Human IL-8 Regulates Smooth Muscle Cell VCAM-1 Expression in Response to Endothelial Cells Exposed to Atheroprone Flow

Nicole E. Hastings, Ryan E. Feaver, Monica Y. Lee, Brian R. Wamhoff, Brett R. Blackman

Objective—Interleukin-8 (IL-8) is a soluble human-specific chemokine implicated in the development of the chronic inflammatory disease atherosclerosis. Recently, we showed that atheroprone hemodynamics induced IL-8 secretion from endothelial cells (ECs) concurrent with increased EC/smooth muscle cell (SMC) VCAM-1 expression in a human hemodynamic coculture model. Despite an IL-8 association with inflammation, we show here that blocking IL-8 activity during atheroprone flow resulted in increased levels of EC/SMC VCAM-1 expression. We tested the hypothesis that IL-8 limits SMC VCAM-1 expression in response to inflammatory stimuli, either atheroprone flow or cytokine interleukin-1β (IL-1β) addition.

Methods and Results—Atheroprone flow increased monocyte adhesion in both EC/SMCs, concurrent with the induction of VCAM-1 protein. VCAM-1 antisera attenuated this response. IL-1β upregulated VCAM-1 in SMCs by 3-fold, a response inhibited by the addition of IL-8 at 24 hours. Neither IL-1β nor IL-8 induced proliferation or migration. Neutralization of the IL-8 receptor, CXCR2, further induced VCAM-1 in the presence of IL-1β, and phospho-p38 was required for NF-κB activation and VCAM-1 expression. Additionally, IL-8 reduced p38 activation and NF-κB activity induced by IL-1β alone.

Conclusions—Together, these findings provide evidence for a novel role whereby IL-8 limits the inflammatory response in ECs/SMCs via VCAM-1 modulation. (Arterioscler Thromb Vasc Biol. 2009;29:725-731.)

Key Words: endothelial smooth muscle interleukin-8 VCAM-1 hemodynamics

Initiating phases of atherosclerosis in large arteries are marked by chronic inflammation and are localized to hemodynamically defined geometries where blood flow is both compromised and disturbed. Chemokines and cytokines are locally secreted in “atheroprone” regions and are known to play important roles in driving the inflammatory response (for reviews see Tedgui and Mallat1 and von der Thüsen et al2). Endothelial cells (ECs) and smooth muscle cells (SMCs) can both contribute and respond to cytokine production, thus promoting atherosclerosis. Recently, we demonstrated using a novel in vitro EC/SMC coculture hemodynamic flow system that the human chemokine interleukin-8 (IL-8/CXCL8) is secreted at higher levels by ECs exposed to an atheroprone flow environment, compared to atheroprotective flow.3 Also, atheroprone flow caused ECs/SMCs to undergo inflammatory priming, whereby the adhesion molecule, vascular cell adhesion molecule-1 (VCAM-1), was upregulated in both cell types compared to atheroprotective flow.

VCAM-1 expression, often resulting from NF-κB signaling, is observed in intimal SMCs in humans, in vivo animal models and cultured SMCs by inflammatory cytokines, including IL-1β.4-10 A recent study examined VCAM-1 expression in mouse intimal cells in the lesser curvature (ie, atheroprone) of the aortic arch and determined that VCAM-1 levels were significantly higher compared to the greater curvature (ie, atheroprotective) region.11 Cai et al showed leukocytes bound to SMCs were more antiapoptotic attributable in part to SMC VCAM-1 expression.12 SMC VCAM-1 expression may be important for trapping monocytes within a developing lesion.13,14 Furthermore, the inflammatory SMC phenotype is differentially regulated by specific collagen matrix components.11,15 and SMC VCAM-1 expression has been linked to proliferation16 and migration.17

IL-8 is an ELR (glutamic acid-leucine-arginine) CXC chemokine, expressed in humans, but not rodents, and is known to activate neutrophils.18 IL-8 is implicated in atherosclerosis via leukocyte attraction and induction of firm adhesions of monocytes to endothelium localized to developing lesions.19-21 Production of IL-8 in ECs occurs under inflammatory conditions, including stimulation with potent
inflammatory mediators such as IL-1β, TNF-α, and exposure to “atheroprone” shear stresses. IL-8 induction is associated with proinflammatory stimuli and disease processes in vivo; however, direct localized inflammatory effects of this molecule on the vasculature are largely unexplored. Because rodents do not express IL-8, further understanding its role in atherosclerosis has been challenging.

We currently demonstrate that blocking IL-8 activity in our hemodynamic coculture system further enhances human EC/SMC VCAM-1 protein during atheroprone flow and that IL-1β-induced VCAM-1 in monocultured SMCs can be inhibited by costimulation with IL-8. Contrary to our hypothesis that IL-8 has proinflammatory effects on the vasculature, we show herein that IL-8 limits SMC VCAM-1 expression in response to inflammatory stimuli by reducing activation of p38 and NF-κB, and thereby decreasing VCAM-1 expression and suppressing monocyte adhesion.

Methods
Please see http://atvb.ahajournals.org for expanded Methods section.

Human Cell Culture and Hemodynamic Flow Model
Human EC/SMC cell culture and coculture plating conditions were used for flow experiments and performed as previously described. The two hemodynamic flow patterns were run in parallel for each EC/SMC subpopulation, or identical flow patterns were run in parallel in the presence or absence of a treatment condition.

IL-8 Inhibition During Flow
Cocultured ECs/SMCs were incubated with IL-8 antisera (1:300) raised in rabbit, donated by R.M. Strieter, MD (University of Virginia, Charlottesville) or with normal rabbit serum control, 10 minutes before the onset of atheroprone or atheroprotective flow and throughout the duration of the experiment. IL-8 siRNA (ON-TARGETplus SMARTpool, Thermo Scientific Dharmacon) was optimized in HUVECs (EC-siIL8) using Oligofectamine Reagent (Invitrogen) and Optimem Media.

Results
Human Coronary Arteries Express VCAM-1 and CXCR2 in All Vessel Layers
Whereas previous literature established the general presence of SMC VCAM-1 expression in human atherosclerotic tissue, we wanted to focus on the correlation between coexpression of VCAM-1 and SMCs in the neointima and medial layers as a function of intimal thickness. Cross-sections of human coronary arteries (n=10 patient samples) were analyzed for VCAM-1 expression in ECs and in intimal/medial layers. Distinct VCAM-1 expression was observed in ECs and intimal and medial regions (Figure 1A and 1B). In general, VCAM-1 was regionally heterogeneous across the tissue and each vascular cell type. In regions where SMCs expressed higher levels of VCAM-1, intimal thickening was present. Similarly, when the intima remained thin, VCAM-1 was not expressed in the deeper layers of the vessel wall. Smooth muscle α-actin (SMαA) staining demonstrated that SMCs were present in the intima, in addition to the media and this SMαA expression colocalized with VCAM-1 (Figure 1C). To understand how IL-8 signaling may be influencing VCAM-1, expression of CXCR2, a receptor which binds IL-8, was also identified in ECs and intima/media layers of the vessel wall (Figure 1D); however, no correlation between intimal thickness and CXCR2 expression patterns was observed. See supplemental figure for additional images (supplemental Figures I through IV). Data suggest that local secretion and signaling of IL-8, rather than differential CXCR2 expression, may regulate the differential VCAM-1 expression.

Atheroprone Hemodynamics Cause Increased Monocyte Binding to ECs/SMCs
Our previous work showed that atheroprone flow induced the expression of VCAM-1 in both ECs and SMCs, relative to atheroprotective flow. VCAM-1 is known to play a role in monocyte adhesion. To demonstrate a functional consequence of this response, monocyte adhesion studies were performed after exposure of the EC/SMC coculture to 24 hours of flow. After cessation of shear stress, labeled MM6 monocytes were seeded on to the EC or SMC layer and examined for number of bound monocytes. Atheroprone flow exhibited 2-fold greater monocyte adhesion to ECs and SMCs compared to atheroprotective flow (Figure 2A and 2B), a response attenuated on incubation with a VCAM-1 blocking antibody (Figure 2B). Although it is possible that cessation of
flow caused VCAM-1 degradation and thus reduced monocyte adhesion, it is unlikely since the half-life of VCAM-1 is on the order of hours.33–35 This is the first model to directly compare this functional consequence after atheroprone and atheroprotective flow on cocultured ECs/SMCs.

Blocking Interleukin-8 During Atheroprone Flow Enhances SMC VCAM-1 Expression

Previous work further established greater amounts of EC-secreted IL-8 at 24 hours of atheroprone relative to atheroprotective flow.3 To test whether atheroprone flow–induced secretion of IL-8 is regulating vascular cell VCAM-1 expression, flow was applied to the coculture system for 24 hours in the presence of IL-8 antisera or control serum. The IL-8 antisera during atheroprone flow enhanced VCAM-1 mRNA by 2.86-fold (Figure 3A). Myocardin was reduced by 25% because of IL-8 antisera and atheroprone conditions, but SMαA was not significantly downregulated (Figure 3A). Myocardin is a critical transcription factor required for serum response factor (SRF)-dependent transcription of SMC differentiation markers containing CArG cis elements in their promoters.36 Changes in myocardin suggest that cell remodeling is still occurring at this time point. In contrast, no change was observed for SMC myocardin or VCAM-1 expression in response to atheroprotective flow with IL-8 antisera, though SMαA was downregulated (Figure 3A). The discrepancy between changes in SMαA and myocardin are unexpected, but could be attributable to other factors inhibiting or competing against myocardin binding to SRF, (ie, KLF-4, Elk-1). Interestingly, blocking IL-8 activity during atheroprone flow further enhanced VCAM-1 protein expression in both ECs and SMCs, indicating that IL-8 may be serving to limit the extent of inflammation (Figure 3B).

Interleukin-8 Limits the Interleukin-1β–Induced SMC Inflammatory Response

To understand how IL-8 might be influencing the SMC VCAM-1 expression, studies were performed with human SMC monocultures. Previous work demonstrated that IL-1β induced VCAM-1 expression in vascular SMCs14 and caused IL-8 secretion in airway SMCs.37 We examined the release profile of IL-1β–treated human vascular SMCs to establish the amount and timing of IL-8 intervention. ELISA analysis showed IL-8 secretion at levels 5.5-fold greater than control after 24 hours, with sustained levels at 48 hours (Figure 4A).

To determine the effect of IL-8 on cytokine stimulated SMCs, cells were treated with IL-1β, IL-8, or the combination to understand the temporal regulation of VCAM-1 expression. At the mRNA level, IL-1β caused significant VCAM-1 upregulation by 4 hours. At 24 hours, VCAM-1 mRNA returned to basal levels (Figure 4B). IL-8 alone did not induce VCAM-1 expression. Changes in myocardin and SMαA were also analyzed, where only myocardin was significantly reduced by 39% at 4 hours after IL-1β treatment (supplemental Figure V).

At the protein level, 4 hours of treatment with IL-1β and IL-1β/IL-8 induced VCAM-1 expression, by 2.33 and 2.16-fold, respectively; however, no significant differences were observed between the conditions (Figure 4C). The most dramatic response was observed after 24 hours of IL-1β/IL-8 treatment, which reduced VCAM-1 levels by 42.6% relative to IL-1β treatment alone (Figure 4C). By 48 hours, VCAM-1 expression returned to basal levels for all conditions. Analysis...
in ECs showed no change in VCAM-1 expression for the IL-1β/IL-8 condition compared to IL-1β alone after 24 hours at the same and higher (100 ng/mL) concentrations of IL-8, indicating SMC-specific responsiveness (Figure 4D). SMCs exposed to IL-1β increased monocyte adhesion, inhibited by the presence of exogenous IL-8 (Figure 4E), demonstrating a functional role for IL-8 to reduce monocyte adhesion to SMCs in the presence of inflammatory mediators.

**Interleukin-8 and Interleukin-1β Do Not Promote SMC Proliferation or Migration**

Because IL-8 has a role in resolving the SMC inflammatory response, proliferation and migration resulting from IL-8 combinatorial signaling were also investigated. Growth arrested SMCs were treated with 10%FBS, IL-1β, IL-8, or IL-1β/IL-8 for 24 hours. Cells were analyzed for proliferative responses via flow cytometry or for chemotactic responses via Boyden Chamber assays. Cells treated with 10% FBS both displayed strong induction of proliferation and migration at this time point; however, the effects of IL-1β, IL-8, or IL-1β/IL-8 were modest and remained insignificant, near control levels (Figure 4F and 4G).

**CXCR2 Signaling Promotes IL-8 Resolution of VCAM-1**

IL-8 binds to 2 G protein–coupled receptors, CXCR1 and CXCR2, both expressed by airway SMCs. We tested the hypothesis that blocking CXCR2 will potentiate the IL-1β–induced expression of VCAM-1 (ie, block the IL-1β/IL-8 reduction in VCAM-1). Blocking CXCR2 via neutralization abolished IL-8–induced ERK phosphorylation, an established response to IL-8 (Figure 5A). SMCs were treated with IL-1β and CXCR2 antibody for 24 or 48 hours. Blocking CXCR2 in the presence of IL-1β further enhanced VCAM-1 expression by 24 hours compared to IL-1β treatment alone (Figure 5B). This is in direct contrast to the response of IL-1β and IL-8, where VCAM-1 was reduced by 42.6% compared to IL-1β alone, as previously shown above in Figure 4C. Further, SMCs were treated with the CXCR2 antibody in the presence of IL-1β/IL-8. This combinatorial response no longer reduced VCAM-1 expression under these conditions, and instead caused a 1.76-fold induction from IL-1β (Figure 5C). We tested the specificity of the CXCR2 receptor for the observed responses using a different CXCR2-specific ligand, Gro-α. Of interest, the combination of Gro-α and IL-1β did not show a reduction in VCAM-1 expression by 24 hours (Figure 5D).

**IL-8 Attenuates IL-1β–Induced p38 and NF-κB Activation**

IL-1β upregulates VCAM-1 in part, because of activation of MAPK signaling molecule p38, shown previously in human tracheal SMCs. In the current monoculture system with vascular SMCs, p38 inhibition (SB202190) reduced VCAM-1 expression in response to IL-1β (Figure 5E). We hypothesized IL-8 may be blocking IL-1β–induced VCAM-1 expression by reducing p38 activation. Combined effects of IL-1β and IL-8 yielded a 25% reduction in phospho-p38, compared to IL-1β alone (Figure 5F), which is on the same order of reduction of VCAM-1 expression attributable to IL-1β/IL-8 treatment. Additionally, as IL-1β is known to activate the inflammatory transcription factor NF-κB, SMCs treated with IL-1β/IL-8 reduced Ad-NF-κB-luc activity after 9 hours, as did SB202190, consistent with the downstream changes in VCAM-1 (Figure 5G). Further, blocking CXCR2
with a neutralizing antibody in the presence of IL-1β/IL-8 inhibited the reduction in NF-κB activity as seen with the IL-1β/IL-8 reduced NF-κB activity.

**Knockdown of IL-8 in Endothelial Cells Enhances Atheroprone-Induced VCAM-1 Expression**

To determine whether EC-derived IL-8 caused atheroprone regulation of SMC VCAM-1 expression, EC expression of IL-8 was knocked down using IL-8 specific siRNA oligonucleotides. IL-8 siRNA was optimized at the mRNA and protein levels by treating transfected ECs with IL-1β and comparing to nonstimulated controls. Figure 6A shows a 65% reduction of IL-8 mRNA, and Figure 6B shows a 72% knockdown of secreted IL-8 in response to IL-1β stimulation for 4 hours compared to control.

Optimal EC-siIL8 conditions were used in the EC/SMC coculture model. EC-siIL8 exposed to atheroprone flow upregulated VCAM-1 1.6-fold and 2-fold in SMCs and ECs, respectively, compared to control (Figure 6C). Downregulation of IL-8 by ECs showed transfection efficiency maintained throughout the flow experiment, which was on average 65% knockdown of expression. EC-siIL8 did not significantly influence SMC IL-8 mRNA levels (P=0.35).

**Discussion**

During atherogenesis, EC/SMC cross-communication via secreted paracrine factors is proposed to promote the chronic inflammatory response localized to hemodynamically compromised regions of arteries. As shown previously, VCAM-1 is expressed in human SMCs in proximity to human ECs exposed to atheroprone flow and IL-8 was upregulated and secreted at higher levels by ECs exposed to atheroprone flow. The functional role of IL-8 in regulating vascular cell phenotype is unknown. A major challenge for the field is that the cytokine IL-8 is not expressed in rodents. Thus, we used a model that recalibrates human in vivo vascular cell phenotypes in vitro to unmask a novel role for IL-8 in regulating SMC phenotype. Herein, we show that IL-8 attenuates a proinflammatory SMC phenotype by reducing VCAM-1 expression via CXCR2, p38, and NF-κB mechanisms. We also demonstrate that atheroprone flow induces VCAM-1-dependent monocyte adhesion.

The proinflammatory phenotype promoted during atheroprone flow translated to observations of VCAM-1 in human coronary artery tissue. Strong expression of VCAM-1 was confined to intimal/medial regions of human coronary arteries, coinciding with intimal thickening in arteries of patients.
The current study is, to our knowledge, the only one showing distinct heterogeneous VCAM-1 expression in entire cross-sections of human intimal and medial layers, colocalizing with SMαA-positive cells.

To understand the role of IL-8 on VCAM-1 expression in SMCs, we performed monoculture experiments where cells were activated via IL-1β. IL-8 decreased IL-1β-induced VCAM-1 protein more quickly than when cells are treated with IL-1β alone. Further, VCAM-1 downregulation occurred via CXCR2 and reduced p38 activation. This is the first report of an IL-8 role in influencing adhesion molecule expression in any cell type. Unmasking the role of IL-8 in atherogenesis is experimentally challenging because mice do not express IL-8. Results herein support the hypothesis that upregulation of IL-8 serves, in part, to maintain overall lower levels of SMC VCAM-1 via reduced p38 and NF-κB activation, thus reducing monocyte adhesion. IL-1β is a well-established activator of NF-κB, and thus VCAM-1, in SMCs; however, it was previously unknown that IL-8 can reduce NF-κB activity when combined with an inflammatory stimulus. Others have reported that inhibition of p38 activation can reduce NF-κB activity in human tracheal SMCs.4,39 This response in our monoculture system further supports this mechanism by which IL-8 is acting to reduce VCAM-1 expression.

SMC phenotypic modulation in response to shear stress exposure on ECs has not been widely explored.5,40 One recent study analyzed SMC paracrine effects on EC adhesion molecule expression, where ECs presheared with high steady flow (12 dynes/cm²) for 24 hours were subsequently cocultured with SMCs after cessation of flow. Authors concluded E-selectin expression induced by culturing SMCs with ECs occurred via IL-6- and IL-1β-dependent mechanisms.40 Although these findings are interesting, this model used methods with potentially confounding factors, including a lack of physiological recapitulation, no serum withdrawal methods with potentially confounding factors, including a lack of physiological recapitulation, no serum withdrawal conditions for SMCs, and short time between plating and experimentation. On examination of IL-1β secretion by ELISA in our flow system (data not shown), we observed no detectable levels of the cytokine, which we suggest is attributable to a more quiescent SMC phenotype.

The effects of IL-8 on EC adhesion molecule expression in the context of inflammation were also examined. We observed similar results where blocking IL-8 activity by IL-8 antisera or attenuating its secretion (EC-siIL8) enhanced EC VCAM-1 during atheroprobe flow. This was interesting because the EC response to IL-1β/IL-8 treatments under static conditions had no effect in comparison, revealing the significance of studying EC/SMC communication in the presence of physiologically relevant hemodynamics. We anticipate similar mechanisms exist for EC/SMC responses to flow-induced inflammation, demonstrating that IL-8 may play a more important role than previously appreciated.

Interestingly, despite its association with inflammation, IL-8 protective effects have been described in several studies, specifically examining the response of IL-8 on neutrophil adhesion to ECs.22,24,42 IL-8 reduces neutrophil binding to IL-1β activated ECs, thus promoting antiinflammatory effects,41 and inhibition of neutrophil adhesion could be expected by an IL-8 role to reduce adhesion molecules on ECs. The cytokine IL-6, often upregulated simultaneously with IL-8, has been shown to be atheroprotective. IL-6+ /− and IL-6− /− mice crossed with ApoE +/− mice resulted in increased lesion formation, coinciding with increased macrophages and reduced SMCs.43 Increased serum levels of soluble VCAM-1 were detected in ApoE +/− /IL-6− /− . Clearly, the role of IL-8 may be dual in its capabilities of promoting and protecting against inflammation, and the current study further emphasizes the complexity of this chemokine.

The hemodynamic environment is known to prime ECs/SMCs toward an inflammatory phenotype, but we now understand a previously unknown compensatory mechanism involved in limiting inflammation via paracrine effects between these 2 cell types. We have described a novel role for IL-8 to limit IL-1β-induced VCAM-1 expression via reduction of p38 and NF-κB activation, without alteration of contractile, migratory, or proliferative phenotypes. We speculate IL-8 induction is one of many pathways that has evolved to maintain homeostasis in the blood vessel wall, and such pathways will continue to unfold as we further our understanding of human EC/SMC communication in systems that recreate critical components of the human vasculature, including EC/SMC spatial orientation and critical hemodynamic environments. In conclusion, the induction of IL-8 in response to atheroprobe hemodynamics may maintain lowered levels of VCAM-1, preventing further inflammation localized to the vessel wall during atherogenesis.

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Disclosures

None.

References


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SUPPLEMENTAL MATERIAL

MATERIALS AND METHODS

Immunofluorescent Labeling of Human Coronary Arteries

Coronary artery tissue was obtained from autopsy material that was destined to be disposed according to university health system policy. Tissue was taken from autopsy patients where the autopsy consent specifically approved use of tissue for research purposes. Details on each patient are reported in Supplemental Table I. Left anterior descending coronary arteries were excised up to 1 cm past bifurcation after prolonged fixation (1 week to 2 years). Arteries were fixed in 10% neutral buffered formalin, pH of 6.8 to 7.2. Tissue was then prepared for sectioning by dehydration, and was oriented for transverse sectioning in paraffin. Sections 5µm thick were cut and mounted onto slides. Samples were immunofluorescently labeled, as previously described. Samples were incubated with primary antibody for VCAM-1 (BBA19, R&D Systems, 1:100 and secondary AlexaFluor546 donkey anti-goat IgG (Molecular Probes, 1:300), CXCR2 (ab24963, Abcam, 10µg/mL) and secondary AlexaFluor546 goat anti-mouse IgG (Molecular Probes, 1:300), or SMαA (Clone 1A4-Cy3, Sigma, 1:500). Imaging was performed on a confocal microscope (Nikon Eclipse Microscope TE2000-E2 and Melles Griot Argon Ion Laser System no. 35-IMA-840). Montages of cross-sections were reconstructed using Adobe Photoshop software.

Human Cell Isolation and Culture Plating Conditions

Primary human ECs were isolated from umbilical cord veins, expanded and used as previously described. Human umbilical vein SMCs were purchased from Cell
Applications, Inc. at passage 2 and used until passage 10, as previously described.\textsuperscript{2} All tissue procurement was approved by the Human Investigation Committees of the University of Virginia and Martha Jefferson Hospital (#10486).

EC/SMC co-culture plating conditions were used for all flow experiments and performed as previously described.\textsuperscript{2} Briefly, porous transwell membranes are treated with 0.1% gelatin and SMCs are seeded onto an inverted well and grown to confluence for 48h in the transwell holding dish in reduced serum growth medium. ECs are then plated on the top surface of the membrane under the same media conditions for an additional 24 hours to ensure confluence. Hemodynamic flow patterns are derived from magnetic resonance imaging (MRI) of human common carotid artery and internal carotid sinus and applied to the EC surface of the dish to simulate atheroprotective and atheroprone shear stress patterns \textit{in vitro}, respectively.\textsuperscript{3} The two hemodynamic flow conditions were run in parallel for each EC/SMC subpopulation or identical flow conditions were used in parallel in the presence and absence of a treatment condition.

For monoculture SMC experiments, cells were plated in 12 well or 6 well plates at a density of 10,000 cells/cm\textsuperscript{2} in low serum containing media (M199 supplemented with 2% FBS, 2mM L-glutamine, and 100 U/ml penicillin-streptomycin). Twenty-four hours later, cells were washed twice with PBS and then serum starved for 48 hours prior to treatment.

\textbf{IL-8 Inhibition during Flow}

Co-cultured EC/SMCs were incubated with IL-8 antisera (1:300) raised in rabbit, kindly donated by R.M. Strieter, M.D. (University of Virginia) or with normal rabbit
serum 10 minutes prior to the onset of atheroprone or atheroprotective flow. Fresh media containing the IL-8 antisera or vehicle was perfused into the system throughout the flow experiment for 24h, and cells were isolated for mRNA or protein analysis.

Additionally, siRNA for IL-8 (ON-TARGETplus SMARTpool, Thermo Scientific Dharmacon) was optimized in HUVEC (EC-siIL8) using Oligofectamine Reagent (Invitrogen) and Optimem Media. ECs were plated at 60,000 cells/cm² in normal media overnight. Then cells were rinsed twice with PBS and treated with ON-TARGETplus™ Control Pool (EC-CP, Thermo Scientific Dharmacon) or EC-siIL8 oligonucleotides, Oligofectamine, and Optimem for five hours, after which an equal volume of HUVEC media with 20% FBS was added to the dish. For flow experiments, ECs were plated in two 100mm dishes at 60,000 cells/cm² for one day, then transfected with either EC-CP or EC-siIL8 (185 pmol) for one day. Twenty-four hours before the onset of atheroprone flow, transfected ECs were trypsinized and replated onto the inner surface of the transwell membranes. After one day of co-culturing, transfected ECs were exposed to atheroprone flow in parallel. SMC and EC genes were analyzed for modulation of VCAM-1 and IL-8 mRNA.

Monocyte Adhesion Assay

Atheroprone and atheroprotective flow patterns were applied to EC/SMC co-culture dishes for 24 hours, after which calcein AM (Molecular Probes) labeled human MM6 monocytes donated by C. L. Hedrick, Ph.D. (University of Virginia) were seeded onto either the EC surface of the transwell dish or the SMC surface at 100,000 cells/cm² and allowed to adhere for 25 minutes at 37°C. Cells were then fixed in 1%
glutaraldehyde. For MM6 adhesion to SMCs, a barrier was placed around the exterior of the inverted transwell using parafilm to contain monocytes on the transwell surface. To determine specificity of binding to VCAM-1, a neutralizing antibody for VCAM-1 (R&D, BBA5) was incubated on ECs or SMCs for 45 minutes, prior to seeding labeled monocytes.

For each condition a minimum of three sections approximately 1cm² area were cut from the membrane and mounted with DAPI reagent for en face imaging via confocal microscopy. Each section was then imaged at least three times in different locations and an average number of monocytes per field of view (total of nine) were counted using a thresholding function via ImageJ (NIH) software.

**IL-1β/IL8 Combinatorial Effects**

Growth arrested SMCs were treated with human recombinant IL-1β (5ng/mL, Peprotech, 200-01B), IL-8 (10ng/mL, Peprotech, 200-08M) or the combination of both. A neutralizing antibody for CXCR2 (2ug/mL, Abcam, ab24963) was used to inhibit IL-8 signaling through this receptor. A p38 inhibitor SB202190 (Sigma, 5µM) was used to block p38 activation. To determine NF-κB activity, cells were infected with Ad-NF-κB-luc reporter construct (Vector Biolabs, 7.3 MOI) during serum starvation.

**SMC Proliferation and Migration Assays**

SMCs were growth arrested and then treated with 10% FBS, IL-1β, IL-8 or IL-1β/IL-8 for 24 hours. SMC proliferation was assayed via Click-iT™ EdU Flow Cytometry Kit (Invitrogen, C35002). Cells were pulsed with EdU (10µM)
simultaneously with treatment. Cells were then trypsinized, fixed and permeabilized, prior to detection of S-phase gated cells using flow cytometry. Alexa Fluor-488 azide was used for S-phase detection in conjunction with Alexa Fluor-633 for cell cycle detection. Negative controls with no EdU treatment plus dye and no EdU treatment without dye were also examined.

Migration of SMCs due to 10% FBS, IL-1β, IL-8 or IL-1β/IL-8 treatment for 24 hours was determined with a Boyden Chamber Assay. SMCs were initially plated on top of a porous polycarbonate transwell membrane (6.5mm diameter, 8.0µm pore diameter, Corning #3422) and allowed to adhere before potential chemo-attractant agents were placed in the bottom of the transwell. The cells were fixed and stained with 0.02% w/v Crystal Violet in 10% EtOH/90% diH2O to quantify cell chemotactic behavior. The bottoms of the transwells were imaged and intensity values per area were measured as an indicator for relative cell chemotaxis.

**Real-time reverse transcriptase polymerase chain reaction**

Total RNA is extracted using PureLink™ Micro-to-Midi™ Total RNA Purification System (Invitrogen, Cat#12183018) and reverse transcribed using the iScript cDNA Synthesis Kit (BioRad, Cat#170-8897). Previously designed primer sequences are used for the genes SMαA, myocardin, and VCAM-1.² The expression of mRNA was analyzed via real-time reverse transcriptase polymerase chain reaction (RT-PCR) using AmpliTaq Gold (Applied Biosystems), SYBR Green (Invitrogen) and an iCycler (BioRad). Real-time RT-PCR results are reported as relative quantity of mRNA and normalized to endogenously expressed gene β-2-microglobulin.
**Western Blot**

Cells were lysed in MAPK buffer (63.5mM Tris HCL pH 6.8, 2% w/v SDS, 10% glycerol, 50mM DTT, 0.01% bromophenol blue). Total protein was collected and lysates resolved on a 7.5% SDS-PAGE gel and blotted on a polyvinyl derivative (PVD) membrane. Primary antibodies [VCAM-1 (R&D systems, 1:500), SMαA (Sigma, 1:1000), and actin (Sigma, 1:1000)] were incubated with the blot for 1 hour at room temperature or overnight at 4°C. Horseradish peroxidase conjugated secondary antibodies [goat anti-rabbit, goat anti-mouse, donkey anti-goat (Santa Cruz, 1:5000)] were incubated with the blot for 1 hour at room temperature. An Alphalmager 8900 and AlphaEaseFC software were used for acquisition of blot image and densitometry analysis, respectively.

**Interleukin-8 Secretion**

Growth arrested SMCs were treated with IL-1β for 4, 24, or 48 hours. Cell culture supernatants were collected and frozen, until assayed as previously described.²

**Data analysis and statistics**

Real-time RT-PCR results for flow experiments were reported as the fold induction of cycle amplification times for paired experiments of treated flow samples compared with control flow samples and normalized to endogenously expressed gene β2-microglobulin. Student's t-test or ANOVA was conducted for all experiments. Data from at least three independent experiments per condition were used for analysis and evaluated at p < 0.05.
REFERENCES


SUPPLEMENTAL FIGURES

Supplemental Figure I. VCAM-1 expression is heterogeneous, correlating with SM\textalpha\textsubscript{A} localization. Coronary artery cross-sections show SM\textalpha\textsubscript{A} (A,B) where white box highlights detail (B). Images co-stained for VCAM-1 (blue, C), SM\textalpha\textsubscript{A} (red, D) indicate expression overlap localization. Patient A08.4. (L, lumen; I, intima, M, media). Bars: 50\mu m.

Supplemental Figure II. VCAM-1 and CXCR2 is present in intimal and medial layers of human coronary arteries. Cross-sections of human patients labeled for VCAM-1 or CXCR-2 in coronary arteries. (Top, Left) A montage shows a coronary cross-section stained for VCAM-1. (A) VCAM-1 staining was present in ECs and intimal/medial layers. (B) Within the same section, a region indicates stronger expression in ECs than SMCs. (C, D) Higher magnification of the image in (A) shows
distinct VCAM-1 staining of intimal cells (white arrows) and expression in the media. 

(E) VCAM-1 secondary only control indicates low non-specific staining. (F) CXCR-2 was present in ECs and intimal/medial layers. See supplement for data from additional donors. (EC, endothelial cell; I, intima; M, media. n=5). Bars: 50µm.

**Supplemental Figure III. Human coronary artery expression of VCAM-1.** Images show additional data from coronary arteries of different human patients, labeled for VCAM-1 expression to identify its localization to endothelium, intima, and media. See **Supplemental Table I** for specific patient information. Bars: 50µm (L,lumen; I, intima, M, media)

**Supplemental Figure IV. Human coronary artery expression of SMαA.**

Images show coronary arteries from different human patients, labeled for SMαA expression to highlight SMC presence in the intima and media. Autofluorescence of matrix components (green) show further detail of vessel composition. See **Supplemental Table I** for specific patient information. Bars: 50µm (L,lumen; I, intima, M, media)

**Supplemental Figure V.** SMCs treated with IL-1β (5ng/mL), IL-8 (10ng/mL) or IL-1β/IL-8 were examined for mRNA changes of SMαA and myocardin via real-time RT-PCR and was normalized to the housekeeping gene β-2-microglobulin. (Mean±SE, n=3, *p<0.05).
**Supplemental Table I.** Autopsy number, age, and cause of death of patients whose coronary artery samples were used in immunofluorescent labeling.

<table>
<thead>
<tr>
<th>Autopsy No.</th>
<th>Age</th>
<th>Cause of Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>A04</td>
<td>151</td>
<td>55 yr</td>
</tr>
<tr>
<td>A07</td>
<td>112</td>
<td>15 yr, encephalocele, meningitis</td>
</tr>
<tr>
<td>A07</td>
<td>186</td>
<td>6 m, complex congenital heart disease</td>
</tr>
<tr>
<td>A07</td>
<td>17.1H</td>
<td>73 yr, necrotizing pancreatitis</td>
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<tr>
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<td>25.2D</td>
<td>79 yr, pulmonary fibrosis</td>
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<td>A07</td>
<td>28.4D</td>
<td>49 yr, ischemic heart disease</td>
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<td>A07</td>
<td>37</td>
<td>29 yr, toxic shock syndrome</td>
</tr>
<tr>
<td>A08</td>
<td>4</td>
<td>41 yr, arrhythmia</td>
</tr>
<tr>
<td>A08</td>
<td>48</td>
<td>75 yr, arrhythmia</td>
</tr>
</tbody>
</table>
Supplemental Figure I.
Supplemental Figure II.

VCAM-1 A07.186

EC

M

A

B

EC

I

M

A,C,D

VCAM-1

A07.186

EC

VCAM-1

A07.186

I

C

M

D

VCAM-1

Control

CXCR-2 A07.186

EC

I

M

EC

E

VCAM-1 Control

F

EC

I

M

CXCR-2 A07.186
Supplemental Figure III.
Supplemental Figure IV.

SMA A07.25.2D

SMA A07.17.1H

SMA A08.4

SMA A07.186

SMA A07.28.4.D
Supplemental Figure V.

**SMαA**

**Myocardin**

Normalized mRNA Quantity

Time (hr)

- Cntl
- IL-1
- IL-8
- IL-1/IL-8

* denotes statistical significance.