REVERSE TRANSENDOTHELIAL MIGRATION OF INTIMAL CD11c+ DENDRITIC CELLS: ROLE IN INTRACELLULAR PATHOGEN REMOVAL AND EFFECT ON ATHEROSCLEROTIC LESIONS

By

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

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ABSTRACT

Dendritic cells are antigen presenting cells that play a key role in the initiation of the adaptive immune response and in chronic inflammatory diseases such as atherosclerosis. Resident intimal CD11c+ cells are abundant in the normal arterial intima of mice, in regions predisposed to atherosclerosis. Upon induction of hypercholesterolemia, these cells engulf lipids and become the first foam cells in nascent lesions. However, their function in the normal aorta remains poorly understood. The purpose of this study is to show that their function is to protect the arterial intima against Chlamydia (C.) muridarum infection, and potentially against other intracellular and extracellular bacteria. This protection is achieved when intimal CD11c+ cells undergo reverse transendothelial migration (RTM) through the endothelium into the arterial circulation to remove the pathogen from the vessel wall.

Systemic pathogens and stimulation of pattern recognition receptors trigger two waves of RTM of intimal CD11c+ cells, each was followed by recovery through proliferation of the remaining cells. Both waves of RTM were dependent on up-regulated expression of CCR7 and its ligand CCL19 by intimal CD11c+ cells.
RTM of intimal CD11c+ cells

*C. muridarum* enters the arterial intima when infected circulating monocytes are recruited. The second wave of RTM removes the pathogen from the vessel wall. Inhibition of RTM (e.g. in Ccr7−/− mice) results in accumulation of *C. muridarum* in the vessel wall, which induces a local inflammatory response, that could be harmful to the arteria intima.

Hypercholesterolemia and lipid loading of intimal CD11c+ cells by feeding Ldlr−/− mice high fat diet for one week results in inhibition of RTM of CD11c+ foam cells. Lipid loading did not affect CCR7 or CCL19 induction in intimal cells and incubation with CCL19 did not rescue RTM in atherosclerotic lesions.

Studies in Asc−/− and Casp1−/− mice that are deficient in the production of cleaved IL-1β, as well as antibody blockade and rescue experiments revealed that RTM is dependent on IL-1β production from the arterial endothelial cells. In addition, experiments with Il-1r1−/− mice suggest that RTM is dependent on receptor expression by the endothelial cells.

Future studies will continue to investigate other factors and mechanisms that might play a role in RTM of intimal CD11c+ cells, and explore how they can be used to induce RTM in atherosclerotic lesions as a potential preventative therapy to reduce atherosclerotic plaque burden.
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Abbreviations

- 16s rRNA – 16s ribosomal RNA
- ANOVA – Analysis of variance
- APCs – Antigen presenting cells
- Apoe – Apolipoprotein E
- ASC – Apoptosis-associated speck-like protein containing a CARD domain
- BM – Bone marrow
- BMDM – BM-derived macrophages
- BMT – Bone marrow transplantation
- BrdU – 5-Bromo-2’-deoxyuridine
- C. muridarum – Chlamydia muridarum
- CARD – caspase activation and recruitment domain
- Casp1 – Caspase 1
- CCL19 – CC-chemokine ligand 19
- CCL21 – CC-chemokine ligand 21
- CCR7 – CC-chemokine receptor 7
- CFUs – Colony forming units
- CL – Clodronate liposomes
- CRD – Cholesterol rich diet
- CTLs – cytotoxic T cells
- CVD – Cardiovascular diseases
- DAMPs – Danger-associated molecular patterns
- DCs – Dendritic cells
RTM of intimal CD11c⁺ cells

- DT – Diphtheria toxin
- Dtr – Diphtheria toxin receptor
- *E. coli* – *Escherichia coli*
- EB – elementary bodies
- EC – Endothelial cells
- ELISA – Enzyme-linked immunosorbent assay
- eNOS – endothelial nitric oxide synthase
- Flt3 – Fms-like tyrosine kinase 3
- GC – Greater curvature
- H₂O₂ – Hydrogen peroxide
- HDL – High density lipoproteins
- HEVs – High endothelial venules
- HP – High probability region
- IFNγ – interferon gamma
- IFUs – Inclusion/infection forming units
- IL-1 – Interleukin-1
- IL-1R1 – Interleukin-1 receptor 1
- IL-1ra – IL-1 receptor antagonist
- IL-1RAcP – IL-1 receptor accessory protein
- iNOS – Inducible nitric oxide synthase
- IV – intravenous
- JAM – Junctional adhesion molecule
- LC – Lesser curvature
- LDL (r) – Low density lipoprotein (receptor)
- LP – Low probability region
RTM of intimal CD11c+ cells

- LPS – Lipopolysaccharide
- MIG – Monokine induce by γ interferon
- MOI – multiples of infection
- NA – Numerical aperture
- ND – Not detected
- NF-κB – Nuclear factor kappa B
- NLRs – Nod-like receptors
- NOD – nucleotide-binding and oligomerization domain
- PAMPs – Pathogen-associated molecular patterns
- PECAM1 – platelet endothelial cell adhesion molecule 1 or CD31
- PFA – Paraformaldehyde
- PL – PBS liposomes
- Poly(I:C) – Polynosine-polycytidylic acid
- PRR – Pattern recognition receptor
- PTx – Pertussis toxin
- PYD – Pyrin domain
- qPCR – quantitative polymerase chain reaction
- R.t. – Room temperature
- RB – Reticular bodies
- RES – Reticulo-endothelial system
- RT – Reverse transcription
- RTM – Reverse Transendothelial Migration
- S1P – Sphingosine-1-phosphate
- S1Pr – S1P receptor
- SDF-1 – Stromal cell-derived factor 1
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- SEM – Scanning electron microscopy
- SEM – Standard error of the mean
- SphK1/2 – Sphingosine kinase 1/2
- Std. diet – Standard diet
- TCM – T-cells central memory
- Tdtomato – tandem dimer tomato fluorescent protein
- TIR domain – Toll IL-1 receptor domain
- TLRs – Toll-like receptors
- TNF-α – Tumour necrosis factor alpha
- TUNEL - Terminal deoxynucleotidyl transferase dUTP nick end labeling
- WT mice – Wild type mice
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Chapter 1

Introduction
1.1 Cardiovascular diseases and atherosclerosis

Cardiovascular diseases (CVD) represent one of the most important health challenges for modern societies and ranks amongst the top causes of mortality. Atherosclerosis, the underlying pathology of CVD, is increasing in prevalence worldwide due to the adoption of Western life-styles\(^1\). Risk factors include obesity, hypercholesterolemia, hypertension, smoking, diabetes, advanced age, and family genetic history\(^1\). Atherosclerosis is a disease that affects elastic and muscular arteries, and is the principal antecedent to myocardial, cerebral infarctions and ischemia of the extremities\(^3\). It is characterized by formation of plaques / lesions, due to the accumulation of lipid-loaded myeloid cells (foam cells), smooth muscle cells and extracellular matrix in the innermost layer of the artery wall\(^3\). Atherosclerotic plaques contain large amounts of cholesterol, cholesteryl esters and cholesterol crystals, which are derived from circulating high levels of blood cholesterol\(^5\). In addition, atherosclerotic lesions harbor all classes of immune cells, and it has become clear that the innate and adaptive immune systems are involved in the initiation and progression of the disease\(^6\). Atherosclerosis is therefore considered a chronic inflammatory disease, and several clinical trials are currently underway to determine whether anti-inflammatory therapies can reduce the risk of cardiovascular events in atherosclerotic patients\(^7\).

Several studies have linked infections to atherosclerosis and CVD\(^8\). The risk of developing atherosclerosis was correlated with chronic infections by various pathogens including bacteria, such as *Chlamydia (C.) pneumoniae*, *Helicobacter (H.) pylori*, and *Porphyromonas (P.) gingivalis*, and viruses, such as Cytomegalovirus (CMV), Human immunodeficiency virus (HIV) and Influenza A virus\(^8\). Studies have shown accelerated atherogenesis following respiratory infection with *Chlamydia*
and it was suggested that *C. pneumoniae* may contribute to atherogenesis via mimicry between bacterial and self-antigens and activation of an inflammatory response\(^\text{11}\). Although *Chlamydia* infection does not cause atherosclerosis *per se*, it may accelerate disease progression and plaque formation\(^\text{12,13}\). These studies suggest that *Chlamydia* and other microbes can influence atherogenesis by direct or indirect means, and therefore should be taken into account as contributors to atherosclerosis progression.

### Stages of atherogenesis

Atherosclerotic plaques form in the inner layer of the artery wall (the intima) over four stages\(^\text{14}\). In the first “initiation” stage, immune cells that reside in the intima take up lipids and transform into the initial foam cells, called fatty streaks\(^\text{15-17}\). They appear shortly after sustained hypercholesterolemia and are clinically asymptomatic. In the second stage, “early lesions” are composed almost entirely of newly accumulated foam cells, and their formation and lateral expansion are critically dependent on the recruitment of circulating monocytes and macrophages\(^\text{18-21}\). In the third stage, “complex lesions” arise when foam cells accumulate and are surrounded by a fibrous cap\(^\text{3,4}\). Smooth muscle cells migrate into the intima and synthesize constituents of the fibrous cap such as collagen and other extracellular matrix proteins\(^\text{3,4}\). Initially the artery outwardly remodels, permitting the plaque to grow without impinging on the lumen and compromising blood flow\(^\text{22}\). Overtime, it can lead to ischemia as a result of progressive narrowing of the vessel lumen\(^\text{22}\). In the fourth stage, “advanced lesions” form when foam cells die and leave behind an acellular lipid rich region referred to as a necrotic core\(^\text{23}\). Advanced lesions also develop thinning of the fibrous caps by metalloproteinases produced by cells in the plaque, which degrade
the collagen in the fibrous cap, and promote its rupture\textsuperscript{24}. When a plaque ruptures, the thrombogenic substances in the plaque contents such as tissue factor, extracellular matrix and cellular debris from necrotic cells induce thrombosis of the blood\textsuperscript{3,4}. If the thrombus is large enough it may occlude the lumen or embolize downstream, and the ensuing ischemia results in tissue damage\textsuperscript{3,4}.

**Response to retention hypothesis**

Multiple mechanisms play a role in atherogenesis. One key permissive factor for atherogenesis is an elevated level of low density lipoproteins (LDL), which are cholesterol enriched lipoprotein particles that transport lipids in the circulation. The response to retention hypothesis proposes that foam cell formation is dependent on the retention and modification of proatherogenic plasma lipoproteins (e.g. LDL) in the arterial subendothelial layer\textsuperscript{25}. Potential modifications of the retained lipoproteins include oxidation, aggregation, glycation, immune complex formation, proteoglycan complex formation, and conversion to cholesterol-rich liposomes\textsuperscript{26}. Macrophages contribute to LDL modification in the intima by producing reactive oxygen species\textsuperscript{26}. The infiltration, retention and modification of LDL particles in the arterial intima initiate an inflammatory response in the artery wall\textsuperscript{27}. Modified LDL activates endothelial cells (EC) to increase the expression of adhesion molecules and inflammatory genes\textsuperscript{28,29}. Circulating monocytes and macrophages migrate into the intima in response to endothelial cell activation and secretion of cytokines and chemokines by leukocytes residing in the intima\textsuperscript{18,30}. The recruitment of monocytes to the arterial wall may initially serve a protective function by removing cytotoxic and proinflammatory modified LDL particles. However, their progressive accumulation ultimately leads to lesion formation.
Mouse models of atherosclerosis

Transgenic models have been developed because normal mice are resistant to developing diet-induced hypercholesterolemia. The apolipoprotein E-deficient (Apoe-/-) and low density lipoprotein receptor-deficient (Ldlr-/-) models develop marked hypercholesterolemia and are used most frequently. ApoE is a component of LDL and is essential for recognition and clearance of serum lipids by lipoprotein receptors. LDL is cleared from the bloodstream and taken up by cells via its receptor, LDLR, a process that occurs largely in the liver. Mice deficient in Apoe or Ldlr cannot clear circulating LDL and develop atherosclerotic lesions throughout the aorta, with morphological features that resemble human atherosclerosis, suggesting that pathogenic mechanisms similar to human atherosclerosis may be involved. The rate of lesion progression is directly proportional to the magnitude of hypercholesterolemia. Ldlr-/- mice fed normal chow (no cholesterol) have only a two-fold elevation in plasma cholesterol and develop lesions at a very slow rate. On a cholesterol-rich diet, these mice develop marked hypercholesterolemia and rapidly develop lesions throughout the aorta. Apoe-/- mice develop hypercholesterolemia (400-500 mg/dL) and atherosclerotic lesions when fed a normal chow diet; however, if fed a “Western-type” diet (0.15% cholesterol, 21% fat), the hypercholesterolemia is >1500 mg/dL and lesions develop more rapidly. Therefore, studying the onset and early stages of atherogenesis is difficult to pinpoint in Apoe-/- mice. The use of Ldlr-/- mice allows more control on the initiation of the disease, since cholesterol-rich diet (CRD) acts as a stimulus for atherogenesis. In this way, the onset of disease can be controlled, and early stages are readily studied. The qualitative features of lesions at different stages of formation are similar in Apoe-/- and Ldlr-/- mice, but the progression rate of lesions varies depending on the magnitude of hypercholesterolemia.
1.2 Regions of the mouse aorta predisposed to atherosclerosis

Despite the fact that all regions of the arterial system are exposed to the same systemic risk factors for atherosclerosis, such as hypercholesterolemia, only certain regions of the artery are predisposed to lesion formation\textsuperscript{40}. The “high probability” (HP) regions are found at branch points or curvatures (e.g. the aortic root and the lesser curvature (LC) of the aortic arch). In contrast, the greater curvature (GC) of the aortic arch and regions in the descending thoracic aorta distant from intercostal artery ostia are relatively protected from atherosclerosis (low probability (LP) regions).

Endothelial cells (EC) form the interface between blood and tissues, and have important homeostatic functions, including maintenance of an antithrombotic and non-adhesive surface, and regulation of protein and lipoprotein permeability\textsuperscript{41}. Endothelial cells can respond to stimuli derived from blood as well as the vessel wall. Examples include inflammatory cytokines, oxidatively modified lipoproteins trapped in the sub-endothelium and even mechanical forces exerted by flowing blood\textsuperscript{42}. Many of these stimuli can induce a change in the endothelial phenotype. EC in the HP regions are exposed to oscillatory hemodynamic shear stress, and tend to exhibit random shape and orientation, whereas EC in LP regions are elongated in shape and oriented in the direction of blood flow\textsuperscript{43,44}. EC in LP regions are exposed to uniform laminar flow which modulates the expression of several genes that have anti-inflammatory and antioxidant properties, and down-regulates endothelial responses to inflammatory cytokines\textsuperscript{45,46}. For example, the expression of endothelial nitric oxide synthase (eNOS) – a potential atheroprotective gene – was lower in HP regions\textsuperscript{47}.

Local hemodynamic differences may also heighten or suppress signaling in response to systemic risk factors by altering the expression of signaling components and/or regulators of other pathways\textsuperscript{48}. EC in HP regions exhibit priming of the nuclear
factor (NF)-κB pathway, production of proinflammatory cytokines, and expression of other key molecules for inflammatory cell recruitment, such as adhesion molecules. NF-κB is a family of transcription factors that induce the expression of numerous genes relevant to atherosclerosis. Expression of p65/RelA (a component of NF-κB) was significantly higher in the cytoplasm of EC in HP regions compared to LP regions, but NF-κB activation (determined by nuclear translocation of p65) was present in only a minority of cells. When mice were exposed to systemic endotoxin or hypercholesterolemia, NF-κB activation and up-regulated expression of NF-κB-responsive genes was found preferentially in this region. These data illustrate how a topographic difference in endothelial signaling mediates accentuated regional target gene expression in response to systemic risk factors for atherosclerosis. Regional activation of endothelial NF-κB by systemic stimuli, such as hypercholesterolemia, may contribute to the localization of atherosclerotic lesions at sites with high steady-state expression levels of NF-κB components.

EC in HP regions had elevated expression of endothelial leukocyte adhesion molecules, such as VCAM-1, as well as several proinflammatory chemokines and cytokines. NF-κB is a key transcriptional regulator of proinflammatory molecules, and it is possible that low-level NF-κB activation in the lesser curvature promotes their expression, and mediates low-level inflammation that modulates the cellular composition of the arterial intima at sites predisposed to atherosclerosis.
1.3 Intimal dendritic cell reside in the arterial intima at predisposed sites

Dendritic cells (DCs) are professional antigen-presenting cells in the immune system\textsuperscript{53}, and form a critical interface between innate and adaptive immunity\textsuperscript{54,55}. DCs are present in tissues that are in contact with the external environment, and are also found in an immature state in the blood\textsuperscript{56,57}. DCs are subdivided into classical (cDCs) and plasmacytoid (pDCs). cDCs can be divided into two main subsets characterized by either CD8$\alpha$ and CD103 expression or CD11b expression\textsuperscript{58}. Both subsets can be found in lymphoid tissues, including spleen, lymph nodes, and bone marrow, as well as most non-lymphoid tissue\textsuperscript{58}. Murine cDCs were defined based on functional and morphological features that distinguish them from monocytes and macrophages\textsuperscript{59}. These include: (a) nonadherent cells with probing morphology (continuously forming and retracting processes), (b) preferential localization to T cell areas thereby facilitating clonal section of antigen-specific T cells, (c) strong stimulators of the mixed leukocyte reaction that measures the capacity for stimulating allogeneic T cells, (d) superior antigen presentation to CD4 and CD8 T cells relative to other myeloid cells in the context of major histocompatibility complex (MHC) class II (MHC-II) and MHC-I, (e) lower phagocytic activity compared with monocytes and macrophages, and (f) loss of monocyte and macrophage markers.

cDCs play diverse functions, such as induction of tolerance against self-antigens and promoting antigen-specific immunity upon exposure to pathogens\textsuperscript{59}. Under normal conditions, DCs function to induce CD4$^{+}$CD25$^{+}$Foxp3$^{+}$ T regulatory cells (Tregs), which are critical for maintenance of peripheral self-tolerance\textsuperscript{60}. In response to pathogens, DCs in tissue upregulate chemokine receptors such as CCR7, migrate via lymphatics into T cell-enriched areas of lymph nodes and undergo maturation by upregulating T cell costimulatory molecules including CD40, CD80/B7-1 and
CD86/B7-2\(^{61}\). To fulfill their immunogenic functions, DCs possess machinery to capture, process and present antigens to T cells (i.e. MHC molecules\(^{62,63}\)). Conventionally, exogenous antigens are presented by MHC class II molecules and recognized by CD4\(^+\) T lymphocytes, while MHC class I molecules are loaded with endogenous/self-antigens and recognized by CD8\(^+\) T lymphocytes\(^{64}\). Antigen presentation can also occur in peripheral tissues, but is stochastic due to the lack of a full T cell repertoire. However, re-exposure to a previously encountered antigen significantly increases the chances of DC antigen presentation to a memory T cell in peripheral tissues, since the initial exposure will produce long-lived antigen-specific T cells that traffic through tissues.

Consistent with the expression of proinflammatory genes by endothelial cells in the atherosclerosis-prone lesser curvature of the normal mouse aorta, leukocytes accumulated in the lesser, but not in the greater, curvature\(^{15,16}\). The morphology of the majority of these leukocytes is characteristic of DCs. Several studies reported that arteries from healthy children and young adults displayed enrichment of dendritic cells that are CD1a\(^+\) S100\(^+\) I\(^{ag}\) CD31\(^-\) CD83\(^-\) CD86\(^-\) and resemble Langerhan’s cells\(^{65-67}\). Intimal DCs have also been reported in atherosclerosis-susceptible regions of the rabbit and mouse aortae\(^{68,69}\). In the human aorta, structurally low-differentiated DCs are predominantly found in close proximity to the endothelium within the normal intima, whereas DCs with a moderately developed tubulovesicular system are localized throughout the thickness of the tunica intima, mostly being concentrated in the subendothelial space\(^{70}\), suggesting distinct structural and probably functional differences between subsets of DCs located at different areas of the aortic wall.

In the healthy mouse aorta, CD11c\(^+\) (integrin \(\alpha_x\)) CD68\(^+\) (myeloid marker, macrosialin) MHC-II\(^{\text{high}}\) (antigen presentation molecule) DCs, but not CD68\(^+\)
macrophages, are abundant within the lesion-susceptible lesser curvature of the aortic intima\textsuperscript{15,16}. The site-specific localization of intimal CD11c\textsuperscript{+} cells occurs independent of circulating cholesterol levels, highlighting the importance of blood flow patterns rather than plasma lipid levels for DC localization within the aorta. Additional characterization of DCs revealed preferential accumulation of these cells within the cardiac valve and aortic sinus of mice\textsuperscript{71}. These aortic DCs expressed low levels of CD40 and were positive for CD1d and co-stimulatory molecules (CD80 and CD86), suggesting that they possess an immature DC phenotype\textsuperscript{71}.

DCs are generated by at least two major pathways that differ in their requirement for the Flt3 (CD135) / Flt3 ligand (Flt3L) axis. Development of DCs from monocyte-independent precursors is Flt-3/Flt3L-dependent\textsuperscript{72-74}, whereas the generation of DCs from monocytes is Flt3/Flt3L-independent\textsuperscript{75}. Flt3 treatment resulted in an expansion of CD11c\textsuperscript{+} cells within the intima and adventitia of \textit{C57BL/6} mice suggesting a DC origin of CD11c\textsuperscript{+}MHC-II\textsuperscript{high} cells\textsuperscript{76}. Additional studies\textsuperscript{15-17,71,76-78} have also demonstrated the existence of two major subsets of DCs as CD11c\textsuperscript{+}CD11b\textsuperscript{+}F4/80\textsuperscript{+} and CD11c\textsuperscript{+}CD11b\textsuperscript{−}F4/80\textsuperscript{−} cells within the aortas of \textit{C57BL/6} mice. CD11c\textsuperscript{+}CD11b\textsuperscript{−}F4/80\textsuperscript{−} cells possessed a distinct phenotype characterized by CD103 and CD207 expression, and were negative for CD8, CD205, CX3CR1, and 33D1. CD11c\textsuperscript{+}CD11b\textsuperscript{+}F4/80\textsuperscript{+}CD103\textsuperscript{−} DCs expressed the CD14 co-receptor for TLR4 and DC-SIGN antigen. Development of these two subsets of DCs was considerably different: CD11c\textsuperscript{+}CD11b\textsuperscript{+}F4/80\textsuperscript{+}CD103\textsuperscript{−} DCs were M-CSF-dependent, and likely monocyte-derived DCs. In contrast, CD11c\textsuperscript{+}CD11b\textsuperscript{−}F4/80\textsuperscript{−}CD103\textsuperscript{+} DCs were Flt3-dependent DCs\textsuperscript{76}.

The abundance and homeostasis of intimal DCs may be dependent on recruitment from the circulation, proliferation of resident DCs, their survival versus
apoptosis, and/or exodus from the intima. Proliferation of intimal DCs is very low in C57BL/6 mice under steady-state conditions. DC precursors could be recruited to the intima and differentiate into DCs. Bone marrow-derived circulating Ly6C^{hi} monocytes may be recruited into the intima and differentiate into monocyte-derived DCs under the control of M-CSF and GM-CSF. Intimal DC numbers were reduced in Vcam-1 deficient mice, suggesting that mechanisms of leukocyte accumulation in the intima of the normal aorta are analogous to those in atherosclerosis. In addition to VCAM-1, endothelial cells in atherosclerosis-susceptible regions of the aorta also express higher levels of ICAM-1, which can contribute to monocyte recruitment. Blood Ly6C^{low} monocytes could also express CD11c, which contributes to monocyte arrest on endothelial cells by cooperating with CD49d binding to VCAM-1. Moreover, activated endothelial cells and leukocytes express CX3CL1 (Fractalkine), a transmembrane protein that functions as an adhesion molecule, and a chemokine when cleaved by membrane proteases. Its receptor CX3CR1 is preferentially expressed by Ly6C^{low} monocytes and intimal DCs. CX3CR1 deficiency significantly reduced the number of Ly6C^{low} monocytes in the circulation and the abundance of intimal DCs. In-vitro experiments identified CX3CL1 as a mediator of monocyte arrest and migration on endothelial cells, suggesting that CX3CL1/CX3CR1 promote Ly6C^{low} monocyte recruitment to the normal intima, and implicate these cells in intimal DC homeostasis. CX3CL1/CX3CR1 signaling promotes atherogenesis and the survival of Ly6C^{low} monocytes, and thus, may also be important for DC survival.
1.4 Potential functions of Intimal dendritic cells in the normal aorta

The function of intimal DCs within healthy arteries remains unclear. Recent studies suggest that they may play a role in the maintenance of vascular homeostasis by forming continuous networks via long cell processes, which play a role in the surveillance and protection against dangerous stimuli\textsuperscript{70}. CD11c\textsuperscript{+} DCs play an active role during the initial stages of atherosclerosis through active uptake of neutral lipids in hypercholesterolemic mice\textsuperscript{16}. A key function of dendritic cells is to present antigens to T cells. DCs isolated from the aorta and valves of wild-type mice have the capacity to cross-present antigens to CD8\textsuperscript{+} T cells \textit{in vitro}\textsuperscript{71}, indicating that intimal DCs are functionally competent DCs with antigen presentation capacity. However, given the scarcity of T cells in the mouse and human aortic intima\textsuperscript{15}, it is unlikely that antigen presentation is their primary function. T-cells are more abundant in the arterial adventitia and their recruitment to this location is dependent on L-selectin\textsuperscript{77}. Intimal DCs may survey the arterial intima for invading pathogens and present antigens to T cells outside the intima, but it remains to be determined whether aortic DCs are capable of migrating out of the intima analogous to a subpopulation of gut and lung dendritic cells that express the αEβ7 integrin (CD103)\textsuperscript{89,90}. These cells have constitutive CCR7-dependent migratory capacity and are found in draining lymph nodes both in steady-state conditions and after bacterial infection\textsuperscript{89}. Alternatively, T-cells that enter the intima may be tolerized against arterial antigens by intimal DCs\textsuperscript{91,92}. Intimal DCs are in close contact with the overlying endothelium\textsuperscript{66,71,93}, which may facilitate cytokine-mediated crosstalk between these cells. DCs can produce both pro-inflammatory and anti-inflammatory cytokines\textsuperscript{94-96}, but further studies will be required to elucidate the nature of communication between intimal cells, and the role of cytokines and cell surface molecules.
1.5 CCR7 – CCL19/21 mediated migration of DCs

Following activation by infectious or inflammatory insults, DCs undergo maturation characterized by the upregulated expression of MHC-II, and co-stimulatory molecules such as CD80, CD83, and CD86, as well as the CC-chemokine receptor 7 (CCR7)\textsuperscript{97-99}. CC-chemokine ligand 19 (CCL19) and CCL21 are the soluble ligands for CCR7, and are generally constitutively expressed and control movement of various subsets of immune cells during homeostasis\textsuperscript{100}. CCR7 and its ligands are involved in the homing of various subpopulations of T cells and antigen-presenting DCs to the lymph nodes. Within lymph nodes, T cells establish close physical contacts with DCs, which allow their antigen-specific activation\textsuperscript{100}.

Unlike CCL19, CCL21 has a uniquely long positively charged C-terminal that allows binding to various matrix and cell surface proteoglycans (e.g. glycosaminoglycans and other molecules such as type IV collagen), which may be required for efficient presentation of CCL21 on the surface of endothelial cells\textsuperscript{101-104}. Two mouse genes encode functional CCL21 variants. CCL21-Leu is expressed by lymphatic vessels of non-lymphoid organs such as the lung, colon, stomach, heart and skin, whereas CCL21-Ser is expressed in lymphoid organs such as the thymus, lymph nodes and spleen\textsuperscript{105}. Both CCL19 and CCL21 are secreted by DCs and stromal cells in the lymph node, but only CCL21 is expressed by the lymphatic vessel endothelium\textsuperscript{106}. In secondary lymphoid organs, CCL21 is produced by fibroblastic reticular cells and by high endothelial venules (HEVs)\textsuperscript{107}. In lymph nodes, CCL19 is produced by reticular cells and by activated DCs that are recruited into lymph nodes under inflammatory conditions\textsuperscript{108}.

Similar to all chemokine receptors, CCR7 contains seven-transmembrane-spanning domains and mediates its signals through heterotrimeric G proteins and their
downstream effectors. CCR7 is expressed by semi-mature and mature DCs\textsuperscript{109}, thymocytes during defined stages of their development\textsuperscript{110}, naive B and T cells\textsuperscript{111,112}, T-reg cells\textsuperscript{113} and a subpopulation of memory T cells known as central memory T (TCM) cells\textsuperscript{112}. CCR7 is also expressed by different non-immune cells, most notably in various malignancies\textsuperscript{114}.

CCL19 and CCL21 have similar binding affinities for CCR7\textsuperscript{101,115} and similar chemotactic potential for DCs and T cells\textsuperscript{116,117}. Despite their similar affinities to CCR7, CCL19 and CCL21 induce different signalling effects through this receptor. CCL19, but not CCL21, effectively stimulates CCR7 phosphorylation and internalization\textsuperscript{117,118}, leading to receptor desensitization. CCL21 signals without requirement for internalization\textsuperscript{119}. This implies that CCR7-mediated cell responses to CCL19 may have a shorter time-span than responses to CCL21. Also, CCL19 can desensitize the receptor towards subsequent responses to CCL21 ligation, but not vice versa. It is not yet clear how these differential signalling events may impinge on the complex cellular functions of CCR7-bearing cells in the environments that contain both chemokines.

Little is known about the mechanisms that regulate the trafficking of mature DCs to the lymph node via the afferent lymphatics. Gene targeting using CCR7 deficient mice showed that CCR7 is essential for DC mobilization, but it is still not clear where and how CCR7 and its ligands are involved in this process. Recent studies suggested that CCL21 is sufficient to mediate DC migration from the skin, full DC maturation and efficient T cell priming in the absence of CCL19\textsuperscript{120}. CCL21 is constitutively expressed intracellularly by lymphatic endothelial cells in the skin, and is rapidly secreted after exposure to inflammatory stimulus, and stimulates trans-lymphatic DC migration\textsuperscript{121}. CCL21 complexed with collagen IV gets deposited on the basement membrane of initial lymphatics, and activated DCs move directionally
towards it, and migrate through portals into the lymphatic lumen\textsuperscript{122}. Once inside lymphatic vessels, DCs crawl along lymphatic endothelium and, sensing lymph flow, proceed downstream\textsuperscript{122}. On the other hand, CCL19 is involved in driving DC migration into the lymph node and their guidance from the subcapsular sinus to their final destination in the T cell area\textsuperscript{123}. CCL19 and CCL21 might not only drive the migration of DCs but also more directly affect their ability to prime T cells\textsuperscript{124}.

Recent studies have also demonstrated that CCR7 and its ligands are important in coordinating migratory events into, within and out of the thymus during T cell development\textsuperscript{125,126}. CCL21 was detected in the thymus during embryogenesis, suggesting that it is involved in the recruitment of fetal haematopoietic progenitors in the developing organ\textsuperscript{127}. In the adult thymus, CCL19 and CCL21 are detectable in the cortex and in the medulla, and they guide the migration of developing thymocytes through both compartments\textsuperscript{110}. CCR7 expression by mature single positive T cells could be involved in the positioning of mature thymocytes near the blood vessels before they exit the thymus\textsuperscript{125}. CCL19 is highly expressed by endothelial venules in the thymic medulla and is thought to act as a chemoattractant for mature T cells generated within the thymus\textsuperscript{128}. The process of thymocyte egress from the thymus remains unclear and is probably the result of a combination of cell specific chemo-repellents, chemo-attractants, and/or chemo-kinetic agents. Shingosine-1-phosphate (S1P) and its receptor S1PR have been shown to play a significant role in thymic emigration\textsuperscript{129,130}. Other studies implicate the involvement of CXCL12 (SDF-1) and its receptor, CXCR4, in the regulation of T cell egress from the thymus into the vasculature\textsuperscript{131}. 
1.6 Reverse transendothelial migration of leukocytes

Immune responses involve the orchestrated trafficking of leukocytes between the vasculature and tissues. The recruitment of leukocytes from the circulation to tissues has been studied extensively and is the primary reason for accumulation of leukocytes at sites of inflammation. Recruitment involves steps that include rolling over the endothelium, arrest, stable adhesion, intravascular crawling and transendothelial migration or diapedesis. This directed movement is regulated by a complex array of soluble factors in combination with the extracellular matrix environment. Once leukocytes penetrate the endothelium, they undergo directed movement (chemotaxis) along chemotactic gradients in inflamed tissues.

Extravascular leukocytes can undergo reverse transendothelial migration (RTM), also called intravasation, and migrate through the vascular endothelium from the abluminal to the luminal surface and re-enter the blood. Less is understood about what regulates the reverse movement of leukocytes from tissues to the vasculature or to the lymphatics, which is also known as reverse chemotaxis. This process of reverse migration is a key component of normal physiology including the trafficking of leukocytes from the bone marrow to the vasculature and the process of lymphocyte egress from lymphoid tissue to the vasculature or lymphatics during immune surveillance. Reverse migration is also a likely component of pathologic conditions, including tumor invasion and metastasis and the dissemination of intracellular pathogens from infected tissues into the vasculature.

Recent studies implicate reverse chemotaxis and reverse transmigration of leukocytes in the resolution of inflammatory responses involving the innate immune system. For example, blockade of the junctional adhesion protein, JAM-C, at endothelial contact sites, reduced the number of monocytes at extravascular spaces.
by increasing reverse transmigration of monocytes across the endothelium\textsuperscript{140}. In venular endothelial cells, JAM-C expression is decreased by ischemia/reperfusion injury, which facilitates reverse migration of neutrophils\textsuperscript{141}. Time-lapse imaging in zebrafish embryos reveals that neutrophil reverse chemotaxis from sites of tissue wounding back to the vasculature is a key mechanism by which inflammation is resolved after acute injury\textsuperscript{142}, and this process may be impaired under some conditions of chronic inflammation\textsuperscript{143}. A recent study demonstrated that rising concentrations of IL-8 initially serve as a neutrophil chemoattractant in mesentery causing neutrophil extravasation\textsuperscript{144}. However, increasing concentrations ultimately result in neutrophil movement in the opposite direction back toward the venule wall\textsuperscript{144}.

Similarly, T cells can migrate away from a high concentration of the chemokine SDF-1 rather than displaying the chemoattraction induced by lower concentrations of the same factor\textsuperscript{145,146}. Studies using intravital microscopy confirmed that reverse migration plays a role in the egress of lymphocytes from lymph nodes to intra-nodal vascular and lymphoid spaces\textsuperscript{147}.

\textit{In vitro}, monocytes that have migrated through an endothelial monolayer and differentiated into mature DCs under proinflammatory conditions can undergo reverse transmigration since they traverse the collagen/endothelial monolayer from basal to apical direction\textsuperscript{148}. These findings model the migration of antigen-loaded DCs from tissues into the afferent lymph to enter lymph nodes. Yet, it remains to be determined whether intimal DCs can undergo reverse transmigration from the arterial intima to the blood circulation especially since the normal non-atherosclerotic arterial intima and media does not contain lymphatic vessels\textsuperscript{149}.
1.7 **Intracellular bacterial infection**

Although most bacteria are harmless or often beneficial (such as commensal bacteria in the gut), several bacteria are pathogenic and can cause disease and damage to infected tissues. Pathogenic bacteria can be divided into two categories; extracellular bacteria – which do not invade cells and proliferate instead in the extracellular environment which is enriched with body fluids – and intracellular bacteria – which are bacteria that have the capacity to survive within host cells and possess specialized mechanisms to protect themselves from lysosomal enzymes encountered within the cells\(^{150}\).

Intracellular bacteria have evolved the ultimate escape from phagocytes, complement, and antibodies by moving directly inside the host cell and complete their reproduction out of reach of these host defenses\(^{151}\). Some species infect non-immune system host cells such as hepatocytes and epithelial cells, while others show a strong preference for macrophages\(^{151}\). Some intracellular bacteria cannot survive outside of a host cell (called obligate), while others merely make intracellular replication a preference (called facultative)\(^{151}\). Like extracellular bacteria, most intracellular bacteria access the host via breaches in the mucosa and skin, but some are introduced directly into the bloodstream. Intracellular bacteria are generally not very toxic to the host and do not produce tissue-damaging bacterial toxins. Therefore, they have extended incubation times and are difficult to resolve completely\(^{151}\).

Intracellular bacteria enter host cells using a trigger mechanism or a zipper mechanism\(^{152}\). The trigger mechanism is when bacteria interact directly with the eukaryotic cell cytoskeleton by injecting bacterial effectors through a dedicated secretion system. These effectors cause massive cytoskeletal rearrangements and engulfment of the bacterium in an entry vacuole. The zipper mechanism is when
bacteria contact and adhere to the eukaryotic cell through the binding of a bacterial surface protein to a eukaryotic surface receptor, often a transmembrane cell-adhesion protein. Modest membrane extensions and cytoskeletal rearrangements engulf the bacterium in an entry vacuole. Under normal circumstances, the vacuole progressively acidifies as it develops into a mature degradative phagolysosome. Some pathogens survive in this niche either by preventing vacuole–lysosome fusion or by modifying the environment within the phagolysosome. Other bacteria have evolved to escape from the vacuole and continue their life cycle within the cytosol.

Intracellular bacteria can be divided into three general classes: 1) those that arrest normal phagosome maturation, which can occur at an early phagosome-like stage (as in the case of *Mycobacterium*) or at a late phagosome-like stage (as in the case of *Salmonella*); 2) those which escape from the phagosome or the vacuole and become cytosolic bacteria, such as *Shigella, Rickettsia, and Listeria*; 3) those which manipulate the traffic of the host membranes to take-up residence in a non-phagosomal organelle such as *Legionella* (which is secluded in an endoplasmic reticulum-like compartment) or *Chlamydia* (which can produce their own unique compartment, distinct from any of those of the host cell).

Intracellular survival of bacteria also depends on their ability to avoid the host immune response. The bacterial intracellular niche is not an entirely safe haven, as it contains a range of antimicrobial peptides and potentially microbicidal reactive oxygen species. In addition, pathogens inside the cell are not hidden from the host immune response; since intracellular pathogen-associated molecular patterns (PAMPs) are recognized by Nod-like receptors (NLRs), and this activates signalling pathways and inflammatory responses. Another component of the host defense against intracellular pathogens is autophagy, which is a degradative pathway by which
cytosolic content, organelles and pathogens are delivered to lysosomes as part of cellular homeostasis and innate immunity\textsuperscript{161}. It is increasingly recognized that cytosolic bacteria interact with and modify the ATG5-dependent autophagy pathway to promote their survival\textsuperscript{162}.

Another challenge for intracellular bacteria is to disseminate in order to infect neighbouring cells without attracting the attention of the host immune response. In host cells, the bacterium induces actin polymerization and the formation of an actin-based pseudopod, which protrudes into the plasma membrane and extends into neighboring cells\textsuperscript{163}. The neighboring cell engulfs the pseudopod and pinches it off to form a secondary vacuole surrounded by a double plasma membrane\textsuperscript{164}. The bacterium then uses degrading enzymes to forge its way through both sets of host membranes and enters the cytoplasm of the new host cell, free to start the cycle anew\textsuperscript{165}. The beauty of this tactic is that the bacterium is never extracellular, meaning that antibodies can never bind to it, thus, it is not recognized by humoral immunity.

Intracellular bacteria (e.g. \textit{Chlamydia pneumonia}) were detected within cells of human atherosclerotic plaques\textsuperscript{166}, and viable organisms have been isolated from vessel lesions\textsuperscript{167}. However, \textit{Chlamydia} was not detected in the vessel wall of healthy individuals nor in mice on regular diet\textsuperscript{168}, suggesting that pathogens either don’t enter the normal arterial intima or are cleared efficiently. Low-grade chronic inflammation with ongoing blood monocyte recruitment occurs in regions with disturbed hemodynamics\textsuperscript{15}. This provides a mechanism for intracellular pathogen transport\textsuperscript{169} into the arterial intima. Extracellular pathogens may be captured from the circulation by intimal myeloid cells that extend dendrites through the endothelial monolayer into the artery lumen\textsuperscript{71}. Potential mechanisms for pathogen clearance from the artery include efficient pathogen killing and/or migration of infected intimal myeloid cells.
1.8 Innate recognition of intracellular bacteria

Innate immunity is the first line of defense against pathogens and is highly developed in its ability to discriminate between self and foreign pathogens\textsuperscript{170}. This discrimination relies on a family of evolutionarily conserved pattern recognition receptors (PRRs), among which is a group of receptors known as Toll-like receptors (TLRs), which have a crucial role in early host defence against invading pathogens\textsuperscript{171}. Furthermore, activation of the innate immune system is a prerequisite for the induction of the adaptive/acquired immunity\textsuperscript{172}. PRRs possess common characteristics\textsuperscript{170}. First, PRRs recognize microbial components known as pathogen-associated molecular patterns (PAMPs) that are essential for the survival of the microorganism and are therefore difficult for the organism to alter. Second, PRRs are expressed constitutively in the host and detect the pathogens regardless of their life-cycle stage. Third, PRRs are germline encoded, non-clonal, expressed on all cells of a given type, and independent of immunologic memory. Fourth, different PRRs react with specific PAMPs, show distinct expression patterns, activate specific signaling pathways, and lead to distinct anti-pathogen responses. The basic machineries underlying innate immune recognition are highly conserved among species, from plants and fruit flies to mammals\textsuperscript{173}. In addition to PAMPs detection, PRRs can also recognize endogenous danger-associated molecular patterns (DAMPs), which are endogenous molecules that are confined within the cell under normal state but are released after injury\textsuperscript{174-176}.

All TLRs are type I integral membrane glycoproteins and possess amino-terminal leucine-rich repeats, which are responsible for the recognition of PAMPs, and a carboxy-terminal Toll–interleukin-1 (IL-1) receptor (TIR) domain, which is required for initiating intracellular signaling\textsuperscript{177}. 13 members of the TLR family have been identified so far. Based on their primary sequences, TLRs can be further divided into
several subfamilies, each of which recognizes related PAMPs: the subfamily of TLR1, 2, and 6 recognizes lipids, whereas the highly related TLR7, 8, and 9 recognize nucleic acids\textsuperscript{178}. Furthermore, TLRs may be expressed extra- or intracellularly; while certain TLRs (1, 2, 4, 5, and 6) are expressed on the cell surface, other TLRs (3, 7, 8, and 9) are found almost exclusively in intracellular compartments, such as endosomes\textsuperscript{178}.

TLR4 was the first TLR member to be discovered and was demonstrated to be the receptor for bacterial lipopolysaccharide (LPS), the outer membrane component of Gram-negative bacteria\textsuperscript{177}. TLR2 recognizes peptidoglycan, in addition to the lipoproteins and lipopeptides of Gram-positive bacteria and mycoplasma lipopeptide, and it collaborates with its relatives TLR1 and TLR6 to discriminate between the molecular structures of diacyl and triacyl lipopeptides, respectively\textsuperscript{179}. TLR3 is involved in the recognition of double-stranded RNA generated during virus replication\textsuperscript{180}. TLR5 recognizes flagellin, a protein component of bacterial flagella\textsuperscript{181}. TLR7 and TLR8 are responsible for viral single-stranded RNA detection\textsuperscript{182}. TLR9 mediates the recognition of unmethylated 2′-deoxyribo CpG DNA motifs found in bacteria and DNA viruses\textsuperscript{183}.

TLRs activate the same signaling molecules that are used for IL-1R signaling\textsuperscript{184}. After ligand binding, TLRs dimerize and undergo conformational changes required for the recruitment of TIR-domain-containing adaptor molecules to the TIR domain of the TLR. There are four adaptor molecules, namely MyD88, TIR-associated protein (TIRAP)/MyD88-adaptor-like (MAL), TIR-domain-containing adaptor protein-inducing IFN-β (TRIF)/TIR-domain-containing molecule 1 (TICAM1), and TRIF-related adaptor molecule (TRAM)\textsuperscript{185,186}. The differential responses mediated by distinct TLR ligands can be explained in part by the selective usage of these adaptor molecules. MyD88 and TRIF are responsible for the activation of distinct signaling pathways, leading to the production of proinflammatory cytokines and type I IFNs, respectively.
TLRs recognize pathogens at either the cell surface or lysosome/endosome membranes, suggesting that the TLR system is not used for the detection of pathogens that have invaded the cytosol. Studies have revealed a large family of novel PRRs termed the nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs)\textsuperscript{187}. Each family member contains a leucine-rich repeat region (LRR) required for ligand sensing, a NOD domain (also termed NACHT domain), and signaling modules such as the caspase activation and recruitment domain (CARD), pyrin or baculoviral inhibitor of apoptosis repeat (BIR) domains\textsuperscript{178}.

Activation of NLRs by bacterial products can stimulate two major signaling pathways: the nuclear transcription factor (NF)-\(\kappa\)B pathway and the inflammasome. NF-\(\kappa\)B, a heterodimeric transcription factor, is a key regulator of the pro-inflammatory response, activating genes that encode cytokines and co-stimulatory factors, such as pro-IL-1\(\beta\)\textsuperscript{188}. NLR proteins can also activate caspase-1 by way of the inflammasome, a multi-protein complex activated by diverse stimuli and whose primary function is to process the inflammatory pro-IL-1\(\beta\) and pro-IL18 to their mature and active forms\textsuperscript{189}. NOD1 and NOD2 are the archetypal members of the NLR family, and they respond to components of peptidoglycans found on Gram-positive and negative bacteria\textsuperscript{190,191}. NALP1 and NALP3 have similar domain structure to NODs, but contain a PYRIN domain instead of, or in addition to, a CARD domain, and they are sensors of bacterial RNA, bacterial toxins, peptidoglycans, and endogenous danger signals\textsuperscript{158}. NAIP5 and IPAF are unique members among the NLRs and they detect bacterial flagellin\textsuperscript{192,193}.

Interestingly, bacteria and other pathogens are also able to exploit the PRR system to evade host immune responses. For example, certain pathogens have modified forms of their normal TLR ligands, such as the LPSs\textsuperscript{194}. Some pathogens modify TLR signaling pathways for their benefit by inhibiting downstream signaling\textsuperscript{195}. 

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Generally, there are several major mechanisms by which the immune system can eliminate intracellular pathogens (reviewed in\textsuperscript{151}): 1) Phagocytosis of bacteria by neutrophils triggers phagosomal killing via respiratory burst and cytokine secretion. 2) Phagocytosis of bacteria by macrophages initiates phagosomal killing and secretion of cytokines that maintain inflammation, activate NK cells, and promote Th1 differentiation. TLR-mediated endocytosis also triggers cytokine secretion. 3) NK cells activated by IL-12 kill infected host cells by natural cytotoxicity and secrete IFN-\(\gamma\) which activates macrophages and supports Th1 differentiation. 4) Phosphorylated metabolites released by a bacterium activates \(\gamma\delta\) T cells that generate T cell effectors. 5) Infected DCs present bacterial components on CD1 to \(\gamma\delta\) T cells and NKT cells. Once activated, these cells generate cytotoxic- and Th-like effectors. 6) CD8\(^+\) Cytotoxic T cells (CTLs) – recognizing bacterial peptides presented on MHC class I by an infected host cell – kill the cell by perforin- and granzyme-mediated cytotoxicity. The CTL also secrete the anti-microbial molecule granulysin and pro-inflammatory cytokines. 7) Unconventional CTL subsets are activated by bacterial components presented on CD1 by infected DCs. One subset kills infected host cells by Fas killing, while another relies on perforin-mediated cytotoxicity. 8) Infected macrophages present bacterial peptides on MHC class II to CD4\(^+\) T cells. In the presence of IL-27, IL-12, and IL-18, Th1 effectors differentiate and supply cytokines that both support the CTL response and hyperactivate macrophages. 9) Hyperactivated macrophages produce increased levels of pro-inflammatory cytokines and reactive oxygen species that increase killing. Granuloma formation may occur if these measures are insufficient to wall off and confine the pathogen. 10) Bacterial components released from a dying infected cell can activate B cells to produce neutralizing antibodies. These antibodies intercept any bacterium temporarily transiting the extracellular environment.
1.9 Effect of hypercholesterolemia on myeloid cell migration

Resolution of acute inflammation typically involves emigration of monocyte-derived cells out of the inflamed site through nearby lymphatic vessels\textsuperscript{196}. This process appears to be impaired in atherosclerosis and has been attributed, in part, to the cholesterol loading of macrophages which shifts these cells to a more sessile phenotype\textsuperscript{197}. Macrophage emigration has been shown to occur in early atherosclerotic plaques, however the rate of macrophage egress was reported to decrease with atherosclerosis progression\textsuperscript{198}. It is likely that plaque macrophages are subject to both retention and emigration signals, and the balance of these forces contributed to plaque macrophage net accumulation.

Studies have shown that reducing plasma non-high-density lipoprotein (HDL) cholesterol and/or increasing HDL, promotes emigration of macrophages from lesions to regional and systemic lymph nodes\textsuperscript{199}. Using a transplant-based mouse model of atherosclerosis regression, in which plaque-bearing aortic segments are transferred from a hyperlipidemic ApoE-deficient mouse into a normolipidemic wild type (WT) mouse, the emigration of myeloid-derived cells into draining lymph nodes and the systemic circulation ensued within days of a plaque being placed into a healthier environment, concurrent with a marked decrease in the content of macrophage foam cells\textsuperscript{200}. The macrophage emigration process in this model was dependent in part on the induction of CCR7 in the emigrating CD68\textsuperscript{+} cells, implicating the CCR7-specific ligands CCL19 and CCL21 in promoting the egress of cells from the artery wall, since blocking this pathway led to substantial retention in the plaque\textsuperscript{201,202}. These findings were reproduced in another non-surgical model of regression using a genetic switch\textsuperscript{203}. This suggests that there are factors that promote macrophage retention and that migration from the plaque is actively inhibited during hypercholesterolemia.
Recent studies have shown that netrin-1 – and other neuro-immune guidance molecules such as ephrin B2, semaphorin 3A and 3E are abundantly expressed by macrophage foam cells in atherosclerotic lesions and inactivate macrophage migration out of the vessel wall\textsuperscript{204-206}. Macrophage expression of these migration inhibitory molecules are also induced during hypoxia, which is intimately linked to atherosclerosis\textsuperscript{207,208}. Other factors that inhibit cell movement (such as adhesion molecules) or resolution of inflammation are also likely to contribute to the retention of macrophages in the plaque\textsuperscript{209}.

Although RTM of foam cells from atherosclerotic lesions has been observed by SEM\textsuperscript{210}, this is a rare event. In fact, there is evidence that one of the reasons that foam cells accumulate in the lesions in the setting of hypercholesterolemia may be their inability to leave the plaque due to the inhibition of RTM by lipid-loading. In a model of dermal inflammation in Apoe\textsuperscript{-/-} mice, hypercholesterolemia impaired the migration of DC to draining lymph nodes\textsuperscript{211}. oxLDL inhibited TLR4-induced efflux of peritoneal macrophages into the lymphatics\textsuperscript{212}. In vitro, atherogenic mediators such as platelet activating factor or lysophosphatidic acid impaired RTM of monocyte-derived cells\textsuperscript{200}.

On the other hand, other studies suggest that the mode of clearance is more dependent on local death and reduction in monocyte recruitment than on emigration. In the acute inflammatory model of thioglycollate-induced peritonitis, macrophage removal was more dependent upon local death than emigration to lymph nodes\textsuperscript{213}. In an atherosclerotic regression model, no evidence of egress marked by a loss of the bead-based phagocytic tracer in the plaque was observed, but reduction of macrophages in plaques was caused by a profound reduction in monocyte recruitment to plaques\textsuperscript{214}. The discrepancy over whether macrophages do or do not exit plaques during disease regression needs clarifying through additional research.
1.10 Rationale and Hypotheses

Myeloid CD11c+ cells with features of dendritic cells are abundant in the normal arterial intima, in regions that are predisposed to atherosclerosis. Their abundance is enriched in the lesser curvature of the aortic arch, making it an ideal representative site for studying the biology of intimal DCs. In the early phases of atherogenesis, intimal CD11c+ cells engulf lipids and become the initial foam cells prior to increased monocyte and macrophage recruitment; but it remains to be determined whether this promotes inflammation and accelerates atherosclerosis, or protects the artery wall from potentially harmful effects of oxidized lipids. Intimal CD11c+ cells may function as antigen-presenting cells, yet as intimal CD3+ T cells are rare, antigen presentation would not occur in situ. **The second chapter of the thesis** will examine whether intimal CD11c+ cells may be capable of migrating out of the intima (as there is no lymphatic drainage) by reverse transmigration (RTM) through the vascular endothelium from abluminal to the luminal surface. The role of the chemokine receptor CCR7 and its ligands CCL19 & CCL21, as well as S1P and its receptors S1PRs, in RTM will also be investigated. Subsequently, the physiological significance of RTM of intimal CD11c+ cells will be explored. **Chlamydia** infection of the arterial intima has been reported in the context of atherosclerosis, but was not detected in the normal arterial intima suggesting efficient clearance from the vessel wall; **and the third chapter of the thesis** will investigate whether RTM of intimal CD11c+ cells functions in clearance of **Chlamydia** from the arterial intima. **Chlamydia** have been detected in human and mouse atherosclerotic lesions, and since various studies have shown that migration of leukocytes are impaired in atherosclerosis, the focus of **the fourth chapter of the thesis** will be to investigate whether hypercholesterolemia inhibits RTM of intimal CD11c+ cells and study the effect of lipid-loading on signaling involved.
**Hypothesis:**

Reverse transendothelial migration (RTM) of intimal CD11c⁺ myeloid cells – triggered by systemic exposure to proinflammatory stimuli (pathogens or TLR/NOD ligands) – is dependent on CCR7 / CCL19 signaling. RTM protects the arterial intima from intracellular pathogens, but this process is inhibited during atherogenesis.

**Specific Aims:**

1. To examine whether systemic inflammatory stimuli (e.g. LPS) induce reverse transendothelial migration of intimal CD11c⁺ cells and determine receptors and ligands that mediate this process such as CCR7 and its ligands CCL19/21, as well as S1P and its receptors. *(Chapter 2)*

2. To ascertain whether *Chlamydia* infects the normal arterial intima, the mechanism of pathogen entry into the vessel wall, and whether reverse transendothelial migration of intimal CD11c⁺ cells or intracellular killing is the mechanism of pathogen clearance from the normal arterial intima. *(Chapter 3)*

3. To investigate the effect of lipid loading during atherogenesis on reverse transendothelial migration of intimal CD11c⁺ cells and on clearance of *Chlamydia* from the intima. *(Chapter 4)*

4. To elucidate the roles of IL-1β production and signaling in reverse transendothelial migration of intimal CD11c⁺ cells, and explore the effects of IL-1β signaling on endothelial cells junctional proteins. *(Chapter 4)*
Chapter 2

CCR7/CCL19-Dependent Reverse Transendothelial Migration of Intimal CD11c+ cells
2.1 Introduction

DCs, with their long dendritic processes, represent a key organizing element of the vascular cell network, integrating small groups of vascular immune cells and screening vascular tissue and the blood for potential harmful antigens. The vascular DC network seems to play three key roles in resolving inflammation in the artery wall: 1) the uptake and storage of intimal and circulating lipids; 2) the clearance of lipids, apoptotic cells, and debris from the artery wall via migratory mechanisms; and 3) the maintenance of vascular Treg responses.

Migratory inflammatory cells bearing a phenotypic resemblance to CD11b+ DCs have been shown to constitutively leave the artery wall and migrate to draining lymph nodes during atherosclerosis regression, and this was dependent on upregulated expression of the chemokine receptor CCR7 and its ligands CCL19 and CCL21, suggesting that CCR7-mediated DC egress plays a major role in resolving vascular inflammation through the removal of inflammatory lipid components.

Migratory vascular DCs have also been credited with the removal of cell debris and apoptotic cell bodies (a process known as efferocytosis), which is critical for the resolution of inflammation as it prevents post-apoptotic secondary necrosis (at least in the early stages of atherosclerosis), which supplies the artery wall microenvironment with further proinflammatory mediators, leading to necrotic core formation.

However, the contribution of CCR7-dependent migration of vascular DCs to the resolution of inflammation under steady-state conditions has not been demonstrated previously and is one of the main objectives of this chapter. Constitutive CCR7-dependent DC migration has been demonstrated in other tissues such as the skin and the intestines, where the maintenance of local immune tolerance relies on constitutive transport of commensal bacteria and apoptotic cells to draining lymph nodes.
In addition, the intima and the underlying media does not contain lymphatic vessels that would serve as a conduit for intimal cells to migrate to regional lymph nodes\textsuperscript{149}. The alternative is that intimal myeloid cells migrate through the vascular endothelium from the abluminal to the luminal side and into the arterial blood (i.e. undergo reverse migration, or intravasation).

In this chapter, studies will show that stimulation with various TLR and NOD ligands induce a rapid reduction in the abundance of intimal CD11c\textsuperscript{+} cells within one day. This reduction is not due to downregulation of CD11c expression nor increased cell death by apoptosis. The reduction is due to cell egress from the arterial intima towards the vascular lumen via reverse transendothelial migration (RTM). RTM is dependent on induction of CCR7 expression on intimal CD11c\textsuperscript{+} cells. Endothelial cells constitutively expresses CCL21, but functional studies will demonstrate that RTM is dependent on CCL19 expressed by intimal CD11c\textsuperscript{+} cells. Furthermore, TLR stimulation induces a second wave of RTM of intimal CD11c\textsuperscript{+} cells and each wave of RTM is followed by increased proliferation of the remaining cells to establish recovery and reconstitution of the original homeostatic abundance of intimal CD11c\textsuperscript{+} cells. Finally, preliminary studies will demonstrate that sphingosine-1-phosphate (S1P) is another important factor in RTM from the arterial intima. S1P expression by endothelial cells is low and is induced by TLR stimulation, whereas S1P receptors are constitutively expressed by intimal CD11c\textsuperscript{+} cells. This pattern of expression might provide directional cues for RTM into the vessel lumen.
2.2 Materials and methods

2.2.1 Mice and reagents

Male and female mice were used between 6 and 12 weeks of age. C57BL/6 wt (stock No. 000664), Ccr7−/− (stock No. 006621), Plt (stock No. 012866), Cd11c-dtr transgenic (stock No. 004509), and Ubc-gfp transgenic (stock No. 004353) mice were obtained from Jackson Laboratories. Cd11c-hBcl2 transgenic mice were a gift from Dr. P. Lesnik (University of Pierre and Marie Curie, Paris, France)222, and Cd11c-eYFP transgenic mice were a gift from Dr. M.C. Nussenzweig (The Rockefeller University, New York, USA)223. Ccl19−/− bone marrow cells were obtained from Dr. S.A. Luther (University of Lausanne, Switzerland)107. Mice were bred and maintained in a pathogen-free environment at the University Health Network animal facility at TMDT with 12h light-dark cycles. Mice were fed a standard rodent chow. All protocols were performed in adherence to the guidelines of the Canadian Council of Animal Care.

Non-pathogenic Escherichia coli (E. coli) (25922) was obtained from ATCC and were grown either in Luria-Bertani (LB) broth or on LB agar plates at 37ºC. The number of colony-forming units (CFUs) was determined using optical density at 600 nm. E. coli was incubated in fresh broth and samples were collected overtime to measure optical density and for plating on LB agar plates for CFUs enumeration. A standard curve was generated relating optical density to the number of CFUs. Broth was inoculated with E. coli and incubated overnight with shaking at 37ºC to obtain a stationary-phase culture. 1 in 1,000 dilution from this seed culture was inoculated into a fresh flask containing fresh LB broth. The culture was incubated for 2 to 2.5 h at 37ºC with shaking in an orbital shaker (250 rpm) to obtain a log-phase culture. The number of CFUs was determined by optical density at 600 nm and the appropriate concentration was injected intravenously into C57BL/6 wt and Ccr7−/− mice.
Lipopolysaccharide (LPS) from *E. coli* 055:B5 was purchased from Sigma-Aldrich (no. L2880) and ultrapure *E. coli* 011:B4 LPS was purchased from Invivogen (no. tlrl-3pelps). Polyinosine-polycytidylic acid (Poly(I:C)) was purchased from Sigma-Aldrich (no. P9582). CpG oligonucleotide ODN M362 (TLR9 ligand, no. tlr-m362), γ-D-Glu-mDAP (iE-DAP) dipeptide (NOD1 ligand, no. tlr-dap), and Muramyl dipeptide (MDP) (NOD2 ligand, no. tlr-mdp) were purchased from Invivogen.

5-Bromo-2'-deoxyuridine (BrdU) was obtained from Sigma-Aldrich (no. B5002), dissolved in PBS and kept in the dark to avoid light-induced degradation. Pertussis toxin (PTx) from *Bordetella pertussis* (no. 181), Pertussis toxin subunit (B-oligomer, no. 184), and Diphtheria toxin (DT) from *Corynebacterium diphtheriae* (no. 150) were purchased from List Biological Laboratories Inc. PBS and clodronate liposomes were a gift from Dr. Nico van Rooijen (Amsterdam, Netherlands). Recombinant mouse CCL19/MIP-3 (no. 440-M3) and CCL21/6Ckine (no. 457-6C), as well as monoclonal function blocking antibodies against mouse CCL21/6Ckine (no. MAB-457), CCL19/MIP-3 (no. MAB-880), and isotype controls were from R&D systems.

Conjugated antibodies to mouse antigens were used for immunostaining, flow cytometry analysis and flow sorting experiments. Biotinylated anti-CD11c (no. 553800) was from BD Pharmingen, biotinylated anti-CD45 (no. 13-0451) was from eBioscience, and biotinylated anti-BrdU (no. 339809) was from BioLegend. Alexa-Fluor 647 directly conjugated antibodies to CD11c (no. 117312), CD68 (no. 137003), CD45 (no. 103124), were obtained from BioLegend. eFluor 605NC antibody to CD11b (no. 93-0112-41) was from eBioscience and FITC antibody to Gr-1 (no. CL8991F-3) was from Cedarlane labs. Alexa-Fluor 647 anti-CD3e (no. 100324), PE anti-CD4 (no. 116005), Apc/Cy7 anti-B220 (no. 103223), Pacific Blue anti-CD45.2 (no. 109820), and Percp-Cy5.5 anti-CD11c (no. 117327) were purchased from BioLegend.
2.2.2 *En face* immunostaining and confocal microscopy

Mice were perfused through the left ventricle with PBS (1% heparin) to wash out the blood from the vessels, and then perfused with 2% paraformaldehyde (PFA) in PBS to fix tissues. The heart, thymus and aorta were separated from the chest cavity, then the ascending aortic arch – the segment from the aortic sinus to the first branch (innominate artery) – was isolated under a dissecting microscope. Surrounding adipose tissues were cut away from the aorta. Fixation of aortic arches was continued for 20 more minutes in 2% PFA after isolation.

Aortic arches were permeabilized with 0.2% Triton X-100 and 0.1M Glycine for 10 minutes at room temperature (r.t.). TSA™ Fluorescein System – Tyramide Signal Amplification kit (Perkin Elmer) was used for immunostaining when a biotinylated primary antibody was used. Endogenous catalase and peroxidase activity were quenched with 3% hydrogen peroxide (H$_2$O$_2$) in PBS for 45 minutes at r.t. The aortic arch was blocked with blocking buffer (supplied by Perkin Elmer) supplemented with 10 μg/mL non-immune mouse IgG and 10 μg/mL rat IgG (Sigma Aldrich) for 30 minutes at r.t. Biotinylated primary antibodies, as well as other directly conjugated antibodies, were added and staining continued overnight at 4°C. Streptavidin conjugated horseradish peroxidase (supplied in the Perkin Elmer kit) was incubated in reagent blocking buffer (from the kit) for 30 minutes at RT. This was followed by FITC-conjugated Tyramide reagent in amplification solution (both from the Perkin Elmer) for 10 minutes at r.t. Nuclei were counterstained with 10 μg/ml Hoechst 33342 for 30 min at r.t. (Molecular Probes, Invitrogen).

For TUNEL staining, aortae were incubated with TUNEL reaction mixture (TMR red *In situ* Cell Death Detection Kit, Roche Applied Science) for 1h at 37°C after H$_2$O$_2$ quenching and prior to overnight incubation with primary biotinylated anti-CD11c.
For experiments involving BrdU labeling, mice were either injected with 2mg of BrdU intravenously for 3h (to study intimal leukocyte proliferation) or 24h (to study recruitment from the circulation) before harvesting the ascending aorta for immunostaining. Alternatively, BrdU was supplied in the drinking water (1mg/ml/day) for 1 week and the water was changed daily. Subsequently, regular water was supplied for another week before LPS injection and harvesting of the aorta. For immunostaining, the aorta was permeabilized with 0.5% Triton X-100 for 15 minutes. Aortae were incubated with either 1N hydrochloric acid (HCl) for 1 hour or DNase I (1:50, Invitrogen, no. 18047019) in PBS for 1.5 hours prior to primary antibody incubation, in order to allow the antibody access to the nucleus for the detection of BrdU incorporation into DNA. Subsequent incubations with Streptavidin-HRP, Tyramide-FITC, and Hoechst 33342 were performed as described above.

Following staining, the arch was opened longitudinally across the greater curvature and placed flat in a highly reproducible manner. Two small nicks at the region of the aortic valve and one at the distal side were made in the arch to loosen the tension in the curvature and allow flattening. The arch was then mounted on glass slides with the intima facing the cover slip. En face micrographs of the lesser curvature were obtained using a confocal microscope equipped with 40x (NA 1.3) and 60x (NA 1.6) oil objectives and outfitted with 405, 488, 543 and 633 nm lasers (FluoView-1000; Olympus). Overlapping micrographs were taken spanning the entire lesser curvature and later compiled in Adobe Photoshop software. Alternatively in some experiments, the number of intimal CD11c+ cells was counted directly from the eyepiece of the microscope. Z-stack slices (1μm steps through the entire intima, media and adventitia) were reconstructed using the 3-D rendering software, Imaris (Bitplane).
2.2.3 Ex vivo culture, perfusion and imaging experiments

For all ex vivo experiments, mice were perfused with warm PBS, and the ascending aorta was dissected, and placed in RPMI media (10% FBS, 1% Penicillin-Streptomycin, with or without LPS, 50 µg/ml) at 37°C for the period of the experiment.

Ex vivo culture experiments: Mice were injected with PBS or LPS and the ascending aorta was dissected 6 or 54 hours later. Samples were placed in 2 ml of culture media in a 37°C incubator with shaking at 120 rpm for 6 or 12 hours. Samples were then fixed in 2% PFA for 1 hour and stained for CD11c as described above.

Ex vivo perfusion experiments: Mice were injected with PBS or LPS and the ascending aorta was dissected 6 or 54 hours later. The aortic arch was dissected and the branches were tied by sutures to prevent leakage. The aortic arch was cannulated, RPMI media was perfused and the eluate was collected from the descending aorta. The aorta was perfused at a rate of 5 ml/hour using a syringe pump in a 37°C incubator with 5% CO₂. Fractions were collected every 2 hours and placed on ice. Each fraction was spiked with $10^4$ eYFP⁺ U937 cells and spun down at 1,200 rpm for 10 min. Cells were stained with Hoechst 33342 and Alexa-Fluor 647 anti-CD11c for 10 min on ice, washed and resuspended in 30 µl of media. Cells were then transferred into uncoated µ-Slide VI 0.4 (no. 80601, ibidi) and the number of eYFP⁺CD11c⁺ cells was counted.

Time-lapse imaging of explanted aorta: Cd11c-eyfp transgenic mice were injected with PBS or LPS for 6 hours. Alternatively, Cd11c-eyfp⁺dtr⁺ mice (generated by breeding Cd11c-eyfp with Cd11c-dtr mice) were used directly without in vivo treatment. The ascending aortic arch was opened and pinned en face (as previously described) on the top of a small sterile rubber rod with the intima facing upwards. A third 5 mm hole was added in the middle of the channel of the Sticky-slides I 0.8 Luer (no. 80198, ibidi, Germany), then a coverslip was attached to the bottom to seal the
chamber. The opened arch pinned to the rubber rod was inverted onto the hole in the middle of the chamber to allow imaging of the intima. Flow was introduced through the other 2 holes of the slide with fresh RPMI media containing PBS, LPS, or DT, using a syringe pump with a rate of 5 ml/hour for 6 hours. Z stacks (1 μm steps through the entire intima and the inner media) were acquired every 5 min using a 20x air objectives on an inverted FluoView-1000 Olympus microscope equipped with a heated (37°C) and humidified chamber. 3D time-lapse videos were reconstructed using Imaris software (Bitplane).

2.2.4 Scanning electron microscopy (SEM) of the arterial intima

Mice were perfused with ice-cold PBS containing 1% heparin followed by EM fixative solution (2% paraformaldehyde and 2.5% Glutaraldehyde in 0.1M phosphate buffer, pH=7.4). The lesser curvature of the ascending arch was separated from the greater curvature and processed for SEM. Samples were post-fixed in 1% osmium tetroxide (OsO₄) in 0.1M phosphate buffer for 1 hour. Samples were dehydrated through an ascending graded series of ethanol in PBS: 30%, 50%, 70%, 90%, and 100% ethanol. This was followed by drying with a graded hexamethyldisilizane (HMDS) to 100% ethanol: 1:3, 1:1, 3:1, and 2 times 100% HMDS. Samples were left to dry in HMDS overnight in a fume hood. Samples were then mounted on aluminum stubs with the lesser curvature intimal surface facing upwards. Samples were sputter coated using gold-palladium (Bal-Tec SCD 050) and imaged using an Hitachi S-2500 scanning electron microscope operated at 15 kV. Digital images were acquired using a frame grabber and Quartz PCI. Leukocytes on the intimal surface were identified by the approximate diameter of the cell body and the shape including the identification of cell surface ridges/microvilli.
2.2.5 Reciprocal bone marrow (BM) transplantation

Femurs and tibias were isolated from donor mice and flushed with 10 ml of warm RPMI media containing 10% FBS and 1% Penicillin-Streptomycin using a 25G needle. The suspension of BM cells was filtered through 70 μm pore filter to remove debris, and was pelleted centrifuged at 300g for 10 minutes at r.t. The cell pellet was resuspended in fresh RPMI with no additives to generate a 25 million cells/ml suspension.

6 week old recipient mice were exposed to 10 grays of total body gamma irradiation with a gamma irradiator using radioactive cesium 137 as a source to destroy proliferating radiosensitive BM cells. Cesium 137 decays by beta emission to a meta-stable nuclear isomer, Ba-137: Ba-137m; and Ba-137m is responsible for all the emission of gamma rays. 5 million BM cells isolated from donor mice were injected intravenously through the tail vein to reconstitute their hematopoietic cells. Recipients were allowed to recover for 8 weeks. The length of the recovery period is based on intimal CD11c⁺ turnover studies described below (Fig. 1). These experiments revealed that 8 weeks were required to ensure complete turnover of intimal leukocytes and reconstitution with cells derived from the donor bone marrow.

Two types of experiments were carried out in order to determine the length of the period required for complete turnover of intimal CD11c⁺ cells: BrdU pulse-chase and bone marrow transplantation from Ubc-gfp transgenic mice to WT recipients. The abundance of intimal CD11c⁺ cells increases with age and a plateau is reached at 12-14 weeks of age (Fig. 1a). Therefore, 12 week old mice received BrdU in their water for one week, which labels dividing bone marrow cells that are subsequently recruited to the aorta; therefore, the pulse period labels a cohort of intimal cells as BrdU⁺ CD11c⁺. Mice then received regular water, which chased out BrdU⁺ cells from the bone
marrow and circulation within few days, consistent with previously described turnover rates of circulating monocytes\textsuperscript{224}. However, the turnover of intimal BrdU\textsuperscript{+}CD11c\textsuperscript{+} cells was longer and the number of those cells decreased gradually and disappeared by 6 weeks (Fig. 1b,c).

Another approach to quantify the turnover of intimal CD11c\textsuperscript{+} cells was to transplant GFP\textsuperscript{+} bone marrow cells into GFP\textsuperscript{-} mice, which would also be relevant for later bone marrow transplant experiments. The percentage of GFP\textsuperscript{+} cells in the intima (chimerism) increased gradually to 85-95\% between 6-8 weeks after reconstitution (Fig. 1d). It is also worth noting that the total number of intimal CD11c\textsuperscript{+} cells, within 2 weeks, dropped to 50\% of the original number of cells before bone marrow transplantation (Fig. 1e). Intimal CD11c\textsuperscript{+} cells do not normally proliferate, and therefore should not be affected by exposure to gamma irradiation. In fact, the total number of intimal CD11c\textsuperscript{+} cells did not decrease during the 3-4 days following irradiation and bone marrow reconstitution, suggesting this process is not responsible for the decrease in cell number observed at 2 weeks. After irradiation and bone marrow transplantation, there is a lag period required for the new bone marrow cells to establish a niche and replenish hematopoietic cell production. The reduction observed in intimal CD11c\textsuperscript{+} cells might be due to continued turnover of these cells from the vessel wall – through cell death or egress – without reconstitution by newly recruited blood monocytes. Nevertheless, the total number of intimal CD11c\textsuperscript{+} cells gradually recovers overtime, and reaches the original homeostatic number of intimal CD11c\textsuperscript{+} cells that are derived from the donor bone marrow by 8 weeks (Fig. 1e). Notice the low number of CD11c\textsuperscript{+} cells at 2 weeks (Fig. 1f) versus higher density of CD11c\textsuperscript{+} cells at 8 weeks (Fig. 1g) shown in representative confocal images.
Figure 1: Complete turnover of intimal CD11c+ cells occurs within 6-8 weeks.

(a) Abundance of intimal CD11c+ cells in the lesser curvature of wild type C57BL/6 mice was determined at different ages. (b,c) Mice received BrdU in the water between -1 and 0 weeks, and regular water afterwards. (b) Enumeration of intimal CD11c+ BrdU+ cells in the ascending aortic arch. (c) Representative en face confocal image at the 0 week timepoint. 40x objective. BrdU is in green, CD11c is in red, and nuclei are in blue. (d-g) C57BL/6 WT recipient mice were exposed to γ-radiation to destroy BM stem cells, were reconstituted with 5x10^6 BM cells from Ubc-gfp transgenic donor mice, and were dissected at the indicated timepoints. The ascending aortic arch was stained for CD11c (red) and nuclei (blue) and analyzed by en face confocal microscopy. (d) Percent chimerism representing the ratio of GFP+CD11c+ intimal cells to the total number of intimal CD11c+ cells, over 8 weeks after irradiation and BM transplantation. (e) Total number of intimal CD11c+ cells as a percentage of the number of cells before bone marrow reconstitution (time 0). (f,g) Representative images of the GFP to WT bone marrow transplantation experiment. 40x objective. GFP is in green, CD11c is in red and nuclei are in blue. (f) is from the 2 weeks timepoint, whereas (g) is from the 8 week timepoint. For all experiments, means ± SEM were derived from at least three independent experiments with two mice per experimental group (the total number of mice per group was 6 or higher).
Figure 1

(a) RTM of intimal CD11c\(^+\) cells

(b) Intimal BrdU \(\cdot\) CD11c\(^+\) cells per ascending aorta

(c) Micrographs of intimal CD11c\(^+\) cells

(d) % chimerism

(e) Intimal CD11c\(^+\) cells (%)

(f) Micrographs of intimal CD11c\(^+\) cells

(g) Micrographs of intimal CD11c\(^+\) cells
2.2.6 Intimal cell harvest, RNA isolation and real-time qPCR

The heart and aorta were transferred to a dissecting dish with cold 1 mM aurintricarboxylic acid (ATA) solution (which inhibits protein-nucleic acid interactions, thus is a potent inhibitor of ribonuclease) in PBS to isolate the ascending aortic arch (from the root to the innominate artery) with the aid of a dissecting scope. The aorta was pinned down with endothelium facing up, and incubated at 37°C for 10 minutes with 2 μl DNase I and 100 μl of 25 μg/ml of Liberase™ enzyme (research grade, Roche) to digest extracellular matrix and loosen endothelial cells. Digestion was terminated by rinsing with 1 mM ATA, and then 2 μL of 0.1 μm FITC beads suspension (Polysciences) was added to assist in visualizing the endothelial cell surface during intimal cell harvesting and collection. Intimal cells were gently scraped with a 30 gauge needle and transferred directly to lysis buffer (RLT) of RNeasy micro RNA extraction kit (Qiagen). Cells from a single aorta were used for each sample analyzed. Total mRNA was isolated from harvested cells as per the manufacturer’s protocol.

RNA was isolated from mouse blood and homogenized spleen samples using TRIzol reagent (Sigma-Aldrich). 200 μl of chloroform were added to 1 ml of TRIzol reagent containing 1-10x10^6 cells, and incubated at room temperature for 15 minutes before centrifugation at 12,000g for 15 minutes at 4°C. The aqueous phase was transferred to a fresh tube and 0.5 ml of 2-propanol was added for 10 minutes, followed by centrifugation for 10 minutes, as above. The pellet was then washed with 1 ml of 75% ethanol and spun down for 5 minutes and supernatant was removed. The RNA pellet was air dried for 10 minutes and resuspended in 13.2 μl of water.

Reverse transcription (RT) was performed with the isolated mRNA and random primers using the SuperScript III RT kit (Invitrogen). cDNA was diluted 1/10 and mRNA levels were quantified by real-time qPCR using Lightcycler 480 SYBR Green I master
mix (Roche). The cycle settings used were: 95°C for 5 minutes, followed by 45 cycles of 95°C for 10 seconds, 60°C for 20 seconds and 72°C for 30 seconds. Melt curve analysis was carried out at the end of 45 cycles to determine product purity.

Standard curves were generated for each primer pair to determine primer efficiency, with 10 fold serial dilutions of reference mRNA prepared from heart, lung and liver and lymph node tissues of C57BL/6 mouse after injection with 10 μg of LPS for 2 hours. All mRNA expression values were normalized to the housekeeping gene, HPRT. The primer pairs used are listed in the following table. All primer pairs span intron/exon boundaries or are located in adjacent exons.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence 5’- 3’</th>
</tr>
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<tbody>
<tr>
<td>HPRT</td>
<td>Forward</td>
<td>CAAGCTTGCTGGTGAAAGGA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TGAAGTACTCATTATAGTCAAGGGCATATC</td>
</tr>
<tr>
<td>CD11c</td>
<td>Forward</td>
<td>CCACTGTCTGCCTTCTTCTATTTCG</td>
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<td>Forward</td>
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<td>Reverse</td>
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<td></td>
<td>Reverse</td>
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2.2.7 Aorta digest, flow cytometry and cell sorting

For digestion of aortic tissue, the aorta was perfused with 10 ml PBS, dissected, cut into small pieces, and subjected to enzymatic digestion with 450 U/ml collagenase I, 125 U/ml collagenase XI, 60 U/ml DNase I and 60 U/ml hyaluronidase (Sigma-Aldrich) for 40 minutes at 37 °C while shaking. Single-cell suspensions of digested tissues were obtained by homogenization though 40-μm-nylon mesh.

Peripheral blood for flow cytometric analysis was collected from the orbital sinus using EDTA-coated capillary tubes or by cardiac puncture using a 50 mM EDTA solution as anticoagulant. Erythrocytes were lysed using RBC Lysis Buffer (BioLegend). Spleens, popliteal and inguinal lymph nodes were homogenized through a 40-μm-nylon mesh using 1 ml PBS, after which RBC lysis was performed for spleen samples using RBC Lysis Buffer. The total cell count was determined by preparing a 1:10 dilution of the cell suspension and Trypan Blue (Invitrogen); and enumeration was done using a hemocytometer. Samples were treated with Fc-Block (BD Biosciences) for 15 minutes prior to incubation with antibody cocktail for an additional 30 minutes. Flow cytometry data was acquired on an LSRII BGRV analyzer (Becton Dickinson, BD) and analyzed with FlowJo software version 10 (Tree Star, Inc.).

For cell sorting of intimal CD11c<sup>+</sup> cells, whole aortas from 4 mice were isolated and pooled for each experiment. CD45.1 spleen cells (10<sup>6</sup>) were added to minimize cell loss during immunostaining and flow sorting. Intimal and blood leukocytes were sorted using an Aria II, BRV cell sorter (Becton Dickinson, BD). CD45.2<sup>+</sup> CD11c<sup>+</sup> cells were collected directly into RLT lysis buffer for RNA isolation with the RNeasy micro RNA extraction kit (Qiagen). RNA was isolated from unsorted and sorted blood cells using TRIZOL standard protocol for RNA extraction, and reverse transcription and real-time qPCR was carried out as described in section 2.2.6.
2.2.8 Adoptive transfer of lymph node cells

Popliteal and inguinal lymph node cells were isolated from donor *Ubc-gfp* transgenic mice and homogenized through a 40-μm-nylon mesh. WT or *Plt* recipient mice were injected with nonimmune isotype control IgG one hour before injection of twenty million lymph node cells from the GFP donor mice. In different groups, WT recipient mice were injected with either function-blocking antibody to CCL19 or CCL21 1 hour before injection of twenty million lymph node cells from the GFP donor mice. Recipient mice were left for two hours before harvesting blood, spleen, and lymph node samples for flow cytometry as described in section 2.2.7. The percentage of total CD3⁺CD4⁺ T cells that were GFP⁺ was determined in lymph nodes. Data was normalized to the percentage of GFP⁺ cells in the spleen to control for errors due to variability in cell injection.

2.2.9 *In vitro* chemotaxis assay

CD8⁺ T cells were isolated by negative selection (no. 19853, Stemcell Technologies, Vancouver BC) from lymph nodes of *C57BL/6* mice according to the manufacturer’s protocol. 1.0 x 10⁵ CD8⁺ T cells were placed into the upper well of a 96 well chemotactic chamber (Neuroprobe, Gaithersburg, MD) and were allowed to migrate at 37°C for 4 hours towards CCL21. Nonimmune isotype control IgG or function-blocking antibody to CCL19 or CCL21 was added to the cells in the upper well. At the end of the chemotaxis period, cells that did not migrate were aspirated from the top well, washed twice with fresh PBS, and migrated cells were pelleted in the bottom well. Migrated cells were detected by labeling of their DNA with SYTOX green (Invitrogen) in PBS (2% Triton x-100). Fluorescence was determined using a DTX 880 Multimode detector (Beckman Coulter, Brea, CA). The number of migrated
cells was calculated based on a standard curve generated with known quantities of CD8+ T cells.

2.2.10 Statistical analyses

Statistical analysis was conducted using Prism software version 4.03. All data reported are expressed as mean values +/- standard error of the mean (SEM). The unpaired Student's t test without Welch's correction (assuming equal variance) was used when comparing differences between two groups. Multiple group analysis was performed using one-way analysis of variance (ANOVA), with the Tukey-Kramer or Newman-Keuls multiple comparison post-hoc tests to determine differences between multiple groups. Group comparisons were determined to be significant for p values less than 0.05 (* p < 0.05, ** p < 0.01, *** p < 0.001).
2.3.1 Reverse transendothelial migration (RTM) of intimal CD11c+ cells.

2.3.1.1 Systemic stimuli reduce the abundance of aortic intimal CD11c+ cells.

Pathogens that gain access to the circulation have the potential to infect the artery wall and intimal myeloid cells are the first line of defense. The innate immune system utilizes Toll-like receptors (TLRs) and NOD-like receptors (NLRs) to detect and respond to pathogens\textsuperscript{225,226}. Therefore, we investigated the response of intimal CD11c+ myeloid cells to systemic injection of \textit{E. coli}, a common extracellular pathogen, and various TLR and NLR ligands. The lesser curvature of the ascending aorta was evaluated by \textit{en face} confocal microscopy\textsuperscript{40} because intimal CD11c+ cells are consistently found at this location in normal wild-type (WT) \textit{C57BL/6} mice\textsuperscript{15}.

\textit{C57BL/6} WT mice received intravenous (iv) injections with increasing doses of log-phase \textit{E. coli} and the total number of intimal CD11c+ was assessed 24 hours later. \textit{E. coli} reduced the number of intimal CD11c+ cells in the ascending aorta in a dose-dependent manner (Fig. 2a). A similar dose-dependent effect was observed after injection of LPS (TLR4 ligand), Poly(I:C) (TLR3 ligand), CpG (TLR9 ligand), IE-DAP (NOD1 ligand), and MDP (NOD2 ligand) (Fig. 2b,c). We did not further examine other TLR and NOD ligands in this study in order to focus on the mechanism of the rapid reduction observed; nevertheless, we anticipate that systemic injection of other ligands may result in a similar dose-dependent effect on intimal CD11c+ cells. Enumeration of intimal CD11c+ cells was obtained by taking overlapping micrographs spanning the entire lesser curvature and were later compiled in Adobe Photoshop software (Fig 2d).

A time-course with LPS revealed that the maximal decrease occurred between 6 and 12 hours (Fig. 1e). The number of cells remained the same between 0 and 6 hours; this lag phase suggests induction of gene expression. It is also worth noting that the number of cells did not decrease below 50% with any ligands (see discussion).
Figure 2: Systemic stimuli reduce the abundance of aortic intimal CD11c⁺ cells. (a,b) WT mice were injected iv with log-phase live *E. coli* (The number of colony forming units (CFUs) is indicated) (a) or different doses of PRR ligands (b). Intimal CD11c⁺ cells per ascending aorta were enumerated 24 hours after injection. (c) Representative *en face* confocal microscopy images of the mouse ascending aorta 24 hours after iv injection of PBS or LPS (100μg). Nuclei of intimal CD11c⁺ cells (green) and endothelial cells (unstained) are blue. Scale bars represent 50 μm. (d) Composite *en face* confocal images of the ascending aorta lesser curvature harvested from PBS (upper panel) or LPS (lower panel) injected mice for 24 hours and stained for CD11c (green) and nuclei (blue). Scale bar = 400 μm. (e) Time-course analysis showing intimal CD11c⁺ cell abundance over 6 hour time intervals during the first 24 hours after injection of LPS (100 μg). In all panels, representative images as well as means ± SEM were derived from at least three independent experiments with two mice per experimental group (the total number of mice per group was 6 or higher). Asterisks represent statistically significant differences from the PBS control group unless otherwise indicated: * P< 0.05, ** P< 0.01, *** P< 0.001.
RTM of intimal CD11c+ cells

Chapter 2

Figure 2

(a) E. coli

(b) Intimal CD11c+ cells

(c) PBS LPS

(d) Intimal CD11c+ cells

(e) Time (h) after LPS
2.3.1.2 Reduction is not due to loss of CD11c expression but cell removal.

In order to ascertain that the observed reductions in intimal CD11c\(^+\) cell number is not due to loss of CD11c expression, we stained the ascending aorta with the pan leukocyte marker, CD45, and a comparable decrease in the number of intimal CD45\(^+\) cells was observed (Fig. 3a). We also assessed LPS-induced reduction of intimal cells after nuclear labeling of a cohort of intimal CD11c\(^+\) cells with BrdU. WT mice received drinking water supplemented with BrdU for a week, and then during the second week BrdU was removed in order to chase-out labeled leukocytes from the bone marrow and blood (Fig. 3b). Approximately 10% of intimal CD11c\(^+\) cells were labeled by this protocol (Fig. 3c,d). LPS injection reduced the intimal BrdU\(^+\) CD11c\(^+\) cell number (Fig. 3d), indicating that these cells either egress from the intima or undergo apoptosis.

2.3.1.3 Reduction in intimal CD11c\(^+\) cells is not due death by apoptosis.

LPS-induced DC maturation has been shown to initiate DC apoptosis in order to regulate the magnitude of the immune response by limiting antigen availability to T cells\(^{227}\). However, we did not observe an increase in TUNEL\(^+\) CD11c\(^+\) intimal cells within 24h of LPS injection (Fig. 3e,f), particularly between 6 and 12h, the time interval when the maximal drop was observed (Fig. 2e). A positive control for this assay was injection of diphtheria toxin (DT) into Cd11c-DT receptor (Dtr) transgenic mice\(^{16}\), which induced abundant TUNEL\(^+\) CD11c\(^+\) intimal cells (Fig. 3e,g). The role of apoptosis was also assessed by injecting LPS or Poly(I:C) into Cd11c-hbcl2 transgenic mice, since the Bcl2 transgene functions as an anti-apoptotic factor and promotes the survival of CD11c\(^+\) cells\(^{228}\). The magnitude of intimal CD11c\(^+\) cell reduction (Fig. 3h) in Cd11c-hbcl2 aorta was comparable to that in WT mice, further suggesting that LPS and Poly(I:C) do not induce apoptosis of intimal myeloid cells.
Figure 3: Reduction of intimal CD11c$^+$ cell numbers was not due to loss of CD11c expression of cell death by apoptosis. (a) CD45$^+$ cells in the ascending aortic intima of WT mice were enumerated 24h after injection of PBS or LPS. (b-d) Intimal CD11c$^+$ cells were labeled by introducing BrdU into the drinking water of WT mice prior to injection of PBS or LPS (b). (c) A representative image from a PBS-injected mouse shows BrdU-labeled nuclei (green, arrowheads) in CD11c$^+$ cells (red). Scale bars in images represent 50 μm. (d) BrdU$^+$ CD11c$^+$ cells were counted 24h after injection of PBS or LPS. (e) Enumeration of TUNEL$^+$ intimal CD11c$^+$ cells after iv injection of LPS into WT mice, or diphtheria toxin (DT) into CD11c-DTR transgenic mice. (f,g) Representative en face confocal images from either WT mice injected with LPS for 12 hours (f), or CD11c-DTR$^+$ transgenic mice injected with DT for 8 hours (g) to induce apoptosis as a positive control for TUNEL staining. Arrowhead: Intimal TUNEL$^+$ (red) CD11c$^+$ (green) cells, nuclei (blue). Scale bar = 20 μm. (h) Enumeration of intimal CD11c$^+$ cells in CD11c-hbcl2 transgenic mice 24 hours after injection of PBS, LPS or Poly(I:C). In all panels, representative images as well as means ± SEM were derived from at least three independent experiments with two mice per experimental group (the total number of mice per group was 6 or higher). Asterisks represent statistically significant differences from the PBS control group: * $P<0.05$, *** $P<0.001$. 
Figure 3

(a) Intimal CD45^+ cells

(b) PBS or LPS

(c) Analysis

(d) Intimal BrdU^+ CD11c^+ cells per ascending aorta

(e) IV injection (mice):
- LPS (C57BL/6)
- DT (CD11c-DTR)

(f) TUNEL^+ intimal CD11c^+ cells per ascending aorta

(g) 0 6 9 12 18 24 8

(h) Time (h) after iv injection

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2.3.1.4 *In vivo* studies show that LPS induces RTM of intimal CD11c+ cells.

Since LPS did not induce apoptosis of intimal CD11c+ cells, we investigated the other alternative that LPS induces egress of intimal myeloid cells by RTM because conventional lymphatic drainage is lacking in the arterial intima. We reasoned that after undergoing RTM intimal myeloid cells would temporarily adhere to arterial endothelium analogous to DCs crawling along the lymphatic endothelial surface following their migration into afferent lymphatics.

Adherent leukocytes can be detected readily by scanning electron microscopy (SEM) in the ascending aorta of WT mice injected with LPS (Fig. 4a). However, SEM cannot distinguish whether adherent leukocytes had undergone RTM or are adherent blood leukocytes. Therefore, clodronate liposomes (CL) were employed 12h before LPS injection to efficiently deplete circulating monocytes (Fig. 4b), and PBS liposomes (PL) served as a control. Injection of CL did not deplete intimal CD11c+ cells, nor did it prevent LPS-induced reduction in intimal CD11c+ cells (Fig. 4c).

Quantification of SEM images revealed that LPS injection increased the numbers of adherent leukocytes on the endothelial surface at 4 and 9h and that depletion of circulating monocytes by CL reduced these numbers (Fig. 4d). In PL-treated mice, LPS-induced expression of adhesion molecules and chemokines on endothelial cells mediated increased blood monocyte adhesion. At 4h, the number of adherent leukocytes in CL-treated mice was close to baseline, suggesting that RTM does not occur at this time point and all cells were adherent circulating leukocytes. At 9 hours, the time point when LPS-induced reduction of intimal CD11c+ cells is first observed (Fig. 2e), the number of adherent leukocytes in CL-treated mice was significantly elevated above baseline levels (Fig. 4d). These data are consistent with the possibility that LPS induces intimal CD11c+ cell RTM at the 9 hours time point.
**Figure 4:** *In vivo* studies show that LPS induces reverse transendothelial migration of intimal CD11c⁺ cells into the arterial lumen. (a) Representative scanning electron microscope (SEM) images of the ascending aortic lesser curvature 9h after iv injection of PBS or LPS into WT mice. Arrowheads indicate leukocytes adhered to the endothelial monolayer after LPS injection. Left and middle panel scale bar = 75 μm. Right panel shows a representative adherent leukocyte at high magnification (scale bar = 5 μm). (b) Representative flow cytometry plots of blood samples from *C57BL/6* WT mice injected with PBS liposomes (PL) (left panel) or clodronate liposomes (CL) (middle panel). Classical monocytes were identified as CD11b⁺Gr-1intermediate (circled). Absolute quantification of monocytes per ml of blood is shown in the right panel. (c) PBS liposomes (PL) or clodronate liposomes (CL) were injected 12h before PBS or LPS, and the number of intimal CD11c⁺ cells per ascending aorta was determined 24h later. (d) Quantification of adherent leukocytes observed by SEM. (e) Representative images of *Cd11c-eYfp* transgenic mouse ascending aortas 9h after PBS (upper panel) or LPS (lower panel) injection. Z-stacks (1 μm steps) from the intima to the adventitia were obtained by confocal microscopy. Orthogonal views of 3D reconstructed Z-stacks by Imaris software are shown (magnification: 60x). Note the absence of eYFP⁺ (green) or CD68⁺ (red) cells in the media. Nuclei are blue. In all panels, representative images as well as means ± SEM were derived from at least three independent experiments with two mice per experimental group (the total number of mice per group was 6 or higher). Asterisks represent statistically significant differences from the PBS control group unless otherwise indicated: * P < 0.05, *** P < 0.001.
RTM of intimal CD11c+ cells

Figure 4

a) PBS and LPS treatments shown in images.

b) Flow cytometry analysis showing CD11c+ cells (left) and classical monocytes (right).

b) Comparison of intracellular CD11c+ cells in CL and PL.

d) Adhered leukocytes per unit area over time: PL and CL.

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To exclude the possibility that intimal CD11c+ cells migrate through the media, Cd11c-eYFP transgenic mice were injected with LPS and after 9h the entire artery wall was subjected to optical sectioning by confocal microscopy. eYFP+ and CD68+ (a marker for myeloid cells including DCs and macrophages) cells could be observed in the intima and the adventitia but were completely absent from the media (Fig. 4e).

2.3.1.5 Ex vivo studies show that LPS induces RTM of intimal CD11c+ cells.

Direct confirmation of intimal CD11c+ cell RTM requires collection of transmigrated cells from the aortic lumen. Since strategies do not exist to selectively tag intimal myeloid cells in vivo, we set out to develop an ex vivo organ culture model. PBS or LPS was injected in vivo for 6h prior to harvest of the ascending aorta and incubation with PBS or LPS for another 6h (Fig. 5a). This approach minimized the ex vivo incubation time. Incubation with LPS ex vivo induced a reduction in the number of intimal CD11c+ cells in a dose-dependent manner (Fig. 5b). Alternatively, when aortas were incubated for 12h ex vivo with LPS (without injection of LPS in vivo) we also observed a reduction of intimal CD11c+ cells (Fig. 5c). To enable collection of transmigrated cells, aortic arches from Cd11c-eYFP transgenic mice were cannulated and the lumen was continually perfused for up to 6h ex vivo (Fig. 5a,d). The number of collected eYFP+ cells was significantly higher in perfusates containing LPS versus PBS (Fig. 5e,f). Continuous imaging of CD11c-eYFP+ aortas placed in a perfusion chamber revealed that LPS induced a gradual disappearance of CD11c+ intimal cells with features that were distinct from apoptosis, where cells did not disappear, instead cell debris were continually being shed off and ghost dead cells were left behind (Fig. 5g). CD11c+ cells that have undergone RTM may be carried off by the bloodstream, or exhibit crawling behavior analogous to that of non-classical patrolling monocytes229.
**Figure 5:** *Ex vivo* studies show that LPS induces reverse transendothelial migration of intimal CD11c+ cells into the arterial lumen. (a) WT mice were injected with PBS or LPS (100 μg) 6h prior to harvesting of ascending aortas. Aortas were incubated, perfused or imaged for 6h with LPS. (b) Intimal CD11c+ cells were enumerated by *en face* confocal microscopy to determine the optimal dose of LPS to be used in *ex vivo* experiments. 50 μg/ml LPS will be used in the remaining panels. (c) The ascending aortic arch from *C57BL/6* WT mice was incubated *ex vivo* (without *in vivo* treatment) for 12 hours with culture media containing PBS or 50 μg/ml LPS, then the number of intimal CD11c+ cells was determined by *en face* confocal microscopy. (d-f) *Cd11c-eyfp* transgenic mice were injected with PBS or LPS for 6h. Aortic arches were cannulated and perfused *ex vivo* for 6h (a). Arrows indicate the direction of flow. (d) eYFP+ cells in the collected perfusate were quantified over three 2h intervals. (f) Representative image of enumeration of collected cells from LPS-treated aortic arches. Each fraction was spiked with 10^4 eYFP-U937 cells (Blue nuclei that are negative to eYFP and CD11c). Cells were then transferred into uncoated μ-Slide VI 0.4 (ibidi) and the number of eYFP+CD11c+ cells was counted. (g) Representative images from *ex vivo* real-time imaging of the arterial intima of *Cd11c-eyfp* transgenic mice. Arrowheads indicate eYFP+ cells that disappear between 0 and 6 h. Scale bars represent 100 μm. For DT samples, *Cd11c-eyfp*dtr+ aortas were used and the perfusate contained diphtheria toxin (DT) to induce apoptosis. Cells did not disappear, instead cell debris were continually being shed off and ghost dead cells are left behind at the 6 hour time point. In all panels, representative images as well as means ± SEM were derived from at least three independent experiments with two mice per experimental group (the total number of mice per group was 6 or higher). Asterisks represent statistically significant differences: * P< 0.05, ** P< 0.01, *** P< 0.001.
Figure 5

a. *in vivo* and *Ex vivo*
- Injection of PBS or LPS
- Harvest of aorta
- Analysis
- Percussion or Incubation with PBS or LPS
- 6 h

b. Graph showing Intimal CD11c+ cells
-PBS
- LPS (µg/ml) 5 16.5 50
- Percent

[c] Graph showing Percent Intimal CD11c+ cells
- PBS
- LPS

[d] CD11c-eYFP mouse aorta

[e] Graph showing Collected eYFP cells
- Collection interval (h) 0-2
- PBS
- LPS

[f] Images showing eYFP and CD11c

[g] Images showing PBS and LPS treatment at 0h and 6h
- PBS 0h
- LPS 0h
- DT 0h
- PBS 6h
- LPS 6h
- DT 6h
2.3.2 RTM is dependent on CCR7 & CCL19 expression by intimal CD11c\(^+\) cells.

2.3.2.1 Intimal mRNA expression after systemic stimulation with TLR ligands.

TLR signaling induces CCR7 expression in peripheral tissue DCs, enabling them to respond to CCL19 and CCL21 chemokines and migrate to regional lymph nodes\(^{109,230}\). Therefore, we investigated the possibility that CCR7 also mediates intimal CD11c\(^+\) cell RTM. A 6h time point was selected because it immediately precedes LPS-induced reduction of intimal CD11c\(^+\) cells (between 6 and 12h) (Fig. 2e). Intimal expression of CCR7 and CCL19 was not detectable in PBS-injected mice; however, injection of either LPS or Poly(I:C) induced detectable mRNA expression in the ascending aortic lesser curvature (Fig. 6a). In contrast, CCL21 expression was detected basally and did not increase in response to LPS or Poly(I:C). To assess whether intimal CD11c\(^+\) cells produce CCR7, CCL19 and CCL21, these cells were deleted by injecting C\(d11c\)-dtr transgenic mice with DT 24 hours prior to PBS, LPS or Poly(I:C) injection. Depletion virtually abrogated CCR7 induction in response to LPS- and Poly(I:C), significantly reduced CCL19 induction, but had no effect on CCL21 expression levels (Fig. 6b). These data indicate that TLR stimuli induce CCR7 and CCL19 expression by intimal CD11c\(^+\) cells and to some extent CCL19 expression by endothelial cells, whereas endothelial cells constitutively express CCL21. We also performed a detailed time-course assessing the expression of CCR7, CCL19, CCL21, CD45, CD68, and CD11c. CCR7 and CCL19 expression patterns were remarkably similar, with expression increasing to peak levels at 4-8h and progressively decreasing at 10, 12 and 16h (Fig. 6c), whereas CCL21 expression did not change significantly. CD45, CD68 and CD11c expression decreased gradually to 50% between 6-12h (Fig. 6c), similar to results from intimal CD11c\(^+\) cell enumeration by immunostaining (Fig. 2e), which correspond to the time in which CCR7 and CCL19 expression dropped.
Figure 6: Intimal mRNA expression after systemic stimulation with TLR ligands.

(a) CCR7, CCL19, CCL21, and CD11c mRNA expression 6h after injection of PBS, LPS or Poly (I:C) in the ascending aortic lesser curvature of control Cd11c-dtr transgenic mice without intimal CD11c⁺ cell depletion (injected with PBS 1 day before the experiment). Values were normalized to LPS-induced levels designated as 1. ND means not detectable. Statistical values are compared to PBS injected group. (b) The value of each column (undepleted) in (a) was normalized to be 1 (dotted line in (b)). mRNA expression in Cd11c-dtr transgenic mice depleted of intimal CD11c⁺ cells (injected with DT 1 day before the experiment) was expressed as ratio compared to undepleted values (dotted line). Asterisks represent significant differences (unpaired Student’s t test) between depleted versus undepleted groups. (c) A time-course showing intimal mRNA expression after injection of LPS. Data are normalized to the expression values at 6 hours. Asterisks are statistical analysis compared to the 0 hour time point, whereas + signs are statistical analysis compared to the 6 hour time point. In all panels, means ± SEM were plotted, which were derived from at least three independent experiments with two mice per experimental group (the total number of mice per group was 6 or higher). One way analysis of variance was applied to (a) and (c), whereas Student’s t test was used for (b). Asterisks represent statistically significant differences: * P< 0.05, ** P< 0.01, *** P< 0.001.
Figure 6

(a) Relative mRNA expression

(b) mRNA expression ratio (depleted:undepleted)

(c) Relative mRNA expression over time after LPS injection (h)
2.3.2.2 CCR7 and CCL19 are expressed in BM-derived (not vessel wall) cells.

CCR7 is coupled to Goi\textsuperscript{231}; therefore, pertussis toxin (PTx), a Goi inhibitor, was used to assess if CCR7 signaling is required for RTM. Injection of PTx, but not of the inactive control B-oligomer (composed of subunits S2-S5 and lacking the enzymatic S1 subunit\textsuperscript{232}), blocked LPS- and Poly(I:C)-induced reduction of intimal CD11c\textsuperscript{+} cells (Fig. 7a). Subsequent experiments using CCR7\textsuperscript{-/-} and Plt (CCL19- and CCL21-deficient) mice revealed no reduction of intimal CD11c\textsuperscript{+} cells following injection of LPS or Poly(I:C) (Fig. 7b). Collectively these experiments establish that CCR7 and its ligands are required for intimal CD11c\textsuperscript{+} cell RTM.

Isolation of cells for mRNA expression analysis is not functional and is not always conclusive as to which cell type in the arterial intima (endothelial cells versus CD11c\textsuperscript{+} cells) is more significant in RTM. Therefore, reciprocal bone marrow (BM) transplantation studies using WT, CCR7\textsuperscript{-/-}, Plt, and CCL19\textsuperscript{-/-} mice were carried out in order to ascertain whether deficiency of CCR7 and CCL19/21 in BM-derived intimal myeloid cells versus recipient artery wall endothelial and smooth muscle cells affects RTM. Initially, transplantation of WT BM into WT recipients established that lethal irradiation and reconstitution of BM and intimal myeloid cells did not have a detrimental effect on RTM (Fig. 7c). Similarly, a reduction in intimal CD11c\textsuperscript{+} cells in response to LPS / Poly(I:C) was observed when WT BM was transplanted into CCR7\textsuperscript{-/-} or Plt recipients (Fig. 7d,e). Plt mice are deficient in both CCL19 and CCL21 and expression studies implicated induction of CC19 but not CCL21 (Fig. 6); therefore, we obtained Ccl19\textsuperscript{-/-} bone marrow cells in order to ascertain its role. The reduction of intimal CD11c\textsuperscript{+} cells was abrogated when CCR7\textsuperscript{-/-}, Plt or Ccl19\textsuperscript{-/-} BM was transplanted into WT recipients (Fig. 7d-f). These data suggest that expression of CCR7 and CCL19 by BM-derived intimal myeloid cells and not vascular wall cells is required for RTM.
Figure 7: RTM is dependent on CCR7 and CCL19 (but not CCL21) expression by intimal BM-derived CD11c+ cells and not vessel wall cells (endothelial and smooth muscle cells). (a) WT mice were injected iv with either B-oligomer (2 μg) or Pertussis Toxin (PTx, 2 μg) as well as LPS or Poly(I:C) and mice were dissected 24 hours later. A control group was injected with PBS for 24 hours. The number of intimal CD11c+ cells per ascending aorta was quantified by en face confocal microscopy. (b) Ccr7−/− and Plt mice were injected iv with PBS, LPS or Poly(I:C) and intimal CD11c+ cells were quantified at 24h. (c-f) Bone marrow (BM) transplantation studies between donors and recipients as indicated above each graph. Mice were housed for 8 weeks after BM transplantation to ensure complete turnover of intimal CD11c+ cells and reconstitution with donor BM-derived cells. The number of intimal CD11c+ cells was quantified at 24h after injection of TLR ligand and normalized to the corresponding PBS-injected group. Note that the expression of both CCR7 and CCL19 in BM-derived cells is required for the reduction of intimal CD11c+ cells by TLR ligands. As we only received Ccl19−/− BM cells not mice, the reciprocal transplantation with WT mice as donors and Ccl19−/− mice as recipients could not be ascertained; nevertheless, studies from Plt mice suggest that WT BM-derived cells would rescue RTM if it is inhibited in Ccl19−/− recipient mice. In all panels, means ± SEM are plotted, which were derived from at least three independent experiments with two mice per experimental group (the total number of mice per group was 6 or higher). Asterisks represent statistically significant differences compared to PBS injected groups: ** P < 0.01, *** P < 0.001.
Figure 7

a. B-oligomer (control) vs. PTx

b. CCR7−/− mice vs. Plt mice

c. BM donor: WT; Recipient: WT

d. BM donor: CCR7−/−; Recipient: WT

BM donor: WT; Recipient: CCR7−/−

e. BM donor: Plt; Recipient: WT

f. BM donor: WT; Recipient: CCL19−/−
2.3.2.3 Functional studies implicating a role for CCL19 but not CCL21 in RTM.

Reciprocal bone marrow transplantation studies suggested that the critical source of CCL19 for RTM is intimal CD11c\(^+\) cells. However, functional studies were still important to definitely show its role in RTM and exclude potential side effect from exposure to \(\gamma\)-radiation. To further characterize the role of CCL19 versus CCL21, function-blocking antibodies were injected along with LPS into WT mice. Non-immune isotype control IgG and PBS served as controls. Only CCL19 antibody prevented LPS-induced reduction of intimal CD11c\(^+\) cells (Fig. 8a).

In order to establish that the anti-CCL21 blocking antibody was efficient in blocking chemokine function, \textit{in vitro} chemotaxis experiments and \textit{in vivo} adoptive transfer experiments were performed. In \textit{in vitro} chemotaxis experiments, CD8\(^+\) T cells (isolated from mouse lymph nodes) were placed in the upper chamber, along with blocking antibody to CCL19, CCL21 or isotype control IgG; CCL21 was placed in the bottom chamber. Anti-CCL21 antibody blocked migration of CD8\(^+\) T cells to CCL21 (Fig. 8b). Homing of T cells to lymph nodes from the circulation via high endothelial venules (HEVs) is CCL21-dependent\(^{64,233}\). In adoptive transfer experiments, GFP-labeled lymph node cells were injected to \textit{Plt} or WT mice, along with blocking antibody to CCL19, CCL21 or isotype control IgG. Anti-CCL21 antibody blocked migration of GFP\(^+\) T cells to recipient’s lymph nodes (Fig. 8c). Overall, these experiments confirmed that the anti-CCL21 blocking antibody blocked chemokine function.

An alternative to antibody blocking in WT mice is to rescue RTM in \textit{Plt} mice by recombinant chemokines delivery. \textit{Plt} mice injected with PBS or LPS for 12 hours received three iv injections of recombinant mouse CCL19 or CCL21 in the last six hours to determine which chemokine rescues the \textit{Plt} phenotype. Only recombinant CCL19 rescued LPS-induced reduction of intimal CD11c\(^+\) cells (Fig. 8d).
Figure 8: Functional studies showing that LPS-induced RTM of intimal CD11c+ cells is dependent on CCL19 but not CCL21. (a) WT mice were injected with LPS and isotype control IgG or function-blocking antibodies to CCL19 or CCL21. A control group was injected with PBS. The number of intimal CD11c+ cell was determined after 24 hours. (b) In vitro blocking of CCL21-mediated chemotaxis of CD8+ T cells by function-blocking antibodies. CD8+ T cells were purified from WT mouse lymph nodes. The bottom wells contained CCL21 (100 nM), and cells treated with control IgG or blocking antibodies to CCL19 or CCL21 were added to the top wells of the chemotaxis plate. Data represents three independent experiments, each contained triplicate wells, and values were normalized to the IgG-treated control group. (d) In vivo adoptive transfer of UBC-GFP lymph node cells into Plt and WT mice injected with isotype control IgG one hour before cell injection, or into WT mice that were injected with function-blocking antibodies to CCL19 or CCL21 one hour before cell injection. Two hours later, blood, spleen, and lymph nodes were isolated and cell suspension was stained with CD3 and CD4 for flow cytometry. Values represent lymph node GFP+ percentage of total CD3+CD4+ T cells. Data were normalized to spleen percentages to control for variability in cell injection, and expressed relative to control WT group injected with IgG (normalized to 1). (d) Plt mice injected with PBS or LPS at 0 hour received iv injections of PBS, recombinant mouse CCL19 or CCL21 (300 ng) at 6, 8 and 10 hours, consecutively. The number of intimal CD11c+ cells was determined at 12 hours. In all panels, means ± SEM are plotted, which were derived from at least three independent experiments with two mice per experimental group (the total number of mice per group was 6 or higher). Asterisks represent statistically significant differences compared to control groups (white bars): ** P< 0.01, *** P< 0.001.
Figure 8

**RTM of intimal CD11c+ cells**

**a**

WT mice:  
- **PBS**
- **LPS**

**Intimal CD11c+ cells**

- IgG
- anti-CCL19
- anti-CCL21

**Percent**

**b**

- **IgG**
- anti-CCL19
- anti-CCL21

**Migrated CD8+ T cells (normalized)**

**c**

- WT-IgG
- Plt-IgG
- WT-anti-CCL19
- WT-anti-CCL21

**Homing of T cells to peripheral LNs**

**d**

**Plt mice:**  
- **PBS**
- **LPS**

**Intimal CD11c+ cells**

- PBS
- CCL19
- CCL21

**Percent**
2.3.2.4 CCR7 and CCL19 are expressed by exiting intimal CD11c+ cells.

Intimal CD11c+ cells were flow sorted from aortas of mice injected with PBS or LPS for 6 hours and gene expression was assessed by qPCR. These experiments showed that LPS induced the expression of both CCR7 and CCL19 in the same population of intimal CD11c+ cells (Fig. 9a). CCL21 and an endothelial gene (VE-Cadherin) were not detected, indicating that CCL21 in the intima is mainly expressed by endothelial cells, and that isolation of intimal CD11c+ cells was devoid of endothelial cells. Moreover, since only intimal CD11c+ cells are analyzed, we observed around 50 fold enrichment in leukocyte/myeloid gene markers (e.g. CD45, CD68 and CD11c), as well as about 100- and 50-fold enrichment in CCR7 and CCL19, respectively (Fig. 9a), compared to intimal expression levels (from intimal cell isolation that includes both leukocytes and endothelial cells) in WT mice injected with LPS for 6h (Fig. 6a).

CCR7 and CCL19 are induced after LPS injection, and their expression gradually decreases after 12 hours (Fig. 6c), which coincides with cell egress out of the intima. To determine if decreased CCL19 and CCR7 expression at 12 hours was because mRNA expression decreased and returned to baseline, or because intimal cells that expressed these genes exited from the intima, we blocked migration and cell egress by RTM with pertussis toxin (as shown in Fig. 7a) or anti-CCL19 antibodies (Fig. 8a) in LPS-injected mice. Mice injected with LPS and B-oligomer or nonimmune isotype control IgG for 12 hours served as controls. Blockade prevented RTM and reduction in the number of intimal leukocytes, thus, the decrease in the leukocyte / myeloid mRNA (e.g. CD45, CD68, and CD11c) was abrogated at the 12h time point (Fig. 9b). Importantly, blockade of RTM prevented the decrease in CCL19 and CCR7 expression, but had no effect on CCL21 expression levels (Fig. 9b), consistent with intimal myeloid CD11c+ cells being the source of CCR7 and CCL19, but not CCL21.
Figure 9: CCR7 and CCL19 are expressed by the same population of reverse migrating intimal CD11c$^+$ cells.

(a) In each experiment, the aortic arches from four WT mice – injected with either PBS or LPS for six hours – were pooled and digested into a single-cell suspension. CD45$^+$CD11c$^+$ cells were sorted directly into lysis buffer for mRNA isolation and analysis of gene expression. For each gene, data were normalized to total intimal expression level (obtained from intimal cell isolation that includes both leukocytes and endothelial cells) in WT mice injected with LPS for 6h. Data indicate fold enrichment in gene expression in CD11c$^+$ cells. ND: not detectable. Means ± SEM are plotted, which were derived from three independent experiments with four mice (pooled) per experimental group (the total number of mice per group was 12). Asterisks represent statistically significant differences compared to PBS injected groups obtained by individual Student’s $t$ tests: * $P < 0.05$, *** $P < 0.001$.

(b) WT mice were injected with LPS for 6 hours (white bars) or 12 hours (black bars). The latter groups were treated with PTx or CCL19 blocking antibody; and controls were injected with B-oligomer or nonimmune IgG, respectively. Intimal mRNA expression was analyzed and data were normalized to mice injected with LPS for 6 hours (white bars). Reduction in leukocyte markers expression correlated with reduction in CCR7 and CCL19, but not CCL21; and RTM blockade prevented the reduction in cell markers, CCR7 and CCL19, but not CCL21. Means ± SEM are plotted, which were derived from three independent experiments with two mice per experimental group (the total number of mice per group was 6). Asterisks represent statistically significant differences compared to the corresponding control groups by one-way ANOVA followed by Tukey-Kramer post-hoc tests: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. 
Figure 9

a

Relative mRNA expression

- **PBS**
- **LPS**

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<tr>
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<tr>
<td>CD11c</td>
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b

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<tr>
<td>CD11c</td>
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- B-oligomer
- PTx
- IgG
- anti-CCL19

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2.3.3 TLR stimulation induces two waves of reverse transendothelial migration of intimal CD11c⁺ cells, each followed by proliferation of the remaining cells.

2.3.3.1 TLR stimulation induces a second wave of intimal CD11c⁺ cell reduction.

We next investigated how TLR stimulation influences intimal CD11c⁺ myeloid cell abundance over several days. In response to either LPS or Poly(I:C) injection, an initial 50% reduction was observed on day 1, and intimal CD11c⁺ cell numbers recovered to 100% on day 2, which was followed by a second reduction to 50% on day 3 and recovery to 100% on day 4 (Fig. 10a).

The second wave of intimal CD11c⁺ cell reduction was not due increased apoptosis. No increase in TUNEL⁺ CD11c⁺ intimal cells was observed between 2-3 days after LPS injection (Fig. 10b). A positive control for this assay was injection of diphtheria toxin (DT) into Cd11c-DT receptor (Dtr) transgenic mice¹⁶, which induced abundant TUNEL⁺ CD11c⁺ intimal cells. The role for apoptosis was also assessed by injecting LPS into Cd11c-hbcl2 transgenic mice, since the Bcl2 transgene functions as an anti-apoptotic factor and promotes the survival of CD11c⁺ cells²²⁸. The kinetics and magnitude of intimal CD11c⁺ cell reduction (Fig. 10c) was comparable to that in WT mice, suggesting that LPS does not induce apoptosis of intimal myeloid cells.

Reduction in intimal CD11c⁺ cell abundance was not observed in Ccr7⁻/⁻ mice throughout five days following LPS injection (Fig. 10d), suggesting the second wave of cell reduction is either dependent on CCR7 expression or dependent on the first wave. An alternative approach is to block CCR7-dependent migration by injection of functional blocking antibody to CCL19 (injected two days after LPS injection), which prevented the second reduction of intimal CD11c⁺ cells (Fig. 10e), suggesting that the second wave is also dependent on CCR7 and CCL19 signaling, and independent of the first wave of RTM.
Figure 10: TLR stimulation induces a second wave of intimal CD11c⁺ cell reduction, not mediated by apoptosis, but is dependent on CCR7 and CCL19 expression. (a) WT mice were injected with LPS (100 μg) or Poly(I:C) (150 μg) and intimal CD11c⁺ cells per ascending aorta were enumerated at different time points. (b) WT mice were injected with LPS and the number of TUNEL⁺ CD11c⁺ cells was determined at the indicated time points. Positive controls were Cd11c-dtr transgenic mice injected with diphtheria toxin (DT) for 8h. (c) Cd11c-hBcl2 transgenic mice were injected with LPS and the number of intimal CD11c⁺ cells was determined at the indicated timepoints. Intimal CD11c⁺ cells recovered to 100% at day 2 and dropped to 50% on day 3 (comparable pattern to that observed in WT mice). (d) Ccr7⁻/⁻ mice were injected with LPS and intimal CD11c⁺ cells were enumerated at the indicated time points. (e) WT mice were injected with PBS or LPS on day 0, and isotype control IgG or function blocking antibody to CCL19 was injected on day 2. Intimal CD11c⁺ cells were enumerated on day 3. In all panels, means ± SEM were plotted, which were derived from at least three independent experiments with two mice per experimental group (the total number of mice per group was 6 or higher). Asterisks represent statistically significant differences compared to the 0 timepoint or PBS injected groups (white bars): * P< 0.05, ** P< 0.01, *** P< 0.001.
Figure 10

(a) LPS and Poly(I:C) treatment over time.

(b) IV injection (mice):
- LPS (C57BL/6)
- DT (CD11c-DTR)

(c) CD11c-hBcl2 mice

(d) CCR7−/− mice

(e) PBS (3d) vs LPS (3d)
2.3.3.2  _In vivo_ and _ex vivo_ studies show that LPS induces a second wave of reverse transendothelial migration of intimal CD11c\(^+\) cells into the lumen.

In order to confirm that the second of wave of reduction in intimal CD11c\(^+\) cells was due to reverse migration, we performed _in vivo_ and _ex vivo_ experiments similar to those used for the first wave (Fig. 4 and 5). _In vivo_ blocking of migration during the second wave was achieved by pertussis toxin (PTx) injection 2 days after LPS injection (i.e. after intimal CD11c\(^+\) cell numbers recovered to homeostatic levels). PTx prevented the second LPS-induced reduction in intimal CD11c\(^+\) cells (Fig. 11a), suggesting involvement of GPCR signaling, such as CCL19/CCR7. Scanning electron microscopy experiments revealed increased leukocyte adherence to the arterial endothelium relative to baseline 57 hours after LPS injection (Fig. 11b,c). This time point coincides with the second reduction in intimal CD11c\(^+\) cells. Depletion of circulating monocytes with clodronate liposomes (CL) prior to the analysis did not diminish the number of adherent cells (Fig. 11b), which suggests that the majority of adherent leukocytes were intimal cells undergoing RTM and that monocyte recruitment is not increased at this time.

In order to carry out _ex vivo_ perfusion experiments, we first confirmed that a single injection of LPS _in vivo_ was sufficient to reduce intimal CD11c\(^+\) cells during a 6h _ex vivo_ incubation (Fig. 11d,e). There was no need to add LPS to the culture media, otherwise the results would have been confounded with the expectation that what is observed is LPS inducing a new first wave. This was not the case, and _ex vivo_ perfusion experiments – in which aortic arches from _Cd11c-eYfp_ transgenic mice were cannulated and the lumen was continually perfused for up to 6h _ex vivo_ in order to collect transmigrated cells – showed a significant increase in the number of eYFP\(^+\) cells collected in the perfusate of LPS-injected versus PBS-injected mice (Fig. 5f).
Figure 11: *In vivo* and *ex vivo* studies show that LPS induces a second wave of reverse transendothelial migration of intimal CD11c⁺ cells into the lumen. (a) WT mice were injected iv with either B-oligomer (2 μg) as a control or Pertussis Toxin (PTx, 2 μg) to inhibit GPCR-dependent intimal cell migration 2 days after LPS injection and mice were dissected 24 hours later. A control group was injected with PBS for 3 days. The number of intimal CD11c⁺ cells per ascending aorta was quantified by *en face* confocal microscopy. (b) Clodronate liposomes (CL) were injected 45 hours after LPS in order to deplete circulating monocytes. PBS liposomes (PL) served as the control. Leukocytes adhered to the endothelial surface lining the ascending aorta inner curvature were quantified by scanning electron microscopy (SEM). Dotted line represents baseline number of adherent leukocytes in PBS injected mice. Both bars are significantly higher than baseline (*P* < 0.001), but not statically different compared to each other. (c) Representative scanning electron microscopy image of a WT arterial intima 57 hours after LPS injection and 12 hours after CL injection. Magnification = 4,000 times and scale bar = 7.5 μm (d) A schematic illustrates *ex vivo* experiments performed 54 hours after iv injection of LPS or PBS. (e) LPS, but not PBS, reduced intimal CD11c⁺ cell abundance during the 6 hour *ex vivo* incubation. (f) *Cd11c-eyfp* transgenic mice were injected with PBS or LPS for 54 hours. Aortic arches were then cannulated and perfused *ex vivo* for 6 hours without any LPS in the culture media. eYFP⁺ cells in the collected perfusate were quantified over three 2h intervals, and demonstrated that LPS increased the migration of intimal CD11c⁺ cells into the arterial lumen. In all panels, representative images as well as means ± SEM were derived from at least three independent experiments with two mice per experimental group (the total number of mice per group was 6 or higher). Asterisks represent statistically significant differences from the PBS control group: ** *P* < 0.01, *** *P* < 0.001.
Figure 11

(a) RTM of intimal CD11c+ cells

(b) Adhered leukocytes per ascending aorta

(c) Microscope image

(d) In vivo and Ex vivo processes

(e) Intimal CD11c+ cells

(f) Collected eYFP+ cells
Each RTM wave was followed by recovery through proliferation of the remaining intimal CD11c$^+$ cells.

We next investigated the mechanism of intimal CD11c$^+$ cell recovery after days 1 and 3 of LPS injection, which could happen either by recruitment of cells from the circulation or by local proliferation of the remaining cells in the intima. Two experiments were used to assess the role of recruitment in the recovery, in which mice were injected with PBS or LPS on day 0 and injected with PTx or B-oligomer in one experiment, or BrdU on day 1 for another 24 hours (Fig. 12a). Blockade of GPCR-mediated chemokine signaling (that was shown to be essential for monocyte recruitment to the vessel wall$^{132}$) with PTx did not affect the recovery on day 2 (Fig 12b). Moreover, assessment of monocyte recruitment using a 24 hour BrdU pulse-labeling assay showed comparable intimal recruitment in PBS and LPS-injected mice between days 1 and 2 (Fig. 12c). These experiments show that increased recruitment of circulating monocytes does not account for the recovery observed.

We then investigated cell proliferation of the remaining intimal myeloid cells as a mechanism for the recovery. A similar one week BrdU pulse-chase experiment to Fig. 3b-d, in which a cohort of intimal myeloid cells was labeled, was extended to 2 and 3 days. The number of labeled cells recovered on day 2, suggesting proliferation since circulating labelled cells were chased out for one week; and LPS reduced labeled cells on day 1 and 3 (Fig. 12d). We also assessed proliferation directly using 3h BrdU assays throughout the LPS time-course. Increased proliferation was found at 36-48h (peak at 42h) and a second peak at 81h (Fig. 12e). Increased intimal cell proliferation preceded intimal CD11c$^+$ cell recovery observed at 2 and 4 days (Fig. 12e in red). These data are consistent with proliferation of the labeled myeloid cells that remain in the intima as the main mechanism of recovery of intimal CD11c$^+$ cells.
Figure 12: Each RTM wave was followed by recovery through proliferation of the remaining intimal CD11c+ cells, not recruitment of circulation leukocytes. (a) A schematic showing the timing of injections for panels b and c. C57BL/6 WT mice were injected with PBS or LPS at day 0, and injected with PTx (2 µg) or B-oligomer (2 µg) as a control for panel b, or BrdU for panel c on day 1, and mice were analyzed by en face confocal microscopy on day 2. (b) PTx was used to inhibit GPCR-dependent chemokine signaling and monocyte recruitment 1 day after LPS injection and mice were dissected 24 hours later. A control group was injected with PBS for 2 days (white bar). Intimal CD11c+ cells were enumerated on day 2. (c) Monocyte recruitment was assessed using a 24 hour BrdU pulse-labeling assay. The number of intimal CD45+BrdU+ cells was quantified on day 2. (d) WT mice were given BrdU in water for one week followed by another week with regular water. LPS was injected on day 0 and the number of CD11c+BrdU+ cells was determined at the indicated timepoints. (e) Intimal leukocyte proliferation was assessed using a 3 hour BrdU pulse-labeling assay, and CD45 immuno-phenotyping by confocal microscopy was performed throughout the LPS time-course. Superimposed in red is the abundance of intimal CD11c+ cells from (Fig. 10a). In all panels, means ± SEM were plotted, which were derived from at least three independent experiments with two mice per experimental group (the total number of mice per group was 6 or higher). Asterisks represent statistically significant differences compared to the 0 timepoint or PBS injected groups (white bars): *** P< 0.001.
Figure 12

RTM of intimal CD11c⁺ cells

Chapter 2
2.3.4 Role of S1P and its receptors in LPS-induced RTM of intimal CD11c⁺ cells.

The fact that intimal CD11c⁺ cells expressed both the receptor CCR7 and CCL19 would not fully explain the RTM mechanism and would not provide the directionality for cell egress into the arterial lumen. Therefore, we investigated sphingosine-1-phosphate (S1P) and its receptors since they have been previously implicated in migration of DCs and T cells in lymph nodes and the thymus²⁸,³⁰,³⁷. WT mice were treated in vivo or aortas were treated ex vivo with FTY720, which is a pharmacological inhibitor of S1P function, and showed that it blocked LPS-induced reduction in the number of intimal CD11c⁺ cells (Fig. 13a). S1P is the product of phosphorylation of sphingosine by one of two sphingosine kinase enzymes (SphK1 and SphK2), and it interacts with one of five receptors (S1Pr1-5). S1P receptors were constitutively expressed in the intima and only S1Pr1 was mildly induced (~ 4-5 folds) after LPS injection, whereas SphK1 was induced ~ 40 folds (Fig. 13b). Therefore, we analyzed further the expression of SphK1 and S1Pr1 throughout a time-course of 12 hours after LPS injection, and found that SphK1 was induced ~ 30 folds at 6 hours but does not decrease significantly after (Fig. 13c), suggesting endothelial expression. On the other hand, S1Pr1 was induced ~ 4-5 folds, and a significant drop in expression between the 6 – 10 hours timepoints was observed, which coincides with cell egress out of the vessel wall (Fig. 13d), suggesting expression by intimal CD11c⁺ cells. In order to confirm the source of SphK1 and S1Pr1, mRNA expression was analyzed in Cd11c-dtr mice injected with PBS or DT (to deplete intimal CD11c⁺ cells) one day prior to PBS or LPS injection for 6 hours. Depletion did not affect expression of SphK1 nor induction by LPS, which suggests that S1P is most likely produced by vascular endothelial cells. In contrast, depletion significantly decreased S1Pr1 expression both at baseline and after induction by LPS, suggesting expression by intimal CD11c⁺ cells.
Figure 13: RTM is dependent on S1P production by vascular endothelial cells and S1P receptor expression by intimal CD11c<sup>+</sup> cells. (a) S1P inhibition by FTY702 (a pharmacological inhibitor of S1P function) blocked LPS-induced reduction in the number of intimal CD11c<sup>+</sup> cells. DMSO served as a control since FTY720 was dissolved in DMSO. Ex vivo experiments were performed by incubating aortas for 12 hours in culture media with or without LPS prior to fixation and staining. In vivo experiments were performed by injecting PBS or LPS and DMSO or FTY720 for 24 hours prior to tissue harvest and staining. (b) mRNA expression of sphingosine kinases and S1P receptors in WT mice injected with LPS for 6 hours. Data are expressed as fold induction of expression relative to PBS injected mice, set to 1 (dotted line). Statistical differences represent individual Student’s t tests in comparison to the dotted line. (c,d) Time course analysis of Sphingosine kinase 1 (SphK1) and S1P receptor 1 (S1Pr1) mRNA expression after LPS injection into WT mice. Data are expressed as fold induction relative to the 0 hour timepoint. (e,f) SphK1 and S1Pr1 expression with or without intimal CD11c<sup>+</sup> cell depletion for 24 hours prior to PBS or LPS injection for another 6 hours. This shows that both SphK1 and S1Pr1 were significantly induced by LPS injection; however, only S1Pr1 was affected by cell depletion suggesting expression by intimal CD11c<sup>+</sup> cells, whereas SphK1 was not affected by cell depletion, suggesting endothelial expression. This result might provide directionality for RTM of intimal CD11c<sup>+</sup> cells. In all panels, means ± SEM were plotted, which were derived from at least three independent experiments with two mice per experimental group (the total number of mice per group was 6 or higher). Asterisks represent statistically significant differences compared to the PBS injected groups: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. 

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2.4 Discussion

The results in this chapter showed that stimulation of PRRs such as TLR and NOD-receptors by their respective ligands triggers intimal CD11c+ cells to undergo reverse transendothelial migration (RTM) into the arterial lumen. RTM was dependent on induction of CCR7 and CCL19 expression by intimal CD11c+ cells; whereas CCL21 was constitutively expressed by endothelial cells and does not seem to play a significant role in RTM. TLR stimulation also induced a second wave of RTM on day 3, and each wave was followed by a recovery period in which the remaining cells proliferated to reconstitute the original homeostatic abundance of intimal CD11c+ cells. Finally, preliminary studies showed that S1P and its receptors are also significant factors in mediating RTM and studies will explore the mechanism of their involvement.

We were surprised by the fact that intimal CD11c+ cells express both CCR7 and CCL19 required for RTM. Initially, we suspected that endothelial cells produce CCR7 ligands to generate a higher chemokine gradient towards the aortic lumen and provide directionality for cell migration. However, our data were not consistent with that possibility and showed that the receptor and the ligand are made by the same population of cells. A possible mechanism might have been that RTM was a chemorepulsive process (i.e. the direction of migration is from a high to a low concentration of CCL19). However, it does not seem plausible that self-generated chemokines could promote reverse migration since the direction of the interstitial fluid flow in the intima is from the lumen towards the media\textsuperscript{234}. If this were true, CCL19 concentration would have been higher proximal to CD11c+ cells and lower towards the media (not towards the lumen), and cells would migrate toward the media (not exit towards the lumen). This does not fit with our observations that intimal CD11c+ cells migrate towards endothelial cells to enter the lumen; thus, another plausible mechanism is required.
In reviewing the RTM literature, we found that multiple molecules have been implicated in the process of T cell RTM from the thymus. In addition to CCL19 / CCR7\textsuperscript{128}, CXCL12 (stromal cell-derived factor 1, SDF-1) and its receptor CXCR4\textsuperscript{235}, and S1P / S1P1\textsuperscript{130} have been implicated in RTM of T cells in the thymus; and sphingosine kinase expressing neural crest-derived pericytes contribute to this process at the thymic cortico-medullary junction\textsuperscript{236}. Adhesion molecules may also participate in the retention of myeloid cells in the intima, as is the case with hematopoietic stem cells and progenitors in the bone marrow, and chemokine signaling is required for stem cell and mature leukocyte release into the circulation via RTM\textsuperscript{136}. In venular endothelial cells, JAM-C expression is decreased by Ischemia/reperfusion injury, which facilitates RTM of neutrophils\textsuperscript{141}. Future studies will evaluate whether these molecules also participate in the RTM of intimal myeloid cells and provide directional cues. So far, our preliminary results showed that S1P signaling is required for RTM of intimal CD11c\textsuperscript{+} cells, and S1Pr1 is expressed by DCs, whereas S1P is produced by endothelial cells, which might provide the directional cues required for cell egress out of the vessel wall into the intima. CCL19/CCR7 signaling may be required to initiate chemokinesis and change the state of intimal CD11c\textsuperscript{+} cells from stationary to migratory state that would then respond to S1P directional signals. Additional experiments are required in order to confirm this observation, including studies to determine which receptor(s) is critical for RTM, as well as studies with appropriate genetically mutated mice.

We had attempted an approach to visualize CCL19 expression in the intima using \textit{in situ} hybridization by adopting RNAscope (Advanced Cell Diagnostics Inc.), a new and highly sensitive \textit{in situ} hybridization technique\textsuperscript{237}, which enables simultaneous assessment of several mRNAs in conjunction with immunostaining of cross sections. However, for intimal myeloid cell analysis, the \textit{en face} approach is
critical, since these cells are difficult to visualize in cross sections. Therefore, much optimization was required for maintenance of tissue integrity (due to necessary protease digestion of RNA-binding proteins, stringent hybridization conditions and multiple washes). We were successful at visualizing specific CD11c mRNA expression in intimal myeloid cells identified by antibody staining, as well as LPS-induced CCR7 mRNA expression. Unfortunately, the CCL19 probe set produced a low signal and unacceptable nonspecific signal in negative controls (high noise).

We ruled out that apoptosis accounts for the reduction of intimal CD11c⁺ cell numbers after LPS injection using transgenic mice that overexpress the anti-apoptotic gene Bcl2 and showed comparable two RTM waves and recovery of cell numbers. Over the last 25 years, many groups have reported that overexpression of Bcl2 in a variety of cell types has reduced apoptotic cell death triggered by a broad range of pathophysiological stimuli. This includes apoptosis of T cells in response to glucocorticoids, radiation, anti-CD3, and sepsis (Lck-Bcl2)²³⁸,²³⁹, macrophages in atherosclerosis (CD68-Bcl2)²⁴⁰, dendritic cell turnover and LPS-induced immunosuppression (CD11c-Bcl2)²²⁸,²⁴¹, neurons in response to ischemia (NSE-Bcl2)²⁴², hematopoietic cells in response to lethal irradiation (H2K-Bcl2)²⁴³, etc. Although there may be scenarios where Bcl2 overexpression would not protect against apoptotic cell death, our TUNEL staining corroborated that apoptosis does not increase in response to LPS and that RTM is the mechanism involved. If apoptosis accounted for the reduction of intimal CD11c⁺ cell abundance from over 600 to approximately 300 within a 12-hour period after LPS injection, we would certainly detect this by TUNEL staining based on our experiments on DT-induced apoptosis of intimal CD11c⁺ cells in Cd11c-dtr transgenic mice.
LPS and other stimuli induced two waves of RTM. LPS is quickly cleared from the circulation after a bolus injection, and circulating cytokines (e.g., TNFα, IL-1β, and IL-6) produced in response drop before the second wave of RTM. This suggests that local cues lead to CCL19/CCR7 signaling, required for the second wave of RTM. In addition, when we incubated aortas ex vivo with LPS for 12 hours without in vivo stimulation, a similar 50% reduction in the number of intimal CD11c⁺ cells was observed, suggesting that the aorta is self-sufficient and does not require systemic signals to mediate RTM of intimal CD11c⁺ cells.

A rapid recovery in the abundance of intimal CD11c⁺ myeloid cells was observed after the two waves of RTM. The recovery in both cases was mediated by proliferation. Since RTM reduced intimal cells by ~50%, only one round of proliferation would be sufficient to replenish the population. The abundance of intimal CD11c⁺ myeloid cells is tightly regulated. The basal proliferation rate was low and increased in response to RTM. Mechanisms that regulate intimal myeloid cell proliferation and abundance are still poorly understood. CX3CL1/CX3CR1 signaling promotes monocyte/macrophage survival, and the abundance of intimal myeloid cells is reduced in CX3CR1 deficient mice. Growth factors such as CSF-1 (M-CSF) may have a similar pro-survival function. In response to LPS stimulation, vascular endothelial cells produce CSF-1 and CSF-2 (GM-CSF). The latter regulates intimal myeloid cell proliferation in early atherosclerotic lesions and may have a similar role following RTM. The cytokine fms-like tyrosine kinase 3 ligand (Flt3L) and its receptor (Flt3) have also been implicated in the differentiation of intimal myeloid CD11c⁺ cells, and may induce intimal cell proliferation following RTM.

CD11c⁺ cells isolated from the mouse aorta have the capacity to cross-present antigen to CD8⁺ T cells in vitro, indicating that they are fully competent with respect
to antigen presentation. Upon undergoing RTM, intimal CD11c+ cells may enter secondary lymphoid organs via the bloodstream, where they may contribute to an adaptive immune response. Currently, we do not have the tools to label intimal myeloid cells specifically and detection of these cells in the periphery would be impossible due to their low abundance. Nevertheless, their involvement in immune response probably is minimal relative to the initiation of adaptive immunity at the other sites including the primary site infection, since the number of DCs in the aortic intima is several orders of magnitude lower than in other tissues such as the lung, spleen, and lymph nodes.

What is also exciting about reverse transendothelial migration of intimal CD11c+ cells is its role in physiological and pathological processes. Physiologically in steady-state homeostatic conditions, intimal CD11c+ cells may upregulate CCL19/CCR7 expression and other molecules involved in RTM in order to exit the vessel wall, as part of the mechanism for the turnover of intimal CD11c+ cells shown in Fig. 1. This is analogous to a subpopulation of DCs in the gut and lungs that have constitutive CCR7-dependent migratory capacity to draining lymph nodes in steady-state conditions\(^{89,90}\). Pathologically, RTM may play a role in removing intracellular pathogens such as intracellular bacteria and viruses that have infected the vessel wall. Intracellular pathogens could hijack and enter the vessel wall via recruited leukocytes such as neutrophils or blood monocytes; and once in the intima, CD11c+ myeloid cells may remove the pathogen by RTM of those infected cells. However, intimal infection under steady-state condition was not previously documented, either because pathogens do not infect the vessel wall, or because they are efficiently removed by RTM of intimal CD11c+ cells. We favour the latter option and the focus of the next chapter will be to demonstrate that possibility.
Chapter 3
Reverse Transendothelial Migration of Intimal CD11c+ Cells Removes the Pathogen *Chlamydia muridarum* from The Arterial Intima
3.1 Introduction

Pathogens such as intracellular bacteria and viruses have been detected in atherosclerotic lesions\textsuperscript{247-249}, but not normal arteries\textsuperscript{168}. This suggests that pathogens either don’t enter the normal arterial intima or are cleared efficiently. We favor the latter possibility. In normal mice, low-grade chronic inflammation with ongoing blood monocyte recruitment occurs in regions with disturbed hemodynamics\textsuperscript{15}. This provides a mechanism for intracellular pathogen transport\textsuperscript{169} into the arterial intima. Extracellular pathogens may be captured from the circulation by intimal myeloid cells that extend dendrites through the endothelial monolayer into the artery lumen\textsuperscript{71}. Potential mechanisms for pathogen clearance include efficient pathogen killing and/or migration of infected intimal myeloid cells.

\textit{Chlamydia/Chlamydophyla (C.) pneumoniae} is a Gram-negative bacterium and an obligate intracellular parasite (requires eukaryotic cells for production of ATP), associated with community-acquired pneumonia\textsuperscript{250}. \textit{C. muridarum} is the mouse counterpart of \textit{C. pneumoniae}. The life cycle consists of two stages: elementary body, which is able to infect cells but cannot replicate, and the reticulate body, which replicates but is not able to cause new infection (reviewed in \textsuperscript{251}). The elementary body is the dispersal form (analogous to a spore) that induces its own internalization upon encountering a target cell. Once internalized, \textit{Chlamydia} actively modifies the properties of the nascent vacuole, which circumvents normal trafficking through the host endocytic pathway and effectively dissociates it from late endosomes and lysosomes. This prevents phagolysosomal fusion, and allows for intracellular survival of \textit{Chlamydia}. Inside an inclusion body in the cytoplasm, the elementary body undergoes primary differentiation and germinates into a larger reticulate body. After a lag phase of several hours, the reticulate body divides and after hundreds of divisions,
reticulate bodies transform back to the elementary form and are released by exocytosis of the inclusion body or cell lysis.

*Chlamydia* evolved sophisticated mechanisms to evade intracellular destruction by phagolysosomal clearance, especially in epithelial cells, which are the primary cells that *Chlamydia* infects in the lung, genito-urinary tract or conjunctiva. However, myeloid cells are capable of killing *Chlamydia* through mechanisms that include phagocytosis and autophagy\(^{252,253}\), although *Chlamydia* can survive in infected monocytes, macrophages and DCs and these cells can disseminate *Chlamydia* from the primary site of infection\(^{254}\). *C. pneumoniae* can migrate from the lungs via circulating leukocytes\(^{255}\). Therefore, *Chlamydia* utilizes the monocyte as a Trojan horse for the purpose of dissemination.

It is known that dissemination of *Chlamydia* infection occurs via blood mononuclear cells. In humans, *Chlamydia* has been detected in circulating monocytes\(^{256}\). In mice, *Chlamydia* was detected in peripheral blood mononuclear cells but not in the plasma 3 days after intranasal or intraperitoneal inoculation\(^{255}\). In rabbits, infected intratracheally with *Chlamydia*, granulocytes, alveolar macrophages and alveolar epithelial cells acted as host cells, and *Chlamydia* remained detectable for up to 8 weeks\(^{257}\). Macrophages transported *Chlamydia* to the peribronchiolar lymphatic tissue, and subsequently *Chlamydia* entered the spleen and the aorta via dissemination by peripheral blood monocytes\(^{257}\).

In this chapter, studies will show that *C. muridarum* elementary bodies are cleared from the plasma but are transported into the arterial intima via recruited blood monocytes which differentiate and acquire CD11c expression. Studies will also show that the second wave of RTM of intimal CD11c\(^{+}\) cells – not phagocytic clearance or intracellular killing – is the mechanism of pathogen clearance from the intima.

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3.2 Materials and methods

3.2.1 Mice and reagents

Male and female mice were used between 6 and 12 weeks of age. In addition to WT, Ccr7−/−, Plt, Cd11c-dtr, and Cd11c-hBcl2 mice obtained from Jackson Laboratories and listed in section 2.2.1, Rag1−/− mice (stock No. 002216) were a gift from Dr. S.S. Nunes (Toronto General Research Institute, Toronto, Canada), and Nos2a−/− mice (stock No. 002609) were a gift from Dr. J. Gommerman (University of Toronto, Canada). Mice were bred and maintained in a pathogen-free environment in the University Health Network animal facility at Toronto Medical Discovery Tower with 12 hour light-dark cycles. Mice were fed a standard rodent chow. All protocols were performed in adherence to the guidelines of the Canadian Council of Animal Care.

The use of Pertussis toxin, Diphtheria toxin, PBS / Clodronate liposomes, functional blocking antibodies to CCL19/MIP-3 or isotype controls, as well as antibodies used for immunostaining and flow cytometry was described previously in section 3.2. 1400W (hydrochloride), a potent and selective inhibitor of iNOS, (no. 81520) was purchased from Cayman Chemicals, and 10 mg / Kg was injected iv into mice 2 or 3 days after C. muridarum injection.

Functional blocking antibody to Ly6G (no. MLDP6), used for neutrophil depletion, was purchased from R&D systems. Biotinylated antibody to CD3ε (no. 553060) from BD. Biosciences was used for enumeration of intimal T cells after C. muridarum infection. Antibody to ERMP58 (an antigen expressed on all M-CSF-responsive cells in the bone marrow but is rapidly lost upon differentiation in tissues) was purchased from GenWay Biotech (no. GWB-D6DF26); and staining was followed by a secondary goat anti-rat HRP conjugated antibody (Jackson Immunology), then FITC conjugated Tyramide as described before.
3.2.2 Preparation and enumeration of Chlamydia muridarum

*C. muridarum* (ATCC, stock no. VR-123) was cultured in McCoy B cells in αMEM further supplemented with 1 μg/ml cyclohexamide (Sigma-Aldrich) and 8.8% D-glucose (Sigma-Aldrich) as previously reported\textsuperscript{258,259}. *C. muridarum* elementary bodies (EB) were isolated by density centrifugation using a 50% -20% Gastrografin gradient (Roche) and were stored in sucrose phosphate-glutamic acid buffer (8.5 mM Na\textsubscript{2}HPO\textsubscript{4}, 4 mM NaH\textsubscript{2}PO\textsubscript{4}, 220 mM sucrose, 0.5 mM L-glutamic acid, pH 7.4 [Sigma-Aldrich]) at -80°C prior to use. A commercially available PCR-based mycoplasm test (PromoKine) was used as per the manufacturer's instructions to ensure *C. muridarum* and cell lines were mycoplasm negative. Mice were injected iv with 10\textsuperscript{7} EB prepared fresh every time from frozen stock aliquots.

To determine *C. muridarum* infection forming units (IFU) in murine plasma samples or confirm the stock concentration overtime, a modified enumeration assay was employed\textsuperscript{260}. McCoy B cells were plated at 2.5 x10\textsuperscript{5} cells / well in 24-well plates overnight to generate confluent monolayers. Samples were first sonicated at 50% power (50 watt sonicator, Fisher Scientific) for 2 x 15 seconds for lysis infected cells. The lysate was spun at 600 g for 5 minutes to remove cellular debris, prior to sterile ultracentrifugation at 19,500 g (L80-70m, Beckman Coulter) for 30 minutes at 4°C to separate *Chlamydia* from cytokine-containing conditioned media. The *Chlamydia* pellet was serially diluted and McCoy B cells were infected by centrifuging dilutions at 600 g for 20 minutes at 37°C to synchronize infection. After culturing for 24 hours, media was removed and infected cells were fixed with ice cold methanol for 10 minutes. The cells were then stained with FITC-conjugated, anti-*Chlamydia* LPS antibody as per the manufacturer's recommendations (Pathfinder, Biorad). Inclusions were counted per well and the respective dilution factor used to calculate IFU / sample.
3.2.3 LUMINEX Multiplex Elisa

*C57BL/6* WT and *Ccr7*−/− mice were injected with $10^7$ *C. muridarum* EB and serial blood samples were collected daily for 7 days. Cells were sedimented by centrifugation at 1,000 g for 10 minutes at 4°C. The clear serum layer (~50% of the original volume) was transferred from the top into a new fresh tube and stored in -80°C until cytokine and chemokine profiling by multiplex ELISA.

The multiplex ELISA assay was performed in magnetic plate according to Millipore Mouse Cytokine / Chemokine Magnetic Bead Panel Protocol (MCYTMAG-70K-PX32). Briefly, 25 μL magnetic beads, 25 μL assay buffer and 25 μL of (1:2 dilution) sample were incubated in each well overnight at 4°C with shaking. A standard curve (8 points of 1 in 5 serial dilutions of the standard stock) was also included in the assay to obtain exact concentrations of cytokines and chemokines in mouse serum samples. Beads were subsequently washed twice and incubated with 25 μL of Mouse Cytokine/Chemokine Panel Detection Antibody for 1 hour at room temperature. This was followed by incubation with 25 μL of streptavidin-phycoerthyrin for 30 minutes at room temperature. Beads were washed twice and resuspended in 150 μL of Sheath Fluid on a plate shaker for 5 minutes. Assays were read with Luminex 100 Reader and data was analyzed using Bio-plex Manager 6.0. Median fluorescent intensity (MFI) data was converted to concentrations using the slope curve-fitting method from the standard curve included in the assay. Standards were run in duplicates, whereas samples were run in singlets. Analyte concentrations are indicated in pg / ml units.

Accuracy and validity of the assay was confirmed by analyzing one of the cytokines in the panel using an ELISA assay. Mouse IFN-γ ELISA (no. 88-7314-22, eBioscience) was performed on the same mouse serum samples according to the manufacturer’s protocol and using an 8 point standard curve of 1 in 2 dilutions.
3.2.4 Flow cytometry, cell sorting, and mRNA analysis

Antibodies used for flow cytometry and cell sorting as well as details about sample collection and protocols used were listed in section 2.2.7.

mRNA isolation from mouse blood, spleen, and intimal cells following *C. muridarum* injection was performed according to details listed in section 2.2.6. Additional primers are listed in the following table.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence 5’- 3’</th>
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<tr>
<td><em>C. muridarum</em> 16s rRNA</td>
<td>Forward</td>
<td>CGCCTGAGGAGTACACTCGC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CCAACACCTCACCGGCACGAG</td>
</tr>
<tr>
<td>CD11b</td>
<td>Forward</td>
<td>TACTTCGGGCCAGTCTCTGAGTG</td>
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<tr>
<td></td>
<td>Reverse</td>
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<tr>
<td>CCL2</td>
<td>Forward</td>
<td>GCTACAAGAGGATCACGACG</td>
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<td></td>
<td>Reverse</td>
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<tr>
<td>CCL3</td>
<td>Forward</td>
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<tr>
<td></td>
<td>Reverse</td>
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</tr>
</tbody>
</table>
3.2.5 Statistical analysis

Statistical analysis was conducted using Prism software version 4.03. All data reported are expressed as mean values +/- standard error of the mean (SEM). The unpaired Student’s t test without Welch’s correction (assuming equal variance) was used when comparing differences between two groups. Multiple group analysis was performed using one-way analysis of variance (1-way ANOVA), with the Tukey-Kramer or Newman-Keuls multiple comparison pot-hoc test to determine differences between multiple groups. Two-way analysis of variance (2-way ANOVA) – with Bonferroni post-hoc tests to determine differences – was used in time course analysis and comparison between WT and Ccr7^-/- mice in gene expression and ELISA studies. Group comparisons were determined to be significant for p value less than 0.05 (* p < 0.05, ** p < 0.01, *** p < 0.001).
3.3.1 **C. muridarum is transported into the intima by recruited blood monocytes**

3.3.1.1 The first RTM wave of intimal CD11c\(^+\) cells after *C. muridarum* infection does not remove the pathogen from the vessel wall.

*C. muridarum*, the mouse counterpart of *C. pneumoniae*, was injected iv to synchronize the infection and pinpoint the timeline of the intimal response. Injection of *C. muridarum* iv induced a dose-dependent reduction in intimal CD11c\(^+\) cells in WT mice at 24 hours ([Fig. 14a](#)), with the maximal reduction observed using \(10^7\) inclusion forming units (IFUs); therefore, this dose was used in subsequent experiments. Injection of *C. muridarum* iv into *Ccr7*\(^{-}\) and *Plt* mice for 24 hours did not induce the same reduction observed in WT mice and relative to PBS-injected controls ([Fig. 14b](#)), suggesting that *C. muridarum* induces RTM that is dependent on CCR7 and its ligands, similar to TLR ligands and *E. coli*.

We used qPCR to quantify *C. muridarum* 16s ribosomal RNA (rRNA) in the aortic intima\(^{259}\). 16s rRNA levels were not detectable in control mice and increased gradually over 24 hours following iv injection ([Fig. 14c](#)). This was surprising since the number of intimal CD11c\(^+\) is decreasing during the same time frame. Moreover, we found comparable 16s rRNA levels in WT, *Ccr7*\(^{-}\) and *Plt* mice ([Fig. 14d](#)). These data suggest that RTM, which occurs within 24 hours in WT mice following *C. muridarum* infection and is inhibited in *Ccr7*\(^{-}\) and *Plt* mice, does not influence intimal infection; and that infection occurs independently of intimal CD11c\(^+\) cells. To corroborate this statement, complete depletion of intimal CD11c\(^+\) cells in *Cd11c-dtr* mice injected with DT one day prior to injection of *C. muridarum* for another day had no effect on *C. muridarum* accumulation in the intima ([Fig. 14e](#)), suggesting that intimal CD11c\(^+\) cells are not infected directly by *C. muridarum*, and that the pathogen is brought into the intima by other means that are independent of RTM of intimal CD11c\(^+\) cells.
Figure 14: The first RTM wave of intimal CD11c⁺ cells after *C. muridarum* infection does not remove the pathogen from the vessel wall. (a) WT mice were injected iv with the indicated inclusion forming units (IFUs) of *C. muridarum* and the number of intimal CD11c⁺ cells per ascending aorta was quantified at 24 hours by *en face* confocal microscopy. (b) Intimal CD11c⁺ cells per ascending aorta were enumerated in CCR7⁻/⁻ and Plt mice 24 hours after *C. muridarum* injection (10⁷ IFUs). (c) *C. muridarum* 16s rRNA expression in the ascending aortic intima was assessed by qPCR at 0, 6 and 24 hours after injection of WT mice with *C. muridarum* (10⁷ IFU). Data were normalized to HPRT and expressed relative to the 24 hours timepoint. *C. muridarum* 16s rRNA was not detected (ND) in control mice. (d) WT, Ccr7⁻/⁻, and Plt mice were injected with *C. muridarum* (10⁷ IFUs) and 16s RNA expression in the intima was assessed 24 hours later. Data were normalized to HPRT and expressed relative to WT values. (e) Prior to injection of *C. muridarum*, intimal CD11c⁺ cells were depleted by injecting DT into Cd11c-dtr transgenic mice, while controls received PBS. Data were normalized to HPRT and expressed relative to PBS-injected control. In all panels, means ± SEM were plotted, which were derived from at least three independent experiments with two mice per experimental group (the total number of mice per group was 6 or higher). Asterisks represent statistically significant differences compared to control groups: *** P < 0.001.
Figure 14

(a) Intimal CD11c+ cells

WT mice

C. muridarum (IFU)

0 200 400 600 800

PBS 10^5 10^6 10^7

(b) CCR7−/− mice

Plt mice

0 200 400 600

PBS C. muridarum (10^7 IFU) PBS C. muridarum (10^7 IFU)

(c) Relative C. muridarum 16s rRNA expression

WT mice

0 6 24

ND ***

(d) Relative C. muridarum 16s rRNA expression

WT CCR7−/− Plt

0 1

(e) Relative C. muridarum 16s rRNA expression

PBS or DTX C. muridarum Analysis

-24 0 24 h

0 1

PBS DT
3.3.1.2  *C. muridarum* infects neutrophils and mononuclear cells and is transported into the arterial intima by recruited blood monocytes.

We next studied how *C. muridarum* infects the arterial intima. *C. pneumoniae* can migrate from the lungs via circulating leukocytes\(^\text{255}\), therefore, we investigated if *C. muridarum* infect circulating leukocytes and are transported into the intima by blood monocytes that are continually recruited to the intima in the lesser curvature of the aortic arch\(^\text{15}\). Blood leukocytes were isolated from mice one day after *C. muridarum* injection and stained for flow cytometry and cell sorting of B cells, T cells, neutrophils, and classical and non-classical monocytes (Fig. 15a), and mRNA was isolated from sorted and unsorted cells. qPCR analysis showed enrichment of *C. muridarum* in circulating neutrophils and monocytes compared to T and B cells (Fig. 15b), and among monocytes, *Chlamydia* levels were higher in circulating classical monocytes.

In order to verify whether the gradual increase in infection in the first 24 hours following iv injection of *C. muridarum* is due to recruitment of infected monocytes to the arterial intima, two approaches were used. In the first approach, mice were injected with PTx to block GPCR-dependent leukocyte recruitment into the arterial intima, which decreased the infection load compared to B-oligomer-injected controls (Fig. 15c), suggesting that *C. muridarum* enters the vessel wall by infecting the recruited leukocytes. In the second approach, mice were injected with clodronate liposomes to deplete circulating classical monocyte, which also decreased the infection load compared PBS liposomes-injected controls (Fig. 15d), suggesting that *C. muridarum* resides in circulating blood monocytes that are recruited to the vessel wall. These cells do not express CD11c and are not depleted when *Cd11c-dtr* mice are injected with DT (Fig. 14e); and their recruitment and entry into the intima is a continuous process, regardless whether intimal CD11c\(^+\) cells are undergoing RTM.
Figure 15: *C. muridarum* is enriched in circulating neutrophils and mononuclear cells, and is transported into the arterial intima by recruited blood monocytes. (a) Flow cytometry plots showing the sorting strategy for isolating blood B cells, T cells, neutrophils, classical (CD11b⁺ Gr-1<sup>med</sup>) and non-classical monocytes (CD11b⁺ Gr-1<sup>lo</sup>). Blood samples were obtained from *C57BL/6* WT mice 24 hours after *C. muridarum* injection, and stained for CD3, B220, Gr-1, and CD11b. CD3⁻ B220⁻ population from the left plot were gated for Gr-1 and CD11b in the right plot. (b) Blood leukocytes, isolated 24 hours after iv injection of *C. muridarum* into WT mice, were immunophenotyped and sorted. Cells were sorted directly into TRIzol reagent for RNA isolation and detection of *C. muridarum* 16s rRNA. *C. muridarum* 16s rRNA expression was assessed in unsorted and sorted leukocyte subpopulations by qPCR. Data were normalized to unsorted blood leukocytes (dotted line) to show fold enrichment in leukocyte subpopulations. (c) After *C. muridarum* injection, WT mice were treated with PTx to inhibit monocyte recruitment, while controls received B-Oligomer. Intimal accumulation of *C. muridarum* was assessed by qPCR, and normalized to B-oligomer controls. (d) WT mice received clodronate liposomes (CL) before *C. muridarum* injection to deplete circulating monocytes, and controls received PBS liposomes (PL). Intimal accumulation of *C. muridarum* was assessed by qPCR, and normalized to PL controls. In all panels, representative plots as well as means ± SEM were derived from at least three independent experiments with two mice per experimental group (the total number of mice per group was 6 or higher). Asterisks represent statistically significant differences from the control groups: * P < 0.05, ** P < 0.01, and *** P < 0.001.
Figure 15

(a) Flow cytometry plots showing the distribution of B220 and CD3 for B cells and T cells, and CD11b and Gr-1 for classical monocytes, non-classical monocytes, and neutrophils.

(b) Bar graph showing relative C. muridarum 16s rRNA expression in blood leukocytes, B cells, T cells, neutrophils, classical monocytes, and non-classical monocytes. Statistical significance is indicated by *** for p < 0.001, ** for p < 0.01, and * for p < 0.05.

(c) Relative C. muridarum 16s rRNA expression over time with C. muridarum B-Olig or PTx treatment. Analysis at -1, 0, and 24 h.

(d) Relative C. muridarum 16s rRNA expression with C. muridarum PL or CL treatment. Analysis at -1, 0, and 24 h.
3.3.2 The second wave of RTM removes *C. muridarum* from the arterial intima.

3.3.2.1 RTM and clearance of *Chlamydia* in WT mice is inhibited in *Ccr7*\(^{-/-}\) mice.

We next investigated how infection of the arterial intima by *C. muridarum* influences intimal myeloid cell abundance over several days and examined whether *C. muridarum* induces a second wave of RTM, analogous to LPS and Poly(I:C). After *C. muridarum* injection and the initial reduction on day 1, intimal CD11c\(^{+}\) cell numbers recovered on day 2, followed by a second reduction on day 4 and recovery on day 5 (Fig. 16a). However, in *Ccr7*\(^{-/-}\) mice, *C. muridarum* did not reduce the abundance of intimal myeloid cells over a 5 day period (Fig. 16b), similar to the inhibition observed after LPS injection in *Ccr7*\(^{-/-}\) mice, suggesting a second CCR7-dependent RTM wave that happens 4 days following *C. muridarum* infection of the artery wall.

We then investigated whether the second RTM wave removes *C. muridarum* from the arterial intima. In WT mice, intimal infection was detected on days 1 to 3; however, a dramatic drop was observed on day 4 and *C. muridarum* levels remained low on subsequent days (Fig. 16c). In contrast, *C. muridarum* infection of the *Ccr7*\(^{-/-}\) mouse intima did not drop but increased progressively, with peak levels on day 5 being approximately 90-fold higher than on day 1, and declining by day 7 (Fig. 16d). The data in WT mice demonstrated that the dramatic drop in intimal *C. muridarum* observed on day 4 was preceded by RTM that occurred between days 3 and 4. The lack of RTM in *Ccr7*\(^{-/-}\) mice correlated with increased and prolonged intimal infection.

Infection on day 1 was comparable between WT and *Ccr7*\(^{-/-}\) mice (Fig. 14d), suggesting that the increase in bacterial load in *Ccr7*\(^{-/-}\) mice is not due to the initial recruitment of infected blood monocytes. Moreover, infection of blood leukocytes was comparable in both genotypes, although the load was slightly higher in *Ccr7*\(^{-/-}\) mice (Fig. 14e,f). Insights into the unexpected *Ccr7*\(^{-/-}\) phenotype will be discussed later.
is worth noting that infection persisted in the circulation in blood leukocytes throughout the time course, while infection was cleared from the intima of WT mice between days 3 and 4. This suggests that in addition to clearance of the local infection by RTM of intimal CD11c+ cells, leukocyte recruitment into the arterial intima may be suppressed in order to prevent further entry of the pathogen.

**Figure 16: The second RTM wave of intimal CD11c+ cells removed *C. muridarum* infection from the arterial intima in WT mice, but is inhibited in *Ccr7*−/− mice.**

(a) WT mice were injected with *C. muridarum* (10^7 IFUs), and intimal CD11c+ cells per ascending aorta were enumerated at different time points. (b) *Ccr7*−/− mice were injected with *C. muridarum* (10^7 IFU) and intimal CD11c+ cells were enumerated at the indicated time points. (c) WT mice were injected with *C. muridarum* (10^7 IFUs), and intimal cells were isolated for analysis of infection load by qPCR for 16s rRNA. Data were normalized to HPRT, and expressed relative to day 1 values (dotted line). (d) *Ccr7*−/− mice were injected with *C. muridarum* (10^7 IFU), and intimal cells were isolated for analysis of infection load by qPCR for 16s rRNA. Data were normalized to HPRT, and expressed relative to day 1 values (dotted line). It is worth noting that the infection load on day 1 in *Ccr7*−/− mice was comparable to WT mice (Fig. 14d). (e,f) WT or *Ccr7*−/− mice were injected with *C. muridarum* (10^7 IFU), and RNA was isolated from blood leukocytes for 7 days to quantify the infection load by qPCR for 16s rRNA. Data were normalized to HPRT, and expressed relative to day 1. Infection load in *Ccr7*−/− mice was slightly higher than WT mice, but not significant. In all panels, means ± SEM were plotted, which were derived from at least three independent experiments (the total number of mice per group was 6 or higher). Asterisks represent statistically significant differences compared to control groups: * P < 0.05, ** P < 0.01, and *** P < 0.001.
Figure 16

a) WT mice

b) CCR7−/− mice

c) WT mice

d) CCR7−/− mice

e) WT mice

f) CCR7−/− mice
**3.3.2.2 RTM not apoptosis is the mechanism of intimal *C. muridarum* clearance.**

In order to corroborate our observations from *Ccr7*−/− mice, we injected PTx to WT mice in order to inhibit GPCR signaling specifically between days 3 and 4, rather than continuous deficiency of *Ccr7* (Fig. 17a). PTx prevented the reduction of intimal CD11c+ cell numbers (Fig. 17b), and prevented the reduction in intimal *C. muridarum* 16s rRNA (Fig. 17c), suggesting that clearance of *Chlamydia* from the arterial intima is dependent on RTM of intimal CD11c+ cells between days 3 and 4. Moreover, WT mice were injected with functional blocking antibodies to CCL19, which also prevented the reduction in intimal *C. muridarum* 16s rRNA (Fig. 17d). These experiments confirmed that a CCR7-dependent second RTM wave removes intimal *C. muridarum*.

*C. muridarum* infection load was different after PTx treatment compared to blocking of CCL19; i.e. whereas PTx-mediated inhibition of GPCR signaling only led to a two-fold increase in *C. muridarum* 16s rRNA, CCL19 blocking caused more than twenty-fold increase. CCL19 blocking antibody only inhibits CCR7-dependent RTM of CD11c+ cells from the arterial intima, while PTx inhibits GPCR signaling in general, which include RTM of intimal CD11c+ cells and trafficking/recruitment of circulating leukocytes across the vessel wall. This suggests that the phenotype of *C. muridarum* amplification (observed in *Ccr7*−/− mice and with CCL19 blocking in WT mice) is possibly caused by recruitment of infected circulating leukocytes (see section 3.3.3).

We investigated whether *C. muridarum* is removed from the aortic wall through apoptosis of intimal CD11c+ cells. Induction of apoptosis in *Cd11c-dtr* transgenic mice on day 2 of infection eliminated intimal 16s rRNA (Fig. 17e), suggesting that *C. muridarum* resided in CD11c+ cells, either by differentiation of recruited monocytes and upregulation of CD11c expression, or phagocytosis of recruited monocytes, or phagocytosis of free *Chlamydia* EB that have escaped from the recruited monocytes.
Nevertheless, apoptosis was not the natural mechanism of clearance from the intima, since prolonging cell survival in Cd11c-hBcl2 transgenic mice did not affect the clearance of C. muridarum from the intima between days 3 and 4 (Fig. 17f).

Figure 17: RTM blockade prevents the clearance of C. muridarum from the intima, whereas apoptosis inhibition has no effect on pathogen removal. (a) A schematic showing blockade of the second wave of reverse transendothelial migration by PTx administration 3 days after C. muridarum injection into WT mice. B-oligomer served as a control. (b) The number of intimal CD11c+ cells was assessed on day 3 (white bar) or day 4 (black bars) by en face confocal microscopy. (c) C. muridarum 16s rRNA was assessed on day 4 by qPCR. Data were normalized to HPRT and are expressed relative to values from mice injected with C. muridarum for 3 days (white bar). (d) WT mice were injected with C. muridarum, and on day 3 with nonimmune IgG or function-blocking antibodies to CCL19. Intimal 16s rRNA was assessed on days 3 and 4. Data were normalized to HPRT and are expressed relative to day 3 values. (e) Intimal CD11c+ cells were depleted by injecting DT into Cd11c-dtr transgenic mice 2 days after injection of C. muridarum. Controls received PBS instead of DT. Intimal 16s rRNA expression was assessed on day 3. Data were normalized to HPRT and are expressed relative to PBS injected control. (f) Cd11c-hBcl2 transgenic mice were injected with C. muridarum and intimal 16s rRNA expression was assessed after 3 and 4 days. Data were normalized to HPRT and are expressed relative to day 3 values. In all panels, means ± SEM were plotted, which were derived from at least three independent experiments with two mice per experimental group (the total number of mice per group was 6 or higher). Asterisks represent statistically significant differences compared to control groups: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. 
Figure 17

a) C. muridarum, B-Olig or PTx, Analysis 0, 3, 4 d

b) Intimal CD11c+ cells

WT mice
- day 3
- day 4

***

(c) Relative C. muridarum 16s rRNA expression

WT mice
- IgG
- anti-CCL19

***

**

(d) Relative C. muridarum 16s rRNA expression

WT mice
- day 3
- day 4

* * *

(e) C. muridarum, PBS or DTx, Analysis 0, 2, 3 d

CD11c-DTR mice

Relative C. muridarum 16s rRNA expression

PBS, DT

* * *

(f) CD11c-hBcl2 mice

Relative C. muridarum 16s rRNA expression

3, 4 Time (d)

* * *
3.3.2.3 Systemic innate and adaptive immune cells are not recruited to the vessel wall and do not play a role in *C. muridarum* clearance from the arterial intima.

In order to strengthen our conclusion that RTM accounts to intimal clearance of *Chlamydia*, we ruled out the possibility that immune cells are recruited to the intima to participate in the clearance process. The time interval between days 3 and 4 is the when pathogen clearance occurs from the intima. Therefore, we digested the aortic arch into a cell suspension and analyzed the abundance of various leukocyte. Flow cytometry analysis showed very low abundance of constitutive levels of B cells, T cells and neutrophils; whereas macrophages constituted the majority of CD45$^+$ cells (Fig. 18a). Comparable B cell, T cell, neutrophil, and macrophage were observed on days 0, 3 and 4 (Fig. 18a), suggesting that these cells are not recruited to the arterial intima to participate in *Chlamydia* clearance from the vessel wall.

With a maximum of 800 CD11c$^+$ cells in the intima and many, many more leukocytes in the adventitia, it seems highly likely that there would be a massive noise signal from adventitial macrophages; and one might argue that flow cytometric analysis of the aortic arch is mainly identifying adventitial not intimal cells. Therefore, we addressed the role of systemic innate and adaptive immune cells using functional studies. Intimal T cells were analyzed by immunostaining for CD3 (Fig. 18b), and their abundance was very rare and did not change over the time-course following *C. muridarum* injection (Fig. 18c). RAG1 (recombination activating gene 1) is a protein involved in the activation of immunoglobulin V-D-J recombination during B and T cell differentiation from progenitor lymphoblasts; therefore, its deficiency results in deficiency in mature B and T cells. In *Rag1*$^{-/-}$ mice, which lack mature B and T cells, *C. muridarum* infection was cleared from the intima comparable to WT mice (Fig. 18d). Furthermore, depletion of neutrophils with anti-Ly6G antibody had no effect on intimal
clearance of *Chlamydia* (Fig. 18e). Collectively, these data demonstrated that the clearance of *C. muridarum* infection from the intima is critically dependent on the second wave of RTM, but not on acute inflammation or acquired immunity.

**Figure 18: Systemic innate and adaptive immune cells are not recruited to the vessel wall and do not play a role in *C. muridarum* clearance from the intima.** (a) Flow cytometry analysis of cells isolated from the aortic arch. *C57BL/6* WT mice were injected with *C. muridarum* for the indicated times and aortic arches were dissected and digested to obtain single cell suspension for flow cytometry analysis. Data are expressed as percentages of total CD45+ cells. (b) Representative image from WT mice injected with *C. muridarum* for 3 days and the ascending aorta was stained for CD3 (green, arrow) and CD11c (red). Nuclei are in blue. Scale bar = 40 μm. (c) WT mice were injected with *C. muridarum* and intimal CD3+ T cells were enumerated at the indicated timepoints. (d) *Rag1−/−* mice were injected with *C. muridarum* and intimal 16s rRNA expression was assessed after 3 and 4 days. Data were normalized to HPRT and are expressed relative to day 3 values. (e) Blocking antibody to Ly6G was used to deplete neutrophils 3 days after *C. muridarum* injection in WT mice and mice were analyzed on day 4. Isotype control IgG served as control. Data were normalized to HPRT and are expressed relative to day 3 values (white bar). In all panels, means ± SEM were plotted, which were derived from at least three independent experiments with two mice per experimental group (the total number of mice per group was 6 or higher). Asterisks represent statistically significant differences compared to control groups: *** *P* < 0.001.
Figure 18

a) Bar chart showing the percentage of aortic CD45.2+ cells.

b) Image showing Rag1-/- mice.

c) Graph showing WT mice over time.

d) Graph showing Relative C. muridarum 16s rRNA expression for Rag1-/- mice.

e) Graph showing WT mice with IgG and α-Ly6G.
3.3.3 Intracellular killing limits the growth of *C. muridarum*, but reverse transendothelial migration of intimal CD11c$^+$ cells is key to clearance.

In *Ccr7$^{-/-}$* mice intima, the load of *C. muridarum* increased markedly several days following infection ([Fig. 16d](#)), including the times when the pathogen was cleared in the intima of WT mice ([Fig. 16c](#)). While the inhibition of RTM-mediated clearance in *Ccr7$^{-/-}$* mice, the extensive levels of *C. muridarum* in the intima of these mice as compared to the accumulation in WT mice prior to clearance came as a surprise. The purpose of this section is to understand the underlying mechanisms for this level of infection in these mice. More specifically, we investigated whether systemic and/or local factors may account for extensive *C. muridarum* accumulation. Studies will focus on examining the difference in the immune response between WT and *Ccr7$^{-/-}$* mice, and study the role of local intracellular killing of *C. muridarum* by intimal CD11c$^+$ cells in the clearance process.
3.3.3.1 *C. muridarum* free EB phagocytosis is not affected in *Ccr7*−/− mice.

Phagocytic clearance is the first line of defense for removal of bacteria from the bloodstream. This task is carried out by tissue macrophages in the spleen, liver and lymph nodes, called the reticulo-endothelial system (RES). Therefore, we investigated whether the extensive accumulation of *C. muridarum* in the intima of *Ccr7*−/− mice was due to a deficiency in phagocytic clearance. The plasma clearance was rapid (within 1-4 hours) in both genotypes (Fig. 19a), suggesting that phagocytic clearance of extracellular *Chlamydia* is not compromised in *Ccr7*−/− mice. A positive control for this study was depletion of tissue macrophages of the RES by clodronate liposomes, which caused a 60-fold increase in plasma elementary bodies relative to PBS liposomes-treated mice (Fig. 19b).

In addition, the dramatic increase of intimal *Chlamydia* in *Ccr7*−/− mice was not recapitulated in other organs. The load of *Chlamydia* in the spleen of wild type and *CCR7*−/− mice was comparable (Figure 19c), and it increased to a similar extent in both genotypes between days 1 and 4. The load of *Chlamydia* in the spleen was actually much higher than in the intima. We also found comparable *C. muridarum* infection in cultured wild type and *CCR7*−/− bone marrow-derived macrophages (Figure 19d,e). These data suggest that there is no inherent defect in *Ccr7* deficient cells with respect to infection and intracellular growth/survival of *Chlamydia*. 
Figure 19: Phagocytic clearance and uptake of free *C. muridarum* elementary bodies is comparable in WT and *Ccr7*<sup>-/-</sup> mice. (a) WT and *Ccr7*<sup>-/-</sup> mice were injected with *C. muridarum* (10<sup>7</sup> IFUs) and blood samples were collected 5 minutes, 30 minutes, 1 hour, 4 hours, and 24 hours after injection. Plasma was isolated and enumeration of extracellular *C. muridarum* EB was performed using cultured fibroblasts. (b) Positive control for the enumeration assay from plasma samples was done by injecting WT mice with clodronate liposomes to deplete tissue macrophages in the RES prior to *C. muridarum* injection. PBS liposomes-injected mice served as a control. (c) WT and *Ccr7*<sup>-/-</sup> mice were injected with *C. muridarum* (10<sup>7</sup> IFU) and spleens were isolated on day 1 and 4. RNA was isolated from homogenized spleens using TRIzol and *C. muridarum* 16s rRNA was quantified using qPCR. Data were normalized to HPRT and are expressed relative to WT day 1 values. Note that the load of *Chlamydia* in the spleen was several orders of magnitude higher than in the intima. Statistical differences are relative to day 1 values. (d) Cultured bone marrow-derived macrophages (BMDM) from WT and *CCR7*<sup>-/-</sup> mice were infected with *C. muridarum* (2 MOI). After 6 or 24 hours, cultures were washed and RNA was extracted and analyzed for *C. muridarum* 16s rRNA expression. Increased intracellular infection was observed between 6 and 24 hours, but significant differences were not observed between WT and *CCR7*<sup>-/-</sup> cells. (e) At 24 hours, BMDM infected with *C. muridarum* were lysed and *Chlamydia* inclusion-forming units (IFUs) were enumerated using fibroblast cultures. Similar infectivity was observed when comparing lysates of WT and *CCR7*<sup>-/-</sup> cells. In all panels, means ± SEM were plotted, which were derived from at least three independent experiments with two mice per experimental group (the total number of mice per group was 6 or higher). Asterisks represent statistically significant differences compared to control groups: *** *P* < 0.001.
Figure 19

(a) RTM of intimal CD11c+ cells

(b) C. muridarum in plasma (x10^3 IFU/ml)

WT
CCR7-/-

(c) Spleen

Relative C. muridarum 16s rRNA expression

WT
CCR7-/-

(d) Normalized C. muridarum 16s rRNA expression

6h 24h

***

(e) Infection load (IFU)

BMDM:
Wild type
CCR7-/-
3.3.3.2 The systemic production of cytokines and chemokines in response to \textit{C. muridarum} is blunted in \textit{Ccr7}⁻/⁻ mice compared to WT mice.

Intracellular \textit{Chlamydia} within the inclusion body can enter into persistence phase, characterized by restricted growth. This is triggered by a number of inducers that include depletion of iron or certain amino acids, antibiotic therapy and interferon-gamma (IFN \(\gamma\))\textsuperscript{251}. Its production is critical for inhibiting \textit{Chlamydia} growth, and other cytokines also interact synergistically with IFN \(\gamma\) for this inhibition\textsuperscript{261}. Since the lymph node and splenic architecture is profoundly abnormal in \textit{Ccr7}⁻/⁻ mice due to abnormal CCR7-mediated T cell trafficking\textsuperscript{221}, we investigated whether production of cytokines and chemokines is defective in response to an intravenous injection of \textit{C. muridarum}, which could lead to increased replication of reticulate bodies within intimal cells.

We obtained serum samples from WT and \textit{Ccr7}⁻/⁻ mice injected with \textit{C. muridarum} and carried out a time-course analysis of multiple cytokines/chemokines using a multiplex LUMINEX ELISA. Cytokine expression patterns were divided into three general categories: induced equally in both genotypes (INF \(\gamma\), IP-10, MCP-1, MIP-1\(\alpha\), MIP-1\(\beta\), IL-10, IL-12(p70), and TNF\(\alpha\)) (Fig. 20), induced to a significantly lesser extent on day 2 in \textit{CCR7}⁻/⁻ mice (IL-6, IL-12(p40), IL-13, KC, eotoxin, MIP-2, MIG, RANTES) (Fig. 21), and low expression with no significant induction over time (G-CSF, M-CSF, GM-CSF, IL-1a, IL-1b, IL-2, IL-3, IL4, IL-5, IL-7, IL-9, LIF, LIX, IL-15, IL-17, VEGF) (Fig. 22). Day 2 post-infection was the timepoint when cytokine expression peaked in WT mice. We did not find any genes that were expressed at a higher level in \textit{Ccr7}⁻/⁻ mice. Intracellular replication, growth restriction or destruction of \textit{Chlamydia} is regulated by numerous factors that influence cell metabolism and the generation of oxygen radicals\textsuperscript{251,254,262}, and blunted systemic induction of cytokine genes in \textit{Ccr7}⁻/⁻ mice may enhance the growth and survival of intracellular \textit{Chlamydia} in the intima.
Figure 20-22: The systemic production of cytokines and chemokines in response to *C. muridarum* is blunted in *Ccr7*<sup>−/−</sup> mice compared to WT mice. Time-course serum cytokines/chemokines analysis from WT mice (blue solid line) and *Ccr7*<sup>−/−</sup> mice (red dashed line) after *C. muridarum* injection. The results were divided into three groups. (Figure 20) Genes that were significantly induced overtime (mostly on day 2 post-infection) compared to the 0 days timepoint, and induction was comparable in WT and *Ccr7*<sup>−/−</sup> mice. The most notable induction was observed in IFNγ, IP-10 and MCP-1. (Figure 21) Genes that were significantly induced on day 2 to a lesser extent in *Ccr7*<sup>−/−</sup> mice compared to WT mice, indicating blunting of the immune response in *Ccr7*<sup>−/−</sup> mice in response to *C. muridarum*. The most notable was MIG (CXCL9, monokine induced by gamma interferon), which is a T cell chemoattractant secreted mostly by macrophages. Like IP-10, MIG binds to the chemokine receptor CXCR3 in TH1 immune reactions. (Figure 22) Genes that either are not detected, have low expression, or not significantly induced overtime after *C. muridarum* injection compared to the day 0 timepoint. In addition to genes plotted, IL-3, IL-4, IL-17, and VEGF were analyzed in the multiplex panel, but were either not expressed at all or below the detection level of this assay. In all panels, means ± SEM were plotted, which were derived from at least three independent experiments (the total number of mice per group was 5). Statistical analysis was done using a two-way analysis of variance (2-way ANOVA) to examine significance of induction overtime and significant differences between WT and *Ccr7*<sup>−/−</sup> mice serum protein levels at every timepoint. Asterisks represent statistically significant differences between WT and *Ccr7*<sup>−/−</sup> mice at the indicated timepoint. * P < 0.05; ** P < 0.01; *** P < 0.001.
Figure 20

- IFN-γ
- IL-10
- IP-10
- IL-12(70)
- MCP-1
- TNF-α
- MIP-1α
- MIP-1β
Figure 21

- **IL-6**
- **Eotaxin**
- **IL-12(p40)**
- **MIP-2**
- **IL-13**
- **MIG**
- **KC**
- **RANTES**
Figure 22
Local inflammation and increased monocyte recruitment in CCR7-/- mice.

The remarkable increase in C. muridarum load was only observed in the intima, and similar amplification was not observed in the spleen, blood leukocytes or cultured bone marrow-derived macrophages (Figure 19). This suggested a local response specific to the arterial intima. Therefore, we investigated gene expression in the arterial intima in WT and Ccr7-/- mice after C. muridarum infection to examine the effects of persistent intimal infection due to defective RTM in Ccr7-/- mice (Figure 23).

We observed a significant increase in expression of CD45, CD68, CD11b, but not CD11c, in Ccr7-/- mice on day 4 after C. muridarum infection compared to WT mice, suggesting an increase in recruitment of circulating myeloid blood monocytes that are negative for CD11c. The increase in recruitment was not due to increase in adhesion molecules expression such as E-selectin. We observed a mild 2-3 fold increase in VCAM-1 expression in WT mice between days 2 and 3, but not in Ccr7-/- mice, suggesting that increased adhesion molecules expression is not the underlying mechanism for increased monocytes recruitment in Ccr7-/- mice. In contrast, a subset of chemokine genes related to monocyte recruitment (such as MCP-1, CCL3 and CCL5) was increased in Ccr7-/- mice on day 4 after C. muridarum infection, whereas CCL4 and CXCL1 were not different. Immuno-confocal microscopy revealed increased numbers of CD11b+ER-MP58+ cells in the CCR7-/- mouse intima on day 4, indicating that newly-recruited myeloid cells were present. ER-MP58 is expressed on M-CSF-responsive cells in the BM but is rapidly lost upon differentiation in tissues263, and has been used previously to assess monocyte recruitment in atherosclerosis264. Collectively, these data suggests that absence of RTM in the setting of CCR7 deficiency leads to a local inflammatory response and the recruitment of blood monocytes, some of which are infected, and thus augment the load of intimal infection.
Figure 23: Inability to clear *C. muridarum* infection causes local inflammation and increased recruitment of infected blood monocytes in *CCR7*<sup>−/−</sup> mice. Time-course mRNA analysis of intimal genes in WT (dashed red line) and *Ccr7*<sup>−/−</sup> (solid blue line) after *C. muridarum* injection. Data were normalized to HPRT and are expressed relative to day 1 values. CD45, CD68, CD11b, and CD11c are myeloid cell markers; E-selectin and VCAM-1 are adhesion molecules; MCP-1, CCL3, CCL4, CCL5, CXCL1 are chemokines related to monocyte recruitment. **(Last panel)** Enumeration of CD11b<sup>+</sup>ERMP58<sup>+</sup> cells in the intima of *CCR7*<sup>−/−</sup> mice after *C. muridarum* injection. In all panels, means ± SEM were plotted, which were derived from at least three independent experiments with two mice per experimental group (the total number of mice per group was 6 or higher). Asterisks represent statistically significant differences between WT and *Ccr7*<sup>−/−</sup> mice at the indicated timepoint. * P < 0.05; ** P < 0.01; *** P < 0.001.
Figure 23

- ▲ WT 
- ▼ CCR7−/−

**RTM of intimal CD11c+ cells**

**Chapter 3**

**Figure 23**

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**122**
iNOS-mediated intracellular killing limits the growth of *C. muridarum*

Intracellular pathogens, including *Chlamydia*, evolved mechanisms to avoid intracellular killing. Nevertheless, host cells can still control intracellular bacterial loads through a variety of pathways. One of these is inducible nitric oxide synthase (iNOS), a product of the *Nos2a* gene, which generates nitric oxide (NO) and oxygen radicals\textsuperscript{265,266}. We therefore investigated the effects of iNOS deficiency and its inhibition on *Chlamydia* loads in the spleen and the intima.

*Nos2a*\textsuperscript{-/-} and WT bone marrow cells were injected into irradiated WT recipients to deplete iNOS production in intimal CD11c\textsuperscript{+} cells but not in endothelial cells. *Nos2a* deficiency did not affect the reduction in intimal CD11c\textsuperscript{+} cell number on day 4 (**Fig. 24a**), indicating that iNOS deficiency does not affect RTM following *C. muridarum* injection. *Chlamydia* infection was significantly elevated relative to WT controls in the spleen (days 3 and 4) (**Fig. 24b**) and the intima (day 3) of *Nos2a*\textsuperscript{-/-} mice (**Fig. 24c**), confirming that iNOS is important in regulating intracellular killing of *Chlamydia*. Yet on day 4, *Chlamydia* infection in the intima was dramatically reduced in both *Nos2a*\textsuperscript{-/-} and WT mice (**Fig. 24c**). These data demonstrate that pathogen clearance from the intima is efficient regardless of whether intracellular killing is compromised.

Since mice with a genetic deficiency can develop compensatory mechanisms, we administered an established, potent and selective iNOS inhibitor (1400W) to WT mice. 1400W injected 24 hours prior to tissue harvest did not affect RTM on day 4 (**Fig. 24d**), significantly elevated infection load in the spleen (days 3 and 4) (**Fig. 24e**) and the intima (day 3), but did not affect clearance on day 4 in the intima (**Fig. 24f**). Collectively these data suggest that NO-dependent intracellular killing is involved in limiting intracellular growth of *Chlamydia* after infection; however, RTM of intimal CD11c\textsuperscript{+} cells remains the critical mechanism for pathogen removal from the intima.
Figure 24: iNOS-mediated intracellular killing limits the growth of *C. muridarum*, but RTM remains the mechanism of pathogen clearance from the arterial intima. 

(a-c) WT or Nos2a<sup>-/-</sup> bone marrow cells were injected into lethally irradiated WT recipients prior to injection of *C. muridarum* after 8 weeks of recovery. (a) The number of intimal CD11c<sup>+</sup> cells was quantified by *en face* confocal microscopy. Statistical differences are relative to the 0 day timepoint for each genotype. (b) Spleens were homogenized and RNA was isolated by TRlzol for *C. muridarum* infection load analysis by qPCR. Data were normalized to HPRT and are expressed relative to the corresponding WT controls (white bars). (c) Intimal cells were isolated and *C. muridarum* infection load was quantified by qPCR. Data were normalized to HPRT and are expressed relative to the corresponding WT controls (white bars). 

(d-f) WT mice were injected with *C. muridarum* on day 0 and either DMSO or 1400W 24 hours prior to tissue harvest (i.e. on day -1 for day 0 analysis, on day 2 for day 3 analysis, and on day 3 for day 4 analysis). (d) The number of intimal CD11c<sup>+</sup> cells was quantified by *en face* confocal microscopy. Statistical differences are relative to the 0 day timepoint for each genotype. (e) Spleens were homogenized and RNA was isolated by TRlzol for *C. muridarum* infection load analysis by qPCR. Data were normalized to HPRT and are expressed relative to the corresponding DMSO control groups (white bars). (f) Intimal cells were isolated and *C. muridarum* infection load was quantified by qPCR. Data were normalized to HPRT and are expressed relative to the corresponding DMSO controls (white bars). In all panels, means ± SEM were plotted, which were derived from at least three independent experiments with two mice per experimental group (the total number of mice per group was 6 or higher). Asterisks represent statistically significant differences compared to control groups: * P < 0.05; ** P < 0.01; *** P < 0.001.
Figure 24

(a) RTM of intimal CD11c+ cells

(b) Spleen

(c) Intima

(d) RTM of intimal CD11c+ cells

(e) Spleen

(f) Intima

Legend:
- **: p < 0.01
- ***: p < 0.001
3.4 Discussion

Overall studies in this chapter showed that *Chlamydia* is disseminated from the primary site of infection through circulating mononuclear leukocytes, which transport the pathogen into the arterial intima. In the intima, recruited monocytes either differentiate to acquire CD11c expression or *Chlamydia* is transferred from host cells into resident intimal CD11c$^+$ cells. In either situation, *Chlamydia* elementary bodies differentiate into reticular bodies and continue to grow in the intima for few days. During this time, iNOS-mediated intracellular killing – and likely other mechanisms involved in phagocytic clearance of intracellular bacteria, as well as systemic production of cytokines and chemokines critical in the activation of the immune response – limit the growth of *Chlamydia* within intimal CD11c$^+$ cells, but are not capable of completely removing the pathogen from the intima. Between days 3 and 4, intimal CD11c$^+$ cells infected with *Chlamydia* upregulate CCR7 and CCL19 expression – and potentially other factors discussed in chapter 2 such as S1P and its receptors – and undergo RTM back into the arterial lumen to remove the pathogen from the intima. Failure to induce RTM of intimal CD11c$^+$ cells (by CCR7 deficiency or CCL19 blockade, as well as a blunted systemic immune response) results in persistent growth of *Chlamydia* in the intima. This in turn causes a local inflammatory response and local production of chemokines related to monocyte recruitment. Those chemokines lead to recruitment of infected monocytes to the arterial intima, which further augments the infection load, suggesting that RTM is not only important to remove pathogens from the vessel wall, but also to avoid activation of a local inflammatory response in the intima.

We reasoned that the primary function of RTM is to remove pathogens from the arterial intima. Once in the blood, CCR7-expressing cells that have undergone RTM likely preferentially home to secondary lymphoid organs, where CCR7 ligands are
expressed by vascular endothelium, and there they may contribute to antigen presentation and participate in priming an adaptive immune response. Unfortunately, we cannot selectively label intimal CD11c+ cells in order to track their fate after RTM. However, it is probably unlikely that antigen presentation in secondary lymphoid organs is a critical function of intimal CD11c+ cells that have undergone RTM for mounting immune response to Chlamydia. In our experiments, the bloodstream is the route for pathogen entry into the arterial intima and the majority of circulating pathogens would be removed in the spleen. Splenic DCs are very abundant and outnumber arterial intimal DCs by several orders of magnitude. Thus, the immune response initiated in the spleen would likely be much more substantial.

The elementary body (EB) of Chlamydia is the dispersal form that upon internalization establishes intracellular infection. Systemic dissemination of Chlamydia from the primary infection site, usually the lungs or genitourinary tract, occurs via infected mononuclear leukocytes. We injected C. muridarum intravenously in order to synchronize systemic dissemination and achieve reproducible infection of the intima. Phagocytic clearance of injected EB from the plasma by the reticulo-endothelial system (RES) occurred within hours, and subsequently infected leukocytes circulated in the blood. The recruitment or trafficking of classical (Ly6C<sup>high</sup>) monocytes into the normal arterial intima and other tissues represents a form of homeostatic immune surveillance. Chlamydia (and potentially other intracellular pathogens) essentially hijack this process to enter the arterial intima via infected blood monocytes.

It was surprising that blood leukocytes remain infected with Chlamydia throughout the time-course analysed and that infection persisted after day 4 to day 7. Since our results showed that Chlamydia enters the artery wall by infected monocytes, which are constitutively recruited to the intima, then one would expect the infection
load to increase gradually overtime and would re-occur after the clearance observed on day 4. However, our data did not follow those expectations, which suggests that monocyte recruitment was inhibited in response to infection, perhaps due to the secretion of systemic cytokines and chemokines. Future studies will investigate the inhibition of monocyte recruitment occurs after RTM.

Although infected intimal CD11c+ cells egress back into the lumen, they do not likely contribute significantly to the dissemination of *C. muridarum* further through the bloodstream. That is because at day 4 of infection, when intimal CD11c+ cells undergo RTM into the bloodstream, *C. muridarum* has already persisted in the leukocytes in the bloodstream regardless whether RTM occurs or is inhibited in *Ccr7−/−* mice. The infection is present in circulating leukocytes before and after RTM occurs; therefore, it is unlikely that RTM of intimal CD11c+ cells is exacerbating the infection.

We used different approaches to confirm that *C. muridarum* is removed from the intima by RTM of CD11c+ cells, and ruled out the recruitment of systemic immune cells or apoptosis as potential mechanisms of clearance. We used *Cd11c-hBcl2* transgenic mice to rule out apoptosis as a mechanism of *Chlamydia* clearance. It was reported that CCR7 signaling can promote the survival and inhibit apoptosis of mature DCs in peripheral lymph nodes and in cell cultures. The longevity of mature DCs in the draining lymph node influences antigen presentation to naïve T cells and thus can affect the magnitude of an immune response. Therefore, one would expect *Ccr7−/−* cells to be more susceptible to apoptosis, which would lead to a decrease in *Chlamydia* load in the intima. This is opposite to what we observed.

We demonstrated that RTM is the critical mechanism for pathogen clearance from the intima, and ruled out other potential mechanisms including intimal cell apoptosis, NO-dependent intracellular killing, killing by recruited neutrophils and
adaptive immunity. Although phagocytic clearance of *Chlamydia* EBs and the load of *Chlamydia* in the spleen, bone marrow cells and circulating leukocytes was comparable in *Ccr7*−/− and WT mice, the systemic inflammatory response was blunted by in *Ccr7*−/− mice. This may influence the growth and survival of intracellular *Chlamydia* in the intima and partly explain the massive *Chlamydia* load observed in *Ccr7*−/− mice. In addition, persistent intimal infection due to defective RTM also resulted in a local inflammatory response, which results in upregulation of chemokines expression, such as CCL2, 3 and 5. These chemokines induce the recruitment of blood monocytes, some of which are infected and deliver additional *Chlamydia* to the intima. These findings provide insights into why CCR7 deficiency enabled the load of *Chlamydia* to increase dramatically in the intima within days of infection.

The next chapter will examine, the effect of lipid-loading associated with atherogenesis on RTM and pathogen clearance from the intima. Other mechanisms including IL-1β signaling will be studied. The ultimate goal is to explore how RTM can be used as a potential preventative therapy to reduce atherosclerotic plaque burden.
Chapter 4

Reverse Transendothelial Migration (RTM) of Intimal CD11c⁺ Cells is dependent on endothelial IL-1β/IL-1R1, and is inhibited by lipid-loading associated with atherogenesis.
4.1 Introduction

As was described in chapter 1, foam cell egress into the blood is considered to be a rare event during atherogenesis\textsuperscript{200}, due to inability of the cells to migrate or active migration inhibition. The production and secretion of repulsive neuroimmune guidance cues such as netrin-1, ephrin-B, semaphorins 3A and 3E may contribute to lipid-laden macrophage retention\textsuperscript{204-206,208}. Hypercholesterolemia also impairs the migration of dermal DCs to regional lymph nodes through generation of platelet-activating factor\textsuperscript{211}. Oxidized, but not native LDL, inhibits TLR4-induced peritoneal macrophage efflux into lymphatics and \textit{in vitro} migration through a process that involves CD36, inactivation of Src homology 2-containing phosphotyrosine phosphatase (SHP-2), sustained activation of focal adhesion kinase (FAK) and alteration of cytoskeletal dynamics\textsuperscript{212}. Upon reversal of hypercholesterolemia or treatment with statins, the induction of CCR7 in plaque macrophages may promote plaque regression\textsuperscript{201,271-275}. Collectively, these studies suggest that egress of lipid-loaded intimal CD11c\textsuperscript{+} cells through RTM is inhibited, which might prevent pathogen clearance from the intima and explain why intracellular pathogens have been identified in atherosclerotic lesions but not in normal arteries. The focus of the first part of this chapter will be to explore this question.

Various chronic diseases appeared to have an autoimmune component, and Interleukin-1 (IL-1), a proinflammatory cytokine with pleiotropic biological effects, appears to be one of the key players in these diseases\textsuperscript{276}. IL-1 has been linked to atherosclerosis and a clinical trial was launched to investigate the effects of an antibody against IL-1\textbeta on cardiovascular events in patients with atherosclerosis\textsuperscript{277}. On the other hand, mice lacking the receptor for IL-1 unexpectedly have features of advanced atherosclerosis and unstable plaques\textsuperscript{278}. These findings illustrate the complexity of IL-1 signaling.
Although the original IL-1 family comprised only IL-1α and IL-1β, the IL-1 family has expanded considerably to include 11 members\textsuperscript{279}. The IL-1R family has also expanded to 9 distinct genes and includes coreceptors, decoy receptors, binding proteins, and inhibitory receptors\textsuperscript{280}. Both IL-1α and IL-1β activate IL-1 receptor type I (IL-1R1), and IL-1 receptor antagonist (IL-1ra) competitively inhibits their binding. IL-1α remains associated with the plasma membrane, whereas IL-1β is fully secreted and can act on IL-1R1 on cells distant from the cell of origin. Thus IL-1β has been thought to be of greater relevance to human inflammatory diseases. IL-1α or IL-1β bind first to the ligand-binding chain of IL-1R1 (signaling has been reviewed in details in \textsuperscript{281}). This is followed by recruitment of the coreceptor chain, termed the accessory protein (IL-1RAcP). A complex is formed of IL-1RI plus IL-1 plus the coreceptor. The signal is initiated with recruitment of the adaptor protein MyD88 to the Toll-IL-1 receptor (TIR) domain in the IL-1r1 cytoplasmic C-terminal. Several kinases are phosphorylated, NF-κB translocates to the nucleus, and the expression of a large portfolio of inflammatory genes takes place. IL-1 receptor type II (IL-1RII) does not signal because it lacks a cytoplasmic domain, and, without a TIR domain, docking of MyD88 cannot take place. This receptor is expressed mostly on macrophages and B cells. IL-1RII binds IL-1β with a greater affinity than IL-1RI, thereby sequestering IL-1β. Hence, IL-1RII functions as a decoy receptor for IL-1β\textsuperscript{282}.

With the sole exception of IL-1 receptor antagonist (IL-1Ra), each member of the IL-1 family is first synthesized as a precursor without a clear signal peptide for processing and secretion, and none are found in the Golgi\textsuperscript{279}. The precursor form of IL-1β does not bind IL-1r1, and requires cleavage by either intracellular caspase-1 or extracellular neutrophilic proteases\textsuperscript{283}. Caspase-1 is the intracellular cysteine protease that cleaves the N-terminal 116 amino acid from the IL-1β precursor, thus converting
the inactive precursor to the active “mature” cytokine. Oligomerization of a highly specialized group of intracellular proteins termed the “inflammasome,” is required to convert procaspase-1 to an active enzyme. Apoptosis-associated speck-like protein containing a CARD domain (ASC) is an adaptor protein composed of two protein-protein interaction domains: an N-terminal PYD domain (which binds to PYD domain on other adaptor proteins such as MyD88) and a CARD domain (which binds to CARD domain on adaptor proteins that are part of the inflammasome). Therefore a deficiency in either ASC or Caspase-1 prevents the processing and cleavage of IL-1β precursor protein and inhibits IL-1β downstream signaling.

Multiple mechanisms have been reported for the secretion of the processed mature IL-1β; these include the loss of membrane integrity, a requirement for phospholipase C and secretory lysosomes, and the shedding of plasma membrane microvesicles or multivesicular bodies containing exosomes. Pyroptosis is a caspase-1–dependent mechanism for cell death and may account for the release of active IL-1β. The majority of IL-1 producing cells are macrophages, dendritic cells, hepatocytes, fibroblasts, muscle cells, or endothelial cells. The main target cells of IL-1 actions are primarily cells of the immune system such as monocytes, lymphocytes, granulocytes, and dendritic cells, but this cytokine can affect many other cells such as epithelial cells, fibroblasts, endothelial cells or smooth muscle cells.

Recent works have shown that certain bacterial species such as Chlamydia pneumoniae aggravate atherosclerotic lesion development by triggering IL-1 release and increase the risk of myocardial infarction. Therefore, since IL-1β is involved in multiple inflammatory processes, the remainder of this chapter will focus of studying the role of IL-1β in RTM of intimal CD11c+ cells.
4.2 Materials and methods

4.2.1 Mice and reagents

Male and female mice were used between 6 and 12 weeks old. WT, Ldlr−/− (stock No. 002207), Tlr4−/− (stock No. 007227), Casp1−/− (stock No. 016621) mice were obtained from Jackson Laboratories. Asc−/− mice were from Genentech Inc. IL-1r1−/− mice (stock No. 003245) were a gift from Dr. C.J. Paige (University Health Network, Toronto, Canada). Mice were bred and maintained in a pathogen-free environment at the University Health Network animal facility at TMDT with 12 hour light-dark cycles. Mice were fed a standard rodent chow. A cholesterol rich diet (CRD) consisting of 1.25% cholesterol and 40% kcal fat (Research Diets, diet D12108) was used to initiate atherosclerosis lesion formation in Ldlr−/− mice. All protocols were performed in adherence to the guidelines of the Canadian Council of Animal Care.

Recombinant mouse IL-1β (no. 401-ML/CF), TNF-α (no. 410-MT), as well as functional blocking antibodies to IL-1β (no. AB-401-NA), TNF-α (no. AB-410-NA) and isotype controls were purchased from R&D systems. Other reagents and methods used were described in the previous chapters.

4.2.2 mRNA analysis by real-time qPCR

Intimal cell harvest and isolation of RNA for qPCR analysis was performed as described in the previous chapters. Additional primers are listed in the following table.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence 5’-3’</th>
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<td>TLR4</td>
<td>Forward</td>
<td>AGCTTCTCCAATTTCAGAACTTC</td>
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<td></td>
<td>Reverse</td>
<td>TGAGAGGTGGTGTAAGCCATGC</td>
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<tr>
<td>mIL-1β</td>
<td>Forward</td>
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<td>Reverse</td>
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<tr>
<td>SphK1</td>
<td>Forward</td>
<td>GCTTCTGTGAACCACTATGCTGG</td>
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<td></td>
<td>Reverse</td>
<td>ACTGAGCACAGAATAGAGCCGC</td>
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<tr>
<td>SphK2</td>
<td>Forward</td>
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4.2.3 Enzyme-linked immunosorbent assay (ELISA)

Ready-SET-Go ELISA kits for mouse IL-1β (no. 88-7013-22) and TNF-α (no. 88-7324-22) were purchased from eBiosciences and used according to the manufacturer’s protocol. Blood was collected from mice 2 hours after LPS injection, and cells were sedimented by centrifugation at 1,000 g for 10 minutes at 4°C. The clear serum layer (~50% of the original volume) was transferred from the top into a new fresh tube and stored in -80°C until cytokine profiling by ELISA. 100 μl of serum was used for each well and samples were run in duplicates. Cytokine concentration was determined using a standard curve (8 points 2-fold serial dilutions of known standard concentration), and expressed in pg/ml.
4.2.4 Statistical analysis

Statistical analysis was conducted using Prism software version 4.03. All data reported are expressed as mean values +/- standard error of the mean (SEM). The unpaired Student’s t test without Welch’s correction (assuming equal variance) was used when comparing differences between two groups. Multiple group analysis was performed using one-way analysis of variance (ANOVA), with the Tukey-Kramer or Newman-Keuls multiple comparison post-hoc test to determine differences between multiple groups. Group comparisons were determined to be significant for p value less than 0.05 (* p < 0.05, ** p < 0.01, *** p < 0.001).
4.3.1 Hypercholesterolemia and lipid loading inhibits reverse transendothelial migration and clearance of *C. muridarum* from the arterial intima.

Chapters 2 and 3 described reverse transendothelial migration (RTM) as the mechanism by which intimal CD11c+ cells numbers are reduced after LPS and *C. muridarum* injection; and RTM is the mechanism of clearance of *C. muridarum* from the arterial intima. In the following sections, these roles of RTM will be verified under conditions of hypercholesterolemia.

4.3.1.1 Lipid loading inhibits LPS-induced RTM of intimal CD11c+ foam cells.

We investigated whether RTM is inhibited in the setting of hypercholesterolemia by feeding *Ldlr*−/− mice with cholesterol-rich diet (CRD) for one week prior to injection of PBS, LPS or Poly(I:C). The period of one week of diet was selected since we have shown before that local proliferation and monocyte recruitment does not start until 2-3 weeks of CRD. This is important since we wanted to only study the effect of lipid-loading on RTM of intimal CD11c+ cells without any confounding factors such as local proliferation or recruitment of blood monocytes. During this period, resident intimal CD11c+ cells engulf lipids and transform into the initial foam cells in the lesion.

In PBS-injected mice, the abundance of intimal CD11c+ cells was comparable in both dietary groups (standard and cholesterol-rich) (*Fig. 25a*), whereas the surface area was significantly higher in the CRD-fed group compared to the standard diet-fed group (*Fig. 25b*), suggesting that resident cells engulf lipids and expand to cover a bigger area. TLR ligands induced a reduction in intimal CD11c+ cell numbers (*Fig. 25a*) and surface area (*Fig. 25b*) at 24 hours only in standard diet-fed *Ldlr*−/− mice, but not CRD-fed mice. Moreover, LPS induced two waves of intimal CD11c+ cell reduction.
in Ldlr−/− mice fed a standard diet (Fig. 25c) identical to WT mice. These reductions did not occur in mice fed a CRD (Fig. 25d), suggesting that hypercholesterolemia and/or cytoplasmic accumulation of lipid inhibits CCR7-dependent RTM of intimal CD11c+ cells. This inhibition was not due to any changes in CCR7 or CCL19 induction after TLR stimulation. In LPS-injected mice, similar levels of intimal CCR7 (Fig. 25e) and CCL19 (Fig. 25f) expression were observed in standard diet- and CRD-fed Ldlr−/− mice.

**Figure 25: Hypercholesterolemia and lipid loading inhibits LPS-induced reverse transendothelial migration of intimal CD11c+ foam cells from the arterial intima.**

Ldlr−/− mice (10 – 12 weeks old) were fed either a standard (Std.) or a cholesterol-rich (Chol.) diet for 1 week. (a,b) Mice were then injected iv with PBS, LPS (100 µg) or Poly(I:C) (150 µg), and intimal CD11c+ cells were enumerated (a) or CD11c+ area was determined at 24 hours (b). (c,d) Mice were injected iv with LPS, and intimal CD11c+ cells were enumerated at the indicated time points. (e,f) Mice were injected with PBS or LPS for 6 hours and the expression of CCR7 (e) or CCL19 (f) mRNA in intimal cells was assessed by qPCR. In all panels, means ± SEM were plotted, which were derived from at least three independent experiments with two mice per experimental group (the total number of mice per group was 6 or higher). Asterisks represent statistically significant differences compared to PBS-injected groups; *** P < 0.001.
Figure 25

(a) RTM of intimal CD11c^+ cells

(b) CD11c^+ area (x10^4 pixels) in the ascending aorta

(c) Time (d) after LPS injection (Std. diet)

(d) Time (d) after LPS injection (Chol. diet)

(e) CCR7

(f) CCL19

Relative mRNA expression
4.3.1.2 **Lipid loading prevents clearance of* C. muridarum *from the arterial intima.**

We investigated whether lipid loading also inhibits RTM and *C. muridarum* clearance. *Ldlr<sup>-/-</sup>* mice were fed either a standard or a cholesterol-rich diet for one week prior to *C. muridarum* injection for another 5 to 7 days. *C. muridarum* induced two waves of intimal CD11c<sup>+</sup> cell reduction in *Ldlr<sup>-/-</sup>* mice fed a standard diet, but these reductions did not occur in mice fed a cholesterol diet (**Fig. 26a**). We next investigated the consequences of this on intimal accumulation of *C. muridarum*. In standard diet-fed *Ldlr<sup>-/-</sup>* mice, *C. muridarum* infection was detected on days 1 and 3, and was virtually eliminated on days 5 and 7 (**Fig. 26b**). In contrast, the infection in the intima persisted throughout the experiment in cholesterol diet-fed *Ldlr<sup>-/-</sup>* mice (**Fig. 26b**). These data demonstrate that inhibition of intimal CD11c<sup>+</sup> cells RTM in hypercholesterolemic mice impairs an efficient and rapid removal of a pathogen from the arterial intima.

As expected, the infection load on day 1 was comparable in both dietary groups (standard and cholesterol-rich diets) (**Fig. 26b**), since monocyte recruitment has not yet started to increase after one week of hypercholesterolemia. In order to substantiate this notion and further demonstrate that mononuclear cells are the vehicle through which *Chlamydia* infects the arterial intima, *Ldlr<sup>-/-</sup>* mice were fed either a standard or cholesterol-rich diet for 4 weeks instead, when monocyte recruitment occurs along with local proliferation. In contrast to CRD for one week, the infection load in mice that were on CRD for 4 weeks was significantly higher (~10 fold) compared to mice on standard diet 1 day after exposure to *Chlamydia* (**Fig. 26c**). These data are consistent with the findings in chapter 3 showing that *Chlamydia* is carried by blood monocytes to infect the arterial intima. During later stages of atherosclerosis, infection load increases which can cause more harm to the vessel wall, activate a local inflammatory response, and accelerate plaque progression and cardiovascular events.
Figure 26: Hypercholesterolemia and lipid loading prevents clearance of *C. muridarum* from the arterial intima. (a) *Ldlr*−/− mice (10 – 12 weeks old) were fed either a standard (Std.) or a cholesterol-rich (Chol.) diet for 1 week. Mice were then injected iv with *C. muridarum* (10^7 IFUs), and intimal CD11c^+ cells were enumerated at the indicated time points. (b) *Ldlr*−/− mice (10 – 12 weeks old) were fed either a standard (Std.) or a cholesterol-rich (Chol.) diet for 1 week. Mice were then injected iv with *C. muridarum* (10^7 IFUs) and the expression of *C. muridarum* 16s rRNA was assessed by qPCR in intimal cells at the indicated time point. Data were normalized to HPRT and are expressed relative to day 1 values of the standard diet group. (c) *Ldlr*−/− mice were fed either a standard (Std.) or a cholesterol-rich (Chol.) diet for 4 weeks. Mice were then injected iv with *C. muridarum* (10^7 IFUs) and the expression of *C. muridarum* 16s rRNA was assessed by qPCR in intimal cells after 24 hours. Data were normalized to HPRT and are expressed relative to standard diet values. In all panels, means ± SEM were plotted, which were derived from at least three independent experiments with two mice per experimental group (the total number of mice per group was 6 or higher). Asterisks represent statistically significant differences compared to day 0 in (a) and to day 1 in (b); * P < 0.05, *** P < 0.001.
Figure 26

(a) Diet: Std. 

(b) Std. Diet 

(c) Relative C. muridarum 16s rRNA expression
4.3.2 LPS-induced reverse transendothelial migration of intimal CD11c+ cells is dependent on TLR4 expression by vascular endothelial cells.

There are several lines of evidence that endothelial cells express functional TLRs, specifically TLR4 and TLR2. TLR2 expression by endothelium was detected by immunostaining primarily in regions predisposed to atherosclerosis and in early atherosclerotic lesions and reciprocal BM transplantation experiments showed that vessel wall expression of TLR2 was critical for atherogenesis. Therefore, we investigated whether RTM is dependent on TLR4 expression by endothelial cells or intimal CD11c+ cells. Ultrapure LPS did not cause a reduction in the number of intimal CD11c+ cells in Tlr4−/− mice, whereas Poly(I:C) yielded normal reduction (Fig. 27a), suggesting that LPS-induced RTM is TLR4-dependent.

TLR4 mRNA was constitutively expressed in the intima of PBS-injected Cdf11c-dtr transgenic mice, and its expression level did not decrease when CD11c+ cells were depleted with DT injection 24 hours prior to PBS injection (Fig. 27b), suggesting that TLR4 is expressed by vessel wall cells. TLR4 expression decreased significantly after LPS or Poly(I:C) injection, probably as part of a negative control mechanism to control inflammation in the intima. Regardless, depletion of intimal CD11c+ cells still did not reduce TLR4 expression compared to undepleted controls (Fig. 27b). We performed reciprocal bone marrow transplantation (BMT) between WT and Tlr4−/− mice in order to investigate further the expression of TLR4 in different compartments and to verify their importance in RTM. When WT mice were transplanted with Tlr4−/− bone marrow cells, intimal TLR4 expression was normal, while Tlr4−/− mice transplanted with WT bone marrow expressed very low levels of TLR4 mRNA (Fig. 27c), further suggesting that only endothelial cells, not intimal CD11c+ cells, express TLR4. Next, the contribution of myeloid versus endothelial TLR4 in RTM was evaluated (Fig. 27d). As a control in
this experiment, we reconstituted lethally irradiated \textit{Tlr4\textsuperscript{-/-}} mice with \textit{Tlr4\textsuperscript{-/-}} bone marrow cells and showed that LPS did not induce a reduction in the number of intimal CD11c\textsuperscript{+} cells, whereas Poly(I:C) induced the expected reduction in cell abundance similar to \textit{Tlr4\textsuperscript{-/-}} mice that did not undergo BMT. Surprisingly, in both types of reciprocal BMT between WT and \textit{Tlr4\textsuperscript{-/-}} mice, a reduction in the number of intimal CD11c\textsuperscript{+} cells was observed 24 hours after LPS injection (Fig. 27d). This may suggest that TLR4 is expressed by both intimal CD11c\textsuperscript{+} cells and endothelial cells and that they compensate for each other; however, this would contradict the above findings. Alternatively, it is known that systemic pathogens and other PRR ligands induce a systemic inflammatory response, which includes secretion of systemic cytokines that could in turn activate signaling in the intima and upregulate CCL19/CCR7 and other molecules necessary for RTM of intimal CD11c\textsuperscript{+} cells. Therefore, we analysed systemic production of cytokines, for example the levels of IL-1\textbeta in mouse serum after LPS injection in both type of reciprocal BMT recipients. Danger signals in the circulation are recognized by macrophages in the spleen (the reticulo-endothelial system, RES); thus, only recipients of WT bone marrow were able to upregulate IL-1\textbeta secretion (Fig. 27e), which may induce CCR7/CCL19 expression and RTM of intimal CD11c\textsuperscript{+} cells. In contrast, recipients of \textit{Tlr4\textsuperscript{-/-}} bone marrow were not able to upregulate IL-1\textbeta production (Fig. 27e), suggesting that RTM was mediated locally through activation of endothelial cells in the vessel wall. Finally, in order to isolate the systemic from local response, we incubated aortas from both types of reciprocal BMT \textit{ex vivo} in culture media with or without LPS, and showed that a reduction in intimal CD11c\textsuperscript{+} cells is only observed when WT mice were the recipients, which express TLR4 in the vessel wall endothelial cells (Fig. 27f), suggesting that locally LPS-induced RTM of intimal CD11c\textsuperscript{+} cells is dependent on TLR4 expression by vascular endothelial cells.
Figure 27: LPS-induced reverse transendothelial migration of intimal CD11c+ cells is dependent on TLR4 expression by vascular endothelial cells. (a) Tlr4−/− mice were injected with PBS, LPS (100 µg), Poly(I:C) (150 µg) for 24 hours and the intimal CD11c+ cells were enumerated by en face confocal microscopy. (b) TLR4 mRNA expression with or without intimal CD11c+ cell depletion by DT for 24 hours prior to PBS, LPS or Poly(I:C) injection for another 6 hours. This shows that TLR4 expression is not affected by cell depletion suggesting expression by endothelial cells. Data were normalized to HPRT expression and are expressed relative to PBS injected undepleted group. (c) TLR4 mRNA expression in the arterial intima after reciprocal bone marrow transplantation as indicated on the x axis. Data were normalized to HPRT and are expressed relative to values from WT recipients. (d) Reciprocal bone marrow transplantation between WT and Tlr4−/− mice followed by recovery for 8 weeks prior to PBS, LPS or Poly(I:C) iv injection for 24 hours. The number of intimal CD11c+ cells was determined by en face confocal microscopy, and data are expressed as percentages of the control PBS injected group. (e) Mice from the indicated BMT were injected with PBS or LPS for 2 hours and blood samples were collected for serum IL-1β quantification using ELISA. (f) Reciprocal bone marrow transplantation between WT and Tlr4−/− mice was followed by recovery for 8 weeks. Aortas were incubated ex vivo in culture media containing PBS or LPS for 12 hours at 37°C, then fixed in PFA and the number of intimal CD11c+ cells was determined by en face confocal microscopy. Data are expressed as percentages of the control PBS injected group. In all panels, means ± SEM were plotted, which were derived from at least three independent experiments with two mice per experimental group (the total number of mice per group was 6 or higher). Asterisks represent statistically significant differences compared to control groups; * P < 0.05, ** P < 0.01, and *** P < 0.001.
Figure 27

(a) RTM of intimal CD11c+ cells

(b) TLR4

(c) Relative TLR4 mRNA expression

(d) Intimal CD11c+ cells

(e) Serum mIL-1β (pg/ml)

(f) Intimal CD11c+ cells
4.3.3 LPS-induced reverse transendothelial migration of intimal CD11c+ cells is dependent on IL-1β and IL-1R1 expression by vascular endothelial cells.

4.3.3.1 Both IL-1β and TNF-α induce RTM but only blocking IL-1β inhibits RTM.

LPS detection was mediated by endothelial cell TLR4 and intimal CD11c+ cells did not express TLR4 required for RTM, suggesting that secondary mediators (such as IL-1β and TNF-α) secreted from endothelial cells are required for induction of CCL19/CCR7 expression in intimal CD11c+ cells. Therefore, we investigated whether IL-1β and/or TNF-α are required for RTM of intimal CD11c+ cells. IL-1β or TNF-α could mediate RTM locally either directly by inducing CCR7/CCL19 gene expression by intimal CD11c+ cells, or indirectly by activating endothelial cells to secrete other cytokines required for induction of gene expression. Systemic administration of IL-1β induces a 50% reduction in the number of intimal CD11c+ cells after 24 hours (Fig. 28a), comparable to what was previously demonstrated with various TLR and NOD ligands. Ex vivo incubation of the aorta with IL1β also induced a 50% reduction in intimal CD11c+ cell number (Fig. 28b), suggesting that IL-1β is capable of inducing RTM locally independent of the systemic response. A similar dose dependent reduction was observed when mice were injected with TNF-α (Fig. 28c), and ex vivo incubation also induced a comparable reduction in intimal CD11c+ cell numbers (Fig. 28d). Since LPS can upregulate the expression of IL-1β, TNF-α and other cytokines, aortas were incubated ex vivo with LPS and blocking antibodies to IL-1β and TNF-α to investigate whether these cytokines are critical for RTM of intimal CD11c+ cells. Neutralization of IL-1β, but not TNF-α inhibited LPS-induced RTM of intimal CD11c+ cells during both waves, at day 1 (Fig. 28e) and at day 3 (Fig. 28f). Similar results were obtained in mice treated in vivo with the same blocking antibodies (not shown), suggesting that RTM is dependent on local IL-1β production in the arterial intima.
**Figure 28:** IL-1β and TNF-α induce reverse transendothelial migration of intimal CD11c⁺ cells, however only blocking IL-1β inhibits LPS-induced RTM. (a) C57BL/6 WT mice were injected iv with recombinant mouse IL-1β and the number of intimal CD11c⁺ cells in the ascending aortic arch was determined after 24 hours by *en face* confocal microscopy. (b) Aortas were incubated *ex vivo* in culture media containing PBS or 50 ng/ml recombinant mouse IL-1β for 12 hours at 37°C prior to fixation and enumeration of intimal CD11c⁺ cells by *en face* confocal microscopy. (c) WT mice were injected iv with recombinant mouse TNF-α at the indicated doses and the number of intimal CD11c⁺ cells in the ascending aortic arch was determined after 24 hours by *en face* confocal microscopy. (d) Aortas were incubated *ex vivo* in culture media containing PBS or 500 ng/ml recombinant mouse TNF-α for 12 hours at 37°C prior to fixation and enumeration of intimal CD11c⁺ cells by *en face* confocal microscopy. (e) Aortas were incubated *ex vivo* in culture media containing PBS or LPS for 12 hours at 37°C prior to fixation and enumeration of intimal CD11c⁺ cells by *en face* confocal microscopy. LPS treatment was also supplemented with function blocking antibodies to IL-1β, TNF-α or isotype IgG control (100 μg). (f) WT mice were injected with PBS or LPS (100 μg) for 54 hours and aortas were dissected and incubated *ex vivo* in culture media (no addition of LPS) for another 12 hours prior to fixation and enumeration of intimal CD11c⁺ cells by *en face* confocal microscopy. Media also contained functional blocking antibodies to IL-1β, TNF-α or isotype IgG control (100 μg). Similar results to (e) and (f) were obtained when PBS, LPS and antibodies were injected *in vivo* on days 0 and 2, and mice were dissected on days 1 and 3, respectively. In all panels, means ± SEM were plotted, which were derived from at least three independent experiments. Asterisks represent statistically significant differences compared to control groups (white bars); ** P < 0.01 and *** P < 0.001.
RTM of intimal CD11c+ cells Chapter 4

Figure 28

(a) *in vivo* mIL-1β

(b) *ex vivo* mIL-1β

(c) *in vivo* mTNFα

(d) *ex vivo* mTNFα

(e) WT mice (1st wave)

(f) WT mice (2nd wave)
4.3.3.2 IL-1β is an important mediator for LPS-induced RTM of intimal CD11c⁺ cells, but its function is not the induction of CCR7 and CCL19.

To further investigate the role of IL-1β in RTM of intimal CD11c⁺ cells, we used Asc⁻/⁻ mice as a tool for deficiency in IL-1β secretion. ASC is an adaptor protein required for the assembly of the inflammasome leading to caspase-1 activation and cleavage of pro-IL-1β into mature IL-1β; therefore deficiency in ASC inhibits maturation and secretion of IL-1β. LPS injection into Asc⁻/⁻ mice did not reduce the abundance of intimal CD11c⁺ cells after 24 hours (Fig. 29a), further confirming our findings that IL-1β is required for RTM of intimal CD11c⁺ cells. We attempted to rescue RTM of intimal CD11c⁺ cells in Asc⁻/⁻ mice by administering recombinant mouse IL-1β in vivo and TNF-α served as a control. However, given their low molecular weight and their rapid clearance from the circulation, neither cytokine rescued the Asc⁻/⁻ phenotype in vivo (Fig. 29a). Nevertheless, incubation of Asc⁻/⁻ aortas with IL-1β ex vivo reversed and rescued the Asc⁻/⁻ phenotype, whereas LPS or TNF-α did not have an effect (Fig. 29b).

We next investigated whether the deficiency in ASC, and thus the lack of production of mature IL-1β, inhibits RTM by preventing the induction in the intima of molecules we have implicated in RTM such as CCR7 / CCL19 and S1P and its receptors. WT and Asc⁻/⁻ mice were injected with LPS for 6 hours and gene expression was analyzed in the arterial intima. ASC deficiency did not affect the induction of CCR7 and CCL19, and the constitutive expression of CCL21 was not altered (Fig. 29c). ASC deficiency decreased the level of induction of SphK1 by 25% compared to WT mice, and yet no effect on SphK2 expression (Fig. 29d). S1P receptors are constitutively expressed in the intima, and only S1Pr1 is induced ~ 4 fold. Yet, ASC deficiency did not affect the induction and expression of S1Pr1, 2 nor 4; however, we observed a significant reduction in the constitutive expression of S1Pr3 and S1Pr5 (Fig. 29d).
**Figure 29:** IL-1β is an important mediator for LPS-induced RTM of intimal CD11c⁺ cells, but its function is not the induction of CCR7 and CCL19. (a) Asc⁻/⁻ mice were injected iv with PBS, LPS (100 μg), IL-1β (50 ng), or TNF-α (500 ng), and the number of intimal CD11c⁺ cells was determined after 24 hours by en face confocal microscopy. (b) Aortas from Asc⁻/⁻ mice were incubated ex vivo in culture media containing PBS, LPS (50 μg/ml), IL-1β (50 ng/ml), or TNF-α (500 ng/ml) for 12 hours at 37°C prior to fixation and enumeration of intimal CD11c⁺ cells by en face confocal microscopy. (c,d) WT and Asc⁻/⁻ mice were injected with LPS (100 μg) for 6 hours and intimal cells were harvested for RNA isolation and analysis of gene expression by qPCR. Data were normalized to HPRT and are expressed relative to WT values (white bars). In all panels, means ± SEM were plotted, which were derived from at least three independent experiments with two mice per experimental group (the total number of mice per group was 6 or higher). Asterisks represent statistically significant differences compared to control groups (white bars); * P < 0.05 and ** P < 0.01.
Figure 29

(a) Asc⁻/⁻ mice (In vivo)

(b) Asc⁻/⁻ mice (Ex vivo)

(c) CCR7  CCL19  CCL21

(d) LPS 6 hours

Relative mRNA expression

SphK1  SphK2  S1P1  S1P2  S1P3  S1P4  S1P5

WT mice  Asc⁻/⁻ mice
4.3.3.3 RTM is dependent on IL-1β and IL-1r1 expression by endothelial cells.

Having found a role for IL-1β in orchestrating RTM of intimal CD11c+ cells, experiments were carried out to investigate which cell type in the intima is the source of IL-1β. IL-1β was constitutively expressed in PBS-injected *Cd11c-dtr* transgenic mice, and its expression level decreased significantly when CD11c+ cells were depleted with DT injection 24 hours prior to PBS injection (Fig. 30a), suggesting that IL-1β is expressed by intimal CD11c+ cells under steady-state conditions. IL-1β expression increased significantly after LPS injection; however, depletion of intimal CD11c+ cells did not completely eliminate IL-1β expression compared to undepleted controls (Fig. 30a), suggesting that under inflammatory conditions, both intimal CD11c+ cells and endothelial cells express IL-1β.

Since mRNA expression is not a good indicator of actual IL-1β production, because pro-IL-1β still has to be processed by the inflammasome, we used reciprocal bone marrow transplantation between WT and *Asc−/−* mice to investigate which cell type is a more important source of IL-1β. RTM occurred only when WT mice were the recipients (Fig. 30b), suggesting that mature IL-1β mainly secreted by vessel wall endothelial cells is critical for RTM. Since ASC has other functions that are independent of the assembly of the inflammasome293, we confirmed our results in *Caspase-1−/−* (*Casp1−/−*) mice. Results showed that LPS-induced RTM is inhibited in *Casp1−/−* mice (Fig. 30c), further supporting the notion that RTM of intimal CD11c+ cells is in fact mediated by inflammasome-dependent IL-1β production by endothelial cells.

Next we investigated which intimal cell type responds to IL-1β through its receptor, IL-1r1. Reciprocal bone marrow transplantation suggests that RTM occurs only when WT mice were the recipients (Fig. 30d), suggesting that IL-1β signals in intimal endothelial cells not in CD11c+ cells.
Figure 30: RTM of intimal CD11c⁺ cells is dependent on IL-1β production by endothelial cells, which signals through IL-1r1 expressed by the same cells. (a) Cd11c-dtr transgenic mice were injected with either PBS or DT to deplete intimal CD11c⁺ cells 24 hours prior to PBS or LPS injection for another 6 hours. IL1-b mRNA expression was assessed in harvested intimal cells. Data were normalized to HPRT expression and are expressed relative to PBS injected undepleted group. * designate significance compared to undepleted PBS group, whereas # designate significance compared to undepleted LPS group. (b) Reciprocal bone marrow transplantation between WT and Asc⁻/⁻ mice was followed by recovery for 8 weeks. Aortas were incubated ex vivo in culture media containing PBS or LPS for 12 hours at 37°C, then fixed in PFA and the number of intimal CD11c⁺ cells was determined by en face confocal microscopy. Data are expressed as percentage of the control PBS injected group (white bars). (c) Caspase-1⁻/⁻ mice were injected with PBS or LPS and the number of intimal CD11c⁺ cells was determined after 24 hours by en face confocal microscopy. (d) Reciprocal bone marrow transplantation between WT and IL-1r1⁻/⁻ mice followed by recovery for 8 weeks prior to PBS or LPS iv injection for 24 hours. The number of intimal CD11c⁺ cells was determined by en face confocal microscopy. In all panels, means ± SEM were plotted, which were derived from at least three independent experiments with two mice per experimental group (the total number of mice per group was 6 or higher). Asterisks represent statistically significant differences compared to control groups (white bars); * P < 0.05, ** P < 0.01 and *** P < 0.001.
4.4 Discussion

Overall, studies in this chapter showed that lipid-loading associated with hypercholesterolemia and atherosclerosis inhibits RTM of intimal CD11c+ foam cells and clearance of *Chlamydia* from the arterial intima. This inhibition is not due to effects on CCR7 and CCL19 induction, suggesting that lipid-loading influences other mediators that are important in the RTM mechanism. Studies using *Tlr4−/−* mice suggested that LPS is detected by vessel wall endothelial cells, which may secrete secondary signals (cytokines such as IL-1β, TNF-α, etc.) to induce CCR7 and CCL19 expression. These studies led us to investigate the role of IL-1β and we discovered that IL-1β is an important mediator in RTM of intimal CD11c+ cells. However, contrary to our expectations, the role of IL-1β was not to induce CCR7 and CCL19 expression since their induction was normal in *Asc−/−* mice. In addition, our results showed that IL-1β signals through IL-1r1 expressed on vessel wall endothelial cells. This suggests that endothelial cells play a critical role in RTM, which requires further investigations.

We showed that hypercholesterolemia inhibits intimal cell migration in response to LPS and *C. muridarum*. This corroborates findings from others suggesting that macrophage exit from atherosclerotic plaques is inhibited because of active secretion of repulsive neuroimmune guidance cues such as netrin-1, ephrin-B, semaphorins 3A and 3E. Whether these factors play significant roles in inhibiting RTM in response to systemic TLR stimuli will require further investigation. We attempted some preliminary experiments to examine the effects of netrin-1 in our model. Previous studies showed that mRNA expression of netrin-1 and its receptor Unc5b are elevated in atherosclerotic plaques. We examined the expression of these genes in our samples, and observed that they were not elevated after feeding cholesterol-rich diet for one week compared to standard diet-fed mice (data not shown). In addition, we
used a blocking antibody to netrin-1 in an attempt to rescue RTM, but we did not observe any differences in cell numbers or lesion size (data not shown). This suggests either that netrin-1 does not inhibit RTM of lipid-loaded intimal CD11c+ cells, that blocking of multiple inhibitory guidance cues is required in order to rescue RTM, or that inhibition of RTM is due to another mechanism altogether.

Another possibility is that lipid loading affects posttranscriptional or posttranslational modifications of CCR7, which could affect its function. A recent study showed that CCR7 is a sialylated protein and that this modification is important in the regulation of signal transduction and biological function of CCR7 in metastasis of cancer cells. Whether CCR7 sialylation (or another glycoprotein modification) is important for its function in RTM of intimal CD11c+ cells, and whether lipid loading affects this step remains to be investigated.

Moreover, the effects of lipid loading on RTM could potentially be completely independent of CCR7 and CCL19. We have shown in the previous chapter, as well as this chapter, that RTM is also dependent on S1P production and signaling, as well as IL-1β / IL-1r1 signaling. It is possible that oxidative stress from hypercholesterolemia affects the induction of SphK1 in endothelial cells or downstream signaling from the S1Pr1 in intimal CD11c+ cells. Alternatively, lipid-uptake by endothelial cells could affect IL-1β- mediated downstream signaling, and thus inhibit RTM of intimal CD11c+ cells. Before we can predict the players that are involved in the effects of hypercholesterolemia on RTM, further investigations about S1P, IL-1β, and other molecules implicated in reverse migration under standard diet conditions are required. In this respect, we need to elucidate which S1P receptor is required for RTM and identify which cells are expressing it in the intima. Furthermore, we need to understand the role of endothelial cell IL-1β signaling in RTM of intimal CD11c+ cells.
Our studies using Tlr4−/− mice suggested that intimal CD11c+ cells do not express TLR4 and that the response to LPS is mediated by endothelial cells in the intima. This is unusual because it is well established that DCs express TLR4, respond to bacterial LPS and migrate to regional lymph nodes to activate the adaptive immune response. Since the intima is devoid of lymphatic drainage and we have shown that intimal DCs do not migrate through conventional routes but through RTM, we were perplexed with the possibility that they might not express TLR4 but rely on secondary signals from the overlying endothelial cells.

Our data depict a complex picture of how endothelial cells communicate with intimal CD11c+ cells to undergo RTM. We used blocking antibodies to IL-1β or TNF-α individually and only IL-1β blocking inhibited RTM. Blocking antibodies to IL-6 did not affect RTM of intimal CD11c+ cells (data not shown). In addition, the inhibition of RTM by blocking or deficiency in IL-1β signaling was not due its role as a secondary mediator to induce expression of CCR7 and CCL19 on intimal CD11c+ cells, but rather due to effects on endothelial cell activation. These data suggest either that we have not yet identified the molecule responsible for relaying the signal from endothelial cells to CD11c+ cells, or that multiple cytokines are compensatory and blocking each individually would not inhibit CCR7 and CCL19 induction. In order to address the latter possibility, future studies will investigate whether combining multiple blocking antibodies would inhibit induction of CCR7 / CCL19 and RTM of intimal CD11c+ cells.

In order for intimal CD11c+ cells to undergo RTM, cells have to cross the endothelial cell layer, and inter-endothelial contacts have to be reduced to facilitate the migration of CD11c+ cells through endothelial cell junctions132. IL-1β signaling could modulate various mechanisms in endothelial cells that affect cell junctions and facilitate cell egress. For example, leukocyte transmigration is dependent on increased
levels of endothelial intracellular calcium, which opens endothelial-cell contacts via the activation of myosin light-chain kinase and subsequent endothelial-cell contraction\textsuperscript{295}. Molecules that do not support leukocyte migration, and might actually act as an obstacle to emigrating cells (such as VE-Cadherin), may be distributed away from the junctional regions\textsuperscript{296}. A recent study showed that IL-1\(\beta\) signals through the small GTPase, ADP-ribosylation factor (ARF6), and its activator, ARF nucleotide binding site opener (ARNO), which promote endocytic internalization of VE-cadherin, disrupt its localization to the cell junction and facilitate leukocyte transmigration\textsuperscript{297}. In addition, endothelial junctional molecules for which leukocytes express ligands, such as platelet / endothelial cell adhesion molecule 1 (PECAM1 or CD31) and junctional adhesion molecule A (JAM-A), may mobilize to the abluminal surface, thus creating an adhesive haptotactic gradient that guides luminal leukocytes to the junctions\textsuperscript{298}. Consistent with these studies, our preliminary experiments using blocking antibodies to CD31 inhibited RTM of intimal CD11c\(^+\) cells (data not shown). Moreover, a recent study showed that IL-1\(\beta\) lowers functional expression of the adhesion molecule JAM-C at endothelial cell junctions in order to facilitate reverse transendothelial migration of neutrophils into the cremaster vein lumen\textsuperscript{141}. Altogether the literature provides us with multiple potential cell junction targets that could affect reverse transendothelial migration of intimal CD11c\(^+\) cells and may be modulated downstream of IL-1\(\beta\) / IL-1r1 signaling on endothelial cells. Future studies will investigate the involvement of these molecules in the reverse transendothelial migration of intimal CD11c\(^+\) cells through the use of neutralizing antibodies and knockout mouse models. Overall, this chapter suggests that endothelial cells play an active role in mediating RTM of intimal CD11c\(^+\) cells by producing secondary mediators that induce CCR7 and CCL19 expression and through IL-1\(\beta\) production followed by autocrine / paracrine signaling through the IL-1r1.
Chapter 5

General Discussion, Conclusions and Future Directions.
5.1 Overview

The concept of leukocyte reverse transmigration is gathering significant momentum and interest but many aspects of this topic require significant exploration. The purpose of this thesis was to determine the key players in RTM of intimal CD11c+ myeloid cells and the impact that this process has on pathogen clearance and atherosclerotic lesion size. CD11c+ myeloid cells reside in the arterial intima in sites that are predisposed to atherosclerosis. Under steady-state conditions, the abundance of leukocytes in the intima is tightly regulated and limited in the ascending aortic arch to one layer of ~600-800 subendothelial CD11c+ cells, which undergo complete turnover every 6-8 weeks. During turnover, cell removal may be through cell death or through low-grade RTM and migration to secondary lymphoid organs as part of an immunosurveillance mechanism and to mediate T cell tolerance. Systemic pathogens and stimulation of pattern recognition receptors induce RTM of ~50% of intimal CD11c+ cells into the arterial lumen, where they can home into secondary lymphoid organs for antigen presentation and activation of the adaptive immune response. We have shown that RTM is dependent on CCR7 and CCL19 expression by CD11c+ cells and on S1P production and signaling through S1P receptors, as well as IL-1β production and IL-1r-1 expression by endothelial cells. We have also shown that RTM is the mechanism of C. muridarum clearance a few days after the pathogen infects the arterial intima via recruited infected blood monocytes, not phagocytic clearance or intracellular killing. Under hypercholesterolemic conditions, intimal CD11c+ cells engulf lipids, become the initial foam cells, and are unable to undergo RTM in response to PRR stimulation. Future studies will ascertain how lipid loading inhibits RTM so that appropriate interventions can be instituted and RTM can potentially be harnessed to transport accumulated lipids from the artery wall.
5.2 Proposed molecular model

Based on our results and review of the literature, we postulate the following signaling mechanism that mediates LPS-dependent RTM of intimal CD11c⁺ cells. LPS is detected by TLR4 expressed on endothelial cell surface which activates the NF-κB pathway and induction of inflammatory gene expression through MyD88-dependent signaling. Stimulated endothelial cells secrete secondary mediators such as IL-6, TNF-α, IL-1β, etc. or an unidentified mediator, which in turn activate intimal CD11c⁺ cells and induce expression of genes required for reverse migration such as CCR7 and its ligand CCL19 (Fig. 6). The receptor and its ligand in this model are both expressed by the same population of cells, suggesting that reverse migration occurs either through chemo-repulsion (as the highest CCL19 concentration is around intimal CD11c⁺ cells and lowest towards vascular smooth muscle cells), or that CCL19 does not provide directionality for reverse migration, but may only be required to switch intimal CD11c⁺ cells from a stationary to a locomotive state. Activated endothelial cells induce SphK1 expression to produce S1P creating a chemokine gradient and providing directionality for reverse migration of intimal CD11c⁺ cells which express S1Pr1 or another S1P receptor (Fig. 13). In order for migrating cells to cross the endothelial cell barrier, IL-1β secreted from activated endothelial cells signals through the IL-1r1 (autocrine or paracrine) to loosen the tight junctions between endothelial cells. IL-1β signaling could reduce JAM-C expression, change the localization of PECAM-1 from the abluminal to luminal surface to guide migratory cells, or induce internalization of VE-Cadherin through ARF6-ARNO signaling, to allow intimal CD11c⁺ cells to squeeze between endothelial cells. Once outside the intima, intimal CD11c⁺ cells may crawl on the endothelial cell surface, similar to recruited monocytes undergoing extravasation, and the bloodstream transports them to secondary lymphoid organs.
Model for the 2nd RTM wave: We observed that LPS, Poly(I:C) and \textit{C. muridarum} induce a second RTM wave of intimal CD11c+ cells, and our current findings suggest that the same factors (i.e. CCR7, CCL19, IL-1β, S1P, etc.) mediate both RTM waves. LPS and Poly(I:C) are rapidly cleared from the circulation after a bolus injection, and circulating cytokines produced in response drop before the second wave of RTM\textsuperscript{235,236}. In addition, the second RTM wave occurred \textit{ex vivo} when aortas were dissected 2 days after LPS injection and incubated for 12 hours (Fig. 5). This suggests that the stimulus for the second RTM wave is produced locally in the arterial intima, which occurs most probably through reactivation of endothelial cells to secrete the required signaling factors for the second RTM wave of intimal CD11c+ cells. After \textit{C. muridarum} infection, the stimulus for the second RTM wave might be the exit of elementary bodies from the host cell to spread the infection to other cells in the arterial intima. During the life cycle of \textit{C. muridarum}, elementary bodies are internalized, escape phagolysosomal fusion, reside in the cytoplasm, transform into reticular bodies, undergo hundreds of divisions, and transform back to elementary bodies that are released to infect other cells\textsuperscript{251}. This life cycle takes approximately 2-3 days, the time frame during which the second RTM wave of intimal CD11c+ cells is observed. \textit{C. muridarum} infects the arterial intima in the first 24 hours, and 2-3 days later (i.e. between days 3-4), the pathogen is cleared by RTM of CD11c+ cells (Fig. 16). While \textit{C. muridarum} is enclosed in intracellular compartments, it is shielded from recognition by the immune system. Its release into the interstitial space (either by exocytosis or lysis of the host cell) might be the signal required for the reactivation of endothelial cells, secretion of necessary secondary mediators, RTM of intimal CD11c+ cells, and pathogen clearance from the vessel wall, which prevents further spread of the infection. Inhibition of RTM results in continued \textit{C. muridarum} growth and a local inflammatory response (Fig. 16, 23).
5.3 Significance of the dissertation

This thesis provides the first report that systemic stimulation of PRRs induce reverse transendothelial migration of CD11c$^+$ myeloid cells that reside in the arterial intima. This process may potentially protect the arterial intima against bacterial and viral pathogens by transporting them out of the arterial wall. Therefore, this thesis identified a novel innate immune mechanism for efficient pathogen removal from the arterial intima, which explains why infection of normal arteries is rare and has not been reported previously in humans. More importantly, this thesis provides a model (in vivo and ex vivo), as well as various tools and methods that might be used to study the role of specific receptor/ligand complexes, their sites of expression and their roles in RTM.

After discovering that systemic stimulation induces egress of leukocytes from the arterial intima, we postulated that this process could be used to induce lipid loaded cells to migrate out of atherosclerotic plaques and decrease lesion burden. Induction of RTM could be a potential therapy that could be applied in early stages of the disease, which are usually more effective than interventions at later stages. However, this thesis showed that LPS- and C. muridarum- induced RTM of intimal CD11c$^+$ cells is inhibited after these cells accumulated lipid and transformed into foam cells after one week of hypercholesterolemia. Nevertheless, we anticipate that information gained from this study, as well as future studies outlined below, will enable us to overcome the inhibitory mechanisms in lipid-loaded cells in the future. Appropriate interventions could be instituted and RTM can be harnessed to transport accumulated lipids out of the artery wall by exiting lipid-loaded CD11c$^+$ cells. In preliminary experiments, aortas from Ldlr$^{-/-}$ mice fed cholesterol rich diet for one week were incubated ex vivo for 12 hours in culture media containing IL-1$\beta$, which rescued RTM and decreased the number of intimal CD11c$^+$ cells and lipid staining.
5.4 Limitations of the experimental approach

One limitation in our experimental model is the small number of leukocytes in the arterial intima. In the ascending aortic arch, there is a maximum of 800 CD11c\(^+\) cell in the intimal space, and many more myeloid cells in the adventitia. This makes studying intimal cells only by flow cytometry almost impossible because of the dilution of intimal cells by the relatively more abundant adventitial cells in the cell suspension. Alternatively, one might attempt to separate the layers and only isolate cells from the intima; however, our personal experience, as well as our collaborators, suggests that there is a big loss in the number of cells recovered when the aortic layers are separated, making it harder to draw conclusions based on flow cytometry. In this study, flow cytometry was used study to support data obtained by other approaches.

On the other hand, immunostaining of fixed aortas coupled with en face confocal microscopy has been extensively used in this work to examine only the intimal layer and quantify the abundance of intimal CD11c\(^+\) cells. However, confocal microscopy, equipped with a maximum of 4 channels, is limited by the number of antibodies and fluorophores that can be used simultaneously compared to flow cytometry, which can detect up to 17 fluorophores. Moreover, whereas it is relatively easy to find and optimize an antibody for staining cells in cell suspension for flow cytometry, not all antibodies work for immunostaining on fixed tissues for confocal microscopy. Complications arise because fixation may destroy the conformation of an antigen, thereby changing its recognition by specific epitopes. Also, the majority of fluorophores used for flow cytometry are not compatible with microscopy as they photo-bleach after exposure to the microscope laser lights. Finally, the internal elastic lamina creates background noise that might mask low expression of certain antigens and signal amplification is required in these situations.
We have used qPCR in order to analyze gene expression in the intima in response to systemic stimulation. Although this approach was appropriate for analysis of a small set of genes, a more global approach would be required in order to discover all factors that play a role in RTM of intimal CD11c⁺ cells. This goal would be achieved by a microarray analysis of RNA isolated from the arterial intima. However, this technique usually requires a large number of cells to obtain sufficient amount of RNA for hybridization onto the microarray. This would not be compatible with the low number of cells isolated from the arterial intima, and an amplification step would be required to increase the RNA yield. However, the amplification step might mask important targets or produce false negatives if transcripts are not amplified homogenously. Nevertheless, this method is the best option until new sensitive techniques are developed. Once a candidate has been identified, gene expression quantification by qPCR can be used to confirm its intimal expression, as well as the cell type which expresses it by using depletion of intimal CD11c⁺ cells in Cd11c-dtr transgenic mice, or following gene expression kinetics through the RTM time-course.

In addition to gene expression, analysis of a signaling pathway also entails quantification of protein levels, as well as identification of potential posttranslational modifications that may affect protein function. Some proteins and modifications are possible to study using immunostaining, however thorough investigations would be achieved using Western blot analysis or a more global approach using mass spectrometry. In addition, some signaling molecules might be secreted in the interstitial space of the arterial intima and would require analysis by ELISA. However, given the small number of cells and the relatively low concentration of proteins produced in the arterial intima, these kinds of studies are not plausible at the moment and more sensitive techniques need to be developed.
Immunostaining and gene expression studies are analytical and quantitative approaches, but they are not functional studies to prove that a candidate plays an essential role in the RTM signaling pathways. Such functional studies include the use of pharmacological inhibitors, blocking antibodies, or genetically mutated mouse models. Each of those approaches have certain disadvantages. The use of pharmacological inhibitors may generate off target effects that might inhibit other signaling molecules that are important in RTM resulting in false negative observations. Blocking antibodies are more stable and specific since they recognize specific epitopes and antigens, but a positive control is required when a blocking antibody has no effect on RTM. Positive controls could include blocking of cell migration in another system that is known to depend on the factor of interest, but there is no guarantee that the conditions and dose used are relevant to RTM from the aorta. Finally, genetically mutated mouse models, which include transgenic and knock-out models are powerful tools in studying the roles of specific proteins in RTM of intimal CD11c$^+$ cells. However, some genes play an important role during development and alterations in those genes results in embryonic lethality and generating such models would be impossible. Moreover, even if the knock out model is not embryonically lethal, there can be systemic side effects resulting from the deficiency of a particular gene (e.g. the abnormal *C. muridarum* infection observed in *Ccr7$^{-/-}$* mice) that would complicate the interpretation of results in the intima. In order to circumvent these difficulties, mice could be generated which contain a “loxp” site surrounding the gene of interest, which could be crossed with mice expressing a Cre-recombinase under the control of the CD11c promoter in order to knock out a particular gene specifically in CD11c$^+$ cells. Nevertheless, compensatory mechanisms that would not regularly play a role in RTM might be activated over a long term when deficiency starts from the embryo and the
studies are performed on older mice. Tamoxifen-inducible Cre recombinase could be used when a deficiency of a gene is required at a determined point in time. This model is used for lineage-tracing experiments in order to determine the origin of certain cell types, and may be adapted to our studies to functionally investigate the role of certain genes in RTM of intimal CD11c$^+$ cells. Altogether, combining the use of pharmacological inhibitors, functional blocking antibodies and genetically mutated mice would be required in order to mitigate the disadvantages in each method and demonstrate definitively that a specific protein plays a critical role in RTM.

In order to support findings from in vivo experiments, in vitro cell migration models would be needed for corroborative evidences. This is difficult because RTM depends on factors and signaling pathways in both intimal CD11c$^+$ cells and endothelial cells, suggesting that an in vitro model would require co-culturing monocyte-derived DCs with endothelial cells. We pursued in vitro DC migration assays and found that we could recapitulate standard chemotaxis through a porous membrane but not chemo-repulsion. Therefore, first we need a better understanding about the in vivo microenvironment of the arterial intima and how intimal CD11c$^+$ cells and endothelial cells cross-talk, before developing an in vitro assay that would recapitulate the microenvironment of the arterial intima. Finally, the ex vivo model requires further development to allow us to acquire direct imaging evidence of cells moving in a retrograde direction across the intimal endothelial cells.

Overall, like every system and every model used to study pathophysiology, our model has its limitations and disadvantages, which did not hinder the progress of the study when multiple approaches were used to complement each other to reach clear and evident conclusions. Specifically, our model offers valuable tools and opportunities to study mechanisms of reverse migration of leukocytes in other tissues.
5.5 Advantages of the experimental model

Despite the limitations of the techniques used in this thesis, as evident from our findings, the methods still merit many advantages, and would still be used in future experiments in order to advance the understanding of RTM of intimal CD11c+ cells. No single technique is sufficient alone to answer questions about the role of a gene of interest in RTM of intimal CD11c+ cells, but multiple techniques used together provide plausible mechanism of how RTM occurs. Depletion of intimal CD11c+ cells using the DT-injected Cd11c-dtr transgenic mouse model and analysis of gene expression by qPCR allow us to identify whether a gene is expressed/induced in intimal CD11c+ cells and/or endothelial cells. Reciprocal bone marrow transplantation allowed us to functionally determine which cell type express and mediate the signaling of the protein of interest. Pharmacological inhibitors and blocking antibodies allow us to block the function of a certain protein within a specific period, avoid compensatory mechanisms, and answer functional questions for which genetically mutated mice are not available.

Although one might consider studying reverse migration of leukocytes in other organs, we argue that the intima offers a unique system that is not found in other models. In the arterial intima, RTM is dependent on only two cell types, namely intimal CD11c+ cells and endothelial cells. Studies in this thesis outlined multiple approaches (cell depletion or reciprocal bone marrow transplantation) that allowed us to define the critical roles of each cell type in RTM and the potential signaling intermediates. Multiple leukocyte subsets (e.g. T cells, DCs, macrophages, and neutrophils) reside in other organs in which reverse migration was studied and the interference or contribution of each subset is difficult to study independently of each other. We have shown that the arterial intima contains only a uniform population of intimal CD11c+ cells (with two subsets\textsuperscript{76}), and that other leukocyte subsets are not recruited following stimulation.
Moreover, studying reverse migration in the aortic intima offers another advantage, which is that RTM occurs during ex vivo culture of the aortic arch, analogous to in vivo results. Whereas in vivo stimulation and LPS-mediated RTM could be a response to both systemic and local cues, ex vivo culture of the aortic arch with LPS for 12 hours is dependent only on local not systemic mediators. Furthermore, data in this thesis showed that ex vivo aortic culture model was able to answer certain questions, which in vivo assessments were not able to address. For example, development of the ex vivo model was an important step to confirm that intimal CD11c⁺ cells undergo reverse transmigration towards the lumen and can be collected during perfusion experiments (Fig. 5). Ex vivo culture of aortas during the time frame of the second LPS-induced RTM wave (12 hours culture without LPS after 48 hours of in vivo LPS stimulation) showed that the second RTM wave may be dependent on local cues (Fig. 11). Ex vivo culture was important to distinguish between systemic and local recognition of LPS in Tlr4⁻/⁻ and WT reciprocal bone marrow transplantation experiments (Fig. 27), and showed that LPS-mediated RTM depends on TLR4 expression by endothelial cells not intimal CD11c⁺ cells. Ex vivo culture were finally used to show that IL-1β can rescue the inhibition of RTM in Asc⁻/⁻ mice (Fig. 29), since IL-1β in vivo injection did not rescue RTM of intimal CD11c⁺ cells likely because of its low molecular weight and rapid clearance from the circulation. The ex vivo culture model of the aortic arch will continue to be a useful tool in future studies to assess the roles of other signaling intermediates in RTM of intimal CD11c⁺ cells and distinguish between local versus systemic cues. Although we cannot currently replicate our in vivo results with an in vitro model, the ex vivo culture model is one step closer and offers more answers instead of just relying on in vivo experiments. This system is robust and is not complicated by the presence of more than two cell types essential for RTM.
5.6 Future directions

The work described in this thesis could be further developed in a number of directions as described below.

Future studies will continue to investigate signaling molecules that play a role in RTM of intimal CD11c\(^+\) cells, and examine their expression levels in mice fed a cholesterol rich diet to induce atherogenesis. We have preliminary results implicating S1P in RTM of intimal CD11c\(^+\) cells; therefore, as outlined previously, studies will continue with blocking antibodies and mice deficient in each S1Pr to determine which of the S1P receptors mediates S1P signaling and on which intimal cells it is expressed. We will also investigate whether CXCR4 / CXCL12 (SDF-1) signaling pathway plays a role in RTM of intimal CD11c\(^+\) cells. Previous studies suggested that JAM-C expression on endothelial cells is important in reverse migration of neutrophils from the cremaster vessels after IL-1\(\beta\) stimulation. Therefore, future studies will measure JAM-C expression, investigate whether blocking JAM-C inhibits RTM, and study its role allowing intimal CD11c\(^+\) cells to squeeze between endothelial cells during RTM.

After PRR stimulation and in each RTM wave, only 50% of intimal CD11c\(^+\) cell undergo RTM, suggesting that there are two (or more) subsets of CD11c\(^+\) cells in the intima. In the whole aorta, which comprise mostly adventitial cells, CD11c\(^+\) cells were divided into two subsets; CD11b\(^+\) subset derived from a monocytic origin, and CD103\(^+\) subset derived from conventional pre-DCs, as was described for other organs such as the lungs and lamina propria of the small intestines. However, another possibility is that intimal CD11c\(^+\) cells are a uniform population of cells derived from a single origin, and PRR stimulation triggers further differentiation of this population into two subsets, one of which upregulates CCR7 and CCL19 to undergo RTM through the endothelial layer. Infection of intimal CD11c\(^+\) cells with \textit{C. muridarum} could also induce
differentiation of infected cells into the subset that would undergo RTM. Whether two or more intimal CD11c+ subsets exist under steady-state conditions, or whether PRR stimulation induce their differentiation will be investigated in future studies.

Intimal CD11c+ cell homeostasis could be maintained via monocyte recruitment from the circulation, proliferation of a resident precursor in the intima, or recruitment of a precursor and its proliferation in the intima. Proliferation is the mechanism of recovery after each RTM wave of intimal CD11c+ cells after PRR stimulation. In addition, previous studies have shown that proliferation is upregulated in early atherosclerotic lesions and is the mechanism of turnover in advanced atherosclerotic plaques. Therefore, future experiments will investigate the role of proliferation in the homeostasis of intimal CD11c+ cells. We will investigate the role of cell growth factors such as M-CSF, GM-CSF, CX3CL1, etc. in the proliferation of intimal CD11c+ cells after LPS-mediated RTM. Studies will investigate whether neutralizing antibodies will inhibit proliferation of intimal CD11c+ cells and prevent recovery following RTM. DT-mediated depletion of intimal CD11c+ cells as well as reciprocal BM transplantation will assess the source of these factors, i.e. intimal CD11c+ cells or endothelial cells.

Preliminary results suggest that IL-1β is an essential mediator of RTM and that IL-1β signaling is mediated through the IL-1r1 expressed by endothelial cells, suggesting an active role of endothelial cells in RTM. Therefore, we will investigate the hypothesis that the endothelium plays an important active role in the migration of intimal CD11c+ cells, and whether IL-1β may be a master switch in endothelial cells to initiate RTM. Future experiments will investigate the downstream signaling essential for RTM of intimal CD11c+ cells. The role of adhesion and junctional molecules such as JAM-A and C, CD31 (PECAM-1), and VE-Cadherin will be examined using gene expression, immunostaining and blocking antibody studies. Whether pharmacological
inhibitors and blocking antibodies against the ARNO/ARF6-mediated internalization of VE-Cadherin have an effect on RTM of intimal CD11c+ cells will be investigated. A more sensitive live imaging technique of the egress of intimal CD11c+ cells through endothelial cells junctions will be developed in order to visually assess the changes in endothelial junctional molecules during the RTM process and define the role they play in the process.

Preliminary ex vivo experiments, in which aortas from mice fed a cholesterol rich diet for one week were incubated with IL-1β for 12 hours, showed a rescue of RTM of CD11c+ foam cells (not shown). Future experiments will confirm this observation in vivo and explore whether IL-1β is able to rescue RTM during later stages of lesion progression (e.g. after 4, 8, 12, and 16 weeks of diet). Future studies will investigate the effects of hypercholesterolemia on IL-1β production and its downstream signaling in intimal CD11c+ cells and endothelial cells. An in vitro model with co-culture of endothelial cells and DCs will be developed. The ultimate goal is to be able to harness RTM to induce egress of lipid loaded intimal foam cells from the vessel wall, thus, decreasing the lesion burden as a preventative medicine in atherosclerosis.

Future studies will also investigate the origin(s) of intimal CD11c+ cells, whether they are derived from a monocytic or classical pre-DC origin. Parabiosis experiments with WT and Ubc-gfp+ mice can be used to determine the contribution of circulating versus resident precursors to the abundance of intimal CD11c+ cells. Parabiosis between WT and Ccr2−/− mice (or other knock out mouse models) will assess the contribution of circulating Ly6C+ monocytes (or other precursors) to steady-state intimal CD11c+ cell numbers. Lineage-tagging experiments in which the expression of a fluorescent protein (e.g. Tdtomato) can be induced at specific timepoints using tamoxifen will be used to determine the lifespan and derivation of intimal CD11c+ cells.
5.7 Summary and Conclusions

In summary, studies in this dissertation supported the hypothesis that CCR7 / CCL19-dependent RTM of CD11c+ myeloid cells protects the arterial intima from intracellular pathogens, but this process is inhibited during atherogenesis. LPS, *C. muridarum*, and other inflammatory stimuli induced two waves of RTM of intimal CD11c+ cells into the arterial lumen, each followed by a recovery phase mediated by proliferation of the remaining cells. RTM is dependent on multiple receptors and ligands such as CCL19 and its receptor CCR7, S1P and S1P receptors, IL-1β and IL-1r1, and others that will be investigated in the future. *C. muridarum* infects the arterial wall by hijacking the recruitment of circulating blood monocytes into the intima, and the second wave of CCR7 / CCL19 – dependent RTM clears the pathogen by transporting it out of the vessel wall inside egressing CD11c+ cells. Other mechanisms of pathogen clearance such as intracellular killing play a role in limiting the growth of *C. muridarum*, but are not the mechanism of clearance from the arterial intima. Hypercholesterolemia and lipid-loading associated with atherosclerosis inhibit RTM of intimal CD11c+ foam cells and the clearance of *C. muridarum* from the arterial intima, but this was not due to inhibition of CCR7 or CCL19 gene expression. Overall, we showed that infection of the normal artery wall is rare because of an efficient clearance mechanism through RTM, while infection was detected in atherosclerotic plaques because RTM is inhibited. The main objective and relevance of this dissertation is in studying the signaling intermediates and pathways that play an important role in RTM and investigating how lipid-loading interferes with their function. Once specific targets are identified, appropriate strategies could be developed to restore RTM of foam cells to transport accumulated lipids out of the artery wall. These could be used alongside with medicines that lower cholesterol levels to reduce lesion burden.
Chapter 6

References


197. Rong, J.X., et al. Elevating high-density lipoprotein cholesterol in apolipoprotein E-deficient mice remodels advanced atherosclerotic lesions by decreasing


