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Divergent and convergent effects on gene expression and function in acute versus chronic endothelial activation

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¹Departments of Cellular and Integrative Physiology, ²Indiana Center for Vascular Biology and Medicine, ³Department of Biochemistry and Molecular Biology and Center for Medical Genomics, Indiana University School of Medicine, Indianapolis, Indiana; ⁴Max Planck Institute, Bad Nauheim, Germany; and ⁵Roudebush Veterans Administration Medical Center, Indianapolis, Indiana

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Rajashekhar G, Grow M, Willuweit A, Patterson CE, Clauss M. Divergent and convergent effects on gene expression and function in acute versus chronic endothelial activation. *Physiol Genomics* 31: 104–113, 2007. First published June 12, 2007; doi:10.1152/physiolgenomics.00157.2006.—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 compared with 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 reactive oxygen species induction occurred only in these cells. Accordingly, p38 and ERK1/2, two MAP kinases downstream of reactive oxygen species, were activated in stable transmembrane-spanning precursor (tm) TNF-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.

tumor necrosis factor; microarray; Genechip; p38 mitogen-activated protein kinase; migration

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 h. 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 (40). Such endothelial cell activation is essential for many physiological and pathological reactions in blood vessels leading to inflammation, vascular remodeling, and aberrant angiogenesis (14, 25, 34). 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 (38). TNF signaling of endothelial adhesion protein upregulation closely resembles what is known as dysfunctional endothelium as it occurs, for instance, in atherosclerotic plaques (38). It essentially involves the nuclear translocation of the NF- κ B transcription factor but also increased reactive oxygen species (ROS) and decreased nitric oxide (NO) production (2, 7). In addition, TNF induces a complex set of MAP kinase activation involving signals in endothelium that affect cell growth (38). 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 (tmTNF) and soluble TNF. In leukocytes, tmTNF is produced in quiescent cells and increased in activated conditions but requires further stimulation and the protease TNF- α -converting enzyme to generate soluble TNF from its tmTNF precursor (3). Importantly, low endogenous levels of tmTNF 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). To address the role of increased endothelial tmTNF 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). To examine the underlying mechanisms in chronic endothelial activation we analyzed gene expression

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Table 1. *Differential gene expression in tmTNF- vs. TNF-activated endothelial cells*

Affy Gene Symbol	Affy Mouse Source ID*	Description	tmTNF α (Chronic)		TNF α (Acute)	
			Fold Change	P	Fold Change	P
Antiangiogenic genes						
Serpine1	1419149_at	serine (or cysteine) peptidase inhibitor (PAI-1)			18.7	0.02
Thbs1	1460302_at	thrombospondin 1			13.4	0.02
Proangiogenic genes						
Serpine2	1416666_at	serine (or cysteine) peptidase inhibitor, clade E, member 2	50.7	0.01		
Arts1	1416942_at	type 1 TNF receptor shedding aminopeptidase regulator	3.0	0.01		
Cell adhesion genes						
Sele	1421712_at	E-selectin			5.41	0.05
Icam1	1424067_at	intercellular adhesion molecule 1	82.5	0.01	15.2	0.01
Vcam1	1436003_at	vascular cell adhesion molecule 1	19.7	0.05	40.9	0.01
Chemokine/cytokine genes						
Ccl2	1420380_at	chemokine (C-C motif) ligand 2 (MCP-1)	4.76	0.04	6.18	0.03
Ccl5	1418126_at	chemokine (C-C motif) ligand 5 (RANTES)	25.65	0.006		
Ccl7	1421228_at	chemokine (C-C motif) ligand 7 (MCP-3)	30.96	0.014		
Cxcl12	1439084_at	chemokine (C-X-C motif) ligand 12 (SDF-1 α)			28.2	0.02
Cxcl5	1419728_at	chemokine (C-X-C motif) ligand 5 (ENA-78)	92.1	0.01		
Cxcl7	1418480_at	chemokine (C-X-C motif) ligand 7 (NAP-2)			8.63	0.01
Cxcl11	1419698_at	chemokine (C-X-C motif) ligand 11 (ITAC)	54.54	0.03		
Il12rb1	1418166_at	interleukin 12 receptor, beta 1	3.16	0.03	2.48	0.05
Il7	1422080_at	interleukin 7	4.28	0.01	1.82	0.01
Tlr2	1419132_at	Toll-like receptor 2	9.35	0.01	5.84	0.01
Oxidant genes						
Cybb	1436778_at	gp91 Phox (cytochrome b-245, beta polypeptide)	7.27	0.00		
Sod2	1417194_at	superoxide dismutase 2			1.64	0.04
Signaling genes						
Mapk1	1453104_at	Mitogen-activated protein kinase 1-(ERK-1)	-1.65	0.03		
Irak1bp1	1431771_a_at	interleukin-1 receptor-associated kinase 1 binding protein 1-(SIMPL)	3.55	0.02		
Traf1	1423602_at	TNF receptor-associated factor 1			15.4	0.009
Rela	1419536_a_at	v-rel reticuloendotheliosis viral oncogene homolog A (p65 NF- κ B)			1.74	0.009

RNA expression profiles were determined by oligonucleotide array hybridization in 3 independent experiments as described in METHODS. Shown are representative differentially regulated angiogenic, cell adhesion, chemokine/cytokine, oxidant, and signaling genes. *Where there were more than one probe set found, a representative set is shown.

changes in mouse microvascular endothelial cells stably transfected with tmTNF compared with 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 wild-type newborn mice carrying the noncleavable transmembrane mutant form of murine TNF and mock-transfected control cells (43) were cultured in DMEM supplemented with 10% FCS, 2 mM glutamine, and 1 \times penicillin/streptomycin. Cells were transfected with the noncleavable transmembrane mutant form of murine TNF [mTNF Δ 1-9,K(11)E (9)] by retroviral gene transfer: the cDNA for mTNF Δ 1-9,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 8 μ g/ml polybrene were used for infection of endothelial cells for 3 h. Infected endothelial cells were selected with gentamicin-containing medium (1 mg/ml). Fluorescence-activated cell sorting (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, 20 ng/ml) for 4 h. 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 overexpressing endothelial cells were subjected to RNA isolation. To obtain statistical confidence in the data set generated for the microarray hybridization experiments, at least four independent replicates of RNA isolated from at least three independent clones were used for each hybridization reaction. At least 10 μ g 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 were performed at the Center for Medical Genomics, 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.¹

Quantitative real-time RT-PCR. 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 (20 ng/ml, 4 h) 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 (Bio-Rad) was used for cDNA synthesis and PCR amplification as per the manufacturer's instructions using gene-specific primer pairs (Please see online Supplementary Table S3). The amount of target gene transcript normalized to the endogenous β -microglobulin housekeeping gene transcript was computed based on a comparative cycle threshold method as published

¹ The online version of this article contains supplemental material.

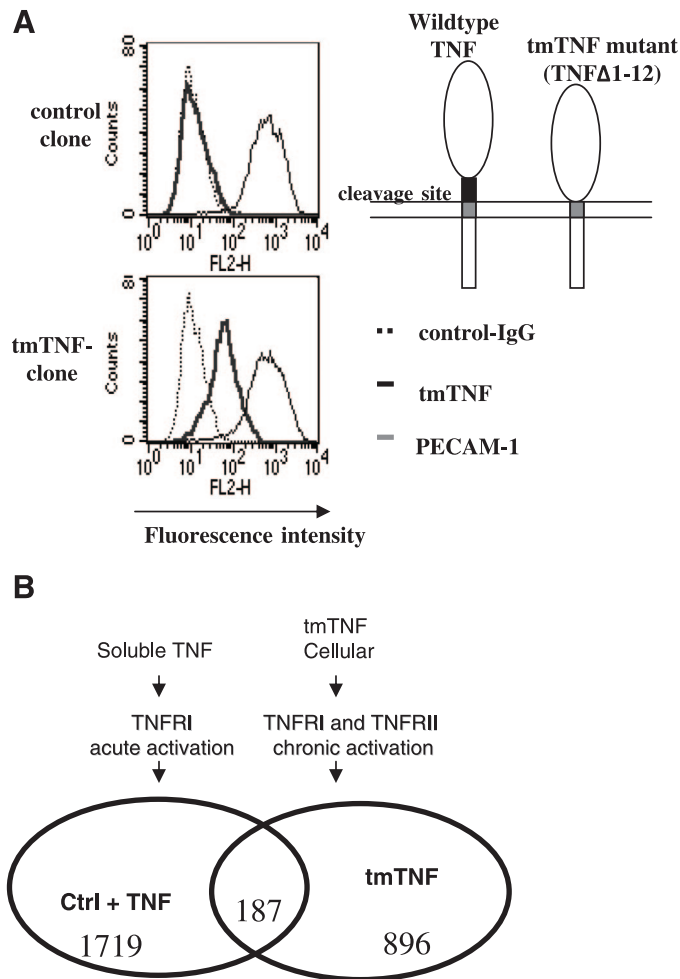


Fig. 1. Overexpression of transmembrane-spanning precursor of TNF (tmTNF) in endothelial cells leads to distinct expression changes and partial overlap with changes induced by acute stimulation with soluble TNF. **A**: a noncleavable transmembrane mutant (TNFΔ1-12) form of murine TNF was introduced into 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 (dark histogram, clone V1q; kind gift of W. Falk, University of Regensburg, Regensburg, Germany) and visualized by incubation with a phycoerythrin-coupled secondary antibody. Fluorescence-activated cell sorting analyses clearly indicated tmTNF overexpression in tmTNF clones compared with control wild-type clones. Platelet endothelial cell adhesion molecule (PECAM)-1 was used as a positive control to demonstrate endothelial phenotype of these cells (gray histogram). **B**: schematic overview of the identified differentially expressed genes (at $P < 0.05$ compared with control cells without treatment) demonstrates that only 187 probe sets were shared as hits between the 2 treatments. Ctrl, control; TNFRI, tumor necrosis factor receptor I; TNFRII, tumor necrosis factor receptor II.

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 confluence, detached with EDTA buffer, and incubated with mouse monoclonal phycoerythrin-labeled anti-ICAM-1 (CD54) or FITC-labeled anti-VCAM-1 (CD106) or mouse monoclonal anti-E-selectin antibodies (R&D Systems) in phosphate buffer supplemented with 2.5% FCS and 0.02% sodium azide for 30 min at 4°C. After being washed, 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, we probed the whole cell extracts from two independent control, control treated with TNF, and three independent tmTNF endothelial cell populations for the presence of ENA-78 (Abcam), thrombospondin-1 (TSP-1, Abcam), gp91^{phox} (BD Biosciences), phospho-p44/42 MAPK (ERK1/2), phospho-p38 MAPK (both from Cell Signaling), and NF-κB (Chemicon) proteins as per the standard procedure described earlier (32, 42). For MAPK phosphorylation studies cells were serum-starved for 4 h and then either treated or untreated with soluble cytokines for 30 min for p38 and 10 min 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, 5-(6)-Carboxy-2',7'-dichlorodihydrofluorescein diacetate by intracellular esterases, and oxidation to the nonfluorescent derivative, 2',7'-dichlorofluorescein (43).

In vitro sprout formation assay. Angiogenesis in vitro was tested as described previously (43). In brief, 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 h, and fixed, and the number of sprouts per 50 beads longer than the average bead size was counted under a phase contrast microscope.

In vitro endothelial cell migration and image analysis. Endothelial cell 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. We subjected the confluent monolayer cells to wound injury with a yellow tip and washed unbound cells, and phase contrast micrograph pictures were taken and labeled as 0 h. Cells were plated with fresh media with and without factors of interest and were cultured for 48 h. We monitored the endothelial cell migration in culture every 12 h by taking at least three different images from each well along the wound with a digital camera under a phase contrast microscope (×10). The cell count within the wound area was measured with NIH Image software 1.6 offline, and results were computed for statistical analysis.

Statistical analysis. For microarray data two-way comparisons of control and experimental groups were performed by 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 < 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 means \pm 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. 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 h) control cells. Assessment of differential gene expression revealed 1,054 probe sets representing 896 genes significantly regulated (at $P < 0.05$) by

stable tmTNF expression compared with control cells, whereas activation with TNF resulted in differential expression detected by 2,026 probe sets representing 1,719 genes. Of these hits, only 187 probe sets were shared as hits between the two treatments (Fig. 1). The complete microarray dataset may be found under the ID number GSE4518 in the Gene Expression Omnibus 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 S2 (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 antiangiogenic genes were regulated both in continuously and acutely activated endothelium. Similar observations 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 fivefold. VCAM-1 was expressed more strongly in acutely than in chronically 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 [monocyte chemoattractant protein (MCP)-1 and interferon- γ -inducible protein-10], two were exclusively regulated by acute TNF and two were only by long-term stimulation (Table 1, see also Table S2

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, gp91^{phox}, in tmTNF-expressing cells, whereas the antioxidant superoxide dismutase-2 was upregulated only in acutely stimulated cells, suggesting that continuous endothelial activation causes pro-oxidative changes. Addressing signaling molecules, we found MAPK1 (ERK1), 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 TNF- α receptor-associated factor-1 appear to be also upregulated in acutely TNF-activated cells (see Table 1, also see Table S2 in supplemental data). Although no further specific changes in either acutely or continuously activated endothelial cells observed, there is a 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, we analyzed several different relationship pathways using PathwayArchitect 1.0.3 software (Stratagene) by loading select sets of significant "hits" from the microarray data. As shown in the 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 12 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 qPCR 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

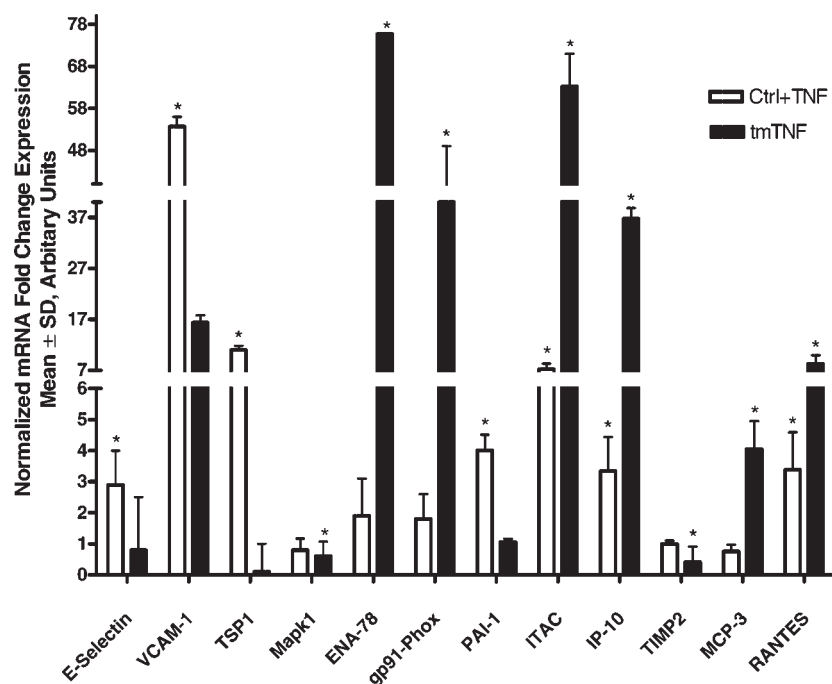


Fig. 2. Confirmation of microarray analysis of representative genes by real-time RT-PCR. For quantitative real-time PCR total cellular RNA was isolated from individual cultured cells and assessed using the iScript one-step qRT-PCR kit with gene-specific primer pairs. The amount of target gene transcript normalized to the endogenous β -microglobulin housekeeping gene transcript was 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 3 independent experiments with duplicates and from 3 different clones for each cell type. *Significant values.

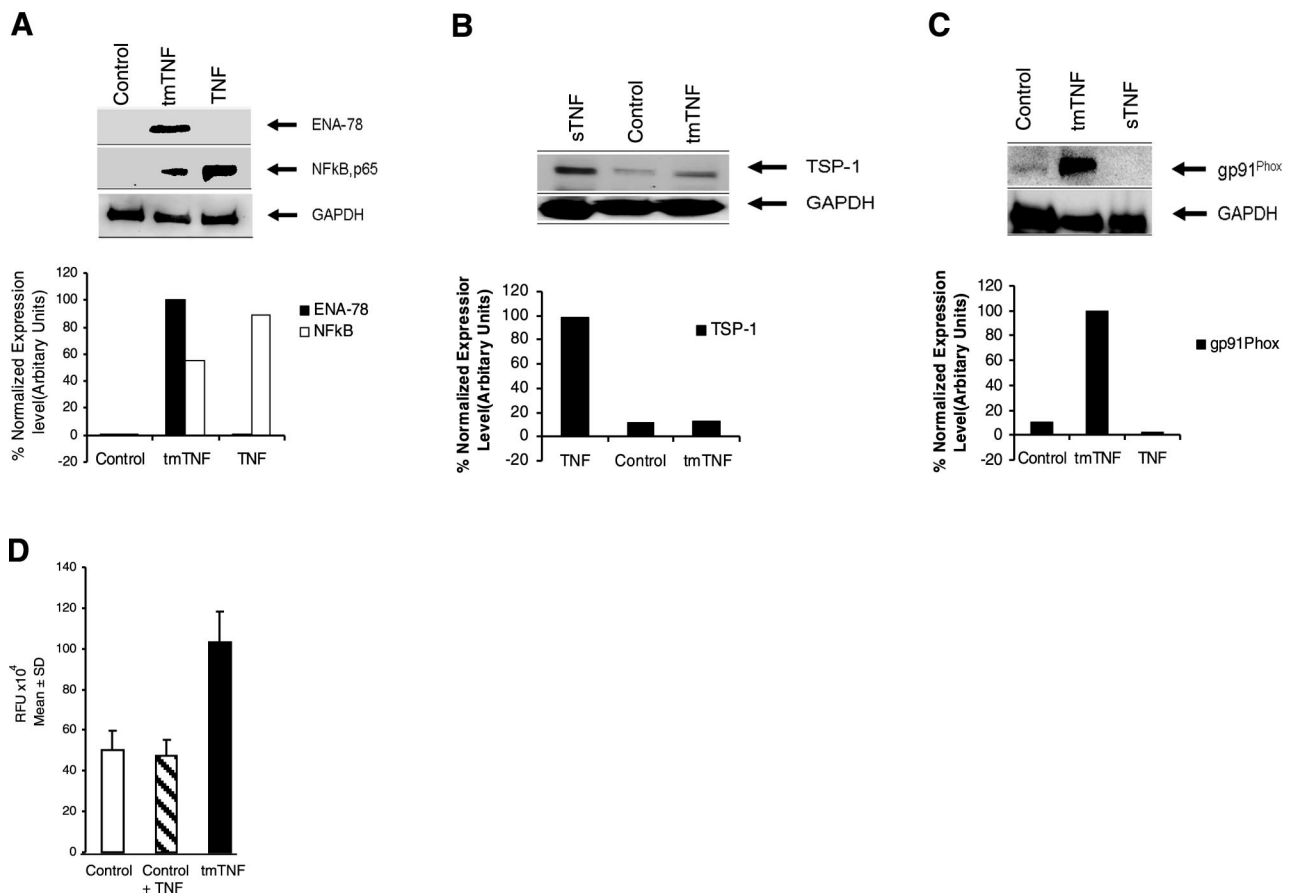


Fig. 3. Confirmation of microarray analysis of representative genes by Western analysis. Immunoblot analysis was performed as described in METHODS with anti-ENA-78 (A), anti-NF- κ B (A), anti-thrombospondin (TSP)-1 (B), and anti-gp91^{Phox} (C) stripped and reprobated with GAPDH. Quantification of fold change in protein expression was analyzed by NIH Image J densitometric analysis software. Data shown are from a representative experiment and repeated at least 3 times with similar results. D: increased reactive oxygen species (ROS) in tmTNF-expressing endothelial cells. Confluent control or tmTNF endothelial cells were tested for ROS production as measured by 2',7'-dichlorofluorescein oxidation. ROS were increased in tmTNF cells (closed bar) compared with 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 were repeated at least 2 additional times.

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 NF- κ B is upregulated in both soluble TNF-treated and tmTNF-expressing cells (Fig. 3A). In addition, we confirmed upregulation of TSP-1, an inhibitor of angiogenesis, in acutely TNF-treated cells (Fig. 3B). Furthermore, a remarkable strong NADPH oxidase subunit gp91^{Phox} 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 gp91^{Phox} we assessed ROS generation. As shown in Fig. 3D, ROS generation after 4 h of incubation with transiently TNF-stimulated cells is only marginally increased, whereas in tmTNF cells a significant effect was observed.

Desensitization of tmTNF overexpression toward 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 compared with control cells, while 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 them with soluble TNF-treated control cells. We embedded endothelial cells grown on microbeads into a fibrin gel and let three-dimensional sprouts form in the presence or absence of TNF. Significantly more sprout formation was found in tmTNF-expressing endothelial

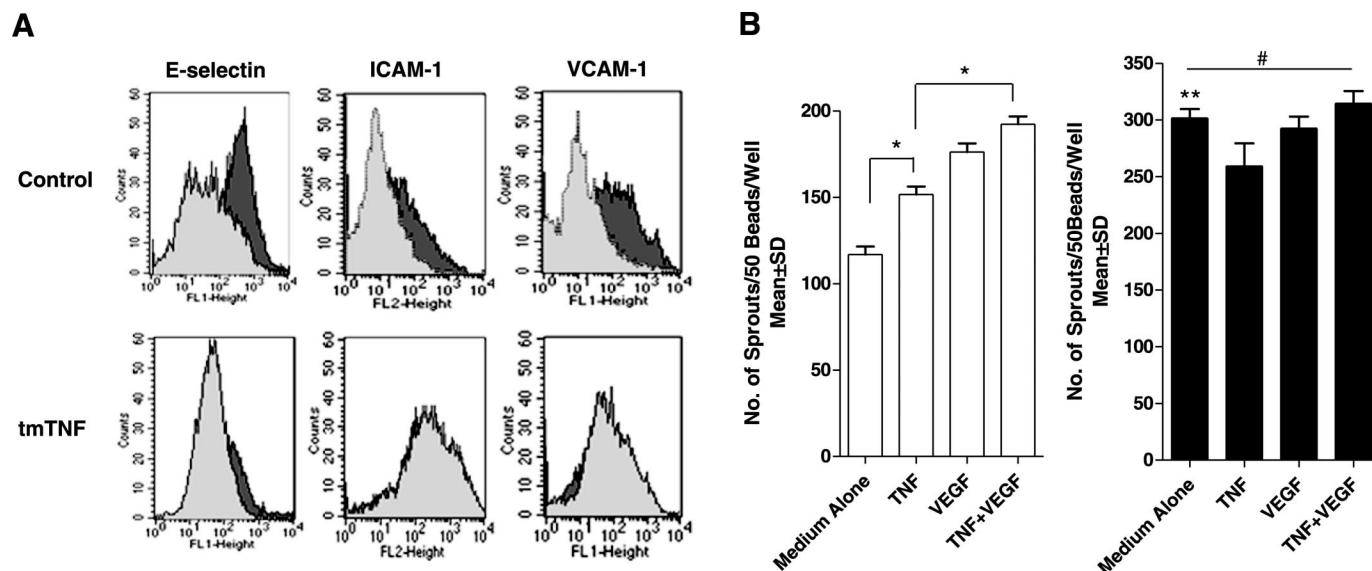


Fig. 4. Divergence of acute stimulation from tmTNF overexpression toward stimulation with soluble TNF. *A*: differential expression of ICAM-1, VCAM-1, and E-selectin in tmTNF-expressing vs. control endothelial cells treated with TNF. Shown are FACS analyses of tmTNF-expressing endothelial cells and control cells before (light histogram) and after treatment (gray histogram) with TNF for 4 h. 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 vs. 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 compared with control cells that was not increased further in the presence of TNF or VEGF ($\#P > 0.05$). 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 4 times independently with similar results.

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 toward stimulation with soluble TNF. Based on the role of p38 and ERK1/2 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 tmTNF- α -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 also leads 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

coincubation 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 with the acute activation. We decided to address acute activation by use of short-term treatment with soluble TNF (4 h) 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 S2). It may be of concern that some genes previously reported to respond to TNF are not found as significant hits in our data.

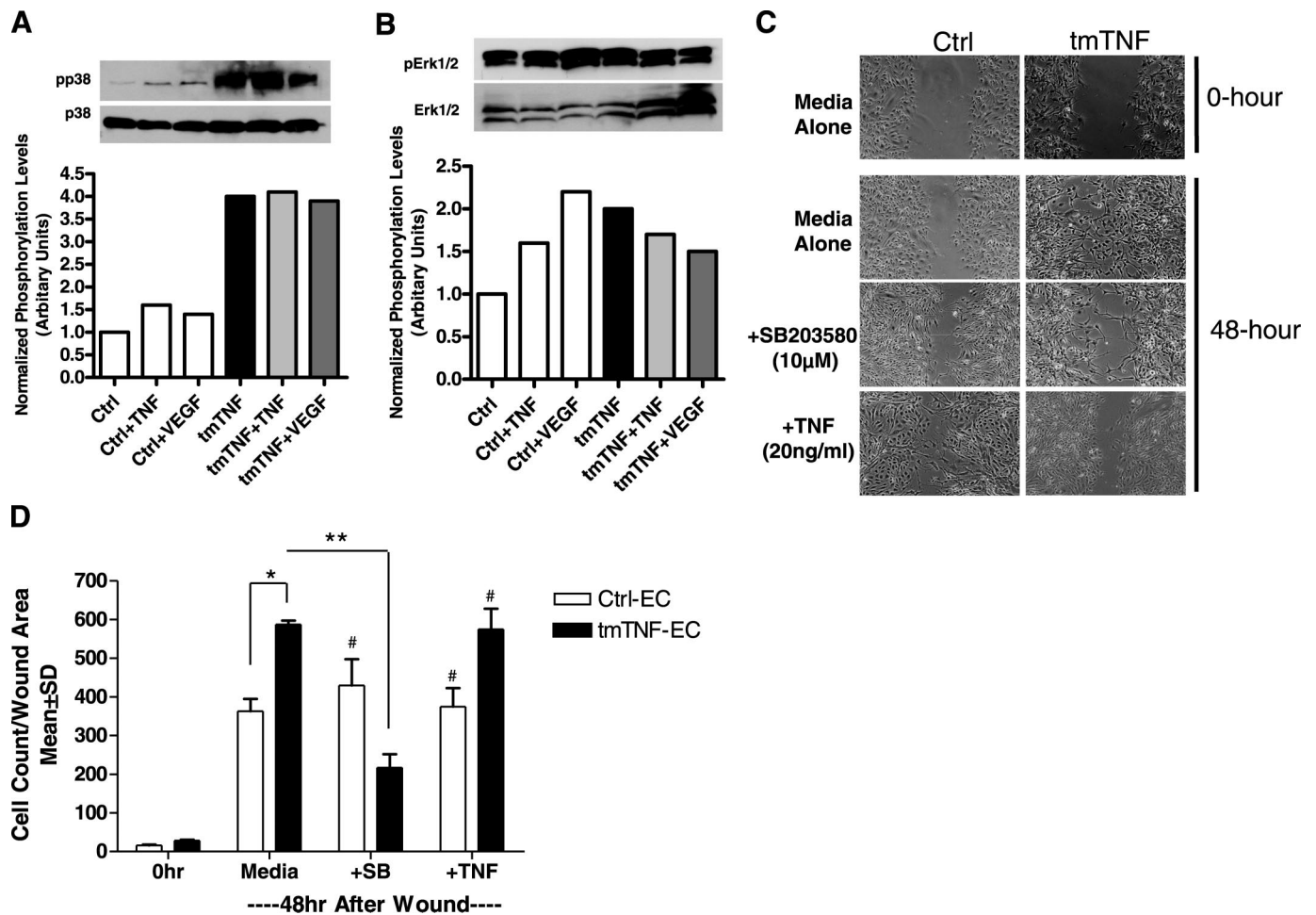


Fig. 5. Increased p38 MAPK activation in tmTNF-expressing cells causes increased migratory activities in these cells. Immunoblot analysis was performed as described in METHODS with antiphospho-p38 (A) or antiphospho-ERK1/2 (B), stripped, and reprobbed with p38 and ERK1/2 antibodies. Quantification of 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 3 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-h) and 48-h postwound for both control and tmTNF-expressing cells with and without p38 MAP kinase inhibitor [SB-203580 (SB)] 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 compared with 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.

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 further contributing to the differences in response to TNF, and 4) mouse endothelial cells used here responding differently from 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 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 upregulated and VCAM-1 was only moderately up-

regulated, 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 as NAP-2 (9-fold), MIP-1 γ (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 TNFR1 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 contin-

uous 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.

In the study of 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, gp91^{phox}. Of interest, the antioxidant 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 atherosomas, 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). On the basis of 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 ERK1/2 MAPK activation. We demonstrated prominent p38 and to a lesser extent also ERK1/2 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 (Figs. 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 also may 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), 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 IFN-inducible T-cell α -chemoattractant (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 gp91^{phox} expression leading to ROS production, which activates MAP kinases and then may feed back to NADPH oxidase as depicted in Fig. 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^{phox} levels, increased p38 MAPK kinase activation, and migration, which is highlighted in the scheme of Fig. 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 being predominately upregulated in continuous activation, resulting in significant

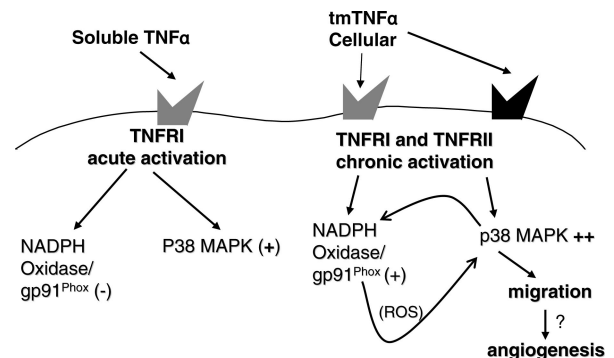


Fig. 6. Schematic diagram of involvement of p38 MAPK activation in acute and chronic activation of endothelial cells. Chronic endothelial cell activation involving the 2 TNF receptors and gp91^{phox} expression leading to enhanced NADPH Oxidase-dependent ROS formation (28) and p38 MAPK activation (our results) compared with acute stimulation with soluble TNF. Of note, p38 MAPK kinase in turn activates the NADPH oxidase complex, suggesting an autocrine loop (37). Our demonstration of a p38 MAPK kinase-dependent increased endothelial migration in tmTNF cells may be a mechanism leading to enhanced angiogenesis as previously reported (43).

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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