IL-25 and B cells in atherosclerosis

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Abstract

Atherosclerosis is a chronic inflammatory disease of medium and large vessels of the body which is mainly characterized by the formation of lipid rich plaques within the arterial tissue. It is the most common cause of cardiovascular disease leading to clinical manifestations of coronary artery and cerebrovascular disease. Immune responses targeting modified low density lipoprotein (LDL) play an important role in atherosclerosis. Moreover, Th1 immune responses have inextricably been associated with disease progression while the role of Th17 and Th2 related immune responses is less clear.

In the present work we report that different B cell subsets could potentially be used as biomarkers for the prediction of future stroke events. Specifically, our findings demonstrate an association between high levels of CD19+CD40+ cells and a decreased risk of stroke while high levels of CD19+CD86+ B cells were associated with an increased risk.

Another main focus was the investigation of the role of IL-25 in atherosclerosis. In order to tackle this, the cytokine was both administered and blocked in hypercholesterolaemic apoE-/- mice. Interestingly, an atheroprotective effect of the cytokine was observed. Regarding exogenous IL-25 administration, the proposed mechanism of atheroprotection implicates the increase of IgM antibodies targeting phosphorylcholine (PC), a major epitope on oxidized LDL (oxLDL) via innate lymphoid cell type 2 (ILC2)-derived IL-5. We suggest that the essential trigger for this mechanism is the expansion of ILC2s.

Additionally, blockade of endogenous IL-25 at the onset of atherosclerosis led to increased plaque formation of unstable phenotype, accompanied by a Th1/Th17 shift of the cytokine balance in the spleen of the mice. The above mentioned findings suggest that signaling through IL-25 during early plaque formation possibly regulates protective immune mechanisms and that disruption of that signaling leads to the dominance of pro-inflammatory immune responses.

Due to the atheroprotective effect of IL-25 in mice we also investigated its effect on human peripheral blood mononuclear cells (hPBMCs). IL-25 was shown to dampen Th17 and Th1-related immune responses in hPBMCs while in the presence of oxLDL it dampens Th1 and promotes Th2-related immune responses. The above mentioned findings indicate that IL-25 could potentially have a protective role in human atherosclerosis.

Key words IL-25, B cells, ILC2s, IL-5, anti-PC IgM, atherosclerosis, stroke, cardiovascular events

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Ithaka

As you set out for Ithaka hope your road is a long one
full of adventure, full of discovery.
Laistrygonians, Cyclops, angry Poseidon don't be afraid of them
you'll never find things like that on your way
as long as you keep your thoughts raised high,
as long as a rare excitement stirs your spirit and your body.
Laistrygonians, Cyclops, wild Poseidon you won't encounter them
unless you bring them along inside your soul
unless your soul sets them up in front of you.

Hope your road is a long one.
May there be many summer mornings when with what pleasure, what joy,
you enter harbors you're seeing for the first time;
may you stop at Phoenician trading stations to buy fine things,
mother of pearl and coral, amber and ebony
sensual perfume of every kind, as many sensual perfumes as you can;
and may you visit many Egyptian cities
to learn and go on learning from their scholars.

Keep Ithaka always in your mind.
Arriving there is what you're destined for.
But don't hurry the journey at all.
Better if it lasts for years,
so you're old by the time you reach the island,

wealthy with all you've gained on the way,
not expecting Ithaka to make you rich.
Ithaka gave you the marvelous journey.
Without her you would not have set out.
She has nothing left to give you now.

And if you find her poor, Ithaka won't have fooled you.
Wise as you will have become, so full of experience
you'll have understood by then what these Ithakas mean.

Constantine P. Cavafy
(1863-1933)

Contents

Introduction

Atherosclerosis is nowadays the leading cause of death and morbidity worldwide [1].

Statins, mediating lipid lowering effects, since their discovery were proven to be a useful therapeutic tool but the necessity of finding new agents and more specialized therapies is on demand.

Besides the disease's lipid-related background, the scientific discoveries of the past few decades in the field of atherosclerosis revealed the great contribution of the immune system in the promotion as well as the resolution of the disease.

The present work is focused on the immune mechanisms mediated by interleukin-25 (IL-25), a novel cytokine the effects of which have not been previously tested in atherosclerosis. Another main focus, is the investigation of the implication of specific B cell subsets in the disease.

The reader will at first be introduced to the world of innate and adaptive immunity with the main focus drawn on atherosclerosis related-pathways but with small interventions from "immune-stories" coming from other diseases. Immunology is a universal language after all…

Further discussion on the methods that have been used as well as on the results obtained will follow. My hope is to make people understand the research conducted, critically reflect on it and my ultimate goal is -if possible- to motivate them.

☺ There is also a paragraph named "Science for everyone" describing the current findings in simple words and with the best of love that I can give for my "non-scientific" friends…

☺ Για τους φίλους μου που έχουν βρει άλλες χαρές στη ζωή εκτός από τις βιοϊατρικές επιστήμες τους παροτρύνω να διαβάσουν την περίληψη «Επιστήμη για όλους»...

Original papers

The thesis is based on the following papers:

I. Mantani PT, Ljungcrantz I, Andersson L, Alm R, Hedblad B, Björkbacka H, Nilsson J and Fredrikson GN. Circulating $CD40^+$ and $CD86^+$ B cell subsets demonstrate opposing associations with risk of stroke. *Arterioscler Thromb Vasc Biol*, **34**:211-218, 2014.

II. Mantani PT, Dunér P, Bengtsson E, Alm R, Ljungcrantz I, Söderberg I, Sundius L, To F, Nilsson J, Björkbacka H and Fredrikson GN. IL-25 inhibits atherosclerosis development in apoE deficient mice. *Submitted.*

III. Mantani PT*, Dunér P*, Bengtsson E, Alm R, Ljungcrantz I, Söderberg I, Sundius L, To F, Nilsson J, Björkbacka H and Fredrikson GN. IL-25 blockade aggravates atherosclerosis development in apoE deficient mice. *Manuscript.*

*Both authors contributed equally.

IV. Mantani PT, Vallejo Mínguez J, Ljungcrantz I, Nilsson J, Björkbacka H and Fredrikson GN. IL-25 reduces Th17 cells and inflammatory responses in human peripheral mononuclear cells. *Manuscript*.

Abbreviations

AAMs	Alternatively Activated Macrophages
ABCA1, ABCG1	ATP-binding cassette A1, G1
AF700	Alexa Fluor 700
AHR	Airway Hypersensitivity Response
APC	Allophycocyanin
APCs	Antigen Presenting Cells
apo	apolipoprotein B
BAL	Broncho-Alveolar Lavage
BCR	B cell receptor
CAD	Coronary Artery Disease
CCL	Chemokine (C-C motif) ligand
CCR	Chemokine (C-C motif) receptor
CD	Cluster of Differentiation
CD40L	CD40 ligand
CE	Cholesterol Esters
C/EBP	CCAAT-Enhancer-Binding Proteins
CETP	Cholesterol Ester Transfer Protein
CIA	Collagen Induced Arthritis
CM	Chylomicrons
CM	Control Medium
CMRs	CM remnants
CRTH2	Chemoattractant receptor TH2
CS	Contact Sensitivity
CTLA4	Cytotoxic T-Lymphocyte Antigen 4
CVD	Cardiovascular disease
CXCL	Chemokine (C-X-C motif) ligand
CXCR	Chemokine (C-X-C motif) receptor
DAMPs	Danger Associated Molecular Patterns
DCs	Dendritic Cells
EAE	Experimental Autoimmune Encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
EL	Endothelial Lipase
FACS	Fluorescence activated cell sorting
FALCs	Fat-Associated Lymphoid Clusters
FC	Flow Cytometry

FcεRI	High-affinity IgE receptor
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead box P3
GATA 3	GATA-binding protein 3
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
HDL	High Density Lipoprotein
HFD	High Fat Diet
HL	Hepatic Lipase
HOCL	Hypochlorous acid
ICAM-1	Intracellular cell adhesion molecule
ICOS	Inducible T-cell costimulator
IDL	Intermediate Density Lipoprotein
ID2	Inhibitor of DNA binding 2
Id3	Inhibitor of differentiation 3
IFNγ	Interferon gamma
Ig	Immunoglobulin
Ih2s	Innate type 2 helper cells
IL1RL1 (or T1/ST2)	Interelukin 1 receptor-like 1
IMT	Intima-Media Thickness
IL	Interleukin
ILCs	Innate Lymphoid Cells
IL-2Rβ	Interleukin-2 receptor beta
IL-17RB	Intereleukin-17 receptor B
iNKTs	invariant Natural Killer T cells
IL-1RAcP	IL-1 Receptor Accessory Protein
LCAT	Lecithin Cholesterol Acyl-Transferase
LDL	Low Density Lipoprotein
Lin	Lineage
LOX-1	Lectin-like oxidized low density lipoprotein receptor1
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
LTi	Lymphoid Tissue inducer
MAPK-AP-1	Mitogen-Activated Protein Kinase Associated Protein 1
MARCO	Macrophage receptor with collagenous structure
MCSF	Macrophage Colony-Stimulating Factor
MDA	Malondialdehyde
MHC	Major Histocompatibility Complex
MLNs	Mesenteric Lymph Nodes
mmLDL	minimally modified LDL
MPPs type 2	Multi-Potent Progenitor cells type 2
MZ	Marginal Zone
NAbs	Natural Antibodies
NBNT	Non B Non T

NF-Kb	Nuclear Factor Kappa-light-chain-enhancer of activated B cells
NHCs	Natural Helper Cells
NK	Natural Killer
NKp46	Natural Killer cell p-46 related protein
NOD	Non-Obese Diabetic
ox	oxidized
PAMPs	Pathogen Associated Molecular Patterns
PB	Pacific Blue
PBMCs	Peripheral blood mononuclear cells
PC	PhosphorylCholine
PD-L1/2	Programmed Death Ligand 1/2
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PL	Phospholipids
PMA	Phorbol 12-myristate 13-acetate
PRRs	Pattern Recognition Receptors
PSOX	Scavenger receptor that binds phosphatidylserine and oxidized lipoprotein
PVAT	Perivascular adipose tissue
Rag	Recombination-activating gene
RORa	Related Orphan Receptor a
Sca1	Stem cell antigen-1
SRA	Scavenger Receptor class A
SR-B1	Scavenger Receptor class B member 1
T-bet	T-box transcription factor
TG	Triglycerides
TGF-β	Transforming Growth Factor beta
Th	T helper
TIA	Transient Ischemic Attack
TLR	Toll-Like Receptor
TNFα	Tumor Necrosis Factor alpha
TRAF6	TNF receptor-associated factor 6
UC	Ulcerative Colitis
VAT	Visceral Adipose Tissue
VCAM-1	Vascular Cell Adhesion Molecule-1
VLDL	Very-Low-Density Lipoprotein
WT	Wild Type
γ_c	gamma chain receptor
4-HNE	4-hydroxynonenal
7AAD	7-aminoactinomycin D
15-LO	15-lipoxygenase

Background

Atherosclerosis

Atherosclerosis is a chronic inflammatory disease of medium and large vessels of the body which is mainly characterized by the formation of lipid rich plaques within the arterial tissue. The word comes from the Greek word athera (ἀθήρα) which means gruel or sticky describing the consistency of the plaques, comprised by a lipid rich and sticky material located in the inner lining of the arteries and the word sclerosis (σκλήρωσις) which refers to arterial stiffness.

Atherosclerosis is the most common cause of cardiovascular disease (CVD) and can lead to clinical manifestations of coronary artery disease (CAD) and cerebrovascular disease depending on the location of the plaque ruptured [2]. Upon such an event the inner parts of the plaque get exposed to the blood flow leading to thrombus formation which occludes the vessel and blocks the blood flow and subsequent supply of the tissues with oxygen and nutrients, a phenomenon called ischemia, possibly leading to death. Myocardial infarctions (MI) usually occur due to rupture of atherosclerotic plaques in the coronary arteries while the manifestation of stroke events is most often due to emboli from ruptured plaques of the carotid arteries. Transient ischemic attacks (TIAs) are caused by emboli in the brain but are followed by complete recovery of the patient [3]. However, patients experiencing TIA have an increased risk of experiencing future cerebrovascular events such as stroke [3]. Besides, the formation of thrombus through plaque rupture the same result can occur through plaque erosion [4]. Upon such a phenomenon the inner parts of the atherosclerotic plaque are "leaking" without the plaque being burst, an event which is enough to induce the formation of a blood clot accompanied by all the adverse effects previously mentioned.

Lipoprotein metabolism

Since certain lipids and lipoproteins are risk factors in CVD a few words concerning lipoprotein metabolism will help us better understand the genesis of atherosclerosis. The most important lipids in our body are cholesterol and triglycerides (TG) but their transport as such units to the tissues in order to serve

various purposes (formation of cell membranes, synthesis of hormones and bile acids for cholesterol and utilization as an energy source for TG) is impossible due to their hydrophobic nature. For that reason they are "packed" to form hydrophilic spheroidal particles called lipoproteins. Lipoproteins are consisted by a hydrophobic core mainly containing cholesterol esters (CE) and varying numbers of triglycerides (TG) and a hydrophilic "membrane" consisting of free cholesterol, phospholipids (PLs) and apolipoprotein molecules.

The body synthesizes by itself most of the circulating cholesterol (~80%) while the rest is diet-derived. Upon the intake of a meal, hydrolysed dietary fats enter the intestine. At that location, reconstituted TG are packed with apolipoprotein B (apo B) isoform B48 to form chylomicrons (CM) which are released to circulate via the lymphatic system. Upon interaction with lipoprotein lipase (LPL), TG hydrolysis takes place leading to the formation of CM remnants (CMRs), taken up by the liver mostly via the LDL receptor. In the liver TG, cholesterol and apoB100 together form the very-low-density lipoprotein (VLDL) particles subsequently being released in the circulation. LPL once more hydrolyses VLDL generating intermediate low density lipoprotein (IDL) which undergoes TG hydrolysis by hepatic lipase (HL) resulting in low density lipoprotein (LDL) formation [5].

The main TG carriers are CMs and VLDL while the main cholesterol-carriers are LDL and high density lipoprotein (HDL). In specific, LDL delivers cholesterol to tissues while HDL transports cholesterol from the tissues to the liver for catabolism into bile acids. Concerning HDL cholesterol metabolism, apolipoprotein A-I (apoA-I) interacts with ATP-binding cassette A1 and G1 (ABCA1, ABCG1 respectively) receiving cholesterol which gets esterified from lecithin cholesterol acyltransferase (LCAT). Next the newly synthesized HDL particle undergoes modification by cholesterol ester transfer protein (CETP) and endothelial lipase (EL) and finally enters into the liver through scavenger receptor class B type I (SRB1) expressed on hepatocytes [5].

Defects in cholesterol metabolism due to genetic factors and lifestyle (lipid-rich diets, lack of exercise) [5, 6] can lead to increased LDL cholesterol levels in the circulation and potentially initiate the formation of an atherosclerotic plaque.

Atherosclerotic plaque – A macroscopic view

A normal vessel is composed by three different layers: intima, tunica media, tunica adventitia (figure 1). The intima layer of the vessel is composed by an endothelial cell lining surrounded by a basement membrane and extracellular matrix. Multiple layers of smooth muscle cells surrounded by basement membrane and extracellular matrix comprise the tunica media while the adventitia is the outer part of the vessel reaching the surrounding connective tissue where *vasa vasorum* exists supplying the tissue with nutrients. This is the place where immune cells

(dendritic cells (DCs), macrophages, T, B cells) can accumulate and form tertiary lymphoid structures upon advanced atherosclerotic plaque formation [7].

The development of atherosclerotic plaques is initiated by the formation of early fatty streaks in the arterial wall accompanied by the presence of macrophages and foam cells through the accumulation of plasma lipoproteins in the subendothelial cell layer. One characteristic event upon such an accumulation is the oxidative modification of the lipids trapped in the intima triggering the immune system to target them as they are recognized as carriers of non-self molecules. Macrophages in their attempt to clear up the surroundings from lipids and activate subsequent immune pathways, engulf them leading to the formation of cholesterol-rich cells that are called foam cells. Under these conditions the endothelium gets further activated leading to the expression of cell adhesion molecules that makes leukocytes, such as T cells, from the periphery to attach to the endothelium and infiltrate the plaques. The endothelial cell activation also leads to the generation of cell signals that lead to the differentiation of monocytes into macrophages that follow the path of lipid engulfment and differentiation into foam cells [2].

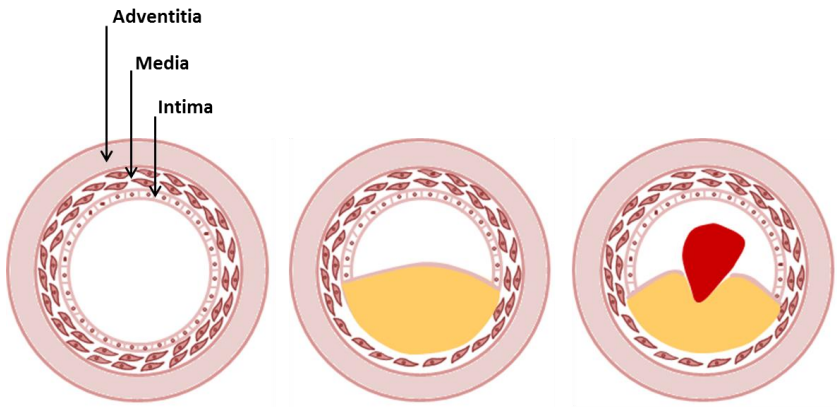

Figure 1. A healthy artery is consisted of three intact layers: intima, tunica media and tunica adventitia (left); Artery containing a lipid-rich atherosclerotic plaque (middle); Plaque rupture and thrombus formation (right).

Vascular smooth muscle cells in their attempt to constrain the altered consistency of the arterial wall form a fibrous cap of variable thickness which is composed mostly by collagen. The continuous accumulation of lipids and engulfment by foam cells in the arterial wall leads to cell apoptosis contributing to the formation of a lipid rich core in the atherosclerotic plaque (figure 1). Besides the formation of a lipid rich core in advanced atherosclerotic plaques, the formation of lipid rich shoulder regions infiltrated by macrophages, T cells and mast cells also takes place [2].

At the event of plaque rupture or erosion the exposure of the atherosclerotic plaque material leads to platelet aggregation and blood coagulation which leads to the formation of a thrombus (figure 1) that can topically occlude the vessel blocking the blood flow or detach and become an embolus blocking the blood flow at a different place than the one that the plaque has ruptured. This event leads to tissue ischemia and possibly to subsequent death [4].

First line of defense - Innate immunity

The first line of defense for an organism against invaders such as pathogens or even altered self-molecules is innate immunity. The triggering of such a response takes place through the recognition of pathogen associated molecular patterns (PAMPS) or danger associated molecular patterns (DAMPs) by pattern recognition receptors (PRRs) expressed by cells such as macrophages. A PAMP or DAMP engagement in PRRs initiates the procedure of inflammation which can be catastrophical for the host in case that the stimulus is persistent, excessive or resistant to eradication. In the case of atherosclerosis one such particle that releases or carries such immunogenic molecules, generated upon its oxidative modification, is LDL [7]. This is a procedure that predominantly takes place in the atherosclerotic plaque due to the lipoprotein's entrapment and retention and upon failure of available antioxidative mechanisms that have evolved in order to prevent the modification of self-components. Further discussion about the generation of oxidized LDL (oxLDL) epitopes takes place in a following section.

Interestingly, because of the fact that many oxLDL epitopes share molecular identity with microbial epitopes, similar innate immune responses can be raised upon presence of microbial PAMPs as well as altered self-components (DAMPs) [8, 9]. As a microbe is recognized by the host as something strange and something that has to be eliminated the same thing happens with epitopes generated from oxidative modifications (on lipoproteins and apoptotic cells). The importance of the generation of such epitopes is crucial in atherogenesis [10]. The activation of endothelial cells by components of oxLDL entails the expression of adhesion molecules (E-selectin, VCAM-1, ICAM-1) and the expression of chemokines (CCL2, CCL5, CXCL10, CX3CL1) that attract cells patrolling the periphery of the body such as monocytes, T cells and DCs. On the other hand, monocytes in the intima are stimulated by macrophage colony-stimulating factor (MCSF) produced by activated endothelial cells to differentiate into macrophages [11]. This step is crucial for the "generation" of macrophages that elicit important roles in the uprising innate immune response triggered by the entrapped lipoproteins within the arterial wall. In that location, macrophages upregulate their scavenger

receptors (SRA-1, SRA-2, MARCO, CD36, SR-B1, LOX-1, PSOX) that can then take up oxLDL [12] but not native LDL [13] or mmLDL.

Scavenger receptors are PRRs that besides microbes can also take up modified LDL particles, internalize them and assist in antigenic presentation. Interestingly, CD36 and SR-A macrophage related expression has been involved in apoptotic cell removal given that apoptotic cells express oxidized phospholipids (oxPLs) which are also expressed by oxLDL [9, 14, 15]. Although *in vitro* it has been shown that CD36 and SR-A mediate extensive oxLDL uptake, genetic deletions of these receptors were proven to be atheroprotective in some of the studies and some others not [16]. One could speculate that although the prevention of foam cell formation is a crucial step in preventing atherogenesis when the disease reaches the point that there is extensive accumulation of lipids in the arterial wall such mechanisms of lipid clearance mediated from cells are important for the constraint/resolution of the disease.

Another set of PRRs expressed by both endothelial cells and macrophages among other cells (DCs, lymphocytes and vascular smooth muscle cells) are toll-like receptors (TLRs) [17]. Knockout studies of hypercholesterolemic mice have demonstrated a major proatherosclerotic role for MyD88 which is a key adaptor protein in the signaling cascades of most TLRs [18]. Oxidized LDL can bind particular TLRs such as TLR2 and induce vascular responses [19, 20] while TLR2 expression by vascular cells may be particularly proatherosclerotic [21]. Interestingly, minimally modified LDL preparations have been shown to bind TLR4 [22] while deficiency of TLR4 or TLR2 in apoE$^{-/-}$ mice reduced aortic intimal lipid accumulation [23].

As the disease proceeds macrophages being dedicated to their role of lipid ingestion, turn into foam cells due to internal cholesterol accumulation.

LDL oxidation and generation of oxLDL specific epitopes

Upon entrance of the lipoprotein to the intimal area of the vessel its retention is taking place by binding of apoB100 to proteoglycans of the extracellular matrix [24], a procedure that is pivotal in early atherogenesis. Trapped LDL particles in the intima get modified by enzymes such as myeloperoxidase and lipooxygenase and by reactive oxygen species (HOCL, phenoxyl, peroxynitrite radicals) generated by the ongoing inflammation in the atherosclerotic plaque. The result of such an attack is the induction of modifications in the lipid and protein content of the lipoprotein generating a vast spectrum of differentially modified LDL particles depending on the extent and the type of modification that they have undergone.

The peroxidation of fatty acid residues in lipid species (PL, CE, TG) generates reactive aldehydes (malondialdehyde (MDA), 4-hydroxynonenal (4-HNE)) and

truncated lipids such as lysophosphatidylcholine (lyso-PC) that can initiate innate inflammatory responses. These lipids activate endothelial cells and macrophages to produce adhesion molecules and chemokines [7]. Additionally, MDA and 4-HNE molecules can also form adducts of lysyl residues of apoB100 and modify it resulting in chemical structures that are immunogenic [7].

Copper induced oxidative modification of LDL in vitro generates a set of heavily oxidized particles characterized by oxidized lipids and modified proteins. Interestingly, it has been shown that *in vitro* prepared oxLDL has almost the same chemical characteristics as the LDL eluted from plaques of rabbits and humans [25]. Although, oxLDL contains abundant lipid peroxidation products such as aldehydes and ketones, minimally modified LDL (mmLDL) contains predominantly early lipid peroxidation products such as polyoxygenated oxCEs [26] formed by 15-lipoxygenase (15-LO) which is an enzyme expressed in cells of the vasculature and secreted under inflammatory conditions.

B1 cells

Genesis and general functions

The term B cells is used to characterize antibody-producing effector cell subsets of either innate and adaptive immunity. Besides the part of B cells of adaptive immunity that need T cell assistance or the induction of co-stimulatory pathways in order to produce antibodies against antigens, there is another part of cells that recognize self-antigens and produce the so-called natural antibodies. Studies have shown that B cells express several toll-like receptors [27-29] indicating the existence of B cell subsets that respond to innate immune signals. Indeed, follicular B cells or B2 cells representing effector cells of adaptive immunity and the largest cell populations of the B cell compartment don't respond that efficiently to lipopolysaccharide (LPS) stimulation compared to splenic marginal zone B cells (MZB cells) and B1 cells. MZB cells and B1a cells have been shown to be involved mainly in innate immune responses [30].

Particularly, B1 cells are the main producers of natural antibodies while their B cell receptors (BCRs) bind to antigens of both self- and microbial origin since their main role is the maintenance of tissue homeostasis and the defense against invading pathogens [31]. B1 cells are further divided into CD5 expressing, known as B1a cells and into CD5 negative, known as B1b cells [32]. Follicular and marginal zone B cells are collectively termed B2 cells and develop from a common bone marrow precursor although nowadays, the term B2 cell is mostly used to describe follicular B cells. Further discussion concerning these cells will follow in a separate section.

B1a cells were shown to be generated before birth and at the first weeks following birth. The fetal liver is considered a great source of B1a cells while in adult mice it has been shown that B1a cells are poorly replenished from the bone marrow [33]. B1b cells on the other hand, are as well reconstituted from fetal liver but can be reconstituted to a greater extent, compared to B1a cells, from bone marrow derived B1 cell precursors [34]. Once B1 cells have resided to the various tissues and under homeostatic conditions the influx of newly-generated B1 cells is restricted [35]. It is believed that B1 cells are maintained in a steady population over time by a process called self-renewal entailing slow proliferative rates targeting replenishment of dying cells [33]. In support of that, it has been shown that peritoneal B1 cells have slow proliferative rates [36]. The maintenance of B1 cells especially early in life are mediated by IL-5 and IL-9 [37, 38] with IL-9 expanding mainly the B1b cell population. Additionally, mice that are deficient for secreted IgM have been shown to have a large B1 cell compartment indicating that to some extent B1 cell development might be regulated by IgM secretion [39]. It also believed that most likely B1 cells undergo positive selection for their reactivity against self-antigens [40] in contrast to T cell development in the thymus where thymocytes with T cell receptors of low-affinity against self-antigens do not undergo apoptosis [41].

B1 cells represent the largest B cell population in pleural and peritoneal cavities while their homing there requires binding to the CXC-chemokine ligand 13 (CXCL13) which is expressed by cells of the peritoneal cavity such as macrophages [42]. Besides the peritoneum, B1a cells can also be found in the spleen but they have been reported to differ in terms of gene expression and antibody production depending on the location that they reside [43, 44]. Interestingly, it has been shown that congenital asplenia and splenectomy in mice results in depletion of peritoneal B1a cells and diminished IgM immune responses against polysaccharide antigens without affecting B1b and B2 cells [45]. It seems that the spleen plays an important role in the generation of peritoneal B1a cells, either by providing essential signals that are important for the development of B1a cells or by being the source of cell progenitors for the subsequent generation of B1a cells the last being suggested from a study in mice [46]. Until now the mechanism through which B1a cell survival depends on splenic presence has not been described.

Besides pathogen associated antigens, B1 cells secrete antibodies against antigens of self origin such as oxidized lipids [15] as well as annexin V expressed by apoptotic cells [47]. It has also been shown *in vitro* that B1-derived IgM antibodies that target apopototic cells promote their phagocytosis by immature DCs [48]. Besides IgM antibodies, B1 cells secrete polyspecific IgA that function as a first defence against pathogens in the gut mucosa [49] while their anti-inflammatory role is further supported by their ability to secrete large amounts of IL-10 [50].

Table 1. B cell subset phenotype in mice; B1 cells as described in the peritoneal cavity. Marginal zone and follicular B cells as described in the spleen. (Table adapted from Perry et al [51])

B cell subsets	Phenotype
B1a	$CD19^{+}$ $B220^{low/mid}$ $IgM^{hi}IgD^{dull}CD43^{+}CD11b^{+}CD5^{+}$
B1b	$CD19^{+}$ $B220^{low/mid}$ $IgM^{hi}IgD^{dull}CD43^{+}CD11b^{+}CD5^{-}$
Marginal zone	$CD19^{+}$ $B220^{+}$ $IgM^{hi}IgD^{dull}CD1d^{high}CD21^{high}CD23^{-}$
Follicular	$CD19^{+}$ $B220^{+}IgM^{dull}IgD^{hi}CD21^{mid}CD23^{+}$

In a recent study the human equivalent of B1 cells has been described as $CD20^{+}CD27^{+}CD43^{+}CD70^{-}$ cells [52]. These cells recognize phosphorylcholine (PC), spontaneously secrete IgM, stimulate T cells and represent ~2–5% in cord blood.

B1 cells – Functional characterisitcs

B1 cells act in different ways depending on the location of stimuli as well as the type of stimuli. One way of action for B1 cells against invaders is the production of high levels of polyreactive IgM antibodies at the site of infection. A nice example of that are the responses taking place upon infection with the influenza virus in the respiratory epithelium. Upon such an event, B1a cells accumulate to regional lymph nodes and differentiate into antibody producing cells conferring early protection against the virus [53]. The accumulated B1a cells are believed to be recruited from locations that they normally reside to such as the body's cavities and the spleen since these cells don't show any proliferative capacity. It is interesting that the vast majority of secreted antibodies are not specific for the influenza virus indicating that this immune response is not triggered and regulated through BCR signaling [53]. B1b cells do not participate in this acute response while it was shown that the accumulation of B1 cell derived antibody production takes place locally and not systemically [53].

Another type of B1 cell response occurs via intraperitoneal or intravenous challenge of certain stimuli such as bacteria [54, 55] and LPS [56] as well as cytokines (IL-5, IL-10) [57]. In response to such stimuli, both B1a cells [55] and B-1b cells [58] migrate from sites of residency (peritoneum) either to the spleen [55, 58] where they differentiate and secrete IgM antibodies or to mucosal tissues where they secrete IgA antibodies [59]. Because of the fact that cytokines can induce this type of response shows once more that signaling through the BCR receptor is not essential for the induction of such a response from B1 cells [57]. The reason for production of such antibodies is the "neutralization" of the target as

well as the clearance of apoptotic bodies. Marginal zone B cells also respond acutely upon innate immune stimulation establishing them alongside with B1 cells as the early defense of the host against pathogens entering through mucosal surfaces and the blood by the production of target related polyreactive antibodies [30].

Another interesting "version" of B1 cell activation has been shown to take place upon immunization of mice with purified pneumococcal polysaccharide type 3 [60]. In that case a B cell population that resembles B1b cells expands. These cells are characterized by high BCR specificity, extensive clonal expansion providing long lasting immunity to the host, factors that totally distinguish them from B1a cells in terms of developmental requirements and function. B1b cell-mediated antibody responses occur independently of T cell help resulting on IgM production or isotype switching to IgG3 or IgA [60, 61].

There are also some reports indicating cooperative actions of B1 cells with cells of the adaptive immunity. Specifically, B1-derived IgM has been shown to promote increased production of IgG by B2 cells [39, 62]. Additionally, peritoneal B cells were shown to inhibit T cell activation [63] and to prevent autoreactive responses in diabetic mice [64]. Lastly, in neonatal mice peritoneal B cells were shown to produce IL-10 and inhibit the priming of IFNγ producing T cells by DCs [65].

Natural antibodies and atherosclerosis

Natural antibodies (NAbs) are considered part of the humoral arm of innate immunity. As previously mentioned B1 cells is the main cell population secreting these antibodies that can be either IgA or IgM. Many studies have indicated an atheroprotective role of natural antibodies targeting oxLDL related epitopes preventing their uptake from macrophages and subsequent foam cell formation while contributing to their clearance from the system.

Splenectomy that has been shown to vastly affect peritoneal B1 cells [45] was shown by another study to aggravate atherosclerosis development in apoE$^{-/-}$ mice [66]. Additionally, B1 cells but not B2 cells were reported to be atheroprotective and that this result was due to B1-derived IgM since transfer of B1 cells that do not secrete IgM did not confer atheroprotection [67]. In addition to that, hypercholesterolaemic LDLr$^{-/-}$ mice that cannot secrete IgM were shown to have increased atherosclerosis compared to control LDLr$^{-/-}$ mice [68] while IgM targeting oxLDL is inversely correlated with CVD in humans [69].

E06 is one of the natural antibodies that has been shown to bind oxLDL, specifically the PC moiety of oxidized phospholipids (oxPL) and to confer atheroprotection [9]. Moreover, E06 has 100% homology with the natural Ab T15 (IgA clone) which has been shown to target PC expressed by microbes and confer

protection from *S. Pneumoniae* infection [70]. In another study the above mentioned natural antibodies T15–IgA and E06–IgM were both shown to recognize the PC moiety of oxPL present in oxLDL and apoptotic cells but did not recognize native LDL [71]. This phenomenon implied that PC is normally a hidden epitope that is probably getting exposed upon oxidative modification of the lipoprotein [71]. Both T15-IgA and E06-IgM are targeting the same antigens and don't seem to "discriminate" between PAMPS (non-self origin) or DAMPS (self origin) upon such challenge. A good example of such statement is the fact that immunizations of $LDLr^{-/-}$ mice with heat-killed *S. pneumoniae* increase plasma E06/T15 levels and reduce atherosclerosis [8]. Lastly, it is also worth mentioning that 20-30% of all IgM bind to oxLDL related epitopes [15] and among them quite many are targeting malondialdehyde-LDL (MDA-LDL) as shown in humans [72].

ILCs

As it will be discussed later on, T cells have been shown to be a great source of cytokines and to direct adaptive immune responses in inflammation upon their orchestration by DCs which is pivotal in this procedure. The recent discovery of analogous cells of innate immunity being great sources of cytokines has opened a whole new door for investigation of their role in various diseases.

As in adaptive immunity T helper effector cells are classified into different categories (Th1, Th2, Th17) depending on the type of cytokines that they produce nearly the same categorization applies for innate lymphoid cells (ILCs) [73]. Nowadays, ILCs are divided into three different subsets; group 1 lymphoid cells that includes ILC1s and natural killer (NK) cells secreting Th1 related cytokines (mainly IFNγ), group 2 lymphoid cells or ILC2s secreting Th2-related cytokines and group 3 ILCs that includes ILC3s ($NKp46^{+}$ and $NKp46^{-}$) and lymphoid tissue inducer (LTi) cells mainly secreting Th17-related cytokines. Since IL-25 has been reported to be a powerful inducer of ILC2s only, I will merely focus on these cells in the following two sections.

ILC2s

Discovery – Anti helminthic function

The possibility of the existence of a cell population that is quite similar in terms of cytokine release with Th2 cells came from two independent studies showing that administration of IL-25 in $Rag2^{-/-}$ mice that lack functional B and T cells induces the release of IL-5 and IL-13 [74, 75]. Later on, another study reported the existence of a non-B non-T cell population (NBNT cells) being responsible for the secretion of IL-5 and IL-13 upon stimulation with IL-25 in $Rag2^{-/-}$ mice [76]. A few years later several studies provided the first concrete evidence of their

existence reporting them as great sources of IL-5 and IL-13 upon activation with IL-25 and IL-33 conferring protection against helminthic infections [77-79].

Different names have been assigned to these cells such as natural helper cells (NHCs) [77], innate type 2 helper cells (Ih2s) [79] and nuocytes [78]. As mentioned previously it has been agreed nowadays that these cells should be denoted as ILC2s [73]. ILC2s were reported to be located in the mesenteric lymph nodes (MLNs) [78, 79], spleen [78, 79], liver [79], blood [78], peritoneal lavage [78, 79], intestine [78] bone marrow [78, 79] and lung [79]. Moro et al [77] reported the presence of ILC2s in fat-associated lymphoid clusters (FALCs) in the mesentery of both mice and humans, but also in adipose tissues around the kidney and genitalia and to a lesser extent in the omentum and in subcutaneous fat tissue [77].

In these studies ILC2s have been reported to expand upon helminthic infection [77-79], to secrete big amounts of IL-5 and IL-13 upon such infection or stimulation with either IL-25 or IL-33 [77-79] and to support B1 cell survival and antibody secretion [77] possibly by the secretion of IL-5 and IL-6 that has previously been shown to regulate B cell antibody production [80, 81]. In an attempt to further designate the biological function and importance of these cells I will briefly discuss the findings of the aforementioned studies.

Moro et al [77] have reported the existence of $Lin^{-}Sca1^{+}cKit^{+}$ cells (proposed as natural helper cells or NHCs) expressing the IL-33 and IL-7 receptor and being organized in clusters of lymphocytes alongside the blood vessels of the mouse mesentery. These clusters although containing mainly lymphocytes ($Lin^{-}Sca1^{+}cKit^{+}$, $CD3^{+}$ T and $B220^{+}$ cells) were distinct from lymph nodes since no fibrous capsule was apparent letting the lymphocytes to be in direct contact with the surrounding adipocytes.

NHCs were shown to express several Th2 related cytokines such as IL-4, IL-5, IL-13 but IL-2, IL-6 and granulocyte macrophage co-stimulatory factor (GM-CSF) as well. Additionally, incubation of the cells with IL-33 and IL-25 induced the release of large amounts of IL-5 and IL-13, with IL-33 being a more potent inducer. Additionally, co-culture of NHCs with splenic B cells induced increased levels of IgA.

Further on, the same study utilized mouse models of adaptive cell deficiency such as the $Rag2^{-/-}$ mouse model and the double knock-out of gamma chain receptor (γ_c) deficiency ($Rag2^{-/-}/\gamma_c^{-/-}$) in which B, T, NK as well as LTi cells are absent since signaling through γ_c is important for their development. Note that the same mouse model contains some IL-25 and IL-33 responders such as mast cells and basophils. Interestingly, administration of IL-25 and IL-33 as well as infection with the helminth Nippostrongylus Brasiliensis (*N. Brasiliensis*) induced the expression of IL-5 and IL-13 in serum and peritoneal fluid only in $Rag2^{-/-}$ mice indicating that the main source of Th2 related cytokines is NHCs. Moreover, helminth infection

(*N. Brasiliensis*) was shown to be responsible for the increase of IL-33 levels in the peritoneal cavity of $Rag2^{-/-}$ mice and subsequent release of IL-13 from NHCs. This resulted in increased goblet cell hyperplasia and mucus production, pathways that are essential for helminthic expulsion as shown previously [76, 82].

In line with the above, Neil et al [78] reported IL-25 and IL-33 as potent inducers of nuocytes (ILC2s) characterized as lineage negative cells expressing CD45, ICOS, T1/ST2 (IL-33-receptor), IL-17RB (IL-25 receptor) and IL7Ra. *In vitro* cultured nuocytes expressed mainly IL-13 and IL-5 alongside with substantial levels of IL-6, GM-CSF and IL-10. Additionally, nuocytes were reported to be the predominant cell population expressing IL-13 upon N. Brasiliensis infection in the MLNs while deficiency of the IL-25 receptor (*IL-17rb*$^{-/-}$) or IL-33 receptor (*Il1rl1*$^{-/-}$) resulted in reduced nuocyte numbers during the early response of such an infection (5 days post infection, d.p.i.) compared to control mice. Later on, in their attempt to combat infection nuocytes arose more rapidly in *Il1rl1*$^{-/-}$ mice compared to *IL-17rb*$^{-/-}$ while in the combined deficiency of these receptors nuocytes failed to expand and expel helminthes. Interestingly upon helminthic infection and transfer of nuocytes in $Rag2^{-/-}$ although the transferred cells showed rapid early expansion (4 d.p.i) they were not maintained in this environment of T and B cell absence for a longer period and failed to expel helminthes. This finding implied that there is a possible "communication" of nuocytes with cells of the adaptive immunity which is crucial for their maintenance and function.

Price et al [79] reported the existence of Lin^- cells that are c-Kit ^{low}, $Sca1^-$, CD122 $(IL\text{-}2R\beta)^{low}$, $Ly5.2^+$, $Thy1^+$ and $CD44^{high}$ in most of the tissues tested, having similar phenotypes while being more prevalent to MLNs, spleen and liver. These cells were shown to respond robustly to IL-25 and IL-33 and to secrete large amounts of IL-5 and IL-13 which were accompanied by eosinophil recruitment. Additionally, with the use of various mouse models such as the previously mentioned $Rag2^{-/-}$ and $Rag2^{-/-}/\gamma_c^{-/-}$, it was shown that these cells were the main IL-13 expressing cells upon helminthic infection with N. Brasiliensis, a cytokine that is necessary for helminthic expulsion.

During the same year Saenz et al [83] reported the induction of a cell population by IL-25 named multipotent progenitor type 2 cells (MPPs (type 2)) mainly in gut associated lymphoid tissues (GALTs) that secreted Th2 related cytokines. The IL25-elicited cell population, termed MPP (type2) cells, being NBNT $IL1RL1^-$ $IL7ra^-c\text{-}Kit^+Sca\text{-}1^{intermediate}$ gave rise to cells of granulocyte and monocyte lineages both *in vitro* and *in vivo*. Adoptive transfer of MPP (type2) cells in $IL\text{-}25^{-/-}$ mice that are normally susceptible to helminthic infection, resulted in the induction of Th2 cytokine responses and conferred protection from such an infection with *Trichuris muris*. These cells although quite similar in function upon helminthic infections seemed to differ from the previously reported NHCs, Ih2s and nuocytes, (nowadays collectively termed ILC2s) because of their ability to differentiate into lineage cells.

Upon the discovery of murine ILC2s the search for the human analogue followed. In 2011 a study [84] was published reporting human ILC2s described as Lin^- $IL7ra^+CD45^{hi}$ that expressed low levels of the RORγt transcription factor as well as transcripts encoding IL-13, the IL-25 and IL-33 receptor (IL-17BR, IL1RL1, respectively) and the prostaglandin D2 receptor 2 (CRTH2). Interestingly, these cells were found in adult lungs and blood as well as in nasal polyps of patients suffering from chronic rhinosinusitis [84].

ILC2s

Development - Implication in disease

A series of studies followed the discovery of ILC2s mainly trying to further characterize them in terms of gene/protein expression, developmental pathways as well as possible implication in various diseases.

As other subsets of ILCs do, ILC2s are dependent on the transcription factor ID2 but were also shown to require the transcription factors GATA-binding protein 3 (GATA 3) [85, 86] and related orphan receptor a (RORa) [87]. RORα seems to be important in defining their phenotype [87] while GATA3 besides promoting ILC2 development [85, 86] has been shown to facilitate IL-13 production from these cells [88]. Among various markers ILC2s have also been shown to express chemokine receptors such as CXCR4, CXCR6 and CCR9 [89], possibly mediating their distribution to the various organs of the body. In contrast to the rest of ILC subsets (ILC1, ILC3) no reports until now have shown conversion of ILC2s to other ILC subsets.

Besides their protective role against helminthic infections [77-79] ILC2s have been reported to promote allergy and asthma and to be implicated in experimental ulcerative colitis (UC) while they have been suggested to promote wound healing and metabolic homeostasis in visceral adipose tissue (VAT).

In specific, upon an allergic response, type 2 cytokines are released leading to recruitment and degranulation of eosinophils and mast cells, increased serum IgE levels, goblet cell hyperplasia, mucus production and smooth muscle cell contraction. Before the discovery of ILC2s it was believed that the main source of these cytokines is Th2 cells. Interestingly, in ovalbumin-induced allergic asthma, ILC2 numbers were increased in the lungs of mice and were proven to be a great source of IL-5 and IL-13 alongside to Th2 cells [90]. Additionally, intranasal administration of IL-25 and IL-33 induced an asthma phenotype and accounted for a robust accumulation of IL-5 and IL-13 expressing ILC2s in the lungs of these mice [90]. In another study intranasal administration of the fungal allergen *Alternaria alternata* to mice induced increased IL-33 levels, accompanied with an

expanded ILC2 cell population in the lungs and increased IL-5 and IL-13 production [91].

ILC2s have also been implicated in promoting airway hyperactivity in virus-infected mice by inducing type 2 immune responses [92] while upon viral lung infection ILC2-derived amphiregulin, was reported to promote tissue repair [93]. Additionally, patients suffering from chronic rhinosinusitis (a typical type 2 immune disease) were shown to have increased ILC2s in nasal polyps [84]. In an experimental mouse model of colitis, characterized as well by a type 2 phenotype, alongside to IL-13 producing NKT cells, IL-13 producing ILC2s were identified indicating a possible involvement of ILC2s in the development of the disease [94]

ILC2s were also reported to be resident in vascular adipose tissue (VAT) and through secretion of IL-5 to sustain eosinophils and alternatively activated macrophages (AAMs) [95] which were reported to promote insulin sensitivity and metabolic homeostasis [96].

APCs - The link between innate and adaptive immunity

The term antigen presenting cells (APCs) refers to a group of cells that have a key role in the initiation of adaptive immune responses and includes dendritic cells (DCs), macrophages and B cells. In specific, DCs can directly activate naïve T cells while the role of B cells and macrophages, alongside with DCs, is to assist in the presentation of antigens merely to effector and memory T cells that were differentiated from naïve T cells.

The process of antigenic presentation from DCs involves engulfment of the target, loading of target-derived antigens to major histocompatibility complex (MHC) molecules and presentation to T cells. Alongside to that, DCs can secrete cytokines and chemokines providing signals to their surroundings and to T cells in specific. DCs reside in most of the tissues of the body and upon the presence of stimuli like PAMPs or DAMPs the innate immune response is initiated involving target ingestion and generation of signals to the surrounding cells that involve the secretion of cytokines and chemokines as well as the presentation of antigens to T cells. Tissue resident DCs can also migrate to draining lymph nodes and present to naïve T cells tissue specific antigens although there are no concrete evidence until now to fully support such a statement. The fact that oxLDL responsive T cells exist in the periphery of the human body [97] could indicate two possibilities: that either the APCs from the vasculature have migrated to draining lymph nodes and have primed antigen specific T cells or that the generation of oxLDL related epitopes have taken place outside the vasculature and T cells have been primed by APCs at that location.

Several studies have described the presence of APCs in the vasculature being in close contact with lesional T cells [98, 99], taking up cholesterol and being capable of antigenic presentation [100]. Interestingly in a recent study it has been shown that $CD11c^+$ cells from mouse aortas can activate T effector cells [101].

Adaptive immunity

T cells

T cells upon activation by APCs can differentiate into effector cells and perform such functions in the vasculature or in the periphery of the human body. Upon activation they can also interact with B cells in lymphoid tissues and stimulate the generation of circulating antibodies that can affect the inflammatory events in the arterial wall. The induction of effector T helper cells from naive $CD4^+$ T cells requires the engagement of the T cell receptor (TCR) as well as signaling through the co-stimulatory molecules expressed by DCs. The type of cytokines that exists in the surroundings, either generated upon a preceding innate immune response or secreted by the DC itself will further determine the type of effector T cell that will be induced.

Briefly, the presence of IFNγ and IL-12 gives rise to Th1 cells that have evolved for the clearance of intracellular pathogens while IL-4 leads to Th2 differentiation which mediates clearance of extracellular pathogens and induction of antibody production from B cells [102]. Concerning Th17 differentiation and as has previously been reviewed [103], the induction of Th17 cells from naïve T cells in mice takes place in the presence of TGF-β and IL-6 or IL-21 while IL-23 is believed to further assist in that procedure. Moreover, in humans TGF-β was reported to be dispensable for the differentiation of human Th17 cells and that the combinations of IL-1β plus IL-6 or IL-1β plus IL-23 could drive Th17. Other studies have indicated that TGF-β is actually needed for the induction of Th17 cells but only when IL-6 and IL-23 or IL-21 are present (reviewed by Korn et al [103]).

Several lines of evidence have demonstrated the presence of T cells in both human [104, 105] and mouse atherosclerotic plaques [106, 107] as well as the presence of systemic T cell activation in hypercholesterolemic mice [108] and in the blood of patients suffering from acute coronary sydromes [109]. $CD4^+$T helper cells in specific are categorized according to the cytokines that they produce into Th1 (mainly secreting IFNγ), Th2 (secreting IL-4, IL-5, IL-13) and Th17 (mainly secreting IL-17A) cells.

Th1

Th1 cells and related cytokines have been associated with the promotion of atherosclerosis. The fact that IFNγ exists in human atherosclerotic lesions [110, 111] and human T cell clones from such material express IFNγ [111], established Th1 cells as an important cell subset for investigation.

The proatherogenic role of IFNγ cells has been confirmed by the use of hypercholesterolaemic mouse models with genetic deletions of the cytokine, its receptor and the Th1 related transcription factor T-bet [112-114] while exogenous IFNγ administration was shown to enhance disease development [115]. Additionally taking one step behind and focusing on the conditions that promote the generation of Th1 cells one would visualize the secretion of IL-12 from APCs to be a key step. Interestingly, it has been shown that treatment of $apoE^{-/-}$ mice with IL-12 enhanced lesion development [116] while IL-12(p40) deficiency in the same mouse model reduced atherosclerosis development [117].

IFNγ and IL-12 were also implicated in the promotion of unstable atherosclerotic plaque formation. IL-12 treatment of $apoE^{-/-}$ mice increased atherosclerotic plaque formation and lesional $CD3^+$ T cell infiltration [116] while exogenous administration of IFNγ to $apoE^{-/-}$ mice increased the lesion size as well as the number of T lymphocytes and MHC-II positive cells within lesions [115].

Th2

The role of Th2 cells and related cytokines (IL-4, IL-5, IL-13) in atherosclerosis is less clear. There is no direct evidence in mouse models of a clear role of Th2 cells in atherosclerosis. However, it has been suggested that there is an association of Th2 immune responses (IL-4) and advanced atherosclerosis in $apoE^{-/-}$ mice [118]. Besides the limited literature concerning the role of Th2 cells in humans, in a prospective clinical study it was shown that high levels of Th2 cells (gated as $CD3^+CD56^-CD4^+IL4^+$ cells) were associated with less carotid intimal media thickness (IMT) and a lower risk of coronary events in women [119].

In mouse studies IL-4 has been shown to have a proatherogenic [117, 120] or antiatherogenic role [121] or no effect [122] on atherosclerosis. IL-5 and IL-13 on the other hand seem to be atheroprotective; low density lipoprotein receptor deficient mice ($LDLR^{-/-}$) deficient in either IL-5 [123] or IL-13 [124] were shown to have increased atherosclerosis. Interestingly, IL-5 has been shown to confer atheroprotection through induction of B cell activation and generation of antibodies (IgM) targeting oxLDL [123]. Exogenous IL-13 administration modulated the morphology of atherosclerotic lesions by increasing the collagen and decreasing the macrophage content of plaques [124].

Th17

The role of Th17 cells, mainly expressing their signature cytokine IL-17A, in atherosclerosis is not very clear until now. In murine studies Th17 cells were reported to be both pro-atherogenic [125-127] and anti-atherogenic [128, 129]. Three studies have indicated a proatherogenic role for IL-17 by utilizing hypercholesterolaemic mouse models being deficient for the cytokine and its receptor [125] as well as by investigating the cytokine's blockade on disease progression [126, 127].

Specifically, it has been shown that apoE$^{-/-}$ mice that are deficient either for IL-17A or the IL-17A receptor (IL-17RA) have smaller atherosclerotic plaques in the aortic arch and aortic roots [125]. Additionally, flow cytometric analysis of IL-17A$^{-/-}$apoE$^{-/-}$ and IL17RA$^{-/-}$apoE$^{-/-}$ murine aortas revealed reduced T cell, neutrophil and macrophage content restricted only to the aortic arch where reduced atherosclerosis was recorded [125].

In another study, IL-17A blockade reduced atherosclerosis in apoE$^{-/-}$ mice and decreased plaque vulnerability and cellular infiltration while this type of treatment downregulated activation markers on the endothelium and immune cells (such as VCAM) and reduced cytokine and chemokine secretion (IL-6, TNFα, CCL5) [126].

It has also been suggested that hypercholesterolaemic conditions promote the differentiation of IL-17 producing T cells since IL-17A–expressing T cells were found to be significantly increased in the aortas, spleen, and lamina propria of aged apoE$^{-/-}$ mice compared with age-matched C57BL/6 mice [127]. Moreover in the same study it has been shown that IL-17A blockade in apoE$^{-/-}$ mice by use of adenovirus-produced IL-17 receptor A, reduced plaque burden and reduced circulating IL-6 and granulocyte colony stimulating factor (GCSF) levels and limited CXCL1 expression and macrophage content within the aortas [127].

From the side of studies that indicated IL-17A to be atheroprotective it has been reported that IL-17A deficiency in apoE$^{-/-}$ mice fed a high fat diet for 8 and 16 weeks accelerated atherosclerotic plaque formation [128]. Mice that were on HFD for a shorter period (8 weeks), representing a model of early plaque formation, had reduced vascular smooth muscle cell and type I collagen content indicating increased plaque vulnerability [128]. Moreover, *in vitro* stimulated splenic CD4^{+} T cells from the aforementioned mice were found to express increased IFNγ and decreased IL-5 levels [128]. In another study it was shown that IL-17 administration in LDLr$^{-/-}$ mice reduced VCAM-1 expression, vascular T cell infiltration and limited atherosclerotic lesion development in the aortic roots of mice [129].

In humans, IL-17A expression has been found in human lesions [129, 130] while in a study focusing on the expression of IFNγ and IL-17 expressing T helper cells,

the existence of double expressing T cells in such lesions was demonstrated [131]. The fact that both Th1 and Th17 cells contribute to the same diseases might indicate that there is a synergistic relationship between these two subsets that allows the differentiation of one subset into the other. Detailed establishment of that possible relationship might give further explanations about the inconsistent data reported about IL-17 on the field of atherosclerosis.

Tregs

Regulatory T cells (Tregs) is a subset of T lymphocytes that recognize among others self-derived antigens in contrast to T effector subsets (Th1, Th2, Th17) and whose main role is to inhibit immune responses. The term Tregs refers to both natural Tregs (nTregs) that develop in the thymus constituting most of the Tregs in the circulation and inducible Tregs (iTregs) that are induced from naïve $CD4^+$T cells in secondary lymphoid organs. In mice Tregs are usually defined as $CD3^+CD4^+CD25^+CD127^{low}FoxP3^+$ with FoxP3 being the lineage-related transcriptional factor. However is has been suggested that not all $FoxP3^+$ T cells are functional Tregs and that T cell induced suppression of immune responses can be induced also in the absence of FoxP3 [132]. The suppressive capacity of Tregs is mediated through a plethora of molecules such as CD25 that captures IL-2 preventing T effector cell replication, CTLA4 which binds CD80 and CD86 on APCs as well as through IL-10, TGF-β and IL-35 expression [132].

Interestingly, Treg depletion in $apoE^{-/-}$ mice by CD25 Ab treatment increased lesion development [133] while treatment with IL-2/anti-IL2 Ab complexes (a preparation which is known to enhance IL-2 binding to CD25) expanded the numbers of Tregs and attenuated atherosclerosis [134].

Cytotoxic T cells

$CD8^+$ T cytotoxic (Tc) cells recognize cytosolic-derived peptides loaded on MHC class I molecules and upon encounter they kill the antigen-expressing cell via a perforin/granzyme B mediated mechanism. These cells have evolved in order to combat viruses, fungi, intracellular bacteria as well as cancer cells. Additionally, through IFNγ production they have been shown to activate macrophages [135].

Interestingly, in human atherosclerotic lesions they were reported to comprise almost half of the T cell population [136] while in mice in the absence of certain immunosuppressive pathways (programmed death ligand, PD-L1/2 deficiency) they accumulate in atherosclerotic plaques, alongside with $CD4^+$ T cells, contributing to disease development [137]. In a recent study, $CD8^+$ T cell depletion was shown to reduce atherosclerosis in $apoE^{-/-}$ mice which was

accompanied by reduced plaque inflammation and cell apoptosis [138]. The peptides that $CD8^+$T cells respond to in atherosclerosis are still unknown.

B2 cells

B2 cells include follicular B cells participating in adaptive immune responses and marginal zone B cells participating in innate immune responses as previously mentioned. Follicular B cells are cells that home mainly in the spleen and lymph nodes where they communicate with T helper cells. Upon activation they have the choice to become plasma cells serving as an antibody pool or become memory B cells that will produce antigen specific antibodies upon re-exposure to the same antigen [139]. Marginal zone B cells reside in the spleen and by being positioned in the outer white pulp, in close proximity to the red, they have the opportunity to immediately respond to antigens from the blood that filters the spleen. Through their high CD1d expression these cells mediate lipid antigen presentation to invariant natural killer T cells (iNKTs) establishing them as a great source of lipid related antibodies [139].

The studies conducted until now assign an atherogenic role to B2 cells. In specific, CD20 Ab treatment of $apoE^{-/-}$ mice was reported to predominantly deplete B2 cells leading to decreased atherosclerosis and lesional macrophages while B2 cell transfer to $apoE^{-/-}$ mice aggravated the disease and increased macrophage accumulation in plaques [140]. Upon B2 cell depletion total IgGs as well as oxLDL-specific IgGs decreased, the last happening for the mice that were for the longest period on HFD and treated at the initiation of the disease [140].

In another study CD20 Ab treatment of hypercholesterolaemic mice reduced atherosclerosis as well as systemic T cell activation and T cell accumulation in plaques [141]. Moreover this type of treatment switched the cytokine balance in the spleen since decreased IFNγ- and increased IL-17- expressing $CD4^+$T cells were observed [141]. CD20 Ab treatment additionally led to decreased levels of oxLDL-specific IgGs while oxLDL-specific IgMs showed a much lower reduction being in accordance with the fact that CD20 Ab treatment mainly depletes B2 and not B1 cells [141].

Additionally, genetic deficiency of the BAFF receptor, abrogating BAFF signaling which is important for B cell survival was reported to mainly affect B2 cell development and was utilized to test the effect of B2 cells in atherosclerosis [142]. In particular bone marrow transfer from BAFF receptor deficient mice ($BAFF\text{-}R^{-/-}$) to $LDLr^{-/-}$ mice reduced atherosclerotic plaque development and T cell plaque infiltration as well as well as T cell activation and T-effector cell proliferation in the spleen. Interestingly plasma IgG1 and IgG2c but not IgM antibodies to MDA-LDL were significantly reduced in mice that were reconstituted with $BAFF\text{-}R^{-/-}$ bone marrow compared to control mice [142].

One thing that seems to be common in the above mentioned studies is the fact that to a bigger or to a lesser extent B2 cell depletion and atheroprotection is accompanied by decreased IgG as well as anti-oxLDL IgG antibodies. Without being necessary as such, one could speculate that either B2 cells contribute in the promotion of atherosclerosis through the secretion of proatherogenic IgGs or through the binding of IgGs to Fcγ receptors which has previously been shown to be pro-atherogenic [143]. Another interesting aspect is that upon B2 cell depletion decreased T cells in the plaques as well as in the periphery of mice were observed indicating that B2 cells potently regulate T cell function.

Bregs – Costimulatory pathways in B cells

The main characteristic of regulatory B cells is the production of IL-10 and their immunosuppressive function [144]. In atherosclerosis IL-10 has been shown to be atheroprotective [145]. IL-10 deficiency was reported to increase lesion formation and to create an unstable plaque phenotype characterized by increased T cell infiltration, decreased collagen content and increased lesional IFNγ expression. Accordingly, IL-10 administration reduced atherosclerosis [145]. Until now the direct effect of Breg derived IL-10 has not been addressed in atherosclerosis.

The identification of B cell subsets secreting IL-10 and being immunosuppressive has been reported in experimental autoimmune encephalomyelitis (EAE), collagen induced arthritis (CIA) and colitis [146-148]. These cells have been characterized throughout the years as expressing various cell surface markers [144], both shared from B1 and B2 cells (Table 1) making quite difficult the firm definition of their phenotype. Besides IL-10 production from Bregs, the induction of such cells requires activation through CD40 [146]. The fact that both IL-10 as well as CD40 signaling lead to both B cell maturation and antibody production [149, 150] seems to be in direct contrast with the reported immunosuppressive functions of Bregs. Additionally, most of the markers used to describe Bregs are used to describe B1a cells as well making things even more complex while still no transcription factor has been assigned to them. Nonetheless, even if we cannot still firmly categorize and define them, the existence of B cell subsets that have immunosuppressive functions is a fact [144].

Interaction of B cell expressed CD40 with the CD40 ligand (CD40L) in T cells induces B cell maturation and antibody secretion *in vivo* [151] while when the engagement is prolonged and stronger in terms of signaling this results in inhibition of antibody secretion [152]. Mice that have B cell-specific CD40 deficiency suffer from severe EAE accompanied by decreased levels of IL-10 and increased IFNγ expression in the spleen [146]. Additionally, administration of agonistic anti-CD40 has been shown to decrease arthritis, inhibit IFNγ and enhance IL-10 production in mice [153]. A few years later it was reported that

treatment of splenic B cells with agonistic CD40 gives rise to a B cell population that produce high levels of IL-10 and low levels of IFNγ [147]. Additionally, transfer of these cells into a mouse model of arthritis prevented and decreased the disease via IL-10 secretion [147].

However, the role of the CD80/CD86 pathway in B cell mediated immune responses does not point to a specific direction. B cell-derived CD80/CD86 interactions as well as IL-10 have been shown to modulate Th1 cell differentiation [154]. In experimental arthritis the expression of CD80/CD86 by B cells was reported to be essential for the activation of autoreactive $CD4^+$T cells [155] while in other studies signaling through these molecules in B cells has been associated with suppression of immune responses [156, 157].

Interleukin-25

Interleukin-25 (IL-25 or IL-17E) is a member of the IL-17 cytokine family comprised by 6 cytokines, from IL-17A to IL-17F. IL-25 shares 16% homology with IL-17A and 80% homology with its human analogue [158]. Briefly, until now IL-25 has been shown to be produced by epithelial cells [159, 160], endothelial cells [161], mast cells [162], eosinophils [163], basophils [163], alveolar macrophages [164], microglia [165], $CD4^+$ and $CD8^+$ T cells [166], skin resident monocyte-derived DCs [167] and Th2 cells [74]. The interleukin's target cell populations are monocytes/macrophages [168, 169], DCs [165], T cells [170], natural killer (NK) T cells [171, 172], epithelial cells [172], ILC2s [77-79] and type 2 MPPs [83].

IL-17RA and IL-17RB comprise the functional heterodimer receptor through which IL-25 signaling is mediated. Although it has been shown that IL-25 binds only to IL-17RB, IL-17RA is still important in signal transduction since mice that are deficient either to IL-17RA or IL-17RB do not respond to IL-25 [173] (reviewed by Gafen S.L.). Upon binding of IL-25 to IL-17RB, Act-1 is initially recruited, followed by TRAF6, leading to the activation of NF-κB, MAPK-AP-1, C/EBP signaling pathways [174]. Amongst the two heterodimers, only IL-17RB contains the cytoplasmic TRAF6-binding motif enabling it to directly interact with TRAF6 (regardless of IL-25 binding) and activate merely NF-κB signaling only upon IL-25 binding.

The cytokine's reported functions include the promotion of type 2 immunity, the induction of Th2 and Th9 immune responses as well as the reduction of Th1 and Th17 immune responses in the different settings of the diseases tested. As extensively discussed in a previous section (ILC2s), IL-25 has been reported to be a potent inducer of ILC2s and type 2 MPPs [77-79, 83] which secrete large amounts of Th2 related cytokines (IL-5, IL-13) mediating type 2 immune

responses that are both beneficial for helminthic expulsion [77-79, 83] and wound healing [93] while pathogenic in allergy and asthma [90].

In specific, upon helminthic infection the IL-25-induced type 2 immune responses downstream the recruitment of ILC2s and the production of Th2-related cytokines are enhanced goblet cell hyperplasia, mucus production and eosinophilia (for more information the reader is encouraged to read the section named "ILC2s"). One of the studies that further support the important role of IL-25 in the eradication of helminthic infections as well as its possible role as a regulator of Th1 and Th17 immune responses reported that IL-25$^{-/-}$ mice fail to develop a type 2 immune response and eradicate helminthic infection, accompanied by increased production of IFNγ and IL-17 by MLN cells [166].

Following the pathway of Th1 and Th17 regulation by IL-25, IL-25$^{-/-}$ mice were reported to be susceptible to EAE which is an experimental model of multiple sclerosis. Interestingly, this was accompanied by increased levels of IL-23 in the periphery of the mice as well as increased IL-17, IFNγ and TNF producing T cells invading the central nervous system [165]. IL-25 has also been reported to decrease Th1 and Th17 (IL-12 and IL-23) cytokine production from CD14^{+} cells of patients suffering from Crohn's disease (CD) and to reduce experimental colitis in mice [168]. Moreover, IL-25 treatment of NOD mice suffering from autoimmune diabetes, regulated disease progression, reduced splenic Th17 cells and increased the frequency of Tregs in pancreatic lymph nodes [175].

As mentioned previously, a pathogenic role has been assigned to IL-25 concerning allergic disorders. This is due to the fact that IL-25 through the secretion of Th2 cytokines from auxiliary cells (ILC2s, T cells) leads to the recruitment of eosinophils and mast cells which is accompanied by increased serum IgE levels, goblet cell hyperplasia and mucus production. Intranasal, IL-25 administration in mice results in the induction of an asthma phenotype due to increased accumulation of IL-5 and IL-13 expressing ILC2s in the lungs and broncho-alveolar lavage (BAL) fluid of the mice [90]. Additionally, in airway hypersensitivity response (AHR), representing an experimental model of asthma, a fraction of CD4^{+} NKT cells expressing the IL-25 receptor (IL-17RB) was reported to secrete Th2 cytokines and especially IL-13 in response to *in vitro* stimulation with IL-25 and to be implicated in the pathogenesis of the disease [172]. Accordingly, in another study IL-25 blockade was reported to prevent AHR which was accompanied by reduced IL-5 and IL-13 levels in mediastinal lymph nodes, reduced goblet cell hyperplasia and eosinophil infiltration in the airways as well as reduced serum IgE levels [176].

IL-25 has also been reported to regulate IL-9 which is considered as a Th2 cytokine both *in vitro* and *in vivo* [177]. In specific, *in vitro* induced IL-9 expressing T cells, obtained upon incubation of T cells with TGF-β and IL-4, expressed high levels of the IL-25 receptor and upon interaction with IL-25

secreted IL-9. Moreover, in the same study IL-25-deficiency in a mouse model of chronic allergic lung disease reduced IL-9 expression and airway inflammation. Since IL-9 was previously reported to contribute in airway inflammation and asthma [178] it seems that IL-25 can also contribute to that by regulating IL-9 secretion.

In regards to atherosclerosis, it has been reported that in human normal arteries IL-25 is expressed by endothelial cells and smooth muscle cells while in atherosclerotic plaques is also expressed by mature B cells and plasma cells [130]. However, the role of IL-25 on atherosclerosis development has not been previously studied.

Interleukin-33

IL-25 and IL-33 have both been reported to be implicated in the initiation of type 2 immune responses as well as in similar manners in anti-helminthic function and exacerbation of allergic lung inflammation as mentioned previously (sections named ILC2s, Interleukin-25). In order to compare, contrast and contemplate over these two cytokines I will also give a short description of IL-33 focusing on the cytokine's origin, cell-targets and anti-atherogenic properties.

As comprehensively reviewed by Pei et al [179], IL-33 is a member of the IL-1 family that includes IL-1 and IL-18. IL-33 is expressed by epithelial and endothelial cells in various organs but also from immune cells like macrophages and dendritic cells. The cytokine's receptor is a heterodimeric complex consisting of ST2 and the IL-1 receptor accessory protein (IL-1RAcP) which is expressed by immune cells like Th2 cells, ILC2s, mast cells, basophils, macrophages, DCs, $CD8^{+}$T cells and B cells as well as non-immune cells like endothelial, epithelial cells and fibroblasts. The main reported functions of IL-33 until now is the induction of type 2 immune responses through the production of IL-5 and IL-13 that have been shown to confer immunity to helminthes, exacerbation of allergic airway inflammation and atheroprotection.

In regards to the latter one, IL-33 was reported to reduce atherosclerosis through the production of IL-5 and oxLDL specific antibodies while IL-33 neutralization (through soluble ST2 administration) resulted in exacerbated atherosclerosis and increased Th1 immune responses [180].

In another study, IL-33 administration in mice was shown to promote cytokine production by vascular adipose tissue (VAT) resident ILC2s leading to rapid increases of tissue specific eosinophils and AAMs contributing to metabolic homeostasis and insulin sensitivity [95]. IL-33 was also reported to activate B1 cells both *in vivo* and *in vitro*, an effect that was accompanied by increased levels

of IL-5, IL-13 and IgM [181]. Additionally, upon administration of IL-33 with an IL-5 neutralizing Ab in wild type (WT) mice, IgM induction was abolished revealing a specific pathway: IL-33-induced IL-5 increases B1 cell-derived IgM levels through which oxazolone-induced contact sensitivity (CS) gets exacerbated [181].

Upon intraperitoneal administration of IL-33 in apoE$^{-/-}$ mice deficient in Id3 (helix-loop-helix protein inhibitor of differentiation 3), peritoneal and serum IL-5 levels were decreased compared to apoE$^{-/-}$ control mice indicating Id3 as a regulator of IL-33-induced IL-5 production [182]. The same study also reported the presence of natural helper cells (ILC2s) in periaortic adventitia and perivascular adipose tissue (PVAT) raising the interesting possibility that ILC2s can also expand topically upon IL-33 administration.

Methods

Various methods have been used in order to obtain the results reported in the present work but here I will focus in some of them that are worth being discussed in more detail.

Malmö Diet and Cancer Study

The Malmö Diet and Cancer Study (MDCS) is a prospective cohort (n=28449) study examining the association between diet and cancer. Subjects born between 1926 and 1945 and living in Malmö were eligible for inclusion in the study. Between October 1991 and February 1994, every other participant was also invited to take part in a sub-study focusing on cardiovascular risk (MDCS cardiovascular cohort, n=6103). We randomly selected 700 subjects participating in the cardiovascular cohort of MDCS, aged 63 to 68 years old in order to study possible associations between B cell subsets with cardiovascular events or carotid IMT.

At baseline examination participants were asked to provide, among others, information about current or previous diseases, lifestyle habits and medication. Blood was collected and mononuclear leukocytes were isolated and stored at -140 ^{o}C while plasma and sera were stored at -80^{o}C. Analysis of common and bulb carotid intima-media thickness (IMT) was performed by B mode ultrasound.

Participants were followed from baseline examination until first event of CVD, emigration from Sweden or death, up until December 31st, 2008. A CVD event was defined as a fatal or non-fatal MI, fatal or non-fatal stroke, or death attributable to underlying CHD, whichever came first. Throughout the follow-up period 150 incident first event CVD cases (84 coronary events and 66 strokes) were recorded.

In order to investigate possible associations of B cell subsets with CVD events or carotid IMT, the mononuclear cells that were stored at -140 ^{o}C were thawed and stained with fluorochrome-labeled antibodies for flow cytometric analysis. This procedure took approximately a year to be completed for all the 700 individuals of this study. The antibodies used were FITC-anti-CD19, FITC-anti-CD14, PerCP-biotin-anti-CD3, PerCP-biotin-anti-CD56, APC-anti-CD40, PB-anti-CD86,

AF700-HLA-DR and PE-B7-H2 (ICOSL). The viability stain 7-Aminoactinomycin D (7AAD) was added in order to detect dead or dying cells. More than 90% of the thawed cells were viable. Stained cells were fixed in 1% paraformadehyde and measured on a CyAn ADP flow cytometer (Beckman Coulter). The analysis was performed with FlowJo7.6 software (Tree Star). The same FITC-channel was used for the $CD14^+$ and $CD19^+$ cells and the lymphocytes were gated based on forward and side scatter parameters. Alive B cells identified as $7AAD^-$, $CD3^-$, $CD56^-$ and $CD19^+$ cells were further gated based on their expression of CD40 or CD86.

Because we wanted to investigate possible associations between B cell subsets and cytokine release from mononuclear cells as well, mononuclear cells were stimulated with CD3/CD28 beads for 72 hours at 37°C. Thereafter, the cell supernatants were stored at -80°C until cytokine analysis with multiplex technology (MesoScale Discovery).

Mouse models

$ApoE^{-/-}$ alongside with $LDLr^{-/-}$ mice are the most commonly used animal models of experimental atherosclerosis. ApoB100-containing particles (LDL) bind to the LDL receptor (LDLr) while particles that mainly consist of apoB48 deliver their cholesterol via binding to apoE. Thus, deficiency of either apoE or LDLr results in different lipoprotein profiles. Mice that lack LDLr accumulate LDL particles while apoE deficiency results in accumulation of larger lipoprotein particles like CMs and CMRs [183]. The $apoE^{-/-}$ mouse model that has been utilized in the present work has been reported to develop complex atherosclerotic plaques even when fed chow diet (cholesterol levels 300-500 mg/dL) while atherosclerotic plaque formation is further enhanced upon feeding with lipid-rich diet (cholesterol levels can exceed 1000 mg/dl) [183]. In specific, $apoE^{-/-}$ mice on normal diet develop in the aortic root foam cell lesions at 8-10 weeks of age, after 15 weeks moderate plaques are formed while beyond 20 weeks atherosclerotic plaques with fibrous cap formation takes place [183]. This procedure is further accelerated on high fat diet were advanced plaques containing necrotic cores and calcifications are evident. In order to study atherosclerosis, we fed $apoE^{-/-}$ mice (on C57BL/6 background) a high fat diet (HFD; 0.15% cholesterol and 21% cocoa butter fat) for 14-15 weeks. We observed atherosclerotic plaque formation in the aortic root as well as in the aorta of the mice, with most of the atherosclerotic burden located in the aortic arch.

Another mouse model that has been used in the present study is the $apoE^{-/-}$ $Rag1^{-/-}$ ($apoE^{-/-}$ and $Rag1^{-/-}$ on C57BL/6 background, crossed in-house). Generally, the recombination activating genes RAG1 and RAG2 are implicated in the

recombination of immunoglobulin gene sequences and T cell receptor sequences. Thus genetic deficiency of either one, results in the production of non-functional B and T cells. More in detail, $Rag1^{-/-}$ mice have been reported not to have $CD3^{+}$ or TCR $\alpha\beta^{+}$ cells while neither the spleen nor the bone marrow contain any IgM or IgD positive cells indicating absence of mature B cells [184]. Deficiency in both Rag1 and apoE genes generates a hypercholesterolaemic mouse model that lacks adaptive immunity.

Mice deficient in IL-5 have also been utilized in the present work. $IL\text{-}5^{-/-}$ mice have been reported to have no defects in B2 cell development, T cell dependent antibody production or cytotoxic T cell responses [185]. However, eosinophilia is impaired upon infection while these mice exhibit impaired $CD5^{+}$ B1 cell development [185].

IL-25 / Anti-IL25 administration - Atherosclerosis

Treatment patterns as well as dosages in order to study the effect of IL-25 and IL-25 neutralization were determined based on previous studies [74, 165, 175]. In specific, Fort et al [74] reported that daily treatment of C57BL/6 mice with 1μg IL-25 (intraperitoneally, i.p.) for approximately a week efficiently induced the induction of Th2 induced eosinophilia. Additionally Emamaullee et al [175] studying the effect of IL-25 in autoimmune diabetes treated NOD mice with daily subcutaneous (s.c.) injections of 1μg IL-25 for 25 days or with i.p. injections of anti-IL17 (100 μg) on alternating days over a period of 12 days. Lastly, Kleinschek at al treated WT mice suffering from EAE with recombinant mouse IL-25 subcutaneously for 12-15 days (the dosage used was not reported) and with neutralizing antibodies against IFNγ (200μg) and IL-17 (1 mg) once a week for ~3 weeks.

Since we wanted to study both the effect of IL-25 and IL-25 blockade at the initiation of atherosclerosis as well as on already established disease we decided to treat young $apoE^{-/-}$ mice for 4 weeks (at the beginning of HFD) or old $apoE^{-/-}$ mice with already established disease for the same time period (last 4 weeks of HFD). The dose chosen for recombinant mouse (rm) IL-25 was 1μg/day or equal volume of control medium (C.M., 4mM HCL)/day (s.c.) and for anti-IL25 as well as the isotype control Ab (Rat IgG1,κ) was 100 μg/week (i.p.).

In the case of rmIL-25 (or C.M.), in order to avoid the great stress that we would induce to the mice if we were to inject them daily we decided to surgically place osmotic pumps subcutaneously (model 1004, ALZET, DURECT Corporation, Cupertino, CA) that delivered the desired amount of substance over the period needed. Specifically, in the experimental session that we investigated the effect of the cytokine at the initiation of the disease we removed the pumps upon expiration

of the treatment period and the mice went on HFD until the termination date, 11 weeks later. When studying the effect of IL-25 on already established disease the pumps were implanted the last 4 weeks of the experimental session and were removed upon euthanization of the mice.

Flow cytometry - Fluorescence activated cell sorting (FACS)

Flow cytometry (FC) is a method that has been extensively used in the present work. It is a powerful technique enabling cell population-profiling that may include analysis of intracellular or extracellular markers on cells, investigation of cell proliferation rates, cell apoptosis/necrosis.

Flow cytometric analysis begins with labeling of the cells with fluorochrome-"carrying" antibodies. This can happen both extracellular and intracellular. Each type of antibody, carrying a specific fluorochrome "targets" a specific type of protein/molecule on the cells. The final step includes the analysis of the stained cells with the flow cytometer. Here, the operator can distinguish the cell populations of interest from the "pool" of cells in the sample according to the antibodies used and also quantify the expression of markers on them or on the whole cell population.

More in detail, upon acquisition of the sample in the flow cytometer, the cell suspension is processed in such a way so that single cells could be able to "get hit" by the beam of laser(s) existing in the flow cytometer. The reason for that is that through excitation of the fluorochromes attached on the cells and subsequent emission of light (fluorescence), the respective detectors are able to receive that light and through a computational system translate it into a signal that the operator can see and quantify. The signal obtained in a specific detector/channel is in reality the relative expression of a marker of interest on the cell population tested.

A sister-technique to FC is the fluorescence activated cell sorting (FACS). The principle is the same with the only difference being that the operator has the opportunity to actually obtain the cell population of interest and not just observe and analyze its computational data.

Briefly, the "fluorochrome-labeled" cell suspension is processed in a single-cell flow stream as described previously. Next, each cell undergoes analysis of its fluorescent profile. Further on, a vibrating mechanism separates the stream of cells in droplets. One cell per droplet is the aim. But just at the point where the stream breaks into droplets, the latter ones get charged according to the fluorescent profile of the cell that they contain. The charged droplets then get diverted with the use of

an electrostatic deflection system into containers according to their charge. The sorted cell populations can then be used for *in vivo* and/or *in vitro* purposes.

Isolation of ILC2s – Adoptive transfer into apoE$^{-/-}$ mice

ILC2s were analyzed in the spleens of apoE$^{-/-}$, apoE$^{-/-}$Rag1$^{-/-}$, IL-5$^{-/-}$ mice upon treatment with rmIL-25 or control medium. The same analysis also took place for apoE$^{-/-}$ mice treated with anti-IL25 or control antibody. Upon preparation of single cell suspensions, ILC2s were immunomagnetically enriched with the use of a "cocktail" of antibodies targeting lineage-related markers (CD3, CD4, CD8, CD11b, CD11c, CD45R, CD19, Gr-1, FcεRI, NK 1.1 and Ter-119). The resultant fraction of cells was enriched in innate lymphoid cells. Next with the use of markers that nuocytes (ILC2s) were previously reported to express [78], further analysis of the lineage depleted cells took place flow cytometrically. These cells were defined as Lin^{-}CD45^{+}IL-17RB^{+}ICOS^{+}IL7ra^{intermediate}.

Additionally, FACS sorting of ILC2s upon lineage depletion took place in order to further characterize them in terms of cytokine expression as well as to perform adoptive transfer of ILC2s into apoE$^{-/-}$ mice.

For cytokine characterization of ILC2s, apoE$^{-/-}$ mice were injected daily with 1μg of rmIL-25 for 7 days. Thereafter the mice were killed, spleens were dissected, ILC2s were enriched by immunomagnetic depletion of lineage positive cells, FACS sorted as Lin^{-}CD45^{+}IL17RB^{+}ICOS^{+}IL7ra^{intermediate} and cultured *in vitro* for 7 and 14 days in the presence of IL-7 (10ng/mL) and IL-33 (10ng/mL). The cell culture medium was renewed every other day. Cytokine analysis of cell culture supernatants at days 7 and 14 took place revealing a previously reported cytokine profile for ILC2s, mainly characterized by high levels of IL-5 and IL-13. Interestingly, even after 14 days of culture the isolated cells retained their cytokine-related characteristics. For the cells that were cultured for 7 days flow cytometric analysis of IL-5 expression also took place revealing that ~70% of the cell population expressed IL-5.

For ILC2 transfer experiments, apoE$^{-/-}$ mice were injected daily with 1μg of rmIL-25 for 7 days. Next, splenic ILC2s were enriched by immunomagnetic depletion of lineage cells, FACS sorted as Lin^{-}CD45^{+}IL17RB^{+}ICOS^{+}IL7ra^{intermediate} and expanded *in vitro* for 9 days in the presence of IL-7 (10ng/mL) and IL-33 (10ng/mL). ILC2s were counted and 0.5 x 10^{6} cells (resuspended in PBS) were transferred i.p. to apoE$^{-/-}$ mice.

Treatment of human PBMCs with IL-25

Human blood leukocyte filters from healthy donors were obtained from the regional Center of Blood (Skane University Hospital, Lund, Sweden) and peripheral blood mononuclear cells (PBMCs) were isolated from them as described previously [186]. Briefly, cells trapped in leukocyte filters were backflushed with 150 ml of PBS (without Ca^{2+}, Mg^{2+}) containing EDTA-Na_2 (5mmol/L) and sucrose 2.5% (w/v). The eluted cells were layered in Ficoll solution and centrifuged at 445xg at 21^{o}C for 35 min. At the end of centrifugation the white layer of mononuclear cells was collected and washed with PBS twice. Next erythrocytes were lysed, PBMCs were washed extensively and were further cultured *in vitro* or used for immunomagnetic isolations of $CD3^+$ T cells or DCs.

The concentration of IL-25 that was used for *in vitro* incubation with PBMCs was in the same range used in previously reported studies investigating the effect of IL-25 on human cells [84, 163, 169, 170]. Specifically, 10^6 cells/mL were incubated with different concentrations (0-100ng/ml) of recombinant human (rh)IL-25 for 20h. Incubation of cells with 4 mmol/L HCL containing 1% bovine serum albumin, the vehicle for rhIL-25, served as a control. PBMCs were also incubated with 10 μg/mL Cu^{2+}-induced oxLDL alone or in combination with IL-25 or the vehicle. Further stimulation of the cells with either PMA, Ionomycin and Brefeldin A took place in order to investigate T helper, cytotoxic or regulatory cell subsets with flow cytometry. Additionally, stimulation with PMA and Ionomycin or CD3/CD28 beads was performed in order to assess cytokine release in cell culture supernatants.

Immunomagnetically isolated $CD3^+$ T cells (1 x 10^6 cells/mL) were incubated with different concentrations of IL-25 (0-50ng/ml) for 24h in the presence of CD3/CD28 and further stimulated with PMA, Ionomycin, and Brefeldin A for flow cytometric analysis. In parallel, stimulation with PMA and Ionomycin was performed for cytokine assessment. Moreover, immunomagnetically isolated DCs (0.5 x 10^6 cells/mL) were incubated with rhIL-25 (50ng/ml) for 20h and further stimulated with LPS for 24h for cytokine assessment.

Results and discussion

Paper I

In this study we investigated the influence of two different B cell subsets on the development of acute cardiovascular events in a prospective study cohort involving 700 randomly selected subjects from the cardiovascular arm of MDCS (Malmö Diet and Cancer Study) with a follow up time of approximately 15 years. B cell subsets were analyzed with the use of flow cytometry. One thing that should be kept in mind is that $CD19^+$ B cells and $CD14^+$ monocytes were stained with the same fluorochrome and then further distinguished according to their side scatter expression; $CD19^+$ cells as being side scatter low and $CD14^+$ cells as being side scatter high. In additional experiments we found that about 6% of the CD40 and CD86 expressing cells in the side scatter low gating are likely to be $CD14^+$ monocytes. We believe that although this represents a limitation in our study it does not significantly influences the conclusions drawn.

Our findings demonstrated an association between high levels of $CD19^+CD40^+$ cells and a decreased risk of stroke while high levels of $CD19^+CD86^+$ B cells were associated with an increased risk (figure 2). The risk was doubled in the presence of high numbers of the latter B cell subset and it seemed to be higher in men. The opposing associations of these B cell subsets with the risk of stroke imply that B cell mediated-immune pathways through CD40 and CD86 might have differential impacts on the possible development of future stroke events. Interestingly, naïve and memory B cells from patients with rheumatoid arthritis were reported to express higher percentage of CD86 than healthy controls [187]. Moreover, previous studies reported that stimulation of CD40 induces the development of immunosuppressive B cell subsets and that absence of CD40 makes B cells unable to regulate immune responses [146, 154]. The existence of B cell subsets with immunosuppressive properties have previously been reported in humans [154, 156] and it will be of interest in future studies to investigate possible associations of the B cell subsets that we report with those.

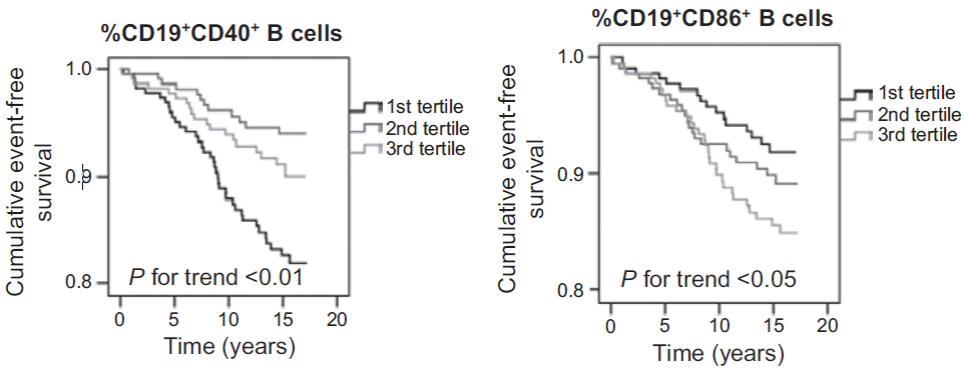

Figure 2. Stroke-event free survival of %CD19$^+$CD40$^+$ and CD19$^+$CD86$^+$ B cells in tertiles during a 15-year follow-up. High levels of CD19$^+$CD86$^+$ B cells (3rd tertile) and low levels of CD19$^+$CD40$^+$ B cells (1st tertile) are associated with an increased risk of stroke.

The fact that only stroke and not coronary artery disease was associated with the reported B cell subsets in our study might indicate that the immune pathways that these B cell subsets serve might have a greater impact on cerebrovascular CVD. The mechanistic background of such observations of course cannot be explained by a clinical study, but such data can always give the initiative for further experimental investigation.

Moreover, mononuclear-derived IL-10 correlated with the fraction of CD19$^+$CD40$^+$ B cells while CD19$^+$CD86$^+$ B cells were associated with a more pro-inflammatory cytokine profile. These data support the notion that CD40 signaling is important in immunosuppressive B cells and that CD86 is important for the induction of Th1 cells [146, 154]. Another interesting observation was the fact that even though B cell subsets correlated strongly with many of the cytokines tested very few associations between the released cytokines and the fraction of CD3$^+$ T cells were detected. These findings indicate that B cells might have a greater impact in regulating cytokine release than T cells.

Overall, this is the first prospective clinical study reporting involvement of B cell subsets in cardiovascular disease.

Paper II

The aim of this study was the investigation of the effect of IL-25 on atherosclerosis. For that reason apoE$^{-/-}$ mice were treated with IL-25 at the initiation of atherosclerosis as well as on already established disease. Treatment with IL-25 inhibited in both treatment patterns atherosclerotic plaque formation in the aortas of the mice.

Upon IL25 treatment of mice with already established plaques, the inhibition of the disease was accompanied by increased numbers of ILC2s gated as Lin^- $CD45^+IL17RB^+ICOS^+IL7ra^{intermediate}$, as well as IgM antibodies targeting PC an epitope expressed on oxLDL [71]. The reason that we did not observe any analogous differences upon IL-25 treatment of mice at the initiation of plaque formation is probably due to the fact that the mice were treated shortly at the initiation of the disease but were left untreated for another 11 weeks until termination. Because of that, it is quite possible that the immune system had the time to get back into a "ground-level" of immunological status still retaining the induced atheroprotection due to early treatment with IL-25.

In order to further investigate the IL-25-induced immunological pathway we treated young and old $apoE^{-/-}$ mice with daily injections of IL-25 for a week. In both cases there were dramatic increases of splenic ILC2s and IL-5 levels (plasma and spleen) accompanied by increased plasma anti-PC IgM levels.

We next questioned whether ILC2s were indeed responsible for the increased IL-5 levels that were observed. For that reason we used the $apoE^{-/-}Rag1^{-/-}$ mouse model that lacks functional cells of the adaptive immunity (B and T cells). Even shorter treatment of these mice with IL-25 (1μg IL-25 every second day instead of every day for a week as happened for $apoE^{-/-}$), IL-5 levels were increased to comparable levels. This implies that ILC2s are indeed a great producer of IL-5 upon IL-25 treatment. In the $apoE^{-/-}Rag^{-/-}$ mouse model two other innate cell populations could have responded to IL-25; basophils and mast cells. We cannot exclude the possibility that part of the increased IL-5 levels could be coming from an interaction of the cytokine with these cells. But the fact that FACS sorted ILC2s from $apoE^{-/-}$ mice expressed extremely high levels of IL-5 compared to $lineage^+$ IL25 responsive cells (sorted as $Lin^+IL17RB^+$) favors our hypothesis.

We next questioned whether ILC2-derived IL-5 could be responsible for the increased anti-PC IgM levels. For that reason we utilized the mouse model of IL-5 deficiency ($IL5^{-/-}$). Upon treatment with IL-25 we observed an increase of ILC2s comparable to the one in $apoE^{-/-}$ mice, but no difference in anti-PC IgM. The aforementioned experiments show that IL-5 is not necessary for the expansion of ILC2s but it is crucial for induction of anti-PC IgM. Collectively, the aforementioned experiments indicate that ILC2s is the main responder of IL-25 and the main producers of IL-5 which is necessary for the induction of the atheroprotective anti-PC IgM (figure 3).

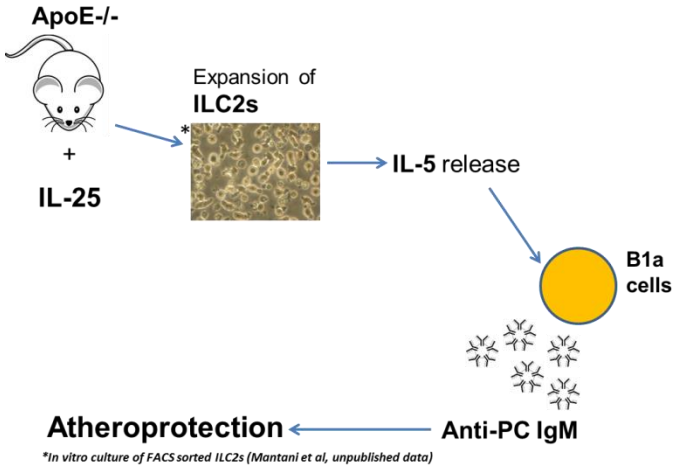

Figure 3. Proposed mechanism of IL-25 induced atheroprotection in apoE$^{-/-}$ mice. Upon IL-25 administration, ILC2-derived IL-5 induces the production of IgM antibodies targeting phsphoryl choline (PC).

In order to investigate the axis between ILC2s and B1a cells we FACS sorted ILC2s, expanded them *in vitro* in order to get a good number of cells and next transferred them intraperitoneally into apoE$^{-/-}$ mice. The reason for transferring ILC2s i.p. is that the peritoneum is a location that most of the B1a cells reside [188]. Three days later, we checked the peritoneum and spleen of the ILC2 recipients for B1a cells. We were not able to detect any B1a cell increases in the peritoneum but we detected increased frequencies of B1a cells in the spleen. This pattern of B1a cell stimulation is in accordance with previous reports. IL-5 injection of mice in the peritoneal cavity has been reported to make B1a cells to migrate from the peritoneal cavity to the spleen [57]. Given that ILC2s serve as an IL-5 pool the latter one possibly have induced such a migration of the cells to the spleen. Conclusively, we suggest that IL-25 through the expansion of ILC2-derived IL-5 activates B1a cells to secrete anti-PC IgM conferring atheroprotection. Although such a mechanism might possibly be taking place topically in the plaque, a parameter that we did not test in our study, it is still a fact that the existence of circulating anti-oxLDL IgM has previously been reported to be associated with atheroprotection in mice [123, 180] and with lower risk for CVD events in humans [72].

Paper III

In this study we questioned whether IL-25 blockade can affect atherosclerosis development in apoE$^{-/-}$ mice both at the initiation of atherosclerosis but on already manifested disease as well.

Anti-IL25 treatment during the early stage of plaque formation aggravated atherosclerosis development in the aortic arch of the mice, a location that most of the atherosclerotic burden is usually observed (figure 4). Moreover, decreased smooth muscle cell content was observed in plaques of aortic root sections indicating increased plaque instability (figure 4). However, no differences in collagen content could be observed. Previously, it has been shown that atherosclerotic plaque development is influenced by abnormal plaque collagen fibril structure [189]. Thus, it could be of interest to evaluate if IL-25 blockade affects the structure of collagen. Aggravation of the disease was also accompanied by a predominance of Th1/Th17 cytokines in the spleen as well as reduced Th2 cytokines in plasma. Previous studies have also associated Th1 and Th17 related cytokines with increased lesion development and plaque instability [113-116, 126].

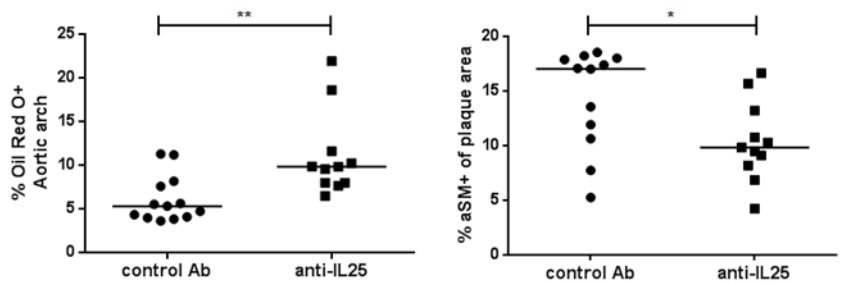

Figure 4. IL-25 blockade at the initiation of plaque formation aggravates disease progression and increases plaque instability in apoE$^{-/-}$ mice.

However anti-IL25 treatment had no effect on already established plaques. The difference in disease out-come depending on the time-point of IL-25 blockade might indicate that disturbance of the cytokine balance into more Th1/Th17 at the initiation of plaque formation is more potent in accelerating disease progression in contrast to already established disease.

Paper IV

The effect of IL-25 on human peripheral blood mononuclear cells (hPBMCs) from healthy individuals was the main focus of this study. Different T cell subsets were analyzed with the use of flow cytometry upon incubation of hPBMCs with IL-25. A consistent effect that was observed after such treatment was decreased frequency of Th17 cells (gated as $CD3^{+}CD4^{+}IL17^{+}$ cells). In order to further investigate this pathway, immunomagnetic isolations of $CD3^{+}$T and dendritic cells (DCs) and subsequent incubations with IL-25 took place. IL-25 treatment of $CD3^{+}$T cells did not affect $CD3^{+}IL17^{+}$ cells indicating that the observed effect of Th17 decrease is not a direct effect of the cytokine on T cells. Therefore, we decided to examine the effects of IL-25 on DCs, a potent antigen presenting cell type that prime T cells and influences the immune responses in either a pro- or anti- inflammatory way depending on the respective environment. Interestingly, IL-25 reduced IL-6 and IL-23 production from DCs, cytokines that have been implicated in induction of human Th17 cells [103] (figure 5).

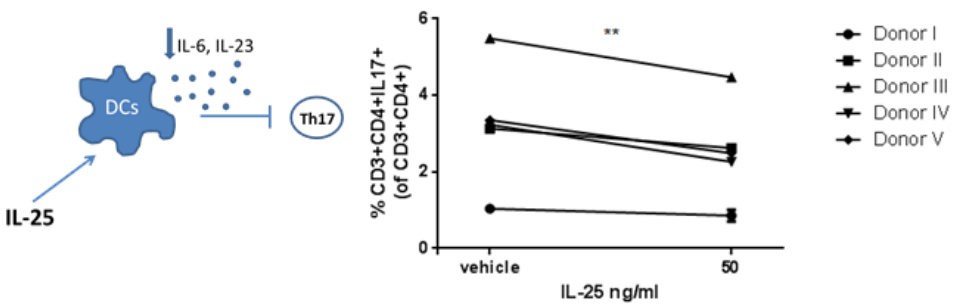

Figure 5. IL-25 reduces Th17 cells in peripheral blood mononuclear cells through inhibition of IL-6 and IL-23 secretion from dendritic cells (DCs).

The effect of IL-25 in the presence and in the absence of oxLDL that served as an atherosclerosis-related, pro-inflammatory stimulus, was also tested. Specifically, the release of IFNγ and IL-6 from hPBMCs was reduced by IL-25 both in the absence and presence of oxLDL. Additionally, IL-25 in the presence of oxLDL increased the release of IL-4 and IL-5 from hPBMCs but decreased IL-13 levels. Although the cell origin of the previously mentioned cytokines was not fully elucidated, some general conclusions can be made. In specific, IL-25 dampens Th17- and Th1- related immune responses in hPBMCs while in the presence of oxLDL it decreases Th1- and promotes Th2- related immune responses. Thus, this raises the interesting possibility that IL-25 might have a protective role in human atherosclerosis.

Conclusions and future perspectives

Alongside to the great need for therapies targeting atherosclerosis there is also a need for new biomarkers predicting the risk for future myocardial infarctions and strokes. The results of paper I, suggest that different B cell subsets could be used as potential biomarkers in order to predict a future stroke event. Because this is the very first study to report such associations more studies confirming the same results could reinforce the possible usage of such B cell subsets as biomarkers in the clinic.

Another purpose of the present work was the investigation of the role of IL-25 on atherosclerosis with the use of hypercholesterolaemic mouse models. In order to tackle this, the cytokine was both administered and blocked in apoE$^{-/-}$ mice. Interestingly, we observed an atheroprotective effect of the cytokine.

Regarding cytokine administration, the proposed mechanism of IL-25-induced atheroprotection implicates the increase of IgM antibodies targeting PC, a major epitope on oxLDL via ILC2-derived IL-5. We suggest that the essential trigger for this mechanism is the expansion of ILC2s. Adoptive transfers of FACS-sorted ILC2s from apoE$^{-/-}$ and IL-5$^{-/-}$ mice to apoE$^{-/-}$ mice will firmly answer to the hot question of whether ILC2s represent a cell subset that mediates protective immune responses in atherosclerosis. I am eagerly looking forward to that…

Additionally, IL-25 blockade at the onset of atherosclerosis led to the formation of increased plaques of unstable phenotype, accompanied by a Th1/Th17 shift of the cytokine balance in the periphery of the mice. It seems that signaling through IL-25 during early plaque formation possibly regulates protective immune mechanisms and that disruption of that signaling leads to the dominance of pro-inflammatory immune responses. Further work focusing on the cell origin of IL-25 *in vivo* and how cells behave upon such a blockade might give a better mechanistic insight. This can also be tested in a hypercholesterolaemic mouse model of genetic IL-25 deficiency like the apoE$^{-/-}$IL-25$^{-/-}$ mouse model that we are currently breeding in our facility.

Generally, it seems that administration of IL-25 induces atheroprotective innate immune responses whereas IL-25 blockade induces to some extent atherogenic adaptive immune responses. These findings will be targeted and further evaluated in the proposed experiments.

Next, the cytokine's effect on human mononuclear cells was tested. Interestingly, IL-25 treatment of human PBMCs decreased pro-inflammatory Th1/Th17 immune responses. Besides the reported IL-25-induced decrease of Th17 cells, it will also be interesting to investigate in more detail the cells that might participate in this pathway. Since IL-25 seems to mediate protective immune responses in atherosclerosis it will be interesting as well to test through clinical studies if it could be used as a future biomarker for cardiovascular disease.

Science for everyone

Atherosclerosis is a disease of medium and large sized vessels of our bodies that is mainly characterized by the deposition of lipids in the vessel walls. Increased lipid levels in the blood of an individual, because of lifestyle habits but also because of genetics, can lead to the formation of an "atherosclerotic plaque". Thus, although lipids are important for the normal function of cells, when in excess can lead to atherosclerosis.

As oil is insoluble in water the same thing happens with the lipids in our blood, making difficult their essential distribution to the various tissues. But our body has found a very efficient solution; the lipids "get packed" to form spherical particles, surrounded by a membrane that enables them to float freely in aqueous "solutions" such as the blood. These particles are called lipoproteins and according to their composition they are categorized into different types. Low density lipoprotein (LDL) particles have mostly been associated with atherosclerosis therefore LDL is known as the "bad cholesterol".

An atherosclerotic plaque is created when the lipids of the blood (mostly LDL) get trapped in the vessel wall and get retained at that location. This gradually increasing, un-physiological homing of lipids and cells leads to the formation of a gruel material that grows within the vascular wall making the vessel less flexible upon physical movement. If the individual that has such a plaque will not regulate his lipid levels through medication and change of lifestyle habits, there is an increased risk that the plaque will increase and rupture. When this happens the material of the plaque gets exposed to the blood flow leading to the formation of a blood clot. This clot can then stop the flow of the blood to the rest of the body and this may lead to death.

When plaques of the heart vessels get ruptured then the individual experiences a heart attack but when the same thing happens for a plaque of the carotid arteries then a stroke event takes place. Unfortunately, nowadays atherosclerosis is the leading cause of death worldwide. Statins are drugs that were discovered in the '70s and they act by decreasing lipid levels in the blood but cannot fully protect from atherosclerosis.

The last few decades, scientists working in the field of atherosclerosis have acknowledged that in addition to the contribution of lipids, the immune system also participates to the progression as well as the resolution of the disease. The

term “immune system” refers to specific cell types and proteins of our bodies which exist in order to protect us from viruses, infections and any microscopic “threat” that the body may encounter.

Some of these cells that work as “soldiers” protecting us, are called B cells, T helper cells and phagocytes. B cells can secrete substances called “antibodies” that can capture anything “unwanted” and remove it from the body. Phagocytes can “eat-up” harmful particles, bacteria and dead cells and remove them from the surroundings while T helper cells can communicate with other cells either through direct contact or by secretion of substances that neighboring cells perceive as signals, telling them to do something very specific.

When lipids increase in the blood and LDL accumulates in the arterial wall, the immune system realizes that something strange and unusual is going on. This accumulation and retention also leads to subsequent modification of LDL. Then the cells of the immune system recognize strange patterns on the modified lipoprotein particles and target them. This is a mechanism of protection but when the control is lost and the accumulation and modification of lipids is great then the promotion of “danger” signals from such cells can lead to increased inflammation and further damage of the arterial wall.

One way to possibly treat atherosclerosis is to try to direct the immune system in such a way so that the inflammation and subsequent disease-promoting immune responses in the atherosclerotic plaque are dampened. Scientists have attempted to do that many times with great success throughout the last decades through administration of specific immune cell types or substances in experimental animal models.

Interleukin-25 (IL-25) is a cytokine (protein) that normally exists in our bodies. In the present work, exogenous IL-25 was administered in mice that have increased lipid levels through genetic modification, thus being prone to atherosclerotic plaque formation. Interestingly, IL-25 administration could decrease atherosclerotic plaques in the aortas of both young and old mice, the latter ones having already established plaques (paper II). This means that IL-25 is effective in preventing to some extent the early formation of atherosclerotic plaques as well as decreasing the progression of already established plaques. We suggest that the way that IL-25 acts in order to confer this protective effect is through the induction of a cell type, the existence of which the scientific community was not aware of, until 2010. This cell type is named ILC2s (innate lymphoid cells type 2) and has the capability to secrete large amounts of another protein called IL-5. IL-5 works as a signal for B cells to get activated and secrete antibodies against unwanted particles which in our case are molecular patterns on modified LDL. By the generation of these antibodies, modified LDL particles get removed thus conferring protection from the disease.

In another set of experiments including the same mouse model we administered an antibody that blocked IL-25, leading to increased atherosclerosis (paper III). This result was observed only when we treated young mice that did not have established plaques. When older mice underwent this treatment we did not observe any difference in the formation of plaques. The results of these experiments indicate that endogenous IL-25 is important in the protection against atherosclerosis during early plaque formation since blockade of the protein led to increased atherosclerotic plaques only in young mice.

Since we established in mice that IL-25 protects against atherosclerosis via the induction of immune mechanisms, we also investigated the effect of the cytokine on human cells of the immune system (paper IV). For that reason we isolated mononuclear cells that exist in the blood of humans. When we incubated the cells *in vitro* (in the lab under sterile conditions) we saw that the cells secreted less pro-inflammatory cytokines. With the term pro-inflammatory we mean promoting inflammation and atherosclerosis-related immune responses. These results indicate that IL-25 can potentially have a protective role in human atherosclerosis.

In order to be able to treat atherosclerosis we also need to find ways to predict future events such as strokes and heart attacks. In paper I we have shown that the existence of specific B cell subsets can be related with increased or decreased risk of a future stroke event. We had the opportunity to characterize and quantify specific B cell subsets in the blood of 700 individuals that were randomly selected from a clinical study called "Malmö Diet and Cancer Study". In this particular study, 28449 individuals were recruited between 1991 and 1994. Some of these individuals were also asked to participate in a sub-study focusing on cardiovascular risk (number of individuals=6103). The 700 individuals that we included in our study (paper I) were randomly obtained from the 6103 participants.

All the participants were followed from baseline examination until the first cardiovascular event such as myocardial infarction (heart attack) or stroke either of them being fatal or non-fatal. Participants were followed until 2008. At the baseline examination, blood was collected from these individuals and the isolated mononuclear cells were frozen at -140^{o}C. In 2011 we thawed the cells that were obtained at the basal examination and analyzed them focusing on B cell subsets with a technique called "flow cytometry". We next had the opportunity to correlate the manifestation of strokes or myocardial infarctions with the amounts of the B cell subsets that were analyzed. Interestingly, we saw that high levels of $CD19^{+}CD86^{+}$ B cells correlated with an increased risk of a future stroke which was greater for men. Additionally, high levels of another B cell subset (identified as $CD19^{+}CD40^{+}$ B cells) were associated with a decreased risk of a future stroke event. This is the very first study to report an association between B cells and cardiovascular events. We hope that in the future other scientists will have similar observations so that this finding could actually be used in the clinic. If so, this would mean that with a blood sample the physician would be able to quantify the

levels of these B cell subsets and depending on the results, the patient could then be categorized as being either in a low or a high risk of experiencing a future stroke event. Patients at high risk will have to be monitored more closely and treated more aggressively in order to prevent such an event.

Επιστήμη για όλους

Η αθηροσκλήρωση είναι μια ασθένεια των αρτηριών του σώματος μας που χαρακτηρίζεται κυρίως από την εναπόθεση λιπιδίων στα αρτηριακά τοιχώματα. Αυξημένα λιπίδια στο αίμα ενός ανθρώπου είτε από κακό τρόπο ζωής (τροφή υψηλή σε λιπαρά, έλλειψη άσκησης, κάπνισμα) είτε λόγω γονιδίων είτε ακόμη από συνδυασμό των προηγουμένων, μπορεί να οδηγήσουν στο σχηματισμό των λεγόμενων «αθηροσκληρωτικών πλακών». Έτσι, παρόλο που τα λιπίδια είναι απαραίτητα για την φυσιολογική λειτουργία των κυττάρων, όταν αυτά βρίσκονται σε υψηλά επίπεδα τότε μπορούν να οδηγήσουν στην «έναρξη» της αθηροσκλήρωσης.

Όπως το λάδι είναι αδιάλυτο στο νερό το ίδιο συμβαίνει με τα λιπίδια του σώματος μας. Το γεγονός αυτό καθιστά δύσκολη την κατανομή τους στα διάφορα όργανα του σώματος μέσω του αίματος το όποιο περιέχει μεγάλη περιεκτικότητα σε νερό. Όμως η βιολογία του οργανισμού μας έχει βρει έναν έξυπνο τρόπο να διανέμει τα απαραίτητα λιπίδια μέσω των λεγόμενων λιποπρωτεϊνών. Οι λιποπρωτεΐνες είναι στην ουσία σωματίδια σφαιρικού σχήματος τα οποία αποτελούνται από «στοιβαγμένα» λιπίδια, τα οποία περιβάλλονται από μια υδρόφιλη μεμβράνη, η οποία τους δίνει την δυνατότητα να «ταξιδεύουν» ελεύθερα μέσω της κυκλοφορίας του αίματος και να «τροφοδοτούν» τα όργανα του σώματος. Γενικά, υπάρχουν διάφορα είδη λιποπρωτεϊνών τα οποία κατηγοριοποιούνται ανάλογα με την σύσταση των λιπιδίων τους. Τα χαμηλής πυκνότητας λιποπρωτεϊνικά σωματίδια (low density lipoprotein (LDL) particles) τα οποία αποτελούν την λεγόμενη χαμηλής πυκνότητας λιποπρωτεΐνη ή αλλιώς LDL ή κακή χοληστερόλη είναι αυτά που έχουν περισσότερο συσχετιστεί με την παθολογία της αθηροσκλήρωσης.

Η αθηροσκληρωτική πλάκα σχηματίζεται όταν τα λιπίδια του σώματος και ειδικά η LDL εισέρχεται στο αρτηριακό τοίχωμα και παραμένει εγκλωβισμένη εκεί. Η συνεχής, μη-φυσιολογική εναπόθεση λιποπρωτεϊνών και κυττάρων στη συγκεκριμένη τοποθεσία οδηγεί στο σχηματισμό ενός λιπιδιακά πλούσιου υλικού το οποίο αναπτύσσεται στον ιστό του αρτηριακού τοιχώματος καθιστώντας το δύσκαμπτο όταν αυτό αναταράσσεται από την ροή του αίματος. Εάν ο ασθενής που φέρει τέτοιες πλάκες δεν αλλάξει τον τρόπο ζωής του και δεν λάβει θεραπευτική αγωγή βρίσκεται σε υψηλό κίνδυνο περαιτέρω ανάπτυξης της αθηροσκληρωτικής πλάκας η οποία μπορεί πιθανά να διαρρηχτεί. Όταν αυτό συμβεί τότε το εσωτερικό, θρομβογενές υλικό της αθηροσκληρωτικής πλάκας

έρχεται σε άμεση επαφή με το αίμα καταλήγοντας στην πήξη του αίματος και στον σχηματισμό θρόμβου ο οποίος μπορεί να φράξει τη ροή του αίματος οδηγώντας σε πιθανό θάνατο.

Συνήθως όταν μια αθηροσκληρωτική πλάκα των αρτηριών της καρδιάς διαρρηχτεί τότε η λεγόμενη «ανακοπή καρδιάς» λαμβάνει χώρα ενώ όταν το ίδιο γεγονός συμβεί σε αθηροσκληρωτική πλάκα των καρωτίδων τότε αυτό μπορεί να οδηγήσει σε εγκεφαλικό επεισόδιο. Δυστυχώς τη σημερινή εποχή, η αθηροσκλήρωση είναι η πρώτη και κύρια αιτία θνησιμότητας παγκοσμίως. Οι στατίνες είναι δραστικές ουσίες που ανακαλύφθηκαν την δεκαετία του ΄70, οι οποίες «ρίχνουν» τα επίπεδα των λιπιδίων στο αίμα χωρίς όμως να παρέχουν πλήρη προστασία από την αθηροσκλήρωση.

Οι επιστήμονες που δουλεύουν στο πεδίο της αθηροσκλήρωσης έχουν ανακαλύψει τις τελευταίες δεκαετίες ότι πέραν της μεγάλης συμμετοχής των λιπιδίων στην έναρξη και ανάπτυξη της αθηροσκλήρωσης, υπάρχει επίσης μια ξεκάθαρη συμμετοχή του ανοσοποιητικού μας συστήματος σε αυτή την διαδικασία. Η συμμετοχή αυτή εμπερικλείει «μηχανισμούς» που μπορεί να οδηγήσουν είτε στην επιδείνωση της ασθένειας είτε στην εξάλειψη της. Ο όρος «ανοσοποιητικό σύστημα» αναφέρεται σε μια πλειάδα κυττάρων (και των επερχόμενων ενεργειών τους) τα οποία μας προστατεύουν από ιούς, λοιμώξεις και κάθε μικροσκοπική απειλή που το σώμα μας μπορεί να αντιμετωπίσει.

Κάποια από τα κύτταρα τα οποία εργάζονται σαν ταγμένοι στρατιώτες στο να μας προστατεύουν ονομάζονται Β κύτταρα, Τ κύτταρα και φαγοκύτταρα. Τα Β κύτταρα μπορούν -μεταξύ άλλων- να εκκρίνουν αντισώματα τα οποία δεσμεύουν οτιδήποτε ανεπιθύμητο για τον οργανισμό και να το απομακρύνουν. Τα φαγοκύτταρα μπορούν να «φάνε» επικίνδυνα για τον οργανισμό σωματίδια, βακτήρια και νεκρά κύτταρα απομακρύνοντας τα από το εκάστοτε περιβάλλον ενώ τα Τ λεμφοκύτταρα μπορούν να επικοινωνούν με άλλα κύτταρα είτε μέσω άμεσης επαφής είτε με την έκκριση ουσιών οι οποίες λειτουργούν σαν μηνύματα για τα γειτονικά κύτταρα κατευθύνοντας τα να υπηρετήσουν ένα συγκεκριμένο βιολογικό σκοπό.

Όταν τα λιπίδια και συγκεκριμένα τα LDL επίπεδα αυξάνονται στη κυκλοφορία του αίματος και συγκεντρώνονται στο αρτηριακό τοίχωμα τότε το ανοσοποιητικό μας σύστημα αντιλαμβάνεται ότι κάτι δεν πάει καλά. Επιπρόσθετα, η συσσώρευση και ο εγκλωβισμός των λιποπρωτεϊνών στο αρτηριακό τοίχωμα οδηγεί επίσης σε μετατροπή της δομής αυτών. Τότε τα κύτταρα του ανοσοποιητικού συστήματος αναγνωρίζουν τις περίεργες τροποποιημένες μοριακές δομές των λιποπρωτεϊνών και τους επιτίθενται σαν κάτι ξένο. Αυτή η διαδικασία αποτελεί ένα μηχανισμό προστασίας αλλά όταν ο έλεγχος χαθεί λόγω της συνεχούς εισαγωγής λιποπρωτεϊνών στο αρτηριακό τοίχωμα και ακόλουθης μετατροπής τους τότε το ανοσοποιητικό σύστημα προωθεί συνεχώς σήματα

κινδύνου δημιουργώντας περεταίρω φλεγμονή και καταστροφή του αρτηριακού τοιχώματος.

Ένας πιθανός τρόπος αντιμετώπισης της αθηροσκλήρωσης είναι η ανακατεύθυνση του ανοσοποιητικού συστήματος με τέτοιο τρόπο ούτως ώστε η φλεγμονή καθώς και τα ακόλουθα μονοπάτια του ανοσοποιητικού συστήματος μέσω των οποίων η ασθένεια επιδεινώνεται να μειωθούν. Πολλές επιστημονικές ομάδες έχουν επιτύχει να κάνουν κάτι τέτοιο κατά την διάρκεια των τελευταίων δεκαετιών μέσω της χορήγησης συγκεκριμένων κυτταρικών ομάδων του ανοσοποιητικού συστήματος ή ουσιών σε πειραματικά μοντέλα ζώων.

Η ιντερλευκίνη-25 (IL-25) είναι μια κυτταροκίνη (πρωτεΐνη) η οποία υπάρχει στον οργανισμό μας και συντίθεται από διάφορα κύτταρα. Στην παρούσα εργασία, εξωγενής IL-25 χορηγήθηκε σε ποντίκια τα οποία έχουν αυξημένα λιπίδια στη κυκλοφορία τους, λόγω γενετικής τροποποίησης, καθιστώντας τα επιρρεπή στη δημιουργία αθηροσκληρωτικών πλακών. Η χορήγηση της ιντερλευκίνης οδήγησε στην μείωση των αορτικών αθηρωματικών πλακών σε νεαρά ποντίκια τα οποία δεν έχουν σχηματισμένες πλάκες αλλά και σε ποντίκια μεγάλης ηλικίας τα οποία ήδη φέρουν τέτοιες πλάκες (εργασία ΙΙ). Αυτό σημαίνει ότι η συγκεκριμένη ιντερλευκίνη είναι ικανή να μειώσει ήδη υπάρχουσα αθηροσκλήρωση αλλά και να αποτρέψει ως ένα βαθμό τον σχηματισμό της. Προτείνουμε ότι ο μηχανισμός της αθηροπροστατευτικής δράσης της ιντερλευκίνης-25 είναι μέσω της επαγωγής ενός κυτταρικού τύπου την ύπαρξη του οποίου η επιστημονική κοινότητα αγνοούσε μέχρι το 2010. Τα συγκεκριμένα κύτταρα ονομάζονται λεμφοκύτταρα φυσικής ανοσίας τύπου 2 (innate lymphoid cells type 2) και ένα γενικό χαρακτηριστικό τους είναι το γεγονός ότι έχουν την δυνατότητα να εκκρίνουν μεγάλες ποσότητες ιντερλευκίνης-5 (IL-5). Η ιντερλευκίνη-5 λειτουργεί ως ένα διεγερτικό σήμα για τα Β λεμφοκύτταρα ενεργοποιώντας τα να συνθέσουν αντισώματα τα οποία αναγνωρίζουν μοριακά μοτίβα της τροποποιημένης LDL, δεσμεύοντας και απομακρύνοντας την.

Σε μια άλλη σειρά πειραμάτων χορηγήσαμε στο ίδιο πειραματικό μοντέλο, ένα τύπο αντισώματος το οποίο δεσμεύει την ιντερελευκίνη-25 εμποδίζοντας έτσι κάθε βιολογική της δράση. Η χορήγηση αυτού του αντισώματος οδήγησε στην αύξηση την αθηροσκλήρωσης σε νεαρά μόνο ποντίκια τα οποία δεν φέρουν αθηρωματικές πλάκες κατά την έναρξη της χορήγησης του αντισώματος (εργασία ΙΙΙ). Τα αποτελέσματα αυτά υποδεικνύουν ότι η ενδογενής IL-25 είναι σημαντική για την προστασία ενάντια στην αθηροσκλήρωση κατά την διάρκεια του σχηματισμού των αθηρωματικών πλακών αφού τα νεαρά ποντίκια που έλαβαν το συγκεκριμένο αντίσωμα κατέληξαν να έχουν μεγαλύτερες αθηροσκληρωτικές πλάκες.

Αφού διαπιστώσαμε ότι η ιντερλευκίνη-25 προστατεύει ενάντια στην αθηροσκλήρωση μέσω μηχανισμών του ανοσοποιητικού συστήματος αποφασίσαμε να μελετήσουμε επίσης την δράση της σε ανθρώπινα κύτταρα του

ανοσοποιητικού συστήματος. Για τον λόγο αυτό απομονώσαμε μονοπύρηνα κύτταρα που βρίσκονται στην κυκλοφορία του ανθρώπινου οργανισμού από υγιείς εθελοντές και μελετήσαμε την επίδραση της ιντερλευκίνης-25 σε αυτά (εργασία IV). Κατόπιν επώασης των κυττάρων με την ιντερλευκίνη-25 διαπιστώσαμε ότι τα κύτταρα εξέκριναν χαμηλότερα επίπεδα προ-φλεγμονοδών κυτταροκινών. Με τον όρο προ-φλεγμονώδη εννοούμε την προαγωγή της φλεγμονής όπως επίσης και μηχανισμών του ανοσοποιητικού που σχετίζονται με την προώθηση της αθηροσκλήρωσης. Τα αποτελέσματα αυτά υποδεικνύουν ότι πιθανά η ιντερελευκίνη-25 θα μπορούσε να έχει ένα προστατευτικό ρόλο στην αθηροσκλήρωση του ανθρώπινου οργανισμού.

Πέρα από την μεγάλη ανάγκη για την εύρεση θεραπειών έναντι της αθηροσκλήρωσης, υπάρχει συνάμα μια μεγάλη ανάγκη για την πρόβλεψη μελλοντικών καρδιαγγειακών περιστατικών (ανακοπές καρδιάς, εγκεφαλικά επεισόδια). Στην εργασία I αναφέρουμε την ύπαρξη συγκεκριμένων Β λεμφοκυττάρων του αίματος και την συσχέτιση τους με είτε αυξημένο είτε μειωμένο κίνδυνο μελλοντικών εγκεφαλικών επεισοδίων. Στην εργασία αυτή είχαμε την δυνατότητα να χαρακτηρίσουμε αλλά και να ποσοτικοποιήσουμε συγκεκριμένες υπο-ομάδες Β λεμφοκυττάρων στο αίμα 700 ανθρώπων οι οποίοι επιλέχθηκαν τυχαία μέσα από μια κλινική μελέτη με την ονομασία "Malmö Diet and Cancer Study". Στη συγκεκριμένη κλινική μελέτη 28449 άνθρωποι στρατολογήθηκαν μεταξύ 1991-1994. Κάποιοι από αυτούς συμμετείχαν σε μια άλλη υπο-μελέτη η οποία εστίαζε στον κίνδυνο των καρδιαγγειακών νοσημάτων (αριθμός συμμετεχόντων=6103). Οι 700 άνθρωποι που μελετήσαμε στην εργασία I επιλέχθηκαν τυχαία από την προαναφερθείσα υπομελέτη.

Όλοι οι συμμετέχοντες παρακολουθήθηκαν από την πρώτη τους εξέταση μέχρι το πρώτο καρδιαγγειακό επεισόδιο το οποίο ορίσαμε ως θανάσιμο ή μη-θανάσιμο καρδιαγγειακό επεισόδιο (ανακοπή καρδιάς ή εγκεφαλικό επεισόδιο). Οι συμμετέχοντες παρακολουθήθηκαν μέχρι το 2008. Κατά την πρώτη εξέταση των ασθενών, αίμα συλλέχθηκε και πραγματοποιήθηκε απομόνωση των μονοπύρηνων κυττάρων τα οποία καταψύχθηκαν στη συνέχεια στους -140^{o}C. Το 2011, αποψύξαμε τα κύτταρα των 700 συμμετεχόντων που επιλέξαμε και αναλύσαμε συγκεκριμένες υπο-ομάδες Β λεμφοκυττάρων με την βοήθεια μιας μεθόδου η οποία ονομάζεται κυτταρομετρία ροής. Μετέπειτα είχαμε την δυνατότητα να συσχετίσουμε την εκδήλωση των καρδιαγγειακών νοσημάτων με τα επίπεδα των υποομάδων των Β λεμφοκυττάρων που αναλύσαμε.

Διαπιστώσαμε ότι υψηλά επίπεδα $CD19^{+}CD86^{+}$ Β λεμφοκυττάρων συσχετίστηκαν σημαντικά με υψηλό κίνδυνο ενός μελλοντικού εγκεφαλικού επεισοδίου ιδιαίτερα για τους άντρες. Επιπρόσθετα, υψηλά επίπεδα $CD19^{+}CD40^{+}$ Β λεμφοκυττάρων συσχετίστηκαν με χαμηλό κίνδυνο για την ανάπτυξη ενός μελλοντικού εγκεφαλικού επεισοδίου.

Η συγκεκριμένη μελέτη είναι η πρώτη που να αναφέρει συσχετισμούς Β λεμφοκυττάρων με την μελλοντική εμφάνιση καρδιαγγειακών νοσημάτων και εγκεφαλικών επεισοδίων συγκεκριμένα. Ελπίζουμε ότι στο μέλλον και άλλες επιστημονικές ομάδες θα επιβεβαιώσουν το ίδιο εύρημα ούτως ώστε να χρησιμοποιηθεί ως εργαλείο στην κλινική. Σε αυτή την περίπτωση, ο γιατρός με απλή δειγματοληψία αίματος θα έχει τη δυνατότητα να εξετάσει τις συγκεκριμένες ομάδες Β λεμφοκυττάρων και να κατατάξει τον εξεταζόμενο ανάλογα με τα αποτελέσματα είτε στην ομάδα υψηλού είτε χαμηλού κινδύνου για ένα μελλοντικό εγκεφαλικό επεισόδιο. Εάν ο εξεταζόμενος ενταχθεί στην ομάδα υψηλού κινδύνου τότε συγκεκριμένες θεραπευτικές οδηγίες πρέπει να δοθούν στον ασθενή για την αποφυγή μελλοντικού εγκεφαλικού επεισοδίου.

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