Role of the CD40 receptor/CD154 ligand dyad in the control of smooth muscle cells phenotype

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Abbreviations

Acc No	accession number
ActD	actinomycin D
AP-1	activator protein-1
APC	antigen-presenting cell
bp	base pair
BSA	bovine serum albumin
cAMP	cyclic adenosine mono-phosphate
CCR	CC chemokine receptor
cDNA	complementary deoxynucleotide acid
C/EBP	CCAAT/enhancer-binding protein
CHX	cycloheximide
COX-2	cyclooxygenase-2
D'MEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
dNTP	deoxynucleotide triphosphate
dODN	decoy oligodeoxynucleotide
ds	double-stranded
ECGS	endothelial cell growth supplement
ECM	extracellular matrix
EDTA	ethylendinitrilo-N, N, N', N'-tetra-acetate
EF	elongation factor
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular-signal-regulated kinase
E-sel	E-selectin
JNK	c-Jun kinase
FCS	fetal calf serum
GM-CSF	granulocyte-macrophage colony-stimulating factor
GRO	growth-regulated oncogene
H89	N-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide
HBSS	Hank's buffered salt solution
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HerbA	herbimycin A
IAP-1	inhibitor of apoptosis protein-1

ICAM-1	intercellular adhesion molecule-1
IFN-γ	interferon gamma
IgM	immunoglobulin M
IKK	IκB kinase
IL	interleukin
LDL	low density lipoprotein
mAb	monoclonal antibody
MAP kinase	mitogen-activated protein kinase
MCP	monocyte chemoattractant protein
M-CSF	macrophage colony-stimulating factor
MEK	MAP kinase kinase
MIP	macrophage inflammatory protein
MHC II	major histocompatibility complex class II
MMP	matrix metalloproteinase
mRNA	messenger RNA
MT-MMP	membrane-type matrix metalloproteinase
NF-κB	nuclear factor kappa B
NIK	NF-κB–inducing kinase
PBS	phosphate-buffered saline
PD 98059	2′-amino-3′-methoxyflavone
PDE IV	phosphodiesterase IV
PECAM-1	platelet endothelial cell adhesion molecule-1
PI-3K	phosphatidylinositol-3 kinase
PKA	protein kinase A
RANTES	regulated upon activation, normal T-cell-expressed and secreted
RNase	ribonuclease
RO 31-8220	3-[1-[3-(amidinothio)propyl-1H-indol-3-yl]-3-(1-methyl-1H-indol-3-yl) maleimide; bisindolylmaleimide IX, methanesulfonate
ROCK	Rho kinase
RPL	ribosomal protein large unit
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
SAPK	stress-activated protein kinase
SB 202190	4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole

SD	standard deviation
SEM	standard error of the mean
SMC	smooth muscle cell
ss	single-stranded
STAT	signal transducer and activator of transcription
TF	transcription factor
TNF-α	tumor necrosis factor-α
TNFAIP	tumor necrosis factor alpha-induced protein
TRAF	TNF receptor associated factor
Tris	Tris-(hydroxymethyl)-aminomethan
U	unit
uPA	urokinase plasminogen activator
uPAR	urokinase plasminogen activator receptor
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
Y27632	(+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl) cyclohexanecarboxamide

1. Introduction

1.1 The CD40/CD154 receptor/ligand dyad

1.1.1 Structure of CD40 and CD154

CD40 is a cell surface receptor that belongs to the tumor necrosis factor receptor (TNF-R) family. The human CD40 gene is mapped to chromosome 20 (q11-q13.2) and consists of 9 exons. The fully maturated protein has 255 amino acids and consists of a 22 amino acid trans-membrane domain connecting a 171 amino acid extracellular domain and a 62 amino acid intracellular domain. CD40 is characterized by a repetitive amino acid sequence pattern of four cystein-enriched sub-domains, typically consisting of six cystein residues forming three disulfide bridges (Stamenkovic et al., 1989).

CD154 is the only known specific ligand for CD40. It belongs to the TNF family of proteins. The CD154 gene is mapped to chromosome X (q26.3-q27.1) and consists of 5 exons. The first exon encodes the intracellular, transmembrane and a small part of the extracellular domain, while exons II-V encode the rest of the extracellular domain. The molecular mass of its membrane-bound form is 39 kDa. However, three biologically active, soluble fragments of 31, 18, and 14 kDa protein derived from proteolytic cleavage of the membrane-bound form have been characterized (Graf et al, 1995; Ludewig et al., 1996; Wykes et al., 1998).

1.1.2 CD40/CD154 expression pattern and chronic inflammation

CD40 and CD154 were first discovered on the surface of B-cells and T-cells, respectively (Paulie et al., 1984). The crucial role of CD154/CD40 interaction in T-cell-dependent B-cell activation and differentiation can best be understood when the consequences of certain genetic polymorphisms in the human CD154 gene are analysed. Several point mutations in the CD154 gene are associated with the so-called ‘family X-linked hyperimmunoglobulin M syndrome’ (Allen et al., 1993). This disease not only is characterized by elevated concentrations of serum IgM and decreased amounts of all other immunoglobulin isotypes, but also by a decreased sensitivity to activation and proliferation of B-cells. Consequently, patients with this syndrome suffer from chronic infections of the upper respiratory tract as well as a

number of opportunistic or viral infections (Durandy et al., 1993). Moreover, Th1-dependent inflammatory reactions are weakened indicating even more important functions of CD40/CD154 interactions in the regulation of the cellular immune response (Grewal and Flavell, 1998).

Apart from the importance of CD154/CD40 co-stimulation for the mounting of an appropriate immune response, more recent studies associated this co-stimulatory pathway with the exaggerated reaction of the immune response in various chronic inflammatory and autoimmune diseases. Moreover, CD154 is also expressed by non-immune cells such as activated platelets (Henn et al., 1998), while CD40 is expressed in epithelial cells, fibroblasts and endothelial cells (Alderson et al., 1993, Yellin et al., 1995, Karmann et al, 1995), as well as in SMC under pro-inflammatory conditions (Krzesz et al., 1999). This broader distribution of CD40 and CD154 also points to the likely involvement of this receptor/ligand dyad in chronic inflammatory diseases such as asthma, rheumatoid arthritis, psoriasis, type I diabetes and multiple sclerosis, to name a few, but also in atherosclerosis (Schonbeck and Libby, 2001).

1.1.3 CD40 signal transduction

CD154 is capable of forming a trimeric structure which, as a consequence, induces trimerization of the CD40 receptor protein (Peitsch et. al, 1993). This trimerization is necessary for optimal CD40 signalling (Pullen et. al, 1999). Although CD40 has no kinase domain, CD40 ligation activates several second messenger systems through (i) activation of separate kinases/phosphatases, and/or (ii) recruitment and activation of several signalling proteins.

Ligation of CD40 on activated B-cells thus results in enhanced tyrosine phosphorylation of four distinct phosphoproteins and induces a rapid increase in the production of inositol 1,4,5-triphosphate (Uckun et al., 1991). Later reports described a wortmannin-sensitive kinase activity, implicating involvement of phosphatidylinositol-3-kinase (PI-3K) in CD40 signalling (Aagaard-Tillery et al., 1996*).* Also, the Src-family tyrosine kinases syk, lyn, and fyk have been associated with CD40 signalling (Tan et al., 2000). Finally, signalling pathways downstream from PI-3/PI-3-K and syk/lyn/fyk result in the activation of various transcription factors, including nuclear factor (NF)-κB, activator protein-1 (AP-1) and nuclear factor of activated T cells (NFAT) (Karmann et al., 1996).

The TNF-R family-associated factors (TRAFs) consist of six known members that share homology in the C-terminal domain. This domain is required for multimerization and binding of the TRAFs to members of the TNF-R family. TRAF3 has been identified as the first protein associated with the cytoplasmic domain of CD40. In addition to TRAF3, TRAF1, TRAF2, TRAF5 and TRAF6 have been associated with CD40 signalling (Ishida et al., 1996). Overexpression of TRAF2, TRAF5, and TRAF6 in B-cells activates stress-activated protein kinases (SAPK) and NF-κB. In addition, CD40/TRAF6 interaction leads to activation of the extracellular signal–regulated kinases (ERK) 1 and 2 as well as p38 mitogen-activated protein kinase (MAPK). This, in turn, results in recruitment of NF-κB–inducing kinase (NIK) which activates NF-κB through IκB-kinase I/II (IKK-I/IKK-II) (Brady et al., 2000). Moreover, TRAF2 and TRAF6 can activate the JNK signalling pathway.

Besides TRAFs, members of the family of signal transducers and activators of trancription (STATs) have been implicated in CD40 signalling (Karras et al., 1997). STAT proteins form homo- and hetero-dimers after phosphorylation, translocate to the nucleus, and bind specific DNA elements, enabling them to directly induce gene expression (Darnell et al., 1994).

Thus, CD40 signalling seems to be rather complex. On the other hand, not all CD40-expressing cells execute all the signal transduction pathways CD40 is principally capable to activate, making it rather difficult to sort out the signalling mechanism in a given cell type that mediates the physiological/pathophysiological response to CD40 activation.

1.2 Atherosclerosis - a chronic inflammatory disease

In principle, atherosclerosis must be considered as a chronic inflammatory disease of the vessel wall that is restricted to arteries and arterioles (Ross, 1993). Because high plasma concentrations of cholesterol, in particular low density lipoprotein (LDL) cholesterol, are among the principal risk factors for atherosclerosis, the process of atherogenesis has been considered by many to consist largely of the accumulation of lipids within the artery wall. However, despite changes in life style and the use of new pharmacological approaches to lower plasma cholesterol concentrations (Shepherd, 1995), atherosclerosis and its consequences (myocardial infarction, heart failure and stroke) continues to be the leading cause of death in industrialized nations.

Atherosclerotic lesions represent a series of cellular responses that can be described as an inflammatory disease. From the start of the disease, inflammatory cells and mediators are major components of the atherosclerotic plaque. Fatty streaks, the so-called early lesions, already contain significant numbers of activated macrophages and T-cells (Emeson et al., 1988). In the course of lesion progression, the number of pro-inflammatory cells in the vessel wall increases even further (Ross, 1999).

1.2.1 Cell-to-cell interactions in atherosclerosis

In atherosclerosis a wide variety of cell-to-cell interactions are important, including leukocyte-endothelial cell, leukocyte-leukocyte, leukocyte-vascular smooth muscle cell, and leukocyte-interstitial cell interactions. The proteins mediating these interactions are adhesion molecules that belong to four major families: (1) selectins, (2) selectin ligands, (3) integrins, and (4) members of the immunoglobulin superfamily (Springer, 1994). A major function of these adhesion molecules is to promote leukocyte recruitment from the circulating blood into the tissue through a series of events, including leukocyte rolling along the endothelial cell surface, firm adhesion and activation and, finally, extravasation into the vessel wall.

The normal endothelium does not in general support rolling and binding of leukocytes. When the endothelial monolyer becomes inflamed, it express selectins and selectin ligands that are mediating leukocyte rolling (Eriksson et al., 2000). Also, activated endothelium express adhesion molecules that bind cognate ligands on leukocytes. In particular, vascular cell adhesion molecule-1 (VCAM-1) binds precisely the types of leukocytes found in early atheroma, the monocyte and T-cells (Li et al., 1993). Once adherent to the endothelium, the leukocytes penetrate into the intima. Recent research has identified candidate chemoattractant molecules responsible for this transmigration. For example, monocyte chemoattractant protein-1 (MCP-1) appears responsible for the direct migration of monocytes into the intima at sites of lesion formation (Boring et al., 1998). In addition to MCP-1, macrophage colony stimulating factor (M-CSF) contributes to the differentiation of the blood monocytes into the macrophage foam cells (Smith et al., 1995). Besides, chemokine binding also increases the cellular production of other cytokines and growth factors further amplifying the inflammatory response.

1.2.2 Role of smooth muscle cells in atherosclerosis

In the initial phase of atherosclerosis, contact between endothelial cells and circulating leukocytes plays a pivotal role. Circulating leukocytes adhere to endothelial cells, migrate through them and enter the vessel wall. This process allows vascular smooth muscle cells (SMC) to come into contact with the infiltrating leukocytes. One hallmark of lesion progression in humans is the focal accumulation of medial SMC within the neointima. In early atherosclerosis, SMC may contribute to lesion formation through the production of pro-inflammatory mediators such as MCP-1 and VCAM-1, and through the synthesis of matrix molecules required for the retention of lipoproteins (Schwartz et al., 1995). However, in later phases of the disease, SMC may also be important in maintaining the stability of the plaque through formation of a firm fibrous cap. Indeed, in lipid-laden lesions in which the fibrous cap is thin or weakened, there is evidence of SMC apoptosis, especially at the 'shoulder' region, that is associated with inflammation (Fuster et al., 1994). In addition, the local inflammatory milieu can induce expression of collagenase and inhibit expression of proteolytic inhibitors, thus rendering the fibrous cap susceptible to rupture (Libby et al., 2002, Ross et al., 1973). The resulting acute thrombosis may lead to complete vessel occlusion and infarction.

In this contest, it is of particular interest that SMC expressing CD40 contribute to the development of atherosclerosis via CD154-CD40 interaction. CD40 activation on SMC leads to SMC activation characterized by the expression of matrix metalloproteinases (MMPs), interleukin-1 beta (IL-1ß) and tissue factor (Horton et al., 2001; Schonbeck et al., 1997; Schonbeck et al., 2000).

1.3 SMC plasticity

Intimal SMC associated with vascular disease are phenotypically distinct from their medial counterparts (Mosse et al., 1985; Campbell et al., 1985). Intimal SMC resemble immature, dedifferentiated SMC that have low levels of contractile proteins but express large amounts of proteins contributing to progression of the atherosclerotic plaque and its degree of inflammation that makes it prone to rupture. Under normal physiological conditions, SMC plasticity is very important. The contractile phenotype is essential for hemodynamic stability. After injury, SMC are able to dedifferentiate, proliferate, produce an appropriate extracellular matrix and,

via this way contribute to repairing the injured vessel wall. Interestingly only two morphologically distinct SMC cell types can be isolated from the media of healthy human arteries (Li et al., 2001). In contrast, several different SMC or myofibroblast-like cells can be characterized in the neointima of atherosclerotic lesions. This SMC phenotype alteration in the course of atherosclerosis is a consequence of prolonged exposure to inappropriate mechanical stress, cell-matrix and cell-to-cell interactions.

1.4 Aims of the project

The hypothesis underlying this thesis is that CD40 signalling in SMC may play an important role in the development of atherosclerosis. However, expression and possible effects of CD40 in this cell type, especially with respect to the situation in humans are only poorly understood. Therefore, the first aim of the thesis was to characterize CD40 expression in human cultured SMC under basal and under pro-inflammatory conditions. After having established near optimal conditions for CD40 expression in these cells, a detailed analysis of the signalling mechanisms underlying CD40-induced gene expression and profiling of CD154-induced gene products were performed in parallel. Finally, the functional consequences of the changes in SMC phenotype were elucidated, paying special attention to the putative interaction between leukocytes and SMC in the vessel wall during atherogenesis.

2. Materials and methods

2.1 Cell biology methods

2.1.1 Medium and substances for cell culture

SMC growth medium 2	Promocell	Karlsruhe
DMEM	Life Technologies	Karlsruhe
M199	Life Technologies	Karlsruhe
RPMI 1640	Life Technologies	Karlsruhe
HBSS	Life Technologies	Karlsruhe
0.05% Trypsin/0.2% EDTA	Life Technologies	Karlsruhe
HEPES	Roth	Karlsruhe
Nystatin	Life Technologies	Karlsruhe
Penisillin	Life Technologies	Karlsruhe
Streptomycin	Life Technologies	Karlsruhe
FCS	Life Technologies	Karlsruhe
Heparin	Sigma-Aldrich	Deisenhofen
Gelatine	Sigma-Aldrich	Deisenhofen
0.15% Collagenase	Sigma-Aldrich	Deisenhofen
Ficoll-Paque®	Amersham	Freiburg

2.1.2 Culturing of human SMC from thymus veins (enzymatic digestion)

Smooth muscle cells were obtained from the thymus of young children (up to 2 years old) undergoing heart surgery. All patients had given their written consent prior to the surgical procedure. The consent was also approved by the Local Ethical Committee. The small veins were excised from the organ, freed of adjacent fat and connective tissue, and transferred to a 60 mm petri dish containing 250 µl collagenase solution and 1.4 ml DMEM medium with 5% FCS. The petry dishes were placed in an incubator at 37°C and 5% CO_2 to allow digestion of the extracellular matrix overnight (14-16 h). Thereafter the isolated cells were centrifuged for 5 min at 1000 rpm and ambient temperature, the pellet was resuspended in 2-3 ml of smooth muscle cell growth medium and allowed to adhere to petri dish previously coated with 2 % gelatine. The medium was changed every 2 days thereafter.

After reaching 90-100% confluence (approximately after 5-6 days) cells were passaged by using trypsin/ EDTA (5 min, 37°C, 5% CO_2). For the experiments described, cells of passage 3 to 4 were used throughout.

At the light microscopy level, their identity with SMC was confirmed by the typical hill-and-valley growth pattern and in addition, by positive immunostaining for smooth muscle cell α-actin, and negative immunostaining for von Willebrandt factor (endothelial cells).

2.1.3 Cultivation of mouse myeloma cells

The mouse myeloma cell line P3xTB.A7 stably expressing human CD154 and the non-transfected P3x63Ag8.653 control cells were kind gifts of Prof. R. Kroczek (Robert-Koch-Institute, Berlin). They were cultured in RPMI 1640 medium with 10% FCS under 5% CO_2 at 37°C. Every three months, cells were selected with gentamycin (G-418 sulphate, Gibco-BRL, Life Technologies, Karlsruhe) to maintain expression of the transgene.

2.1.4 Cultivation of THP-1 cell line

The human premonocytic cell line THP-1 was purchased from the American Type Culture Collection (Rockville, MD). The cells were cultured in RPMI 1640 medium with 10% FCS under 5% CO_2 at 37°C, at a density between $2x10^5$ and $1x10^6$ cells/ml.

2.1.5 Pre-stimulation of SMC with cytokines

The cytokines IFNγ (1000 U/ml) and TNFα (100 U/ml) were used to stimulate CD40 receptor expression in the cultured smooth muscle cells. Incubation times varied from 6-24 h (37°C, 5% CO_2). After this time, the conditioned medium was replaced with fresh medium and cells left for another 12-h period to allow for maximum CD40 protein expression on the surface of the smooth muscle cells.

2.1.6 Activation of CD40 receptor

The cytokine pre-stimulated SMC were co-incubated with the P3xTB.A7 cells (expressing human CD154), P3x63Ag8.653 control cells or with the P3xTB.A7 cells in the presence of a monoclonal anti-CD154 antibody (Pharmingen, Germany, 40 µg/mL) for 12 h in the incubator (37°C, 5% CO_2).

Table 1. Substances used as signal transduction inhibitors

Substance	Final concentration	Effect	Source	Diluted in
Actinomycin D	1 µM	Transcription inhibitor	Sigma	DMSO
Cycloheximide	1 µg/mL	Translation inhibitor	Sigma	DMSO
Forskolin	10 µM	Activator of adenylat cyclase	Calbiochem	DMSO
Herbimycin A	1 µM	Tyrosine kinase inhibitor with Src selectivity	Alexis	Water
H89	1 µM	PKA inhibitor	Alexis	Water
PD98059	50 µM	MEK-1/2 inhibitor	Calbiochem	DMSO
Rapamycin	10 nM	P70 S6 kinase inhibitor	Calbiochem	DMSO
RO31-8220	1 µM	PKC inhibitor	Calbiochem	DMSO
Rolipram	12.5 µM	PDE IV inhibitor	Calbiochem	DMSO
SB202190	10 µM	p38 MAP kinase inhibitor	Calbiochem	DMSO
SP600125	20 µM	JNK inhibitor	Biomol	DMSO
Y27632	10 µM	Rho kinase inhibitor	Calbiochem	Water
Wortmannin	100 nM	PI3K inhibitor	Sigma	Ethanol

To interfere with CD40 signalling to the nucleus in stimulated SMC, the drugs listed in Table 1 were employed. Typically they were pre-incubated with the cultured SMC for 1 h, before addition of P3xTB.A7 cells and left in the medium throughout the remaining period of the experiment. If required, an appropriate solvent control (DMSO, 0.05% v/v) was analysed, too.

2.1.7 Immunofluorescence analysis

α-Actin staining

SMC were washed twice in PBS [140 mM NaCl, 2,7 mM KCL, 9 mM Na_2HPO_4, 1,5 mM KH_2 PO_4 (pH 7,4)] and fixed in 3.3% p-formaldehyde (30 min, at ambient temperature followed by 30 min, at 4°C). To inactivate aldehyde groups cells were treated with 50 mM ammoniumchloride (30 min). Thereafter, cells were subsequently blocked with blocking solution II (0.2% TritonX in blocking solution I, 5 min), and blocking solution I (0.2% fish skin gelatine in PBS, 10 min), followed by incubation with the mouse anti-human α-actin antibody (Sigma) for 30 min. To visualise the bound primary antibody, goat anti-mouse IgG conjugated with Alexa Fluor 488 (R&D systems, Germany) was applied at a dilution of 1:100 (30 min, in the dark). Glass slides were prepared using 15 µl of 50% glycerin and analysed using an inverted epifluorescence microscope (Axiovert S 100; Zeiss, Germany).

Staining for VCAM-1 expression

SMC were washed twice with Hepes-Tyrode and incubated with a monoclonal mouse anti-human VCAM-1 antibody (R&D systems, Germany) at a dilution of 1:100 (60 min, ambient temperature). After 3 washes cells were fixed with 3.3% p-formaldehyde (30 min) followed by incubation with amoniumchloride (10 min). After that 0,1% saponin (diluted in Hepes-Tyrode buffer) was added to avoid unspecific binding (10 min) followed by a 30-min incubation with a goat anti-mouse IgG conjugated with Alexa Fluor 488 at a dilution of 1:100 in the dark. Glass slides were prepared as described above.

2.1.8 Cell interaction assay

For cell interaction studies, cell culture inserts containing PET membranes (BD Falcon, Heidelberg, Germany) with a pore size of 8.0 µm and a density of 1 x 10^5 pores/cm^2 were fitted into 12-well plates (Techno Plastic Products AG, Trasadingen, Switzerland). SMC were allowed to grow on the PET membranes until reaching confluence. Thereafter, SMC were pre-stimulated with IFNγ plus TNFα for 24 h, medium was changed for 12 h and then the SMC were co-incubated with the P3xTB.A7 or P3x63Ag8.653 myeloma cells (1x10^6 cells/mL) for 12 h. Co-incubations

were terminated by removing the supernatant, followed by a thorough washing of the SMC layer with medium M199 to remove the myeloma cells. For subsequent migration studies, $1x10^6$/mL THP-1 cells were presented to the SMC side of the membrane in the presence of 100 ng/mL recombinant MCP-1 (R&D Systems, Wiesbaden, Germany) in lower or sub-SMC compartment (trans) and allowed to migrate for 12 h. In some experiments, SMC were pre-incubated for 90 min with the CD154-neutralizing antibody before addition of the THP-1 monocytes. THP-1 cells that migrated through the SMC layer were quantified by counting under the light microscope, followed by RNA isolation and RT-PCR analysis or IL-1ß protein determination.

2.2 Molecular biology methods

2.2.1 Isolation of total RNA

Total RNA was extracted from cultured smooth muscle cells, THP-1 cells or myeloma cells by using the RNeasy kit® (Qiagen, Germany) according to the manufacturer's instructions. Briefly, cells cultured either in 6-well plates or 12-well plates (TPP, Germany) were washed twice with PBS before being disrupted by the addition of RLT buffer and homogenized by 5 times passing through a 20-gauge needle. Then, 600 µl 70% ethanol were added and mixed immediately by pipetting. The mixture (approximately 700 µl) was applied twice to an RNeasy® mini column to bind the RNA by centrifugation at 13,000 rpm for 15 s. The column was washed once with 700 µl buffer RW1, and twice with 500 µl buffer RPE. After the last washing step, an additional centrifugation at 13,000 rpm for 1 min was performed to dry the membrane. RNA was eluted into 40 µl RNase-free water and stored at -70°C.

2.2.2 Semi-quantitative RT-PCR

For each sample, 1 µg RNA was mixed with 1 µl oligo-$dT_{(15)}$ (500 ng/µl) in a total volume of 14 µl and denatured by incubation at 70°C for 10 min. After brief centrifugation, 6 µl cDNA-Synthesis-Mix [50 mM Tris/HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 2.5 mM dNTP and 200 U MMLV reverse transcriptase] was added and cDNA synthesis continued for 50 min in a water bath at 42°C. The

reaction was stopped by heating to 70°C for 10 min. Each cDNA sample was diluted with 180 μl H_2O and stored at -20°C.

Semi-quantitative PCR analysis was performed by normalizing to the relative amount of cDNA of a house-keeping gene. For this purpose, the ribosomal protein L32 (RPL-32) was chosen as internal standard. The reagents for PCR amplification were premixed to ensure identical conditions in each reaction [20 mM Tris/HCl (pH 8.4), 1.5 mM $MgCl_2$, 0.2 mM dNTP, 20 pmol primers (for both forward and reverse primers) and 1 U Taq DNA polymerase, in a total volume of 50 μl]. The PCR reaction was performed in a model thermocycler (Eppendorf, Germany) programmed as follows:

Pre-denaturation:	2 min at 94°C
Denaturation:	30 s at 94°C
Annealing:	30 s at the temperatures shown in Table 2
Extension:	30 s-1 min (depending on the fragment size) at 72°C
Cycles:	primer-specific number as shown in Table 2
Final extension:	5 min at 72°C

After the amplification, PCR products were separated on 1.7% agarose gels with ethidium bromide staining. The density of the detected bands was determined by using PhotoFinish® imaging system (WordStar Atlanta Technology Center, USA) and One-Dscan® (software, Scananlytics, Billerica, USA). These results permitted adjustments for the volume of cDNA administered in order to obtain (in a PCR reaction of 20 cycles) the identical quantity of the product for the housekeeping gene (RPL-32). With that amount of cDNA fixed, the PCR reactions were performed with specific primers for the genes of interest.

Table 2. RT-PCR primers employed

gene product	GeneBank accession position	Length (bp)	No. of cycles	Annealing temperature	Primer (for/rev)
CCR7	XM_049959 329-794	465	33	60°C	TCAAGACCATGACCGATACC AGGTAACAGAAGCTCATGGC
CD154	X96710 231-559	328	33	55°C	GAGATGCAACACAGGAGAAAGATCC TGGAACAGAAGGTGACTTGGGC
CD40	NM001250 202-582	380	25-27	60°C	CAGAGTTCACTGAAACGGAATGCC TGCCTGCCTGTTGCACAACC
E-sel	M30640 2824-3283	459	31-33	60°C	AGCAAGGCATGATGTTAACC GCATTCCTCTCTTCCAGAGC
GRO-γ	M36821 175-400	225	27	60°C	GAGCGTCCGTGGTCACTGAA TGTCAGTTGGTGCTCCCCTT
IL-1β	M54933 226-555	329	29-31	59°C	GGCATCCAGCTACGAATCTCCG CACTTGTTGCTCCATATCCTGTCCC
IL-8	XM_170504 149-655	506	27-29	58°C	GCCAAGGAGTGCTAAAGAAC CTCCCGTGCAATATCTAGGA
IL-15 receptor α	NM_002189 313-774	461	27	62°C	GAACAAGGCCACGAATGTCG CTTGACTTGAGGTAGCATGC
IL-23p19	NM_016584.1 260-719	459	35-36	61°C	CACAGAAGCTCTGCACACTGGC TAGGGACTCAGGGTTGCTGCTC
MCP-1	X14768 46-376	330	22-24	63°C	GCGGATCCCCTCCAGCATGAAAGTCTCT ACGAATTCTTCTTGGGTTGTGGAGTGAG
MIP-3α	U64197 51-313	262	31-33	60°C	TACCAAGAGTTTGCTCCTGG TTTTACTGAGGAGACGCAC
MMP-3	NM_002422.2 790-1083	293	30-33	60°C	CTCACAGACCTGACTCGGTT CACGCCTGAAGGAAGAGATG
RPL-32	X03342 91-459	368	18-22	60°C	GTTCATCCGGCACCAGTCAG ACGTGCACATGAGCTGCCTAC
VCAM-1	X53051 729-1252	523	22-24	63°C	CATGACCTGTTCCAGCGAGG CATTCACGAGGCCACCACTC

2.2.3 Sequencing of the PCR product

A 10 μl sequencing reaction was set up by mixing 5 μl purified PCR product (100 ng), 3 μl BigDye Terminator DNA-sequencing-mix (Applied Biosystems, Damstadt), 1 μl GSP3 primer (1 pmol/μl) and 2 μl sterilized water. The sequencing PCR was

performed for 25 cycles as follows: denaturation at 94°C for 30 s, annealing at 55°C for 15 s, extension at 60°C for 4 min.

After the sequencing PCR, the products were precipitated by adding 80 μl H_2O, 15 μl 2 M NaAC (pH 4.0), 250 μl 100% ethanol and centrifugation at 14,000×g for 20 min. The pellet was washed once with 250 μl 70% ethanol and then dissolved in 5 μl H_2O. The products were sequenced by using a model 3100 Genetic Analyser (Applied Biosystems, Damstadt).

2.2.4 Decoy ODN technique

Double-stranded (ds) decoy ODN were prepared from the complementary single-stranded (ss) phosphorothioate-bonded ODN (Eurogentec, Germany) by melting at 95°C for 5 min followed by a cool-down phase of 3 h at room temperature. The efficiency of the hybridisation reaction was checked on 2.5 % agarose gels containing 0.1 % ethidium bromide and usually found to exceed 80 %. The ds decoy ODN were pre-incubated with the cultured SMC for 4 h at a concentration of 10 μM. Thereafter, the decoy ODN-containing medium was replaced with fresh medium containing P3xTB.A7 cells for additional 12 h. The ss sequences of the decoy ODN were as follows (orientation 5' to 3', underlined letters denote phosphorothioate-bonded bases): activator protein-1 (AP-1): CGCTTGATGACTCAGCCGGAA, CCAAT/enhancer binding protein (C/EBP) TGCAGATTGCGCAATCTGCA, and NF-κB: AGTTGAGGGGACTTTCCCAGGC.

2.2.5 Western blot analysis

For Western blot analysis of CD40 protein expression, cells were scraped off the plate into 1 ml HBSS and then pelleted by centrifugation at 500×g for 5 min. The cell pellet was resuspended in lysis buffer [50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 1 mM DTT, with Proteinase Inhibitor Mix, 30 μl/well] and lysed by 5 cycles of freeze/thawing in liquid nitrogen and at 37°C, respectively. After centrifugation at 13000 g for 5 min, the supernatant was collected into a new tube. Protein concentration of cell extracts was estimated with the modified Bradford protein assay from BioRad (Munich, Germany) according to the manufacturer's instructions.

Sample protein (30 μg) was denatured by heating to 94°C for 5 min in 1× loading buffer (Roth, Karlsruhe), and then separated on a 12% denaturing polyacrylamide gel. Electrophoresis was performed at constant current (20 mA per gel) for approximately 2 h. Subsequently, the separated proteins were electronically transferred to a BioTrace polyvinylidene fluoride transfer membrane (Pall, Dreieich) with Mini-Trans Blot system (Bio-Rad, Muenchen) at 350 mA for 45 min.

The membrane was blocked with 5% non-fat milk in PBS at room temperature for 1 h followed by incubation with the primary antibody at 4°C overnight. Human CD40 was detected by using a polyclonal rabbit anti-human CD40 antibody (Research Diagnostics, Flanders, NJ) at a dilution of 1:2,000. HRP-conjugated goat anti-rabbit IgG antibody (Sigma, Germany) was used as the secondary antibody at a dilution of 1:10,000. The detected bands were visualized by SuperSignal® Chemiluminescent Substrate (Pierce, Rockford, USA) followed by exposure of the membrane to X-ray film (Kodak, Germany) for 1 min.

2.2.6 DNA microarray analysis

Cytokine pre-stimulated cultured SMC [obtained from passage 3 (p3)] were incubated either with P3xTB.A7 or P3x63Ag8.653 control cells for 12 h. Total RNA was isolated as described in 2.2.1. Human GeneChips® (HG-U95Av2) were purchased from Affymetrix (Santa Clara, CA). Array HG-U95Av2 is comprised of ≈12,000 sequences, most of which are derived from previously characterized full-length genes (≈10,000); each gene is represented by ≈16 non-overlapping oligonucleotide probes (25-mers). RNA conversion to cDNA, labelling with CY3 or CY5 fluorescence dye, hybridization, and signal intensity normalization were carried out at the Core Unit DNA Technologies of the IZKF/University of Leipzig. Data indicating presence or absence of gene expression (presence/absence call, determined by Affymetrix) were sorted, compared, and statistically analyzed by using the Affimetrix Microarray Suite 5.0 software. Genes were considered up-regulated or down-regulated if the expression was changed at least 1.9-fold from control. Fold change in this case indicates the relative difference in signal intensity for the gene between the two samples. Only reliable and consistent mean values from three independent experiments with an appropriate standard deviation were considered. Data with low signal intensity, high background, and high variability among

experiments were eliminated. Genes were designated according to the annotations from Affymetrix and the GenBank databases.

2.2.7 ELISA

For the quantitative determination of IL1-1ß concentrations, THP-1 cells were collected from the lower compartment of the cell-interaction chamber (2.1.8). For detection of a humoral stimulatory factor SMC were cultured in 6-well plates until reaching confluence followed by stimulation with IFNγ (1000 U/ml) plus TNFα (100 U/ml) for 24 h, medium exchange for 12 h and subsequent exposure to the P3xTB.A7 or P3x63Ag8.653 control cells ($1x10^6$ cells/mL) for 12 h. Supernatant was taken, centrifuged, diluted in a ratio of 1:1 with fresh DMEM medium containing 15 % FCS and incubated in a new 6-well plate with THP-1 cells ($1x10^6$ cells/mL) for 12 h. After this time THP-1 cells were collected, washed twice with PBS and lysed with lysis buffer [1 M Tris-HCl (pH 8.0), 5 M NaCl, NP-40, glycerol, proteinase inhibitor mix]. After centrifugation (13000 rpm, 80 min, +4°C), the supernatant containing the cell lysate was taken and used for IL-1ß immunoassay. To this end the human IL-1ß QuantiGlo® ELISA Kit (R&D Systems, Wiesbaden, Germany) was used according the manufacturer's instructions. Standards (dilution series from 0.5 pg/ml to 5000 pg/ml) and samples (100 µl) were pipetted into the wells pre-coated with IL-1ß monoclonal antibody. After incubation on the horizontal orbital micro plate shaker (Ika-Labortechnik, Germany) (0.12" orbit, 500 rpm, 2 h, ambient temperature) and repeated washing steps, an enzyme-linked polyclonal antibody specific for IL-1ß was added to the wells and incubated (for 3 h at ambient temperature with constant shaking). The final step was performed by adding luminol/peroxide substrate solution to the wells for 30 min and light was produced in proportion to the amount of IL-1ß bound in the initial step. A microplate luminometer was used to measure the intensity of the light emitted.

2.3 Statistical analysis

Unless indicated otherwise, results are expressed as mean ± SEM of n independent observations (i.e. samples from different batches of SMC). Statistical evaluation was performed by Student's t-test for unpaired data with the Instat for Windows™ version

3.05 statistics software package (GraphPad Software, San Diego, USA) and a p value < 0.05 was considered statistically significant.

3. Results

3.1 Characterisation of thymus vascular smooth muscle cells

Smooth muscle cells derived from the thymus veins of children (not older than 2 years of age) were the model used throughout this study. The veins were isolated from thymus tissue that had to be resected in the course of heart surgery. The cells were isolated as described and used for experiments up to passage 4. Examination by phase-contrast microscopy revealed that the cells uniformly display the typical 'hill and valley' pattern of cultured SMC and form multilayers upon reaching confluence.

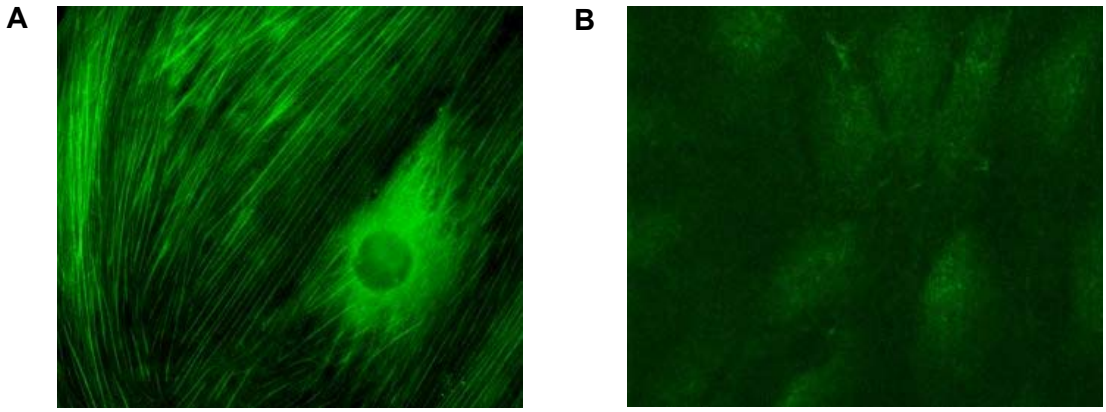

Figure 1. Typical results of immunofluorescence analyses for SMC and endothelial cells markers: Immunofluorescence analysis was performed as described in Materials and methods. A) Smooth muscle α-actin expression in human cultured SMC. B) SMC stained for *von Willebrand* factor (essentially negative). Original x 400.

In addition, immunofluorescence analysis for smooth-muscle α-actin and *von Willebrand* factor, a marker for endothelial cells, was routinely performed. Whereas 98-100% of the cells stained positive for smooth-muscle α-actin with the typical fibrillar pattern, they were essentially negative for *von Willebrand* factor (human cultured endothelial cells were used as a positive control for this factor, not shown).

3.2 Stimulation of SMC with IFNγ/ TNFα induces expression of CD40

As outlined in 1.4, the central aim of this work was to elucidate the functional consequences of CD40 activation in SMC under pro-inflammatory conditions.

To this end, first the expression of CD40 under these conditions was studied. RT-PCR analysis resulted in the amplification of a DNA fragment with the expected size of approximately 380 bp already under control conditions, i.e. in untreated SMC. Subsequent sequence analysis confirmed the identity of the amplified product. The abundance of CD40 mRNA was markedly increased after exposure of the SMC to pro-inflammatory cytokines. Shown is the combination of TNFα (100 U/mL) and IFNγ (1000 U/mL) for 24 h (Fig. 2).

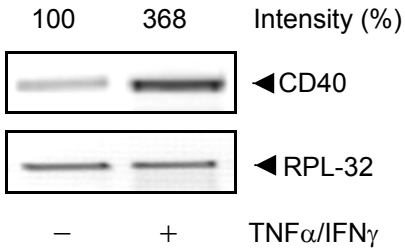

Figure 2. Expression of CD40 mRNA in SMC under basal conditions and following stimulation with TNFα (100 U/mL) and IFNγ (1000 U/mL) for 24 h. The relative intensity (% of control, measured by densitometry) is indicated at the top of the panel. The figure depicts the result of one representative experiment. Similar results were obtained in 2 additional experiments with different batches of SMC. The cDNA of RPL-32 was used as an internal standard to normalize the cDNA load in the individual samples.

Moreover, cytokine induced up-regulation of CD40 in SMC was time-dependent, reaching a plateau phase after approximately 24 h (Fig. 3).

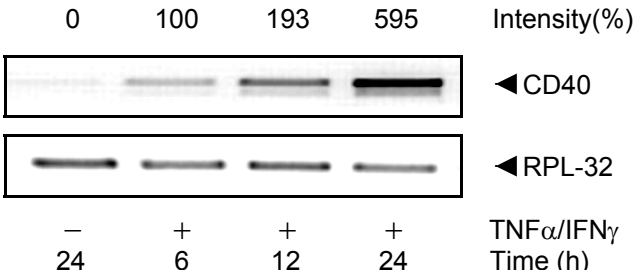

Figure 3. Time-dependent effect of TNFα (100 U/mL) and IFNγ (1000 U/mL) on CD40 mRNA expression. The relative intensity (% of TNFα/IFNγ treatment after 6 h) was measured by densitometry, indicated at the top. The figure depicts the result of one representative experiment. Similar results were obtained in 2 additional experiments with different batches of SMC. RPL-32 was used as an internal standard as described above.

To confirm these data on the protein level, Western blot analyses were performed. CD40 protein was detected as an immunoreactive band corresponding to a

molecular mass of approximately 49 kDa. In contrast to CD40 mRNA, the protein was only detectable upon exposure of the SMC to IFNγ (1000 U/mL) alone or IFNγ (1000 U/mL) plus TNFα (100 U/mL), (Fig. 4).

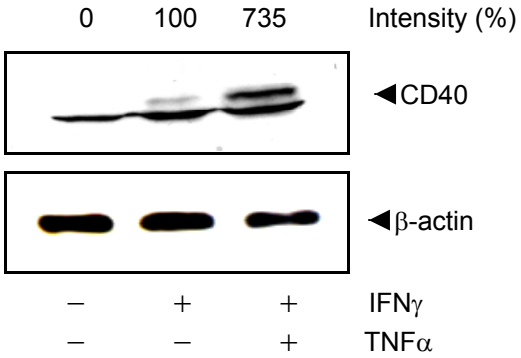

Figure 4. Expression of CD40 protein under basal conditions and following stimulation with IFNγ (1000 U/mL) or IFNγ plus TNFα (100 U/mL) for 24 h. Representative Western blot analysis. Loading and transfer of equal amounts of protein in each lane was verified by reprobing the membrane with an anti-β-actin antibody.

Like CD40 mRNA expression, cytokine-induced CD40 protein expression was rather slow reaching a significant level only after 24 h (Fig. 5). Although higher amounts of TNFα (1000 U/mL plus 100 U/mL IFNγ) resulted in a more prominent induction of CD40 protein expression (Fig. 5 right lane), this cytokine combination was omitted from further experiments as the viability of the cultured SMC was negatively affected (not shown). Thus, for all further experiments the cytokine combination 1000 U/mL IFNγ plus 100 U/mL TNFα was employed.

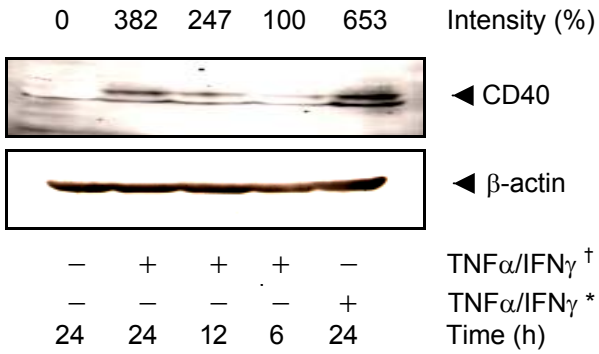

Figure 5. Time course of CD40 protein expression in TNFα/IFNγ primed SMC. Expression of CD40 protein was both time and stimulus dependent († refers to 100 U/ml TNFα and 1000 U/ml IFNγ; * refers to 1000 U/ml TNFα and 100 U/ml IFNγ). Representative Western blot analysis. Loading and transfer of equal amounts of protein in each lane was verified by reprobing the membrane with an anti-β-actin antibody.

3.3 CD40-induced expression of MCP-1 and MMP-3 in SMC

After having characterized cytokine-induced expression of CD40 in cultured human SMC, the next step was to analyse which gene products were expressed upon CD40 activation. For this purpose, SMC were first exposed to the cytokine mixture (100 U/ml TNFα and 1000 U/ml IFNγ) found to optimally induce CD40 expression over 24 h, followed by a 12 h resting phase in which the cytokines were removed from the cell supernatant.

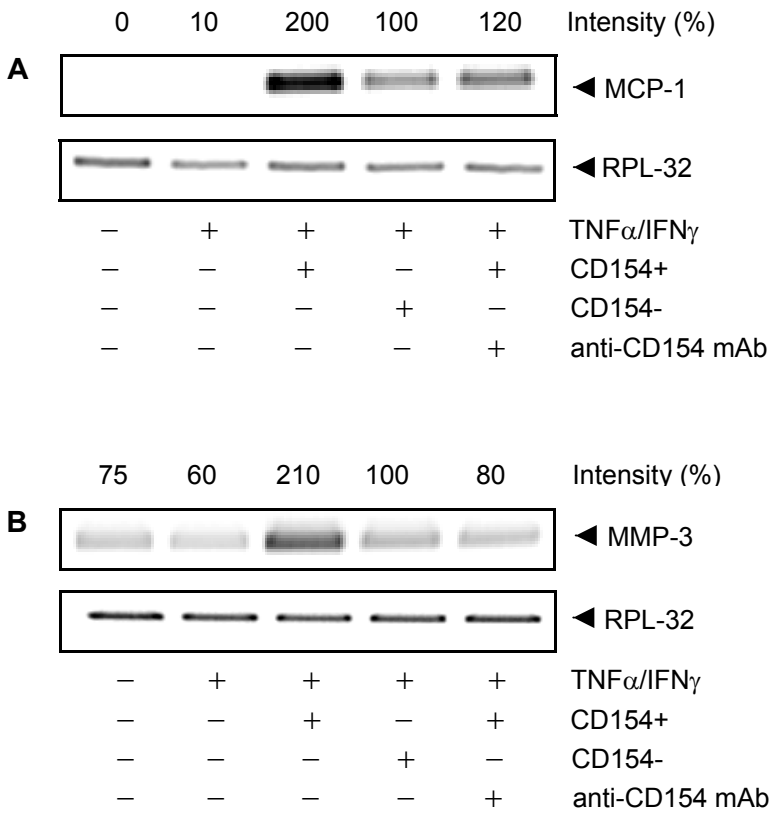

Figure 6. CD154 up-regulation of the expression of (A) MCP-1 and (B) MMP-3 in cytokine-stimulated SMC. SMC pre-stimulated with TNFα/IFNγ for 24 h were co-incubated with P3xTB.A7 cells (CD154+), P3x63Ag8.653 cells (CD154-), or P3xTB.A7 cells plus 40 µg/mL of the neutralizing anti-CD154 antibody. The cDNA coding for RPL-32 was used as an internal standard. MCP-1 and MMP-3 mRNA abundance are expressed as the percentage of the control (i.e. SMC treated with TNFα/IFNγ plus non-transfected myeloma cells). Virtually identical results were obtained in 2 further experiments each with different batches of cells.

To activate CD40, the cytokine-primed SMC were co-incubated with the P3xTB.A7 mouse myeloma cells stably transfected with human CD154 ($1x10^6$ cells/mL). Co-incubations with P3x63Ag8.653 control cells or with P3xTB.A7 cells in the presence

of a neutralising monoclonal anti-CD154 antibody (40 µg/mL) were used as a control. This amount of antibody has previously been determined to completely inhibit CD154-induced CD40-signalling in human cultured endothelial cells (Lienenlueke et al., 2000).

Next, a broad and representative spectrum of gene products known to be up-regulated in SMC under pro-inflammatory conditions was analysed by RT-PCR: the PDGF-receptor α, the matrix proteins fibronectin and tenascin C, the modulator of secondary hemostasis thrombospondin, the chemokine MCP-1 and the matrix metalloprotease MMP-3. Surprisingly, activation of CD40 resulted in the up-regulation of only MCP-1 and MMP-3 expression in the cultured SMC, suggesting a specific and selective capacity of CD40 to change the gene expression profile of these cells.

3.4 Signalling pathway involved in CD40 dependent MMP-3 activation

To study the signalling mechanisms involved in CD40-induced gene expression, MMP-3 was chosen as a readout. SMC were first exposed to several agents known to interfere with cAMP signalling.

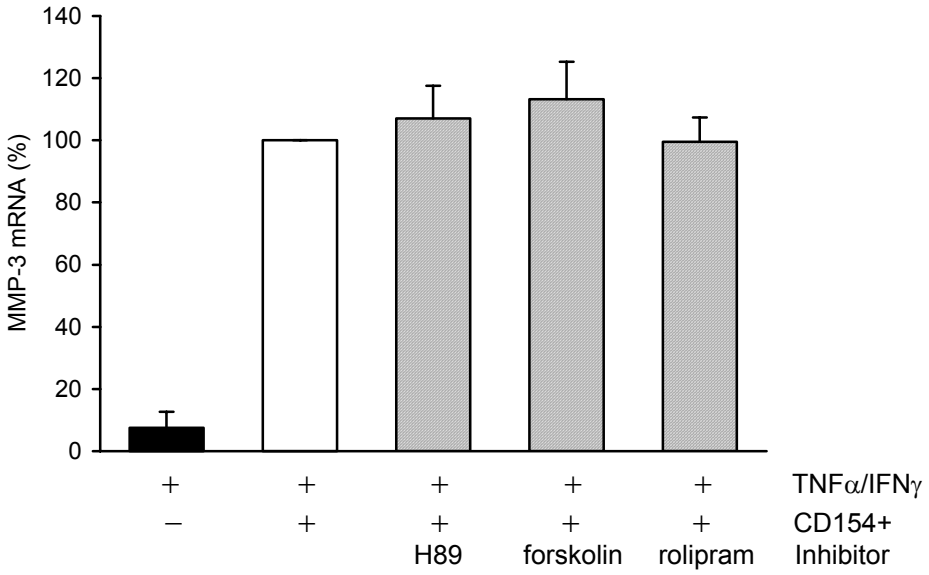

Figure 7. Effects of cAMP pathway inhibitors on CD154-induced MMP-3 expression in cultured SMC. The CD40-expressing cells were pre-treated with H89 (1 µM, n=3), forskolin (10 µM, n=4) or rolipram (12.5 µM, n=4) for 1 h before exposure to the P3xTB.A7 cells (CD154+) as described before (Fig. 6). Statistical summary of MMP-3 mRNA abundance, expressed as percentage of CD40-stimulated SMC; bars show the standard error.

Neither a specific protein kinase A inhibitor (H89) nor an adenylyl cyclase activator (forskolin) or a phosphodiesterase IV inhibitor (rolipram) exerted inhibitory effects on CD154-induced MMP-3 expression, as shown in Fig. 7.

The putative involvement of other protein kinases was examined by using the corresponding inhibitors. These experiments revealed that neither inhibition of the classical protein kinase C (PKC inhibitor RO 31-8220) or MAP-kinase pathway (MEK-1→ ERK1/ERK2; PD 98059 was used as a specific inhibitor of MEK-1) nor that of the Jun kinase/SAPK pathway (JNK inhibitor SP 600125) affected CD154-induced MMP-3 expression in the cultured SMC (Fig. 8).

However, both Src tyrosine kinase (herbimycin A) and p38 MAP kinase (p38-MAPK; specific inhibitor: SB 202190) activation seemed to be essential for CD154-induced MMP-3 expression. Thus each inhibitor reduced CD154-induced MMP-3 expression by approximately 70% (Fig. 8). The PI-3 kinase inhibitor wortmannin, on the other hand, had no effect on CD154-induced MMP-3 expression, while the Rho kinase inhibitor, Y 27632, augmented this almost 2 fold (Fig. 9).

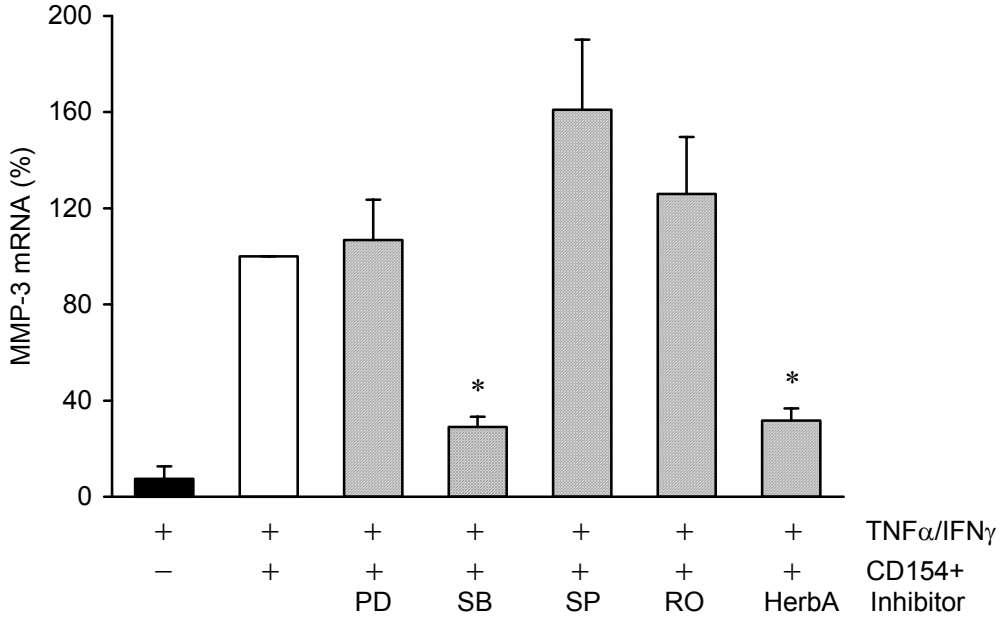

Figure 8. Effects of different protein kinase inhibitors on CD154-mediated MMP-3 expression in the cultured SMC. Cells expressing CD40 were pre-treated with PD 98059 (PD, 50 µM, n=4), SB 202190 (SB, 10 µM, n=5), SP 600125 (SP, 20 µM, n=3), RO 31-8220 (RO, 1 µM, n=5) or herbimycin A (HerbA, 1 µM, n=6) for 1 h before exposure to the P3xTB.A7 cells (CD154+) as described before (Fig. 6). MMP-3 mRNA abundance in the individual samples is compared to that in CD154-stimulated control cells (set to 100%). $^{*}P<0.05$ versus positive control.

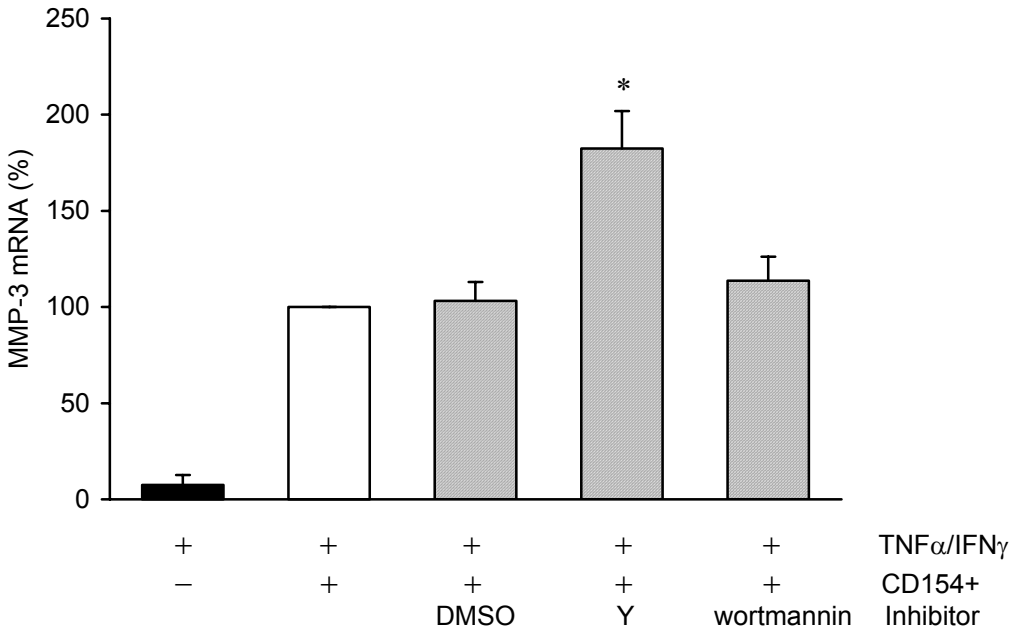

Figure 9. Effects of Y27632 on CD154-induced MMP-3 expression in the cultured SMC. CD40-expressing cells were pre-treated with Y 27632 (Y, 10 µM, n=7), wortmannin (100 nM, n=3) or the vehicle (DMSO, 1 µL/mL, n=3) for 1 h before exposure to the P3xTB.A7 cells (CD154+) as described before (Fig. 6). MMP-3 mRNA abundance in the individual samples is expressed relative to that in CD154-stimulated control cells (set to 100%). $^{*}P<0.05$ versus positive control.

3.4.1 Increase in MMP-3 mRNA expression induced by CD154 stimulation is due to transcriptional activation

Based on the finding that p38 MAPK is involved in CD154-induced MMP-3 expression (Fig. 8), the hypothesis was tested that p38 MAPK exerts this effect through mRNA stabilization (Reunanen et al., 2002) rather than activation of transcription. To determine whether the CD154-induced increase in steady state levels of MMP-3 mRNA is due to a decrease in MMP-3 mRNA degradation, the stability of MMP-3 mRNA was measured in the presence of the inhibitor of transcription actinomycin D (ActD). Cells were treated with P3xTB.A7 mouse myeloma cells for 12 h, after which time ActD (final concentration 1 µM) was added. Total RNA was isolated 0-12 h after the addition of ActD and analysed by RT-PCR. MMP-3 mRNA abundance decreased to approximately 60% over 12 h, indicating that the increase in MMP-3 mRNA observed after CD40 activation is due to increased synthesis (i.e. transcription) rather than stabilization of MMP-3 mRNA.

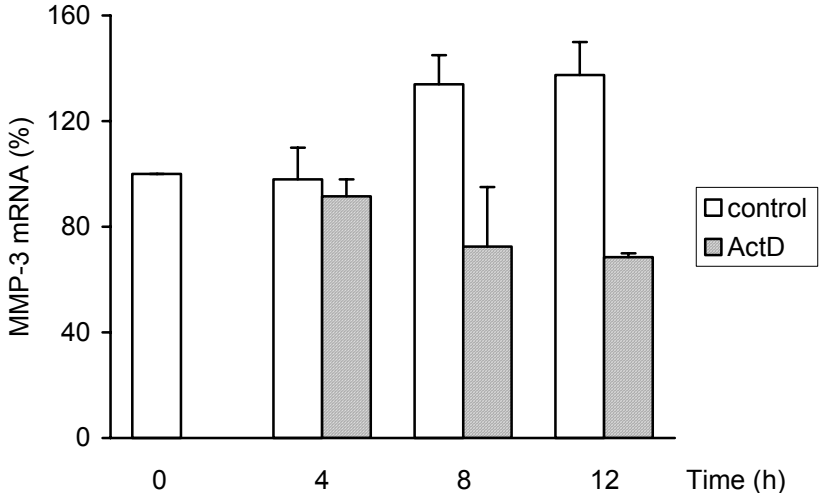

Figure 10. Analysis of MMP-3 mRNA stability. ActD was added after 12 h stimulation of the cultured SMC with CD154+, and cells were collected at the indicated time points. MMP-3 mRNA abundance in the individual samples is expressed relative to that in CD154-stimulated control cells at time 0 (set to 100%).

To confirm that CD154-induced MMP-3 expression is due to an increase in *mmp-3* gene transcription, cells were first treated with ActD (1 μM) for 1 h to inhibit transcription followed by exposure to the P3xTB.A7 myeloma cells for 12 h.

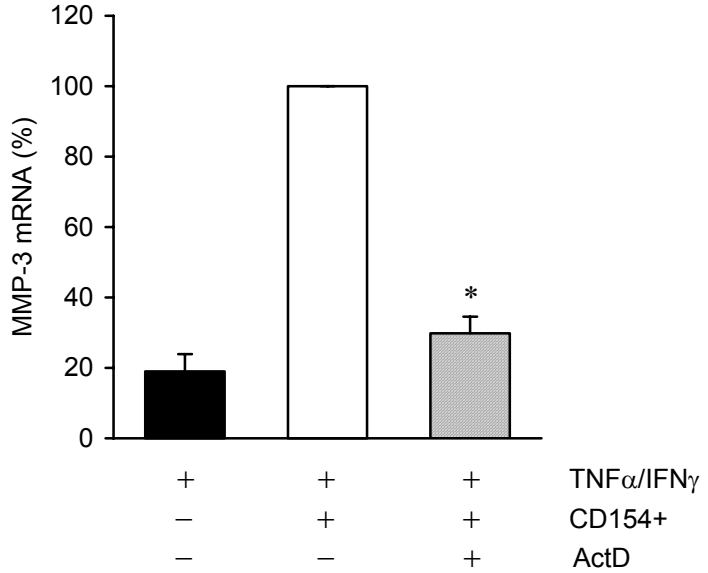

Figure 11. Effect of Actinomycin D (ActD) on *mmp-3* gene transcription. ActD was applied at 1μM to the SMC 1 h prior to CD40 stimulation. Statistical summary of three individual experiments. $^{*}P<0.05$ versus positive control (CD154-stimulated SMC). For experimental details refer to Fig. 6.

CD154-induced MMP-3 expression was almost completely abolished in the presence of ActD when compared to CD40 stimulation only, suggesting that the increase in MMP-3 mRNA expression in response to CD40 activation is indeed due to an increase in *mmp-3* gene transcription.

3.4.2 CD154-induced MMP-3 mRNA expression does not require *de novo* protein synthesis

To evaluate whether the induction by CD154 of *mmp-3* gene transcription requires *de novo* synthesis of a transcription factor, CD40-expressing SMC were treated with cycloheximide (1 µg/mL) 1 h prior to addition of the P3xTB.A7 myeloma cells to stimulate MMP-3 mRNA expression for 12 h. Inhibition of protein synthesis significantly augmented rather than inhibited MMP-3 mRNA expression (Fig. 12), suggesting that a latent transcription factor(s) is involved in CD154 stimulation of *mmp-3* gene transcription.

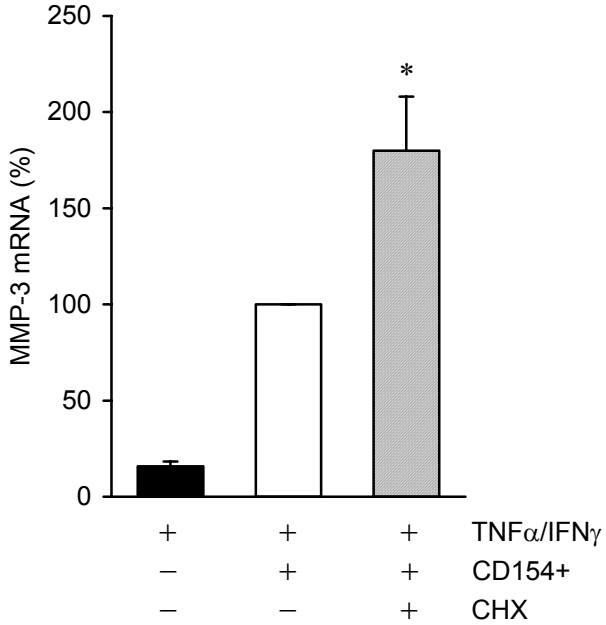

Figure 12. Superinduction by cycloheximide (CHX) of CD154-stimulated MMP-3 mRNA expression. CHX pre-treatment (1 µg/mL) was done for 1 h and MMP-3 mRNA expression monitored by RT-PCR analysis over 12 h in SMC exposed to the P3xTB.A7 mouse myeloma cells (CD154+). Statistical summary (n=3). $^{*}P<0.05$ versus positive control (CD154-stimulated SMC).

3.4.3 Effects of decoy ODN

In an attempt to characterize the transcription factor(s) involved in CD154-induced MMP-3 expression in the cultured SMC, the decoy oligonucleotide (dODN) technique was employed. Decoy-ODNs are short double-stranded DNA molecules that readily enter cells *in vitro* and *in vivo* without any additional transfection reagent. They mimic the binding site of their target transcription factor in the cellular DNA. As a consequence, the transcription factor is effectively neutralised when interacting with the dODN instead of its natural DNA-binding site.

Three different transcription factors were hypothesized to play a role in MMP-3 mRNA expression, namely AP-1, C/EBP and NF-κB, because it is known that they are activated by CD40 or c-src/p38 MAPK. Therefore, dODN against these three factors at a final concentration of 10 µM were applied for 4 h prior to CD154 stimulation. None of them had an effect on MMP-3 mRNA levels (Fig. 13). To check the activity of the dODN applied, MCP-1 mRNA expression was assessed in parallel since it is known that NF-κB and C/EBP are involved in the transriptional regulation of this gene (Schwabe et al., 2001). MCP-1 expression was effectively reduced by the dODN directed against both NF-κB and C/EBP, thus confirming their biological activity.

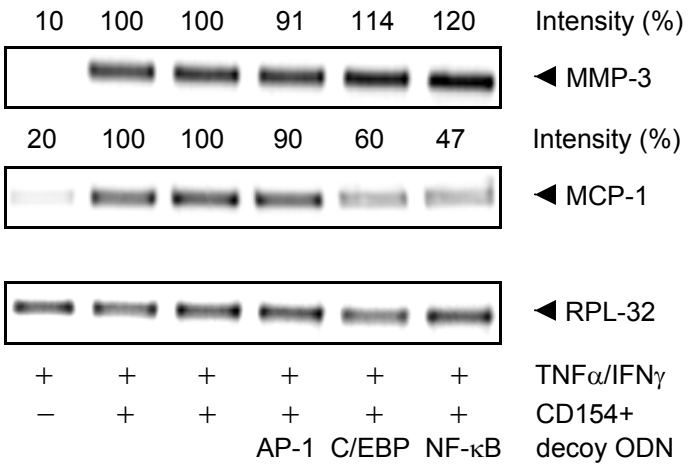

Figure 13. Effects of the indicated consensus decoy ODN on CD40 induced MMP-3 and MCP-1 mRNA expression. The figure is representative of 3 independent experiments. The cDNA encoding RPL-32 was used as an internal standard.

3.5 Gene expression profile in SMC after CD40 ligation analysed by DNA microarray

To obtain a more detailed view of the gene expression profile in CD154-stimulated SMC, a DNA microarray analysis was performed using the human GeneChip Expression Array by Affymetrix which detects approximately 10,000 individual human genes. Differential gene expression was analysed in three independent batches of SMC essentially treated as described in 3.3. In brief, the SMC (passage 3, 10^7 cells collected from 6-well plates to obtain approximately 10 μg of total RNA) were primed with TNFα plus IFNγ (100 and 1000 U/mL, respectively) for 24 h followed by cytokine removal and the exposure to either the P3xTB.A7 mouse myeloma cells (CD154+) or P3x63Ag8.653 control cells (CD154-) for 12 h before lysis and extraction of total RNA.

Analysis of the 3 microarray experiments was designed as follows. To be counted as truly differentially expressed, a given gene product had to accomplish three criteria:

1) Expression of the gene product in question had to be uniformly high in the individual microarray analysis in order to exclude hybridisation artefacts and background signals.
2) The difference (increase or decrease) in signal strength had to be at least 1.9-fold.
3) The two criteria defined above had to be fulfilled in all three independent microarray analyses performed.

As a result, 36 gene products out of 10,000 were scored to be differentially expressed. Of these, 2 were down- and 34 up-regulated. The results are summarized in tables 3 and 4. Interestingly, most of the differentially expressed gene products are associated with inflammatory reactions.

Differentially expressed gene products in CD154-stimulated SMC according to DNA microarray analysis

Table 3: Secreted mediators, cell adhesion molecules, receptors and pro-inflammatory molecules

Gene product	Fold ± S.E.M	Class/Function
MIP-1α	2.7 ± 0.9	C-C Chemokine ligand-3
MIP-3α	2.9 ± 0.3	C-C Chemokine ligand-20
RANTES	2.1 ± 0.3	C-C Chemokine ligand-5
GM-CSF	4.1 ± 2.3	Cytokine involved in monocytes activation
GRO-β	2.0 ± 0.5	Chemokine (C-X-C motif) ligand 2
GRO-γ	3.3 ± 0.5	C-X-C Chemokine ligand-3
MCP-2	1.9 ± 0.5	C-C Chemokine ligand-8
Interleukin-1β	3.0 ± 1.6	Member of the interleukin 1 cytokine family
Interleukin-15	2.1± 0.0	Cytokine of the four-helix bundle family
Interleukin-15rα	1.9 ± 0.4	Binds IL-15 with high affinity
VCAM-1	1.9 ± 0.3	Ligand for VLA-4
E-Selectin	7.2 ± 3.0	Selectin family of cell adhesion molecules
Ninjurin	2.6 ± 0.4	Adhesion molecule
CCR7	4.9 ± 0.8	C-C Chemokine receptor-7
CD40	2.2 ± 0.5	Member of TNF receptor superfamily
TNFAIP6	2.8 ± 0.4	Member of hyaluronan-binding protein family
Fractalkine	1.9 ± 0.2	C-X-3C chemokine
Cox-2	3.4 ± 2.3	Key enzyme in prostaglandin biosynthesis
MMP-12	2.7 ± 0.8	Member of MMP family
VEGF	2.9± 1.6	Mitogen primarily for vascular endothelial cells

Table 4: Intracellular gene products involved in signal transduction and other gene products induced or repressed (printed in italics)

Gene product	Fold ± S.E.M	Class/Function
Jun-B	2.0 ± 0.1	Participates in AP-1 transcriptional activation
p50-NF-κB homolog	2.6 ± 0.5	Related to p50/p105 NF-κB
NF-κB subunit p100	2.1 ± 0.3	Transcription factor
TRAF1	2.8 ± 0.8	Signal transducer
Zinc finger protein 36	2.0 ± 0.8	Involved in growth factor response regulation
RGP4	0.5 ± 0.1	Inhibits G- protein-mediated MAP kinase activation
MacMarcks	2.0 ± 0.3	Signal transducer mediating cell adhesion
Follistatin-related protein	2.3 ± 0.8	Member of the follistatin-module-protein family
Fas/Apo 1	1.9 ± 0.4	Member ot TNF receptor superfamily
Diubiquitin	3.1 ± 1.1	Ubiquitin family
Id1	0.5 ± 0.1	Member of the Id HLH-family of proteins
Hypothetical protein	2.0 ± 0.3	Similar to MT1E metallothionein 1E
TNFAIP2	2.4 ± 0.2	Plays a role in myeloid development
GC20	2.3 ± 0.8	Translation initiation factor
IAP-1	3.3 ± 1.8	Suppressor of apoptosis
Dual-specificity protein phosphatase	2.1 ± 0.1	Member of protein-tyrosine phosphatases

3.5.1 Validation of the DNA microarray experiments

To confirm the DNA microarray data, mRNA expression of seven CD154-inducible gene products selected for their putative involvement in the development of atherosclerosis was analysed further by RT-PCR (Fig. 14). The identity of all PCR fragments was verified by direct sequencing (not shown). The RT-PCR data fully corroborated the changes in gene expression detected by the DNA microarray technique (Fig. 14). Moreover, RT-PCR analysis confirmed that these gene products were up-regulated to nearby the same extent as detected by the DNA microarray (Table 5).

IL15 receptor α-chain mRNA appeared as a double band. As the PCR primer pair used for this analysis amplified a DNA product including exon-2 of the *il-15rα* gene, this may be explained through differential splicing of the mRNA. IL-15rα-chain mRNA can exist in eight different transcripts resulting from exon-splicing mechanisms within the *il-15rα* gene. From these transcripts two main classes of transcripts can be distinguished that do or do not contain the exon 2-coding sequence (Dubois et al., 1999).

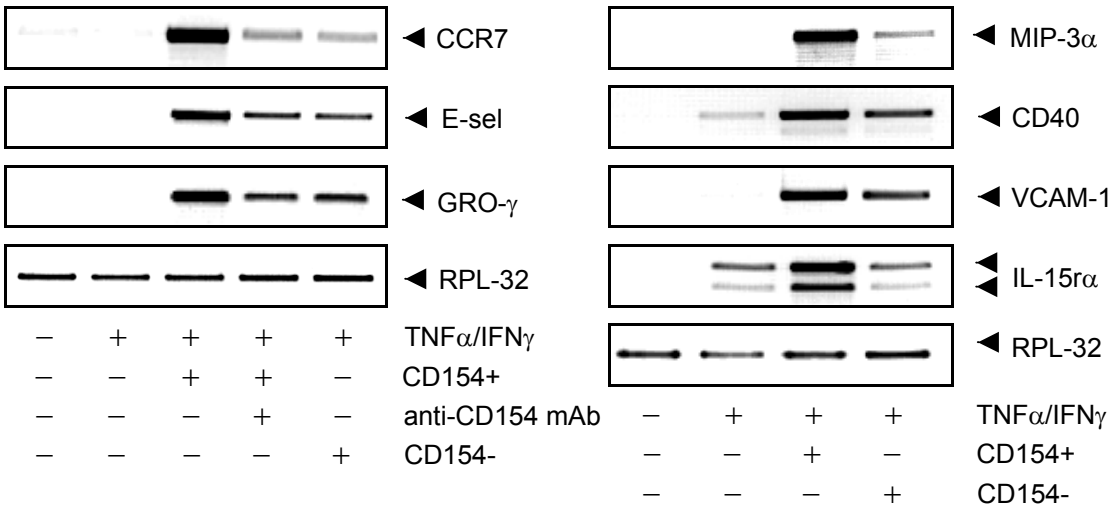

Figure 14. RT-PCR analysis of 7 gene products randomly selected from the list of inducible gene products characterized to be CD154-sensitive in SMC by DNA microarray analysis. For experimental details refer to Fig. 6. Additional incubation with the neutralizing anti-CD154 antibody demonstrates that induction of gene expression is mediated via CD40 activation. The figures are representative of two independent experiments. The cDNA encoding RPL-32 was used as an internal standard.

Interestingly, the microarray results could also be confirmed for CD40 itself, thus CD40 activation also induces its own expression (Fig. 14). The increase in VCAM-1 expression was additionally confirmed on the protein level by immunofluorescence analysis (data not shown).

Table 5. Comparision of data obtained by DNA microarray and RT-PCR analysis with respect to the relative increase in gene expression. Values represent the mean of 3 different experiments (*mean of two experiments).

Gene product	Fold increase by DNA microarray (range)	Fold increase by RT-PCR analysis (*)
CCR7	4.9 (4.0-5.5)	7.1
E-selectin	7.2 (4.8-10.6)	7.5
GRO-γ	3.3 (2.8-3.7)	3.3
MIP-3α	2.9 (2.6-3.1)	4.4
CD40	2.2 (1.6-2.7)	2.2
IL15receptor	1.9 (1.8-2.4)	3.0
VCAM-1	1.9 (1.4-2.4)	1.9

3.6 CD40-mediated SMC activation of monocytes

As described above, CD154 stimulation resulted in an increase in expression of several pro-inflammatory chemokines, their receptors and adhesion molecules in the human cultured SMC. To understand the implications of these findings in the light of vascular inflammation, namely atherosclerosis, a cell interaction assay (Fig. 15) was set-up. SMC cultured on a porous membrane were pre-stimulated with IFNγ plus TNFα followed by co-incubation with the P3xTB.A7 or P3x63Ag8.653 control cells for 12 h. After this time, the SMC were thoroughly washed in order to completely remove the mouse myeloma cells and then co-incubated with THP1 pre-monocytic cells ($1x10^6$ cells/ml). Recombinant MCP-1 (100 ng/mL) was added to the bottom well of the transwell system to serve as a chemoattractant for the monocytes. After 12 h THP-1 cells that had moved to the bottom well were collected and used for further analysis.

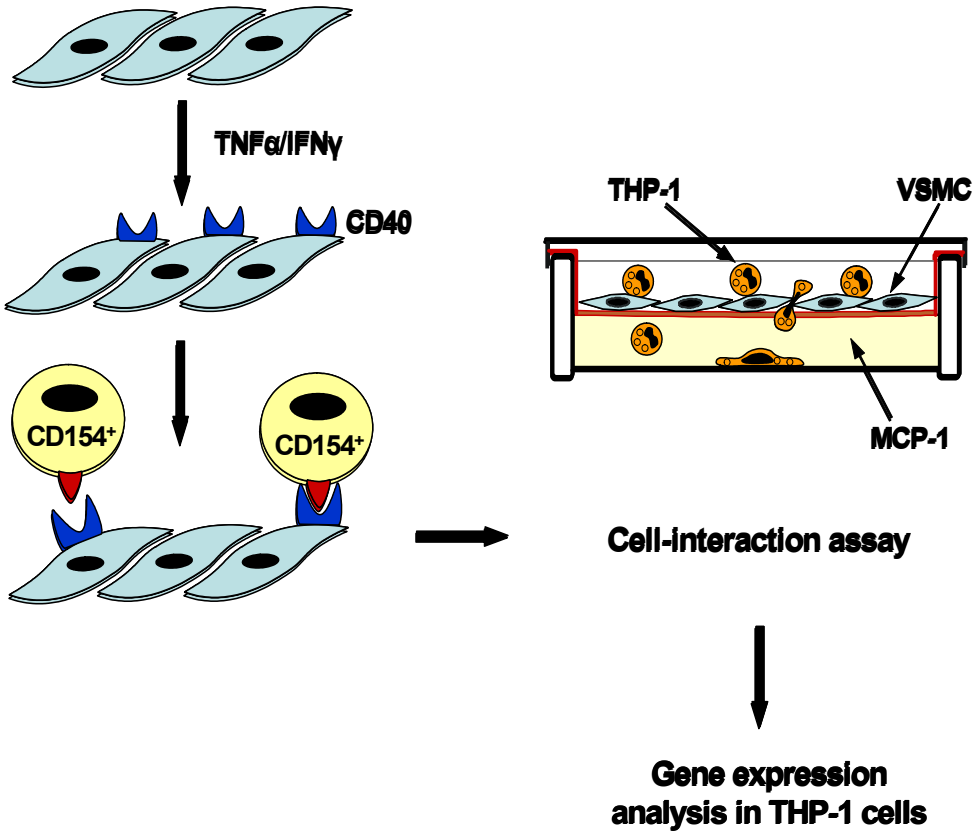

Figure 15. Outline of the cell interaction assay.

Interestingly, no difference was observed in the number of migrating THP-1 cells through the SMC layer irrespective of whether these had been or had not been exposed to CD154. Typically 10%, i.e. approximately 10^5 THP-1 cells migrated into the lower compartment after 12 h of incubation, indicating that the recombinant MCP-1 added to the lower compartment was a highly effective chemoattractant.

RT-PCR analysis revealed that THP-1 cells which came into contact with SMC previously stimulated by CD154, became activated themselves, whereas THP-1 cells interacting with control SMC remained essentially quiescent. As a readout for monocyte activation, IL-23 p19 and IL-1ß mRNA expression was monitored in the transmigrated THP-1 cells and found to be markedly up-regulated (Fig. 16A and B). IL-1ß was also up-regulated on the protein level as demonstrated by ELISA (Fig. 16 C) To avoid a possible contamination with SMC-derived soluble IL-1ß, cell-associated IL-1ß protein was determined in THP-1 cell lysates.

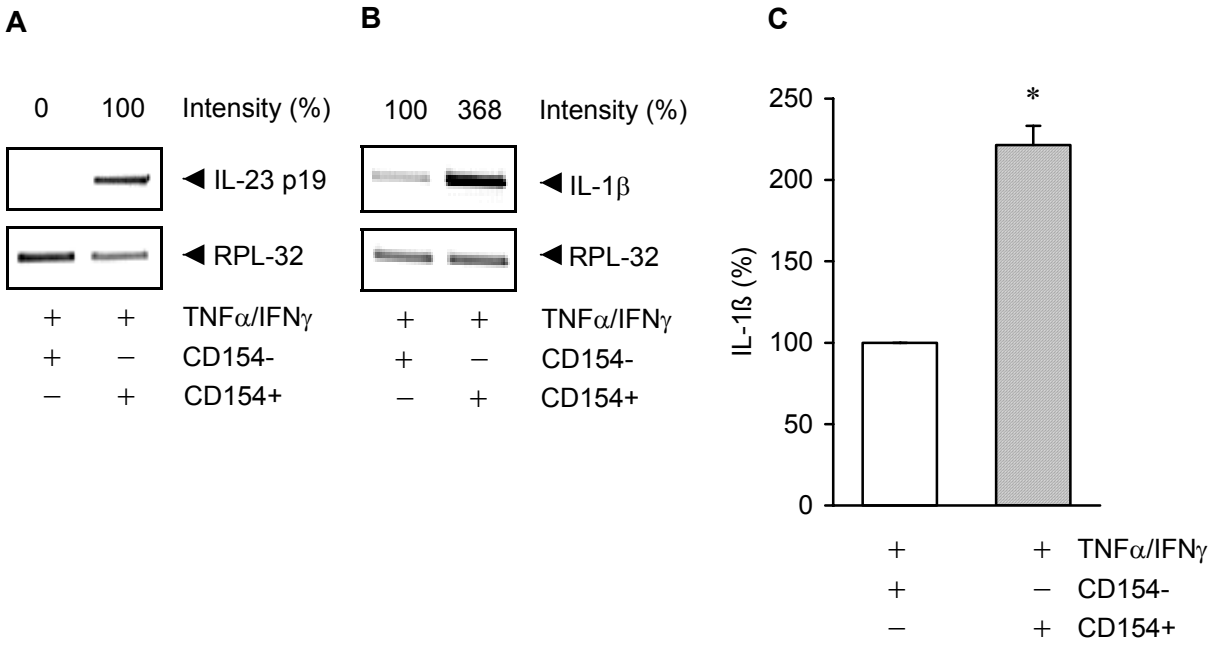

Figure 16. Effects of CD40 activation in a SMC-THP-1 cell-interaction assay. THP-1 cells were harvested 12 h after beginning of the experiment followed by RT-PCR analysis of (A) IL-23 p19 and (B) IL-1ß mRNA expression in the transmigrated THP-1 cells. Data are representative for 5 experiments. (C) ELISA of IL-1ß protein abundance in the lysate of the transmigrated THP-1 cells (n=4). Protein concentration of cell extracts was estimated with the modified Bradford protein assay and equalized for each sample. $^{*}P<0.05$ versus negative control (i.e. SMC treated with TNFα/IFNγ and exposed to the CD154- myeloma cells).

However, CD154 stimulation of the cultured SMC up-regulated the expression of both adhesion molecules (necessary for cell-to-cell interaction) and chemokines (same of which are thought to be highly effective chemoattractants for monocytes).

The next series of experiments, therefore, was designed to discriminate between SMC activation of THP-1 cells through cell-to-cell interaction (mediated by, e.g., adhesion molecules) or via the release of a humoral factor (e.g., a chemokine). To this end, the conditioned medium from SMC pre-stimulated with TNFα plus IFNγ followed by co-incubation with the CD154-expressing P3xTB.A7 cells or the corresponding control cells was collected and incubated with the THP-1 cells for 12 h. Five gene products, i.e. IL-23 p 19, IL-1ß, IL-8, CD40 and MCP-1 were monitored on the mRNA level (Fig. 17). In addition, IL-1ß protein expression was determined (Fig. 18).

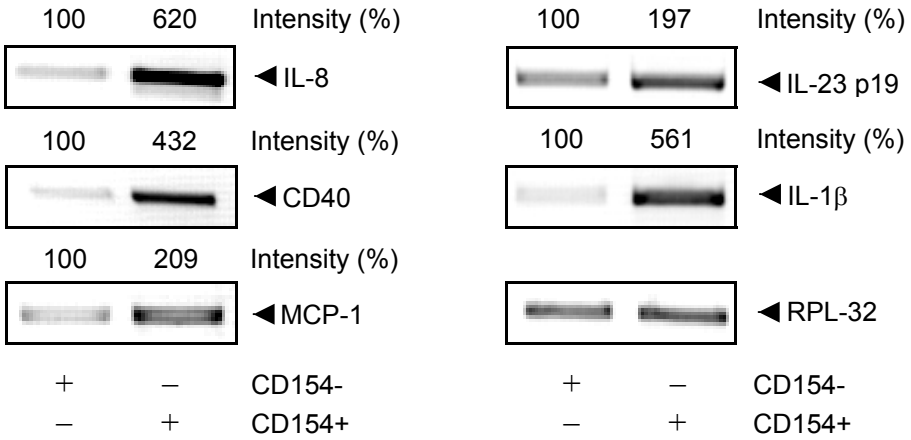

Figure 17: The conditioned medium of CD154-stimulated SMC induces the expression of IL-23 p19, IL-1ß, IL-8, CD40 and MCP-1 in THP-1 cells. CD40-expressing SMC were co-incubated with the CD154-expressing P3xTB.A7 cells or the corresponding control cells for 12 h. Thereafter THP-1 cells were exposed to the conditioned medium diluted 1:1 with normal culture medium for 12 h followed by RT-PCR analysis with RPL-32 as an internal standard. Data are representative for 3 individual experiments.

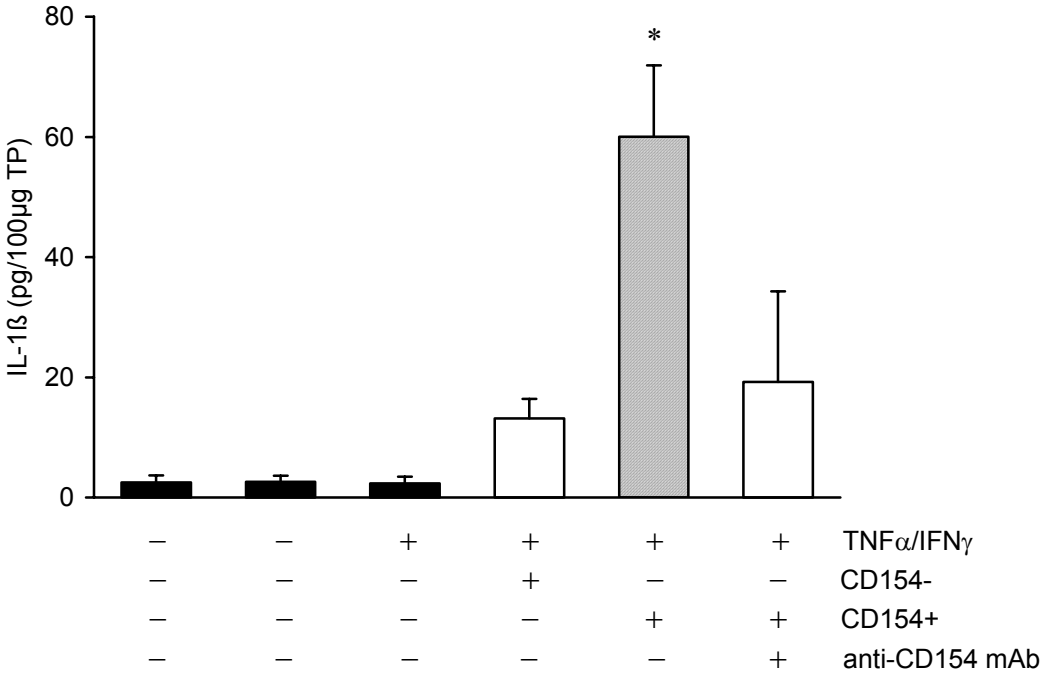

Figure 18. SMC supernatant-induced IL-1ß protein expression in THP-1 cells. Cell-associated IL-1β protein expression was measured by ELISA as the amount (in pg) per 100 µg of total protein (TP). Statistical summary (n=5). For experimental details see Fig. 17. $^{*}P<0.05$ versus negative control (i.e. conditioned medium of non-stimulated SMC).

IL-1β protein expression induced by the conditioned medium of CD154-stimulated SMC was even more pronounced as in the cell interaction assay. This result strongly

suggests, that SMC-induced THP-1 activation is mediated by a soluble factor (i.e. most probably a chemokine and/or a cytokine) produced by SMC in response to CD40 activation and not by direct cell-to-cell interaction.

3.6.1 Effects of GM-CSF and IL-15 on CD40 and IL-8 expression

Which of the soluble factors expressed by SMC in response to CD40 activation are responsible for monocyte activation? To test potential candidates causing CD40 or IL-8 mRNA expression in THP-1 cells, the same experimental approach was chosen as described above except that neutralizing antibodies against GM-CSF (20 µg/mL) and IL-15 (2 µg/mL) were employed in addition. An anti-β-actin antibody was used (20 µg/mL) as a negative control (not shown). The anti-GM-CSF antibody, in contrast to the antibody directed against IL-15, clearly affected IL-8 expression in response to the SMC supernatant while CD40 expression was attenuated only weakly (Fig. 19).

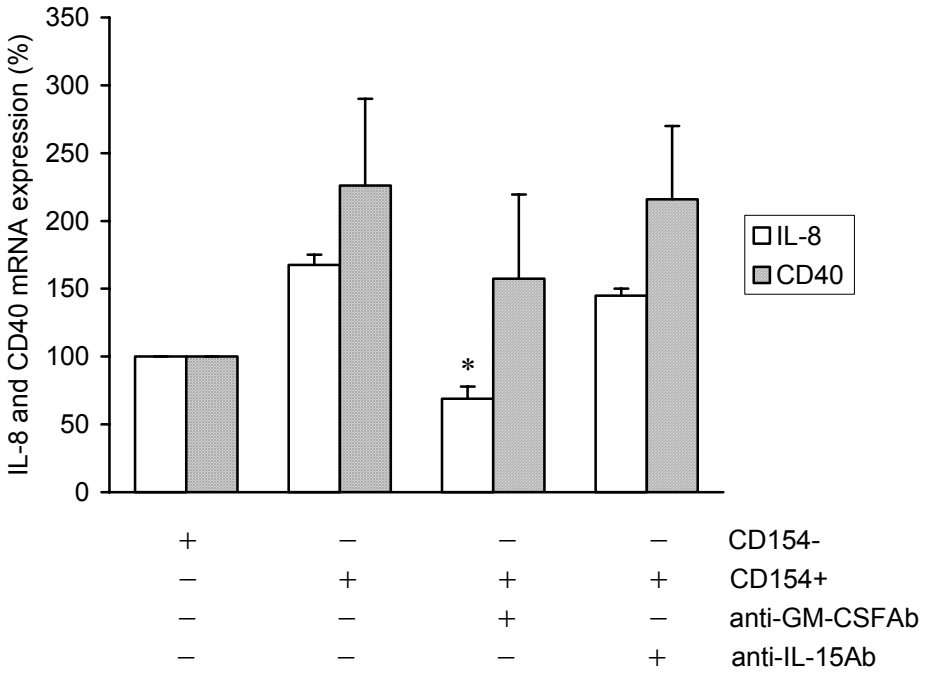

Figure 19. Effects of GM-CSF and IL-15 on CD40 and IL-8 expression. Supernatants of SMC incubated with P3x63Ag8.653 or P3xTB.A7 cells were pre-incubated for 1 h with anti-IL-15 antibody or anti-GM-CSF antibody, applied to THP-1 cells for 12 h as described and the corresponding gene products were detected in the THP-1 cell lysates by RT-PCR. Statistical summary (n=3). $^{*}P<0.05$ versus CD154+.

4. Discussion

4.1 The experimental model

The experimental model used in this study was cultured smooth muscle cells isolated from human thymus veins. To ensure that the cells were not contaminated with associated fibroblasts and endothelial cells, they were routinely monitored for the expression of smooth muscle α-actin, a marker for SMC, and for von Willebrand factor, a marker for endothelial cells. Although fibroblasts also contain smooth muscle α-actin, the staining pattern in fully differentiated SMC is very different as compared to that in fibroblasts. Typically, 100% of the cells stained positive for α-actin with approximately 98% showing the fibrillar pattern typical for SMC. On the other hand, the cells were essentially negative for von Willebrand factor, thereby ruling out a contamination by endothelial cells.

Besides SMC homogeneity, 3 additional criteria had to be met for the experiments described.

First, to achieve an 'activated state' of the SMC associated with a significant expression of CD40, the cells had to be pre-incubated with a combination of pro-inflammatory cytokines (TNFα/IFNγ). On the one hand, these cytokines cause an increase in expression of numerous pro-inflammatory gene products (for review see, e.g., Libby et al., 1995). On the other hand, truly CD40-induced gene products could nevertheless and only be detected in these cells after induction of CD40 expression (see below). Pertaining to the situation *in vivo*, it can be stated that vascular SMC will be exposed to these cytokines under conditions of an inflammatory response in the vessel wall. Thus, cultured SMC exposed to TNFα/IFNγ provide a simple, but relevant model for the analysis of the role of CD40 in the course of vascular inflammation.

Secondly, CD40 activation in SMC had to be achieved by the use of mouse myeloma cells expressing human CD154 because no trimeric soluble ligand or fusion protein was available. As a control, identical cells not expressing CD154 were used in every experiment, evidencing that all effects addressed to CD154 indeed were specific. Moreover, special care was taken that no gene (e.g., human specific PCR primers) or protein derived from myeloma cells was detected.

Thirdly, THP-1 cells were used as monocytic cells to monitor functional consequences of SMC CD40 activation. This cell line is well characterized as a monocyte pre-cursor (Tsuchiya et al., 1980.). Although the use of freshly isolated peripheral monocytes might have been desirable, the inherent variability of cells derived from different donors proves a major problem. Therefore, THP-1 cells were chosen as the second best choice for the cell-to-cell interaction assays described herein.

4.2 CD40 expression in SMC

Activation of CD40 by its specific ligand CD154 present mainly on T-cells and platelets plays an important role in the pathogenesis of atherosclerosis (Mach et al., 1998). Human endothelial cells express functional CD40 under basal conditions *in vitro* (Karmann et al., 1995) and this expression is significantly up-regulated in the presence of pro-inflammatory cytokines (Wagner et al., 2002). As SMC are well characterized to play a major role in the development and progression of atherosclerosis, the aim of this study was to answer the question whether CD40 might also play a role in atherosclerosis-related activation of SMC. Under basal conditions, SMC express only very little CD40 mRNA while CD40 protein was not detectable (Western blot analysis). When exposed to pro-inflammatory cytokines such as IFNγ and/or TNFα, i.e. mimicking an inflammatory response in the vessel wall, CD40 abundance was markedly increased both on the mRNA and protein level, suggesting that expression of CD40 is induced *de novo* by these cytokines in the cultured SMC. This finding is at variancy with a previous study showing basal expression of CD40 protein in human SMC (Mach et al., 1997.). The reason for this difference might be, besides the protocol for isolating the cells, the source of the cells used in this study. Mach et al. used cells derived from saphenous vein grafts left over from aortocoronary bypass surgery. These veins usually stem from elderly patients suffering from atherosclerosis at least in the heart, so that SMC isolated from such vessels might be chronically activated by high levels of circulating pro-inflammatory mediators present in these patients.

However, one important result derived from these experiments was that CD40, as in endothelial cells, is inducible by pro-inflammatory cytokines also in vascular SMC.

4.3 CD40 induced by IFNγ and TNFα is functionally active

The first step assessing the effects of CD40 activation in human SMC was the analysis of MCP-1 and MMP-3 expression. These gene products were chosen because it is well known that both play an important role in macrophage activation and inflammatory *remodelling* processes in human blood vessels (Russell et al., 1993; Galis et al., 1994). Indeed, expression of MMP-3 and MCP-1 in the SMC was up-regulated solely after CD40 activation, because it was induced only by mouse myeloma cells stably transfected with human CD154, but not by the corresponding control cells , and this effect was specifically abrogated in the presence of an anti-CD154 antibody.

Several other gene products, known to be up-regulated in SMC under pro-inflammatory conditions, were tested for CD40 inducibility. However, none of these was up-regulated by CD40 activation, revealing a rather specific pattern of CD40-mediated gene expression in the SMC.

4.4 CD40 induced MMP-3 expression is dependent on Src-like tyrosine kinase and p38 mitogen-activated protein kinase

Mitogen-activated protein kinases (MAPKs) play an important role in regulating cell growth, differentiation, survival and death. To date, three mammalian MAPK pathways, the mitogen-activated ERK1/2 (Raf→MEK1/2→ERK1/2), JNK (MEK kinase1-4→MKK4/7→JNK1-3) and p38 MAPK pathway (MAPKKK→MKK3/6→p38 MAPK), activated by inflammatory cytokines and cellular stress, have been characterized in detail (Westermarck et al.,1999).

Using MMP-3 expression as a readout for CD40 signalling and specific protein kinase inhibitors directed against the aforementioned pathways, it could be demonstrated that p38 MAPK and c-src or a similar protein kinase are involved in CD40 signalling leading to the increased SMC expression of MMP-3. It has already been reported that CD40 activation affects the p38 MAPK pathway (Zhang et al., 2002). Several upstream regulators of p38 MAPK have been characterized in SMC, one of which is protein kinase C (PKC; Igarashi et al., 1999). However, inhibition of PKC with RO 31-8220 did not affect CD154-induced MMP-3 expression indicating that other signalling mechanisms are involved in the upstream regulation of p38 MAPK following CD40 activation.

Another potential activator of p38 MAPK is p60/Src. The inhibitory effect of herbimycin A, a reasonably specific inhibitor of Src-like tyrosine kinase (Uehara et al., 1985), on CD154-induced MMP-3 expression suggested an involvement of Src-like tyrosine kinases in the upstream regulation of p38 MAPK activity in the SMC. Indeed, such a signal transduction pathway has already been documented in neutrophils and fibroblasts, respectively (Mocsai et al. 2000; Domeij et al., 2002).

The rho/ROCK pathway, playing a role in stretch-induced gene expression in SMC via p38 MAPK (Cattaruzza et al., 2001), was tested as a third potential mediator of CD40 signalling in MMP-3 expression. Interestingly, a specific inhibitor, Y27632, increased rather than decreased MMP-3 expression in response to CD154 stimulation, suggesting that the rho/ROCK pathway attenuates rather than contributes to CD40 signalling in the SMC.

One of the transcription factors activated by p38 MAPK and potentially responsible for MMP-3 expression is AP-1. An active consensus binding site for AP-1 has been characterized at position -70 in the *mmp-3* gene promoter (Quinones et al., 1994). Moreover, activation of p38 MAPK was identified as an upstream event involved in the induction of AP-1 components and AP-1-controlled gene expression in SMC (Häcker et al., 1998). The other putative transcription factor involved in MMP-3 expression is NF-κB. This transcription factor is required for cytokine up-regulation of MMP-3 in SMC (Bond et al., 2001). However, there is evidence that p38 MAPK activation alone is sufficient to induce MMP-3 expression in an AP-1-independent manner by stabilization of the mRNA in human fibroblasts (Reunanen et al., 2002).

The question which of the 2 mechanisms plays a role in CD40-induced MMP-3 expression in SMC was tested by inhibition of *de novo* RNA synthesis and application of decoy-oligonucleotides directed against AP-1, NF-κB and other transcription factors. Using the RNA II polymerase inhibitor, actinomycin D, prior to CD40 activation, resulted in a complete abolition of CD154-induced MMP-3 mRNA expression, indicating that the observed increase in MMP-3 mRNA abundance primarily is the result of an increased trancription. Assessing MMP-3 mRNA stability, again by employing actinomycin D, revealed a high half life of more than 12 h. Thus, p38 MAPK-mediated stabilization of MMP-3 mRNA is unlikely to play a role in CD40-mediated MMP-3 expression in the cultured SMC.

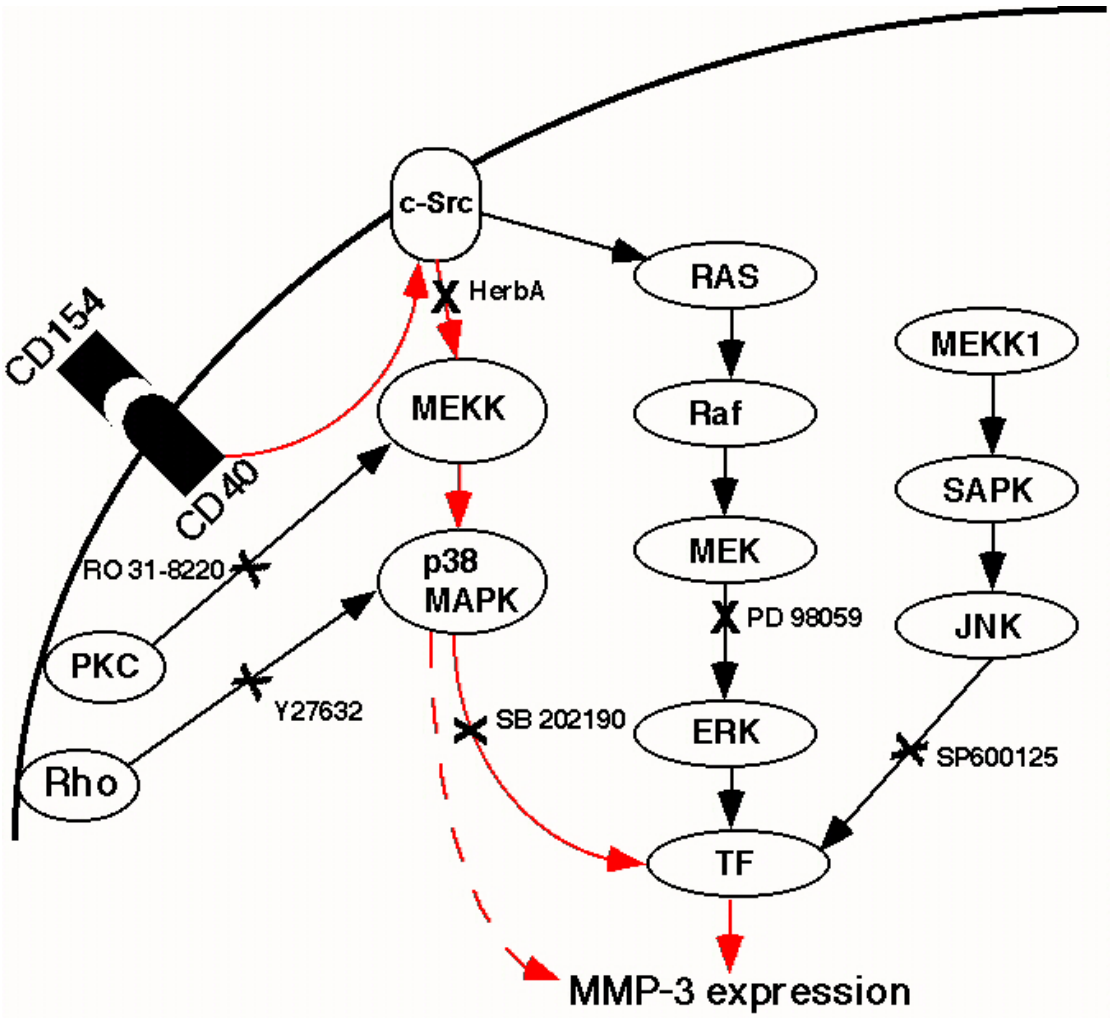

Figure 20. CD40 signalling pathways potentially leading to MMP-3 expression in SMC. The protein kinase pathways tested in this study are outlined. The only pathway that could be connected to CD40-induced MMP-3 expression is the pathway via c-Src and p38 MAPK (highlighted in red). The level of the interaction of inhibitors with the affected kinase pathways is marked by *double-bars* and the name of the respective inhibitor.

Furthermore, pre-treatment with cycloheximide prior to stimulation with CD154 did not block CD154 effects on MMP-3 mRNA expression, indicating that *de novo* synthesis of transcription factors is not essential for the up-regulation of MMP-3 expression. In an attempt to characterise latent transcription factors involved in MMP-3 expression, decoy oligonucleotides against AP-1, NF-κB and other related transcription factors were employed. Their lack of effect on MMP-3 expression, but not that of other gene products, excluded the involvement of these transcription factors in CD154-induced transcription of the *mmp-3* gene. Thus, the exact

mechanism of CD154-induced MMP-3 expression on this level still remains to be elucidated.

The putative upstream signalling mechanisms are summarized in Fig. 20.

4.5 Characterization of CD40-mediated gene expression in SMC by DNA microarray analysis

As stated before, CD40 activation in SMC results in an increase in MMP-3 and MCP-1 expression. To gain a more detailed overview of the changes in CD40-induced gene expression in SMC, a DNA microarray approach was chosen. This technique offers a number of advantages over other potential screening methods for gene expression analysis. Besides giving the possibility of screening the expression of a large number of genes in parallel, another advantage is that relatively small amounts of RNA are required for this assay. The approach chosen here, i.e. performing three individual microarray experiments and applying rigorous selection criteria (i.e. counting only gene products with high quality signals that were up-regulated in response to CD154 in all three experiments) resulted in the characterization of only truly up-regulated gene products. This could be proven by subsequent RT-PCR analysis performed for 7 of gene products detected by DNA microarray to be CD154-inducible. In addition, these gene products were selected on the basis of their relevance for the development of atherosclerosis (see below) and were confirmed to be up-regulated to a similar extent according to RT-PCR analysis as estimated by DNA microarray.

Thus, the list of differentially expressed gene products identified in this study is rather reliable (low abundance of false positive gene products). One disadvantage of the DNA microarray approach, on the other hand, is that several CD154-inducible gene products were not recognized due to the stringent selection criteria applied. According to the DNA microarray data, interaction of CD154-expressing myeloma cells with CD40-expressing SMC resulted in an pronounced pro-inflammatory response in the SMC. Most of the gene products up-regulated in CD40-activated SMC were pro-inflammatory molecules (chemokines and their receptors) including CCR7, GRO-β, GRO-γ, IL-15, IL-15 receptor α, MCP-2, MIP-1α, MIP-3α, MMP-12 and ninjurin. Moreover, it would appear that expression of these gene products in human vascular SMC has been demonstrated for the first time in this study.

Several of these pro-inflammatory molecules have similar biological actions, e.g. chemotactic activity, such as GROβ and GROγ (Arenberg et al., 1997), RANTES (Dieu et al., 1998), fractalkine (Imai et al., 1997; Al-Aoukaty et al., 1998), MIP-1α and MIP-3α (Dieu et al., 1998), and GM-CSF (Dieu et al., 1998). They play a common role in leukocyte trafficking and dendritic cell maturation. One equally interesting CD154-induced ligand-receptor pair was expressed in the cultured SMC, i.e. IL-15 and IL-15 receptor α. The parallel expression of the ligand and its receptor indicates a self-preserved pro-inflammatory process established by CD40 activation in SMC. IL-15 stimulates cytokine release in macrophages and lymphocytes (McInnes et al., 1997; Badolato et al., 1997) and acts as a chemokine for T-cells (Wilkinson et al., 1995). All of these findings, in correlation with the results of this study, imply that IL-15 expression in CD40-activated SMC might not only contribute to local T-cell and monocyte activation and survival but also act on SMC in an autocrine manner. It is of particular interest that activation of T-cells by IL-15 is dependent on direct cell-to-cell contact but independent of specific antigenic stimulation (McInnes et al., 1997). IL-15 expression might thus be relevant for a non-specific chronic inflammation of the vessel wall including the non-specific activation of T-helper cells and SMC in atherosclerotic lesions. However, at this point, IL-15 effects on SMC are merely speculative, as expression of this cytokine and its receptor in SMC has not been described as yet.

Another interesting finding was that, unlike in endothelial cells (Lienenluke et al., 2000), activation of CD40 induces its own expression in SMC. This in turn could promote the interaction between CD154-expressing cells present in the vessel wall during inflammation and SMC, thereby fuelling the pro-inflammatory response.

4.6 Functional significance of CD40 activation in SMC: activation of monocytes

To analyse the effects of CD40 activation in SMC on monocytes, two different experimental approaches were chosen. Essentially they revealed that monocyte activation is dependent on the release of a humoral factor (chemokine or cytokine) from CD154-stimulated SMC rather than a cell-to-cell contact mediated by, e.g., adhesion molecules. This humoral factor or factors released by SMC in response to CD40 activation may be crucial for a full-blown macrophage-mediated vascular

inflammation. Monocyte activation was confirmed by the detection of an increased expression of several marker genes in the THP-1 cells employed both on the mRNA and protein level. The obvious next question was which humoral factor is in fact responsible for monocyte activation? To this end, the list of differentially expressed genes revealed by DNA microarray was consulted first, essentially yielding GM-CSF, GRO-ß, GRO-γ, MCP-2, MIP-1α and MIP-3α as potential candidates as well as IL-1ß and IL-15. Previous findings that GM-CSF up-regulates, e.g., CD40 and IL-8 expression in human monocytes (Paquette et al., 1998; Baqui et al., 1999) prompted the investigation of this protein along with IL-15. By using neutralizing antibodies against these two proteins it could be confirmed that GM-CSF is, at least in part, responsible for the increase in gene expression in THP-1 cells exposed to the supernatant of CD154-stimulated SMC. GM-CSF contributes to monocyte survival and their differentiation to macrophages (Geissler et al., 1989), a process crucial for the development and propagation of the atherosclerotic process. Thus, blockade of CD40-mediated release of GM-CSF may have profound effect on this population of leukocytes during the progression of atherosclerosis.

4.7 A model of the role of SMC CD40 in atherosclerosis

Till now, most research based on the role of inflammation in atherosclerosis was pointed to the interaction of endothelial cells and leukocytes. Indeed, the endothelial cells of the vessel wall are the first barrier for circulating blood cells. Endothelial cell activation leads to the expression of adhesion molecules (e.g. E-selectin and VCAM-1) and chemoattractants (MCP-1), which trigger leukocyte rolling, adhesion and migration into the vessel wall. However, once present within the vessel wall, leukocytes come into close contact with SMC, creating the potential for intercellular communication to occur. The results of this study indicate that CD40 is expressed under pro-inflammatory conditions on the surface of SMC. CD40 activation in SMC inevitably occurs through CD154/CD40 interaction when activated T-cells enter the vessel wall (Schonbeck et al., 1997).

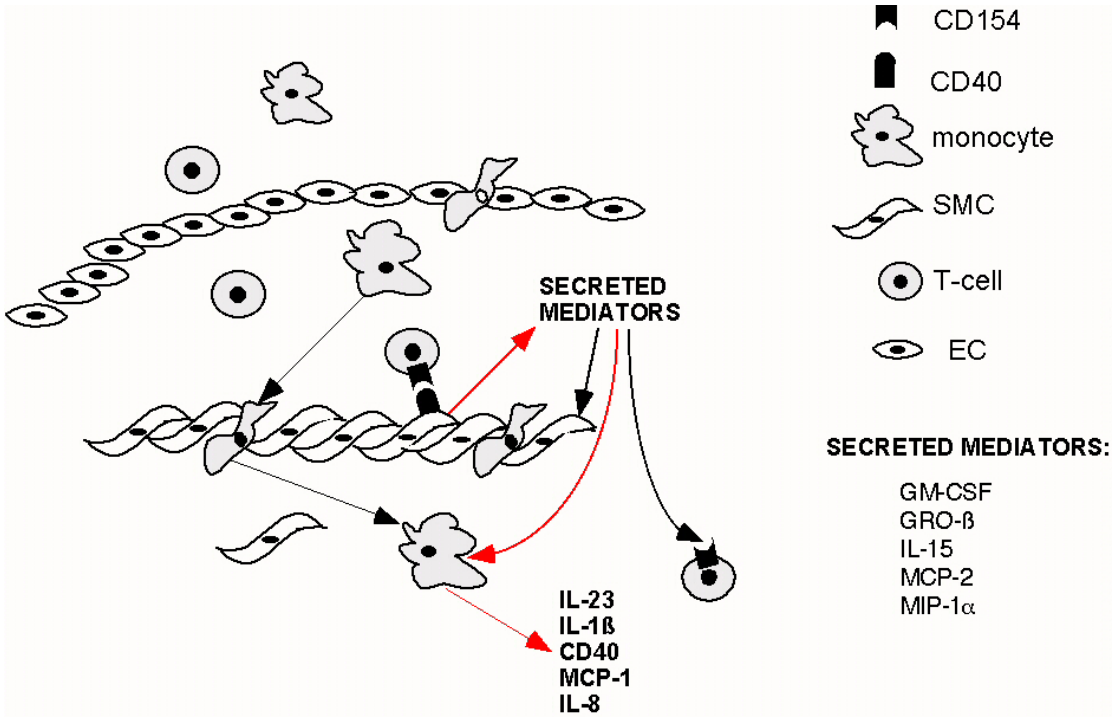

Figure 21. Effects of cell-to-cell interaction between CD40-activated SMC and monocytes. SMC under pro-inflamatory conditions express CD40. T-cells expressing-CD154 interact with CD40-expressing SMC. As a result, SMC produce pro-inflammatory molecules that affect monocytes, T-cells and SMC present in inflamed vessel wall.

As shown here, the response of SMC to CD40 activation is the production of a variety of pro-inflammatory molecules, ranging from chemokines and their receptors to adhesion molecules. Pro-inflammatory molecules secreted from CD40-activated SMC can principally affect 3 different types of cells (Fig. 21). First, molecules like GM-CSF or MCP-1 act as chemoattractants for monocytes and provoke their maturation. Secondly, by secreted mediators from CD40-activated SMC such as, e.g., IL-15 are responsible for T-cell maturation. This cytokine also induces expression of CD154 on activated T-cells, suggesting a positive feed-back loop between activated T-cells and SMC. Thirdly, CD40 activation induces its own expression as well as the expression of several ligand-receptor systems (e.g. IL-15/IL-15 receptor or CCR7 and several ligands) in SMC. CD40 activation in SMC, although strongly activated already by other cytokines such as TNFα plus IFNγ does not seem to be just another activated receptor-ligand system but leads, as could be shown herein for the first time, to the expression of gene products that truly promote the activation of monocytes/macrophages, T-cells and potentially also SMC themselves. Thus, one likely consequence of these changes in gene expression in

the SMC might be the initiation and maintenance of a vicious cycle in which the 3 cell types interact with each other to fuel the inflammatory response. Continuous attraction and activation of inflammatory cells, as well as propagation of a predominant Th1-mediated immune response, might figure among the most prominent CD154-mediated processes contributing to the progression of atherosclerotic lesions toward the vulnerable, rupture-prone plaque. In this context, elucidating the signalling pathway activated following CD40 stimulation in SMC appears to be a therapeutically relevant goal.

5 Summary

In the pathogenesis of atherosclerosis, circulating leukocytes adhere to endothelial cells, migrate through them and enter the vessel wall. CD40, a member of the tumor necrosis factor receptor family, that is expressed by both leukocytes and endothelial cells plays an important role in this process. Upon their emigration into the vessel wall leukocytes come into contact with the vascular smooth muscle cells (SMC). These cells also play a major role in the development and progression of atherosclerosis and express CD40 under pro-inflammatory conditions. The aim of the present study was to answer the question which role CD40 expression in SMC might play in the context of atherosclerosis. To address this complex question, four different experimental approaches were taken: (i) analysis of CD40 expression itself in human cultured SMC, (ii) analysis of CD154-induced gene expression in these cells, as well as (iii) the signal transduction pathways involved therein, and (iv) investigating possible functional consequences of these changes in gene expression.

A first result achieved was that under pro-inflammatory conditions, CD40 expression is markedly up-regulated in human cultured SMC. Moreover, activation of CD40 in these cells resulted in the differential expression of 36 genes, as judged by DNA microarray and confirmed by RT-PCR analysis. Most of these gene products were up-regulated and comprised pro-inflammatory molecules, namely chemokines and their receptors, cytokines and their receptors, and adhesion molecules. The expression of these gene products in human SMC could be demonstrated for the first time.

To elucidate the signalling pathways linking CD40-CD154 interaction to gene expression in the SMC, CD40-induced matrix metalloproteinase-3 (MMP-3) expression was chosen as a readout. By using different pharmacological inhibitors, it was demonstrated that the tyrosine kinase c-Src and the mitogen-activated protein kinase p38 are involved in CD40 signalling to the nucleus in these cells. Moreover, MMP-3 expression was verified to be up-regulated on the level of transcription, although the transcription factor(s) responsible therefor could not be identified as yet.

By using two different experimental approaches, a SMC-monocyte interaction assay and analysing changes in gene expression in these cells upon exposure to the conditioned medium of CD154-stimulated SMC, it was further shown that monocyte activation is mediated by a humoral factor rather than a cell-to-cell contact. Further

investigations, employing neutralizing antibodies, revealed that granulocyte-macrophage colony-stimulating factor is a likely candidate for the monocyte-activating humoral factor.

Collectively, these findings suggest that vascular SMC like endothelial cells contribute to the maintenance of an inflammatory response in the vessel wall as, e.g., in atherosclerosis when stimulated via the CD40/CD154 receptor/ligand dyad. Elucidating the transcriptional mechanism by which CD40 activation in these cells is translated into the release of pro-inflammatory mediators represents a valuable therapeutic goal in this context.

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