Lipid Accumulation in CD11c-expressing Intimal Myeloid Cells Induces Chemokine Production Required for Leukocyte Recruitment to Early Atherosclerotic Lesions

by

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Laboratory Medicine and Pathobiology
University of Toronto

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Abstract
Monocyte recruitment promotes the accumulation of myeloid foam cells in early atherosclerotic plaques. However, initial foam cells form prior to increased monocyte recruitment in hypercholesterolemic Ldlr−/− mice. These initial foam cells are derived from myeloid cells residing in the normal intima, and express integrin alphaX (CD11c). The goal of this thesis was to assess the role of initial foam cells in atherogenesis. The approach was to delete these cells by diphtheria toxin-induced apoptosis in Ldlr−/− bone marrow chimeras. Depletion of CD11c+ leukocytes resulted in significant reductions of intimal lipid accumulation, monocyte recruitment, intimal chemokine expression, but not endothelial cell adhesion molecule expression, at 10 and 21 days of hypercholesterolemia. These data suggest that lipid uptake by resident intimal CD11c-expressing myeloid cells during the earliest stages of atherosclerosis promotes chemokine production that is required for increased monocyte recruitment.
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List of Abbreviations

ADP – adenosine diphosphate
APC – allophycocyanin
ApoB – apolipoprotein B
ApoE – apolipoprotein E
Apoe-/- - apolipoprotein E deficient mice
ATA – aurin tricarboxylic acid
BrdU – 5-bromo-2’-deoxyuridine
BM- bone marrow
CCL2 – Chemokine (C-C) motif ligand 2 aka monocyte chemoattractant protein 1 (abbrev MCP1)
CCL3 – Chemokine (C-C) motif ligand 2 aka macrophage inflammatory protein 1α (abbrev MIP1α)
CCL4 – Chemokine (C-C) motif ligand 4 aka macrophage inflammatory protein 1β (abbrev MIP1β)
CCL5 – Chemokine (C-C) motif ligand 5 aka RANTES
CCL7 – Chemokine (C-C) motif ligand 7 aka monocyte chemoattractant protein 3 (abbrev MCP3)
CCL17 – Chemokine (C-C) motif ligand 17
CCR2 – CC chemokine receptor 2
CCR5 – CC chemokine receptor 5
CD11c – cluster of differentiation 11c, integrin alpha₅
CD36 – cluster of differentiation 36
CD45 – cluster of differentiation 45
CD68 – cluster of differentiation 68 aka macrosialin
CD80 – cluster of differentiation 80 aka B7-1
CD86 – cluster of differentiation 86 aka B7-2
CRD – cholesterol rich diet
CX3CL1 – chemokine (C-X3-C) motif ligand 1 aka fractalkine
CX3CR1- fractalkine receptor or Chemokine C-X3-C motif receptor 1
CXCL2 – Chemokine (C-X-C) motif ligand 2 aka macrophage inflammatory protein 2 (abbrev MIP2)
DC – dendritic cells
DTR – diphtheria toxin receptor
DTx – diphtheria toxin
EDTA – ethylenediaminetetraacetic acid
FBS – fetal bovine serum
FITC – fluorescein isothiocyanate
g/dL – grams per deciliter
GC – greater curvature of the ascending aortic arch
HDL – high density lipoprotein
hrs – hours
ICAM1- intercellular adhesion molecule 1
IDL – intermediate density lipoprotein particles
IFNγ - interferon gamma
IL-1β - interleukin 1 beta
IL-10 – interleukin 10
LC – lesser curvature of the ascending aortic arch
LDL – low density lipoprotein
LDLR – low density lipoprotein receptor
Ldlr-/- : low density lipoprotein receptor deficient mice
LFA1 – lymphocyte function-associated antigen 1 (αLβ2)
LPL – lipoprotein lipase
LPS – lipopolysaccharide
Ly6C – lymphocyte antigen 6C
M – molar
MDP – macrophage DC precursor
MHC I – major histocompatability complex I
MHC II – major histocompatability complex II
mL – milliliter
mM – millimolar
mm² - millimeters squared
mmLDL – minimally modified low density lipoprotein
mmol - millimole
mRNA – messenger ribonucleic acid
ng/g – nanogram per gram
nm – nanometer
oxLDL – oxidized low density lipoprotein
oxPAPC – oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocoline
PAMP – pathogen associated molecular pattern
PAPC – 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocoline
PBS – phosphate buffered saline
PFA – paraformaldehyde
PCR – polymerase chain reaction
PRR – pattern recognition receptor
PSGL1 – P-selectin glycoprotein ligand 1
rcf – relative centrifugal force
SCD – standard chow diet
SEM – standard error
SMase – sphingomyelinase
sPLA- secreted phospholipase A
TGFβ - transforming growth factor beta
TH1 – T helper 1
TH2 – T helper 2
TLR – toll-like receptor
TNFα - tumor necrosis factor alpha
Tregs – regulatory T cells
VCAM1 - vascular cell adhesion molecule 1
VE-cadherin – vascular endothelial cadherin
VLA4 – very late antigen 4 (α4β1 integrin)
VLDL – very low density lipoprotein particles
μg/mL – microgram per milliliter
μL – microliter
μm – micrometer
°C – degrees Celsius
1 Introduction

1.1 Cardiovascular Disease and Atherosclerosis

Cardiovascular disease refers to conditions or illnesses that affect the heart, arteries and veins. Due to its important role in blood circulation, it is not surprising that cardiovascular disease ranks amongst the top causes of mortality. Atherosclerosis is a chronic inflammatory disease that affects elastic and muscular arteries. This disease is characterized by plaques, which are focal accumulations of myeloid foam cells, smooth muscle cells and extracellular matrix in the artery wall. These atheromatous plaques can compromise vascular function and integrity and pose a life-threatening risk. In order to develop better therapies to control and prevent atherosclerosis, further research is necessary to better understand the mechanisms of this disease’s initiation and progression.

Atherosclerotic plaques form in the inner layer of the artery wall called the intima. Initially plaques begin as a collection of myeloid foam cells, called fatty streaks. They appear shortly after sustained hypercholesterolemia and are clinically asymptomatic. The fatty streak develops into a plaque as more foam cells accumulate. Over time as foam cells die, they leave behind an acellular lipid rich region encompassed by dying foam cells referred to as a necrotic core (Stary et al., 1994). Meanwhile, smooth muscle cells migrate into the intima and synthesize constituents of the fibrous cap such as collagen and other extracellular matrix proteins. Initially the artery outwardly remodels, permitting the plaque to grow without impinging on the lumen and compromising blood flow (Ward et al., 2000). Advanced plaques, characterized by large lipid rich necrotic cores and thinning fibrous caps, are prone to rupture because metalloproteinases produced by cells in the plaque, degrade the collagen in the fibrous cap (Shah and Galis, 2001). The thrombogenic substances in the plaque contents such as tissue factor, extracellular matrix and cellular debris from necrotic cells induce thrombosis of the blood when a plaque ruptures. If the thrombus is large enough it may occlude the lumen or embolize downstream, and the ensuing ischemia results in tissue damage.

The experiments by Anitschkow and Chalotow in the early 1900’s, demonstrated that solely feeding rabbits cholesterol purified from egg yolks was sufficient to induce atherosclerosis and
established a correlation between hypercholesterolemia and atherosclerosis. Epidemiological studies and intervention studies involving cholesterol lowering resins and eventually statins confirmed the positive correlation between increased risk of coronary heart disease and hypercholesterolemia (Steinberg, 2004). In the mid 1950’s it was proposed that cholesterol in a particular lipoprotein fraction was responsible for this increased risk of CHD (Gofman et al., 1950). Cholesterol is transported through the circulation in microscopic particles due to its hydrophobic nature. The main classes of cholesterol trafficking lipoprotein particles are chylomicrons, very low density lipoprotein particles (VLDL), intermediate density lipoprotein particles (IDL), low density lipoprotein particles (LDL) and high density lipoprotein particles (HDL) (Breslow, 1993). Dietary lipids and cholesterol are packaged into chylomicrons by the gut epithelium for transport to the liver. VLDL are produced by the liver and mainly distribute cholesterol and triglycerides from the liver to other tissues of the body. As the triglyceride content of VLDL is reduced by lipases, they become IDL. LDL are cholesterol enriched lipoprotein particles formed from further removal of triglycerides from IDL. HDL mediate return of excess cholesterol and lipids from peripheral tissues to the liver for excretion or to steroidogenic organs for the biosynthesis of vitamin D, and steroid hormones (Eckardstein et al., 2001). Each HDL is associated with apoA-I apolipoprotein and enriched in apoE apolipoprotein. VLDL and LDL are associated with a high atherogenic potential while atheroprotective properties have been implicated for HDL.
1.2 Inflammation and Monocyte Recruitment

Although the key permissive factor of atherosclerosis is an elevated level of LDL, we now know that inflammation plays an essential role in the pathogenesis of atherosclerosis (Libby, 2006). An inflammatory response parallels atherosclerotic plaque development, involving cytokines and cells of the immune system. It is a low-grade chronic inflammatory response, which is thought to be induced by the retention of LDL in the intima. Each LDL is associated with an ApoB apolipoprotein, which intrinsically has a high affinity for interacting with endothelial matrix proteoglycans (Boren et al., 1998; Skålén et al., 2002). Retained LDL become biochemically or enzymatically modified in order to induce foam cell formation (Steinberg, 1997). Modified LDL is endocytosed via macropinocytosis, LDL receptor (LDLR) mediated endocytosis or scavenger receptor mediated endocytosis. Macropinocytosis and scavenger receptor mediated endocytosis are more crucial than LDLR mediated endocytosis in the uptake of modified LDL since they are not negatively regulated by cholesterol content of the cell. Lipids and cholesterol esters in modified LDL are degraded by acid hydrolases when the late endosomes fuse with lysosomes. Consequently free cholesterol is converted to cholesterol esters in the endoplasmic reticulum and stored in lipid droplets, imparting the foamy appearance to lipid loaded myeloid cells (Simons, 2000).

Early in vitro experiments demonstrated that LDL must be modified by endothelial cells in order to induce foam cell formation (Steinbrecher et al., 1984). It was later discovered that a mildly oxidized form of LDL, minimally modified LDL (mmLDL) had the same properties as endothelial cell modified LDL. Furthermore mmLDL enhanced monocyte adhesion to the endothelial cell monolayer (Berliner et al., 1990; Cushing et al., 1990; Navab et al., 1991). Additionally, the biologically active component of mmLDL was determined to exist in the lipid component of mmLDL (Quinn et al., 1987). 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (PAPC), a phospholipid commonly found in LDL can be oxidized (Watson, 1997; 1999; Watson et al., 1995) and induce endothelial activation and monocyte adhesion (Furnkranz et al., 2005). Data from in vivo studies reveal that auto-antibodies to oxidized LDL (oxLDL) develop in hypercholesterolemic patients and experimental animals. Genetically modified mice that lack expression of scavenger receptors or enzymes that oxidize unsaturated fatty acids display reduced lesion development (Berliner and Watson, 2005). Although the previously mentioned evidence strongly supports oxidative modification of LDL as an etiological factor of
atherosclerosis, the extent to which lipids or LDL is oxidized in the artery wall and its physiological relevance remains to be determined.

Monocytes differentiate into macrophages and dendritic cells that take up modified LDL in the intima (Rajavashisth et al., 1998; Smith et al., 1995). Monocyte recruitment is an essential process in atherosclerotic plaque formation. In LDL receptor deficient (Ldlr^{-/-}) mice, monocyte recruitment to the artery wall is elevated shortly after foam cells of the fatty streak appear (Zhu et al., 2009). There are two subsets of monocytes characterized by different expression of surface markers and chemokine receptors. Ly6C^{hi} monocytes express high levels of CCR2 and low levels of CX3CR1. Ly6C^{lo} monocytes express low levels of CCR2 but high levels of CX3CR1. In mice the two subsets occur at a 1:1 ratio in the circulation at steady state (Murray and Wynn, 2011). Sustained hypercholesterolemia specifically increased Ly6C^{hi} monocytes in the circulation and their recruitment to atherosclerotic plaques (Swirski et al., 2007). The increase in recruitment of Ly6C^{hi} monocytes has been shown to be correlated with their levels in the circulation (Combadiere et al., 2008).

Adhesion of monocytes to endothelial cells lining the vasculature requires cell to cell contact mediated by endothelial cell adhesion molecules and their cognate binding partners on monocytes. Endothelial selectins bind to glycosylated ligands such as P-selectin glycoprotein ligand 1 (PSGL1) and mediate capture and rolling adhesion of monocytes. Vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) bind to heterodimeric integrins such as \( \alpha_4\beta_1 \) (VLA-4 very-late antigen 4) and \( \alpha_4\beta_2 \) (LFA-1 lymphocyte function-associated antigen 1) respectively and mediate rolling adhesion, firm adhesion and arrest (Ley et al., 2007). After monocytes arrest on the endothelial cells lining the arteries, they transmigrate between endothelial cells into the subendothelial space. Adhesion molecule expression has been reported in plaques isolated from humans (Davies et al., 1993), rabbits (Li et al., 1993), and mice (Iiyama et al., 1999; Nakashima et al., 1994). Furthermore the importance of adhesion molecules in leukocyte recruitment and plaque development is supported by experiments where deficiency of adhesion molecules in atherosclerosis susceptible mice resulted in decreased lipid accumulation and macrophage content in the aorta (Collins et al., 2000; Cybulsky et al., 2001; Dansky et al., 2001).
Monocytes must be activated by chemokines in order to firmly adhere/arrest on the artery wall. Chemokines and chemoattractants bind to G-protein coupled receptors on monocytes and increase the integrin’s affinity resulting in stronger interactions. Chemokines produced by endothelial cells, recruited leukocytes, foam cells, smooth muscle cells and platelets are presented on the luminal surface of endothelial cells by binding to glucosaminoglycans (GAGs) (Ley et al., 2007). Ly6C^hi monocytes express chemokine receptors CCR2, CCR5 and low levels of CX3CR1 (Randolph, 2009). Interruption of one or more of these chemokine receptors interferes with monocyte recruitment to lesions, reducing lipid and myeloid cell accumulation in atherosclerosis susceptible regions. Blockade of CCR2 signaling either by knocking out the expression of the receptor or its ligands such as CCL2 reduced plaque formation in atherosclerosis susceptible mice (Gosling et al., 1999; Gu et al., 1998). Similarly inhibition of CCR5 reduces monocyte recruitment and lipid accumulation (Tacke et al., 2007; Veillard, 2004). Lastly, deficiency in either fractalkine or its receptor CX3CR1 also reduced atherosclerotic plaque growth in mice (Combadiere, 2003; Teupser et al., 2004). However, this may not be due to decreased monocyte recruitment, since CX3CR1 provides a survival signal to monocytes and macrophages (Landsman et al., 2009).

The characteristic and reproducible pattern with which atherosclerotic plaques form in the vasculature has motivated several groups to investigate what other factors contribute to plaque formation other than hypercholesterolemia. Plaques form specifically at branch ostia, outer wall of arterial bifurcations and inner wall of curvatures. These sites are considered to be prone or susceptible to atherosclerosis while the other regions are protected or resistant. The difference in hemodynamic forces exerted by the flow of blood through these regions are responsible for this dichotomy (Traub and Berk, 1998). Due to the vascular architecture, endothelial cells in atherosclerosis susceptible regions experience “disturbed” laminar low time-averaged blood flow while endothelial cells lining linear portions of the vasculature experience uniform laminar high time-averaged blood flow. One study demonstrated that “atheroprotective” flow was able to block IL-1β induced VCAM-1 expression in endothelial cells in vitro (Dai et al., 2004). In another study, reducing the rate of blood flow made normally resistant regions of the aorta susceptible to plaque formation (Zhou et al., 2010).

The endothelial cells in atherosclerosis susceptible regions exhibit a different phenotype than endothelium found in atherosclerosis resistant regions of the vasculature. Endothelial cells in
atherosclerosis susceptible regions are polygonal and demonstrate a dysfunctional phenotype (Liao, 2013). In the lesser curvature of the ascending aortic arch, where lesions form the endothelium express adhesion molecules (Jongstra-Bilen, 2006) and NF-κB family members (Hajra et al., 2000) at a higher level than endothelial cells in the greater curvature. This dysfunctional phenotype has been associated with a lower production of nitric oxide (Won et al., 2007) which has anti-inflammatory properties (De Caterina et al., 1995). Currently the paradigm of plaque formation recognizes that hemodynamic disturbances predispose regions of the vasculature to inflammation induced by hypercholesterolemia.
1.3 Resident Intimal Leukocytes

Leukocytes reside in the intima of atherosclerosis susceptible regions. Since the first report of intimal leukocytes in atherosclerotic plaques and non-diseased arteries in humans (Bobryshev and Lord, 1995) and animal models (Ozmen et al., 2002), they have been better characterized and continue to be studied. The majority of intimal leukocytes in the aorta express integrin $\alpha_X$ (CD11c), myeloid marker: macrosialin (CD68), co-stimulatory molecules: CD80 and CD86, and antigen presentation molecules: MHCII and CD1 (Choi et al., 2009; Jongstra-Bilen, 2006). The morphology of the leukocytes and the pattern of cell marker expression are characteristic of dendritic cells (DCs). Data in published studies suggest that this population of CD11c$^+$ intimal leukocytes are derived from monocytes or at least a circulating precursor and not maintained independent of hematopoietic progenitors in the bone marrow. CD11c$^+$ intimal leukocytes isolated from the aorta demonstrated DC capabilities such as efficiently stimulating naïve T-lymphocytes and cross-antigen presentation (Choi et al., 2009).

DCs specialize in antigen presentation and they are instrumental in activating the adaptive arm of the immune system. The classical DC activation paradigm proposes DCs in the periphery are immature and survey the environment for pathogenic antigens (Banchereau et al., 2000). Upon detecting a pathogen associated molecular pattern (PAMP) via a pattern recognition receptor (PRR) such as a toll-like receptor (TLR), the DC becomes activated, and migrates to the draining lymph node for antigen presentation. During the journey to the lymph node, the DCs mature and increase its expression of co-stimulatory molecules (CD80 and CD86) as well as decorate their cell surface with antigen presentation molecules loaded with peptides from the antigen.

Conventionally, exogenous antigens are presented by MHC class II molecules and recognized by CD4$^+$ T lymphocytes, while MHC class I molecules are loaded with endogenous/self antigens and recognized by CD8$^+$ T lymphocytes (Janeway et al, 2005). It is uncertain if this series of events occur in atherosclerosis, since intimal foam cells are unable to migrate to lymph nodes efficiently and remain in the intima instead (van Gils et al., 2012).

Evidence that an adaptive immune response contributes to the pathogenesis of atherosclerosis is growing. Although T lymphocytes in atherosclerotic plaques are rare, they have been detected. CD4$^+$ T lymphocytes interacting with lipid-loaded foam cells in aortic plaques have also been observed by two separate groups (Koltsova et al., 2012; Weber et al., 2011). Furthermore, a T$_{H1}$
polarized immune response has been associated with atherosclerosis (Gupta et al., 1997; Laurat et al., 2001). The adaptive immune system is unique in the fact that it can learn and has immunological memory of foreign antigens, which is how vaccines work. Vaccination with modified forms of LDL has been proposed as a prophylaxis. One study demonstrated that adoptively transferring bone marrow-derived DCs activated with LPS and pulsed with copper-oxidized LDL into Ldlr−/− mice reduced lipid accumulation due to increased T and B lymphocyte responses specific to oxLDL (Habets et al., 2010).
1.4 Mouse models

1.4.1 Mouse models of Atherosclerosis

Genetically modified inbred strains of mice have become a valuable tool to study atherosclerosis in vivo. Two of the most common atherosclerosis susceptible mouse models are LDL receptor knockout mice and ApoE lipoprotein deficient (ApoE-/-) mice. Ldlr-/- mice express a truncated receptor that is unable to clear LDL from the plasma. As a result their serum cholesterol levels are moderately increased (~200mg/dL) (Veniant et al., 2001) and increase specifically in the LDL fraction of lipoprotein particles. Apoe-/- mice demonstrate a marked increase in serum cholesterol (~450 mg/dL) much greater than Ldlr-/- mice and increase in VLDL fraction of lipoproteins specifically. Ldlr-/- mice do not develop atherosclerotic lesions spontaneously like ApoE-/- mice on a low fat standard chow diet. Instead they must be fed a cholesterol rich diet. This difference between Ldlr-/- and Apoe-/- mice is likely due to the important role of ApoE in VLDL and chylomicron remnant clearance from the plasma by a receptor other than LDLR, often referred to as the remnant receptor (Mahley, 1988). Furthermore ApoE produced by macrophages has been shown to be important for chylomicron remnant and VLDL clearance (Fazio et al., 1997; Linton et al., 1995).

Both Apoe-/- and Ldlr-/- model human atherosclerosis well as they develop lesions ranging from foam cell lesions to advance plaques containing necrotic cores and fibrous caps. The only exception is that neither model recapitulates the final stage of human disease, which is plaque rupture. Despite this fact, target gene knockout, replacement and transgene expression in these atherosclerosis susceptible mice has helped define the genetic determinants that contribute to atherosclerotic plaque formation.

1.4.2 Transgenic CD11c-DTR mice

Diphtheria toxin (DTx) mediates non-inflammatory depletion of target cells. DTx is a heterodimer composed of two subunits, A and B, which are joined by a disulfide bond. DTx enters the cell by receptor-mediated endocytosis, via diphtheria toxin receptor (DTR). In late endocytic vesicles, the acidic environment created by fusion of the lysosome induces a confirmation change in subunit B and it inserts into the membrane to form a pore. Subsequently subunit A translocates to the cytoplasm through this pore. Subunit A is an enzyme that mediates
the ADP ribosylation of eukaryotic elongation factor 2 and effectively inhibits protein synthesis and induces apoptosis via a caspase activation cascade.

Mice are normally resistant to DTx due to a three amino acid difference in the DTR, which reduces the receptor’s affinity for the toxin. Transgenic CD11c-DTR mice allow for the specific deletion of CD11c high expressing cells. This mouse has been genetically modified to express the simian diphtheria toxin receptor (aka heparin binding EGF like growth factor receptor) as a transgene specifically in cells that also express CD11c (Jung et al., 2002). Although CD11c has been used as a marker for DCs in the spleen, other cell types such as monocytes, T cells and resident macrophages have also been reported to express this integrin. One injection of 4 ng/g body weight of DTx is sufficient to deplete >98% of the CD11c+ cells in the aorta. Repopulation of the lesser curvature takes approximately 3 weeks (Paulson et al., 2010). It has been reported that macrophages in the marginal zone of the spleen and sinusoidal lymph node are also deleted (Probst et al., 2005) but not lung macrophages (Hashimoto et al., 2013).
1.5  Rationale, Hypothesis and Approach

Our lab is interested in studying the role of CD11c+ intimal myeloid cells in early atherosclerosis. Paulson et al, (2010) demonstrated these intimal leukocytes promptly ingest retained LDL and became the first foam cells of the fatty streak in the aortic arch of Ldlr−/− mice, detected as early as 5 days on a cholesterol rich diet (CRD), which is prior to monocyte recruitment (Zhu et al., 2009). These CD11c+ intimal leukocytes are required for fatty streak formation and when they are deleted, lipid accumulation in the intima is dramatically reduced. It still remains to be determined how they contribute to the plaque development process. Are CD11c-expressing intimal leukocytes pro-atherogenic or do they mediate a protective response such as sequestering modified LDL to avert endothelial activation?

Currently the data from studies investigating the role of CD11c+ intimal leukocytes in developed plaques have been contradictory. Both protective (Choi et al., 2011; Subramanian et al., 2012) and pro-inflammatory (Weber et al., 2011) roles have been suggested for subsets of CD11c+ leukocytes in plaques. All of the studies demonstrated a link to FoxP3+ regulatory T-cells (Tregs). Tregs are responsible for restricting both TH1 and TH2 responses by producing anti-inflammatory cytokines such as IL-10 and TFGβ, which limit plaque development in atherosclerosis susceptible mice (Ait-Oufella et al., 2006).

We hypothesize that CD11c+ intimal myeloid cells take up LDL and become foam cells, promote monocyte recruitment to the lesser curvature and contribute to lesion expansion by secreting proinflammatory cytokines and chemokines. We expect that the deletion of intimal CD11c+ leukocytes should decrease monocyte recruitment in Ldlr−/− mice fed a CRD.

The aims of my project are:

1) to determine whether monocyte recruitment to the lesser curvature of the aortic arch in Ldlr−/− BM chimeras fed a CRD is affected by lipid accumulation in CD11c+ intimal leukocytes.

2) to determine whether decreasing intimal lipid accumulation and CD11c+ leukocytes affects adhesion molecule and chemokine mRNA expression in the lesser curvature during nascent lesion formation.
2 Methods

2.1 Mice

Male and female C57BL/6, Ldlr⁻/⁻, and Tg CD11c-DTR-EGFP strains of mice were used at ages between 6 weeks and 12 weeks old (Paulson et al., 2010). Tg CD11c-DTR-EGFP mice were kept in the hemizygous state, and newborns were genotyped by PCR using the following primer set to EGFP: forward 5’-GGGACCATGAAGCTGCTGCCG-3'; reverse 5’-TCAGTGGGAATTAGTCATGCC-3’. Mouse colonies were maintained in a pathogen-free environment at the University Health Network animal facility at TMDT with 12h light-dark cycles. Mice were fed a standard rodent chow. A CRD consisting of 1.25% cholesterol and 40% kcal fat (Lichtman et al., 1999) (Research Diets, diet D12108) was used to initiate atherosclerotic lesion formation in Ldlr⁻/⁻ BM chimeras. All protocols were performed in adherence to the guidelines of the Canadian Council of Animal Care.

2.2 Bone marrow cell isolation

Both femurs and tibias were isolated from donor mice (Tg CD11c-DTR-EGFP or C57BL6/J) and flushed with 10 mL of RPMI containing 10% FBS and 1% Pen/Strep. The bone marrow (BM) cell suspension was filtered through 70 µm pore filter to remove debris. 10 µL of filtered BM cell suspension was added to 90 µL of 1% crystal violet and counted. BM cell suspension was pelleted by centrifuging at 500 rcf for 10 minutes. The cell pellet was resuspended in RPMI to generate a 25 million cells/mL suspension.

2.3 Bone Marrow Reconstitution

6 week old Ldlr⁻/⁻ mice were exposed to 10 grays of gamma irradiation with a gamma irradiator using a cesium 137 radioactive source (CS137 decays by beta emission to a meta stable nuclear isomer, Ba 137: Ba-137m, Ba-137m is responsible for all the emission of gamma rays). 5 million BM cells were injected intravenously though tail vein one day after irradiation to reconstitute hematopoietic cells. Recipients were allowed to recover for six weeks.

2.4 Deletion of CD11c⁺ cells

After the six weeks recovery period, Ldlr⁻/⁻ BM chimeras received 4 ng of DTx per gram of body weight in PBS intravenously on the first day and every three days afterwards until day 6 and
mice were analyzed at ten days. For mice analyzed at 21 days, DTx injections were continued every three days until day 18.

2.5 BrdU Pulse Labeling

2 mg of BrdU in 200 μL of PBS was injected intravenously to pulse label cells undergoing cell division.

2.6 Isolation of aortic arch and plasma collection

At the end of the 10 and 21 days diet, mice were anaesthetized with isofluorane. 400 μL of blood was collected by cardiac puncture and 20 μL of 0.5M EDTA was added to prevent coagulation. Blood was centrifuged at 4°C, for 15 minutes at 370 ref. Plasma was collected to determine total plasma cholesterol and triglyceride levels. Aortas were perfused through the left ventricle with PBS to wash out remaining blood and then perfused with 2% PFA in PBS to fix tissues. The heart, thymus and aorta were separated from the chest cavity. Then the ascending aortic arch, the segment from the aortic sinus to the first branch (innominate artery) was isolated under a dissecting microscope (SMZ-U; Nikon). Surrounding adipose tissues were cut away from the aorta.

2.7 Staining Ascending Aortic Arch

Fixation of aortic arches was continued for 20 more minutes in 2% PFA after isolation. Afterwards, aortic arches were permeabilized in 0.5% Triton X-100 in PBS for 15 minutes. Endogenous catalases and peroxidases were quenched with 3% hydrogen peroxide in PBS. TSA™ Fluorescein System –Tyramide Signal Amplification kit (Perkin Elmer) was used for immunostaining. Modifications to the staining protocol were made. The aortic arch was blocked with blocking buffer (supplied by Perkin Elmer) supplemented with 10 μg/mL non-immune mouse IgG and 10 μg/mL rat IgG (Sigma Aldrich). Primary antibodies used included Alexa647 conjugated anti-CD45 (2 μg/mL clone 30-F11) and biotin conjugated anti-BrdU (4 μg/mL clone Bu20a). Streptavidin conjugated horseradish peroxidase was used for secondary with FITC conjugated tyramide reagent (supplied by Perkin Elmer kit). Nuclei were stained with 10 μg/mL HOECHST 33342 (Invitrogen). Lipid was stained with neutral lipid stain Nile Red 5 μg/mL (Sigma).
2.8 Confocal Microscopy

En face micrographs of the lesser curvature were obtained with an Olympus Fluoview 1000 confocal microscope outfitted with 405, 488, 543 and 633 nm lasers, and Olympus FV10-ASW acquisition software. Objective lenses included 4x (NA 0.13), and 40x oil (NA 1.3). Overlapping micrographs were taken spanning the entire lesser curvature and later compiled in Adobe Photoshop software, version CS3. BrdU positive nuclei of CD45\(^+\) leukocytes were counted. Nile Red positive area was determined as percentage of total aorta, with Image J software version 1.46a.

2.9 Isolating intimal mRNA from ascending aortic arch

The heart and aorta were transferred to a dissecting dish with cold 1 mM aurin tricarboxylic acid (ATA) solution in PBS to isolate the ascending aortic arch (from the root to the innominate artery) with the aid of a dissecting scope. The aortic arch was separated into the lesser curvature and greater curvature and pinned down. Both curvatures were incubated at 37°C for 2 minutes with 100 \(\mu\)L of 25 \(\mu\)g/mL of Liberase™ research grade (Roche) to digest extracellular matrix and loosen endothelial cells. Digestion was terminated by rinsing with 1 mM ATA, and then 2 \(\mu\)L of 0.1 \(\mu\)m FITC beads suspension (Polysciences) was added to assist in visualizing the endothelial cell surface during intimal cell harvesting and collection. Tissue collected from the intima was directly transferred to lysis buffer (RLT) of RNeasy micro RNA extraction kit (Qiagen). mRNA isolation proceeded as outlined in the protocol of the kit.

2.10 Real-time PCR

Isolated mRNA was reversed transcribed with High Capacity cDNA reverse transcription kit (Invitrogen). mRNA levels were quantified with Roche Lightcycler 480 using Lightcycler 480 SYBR Green I master mix. The cycle settings used were: 95°C for 5 minutes, followed by 45 cycles of 95°C for 10 seconds, 60°C for 20 seconds and 72°C for 30 seconds. Melt curve analysis was carried out at the end of 45 cycles to determine product purity. Standard curves were generated for each primer pair to determine primer efficiency (Table 1) with 10 fold serial dilution of reference mRNA prepared from heart, lung and liver or lymph node of C57BL/6 mouse injected with 10 \(\mu\)g of LPS. All mRNA expression values were normalized to VE-Cadherin.
2.11 Flow cytometric analysis of circulating monocytes in Ldlr-/-BM chimeras.

Monocyte levels in the blood were determined at the time of sacrifice in chimeras. Blood was obtained by cardiac puncture and 50 μL was analyzed. Blood cells were washed with 450 μL of staining media (PBS + 2% FBS + 2 mM EDTA). Cells were incubated on ice with 100 μL of antibody solution for 30 minutes. Primary antibodies include PE conjugated anti-CD115 (1 μg/mL, clone AFS98), APC conjugated anti-Ly6C (1 μg/mL, clone HK1.4) and FITC conjugated CD11b (1 μg/mL, clone M1/70). Red blood cells were lysed following incubation with antibodies, with a solution containing 0.826 g/dL ammonium chloride + 0.1 g/dL sodium bicarbonate + 26 µM EDTA. Cytometry was carried out on Beckman Coulter FC500 flow cytometer outfitted with 488 nm and 635 nm lasers and tetraCXP software for data acquisition. Data analysis was carried out on FlowJo v10.

2.12 Statistics

Statistical analysis was conducted using Prism software version 4.03. Statistical tests were performed using one-way analysis of variance (ANOVA) with all pairs Tukey-Kramer post-test analysis or two-tailed Student’s t-test.
Table 1: Real-time PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>VE-Cadherin</td>
<td>Forward</td>
<td>GAAAACCAGAGGAAACCGCTGAT</td>
<td>95.68%</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CACTGGTCTTGGCAGATGGA</td>
<td></td>
</tr>
<tr>
<td>E-selectin</td>
<td>Forward</td>
<td>GAACCAAGAAGTCCGGGATGTA</td>
<td>91.05%</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ATGACCAGTGGCAGATGCA</td>
<td></td>
</tr>
<tr>
<td>Icam1</td>
<td>Forward</td>
<td>CTGCCTTGGTACAGGGATTGACTG</td>
<td>97.89%</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AGGACAGGAGCTGAAAAGTTGTAGA</td>
<td></td>
</tr>
<tr>
<td>Vcam1</td>
<td>Forward</td>
<td>GCACAAAGAAGGCTTTGAAGCA</td>
<td>98.84%</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GATTTGAGCAAACGTTTTGTATTAGAG</td>
<td></td>
</tr>
<tr>
<td>Cd45</td>
<td>Forward</td>
<td>TCCACGGGTATTCAGCAAGTT</td>
<td>93.99%</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ACACGTTTATAATCATAGGGAAGAATG</td>
<td></td>
</tr>
<tr>
<td>Cx3cl1</td>
<td>Forward</td>
<td>CAGTGGCTTTGCTCAGTGCA</td>
<td>96.69%</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>AGCCTGGTGATCCAGTGCTC</td>
<td></td>
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<tr>
<td>Ccl3</td>
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<td></td>
<td>Reverse</td>
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<td></td>
</tr>
<tr>
<td>Ccl4</td>
<td>Forward</td>
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<td>96.03%</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>CTGTCTGCTTCTTTTGTCAGG</td>
<td></td>
</tr>
<tr>
<td>Ccl5</td>
<td>Forward</td>
<td>CCTGCTGCTTTGCTCCTACCTTCTC</td>
<td>91.75%</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>ACACACTCGGCGGTCTTCTTGA</td>
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</table>

Table 1: Primer pairs were designed to target two separate exons spanned by an intron for the indicated genes according to mRNA sequences. Efficiencies were determined by serial dilution of mRNA isolated from heart, lung and lymph node of C57BL6 mice stimulated with 10 μg of LPS.
3 Results

In order to study the role of intimal leukocytes in atherogenesis, CD11c-expressing cells were deleted in Ldlr−/− mice. Previously, Paulson et al bred the transgenic CD11c-DTR mice with Ldlr−/− mice and demonstrated that lipid accumulation is reduced when CD11c+ cells are deleted at the initiation of CRD and analyzed 5 days afterward. In mouse models of atherosclerosis, the duration of hypercholesterolemia is directly proportional to the extent of inflammation. Therefore, our goal was to maintain a CRD for longer than 5 days. In order to maintain a low number of CD11c-expressing leukocytes in the aorta during this period multiple DTx administrations would be necessary. However, multiple DTx injections are toxic to transgenic CD11c-DTR mice due to the low level of non-specific transgene expression in non-hematopoietic tissues (Bennett and Clausen, 2007). Therefore, bone marrow chimeras were generated. Ldlr−/− mice were reconstituted with bone marrow from transgenic CD11c-DTR mice. In these Ldlr−/− CD11c-DTR BM chimeras, DTx sensitivity is observed only in bone marrow derived cells that express CD11c and chimeric mice tolerate multiple DTx injections.

3.1 CD11c-expressing leukocytes in the ascending aortic arch are reduced in DTx-sensitive Ldlr−/− BM chimeras

The extent of intimal leukocyte depletion by DTx was determined at the end of 10 or 21 days of CRD or standard chow diet (SCD). Intimal leukocytes in the ascending aortic arch were immunostained with an antibody to CD45, a pan-leukocyte marker, imaged by en face confocal microscopy and counted in both Ldlr−/− BM chimeras (Fig 1B and 1E). A five-fold reduction in intimal leukocyte population was observed in Ldlr−/− CD11c-DTR BM chimeras compared to wild type BM chimeras fed a SCD or CRD for 10 days (Fig 1C). Due to the difficulty of discerning individual CD45 positive cells after 21 days of CRD, the extent of intimal leukocyte reduction was reported in terms of the area positive for CD45 and expressed as a percent of total ascending aorta up to the left carotid artery, opened in a reproducible fashion (Fig 1F). There was a 3.5-fold reduction in CD45 positive area in Ldlr−/− CD11c-DTR BM chimeras compared to wild type BM chimeras fed a CRD for 21 days.
**Figure 1: Intimal leukocytes were reduced in the ascending aortic arch of Ldlr⁻/⁻ CD11c-DTR bone marrow chimeras.**

Intimal leukocytes in the ascending aortic arch were quantified in Ldlr⁻/⁻ BM chimeras. A) schematic of the 10 days experimental timeline. B) representative confocal micrographs of the lesser curvature of the aortic arch of CD11c-DTR or wild type BM chimeras on a CRD for 10 days. Scale Bars: 100 μm; magnification: 40x; colours: green - CD45; blue – Hoechst. C) CD45 positive cells were counted in the ascending aortic arch of Ldlr⁻/⁻ CD11c-DTR BM chimeras fed either a SCD (n=3) or CRD (n=5) for ten days and Ldlr⁻/⁻ wild type BM chimeras fed either a SCD (n=3) or CRD (n=4) for ten days. Values represent mean ± SEM of total CD45⁺ cells in the ascending aortic arch. Significant differences between CD11c-DTR vs. wild type BM chimeras are indicated: ** p<0.01, ***p<0.001; unpaired, two tailed Student’s t-test. D) schematic of the 21 days experimental timeline. E) representative confocal micrographs of CD45 immunostained cells in the lesser curvature of the aortic arch of CD11c-DTR and wild type BM chimeras on a CRD for 21 days. Scale Bars: 100 μm; magnification: 40x; colours: green - CD45; blue – Hoechst. F) CD45 positive area was quantified in the ascending aortic arch of CD11c-DTR (n=10) and wild type (n=14) BM chimeras fed a CRD for 21 days. Values represent mean ± SEM of CD45⁺ area expressed as a percent of ascending aortic arch. Significant differences between CD11c-DTR vs. wild type BM chimeras fed a CRD are indicated: ** p<0.01; unpaired, two tailed Student’s t-test.
A) 10 days Cholesterol Rich Diet

B) 10 Days Cholesterol Rich Diet

Wild type BM (control group)

CD11c-DTR BM (depleted group)

C) Total CD45+ cell count

Bone marrow genotype

Wild type  CD11c-DTR

D) 21 days Cholesterol Rich Diet

E) 21 Days Cholesterol Rich Diet

Wild type BM (control group)

CD11c-DTR BM (depleted group)

F) CD45+ area (%)

Wild type  CD11c-DTR

21 day CRD

***  **
3.2 Deletion of CD11c-expressing leukocytes reduced lipid accumulation in the ascending aortic arch

The effect of depleting CD11c+ leukocytes on lipid accumulation in the aortic arch of Ldlr−/− BM chimeras was assessed after 10 and 21 days on a CRD. Ascending aortic arches were stained with Nile red, a neutral lipid stain, imaged en face by confocal microscopy, and the Nile red positive area quantified with ImageJ. There was significantly less intimal lipid accumulated in the aortic arch of Ldlr−/− CD11c-DTR BM chimeras (depleted of CD11c+ leukocytes) compared to wild type BM chimeras (Fig 2A and 3A). The mean Nile red area in the wild type BM chimeras after 10 days CRD was 5-fold higher than CD11c-DTR BM chimeras (Fig 2B). Similarly after 21 days CRD, there was a 3-fold reduction in intimal lipid accumulation in CD11c-DTR BM chimeras compared to wild type BM chimeras (Fig 3B). Furthermore, lipid accumulation in the ascending aortic arch increased from 10 to 21 days in both Ldlr−/− BM chimeras. These data confirm previous observations by our laboratory (Paulson et al., 2010). Total plasma cholesterol and triglycerides were measured from blood of Ldlr−/− BM chimeras obtained at the time of sacrifice, after 10 and 21 days CRD. BM chimeras depleted of CD11c-expressing leukocytes demonstrated higher total serum cholesterol and triglyceride levels than non-depleted wild type BM chimeras; the differences were not statistically significant (Table 2). This effect was previously described in Ldlr−/− CD11c-DTR BM chimeras (Gautier et al., 2009a). It is likely that CD11c-expressing myeloid cells play a role in LDL clearance since osteopetrotic ApoE−/− mice, who lack myeloid cells due to a deficiency in macrophage colony stimulating factor (CSF1) also have a higher serum cholesterol level compared to Csf1+/+Apoe−/− mice (Smith et al., 1995).
### Table 2: Plasma Biochemistry

<table>
<thead>
<tr>
<th></th>
<th>Wild type</th>
<th>CD11c-DTR</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>10 days</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Cholesterol (mmol/L)</td>
<td>14.89 ± 1.239</td>
<td>20.18 ± 2.607</td>
<td>0.08</td>
</tr>
<tr>
<td>Total Triglyceride (mmol/L)</td>
<td>1.973 ± 0.2113</td>
<td>2.352 ± 0.2590</td>
<td>0.28</td>
</tr>
<tr>
<td>n</td>
<td>7</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td><strong>21 days</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Cholesterol (mmol/L)</td>
<td>14.05 ± 1.902</td>
<td>15.88 ± 1.115</td>
<td>0.43</td>
</tr>
<tr>
<td>Total Triglyceride (mmol/L)</td>
<td>1.637 ± 0.2084</td>
<td>2.098 ± 0.1785</td>
<td>0.12</td>
</tr>
<tr>
<td>n</td>
<td>6</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Total plasma cholesterol and triglycerides of Ldlr⁻/⁻ BM chimeras at the end of 10 and 21 days of CRD. Values are given as mean ± SEM. Unpaired two tailed Student’s t-test.
Figure 2: Intimal lipid accumulation is reduced by depletion of CD11c-expressing leukocytes in Ldlr−/− CD11c-DTR BM chimeras after 10 days on a CRD.

Nile red stained area was determined in aortic arch of Ldlr−/− BM chimeras fed a CRD for 10 days. A) representative composite of confocal micrographs of the lesser curvature of Ldlr−/− wild type and CD11c-DTR BM chimeras fed a CRD for 10 days. Scale bars: 100 μm; magnification: 40x; colours: red – Nile red; blue – Hoechst. B) Nile red area in Ldlr−/− wild type and CD11c-DTR BM chimeras (n=13 per group). Values represent mean ± SEM Nile red positive area as a percentage of ascending aortic arch. Significant differences between Ldlr−/− wild type and CD11c-DTR BM chimeras are indicated: ***p<0.001, unpaired two tailed Student’s t-test.
Figure 3: Intimal Lipid Accumulation is reduced by depletion of CD11c+ cells in Ldlr-/- BM chimeras after 10 days on a CRD.
Figure 3: Intimal lipid accumulation is also reduced in Ldlr<sup>−/−</sup> CD11c-DTR bone marrow chimeras fed a CRD for 21 days.

Nile red stained lipid area was determined in aortic arches of Ldlr<sup>−/−</sup> BM chimeras fed a CRD for 21 days. A) representative composite of confocal micrographs of the lesser curvature of Ldlr<sup>−/−</sup> wild type and CD11c-DTR BM chimeras fed a CRD for 21 days. Scale bars: 100 μm; magnification: 40x; colours: red – Nile red; blue – Hoechst. B) Nile red area in Ldlr<sup>−/−</sup> wild type (n=14) and CD11c-DTR (n=10) BM chimeras. Values represent mean ± SEM Nile red positive area as a percentage of ascending aortic arch. Significant differences between Ldlr<sup>−/−</sup> wild type and CD11c-DTR BM chimeras are indicated: ***p<0.001, unpaired two tailed Student’s t-test.
**A)**

Wild type BM

CD11c-DTR BM

**B)**

<table>
<thead>
<tr>
<th>Nile red positive area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wildtype</td>
</tr>
<tr>
<td>C11c-DTR</td>
</tr>
</tbody>
</table>

**B)***
3.3 Deletion of CD11c\(^+\) cells reduces leukocyte recruitment to the aortic arch

Since the lipid content in the aortic arch is reduced when CD11c\(^+\) leukocytes are deleted, we asked whether this impacted subsequent inflammatory events. In atherosclerosis, hypercholesterolemia induces an inflammatory response in the artery wall. Leukocyte recruitment occurs in response to local inflammation and has been well documented as an essential process in the pathogenesis of atherosclerosis. The rate of leukocyte recruitment to the artery wall was quantified in Ldlr\(^{-/-}\) BM chimeras depleted of CD11c\(^+\) leukocytes and compared to undepleted Ldlr\(^{-/-}\) BM chimeras. To quantify the rate of monocyte recruitment BrdU labeled leukocytes in the aortic arch were determined 24 hours after BrdU administration. BrdU is used to label newly produced Ly6\(^{chhi}\) monocytes. Ly6\(^{chhi}\) monocytes have a half-life of 17 hours and are constantly produced by proliferation and differentiation of macrophage-DC progenitor cells (MDP) (Goto et al., 2003). BrdU is cleared from the blood within an hour after BrdU injection. BrdU-labeled Ly6\(^{chhi}\) monocytes begin to appear in the circulation approximately 6 hours after BrdU injection whereas BrdU-labeled Ly6\(^{chlo}\) monocytes and neutrophils do not appear in the circulation within 24 hour period after BrdU injection (Jongstra-Bilen, 2006; Zhu et al., 2009). BrdU can be incorporated in the DNA by any proliferating cell. Therefore BrdU-labeled leukocytes in the aortic arch prior to the appearance of BrdU labeled monocytes in the circulation arise from locally proliferating leukocytes in the intima. The corollary is BrdU-labeled leukocytes in the aortic arch 24 hours after BrdU injection, consist of either BrdU-labeled monocytes recruited from the circulation or intimal leukocytes that proliferated locally. Hence, BrdU-positive intimal leukocytes were counted in the intima at 3 and 24 hours after BrdU injection in Ldlr\(^{-/-}\) BM chimeras fed a CRD. BrdU-positive nuclei at the 3 hour time point gives an indication of the level of leukocyte proliferation in the aortic arch and is taken into consideration when interpreting the results from the later 24 hour timepoint.

After 10 days on CRD, a significantly reduced number of leukocytes were recruited to the aortic arch in Ldlr\(^{-/-}\) BM chimeras depleted of CD11c\(^+\) expressing cells. The mean number of BrdU-labeled CD45 positive cells in aortic arches isolated 24 hour hours after BrdU injection in Ldlr\(^{-/-}\) wild type BM chimeras was 6.4 compared to 2.6 BrdU\(^+\) nuclei / mm\(^2\) of the total ascending aorta up to the left carotid artery in Ldlr\(^{-/-}\) BM chimeras depleted of CD11c-expressing cells (Fig 5A). The mean number of BrdU-labeled leukocytes of the aortic arch in the Ldlr\(^{-/-}\) CD11c-DTR BM
chimera was not significantly different between 3 and 24 hours after BrdU injection indicating that monocyte recruitment was negligible and the majority of BrdU-labeled leukocytes were derived from locally proliferating cells (Fig 4A). Additionally, despite a five-fold decrease in intimal leukocytes in the aortic arch of CD11c-DTR Ldlr⁻/⁻ BM chimeras (Fig 1C), there were similar numbers of BrdU-positive nuclei per area of the aortic arch between control and depleted chimeras at the 3 hours timepoint (Fig 4A). This indicated that the leukocyte proliferation rate in the intima is greater in Ldlr⁻/⁻ CD11c-DTR BM chimeras, a conclusion that was supported when the proportion of CD45⁺ cells that were BrdU positive were measured (Fig 4B).

Wu et al demonstrated that CD11c⁺ CD204⁺ monocytes in Apoe⁻/⁻ mice fed a high fat diet consisting of 0.15% cholesterol increased CD11c expression (Wu et al., 2009). Usually Ly6Cʰⁱ monocytes express low levels of CD11c unlike Ly6Cᵈᵒ monocytes which express higher levels of CD11c (Tacke and Randolph, 2006). Ly6Cʰⁱ and Ly6Cᵈᵒ monocytes were counted by flow cytometry in the blood at the time of sacrifice, to determine whether or not DTx administration altered either population. Monocyte populations were not significantly different in both groups of Ldlr⁻/⁻ BM chimeras and rules out an inhibition of recruitment due to deletion of Ly6Cʰⁱ monocytes (Fig 4C).

Although not statistically significant the same trend as 10 days CRD was observed after 21 days CRD. BrdU-labeled leukocytes/mm² in the ascending aortic arch of Ldlr⁻/⁻ wild type BM chimeras increased between 3 and 24 hours after BrdU injection. A very small increase was observed in BrdU-labeled leukocytes/mm² in the ascending aortic arch of Ldlr⁻/⁻ CD11c-DTR BM chimeras between 3 and 24 hours after BrdU injection (Fig 4D). The majority of BrdU-labeled leukocytes in Ldlr⁻/⁻ BM chimeras depleted of CD11c-expressing leukocytes were likely proliferating cells since there was only a slight increase in mean BrdU-labeled leukocytes/mm² in aortas isolated 24 vs. 3 hours after BrdU injection (Fig 4D). The level of BrdU-labeled leukocytes in the aortic arch 3 hours after BrdU injection was slightly lower in Ldlr⁻/⁻ BM chimeras depleted of CD11c⁺ leukocytes compared to wild type BM chimeras. Still, the proportion of proliferating leukocytes was higher, since the abundance of intimal leukocytes was reduced by DTx mediated depletion (Fig 4E). Lastly no difference in the level of monocytes of both subsets in the circulation was observed between wild type and CD11c-DTR BM chimeras (Fig 4F). Together these data suggest that CD11c-expressing intimal leukocytes in Ldlr⁻/⁻ wild
type BM chimeras promote lipid retention and the recruitment of BrdU labeled leukocytes after feeding a CRD for 10 and 21 days.
Figure 4: BrdU+ leukocyte recruitment is reduced in Ldlr−/− CD11c-DTR BM chimeras on CRD for 10 and 21 days.

BrdU+ CD45+ leukocytes were immunostained, visualized by en face confocal microscopy and counted in the ascending aortic arches, isolated from Ldlr−/− BM chimeras fed a CRD for 10 or 21 days. BrdU+ CD45+ leukocytes normalized to the area of the ascending aortic arch in Ldlr−/− BM chimeras fed a A)10 or B) 21 days CRD, 3 and 24 hours after BrdU pulse. Wild type BM chimera 3h BrdU n=6; 24h BrdU n=7; CD11c-DTR BM 3h BrdU n=6; 24h BrdU n=7 for 10 days CRD. Wild type BM chimera 3h BrdU n=6; 24h BrdU n=6; CD11c-DTR BM 3h BrdU n=5; 24h BrdU n=5 for 21 days CRD. All values represent mean ± SEM BrdU+ CD45+ leukocytes/mm² of ascending aortic arch. Significant differences between wild type and CD11c-DTR BM chimeras are indicated: * p<0.05, unpaired, two tailed Student’s T-test. BrdU+ CD45+ leukocytes normalized to the total number of CD45+ leukocytes in the ascending aortic arch in Ldlr−/− BM chimeras fed a CRD for C)10 or D) 21 days, 3 hours after BrdU pulse. 10 days CRD wild type BM n=6 and CD11c-DTR BM n=6; 21 days CRD wild type BM n=6 and CD11c-DTR BM n=5. All values represent mean ± SEM BrdU+ CD45+ leukocytes/total CD45+ count or CD45+ area in the ascending aortic arch. Significant differences between wild type and CD11c-DTR BM reconstituted Ldlr−/− mice are indicated: *p<0.05, **p<0.01, unpaired two tailed Student’s t-test. Ly6Chi or Ly6Clow monocytes in wild type and CD11c-DTR BM chimeras fed a CRD for E) 10 or F) 21 days. 10 days CRD wild type BM n=4 and CD11c-DTR BM n=5; 21 days CRD wild type BM n=9 and CD11c-DTR BM n=5. All values represent mean ± SEM circulating monocyte count. Differences in Ly6Chi monocytes between wild type and CD11c-DTR BM reconstituted Ldlr−/− mice were not significant: unpaired, two tailed Student’s t-test.
**A**

10 days CRD

- **Wild type**
- **CD11c-DTR**

![Graph showing BrdU+ CD45+ cells/mm² of ascending aorta](image)

**B**

21 days CRD

- **Wild type**
- **CD11c-DTR**

![Graph showing BrdU+ CD45+ cells/mm² of ascending aorta](image)

**C**

10 days CRD

- **Wild type**
- **CD11c-DTR**

![Graph showing BrdU+ CD45+ cells/100 CD45+ cells](image)

**D**

21 days CRD

- **Wild type**
- **CD11c-DTR**

![Graph showing BrdU+ CD45+ cells/CD45+ area](image)

**E**

10 days CRD

- **Wild type**
- **CD11c-DTR**

![Graph showing Circulating Monocytes (x 10⁵ cells/mL)](image)

**F**

21 days CRD

- **Wild type**
- **CD11c-DTR**

![Graph showing Monocyte Subsets: CD115⁺ Ly6C⁺ and CD115⁺ Ly6C⁺](image)
3.4 Adhesion molecule and chemokine expression in the aortic arch is modulated by CD11c-expressing intimal leukocytes.

Leukocyte recruitment is a complex process that is orchestrated by adhesion molecules and chemokines. Previous studies have demonstrated that the transcription of adhesion molecules by endothelial cells is up-regulated by hypercholesterolemia in murine aortas (Iiyama et al., 1999). Additionally, chemokines such as MCP1/CCL2, RANTES/CCL5 and Fractalkine/CX3CL1, and their cognate receptors CCR2, CCR5 and CX3CR1 are implicated in monocyte recruitment to atherosclerotic lesions (Tacke et al., 2007). Since depletion of CD11c-expressing cells reduced leukocyte recruitment to the aortic arch, the expression of endothelial adhesion molecules and intimal chemokines was assessed in the aortic arch of Ldlr⁻/⁻ BM chimeras with and without CD11c⁺ leukocyte depletion. mRNA was isolated from the intima of the lesser and greater curvatures of the ascending aortic arch. All mRNA levels were determined by real-time PCR and normalized to vascular endothelial (VE) cadherin mRNA expression. This endothelial-specific gene was chosen as a reference since a housekeeping gene is expressed by both endothelial and intimal leukocytes would differ between the control and Ldlr⁻/⁻ BM chimeras depleted of CD11c-expressing cells. No significant differences were observed in VE-cadherin expression neither between the LC and GC nor between the four combinations of Ldlr⁻/⁻ BM chimeras and diet types (Fig 5A).

3.4.1 Adhesion molecule and chemokine expression differs between LC and GC of the ascending aortic arch.

mRNA was isolated from the greater and lesser curvatures of the ascending aortic arch of Ldlr⁻/⁻ BM chimeras separately since the lesser curvature is prone to form atherosclerotic plaques, while the greater curvature is relatively resistant except for the ostia of the innominate artery. This susceptibility to lesion formation is due to local hemodynamic differences and a more activated or dysfunctional endothelial cell phenotype in the lesser curvature compared to the greater curvature (Won et al., 2007). In accordance with previously published data, Vcam1, Icam1 and E-selectin mRNA were expressed at higher level in the lesser curvature (Fig 5B) (Jongstra-Bilen, 2006). Cd45, a leukocyte specific gene was only detected in the lesser curvature, which is consistent with the fact that intimal leukocytes selectively reside in the lesser curvature and not in the greater curvature. Interestingly Ccl3, Ccl4 and Ccl5 expression was only detected in the lesser curvature and corresponded with the pattern of expression of leukocyte specific Cd45,
suggesting that chemokines are likely produced by intimal leukocytes. Fractalkine mRNA expression was detected in both the lesser and greater curvatures and is consistent with an endothelial specific origin.
Figure 5: Region specific expression of adhesion molecules and chemokines in the ascending aortic arch.

VE-Cadherin mRNA expression was determined by real-time PCR in the lesser (LC) and greater (GC) curvatures of the ascending aortic arch of Ldlr<sup>−/−</sup> BM chimeras fed a SCD or CRD for A) 10 or B) 21 days. All values represent mean ± SEM raw unnormalized VE-Cadherin cycle threshold (Ct) values. Ldlr<sup>−/−</sup> CD11c-DTR BM chimeras: 10 days SCD (n=12) or CRD (n=14); 21 days SCD (n=10) or CRD (n=10). Ldlr<sup>−/−</sup> wild type BM chimeras: 10 days SCD (n=12) or CRD (n=13); 21 days SCD (n=11) or CRD (n=11). Differences were not statistically significant by ANOVA. Vcam1, Icam1, Sele, Cx3cl1, Cd45, Ccl3, Ccl4, and Ccl5 mRNA levels were analyzed in the intima of the LC and GC of the ascending aortic arch of Ldlr<sup>−/−</sup> wild type BM chimeras fed a SCD for C) 10 or D) 21 days. All values represent mean ± SEM mRNA expression, normalized to VE-Cadherin expression and relative to the lesser curvature, which was assigned a value of 1. Significant differences in expression between greater and lesser curvature are indicated: nd, not detectable, *p<0.05, ** p<0.01, *** p<0.001; paired, two tailed Student’s t-test.
3.4.2 CRD induces expression of adhesion molecules and especially chemokines in the lesser curvature

The expression of endothelial cell molecules (Vcam1, Icam1, and E-selectin) and chemokines (Cx3cl1, Ccl3, Ccl4 and Ccl5) was quantified in the lesser and greater curvatures of Ldlr^{−/−} wild type BM chimeras fed either a SCD or a CRD for 10 or 21 days. CRD induced the expression of adhesion molecules in Ldlr^{−/−} wild type BM chimeras at both 10 and 21 days timepoints (Fig 6). The increase in Vcam1 and Icam1 mRNA level in the lesser curvature was statistically significant at both 10 and 21 days of CRD. E-selectin expression was increased in the lesser curvature primarily at the 21 days timepoint. There was a substantial induction observed in Ccl3, Ccl4 and Ccl5 mRNA expression at 21 days of CRD in the lesser curvature (Fig 6). However, after 10 days of CRD, chemokine mRNA expression only increased slightly and was not statistically significant. CRD did not affect fractalkine expression at either 10 or 21 day timepoints in Ldlr^{−/−} wild type BM chimeras. The expression of the above adhesion molecules and fractalkine did not change dramatically in the greater curvature upon feeding Ldlr^{−/−} BM chimeras a CRD.

The expression of this group of adhesion molecules and chemokines was also analyzed in the ascending aortic arch of C57BL/6 mice stimulated systemically for 2 hours with the pro-inflammatory cytokine IL-1β. IL-1β induces activation of endothelial cells and leukocytes by binding to the IL-1 receptor, which is a receptor tyrosine kinase that transduces the signals into the cell. Consequently pro-inflammatory gene expression is up-regulated due to activation of Nf-κB and JNK and p38 mitogen activated protein kinase signaling (Weber et al., 2010). Recombinant murine IL-1β induced the expression of Vcam1, Icam1, E-selectin and fractalkine robustly in both the lesser and greater curvatures (Fig 7). Conversely the expression of Ccl3, Ccl4 and Ccl5 was only weakly induced by IL-1β stimulation in the lesser curvature (Fig 7). The expression of these chemokines was not detected in the greater curvature (data not shown). This pattern of expression further supports an endothelial specific origin for adhesion molecules and fractalkine while chemokines Ccl3, Ccl4 and Ccl5 expression is leukocyte derived.
Figure 6: Hypercholesterolemia induces adhesion molecule expression specifically in the lesser curvature of the aortic arch.

Vcam1, Icam1, Sele, Cx3cl1, Ccl3, Ccl4, and Ccl5 expression were measured by real-time PCR in the lesser (LC) and greater (GC) curvatures of the ascending aortic arches in A) Ldlr\(^{-/-}\) wild type BM chimeras fed a SCD or CRD for 10 days. B) Ldlr\(^{-/-}\) wild type BM chimeras fed a SCD and CRD for 21 days. Adhesion molecule and fractalkine expression in Ldlr\(^{-/-}\) wild type BM chimeras fed a SCD (n=12) or CRD (n=13) for 10 days; Ccl3, Ccl4 and Ccl5 expression in Ldlr\(^{-/-}\) BM chimeras fed as SCD (n=4) or CRD (n=3) for 10 days. All gene expression in Ldlr\(^{-/-}\) wild type BM chimeras fed a SCD (n=11) or CRD (n=11) for 21 days. All values represent mean ± SEM mRNA expression normalized to VE-Cadherin and relative to lesser curvature of SCD fed wild type BM chimeras, which was assigned a value of 1. Significant differences in mRNA expression in the lesser curvature between SCD and CRD fed Ldlr\(^{-/-}\) wild type BM chimeras are indicated ** p<0.01, *** p<0.001; two tailed unpaired Student’s t-test.
**Figure 7: Adhesion molecules and fractalkine expression is induced in both the lesser and greater curvatures of the ascending aortic arch by IL-1β.**

Vcam1, Icam1, Sele, Cx3cl1, Ccl3, Ccl4, and Ccl5 expression were measured in the lesser (LC) and greater (GC) curvatures of the ascending aortic arches in C57BL/6 mice stimulated with 300 ng of recombinant murine IL-1β for 2 hours (n=3) or PBS (n=3). All values represent mean ± SEM mRNA expression normalized to VE-cadherin and relative to the expression in the lesser curvature of PBS injected C57BL/6, which was assigned a value of 1. Significant differences in endothelial specific mRNA expression in the lesser curvature of PBS or IL1β stimulated C57BL/6 mice are indicated: **p<0.01, ***p<0.001; two tailed unpaired Student’s t-test.
**IL-1β Stimulated**

**Vcam1**

**Ilcam1**

**Sele**

**Cx3cl1**

**Ccl3**

**Ccl4**

**Ccl5**

Relative mRNA expression

- **PBS**
- **IL-1β**
3.4.3  Depletion of CD11c-expressing leukocytes markedly diminished the expression of Ccl3, Ccl4 and Ccl5

Intimal leukocyte abundance in Ldlr<sup>−/−</sup> CD11c-DTR BM chimeras was determined by quantifying Cd45 expression in the lesser curvature of Ldlr<sup>−/−</sup> BM chimeras fed either a SCD or CRD for 10 or 21 days (Fig 8). Cd45 expression was significantly reduced in Ldlr<sup>−/−</sup> CD11c-DTR BM chimeras fed either a SCD or CRD for 10 and 21 days. Endothelial cell adhesion molecule and fractalkine expression in the lesser curvature of the ascending aortic arch of Ldlr<sup>−/−</sup> CD11c-DTR BM chimeras fed a SCD was not significantly affected (Fig 9). However chemokines Ccl3, Ccl4 and Ccl5 expression was substantially reduced by the depletion of CD11c<sup>+</sup> intimal leukocytes. The increase in endothelial adhesion molecule expression in the lesser curvature induced by CRD was not significantly reduced by the reduction in CD11c<sup>+</sup> intimal leukocytes (Fig 10). However, CRD induced chemokine expression in the lesser curvature was blunted by the deletion of CD11c-expressing intimal leukocytes in Ldlr<sup>−/−</sup> BM chimeras fed a CRD for 10 and 21 days. Although the reduction in fractalkine expression was small, it was statistically significant. This data strongly suggests that intimal leukocytes are responsible for Ccl3, Ccl4 and Ccl5 expression in the intima in the steady state.
Cd45 mRNA expression in lesser curvature of Ldlr\(^{-}\) CD11c-DTR BM chimeras fed either a SCD or CRD for A) 10 or B) 21 days. Ldlr\(^{-}\) CD11c-DTR BM chimeras fed either a 10 days SCD (n=12), CRD (n=14), 21 days SCD (n=10) or CRD (n=10) and Ldlr\(^{-}\) wild type BM chimeras fed either a 10 days SCD (n=12), CRD (n=13), a 21 days SCD (n=11) or CRD (n=11). All values represented as mean ± SEM Cd45 mRNA expression normalized to VE-Cadherin and relative to SCD fed Ldlr\(^{-}\) wild type BM chimeras, which was assigned a value of 1. Significant differences between CD11c-DTR and wild type BM chimeras fed a SCD or CRD are indicated: *p<0.05, ** p<0.01, ***p<0.001, one-way ANOVA with Tukey-Kramer post-test.
A) 10 days

Relative Cd45 mRNA expression

SCD | CRD

Bone marrow genotype

Wildtype
CD11c-DTR

B) 21 days

Relative Cd45 mRNA expression

SCD | CRD

Bone marrow genotype

Wildtype
CD11c-DTR
Figure 9: Depletion of CD11c-expressing leukocytes reduced Ccl3, Ccl4 and Ccl5 but not endothelial cell adhesion molecule or fractalkine expression in Ldlr−/− BM chimeras fed a SCD

Vcam1, Icam1, Sele, Cx3cl1, Ccl3, Ccl4, and Ccl5 expression in the lesser (LC) and greater (GC) curvatures of Ldlr−/− BM chimeras fed SCD for A) 10 days and B) 21 days. Adhesion molecules and fractalkine expression in Ldlr−/− wild type (n=12) and CD11c-DTR (n=12) BM chimeras fed as SCD for 10 days; Ccl3, Ccl4 and Ccl5 expression: Ldlr−/− wild type (n=4), CD11c-DTR (n=4) BM chimeras for 10 days SCD. For 21 days SCD all gene expression in Ldlr−/− wild type (n=11) and CD11c-DTR (n=10) BM chimeras. All values represented as mean ± SEM mRNA expression normalized to VE-Cadherin and relative to expression in the lesser curvature of SCD fed Ldlr−/− wild type BM chimeras, which was assigned a value of 1. Significant differences between wild type and CD11c-DTR BM chimeras are indicated: *P<0.05, and **P<0.01 unpaired two tailed Student’s T test.
Figure 10: CRD induced chemokine expression is reduced by depletion of CD11c expressing leukocytes in Ldlr<sup>−/−</sup> BM chimeras.

Vcam1, Icam1, Sele, Cx3cl1, Ccl3, Ccl4, and Ccl5 expression in the lesser curvature of Ldlr<sup>−/−</sup> BM chimeras fed CRD for A) 10 days or B) 21 days. Adhesion molecules and fractalkine expression in Ldlr<sup>−/−</sup> wild type (n=13) and CD11c-DTR (n=14) BM chimeras fed a CRD for 10 days; Ccl3, Ccl4 and Ccl5 expression in Ldlr<sup>−/−</sup> wild type (n=3), CD11c-DTR (n=4) BM chimeras fed a CRD for 10 days. For 21 days CRD all gene expression in Ldlr<sup>−/−</sup> wild type (n=11) and CD11c-DTR (n=10) BM chimeras. All values normalized to VE-Cadherin and relative to lesser curvature of Ldlr<sup>−/−</sup> wild type BM chimeras fed a SCD, which was assigned a value of 1. Significant differences between wild type and CD11c-DTR BM chimeras are indicated: *P<0.05, and **P<0.01 unpaired two tailed Student’s T test.
A) 10 days CRD

Chimera:
- Wild type
- CD11c-DTR

Relative mRNA expression in LC:
- Vcam1
- Icam1
- Sele

B) 21 days CRD

Chimera:
- Wild type
- CD11c-DTR

Relative mRNA expression in LC:
- Vcam1
- Icam1
- Sele
- Ccl3
- Ccl4
- Ccl5
- Cx3cl1
4 Discussion

CD11c-expressing leukocytes in the intima have been reported in non-diseased and hypercholesterolemic Ldlr−/− (Paulson et al., 2010) and Apoe−/− mice (Bobryshev et al., 2001). Our previous studies showed that leukocytes residing in the intima of atherosclerosis-susceptible regions are essential for lesion initiation because they take up LDL and become the first foam cells of nascent fatty streaks (Paulson et al., 2010). Yet their role in mediating the chronic inflammatory response in the setting of diet induced hypercholesterolemia remains to be elucidated. This is the focus of the current work. These set of experiments investigate whether monocyte recruitment and pro-inflammatory gene expression is modulated by CD11c-expressing leukocytes in Ldlr−/− BM chimeras fed a CRD. I modified Paulson’s intimal leukocyte depletion model to enable chronic depletion (Fig 1) and showed that lipid accumulation in the lesser curvature was substantially reduced after 10 and 21 days of CRD (Fig 2 and 3 respectively) when CD11c-expressing leukocytes were depleted.

Monocyte recruitment is an important inflammatory response that contributes to early plaque development. Monocyte recruitment to the lesser curvature increased shortly after Ldlr−/− wild type BM chimeras were fed a CRD (Fig 4) and this correlated with the increase in intimal lipid content and adhesion molecule and chemokine expression in the lesser curvature of the ascending aortic arch (Fig 6). Consistent with that relationship, a decrease in the recruitment of BrdU labeled leukocytes was observed in Ldlr−/− CD11c-DTR BM chimeras (Fig 4a), which have lower levels of intimal lipid and chemokine expression (Fig 9 and 10).

Both endothelial cells and leukocytes in the intima contribute to the inflammatory gene expression in early atherosclerotic lesions in the lesser curvature of the ascending aortic arch. By depleting CD11c-expressing intimal myeloid cells, endothelial versus leukocyte specific proinflammatory gene expression was deduced. Ccl3, Ccl4 and Ccl5 expression corresponded to a leukocyte specific origin, while adhesion molecules and fractalkine demonstrated an endothelial origin. The depletion of CD11c-expressing cells significantly reduced the expression levels of chemokines regardless of diet type or length, while adhesion molecule and fractalkine expression were not affected significantly. An interesting observation was that Ccl3, Ccl4 and Ccl5 expression was only substantially elevated at 21 days of CRD and the fold induction (relative to baseline expression) was higher than the induction of endothelial cell derived
adhesion molecules and fractalkine (Fig 6). Yet, the leukocyte recruitment rate was reduced at both 10 and 21 days of CRD. First of all, this demonstrates that CRD induced chemokine expression by intimal leukocytes takes time, which is consistent with previously published data demonstrating that recruitment surges a couple of weeks after initiation of CRD. Secondly, that basal expression of chemokines in the lesser curvature is sufficient to permit recruitment at the earlier 10 days timepoint. Lastly, that CRD induced endothelial cell adhesion molecule expression cooperates with leukocyte derived chemokine expression to promote the recruitment of monocytes. These data are consistent with the current paradigm of leukocyte recruitment, which proposes that firm adhesion of leukocytes to endothelium is preceded by an obligatory chemokine dependent activation of leukocytes. Together with the fact that the earliest foam cells originate from CD11c-expressing intimal leukocytes, these data suggest that foam cells derived from CD11c+ intimal myeloid cells are a major source of chemokines in the artery wall. This also implies that systemic hypercholesterolemia and not foam cells account for increased endothelial cell adhesion molecule expression.

The depletion of CD11c-expressing cells did not significantly affect genes specifically expressed by endothelial cells. Ldlr−/− CD11c-DTR BM chimeras fed a SCD did not demonstrate endothelial activation, which would be characterized by increased adhesion molecule expression. One study demonstrated that DTx induced apoptosis in Ldlr−/− CD11c-DTR BM chimeras already fed a western diet for 8 weeks, increased recruitment of monocytes to the ascending aortic arch (Gautier et al., 2009b). Additionally they observed increased expression of chemokines Ccl2, Ccl3, Ccl4 and Cxcl2 (MIP2) as well as endothelial cell adhesion molecule Vcam1. One possible explanation would be that established plaques lose the ability to efficiently clear apoptotic cells, resulting in increased inflammation via secondary necrosis (Tabas, 2009). This was not the case in our study, since CD11c-expressing leukocytes were depleted on the first day of CRD, prior to lesion formation. Although statistically not significant, there was a trend that the mean endothelial cell adhesion molecule expression was lower in Ldlr−/− CD11c-DTR BM chimeras compared to wild type BM chimeras fed a CRD. One limitation of this experiment was the size of the experimental groups of approximately 10-14 mice per group. Standard deviations originating from variability from mouse to mouse made differences in the population mean not significant. Increasing the number of mice per group may decrease the standard deviation, resulting in statistical significance.
The efficiency of depletion was only determined at the end of the diet in the CD11c-DTR Ldlr-/- BM chimeras. It was previously reported in the CD11c-DTR transgenic mouse that >98% of CD11c-expressing intimal leukocytes were deleted in the lesser curvature by a single DTx injection. Seven days after DTx administration, intimal leukocytes had recovered to 25% (Paulson et al., 2010). There was a substantially higher intimal leukocyte count in Ldlr-/- BM chimeras depleted of CD11c-expressing leukocytes. In the 10 day timepoint, there was four days between the last DTx injection and analysis while in the 21 day timepoint, there was three days. In both timepoints, intimal leukocytes had recovered more than 20% suggesting a lower efficiency of depletion. This problem may be inherent to the BM chimera model since the extent of bone marrow reconstitution was not verified. Chimeras were deemed successfully reconstituted if they survived more than two weeks after bone marrow transplant. It is possible that a small proportion of Ldlr-/- recipient’s bone marrow survived the lethal irradiation and continued to produce CD11c+ intimal myeloid cells that are insensitive to DTx. As a result of multiple DTx administrations, these recipient CD11c-expressing leukocytes may have accumulated in the aortic arch of Ldlr-/- CD11c-DTR BM chimeras.
5 Future Directions

Early atherosclerotic lesions consist mainly of endothelial cells and bone marrow-derived CD11c-expressing myeloid cells, and it is difficult to separate the individual contributions of each cell type to the hypercholesterolemia-induced inflammatory response. The Ldlr<sup>−/−</sup>CD11c-DTR BM chimeras allow one to directly measure the endothelial-specific component and indirectly determine how CD11c-expressing myeloid cells contribute to the local inflammation. Future experiments will evaluate the extent of bone marrow reconstitution in the chimeras by determining the proportion of circulating CD11c-DTR transgenic (donor) versus non-transgenic (recipient) leukocytes. These experiments are necessary because the efficiency of depleting intimal CD11c<sup>+</sup> cells in chimeras was not as high as in CD11c-DTR transgenic mice (Paulson et al., 2010). Additionally one can determine the depletion efficiency of a single DTx injection at various lengths of CRD to determine whether just using a single injection is a viable option, in the hopes to prevent selective accumulation of DTx insensitive CD11c-expressing leukocytes derived from host bone marrow cells that survived the lethal irradiation.

The presented experiments suggest that chemokines derived from intimal CD11c-expressing leukocytes play a key role in directing monocyte recruitment to early atherosclerotic lesions. A logical future experiment would be to reconstitute Ldlr<sup>−/−</sup> mice with bone marrow from a mouse that is deficient in one of these chemokines (eg. CCL5/RANTES). In these chimeras, one could assess whether endothelial cells in early lesions are capable of producing CCL5 by determining CCL5 mRNA production (using real-time PCR) and comparing it to other intimal leukocyte-derived chemokines (eg. CCL3 and CCL4). Furthermore, by assessing monocyte recruitment using the BrdU assay, one could determine the function of leukocyte-derived CCL5 in this process.

CCL3, CCL4 and CCL5 are ligands of CCR5, an important chemokine receptor for the recruitment of both subsets of monocytes to atherosclerotic plaques (Tacke et al., 2007). Inhibition of CCR5 with either function blocking anti-CCR5 antibody (Tacke et al., 2007) or competitive inhibitors such as Met-RANTES an unprocessed recombinant CCL5 produced by bacteria (Veillard, 2004) reduced recruitment and atherosclerotic plaque growth. Blockade of CCR5 would also confirm the function of intimal chemokines in monocytes recruitment to early atherosclerotic lesions.
Hypercholesterolemia induces the expression of endothelial cell adhesion molecules, which are essential for the recruitment of monocytes. One commonly proposed model suggests that intimal foam cells produce pro-inflammatory cytokines such as IL-1\(\beta\) and TNF\(\alpha\) that can stimulate the up-regulation of endothelial cell adhesion molecule expression (Moore and Tabas, 2011). In contrast, our experiments show that depletion of intimal leukocytes in early lesions did not dramatically alter the expression levels of endothelial cell adhesion molecules, and suggests that the up-regulation of endothelial cell adhesion molecules may be a direct response to systemic hypercholesterolemia. Therefore, it would be interesting to assess the role of intimal leukocyte derived IL-1\(\beta\) in endothelial cell adhesion molecule expression. IL-1\(\beta\) is generated from inactive pro-IL-1\(\beta\) by caspase-1, which is part of a protein complex known as the inflammasome (Bell, 2002). Ldlr\(^{-/-}\) mice can be reconstituted with bone marrow from caspase-1 deficient mice and confirm that endothelial cell adhesion molecule expression occurs independently of IL-1\(\beta\) produced from bone marrow derived cells.

The response to retention hypothesis proposes that plaque development is initiated by the retention and modification of LDL particles in the intima, which promotes a local inflammatory response (Tabas et al., 2007). Although LDL interaction with subendothelial matrix proteoglycans is an important step for retention, we found that intimal lipid accumulation was dramatically reduced when intimal CD11c-expressing myeloid cells were reduced. Furthermore the correlation between atherosclerosis resistance and the absence of intimal leukocytes in other inbred strains of mice suggests that intimal leukocytes are essential for lipid retention. It remains to be confirmed whether intimal CD11c-expressing myeloid cells also promote the retention of LDL. Currently, studies published on ex vivo model of foam cell formation demonstrated that oxidatively-modified LDL is taken up by scavenger receptors such as CD36 and not LDLR (Kuchibhotla et al., 2008; Kunjathoor, 2002). Reconstituting Ldlr\(^{-/-}\) mice with bone marrow from mice deficient in scavenger receptors CD36 and SRAI/II will reveal the role of these molecules in nascent foam cell formation by intimal CD11c-expressing myeloid cells. Accessory proteins such as lipoprotein lipase, sphingomyelinase and secreted phospholipases, are also implicated in promoting LDL retention in the intima (Pentikainen, 2002). Ldlr\(^{-/-}\) mice reconstituted with bone marrow from mice deficient in lipoprotein lipase reduced lipid accumulation (Babaev, 2000). It is possible that CD11c-expressing intimal myeloid cells also produce these accessory proteins to promote lipid retention.
Another interesting avenue one can pursue and is a burgeoning field is the study of T lymphocytes in atherosclerosis. CD11c-expressing intimal leukocytes are capable of antigen presentation (Choi et al., 2009) and may bridge the innate and adaptive immune responses in atherosclerosis. A mature DC phenotype distinguished by co-stimulatory molecule expression has also been reported in atherosclerotic plaques (Subramanian et al., 2012). Although CD11c+ foam cells are unlikely to exit, CD4+ T lymphocytes have been shown to be recruited to plaques and interact with CD11c+ foam cells and induce proinflammatory cytokine production (IFNγ and TNFα) (Koltsova et al., 2012). Another study demonstrated that CCL17 produced by CD11c+ foam cells recruited and induced apoptosis in Tregs, hence promoting inflammation (Weber et al., 2011). Conversely, two other studies have suggested specific subsets of CD11c expressing leukocytes in the aorta that limit atherosclerotic plaque formation via Treg mediated inhibition of inflammation (Choi et al., 2011; Subramanian et al., 2012). It remains to be determined whether these interactions between T lymphocytes and CD11c-expressing intimal myeloid cells are instances of antigen presentation. Alternatively, these may be interactions with Tregs, which suppress inflammation and have been shown to play a role in atherosclerosis.

As the role of CD11c+ intimal myeloid cells in atherosclerosis is uncovered, the use of the depletion model to study these leukocytes will become limited. Recently a CD11c tissue specific Cre recombinase mouse was made (CD11c-Cre). Cre recombinase mediates site specific deletion of a region of DNA flanked by loxP sites by homologous recombination. This mouse allows one to study the absence of specific genes in CD11c-expressing cells without altering blood cholesterol levels. Subramarian et al., 2013 deleted expression of the TLR adaptor Myd88 in CD11c+ cells by breeding the CD11c-Cre mouse with floxed Myd88 (Myd88fl/fl) mouse. This CD11c-Cre mouse can be a very useful tool to study other genes expressed by intimal myeloid cells already implicated in atherosclerosis.
6 Conclusion

CD11c-expressing myeloid cells residing in the intima of atherosclerosis susceptible regions mediate the first response to hypercholesterolemia in Ldlr−/− mice. Our lab previously showed that these intimal leukocytes shortly after hypercholesterolemia take up lipid and become the first foam cells of nascent lesions. The current work establishes a proatherogenic role for these CD11c-expressing myeloid foam cells in Ldlr−/− mice fed a CRD. Shortly after diet induced hypercholesterolemia, these cells produce chemokines, which facilitate the recruitment of circulating monocytes to early lesions. Monocyte recruitment and chemokine expression in the lesser curvature were substantially reduced when CD11c-expressing leukocytes were depleted by DTx-induced apoptosis. In addition this work revealed that adhesion molecule expression is up-regulated in endothelial cells, which is required for monocytes recruitment occurred independently of intimal leukocytes. The continued study of these leukocytes and their role in the initiation of atherosclerosis will yield novel findings, which one day may form the basis of a therapy for vascular disease.
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