Divergent and Convergent Effects on Gene Expression and Function in Acute versus Chronic Endothelial Activation.

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Short Title: Gene Expression in Acute versus Chronic Endothelial Activation.
Abstract

Activation of the vascular endothelium with cytokines such as TNF is widely used to study the role of the vasculature in proinflammatory disease. To gain insight into mechanisms of prolonged vascular endothelial activation we compared changes in gene expression induced by continuous activation in stable tmTNF expressing cells with changes due to acute TNF challenge in vitro. Affymetrix Genechip® analysis was performed on RNA from control, acute and continuous TNF-activated endothelial cells. Only 36% of the significant changes in gene expression were convergent between the acute and continuously activated endothelial cells in comparison to the control. From the divergently regulated genes, for example the cytokine ENA-78 was specifically induced in chronically activated cells, while E-Selectin, a cell adhesion molecule, was upregulated only in acutely activated endothelial cells. Antioxidant SOD gene induction was noted in acute activation while a regulatory NADPH oxidase subunit was selectively upregulated in continuously activated endothelium in accordance with significant ROS induction occurred only in these cells. Accordingly, p38 and Erk-1/2, two MAP kinases downstream of ROS, were activated in stable tmTNF expressing cells and were refractory to activation with soluble TNF or VEGF. In consequence, the increased p38 MAP kinase activity contributed to increased endothelial cell migration in tmTNF expressing cells. These data suggest that continuous activation of endothelial cells leads to specific expression and functional changes, consistent with alterations observed in dysfunctional endothelium exposed to or involved in chronic inflammation.

Key Words - Endothelial Activation, TNF, Microarray, Genechip, p38 MAPK, migration
Introduction

The inflammatory response consists of changes in blood flow, increased permeability of blood vessels and emigration of leukocytes from the blood into the tissues. Acute inflammation is localized and usually resolves within 24-72 hours. However, persistent or chronic inflammatory signals can result in the inappropriate recruitment of leukocytes and cause localized or disseminated tissue dysfunction and damage. Under these circumstances, the endothelial cells face chronic exposure to leukocytes and membrane bound cytokines such as TNF and CD40L and become activated as an adaptive response. Such endothelial cell activation is essential for many physiological and pathological reactions in blood vessels leading to inflammation, vascular remodeling, and aberrant angiogenesis. These include atherosclerotic plaques in coronary disease and the inflamed synovium in rheumatoid arthritis, in which increased leukocyte emigration, elevated levels of oxidative stress, and angiogenesis of microvessels are commonly observed.

Endothelial cell activation is predominately described in conjunction with its activating cytokines, of which TNF emerges most prominently. TNF treatment of endothelial cells directly induces a series of effects including cytoskeletal reorganization, oxidant production, MAP kinase and transcription factor activation, and gene activation leading to release of cytokines/chemokines and intercellular adhesion protein expression. TNF signaling of endothelial adhesion protein upregulation closely resembles what is known as dysfunctional endothelium as it occurs, for instance, in atherosclerotic plaques. It essentially involves the nuclear translocation of the NFkB transcription factor but also increased reactive oxygen species (ROS) and decreased NO production. In addition, TNF induces a complex set of MAP-Kinase activation involving signals in endothelium that affect cell growth. Although the
ability of TNF to induce endothelial proliferation is controversial (30, 54), TNF may also employ or modulate other angiogenic mechanisms, including release of proteolytic activities and migration(26, 55).

TNF appears in two biologically active forms, the transmembrane spanning precursor (tm TNF) and soluble TNF. In leukocytes, tmTNF is produced in quiescent cells and increased in activated conditions but requires further stimulation and the protease TACE to generate soluble TNF from its tm TNF precursor(3). Importantly, low endogenous levels of tm TNF are present in endothelium in physiological conditions and account for the susceptibility of endothelial cells to increase vascular permeability in response to VEGF in vitro and in vivo(6). In order to address the role of increased endothelial tm TNF expression in vivo, we generated transgenic lines, in which we placed the uncleavable mutant form of tmTNF under the control of the endothelial cell specific tie2 promoter(51). These mice displayed increased multiorgan leukocyte infiltration, resistance to ConA-induced liver necrosis and increased angiogenesis in subcutaneous Matrigel implants(43, 51). In order to examine the underlying mechanisms in chronic endothelial activation we analyzed gene expression changes in mouse microvascular endothelial cells stably transfected with tmTNF in comparison to untreated or to acutely activated with soluble TNF endothelial cells. The resulting gene expression differences between continuously and acutely activated endothelial cells were analyzed with a specific focus on molecules involved in inflammation, cell signaling, and angiogenesis.
Methods

**tmTNF Expressing Endothelial Cells**

Polyoma virus middle T (23) transformed endothelial cells from wildtype newborn mice carrying the non-cleavable transmembrane mutant form of murine TNF and mock transfected control cells (43) were cultured in DMEM supplemented with 10% FCS, 2mM glutamine and 1x penicillin/streptomycin. Cells were transfected with the non-cleavable transmembrane mutant form of murine TNF (mTNFΔ1-9,K(11)E (9) by retroviral gene transfer: The cDNA for mTNFΔ1-9,K(11)E (51) was cloned into the pBABE/neo vector (35) and stably transfected into the retrovirus packaging cell line GP+E86 (31). Culture supernatants of transfected GP+E86 cells supplemented with 8µg/ml polybrene were used for infection of endothelial cells for 3 hours. Infected endothelial cells were selected with gentamycin containing medium (1mg/ml). FACS analysis of selected clones confirmed overexpression of tmTNF. Endothelial cells transfected with the empty vector were treated identically and used as control cells.

**Stimulation of Endothelial Cells and RNA Isolation**

Control endothelial cells in culture were treated with TNF (Sigma, 20ng/ml) for 4 hours. After treatment, the medium was removed, the cells were washed with phosphate-buffered saline, and RNA was isolated using TRIzol reagent (Invitrogen). In addition to the treated samples, untreated samples as well as tmTNF over expressing endothelial cells were subjected to RNA isolation. To obtain statistical confidence in the data set generated for the microarray hybridization experiments, at least 4 independent replicates of RNA isolated from at least three independent clones were used for each hybridization reaction. At least 10 µg of total RNA was harvested for each replicate and submitted for target synthesis, hybridization, and scanning on the mouse genome 430 2.0 Genechip®.
Microarray Processing and Analysis

All microarray processing and analysis was performed at the Center for Medical Genomics (CMG), using current Affymetrix-approved protocols (see http://cmg.iupui.edu and http://www.Affymetrix.com for details). For detailed protocol and validation please refer to online supplementary material.

Real-time Quantitative RT-PCR

In order to validate the differential expression of some representative genes quantitative Real-time PCR was performed with total cellular RNA isolated from individual cultured cells with and without TNF (20ng/ml, 4hours) treatment as well as from tmTNF cells. Quantitative real-time PCR was performed using an MJ Research PTC-200 Chromo4 sequence detector. The iScript one-step qRT-PCR kit with SYBR Green (Biorad) was used for cDNA synthesis and PCR amplification as per the manufacturer’s instructions using gene-specific primer pairs (Please see online supplementary Table 3). The amount of target gene transcript normalized to the endogenous β-microglobulin, house keeping gene transcript were computed based on a comparative Ct method as published previously (41). The results were expressed as fold change in mRNA expression relative to control untreated cells.

Flow Cytometric Analysis of Cell Adhesion Molecules

Flow cytometric analysis (FACS) was performed as described previously (6). Both control and tmTNF expressing endothelial cells were cultured to confluency, detached using EDTA-buffer, and incubated with mouse monoclonal PE labeled anti-ICAM-1 (CD54) or FITC labeled anti-VCAM-1 (CD106) or mouse monoclonal anti-E-Selectin antibodies (R&D Systems Inc) in
phosphate buffer supplemented with 2.5% FCS and 0.02% sodium azide for 30 min at 4°C. After washing, cells were analyzed on a FACStar using CellQuest software (Becton Dickinson) drawing light scatter gates around live endothelial cell populations.

**Western Blot Analysis**

To perform immunoblot analysis the whole cell extracts from two independent control, control treated with TNF and three independent tmTNF endothelial cell populations were probed for the presence of ENA-78 (Abcam), TSP-1 (Abcam), gp91phox (BD Biosciences), phospho-p44/42 MAPK (Erk1/2), phospho-p38 MAPK (both from Cell Signaling), NFκB (Chemicon) proteins as per the standard procedure described earlier (32, 42). For MAPK phosphorylation studies cells were serum starved for 4 hours and then either treated or untreated with soluble cytokines for 30 minutes for p38 and 10 minutes for Erk1/2.

**Real-Time Analysis of Intracellular ROS Production**

ROS from control, control treated with TNF and tmTNF endothelial cell populations were analyzed as described previously based on the uptake and intracellular hydrolysis of the fluorescent dye, H2DCFDA by intracellular esterases, and oxidation to the non-fluorescent derivative, DCFH(43).

**In vitro Sprout Formation Assay**

Angiogenesis in vitro was tested as described previously(43). Briefly, the endothelial cells were grown to confluence on cytodex-3 microcarrier beads and embedded in fibrin gels. Media with or without factors of interest were added to each well and incubated for 24 hours fixed and
the number of sprouts per 50 beads longer than the average bead size was counted under a phase contrast microscope.

**In vitro EC Migration and Image Analysis**

EC migration was modified from the method as described previously (29). In brief, control cells and tmTNF overexpressing cells were plated in six well plates and left overnight for attachment. The confluent monolayer cells were subjected to wound injury with a yellow tip, washed unbound cells and phase contrast micrograph pictures were taken and labeled as 0-hr. Cells were plated with fresh media with and without factors of interest and were cultured for 48 hours. The EC migration in culture was monitored every 12 hours by taking at least three different images from each well along the wound using a digital camera under a phase contrast microscope (10x). The cell count within the wound area was measured using NIH image software 1.6 offline and computed results for statistical analysis.

**Statistical Analysis**

For microarray data two-way comparisons of control and experimental groups were performed using a Welch’s T-test. The results were filtered for present/absent call (generated by the MAS 5 software with numeric values of: Absent (A=0), Marginal (M=0.5), or Present (P=1). All samples with an average “Fraction Present” value of less than 0.5 for each group were excluded from the analysis. Additionally, the results were filtered for statistical significance based on p-value. For in vitro sprout assay and migration assay data are expressed as mean ± SD for each group performed in triplicate. Statistical significance was determined by ANOVA using
Microsoft Excel Statistical Package. A probability value of $p < 0.05$ was considered statistically significant.
Results

tmTNF overexpression in endothelial cells leads to distinct expression changes and partial overlap with changes induced by acute stimulation with soluble TNF

Previously(43) we have identified continuous endothelial cell activation as a proangiogenic mechanism. In order to analyze the effect of endothelial cell activation on gene expression in microvascular endothelial cells, in which inflammation and angiogenesis usually is mediated, we used microarray analysis (see Fig. 1) and compared RNA isolated from tmTNF expressing cells, control cells, and short term (acute) activated (TNF, 4 hours) control cells. Assessment of differential gene expression revealed 1054 probe sets representing 896 genes significantly regulated (at p<0.05) by stable tmTNF expression in comparison to control cells; whereas activation with TNF resulted in differential expression detected by 2026 probe sets representing 1719 genes. Of these hits, only 187 probe sets were shared as hits between the two treatments (figure 1). The complete microarray dataset may be found under the ID number GSE4518 in the GEO database (http://www.ncbi.nlm.nih.gov/geo/) and in the supplementary material (Please see online supplementary material).

Annotation based functional analysis of gene expression changes

To test the hypothesis that specific functions of identified genes correlate with either chronic or acute activation by tmTNF or soluble TNF we performed annotation based comparison of functional groups. Analysis of significant hits indicated divergence between continuous and acute activation as shown in Table 1 and supplemental table 2 (please see online supplementary material). Many angiogenesis regulating genes are induced by either chronic or acute activation,
including thrombospondin, serpins, proteases and protease inhibitors. Of note, pro- and anti-angiogenic genes were regulated both in continuously and acutely activated endothelium. Similar observation were made with genes involved in inflammation. Acute activation with TNF, but not chronic activation with tmTNF expression increased expression of E-Selectin more than 5 fold. VCAM-1 was expressed more strongly in acutely than in chronic activated cells (41 fold over control cells), whereas ICAM-1 revealed the opposite regulation (82 fold over control cells after treatment with TNF). Given the crucial role of chemokine production to trafficking of inflammatory and precursor cells from blood to tissues, we asked how short and long term activation of endothelial cells affected their gene expression. Although few chemokines were activated by both acute and chronic stimulation (MCP1 and IP-10), two were exclusively regulated by acute TNF and two were only by long-term stimulation (table1, see also table 2 in supplementary material). Next, we addressed the effect of acute and chronic activation on genes involved in oxidative stress. Indeed, we found a strong induction of the critical catalytic membrane component, gp91phox, in tmTNF expressing cells whereas the anti-oxidant superoxide dismutase-2 was upregulated only in acutely stimulated cells, suggesting that continuous endothelial activation causes pro-oxidative changes. Addressing signaling molecules, we find MAPK1 (Erk-1), which is associated with differentiation, proliferation and activation of cells, is downregulated, whereas the proinflammatory Irak1bp1 (SIMPL) is selectively upregulated in tmTNF expressing cells. However, other signaling molecules relevant for TNF signaling such as TRAF1 appear to be also upregulated in acutely TNF activated cells (see table 1, also see table 2 in supplemental data). Although no further specific changes in either acutely or continuously activated endothelial cells observed there is trend to pro-oxidative stress inducing genes in the case of tmTNF expressing endothelial cells. To gain further insight into possible links between
groups of regulated genes, several different relationship pathways were generated using PathwayArchitect 1.0.3 software (Stratagene, Inc.) by loading select sets of significant “hits” from the microarray data. As shown in online supplemental data specific links have been demonstrated for genes involved in inflammation, intracellular signaling, and angiogenesis.

**Confirmation of convergent or divergent gene expression and function in tmTNF overexpressing and soluble TNF activated endothelial cells.**

To confirm the microarray results, we chose twelve representative genes equally distributed over their different functional activities and measured their expression with real-time qRT-PCR. As expected all of them showed very high concordance between microarray and quantitative PCR data (see Fig. 2). Next we wanted to know whether the regulatory changes of key molecules of divergent gene expression in chronically and acutely activated endothelial cells are also observed on protein levels. Figure 3 depicts the confirmation of divergent expression at the protein level by Western blot analysis using specific antibodies. The expected 14 kDa protein band for ENA-78 can be only seen in chronically activated cells but not in control cells or cells acutely activated with TNF, whereas the RelA/p65 subunit of NFκB is upregulated in both soluble TNF treated and tmTNF expressing cells (Fig. 3A). In addition, we confirmed upregulation of thrombospondin-1 (TSP-1), an inhibitor of angiogenesis, in acutely TNF treated cells (Fig. 3B). Furthermore, a remarkable strong NADPH oxidase subunit gp91phox upregulation in tmTNF expressing cells (Fig. 3C) was demonstrated in line with the mRNA expression data. To confirm the functional relevance of the upregulated regulatory gp91phox we assessed ROS generation. As shown in Figure 3D, ROS generation after 4 hours of incubation with transiently TNF stimulated cells is only marginally increased whereas in tmTNF cells a significant effect was observed.
Desensitization of tmTNF overexpression towards stimulation with soluble TNF

Based on the fact that effects of soluble TNF are characterized by desensitization (8, 21) we addressed desensitization in tmTNF expressing cells. In this regard, we performed FACS-analysis for vascular adhesion molecule surface expression (Fig. 4A). Cells stimulated with TNF (dark gray histogram) or untreated (light gray histogram) were incubated with antibodies against E-Selectin, VCAM-1, or ICAM-1. In tmTNF expressing endothelial cells, the signals for ICAM-1 and VCAM-1 but not for E-Selectin shifted to the right indicating increased expression; whereas in control cells they remained at basal levels. This implies that the stably transfected tmTNF cells display constitutively increased levels of ICAM-1 and VCAM-1 at the surface in comparison to control cells, whilst E-Selectin did not change. Importantly, stimulation with TNF strongly induced E-Selectin, VCAM-1 and ICAM-1 surface expression in control cells but no further significant upregulation was seen in tmTNF expressing endothelial cells. This suggests that stable tmTNF expressing cells are desensitized to treatment with soluble TNF.

Previously we have identified continuous endothelial cell activation by tmTNF expression resulted in increased angiogenesis in vitro (43). Using this assay we further addressed the effect of desensitization on a functional level in these tmTNF expressing cells and compared with soluble TNF treated control cells. We embedded endothelial cells grown on microbeads into a fibrin gel and let 3-dimensional sprouts form in the presence or absence of TNF. Significantly more sprout formation was found in tmTNF expressing endothelial cells vs. control cells with and without TNF (p<0.01; Fig. 4B). Sprout formation in tmTNF cells was not further increased by TNF, but TNF caused a small increase in sprouting in control cells (p<0.04). Although VEGF was able to increase sprout formation in both control and tmTNF expressing
cells, it did not further support sprout formation together with soluble TNF. This indicates that continuously activated tmTNF expressing cells are refractory to further stimulation with TNF.

**Divergence of acute stimulation from tmTNF overexpression towards stimulation with soluble TNF in endothelial MAPK activation**

Based on the role of p38 and Erk-1/2 MAP kinases in multiple endothelial functions including angiogenesis(19), we have assessed activation of these kinases. Using Western blot analysis with phosphorylation specific antibodies we have compared the phosphorylation of p38 and Erk1/2 MAP kinases in untreated control cells, control cells treated acutely with TNF and continuously tmTNF activated cells (Fig. 5). Figure 5 demonstrates sustained p38 and Erk1/2 MAPK phosphorylation in tmTNF expressing cells. Addition of soluble TNF and VEGF transiently phosphorylates both p38 and Erk1/2 MAP kinases in control cells but fails to further increase phosphorylation in tmTNF transduced cells.

**Increased p38 MAPK activation in tmTNFα expressing cells causes increased migratory activities**

Because the p38 MAPK reportedly is associated with endothelial cell migration (33, 44), we have investigated whether this increased activation in tmTNF expressing cells leads also to increased migration in a wounding assay. Whereas no significantly evaluated migration is observed with soluble TNF, tmTNF expressing cells showed increased migration into wounded areas. This migration is strongly reduced to values below of control cells by co incubation with a p38 MAPK specific inhibitor, indicating that the increased p38 MAPK is involved in the observed increase in migration.
Discussion

Our findings demonstrate that continuous activation of microvascular endothelial cells is possible and results in both overlapping and distinct changes compared to the acute activation. We decided to address acute activation by use of short-term treatment with soluble TNF (4 hours) as published previously which was adequate to detect the pattern of TNF-induced change in gene expression (5, 13). For long term activation we chose stable tmTNF transfected cells as encountered in our transgenic model of tie2- tmTNF expression. In addition, this tmTNF is also likely to resemble the activated endothelium in vivo, where tmTNF is presented on circulating and transendothelial trafficking monocytes and lymphocytes, which are in increased contact with endothelial cells in chronic proinflammatory diseases such as atherosclerosis and rheumatoid arthritis. Because typical characteristics of inflammation such as leukocyte emigration and aberrant angiogenesis occur in the microvasculature, we here addressed changes in gene expression by using immortalized microvascular mouse endothelial cells.

We compared our results to several publications that have used high-throughput gene expression methods to study TNF (5, 13, 22, 36, 45, 46, 50). As ours is the first such report using mouse cells, our comparison was made to the studies involving human endothelial cells (online supplementary table 2). It may be of concern that some genes previously reported to respond to TNF are not found as significant hits in our data. There are a number of possible reasons for this, including but not limited to 1) no representative probe set on the mouse microarray, 2) annotation errors that misidentify genes or prevent the linking of the mouse data to the human data 3) tissue culture conditions and immortalization procedures may further attribute to the differences in response to TNF and 4) mouse endothelial cells used here respond differently than human endothelial cells tested in previous experiments.
We hypothesized that chronic exposure to tmTNF causes a distinct pattern of gene expression, which differs from acute stimulation seen with TNF but remarkably resembles to what is observed in chronic inflammatory disease. In line with this hypothesis, E-Selectin which is characteristically upregulated in acute but not in chronic inflammation (10, 51) is absent in continuously tmTNF-activated cells but induced in soluble TNF treated control cells. Furthermore, these results obtained in vitro are in concordance with our previous findings in vivo in tie2- tmTNF transgenic animals, in which ICAM-1 was strongly and VCAM-1 was only moderately upregulated whereas E-Selectin levels were not at all elevated (51). This could explain the relatively mild proinflammatory phenotype in these animals despite strong vascular adhesion molecule (VCAM-1 and especially ICAM-1) expression. Finally, chemokines such as ENA-78 (92 fold) and MCP-3 (31 fold) were only significantly upregulated in stable tmTNF expressing cells. It was observed previously that ENA-78 is strongly upregulated in rheumatoid arthritis and correlated with the angiogenesis in the diseased joints (15, 24). In contrast other chemokines such NAP-2 (9-fold), MIP-1γ (9-fold) or CXCL-16 (18-fold) were only induced in acutely activated endothelial cells.

Effects of soluble TNF are characterized by desensitization, which is mediated by TNFRI downregulation (8, 21). Accordingly, we demonstrated both on protein expression and functional levels that tmTNF cells do not respond to further activation with soluble TNF (Fig. 4). However, we do not know yet whether this effect is congruent with chronic continuous endothelial activation or restricted to activation with transmembrane TNF. We have not included in this study short term activation with tmTNF and long term activation with soluble TNF. Future experiments employing long term activation with soluble TNF are expected to address this question.
When studying genes involved in endothelial oxidative stress, one striking observation was the increased expression in oxidative stress inducing genes including the critical NADPH oxidase catalytic component Nox-2, gp91phox. Of interest, the anti-oxidant superoxide dismutase gene was only induced upon acute activation with TNF in concordance with previous reports (53). Importantly, oxidative stress reportedly is increased in vessels associated with chronic inflammatory diseases, leading to a quenching of NO and subsequent endothelial dysfunction. Interestingly, in endothelial cells in vitro, hydrogen peroxide and radiation upregulate the membrane form of TNF(11, 49). This could be in line with the finding that endogenous endothelial tmTNF expression in vivo was first found to be upregulated in angiogenic vessels in atheromas, which are known to comprise an environment of increase oxidative stress(1). Thus tmTNF expression could be part of an autocrine loop of increased endothelial activation and dysfunction(43).

NADPH dependent ROS generation has been described to be upstream of intracellular signal transduction pathways, including the MAP kinases (17, 28). Based on this established link between NADPH dependent ROS formation and MAP kinase activation, we tested the hypothesis that long term activation with transmembrane TNF also leads to continuous p38 MAPK and Erk-1/2 MAPK activation. We demonstrated prominent p38 and to a lesser extent also Erk-1/2 MAPK activation in tmTNF expressing cells (Fig. 5). Of note, soluble TNF but also VEGF are refractory to further activation of these MAP kinases, suggesting a possible mechanism for the observed inability of tmTNF expressing cells to functionally respond to additional stimulation with soluble TNF (Fig’s 4 and 5). This sustained p38 MAPK activation in tmTNF expressing cells not only can be a consequence of the gp91 gene expression and subsequent ROS induction but may also participate in the specific gene expression in tmTNF
expressing versus transiently activated endothelial cells. In fact, from the strongest upregulated (> 5 fold) 21 genes, which are induced only in tmTNF expressing cells, 6 out of 10 reports addressing signaling pathways for their gene induction identify the p38 MAPK as the essential upstream activator. One of these p38 inducible gene products, the discoidin domain receptor 2(47), a novel collagen receptor in fibroblasts and smooth muscle cells(27), was shown here to be also expressed in continuously activated endothelial cells. Based on its predicted role in tissue remodeling(12), this receptor may also be involved in the increased angiogenesis observed with tmTNF expressing cells. Another p38 MAP kinase-induced gene product, clusterin (39), was suggested as a novel potent defense mechanism against complement-induced endothelial cell activation (48) was upregulated in tmTNF expressing cells which may explain the protective role of tmTNF in atherosclerosis in ApoE-/- animals(4). Furthermore, p38 inducible gene products which include the chemokines RANTES, MCP-3 and ITAC (16, 20, 52), have been shown to attract monocytes and T-cells. This possibly explains the predominant infiltration of these monocytic cells in our tie2-tmTNF transgenic animals (51). Finally, the ability of p38 MAPK to induce and promote NADPH oxidase activation(37) may in part be a possible autocrine loop that sustains endothelial cell activation. Accordingly, TNF induces gp91phox expression leading to ROS production, which activates MAP kinases and then may feed back to NADPH oxidase as depicted in Figure 6. Further studies testing gene expression changes in the presence of p38 MAPK inhibitors in tmTNF cells will be required to test the suggested role of p38 MAPK in the induction of the above discussed genes and their relevance to biological function.

In addition to gene induction, p38 MAPK has been shown to induce directly endothelial migration and vascular permeability ((19, 33, 44). These functions are shown to be independent of gene induction and to involve activation of hsp 27 leading to actin reorganization (18). In line
with these publications we have demonstrated that tmTNF expressing cells display enhanced migration into wounded areas in a p38 dependent fashion. Again, this effect was not observed with soluble TNF in control cells and could not be enhanced with soluble TNF in tmTNF expressing cells. Although other mechanisms including tmTNF dependent Bmx pathways may also be involved in the observed enhanced migration(55), these studies define a link between the increased gp91\textsuperscript{phox} levels, increased p38 MAP kinase activation and migration, which is highlighted in the scheme of Figure 6. As endothelial cell migration is part of the complex machinery leading to angiogenesis these results provide a mechanism for our previously reported increased angiogenesis in tmTNF expressing endothelial cells in vitro and in vivo(43).

Taking these data together, we were able to demonstrate distinct activation patterns in short term and continuously activated mouse endothelial cells including: 1) Distinctly activated cell adhesion molecules such as E-Selectin which was upregulated only in acutely activated endothelial cells, 2) Distinct and extremely high upregulation of specific chemokines, occurring only in tmTNF expressing cells, 3) Genes involved in generation of oxidative stress were predominately upregulated in continuous activation, resulting in significant chronic ROS induction, 4) Sustained phosphorylation and activation in tmTNF but not in soluble TNF treated cells of the p38 MAP kinase, which is downstream of ROS, 5) p38 MAP kinase dependent increased cell migration in tmTNF expressing endothelial cells.
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References


Table Legends

Table 1. Differential gene expression in tmTNF vs. TNF activated endothelial cells. RNA expression profiles were determined by oligonucleotide array hybridization in three independent experiments as described in Methods. Shown are representative differentially regulated angiogenic, cell adhesion, chemokine/cytokine, oxidant and signaling genes. Genes that were upregulated are highlighted in red while those genes that were downregulated are highlighted in green color. * Where there were more than one probe set found, a representative set is shown.
Figure Legends

**Figure 1.** *over expression of tmTNF in endothelial cells leads to distinct expression changes and partial overlap with changes induced by acute stimulation with soluble TNF* (A) A non-cleavable transmembrane mutant (TNF△1-12) form of murine TNF was introduced into immortalized endothelial cells by retroviral gene transfer. Selected clones were stained either with an isotype control antibody (dotted histogram) or a rat anti-mouse monoclonal antibody directed against TNF (Clone V1q; kind gift by W. Falk, University of Regensburg, Germany; dark histogram) and visualized by incubation with a phycoerythrin coupled secondary antibody. FACS analyses clearly indicated tmTNF over expression in tmTNF clones as compared to control wild type clones. PECAM-1 was used as a positive control to demonstrate endothelial phenotype of these cells (grey histogram). (B) Schematic overview of the identified differentially expressed genes (at p < 0.05 compared to Control cells without treatment) demonstrates that only 187 probe sets were shared as hits between the two treatments.

**Figure 2.** *Confirmation of Microarray analysis of representative genes by Realtime RT-PCR.* For quantitative Real-time PCR total cellular RNA was isolated from individual cultured cells and assessed using the iScript one-step qRT-PCR kit with gene-specific primer pairs. The amount of target gene transcript normalized to the endogenous β-microglobulin, house keeping gene transcript were computed based on a comparative Ct method. The results are expressed as mRNA fold change normalized to control cells. Data shown are the mean of three independent experiments with duplicates and from three different clones for each cell type. Significant values are indicated with asterisks.
Figure 3. Confirmation of Microarray analysis of representative genes by Western analysis. A-C). Immunoblot analysis was performed as described in Methods with anti-ENA-78 (A), anti-NFκB (A) anti-gp91Phox (B) and anti-TSP1(C) stripped and re-probed with GAPDH. Quantification of fold change in protein expression was analyzed by NIH Image J densitometric analysis software. Data shown are from a representative experiment and repeated at least three times with similar results. D) Increased ROS in tmTNF expressing endothelial cells. Confluent control or tmTNF endothelial cells were tested for ROS production as measured by DCFH oxidation. ROS were increased in tmTNF cells (closed bar) compared to control mock transfected cells (open bar) as well as control cells treated with TNF (hatched bar). Of note, no significant further increase was observed in control cells acutely treated with TNF. Tests were performed with 4-8 replicates in each experiment and experiments repeated at least two additional times.

Figure 4. Divergence of acute stimulation from tmTNF overexpression towards stimulation with soluble TNF A) Differential expression of ICAM-1, VCAM-1 and E-Selectin in tmTNF expressing versus control endothelial cells treated with TNF. Shown are FACS-analyses of tmTNF expressing endothelial cells and control cells before (light histogram) and after treatment (grey histogram) with TNF for 4 hours. Of note, in tmTNF expressing cells in addition to lack of E-Selectin staining, TNF did not lead to further significant increase in ICAM-1 or VCAM-1 expression. B) Increased sprouting activity in tmTNF expressing versus control endothelial cells is refractory to further stimulation with TNF, VEGF and or both. Angiogenic sprouting activity in control (open bars) and tmTNF (closed bars) endothelial cells embedded in fibrin gels was quantified. tmTNF endothelial cells demonstrated a significant (**p < 0.01) increase in capillary
like sprouts as compared with control cells which was not increased further in the presence of TNF or VEGF. On the other hand, control cells demonstrated a significant increase (*p<0.04) in sprouting activity in the presence of TNF and VEGF; however, the increase was not comparable to tmTNF cells. The data presented are from a representative experiment repeated four times independently with similar results.

**Figure 5. Increased p38 MAPK activation in tmTNF expressing cells causes increased migratory activities in these cells A-B)** Immunoblot analysis was performed as described in Methods with anti-phospho p38 (A) or anti-phospho Erk1/2 (B), stripped and re-probed with p38 and Erk1/2 antibodies. Quantification of fold change in protein expression was analyzed by NIH Image J densitometric analysis software. Data shown are from a representative experiment and repeated at least three additional times with similar results. C) *Effect of increased p38 MAPK in tmTNF cells resulted in increased migration.* Endothelial cell migration in culture was determined by measuring wound areas in cell monolayers as described in Methods. A representative phase contrast micrograph is shown immediately after wound (0-hr) and 48-hr post wound for both control and tmTNF expressing cells with and without p38 MAP kinase inhibitor (SB203580) and TNF treatment. D) Quantification of cell number in wound area was assessed by NIH Image J analysis as described in Methods. Note a significant (*p<0.03) increase in migration of tmTNF cells as compared to control cells. Of note this increase in migration is significantly (**p<0.01) inhibited by SB inhibitor. TNF treatment had no significant (#p>0.05) effect on migration in both control and tmTNF expressing cells.
Figure 6. Schematic diagram of involvement of p38 MAPK activation in acute and chronic activation of endothelial cells. Chronic endothelial cell activation involving the two TNF receptors and gp91 phox expression leading to enhanced NADPH Oxidase dependent ROS formation\(^{(28)}\) and p38 MAPK activation (our results) in comparison to acute stimulation with soluble TNF. Of note, p38 MAP kinase in turn activate the NADPH Oxidase complex suggesting an autocrine loop\(^{(37)}\). Our demonstration of a p38 MAP kinase dependent increased endothelial migration in tmTNF cells may be a mechanism leading to enhanced angiogenesis as previously reported\(^{(43)}\).
### Table 1

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<td>Thrombospondin 1</td>
<td>13.4</td>
<td>0.02</td>
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<td><strong>Anti-angiogenic Genes</strong></td>
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<td>Serpine2</td>
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<td>Serine (or cysteine) peptidase inhibitor, clade E, member 2</td>
<td>50.7</td>
<td>0.01</td>
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<td>Arts1</td>
<td>1416942_at</td>
<td>Type 1 TNF receptor shedding aminopeptidase regulator</td>
<td>3.0</td>
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<td><strong>Pro-angiogenic Genes</strong></td>
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<td><strong>Cell adhesion Genes</strong></td>
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<td>Sele</td>
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<td>E-Selectin</td>
<td>82.5</td>
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<td>Icam1</td>
<td>1424067_at</td>
<td>Intercellular adhesion molecule 1</td>
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<td>Vcam1</td>
<td>1436003_at</td>
<td>Vascular cell adhesion molecule 1</td>
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<td><strong>Chemokine/Cytokine Genes</strong></td>
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<td>Ccl2</td>
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<td>Chemokine (C-C motif) ligand 2 (MCP-1)</td>
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<td>Ccl5</td>
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<td>Ccl7</td>
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<td>Cxcl12</td>
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<td>Cxcl7</td>
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<td>Cybb</td>
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<td>gp91 Phox (cytochrome b-245, beta polypeptide)</td>
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<td>Superoxide dismutase 2</td>
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<td>Mapk1</td>
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<td>Rela</td>
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<td>v-rel reticulumendotheliosis viral oncogene homolog A (p65 NfXb)</td>
<td>1.74</td>
<td>0.009</td>
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</tbody>
</table>

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Figure 1

A

B

Soluble TNF

Cellular

TNFRI acute activation

TNFRII chronic activation

Ctrl + TNF

1719

tmTNF

187

896

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Figure 3

A

[Graph showing % Normalized Expression Level (Arbitrary Units) for ENA-78, NFkB, and GAPDH under Control, tmTNF, and TNF conditions.]

B

[Graph showing % Normalized Expression Level (Arbitrary Units) for TSP-1 and GAPDH under TNF, Control, and tmTNF conditions.]

C

[Graph showing % Normalized Expression Level (Arbitrary Units) for gp91Phox and GAPDH under Control, tmTNF, and TNF conditions.]

D

[Graph showing RFU x 10^4 Mean ± SD under Control, Control + TNF, and tmTNF conditions.]
Figure 4

A

B
Figure 5

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A

Normalized Phosphorylation Levels (Arbitrary Units)

B

Normalized Phosphorylation Levels (Arbitrary Units)

C

Cell Count/Wound Area Mean±SD

---48hr After Wound---
Figure 6

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