The Role of Interleukin-1 Beta in Mediating LPS-Induced Reverse Transendothelial Migration of Intimal Dendritic Cells

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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Abstract

Intimal dendritic-like cells (DCs) reside in regions of the aorta predisposed to atherosclerosis. LPS and interleukin-1 beta (IL-1β) injections into C57BL/6 mice result in decreased numbers of intimal DCs due to their exit into the blood via reverse transendothelial migration (RTM). This study evaluated the role of IL-1β in RTM induced by intravenous LPS injection. IL-1β mRNA expression was rapidly upregulated in the intima by LPS. Experiments using genetic approaches (Il1b−/−, Casp1−/− and Il1r1−/− mice) and function-blocking antibodies demonstrated a critical role for IL-1β production and signalling in LPS-induced RTM. Reciprocal bone marrow transplantation experiments revealed that IL-1β production and signalling only in the non-hematopoietic compartment, likely endothelium, was critical. Ex vivo studies showed that IL-1β could rescue RTM in the Il1b−/− aorta, implicating sustained intimal IL-1β signalling. These data suggest that intimal IL-1β production and signalling in endothelial cells is required for LPS-induced RTM of intimal DCs.
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List of Abbreviations

°C – degrees Celsius  
µg – microgram  
µL - microlitre  
Ab - antibody  
ANOVA – analysis of variance  
ApoE – apolipoprotein E  
ARC – Animal Resource Centre  
ASC – apoptosis-associated speck like protein containing a caspase recruitment domain, pycard  
ASC<sup>-/-</sup> - apoptosis-associated speck like protein containing a caspase recruitment domain knockout mice  
ASK – apoptosis signal-regulating kinase  
ATA – aurintricarboxylic acid  
ATF6 – activating transcription factor 6  
ATP – adenosine triphosphate  
BrdU – 5-bromo-2’-deoxyuridine  
C5A – complement 5A  
Ca<sup>2+</sup> - calcium ions  

*Cad5-CreER* – transgenic mice containing genes encoding for Cad5 and Cre recombinase on same promoter, Cre gene can be induced by activation of the estrogen receptor  
CANTOS – canakinumab anti-inflammatory thrombosis outcome study  
CARD – caspase recruitment domain  
*Casp1<sup>-/-</sup>* - caspase-1 knockout mice  
*CD11c-Cre* – transgenic mice containing genes encoding for CD11c and Cre recombinase on same promoter  
*CD11c-EYFP* – transgenic mice containing genes encoding for CD11c and EYFP proteins on same promoter  
*CD11c-hBcl2* – transgenic mice containing genes encoding for CD11c and hBcl2 proteins on same promoter  
CCL19 – C-C chemokine ligand 19  
CCL21 – C-C chemokine ligand 21  
CCR7 – C-C chemokine receptor type 7, cluster of differentiation 197  
CD – cluster of differentiation  
CD11c – cluster of differentiation 11c, integrin alpha<sub>x</sub>  
CD45 – cluster of differentiation 45  
CD68 – cluster of differentiation 68, macrosialin  
COX2 – cyclooxygenase 2  
Cre – causes recombination  
CX3CL1 – chemokine (C-X3-C) motif ligand 1, fractalkine  
CX3CL12 – chemokine (C-X3-C) motif ligand 12, stromal-derived factor 1  
CX3CR1 – chemokine (C-X3-C) motif receptor 1, fractalkine receptor  
CX3CR4 – chemokine (C-X3-C) motif receptor 4, cluster of differentiation 184  
DAMP – danger-associated molecular pattern  
DAPI - 4′,6-diamidino-2-phenylindole  
DCs – dendritic cells  
DC-SIGN - Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin, cluster of differentiation 209
DMEM – Dulbecco’s modified eagle’s medium
(c)DNA – (complementary) deoxyribonucleic acid
DT – diphtheria toxin
DTR – diphtheria toxin receptor
EC – endothelial cell
ER – endoplasmic reticulum
EYFP – enhanced yellow fluorescence protein
FBS – fetal bovine serum
Flt3 – fms-like tyrosine kinase 3, cluster of differentiation 135
Flt3L – fms-like tyrosine kinase 3 ligand
g - gram
GC – greater curvature
GM-CSF – granulocyte-macrophage colony-stimulating factor
h - hours
H2O - water
hBcl2 – human B cell lymphoma 2
HEPES – 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HP – high probability
IκB – inhibitor of nuclear factor kappa B
ICAM – intracellular cell adhesion molecule
ICE – interleukin-converting enzyme
IFN-γ – interferon gamma
IgG – immunoglobulin G
IKK – I kappa B kinase
IL – interleukin
IL-18 – interleukin 18
IL-18BP – interleukin 18 binding protein
IL-1α - interleukin 1 alpha (IL-1F1)
Il1α−/− - interleukin 1 alpha knockout mice
IL-1β – interleukin 1 beta (IL-1F2)
Il1β−/− - interleukin 1 beta knockout mice
IL-1R1 – interleukin 1 receptor 1
Il1r1−/− - interleukin 1 receptor knockout mice
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Il1r1CD11cCre+ - mice with IL-1R1 gene knocked out in CD11c+ cells
Il1r1loxP–IL-1R1 floxed mice
IL-1R2 – interleukin 1 receptor 2
IL-1Ra, IL-1RN gene – interleukin 1 receptor antagonist (IL-1F3)
IL-1RAcP – interleukin 1 receptor accessory protein
IP3R – inositol 1,4,5-trisphosphate receptor
IRAK – interleukin-1 receptor-associated kinase 4
IRE1α –inositol-requiring enzyme 1 alpha
IRF8 – interferon regulator factor 8
IV - intravenous
JAM – junctional adhesion molecule
JAX – Jackson Laboratories
JNK – c-Jun N-terminal kinase
K+ - potassium ions
KLF2 – Kruppel-like factor 2
KO – knockout
LC – lesser curvature
LDL – low density lipoprotein
LRR – leucine-rich repeat domain
Ldrlr^−/− – low-density lipoprotein receptor knockout mice
LICAM – neural cell adhesion molecule L1
LOX-1 – lectin-like oxidized low density lipoprotein receptor-1
LP – low probability
LPS – lipopolysaccharide
Ly6C – lymphocyte antigen 6C
M-CSF – macrophage colony stimulating factor
MAPK – mitogen-activated protein kinase
MCP-1 – macrophage chemoattractant protein-1, C-C chemokine ligand 2
MEKK3 – MAP kinase kinase kinase 3
MHCI – major histocompatibility complex class I
MHCII – major histocompatibility complex class II
mL - millilitre
MMP – matrix metalloproteinase
MyD88 – myeloid differentiation primary response 88
NA – numerical aperture
NBD – nucleotide-binding domain
NEMO - NF-κB essential modifier
NF-κB – nuclear factor kappa B
ng - nanograms
NLR – nod-like receptor
NLRP3 – nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3
NTP-Stat1 – non-tyrosine phosphorylated signal transducer and activator of transcription 1
OX40L – cluster of differentiation 252
P2X7 – purinergic receptor P2X
PAMP – pathogen-associated molecular pattern
PBS – phosphate-buffered saline
PECAM-1 – platelet endothelial cell adhesion molecule, cluster of differentiation 31 (CD31)
PERK – protein kinase R-like ER kinase
PFA - paraformaldehyde
PI3K – phosphoinositide-3 kinase
PKR – protein kinase R
PLA2 – phospholipase A2
PolyI:C – polynosinic:polycytidylic acid
PYD – pyrin domain
qPCR, RT-PCR – quantitative real-time polymerase chain reaction
RIDC – resident intimal dendritic cells
ROS – reactive oxygen species
RPMI – Roswell Park Memorial Institute medium
RTM – reverse transendothelial migration
(m)RNA – (messenger) ribonucleic acid
S1P – sphingosine-1 phosphate
S1PR1 – sphingosine-1 phosphate receptor 1
SA-HRP – streptavidin-horseradish peroxidase
SEM – standard error of the mean
SphK1 – sphingosine kinase 1
SLAM – signaling lymphocytic activation molecule
SYK – spleen tyrosine kinase
TAB – TAK1-binding protein
TAK – transforming growth factor beta-activated kinase
Th17 – T helper cell 17
TIR – toll and interleukin 1 receptor-like domain
TLR – toll-like receptor
TMDT – Toronto Medical Discovery Tower
TNFα – tumor necrosis factor alpha
TOLLIP – toll interacting protein
TRAF – TNF receptor associated factor
TRX – thioredoxin
TUNEL – terminal deoxynucleotidyl transferase dUTP nick end labeling
Tyr-FITC – tyramide-fluorescein isothiocyanate
UV – ultraviolet
V – volts
VCAM-1 – vascular cell adhesion molecule 1
VE-CAD – vascular endothelial cadherin, cadherin 5 (Cad5)
WT – wild-type, C57BL/6 mice
1 Introduction

1.1 Atherosclerosis

Atherosclerosis, a chronic inflammatory disease of the large muscular and elastic arteries, is the underlying cause of heart disease and stroke, which are the leading causes of death in North America\(^1\). The process of atherosclerosis begins when low-density lipoprotein (LDL) particles get taken up into the subendothelial layer of the artery wall called the intima. LDL particles are then modified and endocytosed by myeloid cells of the intima forming lipid droplets, which results in their foamy appearance. These “foam cells” appear as fatty streaks, characteristic of early atherosclerotic lesions. Over time, abundant numbers of macrophage foam cells accumulate in atherosclerotic plaques through monocyte recruitment and local proliferation\(^2\)\(^3\). Subsequently, these foam cells die and a mixture of lipids and dead cells form the necrotic core. Smooth muscle cells from the inner layer “media” of the arteries migrate to the intima, where they proliferate and differentiate into cells that synthesize extracellular matrix (ECM) proteins\(^1\). This leads to the formation of the fibrous cap that stabilizes the plaque and isolates the necrotic core from the blood. In advanced stages of atherosclerosis, matrix metalloproteinases (MMPs) degrade collagen that makes up the fibrous cap, and make the plaque prone to rupture\(^4\). Plaque rupture leads to release of thrombogenic proteins, accumulation of clotting factors and formation of thrombi that can block blood flow in arteries. This event can compromise the circulation in major organs such as the heart, leading to myocardial infarction, or the brain, leading to stroke\(^1\). Inflammatory events accompany all stages of atherogenesis.

1.2 Atherosclerosis susceptibility

Atherosclerosis does not develop randomly in arteries; rather, there are certain regions that are predisposed. These regions are typically at curvatures and bifurcations in the arterial tree. The aortic arch is a region of the aorta that connects the ascending and descending regions of the aorta together with the branches of the brachiocephalic artery, the left common carotid artery, and the left subclavian artery (Figure 1). The inner or lesser curvature (LC) of the aortic arch is susceptible to lesion formation, while the outer or greater curvature (GC) is protected. The LC and GC experience different hemodynamics and this is underlying factor for the regional differences in atherosclerosis susceptibility. Disturbed laminar blood flow (disturbed flow) with a low time-averaged shear stress and different directions during each phase of the cardiac cycle (even a reverse direction during diastole)\(^5\)\(^6\) occurs in the LC, whereas uniform
laminar flow (laminar flow) with a high time-averaged shear stress and a uniform direction during the cardiac cycle occurs in the GC. Endothelial cells lining the inner surface of arteries sense hemodynamics. Laminar flow maintains a quiescent endothelium with elongated cells aligned with the direction of blood flow. Disturbed multidirectional flow results in unique signalling mediated by activation of ion channels, primary cilia, junctional signalling complexes, integrins and the cell cytoskeleton, which influences the shape of endothelial cells (polygonal or elongated in multiple directions with nuclear deformations). In addition, relatively higher levels of NF-κB proteins are expressed primarily in the cytosol of endothelial cells from LC regions. Thus, NF-κB signalling is primed for activation upon exposure to pro-inflammatory stimuli such as LPS and hypercholesterolemia\(^7\). Low-grade inflammation occurs in the LC of the aortic arch of wild-type mice, with elevated expression of pro-inflammatory genes coding for adhesion molecules and scavenger receptors, such as VCAM-1, ICAM-1, E-selectin and LOX-1\(^8\)\(^9\). In contrast, transcription factors such as Kruppel-like factor 2 (KLF2) that encode atheroprotective genes are expressed in regions of uniform laminar flow\(^10\)\(^11\).

1.3 Resident Intimal Dendritic Cells (RIDscs) and the Initiation of Atherosclerosis

As early as 1995, it has been shown that lesion-prone regions of the rabbit aorta contain a population of leukocytes. The majority of these cells express macrophage markers, are MHCII\(^+\) and exhibit dendritic cell (DC)-like morphology; other occasional cells present are eosinophils and neutrophils. In the healthy human aorta intimal DC-like cells are S-100\(^+\) and CD1a\(^+\), and are found in atherosclerosis-susceptible regions\(^12\). Normal C57BL/6 mouse aortae display intimal CD45\(^+\), CD68\(^+\), CD11c\(^+\) cells with dendritic-like morphology in atherosclerosis-susceptible areas\(^3\)\(^8\) (Fig. 1C), along with a few T cells\(^8\). These DCs are referred to as resident intimal DCs (RIDscs). The accumulation and homeostasis of these DCs was shown to be fractalkine (CX3CL1)-dependent, as mice deficient in its receptor (CX3CR1) presented with less DCs in this region compared to WT mice\(^13\). In addition, RIDC abundance is VCAM-1-dependent, as VCAM-1 deficient mice presented with less CD68\(^+\) intimal cells\(^8\). A low level of Ly6C\(^{hi}\) monocyte recruitment occurs in the LC of normal mouse aortic arch, as was determined by a 24 hour BrdU assay\(^8\). However, the role of monocyte recruitment in the maintenance of RIDCs remains unknown. Subsequently, Choi et al described two major populations of DCs in the mouse intima\(^14\): classical Flt3-Flt3L signalling-dependent CD103\(^+\)CD11b\(^-\) DCs and
macrophage-colony stimulating factor (M-CSF)-dependent CD14⁺CD11b⁺DC-SIGN⁺ monocyte-derived DCs.

Figure 1: Regions in the aortic arch predisposed to atherosclerosis. (A) Schematic of the aortic arch. The lesser curvature (LC) and greater curvature (GC) of the aortic arch are indicated. Dotted lines indicate the cuts that were made when opening up the aorta. (B) Schematic of the aortic arch after the tissue is flattened. HP indicates the area where there is a high probability of atherosclerosis development; LP indicates the area with low probability of atherogenesis, these regions are present in the LC and GC respectively. (C) En face immunoconfocal image of an aortic arch from a C57BL/6 mouse. The aorta was stained with biotinylated anti-mouse CD11c, and Hoechst. Areas that represent the LC and GC are indicated. Blue: DAPI; Yellow: CD11c⁺ cells accumulated in LC. (A) and (B) are adapted from Iiyama et al., Circulation Research, 1999⁹.
The primary function of DCs is to sense injury and present antigens to T cells. Tissue-resident DCs are considered immature, and detect danger-associated molecular patterns on pathogens (DAMPs) through pattern-recognition receptors (i.e. TLRs, NLRs). DCs internalize the danger molecules and migrate into the lymphatic system in order to reach the lymph nodes, where they can present antigens on either MHC class I molecules to be recognized by CD8⁺ T cells, or on MHC class II molecules to be recognized by CD4⁺ T cells. The process of DC maturation occurs in the lymphatic system, where they up-regulate co-stimulatory markers such as CD80 and CD86 on their surface. Although cultured aortic DCs captured and presented antigens on MHC class I molecules to CD8⁺ T cells in vitro, it is unlikely that DCs actually present antigen in the aortic intima since only a few T cells were found in atherosclerosis predisposed LC regions. In addition, lymphatics are present in the artery adventitia, but are not found in the intimal layer.

Intimal DCs constitutively express pro-inflammatory cytokine mRNA, including IL-1β, TNFα, and IL-6, and depletion of intimal CD11c⁺ cells sharply reduced the intimal mRNA levels of these cytokines. These data suggest that resident intimal DCs play an integral part to the basal pro-inflammatory environment of the atherosclerosis-susceptible regions. It is widely believed that resident intimal DCs participate in the initiation of atherosclerosis. DCs accumulate lipid in Ldlr⁻/⁻ mice fed a high cholesterol diet in just 5 days, prior to the onset of monocyte recruitment and increased local cell proliferation. Depletion of CD11c⁺ cells by injection of diphtheria toxin (DT) into CD11c-DT receptor transgenic mice led to a decrease in intimal lipid area, indicating that the majority of intimal lipid rapidly accumulates within intimal DCs. However, the consequence of this early lipid loading on gene expression is not as yet known. The intimal classical Flt3-dependent DCs exhibited atheroprotective properties, since their depletion lead to increased atherosclerotic lesions by an unknown mechanism. Taken together, the function of resident intimal DCs in the initiation of atherosclerosis remains elusive.

1.4 Migration of Immune Cells

1.4.1 Immune Cell Recruitment from Blood to Tissues

When tissue in an organism get infected or injured, the inflammatory cascade is triggered by the production of pro-inflammatory cytokines and chemokines, as well as adhesion molecules by endothelial cells, resident immune cells or recruited inflammatory cells. Cytokines
induce a wide variety of functions upon binding to their receptors, such as upregulation of adhesion molecules, modulation of cell survival, and changes in dynamics of blood flow that result from vasodilatation and increased permeability at sites of inflammation. Chemokines are chemotactic cytokines, which, as their name suggests, elicit downstream responses upon binding G protein-coupled chemokine receptors and eventually induce chemotaxis of immune cells\textsuperscript{18}. The recruitment of immune cells by chemokines occurs through a series of steps, termed the leukocyte adhesion cascade. The process of tethering captures leukocytes in the bloodstream to loosely adhere to the endothelium. The leukocytes then crawl along the luminal endothelial layer (rolling), and continue to slow down (slow rolling) as they approach the site of injury. Leukocyte tethering, rolling and slow rolling are mediated by selectin molecules. Meanwhile, chemokines activate the endothelium to increase production and expression of adhesion molecules such as ICAM-1, ICAM-2 and VCAM-1. These molecules bind to integrin molecules on leukocytes, causing them to firmly adhere on the endothelium. At this point, junctional molecules such as PECAM1 and JAMs are downregulated, thus opening up endothelial junctions so that leukocytes can subsequently transmigrate into the subendothelial layer. There is also evidence that leukocytes can pass within endothelial cells as opposed to through their junctions, termed transcytosis\textsuperscript{19}.

\subsection*{1.4.2 Immune Cell Migration From Tissues to Lymphatics}

Leukocyte migration from a site of inflammation into afferent lymphatics is critical to the initiation of an immune response and to the resolution of inflammation. DCs and T cells are the typical leukocytes that are involved in this pathway. DCs in tissues internalize and present antigens on their surface, and migrate from tissues into lymph nodes, where they upregulate co-stimulatory molecules, mature completely and interact with T cells\textsuperscript{20}. Four stages are identified in this process. The first stage is termed the interstitial stage, where DCs migrate to the lymphatic endothelium. Here, there are many different mechanisms that have been proposed, including CCR7/CCL19 interactions, interactions between CCL21 on lymphatic endothelium and CCR7 on DCs, interactions between CXCL12 in lymphatic endothelium and CXCR4 on DCs, interactions between CX3CL1 on lymphatic endothelium and CX3CR1 on DCs, S1P(3) and S1P(1), and many other molecules. CCR7 is also present on CD4+ T cells, and is also involved in their egress into lymph nodes\textsuperscript{20}. The second step of cell egress is the actual process of transmigration across the lymphatic endothelium. In DC egress, many of the above molecules mentioned in the leukocyte adhesion cascade are upregulated\textsuperscript{20}, in addition to adhesion molecules VCAM-1, ICAM-1 and L1CAM\textsuperscript{20}. The third stage of lymphatic migration occurs
within lymphatic vessels; CCR7/CCL21 interactions play a major role\textsuperscript{20}. The final stage involves the migration of cells across the sinuses of distal lymph nodes. Key events involve CCR7/CCL19 interactions, along with the involvement of other molecules such as heparin sulfate or CD69\textsuperscript{20}.

1.4.3 Reverse Transendothelial Migration

The field of reverse transendothelial migration (RTM) is a novel area of study. RTM is a potential major mechanism of immune cell homeostasis and regulation. There are multiple mechanisms through which immune cells can be cleared from a site of injury. This includes apoptosis, migration of cells into lymphatics and RTM. These mechanisms are necessary in order to prevent excessive inflammation that could be harmful to healthy tissue and to the organism.

RTM was first shown in cultured EC monolayers, where neutrophils migrated across the monolayer first and then migrated opposite to the initial direction\textsuperscript{21}. One of the earliest \textit{in vivo} studies of RTM was shown in neutrophils that exited the site of injury in zebrafish; the neutrophils transmigrated through the endothelium from the tissue and re-entered the bloodstream\textsuperscript{22, 23}. The process of neutrophil RTM was shown to be initiated by macrophage contact\textsuperscript{24}, and these neutrophils expressed increased ICAM-1 and decreased CXCR1\textsuperscript{23}. In order for neutrophils to undergo RTM, the downregulation of JAM-C at endothelial cell junctions was necessary\textsuperscript{25}. Neutrophils undergo RTM in certain human diseases as well, such as pancreatitis-associated acute lung injury\textsuperscript{26}.

In addition to neutrophils, other leukocytes can also undergo RTM. For example, T cells utilize RTM to exit the thymus into the bloodstream\textsuperscript{27}. In an \textit{in vitro} model, T cells reverse their direction of endothelial transmigration through endothelial signalling of CXCL12\textsuperscript{22, 28}. Monocyte-derived DCs can also undergo RTM. In vitro, blood monocytes differentiated into DCs after migrating through a cultured endothelial monolayer and phagocytosing subendothelial collagen beneath the endothelium, before reverse migrating back to the endothelial surface\textsuperscript{29}. RTM of monocytes is dependent upon signalling by ICAM-1 and CD11/CD18 integrins\textsuperscript{30}. RTM has also been described in tumor cells during cancer metastasis, which involves macrophage signalling, activation of the PI3K cascade, and secretion of proteases that cleave endothelial junction molecules\textsuperscript{23}.

RTM has recently been reported to occur in arteries. The aorta does not contain lymphatics except for the adventitial layer\textsuperscript{15}; therefore, migration of intimal leukocytes into lymphatics would require migration through the entire media. Therefore, RTM of intimal cells
into the bloodstream is the most efficient way to exit the intima. *Chlamydia pneumoniae*, a bacterium transported by monocytes into atherosclerotic lesions, is not found in normal arteries in regions predisposed to atherosclerosis. This suggests that *Chlamydia* are cleared from the normal intima by RTM. Therefore, the Cybulsky lab studied the response of DCs in the intima of the ascending arch predisposed to atherosclerosis, upon exposure to danger signals.

Following a systemic injection of LPS, a decrease in number of CD11c+ intimal DCs was observed in the LC between 12 – 24 hours and 60 – 72 hours post-LPS (Fig. 2A), each followed by recovery of intimal DC numbers as a result of proliferation of the remaining cells. The decrease in intimal DCs was also observed upon activation of the inflammatory response via a TLR3 agonist, PolyI:C (Fig. 2B). The following experiments demonstrated that the decrease in intimal DCs was due to their exit out of the intima into the lumen, via the process of RTM.

Scanning electron micrographs showed that an injection of LPS led to adhesion of leukocytes on the luminal layer of the endothelium of the LC when aortas were harvested 9h post-injection, and these leukocytes were shown to not only be recruited circulating monocytes, as inducing their death by clodronate liposomes did not completely eliminate leukocyte-endothelial adhesion. Furthermore, it was confirmed that intimal DCs exited the intima after stimulation with LPS, when aortas of CD11c-EYFP mice were harvested and cannulated and a flow of media consisting of LPS was passed through the lumen, there was an increase in CD11c+ cells that was detected by EYFP staining (Fig. 2C). In addition, the decrease in intimal DCs was not observed in CD11c-hBcl2 transgenic mice, whose CD11c+ cells consisted of the anti-apoptotic factor hBcl2. Also, TUNEL staining did not indicate any increase in DNA in the intima upon injection of LPS (Fig. 2D, 2E). Together, these results show that the reduction in intimal DCs was not due to apoptosis, but because of RTM, and the decrease in CD11c+ cells at 24h and 72h post-LPS are referred to as 2 waves of RTM. Both waves of RTM were mediated by induced expression of CCL19 and its receptor CCR7 in intimal DCs. An injection of *Chlamydia muridarum* also induced two waves of RTM. Blood monocytes shuttled *Chlamydia muridarum* into the intima, and the second wave of RTM resulted in their clearance. RTM and the clearance of *Chlamydia muridarum* was inhibited in CCR7 deficient and hypercholesterolemic mice. If RTM can be rescued in hypercholesterolemic conditions, this process may be adaptable for the removal of myeloid cells loaded with intracellular lipid from atherosclerotic lesions. Thus, it is imperative to elucidate the mechanism of RTM and its inhibition by hypercholesterolemia. My project focuses on the role of IL-1β in LPS-induced RTM.
Figure 2
**Figure 2:** The TLR4 agonist LPS induces RTM of intimal DCs. (A) An injection of LPS induces a decrease in intimal DCs, represented by number of CD11c<sup>+</sup> cells present in the LC of the aortic arch of WT mice. The decrease in counts occurs at 12 – 24 hours, and 60 – 72 hours post LPS, which indicates that LPS induces 2 waves of RTM. (B) Injection of Poly I:C, a TLR3 agonist, also induces both waves of RTM at similar timepoints (C) CD11c-EYFP mice were sacrificed and their aortas were cannulated ex vivo, before a buffer solution consisting of either LPS or PBS was passed through the lumen for the timepoints indicated on the graph. The number of EYFP<sup>+</sup> cells collected from the buffer, representing intimal CD11c<sup>+</sup> DCs, was graphed. (D) The amount of TUNEL-positive DNA was quantified in the LC of the aortic arch from WT mice at different timepoints after injection of LPS. The positive control group included arches from CD11c-DTR mice injected with DT, which kills intimal DCs. (E) PBS, LPS or PolyI:C was injected into CD11c-hBcl2 mice, and number of CD11c<sup>+</sup> intimal DCs were quantified 24h post-injection. (C) shows that DCs have undergone RTM upon interaction of LPS with the endothelium, while (D) and (E) indicate that apoptosis is not a factor that contributes to the decrease in intimal DCs. Values represent mean ± SEM. n > 6. * p < 0.05; ** p < 0.01; *** p < 0.001. This figure is adapted from Mark Roufaiel’s thesis.

1.5 The Interleukin-1 Family

The Interleukins are a family of cytokines with either pro-inflammatory or anti-inflammatory activity. They are produced by cells exposed to bacterial products such as lipopolysaccharide (LPS), danger associated molecular patterns (DAMPs) or other mediators of inflammation. The interleukins that are of particular interest to this project are IL-1F2 (IL-1β), IL-1F1 (IL-1α) and IL-1F3 (IL-1 receptor antagonist or IL-1Ra)<sup>34</sup>. Their receptors and downstream inflammatory effects are briefly summarized in Table 3. The structure of IL-1β was elucidated, as a tetrahedral structure with 12 beta strands forming many complex hydrogen bonds<sup>35</sup>. IL-1α consists of a β-barrel of 14 beta strands and triple helices<sup>36</sup>. IL-1Ra, whose structure comprises of a β-barrel consisting of 12 protein strands and 3 β-hairpin loops<sup>37</sup>, is a competitive inhibitor of IL-1α and IL-1β, and thus, is considered an anti-inflammatory molecule<sup>34</sup>.

1.5.1 IL-1 production, processing and signalling

The transcription and translation of IL-1α and IL-1β is stimulated by DAMPs and pathogen associated molecular patterns (PAMPs), such as LPS. LPS binds to toll-like receptor 4
(TLR4), a transmembrane receptor with extracellular and cytoplasmic domains. LPS binds to the extracellular domain, resulting in a conformational change in TLR4 and the recruitment of MyD88 to the cytoplasmic Toll- and IL-1R-like (TIR) domain. This step, in turn, recruits multiple proteins that initiate a signalling cascade, which leads to the activation of NF-κB transcription factor and transcription of proinflammatory genes, including members of the IL-1 family. While the IL-1α propeptide can elicit downstream pro-inflammatory functions, it can also be cleaved by calpain to produce IL-1α, also an active cytokine. However, IL-1β is translated as a propeptide that need to be cleaved by the inflammasome to become active. The inflammasome is formed when danger signals induce NLRP3 to recruit ASC and procaspase-1. Procaspase-1 is auto-cleaved to activated caspase-1 (previously known as interleukin-1 converting enzyme or ICE), which in turn cleaves pro-IL-1β to activated IL-1β, which is then secreted

Pro-IL-1β may also be cleaved independently of the NLRP3 inflammasome. Studies dating back to the 1980’s have shown that pro-IL-1β could be cleaved by bacterial proteases. Other proteases such as trypsin and plasmin from human monocytes, Granzyme A from leukocytes, elastin, cathepsin G, collagenase, and chymase in human mast cells also cleave pro-IL-1β. While each of these enzymes cleaves pro-IL-1β at different sites, the products of cleavage results in forms of active IL-1β. NLRP3-independent cleavage of pro-IL-1β primarily occurs in neutrophilic inflammation, such as neutrophil-mediated arthritis of cartilage tissue.

A schematic diagram representing a condensed version of the signalling cascade downstream of IL-1R1 activation is indicated on Fig. 3B. IL-1α and IL-1β bind to IL-1R1, a pro-inflammatory receptor that also has a TIR cytosolic domain capable of recruiting MyD88. The first step of signal transduction includes a conformational change in IL-1R1, required for its dimerization with the IL-1 co-receptor (IL-1RAcP). Both receptors share the same cytosolic TIR domain that recruits MyD88 and IRAK4. IRAK4 undergoes autophosphorylation and then phosphorylates IRAK1 and IRAK2, which causes the recruitment of TRAF6. TRAF6 is an E3 ubiquitin ligase that forms a complex with other E2 ubiquitin ligases and attaches polyubiquitin chains to IRAK1, TAB2, TAB3 and TAK1. The ubiquitination process (as mediated by TRAF6 and other E3 ligases) leads to the binding and oligomerization of TRAF6 with TAK1 and MEKK3, which activates the p38 MAPK, JNK and NF-κB pathways. IKK2 is then activated, which then initiates the formation of a complex consisting of IKK1, IKK2 and the NF-κB essential modifier (NEMO), which binds to polyubiquitin chains on IRAK1 and TAK1. The
inhibitor of nuclear factor B (IκB) is phosphorylated by activated IKK and polyubiquitinated and therefore degraded, which releases p65 and p50, the prototypic NF-κB subunits. Following translocation to the nucleus, the p65/p50 NF-κB complex binds to the promoters of genes coding for IκB and several proinflammatory genes such as TNF-α, IL-6, IL-8, MCP-1 and COX-2. A summary of the IL-1 receptors and their ligands is shown on Table 4.

Table 1: Important IL-1-related cytokines for this project, their receptors, and their overall pro or anti-inflammatory functions. Adapted from Dinarello, 2009.

<table>
<thead>
<tr>
<th>Name</th>
<th>Receptor</th>
<th>Property</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>IL-1R1</td>
<td>Pro-inflammatory</td>
</tr>
<tr>
<td>IL-1β</td>
<td>IL-1R1, IL-1R2</td>
<td>Pro-inflammatory</td>
</tr>
<tr>
<td>IL-1Ra</td>
<td>IL-1R1</td>
<td>Anti-inflammatory</td>
</tr>
</tbody>
</table>

Table 2: Important IL-1 receptors for this project, their common names, and their ligands. Adapted from Dinarello, 2009.

<table>
<thead>
<tr>
<th>Name</th>
<th>Common name</th>
<th>Ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1R1</td>
<td>Primary receptor</td>
<td>IL-1α, IL-1β, IL-1Ra</td>
</tr>
<tr>
<td>IL-1RAcP</td>
<td>Co-receptor</td>
<td>IL-1α, IL-1β, IL-1Ra</td>
</tr>
<tr>
<td>IL-1R2</td>
<td>Decoy receptor</td>
<td>IL-1β, pro-IL-1β</td>
</tr>
</tbody>
</table>
Figure 3
Figure 3: IL-1β production, processing and signalling pathways (A) Pathway by which LPS leads to the production and processing of active IL-1β by the canonical NLRP3 inflammasome. This figure was produced by Kawana et al., 2014\textsuperscript{42}. Permission to use the figure has been obtained from \textit{Austin Journal of Clinical Immunology} (Austin Publishing Group) (B) Signalling pathway downstream of IL-1β binding to IL-1R1. This figure was produced by Vicenova et al., 2009\textsuperscript{43}. Permission to use the figure has been obtained from \textit{Physiological Research}.

1.5.2 Mice deficient in IL-1β production

\textit{ASC} knockout mice (\textit{ASC}\textsuperscript{-/-}) lack the ability to assemble the NLRP3 inflammasome to process pro-IL-1β, and therefore \textit{ASC}\textsuperscript{-/-} peritoneal macrophages are unable to clear \textit{Listeria} bacteria, and do not produce active caspase-1, IL-1β or IL-18 when infected with \textit{Listeria}\textsuperscript{44}. \textit{Caspase-1} knockout mice (\textit{Casp1}\textsuperscript{-/-}) are unable to process pro-IL-1β. Adherent \textit{Casp1}\textsuperscript{-/-} monocytes also exhibited decreased production and no secretion of IL-1β, and decreased secretion of TNF-α and IL-6, 16 hours after stimulation by LPS with nigericin, which induces K\textsuperscript{+} efflux that activates the NLRP3 inflammasome. In certain cases, however, caspase-1 deficient mice do exhibit inflammasome-independent processing of IL-1β\textsuperscript{45}. In addition, these mice are also deficient in the caspase-11 gene\textsuperscript{46}, important for pyroptosis\textsuperscript{47}. Therefore, \textit{Casp1}\textsuperscript{-/-} mice may not be the most accurate model to study IL-1β function. On the other hand, \textit{IL-1β} knockout mice (\textit{Il1b}\textsuperscript{-/-}) are deficient only in IL-1β and are a better model. These mice have reduced inflammatory processes such as fever or expression of COX-2\textsuperscript{48}. \textit{IL-1RI} knockout mice (\textit{Il1r1}\textsuperscript{-/-}) are unable to initiate signalling in response to IL-1 stimulation\textsuperscript{49}, and is therefore also a suitable model to study IL-1 function.

1.5.3 Regulation of the NLRP3 inflammasome

NLRP3 has been shown to be upregulated in neutrophils, monocytes, macrophages, DCs\textsuperscript{50}, and to a lesser degree in non-immune cells\textsuperscript{51}. The NLRP3 inflammasome is part of the set of nod-like receptors (NLRs), which consist of 3 domains: the leucine-rich repeat domain (LRR), the nucleotide-binding domain (NBD), and the pyrin domain (PYD). The LRR binds and recognizes ligands that are responsible for the activation of the inflammasome, while the NBD is responsible for the oligomerization of NLRP3 to form the inflammasomal complex. Apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC) consists of a PYD, which interacts with the PYD of NLRP3 and is thus recruited as part of the inflammasome. ASC also consists of a caspase recruitment domain (CARD), which binds
and interacts with the CARD domain of procaspase-1, which is eventually cleaved such that only the active caspase domain remains. NLRP3 is activated upon the induction of 2 signals: the first one involves proinflammatory transcription factors such as nuclear-factor kappa B (NF-κB), while the second signal involves activation of the inflammasome or phosphorylation of ASC.

NLRP3 is regulated at the level of protein production. For example, ROS activates NF-κB outside of TLR4 signalling and increases production of NLRP3. Post-translational regulation of NLRP3 also occurs. A major regulator of the NLRP3 protein after its translation is its level of ubiquitination or deubiquitination, which would result in the degradation of the protein or inhibition of degradation respectively. Juliana et al. showed that the deubiquitination of NLRP3 by TLR4, ROS, and ATP is a major requirement in order for the inflammasome to elicit downstream functions.

The activation of NLRP3 can be regulated by the following processes: K⁺ efflux, ROS production, endoplasmic reticulum (ER) stress, mitochondrial dysfunction, Ca²⁺ levels, and lysosomal rupture (Fig. 3A). K⁺ efflux is induced by the activation of P2X7 receptors by ATP, which leads to the opening of pannexin hemichannels in the plasma membrane that release K⁺. This activates the NLRP3 inflammasome via downstream signalling pathways that involve PKR, SYK and JNK through mitochondrial adapter proteins. Increased levels of ROS oxidizes the thioredoxin (TRX) enzyme. TRX activates the ASK kinase enzyme to stimulate the MAP kinase and JNK pathways in order to phosphorylate ASC. Along with these mechanisms, increased ROS production oxidizes mitochondrial DNA, which is important in the activation of NLRP3. ROS might also activate double-stranded RNA-dependent protein kinase (PKR), which activates the inflammasome. ER stress is another important factor in NLRP3 activation. ER stress sensors, especially IRE1α, ATF6, PERK and IP3R, are known to increase NF-κB activation, ROS production, and JNK. Lysosomal rupture, which can be upregulated by ROS and cholesterol crystals, leads to the release of cathepsin B, which can either directly bind and activate NLRP3 or JNK.

1.5.4 Regulation of IL-1β production

IL-1β is produced by macrophages, neutrophils, lymphocytes, DCs, endothelial cells, fibroblasts and muscle cells, and the cytokine acts on monocytes, lymphocytes, DCs, granulocytes, epithelial cells, endothelial cells, fibroblasts and smooth muscle cells. IL-1β production can be stimulated through LPS signalling, and other non-TLR ligands such as
complement 5A (C5A).\textsuperscript{38,68} Regulators of NF-κB-mediated transcription downstream of LPS include NTP-Stat1 and IRF-8\textsuperscript{69}. In addition, IL-1α induces transcription of IL-1β\textsuperscript{70}, and the NF-κB response itself can be inhibited by synthesized IκB\textsuperscript{71}. Regulation of IL-1β processing is largely dependent on the NLRP3 inflammasome, which has been covered in detail in the previous section.

Secretion of IL-1β is also important but this process is still poorly understood. IL-1β lacks a signal sequence that allows it to be processed in the ER and Golgi. Therefore, IL-1β is not secreted through conventional pathways, but through an alternate pathway that involves vesicular transport\textsuperscript{72}. Andrei et al. showed that IL-1β secretion occurs through its internalization in endolysosomes or early lysosomes\textsuperscript{73}; their exocytosis was activated by ATP-induced K\textsuperscript{+} efflux\textsuperscript{74}. There is also evidence of IL-1β secretion as a consequence of autophagy, and this process was associated with heat shock proteins\textsuperscript{75} and ATP\textsuperscript{76}. The formation of a non-canonical inflammasome, which involves caspase-4, caspase-5 and caspase-11, activates caspase-11 that cleaves Gasdermin D, which in turn oligomerizes to form pores in the membrane. This process might also mediate IL-1β secretion, especially when cells undergo increased oxidative stress\textsuperscript{77,78}. Another type of cell death, necroptosis, has recently been proposed as a mechanism for ATP and K\textsuperscript{+} dependent IL-1β secretion\textsuperscript{79}.

IL-1β can also be regulated at the level of signalling. IL-1Ra can compete with IL-1α and IL-1β for IL-1R1 binding, but does not activate a downstream signalling response, since the IL-1RAcP is not recruited. In addition, IL-1 receptor type 2 (IL-1R2) lacks the TIR domain and thus sequesters IL-1 proteins without inducing an intracellular signalling response; therefore, it is considered an anti-inflammatory receptor (Table 4)\textsuperscript{74,80}. IL-1R1 can also bind an adaptor toll-interacting protein (TOLLIP) that inhibits signalling cascade molecule IRAK1\textsuperscript{81}, and MAP kinases\textsuperscript{82}.

1.5.5 Downstream functions of IL-1β

IL-1β is known to influence gene expression of many proinflammatory and acute phase genes, such as cytokines and their receptors, growth factors, clotting factors, tissue remodelling factors, extracellular matrix molecules, oncogenes, and mediators of lipid synthesis\textsuperscript{83,84}. IL-1β signalling increases expression of adhesion molecules such as VCAM-1, ICAM-1 and E-selectin, cytokine receptors such as those for IL-2, IL-3, and GM-CSF, and specific enzymes such as COX-2 and PLA2\textsuperscript{83}. IL-1β signalling also leads to the production of iNOS, which inhibits the contractile response in aortic smooth muscle cells\textsuperscript{85}. IL-1β-mediated NOS
production was shown to affect fever levels, sleep patterns, hormone regulation, anabolism, and apoptosis\textsuperscript{83}. IL-1β can alter gene expression profiles and cell markers, thus priming leukocytes to perform their appropriate functions. For example, IL-1β can upregulate CD40, OX40L, SLAM and IL-12 on DCs in order to influence their ability to stimulate T cells. Mast cells respond to IL-1β by secreting TNF-α, IL-3, IL-5 and IL-6 in order to mediate the response to allergens\textsuperscript{86}. IL-1β is also required in order to stimulate Th17 cells and the production of IL-17 by Th17 cells\textsuperscript{87}, in an IL-6-dependent manner\textsuperscript{88}, and in T cell-mediated activation of B cells for antibody production\textsuperscript{89}.

1.5.6 Involvement of IL-1 and related cytokines in atherosclerosis

Multiple studies have shown the involvement of the NLRP3 inflammasome in the process of atherosclerosis. In patients with coronary atherosclerosis, the NLRP3 inflammasome was highly expressed in plaques, and was correlated with risk factors that lead to atherosclerosis\textsuperscript{90}. Other components of the inflammasome, such as ASC and caspase-1, as well as IL-1β and IL-18, were also upregulated\textsuperscript{91}. When LDLR-deficient mice were transplanted with bone marrow deficient in NLRP3 or any of its components, atherosclerotic lesions were decreased\textsuperscript{92, 93}. NLRP3 inflammasomes could be activated by cholesterol crystals in macrophages\textsuperscript{92}. Cholesterol crystals produced neutrophil extracellular traps that primed macrophages to produce IL-1β which exacerbated atherosclerosis\textsuperscript{94}. In addition, deficiency of caspase-1 in ApoE-deficient mice was shown to decrease atherosclerotic lesions, along with a lesser expression of IFN-γ and MHC II within those lesions\textsuperscript{95}. However, another study showed that typical features of atherosclerosis, such as lesion size and macrophage infiltration, was not altered in mice deficient in NLRP3, ASC and caspase-1; these discrepancies could be due to differences in experimental conditions\textsuperscript{96}.

There has been a considerable number of studies that have implicated the role of IL-1β in mediating atherogenesis, in both human and mouse models. Early studies showed that IL-1β was induced by bacterial endotoxin and TNF-α in human vascular endothelial cells\textsuperscript{97} and smooth muscle cells\textsuperscript{98}, and induced proliferation of the smooth muscle\textsuperscript{99}, a common occurrence in late atherosclerotic lesions. It was also found that IL-1β increased procoagulant activity and leukocyte adhesion on vascular endothelial cells\textsuperscript{100}. Later studies showed that lesion size and area were decreased in mice deficient in ApoE and IL-1β\textsuperscript{101}, as well as in IL-1R1-deficient mice\textsuperscript{102}. Lesion size was increased in mice heterozygous for the IL-1RN gene\textsuperscript{103}. Since IL-1β had a critical role to play in the process of atherosclerosis, it was proposed that this cytokine
could be a potential target against cardiovascular disease. Initially, doses of recombinant IL-1Ra (anakinra) was shown to improve the phenotype of patients with various illnesses; however, a much more specific target to IL-1β would be its function-blocking antibody, also known as canakinumab (a monoclonal antibody against IL-1β). The CANTOS trial showed that 3 subcutaneous administrations of canakinumab in humans who have had previous myocardial infarctions led to a decreased level of C-reactive protein (marker of inflammation and cardiovascular disease) and reduced incidence of future myocardial infarctions and strokes\textsuperscript{104}. Although these studies are in agreement with others on the critical role of IL-1β in atherogenesis, a study suggested a protective function for IL-1β signalling since deficiency of interleukin-1 receptor type I (IL-1R1) led to greater plaque instability\textsuperscript{105}.

IL-18 is another cytokine produced by the NLRP3 inflammasome, and is also shown to be involved in atherosclerotic processes. Human studies showed that levels of IL-18 in the plasma are good predictors of cardiovascular diseases\textsuperscript{106}. IL-18 is expressed in human plaques, with greater expression levels in unstable plaques\textsuperscript{107,108}. In addition, when IL-18 was inhibited by binding to its binding protein IL-18BP, lesion and fatty streak development in ApoE-deficient mice was inhibited, along with a decrease in macrophages and T cells\textsuperscript{109}.

1.6 Rationale

A previous student in our laboratory published data showing that an intravenous injection of IL-1β into mice, or \textit{ex vivo} incubation of aortas of mice with IL-1β, elicits RTM of intimal DCs in the lesser curvature of the aortic arch (Fig. 4A, 4B). He also showed that RTM of DCs is inhibited in ASC knockout mice incubated with LPS (Fig. 4C), and IL-1β, but not TNF-α, rescued this inhibition\textsuperscript{33}. Moreover, IL-1β could induce RTM of DCs under hypercholesterolemic conditions (not shown). This work suggests that IL-1β is an essential component for LPS-induced RTM of intimal DCs. Additional experiments are required to confirm these findings. Furthermore, studies are necessary to determine if IL-1β signalling in endothelial cells, DCs or both cell types is required for RTM.

1.7 Hypothesis

IL-1β production and signalling in the arterial intima is required for LPS-induced RTM of intimal DCs.
1.8 Aims

1. Investigate the expression of IL-1β, other IL-1 family members (IL-1α and IL-1Ra) and proinflammatory genes in the aortic intima throughout the process of LPS-induced RTM by an intravenous injection of LPS.

2. Investigate the function of IL-1β in LPS-induced RTM using genetic strategies and function-blocking antibodies.

3. Ascertain whether bone marrow-derived or artery wall cells produce and respond to IL-1β.

4. Investigate the local functions of IL-1β in LPS-induced RTM by an *ex vivo* approach.
Figure 4: IL-1β is able to induce RTM of CD11c⁺ intimal DC. (A) Wild-type (C57BL/6) mice were IV injected with either PBS or different doses of IL-1β. (B) Aortas from wild-type mice were incubated *ex vivo* for 12 hours at 37°C with PBS or IL-1β. (C) Aortas from ASC⁻/⁻ mice were incubated *ex vivo* for 12 hours at 37°C with PBS, 50 µg/mL LPS, 50 ng/mL IL-1β and 500 ng/mL TNF-α. For all graphs, the number of CD11c⁺ cells was enumerated using *en face* immunoconfocal microscopy. All *ex vivo* incubation took place in RPMI, 10% FBS, and 1% penicillin-streptomycin. Values represent mean ± SEM. n > 6. ** p < 0.01; *** p < 0.001. *This figure is adapted from Mark Roufaiel’s thesis.*
2 Methods

2.1 Reagents and Mice

Ultrapure \textit{E. coli} 011:B4 lipopolysaccharide (LPS) was purchased from Invivogen (cat. no. tlrl-pelps). Recombinant mouse IL-1\(\beta\) (cat. no. 401-ML/CF) was purchased from R&D systems. Polyclonal Armenian hamster IgG (cat. no. BE0091), Armenian hamster anti-mouse IL-1\(\alpha\) (clone no. ALF-161, cat. no. BE0243) and Armenian hamster anti-mouse IL-1\(\beta\) (clone no. B122, cat. no. BE0246) blocking antibodies were purchased from BioCell. These reagents were injected intravenously (i.v.) via a tail vein of mice, or used in \textit{ex vivo} incubations of aortas.

For immunostaining experiments, conjugated antibodies to mouse antigens were used. Alexa Fluor 647 conjugated antibodies to CD11c (cat. no. 117312) and CD45 (cat. no. 103124) were purchased from BioLegend. Biotinylated anti-CD11c antibodies (cat. no. 553800) were purchased from BD Pharmingen. The TSA\textsuperscript{TM} Fluorescein System – Tyramide Signal Amplification Kit purchased from Perkin Elmer was used after staining with biotinylated antibodies. The reagents in the kit were FITC-conjugated Tyramide reagent (Tyr-FITC), amplification buffer, and streptavidin-conjugated horseradish peroxidase (SA-HRP). Hoechst 33342 was purchased from Molecular Probes, Invitrogen.

For gene expression analysis by qPCR, intimal cells were harvested (see below) using Liberase\textsuperscript{TM} and DNase I enzymes (research grade, Roche), and FITC beads (Polysciences). RNA was isolated and converted to cDNA using reagents from the RNeasy micro RNA extraction kit (Qiagen) and the SuperScript III RT kit (Invitrogen), and the LightCycler 480 SYBR Green I master mix (Roche) was used in qPCR experiments. In addition, genotyping experiments involved a Long-Range Hot Start PCR kit (KAPA). Isolation of RNA from peritoneal macrophages was performed using the E.Z.N.A. total RNA kit (Omega Bio-Tek).

All mice were used between 11 and 13 weeks of age. \textit{C57BL/6} (WT) mice were either bred in the Animal Resource Centre (ARC) in the Toronto Medical Discovery Tower (TMDT), or purchased from Ontario Cancer Institute or Jackson Laboratories (JAX) (stock no. 000664). Breeding pairs of \textit{Casp1}\textsuperscript{-/-} (stock no. 016621) and \textit{Il1r}\textsuperscript{1/-} (stock no. 003245) mice were purchased from JAX, and breeding pairs of \textit{Il1b}\textsuperscript{-/-} mice were generously provided by the Delaney Lab at McMaster University, Hamilton, Ontario. All mice were housed and bred at the ARC in the TMDT, and were fed a standard rodent chow. For experiments measuring the first wave of RTM (Fig. 6A, 6B), \textit{Il1b}\textsuperscript{-/-} mice were bred, housed and obtained from the quarantine facility in the Ontario Cancer Institute, due to a diagnosis of \textit{Helicobacter} infection. Pups from
infected IIIb−/− mice were negative for Helicobacter infection and were subsequently used for most experiments. All protocols were performed according to the guidelines of the Canadian Council of Animal Care.

2.2 Mouse Injections

It was previously shown that RTM of intimal DCs is not only induced by LPS through TLR4, but by other TLR or NLR agonists. Therefore ultrapure E. coli LPS was used throughout this project to focus on TLR4-mediated processes. The dose of LPS (30 µg) used throughout this work is lower by more than 3 folds compared to the previous published work since this dose was sufficient to elicit a decrease in CD11c+ cells that represented RTM and avoided occasional lethal effects observed with the higher doses. Mice were also injected with 100 µg anti-IL-1β, anti-IL-1α antibodies or IgG, or 50 ng IL-1β, during the appropriate experiments. All reagents were diluted in 200 µL PBS. All injections were performed i.v. via a tail vein.

2.3 Harvesting of aortae

Mice were sacrificed by isoflurane overdose or CO2 prior to perfusion at 1.5 psi, through the left ventricle with cold PBS and 4% paraformaldehyde (PFA) in order to fix the tissues. The entire aortic arch and surrounding tissue was removed from sacrificed mice, and placed in 1 mL of 4% PFA for 1 hour, before the ascending aortic arch was isolated by removing the surrounding fat under a dissection microscope, and cutting along the segments indicated on Figure 1A. For ex vivo procedures, aortic arch samples were not fixed.

2.4 Collection of tissue for genotyping

In order to verify the genotypes of mice, a piece of earlobe was isolated from mice, using an ear puncher. Earlobes were incubated overnight in 55°C with proteinase K in order to digest the proteins. The extracts were heated for 10 min at 95°C, before they were diluted in PCR-grade H2O and centrifuged at 1400 rpm for 10 min.

2.5 Reciprocal Bone Marrow Transplantation

All dissection instruments used in the procedures were sterilized with 70% ethanol.
Recipient mice (5 – 7 week old) were irradiated for 12 min in a gamma cell irradiator by exposure to 10 Grays of gamma irradiation by a radioactive cesium 137 source. Donor mice were sacrificed, and their tibia and femur bones were washed in ethanol and 1X sterile PBS. After the bones were further cleaned from surrounding muscles, RPMI (10% FBS, 1% penicillin-streptomycin) were used to flush out the marrow from the femur and tibia. Cells were layered on a 70 μm strainer and topped to 10 mL. Cells were counted using a hemocytometer, after dilution (1:5) of an aliquot with crystal violet in order to avoid counting red blood cells. After spinning for 10 min at 300 g, 5 X 10⁶ cells in RPMI (300 µL) were injected into the irradiated recipient mice. Experiments were performed after recipient mice recovered for 6 weeks.

2.6 Ex Vivo Experiments

Mice were perfused in the left ventricle with Krebs ringer buffer solution containing 10% FBS, 1% HEPES. Isolation of the aortic arch was performed as described in 2.3, but the aortae were not fixed, and the ascending arches were left intact and placed in 2 mL Krebs ringer buffer containing 10% FBS, 1% HEPES, 1% Penicillin-Streptomycin, with or without 25 µg/mL LPS, or 50 ng/mL IL-1β for up to 18 hours, with shaking at 120 rpm, at 37°C. The aortae were then placed in 4% PFA for 1 hour, and prepared for en face confocal analysis as in 2.3.

2.7 En Face Immunostaining and Confocal Microscopy

All fixed aortae were placed on a shaker in 1.5 mL Eppendorf tubes, where they were immersed in 0.5% Triton X-100 for 5 minutes to permeabilize the cells and 3% hydrogen peroxide for 30 minutes to quench any peroxidase activity. Immunostaining was performed with biotinylated CD11c antibody (1:100) in 10% goat serum overnight followed by SA-HRP (1:200) for 30 minutes, Tyr-FITC (1:100) for 8 minutes, and Hoechst (1:400) for 25 minutes. Alexa Fluor 647 conjugated antibodies were also used in certain experiments (1:150 anti-CD11c, 1:100 anti-CD45). After each incubation step, there were 3 washes in PBS for 4 min each and 5 washes after the steps involving overnight Ab incubation and shaking with Tyr-FITC. The aortae were flattened and mounted on slides, and imaged under the confocal microscope with a Nikon A1R+ Resonance Scanning Confocal 40X oil (N.A. 1.3), and lasers emitted light of wavelengths 405, 488 and 640 nm, for Hoescht, Tyr-FITC and Alexa Fluor 647 staining respectively. Z-stack images (1 μm each) were compiled to a single image and stitched
to cover the LC area (Nikon NIS Elements C). The CD11c+ cells were counted using the ImageJ software (National Institute of Health, version 1.50i).

2.8 Isolation of RNA from Intimal Cells

After perfusion of mice with 10 mL of cold PBS, aortae were cleaned in cold PBS containing 1 mM aurintricarboxylic acid (ATA), a ribonuclease inhibitor, on a silicone-coated dissection dish. Aortae were then pinned down on the dish with intima facing up and 2 µL DNase I and 100 µL of 25 µg/mL Liberase Blendzyme were added for 10 min at 37°C in order to digest extracellular matrix molecules. After washing with PBS/ATA, a needle was used to gently scrape cells off the intima, covered in FITC beads. RNA carrier (0.04%) and β-mercaptoethanol were added prior to RNA isolation using the Qiagen microRNeasy kit, according to the supplier’s protocol.

2.9 RT-qPCR

Random primers were added to RNA samples isolated from the intima and reverse transcription (RT) of mRNA was performed using the SuperScript III RT kit. The resulting cDNA (diluted 1:10) was used for gene expression analysis by real-time PCR (qPCR) using LightCycler 480 SYBR Green I master mix, along with forward and reverse primers that span exon-exon junctions. Primers for qPCR are listed in Table 1. Roche LightCycler 480 was used for qPCR; the settings required for SYBR Green I amplification are: 95°C for 5 minutes initially, then 45 cycles of 95°C for 10 seconds, 60°C for 20 seconds and 72°C for 30 seconds. Analysis of the relative amounts of mRNA was performed using the Roche LightCycler 480 software, and the comparative standard curve method. Standard curves were prepared by measuring 10-fold serial dilutions of mRNA levels from the heart, lungs, liver and lymph nodes of C57BL/6 mice after injections with 10 µg LPS for 2 hours. Relative amounts of mRNA expression values were calculated after normalizing to VE-cadherin.

In order to genotype Casp1−/− and Il1r1−/− mice, DNA samples (section 2.4) were amplified by PCR (GeneAmp PCR System 9700, Applied Biosystems), using the primer sets indicated in Table 2, and reagents from the KAPA Long-Range HotStart PCR kit, as specified in the Jackson Laboratories website. 10X loading dye was added to the amplified PCR samples, before they were loaded onto a 2% agarose gel consisting of a 1:10000 dilution of Gel Red, a UV-activated fluorophore that binds to DNA. After electrophoresis at 100V, gels were placed
under a camera and bands were visualized under UV light (DNR Bio Imaging Systems, MiniBIS Pro). In order to genotype $Il1b^{-/-}$ mice, qPCR was performed. WT and mutant genotypes were determined by distinct melt curve profiles.

2.10 Isolation of Peritoneal Macrophages

Thioglycollate-elicited peritoneal macrophages were harvested by Kenneth Ting (PhD candidate) in our laboratory from C57BL/6 mice, as previously described. Cells ($1 \times 10^6$/well in 24-well plates) were cultured overnight in DMEM (Wisent, QC) containing 10% FBS, 2 mM L-glutamine and 10000U/mL Penicillin-streptomycin. After washing off the non-adherent cells, adhered peritoneal macrophages were incubated in the same media with 10.6 ng/mL IL-1β, with control IgG, anti-IL-1β or anti-IL-1α function blocking antibodies (10 µg) for 1h prior to lysis and RNA purification according to the protocol provided by the supplier of the E.Z.N.A. total RNA kit.
**Table 3:** Primer sequences used to measure mRNA expression levels

<table>
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<tr>
<th>Gene</th>
<th>Primer</th>
<th>5’ – 3’ sequence</th>
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<tbody>
<tr>
<td>VE-CAD</td>
<td>Forward</td>
<td>GAAAACCAGAAGAAACCGCTGAT</td>
</tr>
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<td></td>
<td>Reverse</td>
<td>CACTGCTTTGCGGATGGA</td>
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<tr>
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<tr>
<td></td>
<td>Reverse</td>
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<tr>
<td></td>
<td>Reverse</td>
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<td></td>
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<tr>
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<td>Reverse</td>
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<tr>
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</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CCTTGAAGTTGACGGAAGAGT</td>
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**Table 4:** Primer sequences used for genotyping mice

<table>
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<tr>
<th>Mouse Strain</th>
<th>Primer</th>
<th>5’ – 3’ sequence</th>
</tr>
</thead>
<tbody>
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<td>Casp1&lt;sup&gt;−/−&lt;/sup&gt;</td>
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<tr>
<td>Il1b&lt;sup&gt;−/−&lt;/sup&gt;</td>
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<tr>
<td>Il1r1&lt;sup&gt;−/−&lt;/sup&gt;</td>
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</tr>
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</table>
3 Results

The purpose of this project was to study the role of IL-1β in LPS-induced RTM of intimal DCs. Our laboratory previously published that an injection of LPS into C57BL/6 mice induced 2 waves of RTM of intimal CD11c⁺ myeloid cells, which occurred at 12h - 24h and 60h - 72h (Fig. 2A). The decrease in CD11c⁺ cells was not attributed to programmed cell death, as shown by TUNNEL staining and CD11c⁻hBcl2 mice, and intimal DCs were collected from cannulated aortas exposed to a flow of LPS (Fig. 2C). In this study, genetic and antibody blockade approaches were used in order to study the importance of IL-1β during the two waves of LPS-induced RTM. The importance of IL-1β expression in hematopoietic versus non-hematopoietic compartments was assessed by bone marrow transplantation (BMT) using mice deficient in IL-1β and IL-1R1.

3.1 Intimal expression of IL-1β and other pro-inflammatory genes during LPS-induced RTM of intimal DCs

The mRNA expression of intimal IL-1β as well as other genes of the IL-1 family was assessed at different time points throughout RTM in order to correlate expression with the two distinct waves of RTM. In peritoneal macrophages, it is known that LPS induces a time-dependent production of IL-1β, TNF-α and IL-6. The inflammatory landscape of the intima was further characterized by similar analyses of mRNA expression levels of other pro-inflammatory genes that are typically induced by LPS in other myeloid cell systems.

3.1.1 LPS induces increases in the intimal expression of IL-1 genes during the first wave of RTM

Figure 5A shows that LPS induced intimal mRNA expression of IL-1β rapidly, with two peaks within 12 hours. The first peak (200-fold increase relative to baseline) occurred at 3h followed by a second peak (about 175-fold increase) at 9h. These oscillations in IL-1β induction preceded and coincided with the first wave of RTM, which occurs between 6 and 12h post-LPS (Fig. 5A). Expression levels gradually increased further up to 48h and during the second waves of RTM at 72h; however, this increase was not statistically significant (Fig. 5A). mRNA levels of IL-1α, another cytokine binding to IL-1R1, was induced 20-fold after 3h, coinciding with the first peak of IL-1β mRNA induction, while the second peak appeared more sustained, reaching maximal expression 24h post-LPS (Fig. 5B). These changes were
accompanied by a rapid induction of IL-1Ra mRNA levels which showed a similar profile to IL-1α (Fig. 5C). Expression levels of IL-1α and IL-1Ra mRNA gradually declined and reached baseline levels by 96h. There appeared to be a decrease in IL-1Ra mRNA between 56 and 60 hours, which precedes the second wave of RTM.

The mRNA expression profiles of the IL-1 receptors were also assessed within the duration of the two-waves of LPS-induced RTM. While relatively small induction of IL-1R1 was observed over the first wave of RTM (Fig. 6A), no significant changes were observed in the mRNA levels of IL-1RAcP, the co-receptor (Fig. 6B). Interestingly, a sustained and significant increase of IL-1R2 (decoy receptor) mRNA levels, up to 40-fold, was measured following the first wave of RTM, with peak expression at 48h (Fig. 6C). Subsequently expression declined coincident with the second wave of RTM (between 54 and 72 hours post-LPS) and a second peak was observed at 72h.

3.1.2 LPS induces increases in gene expression of other pro-inflammatory cytokines

The initial bursts that are of intimal IL-1β mRNA expression suggest that it has a critical function in the initiation of LPS-induced RTM. TNF-α is a pro-inflammatory cytokine whose transcription and translation is induced downstream of TLR4 and IL-1R1 signalling. In agreement with a potential requirement for local signalling, LPS-induced TNF-α mRNA expression levels displayed a similar oscillatory characteristic of the two peaks mRNA of IL-1β mRNA expression at 3 and 9h post-LPS (Fig. 7A). The LPS-induced mRNA expression of IL-6 mRNA, another proinflammatory cytokine whose expression is regulated by IL-1β, displayed an induction profile that was similar to that of IL-1α (Fig. 7B). Like IL-1β, IL-18 is a proinflammatory cytokine with the pro-IL-18 cleaved by caspase-1 in the NLRP3 inflammasome complex. Modest increases in IL-18 mRNA expression levels were observed at and beyond 72h post-LPS, following the second wave of RTM (Fig. 7C).

Overall, mRNA of IL-1β and other proinflammatory cytokines (IL-1α, TNFα and IL-6) is induced dramatically in the arterial intima prior to and during the first wave of RTM (6 – 12 hours post-LPS). This coincides with induction of IL-1RA and IL-1R2, which are inhibitors of IL-1 signalling. Although a peak of IL-1β mRNA expression was not observed prior to the second wave of RTM (54 – 72 hours post-LPS), expression was still elevated relative to baseline levels and the expression of IL-1RA and IL-1R2 declined at 54 and 60h post-LPS, which may account for IL-1R1 signalling coincident with the second wave of RTM.
Figure 5: Time-course of gene expression profiles for (A) IL-1β, (B) IL-1α and (C) IL-1Ra in WT mice following LPS injection. Mice were injected iv with 30 µg of LPS, and the intima was micro-dissected at sequential time points. Intimal cell RNA was isolated, and gene expression levels were determined by reverse transcription and qPCR. All values are normalized to the endothelial VE-cadherin (Cad5) mRNA levels and plotted relative to the expression level at 0 h (no injection). Values represent mean ± SEM. n = 5 - 6. Statistically significant differences from the 0 h time point were determined using one-way ANOVA with Dunnett’s multiple comparison test; * p<0.05; ** p<0.01; *** p<0.001.
Figure 6: Time-course of gene expression profiles for (A) IL-1R1, (B) IL-1RAcP and (C) IL-1R2 in WT mice following LPS injection. Mice were injected iv with 30 µg of LPS, and the intima was micro-dissected at sequential time points. Intimal cell RNA was isolated, and gene expression levels were determined by reverse transcription and qPCR. All values are normalized to the endothelial VE-cadherin (Cad5) mRNA levels and plotted relative to the expression level at 0 h (no injection). Values represent mean ± SEM. n = 5 - 6. Statistically significant differences from the 0 h time point were determined using one-way ANOVA with Dunnett’s multiple comparison test; * p<0.05; *** p<0.001.
Figure 7: Time-course of gene expression profiles for (A) TNF-α, (B) IL-6 and (C) IL-18 in WT mice following LPS injection. Mice were injected iv with 30 µg of LPS, and the intima was micro-dissected at sequential time points. Intimal cell RNA was isolated, and gene expression levels were determined by reverse transcription and qPCR. All values are normalized to the endothelial VE-cadherin (Cad5) mRNA levels and plotted relative to the expression level at 0 h (no injection). Values represent mean ± SEM. n = 5 - 6. Statistically significant differences from the 0 h time point were determined using one-way ANOVA with Dunnett’s multiple comparison test; *** p<0.001.
3.2 IL-1β is required to elicit the first wave of RTM induced by LPS

Previous data showed that incubation of aortas from ASC−/− mice with LPS ex vivo did not elicit RTM (Fig. 4C), and the same result was shown in vivo upon injection of LPS (data not shown). IL-1β rescued RTM in ASC−/− mice pointing to a critical role for IL-1β in this process. In this study other mouse models were used that are deficient in IL-1β production, processing and signaling: Il1b−/−, Casp1−/− and Il1r1−/− mice, respectively and compared to WT mice. Aortae from WT mice were harvested at 24h post-LPS injection and stained for CD11c and analyzed by en face confocal microscopy (Fig. 8A, 8B). As shown in a representative confocal image (Fig. 8A) and the cumulative data (Fig. 8B), numbers of CD11c+ cells in the LC of the aortic arch decreased by 31% in LPS injected mice relative to PBS. This decrease was minimal and lacked statistical significance between PBS and LPS-injected groups in Casp1−/−, Il1b−/− and Il1r1−/− mice. It is to note that the baseline level of CD11c+ cells in Casp1−/− mice was significantly lower (Fig. 8B), which suggested a role for Caspase-1 in homeostasis of intimal DCs.

As a second approach, antibody blockade experiments were performed with function-blocking antibodies neutralizing IL-1β or IL-1α or control IgG. First, the specificity of these function blocking antibodies were tested with thioglycollate-elicited peritoneal macrophages that were stimulated with purified recombinant mouse IL-1β for 2h. (Fig. 9A). IL-1β induced IL-1β and TNF-α mRNA in these cells. The induction of these genes was not detectable when cells were pre-incubated with the function-blocking antibodies against IL-1β, when compared to the mRNA levels with control IgG. In contrast, anti-IL-1α blocking Abs did not affect IL-1β and TNF-α gene expression. This experiment confirmed that there is no cross-reactivity between the IL-1α and IL-1β blocking antibodies. Subsequently, WT mice were injected with LPS and the antibodies tested above, and the aortic arches were harvested 24h later (Fig. 9B). As expected, CD11c+ intimal cell numbers were reduced significantly (25%) when LPS was injected along with IgG control for 24h, relative to the intimal cell numbers with IgG alone, indicating that IgG injection did not affect RTM. However, neutralizing IL-1β or IL-1α significantly blocked LPS-induced RTM. These data are consistent with the findings in Figure 8B that signaling through IL-1R1 is required for LPS-induced RTM. Interestingly, these data suggest that IL-1α or IL-1β are equally critical for the first wave of LPS-induced RTM. In the future, the role of IL-1α needs to be tested in Il1a−/− mice.
3.3 IL-1β is also required to elicit the second wave of RTM induced by LPS

Figure 5A showed that gene expression levels of IL-1β were upregulated before the first wave of RTM. During the second wave (60h - 72h) the increase in IL-1β mRNA levels were not statistically significant. However, the mRNA expression profiles for pro-IL-1β do not provide direct information about the levels of mature IL-1β protein that can be actively involved in the subsequent second wave of LPS-induced RTM. Therefore, experiments were performed to determine whether IL-1β is required for the second wave of RTM at 72h post-LPS, using Casp1−/− and Il1b−/− mice (Fig. 8C). Previously published data indicated a decrease in CD11c+ cells at 60 – 72h after an injection of 100 µg LPS (Fig. 2A); an injection of 30 µg of ultrapure LPS produced the same result (data not shown). There was no significant decrease in intimal CD11c+ cell numbers in both Casp1−/− and Il1b−/− mice, suggesting that the second wave of LPS-induced RTM may require IL-1β (Fig. 8C). The drawback from these experiments is that with the knockout mice used, the first wave is already inhibited which may prevent the second wave even if IL-1β was not directly involved. Therefore, a second approach with function-blocking antibodies was used. LPS or PBS was injected to WT mice for 72h and anti-IL-1β or anti-IL-1α blocking antibodies or IgG was injected 48h post-LPS for 24h (Fig. 9C). This protocol allowed for the first wave of LPS-induced RTM to occur prior to adding the blocking antibodies for testing the second wave. The data generated was similar to Figure 9B; RTM of intimal DCs occurred in the aortas of WT mice injected with LPS and IgG, while there was inhibition of RTM when LPS was injected with anti-IL-1β or anti-IL-1α antibodies (Fig. 9C). Overall, these data confirmed that both IL-1β and IL-1α are required for the first and second waves of LPS-induced RTM. The fact that both IL-1β and IL-1α signal through the same receptor, IL-1R1, suggest that common signaling molecule(s) or secreted product(s) produced through the same receptor signaling pathway may be critical for LPS-induced RTM (see the scheme in Discussion).
Figure 8: Both waves of RTM are inhibited in Casp1<sup>−/−</sup>, Il1b<sup>−/−</sup> and Il1r1<sup>−/−</sup> mice
(A) Representative tiled en face confocal microscopy images showing CD11c<sup>+</sup> cells in the entire lesser curvature of the ascending aorta of WT mice injected with either PBS or LPS. The absolute number of CD11c<sup>+</sup> cells was determined in the LC of the aortic arch in WT mice and mice deficient in caspase-1, IL-1β or IL-1R1 at 24 hours (B) and 72 hours (C) after iv injection of PBS or LPS (30 µg). Schematic plan for all experiments performed are above the graphs. Aortas were stained with Alexa-647 anti-CD11c Ab (WT, Casp1<sup>−/−</sup> mice) or biotinylated anti-CD11c Ab (Il1b<sup>−/−</sup>, Il1r1<sup>−/−</sup> mice). Values represent mean ± SEM. n = 5 – 9. Statistically significant differences were determined using one-way ANOVA with the Bonferroni post-hoc test; * p<0.05.
Figure 9
**Figure 9:** Both waves of RTM are inhibited in mice injected with neutralizing antibodies to IL-1β and IL-1α. (A) Gene expression levels of TNF-α and IL-1β after peritoneal macrophages from WT mice were incubated with IgG, anti-IL-1β or anti-IL-1α antibodies and stimulated with IL-1β for 2h. (B) Absolute numbers of CD11c+ cells was determined in WT mice at 24 hours after iv injection of PBS or LPS (30 µg), immediately followed by another injection of IgG, anti-IL-1β or anti-IL-1α antibodies (100 µg). (C) Absolute numbers of CD11c+ cells was determined in WT mice at 72 hours after iv injection of PBS or LPS (30 µg), when IgG, anti-IL-1β or anti-IL-1α antibodies (100 µg) were injected at 48h post-LPS. Schematic plan for all experiments performed are above the graphs. Aortas were stained with biotinylated anti-CD11c Ab. Values represent mean ± SEM. n = 7 – 8. Statistically significant differences were determined using one-way ANOVA with the Bonferroni post-hoc test; * p<0.05.

3.4 The production of IL-1β and signaling through its receptor in non-hematopoietic cells, as opposed to bone marrow-derived cells, is required for the first wave of RTM of intimal DCs

Roufaiel et al. showed that CCL19-CCR7 interactions were essential to mediate RTM of intimal DCs. They also showed that CCL19 production and signalling via CCR7 receptors on DCs that belong to the hematopoietic compartment, as opposed to on artery wall cells, was essential for RTM. Therefore, it was of interest to ascertain whether IL-1β production and signalling in artery wall cells or bone marrow cells was required for RTM. In the following experiments, reciprocal BMT was performed on lethally-irradiated recipient WT, Il1b−/− or Il1r1−/− mice, which were transplanted with WT or knockout bone marrow. After 6-7 weeks of recovery, the mice were injected with LPS or PBS and their aortas were harvested 24h later and stained for CD11c (Fig. 10A). The table in Figure 10A lists the phenotypes of the three types of chimeric mice produced. In WT recipient mice transplanted with WT bone marrow, a decrease in intimal DC numbers was observed 24h after an injection of LPS, which is consistent with RTM. This experiment confirmed that BMT procedure did not affect the process of RTM (Fig. 10B, 10C). In WT recipient mice transplanted with Il1b−/− or Il1r1−/− bone marrow, a decrease in intimal Cd11c+ cells was observed, consistent with RTM, but in Il1b−/− or Il1r1−/− recipient mice transplanted with WT bone marrow, a decrease in intimal Cd11c+ cells was not observed, suggesting that RTM was inhibited (Fig. 10B, 10C). Therefore, these results showed that RTM of intimal DCs was elicited through IL-1β and its receptor IL-1R1 in the non-hematopoietic...
compartment as opposed to bone marrow-derived cells. Since the intima consists of endothelial cells, and leukocytes that are derived from bone marrow, these data suggest that IL-1β production and signalling on endothelial cells, not intimal DCs, was responsible for RTM.

**Figure 10:** Reciprocal bone marrow transplantation experiments between WT and KO mice. (A) Schematic of reciprocal bone marrow transplantation and subsequent assessment of RTM, and table explaining cells expressing deficiency of genes in each group of mice with transplanted bone marrow. The absolute number of intimal CD11c⁺ cells (stained with biotinylated CD11c Ab) in *Il1b⁻/⁻* (B) or *Il1r1⁻/⁻* (C) chimeric mice was enumerated in the LC of the ascending aortic arch. Mice were injected with either PBS or LPS 24h before analysis. Values represent mean ± SEM. n = 3 – 8. Statistically significant differences were determined using the Student’s t-test, between PBS and LPS-injected groups. * p<0.05; ** p<0.01.
3.5. Local effects of IL-1β on intimal cells are required for RTM of intimal DCs

It is most likely that intravenous injection of LPS into mice elicits production of IL-1β and other cytokines not only in the intima but rather systemically, in multiple organs, e.g., liver and spleen, resulting in elevated IL-1β circulating in the blood\textsuperscript{114,115,116}. In fact, it was shown that an injection of LPS in mice led to an increase in serum TNF-α at 1h - 2h, followed by an increase in serum IL-1β and IL-6 at 4h onward\textsuperscript{117,118}. Thus, systemically produced IL-1β may induce other proinflammatory cytokines, which could possibly mediate RTM of intimal DCs. The data presented in Figures 8 and 9 do not address these considerations, i.e., whether other critical cytokines may be induced by LPS or IL-1β signalling systemically or locally in the aorta. While an injection of LPS is not sufficient to induce RTM in II\(1b^{-/-}\) mice, an injection of LPS followed by 2 injections of IL-1β at 3h and 9h post-LPS, corresponding to the increase in local IL-1β gene expression in the intima (Fig. 11A), did elicit a decrease in CD11c\(^+\) cells. This result suggested that local IL-1β signalling in the intima at those time points is required for RTM of intimal DCs. However, unlike in WT mice, where RTM was induced upon a single injection of IL-1β 24h prior to harvesting aortas (Fig. 11B), this injection did not induce RTM in II\(1b^{-/-}\) mice (Fig. 11C). We speculated that this result is because i.v. injected LPS or IL-1β cannot induce local production of IL-1β in II\(1b^{-/-}\) mice, in contrast to WT mice, and furthermore, LPS or IL-1β are cleared rapidly from the blood after a single i.v. injection. Circulating TNFα and IL-1β produced in response to injected LPS are also cleared rapidly\textsuperscript{119,120}. Therefore, \textit{ex vivo} experiments were performed using aortas harvested from II\(1b^{-/-}\) mice and incubated with PBS, LPS or IL-1β. The \textit{ex vivo} incubation will maintain a constant level of IL-1β, unlike after a single i.v. injection \textit{in vivo}. There was no decrease in the number of intimal CD45\(^+\) cells if aortas were incubated with LPS, but IL-1β incubation reduced intimal CD45\(^+\) cells, consistent with RTM (Fig. 11D). Since there is no known apoptosis defect in II\(1b^{-/-}\) mice, this experiment is consistent with IL-1-dependent RTM, as opposed to apoptosis, as the process that accounts for reduced number of intimal myeloid cells. Therefore, these results with II\(1b^{-/-}\) mice suggest that IL-1β acts locally in non-hematopoietic cells to elicit RTM of intimal DCs. A future direction is to identify key downstream mediators produced by IL-1 signalling.
**Figure 11**

**A** RTM rescue experiments in *Il1b⁻/⁻* mice

- **0h**, **3h**, **9h**, **24h**
- PBS
- LPS
- LPS, IL-1β
- IL-1β

**B** *In vivo* mIL-1β (WT mice)

- Dose (ng)
- Intimal CD11c⁺ cells
- Percent

**C** *In vivo* mIL-1β (*Il1b⁻/⁻* mice)

- IL-1β (ng)
- Intimal CD11c⁺ cells
- NS

**D** *Ex vivo* mIL-1β (*Il1b⁻/⁻* mice)

- 0h, 18h
- PBS, LPS, IL-1β
- Fix

- Intimal CD11c⁺ cells
**Figure 11**: IL-1β acts locally in the intima in order to elicit RTM of DCs. (A) RTM rescue experiments performed in Il1b−/− mice. The absolute number of CD11c+ cells was determined in the LC of the aortic arch in WT mice and mice deficient in IL-1β at 24 hours (B) post PBS, LPS (30 µg), or LPS + 2 IL-1β (50 ng per injection) injections. RTM was compared between WT (B) and Il1b−/− mice (C), which were injected with IL-1β and whose aortas were harvested 24h later. The graph in (B) has been adapted from Mark Roufaiel’s thesis. (D) Numbers of CD45+ cells were determined in Il1b−/− mice 18 hours after incubation of aortas *ex vivo* with PBS, LPS (25 µg/mL) or IL-1β (50 ng/mL). Schematic plan for all experiments performed are above the graphs, except for (B) and (C), whose schematics are that of Fig. 8B, except that IL-1β was injected as opposed to LPS. Aortas were stained with biotinylated anti-CD11c Ab or Alexa Fluor 647 anti-CD45 Ab. Values represent mean ± SEM. n = 5 – 9. Statistically significant differences were determined using one-way ANOVA with the Bonferroni post-hoc test; * p<0.05; ** p<0.01; *** p<0.001.
4 Discussion

Previously published data from our laboratory showed that infection of wild type C57BL/6 mice by intranasal or intravenous administration of the intracellular pathogen, *Chlamydia muridarum*, or inflammation by TLR agonists such as PolyI:C or LPS, induced RTM of intimal DCs that are present in atherosclerosis-susceptible regions of the aortic arch. *Chlamydia muridarum* was removed by RTM of DCs, suggesting that RTM is a protective mechanism against infection in the normal aorta. It was proposed that inflammatory cytokines such as IL-1β or TNF-α are produced under conditions that induce RTM and they may have a role in this process. In fact, intravenous administration of IL-1β, or *ex vivo* incubation of the normal arch with IL-1β, did induce RTM of DCs (Fig. 4A, 4B). Since IL-1β is produced downstream of LPS signalling, the aim of this study was to verify whether LPS-induced IL-1β is critical for RTM of intimal DCs and obtain potential mechanistic insights.

This study confirmed that IL-1β expression and signalling are critical for LPS-induced RTM of intimal CD11c+ DCs in C57BL/6 mice. LPS induces RTM in two waves (Fig. 2A) and evidence is presented that IL-1β is required for both waves using mice deficient in caspase-1 or IL-1β, which do not produce IL-1β, or mice deficient in IL-1R1, which lack IL-1β-induced signalling (Fig. 8). Gene expression of IL-1β, its related cytokines and receptors, and other pro-inflammatory cytokines were significantly increased before and during the first wave of RTM (Fig. 5–7). Experiments with function-blocking antibodies against IL-1β further confirmed the findings with the knockout mice and were especially valuable to demonstrate the importance of IL-1β for the second wave of LPS-induced RTM (Fig. 9B, 9C). Moreover, the results with the antibodies negated any potential compensation issue due to IL-1β deficiency in the knockout models that could have contributed to the results. The second wave of RTM occurs between 60-72h. However, LPS and IL-1β are cleared from the bloodstream via the kidneys and liver at 30 min and 90 min respectively, well before the second wave of RTM begins. Thus, the requirement for IL-1β for the second wave is intriguing. There is a slight increase in the expression of IL-1β, TNF-α and IL-6 from 54h to 72h post-LPS (Fig. 5A, 7A, 7B). Whether this second increase in the expression of these genes is in endothelial cells or DCs that proliferated between both waves of RTM and the triggers involved have yet to be discovered. Reciprocal bone marrow transplantation experiments suggested that IL-1β produced by artery wall cells, as opposed to bone marrow-derived cells, was important to elicit the first wave of LPS-induced RTM (Fig. 10).
Previously, it was demonstrated that the reduction in intimal DC numbers during the first
and second wave after injection of LPS into WT mice correlated with their exit from the intima,
rather than apoptosis, as shown by scanning electron microscopy, cannulation experiments and
assessing apoptosis of intimal DCs (Fig. 2, detailed explanation in Introduction)\textsuperscript{33}. For example,
when aortae of CD11c\textsuperscript{-}EYFP mice were cannulated, and exposed to a flow of media containing
LPS, CD11c\textsuperscript{+}EYFP\textsuperscript{+} cells were collected (Fig. 2C). Therefore, a significant reduction in intimal
DCs upon exposure to LPS or IL-1\textbeta in the study by Roufaiel et al.\textsuperscript{33}, and in this thesis, was
attributed to RTM. In the future, key findings of this study will be verified by approaches used
previously to demonstrate RTM or by imaging in real-time, once established in the laboratory.

It is important to note that the baseline numbers of CD11c\textsuperscript{+} cells in Casp1\textsuperscript{-/-} mice was
significantly lower than those of WT mice, and this trend was observed in Il1b\textsuperscript{-/-} and Il1r1\textsuperscript{-/-}
mice (Fig. 8B). In addition, BMT experiments showed that baseline numbers were lower in
recipient Il1b\textsuperscript{-/-} and Il1r1\textsuperscript{-/-} mice transplanted with WT bone marrow cells (Fig. 10). These
results indicate that IL-1\textbeta is important for the homeostasis of intimal DCs. There are 2 types of
DC populations with different markers that were shown to be present in the intima\textsuperscript{14} (described
in the Introduction). It could be envisaged that if there is a specific population of DCs that
preferentially undergoes RTM but missing in mice deficient in IL-1\textbeta or signalling, the lack of
LPS-induced RTM in intimal DCs in these mice may be due to the absence of this population.
However, this was shown not to be the case in Il1b\textsuperscript{-/-} mice, as \textit{in vivo} and \textit{ex vivo} treatment with
IL-1\textbeta rescued RTM, indicating that the intimal DCs in these mice are capable of undergoing
RTM (Fig. 11A, 11D).

An injection of LPS induces IL-1\alpha, IL-1\textbeta, IL-1Ra, TNF-\alpha and IL-6 in macrophages that
reside in the marginal zone of the spleen. These cytokines are then released into the serum, and could mediate pathological effects in multiple organs. In addition, Figure 9A showed
that stimulation of peritoneal macrophages with IL-1\textbeta results in IL-1\textbeta and TNF-\alpha production.
Therefore, it is possible that upon injection of LPS or IL-1\textbeta, systemic production of IL-1\textbeta or
downstream cytokines are responsible for RTM of DCs. It was because of these considerations
that \textit{ex vivo} experiments were undertaken, which showed that incubation of aortas from Il1b\textsuperscript{-/-}
mice with recombinant mouse IL-1\textbeta induced RTM (Fig. 11D). Therefore, combined with the
data from \textit{in vivo} experiments, we conclude that LPS-induced IL-1\textbeta in the intima signals locally
via the IL-1R1 on endothelial cells to induce the production of downstream molecules or
cytokines that mediate the process of RTM of intimal DCs.
Results of gene expression levels of LPS-induced IL-1β showed two rapid peaks of expression at 3h and 9h post-LPS, time points that respectively precede the onset and coincide with, the first wave of RTM (Fig. 5A). While this study showed that endothelial IL-1β is important for RTM (Fig. 10B), previous data from our lab showed that intimal DCs also produce IL-1β. Therefore, it is possible that the oscillating levels of mRNA expression of IL-1β at 3h to 9h post-LPS results from different cells producing IL-1β at different times. Based on the results with BMT showing that endothelial IL-1β production is critical for the first wave of RTM, it is possible that the first wave of IL-1β in endothelial cells induces intimal DCs to produce IL-1β subsequently, corresponding to the second pulse at 9h.

IL-1β was shown to mediate the migration of neutrophils across endothelial cells in a Ca²⁺-dependent manner. Moreover, IL-1β promoted RTM of neutrophils by reducing JAM-C expression in endothelial cells within veins of the cremaster muscle. Further studies have to be performed in order to determine if IL-1β mediates RTM of intimal DCs utilizing these mechanisms. mRNA expression levels of TNF-α followed a very similar pattern to that of IL-1β, with peaks of mRNA expression at 3h and 9h post-LPS (Fig. 5A, 7A). Further studies need to be performed to determine the relationship between LPS-induced IL-1β and TNF-α during LPS-induced RTM of intimal DCs. It is possible that both cytokines might have complementary functions since both IL-1β and TNF-α are pro-inflammatory cytokines that promote monocyte recruitment into tissues and TNF-α was shown to upregulate adhesion molecules such as VCAM-1 and CX3CL1. In addition, CX3CL1 also promotes cell survival. Therefore, TNF-α functions might be complementary to the role of IL-1β in mediating RTM by promoting adhesion molecule expression and survival of the intimal DCs and their increased exit through the endothelium. In fact, it was shown that an injection of TNF-α (500 ng) induced a decrease in intimal DCs.

IL-1α mRNA expression profile is similar to that of IL-6; upregulated from 3h to 24h after LPS injection, which covers the first wave of RTM (Fig. 5B, 7B). Both IL-1α and IL-6 increased the motility and migration capability of cultured melanoma cells. Both cytokines are also involved in the recruitment of leukocytes. IL-1α was shown to induce ICAM-1, VCAM-1, and release CX3CL1 in cultures of brain endothelial cells and increase survival of cultured cancer cells. IL-6 upregulates adhesion molecules such as ICAM-1 and also increased the mobility of cultured melanoma cells, and cancer cells by increasing expression.
of anti-apoptotic proteins\textsuperscript{130}. The functions of IL-1\(\alpha\) and IL-6 in the intima could be to increase the motility and/or survival of intimal DCs during RTM of these DCs. Experiments with neutralizing antibodies against IL-1\(\alpha\) inhibited LPS-induced RTM (Fig. 9B, 9C), which could be associated with its potential functions discussed above.

Previous published data from our laboratory showed that LPS induced expression of CCL19 and CCR7 in myeloid cells is critical for LPS-induced RTM of intimal DCs. LPS-induced expression of CCL19 and CCR7 occurs from 4h to 12h in intimal CD11c\(^+\) cells, just before and during the first wave of RTM. In non-intimal tissues, IL-1\(\beta\) stimulation increased the expression levels of CCL19 in rheumatoid arthritis\textsuperscript{132} and CCR7 in osteoclasts\textsuperscript{133}. Therefore, LPS-induced IL-1\(\beta\) may increase the expression of CCL19 and CCR7 in intimal DCs. However, our results show that endothelial IL-1\(\beta\) and IL-1R1 are required for RTM (Fig. 10), while CCL19 and CCR7 on intimal DCs is required for RTM\textsuperscript{33}; therefore, mechanisms of cross-talk between endothelial cells and DCs are required in order to elicit RTM. A possible mediator of endothelial cell-DC cross-talk is the interaction between S1P and S1PR. SphK1 phosphorylates and activates sphingosine-1 phosphate (S1P), that signals through S1PR1 to mediate RTM of T cells from the thymus\textsuperscript{134}. In addition, experiments using FTY720, an inhibitor of S1P signalling, demonstrated that this pathway is critical for LPS-induced RTM of intimal DCs (Mark Roufaiel’s thesis). S1PR1 appears to be expressed in intimal myeloid cells since depletion of DCs in the DT-injected DTR model, reduced the expression of S1PR1, but not SphK1 (Mark Roufaiel’s thesis). Therefore, these results suggest that S1P production in endothelial cells and S1P signalling on S1PR1 in DCs are required for RTM. Since IL-1\(\beta\) upregulates SphK1 in glioblastoma cells\textsuperscript{135}, IL-1\(\beta\) signalling might induce SphK1 expression that produces S1P in endothelial cells, which in turn signals through S1PR1 in intimal DCs that stimulates RTM by inducing CCL19/CCR7 signalling on DCs (see Model, Fig. 12).

It was previously reported that under conditions of hypercholesterolemia foam cells are retained in the intima and our laboratory showed that LPS-induced RTM is inhibited upon feeding Ldlr\(^{-}\) mice with cholesterol rich diet for 1 week\textsuperscript{33,136,137}. If this inhibition is due to a defect in the function of IL-1\(\beta\) in RTM, understanding the processes downstream of IL-1\(\beta\) signalling will be critical so that interventions which restore RTM of foam cells, in order to remove them from plaques, will circumvent production and signalling of the pro-atherosclerotic cytokine IL-1\(\beta\)\textsuperscript{101,102,103}. 

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Figure 12: Potential mechanisms by which IL-1β mediates LPS-induced RTM of intimal DCs. LPS binds to TLR4 on endothelial cells lining the lumen of atherosusceptible regions. Downstream signalling of TLR4 leads to the production, processing and secretion of IL-1β, which in turn binds to IL-1R1 on endothelial cells. This induces a number of responses that could potentially elicit RTM of DCs. (A) IL-1R1 signalling leads to the transcription and translation of other proinflammatory cytokines, such as TNF-α and IL-6, which in turn could mediate RTM of intimal DCs via multiple processes such as upregulation of adhesion molecules and mediation of survival and motility of DCs. (B) IL-1β signalling upregulates adhesion molecules, which may mediate the migration of intimal DCs out of the endothelial layer while reverse transmigrating. (C) IL-1β signalling downregulates junctional molecules which allow the endothelial junctions to become permissive for the crossing of the intimal DCs into the lumen. (D) IL-1β signalling promotes the production of S1P which may signal through the S1PR, and the upregulation of CCL19 and signaling through CCR7, which mediate the process of RTM of intimal DCs.
5 Future Directions

This study showed that IL-1β is critical for LPS-induced RTM. As mentioned before, intimal CD11c+ cell numbers were used as a surrogate to monitor RTM. Many approaches were previously used that showed a good correlation between DC numbers and exit of DCs from the intima into the lumen and absence of increased apoptosis; thus, key findings of this study will be tested by live cell imaging of DC migration from the intima into the aortic lumen ex vivo.

LPS is a purified component of bacterial endotoxin that can potentially cause sepsis-like symptoms. Although a titrated amount of LPS that does not cause mortality (30 µg/mouse) was used, in the future, LPS could be substituted with another molecule such as Lipid A, the lipid component of the LPS molecule. Synthetic monophosphoryl Lipid A was shown to be virtually non-toxic, as opposed to natural Lipid A, LPS or its derivatives; therefore, systemic toxic effects, such as excessive cytokine production (referred to as a cytokine storm), complement proteins and arachidonic metabolites, disseminated intravascular coagulation, excessive hypotension and even death, that are normally produced by LPS may be minimized.

During reciprocal bone marrow transplantation experiments, lethal irradiation and subsequent loss of bone marrow, or the transplantation of bone marrow of a different genotype, are invasive procedures and may produce compensatory responses from cells other than bone marrow cells, that might affect the results. Therefore, a better experimental procedure would use a conditional (e.g., Cre-Lox) mouse system. An Il1r1loxp mouse model has been generated by Robson et al. These mice can be crossed with a CD11c-Cre or Cad5-CreERT2 mice (Cad5 is the gene that expresses the VE-cadherin protein, a common marker for endothelial cells), where the resulting strains will either be CD11c-Cre; Il1r1loxp or Cdhs-CreERT2; Il1r1loxp mice. In the CD11c-Cre; Il1r1loxp model, when the CD11c transgene is transcribed in DCs (i.e. during the process of DC maturation), the Cre recombinase enzyme is also produced, and binds to the LoxP sites on the IL-1R1 gene in order to “floX” or remove the floxed alleles and thus inhibit production of IL-1R1 in DCs. In the Cdhs-CreERT2; Il1r1loxp mouse model, a similar process takes place, except that it is necessary to induce the production of Cre using tamoxifen, which binds to the estrogen receptor that regulates nuclear translocation of Cre, and subsequently deletes Il1r1 alleles in endothelial cells. In this manner, it would be possible to avoid bone marrow transplantation, and provide results with mice deficient in IL-1R in specifically either endothelial cells or intimal DCs.
While qPCR is the gold standard in sensitivity and efficiency for gene expression studies, and provided valuable information on the intimal expression of IL-1β and other pro-inflammatory cytokines, a broader approach such as RNA-seq could be used that can provide information on a wide range of pro-inflammatory genes and non-coding RNA in the intima in response to LPS. These experiments will ultimately be performed on isolated intimal endothelial and myeloid cell populations or at the level of a single cell analysis. These methodologies are currently being developed in our laboratory.
References


