunction including aberrant angiogenesis. To better understand the endothelial role in proinflammatory angiogenesis, we analyzed signaling pathways in continuously activated endothelial cells, which were either chronically exposed to soluble TNF or the reactive oxygen species (ROS) generating H₂O₂, or express active transmembrane TNF. Testing in an *in vitro* capillary sprout formation assay, continuous endothelial activation increased angiogenesis dependent on activation of p38 MAP kinase, NADPH oxidase and matrix metalloproteinases. p38 MAP kinase- and MMP-9-dependent angiogenesis in our assay system may be part of a positive feed forward autocrine loop because continuously activated endothelial cells displayed up-regulated ROS production and subsequent endothelial TNF expression. The pro-angiogenic role of the p38 MAP kinase in continuously activated endothelial cells was in stark contrast to the anti-angiogenic activity of the p38 MAP kinase in unstimulated control endothelial cells. *In vivo*, using an experimental prostate tumor, pharmacological inhibition of p38 MAP kinase demonstrated a significant reduction in tumor growth and in vessel density, suggesting a pro-angiogenic role of the p38 MAP kinase in pathological angiogenesis *in vivo*. In conclusion, our results suggest that continuous activation of endothelial cells can cause a switch of the p38 MAP kinase from anti-angiogenic to proangiogenic activities in conditions which link oxidative stress and autocrine TNF production.

INTRODUCTION

Angiogenesis, the formation of new blood vessels from existing vessels by sprout formation, is essential for normal physiological processes such as embryonic development, the female reproductive cycle and the repair of injured tissue (Risau, 1997). The main angiogenesis factors responsible for physiological angiogenesis include the vascular endothelial growth factor (VEGF) and the fibroblast growth factor (FGF) (Risau, 1991; Risau, 1997). In this setting and on signal transduction levels, the Akt and the erk-1/2 MAP kinase pathways have been identified as pro-angiogenic principals (Clauss and Schaper, 2000; Zachary, 2003). In contrast, an anti-angiogenic activity of the p38 MAP kinase in response to VEGF and FGF in the chicken chorioallantois membrane (CAM) assay, an *in vivo* model of physiological angiogenesis, and in the *in vitro* model of endothelial capillary sprout formation was demonstrated (Issbrucker et al., 2003; Matsumoto et al., 2002). This finding was explained by inhibition of the erk-1/2 MAP kinase through p38 MAP kinase activated dual specific phosphatases (Bellou et al., 2009; McMullen et al., 2005).

In addition to developmental angiogenesis, excessive angiogenesis is also noted in many diseases such as tumors, rheumatoid arthritis and diabetic retinopathy (Carmeliet, 2003). There is evidence that this pathologic vessel growth, which contributes to exacerbation of the disease state is not only under control of vascular endothelial growth factors such as VEGF (Carmeliet, 2003), but also intimately linked with inflammation characterized by infiltration of monocytes and T cells (Arras et al., 1998; Monaco et al.,

2004). While the proinflammatory microenvironment may release growth factors and cytokines, it also influences the activation of the vascular endothelial cells and may influence signal transduction in these cells. In pursuing the overlap between inflammation and angiogenesis at the level of endothelial cell activation, we have demonstrated that in the absence of leukocytes a continuous stimulus with TNF- α results in endothelial sprout formation in a sprouting model, thus linking inflammation with angiogenesis (Rajashekhar et al., 2006). In a proinflammatory setting, TNF has been recognized *in vivo* as a proangiogenic factor (Balkwill, 2009; Frater-Schroder et al., 1987; Leibovich et al., 1987). *In vitro*, TNF is also proangiogenic (Pandey et al., 1995; van Hinsbergh et al., 1997; Vanderslice et al., 1998), although lower concentrations of either soluble TNF or its transmembrane bound cellular form may be favorable (Fajardo et al., 1992; Rajashekhar et al., 2006). Using such continuously activated endothelial cells we have shown specific gene expression pattern in endothelial cells, consistent with alterations observed in dysfunctional endothelium exposed to or involved in chronic inflammation. These gene changes were associated with elevated reactive oxygen species (ROS) levels, sustained p38 MAP kinase activity and increased migration in tmTNF expressing endothelial cells (Rajashekhar et al., 2007). However, the link of sustained p38 MAP kinase activity in activated endothelial cells with pro-inflammatory angiogenesis is not known.

In this study we analyzed signaling changes and proangiogenic activities in endothelial cells under pro-inflammatory conditions. To model these conditions, we

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employed transmembrane TNF overexpressing endothelial cells as well as exposure of endothelial cells to chronic treatment with low concentrations of soluble TNF or low concentrations of the ROS generator H₂O₂. In all these models subsequent to p38 MAP kinase signaling, in a positive feed forward autocrine loop, chronically activated but not acutely activated endothelial cells displayed up-regulated ROS production and subsequent endothelial TNF expression dependent upon MMP-9 activation leading to increased sprout formation and is susceptible to p38 MAP kinase inhibition. These results *in vitro* suggest a positive regulatory role for p38 MAP kinase in pro-inflammatory angiogenesis. Using a rat prostate tumor model we provide evidence for a pro-angiogenic role of the p38 MAP kinase in pathological angiogenesis. These results suggest that inhibitors of the p38 MAP kinase pathway may selectively inhibit angiogenesis associated with chronic inflammation.

MATERIALS AND METHODS

Cell culture and generation of continuously activated endothelial cells

Mouse endothelial cells transfected with the non-cleavable transmembrane mutant form of murine TNF (tmTNF) and control endothelial cells were cultivated as previously described (Rajashekhar et al., 2006). Primary human microvascular endothelial cells (Lonza Group Ltd, Switzerland) were exposed to low doses of TNF (Sigma-Aldrich, St Louis, MO; 1ng/ml, every 12 hours for up to 72 hr). In contrast, for acute stimulation cells were stimulated with one bolus of soluble TNF (1ng/ml) for 12 hr. To demonstrate ROS induced feed forward TNF mediated angiogenesis, primary microvascular endothelial cells were exposed to 10 μ M H₂O₂ every 12 hours for up to 72hr, while 12 hour one bolus dose served as acute control.

Western Blot Analysis

Primary human microvascular endothelial cells cultured in 6-well plates were serum starved overnight before treatment with low dose TNF. For acute phosphorylation analysis of p38 MAP kinase, protein lysates were prepared at 0, 5, 30 minutes, 1, 2, 6, 12 and 24 hours after one bolus TNF addition (1ng/ml). For chronic treatment, addition of TNF (1ng/ml) is repeated every 12 hours for up to 72 hours. Following this, media was changed and protein lysates were collected at 0, 12 and 24 hours. Control unstimulated cells served as background. In all cases cells were lysed with NP 40 lysis buffer (Invitrogen, Carlsbad, CA) containing protease inhibitor cocktail (Sigma Aldrich, St.

Louis, MO) and 5x lane marker reducing sample buffer (Thermo Scientific, Waltham, MA). The lysates were transferred to a microcentrifuge tube kept on ice followed by sonication for 10-15 seconds. Then the lysates were centrifuged at 14,000g for 10 minutes followed by heating at 95-100° C for 5 min. The samples were cooled to RT, centrifuged and resolved by electrophoresis. This was followed by transfer onto nitrocellulose membrane and incubation with the primary antibody (anti-phospho p38; 1:1000 dilution; Cell Signaling Technology, Danvers, MA) overnight at 4° C. The blots were washed with wash buffer followed by incubation with peroxidase conjugated secondary antibody (anti-rabbit IgG) for 1 hour at RT. The blots were visualized using enhanced chemiluminescence kit (Thermo Fisher Scientific, Rockford, IL). Then the blots were stripped and probed for total p38 MAP kinase using total p38 MAP kinase antibody (1:1000 dilution). Quantification of band intensities was performed using NIH ImageJ Software.

Immunocytochemical and FACS Analysis of tmTNF expression

Primary human umbilical vein endothelial cells exposed to 10 μ M H₂O₂ for 24 hours were detached using EDTA-buffer and incubated with primary anti-tmTNF antibodies (Hycult, HM2010, Cell Sciences Inc., Canton, MA) and assessed by cytofluorometric analysis (FACS) as described previously (Clauss et al., 2001). For confocal immunofluorescent analysis about 10,000 cells were plated in an 8-well chamber slide (LabTek, Nunc, Thermo Fisher Scientific, Rochester, NY) and stimulated with 10 μ M H₂O₂, stained with antibodies as described above. Cells were analyzed by confocal microscopy as described previously (Rajashekhar et al., 2003b).

Real-time Quantitative RT-PCR

In order to demonstrate the differential expression of E-Selectin and gp91^{phox} transcripts, quantitative real-time PCR was performed with total cellular RNA isolated from individual cultured cells with and without soluble TNF treatment as well as from tmTNF cells. Quantitative real-time PCR was performed and analyzed gene expression using a comparative Ct method as published previously (Rajashekhar et al., 2005). The results were expressed as fold change in mRNA expression relative to control untreated cells.

Gelatin Zymography

Endothelial cells post treatment with TNF without and with inhibitors at different time points were assessed for MMP-9 activity in the conditioned media. Ten micrograms of total protein per sample were loaded onto 10% SDS-PAGE gels (Invitrogen, Carlsband, CA) impregnated with 0.1% gelatin and separated using nondenaturing electrophoresis. Gel development was carried out as per manufacturer's protocol using SimplyBlue™ SafeStain (Invitrogen, Carlsband, CA). Gels were imaged on a gel scanner (Epson) using white light transillumination.

MTT assay

The direct effect of p38 MAP kinase inhibitor (SB202190, Sigma) in tumor cells was evaluated using a tetrazolium dye (MTT) assay as described previously (Rajashekhar et al., 2003a). Briefly, prostate tumors cells (767-PA3 and 797-PA3) were incubated in the

presence/absence of different concentrations of SB inhibitor for up to 72 hours, MTT dye working solution (Sigma-Aldrich, 5 mg/ml) was added and dye extracted with DMSO (Sigma-Aldrich) followed by measuring absorbance at 540 nm using an automatic multiwell spectrophotometer. The percentage viability was calculated using the background-corrected absorbance as follows: % Viability = [(A of experimental well)/A of control well] × 100. Each value represents mean ± s.d. of triplicate estimations from four independent experiments.

***In vitro* Sprout Formation Assay**

Angiogenesis *in vitro* was tested as described previously (Rajashekhar et al., 2006) without and with pharmacological inhibitors of p38 MAP Kinase (SB203580 & SB202190, Sigma-Aldrich); Erk1/2 MAP Kinase (PD98056, Sigma-Aldrich); Apocynin (Acetovanillone, Sigma-Aldrich); MMP broad spectrum inhibitor, GM6001 and/or MMP-9 specific inhibitor I (both EMD Chemicals, Gibbstown, NJ). The number of sprouts was counted by a person blinded to the study design.

Recombinant adenoviral Transduction and Sprout formation assay

Replication deficient adenoviruses encoding the DNp38 α MAP kinase is described previously (McMullen et al., 2004). For infection, endothelial cells were grown in six-well plates and incubated overnight for attachment. On day two, wells were washed twice with PBS and incubated at a multiplicity of infection of 100 with the adenoviruses in PBS for 2 hours. Efficiency of infection was greater than 90% as monitored by

immunofluorescent evaluation of the GFP expression. Thereafter, the cells were washed and cultured in complete medium for 3 additional days and proceeded with sprout formation assay as described above.

Angiogenesis *in vivo*

All animal protocols were performed in compliance and with approval from the IACUC of the University of Notre Dame. Mature LW (WI/Lob) rats with subcutaneous PA3 tumors were described previously (Kamocka et al., 2008). Animals were grouped randomly into two groups of six animals each with two tumors. Group one received ip injections of p38 MAP kinase inhibitor (SB202190, Sigma-Aldrich) at 2.5mg/kg body weight every day. The control group received solvent. After 17 days, the tumors were excised, wet weight was taken and tissues were preserved by embedding in Histoprep (Thermo Scientific) for confocal analysis.

Confocal and MetaMorph analysis for angiogenesis

To assess tumor blood vessel density, serial sections were stained for endothelium with HPA lectin. Briefly, 15 μm sections postfixed in 4% paraformaldehyde for 30 minutes were rinsed and blocked with the Image-iT FX signal enhancer (Molecular Probes, Eugene, OR) for 30 minutes. Following this sections were stained with 1.3 $\mu\text{g}/\text{ml}$ of HPA lectin from *Helix pomatia* labeled with Alexa Fluor 647 (Molecular Probes) for 90 minutes. Sections were rinsed with veronal buffer saline and co-stained for nuclei with 1 μM Sytox Green (Molecular Probes), rinsed and mounted with an anti-fading mounting

medium (Biomedex, Foster City, CA). Tissue samples were analyzed by confocal microscopy using a Zeiss UV LSM-510 confocal microscope system (Zeiss). Confocal image analysis was performed using MetaMorph software (Molecular Devices Corporation, Downingtown, PA). All images were background corrected as described previously (Dunn et al., 1989). Briefly, background intensity for each pixel was calculated as the median intensity in a surrounding 32x32 pixel region and only the central 312x312 pixel region of original 512x512 pixel image was further analyzed. Integrated morphometry analysis was performed on all background corrected and thresholded pictures. Total positive pixel areas in red channel for lectin staining were normalized to the number of nuclei for every image in both treated and untreated groups. Values for lectin staining from a total of 6-7 tumors in each group and about 300-500 pictures were analyzed to compute the analysis. All studies were performed in a blinded fashion by an investigator not aware of the treatment groups.

Statistical Analysis

Data are expressed as mean \pm SD for each group performed in triplicate for *in vitro* sprout assay and repeated at least three times. For inter-individual observational differences sprouts were calculated by two independent investigators blinded for the treatment groups. For *in vivo* experiments data are expressed as mean \pm SEM. Comparison between treated and untreated groups was performed with a paired student t-test. Statistical values were calculated using Sigma Plot software (Systat Software, Inc.,

Richmond, CA). Differences were considered statistically significant at $p < 0.05$ with a confidence interval of 95%.

RESULTS

Increased p38 MAP kinase activation and angiogenesis in endothelial cells exposed to prolonged low doses of soluble TNF

In order to generate a continuous endothelial activation model, we supplemented the culture media of primary human microvascular endothelial cells with 1 ng/ml soluble TNF changing media twice daily for three days (see scheme in Fig. 1A). Previously, we have identified a gene expression signature pattern in mouse endothelial cells expressing tmTNF, different from acute stimulation with soluble TNF including increase in gp91^{phox} but absence of E-Selectin induction (Rajashekhar et al., 2007). To further validate that chronic treatment with soluble TNF can mimic the endothelial activation induced by transmembrane TNF, we analyzed transcriptional regulation of gp91^{phox} and E-Selectin. Acute stimulation with soluble low dose TNF (1ng/ml) induced E-Selectin (a preferential marker of acute stimulation) but not gp91^{phox} transcripts after 12 hours (acute TNF, Fig. 1B), whereas chronic treatment with soluble low dose TNF (chronic low dose TNF) failed to induce E-Selectin but induced gp91^{phox} to comparable levels as observed with tmTNF expressing endothelial cells (Rajashekhar et al., 2007) (Fig. 1B). Subsequent to this continuous activation, low dose soluble TNF for 72 hours resulted in sustained levels of p38 MAP kinase phosphorylation even after withdrawal of TNF for further 24 hours (Fig. 1C). In contrast, acute stimulation resulted in a transient increase of p38 phosphorylation, which peaked at 30 minutes and was back to baseline levels after 24 hours.

Next, we wanted to assess this continuous activation results in increased angiogenesis as previously reported by us (Rajashekhar et al., 2006). As expected, the repeated (chronic) activation with soluble TNF resulted in increased sprouting angiogenesis in comparison to untreated control cells (Fig. 1D). Given the evidence that ROS can induce endothelial cell proliferation and tube formation (Rocic et al., 2007) we wanted to know whether chronic activation with soluble TNF induced expression of gp91^{phox}, the regulatory subunit of the ROS generating endothelial NADPH oxidase, is associated with angiogenesis. In fact, sprouting angiogenesis induced by chronic stimulation with soluble TNF for 72 hours (red bars) but not by acute stimulation (green bars) is inhibited by apocynin, an inhibitor of NADPH oxidase activation (Fig. 1D). This suggests that TNF-induced gp91^{phox} gene expression leads to NADPH oxidase activation dependent angiogenesis. Taken together, continuous activation of endothelial cells with soluble TNF induces p38 MAP kinase and ROS and participate in increased angiogenesis.

Increased tmTNF expression and angiogenesis in endothelial cells treated with low doses of hydrogen peroxide

Having shown that chronic treatment with low dose soluble TNF can mimic the transmembrane TNF-induced NADPH oxidase activation and angiogenic sprout formation, we next wanted to test the hypothesis that ROS induced oxidative stress could amplify endothelial activation by inducing TNF expression in endothelial cells. As shown in figure 2A primary human dermal microvascular endothelial cells treated with a low dose bolus application of 10 μ M hydrogen peroxide demonstrated an upregulation of

transmembrane TNF (up to 20%) as assessed by FACS analysis. Staining of endothelial cells with immunofluorescently labeled anti-tmTNF antibodies and confocal microscopy revealed punctuate surface staining (Fig. 2B) as it is also described for other endothelial transmembrane proteins such as the platelet-activating factor receptor (Predescu et al., 1996). In addition, low dose H₂O₂ treatment induced significant sprout formation in human endothelial cells, whereas higher doses demonstrated inhibitory activity (Fig. 2C). These data suggest that oxidative stress can upregulate tmTNF on the endothelial surface, which in turn may produce ROS (Rajashekhar et al., 2006) as part of a positive forward loop to enhance angiogenesis (see scheme in Fig. 6).

The p38 MAP kinase signal transduction pathway in endothelial cells under continuous activation plays a pro-angiogenic role.

Based on our demonstration that continuously activated endothelial cells with H₂O₂ upregulated membrane TNF expression and p38 MAP kinase has been shown as a downstream signaling protein in tmTNF cells, we assessed the role of the p38 MAP kinase in angiogenesis *in vitro* using endothelial cells continuously activated by tmTNF overexpression. Using the *in vitro* endothelial sprout formation in the presence and absence of small molecular weight p38 MAP kinase inhibitors, in control endothelial cells, addition of the p38 MAP kinase inhibitor SB203580 enhanced sprout formation when stimulated with VEGF (Fig. 3A, blue bars), as we have previously reported in primary human microvascular endothelial cells (Issbrucker et al., 2003). In contrast, in continuously activated tmTNF-expressing cells, the p38 MAP kinase inhibitor

(SB203580) strongly reduced sprout formation (Fig. 3A, red bars). Of note, this effect was also confirmed with a dominant negative form of p38 MAP kinase, which reduced sprouting angiogenesis in tmTNF-expressing cells but increased sprouting angiogenesis in control endothelial cells (Supplemental Fig. 1). To rule out Erk1/2 MAP kinase mediated compensatory angiogenesis in tmTNF cells, we also performed pharmacological inhibition with PD98056, an upstream inhibitor of Erk1/2 MAP kinase. Although application of PD98056 (10 μ M) suppressed sprout formation in control cells as published by us previously (Issbrucker et al., 2003) it did not have any effect on tmTNF expressing endothelial cells (Fig. 3B, red bars). These results suggest a pro-angiogenic role in proinflammatory angiogenesis in contrast to an anti-angiogenic role of the p38 MAP kinase in growth factor mediated physiological angiogenesis.

Increased angiogenesis in endothelial cells exposed to prolonged low doses of soluble TNF, hydrogen peroxide and tmTNF is mediated by matrix metalloproteinase-9.

Matrix metalloproteinases (MMP) have been shown to play a role in degradation of basement membrane and to promote capillary angiogenesis (Davis et al., 2001). Previously, ROS and p38 MAP kinase have been shown to act upstream of MMP-9 in non-endothelial cells (Moon et al., 2006; Underwood et al., 2000). Therefore, we examined MMP-9 activation by zymography in supernatant from primary endothelial cells treated with either chronic low dose soluble TNF or low dose H₂O₂. As shown in Fig. 4, stimulation with low doses of either TNF or H₂O₂ for 72 hours resulted in increased active MMP-9 (84kDa) levels and did not change MMP-2 (67kDa) levels (Fig.

4A). These responses were not seen with stimulation of low dose soluble TNF or H₂O₂ for 12 hours (acute). The increased MMP-9 activity is downstream of p38 MAP kinase signaling as the inhibitor SB203580 blocked both TNF and H₂O₂ mediated MMP-9 induction. Importantly, MMP-9 inhibition with a broad spectrum (GM6001) as well as a MMP-9 specific inhibitor I (Calbiochem; data not shown) abrogated completely the soluble TNF induced sprout formation (Fig. 4B). Based on the data suggesting that oxidative stress can upregulate tmTNF on the endothelial surface, in addition, we could also demonstrate elevated MMP-9 expression (Fig. 4C) as well as MMP-inhibitor dependent sprout formation in tmTNF expressing cells (Fig. 4D). Taken together, these data suggest that increased sprout formation in continuously activated endothelial cells is mediated by MMP-9 downstream of p38 MAP kinase.

Inhibition of p38 MAP kinase *in vivo* reduces tumor growth and angiogenesis

Having demonstrated that p38 MAP kinase mediated TNF-induced angiogenesis *in vitro* (Fig. 3A), we tested the hypothesis that p38 MAP kinase is involved in tumor angiogenesis *in vivo*. To this end we tested the effect of a water soluble p38 MAP kinase inhibitor SB202190 in a transplantable prostate tumor model since human prostate tumors demonstrate ROS production (Pani et al., 2009) and display strongly upregulated vascular TNF expression (Supplemental Fig. 2). Seventeen days after treatment the tumor wet weight from the SB inhibitor treated group was reduced to 0.53 \pm 0.34 as compared to 1.75 \pm 0.035g (n=6 per group; p<0.05) for the saline treated control group. This effect was associated with ameliorated angiogenesis as shown by decreased vessel density in

the treated group compared to the untreated group (Fig. 5A). Quantification of vessel density was achieved by normalization of the total immunofluorescence (endothelial staining) to the nuclear pixel density, which demonstrated a significant ($p < 0.0001$) decrease (Fig. 5B) in p38 MAP kinase inhibitor treated rats. This MetaMorph-assisted quantification of diminished microvasculature networks in tumors of SB202190 treated mice was visualized by representative 3D reconstruction of tumor sections using VOXX software (Supplemental movie). Taken together, the data suggest inhibition of p38 MAP kinase suppresses growth of implanted prostate tumors by reducing tumor angiogenesis.

DISCUSSION

This study demonstrates that proinflammatory conditions such as ROS generation by H₂O₂, continuous treatment with soluble TNF or tmTNF expression promote p38 MAP kinase and MMP-9 activation and sprouting angiogenesis in endothelial cells. This is to our knowledge the first demonstration that MMP-9 expression is linked with p38 MAP kinase in endothelial cells leading to increased sprout formation. It is in contrast to physiologic angiogenesis in non-activated conditions of the endothelium, which is mediated by plasminogen activator 1 and negatively regulated by the p38 MAP kinase (Issbrucker et al., 2003; Matsumoto et al., 2002). Therefore, it may be possible to target p38 MAP kinase to inhibit selectively angiogenesis associated with pathological as opposed to physiological processes. Such treatment maybe combined with targeting VEGF using neutralizing anti-VEGF antibodies, which are in clinical trials for treating aberrant angiogenesis in tumor (Kessler et al., 2010) and diabetic retinopathy (Rodriguez-Fontal et al., 2009). Interestingly, the p38 MAP kinase has been shown to be a mediator of vascular permeability in several experimental pathological settings (Clauss et al., 2001; Issbrucker et al., 2003; Kiemer et al., 2002; Lal et al., 2001; Niwa et al., 2001; Petrache et al., 2003). Thus, targeting the p38 MAP kinase may not only inhibit angiogenesis more selectively but may also contribute to normalize the highly leaky vasculature as observed in tumors and macular edema (Anderson et al., 2008; Du et al., 2010; Jain, 2005; Shchors and Evan, 2007).

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Although TNF has been originally identified as a hemorrhagic tumor necrosis inducing factor (Carswell et al., 1975), endothelial cell activation by TNF has been implicated in pro-angiogenic processes including rheumatoid arthritis, retinopathies, tumor formation and lymphatic vessel proliferation and remodeling (Baluk et al., 2009; Feldman and McTiernan, 2004; Hofer et al., 2002; Hurlimann et al., 2002; Ilg et al., 2005; Li et al., 2009; Nakao et al., 2003; Pandey et al., 1995; Sunderkotter et al., 1991; Zhang, 2008). TNF is produced as a transmembrane precursor protein, which had been suggested to be proangiogenic based on studies using transgenic overexpression and receptor knockouts (Luo et al., 2006; Rajashekhar et al., 2006). In fact, in pathological setting forward feedback loops involving ROS production could lead to transmembrane TNF expression in endothelial cells (Fig. 2A&B) explaining the here (Supplemental Fig. 2) and previously described (Clauss et al., 2001) expression of transmembrane TNF in tumor endothelium. Therefore, induction of ROS formation by transmembrane TNF expression and vice versa and subsequent endothelial activation resulting in a forward positive autocrine loop may enhance pathological and proinflammatory angiogenesis (Fig. 6).

The redox state of endothelial cells is usually tightly controlled reflecting a balance of oxidant and antioxidant systems. ROS like superoxide (O_2^-) or hydrogen peroxide (H_2O_2) are generated during a multitude of physiologic reactions such as mitochondrial respiration or by enzymes like xanthine oxidase and NADH/NADPH oxidase. Oxidative stress describes a situation, when the balance is markedly shifted towards the production

of pro-oxidant ROS, thus overwhelming the anti-oxidant system (Forstermann, 2008). This can be induced by inflammation, high glucose levels or hypertension. Previously we have shown a significant increase in the production of ROS in tmTNF cells which was attenuated by the addition of the NADPH oxidase inhibitor apocynin (Rajashekhar et al., 2006). We now demonstrated that continuous stimulation of primary endothelial cells by TNF (1ng/ml, every 12 hr) over a 3 day period mimicked the chronic inflammatory endothelium with significant increases in gp91^{phox} and decreases in E-Selectin transcripts (Fig. 1B). This differential gene expression in settings of chronic inflammation is associated with a significant increase in angiogenesis in an *in vitro* sprout assay model that is abrogated with the NADPH oxidase inhibitor, apocynin. This could be explained by the fact that NADPH dependent ROS generation is upstream of intracellular signal transduction pathways, including the p38 MAP kinase (Hsieh and Papaconstantinou, 2006; Li et al., 2005). Taken together, these lines of evidence suggest that the continuous activation of endothelium by TNF leads to persistently increased activation of gp91^{phox}, which induces a dysfunctional and proinflammatory phenotype, and ultimately increase pathological angiogenesis.

The MMPs are zinc dependent endopeptidases, which break down the extracellular matrix. Through the use of models of human disease in mice with targeted deletions of individual MMPs, it has become clear that MMPs act broadly in inflammation to regulate barrier function, inflammatory cytokine- chemokine activity and angiogenesis (Heymans et al., 1999; Manicone and McGuire, 2008). As shown in Fig 4, activation of MMP-9 but

not MMP-2 occurred with chronic treatment using either low dose soluble TNF or H₂O₂. Furthermore, the activity of MMP-9 was inhibited by pre-treatment with p38 MAP kinase inhibitor. Based on the data from this and the inhibition of sprouting angiogenesis (Fig. 4B&D) in the presence of MMP inhibitor suggests that the MMP-9 mediated sprout formation is downstream of p38 MAP kinase signaling. Our data suggesting that MAP kinases are upstream of MMP activation is in concordance with previous reports in other cell types such as in proteolytic cartilage degradation(Sondergaard et al., 2009) and lung fibroblasts (Underwood et al., 2000). Interestingly, increased MMP-9 expression is correlated with increase in hsp-27 expression (Hansen et al., 2001)which is also downstream of p38 MAP kinase and modulates cell migration and vascular permeability (McMullen et al., 2005).

Inflammation has been suggested as a tumor promoting factor in several human tumors, including prostate cancers, where expression of TNF and its receptors have been documented (de Miguel et al., 2000; Naylor et al., 1990; Royuela et al., 2008). However, several TNF inhibitors and downstream signaling pathway inhibitors have been employed as a modality for therapeutic intervention against tumors with little to no success (Sethi et al., 2009; Szlosarek et al., 2006). In this study, we have demonstrated first evidence that the p38 MAP kinase plays a major proangiogenic role in the here employed rat prostate tumor model (Fig. 5). Since tumor suppression can also occur through tumor cell necrosis directly rather than decreased vascularization, using *in vitro* MTT assay we have excluded the possibility. In this assay the corresponding concentration of p38 MAP

kinase inhibitor we have used *in vivo* did not suppress the proliferation of tumor cells, suggesting that the tumor regression noted in rat model is indeed due to decreased angiogenesis (Supplemental Fig. 3). However, future studies using endothelial specific dominant negative p38 MAP kinase transgenic mouse may provide further support for the hypothesis that the p38 MAP kinase is proangiogenic in a proinflammatory pathological setting. Although such a transgenic mouse is not available yet, it could be constructed in analogy to cardiac specific gene expression of a dominant negative p38 MAP kinase as reported previously (Braz et al., 2003). Together, our results provide several lines of evidence suggesting a novel role p38 MAP kinase in linking inflammation, oxidative stress and MMP-9 activity to promote angiogenesis. These studies further suggest that pharmacological intervention of p38 MAP kinase may be a novel strategy in specifically targeting proinflammatory condition-induced angiogenesis.

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FIGURE LEGENDS

Fig.1. Increased p38 MAP kinase activation, gp91^{phox} expression and angiogenesis in endothelial cells exposed to prolonged low doses of soluble TNF

(Scheme A) Human primary microvascular endothelial cells treated with 1ng/ml TNF either as a bolus application (acute) or repeatedly every 12 hours for up to 72 hours (chronic) and assessed for either gene expression or sprouting assay. **(B)** Differential gene expression in acute and chronic activation of endothelial cells was assessed by real time PCR analysis of E-Selectin and gp91^{phox} gene expression. Endothelial cells exposed to low doses of TNF for up to 72 hours (red bars) showed an increased gp91^{phox} gene expression while acute stimulation for up to 12 hours (green bars) displayed an increased E-Selectin expression comparable to levels of tmTNF cells (black bars). **(C)** At the indicated time points (acute) or 24 hours (chronic) after treatment with TNF at 72 hours, Western analysis using anti-phospho p38 or anti-p38 antibodies was performed. Quantification of fold change in phospho protein expression was analyzed by NIH Image J densitometric analysis software. Chronic TNF activated endothelial cells demonstrated increased phospho p38 MAPK. **(D)** Sprouting assay demonstrated a significant increase within 12 hours of acute stimulation (green bar), which was further increased with chronic activation (red bar). The increased sprout formation observed in chronic activation but not acute activation was significantly blocked with the NADPH oxidase

inhibitor, apocynin (0.1mM). Data shown are from a single experiment performed in triplicates and repeated three times with similar results.

Fig. 2. Increased tmTNF expression and angiogenesis in endothelial cells treated with low doses of hydrogen peroxide. (A) Flow assisted cytofluorometric analysis of human primary microvascular endothelial cells treated with 10 μ M H₂O₂ for 24 hours demonstrated a 15-20% increase in tmTNF expression after staining with anti-tmTNF antibodies. (B) Confocal microscopy revealed a surface tmTNF expression with a punctuate staining on the plasma membrane (bottom panel) compared to unstimulated cells (upper left panel). An isotype IgG ctrl demonstrated the specificity of staining (upper right panel). Scale bar=20 μ m. (C) At low doses H₂O₂ significantly increased sprout formation in these cells while at high doses it decreased sprout formation. Data shown are from a single experiment performed in triplicates and repeated three times with similar results.

Fig.3. The p38 MAP kinase signal transduction pathway in endothelial cells under continuous activation plays a pro-angiogenic role. Control endothelial cells (blue) or stable tmTNF-transfected endothelial cells (red) were used in sprout formation assay. (A) In tmTNF endothelial cells VEGF-induced sprout formation was inhibited by pharmacological inhibition of p38 MAP kinase with 10 μ M SB203580 while in control cells sprout formation is promoted by this treatment. (B) In contrast, in control endothelial cells VEGF-induced sprout formation was inhibited by pharmacological inhibition of Erk1/2 MAP kinase with 10 μ M PD98056, while in tmTNF cells sprout

formation is unaffected. The data shown are from a representative experiment performed in triplicates and repeated at least three times with similar results.

Fig. 4. Increased angiogenesis in endothelial cells exposed to prolonged low doses of soluble TNF, hydrogen peroxide and tmTNF cells is mediated by matrix metalloproteinase-9. (A) Human primary endothelial cells treated with low dose (1ng/ml) of TNF and 10 μ M H₂O₂ repeatedly for up to 72 hours increased active MMP-9 (84kDa) but not MMP-2. Furthermore, this increased expression is abrogated with a p38 MAP kinase inhibitor. In contrast, acute stimulation for 12 hours did not upregulate MMP-9. TNF treated corneal endothelial cells served as positive control (pos) (B) The increased sprout formation observed in low dose soluble TNF mediated chronic activation is significantly blocked with MMP broad-spectrum inhibitor, GM6001. (C) Zymography from conditioned media from tmTNF endothelial cells without and with exogenous soluble TNF but not control endothelial cells demonstrated active MMP-9 (84kDa) and can be blocked by pre-incubation with GM6001. (D) Increased sprout formation in tmTNF cells is blocked with 10 μ M GM6001. Data shown is from a single experiment and repeated three additional times with similar results.

Fig. 5. Inhibition of p38 MAP kinase *in vivo* reduces tumor angiogenesis. (A) Shown is the assessment of vascular density in tumors treated or untreated with the p38 MAP kinase inhibitor SB202190. Confocal microscopy of representative cryosections stained with HPA lectin – Alexa Fluor 647 and cyto16 from vehicle (untreated) and SB202190 (treated) rats demonstrated clear differences with least number of vessels in the treated

group. **(B)** Quantification of the total pixel intensities normalized to total nuclear pixel intensities by MetaMorph software revealed a significant decrease in vascular density in p38 MAP kinase inhibitor treated group compared to untreated group. Data presented is a mean \pm s.e.m of n=6 in each group.

Fig. 6. The ROS - TNF positive feed forward loop as a vicious cycle in continuously activated endothelium. Based on our findings that reactive oxygen species (ROS) induce endothelial tmTNF expression *in vitro* and tmTNF is expressed in endothelium of tumors (Clauss et al., 2001) and atheromas, (Barath et al., 1990) we propose that oxidative stress elicits a novel proangiogenic mechanism involving TNF (Rajashekhar et al., 2006). TNF in turn can induce ROS and thus may elicit “vicious cycles” of endothelial dysfunction linked via p38 MAP kinase signaling in a forward positive autocrine loop, leading to MMP-9 dependent proinflammatory angiogenesis.

SUPPLEMENTAL DATA

Supplemental Figure Legends

Supplemental Figure 1. Pro-angiogenic role of the p38 MAP Kinase signal transduction pathway in tmTNF endothelial cells. Cells were infected with dnp38 α MAP kinase-GFP or GFP-control vector, and embedded into fibrin gel in the presence of 50 ng of VEGF. The number of sprouts formed from tmTNF endothelial cells transfected with dnp38 α MAP kinase demonstrated a significant decrease compared to GFP-control vector. On the other hand, as observed with chemical inhibitor, control endothelial cells demonstrated an increase in number of sprouts. Shown are phase contrast micrographs plus GFP fluorescence. Data shown is from a single experiment performed in triplicates.

Supplemental Figure 2. Expression of TNF in human prostate tumors. Using the standard procedures of immunohistology, TNF expression was assessed in human prostate tumors. Immunohistochemical analysis demonstrated vascular expression adjacent to tumors (arrows; A, 10x). The expression is not restricted to the endothelium but also noted in vascular smooth muscle medial layer (B, 20x). A representative adjacent luminal epithelial tumor is negative for TNF staining (C, 10x). Data shown are from a representative tumor with similar results observed from at least six more patients. Scale bar=10 μ m

Supplemental Figure 3. p38 MAP kinase inhibitor induced tumor regression is not due to decreased tumor cell survival. Using standard procedures of MTT assay

viability of the rat prostate cell lines (767-PA3 and 797-PA3) for upto 3 days was performed. At an increased dose of p38 MAP kinase inhibitor that is relevant to the *in vivo* dose demonstrated a viability of about 90% even at day 3 suggesting the antitumor effect of p38 MAP kinase is acting at the level of vascular regression. Data shown as mean \pm s.d of triplicate measurements of each dose from a single experiment and repeated three additional times with similar results.

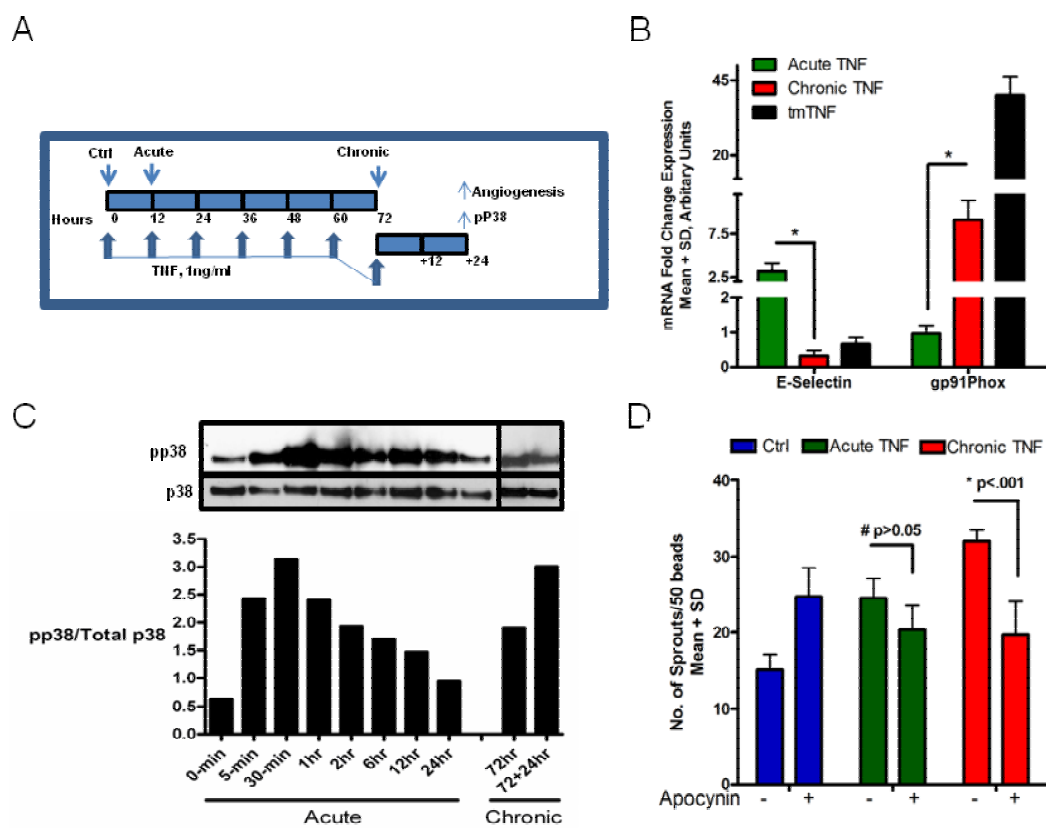


Figure 1

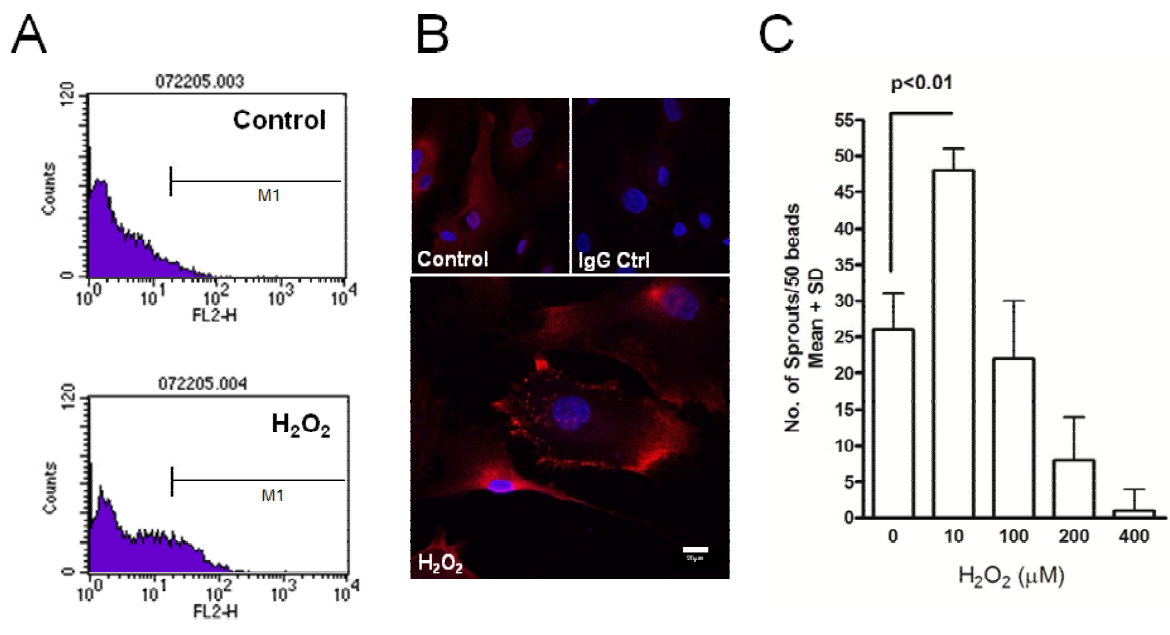


Figure 2

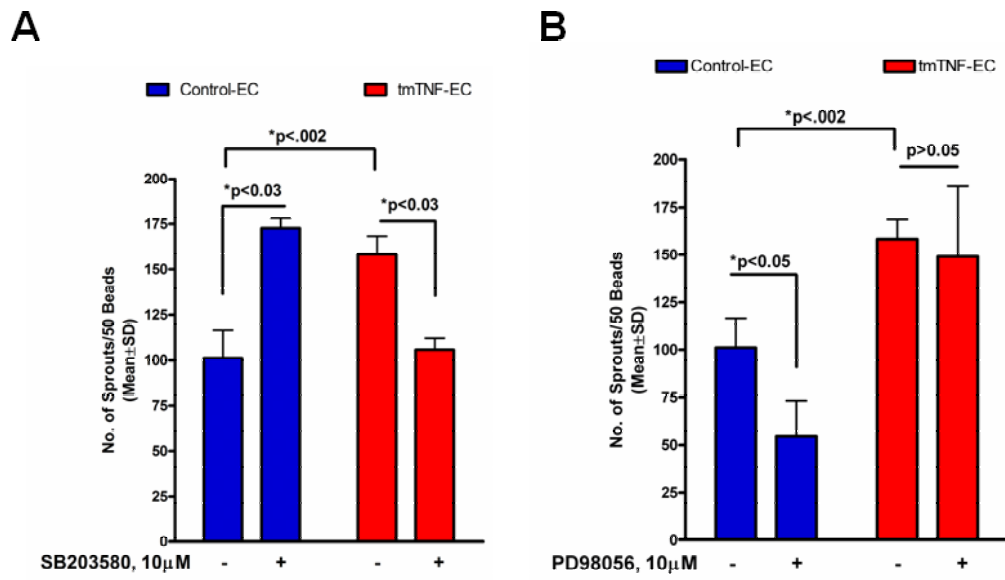


Figure 3

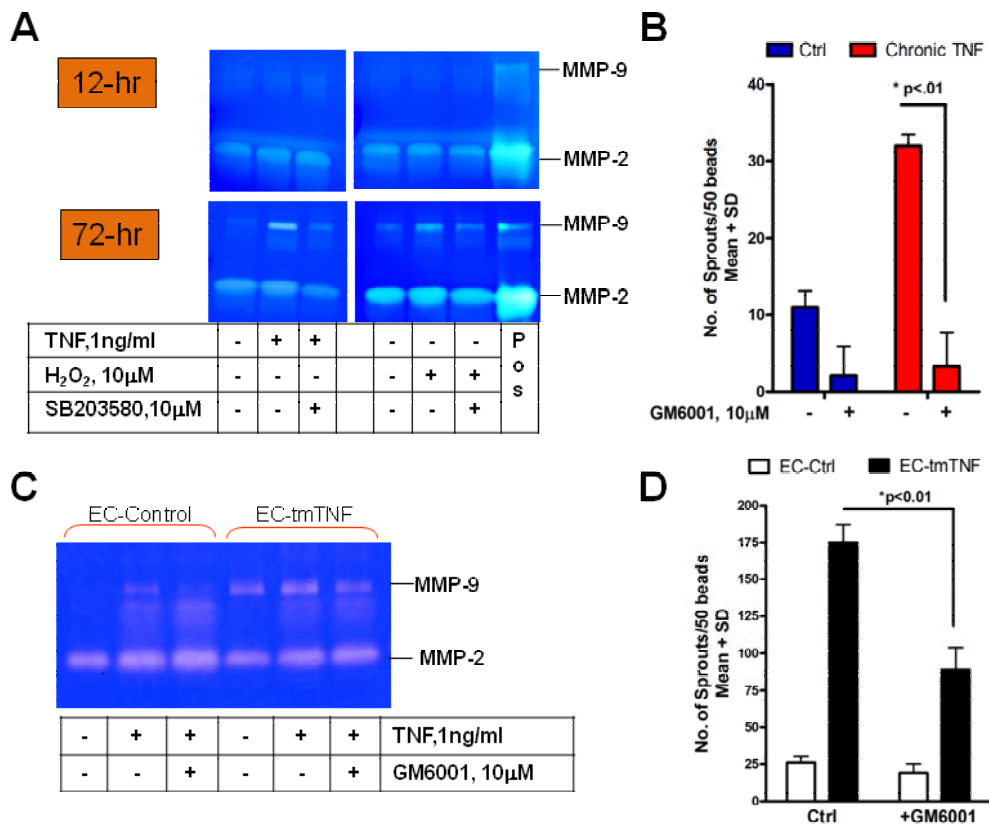


Figure 4

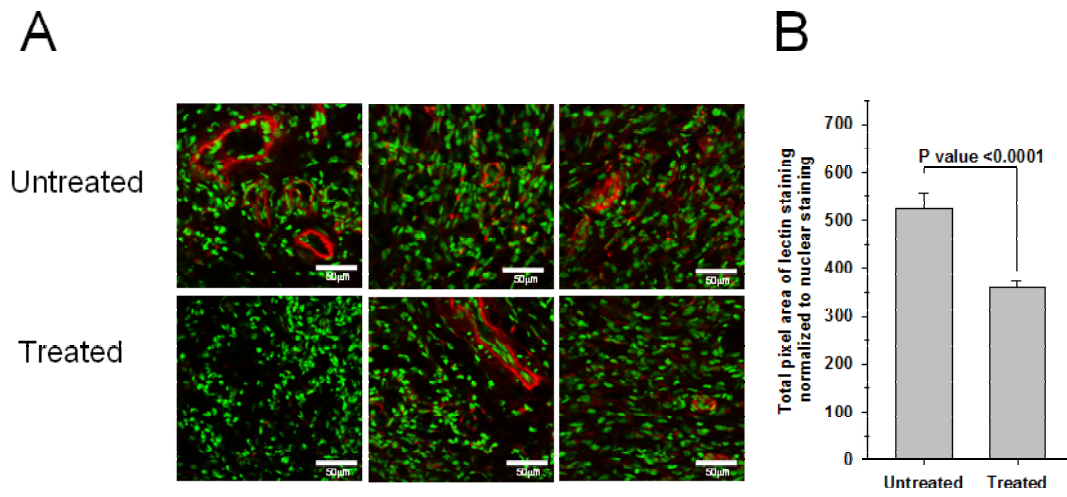


Figure 5

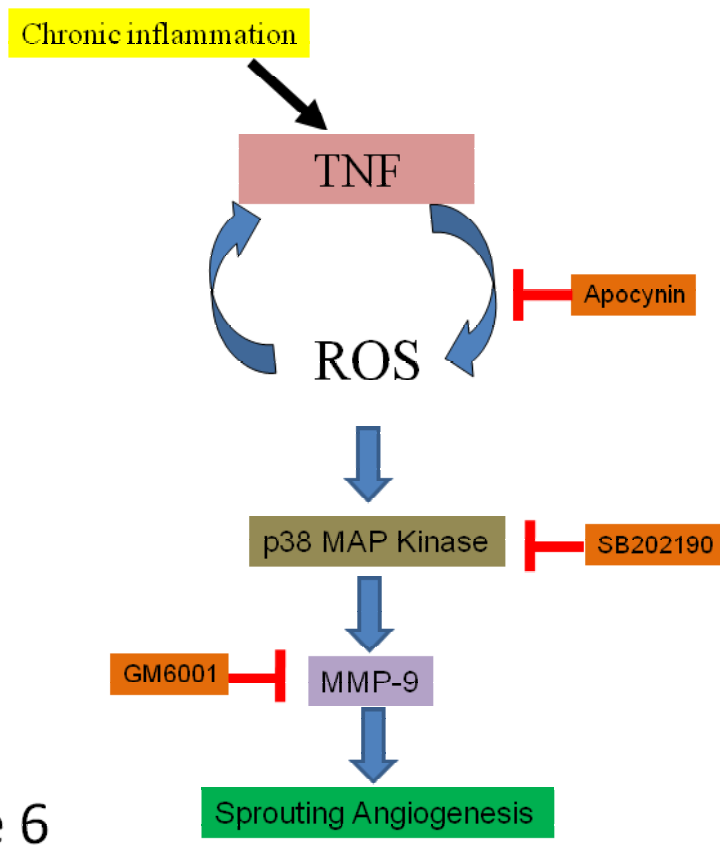


Figure 6