PLoS one

Similar NF-κB Gene Signatures in TNF-α Treated Human Endothelial Cells and Breast Tumor Biopsies

Martine Perrot-Applanat[1,2]*, Sophie Vacher[3], Aurore Toullec[1,2], Irma Pelaez[1,2], Guillaume Velasco[1,2], Françoise Cormier[4,5,6], Hanan El Sheikh Saad[1,2], Rosette Lidereau[3], Véronique Baud[4,5,6], Ivan Bièche[3,6]

1 INSERM U965, Paris, France, 2 Université Paris-Diderot, Sorbonne Paris Cité, Paris, France, 3 INSERM U735, Institut Curie - Hôpital René Huguenin, FNCLCC, Saint-Cloud, France, 4 INSERM U1016, Institut Cochin, Paris, France, 5 CNRS UMR8104, Paris, France, 6 Université Paris Descartes, Paris, France

Abstract

Background: Endothelial dysfunction has been implicated in the pathogenesis of diverse pathologies ranging from vascular and immune diseases to cancer. TNF-α is one of the mediators of endothelial dysfunction through the activation of transcription factors, including NF-κB. While HUVEC (macrovascular cells) have been largely used in the past, here, we documented an NF-κB gene signature in TNFα-stimulated microvascular endothelial cells HMEC often used in tumor angiogenesis studies.

Methodology/Principal Findings: We measured mRNA expression of 55 NF-κB related genes using quantitative RT-PCR in HUVEC and HMEC. Our study identified twenty genes markedly up-regulated in response to TNFα, including adhesion molecules, cytokines, chemokines, and apoptosis regulators, some of them being identified as TNF-α-inducible genes for the first time in endothelial cells (two apoptosis regulators, TNFAIP3 and TNFRSF10B/Trail R2 (DR5), the chemokines GM-CSF/CSF2 and MCF/CSF1, and CD40 and TNF-α itself, as well as NF-κB components (*RELB*, *NFKB1*or *50/p105* and *NFKB2* or *p52/p100*). For eight genes, the fold induction was much higher in HMEC, as compared to HUVEC. Most importantly, our study described for the first time a connection between NF-κB activation and the induction of most, if not all, of these genes in HMEC as evaluated by pharmacological inhibition and RelA expression knock-down by RNA interference. Moreover, since TNF-α is highly expressed in tumors, we further applied the NF-κB gene signature documented in TNFα-stimulated endothelial cells to human breast tumors. We found a significant positive correlation between *TNF* and the majority (85 %) of the identified endothelial TNF-induced genes in a well-defined series of 96 (48 ERα positive and 48 ERα negative) breast tumors.

Conclusion/Significance: Taken together these data suggest the potential use of this NF-κB gene signature in analyzing the role of TNF-α in the endothelial dysfunction, as well as in breast tumors independently of the presence of ERα.

Introduction

Tumor necrosis factor (TNF-α) is a potent multi-functional cytokine produced by various cells, including neutrophils, activated macrophages, T and natural killer cells, and fibroblasts, in response to inflammation, infection, tissue injury and other environmental challenges. TNF-α elicits a large number of biological activities including the control of cell proliferation, differentiation and apoptosis, and is an important mediator of inflammation and immunity [1]. Considerable progress has been made in understanding the molecular mechanisms that mediate TNF-α-induced cellular responses. TNF-α binds to membrane receptors, TNFR1 (or TNF-Rp55) and TNFR2 (or TNF-Rp75), which initiate signalling pathway leading to gene induction *via* activation of transcription factors [2–3]. TNFR1 is ubiquitously expressed, while TNFR2 expression is restricted to endothelial and hematopoietic cells [4].

Through complex signaling cascades and networks, signal transducers lead to the activation of transcription factors (NF-κB, AP-1 and SP1) [2] and their binding to the promoters of specific genes.

The transcription factor NF-κB is a key inducible transcription factor which regulates numerous genes controlling inflammation, immune response, cell growth, tissue differentiation and apoptosis [5–7]. NF-κB-activating signals emanate from cell surface receptors for inflammatory cytokines (such as TNF-α, IL-1, or LPS), growth factors, lymphokines, reactive oxygen intermediates, viral infection, and B or T cell activation [5]. NF-κB factors are homo- and heterodimeric transcription factors that belong to the Rel family; this family is composed of five homologous subunits in mammals: NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p100), RelA/p65, RelB and c-Rel. Biological activity mediated by NF-κB occurs through two main signalling pathways,

the classical and the alternative pathways, the former being specifically induced in response to TNF-α, whereas the latter is induced by other specific stimuli [6–8]. In the classical pathway, the inactive NF-κB complexes are sequestered in the cytoplasm through interaction with specific inhibitors, the IκB proteins (IκBα, IκBβ, especially). Following stimulation by TNF-α, activation of the IκB kinase (IKK) complex leads to the degradation of IκB proteins and subsequent release of mainly RelA/p50 dimers, which subsequently translocate into the nucleus where they bind to specific sites within the promoter of specific target genes. Based on studies on fibroblasts and B cell maturation, biological activity mediated by NF-κB may also occur through alternative pathway involving the activation of IKKα (IKK1) and the activation of RelB/p52 and RelB/p50 dimers [9–11].

Endothelial dysfunction has been implicated in the pathogenesis of diverse pathologic processes ranging from vascular diseases to cancer and metastasis. TNF-α mediated endothelial changes are relevant to acute inflammation, a highly complex biologic cascade-involving cytokines (such as IL6), chemokines IL-8 and MCP-1, and the induction of adhesion molecules (VCAM, ICAM and E selectin) [12–17] that recruit and activate granulocytes, mono-cytes/macrophages, and lymphocytes at the damaged tissue site. NF-κB often plays a major role in these pathways [17–18]. The diversity of the vascular bed is crucially determined by the type of endothelial cells which can be divided into microvascular and macrovascular endothelial cells. Although many reports have addressed the biological responses of human macrovascular endothelial cells (HUVEC) to TNF-α[14–15,19], the detailed dissection of the coordinated expression of TNF-α-inducible genes via NF-κB has not been performed in human microvascular endothelial cells (HMEC).

In this study, we have determined the expression profile of 55 selected key genes in TNF-α-stimulated HMEC, that are all known as being regulated by NF-κB in other cell types in response to various stress responses [20–21]. HMEC were chosen, in addition to HUVEC which have been largely used in the past, because they originate from the microcirculation, their varying thresholds to cytokines have been previously described and they are often used in tumor angiogenesis studies. Using real-time reverse transcription (RT)-PCR, we demonstrated that 36.4 % (20/55) genes are markedly up-regulated (>5-fold) by TNF-α in a dose- and time-dependent pathway in HMEC. Using two NF-κB inhibitors and a direct approach of RelA expression knock-down by RNAi, we demonstrated that the gene signature observed in TNF-α stimulated HMEC is NF-κB-dependent. Intracellular signalling pathways triggered by TNF-α leading to numerous tumors have been partially delineated, with NF-κB playing an important role especially in breast cancer cells [20–25]. However, the stroma of a solid tumor is vital for its survival, and key components in this respect are the blood vessels. We further demonstrated that tumors producing high amount of TNF-α could have similar dysregu-lated NF-κB endothelial pathways.

Materials and Methods

Cell culture experiments

Human microvascular endothelial cells (HMEC) from derma (ATCC, Molsheim, France) were cultured in MCDB 131 medium (Gibco) supplemented with 10 % FBS, hydrocortisone (1 µg/ml; Sigma), epidermal growth factor (0.5%; Sigma), glutamine 2 mM, 100 U/ml penicillin and 100 µg/ml streptomycin. Human umbilical vein endothelial cells (HUVEC) were isolated with Collagenase I [26]. Cells were cultured on 0.2% gelatin-coated dishes in Medium 199 (M199) supplemented with 20% FBS, 2 mM glutamine, 100 µg/ml streptomycin, 15 mM of HEPES, and 0.4% ECGS (Promocell, Heidelberg, Deutschland). Second passaged cells and mix of three cords were used for all experiments. Cell cultures were incubated at 37°C in a humid atmosphere of 5 % CO2-95 % air. Once cells had grown to 80 % confluence, TNF-α (R&D Systems; Minneapolis, MN) was added into the medium at the indicated concentrations (1–80 ng/ml). The NF-κB inhibitors, Bay 11–7082 (10 µM; Calbiochem) [27] and the IκKβ kinase (IKK-2) inhibitor V (3,5-bis-trifluoromethyl-phenyl)5-cloro-2-hydroxybenzamide, Calbiochem) [28] were add-ed in some experiences.

Table 1. Characteristics of the 48 ERα-positive and the 48 ERα-negative breast tumors.

	ERα-positive breast tumors			ERα-negative breast tumors		
	Number of patients	Number of relapses (%)	p-value[a]	Number of patients	Number of relapses (%)	p-value[a]
Age						
≤70	27	14 (51.9)	NS	40	17 (42.5)	NS
>70	21	10 (47.6)	(0.39)	8	2 (25.0)	(0.51)
SBR histological grade[b]						
I + II	38	16 (42.1)	NS	6	5 (83.3)	NS
III	10	8 (80.0)	(0.13)	39	14 (35.9)	(0.058)
Lymph node status						
≤3	32	13 (40.6)	**0.023**	41	16 (39.0)	NS
>3	16	11 (68.7)		6	3 (50.0)	(0.96)
Macroscopic tumor size						
≤30 mm	32	14 (43.8)	NS	27	13 (48.1)	NS
>30 mm	16	10 (62.5)	(0.076)	20	6 (30.0)	(0.15)

[a]Log-rank test. NS: not significant.
[b]Scarff Bloom Richardson classification.
doi:10.1371/journal.pone.0021589.t001

Table 2. List of the 55 selected genes tested.

Gene symbols	Protein symbols	Gene name	Chromosome location	Genbank accession number
NFKB genes (n = 10)				
NFKB1	p50, p105	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	4q24	NM_003998
NFKB2	p52, p100	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2	10q24	NM_002502
REL	C-Rel	v-rel reticuloendotheliosis viral oncogene homolog	2p13-p12	NM_002908
RELA	p65, NFKB3	v-rel reticuloendotheliosis viral oncogene homolog A (p65)	11q13	NM_021975
RELB	I-REL	v-rel reticuloendotheliosis viral oncogene homolog B	19q13.32	NM_006509
CHUK	IκKα	Conserved helix-loop-helix ubiquitous kinase	10q24-q25	NM_001278
IKBKB	IκKβ	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	8p11.2	NM_001556
IKBKG	IκKγ, Nemo	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma	Xq28	NM_003639
NFKBIA	IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	14q13	NM_020529
NFKBIB	IκBβ	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta	19q13.1	NM_002503
Apoptosis (n = 12)				
BCL2A1	BFL1/A1	Baculoviral IAP repeat-containing 2	15q24.3	NM_004049
GADD45B		Growth arrest and DNA-damage-inducible, beta	19p13.3	NM_015675
TNFRSF10B	TRAILR2	Tumor necrosis factor receptor superfamily, member 10b	8p22-p21	NM_003842
FASLG	TNFSF6	Fas ligand (TNF superfamily, member 6)	1q23	NM_000639
BIRC4	XIAP	Baculoviral IAP repeat-containing 4	Xq25	NM_001167
TNFAIP3	A20	Tumor necrosis factor, alpha-induced protein 3	6q23	NM_006290
TRAF2		TNF receptor-associated factor 2	9q34	NM_021138
IER3S		Immediate early response 3, large transcript	6p21.3	NM_003897
IER3L		Immediate early response 3, short transcript	6p21.3	NM_052815
BIRC2	c-IAP1	Baculoviral IAP repeat-containing 2	11q22	NM_001166
MCL1S		Myeloid cell leukemia sequence 1 (BCL2-related), short transcript	1q21	NM_182763
MCL1L		Myeloid cell leukemia sequence 1 (BCL2-related), large transcript	1q21	NM_021960
Immune response (n = 12)				
IL1A		Interleukin 1, alpha	2q14	NM_000575
IL1B		Interleukin 1, beta	2q14	NM_000576
IL6		Interleukin 6 (interferon, beta 2)	7p21	NM_000600
IL12B		Interleukin 12B (natural killer cell stimulatory factor 2)	5q31.1-q33.1	NM_002187
CCL2	MCP-1	Chemokine (C-C motif) ligand 2	17q11.2-q21.1	NM_002982
TNFRSF11A	RANK	Tumor necrosis factor receptor superfamily, member 11a, activator of NFKB	18q22.1	NM_003839
TNFSF11	RANKL	Tumor necrosis factor (ligand) superfamily, member 11	13q14	NM_003701
TNF		Tumor necrosis factor (TNF superfamily, member 2)	6p21.3	NM_000594
TNFRSF1A	TNFR1	Tumor necrosis factor receptor superfamily, member 1A	12p13.2	NM_001065
TNFRSF1B	TNFR2	Tumor necrosis factor receptor superfamily, member 1B	1p36.3-p36.2	NM_001066
CD40		CD40 antigen (TNF receptor superfamily member 5)	20q12-q13.1	NM_001250
CD40LG		CD40 ligand (TNF superfamily, member 5, hyper-IgM syndrome)	Xq26	NM_000074
Cell Proliferation (n = 7)				
CSF1	MCSF	Colony stimulating factor 1 (macrophage)	1p21-p13	NM_000757
CSF1R		Colony stimulating factor 1 receptor, v-fms oncogene homolog	5q33-q35	NM_005211
CSF2	GMCSF	Colony stimulating factor 2 (granulocyte-macrophage)	5q31.1	NM_000758
CCND1		Cyclin D1 (PRAD1 : parathyroid adenomatosis 1)	11q13	NM_053056
CCND2		Cyclin D2	12p13	NM_001759
CCND3		Cyclin D3	6p21.3	NM_001760
CCNG1		Cyclin G1	5q32-q34	NM_004060
Tumor progression (n = 9)				
MMP9		Matrix metalloproteinase 9 (gelatinase B, 92kDa type IV collagenase)	20q11.2-q13.1	NM_004994
MMP11		Matrix metalloproteinase 11 (stromelysin 3)	22q11.23	NM_005931
PLAU	UPA	Plasminogen activator, urokinase	10q24	NM_002658

Table 2. Cont.

Gene symbols	Protein symbols	Gene name	Chromosome location	Genbank accession number
CXCR4		Chemokine (C-X-C motif) receptor 4	2q21	NM_003467
CXCL12	SDF1	Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	10q11.1	NM_000609
ICAM1		Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	19p13.3-p13.2	NM_000201
VCAM1		Vascular cell adhesion molecule 1	1p32-p31	NM_001078
SELE	ELAM1	Selectin E (endothelial adhesion molecule 1)	1q22-q25	NM_000450
TNC	HXB	Tenascin C (hexabrachion)	9q33	NM_002160
Angiogenesis (n = 4)				
IL8		Interleukin 8	4q13-q21	NM_000584
CXCL1	GRO1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	4q21	NM_001511
VEGF	VEGFA	Vascular endothelial growth factor	6p12	NM_003376
PTGS2	COX2	Prostaglandin-endoperoxide synthetase 2	1q25.2-q25.3	NM_000963
Divers (n = 1)				
EGR1		Early growth response 1	5q31.1	NM_001964

doi:10.1371/journal.pone.0021589.t002

Cell transfections

Subconfluent HMEC were transfected in OPTI-MEM medium (Gibco) with Fugene (4 μg per P30 flask; Roche) containing a construct of firefly luciferase ligated to a NF-κB -sensitive promoter of the gene [29]. Twenty hours later, cells were incubated with Bay 11–7082 (10 μM) and stimulated with TNF-α (40 ng/ml) for 4 h in the presence of the inhibitor, before they were harvested; pellets were analyzed for luciferase activity. Results are given as firefly luciferase activity as compared to TNF stimulation

Transient of siRNA. TNFR1 and TNFR2 were silenced using a specific siRNA mix containing three different siRNAs provided by Santa Cruz Biotechnology, as previously described [30]. As a control, siRNA with a nonsense sequence was usedg. Briefly, 10 μM of siRNA was added to the transfection reagent (Oligofectamine, InVitrogen; 1.25 μg) and subsequently transferred to culture plates P100 flask with 4×10^5/flask HMEC. Then, 24 hours after transfection, cells were stimulated with TNF-α for 4 h and the specific experiment was performed.

Plasmid constructs. pTrip-shRNA RelA was generated by subcloning an oligonucleotide designed to target RelA under control of the H1 promoter into pTRIP-ΔU3-MND-GFP lentiviral vector [31]. pTRIP-shRNA control was generated following the same approach using a scrambled control oligonucleotide. Sequences are available upon request.

Lentiviral infection. Production of infectious recombinant lentiviruses was performed by transient transfection of 293T cells, as previously described [31]. For infections, 10^5 cells in 35-mm dishes were transduced with 5000 ng/ml of p24 (HIV-1 capsid protein). 48 h later, cells were washed and fresh medium was added. The culture was then continued as described above.

Electrophoretic Mobility Shift Assays (EMSA)

Nuclear extracts were prepared and analyzed for DNA binding activity using the HIV-LTR tandem κB oligonucleotide as κB probe [32]. For supershift assays, nuclear extracts were incubated with specific antibodies for 30 min on ice before incubation with the labeled probe. Anti-RelA, RelB, cRel, and p105/p50 supershifting antibodies were purchased from Santa Cruz Biotechnology.

Table 3. Effect of tumor necrosis factor (TNF-α treatment on selected NF-κB gene expression in HMEC cells.

Gene	Mean[a] +/− SEM
TNF	1156+/− 336
SELE	1022+/− 102
VCAM	896+/− 246
CSF2/GMCSF	762+/− 89
IL8	726+/− 91
ICAM	357+/− 103
CCL2/MCP1	75+/− 8,2
CXCL1/GRO1	71+/− 8,3
RELB	63+/− 11,4
TNFAIP3/A20	49+/− 7,5
IL1A	31+/− 0,7
IL1B	26+/− 0,5
BCL2A1	21+/− 2,3
NFKB2	20+/− 1,9
NFKBIA	18+/− 0,8
CSF1	14+/− 1,1
NFKB1	8,7+/− 0,4
CD40	5,3+/− 1,1
IL6	5,3+/− 0,6
TNFRSF10B	5,1+/− 0,1
MKI67	0,9+/− 0,1
ESR1/ERα	NQ[b]

[a]Ratio of the mRNA content in stimulated cells for 4 hours by TNF-α to the mRNA content in the unstimulated cells was calculated. This time was chosen from preliminary kinetic analysis on gene candidates (such as E-selectin and IL-8) (see also Table 4). Values shown are calculated from three independent experiments, each experiment in duplicate.
[b]NQ, not quantifiable: very low levels of target gene mRNA that were only detectable, but not quantifiable, by means of the real-time quantitative RT-PCR assay (Ct values >35).
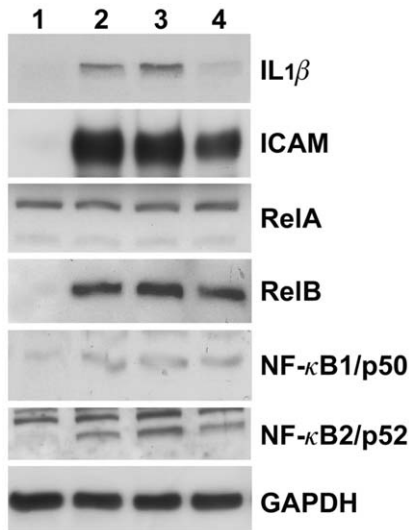doi:10.1371/journal.pone.0021589.t003

Figure 1. Western blot analyses of IL-1β, ICAM, RelA, RelB, NF-κB1/p50 and NF-κB2/p52 proteins in TNF-α stimulated HMEC. HMEC cells were incubated for 4 h with 4–40 ng/ml TNF-α. Whole cell lysates were prepared and subjected to Western blotting using anti-ICAM, anti-IL-1β anti-RelA, anti-RelB, anti-NF-κB1/p50 or anti-NF-κB2/p52 antibodies, as indicated in *Materials and Methods*. As loading controls, total proteins were also analyzed with anti-GAPDH. Each lane contains 50 μg of cellular protein. Lanes 1–4 correspond to control endothelial cells (lane 1), endothelial cells stimulated with 40 ng/ml (lanes 2 and 3) or 4 ng/ml (lane 4) TNF-α. Results were similar in two independent experiments.
doi:10.1371/journal.pone.0021589.g001

Western blotting and Antibodies

Cells were cultured in 100 mm dishes (Falcon) and harvested in lysis buffer (RIPA: PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitors; Santa Cruz). After 30 min incubation on ice, lysates were cleared by centrifugation (12000 rpm, 10 minutes). Samples containing 50 μg of total protein from lysates were separated in an SDS/polyacrylamide gel and transferred onto a nitrocellulose membrane. The rinsed and blocked membrane was then incubated with mouse antibodies against ICAM-1 (Santa Cruz biotechnologies, Inc, Santa Cruz, CA; 1/500), goat anti-IL-1β (R&D 1/500) or anti-GAPDH (Santa Cruz, 1/500), anti-rabbit polyclonal antibodies against RelA, RelB, p50/NF-κB1 or p52/NF-κB2 [32] (1:1000; provided by R Weil and A Israel, Institute Pasteur, Paris), at room temperature for 1 hour. The blot was washed and probed with anti-mouse, -goat, or -rabbit antibodies conjugated with horseradish peroxidase (1:10000; Dako, Glostrup, Denmark) at room temperature for 1 hour. The blot was washed and developed with ECL (Amersham biosciences, Orsay, France).

Patients

To investigate the *in vivo* inter-relationships between mRNA levels of genes of interest, we analyzed 96 (48 ERα positive and 48 ERα negative) breast tumors excised from women at our institution (Centre René Huguenin, St Cloud, France). Samples containing more than 70% of tumor cells were considered suitable for this study. Immediately following surgery, the tumor samples were placed in liquid nitrogen until RNA extraction. The patients met the following criteria: primary unilateral non metastatic breast carcinoma; complete clinical, histological and biological information available; no radiotherapy or chemotherapy before surgery; and full follow-up at our institution. Estrogen receptor alpha status

Table 4. Dose-dependent effect of TNF-α treatment (0–80 ng/ml) on selected NFkB gene expression in HMEC cells.

| Gene | TNF-α concentration | | | | | |
	0.2 ng/ml	1 ng/ml	2.5 ng/ml	10 ng/ml	40 ng/ml	80 ng/ml
TNF	53[a]	225	559	769	1241	1210
SELE	13	65	359	609	1016	1029
VCAM	113	215	610	832	958	937
CSF2/GMCSF	140	314	443	463	714	583
IL8	179	520	691	926	1123	918
ICAM	16	63	114	123	155	139
CCL2/MCP1	16	42	53	55	70	56
CXCL1/GRO1	40	67	76	69	92	71
RELB	30	42	60	55	63	52
TNFAIP3/A20	15	26	25	32	44	32
IL1A	4.6	13	22	25	37	36
IL1B	3.2	7.8	14	14	21	17
BCL2A1	3.3	6.9	14.2	13.8	21	13
NFKB2	6.2	11.4	16	17	20	17
NFKBIA	7.6	10	12	12	19	13
CSF1	4.3	7.8	11	11	13	10
NFKB1	2.4	3.2	3.7	4.1	5.3	4.2
CD40	1.4	1.2	1.7	1.8	5.1	2.5
IL6	1.1	1.9	2.9	3.1	5.7	3.1
TNFRSF10B	2.3	3.2	3.7	3.8	5.1	3.8

[a]Ratio of the mRNA content in cells stimulated with TNF-α for 4 h to the mRNA content in the unstimulated cells.
doi:10.1371/journal.pone.0021589.t004

was determined at the protein level by biochemical method (enzymatic immunoassay) and confirmed by ERα real-time quantitative RT-PCR assay [33]. The malignancy of infiltrating carcinomas was scored according to Scarff Bloom and Richardson's histoprognostic system [34]. The characteristics of these patients are shown in **Table 1**.

RNA extraction

RNA was isolated using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Cergy Pontoise, France). The quality of the RNA samples was determined by electrophoresis through agarose gels and staining with ethidium bromide, and the 18S and 28S RNA bands were visualized under ultraviolet light.

Real-time quantitative-PCR

Transcript quantification of NF-κB selected genes was performed as previously described [35], using the TATA box-binding protein (TBP) as the endogenous RNA control; each sample was normalized on the basis of its TBP content. Primers for TBP and the 55 target genes (**Table 2**) were chosen with the assistance of the Oligo 5.0 computer program (National Biosciences, Plymouth, MN). We searched the dbEST and nr databases to confirm the total gene specificity of the nucleotide sequences chosen as primers, and the absence of single nucleotide polymorphisms. In particular, the primer pairs were selected to be unique relative to the sequences of closely related family member genes or of the corresponding retropseudogenes. To avoid amplification of

Table 5. Kinetics of selected NFkB gene expression in TNF-α treated HMEC.

Gene	Time							
	2 minutes	8 minutes	15 minutes	30 minutes	1 hour	4 hours	24 hours	72 hours
TNF	1.2 [a]	2.0	2.6	5.1	12	1241	335	2.7
SELE	0,9	1,0	1,2	1,2	1,3	1016	58	NQ
VCAM	1,0	1.1	1,2	1.8	27	958	88	6.7
CSF2/GMCSF	1.5	1.2	1.7	19	21	714	219	6.1
IL8	1.5	4.5	11	57	17	1123	331	11
ICAM	0.7	0.8	0.7	0.8	14	155	15	6.5
CCL2/MCP1	0.7	0.7	0.9	1.6	8.6	70	20	3.7
CXCL1/GRO1	1.1	3.2	6.8	11.4	4.2	92	23	2.9
RELB	1.0	1.0	1.0	1.0	25	63	30	18.5
TNFAIP3/A20	0.9	0.9	0.9	2.5	1.7	44	26	3.7
IL1A	0.6	0.6	0.6	0.7	1.0	37	7.6	0.7
IL1B	0.6	0.7	0.6	1.2	3.1	21	15	1.1
BCL2A1	1.1	1.0	1.1	1.1	3.3	21	16	0.9
NFKB2	1.6	1.0	1.6	1.4	8.8	20	12.1	5.7
NFKBIA	0.5	0.6	0.8	1.3	1.2	19	2.0	1.2
CSF1	1.0	0.8	1.0	0.9	3.5	13	5.3	2.9
NFKB1	1.0	0.9	1.1	1.0	1.7	5.0	2.8	1.5
CD40	0.6	0.4	0.6	0.7	1.6	4.7	1.7	1.8
IL6	0.7	0.9	1.0	2.6	1.7	5.4	3.3	1.9
TNFRSF10B	1.0	0.8	1.0	0.9	1.3	5.1	2.1	1.3

[a]Ratio of the mRNA content in cells stimulated with TNF-α (40 ng/ml) for 2, 8, 15, 30 min or 1, 4, 24 and 72 h to the mRNA content in the control cell.
doi:10.1371/journal.pone.0021589.t005

contaminating genomic DNA, one of the two primers was placed at the junction between two exons, if possible. In general, amplicons were between 60 and 120 nucleotides long. Gel electrophoresis was used to verify the specificity of PCR amplicons. For each primer pair we performed no-template control (NTC) and no-reverse-transcriptase control (RT-negative) assays, which produced negligible signals (usually >40 in Ct values), suggesting that primer-dimer formation and genomic DNA contamination effects were negligible. The cDNA synthesis and PCR conditions have been described in detail elsewhere [35]. Results are expressed as N-fold differences in target gene expression relative to TBP gene expression.

Statistical analysis

Statistical analysis of *in vitro* experiments was performed by analysis of mean with the Student t test. The terms n = x is used to indicate the number of independent experiments performed. Correlations between *TNF* and the gene target expressions were tested using the non parametric Spearman rank correlation test (which compared continuous variables). Differences were judged significant at confidence levels greater than 95% ($p < 0.05$).

Results

Identification of TNFα-dependent NF-κB-associated genes in microvascular endothelial cells

To determine the expression pattern of NF-κB-associated genes in response to TNF-α in microvascular endothelial cells, we have performed real-time RT-PCR in HMEC stimulated by TNF-α for

4 h. Our preliminary kinetic analysis on gene candidates (such as E-selectin and IL-8) suggested an induction at 2–4 h time point induction. Among the 55 genes tested (**Table 2**), only one gene, *IL12B*, presented detectable but not reliably quantifiable mRNA expression (Ct>35). Twenty (37 %) of the other 54 genes were up-regulated by 5-fold or more (until 1200-fold) in HMEC stimulated with TNF-α as compared to untreated cells (**Table 3**). These robust selection criteria (a cut-off of 5-fold expression difference in HMEC stimulated with TNF-α) ensure identification of genes with marked interest.

The 20 identified genes belong to several groups according to their biological function: adhesion molecules (*SELE* encoding for E-selectin, *VCAM*, *ICAM*, and *CD40*), chemokines (*CSF2/GMCSF*, *IL8*, *CCL2/MCP1*, *CXCL1/GRO1* and *CSF1*), and cytokines (*TNF*, *IL1A*, *IL1B* and *IL6*) (**Table 3**). Both *TNFR1* and *TNFR2* were expressed at a similar level in endothelial cells, and were not regulated by TNF-α (*not shown*). Interestingly, three anti-apoptotic proteins, TNFAIP3/A20, BCL2A1 and TNFRSF10B/TRAILR2/ DR5 were markedly up-regulated. *BIRC2* was moderately up-regulated (4.4 fold), while other genes involved in apoptosis, such as *FASL*, *TRAF2*, *MCL1* and *BIRC4* were not (*not shown*).

The expression of several components of the NF-κB signalling cascades was also up-regulated in TNF-α stimulated HMEC, as compared to untreated cells (**Table 3**). *RELB* (gene encoding RelB) was strongly up-regulated (63-fold induction). In addition *NFKB2*, *NFKB1* and *NFKBIA*, which respectively encode NF-κB2 (p52/p100), NF-κB1 (p50/p105) and NF-κBIA/inhibitor IκBα were more moderately up-regulated (10 to 20-fold). In contrast, *RELA* (encoding RelA/p65), *REL* (encoding c-Rel) and *NFKB1B*

Table 6. TNF-α up-regulates the expression of the same 20 NF-κB associated genes in HMEC and HUVEC.

Gene	HMEC cells			HUVEC cells		
	Without TNF-α	With TNF-α	Fold change	Without TNF-α	With TNF-α	Fold change
TNF	1	411	**411**	1	198	**198**
SELE	1	743	**743**	23	20123	**875**
VCAM	1	1314	**1314**	553	150276	**272**
CSF2/GMCSF	1.3	1027	**790**	23	3577	**155**
IL8	40	20172	**504**	24086	206710	**8.6**
ICAM	278	89187	**321**	1987	152666	**77**
CCL2/MCP1	1579	108418	**69**	3834	288180	**75**
CXCL1/GRO1	101	12752	**126**	11350	56063	**4.9**
RELB	145	10712	**74**	827	6903	**8.4**
TNFAIP3/A20	643	37685	**59**	3697	84782	**23**
IL1A	11	81	**7.4**	374	2894	**7.7**
IL1B	59	689	**12**	53	664	**13**
BCL2A1	1	27	**27**	19	733	**39**
NFKB2	269	6729	**25**	1191	8898	**7.5**
NFKBIA	1682	35376	**21**	3144	23936	**7.6**
CSF1	540	7385	**14**	2252	24483	**11**
NFKB1	1328	12972	**9.8**	2503	12581	**5.0**
CD40	150	944	**6.3**	1216	2426	2.0
IL6	49	384	**7.8**	290	902	3.1
TNFRSF10B	1186	6357	**5.3**	11253	21756	1.9

Cells were incubated in the presence of TNF-α (40 ng/ml) for 4 h. Gene expression was measured by RT-PCR, as described in *Materials and Methods*. For each gene, mRNA levels were normalized such that the value for the ''basal mRNA level'' (smallest amount of target gene mRNA quantifiable, Ct = 35) was 1. Ratio of the mRNA content in stimulated cells *versus* unstimulated cell mRNA content was calculated. Results are expressed as the mean of two independent experiments.
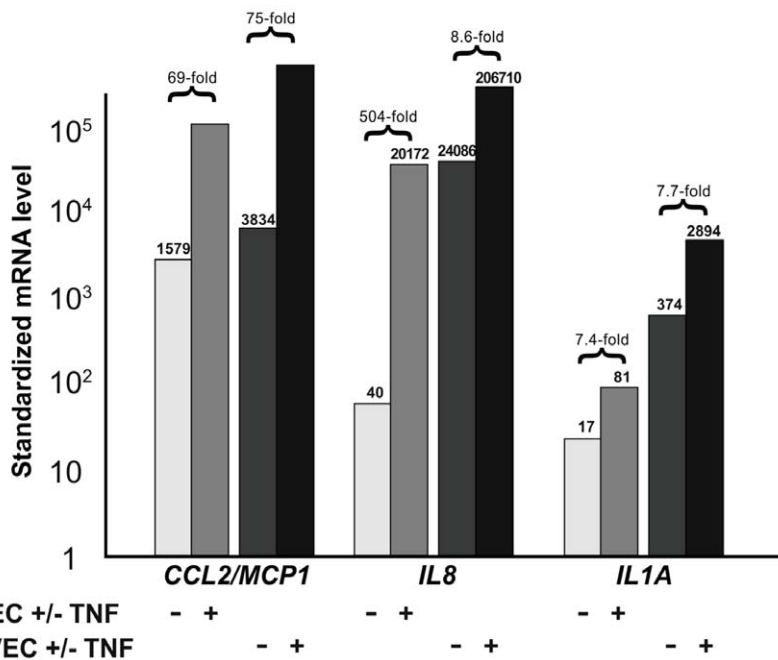doi:10.1371/journal.pone.0021589.t006

Figure 2. mRNA expression of CCL2/MCP1, IL8 and IL1A in TNF-α-stimulated and unstimulated HUVEC and HMEC cells. For each gene, mRNA levels were normalized such that the value for the ''basal mRNA level'' (smallest amount of target gene mRNA quantifiable, Ct = 35) was 1.
doi:10.1371/journal.pone.0021589.g002

Table 7. Inhibitory effect of SiRNA TNFR on TNF-α-induced genes in HMEC endothelial cells.

Gene	Control SiRNA +TNF-α	SiRNA TNFR1 + TNF-α	% inhibition via R1	Control SiRNA +TNF-α	SiRNA TNFR2 + TNF-α	% inhibition via R2
TNF R1	1.7	0.94	**45**	0.95	0.89	**7**
TNFR2	1.41	1.56	**0**	10.3	2.43	**77**
TNF	>225	>158	**30**	185.5	155	**17**
SELE	>19	>9	**ND**	>65	>51	**ND**
VCAM	124	75.6	**40**	>153	>218	**ND**
CSF2/GMCSF	92	52.5	**43**	97	70	**28**
IL8	86	43.5	**50**	59.3	62	**0**
ICAM	288	171.8	**40**	113	101.5	**11**
CCL2/MCP1	162.7	122.3	**25**	61.8	59.4	**4**
CXCL1/GRO1	21.7	17	**22**	18.6	19	**0**
RELB	17.3	14.5	**16**	6.47	6.49	**0**
TNFAIP3/A20	12.6	8.4	**33**	10.1	10.7	**0**
IL1A	11.7	5	**57**	18.8	17.8	**6**
IL1B	81.4	39.7	**51**	25.5	19	**26**
BCL2A1	10.5	5.5	**48**	10	12	**0**
NFKB2	10.6	7.6	**28**	4.8	5	**0**
NFKBIA	8	5.8	**27**	4.43	4.78	**0**
CSF1	15.7	10.9	**30**	7.7	7.9	**0**
NFKB1	6.4	4.5	**30**	3.5	3.5	**0**
CD40	3.3	2.5	**23**	3.3	3.1	**5**
IL6	8.7	5.2	**40**	5.13	5.43	**0**
TNFRSF10B	3.7	2.7	**26**	3.56	2.8	**22**

[a]Results are expressed as ratio (in percentage) of the mRNA content in siRNA TNFR-transfected cells stimulated with TNF-α (40 ng/ml) for 4 h to the mRNA content in unstimulated cells. After transfection with siTNFR1 or siTNFR2 RNAs, TNFR1 and TNFR2 expression were strongly inhibited (45 % and 77 %, respectively). Results are expressed as the mean of two different independent experiments.
doi:10.1371/journal.pone.0021589.t007

were increased only 2 to 3-fold (*not shown*). Genes encoding kinases, *CHUK* (IκKα), *IKBKB* (IκKβ) and *IKBKG* (NEMO or IκKγ) were not up-regulated (*not shown*).

In contrast, TNF-α did not modify the expression of a few set of genes known to be regulated by TNFα in tumor cells (such as MMP9). In the same set of samples, TNF-α did not modify the expression of MKI67 gene encoding the proliferation-related Ki-67 antigen cell proliferation marker.

To confirm that the increase of mRNA expression correlates with induction of protein expression, cell lysates from TNF-α stimulated HMEC were subjected to western immunoblotting using antibodies directed against ICAM, IL-1β or several proteins of the NF-κB and IκB families. As shown in **Fig. 1**, treatment of HMEC with TNF-α strongly up-regulated the expression of IL-1β ICAM and RelB. TNFα also increased NF-κB1/p50 and NF-κB2/p52 expression in endothelial cells, whereas modulation of RelA protein level was not observed, in agreement with findings at the mRNA levels.

TNF-α modulates NF-κB - associated genes in a dose- and time-dependent manner

Expression of the NF-κB - associated genes was up-regulated with TNF-α in a dose-dependent manner (1–80 ng/ml) in HMEC with the same profile of response (maximum degree of response for 40 ng/ml of TNF-α) (**Table 4**). Ten of these genes were up-regulated more than 10-fold at 0.2 ng/ml, as compared to controls, with *VCAM*, *CSF2/GMCSF* and *IL8* being stimulated more than 100-fold.

We also investigated the kinetics of TNF-α-induced mRNA expression. HMEC were treated with TNF-α (40 ng/ml) at different time periods (0 to 72 h). We observed the same profile of response for all genes, with a maximum induction always observed at 4 hours of TNF-α treatment (**Table 5**), associated to a marked decreased at 24 and 72 hours.

Quantitative and qualitative differences in the expression of the NF-κB-associated genes between microvascular and macrovascular endothelial cells

Quantitative RT-PCR was used to compare the expression of NF-κB associated genes in TNF-α stimulated HUVEC and HMEC (40 ng/ml for 4 h). As shown in **Table 6**, TNF-α up-regulated the expression of the same 20 NF-κB associated genes on the two classes of endothelial cells, except for *CD40*, *IL6* and *TNFRSF10B* that are hardly induced in HUVEC. Interestingly, for *VCAM*, *ICAM*, *IL8*, *CSF2*, *CXCL1/GRO1*, *RELB*, *IL6* and *TNFRSF10B* expression, the fold induction was much higher in HMEC, as compared to HUVEC. However, these results were probably due to a lower basal expression level in HMEC compared to HUVEC (**Table 6**). **Fig. 2** shows the mRNA levels of three characteristic genes (*CCL2/MCP1*, *IL8* and *IL1A*) in TNF-α-stimulated HUVEC and HMEC cells. Finally, it is interesting to note that among the 35 genes whose expression was not up-regulated (>5-fold) in stimulated HMEC, no gene was up-regulated in HUVEC (>5-fold) (not shown).

Table 8. Inhibitory effects of Bay 11–7082 and IKK2 inhibitor V on TNF-α-induced genes in HMEC endothelial cells.
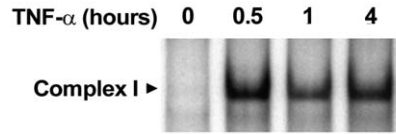
Gene	TNF-α	TNF-α + Bay 11–7082	TNF-α	IKK2+TNF-α 1 μM	IKK2+TNF-α10 μM
TNF R1	0.6	0.8	0.86	0.8	1
TNFR2	0.8	2.4	1.8	1.1	1
TNF	78	NE	34	19	22
SELE	9.5	1.6	4	4	3.7
VCAM	47.5	NE	24.8	24.9	7.3
CSF2/GMCSF	22.8	0.4	136	286	337
IL8	157	2.2	246	419	368
ICAM	332	1.1	450	284	261
CCL2/MCP1	85	0.9	162	114	42.3
CXCL1/GRO1	20	1	33.4	39.5	29.2
RELB	12.4	0.5	28	26.8	22.7
TNFAIP3/A20	11	2.4	17.6	26.6	29.6
IL1A	20.3	0.9	23.4	30.4	57
IL1B	15.6	1.5	29.8	25.6	16.3
BCL2A1	2.6	0.3	9	4.6	3.6
NFKB2	7.5	1	12.7	10.1	10
NFKBIA	5.5	0.6	6.3	6.8	7.7
CSF1	14.9	1.1	16.5	14.7	16.2
NFKB1	4.4	0.8	6	6	5.3
CD40	2.6	1.6	2.1	1.7	1.8
IL6	8	1.2	10.7	10.7	13.4
TNFRSF10B	3	2.2	4.3	3.6	4.4

Cells were pre-treated with pharmacological inhibitors of NF-κB, Bay 11–7082 10 μM or IKK2 inhibitor V (1 and 10 μM), for 30 min before incubation with 40 ng/ml TNF-α for 4 h. mRNA levels were normalized to unstimulated cells which values were set to 1.
doi:10.1371/journal.pone.0021589.t008

Figure 3. RelA knock-down by RNAi blocks TNFα-induced gene expression in HMEC cells. (A) Nuclear extracts from HMEC cells treated with TNF-α for the indicated periods of time were analyzed by EMSA using a ^{32}P-labeled HIV-LTR tandem κB oligonucleotide as a probe. (B) For supershift, nuclear extracts from HMEC cells treated with TNFα for 4 hours were incubated with the indicated antibodies before incubation with the labeled probe. Complex I: RelA/p50. (C) Nuclear extracts from HMEC cells stably transduced with a lentivirus encoding a shRNA targeting either RelA or a scrambled control, and treated by TNFα for the indicated periods of time were analyzed by EMSA as described in (A). doi:10.1371/journal.pone.0021589.g003
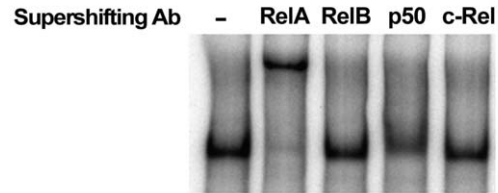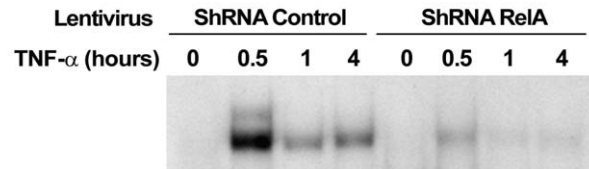
TNFR1 and, to a lesser extent, TNFR2 stimulate the NF-κB -- associated genes

We then analysed the mechanisms of the regulation by TNF-α observed in endothelial cells first using silencing of TNF receptors. Since TNFR1 (or TNF-Rp55) and TNFR2 (or TNF-Rp75) are both present in endothelial cells, we silenced the expression of each receptor by RNA interference in HMEC. In the presence of TNF-α, the expression of TNFR1 was reduced from 45 % in siRNA TNFR1-transfected cells, as compared to siRNA control (**Table 7**). In these cells, the expression of twelve NF-κB associated genes including *VCAM, ICAM, CSF2/GMCSF* and *CSF1*, the cytokines *TNF, IL8, IL1A, IL1B* and *IL6, TNFAIP3, BCL2A1* and *NFKB1* was inhibited more than 30 %, as compared to TNF-α stimulated siRNA control-transfected cells (**Table 7**). In contrast, *TNFR2* expression was not reduced in siRNA TNFR1-transfected cells, suggesting that the decrease of TNFR1 expression was receptor specific. We further tested the putative involvement of TNFR2 in TNF-α response in endothelial cells. The expression of *TNFR2*, but not *TNFR1*, was reduced from 77 % in HMEC transfected with siRNA TNFR2. Only 4 genes including *CSF2/GMCSF, IL1B, TNFRSF10B* and *TNF* were inhibited more than 15 % (28 %, 26 %, 22 % and 17 %, respectively) when *TNFR2* was silenced (**Table 7**). Altogether, these results indicate that TNFR1 and, to a lesser extent, TNFR2 are involved in the control of the NF-κB associated gene signature in TNF-α stimulated endothelial cells.

NF-κB activation is involved in TNF-α-induced gene expression in endothelial cells

We next examined the effects of chemical inhibition of IκB phosphorylation and IkappaB kinase activity on gene expression in TNF-stimulated HMEC (**Table 8**). Bay 11–7082, an inhibitor IκB phosphorylation [27] was first tested on NF-κB activity in endothelial cells using a luciferase reporter gene whose activity depends on a κB element. Luciferase activity was decreased by 99 % after addition of Bay 11–7082 (10 μM) on TNF-α treated endothelial cells (*data not shown*). Most importantly, the addition of Bay 11–7082 resulted in decreased expression of all selected genes in TNF-α treated HMEC (**Table 8**). As a control, TNFR receptor expression which was not changed by TNF-α stimulation was insensitive to Bay 11–7082. The addition of pharmacological IκB kinase (IKK2) inhibitor V [28] also inhibited the expression of *VCAM, ICAM, CCL2/MCP1* and *BCL2A1* and *IL1B* at 1–10 μM (**Table 8**); surprisingly, it activated *CSF2/GMCSF, TNFAIP3, IL1A* and *IL8*. Altogether these experiments using two different inhibitors suggest that canonical NF-κB signalling pathway is involved in TNF-κ-induced gene expression in endothelial cells.

To directly demonstrate the involvement of NF-κB in the gene signature induced by TNF-α in endothelial cells, we first evaluated TNFα-induced activation of NF-κB complexes in HMEC cells by EMSA. As shown in **Fig 3A**, TNF-α treatment in HMEC resulted in

Table 9. Effect of ShRNA RelA on TNF-α-induced genes in HMEC endothelial cells.

Gene	Control ShRNALuc +TNF-α	ShRNA RelA + TNF-α	% inhibition
CCL2	8.4	2.1	74.8
CD40	1.6	1	41.4
CSF1	3.2	2.2	31.9
CSF2	79.7	40.8	48.8
CXCL1	18.6	3.45	81.4
ICAM	17.7	2.1	85.8
IL1A	55.8	12.3	77.9
IL1B	44	4.1	90.5
IL6	17	0	100
IL8	135.9	10.4	92.3
NFKB1	3	1.2	60.5
NFKB2	11.5	6.2	46
NFKBIA	22.3	3.1	85.8
RelB	5.4	1.4	74.6
TNFAIP3	30	6.8	77.1
TNFRSF10B	2.4	2.4	0

For each gene and each sample, mRNA levels were normalized such that the value of the Control shRNALuc sample was 1.The inhibition by ShRNA RelA is expressed as pourcentage of the difference between control shRNA Luc vs shRNA RelA in cells stimulated with TNF-α (40 ng/ml) for 4 h. Genes were classified by alphabetical order.
doi:10.1371/journal.pone.0021589.t009

Table 10. Relationship between expression of *TNF* and expression of the 19 others identified putative TNF-inducible genes in 48 REα negative breast tumors and 48 REα positive breast tumors.

Gene	TNF mRNA level			
	REα negative breast tumors		REα positive breast tumors	
SELE	0,731 [a]	<0,0000001 [b]	0,387 [a]	0,0065 [b]
VCAM	0,690	0,0000002	0,403	0,0045
CSF2/GMCSF	0,589	0,00002	0,373	0,0089
IL8	0,333	0,02	0,202	NS (0,17)
ICAM	0,846	<0,0000001	0,577	0,000031
CCL2/MCP1	0,640	0,000002	0,578	0,000029
CXCL1/GRO1	0,494	0,0004	0,300	0,036
RELB	0,858	<0,0000001	0,619	0,000006
TNFAIP3/A20	0,815	<0,0000001	0,593	0,000024
IL1A	0,489	0,0005	0,248	NS (0,086)
IL1B	0,772	<0,0000001	0,477	0,0007
BCL2A1	0,760	<0,0000001	0,434	0,0022
NFKB2	0,826	<0,0000001	0,570	0,000038
NFKB1A	0,705	<0,0000001	0,592	0,000018
CSF1	0,851	<0,0000001	0,427	0,0026
NFKB1	0,691	0,0000002	0,292	0,041
CD40	0,792	<0,0000001	0,433	0,0022
IL6	0,307	0,03	0,275	NS (0.056)
TNFRSF10B	0,655	0,000001	0,346	0,015
MKI67	0,079	NS (0,60)	−0,036	NS (0.83)
ESR1	-	-	−0,063	NS (0.63)

[a]Spearman correlation coefficient. [b]P value, Spearman rank correlation test.
NS, not significant.
doi:10.1371/journal.pone.0021589.t010

a characteristic biphasic induction of κB DNA binding (complex I). Supershift analysis of nuclear extracts stimulated for 4 h with TNF-α indicated that the NF-κB DNA-binding complexes consist of RelA-p50 dimers (**Fig 3B**).

We then used stable RNA interference knock-down to inhibit RelA activity in HMEC cells. We infected HMEC cells with either a lentivirus carrying a shRNA targeting RelA or a scrambled control. Whereas TNFα stimulation of scrambled control-infected HMEC cells induced RelA DNA binding with kinetics that parallel what is seen in non-infected cells, RelA expression knock-down almost completely abolished RelA-DNA binding activity (**Fig 3C**).

We then examined the mRNA expression levels of 16 genes by real time RT-PCR. The down-regulation of RelA activity clearly inhibited the expression of all of them except TNFRSF10B (**Table 9**), thus indicating that RelA is required for the TNF-α-induced expression of these genes in HMEC cells.

Positive correlation between the expression of TNF-α mRNA and of the 19 other identified putative TNF-inducible genes in 96 human breast tumors

TNF-α is highly expressed in tumors where it has been considered initially as a potent tumor cell killer and an anti-vascular cytokine at high doses [36]. However, low chronic doses of the cytokine are thought to be pro-angiogenic [4]. In order to test the involvement of TNF-α activation/endothelial dysfunction of the breast tumor vasculature, we further explored if the correlations identified in our ex vivo cellular model (endothelial cells) between the expression of TNF-α mRNA and the expression of 19 putative TNF-α- inducible NF-κB target genes were also observed *ex vivo* in human breast tumors.

This study was performed on a series of 96 breast tumors (48 ERα-positive and 48 ERα-negative tumors) (**Table 1**). We observed a positive correlation between *TNF* and 16 (84.2 %) of the 19 endothelial TNF-α-inducible genes in both ERα-negative and ERα-positive breast tumors (**Table 10**). The three remaining genes (i.e., *IL8*, *IL1A* and *IL6*) were significantly linked to *TNF* only in ERα-negative tumors. In the same set of 96 tumors, we also examined the expression of the *ESR1* gene, which encodes ERα as well as the proliferation-associated gene *MKI67*, which encodes the proliferation-related antigen Ki-67. We found no correlation between *TNF* and *ESR1* and *MKI67*, suggesting that the 19 genes were associated to TNF independently to hormonal status and cell proliferation.

Prognostic value of TNF-α mRNA and of the 19 putative TNF-inducible gene mRNAs in human breast tumors

We estimated the prognostic value of the *TNF-α* mRNA and the 19 other identified putative TNF-α-inducible gene mRNAs in our series of 96 breast tumors (48 ERα-positive and 48 ERα-negative tumors). In the cohort of 48 ERα-negative breast tumors, nineteen patients relapsed, whereas in the cohort of 48 ERα-positive breast tumors, twenty-seven patients relapsed (**Table 11**). We used univariate analysis (log-rank test) to study the prognostic value of these 20 genes. For each gene, the 48 ERα-positive (or –negative) breast tumors were divided into two equal groups of 24 tumors

Table 11. Statistical analysis of mRNA expression of genes in 96 breast tumors relative to relapse.

	P-value [a]	
	REα negative breast tumors	REα positive breast tumors
Metastases		
Yes	19	27
No	29	21
Genes		
TNF	0.60 (NS)	0.59 (NS)
SELE	0.83 (NS)	0.99 (NS)
VCAM	0.92 (NS)	0.78 (NS)
CSF2/GMCSF	0.11 (NS)	0.099 (NS)
IL8	0.72 (NS)	**0.022**
ICAM	0.38 (NS)	0.098 (NS)
CCL2/MCP1	0.99 (NS)	0.13 (NS)
CXCL1/GRO1	0.90 (NS)	0.33 (NS)
RELB	0.89 (NS)	0.45 (NS)
TNFAIP3/A20	0.62 (NS)	0.49 (NS)
IL1A	0.62 (NS)	0.27 (NS)
IL1B	0.51 (NS)	0.55 (NS)
BCL2A1	0.25 (NS)	0.78 (NS)
NFKB2	**0.039**	0.34 (NS)
NFKBIA	0.94 (NS)	0.61 (NS)
CSF1	0.43 (NS)	0.70 (NS)
NFKB1	0.65 (NS)	0.84 (NS)
CD40	0.41 (NS)	0.53 (NS)
IL6	0.65 (NS)	0.78 (NS)
TNFRSF10B	0.21 (NS)	0.66 (NS)
MKI67	0.26 (NS)	0.56 (NS)
ESR1	-	0.059 (NS)

[a]Log-rank Test.
doi:10.1371/journal.pone.0021589.t011

with 'low' and 'high' mRNA levels. Univariate analysis showed that only low expression level of *NFKB2* in the cohort of 48 ERα-negative breast tumors and high expression level of *IL8* in the cohort of 48 ERα-positive breast tumors correlated with significantly shorter metastases-free survival, but at the limit of the significance ($p = 0.039$ and $p = 0.022$, respectively) (**Table 11**).

Discussion

This study documents, for the first time, the NF-κB gene expression signature in two different subtypes of TNFα-stimulated endothelial cells, especially in HMEC which originate from the microcirculation and have been much less studied as compared to HUVEC. Twenty genes were up-regulated 5-fold or more (until 1200-fold) in TNFα-stimulated endothelial cells, in a concentration- and time-dependent manner. The up-regulated genes include those controlling cytokines, chemokines and adhesion molecules, relevant to inflammation and apoptosis, as well as factors involved in the immune system. The most highly up-regulated genes in TNFα-stimulated endothelial cells are those encoding adhesion molecules and cytokines/chemokines (*i.e.,* TNF-α, IL-8, MCP-1 and GM-CSF/CSF2). Novel observations concern the up-regulation of *TNFAIP3, CXCL1/Gro1, GM-CSF/CSF2* and *MCF/CSF1, TNFRSF10B/Trail R2 (DR5), CD40* and *TNF* itself in TNFα-stimulated endothelial cells *via* NF-κB, as well as selective expression of NF-κB components (*RELB, NFKB1* and *NFKB2*). Interestingly, while TNFR1 was involved in all the regulations observed, some of the regulated genes were shown to be regulated through both TNFR1 and TNFR2. Moreover, the correlations identified in our *ex vivo* cellular model (endothelial cells) between the expression of TNF-α mRNA and the expression of 19 putative TNF-α- inducible NF-κB target genes were also observed in human breast tumors.

The up-regulation of adhesion molecules (VCAM-1, ICAM-1 and E-selectin) and pro-inflammatory cytokines (IL1α, ILβ and IL-6) in TNFα-stimulated endothelial cells through NF-κB agrees with previous findings reported in HUVEC [16-18].IL-6 expression was poorly modulated by NF-κB activation in these cells. Interestingly, our results show that TNF-α strongly up-regulates *TNF-α* (but not *TNFR1* and *TNFR2* expression), as well as the expression of *DR5* (*TNFRSF10B* or *TRAIL-R2*) and *CD40*, two additional members of the TNF/TNF receptor superfamily involved in the immune system [3]. Such induction occurs through TNFR1 and TNFR2 and *via* NF-κB activation. Interestingly, DR5 has been detected in endothelial cells derived from blood brain barrier (HBEC), where it released MMP9 upon activation [37].

The chemokines IL-8/CXCL8, CXCL1/Gro1, CCL2/MCP-1, GMCSF/CSF2 and MCF/CSF1 were significantly up-regulated more than 5 fold in HMEC and HUVEC. Their expression was up-regulated as early as 30 min-1 h and sustained for up to 24 h, as previously described for IL-8 and CCL2/MCP-1 in HUVEC [15,38]. The up-regulation of IL-8 through NF-κB has not been described before in TNF-α-induced endothelial cells. IL-8, CCL2/MCP-1 and CXCL1/Gro1 facilitate the subset specific recruitment of neutrophils, monocytes, T lymphocytes or NK cells. In addition to their chemotactic activity, the three chemokines possess angiogenic activity *in vitro* [39] and are involved in the regulation of tumoral angiogenesis such as in breast cancer [40-41]. Our study further shows for the first time that GM-CSF/CSF2 and MCF/CSF1 expression is induced through NF-κB in HMEC. CSF1 has been previously shown to be induced in HUVEC by endotoxin [42-43]. This chemokine promotes differentiation of hematopoietic cells to mature granulocytes and macrophages. GM-CSF/CSF2 is involved in the recruitment of macrophages in various inflammatory diseases and was recently found to mobilize bone marrow progenitors [44]. This chemokine is also involved in angiogenesis and is a key target of NF-κB in cancer and metastasis [45].

Bcl2A1 (A1) and TNFAIP3 (also known as A20) are anti-apoptotic proteins expressed in endothelial cells [46-48], and have also been associated with angiogenesis [49]. Their expression was shown to be up-regulated by TNF-α in both HUVEC and HMEC, in contrast to other classical NF-κB-inducible anti-apoptotic proteins, such as MCL1S, MCL1L, BIRC2 (c-IAP1), BIRC4 (XIAP) and TRAF2. To our knowledge, our study indicates for the first time the up-regulation of *TNFAIP3/A20* gene expression by TNF-α through NF-κB.

In our study, several approaches were used to demonstrate the involvement of the NF-κB canonical activation pathway in TNF-α-induced gene expression in endothelial cells. Our observations showing that genes encoding the kinases IκKα (CHUK), IκKβ (IKBKB) and NEMO/IκKγ (IKBKG) were not up-regulated by TNF-α in endothelial cells indicate that changes in the NF-κB target gene expression is not dependent upon changes in the expression of these kinases. The decreased expression of the NF-κB-mediated genes in TNF-α treated HMEC in the presence of Bay11–7082 (a specific inhibitor of cytokine-induced IκBα phosphorylation), is in agreement with the involvement of this

pathway. It is also in agreement with preliminary experiments showing the time-dependent phosphorylation of IκBα and nuclear translocation of p50 (NF-κB1) in TNF-α endothelial cells (L Michel and M Applanat, *not shown*). The most direct demonstration that TNF-α-induced gene expression in HMEC depends on NF-κB activation arises from RelA knock-down experiments, RelA representing the major NF-κB activity in HMEC stimulated by TNFα [50] (see Fig. 3). Nonetheless, the expression of *TNFRSF10B* is not significantly down-regulated by RelA knock-down as compared to the effect observed with the chemical inhibitors.

The significance of the selective increase of expression of *RELB*, *NFKB1* and *NFKB2/p52*, Rel/NF-κB family members, but not *RelA* and *c-rel*, which is demonstrated for the first time in TNFα-stimulated endothelial cells, is unknown. These up-regulations occur at both the mRNA and protein levels, and correlate with nuclear translocation of p52 *(not shown)*. These findings suggest that heterodimers NF-κB2 (p52)/RelA or p52/p52 could also favour TNF-α signalling in endothelial cells. Of note, TNFRSF10B/DR5 has previously been shown to be activated by p52 in some context [51]. The non canonical pathway is known to be activated by stimuli such as lymphotoxin-β, CD40L and BAFF, involves IKKα (IKK1) activation and nuclear translocation of RelB in the form of RelB/p52 and RelB/p50 dimers [9–10,11,51–52].

Our study also contributes to analyze the role of TNFR1 and TNFR2 receptors, which are expressed at a similar level in endothelial cells. The decrease of gene expression is observed in TNFα-stimulated endothelial cells after silencing TNFR1; this receptor is thought to mediate the majority of the biological effects of the TNFα and the classical pathway of TNF-α signalling. However, silencing of TNFR2 also decreases the expression of *IL1B*, *GM-CSF/CSF2*, *TNFRSF10B/Trail R2* and *TNF*, suggesting that both TNFR1 and TNFR2 are involved in TNF-α dependent up-regulation of these genes in HMEC.

TNF-α and its related NF-κB transcription factor are described as critical components of tumor progression [20,22]. NF-κB is deregulated in breast cancer patients and in breast cancer cells [25,53–55]. Several studies have shown that tumor-associated neovascularisation is a prerequisite of rapid growth and metastasis [56]. Although great strides have been made in the elucidation of molecular mechanisms of tumor vasculature, different markers and many essential interactions have to be yet determined. Among genes described in this study, IL8, CCL2/MCP1, CXCL1/Gro1, Bcl2 and TNFAIP3 are known to be pro-angiogenic [38–

40,49,57]. Moreover, one of the consequences for TNF-á activation of tumor endothelial cells is also to recruit numerous cells through chemokines and adhesion molecules, including monocytes, neutrophils, fibroblasts and immune cells inside the tumor.

Our study provides for the first time a significant correlation between TNF-α expression and the expression of putative TNF-α-inducible NF-κB target genes (as identified in microvascular endothelial cells) in human breast tumors (**Table 10**). The highest correlation between TNF-α and NF-κB related genes was observed for ERalpha negative-, as compared to ERpositive-breast tumors. TNF-α expression and IL8, IL1A and IL6 expression were only correlated in ER-negative, but not in ER-positive breast tumors. A distinct pattern of activation of NF-κB subunits (c-Rel, RelB, p50/ NF-κB1, p52/ NF-κB2, and Iκ-Bα), as well as the absence (or low level) of nuclear p65/RelA has also been previously described in breast tumors, as compared to normal adjacent tissue [22,48]. Altogether, these data suggest that dysregulated/TNF-α stimulated endothelial cells through NF-κB are likely to play an important role in breast cancer.

In conclusion, we describe for the first time the profile of cytokines, chemokines, adhesion molecules and NF-κB components induced in endothelial cells stimulated by TNF-α through TNFR and NF-κB. This study provides a NF-κB gene signature associated with TNF-α stimulation in endothelial cells. Some of the newly identified regulated genes, such as those involved in the recruitment of bone marrow or endothelial cell progenitors, will be further explored in a mouse cancer model. The cytokines, chemokines, adhesion molecules and angiogenic factors, as well as factors involved in the immune system, that we have identified in TNF-α stimulated endothelial cells may also promote tumor progression in vivo.

Acknowledgments

Author Contributions

Conceived and designed the experiments: MPA IB VB. Performed the experiments: SV IP GV AT FC HES MPA. Analyzed the data: IB RL MPA VB. Wrote the paper: MPA VB IB.

References

1. Tracey KJ, Cerami A (1993) Tumor necrosis factor, other cytokines and disease. Annu Rev Cell Biol 9: 317–343.
2. Baud V, Karin M (2001) Signal transduction by tumor necrosis factor and its relatives. Trends Cell Biol 11: 372–377.
3. Locksley RM, Killeen N, Lenardo MJ (2001) The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell 104: 487–501.
4. Mocellin S, Rossi CR, Pilati P, Nitti D (2005) Tumor necrosis factor, cancer and anticancer therapy. Cytokine Growth Factor Rev 16: 35–53.
5. Pahl HL (1999) Activators and target genes of Rel/NF-kappa B transcription factors. Oncogene 18: 6853–6866.
6. Karin M, Lin A (2002) NF-kappa B at the crossroads of life and death. Nat Immunol 3: 221–227.
7. Hayden MS, Ghosh S (2004) Signalling to NF-kappa B. Genes Dev 18: 2195–2224.
8. Baud V, Karin M (2009) Is NF-kappaB a good target for cancer therapy? Hopes and pitfalls. Nature Reviews Drug Discovey 8(1): 33–40.
9. Dejardin E, Droin NM, Delhase M, Haas E, Cao Y, et al. (2002) The lymphotoxin-beta receptor induces different patterns of gene expression via two NF-kappaB pathways. Immunity 17(4): 525–535.
10. Claudio E, Brown K, Park S, Wang H (2002) Siebenlist UBAFF-induced NEMO-independent processing of NF-kappa B2 in maturing B cells. Nat Immunol 3(10): 958–965.
11. Derudder E, Dejardin E, Pritchard LL, Green DR, Korner M, et al. (2003) RelB/p50 dimers are differentially regulated by tumor necrosis factor-alpha and

lymphotoxin-beta receptor activation: critical roles for p100. J Biol Chem 278(26): 23278–23284.
12. Vanden Berghe W, Vermeulen L, De Wilde G, De Bosscher K, Boone E, et al. (2000) Signal transduction by tumor necrosis factor and gene regulation of the inflammatory cytokine interleukin-6. Biochem Pharmacol 60: 1185–1195.
13. Krishnaswamy G, Smith JK, Mukkamala R, Hall K, Joyner W, et al. (1998) Multifunctional cytokine expression by human coronary endothelium and regulation by monokines and glucocorticoids. Microvasc Res 55: 189–200.
14. Zhou J, Jin Y, GaoY, Wang H, Hu G, et al. (2002) Genomic-scale analysis of gene expression profiles in TNF-alpha treated human umbilical vein endothelial cells. Inflamm Res 51: 332–341.
15. Zhao RZ, Chen X, Yao Q, Chen C (2005) TNF-alpha induces interleukin-8 and endothelin-1 expression in human endothelial cells with different redox pathways. Biochem Biophys Res Commun 327: 985–992.
16. Hou J, Baichwal V, Cao Z (1994) Regulatory elements and transcription factors controlling basal and cytokine-induced expression of the gene encoding intercellular adhesion molecule 1. Proc Natl Acad Sci U S A 91: 11641–11645.
17. Kuldo JM, Westra J, Asgeirsdottir SA, Kok RJ, Oosterhuis K, et al. (2005) Differential effects of NF-{kappa}B and p38 MAPK inhibitors and combinations thereof on TNF-{alpha}- and IL-1{beta}-induced proinflammatory status of endothelial cells in vitro. Am J Physiol Cell Physiol 289: C1229–1239.
18. Ledebur HC, Parks TP (1995) Transcriptional regulation of the intercellular adhesion molecule-1 gene by inflammatory cytokines in human endothelial cells.

Essential roles of a variant NF-kappa B site and p65 homodimers. J Biol Chem 270: 933–943.

19. Viemann D, Goebeler M, Schmid S, Nordhues U, Klimmek K, et al. (2006) TNF induces distinct gene expression programs in microvascular and macrovascular human endothelial cells. J Leukoc Biol 80(1): 174–185.

20. Karin M, CaoY, Greten FR, Li ZW (2002) NF-kappa B in cancer: from innocent bystander to major culprit. Nat Rev Cancer 2: 301–310.

21. Aggarwal BB (2004) Nuclear factor-kappaB: the enemy within. Cancer Cell 6: 203–208.

22. Coussens LM, Werb Z (2002) Inflammation and cancer. Nature 420: 860–867.

23. Baldwin AS (2001) Control of oncogenesis and cancer therapy resistance by the transcription factor NF-kappaB. J Clin Invest 107: 241–246.

24. Shishodia S, Aggarwal BB (2004) Nuclear factor-kappa B activation mediates cellular transformation, proliferation, invasion angiogenesis and metastasis of cancer. Cancer Treat Res 119: 139–173.

25. Sovak MA, Bellas RE, Kim DW, Zanieski GJ, Rogers AE, et al. (1997) Aberrant nuclear factor-kappa B/Rel expression and the pathogenesis of breast cancer. J Clin Invest 100: 2952–2960.

26. Jaffe EA, Nachman RL, Becker CG, Minick CR (1973) Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J Clin Invest 52: 2745–2756.

27. Pierce JW, Schoenleber R, Jesmok G, Best J, Moore SA, et al. (1997) Novel inhibitors of cytokine-induced IkappaB alpha phosphorylation and endothelial cell adhesion molecule expression show anti-inflammatory effects in vivo. J Biol Chem 272(34): 21096–21103.

28. Sors A, Jean-Louis F, Bégué E, Parmentier L, Dubertret L, et al. (2008) Inhibition of IkappaB kinase subunit 2 in cutaneous T-cell lymphoma down-regulates nuclear factor-kappaB constitutive activation, induces cell death, and potentiates the apoptotic response to antineoplastic chemotherapeutic agents. Clin Cancer Res 14(3): 901–911.

29. Levkau B, Scatena M, Giachelli CM, Ross R, Raines EW (1999) Apoptosis overrides survival signals through a caspase-mediated dominant-negative NF-kappa B loop. Nat Cell Biol.1(4): 227–233.

30. Rossol M, Meusch U, Pierer M, Kaltenhäuser S, Häntzschel H, et al. (2007) Interaction between transmembrane TNF and TNFR1/2 mediates the activation of monocytes by contact with T cells. J Immunol 179(6): 4239–4248.

31. Kieusseian A, Chagraoui J, Kerdudo C, Mangeot PE, Gage PJ, et al. (2006) Expression of Pitx2 in stromal cells is required for normal hematopoiesis. Blood 2: 492–500.

32. Jacque E, Tchenio T, Piton G, Romeo PH, Baud V (2005) RelA repression of RelB activity induces selective gene activation downstream of TNF receptors. Proc Natl Acad Sci U S A 102(41): 14635–14640.

33. Bieche I, Parfait B, Laurendeau I, Girault I, Vidaud M, et al. (2001) Quantification of estrogen receptor alpha and beta expression in sporadic breast cancer. Oncogene 20: 8109–8115.

34. Bloom HJ, Richardson WW (1957) Histological grading and prognosis in breast cancer; a study of 1409 cases of which 359 have been followed for 15 years. Br J Cancer 11: 359–377.

35. Bieche I, Lerebours F, Tozlu S, Espie M, Marty M, et al. (2004) Molecular profiling of inflammatory breast cancer: identification of a poor-prognosis gene expression signature. Clin Cancer Res 10: 6789–6795.

36. Ten haguen TL, Seynhaeve AL, Eggermont AM (2008) Tumor necrosis factor-mediated interactions between inflammatory response and tumor vascular bed. Immunol Rev 222: 299–315.

37. Wosik K, Biernacki K, Khouzam MP, Prat A (2007) Death receptor expression and function at the human blood brain barrier. J Neurol Sci 259(1-2): 53–60.

38. Goebeler M, Gillitzer R, Kilian K, Utzel K, Bröcker EB, et al. (2001) Multiple signaling pathways regulate NF-kappaB-dependent transcription of the mono-cyte chemoattractant protein-1 gene in primary endothelial cells. Blood 97: 46–55.

39. Bernardini G, Ribatti D, Spinetti G, Morbidelli L, Ziche M, et al. (2003) Analysis of the role of chemokines in angiogenesis. J Immunol Methods 273: 83–101.

40. Saji H, Koike M, Yamori T, Saji S, Seiki M, et al. (2001) Significant correlation of monocyte chemoattractant protein-1 expression with neovascularization and progression of breast carcinoma. Cancer 92: 1085–1091.

41. Bieche I, Chavey C, Andrieu C, Busson M, Vacher S, et al. (2007) CXC chemokines located in the 4q21 region are up-regulated in breast cancer. Endocr Relat Cancer; 14: 1039–52.

42. Quesenberry PJ, Gimbrone MA Jr. (1980) Vascular endothelium as a regulator of granulopoiesis: production of colony-stimulating activity by cultured human endothelial cells. Blood 56(6): 1060–1067.

43. Lenhoff S, Olofsson T (1996) Cytokine regulation of GM-CSF and G-CSF secretion by human umbilical cord vein endothelial cells (HUVEC). Cytokine. 8. pp 702–709.

44. Hamilton JA (2002) GM-CSF in inflammation and autoimmunity. Trends Immunol 23: 403–408.

45. Park BK, Zhang H, Zeng Q, Dai J, Keller ET, et al. (2007) NF-kappaB in breast cancer cells promotes osteolytic bone metastasis by inducing osteoclastogenesis via GM-CSF. Nat Med 13: 62–69.

46. Karsan A, Yee E, Harlan JM (1996) Endothelial cell death induced by tumor necrosis factor-alpha is inhibited by the Bcl-2 family member, A1. J Biol Chem 271: 27201–27204.

47. Karsan A, Yee E, Kaushansky K, Harlan JM (1996) Cloning of human Bcl-2 homologue: inflammatory cytokines induce human A1 in cultured endothelial cells. Blood 87: 3089–3096.

48. Stroka DM, Badrichani AZ, Bach FH, Ferran C (1999) Overexpression of A1, an NF-kappa B-inducible anti-apoptotic bcl gene, inhibits endothelial cell activation. Blood 93: 3803–3810.

49. Karl E, Warner K, Zeitlin B, Kaneko T, Wurtzel L, et al. (2005) Bcl-2 acts in a proangiogenic signalling pathway through nuclear factor-kappa B and CXC chemokines. Cancer Res 65: 5063–5069.

50. Marui N, Medford RM, Ahmad M (2005) Activation of RelA homodimers by tumour necrosis factor alpha: a possible transcriptional activator in human vascular endothelial cells. Biochem J 390: 317–324.

51. Schumm K, Rocha S, Caamano J, Perkins ND (2006) Regulation of p53 tumour suppressor target gene expression by the p52 NF-kappaB subunit. EMBO J. 25(20): 4820–4832.

52. Gasparini C, Foxwell BM, Feldmann M (2009) RelB/p50 regulates CCL19 production, but fails to promote human DC maturation. Eur J Immunol 39(8): 2215–2223.

53. Cogswell PC, Guttridge DC, Funkhouser WK, Baldwin AS, Jr. (2000) Selective activation of NF-kappa B subunits in human breast cancer: potential roles for NF-kappa B2/p52 and for Bcl-3. Oncogene. 19: 1123–1131.

54. Kim DW, Sovak MA, Zanieski G, Nonet G, Romieu-Mourez R, et al. (2000) Activation of NF-kappa B/Rel occurs early during neoplastic transformation of mammary cells. Carcinogenesis 21: 871–879.

55. Cao Y, Karin M (2003) NF-kappa B in mammary gland development and breast cancer. J Mammary Gland Biol Neoplasia 8: 215–223.

56. Folkman J (1995) Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat Med 1: 27–31. Review.

57. Yoshida S, Ono M, Shono T, Izumi H, Ishibashi T, et al. (1997) Involvement of interleukin-8, vascular endothelial growth factor, and basic fibroblast growth factor in tumor necrosis factor alpha-dependent angiogenesis. Mol Cell Biol 17: 4015–4023.