CELLULAR MECHANISMS OF THE ANTI-INFLAMMATORY EFFECTS OF INTERLEUKIN-19

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by

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ABSTRACT

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BACKGROUND: Atherosclerotic vascular disease is a significant medical and socioeconomic problem and contributes to mortality in multiple diseases including myocardial infarction (MI), stroke, renal failure, and peripheral vascular disease. Atherosclerosis, as well as other vascular diseases including post-intervention restenosis and allograft vasculopathy, is known to be driven by chronic inflammation and, consequently, pro- and anti-inflammatory cell signaling molecules have been an important target of cardiovascular research. Interleukin (IL)-19 is a recently discovered member of the IL-10 family of anti-inflammatory cytokines. IL-19 is expressed in injured vascular cells, including vascular smooth muscle cells (VSMCs) and endothelial cells (ECs), where it exerts an anti-inflammatory effect. In VSMCs, IL-19 signaling results in inhibition of proliferation, migration, spreading, production of reactive oxygen species (ROSs), and expression of pro-inflammatory genes. In ECs, IL-19 signaling is pro-angiogenic and results in increased EC proliferation, migration, and spreading.

AIMS and HYPOTHESIS: The goal of the present study was to explore the hypothesis that IL-19 mediates anti-inflammatory effects on vascular cells by inhibiting the expression of pro-inflammatory genes, such intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, IL-1β, IL-8, and monocyte chemotactic

protein (MCP)-1, through modulation of the mRNA stability factor HuR by post-transcriptional (e.g., microRNA) and post-translational (e.g., serine phosphorylation) mechanisms.

METHODS and RESULTS: We found that IL-19 can significantly inhibit tumor necrosis factor (TNF)-α-driven ICAM-1 and VCAM-1 mRNA and protein abundance in cultured human coronary artery ECs ($p < 0.01$). IL-19 treatment of ECs, but not monocytes, significantly inhibited monocyte adhesion to cultured EC monolayers ($p < 0.01$). In wild-type mice, systemic administration of IL-19 significantly reduced TNF-α-induced leukocyte rolling and adhesion as quantitated by intravital microscopy ($p < 0.05$). IL-19 failed to inhibit TNF-α-induced nuclear factor (NF)-κB activation in ECs. IL-19 inhibited nuclear-to-cytoplasmic translocation of HuR and significantly reduced mRNA stability of ICAM-1 and VCAM-1 ($p < 0.01$). IL-19 significantly inhibited serine-phosphorylation of HuR, which is required for its translocation, and significantly increased expression of the putative HuR regulator microRNA (miR)-133 in VSMCs.

CONCLUSIONS: These data are the first to report that IL-19 can reduce leukocyte-EC interactions, and to propose reduction in HuR-mediated mRNA stability of ICAM-1 and VCAM-1 as a mechanism. We conclude that expression of IL-19 by ECs and VSMCs may represent an auto-regulatory mechanism to promote resolution of the vascular response to inflammation. These results suggest that IL-19 is anti-inflammatory in vascular cells and, therefore, may be of therapeutic value in atherosclerotic vascular disease.

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LIST OF ABBREVIATIONS

3' UTR	3-prime untranslated region
Act D/Actino D	Actinomycin D
ANOVA	analysis of variance
ApoE	Apolipoprotein E
ARE	Adenosine Uridine (AU)-Rich Element
AUF-1	Adenosine Uridine-Rich Element/Poly-binding Degradation Factor-1
BCECF/AM	2′,7′-bis(2-carboxyethyl)-5(6)-carboxy-fluorescein acetoxymethyl ester
bFGF	basic fibroblast growth factor
C57BL/6	C57 Black/6
CABG	coronary artery bypass graft
CaEC	coronary artery endothelial cell
CAM	cell adhesion molecule
CD36	Cluster of Differentiation 36
cDNA	complementary deoxyribonucleic acid
COX-2	cyclooxygenase-2
DC	dendritic cell
DSS	dextran sucrose sodium

EBM-2	endothelial basal medium-2
EC	endothelial cell
ELAVL1	Embryonic Lethal Abnormal Vision-Like Protein 1
EST	expressed sequence tag
FBS	fetal bovine serum
GAPDH	glyceraldephyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
GM-CSF	granulocyte/macrophage colony stimulating factor
HBEC	human bronchial epithelial cell
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPF	high-power field
Hsc70	heat shock cognate 71 kilodalton protein
Hsp70	heat shock protein, 70 kilodalton
HuR	Human R Antigen
HUVEC	human umbilical vein endothelial cell
HVEC	human vascular endothelial cell
IκB	inhibitor of kappa-B

IgG	immunoglobulin G
ICAM-1	intercellular adhesion molecule-1
IFN-γ	interferon-γ
IL-19	interleukin-19
IL-20Rα/β	interleukin-20 receptor subunit α/β
IP	immunoprecipitation
KGF	keratinocyte growth factor
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemotactic protein-1
mEC	microvascular endothelial cell
MI	myocardial infarction
miR-133	microRNA-133
MLC	myosin light chain
mm-LDL	minimally oxidized low-density lipoprotein

mRNA	messenger ribonucleic acid (RNA)
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
PBMC	peripheral blood monocytic cell
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
qRT-PCR	quantitative real time polymerase chain reaction
RASC	rat arthritis synovial cell
ROS	reactive oxygen species
RT-PCR	real time polymerase chain reaction
SDS-PAGE	sodium dodecyl-sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
siRNA	small interfering ribonucleic acid
SOCS5	suppressor of cytokine signaling 5

STAT	signal transducer and activator of transcription
TCM	T cell conditioned medium
T_h1	Helper T cell, Type 1
T_h2	Helper T cell, Type 2
TNF-α	tumor necrosis factor-α
VCAM-1	vascular cell adhesion molecule-1
VLA-4	very late antigen-4
VEGF	vascular endothelial growth factor
VSMC	vascular smooth muscle cell
WBC	white blood cell

CHAPTER 1

INTRODUCTION

1.1. Cardiovascular disease in the 21st century

The spectacular medical and surgical advancements of the 20th century prompted a dramatic improvement in life expectancy and quality of life in the United States and changed entirely the spectrum of life-threatening illnesses facing the western world. In 1900, life expectancy in the US was 49.2 years and the three leading causes of mortality, accounting together for 31 percent of all deaths, were influenza and pneumonia, tuberculosis, and gastrointestinal diarrheal infections. Cardiovascular disease was only the fourth leading cause of death, accounting for just 8 percent of total deaths[1]. Beginning in 1928, the discovery and consequent availability of powerful anti-microbial agents reshaped western mortality. In 2008, by contrast, life expectancy was 78.1 years. Now, the leading cause of death in the United States, accounting for approximately 30 percent of all deaths annually, is cardiovascular disease[2]. Thus, as the 20th century frontier of biomedical research was microbial disease, the frontier of research as we embark on the 21st century is cardiovascular disease.

Almost half (49.9 percent) of all cardiovascular disease is accounted for by coronary heart disease, which includes acute myocardial infarction (AMI) and angina pectoris and the vast majority of which is secondary to the formation of atherosclerotic vascular lesions in the coronary arteries[3]. The development of effective treatments for atherosclerosis is, therefore, essential to the improvement of modern therapies for cardiovascular disease.

1.2. Atherosclerosis

Atherosclerosis is a chronic vascular lesion which slowly progresses and grows, leading eventually to reduced arterial lumen diameter and compromised distal blood flow (see Figure 1). At advanced stages, the atherosclerotic plaque can rupture and consequent thrombosis can lead to complete vessel occlusion or thromboembolus formation—crucial steps in the pathogenesis of acute myocardial infarction and thromboembolic stroke.

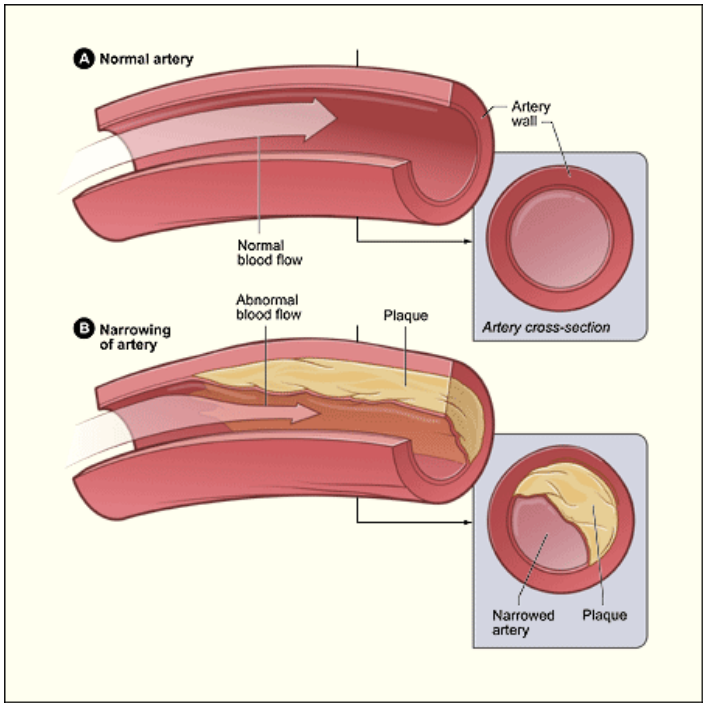

Figure 1. Luminal narrowing from atherosclerosis. Photo Credit: NHLBI.

Advanced atherosclerotic plaques are heavily composed of lipid, including cholesterol- and cholesterol ester-laden macrophages, which historically prompted scientists to favor

the *lipid hypothesis*—the view that increased blood lipid levels was the primary cause of atherosclerosis. Later research cast doubt on the lipid hypothesis by revealing the crucial role of endothelial cell (EC) injury and chronic inflammation in the development of atherosclerosis[4,5]. In the current view of the pathogenesis of atherosclerosis (see Figure 2), endothelial dysfunction resulting from disturbed blood flow (e.g., at branch points in the arteries, hypertension) and injurious agents (e.g., oxidized low-density lipoprotein, ox-LDL; tobacco-related and other toxins; immune reactions; and/or viruses) cause increased localization of macrophages to the sub-endothelial space. The sub-endothelial macrophages take up lipid which leads to the formation of a *fatty streak*. Increasing addition of lipid to the plaque eventually leads to the formation of an *atheroma*, characterized by an *extra*cellular lipid core. Progression of atheromatous plaques proceeds from inflammatory activation of vascular smooth muscle cells (VSMCs) which, ordinarily quiescent and contractile, take on a migratory, proliferative, and secretory phenotype. The VSMCs, thus activated, migrate from the tunica media through a progressively degraded internal elastic lamina into the core of the plaque and secrete pro-inflammatory cytokines and collagen fibers, a key step in the progression of an atheroma to a *fibroatheroma*. Increasing plaque size, central necrosis of plaque cells, and progressive surface defects in the fibrous cap of the plaque lead to the formation of a *complicated lesion*, the full rupture and thrombosis of which can cause vessel occlusion.

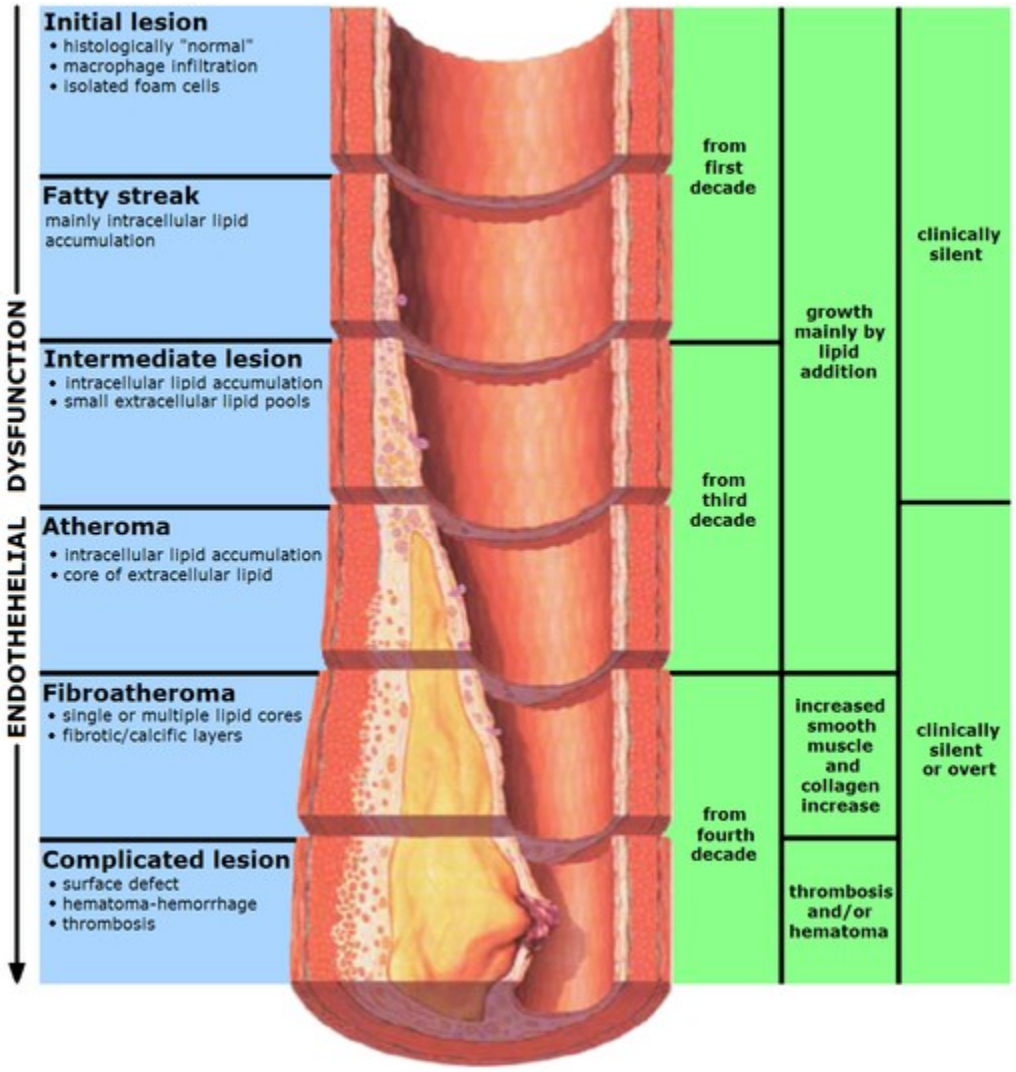

Figure 2. Sequences in Progression of Atherosclerosis. Photo Credit: Grahams Child.

1.3. Inflammation, cytokines, and vascular disease.

Inflammation is a ubiquitous pathological process which is central to the development of multiple cardiovascular diseases. Many vascular diseases such as atherosclerosis, restenosis, and transplant vasculopathy are chronic, progressive processes initiated and propagated by local inflammation of large and medium sized arteries[6]. This inflammation is mediated by a variety of cell types including macrophages, lymphocytes, platelets, endothelial cells (ECs), and vascular smooth muscle cells (VSMCs). The multiple cell types which participate in vascular inflammation have evolved to produce common cytokines and specific membrane receptors allowing them to transmit their effects into the cell, permitting these diverse cell types to communicate by expression and recognition of multiple pro- and anti-inflammatory cytokines. As such, cytokines and their receptors are the currency of inflammation, and represent attractive targets for therapeutic modalities in numerous vascular inflammatory disorders.

Synthesis and recognition of cytokines and receptors by both vascular and immune cells allows bi-directional communication between these two systems, and demonstrates that, under particular conditions, we can consider vascular cells as extended participants in the adaptive immune response. Cytokines often act in synergy with other cytokines and frequently share receptor subunits, which combine into homodimers or heterodimers with receptors of other cytokines. Cytokines can drive multiple, often simultaneous cellular processes including mitogenesis, gene expression, fibrosis, and chemotaxis[7]. This communication initiates a series of receptor-mediated signal transduction cascades including activation of mitogen-activated protein kinases (MAPK), Protein Kinase C (PKC), and transcription factors, often including the signal transducer and activator of transcription (STAT) family[8]. Pro-inflammatory cytokines most often lead to activation of nuclear factor

(NF)-κB which acts as a “master switch” for transcription of numerous pro-inflammatory genes, the expression of which may be appropriate, as in host defense, or maladaptive, as in chronic vascular disease[9,10].

Atherosclerosis is a chronic vascular inflammatory condition mediated by interactions between lymphocytes, macrophages, endothelial cells, and vascular smooth muscle cells which results in local inflammation of the arterial wall. While an excess of serum low density lipoprotein (LDL) is an established risk factor for atherosclerosis, inflammatory mechanisms play an acknowledged role in initiation and propagation of atherogenesis. The inflammatory nature of atherosclerosis has prompted broad investigation into vascular inflammatory processes and, consequently, pro-inflammatory signaling mechanisms in the vascular wall have been well characterized[5,11–13]. Less, albeit increasing, interest has been placed on understanding the potentially protective role of anti-inflammatory cell signaling in the vascular wall[14,15]. Such studies that do exist place a strong emphasis on the role of anti-inflammatory cytokines, e.g., interleukin (IL)-10, in the immune cells of the plaque. Even less investigation has been carried out on the direct effect of anti-inflammatory cytokines on vascular smooth muscle cells and endothelial cells. Consequently, direct effects of anti-inflammatory cytokines constitute an emerging and promising area of study.

1.4. Vascular cell adhesion molecules in inflammation and atherosclerosis

Vascular endothelial cells (ECs) and immune cells actively participate in the pathogenesis of inflammation in general and many vascular diseases in particular[16]. Extravasation of circulating leukocytes, which is important physiologically for immune surveillance, is up-regulated during vascular inflammation through changes in both immune cell and EC activity. In response to injury or pro-inflammatory mediators, ECs express higher levels of

vascular cell adhesion molecules (CAMs) with the capability to tether and firmly adhere to circulating leukocytes. ECs also actively participate in recruitment of leukocytes by generating a chemotactic gradient of chemokines which attract leukocytes to the site of injury[16,17].

Recruitment and migration of leukocytes to the site of injury occurs in well characterized phases[18] (see Figure 3). The capture of an approaching leukocyte is mediated by the low-affinity binding of selectins (e.g., E-selectin, P-selectin, L-selectin) to glycoprotein counter-receptors, which causes the leukocyte to roll along the endothelial cell layer. EC-released chemokines further activate the leukocyte, causing conformational changes in several of its cell adhesion molecules (e.g., LFA-1, VLA-4) allowing for firm adhesion of the leukocyte to the ECs. This step is mediated by the immunoglobulin-like superfamily of cell adhesion molecules, which includes intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1. The final phases of leukocyte migration include adhesion reinforcement and spreading of the leukocyte, intravascular crawling, and, ultimately, migration of the leukocyte into the extravascular tissues.

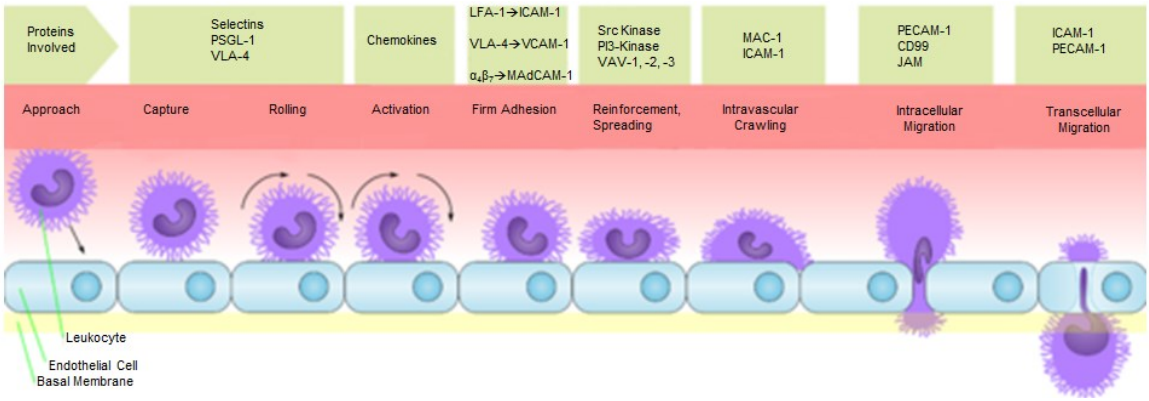

Figure 3. Phases of Leukocyte Migration. Photo Credit: Armin Kubelbeck

1.5. T_h1 and T_h2 interleukins in atherosclerosis.

Interleukins are often classified, according to their effects on lymphocyte function or maturation, as T_h1 (pro-inflammatory, cytotoxic), which promote inflammation, and T_h2 (anti-inflammatory, antibody responses), which generally dampen inflammation[19]. Approximately 10% of the cellular content of human atherosclerotic plaque consists of CD3+ T cells[9]. The overwhelming majority of these are CD4+ helper T cells (T_h), which recognize epitopes on oxidized LDL[20]. Because atherosclerosis is primarily an inflammatory condition, it is not surprising that T_h1 interleukins are much more prevalent in human atherosclerotic lesions than the T_h2 cytokines[13,19]. T_h1 cells drive cell-mediated immunity and are characterized by abundant expression of interferon (IFN)-γ, IL-12, tumor necrosis factor-α, and other pro-inflammatory cytokines, which are also highly expressed in atherosclerotic lesions. In contrast, T_h2 cytokines such as IL-4 and IL-10 are far less

abundant in human and mouse atherosclerotic lesions. T_h2 cytokines dampen the inflammatory response through inhibition of pro-inflammatory genes (including T_h1 genes) and, conversely, T_h1 cytokines reduce expression of T_h2 cytokines. Thus, the very low levels of T_h2 cytokines detected in atherosclerotic lesions are may be due to the elevated levels of T_h1 cytokines. The prevailing hypothesis that atherosclerosis is a T_h1 disease is supported by studies in which mice lacking IFN-γ or the IFN-γ receptor, TNF-α, or the T_h1 transcription factor T-bet have reduced atherosclerosis[21–23]. Further supporting this hypothesis, mice lacking STAT6, which is essential for T_h2 cell differentiation, have increased atherosclerosis[24]. Since so many more pro-atherogenic cytokines and receptors have been identified and characterized, much greater effort has gone into understanding these cytokines and the potential for inhibition of their expression or activity. Far fewer studies have pursued characterization of anti-inflammatory cytokines in vascular disease. Interleukin-10 is the archetypical T_h2 interleukin, a potent immune modulator, and the most studied in terms of vascular disease. Several studies have suggested that IL-10 is athero-protective by several mechanisms including T_h2 T cell polarization and attenuation of inflammatory gene expression in inflammatory cells. For example, IL-10 atherosclerotic plaque burden is reduced in IL-10 transgenic mice, and transfer of bone marrow from these mice into $LDLR^{-/-}$ mice reduced atherosclerosis[25]. As expected, atherosclerosis is increased in IL-$10^{-/-}$ mice and IL-$10^{-/-}$/$ApoE^{-/-}$ double knock-out mice. The mechanism is most likely mediated by inflammatory cells, as transfer of IL-$10^{-/-}$ bone marrow to $LDLR^{-/-}$ polarizes the T lymphocyte T_h2/T_h1 ratio toward a more anti-inflammatory phenotype[26]. Although considered to be a T_h2 interleukin, IL-4 does not appear to be anti-atherosclerotic, as IL-$4^{-/-}$ mice do not have increased atherosclerosis, and administration of IL-4 into $ApoE^{-/-}$ mice does not reduce development of atherosclerotic lesions[27]. Thus, studies report most T_h2 interleukins to be indirectly anti-atherogenic by polarizing the

adaptive immune response to an anti-inflammatory phenotype. Though the number of studies investigating the role of T_h2 cytokines is dwarfed by those investigating T_h1, our current understanding suggests that the balance of these two opposing "forces" can dictate the outcome of the atherosclerotic lesion. Consequently, investigation of the role of T_h2 cytokines as potential therapeutics in vascular inflammatory disorders may be potentially promising as anti-inflammatory therapy for vascular disorders.

1.6. Discovery and Characterization of Interleukin-19.

Interleukin-19 (IL-19) was first identified and cloned by searching Expressed Sequence Tag (EST) databases for IL-10 homologues[28]. The IL-19 gene is located in chromosome 1q32, in an "IL-10 cluster", which includes genes for several other IL-10 family members. IL-19 is a member of a subfamily of the IL-10 family of interleukins and is more broadly classified as a Class II cytokine, a class which includes the IL-10 family members and the interferons (Types I, II, and III)[29–31]. More recently, IL-19 has been classified in a subfamily including IL-19, IL-20, and IL-24, though these subfamily members are recognized by and signal through different combinations of shared receptor chain complexes.

In its secreted form, human IL-19 is a compact α-helical protein composed of 159 amino acids. IL-19 has amino acid identity with IL-10 at 30 residues including 4 cysteines known to be required for correct folding of IL-10 and 41 of the 50 amino acids required for formation of the IL-10 hydrophobic core[28]. The overall IL-19 amino acid sequence shares 20% identify with IL-10, and x-ray crystallography confirms that IL-19 is structurally similar to IL-10 but with key differences[32]. While IL-10 has 6 α-helices (A-F) the last of which (F) contributes to the formation of a stable IL-10 homodimer in solution through its insertion into the core of its paired protein, IL-19 has 7 α-helices (A-G) the last of which (G) is able

to fold back and stabilize IL-19 as a soluble monomer. Further, the homologous region of IL-10 that interacts with the IL-10 receptor chain 1 is far less conserved in IL-19. Together, these properties may explain why IL-19, despite its amino acid identity with IL-10, is not recognized by and cannot signal through the IL-10 receptor complex.

IL-19 shares relatively greater structural similarity with fellow sub-family members IL-20 and IL-24, each of which also forms a stable monomer in solution. The genes for these three proteins are found in a gene cluster with IL-10 on chromosome 1, and have been alternately referred to as the "IL-19 subfamily"[29] or the "IL-20 subfamily"[33]. In addition to their structural similarity, interleukins 19, 20, and 24 all signal through receptor complexes containing the IL-20 receptor β chain (IL-20Rβ)[34]. All three proteins can signal through the heterodimer formed by IL-20Rα and IL-20Rβ. IL-20 and IL-24, but not IL-19, can also signal through the receptor formed by IL-22Rα and IL-20Rβ.

1.7. Expression and Function of IL-19.

Expression of IL-19 was first reported by Gallagher *et al.*[28] in LPS- and GM-CSF-stimulated primary human monocytes and subsequent early reports on IL-19 focused on its role as a product of immune cells[35]. Among immune cells, IL-19 is primarily expressed by monocytes and, to a lesser extent, by B cells, but some investigators have questioned its role in regulating these cells due to the lack of detectable expression of the IL-20Rα chain in lymphocytes[35–38]. Notwithstanding the lack of this receptor, effects of IL-19 have been reported in lymphocytes[39,40], including the notable observation that IL-19 treatment is able to polarize the maturation of human T cells away from the pro-inflammatory T_h1 phenotype to the anti-inflammatory T_h2 phenotype[40,41]. While expression of IL-20Rα and IL-20Rβ chains are reported to be cytokine-regulated, detailed studies on expression of

these peptides in vascular cells or myocytes are lacking. Since its discovery and early characterization, IL-19 expression has been detected in a wide variety of non-immune human peripheral cell types, including keratinocytes[36], bronchial epithelial cells[42,43], synovial tissue[44,45], fetal membranes[46], and vascular endothelial[47] and smooth muscle cells[48] (See Table 1). This suggests a functional role for IL-19 distinct from T_h1/T_h2 polarization. Paradoxically, IL-19 seems to exert both pro-inflammatory and anti-inflammatory properties in a manner contextually governed by tissue-specific and disease-specific factors. The myriad roles of IL-19 in non-cardiovascular tissues and diseases are of qualified interest to the scope of this thesis as other roles of IL-19 could affect its efficacy and desirability as a therapeutic modality in vascular disease. The multiple non-vascular effects of IL-19 have been well reviewed in the past[29] and will be only briefly presented here.

Table 1. Effects of IL-19 on Non-Vascular Cell Types

Tissue Type	Effect	Species	Source
Immune	T_h2 response in T cells	h,m	[40,41,56]
	Inhibits IFN-γ production in T cells	h	[41]
	Induces IL-4 and IL-13 in T cells	h	[41]
	Induces IL-10 in monocytes	h	[110]
	Auto-induces IL-19 expression in PBMCs, DCs	h	[110]
	Induces KGF expression in CD8+ T cells	h	[49]
	Suppresses cell-mediated immunity post-bypass	h	[65]
	Induces IL-6, TNF-α in monocytes	m	[111]
	Induces ROS production, apoptosis in monocytes	m	[111]
Skin	Expressed in keratinocytes in psoriatic skin	h	[36,50]
	STAT3 phosphorylation in HaCat keratinocytes	h	[40]
Airway	HBEC-produced IL-19 induces TNF-α in THP-1	h	[57]
	Induces apoptosis in lung epithelium cells	h	[60]
Colon	Protective against DSS-induced colitis	m	[58]

Table 1. (continued)

Tissue Type	Effect	Species	Source
Cancer	Inhibits proliferation of ovarian carcinoma cells	h	[67]
	Proliferation of oral squamous cell carcinoma cells	h	[112]
	Proliferation of breast cancer cells	h,m	[62]
	Induces pro-inflammatory cytokines in breast cancer	m	[62]
	Induces fibronectin expression in breast cancer	m	[62]
Fetal	Induces IL-6 in fetal membranes	h	[46]
	Inhibits LPS-induced TNF-α in fetal membranes	h	[46]
Liver	Induces ROS production in Huh-7 cell line	h	[60]
Synovium	Inhibits apoptosis in RASC	r	[44]
	Activates STAT3 and induces IL-6 in RASC	r	[44]
	Induces pro-inflammatory cytokines in fibroblasts	r	[61]
Nasal	Inhibits eotaxin expression in nasal fibroblasts	h	[113]

Abbreviations: HBEC: human bronchial epithelial cell, RASC: rat arthritis synovial cell, PBMC: peripheral blood mononuclear cell, DC: dendritic cell, DSS: dextran sucrose sodium, ROS: reactive oxygen species, KGF: keratinocyte growth factor, h: human, m: mouse, r: rat

A putative role for IL-19 has been put forth in the development of psoriasis, a chronic inflammatory skin condition characterized by increased proliferation of keratinocytes leading to the development of plaque-like epidermal lesions. Expression of IL-19, IL20Rα, and IL20Rβ can be detected in psoriatic lesions[36,49–52] and treatment of psoriasis reduces expression of IL-19[50,51]. Current findings suggest a possible feedback loop whereby IL-19 promotes expression of keratinocyte growth factor (KGF) in CD8+ T cells which, in turn, induces increased expression of IL-19 from keratinocytes[49]. However, while IL-20 transgenic mice are reported to have a psoriatic phenotype, IL-19 transgenic mice exhibit no such pathology. A causal role for IL-19 in psoriasis has not been well established, though the data support a stronger implication for the IL-19 subfamily member IL-20 in this disease.

T_h2 cytokines are involved in the pathogenesis of a number of diseases, most notably asthma[53–55], a chronic inflammatory airway disease resulting in bronchospasm and consequent reversible airway obstruction. As expected, given the T_h2 nature of the disease, IL-19 has a demonstrable, though yet unclear, role in the development of asthma. IL-19 expression is increased in the lungs of mice exposed to allergens[56]. Serum IL-19 levels are increased in children with asthma when compared with normal children[56] and airway epithelial cells of asthma patients exhibit increased IL-19 expression[43]. IL-19 expression in airway cells can be modulated by adenosine receptors[57], which play a role in asthma-related cell signaling.

Recent scientific interest in IL-19 has prompted investigators to pursue exploration of IL-19 involvement in various other diseases and tissue types. IL-19 has been indicated as potentially protective against gut inflammation[58,59], representing the potential for therapeutic use in inflammatory bowel disease. IL-19 has a suggested role in promoting

the development of endotoxic ("septic") shock[60] as well as rheumatoid arthritis[45,61]. Recent work demonstrated IL-19 expression in numerous neoplastic cell types, including cells of squamous cell carcinoma of the oral cavity, in which IL-19 promoted proliferation[62]. IL-19 also promoted proliferation and migration of breast cancer cells, and high IL-19 expression was associated with poor outcomes in breast cancer patients[62].

1.8. Expression of Interleukin-19 in Vascular Disease.

Vascular expression of IL-19 was first identified in 2005 through cDNA microarray analysis of cultured human vascular smooth muscle cells treated with inflammatory stimuli[63]. This was unexpected as IL-19 expression had previously been thought to be restricted to leukocytes[28,29,41,64]. Induction of IL-19 expression in vascular cells was further characterized and western blot analysis of cultured human VSMCs demonstrated that IL-19, while not expressed in quiescent (unstimulated) controls, can be induced in VSMCs treated with inflammatory stimuli, including fetal bovine serum (FBS), T-cell conditioned media (TCM), IFN-γ, platelet-derived growth factor (PDGF), and TNF-α[48]. Analysis of endothelial cells produced similar results, showing that microvascular ECs (mECs), coronary artery ECs (CaECs), and human vascular ECs (HVECs) can all be stimulated to express IL-19 by FBS, basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and oxidized LDL (ox-LDL)[47]. In contrast, IL-10 expression could not be detected in VSMCs at the mRNA or protein level[48]. In histological analysis of human coronary arteries, IL-19 expression was undetectable in sections from normal arteries, but was highly expressed in ECs[47], neointimal and medial VSMCs[48], and CD45+ leukocytes in coronary arteries with allograft vasculopathy, a chronic vascular inflammatory syndrome. Similarly, both IL-20 and its receptor subunits are expressed in macrophages and ECs in atherosclerotic plaque, and are induced in these cultured cells when stimulated with

inflammatory factors. IL-20 is not expressed in VSMCs. In contrast, IL-19 is detected in VSMCs[48], ECs[47], and CD45+ leukocytes[48] in atherosclerotic plaque in aortic arch of ApoE$^{-/-}$ mice, but not aortic arch of wild-type mice, further suggesting that IL-19 is only expressed in response to vascular injury.

In another connection to vascular disease, two reports indicate that serum concentrations of IL-19 are increased in patients undergoing coronary artery bypass graft (CABG) surgery with cardiopulmonary bypass[65,66], and that the increased IL-19 levels contribute to the cell-mediated immune suppression frequently observed in these patients[65].

1.9. Pleiotropic effects of IL-19 on vascular cells.

In vascular disease, where the participating cell types are primarily immune cells and vascular cells, IL-19 exerts a pronounced anti-inflammatory effect (Table 2). IL-19 has anti-proliferative effects on the NIH:OVCAR-3 ovarian carcinoma cell line[67] and IL-10 has anti-proliferative effects in vascular cells[68], suggesting that vascular expression of IL-19 in response to injury might represent a novel autocrine or paracrine mechanism for attenuation and regulation of VSMC proliferation. Several experiments were carried out to test this hypothesis and have uncovered multiple potential mechanisms for these effects. Treatment of cultured VSMC with recombinant IL-19 or with adenoviral expression of IL-19 decreased VSMC proliferation compared to controls in a concentration-dependent manner[48]. *In vivo* experiments in rats recapitulated this anti-proliferative effect, demonstrating that adenoviral delivery of IL-19 to balloon angioplasty injured rat carotid arteries decreased neointima formation and number of proliferating (Ki-67-positive) VSMC in this tissue. IL-19 treatment of VSMC evoked a rapid and transient activation of STAT3 as measured by both phosphorylation and nuclear translocation.

Table 2. Effects of IL-19 on Resident Vascular Cells

Tissue Type	Effect	Species	Source
EC	Auto-induces IL-19 Expression	h	[47]
	Activates STAT3, Rac1, MAPK p44/42	h	[47]
	Increases EC proliferation	h	[47]
	Increases EC spreading and migration	h	[47]
	Pro-angiogenic (increases tube, microvessel formation)	m	[47]
VSMC	Inhibits proliferation, hyperplasia	h,r	[48]
	Auto-induces IL-19 expression	h	[48]
	STAT3 phosphorylation, translocation	h	[48]
	Increases SOCS5 expression	h	[48]
	Inhibits MAPKs (p44/42, p38)	h	[48]
	Decreases inflammatory, proliferative proteins, mRNAs	h	[69]
	Decreases HuR protein abundance	h	[69]
	Inhibits HuR nucleocytoplasmic translocation	h	[69]
	Decreases ARE-bearing mRNA stability	h	[69]
	Inhibits PKCα activation	h	[69]
	Inhibits migration, spreading	h	[72]
	Inhibits activation of MLC, cofilin, Hsp70, Rac1, RhoA	h	[72]
	STAT3-dependent increase in HO-1 expression	h	[76]
	Decreases ROS *in vitro* and *in vivo*	h,m	[76]
	Inhibits apoptosis	h	[76]

Abbreviations: ROS: reactive oxygen species, h: human, m: mouse, r: rat

IL-19 was shown to rapidly increase expression of suppressor of cytokine signaling 5 (SOCS5), a STAT-responsive gene, at both the mRNA and protein levels. IL-19-induced SOCS5 expression was dependent on STAT3[48]. There are six SOCS family members that function to suppress cytokine signaling by binding to phosphorylated tyrosine residues on cytokine receptors and cytoplasmic signaling intermediates and targeting them for E3-ubiquitin ligase-mediated degradation. SOCS-mediated signaling inhibition is a mechanism exhibited by numerous cytokines[64]. In VSMC, IL-19 can reduce fetal bovine serum-induced activation of the p44/42 and p38 MAPKs, both mediators of inflammation. IL-19-induced SOCS5 binds the p44/42 and p38 MAPKs, providing at least one probable mechanism for these effects[48]. This indicates that IL-19 can reduce VSMC activation by inhibition of signal transduction. In addition to IL-19, this work also implicates SOCS5 as an important mediator of anti-inflammatory signal transduction.

IL-19 can decrease FBS-mediated induction of protein and mRNA abundance of proliferative and pro-inflammatory genes in VSMC, including cyclin D1, cyclooxygenase-2 (COX-2), IL-1β, and IL-8[69]. Interestingly, this inhibition was selective, and other important regulatory proteins such as proliferating cell nuclear antigen (PCNA), Rac1, and others, were not sensitive to IL-19. Other T_h2 interleukins also reduce inflammatory cytokine expression, and IL-10 in particular reduces inflammatory responses in varied cell types by inhibition of activation of the transcription factor NF-κB. In contrast to IL-10, IL-19 did not inhibit NF-κB activation, as determined by IκB degradation and p65 subunit phosphorylation in VSMC[69]. This surprising finding suggested that IL-19 decreases abundance of inflammatory and proliferative genes in an NF-κB-independent mechanism, and prompted a search for other possible mechanisms whereby IL-19 could decrease inflammatory gene abundance. Notably, many pro-inflammatory genes, including the

genes affected by IL-19, are targeted for preferential degradation by cis-acting AU-rich elements (AREs) in their 3' untranslated regions[70]. Two proteins, Human R Antigen (HuR) and AU-Rich RNA Binding Factor-1 (AUF-1), have been shown to regulate ARE-bearing transcripts by binding to ARE and modifying their mRNA stability[71], with HuR promoting increased mRNA stability and AUF-1 promoting decreased stability. The half-lives of ARE-bearing transcripts may be regulated in a "yin-yang" fashion through competitive binding of HuR and AUF-1 to the same AREs. The ability of HuR to stabilize mRNA corresponds with its translocation from a predominately nuclear location into the cytoplasm[71]. Prompted by the observation that IL-19-inhibited transcripts bear AREs, and that transcripts lacking AREs (e.g., PCNA) are unaffected by IL-19, the effects of IL-19 on HuR, AUF-1, and mRNA stability were explored in VSMCs. It was found that IL-19 reduces HuR translocation in FBS-stimulated VSMCs[69]. AUF-1 abundance and translocation were not affected by IL-19. As expected, IL-19 reduced stability of inflammatory and proliferative mRNA transcripts which contained AREs in VSMCs, as measured using qRT-PCR and with the transcription inhibitor actinomycin D, but failed to affect stability of mRNA lacking AREs. This effect on stability was able to be recapitulated using HuR siRNA[69]. Taken together, these observations suggested that IL-19 signaling in vascular cells is permissive of NF-κB-mediated increases in inflammatory and proliferative gene *transcription*; however, IL-19 produces a *post-transcriptional* decrease in the abundance of these transcripts through inhibition of HuR translocation, thereby mediating a decrease in transcript stability. This represents a second mechanism, in addition to IL-19-induced SOCS5 expression, through which IL-19 may exert its anti-inflammatory effects on vascular cells.

IL-19 has been shown to have a direct effect on VSMC motility. IL-19 inhibited cultured VSMC re-migration into a scratch wound and also inhibited PDGF-induced migration in a Boyden chamber[72]. Molecular analysis revealed that IL-19 inhibits activation of cellular motility proteins, including myosin light chain (MLC), cofilin, Hsp70, and the monomeric G proteins Rac1 and RhoA. The precise molecular mechanism(s) of IL-19 decrease in activation of these important proteins remains to be elucidated.

In contrast to its documented anti-proliferative effects in VSMCs, IL-19 exhibits proliferative, pro-migratory, and pro-angiogenic effects in vascular ECs. Recombinant IL-19 treatment of ECs *in vitro* results in activation of STAT3, Rac1, and MAPK p44/42 with consequent increases in EC proliferation, spreading, and migration. Confirming its pro-angiogenic potential, IL-19 promotes formation of endothelial cell tubes in isolated cultured mouse aortic rings and promotes formation of nascent blood vessels in subcutaneous gel plugs in mice[47]. These functions are independent of bFGF and VEGF expression, and are IL-19-specific, as specific antibody to IL-20 receptor significantly reduces IL-19-driven EC migration[47]. The molecular basis of these intriguing and unexpected observations should uncover interesting distinctions between EC and VSMC processing of anti-inflammatory signals.

Heme oxygenase-1 (HO-1) has powerful anti-inflammatory and anti-apoptotic effects and protects against vascular inflammation through multiple mechanisms including decreasing monocyte arterial transmigration, decreasing VSMC proliferation, and acting as a potent anti-oxidant[73,74]. HO-1 is induced primarily at the transcriptional level by many pro-inflammatory mediators including cytokines, oxidative stress, and some growth factors[75]. IL-19 can induce expression of HO-1 mRNA and protein in cultured VSMCs, but not ECs[76], again, another interesting distinction in IL-19 cell-specific effects. Consistent with

this finding, IL-19 can reduce peroxide-induced apoptosis, and growth-factor-induced reactive oxygen species (ROSs) accumulation in VSMCs. This reduction in ROSs was abrogated when VSMCs were transfected with HO-1-specific siRNA prior to IL-19 treatment. *In vivo*, IL-19 can reduce TNF-α-induced ROS accumulation in murine coronary arteries[76]. While it has been shown that IL-10 can induce HO-1 in monocyte/macrophages[77], induction of HO-1 in vascular cells by any anti-inflammatory cytokine or T_h2 interleukin had not been reported. This provides a third potential molecular mechanism whereby IL-19 can reduce vascular inflammation, and implicates IL-19 as a potential link between two powerful and protective systems, anti-inflammation and reduction of ROSs.

In unpublished experiments, $LDLR^{-/-}$ mice fed an atherogenic diet and injected with as little as 1.0 ng/g/day of recombinant IL-19 demonstrated significantly less atherosclerotic plaque lesion area in the aortic arch compared with PBS-injected control mice[78]. These mice have decreased macrophage infiltrate into the atherosclerotic lesion and increased the ratio+ of T_h2 to T_h1 lymphocytes. This is in contrast to IL-20, which is pro-atherosclerotic[79]. Further, weight gain as well as serum cholesterol and triglyceride levels are identical in IL-19 treated mice compared with PBS controls. This is an important distinction for IL-19, as several studies have shown an association between T_h1/T_h2 balance with hypercholesterolemia[80,81]. Together, these preliminary, but provocative, data suggest that IL-19 can decrease atherosclerosis in susceptible mice not suppressing serum lipid levels. Future studies are necessary to determine the precise molecular and cellular mechanisms for IL-19-mediated decreases in vascular disease.

1.10. Summary

The roles of cytokines in development of vascular inflammatory diseases such as atherosclerosis, restenosis, and coronary artery transplant vasculopathy are very complex. It is clear however that the functions of putative anti-inflammatory cytokines in these disease processes hold potential as therapeutics and require further study to characterize their precise mechanism(s) of action. Interleukin-19 is unique among interleukins and its expression by resident vascular cells may represent an auto-regulatory, autocrine or paracrine mechanism to promote resolution of the vascular response to inflammatory insult. IL-19 is not detectible in naïve artery, but is induced in response to vascular injury and inflammation. Similarly, IL-19 is expressed in ECs and VSMCs when stimulated with inflammatory stimuli, and its addition to these cell types imparts anti-inflammatory effects, with decreases in ROS abundance, migration, proliferation, and expression of inflammatory genes. Function of IL-19 outside of the immune system implies that resident vascular cells may assume a T_h2 phenotype and the pleiotropic mechanisms of IL-19 in vascular cells suggest that IL-19 may be a valuable anti-inflammatory therapeutic modality in acute as well as more chronic vascular inflammatory diseases such as allograft vasculopathy and atherosclerosis.

1.11. Overall Hypothesis

The overall hypothesis of this dissertation is that, given its powerfully anti-proliferative effects in VSMC and the putative anti-inflammatory effects of IL-10 family cytokines, interleukin-19 may exert anti-inflammatory effects in vascular cells by regulating processes relating to the recruitment of leukocytes by vascular cells. In particular, we will

examine synthesis of pro-inflammatory mediators and regulation of the leukocyte-endothelial cell interactions necessary for trafficking of leukocytes into vascular tissues.

In order to test this hypothesis, the following aims will be explored:

1.12. Aims

Aim 1. IL-19 effect on expression of pro-inflammatory genes

Aim 1 Hypothesis: We hypothesize that IL-19 may mediate anti-inflammatory effects through down-regulation of pro-atherosclerotic genes, such as cell adhesion molecules and chemotactic proteins.

Specific Aims:

a. We will characterize the cellular effects of IL-19 treatment on vascular expression of cell adhesion molecules (e.g., ICAM-1, VCAM-1, E-selectin) *in vitro*.
b. We will characterize the cellular effects of IL-19 treatment on leukocyte-EC adhesion *in vitro* and *in vivo*.
c. We will characterize the cellular effects of IL-19 treatment on VSMC and EC expression of chemokines (e.g., MCP-1, IL-8).

Aim 2. IL-19 and HuR in vascular inflammation and atherosclerosis

Aim 2 Hypothesis: We hypothesize that IL-19 mediates anti-inflammatory effects by reducing stability of pro-atherosclerotic mRNAs through modulation of HuR cellular localization.

Specific Aims:

a. We will characterize the effects of IL-19 on HuR nucleocytoplasmic translocation in ECs.
b. We will characterize the molecular mechanism(s) by which IL-19 affects the cellular localization and abundance of HuR, including the possibilities that IL-19 affects serine-phosphorylation of HuR and that it up-regulates HuR-specific microRNAs.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cells and Culture.

Primary human coronary artery vascular endothelial cells (hCaECs) were obtained as cryopreserved secondary culture from Cascade Corporation (Portland, OR) and subcultured as we described[47]. Cells were used from passages 3-5. ECs were incubated in Endothelial Basal Medium-2 (EBM-2; Lonza Group, Switzerland) supplemented with 1.0 % FBS for 24 hours, and then exposed to recombinant human IL-19 (100ng/ml, R&D, Inc. Minneapolis, MN) for various times, then stimulated with recombinant human TNF-α (10ng/ml, Sigma, St. Louis, MO).

2.2 Western blotting.

Cells were cultured and extracts were made as we have described[47]. Briefly, cells were rinsed with PBS and extract proteins separated by SDS-PAGE. Membranes were incubated with a 1:4000-5000 dilution of primary antibody, and a 1:5000 dilution of secondary antibody. Primary antibodies used were ICAM-1(Abcam, Cambridge, England), VCAM-1, HuR, Hsc70, E-selectin (Santa Cruz Inc, Santa Cruz, CA), GAPDH, IkB, phospho-serine, phospho- and total p65 from (Cell Signaling, Danvers, MA) and IL-8 (Biosource, Life Technologies, Carlsbad, CA). Reactive proteins were visualized using enhanced chemiluminescence (Amersham, UK) according to manufacturer's instructions. Relative intensity of bands was normalized to GAPDH and Hsc70, and quantitated by scanning image analysis and the Image J densitometry program.

2.3 Cellular fractionation and Immunoprecipitation.

Cell extracts were fractionated as we have described[69]. Briefly, ECs were washed in PBS, scraped off the plates, pelleted by low-speed centrifugation (4500rpm), lysed by addition of 100µl of ice-cold lysis buffer (10mM HEPES, pH 7.9, 1.0mM EDTA, 60mM KCl, 0.5% NP-40, and complete Mini EDTA-free protease inhibitor cocktail tablet, Roche). Nuclei were pelleted by centrifugation (8000rpm) for 5 min at 4°C and the supernatant isolated as cytoplasmic proteins. Nuclear proteins were isolated by brief sonication of nuclear pellets in SDS sample buffer. HuR immunoprecipitation was performed as described[69]. Briefly, ECs were lysed by addition of 500µl of ice-cold immunoprecipitation (IP) buffer (10mM Tris, pH 7.4, 150mM NaCl, 6mM beta-octyl glucoside, 1mM sodium orthovanadate, 10mM sodium fluoride, and complete Mini EDTA-free protease inhibitor cocktail tablet), cell debris pelleted by centrifugation at 8000 rpm at 4°C for 15 min, supernatant pre-cleared with protein A/G conjugate (Santa Cruz Inc.), and HuR antibody (Santa Cruz, Inc), and Protein A/G conjugate was added to supernatant and incubated with gentle rocking overnight at 4°C. Pellets were washed three times in IP buffer, and western blotting with anti-phospho-serine antibody (Abcam, Inc.) carried out as described above.

2.4 Immunocytochemistry.

hCaECs were grown to confluence in chamber slides, serum starved for 16 hours, and treated with either 16h IL-19, 6h TNF-α, or both. ECs were fixed in 10% NB formalin, permeabilized for 10 minutes in 0.2% triton-X100, and blocked for 25 minutes in 5% goat serum. ECs were incubated with either anti-HuR primary antibody or negative control mouse IgG for 1 hour, followed by incubation with goat anti-mouse Alexa Fluor (R) green

fluorescent secondary antibody (AF-488; Invitrogen) for 30 minutes, and DAPI counterstain for 5 minutes.

2.5 Transfection and siRNA knockdown.

Gene silencing was performed using ON-TARGET plus SMARTpool HuR siRNA , which contains a mixture of four siRNAs which target HuR (30 nM) purchased from Dharmacon, Inc. as we have described[69]. EC transfection was performed using the Human EC Nucleofector™ Kit (Amaxa, Inc.) following manufacturer's instructions. Transfection efficiency was 70-90% as assayed by dual transfection of a GFP reporter plasmid[47]. Lysates were immunoblotted for HuR 72 hours post-transfection and RNA was extracted 72 hours post-transfection.

2.6 Monocyte adhesion assay.

Adhesion was assayed as described[82]. Briefly, hCaECs were cultured on glass coverslips at a density of 6×10^5 cells/chamber. Confluent ECs were treated with IL-19 (100ng/ml) for 16 hours, then in the presence or absence of TNF-α (10ng/ml) for an additional 24 hours, followed by extensive washing with PBS. THP-1 human monocytes were purchased from the American Type Culture Collection (Cat# TIB-202) and cultured according to vendors instructions. THP-1 monocytes (5×10^5 cells/well) were labeled with 2′,7′-bis(2-carboxyethyl)-5(6)-carboxy-fluorescein acetoxymethyl ester (BCECF/AM, 10 μM, Sigma) and incubated with hCaECs for 30 min at 37°C. Unbound THP-1 cells were removed by gently washing twice with PBS, and adherent cells fixed with 4% formalin, photographed by an inverted fluorescent microscope (Eclipse TS-100, Nikon), and counted per high power field. For some experiments, anti-IL-20Rα (R&D Inc. Cat# MAB11762) or negative control antibody was added to ECs 2 hours prior to addition of

IL-19. Results are expressed as percentages of the controls and represent mean ± SEM from triplicate experiments.

2.7 Intravital microscopy, animal care.

Leukocyte rolling and adhesion were assayed in mesenteric post-capillary venules by intravital microscopy as we have described[83]. Mice (20g in weight) were then injected with 10.0ng/g IL-19 i.p. followed 16 hours later by 20.0 ng/g/weight TNF-α i.p. Rolling and adhesion were quantitated 4h following TNF-α injection. Wild-type C57BL/6 mice were injected with 120mg/kg sodium pentobarbital by i.p. injection. Depth of anesthesia was monitored by toe-pinch and blood pressure. Three to four straight, unbranched segments of post-capillary venules with lengths of >100 μm and diameters between 25 and 40 μm were studied in each mouse using an Eclipse FN1 Microscope (Nikon Corp.), and the image recorded and analyzed on a WIN XP Imaging Workstation. Leukocyte rolling was defined as the number of leukocytes rolling past a fixed point per minute; leukocyte adherence was defined as the number of leukocytes firmly adhered to 100-μm length of endothelium for at least 30 seconds. Venular blood velocity (V) was measured using the Microvessel Velocity OD-RT optical Doppler velocimeter (Circusoft Instrumentation) with corresponding software. Venular wall shear rate (γ) was calculated using the formula: $\gamma = 4.9\times8(V_{mean}/D)$, where D is the venule diameter[84]. Euthanasia was by injection of 160mg/kg pentobarbital, then exsanguination under anesthesia. Systemic leukocytes counts were determined using the Leuko-TIC® Kit (Bioanalytic) according to manufacturer instructions with an enhanced Neubauer hemocytometer. All animal procedures followed Temple University IACUC-approved protocols and conformed to the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH, 8th ed., 2011).

2.8 RNA extraction and quantitative RT-PCR.

RNA was isolated using the RNeasy Mini Kit (Qiagen) and reverse transcribed into cDNA using the Superscript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) as we have described, and target genes were amplified using Maxima^{TM} SYBR Green/ROX qPCR Master Mix (Fermentas) in an Eppendorf Realplex4 Mastercycler[69]. Multiple mRNAs (Ct values) were quantitated simultaneously by the Eppendorf software. Primer pairs were purchased from Integrated DNA Technologies, (Coralville, IA). The following primer pairs were used:

GAPDH:

F: CGAGAGTCAGCCGCATCTT,

R: CCCCATGGTGTCTGAGCG;

ICAM-1

F: CTCCAATGTGCCAGGCTTG,

R: CAGTGGGAAAGTGCCATCCT;

VCAM-1

F: TTCCCTAGAGATCCAGAAATCGAG,

R: CTTGCAGCTTACAGTGACAGAGC;

VLA-4 β1 integrin

F: GCGGAAAAGATGAATTTACAACCA,

R: TTCTCCACATGATTTGGCATTTGGCATTTG;

E-selectin

F: TCTGGGAATTGGGACAACG

R: CCTCACAGGTGAAGTTGCAG;

P-selectin

F: TTCCATTGTCTAGAGGGCC

R: AATGGTCCTTGGCAGGTTG;

HuR

F: CCGTCACCAATGTGAAAGTG

R: TCGCGGCTTCTTCATAGTTT;

2.9 MicroRNA

MicroRNAs were isolated in TRIzol® Reagent (Ambion, Life Technologies, Carlsbad, CA) and purified according to manufacturer's instructions. MicroRNA reverse transcription was carried out using the miScript II RT Kit (Qiagen, Hilden, Germany) and quantitation was performed using the miScript SYBR® Green PCR Kit and controlled against the housekeeping gene U6B. Specific primers for human microRNA-133a and microRNA-133b were obtained from Qiagen as well as miScript microRNA mimics and inhibitors for each.

2.10 Luciferase

Measurement of NF-κB activity was carried out using an NF-κB metridia luciferase vector (gift from the laboratory of Dr. Satoru Eguchi). CaECs were co-transfected with NF-κB-met-luc and pSEAP2 (secreted alkaline phosphatase) using the Human EC Nucleofector Kit (Amaxa, Inc.) and then treated for various times with IL-19 (100ng/ml) and TNF-α (10ng/ml). Luciferase activity was quantitated using a luminometer and the Ready-To-Glow™ Secreted Luciferase Reporter System (Clontech, Mountain View, CA).

The VCAM-1 promoter luciferase vector, pGL3-hVCAM1, containing 1.7 kilobase of the region upstream of the VCAM-1 gene, and pGL3-hVCAM1-NFκBMut, containing the same bases but with deletions of the tandem NF-κB elements[85], were obtained as a gift from the laboratory of Dr. Dennis Bruemmer (University of Kentucky) and were co-transfected with renilla luciferase vector (for normalization control) into CaECs using the Human EC Nucleofector Kit (Amaxa, Inc.) Luciferase activity for these vectors was quantitated using a luminometer and the Promega Dual Luciferase® Reporter Assay (Fitchburg, WI)

2.11 Statistical analysis.

Results are expressed as mean ± SEM. Differences between groups were evaluated using one-way ANOVA. Individual mean differences were evaluated using the Newman-Keuls post-test and confirmed by paired t-tests where appropriate. Differences were considered significant at a level of $p < 0.05$.

CHAPTER 3

RESULTS

3.1 IL-19 reduces TNF-α-induced cell adhesion molecule expression in human ECs.

We previously demonstrated that IL-19 is expressed in cultured human endothelial cells in response to inflammatory stimuli[72]. Considering that IL-19 has putative anti-inflammatory effects, we tested if IL-19 treatment could decrease expression of adhesion molecules on human endothelial cells. Human ECs were pre-treated with IL-19 at different times prior to stimulation with TNF-α to induce expression of ICAM-1 and VCAM-1.

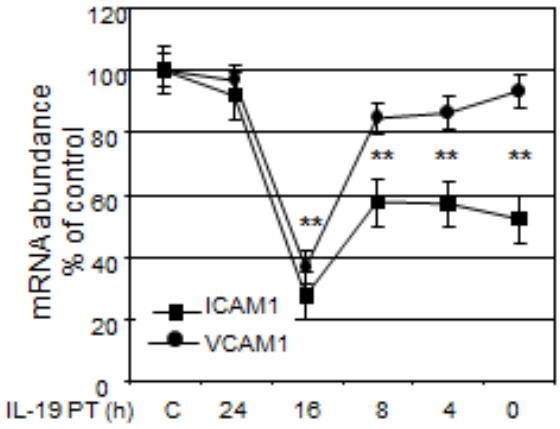

Figure 4. IL-19 reduces ICAM-1 and VCAM-1 mRNA Abundance in ECs. ECs were pre-treated with IL-19, then stimulated with TNF-α. Six hours later, mRNA was quantitated by qRT-PCR and normalized to GAPDH. $p < 0.01$.

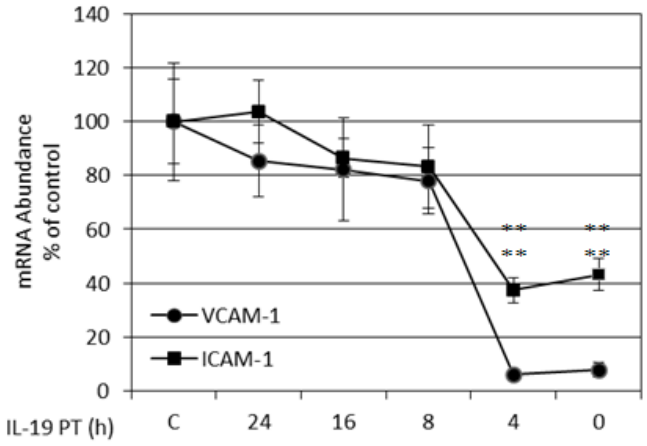

Figure 5. IL-19 reduces ICAM-1 and VCAM-1 mRNA Abundance in VSMCs. VSMCs were pre-treated with IL-19, then stimulated with TNF-α. Six hours later, mRNA was quantitated by qRT-PCR and normalized to GAPDH. $p < 0.01$.

Figures 4 and 5 demonstrate that IL-19 pre-treatment can significantly decrease both ICAM-1 and VCAM-1 mRNA abundance as measured by qRT-PCR in CaECs and VSMCs. In VSMCs, 4 hour pre-treatment (37.5±4.7% and 6.1±1.7% of control cells for ICAM-1 and VCAM-1 respectively, $p < 0.01$)and simultaneous addition (43.2±5.9% and 7.8±2.8% for ICAM-1 and VCAM-1 respectively, $p < 0.01$) addition of IL-19 is most effective in reducing CAM mRNA abundance. In CaECs, 16 hour IL-19 pre-treatment is the most effective time for reduction of mRNA abundance (26.0±7.7% and 36.4±5.2% of control cells for ICAM-1 and VCAM-1 respectively, $p < 0.01$). The inhibitory effect of a single addition of IL-19 to culture media is transient, as the mRNA abundance of both molecules returned to basal levels with 24h IL-19 pre-treatment. In ECs, VCAM-1 mRNA suppression is less sensitive to IL-19 treatment than ICAM-1, as 4 hour pre-treatment

and addition of IL-19 simultaneously with TNF-α have no suppressive effect on VCAM-1 mRNA levels.

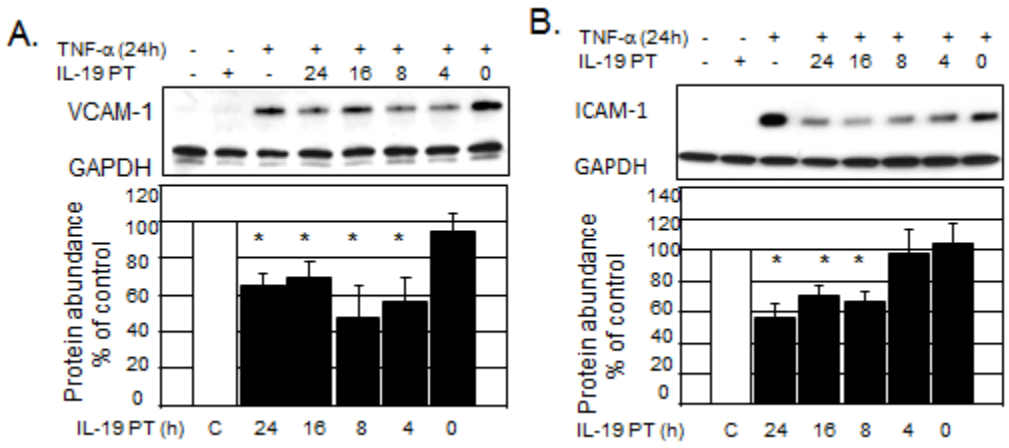

Figure 6. IL-19 reduces ICAM-1 and VCAM-1 protein abundance *in vitro*. ECs were pre-treated with IL-19, then stimulated with TNF-α. 24 hours later, protein extracts were prepared in SDS sample buffer and SDS-PAGE performed. Western blot was quantitated by densitometry from at least 3 experiments. $p < 0.05$

Figures 6A and 6B show that IL-19 pre-treatment between 4 and 24 hours significantly decreases VCAM-1 protein abundance and IL-19 pre-treatment between 8 and 24 hours significantly decreases ICAM-1 abundance on ECs.

*3.2 IL-19 can reduce leukocyte-endothelial cell adhesion***.**

Adhesion molecules are responsible for leukocyte-endothelial cell interactions, and we tested the hypothesis that IL-19 could reduce leukocyte-endothelial cell adhesion using an established endothelial cell monolayer adhesion assay[82].

ECs were cultured on glass cover slides, and were pre-treated with IL-19 for 16 hours (as this was the optimal time for decrease in ICAM-1 and VCAM-1 protein abundance), then with TNF-α for an additional 24 hours. THP-1 human monocytes were fluorescently labeled with BCECF/AM, added to EC monolayers, and adherent cells quantitated by microscopy (images shown in Figure 7A). Figure 7B shows that IL-19 significantly decreases THP-1 adhesion to human ECs (165.3±15.1 vs 68.3±12.3 adherent cells per HPF for TNF-α and IL-19 pre-treated respectively, $p < 0.01$). IL-19 signals through the IL-20 receptor complex[41]. Neutralization of the IL-20 receptor with specific antibody negates IL-19-induced decrease in THP-1 adhesion (87.4±36.1 vs 179±30.4 adherent cells per HPF TNF-α+IL-19 and TNF-α+IL-19 and IL-20R antibody), (Figure 8).

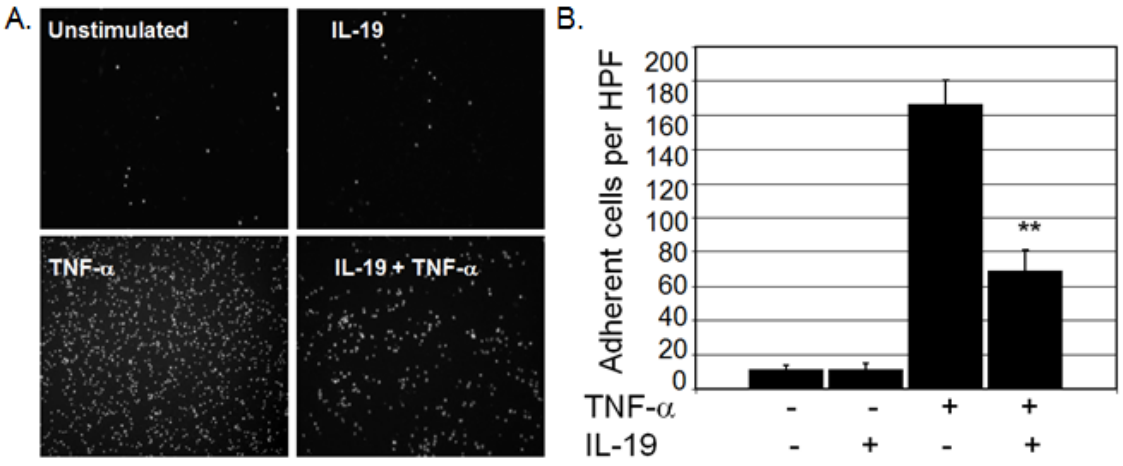

Figure 7. IL-19 reduces leukocyte-EC interactions *in vitro*. ECs were grown on glass coverslips and were treated with IL-19 for 16 hours, followed by treatment with TNF-α. 24 hours later, ECs were incubated with fluorescently labeled THP-1 monocytes for 30 minutes, followed by washing and fixation. A. Adherent THP-1 were quantitated by counting per high-power field. Photograph shown is representative (200X magnification). B. Results represent mean ± SEM for five high-power fields from three independent experiments. $p < 0.01$.

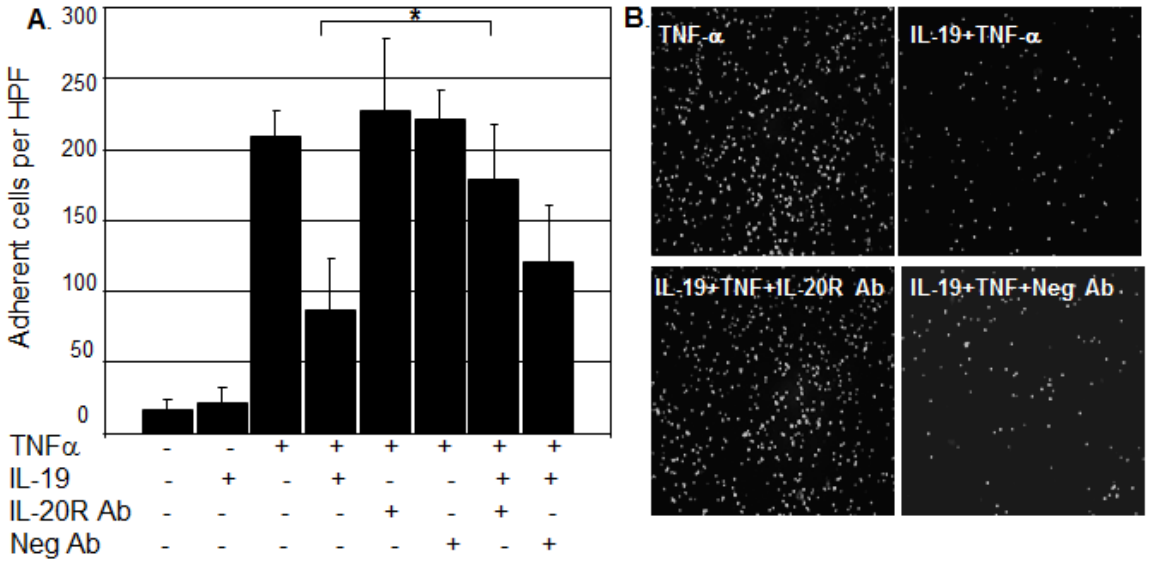

Figure 8. IL20Rα-neutralizing antibody negates IL-19-mediated inhibition of leukocyte-EC interactions. ECs were grown on glass coverslips and were treated with control IgG or IL20Rα-specific IgG (1μg/mL) followed 24 hours later by IL-19 (16 hours, 100ng/ml), followed by treatment with TNF-α (10ng/ml). 24 hours later, ECs were incubated with fluorescently labeled THP-1 monocytes for 30 minutes, followed by washing and fixation. A. Results represent mean ± SEM for five high-power fields from three independent experiments. $p < 0.05$. B. Adherent THP-1 were quantitated by counting per high-power field. Photograph shown is representative (200X magnification).

We also tested the possibility that IL-19 could reduce expression of the VCAM-1 co-receptor VLA-4 on the surface of THP-1 monocytes. THP-1 cells were pre-treated with IL-19, then stimulated with TNF-α for 6 hours to induce expression, and VLA-4 β1 integrin subunit mRNA abundance quantitated by qRT-PCR.

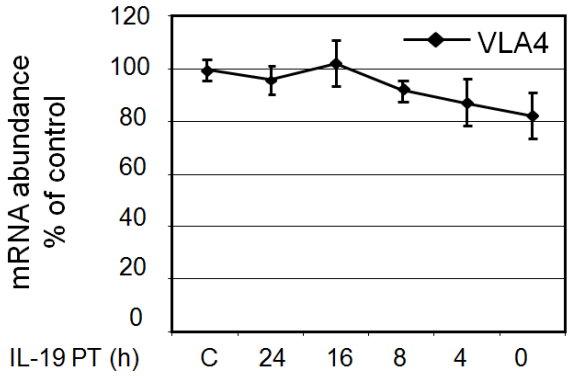

Figure 9. IL-19 does not reduce VLA-4 β1 integrin mRNA abundance in THP-1 monocytes. THP-1 were pre-treated with IL-19, then stimulated with TNF-α. Six hours later, mRNA was quantitated by qRT-PCR and normalized to GAPDH. No significant differences between groups were noted.

Figure 9 shows that IL-19 does not decrease VLA-4 mRNA abundance. Further, IL-19 pre-treatment of TNF-α-stimulated THP-1 cells does not significantly reduce THP-1-EC interaction when THP-1 cells are pre-treated with IL-19, nor does pre-treatment of THP-1

cells with IL-19 significantly decrease adhesion between TNF-α-stimulated THP-1 and TNF-α-stimulated ECs (Figure 10). These data suggest that IL-19 can reduce leukocyte-EC interaction by a reduction of adhesion molecules abundance on ECs.

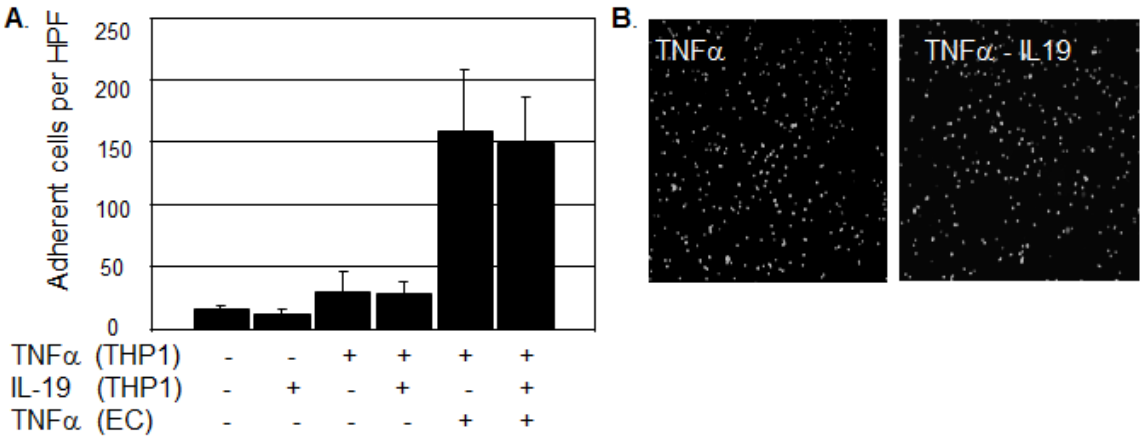

Figure 10. IL-19 pre-treatment of THP-1 cells does not inhibit leukocyte-EC interactions. THP-1 monocytes were treated with IL-19 (100 ng/ml) for 16 hours followed by treatment with TNF-α. Twenty-four hours later, the THP-1 cells were fluorescently labeled and incubated with ECs, grown on glass coverslips and treated with TNF-α for 24 hours. After 30 minutes of co-incubation, coverslips were washed and fixed. A. Results represent mean ± SEM for five high-power fields from three independent experiments. B. Adherent THP-1 were quantitated by counting in five high-power fields. Photograph shown is representative (200X magnification).

3.3. IL-19 reduces leukocyte-endothelial cell adhesion in vivo.

Intravital microscopy was used to determine if IL-19 treatment could reduce leukocyte-EC adhesion *in vivo*. Wild-type C57BL/6 mice were injected with a single dose of 10.0ng/g i.p. IL-19 16 hours prior to injection of TNF-α. Mice were then injected with a single dose of 20.0ng/g TNF-α to elicit leukocyte adhesion to endothelium[86,87]. Four hours later, rolling and adhesion in post-capillary venules were quantitated by intravital microscopy[83].

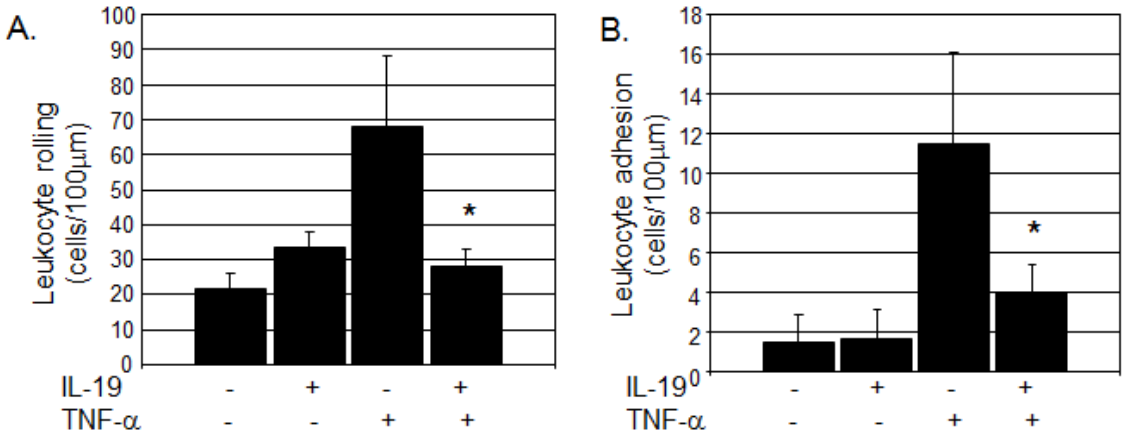

Figure 11. IL-19 reduces leukocyte-EC interactions *in vivo*. Wild-type C57BL/6 mice were treated with a single i.p. injection of IL-19 (10.0ng/g) followed 16 hours later by a single i.p. injection of TNF-α (20.0ng/g). After four hours, leukocyte rolling (A) and adhesion (B) were quantitated by intravital microscopy. IL-19 treatment significantly reduces leukocyte rolling ($p < 0.01$) and adhesion ($p < 0.05$), n=5 mice per group.

Figures 11A and 11B show that, similar to our observations in cultured endothelial cells, IL-19 pre-treatment can significantly reduce TNF-α-stimulated leukocyte rolling (67.6±20.4 vs 28.2±4.5 cells/minute for TNF-α and TNF-α pre-treated with IL-19, $p < 0.01$), and adhesion (11.5±5.6 vs 4.0±1.4 cells/100μm for TNF-α and TNF-α pre-treated with IL-19, $p < 0.05$). There was no statistical difference in adhesion between IL-19 pre-treated and control mice. There were no significant differences in microvascular and hemodynamic parameters between the TNF-α and IL-19 + TNF-α groups of mice (summarized in Table 3).

Table 3. Microvascular and Hemodynamic Parameters

Group	Average blood flow velocity (mm/s)	Wall shear rate(s^{-1})	Average venule diameter (μm)	No. of vessels	wbc (10^6)
Saline	1.12±0.33	1458±425	30.0±0.0	5	3.17±0.58
IL-19	1.16±0.08	1993±428	30.0±8.6	5	2.94±0.09
TNF-a	1.28±0.13	1699±372	32.5±9.5	5	1.48±0.09
IL-19+TNF-a	1.24±0.11	1665±260	30.0±5.0	5	1.57±0.20

Abbreviations: wbc; white blood cells

The reduction in *in vivo* rolling prompted us to investigate if IL-19 could modulate selectin expression in cultured ECs.

IL-19 failed to significantly decrease abundance of P-selectin in ECs at the 16 hour pre-treatment time point (Figure 12) which evoked inhibition of rolling in the *in vivo* model. However, IL-19 moderately, but significantly decreases TNF-α-driven expression of E-selectin mRNA (68.0±6.3% of control) and protein (Figure 13A and 13B) at the 16 hour time point.

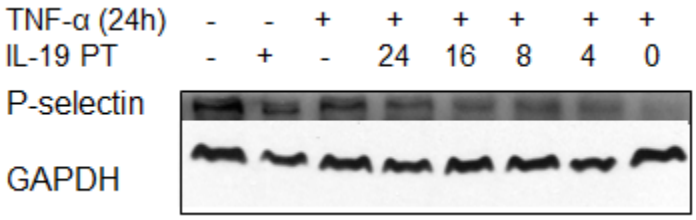

Figure 12. IL-19 does not reduce expression of P-selectin in cultured ECs. ECs were treated with IL-19 (100ng/ml), followed by TNF-α (10ng/ml). A. Six hours post-TNF-α, mRNA abundance was quantitated using qRT-PCR and normalized to GAPDH. $p < 0.5$. B. 24 hours post-TNF-α, protein extracts were prepared in SDS sample buffer and SDS-PAGE/Western Blot performed

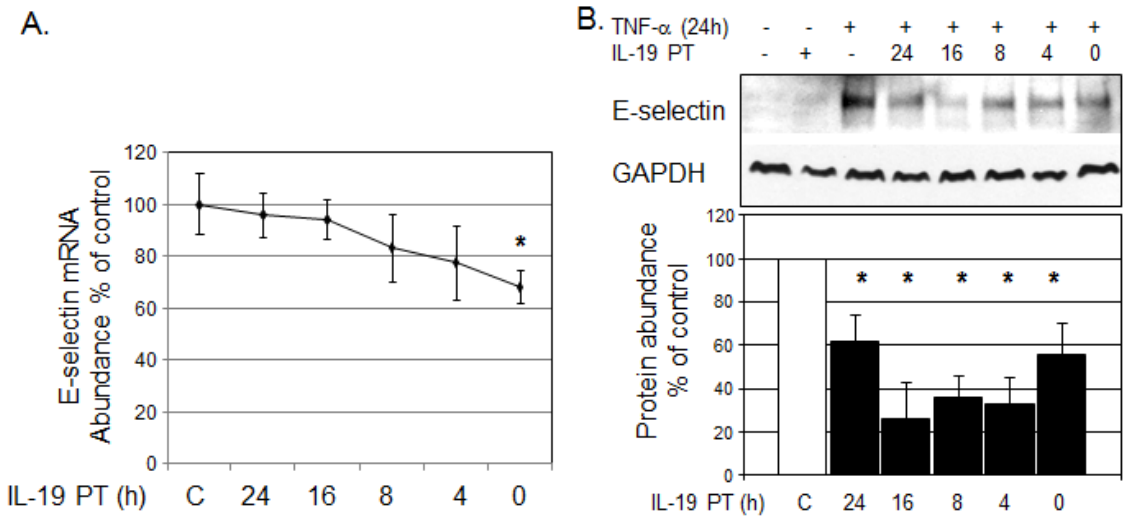

Figure 13. IL-19 reduces expression of E-selectin in cultured ECs. ECs were treated with IL-19 (100ng/ml), followed by TNF-α (10ng/ml). A. Six hours post-TNF-α, mRNA abundance was quantitated using qRT-PCR and normalized to GAPDH. $p < 0.5$. B. 24 hours post-TNF-α, protein extracts were prepared in SDS sample buffer and SDS-PAGE performed. Western blot was quantitated by densitometry of at least 3 experiments. $p < 0.05$.

3.4 IL-19 reduces cell adhesion molecule abundance in an NF-κB-independent mechanism.

To determine a molecular mechanism for IL-19 reduction of EC adhesion molecules, four experiments were performed to determine if IL-19 reduced CAMs expression by inhibition of NF-κB. TNF-α was used to activate NF-κB. First, TNF-α-induced degradation of inhibitor of κB (IκB), which allows activation and nuclear translocation of NF-κB, is not altered by IL-19 treatment (Figure 14).

Second, IL-19 has no inhibitory effect on phosphorylation of the NF-κB p65 subunit (Figure 15).

Third, TNF-α-driven nuclear translocation of p65 was not inhibited by IL-19 pre-treatment (Figure 16).

Fourth, to respond to the possible objection that Figures 14 – 16 do not measure the effect of IL-19 on NF-κB-driven *transcription*, we measured the effect of IL-19 on NF-κB-driven luciferase expression.TNF-α-driven pNF-κB-met-luc luciferase activity failed to be inhibited by IL-19 in cultured CaECs (Figure 17). The pNF-κB-met-luc vector consists of the metridia luciferase gene driven by tandem NF-κB promoter elements but without AU-rich elements in its 3' UTR, meaning that changes in the luciferase activity can be attributed to NF-κB-driven *transcription* of the luciferase gene and not ARE-mediated changes in luciferase mRNA stability.

These data suggest that IL-19 decreases cell adhesion molecule abundance in an NF-κB-independent mechanism, and prompted us to investigate other potential mechanisms for IL-19 inhibitory effects.

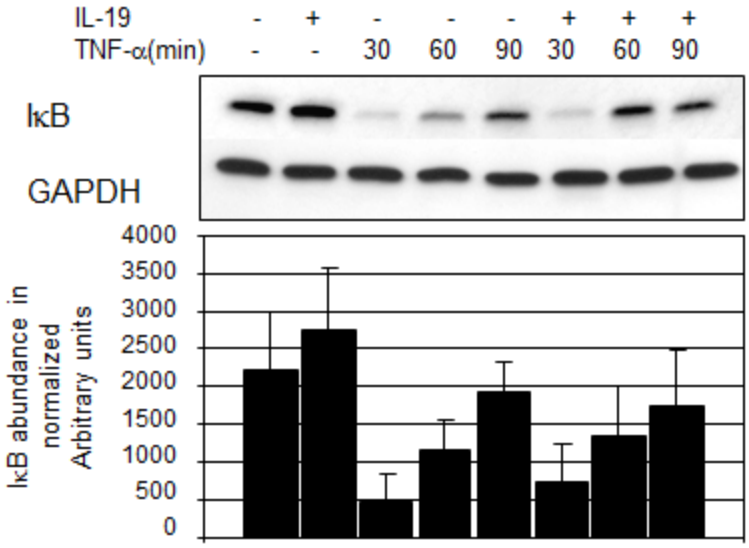

Figure 14. IL-19 does not inhibit IκB degradation. ECs were cultured in serum-reduced medium, pre-incubated with IL-19 for 16 hours, then stimulated with TNF-α. IL-19 does not decrease degradation of IκB in response to TNF-α stimulation; lysates immunoblotted with anti-IκB and GAPDH antibody. There is no statistical difference in band intensity between IL-19-treated and un-treated EC.

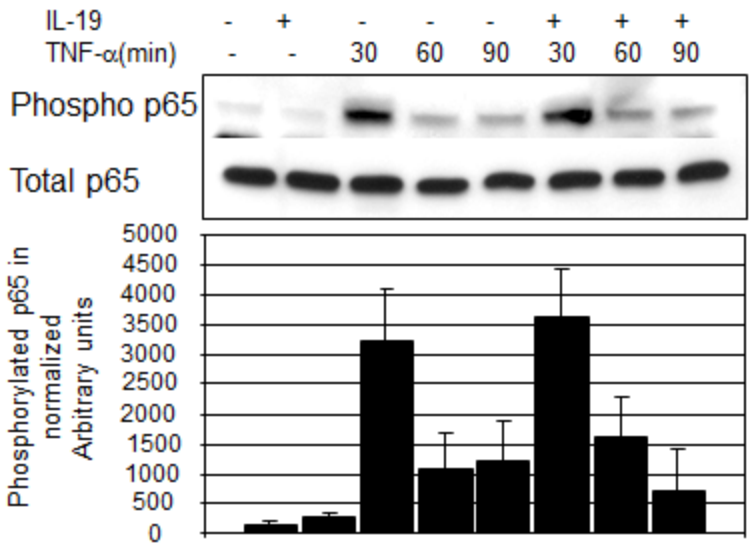

Figure 15. IL-19 does not inhibit phosphorylation of NF- κB p65 subunit. Lysates were blotted with anti-phospho-NF- κB p65 subunit. There is no statistical difference between IL-19-treated and un-treated ECs.

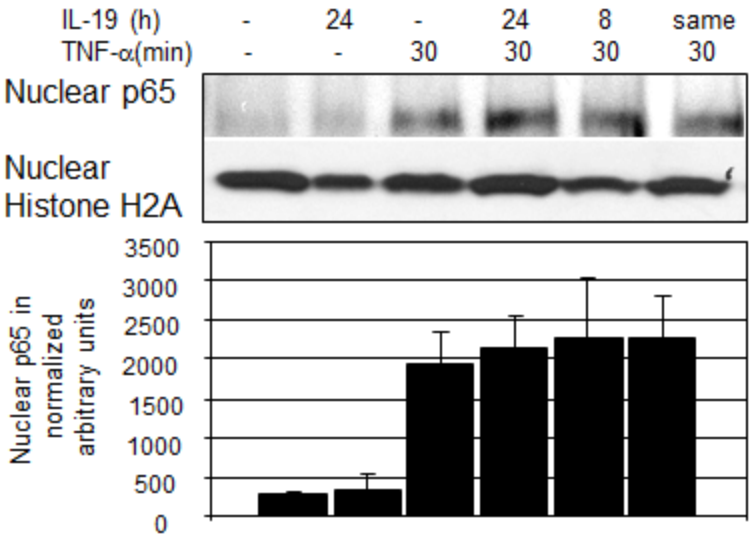

Figure 16. IL-19 does not inhibit nuclear translocation of p65. ECs were pre-treated with IL-19, then stimulated with TNF-α for 30 minutes to induce NF-κB translocation. Lysates were fractionated, and nuclear fraction immunoblotted with p65 antibody. Proteins were detected by western blot. Images are representative of at least 4 experiments. There is no significant difference in intensity of bands for any experiment.

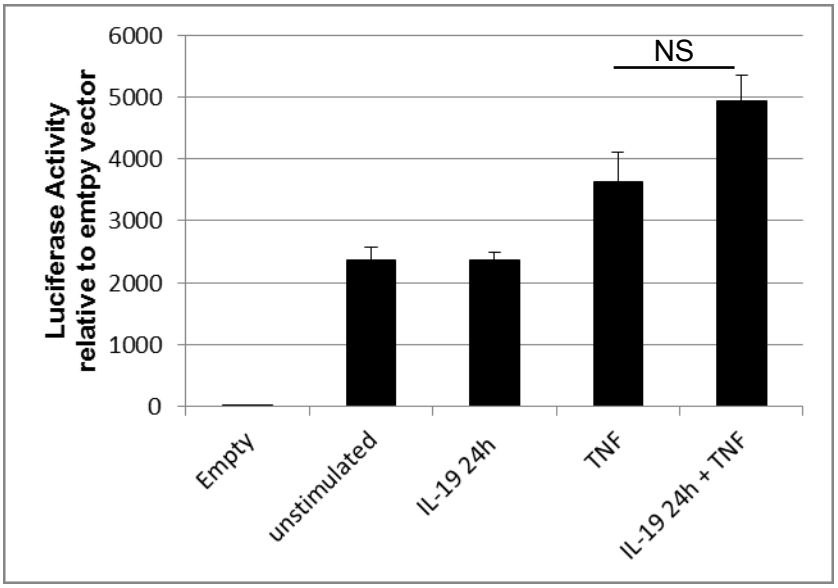

Figure 17. IL-19 does not reduce NF-kB-luciferase activity. Cultured CaECs were co-transfected with pNF-κB-met-luc vector and pSEAP2 vector (as normalization control) and treated for times shown with IL-19 (100ng/ml) and TNF-α (10ng/ml). Luciferase activity was quantitated, normalized to pSEAP2 activity, and plotted relative to the normalized luciferase activity of the empty vector (E) control.

Knowing that NF-κB is but one of the inflammation-responsive transcription factors which can drive CAM expression, we next explored whether IL-19 can reduce VCAM-1 promoter-driven luciferase activity with both intact VCAM-1 promoter and VCAM-1 promoter in which the tandem NF-κB responsive elements were mutated (Figure 18). As expected, mutation of the NF-κB elements caused decreased TNF-α-driven promoter activity. IL-19 was able to significantly reduce VCAM-1 promoter activity in both intact and NF-κB mut ECs, indicating that IL-19 can inhibit VCAM-1 expression at the transcriptional level but suggesting that it may do so by regulating inflammation-responsive transcription factors other than NF-κB.

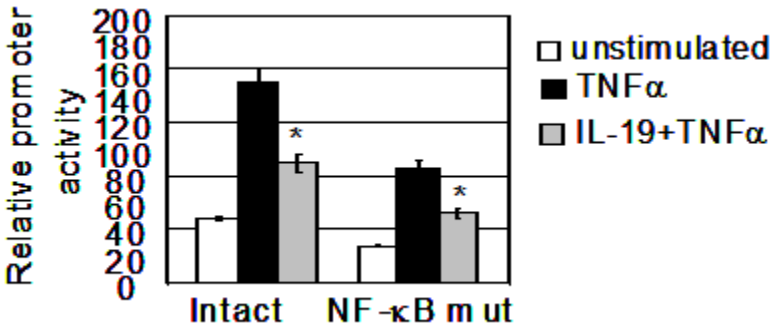

Figure 18. IL-19 Reduces VCAM-1 promoter activity with NF-kB elements intact and mutated. Cultured CaECs were transfected with pGL3-hVCAM-1 luciferase vector ("Intact" in axis label), containing the VCAM-1 promoter upstream of luciferase, and pGL3-hVCAM-1-NFκBMut ("NF-κB mut" in axis label), which contains the VCAM-1 promoter with its tandem NF-κB elements mutated. The CaECs were then treated with a 16 hour pre-treatment of IL-19 (100ng/ml) and then 24 hour stimulation with TNF-α (10ng/ml) as shown in the figure legend. Luciferase activity was quantitated and normalized to renilla luciferase activity and empty vector control. n = 3, $p < 0.05$.

3.5 IL-19 reduces TNF-α-driven HuR translocation and serine phosphorylation.

HuR (Human R antigen) is an mRNA stability protein which regulates the half-life of transcripts which contain AU-rich elements (AREs) in their 3' UTRs[88]. HuR has recently been implicated as a mediator of ICAM-1 and VCAM-1 expression in umbilical vein ECs[89]. Normally sequestered in the nucleus, nuclear-to-cytoplasmic translocation is required for HuR's mRNA stabilizing effects. In the absence of stimulus, HuR is re-located to the nucleus, causing its mRNA-stabilizing effects to be transient[90]. We tested to see if IL-19 can decrease HuR nuclear-to-cytoplasmic translocation.

First, we determined that 6-8 hours were optimal for TNF-α-driven HuR translocation (Figure 19A). Next, ECs were pre-treated with IL-19 for the indicated times, then stimulated with TNF-α for 6 hours, and the cytoplasmic fraction immunoblotted with anti-HuR antibody. Figures 19B and 19C shows that IL-19 can significantly reduce TNF-α-driven HuR cytoplasmic translocation, with 16 and 8 hours of pre-treatment being the most effective (28.0±13.9% and 25.6±17.7% of control for 16 and 8 hours pre-treatment respectively, $p < 0.05$). Immunocytochemistry confirms that IL-19 inhibits HuR nuclear-to-cytoplasmic translocation (Figure 20).

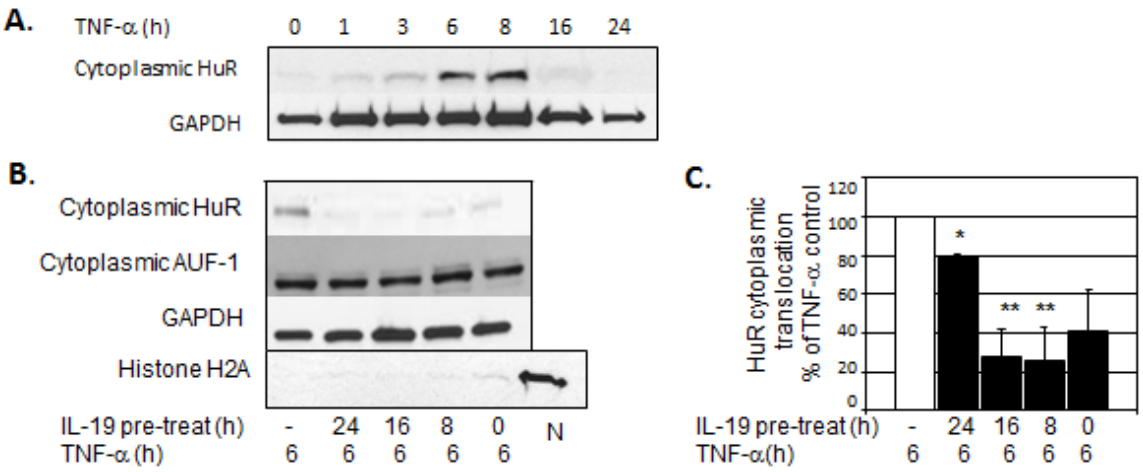

Figure 19. IL-19 reduces TNF-α-driven HuR cytoplasmic translocation in ECs. A. Time course of TNF-α-driven HuR cytoplasmic translocation. B. ECs were pre-treated with IL-19, then stimulated with TNF-α for 6 hours to induce HuR translocation. The cytoplasmic fraction of lysates were immunoblotted with anti-HuR antibody, anti-AUF-1 antibody, and anti-Histone H2A antibody as a nuclear protein control to show potential for unintended mixing of nuclear and cytoplasmic fractions. N is a Histone H2A positive control lane containing only nuclear fraction. C. Densitometry from at least 3 experiments determined significance ($P<0.05$).

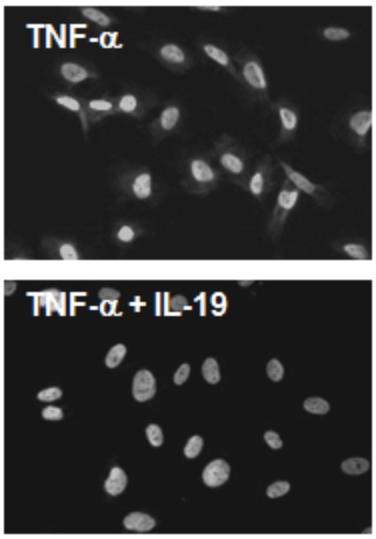

Figure 20. IL-19 reduces TNF-α-driven HuR cytoplasmic immunofluorescence in ECs. Immunocytochemistry showing 16 hour IL-19 pre-treatment reduces HuR cytoplasmic translocation. ECs grown on glass slides were treated for 16 hours with IL-19 prior to treatment with TNF-α. Six hours later, cells were fixed, permeabilized and immunostained using HuR-specific antibody.

IL-19 treatment does not reduce whole cell HuR protein abundance (Figure 21), nor does it increase AUF-1 (Figure 19B), an mRNA destabilizing protein which also regulates ARE-bearing transcripts[71].

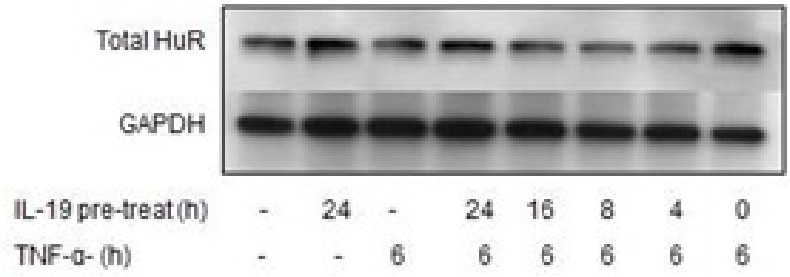

Figure 21. IL-19 does not decrease total HuR abundance in ECs. ECs were pre-treated with IL-19, then stimulated with TNF-α. 24 hours later, protein extracts were prepared in SDS sample buffer and SDS-PAGE performed. Western blot was quantitated by densitometry.

It was important to determine a mechanism for IL-19 inhibition of HuR cytoplasmic translocation. In mesangial cells, HuR cytoplasmic translocation is associated with

phosphorylation on serine residues[91], but this has not been reported in EC. Time course studies in ECs established that 30 minutes of TNF-α-stimulation resulted in maximal HuR phosphorylation (Figure 22A).

To determine if IL-19 treatment could reduce HuR serine phosphorylation, ECs were cultured in serum-reduced media, pre-treated with IL-19, then stimulated with TNF-α for 30 minutes. HuR was immunoprecipitated with HuR antibody, then blotted with phospho-serine antibody. Figure 22B demonstrates that 24 and16 hours of IL-19 pre-treatment significantly reduced TNF-α-driven serine phosphorylation of HuR (48.3±11.1 and 63.0±7.7% of TNF-α-phosphorylated control for 24 and 16 hours pre-treatment respectively).

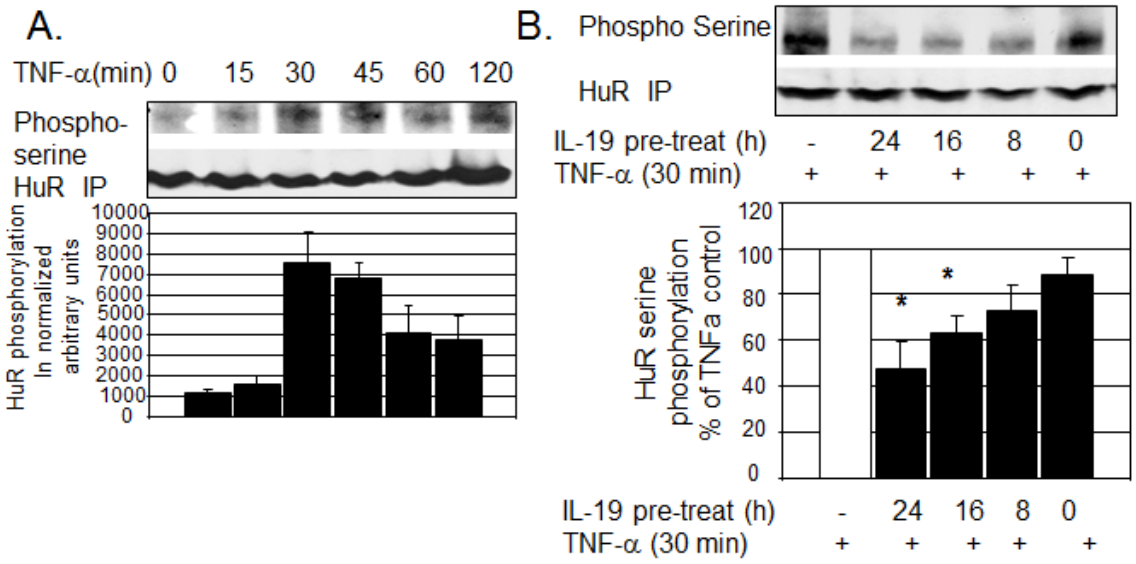

Figure 22. IL-19 reduces TNF-α-driven serine-phosphorylation of HuR. A. TNF-α induces serine phosphorylation of HuR. ECs were serum reduced, then stimulated with TNF-α for the indicated times. HuR was immunoprecipitated with HuR antibody, then blotted with anti-phosphoserine or HuR antibody as a loading control. B. IL-19 decreases HuR serine phosphorylation. Serum-reduced ECs were pre-treated with IL-19, then stimulated with TNF-α for 30 minutes. HuR was immunoprecipitated with HuR antibody, then blotted with anti-phosphoserine or HuR antibody. Blots shown are representative of at least 3 experiments ($p < 0.05$).

3.6 IL-19 decreases ICAM-1 and VCAM-1 mRNA stability.

IL-19 inhibition of HuR translocation suggested that one mechanism whereby IL-19 could reduce CAM abundance was by promoting a decrease in mRNA stability. Three approaches were used to test this hypothesis. First, ECs were pre-treated with IL-19, then stimulated with TNF-α for 6 hours to induce expression of CAM mRNA, then treated with the transcription inhibitor actinomycin D. Target mRNA was quantitated by qRT-PCR.

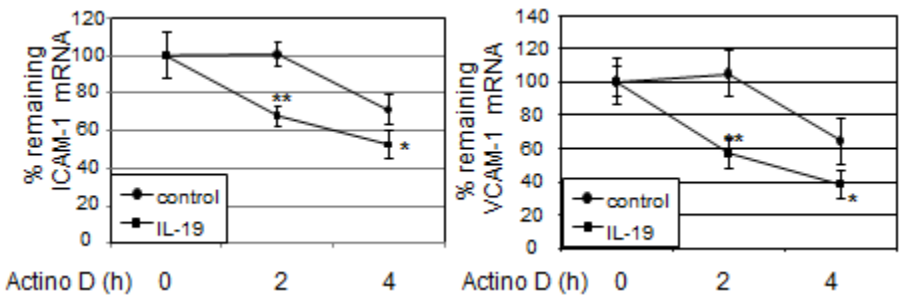

Figure 23. IL-19 reduces ICAM-1 and VCAM-1 mRNA stability in cultured ECs. ECs were pre-treated with IL-19 for 16 hours, stimulated with TNF-α for 6 hours to induce expression of CAM mRNA, then treated with the transcription inhibitor Actinomycin D. mRNA was quantitated by qRT-PCR, normalized to GAPDH at the times indicated. IL-19 reduces stability of CAM mRNA transcripts at all times tested ($P<0.05$ or 0.01 for all).

Figure 23 shows that IL-19 can significantly reduce stability of adhesion molecule mRNA at all times tested (ICAM-1;100.4±6.6% vs 67.8±5.2% for control and IL-19 treated, VCAM-1; 105.7± 14.0% vs 57.3±6.8%, at two hours, $p < 0.05$ or 0.01 for all). To establish a relationship between IL-19 effects on CAM mRNA stability and HuR function, ECs were transfected with HuR siRNA or scrambled control siRNA, then treated with actinomycin D as described in the preceding section.

Figure 24C shows that HuR siRNA was effective in reducing HuR protein, and Figure 24A demonstrates that ICAM-1 and VCAM-1 mRNA stability is reduced when HuR abundance is decreased (ICAM-1; 96.8±11.8% vs 79.1±1.7% for control and siRNA transfected, VCAM-1; 57.2±6.8% vs 36.5±2.7% at four hours, $p < 0.05$ or 0.01 for all). To strengthen the relationship between HuR and leukocyte-EC adhesion, we reduced HuR protein abundance by siRNA, then assayed THP-1 adhesion. Figure 24B shows that siRNA knock down of HuR results in significantly less THP-1 adhesion compared with scrambled controls (137.1.3±17.1 vs 75.1±15.3 adherent cells per HPF for scrambled versus HuR siRNA transfected, respectively $p < 0.05$). Considering that IL-19 may reduce CAM abundance through mechanisms *other* than HuR modulation, we sought to discover whether IL-19 treatment could *further* reduce leukocyte-EC interactions *after* HuR knockdown. Therefore, to demonstrate a fundamental role for HuR in *IL-19-mediated* abolition of leukocyte-EC interaction, we reduced HuR content in ECs by siRNA. Some were pre-treated with IL-19 for 16 hours, then stimulated with TNF-α. We then assayed THP-1 adhesion using the previously described in vitro adhesion assay.

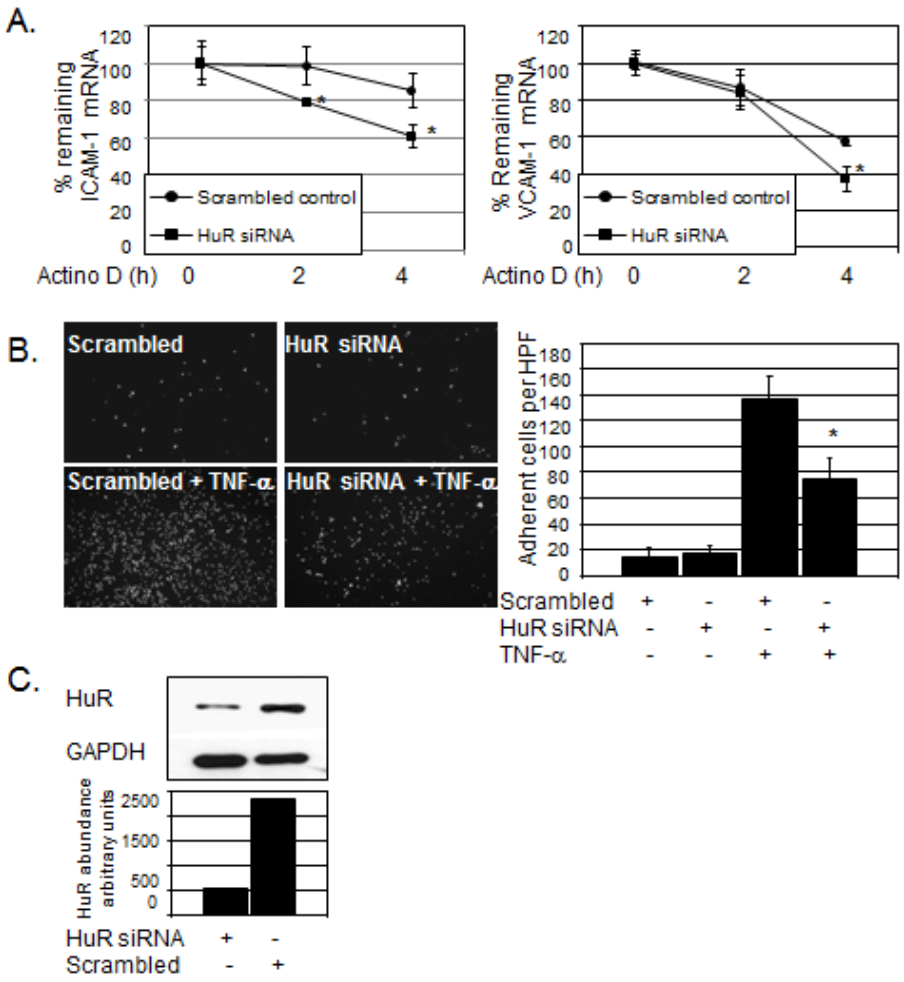

Figure 24. HuR siRNA reduces ICAM-1 and VCAM-1 mRNA stability and leukocyte-EC interactions *in vitro*. A. ECs were transfected with HuR siRNA or scrambled siRNA control, treated with Actinomycin D, and mRNA quantitated by qRT-PCR. ICAM-1 and VCAM-1 mRNA stability is significantly reduced when HuR abundance is decreased by siRNA, ($P<0.05$ or 0.01). B. HuR knock down reduces leukocyte-endothelial cell interaction. Experiment performed as described for Figure X, image shown is representative of 3. C. Representative western blot and densitometry of HuR knock down by specific siRNA.

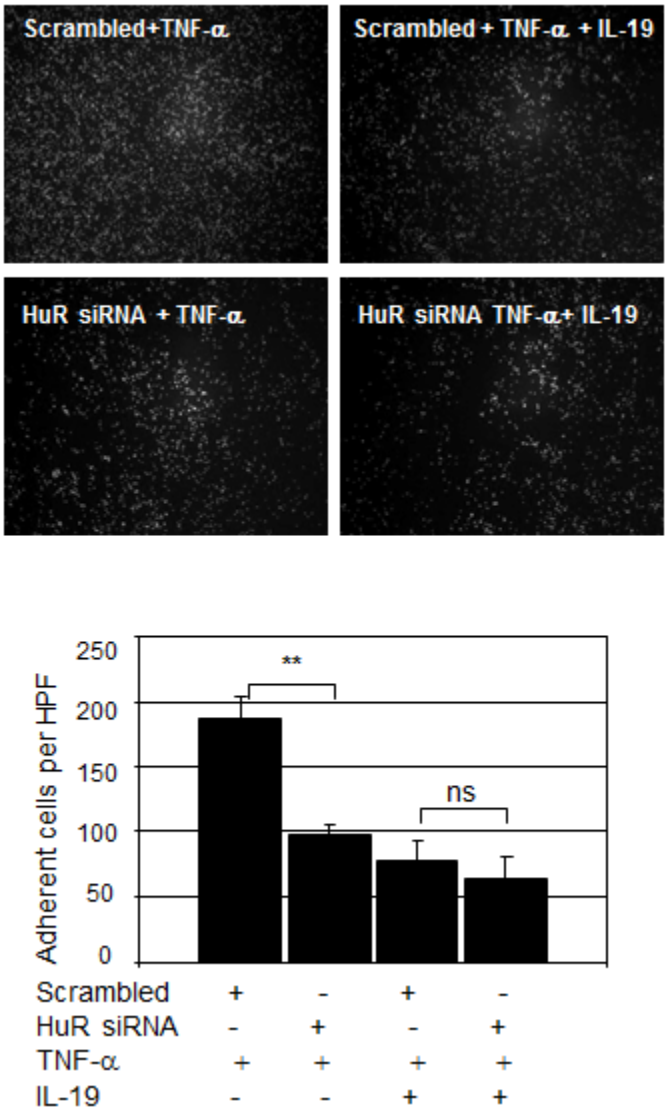

Figure 25. IL-19 does not further inhibit leukocyte-EC interactions in HuR-depleted ECs. ECs were transfected with scrambled or HuR siRNA. Some ECs were pre-treated with IL-19 prior to stimulation with TNF-α. Adhesion was performed as for Figure, $p < 0.01$. Adhesion in ECs pre-treated with IL-19 was not significantly different from HuR siRNA ECs not treated with IL-19. Image shown is representative of at least 3.

As expected, Figure 25 shows that IL-19 reduced leukocyte-EC interaction, as did HuR siRNA knock down. Importantly, however, no *additional* decrease in THP-1-EC adherence is observed after HuR is knocked-down (77.6±14.7 vs 64.0±17.3 adherent cells per HPF for HuR siRNA + TNF-α and HuR siRNA + IL-19 + TNF-α) suggesting that IL-19 acts to inhibit adhesion primarily through its modulation of HuR. Together, these results implicate HuR activity with stability of ICAM-1 and VCAM-1 mRNA, and leukocyte-EC interactions, and suggest that IL-19-mediated decreases in leukocyte-EC interactions are overwhelmingly mediated by modulation of HuR.

3.7 IL-19 up-regulates the HuR-targeting microRNA, miR-133

A previous report from our group has shown that IL-19 treatment of VSMCs causes a transient decrease in total cellular HuR *abundance* (mRNA and protein) as well as inhibition of HuR nucleocytoplasmic translocation[69]. Our results in CaECs have shown only inhibition of HuR translocation, but no transient decrease in total cellular HuR abundance. Exploring the possibility that IL-19 may up-regulate a VSMC-specific HuR-regulating factor, we discovered a putative binding site for the muscle-specific microRNA miR-133 in the 3' UTR of the HuR mRNA.

Figure 26 shows that IL-19 treatment up-regulates microRNA-133a in VSMCs, but not in CaECs—consistent with reports that miR-133 is a muscle-specific miRNA[92]. MicroRNA-133a is maximally increased at 4 hours (13.1±2.3-fold increase, $p < 0.01$), 6 hours (2.8±0.3-fold increase, $p < 0.05$), and 16 hours (4.3±1.0-fold increase, $p < 0.05$) treatment with IL-19.

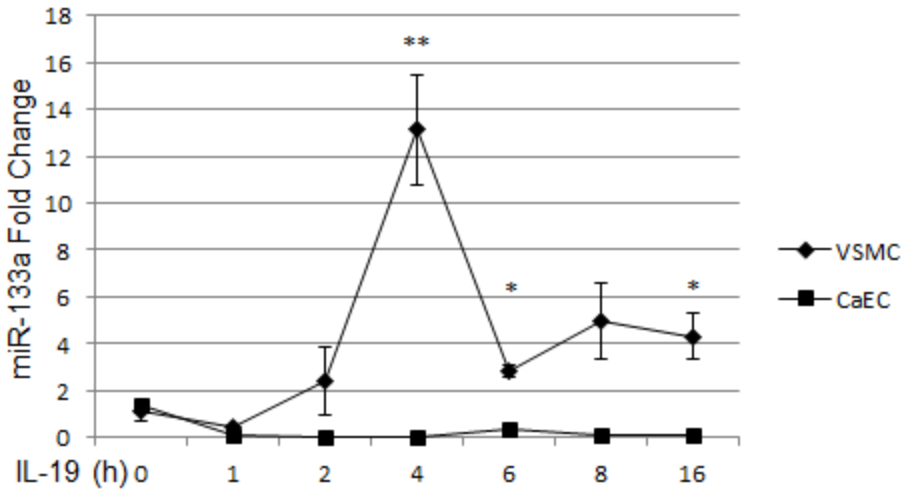

Figure 26. IL-19 increases vascular expression of miRNA-133a in VSMCs but not CaECs. Cultured VSMCs and ECs were treated with IL-19 (100ng/ml) for 16 hours, after which small RNAs were isolated, purified, and reverse transcribed. miR-133a miRNA abundance was measured by qRT-PCR using specific primers (Qiagen) and normalized to U6B. miRNA abundance is graphed relative to levels in unstimulated cells. n = 3.

To confirm the hypothesis that miR-133a can target and prompt the degradation of HuR mRNA, we transfected cultured VSMCs with miR-133a mimic (Qiagen). Figure 27 shows that miR-133a mimic over-expression produces a significant ($p < 0.01$) decrease in HuR mRNA abundance at 24 hours (69.1±5.0% of mock) and 48 hours (56.6±10.8% of mock) post-transfection.

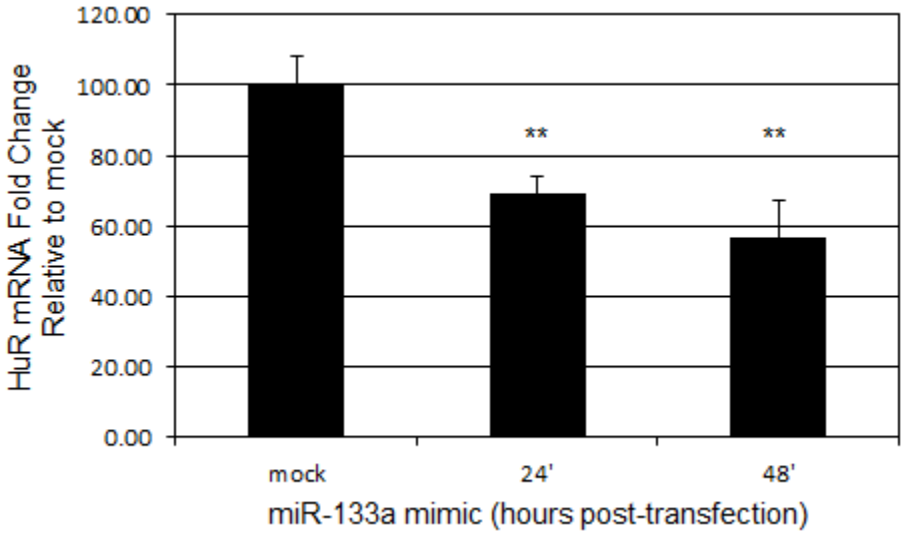

Figure 27. miR-133a over-expression causes reduction in HuR mRNA abundance. VSMCs were transfected with miR-133b mimic (Qiagen) and RNA extracted at the indicated time points post-transfection. RNAs were reverse transcribed and quantitated using qRT-PCR and normalized to GAPDH abundance. n = 3, $p < 0.05$.

3.8 IL-19 reduces TNF-α-driven vascular expression of chemotactic proteins

In order to further explore the anti-inflammatory effects of IL-19 in vascular cells, we examined the effects of IL-19 pre-treatment on TNF-α-driven expression of chemokines, MCP-1 and IL-8, and inflammatory mediator IL-1β.

Figure 28 demonstrates that IL-19 pre-treatment can significantly decrease IL-1β and IL-8 mRNA abundance as measured by qRT-PCR in CaECs. In CaECs, 16 hour IL-19 pre-treatment is the most effective time for reduction of mRNA abundance (15.4±8.9% and 16.1±3.7% of control cells for IL-1β, $p < 0.001$, and IL-8, $p < 0.05$, respectively). MCP-1 expression is reduced in IL-19 pre-treated ECs but not with statistical significance.

Figure 29 demonstrates that IL-19 pre-treatment can significantly decrease IL-1β, IL-8 and MCP-1 mRNA abundance as measured by qRT-PCR in VSMCs. In VSMCs, 4 hour and simultaneous IL-19 pre-treatment is the most effective time for reduction of mRNA abundance ($p < 0.01$). At 4 and 0 hours IL-19 pre-treatment, IL-1β mRNA abundance is reduced to 11.8±3.3% and 7.5±1.5% of control levels. At 4 and 0 hours IL-19 pre-treatment, IL-8 mRNA abundance is reduced to 5.0±0.0% and 2.7±0.4% of control levels. Finally, at 4 and 0 hours IL-19 pre-treatment, MCP-1 mRNA abundance is reduced to 23.0±5.1% and 19.8±3.2% of control levels.

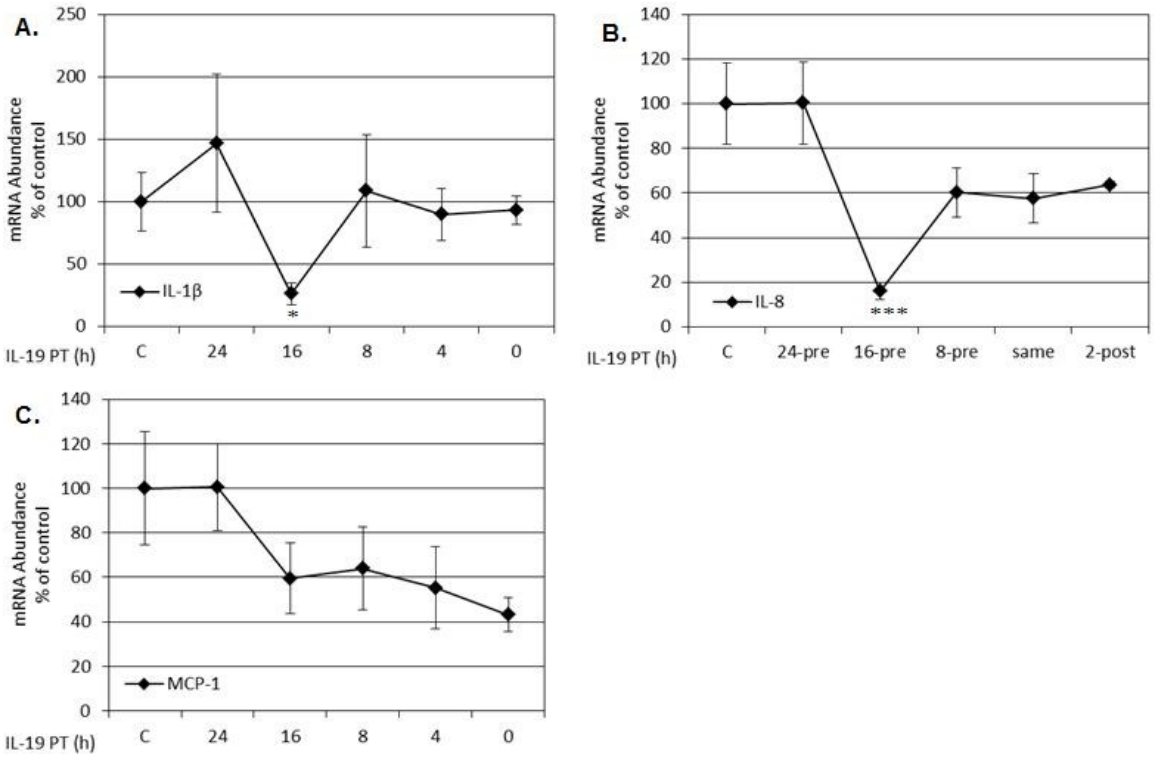

Figure 28. IL-19 inhibits TNF-α-driven chemotactic gene expression in CaECs. ECs. ECs were pre-treated with IL-19, then stimulated with TNF-α. Six hours later, mRNA was quantitated by qRT-PCR and normalized to GAPDH. $p < 0.01$.

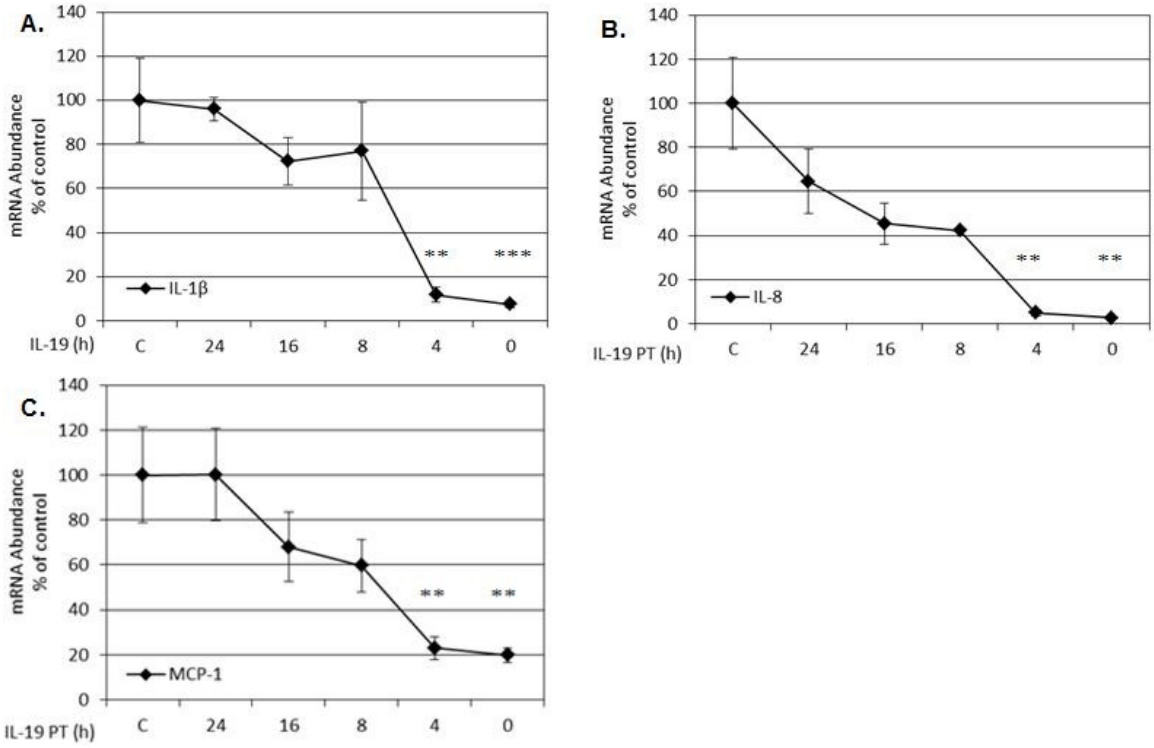

Figure 29. IL-19 inhibits TNF-a-driven chemotactic gene expression in VSMCs. ECs. VSMCs were pre-treated with IL-19, then stimulated with TNF-α. Six hours later, mRNA was quantitated by qRT-PCR and normalized to GAPDH. $p < 0.01$.

CHAPTER 4

DISCUSSION

Little has been reported on the direct effect of T_h2 interleukins on endothelial cell pathophysiology, and this is the first study to report that IL-19 can decrease expression of cell adhesion molecules, decrease leukocyte-endothelial cell interaction *in vivo*, diminish HuR activity in ECs, and regulate expression of microRNA-133. We propose IL-19 reduces leukocyte-EC adhesion through an HuR-mediated decrease in ICAM-1 and VCAM-1 mRNA stability, leading to a reduction in protein levels of these adhesion molecules.

T_h1 interleukin expression predominates in vascular inflammatory lesions[26]. However, we have previously reported that IL-19 was expressed in endothelium in murine atherosclerotic plaque and human coronary arteries from rejected cardiac allografts[48], suggesting a function for this cytokine in vascular inflammatory conditions. ICAM-1 and VCAM-1 are induced in ECs by pro-inflammatory conditions and subsequently promote the adhesion of leukocytes to the endothelium. It is also well known that ICAM-1, VCAM-1, and E-selectin are pro-atherosclerotic genes. ICAM-$1^{-/-}$ mice fed a western diet have reduced atherosclerotic lesion size when compared to wild-type C57BL/6 control mice[18,93]. VCAM-$1^{-/-}$ imparts an embryonic lethal phenotype, but effective experiments have been performed using a mutated VCAM-$1^{D4D/D4D}$, in which the fourth immunoglobulin domain is deleted yielding VCAM-1 mRNA and protein levels 8 percent of wild-type levels. VCAM-$1^{D4D/D4D}$ mice have reduced atherosclerotic lesion size when compared to wild-type controls[18,94,95]. Moreover, VCAM-$1^{D4D/+}$ mice have greater lesion size than homozygous VCAM-$1^{D4D/D4D}$ but still reduced lesion size when compared with

wild-type mice, indicating that VCAM-1 has a dose-dependent effect on atherosclerosis development[96]. Double knockout (DKO) of E-selectin and apolipoprotein E (Sele$^{-/-}$, ApoE$^{-/-}$) prompts a modestly reduced lesion size[97] when compared with littermate controls, and triple knockout (TKO), P-selectin null (Sele-/-, Selp-/-, ApoE-/-) mice have up to 80 percent reductions in atherosclerotic lesion size[98] when compared to littermate controls.

Little has been reported on direct effects of T_h2 interleukins on EC expression of adhesion molecules, and most work in this area focuses on regulation of *leukocyte*, not EC, CAM expression. The prototypical T_h2 cytokine, IL-10, did reduce IFN-γ-driven ICAM-1 mRNA expression in human monocytes at the transcriptional level[99]. In the present study, pre-treatment of human coronary ECs with IL-19 significantly decreases TNF-α-induced expression of ICAM-1 and VCAM-1 mRNA and protein. By contrast, a single IL-19 *post*-treatment after a single TNF-α-stimulation had no inhibitory effect on mRNA abundance, similar to our observations in VSMC[48,69,72]. IL-19-mediated decreases in endothelial CAM expression was reflected functionally, as IL-19 can significantly reduce THP-1-EC interactions. IL-19 effects were EC-specific, as IL-19 treatment of THP-1 cells did not reduce expression of monocyte VLA-4, nor was THP-1 adhesion to ECs affected in the reverse assay in which only THP-1 cells were treated with IL-19. This study is the first to show a function for IL-19 in regulation of ICAM-1, VCAM-1, and E-selectin and strongly suggests the possibility that IL-19 has anti-atherosclerotic effects.

The limited time range in which IL-19 acts to regulate adhesion molecules (8-24 hours prior to stimulation in most experiments) should not be too readily interpreted as a limitation of the therapeutic potential of IL-19 to inhibit atherogenesis as, in these experiments, IL-19 was administered in a single dose and, thus, would be expected to

have a time point of peak efficacy consistent with its signaling kinetics. There is reason to expect IL-19 to produce therapeutic effects *in vivo*, both IL-19 and pro-inflammatory cytokines would be *continuously* expressed by inflamed ECs as well as inflammatory cells, and so a consistent fraction of IL-19 signaling would be expected to be reaching peak efficacy at any given time.

No data concerning IL-19 involvement in reduction of leukocyte-EC interaction have previously been reported. The IL-19 "relative," IL-10 can reduce IL-1β-driven monocyte-endothelial cell adhesion[100], although in this assay it was the monocytes that were treated, rather than the endothelial cells. Treatment of ECs with IL-10 had no inhibitory effect on leukocyte-HUVEC adhesion[70]. This is in contrast with our results indicating that IL-19 has no effect on adhesion when THP-1 cells are treated, further distinguishing IL-19 from IL-10 activity. In one interesting report, IL-10 did decrease ICAM-1 expression in ECs stimulated with LPS, but *not* when they were stimulated with TNF-α or IL-1β[101]. Treatment of human ECs with IL-10 can inhibit minimally oxidized LDL (MM-LDL)–induced monocyte-endothelium interaction and, similar to IL-19, 18 hours of pre-treatment were necessary for significant reduction[68]. However, in this manuscript, neither CAM expression levels, nor a mechanism for these effects were elucidated. Taken in total, our present data are remarkable in their demonstration of a mechanism whereby a T_h2 interleukin can have direct anti-inflammatory effects on cells *outside* of the T cell lineage.

Extending these data into *in vivo* studies, we determined that IL-19 could reduce TNF-α-induced leukocyte rolling and adhesion as quantitated by intravital microscopy. This is likely due to effects on ECs, as IL-19 does not decrease counter-receptor abundance on THP-1 cells, nor does it reduce adhesion when THP-1 cells are incubated with IL-19. It is

not yet known whether IL-19 can specifically affect adhesion molecule expression in neutrophils, which are known to comprise a portion of rolling and adhering cells in our model[102,103]. The reduction in *in vivo* rolling prompted us to investigate if IL-19 could modulate selectin expression on cultured ECs. P-selectin is constitutively expressed and not subject to strong regulatory control by cytokines[18] and, not surprisingly, TNF-α could not increase nor could IL-19 significantly decrease abundance of P-selectin in ECs. IL-19 did slightly, but significantly reduce E-selectin mRNA and protein in ECs, providing a possible mechanism for reduced rolling *in vivo*. The possibility exists that IL-19 could effect a conformational change in either of these molecules, or inhibit an additional molecule on ECs or leukocytes. While the *in vitro* reverse-adhesion assay suggested that IL-19 did not directly regulate monocyte CAM expression, this assay cannot measure the loose adhesion characteristic of the rolling phase of leukocyte migration. It, therefore, remains for future experiments to explore whether IL-19 may directly regulate a leukocyte-borne rolling molecule (e.g., L-selectin, PSGL-1) in an effort to determine the precise mechanism of IL-19 reduction in leukocyte rolling. It is also important to recognize that because ECs are heterogeneous, results obtained in mesentery may not exactly mimic those in arterial ECs. Nevertheless, because of their transparency, they provide utility and useful information on basic mechanisms which cannot be obtained on arterial vascular beds which are not accessible to study by intravital microscopy.

It is well understood that the major mechanism for transcription of ICAM-1 and VCAM-1 is activation of NF-κB[99]. Though none performed in ECs, other studies have shown that the anti-inflammatory effects ascribed to IL-10 are mediated by inhibition of NF-κB activity[104,105], and IL-10 can decrease expression of NF-κB-dependent gene transcripts in both monocytes and VSMC[105–107]. IL-10 can inhibit IFN-γ-induced NF-κB activation in

monocytes, but, in contrast to our study, did not enhance the rate of ICAM-1 mRNA degradation, suggesting that IL-10 does not alter ICAM-1 mRNA stability[99]. It was therefore somewhat unexpected that IL-19 did not inhibit or reduce TNF-α-driven NF-κB activation while nevertheless reducing transcript abundance by means of reducing transcript half-life. HuR is an mRNA stability protein which is a member of the ELAV family of mRNA stability proteins which regulate half-life[88] of mRNAs which bear conserved AU-rich elements in their 3' UTRs. The ability of HuR to stabilize mRNA corresponds with its translocation from a predominately nuclear location to the cytoplasm. IL-19 inhibition of TNF-α-driven nuclear-to-cytoplasmic translocation peaked at 16 hours pre-treatment, which correlates very well with the observed 16 hours of pre-treatment necessary for efficient inhibition of ICAM-1 and VCAM-1 mRNA abundance. Interestingly, IL-19 treatment does not decrease the overall abundance of HuR, which is in contrast to our previous report using VSMC and possibly reflects cell-specific differences[69]. Nuclear-to-cytoplasmic translocation is essential for HuR activity, and is regulated by serine phosphorylation[26,71,100]. A least one mechanism whereby IL-19 can decrease HuR translocation is by reduction of its serine phosphorylation, as IL-19 pre-treatment can transiently decrease serine phosphorylation of HuR. This decrease requires at least 16 hours of pre-treatment and is transient, implying that synthesis of a labile factor or factors is necessary for the detected decrease in phosphorylation. Future studies are necessary to characterize IL-19-sensitive signaling pathways which may affect HuR cytoplasmic shuttling.

Figures 30 and 31 provide schematic representations of the proposed mechanisms of TNF-α-driven CAM expression (Figure 30) and IL-19-mediated CAM inhibition (Figure 31).

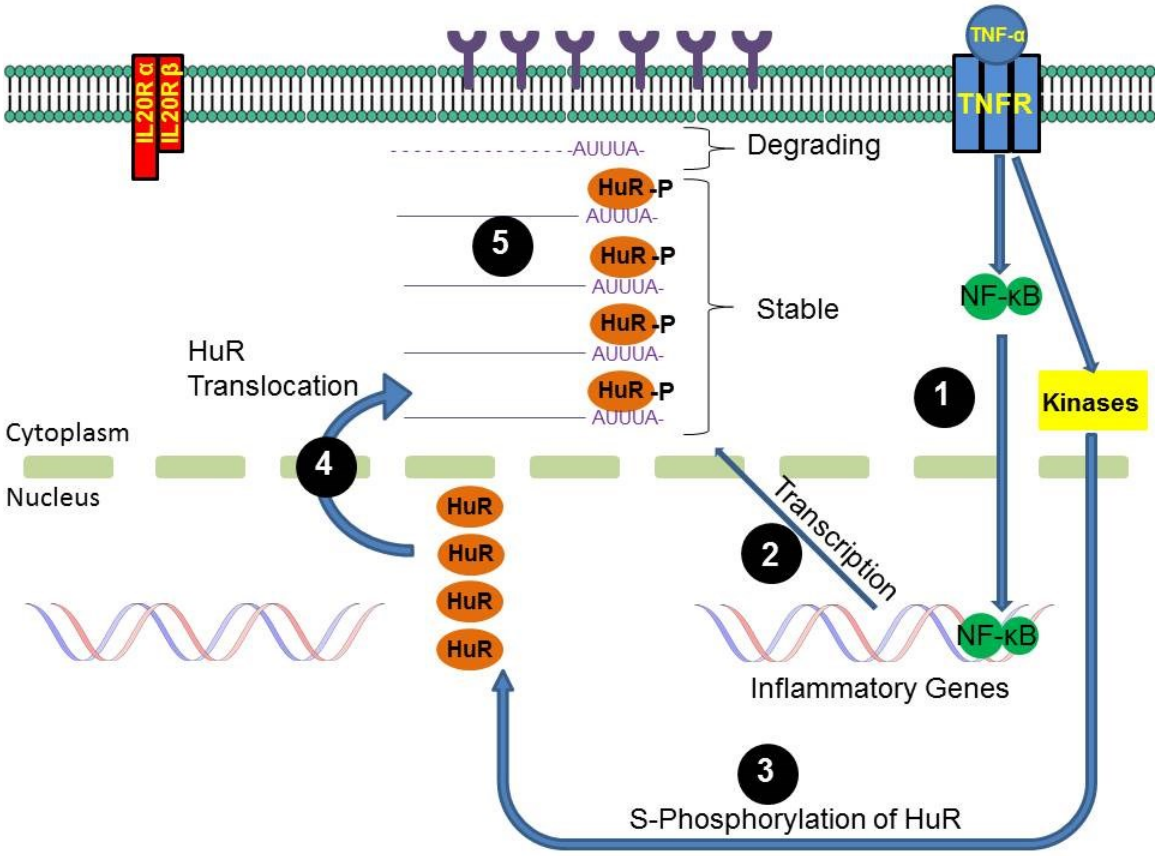

Figure 30. Mechanism of TNF-α-driven CAM expression. (1) Binding of TNF-α to the TNF receptor prompts the activation and translocation of the pro-inflammatory transcription factor NF-κB. (2) NF-κB transcribes inflammatory genes, including CAMs, which transcripts are shuttled into the cytoplasm. (3) TNF-α signaling also activates kinases which phosphorylate HuR on serine residues leading to (4) the translocation of nuclear HuR into the cytoplasm. (5) Cytoplasmic HuR stabilizes the NF-κB-transcribed CAM mRNAs, leading to the synthesis of CAM proteins.

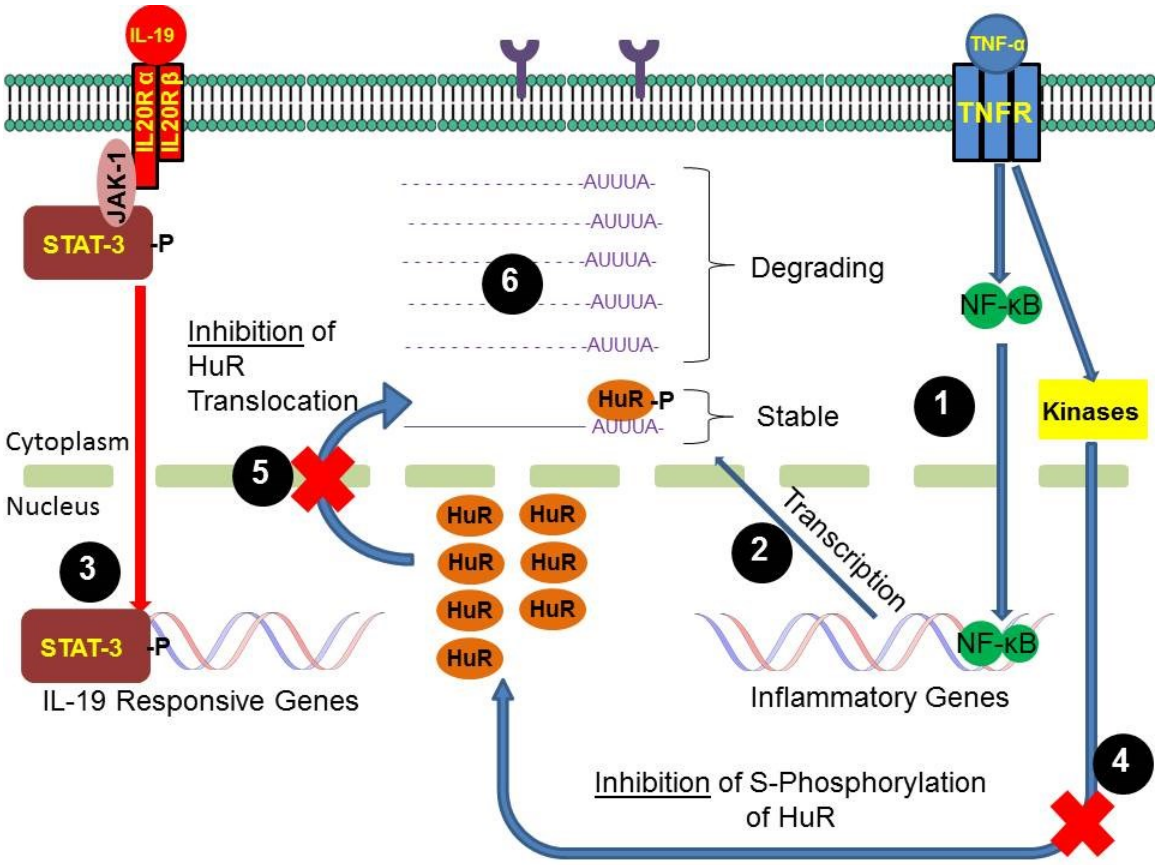

Figure 31. Mechanism of IL-19-mediated CAM inhibition. (1) Binding of TNF-α to the TNF receptor prompts the activation and translocation of the pro-inflammatory transcription factor NF-κB. (2) NF-κB transcribes inflammatory genes, including CAMs, which transcripts are shuttled into the cytoplasm. (3) Binding of IL-19 to the IL20Rα/IL20Rβ prompts JAK-1-mediated activation of STAT3, which moves to the nucleus and transcribes IL-19 mediated genes which result in (4) inhibition of serine-phosphorylation of HuR and (5) inhibition of translocation of HuR into the cytoplasm. (6) The lack of HuR in the cytoplasm causes NF-κB-transcribed CAM mRNAs to be unstable, leading to decreased expression of CAM protein.

While our group has previously shown that HuR nucleocytoplasmic translocation is inhibited in VSMCs, as well as the present data showing this to be the case in ECs, a remarkable difference exists in the responsiveness of total cellular HuR abundance to IL-19 treatment in the two cell types. VSMCs treated with IL-19 show a marked reduction in total HuR mRNA and protein abundance[69], whereas IL-19 produces no change in total HuR abundance in ECs. In seeking a probable mechanism for this disparity, we explored the possibility that a muscle-specific, HuR-targeting microRNA, miR-133, may be up-regulated by IL-19 in vascular smooth muscle and may there be the mediator of reduced HuR abundance. We have shown that microRNA-133a is up-regulated by IL-19 in VSMCs but not in ECs. We also showed that miR-133a does indeed possess the ability to regulate the HuR gene (previously only a prediction based on sequence homology) by transfecting VSMCs with miR-133a mimic and measuring consequent decreased HuR mRNA abundance.

In a broad sense, this study implicates HuR as a potential target of anti-inflammatory therapy. The 3′ UTRs of many inflammatory transcripts associated with inflammation contain AU-rich elements (AREs) which are target sites for HuR[70]. It is not entirely unexpected that ICAM-1 and VCAM-1 transcripts are sensitive to HuR modulation, as both possess numerous Class 1 AU-rich elements, consisting of conserved AUUUA pentamers, in their respective 3' UTRs (see Table 4). AU-rich elements are known to impart an extremely short half-life to transcripts which bear them. ICAM-1 and VCAM-1 are no exception to this principle. ICAM-1 mRNA has an extremely short half-life of ~50 minutes in unstimulated ECs—a half-life which increases to > 2 hours in cells treated with pro-inflammatory mediators IFN-γ and PMA[108], which evoke an upregulation of transcript-stabilizing cellular machinery, including translocation of HuR. VCAM-1 mRNA

has so short a half-life in unstimulated cells that it is not even detectable, while stimulation of ECs with TNF-α increases the half-life to 5 hours[109].

AREs are crucial in the ability of cytokines to increase the half-life of transcripts. When the AREs are removed from the ICAM-1 UTR, for instance, the transcript-stabilizing effects of IFN-γ and PMA are substantially abridged[108]—leading to a marked decrease in the ability of these cytokines to induce ICAM-1 expression.

Table 4. AU-Rich Element Loci in CAM mRNA Sequences

Gene	Accession	mRNA length (kb)	3'UTR	ARE loci
ICAM-1	NM_000201	3249	1918-3249	2419-23
				2446-50
VCAM-1v1	NM_001078	3220	2442-3220	1951-55
				2370-74
				2473-77
				2757-61
				2798-803
VCAM-1v2	NM_080682	2944	2166-2944	2094-98
				2197-201
				2523-27
VCAM-1v3	NM_001199834	3034	2256-3034	2184-88
				2287-91
				2613-17

Abbreviations: v1: transcript variant 1, v2: transcript variant 2, v3: transcript variant 3

While many inflammatory cytokines have been shown to stabilize inflammatory gene transcripts, few have been shown to *de*stabilize mRNA. This is the first report showing inhibition of HuR by an anti-inflammatory cytokine in ECs. There is one important study that demonstrated that siRNA depletion of HuR in HUVEC resulted in a significant decrease in lippopolysaccharide (LPS)-stimulated HUVEC-monocyte binding and LPS-*induction* of ICAM-1 and VCAM-1 mRNA transcripts[89]. Our present study in coronary artery EC extends this interesting work in two ways. First, it demonstrates that inhibition of HuR by IL-19 or HuR siRNA also decreases the *stability* of ICAM-1 and VCAM-1 mRNA transcripts. Second, our study also places HuR activity in a physiological context, with use of coronary artery ECs rather than human umbilical vein ECs, with ECs stimulated with the cytokine TNF-α rather than LPS, and inhibition of HuR translocation by an anti-inflammatory cytokine, rather than by knock-down with transfected siRNA. It is interesting to note that this same report suggested that HuR also decreased NF-κB activation, specifically through inhibition of NF-κB p65 phosphorylation, in contrast to our study in which IL-19 did not inhibit NF-κB p65 phosphorylation. One explanation may be that, in that study, total levels of HuR were decreased by specific siRNA, whereas IL-19 inhibits HuR nuclear-to-cytoplasmic translocation without reducing overall cellular HuR levels, which may allow remaining *nuclear* HuR to modulate NF-κB. One limitation of our present study is that other transcription factors which may play a role in adhesion molecule expression, such as Sp1, AP-1, IRF-1, and GATA-binding proteins[85], have not been completely eliminated as potentially sensitive to IL-19 effects. Indeed, when the NF-κB binding elements in the VCAM-1 promoter are mutated, IL-19 still showed a capacity to reduce transcription of VCAM-1 promoter-driven luciferase, suggesting that IL-19 may have an effect on one or more other transcription factors. However, if these other transcription factors are targets of IL-19, their contribution is likely very small,

because when HuR is knocked down by siRNA, IL-19 pre-treatment does not potentiate loss of adhesion, suggesting IL-19 actions are overwhelmingly mediated by HuR. NF-κB activation is necessary for mediating the response to inflammatory stimuli, and our results suggest that IL-19 actions on mRNA are distal to NF-κB and post-transcriptional. Nevertheless, this study does demonstrate the interesting and unanticipated findings that NF-κB is not sensitive to IL-19 inhibition, and that HuR and mRNA stability are sensitive to IL-19.

In summary, there are several novel findings from this study. First, IL-19 directly can decrease TNF-α-induced ICAM-1 and VCAM-1 mRNA and protein abundance in ECs. Second, IL-19 reduces adhesion molecule abundance by a post-transcriptional mechanism through inhibition of HuR and mRNA stability. Third, IL-19 can reduce HuR phosphorylation and subsequent cytoplasmic shuttling. Fourth, IL-19 reduces leukocyte-endothelial cell interaction *in vitro* and *in vivo*. Fifth, IL-19 can up-regulate the HuR-targeting microRNA-133a in VSMCs but not in ECs. Together, these data imply that IL-19 can impart a T_h2 phenotype on ECs, and also support the hypothesis that IL-19, or IL-19 signaling targets, may be a valuable anti-inflammatory therapeutic modality.

Our overall hypothesis that interleukin-19 may exert anti-inflammatory effects in vascular cells is confirmed by our data. With respect to Aim 1 of this dissertation, our hypothesis that IL-19 may mediate anti-inflammatory effects through down-regulation of pro-inflammatory genes is strongly supported by our data, which shows that IL-19 has the capacity to down-regulate cell adhesion molecules (ICAM-1, VCAM-1, E-selectin) and chemokines (IL-1β, MCP-1, IL-8). The hypothesis of Aim 2 of this dissertation, that IL-19 mediates anti-inflammatory effects by reducing stability of pro-inflammatory mRNAs through modulation of HuR cellular localization, has likewise been supported by our

data. Additionally, we have shown that IL-19 may regulate HuR through inhibition of serine phosphorylation of HuR and, in VSMCs, through up-regulation of miR-133a.

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