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INNATE IMMUNE MECHANISMS OF ATHEROSCLEROSIS

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Karolinska Institutet

Stockholm 2019

Published by Karolinska Institutet.

ISBN 978-91-7831-461-4
Printed by Eprint AB 2019

INNATE IMMUNE MECHANISMS OF ATHEROSCLEROSIS

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Now this is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning. (Winston Churchill)

To my family

ABSTRACT

Atherosclerosis is a multi-factorial immune mediated disease in arterial wall characterized by lipid driven inflammation through activation of the immune system. Chronic vascular inflammation is an important component that modulates atherosclerosis evolution and its complications. The interaction of innate immune activators from both host and environment with innate immune receptors has been considered as one of fundamental mechanisms accounting for the inflammatory responses that affect multiple pathogenic processes during atherosclerosis. The aim of the thesis is to improve our understanding of innate immune mechanisms in atherosclerosis. The objective of the thesis is to investigate the cellular mechanism of NOD1 and TRIM21, to identify the innate immune phenotype of intimal vascular smooth muscle cells (SMC) and to elucidate the activity and clinical relevance of inflammasome-IL-1 signaling in atherosclerosis.

Paper I addresses how the local NOD1 signaling in vascular wall contributes to atherosclerosis and vascular inflammation. We report that a phenotypically distinct subpopulation of VSMC imprinted by NOD1^{high}, a member of NOD-like receptor family, have unique functions in promoting vascular inflammation and lesion development.

Paper II reports the identification of a SMC subpopulation with typical innate immune features in human atherosclerosis lesion and rat neointimal lesion. Functional studies and numerical quantifications further establish that these SMCs as important source of arterial resident innate immune effector cells.

Paper III investigates inflammasome function and IL-1 generation in human atherosclerosis lesion. IL-1α/β production is a common feature of advanced lesion, and is linked with the regulation of multiple canonical and non-canonical inflammasome. Plaque IL-1β increases in complex plaques and in the patients with hyperlipidemia and no or low-dose statin therapy.

Paper IV elucidates the mechanisms of Trim21, an ubiquitin E3 ligase with potent regulatory function in innate immune responses, in the pathogenesis of atherosclerosis. TRIM21 deficiency drives the generation of non-pathogenic Th17 in a cell-intrinsic manner and leads to a more stable plaque phenotype with higher collagen content.

This thesis illustrates the involvement and regulation of different modules in innate immunity in the pathogenesis of atherosclerosis. These notions may provide novel understandings in the inflammatory hypothesis of atherosclerosis and lead to new therapeutic strategies.

LIST OF SCIENTIFIC PAPERS

Zhang X*, Johansson ME*, Jiang X*, Ding Y, Gisterå A, Religa P, Hansson GK, Yan ZQ.
Implication of NOD1-high smooth muscle cell in vascular inflammation and injury.
(Manuscript)

Jiang X, Hedin U, Halle M, Hansson GK, Yan ZQ.
Evidence for a subtype of human vascular smooth muscle cell as arterial resident innate immune cell.
(Manuscript)

Jiang X*, Wang F*, Wang Y, Roy J, Paulsson-Berne G, Hedin U, Lerman A, Hansson GK, Herrmann J§, and Yan ZQ§.
Inflammasome-driven interleukin-1α and -1β production in atherosclerotic plaques relates to hyperlipidemia and plaque complexity.
(Manuscript accepted by *JACC Basic Transl Sci*)

Brauner S, Jiang X, Thorlacius GE, Lundberg AM, Östberg T, Yan ZQ, Kuchroo VK, Hansson GK, Wahren-Herlenius M.
Augmented Th17 differentiation in Trim21 deficiency promotes a stable phenotype of atherosclerotic plaques with high collagen content.
Cardiovasc Res. 2018 Jan 1;114(1):158-167

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Other publications not included in the thesis:

Wang F, Zhang S, Jeon R, Vuckovic I, Jiang X, Lerman A, Folmes CD, Dzeja PD, Herrmann J. **Interferon Gamma Induces Reversible Metabolic Reprogramming of M1 Macrophages to Sustain Cell Viability and Pro-Inflammatory Activity.** *EBioMedicine*. 2018 Apr;30:303-316. doi: 10.1016/j.ebiom.2018.02.009.

Laguna-Fernandez A, Checa A, Carracedo M, Artiach G, Petri MH, Baumgartner R, Forteza MJ, Jiang X, Andonova T, Walker ME, Dalli J, Arnardottir H, Gisterå A, Thul S, Wheelock CE, Paulsson-Berne G, Ketelhuth DFJ, Hansson GK, Bäck M. **ERV1/ChemR23 Signaling Protects Against Atherosclerosis by Modifying Oxidized Low-Density Lipoprotein Uptake and Phagocytosis in Macrophages.** *Circulation*. 2018 Oct 16;138(16):1693-1705. doi: 10.1161/CIRCULATIONAHA.117.032801.

CONTENTS

LIST OF ABBREVIATIONS

ACS	Acute coronary syndrome
AIM2	Absent in melanoma 2
Apo	Apolipoprotein
ASC (PYCARD)	Apoptosis-associated speck-like protein containing A CARD (PYD And CARD domain-containing protein)
CARD	The caspase activation and recruitment domain
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
CRP	C-reactive protein
CVD	Cardiovascular disease
CXCL	C-X-C motif ligand
DAMP	Damage-associated molecular pattern
DAP	g-D-glutamyl-meso-diaminopimelic acid
ERK	Extracellular signal-regulated protein kinase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HIF	Hypoxia inducible factor
HSP	Heat shock protein
IFN	Interferon
Ig	Immunoglobulin
IκB	Inhibitor of NF-κB
IL	Interleukin
ILCs	Innate lymphoid cells
INOS (NOS2)	Inducible nitric oxide synthase (Nitric oxide synthase 2)
IRF	Interferon regulatory factor
JNK	c-Jun N-terminal kinase
KLF	Krüppel-like factor
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase

MCP	Monocyte chemoattractant protein
MDP	Muramyl dipeptide
MMP	Matrix metalloproteinase
MyD88	Myeloid differentiation primary-response protein 88
NETs	Neutrophil extracellular traps
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	NOD-like receptor or Nucleotide-binding domain, leucine-rich repeat containing protein
NLRC4	NLR family CARD domain containing 4
NLRP3	NLR family Pyrin domain containing 3
NO	Nitric oxide
oxLDL	Oxidized low-density lipoprotein
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PRR	Pattern recognition receptor
P2RX7	Purinergic receptor P2X7
RIP2	Receptor interacting protein 2
ROS	Reactive oxygen species
SM-α-actin (SMA)	smooth muscle cell α-actin
SR	Scavenger receptor
STAT	Signal transducer and activator of transcription
TG	Triglyceride
TGF	Transforming growth factor
Th cells	T helper cells
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRIM21	Tripartite motif containing 21
VSMC	Vascular smooth muscle cells
VCAM-1	Vascular cell adhesion molecule 1

1 INTRODUCTION

1.1 THE ADVANCES IN UNDERSTANDING OF ATHEROSCLEROSIS PATHOGENESIS

Atherosclerosis is the underlying cause of cardiovascular diseases (CVDs), including ischemic heart disease and stroke, the leading cause of death worldwide[1]. The development of atherosclerosis has been linked with one or more risk factors, such as age, obesity, hypertension, diabetes mellitus, tobacco use, genetic background, and in particular, high plasma concentrations of low-density lipoprotein (LDL)[2, 3].

The understanding on the pathogenesis of atherosclerosis has over the years been improved from being a lipid accumulation disorder to a chronic inflammatory disease, characterized by a sustained activation of immune responses that triggered by so called ‘‘danger signals’’ developed locally in the lesion along with atherosclerosis progression[4, 5]. The inflammatory hypothesis was first proposed in 1994 on the basis of circulating acute-phase reactants C-reactive protein (CRP) in the prediction of acute coronary syndrome (ACS)[6]. Nonetheless, this hypothesis was yet validated until the very recent results from the Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS) were published. It shows for the first time a significantly lower rate of recurrent cardiovascular events by targeting the interleukin-1β (IL-1β) innate immunity pathway, independent of lipid-level lowering[7]. Thus, inflammation itself as an independent causative pathogenesis in human atherosclerosis has been finally established.

Atherosclerosis develops in the sub-endothelial area of large and medium sized artery and more frequently at the vessel bifurcation region with disturbed blood flow[2], initiated by endothelial dysfunction and followed by sub-endothelial lipoprotein retention[2]. Activated endothelial promotes the entry of circulating monocytes and other leukocytes into the intima by up-regulating adhesive molecule expression and chemokine secretion. Infiltrated monocytes differentiate into macrophages and undergo a transformation into foam cells after a continuous engulfment of lipoproteins, which builds up the lipid-laden plaques. In parallel, accumulated lipoproteins are subjected to complex modifications on both lipid and protein components, partially owing to oxidations and protease and lipase mediated reactions in the lesion. This process transforms lipoproteins, leading to generation of “danger signals” that elicit a series of inflammatory response and immune activation, which involves both innate and adaptive immune system. In addition, vascular smooth muscle cells (VSMCs) also

response to the vessel injuries and inflammation by migrating to the intima and contributing to the fibrous cap formation[8, 9].

As a consequence of the chronic maladaptive inflammatory response with impaired resolution, atherosclerosis lesion is characterized by severe necrosis and plaque destruction, and eventually causes arterial thrombosis and end-organ ischemia[10]. The pathological features of clinical vulnerable atherosclerosis plaques comprise thinning of fibrous cap, large necrotic area and severe inflammation, which displaying a high risk of plaque rupture and fissure formation[11]. Additionally, acute thrombotic vascular events also occur in plaques with superficial erosion, characterized by endothelial detachment and neutrophil and platelet activation[12, 13]. Of note, due to the intensive lipid-lowering treatment, a decreased occurrence of plaque rupture caused acute thrombotic vascular events has been achieved. On the other hand, plaque erosion has been accounted for causing more than 30% of total ACS, which contributes considerably to the residual burden of risk[13, 14].

1.1.1 The innate immune system and atherosclerosis

In general, the arteries can be seen as an integral components of the immune system[15]. Innate immunity, first line of host defense and capable to mobilize in minutes[2], plays important roles in both vessel homeostasis and the pathogenesis of atherosclerotic development. Innate immune regulation starts from endothelial activation characterized by adhesion molecule expression and chemokine secretion, followed by immune cell recruitment and complex cellular interactions in atherosclerotic lesion[2, 16]. A great number of studies have shown that disruption of key innate immune components can considerably influence atherosclerosis burden[17].

In atherosclerosis lesion, molecular patterns from both host and exogenous sources are shown to promote lesion development. Accumulation of modified LDL promotes the inflammatory response throughout lesion development[16]. On the other hand, pathogenic infection and tissue injury mediated danger signals have also been implicated as inducers of lesion inflammation[16].

Several different cell types are implicated in the innate immune response in atherosclerosis, most importantly those of the mononuclear phagocyte lineage such as macrophages[15]. Activation of the innate immune cells relies on the expression of a group of innate immune receptors, which comprises scavenger receptors (SRs), Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) etc[16]. The receptors recognize various pathogen-associated molecular patterns (PAMPs) from exogenous invaders

and host-derived damage-associated molecular patterns (DAMPs) that are formed at sites of sterile inflammation[16]. So far, compelling evidence has shown the innate immune recognition and activation play pivotal roles in the development of the disease[18-20].

1.1.2 The adaptive immune system and atherosclerosis

Compared to innate immunity, adaptive immunity is characterized by its exquisite specificity and long-lasting memory that favours recognition of millions of different molecular structures. As the main constituents of adaptive immune system, T and B cells are essential participators in atherogenesis and plaque stability. The adaptive immune response targeting auto-antigenic components derived from LDL particles orchestrate plaque development together with innate immune response. The specificity of adaptive immune recognition relies on the expression of T-cell receptor and the B-cell receptor, which is generated by germ-line rearrangement that governed by key enzymes including recombination-activating genes (RAGs)[5, 9].

T cells are present in all stages of disease in mice and humans, whereas B cells are occasionally found in plaques but more in the adventitial layer of the arterial wall[5, 21-23]. Global deficiency of T and B cells leads to a substantial reduced atherosclerosis, which has been shown in hypercholesteraemic mice lacking RAG1 or RAG2, two essential enzymes for T and B cell maturation[24, 25]. However, dissecting the roles of different subsets of T and B cells in atherosclerosis suggests a diverse and complex network.

$CD4^+$ T helper cells (Th cells) are the main adaptive immune cells in atherosclerosis and different Th cell subsets have substantial distinct roles in disease development[26]. As the major Th subset in atherosclerosis plaque, Th1 cells have been well demonstrated as a proatherogenic population. Indeed, mice lacking T-bet (key transcription factor for Th1 differentiation) or IFN-γ (signature cytokine for Th1) displayed remarkable reduced atherosclerosis[27-29]. On the contrary, regulatory T cells (Tregs) display athero-protective roles which partially mediated by cytokines like IL-10 and TGFβ[30-32]. In addition, conflicting results on the role of Th17 cells and its signature cytokine IL-17 have been shown in disease development and plaque stability[33]. Other T cell subpopulations have also been described in atherosclerosis, including Th2, $CD8^+$ T cell, TCRγδ+ T cell and NKT cell. However, the functions and importance of these minor subsets in atherosclerosis is still ambiguous[5, 9].

Similar to T cells, opposing roles from different B cell subsets have been described in atherosclerosis[5, 34]. Conventional B2 cells are suggested to be proatherogenic, as an attenuated atherosclerosis formation was observed after B2 cells depletion by anti-CD20

antibody (rituximab) in hyperlipidaemia mice[35]. By contrast, B1 cells with the germline-encoded natural antibodies and a newly described regulatory B-cell subtype show protective effect against atherosclerosis[36, 37]. However, mechanistic clarification of how these cells influence atherogenesis is still uncompleted.

1.2 INNATE IMMUNE MECHANISMS IN ATHEROSCLEROSIS

1.2.1 Innate immune activators

1.2.1.1 Endogenous innate immune activators

In the context of atherosclerosis, plaque-accumulated LDL has been considered to constitute the primary endogenous innate immune activator in terms of both quantity and immune activity and is considered a relative causal risk factor according to the modified Koch's postulates[1, 38]. LDL levels are correlated with augmented individual susceptibility to atherosclerosis and its complications[1]. Several interventions that lowering LDL levels by independent mechanisms diminish the incidence of cardiovascular events[1].

However, although LDL has an indispensible role in plaque formation and development, the mechanistic links between high LDL level and atherogenesis remains elusive. Despite oxidized LDL (oxLDL) and its early form induced by mild oxidation, minimally modified LDL (mmLDL) have been shown to trigger a cellular response in macrophages[39], key question remains on identification of the bioactive component of LDL particles that triggers innate immune activation in atherosclerosis. Recent studies by Ketelhuth et al. identified a distinct native peptide derived from apolipoprotein B100 that displays sequence-specific proinflammatory bioactivity[40]. This study provided new mechanistic insights to better understand the disease-promoting effects of LDL in the pathogenesis of atherosclerosis.

Cholesterol crystals have also been demonstrated to be a host derived danger signal, and their accumulation in the arterial wall is considered as an early cause of inflammation in atherosclerosis, rather than a consequence[41,42]. Mechanistically, cholesterol crystals employ the complement system to activate NLR family pyrin domain-containing 3 (NLRP3)-inflammasome and result in mature IL-1β production[43].

In addition, phospholipids, another type of essential component in the LDL particle, also play important roles in atherogenesis. Phosphocholine (PC)-containing oxidized phospholipids (OxPL) have been shown to be highly immunogenic and pro-inflammatory and are found in

atherosclerosis lesions in humans, particularly in vulnerable and disrupted plaques[44]. A recent study also suggested that elevated OxPL levels can be used as a biomarker for predicting cardiovascular outcomes in patients with established stable coronary heart disease[45].

Moreover, endogenous danger signals can also originate from the products of lesional inflammation. Heat shock proteins (HSPs) generated from cell necrosis trigger the production of proinflammatory cytokines and is mediated by TLR2 and TLR4 signaling[46]. High-mobility group box 1 (HMGB1) produced by macrophages and vascular cells or released through passive diffusion from surrounding necrotic cells has been shown to promote a local inflammatory response through activation of TLR2 and TLR4[47].

Nevertheless, recent discoveries regarding neutrophil extracellular traps (NETs) as a novel host-derived pro-atherogenic danger signal. NETs are net-like chromatin fibers that are released from dying neutrophils and comprise nuclear chromatin in association with nuclear histones and granular antimicrobial proteins[48]. During activation, the release of NETs is triggered along with reactive oxygen species (ROS) production[49]. The presence of NETs in the luminal portion of atherosclerotic lesions has been reported in both human and mice[50, 51]. A recent study by Warnatsch et al. showed that NET-deficient mice exhibited a reduction in atherosclerotic lesion growth with dampened IL-1 production. Mechanistically, the release of NETs triggered by cholesterol crystals can function as a danger signal for priming IL-1 transcription in macrophages, activating Th-17 cells and amplifying immune cell recruitment in atherosclerotic plaques[52].

1.2.1.2 Exogenous innate immune activators

A number of pathogens have been reported in epidemiological studies to be associated with an elevated risk of atherosclerosis, including Chlamydia pneumoniae (C.pn), Helicobacter pylori, and Porphyromonas gingivalis[53, 54]. In addition, previous studies have shown the presence of pathogen derived materials in human atherosclerotic plaque, suggesting a direct etiological role[55]. A pro-atherogenic effect of C.pn infection in *ApoE*-/- mice has been shown and mainly mediated via a TLR2/TLR4-driven pathway[56]. Recent study also discovered that NLRP3 inflammasome-IL-1 signaling was highly involved in C.pn-induced atherosclerosis by dampening cholesterol efflux[57]. Nevertheless, during acute infections, CVD events including myocardial infarction and stroke are increased[58]. However, a direct causative effect rather than an immune-mediated systemic inflammation for the suggested pathogens within the lesion still needs to be established[16]. In summary, despite its strong association with disease occurrence, pathogen infection cannot be confirmed to be the causative agent or the

specific pathogenesis of atherosclerosis, and the contribution of infection to atherosclerosis remains to be defined.

In addition, recent studies suggested that intestinal microbiota is a key component in the pathogenesis of metabolic disorders and cardiovascular diseases. Gut microbiota is considered as an endocrine organ and interacts with the host through many pathways. Specifically, trimethylamine *N*–oxide (TMAO), a oxidation product of the microbial metabolite trimethylamine TMA, is shown to be a potential promoter of atherosclerosis and cardiometabolic diseases[59].

On the other hand, metabolism-independent processes of intestinal microbiota have also been implied to contribute to cardiovascular disease. In particular, impaired intestinal barrier function is considered to contribute to bacterial translocation. Thus, the invasion of bacterial products (e.g., lipopolysaccharide and peptidoglycans) in the systemic circulation and local tissue stimulates and instructs the host immune response though innate immune receptors, thus heightening the inflammatory state in atherosclerosis[60]. Alteration of the gut microbial composition in atherosclerosis patients has been shown by Karlsson et al. using metagenomic sequencing, indicating a microbiome featured by producing proinflammatory peptidoglycans rather than anti-inflammatory carotenes[61]. Modulation of gut microbiota through diet, prebiotic and probiotic use and transplantation may favor the host metabolic profile in a desired direction[60].

1.2.2 Innate immune receptors

1.2.2.1 The orchestrated PRR network

A wide range of pathogen recognition receptors (PRRs) expression profiles has been observed in human atherosclerotic lesions[62]. Moreover, atherosclerotic plaque contains a mixture of various PRR ligands as described above. Therefore, it is likely that multiple innate immune pathways are activated either simultaneously or alternatively along with the disease progression[16].

Two principal classes of PRRs have been proposed to function in atherosclerosis: endocytic receptors and signaling receptors. Endocytic receptors include scavenger receptors (SRs), C-type lectins, and opsonic receptors, which are responsible for the metabolism of lipoprotein, the clearance of apoptotic cells, the elimination of pathogens, and antigen uptake and presentation[63]. The signaling receptors consist of TLRs and NLRs that are critical for the activation of proinflammatory pathways upon infection. TLRs are membrane-anchored and

consist of a leucine-rich repeats domain and a Toll/IL-1R homology domain, which are responsible for ligand sensing and signaling transduction respectively. NLRs are a family of cytoplasmic PRRs and have a NATCH domain responsible for self-oligomerization in addition to leucine-rich repeats domain and effector domain[63]. An indispensible basis of TLR and NLR signaling depends on the recruitment of several adaptors. Adaptor-like myeloid differentiation factor 88 (MyD88) and TIR-domain-containing adaptor protein inducing IFN-β (TRIF) are the adaptor proteins for most TLRs, whereas receptor-interacting serine-threonine kinase 2 (RIP2) for NOD1 and NOD2. Once activated, these adaptor molecules relay the downstream kinases and transcription factors to modulate inflammatory response[64].

As a complex milieu, multiple innate immune mechanisms are in play simultaneously or sequentially in atherosclerotic lesions, which inevitably lead to a crosstalk between different signaling pathways. This likely determines the specificity of innate immune response in atherosclerosis. For instance, CD36 and lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) function as co-receptors with TLR2 and regulate inflammatory response[65, 66]. In return, activation of TLR3, TLR4, and TLR9 in macrophages induce the scavenger receptors expression thus leading to an increased phagocytosis[67].

On the other hand, the activation of TLRs or NLRs can trigger shared signaling cascades, such as MyD88 or RIP2, and result in NFκB activation, which increases the levels of transcription mediators such as pro-IL-1β and pro-IL-18[16]. Nonetheless, bioactive form of these cytokines requires processing by NLRP3 inflammasome activation, which can be considered a second signal (Figure 1). With these two signals, these potent proinflammatory cytokines can induce inflammation, cause tissue damage, enhance cascade signaling and play an important role in the pathogenesis of atherosclerosis[68, 69]. As a consequence, the loop effect contributes to an irreversible and chronic inflammation lesion.

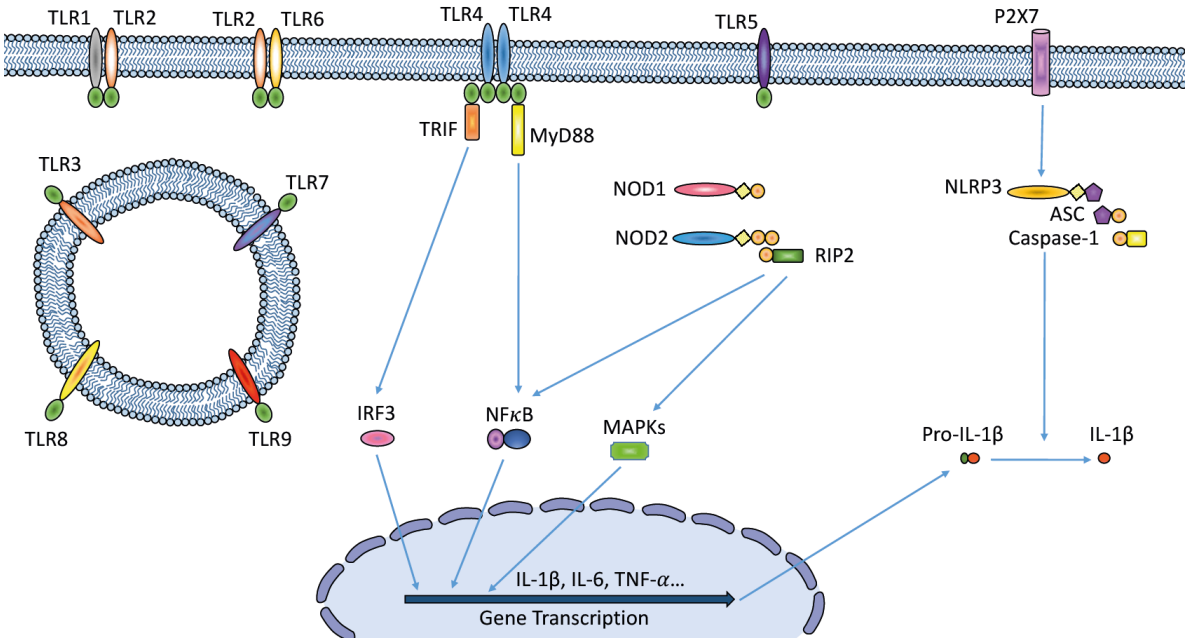

Figure 1. TLR and NLR signaling pathways. Activation of TLRs (e.g., TLR4) and NLRs (e.g., NOD2) facilitates the recruitment of adaptor protein MyD88/TRIF and RIP2, respectively. The activated MyD88, TRIF and RIP2 pathways lead to the activation of NF-κB, IRF3 and MAPKs pathways, which drives the production of inflammatory mediators. Furthermore, activation of NLRP3 triggers the forming of inflammsomes, which is essential for IL-1β maturation.

1.2.2.2 Toll-like receptors

So far, 10 human TLRs and 13 mouse TLRs have been characterized[19]. They are capable of recognizing a wide spectrum of microbial and host-derived danger signals. The location of most TLRs is found on the cell surface, however TLR3 and TLR7-9 are positioned in intracellular compartments[19]. As the earliest and widely investigated TLRs in atherosclerosis, TLR2 and TLR4 have been shown to be pro-atherosclerotic in hyperlipidemia mice in the absence of exogenous stimulation[70,71]. TLR4 deficient *ApoE*-/- mice showed attenuated atherosclerosis lesion and macrophage content after high fat diet[70]. Similarly, *TLR2* deficiency in *Ldlr*-/- mice on high-fat diet displayed reduced lesion size and decreased inflammatory cytokine levels[71]. Recent investigations on the roles of the remaining TLRs in atherosclerosis demonstrate the diversity and complexity of this receptor family.

TLR3

TLR3 is carried by both myeloid cells such as macrophages and dendritic cells and vascular cells such as SMCs and recognizes viral double-stranded RNA (dsRNA)[72]. Both pro- and anti-atherogenic roles have been characterized for TLR3 in atherosclerosis. The observation by Cole et al. that TLR3 deficiency accelerated the onset of atherosclerosis in *ApoE*-/- mice

suggested a protective role of TLR3 in early atherosclerosis (at 15 weeks of age) but not in advanced atherosclerosis (at 30 weeks of age)[72]. In addition, another study by Ishibashi et al. using *Tlr3*-/-*Ldlr*-/- mice showed no change in aortic root lesion area compared to *Ldlr*-/- mice, despite a significant increase in the cap thickness with higher collagen and SMC content, together with the suppression of MMP-2/9 activity[73]. Using a bone-marrow transplantation strategy in *Ldlr*-/-, Lundberg et al. demonstrated that deleting TLR3 or its essential signaling adaptors in immune cells significantly reduced both aortic inflammation and atherosclerotic burden[74]. Taken together, the role of TLR3 is prone to be pro-atherogenic. However, the TLR3-activating ligands in human atherosclerotic lesions remain to be identified. The presence of cell death material such as host-derived RNA in the necrotic core makes this a plausible means of TLR3 activation within the lesion.

TLR5

TLR5 is a cell surface receptor for bacterial flagellin and is ubiquitously expressed. Although there is a enrichment in TLR5 expression in human carotid plaque compared to normal arteries[62], the functional relevance of TLR5 to atherosclerosis has remained unknown until recently. A study by Ellenbroek et al. suggested that TLR5 deficiency in the myeloid cells limited atherogenesis due to decreased macrophage accumulation, reduced necrotic core and impaired T-cell responsiveness[75]. Kim et al. further demonstrated that activation of the TLR5-Nox4 cascade contributes to atherogenesis development. Challenge of recombinant FliC (rFliC) in *ApoE*-/- mice showed a marked increase in atherosclerosis, which is NOX4 dependent[76].Based on both loss of function and gain of function studies, the role of TLR5 in atherosclerosis seems to be consistently deleterious.

TLR7

However, a deleterious role of TLR activation in atherosclerosis does not apply to all the members in this family. TLR7, another endosomal receptor, recognizes single-stranded RNA from both viral and host. It is also a ubiquitously expressed receptor carried by macrophages, T cells and capillary endothelial cells within human advanced carotid atherosclerotic lesions[77]. The role of TLR7 in atherosclerosis has been described as protective[78]. TLR7 deficiency in *ApoE*-/- mice showed increased atherosclerosis lesion in comparison to the control mice. This has been attributed to a TLR7-related constraining of inflammatory macrophage activation and cytokine production[78]. A recent study on TLR7 in human atherosclerotic plaques demonstrated an association of high TLR7 expression with better clinical outcome. Increased TLR7 transcript in the plaque from patients who underwent

carotid endarterectomy was linked with fewer adverse cardiovascular events[77]. In addition, the study further strengthened the function of TLR7 in atheroprotective cytokine IL-10 production, presumably through alternative macrophages (M2) and T cells[77]. However, the underlying mechanism needs further investigation.

TLR9

TLR9, another endosomal TLR, recognizes the CpG motif in both bacterial and host DNA[79]. This receptor is expressed by various cell types, including macrophages, B-cells, and plasmacytoid dendritic cells (pDCs)[79, 80]. Like TLR3, TLR9 has been described as causing both pro- and anti-atherosclerotic effects. An athero-protective role for TLR9 has been shown in both *Tlr9*-/-*ApoE*-/- mice and TLR9 agonist type B CpG oligodeoxynucleotide (CpG ODN)-treated *ApoE*-/- mice, in which CD4+ T cells were identified as potential mediators[81]. However, in another study using a higher dose CpG ODN as a TLR9 agonist in *ApoE*-/- mice, impaired re-endothelialization upon acute vascular injury and increased atherosclerotic plaque development were observed[82]. Moreover, a recent study also showed TLR9 deficiency attenuated atherogenesis in an angiotensin II-infused mouse model[83]. In addition, HMGB1, a potential endogenous risk factor for atherosclerosis[84], has been implicated in lesion development in a TLR9 dependent pathway under vascular injury[85]. The discrepancies between these different models might largely be due to the diverse and complex roles of TLR9 in various cell populations in atherosclerosis.

1.2.2.3 NOD-like receptors

So far, more than 20 NLR genes have been found in humans. NLRs can be categorized generally into two major subgroups on the basis of the N-terminal effector domain, including NLR family caspase recruitment domain (CARD)-containing (NLRC) group and pyrin domain-containing (NLRP) group[19]. One of the most distinct features of NLRP is the assembly of inflammasome, a multi-protein complex formed in response to stimulation[19, 86]. Compared to TLRs, the roles of NLRs are less investigated in atherosclerosis. However, several recent studies have demonstrated a critical role for NLRs in lesion development.

NOD1

NOD1 is a cytosolic innate immune receptor that mediates bacterial peptidoglycan-induced immune activation. NOD1 expression has been found in a wide array of cell types, including arterial endothelial cells and VSMCs[87, 88]. NOD1 recognizes D-glutamyl-meso-diaminopimelic acid (meso-DAP), a dipeptide that occurs in the peptidoglycans of many

Gram-negative bacteria and certain Gram-positive bacteria[89, 90]. Kanno et al. have shown a pro-atherogenic role of NOD1 in the oral administration of a synthetic NOD1 ligand FK565 in *ApoE*-/- mice[91]. Moreover, NOD1 has also been demonstrated to play a pivotal role in site-specific vascular inflammation, particularly coronary arteritis and valvulitis[88]. Mechanistically, endothelial NOD1 signaling promotes the recruitment of cardiac $CD11c^+$ macrophages via VCAM-1 up-regulation, which exacerbates the inflamed micro- and macrovasculature[92, 93]. However, the cellular basis of NOD1 signaling in the context of vascular inflammation remains to be elucidated.

NOD2

Unlike NOD1, NOD2 is preferentially found in myeloid immune cells, intestinal epithelial cells and Paneth cells[94]. NOD2 recognizes muramyl dipeptide (MDP), a bioactive motif of peptidoglycans that occurs in both gram-positive and gram-negative bacteria[94]. Alterations in NOD2 immune function drive a higher incidence of autoimmune diseases such as Crohn's disease[95, 96].

A pro-atherogenic effect of NOD2 immunity was suggested on the basis that human culprit atherosclerotic plaques with enhanced inflammatory responses were enriched with NOD2 ligands[97] and was further supported by studies in experimental models of atherosclerosis, which demonstrated that the ligation of NOD2 in vivo with its ligand aggravates atherosclerosis, as indicated by increased lesion size with enlarged lipid-rich necrotic cores and heightened vascular inflammatory responses. These pro-atherogenic effects are dependent on NOD2 immunity carried by myeloid immune cells because the myeloid-specific ablation of NOD2 restrains the expansion of the lipid-rich necrotic core[98]. Moreover, in human carotid plaques, NOD2 activation unregulated inflammatory lipid pathways, preferentially the COX-prostaglandin E2 pathway[99], and triggered IL-1β production[98], thus confirming the functional relevance of NOD2 in human atherosclerosis.

On the other hand, NOD2 immunity reportedly provides protection against *P*. gingivalis-induced atherosclerosis in *ApoE*-/- mice[100]. The discrepancy of NOD2 function in atherosclerosis between this study and others leads to the caveat that the role of innate immunity in atherosclerosis may change in accordance with environmental cues.

NLRP3

Another important member in the NLR family is inflammasome, a multimeric protein complex that senses not only microbial infection but also a wide array of host-derived danger

signals and is essential for inflammatory cytokine production[101, 102]. As the most-studied inflammasome, NLRP3 inflammasome induces the assembly of apoptosis-associated speck-like protein containing a CARD (ASC), then trigger the cleavage of pro-caspase 1[101, 102]. The cleaved caspase-1 promotes the maturation and secretion of IL-1β and IL-18[101, 102].

The NLRP3 inflammasome was first identified as a critical component of the inflammatory process that causes the inherited Muckle–Wells syndrome (MWS) and familial cold autoinflammatory syndrome[103]. The NLRP3 inflammasome is also implicated in several other autoinflammatory diseases including gout, Alzheimer's disease, T2 diabetes, and atherosclerosis[103]. Cholesterol crystals and oxidized LDLs, which are commonly deposited with atherosclerotic plaques, have been reported to be endogenous danger signals that trigger NLRP3 inflammasomes activation in macrophages[104,105].

Current studies propose several models for the activation of the NLRP3 inflammasome. First, extracellular ATP induces the caspase 1-dependent release of IL-1β through the activation of P2X7 receptor[106], subsequently activating potassium efflux. High concentrations of potassium in the cell culture medium have been shown to inhibit potassium efflux and thus suppress inflammasome activation[107, 108]. Second, for crystalline materials and peptide aggregates such as silica and crystalline or amyloid-β, phagocytosis is needed for inflammasome activation since the secretion of IL-1β is suppressed by cytochalasin D, which is an inhibitor of phagocytosis. Upon uptake, lysosomal membrane integrity is disrupted, resulting in the leakage of lysosomal proteases into the cytosol where they activate the NLRP3 inflammasomes[109]. Third, the dysfunction of mitochondria and the generation of ROS by the mitochondrial respiratory chain have been shown to participate in inflammasome activation[110]. Takeshi Ichinohe et al. showed that the mitochondrial protein mitofusin 2 plays essential roles in RNA virus infection triggered NLRP3 activation[111]. Fourth, a recent discovery shows that cAMP acts as a key molecular regulator that binds to NLRP3 and promotes its ubiquitination and degradation, thus dampening its activation[112].

However, the importance of the NLRP3 inflammasome in atherosclerosis has been challenged by its controversial role in animal models. *Ldlr*-/- mice with NLRP3-deficient bone marrow exhibit a 69% reduction in atherosclerotic lesion size compared to wild-type bone marrow recipients[41]. Moreover, a recent study showed that silencing NLRP3 in *ApoE*-/- mice effectively decreased lipid and macrophage contents in atherosclerotic lesions while increasing smooth muscle cell and collagen contents, thus increasing the stability of atherosclerotic lesions[113]. In contrast, another study in *ApoE*-/- mice crossing *Nlrp3*-/-, *Asc*-/-, or *Caspase1*-/- showed mild differences between NLRP3 inflammasome component-deleted

mice and control mice[114]. A possible explanation is that NLRP3 inflammasome priming or even activation requires extra damage in the vasculature, such as radioactive damage in the bone marrow transplantation model or viral infection in the NLRP3-silencing model, respectively. In comparison, the endogenous danger signals in low-grade chronic inflammatory processes such as atherosclerosis might be insufficient for NLRP3 inflammasome activation. Thus, the discrepancies between these mouse models await a direct clarification of NLRP3 inflammasome function in human atherosclerosis.

1.2.3 Innate immune cells

A key pathology of atherosclerosis lesion is featured by the persistence of inflammation and different innate immune cell populations including macrophage, mast cell, neutrophil and natural killer cell[18]. These innate immune effector cells orchestrate the inflammatory responses and contribute to plaque evolution[115, 116]. In addition to the classical hematopoietic innate immune cell, vascular cells (endothelial cells and VSMCs) also respond to both exogenous and host derived innate immune activators, indicative of innate immune capability[117].

1.2.3.1 Macrophages and monocytes

Lesional macrophage is considered to be primarily derived from circulating monocyte[118]. Monocytes adhere and infiltrate through the activated endothelium, differentiate into macrophages in the sub-endothelial space[118]. On the other hand, local proliferation of resident macrophage proliferation is likely an alternative mechanism accounting for macrophage accumulation in atherosclerosis[119], adding a new dimension to our understanding of lesional leukokinetics. However, more mechanistic dissection is needed to understand the orchestration of monocyte recruitment and macrophage self-renewal at various stages of atherosclerosis.

The abundance of monocytes in the circulation is strongly associated with atherosclerotic vascular disease[3, 120, 121]. In *ApoE-/-* mice, continuous influx of monocytes is observed throughout all stages of atherogenesis, which is considerably governed by chemokines and the receptors like CCR2, CX3CR1 and CCR5[122]. Depletion of $CCR2^{+}Ly6C^{high}$ monocytes in *ApoE-/-* mice leads to a decreased plaque formation[123, 124, 125]. In addition, *ApoE-/-* mice lacking CX3CL1 or CX3CR1 also show attenuated atherosclerosis[126-128]. Thus, the recruitment of circulating monocytes into plaques is a key step for lesion development.

Once the monocytes into the lesion, a differentiation process into macrophages takes place rapidly in response to the stimuli in the microenvironment[124]. As the most studied innate immune cell in atherosclerosis lesion, macrophage has been considered as the signature cell and the major effector cell responsible for innate immunity mediated inflammation. Excess lipoprotein uptake and impaired exocytosis of lipids by macrophages is central in the development of foam cells. In addition, dysregulated lipid metabolism and the resulting endogenous danger signals that in plaques trigger PRRs, thereby activating the inflammatory response by secreting cytokines such as IL-1β and IL-12, chemokines including monocyte chemoattractant protein-1 (MCP-1) and chemokine (C-C motif) ligand (CCL) 2, proteases and other potent immune effectors such as nitric oxide and reactive oxygen species[3, 129]. A chronic inflammatory response then leads to a further expansion of specific cellular components as well as severe cell death, and promotes detrimental plaque morphological changes like necrosis core formation and fibrous cap thinning[3].

The diversity and plasticity of macrophage phenotype have been well recognized for a long time. Homeostatic imbalance in microenvironmental cues and the intracellular proinflammatory versus proresolving pathways affects macrophage phenotype and function considerably[118, 130]. Compared to the classical activated macrophages (M1) that served as the major inflammatory cells in atherosclerosis progression, several subsets of alternatively activated macrophages (M2, M(Hb), Mhem, Mox, M4) have been implicated in inflammation resolving and tissue repair[118, 130-132]. However, due to the remarkable plasticity and complicated regulatory network, lesional macrophages with different phenotypes may not be classified into predetermined subsets, but rather function on a spectrum from inflammatory to resolution and repair[118].

1.2.3.2 Neutrophils

Pleiotropic roles of neutrophils have been linked to plaque development in atherosclerosis. The proteolytic enzymes release, including myeloperoxidase (MPO), elastase and matrix metalloproteinase (MMP), contributes to tissue damage and plaque destabilization[133].

Neutrophils are detected in human atherosclerotic lesion, albeit in much lower numbers compared to macrophages[134, 135]. The presence of neutrophils in the plaque is shown to be associated to the features of plaque rupture and erosion[135, 136], which goes in line with a prognostic role of peripheral neutrophil counts in cardiovascular events[137, 138]. In *ApoE-/-* mice, neutrophils have been implicated in early atherosclerosis formation[139]. Moreover, Zernecke et al. showed that neutrophil depletion by anti-PMN antibody leads to a reduced

plaque formation in *ApoE-/-* mice[140]. Of note, recent study by Franck et al. showed the interplay between neutrophils and endothelial cells in regions of disturbed arterial flow as mechanisms that contribute to plaque erosion in *ApoE-/-* mice[136], which may serve as a potential therapeutic target in controlling plaque erosion caused CVD risk.

1.2.3.3 Mast cells

Mast cells are present in human atherosclerotic lesions at sites of plaque erosion, rupture or hemorrhage[2, 141-143]. Accumulating evidence establish the contribution of the mast cell to atherosclerosis plaque progression and destabilization, through its role in lipoprotein metabolism and inflammation[144, 145]. Specific proteases released from activated mast cells such as chymase and tryptase result in matrix degradation, thus have detrimental effects on the vessel wall. In addition, mast cells serve as a big source of growth factors, histamine and chemokines, thereby actively contributing to lesion development[146].

Indeed, lacking of mast cells in the mast cell-deficient mice (*Ldlr-/-Kit*$^{W\text{-}sh/W\text{-}sh}$) was linked with a reduction in atherosclerosis lesion, which was attributed to an attenuation in both hyperlipidaemia and vascular inflammation[145, 147]. Consistently, pharmacological stabilization of mast cells significantly attenuates atherogenesis and plaque destabilization in *Ldlr-/-* mice [88, 148].

1.2.3.4 Innate lymphoid cells

Innate lymphoid cells (ILCs) is a recently identified member in the lymphoid lineage, reveal important roles in host immunity, tissue homeostasis and inflammation[149, 150]. ILCs represent a heterogeneous population being composed of three subgroups: ILC1, ILC2 and ILC3. The classification of these 3 subgroups is mainly based on their capacity to produce a number of Th1, Th2 and Th17 cell-associated cytokines, which mirrors the adaptive Th responses respectively[151]. The classically defined natural killer cells (NK cells), a potent innate immune effector cell population, has recently been categorized into ILC1[152].

ILC populations rely on common key developmental signals for their maintenance, as through the cytokine receptor γ-chain (also known as IL-2Rγ), the transcriptional repressor inhibitor of DNA binding 2 (ID2) and interleukin-7 receptor subunit-α (IL-7Rα)[153-155]. An essential criterion is that they do not possess other immune cell lineage markers on the cell-surface, as negative for CD11c, CD14, CD3, TCRαβ, TCRγδ and CD19 etc[149, 151].

Although a global depletion of all the ILC populations showed no effect in atherosclerosis development in *Ldlr-/-Rag1-/-* mice, an expansion of ILCs successfully reduced

atherosclerosis in these mice[156]. Moreover, studies targeting specific ILC populations further suggest a diverse role of each cell subsets in atherosclerosis.

As a prototypical member of ILC1s, NK cells have been discovered in human and mouse atherosclerosis lesions[157, 158]. Although the function of NK cells in atherosclerosis remains controversial, an atherogenic role of these cells is shown from most of the studies[159, 158, 160, 161]. Recent study by Selathurai et al. showed that specific depletion of NK cells by adopting anti-Asialo-GM1 antibody reduced atherogenesis in *ApoE-/- mice.* On the other hand, reconstituting NK cells in *ApoE-/-Rag2-/-IL2rg-/-* mice that deficient in all lymphocytes augmented atherosclerosis[162]. However, the contribution of non-cytotoxic IFN-γ secreting ILC1s has not been addressed.

The role of ILC2s and ILC3s has been mainly studied in tissue homeostasis and inflammation at mucosal and barrier surfaces. Proinflammatory and tissue reparative functions of ILC2s have been reported in disorders like atopic dermatitis, Crohn's disease and chronic rhinosinusitis[152, 163-165]. Recently, studies investigating the role of ILC2 in hyperlipidaemia mice attracted big attention to these cells in atherosclerosis[156, 166]. A very recent study from Engelbertsen et al. showed a presence of ILC2s in para-aortic adipose tissue and lymph nodes in *ApoE-/-* mice[166]. Moreover, a selective ablation of ILC2s (*Staggerer/Ror*α^{Flox}*-Cd127*^{Cre} mice) in *Ldlr-/-* mice significantly accelerated atherosclerosis, which was mediated by IL-5 and IL-13 production[166]. These data indicate ILC2s as an important source of atheroprotective immunity. The presence and role of ILC3s in murine and human atherosclerotic lesions remains unclear.

1.3 VSMC IN ATHEROSCLEROSIS

1.3.1 VSMC heterogeneity

The mature VSMC is a highly specialized cell type whose major physiological function is contraction to maintain the vessel tone and homeostasis of blood flow. However, VSMC is not terminally differentiated and retains remarkable plasticity that allows rapid adaptations to fluctuating environmental cues[167], which differs them from skeletal and cardiac muscle cells [168].

During the latest two decades, increasing number of publications demonstrate that the presence of VSMC with distinct phenotypes in arteries of various species, including humans. Even though the origin remains debated, the presence of phenotypically heterogeneous

VSMC has been observed in the arterial wall that under both normal and atherosclerotic conditions[167, 169]. Numerous attempts have been made to isolate VSMC subpopulations with distinct phenotypes from both normal and disease arterial vessels, which established an even broader scope of VSMC heterogeneity.

1.3.1.1 Contractile and synthetic VSMC phenotype

The provocative paradigm depicts that during atheromatous plaque formation or restenosis after angioplasty, the complex network of growth factors, cytokines, chemokines and proteolytic agents generated in the lesion induces medial SMC migrating to the subendothelial space, where they proliferate and undergo a phenotypic switch from a contractile to a synthetic phenotype[170].

Contractile VSMCs (conventional VSMCs) are elongated or spindle-shaped, whereas synthetic VSMCs (unconventional VSMCs) have an epithelioid, rhomboid or cobblestone-like morphology. Synthetic VSMCs contain increased number of organelles involved in protein synthesis, in contrast to prevailing contractile filaments in contractile VSMCs. In particular, synthetic VSMCs have several distinguished features when compared with contractile SMCs: (1) high proliferative capability[171, 172]; (2) profound migratory activity[172, 173]; (3) enhanced proteolytic activity[172, 174]; (4) poorly differentiated[172, 173]; and (5) distinguished sensitivity to apoptotic stimuli[170] (Figure 2).

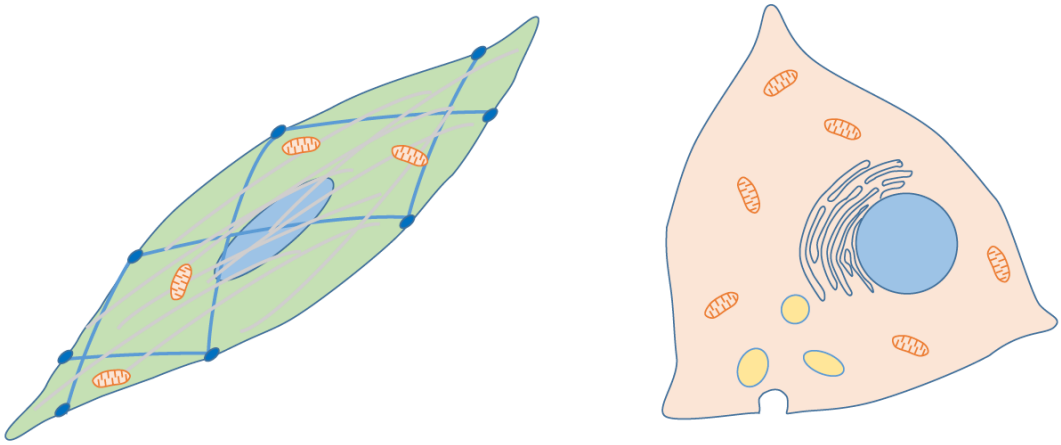

Figure 2. Characteristics of conventional and unconventional VSMCs.

The contractile and synthetic VSMCs represent the two extremes of a spectrum of VSMC phenotypes[175]. Numerous explorations have so far been carried out to define the markers of differential VSMC subtypes. Apart from classical contractile proteins like smooth muscle cell α-actin (SMA), myosin heavy chain 11 and SM22, etc., newly identified transforming growth factor-1-induced transcript 1(TGFB1I1) is a novel marker for the contractile phenotype of VSMC[176]. TGFB1I1 is tightly regulated by SRF/myocardin and is essential for maintaining contractile phenotype in SMCs[176]. On the other hand, S100A4, a low molecular weight calcium-binding proteins, was barely detectable in human coronary artery media but markedly expressed in VSMCs of atheromatous and restenosis coronary artery lesions[177]. Additionally, CRBP-1, Flt-1, c-kit, Calmodulin and Heparanase has also been defined as specific signature of VSMC synthetic phenotype under aging or pathological conditions[178, 179, 180, 181]. However, due to the phenotype plasticity and technical limitations, it is difficult to address the question in vivo whether these markers represent an intrinsic phenotype or a transient expression in response to the environmental stimulation.

1.3.1.2 VSMC contribute to foam cell formation in atherosclerosis

Unlike macrophages, the contribution of lesional SMCs to foam cell formation in human atherosclerosis has received inadequate attention until recently Allahverian et al showed about 50% of the total foam cells were VSMC derived in human coronary artery[182]. Similar result was also observed in advanced atherosclerosis lesion in *ApoE*-/- mice[183].

It is conceivable that under atheromatous conditions, VSMCs, one of the major cell populations, may develop or activate the lipid metabolism pathway and contribute to the total foam cell population. Although atherogenic lipoproteins uptake is mainly mediated by scavenger receptors typically seen in macrophages, the expression of scavenger receptors have also been found in VSMCs in both human and mouse atherosclerosis lesions[184, 185, 186]. On the other hand, intracellular cholesterol removal is mediated by ATP-binding cassette (ABC) transporters and scavenger receptor class B type I (SR-BI), which are lower expressed in human coronary artery intimal SMCs compared with medial SMCs[187]. These descriptive and comparative studies provide new insights that intimal SMCs serve as an important source of lesional foam cells, differing from the differentiated SMCs in the tunica media.

Furthermore, the consequences of the increase in VSMC derived foam cells leads to not only the enlargement of lesion size, but also an alteration of lesion microenvironment and plaque stability. Lipid loading of VSMCs is associated with acquirement of macrophage markers, pro-inflammatory cytokines[188-190], phagocytic properties[188], migration and calcification

properties[191] and impairment in collagen and fibronectin assembly[192]. Overall, these alternations synergistically increase the vulnerability of atherosclerotic plaques.

1.3.1.3 VSMC-macrophage trans-differentiation in atherosclerosis

Along with the re-evaluation of VSMC derived foam cells in atherosclerosis, studies by Allahverdian et al. also proposed that nearly 40% of $CD68^+$ cells co-expressed SMA in human coronary atherosclerosis lesion[182]. This has triggered intense discussions on the phenotypic transition between VSMCs and macrophages, and has brought a tricky question regarding the ambiguous origin for the cells in atherosclerosis lesion.

A trans-differentiation from VSMC to macrophage was suggested by using SM22α lineage tracing technique in *ApoE-/-* mice, which showed medial SMCs underwent clonal expansion, converted to MAC-2^+ and $CD68^+$ cells and lost classic SMC marker expression[193]. These in vivo observations are consistently with the in vitro observation showing that cholesterol loading of VSMC activates macrophage markers expression[188]. However, the functionality and pathological relevance of these VSMC-derived macrophage-like cells compared to classic monocyte-derived plaque macrophages require further studies.

On the other hand, in a human cross-gender bone marrow transplantation study, 10% SMA^+ cells in atherosclerosis lesion showed a myeloid origin[194]. Similar phenomenon was also observed in mice subjected to wire-induced arterial injury, which showed a fraction of bone marrow derived cells express SMA after migrated into the injured vessel wall. However, further lineage identifications demonstrated that these myeloid derived SMA^+ cells do not acquire a fully differentiated VSMC phenotype, which requires SM-MHC and calponin expression[195]. Moreover, multiple cell types other than SMC can also express SMA, which requires more comprehensive lineage identification. Thus, despite the trans-differentiation of VSMCs from bone marrow or peripheral mononuclear cells has been shown in vitro[196, 197], the in vivo evidence supporting a myeloid origin of VSMCs is still missing.

1.3.2 Role of VSMC in inflammation

1.3.2.1 Innate immune receptors expressed by VSMC

Some of TLRs and NLRs, which are the classical innate immune sensor, are also expressed by VSMCs and play functional roles in SMC biology. TLR2 is important for the recognition of bacterial lipotelchoic acid and lipoprotein, and to promote inflammation in VSMCs in *ApoE*-/- mice[198, 199]. Moreover, Lee et al. showed that MMP2 and pro-inflammatory cytokines can be produced in TLR2-Nox1-dependent manner in VSMCs, leading to increasing

monocyte-endothelial cell adhesion and trans-endothelial migration of monocytes[200]. In addition, TLR4 mediates LPS induced nitric oxide production in human aortic VSMCs[201]. However, a comprehensive characterization on the immune phenotype of VSMC is still lacking.

1.3.2.2 VSMC produce immune effective mediators

Cytokines, chemokines and other proinflammatory mediators are potent immune effectors in maintaining or commoving tissue homeostasis. Within the vessel wall, VSMCs can significantly contribute to the cytokine dependent inflammatory network[202].

A classic example is the discovery of inducible nitric oxide synthase (iNOS) expression and nitrite oxide (NO) production in VSMCs. Aside from the important roles in the physiological regulation of vascular tone and platelet aggregation and adhesion, NO serves as an important innate immune effector produced by iNOS in host defense. INOS, which was originally identified in cytokine-activated macrophages, can also be rapidly induced in VSMCs in vivo during the formation of the neointima in a rat balloon injury model. Moreover, intimal SMCs produced remarkably higher levels of NO than medial SMCs upon stimulation with IFNγ and LPS[203].

Recently, Kiyan et.al showed oxLDL impaired the expression of contractile proteins and myocardin in VSMC and promoted NFκB dependent colony-stimulating factors (G-CSF and GM-CSF) expression and other proinflammatory cytokines like IL-8 upregulation[204]. Zeiffer et.al also have identified a NFκB-mediated proinflammatory phenotype of neointimal SMCs that is characterized by increased P-selectin and chemokine expression and thereby effectively supports leukocyte recruitment[205]. In addition, VSMCs have shown the competence of producing TNF-α, IL-6, MMP2, MCP-1 and CX3CL-1, despite many of them are traditionally described as myeloid immune cells derived cytokines or chemokines[126, 206, 207, 208-210].

1.3.2.3 Crosstalk between VSMC and leucocytes

Lesional SMCs often reside in close proximity to invading macrophage and T cell clusters, constituting inflammation niches[211]. Interaction of SMCs with these leucocytes or activated endothelial cells may result in many subsequent enhancements of cellular responses, thereby perpetuating the atherosclerotic development. Ostriker et al. showed that SMC-derived transforming growth factor beta (TGF-β) modulates the phenotype of maturing macrophages in vitro[212]. In return, SMC-modulated macrophages can further promote SMC activation to a

greater extent compared to unmodulated macrophages[212]. Meanwhile, macrophage–SMC co-culture experiments demonstrated the macrophage differentiation in the presence of M-CSF produced by smooth muscle cells[213].

In addition, VSMCs have been shown to orchestrate the homeostasis of both innate and adaptive immunity in atherosclerosis through regulation of artery tertiary lymphoid organs (ATLOs) development[23]. ATLOs refer to the lymphoid aggregates with varying degrees of complexity ranging from lymphocyte clusters to well-structured unencapsulated lymph node-like tissues in the adventitia of the diseased arteries. In *ApoE-/-* mice, ATLOs are formed in the adventitia adjacent to atherosclerotic plaques during aging. ALTOs have been suggested as powerhouses of advanced atherosclerosis immunity as the size and structure of ATLOs is correlated with the disease severity[214]. On the other hand, ATLOs seemingly also can afford protection from advanced atherosclerosis[214, 215]. This is supported by a recent study from Habenicht et al. that showed VSMC lymphotoxin b receptors (LTbRs) protected against atherosclerosis development by maintaining the morphology and function of ATLOs. Atherosclerosis was markedly exacerbated in *Ltbr-/- ApoE-/-* mice, which has been linked to the key role of LTbR signaling in initiating the transdifferentiation of VSMCs to a lymphoid tissue organizer-like phenotype[215]. Their data suggest that the immune system employs VSMC-LTbRs to maintain the immune homeostasis in the vasculature via ATLOs.

1.3.3 Transcriptional regulation of VSMC phenotype

Extensive studies have demonstrated that vascular SMC differentiation and phenotypic modulation are governed by a delicate network of transcription regulatory mechanisms and controlled by a dynamic array of environmental cues[216]. Moreover, recent advances in this area have provided significant insight into master transcriptional pathways that control SMC phenotype in atherosclerosis and restenosis (Table1).

Myocardin is a muscle restricted transcriptional co-activator of serum response factor. By interaction with serum response factor, myocardin binds and selectively activates the degenerate CC(A/T-rich)$_6$GG (CArG) cis-elements of virtually all the CArG-dependent SMC marker genes that confer contractile, morphological and structural properties of conventional VSMC[217-219].

Matthew et al. recently showed myocardin deficiency accelerates atherogenesis in *ApoE*-/- mice[220]. Mechanistically, increased myocardin potently abrogates the production of a large number of inflammatory molecules in VSMCs[220]. This has been putatively attributed to the inhibition of intracellular proinflammatory pathway mediators CCAAT/enhancer-binding

protein (C/EBP) transcription factor (CEBPB) and CEBPD CCAAT/enhancer-binding protein (C/EBP) transcription factor delta (CEBPD) by myocardin[220, 221]. CEBPB and CEBPD have been reported to synergistically increase and sustain the inflammatory gene expressions in VSMCs[220, 221]. Taken together, myocardin displays a critical regulatory role in vessel inflammation.

Recently, the advances in SMC specific gene modified mice have enormously improved our understanding in VSMC origin and function[222]. Krüppel-like factor 4 (KLF4), a stem cell pluripotency gene, is a transcription factor (TF) that plays a crucial role in regulating phenotypic transitions of SMCs[223, 224]. Strikingly, SMC-specific conditional knockout of KLF4 resulted a marked reduction in lesion size and increases in plaque stability. This is associated with a selective reduction of SMC-derived macrophage-like cells in the lesion[223, 224], which strongly indicates that KLF4-dependent SMC phenotype transitions are critical in lesion pathogenesis. Intriguingly, another pluripotency gene octamer-binding transcriptional factor 4(OCT4) has recently shown to be atheroprotective. SMC specific Oct4 knockout within *ApoE*-/- mice resulted in increased lesion size and decreased plaque stability, including increased necrotic core and intra-plaque hemorrhage with a thinner fibrous cap. These changes were linked to an impaired SMC migration related to fibrous cap formation[225]. Thus, the roles of these two key pluripotency genes, KLF4 and OCT4, turned to be contradictory in atherosclerosis. This may reflect a counterbalanced and complex regulation of these genes in VSMC phenotype. Nevertheless, even though striking discoveries have been made on these mice, how to translate the knowledge to human atherosclerosis is another challenging task.

Table 1. Transcriptional regulation of VSMC phenotype in vascular diseases

TF	Model	Lesion phenotype	Reference
Myocardin	*ApoE*-/-*Myocd*+/- mice	Lesion↑, macrophage-like cell↑	220
KLF4	*ApoE*-/-*Myh-Klf4*-/- mice	Lesion↓, cap↑	226
OCT	*ApoE*-/-*Myh-Oct4*-/- mice	Lesion↑, SMC migration↓	225
TET2	Locally *Tet2*-/- mice	Neointima↑, MyH11↑	227
NF-κB	*Sm22α-IκB*-/- mice	Neointima↓, SMC proliferation↓	228
IRF7	*Irf7*-/- rat	Neointima↑, SMC proliferation↑	229

IRF8	*Irf8*-/- mice	Neointima↓, SMC differentiation ↑	[230]
IRF9	*Irf9*-/- mice	Neointima↓, SMC proliferation↓	[231]
HIFα	Locally *Hifα*-/- mice	Neointima↓, VEGF-A; Flt-1↓	[232]
GATA6	Locally GATA6 inhibition mice	Neointima↓, SMC proliferation↓	[233]
STAT3	Locally STAT3 inhibition mice	Neointima↓	[234]

TET2 (Ten-eleven translocation-2); HIF (Hypoxia-inducible factors); GATA6 (GATA-binding factor 6); STAT3 (Signal transducer and activator of transcription 3).

2 AIMS

The studies included in this thesis aimed to improve our understanding of innate immune mechanisms in atherosclerosis.

The specific aims were to:

I. Investigate the role and cellular mechanism of NOD1 in atherosclerosis
II. Identification of the innate immune phenotype of intimal SMCs and its relevance in atherosclerosis
III. Elucidate the activity and clinical relevance of IL-1 and inflammasome in human atherosclerosis plauqe
IV. Explore the role and cellular mechanism of TRIM21 in atherosclerosis

3 METHODOLOGICAL CONSIDERATIONS

3.1 MOUSE MODELS OF ATHEROSCLEROSIS

Mouse models are useful for exploring the role of target molecules in atherosclerosis. Compared with the complexity of human population, animal models provide a rather identical genetic and environmental background, which enables a better observation of certain biological phenomena without being influenced by many unpredictable factors. Moreover, mouse models allow mechanistic investigations by pharmacological interventions and genetic modifications[235].

However, wild-type mice like C57BL/6 strain are generally resistant to atherosclerosis due to a HDL dominant serum cholesterol profile. Therefore, genetically modified hyperlipidemia mouse models such as *Ldlr*-/- and *ApoE*-/- on the C57BL/6 background are more widely used as experimental models of atherosclerosis[236].

LDLr is expressed mainly on hepatocytes and binds to ApoE and ApoB-100 on the lipoproteins, thus functions as the main mechanism to remove intermediated density lipoprotein (IDL) and LDL from the plasma[237]. ApoE is an apolipoprotein mainly found in IDL, and mediates cholesterol metabolism by binding to LDLr and chylomicron remnant receptor. The lack of either LDLr or ApoE severely disturbs the lipid metabolism and leads to hyperlipidemia. Compared to LDLr, ApoE is produced by both hepatocytes and bone marrow cells. Thus, transplantation of bone marrows with wild-type ApoE to *ApoE*-/- mice will rescue the hyperlipidemia and atherogenesis[238]. Therefore *Ldlr*-/- mice were used as the recipients in the bone marrow transplantation model in this thesis.

To study the role of hematopoietic NOD1 and TRIM21 in a hyperlipidemia condition in vivo, *Nod1*-/- or *Trim21*-/- mice (C57BL/6 background) were used as donors in a bone marrow transplantation model in **Paper I and IV**. 6-9 weeks old *Ldlr*-/- mice were irradiated with lethal doses and received bone marrow cells lacking NOD1 or TRIM21 from the donor mice. A high fat diet for 6-12 weeks was used for atherosclerosis development after 4 weeks recovery from the transplantation.

Bone marrow transplantation model is hematopoietic-specific and rather time-efficient compared to double knockout mouse model. However, total body irradiation causes not only acute but also long term persistent multiple tissue and organ damages, adding a new confounding variable to LDL-induced atherosclerosis. Thus, data need to be interpreted with appropriate caveats and proper controls.

On the other hand, a pharmacologically synthesized ligand of NOD1, FK565, was used to investigate the direct effect of NOD1 activation in *Ldlr*-/- mice in **Paper I**. Pharmacologically intervention provides an exogenous activation or blockage of targeted signaling pathway in addition to the endogenous regulation network. This allows an efficient dose- and time-dependent investigation, and avoids the compensatory effect in genetic modified mouse models. However, the efficacy and specificity of the ligands or inhibitors need to be critically evaluated, and the gap between the exogenous intervention and the endogenous regulation need to be kept in mind.

Despite the rapid advances in experimental atherosclerosis from mouse models have provided us striking insights in the disease pathogenesis, the translation of the data from mice to human is still a big task due to the inherent differences between species. Thus, it is very important to put more considerations into the animal model selection, data interpretation and method standardization[239].

3.2 RAT CAROTID ARTERY BALLOON INJURY AND VSMC CULTURE

A rat carotid balloon injury model was used in **Paper I** and **II**. Male adult Sprague-Dawlery rats (3 months old) were subjected to angioplasty injury by repeatedly forwarding and withdrawing an inflated F2 balloon catheter to left common carotid artery under general anesthetization[240]. This well-characterized model provides an ideal tool to investigate the biological behaviors of VSMCs under neointima formation.

Three different types of rat VSMCs were used in the studies. They were normal adult medial SMC, intimal SMC and neonatal arterial SMC. The normal medial SMC were isolated from the medial layer of the thoracic aorta of male adult Sprague-Dawley rats, and intimal SMC were derived from the carotid artery of male adult rats 2 weeks after balloon angioplasty. At this time point, the intima could be easily identified and separated from the media under a dissection microscope. The neonatal SMC were derived from the thoracic aorta of rat pups (4 days). All three types of VSMC were prepared from tissues by using enzymatic dissociation techniques or by explanation. Cells were maintained under standard cell culture conditions (+37°C, 5% CO2). Rat medial, intimal and newborn SMCs were grown in DMEM supplement with 10% (vol/vol) FCS, 1 mmol/L L-glutamine and antibiotics (penicillin G 100 U/mL, and streptomycin 100 μg/mL). Cells were passaged with trypsin, and used from passages 5 to 10 for all experiments.

The comparisons among three SMC populations give us a comprehensive view of the similarities and diversities of these SMCs, which enables a better understanding the role of VSMC under pathological conditions like vascular injury, inflammation and atherosclerosis. However, it is important to adjust certain factors like proliferation rate among different populations before analyzing other cell behaviors such as SMC migration. Another potential issue of using primary VSMC culture is the plasticity or phenotypic transformation of the SMC in vitro. Thus, it is necessary to routinely evaluate of the cell morphology and other biological behaviors, and examine the expressions of the cell linage markers.

3.3 HUMAN CAROTID ATHEROSCLEROSIS PLAQUE CULTURE

Human atherosclerotic plaque tissue ex vivo culture has been used to determine the functional relevance of the innate immune receptors in human atherosclerosis[98, 99]. This has been so far, one of the most useful tools to understand the biological and pathological functions of target molecules or pathway in human atherosclerosis lesion. Compared with cell culture experiments, tissue culture keeps a rather intact lesion microenvironment and cell-cell interaction. The variety of cell types and extracellular components, the abundant of cytokines or chemokines, to the most extent mimics the complex inflammatory milieu in vivo with high clinical relevance. This model was used to investigate the function and regulation of NOD1 and inflammasome in human atherosclerotic plaques in **Paper I and III**.

Of note, the heterogeneity of the plaque may cause big variations between individuals. Thus, a standardized protocol and adequate number of replicates is essential to show the real biological difference. On the other hand, the plaque heterogeneity may link to other biological phenomena or clinical presentations, which provides important information to understand the clinical relevance of the observation.

However, the complexity of the plaque tissue may bring difficulties for certain analysis like western blot. The enrichment of various extracellular matrixes considerably dilutes the target proteins and also brings trouble for the normalization. To remove the noise from non-cellular components, plaque isolated cells were used in some experiments.

3.4 BIOBANK OF HUMAN ATHEROSCLEROSIS PLAQUES

The Biobank of Karolinska Endarterectomies (BiKE) was established in 2001 as a collaborative research effort between the Experimental Cardiovascular Research Unit at Karolinska Institutet and the Department of Vascular Surgery at Karolinska University Hospital[241-243]. The biobank consists of more than 400 carotid plaques from patients

undergoing carotid endarterectomies at Karolinska University Hospital upon informed consent. Carotid endarterectomy is recommended in cases with more than 70% stenosis as determined by ultrasonography. The atherosclerosis specimens were snap frozen followed by RNA purification for gene expression microarrays, or tissue fixation for histological analysis. So far, total gene expression profiling was performed on RNA samples from 127 plaques by Affymetrix Gene Array U133 Plus 2.0 (**Paper I, III** and **IV)**. Non-atherosclerotic normal arteries were obtained from 10 macroscopically disease-free iliac arteries or aorta from organ donors without a history of cardiovascular disease at Karolinska University Hospital. The vessels were dissected and the intima and media used for RNA isolation and microarrays.

A critical limitation of this biobank is the lack of ideal control tissues. It is noteworthy that the distinct gene expression profile between plaques and lesion-free arteries may considerably due to the difference in cell composition and the complexity of the tissue environment. Another noteworthy point is the plaques used here only represent the advanced stages of atherosclerosis lesion, and the majority of the patients were under Statins therapy. Therefore, the data may not provide the whole picture of disease development, and the effect of statins on plaque progression may remain unrevealed and require validation in a larger cohort.

3.5 MOLECULAR BIOLOGICAL TECHNIQUES

3.5.1 mRNA expression analysis

Quantification of mRNA concentrations was performed by real-time quantitative PCR (qPCR and microarray analysis. For qPCR, mRNA was extracted and reversely transcribed to complimentary DNA (cDNA), which was amplified and quantified “real-time” in a PCR reaction utilizing the SYBR green or Taqman system (**PaperI, II and IV)**. Normalization was performed by comparing the expression values to the housekeeping genes. Chip-based microarray analyses, utilizing hybridization techniques to quantify the transcriptome, were performed in human carotid atheroma (**Paper I, III and IV**). Data was normalized using robust multi-array analysis (RMA), utilizing light intensity comparisons inter- and intra-chip.

3.5.2 protein expression analysis

Analysis of protein expressions was performed by western blot, enzyme-linked immunosorbent assay (Elisa), immunostaining and flow cytometry analysis. Western blot was used as a classic and important technique for protein identification and semi-quantification in tissues and cells (**Paper I and III)**. Elisa was used for measurement of protein concentrations in serum, plasma and cell culture supernatants (**Paper I-IV)**. Immunocytochemistry or immunofluorescence staining was used for analysis of protein

localization and quantification in the fixed human or mouse tissues (**Paper I- IV)**. Double or triple immunofluorescence staining analyzed by confocal microscopy allowed us to further investigate the relationship between different proteins, thus providing valuable information to understand the cell biology. In addition, multicolor flow cytometry allowed a cellular analysis investigating multiple extracellular and intracellular protein expression simultaneously (**Paper IV).**

Of note, all these antibody-techniques above require a high specificity of the antibodies and well-reserved antigen epitopes. Appropriate sample preservation, adequate background blotting, optimized antibody concentration and proper positive/negative controls are essential for the data quality. For multicolor confocal imaging and flow cytometry, the compensation and adjustment of voltage between different channels is of utmost importance for a well-performed analysis.

4 RESULTS AND DISCUSSION

4.1 NOD1^{HIGH} VASCULAR SMOOTH MUSCLE CELL IS IMPLICATED IN VASCULAR INFLAMMATION AND DAMAGE

Paper I investigated the molecular mechanism of NOD1 in the pathogenesis of atherosclerosis by using *Ldlr*$^{-/-}$ mice fed with high-fat diet and challenged for 10 weeks with a chemically synthesized NOD1-specific ligand FK565. Severe atherosclerosis was observed in mice that were treated with NOD1 ligand, demonstrated by a 3-fold increase of atherosclerotic plaque area in the aortic arch compared to the control mice (Figure 3A). Of note, stimulation of NOD1 resulted in the development of occlusive atherosclerosis in the coronary and innominate artery, causing fatal lumen occlusion with a large amount macrophage infiltration (Figure 3B). These findings indicate a crucial role of NOD1-mediated signaling in accelerating atherogenesis and development of severe coronary inflammation.

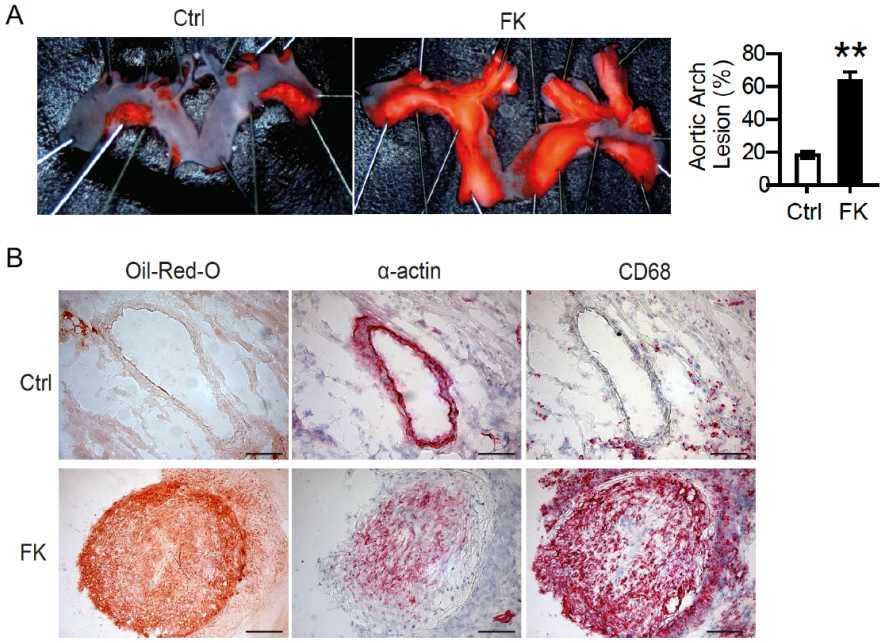

Figure 3. Exposure to NOD1 ligand in drinking water exacerbates development of atherosclerosis and results in occlusive coronary arteritis in *Ldlr-/-* mice. High-fat diet fed *Ldlr-/-* mice were treated for 10 weeks with NOD1 ligand FK565 (FK, 21 µg/ml) or without the ligand (Ctrl) in drinking water. (A) Representative images and quantifications of atherosclerotic lesion in aortic arch stained with Sudan IV. Data are presented as mean ± SEM, FK n = 5 and Ctrl n = 10. Mann-Whitney test, ** $p < 0.01$. (B) Representative micrographs of Oil Red O and immunohistochemistry staining for SM-α-actin and CD68 in coronary artery from the mice as in (A). Scale bar, 100 µm.

Despite being extensively used for studying the pathogenesis of atherosclerosis, the *Ldlr-/-* mouse fed with high-fat diet does not usually develop the culprit coronary atherosclerosis as

shown in the NOD1-ligand treated *Ldlr-/-* mice. Furthermore, immunohistochemistry examination of the aortic root lesion in NOD1 ligand treated mice demonstrated heavy damage of elastic lamellae including internal elastic lamina, and exaggerated infiltration of $CD68^+$ macrophages, $Ly6G^+$ neutrophils and $CD3^+$ lymphocytes across atherosclerotic lesion into tunica media (Figure 4),

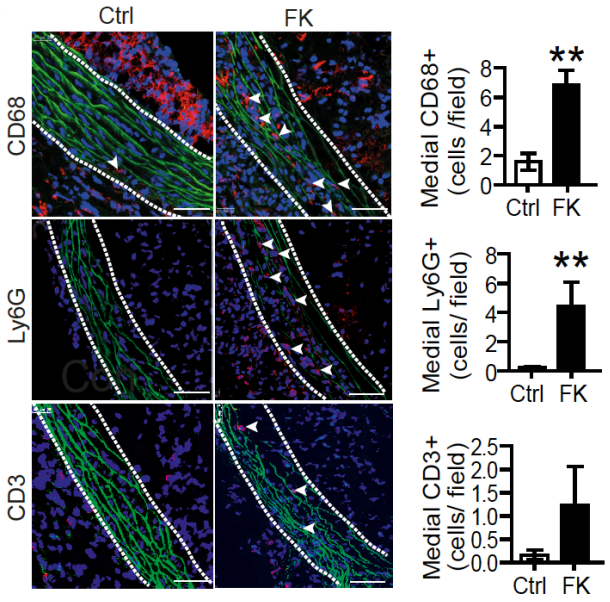

Figure 4. Arterial wall destruction accompanied by inflammatory infiltration into arterial media characterizes the functional role of NOD1 activation. Immunofluorescence images of macrophage (CD68), neutrophil (Ly6G), and T lymphocyte (CD3) infiltrate in aortic root from the mice as in Figure 3. Scale bars, 50 μm. Numeric analyses of inflammatory cell infiltrates in the media are presented at right of corresponding images. Dotted lines mark media of aorta. Data are presented as mean ± SEM, FK n = 4-6 and Ctrl n = 10. Mann-Whitney test, ** $p < 0.01$.

Given the fact that NOD1 is implicated in vascular inflammatory disorders, the cellular basis of NOD1 signaling in the context of vascular inflammation remains to be elucidated. While myeloid NOD1 pathway has been shown as irrelevance to disease progression, endothelial NOD1 signaling is regarded as necessary for the recruitment of myeloid cells, thus contributing to the pathogenesis of acute coronary arteritis and atherosclerosis[92, 93]. In our study, immunostaining for NOD1 protein noted that a number of SMC (α-actin positive) in brachiocephalic artery were distinguished from other α-actin positive SMC by expressing high levels of NOD1, hereafter termed $NOD1^{high}$ SMC. Nevertheless, activated NF-κB, one defined canonical signal of NOD1 activation, was exclusively associated $NOD1^{high}$ SMC in atherosclerotic lesion of *Ldlr-/-* mice subjected to NOD1 ligand (Figure 5). These observations suggest $NOD1^{high}$ SMC as the main cellular basis in the NOD1-ligand induced transvascular inflammation.

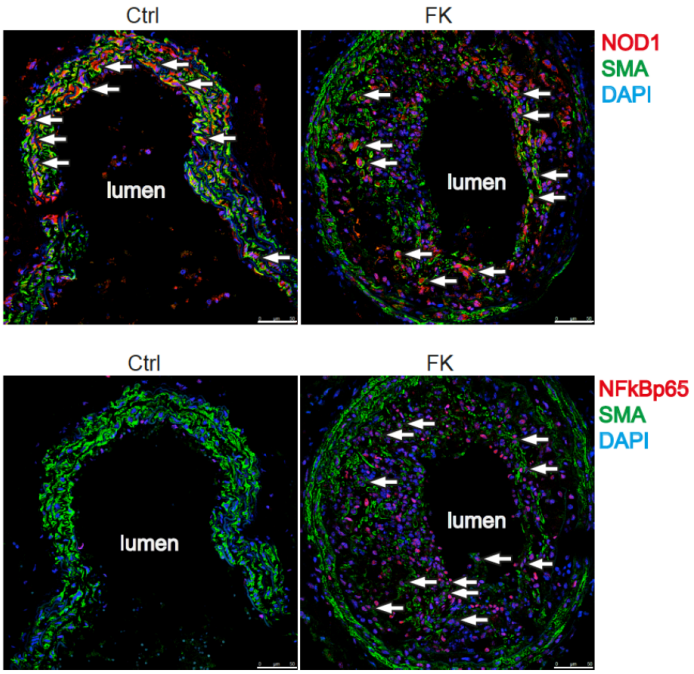

Figure 5. Involvement of NOD1^{high} SMC in atherosclerosis in *Ldlr-/-* mice. Immunofluorescence micrographs of NOD1 and NF-κB p65 in the brachiocephalic artery from NOD1-ligand treated (FK) and untreated mice (Ctrl) as in Figure 3. Arrowheads denote NOD1 or NF-κB (red) expression in Sm-α-actin (SMA, green) positive SMC. Scale bar, 50 μm. Data are representative of 3 independent experiments.

To better understand the role of SMC NOD1 signaling, we used rat carotid balloon injury model which developed neointima resembles many aspects of accelerated atherogenesis and restenosis. Immunostaining for NOD1 expressing cells further supported the occurrence of a phenotypically distinctive population of NOD1^{high} SMC in neointima (Figure 6A). Genotyping of the SMC confirmed that constitutive NOD1 mRNA was three-fold higher in neointima-derived SMC vs medial SMC (Figure 6B). Of note, NOD1^{high} SMC was characterized by spontaneous releasing of CCL5 in the absence of extra stimulation (Figure 6C). These findings in combination suggest that NOD1^{high} SMC have unique capability in producing chemokine thereby contributing to NOD1-induced transvascular inflammation and accelerated atherosclerosis.

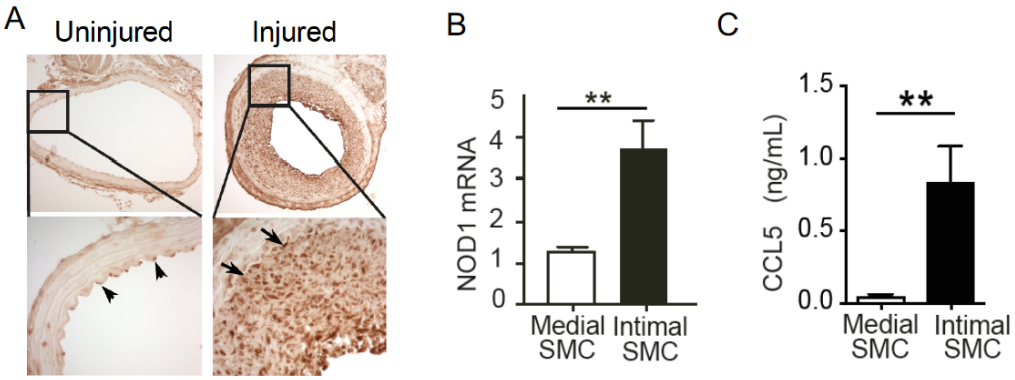

Figure 6. NOD1^{high} SMCs function as NOD1 effector cell. (A) Immunohistochemistry staining of NOD1 in rat carotid arteries 14 days after balloon injury. Arrowheads indicate endothelium. Arrows indicate internal elastic lamina. (B) NOD1 mRNA in rat primary SMC isolated from media of uninjured carotid artery (Medial) or neointima of injured carotid artery (Intimal) measured by RT-PCR. (C) ELISA assessment of CCL5 production by the SMCs as in (B) in the absence exogenous stimuli for 24 h. Results are presented as mean ± SEM. Mann-Whitney test, ** $p < 0.01$. Data are representative of 3 independent experiments.

Furthermore, NOD1 was also found expressed in a notable number of SM α-actin positive SMC in human atherosclerotic lesion. Stimulation of ex vivo atheromatous tissue culture with NOD1 ligand resulted in MAPK p-38 and JNK dependent generation of IL-1b, IL-6 and IL-8 and IL-10 (Figure 7). These data demonstrate for the first time a SMC based NOD1 pathway with inflammatory activities in human atherosclerosis.

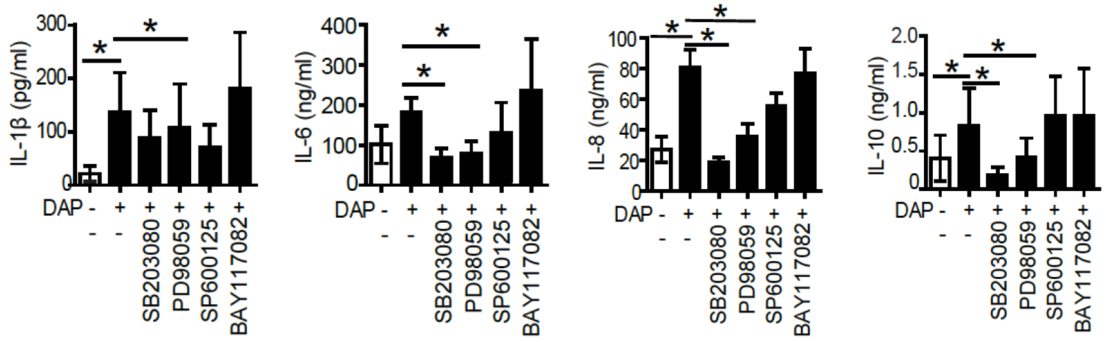

Figure 7. NOD1 mediates innate immune inflammation in human atherosclerosis. NOD1 induced IL-1β, IL-6, IL-8 and IL-10 production by human carotid plaques pre-treated with or without p38 inhibitor (SB203080), ERK inhibitor (PD98059), JNK inhibitor (SP00125), or NF-κB inhibitor (BAY117082) 30 min in prior to DAP. The cytokines in supernatant were determined by ELISA 24 h after the treatments. Data are presented as mean ± SEM, n = 7. Wilcoxon matched-pairs signed rank test, * $p < 0.05$.

In summary, we propose a subpopulation of vascular SMC defined by NOD1^{high} imprint in humans and rodents have unique function in promoting transvascular inflammation and lesion development in response to infection and vascular injury. However, whether bacterial peptidoglycans alone are responsible for the activation of NOD1 or NOD2 in atherosclerosis or require additional endogenous danger signals remains to be elucidated.

4.2 INTIMAL VSMCS HAVE AN INNATE IMMUNE EFFECTOR CELL PHENOTYPE IN ATHEROSCLEROSIS

Paper II further analyzed the innate immune phenotype of human plaque intimal SMCs with NOD1^{high} imprint, pointed to the existence of such SMCs with innate immune capacity in human atherosclerosis plaque characterized by the constitutively expression of a panel of PPRs including TLR2, NOD2 and NLRP3 which typically observed in macrophages (Figure

8A, 8B). The distinguished activated NFκB, iNOS and chemokine expression featured in these intimal SMCs may qualify them a considerable competence as innate immune effector cells. Notably, these TLR2^{+}NOD2^{+}NOD1^{high} SMCs surrounded by macrophages and T cells promote severe inflammation niches formation and further exacerbate the stenosis in human plaque (Figure 8C).

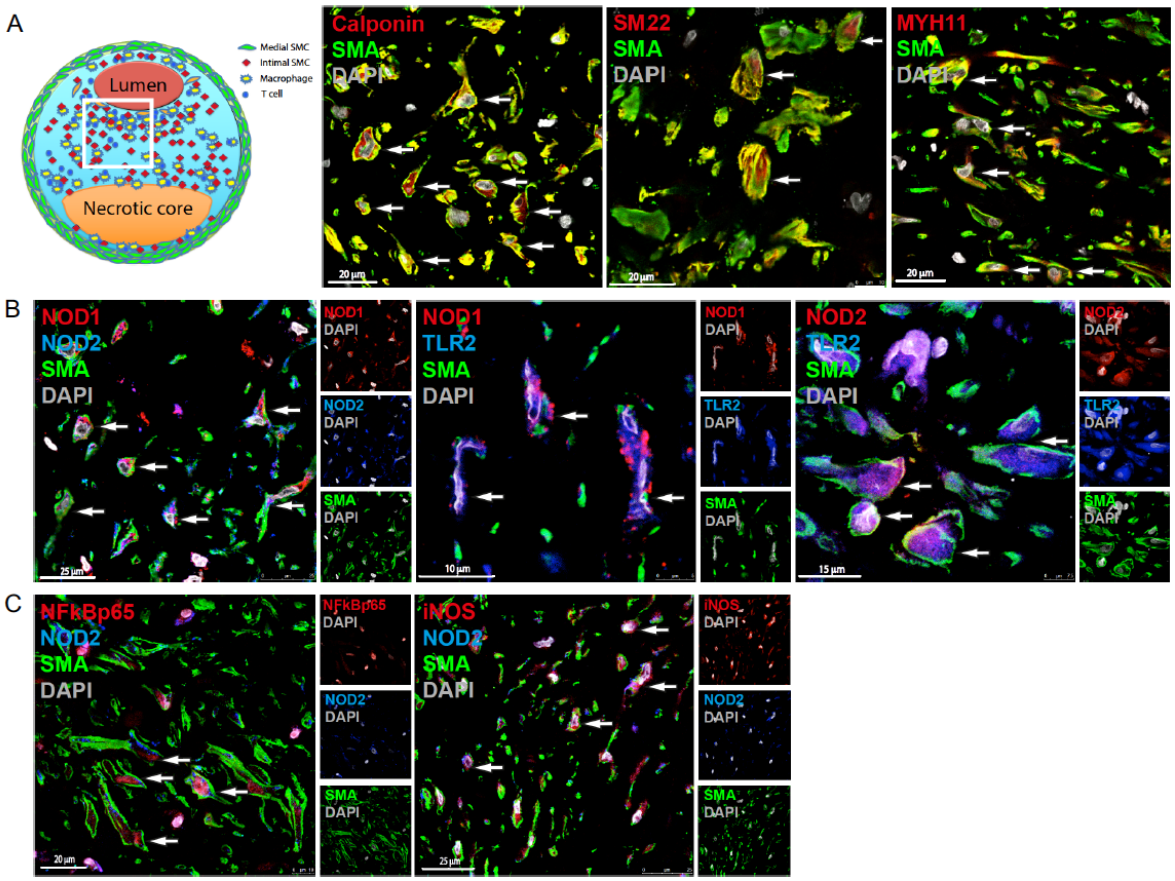

Figure 8. Identification of lesional SMCs with innate immune phenotype in human atherosclerosis. (A) Immunofluorescence micrographs of SM-α-actin (SMA), Calponin, SM22 or MYH11 in human carotid plaques, images are captured from the region indicated in the white-box of the schematic plaque. (B-C) Immunofluorescence micrographs of (B) NOD1, NOD2 or TLR2 and (C) NOD2, NFκBp65 or iNOS in SM-α-actin positive cells from adjacent sections in human carotid plaques in A. Nuclei are stained by DAPI. Arrows show the cells positive for corresponding markers indicated in each image. Images are representative from 5 individuals.

In line with the observation in human plaque, examination of innate immune phenotype of rat neointimal NOD1^{high} SMCs suggested a similar innate immune phenotype to human intimal SMCs. Besides NOD1, rat neointimal SMCs were characterized by constitutively expressing a broad profile of innate immune receptor such as NOD2, NLRP3 and TLR2-5, some of which are typically only seen in myeloid cells. Intriguingly, IFIT-1, normally silent or expressed at very low constitutive levels, was also remarkably high expressed in rat

neointimal SMCs (Figure 9). IFIT-1 has been shown to specifically recognize and bind mRNAs or proteins of a number of viruses, thus play important role in host defense against viral infection[244]. This may considerably compensate role of TLR7-9 that are lower expressed in intimal SMCs than myeloid cells.

Thus, these data suggest that rat NOD1^{high} SMC recapitulate the immune phenotype of human NOD1^{high} SMC in atherosclerosis and distinguished from medial SMCs and macrophages by the TLR2^{+}NOD2^{+}NOD1^{high} signature, thus being an ideal model for deciphering their immune function.

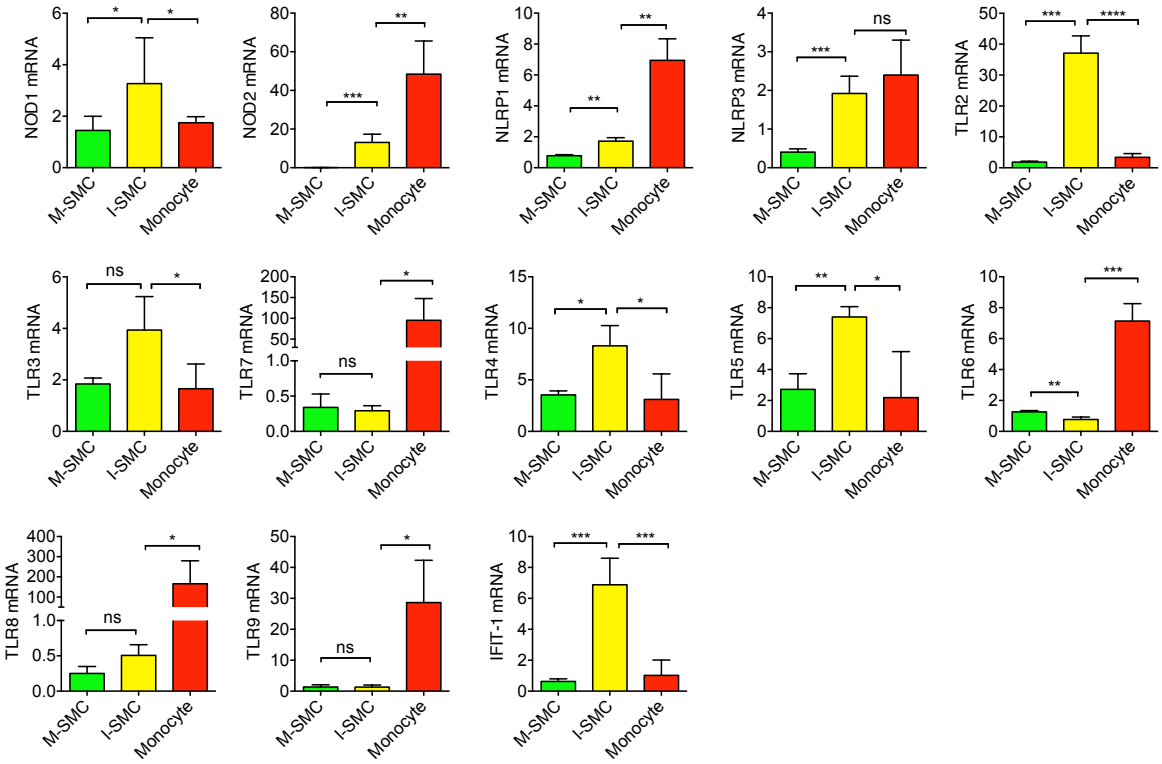

Figure 9. Rat neointimal SMCs display a unique innate immune signature. qPCR analysis for ()*Nod1-2*; *Nlrp1/3*; *P2rx7*; *Tlr2-9* and *Ifit-1* in the rat medial (M-SMC) and neointimal SMC (I-SMC) populations and peripheral blood derived monocytes. Data is presented as mean ± SEM, *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001, unpaired t test, n=3-6.

Functional studies demonstrated that this innate immune signature confer rat TLR2^{+}NOD2^{+}NOD1^{high} SMC the ability to react to an extensive list of microbial components, leading to production of inflammatory mediators including nitric oxide, cytokines and chemokine (Figure 10A). Moreover, rat TLR2^{+}NOD2^{+}NOD1^{high} SMC possess intrinsic innate effector activity, spontaneously producing leukocyte chemokine CCL-5 in the absence of exogenous stimuli (Paper I), and the activity will be further enhanced when innate immune receptor NOD1 and NOD2 are activated. CCL-5 has been recognized as an

important chemokine in immune cell recruiting, however, a recent study identified another role of CCL-5/CCR5 axis in promoting SMC proliferation and phenotype transition from contractile to synthetic[245]. Thus, the production of CCL-5 from these SMC may serve as an important therapeutic target in the context of atherosclerosis.

Additionally, our current and previous studies have also showed that rat $TLR2^{+}NOD2^{+}NOD1^{high}$ SMC distinguish from medial SMC by spontaneously activated NFκB signals and hyper-responsiveness to pathogen derived stimuli[203] (Figure 10B). Of note, since NLRP3 inflammsome components are highly expressed in intimal SMCs, we could not observe IL-1 production upon inflammsome activation. One explanation could be the presence of constitutive inhibitory mechanisms that impedes inflammasome mediated IL-1 production in SMC[246]. However, a recent study implied a non-canonical role of NLRP3 inflammasome in promoting foam cell formation in SMCs, which provides new insights in understating the SMC immune phenotype in the context of atherosclerosis[247]. In all, these findings from rat $TLR2^{+}NOD2^{+}NOD1^{high}$ SMCs strongly support the innate competence of intimal SMCs in human atherosclerosis, based on their similarities in the immune phenotype.

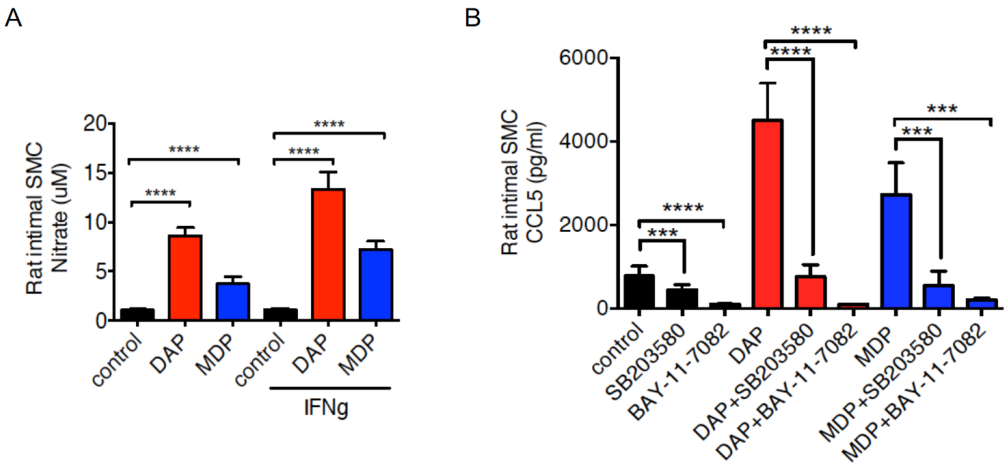

Figure 10. NOD-like receptors driven iNOS expression and chemokine production in rat $TLR2^{+}NOD2^{+}NOD1^{high}$ SMC. (A) Nitrite oxide production in rat intimal SMCs after DAP or MDP (1µg/ml) with or without IFNγ (100U/ml) stimulation for 72-hour is analyzed in the supernatant by griess reagent system. (B) CCL5 production in rat neointimal SMCs after 24-hour DAP or MDP (1µg/ml) with or without Rip2 or NFκB inhibitors (SB203580 1µg/ml, BAY-11-7082 10nM) is measured in the supernatant by Elisa. Data is presented as mean ± SEM, ***$p < 0.001$; ****$p < 0.0001$, one-way ANOVA followed by Tukey's multiple comparisons test, n=3-6.

Of note, the numerical quantification of human plaque cells showed comparable amount of $TLR2^{+}NOD2^{+}NOD1^{high}$ SMCs and macrophages with similar innate immune phenotype (Figure 11). Together with the phenotypic characterization of human intimal SMCs and

functional investigations in rat neointimal SMCs, these data strongly support a distinguished innate immune capacity of the TLR2^{+}NOD2^{+}NOD1^{high} SMCs in inflammation initiation and propagation. The innate immune receptors equipped by these SMCs enables them to direct interact with environmental danger signals, triggering the activation of intracellular signaling like NFκB and RIP2, and mediating the production of NO, chemokines and cytokines. In comparison, lesional macrophages, the classic innate immune effector cell in atherosclerosis lesion, with the expression of most innate immune receptors except NOD1, may serve as the dominant effector cell in phagocytosis, efferocytosis and the cytokine production such as IL-1β and TNF-α etc.

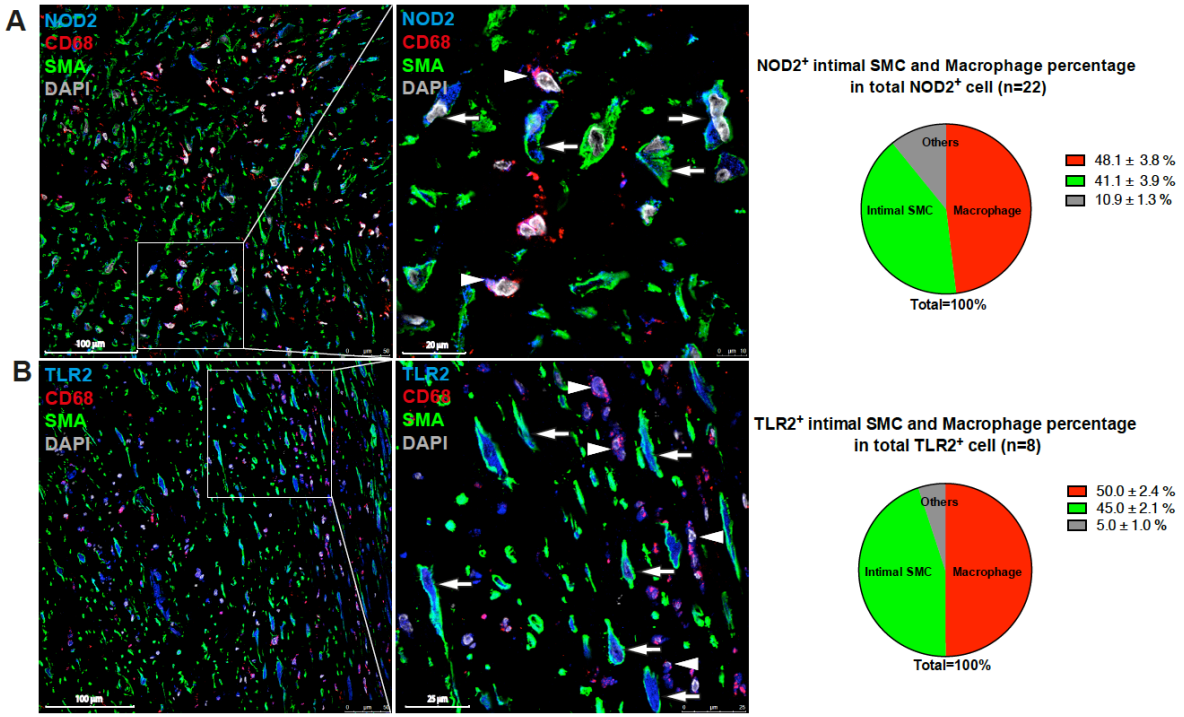

Figure 11. TLR2^{+}NOD2^{+}NOD1^{high} SMCs serve as potent innate effector cells in human atherosclerosis. (A-B) Immunofluorescence micrographs of (A) NOD2 and (B) TLR2 expressing SMCs (SM-α-actin+) and macrophages (CD68+) in human carotid plaques. Images are captured from the region indicated in the white-box of the schematic plaque in Figure 8A. Arrows show NOD2/TLR2 expressing SMCs and arrowheads show NOD2/TLR2 expressing macrophages. Pie charts show the percentage of NOD2/TLR2 positive SMCs and macrophages in total NOD2/TLR2 expressing cells (quantified as the average of five fields for each specimen, n=8-22).

In conclusion, the identification of the intimal SMC population with TLR2^{+}NOD2^{+}NOD1^{high} imprint in human plaque highlights the importance of SMCs in atherosclerotic inflammation and stabilization, arguing an indispensable role for SMCs in addition to monocyte-derived macrophages and other myeloid lineage cells. Moreover, the clarification of innate immune phenotype of SMCs provides a mechanistic explanation of the pro-inflammatory properties in

intimal SMC that has been implicated previously. Instead of passively recognized inflammatory activity, SMCs may function as potent innate immune effector cells in vascular inflammation and immune homeostasis.

4.3 INFLAMMASOME DRIVEN IL-1 PRODUCTION IS LINKED TO HYPERLIPIDEMIA AND PLAQUE COMPLEXITY

Despite the CANTOS trial demonstrated IL-1β as an appealing therapeutic target for human atherosclerosis and related complications, there are still serious gaps in our understanding of IL-1 production in atherosclerosis. **Paper III** investigated the inflammasome-IL-1 activity and regulation in advanced atherosclerosis using transcriptome analysis, immunostaining and ex vivo culture in human atherosclerotic plaques. Firstly, a heat map generated from microarray based gene expression analysis in carotid plaques revealed that atherosclerotic plaques were enriched with a broad reservoir of inflammasome transcripts, characterized by top expression of both canonical inflammasome sensors *Nlrp1-3*[248], *Nlrp8-9*, *Nlrp11-12*, *Nlrc3-5*, *Naip, Pyrin* and *Absent in melanoma 2*, (*Aim2),* and also non-canonical inflammasome components *caspase-4, -5*. Of them, *Nlrp6, Nlrp12, Nlrc4, caspase-4* and the recently reported *Nlrp3*[248] were highly expressed in symptomatic plaques compared to asymptomatic plaques (Figure 12). These highly suggest that apart from NLRP3, there are possibly additional inflammasome pathways relevant to IL-1 signaling in atherosclerosis.

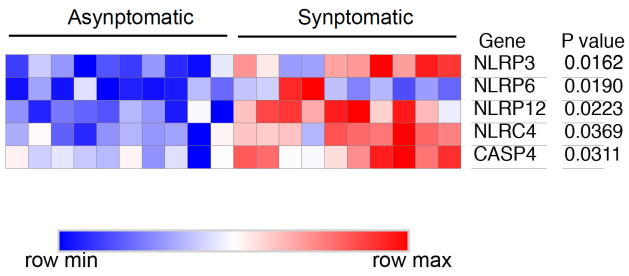

Figure 12. Selected inflammasome components are associated with symptomatic atherosclerotic plaques. Heat map representation of the top 5 differentially expressed inflammasome genes in atherosclerotic plaques from patients without clinical symptoms (asymptomatic, n = 40) and patients with clinical symptoms (symptomatic, n=85). Gene expression was determined by ribonucleic acid microarray analysis. P values are based on Mann-Whitney U test. The scale bar shows color-coded differential expression, with red indicating higher levels of expression and blue indicating lower levels of expression.

Consistently, the markedly elevated levels of NLRP3 and NLRC4 in atherosclerotic plaques were furthermore underscored by the IL-1β production in response to their external ligands (ATP and S.typh, respectively) in plaque-derived tissue (Figure 13A). Despite a proatherosclerotic role of AIM2 in mice has been proposed from recent studies[249, 250], we did not observe AIM2 induced IL-1β response in our current plaque culture model. However,

other potential effectors from AIM2 activation had not been evaluated in our study. Thus, the contribution of AIM2 in human atherosclerosis needs to be further investigated. Additionally, increased plaque caspase-4 expression and LPS stimulation boosted IL-1β together pointed to the direction of non-canonical inflammasome activation, an alternative mechanism that has been demonstrated for IL-1β in murine macrophages in vitro but has not been shown in human atherosclerotic plaques.

Of note, an equivalent quantity of IL-1α production upon LPS stimulation was also observed in human plaques (Figure 13B). Given the pro-atherosclerotic role of IL-1α from previous study[251], an uncontrolled IL-1α generation can be as important as IL-1β in the pathogenesis of atherosclerosis. In all, these data suggest that most advanced atherosclerotic plaques retain a considerable inducible IL-1 capacity, being constituted by canonical and non-canonical inflammasomes.

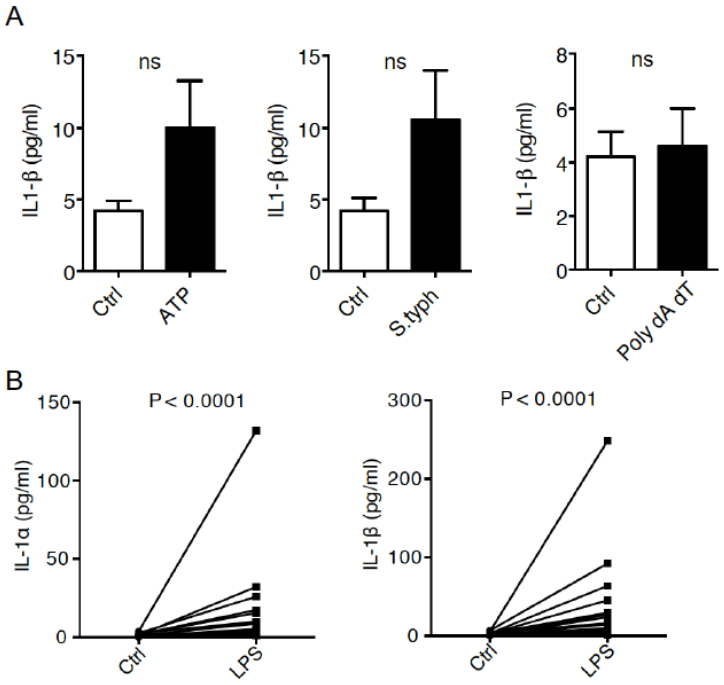

Figure 13. Canonical and non-canonical inflammasome activity in atherosclerotic plaques. (A) IL-1β production by atherosclerotic plaque samples in response to NLRP3 activators ATP (5 mmol/l), NLRC4 activator Salmonella typhimurium (S. typh) (10 mmol/l), or AIM2 activator (poly dA dT) (10 mg/ml). The concentration of IL-1β in the supernatant was quantified using ELISA. Data are shown as mean±SEM; n= 3 to 7. (B) Release of IL-1 cytokines from atherosclerotic plaque samples upon lipopolysaccharide (LPS) challenge (100 ng/ml for 24 h). IL-1α (n=16) and IL-1β (n=24) concentrations in the supernatant were measured by ELISA. P values are based on Mann-Whitney's U test. ns, not significant.

An important observation of the present study is the relationship between plaque IL-1 signaling and the classic proatherogenic risk factor LDLc. Compared with individuals with

circulating LDLc below 130 mg/dl, the average of LPS inducible IL-1β was more than two-fold higher in the individuals whose circulating LDLc over the borderline high (>130 mg/dl), indicative of a potential role of LDL in the regulation of plaque IL-1 signaling (Figure 14A). Indeed, danger signals derived from LDL like oxidized LDL and cholesterol crystals has been shown to provide both signal 1 and signal 2 for priming and activating inflammasome and promote IL-1 production in macrophages[41, 104, 252]. LDLc levels may thus, indeed, serve as an important parameter for plaque IL-1 signaling and identification of the patients prone to inflammasome activation. Furthermore, we found that atherosclerotic plaque tissues from patients on no or low-intensity statin therapy mounted much higher IL-1β production upon stimulation. Taken together, these data lend support for an aggressive lipid-lowering therapy in patients with advanced atherosclerosis as an important translational aspect. However, optimal LDLc goals must be carefully evaluated due to the individualized risk factors in clinical practice. The LDLc values provide clinicians with actionable information to help further optimize medications and improve outcomes.

Another important finding is that IL-1 signaling was significantly upregulated in the culprit lesions featured by imaging signs of one of the complexities as hemorrhage, ulceration or calcification (Figure 14B). In combination with increased expression of inflammasome components in symptomatic plaques, these data suggest inflammasome pathways are primed and prone to activation in more complex and biologically active plaques.

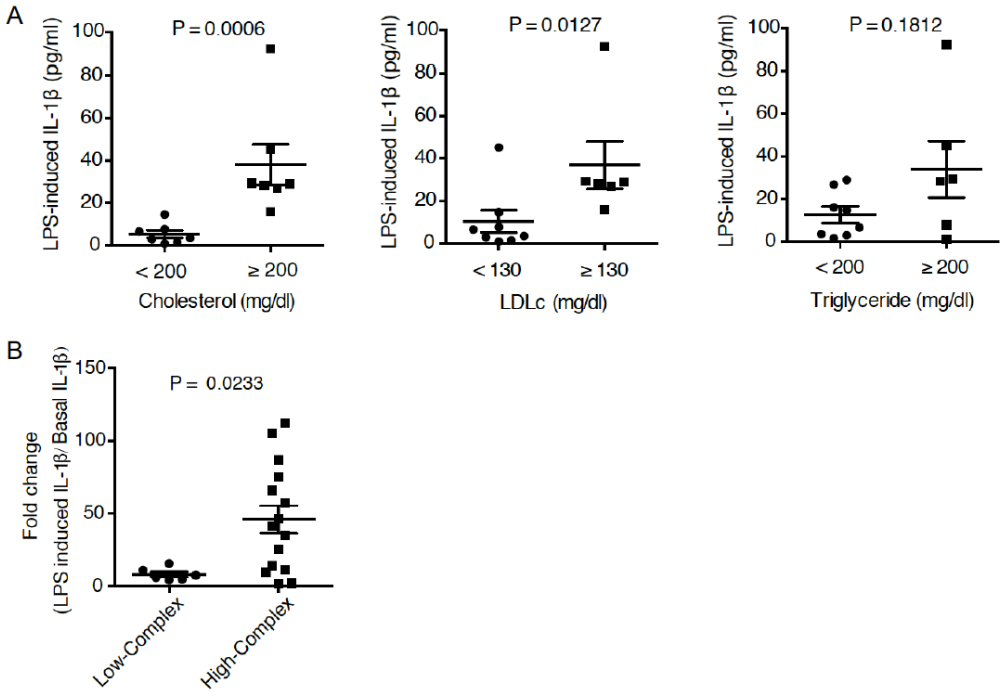

Figure 14. Plaque high IL-1 activity is linked with uncontrolled hypercholesterolemia and disease complexity. (A) Analysis of the relationship between LPS induced plaque IL-1 responses and serum total

cholesterol, LDLc and triglycerides. P-values based on Mann-Whitney's U test, data shown as mean ± SEM, n=14. (B) IL-1β fold-increase by LPS stimulation (100 ng/ml, 24 hours) in cultured carotid plaques with low or high degree of complexity measured by ELISA in supernatant. P-values based on Mann-Whitney's U test, data shown as mean ± SEM, n=6-15.

At last, we show that LPS-triggered IL-1β and IL-1α secretion could be effectively inhibited by MCC950, a recently developed NLRP3 inhibitor[253] (Figure 15). This is in keeping with an experimental in vivo study noting a reduction in atherosclerotic lesion development by inhibition of the inflammasome with MCC950[254]. On the other hand, since the current IL-1β-blocking antibody still has the drawbacks such as heightened risk for infection and high economic cost[7], targeting the inflammasomes with MCC950 may be a promising therapeutic alternative to inhibit the IL-1 signaling in atherosclerosis.

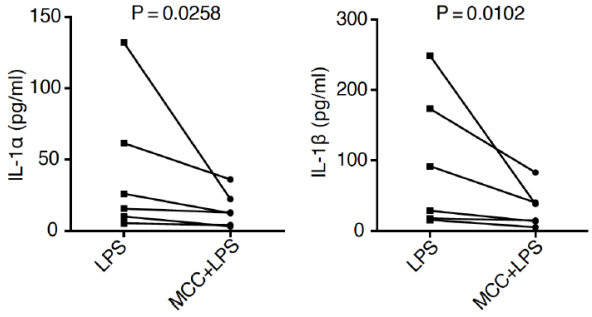

Figure 15. Inflammasome inhibitor dampens plaque IL-1 activity. ELISA assessment of LPS induced IL-1α and IL-1β production in the carotid artery plaque tissues pre-treated with or without MCC950 (100 nM). MCC, MCC950; n=6, Wilcoxon matched-pairs test.

4.4 TRIM21 INFLUENCES ATHEROSCLEROSIS VIA REGULATION OF TH17 RESPONSES

Previous studies have shown that TRIM21 is predominantly expressed in haematopoietic cells and play important roles in controlling innate immune responses and tissue inflammation[255, 256]. However, the role of TRIM21 in atherosclerosis is unknown. **Paper IV** investigated the role and cellular mechanism of TRIM21 in atherosclerosis using a bone marrow transplantation mouse model. A myeloid TRIM21 deficiency was achieved by reconstituting bone marrow from *Trim21-/-* mice into *Ldlr-/-* mice, which followed by a high-fat diet kept for 6 or 12 weeks after the transplantation. Herein, *Trim21-/-* bone marrow chimeras developed significantly larger atherosclerotic plaques (Figure 16A). Intriguingly, multiple signs of increased plaque stability were observed in these *Trim21-/-Ldlr-/-* chimeric mice, indicated by increased collagen content and fibrous cap thickness (Figure 16B). In addition, increased $CD4^+$ T cells with higher IL-17A production instead of IFNγ was also

observed in the lesion, implicating a Th17 response driven by TRIM21 deficiency in disease development (Figure 16C, 16D).

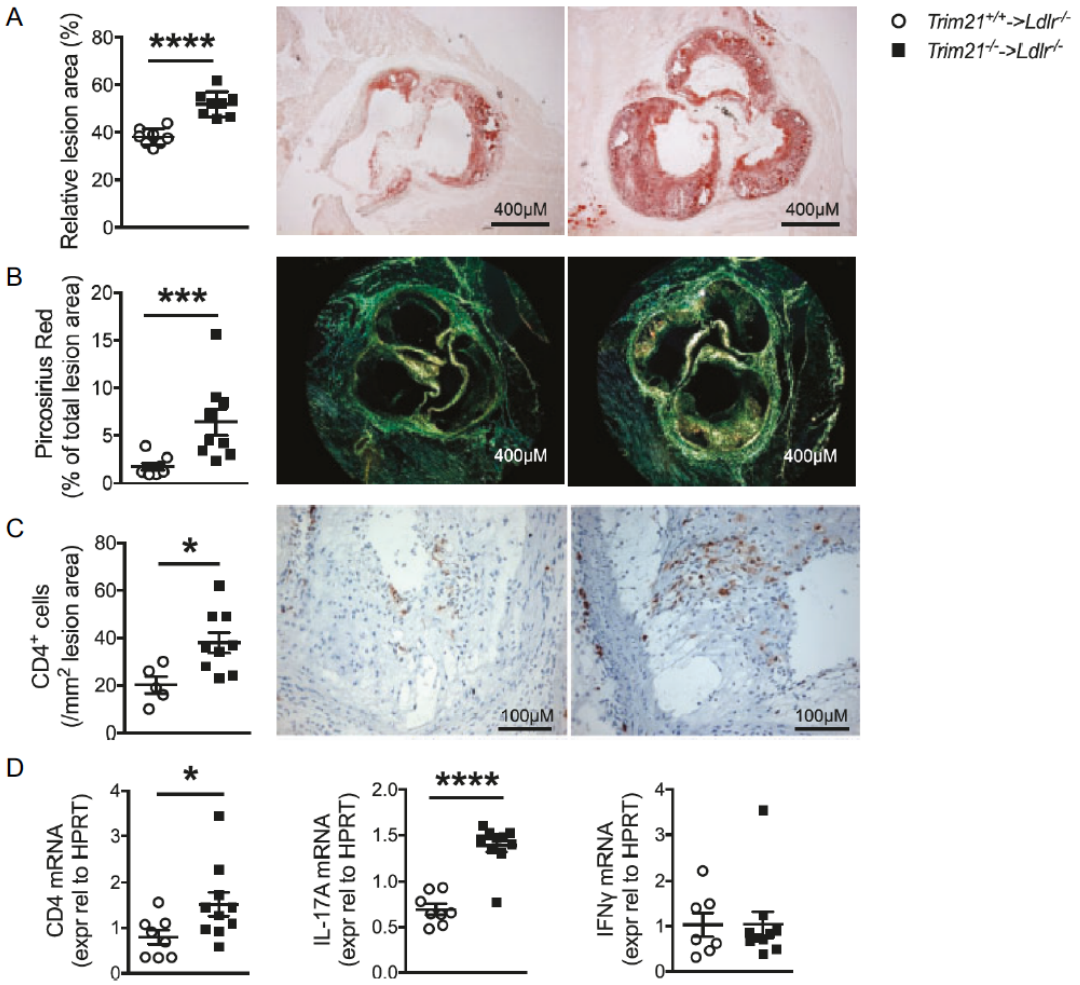

Figure 16. Trim21 deficiency increases atherogenesis and fibrous content with enhanced local Th17 response in plaques. (A) Lethally irradiated *Ldlr-/-* mice were transplanted with bone marrow from *Trim21+/+* or *Trim21-/-* mice, and fed a high-fat diet for 12 weeks (n= 8; 10). Lesion size was measured by Oil Red-O staining at the aortic root. (B) Collagen content visualized in polarized light after Picrosirius Red staining in the aortic root. (C) CD4 Positive cells detected by immunohistochemistry staining in the aortic root. (D) Gene expression of CD4, IL-17A, and IFNγ in the aorta assessed by RT-PCR relative to HPRT. *Trim21+/+->Ldlr-/-* depicted with open circles and *Trim21-/-->Ldlr-/-* with black boxes. P-values based on Mann-Whitney U test, *P<0.05, ***P< 0.001, ****P< 0.0001, data shown as mean ± SEM.

To understand how TRIM21 is influencing T cell biology, an in vitro T cell differentiation experiment was performed using naïve T cells. Consistently, instead of Th1 or Th2 differentiation, Trim21 deficient T cells differentiated into Th17 cells as shown by both intracellular cytokine staining and quantitative RT-PCR (Figure 17A-C). These data for the first time demonstrated that Trim21 directly regulates the generation of Th17 cells in a cell-intrinsic manner. Moreover, further investigation of the phenotype of Th17 cells generated by

Trim21-/- cells indicated a rather nonpathogenic phenotype based on gene expression profiling[257, 258]. Due to the mysterious role of Th17 in atherosclerosis, our data may bring new clues for future studies.

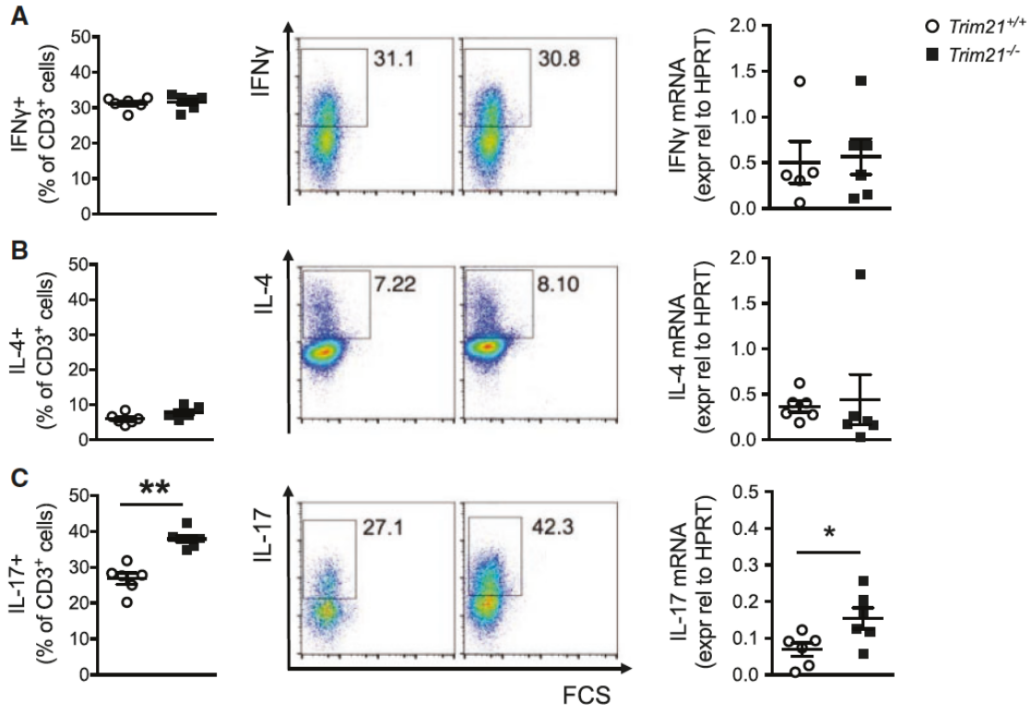

Figure 17. Enhanced Th17 differentiation of naïve *Trim21-/-* CD4$^+$ T cells. CD4$^+$CD44$^-$CD62L$^+$ naïve T cells were cultured under conditions promoting T helper cell differentiation for 5 days. Cells were analyzed by flow cytometry and RT-PCR. For flow cytometric analysis, live CD3$^+$ T cells were stained and analyzed for the expression of lineage specific cytokines. (A) Cells differentiated into Th1, (B) Th2 cells, and (C) Th17 cells. *Trim21*+/+ open circles and *Trim21-/-* black boxes (n= 6 in each group), representative data of three independent experiments. P-values based on Mann-Whitney U test, *P<0.05, **P< 0.01, data shown as mean ± SEM.

To elucidate the clinical relevance of TRIM21 in human atherosclerosis and translate our findings from mouse experiments, we analyzed the transcription level of TRIM21 and Th17-associated genes in human atherosclerosis plaque using BiKE cohort. Surprisingly, almost all the essential regulators of Th17 response were inversely correlated with TRIM21 expression, including key transcriptional factors as IRF4 and RORγT, crucial cytokines for Th17 cells maintaining as GM-CSF and IL-23, and the signature cytokine IL17A. Furthermore, inverse correlation was also found between TRIM21 and Collagen type 1, which went in line with the increased collagen content in the plaques of Trim21-deficient mice (Figure 18).

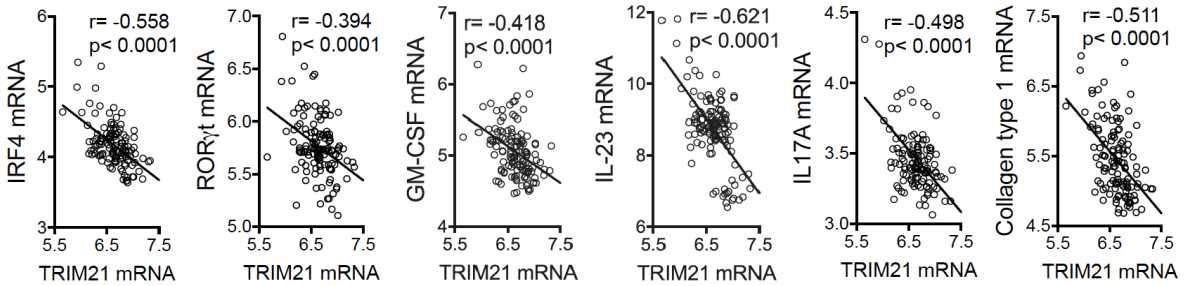

Figure 18. TRIM21 expression correlates to the Th17 pathway in human atherosclerotic plaques. Pearson correlation analysis between gene expression of TRIM21 and IRF4, RORγt, GM-CSF, IL-23, IL-17A and Collagen type 1 in atherosclerotic plaques (obtained from 127 patients undergoing carotid endarterectomy), respectively.

In conclusion, we here demonstrate a potent regulatory role and provide a mechanistic insight of TRIM21 in atherosclerosis development. TRIM21 deficiency leads to a more stable plaque phenotype with higher collagen content, which is linked to a newly discovered role for TRIM21 as an intrinsic negative regulator of Th17 differentiation.

4.5 CONCLUDING REMARKS

This thesis illustrated the roles of NOD1, intimal SMCs, NLRP3 inflammasome and TRIM21 in contribution to the complex pathogenesis of atherosclerosis (Figure 19). Specific conclusions include the following:

Paper I provides key insights on the role of NOD1 in vascular biology and its molecular mechanisms. Activation of NOD1 causes extensive atherosclerosis throughout aorta and striking occlusive atherosclerosis in both coronary and innominate arteries that are accompanied by transmural infiltrates and arterial wall demolition. $NOD1^{high}$ SMCs are likely the vascular innate immune cell capable of detecting vascular infection and injury, transducing danger signals into inflammatory responses and accelerating atherosclerosis.

On the basis of the study in $NOD1^{high}$ SMCs, **Paper II** further identifies a SMC subpopulation in human atherosclerosis lesion possessing a $TLR2^{+}NOD2^{+}NOD1^{high}$ imprint with distinguished proinflammatory activity. The considerable numeral capacity and functional potential of this SMC population suggest them as an important source of arterial resident innate immune effector cells in human atherosclerosis that may have been underestimated.

On the other hand, the role of NLRP3 inflammasome and IL-1 signaling seems to be dominated by macrophages. In **Paper III,** the expression and activity of multiple canonical and non-canonical inflammasome pathways in atherosclerotic plaques have been investigated. Producing IL-1 has been shown as a hallmark of advanced atherosclerosis, while IL-1β yield is markedly increased in more complex plaques and in individuals with higher levels of circulating LDL cholesterol or receiving no or low statin treatment. Moreover, IL-1generation in human atherosclerotic plaques is suppressible by inhibition of the inflammasome, providing a novel angle to the reduction of plaque inflammation.

Last but not least, **Paper IV** strongly supports the notion that Trim21 negatively regulates the generation of non-pathogenic Th17 cells that proposes a novel mechanism of the stabilization of atherosclerotic plaque.

Taken together, the innate immune system is presented as a diverse and coordinated regulatory network, which plays important roles in the pathogenesis in atherosclerosis.

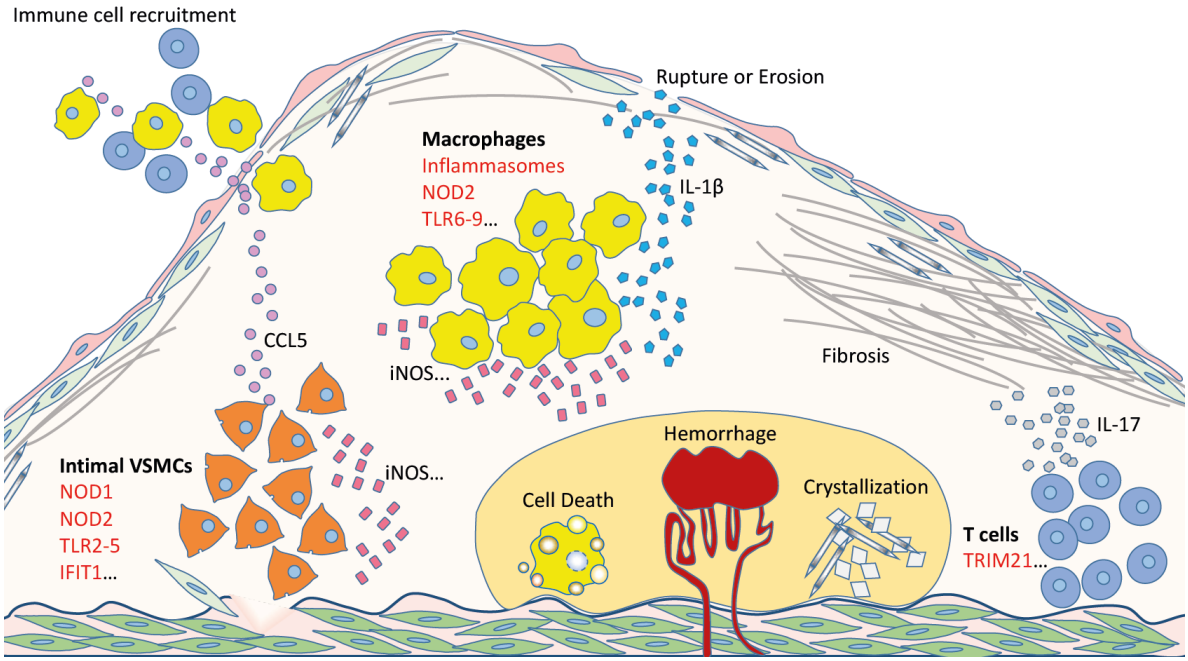

Figure 19. Schematic summary of the innate immune mechanisms in atherosclerosis. Activation of NOD1 and other innate immune receptors in intimal VSMCs leads to a potent chemokine production, which drives a continuous macrophage and T cell recruitment into the lesion. Lesional macrophages sense the danger signals generated in the plaque that triggers the activation of inflammasomes, which controls IL-1 production and is associated with disease activity. In addition, non-pathogenic Th17 cells regulated by Trim21 promote fibrous cap formation, thus contribute to plaque stabilization.

5 ACKNOWLEDGEMENTS

This 5 years journey of my PhD has been an adventure in my life, full of joy and challenges. Thanks to all the people who shared a part of the journey with me and every tiny achievements and difficulties we've been through together. All of these make who I am today.

I would like to thank my main supervisor, **Zhongqun Yan**, for bring me to the world of science, for training me to gain scientific independence and for your tremendous support and trust. Most importantly, you showed me a good example of being a scientist, that is always being curious, honest and critical. You have been an excellent guide on these five years' wonderful adventure.

Göran K Hansson, my co-supervisor, thank you for providing me an opportunity to start my PhD in the lab. I appreciate every discussion we had in the raw-data meeting, thank you for bringing up interesting questions and sharing your knowledge and ideas with me.

Joerg Herrmann, my supervisor at Mayo Clinic, thank you for the support for our projects. I enjoy every discussion with you, your positive attitude and great passion about science and clinic always give me energy to continue. The 3 months research visiting in Mayo was a fruitful and unforgettable experience to me. I survived the -20 degrees in Rochester with your warm welcome and a fantastic Christmas party with your family.

Peder Olofsson, thank you for your support and encouragement, especially in the later part of my PhD study. You shared your experiences to me and helped me to find the direction in my career. **Eva Sundman Olofsson**, thank you for letting me get to know the Swedish medical care system in your clinic, you are absolutely a model that I want to become in the future.

Per Eriksson, thank you for your support, ideas and knowledge. I enjoyed a lot involving in the joint activities in our cardiovascular research unit.

Harvest Gu, my mentor, thank you for being there whenever I need your advices and tips.

Gabrielle Paulsson-Berne, thank you for the effort in maintaining the routine in the lab and sharing the Swedish culture (sweet rules) with us. Thank you so much for the support and suggestions for pushing my PhD study forward.

Daniel Ketelhuth, thank you for sharing your knowledge in my projects and advices for my future career.

Magnus Bäck, thank you for the input and comments for my projects and giving me the opportunity to present my project in the KI-Mayo meeting.

Stephen Malin, thank you for sharing me your great knowledge in science and every tip you gave me to improve myself. Thank you for the effort for organizing the journal clubs and the seminars.

Ingrid Törnberg, I feel always being taken care of by you, really miss you a lot! You can always fix the problem I had in my experiments. I really miss the music from your radio, and the Swedish strawberries from your garden and your sense of humor.

Anneli Olsson, thank you for all the great help under these years. It had been a while that we two were the earliest in the lab, and it's a good start to begin the days with a little Swedish conversation.

Linda Haglund, I appreciate a lot for your help with my projects, for teaching every basic techniques step by step from the scratch, for analyzing every possible mistake in the data.

André Strodthoff, thank you for your help in the projects and showing me the technique.

Eva Palmaeus, Ann Hellström and Lillemor Melander, thanks a lot for the help with handling the tons of tedious administration work, which saved a lot of time and energy for us.

Xiaoying Zhang (Cheryl) and **Yajuan Wang,** thank you for helping me to get adapted to the new environment quickly and smoothly, for showing me the basic knowledge and technique and sharing me the experiences in your studies. **Pei Zhao**, thank you for the accompany in the lab for 3 months. I miss the time that we designed experiments, analyzing data and also exploring Stockholm and Athens.

Susanna Brauner, it's always been a wonderful pleasure to work with you! Your clear mind and efficient way of working impress me a lot. We've been through a lot for our TRIM21 paper, which is a precious learning experience for me. I hope we'll be co-workers again. **Marie Wahren-Herlenius** and **Gudny Ella Thorlacius,** thank you for all the contributions in the project.

Ulf Hedin and **Joy Roy**, thank you for the contribution for my projects and sharing the knowledge under all the discussions. **Ljubica Perisic, Malin Kronqvist** and **Mette Lengquist,** thanks a lot for your help for these years.

Martin Halle and **Tina Christersdottir,** thank you for the great help for my projects and the time we spent under the years.

Cecilia Österholm Corbascio and **Heidi Wähämaa,** thank you for the generosity for providing us the material or methods for my experiments.

Feilong Wang, perhaps I would not be able to survive the winter in Rochester without your great help. I'm so grateful to have you there as a generous colleague and a thoughtful friend. **Lisa Nesbitt** and **Darrell Loeffler,** thanks a lot for your help for my 3 months at Mayo.

Andrés Laguna Fernández, I feel so lucky to have you as my colleague, my friend and my mentor in the past years. You cheered me up in my difficult times and shared my joy when I made progress.

Anton Gisterå, it's always nice to have you around, I can't remember how many weekends we both worked in the office, how many lab techniques you taught me (western blot and en-face) and how many gym trainings after work.

Konstantinos Polyzos, my great labmate! I really enjoyed the time we spent together in learning new techniques, sharing thoughts on each other's projects and cheering each other up after a long day.

Glykeria Karadimou, sweet Glykeria! Thank you for always being on my side (office desk and support). The lab is always full of joy when you're here.

Monica Centa, it's so nice to have you around! We've been helping each other a lot at work and sharing a lot feelings in life. And thank you so much for the help with my dissertation application with Glykeria.

Martin Berg, always calm and gentleman! Thanks a lot for the support in lab and also the help in correcting my Swedish homework.

Marcelo Petri, thanks a lot for your generous help in the lab, Swedish course and the information for exam preparations.

Miguel Carracedo, I always feel happy to hear you call my Chinese name, and to have you as my colleague and friend. Most importantly, to help me get into the lab building every time I forgot my access card at weekends.

Gonzalo Artiach Castañón, it's hard to believe a hardworking PhD student like you can also be a talented pianist!

Katrin Habir, thank you for your support during the years and helping me with my Swedish.

John Pirault, it's so nice to have you sitting beside me for almost 4 years for answering my every tiny silly question about the experiments and results.

Reiner Mailer, I was always impressed by your hard-working and the informative FACS data (>20 graphs on one A4 paper) as you presented in our morning meetings.

Roland Baumgartner, my generous colleague and friend (like you said, Austrians always help)! Thanks a lot for the support and your homemade beers and tiramisu!

Maria Forteza de los Reyes, I appreciate a lot of our discussions about the projects, and so many interesting conversations about music and movies.

Silke Thul, your warm smile and fantastic cakes are the sweetest we have in the lab.

Hildur Arnardottir, thank you for the time we spent in the lab and being my schoolmate in Swedish learning.

April Caravaca, the queen of energy! Memories always come to the happy moments with you, CMM pubs, our small Halloween parade…

Laura Tarnawski, thanks for every small discussion about science in the lab and the nice company when we hang out.

Karolina Ahlsten, it's great to have you around for your warm smile and your tips for Gotland!

Sven-Christian Pawelzik, every time when I stayed a bit late in the lab, there's always you who stayed longer, perhaps much longer.

Nikolas Skenteris, Ilona Kareinen, Sanna Hellberg, Alessandro Gallina, Bianca Suur, Lisa Hofste, Maria Rosén Klement, Hanna Agardh, Olga Ovchinnikova, Anna Lundberg, Daniel Johansson, Daniela Strodthoff, Leif Söderström, Lasse Folkersen, Kajsa Prokopec, Albert Dahdah, Jasmin Barimani, Teodora Andonova, Tanize Faulin, Kathryn Anne Potter, Carolin Dreyer, Martina Walter, thank you all for sharing parts of my PhD journey in CMM L8:03. I appreciate our every conversation about science, culture and life. Being a member of the group together with you has been a wonderful time.

Lars Maegdefessel, thank you for clarifying the experimental models and organizing the interesting journal clubs. **Hong Jin, Suzanne Eken, Ekaterina Chernogubova, Yuhuang**

Li (Daniel), Yan Chang, thank you for the help and support under the years. Special thanks to **Hong Jin** for your kindness and generosity, I feel close to you since the first day we met.

Ewa Ehrenborg, thank you for all the encouragements and inspiring comments under my PhD studies. Thank you for the nice company in the EAS meeting in Glasgow.

Angela Silveira, thanks a lot for the effort in many management works, especially for the moving of our unit.

Ning Xu Landén, thank you for always being there whenever I need your help and advice since the first day I arrived in Stockholm. I still remember you and **David Landén** helped me a lot with my moving even when you still had the cold, and all the fantastic Christmas dinners we had together.

Nailin Li, thank you for your encouragements under my PhD studies. Thank you for the course organizing.

Andrew H. Lichtman and Sheila Leavitt, I'll always remember our beautiful cruise trip in Stockholm and the inspiring talk in Harvard.

Hong Chen and Rong Mu, thank you for the supervision during my MD, and the support for my PhD studies.

Jingyi Ren, it's my great pleasure to have you as my supervisor, mentor and friend. My PhD journey wouldn't start without your tips and recommendations. You always care about my study, career and life no matter where I am, which makes me feel so warm even in the cold and dark Swedish winter.

Zhengguo Qiao, thank you for starting our connections between Beijing and Stockholm at the beginning, and your help with my early research plans.

Jonas Ludvigsson, thank you for the refreshing morning walks and the interesting conversations in Swedish.

My friends in CMM, Helena Costa, Abdul-Aleem Mohammad, Anna Martinez Casals, Ewa Kurzejamska, Sharan Ananthaseshan, KC Yaiw, Xinling Xu, Huanhuan Cui (Leah), Masany Jung, Afsar Rahbar, Piotr Religa, Belghis Davoudi, Natalia Landázuri Sáenz, Mattia Russel Pantalone, Peter Saliba Gustafsson, Jesper Gådin, Mona Ahmed, Björn Gustafsson, Marita Wallin, Jiangning Yang, Zhichao Zhou, Ya-Ting Chang, Chi-Nan Tseng (Jack), Silvia Aldi, Lei Du, Louisa Cheung, Hoyin Lam, Varsha Prakash, it's

my great pleasure to have you around, every talk at lunch or Fika with you refuel the rest of my day. Thank you for all the help and support, for every sunny smile in the corridor and every warm greeting to start a day.

All my friends in Sweden, China and USA, Jiangrong Wang, Xiaoyuan Ren, Chang Liu, Xiao Tang, Tian Li, John, Ran Yang, Tong Gong, Jie Song, Yanpeng Yang, Quan Tang, Yanying Liu, Xingmei Zhang, Mingmei Shang, Jing Guo, Ming Liu, Yiqiao Wang, Meng Chen, Jingru Yu, Yuanyuan Zhang, Dongmei Tong, Aoxue Wang, Xi Li, Dongqing Li, Tiantian Liu, Min Yang, Yuanjun Ma, Donghao Lyu, Benjamin Liu, Yuan Xu, Tenghao Zheng, Xiaonan Zhang, Lidi Xu, Yixin Wang, Ying Qu, Xin Wang, Hongya Han, Xinyan Miao, Min Guo, Huang Huey-Jy, Xiaofei Li, Limin Ma, Xuan Li, Shuyang Yao, Xinsong Chen, Ran Ma, Bojing Liu, Xicong Liu, Yabin Wei … So many delightful memories come to my mind when I think of you guys, thank you for all the moments we shared together. Distances between us nowadays might be thousand miles away, however the friendship is still as fresh as yesterday and is the treasure that I'll always keep.

Jingcheng Xie, thank you for the encouragement at the beginning and the support over the years.

Gunilla Colldahl and **Tore Scherstén**, thank you for the support, for reading my long emails and giving me the valuable advises, for all the joyful moments we had together.

Fredrik, thank you for your support and understanding. My anxiety and worry goes away as long as you're around, you make me the luckiest girl.

My parents Yu Jiang and **Fengqin Lyu,** **爸爸妈妈，谢谢您们赐予我生命，一直以来的支持和关爱，给我追寻梦想的自由和勇气。**

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