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**NOVEL IMMUNOLOGIC CELLULAR
MECHANISMS IN ATHEROSCLEROSIS AND
POTENTIAL THERAPEUTIC IMPLICATIONS**

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Novel Immunologic Cellular Mechanisms in Atherosclerosis and Potential Therapeutic Implications

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ABSTRACT

Atherosclerosis, an arterial ailment, is a major cause of cardiovascular diseases (CVD), which cause more than 17 million deaths each year and the number is expected to rise. In recent years atherosclerosis has been shown to be an inflammatory condition involving activated immunocompetent cells, including T-cells, macrophages and dendritic cells (DC), but the mechanism by which these cells are activated remains to be elucidated in detail. Treatment of atherosclerosis is still not satisfactory, primarily due to the complex underlying mechanisms, especially with respect to inflammation and immunity. An additional characteristic of atherosclerosis is the accumulation of dead cells in a necrotic core in the plaques, as well as of oxidized forms of low density lipoprotein (Ox-LDL). Interestingly, the prevalence of atherosclerotic plaques and CVD are elevated among individuals with systemic inflammatory diseases, such as systemic lupus erythematosus (SLE).

These studies focused on the responses of major immunocompetent cells, such as T-cells, DC and macrophages to potential antigens, including heat shock protein (HSP) 60 and 90, phosphorylcholine (PC) and malondialdehyde (MDA), of which the latter two are components of Ox-LDL. Such investigations are related to potential links between the prototypical autoimmune disease SLE and CVD, as well as the development of novel therapies against atherosclerosis.

For these purposes, we examined peripheral blood cells from healthy donors and patients with SLE, as well as cells obtained from human atherosclerotic plaques in connection with operations for CVD. We also studied a well-characterized cohort of SLE-patients, SLEVIC.

Overall, we found that antibodies against phosphorylcholine (PC) and MDA were correlated with a lower prevalence of atherosclerosis among patients with SLE. Potential mechanisms involve enhanced uptake of apoptotic cells and a reduction in oxidative stress. Furthermore, anti-PC antibodies promoted polarization of T-reg cells, which may protect against both SLE and atherosclerosis. Surprisingly, production of these antibodies was dependent on T-cells.

HSP60 and HSP90 exerted pro-inflammatory effects on DCs and DCs stimulated in this manner induced pro-inflammatory activation of T-cells obtained from human peripheral blood and atherosclerotic plaques. Expression of HSP60 was induced by OxLDL.

A conjugate of MDA with human serum albumin (MDA-HSA) promoted pro-inflammatory activation of DCs and, subsequently, of T-cells obtained from human peripheral blood and atherosclerotic plaques via these DCs. Importantly, MDA-HSA also activated such T-cells directly. Both MDA-HSA itself and T-cells exposed to MDA-HSA promoted polarization of pro-inflammatory M1 macrophages. Annexin A5 inhibited the

pro-inflammatory effects of HSP60 (in line with our previous identification of this substance as potentially protective against atherosclerosis) and anti-MDA antibodies or an inhibitor of mitochondrial production of reactive oxygen species (ROS) attenuated the activation of T-cells by MDA-HSA.

Taken together, immunity plays an important role in connection with atherosclerosis and atherosclerotic plaques. Potential links between autoimmunity and atherosclerosis were identified. In particular, HSP60 and MDA may be important activators of immune cells in connection with atherosclerosis and, importantly, antibodies against PC and MDA, Annexin A5 and inhibitors of ROS could prove to be of value for prevention of and/or therapy against atherosclerosis.

LIST OF SCIENTIFIC PAPERS

- I. Rahman M, Sing S, Golabkesh Z, Fiskesund R, Gustafsson T, Jogestrand T, Frostegård AG, Hafström I, Liu A, Frostegård J IgM antibodies against malondialdehyde and phosphorylcholine are together strong protection markers for atherosclerosis in systemic lupus erythematosus: Regulation and underlying mechanisms. Clin Immunol. 2016 May;166-167:27-37.
- II. Mizanur Rahman, Johnny Steuer, Peter Gillgren, Assim Hayderi, Anquan Liu, and Johan Frostegård. Induction of Dendritic Cell–Mediated Activation of T Cells from Atherosclerotic Plaques by Human Heat Shock Protein 60. J Am Heart Assoc. 2017 Nov; 6(11).
- III. Sun J, Lundström SL, Zhang B, Zubarev RA, Steuer J, Gillgren P, Rahman M, Ajeganova S, Liu A, Frostegård J. IgM antibodies against phosphorylcholine promote polarization of T regulatory cells from patients with atherosclerotic plaques, systemic lupus erythematosus and healthy donors. Atherosclerosis. 2018 Jan;268:36-48.
- IV. Mizanur Rahman, Johnny Steuer, Peter Gillgren, Ákos Végvári, Anquan Liu and Johan Frostegård. Malondialdehyde-conjugated with albumin induces a pro-inflammatory activation of T cells from human atherosclerotic plaques through both a direct and a dendritic cell-mediated mechanism. (Manuscript).

Related paper (Not included in the thesis)

- I. Thiagarajan D, Frostegård AG, Singh S, Rahman M, Liu A, Vikström M, Leander K, Gigante B, Hellenius ML, Zhang B, Zubarev RA, de Faire U, Lundström SL, Frostegård J. Human IgM Antibodies to Malondialdehyde Conjugated With Albumin Are Negatively Associated With Cardiovascular Disease Among 60-Year-Olds. J Am Heart Assoc. 2016 Dec 20;5(12).

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LIST OF ABBREVIATIONS

CANTOS	Canacinumab anti-inflammatory thrombosis outcome study
CVD	Cardio vascular disease
CD	Cluster of differentiation
DAMP	Danger associated molecular pattern
DC	Dendritic cell
ELISA	Enzyme linked immunosorbent assay
ELISPOT	Enzyme linked immunosorbent spot
FACS	Fluorescence absorbance cell sorting
FOX-P3	Forkhead box –P3
HDL	High density lipoprotein
HLA-II	Human leukocyte antigen II
HSA	Human serum albumin
HSP	Heat shock protein
MAPK	Mitogen activated protein kinase
MDA	Malondialdehyde
MI	Myocardial infarction
MS	Mass spectrometry
Ox-LDL	Oxidized –low density lipoprotein
PAMP	Pathogen associated molecular pattern
PBMC	Pepripheral blood mononuclear cell
PC	Phosphorylcholine
PCSK9	Proprotein convertase subtilisin/kexin type 9
PRR	Pattern recognition receptor
ROS	Reactive oxygen species
RORC	RAR related orphan receptor C
SiRNA	Small interfering ribonucleic acid

ShRNA	Short hairpin ribonucleic acid
SLE	Systemic lupus erythematosus
Tbet	T-box transcription factor
Th	T helper
T-reg	T regulatory cell

1. Introduction

Atherosclerosis, start with the formation of lesions in the arterial wall, similar to those present in mummies more than 1000 years old¹. Already in the 19th century, Rudlof Virchow and Carl von Rokitansky described cellular inflammatory changes, such as leukocyte diapedesis in the atherosclerosis vessel wall, although Rokitansky thought these changes were secondary, whereas Virchow proposed that this inflammation was a primary cause of disease². In 1910 Windaus demonstrated the presence of calcified and connective tissue in atherosclerotic plaque and three years later Anitschkow and Chaltow reported that a cholesterol-rich diet is a risk factor for atherosclerosis². The role played by inflammation in atherosclerosis, is now extensively documented²⁻⁴.

Inflammatory diseases are primarily characterized by immunological changes⁴. Inflammation is a body's defensive system but now it is well known that chronic inflammation can cause disease or associated with disease, e.g., cardiovascular-disease (CVD)^{5,6}. The prevalence of chronic inflammatory diseases has risen during the past few decades but reason not yet clearly understood. Association between inflammation and diseases can be explainable only when the causes of the disease are the dysregulation of inflammatory response⁴. As revealed by epidemiological and clinical studies, atherosclerosis is in the turn the major cause of CVD, although the exact relationship or precise chain of events remain to be elucidated^{7,8}. Atherosclerosis is the major cause of CVD^{6,9}, where inflammation plays a role in atherogenesis¹⁰. The ischemic heart diseases, stroke and hypertension very often caused by atherosclerosis¹¹, were not a serious burden until the beginning of the 20th century, but it has been long now that these became a major health problem in Western countries, such as Europe or US¹². These inflammatory diseases are the leading cause of mortality in the Western world^{12,13}. In Europe, cardiovascular mortality is twice that from cancers¹⁴, and CVD are also becoming a problem in developing countries^{12,15}. It has been estimated that each year 17.3 million die from both ischemic heart disease and stroke. Recent data shows death from CVD in the year 2013 was 31.5% of total deaths, and expected to increasae^{16,17}.

The many risk factors for atherosclerosis or CVD are classified as classic or traditional¹⁸, non-traditional or novel¹⁹, modifiable²⁰ and non-modifiable²¹. Atherosclerotic CVD include age, sex, cigarettes smoking, diabetes²² melitus, hypertension, dyslipidemia, obesity and inherited risk factors^{22,23}. Although used for generalized evaluation, it may be inappropriate to apply this factors in the assessment of any individual patient²³. Non-traditional risk factors, some still controversial include genetic variation, malnutrition, vascular calcification, thrombosis markers, platelet-related factors, lipid-related factors, inflammation, homocysteine, circulating DNA and oxidative stress, and have not been examined as extensively as traditional risk factors^{23,24}. Certain other diseases, including systemic lupus erythematosus, chronic kidney diseases, arthritis, vasculitides, human

immunodeficiency virus and malignancy, as well as heavy alcohol consumption have also been proposed to enhance the risk for premature atherosclerosis²³. Lifestyle can be considered as modifiable risk factors whereas sex, age and family history are considered as non-modifiable risk factors^{20, 21}. Most of these risk factors are related in one way or another to inflammation and thus body's immune system can play diverse functions.

1.1 The immune system

Immunity is the body's defensive system against infections, diseases or unwanted particles²⁵. Immunity is a system or network of cells, tissues or organs. In general, foreign particles or microorganisms induce the immune response. The particles or molecules capable of inducing an immune response are called antigen. The antigen can be a protein, lipid, phospholipid or polysaccharide. Certain small molecules called hapten, which cannot themselves induce immunity but in conjugation with a carrier (protein, carbohydrate, lipid), act as antigen²⁶. The two types of immune responses are referred to as innate and adaptive^{27, 28}. Innate immunity, the body's first line of defense, is non-specific and short term; whereas the adaptive immune system is specific. A variety of cells participate together and secrete products such as cytokines, chemokines or antibodies.

The immune cells (white blood cells) include B, T, dendritic cells (DCs), macrophages and neutrophils. The T-cells that are produced and mature in the thymus, where they are taught to detect non-self-antigen, are mainly of two types, i.e., helper T-cells, and cytotoxic T-cells. Cytotoxic T-cells, also called killer T-cells, can kill the infected cells or cells that express certain antigens. Helper T-cells help B-cells to produce antibodies against the antigen and also, help cytotoxic T-cells to kill the affected cells and activate macrophages and DCs. These subset of T-cells are characterized by unique markers CD8⁺ (cytotoxic T cell) or CD4⁺ (helper T cell).

CD4⁺ T-cells are divided further into different subtypes, including Th1, Th2, Th17, and T-regulatory(T-reg) cells. Th1 and Th17 cells are pro-inflammatory whereas Th2 are anti-inflammatory. T-regs suppress pro-inflammatory responses. DCs are the best-known antigen presenting cells, main function is to present antigen to T-cells and thereby activate the immune system, so that their maturation is a key aspect of immune regulation. Macrophages phagocytize infectious agents, dead cells and the body's unwanted debris and also act as antigen-presenting cells.

Adaptive immunity is mediated either by cells or antibodies (so called humoral immunity). B-cells producing antibodies, while also acting as antigen-presenting cells. They interact with T-cells through antigen and produce antibodies, which is T-cell dependent, but also can produce antibodies without the help of T-cells, so called T-independent antibodies, referred to as TI5 antibodies. T-independent antibodies are mainly natural antibodies. Both innate and adaptive immunity can under certain circumstances, lead to inflammation and autoimmunity. After the anatomical barrier, inflammation is the first response of the

immune system. Short-term inflammation is called acute and a long-lasting inflammation is called chronic inflammation ⁵.

1.2 Inflammation: cause, and consequences

At the tissue level, inflammation mainly develops through steps of redness, swelling heat, and pain in response to microbial or non-microbial stimuli (Table-1). Certain non-microbial endogenous inducers such as tissue injury or damage link to microbial inducers. Depending on the location and type of stimulus, the inflammatory response can vary but certain steps are always involved: 1) recognition of the stimulus by pattern recognition receptors (PRRs); 2) activation of inflammatory pathway; 3) release of inflammatory markers; and 4) recruitment of inflammatory cells to the site of injury²⁹.

Acute inflammation results in healing or repair of tissue damage whereas in attempt to repair, chronic inflammation may lead to the tissues destruction and thus disease. In addition to infection, tissue injury or destruction causes inflammation, but depending on circumstances effect can vary³⁰. The two main phases of acute inflammation are referred to as initiation and resolution³¹. Immune cells recruited to the site of injury by cellular products such as prostaglandin and leukotrienes, begin their activities there, primarily clearance and repair of damaged tissue. Acute inflammation can sometimes develop into chronic inflammation, and both can continuously reinitiate and thus co-exist, but the main question is the frequency and fate of inflammation.

It remains unclear exactly when inflammation resolves and when it fails³². A globally enormous number of diseases including atherosclerosis, involve chronic or non-resolving inflammation³². For the inflammation initiation or regulation, 4 different factors are involved, defined as inducer, sensor, mediator, and effector. Some exogenous and endogenous inducers are presented in the table -1. Toxic substances, chemical, allergens or different types of foreign bodies are belonging to non-microbial exogenous inflammatory inducers³⁰. The pathogen-associated molecular pattern are recognized mostly by pattern recognition receptors (PRRs) and triggers inflammatory signals. Danger-associated molecular pattern (DAMPs) mainly non-microbial molecules but can be recognized also by PRRs. The different types of PRRs include toll-like receptors (TLRs), retinoic-inducible gene I-like receptors, C-type lectin receptors and NOD-like receptors. Myeloid differentiation factor-88 mediates the transmission of PAMPs and DAMPs from TLRs, activating nuclear intracellular signaling pathways, involve NF-kB and MAP kinase, and janus kinase-signal transducer and activation of transcription. Nuclear translocation of specific transcriptions factors triggers the inflammatory pathway²⁹.

Some non-microbial inducers of inflammation can also be detected by TLRs but in most cases their detectors are still unknown. Like exogenous inducers, endogenous inducers can play a major role in both acute or chronic inflammation. Inducers of chronic inflammation include crystals of monosodium urate, calcium pyrophosphate dihydrosate, and Ox-LDL,

or by-products of Ox-LDL and epitopes of these^{5, 33-35}. The inducers trigger or produce various mediators include components of complement, lipid mediators, cytokines, chemokines, and proteolytic enzymes. In addition, irregular production of reactive oxygen species (ROS) in response to inducers or mediators can initiate an endogenous inflammatory response.

Table-1. Microbial and non-microbial inducers of inflammation.

Microbial inducers	Non-microbial inducers	
<u>Bacterial lipopolysachraide</u> ³⁶ <u>Microbial peptides</u> ³⁷ <u>Glycolipid</u> ³⁷ <u>Bacterial short-chain fatty acids</u> ³⁸ <u>Bacterial Lipoteichoic acid</u> ³⁹ <u>DNA</u> ³⁹ <u>Fungi, virus</u> ⁴⁰	Exogenous	Endogenous
	Smoking ⁴¹ Air particles ⁴² Nano particles ⁴³ Nitrogen oxide ⁴⁴	Ox-LDL ⁴⁵ Fatty acids ⁴⁶ Self-DNA ⁴⁷ ROS ⁴⁸

1.3 ROS and inflammation

By products of aerobic metabolisms⁴⁹, including (O₂⁻) superoxide, (OH⁻) hydroxyl radical and (H₂O₂) hydrogen peroxide are classified as ROS and react with biological molecules to produce results that are usually deleterious. ROS production is associated with oxidative stress and may damage DNA, lipids or proteins, induce cellular dysfunction and thus contribute to inflammation⁴⁸. Indeed, metabolic and inflammatory diseases, including atherosclerosis, are associated with excessive ROS production or imbalance in redox pathways. At the same time normal biological processes are also regulated by ROS⁵⁰.

Inducers often stimulate inflammatory signaling via ROS. NF-kB signaling plays a vital role in inflammation and has also been reported to be involved in insulin resistance associated with obesity and atherosclerosis. In response to inflammatory inducers, ROS activates NF-kB signaling in a manner dependent on the location and/or amount and nature of the ROS.

ROS are generated in mitochondria, cytoplasm, and peroxisome. Mitochondrial H₂O₂ is involved in NF-kB activation of endothelial cells in rat arteries⁵¹ and, furthermore, activation of NF-kB by hypoxia was abolished by inhibiting mitochondrial ROS⁵². It is assumed that secondary products of ROS can also play a role in inflammatory signaling. Lipid peroxidation generates various products e.g., oxidized phospholipid, which can activate NF-kB or other inflammatory signaling and also regenerate ROS.

It is still not clear whether secondary ROS is protective or provoke the inflammatory response, this probably depends on the amount of ROS and cell and/or nature of the stressor. However, in the case of atherosclerosis, ROS or oxidative stress have been linked to certain endogenous inflammatory inducers⁵³. Endogenous inducers play a major role in atherosclerosis. In atherosclerosis, Ox-LDL derived lipid by-products considered as the main signaling initiator or inflammatory inducer⁴⁵.

1.4 Inflammatory inducers or oxidation-specific epitopes in Atherosclerosis

Native LDL deposited in the artery can be modified/oxidized to yield a set of complex products^{54, 55}, including free and esterified fatty acids, aldehydes such as malondialdehyde, oxidized phospholipids, and protein carbonyls, among many others depending on the type and duration of oxidation⁵⁴. It has been proposed that Ox-LDL contains 600 molecules of free cholesterol, 700 phospholipids, 1600 cholesteryl esters, 185 triglycerides and one molecule of Apo lipoprotein B100⁵⁵. Specific oxidized epitopes of LDL constitute a danger-associated molecular pattern (DAMP)⁵⁶, and are recognized by pattern recognition receptors⁵⁷. It is possible that not all Ox-LDL expose the same epitopes, which can result in varying consequences.

Phospholipids are among the epitopes or products of Ox-LD most studied in the context of inflammation⁵⁸. Phosphatidylcholine (PTC) is an important component in LDL⁵⁹, also it can be found in an outer layer of the cell membrane. In the chemical structure of PTC, the unsaturated fatty acid is also present in addition to a saturated fatty acid with glycerol. Glycerol also links to phosphorylcholine (PC) comprised of a phosphate group and a choline group. Another phospholipid, lysophosphatidylcholine (LPC) is a derivative of PTC. The LPC produced from PTC by removing an unsaturated fatty acid.

The unsaturated fatty acid region in PTC prone to oxidation and impaired biosynthesis of PTC is linked to atherosclerosis^{59, 60}, where impaired biosynthesis or lack of phosphatidylcholine attenuates atherosclerosis. On the other hand, one common head group of PTC, phosphorylcholine (PC), is anti-inflammatory according to several studies^{28, 61}, a curious finding in light of the fact that PTC is pro-inflammatory. Perhaps when PC is linked to platelet activating factor, protein, carbohydrate or some other molecule it acts in a different manner^{62, 63}. Another possibility is that the fatty acids moieties in PTC are involved in inflammatory properties^{64, 65}.

Malondialdehyde (MDA), a product of lipid peroxidation that induces oxidative stress, is involved in the development of various diseases including atherosclerosis^{55, 66, 67}. MDA can covalently modify proteins or lipids and thus generate more complex products. MDA acetaldehyde and MDA-modified LDL are produced by MDA. The MDA epitope of Ox-LDL modified in this manner can differ with respect to immunogenicity or reactivity to antibody⁶⁸⁻⁷¹. Understanding MDA-induced inflammation can be useful in connection with therapeutic approaches to atherosclerosis, but the underlying mechanism(s) remains to be

identified⁶⁶. In addition to Ox-LDL or their related products, some other molecules have been shown to be important inflammatory regulators in atherosclerosis. In this regard, heat shock proteins are under investigation.

1.5 Heat shock proteins in connection with atherosclerosis

Heat shock proteins (HSP), also known as stress proteins, are expressed in response to heat or toxic substances. On the basis of their molecular weight, the HSP is classified into HSP10, HSP40, HSP60, HSP70, HSP90 and so on⁷² and several studies suggest that they play a role in cardiovascular diseases⁷³⁻⁷⁶. All organisms express HSP60, and the prokaryotic and eukaryotic (including human) forms, display > 90% sequence homology and overall homology in protein and DNA level is more than 50 percent, even in some cases it is 70 percent. Microbial materials acquired by infections or vaccination induce immunity in humans against bacterial HSP60. Because of sequence homology, it can cause cross-reactivity with endothelial cells-produced autologous HSP60⁷⁷. Involvement of HSP60 in atherosclerosis was originally proposed by G. Wick and co-workers in 1990s on the basis of their findings of induction of atherosclerosis in animal models, induced by HSP65 immunization⁷⁸. Since then this proposal has been examined in other animal models as well as in humans. Antibodies against HSP60 may be pathogenic and thus associated with CVD and/or atherosclerosis^{79, 80}. Although the detailed adaptive response to this protein is not known and even less is known about the innate immune response. Our previous research has shown that in connection with an inflammatory Ox-LDL-induced response, heat shock proteins acts as co-stimulatory factors. T-cells activation by Ox-LDL was inhibited by silencing HSP60 and HSP90, suggesting their involvement in atherosclerosis progression and/or development⁸¹.

1.6 Initiation, progression and development of atherosclerosis

Atherosclerotic lesions (plaques) contain activated cells, in particular immune cells, which including macrophages, dendritic cells and T-cells as well as an abundance of dead cells. Although lipid deposition in the artery might initiate atherosclerosis, the initiation and progression of atherosclerosis remains poorly understood. It is widely thought that inflammation caused by Ox-LDL is an important initiator of atherosclerotic plaque formation⁸² that may develop in response to inflammation⁸³. However, even though inflammatory components in plaque development and rupture has been implicated⁸⁴ and inflammatory mediators of adaptive and innate immunity e.g., various receptors, cytokines, and chemokines appear to be involved in plaque formation and rupture, the detail mechanisms need to be elucidated⁸⁵. The artery is the blood vessel, carries out blood, nutrients and some other molecules including cholesterol. Lipoproteins that carry out cholesterol are mainly 2 types the low-density lipoprotein (LDL) and high-density lipoprotein(HDL)⁸⁶. In the LDL, the protein content is low, whereas HDL has high protein content. HDL considered being “good cholesterol”, whereas LDL is “bad cholesterol”. In

HDL, ApoA-1 is the major component, functioning as an anti-inflammatory factor in atherosclerosis.

It is generally believed that atherosclerosis is initiated by the deposition of the LDL in the artery. Tunica externa comprise of connective tissue, tunica media made up of smooth muscle cells and tunica intima is made up of endothelial cells. During transportation, some of the circulating LDL attached to the artery wall and it is probably the endothelial cells facilitate the entry of LDL into the vessel intima, where it is oxidized or modified enzymatically.

Ox-LDL is then aggregate there and phagocytized by macrophages. Irregular uptake of Ox-LDL leading to the formation of foam cell and thus plaque progression (fig:1). The composition of plaques and thereby their stability differ⁸⁷. Depending on this composition or type, plaque can be more unstable or vulnerable, these plaques are more prone to rupture^{84, 87, 88}. The first plaque rupture was reported in 1844.

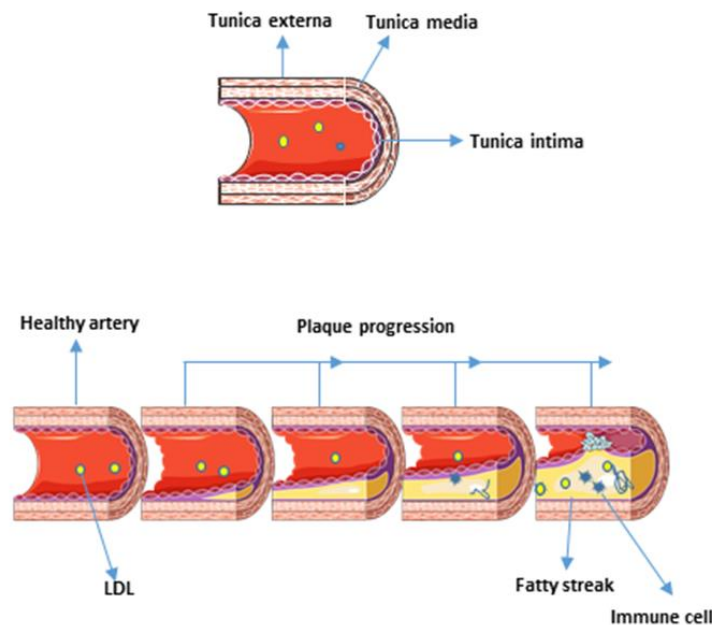


Fig-1: A healthy and an atherosclerotic artery.

On the other hand, HDL or Apo-A1 is athero-protective in several ways, mainly by inhibiting the early stage of atherosclerosis, suppressing adhesion molecules; inhibiting secretion of inflammatory cytokines⁸⁹ or chemokines⁹⁰; as well as attenuating LDL oxidation⁹¹; and influencing monocyte chemotaxis⁹⁰.

HDL may also protect later on by promoting cholesterol efflux from foam cells⁹², but this has not been much investigated the role of HDL in late stage of atherosclerosis.

The concern about the HDL recently published⁹³. The predictive effect of HDL effect depends mainly on age, with more pronounced protection in young than older mice. Moreover, HDL did not reduce atherosclerosis or cardiovascular events in elderly patients.⁹³ Further characterization revealed that a deficiency in scavenger receptor-B1, HDL receptor, aggravates atherosclerosis, even in the presence of a higher level of HDL,⁹⁴ indicating the importance of the receptors on the cell surface. All the recent advances in cellular involvement highlight in to the understanding more in cellular biology of different immune cell types in atherogenesis.

1.7 Different types of immune cells and Atherosclerosis

As already mentioned, atherosclerosis is characterized by activated cell types including macrophages, DCs, T-cell and B-cell. These activated cells play inflammatory roles in atherosclerosis⁹⁵.

1.7.1 Macrophages

Macrophages clear Ox-LDL, a beneficial activity that can also exert adverse effects⁹⁶. Foam cells derived from macrophages that have taken up Ox-LDL, trigger an inflammatory response and can become inert and/or undergo apoptosis and/or necrosis. Macrophages are the most abundant cells in plaques, where they play a vital role in connection with atherosclerosis. Macrophage-secreted inflammatory molecules activate other cells types, and attract monocytes.

Among other activities, macrophages proliferate in atherosclerotic lesions, but number of macrophages that come from the recruitment of circulating monocytes and subsequent differentiation into macrophages is still a puzzle⁹⁷. These monocytes might be polarized into M1 macrophages that secrete pro-atherosclerotic cytokines and induce production of reactive species of oxygen and nitrogen. Another type, M2 macrophages also present in atherosclerotic plaques⁹⁸. In vitro and animal studies suggest that plaque inflammation is caused by M1 and resolved by the M2 macrophages also present in lessions⁹⁶. There are also some resident macrophages in the aorta.

The macrophage (macro=big, phage =eater) is well known as a phagocytic cell. Phagocytosis of apoptotic or dead cells in the context of atherosclerosis is not well explained, although such inhibits secretion of pro-inflammatory cytokines. Apoptosis or dead cells are one of the major characteristic features of atherosclerosis, and impaired phagocytosis has been proposed to make plaques more vulnerable to rupture. Although, the effect of phagocytosis in atherosclerosis is not well understood⁹⁹, but improved understanding of macrophage activation may help in the development of target-specific safer drugs¹⁰⁰.

1.7.2 Dendritic cell

With their dendron (the Greek word for tree)-like structure, dendritic cells (DCs) are the best known for antigen presentation, the initial step in adaptive immune responses¹⁰¹. These cells constitute a bridge between innate and adaptive immunity. DC originates in bone marrow from common progenitors and has three stages as defined precursors, immature and mature stage. Precursors DCs leave the bone marrow and circulate in the bloodstream for a different time and develop into the immature stage in several ways. Tissue monocytes can differentiate into DCs and play an important role in immunity¹⁰². According to the origin and functional disposition, DCs can be sub divided into myeloid and plasmacytoid lymphoid DCs, suggested to be involved T-cell activation and T-cell tolerance, respectively¹⁰³⁻¹⁰⁶. The types of DCs including those derived from monocytes, in healthy and atherosclerotic mice differ. DCs are also present in healthy human arterial intima¹⁰⁷, with elevated numbers in atherosclerotic plaques¹⁰⁸. DCs may activate T-cells and thus destabilize plaque¹⁰⁸. In advanced plaques, most of the DCs are activated and cluster with T-cells, which suggest that DCs activation within arterial wall, and plaques growth and inflammation are associated with activated DCs in the arterial wall⁷⁶. On the other hand, tolerogenic DCs may be as athero-protective¹⁰⁹, improving plaque stability¹¹⁰. However, DCs phenotype in the context of clinical importance still unknown. The recruitment and proliferation of DCs in atherosclerotic lesions are lots remain to be characterized¹¹¹.

1.7.3 T-cell

T-cells are generated in the thymus, and the most abundant cells in atherosclerotic plaques¹¹² consisting of 10 % of the total cells¹⁰. Seventy percent of these T-cells are CD4⁺ T-cells and the remaining CD8⁺ T-cells¹¹³. Most of the CD4⁺ T-cells in plaques are pro-inflammatory Th1 T-cells, although Th2, Th17, T-reg, and NKT cells are also present in plaques. A different subset of T-cells has a different role in atherosclerosis, as for example Th1 and T-reg have the opposite effect on atherosclerosis. Th1 cells have been proposed to accelerate atherosclerosis whereas T-reg reduces inflammation and plaque formation.

In mice, deficiency in CD4⁺ T-cells provided athero-protection, whereas disease was accelerated by transfer of such T-cells^{114, 115}. CD8⁺ T cells may not play any important role in atherosclerosis, since a deficiency in these cells did not affect the size of atherosclerotic plaque¹¹⁵. However, in one study transfer of CD8⁺ T cells in mice was atheroprotective¹¹⁶. Th2 cells were believed to be protective, but this is now controversial. Deletion of IL-5 and IL-13, cytokines secreted by Th2 cells, accelerate the atherosclerosis^{117, 118} while IL-4 deficiency reduce severity of atherosclerosis^{119, 120}.

The newly identified IL-17-producing Th17 cells have been shown to be involved in atherogenesis, being present in the aortic sinus of Ldlr^{-/-} (low-density lipoprotein receptor-deficient mice) and ApoE^{-/-} mice¹²¹, IL-17 appears to be protective together with higher

levels of IL-10, but are pro atherogenic in the presence of higher levels of INF gamma cytokines.

Th17 cells produce IL-17, is pro-atherogenic and depletion of this cytokine is shown to be athero-protective¹²²⁻¹²⁵ but other studies showed slow progression of atherosclerosis by IL-17^{126, 127}. The complex pathway of plaque rupture is not yet completely understood but the role of T-cells has been implicated in this process¹⁰⁹.

1.7.4 B-cell

The B-cell, is developed in the bone marrow and finally matured in the spleen¹²⁸. Because of different types of antibodies production, their role in connection with atherosclerosis still controversial¹⁰. Depletion of B-cells augmented plaque formation whereas transfer of these cells attenuated development of atherosclerosis¹²⁹. In some other studies, an effect of B-cells deficiency¹³⁰ and beneficial role of IgM antibodies produced by B1 cells on vessel wall¹³¹ or protective role against atherosclerosis were reported¹³². Among the 5 different isotypes of antibodies, IgM can be generated without previous infection or immunization and are therefore called natural antibodies¹³³. Such natural antibodies are believed to be T-cell independent antibodies, while providing bridging between innate and adaptive immunity¹³⁴. Studies indicate that B1 cells are athero-protective and B2 cells are pro-atherogenic^{135, 136}.

Although the overall role of B-cells in connection with atherosclerosis appears to be protective but subpopulation may play different roles. CD19⁺ CD40⁺ B cells were negatively associated with stroke whereas CD19⁺ CD86⁺ B cells showed a positive association with stroke¹³⁷. Furthermore, B-cells switched or unswitched were protective against secondary CVD event¹³⁸.

However, anti-PC antibodies believed to be natural antibodies and has been shown to be negatively correlated with CVD¹³⁹. It has been proposed that these antibodies bind to PC epitopes in Ox-LDL as well as to apoptotic cells, and even to microorganisms exposing PC epitopes^{140, 141}. In animal study, showed immunization of PC-IgM reduces atherosclerosis progression¹⁴². In addition, IgM antibodies against MDA were recently shown by our group to be a marker of protection and potential regulator of CVD¹⁴³.

Levels of certain natural antibodies provide a link between atherosclerosis and autoimmune diseases¹⁴⁴.

1.8 The relationship between autoimmune and cardiovascular diseases

The concept of autoimmunity was introduced in 1900s. The main pathogenic factor, antibody involved in autoimmune disease was also discovered in the early 1900s but until 1940 the concept of autoimmunity was an over shadow¹⁴⁵. Waaler in 1940 discovered rheumatoid factor, later on, identified IgM type antibodies¹⁴⁶. In the past, autoimmune disease considered as rare diseases but today 3-5% of world's population suffers from

around 100 types of autoimmune diseases¹⁴⁷. The immune system has a sense for distinguishing self and non-self-particles but when this signal is misguided, it recognizes body's own cells or particles as antigen. Body's immunity against own particles leads to autoimmune diseases¹⁴⁸ and leads to organ damage and thus promotes different diseases (Fig: 2). Autoimmune diseases are also one of the major health problems with the significant cause of morbidity and mortality¹⁴⁹, even more, also a major complication is that these diseases have a link with CVD. Multiple rheumatic diseases including systemic lupus erythematosus (SLE) have a higher risk of developing CVD or atherosclerosis^{150, 151}.

SLE is characterized by the formation of pathogenic autoantibodies, including antibodies against DNA, also antibodies against cardiolipin considered as a disease marker, which can contribute to diseases complications or pathogenesis¹⁵². SLE is also called butterfly rash due to the visible butterfly-shaped inflamed area on the face. Increased risk of CVD in SLE was shown by Urowitz et al. for the first time in 1976¹⁵³. The risk of CVD in SLE is already well known, which is mostly shown by epidemiological association studies. The incidence of atherosclerosis in women with SLE in comparison to women with no SLE is 5-9 times higher¹⁵⁴. Moreover, the risk of myocardial infarction is 50 times higher in individuals with SLE in comparison to individuals without SLE¹⁵⁵ but the mechanism has not been elucidated. Increased atherosclerosis was also shown in the autoimmunity animal model^{156, 157}. TNF ligand or TNF receptor superfamily ligand was shown to be linked with defective clearance of apoptotic cells and atherosclerosis¹⁵⁶, as well as to the inflammation in the arterial wall associated with increased atherosclerosis¹⁵⁷. In this same study, plaque progression was associated with activation of T, B, and endothelial cells and also monocyte recruitment and accumulation of apoptotic debris.

The mechanism(s) by which autoimmunity elevates the risk of CVD has not yet been established. *In vitro*, *ex vivo*¹⁵⁸ or animal studies¹⁵⁹ could be more important for mechanistic studies of the link between autoimmunity and CVD. The link between these 2 diseases is firmly established but more research is necessary to understand the mechanism of cause¹⁵⁴. Although the animal model of lupus displays enhanced atherosclerosis¹⁶⁰ the animal model of SLE develops only a few features relevant to human disease¹⁵⁴.

1.9. An animal model of atherosclerosis

The animal model is becoming a useful tool for the study of atherosclerosis^{161, 162}. Several animal models have been developed in atherosclerosis studies, for an understanding of disease initiation and progression. In 1908, Ignatowski in a rabbit model reported atherogenesis¹⁶¹. Since then subsequent studies of atherosclerosis on the animal models are ongoing and also genetically modified animals are available for specific studies. Mice, rats, non-human primate, pigs, birds and some other animal models are used for atherosclerosis studies.

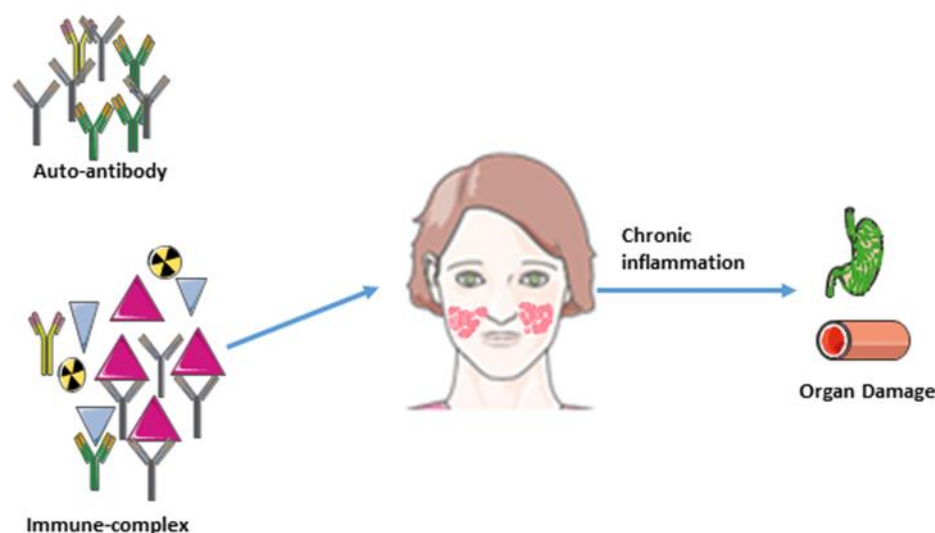


Figure: The General relationship between autoimmune and other disease. The immune system produces antibodies against the body's own components and these form immune complex that will trigger autoimmune diseases. Eventually, chronic inflammation damages to different organs and induces other diseases.

Most of the information on the underlying pathophysiological mechanism of diseases is provided by rodents, swine, and rabbits. Animal models are extensively used for cardiovascular or atherosclerosis studies but consideration of a proper animal model is important. One of the important features of an ideal or proper animal model is resemblance to human conditions. Despite extensive studies on animal models, each animal model for atherosclerosis has limitations. At present, mice are mostly used than another animal for atherosclerosis studies. Some of the advantages and limitations of mice models used in atherosclerosis research are listed in Table-2. The limitations or gap between *in vitro* and animal studies can be bridged by *ex vivo* studies¹⁶³ for atherosclerosis and thus cardiovascular research.

Table-2. Advantages and limitations of the animal models of atherosclerosis.

Advantages	Limitation/Disadvantages
<ul style="list-style-type: none"> Genetic manipulation Low cost Comparatively little time required for study Availability 	<ul style="list-style-type: none"> Induction of atherosclerosis in wild-type mice requires a toxic diet Circulating cholesterol HDL, not LDL Cardiovascular anatomy and physiology are different from those of humans Genetically modified mice needed

1.10 Cardiovascular research: A story of a multiple sides

Already mentioned that death rate in cardiovascular diseases is still higher but comparatively the rate declined significantly, even though prevalence has increased¹⁶⁴. In addition to the development of several areas in CVD, scientific research plays important role in preventing the death rate of CVD, although identification and development of proper therapy or medication are still challenging. The research from bench to bench side has lunched therapeutic to prevent atherosclerotic disorder or heart failure. Translational research is explained in many ways but it is generally accepted that the final target of translational research is the patient and to improve care¹⁶⁴. The traditional explanation of translational research simply identifying a new mechanism and discovery of potential therapeutic in a basic or experimental research setting, the approach validation in a clinical setting and introduce it in clinical practice. It is true that there are many success stories of CVD research but at the time unexpected difference has been observed from different research. As for example between genomic studies and animal models show the discrepancy. Hence introduction a new therapeutic becoming more unrealistic. In recent decades' developments in biological and genetic research have unraveled some of the pathological features of atherosclerosis, but there still is a gap between basic research and clinical practice that must be bridged¹⁶⁵.

The methodology employed in CVD research has not improved significantly in recent years. For example, miRNA research is attracting attention, but methodologies are not optimized. RT-PCR, droplet-based PCR and loop-based digital PCR microarray, RNA seq are used to quantify miRNA. However to reduce variability, normalization to a common set of miRNAs as well as an internal control and appropriate statistical analysis, are necessary¹⁶⁶. The most valuable question is what is the next best in the research of cardiovascular diseases¹⁶⁷. Different focuses on cardiovascular research topics include, cardiac fibrosis, metabolic dysfunction, signaling associated with heart failure, oxidative stress and RNA biology, as well as most importantly include novel therapies. Many such approaches appear promising, but the main limitation is irreproducibility and improper study design is also a major concern in the connection with cardiovascular research, still none of approaches could not reach on the stage to pay attention for refocusing. In clinical trial, those who experience adverse side-effects are often excluded and discontinue the medication¹⁶⁸. In this case, a clinical trial could be misinterpreting. One article published recently, screened 28636 publications but finally according to their inclusion criteria, included only 3396 in their analysis¹⁶⁹. One-third of the studies, has focused on therapeutics, mice or rat were mostly used, accounted to be 89.8% cases. One of the main issues in cardiovascular research is lack of reproducibility, in addition, discussed earlier that animal relevance to human physiology is not highly satisfied.

Although cardiovascular disease is considered primarily to affect men¹⁷⁰. Since 1984 more women than men have died from heart disease. The risk factors are likely to be the same

for men and women, but the prevalence and relative risk vary. For example, SLE is more common among women than men. Proper study design in a clinical trial must include both genders, as well as subjects of appropriate age and proper statistical design¹⁷⁰. As mentioned at the beginning of the introduction, the term vulnerable plaque also high risk or unstable plaque indicates a higher risk of disruption. However, this concept is based primarily on cross-sectional studies or animal models, which have their limitations. Many of the techniques of both invasive and non-invasive, used for detection of plaque vulnerability¹⁷¹, are not reliable, yet are being utilized more and more.

Basic research characterized plaque and a possible reason for plaque vulnerability as well as identified atherogenic and athero-protective function in atherosclerosis. Atherogenic and athero-protective function in atherosclerotic plaque leads to another new approach in cardiovascular research, the immunomodulation of the inflammation via active immunization or vaccination.

1.11 Vaccination approach in atherosclerosis

The vaccine is one of the major advances to eradicate the many infectious diseases. Vaccination against chronic inflammatory diseases, including atherosclerosis is being considered¹⁷². Indeed, research on atherosclerosis has led to the discovery of new aspects of adaptive and innate immunity. The idea that specific antigen plays a role in atherosclerosis, suggests anti-inflammatory treatment or raising protective antibodies. Several animal studies suggest that vaccine against atherosclerosis might be effective¹⁷³⁻¹⁷⁷.

Although Ox-LDL, HSP and Apo-B are generally considered to be potential immunogens in this connection, recent focus has been placed on PCSK9 as well¹⁷⁸. Most animal studies have shown that vaccination with specific immunogens reduced atherosclerosis at early stage or attenuated the progression of atherosclerosis, but some controversial results have also been obtained. For example, immunization with HSP65 promoted atherosclerosis. Vaccine can be administered mucosally, orally or nasally, and interestingly, mucosal administration of HSP65 appeared to be athero-protective while perinatal administration is atherogenic¹⁷⁹. Immunization with HSP60/65 peptides effective, increases the number of T-reg cells, but, surprisingly, detail study is missing since the od of the peptide P210 is not recognized by mouse MHC II¹⁷⁹. The potential risks of long-term immunization are not well known at present. In addition, many other challenges in concerning vaccination, against atherosclerosis remain, including formulation, route of administration, and the safety, stability and duration of immunization as well as appropriate selection of patients for testing.

The potential role of immunogen to atherosclerosis, needs to be addressed first, as does the cellular distinction or cellular diversity that plays an important role in inflammation.

Vaccination idea can be an avenue against atherosclerosis but, it is still a long journey to reach to the destination.

Much can be learned from patients' samples or data from patients¹⁷². Despite all these challenges, researchers are optimistic about vaccination or immunomodulation as prevention or treatment for atherosclerosis.

1.12 Treatment of CVD/atherosclerosis: present, future, and challenges

Many preventive strategies involving lifestyle intervention, and therapies have been designed to reduce the burden of CVD or atherosclerosis¹⁸⁰, including hypolipidemic drugs, anti-hypertensive therapy, inhibitor of the angiotensin-converting enzyme, blockers of the angiotensin-receptor, beta, calcium-channel and anti-platelet/anti-thrombotic therapies¹⁸¹. On the basis of clinical and public health concern and in consideration of healthy long lives, the burden of cardiovascular disease is the highest than another global challenge despite all technological development or advanced treatment¹⁸² in compare to past. Clinical complications and the unclear pathophysiology of this disease can be major reasons for the limited success to date.

Lipid-lowering drugs are more used for prevention of CVD^{183, 184}, some of these drugs mainly are of value for symptomatic but not asymptomatic cases¹⁸⁴. Statin, the most widely used of these drugs for more than 2 decades but has many clinical complications¹⁸⁵, and in 2012 Food and Drug Administration warned against the elevated risk of diabetes¹⁸⁶ and other side effects or limitations¹⁸⁷. Another limitation is cost in much of the world, statins, blood pressure-lowering agents even aspirin are still not affordable¹⁸⁸. It has also been estimated that the cost for the CVD will increase to 749 billion dollars in 2035.

Another approved class of lipid-lowering drugs class is inhibitor of PCSK9 (proprotein convertase subtilisin/kexin type 9) is now receiving more attention. It was first described in 2003¹⁸⁹ as a potential lipid-lowering therapeutic^{187, 190} with a different mechanism of action¹⁹⁰. Monoclonal anti-PCSK9 antibodies are at present in phase III clinical trials¹⁹⁰ and some of the trials already completed and results have already been reported in 2017¹⁹¹. Some questions that arise regarding treatment with monoclonal antibodies¹⁸⁵. Concern the risk of an immune reaction against the monoclonal antibodies, As well as adverse side effects such as target specific effect and cardiotoxicity¹⁹². Accordingly, use of aggressive statin or other lipid-lowering drugs still remain the treatment of choice for atherosclerosis. Although the mechanisms linking of inflammation to atherosclerosis have not yet been elucidate in detail, targeting inflammation has produced positive results. For example, a recent randomized trial focusing on canakinumab thrombosis outcome study (CANTOS), concluded that anti-inflammatory therapy can reduces mortality from CVD¹⁹³. At the same time, another recent investigation has raised a question about which anti-inflammatory drugs to use¹⁹⁴. IL-1 beta is thought to be one of the key regulators of the plaque formation, and CANTOS trial demonstrated treatment with anti-IL-1beta antibody is beneficial¹⁹³.

However, Gomez and colleagues found that IL-1 beta plays different role at different stages of atherosclerosis¹⁹⁴, suggesting alternative anti-inflammatory therapy¹⁹⁵.

The CANTOS trail involved participants 10,061 who had developed myocardial infarction and inflammation with blood levels of C-reactive proteins $>2 \mu\text{g/ml}$. Different doses (50, 150 or 300 mg) of canakinumab were administered and the primary outcomes included non-fatal stroke and non-fatal MI.

Mortality in the canakinumab and placebo groups did not differ significantly but the secondary effect was the composite of primary effect as well as urgent vascularization, and a significantly higher risk for fatal infection.

In addition, others anti-inflammatory therapies have been proposed and some are now in phase III clinical trial. Methotrexate targets a variety of cytokines and treatment of patients with arthritis with this drug lower their risk of CVD. However, application is limited only to arthritis patients group.

Another candidate, colchicine, has multiple targets, including neutrophils, macrophages T-cells, and mast cells, although, safety with long term use of this medication is still a concern. Its mechanism of action mimics in part that of canakinumab. Various studies have reported that colchicine reduces CVD but a meta-analysis showed colchicine trial was only for short term use to prevent primary or secondary event, whereas an effect on CVD requires longer use. The side effects reported, were minimal, but individuals with such inside effects were removed at an early stage from the study¹⁶⁸.

Furthermore, micro RNA-based therapy for CVD has been suggested. This is has been effective in an animal model but again, in a long time, effect and the efficiency of avoiding off-target still a question¹⁹⁶. The complex mechanism in atherosclerosis especially inflammatory response still much remains to identify¹⁹⁷. A deeper understanding of cellular involvement in atherosclerosis will be of key importance for development of novel drugs/treatment strategies³¹.

2. Aim of the study

My Ph.D. thesis works focus mainly on immune and/or inflammatory mechanisms involved in the initiation and/or progression of atherosclerosis. Specifically, the aim was to improve our understanding of the involvement of immunocompetent cells in atherosclerosis and the potential therapeutic actions against these pro-inflammatory effects. In addition, one of our major interests is the risk of atherosclerosis and CVD associated with systemic lupus erythematosus.

To gain insights into the inflammatory mechanisms underlying the formation and/or rupture of atherosclerotic plaques and link between autoimmunity and CVD or atherosclerosis, our specific aims were to examine the following –

1. The role of anti-PC and anti-MDA antibodies against atherosclerosis in patients with SLE
2. The mechanisms by which, HSP60 and HSP90 induced inflammatory mechanisms in connection with atherosclerosis
3. The role of anti-PC antibodies in the polarization of T-reg cells
4. The potential involvement of MDA in atherosclerosis

3. Methods

The methods and materials employed are described in detail in the papers on which the thesis is based and an overview is provided here.

3.1 Cell cultures

In the 18th century when the cell theory was first proposed, it was explained in both plants and in the animals at a similar time. Theodor Schwann described the cell as an fundamental unit of tissues and the formation of the cells is the main principle of organisms¹⁹⁸. Today, cell cultures are a widely used and important tool in scientific research, providing disease models and production of therapeutic protein.

Primary cell cultures hold numerous advantages over cell lines, being more physiologically relevant^{199, 200} but they are more sensitive in comparison to the cell lines and require extra care. We used primary cells from healthy individuals and patients who had developed atherosclerotic plaques, and suffered from SLE.

3.2 Cells from healthy donors

Buffy coats (concentrated blood with more number of cells with the reduced portion of plasma) collected from Karolinska University Hospital, Stockholm, Sweden were handled in accordance with rules and regulations of the hospital (ref. number- 1-485/2013). Peripheral blood mononuclear cells (PBMCs) were then separated on a Ficol-paque density gradient²⁰¹. In the study, B-cells, T-cells, DCs and macrophages were used for different study purposes. PBMCs derived monocytes were differentiated into DCs or macrophages by treatment with GM-CSF and IL-4 or GM-CSF alone respectively. B-cells and T-cells were separated from whole buffy coats or PBMCs with B or T-cell enrichment cocktails or positive selection kits. Cells from healthy donors were used in all of the studies.

3.3 Cells from patients with SLE

Study with SLE patients` cells was approved by the Karolinska University ethical committee and all the patients gave their informed consent. Whole blood from SLE patients with SLE were collected from Karolinska University Hospital; PBMCs were isolated as described above; and T-reg cells investigated in the third study.

3.4 Cells from atherosclerotic patients

Ex vivo study with cells from atherosclerotic plaques provides plenty of advantages, including the presence of all the important types of immune cells and the structure, can be used to explore lesion biology or atherogenesis²⁰². One difficulty working with plaques` cells, involves separation of the cells from the fatty streaks. Plaques were collected at Södersjukhuset Hospital, Stockholm, Sweden, from patients following endarterectomy of carotid or femoral arteries and blood was also collected from the same area undergoing surgery. PBMCs were isolated as above. The atherosclerotic plaques were cut into small pieces and cells separated after enzymatic treatment. T-cells from total plaque cells were

separated by T-cell positive selection kit and utilized in studies II, III and IV to characterize mechanisms of immune activation in atherosclerotic plaques.



Fig-3: Human atherosclerotic plaques from a carotid artery

3.5 Trans-well co-culture

Cell co-culture allows to examine cell-cell interactions²⁰³, which is important for atherosclerosis, where different cell types play important roles. We used trans-well co-cultures to study B-T-cells interactions in connection with the production of antibodies against PC and MDA (study I) and DC-T-cell interactions involvement in connection with the HSP60-induced DC-mediated activation T-cells. (study II).

3.6 Gene silencing

Gene is considered as a basic unit for the biological system. Genetic information is carried out through DNA or RNA and finally turn into protein²⁰⁴. Gene silencing has helped unraveled the function of individual genes for more than two decades now. The most common tools for gene silencing involves siRNA (small interfering RNA) and shRNA (short hairpin RNA), which have both similarities and difference²⁰⁵ but shRNA has certain advantages. Silencing with shRNA is more stable than siRNA, lasting for a year, whereas with siRNA mediated silencing, the silenced gene can begin to be expressed again after 48-72 hours. In addition, shRNA mediated silencing is more specific.

In my first study, shRNA was utilized to silence the CD40 and CD1d genes in B-cells and cultured for 6-7 days to study the involvement of these molecules in the production of anti-PC and anti-MDA IgM antibodies. In the fourth study the TLR4 gene was silenced in T cells with shRNA, control shRNA in both cases was used to confirm the specific function of the molecules. CD40, CD1d or TLR4 silencing was investigated by RT-qPCR or flow cytometry at the gene and the protein level respectively.

3.7 Blood serum and plasma

In biological research, serum and plasma are most commonly used materials²⁰⁶. The serum has a higher concentration of metabolites whereas plasma has all the constituents of blood, although measuring specific components from both plasma and serum give similar results²⁰⁶. We used serum to measure IgM antibodies against PC and MDA. In the case of atherosclerotic patient, we separated plasma, and the plasma was used for peptide modification by MDA from human serum albumin. Serum from patients with SLE was collected from Karolinska University Hospital, Stockholm, Sweden and kept under -80 C°. Blood plasma from atherosclerotic patients were collected at the same time of cell separation from blood and was preserved at the same condition as serum.

3.8 Ultrasound

Ultrasound (US) is one of the techniques for detection of plaque¹⁷¹. Although limited in sensitivity, is inexpensive, high resolution, user-friendly, no radiation, rapid and functional. A duplex scanner of 6 MHz was used to detect plaques in the left and right arteries of patients with SLE

3.9 ELISA/ELISPOT

ELISA developed by Peter and Eva²⁰⁷ and they published their first paper in 1971 on the ELISA measuring IgG antibodies²⁰⁸. Since then, ELISA procedures have been developed extensively, and highly sensitive measurement of various proteins, including antibodies and cytokines. In our study, we used ELISA for the detection of antibodies and cytokines in serum and the medium from cells. Antibodies against PC and MDA were measured with protocol developed in house and total IgM antibodies or cytokines with commercially available kits. ELISPOT which is similar to ELISA, measures antibody or cytokine secreting cells. In the first study, ELISPOT was utilized to detect anti-PC and-MDA producing B-cells.

3.10 Flow cytometry/Fluorescence absorbance cell sorting

This technique was invented in 1960 by Bonner, Sweet, Hullet, Herzenberg and machines for fluorescence absorbance cell sorting (FACS) become commercially available 10 years later. This highly sensitive technique can be used to analyze the expression of multiple proteins and has a major impact on research in biology, especially in the area of immunology²⁰⁹. We used flow cytometry for detection of different cell types and/or their activation as well as for the detection of inflammatory pathways, apoptosis, and generation of ROS. Flow cytometric data were analyzed with the FlowJo software.

3.11 RTqPCR

The polymerase chain reaction (PCR) has a revolutionary impact on the detection and quantification of individual gene. Mullis in 1984, invented PCR and it leads to developing real-time PCR²¹⁰. RT-qPCR was performed using TaqMan or Sybr green reagent as a master mix. The level of GAPDH gene expression was used as an internal control and the

level of gene expression calculated by $\Delta\Delta CT$ method. RT-qPCR was used to detect the expression level of transcription factors for T-cells (study II, III and IV). In addition, CD40 and CD1d gene expression in study I, and in study II expression of HSP60 in gene level were detected by RT-qPCR.

3.12 Mass spectrometry

In the early 20th century, mass spectrometry (MS) was used by physicists to determine an atom's mass, and later on expanded branch to chemistry. During the last two decades this tool has exerted a very great impact on biological research, allowing characterization of molecules and diagnosis of diseases. The principle was discovered by Joseph James Thomson and later Francis Weston designed mass spectrometer. MS widely used in the proteomic study. In my studies, we used LC-MS/MS analysis to characterize the peptide sequence of anti-PC antibodies (study III) and modification of peptides of human serum albumin by MDA in atherosclerotic patients' plasma or in vitro (study IV). Raw data were analyzed using DeMix- Q workflow (study III) Mascot v.2.4 database search engine (study IV).

3.13 Microscopy

The history of invention of the microscope is long but August Kohler's invention of the ultraviolet absorption microscope in 1904 led to the fluorescence microscope. Today's this an extremely important tool in biological research, allowing identification of single specific molecules. In study III, fluorescence microscope was used to detect the binding or localization of surface proteins, specifically the binding of IgM anti-PC antibodies to CD40 on dendritic cells. Microscopic images were analyzed with the Image J software. In addition, light microscope was used regularly to monitor the cellular morphology in all my projects.

3.14 Chromatographic column

Chromatography was first employed by Mikhail Tsvet in 1903s but a different version was developed from 1930-1940. Proteins differ in their size, shape and/or charge, and can be separated or purified by chromatographic methods. Column chromatography, which is most commonly used for this purpose, has the largest stationary phase ²¹¹. Although reproducibility is comparatively difficult by column chromatography but a cost-effective method. We used cation-exchange high-trap and gel chromatographic columns for the separation and purification of antibody specific for malondialdehyde (study I) or phosphorylcholine (study III). Concentration and/or purity of these antibodies was assessed by ELISA.

3.15 BrdU incorporation

BrdU, an analog of thymidine, is incorporated into specifically into newly synthesized DNA during the S phase of cell division and this property has been exploited since the 1980s to monitor cellular proliferation. This technique is relatively inexpensive and rapid,

as well as the working labeling concentration is not toxic. One limitation is that this can be incorporated into nuclear DNA during cell repair or cellular regeneration, but at a rate much lower than during cell divisions²¹². We utilized BrdU incorporation to assess the proliferation of dendritic or T-cells in response to HSP60 (study II) or MDA (study IV).

3.16 Statistical analysis

In biological research, experimental results have a risk for error, unreliability or uncertainty²¹³ but with statistical methods errors can be reduced. A statistical test of significance determines the effect of experimental achievement. A statistical significance is not the scientific significance but provides a justification to trust on the data, it mainly based on probability calculation²¹³.

Epidemiological data from a case-control study are analyzed by statistically be comparing the disease group (case) to the control group (without disease) to obtain odds ratio.

In study I, conditional logistic regression was performed to determine the prevalence of atherosclerosis in patients with SLE, using the SAS 9.4 software. In the regression model, relative risk takes into consideration factors that may influence the relationship between prevalence or frequency²¹⁴. In the experimental part, of all studies, the two tailed Student T-test was applied and p-value <0.05 was considered as statistically significant.

4. Results

Results from all the studies are summarized here. Each result is described with a figure or a table in each constituent papers.

4.1 Study I

The potential impact of anti-PC and anti-MDA antibodies has been reported previously in various cohort studies. Here, the first question in our study, was to identify the relationship between these antibodies and risk of CVD in patients with SLE. Logistic regression analysis revealed that anti-PC and anti-MDA IgM antibodies together protect against the progression of atherosclerosis; levels above the 60th percentile were associated with protection, while below the 33rd or 25th percentile the risk was higher. Subsequently, laboratory experiments were performed to elucidate the underlying mechanism(s). Uptake of apoptotic cells by macrophages was increased in presence of anti-PC or anti-MDA IgM antibodies. Moreover, induction of oxidative stress in PBMCs or in monocytes by MDA was inhibited by anti-MDA antibodies. The production of these antibodies regulated by T-cells. Thus, in the absence of T-cells, B-cells did not produce expected levels of either of these antibodies but in the presence of T-cells in a mix culture system produced an abundant amount. B and T-cells in trans-wells co-culture system did not produce the same amount of antibodies as like as mix B and T-cells culture, indicating B-T cells interactions are essential for such production.

Investigation of the molecular mechanism confirmed that production of these antibodies is dependent on interactions. To identify the specific molecules involved, CD40, CD1d and HLA-II were silenced or inhibited, and silencing or inhibition of CD40 or HLA-II molecules, the production of antibodies was attenuated, indicating that adaptive immunity plays a role in regulating the production of anti-PC and anti-MDA IgM antibodies.

4.2 Study II

The second study, designed to elucidate the potential role and mechanism of heat shock protein(HSP) 60 and HSP90 in immune activation in connection with atherosclerotic plaques, and underlying mechanism revealed that both proteins promote the activation and maturation of DCs. When HSP60 or HSP90 treated DCs were co-cultured with T-cells from the same individual, only the former activated-cells. HSP60 elicited hyperactive DCs that produced higher levels of pro-inflammatory cytokines, although a mild elevation of the level of the anti-inflammatory cytokine IL-10 was also observed. Similar results were found when DCs from the peripheral blood of atherosclerotic patients were exposed to HSP60 and subsequently co-cultured with T-cells obtained from the same individual's plaques.

To identify the type of T-cells activation, cytokines levels were determined and the levels of the Th1 and Th17-type cytokines INF gamma and IL-17, respectively found to be elevated in response to HSP60. This observation was supported by the upregulation of the transcription factors T-bet (for Th1) and RORc (for IL-17) by HSP60. Because of the low volume of cell culture supernatant, we could only measured INF-gamma in the case patients` and the findings were similar.

More detail study of T-cell activation revealed that HSP60 is a classical antigen, since HLA-II inhibiting antibodies prevented T-cell activation by DCs pre-exposed to HSP60. In addition, T-cell activation was inhibited when DCs exposed to HSP60 in presence of annexin A5. To investigate the mechanism, we measured HSP60 in the presence or absence of annexin A5 and the levels of HSP60 detection was reduced in the presence of annexin A5, indicating annexin A5 interacts with HSP60. Furthermore, annexin A5 inhibited Ox-LDL induced HSP60 production in dendritic cells.

4.3 Study III

In the third study the role of anti-PC IgM antibodies was investigated further. First, we were curious whether these antibodies induce T-reg cells derived from healthy donors.

When PBMCs from healthy donors were cultured in the presence of different concentrations of anti-PC IgM or anti-PC flow through (control antibodies) antibodies, the proportion of T-reg cells rose in response to anti-PC antibodies, especially with concentrations of 2.5 or 5 µg/ml, but 0.2µg, 1µg or 10µg/ml anti-PC antibodies did not influence T-reg polarization.

When we investigated T-reg and Th17 cells in CD4 T-cells from atherosclerotic plaques, because of limited number of plaque cells we had to choose a single concentration (5 µg/ml) of anti-PC antibodies or control antibodies. Again, anti-PC antibodies enhanced the number of T-reg cells but not Th17 cells.

In addition, in the peripheral blood of patients with SLE, the number of T-reg cells was lower than in control subjects matched for age and sex. In both groups anti-PC IgM induced T-reg cells polarization. In the control group, the number of Th17 was significantly lower but neither anti-PC or control antibodies did not alter this amount in either group.

Analysis of transcription factors supported these findings on T-reg and Th17 cells polarization. Anti-PC IgM antibodies upregulated the T-reg transcription factor Fox-P3, while the transcription factor RORC for Th17 was unaffected.

Furthermore, anti-PC IgM antibodies did not affect the proinflammatory cytokines TNF-alpha and IL-17 secretion, although, interestingly, control antibodies induced the secretion of both, indicating the pro-inflammatory effects of IgM antibodies those directed to other than PC.

When T-cells alone were stimulated with anti-PC antibodies, the proportion of T-reg cells was not influenced. We, therefore, decided to investigate dendritic cells to investigate as potential mediators of T-reg cells polarization induced by anti-PC antibodies. These antibodies inhibited activation or maturation of DCs, as reflected in expression of the markers CD80, CD86, CD83, CD11C, and HLA-II. These also attenuated the activation of NF-kB pathway.

Subsequent immunocytochemistry analysis revealed that anti-PC IgM antibodies binds to DCs in co-localized with CD40. De novo protein sequencing identified the more heterogenous variable region of control antibodies (Non anti-PC) in comparison to anti-PC antibodies. In comparison to control antibodies, peptides of lamda variable was less in anti-PC antibodies. Interestingly 7 peptides of HV chain in CDR2 and CDR3 regions were more abundant in the anti PC IgM antibodies in comparison to control antibodies.

4.4 Study IV

Here, we investigated activation of immune cells in response to MDA-HSA, with a focus on atherosclerosis. We stimulated PBMCs with MDA-HSA and observed activation of T-cells. Next, we stimulated DCs with MDA-HSA and found activation of DCs. The DCs activated by MDA-HSA, secreted pro-inflammatory but not anti-inflammatory cytokines. When MDA-HSA-stimulated DCs were co-cultured with T-cells, activation of the latter was not pronounced when PBMCs were exposed directly. Next, T-cells stimulated with MDA-HSA directly, promoted the activation, in a pattern, as like as PBMCs induced activation of T-cells.

Furthermore, we found that pro-inflammatory Th1 whereas anti-inflammatory Th2 cells were not activated in response to direct stimulation. Anti-MDA antibodies, inhibited the activation of T-cells presumably by preventing MDA-HSA from binding to or entering T-cells.

Moreover, MDA-HSA induced generation of ROS in T-cells and inhibition of this generation attenuated this activation, indicating mitochondrial ROS involved in MDA-HSA induced activation of T-cells. In response to stress, induced by MDA-HSA, DCs and in particular, T-cells secreted more HSP60.

Both MDA-HSA itself and T-cells treated with MDA-HSA, stimulated polarization of macrophages towards M1 phenotype. MDA-HSA with its danger associated molecular pattern, enhanced expression of the pattern recognition receptors TLR2 and TLR4 and, also activated the inflammatory pathway p38 MAPK in both T-cells and DCs.

To understand the immunogenic properties of MDA-HSA, we identified modified peptides in human serum albumin conjugated with MDA. There were 30 such peptides modified by MDA in vitro. Moreover, such modification of 9 peptides in the plasma of atherosclerosis patients were found, two of which were similar to those generated in vitro.

5. Discussion

The disease etiology of atherosclerosis is complex; it can vary individual to individual depending on the inflammatory risk markers. The study suggests the effects depend on the age, sex or genetic variation²¹⁵. Atherosclerosis starts with the damage to the endothelial cell, represents difference cellular and molecular responses²¹⁶.

My study focused on the immune cells in connection with atherosclerosis. Antibodies are produced by B cells, control body's unpleasant situation in case of infection or in presence of unwanted components. The antibodies itself in unregulated condition cause damage to bodies own system depending on the level of the antibodies²¹⁷. First, we studied antibodies against phosphorylcholine (PC) and malondialdehyde (MDA). There are, several reports on the adverse effect of MDA in both healthy and pathological situations^{55, 67, 218}, although the specific epitopes recognized by anti-MDA antibodies are not exactly identified.

Here we found that antibodies against both PC and MDA are together strongly associated with protection against the formation of atherosclerotic plaques and, of high levels against the prevalence of vulnerable plaques. A similar finding in a previous study, was observed in our lab that anti-PC together with antibodies against MDA-modified-LDL are protective against the development of atherosclerosis²¹⁹. In addition, lower levels (especially below 33rd and 25th percentile) were associated with a higher risk of atherosclerosis progression in patients with SLE.

The risk of CVD is higher in patients with SLE and indeed, Manzi and co-workers found¹⁵⁵ fifty fold elevation in this risk. Furthermore, the risk of atherosclerosis in patients with SLE is higher²²⁰ and plaque rupture and vulnerability are the major causes of CVD⁶. Vulnerable plaques most likely represent by echolucent plaques^{221, 222} and in SLE, the presence of the echolucent plaques are common¹⁴⁴.

Ox-LDL comprised of around three thousand molecules including phospholipids, and antibodies against some of their epitopes have been reported. Antibodies against Apo-B or MDA-modified-LDL are found to be as risk factors²²³⁻²²⁶ whereas protective effects were observed in others²²⁷.

Such contradictory results might reflect differences in the isotypes of the antibody or the nature of Ox-LDL. Generation of Ox-LDL is complex, can be modified in different ways, and expression of epitopes can vary and thus produced different types of antibodies. IgM antibodies are more likely to be protective. However, we found antibodies against PC and MDA to be protective. In previous study, suggested a potential mechanism for the protective role of anti-PC while anti-PC inhibited uptake of Ox-LDL²²⁸, further, death caused by lysophosphatidylcholine (LPC) was inhibited by anti PC antibodies²¹⁹.

Here, anti-PC and anti-MDA IgM promoted the uptake of apoptotic cells, which is more in line with earlier findings, that both increased uptake of apoptotic cells and reduce uptake of Ox-LDL attenuate inflammation.

Clearance of apoptotic cells is the major issue in patients with SLE, which cause a pro-inflammatory effect. Dead cells are one of the major characteristics of atherosclerotic plaques and defective clearance of apoptotic or dead cells has been implicated¹⁵⁶. Although, inhibition of Ox-LDL uptake by anti-PC antibodies is beneficial but Ox-LDL generate MDA during lipid peroxidation or release more MDA while circulating in the blood, and in this context, anti-MDA could be protective. Thus both antibodies could be considered as co-operative.

Comparatively, role of anti-MDA is not much uncovered. In autoimmune diseases, including SLE, oxidative stress and the exponents of MDA is increased, and thus increase the risk of different diseases including atherosclerosis. Here the finding MDA induced oxidative stress inhibition by anti-MDA suggests that anti-MDA balance the oxidative stress and thus could play important role in redox balance.

Anti-PC and anti-MDA antibodies are believed to be T-cell independent, so that one most surprising finding is that in the presence of T-cells, the levels of these antibodies increased enormously, also confirmed that they are HLA-II mediated, T-cell dependent.

Soon after we made this observation, at least two other studies provided support for our findings^{229 230}, in one, inhibiting or knocking out CD4 T-cells reduced the levels of these antibodies; and the other demonstrated that production of anti-PC antibodies is dependent on IL-5.

Combination antibody therapy of cancer appear promising²³¹ and multi-domain antibodies provide better protection against influenza infection²³². Anti-PC and anti-MDA antibodies together may offer a novel therapeutic approach against atherosclerosis. Although the level of these two antibodies regulated still remain to be elucidated, but our finding shows new avenue for future research¹⁵².

However, in addition to Ox-LDL and dead cells, other factors, including HSP, may be involved in the inflammation associated with atherosclerotic plaques. In the second study, we investigated the potential role of HSP90 and, especially HSP60. In this context, T-cell in atherosclerotic plaques has been suggested in several studies²³³ in particular T-cells specific for HSP60 epitopes were identified at an early stage of atherosclerotic plaques²³⁴, but activation triggering factors are essential to be identified.

In the context of activation of T-cells, DCs as an antigen presenting cells are important. DCs are present in human at early or late stage of plaques also it has been found more in vulnerable plaques^{107, 108}.

Co-localization of DCs and T-cells in plaques indicating the immune reaction²³⁵. In addition to human, DCs are also present in mouse at all stages of atherosclerotic plaques, participating in plaques pathogenesis^{236, 237}. Although in mice, DCs induce thrombogenesis, activation of T-cells and pro-inflammatory activity was reported²³⁸, the HSP60 mediated underlying immune mechanism especially in connection with atherosclerosis was not much clear.

A role for HSP, in the immune reactions associated with atherosclerosis has been indicated in several findings. For example, HSP60 stimulated DCs promote Th1 phenotype^{239, 240}, here, our study, shows the extension of those findings, especially in the context of atherosclerosis. The controversial role of IL-17 in connection with atherosclerosis, clarifies in some extent according to previous findings¹²¹. In presence of higher levels of IL-10, IL-17 may have protective roles by reducing INF-gamma whereas in the presence of higher level of INF-gamma, IL-17 elevated risk for progression of atherosclerosis and vulnerable plaques. In this context, HSP60 induced IL-17 and also could be a pro-atherogenic under circumstances, including with the higher levels of INF-gamma.

Indeed, antibodies against HSP65 are associated with atherosclerosis and hypertension. Our present demonstration indicates that the production of HSP60 antibodies is dependent on T-cells, in this context, could also indicate a link to autoimmunity.

Simultaneously, another study²⁴¹ reported autoimmunity to HSP60 in obese mice, with an association of the proliferation of T-cells and production of IgG1 and IgG2 antibodies against HSP60.

However, HSP60 might participate in pro-inflammatory activity and thus in atherosclerosis in several ways. Ox-LDL promotes pro-inflammatory activity by inducing HSP, and the immune action of Ox-LDL is dependent on HSP60/90⁸¹, although dependence on HLA-II was not shown.

We found that both HSP60 and HSP90 activate DCs but HSP90 stimulated DCs cannot activate T-cells. Possibly, peptides of HSP90 are not immunogenic or lose their immunogenicity after endocytosis by DCs. Even if HSP90 acts via another mechanism, both HSP60 and HSP90 are circulating in plasma, and have been proposed to be risk markers of CVD.

Annexin A5 is a plasma protein also has an anti-thrombotic effect. Previously our group identified anti-atherosclerosis properties of annexin A5 in both *in vivo* and *ex vivo*. *Ex vivo*, pro-inflammatory activation of T-cells by Ox-LDL was inhibited by annexin A5, and T-reg cells polarized in response to protein^{81, 242}. In the present study, this might reflect the complex structure of Ox-LDL, so it could explain from a different corner. Ox-LDL induced immune activation could involve HSP60 as one mechanism. Annexin A5 was anti-

inflammatory in both cases but no polarization of T-reg cells was observed in the present study.

Importantly, both studies confirmed that annexin A5 does not cause polarization of T-reg cells under healthy conditions. Here, annexin A5 showed indication of interacting with HSP60, and if so, might suppress HSP60-induced immune activation by competing for its receptors, including those on DCs. Anti-HSP60 antibodies as an immune complex are pro-inflammatory, in this context it also possible that annexin A5 inhibits binding of this antibodies to HSP60 and thus the pro-inflammatory effect.

In the third study, anti-PC IgM induced polarization of T-reg cells, so it will be interesting to study HSP60 induced immune activation in presence of anti-PC IgM antibodies.

Antibodies against PC could promote the polarization of T-reg cells, but not IL-17 T-cells in individuals with SLE and, sex and population-based control group. This finding shows significance in the fields of autoimmune disease as well atherosclerosis. T-reg cell has more importance in autoimmune diseases, the immunomodulatory function of T-reg cell control the immune activation but in an impaired situation when T-reg cell's function is interrupted can lead to autoimmune disease. Induction of T-reg cells in different diseases including CVD was suggested as therapeutic option²⁴³. The proportion of T-reg cells in the SLE group found to be less than control group²⁴⁴ also was confirmed by other study²⁴⁵. It is still not identified the reason for the reduced proportion of T-reg cells in SLE but may be potentially the affected circulatory environment in SLE. The proportion of T-reg in similar line in our experiment, the proportion of T-reg cells was less in patients with SLE was restored to normal by anti-PC IgM antibodies.

We also found that polarization of IL-17 and T-reg cells is the opposite in healthy individuals and those with SLE. As shown in first study, antibodies against PC and MDA protect against atherosclerosis and this may be the explanation with polarization of T-reg cells, suggesting multiple activities of anti-PC antibodies.

In addition, production of these antibodies was T-cell- dependent, showing the involvement of adaptive immunity, whereas the third project demonstrated that anti-PC antibodies are involved in controlling-adaptive immunity, perhaps creating a feedback loop.

Cytokines of the Th1²⁴⁶ and Th17^{247, 248} types are abundant in both advanced and vulnerable plaques. The regulation of Th17 and T-reg cells, is in general, negatively correlated, and in this context T-reg with a lower number could promote atherosclerosis.

In this manner, these later findings may be relevant to the first study, wherein patients with SLE, circulating levels of anti-PC antibodies and prevalence of atherosclerosis were negatively correlated. In combination with the second study, this also indicates an important role for DCs in connection with atherosclerosis.

Induction of T-reg cells polarization by anti-PC antibodies appeared to be dependent on other cells types, at least DCs. These antibodies on T-cells alone did not promote such polarization whereas PBMCs stimulated with these antibodies did. Anti-PC antibodies affected the maturation and activation of DCs, reduced basal inflammatory condition, which may explain these findings. Although CD40 on DCs, the potential binding site for anti-PC antibodies, more investigation remains to establish and identify additional potential site.

Since, antibodies other than those against PC do not exhibit similar properties, using de novo protein sequencing, we characterized their peptides. The peptides of variable region of control antibodies was more heterogenous, indicating anti-PC structure is less complex and perhaps more specific.

Although, we did not characterize sequence homology between anti-PC and anti-MDA antibodies, the anti-MDA antibodies probably act in a different manner, at least with respect to T-reg polarization, even though they were also a protective in the first study.

In our fourth study, anti-MDA antibodies inhibited pro-inflammatory activation of T-cells by MDA-HSA. Effects of MDA on different cell types in healthy and atherosclerotic individuals was investigated. The adverse effects of Ox-LDL are still not clear, and a concentration-dependent beneficial role has also been reported²⁴⁹. Its effects, could also depend on the nature of Ox-LDL, as well as the products of lipid peroxidation e.g., MDA.

Ox-LDL induced activation of T-cells from plaques confirmed in several studies^{81, 250}. In addition to peripheral blood T-cells, here, we studied also T-cells obtained from plaques, induced by MDA-HSA. Our results suggest that T-cells in peripheral blood or in plaques can be activated by MDA more strongly without DCs. This activation leads to pro-inflammatory differentiation into mainly Th1 type T cells, without cells proliferation upon direct activation, in line with the previous finding of Ox-LDL⁸¹. Activation of pro-inflammatory T-cells and DCs activation are considered to be a major cause of plaque rupture, as well as common phenomenon in vulnerable plaques^{108, 109}. Accordingly, our finding also reveals the factor responsible for plaque rupture and thus the progression of CVD.

The MDA, a danger associated molecular pattern, its presence in atherosclerotic plaques has long been known and it is considered to be a marker of stress in connection with various diseases, including atherosclerosis and SLE.

Immunization with MDA-modified-LDL in mice has been shown to be athero-protective, MDA can form adducts with different proteins, including serum albumin produced in the liver but most abundant protein in blood plasma²⁵¹. Here, we found that MDA modified peptides derived from HSA both *in vitro* and in the plasma of atherosclerotic patients, demonstrating immunogenicity. Interestingly, 2 of these peptides modified were similar in

both cases. The possibilities that MDA also modifies HSA and causes immune activation in healthy individuals as well cannot be excluded.

Both antibodies anti-PC and anti-MDA antibodies are protective and their production depend on T-cells, since among 2 epitopes of Ox-LDL, MDA is pro-inflammatory, we focused on these and especially T-cells activation. Moreover, Ox-LDL induces immune activation through HSP60, we, showed that HSP60 is a classical T-cell antigen.

MDA induced HSP60 more potently in T-cells than DCs derived from both blood and plaques. May be therefore, MDA-HSA-induced DCs-mediated activation of T-cell does not follow the same mechanism as Ox-LDL did, but MDA-HSA- induced secreted HSP60 by T-cells could be processed by DCs and thus can be recycled to T-cells as antigen. Since according to the second study, HSP60 induces pro-inflammatory T-cells, MDA could contribute to plaque inflammation in this manner.

In addition, HSP60 is induced by toxic or stressful conditions that elevate the levels of ROS, here we found that activation of T-cells by MDA-HSA is partly dependent on ROS, especially those generated in mitochondria, a novel insight, and thus may play role in cell death in plaques.

TLR acts as an agonist for many microbial or non-microbial epitopes, but in our study MDA-HSA was not recognized by at least TLR2 or TLR4 in the T-cells. Possibly MDA-HSA bind directly to membrane proteins on T-cells and/or enters T-cells though diffusion. In the case of DCs and macrophages, MDA-HSA can be recognized by CD36 on the cells surface but highly important question of recognition by T-cells remains to be identified.

Another important finding was the effect of MDA on macrophages. In connection with atherosclerosis, macrophages play key role at all stages. In general, M1 macrophages, considered to be more pro inflammatory, are associated with type Th1 cellular responses and induce proinflammatory cytokines; whereas M2 macrophages are considered to be involved in the repair process associated with Th2 cells.

TLR2 and TLR4 has been implicated in chronic inflammatory conditions, and inhibition of them has been proposed as a therapeutic approach to cardiovascular disease. Although, activation of T-cells by MDA-HSA was not dependent on them, induced the expression of both TLR2 and TLR4 in both DCs and T-cells. In this manner, MDA-HSA might accelerate inflammation in atherosclerosis plaques and promote CVD through innate immune reaction.

Again, it has been believed that all autoantibodies in autoimmune diseases, including SLE, are pathogenic factors but some antibodies appeared to be protective in connection with atherosclerosis¹⁵² and it is important to clarify which is which, among other reasons, therapeutic purposes. Our study, an unexpected finding of T-cell involvement in the

production of autoantibodies, suggests a role of adaptive immunity in the production of antibodies against product of lipid peroxidation. The different levels of such antibodies in different individuals, might reflect different numbers of CD4 T-cells and/or levels of pro-inflammatory cytokines. In addition, immunosuppressive role of T-reg is important function to control immune reaction against self-antigens.

The observation in our second study that HSP60 is a classical T-cell antigen, interesting in light of a report on autoimmune response against this protein ²⁴¹. In this regards, anti-PC IgM antibodies could control the adaptive response against HSP60. In our third study, anti-PC antibodies, interrupted both DCs maturation and the NF-kB inflammatory pathway. In this manner, HSP60 and HSP90 induced activation and maturation of DCs may be potentially limit by the anti-PC antibodies. Influence of anti-PC antibodies on HSP60 stimulated activation of T-cells remains to be identified.

Antibodies to MDA induced phagocytic activity, and inhibited MDA induced stress without influencing polarization of T-reg cells.

Our other study on a cohort of 60-year-olds revealed that anti-MDA IgM protects against atherosclerosis and lower the risk of CVD¹⁴³. This finding may reflect increased clearance of apoptotic cells in the first study. Apoptosis, mechanism still not fully uncovered but in the elderly the rate of apoptosis is relatively higher, reducing myocytes by 30% ²⁵². Perhaps due to, at least in part to a higher levels of oxidative stress.

However, reports ^{253 254} documented a correlation between the elevated levels of MDA IgG antibodies and disease activity in connection with arthritis or SLE, but no explanation provided. Possibly, in stress situation with the higher levels of MDA, antibodies increased, but it is important to identify the exact functional role and regulation of anti-MDA IgG antibodies in those condition. However, in our study MDA appeared to be more important component to induce pro-inflammatory immune cells activation.

Figure 4 based on our findings together, illustrates possible pro-inflammatory or anti-inflammatory mechanisms that may be involved in atherosclerosis prevalence or atherosclerosis.

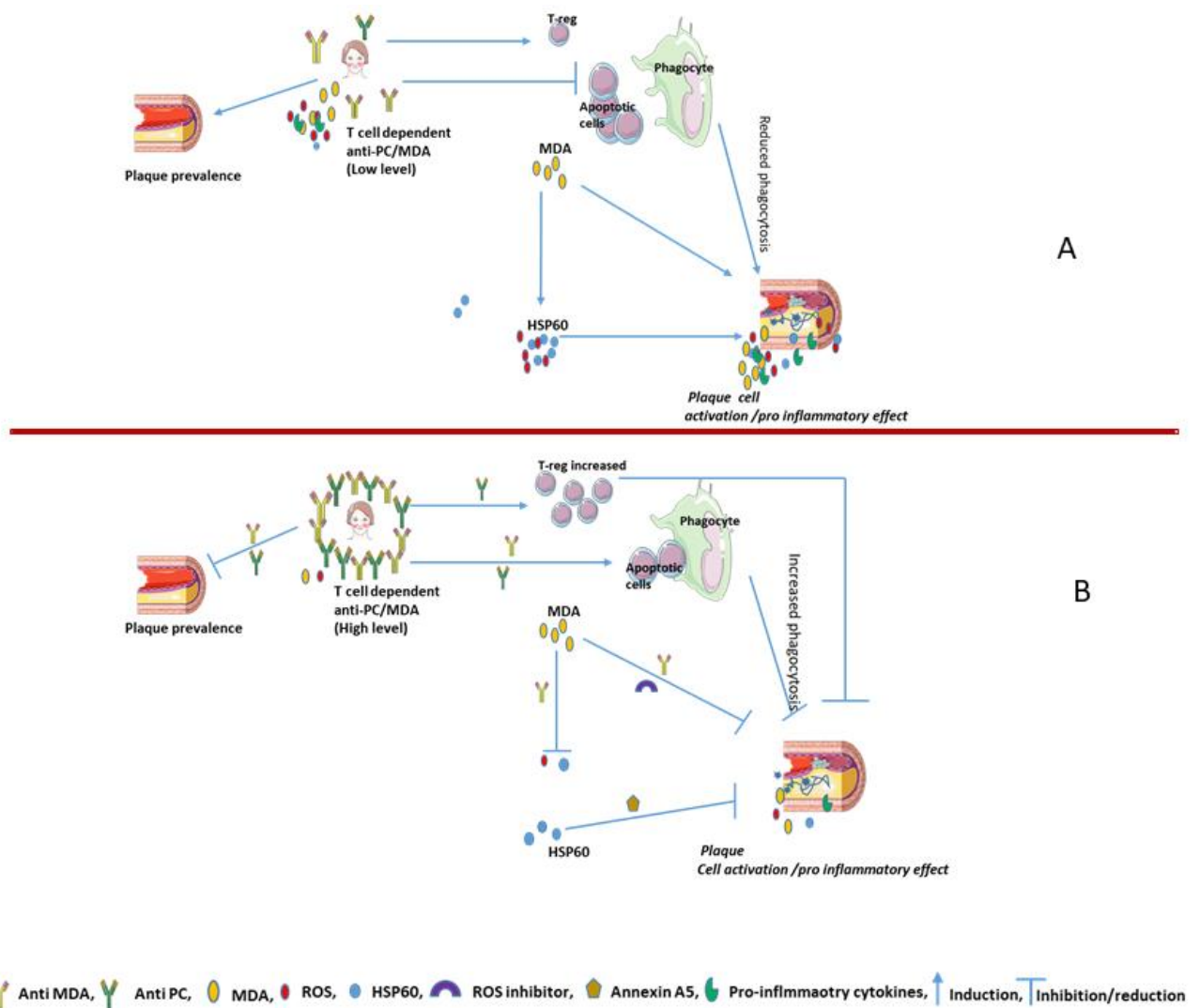


Fig-4: Inflammatory effect in the context of atherosclerosis in individuals with or without SLE and low (A) or high (B) levels of anti PC and anti MDA IgM antibodies, or with (A) or without (B) annexin A5.

6. Conclusion and future perspective

According to the latest update in 2018²⁵⁵, global mortality from CVD was 17.9 million in 2015,¹⁸⁸ and is expected to rise 23.6 million by 2030, with associated medical costs, estimated to be 749 billion dollars in 2035. Thus CVD accounted for 31% of all deaths and 45% from those noninfectious diseases.

Information gathering on diseases epidemiology, clinical, scientific basic research, and some other related data pointing mostly on the complexity of the mechanism of the disease, and in this context, cellular inflammation has receiving more attention.

Here, in my study, we show that anti-PC and anti-MDA IgM antibodies protects against CVD, and act against plaque inflammation. Protective role of these antibodies in autoimmune diseases, in connection with atherosclerosis, is necessary to investigate in larger cohort. Effect of pro-inflammatory cytokines in the regulation of anti-PC and anti-MDA antibodies may be interesting for investigation.

Furthermore, we identified HSP especially HSP60 and MDA as two important pro-inflammatory components in connection with atherosclerosis, and annexin A5 and anti-MDA antibodies and/or ROS are potential inhibitors respectively.

HSP60-annexin A5 interaction, further study with different method is important, which might will clarify whether they interact each other or compete for a binding site. In our study, the impact of HSP90 in connection with atherosclerosis, did not provide detail information, further investigation required.

Identifying MDA-modified immunogenic effect of certain other proteins such as Apo-B or collagen will be interesting in connection with atherosclerosis and/or in autoimmune diseases. MDA is pro-inflammatory in different ways; direct effect of activation of T-cells might be controlled by anti-MDA antibodies. Remaining concern on MDA induced activation of DCs and thus T-cells, possibly, anti-PC might be useful to suppress the MDA induced DCs activation primarily. Potential role of other immune cell types including neutrophil in connection with atherosclerosis may will reveal further information, since, Ox-LDL induced neutrophil extracellular trap formation already reported, in this context, may be MDA have possible effect on them.

Anti-PC and anti-MDA antibodies are protective in different ways and in this context, immunization against PC and/or PC and MDA might help to prevent CVD, although all other isotypes of these antibodies remains to be studied more.

Although several studies suggest anti-inflammatory properties of PC epitopes, detailed investigation of the role of PC in CVD is highly motivated. Investigation of possible correlation between annexin A5 and autoantibodies could provide valuable information.

However, others potential therapeutics such as inhibitor of IL1-beta or PCSK9 may still be considered in certain situation, e.g., depending on the disease context. These all potentials soldiers, including anti-PC and anti-MDA antibodies, ROS inhibitor and annexin A5 might fight unfavorable immune responses together or in appropriate manner.

However, it is general but again, proper study plan, inclusion of all-important factors in the study plan should be followed, reproducibility especially reliability is much important. Reliable and strong research should be our commitment for a constructive cardiovascular research.

My findings point a picture of immunologic cellular inflammation and potential therapeutic possibilities in connection with atherosclerosis. Further investigation studies including in vivo are necessary to confirm some of these findings.

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