INDUCTION OF INTERLEUKIN-37 EXPRESSION IN MACROPHAGES OF HYPERLIPIDEMIC MICE TO ATTENUATE ATHEROSCLEROSIS

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ABSTRACT

Cardiovascular diseases (CVD) are the main cause of death worldwide, leading to massive medical and economic burdens on society. Atherosclerosis, the primary cause of CVD, is the progressive buildup of plaque within arterial blood vessels that can lead to fatal downstream events such as heart attack or stroke. A key event contributing to the pathogenesis of atherosclerosis is the infiltration of monocytes and the formation of lipid-laden macrophage foam cells within the vessel wall. This leads to inflammation and mass deposition of lipids within the artery wall, driving the disease progression. It is therefore extremely important to explore therapeutic options aimed at slowing the disease progression by preventing macrophage inflammatory signaling or controlling macrophage cholesterol homeostasis.

I have investigated the role of macrophage-expressed Interleukin-37 (IL-37) in reducing the production and effects of pro-inflammatory cytokines and preventing foam cell formation. A retroviral vector with a region of the CD68 promoter upstream of my gene of interest was utilized to obtain robust, macrophage-specific expression of either IL-37 or EGFP. Experiments exploring the effect of IL-37 expression on macrophage inflammatory response, transmigration, and cholesterol homeostasis were performed in vitro. Macrophage IL-37 expression in vitro resulted in decreased mRNA and secreted protein production of key inflammatory cytokines, inhibited macrophage transmigration, as well as reduced lipid uptake compared to controls. The stable integration of the IL-37 or EGFP genes was necessary to execute a long-term atherosclerosis mouse study in vivo. The in vivo effects of macrophage-expressed IL-37 were investigated through bone marrow transplantation of transduced hematopoietic stem cells (HSC)
into irradiated, atherosclerosis-prone \textit{Ldlr}^{-/-} mice. After 10 weeks on a high fat diet, I found that mice with IL-37-expressing macrophages showed reduced disease pathogenesis, which was demonstrated by significantly less arterial plaque development compared to control mice. The athero-protective effect of macrophage-expressed IL-37 has implications for development of future therapies to treat atherosclerosis as well as other chronic inflammatory diseases.
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LIST OF ABREVIATIONS

EGFP: Enhanced green fluorescent protein

HA-tag: human influenza hemagglutinin sequence

Tg: Transgenic

PH-E: Phoenix cells - Ecotropic

MMLV: Murine molony leukemia virus

CMV: Cytomegalovirus

°C: degree Celsius

PBS: phosphate buffered saline

HRP: Horse radish peroxidase

EDTA: ethylenediaminetetraacetate

FBS: fetal bovine serum

HEPES: 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid

DMSO: dimethyl sulfoxide

PVDF: polyvinyl difluoride

AcLDL: Acetylated low density lipoprotein

OxLDL: Oxidized low density lipoprotein

RCT: Reverse cholesterol transport

SMA: Smooth muscle actin

PE: phycoerythrin
CHAPTER 1: INTRODUCTION

1.1 Cardiovascular Disease Burden

Cardiovascular diseases (CVD), a group of diseases affecting the heart and circulatory system, are the main cause of human mortality worldwide. CVD accounted for 17.5 million deaths, or 35% of total deaths in 2012 [1], and is a significant burden to the global economy with an estimated $863 billion spent annually to treat the disease [2]. Atherosclerosis is the underlying cause of most of CVD which can become fatal when large, vulnerable plaques within medium and large arteries rupture and lead to impeded blood flow to important organs such as the heart or brain [3]. In the United States (US), it is estimated that a heart attack occurs every 43 seconds, and someone dies from a heart disease related event every minute [4]. CVD accounts for 17% of US annual national healthcare costs [5], with coronary heart disease (CHD) alone costing $108.9 billion in healthcare, medication and lost productivity [6]. Overall, CVD mortality has declined dramatically in the last 4 decades, however this decrease is most evident for the older population (<65 years of age), mainly due to the decline in cigarette smoking, use of pharmacologic treatment of high blood pressure and cholesterol levels, as well as general increased awareness about causes and treatments of complications from the disease [7]. The trend for younger adults has not been as optimistic with only about 1% annual decrease over the last 2 decades in mortality for men and women <55 years of age [8].

While advances in pharmacology and medical technology have helped to reduce the overall mortality of CVD, this plateau in the movement to reduce mortality highlights the relationship between environmental risk factors and disease incidence in young people. Understanding the disease process of atherosclerosis and discovering innovative therapies to
treat and reduce morbidity and mortality, both within the United States and throughout the world, are crucial to improving population health and reducing the enormous medical and economic burdens of CVD.

1.2 Atherosclerosis - Causes and Risk Factors

Atherosclerosis was once thought to be a passive disease of excessive lipid accumulation, however, research over the last several decades has revealed that inflammation and the immune response play a prominent role in its pathogenesis. Key environmental risk factors known to contribute to the development of atherosclerosis include hypertension, hyperlipidemia (specifically high LDL cholesterol), and smoking, with roughly half of the US population having at least one of these three factors [9]. Other prominent risk factors include obesity, diabetes, tobacco use, excessive alcohol consumption, and a sedentary lifestyle, leading to dyslipidemia and inflammation in people at high risk for developing atherosclerosis and its complications [10].

Atherosclerosis is caused by the accumulation of lipids and immune cells within the vessel wall, which thickens and hardens to form atherosclerotic plaques. A build-up of these plaques can make the vessel wall narrow and less flexible, leading to ischemia from impeded blood flow. As plaques advance, certain areas, especially the shoulder regions, can become weakened and rupture, leading to thrombus formation. When the thrombus blocks blood flow through critical arteries such as those of the brain or the heart, it can lead to devastating downstream events such as stroke or myocardial infarction, respectively [3]. Drugs are used during myocardial infarction or stroke to dissolve the thrombus and restore blood flow, however,
time is of essence in these acute cases and treating the symptoms at this point is often too late to avoid tissue damage or even death due to deprivation of oxygen and other key nutrients [11, 12].

Although most risk factors are environmental, there are inherited or acquired genetic mutations that predispose those affected to atherosclerosis. The closest links between genetic mutations and disease acceleration involve genes that control circulating cholesterol levels. About 1 in 1,000,000 people inherit homozygous familial hypercholesterolemia (FH), which is caused by a mutation in both copies of the low density lipoprotein receptor (Ldlr) gene that prevents all LDLR protein expression in the liver [13]. This prevents uptake of LDL by the liver and causes circulating total cholesterol levels to be >800mg/dL, over 4 times the healthy limit recommended by the American Heart Association (AHA) and Centers for Disease Control and Prevention (CDC) [14, 15]. Individuals heterozygous for the mutation, about 1 in 500, have one mutated and one functional copy of the gene, producing 50% of the normal amount of LDLR. However, at around 300mg/dL, the circulating total cholesterol level of those affected with heterozygous familial hypercholesterolemia is still twice as high as the recommended limit and treatment with lipid-lowering drugs such as statins are necessary to prevent premature death caused by atherosclerosis. The incidence of FH in the world population warrants treatment and diagnosis of the disease by the World Health Organization [16]. Mutations in other important genes, such as those involved in forming HDL [17] or those that regulate blood pressure [18] can also predispose individuals to atherosclerosis.
1.3 Molecular Mechanisms of Atherosclerosis

In humans, the initiation of atherosclerosis occurs when circulating LDL particles become trapped within the arterial wall and become modified, eliciting an inflammatory response. Signaling molecules produced by cells of the vessel wall, such as endothelial and smooth muscle cells, lead to activation of the innate immune response (Figure 1B). There is now mounting evidence in support of a dysfunctional endothelial lining associated with factors such as hypertension [19], dyslipidemia [20] and smoking [21], all increasing atherosclerosis disease risk. Inflamed endothelial cells lining the vessel wall express adhesion molecules such as vascular cell adhesion molecule (VCAM) and intracellular adhesion molecule (ICAM) on their luminal surface [22, 23]. These adhesion molecules along with chemokines orchestrate the chemotaxis of circulating monocytes, allowing them to exit circulation at the site of inflammation and transmigrate into the vessel wall [24, 25].
Figure 1: Stages of atherosclerosis development.

(A) The normal anatomy of an artery consists of an inner lining of endothelial cells, separating the lumen from the rest of the vessel, followed by a medial layer of smooth muscle cells and matrix proteins such as collagen and elastin, which is covered by an adventitial layer containing fibroblasts, nerve endings and microvessels. (B) The first stage of atherosclerosis begins with the attraction of immune cells, specifically monocytes, to an area of inflamed endothelial lining. The monocytes transmigrate through the endothelium to the intima, where they encounter and uptake modified lipids, leading to foam cell formation. This stage is termed the “fatty streak”. (C) Intermediate lesions are characterized by the formation of a fibrous cap, which consists of migrated smooth muscle cells (SMC) and deposition of matrix proteins to protect and stabilize the plaque. SMC and macrophages become apoptotic or necrotic, releasing their stored lipid, which contributes to the formation of a lipid and necrotic core of dead cells and debris. (D) An advanced plaque is prone to rupture if the fibrous cap is degraded, which leads to thrombus formation and potentially lethal blockage of blood flow.
Plaque progression is exacerbated primarily by monocytes, which differentiate into macrophages and take up modified and aggregated lipoproteins beneath the endothelium, leading to the formation of macrophage foam cells, named due to the “foamy” appearance of lipid droplets within the cell [26, 27]. The early stage of atherosclerosis development is termed “fatty streak” due to the prominent presence of lipid-laden foam cells within the vessel wall and the relatively thin and simple composition of the plaque (Figure 1B). Continuous recruitment and activation of immune cells ultimately causes a state of chronic inflammation and leads to significant plaque growth over time [28]. Monocytes and macrophages can account for over 40% of the mass of advanced atherosclerotic plaques [29], although dendritic cells, T cells, and neutrophils are also attracted to lesion areas via specific chemoattractant molecules, and can also be found within developed plaques [30].

In advanced plaques, a necrotic core region forms, composed primarily of apoptotic foam cells, extracellular lipid droplets as well as cholesterol crystals [31]. This necrotic core is surrounded by a fibrous cap following the proliferation and migration of smooth muscle cells (SMCs) from the media and subsequent deposition of a collagen-rich matrix that stabilizes the plaque [32]. The fibrous cap plays a crucial role in preventing contact between the circulating blood and the contents of the lesion, however if the cap is weakened, plaque rupture and thrombus formation can occur [30, 33]. Apoptosis of SMC in atherosclerosis can lead to plaque vulnerability via thinning of the fibrous cap and subsequent plaque destabilization [34] (Figure 1C). Recent studies have shown that expression of matrix metalloproteinases (MMPs) can lead to fibrous cap thinning via degradation of the matrix proteins that give the cap its structure [35-37].
Specific MMP family members are detrimental while others appear to be beneficial for preventing plaque rupture [38]. Expression of MMP inhibitor proteins, tissue inhibitor of metalloproteinases (TIMPs) [39-41], leads to reduced atherosclerosis development, indicating that when left unchecked, MMPs are generally harmful in the context of atherosclerosis. Destruction of collagen fibrils by proteinases are associated with areas rich in macrophage foam-cells within the plaque, especially at vulnerable “shoulder regions” [3]. Macrophages have been identified as a key source of destructive MMPs [42, 43], as well as other inflammatory mediators, leading to progressive weakening of the plaque and subsequent rupture (Figure 1D). Due to their prominent role in the disease process, macrophages have been a topic of intense study in the context of atherosclerosis, not only to better understand the mechanisms of the disease process, but also as a potential therapeutic target to treat or prevent the disease.

1.4 Macrophages in Atherosclerosis

The role of macrophages in atherosclerosis initiation, progression, and clinical manifestation is complicated. As phagocytic antigen presenting cells (APC), macrophages play a key role in the innate immune system and are critical for the first line of defense against pathogens, orchestration of tissue repair, and clearance of apoptotic cells and debris to prevent necrosis and excessive inflammation [44]. Under typical conditions of inflammation, the pathogenic trigger of the immune response would be combatted by the immune system and eventually cleared from the body, followed by a resolution phase in which anti-inflammatory signaling molecules and growth factors are produced, allowing tissue repair and remodeling to occur [45]. During atherogenesis, the chronic inflammatory microenvironment of the developing
plaque, along with elevated circulating LDL cholesterol levels, leads to an influx of immune cells, perpetuating the cycle of inflammation and plaque growth.

1.4.1 Inflammatory Potential of Macrophages

A specific inflammatory subset of circulating monocytes characterized by high expression of the surface marker Ly-6C is targeted to the site of vessel inflammation [46]. The non-classical, resident Ly-6C\textsuperscript{lo}, and the classical, inflammatory Ly-6C\textsuperscript{hi} monocytes are now accepted to be two independent subsets that are each predisposed to express a different variety of chemokine receptors and signaling molecules. Inflammatory Ly-6C\textsuperscript{hi} monocytes increase in number during hyperlipidemia and are the main monocyte subset that infiltrate into the growing atherosclerotic plaque [46]. Drawn in by chemoattractant molecules such as CCL2, CXCL1, and CCL5 [47], the monocytes transmigrate into the intima of the of the vessel well beneath the endothelial monolayer where they differentiate into macrophages.

Depending on the particular microenvironment, macrophages can be polarized into a variety of subsets (Figure 2). The phenotypes were originally designated as either M1 or M2 to describe macrophage activation states ranging from pro-inflammatory to anti-inflammatory, respectively. This nomenclature stems from the helper T cell Th1/Th2 paradigm, in which the cells are primed for responses to various infectious attacks to the body depending on the particular stimuli encountered [48]. M1, or classically activated macrophages (CAM), are polarized to an inflammatory state after exposure to TLR or IFN signals, as well as various other inflammatory mediators, typically in response to bacterial or viral infection. M2, or alternatively activated macrophages (AAM), are polarized in response to cytokines such as IL-13, IL-10 and
IL-4, as well as growth factors, which are mainly produced by Th2 cells to fight off extracellular pathogens [49]. The specific subclasses of M2b and M2c are termed “regulatory macrophages”, and are induced in response to various stimuli including IL-10, immune complexes, glucocorticoid hormones, and the uptake of apoptotic cells [50]. M2d macrophages are considered pro-angiogenic and pro-tumoral [51]. In general, regulatory macrophages are vital for inducing the reparative resolution phase following an inflammatory response. Additional macrophage polarization subtypes that have been described in the context of atherosclerosis include Mhem and M(Hb), which are activated in response to hemoglobin encountered after intraplaque hemorrhage [52]. These subtypes are considered athero-protective because of their reduced ROS production as well as increased cholesterol efflux [51]. Opposing this subtype is the pro-inflammatory phenotype termed “Mox”, which is activated in response to oxidized lipids, and is generally considered pro-atherogenic [53].

The various macrophage polarization states are important in the context of atherosclerosis, each present within the plaque environment, although M1 and Mox inflammatory macrophages have the most dominant presence during disease pathogenesis (Figure 2). Controlling macrophage-induced inflammation is a key therapeutic goal in the treatment and prevention of a wide variety of human diseases, especially atherosclerosis [54], but also including asthma [55], rheumatoid arthritis [56], cancer [57], and obesity-induced insulin resistance [58]. Diseases that are caused by excessive inflammation such as human immunodeficiency virus (HIV), systemic lupus erythematosus (SLE), and rheumatoid arthritis (RA), lead to a 2-3 fold higher risk of developing atherosclerosis, independent of high
cholesterol levels, indicating an essential role for inflammation in atherogenesis [59]. This is supported by the finding that mice that are severely hypercholesterolemic, but deficient in macrophages are resistant to atherosclerosis development [60].

Figure 2: Activation and polarization of macrophages in atherosclerosis.
Monocytes are drawn to the site of plaque development where they differentiate into macrophages and can be polarized to various phenotypes. M1, M2, Mhem, M(Hb), Mox, and M4 macrophage subtypes are shown with their potential effects within a plaque environment. M1 and Mox macrophages dominate during inflammation and are pro-atherogenic, while M2 and Mhem macrophages suppress inflammation and prevent foam cell formation, making them atheroprotective.
1.4.2 Cholesterol Homeostasis in Macrophages

Dysregulation of cholesterol metabolism by macrophages within the plaque promotes atherogenesis. In hyperlipidemic conditions, the supply of modified lipoproteins is overly abundant while macrophage uptake of lipoproteins within the intima is essentially uninhibited due to the lack of negative feedback, quickly leading to macrophage foam cell formation. Macrophages encounter and take up modified lipoproteins via scavenger receptors [61], or through receptor-independent phagocytosis or macropinocytosis [62]. Uptake of modified lipoproteins and cholesterol crystals within the plaque leads to activation of pattern recognition receptors (PRR) and upregulation of Toll like receptor (TLR) expression. This promotes the production of reactive oxygen species and other inflammatory immune mediators [63, 64], which leads to impaired cholesterol efflux [65], compounding the negative effects of foam cell formation (Figure 3).
Intracellular accumulation of modified LDL in macrophages can activate inflammasome formation, leading to secretion of inflammatory mediators such as IL-1β. Macrophages that encounter modified LDL also elicit an inflammatory response when TLRs on the cell surface are activated, leading to downstream inflammatory gene transcription and the release of cytokines, proteases, and other inflammatory mediators that drive plaque progression.

Studies aimed at manipulating macrophage cholesterol homeostasis have shown that increasing cholesterol efflux, or preventing cholesterol uptake in macrophages can attenuate atherosclerosis development [66]. Cholesterol efflux to ApoA1 or nascent HDL via the efflux proteins ATP-binding cassette transporter A1 (ABCA1) and ABCG1, respectively, is an
important mechanism for clearing cholesterol from the periphery, formation of HDL, and preventing foam cell formation, all of which reduce atherosclerosis development [67]. High HDL levels are known to be protective against atherosclerosis in humans [68], and treatment with exogenous HDL has been shown to reduce atherosclerosis in animal models due to increased cholesterol efflux and decreased foam cell formation [69]. In addition, HDL has been shown to be anti-inflammatory [70], increasing its value as an athero-protective molecule. Defects in either ABCA1 or ABCG1 lead to massive foam cell accumulation and increased atherosclerosis in mice [71, 72]. Genetic mutations in the human ABCA1 gene cause the autosomal dominant Tangier disease, characterized by low HDL levels, excessive deposition of cholesterol esters in non-adipose tissue throughout the body, and a significant increased risk of atherosclerosis [73]. On the other hand, enhancing ABCA1 leads to an increase in circulating HDL levels and attenuates atherosclerosis development [74].

Additionally, the intracellular breakdown of lipoproteins by lipoprotein lipase and cholesteryl ester hydrolases leads to cholesterol-induced cytotoxicity [31] and endoplasmic reticulum (ER) stress in foam cells, which in turn initiate the unfolded protein response (UPR), Caspase-3 activation, ultimately resulting in cell death by apoptosis [75, 76]. Clearance of apoptotic cells, which is termed efferocytosis, is an important protective function of macrophages, especially in the context of atherosclerosis. This clearance, observed mainly in early plaques, triggers the release of anti-inflammatory molecules such as IL-10 and growth factors, which helps prevent plaque development [77]. Impaired efferocytosis in more advanced plaques leads to necrotic cell death and development of the acellular necrotic core [78]. This
vicious cycle of inflammation, uncontrolled cholesterol uptake, apoptosis, and promotion of inflammation due to necrosis is the principal sequence of plaque growth over time.

1.4.3 Potential Therapies to Mitigate Atherogenesis

The main therapeutic options targeting macrophages in atherosclerosis involve reduction of inflammation and monocyte migration to the plaque, inhibition of cholesterol uptake, and activation of cholesterol efflux.

Inhibiting inflammation has been a central tenet in the pursuit of innovative therapies to treat atherosclerosis. The top biomarkers for predicting cardiovascular disease are markers of inflammation, including IL-6, soluble ICAM-1 and C-reactive protein (CRP) [79], emphasizing the intimate relationship between inflammation and atherosclerosis. One method of reducing inflammation in autoimmune disorders, TNFα blockade, has also proven to be beneficial in reducing the risk of atherosclerosis [80]. TNFα antagonists given to RA patients reduce inflammation by preventing the production of inflammatory cytokines such as Interleukin (IL)-1β, IL-6, monocyte chemoattractant protein (MCP)-1 and various MMPs [81], significantly reducing the incidence of deaths from plaque rupture and myocardial infarction or stroke [82]. Other treatment options currently undergoing clinical trials to reduce cardiovascular inflammation include the drug methotrexate, which targets the IL-6 pathway [83], canakinumab, a monoclonal antibody inhibitor of IL-1β [84], and various leukotriene inhibitors, which limit oxidative activity of leukocytes within the plaque [85].

By far the most commonly utilized class of drugs against CVD is 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, also known as statins. They are very
effective at lower circulating cholesterol levels by preventing cholesterol production by the liver [86], acting to reduce the main risk factor of atherosclerosis. As a secondary effect statins have been shown to be effective in tapering inflammation. Clinical trials have shown a direct link between statins and reduction of inflammatory markers, especially CRP [87]. In fact, even in the absence of hyperlipidemia, patients on the drug Rosuvastatin showed decreases in CRP levels and reduced incidence of major adverse cardiovascular events [88]. However, statins have been shown to cause some side effects such as increased risk of diabetes, cataracts, as well as muscular side effects, although they have been very successful in the primary and secondary prevention of atherosclerosis and remain the main drug of choice by clinicians [86].

In addition to lowering circulating cholesterol, increasing cholesterol efflux or preventing cholesterol uptake by macrophages is an effective method for preventing foam cell formation. Activation of the TGFβ pathway has been shown to prevent macrophage foam cell formation by downregulating scavenger receptors and reducing cholesterol uptake [89]. Peroxisome proliferator-activated receptors (PPARs) are involved in regulation of lipid metabolism. PPAR-α and PPAR-γ agonists have been shown to be important in reverse cholesterol transport through upregulation of the cholesterol transporter, ABCA1 [90]. In mice, ABCA1, ABCG1, and SR-BI play major roles in macrophage cholesterol efflux, while in humans, ABCG1 is less important [91]. However, treatments that elevate expression and efflux capacity of these proteins would have great therapeutic potential for preventing atherosclerosis.

Overall, therapies to treat or prevent atherosclerosis have focused on reducing inflammation, preventing LDL cholesterol uptake, and increasing cholesterol efflux to HDL. In
general, pharmaceutical approach is via systemic treatment, and targeting the atherosclerotic plaque itself has been a challenge. Future therapies that directly target the mechanisms of macrophage inflammation and cholesterol homeostasis may prove to be beneficial with fewer side effects affecting other organs and tissues than pharmaceutical options. Since macrophages are central to disease pathogenesis in various ways from early to late stages of atherosclerosis, they could potentially provide a vehicle for delivery of anti-inflammatory mediators to inhibit plaque development.

1.5 Interleukin-37

Interleukin-37 (IL-37) was identified in 2000 by several independent groups using computational screening of human cDNA and genomic databases. Sequence homology to other IL-1 family members led to the designation of the various newly discovered isoforms with names such as IL-1 family member 7 (IL-1F7), and IL-1 homolog 4 (IL-1H4). Human IL-37, for which there is no mouse homologue as yet, has five known splice variants (a-e) [92] (Figure 4), which are expressed in a variety of tissues throughout the body including lymph nodes, thymus, skin, bone marrow, lung, heart, brain and testis [93, 94]. IL-37b is the best-characterized IL-37 isoform, and has the longest sequence consisting of 218 amino acids. This splice variant is found predominantly in cells of the immune system, including human peripheral blood mononuclear cells (PBMC), natural killer cells (NK), and stimulated B cells [94]. The IL-37 protein contains the 12 \( \beta \)-strands that are typical of IL-1 family ligands, which are essential for the \( \beta \)-trefoil secondary structure that makes it biologically functional [95].
**Figure 4: Human Interleukin-37 splice variants.**

The human IL-37 gene is depicted with exon and intron lengths noted for each segment. The 5 isoforms and their spliced mRNA structure are shown below the full gene. Exon 1 contains a caspase-1 cleavage site and exons 4-6 compose the 12 β-strands that compose the typical β-trefoil structure of the IL-1 family.

1.5.1 Role in immune response

The IL-1 family of cytokines and receptors are central in modulating innate immune responses [96]. The cytoplasmic domain of the 10 IL-1 family type I receptors are highly homologous to another large family of receptors, Toll-like receptors (TLRs), which also play a
significant role in activating the innate immune system to defend against invading pathogens. Unlike the TLR family, however, which acts only to activate the inflammatory response, the IL-1 family contains receptors that inhibit inflammatory activation as well. The anti-inflammatory IL-1 family receptors and cytokines control against excessive inflammation and are essential for prevention of autoimmune and allergic responses. IL-1 family receptors that inhibit inflammation include decoy receptors that bind the intended inflammatory ligands, but fail to transmit a pro-inflammatory signal, such as IL-1RII and TIR8/SIGIRR [97]. Of the 11 ligands of the IL-1 family, there are four that are anti-inflammatory, including IL-1 receptor antagonist (IL-1Ra), IL-36Ra, IL-38 and IL-37. The precursor IL-37b isoform is expressed at low levels under steady state conditions due to an RNA-instability element in exon 5 of the transcript [98]. Transcripts are stabilized and expression of IL-37b protein is upregulated under inflammatory conditions induced by LPS and other TLR ligands [99].

1.5.2 Extracellular Mechanism of IL-37 Function

Investigation into the function of IL-37 has revealed a dual role in modulating the immune response, with both extracellular and intracellular functions. The study of the extracellular function of IL-37 gained momentum after it was discovered that IL-37b binds the IL-18Rα chain and also the IL-18 binding protein (IL-18bp). IL-37 is structurally similar to IL-18 and fits within the binding site of IL-18Rα to competitively inhibit binding of IL-18 and subsequent recruitment of the co-receptor, IL-18Rβ [100]. It is not surprising that IL-37 also shares significant homology with another anti-inflammatory member, IL-1 receptor antagonist (IL-1Ra), which competitively inhibits IL-1β from binding the IL1R [101]. A similar
antagonistic role for extracellular IL-37 was investigated and confirmed by multiple findings. Although no murine homolog for IL-37 has been identified, the human protein is functional in the mouse and studies have been carried out using an IL-37b-expressing transgenic mouse model (IL-37Tg) [99]. Treating IL-37Tg mice with neutralizing antibodies against IL-37 resulted in reversal of its anti-inflammatory effect on levels of pro-inflammatory cytokines in the serum after stimulation with LPS [102]. Another study using neutralizing antibodies against IL-37 in human PBMCs has confirmed this result, supporting a clear role for IL-37 in quelling inflammation though an extracellular mechanism [103].

Recently it has been discovered that a complex consisting of IL-37, IL-18Rα, and the decoy receptor SIGIRR, is necessary for IL-37 to inhibit inflammatory signaling [104]. Complexing of the three proteins was visualized by bioluminescence resonance energy transfer (BRET) in fresh human PBMCs. The disruption of the complex formation by inhibiting either IL-18Rα or SIGIRR in THP-1 macrophages or PBMCs resulted in significant reversal of the anti-inflammatory effects of IL-37 in vitro and in vivo [104]. Nold-Petry et al. compared total phosphorylated proteins in LPS-stimulated macrophages and dendritic cells (DC) isolated from wild-type, IL-37Tg, or IL37Tg/SIGIRR-deficient mice. They demonstrated that effective IL-37 signaling through the IL-18Rα and SIGIRR receptor complex leads to reduced phosphorylation of TAK1, Fyn, and the NF-κB pathways mediators IkBε, p65 and p105. Additionally, extracellular IL-37 signaling was shown to increase phosphorylation of anti-inflammatory mediators Stat3, Mer, PTEN and p62 [104]. This set of changes in phosphorylation of key signaling proteins acts in suppressing the inflammatory response. Interestingly, Nold-Pety et al.
also observed anti-inflammatory effects of IL-37 that were independent of extracellular signaling through SIGIRR, including reduced phosphorylation of various MAP kinases, the focal adhesion kinase FADK, and the NF-κB kinase IKKβ. This supports the hypothesis that IL-37 plays a dual role in suppressing inflammation through both extracellular as well as intracellular mechanisms.

1.5.3 Intracellular Mechanism of IL-37 Function

It has been shown that IL-37, similar to other IL-1 family cytokines such as IL-1β, IL-18, and IL-33, is processed by caspase-1 within the cell to produce the mature IL-37 cytokine after the N-terminal pro-domain is cleaved. Mature IL-37 can then be secreted to act extracellularly, or like IL-1α and IL-33, can translocate to the nucleus and act as a transcription factor [102, 105, 106]. Secretion of both the full length and mature IL-37 occurs in stimulated macrophages, although only mature IL-37 secretion requires ATP [102]. Cleavage by Caspase-1 is not necessary for IL-37 to function as an extracellular cytokine since both pro-IL-37 and mature IL-37 bind the IL-18Rα chain, although the mature form was shown by immunoprecipitation to bind with stronger affinity than the uncleaved form [105]. To function within the nucleus however, Caspase-1-mediated cleavage is required. Mutation of the caspase-1 cleavage site in the first exon of IL-37b, or use of a pharmacologic caspase-1 inhibitor, leads to inhibition of nuclear translocation, [102, 106]. In addition, blocking nuclear translocation of IL-37b resulted in reversal of its suppressive effects on inflammatory gene and protein expression [102, 106], confirming the importance of pro-domain cleavage for proper function of IL-37 as a transcription factor.
Further investigation into the intracellular role of IL-37 was inspired by the identification of IL-37 through a proteomics screen as a potential binding partner of the transcription factor, Smad3 [107]. Nold et al. confirmed this link through co-localization and immunoprecipitation experiments using an epithelial cell line, demonstrating that binding between IL-37 and Smad3 occurs in vitro after IL-1β stimulation [99]. In the same study, IL-37 was shown to act through a SMAD3-dependent mechanism in both human THP-1, and mouse RAW264.7 macrophages in vitro. Inhibition of SMAD3 expression by siRNA, or its activity by the SMAD3 inhibitor SIS3, led to reversal of the anti-inflammatory effects of intracellularly-expressed IL-37b [99]. The contribution of Smad3 to IL-37 function in vivo was evaluated using the IL-37b transgenic mice created by the authors for the same publication [99]. Smad3 knockdown by siRNA treatment in the lungs of IL-37Tg mice challenged with LPS led to increased inflammatory gene and protein expression compared to IL-37Tg treated with scrambled control siRNA [99], supporting an essential role of Smad3 in the anti-inflammatory mechanism of IL-37. This landmark study by Nold et al. in 2010 paved the way for many more studies to follow investigating the broad, anti-inflammatory effects of IL-37 in various disease settings.

The discovery that IL-37 is dependent on the transcription factor Smad3 and the finding of robust IL-37 expression induced by TGFβ provides evidence for a potential role of IL-37 in regulating macrophage cholesterol homeostasis. Smad3 is a classical mediator of the TGFβ signaling pathway, which plays a critical role in the resolution of the inflammatory response [108, 109]. Smad3 has also been shown to inhibit MCP-1 expression in macrophages by antagonizing AP-1/DNA binding in macrophages [110]. Furthermore, TGFβ-Smad3 signaling
axis has been shown to participate in reducing macrophage foam cell formation [111, 112]. Taken together, these results provide strong support for a potential role of IL-37 in regulating macrophage inflammation as well as lipid homeostasis, both key drivers of atherosclerosis.

1.5.4 Therapeutic potential of IL-37 in inflammatory diseases

Since the discovery that IL-37b expression has broad protective effects against inflammation, many inflammation-related diseases and conditions have been evaluated in vivo using the IL-37Tg mice described above. IL-37Tg mice have been shown to be protected from endotoxic shock, displaying reduced levels of hypothermia, metabolic acidosis and liver damage compared to littermate controls [99]. Clinical disease scores of intestinal colitis were 50% lower in IL-37Tg mice compared to wild-type control mice [113]. Interestingly, the reduction in colon damage was also seen after bone marrow transplantation from IL-37Tg to wild-type mice, indicating the protective effects of IL-37 were due to leukocyte-specific suppression of inflammation [113]. Ballak et al. show that IL-37Tg mice are protected from obesity-induced inflammation and insulin resistance, indicating a potential protective role of IL-37 in preventing type 2 diabetes [114]. A role for IL-37 in suppressing adaptive immunity is highlighted in a study focusing on dendritic cell response in IL-37Tg mice after induction of skin contact hypersensitivity (CHS). Dendritic cells (DC) isolated from CHS-challenged IL-37Tg mice showed impaired antigen presentation, reduced production of inflammatory cytokines and activation of T-cells, and increased induction of regulatory T-cells compared to wild-type control DC [115]. This is the first study to provide evidence for the importance of IL-37 in regulating
adaptive immunity, in addition to its well-established role in suppressing the innate immune response.

The recognition of IL-37 as a potential therapeutic cytokine has led to its investigation in human populations, and its expression has been strongly associated with many human diseases, including psoriasis [116], rheumatoid arthritis [117], systemic lupus erythematosis [118], inflammatory bowel disease [119, 120] and HIV infection [121]. Elevated plasma IL-37 has also been found in patients with acute coronary syndrome [122]. Reperfusion with recombinant IL-37 protein after ischemia has been shown to reduce damage and improve heart function in mice after ischemia/reperfusion injury [123]. Although the anti-inflammatory benefits of IL-37 have been widely accepted, its potential as a therapeutic measure for reducing the chronic inflammation that drives the pathogenesis of atherosclerosis has been suggested [124], yet never been explored.

1.6 Retroviral Gene Transfer

The stable transfer of genes to specific cells via retroviral delivery has therapeutic value in treating human diseases, especially monogenic disorders such as primary immunodeficiency syndromes [125, 126]. The use of a retrovirus over other viral gene delivery methods carries the advantage of stable gene expression and high infection rates, although the target cells need to be proliferating for adequate infection [127]. The advent of retroviral packaging cells, which separates the viral packing genes gag and pol from env, and also from the viral construct containing the gene of interest, has allowed the production of high titers of virus that are able to infect the target cells, but unable to replicate [128, 129]. Drawbacks to retroviral gene therapy
that have prevented its widespread use involve the risk of mutation associated with random transgene integration into the host genome [130]. However, current research is aimed at resolving this issue by guiding the gene integration to specific benign sites within the genome [131]. Retroviral gene transfer has also become a powerful tool in research and has been used to achieve stable gene expression in target cells by integration of specific promoters before the gene of interest. This method is also suitable for expressing genes that are not endogenously found in the target cells or organism, such as expression of IL-37 within the mouse.

Retroviral transduction of hematopoietic stem cells with a macrophage-specific transgene has already been successfully carried out to study the effects of transgene expression on atherosclerosis development in mice [132, 133]. Gough and Raines have created and characterized two different self-inactivating (SIN) retroviral vectors for use in HSC transduction, with the long terminal repeats (LTR) and packaging signal (Ψ) sequences derived from the Moloney murine leukemia virus (MMLV) [132]. The promoter and enhancer elements for driving expression of the virus typically reside in the 5’ LTR, while the genes used for replication of the virus can be found in the 3’ LTR. However, SIN retroviral vectors contain one or more deletions within the LTRs that prevent the virus from replicating after infecting a target cell.
Figure 5: Retroviral vector designs for macrophage-specific gene expression.

(A) The retroviral constructs tested by Raines and Gough for macrophage-specific expression of target genes are depicted in schematic diagrams. $\Psi^+$ represents the packaging signal, and the CD68 promoter sequence with the cDNA of interest is inserted in the opposite direction (black arrow). LTRs are shown flanking the entire sequence packaged into the virus, with a deletion in the 3’LTR making the retrovirus produced self-inactivating. (B) The control RV (PBM-I-EGFP) and the two version of the CD68 promoter, short (S) and long (L), were tested by transducing BMDM. % EGFP-positive cells are shown, with the short version of the promoter more effective at driving EGFP expression.
The retroviral constructs created by Raines and Gough contain a deletion in the 3’ LTR that is transferred to the 5’ LTR upon conversion of the RNA to cDNA within the target cell, preventing viral replication and leading to gene expression solely from the internal promoter. One of the two SIN RV vectors created by Raines and Gough contains a 2.9-kilobase (kb) long fragment of the human CD68 promoter region, while the other contains a 342-base pair (bp) short fragment, both including the first intron (IVS-1) as well as additional sequence located 5’ of the ATG start codon [132]. The internal promoter region and target gene were intentionally designed in the 3’ to 5’ orientation to prevent unwanted splicing of the IVS-1 sequence during initial synthesis of the virus, as shown by the left-facing arrow in the schematic diagrams of the vectors tested by Raines and Gough (Figure 5A). The retroviral vector with the short promoter (CD68S) proved more efficient at driving HA-EGFP expression in macrophages differentiated from the transduced HSC (Figure 5B). The CD68S construct was therefore chosen for the authors’ subsequent atherosclerosis study in which HSC transduced with the apolipoprotein E (ApoE) gene were transplanted into lethally irradiated, atherosclerosis-prone ApoE-deficient mice, which resulted in successful prevention of atherogenesis [132].

The CD68S-HA-EGFP construct as well as a detailed protocol for its use were generously donated to our laboratory by E. Raines, and has since been modified with the cloning of our own various genes of interest in the place of HA-EGFP. Han *et al.* used the CD68S retroviral construct to investigate the effects of macrophage-expressed IL-10 in the context of atherosclerosis via bone marrow transplantation of CD68S-IL-10-transduced HSC into irradiated *Ldlr*−/− mice, which resulted in decreased inflammation and reduced atherosclerosis development.
Most recently, the CD68S construct was used to obtain stable, macrophage-specific expression of IL-37b for both the in vitro and in vivo research described in this dissertation.

1.7 Hypothesis and Aims

Given the importance of inflammation and cholesterol regulation in the pathogenesis of atherosclerosis, the study of IL-37 in the context of atherosclerosis has great potential for the discovery of novel therapeutic options. The human IL-37 has been shown to have potent anti-inflammatory effects when expressed within either human or mouse macrophages, reducing the production of inflammatory cytokines and other immune mediators. In addition, IL-37 may potentially play a role in macrophage cholesterol homeostasis through the TGFβ/Smad3 axis. Its protective effects and dual mechanisms of action in preventing inflammation have been demonstrated in vivo using mice transgenic for IL-37, although a macrophage-specific expression system has not yet been tested. The hypothesis that macrophage IL-37 expression will be protective against atherosclerosis will be tested with the following aims:

Aim 1: Express IL-37b specifically in macrophages using retroviral transduction of HSC.

Aim 2: Determine the effects of macrophage-expressed IL-37b on inflammatory gene and protein expression, cholesterol uptake and efflux, and macrophage transmigration.

Aim 3: Investigate the role of macrophage-expressed IL-37b in a mouse model of atherosclerosis using bone marrow transplantation of CD68S-IL-37b transduced HSC.
Chapter 2: MATERIALS AND METHODS

2.1 Mouse strains and cell lines

Wild-type C57BL/6 mice, as well as Ldlr<sup>−/−</sup> mice on the C57BL/6 background, were originally purchased from Jackson Laboratories (Bar Harbor, Maine) and colonies were maintained at the John A. Burns School of Medicine animal facility. Male 8-10 week old wild-type mice were used for isolating bone marrow for both in vitro and in vivo experiments. 6-8 week old male Ldlr<sup>−/−</sup> were used as bone marrow transplantation recipient mice for the in vivo atherosclerosis study. All animal protocols were approved by the University of Hawaii Institutional Animal Care and Use Committee.

Phoenix-ECO (ATCC® CRL-3214™) retroviral packing cells were purchased from ATCC (Manassas, Virginia) and cultured according to the company’s recommendations in a humidified incubator with 5% CO<sub>2</sub> at 37°C. The recommended basic growth media, composed of DMEM (Gibco, Invitrogen) supplemented with 10% FBS (HyClone) and 1% penicillin/streptomycin (10,000 U/mL) antibiotic mix (Gibco) was used for all experiments. Cells were split no more than 1:5 when they reached approximately 70-80% confluence. To passage cells, they were first washed with PBS, and then detached with 0.25% (w/v) Trypsin- 0.53 mM EDTA, which was then quenched with complete growth medium to inhibit the Trypsin before splitting the cells into new flasks. Early passages (1-5) were cryopreserved for future use in freezing medium (90% FBS / 10% DMSO) at 1x10<sup>6</sup> cells per milliliter and stored at -150°C or in a liquid nitrogen dewar in the vapor phase.
2.2 Bone marrow derived macrophage cell isolation and in vitro culture

Male wild-type mice, 8-10 weeks old, were sacrificed by CO\textsubscript{2} asphyxiation and sprayed with 70% EtOH to disinfect the skin. The femur and tibia bones were isolated under a biosafety cabinet to prevent contamination and were flushed with ice cold PBS using a 30-guage needle into a 50-mL Falcon tube kept on ice. The isolated bone marrow was broken up by pipetting with a 10-mL pipette, passed through a 40-µm cell strainer into a new 50-mL tube, and pelleted by centrifugation at 1300 rpm (~350 x g) for 5 minutes. The PBS was aspirated and the pellet was resuspended in BMDM differentiation medium (DMEM/F12 (Gibco) supplemented with 10% FBS 20% L929 conditioned medium (described below), and 1% pen/strep (Gibco)). A small, 10-µL aliquot of cells were stained with bromophenol blue to exclude dead cells, and were counted using a hemacytometer. The cells were cultured at a density of 1x10\textsuperscript{6} cells per mL and plated in 15-cm tissue culture treated plates (Corning) in 25 mL medium each. 5-10 mL of medium was added to each plate every other day for a total of 7 days. Adherent differentiated macrophages were detached using the non-enzymatic solution, Cell Stripper (Gibco), counted, and replated into the appropriate wells for each in vitro experiment.

L929-conditioned medium was produced by culturing 4.7x10\textsuperscript{5} L929 cells (ATTC) per T-75 flask in 50 mL medium (DMEM/F12, 10% FBS, 1% HEPES (Gibco), and 1% pen/strep) for 1 week before collecting, sterile filtering, and storing the supernatant at -80°C (long-term) or -20°C (short term) in 50-mL aliquots.
2.3 Molecular cloning

2.3.1 Amplification of IL-37 cDNA from the pCMV6-entry vector

An IL-37b human cDNA (reference sequence: NM_014439) open reading frame clone with a C-terminal Myc-DDK tag was purchased from OriGene. A plasmid map for pCMV6-IL-37b is shown in Figure 6A. Primers were designed flanking the IL-37b cDNA and Myc-DDK tag, with HindIII restriction enzyme sequences added to the 5’ end of the forward primer and 3’ end of the reverse primer.Polymerase chain reaction (PCR) was performed on the linearized pCMV6-IL-37b-Myc-DDK plasmid using TaKaRa LA Taq polymerase (Clonetech). The amplified product was then subjected to DNA gel electrophoresis through a 1% agarose gel using the non-toxic, SYBR Safe DNA stain (Thermo). The single 800-bp band of interest visualized with a UV box was excised using a clean razor blade, and the gel fragment was subjected to DNA purification using the Wizard® SV Gel and PCR Clean-Up System (Promega). The insert was subsequently used for subcloning into the CD68S retroviral vector (Figure 6C).
Figure 6: Vector maps of pCMV6-entry vector and CD68S-EGFP-HA retroviral vector

(A) The pCMV6-Entry vector used for amplification of the IL-37b mRNA, including the adjacent myc and DDK tags. (B) The CD68S-EGFP-HA retroviral vector donated by E. Raines with intact NotI and HindIII sites. (C) The CD68S-IL-37-Myc-DDK retroviral vector created by subcloning the IL-37b cDNA with tags into the CD68S vector.
2.3.2 Bacterial transformation and DNA purification.

The CD68-EGFP-HA retroviral vector donated by E. Raines (Figure 6B) was reconstituted from filter paper with nuclease-free water and analyzed for DNA concentration and purity using a NanoDrop (Thermo Scientific). The vector DNA was then transformed into One Shot competent E. coli (Invitrogen). 2 ng of vector DNA was added to the frozen competent bacteria, which were thawed on ice for 30 minutes, heat-shocked in a 42°C water bath for 30 seconds, and then put back on ice for 2 minutes. 500 µl of nutrient-rich SOC medium were then added to each vial of bacteria, which was incubated for 1 hour in a 37°C shaker. The vials were then centrifuged at 350 x g for 5 minutes and 400 µl of broth was removed. The bacteria were then resuspended in the remaining broth and spread onto LB-agar plates containing 1mg/mL ampicillin (100 mg/mL stock, Sigma) overnight at 37°C for the growth of colonies. Colonies were selected and grown in 250 mL of LB growth medium + ampicillin overnight, followed by purification of the vector DNA with the PureYield™ Plasmid Maxiprep System (Promega) using vacuum filtration according to the company’s protocol. Purified plasmid DNA was stored at -20°C until further use.

2.3.3 Cloning of IL-37b cDNA into the CD68-EGFP-HA vector

Sticky-end cloning was used to insert the IL-37b-Myc-DDK cDNA into the CD68 retroviral plasmid. First, the CD68-EGFP-HA vector was digested with the restriction enzyme NotI (New England Biolabs) to remove the EGFP-HA insert. The digested DNA was separated on a 1% agarose gel and the linearized vector backbone was isolated and purified before being re-ligated with T4 DNA ligase (NEB). The product was then digested with the restriction
enzyme HindIII to create the sticky ends necessary for subcloning of the IL-37 insert into the CD68S vector, and then treated with Antarctic Phosphatase (NEB) to prevent re-circularization of the vector. The IL-37b PCR product amplified from the pCMV6 plasmid was also digested with the restriction enzyme HindIII (NEB) according to the company’s protocol for 1 hour at 37°C. Next, the vector and insert were combined in a 1:1 ratio (100ng each) and ligated with T4 DNA ligase. 2µl of the reaction mixture was transformed into competent bacteria and colonies were grown on LB-agar plates containing ampicillin. Colonies were isolated and grown overnight in 2mL LB growth medium + 2 µl ampicillin per clone for mini-prep purification (Promega). Clones were then screened by HindIII digest and were considered positive for the IL-37b-Myc-DDK insert if an 800-bp band was visible. Positive clones were then further screened by sequencing to ensure correct orientation of the insert within the CD68S vector.

2.4 Retroviral transduction of hematopoietic stem cells

2.4.1 Reagents for CaCl₂/HBS transfection of PH-E cells

1. 2X Hepes-buffered saline (HBS):

   1.5 mM sodium phosphate 106.5 mg Na₂HPO₄
   12 mM dextrose 1.08 g
   50 mM Hepes 6.51 g
   280 mM NaCl 8.18 g
   10 mM KCl 372.8 mg

   Add all ingredients to 450mL sterile H₂O. Adjust pH to exactly 7.05 using hydrochloric acid and fill up to 500 mL total with sterile H₂O. Sterile filter and store in 1.75 mL aliquots at -20°C.
2. **2M CaCl$_2$:** Prepare in sterile water and sterile filter. Store in 200-µl aliquots at -20°C.

3. **25mM Cholroquine:** Prepare in PBS and sterile filter. Store as 15-µl aliquots at -20°C.

4. **Puromycin:** Prepare as 10 mg/mL (5000x) stock in sterile PBS. Store as 120-µl aliquots at -20°C. Add 2 µg/mL of growth medium for selection of PH-E cells.

**2.4.2 Stable transfection of Ecotropic Phoenix cells**

Ecotropic Phoenix (PH-E) cells were grown in vented-cap T-75 flasks (Corning) in the growth media described above. According to the protocol from E. Raines, 6x10$^6$ PH-E cells were plated in a 10-cm tissue culture dish. 24 hours later, the cells were transfected with the CD68S-EGFP-HA retroviral vector. 24 µg of vector DNA was added to a total of 1.314 mL of sterile water and gently mixed by vortexing. 186 µl of 2M CaCl$_2$ was then added to the DNA and mixed again. At this time, 10 µl of 25mM chloroquine (1:1000) was added to each 10-cm dish of PH-E cells and swirled gently to mix. 1.5 mL of 2X HEPES-buffered saline (HBS) was added using a Pasteur pipette and the mixture was bubbled vigorously for 10 seconds. The mix was then added dropwise to the 10-cm dish of PH-E cells, swirling gently to mix. The cells were then placed back in the incubator for 8-9 hours. After incubation, the medium was aspirated and 10 mL of fresh medium was added.

The following day, cells were detached with Trypsin/EDTA and 80% of the cells were replated in a T-75 flask with 2 µg/mL puromycin added to the growth medium to select for transfected cells. Cells were maintained for 2 weeks on puromycin-containing medium for full selection. The selected PH-E cells were then frozen for future use, or grown in a T-150 for production of retrovirus for transduction of mouse HSC. A confluent T-150 was split allocating
1/3 of the total PH-E cells each into 2 10-cm dishes for the first round of transduction and 1/6 each into 2 additional 10-cm dishes for the second round of transduction. 24 hours before each round of transduction the medium on the PH-E cells was replaced with puromycin-free medium.

2.4.3 Detecting retroviral copy number using an SG-PERT assay

A SYBR Green qPCR-based product-enhanced reverse transcriptase assay (SG-PERT) was performed on retroviral supernatants from PH-E cells to determine the concentration of viral particles based on the activity units of the virus’ reverse transcriptase (RT) enzyme. RNA from the bacteriophage MS2 served as a template for the RT enzyme and was purchased from Roche. The 2X lysis buffer used to release the RT from the virus in each sample of supernatant was prepared with 0.25% Triton X-100, 50 mM KCL, 100 mM TrisHCL (pH 7.4) in 40% glycerol. Ribolock RNase inhibitor (ThermoFisher) was added to the lysis buffer at a dilution of 1:50 just prior to use. The primers used to amplify the MS2 cDNA had the forward sequence 5′-TCCTGCTCAACTTCCTGTCGAG-3′, and the reverse sequence 5′-CACAGGTCAAAACCTCCTAGGAATG-3′ (Integrated DNA Technologies). Recombinant HIV Reverse Transcriptase (rHIV-RT) (Worthington Biochemical Corp, 27 U/µl, Specific activity 21,600 U/mg) was used as a standard for RT activity. 10-fold serial dilutions of rHIV-RT from 1 to $10^{10}$ pU in 5 µl were made using DMEM. Retroviral supernatants collected from CD68S-EGFP-HA-transfected PH-E cells were centrifuged at 350 x g for 10 minutes to pellet any contaminating cells or debris. 5 µl of the cell supernatant or rHIV-RT or were added to a 96-well round bottom plate and lysed with 5 µl of 2X lysis buffer for 10 minutes at RT. The samples were then diluted with 90 µl of nuclease-free water and 9.6 µl of the lysate was added to a qPCR
384-well plate along with 10.4 µl of a master mix consisting of 10 µl SYBR Green (Roche), 0.1 µl each of the forward and reverse primers (both at 100 µM) and 0.1 µl MS2 RNA. The samples, including a no template control, were run in duplicate on an Applied Biosystems 7900HT Fast Real-Time PCR System with the following program:

<table>
<thead>
<tr>
<th>Name</th>
<th>Time</th>
<th>Temperature</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>RT Reaction</td>
<td>20 min</td>
<td>42°C</td>
<td></td>
</tr>
<tr>
<td>Polymerase activation</td>
<td>2 min</td>
<td>95°C</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>5 sec</td>
<td>95°C</td>
<td></td>
</tr>
<tr>
<td>Annealing and Acquisition</td>
<td>30 sec</td>
<td>60°C</td>
<td>40 cycles</td>
</tr>
<tr>
<td>Elongation</td>
<td>15 sec</td>
<td>72°C</td>
<td></td>
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</tbody>
</table>

The cycle of quantification (Cq) value indicates the amount of RT enzyme in the sample, which can be used to determine the number of virion particles per sample volume based on the standard curve of recombinant HIV RT activity.

2.4.4 Transient transfection of retroviral packaging cells

Transient transfection of PH-E cells was achieved by transfecting the cells with the retroviral construct as described above, however the cells were plated at 4x10^6 cells per T-75 flask the night before, with 2 flasks per construct prepared. Transfection of the cells with 24 µg of DNA per flask was performed by CaCl₂ precipitation exactly as described in the previous section. However, the day following the transfection, the cells were not split and were not selected for puromycin resistance. The medium was changed once at the end of the 8-9 hour transfection, and again the evening before each transfection. Retroviral-containing supernatant
was collected 48 and 72 hours post-transfection for use in the two rounds of HSC transduction described below.

2.4.5 Hematopoietic stem cell isolation and transduction

Day 1

As described above for isolation of bone marrow derived macrophages, 2 male 8-10 week old C57BL/6 mice per construct were sacrificed and the bone marrow was isolated. After straining through a 40-µm filter, the bone marrow cells were pelleted by centrifugation and then resuspended in 5 mL of red blood cell lysis buffer (eBioscience) for 2-3 minutes. 20 mL of PBS were added to the tube and cells were pelleted and washed with an additional 20 mL of PBS before being resuspended in stem cell complete medium (described below) containing stem cell factor (SCF) as well as IL-6 and IL-3 to induce cycling of stem cells. The cells were plated at a density of 25x10⁶ cells per 10-cm non-tissue culture plastic dish in 10 mL of stem cell complete medium.

Stem cell complete medium:

- DMEM/F12
- 1% pen/strep
- 15% FBS
- 100 ng/ml recombinant mouse SCF
- 20 ng/ml recombinant human IL-6
- 10 ng/ml recombinant mouse IL-3
Day 2

The day following HCS harvest, one 6-well plate per construct was coated with 1-1.25 mL per well of endotoxin-free human fibronectin (Invitrogen) at a concentration of 25 µg / mL in PBS and incubated at 4°C overnight. The old medium was aspirated and new growth medium was added to the flasks containing the transfected PH-E cells. Stably transfected PH-E were washed with PBS before addition of fresh growth medium, which did not contain puromycin.

Day 3

The following morning, the fibronectin-coated plates were brought to room temperature and the HSC were harvested from the 10-cm dishes. First the medium containing the non-adherent cells was collected into an appropriate number of 50 mL polypropylene Falcon tubes. Next, the dishes were washed with 3-4 mL PBS, which was also added to the 50 mL tubes. The cells were then treated with 1 mL of cell stripper per dish at 37°C for 5 minutes. The remaining cells were carefully detached from the dishes with cell lifters (Corning), and were also added to the 50 mL tubes. One final PBS wash was performed and added to the 50-mL tubes. The tubes were then were centrifuged at 350 x g, the supernatant was aspirated, and the cells were resuspended in 5 mL stem cell medium per construct without added cytokines.

The retroviral supernatant from the PH-E cells for round 1 was collected and passed through a 0.45 µm filter which is small enough to avoid contamination with PH-E cells, but large enough not to damage the virus due to sheering. The retroviral supernatant, 19 mL per construct, was then added to the 5 mL of resuspended HSCs. Also added to the mix was 1.25mL HEPES buffer solution (Invitrogen), 12 µl polybrene (8 mg/mL stock, Sigma), 12 µl SCF (0.27 µg/mL),
0.8 µl IL-6 (1 µg/mL), and 1.8 µl IL-3 (0.19 µg/mL) (all from Peprotech). The fibronectin solution was aspirated from the 6-well plates and the infection mix was distributed evenly between the wells, with enough for about 4.2 mL per well. The plates were centrifuged at 2500 rpm in a 37°C pre-warmed centrifuge for 2 hours, with the break set to half-speed to prevent spilling from the plates. Following centrifugation, the cells were returned to the incubator overnight. In the evening, the medium on the PH-E cells was changed for the second transduction the following day.

Day 4

For round 2 of transduction, a new infection mix was created consisting of 5 mL stem cell medium (no cells), 19mL retroviral supernatant, 1.25 mL HEPES, 12 µl polybrene, 12 µl SCF, 0.8 µl IL-6 and 1.8 µl IL-3. Next, the infection mix from the previous day was removed from the 6 wells and collected into 50-mL Falcon tubes. The wells were immediately covered with 2-3 mL of the new infection mix, while the tubes with the old mix were centrifuged at 350 x g for 5 minutes to pellet the stem cells. The supernatant was aspirated and the pelleted cells were resuspended in the remaining new infection mix, which was then distributed evenly among the wells of the appropriate plates. The plates were then centrifuged at 2500 rpm at 37°C for 2 hours as done the previous day for the first round of transduction. Following centrifugation, the plates were again incubated overnight at 37°C.

Day 5

The day following the second round of transduction, the medium and transduced HSCs from the 6-well plates were collected in 50-mL tubes and the PBS used to wash the wells was
also collected in the same tubes. The adherent cells left within the wells were detached using cell stripper, which along with another PBS wash, was added to the collection tubes and centrifuged at 350 x g for 5 minutes to pellet the transduced cells. The cells were washed once with PBS and either differentiated by resuspending and plating the cells in BMDM differentiation medium to use the transduced macrophages for in vitro experiments, or resuspended in PBS for injection into irradiated Ldlr<sup>−/−</sup> recipient mice for the in vivo BMT atherosclerosis study.

![Diagram](image_url)

**Figure 7: Schematic diagram of HSC transduction and BMT into irradiated Ldlr<sup>−/−</sup> mice.**

An overview of the methods for transduction of HSC begins with transfection of the retroviral constructs into viral packaging cells (PH-E). The donor bone marrow is harvested and the HSCs are transduced using the viral supernatant from the PH-E cells. Next the cells are either differentiated for in vitro experiments (not shown), or are transplanted into irradiated recipient mice via tail vein injection.
2.5 Western blot

2.5.1 Preparation of protein lysates from cells and tissues

RIPA buffer (10 mM Tris-Cl (pH 7.6), 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl), supplemented with 1X Halt protease and phosphatase-inhibitor cocktails (Thermo) was used for all lysate preparations. Cells grown in 6-well plates were prepared by first washing the wells with PBS, and then completely aspirating the liquid in each well before adding 100 µl of ice cold lysis buffer supplemented with protease and phosphatase inhibitor cocktails (both diluted 1:100) (Roche). The plates were kept on ice for 15 minutes before the wells were scraped with a cell scraper and the lysates transferred to 1.5 mL Eppendorf tubes on ice. Samples were then sonicated at a power level of 2.5 for 10 pulses using a probe sonicator (ThermoFisher) and centrifuged at 400 x g for 5 minutes at 4°C. The lysates were then transferred to a new tube and the cell debris pellets were discarded. The protein concentrations of the cell lysates were determined with the Pierce BCA protein assay kit (ThermoFisher) using a 1:10 dilution of the samples.

2.5.2 Polyacrylamide gel electrophoresis and Western blot transfer

Precast NuPAGE® Novex® 4-12% Bis-Tris Mini or Midi Protein Gels (Invitrogen), with 12 or 20-wells respectively, were used with a mini or midi gel box for electrophoresis of proteins. An appropriate volume of 4x SDS loading buffer, (0.1 M Tris-HCl pH 6.8, 0.7% SDS, 33% glycerol, 0.01% bromophenol blue, 8% b-mercaptoethanol), was added to 10-20 µg of protein and heated to 95°C for 5 minutes. Samples were added to each well and 4 µl of the Licor one-color protein marker was added as a size reference. Gels were run in 1x NuPAGE® MES
SDS Running Buffer (Invitrogen) at a constant voltage of 150 V until the loading dye reached the bottom of the gel. The gel was then removed and placed on the negative side of a low fluorescence PVDF membrane that was activated with 100% methanol and soaked in 1x Efficient™ Western Transfer Buffer (G-Biosciences® #786-019) with 20% methanol for 5 minutes. The gel and membrane were sandwiched between sheets of filter paper soaked in transfer buffer and packaged tightly within a transfer cassette. The transfer chamber containing an ice pack and stir rod was packed in ice and was placed on a stir plate to ensure that the transfer buffer remained at a cool temperature. The transfer was carried out at a power of 100 volts for 1 hour.

2.5.3 Incubation with antibodies and scanning of membrane

The membranes were then blocked in Licor blocking buffer diluted 1:1 with PBS for 1 hour on an orbital shaker. Primary antibodies were diluted in Licor blocking buffer and incubated at RT for 1 hour or at 4°C overnight. The membranes were then washed 3 times with 0.1% Tween20 in PBS. The secondary antibodies were also diluted in Licor blocking buffer and incubated at RT for 1 hour in the dark. After 3 more 5-minute washes with 0.1% PBST, the membranes were visualized on a Licor Odyssey infrared scanner and the images were analyzed using the Licor Image Studio software (both, Licor Biosciences).
Table 1: List of antibodies used for Western blotting

<table>
<thead>
<tr>
<th>Name</th>
<th>Species</th>
<th>Company</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-37</td>
<td>Goat α Human</td>
<td>R&amp;D Systems</td>
<td>1:1000</td>
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<tr>
<td>EGFP</td>
<td>Rabbit α EGFP</td>
<td>AbCam</td>
<td>1:2000</td>
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<tr>
<td>β-actin</td>
<td>Rabbit α Mouse</td>
<td>Sigma</td>
<td>1:2000</td>
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<td>IRDye 800CW</td>
<td>Donkey α Goat</td>
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<td>Donkey α Rabbit</td>
<td>Licor</td>
<td>1:10,000</td>
</tr>
<tr>
<td>IRDye 680CW</td>
<td>Donkey α Mouse</td>
<td>Licor</td>
<td>1:10,000</td>
</tr>
</tbody>
</table>

2.6 Detecting IL-37 protein by ELISA

A Duoset ELISA kit for IL-37 (R&D) was used to analyze cell culture supernatants or mouse serum samples for IL-37 protein. Clear, flat-bottom 96-well plates (Corning) were coated with the IL-37 capture antibody provided with the kit. After an overnight incubation at RT, the plate was washed 3 times with 200 µl of 0.05% Tween-20 in PBS (PBST). The wells were then blocked with 100 µl of 1% BSA in PBS for 1 hours, followed by 3 washes with PBST. Recombinant IL-37 protein provided in the kit was used to make a 7-point standard with 2-fold dilutions, starting with 2000 pg/mL. 100 µl of IL-37 standard protein and either undiluted cell culture supernatant, or mouse serum samples diluted 1:4 with 1% BSA in PBS, were added to the plate in duplicate, which was then incubated for 2 hours at RT. Following 3 more washes, 100 µl the biotinylated detection antibody provided with the kit was added to each well and incubated for 2 hours at RT. The plate was washed 3 times and then 100 µl of streptavidin-HRP was added to each well for 20 minutes in the dark. After 3 washes, 100 µl of substrate solution was added to each well for 20 minutes in the dark, followed by 50 µl of stop solution. The plate was analyzed
for optical density (OD) with a plate reader set to 450 nm. OD readings at 540 nm were subtracted from each value to account for any changes due to imperfections in the plate.

### 2.7 Reverse transcriptase quantitative PCR (RT-qPCR)

Total RNA was recovered from cells or tissues by the addition of 500 µl - 1 mL TRIzol Reagent (Life Technologies) per sample in a 1.5 mL tube. After vortexing the cells or tissue until dissolved, a volume of chloroform equal to 20% the volume of TRIzol was added and samples were inverted vigorously to mix. The samples were then centrifuged at 12,400 rpm for 15 minutes in a pre-cooled centrifuge set to 4°C. The aqueous phase was then carefully pipetted into a new tube and an equal volume of nuclease-free 70% ethanol was added. Samples were mixed well and run though the RNA purification columns provided with the Qiagen RNeasy RNA extraction kit. An on-column DNA digest was performed for 15 minutes at RT and the appropriate wash steps were taken, followed by complete drying of the column. RNA was eluted from the columns using 30-50 µl nuclease-free water and RNA concentration and quality was measured on a NanoDrop 2000. If not used immediately, the RNA was stored at -80°C.

1 µg of total RNA per sample was transcribed to cDNA using the qScript cDNA synthesis kit (Quanta Biosciences). The recommended protocol was followed using a thermocycler to set the program. Following synthesis, 80 µl of nuclease-free water were added to each sample, making the total volume of synthesized cDNA 100 µl. For RT-qPCR, 4 µl cDNA were added to each well, followed by a master mix consisting of 12.5 µl SYBR Green 2X master mix with ROX (Roche), 0.5 µl each of forward and reverse primers (10 µM), and 7 µl nuclease-
free water. 10 µl of the reaction mix was transferred in triplicate to a 384-well qPCR plate and run on an Applied Biosystems 7900HT Fast Real-Time PCR System.

**Table 2: List of PCR primers**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward sequence (5’ – 3’)</th>
<th>Reverse Sequence (5’ – 3’)</th>
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<tbody>
<tr>
<td>Gapdh</td>
<td>GGCAAATTCAACGGCACAGT</td>
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<td>IL-37</td>
<td>AACCCCATGTGTGTCTTAGAA</td>
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<td>IL-1α</td>
<td>GTTCTGCCATTGACCATCTCTC</td>
<td>CACTGAAACTCAGCCGTCTCTT</td>
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<tr>
<td>IL-1β</td>
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<td>IL-4</td>
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<td>iNOS</td>
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<td>CTGTAAGCCACGTCGAGC</td>
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<td>TIMP1</td>
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<td>MCP-1</td>
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<td>Smad3</td>
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<td>SR-II</td>
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<tr>
<td>CD36</td>
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2.8 Cholesterol homeostasis assays

2.8.1 Cholesterol uptake using Dil-labeled cholesterol

For cholesterol uptake, transduced and differentiated macrophages (empty vector vs. IL-37b) were plated at 2.5x10^5 cells per well of an 8-well chambered glass slide for analysis by microscopy, or 1x10^6 cells per well of a 12-well plate for analysis by flow cytometry. The macrophages were treated with or without LPS to induce IL-37 expression, and were also treated with 50 µg/mL Dil-labeled Ac-LDL or OxLDL for 4 hours. The cells in the 12-well plates were detached with Cell Stripper and fixed for 10 minutes with 4% PFA before being analyzed by flow cytometry for intensity of Dil fluorescence. The cells treated in the glass slides were also fixed for 10 minutes with 4% PFA and then analyzed with an epifluorescence microscope for Dil uptake.

2.8.2 Cholesterol efflux using [3H]-cholesterol

Transduced and differentiated macrophages were seeded in 24-well plates and incubated overnight. The following morning, the cells labeled with loading medium (DMEM, glutamine, penicillin, streptomycin, 0.2% fatty acid-free bovine serum albumin, AcLDL (50 µg/ml), and 1 μCi/μl [3H]cholesterol (NEN Life Science products)) for 36 hours. After washing twice with PBS, the cells were incubated in DMEM/0.2% BSA during an equilibration period of 2 hours. Cells were then washed with PBS and 0.5 ml efflux media, (DMEM/0.2% BSA) with or without 20µg/ml apoA1 (acceptor for ABCA1-mediated efflux) or 50 µg/ml HDL (acceptor for ABCG1-mediated efflux), was be added to each well. Cells were then incubated for 7 hours, and the
supernatant was analyzed for radioactivity to measure macrophages efflux of radiolabeled cholesterol to HDL or ApoA1 and presented as percent HDL- or ApoA1-specific efflux.

2.9 Transmigration of macrophages

Using a 5µm pore filter (Costar), transduced and differentiated BMDM were plated in the upper chamber of the transwell filter (100,000 cells/well of a 24-well filter plate) in DMEM/F12 + 0.2% BSA medium. Cells were given 2 hours to become adherent before the medium in the lower chamber was replaced with new medium containing the chemoattractant MCP-1 (25ng/mL) to stimulate the transmigration of cells through the filter. After overnight incubation in a humidified incubator at 37°C with 5% CO2, the top filter chamber was carefully and completely swiped with a Q-tip to remove non-migrated cells. The filter was fixed with 4% PFA for 10 minutes and stained with the nuclear stain DAPI to visualize and quantify the number of cells that had migrated through the filter. Photographs of the lower side of the filter (3 photos per well) were analyzed with ImageJ. Final counts were compared between control and IL-37-expressing macrophages. All conditions were run in triplicate with N=3 separate experiments.

2.10 Mouse atherosclerosis study

2.10.1 Irradiation of donor mice and bone marrow transplantation

The day before the transduced bone marrow cells were harvested, the recipient Ldlr−/− mice were irradiated with 1000 rads of whole body irradiation to destroy their endogenous HSC [132]. Half of the irradiated mice (n=15) received 1-2x10⁶ HSC transduced with CD68-EGFP and the other half received HSC transduced with CD68-IL-37b via tail vein injections. Injected mice were allowed 4-week recovery period for full reconstitution of their immune systems with
the transduced stem cells before the atherogenic diet containing 15.8% fat (w/w) and 1.25% cholesterol (w/w) (Harlan Teklad) was initiated for an additional 10 weeks. After the 4-week recovery period, 100 µl of blood was collected by submandibular bleeding to establish baseline cholesterol and triglyceride levels, and to confirm immune cell reconstitution by flow cytometry of blood leukocyte populations.

2.10.2 Blood collection and sacrifice of study animals

Submandibular bleeding of each mouse was performed at the start, the midpoint, and at the completion of the study to collect whole blood for analysis of leukocyte population by flow cytometry. A sample of the blood collected was centrifuged at 1500 x g for 15 minutes to obtain plasma for the purpose of measuring lipid levels as well as pro-inflammatory and anti-inflammatory cytokine levels by ELISA or a Luminex bead assay. At the end of the study, the animals were euthanized by CO2 asphyxiation and total blood was collected by cardiac puncture of the left ventricle. The mice were then perfused with ice cold PBS followed by 4% PFA/5% sucrose. The hearts and aortas were collected and analyzed for plaque as described below.

2.10.3 Analysis of blood cell populations by flow cytometry

Blood was collected in EDTA tubes, mixed by gently flicking the tubes, and put on ice. Red blood cells were lysed with 5 mL red blood cell (RBC) lysis buffer (eBioscience) for 15 minutes at RT. 5 mL of PBS were added and the tubes were centrifuged at 350 x g for 5 minutes. If the cell pellets were still very red, a second round of RBC lysis was performed with 1 mL of lysis buffer for an additional 5 minutes before adding PBS and centrifuging again. The cell pellet was then resuspended in 200 µl of Hanks complete buffer (recipe below) and transferred to a
round-bottom 96-well plate and centrifuged again. The supernatant was decanted by quickly inverting the plate. The antibody mix was prepared by adding 0.5 µl of each antibody and filling up to 50 µl total with 50% flow blocking buffer (recipe below) and 50% Hanks complete buffer for each sample. A master mix was made for 7 mice per group, totaling 14 samples. 50 µl of the antibody mix was used to resuspend each cell pellet and the plate was incubated for 20 minutes on ice in the dark. The cells were washed with 150 µl of Hanks complete buffer and centrifuged. The pellets were resuspended in 200 µl of 2% PFA in Hanks complete buffer, transferred into flow cytometry tubes, and kept on ice before being analyzed on a FACSAnia flow cytometer (BD Biosciences).

**Table 3: List of flow cytometry antibodies**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Fluorophore</th>
<th>Company</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ly6G</td>
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<td>Biolegend</td>
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<td>PE-Cy7</td>
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<td>AlexaFluor700</td>
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<td>BV605</td>
<td>Biolegend</td>
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</table>
2.10.4 Analysis of serum cholesterol and triglyceride levels

Blood cholesterol and triglyceride measurements were performed at the end of the BMT atherosclerosis study from serum samples of all mice from each group (N=14). Total cholesterol was measured using a fluorometric assay kit (Cayman Chemical, Item No. 10007640) according to the company’s instructions. Samples were diluted 1:200 with assay buffer in duplicate in a 96-well plate. The mouse serum samples were also analyzed for triglyceride levels using a colorimetric assay kit (Cayman Chemical, Item No. 10010303) according to the company’s instructions. The final fluorescence or absorbance measurements were recorded using a SpectraMax Microplate Reader. The sample values were calculated from a standard curve for each kit.

2.10.5 Analysis of circulating cytokine levels with a Luminex magnetic bead assay

A multi-plex magnetic bead assay was used to detect cytokines in EGFP or IL-37 mouse serums samples. 50 μl of the premixed microparticle cocktail was added to each well along with 50 μl of each serum sample previously diluted 1:2 in calibrator diluent provided in the kit. The plate was incubated for 2 hours at RT on an orbital shaker set to 750 rpm. After following the recommended wash steps using a magnetic to pull down the beads, the plate was incubated with 50 μl of a premixed biotin antibody cocktail for 1 hour at RT on a shaker. The plate was then washed and 50 μl of streptavidin-PE was added to each well for 30 minutes at RT on a shaker. After repeating the wash steps, the microparticles were resuspended in 100 μl wash buffer and the median fluorescence intensity (MFI) was recorded on a Luminex analyzer with a threshold of 50 events per bead and a flow rate of 60 μl/minute.
2.10.6 Analysis of lesion area of whole aortas and aortic root cryosections

The aortas were cleaned of the adventitial fat and any branching arteries, cut open longitudinally, excised and pinned to a wax-lined dissecting tray. A stock solution of Oil-red-O was prepared by dissolving 1 gram of Oil-Red-O powder (Sigma) in 300 mL of 99% isopropanol. The working solution was then prepared fresh for each stain by mixing 180 mL of the stock solution with 120 mL of Milipor water. The mixture was left for 1 hour before filtering through Whatman filter paper into a new glass bottle. The pinned aortas were washed once with 60% isopropanol and then incubated completely covered with Oil-red-O working solution for 15 minutes. The aortas were then washed with 60% isopropanol until the wash fluid was no longer pink (3-4 washes). Each aorta was then photographed and analyzed for plaque area using ImageJ. The thoracic and abdominal areas were also analyzed separately to see any potential differences in areas of plaque abundance.

The fixed hearts collected from the study mice at sacrifice were mounted in O.C.T. (Tissue-Tek) and frozen at -80°C. The OCT-embedded hearts were sectioned sagittally using a cryostat through the aortic valve from the point where all 3 leaflets were visible. Serial sections of 5 mm thickness were made, and 5 representative sections were selected from each mouse heart and stained with Oil-red-O to visualize the lesion area. The cryosections were incubated in PBS for 5 minutes and air-dried. Sections were then dipped 10 times in 60% isopropanol and then incubated in fresh Oil-red-O working solution for 15 minutes. Excess solution was removed and the slides were again dipped 10 times in isopropanol and rinsed for 5 minutes in running tap water. The slides were embedded using mounting media (Sigma) and dried overnight laying flat.
5x photos of the sections were analyzed for lesion area using ImageJ and expressed as the percentage of total aortic area.

2.10.7 Immunofluorescent staining of aortic root cryosections

Three equally spaced aortic root sections per mouse were subjected to immunohistochemical staining to identify macrophage and smooth muscle cells within the lesions. Moma-2 was used for detection of macrophages and Smooth-muscle Actin (SMA), for identification of smooth muscle cells [134]. First, sections were washed for 5 minutes in PBS to dissolve the OCT and air-dried. The slides were then washed once in 0.2% PBST and placed in a dark incubation chamber surrounded by water for humidity. The tissue section was circled with a PAP pen, and incubated 0.05% Trypsin / 0.1% CaCl₂ for 15 minutes at 37°C for antigen retrieval. Following 2 washes with 0.2% PBST, the slides were incubated in blocking buffer (5% normal goat serum in 2% BSA/PBS) for 1 hour at RT. The primary antibodies Moma-2 (rat) and SMA (rabbit) were diluted 1:100 in 2% BSA/PBS and added to separate sections on the same slide for 1 hour at RT. The slides were then washed three times with 0.2% PBST and the secondary antibodies were added. The Moma-2 sections were incubated with a goat anti-rat Cy3 secondary, while the SMA sections were incubated with a goat anti-rabbit Cy3 secondary, both diluted 1:300 in 2% BSA/PBS. 5 minutes prior to the end of the 1-hour secondary antibody incubation, a drop of DAPI nuclear stain was added to each section. The slides were then washed twice using 0.2% PBST and mounting using fluorescent mounting media (DAKO) and left flat in a dark box at 4°C to dry overnight. The sections were then imaged at a magnification of 20x using an epifluorescent microscope, and MOMA-2 or SMA positive areas were analyzed with
ImageJ and expressed as a percentage of the total plaque area, previously measured by Oil-red-O.

Additional staining of aortic root sections for IL-37 and EGFP proteins was performed using primary antibodies against Myc and EGFP respectively. Antigen retrieval was performed with 0.05% Trypsin / 0.1% CaCl₂, incubated at 37°C for 15 minutes. Sections were washed 2x with 0.2% PBST and then blocked with 5% goat or donkey serum in 2% BSA/PBS for 1 hour at RT. Primary antibodies were diluted as shown in in Table 4

Table 4: List of antibodies used for immunohistochemistry staining

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Fluorophore</th>
<th>Company</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat-α-MOMA-2</td>
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<td>R&amp;D</td>
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</tr>
<tr>
<td>Goat-α-Rabbit</td>
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<td>Donkey-α-Goat</td>
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### 2.11 Statistical analyses

For *in vitro* experiments, at least 3 biological replicates were analyzed, with 2 or 3 technical replicates run for each assay as indicated. cDNA was analyzed for transcript abundance via qPCR analysis. Ct values were first normalized to Gapdh for each sample, and then expressed as the average fold change relative to EGFP controls for each treatment, with error bars representing the SEM. For *in vitro* protein secretion measured by antibody array, cell culture
supernatants from 3 separate experiments were pooled for each treatment with cytokine abundance measured in duplicate. Values represent the average of the duplicate measurements ± SEM.

*In vitro* cholesterol uptake was determined by measuring the intensity of DiI fluorescence per cell by flow cytometry with 10,000 events counted for each sample in duplicate. The shift in mean fluorescence intensity (MFI) from baseline autofluorescence for IL-37 samples was expressed as a percentage relative to EV cells with averages from 3 separate experiments shown ± SEM. For *in vitro* cholesterol efflux, conditions were run in triplicate for each group, with 3 separate experiments performed. Baseline cholesterol efflux (no acceptor), or efflux to ApoA1 or HDL, is displayed as a percentage of total cholesterol loaded, with bars representing the average ± SEM.

Transmigration experiments were run three times, each in triplicate. For each replicate well, three photos were taken, and the number of transmigrated macrophages were analyzed with ImageJ. The 3 cell counts for each well were averaged and used as the values for each replicate, which were then analyzed with Prism. For all *in vitro* experiments, differences between IL-37 and control samples were analyzed using Prism and p-values were calculated using the two-tailed Student’s T-test with p<0.05 set for the threshold of significance.

For the *in vivo* mouse study comparing 2 groups with 12 mice per group and a resulting degree of freedom of 22, a student t-distribution of 2.07 is used as the limitation for statistical significance with alpha=0.05 (two-tailed test). With standard deviation within groups less than 25% from the mean, which is typical for the plaque and other analyses, a minimum of 10 mice
per group is necessary to detect significant differences of at least 20% between groups. Accounting for the potential loss of one or more mice during the study and the unknown effects of IL-37b expression within the mice, 15 mice per group were used for the in vivo mouse study, with 14 mice per group surviving until the day of sacrifice.

For some experimental measurements from the study mice, including circulating leukocyte analysis, BMDM and spleen analysis for expression of IL-37 or EGFP by Western blot, and LN transcript analysis, the standard deviation was small enough to detect significant differences using 7 individual samples per study group, although all other assays were run with N=14. The Student’s T-test was used to compare differences between control and IL-37 groups with significance set at p<0.05. The p-values for all experiments were calculated using Prism (Graph Pad), with * indicating p<0.05, ** indicating p<0.001, and *** indicating p<0.0001.
Chapter 3: RESULTS

3.1 Optimization of retroviral transduction

The first transduction experiments performed to express my genes of interest in macrophages began with optimization of retrovirus production by PH-E cells transfected with the CD68S-EGFP-HA retroviral construct (Figure 6B). PH-E cells were stably transfected with the CD68S-EGFP-HA construct using puromycin selection. The first few attempts to transduce HSC with this method resulted in very low EGFP expression in macrophages differentiated from the transduced HSC. To determine if this was due to defective retrovirus production by the PH-E cells, a SYBR Green qPCR-based product-enhanced reverse transcriptase assay (SG-PERT) was performed.

3.1.1 Retroviral copy number determination by reverse transcriptase assay

The SG-PERT assay, as described in section 2.4.2 and previously published [135], was used to determine whether the PH-E cells stably transfected with the CD68S-EGFP-HA vector were producing high enough retroviral titers to adequately transduce the HSC. A standard curve for reverse transcriptase (RT) activity was prepared by making 10-fold serial dilutions of recombinant HIV RT enzyme with known activity (Figure 8A). The regression line was used to calculate the number of infectious retroviral particles produced per mL of PH-E cell supernatant based on the Cq value of each viral supernatant tested. The supernatants from stably-transfected PH-E cells had very low reverse transcriptase activity, indicating insufficient production of retroviral particles (Figure 8B). The untransfected PH-E cells showed low RT activity using this assay, which we attributed to the low level of RT enzyme found in the cell culture media. Further
analysis of PH-E supernatant after using a transient transfection method resulted in significantly increased virus production (Figure 8C).

![Graph showing viral titer](image)

**Figure 8: SG-PERT assay to determine viral titer from reverse transcriptase activity.**

(A) Recombinant HIV reverse transcriptase enzyme was prepared in 10-fold serial dilutions to create a standard curve of known activity units from 1 to $10^{10}$ pU. (B) Stably-transfected PH-E grown for 2 weeks under puromycin selection or untransfected control PH-E were tested for virus production. The RT activity was used to calculate the number of retroviral particles as the number of virions/mL. C) PH-E cells were transfected with the CD68S-EGFP-HA vector using HBS/CaCl$_2$ or left untransfected. After 48 hours, supernatants were tested for RT activity, which was then used to calculate the number of retroviral particles, which were significantly increased over the stable-selection method.

### 3.1.2 Transfection of retroviral packaging cells

We next questioned if the low virus production was due to inefficient transfection of the PH-E cells with the retroviral constructs. We tested the HBS/CaCl$_2$ transfection method against Lipofectamine 2000 (Invitrogen), which we had successfully used for previous difficult transfections, to ensure that the cells were being adequately transfected with the large retroviral constructs. A control retroviral vector, pQ-EGFP [136], of similar size to the CD68S-EGFP-HA
with EGFP expression driven by a CMV promoter was transfected into the PH-E cells. Figure 9A shows EGFP expression 48 hours after transfection using Lipofectamine (top) or HBS/CaCl2 (bottom), visualized with fluorescence microscopy (right) and flow cytometry (left). Figure 9B shows bright field (left) and green channel fluorescence (right) images of control PH-E cells transfected with an empty vector, which did not express EGFP. This finding suggests that the transfection efficiency of HBS/CaCl2 is comparable to that of Lipofectamine 2000 in PH-E cells.
Figure 9: PH-E transfection shown by EGFP expression.

EGFP expression by PH-E transfected with the pQ-EGFP control vector using (A) Lipofectamine 2000 or (B) HBS/CaCl₂ was measured by flow cytometry (left) or fluorescence microscopy (right). Empty vector-transfected control cells were EGFP-negative, as shown in (C) with a bright field image on the left corresponding to the fluorescence image on the right.
3.1.3 EGFP expression in transduced cells

Analysis of the retrovirus produced by PH-E cells transiently-transfected with CD68S-EGFP-HA or pQ-EGFP retroviral constructs using either Lipofectamine-2000 or HBS/CaCl₂ is shown in Figure 10. Differentiated BMDM from transduced-HSC were analyzed by flow cytometry for EGFP expression (top panels). Using EV control BMDM as baseline, the number of EGFP-positive macrophages was measured and is shown in Figure 10B. The CD68S-EGFP-HA transduced macrophages had higher expression of EGFP than the less-specific pQ-EGFP transduced cells. The transient transfection of PH-E cells was chosen as the method for all future transductions for this research.
Figure 10: BMDM show significant EGFP expression after CD68S-EGFP-HA transduction. (A) Macrophages differentiated from CD68S-EGFP-HA-transduced HSC show EGFP expression as measured by flow cytometry. (B) Retrovirus produced in Lipofectamine- or HBS/CaCl₂-transfected PH-E cells were both successful in transducing HSC with pQ-EGFP retrovirus leading to slightly less EGFP expression (28.8% and 26.9% respectively) in macrophages compared to CD68S-EGFP-HA retrovirus (36.1% and 36.5% respectively).

3.1.4 Subcloning of IL-37b into the CD68S retroviral vector

The cDNA sequence for the IL-37b isoform gene was obtained as part of the pCMV6-Entry shuttle vector (OriGene), with the stop codon of the original IL-37b sequence mutated to allow the addition of a Myc and DDK tag within the open reading frame. The cloning scheme
recommended by E. Raines for use of the CD68S retroviral vector involved directional cloning using the NotI and HindIII restriction enzyme sites (RE) provided. However, the CMV6-Entry vector contained a NotI site right between the IL-37b gene and the Myc-DDK tag (Figure 7A), so an alternative cloning scheme had to be created. PCR amplification of the entire IL-37b-Myc-DDK sequence using primers with added HindIII RE sites at the ends was performed using a high fidelity Taq polymerase to increase efficiency and reduce the error rate of transcription. The CD68S retroviral vector, and the IL-37b-Myc-DDK PCR product were digested with the restriction enzyme HindIII to produce compatible ends for ligation with T4 DNA ligase. After transformation into competent bacteria, positive clones were screened for the presence of IL-37b-Myc-DDK insert with the correct orientation. Figure 11 shows the sequence of the IL-37 clone used for all future experiments.

![Sequence verification of IL-37b-Myc-DDK after cloning into the CD68S vector.](image)

The IL-37b ORF is shown in turquoise with the ATG start codon in dark blue, the last coding amino acid shown in yellow, and the TAA stop codon shown in red. The Myc and DDK tags are marks with green and purple, respectively. HindIII restriction enzyme sites are shown in pink. The orange highlighted cytosine nucleotide is the only change from the IL-37 transcript variant 1 mRNA sequence found in the NCBI nucleotide database (NM_014439.3), although it does not change the amino acid sequence.
3.2 Effects of IL-37b expression on macrophage function

After successful subcloning of IL-37b-Myc-DDK into the CD68S vector, in vitro experiments to investigate the effects of IL-37b expression on macrophage inflammation, cholesterol homeostasis and transmigration. PH-E retrovirus production was achieved after transient transfection with the CD68S-EGFP-HA or CD68S-IL-37b-Myc-DDK vectors. Rounds 1 and 2 of HSC transduction were performed with retroviral supernatants 48- and 72-hours post-PH-E transfection. After differentiating the transduced HSC into macrophages for 1 week using DMEM/F12 supplemented with 20% L929-conditioned medium, the adherent macrophages were replated for in vitro experiments.

3.2.1 Transduced macrophages show robust EGFP and IL-37b expression in vitro

Transduced and differentiated macrophages expressing either EGFP or IL-37b were replated into 6-well plates for treatment with AcLDL, IFNγ or TNFα overnight. The cells were then used for RNA extraction or protein lysate preparation. Quantitative PCR (qRT-PCR) was performed on EGFP or IL-37 macrophage cDNA. IL-37 transcript was detected at Ct values similar to GAPDH in IL-37b-transduced macrophages while the transcript was not detected at all in EGFP macrophages (data not shown). EGFP or IL-37b protein expression was achieved as evidenced by Western blot analysis (Figure 12). A band corresponding to the predicted molecular weight of uncleaved IL-37b-Myc-DDK was visible at 28 kDa exclusively in IL-37b-transduced macrophages. The cleaved, mature form of the protein, visible as a 24 kDa band, was present under all conditions, but it was especially prominent after IFNγ treatment. EGFP expression was clearly visible in macrophages treated with or without AcLDL or IFNγ. Robust
and specific expression of each protein was achieved in macrophages differentiated from transduced HSC, indicating that the method was successful and further in vitro experiments could be initiated.

Secretion of IL-37 protein from transduced BMDM was verified by enzyme-linked immunosorbant assay (ELISA). Cell culture supernatants were collected 24 hours after treatment with IFNγ or AcLDL. IL-37 protein secretion was observed exclusively in IL-37 samples with no detectible signal (ND) from EGFP control samples (Figure 13). Interestingly, IFNγ-mediated IL-37 secretion was markedly decreased compared to untreated cells expressing IL-37. This is in contrast to the increase in intracellular mature IL-37 protein levels found in IFNγ-treated cells by Western blot (Figure 12), indicating a possible link between increased processing of pro-IL-37 to mature-IL-37 and decreased secretion.
Figure 12: IL-37b and EGFP protein expression in transduced BMDM

(A) Western blots for IL-37b or EGFP protein show robust expression of each protein in the respective transduced macrophages. Pro-IL-37b-Myc-DDK shows a band at approximately 28 kDa as expected. The mature, cleaved form is visible at approximately 24 kDa, with higher expression after IFNγ treatment. Another band representing IL-37 protein is visible at 35 kDa. EGFP expression is visible under all treatment conditions at its expected 28 kDa. β-actin was used as a loading control and is visible at 42 kDa. (B) IL-37 protein Western blot quantification from IL-37-transduced BMDM for pro-IL-37b and mature-IL-37b. N=3 with ***p<0.0001.
Cell culture supernatants from transduced BMDM were left untreated or treated with IFNγ or AcLDL for 24 hours. IL-37 protein secretion was analyzed by ELISA. EGFP-transduced BMDM supernatants showed no detectable (ND) IL-37 protein. N=6 with *p<0.05.

3.2.2 IL-37b expression reduces macrophage inflammation

The effects of IL-37 on macrophage inflammatory response were tested by challenging the transduced BMDM with various inflammatory stimuli. Overnight treatment of EGFP- or IL-37-expressing macrophages with AcLDL, IFNγ, or TNFα, followed by RNA extraction and RT-qPCR analysis, revealed suppression of the inflammatory response by IL-37 (Figure 14). IL-37 expression in unstimulated cells showed reduced expression of inflammatory cytokines and mediators such as IL-1α, IL-1β, IL-6, IL-12, and TNFα compared to EGFP control cells (Figure 14A). Macrophages were treated with AcLDL to induce inflammation typical of a macrophage encountering modified LDL within the plaque. AcLDL-treated IL-37-expressing macrophages showed reduced IL-1α, IL-1β, IL-6 and IL-12 gene expression (Figure 14B). Transduced
macrophages were also treated with IFNγ or TNFα, inflammatory molecules also found in the plaque microenvironment. IL-37 expressing cells treated with IFNγ showed reduced IL-1β, IL-6, IL-12 and MIP-1β gene expression compared to EGFP controls (Figure 14C), while IL-37 cells treated with TNFα showed reduced IL-1α, IL-1β and IL-6 gene expression (Figure 14D). Under conditions of inflammation that would be found in the plaque microenvironment, IL-37 expression effectively suppressed inflammation in macrophages.
Figure 14: Macrophage-expressed IL-37b reduces pro-inflammatory gene expression

Macrophages transduced with EGFP or IL-37b were left untreated (A), or treated with 20µg/mL AcLDL (B), 20ng/mL IFNγ (C), or 10ng/mL TNFα (D) for 24 hours. cDNA prepared from total cellular RNA was subjected to qPCR analysis for the genes displayed using Gapdh as housekeeping control. The graphs show gene expression of IL-37 transduced macrophages compared to EGFP control cells under basal or inflammatory treatment conditions. N=3, with values representing mean fold change in transcript abundance ± SEM. *p<0.05, **p<0.001, ***p<0.0001)
Figure 15: Macrophage inflammatory protein production is inhibited by IL-37b expression.

A mouse cytokine antibody array was used to detect protein expression in the supernatants of EV or IL-37 BMDM after treatment with or without AcLDL (20µg/mL) for 48 hours. (A) Cytokine secretion from unstimulated EV vs IL-37 BMDM. (B) Cytokine secretion from EV vs IL-37 BMDM after AcLDL stimulation. N=3 pooled samples per group, each run in duplicate. Values represent the mean ± SEM. *p<0.05, **p<0.001, ***p<0.0001.
To determine if the anti-inflammatory effects of IL-37 on macrophage gene expression were also true for the synthesis and secretion of cytokine proteins, an antibody array was performed on cell supernatants comparing EV to IL-37 BMDM for both unstimulated and AcLDL-treated cells (Figure 15). The nitrocellulose membranes supplied with the array kit were pre-incubated with capture antibodies for both pro-inflammatory and anti-inflammatory cytokines as well as chemokines. The pooled supernatants (N=3) of EV- or IL-37-transduced macrophages that were either left untreated, or treated with 20µg/mL AcLDL for 48 hours were incubated with a biotin-labeled detection antibody cocktail before being added to nitrocellulose membranes pre-dotted in duplicate with capture antibody for protein detection by chemiluminescence.

IL-37 expression resulted in the suppression of inflammatory protein production, as seen in Figure 15. Cytokines such as IL-16, M-CSF and MIP-1α were all downregulated by IL-37 expression in untreated macrophages (Figure 15A). A more potent anti-inflammatory effect of IL-37 expression is seen after Ac-LDL treatment in which a majority of cytokines tested were downregulated, including IL-1α, IL-17, IFNγ, CXCL9, MIP-1α, MIP-1β and M-CSF (Figure 15B). This provides new insight into the role of IL-37 in regulating the macrophage inflammatory response to modified LDL uptake.

3.2.3 Effect of IL-37 expression on cholesterol metabolism-related gene transcription

To further investigate the effect of IL-37 on expression of macrophage lipid response genes, RT-qPCR analysis of transduced BMDM with or without AcLDL treatment for 24 hours was performed. The results shown in Figure 16 reveal a significant increase in gene expression
of the cholesterol transporter ABCA1 in untreated IL-37-transduced cells compared to EGFP controls. However, this difference was not observed with AcLDL treatment. Transcript levels of the cholesterol transporter ABCG1, and the nuclear receptor PPARγ, were not significantly changed. Although the expression of ABCA1 is increased by IL-37 expression, under cholesterol-rich conditions, macrophage expression of IL-37 does not appear to alter expression of genes involved in macrophage cholesterol efflux. Cholesterol uptake genes, including C36 and the scavenger receptors, SR-AI and SR-AII were also not significantly changed in untreated or AcLDL-treated macrophages. However, there was a strong trend towards decreased CD36 expression in untreated IL-37-transduced compared to EGFP-transduced macrophages (p=0.06).
Figure 16: Cholesterol-related gene transcription in EGFP vs. IL-37 transduced macrophages.

Macrophages transduced with EGFP or IL-37b were left untreated (A), or treated with 20µg/mL AcLDL (B), 20ng/mL IFNγ (C), or 10ng/mL TNFα (D) for 24 hours. cDNA prepared from total cellular RNA was subjected to qPCR analysis for the genes displayed. IL-37 expression downregulates inflammatory gene expression compared to EGFP control cells under basal or inflammatory treatment conditions. N=3, with values representing fold change in transcript abundance ± SEM. *p<0.05, **p<0.001, ***p<0.0001

3.2.4 Macrophage cholesterol uptake is reduced by IL-37b expression

The uptake of modified lipoprotein within the plaque is central to foam cell formation. Inhibiting cholesterol uptake helps to prevent foam cell formation and protects against plaque growth and development. To investigate whether IL-37 had a role in the macrophage’s capacity to take up cholesterol, we used Dil-labeled modified lipoproteins to visualize the uptake by...
macrophages using fluorescence microscopy and flow cytometry. For these experiments, the control cells used were switched from EGFP to empty vector (EV) transduced to avoid fluorescence spill over from the EGFP protein into the Dil channel of the microscope and flow cytometer.

Figure 17 shows Dil-AcLDL and Dil-OxLDL uptake in IL-37b- and EV-transduced macrophages treated in parallel with or without 20ng/mL IFNγ for 4 hours. The cells used for fluorescence analysis by microscopy were fixed in 2% PFA for 15 minutes and then stained with DAPI to visualize the nuclei. The images in Figure 17A taken at 20x magnification show the macrophages with nuclei stained in blue and Dil-LDL visible in red. Quantitative analysis performed using flow cytometry is presented in Figure 17B and C for Dil-AcLDL and Dil-OxLDL, respectively. IL-37 expression reduced the amount of modified LDL uptake compared to controls with a significant decrease in both AcLDL and OxLDL uptake. IFNγ treatment showed a slight trend towards increasing OxLDL uptake in EV and IL-37 macrophages, as well as AcLDL in EV macrophages, although the changes were not significant. In contrast, IFNγ treatment showed a trend towards decreased AcLDL uptake in IL-37-expressing macrophage, but the difference was also not significant. Overall, IL-37 expression in macrophages functions to prevent AcLDL and OxLDL uptake, implying a protective role for IL-37 in preventing foam cell formation.
**Figure 17:** IL-37b expression leads to reduced macrophage uptake of modified LDL.

IL-37 or EV control macrophages were treated with Dil-AcLDL or Dil-OxLDL (both at 20µg/mL) ± IFNγ (20ng/mL) treatment for 4 hours. (A) Fluorescent images were taken on an epifluorescent microscope at 20x. Nuclei were stained with DAPI (blue) and Dil-LDL is shown in red. (B) Flow cytometry analysis of treated macrophages shows differences in uptake between IL-37 and EV control cells. Results were normalized to the untreated, EV control. Experiments were performed in triplicate for each run with N=3. (Values represent the mean ± SEM with ***p<0.0001, **p<0.001 and *p<0.05.)
3.2.5 Macrophage cholesterol efflux is not affected by IL-37b expression

The two principal mechanisms in prevention of foam cell formation include reducing cholesterol uptake and enhancing cholesterol efflux. The effect of IL-37 expression on macrophage cholesterol efflux was tested by first loading transduced macrophages with tritium-labeled cholesterol for 36 hours, then allowing the intracellular cholesterol to efflux to the cholesterol acceptors, ApoA1 or HDL, for 7 hours. To determine the % efflux to each acceptor, the level of radioactivity in the cell culture supernatant following efflux was expressed as a percentage of the total loaded cholesterol within the cells. IL-37 expression showed no significant effect on macrophage cholesterol efflux compared to controls, although there was a slight trend towards increased basal and ApoA1 efflux (Figure 18).

Figure 18: Macrophage cholesterol efflux is not affected by IL-37 expression.

Macrophages expressing EGFP or IL-37 were loaded with 1µCi/mL along with 50 µg/mL AcLDL in 24-well plates using media with 10% lipoprotein deficient serum for 36 hours. ApoA1 or HDL served as cholesterol acceptors in a 7-hour incubation. No significant differences in efflux to either acceptor were observed comparing IL-37 and EGFP expressing macrophages. N=3, each in triplicate. (Values represent the mean ± SEM)
3.2.6 Macrophage transmigration is inhibited by IL-37b expression

Chemotaxis of immune cells to developing plaque is achieved in part by the production of chemoattractants. The migratory response of monocytes and macrophages towards chemoattractants, especially MCP-1, plays a central role in the pathogenesis of atherosclerosis. To determine if IL-37 has any effect on the migration of macrophages towards the chemoattractant MCP-1, a trans-well filter assay was performed. EV- or IL-37-transduced macrophages (left untreated or pretreated overnight with 25ng/mL IFNγ) were added to the top chamber of 0.5µm transwell filters and allowed to migrate overnight towards MCP-1-containing medium in the bottom chamber of the wells. The cells on the underside of the filters were stained with DAPI and fluorescent images were analyzed with ImageJ to determine the number of transmigrated macrophages in each group. IL-37 expression significantly reduced macrophage transmigration compared to EV controls (Figure 19). IFNγ pretreatment increased EV control macrophage transmigration, but did not have a significant effect on IL-37 macrophages.
Figure 19: Macrophage-expressed IL-37 leads to reduced transmigration of macrophages.

IL-37- or EGFP-transduced macrophages were left untreated, or pretreated with or without IFNγ for 4 hours and then plated and allowed to transmigrate towards MCP-1 overnight through a transwell filter. Transmigrated cells were analyzed with ImageJ and quantified for EV control and IL-37b-expressing macrophages. Experiments were performed in triplicate and repeated for an N=3. (Values represent the mean ± SEM with **p<0.001).

3.3 Macrophage-expressed IL-37b protects against atherosclerosis in vivo

Taken together, the in vitro data that 1) IL-37 expression reduced levels of inflammatory gene and protein expression; 2) inhibited modified LDL uptake; and 3) reduced macrophage transmigration towards the chemoattractant MCP-1 support a strong athero-protective role for macrophage-expressed IL-37. To test whether these in vitro findings hold true in a disease setting, an in vivo mouse atherosclerosis development study was set up. EGFP- or IL-37b-transduced HSC were transplanted into lethally irradiated male Ldlr−/− mice, 6-8 weeks old. A portion of the transduced HSC were cultured and differentiated into macrophages for analysis of EGFP or IL-37 expression. After tail vein injection of 1-2x10⁶ transduced HSC, the irradiated recipient mice were allowed to reconstitute with the donor marrow for 4 weeks, after which
blood samples were collected from all study mice to verify that the transduction and transplantation were successful.

3.3.1 Verification of HSC transduction in vitro

A portion of the HSC used to inject the irradiated recipient mice were cultured and differentiated into macrophages. Macrophage expression of EGFP or IL-37 was shown by flow cytometry and Western blot (Figure 19). EGFP (green) and IL-37 (orange) -transduced cells were compared for EGFP expression (Figure 20A) and shows a clear EGFP-positive population not present in IL-37 cells. Cells used for analysis of IL-37 protein expression were first fixed and permeabilized before incubation with an IL-37 primary antibody followed by a Cy3 α-Goat secondary antibody. IL-37-stained EGFP cells and Cy3 secondary stained IL-37 cells served as negative controls. IL-37 cells stained positive for IL-37 protein, indicating a successful transduction of the HSC used for repopulation of the irradiated recipient Ldlr<sup>−/−</sup> mice. Figure 20B and C show IL-37 expression by PCR amplification of IL-37 transcript and detection of IL-37 protein by Western blot respectively. Western blot analysis of lysates from the cultured BMDM shows both EGFP and IL-37 protein expression exclusively in their respective groups.
Figure 20: IL-37 and EGFP expression in transduced HSC used for BMT.

Transduced HSC kept from each group for macrophage differentiation in vitro were split into separate wells for each assay. EGFP and IL-37 expression were analyzed by flow cytometry (A), PCR (B) or Western blot (C). EGFP expression is shown by flow cytometry using unstained IL-37 cells as a control (A). IL-37 expression was measured using a Goat-α-IL-37 primary antibody with an α-Goat Cy3 secondary antibody (orange line). A Cy3 isotype control (teal line), and IL-37-Cy3-stained EGFP cells (green line) were used as negative controls. (B) IL-37 cDNA abundance in IL-37 or EGFP BMDM shown by PCR. (C) Western blot analysis of BMDM differentiated from transduced HSC show EGFP or IL-37 protein. N=1.

Serum levels of IL-37 protein were detected in IL-37 but not EGFP BMT mice. Following bone marrow transplantation and 4 weeks of recovery, blood serum was collected from EGFP or IL-37 mice and analyzed for IL-37 protein by ELISA. As shown in Figure 21, IL-
37 mice showed levels of circulating IL-37 protein around 2000 pg/mL on average. This confirmed that IL-37 expression in vivo was successful, and also that IL-37 was effectively secreted by monocytes/macrophages.

![IL-37 protein detected in sera of IL-37 mice 4 weeks post-BMT.](image)

**Figure 21: IL-37 protein detected in sera of IL-37 mice 4 weeks post-BMT.**

Sera collected from EGFP and IL-37 mice following 4-week recovery period, prior to HFD initiation was analyzed by ELISA for IL-37 protein abundance. Protein concentration is expressed as pg/mL serum with the mean ± SE shown by horizontal lines. No IL-37 protein was detected in EGFP serum samples (ND). N=14 samples per group.

### 3.3.2 Verification of in vivo HSC reconstitution in Ldlr<sup>−/−</sup> mice following BMT

Blood was collected from each study mouse at the end of the 4-week recovery period following irradiation and BMT, just before the start of the high fat diet. 100 µl of whole blood was incubated in RBC lysis buffer and then stained with a panel of conjugated flow cytometry antibodies (listed in 2.9.4) to analyze the abundance of the various circulating immune cell populations. As shown in Figure 22, the populations of T-cells, monocytes and neutrophils were detected by flow cytometry, indicating a successful repopulation with the donor marrow. There were no differences in the percentages of each leukocyte population found between EGFP and IL-37 mice. At this stage, the mice were switched from a normal chow diet to a high fat diet.
containing 15.8% (wt/wt) fat and 1.25% cholesterol (wt/wt) (diet 94059; Harlan Teklad Laboratories, Indianapolis, IN, USA) for 10 weeks to induce atherosclerosis development.

**Figure 22: Reconstituted leukocyte populations in study mice after BMT.**

Mouse blood was collected 4 weeks after HSC transplantation. Circulating leukocyte populations were analyzed by flow cytometry. Monocytes, neutrophils, CD4 T-cells and CD8 T-cells were identified by differential gating and expressed as a percentage of total CD45+ cells. N=7 per group.
3.3.3 Initial characterization of BMT mice post-HFD

Following 10 weeks on a high fat diet, blood was collected from the 14 study mice from each group before sacrifice. The serum cholesterol and triglyceride levels were measured using commercially available fluorometric and colorimetric kits (Cayman Chemical), which revealed no difference in cholesterol or triglycerides between EGFP and IL-37 mice (Figure 23A&B). The body weight of each mouse measured before and after the 10 weeks of high fat diet showed significant weight gain between the start and end of the experiment, with no differences observed between EGFP and IL-37 groups (Figure 23C). The absence of differences between EGFP and IL-37 mice indicate that IL-37 did not likely affect the overall metabolism of the mice.
Figure 23: Cholesterol, triglyceride and body weight measurements of EGFP and IL-37 mice.
Total cholesterol (A) and triglycerides (B) were measured using 1:200 dilutions of blood serum from each study mouse, with N=14 per group. (C) Body weights were measured before and after 10 weeks of high fat diet. N=14 per group. *p<0.05, **p<0.001.

For analysis of peripheral leukocyte populations after 10 weeks of HFD, 100µl of whole blood was prepared for staining with flow cytometry antibodies as described previously. Figure 24 shows the various circulating leukocyte populations, including monocytes, neutrophils, B-cells and T-cells, from EGFP and IL-37 mice. No significant differences in any leukocyte
population were detectable between groups, indicating that IL-37 expression did not affect the survival or turnover of the various circulating immune cells.

To test whether IL-37 was present in circulation of the BMT mice post-HFD, sera samples from blood collected at sacrifice were tested for IL-37 protein abundance by ELISA. IL-37 BMT mice showed wide-ranging IL-37 protein levels in the blood while EGFP BMT serum showed no detectable (ND) IL-37 protein, as expected (Figure 25).

**Figure 24: Circulating leukocyte populations in study mice after 10 weeks HFD.**
Mouse blood was collected at sacrifice and circulating leukocyte populations were stained and analyzed by flow cytometry. Monocytes, neutrophils, CD4 T-cells, CD8 T-cells, and B-cells were identified by differential gating and expressed as a percentage of total CD45+ cells. No significant difference in leukocyte populations was observed between groups. N=8 per group.
3.3.4 Robust expression of IL-37 or EGFP in BMDM and spleens lysates

At sacrifice, the femurs from 7 mice per group were harvested and the bone marrow was cultured for analysis of bone marrow derived macrophages. As seen in Figure 26, clear expression of IL-37 or EGFP was detectable by flow cytometry and Western blot in each respective group. EGFP expression measured by flow cytometry (Figure 26A) is shown in a scatter plot with the percentage of EGFP-positive macrophages from the 7 mice tested from each group (left) as well as a flow cytometry graph of EGFP expression from 3 representative mice per group (right). The same analysis was performed for IL-37 expression, shown in Figure 26B. IL-37 was detected in macrophages by flow cytometry using a primary and Cy3 secondary antibody as described previously. Figure 26C shows a Western blot for EGFP or IL-37 protein expression in macrophage cell lysates from the same 7 BMDM samples per group. Both groups displayed strong expression of their respective proteins, but the level of expression was quite variable among the BMDM samples analyzed (Figure 26C).
Figure 26: BMDM isolated from mice 14 weeks post-BMT show IL-37 and EGFP expression.

After 4 weeks of recovery and 10 weeks of HFD, IL-37 or EGFP bone marrow was isolated from 7 mice per group at the time of sacrifice. Macrophages were analyzed for EGFP or IL-37 expression by flow cytometry (A&B) or Western blot (C). Strong EGFP or IL-37 expression was detected in BMDM from each respective group.
IL-37 protein levels in spleen lysates collected EGFP or IL-37 mice at sacrifice are shown in Figure 27. The IL-37 pro-form (28kDa) as well as a strong 35kDa band can be seen for all IL-37 samples tested. EGFP expression is shown only in EGFP samples, confirming that the transgenes were expressed as expected.

Figure 27: EGFP and IL-37 protein in mouse spleen lysates 14 weeks post-BMT.
Lysates prepared from EGFP or IL-37 mouse spleens were analyzed by Western blot for EGFP or IL-37 protein abundance. EGFP or IL-37 protein was detected exclusively in their respective groups. IL-37 pro-form (28 kDa) shown below the 35 kDa band. EGFP was observed at the expected 27 kDa. β-actin was used as a loading control. N=7 per group.

3.3.5 EGFP-positive cells are visible in the plaques of EGFP but not IL-37 BMT mice.
Aortic root heart sections were analyzed for EGFP or IL-37 positive plaque area with an epifluorescence microscope. The exposure times for photos taken of each slide were set to a constant for EGFP and IL-37 and their respective secondary antibody control stains to prevent differences due to photo bleaching. Sections from EGFP BMT mice were stained with an EGFP primary antibody followed by a Cy3 fluorescent secondary antibody. EGFP protein was detected in EGFP mouse plaques (Figure 28, left panel) with secondary antibody control staining shown
Aortic root sections from IL-37 BMT mice were stained with a primary antibody against the Myc tag found on the IL-37 protein, followed by a Cy3 fluorescent secondary antibody. IL-37 was detected in the plaques of IL-37 BMT mice (Figure 29) with secondary antibody control staining shown for comparison (right panel). There was noticeably less IL-37 positive staining in IL-37 mice than EGFP staining in EGFP BMT mice, indicating a reduced infiltration of IL-37-expressing macrophages, although the results are not quantitative. The staining of EGFP and IL-37 proteins within the respective BMT mice confirms the presence of transduced macrophages within the atherosclerotic plaque.
**Figure 28: EGFP is detectable in plaques of EGFP BMT mice.**

Aortic root sections from EGFP mice were stained using a primary antibody against EGFP followed by a Cy3 secondary antibody. Sections were analyzed with an epifluorescence microscope using DAPI and Rhodamine filters. EGFP-positive areas were detected in the plaque (left panel). Secondary antibody control staining shown for comparison (right panel).
Figure 29: IL-37 is detectable in plaques of IL-37 BMT mice.

Aortic root sections from IL-37 mice were stained for IL-37 using a primary antibody against the Myc tag followed by a Cy3 secondary antibody. Sections were analyzed with an epifluorescence microscope using DAPI and Rhodamine filters. IL-37-positive areas were detected in Myc-stained sections (left panel). Secondary antibody control staining shown for comparison (right panel).
3.3.6 Serum cytokine levels and IL-37 protein expression in vivo

Serum samples from EGFP and IL-37 study mice were analyzed with a Luminex magnetic bead assay for measurement of 12 cytokines, including TNFα, IL-12p70, MCP-1, IL-1α, IL-1β, IL-4, IL-6, IL-10, IFNγ, M-CSF, MIP-1α, and MIP-2. Of the cytokines analyzed, only M-CSF, MIP-1α, MIP2, TNFα, and IL-4 were detectable in all samples tested. IL-37 BMT mouse sera showed a trend towards decreased circulating M-CSF compared to EGFP control mice (p=0.07), however none of the circulating inflammatory cytokine levels measured were significantly different between IL-37 and EGFP mice (Figure 30).
Figure 30: Cytokine expression in EGFP or IL-37 mouse sera 10 weeks post-HFD.

Sera from EGFP or IL-37 mice at sacrifice were analyzed with a magnetic 12-plex Luminex assay. The abundance of circulating cytokines is shown for EGFP and IL-37 mice. N=14 per group with the mean ± SE shown for each.

To determine if there was a correlation between circulating IL-37 protein (measured by ELISA) and the levels of inflammatory cytokines (measured by Luminex), linear regression analysis was performed for each cytokine. Figure 31 shows the relationship between serum abundance of IL-37 and the levels of M-CSF, MIP-1α, MIP2, TNFα, or IL-4, with the best-fit-line and Pearson correlation coefficient shown for each graph. The analysis revealed a significant negative correlation between IL-37 protein abundance and M-CSF levels in the serum of IL-37 mice ($R=-0.7071, p=0.0047$), implying a systemic anti-inflammatory role for IL-37 in vivo.
There was no significant correlation for each of the other cytokines analyzed, although there was a positive trend between IL-37 and IL-4 serum concentrations (Figure 31).

Figure 31: Correlation of circulating cytokine levels with IL-37 serum concentration
Linear regression analysis was carried out with Prism (GraphPad) comparing mouse serum cytokine levels with IL-37 serum concentration from the IL-37 mice in the study. Pearson correlation coefficient values (R) are shown for each graph with p values shown only for significant correlations (p<0.05). N=14.

To further investigate the effects of IL-37 on inflammation within the BMT mice, lumbar lymph nodes collected from IL-37 or EGFP mice at sacrifice were analyzed for inflammatory gene expression by RT-qPCR. Lymph node transcript from IL-37 mice showed a significant
reduction in IL-6 expression, and a strong trend (p=0.06) towards reduced IL-1β expression (Figure 32A&B). There were also trends towards reduced IL-12α, TNFα, and CCL4 in IL-37 vs. EGFP lymph nodes (Figure 32C-E), although these differences were not significant. The reduction in lymph node inflammatory gene expression indicates that IL-37 expression acts systemically in vivo.

Figure 32: Inflammatory gene expression in the lymph nodes of IL-37 and EGFP mice
RNA extracted from lumbar lymph nodes collected at sacrifice from EGFP and IL-37 mice was transcribed to cDNA and analyzed for gene transcript abundance by qPCR. Gapdh was used as housekeeping control. Fold change in transcript levels for each gene was then compared to the average obtained from EGFP controls. N=7 per group with *p<0.05.
3.3.7 Macrophage-expressed IL-37b reduces total plaque area in Ldlr\(^{-/}\) mice

At sacrifice, the study mice were perfused with PBS followed by 4% PFA/5% sucrose. The heart and other organs were collected for further analysis. The whole aortas, from the aortic arch down to the iliac bifurcation, were meticulously cleaned of adventitial fat and cut open longitudinally to display the inner surface. Once all 14 aortas were prepared from each group, they were stained with Oil-red-O to visualize plaque area. Photos were taken of each aorta and Oil-red-O-positive plaque areas with 5 aortas from each group shown as red areas (Figure 33A). Whole aortas as well as the thoracic segment alone (measured from the arch to the branching point of the renal arteries) were analyzed for plaque area, which was quantified using ImageJ (Figure 33B). Whole aortas as well as thoracic segments alone revealed significantly less plaque area in IL-37 compared to EGFP mice, demonstrating that macrophage-expressed IL-37b protected against atherosclerotic plaque development in lesion-prone Ldlr\(^{-/}\) mice.
**Figure 33: Aortic plaque area is significantly reduced in IL-37 mice compared to controls**

*(A)* Whole aortas from EGFP or IL-37 mice were stained with Oil-red-O to visualize plaque area. *(B)* Quantification of plaque areas was performed using ImageJ software and expressed as a percentage of total aorta area. Significantly less plaque was detected in IL-37 aortas compared to EGFP mice. *(N=14. **p<0.001 with mean ± SEM displayed.)*

The hearts collected from EGFP and IL-37 mice at sacrifice were embedded and frozen in OCT before being cryosectioned at a thickness of 5µm. Serial sections of the aortic root were collected at the point where the 3 valve leaflets were visible. 5 sections per heart spanning the aortic root were stained with Oil-red-O and analyzed for atherosclerotic plaque area using ImageJ. The results of this analysis, shown in Figure 34, revealed less plaque area in IL-37 compared to EGFP heart sections. These data are in agreement with the plaque data in the aorta (Figure 33), and provide further evidence for the athero-protective role of IL-37.
Figure 34: Atherosclerotic plaque in aortic root cryosections stained with Oil-red-O

(A) Aortic root cryosections of EGFP and IL-37b mouse hearts were stained with Oil-red-O. A representative image is shown for each group. 5 images per heart with N=14 hearts per group were analyzed with ImageJ software. Quantification of plaque area expressed in mm$^2$ is shown in (B), revealing significantly less plaque in IL-37 compared to EGFP heart sections. (N=14. *p<0.05 with mean ± SEM displayed.)
3.3.8 Presence of macrophages and SMC in the atheroma was not affected by IL-37b

Aortic root sections immediately adjacent to sections used for Oil-red-O staining were selected for immunofluorescent staining to detect macrophages or smooth muscle cells within the plaque. Each aortic root section was imaged at 20x magnification in multiple overlapping shots, which were then stitched together using Photoshop (Adobe) and analyzed with ImageJ to determine macrophage- or SMC-positive areas. Smooth muscle actin staining revealed little to no SMC-positive area within the plaque and was not quantifiable, likely due to the relatively early stage of plaque development after only 10 weeks of HFD (data not shown). MOMA2 staining showed robust infiltration of macrophages with representative images shown for EGFP and IL-37 sections in Figure 35A. The macrophage-positive area was then divided by the total plaque area, which was previously calculated from Oil-red-O-stained adjacent sections, and expressed as a percentage of total plaque area. Although the total plaque area was reduced in IL-37 mice, there was no difference observed in macrophage content per plaque area between EGFP and IL-37 groups (Figure 35B).
Figure 35: Aortic root sections show no difference in macrophage content per plaque area.
(A) Aortic root heart sections were subjected to immunofluorescent staining for MOMA-2 (red), with DAPI-positive nuclei shown in blue and the auto-fluorescence of the tissue in green. (B) Quantification of the MOMA-2 positive area was calculated using ImageJ. The total plaque areas measured from Oil-red-O sections were then used to calculate the macrophage content as a percent of total plaque for each mouse.
CHAPTER 4: DISCUSSION

4.1 Summary and interpretation of results

The experiments detailed within this dissertation demonstrate that macrophage-specific IL-37 expression leads to suppression of inflammation, reduction of cholesterol uptake, and inhibition of macrophage transmigration in vitro. Bone marrow transplantation of IL-37b-transduced HSC resulted in reduced plaque development in atherosclerosis-prone Ldlr<sup>-/-</sup> mice compared to controls. This is the first study to investigate the role of IL-37 in the context of atherosclerosis, providing key evidence to further explore its potential therapeutic value in the treatment and prevention of the disease.

Retroviral gene transfer was optimized (Figure 8, Figure 12) to stably express IL-37b in monocytes/macrophages differentiated from mouse HSC. IL-37 transcript and protein expression were detected exclusively in IL-37-transduced cells by qPCR, Western blot, and ELISA. Interestingly, IL-37 macrophage lysates showed a significant increase in protein after IFN<sub>γ</sub> treatment (Figure 12), while analysis of cell culture supernatants by ELISA showed a significant decrease in secreted protein (Figure 13). Although it can only be speculated with the data obtained, the reduced secretion of IL-37 protein by macrophages treated with IFN<sub>γ</sub> could possibly be linked to the increased processing of pro-IL-37 to mature IL-37 to signal intracellularly. Alternatively, IL-37 protein secretion may be reduced due to competition with other cytokines produced and secreted as part of the macrophage’s normal response to IFN<sub>γ</sub> stimulation. It remains to be investigated whether the macrophage secretion of pro- and mature-IL-37 forms differs based on the particular inflammatory stimuli. In any case, the production of
both intracellular and secreted IL-37 protein was successfully executed with the retroviral gene transfer method used for the experiments detailed within this dissertation.

4.1.1 Macrophage inflammatory response

The *in vitro* experiments performed with IL-37-transduced macrophages demonstrate that IL-37 expression leads to suppression of inflammation under basal as well as inflammatory conditions. Since the main driver of atherosclerosis is the chronic inflammation that continually attracts immune cells to the site of plaque development, the primary athero-protective effects of macrophage IL-37 expression *in vivo* are likely attributable to its anti-inflammatory properties.

The innate immune response that drives atherosclerosis is led primarily by monocyte recruitment to the intima of the vessel wall, followed by macrophage uptake of lipids and production of inflammatory mediators. Macrophage expression of inflammatory mediators, including potent chemokines responsible for recruiting various leukocytes to the plaque, was reduced by expression of IL-37. M-CSF gene expression has long been known to be linked to atherosclerosis development [137], and its expression within the plaque leads to the differentiation of macrophages and expression of scavenger receptors [138]. M-CSF deficiency in atherosclerosis-prone ApoE knockout mice reduces plaque development [60], emphasizing the importance of limiting its expression to prevent the disease. IL-37 expression in macrophages reduced M-CSF protein production in both unstimulated and Ac-LDL-stimulated macrophages in *vitro* (Figure 15). In addition, circulating levels of M-CSF in IL-37-transduced BMT mice were negatively correlated with serum IL-37 protein concentration (Figure 31), implicating a strong relationship between IL-37 expression and reduction of inflammatory response in vivo. Since M-
CSF leads to enhanced macrophage phagocytosis, chemotaxis and production of certain cytokines including IL-6, IL-18 and TNFα [139], reducing its expression has wide-reaching effects on inflammation in vivo. The reduction of this key cytokine by IL-37 could help explain the observed decrease in lipid uptake as well as transmigration. The relationship between IL-37 expression and decreased M-CSF levels agrees with the observed decreased plaque formation in IL-37-tranduced BMT mice compared to controls (Figure 33 and Figure 34).

Other potent chemoattractant molecules, such as MCP-1, CXCL9, MIP-1α, MIP-1β, and KC were downregulated by macrophage IL-37 expression in vitro. mcp-1/Ldlr double knockout mice have 86% less lipid deposition and plaque formation in their aortas compared to control Ldl r+/− mice [140] emphasizing the athero-protective effects of reducing MCP-1 in vivo. CXCL9, also known as monokine induced by IFNγ (MIG), is one of the key chemoattractants that leads T-cells to the site of plaque development [141]. Macrophages expressing IL-37 show downregulation of CXCL9 protein secretion after AcLDL stimulation in vitro (Figure 15), implying a potential role of IL-37 in reducing macrophage-driven T-cell recruitment to the plaque in vivo.

The macrophage inflammatory proteins MIP-1α, MIP-1β, and MIP-2, also known as CCL3, CCL4, and CXCL2, are secreted in response to inflammatory stimulus and induce leukocyte chemotaxis. MIP-1α induces expression of ICAM [142], and acts as a chemoattractant for B-lymphocytes, natural killer (NK) cells [143], eosinophils [144] and activated CD8+ T-cells. MIP-1β is a chemoattractant for CD4+ T-cells [145], while MIP-2 is known to be important for neutrophil migration [146]. Preventing T-cell migration to the plaque is especially important
given that T-cells within the plaque are more reactive and inflammatory than circulating T-cells [147]. Both MIP-1 chemokines were downregulated by IL-37 expression in AcLDL-treated macrophages (Figure 15). In addition, there was a trend towards reduced MIP-1β (but not MIP-1α) transcript levels in the lymph nodes of IL-37 vs. control mice (Figure 32). Although no significant differences were found comparing circulating levels of MIP-1α, MIP-1β or MIP-2 in the serum of EGFP vs. IL-37 BMT mice (Figure 30), linear regression analysis of IL-37 and levels of each cytokine revealed a trend towards decreased MIP-1 and MIP-2 protein abundance with increasing IL-37 abundance (Figure 31). Downregulation of these chemokines is important for preventing leukocyte attraction to potential sites of atherosclerosis development, which could be a contributing mechanism concerning the athero-protective role of macrophage-expressed IL-37 in vivo.

Expression of various inflammatory cytokines, including IL-1α, IL-1β, IL-6, IL-12 and IL-18, all shown to be detrimental in the context of atherosclerosis, were reduced by macrophage IL-37 expression in vitro (Figure 15). IL-1α and IL-1β are central signaling mediators of the innate immune response expressed mainly by monocytes, macrophages and dendritic cells. IL-1 signaling is responsible for the production of reactive oxygen species and the expression of adhesion molecules such as ICAM and VCAM, ultimately resulting in the activation and recruitment of other immune cells to the site of inflammation [148]. It has been shown that within minutes of macrophage inflammatory activation, IL-37 expression closely follows that of IL-1α and IL-1β [99]. Interestingly, the gene regulatory regions of all three genes, found in the IL-1 locus on chromosome 2, come into close proximity following LPS stimulation, implying
co-regulation [149]. This programmed expression of IL-37 during the inflammatory response acts as a break on excessive inflammation. IL-37-induced reduction of IL-1α and IL-1β gene and protein expression (Figure 14 and Figure 15) provides strong support for its protective role against inflammation in vivo in the context of atherosclerosis.

Another central mediator of the innate immune response, IL-6, is associated with unstable angina in humans [150], and is known to stimulate production of matrix-degrading enzymes by macrophages, which increases the risk of atherosclerotic plaque rupture [151]. IL-37 expression in macrophages consistently reduced IL-6 transcript and protein expression both in vitro, as shown in Figure 14 and Figure 15 respectively, and also in vivo, as evidenced by reduced IL-6 transcript in lymph nodes isolated from IL-37 mice compared to controls (Figure 32). Taken together, this indicates that the reduction of IL-6 expression by IL-37 is likely a local effect and not systemic. Sera from EGFP or IL-37 mice were tested for IL-6 concentration with the Luminex assay, but the levels were below detection for both groups (data not shown) and potential differences between groups could not be determined.

Another important inflammatory cytokine, IL-12, known to be produced by macrophages during atherogenesis [152], was also downregulated by IL-37. Blockade of IL-12 in ApoE−/− mice leads to nearly 70% reduced atherosclerosis development [153], highlighting the therapeutic value in reducing its expression to prevent plaque development. IL-12α gene transcript levels were reduced in IL-37-transduced unstimulated BMDM, and the reduction was much more precipitous after stimulation with various inflammatory mediators (Figure 14). Lymph nodes
collected from EGFP or IL-37 study mice at sacrifice showed a trend towards reduced IL-12α transcript levels, but the change was not significant.

IL-18 expression was also reduced in IL-37-expressing macrophages on both the transcript and protein level. Known to be expressed by plaque macrophages in response to IFNγ [154], IL-18 is upregulated in unstable, symptomatic human plaques compared to stable plaques [155]. Mallat et al. show that transfection of the IL-18 inhibitor, IL-18bp, in vivo reduces atherosclerosis [156]. Since IL-37 is known to enhance the ability of IL-18bp to inhibit IL-18 signaling [100], the reduction of IL-18 production by IL-37-expressing macrophages (Figure 14 and Figure 15) further supports the role of IL-37 in reducing IL-18 inflammatory signaling, providing a stronger basis for the anti-atherogenic role of IL-37 in vivo.

An athero-protective role for macrophage-expressed IL-37 via reduction of the inflammatory response in vivo is supported by the results discussed above. The clear anti-inflammatory role of IL-37 in vitro, along with the reduction of IL-6 transcript in lymph nodes and reduced circulating M-CSF in IL-37 mice in vivo, all support the anti-inflammatory function of IL-37 that likely contributed to the reduction in atherosclerosis in mice expressing IL-37.

4.1.2 Macrophage cholesterol homeostasis

In our study, IL-37 expression in macrophages led to reduction of modified LDL uptake (Figure 17) shown by reduced Dil-labeled AcLDL and OxLDL content of the cells after 4 hours. Aside from the obvious benefit that this would have in reducing foam cell formation, it may have anti-inflammatory benefits as activated plaque macrophages become especially pathogenic when they take up modified LDL and become lipid-laden foam cells. It has been shown that TNFα, IL-
1β, MCP-1, ICAM-1, MMP3, and MIP-1α are upregulated in macrophages after treatment with VLDL [157], or modified lipoproteins via scavenger receptors [61], connecting cholesterol uptake to the inflammatory response and supporting the cycle of foam cell formation and the resulting inflammatory signaling found within the plaque. Macrophage uptake of modified LDL or cholesterol crystals within the plaque leads to activation of pattern recognition receptors (PRR) and Toll like receptors (TLR), enhancing the inflammatory response (Ref). CD36 recognizes modified lipoprotein and coordinates inflammasome formation in the context of sterile inflammation [158]. Macrophages expressing IL-37 express significantly less CD36 transcript (Figure 16), supporting the finding that IL-37 expression reduces lipid uptake in vitro. Interestingly, THP-1 macrophages treated with TGFβ show decreased mRNA and surface expression of CD36 [159], which is in accordance with the known role of TGFβ/Smad3 signaling upstream of IL-37.

Although IL-37 expression significantly downregulated CD36 expression, it had no effect on SR-AI or SR-AII genes. There was, however, a trend towards increased PPARγ gene expression in IL-37 macrophages compared to controls (Figure 16). PPARγ is a transcription factor that regulates macrophage lipid processing genes, and although it increases expression of CD36, it is also important for expression of genes involved in cholesterol efflux and genes responsible for limiting inflammation [160]. Ldlr<sup>−/−</sup> mice transplanted with PPARγ<sup>−/−</sup> bone marrow show increased atherosclerosis development, indicating its importance in the context of the disease. The strong tendency towards increased PPARγ expression by IL-37 indicates a protective regulation of macrophage lipid metabolism. Of note, ABCA1, responsible for
macrophage cholesterol efflux to the lipid-poor ApoA1 [161], is upregulated by IL-37 expression at the transcript level (Figure 16). Although this led to the expectation that cholesterol efflux from IL-37 expressing macrophages would be increased, no significant changes were observed in efflux capacity, only a slight trend towards more efflux (Figure 18). The reason for this could be due to the time point chosen to measure efflux (7 hours). Perhaps a longer efflux time is necessary, or the treatment conditions of the macrophages may play a role, given that the increase in gene expression of ABCA1 observed in unstimulated cells was no longer observed when macrophages were treated with AcLDL for 24 hours. It is also possible that there were less intracellular cholesterol to efflux to begin with in the IL-37 macrophages due to reduced uptake. Taken together the reduction of modified lipid uptake by IL-37 expression coupled with its well-established role in preventing inflammation, are likely the two key components of its protective mechanism against the development of atherosclerosis in vivo.

4.1.3 Macrophage transmigration

The observation that IL-37 expression reduces macrophage transmigration towards MCP-1 (Figure 19) corresponds with the reduction of inflammatory cytokines and chemoattractant molecules seen in IL-37-expressing macrophages (Figure 14 and Figure 15). The receptor for MCP-1, CCR2, is upregulated in atherosclerosis and other inflammatory conditions and responsible for the recruitment of monocytes/macrophages to the inflamed area. Since the expression of CCR2 is enhanced by inflammatory cytokines, IL-37 may be downregulating the expression of the receptor indirectly by inhibiting inflammatory cytokine production. Another mechanism by which migration is inhibited could involve IL-37-mediated decrease in
phosphorylation of various kinases and transcription factors as shown by Nold-Petry et al. to physically decrease the chemotactic response of the macrophages. IL-37 expression has been shown to decrease phosphorylation of mTOR, various MAP kinases, and NF-κB pathway members including IκBε, p65 and p105 [104], all of which are known to be involved in promotion of inflammatory and chemotactic gene expression [162-164]. Of note, the macrophage content of the aortic root plaque did not differ between EGFP and IL-37 BMT mice (Figure 35), indicating that the composition of the plaques were similar. However, the total plaque area was reduced in IL-37 BMT mice, indicating a general reduction of macrophage infiltration.

4.1.4 In vivo expression of IL-37 during atherogenesis

IL-37 has been studied in the context of many inflammatory diseases and conditions, yet its role in the chronic inflammatory disease of atherosclerosis has not yet been elucidated. Given the central role of macrophages in the initiation and development of atherosclerosis via inflammation and foam cell formation, I sought to determine whether macrophage-expressed IL-37 was protective against atherosclerosis development. The use of the CD68S macrophage-specific retroviral vector to express IL-37 in transduced HSC was successfully executed for all in vitro experiments as well as the in vivo BMT atherosclerosis study. Analysis of plaque formation in Ldlr<sup>−/−</sup> mice after BMT from IL-37- or EGFP-transduced HSC revealed a protective role for macrophage-expressed IL-37 in atherosclerosis, as evidenced by the decrease in plaque area of aortas by 34.3% as well as aortic root sections by 26.7% compared to EGFP controls (Figure 33 and Figure 34 respectively).
The expression of IL-37 and EGFP in vivo was sustained throughout the 4 weeks of recovery after BMT and the subsequent 10 weeks of HFD feeding, as evidenced in macrophages isolated from the bone marrow of IL-37 or EGFP mice at sacrifice (Figure 26). Although the goal of expressing IL-37 specifically in macrophages was to deliver the anti-inflammatory cytokine to the plaque microenvironment, the relatively high concentration of IL-37 protein in the sera of IL-37 mice at the 4-week and 14-week time points implicates a possible systemic role for IL-37 in reducing atherosclerosis as well. The protein was detected in both the dimer (~30kDa) and the uncleaved form (28kDa) in BMDM as well as in spleens isolated from IL-37 BMT mice (Figure 27, respectively), with very little detected in the mature, cleaved form. This could be due to the relatively small amount of IL-37 that is cleaved to act intracellularly within the nucleus compared to the large amount of cytokine produced and secreted from the cells, as shown by ELISA in Figure 21Figure 25.

Circulating IL-37 protein could have influenced the function of various other immune cells, thereby reducing the systemic inflammatory response, which may have contributed to the reduction in atherosclerosis development. The number of circulating monocytes, CD4+ and CD8+ T-cells, neutrophils, and B-cells between groups was not significantly different (Figure 24), although the inflammatory state or infiltration of the cells into the plaque could have been affected by IL-37 expression. The body weight, serum cholesterol and triglyceride levels were also not different between EGFP and IL-37 mice (Figure 23), indicating no effect of IL-37 on general metabolism or systemic cholesterol processing. Surprisingly, only one inflammatory cytokine, M-CSF, showed a significant negative correlation with IL-37 serum concentration
(Figure 31), with a strong trend towards lower concentration in IL-37 compared to EGFP mice overall (Figure 30). MIP-1α, MIP-2 and TNFα showed no difference between the groups, while seven other inflammatory cytokines tested were below detection for the assay, supporting a stronger local effect of IL-37 in preventing inflammation directly within the plaque. Alternatively, the low detection of circulating inflammatory cytokines could be due to the relatively short HFD period of 10 weeks compared to other advanced studies of 12-16 weeks. Even though the systemic reduction of inflammation was not pronounced, the reduction in M-CSF abundance likely played a valuable role in preventing the recruitment of monocytes/macrophages to the plaque and in turn reducing atherosclerosis development.

4.2 Limitations and future directions

The research described within this dissertation is the first to investigate the role of macrophage-expressed IL-37 in atherosclerosis. The expression of IL-37 specifically by monocytes/macrophages was successfully achieved in vivo and resulted in the suppression of atherosclerosis development in a mouse model of the disease. The conclusions drawn from this research must be evaluated with the consideration that many differences exist between the immune systems of the model used compared to humans. Although the human IL-37 gene was used for all experiments, the athero-protective effect of IL-37 observed within the mouse may not directly correlate with human atherosclerosis development. It is interesting that IL-37, which has no known mouse homolog, is so effective in reducing inflammation within the mouse. Nold-Petry et al. show that the extracellular function of IL-37 relies on the orphan receptor SIGIRR and IL-18Rα, using IL-37Tg mice crossed with SIGIRR−/− mice to show the signaling mechanism.
as well as its downstream effects [104]. This implies that another cytokine must exist within the mouse with functional overlap with IL-37. Future investigation into binding partners for SIGIRR and IL-18Rα within the mouse would help to elucidate any endogenous pathways used in the prevention of excessive inflammatory signaling.

IL-37 has been shown to be protective against inflammation in animal models, and has been strongly associated with many human diseases including colitis [120], psoriasis [116], rheumatoid arthritis [117], systemic lupus erythematosis [118], inflammatory bowel disease [119] and HIV infection [121]. IL-37 is elevated in patients with acute coronary syndrome [122], and reperfusion with recombinant IL-37 protein after ischemia has been shown to reduce damage and improve heart function in mice [123]. Additionally, IL-37 has been shown to suppress the adaptive inflammatory response of dendritic cells after induction of skin contact hypersensitivity [115], implicating broad therapeutic value due to its ability to quell both the innate and adaptive immune response.

It is now clear that treatment with IL-37 is a viable therapeutic option for reducing the chronic inflammation that drives the pathogenesis of atherosclerosis, although translation to treatment of human patients will need further investigation as to possible side-effects, dosing, stability of the protein, etc. The expression of IL-37 in macrophages in vivo is possible given the CD68S macrophage-specific retroviral vector used for this work, which actually contains the human CD68 promoter region. However, current limitations and safety concerns with using retroviral gene therapy in humans regarding the genomic location of transgene insertion remain an issue. Transient treatment with recombinant IL-37 protein may be viable for short-term
prevention of inflammation, which would be especially useful in the context of inflammatory diseases characterized by “flare ups”, such as rheumatoid arthritis, fibromyalgia, and inflammatory bowel disease. The long-term treatment of the chronic inflammation associated with atherosclerosis would require stable expression of IL-37, ideally at the site of plaque development. This may be possible in the future as specific methods of drug delivery, such as targeting inflamed endothelial cells with ICAM-1 expression, are gaining great interest and progress in the field is expanding rapidly [165]. It is possible that in the future, targeted delivery of IL-37 to inflamed endothelial sites of plaque formation will be a viable treatment in the prevention of atherosclerosis.
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