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**INNATE IMMUNE MODULATION IN
ATHEROSCLEROSIS – WITH FOCUS ON
APOB100 DERIVED DANGER
ASSOCIATED SIGNAL 1 (APOBDS-1)**

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Innate immune modulation in atherosclerosis – with
focus on ApoB100 derived danger associated signal 1
(ApoBDS-1)
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my dearest family

ABSTRACT

Elevated level of LDL is the most important risk factor for atherosclerosis. ApoB100 is the only unexchangeable protein in LDL particle. Recent reports have shown that native peptides of ApoB100 trigger activation of adaptive immune responses. Whether ApoB100 can activate innate immune response is less-known.

In this thesis, we identified a native ApoB100 peptide from human ApoB100, named ApoB100 danger associated signal-1 (ApoBDS-1), given its biological nature to trigger innate proinflammatory responses in monocytes and macrophages. Besides macrophages, ApoBDS-1 can also activate platelets and endothelial cells, eliciting proinflammatory mediators and promoting platelet-leukocyte aggregates through complex molecular mechanisms involving Ca^{2+} flux, ROS production, MAPKs activation, PI3K-Akt activation, and microRNA regulations. ApoBDS-1 contributes to the activation of inflammatory signaling in human atherosclerotic plaque. We showed that ApoBDS-1 exists in human carotid plaques by immunofluorescence staining. Size-exclusion chromatography and Western blot confirmed that some low molecular weight fractions isolated from plaque contain ApoBDS-1 epitopes and possess ApoBDS-1-like bioactivity for induction of IL-8. These findings suggest that active ApoBDS-1 presents in atherosclerotic lesions. Analysis of BiKE database indicates that inflammasome pathways are involved in atherosclerosis and associated with the disease severity. Our studies show that ApoBDS-1 is an endogenous activator of NLRP3 inflammasome, inducing IL-1 β in monocytes and macrophages via NLRP3-dependent caspase-1 activation. We also found that ApoBDS-1 could induce NLRP3 inflammasome complex formation in vivo, and activate NLRP3 inflammasome by induction of K^+ efflux. Lastly, we explored the receptor/interacting protein for ApoBDS-1 using far Western blot and 2-D electrophoresis and identified TNF receptor associated protein 1 (Trap1) as an ApoBDS-1 specific interacting protein. Trap1 and ApoBDS-1 are colocalized mainly in cytoplasm and also on cell surface membrane. Biacore SPR analysis suggests that ApoBDS-1 binds to Trap1 with a medium affinity depending on the last 5 amino acids in its C-terminal domain. Trap1 is indispensable for ApoBDS-1 function since ApoBDS-1 induced cytokine secretion and reactive oxygen species can be inhibited by Geldanamycin, an inhibitor of Trap1 or by knocking down of Trap1 using specific shRNA.

Taken together, we have identified ApoBDS-1 as the innate immune activator in ApoB100. Blocking the interaction of ApoBDS-1 and Trap1, or inhibition of ApoBDS-1 induced signaling pathways may represent new therapeutic options for atherosclerosis treatment.

LIST OF SCIENTIFIC PAPERS

- I. Ketelhuth DF, Rios FJ, **Wang Y**, Liu H, Johansson ME, Fredrikson GN, Hedin U, Gidlund M, Nilsson J, Hansson GK, Yan ZQ. **Identification of a danger-associated peptide from apolipoprotein B100 (ApoBDS-1) that triggers innate proatherogenic responses.** *Circulation*. 2011;124(22):2433-2443, 2431-2437.
Note: the first two authors contributed equally.
- II. Assinger A, **Wang Y**, Butler LM, Hansson GK, Yan ZQ, Soderberg-Naucler C, Ketelhuth DF. **Apolipoprotein B100 danger-associated signal 1 (ApoBDS-1) triggers platelet activation and boosts platelet-leukocyte proinflammatory responses.** *Thromb Haemost*. 2014;112(2):332-341.
- III. **Yajuan Wang**, Huiqing Liu, Xintong Jiang, Haiqing Gao, Göran K Hansson, Zhong-qun Yan. **Activation of the NLRP3 inflammasome by a danger-associated peptide from Apolipoprotein B100 (ApoBDS-1) provides a mechanism for IL-1 β production in atherosclerosis.** *Manuscript*
- IV. **Yajuan Wang**, Daniel Ketelhuth, Sanela Kjellqvist, Haiqing Gao, Per Eriksson, Göran Hansson, Zhong-qun Yan. **A danger-associated peptide from Apolipoprotein B100 induces macrophage inflammatory responses by targeting the Tumor necrosis factor type 1 receptor-associated protein 1.** *Manuscript*

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LIST OF ABBREVIATIONS

aa	Amino acid
Acetyl-LDL	Acetylated LDL
AGE	Advanced glycation end product
AIM2	Absent in melanoma 2
ApoBDS-1	ApoB100 derived danger associated signal 1
ASC	Apoptosis-associated speck-like protein containing a CARD
BiKE	Biobank of Karolinska Carotid Endarterectomies
CARD	The caspase activation and recruitment domain
CCL2 (MCP-1)	Monocyte chemotactic protein 1
DAMP	Damage associated molecular pattern
DTT	Dithiothreitol
EC	Endothelial cell
ELISA	The enzyme-linked immunosorbent assay
HEK 293	Human embryonic kidney 293 cells
HDL	High density lipoprotein
HSP, Hsp	Heat shock protein
IDL	Intermediate density lipoprotein
IEF	Isoelectric focusing
IFI16	IFN γ -inducible protein 16
IFN	interferon
IL	Interleukin
IL-1ra	IL-1 receptor antagonist
IMT	Intima-media thickness
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
LRP6	LDL receptor-related protein 6
Lyso-PC	Lysophosphatidylcholine
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemoattractant protein
MDA	Malondialdehyde

miRNA	microRNA
mmLDL	Minimally modified LDL
MMP	Matrix metalloproteinase
NF- κ B	Nuclear factor- κ B
NLR	NOD-like receptor
NOD	Nucleotide-binding oligomerization domain
oxLDL	Oxidized LDL
Ox-PAPC	Oxidized 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphocholine
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PC	Phosphatidylcholine
<i>P. gingivalis</i>	<i>Porphyromonas gingivalis</i>
PLC	Phospholipase C
PPAR γ	Peroxisome proliferator-activated receptor γ
PYHIN	pyrin and HIN domain-containing protein
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
ROS	Reactive oxygen species
shRNA	Small hairpin or short hairpin RNA
siRNA	Small interfering RNA
SMC	Smooth muscle cell
SPR	Surface plasmon resonance
TLR	Toll-like receptor
TG	Triglyceride
TRAP1, Trap1	TNF receptor associated protein 1, also referred to as Heat shock protein 75, Hsp75
VLDL	Very low density lipoprotein
2-D	Two-dimensional electrophoresis

1 INTRODUCTION

Cardiovascular diseases such as coronary heart disease, stroke and peripheral vascular disease are the number one killer world-wide today. These cardiovascular diseases are caused primarily by atherosclerosis, which inflicts the large and medium sized arteries¹.

Development of atherosclerotic plaque initiates as sub-endothelial accumulation of low-density lipoprotein (LDL) and modification of LDL within the vessel wall². On the basis of current understanding vascular inflammation in the early stage of disease may result from LDL derived oxidized phospholipids and cholesterol crystals³⁻⁶. However, LDL has also a large protein molecule, ApoB100, which is the unexchangeable protein of LDL and subjective to enzymatic degradation in the vessel wall. The role of ApoB100 in the atherosclerosis had been neglected for many years. Only in recent past ApoB100 was no longer considered as a by-stander. Fredrikson et al, 2003 revealed that IgM or IgG circulating autoantibodies from atherosclerotic patients could recognize in a sequence specific manner over one hundred different native peptide fragments of ApoB100⁷. These findings indicate that ApoB 100 derived peptides contribute to adaptive immune responses in atherosclerosis. Since both innate immunity and adaptive immunity take part in the pathogenesis of atherosclerosis, it is interesting to investigate the relevance of ApoB 100 peptides to the innate immune responses.

This thesis is focused on the identification of an ApoB100 derived danger associated signal (ApoBDS-1), the effect of ApoBDS-1 on monocytes/macrophages and platelets, the mechanisms behind ApoBDS-1-induced inflammation, and the investigation of interacting protein mediating the effect of ApoBDS-1. First, an overview of innate immunity in atherosclerosis is provided, followed by an emphasis on the innate immune regulatory role of LDL. In the end the importance of our studies is discussed in this context.

1.1 ATHEROSCLEROSIS IS AN INFLAMMATORY DISEASE

Atherosclerosis was for long time believed to occur due to sub-endothelial passive accumulation of cholesterol⁸. Today the picture is much more complex and it is generally accepted that atherosclerosis is a chronic inflammatory disease^{9, 10}. Atherosclerosis initiates with the retention of LDL in the intima of large or medium sized arteries, mainly because of interactions between LDL and extracellular matrix^{11, 12}. Early atherosclerotic lesions are characterized by subendothelial depositions of lipids, cholesterol loaded foam cells and T cells. The atherosclerotic lesion gradually progresses to a more complex plaque, with apoptotic and necrotic cells, cell debris and cholesterol crystals in the core covered by a fibrous cap composed of smooth muscle cells and collagen. The shoulder regions of the plaque are usually infiltrated by activated T cells, macrophages and mast cells, which are the sources of proinflammatory mediators and enzymes⁹ (Figure 1).

Plaques with thin fibrous cap and intense inflammatory activity are prone to rupture. When a plaque ruptures, the thrombogenic material from the core is exposed to the blood stream and this triggers platelet aggregation, activation of humoral coagulation and thrombus formation,

contributing to artery occlusion which causes ischemia in the heart, the brain or peripheral organs and results in development of myocardial infarction, ischemic stroke or transitory ischemic attacks, and peripheral artery disease, respectively⁹.

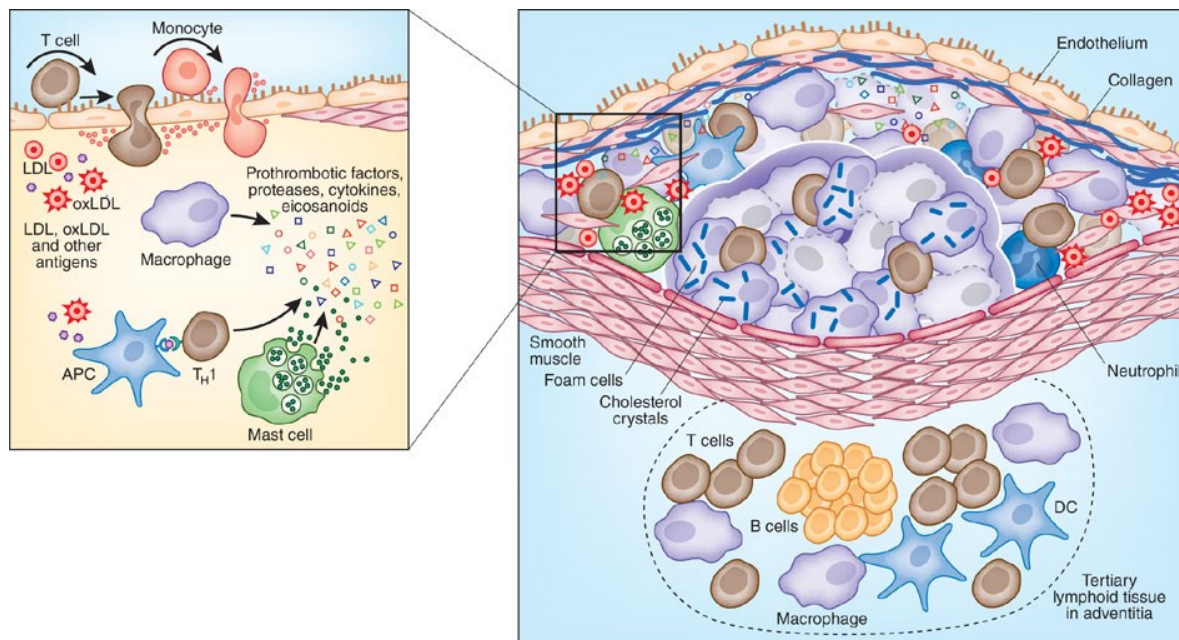


Figure 1: Pathogenesis of atherosclerosis. (adapted from Figure 1 in reference⁹; reprinted by permission)

1.2 INNATE IMMUNITY IN ATHEROSCLEROSIS

Both innate and acquired immunity are involved in all stages of atherosclerosis. The atheroma contains abundant macrophages which are the major player in innate immunity. Innate immunity is the first line of immune defense against microbes or danger signals. The germline genome encodes the mediators of innate immunity, including pattern recognition receptors which are constitutively expressed¹³. Thus, innate immunity can be mobilized in minutes to hours. Rapidly mobilized arms of innate immunity involve phagocytic leukocytes, complement, and proinflammatory cytokines, all that can contribute to atherogenesis¹³.

LDL is an endogenous activator of adaptive immunity. LDL was viewed merely as an autoantigen. But LDL can also activate innate immunity.

1.2.1 Danger signal and alarming

Innate immune responses are initiated when the body encounters danger signals and recognizes signature molecules. In the past, innate immunity was thought to be the first line of defense against foreign molecules and indeed many molecules that are recognized by innate immunity are derived from microbes, i.e. pathogen-associated molecular patterns (PAMPs). Later it was found that molecules from within the body can also cause immune responses, such as self-molecules that share similar patterns as pathogens, or self-molecules that are exposed or accessible to the immune system during cell injury or cell death, i.e., damage-associated molecular patterns (DAMPs)^{14, 15}. DAMPs include molecules that are

released in response to injury, such as heat shock proteins (HSPs) or high mobility group box 1 protein, or molecules that are newly generated or modified, such as advanced glycation end products (AGEs)¹⁴.

In the context of atherosclerosis, LDL can be modified or oxidized to be oxidized LDL (oxLDL) or be degraded to fragments. We identified a peptide from native ApoB100 protein that induces proinflammatory innate immune response, which was named ApoB100 danger associated signal 1 (ApoBDS-1)¹⁶. These are examples of DAMPs.

1.2.2 Activation of the innate immune system in response to LDL

Innate immunity comprises of several different cell types and cells of the mononuclear phagocyte lineage are considered to be the most important effectors¹³.

1.2.2.1 Monocytes

Monocytes are the main component of innate immune system and are involved in the initiation and progression of atherosclerosis¹⁷. Monocytes secrete proinflammatory cytokines and chemokines in response to bacteria, viruses and endogenous nucleic acids¹⁸. They phagocytose microbes, apoptotic cells and damage-associated molecules such as oxLDL or otherwise modified LDL. Monocytes can be differentiated into macrophages, dendritic cells, or endothelial progenitor cells. In male non-smokers, the presence of carotid atherosclerosis has been associated with increased counts of all leukocyte, neutrophil and monocyte¹⁹. Particularly, the peripheral monocyte count has emerged as an independent predictor of carotid intima-medial thickness (IMT) and future plaque formation in healthy population^{20, 21}.

Human monocytes can be classified as inflammatory/classical Mon1 (CD14⁺⁺CD16⁻), intermediate Mon2 (CD14⁺CD16⁺) and resident/nonclassical Mon3 (CD14⁺CD16⁺⁺). Mouse monocytes are distinguished into 2 main subpopulations, Ly6-C^{hi} and Ly6-C^{low}. The majority of monocytes that accumulate in atherosclerotic plaque are described to be the inflammatory Mon1 or Ly6-C^{hi} monocytes¹⁷. The CD16⁻ human monocytes and Ly6-C^{high} murine monocytes express high level of CCR1 and CCR2 and are attracted to the atherosclerotic site by MCP-1 secreted by resident macrophages¹⁷. The human CD16⁺ monocytes and mouse Ly6-C^{low} monocytes express high level of CX3CR1 and are attracted to endothelial cells by CX3CL1¹⁷. In mice, the Ly6-C^{hi} monocytes represent 50% of monocytes normally while the corresponding CD14⁺⁺CD16⁻ monocyte subset account for 95% of monocytes in humans²². Besides this, human monocytes and mouse monocytes have other discrepancies. In a comparative transcriptome analysis of human and mouse monocyte subsets, the authors found that some molecules from these two species were conversely expressed, including CD36, CD9, CXCR4 and TREM-1 (The triggering receptor expressed on myeloid cells 1), while others, e.g. the Peroxisome proliferator-activated receptor γ (PPAR γ) signature is prominent in mouse monocytes but not in humans²³. Furthermore, a major difference lies on the classical scavenger receptors and apoptotic cell recognition molecules. They are expressed more on Ly6-C^{low} mouse cells while in human cells they are more highly expressed (like signal-regulatory protein alpha (SIRP α), macrophage scavenger receptor 1 (MSR-1),

CD36, thrombospondin 1/Thbs 1) on CD16⁻ cells or not differentially expressed²³. The markedly different expression pattern of phagocytic recognition receptors may help explain the different phagocytic capacity and functional relevance between human and mouse monocyte subsets.

1.2.2.2 Macrophages

Monocytes can transmigrate into the subendothelial space where the cells differentiate into macrophages and take up accumulated lipoproteins becoming foam cells. Macrophages express receptors that recognize a broad range of PAMPs and DAMPs. These pattern recognition receptors include various scavenger (ScRs) and Toll-like receptors (TLRs)¹³. Whereas ligation of scavenger receptors leads to endocytosis and lysosomal degradation of the molecules, engagement of TLRs usually activates NF- κ B and mitogen-activated protein kinase (MAPK) pathways which in turn leads to upregulated expression of genes involved in leukocyte recruitment, reactive oxygen species production and phagocytosis¹³. Activation of TLRs will also elicit production of cytokines and induce apoptosis¹³. Macrophages can also synthesize proteases such as matrix metalloproteinases (MMPs), induce SMC apoptosis and contribute to plaque destabilization²⁴.

Macrophages engulfing LDL to become lipid-laden foam cells is an important step in the development of atherosclerosis²⁵. In this process, different ScRs are engaged when it encounters different particles, for example, Acetyl-LDL is recognized by SR-A, MARCO, SR-EC while oxLDL is recognized by SR-A1, MARCO, CD36, SR-B1, SR-PSOX, LOX-1²⁶.

1.2.2.3 Neutrophils

The number of neutrophils in atherosclerotic lesions is not as many as macrophages. Yet, they have an important role in plaque destabilization especially due to their capacity to release proteases and mediators that can degrade collagens and attract platelets. Thus, neutrophil infiltration has been found to be associated with acute coronary events²⁷. Further, neutrophil numbers are strongly associated with the histopathologic features of rupture-prone atherosclerotic lesions, suggesting a role for neutrophils in plaque destabilization²⁸. Indeed, neutrophils, but not eosinophils, basophils, monocytes, lymphocytes, or the total leukocyte count have been shown to be significantly associated with long-term mortality²⁹.

1.2.2.4 Endothelial cells (ECs)

The normal endothelium regulates vascular tone and structure and exerts anticoagulant, antiplatelet and fibrinolytic properties³⁰. Endothelial dysfunction is an early marker for atherosclerosis. OxLDL can induce endothelial dysfunction, stimulates the expression of adhesion molecules and leads to endothelial cell death. Activated endothelial cells can secrete chemokines and promote monocyte recruitment.

1.2.2.5 Platelets

Platelets play a critical role in innate immune response. The majority of TLRs are expressed on the surface of platelets, such as TLR4, TLR9 and TLR2³¹.

Platelets participate not only in the end stage of atherothrombosis, but also in the initial and progressive phases of the disease. Platelets facilitate monocyte recruitment to endothelial cells and monocyte-platelet aggregate formation was significantly increased in patients with stable coronary artery disease³². Platelets synthesize and secrete many proinflammatory mediators and interact with endothelial cells, smooth muscle cells (SMCs) and circulating leukocytes. Platelet interaction with oxLDL results in platelet activation, morphological changes and aggregation³³. Phospholipase C (PLC) signaling and calcium mobilization are proposed to be the common mechanism for platelet activation³³.

1.3 INNATE IMMUNE RECEPTORS INVOLVED IN LDL INDUCED INNATE IMMUNE SIGNALING

1.3.1 Toll like receptors (TLRs) and NOD-like receptors (NLRs)

TLRs and NLRs (nucleotide-binding oligomerization domain (NOD)-like receptors) are currently extensively studied innate immune receptors in atherosclerosis.

TLRs were the first discovered signaling pattern recognition receptors in mammals³⁴. At least 14 TLRs have been identified in human. Among them, TLR1, TLR2, TLR4, TLR5 and TLR6 are cell surface receptors, whereas the others are associated with the membrane of intracellular organelles such as endosomal vesicles³⁵. Several TLRs including TLR4, 2, 3, 7 and 9 have been shown with crucial roles in the pathogenesis of atherosclerosis³⁶⁻⁴⁰. However the ligands responsible for TLR activation in atherosclerosis remain to be defined. Minimally modified LDL (mmLDL) induces TLR4 and TLR2 activation in macrophages leading to release of cytokines and production of reactive oxygen species. OxLDL activates TLR4 in macrophages and promotes cell migration. Furthermore, oxLDL induces calcification within the plaque through TLRs⁴¹. TLR4, TLR6 in cooperation with scavenger receptor CD36 mediate the chemokine expression and IL-1 β production induced by oxLDL⁴². The recognition of endogenous DAMPs like oxLDL by CD36 triggers the assembly of TLR4 and TLR6, leading to increased nuclear factor- κ B (NF- κ B) activation which underlies pro-inflammatory responses in atherosclerosis⁴³. The induction of apoptosis by oxLDL involves CD36 and TLR2⁴⁴.

NLRs are cytoplasmic PRRs. There are two main subgroups of NLRs based on their different N-terminal effector domains: the caspase recruitment domains (CARD)-containing NLRC group and the pyrin domain containing NLRP group⁴⁵. NOD1 and NOD2 from the NLRC family are best characterized. The expression of NOD2 is increased in atherosclerotic lesions⁴⁶, and stimulation of NOD2 promotes vascular inflammation and development of necrotic core in atherosclerosis-prone mice⁴⁷. Interestingly, several NLRs can form

inflammasome which is involved in the activation of caspase-1 and production of the IL-1 family of inflammatory cytokines including IL-1 β , IL-18 and IL33^{48, 49}.

1.3.2 Inflammasome activation and the role of IL-1 β in atherosclerosis

Inflammasome is a multimeric protein complex usually consisting of an inflammasome sensor molecule (mostly a NLR), procaspase-1 and an adaptor protein called Apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (CARD), i.e. ASC or PYCARD. The inflammasome is formed in response to microbial molecules or endogenous “danger” molecules⁵⁰⁻⁵². The assembly of inflammasome aggregates, in which the NALP interacts with ASC via the pyrin domain and the ASC interacts with pro-caspase-1 via CARD, initiates procaspase-1 self-cleavage to generate active caspase-1, which in turn proteolytically processes pro-IL-1 β to generate mature IL-1 β and induces their release via a non-classical secretion pathway⁴⁸. The generation of bioactive IL-1 β is regulated by at least two signals. The first signal comes from various PAMPs through activation of TLRs, resulting in the upregulation of pro-IL-1 β . The secondary signal usually provided by DAMPs leads to inflammasome formation and activation of caspase-1^{53, 54}. Besides NLRs, PYHIN (pyrin and HIN domain-containing protein) family members can be included in inflammasomes such as the inflammasome absent in melanoma 2 (AIM2) and the inflammasome IFN γ -inducible protein 16 (IFI16). In addition to inflammasomes that are involved in generating IL-1 β , the Retinoic acid-inducible gene I (RIG-I) inflammasome and IFI16 inflammasome induce type I interferon (IFN) response⁴⁸.

Caspase 1 is one of the inflammatory caspases and is the main caspase recruited to inflammasome. Additionally, NLRP1 can recruit and activate caspase 5⁵⁵. Caspase 8 can also mediate IL-1 β processing⁵⁶.

Of all the inflammasomes, nucleotide-binding leucine-rich repeat-containing pyrin receptor 3 (NLRP3) inflammasome is the best characterized and has been much investigated in atherosclerosis. The NLRP3 inflammasome signaling promotes the disease progression⁴⁴. Chimeric mice with NLRP3-deficient bone marrow exhibit 69% reduction in atherosclerotic lesion size at aortic sinus compared with the mice that received wild-type bone marrow⁵⁷. Contradictorily, global deletion of NLRP3 in atherosclerosis-prone Apoe^{-/-} mice did not affect lesion size⁵⁸. However, studies showed that NLRP3 inflammasome was activated in the infiltrated macrophages and neutrophils in ischemic myocardium and that statins inhibited in a direct manner the formation of cholesterol crystals *in vivo*⁴⁴. Thus, compiling data argue for a crucial role of NLRP3 inflammasome in atherosclerosis⁴⁴.

A wide variety of microbial or endogenous stimuli can activate NLRP3 inflammasome, like lipopolysaccharide (LPS), extracellular ATP, monosodium urate (MSU) crystal⁵⁹, calcium pyrophosphate dehydrate (CPPD)⁵⁹, cholesterol crystals⁶, and oxidized LDL. OxLDL induces the upregulation of pro-IL-1 β and promotes the formation of cholesterol crystals and therefore provides the both signals required for NLRP3 inflammasome activation⁶⁰.

Moreover, oxLDL induces production of ROS and causes lysosomal damage which are also linked with NLRP3 inflammasome⁶⁰.

Several hypotheses for the activation of inflammasome have been proposed. Mitochondria plays an important role in inflammasome activation. Mitochondria provides a platform for inflammasome components and mitochondria-derived mROS, mitDNA and cardiolipin⁶¹ are suggested to be essential in inflammasome activation⁶². Mitochondrial antiviral-signaling protein (MAVS)⁶³ is involved in NLRP3 relocation from the cytosol to mitochondria and guanylate binding protein 5 (GBP5) promotes NLRP3 signaling to ASC⁶². Considering the wide array of activators for NLRP3, it is generally accepted that these PAMPs and DAMPs cause disturbance of cellular homeostasis which in turn activates NLRP3 inflammasome⁶². One of these cell stresses is endoplasmic reticulum (ER) stress which activates NLRP3 inflammasome through a pathway that is independent of unfolded protein response⁶². Besides, increased intracellular Ca^{2+} and/or decreased intracellular K^+ are also proposed to be required for NLRP3 inflammasome activation^{62, 64, 65}.

Activation of inflammasome results in production of IL-1 β . Apart from exogenous innate immune activators such as lipopolysaccharides (LPS) from microbes, endogenous stimuli such as oxLDL, cholesterol⁵⁷, chylomicrons⁶⁶, triglycerides⁶⁷ and high glucose have also been shown to influence IL-1 β generation⁶⁸. IL-1 β is a potent proinflammatory cytokine. It has important effects on crucial cellular processes in the development of atherosclerotic lesions, among which IL-1 β can activate endothelial cells, promote foam cell formation, induce smooth muscle cell proliferation, induce IL-6, TNF and C-reactive protein and attract inflammatory cells to the plaque^{59, 69}. Lack of IL-1 β or inhibition using anti-IL-1 β depleting antibodies in ApoE^{-/-} mice results in diminished atherosclerotic lesions and reduced vascular inflammation compared with controls^{70, 71}. Individuals bearing the -1473 CC IL-1B polymorphism likely have a pronounced atherosclerotic process because this single nucleotide polymorphism is associated with increased fasting lipids and IL6 (effector of IL-1 β) in the elderly population^{72, 73}.

Contradictory evidence for the detrimental role of IL-1 β also exist. In humans, neutralization of IL-1 β in the acute phase of myocardial infarction has been shown to promote the progression of left ventricular remodeling, suggesting that IL-1 β is protective in the acute phase of the disease⁷⁴. However, this result was also proposed to be due to effects mediated by IL-1 α . IL-1ra is a natural antagonist for both IL-1 α and IL-1 β . In another study, the MRC ILA-HEART study, patients with NSTEMI were given daily subcutaneous injection of IL-1ra for 2weeks and had a trend decrease in myocardial infarction and major adverse cardiac events compared with placebo⁷⁵. Yet, the precise role of IL-1 β and IL-1ra needs to be clarified further.

Canakinumab Anti-Inflammatory Thrombosis Outcomes Study (CANTOS), an ongoing clinical trial of IL-1 β blockade in cardiovascular disease⁶⁹, already shows encouraging results. The inhibition of IL-1 β leads to reduced hsCRP, IL-6 and fibrinogen without an apparent impact on LDLC or HDLC⁷⁶. Once completed, it will provide critical insights into

the inflammatory mechanisms in atherothrombosis and probably widen the current range of therapies for cardiovascular disease.

1.4 THE IMMUNE REGULATORY ROLE OF LDL IN ATHEROSCLEROSIS

1.4.1 Lipoproteins, LDL and ApoB

Cholesterol and triglycerides are transported in the circulation by water-soluble lipoprotein particles. Lipoprotein particles have a highly hydrophobic core which is packed with apolar lipids, mainly cholesteryl esters and triglycerides. The surface of lipoproteins consists of polar molecules, primarily free cholesterol, phospholipids and apolipoproteins⁷⁷ (Figure 2).

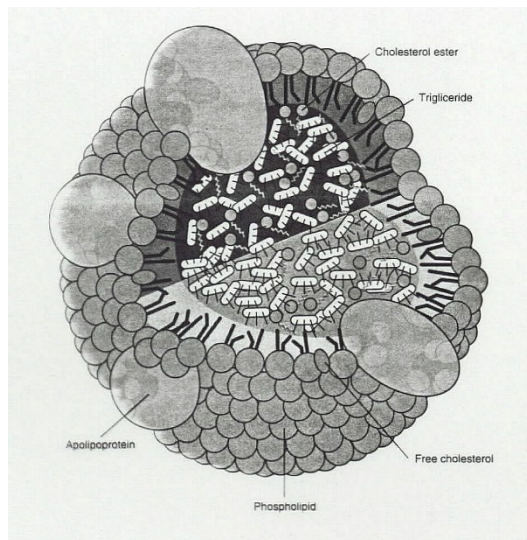


Figure 2: Structure of lipoprotein (adopted from Figure 1 in reference⁷⁸)

The plasma lipoproteins have different density, size, electrophoretic mobility, lipid content and apolipoprotein composition. According to the density, lipoproteins can be divided in chylomicrons, very low-density lipoproteins (VLDL), low-density lipoproteins (LDL), intermediate density lipoproteins (IDL) and high-density lipoproteins (HDL).

Dietary fat is absorbed in the intestine, packaged into chylomicrons and secreted into the lymph to enter the general circulation. The chylomicrons contain apolipoprotein B48, apoE and triglyceride, etc. In the circulation the particles are hydrolyzed and become smaller and finally taken up by the liver. In the endogenous pathway in lipid metabolism, cholesterol and triglycerides are secreted from the liver to the circulation in VLDL particles which also contain apoB100, apoC and apoE. VLDL is hydrolyzed and cholesterol is enriched. VLDL becomes IDL and gradually LDL is formed. LDL is rich in cholesteryl esters and contains apoB100 which is the only unexchangeable lipoprotein. In the end the apoB100 on the LDL surface is recognized by LDL receptors to mediate the uptake of LDL in the liver or in other tissues. Finally cholesterol from extrahepatic tissues can be reversely transported back to the liver by help of HDL particles.

In humans about two thirds of the plasma cholesterol is transported in LDL particles. The LDL particle has an outer surface layer composed of phospholipid headgroups, an interfacial layer of interpenetrating core and surface lipid fatty acids and cholesterol, and a core of randomly oriented hydrophobic lipids, mainly triglycerides and cholesterol esters⁷⁷. The LDL

particle has a single copy of ApoB100 protein and ApoB100 is predicted to have some parts in each of these layers of the LDL particle⁷⁷.

In general, LDL particles assume a globular shape with an average particle diameter of about 22nm (range from 18 to 25nm)⁷⁹. LDL can be quite heterogeneous and consists of 3 subclasses based on density: large LDL (LDL1 and LDL2 with $d=1.018-1.030\text{g/mL}$), intermediate LDL (LDL3 with $d=1.030-1.040\text{g/mL}$), and small, dense LDL (LDL4 and LDL5 with $d=1.040-1.065\text{g/mL}$)⁸⁰. It has been proposed that small, dense LDL can penetrate more easily into the subendothelial space of the vascular wall and are more prone to oxidation⁸⁰.

There are two forms of ApoB: apoB100 synthesized by the liver and apoB48 synthesized by the intestine. ApoB100 and ApoB48 are encoded by the same gene and the ApoB48 is generated via RNA editing by the APOBEC1 enzyme⁸¹. Thus the ApoB48 corresponds to the N-terminal 48% of the full length apoB100. ApoB 100 is a large protein and has 4536 amino acids. ApoB100 mediates the interaction of LDL and LDL receptor via the two regions on ApoB100, namely aa 3147-3157 and aa 3359-3369⁸². Researches on those two regions are extensive. For example, the peptide aa 3359-3369 was found to be able to trigger transient activation of p38MAPK and cytosolic phospholipase A2 on platelets⁸³.

1.4.2 Modified LDL

1.4.2.1 Generation of modified LDL

LDL accumulated in the arterial wall is susceptible to modifications such as oxidation, enzymatic and non-enzymatic cleavage, glycation, lipolysis and aggregation.

LDL particles undergo oxidation by the lipoxygenase and myeloperoxidase pathways. Various components in LDL can be oxidized, such as apoB, phospholipids, cholesterol and unsaturated fatty acids⁸⁰. Cholesterol can be oxidized to form oxysterols, especially at the 7-position⁸⁰. The polyunsaturated fatty acids in cholesterol esters, phospholipids, and triglycerides (TG) can be subject to free radical-initiated oxidation⁸⁰. Lipid peroxides can undergo a second oxidation and breakdown, eventually giving rise to degradation molecules (3-9 carbon long fragments), including reactive aldehydes, such as malondialdehyde (MDA)⁸⁰. These modified products can react with ApoB. The aldehydes can form Schiff bases with the ϵ -amino groups of lysine residues, creating cross-links between lipid and protein or among lipid molecules⁸⁰. There are assays to detect MDA-modified LDL, copper-oxidized LDL (oxLDL), N ϵ (carboxymethyl) lysine (CML)-modified LDL, and advanced glycosylation end product (AGE)-modified LDL⁸⁴. Studies showed that oxLDL and AGE-LDL levels in immune complex were strong predictors of increased progression of carotid IMT in patients with type 1 diabetes, and that levels of MDA-LDL in immune complex predict future MI and acute cardiovascular events in patients with type 2 diabetes⁸⁵. Oxidation of LDL can generate a number of new innate immune activators, including lipid oxidation products such as lysoPC, 4-hydroxy-2-nonenal (4HNE)⁸⁶. Oxidation of LDL makes

the particle more negatively charged, increasing its affinity to scavenger receptors on macrophages, and contributing to foam cell formation⁹.

Besides oxLDL, there are other oxidized low-density lipoprotein markers for cardiovascular disease such as the serum amyloid A-LDL (SAA-LDL) complex which is formed from the oxidative interaction between SAA and LDL, and the α 1-antitrypsin-LDL (AT-LDL) complex which is formed by the binding between oxidized α 1-antitrypsin and LDL in the arterial intima⁸⁷.

Numerous hydrolytic enzymes and pro-oxidative agents are present in atherosclerotic lesions⁷⁷. These modifying substances transform the LDL particles into the extracellular lipid droplets and vesicles in the arterial intima⁷⁷. The aggregation and fusion of the particles strengthen the binding to extracellular matrix and promote atherosclerosis⁸⁸.

1.4.2.2 Relevance of modified LDL to vascular inflammation in atherosclerosis

The atherogenic property of LDL has been ascribed to the modification of LDL particles. Accumulating data have shown that immune responses to modified LDL such as oxLDL are important in atherosclerosis^{9, 12, 89, 90}. OxLDL acts as an early activator of vascular inflammation⁸⁶. Modified LDL can induce endothelial cell activation, impairs endothelium-dependent arterial relaxation, and is cytotoxic to endothelial cells⁹¹. OxLDL is a chemoattractant for circulating monocytes and induces monocyte recruitment to the atherosclerotic site which is supported by the observation that macrophages can be often detected close to regions rich in oxLDL-derived epitopes⁸⁶. OxLDL induces monocyte differentiation into macrophages, promote foam cell formation and increases macrophage growth⁹¹. OxLDL can induce abundant amounts of proinflammatory mediators, activate ECs, SMCs, macrophages, lymphocytes and other leukocytes to foster cytokine production, destabilize plaques and trigger thrombosis⁸⁰.

The degree of oxidation in oxLDL varies. The lipid components have been suggested to undergo oxidation in the initial phase, followed by oxidative changes of amino acid side chains and finally the cleavage of peptide bonds⁹². Minimally modified LDL (mmLDL) may contain lipid oxidation products without extensive protein modification⁹². Mm LDL induces IL-1 β , IL-6 and IL-10 secretion in human monocytes and macrophages through activation of CD14, TLR4 and TLR2⁹³. MmLDL (acetylated LDL) can also prime NLRP3 inflammasome and increase the expression of pro-IL-1 β in macrophages^{57, 60}. Electronegative LDL, a minor modified LDL in the circulation, induces cytokine release in monocytes via CD14 and TLR4⁹⁴.

The inflammatory responses may be elicited in part by the oxidation of LDL derived phospholipids such as 1-palmitoyl-2(5-oxovaleroyl)-sn-glycero-3-phosphorylcholine (POVPC), 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphorylcholine (PGPC), 1-palmitoyl-2(5, 6-epoxyisoprostane E2)-sn-glycero-3-phosphorylcholine (PEIPC)⁹⁵. POVPC and PEIPC induce monocyte recruitment and PGPC induces monocyte and neutrophil recruitment to endothelial cells⁹⁵. These oxidized phospholipids are increased in atherosclerotic lesions and

induce production of MCP-1 and IL-8 in endothelial cells through the lipid-dependent transcription factor peroxisome proliferator-activated receptor- α ⁹⁵. OxLDL also contains platelet-activating factor (PAF)-like phospholipids that can activate PAF receptors on platelets, monocytes and leukocytes⁹⁶.

Phosphatidylcholine (PC) is the major phospholipid in LDL. Oxidized PC-containing phospholipids such as oxidized 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphocholine (Ox-PAPC) are strong regulators of endothelial cell function⁹⁷. OxPAPC is present in minimally modified LDL and is found in atherosclerotic plaques. Ox-PAPC regulates genes involved in unfolded protein response, redox signaling, inflammation and angiogenesis which lead to plaque instability⁹⁷. POVPC induces foam cell formation and ox-PAPC induces cytokines from macrophages⁹⁷. Ox-phospholipids also induce thrombotic processes⁹⁷. Although the phospholipids (PL) have some protective role, the net effect is pro-atherosclerotic. CD36, TLR2 and TLR4 are among the most well studied receptors for ox-PL⁹⁷.

1.4.3 ApoB degradation

ApoB100 is mainly synthesized in the liver and the majority of newly synthesized apoB undergoes rapid intracellular degradation in a pre-Golgi or ER compartment⁹⁸. The posttranslational degradation of apoB may involve at least two steps⁹⁸. The first step occurs while apoB is partially translocated from ER to the Golgi and is sensitive to N-acetyl-leuciny-leuciny-norleucinal (ALLN). And the second step takes place in the ER lumen and is dithiothreitol (DTT)-sensitive⁹⁸. The degradation of partially translocated apoB generates a 70-kDa amino-terminal fragment that is mainly degraded in the ER lumen by a DTT-sensitive pathway⁹⁸.

The degradation of ApoB100 can be modulated by multiple factors. Docosahexaenoic acid (DHA), a dietary polyunsaturated fatty acid (PUFA) lowers VLDL secretion by inducing presecretory ApoB100 degradation which is dependent on PUFA-derived lipid peroxides like superoxide (SO)⁹⁹. The lipid lowering drug Niacin increases intracellular ApoB degradation by inhibiting triacylglycerol synthesis¹⁰⁰. Protease inhibitors lead to hyperlipidemia in HIV patients because they not only inhibit proteasomal degradation of nascent apoB but also inhibit the secretion of apoB which is associated with inhibition of cholesteryl-ester synthesis and microsomal triglyceride transfer-protein activity¹⁰¹.

ApoB100 is also subjected to degradation in atherosclerotic lesions. There are numerous proteases in atherosclerotic plaques such as cathepsin cysteine proteases¹⁰², secretory sphingomyelinase (SMase)¹⁰³, phospholipases¹⁰⁴, plasmin^{105, 106}, chymotrypsin, thrombin, kallikrein¹⁰⁷, metalloproteinase MMP-12¹⁰⁸ that can degrade ApoB100 and promote aggregation of modified LDL. By Western blot, Nishi et al showed that while intact apoB100 band can be detected in patients' plasma and control plasma, the 500-kD apoB band disappears in macrophage-rich carotid plaque homogenates with smear-staining in the lower molecular weight range (200kD to less than 20kD), indicating that ApoB100 is partially

degraded¹⁰⁹. It has been shown that the degradation of ApoB100 is associated with macrophages and oxLDL level, and that the degraded apoB is accumulated in lysosomes¹⁰⁹.

In addition to endogenous proteases, apoB100 can be degraded by proteases secreted from pathogens. *Porphyromonas gingivalis* (*P. gingivalis*) is the primary pathological agent in adult periodontitis which is associated with increased atherosclerosis supported by epidemiological studies. Intravenous injection of wild-type *P. gingivalis* increased atherosclerotic lesions and increased the serum LDL cholesterol which is due to selective proteolysis of apoB100 in LDL particles by cysteine proteases like Arg-gingipain (Rgp) produced by *P. gingivalis*¹¹⁰.

1.4.4 Heat shock proteins are crucial for ApoB protein stability

Heat shock proteins (Hsp) are molecular chaperones that play an important role in protein homeostasis and quality control. They facilitate protein folding and prevent misfolding and aggregation of nascent polypeptides. HSPs can bind exposed hydrophobic residues of substrate proteins or peptides and thus deliver antigenic peptides to MHC class I molecules for presentation to lymphocytes¹¹¹. HSPs are also involved in TLR recognition for hydrophobic ligands like lipid molecules or hydrophobic residues from lipoproteins¹¹¹. Despite different molecular masses, cellular locations and specificity for protein binding, the HSPs share common functional domains: one adenine nucleotide-binding domain which interacts and hydrolyzes ATP, and the other peptide-binding domain which interacts with exposed hydrophobic residues of substrate proteins¹¹¹.

Hsps have been implicated in ApoB100 degradation or stabilization. Studies showed that Hsp 70s and Hsp 90s facilitate apoB degradation¹¹². Through the increase of the ER chaperones, 78-kDa glucose-regulated protein (Grp78 or Heat shock 70kDa protein 5, HSPA5) and Grp94, glucosamine treatment leads to proteasomal degradation of ApoB100 dramatically¹¹³. Grp78 reduces the translocational efficiency of apoB100 in the ER and promotes proteasomal degradation of apoB100, thus decreasing both cellular and secreted apoB100 significantly¹¹³.

Hsp110 is an Hsp70 homolog and is efficient in preventing protein aggregation but lacks the folding activity of Hsp70¹¹⁴. The Hsp110 and Hsp70 differs in peptide substrate binding properties that the Hsp110 exhibits fast kinetics of substrate binding and release and prefers aromatic residues¹¹⁴.

Hsp110 associates with and stabilizes apoB. Overexpression of Hsp110 enhanced apoB secretion¹¹⁵. It is likely that chaperones within different complexes can play distinct roles during ER-associated degradation.

1.4.5 The immune regulatory role of ApoB100 in atherosclerosis

ApoB100 is present in VLDL, IDL, LDL and lipoprotein (a) [Lp(a)], all of which are atherogenic or potentially atherogenic¹¹⁶. The level of ApoB100 predicts ischemic cardiovascular events and it is better than LDL cholesterol alone^{117, 118}.

Both native apoB100 and modified ApoB100 can be target of the immune responses¹¹⁹. Immunization of ApoB100 and Hsp60 peptides effectively inhibited atherosclerosis in animal models¹²⁰. ApoB100 can also be modified or oxidized, resulting in loss of recognition by LDL receptor and generating epitopes for recognition by the scavenger receptor¹²¹. Moreover, due to a conformational change that occurs during sphingomyelin hydrolysis, ApoB100 mediates the formation of LDL aggregates that may contribute to retention of LDL in the arterial intima¹⁰³.

Researchers in Lund University created a peptide library consisting of 302 peptides that cover the full-length of apoB100⁷. They found that the level of circulating autoantibodies against apoB100 peptides is associated with the severity of atherosclerosis¹²²⁻¹²⁴. Treatment with apoB100 peptide vaccines inhibited the development of atherosclerosis¹²⁵⁻¹²⁹.

We did a screening of the library and found that the peptide at the position aa 3226-3245 is the most potent one that can activate monocytes/macrophages and other innate immune cells^{16, 33}. We name it ApoB100 danger associated signal 1 (ApoBDS-1). The thesis is about the identification of ApoBDS-1, the inflammatory effects of ApoBDS-1 on monocytes and platelets, the activation of inflammasome pathway by ApoBDS-1 and finally the interacting protein of ApoBDS-1.

2 HYPOTHESIS AND AIMS

The studies included in this thesis aimed to investigate the role of LDL in the modulation of innate immune responses in atherosclerosis.

More specifically the aim of the studies was to:

- Identify the innate immune stimuli in ApoB100 peptides (**paper I**)
- Examine the effect of ApoBDS-1 on platelets (**paper II**)
- Investigate the impact of ApoBDS-1 on inflammasome activation (**paper III**)
- Define the molecular basis (receptor or interacting protein) that mediates the activity of ApoBDS-1 (**paper IV**)

3 MAIN METHODOLOGY

This chapter highlights the state-of-the-art and interdisciplinary methods in protein/peptide research and molecular biology that we employed to conduct the thesis work.

3.1 APOB100 PEPTIDE LIBRARY

ApoB100 is the largest protein component in human lipoproteins^{130, 131}. Previous studies have focused on the sequences on ApoB100 that are crucial for the LDL receptor binding, i.e. aa 3147 – 3157 and aa 3359-3367¹³². However, the function of the other sequences of the ApoB100 with its 4536 amino acids⁷⁷ has largely been undetermined. Peptide library is a technique for protein related study. Fredrikson et al⁷ have created an ApoB100 peptide library with 302 peptides covering the full-length of ApoB100. Each peptide has 20 amino acid with 5 aa overlapping with the neighbor peptide (Figure 3).

Detailed sequences of the ApoB100 peptides are found in papers^{7, 16, 33}.

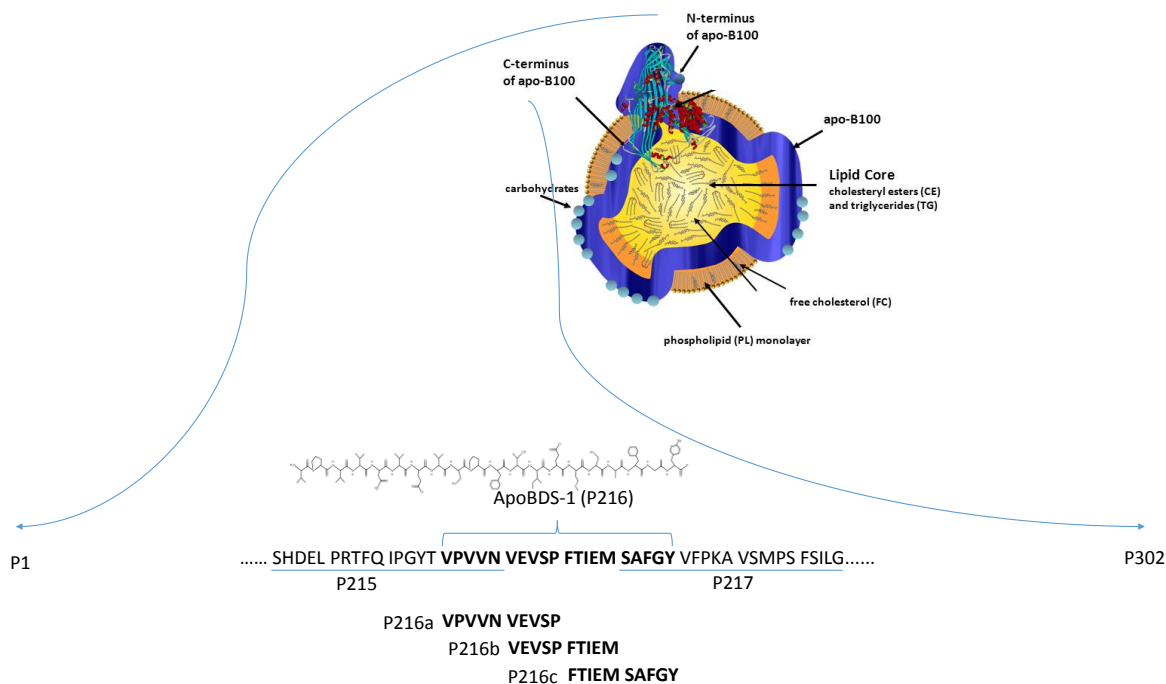


Figure 3: Schematic representation of the ApoB100 peptide library with the sequence for ApoBDS-1 (aa 3226-3245) and truncate peptides. (modified from Figure 1 in reference¹³³)

3.2 EX VIVO PLAQUE CULTURE

To identify the innate immune activating ApoB100 peptide and to characterize its effect on inflammatory response, we used monocytic cell line THP-1, human PBMC and *ex vivo* human carotid plaque cultures. Human atherosclerotic lesions have a variety of cell types creating a complex inflammatory milieu. The atherosclerotic plaques are rich in CD68+CD14+ macrophages which are not always the same with human macrophages in culture¹³⁴. Besides macrophages, there are smooth muscle cells, endothelial cells, fibroblasts,

collagens, lipoproteins, proteases, extracellular matrix, abundant cytokines and chemokines in plaques which have more diverse effect than isolated cells^{1, 135-138}. The *ex vivo* plaque cultures provide a method to study the impact of the agent molecule in a systemic and complex inflammatory milieu with high clinical relevance. Like any other experiment using human tissues, the heterogeneity of the plaques and/or plaque related cells is unavoidable although equal amount of tissue (about 0,1g per well in 48-well plate) was seeded in plaque culture. And the *ex vivo* culture per se may select certain cells and eliminate other cells which are short-lived and prone to environmental change. To mimic the in vivo situation as much as possible, extra care should be taken when doing the plaque culture and the total *ex vivo* time should not be too long. Therefore, *ex vivo* culture of plaques may be a strong complementary tool to the traditional cell line and primary cell cultures.

For the work presented in this thesis, plaques were obtained from Biobank of Karolinska Carotid Endarterectomies (BiKE). Detailed information about BiKE is found in ¹³⁹⁻¹⁴³.

The *ex vivo* plaque culture method was used in **paper I**¹⁶ and **paper III**.

3.3 IMMUNOFLUORESCENCE STAINING

Immunofluorescence staining is a widely used method to visualize cellular proteins or tissue structures in situ. We used it to localize ApoBDS-1 in human carotid plaque sections or in monocytes/macrophages (**paper I, III**) and to investigate the interaction between ApoBDS-1 and other proteins including Trap1 (**paper IV**).

Apart from qualitative visual inspection of immunofluorescence images which showed highly specific immunostaining of proteins under study with high sensitivity due to fluorescence signals, quantitative analysis of these images was also performed to facilitate sample comparisons.

For quantification of fluorescence parameters in immunofluorescence images, two major computational methods using MATLAB 2010a scripts (MathWorks, Massachusetts, USA) were employed in the thesis. In **paper I**, to evaluate the expression levels of proteins inside cells, images with comparable areas of interests were selected for comparison, and for a particular image, fluorescent intensities at all pixels that passed an automatically-set threshold to separate noises from signals by Otsu's method¹⁴⁴ were summed together. The advantages of Otsu's method for noise and signal separation include that no human supervision is needed therefore it is not a subjective approach and it is purely statistical based on the images themselves that the algorithm searches for the optimum threshold separating the two classes (noises and signals) at which the intra-class variance is minimal. Intensities below the threshold were discarded as noises to minimize their impact on protein expression level comparison. The intensity noises were likely due to photon noises of detectors or very weak unspecific binding of fluorescently labeled antibodies to the specimens or glass surfaces. In **paper III**, to evaluate the degree of protein interactions in cells imaged by immunofluorescence staining, Pearson correlation coefficients¹⁴⁵ for protein pairs under study were calculated. The advantage of Pearson correlation coefficient for co-localization

determination is that it calculates the correlation of two signals instead of relies on the absolute intensities and thus not very sensitive to the strength of either signal.

3.4 FAR-WESTERN BLOT

Western blot is another commonly used method in protein analysis and is one of the methods I used most in the thesis work. Far Western blot is a method for detection of protein-protein interactions described by Wu Y et al¹⁴⁶. The protein blotting procedure is similar to that of regular Western blot but with additional denature and renature steps so that the prey protein on the membrane can retain natural spatial structures and thus possible to bind the bait protein which is added in this step. And then using an antibody against the bait protein, the prey protein is detected on the membrane because of the bait-prey protein complex. After that, the membrane is stripped and the protein complex is thereby destroyed. The prey protein can be detected again using antibody against it. Here the prey protein can be used as endogenous control as the two images from the far western blot should have identical molecular weight.

A comparison of commonly used methods to detect protein-protein/peptide interaction is provided in Table 1.

Methods	Advantages	Disadvantages	Time
Co-immunoprecipitation (co-IP)	Cell lysates, can detect binding in a complex	A tag is usually needed and false-positive or false-negative can occur. Cannot distinguish direct binding or binding in a complex	2-3days
Far western blot	Cell lysates, detect direct binding	Purified bait protein is needed	2-3days
Fluorescent resonance energy transfer (FRET)	Live cells	Weeks to months to construct the expression vectors and optimize the transfection of fluorescence labeled proteins	30min
Biacore surface plasmon resonance (SPR)	Monitor the interaction in real time and label-free. Can be used for screening	Purified protein, sensor chips and special instrument are needed	30min

Table 1: Comparison of methods to detect protein-protein/peptide interactions

In this thesis, we used a biotin labeled ApoBDS-1 peptide in Far western blot to find out ApoBDS-1 receptor or interacting protein (**paper IV**). The advantage of far western in the project is that we can study the native state of the interaction in addition to the precision controlled by the molecular weight.

3.5 TWO DIMENSIONAL ELECTROPHORESIS

Two-dimensional (2-D) electrophoresis is a standard method to separate proteins from biological samples in proteomics¹⁴⁷. Proteins are separated first according to the charge by isoelectric focusing (IEF) and then according to the size by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)¹⁴⁸. Identification of protein is done by mass spectrometry which employs spectra of the mass-to-charge ratio and bioinformatics.

We applied 2-D electrophoresis and mass spectrometry to identify the ApoBDS-1 interacting protein in **paper IV**. We used total protein from THP-1 cells. Alternatively we could extract membrane protein and investigate membrane protein exclusively. But it is complicated to isolate membrane proteins and we don't know whether the interacting protein is a membrane protein or a protein in the cytosol or other compartment. So we used whole protein lysates.

At first we tried to stain the 2-D gel after electrophoresis with fluorescence dye and then did far western blot using the same gel. After the staining and scanning, the gel is detached from the plastic backing for following far western blot. Then the gel becomes delicate for handling since it is large (26cm×20cm) and thin (less than 0,65cm), and lacks plastic backing. It easily breaks during washing. Therefore we used parallel gels which we performed under exactly the same conditions. One gel is for silver staining which gives more protein spots than the Coomassie blue staining. The other is for far western blot. Although extra care is taken during all the experiments, systemic errors can occur especially during the alignment of the images. For example, when the 2-D gel is detached from the plastic backing, the size of the gel can be changed slightly. When the proteins on the gel are transferred to the PVDF membrane, the size can be slightly changed. Then when we visualize the bands using ECL and the film, the image on the film can slightly differ from the position of the membrane. To increase the precision, we used the marker, the edge of the gel and the membrane, and beta actin from western blot image as alignment help. The location information of Coomassie blue stained gel image and Western blot image of ERK was also compared to minimize the imprecision.

The detailed method for 2-D electrophoresis and mass spectrometry can be found in **paper IV** and reference¹⁴⁷.

3.6 BIACORE SPR

Biacore's surface plasmon resonance (SPR)-based protein interaction technology provides a method to monitor protein-protein or protein-peptide interaction in real time¹⁴⁹.

Experimentally, the method consists of immobilizing one molecule of a binding pair on the sensor chip surface ("ligand") and injecting a partner molecule ("analyte") over the surface. Alterations in the index of refraction at the binding surface are detected and recorded in

resonance unit (RU). The comparison of Biacore SPR and other methods is provided in Table 1.

We did Biacore experiment to examine the binding of ApoBDS-1 and Trap1 and characterized the kinetics/affinity of the binding in **paper IV**. There are 2 or 4 flow cells on the sensor chips. The 1st flow cell has no protein immobilized. Trap1 was immobilized on the surface of the 2nd flow cell as “ligand”. “Analyte” was ApoBDS-1 or running buffer that was flowed over the both surfaces sequentially, first control flow cell, then Trap1 flow cell. Then the sensorgram from the trap1 flow cell was subtracted by the sensorgram from the blank flow cell, to get the adjusted sensorgram.

The method is very sensitive. Therefore in experiments with DMSO added to the PBS-P+ buffer, the concentrations of DMSO in the buffer and samples were matched or solvent corrections were performed.

The detailed method is found in **paper IV**.

3.7 GENE SILENCING, RECONSTITUTION AND OTHER METHODS

Gene silencing is a commonly used method to study the regulation of gene expression. Gene silencing can occur on the transcription or translation level. I used NLRP3 siRNA in **paper III** and Trap1 shRNA in **paper IV** to determine whether NLRP3 or Trap1 is required in ApoBDS-1 induced proinflammatory response. Detailed methods can be found in respective papers.

Besides gene silencing and inhibition experiments, other methods to study the function of gene expression include over-expressing, and gene reconstitution and alike. In **paper III**, I used HEK 293 cells which lack the inflammasome components and reconstituted the inflammasome machinery with expression plasmids. Detailed methods for gene reconstitution are found in **paper III** and references^{150, 151, 152}.

Other methods used in the thesis included cell culture experiments of primary cells and cell lines (**paper I-IV**), ELISA to measure the concentration of cytokines and chemokines (**paper I-IV**), Western blot to investigate intracellular signaling pathways (**paper I-IV**), flow cytometry for surface expression of leukocyte markers and functional assays of ROS production and caspase-1 activity (**paper II-IV**), BiKE database analysis for inflammasome related genes (**paper III**), and so on. Detailed methods can be found in respective papers.

4 RESULTS AND DISCUSSION

4.1 IDENTIFICATION OF APOBDS-1 (PAPER I)

In order to find the innate immune activator(s) in ApoB100 protein, a screening of the ApoB100 peptide library for the secretion of IL-8 in THP-1 cells was performed. A strong effect on IL-8 production was found specifically with the peptide p216 (aa 3226-3245) which was later named ApoB100 danger associated signal 1 (ApoBDS-1). The bioactivity of ApoBDS-1 was confirmed using different concentrations in THP-1, human peripheral blood mononuclear cells (PBMCs), monocyte-differentiated macrophages, also in *ex vivo* plaque cultures. We also found that MDA modification debilitated its effect. Furthermore the truncated fragments of ApoBDS-1 failed to elicit IL-8 production (Figure 4).

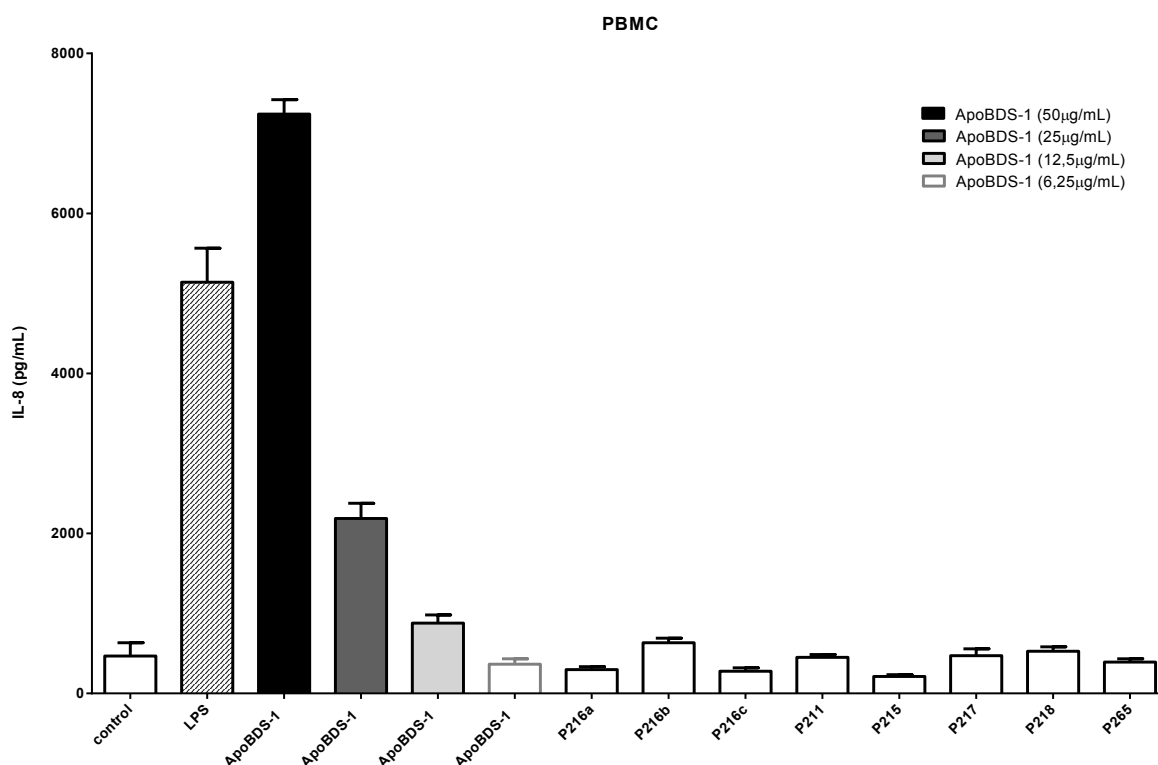


Figure 4: ApoBDS-1 is capable of inducing IL-8 secretion from PBMCs and the bioactivity is sequence dependent

To exclude the possibility that the effect of ApoBDS-1 was due to contamination of lipopolysaccharide (LPS), we first tested 4 independent batches of synthetic ApoBDS-1 and found no evidence for coincidental LPS contamination. Furthermore, we showed that ApoBDS-1 induced IL-8 production was not affected by the LPS inhibitor polymyxin B¹⁵³ (Figure 5). Lastly, we demonstrated that ApoBDS-1 alone could not induce IL-8 in TLR4¹⁵⁴ or TLR2¹⁵⁵ transfected human embryonic kidney cells (HEK293) (Figure 5). These results confirmed that the effect of ApoBDS-1 was not attributed to LPS contamination.

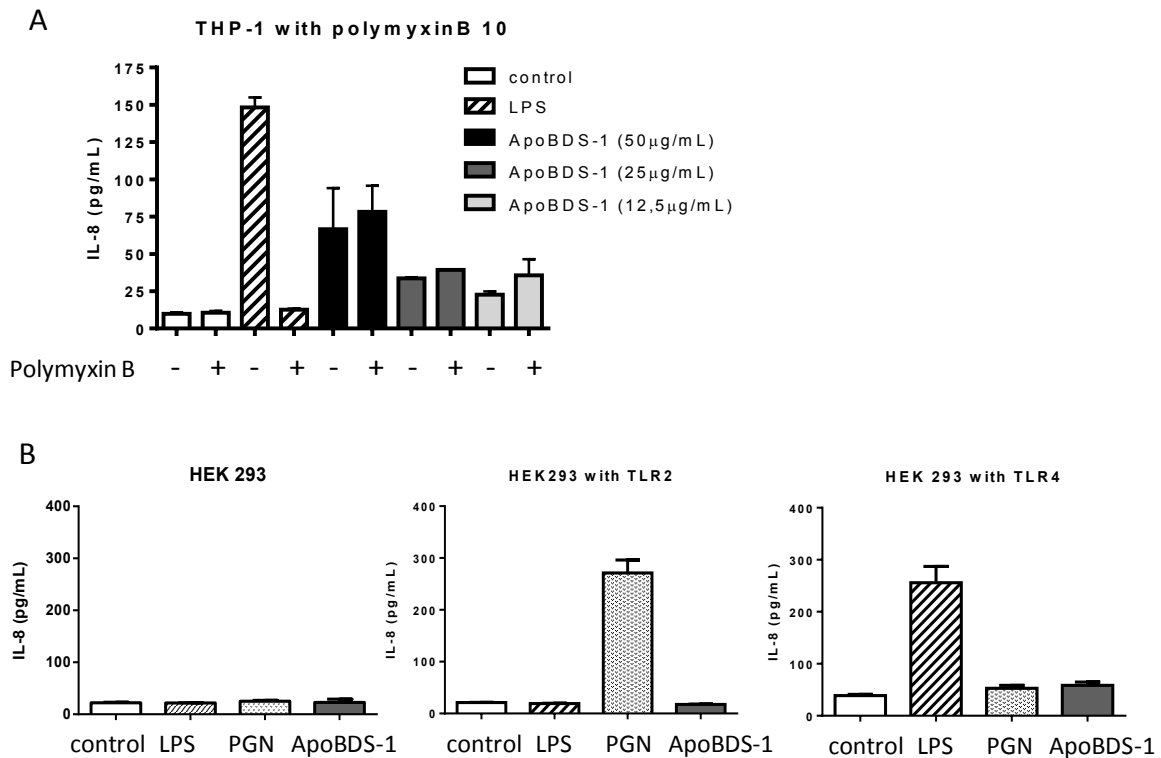


Figure 5: The effect of ApoBDS-1 is not due to LPS contamination. A: Polymyxin B (10µg/mL) didn't inhibit ApoBDS-1 induced IL-8 production in THP-1 cells. B: ApoBDS-1 didn't induce IL-8 secretion in HEK293 cells without/ with TLR2 or TLR4 transfection. LPS or peptidoglycan (PGN) used as positive control.

Previous studies have identified native and MDA modified ApoB100 peptides that can be recognized by circulating IgM or IgG autoantibodies from patients with atherosclerosis⁷. In this project, we identified a native ApoB100 peptide ApoBDS-1 with potent activity for induction of inflammatory responses in monocytes and macrophages. This finding suggests that fragmented ApoB100 may generate danger signals contributing to the regulation of innate immune responses in atherosclerosis. It is interesting that neither neighbour peptides nor truncate peptides of ApoBDS-1 have the bioactivity although they also share some amino acids with ApoBDS-1, raising a question as to the core sequence and the fundamental structure that determine ApoBDS-1 bioactivity.

4.2 THE INFLAMMATORY PROPERTIES OF APOBDS-1 (PAPER I-IV)

To extend the knowledge of ApoBDS-1, we characterized the responses of monocytes/macrophages, endothelial cells and platelets to ApoBDS-1.

4.2.1 ApoBDS-1 activates monocytes/macrophages, endothelial cells and platelets

4.2.1.1 The effect of ApoBDS-1 on monocytes/macrophages

To study the responses of monocyte/macrophage to ApoBDS-1, 12 chemokines and cytokines were measured by cytometric bead array and ELISA in PBMCs, THP-1 cells and

PMA-differentiated THP-1 cells treated with ApoBDS-1 at the indicated time points. Interestingly, ApoBDS-1 seems responsible for a selective innate immune signature¹⁶, characterized by a preferential activity for induction of IL-6, IL-8, CCL2 (monocyte chemotactic protein-1, MCP-1) and IL-1 β . And the impact of ApoBDS-1 is not equal on different cytokines and chemokines. While concentration of secreted IL-1 β induced by ApoBDS-1 is usually less than 100pg/mL, the amount of IL-6 can be 10 times more, and the strongest effect is seen on IL-8 and MCP-1 which can be as much as more than 100 times of IL-1 β (Figure 6). Interestingly, IL-1receptor antagonist (IL-1ra) preincubation in ApoBDS-1 stimulated ex vivo plaque cultures tended to decrease IL-6 and IL-8, suggesting that the production of the subset of chemokines could be a secondary response to IL-1 β .

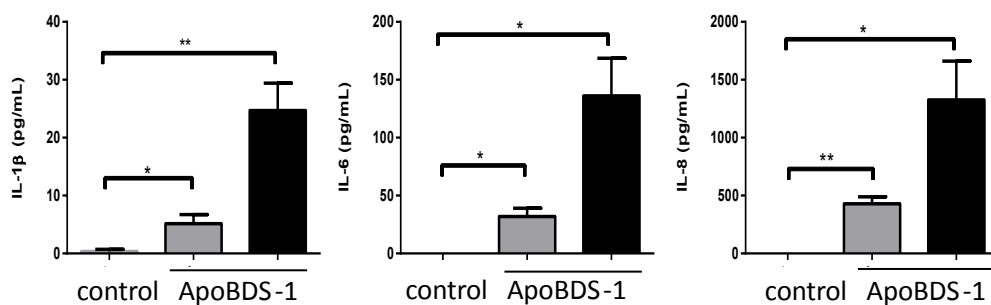


Figure 6: ApoBDS-1 induces cytokines and chemokines in PBMCs at 8h. grey: ApoBDS-1 (25 μ g/mL). black: ApoBDS-1 (50 μ g/mL)

In connection to inducing cytokines and chemokines in monocytes and macrophages, ApoBDS-1 in the range 10-100 μ g/ml could elicit concentration-dependent transwell migration of PBMCs.

Taken together, these data propose that ApoBDS-1 can attract monocytes and neutrophils to atherosclerotic lesion by induction of chemokines.

4.2.1.2 The effect of ApoBDS-1 on endothelial cells

Human aortic endothelial cells respond to ApoBDS-1 by increased expression of IL-6, MCP-1, ICAM-1, VCAM-1, COX2, MCP-1, CCL5, CCL20 mRNA and secretion of IL-6 and MCP-1. However, ApoBDS-1 didn't induce IL-8 secretion in endothelial cells, which is different from that in monocytes and macrophages. And the ApoBDS-1 is not the only peptide that induces proinflammatory response in endothelial cells, as the neighbor peptide p217 showed stronger effect than ApoBDS-1 in the induction of IL-8, VCAM-1, CHOP and XBPs.

4.2.1.3 The effect of ApoBDS-1 on platelets

ApoBDS-1 also exerts prothrombotic effects and proinflammatory effects on platelets. This has been demonstrated by the facts that ApoBDS-1 increased platelet aggregation, GPIIb/IIIa activation, fibrinogen binding and surface expression of procoagulant phosphatidylserine. ApoBDS-1 also induced P-selectin (CD62P), CD40L, IL-1 β and CD11b. ApoBDS-1

facilitated platelet-neutrophil and platelet-monocyte aggregates (Figure 7), and increased neutrophil adhesion and transmigration.

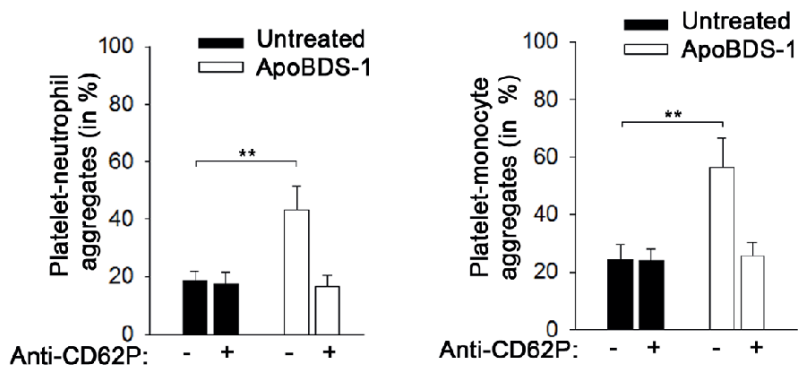


Figure 7: ApoBDS-1 increased P-selectin-dependent platelet-neutrophil aggregates and platelet-monocyte aggregates. (adopted from reference³³)

4.2.2 Mechanisms for ApoBDS-1 induced proinflammatory response

As summarized in Figure 8, our studies suggest that multiple signaling pathways are involved in ApoBDS-1 induced proinflammatory responses. The signaling mechanism of ApoBDS-1 seems more complex than initially thought.

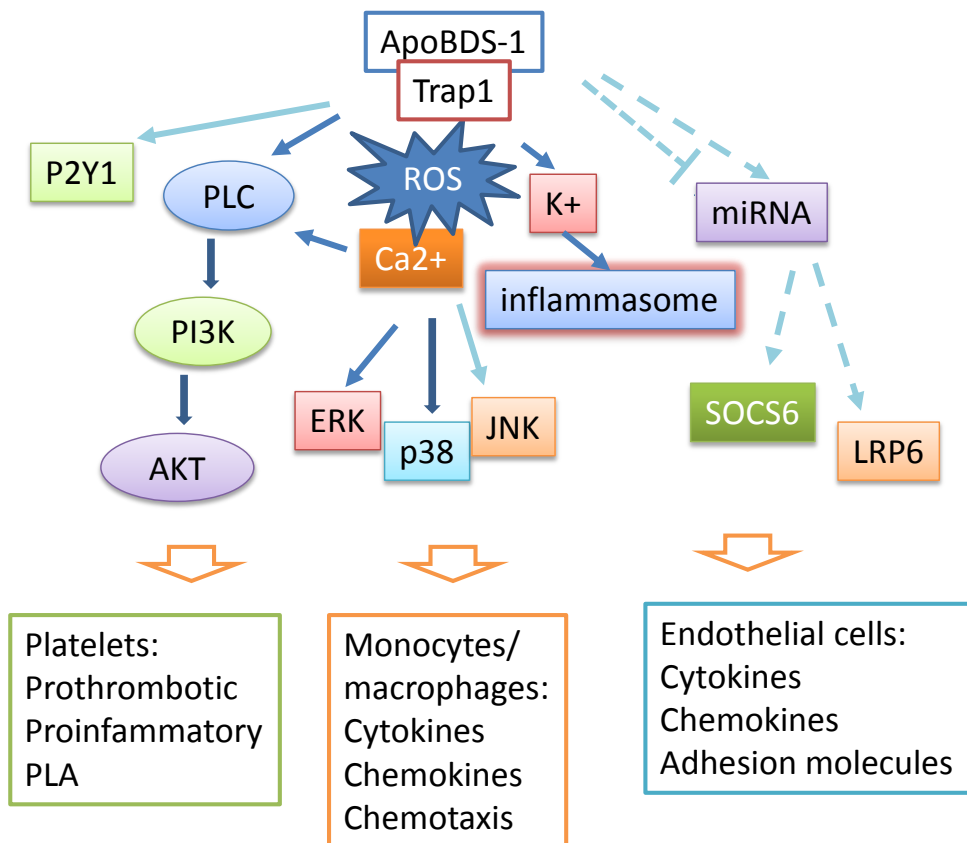


Figure 8: Multiple signaling pathways are involved in ApoBDS-1 induced proinflammatory response

4.2.2.1 *Ca²⁺ mobilization*

Calcium signaling is critical for the activation of transcriptional regulatory pathways¹⁵⁶. ApoBDS-1 treatment rapidly increased Ca^{2+} flux within 30 seconds in PBMCs, suggesting Ca^{2+} flux as an initial signaling in response to ApoBDS-1. And ApoBDS-1 induced IL-8 production was completely abrogated in the presence of BAPTA, a calcium chelator. Ca^{2+} flux was shared in the process of platelet activation upon ApoBDS-1 stimulation. However, mechanisms by which ApoBDS-1 triggers Ca^{2+} signaling have remained elusive.

4.2.2.2 *MAPK*

Ca^{2+} signaling is implicated in the modulation of MAPK pathways. Incubation of ApoBDS-1 with THP-1 cells led to activation of ERK1/2 and JNK at 10min and 60min, followed by p38 which was fully activated at 60min. When MAPK inhibitors were used, only the inhibitor of p38 MAPK completely abolished ApoBDS-1 induced IL-8 mRNA and protein, suggesting that p38 MAPK mediates ApoBDS-1 induced IL-8 production in monocytes.

Platelets are more sensitive to stimuli and react swiftly. The activation of ERK was detected already after 1min of ApoBDS-1 stimulation (Figure 9). Subsequently, activation of Akt and p38 occurred in platelets within 5 min. In different from monocytes, JNK was not affected in platelets when treated with ApoBDS-1.

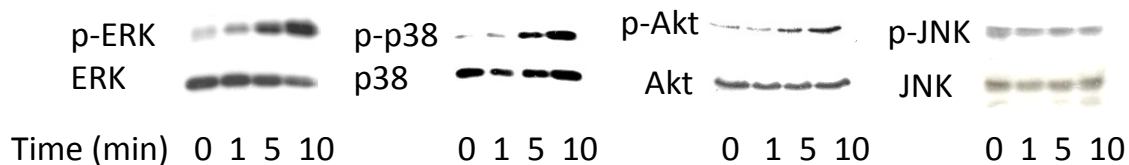


Figure 9: ApoBDS-1 induced p-ERK, p-p38 and p-Akt in platelets.

4.2.2.3 *PLC-PI3K-AKT*

Pharmacological inhibition demonstrated that activation of PI3K, Akt and PLC is critical for mediating ApoBDS-1-induced inflammatory responses monitored by P-selectin expression.

4.2.2.4 *ROS*

Reactive oxygen species (ROS) is involved in inflammatory pathways. We found that ApoBDS-1 dose-dependently increased ROS in platelets and THP-1 monocytes.

4.2.2.5 *miRNA*

In order to find out the mechanism for ApoBDS-1 induced inflammatory response in endothelial cells, we performed microRNA (miRNA) array analysis and compared regulated miRNA in presence of ApoBDS-1. We found that hsa-miR-494, hsa-miR-382, hsa-miR-15a are decreased by ApoBDS-1 treatment while hsa-miR-126, hsa-miR-146a¹⁵⁷, hsa-miR-196b are increased by ApoBDS-1 treatment. Target gene analysis indicated that SOCS6 may be a target of these microRNA. Hence, by qPCR analysis we confirmed that SOCS6 mRNA was upregulated by ApoBDS-1 (Figure 10). SOCS6 was previously found to be regulated by JAK-STAT3 signaling and involved in inflammation.

Another possible target from microRNA array and chemokine array is LDL receptor-related protein 6 (LRP6), which is involved in Wnt signaling and chemokine production. ApoBDS-1 treatment increased LRP6 mRNA in endothelial cells (Figure 10). These findings may suggest a potential role for ApoBDS-1 involved in the regulation of endothelial cell function.

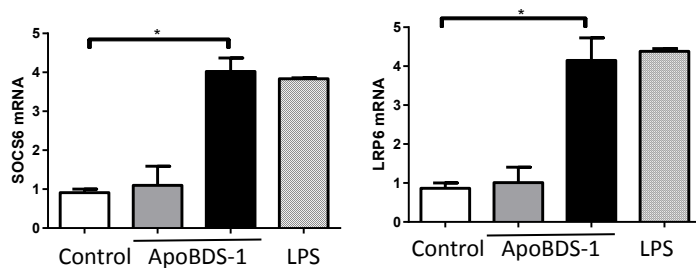


Figure 10: ApoBDS-1 increased gene expression of SOCS6 and LRP6 in HAECs. grey: ApoBDS-1 (25µg/mL). black: ApoBDS-1 (50µg/mL)

4.2.2.6 *Inflammasome (see 4.2.3 ApoBDS-1 activates inflammasome)*

4.2.2.7 *TRAP1 (see 4.3 the receptor/ interacting protein for ApoBDS-1)*

4.2.2.8 *Phagocytosis*

ApoBDS-1 can be taken up by monocytes and macrophages. We incubated PMA-differentiated THP-1 cells or PBMCs with biotin conjugated ApoBDS-1 and detected presence of ApoBDS-1 inside the cells after 30min by immunofluorescence staining (Figure 11A). And ApoBDS-1 was colocalized with mitochondria (Figure 11B). Using cytochalasin D which inhibits phagocytosis, we found that ApoBDS-1 induced IL-6 secretion was suppressed, suggesting that phagocytosis may be partly attributed to ApoBDS-1 bioactivity.

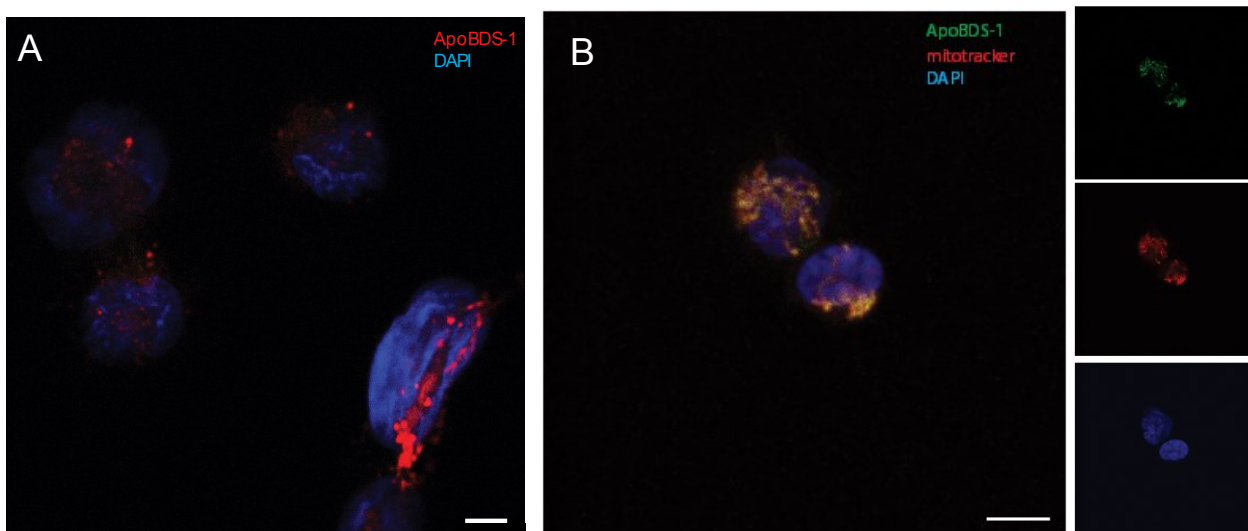


Figure 11: ApoBDS-1 can enter cells and is colocalized with mitochondria. Scale bar: 5µm

4.2.2.9 *Other mechanisms*

Our results suggest that ApoBDS-1 induced platelet activation involves platelet ADP signaling and TxA2 signaling since inhibition of TxA2 production using acetylsalicylic acid

or inhibition of purinergic receptors P2Y1 and P2Y12 partially suppressed ApoBDS-1 induced P-selectin surface expression on platelets.

4.2.3 ApoBDS-1 activates inflammasome (paper III)

IL-1 β is an important cytokine in atherosclerosis and other inflammatory diseases. We found that ApoBDS-1 induces IL-1 β release in human monocytes and macrophages. Bioactive IL-1 β is generated from a precursor by caspase-1 cleavage. We then investigated whether caspase-1 is activated by ApoBDS-1. First we examined caspase-1 enzymatic activity in THP-1 cells by flow cytometry based quantitative analysis of cleaved FAM-YVAD-FLICA, a caspase-1 selective substrate. ApoBDS-1 increased the caspase-1 activity as early as 30min and the activity remained high with time. Then we confirmed the results using another fluorometric assay. ApoBDS-1 treatment significantly activated caspase-1 which is reflected by the fluorescence of free AFC cleaved from YVAD-AFC. The third method we used to measure caspase-1 activity is Western blot. Caspase-1 is proteolytically activated from a 45kDa proenzyme to generate a tetramer of its two active subunits, p20 and p10. We showed that ApoBDS-1 treatment increased the active caspase-1 p20 significantly at 2h (Figure 12). Inhibition of caspase-1 by z-YVAD-FMK abolished ApoBDS-1 induced IL-1 β in both PMA-differentiated THP-1 cells and PBMCs, suggesting that ApoBDS-1 triggered IL-1 β production is dependent of caspase-1 activity. RIP2 is involved in IL-1 β production but RIP2 inhibitor Necrostatin did not affect the IL-1 β secretion induced by ApoBDS-1.

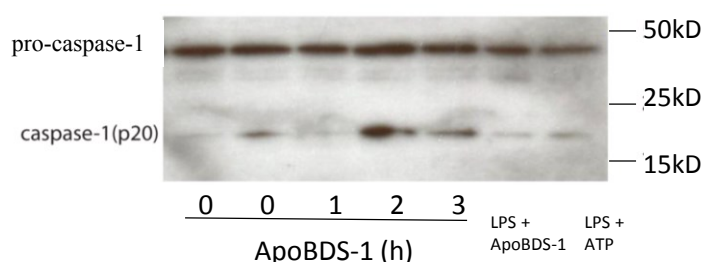


Figure 12: ApoBDS-1 activates caspase-1 in macrophages as early as 2h.

Caspase-1 is usually activated by inflammasome. Of them, NLRP3 inflammasome has been suggested to be activated by oxLDL¹⁵⁸ and cholesterol crystals⁶. By searching Biobank of Karolinska Carotid Endarterectomies (BiKE)¹³⁹⁻¹⁴³ database, we analyzed mRNA levels of genes encoding the currently defined components of inflammasome pathways in 125 human carotid plaques. The expression of inflammasome genes was generally very low in the arteries without evident atherosclerosis. In contrast, all inflammasome related genes were highly expressed in the plaque. In specific, carotid plaques were characterized by increased expression of major canonic inflammasome sensors including NLRP1, NLRP3, NLRC4, AIM2, but also IFI16, a non-canonic inflammasome sensor which is a host DNA sensor with critical roles in the generation of IFN- β and IL-1 β ^{159, 160}. Importantly, NLRP3, NLRC4 and IFI16 inflammasome genes are associated with the severity of atherosclerosis. We also noted that monocyte/macrophage marker CD14 was positively correlated with NLRP3, ASC, caspase-1 and IL-1 β . Immunofluorescence staining confirmed that NLRP3, ASC and CD68

were colocalised in plaque lesional macrophages, implying that NLRP3 inflammasome seems in an activated state in atherosclerotic plaques.

To determine whether NLRP3 inflammasome is involved in ApoBDS-1 induced proinflammatory response, we performed siRNA silencing experiment. Knock down of NLRP3 led to complete inhibition of IL-1 β production in ApoBDS-1 stimulated PBMCs and PMA-primed THP-1 cells (Figure 13).

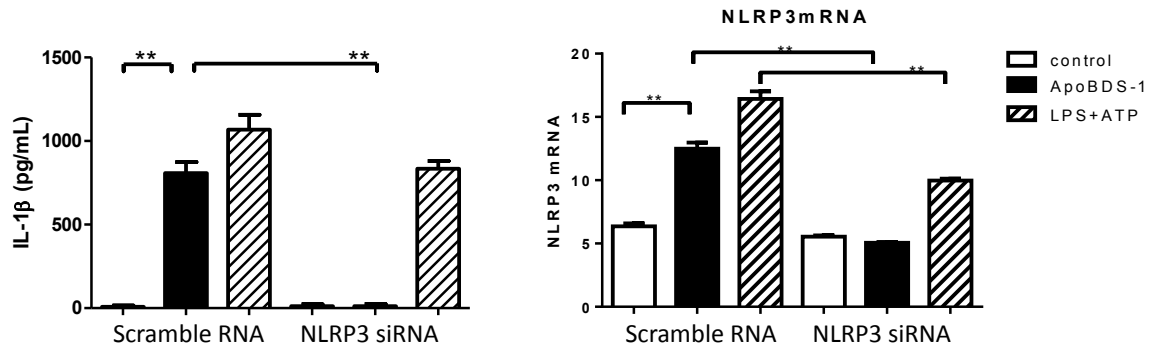


Figure 13: Knock down of NLRP3 abolished ApoBDS-1 induced IL-1 production in PMA differentiated THP-1 cells.

We then did NLRP3 inflammasome reconstitution by transfection of HEK293 cells with expression plasmids encoding pro-caspase-1, pro-IL-1 β , ASC and NLRP3. We showed that ApoBDS-1 stimulated in a concentration dependent manner the NLRP3 inflammasome-mediated IL-1 β generation (Figure 14).

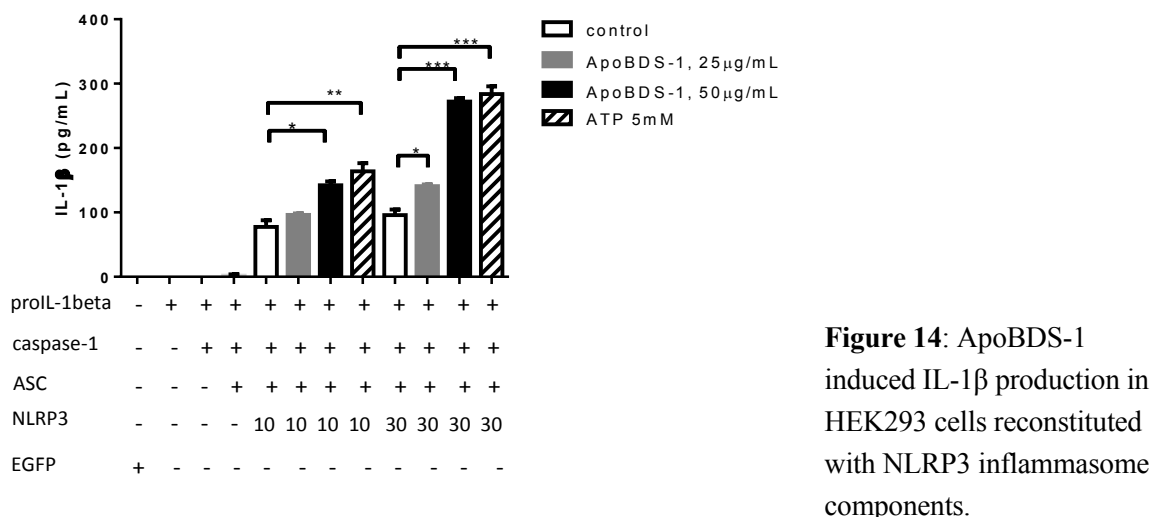


Figure 14: ApoBDS-1 induced IL-1 β production in HEK293 cells reconstituted with NLRP3 inflammasome components.

When inflammasome is activated, the formation of a multiprotein complex can be visualised in the form of ASC specks. Immunofluorescence staining of ASC and NLRP3 in different time points of ApoBDS-1 treatment showed that intracellular ASC and NLRP3 were increased as early as 1h in response to ApoBDS-1 in PMA-differentiated THP-1 cells. Quantification of the colocalization of ASC and NLRP3 also showed that ApoBDS-1 treatment increased their colocalization significantly (Figure 15).

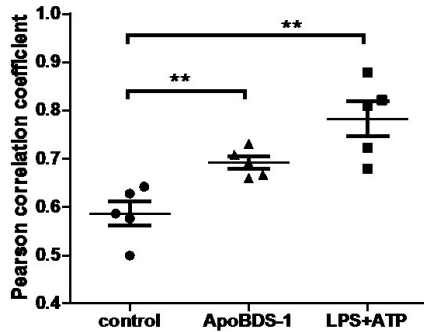
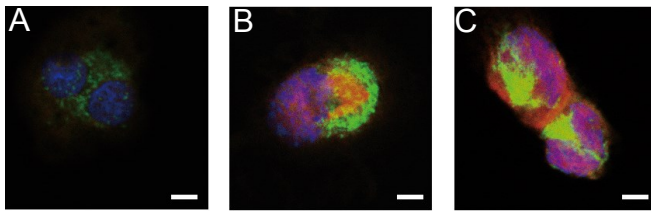


Figure 15: ApoBDS-1 increased ASC and NLRP3 at 1h and enhanced the colocalization of ASC and NLRP3 in PMA-differentiated THP-1 cells (B) compared with control (A) or cells treated with LPS+ATP (C). Red: ASC. Green: NLRP3. Blue: DAPI. Scale bar: 5μm.

Potassium (K^+) efflux is proposed as a common mechanism whereby a variety of substances activate NLRP3 inflammasome⁶⁵. We showed that blocking efflux of intracellular K^+ virtually abolished the stimulatory effect of ApoBDS-1 on IL-1 β production, suggesting that K^+ efflux is a central mechanism underlying ApoBDS-1 activated inflammasome.

4.3 THE RECEPTOR/ INTERACTING PROTEIN FOR APOBDS-1 (PAPER IV)

We have known that ApoBDS-1 stimulates macrophages inducing inflammatory cytokine and chemokine responses, and activates platelets boosting their inflammatory responses. The next question is whether there is a receptor or interacting protein for ApoBDS-1.

Performing far western blot, we assessed the ApoBDS-1 interacting proteins in macrophage lysates and noted that one band near 75 kDa could be constantly recognized by the ApoBDS-1-biotin. Interestingly, this protein seems only detected by far western blot but not in regular western blot. Based on these results, this ApoBDS-1-recognized protein has to be in a properly re-natured structure when interacting with ApoBDS-1. Furthermore the observed interaction with ApoBDS-1 was highly specific as confirmed by competing with 10-fold excess concentration of unlabeled ApoBDS-1 (Figure 16 left).

	75kD			
ApoBDS-1-biotin	+	+	+	+
ApoBDS-1	-	-	+	+

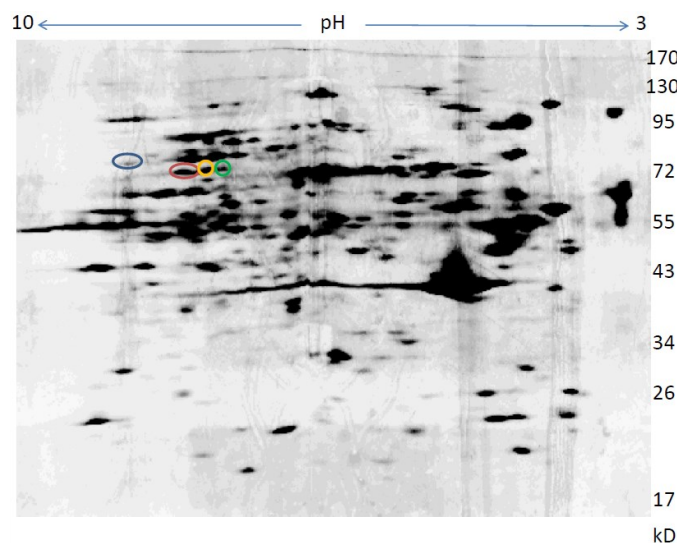


Figure 16: Left: ApoBDS-1 interacting protein was detected by far western blot and the interaction was inhibited by incubation with 10-times excess concentration of unlabeled ApoBDS-1. Right: Representative of 2-D electrophoresis that illustrates the far western blot mapped ApoBDS-1 reactive spots.

Subsequently, the identity of ApoBDS-1 interactive proteins was verified by 2-Dimensional (2-D) electrophoresis and mass spectrometry. 2-D electrophoresis of THP-1 lysate generated a high resolution of cellular protein profile (Figure 16 right). Thereafter, the ApoBDS-1 interactive proteins were probed, and mapped to a parallel 2-D gel stained with silver. By this approach, in total 5 ApoBDS-1 interactive protein spots from two independent 2D gels were retrieved and identified by MALDI-TOF mass spectrometry and Peptide mass fingerprint analysis (PTMs).

Among the identified proteins, TRAP1 (Tumor necrosis factor receptor-associated protein 1) was characterized with the highest score to be ApoBDS-1 interactive protein.

Trap1 was originally found to interact with TNF receptor. It resides mainly in mitochondria. Previous researches revealed that Trap1 can also exist in the ER¹⁶¹, cytosol or extracellular space¹⁶². Trap1 and TBP7 were found to interact directly in the ER, indicating that Trap1 is involved in protein quality control for mistargeted/misfolded mitochondria-destined proteins¹⁶¹. Trap1 may facilitate the ER-mitochondria crosstalk. Trap1 interacts with client proteins that play key role in signaling pathways. For instance, by interacting with Sorcin¹⁶³, a calcium sensor, whereby it is implicated in the regulation of Ca²⁺ homeostasis in the heart. Perturbation of Trap1 activity has been linked to neurodegenerative diseases^{164, 165}, vascular disorder¹⁶⁶, several cancers^{167, 168} and autoimmune disease¹⁶⁹. But the relevance of Trap1 to atherosclerosis is ambiguous.

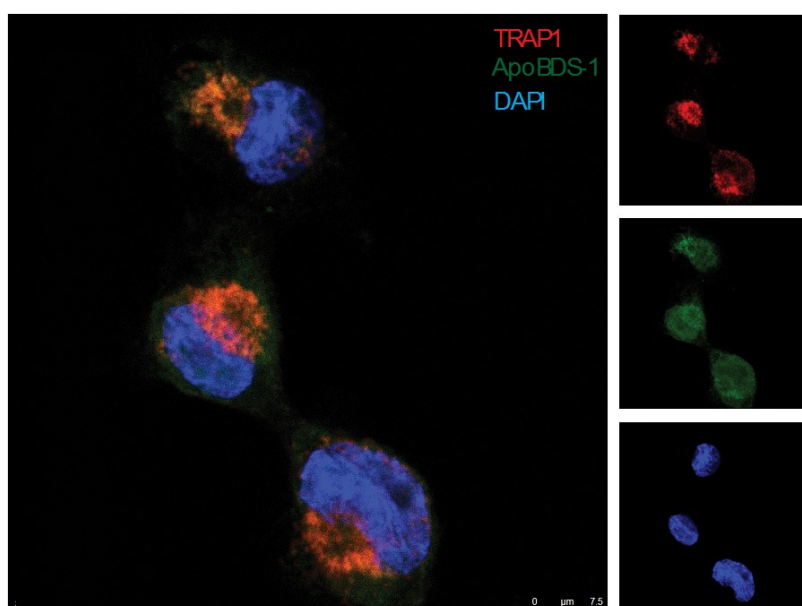


Figure 17: Colocalisation of ApoBDS-1 and Trap1 in PMA-differentiated THP-1 cells. Scale bar: 7.5µm

Bioinformatics indicated that ApoBDS-1 has a mitochondrion targeting sequence. Indeed, we found that ApoBDS-1 was colocalised with mitochondria, and also colocalised with Trap1 intracellularly and on the membrane (Figure 17). It is possible that ApoBDS-1 travels into the

cell by forming complex with TRAP1. The majority of the colocalisation exist in the cytosol suggesting that the binding mostly takes place inside the cells. Since the hydrophobic property of ApoBDS-1, it is likely that TRAP1 shields ApoBDS-1 from being aggregated and degraded quickly, maintaining ApoBDS-1 in an active form in the cells.

We then characterized the interaction of ApoBDS-1 and Trap1 using Biacore surface plasma resonance analysis. By immobilizing TRAP1 on biosensor surface and flowing ApoBDS-1 and related peptides or molecules over the blank control and the immobilized TRAP1, we demonstrated that ApoBDS-1 directly interacted with TRAP1 with a binding strength of 8 resonance unit (RU). The binding specificity was controlled using a third flow cell immobilized with Conalbumin, an unrelated protein with the same molecular weight as TRAP1 (75 kDa). And the binding of ApoBDS-1 to its specific antibody was used as positive control. Furthermore, two neighboring peptides of ApoBDS-1, p215 and p217 showed minimal binding to TRAP1. Collectively, these data indicate that the interaction with TRAP1 is a unique property of ApoBDS-1.

To find out which domain is critical in the interaction with Trap1, we tested ApoBDS-1 truncate peptides. P216c, which spans the last 10 amino acids in the C-terminus of ApoBDS-1, exhibited the strongest affinity to TRAP1 in contrast to p216a and p216b (Figure 18). These results suggest that the binding of ApoBDS-1 to TRAP1 relies primarily on the C-terminal domain of ApoBDS-1.

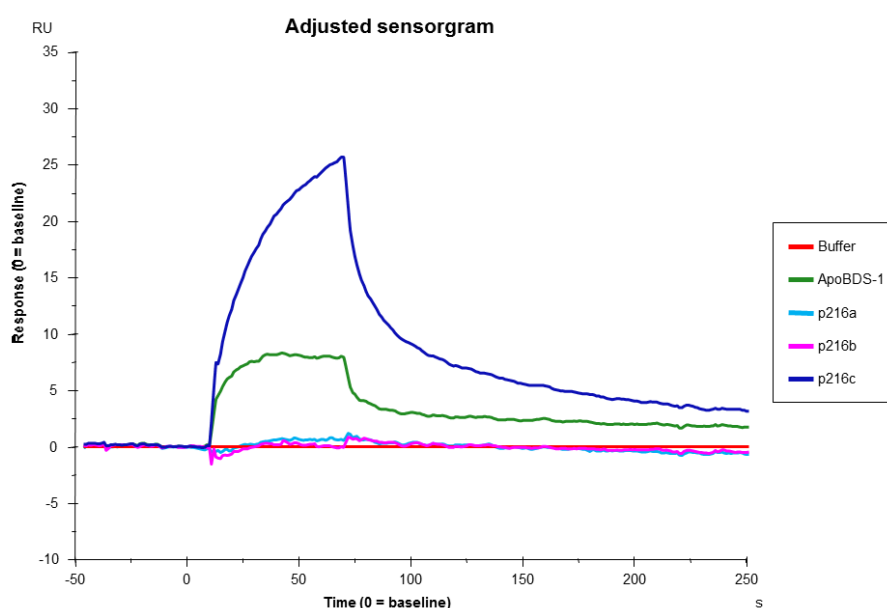


Figure 18: Biacore SPR showed that ApoBDS-1 interacts with Trap1 and p216c is the critical sequence.

Next we determined the kinetics/affinity of the truncate peptide p216c. The interaction of p216c and TRAP1 has some heterogeneity with one portion dissociating slowly (Figure 19). Therefore it could not be simulated by 1:1 binding algorithm. To estimate the kinetics, we performed steady state affinity analysis, and the dissociation constant (KD) was estimated as $28.77 \pm 0.833 \mu\text{M}$ ($\text{KD} \pm \text{SEM}$) with increased concentrations ranging from 1.875 to $30 \mu\text{M}$.

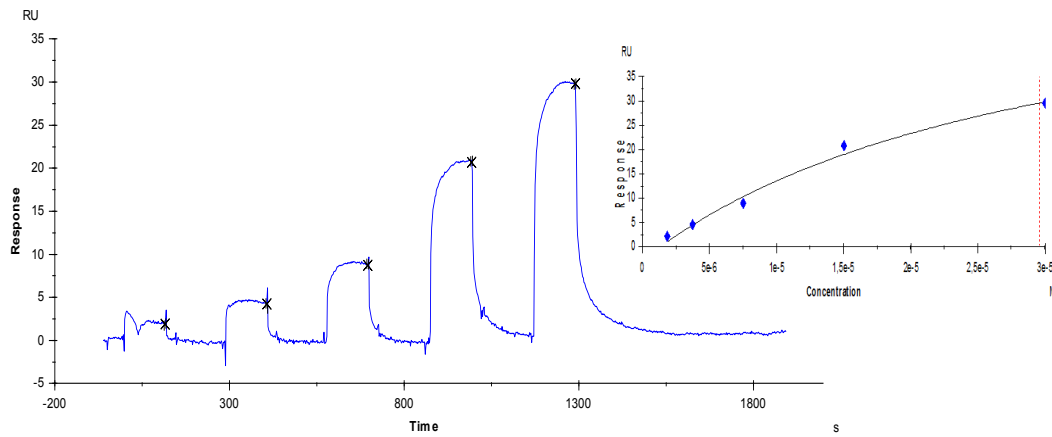


Figure 19: Kinetics of p216c binding to TRAP1. Steady state affinity analysis was performed.

We also characterized ApoBDS-1 binding site in TRAP1. Like other proteins in the HSP90 family, TRAP1 has ATPase activity and 4 ATP binding sites. Hydrolysis of ATP will lead to a conformational change in TRAP1, as a consequence, the rearrangement of client protein binding site. Utilizing Geldanamycin, a HSP90/TRAP1 ATPase domain inhibitor¹⁷⁰ we showed that ApoBDS-1 did not compete with Geldanamycin on the binding to TRAP1, indicating that the interaction between ApoBDS-1 and TRAP1 leaves its ATPase domain intact.

Having confirmed the interaction between ApoBDS-1 and TRAP1, we investigated the functional relevance of TRAP1-ApoBDS-1 interaction. TRAP1 protein was silenced in PBMC using TRAP1 specific shRNAs. This led to 40% reduction of TRAP1 protein from the basal level in PBMC and nearly 80% reduction in the ApoBDS-1 treated PBMCs (Figure 20). Coincided with knocking down TRAP1, ApoBDS-1 induced IL-1 β production was completely inhibited (Figure 20).

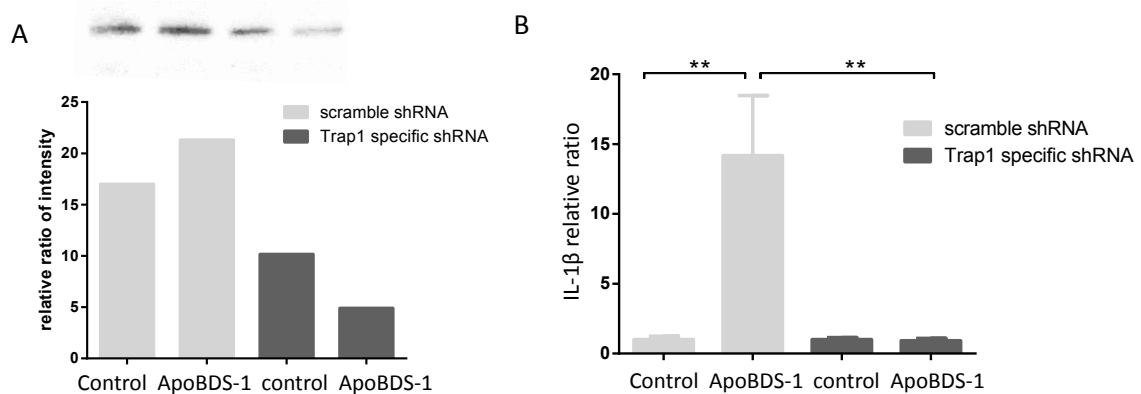


Figure 20: Trap1 knock down inhibited ApoBDS-1 induced IL-1 β production in PBMCs.

Besides knock down experiment, we also used pharmacological inhibitors to test the function of TRAP1. Preincubation of PBMCs or THP-1 cells with Geldanamycin inhibited ApoBDS-1 induced IL-1 β , IL-8 and MCP-1 production, suggesting that TRAP1 is required in ApoBDS-1 induced proinflammatory response.

Mitochondria generated reactive oxygen species (ROS) have important physiological functions and are involved in cytokine and chemokine production. We observed that ApoBDS-1 promoted in a concentration dependent manner an increase in the generation of ROS in the monocytic cells and platelets. It is worth of noting that ApoBDS-1-induced ROS was also blocked by Geldanamycin (Figure 21), suggesting that TRAP1 plays a regulatory role in the process of ROS production, which in turn may mediate ApoBDS-1 induced proinflammatory response.

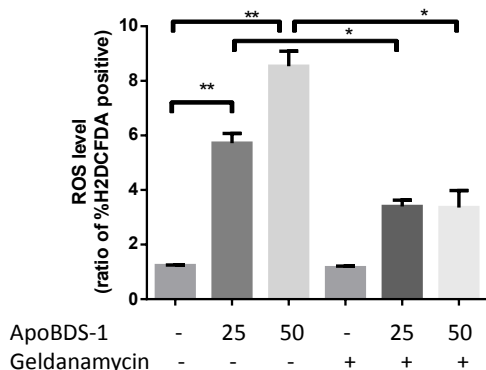


Figure 21: ApoBDS-1 induced ROS production was inhibited by Geldanamycin.

Lisanti et al recently report that deletion of TRAP1 results in a compensatory metabolic reprogramming, with a compensatory increased recruitment of cytoprotective chaperones Hsp90 and Hsp27 to mitochondria, a global compensatory upregulation of an oxidative phosphorylation transcriptome, increased aerobic glycolysis and ATP production¹⁷¹.

Consistent with their studies, we found that knock-down of TRAP1 using shRNA silencing abolished ApoBDS-1 induced proinflammatory IL-1 β secretion. And Geldanamycin, an inhibitor of TRAP1 by inhibiting ATPase activity also blunted ApoBDS-1 induced ROS production and IL-1 β , IL-8 and MCP-1 production. It is possible that these recruited protective chaperones can neutralize or hinder the proinflammatory effects of ApoBDS-1. But whether ApoBDS-1 interacts with Hsp90 or Hsp27 directly or how these chaperones crosstalk with each other remain to be determined.

Based on our data, we propose a function model for TRAP1 in the regulation of ApoBDS-1 activity. Our model suggests ApoBDS-1 recruitment to TRAP1 as a crucial process that keeps intracellular ApoBDS-1 in a stabilized and bioactive state. Perturbation of TRAP1 function then leads to the dissociation of ApoBDS-1, subsequently resulting in ApoBDS-1 ubiquitinated and degraded. An unsolved question of the present study is how ApoBDS-1 is degraded after dissociation from TRAP1. We speculate that it is likely by a similar mechanism responsible for ApoB100 degradation. ApoB100 is regulated mainly by the co-translational degradation mechanism via the ubiquitin-proteasomal pathway¹⁷². During translation, the N terminus of ApoB is translocated into the ER lumen^{173, 174} where BiP, a chaperone protein, stabilizes hydrophobic sites^{174, 175}.

NLR orthologues in plants, which lack adaptive immunity, are R proteins that form a complex with suppressor of G2 allele of S-phase kinase-associated protein 1 (SGT1) and

HSP90 for detection of pathogens¹⁷⁶. The complex formation is required for R protein stability and activation of signaling. Several mammalian NLRs, including NOD2, IL-1 β -converting enzyme protease-activating factor (IPAF), NALP3 and Monarch-1 (NLRP12), also form a pre-activation complex with homologues of SGT1 and HSP90¹⁷⁶. It is proposed that NLRs need to form complex and indirectly sense PAMPs¹⁷⁶. It is possible that NLRP3 and Trap1 form a complex and facilitate ApoBDS-1 activation of inflammasome. This needs to be investigated further.

4.4 THE RELEVANCE OF APOBDS-1 WITH ATHEROSCLEROSIS (PAPER I, III)

We showed that ApoBDS-1 is capable of inducing proinflammatory responses in monocytes/macrophages, platelets, endothelial cells. We also found that ApoBDS-1 elicited IL-6, IL-8, and PGE2 in ex vivo atherosclerotic plaque cultures.

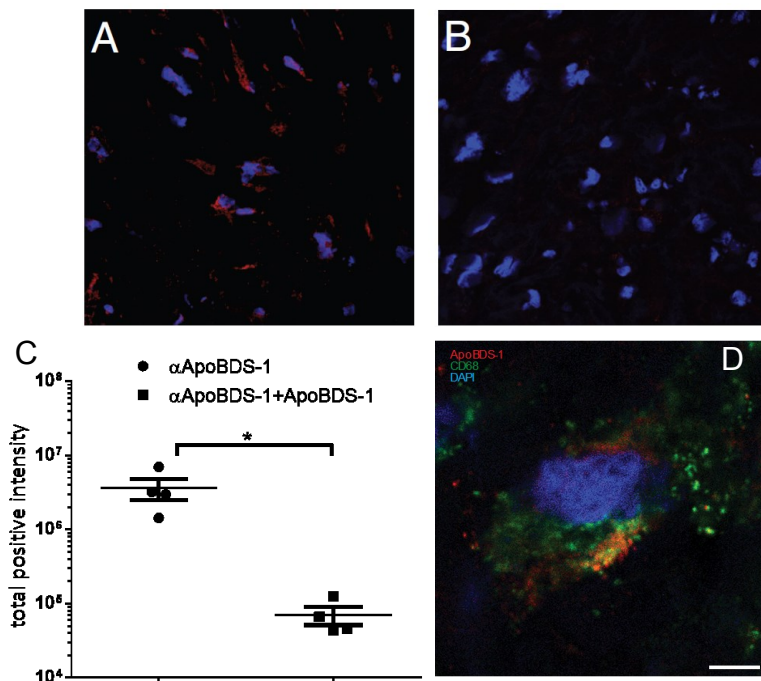


Figure 22: ApoBDS-1 is present in atherosclerotic plaques (A) and the signal is inhibited by incubation with both ApoBDS-1 antibody and excess ApoBDS-1 peptide (B). C shows the quantification of fluorescence intensity. D shows ApoBDS-1 (red) is colocalized with CD68 (green).

To further explore the relevance of ApoBDS-1 to atherosclerosis, we examined whether ApoBDS-1 is present in atherosclerotic lesions. Using ApoBDS-1 specific antibody, we showed that ApoBDS-1 exists in the macrophage-rich region of atherosclerotic plaque sections. ApoBDS-1 signals were observed in extracellular and intracellular spaces (Figure 22), and the intracellular ApoBDS-1 was predominantly colocalized with CD68 positive macrophages.

To further verify the presence of ApoBDS-1 in atherosclerotic plaques, we analyzed plaque homogenates by western blot and size-exclusion chromatography and found that several bands/fractions between 10kDa and 42kDa from the plaque homogenates can be recognized by ApoBDS-1 specific antibody. We then collected these ApoBDS-1 positive chromatographic fractions. Incubation of PBMCs with these fractions elicited similar IL-8 production as ApoBDS-1 and the response can be inhibited by ApoBDS-1 truncate peptide

p216c (Figure 23), implying that fragments with ApoBDS-1 bioactivity can be found in atherosclerotic plaques.

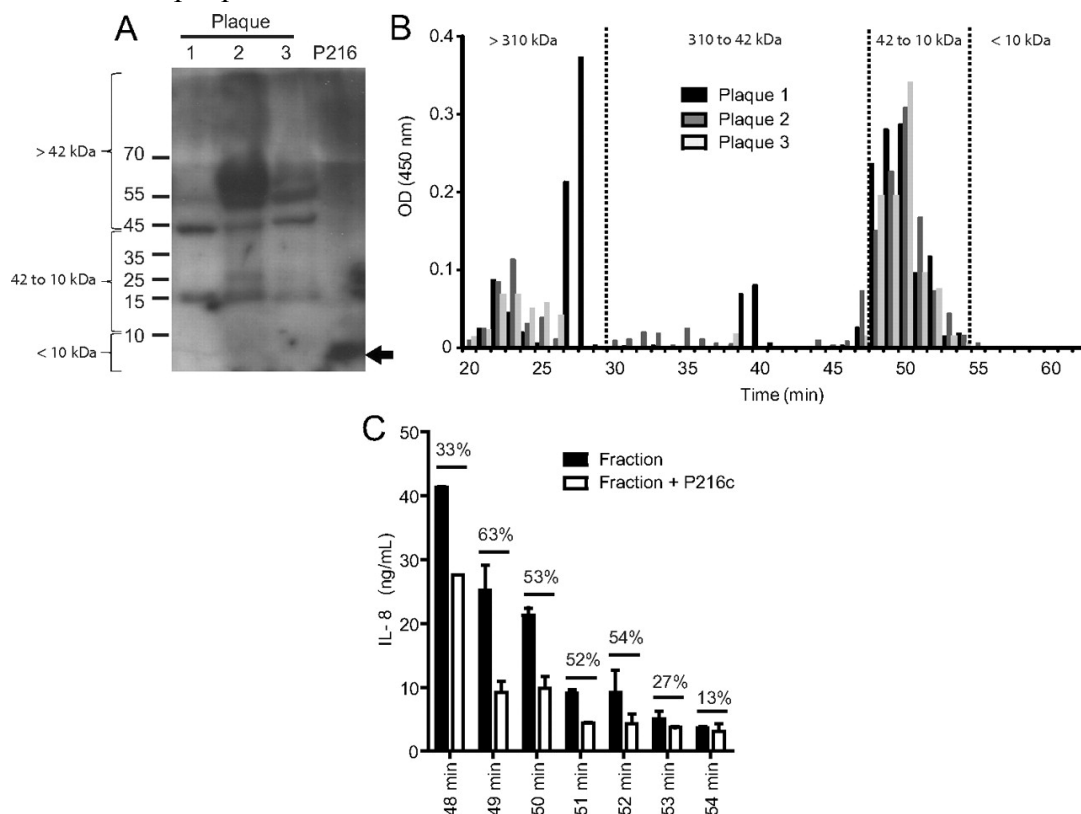


Figure 23: ApoBDS-1 positive peptides exist in atherosclerotic plaque homogenates. A. western blot. B. size-exclusion chromatography. C. ApoBDS-1 positive fragments induce IL-8 in PBMCs and the response can be inhibited by truncate peptide.

These data suggest that ApoBDS-1 positive fragments are present in atherosclerotic plaques and these fragments also have proinflammatory bioactivity like ApoBDS-1.

The analysis of BiKE database suggests that several inflammasome pathways may be active in atherosclerotic lesions. NLRP3 inflammasome related genes and caspase-1 are dominant in plaques and have obvious correlation with CD14. Ex vivo plaque culture showed that ApoBDS-1 induced IL-1 β at 100 μ g/mL. It is possible that ApoBDS-1 also plays a role in inflammasome activation in atherosclerotic lesions.

ApoBDS-1 is a synthesized peptide. So far we haven't found the evidence that it exists as a free form in peripheral blood, which might be beneficial as the body avoids being activated continuously by an innate immune activator. We did find several ApoBDS-1 containing fragments in plasma after we incubated plasma with neutrophils or macrophages. We also generated ApoBDS-1 positive fragment about 10kD from ApoB100 incubated with trypsin or neutrophil elastase. Neutrophil elastase can be produced by monocytes, macrophages and vascular endothelial cells and is found in macrophage-rich shoulders of atheromatous vulnerable plaques¹⁷⁷. It is possible that LDL or oxLDL particles which are entrapped in intima undergo a series of modification including enzymatic degradation of neutrophil elastase and other proteases generating neo-fragments that may contain ApoBDS-1 or ApoBDS-1 epitopes. The relevance of ApoBDS-1 to atherosclerosis has been suggested by

the existence of endogenous ApoBDS-1 containing peptides with pro-inflammatory property in human carotid plaques¹⁶. Yet, it still needs to be investigated in which form the ApoBDS-1 sequence exists in atherosclerotic lesions, whether it is the same sequence of ApoBDS-1 in vivo, and how it is generated in vivo, etc.

5 CONCLUDING REMARKS

We identified a native peptide ApoBDS-1 from ApoB100 protein of LDL particle that induces innate immune responses. ApoBDS-1 is present in atherosclerotic plaques. ApoBDS-1 activates monocytes/macrophages, endothelial cells and platelets via multiple pathways. ApoBDS-1 is capable of activating NLRP3 inflammasome leading to IL-1 β production which may elicit cytokine and chemokine secretion. We identified Trap1 as ApoBDS-1 interacting protein and targeting Trap1 may become therapeutic option for atherosclerosis in the future.

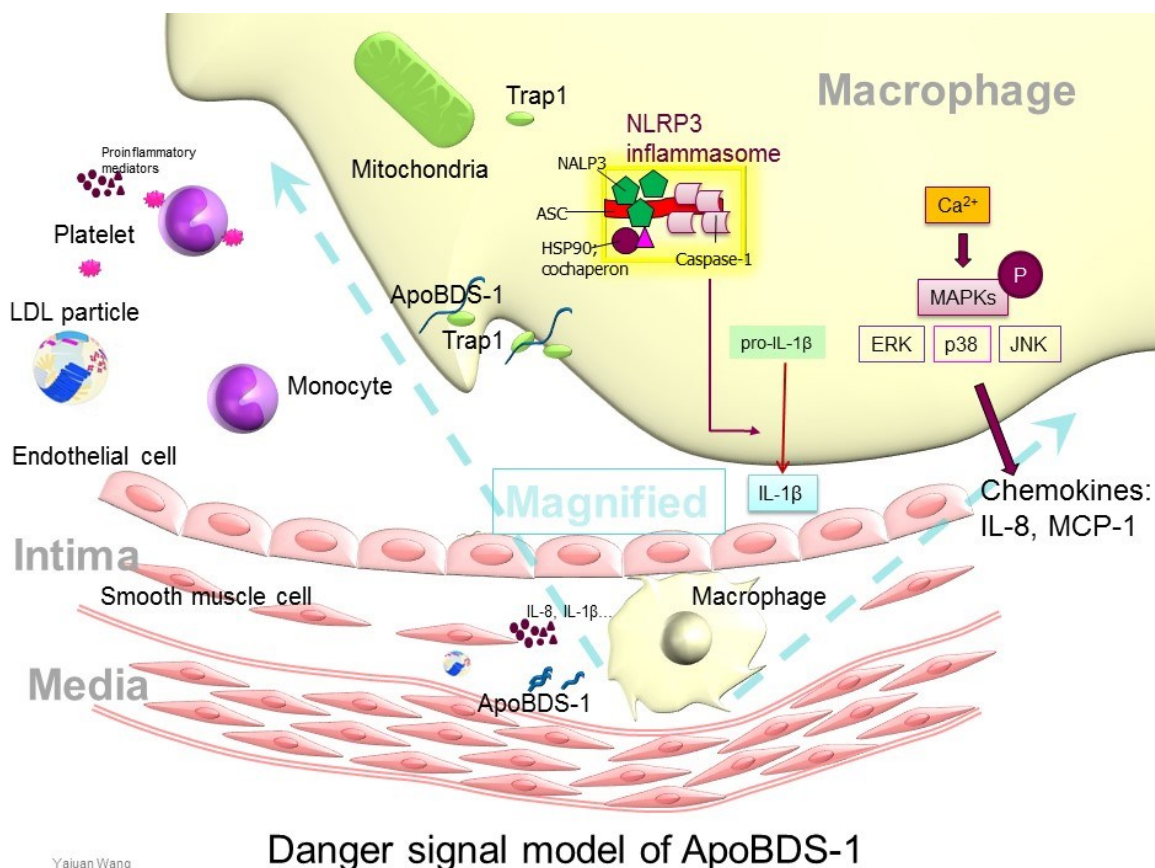


Figure 24: The role of ApoBDS-1 in atherosclerosis.

In the presence of risk factors for atherosclerosis, LDL particles are trapped in the intima and become modified by enzymes or oxidative stress. ApoB100, the unexchangeable protein of LDL, can be broken down by extracellular proteases or intracellular machinery, releasing or exposing ApoBDS-1. ApoBDS-1 interacts with chaperone protein Trap1 to initiate the innate immune response. ApoBDS-1 induces Ca^{2+} flux, increases ROS production and increases MAPKs phosphorylation. ApoBDS-1 also activates caspase-1 via NLRP3, leading to IL-1 β secretion. The consequences are that ApoBDS-1 induces endothelial cells to secrete MCP-1 and other chemokines to attract more leukocytes. ApoBDS-1 activates platelets and promotes platelet-monocyte aggregates or platelet-neutrophil aggregates. ApoBDS-1 induces monocytes and macrophages to secrete IL-8, IL-6, MCP-1 and other chemokines and attract leukocytes to the site, which propagate the inflammation and form a vicious circle. Thus ApoBDS-1 may represent a new LDL derived innate immune activator which aggravates

atherosclerosis. Blockage of ApoBDS-1 pathways, like targeting Trap1, accelerating ApoBDS-1 degradation, or neutralizing ApoBDS-1, may provide new therapeutic alternatives.

6 SUMMARY OF PAPERS AND AUTHOR CONTRIBUTIONS

Paper I

Identification of a Danger-Associated Peptide From Apolipoprotein B100 (ApoBDS-1) That Triggers Innate Proatherogenic Responses

Daniel F.J. Ketelhuth*; Francisco J.O. Rios*; YAJUAN WANG; Huiqing Liu; Maria E. Johansson; Gunilla N. Fredrikson; Ulf Hedin; Magnus Gidlund; Jan Nilsson; Göran K. Hansson; Zhong-qun Yan (*contribute equally)

Circulation. 2011;124:2433-2443.

Summary: Background—Subendothelial deposited low-density lipoprotein particles are a known inflammatory factor in atherosclerosis. However, the causal components derived from low-density lipoprotein are still poorly defined. Apolipoprotein B100 (ApoB100) is the unexchangeable protein component of low-density lipoprotein, and the progression of atherosclerosis is associated with immune responses to ApoB100-derived peptides. In this study, we analyzed the proinflammatory activity of ApoB100 peptides in atherosclerosis.

Methods and Results—By screening a peptide library of ApoB100, we identified a distinct native peptide referred to as ApoB100 danger-associated signal 1 (ApoBDS-1), which shows sequence-specific bioactivity in stimulation of interleukin-8, CCL2, and interleukin-6. ApoBDS-1 activates mitogen-activated protein kinase and calcium signaling, thereby effecting the expression of interleukin-8 in innate immune cells. Ex vivo stimulation of carotid plaques with ApoBDS-1 enhances interleukin-8 and prostaglandin E2 release. Furthermore, we demonstrated that ApoBDS-1-positive peptide fragments are present in atherosclerotic lesions using immunoassays and that low-molecular-weight fractions isolated from plaque show ApoBDS-1 activity inducing interleukin-8 production.

Conclusions—Our data show that ApoBDS-1 is a previously unrecognized peptide with robust proinflammatory activity, contributing to the disease-promoting effects of low-density lipoprotein in the pathogenesis of atherosclerosis

Author contribution: Yajuan participated in the hypothesis formulation, experimental design, project planning and results interpretation, manuscript writing etc. Specifically, she carried out experiments regarding: immunofluorescence detection of ApoBDS-1 in carotid

atherosclerotic plaques using immunofluorescence staining, confocal microscopy and quantification analysis for the images, and analysis of ApoBDS-1 in carotid plaque homogenates using Western blot, assessment of the activity of ApoBDS-1-positive fractions using cell experiment. She also did experiment to verify the specificity of ApoBDS-1 dependent bioactivity, and the specificity of the ApoBDS-1 antibody.

Paper II

Apolipoprotein B100 danger-associated signal 1 (ApoBDS-1) triggers platelet activation and boosts platelet-leukocyte's proinflammatory responses

Alice Assinger, YAJUAN WANG, Lynn M Butler, Goran K Hansson, Zhong-qun Yan, Cecilia Soderberg-Naucler, Daniel FJ Ketelhuth

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Summary: Low-density lipoproteins (LDL), occurring in vivo in both their native and oxidative form, modulate platelet function and thereby contribute to atherothrombosis. We recently identified and demonstrated that 'ApoB100 danger-associated signal 1' (ApoBDS-1), a native peptide derived from Apolipoprotein B-100 (ApoB100) of LDL, induces inflammatory responses in innate immune cells. Platelets are critically involved in the development as well as in the lethal consequences of atherothrombotic diseases, but whether ApoBDS-1 has also an impact on platelet function is unknown. In this study we examined the effect of ApoBDS-1 on human platelet function and platelet-leukocyte interactions in vitro. Stimulation with ApoBDS-1 induced platelet activation, degranulation, adhesion and release of proinflammatory cytokines. ApoBDS-1-stimulated platelets triggered innate immune responses by augmenting leukocyte activation, adhesion and transmigration to/through activated HUVEC monolayers, under flow conditions. These platelet-activating effects were sequence-specific, and stimulation of platelets with ApoBDS-1 activated intracellular signaling pathways, including Ca²⁺, PI3K/Akt, PLC, and p38- and ERK-MAPK. Moreover, our data indicates that ApoBDS-1-induced platelet activation is partially dependent of positive feedback from ADP on P2Y₁ and P2Y₁₂, and TxA₂. In conclusion, we demonstrate that ApoBDS-1 is an effective platelet agonist, boosting platelet-leukocyte's proinflammatory responses, and potentially contributing to the multifaceted inflammatory-promoting effects of LDL in the pathogenesis of atherothrombosis.

Author contribution: Yajuan participated in the project discussion, performed Western blot experiments dealing with ApoBDS-1 signaling mechanisms in platelets, wrote the corresponding method and results, and performed experiments to help answer the reviewers.

Paper III

Activation of the NLRP3 inflammasome by a danger-associated peptide from Apolipoprotein B100 (ApoBDS-1) provides a mechanism for IL-1 β production in atherosclerosis

YAJUAN WANG, Huiqing Liu, Xintong Jiang, Haiqing Gao, Göran K Hansson, Zhong-qun Yan

(manuscript)

Summary: Interleukin (IL)-1 β is one of the major culprits in atherosclerosis, metabolic syndrome, and insulin resistance, resulting from activation of macrophage inflammasome by oxidized low density lipoprotein (oxLDL). However, a comprehensive understanding of inflammasome pathways in human atherosclerotic lesion is lacking, and the molecular identity responsible for oxLDL-induced IL-1 β production is still unclear. We recently discovered that LDL contains an alarming signal, called Apolipoprotein B 100 danger associated signal 1 (ApoBDS-1) given its activity in triggering macrophage-mediated innate immune responses in atherosclerosis. Here we report that NALP3, NLRP4 and IFI16 inflammasome pathways are linked with severe human atherosclerosis, and that LDL related peptide ApoBDS-1 is an activator of NALP3 inflammasome, possibly contributing to oxLDL induced IL-1 β production in atherosclerosis. Information from Biobank of Karolinska Carotid Endarterectomies (BiKE) database showed that many inflammasome genes are expressed in atherosclerotic plaques, among which NLRP3 inflammasome is positively correlated with monocytes/macrophages. Using fluorometric assay and flow cytometry we found that ApoBDS-1 is capable of activating caspase-1 in monocytes and macrophages, leading to caspase-1 dependent IL-1 β production. Immunofluorescence staining analysis demonstrated that ApoBDS-1 could induce assembly of NLRP3 inflammasome in macrophages. Knocking down NLRP3 gene abolished ApoBDS-1 induced IL-1 β production. Further, by reconstituting NLRP3 inflammasome in HEK 293 cells, we confirmed that NLRP3 inflammasome is indispensable to ApoBDS-1 induced IL-1 β production. Mechanistically, ApoBDS-1 activates NALP3 inflammasome as a consequence of altered potassium efflux and mitochondrial membrane potential. Collectively, these findings reveal this ApoB100-derived peptide as an LDL originated activator of NALP3 inflammasome, possibly contributing to the inflammatory roles of oxLDL in atherosclerosis.

Author contribution: Yajuan participated in the formation of the idea/hypothesis, the experimental design, performed the cell culture experiments and ELISA to show ApoBDS-1 induces IL-1 β in monocytes and macrophages, the BiKE database searching and data analysis to show involvement of inflammasome signaling, performed fluorometric assay, flow cytometry, Western blot and inhibitor experiment to show caspase-1 activation is necessary, performed immunofluorescence staining, siRNA knock down and plasmid reconstitution experiments to show NLRP3 inflammasome is activated and essential, also performed cell culture experiments to show potassium efflux and mitochondrial membrane potential are important. Yajuan even wrote scripts using octave (equivalent to Matlab) to facilitate data analysis for ELISA and qPCR. Yajuan analyzed all the data, interpreted the results and wrote the manuscript.

Paper IV

A danger-associated peptide from Apolipoprotein B100 induces macrophage inflammatory responses by targeting the Tumor necrosis factor type 1 receptor-associated protein 1

YAJUAN WANG, Daniel Ketelhuth, Sanela Kjellqvist, Haiqing Gao, Per Eriksson, Göran Hansson, Zhong-qun Yan

(manuscript)

Summary: ApoBDS-1 or Apolipoprotein B 100 danger associated signal 1 is a 20-aa peptide derived from ApoB100 protein. Our previous data have shown that ApoBDS-1 triggers calcium efflux and MAPK signaling, activates NLRP3 inflammasome, and induces proinflammatory cytokines and chemokines in monocytes/macrophages but also platelets and neutrophils. This leads to an inevitable question, is there a receptor or interacting protein for ApoBDS-1.

In this paper, using Far western blot, we found that ApoBDS-1 probed a 75 kD protein in THP-1 cell lysate. Subsequently, performing 2-Dimensional (2-D) electrophoresis, we identified five corresponding spots for sequencing by mass spectrometry assay. Of the candidate proteins, Trap1 (Tumor necrosis factor receptor-associated protein 1) ranked high on the list. Immunofluorescence staining suggested that Trap1 and ApoBDS-1 colocalized in THP-1 cells. Surface plasma resonance analysis showed that ApoBDS-1 interacted with Trap1 with a medium affinity ($KD \pm SEM = 28,77 \pm 1,443 \mu M$). We further identified that 5 amino acids at c-terminus of ApoBDS-1 are crucial for the binding with Trap1, confirming a sequence dependent specific interaction between ApoBDS1 and Trap1. Cell culture experiments showed that ApoBDS-1 induced cytokine secretion and reactive oxygen species can be inhibited by geldanamycin, an inhibitor of Trap1. Knocking down of Trap1 using specific shRNA also suppressed the ApoBDS-1 induced IL-1 β production. In summary, we identified Trap1 as an ApoBDS-1 interacting protein that mediates ApoBDS-1 induced proinflammatory response.

Author contribution: Yajuan participated in formulation of the hypothesis and experimental design, performed all the far western blot, all the 2-D electrophoresis, immunofluorescence staining for Trap1 and ApoBDS-1 and mitochondria in cells and plaque sections, cell culture experiments to knock down NLRP3 and NLRP1 and inhibitor experiments using Geldanamycin, all the ELISAs to measure IL-1 β and other molecules in supernatants, Western blot and qPCR to measure Trap1, all the Biacore surface plasma resonance analysis, flow cytometry to detect ROS, and analyzed all the data except for mass spectrometry and part of 2-D experiment. Yajuan proposed to investigate the role of Trap1 in ApoBDS-1 induced proinflammatory responses. Yajuan and Zhong-qun interpreted the data and Yajuan wrote the manuscript together with Zhong-qun.

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