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INNATE IMMUNITY IN ATHEROSCLEROSIS — THE ROLE OF PATTERN RECOGNITION RECEPTORS

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The fruitage of the spirit is love, joy, peace, patience,

kindness, goodness, faith, mildness, self-control.’

(Galatians 5:22-23)

ABSTRACT

The pathogenesis of atherosclerosis is greatly influenced by the activities of both innate and adaptive immunity. Danger signals such as cholesterol crystals, oxidized LDL, and modified phospholipids may trigger sterile inflammation in atherosclerosis. Systemic infection or transient release of pathogen associated molecules in the circulation might also activate immune system and affect atherosclerosis. Activation of the innate immunity relies on a set of pattern recognition receptors (PRRs). Thus, PRRs are fundamental for activating the innate immunity in atherosclerosis.

This thesis focuses on the role of three different PRRs in atherosclerosis, including NOD1, NOD2 and TLR9. We hypothesized that these PRRs regulate immune responses in the pathogenesis of atherosclerosis.

We found that NOD2 is expressed in endothelial cells and macrophages in atherosclerotic plaques, and lesional NOD2 signal leads to activation of PGE2 pathway via NF-kB and MAPK p38. NOD2 activation *in vivo* promotes the development of vulnerable atherosclerotic plaques, characterized by enlarged necrotic core in the atherosclerotic plaques and enhanced vascular inflammation. Furthermore, NOD2 induces lipid retention in macrophages may contribute to the necrotic core formation.

Although belonging to the same family, NOD1 signal promotes another lesional phenotype characterized by occlusive atherosclerosis with elastin degradation and vascular smooth muscle cell (VSMC) activation. *In vitro* stimulation of SMCs with NOD1 ligand induces chemokine and MMP production as well as enhances migration ability. Our data point to a possible mechanism via NOD1 in the development of occlusive atherosclerotic lesions.

Unlike NOD1 and NOD2, TLR9 stimulation decreases atherosclerosis and necrotic core albeit activates local and systemic inflammation. Two important anti-inflammatory mediators IL-10 and IDO are induced by TLR9 activation and are potential contributors to the mechanisms that TLR9 restrains atherosclerosis.

In summary, we identified three innate immune pathways linked to the distinct features of atherosclerosis. NOD2 leads to formation of vulnerable plaques with big necrotic cores. NOD1 promotes severe occlusive atherosclerosis. TLR9 signal restrains the development of atherosclerosis.

LIST OF SCIENTIFIC PAPERS

1. Liu HQ, Zhang XY, Edfeldt K, Nijhuis MO, Idborg H, Bäck M, et al. NOD2-mediated innate immune signaling regulates the eicosanoids in atherosclerosis. Arterioscler Thromb Vasc Biol 2013;33:2193-2201.

2. Johansson ME*, Zhang X-Y*, Edfeldt K, Lundberg AM, Levin MC, Borén J, et al. Innate immune receptor NOD2 promotes vascular inflammation and formation of lipid-rich necrotic cores in hypercholesterolemic mice. Eur J Immunol. 2014 Jul 17. doi: 10.1002/eji.201444755. [Epub ahead of print]

3. Zhang X-Y, Johansson ME, Jiang X-T, Hansson G, Yan Z-Q. Innate immune receptor NOD1 provides a mechanistic link to inflammatory destruction of arterial wall and development of severe atherosclerosis. Manuscript.

4. Zhang X-Y, Qiao Z-G, Berg M, Ketelhuth D, Yan Z-Q. CpG induces potent immune regulatory mechanisms that inhibit progression of atherosclerosis in hyperlipidemic mice. Manuscript.

* Equal contribution

CONTENTS

LIST OF ABBREVIATIONS

ACS	acute coronary syndrome
AGPAT9	1-acylglycerol-3-phosphate O-acyltransferase 9
ApoE	apolipoprotein E
CPT1	carnitine palmitoyltransferase 1
CVD	cardiovascular disease
DAMP	damage-associated molecular pattern
DAP	g-D-glutamyl-meso-diaminopimelic acid
DGAT2	diacylglycerol O-acyltransferase 2
EC	endothelial cells
EDA	extro-cellular domain A of fibronectin
EPC	endothelial progenitor cells
ER	endoplasmic reticulum
ERK	extracellular signal-regulated protein kinase
GLUT1	glucose tranporter 1
GWAS	genome-wide association study
iE-DAP	D-γ-glutyamyl-meso-diaminolimelic acid
IRAK	IL-1 receptor-associated kinase
JNK	c-Jun N-terminal kinase
LDL	low-density lipoprotein
Mal1	maltase alpha-glucosidase
MAPK	mitogen-activated protein kinase
M-CSF	macrophage colony-stimulating factor
MDP	muramyl dipeptide
NLR	NOD-like receptor or Nucleotide-binding domain, leucine-rich repeat-containing proteins
NOD1	nucleotide-binding oligomerization domain containing 1
NOD2	nucleotide-binding oligomerization domain containing 2
PAMP	pathogen associated molecular pattern
PI3K	phosphoinositide 3-kinase
PPAR	peroxisome proliferator-activated receptor
PRR	pattern recognition receptor
RIG-I-like receptor	retinoic acid-inducible gene like receptor
RIP3	receptor interacting protein 3
ROS	reactive oxygen species
SDMA	symmetric dimethylarginine
SMC	smooth muscle cells
TAK	transforming growth factor beta-associated kinase 1
TIR domain	Toll/IL-1R homology domain
TLR	Toll-like receptor
VSMC	vascular smooth muscle cell
WHO	world health organization
αP2	adipocyte fatty acid-binding protein

1 INTRODUCTION

1.1 ATHEROSCLEROSIS

Cardiovascular disease (CVD)

Cardiovascular diseases (CVD) including ischemia heart disease and stroke are the leading cause of death worldwide [1]. In Sweden, CVD caused 42% of total deaths, and in China the figure is 38% of total death as reported by World Health Organization (WHO) in 2010. Although the mortality has declined in many European countries over the decades, it is rapidly increasing in developing countries, where more than 80% of CVD mortality occurs.

Multiple genetic factors contribute to CVD. The evidence includes that several Mendelian dyslipidemia syndromes cause familial prevalence of early-onset CVD. Besides, premature atherosclerotic CVD in one parent confers a 3-fold increased risk in the offspring. Furthermore, genomic DNA variants confer risk for CVD as so far over 5,500 SNPs have been associated with CVD at $p < 10^{-5}$ by genome-wide association study (GWAS) [2]. GWAS study may provide relevant hints for understanding human disease, however the disease-causing SNPs remains to be verified in functional studies.

Environmental risk factors of CVD include the use of tobacco, inadequate physical activity, unhealthy diet, and psychosocial stress. Life style changes require intensive public health and individual life-long preventive efforts. It is uncertain whether CVD can be avoided completely by preventive efforts [3].

Atherosclerosis

Atherosclerosis develops insidiously throughout life from fatty streaks to advanced lesion and some of the plaques, not all, progress to cause thrombotic complications including acute coronary syndromes and stroke, two most common CVDs. The term ‘athero-sclerosis’, originated from Latin, refers to lipid core and fibrotic cap which are the structure of advanced atherosclerotic plaques in the intima of arteries. The transition from asymptomatic atherosclerosis to the sudden thrombosis complications is intensively discussed and reviewed in [4]. The paradigm shifts over the last decades from 1) the development of atherosclerotic plaques causes progressive stenosis of the lumen, and finally the critically stenotic lumen is occluded by thrombus, to 2) atherosclerosis is a chronic inflammatory disease developing on the basis of sub-endothelial lipid retention [5]. Culprit lesion does not cause stenosis, but

rather undergoes rupture driven by inflammation or superficial erosion which account for the thrombotic complications [4]. Vulnerable or unstable atherosclerotic plaques are histological characterized as large lipid-rich necrotic core, thin fibrous cap, massive inflammatory macrophages and few VSMCs in fibrous cap, outward remodeling, intraplaque vasa vasorum, intraplaque hemorrhage, and calcification [6]. The characterization of vulnerability in carotid plaques predicts the risk of cardiovascular disease outcome [7, 8]. Statin therapy increases fibrous cap thickness and stabilizes the vulnerable plaques [9, 10], but is still far from eliminating the disease, which brings two challenges to the future research: understanding pathogenesis of the disease in the statin era as the disease changes over the time [11], and delineating the complexity of the pathogenesis accounting for atherothrombotic complications. Interestingly, superficial erosion of plaques, lacking endothelial layer but without features of vulnerability nor conclusiveness, is estimated to account for a third of cardiovascular events [12]. In the light of this paradigm shift, this thesis work discusses the functions of pattern recognition receptors (PRRs) with regard to not only the changes in lesion size, but also the composition of the atherosclerotic plaque and the arterial inflammatory responses.

1.2 INNATE IMMUNITY IN ATHEROSCLEROSIS

Inflammation and immune mechanisms are crucial in the pathogenesis of atherosclerosis and link many traditional risk factors to altered arterial functions [13]. Innate immune cells such as monocytes and macrophages, neutrophils [14, 15], dendritic cells [16, 17], and mast cells [18, 19] are critical to atherosclerosis development. Non-professional immune cells in the vasculature such as EC and VSMC also take part in the disease not only as physical barrier but also by acquiring innate immune functions. In this part we will mainly focus on the recent progress on monocytes and macrophages in atherosclerosis, and EC, VSMC and mast cells are discussed under Section 1.4. A more comprehensive review on innate immunity in atherosclerosis is found in [20-22].

Monocytes in atherosclerosis

Monocytes are currently classified into pro-inflammatory and anti-inflammatory monocytes, which are CD14+CD16+ and CD14++CD16- monocytes in human or CCR2+CX3CR1^{lo}Ly6C^{hi} and CCR2-CX3CR1^{hi}Ly6C^{lo} monocytes in mice [23, 24]. Ly6C^{hi} monocytes are differentiated from hematopoietic stem cells and progenitor cells which relocate from bone marrow to extramedullary sites such as spleen [25]. Ly6C^{hi} monocytes

adhere to activated endothelium and accumulate and differentiated into macrophages rapidly (<24 hours) in atherosclerotic plaques [26]. Ly6C^{hi} monocytes rely on CX3CR1, CCR2 and CCR5 to be recruited to atherosclerotic plaques, while Ly6C^{lo} monocytes recruitment is partly dependent on CCR5 [27].

Ly6C^{hi} monocytes seem to be more important to atherosclerosis because ApoE$^{-/-}$ mice fed a high-fat diet increase the number of circulating Ly6C^{hi} monocytes gradually and dramatically, and they infiltrate more than Ly6C^{lo} monocytes into atherosclerotic plaques [26]. CCR2$^{-/-}$ ApoE$^{-/-}$ mice in absence of Ly6C^{hi} monocytes develop less atherosclerosis than ApoE$^{-/-}$ mice [26]. Moreover, myocardial infarction in ApoE$^{-/-}$ mice with chronic Ly6C^{hi} monocytosis results in increased debris and necrotic tissue and decreased α-actin and collagen compared with ApoE$^{+/+}$ mice [28]. Ly6C^{hi} monocytes exhibit proteolytic and inflammatory function, and Ly6C^{lo} monocytes express higher levels of vascular endothelial growth factor in myocardial infarction [28]. Ly6C^{lo} monocytes scavenge microparticles and recruit neutrophils to mediate necrosis of endothelial cells in kidney cortex [29]. CX3CR1-CX3CL1 interactions are an essential survival signal to Ly6C^{lo} monocytes, and CX3CR1 or CX3CL1-deficience are protective from atherosclerosis [30, 31]. But since CX3CR1 are expressed in all blood monocytes with different levels in the two subsets, this is not conclusive for the role of Ly6C^{lo} in atherosclerosis.

Macrophages in atherosclerosis

Macrophages are heterogeneous and the subsets included at least classical activated macrophages (M1), alternatively activated macrophages (M2), Mox, M4, MHem, MHb, based on the current understanding [32]. In response to LPS and IFN-γ *in vitro*, macrophages become M1 which produce IL-12 and reactive nitrogen and oxygen intermediate, while in response to IL-4, macrophages become M2 which express high levels of scavenger, mannose and galactose receptors, and some M2 produce IL-10 [24]. Various other stimuli may take part in polarization of macrophages. For example, M-CSF and GM-CSF favor polarization toward M2 and M1 respectively [33]. In human atherosclerotic plaques, rupture-prone shoulder regions are rich in M1, fibrous caps have equal amount of M1 and M2, foam cells incorporate individual M1 and M2 markers, adventitia are rich in M2 [34]. Macrophages up-regulate the expression of scavenger receptor and secretion of ApoE, now classified as M2 subsets, in response to M-CSF secreted by endothelial cells (ECs) and smooth muscle cells (SMCs) upon inflammatory stimulation such as LPS, IL-1α, and TNF-α [35]. Oxidized

phospholipid induces Mox by up-regulation of Nrf2-mediated expression of stress response genes [36].

The association between macrophage subsets and atherosclerosis is an attractive question. In aortas from $Ldlr^{-/-}$ mice fed an atherogenic diet for 30 weeks, M1, M2, Mox, comprise about 40%, 20%, 35% of F4/80+CD11b+ macrophages. Interestingly, 15% of macrophages express both Mox and M1 markers, and 5% macrophages express Mox/M2 markers [36]. CD68+ macrophages in regressing plague up-regulate genes associated with M2 phenotype (Arginase I, CD163, and C-lectin receptor) and contractile apparatus, and down-regulate genes related to adhesion [37]. Proof-of-principle experiments on the function of various macrophage subsets are needed in the future.

Evidences on lesional macrophage origin are starting to emerge. Although $Ly6C^{hi}$ monocytes can be recruited into atherosclerotic plaques at a higher level than $Ly6C^{lo}$ monocytes [27], lesional macrophages have been suggested to originate mainly from local proliferation of resident macrophages instead of differentiation from infiltrated monocytes [38].

1.3 PATTERN RECOGNITION RECEPTORS IN ATHEROSCLEROSIS

The innate immune system recognizes the structures shared by classes of microbes (pathogen associated molecular patterns (PAMPs)) or damaged cells (damage-associated molecular patterns (DAMPs)). The receptors recognizing these structures are named pattern recognition receptors (PRRs) [39]. PRRs are expressed on phagocytes, dendritic cells, lymphocytes, epithelial cells, and endothelial cells, and are located on cell surface, endosome, or in cytosol [39].

The history of recognition of PRRs as immune sensors is inspiring and provides a good example of using knowledge from model system. In early and mid-90th, immunologists recognized cytokine triggered NF-κB as an important pathway in pathogen-induced inflammation, but were puzzled by how immune response is triggered at the first place. By paralleling the mammal IL-1 induced NF-κB activation and Drosophila dorsoventral pathway (illustrated in table 1, summarized from [40]), they proposed that dorsoventral pathway, previously recognized to mediate embryonic dorsoventral polarity, was involved in Drosophila immune response [41].This hypothesis was supported later by the observation that overexpression of Toll, which shared the similar domain with IL-1R (later named TIR), up-regulates anti-microbial peptide production in Drosophila blood cells [42]. Jules Hoffmann *et al* provided a key evidence of by using Toll mutant Drosophilla, confirming that

Toll is a sensor of fungi for activating host defense in Drosophila [40]. This discovery was soon translated back from insects to human. Medzhitov and Janeway et al cloned human homologue of the Drosophila Toll (now named TLR4) and verified that TLR4 activates NF-κB pathway and thereby induce inflammatory cytokines and co-stimulatory molecules [43]. Bruce Beutler *et al* published that the LPS-resistant mouse strains C3H/HeJ and C57BL/10ScCr harbor missense or null mutation of TLR4 gene [44]. Jules Hoffmann and Bruce Beutler were awarded with 2011 Nobel Prize in Physiology or Medicine 'for or their discoveries concerning the activation of innate immunity'[45].

Table 1. Discovery of TLRs by paralleling mammal NF-κB pathway with Drosophila dorsoventral pathway.

	NF-κB	**Dorsoventral Pathway**
Species	Mammal	Drosophila
Family	Rel*	Rel*
Translocation	Cytoplasma to nucleus	Cytoplasma to nucleus
Inhibitor	I κB	Cactus2#
Activator	IL-1R, Toll-like receptor?	Toll-receptor
Binding site	NF- κB	NF- κB- like site
Downstream	IRAK (protein kinase)	Pelle (protein kinase)
Gene product	Host defense	Host defense

*Rel: rapidly inducible transactivators, #Cactus 2 is structrually related with I κB.

PRRs are expanding rapidly since the discovery of TLR4. PRRs include scavenger receptors (SRs), TLRs, NLRs, C-type lectin receptors, pyrin, HIN domain-containing family members, and RIG-I-like receptors, and a range of newly described cytosolic nucleic acid sensors as reviewed in [46]. To date, 10 members have been identified in TLR family, and 22 intracellular proteins has been identified in NLR family in human.

A more general review on pattern recognition receptor in atherosclerosis can be found in [47, 48]. Among PRRs, SRs are involved in phagocytic clearance by macrophages and thus extensively studied in foam cell formation [49]. TLR2 and TLR4 are the best characterized

signaling receptors in the context of atherosclerosis [48]. Studies on NLR members in atherosclerosis, such as NLRP3, are emerging because of their role in sensing cholesterol crystal and mediating sterile inflammation [50]. NOD1 and NOD2 are intensively studied in inflammatory bowel disease which is another chronic inflammatory disease [51, 52]. The following part will focus on the role of TLRs and NLRs in atherosclerosis.

1.4 TLRS IN ATHEROSCLEROSIS

1.4.1 Expression of TLRs

TLRs expression in professional immune cells is summarized from [53, 54] in table 2. In general, innate immune cells express a broader number of TLRs than adaptive immune cells [53]. TLRs are therefore expressed at high levels in tissues that are rich in immune cells, such as peripheral blood leukocytes and spleens, as well as in tissues exposed to the external environment, such as lungs [54]. Some TLRs are also highly expressed in pancreas, placenta and ovaries [54]. Non-professional immune cells such as endothelial cells also express low levels of TLRs [55]. Of note, TLR expression is inducible in pathophysiological conditions. For example, patients with hepatitis C have increased TLR7 and TLR9 expression on CD4+ T cells compared with healthy controls, and increased TLR2, TLR4 and TLR9 expression on all T cells [56].

Table 2. The expression of TLRs in immune cells and in tissue.

TLRs	Immune cell Expression	Tissue Expression
TLR1	Mo, Mac, DC, B	PBL, Sp, Lu, Pa
TLR2	Mo, Mac, DC, B	PBL, Sp, Lu, Pa, Ov
TLR3	Mo, Gr, B, T, NK, DC, IE	Pl, Te, Lu, Pa
TLR4	Mo, Mac, DC, MC, IE	PBL, Sp, Lu, Pl
TLR5	Mo, Mac, DC, IE	Ov, PBL, Lu, Pr
TLR6	Mo, Mac, DC, B	PBL, Sp, Lu
TLR7	Mo, Mac, DC, B, T	Pl, Lu, PBL, Sp
TLR8	Mo, Mac, DC, MC	PBL, Lu, Sp, Pl
TLR9	Mo, Mac, DC, B, T	Sp, Ov, PBL, Th
TLR10	Mo, Mac, DC	Sp

Mo, monocytes; Mac, macrophages; DC, dendritic cells; Gr, granulocytes; MC, mast cells; B, B cells; T, T cells; IE, intestinal epithelium. Lu, lung; GI, gastrointestinal tract; PBL,

peripheral blood leukocytes; Sp, spleen; Pa, Pancreas; Ov, ovary; Te, testis; Pl, placenta; Pr, Prostate; Th, Thymas.

The detection of TLR expression in atherosclerotic tissue is of interest because it provides the first hints for the relevance of TLRs in atherosclerosis. TLR4 was the first recognized PRR in atherosclerotic plaques. It is mainly expressed in macrophages and can be up-regulated by ox-LDL [57]. Our group showed previously that the mRNA of TLR1, TLR2, TLR6, TLR7, and TLR8 are significant increased, and TLR3, TLR4 and TLR5 have an increased tendency, but TLR9 expression has a decreased tendency in carotid atherosclerotic plaques compared with internal mammary arteries as controls [55]. TLR2 and TLR4 mRNA expression increase with age in atherosclerosis-prone aortic arch of $ApoE^{-/-}$ mice. After 15-week age, the mRNA levels in $ApoE^{-/-}$ mice are higher than wild-type mice [58]. Moreover, monocyte TLR4 expression is associated with plaque stability because the expression of TLR4 and TLR common adaptor protein MyD88 in circulating monocytes is increased from patients with acute coronary syndrome (ACS), including myocardial infarction and unstable angina, than healthy individuals and patients with stable angina [59]. Furthermore, monocytes from acute coronary syndrome patients have higher response to LPS in the forms of secreting pro-inflammatory cytokine IL-12 and expression of co-stimulating molecue B7-1[59]. As the knowledge of monocyte heterogeneity gathered [60], the increased TLR4 expression in monocyte subsets in acute myocardial infarction patients was further dissected to be mainly on CD14+CD16+ pro-inflammatory monocytes [61]. Furthermore, the expression of TLR4 is in ACS thrombi >ACS blood >healthy control blood, indicating a enrichment of monocytes bearing TLR4 in thrombi [62]. It remanins unclear whether theseTLR4- rich monocytes have a systemic or local effect on plaque rupture. In contrast to these results, a recent study found no correlation of TLR4+ monocytes with cardiovascular events or cardiovascular death in patients with chronic kidney disease stage V receiving dialysis, indicating that additional pathogenic pathways may cause cardiovascular events in this high risk group of patients [63].

1.4.2 TLR downstream signals

TLRs belong to TLR/IL-1 receptor superfamily because TLRs contain a TIR domain which is similar to IL-1 receptor. Ligation of TLR/IL-1 receptor recruits TIR domain-containing adaptor protein MyD88, and initiates formation of a complex containing protein kinases including IL-1 receptor-associated kinase (IRAK) 1, IRAK4, and transforming growth factor beta-associated kinase (TAK) 1. The complex further activates NF- κB and mitogen-activated protein kinase (MAPK) pathways. Ligated TLR3/4 are also able to interact with another TIR

domain containing adaptor protein, TRIF, and activate IRF-3. TLR7/8/9 can activate IRF-7 via MyD88/TRAF3/TBK1-dependent pathway [48]. Activation of TLRs eventually lead to activation of anti-microbial killing mechanisms, production of cytokines and chemokines, maturation of antigen presenting cells, and recruitment of the adaptive immune response in the context of infection [64].

In atherosclerotic plaques, TLR2 and TLR4 induce NF- κB activation[55]. In endothelial cells, this leads to upregulation of adhesion molecule VCAM-1 which promotes the adhesion of moncytes [65]. In macrophages, this provides pro-survival signals in macrophages, whereas inhibiting macrophage NF-κB results in increased cell death and accelerated atherosclerosis [66]. It is rather complex to predict the overall effect on atherosclerosis of the different signal pathways elicited by TLRs ligation in various cell types.

1.4.3 Regulation of TLRs

TLR activation is under tight control. One example is that TLRs are normally not over-activated on intestinal epithelial cells which are in direct contact with microbiota [64]. Another example is that LPS transiently supress TLR4 mRNA expression in macrophages, which may contribute to endotoxin tolerance [44].

In the context of atherosclerosis, increasing evidences suggest the regulation of TLR expression or signaling by hypercholesterolemia. Cholesterol efflux gene ABCA1 and ABCG1 supress the expression and the function of TLR2 and TLR4 [67]. $ABCA1^{-/-}ABCG1^{-/-}$ macrophages express higher levels of TLR2 and TLR4 and have higher response to LPS stimulation compare with wild-type macrophages [67]. The effect is mediated by membrane cholesterol since it is enhanced by increasing membrane cholesterol level and abolished or decreased by delpleting membrane cholesterol by cyclodextrin [67]. Interestingly, ABC transporters deficient macrophages form more caveolae upon acetylated-LDL loading [67]. The role of caveolae lies not only in transcellular movement of molecules but also as a mediator in cell signaling, notably as a regulator of TLR4 signaling through eNOS and IRAK4[68]. However the relevence remains to be confirmed *in vivo.*

Another class of lipid-related negative TLR regulators are peroxisome proliferator-activated receptors (PPAR) ligands including unsaturated fatty acid. PPAR ligands exert anti-inflammatory effect on variaties of cell types including macrophages, T cells, dendritic cells, endothelial cells and smooth muscle cells, and the molecular mechanisms varies among cell types and PPAR members [69]. In macrophages [70] and smooth muscle cells [71], PPAR

gamma ligand inhibit TLR-stimulated inflammatory gene expression by interfering IRF3 signaling.

1.4.4 TLR ligands

PRR recognize pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs). Bacterial PAMPs includes LPS, flagellin, peptidoglycan, cyclic dinucleotide. Viral PAMPs includes viral fusion glycoproteins, dsRNA, ssRNA, and viral DNA (summarized in Table 3). Several pathogens have been associated with elevated risk of atherosclerosis, such as Clamydia pneumonia, Helicobacter pylori, Porphyromonas gingivalis, Cytomegalovirus, Epstein-Barrr-virus, Human immunodeficiency virus, Herpes simplex virus 1 and 2, Hepatitis A and B, and Influenza A virus [72]. In line with this, vaccination against influenza virus decreases the risk of acute coronary syndrome [48]. Gut commensal bacteria release peptidoglycan into blood stream during bacteria amplification, and atherosclerotic plaques contain detactable peptidoglycan, the ligand for TLR2, NOD1 and NOD2 [73]. These observations lead to the hypothesis that PAMPs may be involved in pathogenesis of atherosclerosis.

The proposed role for PAMPs in atherosclerosis is tested in experimental atherosclerotic models. Intraperitoneal injection of *Lactobacillus casei* cell wall extract is able to induce vasculitis and myocarditis after 1-2 weeks. Coronary lesions could be treated by IL-1 receptor antagonist if administrated less than 3 days after injection of cell wall extract [74]. Furthermore, *Lactobacillus casei* cell wall extract could accelerate atherosclerosis in $ApoE^{-/-}$ or $Ldlr^{-/-}$ mice with high fat diet. Administration of IL-1 receptor antagonist from day 1 to day 5 inhibits the acceleration of atherosclerosis [75]. However, atherosclerosis in germ-free $ApoE^{-/-}$ mice is not different from animals raised in ambient levels of microbial challenges, indicating that commensal bacteria is not necessary for the development of atherosclerosis in immune sufficient mice [76]. Since the housing conditions of mice differ between animal houses, contributions from commensal microbiota to atherosclerosis cannot be completely excluded.

On the other hand, the endogenous ligands of PRRs are hypothesized to contribute to atherogenesis. Atherosclerotic plaques contains large amount of cholesterol crystals, modified proteins and lipids, and cell debris, and degraded extracellular matrix due to intensive tissue remodeling. These DAMPs could act as endogenous ligands to PRRs and elicit inflammation in atherosclerosis. It has been shown that oxidized LDL (oxLDL) and modified phospholipids activate TLR4, and cholesterol crystals activate NLRP3 inflammasome. Heat shock proteins

(Hsp60, Hsp70, Gp96) and high-mobility group box 1 (HMGB1) released by stressed cells or necrotic cells can be detected by TLR2 and TLR4 [77-80]. Endosomal TLRs such as TLR3 can detect mRNA [81], while TLR7/9 can detect RNA/DNA-containing immune complex [82]. Extracellular matrix protein fibronectin derived extra domain A activates TLR4 [83]. These are the potential endogenous ligands to PRRs that affect atherosclerosis as reviewed in [48].

Modified LDL has caught great attention as endogenous ligands. Biotin-mmLDL (minimally modified low-density lipoprotein, endotoxin< 0.1ng/ml or 1EU) binds to macrophages in a TLR4/MD-2 and CD14-dependent manner [84]. mmLDL (endotoxin< 2.5 pg/ml or 0.025 EU) stimulating murine peritoneal cells induces RANTES secretion at a relatively low magnitude (100 pg/ml) and with the peak at 2 hours [85]. mmLDL also induces IL-6 (<10 pg/ml), TNF-α (<100 pg/ml) within one hour [86]. Cholesterol ester hydroperoxide in mmLDL is identified as an endogenous ligand for TLR4 [87]. Moreover, mmLDL stimulation increases F-actin concentration, a liner polymer microfilament and are essential for cell mobility, in a TLR4, CD14- spleen tyrosine kinase (Syk)-dependent pathway [88, 89]. mmLDL induced cytoskeleton rearrangement is accompanied by macropinocytosis, a process that facilitate small molecule or native LDL uptake[87], and leads to decrease phagocytosis of apoptotic cells, and increased uptaken of monomeric oxLDL [84]. mmLDL can also activate reactive oxygen species (ROS) production via Syk, PLCγ1, protein kinase C, and NOS2 pathway in a TLR4-dependant but MyD88-independent manner [85]. Furthermore, mmLDL activates TLR4- independent PI3K pathway through unknown PRRs [86]. It is possible that complex molecules such as modified LDL embeds several endogenous ligands that bind to more than one PRR.

In contrary to the majority of the results showing mmLDL as endogenous PRR ligands, Kannan et al found endotoxin free mmLDL alone is not able to acitivate and even supress cytokine production in human monocytes or macrophages cultured for 3 and 6 hours [90]. The discrepancy is unlikely because of containmination of TLR4 agonist/antagonists, as both studies have performed endotoxin test and found rather low levels of enodtoxin. However, this study brings up a key question that needs to be addressed in this emerging field that whether or not the proposed endogenous ligands may posesss genuine TLR-activating potential or instead reflect the contamination of exogenous ligands such as LPS.

Table 3. Exogenous and endogenous ligands of selected number of TLRs, modified from [91] and [48].

TLR	cellular compartment	Exogenous Ligand	Endogenous Ligand
TLR1	Plasma membrane	Triacyl lipoprotein	*
TLR2	Plasma membrane	Lipoprotein	HSPs, HMGB1, ApoCIII, SDMA
TLR3	Endo/lysosome	dsRNA	mRNA
TLR4	Plasma membrane	Lipopolysaccharide	mmLDL, oxLDL, modified phospholipids, HSPs, HMGB1, Fibornectin-derived extra domain A
TLR5	Plasma membrane	Flagellin	*
TLR6	Plasma membrane	Diacyl lipoprotein	*
TLR7/ TLR8	Endo/lysosome	ssRNA	RNA/DNA Immune complex
TLR9	Endo/lysosome	CpG-DNA	RNA/DNA Immune complex
TLR10	Endo/lysosome	*	*
TLR11	Plamsa membrane	Profilin-like molecule	

* remains to be determined.

1.4.5 TLRs in regulation of foam cell formation

Cellular lipids come from cholesterol-rich lipoprotein particles such as LDL, oxLDL or mmLDL, fatty acid catalyzed from tryglycerides-rich lipoprotein particles (VLDL) by endothelial lipase, or *de novo* lipid synthesis. Insufficient HDL removal of extra triglycerides or cholesterol esters contributes to the accumulation and formation of lipid droplets in macrophags, namely foam cell formation [92]. Several studies have shown that TLR4 activation promotes cholesterol esters accumulation but the mechanistic explanations are controversial. The possible mechanisms are summarized in figure 1.

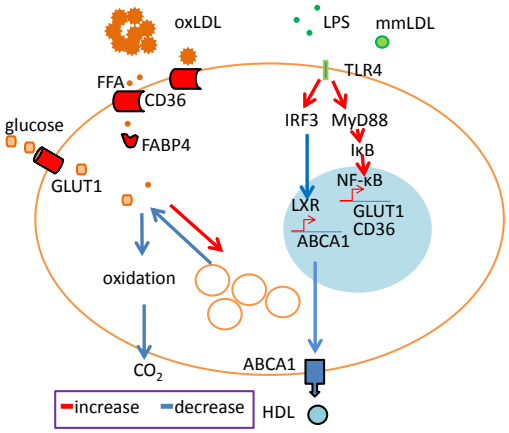

Figure 1. Possible mechanisms of TLR4 regulating foam cell formation. LPS or mmLDL activate TLR4 and downstream MyD88-NF-kB or IRF3 pathways. TLR4 activation increases CD36, glucose transporter 1 (GLUT1) and suppresses LXR-regulated genes such as ABCA1. This leads to increased uptake of oxLDL, fatty acid and glucose, decreased oxidation of glucose and fatty acid, decreased hydrolysis of triglycerides and decreased efflux of cholesterol.

TLR4 activation can increase lipid uptake which contributes to foam cell formation. Oiknine et al showed that LPS increased LDL uptake, and cellular cholesterol synthesis, but doesn't alter HDL-mediated efflux, and lead to accumulation of triglycerides and cholesterol esters in macrophages [93]. Miller et al showed that mmLDL activate TLR4/MD-2 and increases scanvenger receptor CD36 and thus increases monomeric oxLDL uptake [84]. For fatty acid uptake, LPS induces the expression of fatty acid-binding protein (FABP)s FABP4 (also named αP2) [94] and FABP5 (also named Mal1) [95] in macrophages, which may lead to increased uptake of fatty acid and thus accelerate atherosclerosis. Boord et al showed that FABP4$^{-/-}$FABP5$^{-/-}$ApoE$^{-/-}$ mice develop less atherosclerosis in early and advanced stage than ApoE$^{-/-}$ mice. These mice also have decreased plasma cholesterol and triglycerides, and improved insulin sensitivity and glucose tolerance. [96].

Insufficient cholesterol efflux is also an essential mechanism in foam cell formation. Castrillo et al showed that TLR3 and TLR4 ligation by Poly I:C or LPS inhibite the binding of nuclear receptor liver X receptor (LXR) to LXR element on the promoter of the efflux genes such as ABCA1, ABCG1, ApoE, SREBP-1c, fatty acid synthase (FAS). This leads to decreased HDL-dependant cholesterol efflux and increased foam cell formation. TLR3 and TLR4 induced repression of LXR seems independent of adaptor MyD88, NF-κB activity, or cytokines such as TNF-α, IL-1β, or IFNs, but dependent on up-regulation and activation of IRF3 expression [97]. TLR3 or TLR4 ligation inhibits LXR but not PPARγ or PPARδ, indicating a specific effect of TLR signaling on LXR [97]. In reverse, ABC transporter-

deficient macrophages increase signaling via TLR, MyD88/TRIF and expression of inflammatory genes [67].

Another possible mechanism of TLR4-indcued foam cell formation is that TLR4 stimulation can alter cell metabolism process such as glucose metabolism, lipid oxidation, *de novo* synthesis, and lipolysis and thus promote accumulation of triglycerides resulting in foam cell formation. Funk et al found that LPS alone can induce triglyceride accumulation even without exogenous lipid addition to macrophage culture. LPS can further enhance free fatty acid loading induced foam cell formation [98]. Later, the same group addressed several different mechanisms by which LPS induced triglycerides accumulation and thus foam cell formation. First, LPS through TLR4 and MyD88 up-regulates the expression of glucose tranporter 1 (GLUT1), and induces the accumulation of GLUT1 protein on plasma membrane, which increased uptake of glucose in macrophages. Second, LPS decreases glucose oxidation to CO_2, and increases glucose metabolized into lactate, which may also metabolized into lipid and accumulate in the cells. Third, LPS up-regulates the expression of scavenger receptor CD36, and increases uptake of fatty acid, as mentioned above in [84]. Similar as glucose, LPS decreased fatty acid oxidation. LPS down-regulates the expression of carnitine palmitoyltransferase 1(CPT1)α and CPT1β which mediate carnitine-dependent transport of fatty acid to mitochondria for oxidation. Instead, fatty acids are subjected to synthesize into glycerol lipid by upregulated enzymes 1-acylglycerol-3-phosphate O-acyltransferase 9 (AGPAT9) and diacylglycerol O-acyltransferase 2 (DGAT2) upon TLR4 activation. TLR2 and TLR3 ligation also induce upregulation of AGPAT9 and DGAT2. However, the signaling pathway is less clear since the effect is independent of MyD88 or cytokines such as TNF-α or IL-1. Fourth, LPS decreases hydrolysis of accumulated triglycerides [99]. TLR4 regulated metabolism may be a general mechanism of pathophysiology applicable to other cells than macrophages. However, knockout/knock-down study is needed to verify the key mechanisms.

Howell et al showed that LPS enhances oxLDL-induced foam cell formation, and the effect is mediated via TLR4 in macrophages. They also observed LPS treated foam cells tends to cluster together, indicating that cell-cell interaction may take place [100].

1.4.6 TLRs and endothelial dysfunction

Endothelial cells (ECs) are located at the interface in direct contact with the blood flow and sense the physical hydrodynamics and chemical mediators of the blood flow. ECs produce nitric oxide which induces vasodilation by opposing EC-derived vasoconstrictor angiotensin

II and endothelin, reduces platelet and leukocyte adhesion, and inhibits VSMC proliferation [101]. Sheer stress, oxidized lipids and inflammatory stimuli induce endothelial dysfunction and lead to increased endothelial permeability, platelet aggregation, and leukocyte adhesion which exacerbate atherosclerosis [101]. Many risk factors of atherosclerosis cause endothelial dysfunction, such as smoking [102], type 2 diabetes and hypertension. Elevated sE-selectin, a marker of endothelial dysfunction, predicts type 2 diabetes [103]. Endothelial cells sense abnormal HDL via TLR2 and thereby increase superoxide and reduce nitric oxide production, which mediate endothelial dysfunction [104]. Thus endothelial dysfunction links many risk factors to atherosclerosis at cellular and molecular levels. Recently, the role of vasa vasorum, the microvasculature in adventitia and expanding from adventitia to intima in response to vascular injury, receives more and more attention in atherosclerosis research. Whether this is the result or the cause of atherosclerosis remains to be investigated and the current understanding is reviewed in [105].

ECs express functional pattern recognition receptors (PRRs), which sense pathogen associated molecular patterns (PAMPs), and release inflammatory cytokines [106-108]. Ablation of proinflammatory NF-κB pathway in ECs substantially reduces atherosclerosis [65]. Ligation of ECs TLR4 induces IL-6 production and enhances cross talk with monocytes [108]. One difference between macrophages and endothelial cells responses to TLR4 is that macrophages activate both MyD88 and TRIF pathways, while endothelial cells lacks protein TRAM and therefore incapable of activating TRIF pathway. For example, LPS stimulation in endothelial cells doesn't induce TRIF-dependent gene, for example, CXCL10 [109].

TLR2 regulates endothelial functions such as repair after injury and anti-inflammatory capacity. TLR2 activation by SDMA in endothelial cells reduces NO production via reduced phosphorylation of Akt (Ser473) and subsequently enhanced phosphorylation of eNOS-inhibiting phosphorylation (Thr495) and reduced eNOS-activating phosphorylation (Ser1177), in a TLR1, TLR6, NF-kB independent pathway [104], indicating TLR2 activation leads to endothelial dysfunction by impairing NO production. Furthermore, endothelial TLR2 activation induces NAPDH oxidase to promote reactive oxygen species (ROS) production. Both exogenous TLR2 ligand Pam3CSK4 and symmetric dimethylarginine (SDMA), an endogenous TLR2 ligand in abnormal HDL from chronic kidney disease patients, induce ROS production in endothelial cells [104].

In contrast, TLRs seem to play a protective role in endothelial progenitor cells (EPC). Circulating EPCs are considered to contribute to re-endothelialization and may be benificial

to cardiovascular disease. Endothelial progenitor cells express TLR4, CD14, and MyD88, and LPS treatment promote EPC proliferation [110]. *P. gingivalis*, a periodontal pathogen, stimulates the mobilization of EPC from bone marrow to periferal and thus improve endothelial function and re-endothelization in a TLR2-dependent pathway [111].

1.4.7 TLRs in VSMC phenotype alteration

Similar as ECs, VSMCs also express functional PRRs. Therefore VSMC respond to inflammatory stimuli as innate immune cells in atherosclerosis. In response of IL-1, TNF-α, and LPS, VSMCs produce substantial M-CSF which promotes macrophage proliferation, differentiation and survival [35]. PRRs such as NOD2 are involved in VSMC homeostasis as $NOD2^{-/-}$ mice have increased neointimal hyperplasia formation after artery injury, and NOD2 is essential for VSMC proliferation and migration in response to PDGF-BB [112]. Direct contact between VSMCs and macrophages via CX3CL1/CX3CR1 has a synergistic effect in production of pro-inflammatory cytokines, chemokines, and MMP9 in both cell types [113].

VSMC apoptosis is induced by mast cell activation and subsequent release of chymase in the cap region of atherosclerotic lesions, and thus is important in plaque vulnerability. It has been shown that although TLR4 signal does not alter mast cell numbers but it is required for mast cell activation and IL-6 and chymase secretion. IL-6 acts in a autocrine and paracrine way and further promotes chymase production eventually leading to more apoptosis of smooth muscles cells [114].

In normal artery, VSMCs are present only in the media, however, in atherosclerotic lesions, they are also present in the intima. VSMCs incubated with free cholesterol decrease the expression of smooth muscle α actin, increase CD68 and MCP-1 expression, and accumulate intracellular lipids. The effect of cholesterol-induced up-regulation of CD68 and MCP-1 was partially mediated by TLR4 [115]. Besides, upon oxLDL stimulation, the expression of VSMC contract proteins such as smooth muscle α actin, calponin, myocardin, and SM22a is also shown to be down-regulated, and G-CSF and GM-CSF production are increased, mediated by TLR4 and CD36 [116]. These data indicates that TLR4 signaling is linked to the alteration of VSMC from contractile to proinflammatory phenotye in response to atherogenic stimuli. However, little is known about the relevance of the proinflammatory phenotype of VSMCs for atherosclerosis.

1.4.8 TLRs in necrotic core development

Endoplasmic reticulum (ER) stress is a proapoptotic stimuli of macrophage in advanced atheroma [117]. Exogenous TLR4 ligand LPS stimulates ER stress as evidenced by up-regulation of activating transcription factor 6 protein [118, 119] . It is partly medicated by insufficient chaperone GRP94 and GRP78 availability upon long-term (24-48 hours) LPS stimulation [118]. Endogenous PRR ligands such as oxidized phospholipids, oxLDL, saturated fatty acids, and lipoprotein(a) trigger CD36 and TLR2 and generate ROS, which results in apoptosis in ER-stressed macrophages. $CD36^{-/-}TLR2^{-/-}$ macrophages are more resistant to apoptosis induced by saturated fatty acids-rich diet. Furthermore, $TLR2^{-/-}TLR4^{-/-}$ bone marrow transplanted $Ldlr^{-/-}$ mice fed on high fat diet develop less macrophage apoptosis and plaque necrotic core than wild-type bone marrow transplanted mice [120].

Insufficient clearance of apoptotic cells could lead to secondary necrosis. As mentioned above, mmLDL induced TLR4 activation leads to macrophage cytoskeleton rearrangement and inhibites phagocytosis of apoptotic cells [84].This could lead to necrotic cell death in advanced atherosclerotic lesions.

Recently, TLR2, TLR3, TLR4, TLR5 and TLR9 was described to trigger a type of programmed cell death named necroptosis in the absense of caspase-8. The signaling pathway for TLR3 and TLR4 was explored in fibroblasts. TLR3 and TLR4 activate TRIF, which interacts with receptor interacting protine (RIP)3 kinase through a RIP homotypic interaction, and activates RIP3 downstream protein mixed linage kinase domain-like protein (MLKL), and results in necroptosis [121]. However, the relevence of necroptosis in atherosclerosis remains to be investigated.

1.4.9 TLR functions in atherosclerosis

TLR4 is the first innate immune receptor studied in atherosclerosis *in vivo*. $TLR4^{-/-}ApoE^{-/-}$ mice fed a western diet develope less atherosclerosis than $ApoE^{-/-}$ mice, albit no change in serum cholesterol. TLR4 deficiency is associated with reduced macrophage infiltration and activation in the lesion, and decreased CCL2 and IL-12 in the circulation [122]. Deficiency of MyD88, downstream adaptor of most TLRs, IL-1R and IL-18R, also decreases atherosclerosis accompanied by decreased chemokines and macrophages in the lesion in $ApoE^{-/-}$ mice fed a western diet [122, 123]. However, knocking-out of CD14, a co-receptor of TLR4, does not alter atherosclerosis [123]. In rat femoral cuff model, LPS-containing gel

increases atherosclerotic plaque size and external elastin lamina (EEL) area, indicating a local direct deleterious effect of TLR4 stimulation in atherosclerosis and vesular remodeling [124].

The role of TLR4 differs in various two-disease models. In atherosclerotic mice fed with a diabetogenic diet, atherosclerosis and LDL and VLDL levels in $TLR4^{-/-}Ldlr^{-/-}$ mice was decreased than $Ldlr^{-/-}$ mice, but glucose intolerance and obesity was not improved, indicating a deleterious role of TLR4 in atherosclerosis but not in diabetes and obese [125]. Since periodontitis is associated with atherosclerosis, Hayashi et al explored atherosclerosis in $TLR4^{-/-}$ $ApoE^{-/-}$ mice fed a chow diet infected with *Porphyromonas gingivalis,* a deleterious bacterial species in chronic periodental disease. The infection significantly exacerbates atherosclerosis in both $ApoE^{-/-}$mice and $TLR4^{-/-}ApoE^{-/-}$ mice fed a chow diet. Suprisingly, they found a larger increase in atherosclerosis in infected $TLR4^{-/-}ApoE^{-/-}$ mice compared with infected $ApoE^{-/-}$ mice [126]. These results indicate that TLR4 overall plays a protective role in atherosclerosis in the subjects with chronic infection, probably because of the protective effect of TLR4 in host defense.

The role of TLR2 in atherosclerosis is elucidated in a rigorous study by Mullick et al using four different models. In the first experiment, without any exogenous ligand stimulation, $TLR2^{-/-}$ $Ldlr^{-/-}$ knockout mice fed a high fat diet for 10 or 14 weeks develop less atheroslcerosis than $Ldlr^{-/-}$ mice. Secondly, to elucidate the role of different cellular TLR2 signal, they performed bone marrow transplantation from $TLR2^{-/-}$ or $TLR2^{+/+}$ mice to $TLR2^{-/-}$ $Ldlr^{-/-}$ or $Ldlr^{-/-}$ mice. They found regardless of bone marrow cell genotype, TLR2-deficiency on non-bone marrow derived cells protects from atherosclerosis. In the third experiment, they stimulated $Ldlr^{-/-}$ mice with TLR2 exogenous ligand Pam3CSK4, and found that the mice developed pronounced increased atherosclerosis than unstimulated $Ldlr^{-/-}$ mice, while there is no difference in response to Pam3CSK4 stimulation in $TLR2^{-/-}$ $Ldlr^{-/-}$ mice as expected. In the fourth experiment, to explore whether the response to exogenous ligand comes from bone marrow cell TLR2 signal, they performed bone marrow transplatation from $TLR2^{-/-}$ or $TLR2^{+/+}$ bone marrow cells to $Ldlr^{-/-}$ mice, and stimulate the chimeras with Pam3CSK4. They found that $TLR2^{+/+}$ chimeras respond to TLR2 stimulation similarly as $Ldlr^{-/-}$ mice in the third experiment, but $TLR2^{-/-}$ chimeras ($TLR2^{-/-}\rightarrow Ldlr^{-/-}$) lost the effect, indicating the bone marrow cell TLR2 signaling is important for exogenous ligand stimulated augmentation of atherosclerosis [127]. The deleterious role of TLR2 in atherosclerosi is verified in another atherosclerotic mouse model $ApoE^{-/-}$ mice [128]. Similar to TLR4, TLR2 stimulation with Pam3CSK4 increased neointima formation in C57/B6 mice and atherosclerosis in $ApoE^{-/-}$ mice in femoral cuff model [129]. However, a recent study shows that maybe TLR2 is not

important in advanced atherosclerosis. TLR2$^{-/-}$ApoE$^{-/-}$ mice develop less atherosclerosis in early age (18-week old) than ApoE$^{-/-}$ mice on chow diet or western diet, however, there difference disapears in a later age (36-week old) [115].

As discussed in previous chapters, TLR2 and TLR4 both can be activated by atherosclerosis-related endogenous ligand and signal through MyD88, and thus it is possible that they interact with each other. Overexpressing human TLR2 and TLR4 increased atherosclerosis in rabbit fed high cholesterol diet, but the effect of overexperssing either TLR2 or TLR4 is not detectable, indicating a synergistic effect of TLR2 and TLR4[130].

Unlike TLR2 and TLR4, TLR3 and TLR7 exert a atheroprotective role. TLR3$^{-/-}$ApoE$^{-/-}$ mice developed more atherosclerosis than ApoE$^{-/-}$ mice when fed a chow diet till age 15-week, but no change till age 30-week. In collar injury induced atherosclerosis model, TLR3$^{-/-}$ mice did not change intima/media ratio, although there is a increase in breaks in elastin lamina than C57/B7 [131]. The reason behind the protective effect of TLR3 in early atherosclerosis is not yet known. Similarly, TLR7$^{-/-}$ApoE$^{-/-}$ mice develop more atherosclerosis than ApoE$^{-/-}$ mice when fed a chow diet both till aged 18-week and age 26-week, indicating a protective role of TLR7 in both early and advanced atherosclerosis. The increased atherosclerosis in TLR7$^{-/-}$ ApoE$^{-/-}$ mice is associated with increased M1 macrophages and necrotic core, decreased fibrous cap, as well as increased Ly6C^{hi} monocytes in blood and spleen. Since peritoneal macrophages from TLR7$^{-/-}$ ApoE$^{-/-}$ mice secrete more inflammatory cytokines than ApoE$^{-/-}$ macrophages upon TLR2 stimulation, the author proposed that the mechanistic explanation is probably due to the compensation effect of up-regulation of other TLR siganalings [132]. However, the hypothesis needs to be examined *in vivo*.

1.4.10 Genetic evidence for TLRs in atherosclerosis

Despite of the massive experimental studies suggesting the importance for TLRs in athoersclerosis, affirming their pathogenic relevance to human atherosclerosis remains a challenge. Two functional polymorphisms of TLR4, Asp299Gly and Thr399Ile, were associated with lower risk of carotid atherosclerosis and myocardial infarction in some studies but not reproducible in others [133]. The interpretation of the negative results should take into consideration that the function of the investigated gene is not totally abolished and possibly even compensated. Also, cardiovascular events are influenced by multiple factors and thus may be not sensitive as an endpoint.

1.5 NLRS IN ATHEROSCLEROSIS

1.5.1 NLR subfamily

Nucleotide-binding domain, leucine-rich repeat-containing proteins (NLRs), represent a group of key sensors which functions as *bona fide* PRRs or adaptor molecules or regulators of signal transduction. NLR family includes at least 22 proteins in human and 33 proteins in mice. This section will focus on the role of NLR in inflammation and especially on the relevance of NOD1 and NOD2 to chronic inflammatory diseases such as atherosclerosis.

As its name implies, NLRs contain central nucleotide-binding and oligomerization domain and C-terminal leucine-rich repeat (LRR) domain. According to the structures of N-terminal domain, NLRs are classified into several subfamilies including the best characterized NLRC and NLRP. NLRC subfamily is characterized by consisting of a caspase activation and caspase-recruitment (CARD) domain at N-terminal, including nucleotide-binding oligomerization domain containing (NOD) 1, NOD2, and NLRC3-5. NLRP subfamily contains a pyrin domain (PYD) as N-terminal effector domain and includes NLRP1-14. Both CARD and PYD domain are involved in both apoptosis and inflammation [134]. According to the current knowledge of the main function, NOD1 and NOD2 are considered to mainly function as PRR, and NLRC4, NLRP1, and NLRP3, etc are considered inflammasome-forming NLRs due to their role in forming inflammasome.

1.5.2 NLR expression

Both NOD1 and NOD2 are expressed in a wide variety of tissue types. In adult humans, NOD1 mRNA is expressed abunduntly in heart, skeletal muscle, spleen, ovary, and to a lesser extent in placenta, lung, liver, kidney, thymus, small intestine, colon, and peripheral blood leukocytes. At stage 15.5 (day 15.5), mouse embryo express NOD1 mRNA in liver, thymus, cortical region of kidney, lung, gut epithelium and in certain regions of central nervous system [135]. Unlike NOD1, the NOD2 mRNA expression seems absence or low in various human tissues, except in peripheral blood leukocytes [136], however, NOD2 protein could be detected in skin, small intestine, colon, trachea, salivary gland, kidney, and bone marrow [137].

At cellular level, unlike NOD1, which is widely expressed, NOD2 is found in restricted cell types including monocytes in peripheral blood [136], peneth cells in small intestine [138], various epithelial cells in digestrion tract [139], and keratinocytes [137] in humans. The expression of NOD1 and NOD2 are inducible even in the cells that normally express none or

little of these genes. For example, NOD1 expression can be induced by *S. aureus* and Salmonella in osteoblast while NOD2 can only be induced by Salmonella [140]. Subcellularly, most NLRs are expressed in cytosol, with the exception of NLRX1 (NOD9) which is localized in the outer membrane of mitochondria [141].

1.5.3 NLR Ligands

NOD1 and NOD2 sense different structures in bacterial peptidoglycan. The minimum NOD1 stimulating structure is D-γ-glutyamyl-meso-diaminolimelic acid (iE-DAP) [142, 143], and the minimum stimulating structure for NOD2 is muramyl dipeptide (MDP). However, although these are generally called NOD1 or NOD2 ligand, there is no evidence of direct binding in a manner consistant with other PRRs [144].

Evidences indicate that NOD1 and NOD2 expressed in the vessels or unexposed organs could be stimulated even without systemic bacterial infection in humans. NOD1 and NOD2 stimulatory molecules were abundant in foods and soil. For example, high human NOD1 stimulatory acitivity and some human NOD2 stimulation have been detected in Natto, a traditional Japanese food product derived from soybeans fermented with *Bacillus. subtilis* natto, while *Lactobacillus plantarum* contain iE-DAP structure but does not have NOD1-stimulatory activity [145]. NOD1 ligands were highly stable at extreme pH (acidic or basic) and boiling conditions. Recycling and turnover of bacterial cell wall peptidoglycan results in release of peptidoglycan fragment into the environment [146]. Bacteria culture supernatant exhibited higher NOD1 stimulatory activities than cell bodies, indicating the possibility of a stimulatory effect even without bacteremia[145]. Peptidoglycans can translocate from gut to circulation and bone marrow and activate oxidative and non-oxidative killing by neutrophils [147].

Synthetic NOD1 and NOD2 ligands are essential tools to study the function of the receptors for at least two reasons. First, many mechanistic studies use laboratory mice raised under specific pathogen free environment and thus the presence of NOD1 or NOD2 ligand in the circulation is uncertain. Second, the published studies on $NOD1^{-/-}$ or $NOD2^{-/-}$ mice often requires an additional triggers, such as bacterial infection, to induce a specific phenotype. Interestingly, NOD1 ligand was initially synthesized and used in vaccine research even before NOD1 was characterized. Early in 1982, during screening for immunostimulants by Fujisawa Research Laboratory, FK156 was isolated and found to be a potent immunostimulatnt, and FK565 was synthesized with similar structure to mimic peptidoglycan

fragments [148]. Thirty years later FK565 was found to be NOD1-specific agonist [146] and frequently used as NOD1-specific ligand in functional studies since then.

Peptidoglycans are associated with atheroslcerosis. Gut metagenome study shows that patients with symptomatic carotid plaques leading to vascular events have enriched genes encoding peptidoglycan synthesis of gut microbiota compared with age- and sex- matched controls without cardiovascular health problem [149]. Peptidoglycans are present in some atherosclerotic plaques in carotid artery, femoral artery and coronary arteries. Peptidoglycan positive plaques are associated with vulnerable features such as high macrophage content, and more than 50% atheroma, and less smooth muscle cells in cap and shoulder area [73].

Inflammasome-forming NLRs, such as NLRP1 and NLRP3, can be activated by a large variaties of activators including self activators and pathogen activators. Self activators, or sterile activators includes self-derived activators such as ATP, cholesterol crystals, glucose, amyloid β, monosodium urate or calcium pyrophosphate dihydrate crystals, and hyaluronan, and environment-derived activators such as alum, asbestos, sillica, Alloy particles, skin irritants, and UV radiation. Pathogen activators include bacteria-derived pore-forming toxins, lethal toxin, flagellin/rod proteins, MDP, RNA, DNA, virus-derived RNA, M2 protein, Fungus –derived β-glucans, hyphae, mannan, zymosan, and protozoa-derived hemozoin. [150].

Cholesterol crystals are atherosclerosis-related inflammasome activators. In atherosclerotic lesions of high-cholesterol diet fed ApoE$^{-/-}$ mice, cholesterol crystals are present as early as two weeks on high fat diet and accumulate further with age. Cholesterol crystals locate both in the necrotic core and also in subendothelial area, both intracellularly and extracellularly. Intracellular cholesterol crystals are located both inside and outside phagosome. Cholesterol crystals are able to activate caspase-1and lead to IL-1β production in LPS-primed human PBMCs. This process is dependent on NLRP3 inflammasome [50].

Internalization of NOD2 ligand MDP may be involved the following pathways as reviewed in [151]. First, membrane protein transports, such as human peptide transporter 1 and pannexin-1 may involved in uptake of extracellular MDP which was cleaved out during bacterial peptidoglycan turn over. Second, MDP may be internalized by endocytosis via clathrin and dynamin. Third, after phagocytes ingest whole bacteria, peptidoglycans are digested in phagolysosome, and the resultant MDP may be transported to cytosol. Two endo-lysosomal peptide transporters, SLC15A3 and SLC15A4, are selectively required for NOD2 sensing

endosomal MDP as recently reported [152]. Last, peptidoglycan turn over of the ingested bacteria may release MDP into infected cells [151].

1.5.4 NLR downstream signals

NOD1 and NOD2 were the first identified NLR members leading to activation of canonical NF-κB and MAPKs pathways [141]. Upon activation, NOD 1 and NOD2 self-oligomerize and interact with the serine-threonine kinase RICK (also known as RIP2) via a hemophilic CARD-CARD interaction and ubiquitination to activate NF-κB signaling pathway [136, 153] and MAPKs including p38, ERK and JNK pathways [154]. NOD2 activates MAPK and cytokine secretion in human macrophages in IL-1β-dependent way [155].

RIP2 is a crucial adaptor protein medicating activation of NF-κB and MAPKs by NOD1 and NOD2 ligation. RIP2-deficiency will abolish the cytokine response from NOD2 stimulation but leave the effect from purified TLR4 agonist stimulation untouched. NOD2 utilizes RIP2 to cooperate with TLR4 for pro-inflammatory cytokine production. Furthermore, NOD1 and NOD2 compensate each other in the sense of cytokine production upon pathogen stimulation due to the common downstream adaptor RIP2 [154].

NLRs are also involved in signaling regulating anti-viral type I IFN production. For example, NOD2 can sense ssRNA and interact with mitochondrial antiviral signaling protein (MAVS), and subsequently activate transcription factor IRF3, consequently leading to increased IFN-β production [156]. Other NLRs are found to negatively regulate type I IFN production. NLRX1, the only known NLR expressed in mitochondria, inhibits RIG-I/MDA5-MAVS-mediated production of antiviral IFN-β [157].

NLRs take part in forming inflammasomes. Inflammasome is a multi-protein platform which activates caspase-1 and consequently mediates cytokine maturation (IL-1β and IL-18) and cell death. For NLRC subfamily, CARD domain at N-terminal can probably interact directly with pro-caspase-1 in CARD-CARD homophilic interaction, and lead to processing of caspase-1 [150]. For example, NOD2 stimulation by MDP induces IL-1β secretion in macrophages. Stimulated NOD2 binds to and activates caspase-1 probably with its N-terminal CARD domain, while the C-terminal LRR domain of unstimulated NOD2 prevent caspase-1 activation [158]. NLRP subfamily containing contains a PYD domain at N-terminal instead of CARD domain, however, inflammasome can be formed together with adaptor protein ASC. ASC contains both PYD domain to interact with NLRPs and CARD domain to interact with pro-caspase-1. For example, NLRP2 and NLRP3 associate with ASC,

CARD8 (also named Cardinal) and caspase-1 and form an inflammasome [159]. NLRP1 is special in that it contains a CARD domain at C-terminal, which can bind caspase-1 directly and assemble inflammasome [160]. ASC is not required in NLRP1 inflammasome but can enhance inflammasome activation [161].

Cross-talk between NLR may exist in forming inflammasome. MDP induces IL-1β secretion in a NOD2-RIP2-dependent manner in macrophages. [158]. It has also been observed by gel filter assay that NOD2-NALP1-caspase-1 formed a complex [158]. To further complicate the picture, MDP-induced IL-1β secretion is shown to be NALP3-dependent [162]. Unlike most other studies performed in cells, Faustin et al also showed that MDP, but not LPS or γ-tri-DAP, directly activate reconstituted NALP1 inflammasome in cell-free environment [161]. These data indicate that multiple NLRs may associate with each other in response to one stimulus to induce inflammasome activation.

1.5.5 Regulation of NLR signal

Dysregulation of inflammasome activation might reduce the host defense in infectious disease, or promote sterile inflammation in chronic inflammatory diseases. Activation of inflammasome results in maturation of an essential pro-inflammatory cytokine IL-1β. IL-1β acts in an auto-crine manner and can further promote the production of other pro-inflammatory cytokines. Furthermore, inflammasome is involved in necroptosis, a newly defined programmed cell death, and leading to the leakage of cellular content which will induce more inflammation.

Several mechanisms have been hypothesized to involve in negative regulation of inflammasome activation. For example, NOD2 and NALP1 alternative splicing variants can regulate full-length isoforms. Also, pyrin-only proteins and CARD-only proteins can act as dominant-negative regulators [150]. RIP3 and RIP1-dependent NLRP3 inflammasome-mediated necroptosis is regulated by caspase-8, which is a switch between apoptosis and necroptosis [163].

NLR functions

NOD1 and NOD2 trigger innate and adaptive immunity. NOD1 ligand FK565 can directly activate mouse macrophages [164]. NOD1 is indespensible for initiation of adaptive immunity, for example, priming antigen-specific Th1 and Th17 cell immunity and subsequent antibody responses [165]. NOD1 agonist alone plus OVA antigen elicit priming

of antigen-specific T and B cell immunity with a predominant Th2 cell polarization profile [165]. NOD2 is indispensable for Th2 polarization of antigen-specific adaptive immune response[166].

NOD1 and NOD2 interact with RIP2 and are involved in autophagy. Autophagy is a self degradation process in which portions of cytoplasma, damaged organelles or long-lived proteins are sequestered into double-membrane bounded vesicles and delivered to lysosome for degradation. NOD1 and NOD2 are found to be intracellular sensors that responsd to invasive bacteria by recruiting autophagy protein ATG16L1 to plasma membrane at bacterial entry site. This is crucial process for antigen presentation to CD4+ T cells by dendritic cells. [167, 168].

NOD1 and NOD2 are essential for mucosal host defense. NOD1 signaling in nonhematopoietic cells are involved in host defense against *Listeria monocytogenes[169]*. In line with this, NOD1-deficient mice are susceptible to Gram positive bacteria *Clostridium difficile* infection with antibiotics treatment, which is associated with reduced neutrophil recruitment and impaired production of CXCL1[170]. NOD2 are involved in host defense also by inducing an inflammatory cytokine IL-32 and therefore promote rapid monocyte differentiation into dendritic cells and a specific DC programming to CD1b+ DC with enhanced ability of MHC class I-restricted antigen presentation to CD8+ T cells [171]. Although IL-32 receptor has not been identified, it has been implicated to be expressed in human atherosclerotic arteries and human IL-32γ-expressing transgenic mice develop vascular inflammation manifested as smooth muscle cell hyperplasia and immune cell infiltration in the adventitia of aortas [172]. NOD1 and NOD2 double knock-out mice have decreased inflammatory response and increased *Salmonella* colonization of the mucosal tissue compared with wild-type mice [173]. However, there is no difference in proliferation and activity of lymph node-derived T cells in $NOD1^{-/-}$ or $NOD2^{-/-}$ mice compared with wild-type [174], probably because of the compensation between the NOD1 and NOD2 as they both signal through RIP2.

NOD1 and NOD2 are involved in pathogenesis of autoimmune and chronic inflammatory diseases which may share pathological mechanisms with atherosclerosis**.** NOD1 variants are associated with autoimmune diseases such as such as asthma [175, 176], atopic eczema [177], and NOD2 variants are associated with chronic inflammaotry disease such as Crohn's disease [52, 178]. The imbalance between protective and harmful bacteria and the decreased complexicity of gut bacteria is observed in Crohn's disease [179]. One attracting hypothesis

is that the role of NOD1 or NOD2 in IBD is mediated by the disturbance of gut microbiota, however, the causitive relation between IBD and microbiota disturbance is under debate.

NOD2 has been shown to induce an important inflammatory disease associated micro-RNA miR-29. NOD2 combined with TLR2 or TLR5 stimulation in dendritic cells increases miR-29. Neither TLR2 or TLR5 alone or the combination of TLR2 and TLR5 does not result in miR-29 upreguation, indicating a essential role of NOD2 signaling in induction of miR-29 [180]. miR-29 exerts dual funcitons in inflammatory diesease. First, miR-29 can down regulate IL-12p40 directly and IL23p19 indirectly, which are two subunit of IL-23. IL-23 and IL-6 are required for induction of Th17 cells, which is important for antimicrobial immunity at mucosa and a hallmark of the inflammatory response in Crohn's disease. This provide a potential mechanism for the role of NOD2 in Crohn's disease [180]. Second, miR-29b mediates epigenetic regulations that are involved in atherosclerosis. In human aortic smooth muscle cell cultrue, oxLDL upregulates miR-29 expression and induce miR-29-dependent down-regulation of DNA methyltransferase 3b and consequently upregulate MMP-2/MMP-9 genes [181].

Knowledge is merging on the role of NLRs in atherosclerosis. NLRP3 forms the caspase-1 activating cytoplasmic complexes, NLRP3 inflammasome, upon the stimulation of cholesterol crystals, and $Ldlr^{-/-}$ mice reconstituted with $NLRP3^{-/-}$, $ASC^{-/-}$ or $IL\text{-}1\alpha^{-/-}IL\text{-}1\beta^{-/-}$ bone marrow and fed a high-cholesterol diet have reduced atherosclerosis and necrotic core area compared with wild-type bone marrow transplanted mice [50]. However, Menu and co-workers later used $NLRP3^{-/-}/Apoe^{-/-}$ mice and showed that atherosclerosis progresses independently of the NLRP3 inflammasome [182]. NOD1 activation induces cardiac dysfunction and this is associated with increases cardiac fibrosis and apoptosis[183]. NOD1 ligand also induce vascular inflammation manifested by coronary arteritis and valvulitis. The inflammation is associated with high expression of chemokines/cytokines and matrix metallopeptidases [184]. The association between NOD2 polymorphisms and cardiovascular diseases was shown in an retrospective study comparing angiografically documented patients and healthy controls[185], but failed to be identified in a prospective study using cardiovascular disease as a readout [186].

2 METHODOLOGICAL CONSIDERATIONS

2.1 HUMAN CAROTID ATHEROSCLEROTIC PLAQUE MODEL

Human atherosclerotic plaques collected from carotid endarterectomy were used in this thesis to analyze the mRNA and protein expression of the genes of interest. Using carotid plaques as a surrogate for characterizing the culprit plaques in coronary or cerebral events have both advantage and disadvantage. The advantages include that it is the easiest plaque to access in humans. Moreover, plaque characteristics show a certain degree of similarities among vascular territories because systemic factors influences atherosclerotic burden and the presence of necrotic core [187]. However, inflammation may distributed non-homogenously in atherosclerotic arteries [187]. Thus, the results require to be supported by other models.

Human atherosclerotic plaque culture was used to determine the functional relevance of the receptors in human atherosclerosis. The advantage of this model compared with cell culture experiments are that it can be used to analyze the responses of all cell types in the intima of human atherosclerotic plaque to a certain stimulus. Secretory proteins or lipids in the supernatant, as well as mRNA or protein, including phosphorylated proteins in the tissue were analyzed in response to PRR stimulation. The disadvantage is that heterogeneity of the plaque tissue and various severity of the disease caused big variations, thus, normalization to the untreated plaque tissue from the same patient and adequate number of replicates are necessary.

2.2 MOUSE MODELS OF ATHEROSCLEROSIS

Animal models are useful for exploring the function of PRRs in atherosclerosis. Compared with human carotid plaque models, animal models provide more mechanistic insights with pharmacological treatment and genetic modifications. Multiple species has been used as animal models of human atherosclerosis, including mice, rabbits, pigs, non-human primates, dogs, hamsters, guinea pigs, and birds. However, mouse models have the advantage of rapid breeding, affordability, and genetic modifiablility. Mice are generally resistant to atherosclerosis, but genetically modified mice are widely used as model of human hyperlipidemia and atherosclerosis. Cautions are needed when extrapolate the results to human disease due to the differences between species. In this section, I will mainly discuss the pros and cons of the three mouse models that were used in this thesis, the other mouse models were well reviewed in [188].

ApoE$^{-/-}$ mice

ApoE is a lipoprotein found in chylomicrons, chylomicron remnants, VLDL, and some isotypes of HDL, and mediate cholesterol metabolism by binding to LDL receptor and chylomicron remnant receptor [189]. Except for its role of decreasing the absorption of dietary cholesterol and increasing biliary cholesterol excretion [190], ApoE can also inhibit LDL oxidation, and has a dual role in inflammation as reviewed in [191] and [192].

ApoE$^{-/-}$ mice develop spontaneous atherosclerosis due to accumulation of atherogenic cholesterol–rich remnants in the plasma [193]. Although the plasma cholesterol level of wild-type mice does not alter when fed with chow diet (0.02% (wt/wt) cholesterol) or high fat diet (0.5% (wt/wt) cholesterol) for 3 weeks, 10-13 weeks old ApoE$^{-/-}$ mice have 6 times higher plasma cholesterol than wild-type mice of the same age when both are fed chow diet, and 12-fold higher when both are fed with high fat diet [190]. ApoE$^{-/-}$ mice fed chow diet develop foam cell accumulation in aortic sinus at 12 weeks old [193]. In our experiment we used mice from 12-14 weeks age and measured atherosclerotic lesion development at 20-22 weeks age to allow the analysis of lesion composition.

ApoE is expressed mainly in hepatocytes, but also in bone marrow cells. Transplantation of bone marrow cells with wild-type ApoE fully rescue dyslipidemia and prevent atherogenesis in ApoE$^{-/-}$ mice [194]. Thus, ApoE$^{-/-}$ mice were not used as recipients in bone marrow transplantation experiments.

Ldlr$^{-/-}$ mice

LDL receptor (Ldlr) is expressed mainly on hepatic cells. It binds with a high affinity to ApoE on intermediated density lipoprotein (IDL), derived from triglycerides-rich VLDL by lipase, and binds with a low affinity to ApoB-100 on LDL. It functions as one of the main mechanisms to remove IDL and LDL from the plasma [195].

Ldlr$^{-/-}$ mice developed atherosclerosis when fed a high-cholesterol diet due to hypercholesterolemia [196]. Ldlr$^{-/-}$ mice has two-fold increased plasma cholesterol compared with wild-type mice at 7-8 weeks age fed on normal chow diet [195, 196], while with 2 weeks of high fat diet (1.25% cholesterol), the differences enlarges to 12 fold between male mice and 14 fold between females [196]. Ldlr$^{-/-}$ mice developed xanthomatosis when fed with a western diet for more than 12 months [196].

2.3 STRATEGIES TO STUDY PRRS IN ATHEROSCLEROSIS

Pharmacological intervention and genetic modification are used to study pattern recognition receptors in atherosclerosis in mouse models. The advantage of genetic modification is the specificity of the interrupted receptor and the targeted cell population. However, the compensatory effect due to disruption of the gene since born may influence the interpretation of the result. Thus, we also used pharmacologically synthesized ligand of the PRRs to investigate the direct effect within the controlled time period.

Transplantation of bone marrow with genetically modified PRR

Bone marrow transplantation was performed from a genetic modified donor to $Ldlr^{-/-}$ recipient to study the role of PRRs (mutated in donors) in bone marrow cells in atherosclerosis. 6-9 weeks old $Ldlr^{-/-}$ mice were irradiated with lethal doses (2 doses of 700 rad 3 hours apart) and receive bone marrow cells from the donor mice. The transplanted mice were recovered for 4 weeks, and fed a high fat diet for 8 weeks for analysis of atherosclerosis development.

Pharmacological interventions with PRR ligands

Commercially available NOD2 ligands includes MDP derivatives and muramyl tripeptide. In paper 1-2 we used minimal bioactive motif of peptidoglycan, MDP, as NOD2 ligand. MDP is muramyl dipeptide, N-Acetylmuramyl-L-Alanyl-D-Isoglutamine (L-D isoform).

Commercially available NOD1 ligands include iE-DAP and its derivative C12-iE-DAP. In pilot experiment we found iE-DAP did not give a satisfactory response (data not shown), thus, C12-iE-DAP was chosen to get a strong effect. Later, we acquired FK565 as a gift from Astellas, Tsukuba, Japan, which has been reported to be even stronger NOD1 stimulator than C12-iE-DAP in endothelial cells [184]. Therefore FK565 was used in animal experiments.

CpG oligonucleotides are also been used as TLR9 ligands, including CpG group A, B and C. Group A is efficient in type I IFN production, group B is efficient in inducing B cell activation, and group C induces both effects. In paper 4 we use CpG-ODN 1826 as TLR9 agonist. It is a 20-mer synthetic single-stranded DNA containing a completely phosphorothioated backbone and two CACGTT motifs, namely CpG motifs, which contains unmethylated CpG dinucleotides. CG was switched as a DNA control. However, the control has been reported to induce the activation of TLR7/TRIF pathway [197]. Thus, PBS was also used as control.

3 RESULTS AND DISCUSSIONS

3.1 NOD2 IS EXPRESSED AND FUNCTIONAL IN HUMAN ATHEROSCLEROSIS

The main aim of paper 1 was to investigate the role of NOD2 in human atherosclerosis. NOD2 is of interest in human atherosclerosis because peptidoglycan, the natural ligand of NOD1, NOD2, and TLR2 is present in human atherosclerotic plaques and associated with unstable plaque phenotype [73]. The function of TLR2 in human atherosclerosis has been addressed in many studies, for example, [55, 73], but not NOD1 or NOD2. Given the importance of eicosanoids in inflammation and atherosclerosis [198-200], we hypothesized that NOD2 signal affects eicosanoids metabolism in human atherosclerosis plaques.

As a basis, we characterized the expression of NOD2 in human atherosclerotic plaque tissue. In endothelial cells, NOD2 expression is induced by inflammatory mediators, such as IL-1β and TNF-α [201] [107]. The mRNA expression of NOD2 is regulated by NF-κB pathway, as the TNF-α induced NOD2 expression is mediated by transcriptional activation of NOD2 promoter by p50 and p65 subunits of NF-κB [202]. TNF-α induced NOD2 expression is a rapid process which takes place in less than 3 h. In immune cells, NOD2 expression is up-regulated in granulocyte and monocytes/macrophages in peripheral blood after differentiation from hematopoietic progenitor cells [202]. We found that the expression of NOD2 is higher in atherosclerotic plaques tissue than normal artery control internal mammary arteries in mRNA level measured by real-time PCR and microarray, and in protein level measured by western blot and immunostaining (Figure 2). Moreover, NOD2 protein is located mainly in necrotic core and endothelial layer in the atherosclerotic plaques, expressed by endothelial cells and macrophages. Besides inflammatory cytokines, NOD2 expression is also up-regulated by TLR4 or NOD2 ligation with LPS and MDP in *ex vivo* plaque tissue culture. These results indicate that NOD2 is expressed in atherosclerotic plaques and the expression may be further up-regulated with the plaque development. These data serve the basis for the function of NOD2 in regulating eicosanoid pathway in atherosclerosis.

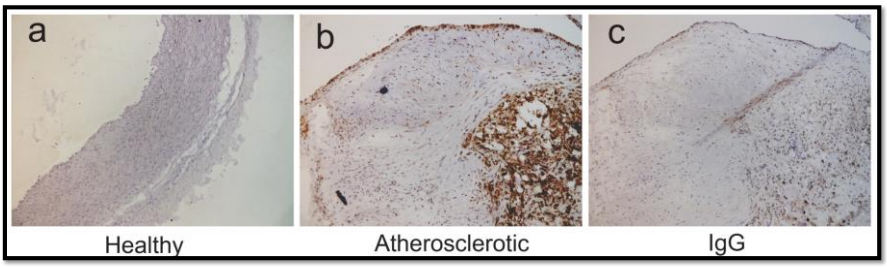

Figure 2. Enhanced expression of NOD2 in atherosclerotic lesions.
Immunohistochemical analysis of NOD2 protein in (a) internal mammary arteries and (b) the atherosclerotic plaques stained by NOD2 antibody or (C) isotype control antibody (IgG).

Another main finding of paper 1 was NOD2 signal specifically regulates COX2-PGE2-EP2/EP4 axis among the eicosanoid pathways in atherosclerotic plaque tissue. We found that atherosclerotic plaque tissue culture release high level of HETEs including 12-HETE, 5-HETE, and 13-HODE, and to a less extent prostanoids in the rest state, and a considerable level of leukotrienes LTB4 and LTE4. Among the eicosanoids that we analyzed, only PGE2 is increased by NOD2 stimulation. The enzymes COX-2 and mPGES-1 as well as the receptors for PGE2, EP2 and EP4, were also increased both in atherosclerotic plaque culture and in monocytes/macrophages, indicating that NOD2 activates COX2-PGE2 pathway in atherosclerosis (Figure 3). Furthermore, we elucidated that NOD2-induced PGE2 is mediated by MAPK p38 activation, and NOD2-induced IL-1β and TNF-α contribute at least part to the COX-2--PGE2 axis. One limitation of this experiment was that 15-HETE and cystainyl leukotrienes, which are abundant in atherosclerosis [203], were not measured due to the limited amount of the material.

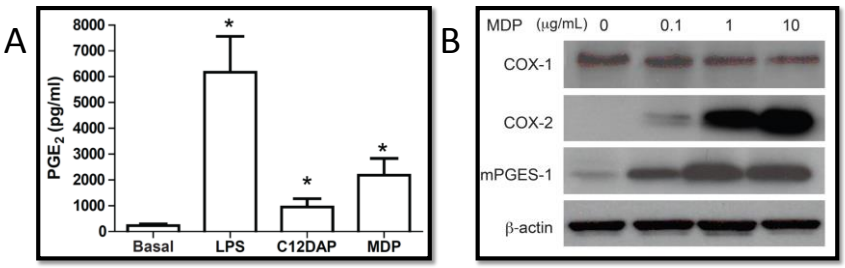

Figure 3. NOD2 induces COX-2/PGE_2 pathway in atherosclerotic plaques. (A) PGE_2 released from *ex vivo* cultures of human carotid atherosclerotic plaque stimulated with TLR4 agonist LPS, NOD1 agonist 12CDAP or NOD2 agonist MDP. (B) Protein levels of COX-1, COX-2, mPGES-1 in the MDP stimulated macrophages determined by western blot.

This result is of interest because eicosanoids including leukotrienes, prostaglandins, lipoxins and hydroxyeicosatetraenoic acids (HETEs), exert broad functions in atherosclerosis [198-200]. For instance, leukotriene LTB4 is potent chemoattractant [204, 205], 15-HETE inhibits neutrophil activation and 13-HODE inhibits cell adhesion to endothelium [206]. The function

of plaque PGE2 varies between either stimulation or inhibition on platelet and athero-thrombosis depending on stimulation of either EP3 or EP4 [207, 208].

Besides eicosanoids pathways, we also explored cytokine production induced by NOD2 in monocytes/macrophages and in atherosclerotic plaques. We found that NOD2 induces remarkable production of inflammatory cytokines TNF-α, IL-8, IL-1β, IL-10 and IL-6 mediated by NF-κB and MAPK in human monocytes/macrophages [209-211]. In consistent with these findings, we found NOD2 stimulation by MDP also induces the production of IL-8, IL-1β, IL-10 and IL-6 via MAPK p38 pathway in atherosclerotic plaques.

NOD2 stimulation by bacterial peptidoglycan component MDP induces NF-κB [136] and MAPK activation [209] in monocytes/macrophages. Over-expression of NOD2 activates NF-κB pathway [107]. NOD2 signal requires autocrine IL-1β to exacerbate MAPK activation [155, 211]. In accordance with the previous findings in monocytes/macrophages, we confirmed activation of NF-κB and MAPK induced by MDP in both human monocyte cell line THP-1 cells and in human atherosclerotic plaques at a relatively late time point, 1.5 h after NOD2 stimulation. The pharmacological inhibition of the NF-κB and MAPK pathway shows that in plaque tissue NOD2 induced cytokine response are dependent on MAPK p38 pathway.

A limitation of using pharmacological inhibitors to delineate signal pathways is that most inhibitors interfere with more than one signal pathways. The p38 inhibitor SB203580 used in our study, as well as other p38 inhibitors SB220025 and PD169316, inhibits auto-phosphorylation of RIP2, an adaptor of NOD2 induced signal [212], at concentrations comparable to those used to inhibit p38 [213], and thus make it difficult to delineate the role of p38 and RIP2 in NOD2 signal with the inhibitory effect of SB203580 on the NOD2-induced cytokine production. On the other hand, NF-κB inhibitor BAY-11-7082 can also activate p38 [214], which make it difficult to delineate the role of NF-κB by a strong MAPK p38 inducer. Alternatively, siRNA might be another available strategy for the inhibition.

Taken together, this study elucidated that NOD2 is highly expressed in human atherosclerotic plaques and NOD2 induces PGE2 production in atherosclerosis. These results identified the PRR signaling that selectively govern PGE2 pathway in atherosclerosis. This also adds evidence to and illustrates a mechanism of how pattern recognition receptors and innate immune immunity involves in atherosclerosis. Further work is expected to determine the functional role of NOD2 in atherosclerosis *in vivo*.

3.2 NOD2 INDUCES VULNERABLE ATHEROSCLEROTIC PLAQUES

The main aim of paper 2 was to investigate the function of NOD2 in atherosclerosis *in vivo*. Bacterial peptidoglycan can be detected in the circulation in systemic infection [215], and even in milder circumstances such as alcohol intake [216]. NOD2 is important sensor for peptidoglycan. The motivation also comes from paper 1 where we found NOD2 leads to rapid activation of prostaglandin E2 in *ex vivo* human atherosclerotic plaques culture [217]. We hypothesized that NOD2 signaling may lead to vascular inflammation and accelerate atherosclerosis.

The main approach of this paper is to evaluate atherosclerosis in *Ldlr*$^{-/-}$ mice w/o NOD2 stimulation and w/o NOD2-deficiency. The first major observation was that when we i.p. injected NOD2 ligand MDP, the minimal bioactive peptidoglycan motif, into high fat diet fed *Ldlr*$^{-/-}$ mice, and compared with PBS controls, we found that NOD2 stimulation aggravated atherosclerosis and vascular inflammation, and that NOD2 signal remarkably enlarged lipid-rich necrotic core in the lesion (Figure 4). The second major observation was that *Ldlr*$^{-/-}$ mice transplanted with bone-marrow from *Nod2*$^{-/-}$ mice have similar atherosclerotic lesion area but reduced necrotic core compared with the mice reconstituted with wild-type bone marrow (Figure 5). This result suggests that NOD2 signaling of myeloid derived cells have a critical role in regulation of necrotic core formation in atherosclerotic plaques. Since NOD2 is also expressed abundantly in endothelial cells, NOD2 signal in non-myeloid cells may also be involved in the development of atherosclerosis, and remains to be investigated.

In this study we identify NOD2 as a novel signal involved in the formation of lipid-rich necrotic core. Necrotic core refers to the core area of atheroma rich in foam cells, cell debris and extracellular lipids. Previously it has been recognized that both apoptosis and necrosis of macrophage foam cells are present in the necrotic core in human atherosclerotic lesion as identified by transmission electron microscopy and nick end-labelling using terminal deoxynucleotidyl transferase (TUNEL) [218]. It has also been proposed that stimuli in the atherosclerotic plaques, such as oxidized low-density lipoprotein (LDL), induce macrophage apoptosis based on the *in vitro* findings that human monocytes-macrophages underwent apoptosis with 50μg/ml or higher concentration of ox-LDL after stimulation for 24 hours [219]. However, early morphological observations suggest that the abundant extracellular lipids may not necessarily result from cell necrosis, because the size of extracellular lipid droplets are much smaller than intracellular ones, and extracellular lipid droplets make up 40% of lipid-rich core volume in early human fibro-lipid lesions [220]. However, the relative

importance and causal relationship among foam cell formation, extracellular lipid accumulation, macrophage egress and cell death in the formation of necrotic core remains to be understood.

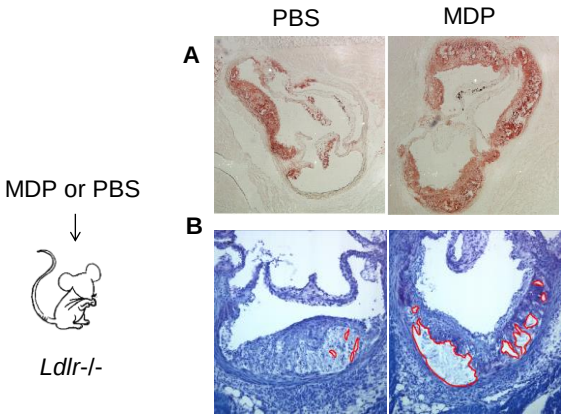

Figure 4. NOD2 stimulation increases atherosclerosis. (A) Oil Red O staining of the lipids in aortic root cryosection showing increased atherosclerosis in MDP (NOD2 ligand) than PBS-treated Ldlr-/- mice fed with high fat diet. (B) Nuclei staining with toluidine blue in aortic root sections showing that MDP (NOD2 ligand) increases the area of necrotic core (area in absence of nuclei, circled in red line) in the same mouse model.

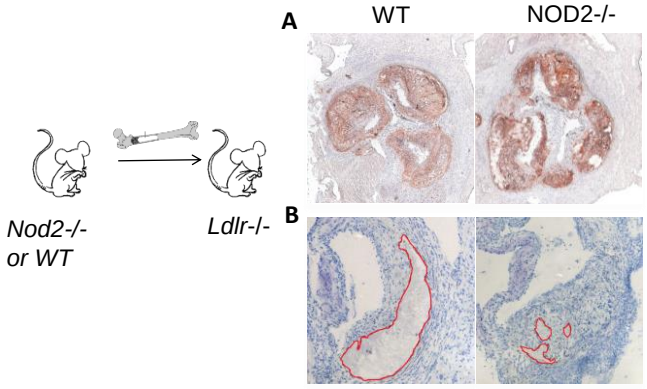

Figure 5. Myeloid cell NOD2 signaling regulates necrosis in atherosclerotic lesions. (A) Oil Red O staining in aortic root cryosection of Ldlr$^{-/-}$ mice transplanted with wild-type or NOD2$^{-/-}$ bone marrow showing equal lesion size. (B) Toluidine blue staining in aortic root sections showing that Ldlr$^{-/-}$ mice transplanted with NOD2$^{-/-}$ bone marrow has smaller necrotic area in the atherosclerotic lesions.

Several molecular mechanisms have also been shown to alter necrotic core formation in atherosclerosis. For example, insulin receptor-deficient mice develop bigger lesions with increased necrotic core and increased number of apoptotic cells [221]. Protease inhibition in advanced atherosclerotic plaques results in a significant decrease in collagen content and a significant enlargement of the necrotic core [222]. However, a schematic picture of the molecular mechanisms leading to necrotic core are yet unrevealed.

Several possibilities are raised on how NOD2 signal promotes necrotic core formation. First, NOD2 promotes lipid accumulation of macrophages by up-regulating the oxidized LDL uptake and down regulating cholesterol efflux. This is related to the up-regulation of the

expression of scavenger receptor gene SRA1/2 and the down-regulation of cholesterol transporter ABCA1 protein. Second, NOD2 facilitate the formation of autophagosome in the context of bacterial infection [168], and fusion of autophagosome with lysosomes is essential for lysosomal acid lipase to hydrolyze cholesteryl esters into free cholesterol for ABCA1-dependent efflux [223]. Future work is needed to elucidate whether NOD2 activation contributes to lipid retention mediated by autophagy pathway. Third, necrotic core is composed of foam cells with large lipid droplets, apoptotic and necrotic cells [224], extracellular lipid including aggregated LDL, and extracellular matrix [225]. Although NOD2 does not affect apoptosis in the necrotic core, this does not rule out the possibilities that MDP affects other type of cell death in the necrotic core. Since MDP induce caspase-1 dependent IL-1β secretion in the plaques, and Caspase-1 activation induced by NLRC4 leads to pyroptosis, a programmed cell death with rapid loss of cell membrane integrity and leakage of cytosolic contents [226] and was proposed to exist in atherosclerosis [227], future work needs to be done on whether NOD2 induced caspase-1-dependent pyroptosis contribute to NOD2-induced necrotic core formation in atherosclerosis.

Necroptosis, meaning programmed necrosis, may be an important signal in necrotic core formation in atherosclerosis. Necroptosis can be induced by TLR or cytokine stimulation in combination with caspase inhibition. Receptor interacting protein 3 (RIP3) is required for necroptosis of macrophages. RIP3 deletion prevent macrophage necroptosis in response to oxidized LDL under caspase inhibition *in vitro*. Furthermore, $RIP3^{-/-}Ldlr^{-/-}$ mice have reduced macrophage necroptosis as well as lesion burden than $Ldlr^{-/-}$ mice [228]. It is a limitation in our study that we could not identify the role of NOD2 in macrophage necroptosis, neither could we identify whether this is possible mechanisms for NOD2 accelerated necrotic core formation in mouse atherosclerosis.

Intraplaque hemorrhage (IPH) is a common phenomenon associated with vulnerability of atherosclerotic plaques. Carotid plaque with IPH or marked intraplaque vessel formation demonstrated an increased risk of cardiovascular outcome including vascular death, nonfatal stroke, nonfatal myocardial infarction, and vascular intervention [7]. IPH is barely detectable in early intimal thickening, frequently present and increased in fibro-atheroma with necrotic core from early to late stage, and maximized in thin-cap fibro-atheroma. Quantification of the degree of intraplaque hemorrhage by staining of iron and erythrocyte-specific glycophorin A is associated with the size of necrotic core as well as the extent of macrophages in plaques [229]. It has been proposed that free cholesterol in erythrocytes membrane may contribute to

cholesterol clefts in the necrotic core or even acts as immune stimuli. It is a limitation that IPH was not described in our study.

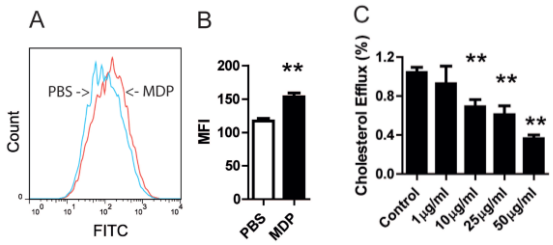

Figure 6. NOD2 stimulation increased ox-LDL uptake and cholesterol efflux in macrophages. (A) In vitro analysis of FITC-labeled oxidized LDL uptake in the J774 cell line after 24 hours MDP (1μg/mL) or PBS treatment by flow cytometry. (B) Quantification of oxidized LDL uptake by median fluorescent intensity (MFI), using data collected as in (A). (C) Cholesterol efflux from Raw 264 cells to apoA-I after treatment with the indicated doses of MDP.

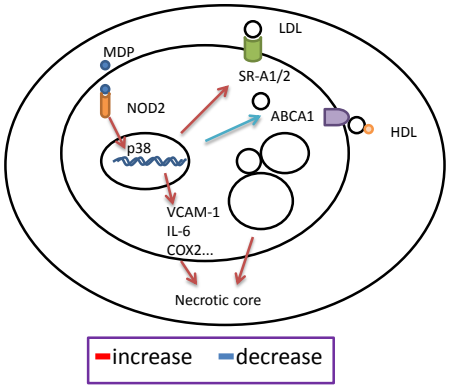

Figure 7. NOD2 promotes necrotic core formation. Atherosclerosis is an inflammatory disease involves activation of innate immunity via Toll-like receptors and Nucleotide-binding oligomerization domain-containing protein (NOD)-like receptors. NOD2 is a crucial signal exacerbates atherosclerosis with enlarged necrotic core in the lesion. Myeloid-specific ablation of NOD2 restrained necrotic core formation. We proposed two mechanisms: i) Activation of NOD2 leads to vascular inflammation mediated by MAPK p38 and NF-κB pathways and ii) NOD2 increases lipid accumulation in macrophages by enhancing the uptake of oxidized low density lipoprotein and impairing cholesterol efflux.

Of note, a recent study showed the protective role of NOD2 for *Porphyromanas gingivalis* infection-mediated vascular inflammation and atherosclerosis [230]. The explanation of the discrepancy may be similar to the TLR4 results [122, 126] as discussed in page 17 paragraph 2. PRRs play important role in host defense. Deficiency in PRR signaling leads to deficiency in host defense, which may result in increased activity and replication of pathogenic bacteria or disturbance of commensal bacteria homeostasis. Certain bacterial infection is deleterious for atherosclerosis. Infection may trigger a NOD2-independent pathway, for example, through stimulation of other PRRs, which lead to accelerating atherosclerosis, and this alternative pathway is stronger than the protective effect of NOD2-deficiency. This should be put into consideration when targeting deleterious PRR signaling in the treatment of atherosclerosis.

In summary, our results provide the first evidence that the direct NOD2 activation promotes atherosclerosis, and suggest an important mechanism of enhanced vascular inflammation and necrotic core formation mediated by NOD2 (Figure 7). Enhanced inflammation, enlarged necrotic core, and thin fibrous cap are recognized as features of vulnerable plaques [225], and predict the risk of cardiovascular disease outcome in human [7, 8], thus, our findings may be of clinical importance.

3.3 NOD1 PROMOTES OCCLUSIVE ATHEROSCLEROSIS

The aim of paper 3 was to investigate the role of NOD1 in atherosclerosis. The motivation is similar to paper 1 and 2 in the sense that peptidoglycans, which are sensed by NOD1, NOD2 and TLR2, are present in human atherosclerotic plaques and are associated with unstable plaques [73]. NOD1 and NOD2 both belong to NLR family and share similarities in structure, natural ligands, downstream signaling pathways (RIP2-NF-κB or MAPKs), but also has distinctive expression profile. Another difference is that only NOD2 ligand MDP was shown to activate NALP3/cryopyrin inflammasome which is independent of RIP2 [231]. As described in paper 2, NOD2 exacerbates lipid retention in foam cells, enhances vascular inflammation, and promotes necrotic core formation a in atherosclerosis [232]. We thus asked what the role of NOD1 is in atherosclerosis.

The first major observation was that NOD1 stimulation with NOD1 ligand C12-iE-DAP induced inflammatory responses with increased production of proinflammatory cytokines IL-1β, IL-8 and IL-6 production and anti-inflammatory cytokine IL-10 mediated by p38 and ERK pathways in human plaque tissue culture (Figure 8). Previous reports showed that NOD1 ligand iE-DAP stimulate NF-kB activation and TNF-α and IL-6 production in macrophages, however, whether the cytokine response is dependent on NF-kB activation was not verified [143]. *Listeria monocytogenes* induces IL-8 secretion by NOD1 in endothelial cells, and this is dependent on p38 pathway [233]. Our study is consistent with these results and contributes to the understanding of the function of NOD1 in atherosclerotic tissue which is a complex inflammatory tissue composed of multiple types of activated cells.

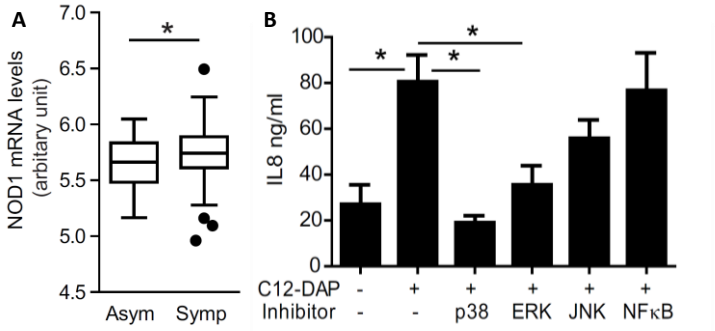

Figure 8. Relevance of NOD1 with human atherosclerosis. (A) Levels of NOD1 mRNA in carotid plaque of symptomatic (n=85) and asymptomatic (n=40) patients in Bike biobank. Symptoms include amaurosis fugax, transient ischemic attack and stroke. *P<0.05.
(B) Plaque IL8 response to NOD1 stimulation. Fresh human carotid plaques were pretreated with p38, MEK 1, JNK, NF-kB inhibitor or control for half an hour, and then treated with C12-DAP or medium for 20 h. Wilcoxon matched-pairs signed rank test, * P<0.05, **P<0.01.

The second major observation was that NOD1 stimulation lead to accelerated atherosclerosis with severely occlusive lesions (Figure 9) in *Ldlr*$^{-/-}$ mice, accompanied by arterial elastin degradation, SMC phenotype alteration, and distinctive systemic and lesional inflammatory responses (Figure 10). Although both accelerated atherogenesis and vascular inflammation, NOD1 and NOD2 stimulation resulted in different atherosclerotic plaque features. NOD2 induced atherosclerotic plaques with remarkably enlarged necrotic core, while NOD1 increased cellular content in the plaques. Moreover, myeloid depletion of NOD1 in *Ldlr*$^{-/-}$ mice does not alter atherosclerosis or vascular inflammation. Unlike NOD2 preferentially expressed in myeloid cells, NOD1 is ubiquitously expressed by multiple cells such as vascular SMC, endothelial cells, and epithelial cells [184, 234]. Thus, we hypothesized that NOD1 in non-myeloid cells may exert a more important role in atherosclerosis.

The observations in this study raised several interesting questions. *In vivo* NOD1 stimulation induced occlusive atherosclerosis lesion which resembles intima hyperplasia. However *in vitro* study failed to show any effect of NOD1 on proliferation of VSMC. A previous study showed that NOD1 acts as gate-keeper for the activation state of Rho GTPase by sensing virulence factors [235]. Rho GTPase activation is required for up-regulation of Skp2 that promotes degradation of p27Kip1, a checkpoint protein in G1 phase, which will lead to VSMC proliferation and intima formation [236]. This raised the possibility for NOD1 directly induce VSMC proliferation. Although we did not observe the effect *in vitro*, the hypothesis requires to be tested *in vivo*.

Another question is the contribution of inflammation induced elastin degradation in the development of severe occlusive atherosclerosis. Studies showed that elastin is essential in VSMC homeostasis. Lack of elastin induces VSMC proliferation and migration [237] and

severe stenosis of aorta, and $Eln^{+/-}$ mice also have reduced aorta cavity with more numerous but thinner elastin lamellae [238]. Deficiency of cathepsin K, one of the most potent elastases and collagenase, decreases atherosclerosis with concomitant decreased elastin breaks in the media underlying advanced atherosclerotic plaques [239]. Unfortunately we could only test the mRNA expression of several elastinolytic MMPs, but measurement of other elastase activity and natural inhibitors of elastases such as tissue inhibitor of metalloproteinase TIMP are also of interest.

How does NOD1 signal accelerate atherosclerosis? Hypothetic mechanisms based on our findings and others are summarized in figure 11. NOD1 activation induces chemokines CCL2, CCL5 and CX3CL1 in smooth muscle cells. This is interesting because CCL2 promotes mobilization of monocytes from bone marrow [240] and CX3CL1 promotes survival of monocytes in the circulation [30, 241], both of which may contribute to monocytosis in the blood. CCL5 facilitate the recruitment of monocytes [27] and neutrophils [242] into the intima and media, where monocytes are activated and differentiated into macrophages. Thus, NOD1 stimulation promotes monocyte mobilization from bone marrow, increases monocyte survival and recruitment to the lesion, contributing to lesion development. The cross-talk between macrophages and smooth muscle cells via CX3CL1/CX3CR1 activates both cell types [113] and lead to production of pro-inflammatory cytokines and elastases including MMP9, MMP10 and MMP12. PDGF-BB, PDGF-DD or oxidized phospholipids repress the transcriptional expression of VSMC marker gene dependent on binding of Kruppel-like factor-4 to the G/C repressor element in the SM22α promoter [36]. NOD1 act as an additional signal promoting SMC phenotypic switching. NOD1 stimulated smooth muscle cells switches phenotype by downgrading α-actin expression and upgrading MMP9 expression and migration ability. Activated MMP9, MMP10 and MMP12 acts as elastases that degrade elastin lamellae. Loss of elastin lamellae enhances smooth muscle cells activation and phenotype switching [237], and also facilitates the infiltration of macrophages and neutrophils into the medial layer of the artery.

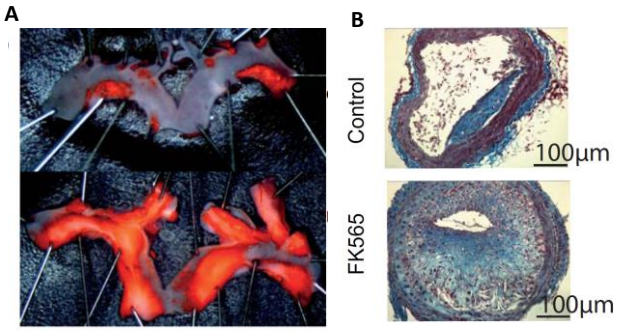

Figure 9. Atherosclerosis in NOD1 stimulated Ldlr$^{-/-}$ mice. (A) Sudan IV staining of neutral lipids in the aortic arch shows that FK565-treated mice developed 2.5-fold larger lesions compared to controls. (B) Masson Trichrome staining of the innominate artery shows occlusive lesions in FK565-treated group but not in the control.

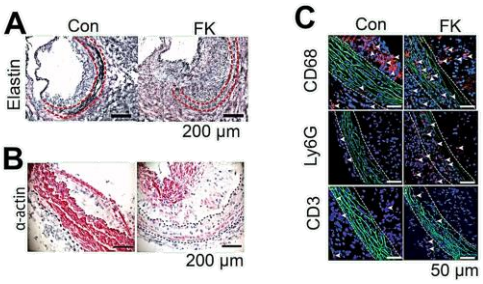

Figure 10. Characteristics of NOD1 stimulated atherosclerosis. Ldlr$^{-/-}$ mice were stimulated with NOD1 ligand FK565 or control water. (A) aortic root sections stained for elastin (Verhoeff-Van Geison staining). (B) Immunostaining of smooth muscle α-actin in innominate artery. (C) Immunostaing of macrophages (CD68), neutrophils and T cells in the media of aortic root.

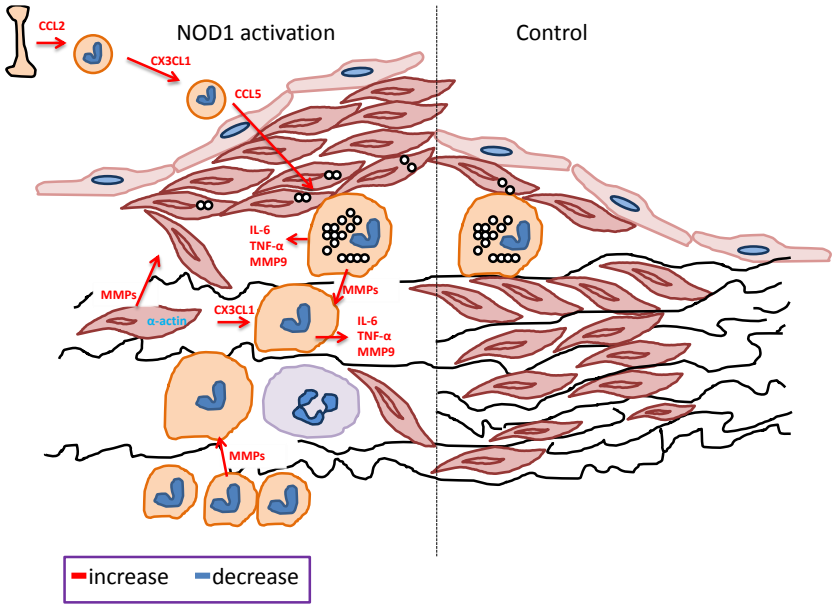

Figure 11. Hypothetic mechanisms of NOD1 accelerated atherosclerosis. NOD1 activation induces chemokines CCL2, CCL5 and CX3CL1 in VSMC, which promotes mobilization of monocytes from bone marrow, survival of monocytes in the circulation, and facilitate the recruitment of monocytes and neutrophils into the intima and media, where monocytes are activated and differentiated into macrophages. The cross-talk between macrophages and smooth muscle cells via CX3CL1/CX3CR1 activates both cell types and lead to production of pro-inflammatory cytokines and elastases including MMP9, MMP10 and MMP12. Smooth muscle cells switches phenotype by downgrading α-actin expression and upgrading MMP9 expression and migration ability by direct NOD1 stimulation. Activated elastases can degrade elastin and further enhance smooth muscle cell phenotype switching and facilitate macrophage migration.

Taken together, we identified NOD1 as a danger signal in atherosclerosis. This study also point out the remarkable difference in the phenotype of atherosclerotic lesions between NOD1 and NOD2 stimulated hyperlipidemic mice. In large arteries, the majority of plaque ruptures are asymptomatic. The current paradigm is that the erythrocyte-rich thrombus is incorporated into the plaques and resolved by the formation of the fibrous cap composed of migrated and proliferated VSMCs and its glycosaminoglycan and collagens. In smaller arteries, this healing process leads to narrowing of the lumen (stenosis) [243]. However, symptomatic plaque ruptures trigger thrombosis which severely and rapidly restricts the vessel lumen, and the emboli break off and block the downstream vessels. This leads to severe consequences such as myocardial infarction and stroke. Thus, it is of importance to understand the development of atherosclerotic plaque and the conversion of a stable, asymptomatic plaque to an unstable, vulnerable plaque. The results of our animal study points out NOD2 as an important signaling in development of unstable plaques, while NOD1 seems to be an important signal in arterial stenosis. This work contributed to the understanding of the complexity of different roles of pattern recognition receptor in atherosclerosis.

3.4 TLR9 RESTRAINS ATHEROSCLEROSIS

The aim of paper 4 was to investigate the role of TLR9 signaling in atherosclerosis. TLR9 is expressed in human atherosclerotic tissue. Stimulation of atherosclerotic tissue culture with TLR9 ligand CpG DNA up-regulates IFN-α and modulates CD4+ T cell function [244]. At the time of the study design (year 2005), TLR9 was found to mediate the activation of B cells and pDC by DNA immune complex in SLE patients [245], a population with extremely high risk for MI [246]. However, TLR9 was found to be protective against SLE in various murine SLE models [82].Thus, we asked the question of the role of TLR9 in atherosclerosis.

The fundamental approach of the study was to stimulate hypercholesterolemic mice with synthesized TLR9 ligand CpG (a type B CpG, murine TLR9 ligand) or PBS for 8 weeks. The first major observation was that TLR9 stimulation enhanced both systemic and local inflammatory response (Figure 12), however, the atherosclerotic burden is reduced as well as the necrotic core (Figure 13). There is no remarkable differences in the cholesterol levels between the groups that could explain the discrepancy of inflammatory responses and atherosclerotic burden.

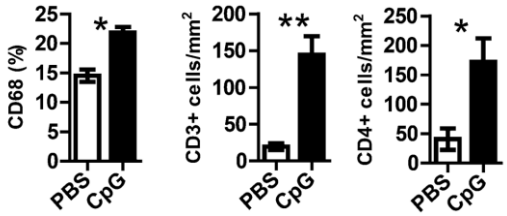

Figure 12. Increased local inflammation in CpG-treated *Apoe*$^{-/-}$ mice. Immunohistochemistry analysis of cellular composition in the lesion. Aortic roots were stained for macrophages (CD68, p=0.0139), T cells (CD3, p<0.0001, CD4, p=0.0381) and smooth muscle cells (α-actin) in CpG (n=4-9) and PBS (n=6-9)-treated *Apoe*$^{-/-}$ mice.

Like TLR9, other TLRs, TLR3 and TLR7, also exert athero-protective roles, however, since these TLRs are activator of inflammation, and inflammation is an important mechanism in atherosclerosis [5], the mechanism of the protective TLRs in atherosclerosis remains puzzling. TLR3 stimulation with its ligand Poly (I:C) activates both pro- and anti-inflammatory response in smooth muscle cells but decreases atherosclerosis [131]. Myeloid ablation of TLR3 in Ldlr$^{-/-}$ mice decreased atherosclerosis indicating a pro-atherogenic role for TLR3 in haematopoietic immune cells [247]. TLR7-deficiency is pro-atherogenic and the mechanisms is proposed as that TLR7 restrains the activation of classical/inflammatory macrophages (M1) by TLR2 and TLR4 ligands [132]. A recent study by Koulis *et al* also showed a protective role of TLR9 in atherosclerosis, and the authors proposed CD4+ T cells activation is responsible for the accelerated atherosclerosis in TLR9-deficient mice [248]. However, we and others showed that TLR9 signal activate adaptive immunity and promotes Th1 responses [249, 250], therefore, it is unlikely that CD4+ T cell is the mechanism for the protective effect of TLR9 in atherosclerosis. In paper 4 we described a remarkable induction of anti-inflammatory cytokine IL-10 and anti-inflammatory mediator indoleamine 2, 3-dioxygenase (IDO) upon TLR9 stimulation (Figure 14). IDO, an enzyme that degrades tryptophan to kynurenine, has an immune regulation function. CpG induces splenic marginal zone CD19+ DCs to produce IDO which suppress T cell response, dependent on Type 1 IFN [251] as well as PD-1/PD-1 ligand and CTLA4/B7 co-inhibitory interactions [252]. Up-regulation of IDO was found to be pronounced in human atherosclerotic plaques compared with non-atherosclerotic artery.[253]. IDO activity has a positive correlation with carotid artery intima/media thickness, an early marker of atherosclerosis [254]. Furthermore, given the potent effect of IL-10 on inflammation resolution, immune suppression and tissue repairing, our observation might provide a mechanistic insights to this puzzle. Further work is needed on whether TLR9 stimulation evoked anti-inflammatory mechanism is responsible for decreased atherosclerosis.

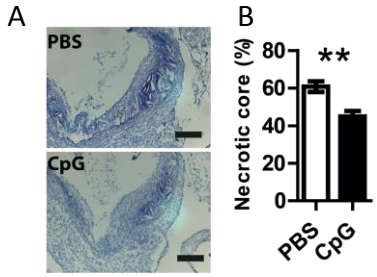

Figure 13. Decreased necrotic core in atherosclerotic lesions of CpG-treated *Apoe*$^{-/-}$ mice.(A)Representative histological analysis of aortic root stained with toluidine blue. 10* magnification. Scale bar, 0.2mm. (B) Percentage of necrotic core in the lesions in aortic root of PBS (n=8) and CpG (n=9)-treated *Apoe*$^{-/-}$ mice. Area in the absence of nuclear staining in the lesion was quantified as necrotic core. p=0.007.

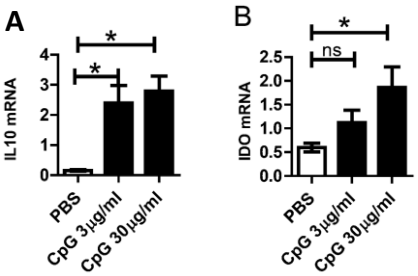

Figure 14. Increased the expression of IDO and IL10 in the aorta of CpG-treated *Apoe*$^{-/-}$ mice. (A) mRNA expression of IDO and (B) IL-10 in the aorta of *Apoe*$^{-/-}$ mice 3days after a single injection of PBS (n=4) or CpG (3 µg/ml, n=5 or 30 µg/ml, n=4). Mann Whitney test, * $p < 0.05$, ** $p < 0.01$.

4 CONCLUSIONS

This thesis illustrated the distinct roles of TLRs and NLRs in contribution to the complex pathogenesis of atherosclerosis (Figure 15). Specific conclusions include the following:

NOD2 is abundantly expressed in endothelial cells and macrophages in human atherosclerotic plaques. NOD2 specifically activates COX2-PGE2 axis via NF-κB and MAPK p38 pathway in human atherosclerotic tissue. NOD2 activation promotes atherosclerosis *in vivo*, which is associated with enlarged necrotic core in the atherosclerotic plaques and enhanced vascular inflammation. NOD2 induced lipid retention in macrophages may contribute to the necrotic core formation, and thereby contribute to the development of vulnerable atherosclerotic plaques.

NOD1 induces cytokine production in human atherosclerotic plaques. Activation of NOD1 enhances the development of occlusive atherosclerosis with elastin degradation and smooth muscle cell activation. NOD1 induces smooth muscle cell activation manifested by increased chemokine and MMP production, which may contribute to the mechanism of NOD1-induced occlusive atherosclerosis.

TLR9 stimulation *in vivo* decreases atherosclerosis and necrotic core although activates inflammatory responses in arteries and blood. Two anti-inflammatory mediators IL-10 and IDO are induced by TLR9 stimulation and probably contribute to the protective mechanisms of TLR9 in atherosclerosis.

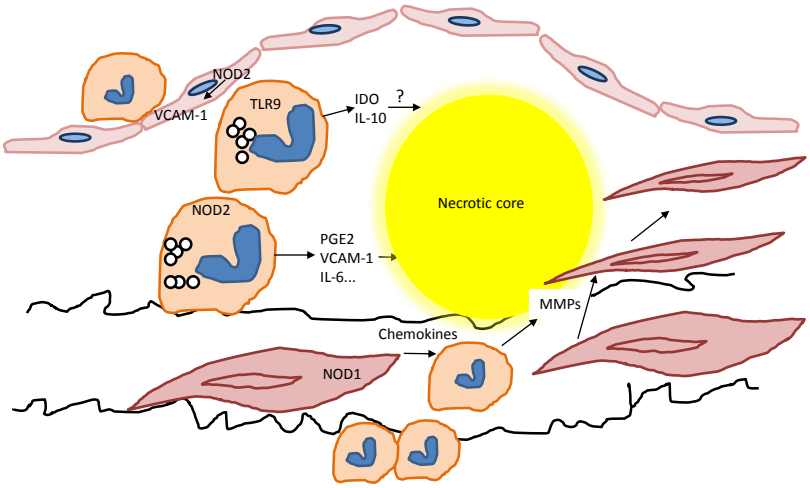

Figure 15. The role of NOD2, NOD1, and TLR9 in atherosclerosis. NOD2 in Ecs and macrophages promotes vascular inflammation and necrotic core formation and thereby promotes atherosclerosis. NOD1 signal in smooth muscle cells enhance chemokine and MMP production, elastin degradation and thus results in occlusive atherosclerosis. TLR9 stimulation decreases necrotic core formation and atherosclerosis probably through IL-10 and IDO.

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6 REFERENCES

1. Murray, C.J., et al., *Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010.* Lancet, 2012. **380**(9859): p. 2197-223.

2. Kathiresan, S. and D. Srivastava, *Genetics of human cardiovascular disease.* Cell, 2012. **148**(6): p. 1242-57.

3. Perk, J., et al., *European Guidelines on cardiovascular disease prevention in clinical practice (version 2012). The Fifth Joint Task Force of the European Society of Cardiology and Other Societies on Cardiovascular Disease Prevention in Clinical Practice (constituted by representatives of nine societies and by invited experts).* Eur Heart J, 2012. **33**(13): p. 1635-701.

4. Libby, P., *Mechanisms of acute coronary syndromes and their implications for therapy.* N Engl J Med, 2013. **368**(21): p. 2004-13.

5. Hansson, G.K. and P. Libby, *The immune response in atherosclerosis: a double-edged sword.* Nat Rev Immunol, 2006. **6**(7): p. 508-19.

6. Virmani, R., et al., *Pathology of the vulnerable plaque.* J Am Coll Cardiol, 2006. **47**(8 Suppl): p. C13-8.

7. Hellings, W.E., et al., *Composition of carotid atherosclerotic plaque is associated with cardiovascular outcome: a prognostic study.* Circulation, 2010. **121**(17): p. 1941-50.

8. Hatsukami, T.S., et al., *Carotid plaque morphology and clinical events.* Stroke, 1997. **28**(1): p. 95-100.

9. Hattori, K., et al., *Impact of statin therapy on plaque characteristics as assessed by serial OCT, grayscale and integrated backscatter-IVUS.* JACC Cardiovasc Imaging, 2012. **5**(2): p. 169-77.

10. Takarada, S., et al., *Effect of statin therapy on coronary fibrous-cap thickness in patients with acute coronary syndrome: assessment by optical coherence tomography study.* Atherosclerosis, 2009. **202**(2): p. 491-7.

11. van Lammeren, G.W., et al., *Time-dependent changes in atherosclerotic plaque composition in patients undergoing carotid surgery.* Circulation, 2014. **129**(22): p. 2269-76.

12. Otsuka, F., et al., *Clinical classification of plaque morphology in coronary disease.* Nat Rev Cardiol, 2014. **11**(7): p. 379-89.

13. Libby, P., A.H. Lichtman, and G.K. Hansson, *Immune effector mechanisms implicated in atherosclerosis: from mice to humans.* Immunity, 2013. **38**(6): p. 1092-104.

14. Doring, Y., et al., *Neutrophils in Atherosclerosis: From Mice to Man.* Arterioscler Thromb Vasc Biol, 2014.

15. Bot, I., et al., *CXCR4 blockade induces atherosclerosis by affecting neutrophil function.* J Mol Cell Cardiol, 2014. **74**: p. 44-52.

16. Paulson, K.E., et al., *Resident intimal dendritic cells accumulate lipid and contribute to the initiation of atherosclerosis.* Circ Res, 2010. **106**(2): p. 383-90.

17. Christ, A., et al., *Dendritic cells in cardiovascular diseases: epiphenomenon, contributor, or therapeutic opportunity.* Circulation, 2013. **128**(24): p. 2603-13.

18. Libby, P. and G.P. Shi, *Mast cells as mediators and modulators of atherogenesis.* Circulation, 2007. **115**(19): p. 2471-3.

19. Sun, J., et al., *Mast cells promote atherosclerosis by releasing proinflammatory cytokines.* Nat Med, 2007. **13**(6): p. 719-24.

20. Packard, R.R., A.H. Lichtman, and P. Libby, *Innate and adaptive immunity in atherosclerosis.* Semin Immunopathol, 2009. **31**(1): p. 5-22.

21. Bjorkbacka, H. and J. Nilsson, *Innate immunity in atherosclerosis.* J Innate Immun, 2010. **2**(4): p. 305-6.

22. Bekkering, S., et al., *Trained innate immunity and atherosclerosis.* Curr Opin Lipidol, 2013. **24**(6): p. 487-92.

23. Ziegler-Heitbrock, L., *The CD14+ CD16+ blood monocytes: their role in infection and inflammation.* Journal of Leukocyte Biology, 2007. **81**(3): p. 584-592.

24. Mantovani, A., C. Garlanda, and M. Locati, *Macrophage diversity and polarization in atherosclerosis: a question of balance.* Arterioscler Thromb Vasc Biol, 2009. **29**(10): p. 1419-23.

25. Robbins, C.S., et al., *Extramedullary hematopoiesis generates Ly-6C(high) monocytes that infiltrate atherosclerotic lesions.* Circulation, 2012. **125**(2): p. 364-74.

26. Swirski, F.K., et al., *Ly-6Chi monocytes dominate hypercholesterolemia-associated monocytosis and give rise to macrophages in atheromata.* J Clin Invest, 2007. **117**(1): p. 195-205.

27. Tacke, F., et al., *Monocyte subsets differentially employ CCR2, CCR5, and CX3CR1 to accumulate within atherosclerotic plaques.* J Clin Invest, 2007. **117**(1): p. 185-94.

28. Nahrendorf, M., et al., *The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions.* J Exp Med, 2007. **204**(12): p. 3037-47.

29. Carlin, L.M., et al., *Nr4a1-dependent Ly6C(low) monocytes monitor endothelial cells and orchestrate their disposal.* Cell, 2013. **153**(2): p. 362-75.

30. Landsman, L., et al., *CX3CR1 is required for monocyte homeostasis and atherogenesis by promoting cell survival.* Blood, 2009. **113**(4): p. 963-72.

31. Combadiere, C., et al., *Decreased atherosclerotic lesion formation in CX3CR1/apolipoprotein E double knockout mice.* Circulation, 2003. **107**(7): p. 1009-16.

32. De Paoli, F., B. Staels, and G. Chinetti-Gbaguidi, *Macrophage phenotypes and their modulation in atherosclerosis.* Circ J, 2014. **78**(8): p. 1775-81.

33. Brocheriou, I., et al., *Antagonistic regulation of macrophage phenotype by M-CSF and GM-CSF: implication in atherosclerosis.* Atherosclerosis, 2011. **214**(2): p. 316-24.

34. Stoger, J.L., et al., *Distribution of macrophage polarization markers in human atherosclerosis.* Atherosclerosis, 2012. **225**(2): p. 461-8.

35. Clinton, S.K., et al., *Macrophage colony-stimulating factor gene expression in vascular cells and in experimental and human atherosclerosis.* Am J Pathol, 1992. **140**(2): p. 301-16.

36. Kadl, A., et al., *Identification of a novel macrophage phenotype that develops in response to atherogenic phospholipids via Nrf2.* Circ Res, 2010. **107**(6): p. 737-46.

37. Feig, J.E., et al., *Regression of atherosclerosis is characterized by broad changes in the plaque macrophage transcriptome.* PLoS One, 2012. **7**(6): p. e39790.

38. Robbins, C.S., et al., *Local proliferation dominates lesional macrophage accumulation in atherosclerosis.* Nat Med, 2013.

39. Abbas, A.K., A.H. Lichtman, and S. Pillai, *Basic immunology : functions and disorders of the immune system*. Fourth edition. ed. 2014, Philadelphia, PA: Elsevier/Saunders. ix, 320 pages.

40. Lemaitre, B., et al., *The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults.* Cell, 1996. **86**(6): p. 973-83.

41. Ip, Y.T. and M. Levine, *Molecular genetics of Drosophila immunity.* Curr Opin Genet Dev, 1994. **4**(5): p. 672-7.

42. Rosetto, M., et al., *Signals from the IL-1 receptor homolog, Toll, can activate an immune response in a Drosophila hemocyte cell line.* Biochem Biophys Res Commun, 1995. **209**(1): p. 111-6.

43. Medzhitov, R., P. Preston-Hurlburt, and C.A. Janeway, Jr., *A human homologue of the Drosophila Toll protein signals activation of adaptive immunity.* Nature, 1997. **388**(6640): p. 394-7.

44. Poltorak, A., et al., *Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene.* Science, 1998. **282**(5396): p. 2085-8.

45. *Nobel Prize Website.* 2011.

46. Broz, P. and D.M. Monack, *Newly described pattern recognition receptors team up against intracellular pathogens.* Nat Rev Immunol, 2013. **13**(8): p. 551-65.

47. Yan, Z.Q. and G.K. Hansson, *Innate immunity, macrophage activation, and atherosclerosis.* Immunol Rev, 2007. **219**: p. 187-203.

48. Lundberg, A.M. and G.K. Hansson, *Innate immune signals in atherosclerosis.* Clin Immunol, 2010. **134**(1): p. 5-24.

49. Li, A.C. and C.K. Glass, *The macrophage foam cell as a target for therapeutic intervention.* Nat Med, 2002. **8**(11): p. 1235-42.

50. Duewell, P., et al., *NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals.* Nature, 2010. **464**(7293): p. 1357-61.

51. McGovern, D.P., et al., *Association between a complex insertion/deletion polymorphism in NOD1 (CARD4) and susceptibility to inflammatory bowel disease.* Hum Mol Genet, 2005. **14**(10): p. 1245-50.

52. Ogura, Y., et al., *A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease.* Nature, 2001. **411**(6837): p. 603-6.

53. Applequist, S.E., R.P. Wallin, and H.G. Ljunggren, *Variable expression of Toll-like receptor in murine innate and adaptive immune cell lines.* Int Immunol, 2002. **14**(9): p. 1065-74.

54. Zarember, K.A. and P.J. Godowski, *Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines.* J Immunol, 2002. **168**(2): p. 554-61.

55. Edfeldt, K., et al., *Expression of toll-like receptors in human atherosclerotic lesions: a possible pathway for plaque activation.* Circulation, 2002. **105**(10): p. 1158-61.

56. Hammond, T., et al., *Toll-like receptor (TLR) expression on CD4+ and CD8+ T-cells in patients chronically infected with hepatitis C virus.* Cell Immunol, 2010. **264**(2): p. 150-5.

57. Xu, X.H., et al., *Toll-like receptor-4 is expressed by macrophages in murine and human lipid-rich atherosclerotic plaques and upregulated by oxidized LDL.* Circulation, 2001. **104**(25): p. 3103-8.

58. Schoneveld, A.H., et al., *Atherosclerotic lesion development and Toll like receptor 2 and 4 responsiveness.* Atherosclerosis, 2008. **197**(1): p. 95-104.

59. Methe, H., et al., *Expansion of circulating Toll-like receptor 4-positive monocytes in patients with acute coronary syndrome.* Circulation, 2005. **111**(20): p. 2654-61.

60. Ziegler-Heitbrock, L., *The CD14+ CD16+ blood monocytes: their role in infection and inflammation.* J Leukoc Biol, 2007. **81**(3): p. 584-92.

61. Kashiwagi, M., et al., *Differential expression of Toll-like receptor 4 and human monocyte subsets in acute myocardial infarction.* Atherosclerosis, 2012. **221**(1): p. 249-53.

62. Wyss, C.A., et al., *Cellular actors, Toll-like receptors, and local cytokine profile in acute coronary syndromes.* Eur Heart J, 2010. **31**(12): p. 1457-69.

63. Lorenzen, J.M., et al., *TLR-4+ peripheral blood monocytes and cardiovascular events in patients with chronic kidney disease--a prospective follow-up study.* Nephrol Dial Transplant, 2011. **26**(4): p. 1421-4.

64. Shibolet, O. and D.K. Podolsky, *TLRs in the Gut. IV. Negative regulation of Toll-like receptors and intestinal homeostasis: addition by subtraction.* Am J Physiol Gastrointest Liver Physiol, 2007. **292**(6): p. G1469-73.

65. Gareus, R., et al., *Endothelial cell-specific NF-kappaB inhibition protects mice from atherosclerosis.* Cell Metab, 2008. **8**(5): p. 372-83.

66. Kanters, E., et al., *Inhibition of NF-kappaB activation in macrophages increases atherosclerosis in LDL receptor-deficient mice.* J Clin Invest, 2003. **112**(8): p. 1176-85.

67. Yvan-Charvet, L., et al., *Increased inflammatory gene expression in ABC transporter-deficient macrophages: free cholesterol accumulation, increased signaling via toll-like receptors, and neutrophil infiltration of atherosclerotic lesions.* Circulation, 2008. **118**(18): p. 1837-47.

68. Mirza, M.K., et al., *Caveolin-1 Deficiency Dampens Toll-Like Receptor 4 Signaling through eNOS Activation.* American Journal of Pathology, 2010. **176**(5): p. 2344-2351.

69. Straus, D.S. and C.K. Glass, *Anti-inflammatory actions of PPAR ligands: new insights on cellular and molecular mechanisms.* Trends Immunol, 2007. **28**(12): p. 551-8.

70. Zhao, W., et al., *Peroxisome proliferator-activated receptor gamma negatively regulates IFN-beta production in Toll-like receptor (TLR) 3- and TLR4-stimulated macrophages by preventing interferon regulatory factor 3 binding to the IFN-beta promoter.* J Biol Chem, 2011. **286**(7): p. 5519-28.

71. Ji, Y., et al., *PPARgamma agonist rosiglitazone ameliorates LPS-induced inflammation in vascular smooth muscle cells via the TLR4/TRIF/IRF3/IP-10 signaling pathway.* Cytokine, 2011. **55**(3): p. 409-19.

72. Sessa, R., et al., *Infectious burden and atherosclerosis: A clinical issue.* World J Clin Cases, 2014. **2**(7): p. 240-9.

73. Laman, J.D., et al., *Significance of peptidoglycan, a proinflammatory bacterial antigen in atherosclerotic arteries and its association with vulnerable plaques.* Am J Cardiol, 2002. **90**(2): p. 119-23.

74. Lee, Y., et al., *Interleukin-1beta is crucial for the induction of coronary artery inflammation in a mouse model of Kawasaki disease.* Circulation, 2012. **125**(12): p. 1542-50.

75. Chen, S., et al., *Marked acceleration of atherosclerosis after Lactobacillus casei-induced coronary arteritis in a mouse model of Kawasaki disease.* Arterioscler Thromb Vasc Biol, 2012. **32**(8): p. e60-71.

76. Wright, S.D., et al., *Infectious agents are not necessary for murine atherogenesis.* J Exp Med, 2000. **191**(8): p. 1437-42.

77. Chen, X., et al., *Study on the relationship between heat shock protein 70 and toll-like receptor-4 of monocytes.* J Huazhong Univ Sci Technolog Med Sci, 2004. **24**(6): p. 560-2.

78. Ohashi, K., et al., *Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex.* J Immunol, 2000. **164**(2): p. 558-61.

79. Vabulas, R.M., et al., *The endoplasmic reticulum-resident heat shock protein Gp96 activates dendritic cells via the Toll-like receptor 2/4 pathway.* J Biol Chem, 2002. **277**(23): p. 20847-53.

80. Yu, M., et al., *HMGB1 signals through toll-like receptor (TLR) 4 and TLR2.* Shock, 2006. **26**(2): p. 174-9.

81. Kariko, K., et al., *mRNA is an endogenous ligand for Toll-like receptor 3.* J Biol Chem, 2004. **279**(13): p. 12542-50.

82. Celhar, T., R. Magalhaes, and A.M. Fairhurst, *TLR7 and TLR9 in SLE: when sensing self goes wrong.* Immunol Res, 2012. **53**(1-3): p. 58-77.

83. Okamura, Y., et al., *The extra domain A of fibronectin activates Toll-like receptor 4.* J Biol Chem, 2001. **276**(13): p. 10229-33.

84. Miller, Y.I., et al., *Minimally modified LDL binds to CD14, induces macrophage spreading via TLR4/MD-2, and inhibits phagocytosis of apoptotic cells.* J Biol Chem, 2003. **278**(3): p. 1561-8.

85. Bae, Y.S., et al., *Macrophages generate reactive oxygen species in response to minimally oxidized low-density lipoprotein: toll-like receptor 4- and spleen tyrosine kinase-dependent activation of NADPH oxidase 2.* Circ Res, 2009. **104**(2): p. 210-8, 21p following 218.

86. Miller, Y.I., et al., *Toll-like receptor 4-dependent and -independent cytokine secretion induced by minimally oxidized low-density lipoprotein in macrophages.* Arterioscler Thromb Vasc Biol, 2005. **25**(6): p. 1213-9.

87. Choi, S.H., et al., *Lipoprotein accumulation in macrophages via toll-like receptor-4-dependent fluid phase uptake.* Circ Res, 2009. **104**(12): p. 1355-63.

88. Choi, S.H., et al., *Spleen tyrosine kinase regulates AP-1 dependent transcriptional response to minimally oxidized LDL.* PLoS One, 2012. **7**(2): p. e32378.

89. Miller, Y.I., et al., *Toll-like receptor-4 and lipoprotein accumulation in macrophages.* Trends Cardiovasc Med, 2009. **19**(7): p. 227-32.

90. Kannan, Y., et al., *Oxidatively modified low density lipoprotein (LDL) inhibits TLR2 and TLR4 cytokine responses in human monocytes but not in macrophages.* J Biol Chem, 2012. **287**(28): p. 23479-88.

91. Takeuchi, O. and S. Akira, *Pattern recognition receptors and inflammation.* Cell, 2010. **140**(6): p. 805-20.

92. Barter, P.J., *Cardioprotective effects of high-density lipoproteins: the evidence strengthens.* Arterioscler Thromb Vasc Biol, 2005. **25**(7): p. 1305-6.

93. Oiknine, J. and M. Aviram, *Increased susceptibility to activation and increased uptake of low density lipoprotein by cholesterol-loaded macrophages.* Arterioscler Thromb, 1992. **12**(6): p. 745-53.

94. Kazemi, M.R., et al., *Adipocyte fatty acid-binding protein expression and lipid accumulation are increased during activation of murine macrophages by toll-like receptor agonists.* Arterioscler Thromb Vasc Biol, 2005. **25**(6): p. 1220-4.

95. Feingold, K.R., et al., *ADRP/ADFP and Mal1 expression are increased in macrophages treated with TLR agonists.* Atherosclerosis, 2010. **209**(1): p. 81-8.

96. Boord, J.B., et al., *Combined adipocyte-macrophage fatty acid-binding protein deficiency improves metabolism, atherosclerosis, and survival in apolipoprotein E-deficient mice.* Circulation, 2004. **110**(11): p. 1492-8.

97. Castrillo, A., et al., *Crosstalk between LXR and toll-like receptor signaling mediates bacterial and viral antagonism of cholesterol metabolism.* Mol Cell, 2003. **12**(4): p. 805-16.

98. Funk, J.L., et al., *Lipopolysaccharide stimulation of RAW 264.7 macrophages induces lipid accumulation and foam cell formation.* Atherosclerosis, 1993. **98**(1): p. 67-82.

99. Feingold, K.R., et al., *Mechanisms of triglyceride accumulation in activated macrophages.* J Leukoc Biol, 2012. **92**(4): p. 829-39.

100. Howell, K.W., et al., *Toll-like receptor 4 mediates oxidized LDL-induced macrophage differentiation to foam cells.* J Surg Res, 2011. **171**(1): p. e27-31.

101. Davignon, J. and P. Ganz, *Role of endothelial dysfunction in atherosclerosis.* Circulation, 2004. **109**(23 Suppl 1): p. Iii27-32.

102. Messner, B. and D. Bernhard, *Smoking and cardiovascular disease: mechanisms of endothelial dysfunction and early atherogenesis.* Arterioscler Thromb Vasc Biol, 2014. **34**(3): p. 509-15.

103. Thorand, B., et al., *Elevated markers of endothelial dysfunction predict type 2 diabetes mellitus in middle-aged men and women from the general population.* Arterioscler Thromb Vasc Biol, 2006. **26**(2): p. 398-405.

104. Speer, T., et al., *Abnormal high-density lipoprotein induces endothelial dysfunction via activation of Toll-like receptor-2.* Immunity, 2013. **38**(4): p. 754-68.

105. Mulligan-Kehoe, M.J. and M. Simons, *Vasa vasorum in normal and diseased arteries.* Circulation, 2014. **129**(24): p. 2557-66.

106. Reed, D.M., et al., *Pathogen sensing pathways in human embryonic stem cell derived-endothelial cells: role of NOD1 receptors.* PLoS One, 2014. **9**(4): p. e91119.

107. Davey, M.P., et al., *Human endothelial cells express NOD2/CARD15 and increase IL-6 secretion in response to muramyl dipeptide.* Microvascular Research, 2006. **71**(2): p. 103-107.

108. Lu, Z., et al., *Toll-like receptor 4 activation in microvascular endothelial cells triggers a robust inflammatory response and cross talk with mononuclear cells via interleukin-6.* Arterioscler Thromb Vasc Biol, 2012. **32**(7): p. 1696-706.

109. Harari, O.A., et al., *Absence of TRAM restricts Toll-like receptor 4 signaling in vascular endothelial cells to the MyD88 pathway.* Circ Res, 2006. **98**(9): p. 1134-40.

110. He, J., et al., *The expression of functional Toll-like receptor 4 is associated with proliferation and maintenance of stem cell phenotype in endothelial progenitor cells (EPCs).* J Cell Biochem, 2010. **111**(1): p. 179-86.

111. Kebschull, M., et al., *Mobilization of endothelial progenitors by recurrent bacteremias with a periodontal pathogen.* PLoS One, 2013. **8**(1): p. e54860.

112. Kwon, M.Y., et al., *Nucleotide-binding oligomerization domain protein 2 deficiency enhances neointimal formation in response to vascular injury.* Arterioscler Thromb Vasc Biol, 2011. **31**(11): p. 2441-7.

113. Butoi, E.D., et al., *Cross talk between smooth muscle cells and monocytes/activated monocytes via CX3CL1/CX3CR1 axis augments expression of pro-atherogenic molecules.* Biochim Biophys Acta, 2011. **1813**(12): p. 2026-35.

114. den Dekker, W.K., et al., *Mast cells induce vascular smooth muscle cell apoptosis via a toll-like receptor 4 activation pathway.* Arterioscler Thromb Vasc Biol, 2012. **32**(8): p. 1960-9.

115. Higashimori, M., et al., *Role of toll-like receptor 4 in intimal foam cell accumulation in apolipoprotein E-deficient mice.* Arterioscler Thromb Vasc Biol, 2011. **31**(1): p. 50-7.

116. Kiyan, Y., et al., *oxLDL induces inflammatory responses in vascular smooth muscle cells via urokinase receptor association with CD36 and TLR4.* J Mol Cell Cardiol, 2013.

117. Feng, B., et al., *The endoplasmic reticulum is the site of cholesterol-induced cytotoxicity in macrophages.* Nat Cell Biol, 2003. **5**(9): p. 781-92.

118. Coope, A., et al., *Chaperone insufficiency links TLR4 protein signaling to endoplasmic reticulum stress.* J Biol Chem, 2012. **287**(19): p. 15580-9.

119. Yao, S., et al., *Minimally modified low-density lipoprotein induces macrophage endoplasmic reticulum stress via toll-like receptor 4.* Biochim Biophys Acta, 2012. **1821**(7): p. 954-63.

120. Seimon, T.A., et al., *Atherogenic lipids and lipoproteins trigger CD36-TLR2-dependent apoptosis in macrophages undergoing endoplasmic reticulum stress.* Cell Metab, 2010. **12**(5): p. 467-82.

121. Kaiser, W.J., et al., *Toll-like receptor 3-mediated necrosis via TRIF, RIP3, and MLKL.* J Biol Chem, 2013. **288**(43): p. 31268-79.

122. Michelsen, K.S., et al., *Lack of Toll-like receptor 4 or myeloid differentiation factor 88 reduces atherosclerosis and alters plaque phenotype in mice deficient in apolipoprotein E.* Proc Natl Acad Sci U S A, 2004. **101**(29): p. 10679-84.

123. Bjorkbacka, H., et al., *Reduced atherosclerosis in MyD88-null mice links elevated serum cholesterol levels to activation of innate immunity signaling pathways.* Nat Med, 2004. **10**(4): p. 416-21.

124. Hollestelle, S.C., et al., *Toll-like receptor 4 is involved in outward arterial remodeling.* Circulation, 2004. **109**(3): p. 393-8.

125. Ding, Y., et al., *Toll-like receptor 4 deficiency decreases atherosclerosis but does not protect against inflammation in obese low-density lipoprotein receptor-deficient mice.* Arterioscler Thromb Vasc Biol, 2012. **32**(7): p. 1596-604.

126. Hayashi, C., et al., *Protective role for TLR4 signaling in atherosclerosis progression as revealed by infection with a common oral pathogen.* J Immunol, 2012. **189**(7): p. 3681-8.

127. Mullick, A.E., P.S. Tobias, and L.K. Curtiss, *Modulation of atherosclerosis in mice by Toll-like receptor 2.* J Clin Invest, 2005. **115**(11): p. 3149-56.

128. Liu, X., et al., *Toll-like receptor 2 plays a critical role in the progression of atherosclerosis that is independent of dietary lipids.* Atherosclerosis, 2008. **196**(1): p. 146-54.

129. Schoneveld, A.H., et al., *Toll-like receptor 2 stimulation induces intimal hyperplasia and atherosclerotic lesion development.* Cardiovasc Res, 2005. **66**(1): p. 162-9.

130. Shinohara, M., et al., *Local overexpression of toll-like receptors at the vessel wall induces atherosclerotic lesion formation: synergism of TLR2 and TLR4.* Arterioscler Thromb Vasc Biol, 2007. **27**(11): p. 2384-91.

131. Cole, J.E., et al., *Unexpected protective role for Toll-like receptor 3 in the arterial wall.* Proc Natl Acad Sci U S A, 2011. **108**(6): p. 2372-7.

132. Salagianni, M., et al., *Toll-like receptor 7 protects from atherosclerosis by constraining "inflammatory" macrophage activation.* Circulation, 2012. **126**(8): p. 952-62.

133. Frantz, S., G. Ertl, and J. Bauersachs, *Mechanisms of disease: Toll-like receptors in cardiovascular disease.* Nat Clin Pract Cardiovasc Med, 2007. **4**(8): p. 444-54.

134. Chaput, C., et al., *NOD-Like Receptors in Lung Diseases.* Front Immunol, 2013. **4**: p. 393.

135. Inohara, N., et al., *Nod1, an Apaf-1-like activator of caspase-9 and nuclear factor-kappaB.* J Biol Chem, 1999. **274**(21): p. 14560-7.

136. Ogura, Y., et al., *Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB.* J Biol Chem, 2001. **276**(7): p. 4812-8.

137. Voss, E., et al., *NOD2/CARD15 mediates induction of the antimicrobial peptide human beta-defensin-2.* J Biol Chem, 2006. **281**(4): p. 2005-11.

138. Ogura, Y., et al., *Expression of NOD2 in Paneth cells: a possible link to Crohn's ileitis.* Gut, 2003. **52**(11): p. 1591-7.

139. Uehara, A., et al., *Various human epithelial cells express functional Toll-like receptors, NOD1 and NOD2 to produce anti-microbial peptides, but not proinflammatory cytokines.* Mol Immunol, 2007. **44**(12): p. 3100-11.

140. Marriott, I., et al., *Induction of Nod1 and Nod2 intracellular pattern recognition receptors in murine osteoblasts following bacterial challenge.* Infect Immun, 2005. **73**(5): p. 2967-73.

141. Chen, G., et al., *NOD-like receptors: role in innate immunity and inflammatory disease.* Annu Rev Pathol, 2009. **4**: p. 365-98.

142. Girardin, S.E., et al., *Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan.* Science, 2003. **300**(5625): p. 1584-7.

143. Chamaillard, M., et al., *An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid.* Nat Immunol, 2003. **4**(7): p. 702-7.

144. Ting, J.P., J.A. Duncan, and Y. Lei, *How the noninflammasome NLRs function in the innate immune system.* Science, 2010. **327**(5963): p. 286-90.

145. Hasegawa, M., et al., *Differential release and distribution of Nod1 and Nod2 immunostimulatory molecules among bacterial species and environments.* J Biol Chem, 2006. **281**(39): p. 29054-63.

146. Fujimoto, Y., et al., *Peptidoglycan as Nod1 ligand; fragment structures in the environment, chemical synthesis, and their innate immunostimulation.* Nat Prod Rep, 2012. **29**(5): p. 568-79.

147. Clarke, T.B., et al., *Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity.* Nat Med, 2010. **16**(2): p. 228-31.

148. Mine, Y., et al., *Immunoactive peptides, FK-156 and FK-565. I. Enhancement of host resistance to microbial infection in mice.* J Antibiot (Tokyo), 1983. **36**(8): p. 1045-50.

149. Karlsson, F.H., et al., *Symptomatic atherosclerosis is associated with an altered gut metagenome.* Nat Commun, 2012. **3**: p. 1245.

150. Davis, B.K., H. Wen, and J.P. Ting, *The inflammasome NLRs in immunity, inflammation, and associated diseases.* Annu Rev Immunol, 2011. **29**: p. 707-35.

151. Salem, M., et al., *Muramyl dipeptide responsive pathways in Crohn's disease: from NOD2 and beyond.* Cell Mol Life Sci, 2013. **70**(18): p. 3391-404.

152. Nakamura, N., et al., *Endosomes are specialized platforms for bacterial sensing and NOD2 signalling.* Nature, 2014. **509**(7499): p. 240-4.

153. Hasegawa, M., et al., *A critical role of RICK/RIP2 polyubiquitination in Nod-induced NF-kappaB activation.* EMBO J, 2008. **27**(2): p. 373-83.

154. Park, J.H., et al., *RICK/RIP2 mediates innate immune responses induced through Nod1 and Nod2 but not TLRs.* J Immunol, 2007. **178**(4): p. 2380-6.

155. Hedl, M. and C. Abraham, *Distinct roles for Nod2 protein and autocrine interleukin-1beta in muramyl dipeptide-induced mitogen-activated protein kinase activation and cytokine secretion in human macrophages.* J Biol Chem, 2011. **286**(30): p. 26440-9.

156. Sabbah, A., et al., *Activation of innate immune antiviral responses by Nod2.* Nat Immunol, 2009. **10**(10): p. 1073-80.

157. Moore, C.B., et al., *NLRX1 is a regulator of mitochondrial antiviral immunity.* Nature, 2008. **451**(7178): p. 573-7.

158. Hsu, L.C., et al., *A NOD2-NALP1 complex mediates caspase-1-dependent IL-1beta secretion in response to Bacillus anthracis infection and muramyl dipeptide.* Proc Natl Acad Sci U S A, 2008. **105**(22): p. 7803-8.

159. Agostini, L., et al., *NALP3 forms an IL-1beta-processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder.* Immunity, 2004. **20**(3): p. 319-25.

160. Martinon, F., K. Burns, and J. Tschopp, *The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta.* Mol Cell, 2002. **10**(2): p. 417-26.

161. Faustin, B., et al., *Reconstituted NALP1 inflammasome reveals two-step mechanism of caspase-1 activation.* Mol Cell, 2007. **25**(5): p. 713-24.

162. Pan, Q., et al., *MDP-induced interleukin-1beta processing requires Nod2 and CIAS1/NALP3.* J Leukoc Biol, 2007. **82**(1): p. 177-83.

163. Kang, T.B., et al., *Caspase-8 blocks kinase RIPK3-mediated activation of the NLRP3 inflammasome.* Immunity, 2013. **38**(1): p. 27-40.

164. Watanabe, Y., et al., *Immunoactive peptides, FK 156 and FK 565. IV. Activation of mouse macrophages.* J Antibiot (Tokyo), 1985. **38**(12): p. 1781-7.

165. Fritz, J.H., et al., *Nod1-mediated innate immune recognition of peptidoglycan contributes to the onset of adaptive immunity.* Immunity, 2007. **26**(4): p. 445-59.

166. Magalhaes, J.G., et al., *Nod2-dependent Th2 polarization of antigen-specific immunity.* J Immunol, 2008. **181**(11): p. 7925-35.

167. Cooney, R., et al., *NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation.* Nat Med, 2010. **16**(1): p. 90-7.

168. Travassos, L.H., et al., *Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry.* Nat Immunol, 2010. **11**(1): p. 55-62.

169. Mosa, A., et al., *Nonhematopoietic cells control the outcome of infection with Listeria monocytogenes in a nucleotide oligomerization domain 1-dependent manner.* Infect Immun, 2009. **77**(7): p. 2908-18.

170. Hasegawa, M., et al., *Nucleotide-binding oligomerization domain 1 mediates recognition of Clostridium difficile and induces neutrophil recruitment and protection against the pathogen.* J Immunol, 2011. **186**(8): p. 4872-80.

171. Schenk, M., et al., *NOD2 triggers an interleukin-32-dependent human dendritic cell program in leprosy.* Nat Med, 2012. **18**(4): p. 555-63.

172. Heinhuis, B., et al., *Towards a role of interleukin-32 in atherosclerosis.* Cytokine, 2013. **64**(1): p. 433-40.

173. Geddes, K., et al., *Nod1 and Nod2 regulation of inflammation in the Salmonella colitis model.* Infect Immun, 2010. **78**(12): p. 5107-15.

174. Vieira, S.M., et al., *Joint NOD2/RIPK2 signaling regulates IL-17 axis and contributes to the development of experimental arthritis.* J Immunol, 2012. **188**(10): p. 5116-22.

175. Hysi, P., et al., *NOD1 variation, immunoglobulin E and asthma.* Hum Mol Genet, 2005. **14**(7): p. 935-41.

176. Eder, W., et al., *Association between exposure to farming, allergies and genetic variation in CARD4/NOD1.* Allergy, 2006. **61**(9): p. 1117-24.

177. Weidinger, S., et al., *Association of NOD1 polymorphisms with atopic eczema and related phenotypes.* J Allergy Clin Immunol, 2005. **116**(1): p. 177-84.

178. Hugot, J.P., et al., *Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease.* Nature, 2001. **411**(6837): p. 599-603.

179. Manichanh, C., et al., *The gut microbiota in IBD.* Nat Rev Gastroenterol Hepatol, 2012. **9**(10): p. 599-608.

180. Brain, O., et al., *The intracellular sensor NOD2 induces microRNA-29 expression in human dendritic cells to limit IL-23 release.* Immunity, 2013. **39**(3): p. 521-36.

181. Chen, K.C., et al., *OxLDL up-regulates microRNA-29b, leading to epigenetic modifications of MMP-2/MMP-9 genes: a novel mechanism for cardiovascular diseases.* FASEB J, 2011. **25**(5): p. 1718-28.

182. Menu, P., et al., *Atherosclerosis in ApoE-deficient mice progresses independently of the NLRP3 inflammasome.* Cell Death Dis, 2011. **2**: p. e137.

183. Fernandez-Velasco, M., et al., *NOD1 activation induces cardiac dysfunction and modulates cardiac fibrosis and cardiomyocyte apoptosis.* PLoS One, 2012. **7**(9): p. e45260.

184. Nishio, H., et al., *Nod1 ligands induce site-specific vascular inflammation.* Arterioscler Thromb Vasc Biol, 2011. **31**(5): p. 1093-9.

185. Galluzzo, S., et al., *Association between NOD2/CARD15 polymorphisms and coronary artery disease: a case-control study.* Hum Immunol, 2011. **72**(8): p. 636-40.

186. Yazdanyar, S. and B.G. Nordestgaard, *NOD2/CARD15 genotype, cardiovascular disease and cancer in 43,600 individuals from the general population.* J Intern Med, 2010. **268**(2): p. 162-70.

187. Vink, A., et al., *Plaque burden, arterial remodeling and plaque vulnerability: determined by systemic factors?* J Am Coll Cardiol, 2001. **38**(3): p. 718-23.

188. Kapourchali, F.R., et al., *Animal models of atherosclerosis.* World J Clin Cases, 2014. **2**(5): p. 126-32.

189. Ishibashi, S., et al., *The two-receptor model of lipoprotein clearance: tests of the hypothesis in "knockout" mice lacking the low density lipoprotein receptor,*

apolipoprotein E, or both proteins. Proc Natl Acad Sci U S A, 1994. **91**(10): p. 4431-5.

190. Sehayek, E., et al., *Apolipoprotein E regulates dietary cholesterol absorption and biliary cholesterol excretion: studies in C57BL/6 apolipoprotein E knockout mice.* Proc Natl Acad Sci U S A, 2000. **97**(7): p. 3433-7.

191. Jofre-Monseny, L., A.M. Minihane, and G. Rimbach, *Impact of apoE genotype on oxidative stress, inflammation and disease risk.* Mol Nutr Food Res, 2008. **52**(1): p. 131-45.

192. Guo, L., M.J. LaDu, and L.J. Van Eldik, *A dual role for apolipoprotein e in neuroinflammation: anti- and pro-inflammatory activity.* J Mol Neurosci, 2004. **23**(3): p. 205-12.

193. Zhang, S.H., et al., *Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E.* Science, 1992. **258**(5081): p. 468-71.

194. Linton, M.F., J.B. Atkinson, and S. Fazio, *Prevention of atherosclerosis in apolipoprotein E-deficient mice by bone marrow transplantation.* Science, 1995. **267**(5200): p. 1034-7.

195. Ishibashi, S., et al., *Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery.* J Clin Invest, 1993. **92**(2): p. 883-93.

196. Ishibashi, S., et al., *Massive xanthomatosis and atherosclerosis in cholesterol-fed low density lipoprotein receptor-negative mice.* J Clin Invest, 1994. **93**(5): p. 1885-93.

197. Volpi, C., et al., *A GpC-rich oligonucleotide acts on plasmacytoid dendritic cells to promote immune suppression.* J Immunol, 2012. **189**(5): p. 2283-9.

198. Funk, C.D., *Prostaglandins and leukotrienes: advances in eicosanoid biology.* Science, 2001. **294**(5548): p. 1871-5.

199. Ricciotti, E. and G.A. FitzGerald, *Prostaglandins and inflammation.* Arterioscler Thromb Vasc Biol, 2011. **31**(5): p. 986-1000.

200. Haeggstrom, J.Z. and C.D. Funk, *Lipoxygenase and leukotriene pathways: biochemistry, biology, and roles in disease.* Chem Rev, 2011. **111**(10): p. 5866-98.

201. Oh, H.M., et al., *Induction and localization of NOD2 protein in human endothelial cells.* Cell Immunol, 2005. **237**(1): p. 37-44.

202. Gutierrez, O., et al., *Induction of Nod2 in myelomonocytic and intestinal epithelial cells via nuclear factor-kappa B activation.* J Biol Chem., 2002. **277**(44): p. 41701-5. Epub 2002 Aug 22.

203. Gertow, K., et al., *12- and 15-lipoxygenases in human carotid atherosclerotic lesions: associations with cerebrovascular symptoms.* Atherosclerosis, 2011. **215**(2): p. 411-6.

204. Peters-Golden, M. and W.R. Henderson, Jr., *Leukotrienes.* N Engl J Med, 2007. **357**(18): p. 1841-54.

205. Riccioni, G. and M. Back, *Leukotrienes as modifiers of preclinical atherosclerosis?* ScientificWorldJournal, 2012. **2012**: p. 490968.

206. Wittwer, J. and M. Hersberger, *The two faces of the 15-lipoxygenase in atherosclerosis.* Prostaglandins Leukot Essent Fatty Acids, 2007. **77**(2): p. 67-77.

207. Schober, L.J., et al., *The role of PGE(2) in human atherosclerotic plaque on platelet EP(3) and EP(4) receptor activation and platelet function in whole blood.* J Thromb Thrombolysis, 2011. **32**(2): p. 158-66.

208. Gross, S., et al., *Vascular wall-produced prostaglandin E2 exacerbates arterial thrombosis and atherothrombosis through platelet EP3 receptors.* J Exp Med, 2007. **204**(2): p. 311-20.

209. Windheim, M., et al., *Molecular mechanisms involved in the regulation of cytokine production by muramyl dipeptide.* Biochemical Journal, 2007. **404**(2): p. 179-190.

210. Li, J., et al., *Regulation of IL-8 and IL-1β expression in Crohn's disease associated NOD2/CARD15 mutations.* Human Molecular Genetics, 2004. **13**(16): p. 1715-1725.

211. Hedl, M. and C. Abraham, *Nod2-Induced Autocrine Interleukin-1 Alters Signaling by ERK and p38 to Differentially Regulate Secretion of Inflammatory Cytokines.* Gastroenterology, 2012. **143**(6): p. 1530-1543.

212. Park, J.H., et al., *RICK/RIP2 mediates innate immune responses induced through Nod1 and Nod2 but not TLRs.* J Immunol, 2007. **178**(4): p. 2380-6.

213. Argast, G., N. Fausto, and J. Campbell, *Inhibition of RIP2/RICK/CARDIAK activity by pyridinyl imidazole inhibitors of p38 MAPK.* Molecular and Cellular Biochemistry, 2005. **268**(1-2): p. 129-140.

214. Langereis, J.D., et al., *Abrogation of NF-κB signaling in human neutrophils induces neutrophil survival through sustained p38-MAPK activation.* Journal of Leukocyte Biology, 2010. **88**(4): p. 655-664.

215. Kobayashi, T., et al., *Detection of peptidoglycan in human plasma using the silkworm larvae plasma test.* FEMS Immunol Med Microbiol, 2000. **28**(1): p. 49-53.

216. Tabata, T., et al., *Bacterial translocation and peptidoglycan translocation by acute ethanol administration.* J Gastroenterol, 2002. **37**(9): p. 726-31.

217. Liu, H.Q., et al., *NOD2-mediated innate immune signaling regulates the eicosanoids in atherosclerosis.* Arterioscler Thromb Vasc Biol, 2013. **33**(9): p. 2193-201.

218. Hegyi, L., et al., *Foam cell apoptosis and the development of the lipid core of human atherosclerosis.* J Pathol, 1996. **180**(4): p. 423-9.

219. Hardwick, S.J., et al., *Apoptosis in human monocyte-macrophages exposed to oxidized low density lipoprotein.* J Pathol, 1996. **179**(3): p. 294-302.

220. Bocan, T.M., T.A. Schifani, and J.R. Guyton, *Ultrastructure of the human aortic fibrolipid lesion. Formation of the atherosclerotic lipid-rich core.* Am J Pathol, 1986. **123**(3): p. 413-24.

221. Han, S., et al., *Macrophage insulin receptor deficiency increases ER stress-induced apoptosis and necrotic core formation in advanced atherosclerotic lesions.* Cell Metab, 2006. **3**(4): p. 257-66.

222. Van Herck, J.L., et al., *Proteasome inhibitor bortezomib promotes a rupture-prone plaque phenotype in ApoE-deficient mice.* Basic Res Cardiol, 2010. **105**(1): p. 39-50.

223. Ouimet, M., et al., *Autophagy regulates cholesterol efflux from macrophage foam cells via lysosomal acid lipase.* Cell Metab, 2011. **13**(6): p. 655-67.

224. Crisby, M., et al., *Cell death in human atherosclerotic plaques involves both oncosis and apoptosis.* Atherosclerosis, 1997. **130**(1-2): p. 17-27.

225. Falk, E., P.K. Shah, and V. Fuster, *Coronary plaque disruption.* Circulation, 1995. **92**(3): p. 657-71.

226. Miao, E.A., J.V. Rajan, and A. Aderem, *Caspase-1-induced pyroptotic cell death.* Immunol Rev, 2011. **243**(1): p. 206-14.

227. Chang, W., et al., *Pyroptosis: an inflammatory cell death implicates in atherosclerosis.* Med Hypotheses, 2013. **81**(3): p. 484-6.

228. Lin, J., et al., *A role of RIP3-mediated macrophage necrosis in atherosclerosis development.* Cell Rep, 2013. **3**(1): p. 200-10.

229. Kolodgie, F.D., et al., *Intraplaque hemorrhage and progression of coronary atheroma.* N Engl J Med, 2003. **349**(24): p. 2316-25.

230. Yuan, H., et al., *Pivotal role of NOD2 in inflammatory processes affecting atherosclerosis and periodontal bone loss.* Proc Natl Acad Sci U S A, 2013. **110**(52): p. E5059-68.

231. Martinon, F., et al., *Identification of bacterial muramyl dipeptide as activator of the NALP3/cryopyrin inflammasome.* Curr Biol, 2004. **14**(21): p. 1929-34.

232. Johansson, M.E., et al., *Innate immune receptor NOD2 promotes vascular inflammation and formation of lipid-rich necrotic cores in hypercholesterolemic mice.* Eur J Immunol, 2014.

233. Opitz, B., et al., *Listeria monocytogenes activated p38 MAPK and induced IL-8 secretion in a nucleotide-binding oligomerization domain 1-dependent manner in endothelial cells.* J Immunol, 2006. **176**(1): p. 484-90.

234. Moreno, L., et al., *Nucleotide oligomerization domain 1 is a dominant pathway for NOS2 induction in vascular smooth muscle cells: comparison with Toll-like receptor 4 responses in macrophages.* Br J Pharmacol, 2010. **160**(8): p. 1997-2007.

235. Keestra, A.M., et al., *Manipulation of small Rho GTPases is a pathogen-induced process detected by NOD1.* Nature, 2013. **496**(7444): p. 233-7.

236. Bond, M., et al., *Rho GTPase, Rac1, regulates Skp2 levels, vascular smooth muscle cell proliferation, and intima formation in vitro and in vivo.* Cardiovasc Res, 2008. **80**(2): p. 290-8.

237. Karnik, S.K., et al., *A critical role for elastin signaling in vascular morphogenesis and disease.* Development, 2003. **130**(2): p. 411-23.

238. Li, W., et al., *Rapamycin inhibits smooth muscle cell proliferation and obstructive arteriopathy attributable to elastin deficiency.* Arterioscler Thromb Vasc Biol, 2013. **33**(5): p. 1028-35.

239. Lutgens, E., et al., *Disruption of the cathepsin K gene reduces atherosclerosis progression and induces plaque fibrosis but accelerates macrophage foam cell formation.* Circulation, 2006. **113**(1): p. 98-107.

240. Serbina, N.V. and E.G. Pamer, *Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2.* Nat Immunol, 2006. **7**(3): p. 311-7.

241. Poupel, L., et al., *Pharmacological inhibition of the chemokine receptor, CX3CR1, reduces atherosclerosis in mice.* Arterioscler Thromb Vasc Biol, 2013. **33**(10): p. 2297-305.

242. Drechsler, M., et al., *Hyperlipidemia-triggered neutrophilia promotes early atherosclerosis.* Circulation, 2010. **122**(18): p. 1837-45.

243. Michel, J.B., et al., *Pathology of human plaque vulnerability: Mechanisms and consequences of intraplaque haemorrhages.* Atherosclerosis, 2014. **234**(2): p. 311-319.

244. Niessner, A., et al., *Pathogen-sensing plasmacytoid dendritic cells stimulate cytotoxic T-cell function in the atherosclerotic plaque through interferon-alpha.* Circulation, 2006. **114**(23): p. 2482-9.

245. Means, T.K. and A.D. Luster, *Toll-like receptor activation in the pathogenesis of systemic lupus erythematosus.* Ann N Y Acad Sci, 2005. **1062**: p. 242-51.

246. McMahon, M., B.H. Hahn, and B.J. Skaggs, *Systemic lupus erythematosus and cardiovascular disease: prediction and potential for therapeutic intervention.* Expert Rev Clin Immunol, 2011. **7**(2): p. 227-41.

247. Lundberg, A.M., et al., *Toll-like receptor 3 and 4 signalling through the TRIF and TRAM adaptors in haematopoietic cells promotes atherosclerosis.* Cardiovasc Res, 2013. **99**(2): p. 364-73.

248. Koulis, C., et al., *Protective Role for Toll-Like Receptor-9 in the Development of Atherosclerosis in Apolipoprotein E-Deficient Mice.* Arterioscler Thromb Vasc Biol, 2014.

249. Hemmi, H., et al., *A Toll-like receptor recognizes bacterial DNA.* Nature, 2000. **408**(6813): p. 740-5.

250. Landrigan, A., M.T. Wong, and P.J. Utz, *CpG and non-CpG oligodeoxynucleotides directly costimulate mouse and human CD4+ T cells through a TLR9- and MyD88-independent mechanism.* J Immunol, 2011. **187**(6): p. 3033-43.

251. Mellor, A.L., et al., *Cutting edge: CpG oligonucleotides induce splenic CD19+ dendritic cells to acquire potent indoleamine 2,3-dioxygenase-dependent T cell regulatory functions via IFN Type 1 signaling.* J Immunol, 2005. **175**(9): p. 5601-5.

252. Baban, B., et al., *Physiologic control of IDO competence in splenic dendritic cells.* J Immunol, 2011. **187**(5): p. 2329-35.

253. Niinisalo, P., et al., *Activation of indoleamine 2,3-dioxygenase-induced tryptophan degradation in advanced atherosclerotic plaques: Tampere vascular study.* Ann Med, 2010. **42**(1): p. 55-63.

254. Niinisalo, P., et al., *Indoleamine 2,3-dioxygenase activity associates with cardiovascular risk factors: the Health 2000 study.* Scand J Clin Lab Invest, 2008. **68**(8): p. 767-70.