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Objective—Atherosclerosis is a focal disease that occurs predominantly at branches and bends of the arterial tree. Endothelial cells (EC) at atherosusceptible sites are prone to injury, which can contribute to lesion formation, whereas EC at atheroprotected sites are resistant. The c-Jun N-terminal kinase (JNK) is activated constitutively in EC at atherosusceptible sites but is inactivated at atheroprotected sites by mitogen-activated protein kinase phosphatase-1 (MKP-1). Here, we examined the effects of JNK activation on EC physiology at atherosusceptible sites.

Methods and Results—We identified transcriptional programs regulated by JNK by applying a specific pharmacological inhibitor to cultured EC and assessing the transcriptome using microarrays. This approach and subsequent validation by gene silencing revealed that JNK positively regulates the expression of numerous proapoptotic molecules. Analysis of aortae of wild-type, JNK1$^{-/-}$, and MKP-1$^{-/-}$ mice revealed that EC at an atherosusceptible site express proapoptotic proteins and are primed for apoptosis and proliferation in response to lipopolysaccharide through a JNK1-dependent mechanism, whereas EC at a protected site expressed lower levels of proapoptotic molecules and were protected from injury by MKP-1.

Conclusion—Spatial variation of JNK1 activity delineates the spatial distribution of apoptosis and turnover of EC in arteries. **(*Arterioscler Thromb Vasc Biol*. 2010;30:546-553.)**

Key Words: apoptosis ■ arterial endothelium ■ atherosusceptibility ■ c-Jun N-terminal kinase ■ mitogen-activated protein kinase phosphatase-1

Atherosclerosis is characterized by the accumulation of cells, lipids, and extracellular matrix in the wall of an artery, which can result in occlusion of the vessel lumen. It develops predominantly at branches and bends that are exposed to disturbed patterns of blood flow, whereas regions exposed to uniform flow are protected.[1–4] The molecular mechanism underlying the distinct spatial distribution of lesions is likely to involve apoptosis. Regions that are predisposed to atherosclerosis are characterized by relatively high rates of endothelial cell (EC) injury and turnover,[5–7] and apoptosis can be induced in cultured EC by the application of atheroprone flow patterns.[8,9] A causal relationship between apoptosis and atherosclerosis was established by enforcing expression of a proapoptotic molecule in arterial EC, which enhanced the accessibility of lipoproteins and leukocytes to arteries and initiated lesion formation in hypercholesterolemic mice.[10] In addition, focal endothelial apoptosis/injury in atherosclerotic lesions can, in turn, lead to endothelial denudation and exposure of a procoagulant vascular wall, a major cause of coronary thrombosis.[11,12] Several proatherogenic agents are known to induce EC apoptosis, including oxidized low-density lipoproteins,[13] reactive oxygen intermediaries (eg, H_2O_2[14]) and proinflammatory mediators (eg, lipopolysaccharide [LPS],[15] tumor necrosis factor [TNF]-α[16]) by activating distinct signaling pathways that converge to cleave procaspase-3 into the active form of caspase-3. Cleaved caspase-3, in turn, executes apoptosis by activating numerous downstream effectors that trigger DNA fragmentation and other hallmarks of apoptotic cell death.[17]

We recently studied EC in the murine aorta and demonstrated that c-Jun N-terminal kinase (JNK), a mitogen-activated protein kinase, can be activated by proinflammatory stimuli at atherosusceptible sites but not at atheroprotected sites.[18] The spatial distribution of JNK activity in the arterial tree is controlled by mitogen-activated protein kinase phosphatase-1 (MKP-1), a negative regulator of JNK, which is induced by blood flow at atheroprotected sites.[18] Here, we demonstrate that JNK induces numerous proapoptotic molecules in EC and that the spatial variation of JNK activation in the arterial tree delineates the spatial distribution of apoptosis and turnover of EC.

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Materials and Methods

Reagents and Antibodies

A pharmacological inhibitor of all 3 JNK isoforms (CT536706) was prepared as described previously[19] and dissolved in DMSO (10 mmol/L stock). LPS (R&D), TNF-α (R&D), and antibodies that recognized phosphorylated JNK Tyr183/Thr185 (Cell Signaling Technology), JNK (Cell Signaling Technology), CD31 (BD Biosciences Pharmingen), Receptor interacting protein 1 (RIP1; BD Bioscience), tubulin (Sigma Aldrich), procaspase-3 (Abcam), active cleaved caspase-3 (Cell Signaling Technology), and Ki-67 (Abcam) were obtained commercially. TUNEL staining was performed using the in situ cell death detection kit (Roche). Other reagents were purchased from Sigma Aldrich unless otherwise stated.

Endothelial Cell Culture

Human umbilical vein endothelial cells (HUVEC) were collected using collagenase and cultured as described previously.[20]

Microarray Analysis

Total RNA was prepared from HUVEC using a commercially available kit (Omega Bio-Tek), and the purity and integrity of total RNA samples were assessed using a Bioanalyser (Agilent). High-quality samples were used to generate labeled cRNA using commercially available kits (Affymetrix). Labeled cRNA was hybridized to Human Gene 1.0 ST Arrays (Affymetrix) following standardized procedures. This array contains probes that identify 764 885 transcripts that represents 28 869 genes. Raw data were analyzed using Resolver software (Rosetta) to quantify changes in transcripts differentially expressed in the experimental samples. Functional annotation of coregulated genes was performed using Database for Annotation, Visualization, and Integrated Discovery (DAVID) software.

Gene Silencing

RNA interference was performed using 2 different JNK1-specific siRNA or c-Jun–specific siRNA (Applied Biosystems). Nontargeting scrambled control sequences were also synthesized (Qiagen). Cell cultures that were 70% to 80% confluent were transfected with siRNA (5 μmol/L final concentration) by electroporation (Amaxa) following the manufacturer's instructions and then incubated in complete growth medium for 24 to 48 hours before analysis.

Comparative Real-Time Polymerase Chain Reaction

Transcript levels were quantified by comparative real-time polymerase chain reaction using gene-specific primers (Supplementary Table I, available online at http://atvb.ahajournals.org) as described previously.[21] Relative gene expression was calculated by comparing the number of thermal cycles that were necessary to generate threshold amounts of product for the genes of interest and for the housekeeping gene β-actin.

Western Blotting

Levels of JNK1, RIP1, and procaspase-3 were measured in total cell lysates prepared using radioimmunoprecipitation assay buffer (150 mmol/L NaCl, 1.0% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mmol/L Tris, pH 8.0) by Western blotting using specific primary antibodies, horseradish peroxidase-conjugated secondary antibodies, and chemiluminescent detection. Tubulin levels were measured to control for equal loading.

c-Jun Activity Assay

The levels of phosphorylated c-Jun and total c-Jun were determined using enzyme-linked immunosorbent assay kits following the manufacturer's instructions (ActiveMotif). The ratio of phosphorylated c-Jun-to-total c-Jun, which is a measure of c-Jun activity, was calculated for each sample.

Animals

Male mice between 2 and 3 months of age were used. Wild-type C57BL/6 mice were obtained from Charles River Laboratories, UK. The MKP-1 knockout mouse strain (MKP-1$^{-/-}$ [C57BL/6]) was obtained from Bristol-Myers Squibb and the JNK1$^{-/-}$ strain was obtained from Professor Roger Davis, University of Massachusetts.[22] Experiments were performed using groups of at least 4 animals. All experiments were performed within guidelines set by the Federation of European Laboratory Animal Science Associations.

En Face Immunostaining

The expression levels of specific proteins were assessed in EC at regions of the murine aorta that are known to be susceptible to (inner curvature of the arch) or protected from (outer curvature) atherosclerosis by en face staining, followed by laser-scanning confocal microscopy as described previously.[18,23] Aortae were tested by immunostaining using specific primary antibodies and Alexafluor568-conjugated secondary antibodies. EC were identified by costaining using anti-CD31 antibodies conjugated to Alexafluor488, and nuclei were costained using Draq5 (Biostatus). The expression of particular proteins at each site was assessed by quantification of fluorescence intensity for multiple cells (at least 100 per site) using LSM 510 software (Zeiss).

En Face TUNEL Staining

DNA fragmentation was measured in EC in murine aortae by en face TUNEL staining, followed by laser-scanning confocal microscopy. Animals were euthanized with CO_2 inhalation and aortae were perfused in situ with phosphate-buffered saline and then perfusion-fixed with 2% formalin before harvesting, as described previously.[18] Fixed aortae were tested by TUNEL staining following the manufacturer's recommendations (Roche). EC were identified by costaining using *Griffonia* lectin conjugated to Rhodamine (Vectorlabs) or by using anti-CD31 conjugated to phycoerythrin, and nuclei were costained using Draq5 (Biostatus). Staining was assessed by confocal microscopy and apoptotic EC, characterized by TUNEL staining colocalized with pyknotic or fragmented nuclei, were quantified in multiple fields of view.

Statistics

Differences between samples were analyzed using an unpaired Student t test or a χ^2 test (*$P<0.05$; **$P<0.01$; ***$P<0.001$).

Results

JNK Acts as a Positive Regulator of Proapoptotic Molecules in Cultured EC

We examined the physiological role of JNK in cultured EC using a pharmacological inhibitor (CT536706).[19] Pretreatment of HUVEC with CT536706 suppressed subsequent phosphorylation of c-Jun by TNF-α, confirming that this compound can inhibit JNK activation in cultured EC (Supplementary Figure I, available online at http://atvb.ahajournals.org). We studied the transcriptional programs regulated by JNK using microarrays. Treatment of HUVEC with CT536706 altered the constitutive expression of 1123 transcripts (116 upregulated and 1007 downregulated) that belong to several functional groups, including inflammation, proliferation, apoptosis, and cell signaling (Supplementary Table II, Supplementary Figure IIA), suggesting that JNK regulates the basal expression of numerous molecules that possess diverse physiological functions. Of particular note, proapoptotic transcripts were significantly enriched in the JNK-dependent group. Treatment of HUVEC with TNF-α altered the expression of 241 transcripts (165 upregulated and 76 downregulated), including regulators of apoptosis, leuko-

Table. Comparison of Microarray Data With Quantitative Polymerase Chain Reaction for Selected Genes

		Genes	Basal state		TNFα-treated		
			Effects of JNK inhibitor		Induction	Effects of JNK inhibitor	
			Array	q-PCR		Array	q-PCR
Apoptosis	Inflammation	Vascular cell adhesion molecule 1 (VCAM-1)			Yes		
		E-selectin			Yes		
		Interleukin 8 (IL-8)			Yes		
		Toll-like receptor 4 (TLR4)			No	n.a.	n.a.
		Caspase recruitment domain family 6 (CARD6)			No	n.a.	n.a.
		Receptor interacting protein 1 (RIP1)			No	n.a.	n.a.
		Bone morphogenetic protein 2 (BMP2)			Yes		n.t.
		Bone morphogenetic protein 4 (BMP4)			No	n.a.	n.a.
		Dickkopf1 (DKK1)			No	n.a.	n.a.
		Caspase 3			Yes		n.t.
		Serine threonine kinase (Mst-1)			No	n.a.	n.a.
		Protein phosphatise 2A (PP2A)			Yes		
		D4, zinc and double PHD fingers family 2 (DPF2)			No	n.a.	n.a.
		Zinc finger protein 346 (JAZ)			No	n.a.	n.a.
	Survival	Growth arrest and DNA damage-inducible (GADD45β)			Yes		n.t.
		Bcl-2 related protein A1			Yes		

JNK regulated
Positively
Negatively
Unaltered

HUVEC were treated with CT536706 (JNK inhibitor; 1 μmol/L for 5 hours), with vehicle alone, or remained untreated. Cultures were costimulated with TNF-α (10 ng/mL) for the final 4 hours or remained untreated. Each experimental condition was performed in triplicate. Transcript levels in each sample were assessed at a genomic level using Affymetrix microarrays. Proinflammatory and proapoptotic transcripts that were positively regulated by JNK in all 3 isolates, either in the basal state or in response to TNF-α treatment, were identified using Rosetta-resolver and DAVID software. The effects of the JNK inhibitor or TNF-α on the expression levels of selected transcripts were subsequently validated by comparative real-time polymerase chain reaction (quantitative polymerase chain reaction) in 3 additional independent experiments. A summary of array and quantitative polymerase chain reaction results are shown for selected genes that regulate inflammation, apoptosis, or cell survival. Black, positively regulated by JNK; White, negatively regulated by JNK; grey, not regulated by JNK.

NA indicates not applicable; NT, not tested.

cyte adhesion, and cell signaling (Supplementary Table II, Supplementary Figure IIB), a finding that is consistent with previous studies.[24,25] Pretreatment of HUVEC with the JNK inhibitor suppressed the induction by TNF-α of 134 transcripts, and this group was enriched with molecules with proapoptotic or proinflammatory functions (Supplementary Table II, Supplementary Figure IIB). TNF-α also induced antiapoptotic molecules, but the majority of them (60%) were not influenced by CT536706 (Table and data not shown). In summary, our findings suggest that JNK regulates proinflammatory, proapoptotic, and other physiological processes in both quiescent and TNF-α–treated EC.

The microarray study was validated by independent quantitative reverse-transcription polymerase chain reaction experiments that confirmed that pharmacological inhibition of JNK leads to reduced basal expression of molecules that positively regulate apoptosis, eg, caspase-3, DKK1 (Table and Figure 1A), proinflammatory activation, eg, vascular cell adhesion molecule-1, E-selectin (Table, Supplementary Figure III), or both of these processes, eg, Toll-like receptor (TLR) 4, RIP1 (Table, Figure 1A). We focused on a subset of proapoptotic molecules and demonstrated by Western blotting that pharmacological inhibition of JNK reduced the expression of RIP1 and procaspase-3 proteins in HUVEC (Figure 1B). Further validation was obtained using JNK1-specific siRNA, which suppressed the expression of JNK1 but did not influence JNK2 (Figure 1C). Silencing of JNK1 significantly reduced the expression of RIP1 and caspase-3, whereas a scrambled, nontargeting control had no effect (Figure 1C). Silencing of the downstream transcription factor

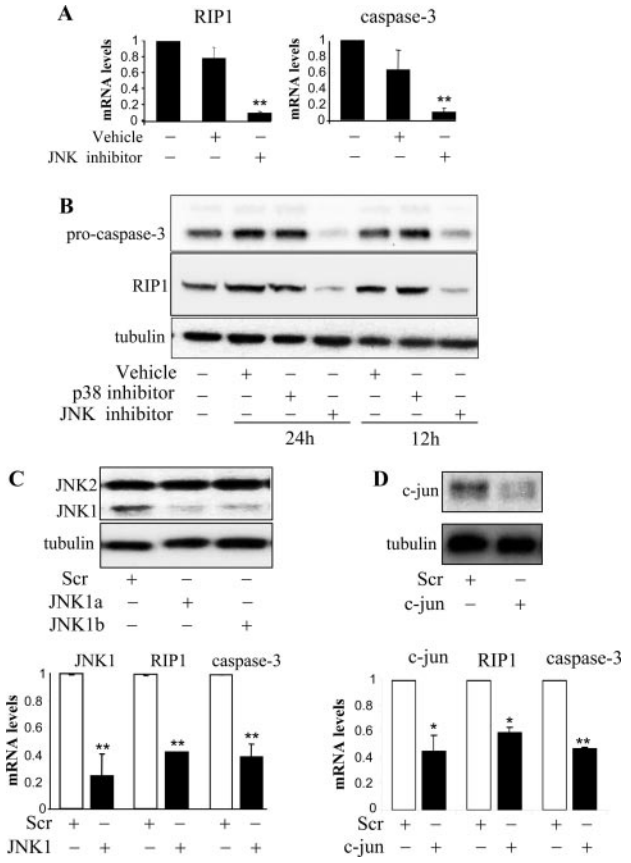

Figure 1. JNK regulates the constitutive expression of proapoptotic molecules in EC. HUVEC were exposed to CT536706 (1 μmol/L) for 5 hours (A) or for 12 to 24 hours (B), or were treated with vehicle alone or remained untreated. A, Transcript levels were quantified by real-time polymerase chain reaction. Mean values (±SD) were calculated from data pooled from 3 independent experiments. B, Procaspase-3, RIP1, and tubulin levels were assessed by Western blotting. Representative of 2 independent experiments. C and D, HUVEC were treated with JNK1-specific siRNA (JNK1a or JNK1b), c-jun–specific siRNA, or with a scrambled nontargeting sequence (Scr) and were incubated for 24 hours. JNK1, JNK2, c-Jun, and tubulin levels were determined by Western blotting. Transcript levels were quantified by real-time polymerase chain reaction. Mean values (±SD) were calculated from data pooled from 3 (C) or 2 (D) independent experiments.

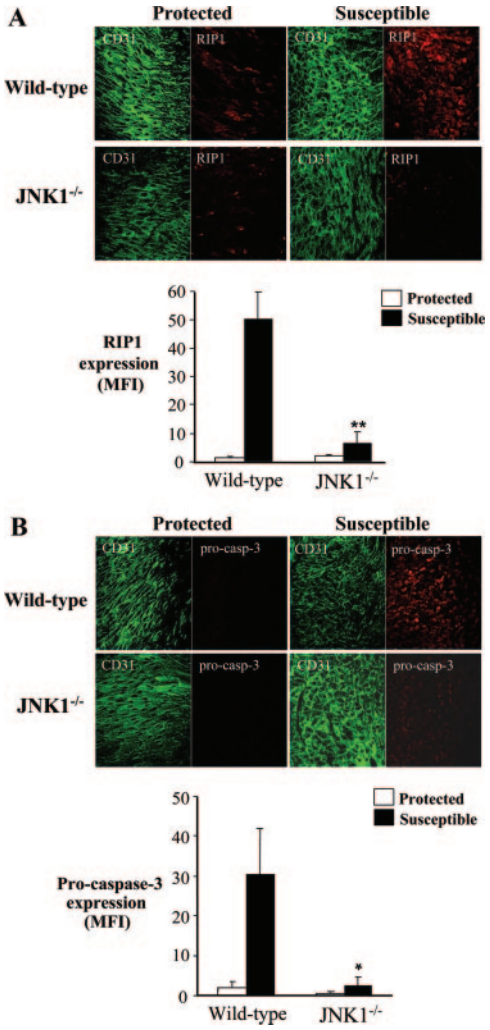

Figure 2. JNK1 positively regulates RIP1 and procaspase-3 expression in EC at an atherosusceptible site. RIP1 (A) and procaspase-3 (B) expression levels were assessed by en face staining of susceptible or protected regions of the aorta in wild-type or JNK1$^{-/-}$ mice (red; n=3 per group). Endothelial marker is CD31 (green). Representative images and quantitation of RIP1 (A) or procaspase-3 (B) expression (mean±SD) are shown. MFI indicates mean fluorescence intensity.

c-jun also led to a significant reduction in RIP1 and caspase-3 levels (Figure 1D). Thus, we conclude that JNK-c-Jun signaling positively regulates the expression of proapoptotic molecules in cultured EC.

JNK1 Regulates the Expression of Proapoptotic Molecules in the Arterial Tree

We previously demonstrated that levels of phosphorylated JNK are higher in EC at an atherosusceptible site compared to an atheroprotected site,[48] whereas endothelial expression of total JNK1 is similar in these 2 regions (data not shown). We concluded that JNK is preferentially activated at the atherosusceptible region and therefore predicted that JNK-dependent proapoptotic signaling molecules will be expressed preferentially at this site. Consistent with this hypothesis, en face staining of wild-type mice revealed that EC in the susceptible site express higher levels of RIP1 (Figure 2A) and procaspase-3 (Figure 2B) compared to EC in the protected site. Genetic deletion of JNK1 reduced RIP1 (Figure 2A) and procaspase-3 (Figure 2B) levels in the susceptible site, indicating that JNK1 is essential for their expression in this region.

JNK1 Delineates the Spatial Distribution of Caspase-3 Activation in the Arterial Tree

We examined whether JNK1 regulates caspase-3 activation at the susceptible site by studying EC in aortae of wild-type, JNK1$^{-/-}$, and MKP-1$^{-/-}$ mice. The cleaved, activated form of caspase-3 was barely detected in untreated wild-type mice but could be induced at the susceptible site by LPS treatment (Figure 3, compare panels 2 and 4). Genetic deletion of JNK1 protected EC at the susceptible site from caspase-3 activation (Figure 3, compare panels 4 and 6), indicating that JNK1 is required for caspase-3 activation in EC at this region. Caspase-3 was not activated in EC at the protected site of

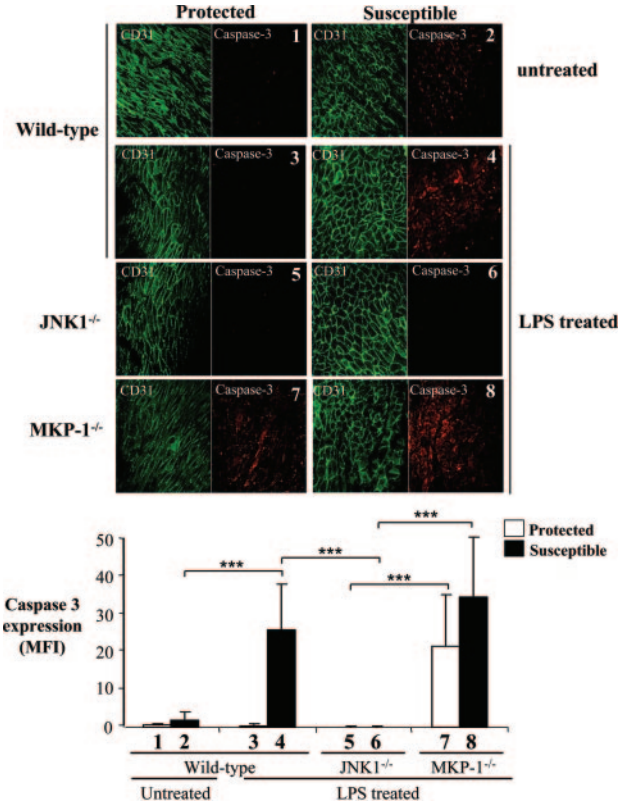

Figure 3. The spatial distribution of caspase-3 activation is regulated by JNK1 and MKP-1. Caspase-3 activation was measured by en face staining of susceptible or protected regions of the aorta in wild-type, JNK1$^{-/-}$, or MKP-1$^{-/-}$ mice that were either treated with LPS (intraperitoneal injection; 4 mg/kg for 6 hours) or remained untreated (red; n=3 per group). Endothelial marker is CD31 (green). Representative images and quantitation of active caspase-3 (mean±SD) are shown.

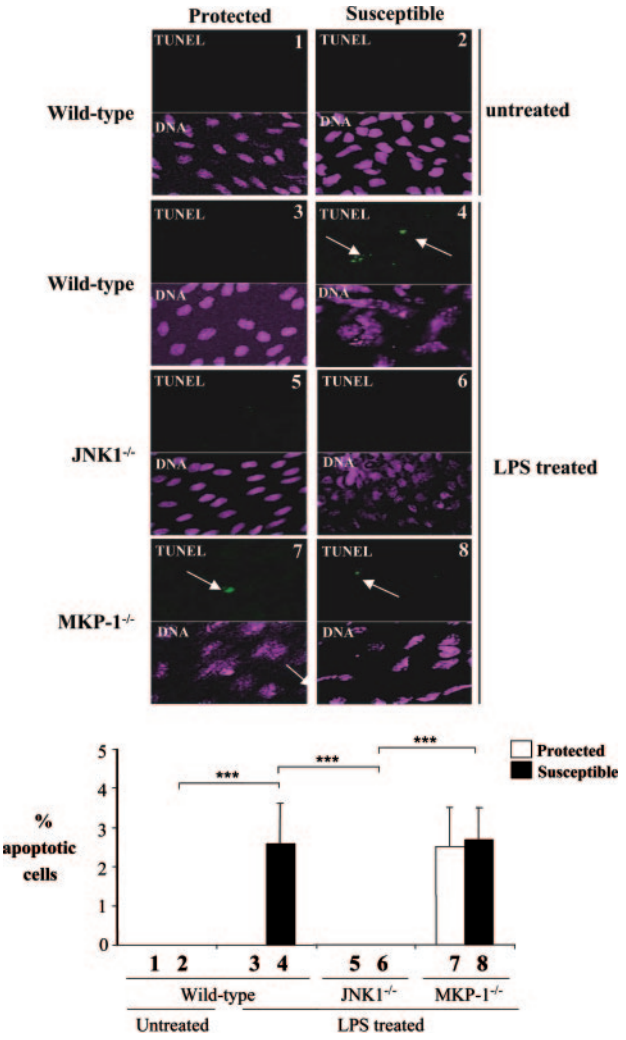

Figure 4. The spatial distribution of EC apoptosis is regulated by JNK1 and MKP-1. DNA fragmentation in EC was measured by TUNEL staining (green) of susceptible and protected regions of the aorta in wild-type, JNK1$^{-/-}$, or MKP-1$^{-/-}$ mice that were either treated with LPS (intraperitoneal injection; 4 mg/kg for 6 hours) or remained untreated (n=4 per group). EC were detected by counterstaining using *Griffonia* lectin-rhodamine (not shown), and nuclei were also counterstained (DNA, purple). Apoptotic EC, characterized by TUNEL staining colocalized with pyknotic or fragmented nuclei, were quantified in multiple fields of view. Representative images and the proportion of TUNEL-positive EC (mean±SD) are shown.

LPS-treated wild-type animals (Figure 3, panel 3). Because MKP-1 suppresses JNK activation at the protected site,[18] we examined whether it is required for the suppression of caspase-3 activation. Genetic deletion of MKP-1 enhanced caspase-3 activation in response to LPS at the protected site (Figure 3, compare panels 3 and 7), indicating a cytoprotective role for MKP-1.

JNK1 Activity Delineates the Spatial Distribution of EC Turnover in the Arterial Tree

EC apoptosis was assessed by detecting DNA fragmentation by TUNEL staining. To ensure that apoptotic cells were of endothelial origin, we performed 2 series of experiments in which EC were costained using either *Griffonia* lectin (Figure 4, Supplementary Figure IV) or anti-CD31 (Supplementary Figure V). TUNEL-positive nuclei were not detected in EC at the susceptible site in untreated wild-type mice but could be induced at this site by LPS (Supplementary Figure IV, Figure 4, Supplementary Figure V, compare panels 2 and 4). Genetic deletion of JNK1 suppressed DNA fragmentation in response to LPS (Figure 4, Supplementary Figure V, compare panels 4 and 6). These data suggest that although JNK1 regulates constitutive expression of proapoptotic proteins at the susceptible site, it is not sufficient to induce EC apoptosis. However, EC in this region are primed for apoptosis in response to LPS through a JNK1-dependent mechanism. In wild-type animals, EC at the protected site were resistant to the induction of apoptosis by LPS (Figure 4, Supplementary Figure V, panel 3). Genetic deletion of MKP-1 enhanced DNA fragmentation in response to LPS in EC at the protected site (Figure 4, compare panels 3 and 7), indicating that MKP-1 is required for the maintenance of EC viability in this region. Genetic deletion of MKP-1 did not influence apoptosis in the susceptible region, which is consistent with our previous observation that MKP1 is not expressed at this site.[18]

To examine whether apoptosis at the susceptible site is associated with EC proliferation, we measured the expression of Ki-67 protein (a marker of proliferation[7]) by en face staining. Ki-67–positive EC nuclei were detected at a low rate

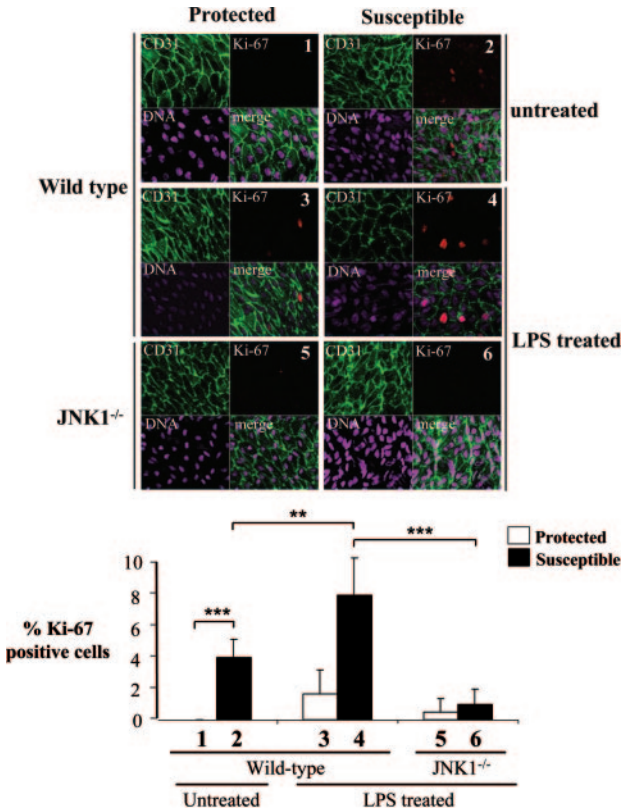

Figure 5. The spatial distribution of endothelial proliferation is regulated by JNK1. The expression of a marker of cell proliferation (Ki-67) was assessed by en face staining of susceptible or protected regions of the aorta in wild-type or JNK1$^{-/-}$ mice that were either treated with LPS (intraperitoneal injection; 4 mg/kg for 6 hours) or remained untreated (red; n=3 per group). Endothelial marker is CD31 (green). Nuclear were counterstained (DNA, purple). Representative images and the proportion of Ki-67–positive EC (mean±SD) are shown.

at the susceptible site in untreated mice but could be induced at this region by LPS (Figure 5, compare panels 2 and 4). By contrast, EC at the protected site did not express Ki-67 constitutively and were relatively resistant to Ki-67 induction by LPS (compare panels 3 and 4). Genetic deletion of JNK1 suppressed Ki-67 expression at the susceptible site (compare panels 4 and 6), indicating that JNK1 is required for EC proliferation at this region. Thus, we conclude that JNK1 activation enhances EC turnover at the susceptible site by positively regulating apoptosis and proliferation.

Discussion

The physiology of EC varies considerably according to their location in the arterial tree. EC at atherosusceptible sites are characterized by enhanced proinflammatory activation and higher rates of injury and turnover compared to EC at atheroprotected sites.[5–7] Thus, their phenotype enhances the accessibility of the vessel wall to leukocytes and lipoproteins, which are key drivers of atherogenesis. Injury of the vascular endothelium can arise through several processes, including apoptosis.[10] Here, we demonstrated for the first time to our knowledge that EC at atherosusceptible sites are primed for apoptosis in response to a noxious stimulus, whereas EC at atheroprotected sites are resistant. This principle was proven using a model noxious agent, LPS,[15] and it will be important in future studies to examine whether EC at atheroprone sites are primed for apoptosis in response to other proatherogenic molecules such as oxidized low-density lipoproteins[13] and during early atherogenesis.

We discovered that the molecular mechanism for priming of EC for apoptosis involves JNK1, which regulates the basal expression of proapoptotic molecules in EC and governs the spatial distribution of EC apoptosis in the arterial tree. A previous study by our group[18] demonstrated that JNK is activated constitutively in EC at atherosusceptible sites but is expressed in an inactive form at atheroprotected sites. Here, we used cell culture and in vivo models to examine whether JNK can influence proatherogenic processes in EC and potentially contribute to local atherosusceptibility. The function of JNK was investigated in cultured EC in the presence or absence of a specific pharmacological inhibitor using a genomics platform followed by functional annotation. This approach and subsequent validation by gene silencing confirmed previous reports that JNK positively regulates the induction of proinflammatory transcripts,[26,27] which influence atherosclerosis by regulating the recruitment of leukocytes to the vessel wall. Moreover, analysis of wild-type and JNK1$^{-/-}$ mice revealed that JNK1 plays an essential role in vascular cell adhesion molecule-1 expression in EC in vivo (data not shown). We also demonstrated for the first time to our knowledge that JNK positively regulates endothelial expression of TLR4, bone morphogenetic protein (BMP) 4, and RIP1, which are molecules known to trigger dual proinflammatory and proapoptotic/necrotic signaling pathways.[28–30] TLR4, a component of the innate immune system, has been implicated in atherosclerosis because its expression in EC is enhanced in atherosclerotic lesions,[31] a polymorphism that attenuates TLR4 activity is associated with a decreased risk of atherosclerosis in humans,[32] and TLR4 signaling in EC can be activated by oxidized low-density lipoprotein,[33] which is known to accumulate in plaques. BMP4 may also contribute to EC activation and apoptosis during atherogenesis because its expression in EC is greatly increased at atherosusceptible sites compared to protected sites.[28] In addition, RIP1 may influence numerous physiological activities in EC, including proliferation, activation, and viability by signaling downstream from TNF receptors.[29,34] Our finding that JNK regulates the expression of numerous proapoptotic molecules in EC is consistent with previous observations that JNK1 can activate proapoptotic signaling pathways[35,36] and induce proapoptotic transcripts by activating the transcription factor c-Jun.[37,38]

En face staining of the murine aorta revealed that JNK regulates the basal expression of procaspase-3 and RIP1 at an atherosusceptible site. Although proapoptotic proteins were expressed at enhanced levels at susceptible sites, EC apoptosis was not detected in untreated animals. However, EC apoptosis was induced at the susceptible site in wild-type animals by LPS through a JNK1-dependent mechanism. Under these conditions, EC apoptosis occurred in ≈3% EC at the susceptible site, a rate that is consistent with previous studies of EC viability in LPS-treated rodents.[6] We suggest that JNK1 primes EC in the susceptible region for apoptosis

by elevating expression of proapoptotic signaling molecules such as procaspase-3 and RIP1, which can be subsequently activated by noxious stimuli. Although this is the first evidence to our knowledge that EC can be primed for apoptosis by elevated expression of proapoptotic proteins, an analogous mechanism has been described for the induction of apoptosis in T lymphocytes in response to T-cell receptor signaling, which upregulates procaspase-3 at a transcriptional level.[39] In addition to the induction of proapoptotic transcripts, JNK1 can regulate apoptosis by alternative mechanisms, including cleavage of Bid (a member of the Bcl2 family) into jBid, which alters mitochondrial permeability to release the proapoptotic factor Smac/Diablo, which activates the initiator caspase-8,[35] and by activation of the E3 ubiquitin ligase Itch, which ubiquitinates the cytoprotective protein c-FLIP, leading to its degradation.[36] Therefore, it is plausible that JNK1 regulates EC apoptosis at susceptible sites through mechanisms that operate at both transcriptional and nontranscriptional levels.

Our study also revealed that EC at atherosusceptible sites were primed for proliferation in response to LPS via a JNK1-dependent mechanism. Although it is plausible that JNK1 influences EC proliferation directly, a previous study[40] suggested that JNK is a negative regulator of EC division. Instead, we favor the possibility that JNK1 influences proliferation indirectly by driving EC apoptosis, which in turn causes neighboring EC to proliferate because of loss of EC–EC contact inhibition.[41] Thus, increased EC proliferation may compensate for EC loss attributable to apoptosis and provide an important repair mechanism at atherosusceptible sites. Nevertheless, EC turnover is likely to influence atherosclerosis by causing transient, local loss of EC, which may increase the accessibility of the artery wall to lipoproteins and inflammatory cells.

We observed that EC at the atheroprotected site were resistant to the induction of apoptosis and proliferation by LPS. The underlying mechanism is likely to involve blood flow, which varies in magnitude and direction according to vascular anatomy and alters the physiology of endothelial cells. The application of unidirectional laminar flow (mimicking atheroprotective conditions) can suppress JNK activity, apoptosis, and proliferation in cultured EC,[42–47] whereas the application of disturbed flow (mimicking atheroprone conditions) can have the opposite effects.[8,9,48] Here, we demonstrate that suppression of EC apoptosis at the atheroprotected site relies on MKP-1, a negative regulator of JNK that is induced by unidirectional laminar flow in cultured EC and is preferentially expressed by EC in the atheroprotected region.[18] Thus, our novel findings suggest that local hemodynamics maintain EC viability and reduce EC turnover at atheroprotected sites by inducing persistent expression of MKP-1, which inactivates JNK and suppresses proapoptotic signaling pathways.

A recent study revealed that genetic deletion of JNK2, but not JNK1, suppressed uptake of modified low-density lipoprotein by macrophages and reduced lesion development after exposure of apolipoprotein $E^{-/-}$ mice to a high-fat diet.[49] This latter study does not, however, preclude a role for JNK1 in endothelial injury during the initiation of atherosclerosis, because this phase of the disease was not studied. In addition, any protective effects of JNK1 genetic deletion may have been overwhelmed by the high atherogenic drive in the apolipoprotein $E^{-/-}$ model. Thus, we suggest that further studies are required to examine the potential role of JNK1 in endothelial injury, lipid deposition, and vascular inflammation during early atherogenesis.

Conclusion

In summary, differential activation of JNK1 delineates the spatial variation in proapoptotic gene expression, apoptosis, and turnover in arterial EC, and may govern the spatial distribution of atherosclerotic plaques. Our conclusion has implications for the therapeutic targeting of JNK1 to suppress vascular injury.

Acknowledgments

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Correction

In the article, "c-Jun N-terminal Kinase Primes Endothelial Cells at Atheroprone Sites for Apoptosis" by Chaudhury et al, which appeared in the March 2010 issue of the journal (*Arterioscler Thromb Vasc Biol*. 2010;30:546–553; DOI: 10.1161/ATVBAHA.109.201368), the following Acknowledgment was missing from the article: The work was funded by the British Heart Foundation and the National Heart and Lung Institute Foundation.

The online version has been corrected.

The authors regret the error.

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***Arterioscler Thromb Vasc Biol* is available at http://atvb.ahajournals.org**

SUPPLEMENT MATERIAL

LEGENDS FOR SUPPLEMENTARY TABLES AND FIGURES

Supplementary Table I

Gene-specific primer sequences for quantitative real-time PCR.

Supplementary Table II

Biological classification of differentially expressed genes regulated by JNK in untreated (basal state) or TNFα-treated EC.

Supplementary Figure I

CT536706 suppresses c-Jun phosphorylation. HUVEC were treated with CT536706 (JNK inhibitor; 1 μM for 1 hour), with vehicle alone or remained untreated, and then stimulated with TNFα (10 ng/ml for 15 minutes) or remained untreated. Levels of phosphorylated c-Jun and total c-Jun were determined by ELISA. Mean ratios (+/- SD) of phosphorylated c-Jun/total c-Jun calculated from two independent experiments are shown.

Supplementary Figure II

Heat maps depicting the effects of CT536706 on the endothelial transcriptome. HUVEC were treated with CT536706 (JNK inhibitor; 1 μM for 5 hours), with vehicle alone or remained untreated. Cultures were co-stimulated with TNFα (10 ng/ml) for the final 4 hours or remained untreated. Each experimental condition was performed in triplicate. Transcript levels in each sample were assessed at a genomic level using Affymetrix® microarrays and analysed using

Rosetta-resolver® and DAVID software. Pro-inflammatory and pro-apoptotic transcripts that were positively regulated by JNK, either in the basal state (A) or in response to TNFα-treatment (B), were identified. Their patterns of expression are superimposed on heat maps where green indicates suppression by the JNK inhibitor and red indicates enhancement.

Supplementary Figure III

JNK positively regulates constitutive and TNFα-inducible expression of pro-inflammatory molecules in EC. HUVEC were treated with CT536706 (JNK inhibitor; 1 μM for 5 hours), with vehicle alone or remained untreated. Cultures were co-stimulated with TNFα (10 ng/ml) for the final 4 hours or remained untreated. Levels of pro-apoptotic (A), anti-apoptotic (B) or pro-inflammatory (C) transcripts were quantified by real-time PCR. Mean values (+/- SD) calculated from three independent experiments are shown.

Supplementary Figure IV

Multiple optical slices revealed TUNEL positive EC in the susceptible region of the aorta of LPS-treated mice.

EC apoptosis was assessed by TUNEL staining (green) at a susceptible region of the aorta in wild-type mice treated with LPS (4 mg/kg for 6 hours; intraperitoneal injection). Endothelial marker (lectin); nuclear counterstain (DNA). Images are shown that were generated from a single site within the susceptible region of an LPS treated wild-type mouse. A single confocal slice is shown (top left). Multiple optical slices were generated at different points along the z-axis from the luminal side (image 1) towards the media (z-stack; lower panels). Z-stacks were compiled and are shown in relation to the x- or y-axes (top right). Apoptosis was characterised by TUNEL-

positive pyknotic or fragmented nuclei (arrows).

Supplementary Figure V

The spatial distribution of EC apoptosis is regulated by JNK1.

DNA fragmentation in EC was measured by TUNEL staining (green) of susceptible and protected regions of the aorta in wild-type or JNK1$^{-/-}$ mice that were either treated with LPS (intraperitoneal injection; 4 mg/kg for 6 hours) or remained untreated (n=4 per group). EC were detected by counterstaining using anti-CD31 conjugated to PE (red) and nuclei were also counterstained (DNA, purple). Apoptotic EC, characterized by TUNEL staining, were quantified in multiple fields of view. Representative images and the proportion of TUNEL-positive EC (mean +/- SD) are shown.

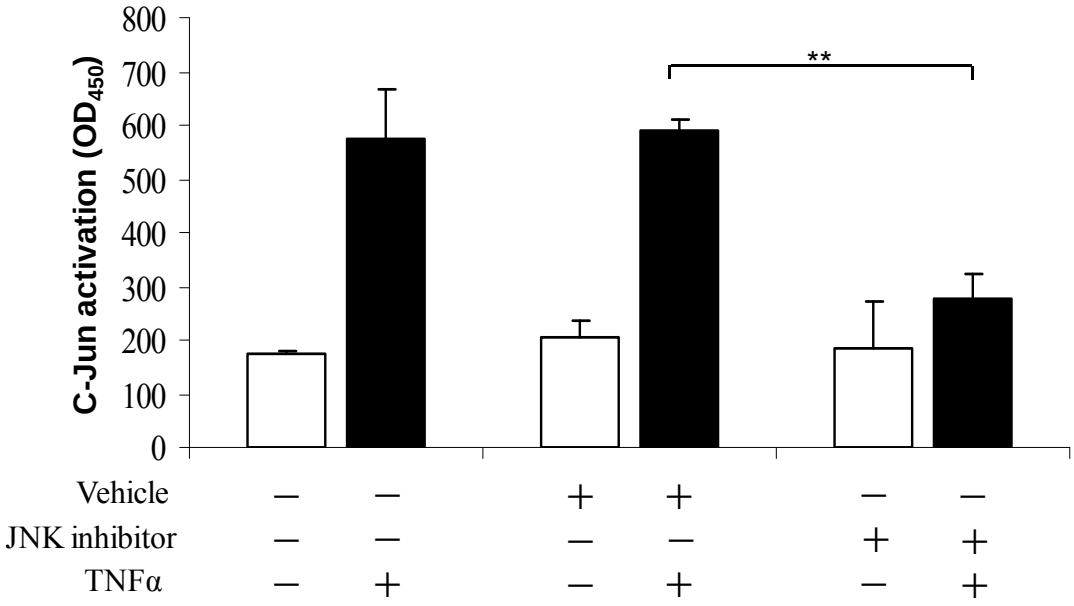
Supplementary Table I

Gene	Forward	Reverse
VCAM-1	5’-GGTGGGACACAAATAAGGGTTTTGG-3’	5’-CTTGCAATTCTTTTACAGCCTGCC-3’
E-selectin	5’-GCTCTGCAGCTCGGACAT-3’	5’-GAAAGTCCAGCTACCAAGGGAAT-3’
IL-8	5’-TGCCAAGGAGTGCTAAAG-3’	5’-CTCCACAACCCTCTGCAC-3’
TLR4	5’-AGACTTTATTCCCGGTGTGG-3’	5’-AAAGATACACCAGCGGCTCT-3’
CARD6	5’-CAGGCTCTAATGCCAATTCA-3’	5’-TGGAAAGAGCCAGGAGTTCT-3’
RIP1	5’-ATATCCCAGTGCCTGAGACC-3’	5’-AGATTCATCTGTTGGTGGCA-3’
BMP2	5’-GGTTCCTAAGGAGGACGACA-3’	5’-CCAAGTGCTGACACACAACA-3’
BMP4	5’-GGCTAGCCATTGAGGTGAACT-3’	5’-TCCCACTCCCTTGAGGTAAC-3’
DKK1	5’-TTCTGAGATGATGGCTCTGG-3’	5’-TGAGAACCGAGTTCAAGGTG-3’
Caspase-3	5’-TAAATGAATGGGCTGAGCTG-3’	5’-ATGGAGAAATGGGCTGTAGG-3’
Mst-1	5’-CCTTTCAGCATTGTTCTAGGTG-3’	5’-AAGACCAGGAATCACTAAATCTGTC-3’
PP2A	5’-CTCGTGGACCATGTATACGC-3’	5’-GGCCCACTCTGTACCAAACT-3’
DPF2	5’-CCCTTCTTCCTCCTCTCCTT-3’	5’-GTTGCTCCTTTCTCCCTGAG-3’
JAZ	5’-CATCCACCAGGTGATTTCTG-3’	5’-GTTGCTCCTTTCTCCCTGAG-3’
GADD45β	5’-GTCGGCCAAGTTGATGAATGT-3’	5’-GGATTTGCAGGGCGATGT-3’
A1	5’-CACAGGAGAATGGATAAGGCAAA-3’	5’-AGTCATCCAGCCAGATTTAGGTTC-3’
JNK1	5’-GAAGCTCCACCACCAAAGAT-3’	5’-GGTTCTCTCCTCCAAGTCCA-3’
β-actin	5’-CTGGAACGGTGAAGGTGACA-3’	5’-AAGGGACTTCCTGTAACAATGCA-3’

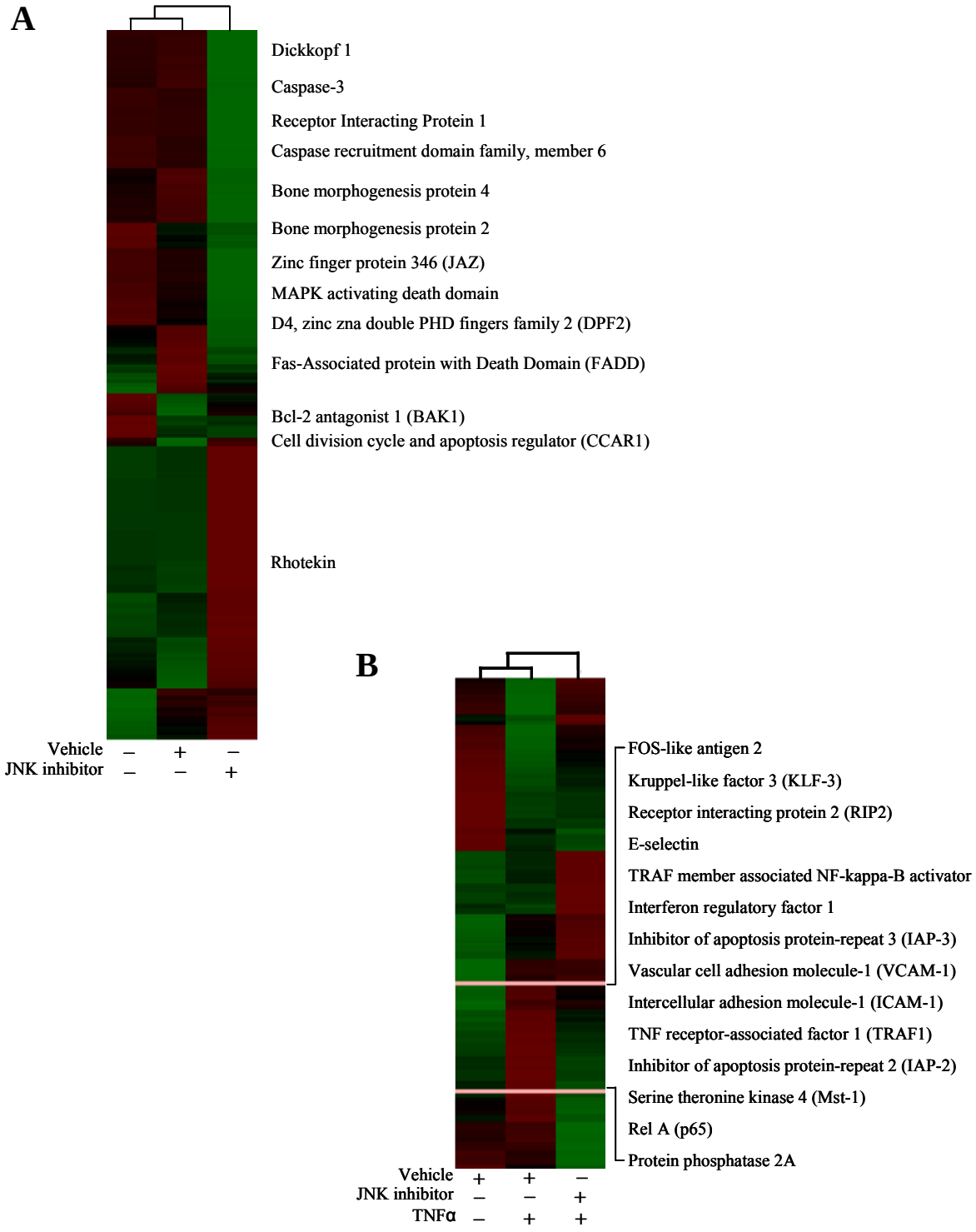
Supplementary Table II

Classification	Basal state				TNFα-induced genes			
	Total number	JNK-dependent	%	p-value	Total number	JNK-dependent	%	p-value
Apoptosis	483	79	16	<0.001	27	17	63	<0.008
Inflammation	1428	40	3	n.s	34	29	85	<0.001
Transcriptional regulation	791	207	26	<0.001	17	14	82	<0.001
Post-translational modification	713	132	19	<0.001	0	0	0	n.s
Proliferation	1299	95	7	n.s	0	0	0	n.s
Signal transduction	2459	217	9	<0.001	21	18	86	<0.001
Cytoskeleton	1755	165	9	<0.001	0	0	0	n.s
Immune response	1007	39	4	n.s	0	0	0	n.s
Oxidative mechanism	1769	45	3	n.s	0	0	0	n.s
NFκB signaling	119	34	29	<0.001	4	3	75	<0.001
Cell adhesion	2082	11	1	n.s	4	3	75	<0.001

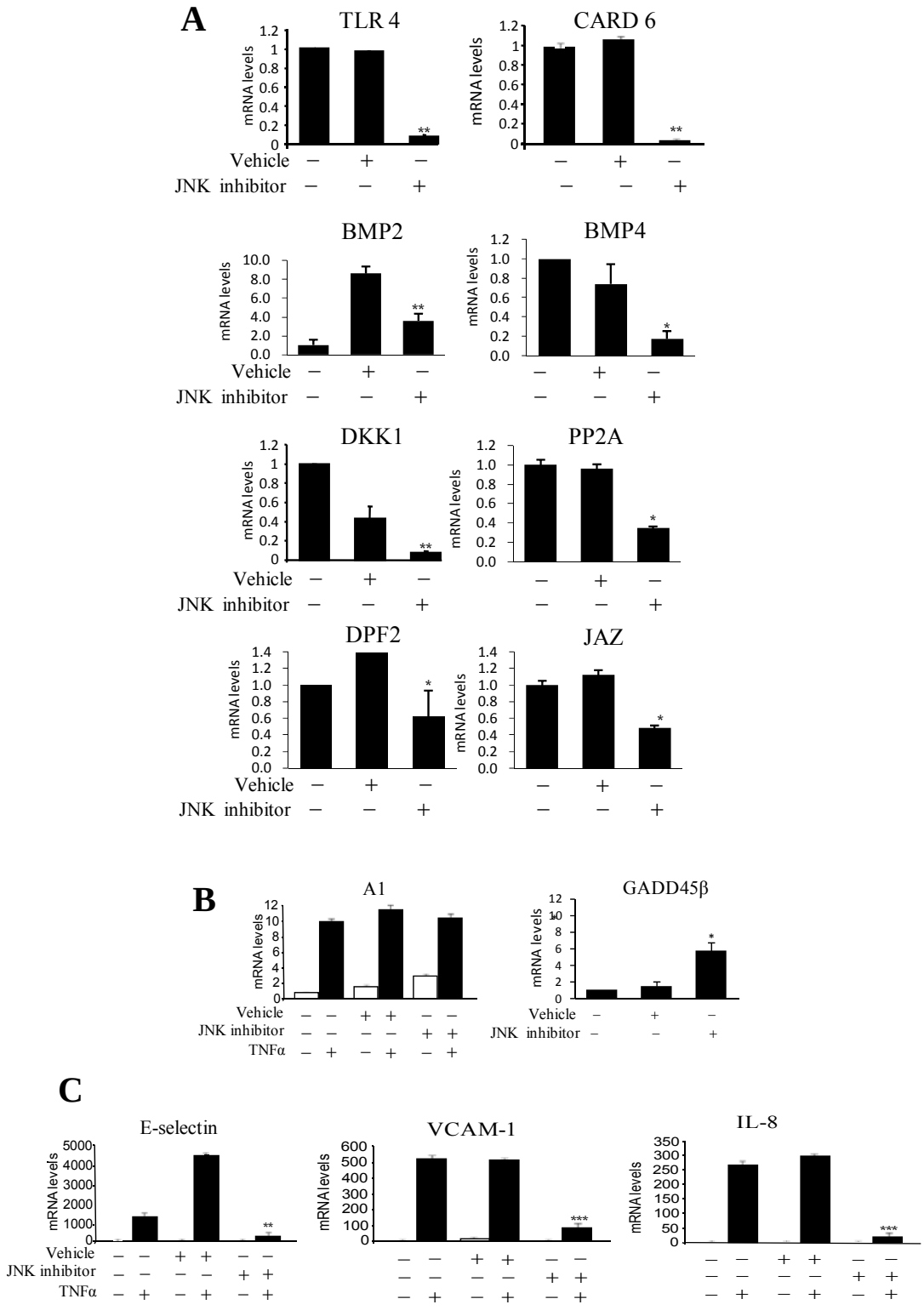
Supplementary Figure I



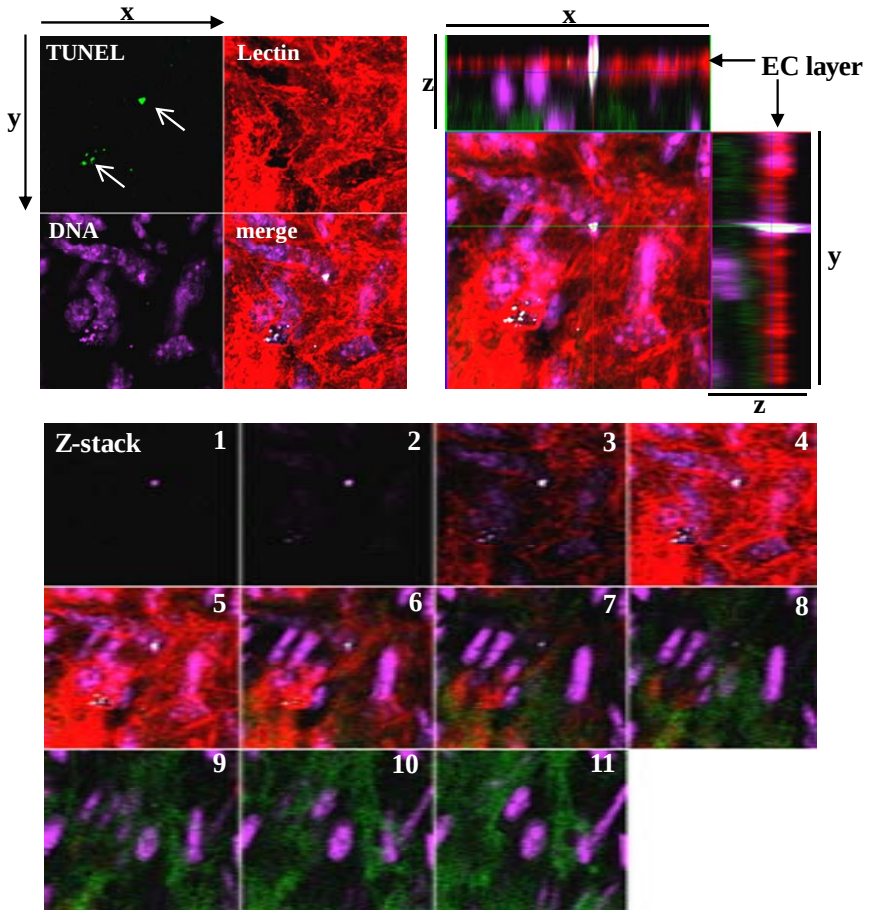
Supplementary Figure II



Supplementary Figure III



Supplementary Figure IV



Supplementary Figure V