THE EFFECTS OF INTERLEUKIN-19 ON ATTENUATION OF THE VASCULAR RESPONSE TO INJURY

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by

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ABSTRACT

THE EFFECTS OF INTERLEUKIN-19 ON ATTENUATION OF THE VASCULAR RESPONSE TO INJURY

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Doctor of Philosophy

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BACKGROUND: Despite aggressive dietary modification, lipid lowering medications, and other medical therapy, vascular proliferative diseases continue to account for 50% of all mortality in the United States. It is a significant medical and socioeconomic problem contributing to the mortality of multiple diseases including myocardial infarction (MI), stroke, renal failure, and peripheral vascular disease. With a growing number of children becoming obese and an increase in the number of patients with co-morbidities such as metabolic syndrome and Type 2 diabetes mellitus, epidemiological studies project the morbidity and mortality of these diseases to increase. Among these vascular proliferative diseases are primary atherosclerosis, vascular restenosis, and allograft vasculopathy, all of which are the result of chronic inflammation believed to stem from initial endothelial injury. Once activated by any number of potential injurious agents, endothelial cells (ECs) secrete cytokines that act on multiple cell types. Stimulation of resident vascular smooth muscle cells (VSMCs) results in a phenotypic switch from a normally contractile state to a proliferative state. Following this phenotypic shift, VSMCs migrate from the media to the intima of the artery where they begin secretion of both pro- and anti-inflammatory cytokines. Vascular proliferative disease ensues as a result of the autocrine and paracrine signaling of these cytokines between many different cell types including ECs, VSMCs, macrophages, and T-cells. As a result of the integral role pro-

and anti-inflammatory cytokines play in the development of vascular proliferative diseases, they have become the subject of intense study in the field of cardiovascular research. Interleukin-19 (IL-19) is a newly described member of the IL-10 sub-family of anti-inflammatory cytokines. Discovered in 2000, it was originally only thought to be basally expressed in monocytes and lymphocytes, however in 2005 our lab discovered that while uninjured arteries have no detectable IL-19, arteries of patients with vascular proliferative diseases have notable IL-19 expression. Since its discovery in multiple cell types of injured arteries, our lab has subsequently shown that IL-19 inhibits proliferation, migration, spreading, production of reactive oxygen species (ROSs), and expression of pro-inflammatory genes in VSMCs, while in ECs IL-19 has been shown to promote angiogenesis, proliferation, migration, and spreading.

AIMS and HYPOTHESIS: The first aim of the current study is to show that IL-19 is expressed in atherosclerotic plaque, and to test that IL-19 can reduce experimental atherosclerosis in susceptible mice. The second aim of the study is to show that IL-19 can regulate development of intimal hyperplasia in a murine model of restenosis. For both aims, we sought to identify potential intracellular signaling mechanisms of IL-19 which produce the observed effect. These aims directed our overall hypothesis that the anti-inflammatory properties of IL-19 can attenuate the vascular response to injury in various animal models of vascular proliferative disease.

METHODS and RESULTS: The first aim of this dissertation showed that $LDLR^{-/-}$ mice fed an atherogenic diet and injected with either 1.0ng/g/day or 10.0ng/g/day rmIL-19 had significantly less plaque area in the aortic arch compared with controls ($p<0.0001$). Weight gain and serum lipid levels were not significantly different. IL-19 could halt, but not reverse expansion of existing plaque. Gene expression in splenocytes from IL-19

treated mice demonstrated immune cell T_h2 polarization, with decreased expression of T-bet, IFNγ, IL-1β and IL-12β, and increased expression of GATA3 messenger ribonucleic acid (mRNA). A greater percentage of lymphocytes were T_h2 polarized in IL-19 treated mice. Cellular characterization of plaque by immunohistochemistry demonstrated IL-19 treated mice have significantly less macrophage infiltrate compared with controls (p<0.001). Intravital microscopy revealed significantly less leukocyte adhesion in wild-type mice injected with IL-19 and fed an atherogenic diet compared with controls. Treatment of cultured EC, VSMC, and bone marrow-derived macrophages (BMDM) with IL-19 resulted in a significant decrease in chemokine mRNA, and in the mRNA-stability protein HuR.

In the second aim of this dissertation we showed that IL-19 attenuates vascular restenosis in response to carotid artery ligation. Carotid artery ligation of hyper-responsive friend leukemia virus B (FVB) wild-type mice injected with 10ng/g/day rIL-19 had significantly lower neointima/media ratio (I/M) compared with phosphate buffered saline (PBS) controls (p=0.006). Conversely, carotid artery of IL-19$^{-/-}$ mice demonstrated significantly higher I/M ratio compared with wild-type mice (p=0.04). Importantly, the increased I/M ratio in the knockout mice could be rescued by injection of 10ng/g/day IL-19 (p=0.04). VSMC explanted from IL-19$^{-/-}$ mice proliferated significantly more rapidly compared with wild-type (p=0.04). Surprisingly, in this model, IL-19 does not modulate adoptive immunity. Rather, addition of IL-19 to cultured wild-type VSMC did not significantly decrease VSMC proliferation, but could rescue proliferation in IL-19$^{-/-}$ VSMC to wild-type levels (p=0.02). IL-19$^{-/-}$ VSMC expressed significantly greater levels of inflammatory mRNA including IL-1β, TNFα, and MCP-1 in response to TNFα stimulation (p<0.01 for all). No polarization of adaptive immunity was noted in these mice.

CONCLUSIONS: These data are the first to report that IL-19 is a potent inhibitor of experimental atherosclerosis via diverse mechanisms including immune cell polarization, decrease in macrophage adhesion, and decrease in gene expression. In addition, these data are also the first to show that IL-19 plays a previously unrecognized protective role in vascular restenosis. Together, these data suggest IL-19 is both anti-atherogenic and anti-restenotic and may identify IL-19 as a novel therapeutic to limit vascular inflammation.

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DEDICATION

This thesis is dedicated in loving memory of Rachel Klitzman.

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LIST OF ABBREVIATIONS

3' UTR	3-prime untranslated region
ANOVA	analysis of variance
$ApoE^{-/-}$	Apolipoprotein E knockout
ARE	Adenosine Uridine (AU)-Rich Element
BMDM	bone marrow derived macrophages
BrdU	5-bromo-2'-deoxyuridine
C57BL/6	C57 Black/6
CABG	coronary artery bypass graft
CAV	coronary allograft vasculopathy
CaEC	coronary artery endothelial cell
CAM	cell adhesion molecule
CD36	cluster of differentiation 36
CD45	cluster of differentiation 45/common leukocyte antigen
cDNA	complementary deoxyribonucleic acid
COX-2	cyclooxygenase-2
CXCL16	chemokine ligand 16
DC	dendritic cell

DSS	dextran sucrose sodium
EC	endothelial cell
EEL	external elastic lamina
EST	expressed sequence tag
FBS	fetal bovine serum
FVB	friend leukemia virus B
GAPDH	glyceraldephyde-3-phosphate dehydrogenase
GATA3	trans-acting T-cell-specific transcription factor GATA-3
hCaECs	human coronary artery vascular endothelial cells
hCaVSMCs	human coronary artery vascular smooth muscle cells
HBEC	human bronchial epithelial cell
HO-1	heme oxygenase-1
Hsp70	heat shock protein, 70 kilodalton
HuR	Human R Antigen
HVEC	human vascular endothelial cell
I/M	intima/neointima to media ratio
IκB	inhibitor of kappa-B

IEL	internal elastic lamina
IFN-γ	interferon-γ
IL-1	interleukin-1
IL-8	interleukin-8
IL-10	interleukin-10
IL-12	interleukin-12
IL-19	interleukin-19
IL-20	interleukin-20
IL-24	interleukin-24
IL-20Rα/β	interleukin-20 receptor subunit α/β
IL-22Rα/β	interleukin-22 receptor subunit α/β
IQR	interquartile range
ISR	in-stent restenosis
KGF	keratinocyte growth factor
$LDLR^{-/-}$	low-density lipoprotein receptor knockout
LOX-1	lectin-like oxidized low-density lipoprotein receptor-1

LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemotactic protein-1
mEC	microvascular endothelial cell
MI	myocardial infarction
MLC	myosin light chain
mRNA	messenger ribonucleic acid (RNA)
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
OCT	optimal cutting temperature
oxLDL	oxidation of low-density lipoprotein
PBMC	peripheral blood monocytic cell
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PECAM	platelet endothelial cell adhesion molecules
PKC	protein kinase C

PTCA	percutaneous transluminal coronary angioplasty
qRT-PCR	quantitative real time polymerase chain reaction
Rac1	ras-related C3 botulinum toxin substrate 1
RASC	rat arthritis synovial cell
ROS	reactive oxygen species
siRNA	small interfering ribonucleic acid
SOCS5	suppressor of cytokine signaling 5
SM-αA	smooth muscle α-action
SM-MHC	smooth muscle myosin heavy chain
SRA	scavenger receptor A
STAT3	signal transducer and activator of transcription 3
STAT6	signal transducer and activator of transcription 6
T-bet	T-box transcription factor TBX21
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

VSMC	vascular smooth muscle cell

CHAPTER 1

INTRODUCTION

1.1. Overview: Vascular Proliferative Disease

Despite aggressive dietary modification, lipid lowering medications, and other groundbreaking medical therapy, cardiovascular disease continues to be the leading cause of morbidity and mortality in the United States with an estimated 79 million Americans being afflicted[1]. While risk factors such as race, gender, age, and hereditary predisposition are unavoidable, many factors such as smoking, high cholesterol, high blood pressure, and physical inactivity are preventable[2]. However, with increasingly sedentary lifestyles, especially among children, epidemiological studies project a greater number of patients with co-morbid conditions such as metabolic syndrome and Type 2 diabetes mellitus resulting in an increase in the overall morbidity and mortality of cardiovascular diseases[3].

Atherosclerosis and the diseases it underlies such as coronary artery disease, peripheral vascular disease, Type 2 diabetes mellitus, and stroke are responsible for approximately 50% of all cardiovascular related deaths in the Western world[1,4]. Atherosclerosis is a complex pathophysiologic process that involves initial endothelial dysfunction resulting in secretion of various cytokine mediators of inflammation from ECs. VSMCs respond to these pro- and anti-inflammatory cytokines by migrating to the tunica intima of the artery where they begin proliferation and secretion of additional inflammatory modulators. Response to cytokine mediators of inflammation is not only found in the development of atherosclerosis, but also as the pathophysiological basis of other very important vascular

proliferative diseases including restenosis, vein bypass graft failure, and transplant vasculopathy[5,6]. As a result of the integral role pro- and anti-inflammatory cytokines play in the development of vascular proliferative diseases, they have become the subject of intense study in the field of cardiovascular research.

1.2. Atherosclerosis

Peripheral vascular disease, coronary artery disease, and stroke are among the leading causes of cardiovascular morbidity and mortality. Each of these critical vascular events has foundations in the natural inflammatory responses associated with the pathological condition of atherosclerosis[7].

While the pathogenesis of atherosclerosis is not fully understood, it is a chronic inflammatory disease that remains asymptomatic for decades[8]. The initial inflammatory reactions underlying atherosclerosis often begin as cholesterol deposits build within the intima of arteries causing endothelial injury and activation, and oxidation of low-density lipoprotein (oxLDL). Expression of chemokines and adhesion molecules by injured endothelial cells and VSMCs employ circulating lymphocytes and monocytes to activate in the area of irritation[9]. Upon integration into the intima of the artery, monocytes are converted to macrophages by a variety of pathways, including via oxLDL interaction. Macrophages then begin uptake of oxLDL and, with continued invasion of the intima by immune cells, advancement of the disease results in the eventual formation of initial atherosclerotic lesions called foam cells[8] (Figure 1). Simultaneously, many different cell types of the atherosclerotic lesion continue to secrete cytokines that further injure VSMCs. VSMCs respond via undergoing a phenotypic shift from a contractile to a synthetic state, where they migrate from the intima to the media of the artery and gain

the ability to express genes for increased proliferation and for specific cytokine production[8].

Although slowly progressive, as atherosclerotic lesions continue to grow and remodel over time, clinically more advanced and sometimes symptomatic lesions begin to develop. These are characterized into stable and unstable (or complicated) plaques[10] (Figure 1). Stable plaques consist of high concentrations of both VSMCs and extracellular matrix and are typically unlikely to rupture. On the other hand, unstable plaques have a high density of oxLDL filled macrophages called foam cells and usually have only a thin layer of extracellular matrix covering the lesion (known as the fibrous cap)[11] (Figure 1). In either case, if atherosclerotic lesions rupture, breakdown of the fibrous cap exposes underlying matrix materials such as collagen, phospholipids, and platelet adhesive matrix molecules to pro-thrombotic molecules freely circulating within the blood, which can induce thrombus formation in the arterial lumen at the site of rupture. Once formed, the thrombus can occlude the artery at the site of formation or detach with the potential to become lodged in smaller downstream arteries and block blood flow to vital organs such as the heart and brain, resulting in either MI or stroke, respectively[12,13].

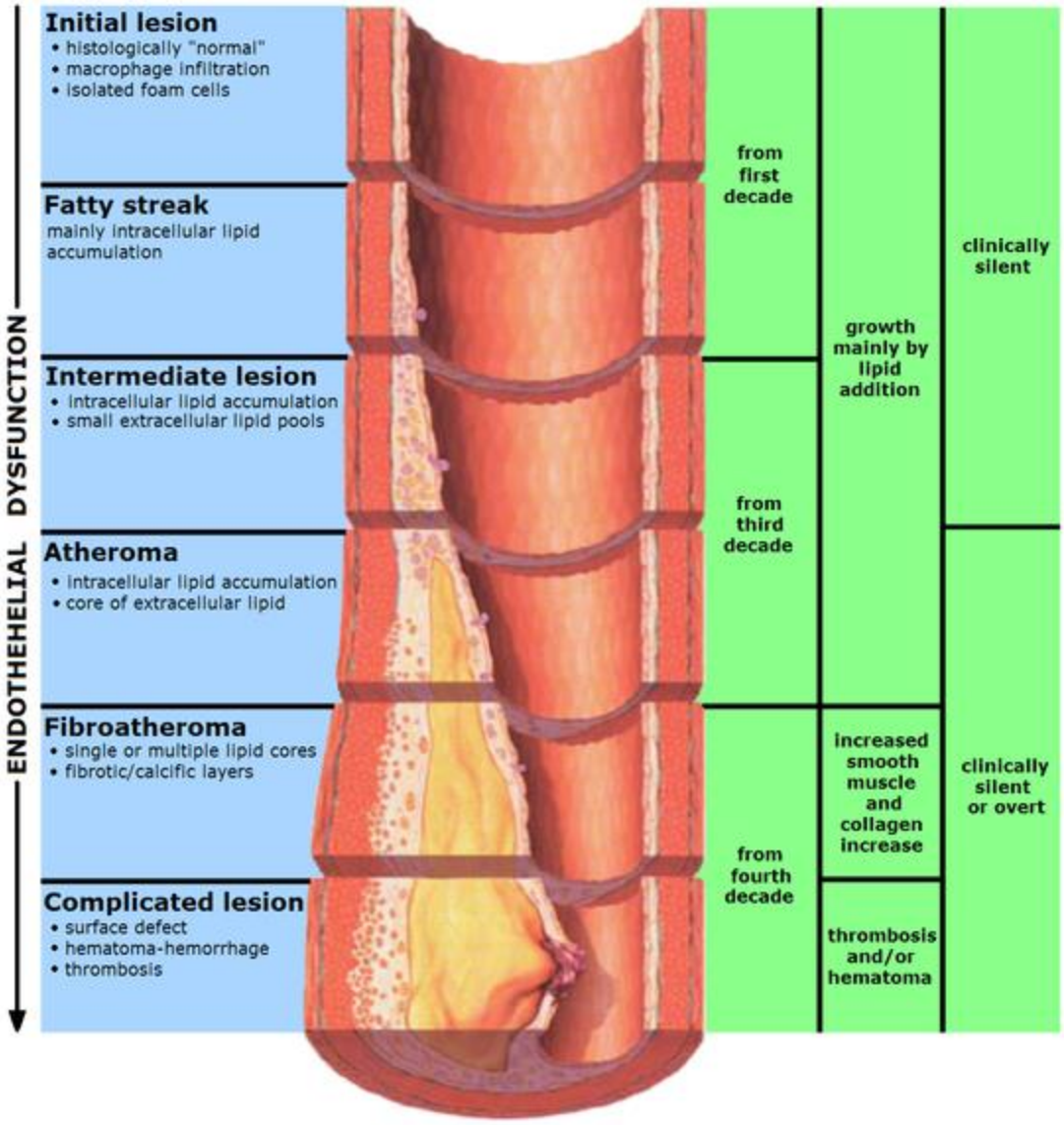

Figure 1. Progression of Atherosclerosis. Photo Credit: Grahams Child.

1.3. Role of Smooth Muscle Cells in Atherosclerosis

Studies have shown that a multitude of cells types play a role in the formation of atherosclerotic lesions, with the majority of these studies focusing primarily on the effects of endothelial and immune cells on the initiation of atherosclerosis. Previous dogma indicates that VSMCs only play a role later in atherosclerosis as they begin forming a neointima via migration from their normal location in the media of the artery[14–19]. More recently, however, studies from autopsy specimens of human coronary arteries from patients ranging in age from infants to adults provide evidence that greater numbers of VSMCs are inherently present in areas prone to develop atherosclerosis, such as at branch points of large and medium sized arteries, while there are significantly less VSMCs in areas less prone to plaque accumulation[20–22]. This raises the question if VSMCs may be playing a pathogenic role in the development of early atherosclerotic lesions.

In uninjured arteries, VSMCs are found predominately in the media of arteries and express proteins necessary for contraction such as smooth muscle α-action (SM-αA) and smooth muscle myosin heavy chain (SM-MHC). Activated VSMCs found in the neointima, however, undergo a phenotypic change during which they become more "synthetic" and less "contractile". Studies have shown that *in vitro* rat and mouse VSMCs can switch between these "contractile" and "synthetic" phenotypic states when challenged by various atherogenic stimuli such as oxLDL[23,24]. This phenotypic change results in decreased expression of contractile proteins and an increase in expression of proliferative and inflammatory cytokines, extracellular matrix, and proteases[19,25,26] (Table 1)[27]. In addition, "synthetic" VSMCs migrate more readily than their "contractile" counterparts and also express a much greater number of scavenger receptors including

scavenger receptor A (SRA), cluster of differentiation 36 (CD36), lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), and chemokine ligand 16 (CXCL16), all of which are directly involved in lipid uptake and foam cell formation[28,29]. These initial VSMC derived foam cells are now thought to play a significant role in the steps before, during, and after atheroma lesion formation[30,31] (Table 2)[27].

Table 1. Atherogenic Cytokines Elaborated by "Synthetic" Smooth Muscle Cells[27]

Cytokine	Cellular Sources	Effects
IFNγ	SMC, M, T	↑ SMC migration and proliferation
		↑ ECM remodeling
		↑ adhesion molecule expression
IL-1	SMC, EC, M, T, B	↑ SMC migration and proliferation
		↑ monocyte accumulation
		↑ adhesion molecule expression
IL-18	SMC, EC, M	↑ adhesion molecule expression
		↑ SMC accumulation
		↑ ECM remodeling
MCP-1	SMC, EC, M, T	↑ recruitment of monocytes
		↑ ECM remodeling
MIF	SMC, EC, M, T	↑ recruitment of monocytes
		↑ SMC migration
		↑ ECM synthesis and remodeling
PDGF-BB	SMC, EC, M	↑ SMC migration and proliferation
TGFβ	SMC, EC, M, T	↑ SMC migration and proliferation
		↑ ECM synthesis

Abbreviations: SMC: smooth muscle cell, EC: endothelial cell, M: macrophage, T: T lymphocyte, B: B lymphocyte.

Table 2. American Heart Association Classification of Atherosclerotic Lesions[27]

	Lesion Type	Cellular Composition
I	Initial change	Isolated macrophage foam cells
II	Minimal change	Multiple layers of foam cells
	IIa: Progression prone; Abundant SMCs	Few lymphocytes
	IIb: Progression resistant; Few SMCs	Isolated mast cells
III	Preatheroma	Isolated pools of densely packed extracellular lipids
		SMCs accumulate lipid droplets
IV	Atheroma	Confluent core of extracellular lipids
		Increased No. of lymphocytes
		SMCs decrease in number, remaining SMCs have thick basement membranes
V	Fibroatheroma	Fibrous tissue and collagen added
		Intimal SMCs increase in number
VI	Hemorrhagic/thrombotic lesion	Lesion becomes fissured and/or thrombotic
VII	Calcific lesion	Calcification predominates
VIII	Fibrotic lesion	Fibrous tissue changes predominate
		Lipid core is nearly absent

1.4. Role of Vascular Cell Adhesion Molecules in Atherosclerosis

Under normal physiologic conditions, freely circulating blood leukocytes are actively involved in host defense and immune surveillance in order to protect against disease through the inhibition or promotion of a wide variety of inflammatory responses. During pathologic processes of chronic inflammation present in vascular proliferative diseases such as atherosclerosis, leukocytes extravasate from the lumen of the artery into the surrounding tissue at sites of vascular inflammation. Upon injury, ECs begin secretion of a variety of cytokines and chemokines such as IL-1β, monocyte chemotactic protein 1 (MCP-1), and interleukin-8 (IL-8), that resident ECs respond to in both an autocrine and paracrine fashion. Along with creating a chemotactic gradient that attracts circulating leukocytes, ECs also begin upregulation of vascular cell adhesion molecules (CAM) to which these circulating leukocytes begin to bind[32,33].

The process of leukocyte extravasation has been the source of a great number of studies and is well documented. The initial stages begin with the binding of carbohydrate ligands on the surface of leukocytes to single chain transmembrane glycoproteins (known as selectins) on the luminal side of endothelial cells. This process has only marginal binding affinity resulting in leukocytes beginning to roll along the inner surface of the artery wall as multiple transitory bonds are broken and reformed between selectins and their ligands. Simultaneously, chemokines in the region cause integrin molecules on the surface of leukocytes to switch toward a high-affinity binding state allowing for leukocytes to bind tightly to their CAM receptors on the surface of endothelial cells. While firmly adhering to endothelial cell CAMs, leukocytes undergo a cytoskeletal rearrangement that allows for extension of pseudopodia between adjacent endothelial cells. This process of transmigration occurs as specific platelet endothelial

cell adhesion molecules (PECAM) present on both endothelial cells and leukocytes tightly bind and pull the leukocyte from the luminal surface through gaps between endothelial cells and into the surrounding tissue[34].

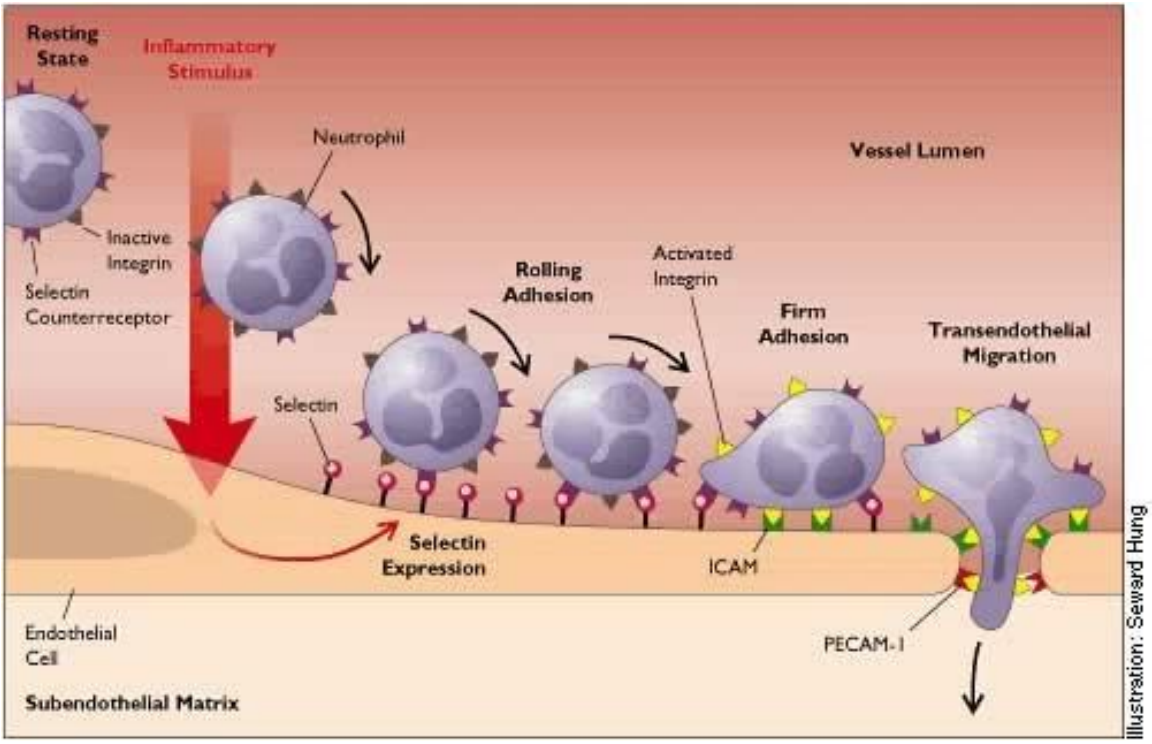

Figure 2. Leukocyte Migration. Photo Credit: Sewerd Hung.

1.5. Restenotic Vascular Proliferative Diseases

Angioplasty refers to a procedure during which a catheter is inserted through a major blood vessel in either the arm or groin of a patient and is subsequently guided through the aorta into the occluded coronary arteries. At the tip of the catheter lies a balloon which is inflated to compress material occluding the vessel away from the lumen, allowing for greater blood flow. Stent placement involves a similar procedure except a small metallic or mesh spring-like device is deployed at the site of occlusion in an attempt to keep the arterial lumen open for maximal periods of time.

Initially when stents are placed, endothelial cells grow around the stent which allows for smoother blood flow and aids in the prevention of clotting. However, as is frequently the case, injury to the vessel wall occurs as a result of the invasive procedure causing activation of VSMCs. This leads to increased migration and proliferation of VSMCs and neointimal formation known as in-stent restenosis (ISR). Similar activation of VSMCs causes restenosis following percutaneous transluminal coronary angioplasty (PTCA).

Restenosis itself is broadly defined as a re-narrowing of a blood vessel, leading to restricted blood flow usually referring to an artery that has become narrowed, received treatment to clear the blockage, and subsequently become re-occluded[35,36]. PTCA has a 40% chance and stent placement has a 25% chance of restenosis, respectively[37]. Even though intra-coronary stents are more effective than PTCA in decreasing restenosis in up to 35% of cases, ISR often still occurs within 6 to 9 months. Further, the incidence of restenosis in selected patient populations, such as diabetics and those with complex lesions can exceed 50%, significantly limiting the success of this modality[38–40].

Coronary artery bypass grafting (CABG) involves harvesting a blood vessel, typically

either the saphenous vein or internal mammary artery, to redirect blood flow around a site of extreme coronary artery atherosclerotic occlusion. In the 12 months following graft placement, neointimal hyperplasia is the main cause of graft failure. As opposed to PTCA and stent placement, this restenosis is a result of the donor vessel's inability to adapt to much higher arterial pressures causing denudation of the endothelial cell layer and subsequent activation of VSMCs. After 12 months, atherosclerosis becomes the main cause of graft failure. CABG failure rates range from 10-30% per year with the percentage increasing for each year post operation[41].

With the continued advancement of our understanding and knowledge of the pathogenesis of diseases such as atherosclerosis and restenosis, solid organ transplantation has become increasing common as a last resort treatment for end-stage heart failure. Unfortunately the risk of coronary allograft vasculopathy (CAV) is approximately 10% per year, significantly limiting the 5-year survival of transplant patients. In CAV, small vessels distal to the actual site of solid organ transplant begin to undergo the same process of VSMC activation and ensuing neointimal hyperplasia. Although the mechanism is still not well understood, occlusion of these distal vessels leads to localized ischemia and eventually results in upstream organ failure. Longer term complications are unlike the typical accelerated atherosclerosis seen in CABG patients where here a diffuse, fibrous intimal thickening occurs and ultimately results in allograft failure[42].

1.6.T-cell Phenotypes

T-cells play an important role in the chronic inflammatory processes of vascular proliferative diseases such as atherosclerosis and restenosis. As part of the

pathophysiology of these diseases, resident T-cells of the vascular lesion produce a wide variety of proteins that can act to either accentuate (pro-inflammatory and pro-proliferative) or attenuate (anti-inflammatory and anti-proliferative) the vascular disease process[43].

Interleukins secreted at the site of vascular injury are commonly categorized based on their effect on lymphocyte maturation. The type 1 helper T-cell (T_h1) arm of adaptive immunity is characterized by secretion of pro-inflammatory, cytotoxic cytokines. These T-cells act to promote inflammation via secretion of molecules such as interleukin-1 (IL-1), interleukin-12 (IL-12), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ), causing activation of macrophages and VSMCs. In contrast, the type 2 helper T-cell (T_h2) arm of immunity elaborates anti-inflammatory interleukins such as interleukin-4 (IL-4) and interleukin-10 (IL-10) which tend to limit the magnitude of the inflammatory response and promote anti-vascular proliferative immune reactions[44,45].

Activated VSMCs have been shown to secrete a variety of pro-inflammatory cytokines that act in both an autocrine and paracrine fashion to accelerate and sustain vascular proliferative diseases in a T_h1-dependent manner[3]. Atherosclerosis in particular has been described as a T_h1 driven inflammatory disease[46]. Since atherosclerosis is widely considered primarily to be a disease of chronic inflammation, it comes as no surprise that a large number of studies advocate the importance of T_h1 interleukins in the atherosclerotic disease process based on the predominance of T_h1 lymphocytes and their cytokines in both human and mouse atherosclerotic lesions[47–49]. Studies in support of this hypothesis have shown that mice lacking IFN-γ, TNF-α, or the T_h1 T-box transcription factor TBX21 (T-Bet) have significantly reduced atherosclerosis[50–52], while mice lacking signal transducer and activator of transcription 6 (STAT6) (a required

transcription factor for T_h2 differentiation) have increased atherosclerotic plaque burden[53].

By comparison, a much smaller number of studies focus on the role of anti-inflammatory cytokines in atherogenesis. The majority of these studies have focused on the archetypical T_h2 interleukin IL-10 and its potent immune modulation. In one study, IL-10 transgenic mice had significantly less atherosclerotic plaque burden than controls. Bone marrow transfer from these IL-10 transgenic mice into atherosclerotic susceptible $LDLR^{-/-}$ mice caused a polarization of the T_h2/T_h1 ratio toward a more anti-inflammatory phenotype and a significant inhibition of plaque production[54,55]. Furthermore, in $IL\text{-}10^{-/-}$ /$ApoE^{-/-}$ double knockout mice, atherosclerosis is significantly increased[55]. Unlike IL-10, $IL\text{-}4^{-/-}$ mice do not have increased atherosclerosis and chronic administration of supra-physiologic levels of IL-4 in $ApoE^{-/-}$ does not reduce plaque burden[56].

Because the number of identified and characterized pro-atherogenic cytokines greatly dwarfs their anti-atherogenic cytokine counterparts, there remains a gap in our knowledge concerning the potential for direct protective effects of T_h2 interleukins on reducing vascular inflammation and lesion formation in diseases such as atherosclerosis and restenosis. Moreover, very little has been published regarding potential protective effects of T_h2 interleukins on resident vascular cells (ECs and VSMCs) in addition to inflammatory cells. Recognition of other T_h2 interleukins with anti-atherosclerotic effects, particularly those which may have direct anti-inflammatory effects on resident vascular cells could have immediate clinical impact.

1.7. Interleukin-19: Characterization, Expression, and Function.

IL-19 is a newly described member of the IL-10 subfamily of anti-inflammatory interleukins. It was first discovered and cloned by Gallagher *et al*. while searching Expressed Sequence Tag (EST) databases for IL-10 homologues. It is located in a cluster of IL-10 like interleukins on chromosome 1 which also includes interleukin-20 (IL-20) and interleukin-24 (IL-24). IL-19 has 20% overall amino acid homology with IL-10, sharing 41 of 50 amino acids required for formation of the IL-10 hydrophobic core and 30 residues including 4 cysteines required for correct folding of the protein[57]. X-ray crystallography shows that while IL-10 and IL-19 are structurally similar, they in fact have fundamental differences. IL-19 consists of 7 α-helices, the last of which can fold back on itself to stabilize IL-19 as a soluble monomer. IL-10, on the other hand, has only 6 α-helices, the last of which allows for the formation of a stable IL-10 homodimer in solution through insertion into the core of its paired protein. In addition, the region of the IL-10 receptor chain 1 that interacts with IL-10 is not very well conserved in IL-19, potentially explaining why IL-19 cannot signal through the IL-10 receptor in spite of its amino acid identity with IL-10[58].

Although IL-19 shares 20% amino acid homology with IL-10, it has recently been found to share greater structural similarity with other members of the IL-10 subfamily, namely IL-20 and IL-24. Unlike IL-10, these too can form stable monomers in solution. Furthermore, IL-19, IL-20, and IL-24 all signal through receptor complexes that contain the IL-20 receptor β chain (IL-20Rβ). All three proteins can signal through the heterodimer formed by the IL-20 receptor α chain (IL-20Rα) and IL-20Rβ, however distinct from IL-19, IL-20 and IL-24 can also signal through the receptor formed by IL-22 receptor α chain (IL-22Rα) and IL-20Rβ[58,59].

IL-19 expression was first reported in 2001 in primary human monocytes stimulated with lipopolysaccharide[57]. During its characterization, IL-19 was originally thought to be expressed exclusively by monocytes, B cells, and T-cells, and as a result, initial studies of IL-19 focused primarily on its role as a product of immune cells. Although some studies question whether IL-19 does in fact play a role in regulating these cells types due to a lack of the IL-20Rα chain, it has been definitively shown in multiple studies that treatment of maturing T-cells with IL-19 results in an increase in the anti-inflammatory T_h2/T_h1 ratio[60,61].

Expression of IL-19 in non-immune cells was first discovered in 2005 through complementary deoxyribonucleic acid (cDNA) microarray analysis of VSMCs treated with inflammatory stimuli[62]. Further study indicated that IL-19 expression was absent in quiescent VSMCs, but could be induced in VSMCs treated with various inflammatory stimuli including TNF-α, IFN-γ, platelet derived growth factor (PDGF), T-cell conditioned media (TCM), and fetal bovine serum (FBS)[63]. Subsequent analysis of microvascular EC (mEC), coronary artery EC (CaEC), and human vascular EC (HVEC) showed similar results where IL-19 expression could be incited by treatment with various inflammatory stimuli[64]. Histological analysis of human coronary arteries taken from patients with allograft vasculopathy confirmed that IL-19 was highly expressed in ECs[64], VSMCs[63], and CD45+ leukocytes[63], however, IL-19 expression was undetectable in normal, uninjured arteries[64]. Identical results were found during histological analysis of atherosclerotic plaques from the aortic arch of $ApoE^{-/-}$ mice, but not in the aortic arch of wild type animals[63,64]. Interestingly, neither IL-10 nor IL-20 expression could be detected in any of these cells types in stimulated or unstimulated conditions at the mRNA or protein level[63]. There are only two publications to date to report serum concentrations of

IL-19 in human subjects. It was found that patients undergoing CABG surgery have increased serum IL-19 levels correlating to cell mediated immune suppression often seen in these patients[65,66]. The compilation of these studies strongly suggest IL-19 plays a unique role in regulating vascular inflammation as it is only expressed as a response to vascular injury.

1.8. Effects of IL-19 on Vascular Cells

IL-19 has profound anti-inflammatory effects in vascular disease (Table 3)[58]. *In vitro* adenoviral delivery of IL-19 or treatment with recombinant IL-19 decreases VSMC proliferation in a concentration dependent manner[63]. Treatment of VSMCs with IL-19 causes activation of signal transducer and activator of transcription 3 (STAT3) as measured by both nuclear translocation and phosphorylation, as well as an increase in expression of suppressor of cytokine signaling 5 (SOCS5), a STAT-responsive gene, at both the mRNA and protein levels[63]. As the name indicates, the SOCS family of proteins act to suppress cytokine signaling by targeting cytokine receptors and cytoplasmic signaling intermediates for E3-ubiquitin ligase-mediated degradation[67]. In VSMC, SOCS5 binding directly inhibits mediators of inflammation such as the FBS-induced activation of the p38 and p44/42 mitogen-activated protein kinases (MAPK). *in vivo* experiments confirmed these anti-proliferative and anti-inflammatory effects where adenoviral delivery of IL-19 to rat carotid arteries injured by balloon angioplasty caused a decrease in the total number of proliferating (Ki-67-positive) VSMCs and overall neointimal formation[63]. These experiments clearly implicate IL-19 as an important mediator of anti-inflammatory signal transduction through activation of SOCS5 and subsequent inhibition of pro-inflammatory signal transduction.

IL-19 selectively inhibits VSMC pro-inflammatory and proliferative genes such as IL-8, IL-1β, cyclooxygenase-2 (COX-2), and Cyclin D1, but not others such as proliferating cell nuclear antigen (PCNA), and ras-related C3 botulinum toxin substrate 1 (Rac1)[68]. Interestingly, studies show that unlike the prototypical anti-inflammatory cytokine IL-10, IL-19 does not inhibit activation of the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), as determined by p65 phosphorylation and inhibitor of kappa-B (IκB) degradation[68]. Since NF-κB is often considered the master transcription factor switch for upregulation of pro-inflammatory gene expression, this finding provoked a search for other potential mechanisms by which IL-19 may cause selective inhibition of gene expression without affecting transcription[58].

Besides IL-10's well-known inhibitory effects on NF-κB, it has also been shown to affect gene expression by down regulating human antigen R (HuR), a stabilizing RNA-binding protein important for post-transcriptional processing of highly regulated mRNA[69]. HuR is ubiquitously expressed and predominately found in the nucleus where it translocates to the cytoplasm with the help of chaperone proteins. Binding of HuR to specific mRNA containing cis-acting AU-rich elements (AREs) in their 3' untranslated regions increases the transcript's stability. Once in the cytoplasm, the HuR-bound mRNA is delivered to the translational apparatus for protein synthesis[70,71]. Interestingly, it was discovered that many pro-inflammatory transcripts affected by IL-19 contain AREs, while most transcripts unaffected by IL-19 lack ARE. Studies in VSMCs found that IL-19 inhibits translocation of HuR from the nucleus to the cytoplasm in FBS-stimulated cells, thus inhibiting the ability of HuR to stabilize many pro-inflammatory gene transcripts. HuR small interfering RNA (siRNA) studies successfully recapitulated this effect[68]. Taken together, these studies provide insight into an important mechanism by which IL-19

causes post-transcriptional decreases in the abundance of many pro-inflammatory transcripts without directly affecting gene transcription.

Along with IL-19's anti-proliferative effects in VSMCs, it has recently been shown that it also has direct effects on VSMC motility. In two experiments, IL-19 treatment of cultured VSMC inhibited PDGF-induced migration in a Boyden chamber and re-migration into a scratch wound. Subsequent analysis showed that IL-19 inhibits the activation of motility proteins such as heat shock protein 70 (Hsp70), myosin light chain (MLC), cofilin, and the monomeric G proteins Rac1 and RhoA, however, the exact molecular mechanisms by which IL-19 causes these observed changes is not well understood[58,72].

IL-19 can induce expression of other powerful anti-apoptotic and anti-inflammatory mediators of vascular inflammation such as heme oxygenase-1 (HO-1) at both the mRNA and protein level in VSMCs[73]. Once induced primarily at the transcriptional level by pro-inflammatory mediators such as growth factors, oxidative stress, and cytokines[74], HO-1 helps protect against inflammation by acting as an anti-oxidant, decreasing monocyte arterial transmigration, and decreasing VSMC proliferation[75,76]. Treatment with IL-19 upregulates HO-1 production resulting in decreased peroxide-induced apoptosis and growth factor-induced ROS accumulation in VSMCs. When VSMCs were transfected with HO-1 siRNA prior to treatment with IL-19, the decrease in ROS production was abolished. *In vivo*, IL-19 treatment can significantly reduce TNF-α induced ROSs accumulation in mouse coronary arteries[73]. While many studies have shown that IL-10 can upregulate HO-1 in immune cells, namely monocytes/macrophages[77], IL-19 is the first of any anti-inflammatory cytokine or T_h2 interleukin to show induction of HO-1 in VSMC[58].

While the primary focus thus far has been on the effects of IL-19 on VSMC activity in vascular disease, there are distinct differences between its effects on VSMCs and ECs that require attention. In ECs, contrary to that reported in VSMCs, IL-19 has pro-proliferative, pro-migratory, and pro-angiogenic effects. Treatment of cultured ECs causes activation of MAPK p44/42, STAT3, and Rac1 resulting in EC spreading, migration, and proliferation. In addition, IL-19 treatment causes formation of endothelial cell tubes in cultured mouse aortic ring studies and the formation of nascent blood vessels in subcutaneous gel plugs in mice. Treatment with IL-20 receptor antibody significantly reduces IL-19-driven EC migration, indicating that these effects are IL-19 specific. The unique nature of the molecular consequences of treatment of VSMCs and ECs with IL-19 is still being elucidated but hopefully futures studies will expand the current knowledge base on how different cells respond to anti-inflammatory signals[58,64].

Table 3. Effects of IL-19 on Resident Vascular Cells. Adapted from England *et al*.[58]

Tissue Type	Effect	Species
EC	Auto-induces IL-19 Expression	h
	Activates STAT3, Rac1, MAPK p44/42	h
	Increases EC proliferation	h
	Increases EC spreading and migration	h
	Pro-angiogenic (increases tube, microvessel formation)	m
	Inhibits HuR nucleocytoplasmic translocation	h
VSMC	Inhibits proliferation, hyperplasia	h,r
	Auto-induces IL-19 expression	h
	STAT3 phosphorylation, translocation	h
	Increases SOCS5 expression	h
	Inhibits MAPKs (p44/42, p38)	h
	Decreases inflammatory, proliferative proteins, mRNAs	h
	Decreases HuR protein abundance	h
	Inhibits HuR nucleocytoplasmic translocation	h
	Decreases ARE-bearing mRNA stability	h
	Inhibits PKCα activation	h

Table 3. (continued)

Tissue Type	Effect	Species
	Inhibits migration, spreading	h
	Inhibits activation of MLC, cofilin, Hsp70, Rac1, RhoA	h
	STAT3-dependent increase in HO-1 expression	h
	Decreases ROS *in vitro* and *in vivo*	h,m
	Inhibits apoptosis	h

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

1.9. IL-19: A Potential Link to Non-Vascular Human Diseases

IL-19 expression is found not only in VSMCs[63] and vascular ECs[64], but also in a wide variety of non-vascular human peripheral cells types such as fetal membranes[78], synovial tissue[79,80], bronchial epithelial cells[81,82], and keratinocytes[83] (Table 4)[58]. Although not yet clearly elucidated, the role of IL-19 in asthma is a topic of great interest. *In vivo*, mice exposed to allergens have increased expression of IL-19[84]. Moreover, both serum[84] and bronchial airway epithelial cells[82] have increased expression of IL-19 in children with asthma as compared to non-asthmatics. A more putative role of IL-19 has been studied in the disease process of psoriasis, an inflammatory skin condition defined

by plaque-like epidermal lesions resulting from an increase in proliferation of keratinocytes. Within psoriatic lesions, expression of IL-19, IL-20Rα, and IL-20Rβ is readily detectable[83,85–88], and treatment for psoriasis, namely with corticosteroids, reduces the expression of IL-19[86,87]. Current studies suggest that IL-19 may promote expression of keratinocyte growth factor (KGF) in CD8+ T-cells which causes increased expression of IL-19 in keratinocytes, indicating a potential positive feedback loop[85]. Most recently, a considerable amount of interest has been placed on exploration of IL-19 in human diseases of inflammation involving peripheral tissues such as inflammatory bowel disease[89,90], endotoxic shock[91], rheumatoid arthritis[80,92], and various types of cancers[93].

Table 4. Effects of IL-19 on Non-Vascular Cell Types. Adapted from England *et al*.[58]

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

Table 4. (continued)

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

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, h: human, m: mouse, r: rat

1.10. Summary

Despite aggressive dietary modification, lipid lowering medications, and other medical therapy, vascular proliferative diseases continue to be the leading cause of mortality in the western world. Among these vascular proliferative diseases are primary atherosclerosis, vascular restenosis, and allograft vasculopathy, all of which stem from chronic inflammation. As a result of the integral role that pro- and anti-inflammatory cytokines have been shown to play in chronic inflammation, they have become the subject of intense study in the field of cardiovascular research. Over the past few decades, much attention has been paid to the negative effects that pro-inflammatory cytokines play in the pathogenesis of vascular proliferative diseases, however, almost no attention has been paid to the potential protective role of anti-inflammatory cytokines. IL-19 is a newly discovered anti-inflammatory cytokine that is undetectable in uninjured arteries, but its expression is induced by vascular injury and inflammation. *In vitro*, it is expressed by VSMCs and ECs activated by inflammatory stimuli resulting in tissue specific anti-inflammatory effects in both VSMCs and ECs, including changes in proliferation, migration, ROS abundance, and expression of inflammatory genes. IL-19's unique expression by resident vascular cells may represent an auto-regulatory mechanism by which the vasculature attenuates its response to injury and inflammation. This is of potential clinical importance because IL-19 may represent a novel therapeutic for the treatment of chronic vascular inflammatory and proliferative diseases such as atherosclerosis, vascular restenosis, and allograft vasculopathy.

1.11. Overall Hypothesis

The overall hypothesis of this dissertation is that the anti-inflammatory properties of IL-19 can attenuate the vascular response to injury in various animal models of vascular proliferative disease.

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

1.12. Aims

Aim 1. IL-19 effect on experimental atherosclerosis

Aim 1 Hypothesis: We hypothesize that IL-19 will attenuate experimental atherosclerosis via direct effect on resident cells at the site of vascular injury.

Specific Aims:

a. We will show that IL-19 is expressed in both human and murine atherosclerotic plaque but not in normal tissue.
b. We will explore the effect of chronic recombinant IL-19 administration on inhibition of atherosclerotic plaque burden in two animal models of experimental atherosclerosis.
c. We will explore the effect of IL-19 on regression of pre-existing atherosclerotic plaque burden in the same atherosclerotic susceptible animal models.
d. We will explore the effects of chronic recombinant IL-19 treatment on polarization of T-cell phenotype.
e. We will explore the effects of chronic recombinant IL-19 treatment on the cellular composition of atherosclerotic lesions.

f. We will explore the effects of chronic recombinant IL-19 treatment on the expression of pro- and anti-inflammatory molecules.

Aim 2. IL-19 effect on vascular restenosis

Aim 2 Hypothesis: We hypothesize that IL-19 will attenuate vascular restenosis and modulate the VSMC response to injury in mice having undergone carotid artery ligation.

Specific Aims:

a. We will explore the effects of recombinant IL-19 treatment on development of intimal hyperplasia in wild type mice.
b. We will explore development of intimal hyperplasia in the absence of IL-19 via comparison of wild type and IL-19$^{-/-}$ mice.
c. We will explore the effect of recombinant IL-19 administration on IL-19$^{-/-}$ mice to determine if treatment rescues intimal hyperplasia development.
d. We will explore the effects of recombinant IL-19 treatment on polarization of T-cell phenotype.
e. We will explore the effects of the absence of IL-19 on VSMC proliferation by comparing wild type and IL-19$^{-/-}$ both *in vivo* and *in vitro*.
f. We will explore the effects of the absence on IL-19 on inflammatory gene expression in VSMC.

CHAPTER 2

MATERIALS AND METHODS

2.1. Mice and Study Design.

LDLR$^{-/-}$ mice (stock #002207) or ApoE$^{-/-}$ (stock #002052) of both sexes on the C57 Black/6 (C57Bl/6) background were purchased from Jackson Labs, housed in an ALAC-approved facility, and maintained on a standard chow diet until study commencement. Excluding one study, LDLR$^{-/-}$ were primarily used because unlike ApoE$^{-/-}$ these mice do not develop atherosclerotic lesions until fed a high fat diet, allowing us to synchronize initiation of atherosclerosis with IL-19 administration. At 3-4 months of age, normal chow was replaced with an atherogenic diet (42% Fat, 0.2% cholesterol, Harlan atherogenic diet TD.88137) and injected i.p. with 1ng or 10ng/g/day mouse recombinant IL-19 (R&D Inc, Minneapolis, MN) or an equal volume of PBS for 5 days per week for 13 weeks for atherosclerotic studies, and 12 weeks for intravital microscopy analysis. Wild-type C57Bl/6 mice purchased from Jackson Labs were used for intravital microscopy and were similarly fed and treated. For the regression study, all LDLR$^{-/-}$ mice were fed an atherogenic diet for 14 weeks. One cohort was euthanized to establish baseline lesion accumulation, and the remaining mice were treated either with PBS or 10ng/g/day IL-19 for an additional 8 weeks and continued to consume an atherogenic diet. No mice were excluded from analysis. All animal procedures followed IACUC approved protocols.

IL-19 null mice were generated using the VelociGene method as described[40,89]. Homozygous IL-19$^{-/-}$ mice were identified by genotyping of tail DNA by polymerase chain reaction (PCR) using specific primers. Age and sex-matched male and female littermates were used for these studies. The shear-stress ligation model of injury was

performed as we described[94]. Mice were anesthetized by injection of ketamine and xylazine. The left common carotid artery was dissected and ligated near the bifurcation for 28 days. Severity of hyperplasia is strain dependent; the FVB lineage develops a robust, and the C57Bl/6 develops a limited intimal hyperplasia in response to carotid ligation[95,96]. Some mice were injected i.p. with 10ng/g/day murine rIL-19 (eBioscience) or an equivalent volume of PBS five days per week for the duration of the study. After 28 days, mice were euthanized and tissue prepared for immunohistochemistry and morphological analysis. All animal procedures followed IACUC approved protocols.

2.2. Atherosclerotic Lesion Analysis.

Atherosclerotic plaque was determined in the aortic intimal surface by en face staining with Sudan IV and in the aortic root by Oil Red O staining as we described[94]. Lesion size in the aortic arch was quantitated by quantitative morphometry using the Image Pro Plus program. Aortic root was frozen in optimal cutting temperature (OCT) medium and sectioned. Four transverse serial sections spaced 70-100μm apart from the aortic sinus to disappearance of valve cusps per aortic root from mice in each group were stained with Oil Red O, and positive stained areas quantitated as a percentage of total area by quantitative morphometry[97].

2.3. VSMC Culture and Proliferation.

Primary human coronary artery vascular endothelial cells (hCaECs), and human coronary artery vascular smooth muscle cells (hCaVSMCs) were obtained as cryopreserved secondary culture from Cascade Corporation (Portland, OR) and maintained as we described[64,68]. Cells were used from passage 3-5. For BMDM, femurs and tibiae were flushed with sterile DMEM, collected cells washed, resuspended

in DMEM+5% FBS, and cultured overnight to remove adherent cells. Non-adherent cells were cultured for 6 days in DMEM+10% FBS in the presence of 100ng/ml M-CSF (Peprotech). The adherent cells were then detached by incubation with Versene solution (GIBCO). For gene expression analysis, cells were pretreated with 100ng/ml IL-19 (R&D, Inc. Minneapolis, MN) for various times, then stimulated with 20ng/ml TNFα (Sigma St. Louis, MO). Some samples remained untreated and used as controls.

Abdominal aorta from wild-type and knockout mice were removed, endothelial layer scraped off, and VSMC isolated as described[94]. VSMCs were cultured in DMEM supplemented with 20% FBS. Greater than 95% of isolated cells were SMC actin positive, and VSMC from passage 3 to 5 were used. Proliferation was performed as described[72]. Briefly, equal numbers of VSMC were seeded into 24 well plates at a density of 5000 cells/ml, in the presence or absence of 100ng murine rIL-19 (eBioscience, Inc). Medium was changed on the fourth day, and after one, four, and seven days, cells were trypsinized and counted in triplicate using a standard hemocytometer. Experiments were performed in triplicate from VSMC isolated from three different knockout and wild-type mice. In a second experiment, wild-type and IL-$19^{-/-}$ mice were injected with 2.5mg/ml 5 days per week i.p. with the nucleotide analogue 5-bromo-2'-deoxyuridine (BrdU) following ligation injury. After fourteen days, carotid arteries were recovered, immunostained, and positive cells were quantitated.

2.4. Immunohistochemistry.

For atherosclerosis studies, human coronary and carotid vessels were graded by a board-certified pathologist and taken from a bank of human vessels excised post-mortem during routine autopsy at the LSU Health Sciences Center in Shreveport,

Louisiana[98]. All experiments using human tissue were deemed non-human research by the LSU Institutional Review Board due to the exclusive use of postmortem samples. Immunofluorescence on human coronary artery sections was performed as described[63]. Briefly, primary antibody incubation was followed by a 30-minute incubation with secondary antibody conjugated to AlexaFluor 568 (red) and AlexaFluor 488 (green) (Molecular probes, Inc., Eugene, OR). IL-19 antibody (Abcam, Inc., Cambridge, MA), GATA3, CD3, SMC actin, Von Willebrand, and leukocyte common antigen (CD45) (NeoMarkers, Inc, Burlingame, CA), were used at 1.0μg/ml as described[63]. For mouse aortic root, five micrometer sections from aortic root fixed in OCT were blocked in 10% goat serum. Sections were incubated with primary antibody at 1μg/ml in 1%BSA/PBS and were applied for one hour, followed by biotinylated secondary antibody (1:200) and then by avidin-biotin peroxidase complex for 30 min each. Non-specific isotype antibodies were used as negative controls. For quantitation of macrophage infiltrate, four transverse serial sections spaced 70-100μm apart from the aortic sinus to disappearance of valve cusps per aortic root from at least 9 mice in each group were immunostained with anti-F4/80 or CD3 purchased from NeoMarkers, Inc. Quantitative F4/80 and CD3 immunoreactivity was quantitated using the Image Pro Plus program as the percent of each lesion area which stains positive[97].

For restenosis studies, digitized images were measured and averaged from at least three representative 5 micron-thick stained tissue sections at least 75-100 microns apart per carotid artery using Image Pro Plus (Media Cybernetics) as we have described[63,94]. At least 6 mice were used for morphology, and 3 for IHC. The circumference of the lumen, the area encircled internal elastic lamina (IEL), and the external elastic lamina (EEL) were quantitated. The medial area was calculated by subtracting the area defined

by the IEL from the area defined by the EEL, and intimal area calculated as the difference between the area inside the IEL and the luminal area. Tissue fixation, processing, IL-19 antibody, and immunohistochemical staining was performed as described[72].

2.5. Intravital Microscopy.

Leukocyte adhesion was assayed in mesenteric post-capillary venules by intravital microscopy as we described[99]. Wild-type C57Bl/6 mice were fed an atherogenic diet for 12 weeks, receiving either PBS or 10ng/g/day rIL-19 i.p. 5 days per week. Three to four relatively straight, unbranched segments of post-capillary venules with lengths of >100 μm and diameters between 25 and 40 μm were randomly studied in each mouse using a Physiostation Microscope (Nikon Corp.), and the image recorded on A WIN XP Imaging Workstation. All data were analyzed using computerized imaging software. Leukocyte adherence was defined as the number of leukocytes firmly adhered to 100-μm length of endothelium for at least 30 seconds collected from at least 4 mice per group. Blood pressure measured by carotid artery cannulation using a blood pressure monitor (World Precision Instruments, Inc,. Sarasota, FL) was identical for all mice.

2.6. Serum Lipid Analysis.

Fasting lipid content in mouse sera was analyzed by Charles River Research Animal Diagnostic Services (Wilmington, MA 01887 USA).

2.7. RNA Extraction and Quantitative RT-PCR.

For the specified studies, VSMCs were serum-starved in 0.5% FBS for 48 hours, then stimulated with 10ng/ml TNFα for the indicated times. RNA from these cultured cells or

extracted spleen were 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 amplified using an Eppendorf Realplex4 Mastercycler[68]. Multiple mRNAs (Ct values) were quantitated simultaneously by the Eppendorf software. Primer pairs were purchased from Integrated DNA Technologies, (Coralville, IA), and SYBR green was used for detection. The following primer pairs were used:

Human glyceraldephyde-3-phosphate dehydrogenase (GAPDH):

F: CGAGAGTCAGCCGCATCTT

R: CCCCATGGTGTCTGAGCG

Human MCP-1:

F: AGCAGAAGTGGGTTCAGGATT

R: TGTGGAGTGAGTGTTCAAGTCT

Human IL-1β:

F: TCCCCAGCCCTT TTGTTG A

R: TTAGAACCAAATGTGGCCGTG

Human IL-8:

F: CCAGGAAGAAACCACGGA

R: GAAATCAGGGCTGCCAAG

Mouse GAPDH:

F: GCAAGGACACTGAGCAAGAG

R: GGGTCTGGGATG GAAATTGT

Mouse MCP-1:

F: TTAAAAACCTGGATCGGAACCAA

R: GCATTAGCTTCAGATTTACGGGT

Mouse IL-1β:

F: CTAATAGGCTCATCTGGGATCC

R: GGTCCGTCAACTTCAAAGAAC

Mouse IFNγ:

F: CCTAGCTCTGAGACAATGAACG

R: TCAATGACTGTGCCGTGG

Mouse GATA3:

F: TACCACCTATCCGCCCTATG

R: CTCGACTTACATCCGAACCC

Mouse T-bet:

F: CCTGTTGTGGTCCAAGTTCAAC

R: CACAAACATCCTGTAATGGCTTGT

Mouse IL-19:

F: AAATCTCTGGAGCGATGTCAG

R: GGCTAAAAGTATGTTCAGTTCTCC

Mouse IL-12p40:

F: GTGAAGCACCAAATTACTCCG

R: AGAGACGCCATTCCACATG

Mouse CXCL2 (human IL-8 homolog):

F: CAGAAGTCATAGCCACTCTCAAG

R: CTCCTTTCCAGGTCAGTTAGC

Mouse CXCL1 (human IL-8 homolog):

F: AGAACATCCAGAGCTTGAAGG

R: CAATTTTCTGAACCAAGGGAGC

Mouse TNFα:

F: CTTCTGTCTACTGAACTTCGGG

R: CAGGCTTGTCACTCGAATTTTG

2.8. Western Blotting.

Western blotting for HuR was performed as we described[68]. Briefly, cultured EC, VSMC, and BMDM were treated with 100ng IL-19/ml continuously for 4 days, changing the

media and adding fresh IL-19 every 24 hours. Extracts were prepared as described[48], and lysates frozen until use. Membranes were incubated with a 1:5000 dilution of HuR (Santa Cruz, Inc.) antibody, and a 1:10,000 dilution of secondary antibody. Reactive proteins were visualized using enhanced chemiluminescence (Amersham) according to manufacturer's instructions.

2.9. Statistical Analysis.

Results are expressed as mean ± standard error. Differences between groups were evaluated with the use of analysis of variance (ANOVA), with the Newman-Keuls method applied to evaluate differences between individual mean values and by paired *t*-tests where appropriate, respectively. Interquartile range was determined by the GraphPad Prizm statistical analysis program. Differences were considered significant when $p<0.05$.

CHAPTER 3

RESULTS

3.1. *IL-19 is Expressed in Human Atherosclerotic Plaque*.

The atherosclerotic plaque microenvironment is biased to T_h1 activation in humans and hypercholesterolemic mice[52]. IL-19 content in atherosclerotic plaque has never been reported. Human coronary arteries obtained post-mortem were immunostained with IL-19 antibody to characterize the cellular distribution in atherosclerosis. Very little IL-19 immunoreactivity was detected in normal artery, but surprisingly, abundant IL-19 immunoreactivity localized within plaque from a human patient with Stary classification type 4 plaque[100] (Figure 3). Very little to no IL-19 immunoreactivity was detected in medial VSMC in this artery, or in human artery from a patient with no atherosclerotic plaque. We did not observe IL-19 expression in Stary plaque classification types 1 or 2. We did observe consistent immunodetection in leukocyte, EC, and VSMC in Stary plaque types 4 and 5. IL-19 cell-specific expression in this plaque was established in EC, VSMC, and inflammatory cells by immunoreactive co-localization with the EC marker Von Willebrand, smooth muscle cell α actin, and leukocyte common antigen CD45 (Figure 4). This is the first description of IL-19 expression in atherosclerotic plaque. IL-19 expression in plaque, but not medial VSMC suggested a role for this cytokine in regulation of atherosclerosis.

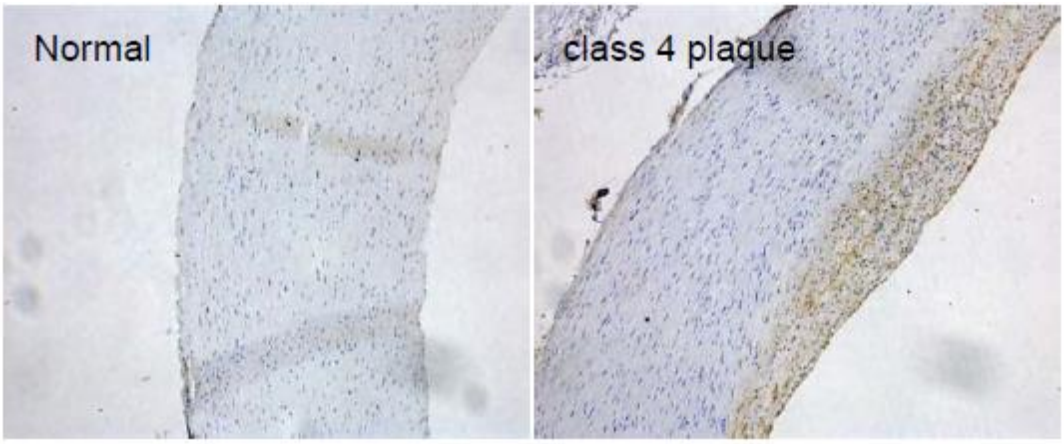

Figure 3. IL-19 is Expressed in Injured but Not Uninjured Human Artery . Immunohistochemical analysis of sections from vessels obtained from a normal, and a human atherosclerotic coronary (Stary Class 4) artery immunostained with anti-IL-19 antibody. Red-brown staining indicates antibody recognition. Magnification 200X.

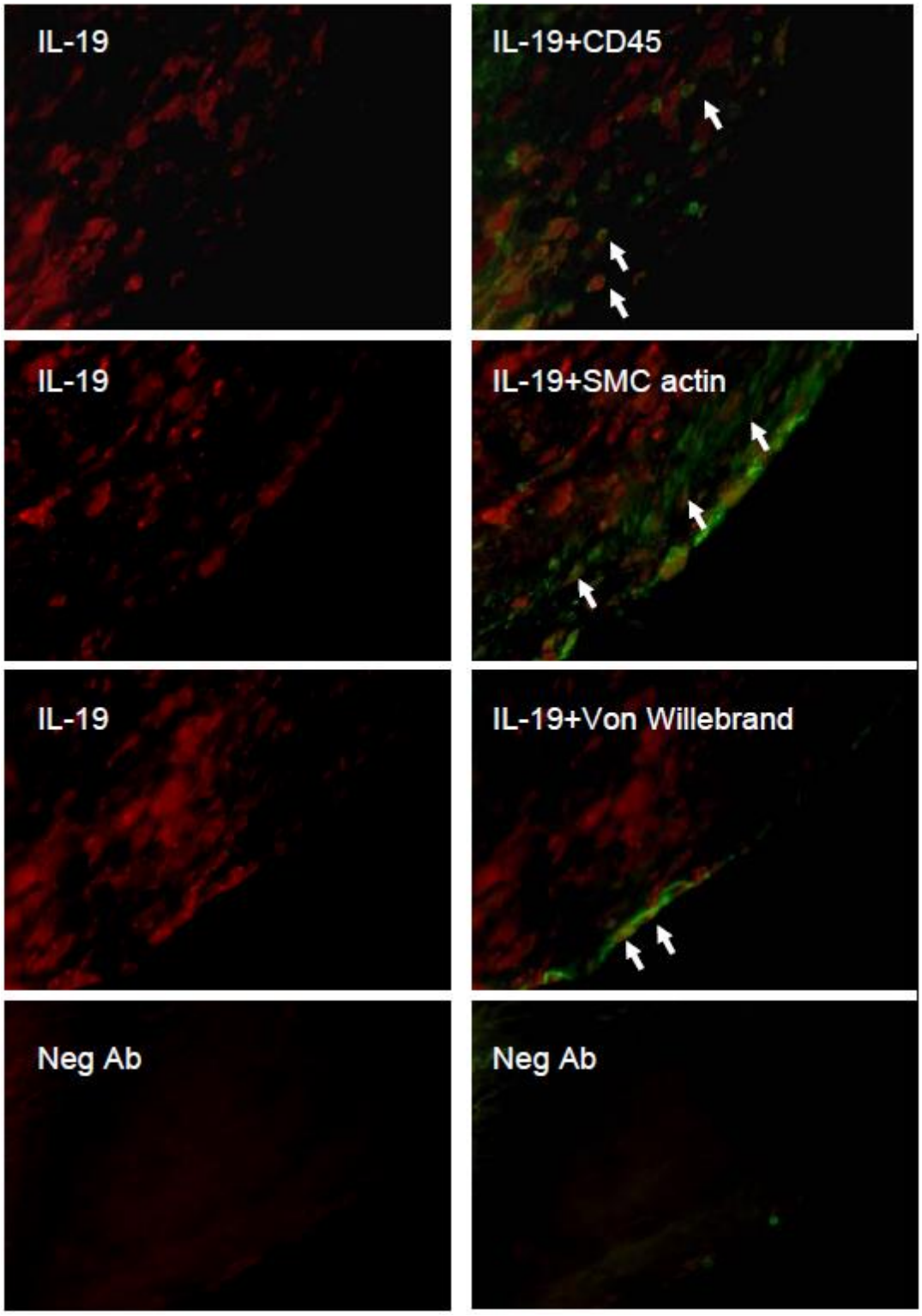
IL-19
IL-19+CD45
IL-19
IL-19+SMC actin
IL-19
IL-19+Von Willebrand
Neg Ab
Neg Ab

Figure 4. IL-19 is Expressed in Multiple Cell Types in Human Atherosclerotic Plaque. Immunohistochemical analysis of serial sections from vessels obtained from a normal, and a human atherosclerotic coronary (Stary Class 4) artery immunostained with anti-IL-19 antibody. Red-brown staining indicates antibody recognition. Sections were counterstained with hematoxylin. For cell-specific co-staining, immunofluorescence immunohistochemistry of an artery from a human patient with atherosclerosis was co-stained with anti-IL-19 (red) and: pan-leukocyte antigen CD45 (green), SMCα actin (green), or Von Willebrand (green). Arrows define merged localization. Magnification 600X.

3.2. IL-19 Decreases Atherosclerotic Plaque Area in LDLR$^{-/-}$ Mice.

We hypothesized that systemic administration of IL-19 would be protective and decrease atherosclerosis. Unless otherwise noted, LDLR$^{-/-}$ mice were utilized for inhibition experiments because, unlike ApoE$^{-/-}$ mice, they do not develop atherosclerotic lesions until fed a high fat diet, allowing synchronization of initiation of atherosclerosis with IL-19 administration. Mice were injected i.p. with 10ng/g/day murine recombinant IL-19, or an equal volume of PBS for 5 consecutive days per week for 13 weeks. Surface lesion area was determined by en face staining of aortic arch with Sudan IV and quantitative morphometry. Figure 5 shows representative pictures and graphical analysis of aortic arch indicating a significant reduction in lesion area between the PBS control and IL-19-treated mice (13.9±0.9% vs. 2.9±0.25%, respectively; $p<0.0001$, n=13 in each group).

Comparable results were obtained using ApoE$^{-/-}$ mice fed an atherogenic diet for 12 weeks (26.78±3.41% vs. 10.36±0.94%, p<0.0001, for PBS and IL-19-treated mice, respectively) (Figure 6). Similarly, morphometric analysis of lesion area in multiple serial transverse sections of Oil Red O stained aortic root was significantly reduced in IL-19 injected mice compared with PBS controls (24.7±1.8% vs. 16.6±1.5% for PBS and IL-19 treated, respectively, p<0.01) (Figure 7). In a second cohort we determined that systemic administration of as little as 1ng/g/day IL-19 could significantly reduce lesion area in the aortic arch (17.7±1.7% vs. 5.3±1.2% for PBS and IL-19 treated mice p<0.0001, n=11 and 13, respectively) (Figure 8). Analysis of lesion area in Oil Red O stained aortic root was also significantly lower in 1ng/g/day IL-19-injected mice compared with PBS controls (27.4±1.1% vs. 18.6±1.1% for PBS and IL-19 treated, respectively, p<0.01) (Figure 8). There was no significant difference in lesion area between sexes in any study. There was no significant difference in serum lipid profiles (Figure 9). There was no significant difference in weight gain (11.29±0.8 vs. 10.95±0.7 grams for PBS and IL-19 in the 10ng/g/day, or 11.99±1.4 vs. 8.9±0.9 grams for PBS and IL-19 in the 1ng/g/day LDLR$^{-/-}$ studies, respectively) (Figure 10).

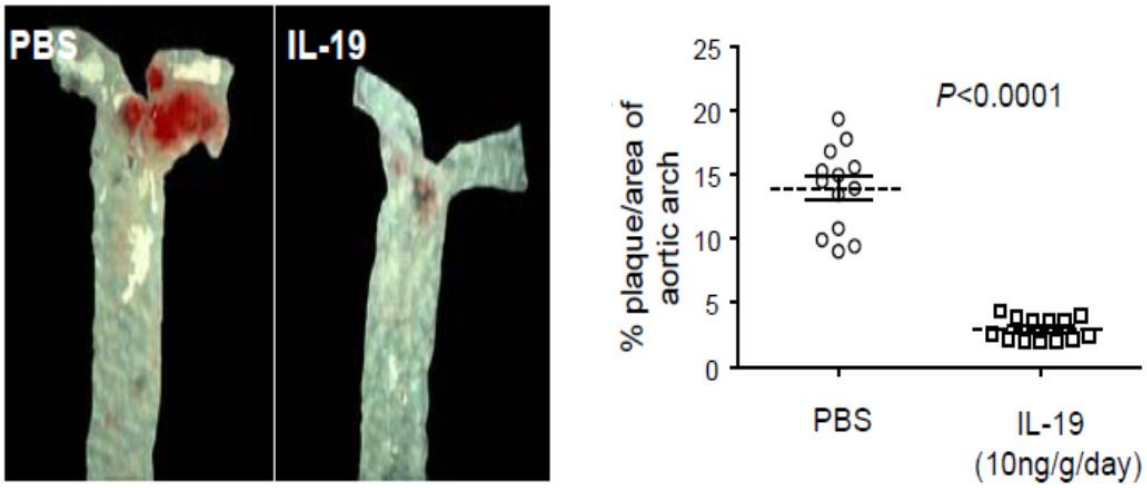

Figure 5. Administration of 10ng/g/day rIL-19 Inhibits Atherosclerotic Plaque Burden in Aortic Arch of LDLR$^{-/-}$ Mice. Representative photomicrograph of aortic arch surface lesion en face stained with Sudan IV. Graphic depiction of atherosclerotic lesion size quantitated from en face stained aortic arches from LDLR$^{-/-}$ mice after consuming atherogenic diet for 13 weeks, injected with either PBS or 10ng/g/day IL-19 (n=13 each).

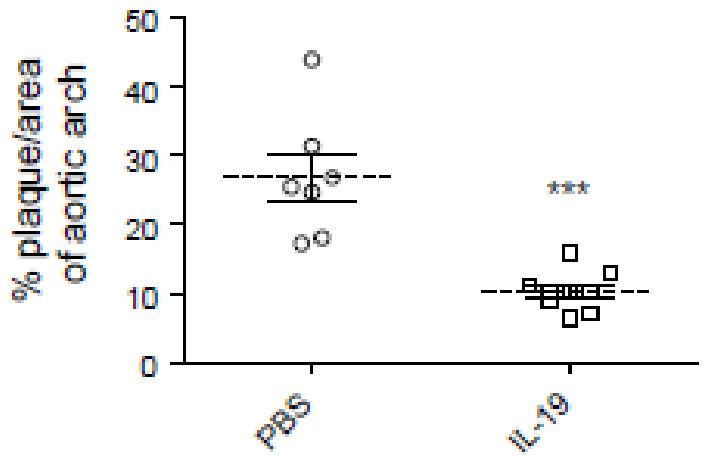

Figure 6. Administration of 10ng/g/day rIL-19 Inhibits Atherosclerotic Plaque Burden in Aortic Arch of ApoE$^{-/-}$ Mice. Graphic depiction of atherosclerotic lesion size quantitated from en face stained aortic arches from 3 month old ApoE$^{-/-}$ mice after consuming atherogenic diet for 12 weeks, injected with either PBS or 10ng/g/day IL-19. n=7 (PBS) and 9 (IL-19).

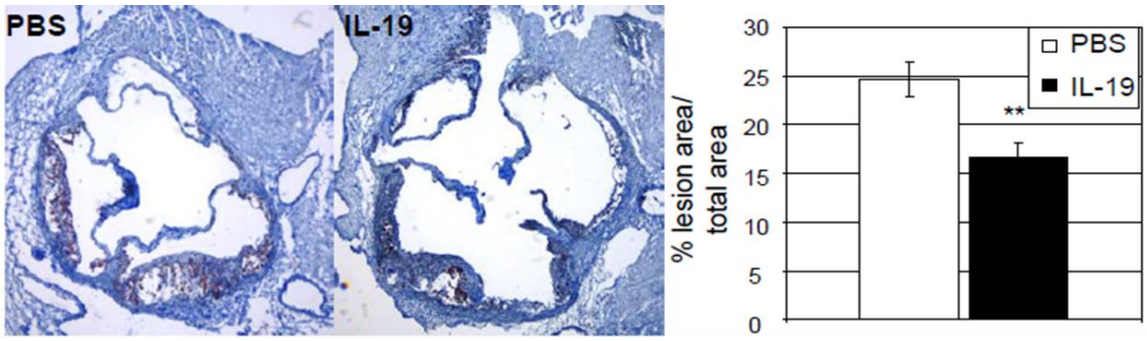

Figure 7. Administration of 10ng/g/day rIL-19 Inhibits Atherosclerotic Plaque Burden in Aortic Root of LDLR$^{-/-}$ Mice. Representative photomicrographs of aortic root stained with Oil Red O. Quantitation of lesion area from four transverse serial sections from the aortic sinus to disappearance of valve cusps per aortic root from mice were stained with Oil Red O, and positive stained areas quantitated. Mice received either PBS or 10ng/g/day IL-19 i.p. 5 days per week for 13wks.

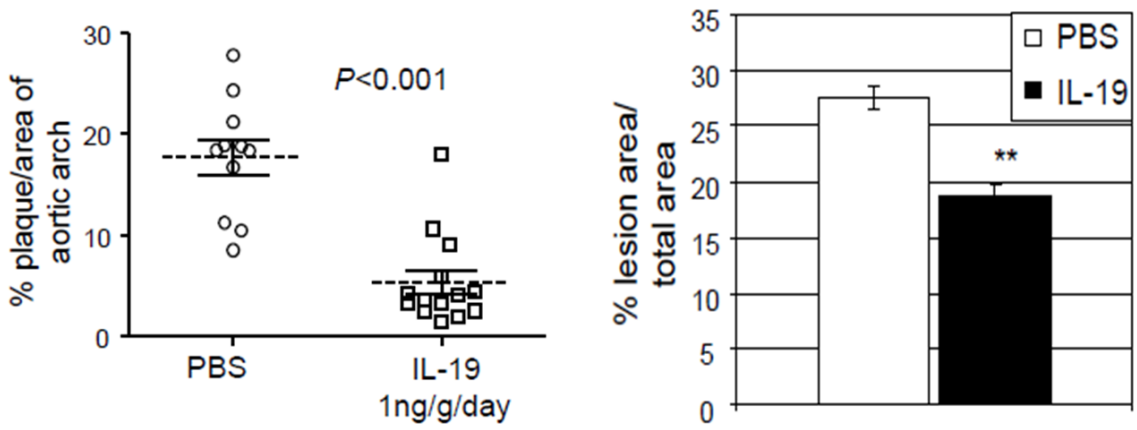

Figure 8. Administration of 1ng/g/day rIL-19 Inhibits Atherosclerotic Plaque Burden in Both Aortic Arch and Aortic Root of LDLR$^{-/-}$ Mice. Graphic depiction of atherosclerotic lesion size quantitated from aortic arches from LDLR$^{-/-}$ mice after consuming atherogenic diet for 13 weeks, injected with either PBS or 1ng/g/day IL-19 (n=11, n=13, respectively). Quantitation of lesion area from four transverse serial sections from the aortic sinus to disappearance of valve cusps per aortic root from mice were stained with Oil Red O and positive stained areas quantitated.

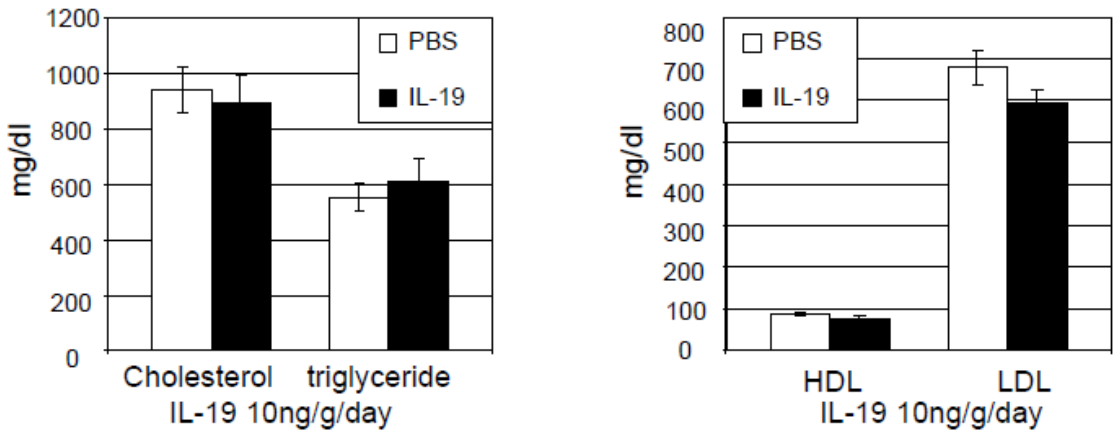

Figure 9. No Significant Difference in Lipid Profile of IL-19 or PBS Treated Animals. Cholesterol, triglycerides, HDL, and LDL in mice fed atherogenic diet for 13 weeks receiving either PBS or 10ng/g/day IL-19, at time of euthanasia do not statistically differ between control and IL-19 groups (n=9 each).

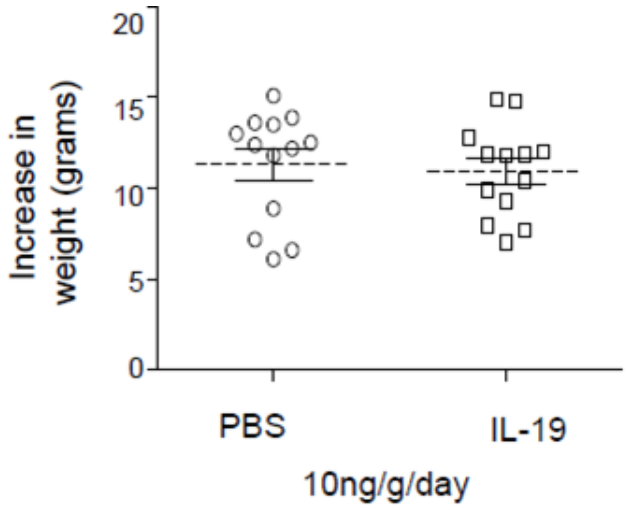

Figure 10. No Significant Difference in Weight Gain of IL-19 or PBS Treated Animals. Representative increase in weight gain of mice receiving either PBS or 10ng/g/day IL-19 i.p. 5 days per week for 13wks (n=13 each). No significant difference in any of the 1ng/g/day or 10ng/g/day studies was observed for animal weight changes.

3.3. IL-19 Can Halt, But Not Reverse Existing Plaque.

We hypothesized that IL-19 might reverse existing plaque. For this study, $LDLR^{-/-}$ mice were fed an atherogenic diet for 14 weeks. The aortic arch from a cohort of mice were examined to establish baseline lesion accumulation, and the remaining mice were treated i.p. either with PBS or 10ng/g/day IL-19 for an additional 8 weeks, continuing to consume an atherogenic diet. Figure 11 shows that baseline mice had 26.7±1.7% lesion area. After 8 additional weeks, IL-19 treated mice had 23.7±2.6% lesion area, in

contrast to PBS controls, which had 41.0±3.1% lesion area. There was no significant difference in lesion area between baseline controls and IL-19 treated mice even though they received atherogenic chow for 8 additional weeks. The difference between PBS controls and IL-19 treated mice was statistically significant (p=0.002) (Figure 11). While IL-19 could not significantly reduce the overall lesion area, it did prevent additional lesion burden from forming. Median and interquartile range (IQR) values for all *in vivo* studies are summarized in Table 5. This is the first report demonstrating that systemic administration of IL-19 is anti-atherogenic, and the remainder of the study was directed toward elucidating mechanisms for this effect.

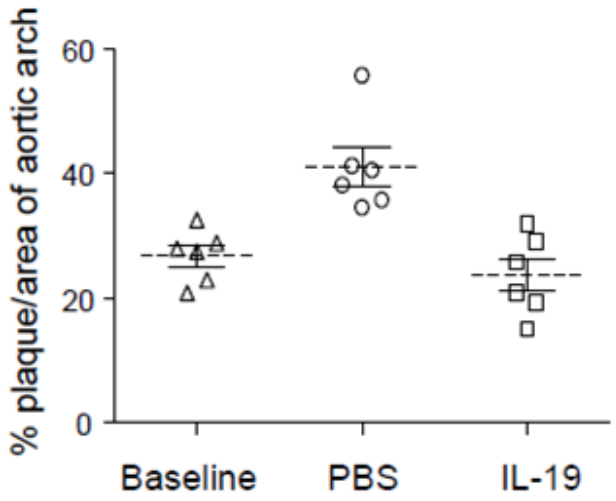

Figure 11. IL-19 Can Halt Progression of Plaque Formation. IL-19 can halt, but not reverse existing atherosclerotic plaque. $LDLR^{-/-}$ mice were fed an atherogenic diet for 14 weeks to establish baseline lesion accumulation, and the remaining mice were treated i.p. either with PBS or 10ng/g/day IL-19 for an additional 8 weeks (n=6 each). There was no statistical difference between baseline and IL-19 treated cohorts, but PBS treated mice were statistically different from baseline.

Table 5. IQR Values for All Studies

Study	mouse	condition	median (%)	IQR (%)
10ng/g/day	($LDLR^{-/-}$)	PBS	16.83	14.73-21.72
		IL-19	2.00	1.32- 2.75
1ng/g/day	($LDLR^{-/-}$)	PBS	18.41	11.27-27.81
		IL-19	3.81	2.50- 6.72
10ng/g/day	($ApoE^{-/-}$)	PBS	25.45	18.06-43.92
		IL-19	10.24	8.05-12.06
Regression 10ng/g/day	($LDLR^{-/-}$)	Baseline	27.71	22.45-29.77
		PBS	39.45	35.52-44.91
		IL-19	23.42	18.27-29.86

3.4. IL-19 Polarizes Leukocytes to a T_h2 Profile.

Atherosclerosis is highly influenced by the Th1/Th2 balance, and the interplay between these two phenotypes determines severity[45,47]. IL-19 is considered a T_h2 interleukin, and one potential mechanism for IL-19 anti-atherogenic effect is polarization of the immune response to the T_h2, T-regulatory phenotype. The immunological status of these mice was determined by quantitation of T_h1 and T_h2 marker expression in splenocytes immediately removed from mice at the termination of the study. Quantitative RT-PCR demonstrates that splenocytes from IL-19 injected mice had significantly lower mRNA levels of the T_h1 markers T-bet (0.82±0.05 vs. 0.66± 0.04 for PBS and IL-19 treated, respectively, $p<0.05$), and IFNγ (0.89±0.05 vs. 0.68±0.02 for PBS and IL-19 treated,

respectively, $p<0.05$). mRNA levels for IL-1β and IL-12p40, both potent pro-inflammatory cytokines, were also significantly decreased in IL-19 injected mice, (0.78±0.28 vs.0.61±0.5 for IL-1β, $p<0.001$, and 0.88±0.30 vs. 0.72±0.04 $p<0.01$, for PBS and IL-19, respectively), suggesting decreased inflammatory background in these mice (Figure 12). Concordantly, splenocytes from IL-19 injected mice had significantly higher mRNA levels of the T_h2 markers GATA3 (1.06±0.08 vs. 1.62±0.22 for PBS and IL-19 respectively $p<0.001$). Interestingly, IL-19 mRNA was increased in mice injected with IL-19 indicating a potential positive feedback mechanism (Figure 13).

Multiple serial sections throughout the aortic root from IL-19 treated and control mice were immunostained using CD3 antibody to detect T lymphocyte. Positively stained areas were determined as a percentage of total area by quantitative morphometry. While IL-19 treated mice trended lower, no significant difference in absolute numbers of CD3 positive lymphocytes in plaque was noted in IL-19-treated compared with PBS mice (28.91±3.24 vs. 23.27±4.35% for PBS and IL-19). However, a significant increase in the percentage of GATA3 positive T lymphocyte infiltrate was noted in IL-19 treated mice, compared with PBS injected controls (75.10±4.70% vs. 56.15±6.15 for IL-19 and PBS, respectively, $p<0.05$) (Figure 14). Taken together, these data indicate that systemic injection of IL-19 can impart a T_h2 profile in these mice, and suggests that one mechanism for IL-19 protective effects is polarization of the immune system to a T regulatory profile.

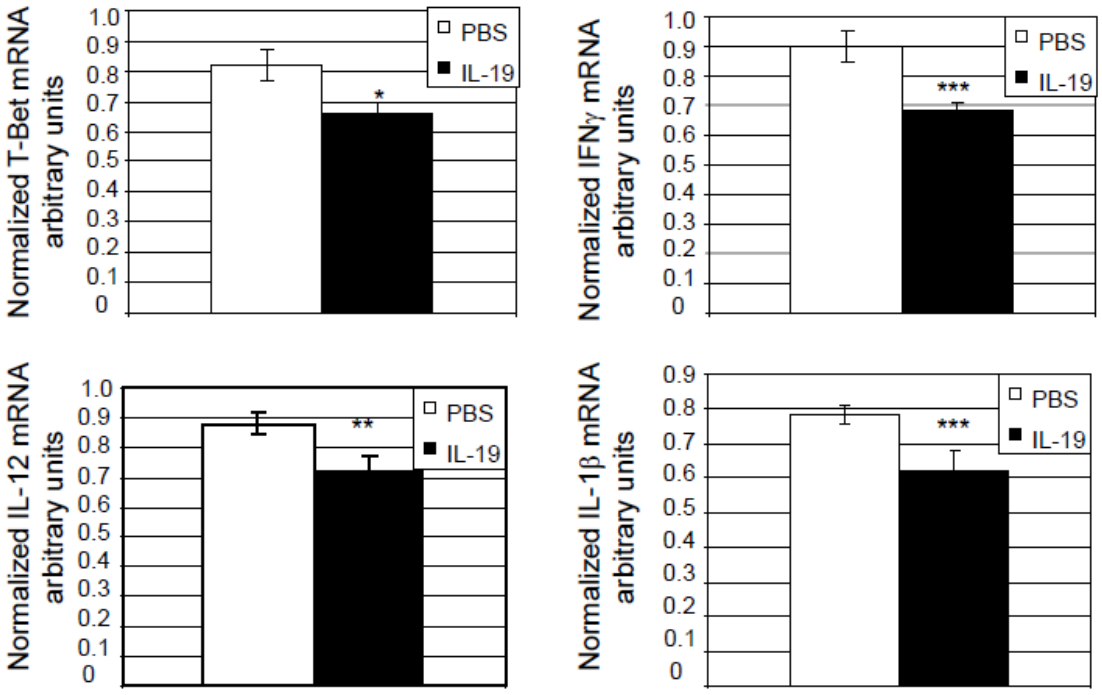

Figure 12. IL-19 Dampens the T_h1 Adaptive Immune Response. Quantitative RT-PCR on RNA extracted from spleen from mice receiving either PBS or 10ng/g/day IL-19. Spleens were removed at the time of euthanasia, RNA extracted and reverse transcribed, and amplified using the primer pairs shown. $p<0.05$, 0.01, or 0.001, as shown. n=9 spleen each group.

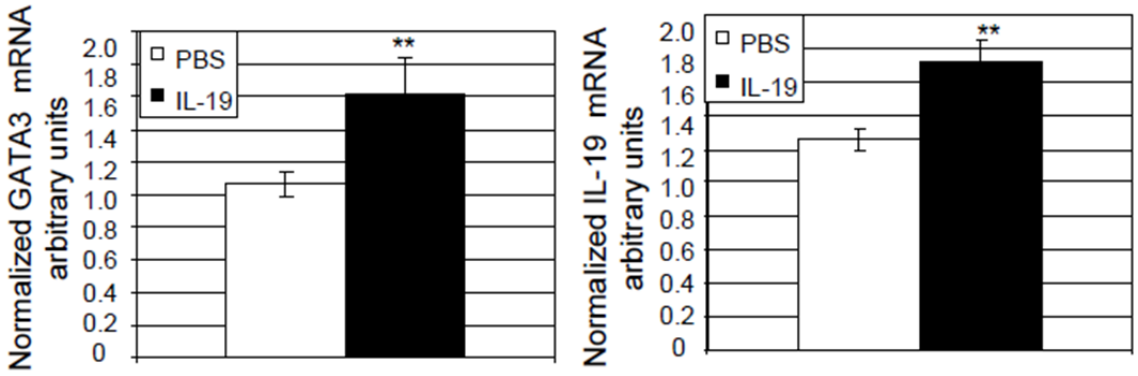

Figure 13. IL-19 Polarizes the Adaptive Immune Response to T_h2. Quantitative RT-PCR on RNA extracted from spleen from mice receiving either PBS or 10ng/g/day IL-19. Spleens were removed at the time of euthanasia, RNA extracted and reverse transcribed, and amplified using the primer pairs shown. $p<0.01$. n=9 spleen each group.

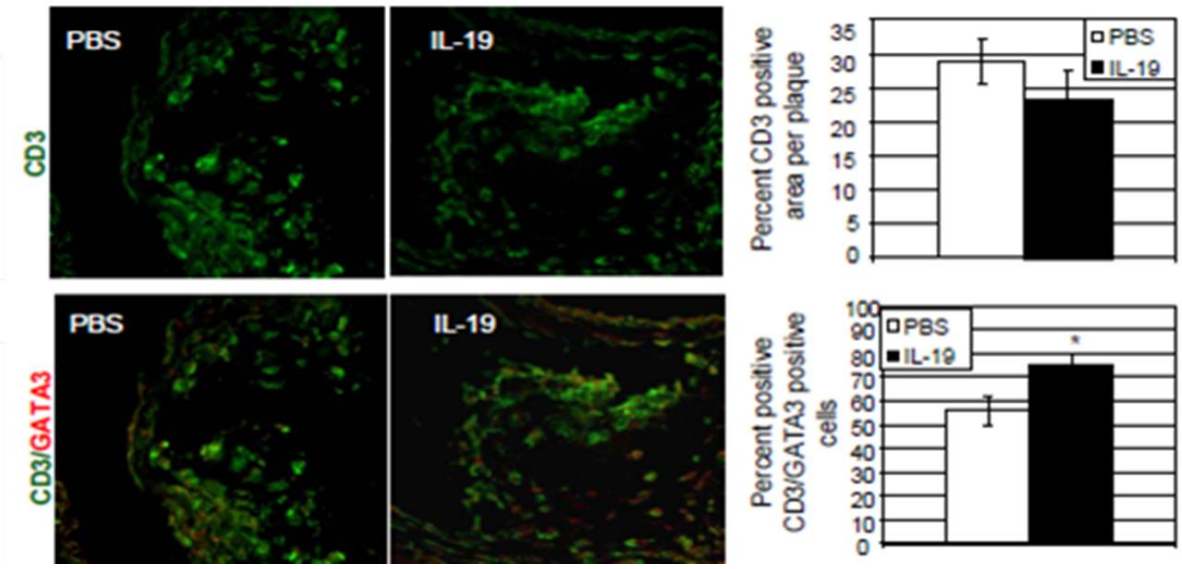

Figure 14. T-Lymphocyte Infiltrate Into Atherosclerotic Lesions. Representative photomicrograph of immunohistochemical characterization of atherosclerotic lesions indicates no significant difference in numbers of CD3 positive lymphocytes in IL-19-treated compared with PBS mice. Representative photomicrograph showing a significant increase in GATA3 positive T lymphocyte infiltrate in IL-19 treated mice compared with PBS injected controls. Multiple serial sections of the aortic root from the aortic sinus to disappearance of valve cusps per root of 10ng/g/day IL-19 treated and PBS control mice were sectioned and immunostained using CD3 and GATA3 antibody. Positively stained areas were quantitated as a percentage of total lesion area by quantitative morphometry. $p<0.001$, n=8 mice, using at least three sections per aortic root.

3.5. IL-19 Decreases Macrophage Accumulation in Atherosclerotic Lesions.

To further characterize the cellular content of atherosclerotic lesions, macrophage infiltrate was assessed by immunohistochemistry. Multiple serial sections throughout the aortic root from IL-19 treated and control mice were immunostained using CD68 or F4/80 antibody to detect macrophage. Positively stained areas were quantitated as a percentage of total area by quantitative morphometry. Immunohistochemical analysis of serial sections of atherosclerotic plaque from $LDLR^{-/-}$ mice fed an atherogenic diet for 6 weeks indicate that IL-19 is expressed in monocyte/macrophages in early atherosclerotic lesions (Figure 15). In $LDLR^{-/-}$ mice fed an atherogenic diet for 12 weeks significantly less macrophage infiltrate in plaque from IL-19 injected mice was observed compared with PBS control mice (20.0±2.7% vs. 36.5±2.2%, respectively, $p<0.001$) (Figure 16). These data suggest that at least in this experimental model of atherosclerosis, IL-19 does not polarize macrophage to the alternative, M2 phenotype, but does indicate that IL-19 can reduce macrophage accumulation.

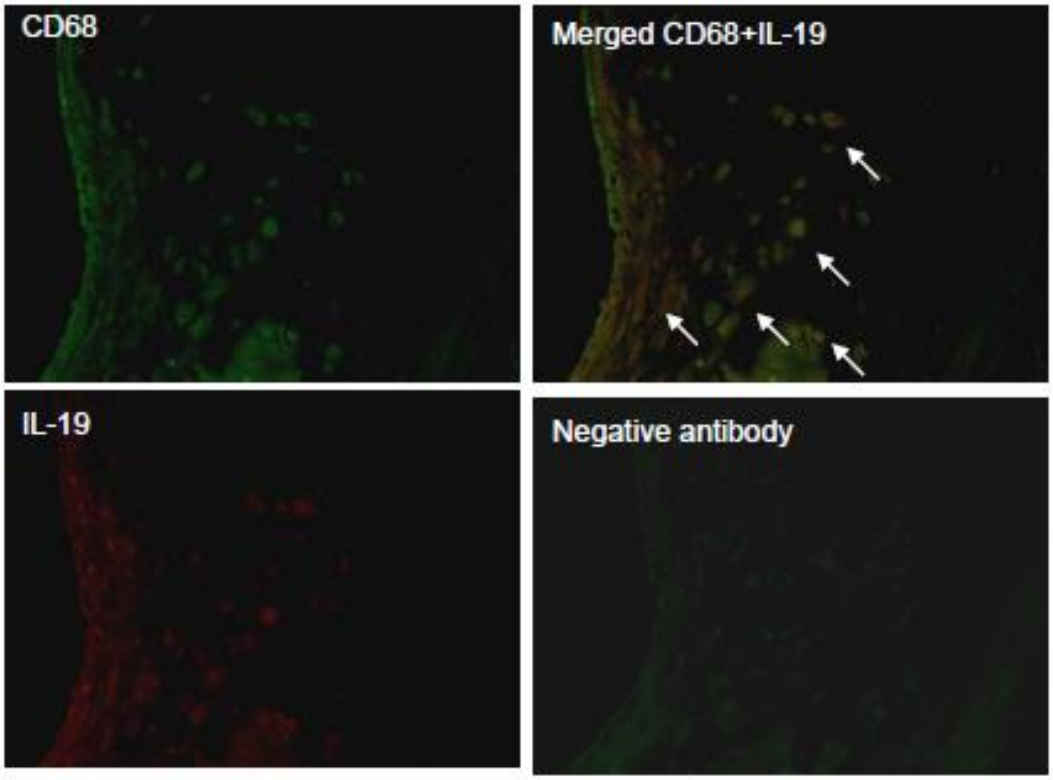

Figure 15. IL-19 is Expressed in Monocyte/Macrophages in Early Atherosclerotic Plaque in LDLR$^{-/-}$ Mice. Immunohistochemical analysis of serial sections of atherosclerotic plaque from LDLR$^{-/-}$ mice fed an atherogenic diet for 6 weeks immunostained with IL-19 antibody (red), and cluster of differentiation 68 (CD68) (green). Arrows depict specific examples of co- staining.

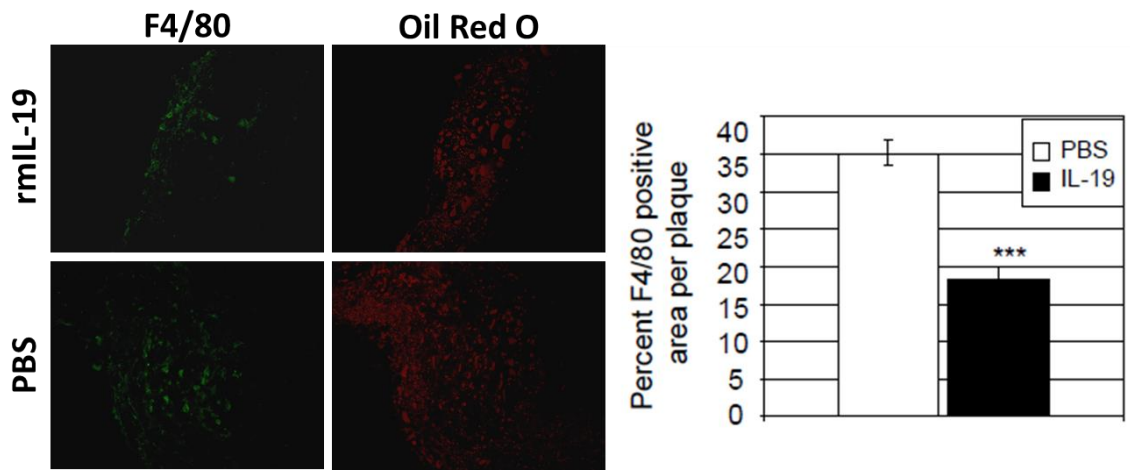

Figure 16. IL-19 Reduces Macrophage Infiltrate in Atherosclerotic Lesions. Representative fluorescent photomicrographs of aortic root immunostained with F4/80 antibody. Multiple serial sections of the aortic root from the aortic sinus to disappearance of valve cusps per root of 10ng/g/day IL-19 treated and PBS control mice were sectioned and immunostained. Positively stained areas were quantitated as a percentage of total lesion area by quantitative morphometry. $p<0.001$, n=8 mice, using at least three sections per aortic root.

3.6. IL-19 Decreases Leukocyte-Endothelial Adhesion in Mice Fed an Atherogenic Diet.

Decreased macrophage infiltrate in atherosclerotic lesion suggested that IL-19 could reduce leukocyte-EC interaction induced by a chronic atherogenic diet *in vivo*. For these experiments, wild-type C57Bl/6 mice were fed an atherogenic diet for 12 weeks, and injected i.p. 5 consecutive days per week with 10ng/g/day IL-19 or PBS. Leukocyte-EC

interaction was assessed *in vivo* by quantitative intravital microscopy. Figure 17 shows that IL-19 significantly reduced leukocyte adhesion induced by an atherogenic diet (2.1±0.4 for no atherogenic diet, and 3.5±.78 vs. 1.5±0.5 cells/100μm for PBS and IL-19 treated mice, respectively, $p<0.05$). There was no statistical difference in rolling between untreated and IL-19 treated mice. This suggests that a second mechanism for IL-19 anti-atherosclerotic effects is a reduction in leukocyte-EC interactions.

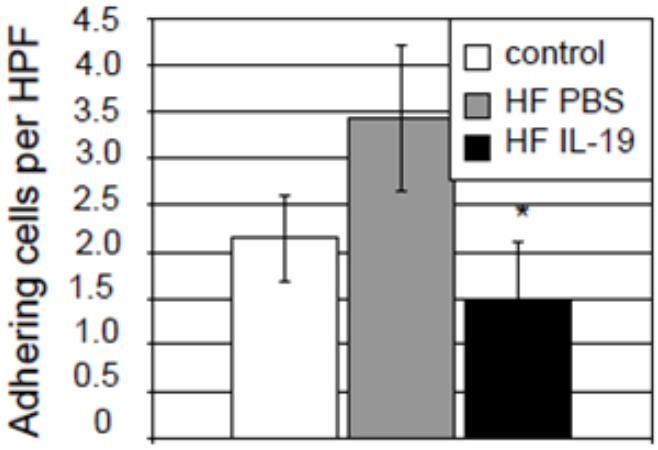

Figure 17. IL-19 Reduces Leukocyte-Endothelial Interactions *In Vivo*. Wild-type C57Bl/6 mice were fed an atherogenic diet for 12 weeks, during which time they were injected i.p. with 10ng/g/day IL-19 or PBS. Leukocyte adhesion was quantitated by intravital microscopy. IL-19 treatment significantly reduces leukocyte adhesion ($p<0.05$, n=5 mice per group).

3.7. IL-19 Directly Decreases Expression of Chemokines in Cultured EC, VSMC, and Macrophage.

Leukocyte homing to the atherosclerotic lesion is mediated by the local chemokine gradient. To test the hypothesis that IL-19 would have direct anti-inflammatory effects on EC, VSMC, and macrophage, we investigated if IL-19 could decrease expression of chemokines in these cells. Cultured human coronary artery EC, VSMC, and mouse BMDM were pre-treated with IL-19 for varying times, then challenged with TNFα. Cytokine mRNA was determined by quantitative RT-PCR. Figure 18 demonstrates that in all cell types tested, IL-19 has a potent inhibitory effect on IL-1β, IL-8, and MCP-1 mRNA accumulation, each of which is a potent leukocyte chemoattractant. Interestingly, efficacy of IL-19 inhibition of mRNA varied for different cell types. Differential sensitivity to IL-19 implies complex and perhaps cell type-specific regulatory mechanisms for IL-19 inhibitory effects. Together, this demonstrates that IL-19 can have direct anti-atherogenic effects on resident vascular cells as well as immune cells, suggesting a third mechanism for IL-19 anti-atherosclerotic effects. We previously reported that IL-19 does not inhibit NF-κB activity, but transiently decreases activation of the mRNA stability factor HuR[68]. Each of the chemokines tested contains an ARE in its 3-prime untranslated region (3'UTR), which is recognized by HuR. To determine potential molecular mechanisms for IL-19 anti-inflammatory effects, we chronically treated cultured EC, VSMC, and BMDM with IL-19 continuously for 4 days, changing the media and adding fresh IL-19 every 24 hours. Figure 19 shows that chronic stimulation of each of these cell types with IL-19 decreases protein abundance of HuR. This is significant as HuR preferentially stabilizes inflammatory transcripts.

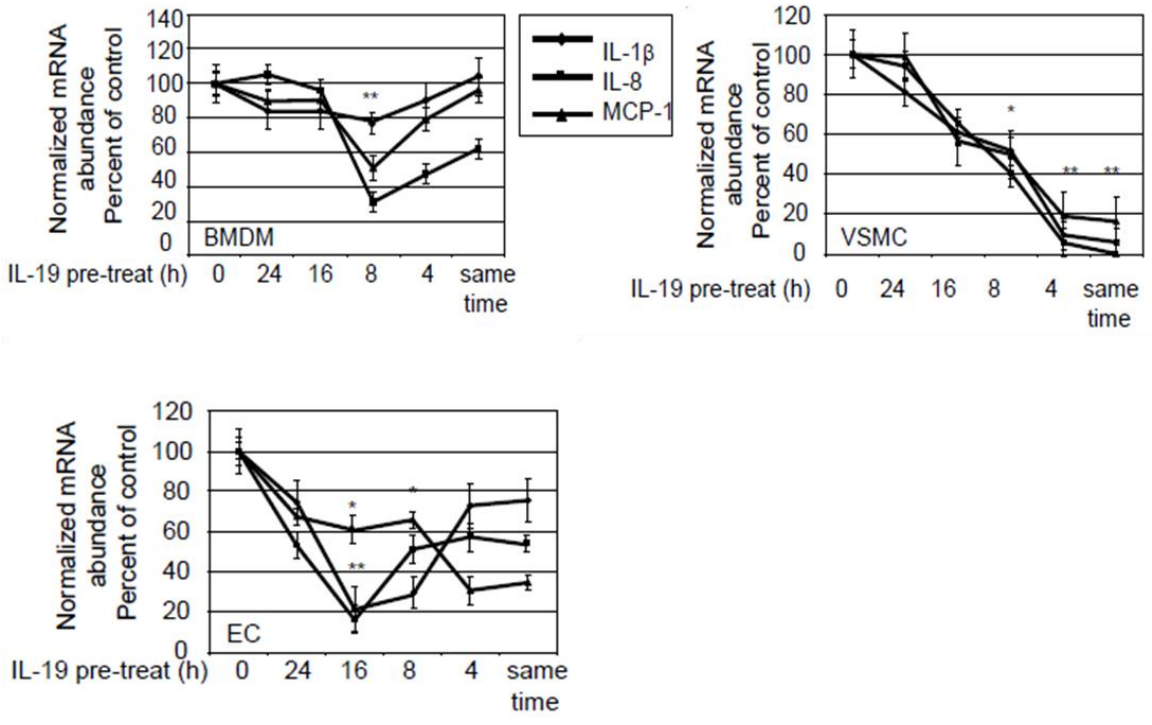

Figure 18. IL-19 Decreases mRNA Abundance of Chemotactic Cytokines in Resident Vascular and Immune Cells. Cultured VSMC or EC were serum-starved 24 hours. BMDM were isolated from C57Bl/6 mice. All cells were pre-treated with IL-19 for the times shown, then stimulated with 10ng/ml TNFα for four hours. Total RNA was reverse transcribed and target mRNA quantitated by quantitative real time polymerase chain reaction (qRT-PCR). mRNA was normalized to GAPDH. Differences in IL-19 pretreated vs. untreated treated controls are significant where indicated ($p<0.05$ or 0.01, n=3 experiments), asterisk are for all targets at that time, unless otherwise noted.

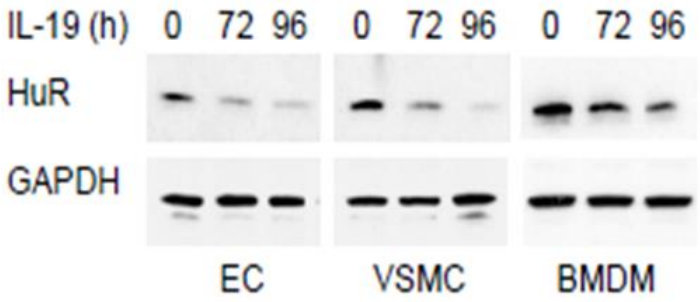

Figure 19. Chronic Treatment with IL-19 Reduces HuR Protein. Cultured VSMC or EC were serum-starved 24 hours. BMDM were isolated from C57Bl/6 mice. Cells were stimulated with IL-19 continuously for 4 days, changing the media and adding fresh IL-19 every 24 hours. At the indicated times lysates were immunoblotted with HuR and GAPDH antibody. Western blot is representative of at least 3 independent experiments, with identical results.

3.8. Injection of Recombinant IL-19 Inhibits Intimal Hyperplasia in Wild-Type Mice.

Based on its potent anti-inflammatory properties, we hypothesized that IL-19 would decrease intimal hyperplasia subsequent to carotid artery ligation. FVB wild-type mice were used for these studies as it has been shown that this strain has a robust response to this type of arterial injury[95,96]. The left common carotid artery was ligated near the carotid bifurcation. One cohort of mice were injected i.p. with 10ng/g/day rIL-19 5 days per week, and the other with an equivalent volume of PBS as controls. After 28 days, arteries were recovered, sectioned, and vascular compartments quantitated by

morphological analysis. Quantitative morphological analysis determined a significant decrease in I/M ratio in IL-19 injected mice (0.87+/-0.20) compared with PBS controls (2.14+/-0.33), (n=10 for rIL-19, 9 for PBS, $p<0.01$). The percent stenosis was significantly decreased in IL-19-injected mice (62.00+/-4.9%) compared with PBS controls (85.01+/-3.6%), ($p<0.001$) (Figure 20). No statistical differences were noted in the overall diameter or medial area of these arteries, suggesting that IL-19 can attenuate intimal hyperplasia.

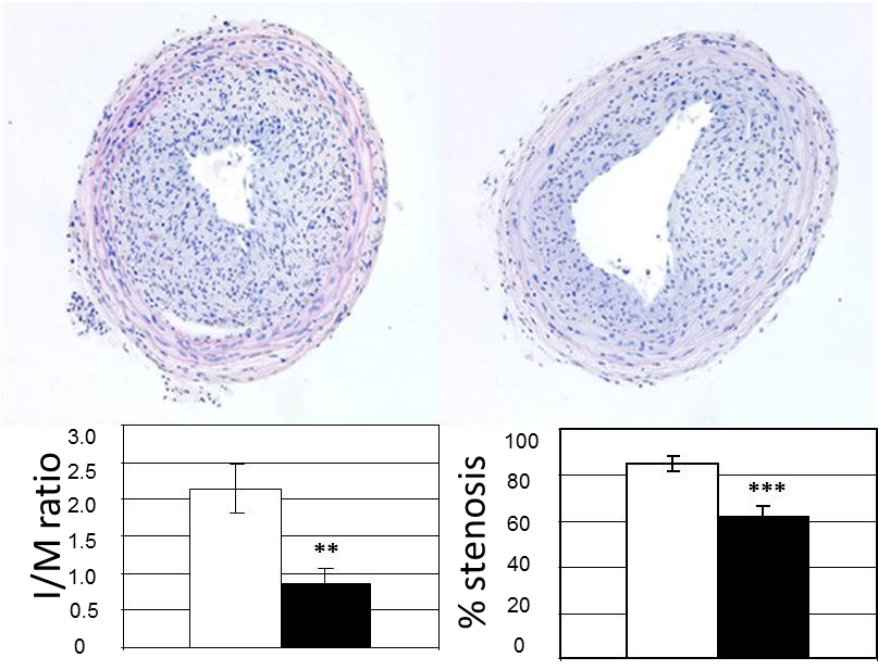

Figure 20. IL-19 Reduces Intimal Hyperplasia in FVB Mice after Carotid Artery Ligation. Representative photomicrographs stained with hematoxylin/eosin. Quantitative morphological analysis determined a significant decrease in the I/M ratio and overall percent stenosis of FVB wild type mice receiving i.p. 10ng/g/day rIL-19 (n=10) compared with PBS controls (n=9) (p<0.01 or 0.001, as shown). Magnification 200X.

3.9. Lack of IL-19 Exacerbates Intimal Hyperplasia.

To further define a role for IL-19 in neointimal hyperplasia, we utilized IL-19 knockout mice[89]. Age and sex-matched littermates in the C57Bl/6 background were subject to carotid artery ligation, and vascular compartments quantitated after 28 days. Figure 21

shows that the I/M ratio was significantly less in wild-type mice (0.50+/-0.12) compared with IL-19 knockout mice (0.83+/-0.09) (n=9 for wild-type, n=13 for IL-19$^{-/-}$, p<0.05). Similarly, the percent stenosis was also significantly less in wild type mice (49.04+/-8.1%) compared with IL-19 knockout mice (74.59+/-4.55%), (p<0.01). The robust response to ligation injury in the IL-19 knockout mice is noteworthy considering the C57Bl/6 strain is particularly resistant to this type of injury[95,96].

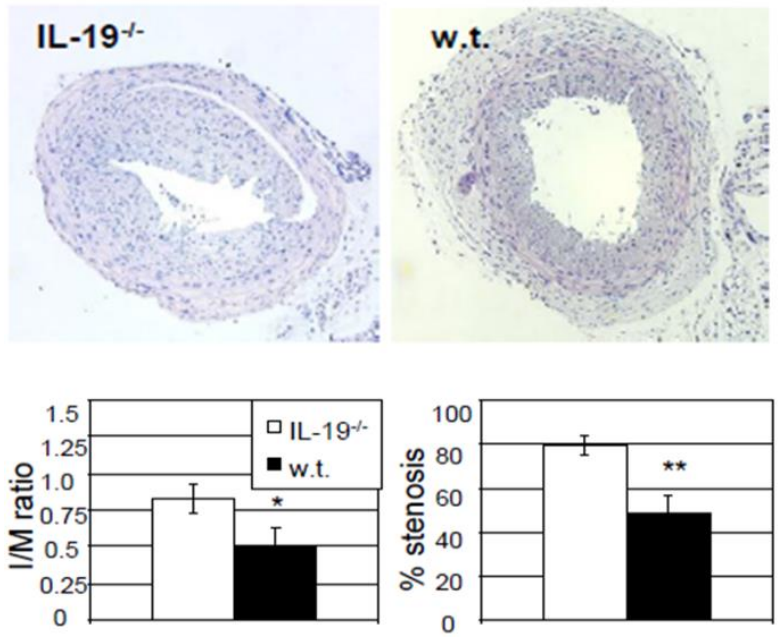

Figure 21. IL-19$^{-/-}$ Mice Exhibit an Exacerbated Response to Ligation Injury. Representative photomicrographs stained with hematoxylin/eosin. Quantitative morphological analysis determined a significant increase in I/M ratio and percent stenosis in IL-19$^{-/-}$ mice (n=13) compared with wild-type mice (n=9) (p<0.05 or 0.01, as shown). IL-19$^{-/-}$ and wild-type aged matched littermates are in the C57Bl/6 background. Magnification 200X.

3.10. Injection of Recombinant IL-19 into IL-19$^{-/-}$ Mice Rescues Intimal Hyperplasia.

To confirm the role of IL-19 in regulation of neointimal hyperplasia, IL-19 knockout mice were subject to carotid artery ligation. Some mice were injected i.p. with 10ng/g/day rIL-19 for 5 consecutive days per week, others with equivalent volume of PBS as controls. Vascular compartments were quantitated after 28 days. Figure 22 shows that IL-19 knockout mice injected with IL-19 had significantly lower I/M ratio (0.40+/-0.11) compared with PBS control mice (1.20+/-0.37) (n=11 for rIL-19, 10 for PBS, $p<0.05$). Likewise, percent stenosis was significantly less in IL-19 injected mice (38.36+/-5.86%) compared with PBS control mice (60.60+/-5.96%) ($p<0.01$). Since the absence of IL-19 is associated with increased intimal hyperplasia and addition of rIL-19 to IL-19$^{-/-}$ mice rescues development of this intimal hyperplasia, these data strongly suggest that IL-19 can regulate carotid artery ligation-induced carotid artery restenosis.

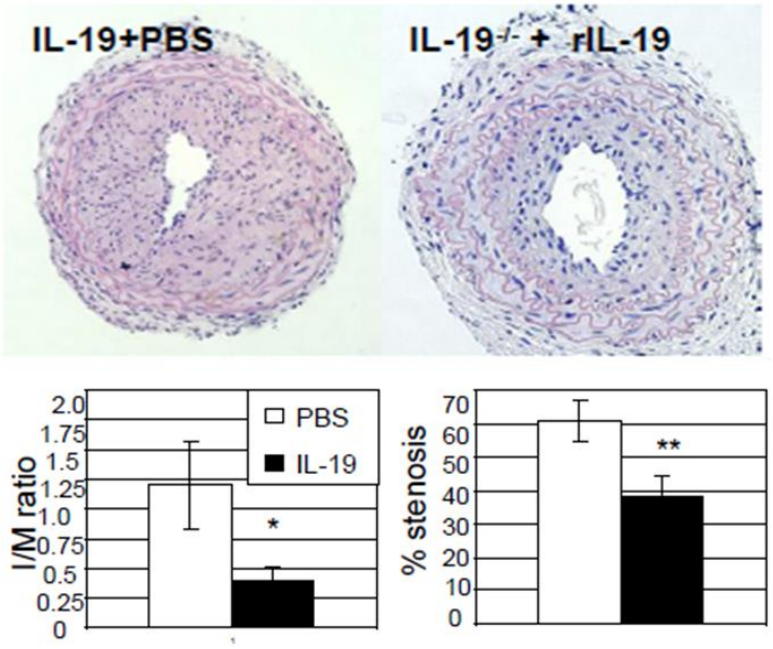

Figure 22. Injection of Recombinant IL-19 into IL-19$^{-/-}$ Mice Can Rescue the IL-19$^{-/-}$ Response to Injury Phenotype. Representative photomicrographs stained with hematoxylin/eosin. Quantitative morphological analysis determined significantly decreased I/M ratio and percent stenosis in mice injected 5 days per week for 28 days with 10ng/g/day rIL-19 i.p. (n=11) as compared to PBS controls (n=10) (p<0.05). Magnification 200X.

3.11. IL-19 Does Not Alter T_h2/T_h1 Polarization in Ligated Mice.

IL-19 is considered a T_h2 interleukin, and we hypothesized that polarization of adaptive immunity to the T_h2 phenotype might account for decreased intimal hyperplasia

observed in IL-19-treated mice[60,61,101]. Two experiments were performed to determine IL-19 effects on immune cells. First, the immunological status of these mice was determined by quantitation of T_h1 and T_h2 marker expression in splenocytes immediately removed from mice at the termination of the study. Quantitative RT-PCR demonstrates that mRNA levels of the T_h1 marker T-bet in splenocytes from FVB mice injected with IL-19 trended lower, but were not significantly different from PBS-injected controls (1.24+/-0.12 versus 1.58+/-0.27, respectively). IFNγ and IL-12β also lacked statistically significant differences in mRNA levels. Concordantly, IL-19 injected mice trended, but did not have statistically higher mRNA levels of the T_h2 marker trans-acting T-cell-specific transcription factor GATA-3 (GATA3) (0.67+/-0.08 versus 0.87+/-0.19, for PBS and IL-19 treated, respectively) (Figure 23). Similarly, transcripts in wild type C57Bl/6 and IL-$19^{-/-}$ mice were not statistically different. Second, serum cytokine levels for IFNγ, IL-1β, TNFα, and IL-10 were also not significantly different in IL-19-injected mice compared with controls, or in wild-type C57Bl/6 compared with IL-$19^{-/-}$ mice. Taken together, these data indicate that injection of IL-19 over a four week period does not polarize the immune system to a T_h2 anti-inflammatory profile, suggesting that immune cell polarization is not a major mechanism for reduced intimal hyperplasia in IL-19 treated mice.

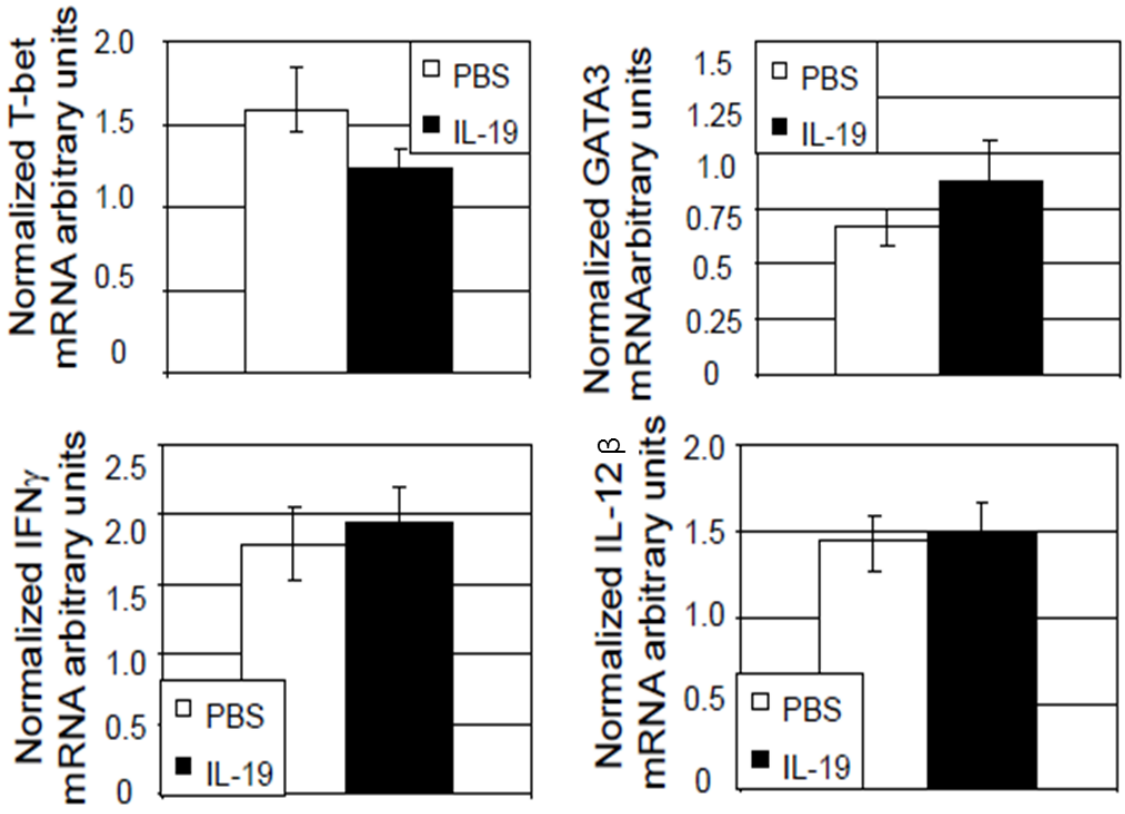

Figure 23. IL-19 Does Not Polarize the Adaptive Immune Response Toward a T_h2 Phenotype in Ligated Mice. Quantitative RT-PCR on RNA extracted from spleen from ligated mice receiving either PBS or 10ng/g/day IL-19 for 28 days. Spleens were removed at the time of euthanasia, RNA extracted, reverse transcribed, and amplified using the primer pairs shown. No significant difference was noted in mRNA abundance for any of the genes shown, n=8 spleen each group.

3.12. *IL-19$^{-/-}$ VSMC Proliferate More Rapidly Than Wild Type VSMC.*

Proliferating VSMC constitute a major cellular event in development of intimal hyperplasia. We hypothesized that the increase in neointimal hyperplasia observed in IL-19$^{-/-}$ mice would be due to increased VSMC proliferation. To test this hypothesis, two different, but complementary experiments were performed. First, to determine if IL-19 expression played a role in VSMC proliferation *in vivo*, IL-19$^{-/-}$ and C57Bl/6 mice were injected daily with 25mg/kg of the nucleotide analogue BrdU. Fourteen days after ligation injury, carotid arteries were recovered, serial sections immunostained, and positive cells quantitated. Figure 24 shows that IL-19$^{-/-}$ mice had significantly increased number of proliferating cells in the neointima compared with wild-type mice (20.55+/-12% versus 55.78+/-4.2%, for wild-type and IL-19$^{-/-}$, respectively; $p<0.01$).

In a second experiment, VSMC cultures were established from explants of aorta from age-matched wild-type or IL-19$^{-/-}$ mice. VSMCs were seeded into 24 well plates and grown in DMEM media. Some were treated with IL-19. At four and seven days, cells were recovered and counted. Figure 24 shows that VSMCs isolated from IL-19 knockout mice proliferate more rapidly than do wild-type VSMCs at both four and seven days post-seeding (24.2±3.1 vs 34.2±1.7 x10^4 cells/ml for wild-type and IL-19$^{-/-}$, $p<0.01$, for seven days). Importantly, the addition of recombinant IL-19 to cultures could significantly reverse this (34.2+/-1.7 vs 25.07+/-2.0 x10^4 cells/ml for IL-19$^{-/-}$ and IL-19$^{-/-}$ VSMCs plus 100ng/ml rIL-19, $p<0.05$). Addition of recombinant IL-19 could also reduce proliferation of wild-type VSMCs (24.2+/-3.1 vs 19.07+/-1.6 x10^4 cells/ml, but not significantly ($p=0.20$). When taken together, these experiments point to direct anti-proliferative effects of IL-19 on proliferation both *in vitro* and *in vivo*.

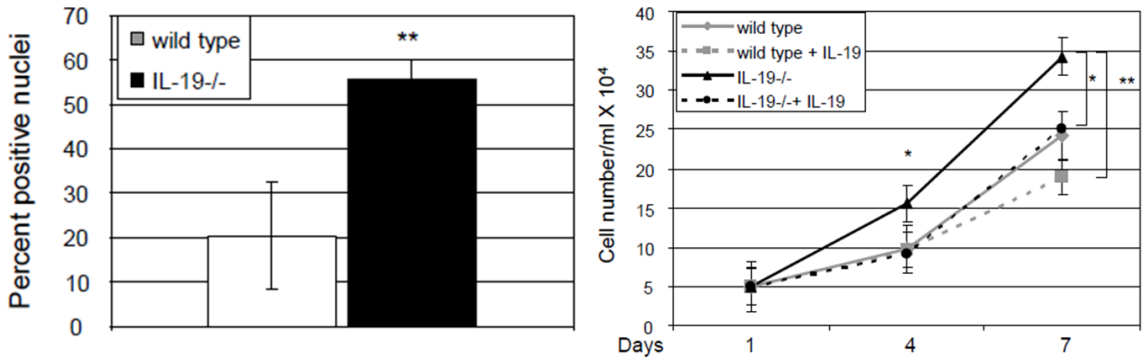

Figure 24. IL-19 Regulates VSMC Proliferation. Left carotid artery ligated IL-19$^{-/-}$ and C57Bl/6 mice received 14 consecutive days of BrdU. At 14 days, serial sections of injured carotids were immunostained and positive cells quantitated by morphological analysis (n=4 for C57Bl/6, n=5 for IL-19$^{-/-}$, p=0.02). Equal numbers of age-matched wild-type or IL-19$^{-/-}$ VSMCs were seeded into 24 well trays, and counted at the indicated days post-seeding. For some samples, rIL-19 was added. Addition of rIL-19 significantly reduced proliferation of IL-19$^{-/-}$ VSMC, but not of wild-type VSMC. p<0.05 or 0.01, as shown.

3.13. Lack of IL-19 Increases Inflammatory Gene Expression in VSMC.

Expression of inflammatory genes by activated VSMC contribute to intimal hyperplasia in multiple ways. We tested if VSMCs from IL-19 knockout mice responded to inflammatory stimuli more robustly than wild-type VSMCs. Aortic VSMCs were isolated from age and sex-matched littermates, cultured, then challenged with the pro-

inflammatory cytokine TNFα. Inflammatory cytokine mRNA expression was determined by quantitative RT-PCR. Figure 25 shows that expression of TNFα, IL-1β, and MCP-1, all potent pro-inflammatory cytokines, were significantly increased in VSMC explanted from IL-19$^{-/-}$ mice compared with VSMCs from wild-type mice, suggesting an enhanced response of IL-19$^{-/-}$ VSMCs to inflammatory stimuli.

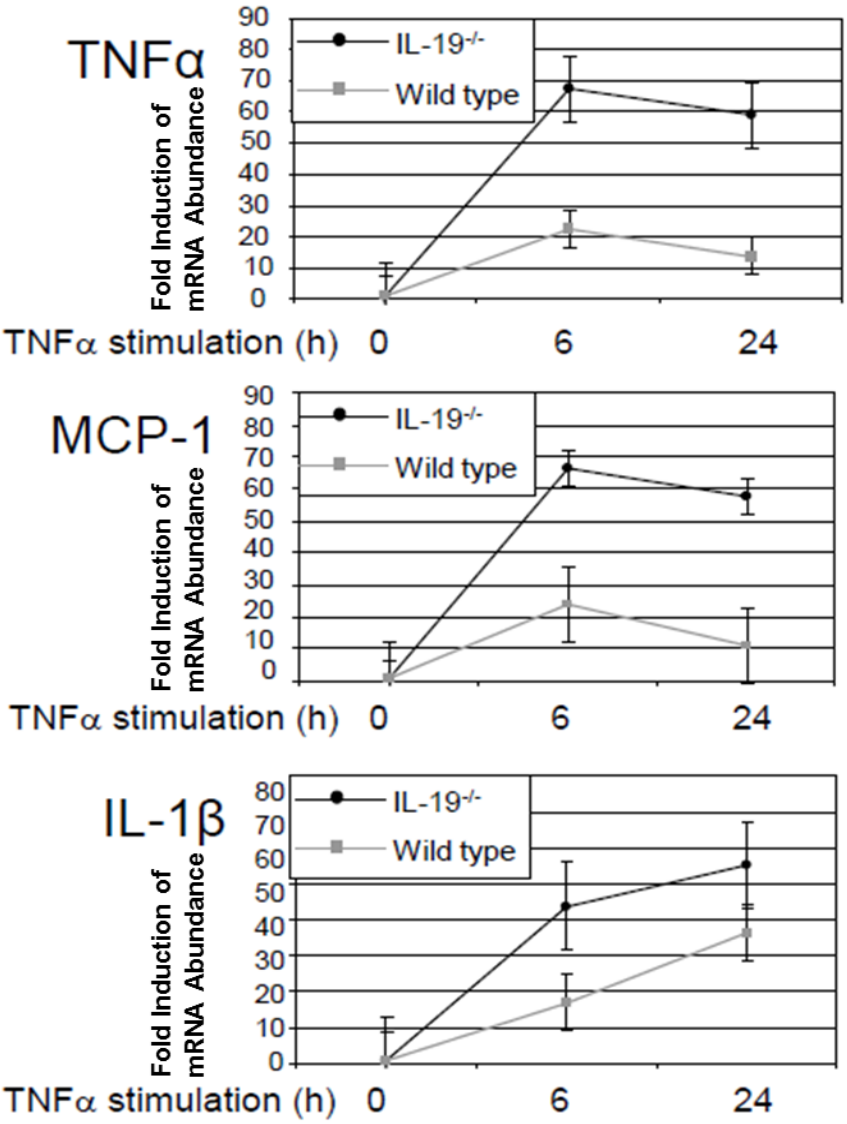

Figure 25. Increased Inflammatory Gene Expression in IL-19$^{-/-}$ VSMCs. VSMCs from wild-type and IL-19$^{-/-}$ mice were serum starved in 0.5% FBS for 48 hours, then stimulated with 10ng/ml TNFα for the times indicated. Total RNA was reverse transcribed and target mRNA quantitated by qRT-PCR. mRNA was normalized to GAPDH. Differences in wild-type vs. IL-19$^{-/-}$ VSMC are significant where indicated (p<0.05 or 0.01, n=3 experiments), asterisk are for all targets at that time, unless otherwise noted.

CHAPTER 4

DISCUSSION

Vascular proliferative diseases continue to account for 50% of all mortality in the United States, despite aggressive dietary modification, lipid lowering medications, and other medical therapy. It is a significant systemic problem contributing to mortality of multiple diseases including myocardial infarction, stroke, renal failure, and peripheral vascular disease, and will worsen with an increasing growing number of patients with co-morbidity such as obesity, metabolic syndrome, and Type 2 diabetes mellitus; conditions linked with atherosclerotic vascular disease. Even though intra-coronary stents are more effective than PTCA alone in decreasing restenosis, in up to 35% of cases, in-stent restenosis occurs between 6 and 9 months[5,38,39]. Further, the incidence of restenosis in selected patient populations, such as diabetics and those with complex lesions can exceed 50%, significantly limiting the success of this modality. In Aim 1 of this dissertation, we show that IL-19 has unprecedented anti-atherogenic effects through direct effects on both adaptive immunity and on resident vascular cells. In Aim 2 of this dissertation, we show that IL-19 has potent inhibitory effects on clinically relevant vascular restenosis that often follows invasive surgical procedures geared at subduing progression of atherosclerosis, including intra-coronary stent placement and PTCA.

In Aim 1 of this dissertation, the major finding is that systemic administration of IL-19 attenuates atherosclerosis in $LDLR^{-/-}$ mice. T_h2 adaptive immune polarization, reduction in macrophage infiltration, and a decrease in macrophage, EC, and VSMC inflammatory cytokine gene expression are likely mechanisms. This is the first description of IL-19 inhibition of atherosclerosis, and is conceptually novel in that IL-19 can have direct

atheroprotective effects on non-immune cells. Our current understanding indicates that T_h1 cytokine expression dominates in atherosclerotic plaque, in contrast to T_h2 cytokines which are far less prevalent in human atherosclerotic lesions[45,47,49]. Thus, it was somewhat surprising that we were able to detect IL-19 protein in EC, VSMC, and leukocyte infiltrate in atherosclerotic plaque from human patients. It was particularly interesting that IL-19 immunoreactivity localized primarily to the plaque, and little to none was detected in medial VSMC. Expression of IL-19 in atherosclerotic plaque suggested a role for this cytokine in plaque pathology, and its expression in resident vascular cells implied a potential novel function for IL-19 in modulating the immune response independent of lymphocyte T_h2 polarization.

Recently, serum IL-19 levels in humans have been reported[65]. In patients selected for CABG, IL-19 levels were 34.4±17.6 ng/ml prior to surgery. IL-19 rose to 541.3±110.4 ng/ml 24 hours post-surgery, and declined to 77.2±24.9 ng/ml 96 hours after surgery. In our study, systemic administration of 10ng/g/day IL-19 almost completely inhibited plaque formation in the aortic arch, and as little as 1ng/g/day IL-19 decreased plaque area by 70%, suggesting potent anti-atherosclerotic effects of IL-19. Importantly, IL-19 can halt expansion of existing plaque even when mice continue to be fed an atherogenic diet, which has important clinical implications. This is a straight-forward study design that does not rely on any genetic modification of IL-19, and suggests utility for IL-19 as a therapeutic. IL-19 reduction of plaque size is likely not due to reduction in lipid as there is no significant difference in total serum cholesterol, triglycerides, HDL or LDL in IL-19 treated mice compared with controls, nor is there any difference in weight gain between these two populations. In contrast to IL-19, IL-10 does appear to have lipid-lowering

effects, as serum cholesterol is significantly reduced in mice receiving IL-10, which also may have contributed to decreased plaque in that study[102].

Other T_h2 interleukins have been shown to decrease atherosclerosis in mice; albeit with less potency than IL-19. Systemic IL-10 gene expression mediated by adenovirus injection reduced atherosclerosis by immune cell modulation and reduction in inflammatory cell infiltrate in plaque[102]. Transfer of bone marrow from IL-$10^{-/-}$ mice into $LDLR^{-/-}$ mice resulted in increased atherosclerotic plaque formation compared with controls, which was attributed to a decrease in macrophage foam cell apoptosis and monocyte activation[103]. Similarly, $LDLR^{-/-}$ mice receiving bone marrow from IL-10 transgenic mice showed a significant decrease in plaque area[54]. The synthesis of these studies point to IL-10 as a potent immune modulator, and the authors conclude that IL-10 anti-atherogenic effects are mediated by modulation of the adaptive immune response. Intimal area of cross sections of atherosclerotic plaque was reduced in $ApoE^{-/-}$ mice injected with 1µg of IL-33 bi-weekly; however, lesion area was not measured in this study[104,105]. Studies with IL-4 are more ambiguous. IL-$4^{-/-}$ mice do not have increased atherosclerosis, and subcutaneous injection of 1.1/ng/day recombinant IL-4 into $ApoE^{-/-}$ mice does not reduce development of atherosclerotic lesions[56,106]. Further, lesions were actually reduced in area in IL-$4^{-/-}$/$ApoE^{-/-}$ double knockout mice, and reconstitution of $LDLR^{-/-}$ mice with IL-$4^{-/-}$ bone marrow also reduces lesion formation.

A tip in the T_h1/T_h2 balance of these seemingly opposing forces toward the T_h2 expression profile has been proposed to have anti-atherosclerotic effects[46–48]. Gene expression in splenocytes from IL-19 injected mice indicated that IL-19 appeared to induce T_h2 polarization, as T_h1 markers T-bet and IFNγ, as well as the pro-inflammatory cytokines IL-1β and IL-12p40 were decreased, and T_h2 markers such as GATA3 were

increased in IL-19-injected mice. An increase in IL-19 mRNA in IL-19-treated mice suggests a positive feedback mechanism. A significantly greater percentage of infiltrating T cells in IL-19 treated mice were GATA3 positive as well. This shift in immune system polarization may provide at least one mechanism for IL-19's anti-atherosclerotic effects. This is consistent with reports in which IL-10 expression was driven by the IL-2 promoter and restricted to T cells[54]. In that study atherosclerotic plaque was reduced in $LDLR^{-/-}$ mice, suggesting that inhibition was mediated by immune system T_h2 polarization.

Monocyte adhesion constitutes a key cellular event in initiation of atherosclerosis[34]. Cellular characterization of plaque determined significantly less macrophage infiltrate in IL-19 injected mice compared with PBS controls. To extend these data, we used quantitative intravital microscopy to show that leukocyte adhesion is decreased in wild-type mice fed atherogenic diet, confirming that IL-19 can reduce leukocyte/EC interactions *in vivo*. We did not observe any difference in macrophage phenotypic modulation in aortic root in IL-19 treated compared with control mice. Together, these approaches represent a second mechanism whereby IL-19 may attenuate plaque formation.

Chemokine expression by vascular cells participates in leukocyte recruitment to the atherosclerotic lesion. IL-19 expression in EC, VSMC, and macrophage in atherosclerotic plaque led us to hypothesize that IL-19 would have anti-inflammatory effects on these cells. Pretreatment of each of these cells with IL-19 prior to TNFα stimulation lead to a significant decrease in mRNA for MCP-1, IL-8, IL-1β, all potent chemoattractants. This may account for the observed decreased macrophage accumulation in plaque, and also decreased adhesion assayed by intravital microscopy.

Similarly, while EC and VSMC respond to IL-10 through receptor-ligand binding, these cells do not express IL-10, precluding bi-directional paracrine signaling between activated resident vascular and inflammatory cells. In cultured EC, IL-10 has no attenuating effect on expression of inflammatory cytokines. IL-10 is acknowledged to reduce NF-κB activity, but did not reduce TNFα or IL-1β-induced expression inflammatory genes[107]. In the present study, IL-19 inhibition of mRNA varied for different cell types. Cell specific sensitivity to IL-19 suggest complex regulatory mechanisms for IL-19 inhibitory effects. Taken in total, our present data are particularly intriguing in their demonstration that a T^h2 interleukin can have direct anti-inflammatory effects on cells outside of the T cell lineage, particularly EC and VSMC.

A plausible mechanism for IL-19 induced decrease in these transcripts is its effect on HuR, an inflammation-specific mRNA stability factor[108]. We reported that in cultured, primary human VSMC, IL-19 inhibitory effects are NF-κB-independent, and use of transcription inhibitors demonstrated that IL-19 decreased the mRNA stability of inflammatory transcripts[68]. IL-19 reduced the nuclear to cytoplasmic translocation of HuR, which is significant because HuR translocation from a predominately nuclear location to the cytoplasm is required for its mRNA stabilizing effects[108]. The present study extends that report, showing that a single addition of IL-19 can rapidly and transiently decrease mRNA abundance of chemokine transcripts in multiple cell types. We then attempted to mirror the *in vivo* scenario in which IL-19 is injected into mice on a daily basis by multiple additions of IL-19 to these cells and demonstrated HuR protein abundance was reduced as well. Thus, both reduction in HuR cytoplasmic translocation as well as protein abundance is induced by IL-19. IL-19 regulation of HuR is of note because the modulation of mRNA stability is recently gaining attention as a potential

novel therapeutic modality[70]. Furthermore, a recent report suggests that HuR mediated post transcriptional control plays an important role in T cell development and T^h2 polarization[109]. Decreased HuR protein abundance, with subsequent reduction in inflammatory gene mRNA provides a third mechanism for IL-19 attenuation of atherosclerosis.

In summary, IL-19 is expressed in multiple cell types in human atherosclerotic plaque, and we used a relatively straight-forward study design to demonstrate the efficacy of IL-19 to reduce atherosclerotic plaque. The probable mechanisms are a polarization of adaptive immunity to the T_h2 phenotype, a decrease in macrophage infiltrate into the plaque, and a decrease in inflammatory gene expression in EC, VSMC, and macrophage. What is particularly novel about this study is the ability of EC and VSMC to express and respond to IL-19. In addition to being a modulator of adaptive immunity, a second unique property of this interleukin is its direct anti-inflammatory effects on resident vascular cells. This is potentially paradigm altering as it suggests that resident vascular cells can respond to IL-19 and assume a T_h2-like, anti-inflammatory phenotype. Endogenous expression of IL-19 by inflamed EC and VSMC may represent a protective autocrine or paracrine mechanism to promote resolution of the local vascular response to injury. A limitation of the present study is that it cannot identify if the protection imparted by IL-19 is mediated primarily by adaptive immune system polarization, a decrease in macrophage infiltrate into the lesion, or by direct anti-inflammatory effects on resident vascular cells. Future studies will elucidate the cellular mediator of IL-19 effects. This will identify IL-19, or IL-19 pathway components as novel therapeutics, or targets of intervention to limit vascular inflammation.

Intimal hyperplasia subsequent to mechanical and immunological insult remain clinically significant obstacles limiting the success of vascular interventions and solid organ transplantation[38,39]. In Aim 2 of this dissertation, we demonstrate a role for IL-19 in regulation of the VSMC response to ligation injury. We hypothesized that IL-19 would decrease intimal hyperplasia subsequent to carotid artery ligation. Addition of 10ng/g/day recombinant IL-19 for 28 days to high-responder FVB wild-type mice significantly reduced intimal hyperplasia as compared to PBS treated controls. Quantitative morphological analysis determined a significant decrease in both I/M ratio and percent stenosis. To further define a role for IL-19 in neointimal hyperplasia, we utilized C57Bl/6 wild type and IL-19$^{-/-}$ mice for an identical 28 day carotid artery ligation study[89]. As expected, quantitative morphological analysis showed that I/M ratio and percent stenosis were significantly less in wild-type mice compared with IL-19$^{-/-}$ mice. In this case, the robust response to ligation injury in the IL-19$^{-/-}$ mice is noteworthy considering the C57Bl/6 strain is particularly resistant to this type of injury[95,96]. Lastly, to confirm the role of IL-19 in regulation of neointimal hyperplasia, IL-19$^{-/-}$ mice were subject to carotid artery ligation, some of which were rescued with 10ng/g/day rIL-19 5 days per week, others with equivalent volume of PBS as controls. At the conclusion of the study, IL-19$^{-/-}$ mice injected with IL-19 had a significantly lower I/M ratio and percent stenosis than IL-19$^{-/-}$ mice injected with PBS. In summary, addition of IL-19 reduces, while absence of IL-19 increases intimal hyperplasia, and addition of IL-19 to IL-19$^{-/-}$ mice rescues intimal hyperplasia development. Moreover, no statistical differences were noted in the overall diameter or medial area of these arteries in any study. When taken together, these data strongly suggest that IL-19 regulates carotid artery ligation-induced restenosis.

The ligation-injury model is primarily proliferative, rather than inflammatory in character, and VSMC are the major effector cell involved in intimal hyperplasia. Perhaps not surprisingly then, little has been reported on effects of T_h2 interleukins in this injury model. IL-19 is considered a T_h2 interleukin, and we hypothesized that polarization of adaptive immunity to the T_h2 phenotype might account for decreased intimal hyperplasia observed in IL-19-treated mice[60,61,101]. Two experiments were performed to determine IL-19 effects on immune cells. First, the immunological status of these mice was determined by quantitation of T_h1 and T_h2 marker expression in splenocytes immediately removed from mice at the termination of the study. Quantitative RT-PCR demonstrates that splenocytes from IL-19 injected wild type FVB mice trended lower, but did not have significantly different mRNA levels of the T_h1 marker T-bet for PBS and IL-19 treated animals. Concordantly, IL-19 injected mice trended, but did not have significantly higher mRNA levels of the T_h2 marker GATA3. Similarly, transcripts in wild type C57Bl/6 and IL-19$^{-/-}$ mice were not statistically different. Second, serum cytokine levels for IFNγ, IL-1β, TNFα, and IL-10 were also not significantly different in IL-19-injected mice compared with controls, or in wild-type C57Bl/6 compared with IL-19$^{-/-}$ mice. Taken together, these data indicate that injection of IL-19 over a four week period does not polarize the immune system to a T_h2 anti-inflammatory profile, suggesting that immune cell polarization is not a major mechanism for reduced intimal hyperplasia in IL-19 treated mice.

Proliferating VSMC constitute a major cellular event in development of intimal hyperplasia. We have previously shown that IL-19 is anti-proliferative for human VSMC[64]. We hypothesized that the increase in neointimal hyperplasia observed in IL-19$^{-/-}$ mice would be due to increased VSMC proliferation. To test this hypothesis, two

different, but complementary experiments were performed. First, to determine if IL-19 expression played a role in VSMC proliferation *in vivo*, IL-19$^{-/-}$ and C57Bl/6 mice were injected daily with the nucleotide analogue BrdU. Following 14 days of ligation injury, IL-19$^{-/-}$ mice had significantly increased number of proliferating cells in the neointima compared with wild-type mice. In a second experiment, VSMC cultures were established from wild-type or IL-19$^{-/-}$ mice, seeded into 24 well plates, and some treated with IL-19. At four and seven days post seeding, VSMCs isolated from IL-19$^{-/-}$ mice proliferate more rapidly than do wild-type VSMCs. Importantly, the addition of recombinant IL-19 to cultures significantly reversed this outcome. Addition of recombinant IL-19 could also reduce proliferation of wild-type VSMCs. When taken together, these experiments point to direct autocrine anti-proliferative effects of IL-19 on proliferation of VSMCs both *in vitro* and *in vivo*.

Ligation of the murine carotid artery, like its clinical counterpart in humans, elicits expression of growth factors and inflammatory cytokines from resident vascular cells as well as infiltrating inflammatory cells. This response to injury initiates and maintains VSMC proliferation and migration, which is at the center of the pathogenesis of intimal hyperplasia. It was important to determine if lack of IL-19 resulted in an enhanced response of VSMC to inflammatory stimuli. Similar to proliferation assays, IL-19$^{-/-}$ VSMC expressed greater amounts of IL-1β, TNFα, and MCP-1, all potent chemoattractants, suggesting an enhanced response of IL-19$^{-/-}$ VSMC to inflammatory stimuli.

Our overall hypothesis that the anti-inflammatory properties of interleukin-19 can attenuate the vascular response to injury in various animal models of vascular proliferative disease is confirmed by our data. With respect to Aim 1 of this dissertation, our hypothesis that IL-19 will mitigate experimental atherosclerosis through effects on

resident cells at the site of vascular injury is strongly supported by our data, which indicates IL-19 does so via diverse mechanisms including immune cell polarization, decrease in macrophage adhesion, and decrease in gene expression. The hypothesis of Aim 2 of this dissertation, that IL-19 will attenuate vascular restenosis and modulate the VSMC response to injury in mice having undergone carotid artery ligation, has likewise been supported by our data.

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