American Journal of Immunology 3 (2): 56-73, 2007
ISSN 1553-619X

Microarray Analysis of Human Vascular Smooth Muscle Cell Responses to Bacterial Lipopolysaccharide

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Abstract: Accumulating evidence suggest a causal role of bacterial and viral infections in atherogenesis. Bacterial lipopolysaccharide (LPS) has been shown to stimulate resting vascular smooth muscle cells (SMC) with the production of inflammatory cytokines and modulation of quiescent cells to the proliferative and synthetic phenotype. To comprehensively identify biologically important genes associated with LPS-induced SMC phenotype modulation, we compared the transcriptomes of quiescent human coronary artery SMC and cells treated with LPS for 4 and 22 h. The SMCs responded robustly to LPS treatment by the differential regulation of several genes involved in chromatin remodeling, transcription regulation, translation, signal transduction, metabolism, host defense, cell proliferation, apoptosis, matrix formation, adhesion and motility and suggest that the induction of clusters of genes involved in cell proliferation, migration and ECM production may be the main force that drives the LPS-induced phenotypic modulation of SMC rather than the differential expression of a single gene or a few genes. An interesting observation was the early and dramatic induction of four tightly clustered interferon-induced genes with tetratricopeptide repeats (IFIT1, 2, 4, 5). siRNA knock-down of IFIT1 in SMC was found to be associated with a remarkable up-regulation of TP53, CDKN1A and FOS, suggesting that IFIT1 may play a role in cell proliferation. Our data provide a comprehensive list of genes involved in LPS biology and underscore the important role of LPS in SMC activation and phenotype modulation which is a pivotal event in the onset of atherogenesis.

Key words: Vascular smooth muscle cells, lipopolysaccharide, transcriptome, microarray, quantitative PCR, siRNA, IFIT1

INTRODUCTION

Atherosclerosis is a complex, chronic inflammatory disease of the arterial vessel wall manifested at predilected locations of the vasculature[1]. It is initiated by multiple local and systemic risk factors (including mechanical shear stress due to hemodynamic changes, hypercholesterolemia, hypertension, high plasma levels of inflammatory markers, etc.) which induce endothelial dysfunction and vascular injury[1]. The disease is characterized by complex interactions between a variety of lipids, mononuclear phagocytes and their soluble mediators in the intima and by intimal hyperplasia. The accumulation of excess cholesterol and cholesteryl esters by macrophages at these sites results in the formation of foam cells that are the hallmarks of early fatty streak lesions and atheroma development[1,2]. Accumulating evidence suggests that chronic viral or bacterial infections, particularly infections with the ubiquitous gram-negative respiratory pathogen, *Chlamydia pneumoniae*, may be an additional risk factor that may initiate and/or promote atherosclerosis. Patients with acute myocardial infarction and coronary artery disease have been reported to have significantly higher titers of antibodies against *C. pneumoniae* compared to healthy controls and *C. pneumoniae* has been localized to the intima of vascular smooth muscle cells (SMCs) in many atherosclerotic lesions[3,4]. *C. pneumoniae* has been shown to infect and multiply within macrophages, endothelial cells and SMCs[3] and *C. pneumoniae* and Chlamydial lipopolysaccharide (LPS) have been reported to induce the release of inflammatory cytokines from mononuclear cells, endothelial cells and SMCs[5,6] and to stimulate

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oxidation of low-density lipoprotein and formation of macrophage foam cells[7,8]. Failure of coronary artery bridge grafts or restenosis has been associated with chronic inflammation induced by LPS[9,10]. Systemic inflammatory responses induced by LPS have been shown to increase neointimal formation after ballon injury and stent implantation and the induced proliferation of SMCs may play a key role in atherogenesis[11].

LPS is a complex glycolipid component of the gram-negative bacterial cell wall composed of a hydrophilic polysaccharide and a hydrophobic Lipid A moiety[12]. The interaction of serum LPS-binding protein (LBP) with LPS facilitates the binding of the lipid A moiety to CD14, a 55 kDa glycoprotein present as a soluble factor (sCD14) or as a glycosylphosphatidylinositol (GPI)-anchored membrane protein[13,14,15]. This complex, which also contains MD-2, interacts with Toll-like receptor 4 (TLR4) which mediates the cellular activation by LPS. TLR4 signaling induces the activation of NFkB and MAPK pathways and proliferation of SMCs[16,17,18,19,20,21]. Recently, Engelmann *et al*.[22,23] have reported that repeated perivascular administration of LPS or inoculation of *Chlamydophila pneumoniae* into rabbits induce formation of atheromatous lesions *in vivo*.

The ability of VSMCs in the media of arteries to undergo phenotype modulation from the quiescent and contractile state to the proliferative, migratory and synthetic phenotype that elaborates extracellular matrix underlies their crucial role in the development and progression of vascular pathology such as atherosclerosis and restenosis. Phenotype modulation involves a cascade of events in which different genes are turned on or off in a regulated manner. Thus to gain further insight into the molecular events associated with LPS-mediated activation of SMCs, we have analyzed the transcriptomes of quiescent and LPS-treated human coronary artery SMCs using oligonucleotide microarray analysis. Our results show that the transcriptional effects of LPS on SMCs were particularly far-reaching and many genes that are involved in various biological mechanisms such as chromatin remodeling, transcriptional regulation, translation, signal transduction, metabolism, innate and adaptive immune responses, cell proliferation, apoptosis, matrix formation, cell adhesion and motility, were regulated in a coordinated fashion. The LPS effect appeared to be mediated via the transcriptional induction of proinflammatory cytokines and growth factors and these in turn initiated multiple signal transduction pathways that induced transcriptional activation of genes that are effectors of cell proliferation, migration and extracellular matrix formation. The data presented is thus in agreement with accumulating evidence that LPS may be a risk factor that initiates and/or promotes atherosclerosis.

MATERIALS AND METHODS

Smooth muscle cell culture and reagents: Human coronary artery SMCs (Clonetics, Walkersville, MD) were cultured in SMC basal medium (SmBM) containing SmBM-3 growth supplements [FBS (5%), bovine insulin (50 ng mL^{-1}), human recombinant (hr)-EGF (5.0 ng mL^{-1}), hr-FGF-B (20 ng mL^{-1}) and GA-1000 (Gentamicin, Amphotericin B)] supplied by BioWhittaker Inc. Walkersville, MD. SMCs were characterized by their typical hill and valley growth pattern, by staining with anti-SM-α-actin antibody (Dako Diagnostics) and by the lack of staining of Factor VIII related antigen, an endothelial cell marker, using anti-factor VIII antibody (Dako Diagnostics). SMCs at passage 4 were seeded in growth supplemented SmBM-3 medium for 24 h and the medium was replaced with basal mediun containing 0.5% FBS for 48 h to induce quiescence before treated with LPS (E. coli 055.B5, Sigma/Aldrich, St. Louis, MO). Human AB serum was purchased from Sigma/Aldrich, St. Louis, MO. For siRNA experiments, human aorta SMCs (ATCC, Manassas, VA) were cultured in growth supplemented F12K medium according to the instructions of the supplier.

RNA isolation and gene expression profiling: Total RNA was extracted from cells using TRIzol reagent (Invitrogen Life Technologies, Inc. Burlington, ON) and contaminating chromosomal DNA was removed using Rnasefree DNase Message Clean Kit (GenHunter Corp., Nashville, TN). The integrity of RNA preparations was assured by comparing the intensities of 28 S and 18 S rRNA bands on 1% formamide-aldehyde agarose gel. mRNA was isolated from the total RNA using Oligotex according to the manufacturer's instructions (Qiagen Inc., Mississauga, ON). Biotinylated complementary RNA (cRNA) samples for chip hybridization were prepared according to protocols supplied by Affymetrix (Affymetrix, Santa Clara, CA) and then hybridized to HG-U133A oligonucleotide array Gene Chip (Affymetrix, Santa Clara, CA) following the manufacturer's protocol. The arrays were washed, stained with streptavidin-phycoerythrin and scanned. Data files were analyzed using Affymetrix GeneChip® Operating Software (GCOS) version 1.0 (Affymetrix, Santa Clara, CA).

Gene cluster analysis: Gene expression pattern analysis was performed using GENESIS ver. 1.3.0. Raw expression values of differentially regulated genes (above signal log ratio of 1.0) from microarray analysis were adjusted with log 2 transform data and 'median centre genes, and clustered I nto 40 groups using k-mean clustering according to the method of Eisen *et al*.[24] Groups with similar gene expression pattern were combined together to generate three gene expression patterns: up-regulated at both 4 and 22 h, up-regulated only at 4 h and down-regulated only at 4 h.

Quantitative real-time PCR: Quantitative real-time PCR was performed with an ABI Prism 7900HT Sequence Analyzer using the manufacturer's recommended protocol (Perkin-Elmer Applied Biosystems, Foster City, CA) to validate differential expression of selected genes. Two different primer sets were designed for each investigated gene using Primer Express version 2.0 (Perkin-Elmer Applied Biosystems, Foster City, CA). Each reactions was run in triplicates in 10 μL volumes containing 4 μL of diluted first strand cDNA template, 5 μL of SYBR Green PCR Master Mix, 0.1 μL (50 μM) of each forward and reverse primers and 0.8 μL H_2O. Samples were incubated at 95 ^oC for 3 min to activate Taq polymerase and 40 cycles were performed at 95 ^oC for 10 sec, at 65 ^oC for 15 sec and at 70 oC for 20 sec. A standard curve of each primer set was generated using human tonsil genomic DNA[25]. Sequences for the primers used in this study are available on request.

siRNA preparation and transfection: Cell cultures at 60-80% confluence were transfected with custom designed and synthesized IFIT1-specific siRNA primers (Sense: GGC UGU CCG UUU AAA UCC A; Antisense: UGG AUU UAA GCG GAC AGC C) using RNAi Human/Mouse Control Kit and instructions supplied by Qiagen. siRNA (30 μL of 20 μM solution), RNAiFect (22.5 μL) and buffer EC-R buffer (70 μL) were mixed together and incubated for 15 min at room temperature to allow formation of tranfection complexes. The complexes were then added drop-wise onto the cells in the fresh medium. A scrambled non-silencing siRNA that has no known homology with mammalian genes was used to control for non-specific silencing effects.

RT-PCR: RT-PCR was performed using the Superscript first strand synthesis system (Invitrogen), Hotstart Taq Master Mix Kit (Qiagen) and gene specific primers designed from published cDNA sequences. IFIT1 forward primer (5'TCAGAATTCGGATCCATGAGTGAAATTCGTA AG3') and reverse primer (5'CAAGCTTGCCTGCAGTTAAATGGAAAGTCGC AG 3') amplifies a 1.5 kb cDNA fragment. GAPDH forward primer (5'CCACCCAGAAGACTGTGGAT3') and reverse primer (5'CCCCTCTTCAAGGGGTCTAC3') amplifies a 600 bp cDNA fragment. PCR amplification was initiated with a hot start at 95^oC for 15 min followed by 35 cycles of denaturation at 94^oC for 3 min, annealing at 56 ^oC for 1 min and extension at 72^oC for 3 min and ended with an extension phase at 72 ^oC for 10 min. PCR products were collected and analyzed by electrophoresis on 1% agarose gel to determine the levels of IFIT1 and GAPDH.

RESULTS AND DISCUSSION

Effect of LPS on the proliferation of SMC: In order to access the effect of LPS on SMC proliferation, quiescent SMCs in microtiter plates were stimulated with LPS (100 ng mL^{-1}) in the presence of 0.5% FBS or 5% AB serum as source of sCD14[26]. The culture media were replaced at day 2 and the cells were detached at day 5 and counted. The mean and standard error of the mean were determined for three experiments, each performed in quadruplicates. LPS stimulated SMC proliferation as determined by the increase in cell number over a 5 day culture period Fig. 1. The increase in cell number in cultures containing 0.5% FBS or 5% AB serum, were comparable. In the presence of 0.5% FBS and 100 ng mL^{-1} LPS, SMC proliferation was increased 2.4 fold relative to cells in basal medium containing 0.5% FBS alone and SMC proliferation in medium containing 5% AB serum and 100 ng mL^{-1} LPS was 5.1 fold greater than in the presence of AB serum alone. Our results on the mitogenic effect of LPS on SMCs is in agreement with earlier reports[27, 28].

Microarray analysis of differential gene expression in LPS-treated SMCs: Confluent SMCs cultures were synchronized to quiescence by incubation for 48 h in SmBM+0.5% FBS. To obtain a global profile of the transcriptional changes induced by LPS, we examined the gene expression responses of human coronary artery SMCs stimulated with 5% AB serum alone or AB serum containing LPS (100 ng mL^{-1}) for 4 and 22 h using Affymetrix oligonucleotide arrays (HG-U133A). Of the 18,400 transcripts (14,500 well-characterized genes) present on the chips, 1015

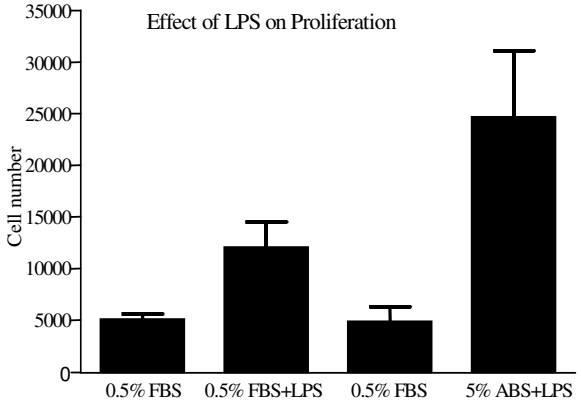

Fig. 1: Effect of LPS on SMC proliferation. 5×10^4 VSMC were seeded in multi-well culture plates and incubated and incubated for 24 h in basal medium containing 0.5% FBS to induce quiescence. The cultures were then grown in four different culture conditions (0.5% FBS, 0.5% FBS+100 ng mL^{-1} LPS, 5% AB serum and 5% AB serum+100 ng mL^{-1} LPS) for 5 days and the cell numbers were measured. The mean and standard error of the mean were determined for three experiments, each performed in quadruplicates

and 3749 genes (including ests and redundant gene probes) were found to be differentially regulated by LPS at 4 and 22 h, respectively. 469 genes were induced and 546 were suppressed at 4 h and 2526 were induced and 1223 were suppressed at 22 h.

Quantitative real-time pcr validation of results of gene array analysis: To validate the gene array results, the expression of 13 regulated genes was analyzed using reverse transcription followed by quantitative real-time PCR. The expression levels of human GAPDH and beta-2-microglobulin were used as internal housekeeping gene controls to normalize technical variability between samples. The expression of 9 genes was shown to correlate well in microarray and real-time PCR whereas the magnitudes of differential expression were somewhat different for 4 genes (data now shown). Overall, the real-time PCR data was more sensitive than the microarray data.

Scatter plot analysis: Scatter plots of log-transformed expression values from microarray analysis for pairwise comparisons of AB serum+LPS treated SMCs (ordinate) versus the control cells treated with AB serum alone (abscissa) for 4 and 22 h were used to display the regulated genes (Fig. 2). The data points along the diagonal represent genes expressed at comparable levels in both sets whereas those that lie off the diagonal represent genes differentially expressed between the samples identified by the two axes. The data indicate that the number of regulated genes increased with time. Also, whereas the number of induced and repressed genes at 4 h was comparable, the number of induced genes at 22 h was much greater than that of the suppressed genes. The plots confirmed that LPS induced and suppressed a greater numbers of genes at 22 h compared to 4 h (Fig. 2A and B). Scatter plot analysis of SMC treated with AB serum for 4 and 22 h shows that AB serum induces regulation of genes (Fig. 2C). This effect was more pronounced at 4 h and appeared to subside by 22 h. Comparison of the scatter plots of SMC treated with AB+LPS for 4 and 22 h showed that the magnitude of expression appeared to be similar for most genes. This suggests that the robust induction of genes in LPS 22 h compared to AB 22 h may be due to the prolonged and sustained activation of genes by LPS which compensates for the diminished AB serum effect at 22 h (Fig. 2D).

Profile of down-regulated genes: Since it is possible that the SMC genes that were down-regulated by LPS may be implicated in the maintenance of the quiescent phenotype, we have displayed in Tables 1A and 1B the induction and functional profiles of the top 10 SMC genes down-regulated by LPS treatment at 4 and 22 h. Two transcripts with the greatest fold decrease in expression in 4 h were albumin and cannabinoid receptor 1 (CNR1). Albumin is the serum carrier protein that plays an important role in stabilizing extracellular fluid volume and is known to be down-regulated during acute inflammation. CNR1 is a G-coupled receptor in brain and some peripheral tissues that recognize plant derived cannabinoids (marijuana) as well as endogenous lipid-like mediators (such as arachidonyl ethanolamide [anandamide] and 2-arachidonyl glycerol) that are potent modulators of neurobehavioral functions[29]. CNR1 and its endogenous ligands have role in regulating cerebral arterial tone and reactivity by modulating influx of calcium through L-type calcium channels. Todate, the role of CNRs in SMC phenotype modulation is unknown. Among the down-regulated genes were two prostanoid receptors, PTGDR and PTGER4, a transcription-related gene (HCAP-G), two structural-related genes (D2LIC, PPFIA3), a chloride transporter (CLCN3) and negative regulators of cell proliferation and apoptosis (GW112[30], INHBE[31]).

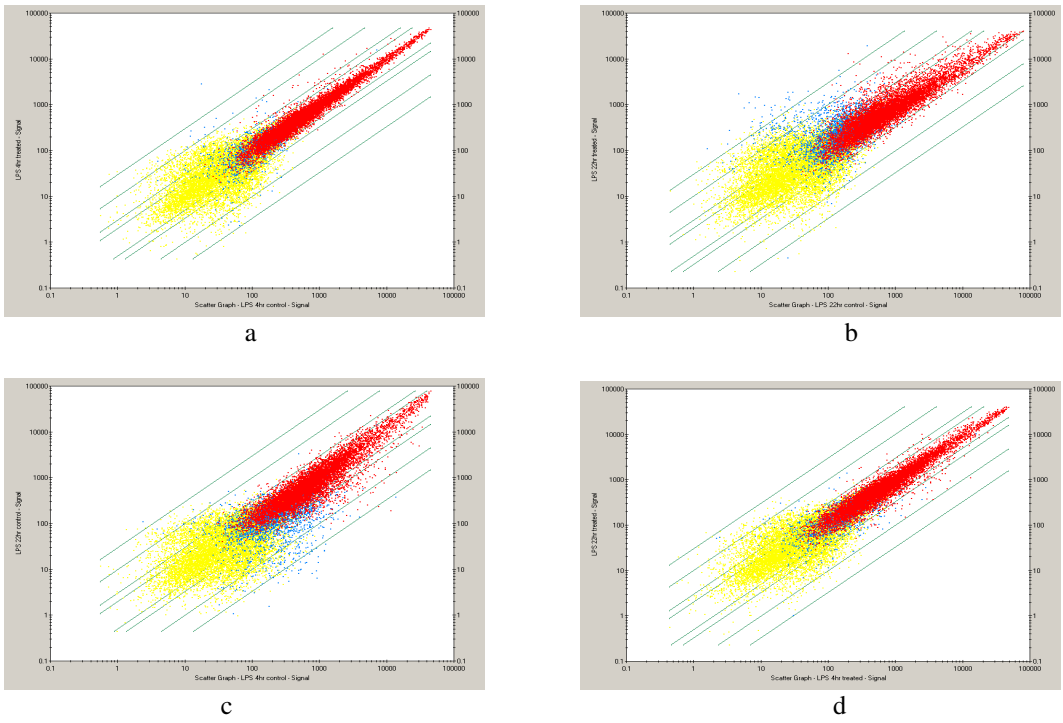

Fig. 2: Scatter Plot analysis of gene expression in VSMCs after 4 and 22 h treatment with AB serum (control) or with AB serum in the presence of LPS. The data points along the diagonal represent genes expressed at comparable levels in both samples whereas those that lie off the diagonal represent genes differentially expressed between the samples identified by the two axes. (A) LPS 4hr control (5% AB serum, 4 h treatment) vs LPS 4 h treated (5%AB serum+100 ng mL^{-1} LPS, 4 hour treatment) (B) LPS 22hr control (5% AB serum, 22 h treatment) vs LPS 22hr treated (5% AB serum+100 ng mL^{-1} LPS, 22 h treatment) (C) LPS 4 h control (5% AB serum, 4 h treatment) vs LPS 22hr control (5% AB serum, 22 h treatment) (D) LPS 4 h treated (5% AB serum+100 ng mL^{-1} LPS, 4 hour treatment) vs LPS 22hr treated (5% AB serum+100 ng mL^{-1} LPS, 22 h treatment)

The expression of the top 10 down-regulated genes at 4 h had returned to near normal levels at 22 h and thus the profile of the genes repressed at 22 h was different from the genes repressed at 4 h (Table 1). Three of the top 10 genes that were most highly down-regulated in 22 h were signal transduction-related genes (KRAS2, PAK1IP1, SH3BP2). This group also contained genes involved in various functions such as transcription repression (HDAC4), ribosome macromolecular assembly (RPS10), cytochrome c oxidase respiratory chain complex (COX5), glycine metabolism (GCSH), actin polymerization and cell adhesion to extracellular matrix (ITGB5) and two hypothetical genes (C6orf79, KIAA0507).

Profile of up-regulated genes: SMC genes up-regulated by LPS treatment may be associated with anti-bacterial or anti-viral activity and inflammation and/or the induction of the proliferative, migratory and synthetic phenotype. The top 10 up-regulated genes in SMC treated with LPS for 4 h were mostly LPS-, interferon-, TNF- or IL1-inducible proinflammatory and antiviral genes that participate in host defense (Table 2). The level of expression of many of the genes up-regulated at 4 h was found to have subsided by 22 h. The gene that was most highly up-regulated in 4 h LPS-stimulated SMCs was IFIT1. It has been suggested that IFIT1 is an anti-viral protein; however, its exact mechanism of action is at present unclear[32]. IFIT1 has recently been reported to bind to Rho guanine nucleotide exchange factor 10[33] and also to block the interaction between eukaryotic translation initiation factors, eIF2 and eIF3 and thereby regulate translation initiation and protein synthesis[34]. IFIT4 which is a member of IFIT1 family of closely linked genes was also highly up-regulated[35]. Other genes encoding

Table 1: Ten SMC genes highly down-regulated by LPS treatment at 4 h (A) and 22 h (B). (All numbers are expressed as signal log ratio.)

Symbol	LPS 4h	LPS 22h	Gene
CNR1	-3.5	1.3	cannabinoid receptor 1 (brain)
ALB	-3.2	0	albumin
HCAP-G	-3.1	-1.1	chromosome condensation protein G
GW112	-3.1	0.1	differentially expressed in hematopoietic lineages
PTGDR	-2.9	0.1	prostaglandin D2 receptor (DP)
CLCN3	-1.8	0.5	chloride channel 3
INHBE	-1.7	-0.4	activin beta E
D2LIC	-1.6	-0.9	dynein 2 light intermediate chain
PTGER4	-1.6	-0.6	prostaglandin E receptor 4 (subtype EP4)
PPFIA3	-1.4	-1.9	protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein (liprin), alpha 3
KRAS2	-0.2	-3.3	v-Ki-ras2 Kirsten rat sarcoma 2 viral oncogene homolog
KIAA0507	0.3	-2.4	Homo sapiens mRNA, chromosome 1 specific transcript KIAA0507.
HDAC4	0.3	-2.2	histone deacetylase 4
COX5A	-0.2	-2.1	cytochrome c oxidase subunit Va
PAK1IP1	-0.1	-2.1	PAK1 interacting protein 1
ITGB5	0.3	-2	integrin, beta 5
SH3BP2	2.1	-2	SH3-domain binding protein 2
GCSH	-0.2	-1.9	Homo sapiens transcribed sequence with strong similarity to protein pir:GCHUH (H.sapiens) GCHUH glycine cleavage system protein H precursor – human
C6orf79	0.1	-1.9	chromosome 6 open reading frame 79
RPS10	0.3	-1.9	ribosomal protein S10

Table 2. Ten SMC genes highly up-regulated by LPS treatment at 4 h (A) and 22 h (B). (All numbers are expressed as signal log ratio.)

Symbol	LPS 4h	LPS 22h	Gene
IFIT1	7.3	5	interferon-induced protein with tetratricopeptide repeats 1
USP18	4.3	5	ubiquitin specific protease 18
SAMHD1	3.9	3.1	SAM domain and HD domain 1
cig5	3.8	0.9	viperin
MX1	3.7	3.8	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)
MDA5	3.7	1.9	melanoma differentiation associated protein-5
IFIT4	3.6	1.6	interferon-induced protein with tetratricopeptide repeats 4
CCL3	3.6	1.3	chemokine (C-C motif) ligand 3
CCL20	3.4	4.3	chemokine (C-C motif) ligand 20
IFI44	3.4	2.6	interferon-induced protein 44
LOXL2	0.1	7	lysyl oxidase-like 2
POLR2E	0	6.8	polymerase (RNA) II (DNA directed) polypeptide E, 25kDa
C1orf29	3.1	6.2	chromosome 1 open reading frame 29
COL6A2	-0.1	6.1	collagen, type VI, alpha 2
IFI27	2.4	5.7	interferon, alpha-inducible protein 27
TETRAN	-0.5	5.7	tetracycline transporter-like protein
EMILIN1	-0.7	5.7	elastin microfibril interfacer 1
MAZ	0.2	5.6	MYC-associated zinc finger protein (purine-binding transcription factor)
PLEC1	-0.4	5.4	plectin 1, intermediate filament binding protein 500kDa
BTBD2	0	5.3	BTB (POZ) domain containing 2

antiviral proteins that were highly induced in 4 h include MX1, cig5 and MDA5. Pro-inflammatory cytokines and chemokines known to be induced in response to bacterial and viral infections (CCL3, CCL20) were upregulated in 4 h LPS-stimulated SMCs. Some of the cytokine up-regulated genes may not be directly involved in defense response and these include USP18 (deubiquitinating protease)[36] and SAMHD1 (implicated in shifting dendritic cells from antigen uptake and processing phenotype to the antigen presentation phenotype)[37]. Two cytokine inducible genes of unknown functions (IFIT4, IFI44) were also upregulated at 4 h. The overall profile of the top 10 genes regulated in 4 h LPS-stimulated SMC is reminiscent of the induction of acute inflammation and a proliferative response.

In contrast with the profile of the genes induced by LPS in 4 h, most of the genes up-regulated in 22 h were probably not the direct result of cytokine activity but rather the effects of induction or activation of transcription factors. The top 10 over-expressed genes were predominantly involved in transcriptional activation, extracellular matrix formation and cell proliferation. LOXL2, a copper-dependent enzyme that

initiates cross-linking of collagens and elastin[38], had the greatest expression among the top 10 highly up-regulated genes in 22 h LPS-treated SMCs (Table 2). Recently a direct link has been established between LOXL2 and SNAIL, a transcription factor that represses E-cadherin expression, in inducing epithelial-mesenchymal cell transitions and carcinoma progression[39]. Thus there is a possibility that the up-regulated expression of LOXL2 in LPS treated SMC may be associated with the induction of the proliferative response. Several genes involved in extracellular matrix formation (COL6A2, EMILIN1, PLEC1) were also highly up-regulated. The other genes in the 22 h top 10 up-regulated category include genes involved in transcription regulation or chromatin modification (POLR2E, MAZ, BTBD2), IFN-inducible genes (IFI27), tetracyclin transporter-like protein (TETRAN) and the hypothetical C1orf29 gene.

Functional characterization of the LPS regulated genes: In order to discover the classes of genes that were involved in LPS-mediated SMC activation, we applied a signal log ratio cut-off of >±1.0, eliminated all redundant probes that had less significant statistical values for each gene and categorized the remaining 1541 differentially regulated genes into functional groups/subgroups based on gene annotation information from Affymetrix database. Genes of unknown or miscellaneous functions were grouped together as Others. As shown in Figure 3, differential expression of genes belonging to functional categories such as transcription and chromatin remodeling, translation, signal transduction, host defense, proliferation, apoptosis, metabolism, ubiquitination, transporters and structural proteins indicates a robust SMC response to LPS treatment. The complete tables of the functional categories of LPS regulated SMC genes are available on request Table 3. A detailed discussion of all regulated genes is beyond the scope of this report and instead we focus on the specific sets of genes that are involved in host defense, transcription and chromatin remodeling, signal transduction, proliferation, apoptosis and cytoskeleton and matrix formation which we believe drive SMC phenotype modulation.

Host defense: Treatment of SMC with LPS induced the expression of several ligands and receptors of C-C and C-X-C chemokines and interleukins. Although the changes in the expression of various interferon subtypes were not deemed significant when analyzed using GCOS version 1.0, several interferon-inducible genes (IFIT1, IFIT2, IFIT4, IFI27, STIP1, G1P2, G1P3,

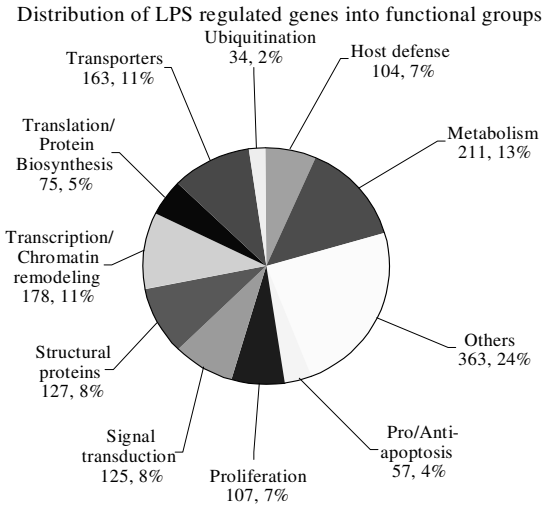

Fig. 3: Functional categories of LPS-regulated VSMC genes. We applied a signal log ratio cut-off of >+1.0 to all the genes that were differentially regulated by LPS treatment by 4 h and 22 h and all redundant probes that had less significant statistical values for each gene were also eliminated. Then the remaining 1541 differentially regulated genes were categorized into functional groups/subgroups based on gene annotation information from Affymetrix database. Genes of unknown or miscellaneous functions were grouped together as Others.

IFI44, IFI35, OAS1-3, MX1), granulocyte colony stimulating factor 3 (CSF3), neutrophil chemokines, IL8, CCL20, CCL3, CXCL3, CXCL10 and MIF were highly expressed. Other inflammatory and immune-related genes such as the toll-interacting protein (TOLLIP)[40] and antiviral proteins (OAS1, 2, 3) were more up-regulated at 22 h compared to 4 h. Serine proteinase inhibitor (SERPINB2) was induced possibly to protect the SMCs from excessive inflammation. Taken together, the results suggest that LPS induces SMCs to express inflammatory cytokines, chemokines and cell surface receptors that could induce chemotactic attraction of hematopoietic cells, neutrophils and mononuclear cells which contribute to inflammatory and immune responses. This is consistent with the well known function of LPS as a potent activator of inflammation and immune response.

Transcription and chromatin remodeling: LPS regulated the expression of many transcription factors and molecules involved in chromatin remodeling. The most highly up-regulated transcription-related gene was

Table 3. VSMC genes regulated by LPS treatment at 4 and 22 h. (Gene expressions are shown in signal log ratio.)

Probe Set ID	Gene	Symbol	4h	22h
A. Genes involved in host defense				
IFN Inducible				
202411_at	interferon, alpha-inducible protein 27	IFI27	2.4	5.7
203153_at	interferon-induced protein with tetratricopeptide repeats 1	IFIT1	7.3	5
212009_s_at	stress-induced-phosphoprotein 1 (Hsp70/Hsp90-organizing protein)	STIP1	-0.1	4.6
205483_s_at	interferon, alpha-inducible protein (clone IFI-15K)	G1P2	2.3	3.9
202086_at	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	MX1	3.7	3.8
204972_at	2'-5'-oligoadenylate synthetase 2, 69/71kDa	OAS2	1.8	2.9
205552_s_at	2',5'-oligoadenylate synthetase 1, 40/46kDa	OAS1	2.5	2.8
217502_at	interferon-induced protein with tetratricopeptide repeats 2	IFIT2	2.3	2.8
218400_at	2'-5'-oligoadenylate synthetase 3, 100kDa	OAS3	1.1	2.8
204415_at	interferon, alpha-inducible protein (clone IFI-6-16)	G1P3	1.3	2.7
214453_s_at	interferon-induced protein 44	IFI44	3.4	2.6
209417_s_at	interferon-induced protein 35	IFI35	0.6	2
204747_at	interferon-induced protein with tetratricopeptide repeats 4	IFIT4	3.6	1.6
Innate Immunity and Inflammation				
205476_at	chemokine (C-C motif) ligand 20	CCL20	3.4	4.3
204614_at	serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 2	SERPINB2	1.3	3.2
217871_s_at	macrophage migration inhibitory factor (glycosylation-inhibiting factor)	MIF	-0.1	2.9
217930_s_at	toll interacting protein	TOLLIP	0.1	2.8
211506_s_at	interleukin 8	IL8	1.9	2.6
207442_at	colony stimulating factor 3 (granulocyte)	CSF3	2.6	1.6
205114_s_at	chemokine (C-C motif) ligand 3	CCL3	3.6	1.3
204533_at	chemokine (C-X-C motif) ligand 10	CXCL10	2.6	0.9
207850_at	chemokine (C-X-C motif) ligand 3	CXCL3	2.5	0.2
B. Genes involved in transcription and chromatin remodeling				
Chromatin Remodeling				
207824_s_at	MYC-associated zinc finger protein (purine-binding transcription factor)	MAZ	0.2	5.6
218045_x_at	parathymosin	PTMS	-0.1	4.9
218064_s_at	neighbor of A-kinase anchoring protein 95	NAKAP95	-0.3	4.9
204805_s_at	H1 histone family, member X	H1FX	-1.1	4
208180_s_at	histone 1, H4h	HIST1H4H	1.5	3
207645_s_at	chromodomain helicase DNA binding protein 1-like	CHD1L	0.3	3
208527_x_at	histone 1, H2be	HIST1H2BE	-0.1	2.4
214290_s_at	histone 2, H2aa	HIST2H2AA	0	2.3
Transcription Regulation				
209636_at	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	NFKB2	0.7	4
209878_s_at	v-rel reticuloendotheliosis viral oncogene homolog A, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3, p65 (avian)	RELA	0.4	3.9
201332_s_at	signal transducer and activator of transcription 6, interleukin-4 induced	STAT6	0.4	3
208436_s_at	interferon regulatory factor 7	IRF7	1.5	2.5
Basal TF				
213887_s_at	polymerase (RNA) II (DNA directed) polypeptide E, 25kDa	POLR2E	0	6.8
203239_s_at	CCR4-NOT transcription complex, subunit 3	CNOT3	-0.2	4.1
215357_s_at	polymerase delta interacting protein 46	PDIP46	0.3	3.6
202996_at	polymerase (DNA-directed), delta 4	POLD4	0	3.5
208960_s_at	core promoter element binding protein	COPEB	0.6	2.6
205966_at	TAF13 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 18kDa	TAF13	0	2.6
210892_s_at	general transcription factor II, i	GTF2I	-0.2	2.2
C. Genes involved in signal transduction				
MAPK				
213490_s_at	mitogen-activated protein kinase kinase 2	MAP2K2	0.2	4
215050_x_at	mitogen-activated protein kinase-activated protein kinase 2	MAPKAPK2	0	3.2
RAS				
209991_x_at	G protein-coupled receptor 51	GPR51	-0.6	5.2
215807_s_at	plexin B1	PLXNB1	0	4.9
207419_s_at	ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2)	RAC2	0.5	4.4
217782_s_at	G protein pathway suppressor 1	GPS1	-0.3	3.9
203267_s_at	developmentally regulated GTP binding protein 2	DRG2	-0.1	3.7
208009_s_at	Rho guanine exchange factor (GEF) 16	ARHGEF16	0.1	3.4
201167_x_at	Rho GDP dissociation inhibitor (GDI) alpha	ARHGDIA	0.8	3.2

206971_at	G protein-coupled receptor 161	GPR161	0.5	3.1
203136_at	Rab acceptor 1 (prenylated)	RABAC1	0.1	2.7
215249_at	mucin 20	MUC20	0.1	2.2
212932_at	RAB3 GTPase-ACTIVATING PROTEIN	RAB3GAP	-0.3	2.1
206673_at	putative G protein coupled receptor	GPR	-0.1	2
200852_x_at	guanine nucleotide binding protein (G protein), beta polypeptide 2	GNB2	-0.4	2
PI3K				
210969_at	protein kinase C-like 2	PRKCL2	0.3	3.9
202545_at	protein kinase C, delta	PRKCD	-1.8	3.8
210417_s_at	phosphatidylinositol 4-kinase, catalytic, beta polypeptide	PIK4CB	-0.2	2.2
NFkB				
58994_at	putative NFkB activating protein	FLJ20241	-0.2	2.7
214398_s_at	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon	IKBKE	0.2	2.3
TGFB Related				
203085_s_at	transforming growth factor, beta 1 (Camurati-Engelmann disease)	TGFB1	-0.3	3.3
209920_at	bone morphogenetic protein receptor, type II (serine/threonine kinase)	BMPR2	-0.4	2
D. Genes involved in proliferation				
Cell Cycle				
221427_s_at	cyclin L2	CCNL2	0	5.1
204247_s_at	cyclin-dependent kinase 5	CDK5	-0.6	4.3
204857_at	MAD1 mitotic arrest deficient-like 1 (yeast)	MAD1L1	-0.8	3.7
211017_s_at	neurofibromin 2 (bilateral acoustic neuroma)	NF2	-0.4	3.5
209754_s_at	thymopoietin	TMPO	0.1	3.1
207428_x_at	cell division cycle 2-like 2	CDC2L2	-0.4	2.8
203252_at	tumor suppressor deleted in oral cancer-related 1	DOC-1R	0	2.7
209414_at	Fzr1 protein	FZR1	0.5	2.4
210622_x_at	cyclin-dependent kinase (CDC2-like) 10	CDK10	0.2	2.4
209953_s_at	CDC37 cell division cycle 37 homolog (S. cerevisiae)	CDC37	-0.4	2.4
208721_s_at	anaphase promoting complex subunit 5	ANAPC5	-0.2	2.3
216277_at	BUB1 budding uninhibited by benzimidazoles 1 homolog (yeast)	BUB1	1.4	2
209112_at	cyclin-dependent kinase inhibitor 1B (p27, Kip1)	CDKN1B	-1.2	-0.7
204170_s_at	CDC28 protein kinase regulatory subunit 2	CKS2	-0.2	-1
202284_s_at	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	CDKN1A	-0.3	-1
204159_at	cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	CDKN2C	-0.7	-1
207039_at	cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	CDKN2A	-0.1	-1.6
GF and Angiogenesis				
219922_s_at	latent transforming growth factor beta binding protein 3	LTBP3	-0.5	4.5
207334_s_at	transforming growth factor, beta receptor II (70/80kDa)	TGFBR2	0.6	4
208231_at	neuregulin 1	NRG1	-0.1	2.9
206796_at	WNT1 inducible signaling pathway protein 1	WISP1	-0.3	2.9
206814_at	nerve growth factor, beta polypeptide	NGFB	0.8	2.6
210513_s_at	vascular endothelial growth factor	VEGF	0.4	2.6
210973_s_at	fibroblast growth factor receptor 1 (fms-related tyrosine kinase 2, Pfeiffer syndrome)	FGFR1	0.3	2.2
213807_x_at	met proto-oncogene (hepatocyte growth factor receptor)	MET	0.9	2
202290_at	PDGFA associated protein 1	PDAP1	0.2	2
Differentiation				
210756_s_at	Notch homolog 2 (Drosophila)	NOTCH2	0.6	2
Others				
200001_at	calpain, small subunit 1	CAPNS1	-0.2	2.7
218663_at	chromosome condensation protein G	HCAP-G	-3.1	-1.1
E. Genes involved in apoptosis				
Pro-apoptotic				
211833_s_at	BCL2-associated X protein	BAX	-0.2	4.1
214667_s_at	tumor protein p53 inducible protein 11	TP53I11	0.6	3.8
209941_at	receptor (TNFRSF)-interacting serine-threonine kinase 1	RIPK1	-0.1	3.6
1861_at	BCL2-antagonist of cell death	BAD	0.1	3.2
206133_at	XIAP associated factor-1	HSXIAPAF1	1.2	2.4
203890_s_at	death-associated protein kinase 3	DAPK3	-0.1	2
214617_at	perforin 1 (pore forming protein)	PRF1	-0.6	2
219209_at	melanoma differentiation associated protein-5	MDA5	3.7	1.9
220518_at	target of Nesh-SH3	TARSH	2	0.8
Anti-apoptotic				
200796_s_at	myeloid cell leukemia sequence 1 (BCL2-related)	MCL1	0.7	5
210538_s_at	baculoviral IAP repeat-containing 3	BIRC3	2.7	4.9

218813_s_at	SH3-domain GRB2-like endophilin B2	SH3GLB2	0	2.9
211546_x_at	synuclein, alpha (non A4 component of amyloid precursor)	SNCA	0.8	2
218943_s_at	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide	RIG-I	3.4	1.6
202510_s_at	tumor necrosis factor, alpha-induced protein 2	TNFAIP2	2.6	1.2
205681_at	BCL2-related protein A1	BCL2A1	2.6	0.6
212768_s_at	differentially expressed in hematopoietic lineages	GW112	-3.1	0.1
TNF Related				
210654_at	tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain	TNFRSF10D	0.3	4.1
206508_at	tumor necrosis factor (ligand) superfamily, member 7	TNFSF7	2.9	1.2
202688_at	tumor necrosis factor (ligand) superfamily, member 10	TNFSF10	2.1	-0.3
F. Genes involved in structural proteins				
ECM				
202997_s_at	lysyl oxidase-like 2	LOXL2	0.1	7
209156_s_at	collagen, type VI, alpha 2	COL6A2	-0.1	6.1
204163_at	elastin microfibril interfacer 1	EMILIN1	-0.7	5.7
217430_x_at	collagen, type I, alpha 1	COL1A1	0.5	5.1
212091_s_at	collagen, type VI, alpha 1	COL6A1	0.2	4.7
211966_at	collagen, type IV, alpha 2	COL4A2	0	4.7
201654_s_at	heparan sulfate proteoglycan 2 (perlecan)	HSPG2	0.6	4.5
201262_s_at	biglycan	BGN	0.7	4.4
202827_s_at	matrix metalloproteinase 14 (membrane-inserted)	MMP14	0.7	4.3
201286_at	syndecan 1	SDC1	0.1	3.4
209561_at	thrombospondin 3	THBS3	-0.1	2.6
205746_s_at	a disintegrin and metalloproteinase domain 17 (tumor necrosis factor, alpha, converting enzyme)	ADAM17	0.1	
Cytoskeleton				
216971_s_at	plectin 1, intermediate filament binding protein 500kDa	PLEC1	-0.4	5.4
209372_x_at	tubulin, beta polypeptide	TUBB	0	4.3
214040_s_at	gelsolin (amyloidosis, Finnish type)	GSN	0.2	4.2
214752_x_at	filamin A, alpha (actin binding protein 280)	FLNA	-0.3	3.3
217817_at	actin related protein 2/3 complex, subunit 4, 20kDa	ARPC4	-0.2	3.2
210978_s_at	transgelin 2	TAGLN2	0.2	3.1
215074_at	myosin IB	MYO1B	-1.3	3
220800_s_at	tropomodulin 3 (ubiquitous)	TMOD3	0	2.1
Adhesion and Migration				
217234_s_at	villin 2 (ezrin)	VIL2	-0.1	3.9
218182_s_at	claudin 1	CLDN1	1.1	2.7
209693_at	astrotactin 2	ASTN2	-0.7	2.7
204627_s_at	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	ITGB3	0.2	2.6
207788_s_at	vinexin beta (SH3-containing adaptor molecule-1)	SCAM-1	0.4	2.5
200808_s_at	zyxin	ZYX	-0.1	2.4
202071_at	syndecan 4 (amphiglycan, ryudocan)	SDC4	1	2.3
210841_s_at	neuropilin 2	NRP2	0.6	2.2
215836_s_at	protocadherin gamma subfamily C, 3	PCDHGC3	0.3	2.1
206908_s_at	claudin 11 (oligodendrocyte transmembrane protein)	CLDN11	0.3	2.1
202638_s_at	intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	ICAM1	1.7	2
204306_s_at	CD151 antigen	CD151	0	2
208850_s_at	Thy-1 cell surface antigen	THY1	0	2

RNA polymerase II polypeptide E (POLR2E), a component of the multisubunit eukaryotic DNA-dependent RNA polymerase II complex[41]. Several other components of the POLR2 holoenzyme were up-regulated. These include DNA polymerases (PDIP46, POLD4), core promoter binding proteins (COPEB, TAF13), initiator binding protein (GTF2I) and transcription complex subunit 3 (CNOT3).

Parathymosin (PTMS) and chromodomain helicase DNA binding proteins (CHD1L) and several nucleosomal histones (H1FX, HIST1H4H, HISTH2BE, HIST2H2AA) were highly up-regulated in LPS-treated cells. Particularly PTMS affects the interaction of linker histone H1 which is the major factor that stabilizes higher order chromatin structure with nucleosomes and thereby modulates the action of chromatin-remodeling enzymes[42]. Zinc finger protein transcriptional regulators (NAKAP95 and MAZ) and members of NF-kB family (NFKB2, RELA) were induced at 22 h. IRF7 (a regulatory factor of viral/interferon inducible genes) and a member of the STAT family of transcription activators (STAT 6) were induced at 22 h. These results

are in agreement with previous reports of activation of the JAK-STAT signaling pathway in LPS treated cells. The above compendium of LPS-mediated differential expression of several chromatin remodeling genes and transcription regulators have profound effects on gene expression in LPS treated SMCs and subsequently on inflammation, proliferation, apoptosis, ECM production, cell adhesion and cell migration responses and the modulation of the quiescent cells to the activated SMC phenotype.

Signal transduction: Several genes involved in a variety of signal transduction pathways were up-regulated in SMCs following treatment with LPS for 22 h. The Ras/Rho/CDC42 signaling pathway was activated as judged by the increase in the expression of genes encoding Rho guanine nucleotide exchange factors and dissociation inhibitors (ARHGEF16, ARHGDIA), G-protein coupled receptors (GPR, GPR51, GPR161, MUC20) and GTP-binding proteins of the Ras superfamily (GNB2, GPS1, DRG2, RABAC1, RAB3GAP, RAC2). Plexin B1 (PLXNB1) was the second highest induced gene in this category. PLXNB1 has been reported to mediate Sema4D-induced repulsive axon guidance signaling by acting as a GTPase activating protein for R-Ras, a member of the Ras superfamily of small GTP-binding proteins, which plays a key role in cell adhesion by activating integrins and promoting cell migration and neurite outgrowth[43, 44].

The transforming growth factor signaling pathway was recruited as judged by the induction of TGFB1, TGFBR2, LTBP3 and BMPR2. Binding of TGFB or BMPs to cognate type II receptors leads to the phosphorylation of the type I subunit by the constitutively active serine-threonine kinase type II receptor. Phosphorylated receptor I is an active kinase that subsequently phosphorylates and propagates intracellular downstream signals, including Smad proteins and mitogen-activated protein kinases[45, 46]. By the regulation of TGF inducible genes, the TGFB signaling pathway may mediate regulatory effects on a multitude of cellular processes in the LPS-treated SMCs including proliferation, differentiation, migration and extracellular matrix accumulation[47].

LPS also stimulated the expression of several transcriptional activators. The NF-kB subunits NFKB2 (p49/p100) and RELA (p65) as well as a putative NF-kB activating protein (FLJ20241) and an inhibitor of inducible I-kappa B-kinase epsilon (IKBKE) were up-regulated and their activation suggests NFkB pathway signaling[48]. The MAPK pathway was activated in LPS-treated SMCs as judged by the increased expression of MAPK enzymes[48] (MAP2K2, MAPKAPK2). The phosphatidylinositol-3-kinase (PI3K) signaling pathway was also activated as judged by the increased expression of PIK4CB and protein kinase isotypes (PRKCD, PRKCL2).

Proliferation: Several growth factor genes (including VEGF, NRG1, NGFB) and growth factor receptor genes (e.g. FGFR1, MET, PDAP1) were induced by LPS at 22 h. In mammals, the progression through the cell cycle is regulated by the association of cyclins and their cyclin-dependent kinase (CDK) partners. Activity of these cyclin/cyclin-dependent kinase complexes is inhibited by the action of cyclin-dependent kinase inhibitors (CDKIs) that bind to these complexes and thereby inhibit progression through the restriction points of the cell cycle and cellular proliferation. Treatment of SMCs with LPS for 22 h resulted in up-regulation of several cell cycle-dependent genes (CCNL2, CDC2L2, CDC37, CDK5, CDK10), cell cycle regulatory genes (BUB1, human DOC-1R, NF2, FZR1, WISP1) and regulators of proliferation (MAD1L1, TMPO, CAPNS1, ANAPC5, NOTCH2). In contrast, negative regulators of growth such as cell cycle arrest genes including HCAP-G (mitosis-related chromosome condensation protein G), MAD1L1 (a G1 to S cell cycle checkpoint protein) and several cyclin-dependent kinase inhibitors (CKS2, p27, p21, p18, p16) were repressed at 4 h. These results are consistent with the pro-proliferative effect of LPS on SMCs.

Apoptosis: Six anti-apoptotic genes (BIRC3, RIG-I, BCL2A1, TNFAIP2, TNFSF7, TNFSF10) and 3 pro-apoptotic genes (HSXIAPAF1, MDA5, TARSH) were up-regulated within 4 h of LPS treatment whereas GW112 were repressed at 4 h. Pro-apoptotic genes such as BAX, RIPK1, TP53I11, BAD, DAPK3, PRF1, MCL1, SH3GLB2, SNCA and TNFRSF10D were induced at 22 h and HSXIAPAF1 was induced at both 4 and 22 h.

Structural proteins: The changes in expression levels of structural genes occurred predominantly at 22 h. Among the up-regulated genes, extracellular matrix collagen 6A2, 6A1, 1A1 and 4A2 were greatly increased. The different types of collagens i) may be associated with laminin, entactin and heparan sulfate proteoglycans to form the sheet-like basement membranes that separate epithelium from connective tissue or ii) may be components of microfibrillar structures in many tissues that localize close to cells

where they may provide an anchoring function and may be involved in cell migration, differentiation and embryonic development. Several non-collagenous ECM components were also up-regulated. LOXL2, a copper-dependent enzyme that initiates cross-linking of collagens and elastin, was among the highly up-regulated structural genes in 22 h LPS-treated SMCs (Table 1B). Genes encoding microfibril associated proteins (EMILIN1, THBS3) and heparin sulfate proteoglycans of basement membranes (HSPG2, BGN, SDC1, SDC4) were also up-regulated at 22 h. These proteins may play a role in cell adhesion, cell morphology, intracellular communication and anchorage of SMC to elastic lamellae. The expression of several genes encoding cytoplasmic cytoskeletal proteins involved in actin reorganization (PLEC1, TUBB, GSN, NF2, VIL2, FLNA, ARPC4, TAGLN2, MYO1B, NRP2, TMOD3) were up-regulated after 22 h of LPS stimulation. These cytoskeletal proteins are central to the modulation of cell shape, adhesion, migration and the organization of cell surface structures.

Cell adhesion is an essential requirement for cell migration and morphogenesis during development. Genes encoding integral membrane proteins localized at focal adhesions and cell-cell junctions, such as VIL2, ASTN2, CLDN1, CLDN11, ITGB3, SCAM-1, ZYX, PCDHGC3, THY1, CD151, ICAM1 were up-regulated at 22 h in LPS stimulated SMCs. These proteins regulate a variety of cellular events, including cell adhesion, spreading and migration, cell proliferation and differentiation and anchorage dependence of signal transduction that may lead to cell proliferation, migration or transformation and metastasis[49,50,51]. Secretory metalloproteinases (such as MMP14, ADAM17) which are Zn(2+)-binding endopeptidases that degrade various components of the extracellular matrix such as collagens, pro-collagens and hyaluronan binding proteoglycans and probably facilitate SMC migration were over-expressed in LPS stimulated SMC.

Studies using the candidate gene approach has shown that bacterial lipopolysaccharide induces differential induction of the expression of several VSMC genes that encode cytokines (IL-1[52], IL-6[53], TNF-alpha[54], IFN[55]), chemokines (MCP-1[56], IP-10[57], IL-8, MIP-2[58]), enzymes involved in prostanoid synthesis (PLA2[59], COX-2[60]), adhesion molecules (VCAM-1[61], E-selectin[62], ICAM-1[63]), LPS-binding proteins (LBP[64], CD14[65], TLR4[17]), human antigen R[16] (an RNA-binding protein involved in LPS cell activation effects), adrenomedullin[66], heme oxygenase-1[67], low-density lipoprotein receptor-1 (LOX-1)[68], complement components and regulators (C3, C4, DAF, MCP, CD59[69]), iNOS[70] and NO production[71], endothelin-receptor A and B[72], antiviral cytomegalovirus inducible gene 5 (viperin)[73], tissue factor and urokinase-type plasminogen activator (u-PA)[74]. LPS has also been shown to mediate some of its effects by the regulation of cytokines[55], activation of NADPH oxidase[17] and MAPK[17,75] and NFKB[70] signaling pathways. The data reported in this study is in agreement with most of these findings and also provides a comprehensive list of genes that have thus far not been implicated in LPS biology.

Clustering analysis of genes displaying similar expression: Differentially regulated genes were grouped according to their expression patterns following treatment with AB (4 h), AB+LPS (4 h), AB (22 h) and AB+LPS (22 h) to determine if a correlation exists between gene function and expression pattern in response to LPS stimulation. Gene expression pattern analysis was performed using GENESIS ver. 1.3.0. Raw expression values of all differentially regulated genes (above signal log ratio of 1.0) from microarray analysis were adjusted with 'log 2 transform data' and 'median centre genes' and clustered into 40 groups using k-mean clustering. We focused on regulation at 4 h because we reasoned that this early time period may better represent direct signaling effect of LPS on gene transcription. Clusters with similar gene expression pattern were combined together to generate 3 groups: up-regulated at both 4 and 22 h, up-regulated at 4 h and down-regulated at 4 h. Detailed results of clustering analysis are available on request.

The majority of 83 genes up-regulated at both 4 and 22 h are IFN- or TNF-inducible genes (IFI-15K, IFIT1, IFN p78, IFNAI27, IFNR2, ISTF3, IFNIP44, IFIT4, OAS1-3, IRF7, TNFSF7, 10, TNFAIP2, etc.) or cytokine/chemokine genes (CCL3, CXCL3, CSF3, CXCL10, CCL8, IL8, etc.) associated with the inflammatory response. This observation re-emphasizes the important role of LPS in the regulation of SMC activation and phenotype modulation and the initiation and/or augmentation of inflammation in cardiovascular diseases.

Fourteen out of 41 genes in the 4 h up-regulated group belong to a subgroup containing growth factors (FGF2, BMP2, LIM), cytokines (IL-1b, IL-15) and cytokine-induced genes (IFIT5, VCAM1, TNFAIP6, IKKa, CC2, CC7, CXC11, OASL, SOD2). The 4 h down-regulated gene group (116 genes) contained genes that mediate diverse biological functions such as chromosomal modification, signal transduction, growth and differentiation, metabolism, translation, and nuclear transport.

siRNA knockdown of IFIT1: Since IFIT1 was an early response gene that was most dramatically induced by LPS, we hypothesized that it may have an important role in the early events of the activation and proliferation of SMCs. Thus to gain insight into the biological significance of IFIT1 in LPS-induced SMCs, we knocked down IFIT1 transcripts using small interference RNA and then compared the expression of candidate genes in knock-down cells with wild type cells using real-time PCR. Human aortic SMCs (T/G HA-VSMC) were transfected with scrambled siRNA or IFIT1 siRNA as described and IFIT1 expression was assessed using semi-quantitative RT-PCR. Aliquots of the amplification mixtures were collected after 25, 30 and 35 cycles and analyzed by 1% agarose gel electrophoresis. IFIT1 expression was almost completely suppressed by siRNA relative to scrambled siRNA control (data not shown). To gain further insight into the biological functions of IFIT1, the effect of IFIT1 knock-down on the expression of 14 different candidate genes such as IFIT1, IFIT4, FOS, B2M, cytokines (TNF, TGFβ), adhesion molecules (ICAM1, VCAM), cell cycle progression/control (CCND1, p53, p15, p16, p21, p27) was analyzed. The expression of each gene in IFIT1 knock-down (KD) SMCs was calculated relative to the expression level in control cells treated with the scrambled siRNA, which was taken as 100%. The expression of IFIT1 in SMC IFIT1 knock-down SMCs was reduced to 8% and interestingly the expression of the closely linked paralogous gene, IFIT4, was also coordinately down-regulated to 35% (Fig. 4A). The expression of TGFβ, ICAM1, VCAM, p15, p16, p27 and B2M was not significantly affected by IFIT1 KD. While TNF was moderately down-regulated to 65% (Fig. 4A), IFIT1 knock-down resulted in a remarkable up-regulation of FOS (8 fold), TP53 (11 fold), CDKN1A/p21 (866 fold) (Fig. 4B). The human c-fos oncogene heterodimerizes with c-jun to form the immediate-early transcription factor AP-1 that stimulates the transcription of genes containing AP-1 regulatory elements[76]. The tumor suppressor protein, TP53, which is undetectable or present in low levels in resting cells but is generally found in increasing amounts in proliferating non-transformed and transformed cells, is up-regulated in response to stress signals, DNA damage and is transcriptionally induced by IFNA/B through ISGF3[77]. It has been proposed that TP53 binds as a tetramer to a p53-binding site and activates the transcription of downstream target genes that induce apoptosis or cell cycle arrest and/or invasion[78,79]. The cyclin-dependent kinase inhibitor, CDKN1A/p21,

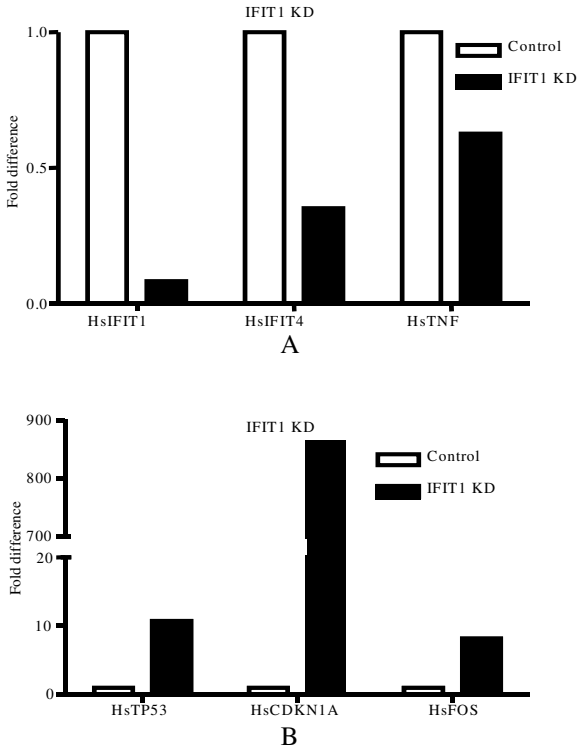

Fig. 4: Effect of IFIT1 knock-down on VSMC gene expression. The expression of IFIT1 was knocked down using a siRNA specific to IFIT1 (IFIT1 KD-black bars) and a scrambled siRNA as a control (Control-white bars). The expression of candidate genes was measured using real-time quantitative PCR. (A) The effect of IFIT1 knock-down on the expression of IFIT1, IFIT4, TNF. (B) The effect of IFIT1 knock-down on the expression of TP53, CDKN1A, FOS.

participates in the regulation of the G2 checkpoint of the cell cycle and probably serves as the effector of TP53 cell cycle control[76]. The up-regulation of these genes in IFIT1 knock-down cells suggests that IFIT1 may be implicated in the regulation of the expression of the above genes during SMC proliferation.

CONCLUSION

In this study, LPS was found to have a profound effect on SMC gene expression profile and the induction of many genes involved in cellular transcription, translation, signal transduction and metabolism indicates the activated state of SMC. This global change in genetic profile may be the main force

that drives the LPS-induced phenotypic transition of SMC rather than the differential expression of a single gene or a few genes. The induction of genes involved in cell proliferation, adhesion, motility and matrix formation suggests that LPS mediated SMC transition from the quiescent to proliferative, synthetic and migratory phenotype. Many genes associated with apoptosis, both pro- and anti-, were also differentially regulated by LPS suggesting that the balance in cell survival and death was somehow tilted although the direction is not clear. Since LPS is the ligand for TLR4 that is a part of host innate immune system, genes involved in host defense are activated expectedly. The LPS response is not due solely to primary TLR4 signaling pathway but also the combinatory effect of various signaling pathways activated by the interaction of several ligands with their cognate receptors that are induced in this system. This will consist of LPS interacting with TLR4 in the presence of CD14 and LBP as well as the interaction of growth factors, interleukins, interferons and TGFb with their receptors.

ACKNOWEDGMENTS

This work was supported by grants from the University of Toronto, Faculty of Medicine, Dean's Research Fund (to J.M) and Genome Canada (to S.D).

REFERENCES

1. Ross, R., 1999. Atherosclerosis--an inflammatory disease. N. Engl. J. Med., 340 (2): 115-126.
2. Lusis, A.J., 2000. Atherosclerosis. Nature, 407 (6801): 233-241.
3. Gaydos, C.A., J.T. Summersgill, N.N. Sahney, J.A. Ramirez and T.C. Quinn, 1996. Replication of Chlamydia pneumoniae in vitro in human macrophages, endothelial cells and aortic artery smooth muscle cells. Infect. Immun., 64 (5): 1614-1620.
4. Shor, A. and J.I. Phillips, 1999. Chlamydia pneumoniae and atherosclerosis. Jama, 282 (21): 2071-2073.
5. Detmer, K., Z. Wang, D. Warejcka, S.K. Leeper-Woodford and W.H. Newman, 2001. Endotoxin stimulated cytokine production in rat vascular smooth muscle cells. Am. J. Physiol. Heart Circ. Physiol., 281 (2): H661-668.
6. Kaukoranta-Tolvanen, S.S., A.M. Teppo, K. Laitinen, P. Saikku, K. Linnavuori and M. Leinonen, 1996. Growth of Chlamydia pneumoniae in cultured human peripheral blood mononuclear cells and induction of a cytokine response. Microb. Pathog., 21 (3): 215-221.
7. Kalayoglu, M.V. and G.I. Byrne, 1998. A Chlamydia pneumoniae component that induces macrophage foam cell formation is chlamydial lipopolysaccharide. Infect. Immun., 66 (11): 5067-5072.
8. Liao, W., 1996. Endotoxin: Possible roles in initiation and development of atherosclerosis. J. Lab. Clin. Med., 128 (5): 452-460.
9. Libby, P., P.M. Ridker and A. Maseri, 2002. Inflammation and atherosclerosis. Circulation, 105 (9): 1135-1143.
10. Rice, J.B., L.L. Stoll, W.G. Li, G.M. Denning, J. Weydert, E. Charipar, W.E. Richenbacher, F.J. Miller, Jr. and N.L. Weintraub, 2003. Low-level endotoxin induces potent inflammatory activation of human blood vessels: inhibition by statins. Arterioscler. Thromb. Vasc. Biol., 23 (9): 1576-1582.
11. Danenberg, H.D., F.G. Welt, M. Walker, P. Seifert, G.S. Toegel and E.R. Edelman, 2002. Systemic inflammation induced by lipopolysaccharide increases neointimal formation after balloon and stent injury in rabbits. Circulation, 105 (24): 2917-2922.
12. Seydel, U., A.B. Schromm, R. Blunck and K. Brandenburg, 2000. Chemical structure, molecular conformation and bioactivity of endotoxins. Chem. Immunol., 74: 5-24.
13. Hailman, E., H.S. Lichenstein, M.M. Wurfel, D.S. Miller, D.A. Johnson, M. Kelley, L.A. Busse, M.M. Zukowski and S.D. Wright, 1994. Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14. J. Exp. Med., 179 (1): 269-277.
14. Simmons, D.L., S. Tan, D.G. Tenen, A. Nicholson-Weller and B. Seed, 1989. Monocyte antigen CD14 is a phospholipid anchored membrane protein. Blood, 73 (1): 284-289.
15. Wright, S.D., R.A. Ramos, P.S. Tobias, R.J. Ulevitch and J.C. Mathison, 1990. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science, 249 (4975): 1431-1433.
16. Lin, F.Y., Y.H. Chen, Y.W. Lin, J.S. Tsai, J.W. Chen, H.J. Wang, Y.L. Chen, C.Y. Li and S.J. Lin, 2006. The role of human antigen R, an RNA-binding protein, in mediating the stabilization of toll-like receptor 4 mRNA induced by endotoxin: a novel mechanism involved in vascular inflammation. Arterioscler. Thromb. Vasc. Biol., 26 (12): 2622-2629.

17. Lin, F.Y., Y.H. Chen, J.S. Tasi, J.W. Chen, T.L. Yang, H.J. Wang, C.Y. Li, Y.L. Chen and S.J. Lin, 2006. Endotoxin induces toll-like receptor 4 expression in vascular smooth muscle cells via NADPH oxidase activation and mitogen-activated protein kinase signaling pathways. Arterioscler. Thromb. Vasc. Biol., 26 (12): 2630-2637.
18. Sasu, S., D. LaVerda, N. Qureshi, D.T. Golenbock and D. Beasley, 2001. Chlamydia pneumoniae and chlamydial heat shock protein 60 stimulate proliferation of human vascular smooth muscle cells via toll-like receptor 4 and p44/p42 mitogen-activated protein kinase activation. Circ. Res., 89 (3): 244-250.
19. Schletter, J., H. Brade, L. Brade, C. Kruger, H. Loppnow, S. Kusumoto, E.T. Rietschel, H.D. Flad and A.J. Ulmer, 1995. Binding of lipopolysaccharide (LPS) to an 80-kilodalton membrane protein of human cells is mediated by soluble CD14 and LPS-binding protein. Infect. Immun., 63 (7): 2576-2580.
20. Stoll, L.L., G.M. Denning, W.G. Li, J.B. Rice, A.L. Harrelson, S.A. Romig, S.T. Gunnlaugsson, F.J. Miller, Jr. and N.L. Weintraub, 2004. Regulation of endotoxin-induced proinflammatory activation in human coronary artery cells: expression of functional membrane-bound CD14 by human coronary artery smooth muscle cells. J. Immunol., 173 (2): 1336-1343.
21. Zhang, G. and S. Ghosh, 2000. Molecular mechanisms of NF-kappaB activation induced by bacterial lipopolysaccharide through Toll-like receptors. J. Endotoxin Res., 6 (6): 453-457.
22. Engelmann, M.G., C.V. Redl and S. Nikol, 2004. Recurrent perivascular inflammation induced by lipopolysaccharide (endotoxin) results in the formation of atheromatous lesions *in vivo*. Lab. Invest., 84 (4): 425-432.
23. Engelmann, M.G., C.V. Redl, J. Pelisek, C. Barz, J. Heesemann and S. Nikol, 2006. Chronic perivascular inoculation with Chlamydophila pneumoniae results in plaque formation *in vivo*. Lab. Invest., 86 (5): 467-476.
24. Eisen, M.B., P.T. Spellman, P.O. Brown and D. Botstein, 1998. Cluster analysis and display of genome-wide expression patterns. Proc. Natl. Acad. Sci. U.S.A., 95 (25): 14863-14868.
25. Yun, J.J., L.E. Heisler, Hwang, II, O. Wilkins, S.K. Lau, M. Hyrcza, B. Jayabalasingham, J. Jin, J. McLaurin, M.S. Tsao and S.D. Der, 2006. Genomic DNA functions as a universal external standard in quantitative real-time PCR. Nucleic Acids Res., 34 (12): e85.
26. Loppnow, H., F. Stelter, U. Schonbeck, C. Schluter, M. Ernst, C. Schutt and H.D. Flad, 1995. Endotoxin activates human vascular smooth muscle cells despite lack of expression of CD14 mRNA or endogenous membrane CD14. Infect. Immun., 63 (3): 1020-1026.
27. Barbosa de Oliveira, L.C., C.J. Rocha Oliveira, D.M. Fries, A. Stern and H.P. Monteiro, 2002. Effects of lipopolysaccharide on low- and high-density cultured rabbit vascular smooth muscle cells: differential modulation of nitric oxide release, ERK1/ERK2 MAP kinase activity, protein tyrosine phosphatase activity and DNA synthesis. Braz. J. Med. Biol. Res., 35 (2): 181-190.
28. Lin, F.Y., Y.H. Chen, Y.L. Chen, T.C. Wu, C.Y. Li, J.W. Chen and S.J. Lin, 2007. Ginkgo biloba Extract Inhibits Endotoxin-Induced Human Aortic Smooth Muscle Cell Proliferation via Suppression of Toll-like Receptor 4 Expression and NADPH Oxidase Activation. J. Agric. Food Chem., 55 (5): 1977-1984.
29. Golech, S.A., R.M. McCarron, Y. Chen, J. Bembry, F. Lenz, R. Mechoulam, E. Shohami and M. Spatz, 2004. Human brain endothelium: coexpression and function of vanilloid and endocannabinoid receptors. Brain Res. Mol. Brain Res., 132 (1): 87-92.
30. Kobayashi, D., S. Koshida, R. Moriai, N. Tsuji and N. Watanabe, 2007. Olfactomedin 4 promotes S-phase transition in proliferation of pancreatic cancer cells. Cancer Sci., 98 (3): 334-340.
31. Chabicovsky, M., K. Herkner and W. Rossmanith, 2003. Overexpression of activin beta(C) or activin beta(E) in the mouse liver inhibits regenerative deoxyribonucleic acid synthesis of hepatic cells. Endocrinology, 144 (8): 3497-3504.
32. Nicholl, M.J., L.H. Robinson and C.M. Preston, 2000. Activation of cellular interferon-responsive genes after infection of human cells with herpes simplex virus type 1.J. Gen. Virol., 81 (Pt 9): 2215-2218.
33. Ye, S., H. Pang, Y.Y. Gu, J. Hua, X.G. Chen, C.D. Bao, Y. Wang, W. Zhang, J. Qian, B.P. Tsao, B.H. Hahn, S.L. Chen, Z.H. Rao and N. Shen, 2003. Protein interaction for an interferon-inducible systemic lupus associated gene, IFIT1. Rheumatology (Oxford), 42 (10): 1155-1163.
34. Hui, D.J., C.R. Bhasker, W.C. Merrick and G.C. Sen, 2003. Viral stress-inducible protein p56 inhibits translation by blocking the interaction of eIF3 with the ternary complex eIF2.GTP.Met-tRNAi. J. Biol. Chem., 278 (41): 39477-39482.

35. Bluyssen, H.A., R.J. Vlietstra, P.W. Faber, E.M. Smit, A. Hagemeijer and J. Trapman, 1994. Structure, chromosome localization and regulation of expression of the interferon-regulated mouse Ifi54/Ifi56 gene family. Genomics, 24 (1): 137-148.
36. Schwer, H., L.Q. Liu, L. Zhou, M.T. Little, Z. Pan, C.J. Hetherington and D.E. Zhang, 2000. Cloning and characterization of a novel human ubiquitin-specific protease, a homologue of murine UBP43 (Usp18). Genomics, 65 (1): 44-52.
37. Li, N., W. Zhang and X. Cao, 2000. Identification of human homologue of mouse IFN-gamma induced protein from human dendritic cells. Immunol. Lett., 74 (3): 221-224.
38. Csiszar, K., 2001. Lysyl oxidases: a novel multifunctional amine oxidase family. Prog. Nucleic Acid Res. Mol. Biol., 70: 1-32.
39. Peinado, H., M. Del Carmen Iglesias-de la Cruz, D. Olmeda, K. Csiszar, K.S. Fong, S. Vega, M.A. Nieto, A. Cano and F. Portillo, 2005. A molecular role for lysyl oxidase-like 2 enzyme in snail regulation and tumor progression. Embo J., 24 (19): 3446-3458.
40. Didierlaurent, A., B. Brissoni, D. Velin, N. Aebi, A. Tardivel, E. Kaslin, J.C. Sirard, G. Angelov, J. Tschopp and K. Burns, 2006. Tollip regulates proinflammatory responses to interleukin-1 and lipopolysaccharide. Mol. Cell. Biol., 26 (3): 735-742.
41. Thomas, M.C. and C.M. Chiang, 2006. The general transcription machinery and general cofactors. Crit. Rev. Biochem. Mol. Biol., 41 (3): 105-178.
42. Martic, G., Z. Karetsou, K. Kefala, A.S. Politou, C.R. Clapier, T. Straub and T. Papamarcaki, 2005. Parathymosin affects the binding of linker histone H1 to nucleosomes and remodels chromatin structure. J. Biol. Chem., 280 (16): 16143-16150.
43. Nakamura, F., R.G. Kalb and S.M. Strittmatter, 2000. Molecular basis of semaphorin-mediated axon guidance. J. Neurobiol., 44 (2): 219-229.
44. Tamagnone, L., S. Artigiani, H. Chen, Z. He, G.I. Ming, H. Song, A. Chedotal, M.L. Winberg, C.S. Goodman, M. Poo, M. Tessier-Lavigne and P.M. Comoglio, 1999. Plexins are a large family of receptors for transmembrane, secreted and GPI-anchored semaphorins in vertebrates. Cell, 99 (1): 71-80.
45. Gambaro, K., E. Aberdam, T. Virolle, D. Aberdam and M. Rouleau, 2006. BMP-4 induces a Smad-dependent apoptotic cell death of mouse embryonic stem cell-derived neural precursors. Cell Death Differ., 13 (7): 1075-1087.
46. Varga, A.C. and J.L. Wrana, 2005. The disparate role of BMP in stem cell biology. Oncogene, 24 (37): 5713-5721.
47. Yu, P.B., H. Beppu, N. Kawai, E. Li and K.D. Bloch, 2005. Bone morphogenetic protein (BMP) type II receptor deletion reveals BMP ligand-specific gain of signaling in pulmonary artery smooth muscle cells. J. Biol. Chem., 280 (26): 24443-24450.
48. Cloutier, A., T. Ear, E. Blais-Charron, C.M. Dubois and P.P. McDonald, 2007. Differential involvement of NF-{kappa}B and MAP kinase pathways in the generation of inflammatory cytokines by human neutrophils. J. Leukoc. Biol., 81 (2): 567-577.
49. Pennica, D., T.A. Swanson, J.W. Welsh, M.A. Roy, D.A. Lawrence, J. Lee, J. Brush, L.A. Taneyhill, B. Deuel, M. Lew, C. Watanabe, R.L. Cohen, M.F. Melhem, G.G. Finley, P. Quirke, A.D. Goddard, K.J. Hillan, A.L. Gurney, D. Botstein and A.J. Levine, 1998. WISP genes are members of the connective tissue growth factor family that are up-regulated in wnt-1-transformed cells and aberrantly expressed in human colon tumors. Proc. Natl. Acad. Sci. U.S.A., 95 (25): 14717-14722.
50. Suwa, A., M. Mitsushima, T. Ito, M. Akamatsu, K. Ueda, T. Amachi and N. Kioka, 2002. Vinexin beta regulates the anchorage dependence of ERK2 activation stimulated by epidermal growth factor. J. Biol. Chem., 277 (15): 13053-13058.
51. Xu, L., R.B. Corcoran, J.W. Welsh, D. Pennica and A.J. Levine, 2000. WISP-1 is a Wnt-1- and beta-catenin-responsive oncogene. Genes Dev., 14 (5): 585-595.
52. Libby, P., J.M. Ordovas, L.K. Birinyi, K.R. Auger and C.A. Dinarello, 1986. Inducible interleukin-1 gene expression in human vascular smooth muscle cells. J. Clin. Invest., 78 (6): 1432-1438.
53. Aarden, L., M. Helle, L. Boeije, D. Pascual-Salcedo and E. de Groot, 1991. Differential induction of interleukin-6 production in monocytes, endothelial cells and smooth muscle cells. Eur. Cytokine Netw., 2 (2): 115-120.
54. Van Dervort, A.L., L. Yan, P.J. Madara, J.P. Cobb, R.A. Wesley, C.C. Corriveau, M.M. Tropea and R.L. Danner, 1994. Nitric oxide regulates endotoxin-induced TNF-alpha production by human neutrophils. J. Immunol., 152 (8): 4102-4109.
55. Lo, Y.C., C.C. Wang, K.P. Shen, B.N. Wu, K.L. Yu and I.J. Chen, 2004. Urgosedin inhibits hypotension, hypoglycemia and pro-inflammatory mediators induced by lipopolysaccharide. J. Cardiovasc. Pharmacol., 44 (3): 363-371.

56. Ikeda, U., K. Okada, S. Ishikawa, T. Saito, T. Kasahara and K. Shimada, 1995. Monocyte chemoattractant protein 1 inhibits growth of rat vascular smooth muscle cells. Am. J. Physiol., 268 (3 Pt 2): H1021-1026.
57. Wang, X., T.L. Yue, E.H. Ohlstein, C.P. Sung and G.Z. Feuerstein, 1996. Interferon-inducible protein-10 involves vascular smooth muscle cell migration, proliferation and inflammatory response. J. Biol. Chem., 271 (39): 24286-24293.
58. Yoshimura, T., M. Takeya, H. Ogata, S. Yamashiro, W.S. Modi and R. Gillitzer, 1999. Molecular cloning of the guinea pig GRO gene and its rapid expression in the tissues of lipopolysaccharide-injected guinea pigs. Int. Arch. Allergy Immunol., 119 (2): 101-111.
59. Nakano, T., O. Ohara, H. Teraoka and H. Arita, 1990. Group II phospholipase A2 mRNA synthesis is stimulated by two distinct mechanisms in rat vascular smooth muscle cells. FEBS Lett., 261 (1): 171-174.
60. Ermert, L., M. Ermert, M. Merkle, M. Goppelt-Struebe, H.R. Duncker, F. Grimminger and W. Seeger, 2000. Rat pulmonary cyclooxygenase-2 expression in response to endotoxin challenge: Differential regulation in the various types of cells in the lung. Am. J. Pathol., 156 (4): 1275-1287.
61. Li, H., M.I. Cybulsky, M.A. Gimbrone, Jr. and P. Libby, 1993. Inducible expression of vascular cell adhesion molecule-1 by vascular smooth muscle cells in vitro and within rabbit atheroma. Am. J. Pathol., 143 (6): 1551-1559.
62. Chen, X.L., P.E. Tummala, L. Olliff and R.M. Medford, 1997. E-selectin gene expression in vascular smooth muscle cells. Evidence for a tissue-specific repressor protein. Circ. Res., 80 (3): 305-311.
63. Beck-Schimmer, B., R.C. Schimmer, R.L. Warner, H. Schmal, G. Nordblom, C.M. Flory, M.E. Lesch, H.P. Friedl, D.J. Schrier and P.A. Ward, 1997. Expression of lung vascular and airway ICAM-1 after exposure to bacterial lipopolysaccharide. Am. J. Respir. Cell Mol. Biol., 17 (3): 344-352.
64. Wong, H.R., B.R. Pitt, G.L. Su, D.P. Rossignol, A.R. Steve, T.R. Billiar and S.C. Wang, 1995. Induction of lipopolysaccharide-binding protein gene expression in cultured rat pulmonary artery smooth muscle cells by interleukin 1 beta. Am. J. Respir. Cell Mol. Biol., 12 (4): 449-454.
65. Choi, H.C. and K.Y. Lee, 2004. CD14 glycoprotein expressed in vascular smooth muscle cells. J. Pharmacol. Sci., 95 (1): 65-70.
66. Shoji, H., N. Minamino, K. Kangawa and H. Matsuo, 1995. Endotoxin markedly elevates plasma concentration and gene transcription of adrenomedullin in rat. Biochem. Biophys. Res. Commun., 215 (2): 531-537.
67. Yet, S.F., A. Pellacani, C. Patterson, L. Tan, S.C. Folta, L. Foster, W.S. Lee, C.M. Hsieh and M.A. Perrella, 1997. Induction of heme oxygenase-1 expression in vascular smooth muscle cells. A link to endotoxic shock. J. Biol. Chem., 272 (7): 4295-4301.
68. Nagase, M., J. Abe, K. Takahashi, J. Ando, S. Hirose and T. Fujita, 1998. Genomic organization and regulation of expression of the lectin-like oxidized low-density lipoprotein receptor (LOX-1) gene. J. Biol. Chem., 273 (50): 33702-33707.
69. Li, W., T. Tada, T. Miwa, N. Okada, J. Ito, H. Okada, H. Tateyama and T. Eimoto, 1999. mRNA expression of complement components and regulators in rat arterial smooth muscle cells. Microbiol. Immunol., 43 (6): 585-593.
70. Matsumura, M., H. Kakishita, M. Suzuki, N. Banba and Y. Hattori, 2001. Dexamethasone suppresses iNOS gene expression by inhibiting NF-kappaB in vascular smooth muscle cells. Life Sci., 69 (9): 1067-1077.
71. Wei, C.Y., K.C. Huang, Y.H. Chou, P.F. Hsieh, K.H. Lin and W.W. Lin, 2006. The role of Rho-associated kinase in differential regulation by statins of interleukin-1beta- and lipopolysaccharide-mediated nuclear factor kappaB activation and inducible nitric-oxide synthase gene expression in vascular smooth muscle cells. Mol. Pharmacol., 69 (3): 960-967.
72. Bucher, M. and K. Taeger, 2002. Endothelin-receptor gene-expression in rat endotoxemia. Intensive Care Med., 28 (5): 642-647.
73. Olofsson, P.S., K. Jatta, D. Wagsater, S. Gredmark, U. Hedin, G. Paulsson-Berne, C. Soderberg-Naucler, G.K. Hansson and A. Sirsjo, 2005. The antiviral cytomegalovirus inducible gene 5/viperin is expressed in atherosclerosis and regulated by proinflammatory agents. Arterioscler. Thromb. Vasc. Biol., 25 (7): e113-116.
74. Wu, S.Q. and W.C. Aird, 2005. Thrombin, TNF-alpha and LPS exert overlapping but nonidentical effects on gene expression in endothelial cells and vascular smooth muscle cells. Am. J. Physiol. Heart Circ. Physiol., 289 (2): H873-885.

75. Ohashi, N., A. Matsumori, Y. Furukawa, K. Ono, M. Okada, A. Iwasaki, T. Miyamoto, A. Nakano and S. Sasayama, 2000. Role of p38 mitogen-activated protein kinase in neointimal hyperplasia after vascular injury. Arterioscler. Thromb. Vasc. Biol., 20 (12): 2521-2526.
76. Glover, J.N. and S.C. Harrison, 1995. Crystal structure of the heterodimeric bZIP transcription factor c-Fos-c-Jun bound to DNA. Nature, 373 (6511): 257-261.
77. Takaoka, A., S. Hayakawa, H. Yanai, D. Stoiber, H. Negishi, H. Kikuchi, S. Sasaki, K. Imai, T. Shibue, K. Honda and T. Taniguchi, 2003. Integration of interferon-alpha/beta signalling to p53 responses in tumour suppression and antiviral defence. Nature, 424 (6948): 516-523.
78. Rinaldo, C., A. Prodosmo, F. Mancini, S. Iacovelli, A. Sacchi, F. Moretti and S. Soddu, 2007. MDM2-regulated degradation of HIPK2 prevents p53Ser46 phosphorylation and DNA damage-induced apoptosis. Mol. Cell, 25 (5): 739-750.
79. Vogelstein, B. and K.W. Kinzler, 1992. p53 function and dysfunction. Cell, 70 (4): 523-526.