

# Heterogeneity in Endothelial Responsiveness to Cytokines, Molecular Causes, and Pharmacological Consequences

Grietje Molema, Ph.D.<sup>1</sup>

## ABSTRACT

Microvascular endothelial cells play an essential role in inflammatory diseases. Functional heterogeneity between microvascular segments in normal organ homeostasis has been appreciated for a long time, and more recent studies have revealed heterogeneity in endothelial reactivity to inflammatory stimuli as well. This review summarizes the state-of-the-art knowledge regarding endothelial cell responses to the proinflammatory cytokines tumor necrosis factor  $\alpha$ , interleukin- $1\beta$ , and the bacterial product lipopolysaccharide. It focuses on similarities and differences in reactivity between endothelial cell subsets in vitro and endothelial cells in their pathophysiological environment in vivo, and culminates into a mainly theoretical model of possible intracellular control mechanisms that can assist to ultimately explain the molecular causes of endothelial heterogeneity. The last part of this review contains some pharmacological considerations, and, with the aim to further unravel the molecular basis of in vivo endothelial heterogeneity, descriptions of new techniques that will be essential to achieve this.

**KEYWORDS:** Inflammation, microvascular endothelial cells, endothelial heterogeneity, gene expression control, pharmacology

The vasculature consists of large (conduit) arteries and veins, smaller arterioles and venules, and capillaries, each of them exerting specific functions in controlling whole-body homeostasis and hemostasis. Whereas the various vascular segments differ in architecture and functionality, they all share the fact that endothelial cells (EC) line the inner walls, directly interact with blood and its constituents, and actively engage in pathophysiological processes.

With advancements in cellular, biochemical, and molecular techniques, we have been able to decipher in

greater detail the molecular basis of endothelial behavior in health and disease. The general concept describes endothelial cells to actively engage in inflammatory diseases by recruitment of leukocytes from the blood via the production of thrombotic factors, cytokines, chemokines, adhesion molecules, and other proteins. The aim of the current review is to provide an overview of what we have learned in the last decades from in vitro and in vivo studies regarding endothelial cell responsiveness to proinflammatory cytokines, with emphasis on tumor necrosis factor (TNF) $\alpha$ , interleukin (IL)- $1\beta$ , and

<sup>1</sup>Department Pathology & Medical Biology Section, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands.

Address for correspondence and reprint requests: Grietje Molema, Ph.D., University Medical Center Groningen, Department Pathology & Medical Biology, Medical Biology Section, IPC EA11, Hanzeplein 1, 9713 GZ Groningen, The Netherlands (e-mail: g.molema@med.umcg.nl).

Under-Recognized Significance of Endothelial Heterogeneity: Hemostasis, Thrombosis, and Beyond; Guest Editors, William C. Aird, M.D., and Hau C. Kwaan, M.D., Ph.D.

Semin Thromb Hemost 2010;36:246–264. Copyright © 2010 by Thieme Medical Publishers, Inc., 333 Seventh Avenue, New York, NY 10001, USA. Tel: +1(212) 584-4662.  
DOI: <http://dx.doi.org/10.1055/s-0030-1253448>.  
ISSN 0094-6176.

the bacterial product lipopolysaccharide (LPS). Both intracellular signaling pathways and effects on gene expression and cell behavior are discussed. Next, focus is on the origin of the endothelial cell in relation to its responsiveness and putative molecular mechanisms underlying the heterogenic behavior of different endothelial subsets. The pharmacological consequences of endothelial heterogeneity is briefly addressed because it not only may pose a threat to the one-drug-fits-all concept but also provides a unique opportunity for selective delivery of drugs into diseased endothelial cells using targeted drug delivery strategies. New technological advances and research tools, partly available and partly under development, conclude this review. They are expected to assist in revealing the bigger molecular map that underlies endothelial heterogeneity.

### ENDOTHELIAL CELL FUNCTION IN INFLAMMATORY REACTIONS

Leukocyte recruitment is a process that should be executed in any organ upon demand in response to invading organisms or damage. In almost all organs, the preferred site for leukocyte transmigration into the underlying tissue upon an inflammatory challenge is the postcapillary venules, in which the endothelial cells form tight junctions between each other.<sup>1</sup> By this means, a local immune response can be mounted without directly compromising capillary function as part of organ homeostasis. The dimensions of both leukocytes and these first segments of the postcapillary venules furthermore force both cell types to physically interact, allowing efficient intercellular communication. Moreover, vessel wall architecture driven preferential leukocyte transmigration may also play a role, as has been described for neutrophils.<sup>2</sup>

Upon an inflammatory insult, one of the first reactions of the endothelial cells is to exocytose the stored, ready-to-release contents of Weibel-Palade bodies, including the blood coagulation factor von Willebrand factor (VWF) and the adhesion molecule P-selectin.<sup>3</sup> By this means, a rapid interaction among the activated endothelium, platelets, and neutrophils is created that facilitates leukocyte rolling.<sup>4</sup> Directly afterward, the endothelium produces E-selectin, which interacts with the tetrasaccharide sialyl-Lewis X expressed on immune cells, leading to rolling adherence of leukocytes to the endothelium. E-selectin is also implicated in the generation of activated integrin microdomains at the leading edge of neutrophils via E-selectin ligand-1, as a consequence of which erythrocytes and platelets can be captured to create additional inflammatory damage.<sup>5</sup> Firm arrest of leukocytes to the endothelium is next facilitated by adhesion molecules of the immunoglobulin superfamily such as vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1, and local production of chemo-

kines and cytokines. The latter molecules rapidly induce integrin activation on the leukocytes via binding to G-protein coupled receptors, prostaglandin, and nitric oxide (NO) production, and actin filament rearrangement leading to permeability changes in the endothelial compartment.<sup>6</sup> The last step is leukocyte transendothelial migration, which is triggered by endothelial integrin adhesion molecule engagement and clustering that affects an array of downstream effector molecules, as recently reviewed by Wittchen.<sup>7</sup>

Because proinflammatory cytokines TNF $\alpha$ , IL-1 $\beta$ , and LPS are best studied for their effects on endothelial cells, these are the main activators discussed in more detail here. Emphasis is on their molecular effects on the endothelium and on our current knowledge regarding endothelial responsiveness in relation to the vascular origin of the endothelial cells.

### EFFECTS OF TUMOR NECROSIS FACTOR $\alpha$ ON ENDOTHELIAL CELLS

Human umbilical vein endothelial cells (HUVEC) express both TNF $\alpha$ -receptors TNFR1 (CD120a, p55) and TNFR2 (CD120b, p75), with TNFR1 more abundant than TNFR2. Because TNFR2 protein is located primarily on the cell membrane whereas TNFR1 mainly localizes to the Golgi apparatus,<sup>8</sup> TNFR2-mediated signal transduction would be the preferred route to direct the signal into the endothelial cells. In the intact organ, however, the localization and expression patterns of the receptors were shown to deviate significantly from those observed *in vitro*. For example, in normal human kidney biopsies, TNFR1 is strongly expressed by the glomerular endothelium, and TNFR2 is profoundly absent from the vascular compartment.<sup>9</sup> In contrast, in normal human myocardium, both TNFR1 and TNFR2 mRNA and protein localize to the vascular endothelial cells.<sup>10</sup> Rat and human lung follow the kidney profile in that TNFR1 is prominently present in the (larger) vessel endothelial cells, and TNFR2 is much less expressed.<sup>11</sup> TNFR1 therefore seems to be the most likely receptor for TNF $\alpha$  to interact with, although a role for TNFR2 in TNF $\alpha$ -induced endothelial cell activation does exist. This was demonstrated in a series of experiments in which aortic endothelial cells from different TNFR knockout mice and blood vessels in the cremaster muscle *in vivo* were challenged by TNF $\alpha$ .<sup>12</sup>

Endothelial cell exposure to TNF $\alpha$  activates intracellular signaling cascades involving NF $\kappa$ B, the mitogen-activated protein kinases (MAPK) p38, JNK or c-Jun N-terminal kinase, extracellular signal-regulated kinases ERK1/2, and PI3 kinase,<sup>13-17</sup> with reactive oxygen species and ceramide-based lipid second messengers also contributing to the signaling.<sup>18,19</sup> Because both TNF $\alpha$  receptors do not have intrinsic kinase activity, adapter proteins TRAF2 (TNF receptor-associated

factor 2), RIP1 (receptor interacting protein 1) and TRADD (TNF receptor-associated death domain) are recruited upon TNF $\alpha$  binding to relay the signal into the cell's interior.<sup>6</sup> Recently, Pincheira and colleagues reported on the association of TNFR1 with nonreceptor tyrosine kinase proteins Jak and c-Src that function as adapters to direct the message into specific kinase routes. TNFR1/c-Src played a role in activating Akt and NF $\kappa$ B, but not JNK or p38 MAPK; TNFR1/Jak2 relayed the signal to activate p38 MAPK, JNK, Akt, as well as signal transducer and activator of transcription (STAT)-3.<sup>20</sup> Whether such adapter protein-based guidance of signals into specific kinase pathways also takes place in endothelial cells, or in subsets of endothelial cells in different vascular segments, is not known.

Table 1 provides an overview of *in vitro* studies reporting on TNF $\alpha$  effects on endothelial cells from different vascular sources and the molecular mechanisms involved. Besides affecting expression of adhesion molecules, chemokines and cytokines, and various other proteins involved in cell survival and feedback mechanisms that inhibit intracellular signaling,<sup>21</sup> TNF $\alpha$  activation also affects endothelial permeability. This is partly attributed to the loss of VE-cadherin from the cell membrane in response to TNF $\alpha$  exposure. VE-cadherin is an endothelial-restricted adhesion molecule present in adherent junctions that through its cytoplasmic tail anchors to actin microfilaments via cytoskeletal proteins.<sup>22</sup> Enhanced phosphorylation of VE-cadherin, mediated for example by intracellular oxidant production by NADPH oxidase or by inhibition of phosphatases, and enhanced VE-cadherin internalization and cleavage from the cell membrane can all contribute to this process.<sup>23,24</sup> p38 MAPK, not ERK activation, is in part responsible for the TNF $\alpha$ -induced VE-cadherin loss.<sup>25,26</sup> It is interesting to note that in adult mice, only lung and uterus, and to a lesser extent, ovary vasculature constitutively express low levels of phosphorylated VE-cadherin.<sup>27</sup> These endothelial subsets may experience a microenvironment that contains specific factors that facilitate continuous activation, the functional consequences of which are not known. Another mechanism of enhanced permeability was described in calf pulmonary artery endothelial cells, in which an increase in the rate of  $\alpha 5\beta 1$  integrin internalization and recycling to the membrane was observed after TNF $\alpha$  exposure. This modifies cell-matrix interactions and as such can affect monolayer integrity.<sup>28</sup>

### INTERLEUKIN-1 EFFECTS ON ENDOTHELIAL CELLS

IL-1 has a lot in common with TNF $\alpha$  with regard to the transcriptional profile launched in endothelial cells, although the upstream signaling pathways are partly dissimilar. IL-1 consists of two major forms, IL-1 $\alpha$  and IL-1 $\beta$ , both able to bind IL-1 receptor type I and

II. IL-1RI activation leads to IL-1 receptor accessory protein, MyD88, IRAK, and TRAF6 recruitment to form a receptor complex. As a consequence, NF $\kappa$ B becomes activated, as shown in a variety of endothelial cell studies *in vitro* as well as *in vivo* in blood vessels of the rat brain exposed to rat IL-1 $\beta$ .<sup>51</sup> Furthermore, p38 MAPK, ERK1/ERK2, AP-1, ATF-2, PKC, and phospholipase A2 become activated by IL-1RI signal transduction.<sup>14,41,52-54</sup> Activation of the PI3K/Akt pathway furthermore inhibits apoptosis, similar to the effect induced by TNF $\alpha$  stimulation.<sup>16</sup>

Molecules reported to be induced by endothelial cells upon IL-1 exposure include E-selectin, VCAM-1 and ICAM-1, MCP-1 (monocyte chemotactic protein 1), IL-8, COX-2 (cyclooxygenase-2), iNOS (inducible NO synthase), and HO-1 (hemoxygenase 1). In a recent study, Williams and colleagues employed microarray analysis to determine the effects brought about in HUVEC by incubation with 0.1 ng/mL IL-1 $\beta$ . Some 2500 genes were differentially expressed. Most were functionally grouped according to gene ontology to apoptosis, cell cycle, NF $\kappa$ B signaling, chemotaxis, and immune response-related events.<sup>55</sup> Of note is the observation that transmigration of PMN only led to a relatively small perturbation of endothelial cells when compared with the effects of proinflammatory cytokine activation. A direct comparison between TNF $\alpha$  and IL-1 activation of HUVEC using a 4000 gene array revealed 58 and 33 genes, respectively, to be significantly regulated, 25 of which were shared between both activators.<sup>56</sup>

These and other studies confirmed that both cytokines, produced early in an inflammatory reaction, show considerable overlap in the nature of endothelial cell activation, although also some differences have been reported. For example, human dermal microvascular endothelial cells only expressed VCAM-1 in response to TNF $\alpha$ , not IL-1. This was related to a dermal microvascular endothelial cell-type specific upstream VCAM-1 gene regulatory region that functions as a transcriptional repressor.<sup>57</sup> In a rat pulmonary artery endothelial cell line, the production of the antioxidant manganese superoxide dismutase (MnSOD) was shown to be regulated differently in response to the two cytokines. Whereas TNF $\alpha$  induced this enzyme via a phospholipase A2 and reactive oxygen species (ROS)-dependent mitochondrial pathway, IL-1 $\beta$  activation led to NF $\kappa$ B-dependent expression of MnSOD.<sup>58</sup> The extent of gene expression induction by TNF $\alpha$  and IL-1 $\beta$  can also be different, as demonstrated in our laboratory. Exposure of HUVEC to TNF $\alpha$  induced adhesion molecule expression to a higher level than exposure to IL-1 $\beta$ , whereas the latter cytokine affected COX-2, IL-6, and IL-8 mRNA levels more extensively.<sup>59</sup> In human pulmonary artery endothelial cells, a similar preference of COX-2 expression upon IL-1 $\beta$  activation was reported. Whereas IL-1 $\beta$  exposure directly activated p38 MAPK, which was

**Table 1 Overview of in Vitro Studies addressing the Cellular Consequences of Exposure of Endothelial Cells from Different Sources to Tumor Necrosis Factor  $\alpha$** 

Cell Type	TNF $\alpha$		Major Outcome	Drugs/Inhibitors of Pathways Used	Reference
	Concentration (ng/mL)	Exposure Time			
HUVEC	1–30	1 h	Nur77 expression is induced by TNF $\alpha$ Nur77 directly activates I $\kappa$ B $\alpha$ promoter and gene expression, which attenuates NF $\kappa$ B activation	Ad-dh Nur77	You et al <sup>29</sup>
HUVEC	10	4 h	E-selectin, VCAM-1, and ICAM-1 in umbilical cord are more extensively expressed by venous than artery endothelium upon TNF $\alpha$ activation in situ	—	Liu et al <sup>30</sup>
HUAEC			No major differences in NF $\kappa$ B, c-jun, and ATF2 activity was observed, but binding of transcription factors and cp300 coactivator to E-selectin enhancer was lower in HUAEC		
BAEC HUVEC	2	90 min–3 h	TNF $\alpha$ induces homeobox transcription factor HOXA9, which controls E-selectin expression	—	Bandyopadhyay et al <sup>31</sup>
HUVEC	5	16 h	HOXA9 inhibits TNF $\alpha$ -induced E-selectin, VCAM-1, and ICAM-1 expression	HDAC inhibitor Trichostatin	Trivedi et al <sup>32</sup>
BAEC Mouse EC			HOXA9 interferes with NF $\kappa$ B-DNA binding		
			HOXA9 expression induces HDAC activity, and Trichostatin rescues HOXA9-mediated NF $\kappa$ B inhibition		
HUVEC	0.05–0.02	4 h	2000 ng/mL Ang2 sensitizes EC to low concentration TNF $\alpha$ Both VCAM-1 and ICAM-1 expression are increased by cocubation of Ang2 and TNF $\alpha$	—	Fiedler et al <sup>33</sup>
HUVEC	50	1–60 min	TNF $\alpha$ -induced expression of eNOS requires Akt This is mediated by neutral sphingomyelinase-2 and sphingosine kinase-1 that generates sphingosine-1-phosphate NO generated via this mechanism inhibits expression of E-selectin	Pertussis toxin (PTx–Gi-coupled receptor inhibitor), LY294002 and wortmannin (PI3K inhibitors)	De Palma et al <sup>19</sup>
HUVEC	10	90 min–6 h	COX-2 expression is induced by TNF $\alpha$ via ROS and p38 MAPK PI3K activation plays basal regulatory role in COX-2 synthesis in HUVEC	NAC, RO31–8220 (PKC inhibitor), fluvastatin (HMGCo reductase inhibitor), p38 MAPK resp. MEK1/ERK inhibitors SB203580 and PD98059, wortmannin	Eigini et al <sup>34</sup>
HUVEC BAEC	10	0.5–4 min–15 h	TNF $\alpha$ inhibits KLF2 expression This is controlled via activation of NF $\kappa$ B and HDAC4 that inhibit MEF2 factors to induce KLF2 expression	Trichostatin (HDAC inhibitor)	Kumar et al <sup>35</sup>
BBMEC	0.1–100	30 min–12 h	TNF $\alpha$ -induced RelA NF $\kappa$ B activation is associated with permeability increase and PGE <sub>2</sub> production	NF $\kappa$ B inhibitors BAY11–7085, CAPE, and PDTC; lactacystin (proteasome inhibitor)	Trickler et al <sup>36</sup>

**Table 1** (Continued)

Cell Type	TNF $\alpha$		Major Outcome	Drugs/Inhibitors of Pathways Used	Reference
	Concentration (ng/mL)	Exposure Time			
HUVEC	2	30 min–5 h–16 h	58 of 13,000 genes are upregulated by NF $\kappa$ B and encode proinflammatory, antiapoptotic, and NF $\kappa$ B inhibitory genes Only 13 genes are under the control of p38 MAPK	P38 MAPK inhibitor, SB202190, retrovirus encoding dn IKK2	Viemann et al <sup>21</sup>
HUVEC b.End	5	10 min–6 h	TNF $\alpha$ induces PI3K $\delta$ with downstream kinases Akt and PDK1	PI3K $\delta$ inhibitor IC87114	Puri et al <sup>37</sup>
HAEC	100 U/mL	4–16 h	TNF $\alpha$ induces cell adhesion molecule expression via Rac1-facilitated, NF $\kappa$ B-mediated transactivation Superoxide production partially affects CAM expression	Ad.N17Rac1 encoding dn Rac1	Chen et al <sup>18</sup>
HMEC					
Human iliac and renal artery EC, BAEC	40	20 min–20 h	Proteasome inhibition blocked TNF $\alpha$ -induced NF $\kappa$ B activation, with consequent inhibition of MCP-1 but enhanced IL-8 expression Proteasome inhibition led to functional AP-1 induction via enhanced c-jun phosphorylation that can transactivate IL-8 gene expression	MG-132 and lactacystin	Hipp et al <sup>38</sup>
BAEC	10	5–60 min/6–20 h	TNF $\alpha$ induced activation of MEK1 and Akt protects cells from apoptosis	MEK1 inhibitor U0126, PI3K inhibitor LY294002	Zhang et al <sup>17</sup>
HUVEC	10	5–60 min	Pre-exposing cells to flow for 10 min inhibited TNF $\alpha$ -induced JNK activation, not ERK1/2 and p38 MAPK Inhibition of JNK is mediated via ERK1/2	PD98059	Surapitschat et al <sup>15</sup>
HUVEC ECV304	20	15 min–16 h	Ephrin A1 induction by TNF $\alpha$ is controlled via p38 MAPK and JNK, not via ERK1/2 and NF $\kappa$ B	Adm1kB $\alpha$ , Ad.TRAF2.DN, JNK inhibitor DMAP, SB20358, PD98059	Cheng and Chen <sup>39</sup>
HPAEC	100 U/mL	60 min	Critical role for PKC- $\zeta$ in TNF $\alpha$ oxidant generation and NF $\kappa$ B activation leading to ICAM-1 expression	Antisense PKC- $\zeta$ , PKC- $\zeta$ mutants, calphostin, staurosporin	Rahman et al <sup>40</sup>
HUVEC	10–50	5–60 min/18 h	TNF $\alpha$ activates antiapoptotic kinase Akt via PI3K pathway Akt does not affect NF $\kappa$ B pathway but inhibits MAPK pathway	LY294002 and wortmannin	Madge and Pober <sup>16</sup>
HUVEC	500 U/mL	4 h	TNF $\alpha$ induces HO-1 gene expression via PKC phosphorylation, phospholipase A2 (PLA2) activation, and oxidant generation	PLA2 inhibitor Mepacrine, Ca-chelator BAPTA-AM, Ca-ionophore A23–187	Terry et al <sup>41</sup>
HPAEC	100	5–60 min/24 h	TNF $\alpha$ induces IL-8 gene expression via p38 MAPK	SB203580	Hashimoto et al <sup>42</sup>
HUVEC	1–5	10–240 min/12 h	TNF $\alpha$ activates ERK, p38, and JNK MAPKs Raf/MEK/ERK and JNK have no effect on MCP-1 expression; MKK6/p38 MAPK did have an effect	SB203580 and SB202190, PD98059, dominant negative kinase mutants	Goebeler et al <sup>43</sup>



**Table 1** (Continued)

Cell Type	TNF $\alpha$ Concentration (ng/mL)	Exposure Time	Major Outcome	Drugs/Inhibitors of Pathways Used	Reference
HPAEC	100 U/mL	1–8 h	TNF $\alpha$ induces NF $\kappa$ B activation and resulting E-selectin expression by a pathway that involves ROS production	NAC and PDTTC	Rahman et al <sup>44</sup>
HUVEC	100 U/mL	5–120 min	p42 MAPK is activated by TNF $\alpha$ PKC is not involved in this activation Unknown upstream protein tyrosine kinase is involved	PKC inhibitor Ro-31–8220, broad protein tyrosine kinase inhibitors genistein and dadzein	May et al <sup>14</sup>
HMEC-1	100 U/mL	15–120 min	c-fos and c-jun are rapidly and transiently induced by TNF $\alpha$ AP-1 proteins mediate effects of TNF $\alpha$ on VCAM-1 expression via interaction with NF $\kappa$ B factors	AP-1 inhibitors curcumin and NDGA	Ahmad et al <sup>45</sup>
PAEC	5	10–120 min	Arachidonic acid (AA) blocks degradation of I $\kappa$ B by inhibition of I $\kappa$ B phosphorylation and degradation TNF $\alpha$ -induced E-selectin, ICAM-1, and IL-8 gene expression are concurrently inhibited	AA and stable AA analogue ETYA	Stuhlmeier et al <sup>46</sup>
HSVEC	1000 U/mL	15 min/2–24 h	NO inhibits TNF $\alpha$ -induced VCAM-1 expression NO inhibits NF $\kappa$ B activation; augments I $\kappa$ B $\alpha$ resynthesis and nuclear translocation	NO donors	Spiecker et al <sup>47</sup>
HUVEC	100 U/mL	10 min–6 h	TNF $\alpha$ activates MAPKAPK2 and Hsp27 via p38 MAPK VCAM-1 expression is posttranscriptionally controlled	SB203580	Pietersma et al <sup>48</sup>
HUVEC	0.3–100	4–24 h	Tissue Factor induces tissue factor, E-selectin, ICAM-1, IL-8 expression Effect is not exclusively attributable to TNFR1	TNFR1- and TNFR2-specific antibodies	Paleolog et al <sup>49</sup> Leeuwenberg et al <sup>50</sup>

\*The table covers a limited selection of studies with the aim to provide an overview of the diversity of molecular processes being activated upon TNF $\alpha$ -induced endothelial activation, and of the (chemical and biological) compounds that have been used to demonstrate specificity of the processes involved.  
Ad, adenovirus; BAEC, bovine aortic endothelial cells; BBMEC, bovine brain microvessel endothelial cells; CAPE, caffeic acid phenethyl ester; Dn, dominant negative; HAEC, human aortic endothelial cells; HDAC, histone deacetylase; HMEC-1, immortalized human dermal microvascular endothelial cells; HPAEC, human pulmonary artery endothelial cells; HSVEC, human saphenous vein endothelial cells; HUVEC, human umbilical artery endothelial cells; HUVEC, human umbilical vein endothelial cells; NAC, N-acetyl-L-cysteine; NDGA, nordihydroguaiaretic acid; PAEC, porcine aortic endothelial cells; PDTTC, pyrrolidine dithiocarbamate; PKC, protein kinase C; ROS, reactive oxygen species.

sufficient to induce the expression of this cyclooxygenase, TNF $\alpha$  was unable to do so on its own.<sup>60</sup>

### **LIPOPOLYSACCHARIDE EFFECTS ON ENDOTHELIAL SIGNAL TRANSDUCTION**

LPS is one of the main components of the cell wall of gram-negative bacteria. In endothelial cells, LPS signals mainly via one of the family members of the toll-like pattern recognition receptors, toll-like receptor (TLR)-4. For this interaction, LPS binding to LPS binding protein (LBP) and subsequent formation of a complex with LPS receptor CD14 is a prerequisite. This facilitates transfer of LPS to the LPS receptor complex that consists of TLR4 and MD2. Because endothelial cells lack the GPI-anchored membrane version of the CD14 molecule, they rely on soluble CD14 for complex formation and subsequent intracellular signaling.<sup>61</sup> The observation that numerous endothelial cells *in vitro* are responsive to LPS indicates that these cells are likely equipped with the required receptors. As for TNF $\alpha$  and IL-1 receptors, neither of them are restricted to the endothelial compartment.

Endothelial cell exposure to LPS leads to activation of NF $\kappa$ B via PI3K/Akt, and of the MAPKs p38, JNK, and ERK1/2, and they are mediated via adapter proteins that include MyD88, IRAK-1 and -2, and TRAF6 that interact with the intracellular domain of TLR4.<sup>61-65</sup> Induced expression of FLICE-like inhibitory protein (FLIP) and the antiapoptotic proteins A1 and A20 have a role in protecting endothelial cells from LPS-induced apoptosis as well as in creating a suppressive effect on LPS-induced NF $\kappa$ B activation.<sup>66,67</sup> For an overview on the more detailed molecular interactions of these signaling pathways, the reader is referred to two excellent reviews.<sup>61,65</sup>

The functional consequences of LPS-mediated endothelial cell activation are widespread and include engagement in leukocyte recruitment, expression of procoagulant activity, and endothelial sprouting.<sup>68</sup> Exposure of rat thoracic aorta endothelium to LPS for short periods (6 hours) or longer periods (72 hours) regulated metabolic pathways, genes related to proliferation, atherogenesis, inflammation, and NF $\kappa$ B-mediated apoptosis, as well as antiapoptosis and anti-inflammatory and antioxidation genes.<sup>69</sup> Furthermore, LPS exposure induced loss of endothelial barrier function in human dermal microvascular endothelial cells. This was associated with fragmentation of VE-cadherin and loss of claudin 5, and related to a decrease in cAMP levels.<sup>70</sup>

### **ENDOTHELIAL HETEROGENEITY IN REACTION TO INFLAMMATORY ACTIVATION IN VITRO**

To better understand how vascular origin relates to endothelial responsiveness to cytokines and LPS, one

could subject the vast number of *in vitro* studies reported in the literature to a detailed comparison. Several obstacles complicate such a comparison, and include the often limited information provided on cell confluency status and passage number used in the experiments. Moreover, when using endothelial cell lines such as mouse brain endothelial cells b.End3,<sup>71</sup> mouse heart H5V,<sup>72</sup> human umbilical cord vein derived ECV304, EVLC2,<sup>73</sup> and Ea.hy926,<sup>74</sup> human dermal microvascular derived HMEC-1,<sup>75</sup> and hTERT based cell lines,<sup>76</sup> the extensive passage of the cells may have significantly altered the molecular basis of their behavior. An example of this was provided by Viemann and colleagues, who compared TNF $\alpha$  responsiveness of HUVEC and HMEC-1.<sup>77</sup> The upregulated genes in HUVEC that did not respond in HMEC-1 mainly included those encoding for adhesion molecules, cytokines, and chemokines, molecules expected to be regulated in endothelial cells in proinflammatory conditions. In contrast, in the HMEC-1 cell line the differentially upregulated genes represented signaling, transcription factor, apoptosis, and proliferation-associated genes. Another factor that complicates direct comparison of published studies is that readout technology such as real-time transcriptase polymerase chain reaction (RT-PCR), fluorescent-activated cell sorting (FACS) analyses, Western blotting, and array-based assays are often executed using at the beginning and end of standard operating procedures yet employ different antibodies, amplification conditions, housekeeping genes, and normalization procedures. As a consequence, only those studies reporting on a direct comparison between endothelial cells from different vascular origins can readily assist in creating a better understanding of endothelial heterogeneity in response to an inflammatory stimulus. The following sections therefore mainly focus on these direct-comparison-investigations.

The extent of quiescent endothelial diversity was for the first time described by Chi and colleagues, who used DNA microarrays to analyze 53 cultured endothelial cell types isolated from different origins in the body. They uncovered persistent differences in gene expression profiles that distinguish large vessel endothelium from endothelium located in the microvasculature, groups of genes specific for arterial and venous endothelium, and tissue-specific expression patterns in different endothelial subsets.<sup>78</sup> One of the earlier studies that directly compared responsiveness of endothelial cells from different vascular origins to TNF $\alpha$  and IL-1 investigated VCAM-1 expression. Although both cytokines induced VCAM-1 in HUVEC, human arterial endothelial cells (HAEC) only expressed this adhesion molecule upon exposure to IL-1.<sup>79</sup> Another study compared TNF $\alpha$  responsiveness of human endothelial cells derived from glomeruli (GEC), dermal microvasculature (MVEC), and umbilical vein (HUVEC). All three endothelial subsets exhibited a reduction in the expression

of CD31 and strong induction of E-selectin, yet VCAM-1 was only (moderately) upregulated in GEC and HUVEC.<sup>80</sup> Using rat pulmonary microvascular and rat pulmonary arterial endothelial cells, a major difference was observed between the two cell types in reaction to TNF $\alpha$  and neutrophil adherence. Both exerted a similar increase in ICAM-1 expression and redistribution upon ICAM-1 cross-linking by exposure to neutrophils or antibodies. However, only the microvascular endothelial subset responded with induced p38 MAPK phosphorylation, ROS production, and F-actin formation.<sup>81</sup>

In an attempt to better understand whether molecular differences in responsiveness to atherogenic stimuli underlie arterial susceptibility to atherosclerosis, Deng and colleagues compared human saphenous vein endothelial cells (HSVEC) and human coronary artery endothelium (HCAEC) gene expression profiles in quiescent conditions and in reaction to oxidized LDL, TNF $\alpha$ , and IL-1 $\beta$ . In quiescent conditions, HSVEC expressed 285 genes to a higher extent than the artery endothelium. These genes were mainly related to anti-inflammatory responses, cell growth, and homeostasis functions. Atherogenic oxidized LDL preferentially induced cellular proliferation and adhesion pathways in CAEC, whereas in HSVEC, focal adhesion, inflammatory response, and apoptosis and NF $\kappa$ B pathway genes were downregulated. TNF $\alpha$  and IL-1 $\beta$  activation, in contrast, induced apoptosis and downregulated anti-inflammatory genes in CAEC, whereas in HSVEC both antiapoptotic and antiatherogenic genes were induced.<sup>82</sup>

Organ-encoded differences in response to inflammatory stimuli are already visible in endothelial cells derived from fetal tissues.<sup>83</sup> TNF $\alpha$  exposure activated the heart, kidney, and aorta endothelium derived from 12-week-old human embryos to express E-selectin, whereas IL-1 $\beta$  treatment only induced this adhesion molecule in fetal kidney and aorta endothelium. ICAM-1 expression was most pronounced in heart, liver, and lung endothelium when exposed to IL-1 $\beta$  and induced to a limited extent in lung endothelium when exposed to TNF $\alpha$ . VCAM-1 induction was lacking in fetal brain-derived EC, irrespective of the stimulus, although it was a late event in all but the heart-derived endothelium upon TNF $\alpha$  exposure. Its expression was only increased by IL-1 $\beta$  in fetal heart EC at a later time point.<sup>83</sup>

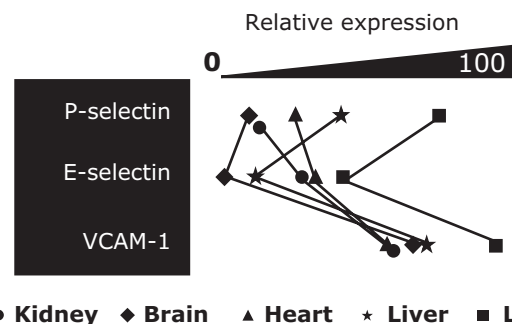
In a study by Methe et al, HSVEC and HCAEC were exposed to venous and coronary artery flow patterns, respectively 2.2 dyne/cm<sup>2</sup> (static) and 17 dyne/cm<sup>2</sup> (at 1 Hz) in combination with TNF $\alpha$  challenge.<sup>84</sup> Under static conditions, an induced expression of VCAM-1 could only be observed in the venous endothelial cells in response to TNF $\alpha$  treatment. This difference in expression induction between the cell

types was not observed for E-selectin and ICAM-1. In the saphenous vein endothelium, exposure to both venous and artery flow conditions increased TNF $\alpha$ -induced E-selectin and ICAM-1 expression, whereas only coronary artery flow affected TNF $\alpha$ -induced VCAM-1 upregulation. In contrast, venous and coronary artery flow created a completely different effect on the coronary artery endothelium. Although either flow type attenuated TNF $\alpha$ -induced E-selectin and VCAM-1 expression, TNF $\alpha$ -induced ICAM-1 expression was only increased further by coronary artery flow, not by venous flow.<sup>84</sup>

Other inflammation-related endothelial activators have been studied with regard to endothelial subset specific responsiveness. These studies include protein kinase C activation,<sup>85</sup> nucleotide-induced P2Y(2) receptor desensitization,<sup>86</sup> and angiotensin-1 and -2 activation of Tie-2,<sup>87</sup> which are not discussed further because they are beyond the scope of this review.

## ENDOTHELIAL HETEROGENEITY IN ADHESION MOLECULE EXPRESSION IN VIVO

In vivo, endothelial heterogeneity in adhesion molecule expression is already visible in quiescent microvascular segments. For example, Eppihimer and colleagues showed that E-selectin protein expression is completely absent throughout the vasculature in healthy cytokine-naive mice,<sup>88</sup> an observation that was corroborated at the mRNA level.<sup>89</sup> At the same time, P-selectin is expressed to a significant extent in the lungs, mesentery,



**Figure 1** Interorgan differences in endothelial restricted adhesion molecule expression in quiescent conditions. Semi-quantitative representation of mRNA levels of P- and E-selectin, and VCAM-1 in C57bl/6 mouse organs as assessed by quantitative RT-PCR and related to housekeeping gene GAPDH. Lowest and highest expression levels were set at 0 and 100, respectively; others were calculated accordingly. The graph shows that brain, liver, and lung exert a comparable profile, with E-selectin expression the lowest of the three. In kidney and heart, in contrast, E-selectin mRNA levels are higher than those of P-selectin, the functional meaning of which is not known (Kuldo and Molema, unpublished data).

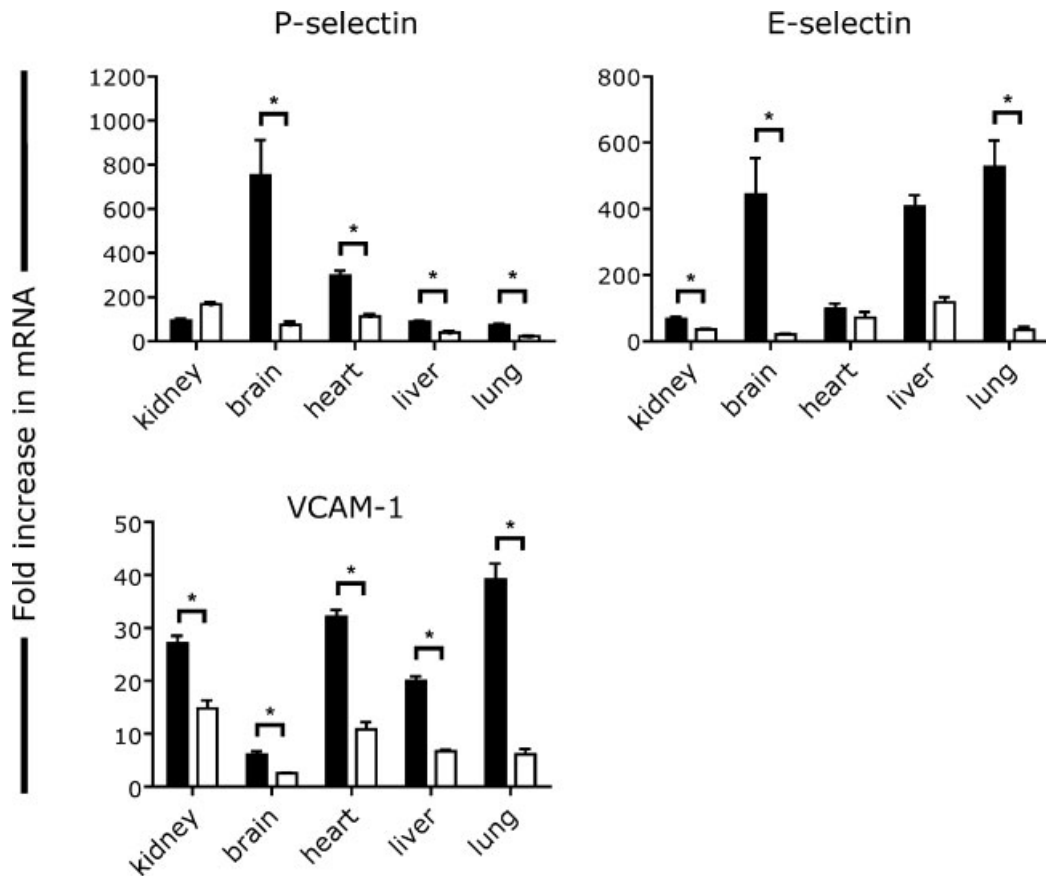


small intestine, and pancreas.<sup>88</sup> Northern Blot analysis furthermore demonstrated that, especially in the lungs, both VCAM-1 and ICAM-1 are constitutively expressed.<sup>89</sup> With the advent of RT-PCR technology, one can now obtain a more quantitative view on basal expression profiles of adhesion molecules in the different organs and the heterogenic character thereof (Fig. 1), the functional meaning of which remains to be established. Even more striking from a heterogeneity point of view is the observation that within one organ, different expression levels of adhesion molecules prevail in different vascular segments in healthy conditions. For example, quiescent mouse kidney, arteriolar and peritubular endothelium (EC) express significant levels of VCAM-1 protein, while at the same time glomerular endothelium is completely devoid of this adhesion molecule (Kuldo and Molema, manuscript in preparation).

Considering the heterogeneity in TNFR expression within the quiescent vasculature, it is not surprising that activation of these receptors results in variable responses in the different vascular segments. Using

competitive RT-PCR, it was shown that intravenous injection of 3000 U TNF $\alpha$  in mice mainly induced E- and P-selectin expression in heart, lung, kidney, liver, and brain but not or to a lesser extent in spleen.<sup>90</sup> A similar difference in microvascular reactivity was reported by Tamaru and colleagues in response to IL-1 $\beta$ . Using Northern blot it was shown that adhesion molecule induction after IL-1 $\beta$  administration was mainly present in heart and lung, whereas it was less prominent in liver and kidney and even lower or absent, respectively, in brain and skin.<sup>89</sup> Using RT-PCR, a quantitative representation of the complexity of organ-specific microvascular reactions to inflammatory cytokines such as TNF $\alpha$  and IL-1 $\beta$  can be created (Fig. 2).

In addition to organ-specific control of gene expression, Tamaru and colleagues also revealed the existence of intraorgan variation in endothelial responsiveness by showing that systemic IL-1 $\beta$  administration mainly induced VCAM-1 mRNA in the larger vessels of the lungs and the heart, whereas small



**Figure 2** Interorgan differences in endothelial responsiveness to systemic tumor necrosis factor (TNF $\alpha$ ) and interleukin (IL-1 $\beta$ ) administration. C57b1/6 mice were injected intravenously with 200 ng TNF $\alpha$  (black bars) or IL-1 $\beta$  (white bars) per mouse and sacrificed 2 hours later. mRNA isolated from snap-frozen organs was analyzed by quantitative reverse transcriptase polymerase chain reaction with GAPDH as the housekeeping gene. Mean values  $\pm$  standard error of mean. \* Statistically significant,  $<0.05$ . The endothelial cells in all organs responded to the inflammatory cytokines, although in general TNF $\alpha$  administration induced higher adhesion molecule expression than IL-1 $\beta$ . Each organ displayed its unique pattern of extent of adhesion molecule induction, with liver and lung displaying the most comparable response. VCAM, vascular cell adhesion molecule. Data from Kuldo and Molema (unpublished).

capillaries were devoid of such an upregulation.<sup>89</sup> Other systemic inflammation models have also revealed intra-organ microvascular segment specific reactivity to inflammatory stress. For example, we showed that induction of hemorrhagic shock by withdrawal of blood to 30 mm Hg and maintenance thereof for 90 minutes led to expression of E-selectin in glomerular endothelial cells in the kidney, whereas it was much less pronounced or even absent in the endothelium of afferent and efferent arterioles, and in the peritubular and postcapillary venule segments. In contrast, VCAM-1 expression was induced in all vascular segments except in glomerular endothelium.<sup>91</sup>

Intravenous or intraperitoneal administration of LPS leads to a rapid induction of systemic TNF $\alpha$  levels besides having direct effects on cells via TLR family members. In the microvasculature of main organs such as the heart, lung, brain, and liver, the expression of E- and P-selectin and ICAM-1 are all significantly induced in response to systemic LPS treatment. Microvascular heterogeneity in response to LPS was, however, observed with regard to the regulation of VCAM-1 because both brain and liver microvasculature did not upregulate this adhesion molecule.<sup>92,93</sup> Interestingly, in this model, the skin microvasculature showed a rather aberrant gene induction profile compared with the other organs,<sup>93</sup> thus questioning the validity of using skin biopsies as surrogate readout of endothelial activation in other tissues. In mouse cremaster venules, LPS induced enhanced thrombosis that was mediated by von Willebrand factor (VWF).<sup>94</sup> Because VWF is highly heterogeneously expressed throughout the vascular bed,<sup>95</sup> it is conceivable that LPS-induced enhancement of thrombotic events mediated by VWF is a vascular segment restricted feature.

Another interesting example of intraorgan endothelial heterogeneity in reaction to LPS was reported by Carrithers and colleagues. In CD31-deficient mice, LPS administration selectively induced microvascular endothelial cell apoptosis in the peritubular capillaries of the kidney while not affecting any of the other microvessel endothelial cells.<sup>96</sup> This effect was not seen in CD31-competent mice, implying that only the peritubular endothelial cells in the kidney rely on a CD31-mediated survival program when being exposed to LPS.

Long-term exposure to systemic disease conditions can also alter the basal gene expression pattern and functional behavior of endothelial cells in an endothelial subset specific manner. Using the type 2 diabetic Goto-Kakizaki rat model, Wang et al demonstrated that myocardial microvascular endothelial cells expressed decreased protein levels of VEGF (vascular endothelial growth factor), VEGF receptor 1 (VEGFR1), and VEGFR2, and exhibited decreased phosphorylation of the receptors compared with their healthy controls, whereas aortic endothelial cells from the diabetic rats did not exhibit such an altered phenotype.<sup>97</sup>

## MECHANISMS UNDERLYING HETEROGENIC ENDOTHELIAL RESPONSIVENESS

Microenvironmental conditions including shear stress, biomechanical forces, leukocyte-endothelial cell interactions, and local production of specific factors by resident cells of the tissues and the microvascular walls can all influence in vivo endothelial cell behavior. How they in concert affect endothelial molecular makeup, to explain heterogenic responsiveness to inflammatory stimuli, is almost exclusively a hypothetical exercise at this moment because only a limited number of studies have addressed this issue. It is nevertheless challenging, and rewarding at the same time, to draw a picture of the molecular steps that lie in between the initiation of cell activation, induced by receptor activation, and the final outcome, changes in expression of genes and proteins. These relationships makes one realize that an integrated approach will be needed to answer the question of how this heterogeneity is brought about.

The first stage of cell activation is at the level of receptor expression and the local concentration of the ligand, which can be different for different vascular segments. In normal kidney, for example, TNFR1 is only expressed in the glomerular and peritubular capillary endothelium.<sup>98</sup> In contrast, in the normal human heart, TNFR1 and TNFR2 are both expressed by the vascular endothelial cells, whereas TNF $\alpha$  mainly localizes to microvessels.<sup>10</sup> Disease conditions such as allograft rejection markedly altered these expression patterns, with TNFR1 expression being downregulated and TNFR2 and TNF $\alpha$  expression being upregulated.<sup>10,98</sup>

The next level of control lies in the signaling pathways employed. Kinases may be differentially expressed in different endothelial subsets, as may be the scaffolds that guide the kinases involved in final transcription factor activation. An example of such a differentially employed signaling pathway was recently provided in a study that investigated endothelial responsiveness to chemokine receptor CXCR2 ligation by macrophage inflammatory protein (MIP)-2. Although both mouse aortic and pulmonary artery endothelial cells expressed comparable levels of the receptor, only in aortic endothelial cells did exposure to MIP-2 result in RhoA activation and enhanced chemotaxis.<sup>99</sup>

The outcome of signal transduction is activation of transcription factors. For many constitutively expressed endothelial restricted genes such as VWF, eNOS, VE-cadherin, and Tie-2, their promoter regions are enriched for Ets and GATA family members, Sp1 family members, AP-1 and Octamer transcription factors, which (by not fully understood mechanisms) lead to endothelial restricted expression.<sup>100,101</sup> The homeobox gene HOXA9 also belongs to this group of transcription factors that have a preferential function in endothelial gene expression control.<sup>102</sup> Because

HOXA9 is obligatory in mediating cytokine induced E-selectin expression,<sup>31</sup> the described heterogenic E-selectin expression in response to systemic TNF $\alpha$  administration or hemorrhagic shock can possibly be explained by vascular segment restricted expression of HOXA9. Liu et al furthermore revealed by in situ exposure of umbilical cord artery and vein to TNF $\alpha$  that the induction of adhesion molecule expression was remarkably restricted to the vein segment.<sup>30</sup> This difference could not be explained by differences in TNFR expression levels nor in the extent of activation of NF $\kappa$ B and AP-1, as assessed by phospho-p65 and phospho-c-jun immunohistochemistry. Possibly, the higher levels of phospho-ATF2 in the vein endothelial cells (in part) contribute to the differential adhesion molecule expression patterns observed.

Differences in epigenetic regulatory mechanisms, affecting methylation status of gene promoters and posttranslational histone modifications such as acetylation, methylation, phosphorylation, ubiquitylation, and sumoylation, may partially explain endothelial subset preference of gene expression. That epigenetic marks are not a static feature of endothelial cells but can dynamically vary in time was recently shown for eNOS, whose histone marks were reset due to hypoxia, thereby affecting gene expression.<sup>103</sup> Also, DNA sequences proximal and distal from the promoter region significantly affect gene expression, and they can in theory be epigenetically controlled to a variable extent in endothelial subsets along the vascular tree.

Noncoding RNAs add an additional level of control of gene expression and VCAM-1 expression<sup>104-106</sup> and miR-296 regulates were shown to represent a missing link for some hitherto difficult to explain observations. Several microRNAs have been reported to be restrictedly expressed in the endothelial compartment and/or to exert a function in the vasculature. For example, miR-126 regulates angiogenesis, vascular integrity, and VCAM-1 expression,<sup>104-106</sup> miR-296 regulates the expression of various growth factors in angiogenic endothelial cells.<sup>107</sup> Suárez and colleagues reported that TNF $\alpha$ -induced miR-31 and miR-17-3p can provide negative feedback control of inflammation by affecting respectively E-selectin and ICAM-1 protein expression.<sup>108</sup> More recently, the large intervening noncoding, or lincRNAs, entered the stage.<sup>109</sup> Whereas small miRNAs hybridize with their target mRNAs to either suppress or completely silence gene expression at the posttranscriptional level, lincRNAs, transcriptionally activated by among others p53 and NF $\kappa$ B, regulate the expression of neighboring protein-coding genes at the level of chromatin modification, transcription, and posttranscriptional processing.<sup>109-111</sup>

Another mode of posttranscriptional control that has been described for the MAPK family member MAPK-activated kinase-2 (or MK2 or MAPKAP-K2)

deals with compartmentalization of proteins in the cell. In conjunction with heat-shock protein 27 (Hsp27), MK2 prevented nuclear retention of p38 MAPK by sequestering it in the cytosol. As a result, MK2 inhibited excessive mitogen- and stress-activated protein kinase, or MSK-1, and NF $\kappa$ B phosphorylation, and hence I $\kappa$ B production, thereby preventing I $\kappa$ B-driven NF $\kappa$ B transport out of the nucleus and regulating NF $\kappa$ B transcriptional activity.<sup>112</sup>

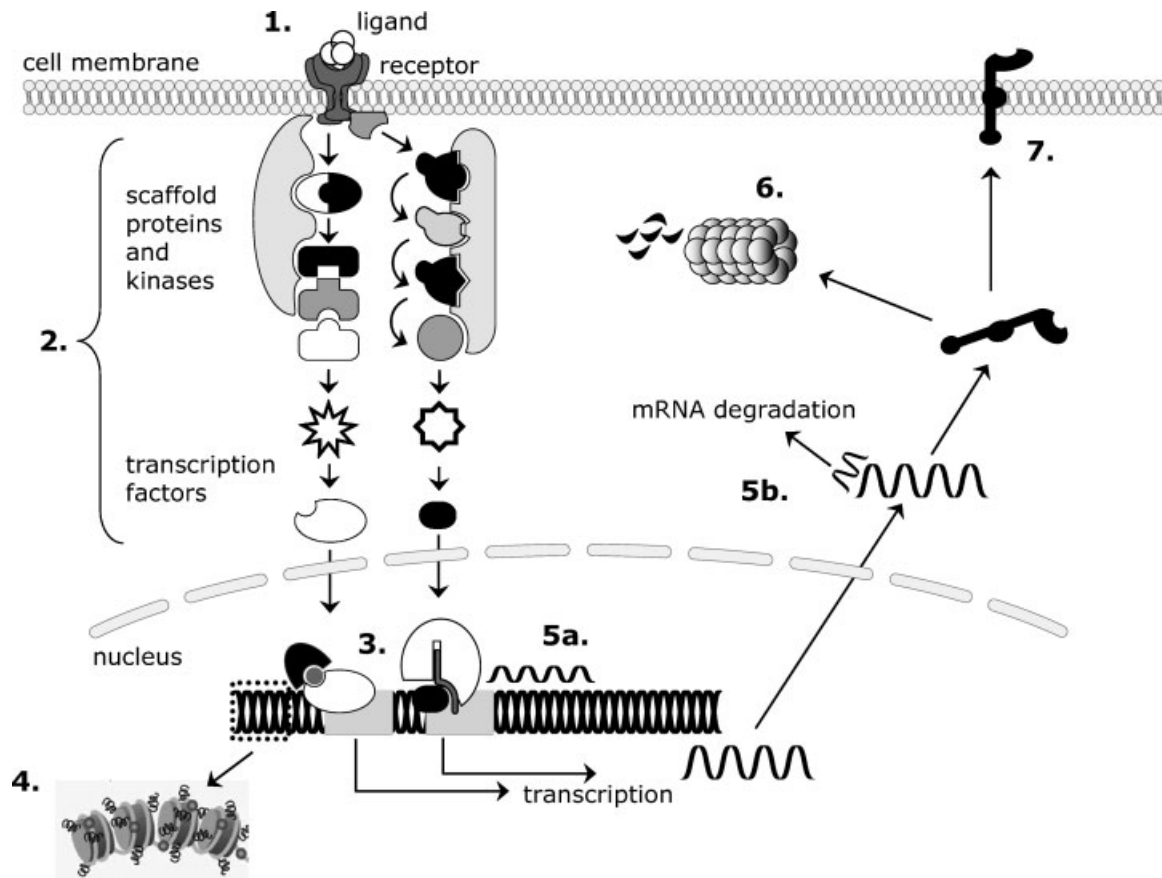
For many proteins, once formed they will shuttle to the desired cellular compartment or be exocytosed for extracellular function. Some proteins that need to be rapidly active in situations of stress are continuously produced but almost immediately afterward are subjected to proteasomal degradation, creating a situation in which mRNA levels are significant yet protein levels minor. Upon exposure to a stressor, proteasomal degradation is instantly halted as a consequence of which the protein becomes instantly available.

Fig. 3 summarizes at which levels within endothelial cells heterogenic behavior may be controlled. Only a few studies have addressed these issues so far, and only to a limited extent. Future research is expected to shed light on their significance in controlling endothelial heterogenic behavior in either quiescent conditions or in response to inflammatory stimuli.

## OTHER FACTORS ADDING TO THE COMPLEXITY OF HETEROGENIC RESPONSIVENESS

This review has so far summarized a selection of studies that have addressed endothelial responsiveness to proinflammatory cytokines in vitro and in vivo. It has focused on TNF $\alpha$ , IL-1, and LPS, and thus neglected other important proteins that can also strongly affect endothelial behavior due to their presence in the microenvironment within vascular segments in vivo. Such proteins are VEGF and its receptors, known for their widespread function in vascular homeostasis and angiogenic sprouting of blood vessels (e.g., in tumor growth), and that have now also been extensively studied for their role in inflammatory reactions. One effect of VEGFR2 signaling is the induction of VE-cadherin phosphorylation, with consequent changes in vascular permeability, as elegantly shown by Weis in an in vivo model of myocardial infarction.<sup>113</sup> In addition, membrane-bound TNF $\alpha$  was shown to sensitize endothelial cells for VEGF-induced permeability.<sup>114</sup>

Combinations of proteins can furthermore strongly affect each other's receptor and intracellular signaling dynamics. Adiponectin, for example, binds in a saturable manner to endothelial cells and selectively inhibited TNF $\alpha$ -induced NF $\kappa$ B activation without affecting JNK, p38 MAPK, and Akt.<sup>115</sup> Moreover, cytokine activation can lead to the production of new



**Figure 3** Simplified scheme of cellular events that may be differentially regulated in endothelial subsets and can contribute to endothelial heterogeneity in response to inflammatory stimuli. Microvascular endothelial cells are exposed to microenvironmental factors that differ from one vascular segment to the other, as a consequence of which their basic phenotype may vary. The following levels of control can in theory be involved in the heterogenic response of endothelial subsets to proinflammatory cytokines such as  $\text{TNF}\alpha$  or  $\text{IL-1}\beta$ . **1.** Variation in local levels of cytokines and endothelial expression levels of the receptors for the cytokines. **2.** Differences in expression levels of adapter, scaffold, and kinase proteins that lead to activation of a preferred intracellular signal transduction cascade. **3.** The route of signaling may affect the nature of the transcription factors that become activated and thus the type of genes that will be expressed. **4.** Epigenetic marks such as histone modifications and DNA methylation may vary between endothelial cell subsets to explain differences in gene expression. **5.** Noncoding RNAs, including long intervening noncoding or lincRNAs (**5a**) and microRNAs (**5b**) can affect gene expression at the transcriptional as well as the posttranscriptional level. **6.** Via ubiquitinylation and subsequent proteasomal degradation, proteins can be eliminated from the cell (before or after they have exerted their function). **7.** When fully matured, proteins can be shuttled to their target compartment in the cell, or in the cell membrane, or to the outside of the cell. For clarity, the intracellular transport systems are not depicted in the scheme.

proteins to facilitate an additional wave of activation, as described for  $\text{TNF}\alpha$  and soluble VCAM-1.<sup>116</sup>

The signal transduction section of this review has only paid attention to the class of proteins that contain substrate phosphorylation activity, the kinases, although nowadays the role of phosphatases as key coenzymes that fine-tune signaling cascades and cellular responses is also appreciated.<sup>117</sup> Protein tyrosine phosphatase 1B, or PTP1B, for example, negatively regulated VEGFR2 autophosphorylation and at the same time inhibited VE-cadherin phosphorylation in HUVEC,<sup>118</sup> implying a role in stabilizing cell-cell contact in the vasculature. Similarly, in HUVEC, the dual-specificity phosphatase MKP-1, rapidly induced

upon  $\text{TNF}\alpha$  or  $\text{IL-1}\beta$  activation, was shown to be involved in limiting cytokine-induced p38 MAPK and JNK signaling, and as such may play a role in limiting inflammatory effects.<sup>54</sup> Other negative feedback mechanisms to silence the endothelial inflammatory signaling response such as the rapid expression of family members of the ubiquitin editing enzyme A20<sup>119</sup> have also only been briefly mentioned, yet represent an additional level of control of endothelial activation that should be taken into account when trying to decipher the molecular mechanisms of heterogeneity in response to inflammatory stimuli.

A final remark on neglected subjects in this review deals with interindividual differences in endothelial



responsiveness. This represents a more technical issue that is of importance when working with primary (human) endothelial cell isolates to compare cellular and molecular reactions to cytokines between endothelial cell subtypes. Using 30 different endothelial isolates from human umbilical cord veins, and subjecting them for 24 hours to LPS stimulation, Beck and colleagues showed that these could be divided into low and high responders based on IL-8 production levels. Similar to reactivity to LPS, low responders also responded less extensively to TNF $\alpha$  and were found to require a higher concentration of activator to achieve maximal NF $\kappa$ B activity.<sup>120</sup>

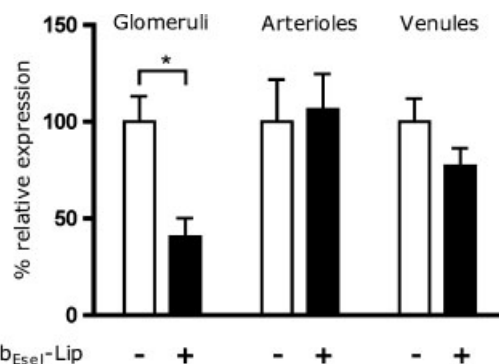
### PHARMACOLOGICAL CONSEQUENCES OF ENDOTHELIAL HETEROGENEITY

Kinases are a class of intracellular proteins that are critical for a variety of cellular responses in pathological conditions, and they are considered important targets for therapeutic intervention. Different classes of kinase inhibitors have been used as research tools, as described in Table 1, and many of these, or their derivatives, have been further developed for clinical application, with NF $\kappa$ B and p38 MAPK inhibitors being the most extensively studied.<sup>121-125</sup> One important pharmacological consequence of the existence of a complex network of different proteins interacting in concert to mount the desired reaction is that inhibition of only one pathway may lead to a more extensive activation of others. One example of such an adaptive response in endothelial cells is the effect of proteasome inhibitor MG-132 on TNF $\alpha$ -mediated cytokine production in human iliac and renal artery endothelial cells. Whereas MG-132 completely blocked TNF $\alpha$ -induced NF $\kappa$ B activation and concurrent MCP-1 expression, IL-8 expression was increased severalfold due to enhanced AP-1 transcription factor activity.<sup>38</sup> A combination of inhibitors is likely to be more effective in shutting down cell activation, as we showed for p38 MAPK/thioredoxin-NF $\kappa$ B inhibition in HUVEC challenged by TNF $\alpha$  or IL-1 $\beta$ .<sup>59</sup>

The effects of these kinase inhibitors and other anti-inflammatory drugs on endothelial cells in culture systems are undisputed, yet only limited data are available on how they affect microvascular endothelial cells in vivo. From the examples of similarities and differences in endothelial responsiveness it can be concluded that vascular segments respond to an inflammatory stimulus in a microenvironment-controlled manner. Although the outcome may be in part a vascular segment overlapping gene expression repertoire, different intracellular modes of relaying the signals to achieve this repertoire likely prevail. This implies that inhibitors of signal transduction such as the kinase inhibitors may be able to affect endothelial cells pharmacologically in one segment while being

ineffective in others. This concept challenges the one-drug-fits-all model in which an inhibitor of NF $\kappa$ B or p38 MAPK could be used as a treatment of any inflammatory disease in the body to counteract microvascular endothelial cell activation irrespective of the location of the endothelial cells.

Microvascular segment specific control of engagement in disease can also provide an opportunity for endothelial selective intervention. Once we know the molecular basis for the control for each vascular bed in its pathophysiological context, drugs can be chosen accordingly. Selectivity can furthermore be achieved by targeted drug delivery, using antibodies or peptides specific for adhesion molecules,<sup>126,127</sup> integrins,<sup>128,129</sup> or growth factor receptors<sup>130</sup> as homing ligands for carrier systems that contain the drug cargo. For example, in our laboratory we designed high payload liposomal carriers harnessed with anti-E-selectin antibody as a homing ligand to selectively deliver drugs into endothelial cells engaging in inflammatory reactions. By this means we were able to interfere pharmacologically with microvascular endothelial cells in the glomerular compartment of kidneys of mice suffering from glomerulonephritis while upstream arteriolar and downstream postcapillary venule endothelial cells were not affected (Fig. 4).<sup>131,132</sup>



**Figure 4** Endothelial heterogeneity in response to inflammatory stimuli allows for vascular segment specific therapeutic intervention by targeted drug delivery strategies. In the antiglomerular basement membrane mouse model of glomerulonephritis, endothelial cells in the affected glomeruli in the kidney express significant levels of E-selectin at an early stage of disease. Systemic administration of anti-E-selectin antibody modified liposomes containing the anti-inflammatory corticosteroid dexamethasone (Dexa-Ab<sub>Esel</sub>-Lip) resulted in accumulation of the drug delivery preparation in the glomeruli (not shown), and in local inhibition of VCAM-1 expression in the glomerular compartment. Neither VCAM-1 expression in the arteriolar nor in the postcapillary venule compartments of the kidney were affected by the treatment. Compartmentalization of drug effects was determined by laser microdissection of the vascular segments prior to analysis of gene expression by real time RT-PCR.<sup>131,132</sup>



## ADDRESSING ENDOTHELIAL HETEROGENEITY IN VIVO REQUIRES NEW TECHNOLOGIES

In an elegant study, Liu and colleagues showed that endothelial cells taken from their *in vivo* context rapidly lose their specific reactivity to an inflammatory stimulus.<sup>30</sup> Absence of VWF in glomerular endothelial cells *in vivo*<sup>133</sup> versus extensive VWF expression in isolated glomerular endothelial cells *in vitro*<sup>134</sup> is just another example of the molecular differences between endothelial cells in their *in vivo* context and *in vitro* culture conditions. Although *in vitro* studies are invaluable for generating new knowledge on endothelial behavior, the challenge for the coming decade will be to unravel the molecular basis of endothelial behavior in health, disease, and in response to drug treatment in their (patho)physiological context *in vivo*. To this end, one can isolate the endothelial cell subsets from an organ and directly map their molecular makeup using widely available gene and protein expression array platforms. Fluorescence-activated or magnetic bead-based cell sorting, for example, is a feasible technology to isolate peritubular endothelial cells from human kidneys by virtue of the fact that this endothelial subset selectively expresses MHC class II.<sup>135</sup> This is, however, a rare example of a molecule that is expressed by one subset of endothelium only.

Transgene mouse models that overexpress, or lack genes of interest, or express reporter genes in the endothelial cell compartment are instrumental in our endeavor to understand endothelial behavior *in vivo*.<sup>95,136–138</sup> The use of such models recently revealed the role of DSCR-1s (Down syndrome critical region gene 1, short variant) in heterogenic endothelial responsiveness to an inflammatory stimulus.<sup>139</sup> DSCR-1s promoter-lacZ mice showed enhanced promoter activity after LPS challenge in major organs. Upon LPS challenge of *Dscr-1*  $-/-$  mice, superinduction of E-selectin and ICAM-1 was observed in heart, while enhanced expression of VCAM-1 was observed in lung vasculature only. These data elegantly revealed a role for DSCR-1s in a negative feedback loop of endothelial cell gene expression control that is differentially regulated throughout the vascular system.

Laser microdissection (LMD) of endothelial cells from microvascular segments in tissues does not depend on advanced transgene animal models. It can be applied to both animal and human tissues, it does not affect RNA, protein and DNA integrity, and it allows for enrichment of endothelial cells from predestined (micro)vascular segments that can be prior assessed for disease activity. As such, it is an important technology for studying *in vivo* endothelial heterogeneity issues. Using this technique, basal and inflammation-related renal vascular compartmentalization of gene expression and local consequences of drug treatment on vascular segments could be revealed by us and others.<sup>131,140,141</sup>

Also, epigenetic marks in specific vascular compartments can be interrogated using LMD-based vascular segment enrichment prior to sample analysis.<sup>142</sup> Combining targeted drug delivery systems that carry siRNA as a cargo to selectively knock out genes in restricted microvascular segments<sup>143</sup> with LMD-based validation of knockout and analysis of downstream molecular consequences in the target endothelial compartment furthermore represents a new, powerful multidisciplinary approach for *in vivo* endothelial cell biology studies.

A vast array of kinase inhibitors has been developed as potential drugs for the treatment of inflammatory diseases. For the design of effective drug regimen, knowledge about the kinase activity status in the tissues, and more specifically in the endothelial cells in the different microvascular segments, is of crucial importance. For this, availability of antibodies to detect phosphoproteins on tissue sections with intact morphology is a prerequisite.<sup>144</sup> Considering the fact that endothelial cells are numerically underrepresented in almost all tissues and are furthermore often difficult to discriminate morphologically from other cells, these phosphokinase antibodies should ideally be suitable for use in immunofluorescence double staining protocols and in tissue quantitation techniques. A limited number of antibodies are at present available for this purpose, and some of them have been instrumental in detecting activated p38 MAPK in endothelial cells in human glomerulonephritis<sup>145</sup> and in determining the consequences of drug treatment on kinase activity in tumor endothelium.<sup>146</sup> Novel, superior performance phosphoprotein-specific antibodies are needed to expand the number of kinases and transcription factors to be analyzed. Combining these techniques will provide a wealth of new information on endothelial involvement in human and mouse pathology, and when including careful inspection of endothelial cells in microvascular segments, will allow us to create a view of *in vivo* endothelial heterogeneity to its full extent.

## CONCLUDING REMARKS

Endothelial cells in the body microvasculature actively engage in inflammatory reactions. In quiescent conditions, endothelial cells exert a heterogenic phenotype while the existence of endothelial subset specific responsiveness to proinflammatory cytokines is now also gradually revealed. The nature of responsiveness is clearly vascular segment dependent, and it represents an intriguing and, from a molecular point of view, a mysterious feature of an organ that is situated within all organs of the body and that was known to exist since the 17th century. Unraveling the molecular control underlying endothelial heterogeneity will be essential for a better understanding of the role of endothelial cells in pathophysiological processes and for the design of

effective pharmacological intervention of inflammatory diseases.

## REFERENCES

- Aird WC. Phenotypic heterogeneity of the endothelium: I. Structure, function, and mechanisms. *Circ Res* 2007;100(2):158–173
- Wang S, Voisin MB, Larbi KY, et al. Venular basement membranes contain specific matrix protein low expression regions that act as exit points for emigrating neutrophils. *J Exp Med* 2006;203(6):1519–1532
- Lowenstein CJ, Morrell CN, Yamakuchi M. Regulation of Weibel-Palade body exocytosis. *Trends Cardiovasc Med* 2005;15(8):302–308
- Zarbock A, Polanowska-Grabowska RK, Ley K. Platelet-neutrophil-interactions: linking hemostasis and inflammation. *Blood Rev* 2007;21(2):99–111
- Hidalgo A, Chang J, Jang JE, Peired AJ, Chiang EY, Frenette PS. Heterotypic interactions enabled by polarized neutrophil microdomains mediate thromboinflammatory injury. *Nat Med* 2009;15(4):384–391
- Pober JS, Sessa WC. Evolving functions of endothelial cells in inflammation. *Nat Rev Immunol* 2007;7(10):803–815
- Wittchen ES. Endothelial signaling in paracellular and transcellular leukocyte transmigration. *Front Biosci* 2009;14:2522–2545
- Bradley JR, Thiru S, Pober JS. Disparate localization of 55-kd and 75-kd tumor necrosis factor receptors in human endothelial cells. *Am J Pathol* 1995;146(1):27–32
- Al-Lamki RS, Wang J, Skepper JN, Thiru S, Pober JS, Bradley JR. Expression of tumor necrosis factor receptors in normal kidney and rejecting renal transplants. *Lab Invest* 2001;81(11):1503–1515
- Al-Lamki RS, Brookes AP, Wang J, et al. TNF receptors differentially signal and are differentially expressed and regulated in the human heart. *Am J Transplant* 2009;9(12):2679–2696
- Ermert M, Pantazis C, Duncker HR, Grimminger F, Seeger W, Ermert L. In situ localization of TNF $\alpha$ /beta, TACE and TNF receptors TNF-R1 and TNF-R2 in control and LPS-treated lung tissue. *Cytokine* 2003;22(3-4):89–100
- Chandrasekharan UM, Siemionow M, Unsal M, et al. Tumor necrosis factor alpha (TNF-alpha) receptor-II is required for TNF-alpha-induced leukocyte-endothelial interaction in vivo. *Blood* 2007;109(5):1938–1944
- Collins T, Read MA, Neish AS, Whitley MZ, Thanos D, Maniatis T. Transcriptional regulation of endothelial cell adhesion molecules: NF-kappa B and cytokine-inducible enhancers. *FASEB J* 1995;9(10):899–909
- May MJ, Wheeler-Jones CP, Houlston RA, Pearson JD. Activation of p42mapk in human umbilical vein endothelial cells by interleukin-1 alpha and tumor necrosis factor-alpha. *Am J Physiol* 1998;274(3 Pt 1):C789–C798
- Surapitschat J, Hoefen RJ, Pi X, Yoshizumi M, Yan C, Berk BC. Fluid shear stress inhibits TNF-alpha activation of JNK but not ERK1/2 or p38 in human umbilical vein endothelial cells: inhibitory crosstalk among MAPK family members. *Proc Natl Acad Sci U S A* 2001;98(11):6476–6481
- Madge LA, Pober JS. A phosphatidylinositol 3-kinase/Akt pathway, activated by tumor necrosis factor or interleukin-1, inhibits apoptosis but does not activate NFkappaB in human endothelial cells. *J Biol Chem* 2000;275(20):15458–15465
- Zhang L, Himi T, Morita I, Murota S. Inhibition of phosphatidylinositol-3 kinase/Akt or mitogen-activated protein kinase signaling sensitizes endothelial cells to TNF-alpha cytotoxicity. *Cell Death Differ* 2001;8(5):528–536
- Chen XL, Zhang Q, Zhao R, Ding X, Tummala PE, Medford RM. Rac1 and superoxide are required for the expression of cell adhesion molecules induced by tumor necrosis factor-alpha in endothelial cells. *J Pharmacol Exp Ther* 2003;305(2):573–580
- De Palma C, Meacci E, Perrotta C, Bruni P, Clementi E. Endothelial nitric oxide synthase activation by tumor necrosis factor alpha through neutral sphingomyelinase 2, sphingosine kinase 1, and sphingosine 1 phosphate receptors: a novel pathway relevant to the pathophysiology of endothelium. *Arterioscler Thromb Vasc Biol* 2006;26(1):99–105
- Pincheira R, Castro AF, Ozes ON, Idumalla PS, Donner DB. Type 1 TNF receptor forms a complex with and uses Jak2 and c-Src to selectively engage signaling pathways that regulate transcription factor activity. *J Immunol* 2008;181(2):1288–1298
- Viemann D, Goebeler M, Schmid S, et al. Transcriptional profiling of IKK2/NF-kappa B- and p38 MAP kinase-dependent gene expression in TNF-alpha-stimulated primary human endothelial cells. *Blood* 2004;103(9):3365–3373
- Dejana E, Orsenigo F, Lampugnani MG. The role of adherens junctions and VE-cadherin in the control of vascular permeability. *J Cell Sci* 2008;121(Pt 13):2115–2122
- Nwariaku FE, Liu Z, Zhu X, et al. NADPH oxidase mediates vascular endothelial cadherin phosphorylation and endothelial dysfunction. *Blood* 2004;104(10):3214–3220
- Dejana E, Tournier-Lasserre E, Weinstein BM. The control of vascular integrity by endothelial cell junctions: molecular basis and pathological implications. *Dev Cell* 2009;16(2):209–221
- Nwariaku FE, Chang J, Zhu X, et al. The role of p38 map kinase in tumor necrosis factor-induced redistribution of vascular endothelial cadherin and increased endothelial permeability. *Shock* 2002;18(1):82–85
- Petrache I, Birukova A, Ramirez SI, Garcia JG, Verin AD. The role of the microtubules in tumor necrosis factor-alpha-induced endothelial cell permeability. *Am J Respir Cell Mol Biol* 2003;28(5):574–581
- Lambeng N, Wallez Y, Rampon C, et al. Vascular endothelial-cadherin tyrosine phosphorylation in angiogenic and quiescent adult tissues. *Circ Res* 2005;96(3):384–391
- Gao B, Curtis TM, Blumenstock FA, Minnear FL, Saba TM. Increased recycling of (alpha)5(beta)1 integrins by lung endothelial cells in response to tumor necrosis factor. *J Cell Sci* 2000;113(Pt 2):247–257
- You B, Jiang YY, Chen S, Yan G, Sun J. The orphan nuclear receptor Nur77 suppresses endothelial cell activation through induction of IkappaBalpha expression. *Circ Res* 2009;104(6):742–749
- Liu M, Kluger MS, D'Alessio A, Garcia-Cardena G, Pober JS. Regulation of arterial-venous differences in tumor

- necrosis factor responsiveness of endothelial cells by anatomic context. *Am J Pathol* 2008;172(4):1088–1099
31. Bandyopadhyay S, Ashraf MZ, Daher P, Howe PH, DiCorleto PE. HOXA9 participates in the transcriptional activation of E-selectin in endothelial cells. *Mol Cell Biol* 2007;27(12):4207–4216
  32. Trivedi CM, Patel RC, Patel CV. Homeobox gene HOXA9 inhibits nuclear factor-kappa B dependent activation of endothelium. *Atherosclerosis* 2007;195(2):e50–e60
  33. Fiedler U, Reiss Y, Scharpfenecker M, et al. Angiopoietin-2 sensitizes endothelial cells to TNF-alpha and has a crucial role in the induction of inflammation. *Nat Med* 2006;12(2):235–239
  34. Eligini S, Barbieri SS, Cavalca V, et al. Diversity and similarity in signaling events leading to rapid Cox-2 induction by tumor necrosis factor-alpha and phorbol ester in human endothelial cells. *Cardiovasc Res* 2005;65(3):683–693
  35. Kumar A, Lin Z, SenBanerjee S, Jain MK. Tumor necrosis factor alpha-mediated reduction of KLF2 is due to inhibition of MEF2 by NF-kappaB and histone deacetylases. *Mol Cell Biol* 2005;25(14):5893–5903
  36. Trickler WJ, Mayhan WG, Miller DW. Brain microvessel endothelial cell responses to tumor necrosis factor-alpha involve a nuclear factor kappa B (NF-kappaB) signal transduction pathway. *Brain Res* 2005;1048(1-2):24–31
  37. Puri KD, Doggett TA, Douangpanya J, et al. Mechanisms and implications of phosphoinositide 3-kinase delta in promoting neutrophil trafficking into inflamed tissue. *Blood* 2004;103(9):3448–3456
  38. Hipp MS, Urbich C, Mayer P, et al. Proteasome inhibition leads to NF-kappaB-independent IL-8 transactivation in human endothelial cells through induction of AP-1. *Eur J Immunol* 2002;32(8):2208–2217
  39. Cheng N, Chen J. Tumor necrosis factor-alpha induction of endothelial ephrin A1 expression is mediated by a p38 MAPK- and SAPK/JNK-dependent but nuclear factor-kappa B-independent mechanism. *J Biol Chem* 2001;276(17):13771–13777
  40. Rahman A, Anwar KN, Malik AB. Protein kinase C-zeta mediates TNF-alpha-induced ICAM-1 gene transcription in endothelial cells. *Am J Physiol Cell Physiol* 2000;279(4):C906–C914
  41. Terry CM, Clikeman JA, Hoidal JR, Callahan KS. TNF-alpha and IL-1alpha induce heme oxygenase-1 via protein kinase C, Ca<sup>2+</sup>, and phospholipase A2 in endothelial cells. *Am J Physiol* 1999;276(5 Pt 2):H1493–H1501
  42. Hashimoto S, Matsumoto K, Gon Y, et al. p38 Mitogen-activated protein kinase regulates IL-8 expression in human pulmonary vascular endothelial cells. *Eur Respir J* 1999;13(6):1357–1364
  43. Goebeler M, Kilian K, Gillitzer R, et al. The MKK6/p38 stress kinase cascade is critical for tumor necrosis factor-alpha-induced expression of monocyte-chemoattractant protein-1 in endothelial cells. *Blood* 1999;93(3):857–865
  44. Rahman A, Kefer J, Bando M, Niles WD, Malik AB. E-selectin expression in human endothelial cells by TNF-alpha-induced oxidant generation and NF-kappaB activation. *Am J Physiol* 1998;275(3 Pt 1):L533–L544
  45. Ahmad M, Theofanis P, Medford RM. Role of activating protein-1 in the regulation of the vascular cell adhesion molecule-1 gene expression by tumor necrosis factor-alpha. *J Biol Chem* 1998;273(8):4616–4621
  46. Stuhlmeier KM, Kao JJ, Bach FH. Arachidonic acid influences proinflammatory gene induction by stabilizing the inhibitor-kappaBalpha/nuclear factor-kappaB (NF-kappaB) complex, thus suppressing the nuclear translocation of NF-kappaB. *J Biol Chem* 1997;272(39):24679–24683
  47. Spiecker M, Peng HB, Liao JK. Inhibition of endothelial vascular cell adhesion molecule-1 expression by nitric oxide involves the induction and nuclear translocation of Ikappa-Balpha. *J Biol Chem* 1997;272(49):30969–30974
  48. Pietersma A, Tilly BC, Gaestel M, et al. p38 mitogen activated protein kinase regulates endothelial VCAM-1 expression at the post-transcriptional level. *Biochem Biophys Res Commun* 1997;230(1):44–48
  49. Paleolog EM, Delasalle SA, Buurman WA, Feldmann M. Functional activities of receptors for tumor necrosis factor-alpha on human vascular endothelial cells. *Blood* 1994;84(8):2578–2590
  50. Leeuwenberg JF, van Tits LJ, Jeunhomme TM, Buurman WA. Evidence for exclusive role in signalling of tumour necrosis factor p55 receptor and a potentiating function of p75 receptor on human endothelial cells. *Cytokine* 1995;7(5):457–462
  51. Nadjar A, Combe C, Layé S, et al. Nuclear factor kappaB nuclear translocation as a crucial marker of brain response to interleukin-1. A study in rat and interleukin-1 type I deficient mouse. *J Neurochem* 2003;87(4):1024–1036
  52. Singh K, Balligand JL, Fischer TA, Smith TW, Kelly RA. Regulation of cytokine-inducible nitric oxide synthase in cardiac myocytes and microvascular endothelial cells. Role of extracellular signal-regulated kinases 1 and 2 (ERK1/ERK2) and STAT1 alpha. *J Biol Chem* 1996;271(2):1111–1117
  53. Apostolakis S, Vogiatzi K, Krambovitis E, Spandidos DA. IL-1 cytokines in cardiovascular disease: diagnostic, prognostic and therapeutic implications. *Cardiovasc Hematol Agents Med Chem* 2008;6(2):150–158
  54. Wadgaonkar R, Pierce JW, Somnay K, et al. Regulation of c-Jun N-terminal kinase and p38 kinase pathways in endothelial cells. *Am J Respir Cell Mol Biol* 2004;31(4):423–431
  55. Williams MR, Kataoka N, Sakurai Y, Powers CM, Eskin SG, McIntire LV. Gene expression of endothelial cells due to interleukin-1 beta stimulation and neutrophil transmigration. *Endothelium* 2008;15(1):73–165
  56. Zhao B, Stavchansky SA, Bowden RA, Bowman PD. Effect of interleukin-1beta and tumor necrosis factor-alpha on gene expression in human endothelial cells. *Am J Physiol Cell Physiol* 2003;284(6):C1577–C1583
  57. Gille J, Swerlick RA, Lawley TJ, Caughman SW. Differential regulation of vascular cell adhesion molecule-1 gene transcription by tumor necrosis factor alpha and interleukin-1 alpha in dermal microvascular endothelial cells. *Blood* 1996;87(1):211–217
  58. Rogers RJ, Monnier JM, Nick HS. Tumor necrosis factor-alpha selectively induces MnSOD expression via mitochondria-to-nucleus signaling, whereas interleukin-1beta utilizes an alternative pathway. *J Biol Chem* 2001;276(23):20419–20427
  59. Kuldo JM, Westra J, Asgeirsdóttir SA, et al. Differential effects of NF-kappaB and p38 MAPK inhibitors and combinations thereof on TNF-alpha- and IL-1beta-induced proinflammatory status of endothelial cells in vitro. *Am J Physiol Cell Physiol* 2005;289(5):C1229–C1239



60. Said FA, Werts C, Elalamy I, Couetil JP, Jacquemin C, Hatmi M. TNF- $\alpha$ , inefficient by itself, potentiates IL-1 $\beta$ -induced PGHS-2 expression in human pulmonary microvascular endothelial cells: requirement of NF- $\kappa$ B and p38 MAPK pathways. *Br J Pharmacol* 2002;136(7):1005–1014
61. Dauphinee SM, Karsan A. Lipopolysaccharide signaling in endothelial cells. *Lab Invest* 2006;86(1):9–22
62. Hull C, McLean G, Wong F, Duriez PJ, Karsan A. Lipopolysaccharide signals an endothelial apoptosis pathway through TNF receptor-associated factor 6-mediated activation of c-Jun NH2-terminal kinase. *J Immunol* 2002;169(5):2611–2618
63. Li X, Tupper JC, Bannerman DD, Winn RK, Rhodes CJ, Harlan JM. Phosphoinositide 3 kinase mediates Toll-like receptor 4-induced activation of NF- $\kappa$ B in endothelial cells. *Infect Immun* 2003;71(8):4414–4420
64. Chen JX, Berry LC, Christman BW, Meyrick B. Glutathione mediates LPS-stimulated COX-2 expression via early transient p42/44 MAPK activation. *J Cell Physiol* 2003;197(1):86–93
65. Kaur J, Kubes P. Endothelium—a critical detector of lipopolysaccharide. In: Aird WC, ed. *Endothelial Biomedicine*. Cambridge, United Kingdom: Cambridge University Press; 2007:410–418
66. Hu X, Yee E, Harlan JM, Wong F, Karsan A. Lipopolysaccharide induces the antiapoptotic molecules, A1 and A20, in microvascular endothelial cells. *Blood* 1998;92(8):2759–2765
67. Bannerman DD, Eiting KT, Winn RK, Harlan JM. FLICE-like inhibitory protein (FLIP) protects against apoptosis and suppresses NF- $\kappa$ B activation induced by bacterial lipopolysaccharide. *Am J Pathol* 2004;165(4):1423–1431
68. Pollet I, Opina CJ, Zimmerman C, Leong KG, Wong F, Karsan A. Bacterial lipopolysaccharide directly induces angiogenesis through TRAF6-mediated activation of NF- $\kappa$ B and c-Jun N-terminal kinase. *Blood* 2003;102(5):1740–1742
69. Tseng HW, Juan HF, Huang HC, et al. Lipopolysaccharide-stimulated responses in rat aortic endothelial cells by a systems biology approach. *Proteomics* 2006;6(22):5915–5928
70. Schlegel N, Baumer Y, Drenckhahn D, Waschke J. Lipopolysaccharide-induced endothelial barrier breakdown is cyclic adenosine monophosphate dependent in vivo and in vitro. *Crit Care Med* 2009;37(5):1735–1743
71. Montesano R, Pepper MS, Möhle-Steinlein U, Risau W, Wagner EF, Orci L. Increased proteolytic activity is responsible for the aberrant morphogenetic behavior of endothelial cells expressing the middle T oncogene. *Cell* 1990;62(3):435–445
72. Garlanda C, Parravicini C, Sironi M, et al. Progressive growth in immunodeficient mice and host cell recruitment by mouse endothelial cells transformed by polyoma middle-sized T antigen: implications for the pathogenesis of opportunistic vascular tumors. *Proc Natl Acad Sci U S A* 1994;91(15):7291–7295
73. van Leeuwen EBM, Wisman GBA, Tervaert JW, et al. An SV40 large T-antigen immortalized human umbilical vein endothelial cell line for anti-endothelial cell antibody detection. *Clin Exp Rheumatol* 2001;19(3):283–290
74. Edgell CJ, McDonald CC, Graham JB. Permanent cell line expressing human factor VIII-related antigen established by hybridization. *Proc Natl Acad Sci U S A* 1983;80(12):3734–3737
75. Ades EW, Candal FJ, Swerlick RA, et al. HMEC-1: establishment of an immortalized human microvascular endothelial cell line. *J Invest Dermatol* 1992;99(6):683–690
76. Yang J, Chang E, Cherry AM, et al. Human endothelial cell life extension by telomerase expression. *J Biol Chem* 1999;274(37):26141–26148
77. Viemann D, Goebeler M, Schmid S, et al. TNF induces distinct gene expression programs in microvascular and macrovascular human endothelial cells. *J Leukoc Biol* 2006;80(1):174–185
78. Chi JT, Chang HY, Haraldsen G, et al. Endothelial cell diversity revealed by global expression profiling. *Proc Natl Acad Sci U S A* 2003;100(19):10623–10628
79. Kalogeris TJ, Kevil CG, Laroux FS, Coe LL, Phifer TJ, Alexander JS. Differential monocyte adhesion and adhesion molecule expression in venous and arterial endothelial cells. *Am J Physiol* 1999;276(1 Pt 1):L9–L19
80. Murakami S, Morioka T, Nakagawa Y, Suzuki Y, Arakawa M, Oite T. Expression of adhesion molecules by cultured human glomerular endothelial cells in response to cytokines: comparison to human umbilical vein and dermal microvascular endothelial cells. *Microvasc Res* 2001;62(3):383–391
81. Wang Q, Pfeiffer GR II, Stevens T, Doerschuk CM. Lung microvascular and arterial endothelial cells differ in their responses to intercellular adhesion molecule-1 ligation. *Am J Respir Crit Care Med* 2002;166(6):872–877
82. Deng DX, Tsalenko A, Vailaya A, et al. Differences in vascular bed disease susceptibility reflect differences in gene expression response to atherogenic stimuli. *Circ Res* 2006;98(2):200–208
83. Invernici G, Ponti D, Corsini E, et al. Human microvascular endothelial cells from different fetal organs demonstrate organ-specific CAM expression. *Exp Cell Res* 2005;308(2):273–282
84. Methe H, Balcells M, Alegret MdelC, et al. Vascular bed origin dictates flow pattern regulation of endothelial adhesion molecule expression. *Am J Physiol Heart Circ Physiol* 2007;292(5):H2167–H2175
85. Mason JC, Yarwood H, Sugars K, Haskard DO. Human umbilical vein and dermal microvascular endothelial cells show heterogeneity in response to PKC activation. *Am J Physiol* 1997;273(4 Pt 1):C1233–C1240
86. Sanabria P, Ross E, Ramirez E, et al. P2Y2 receptor desensitization on single endothelial cells. *Endothelium* 2008;15(1):43–51
87. Nguyen VP, Chen SH, Trinh J, Kim H, Coomber BL, Dumont DJ. Differential response of lymphatic, venous and arterial endothelial cells to angiotensin-1 and angiotensin-2. *BMC Cell Biol* 2007;8:10
88. Eppihimer MJ, Wolitzky B, Anderson DC, Labow MA, Granger DN. Heterogeneity of expression of E- and P-selectins in vivo. *Circ Res* 1996;79(3):560–569
89. Tamaru M, Tomura K, Sakamoto S, Tezuka K, Tamatani T, Narumi S. Interleukin-1 $\beta$  induces tissue- and cell type-specific expression of adhesion molecules in vivo. *Arterioscler Thromb Vasc Biol* 1998;18(8):1292–1303
90. Yao L, Setiadi H, Xia L, Laszik Z, Taylor FB, McEver RP. Divergent inducible expression of P-selectin and E-selectin in mice and primates. *Blood* 1999;94(11):3820–3828

91. van Meurs M, Wulfert FM, Knol AJ, et al. Early organ-specific endothelial activation during hemorrhagic shock and resuscitation. *Shock* 2008;29(2):291–299
92. Yano K, Okada Y, Beldi G, et al. Elevated levels of placental growth factor represent an adaptive host response in sepsis. *J Exp Med* 2008;205(11):2623–2631
93. Shapiro NI, Yano K, Sorasaki M, Fischer C, Shih SC, Aird WC. Skin biopsies demonstrate site-specific endothelial activation in mouse models of sepsis. *J Vasc Res* 2009;46(5):495–502
94. Patel KN, Soubra SH, Bellera RV, et al. Differential role of von Willebrand factor and P-selectin on microvascular thrombosis in endotoxemia. *Arterioscler Thromb Vasc Biol* 2008;28(12):2225–2230
95. Minami T, Donovan DJ, Tsai JC, Rosenberg RD, Aird WC. Differential regulation of the von Willebrand factor and Flt-1 promoters in the endothelium of hypoxanthine phosphoribosyltransferase-targeted mice. *Blood* 2002;100(12):4019–4025
96. Carrithers M, Tandon S, Canosa S, Michaud M, Graesser D, Madri JA. Enhanced susceptibility to endotoxic shock and impaired STAT3 signaling in CD31-deficient mice. *Am J Pathol* 2005;166(1):185–196
97. Wang XH, Chen SF, Jin HM, Hu RM. Differential analyses of angiogenesis and expression of growth factors in micro- and macrovascular endothelial cells of type 2 diabetic rats. *Life Sci* 2009;84(7–8):240–249
98. Al-Lamki RS, Wang J, Vandenabeele P, et al. TNFR1- and TNFR2-mediated signaling pathways in human kidney are cell type-specific and differentially contribute to renal injury. *FASEB J* 2005;19(12):1637–1645
99. Moldobaeva A, Baek A, Wagner EM. MIP-2 causes differential activation of RhoA in mouse aortic versus pulmonary artery endothelial cells. *Microvasc Res* 2008;75(1):53–58
100. Minami T, Aird WC. Endothelial cell gene regulation. *Trends Cardiovasc Med* 2005;15(5):174–184
101. Fish JE, Marsden PA. Endothelial nitric oxide synthase: insight into cell-specific gene regulation in the vascular endothelium. *Cell Mol Life Sci* 2006;63(2):144–162
102. Matouk CC, Marsden PA. Epigenetic regulation of vascular endothelial gene expression. *Circ Res* 2008;102(8):873–887
103. Fish JE, Yan MS, Matouk CC, et al. Hypoxic repression of endothelial nitric-oxide synthase transcription is coupled with eviction of promoter histones. *J Biol Chem* 2010;285(2):810–826
104. Fish JE, Santoro MM, Morton SU, et al. miR-126 regulates angiogenic signaling and vascular integrity. *Dev Cell* 2008;15(2):272–284
105. Harris TA, Yamakuchi M, Ferlito M, Mendell JT, Lowenstein CJ. MicroRNA-126 regulates endothelial expression of vascular cell adhesion molecule 1. *Proc Natl Acad Sci U S A* 2008;105(5):1516–1521
106. van Solingen C, Seghers L, Bijkerk R, et al. Antagomir-mediated silencing of endothelial cell specific microRNA-126 impairs ischemia-induced angiogenesis. *J Cell Mol Med* 2009;13(8A):1577–1585
107. Würdinger T, Tannous BA, Saydam O, et al. miR-296 regulates growth factor receptor overexpression in angiogenic endothelial cells. *Cancer Cell* 2008;14(5):382–393
108. Suárez Y, Wang C, Manes TD, Pober JS. Cutting edge: TNF-induced microRNAs regulate TNF-induced expression of E-selectin and intercellular adhesion molecule-1 on human endothelial cells: feedback control of inflammation. *J Immunol* 2010;184(1):21–25
109. Guttman M, Amit I, Garber M, et al. Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals. *Nature* 2009;458(7235):223–227
110. Mercer TR, Dinger ME, Mattick JS. Long non-coding RNAs: insights into functions. *Nat Rev Genet* 2009;10(3):155–159
111. Khalil AM, Guttman M, Huarte M, et al. Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. *Proc Natl Acad Sci U S A* 2009;106(28):11667–11672
112. Gorska MM, Liang Q, Stafford SJ, et al. MK2 controls the level of negative feedback in the NF-kappaB pathway and is essential for vascular permeability and airway inflammation. *J Exp Med* 2007;204(7):1637–1652
113. Weis S, Shintani S, Weber A, et al. Src blockade stabilizes a Flk/cadherin complex, reducing edema and tissue injury following myocardial infarction. *J Clin Invest* 2004;113(6):885–894
114. Clauss M, Sunderkötter C, Sveinbjörnsson B, et al. A permissive role for tumor necrosis factor in vascular endothelial growth factor-induced vascular permeability. *Blood* 2001;97(5):1321–1329
115. Ouchi N, Kihara S, Arita Y, et al. Adiponectin, an adipocyte-derived plasma protein, inhibits endothelial NF-kappaB signaling through a cAMP-dependent pathway. *Circulation* 2000;102(11):1296–1301
116. Nakao S, Kuwano T, Ishibashi T, Kuwano M, Ono M. Synergistic effect of TNF-alpha in soluble VCAM-1-induced angiogenesis through alpha 4 integrins. *J Immunol* 2003;170(11):5704–5711
117. Tonks NK. Protein tyrosine phosphatases: from genes, to function, to disease. *Nat Rev Mol Cell Biol* 2006;7(11):833–846
118. Nakamura Y, Patrushev N, Inomata H, et al. Role of protein tyrosine phosphatase 1B in vascular endothelial growth factor signaling and cell-cell adhesions in endothelial cells. *Circ Res* 2008;102(10):1182–1191
119. Enesa K, Zakkar M, Chaudhury H, et al. NF-kappaB suppression by the deubiquitinating enzyme Cezanne: a novel negative feedback loop in pro-inflammatory signaling. *J Biol Chem* 2008;283(11):7036–7045
120. Beck GC, Rafat N, Brinkkoetter P, et al. Heterogeneity in lipopolysaccharide responsiveness of endothelial cells identified by gene expression profiling: role of transcription factors. *Clin Exp Immunol* 2006;143(3):523–533
121. Kůřdo JM, Ogawara KI, Werner N, et al. Molecular pathways of endothelial cell activation for (targeted) pharmacological intervention of chronic inflammatory diseases. *Curr Vasc Pharmacol* 2005;3(1):11–39
122. Gilmore TD, Herscovitch M. Inhibitors of NF-kappaB signaling: 785 and counting. *Oncogene* 2006;25(51):6887–6899
123. Keri G, Orfi L, Eros D, et al. Signal transduction therapy with rationally designed kinase inhibitors. *Curr Signal Transduct Ther* 2006;1:67–95
124. Fabian MA, Biggs WH III, Treiber DK, et al. A small molecule-kinase interaction map for clinical kinase inhibitors. *Nat Biotechnol* 2005;23(3):329–336
125. Fedorov O, Marsden B, Pogacic V, et al. A systematic interaction map of validated kinase inhibitors with Ser/Thr



- kinases. *Proc Natl Acad Sci U S A* 2007;104(51):20523–20528
126. Everts M, Kok RJ, Asgeirsdóttir SA, et al. Selective intracellular delivery of dexamethasone into activated endothelial cells using an E-selectin-directed immunoconjugate. *J Immunol* 2002;168(2):883–889
  127. Koning GA, Schiffelers RM, Wauben MHM, et al. Targeting of angiogenic endothelial cells at sites of inflammation by dexamethasone phosphate-containing RGD peptide liposomes inhibits experimental arthritis. *Arthritis Rheum* 2006;54(4):1198–1208
  128. Schraa AJ, Kok RJ, Berendsen AD, et al. Endothelial cells internalize and degrade RGD-modified proteins developed for tumor vasculature targeting. *J Control Release* 2002;83:241–251
  129. Schraa AJ, Kok RJ, Moorlag HE, et al. Targeting of RGD-modified proteins to tumor vasculature: a pharmacokinetic and cellular distribution study. *Int J Cancer* 2002;102(5):469–475
  130. Janssen AP, Schiffelers RM, ten Hagen TL, et al. Peptide-targeted PEG-liposomes in anti-angiogenic therapy. *Int J Pharm* 2003;254(1):55–58
  131. Asgeirsdóttir SA, Kamps JAAM, Bakker HI, et al. Site-specific inhibition of glomerulonephritis progression by targeted delivery of dexamethasone to glomerular endothelium. *Mol Pharmacol* 2007;72(1):121–131
  132. Asgeirsdóttir SA, Zwiers PJ, Morselt HW, et al. Inhibition of proinflammatory genes in anti-GBM glomerulonephritis by targeted dexamethasone-loaded AbE-sel liposomes. *Am J Physiol Renal Physiol* 2008;294(3):F554–F561
  133. Pusztaszeri MP, Seelentag W, Bosman FT. Immunohistochemical expression of endothelial markers CD31, CD34, von Willebrand factor, and Fli-1 in normal human tissues. *J Histochem Cytochem* 2006;54(4):385–395
  134. Satchell SC, Tasman CH, Singh A, et al. Conditionally immortalized human glomerular endothelial cells expressing fenestrations in response to VEGF. *Kidney Int* 2006;69(9):1633–1640
  135. Muczynski KA, Ekle DM, Coder DM, Anderson SK. Normal human kidney HLA-DR-expressing renal microvascular endothelial cells: characterization, isolation, and regulation of MHC class II expression. *J Am Soc Nephrol* 2003;14(5):1336–1348
  136. Maharaj AS, Saint-Geniez M, Maldonado AE, D'Amore PA. Vascular endothelial growth factor localization in the adult. *Am J Pathol* 2006;168(2):639–648
  137. Monvoisin A, Alva JA, Hofmann JJ, Zovein AC, Lane TF, Iruela-Arispe ML. VE-cadherin-CreERT2 transgenic mouse: a model for inducible recombination in the endothelium. *Dev Dyn* 2006;235(12):3413–3422
  138. Gareus R, Kotsaki E, Xanthoulea S, et al. Endothelial cell-specific NF-kappaB inhibition protects mice from atherosclerosis. *Cell Metab* 2008;8(5):372–383
  139. Minami T, Yano K, Miura M, et al. The Down syndrome critical region gene 1 short variant promoters direct vascular bed-specific gene expression during inflammation in mice. *J Clin Invest* 2009;119(8):2257–2270
  140. Zhang L, Zhang ZG, Liu XS, Hozeska-Solgot A, Chopp M. The PI3K/Akt pathway mediates the neuroprotective effect of atorvastatin in extending thrombolytic therapy after embolic stroke in the rat. *Arterioscler Thromb Vasc Biol* 2007;27(11):2470–2475
  141. van Meurs M, Kurniati NF, Wulfert FM, et al. Shock-induced stress induces loss of microvascular endothelial Tie2 in the kidney which is not associated with reduced glomerular barrier function. *Am J Physiol Renal Physiol* 2009;297(2):F272–F281
  142. Hellebrekers DMEI, Castermans K, Viré E, et al. Epigenetic regulation of tumor endothelial cell anergy: silencing of intercellular adhesion molecule-1 by histone modifications. *Cancer Res* 2006;66(22):10770–10777
  143. Asgeirsdóttir SA, Talman EG, de Graaf IA, et al. Targeted transfection increases siRNA uptake and gene silencing in primary endothelial cells in vitro—a quantitative study. *J Control Release* 2010;141(2):241–251
  144. Pham NA, Schwock J, Iakovlev V, Pond G, Hedley DW, Tsao MS. Immunohistochemical analysis of changes in signaling pathway activation downstream of growth factor receptors in pancreatic duct cell carcinogenesis. *BMC Cancer* 2008;8:43
  145. Stambe C, Nikolic-Paterson DJ, Hill PA, Dowling J, Atkins RC. p38 Mitogen-activated protein kinase activation and cell localization in human glomerulonephritis: correlation with renal injury. *J Am Soc Nephrol* 2004;15(2):326–336
  146. Kuwai T, Nakamura T, Sasaki T, et al. Phosphorylated epidermal growth factor receptor on tumor-associated endothelial cells is a primary target for therapy with tyrosine kinase inhibitors. *Neoplasia* 2008;10(5):489–500