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THE INTERPLAY BETWEEN LIPOPROTEINS, IMMUNITY AND TRYPTOPHAN METABOLISM IN ATHEROSCLEROSIS

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Atherosclerotic cardiovascular disease (CVD) is the leading cause of mortality worldwide. Atherosclerosis is initiated by the infiltration and accumulation of low-density lipoprotein (LDL) cholesterol in the vascular wall, which activates the innate and adaptive arm of immunity, thereby causing chronic vascular inflammation. The LDL particle is immunogenic, as it not only activates lesional macrophages but is also recognized by T cells, and it elicits B cell-mediated antibody responses. Animal immunization studies suggest that anti-LDL antibodies inhibit atherosclerosis, but concerns exist about the potential proinflammatory role of lesional LDL-reactive T cells. In addition to lipoproteins, amino acids and their metabolites can shape immune cell responses, which has been the subject of intense research in the emerging field of immunometabolism. Current clinical practice guidelines on the prevention of CVD focus on controlling traditional risk factors, such as hypercholesterolemia, which indirectly influence inflammation in the vascular wall. Despite optimal management, however, residual inflammatory risk persists and underscores the need for novel therapeutics that directly target vascular inflammation.

In **Paper I**, we generated mouse strains bearing T cell receptor (TCR) transgenic T cells that react to human LDL. Adoptive transfer of these autoreactive T cells or the intercross of TCR transgenic mice with animals expressing human apolipoprotein B-100 (apoB100) on the LDL receptor$^{-/-}$ (LDLR$^{-/-}$) background led to reduced vascular inflammation and atherosclerosis. Interestingly, a significant proportion of LDL-reactive T cells differentiated into T follicular helper cells, which helped B cells produce anti-LDL antibodies that formed immune complexes with circulating LDL, thereby reducing plasma cholesterol. In **Paper II**, we employed dendritic cell (DC) based immunotherapy in an attempt to induce apoB100-specific regulatory T (T_{reg}) cells that can exert anti-inflammatory functions in developing plaques. The vaccine was prepared using bone marrow-derived DCs, which were loaded with apoB100 in the presence of the anti-inflammatory cytokine transforming growth factor beta 2 (TGF-β_2). Immunotherapy with these DCs promoted an immune response to apoB100 that favoured the accumulation of T_{reg} cells in atherosclerotic plaques, increased vascular expression of the immunomodulatory enzyme indoleamine 2,3-dioxygenase 1 (IDO1), and ameliorated atherosclerosis. *In vitro* experiments suggested that the T_{reg} molecule cytotoxic T-lymphocyte–associated antigen-4 (CTLA-4) regulates IDO1 expression in macrophages and vascular cells.

In Paper III, we studied the role of IDO1-mediated tryptophan metabolism in atherosclerosis using an inhibitor of IDO1 enzyme, 1-methyl-tryptophan. *In vivo* and *in vitro* data indicated that IDO1 regulates vascular inflammation, particularly in smooth muscle cells, and inhibits atherosclerosis possibly via the generation of the metabolite 3-hydroxyanthranilic acid (3-HAA). In **Paper IV**, we investigated the effects of increased endogenous 3-HAA levels on plasma lipids and atherosclerosis using an inhibitor of the enzyme 3-hydroxyanthranilic acid 3,4-dioxygenase (HAAO). Our data suggested that 3-HAA can lower plasma lipids via inhibition of the sterol regulatory element binding protein-2 (SREBP-2) pathway in hepatocytes and suppress inflammation via inhibition of the nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 3 (NLRP3) inflammasome in macrophages.

The studies included in the present thesis illustrate the intricate interplay between metabolism and immunity in atherosclerosis. It is my belief that our findings will contribute to the development of effective immunomodulatory strategies directly targeting vascular inflammation and addressing the residual inflammatory cardiovascular risk.

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I. Gisterå A, Klement ML, Polyzos KA, Mailer RK, Duhlin A, Karlsson MCI, Ketelhuth DFJ, Hansson GK.
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* In Paper IV, the two first authors contributed equally.

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CONTENTS

LIST OF ABBREVIATIONS

CVD	Cardiovascular disease
LDL	Low-density lipoprotein
T_{reg}	Regulatory T
DC	Dendritic cell
ApoB100	Apolipoprotein B-100
TGF-β_2	Transforming growth factor beta 2
IDO1	Indoleamine 2,3-dioxygenase 1
CTLA-4	Cytotoxic T-lymphocyte–associated antigen-4
3-HAA	3-hydroxyanthranilic acid
SREBP	Sterol regulatory element binding protein
NOD	Nucleotide-binding oligomerization domain
NLRP3	NOD-like receptor family pyrin domain containing 3
CRP	C-reactive protein
TNF	Tumor necrosis factor
IL	Interleukin
MCP-1	Monocyte chemoattractant protein-1
hsCRP	High-sensitivity CRP
IL-6R	IL-6 receptor
RCT	Randomized controlled trial
CANTOS	Canakinumab Anti-Inflammatory Thrombosis Outcome Study
WBC	White blood cell
Fc	Fragment crystallizable
NET	Neutrophil extracellular trap
CCR2	C-C chemokine receptor 2
CX3CR1	CX3C chemokine receptor 1
CD	Cluster of differentiation
NO	Nitric oxide
IFN-γ	Interferon gamma
ROS	Reactive oxygen species
CDP	Common dendritic cell progenitor
pDC	Plasmacytoid dendritic cell
cDC	Classical dendritic cell
MDP	Monocyte-dendritic cell progenitor
GMP	Granulocyte monocyte progenitor

CMP	Common myeloid progenitor
mo-MΦ	Monocyte-derived macrophage
mo-DC	Monocyte-derived dendritic cell
MP	Monocyte progenitor
GP	Granulocyte progenitor
TLR	Toll-like receptor
APC	Antigen-presenting cell
MHC	Major histocompatibility complex
NK	Natural killer
Ig	Immunoglobulin
PAMP	Pathogen-associated molecular pattern
DAMP	Damage-associated molecular pattern
PRR	Pattern recognition receptor
NLR	NOD-like receptor
SR	Scavenger receptor
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
MAPK	Mitogen activated protein kinase
IRF	Interferon regulatory factor
ASC	Apoptosis-associated speck-like protein containing CARD
ATP	Adenosine triphosphate
mtROS	Mitochondrial reactive oxygen species
T_H	T helper
CTL	Cytotoxic T lymphocyte
TCR	T cell receptor
CD40L	CD40 ligand
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death ligand 1
PD-L2	Programmed cell death ligand 2
T_{FH}	T follicular helper
T-bet	T-box–containing protein expressed in T cells
GATA-3	GATA-binding protein 3
Foxp3	Forkhead box p3
ICOS	Inducible T-cell costimulator
BCR	B cell receptor
MZ	Marginal zone

oxLDL	Oxidized low-density lipoprotein
IRA	Innate response activator
LPS	Lipopolysaccharide
GM-CSF	Granulocyte-macrophage colony-stimulating factor
apo	Apolipoprotein
FoxO3	Forkhead box O3
LAG3	Lymphocyte activation gene 3
CoA	Coenzyme A
HMG	3-hydroxy-3-methylglutaryl
NPC1L1	Niemann-Pick C1 Like 1
VLDLR	Very low-density lipoprotein receptor
VLDL	Very low-density lipoprotein
MTTP	Microsomal triglyceride transfer protein
LDLR	Low-density lipoprotein receptor
HSPG	Heparan sulfate proteoglycan
LRP1	LDLR-related protein 1
IDL	Intermediate-density lipoprotein
LPL	Lipoprotein lipase
PCSK9	Proprotein convertase subtilisin/kexin type 9
SCAP	SREBP cleavage-activating protein
ER	Endoplasmic reticulum
S1P	Site-1 protease
S2P	Site-2 protease
INSIG	Insulin induced gene
FAS	Fatty acid synthase
ACC	Acetyl-CoA carboxylase
HDL	High-density lipoprotein
ApoA1	Apolipoprotein A1
ABC	ATP-binding cassette
ABCA1	ATP-binding cassette A1
ABCG1	ATP-binding cassette G1
LCAT	Lecithine cholesterol acyl transferase
CETP	Cholesterol ester transfer protein
CYP	Cytochrome P450
5-HTP	5-hydroxytryptophan

5-HT	5-hydroxytryptamine
TDO	Tryptophan 2,3-dioxygenase
KAT	Kynurenine aminotransferase
KYNA	Kynurenic acid
P5P	Pyridoxal 5-phosphate
KMO	Kynurenine monoaminooxidase
HAAO	3-hydroxyanthranilic acid 3,4-dioxygenase
QPRT	Quinolinate phosphoribosyltransferase
ACMSD	ACMS decarboxylase
AMSD	AMS-dehydrogenase
SMC	Smooth muscle cell
STAT	Signal transducer and activator of transcription
PKR	Protein kinase R
COX-2	Cyclooxygenase 2
PGE2	Prostaglandin E2
PI3K	Phosphoinositide 3-kinase
mTOR	Mammalian target of rapamycin
PKC	Protein kinase C
GSK3b	Glycogen synthase kinase-3b
GCN2	General control nonderepressible 2
1-MT	1-methyl-tryptophan
JNK	c-Jun N-terminal kinase
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
PKG	Protein kinase G
AHR	Aryl-hydrocarbon receptor
PDK1	Phosphoinositide-dependent protein kinase 1
GPR35	G protein-coupled receptor 35
PBMC	Peripheral blood mononuclear cell
PPAR	Peroxisome proliferator-activated receptor
M-CSF	Macrophage colony-stimulating factor
HSP	Heat-shock protein
MIF	Migration inhibitory factor
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
Nrf2	Nuclear factor erythroid 2-related factor 2

HO-1	Heme oxygenase 1
MyD88	Myeloid differentiation primary response 88
OSE	Oxidation specific epitope
ERK	Extracellular-signal-regulated kinase
HLA	Human leukocyte antigen
CXCL1	CXC ligand 1
Rag2	Recombination activating gene 2
FcγR	Fcγ receptor
BAFF	B-cell activating factor
BAFFR	B-cell activating factor receptor
scid	Severe combined immunodeficiency
MDA	Malondialdehyde
nLDL	Native LDL
GFP	Green fluorescent protein
PBS	Phosphate-buffered saline
EDTA	Ethylenediaminetetraacetic acid
BHT	Butylated hydroxytoluene
SDS	Sodium dodecyl sulfate
ELISA	Enzyme-linked immunosorbent assay
TBS	Tris-buffered saline
FITC	Fluorescein isothiocyanate
HRP	Horseradish peroxidase
ORO	Oil Red O
ANOVA	Analysis of variance
SEM	Standard error of the mean
TRBV	TCR variable β chain
TRAV	TCR variable α chain
HUVEC	Human umbilical vein endothelial cell
VCAM-1	Vascular cell adhesion protein 1
GC	Germinal center
nSREBP-2	Nuclear SREBP-2

1 THE BURDEN OF CARDIOVASCULAR DISEASE

Cardiovascular diseases (CVDs), a group of disorders of the heart and blood vessels, are the leading cause of mortality worldwide, accounting for one-third of global deaths. Seventy-eight percent of cardiovascular deaths are due to heart attacks and strokes, which, in the majority of cases, are acute manifestations of coronary heart disease and cerebrovascular disease, respectively. In 2015, there was a global estimate of 422.7 million cases of CVD and 17.9 million CVD-related deaths.[1] In Sweden, 31,616 CVD-related deaths (35% of total deaths) were reported in 2016, while it is estimated that every fifth person lives with some sort of cardiovascular condition.[2] The high rates of CVD-related morbidity and mortality are translated into significant socioeconomic costs, resulting from increased healthcare costs, productivity losses and informal care of individuals with CVDs. The total annual cost of CVDs in the European Union and the United States is estimated at €210 and $316 billion, respectively.[3,4]

The main underlying cause of CVDs is the asymmetric focal deposition of lipids—also known as the "atheroma" or "plaque"— on the inner lining of the arteries. This pathological process is known as atherosclerosis, it starts early in life, progresses slowly and can be influenced by various factors including genetic predisposition, smoking, hyperlipidaemia, hypertension, sedentary lifestyle, poor diet, obesity, and diabetes.[5-8] Population-wide strategies aimed at modifying behavioural factors, the use of lipid-lowering drugs, antihypertensive and antidiabetic medications, as well as the improvement and wide availability of invasive interventions, have reduced the burden of CVDs in high-income countries.[9] However, substantial residual risk remains, which underscores the need for novel therapies directly targeting the pathogenic mechanisms underlying the atherosclerotic cardiovascular disease in the vascular wall.[10]

2 INFLAMMATION IN ATHEROSCLEROTIC CARDIOVASCULAR DISEASE

Over the past decades, histopathological studies of human plaques, experimental studies in genetically engineered mice, and epidemiological data have illuminated the central role of inflammation and the immune system in atherosclerotic cardiovascular disease. First and foremost, a significant proportion of atherosclerotic plaques consist of immune cells, especially macrophages and T cells. Epidemiological studies have shown positive correlations between several inflammatory molecules, such as C-reactive protein (CRP),

tumor necrosis factor (TNF), interleukin (IL)-6 and monocyte chemoattractant protein-1 (MCP-1), and cardiovascular morbidity and mortality.[11,12] Experimental studies suggest causality for some of these associations (e.g., for TNF and MCP-1) but not others (e.g., for CRP).[13-15] Most importantly, studies on genetically targeted mice lacking cells or molecules of the immune system confirmed the causal role of various components of the immune system in atherosclerosis.

CRP is the most frequently used marker of systemic inflammation in the clinic. Mendelian randomization studies and experimental data do not support a causative role of CRP in atherothrombosis.[16] Nevertheless, high-sensitivity CRP (hsCRP) predicts myocardial infarction, stroke and cardiovascular death independently of traditional cardiovascular risk factors. HsCRP has been used in clinical trials to select patients who would benefit from therapies with anti-inflammatory effects. For instance, the effectiveness of acetylsalicylic acid in the primary prevention of myocardial infarction is particularly pronounced in individuals with high baseline CRP.[17] Statins, drugs with pleiotropic anti-inflammatory properties, lower plasma CRP levels independently of plasma cholesterol, and their beneficial effects relate to both cholesterol and CRP lowering. These drugs reduce major cardiovascular events not only in individuals with high baseline cholesterol levels but also in those with low plasma cholesterol and high CRP.[18] The 2019 American Heart Association guidelines on the primary prevention of CVD recommend that elevated hsCRP ($\geq$ 2 mg/L) may be used to guide decision-making to initiate statin therapy.[19]

CRP is secreted by the liver in response to IL-6, a cytokine that is also associated with an increased risk for cardiovascular events. Mendelian randomization studies have found that two common variants of the IL-6 receptor (IL-6R), which are known to impair IL-6R signalling, are associated with lower plasma CRP levels and a reduced risk for cardiovascular outcomes.[20,21] These findings have rendered the IL-6R signalling pathway a promising target for the prevention of coronary heart disease. A cytokine lying upstream of the IL-6–CRP pathway is IL-1β. This cytokine assumed extensive interest after it was demonstrated that cholesterol crystals in atherosclerotic plaques can activate the nucleotide-binding oligomerization domain (NOD)-like receptor family pyrin domain containing 3 (NLRP3) inflammasome in macrophages, thereby promoting IL-1β release.[22,23] Interestingly, inflammasome activation can be induced in macrophages by plaque hypoxia[24] and in endothelial cells at arterial segments exposed to turbulent blood flow.[25]

Despite the large body of evidence implicating inflammation in atherosclerotic cardiovascular disease, it was only in 2017 that a randomized controlled trial (RCT) showed that a therapy

directly targeting inflammation could reduce major cardiovascular events. In this trial (CANTOS), targeting innate immunity with canakinumab, a monoclonal antibody against IL-1β, reduced hsCRP and cardiovascular events in patients with previous myocardial infarction and baseline hsCRP greater than or equal to 2 mg/L.[26] A secondary analysis of canakinumab-treated patients showed that only those who achieved a hsCRP below 2 mg/L had reduced cardiovascular events and mortality.[27] Another patient population with chronic low-grade inflammation that may benefit from anti-inflammatory therapies includes patients with rheumatic diseases. For instance, meta-analyses of RCTs suggest that TNF inhibitors and methotrexate reduce cardiovascular events in patients with rheumatoid arthritis.[28]

Atherosclerosis is now widely accepted as a chronic inflammatory disease of large- and medium-sized arteries, where diverse metabolic pathways, such as lipoprotein and amino acid metabolism, influence the balance between proinflammatory and anti-inflammatory immune cells and subsequently clinical outcomes. Over the past decades, the standard of care for primary and secondary prevention of CVDs has focused on managing hypercholesterolemia and other traditional risk factors. The study of the immune system in the context of atherosclerosis will allow the discovery of immunomodulatory drugs directly targeting vascular inflammation, thus providing the medical community with a completely new treatment strategy for the prevention and treatment of atherosclerotic cardiovascular disease.

3 THE IMMUNE SYSTEM

The immune system is the collection of tissues, cells and molecules with a primary function of providing protection against infections. It also prevents the growth of cancer cells and contributes to tissue repair. In contrast to these beneficial functions, abnormal immune reactions against foreign and self-antigens can cause harmful allergic, autoinflammatory and autoimmune diseases. The immune system is divided into the innate immune system, which acts rapidly and provides the first line of defence against invading organisms, and the adaptive immune system, which takes days to develop but is more prevailing and generates responses that are specific to the different pathogens that might be encountered.[29]

3.1 THE INNATE IMMUNE SYSTEM

The principal function of innate immunity is to prevent microbial invasion, induce inflammation, provide antiviral immune mechanisms, and activate the adaptive immune

system. The innate immune system consists of epithelial cells, various innate immune cell types and a large number of secreted molecules.

3.1.1 Epithelial barriers

Epithelial cells of the skin, respiratory tract, gastrointestinal tract and genitourinary tract provide a physical barrier that prevents microbes from penetrating our bodies. In addition, epithelial cells produce mucus, secrete antimicrobial enzymes and peptides and are equipped with cilia that remove pathogens trapped in the mucus.

3.1.2 Innate immune cells

3.1.2.1 Polymorphonuclear cells

Polymorphonuclear cells or granulocytes are bone marrow-derived white blood cells (WBCs) characterized by a lobed nucleus and the presence of intracellular granules that are released upon infection, allergic reactions and asthma. Depending on their staining pattern, these cells are divided into neutrophils, eosinophils and basophils. Neutrophils, the most abundant leukocyte type in the circulation (40-80% of WBCs in humans), patrol tissues and initiate prompt immune responses upon infection or sterile inflammation.[30] These cells are equipped with various receptors (pattern recognition receptors, antibody Fc receptors, complement receptors) that help them phagocytose and kill invading pathogens. Other neutrophil effector mechanisms include the release of reactive chemicals and proteolytic enzymes as well as the release of neutrophil extracellular traps (NETs), which are web-like structures consisting of DNA and enzymes that bind to and kill microbes.[31]

Eosinophils, which are present in limited numbers in the circulation (1-6% of WBCs in humans), are recruited to tissues, where they degranulate antimicrobial proteins and play an important role in parasitic infections and during the late phase of type I hypersensitivity reactions. Basophils, the least abundant WBC type in peripheral blood (<1-2% of WBCs in humans), are recruited to peripheral tissues upon infection and hypersensitivity reactions, release their granular contents and promote inflammation.

3.1.2.2 Monocytes

Similar to granulocytes, monocytes (2-10% of WBCs in human peripheral blood) are myeloid cells derived from bone marrow progenitors, and their production increases significantly in response to emergency situations such as infection. Two major populations of monocytes have been described, classical or inflammatory ($Ly6C^+$ $CCR2^+$ $CX3CR1^{low}$ cells in mice and $CD14^+$ $CD16^-$ in humans) and the non-classical ($Ly6C^-$ $CCR2^-$ $CX3CR1^{high}$ in mice and

$CD14^{low}$ $CD16^{+}$ in humans) monocytes.[32] It has been suggested that the short-lived (half-life ~ 20 hours) inflammatory monocytes differentiate in the circulation into non-classical (half-life ~ 5 days) monocytes.[33] The different monocyte subsets are believed to exert distinct functions. Classical monocytes are readily recruited to sites of inflammation and generate macrophages and dendritic cells (DCs), whereas non-classical monocytes are believed to crawl on the luminal surface of endothelial cells, survey endothelial integrity, recruit neutrophils and clear cell debris.[34,35]

3.1.2.3 Macrophages

Macrophages are a heterogeneous population of tissue cells that exert multiple functions, including phagocytosis, antigen presentation, cytokine secretion, resolution of inflammation, and tissue repair. In the steady state, the majority of tissue resident macrophages (e.g., brain microglia, Kupffer cells, skin Langerhans cells, lung macrophages, peritoneal macrophages) come from embryonic progenitors that are seeded into tissues before birth.[33,36] The maintenance of tissue macrophages in the steady state relies on self-renewal. Exceptions include intestinal macrophages, spleen marginal zone (MZ) macrophages and a population of dermal macrophages, which are maintained through classical monocyte recruitment.[34] Upon inflammation, classical $Ly6C^{+}$ monocytes are abundantly recruited and differentiate into monocyte-derived macrophages, thus contributing substantially to the pool of tissue macrophages. In certain inflammatory settings, such as atherosclerosis, the recruitment of classical monocytes and differentiation into macrophages plays a crucial role in the pathogenesis of the initial stages of disease. The maintenance and expansion of lesional macrophages, however, depends on the local proliferation of monocyte-derived macrophages rather than continuous monocyte influx.[37]

Depending on the tissue microenvironment, monocyte-derived macrophages polarize through different differentiation programmes, thereby exerting distinct functions. During the initial phase of inflammation, macrophages shift arginine metabolism towards the generation of nitric oxide (NO) and citrulline, which enhances inflammation and host defence against invading pathogens (M1 polarization). *In vitro*, M1 polarization is induced by microbial products (e.g., lipopolysaccharide), endogenous danger signals (e.g., oxidized lipids) and inflammatory cytokines [e.g., interferon (IFN)-γ, TNF]. M1 macrophages are characterized *in vitro* by low expression of IL-10 and increased production of proinflammatory cytokines (TNF, IL-1β, IL-6, IL-12, IL-23), NO and reactive oxygen species (ROS).

During the resolution phase of inflammation, macrophage metabolism of arginine shifts towards ornithine and polyamines (M2 polarization), promoting collagen secretion, fibrosis, angiogenesis, tissue repair and remodelling. Four M2 subclasses have been described: M2a macrophages, which are induced by IL-4 and IL-13; M2b macrophages, which are induced by immune complexes in combination with IL-1β or Toll-like receptor (TLR) ligands; M2c macrophages, which are induced by IL-10, transforming growth factor (TGF)-β or glucocorticoids; and M2d macrophages, which are induced by costimulation with TLR and adenosine A_{2A} agonists. M2 macrophages express high levels of IL-10, TGF-β and scavenger receptors but low levels of IL-12 and IL-23. From a functional perspective, M2 macrophages are involved in defence against parasites, immunoregulation, scavenging of apoptotic debris, resolution of inflammation, and wound healing. Apart from M1 and M2 macrophages, additional macrophage subsets have recently been described in the context of atherosclerosis, namely Mox, M(Hb), Mhem and M4.[38,39]

It is believed that, in the steady state, tissue resident macrophages have a default M2-like phenotype, whereas monocyte-derived macrophages can differentiate along different polarization programmes depending on the stage of the ongoing inflammatory reaction. Whether the different monocyte subsets show an intrinsic preference for polarization towards distinct polarization programmes remains to be investigated. Interestingly, it has been suggested that macrophages show plasticity and may transdifferentiate from one phenotype to another depending on the dynamic changes in the tissue microenvironment. Because of the paucity of data on the role of macrophage subsets *in vivo*, the M1/M2 paradigm should be viewed as a simplified conceptual framework where M1 and M2 cells represent the extremes of a continuous spectrum of macrophage functional phenotypes.[40]

3.1.2.4 Dendritic cells

DCs arise from a common dendritic cell progenitor (CDP), which can give rise to terminally differentiated plasmacytoid DCs (pDCs) in the bone marrow or precursors of classical DCs (cDCs) that are transferred to peripheral tissues and lymphoid organs where they differentiate into cDCs. In mice, classical DCs are subdivided into type 1 cDCs ($CD8\alpha^+$ $CD11b^-$ cDCs in lymphoid tissues; $CD103^+$ $CD11b^-$ cDCs in non-lymphoid tissues) and type 2 $CD11b^+$ cDCs. Another population of DCs in peripheral tissues (monocyte-derived DCs), which becomes particularly prominent upon inflammation, derives from circulating monocytes (**Figure 1**).[41-44]

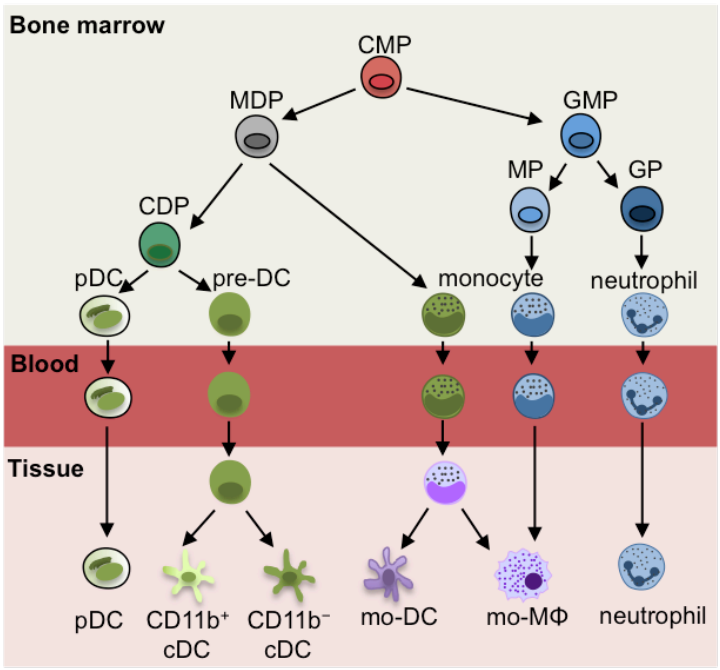

Figure 1. Bone marrow generation of myeloid cells. Monocyte-dendritic cell progenitors (MDPs) and granulocyte monocyte progenitors (GMPs) are thought to derive from common myeloid progenitor (CMP) cells. MDPs differentiate further into monocytes or common dendritic cell progenitor (CDP) cells, which give rise to plasmacytoid dendritic cells (pDCs) and classical dendritic cells (cDCs). MDP-derived monocytes can migrate to tissues and differentiate into macrophages (mo-MΦ) or DCs (mo-DCs). Monocytes can also be generated by GMPs via committed monocyte progenitor (MP) cells. GMPs generate neutrophils via committed granulocyte progenitor (GP) cells.[45]

Plasmacytoid DCs resemble plasma cells, express high levels of TLR7 and TLR9 and produce large amounts of type I interferon in response to viral infection. Classical DCs are professional antigen-presenting cells (APCs) that sample the tissue microenvironment and recognize foreign material to be presented on molecules of the major histocompatibility complex (MHC) to adaptive immune cells. In peripheral tissues, immature DCs take up microbes and self-antigens and home to the T cell zone of draining lymph nodes. Upon infection, microbial products and inflammatory cytokines promote the maturation of DCs, which upregulate MHC, costimulatory molecules and the cytokine IL-12. Important costimulatory molecules include the B7-1 (CD80) and B7-2 (CD86) proteins, which bind to CD28 or cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) on the surface of T cells.[46,47] Antigen presentation in the presence of adequate costimulation results in lymphocyte activation and proliferation. In the absence of infection, however, DCs present antigens to self-reactive T cells without adequate costimulation, thereby leading to T cell functional inactivation or death.[29,48-50]

3.1.2.5 Other innate immune cells

Other cells of the innate immune system include mast cells, natural killer (NK) cells, γδ T cells and NK-T cells. Mast cells are granulocytes that are present in the skin and mucosal tissues and play a role in type I hypersensitivity reactions as well as in the defence against parasites. NK cells have the capacity to kill infected and tumor cells via the secretion of apoptosis-inducing proteins and to stimulate the phagocytic efficiency of macrophages via the

secretion of the cytokine IFN-γ. γδ T cells, a population of T lymphocytes bearing receptors with limited diversity, are found in epithelial layers participating in the early defence against pathogen invasion. Finally, NK-T cells expressing surface molecules found on both NK cells and T cells recognize microbial lipids and glycolipids presented on the antigen-presenting molecule CD1d.[29]

3.1.3 Humoral innate immunity

Apart from epithelial barriers and cellular components, the innate immune system also has a humoral component, including the complement system, cytokines, acute phase reactants and natural antibodies. The complement system, a collection of circulating and membrane-bound proteins produced by the liver, participates in antimicrobial defence via different mechanisms: opsonization of microbes and facilitation of phagocytosis, leukocyte recruitment, and direct lysis of microbial cells via the formation of a multi-protein membrane attack complex.

Upon infection, innate immune cells as well as other cell types secrete cytokines and chemokines, soluble proteins that mediate many of the actions of innate immune cells. For instance, TNF and IL-1 activate the endothelium and facilitate the recruitment and activation of neutrophils and monocytes at the site of infection. In addition, these cytokines, as well as IL-6, induce hepatic secretion of acute phase proteins, such as CRP. These proteins bind to pathogens and apoptotic cells and facilitate their clearance via recognition by Fcγ receptors (FcγRs) of phagocytes and activation of the complement system. Finally, humoral innate immune responses are enhanced by circulating natural antibodies, germline-encoded antibodies—most commonly of the immunoglobulin (Ig) M class— that are produced without previous exogenous antigen stimulation and contribute to antimicrobial defence.[51]

3.1.4 Receptors of innate immune cells: Toll-like receptors, scavenger receptors, NOD-like receptors

Innate immune cells recognize microbial structures, known as pathogen-associated molecular patterns (PAMPs), as well as products that are released by damaged cells and modified self-antigens, collectively termed damage-associated molecular patterns (DAMPs), via germline-encoded pattern recognition receptors (PRRs) localized in different cellular compartments.[52] PRRs are expressed not only by innate immune cells but also by other cell types including epithelial cells, endothelial cells and lymphocytes. Major PRR families include Toll-like receptors, NOD-like receptors (NLRs), and scavenger receptors (SRs).[29]

The TLR family includes 13 receptors (TLR1 to TLR13), although TLR11, TLR12 and TLR13 are expressed only in mice. TLRs are present on the plasma membrane (TLR1, TLR2, TLR4, TLR5, TLR6, TLR11) or in intracellular vesicles (TLR3, TLR7, TLR8, TLR9). TLRs sense microbial components or endogenous ligands, thereby initiating a signal transduction cascade that results in the expression of proteins involved in antimicrobial defence.[53,54] Scavenger receptors are membrane-associated PRRs that were initially recognized as taking up modified lipoprotein particles but were later identified to recognize microbial structures and self-molecules. Scavenger receptors participate in the phagocytosis and clearance of pathogens, clearance of foreign molecules and apoptotic bodies, as well as in fatty acid uptake and cholesterol clearance.[55]

NLRs comprise a family of evolutionarily conserved cytosolic PRRs that recognize various structurally unrelated molecules, including microbial substances, products of damaged cells, and endogenous noxious substances. NLRs are composed of an N-terminal signalling domain, a central NOD domain and a C-terminal domain involved in ligand binding. Mammalian NLRs are subdivided into 4 subfamilies characterized by different N-terminal domains: NLRA, NLRB, NLRC and NLRP. Upon activation, NLRs form oligomerization complexes that can either recruit/activate proinflammatory caspases or initiate inflammatory signalling pathways, including the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), mitogen activated protein kinase (MAPK) and interferon regulatory factor (IRF) pathways. NLR complexes that recruit and activate caspase-1 are widely known as inflammasomes. Caspase-1 cleaves pro-IL-1β and pro-IL-18 into mature cytokines that induce an inflammatory form of cell death known as pyroptosis.[56,57]

One of the best-characterized inflammasome platforms is the NLRP3 inflammasome, which is biochemically defined by the NOD-like receptor NLRP3, the adaptor protein apoptosis-associated speck-like protein containing CARD (ASC), and caspase-1. The NLRP3 inflammasome is activated by a two-step process: the first signal (priming) results in NF-κB activation and transcriptional induction of inflammasome components, immature pro-IL-1β and pro-IL-18. The second signal is provided by diverse exogenous or endogenous PAMPs or DAMPs [bacteria, viruses, extracellular adenosine triphosphate (ATP), particulate matter, monosodium urate and calcium pyrophosphate crystals, cholesterol crystals, palmitate, ROS, oxidized mitochondrial DNA] and promotes the assembly of NLRP3, ASC and pro-caspase-1, thus leading to the activation of this inflammasome complex and production of IL-1β and IL-18.[58] Some widely recognized molecular mechanisms leading to NLRP3 inflammasome

activation include K^+ efflux, mitochondrial ROS (mtROS) production, and lysosome disruption and the release of lysosomal enzymes (**Figure 2**).[59]

Gain-of-function mutations in NLRP3 have been identified as the cause of hereditary periodic fever syndromes, while single nucleotide polymorphisms in the NLRP3 locus have been associated with various diseases, including type 1 diabetes, celiac disease and Alzheimer's disease. Moreover, activation of the NLRP3 inflammasome has been implicated in gout, insulin resistance, obesity and atherosclerosis.[58]

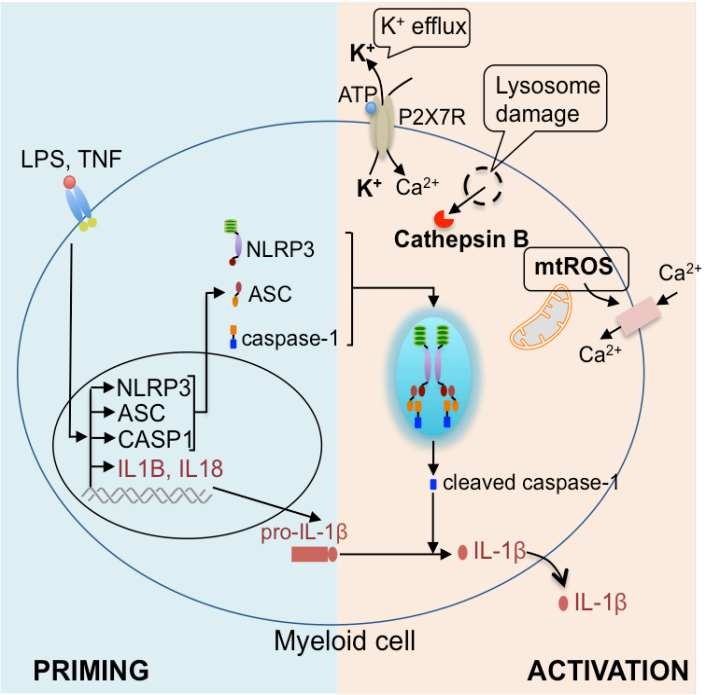

Figure 2. Activation of the NLRP3 inflammasome. Activation of the inflammasome requires two steps. The priming signal results in the activation of the NF-κB pathway, thus inducing the expression of inflammasome components, pro-IL-1β and pro-IL-18. The second signal is provided by microbial molecules and diverse DAMPs, such as ATP, cholesterol crystals, and fatty acids. Molecular mechanisms involved in inflammasome activation include K^+ efflux, lysosome destabilization/ release of cathepsin B, and generation of mitochondrial ROS (mtROS).

3.2 THE ADAPTIVE IMMUNE SYSTEM

The adaptive immune system consists of lymphocytes and their products. Adaptive immunity is divided into humoral immunity, which is mediated by antibodies produced by B lymphocytes and fights extracellular microbes, and cell-mediated immunity, which is conferred by T lymphocytes and protects against intracellular pathogens. Lymphocytes express an extremely diverse repertoire of antigen receptors that can recognize millions of different microbial molecules and other exogenous or endogenous substances, collectively called antigens. Lymphocytes that have not yet encountered their antigen are called naïve lymphocytes. Upon antigen recognition, lymphocytes proliferate and differentiate into effector cells that help eliminate the antigens, or long-lived memory cells that respond rapidly to re-encounter the same antigen.[29]

3.2.1 T lymphocytes

T lymphocytes or T cells arise from haematopoietic stem cell progenitors in the bone marrow, but their maturation is completed in the thymus. They express the cell surface marker $CD3^+$ and are commonly divided in $CD4^+$ T helper (T_H) cells, which help macrophages and B cells exert their effector functions, and $CD8^+$ cytotoxic T lymphocytes (CTLs), which kill infected, damaged and cancer cells. The majority of T cells recognize peptide antigens bound to MHC molecules. The antigen receptor of T cells, called T cell receptor (TCR), is a membrane heterodimeric receptor consisting of the α chain and the β chain, each chain consisting of a constant (Cα, Cβ) and a variable (Vα, Vβ) region. The variable regions of TCR are responsible for recognition of the antigen-MHC complex. CD4 and CD8 serve as co-receptors that bind to an invariable region of the MHC molecule. Antigen recognition initiates an intracellular signal transduction cascade mediated by a complex of proteins, the CD3 and ζ chains, which together with the TCR form the TCR complex.[29,60]

The total T lymphocyte repertoire consists of millions of different clones with different antigen specificities, meaning that each clone expresses a TCR with unique sequence in the variable region of α and β chains and recognizes a specific antigen. The extreme diversity of TCRs is accomplished during maturation in the thymus via the following two processes: somatic VDJ recombination of gene segments encoding the variable regions of TCR chains (known as combinatorial diversity), and enzymatic addition or removal of nucleotides at the sites of recombination (known as junctional diversity).[29,61]

The maturation of T cells occurs in the thymus, where clones expressing potentially useful TCRs are selected to survive. In the thymus, T cell progenitors are double-negative cells ($CD4^-$ $CD8^-$) that initiate the VDJ recombination process. Successful recombination promotes survival and proliferation and eventually results in the generation of double-positive $CD4^+$ $CD8^+$ cells expressing the complete TCR receptor. The next step is positive selection, where only T cells recognizing self-MHC molecules (presenting self-peptides) with low to moderate affinity are selected for survival. Cells recognizing MHC class I molecules lose expression of CD4 and become single positive $CD8^+$ (MHC-I restricted) T cells, whereas cells recognizing MHC class II molecules lose expression of CD8 and become single-positive $CD4^+$ (MHC-II restricted) T cells. Double-positive T cells that bind to MHC molecules with strong affinity die via apoptosis (negative selection).[61]

After maturation in the thymus, T lymphocytes migrate to secondary lymphoid organs and peripheral tissues in a naïve, inactivated state. Two signals are required for activation, clonal

expansion and differentiation of naïve T cells to effector cells: antigen stimulation (signal 1) and costimulation (signal 2). The latter is provided by the engagement of CD28 molecule on T cells with costimulatory B7 molecules (CD80, CD86) on antigen-presenting cells.

Another molecule that enhances T cell responses is the CD40 ligand (CD40L) on T cells that binds to CD40 on APCs, thereby leading to APC activation (cytokine secretion, upregulation of B7) and indirect T cell activation. Apart from costimulatory molecules, there are also T cell inhibitory receptors that limit T cell responses after invading pathogens have been eliminated. Two such receptors are CTLA-4, which antagonizes CD28 binding to B7 molecules on APCs, and programmed cell death protein 1 (PD-1), which binds to programmed cell death ligand 1 (PD-L1) and programmed cell death ligand 2 (PD-L2) on various cells. CTLA-4 is also expressed by T regulatory (T_{reg}) cells and mediates some of their functions.[62]

Depending on the type of invading pathogen and the cytokines present in the microenvironment, $CD4^+$ T_H cells, upon activation, can differentiate into different effector T cell subsets that secrete different sets of cytokines and exert different effector functions. Among the T_H cell subsets, T_H1, T_H2, T_H17, T_{reg}, and T follicular helper (T_{FH}) cells are the most common subtypes.

T_H1 cells play an important role in the elimination of bacteria and viruses ingested by phagocytes. Differentiation of naïve T cells to T_H1 cells is induced by the cytokines IFN-γ and IL-12. These cells express the transcription factor T-box–containing protein expressed in T cells (T-bet), and their signature cytokine is IFN-γ, which acts on phagocytes, thereby inducing their bactericidal activity.[63]

T_H2 cells are induced by the cytokine IL-4, express the signature transcription factor GATA-binding protein 3 (GATA-3), and secrete IL-4, IL-5 and IL-13, which are important cytokines for protection against parasite infections. IL-4 stimulates antibody class switching and the production of IgE antibodies, IL-5 activates eosinophils to secrete their granule content, whereas IL-13 promotes mucus secretion and intestinal peristalsis. T_H2 responses are also implicated in allergic reactions.

T_H17 cells contribute to the elimination of extracellular bacteria and fungi by recruiting neutrophils and monocytes at the site of infection. The signature cytokines of these cells are IL-17 and IL-22. Cytokines that skew differentiation towards the T_H17 subset include IL-1, IL-6, IL-23 and TGF-β.[64]

T_{reg} cells are immunoregulatory cells that promote immunological self-tolerance and limit excessive immune responses to foreign antigens. Naturally occurring T_{reg} cells are $CD4^+$ $CD25^+$ CTLA-4^+ cells that are generated in the thymus in response to self-antigens and constitutively express the transcription factor forkhead box p3 (Foxp3). The importance of T_{reg} cells in the maintenance of self-tolerance is illustrated by the observation that Foxp3 mutations cause multiorgan autoimmune disorders in mice and humans.[65-67] Induced T_{reg} cells are $CD4^+$ $CD25^+$ CTLA-4^+ cells generated in the periphery by naïve T cells that are induced to express Foxp3 under certain conditions, such as antigen recognition in the presence of TGF-β or antigen presentation by immature tolerogenic dendritic cells.[68-70]

T_{reg} cells can also be generated by naïve conventional T cells of the mucosal immune system via oral immunization. These T_{reg} cells, termed T_H3 cells, secrete TGF-β and various amounts of IL-10 and IL-4 and actively suppress immune responses not only to their specific antigen but also to other antigens in the microenvironment.[71,72] Another regulatory T cell subset is the IL-10 producing regulatory T cell type 1 (Tr1) population, which is induced by the cytokines TGF-β and IL-27. Tr1 cells can be induced *in vitro* by repetitive antigen stimulation of naïve T cells in the presence of IL-10 and *in vivo* by chronic antigen stimulation or mucosal immunization. These cells are particularly present in the gut where they play a central role in mucosal tolerance, as suggested by the observation that T_{reg} specific IL-10 deletion leads to autoimmune gastrointestinal disease.[73,74]

T_{FH} cells represent a subset of effector T cells that, upon activation in secondary lymphoid organs, migrate to the B cell zone of lymphoid follicles and help B cells differentiate into long-lived antibody-producing plasma cells. The generation of T_{FH} cells depends on the expression of inducible T-cell costimulator (ICOS), a member of the CD28 family. Importantly, T_{FH} cells, depending on the type of underlying T cell response, secrete different cytokines (e.g., IFN-γ, IL-4, IL-17) that influence the production of different heavy chain isotype antibodies.

3.2.2 B lymphocytes

Humoral immunity is mediated by antibodies produced by B lymphocytes or B cells. These cells are generated and mature in the bone marrow. The antigen receptors of B cells are membrane-bound antibodies that recognize not only peptide antigens but also native (conformational) non-protein epitopes, including polysaccharides and lipids. The B cell antigen receptor (BCR) is a Y-shaped tetramer composed of 2 identical heavy (H) chains attached to each other via disulphide bonds and 2 identical light (L) chains bound to heavy

chains via disulphide bonds. Each heavy chain has a variant domain (V_H) and 3 or 4 constant domains (C_H), whereas each light chain has a variant (V_L) and a constant (C_L) domain. Every antibody receptor molecule has two antigen binding sites, each consisting of the V_H domain of the heavy chain and the V_L domain of the associated light chain. The extreme diversity of B cell specificities is achieved, as in the case of T cells, via VDJ recombination and junctional nucleotide addition or removal.

Based on the constant region, there are 5 different types of heavy chains (α, γ, δ, ε, μ) that form 5 different antibody classes or isotypes (IgA, IgG, IgD, IgE, IgM). Similarly, there are 2 types of light chains (κ, λ) that differ in their constant regions. Mature B cells in the naïve state express only IgM and IgD receptors on their surface. Upon activation by T_H cells, however, IgD- and IgM-expressing B cells may secrete IgM or antibodies of other isotypes (isotype switching).

As mentioned earlier, B cells can respond to both protein and non-protein antigens. Antibody responses to most protein antigens typically rely on T_H cells and are therefore called T cell-dependent responses. The first step of T-dependent reactions is the recognition of a native epitope of a protein antigen by B cells in lymphoid follicles, and parallel recognition by naïve T cells of a peptide (bound to MHC-II) that is usually derived from the same protein antigen. Effector T_H cells and activated B cells meet at the parafollicular zone, where T cells again recognize the peptide-MHC complex on B cells, secrete cytokines and induce CD40L expression, thus inducing B cell proliferation and differentiation into short-lived antibody-secreting plasmablasts (extrafollicular reaction). A number of activated B cells migrate back to the follicle, where, upon stimulation by T_{FH} cells (e.g., via cytokines, CD40L-CD40 ligation), they proliferate and form germinal centres (GCs). In the germinal centres, B cells undergo isotype switch recombination and somatic hypermutation (affinity maturation) and differentiate into high-affinity, long-lived plasma cells.[75]

B lymphocytes can be subdivided into follicular B cells (also known as B2 cells), MZ B cells and B1 cells. Follicular B cells constitute the main B cell population in the lymphoid follicles of peripheral lymphoid organs (90% of B cells in the spleen). These cells receive T cell help and initially produce IgM and later IgG, IgA or IgE antibodies (upon isotype switching). MZ B cells are found in the marginal zone of the spleen where they respond to blood-borne antigens, while B1 cells are mostly found in the peritoneal and pleural cavities and in mucosal tissues, where they participate in surveillance against environmental pathogens. MZ B cells and B1 cells are known to mediate early (within 3 days) T cell-independent antibody responses, thus bridging innate and adaptive immunity. B1 cells—which are further

categorized as B1a and B1b based on surface markers—secrete germline-encoded natural antibodies (predominantly IgM but also IgA). Natural antibodies can recognize the phosphorylcholine head group present in oxidized phospholipids of oxidized low-density lipoprotein (oxLDL), apoptotic cells and the polysaccharide capsule of *Streptococcus pneumoniae*.[76] An additional B1 cell subset has recently been identified: innate response activator (IRA) B cells. These cells express the immature B cell marker CD93 and respond to lipopolysaccharide (LPS) by secreting granulocyte-macrophage colony-stimulating factor (GM-CSF). Finally, regulatory B cells are also considered a separate B cell subset with a distinct surface marker profile and regulatory functions (e.g., secretion of IL-10).[29,77]

3.3 IMMUNOLOGICAL TOLERANCE

3.3.1 Central and peripheral tolerance

In contrast to the innate immune system, adaptive immune cells have the potential to produce antigen receptors that recognize self- (or altered self) antigens, thereby triggering deleterious immune responses. Such autoimmune reactions do not usually occur, and this unresponsiveness to self is called "immunological tolerance". Immunological tolerance comprises central tolerance, which is induced by exposure of immature lymphocytes to self-antigens in the primary lymphoid organs (i.e., thymus, bone marrow), and peripheral tolerance, which is induced by exposure of mature lymphocytes to self-antigens in the periphery. Central tolerance is induced either by negative selection in primary lymphoid organs or the generation of naturally occurring regulatory T cells. Peripheral tolerance can be achieved via different mechanisms, including anergy or apoptosis of self-reactive lymphocytes and active suppression of self-reactive T cells by T_{reg} cells. The elucidation of peripheral tolerance mechanisms is particularly important for the development of immunomodulatory therapies against autoimmune diseases.[74]

3.3.2 Dendritic cells: orchestrators of immunity and tolerance

The outcome of a T cell response upon antigen recognition is influenced by the DC maturation state and co-signalling molecules—both costimulatory and co-inhibitory—on lymphocytes and DCs. Mature DCs that are induced by innate immune signals and express high levels of MHC-II and costimulatory molecules are considered proinflammatory/immunogenic, whereas immature DCs are tolerogenic.[78] Costimulatory and co-inhibitory molecules on DCs bind to their respective receptors on T cells. Important costimulatory molecules on T cells include CD28, CD40L, ICOS, CD137 and OX40, whereas CTLA-4 and PD-1 are the best-described co-inhibitory receptors.[79] Peripheral

tolerance may occur due to the absence of adequate costimulation or the presence of immunosuppressive cytokines, which can induce T-cell anergy or T_{reg} cell differentiation. Interestingly, CTLA-4 binds to CD80/CD86 (the same ligand for the costimulatory molecule CD28) on DCs.[47,80,81] In experimental atherosclerosis, blockade/deletion of co-inhibitory molecules or activation of co-stimulatory pathways aggravates disease.[82-84] The central role of DCs in the regulation of immunity renders these cells potential targets for immunomodulation.[78]

The function of DCs may also be influenced by the local cytokine milieu. A number of cytokines, especially IL-10 and TGF-β, have been used *in vitro* to generate tolerogenic DCs, which can promote antigen-specific T-cell unresponsiveness via several mechanisms, including induction of anergy, expansion of T_{reg} cells and secretion of immunosuppressive cytokines.[85] Controlling DC function via cytokine modulation is an attractive immunomodulatory approach and has been tested in animal models of autoimmunity. For example, injection of antigen-pulsed TGF-β_2–treated APCs ameliorates experimental autoimmune encephalomyelitis by induction of $CD8^+$ regulatory T cells.[86] In the context of atherosclerosis, IL-10–induced tolerogenic DCs have been shown to reduce atherosclerosis by inducing $CD4^+$ T_{reg} cells that inhibit apolipoprotein (apo) B-specific T cell responses.[87]

3.3.3 Regulatory T cells: mechanisms of immunological tolerance

The capacity of T_{reg} cells to promote immunological self-tolerance depends to a large extent on their ability to inhibit self-reactive T cells that have escaped central deletion in the thymus and exist normally in the periphery as part of the T cell repertoire. Many of the functions of T_{reg} cells are mediated by the secretion of the inhibitory cytokines IL-10 and TGF-β, which act on both APCs and T cells, induce a tolerogenic phenotype in DCs, and suppress T cell responses. These cytokines have been shown to be key players in the generation of induced T_{reg} cells *in vitro* and *in vivo*. IL-35 is another cytokine implicated in the regulatory activity of $Foxp3^+$ T_{reg} cells. T_{reg} cells may also induce contact-mediated cytolysis and apoptosis of effector T cells in a granzyme- and perforin-mediated manner. Other T_{reg} immunoregulatory mechanisms include CD25-mediated depletion of IL-2 and the production of adenosine nucleosides. The latter bind to adenosine receptors on effector T cells, thereby inhibiting IL-6 while promoting TGF-β secretion.[88]

CTLA-4 expressed on T_{reg} cells competes with the CD28 costimulatory molecules of proinflammatory cells for binding to CD80/CD86 of antigen-presenting cells, thereby preventing proinflammatory T cell responses. Moreover, CTLA-4 binding to CD80/CD86

leads to activation of the immunoregulatory enzyme indoleamine 2,3-dioxygenase 1 (IDO1), which leads to tryptophan depletion and production of immunomodulatory metabolites, downregulation of CD80/CD86, and activation of the transcription factor forkhead box O3 (FoxO3), which inhibits cytokine production by antigen-presenting cells. Another regulatory molecule expressed on T_{reg} cells is the lymphocyte activation gene 3 (LAG3), which binds to MHC-II molecules and inhibits dendritic cell maturation.[88]

4 LIPID METABOLISM

Cholesterol is an organic lipid molecule that is an essential component of cell membranes and a precursor of steroid hormones, bile acids and vitamin D. It can be synthesized by most cell types, with the highest production rates occurring in the liver and intestine. Cholesterol biosynthesis starts with the condensation of 2 acetyl-coenzyme A (CoA) molecules into acetoacetyl-CoA by acetoacetyl-CoA thiolase, subsequent condensation of acetoacetyl-CoA with an additional acetyl-CoA into 3-hydroxy-3-methylglutaryl (HMG)-CoA by HMG-CoA synthase, and reduction of HMG-CoA into mevalonate by HMG-CoA reductase, the rate-limiting enzyme of the pathway. Triglycerides, in contrast, are the main constituent of body fat and are used as energy sources and transporters of dietary fat. Cholesterol and triglycerides are transferred throughout the body on lipoprotein particles along three interconnected pathways: the exogenous pathway of dietary lipids, the endogenous pathway, and the reverse cholesterol transport pathway.

4.1 EXOGENOUS PATHWAY

In the gastrointestinal tract, dietary triglycerides are emulsified by bile acids and hydrolysed by pancreatic lipases into free fatty acids and monoacylglycerol that can be readily absorbed along with dietary cholesterol. Cholesterol absorption is regulated by intestinal Niemann-Pick C1 Like 1 (NPC1L1) protein, the molecular target of the lipid-lowering drug ezetimibe.[89] The same protein is also expressed in the canalicular membrane of hepatocytes and promotes the reabsorption of secreted biliary cholesterol.[90] In enterocytes, lipids (free fatty acids, monoacylglycerol, cholesterol and cholesterol esters) are packaged with apoB48 into nascent chylomicrons, which are then excreted in the lymphatics and enter the circulation via the thoracic duct. ApoB48, an obligate structural protein of chylomicrons, is the N-terminal 48% of the full-length apoB100. It is produced by enzymatic editing of a single nucleotide in apoB100 mRNA, which leads to the replacement of glutamine-2153 by a stop codon. Apolipoprotein C-II on circulating chylomicrons activates endothelial lipoprotein lipase

(LPL), which hydrolyses triglycerides into free fatty acids and glycerol that are then taken up by peripheral tissues, especially adipose tissue and muscle. The lipolysis of triglyceride-rich chylomicron particles is also facilitated by very low-density lipoprotein receptor (VLDLR), which is highly expressed on the endothelium of peripheral tissues (adipose tissue, muscle, heart) and has been suggested to enhance the activity of LPL.[91]

4.2 ENDOGENOUS PATHWAY

Hepatocytes synthesize triglycerides and package them with cholesterol esters and apoB100 in very low-density lipoprotein (VLDL) particles. Human liver synthesizes only apoB100, since there is no apoB100 mRNA editing activity. In mice, conversely, approximately 70% of hepatic apoB100 mRNA is edited, thereby yielding apoB48 VLDL particles. The regulation of apoB/VLDL secretion is complex, but the rate limiting step involves the microsomal triglyceride transfer protein (MTTP), which catalyses the lipid loading of nascent apoB in the endoplasmic reticulum.[92] VLDL particles are cleared by various receptors in the liver, including the low-density lipoprotein receptor (LDLR), heparan sulphate proteoglycans (HSPGs), LDLR-related protein 1 (LRP1), and SR-B1.[93,94]

In the periphery, VLDL is hydrolysed by lipoprotein lipase and hepatic lipase to generate free fatty acids and glycerol. VLDL remnants, also known as intermediate-density lipoprotein (IDL) particles, can then be either taken up by the liver or further hydrolysed into low-density lipoprotein (LDL). Apolipoprotein C-III, a lipoprotein secreted by the liver and intestine and present on triglyceride-rich lipoprotein particles, has been suggested to inhibit the clearance of VLDL, IDL and chylomicron particles by inhibiting LPL activity and interfering with the interaction of apoE and apoB with their hepatic receptors. LDL particles are finally removed from the circulation via the interaction of apoB100 with LDL receptors in the liver. The proprotein convertase subtilisin/kexin type 9 (PCSK9) has recently been shown to escort LDLR to lysosomes for degradation, thus regulating the levels of LDLR on the hepatic cell surface and LDL cholesterol concentration in the peripheral blood.

The biosynthesis of cholesterol and unsaturated fatty acids is regulated by a family of membrane-bound transcription factors, the sterol regulatory element binding proteins (SREBPs).[95,96] The inactive precursors of SREBPs are endoplasmic reticulum (ER) membrane-bound proteins. Their activation is regulated by sterols and leads to the cleavage and release of the active nuclear forms. Upon sterol depletion, SREBPs are escorted by the SREBP cleavage-activating protein (SCAP) from the ER to the Golgi apparatus, where they are sequentially cleaved by site-1 protease (S1P) and site-2 protease (S2P). This process

releases the active N-terminal segment of SREBPs, which is translocated to the nucleus and promotes gene transcription. High cellular cholesterol levels induce the association between SCAP and insulin-induced gene (INSIG) protein, leading to the retention of SREBP-SCAP complex in the ER.[97]

There are three SREBP isoforms (SREBP-1a, SREBP-1c and SREBP-2) that are encoded by two genes. SREBP-1a and SREBP-1c are encoded by the same gene but use different promoters. SREBP-1a and SREBP-2 are potent transcription factors, whereas SREPB-1c is relatively weak. For example, SREBP-1c transgenic mice display 2- and 4-fold increases in liver mRNAs of two important fatty acid biosynthetic genes, fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC), respectively, as opposed to 9- and 16-fold increases in SREBP-1a transgenic mice. Similarly, gene expression of LDLR and several cholesterol biosynthetic genes are elevated in SREBP-1a but not in SREBP-1c transgenic mice.[98] Both SREBP-1a and SREBP-2 can induce the LDLR and other genes involved in cholesterol and fatty acid biosynthesis. However, SREBP-1a is relatively more potent in inducing fatty acid biosynthesis, whereas SREBP-2 preferentially induces the cholesterol biosynthetic pathway.[99,100]

4.3 REVERSE CHOLESTEROL TRANSPORT

High-density lipoprotein (HDL) particles, which are mainly composed by phospholipids and apolipoprotein A-1 (apoA1), are synthesized by the liver and the intestine. The hepatic transporter ATP-binding cassette (ABC) A1 (ABCA1) plays an important role in the production of HDL by mediating the efflux of free cholesterol and phospholipids to apoA1. HDL particles can take up cholesterol from peripheral macrophages via macrophage ABCA1 and ABCG1. In the HDL particle, cholesterol is esterified by the enzyme lecithin cholesterol acyl transferase (LCAT), which creates a gradient for free cholesterol transfer from cells to HDL. Cholesterol esters in the mature HDL particle can either be taken up by the liver through the interaction of HDL with the scavenger receptor SR-B1 or transferred to apoB-containing lipoproteins—in exchange for triglycerides— via the enzyme cholesterol ester transfer protein (CETP).[101] Increasing HDL plasma levels by CETP inhibitors has been evaluated in clinical trials but does not seem to reduce cardiovascular events.[102] In the liver, cholesterol can be secreted in the bile or used for bile acid synthesis. Important proteins involved in cholesterol secretion in bile include the heterodimer ABCG5/ABCG8[103] as well as ABCB4[104] and ABCB11.[105] Cholesterol can be metabolized to bile acids via the classic bile acid biosynthetic pathway, which is regulated by the rate-limiting enzyme cytochrome P450 (CYP) 7A1 (CYP7A1), as well as an alternative pathway initiated by CYP27A1.[106]

5 TRYPTOPHAN METABOLISM

Tryptophan is an essential amino acid; it cannot be synthesized de novo and therefore the naturally occurring L-enantiomer must be acquired via the diet. Rich dietary sources of this amino acid include egg white, meat, fish, dairy products, sesame seeds and soybeans. In addition to being used as building block for protein synthesis, tryptophan is a precursor of two important metabolic pathways: the 5-hydroxyindole pathway, which leads to the generation of the neurotransmitter serotonin; and the oxidative/kynurenine pathway, which leads either to complete oxidation to carbon dioxide and water or, to a lower extent, the generation of nicotinamide nucleotides. Tryptophan decarboxylation to tryptamine and transamination to indole pyruvic acid are two additional metabolic fates of quantitatively minor importance.[107]

5.1 THE 5-HYDROXYINDOLE (SEROTONIN) PATHWAY

Once in the circulation, tryptophan is able to traverse the blood brain barrier through transport via the large amino acid receptor. In neurons, enterochromaffin cells, pulmonary endothelial cells and mast cells, serotonin is generated via the hydroxylation of tryptophan to 5-hydroxytryptophan (5-HTP) by the rate-limiting enzyme tryptophan 5-hydroxylase and subsequent decarboxylation of 5-HTP to 5-hydroxytryptamine (5-HT), which is widely known as serotonin. Serotonin is found abundantly in the enteric nervous system and, to a lower extent, in the central nervous system and in circulating platelets. In the pineal gland, serotonin is the substrate for melatonin synthesis.

5.2 THE KYNURENINE PATHWAY

In peripheral tissues, only 1% of dietary tryptophan is metabolized to serotonin, whereas over 95% is metabolized along the kynurenine pathway and generates a number of metabolites that are collectively called kynurenines. The first and rate-limiting step of the pathway, the oxidative cleavage of L-tryptophan to N-formyl-L-kynurenine, is catalysed by 3 different enzymes: tryptophan 2,3-dioxygenase (TDO) in the liver; indoleamine 2,3-dioxygenase 1 (IDO1) in various tissues; and the recently discovered and not well-studied indoleamine 2,3-dioxygenase 2 (IDO2). The level and/or activity of TDO is positively regulated by glucocorticoids and L-tryptophan and negatively by intermediate metabolites of the kynurenine pathway and nicotinamide nucleotides. IDO1 differs from TDO in that it is expressed in various tissues, uses superoxide rather than molecular oxygen to oxidize tryptophan into formylkynurenine and is induced upon inflammatory stimuli.

Formylkynurenine is hydrolysed to L-kynurenine by a formamidase enzyme. L-kynurenine is positioned at a branching point of the kynurenine pathway: it can be transaminated by kynurenine aminotransferases (KATs) to kynurenic acid (KYNA); it can be converted to anthranilic acid and alanine by the pyridoxal 5-phosphate (P5P, the active form of vitamin B6)-dependent enzyme kynureninase; a third alternative is oxidation by kynurenine 3-monooxygenase (KMO) to 3-hydroxykynurenine. The latter may be converted to xanthurenic acid by kynurenine aminotransferases or 3-hydroxyanthranilic acid (3-HAA) by kynureninase. The 3-HAA is further metabolized by 3-hydroxyanthranilic acid 3,4-dioxygenase (HAAO) to yield a metabolite that can either be oxidized completely to carbon dioxide and water or be converted to quinolinic acid. Quinolinate phosphoribosyl transferase (QPRT) metabolizes quinolinic acid further to nicotinamide nucleotides. Tryptophan is therefore an important supplementary source of nicotinamide nucleotides in addition to dietary niacin (nicotinic acid and nicotinamide) (**Figure 3**).[108,109]

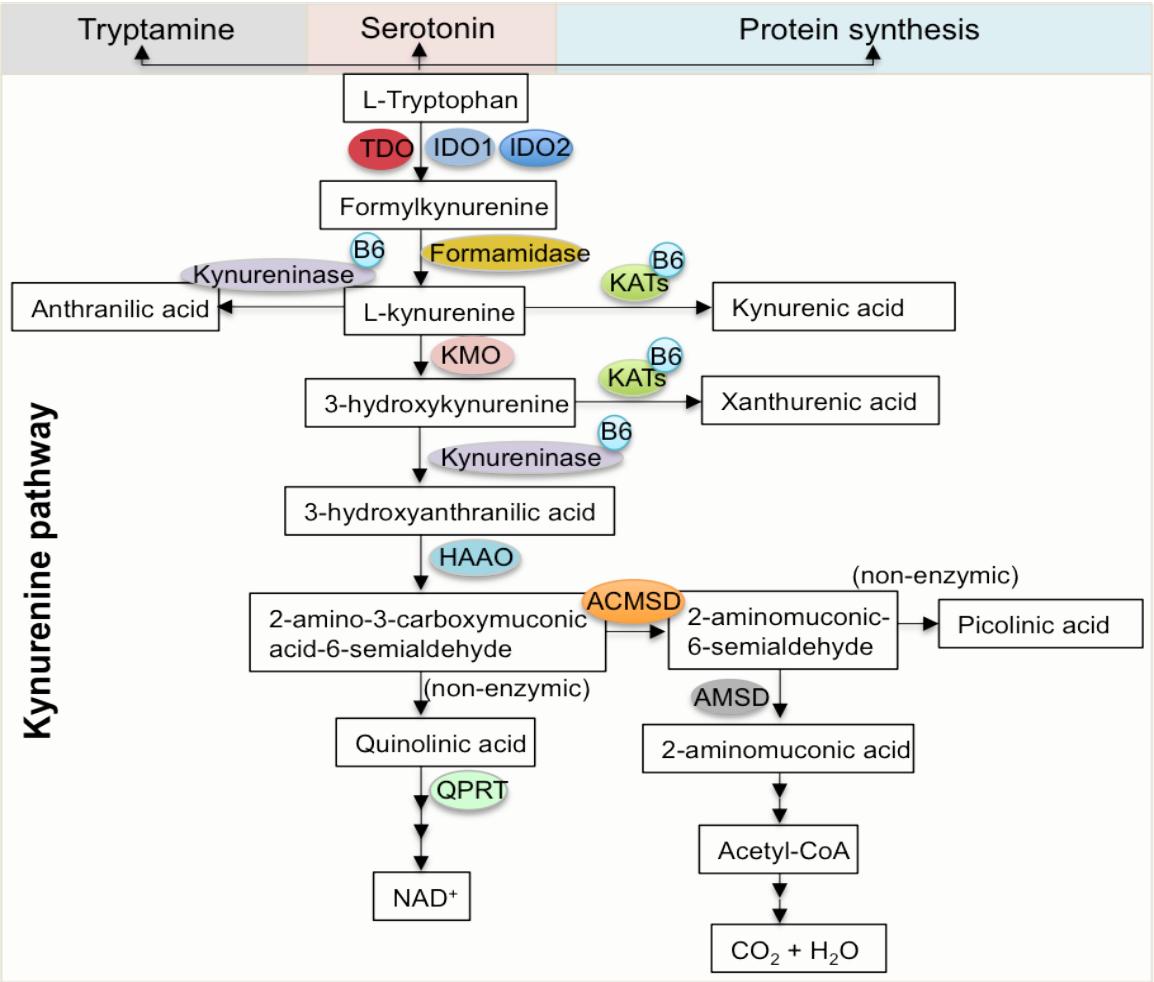

Figure 3. Tryptophan metabolism. IDO1 indoleamine 2,3-dioxygenase 1; IDO2 indoleamine 2,3-dioxygenase 2; TDO tryptophan dioxygenase; KAT kynurenine aminotransferase; B6 vitamin B6; KMO kynurenine 3-monooxygenase; HAAO 3-hydroxyanthranilate 3,4-dioxygenase; ACMSD 2-amino-3-carboxymuconic acid-6-

semialdehyde decarboxylase; AMSD 2-aminomuconic-6-semialdehyde dehydrogenase; QPRT quinolinate phosphoribosyl transferase.

5.2.1 Indoleamine 2,3-dioxygenase 1

Hayaishi and co-workers discovered indoleamine 2,3-dioxygenase in 1967.[110,111] Human IDO1 has relatively high activity in placenta, lung and small intestine, moderate activity in kidney, stomach and spleen and low activity in other tissues.[112] The human enzyme is able to oxidize L-tryptophan but also D-tryptophan and 5-hydroxy-L-tryptophan. In mice, high IDO1 activity is found in epididymis and small intestine, moderate activity in spleen, lung, colon and stomach and lower activity in other organs.[113] At a cellular level, IDO1 is expressed in several cell types, including macrophages, DCs, smooth muscle cells (SMCs) and endothelial cells, and it is induced under inflammatory conditions by various cytokines. IDO1 is induced by IFN-γ and, to a lower extent, by other cytokines, including TNF, IL-1β and type 1 interferons. Signal transducer and activator of transcription (STAT)-1α is necessary for IDO1 induction, whereas NF-κB, protein kinase R (PKR) and IRF-1 may also contribute to the induction/activation of IDO1 following IFN-γ treatment.[114] The cyclooxygenase 2 (COX-2)− prostaglandin E2 (PGE2) pathway has also been implicated in IDO1 expression, as the COX-2 inhibitor celecoxib suppresses IDO1 expression in a breast cancer cell line.[115] As shown in tumor cell lines, COX-2 contributes to the production of PGE2, which induces IDO1 via the PI3K–Akt−mTOR and the PKC− GSK3b− β catenin pathways. Interestingly, COX-2 expression and PGE2 production is induced by IL-1β.[116]

Munn and Mellor discovered that the immunoregulatory role of IDO1 activity in the placenta of pregnant mice prevents T cell-mediated rejection of allogeneic concepti.[117,118] IDO1 expression in APCs suppresses excessive immune responses through different mechanisms, including the induction of T-cell anergy or apoptosis and the generation of T_{reg} cells. Catabolism of tryptophan by IDO1-expressing cells depletes this essential amino acid in the local microenvironment, thus suppressing T cell proliferation and promoting T cell anergy and apoptosis.[118,119] This effect of tryptophan insufficiency is mediated by induction of the general control nonderepressible 2 (GCN2) stress response pathway in T cells.[119] Tryptophan depletion may also inhibit the mTOR and PKC-θ kinase pathways, thus inducing autophagy.[120] Moreover, there seems to be a bidirectional connection between IDO1 and T_{reg} cells. The expression of IDO1 in APCs can induce T_{reg} cells,[121,122] and T_{reg} cells have been shown to induce IDO1.[123] Experimental studies have employed either genetic ablation of IDO1 or the pharmacological inhibitor 1-methyl-tryptophan (1-MT) to show the importance

of IDO1 in peripheral tolerance[124-127] but also its deleterious effects in cancer immune escape.[128-130]

5.2.2 The pharmacological inhibitor 1-methyltryptophan (1-MT)

1-methyl-tryptophan (1-MT) is a competitive inhibitor of IDO1 that is currently being investigated in clinical trials investigating cancer immunotherapy. The inhibitor exists as two stereoisomers, L-1-methyl-tryptophan (L-1-MT) and D-1-methyltryptophan (D-1-MT), with distinct biological activities. The L isomer is more potent in cell-free enzyme assays as well as in a number of cell lines. However, the D isomer has been shown to be at least equally effective in inhibiting IDO1 activity in mouse and human dendritic cells. Interestingly, only the D isomer exhibits a significant *in vivo* antitumor effect, which is lost when the inhibitor is used in IDO1-knockout mice.[131] Interestingly, Metz and co-workers showed that only D-1-MT, but not L-1-MT, can inhibit IDO2-mediated tryptophan degradation.[132] A later study, however, showed that L-1-MT is a more potent inhibitor of both IDO1 and IDO2 activity.[133] At supraphysiological concentrations (>250 μM), D-1-MT has been shown to induce IDO1 mRNA and kynurenine production via the p38 MAPK and c-Jun N-terminal kinase (JNK) signalling pathways.[134]

5.2.3 Kynurenines: tryptophan metabolites with biological effects

In addition to tryptophan deletion, IDO1 activation results in the generation of several kynurenine metabolites with immunomodulatory functions. Particularly, L-kynurenine, 3-hydroxykynurenine, 3-HAA and quinolinic acid have been shown to inhibit T cell proliferation or to induce T cell apoptosis[135,136] via different mechanisms, including caspase 8 activation,[136] impairment of calcium signalling,[137] generation of ROS and glutathione depletion.[138,139] Upon systemic inflammation, endothelial cell-derived L-kynurenine induces SMC relaxation, vasodilation, and reduction of arterial blood pressure via activation of the adenylate cyclase−cAMP pathway as well as the soluble guanylate cyclase− cGMP−PKG pathway.[140] In addition, L-kynurenine can activate the aryl-hydrocarbon receptor (AHR) and induce differentiation of naïve T cells into T_{reg} cells.[141] 3-HAA can also induce T_{reg} differentiation in a dose-dependent manner.[142] In T cells, 3-HAA has been shown to inhibit NF-κB activation upon TCR engagement, thereby suppressing T cell function and inducing apoptosis. This effect is mediated by inhibition of phosphoinositide-dependent protein kinase 1 (PDK1), a key signal transducer downstream of CD28 that leads to NF-κB translocation to the nucleus.[143]

Another kynurenine metabolite with biological effects is KYNA, which has been shown to be a direct ligand for AHR and G-protein-coupled receptor 35 (GPR35).[144,145] KYNA treatment increases IL-1−induced expression of IL-6 in MCF-7 breast tumor cells in an AHR-dependent manner.[144] In an *in vitro* vascular flow model, KYNA was observed to trigger the adhesion of monocytes to endothelial cells, an effect that was partly mediated by GPR35.[146] In contrast to these proinflammatory actions, KYNA inhibits LPS-induced secretion of TNF by human peripheral blood mononuclear cells (PBMCs) or $CD14^+$ monocytes.[145] In the central nervous system, KYNA can block nicotinic α7 acetylcholine receptors and *N*-methyl-D-aspartate (NMDA) glutamate receptors,[147,148] thus opposing the effects of quinolinic acid, a known NMDA receptor agonist with pro-oxidant activity.[149,150] These metabolites have been implicated in a number of neurological disorders. Interestingly, a shift of the kynurenine pathway from L-kynurenine to KYNA through exercise training has been shown to protect against stress-induced depression.[151]

6 PATHOGENESIS OF ATHEROSCLEROSIS

Atherosclerosis refers to the build-up of a plaque in the artery wall, which leads to a progressive thickening of the intima and narrowing of the arteries supplying oxygen and nutrients to the heart, brain, extremities and peripheral organs. It is a disease of large- and medium-sized arteries, and it is initiated by the accumulation of apoB-containing LDL particles in the subendothelial space—also known as the intima— of arterial segments exposed to low wall shear stress (e.g., at branch points).[152]

Lipoprotein particles can pass the endothelial cell barrier through transcellular or paracellular transport.[153] In the intima, apoB-containing lipoproteins get trapped via ionic interactions with matrix proteoglycans, which is considered an initiating event in atherogenesis.[154-156] Following subendothelial retention, LDL undergoes various modifications, including oxidation, degradation and aggregation.[157] Modified LDL, in concert with local haemodynamic forces, induces endothelial cell expression of leukocyte adhesion molecules and secretion of cytokines and chemokines.[158,159] Oxidized phospholipids activate peroxisome proliferator-activated receptor (PPAR)-α and induce endothelial cell cytokine secretion and endothelial cell-monocyte interactions.[160] Another factor that contributes to the activation of endothelial cells in atherosclerosis-prone areas is the adhesion of platelets to endothelial cells via glycoprotein (GP) Ibα and GPIIb-IIIa.[161] Upon endothelial cell activation, circulating monocytes adhere to the endothelium and transmigrate towards a

chemokine gradient produced in the inflamed intima. T cells, dendritic cells and mast cells are also attracted into the intima, where they participate in a maladaptive immune response to the retained lipoproteins.[162,163]

In the intima, monocytes, under the influence of the macrophage colony-stimulating factor (M-CSF) and GM-CSF, differentiate into macrophages.[164] Macrophage accumulation in atherosclerotic plaques is caused by both monocyte recruitment and local macrophage proliferation.[37,165] In the intima, macrophages upregulate diverse PRRs, among which scavenger receptors are involved in the uptake of modified LDL. Over time, macrophages accumulate lipids, as scavenger receptors are not downregulated by increased intracellular cholesterol content.[166] LDL uptake is also mediated by fluid-phase endocytosis (pinocytosis) of native LDL (at much higher concentrations than oxLDL) and by phagocytosis or patocytosis of aggregated LDL.[167] Local inflammation impairs cholesterol efflux from macrophages via the downregulation of ABCA1 and ABCG1.[168,169] Altogether, these mechanisms lead to the cytosolic accumulation of large amounts of cholesterol esters in macrophages, which become lipid-laden "foam cells". Foam cells are the pathologic hallmark of early atherosclerotic lesions or "fatty streaks".[170] Throughout lesion development, many foam cells die, thereby producing apoptotic bodies that can later undergo secondary necrosis. Failure to remove the apoptotic material (impaired efferocytosis) from atherosclerotic plaques results in the formation of a necrotic core, which is associated with plaque vulnerability and atherothrombotic complications.[171]

In addition to macrophages, various immune cells, including T cells, dendritic cells, mast cells and neutrophils, have been described in atherosclerotic plaques.[163,172] T lymphocytes may account for up to 20% of infiltrating cells in certain regions of human plaques. Importantly, T cells recognizing foreign or modified self-antigens [e.g., oxLDL, chlamydia proteins, heat-shock protein (HSP)-60] have been isolated from atherosclerotic plaques, which indicates that T-cell mediated responses can influence the development of atherosclerosis.[173,174] In human plaques, $CD4^+$ T cells predominate over $CD8^+$ T cells and express the αβ T cell receptor. Most of these cells are $CD45RO^+$ memory (previously activated) cells located in the shoulder region or the interface between the fibrous cap and the lipid core, and they are often in an activated state.[175,176] T_H1 cells predominate in atheromata and secrete proinflammatory cytokines that activate both vascular and innate immune cells.

In addition to leukocytes, SMCs of the media layer also contribute to lesion pathogenesis. These cells can expand clonally, transdifferentiate into macrophage-like cells and migrate to the intima, where they can take up lipoprotein particles and become foam cells.[177] In fact,

SMC-derived cells comprise 30% of the total cells in mouse atherosclerotic lesions.[178] Intimal SMCs proliferate and produce extracellular matrix macromolecules, thereby forming a fibrous cap that surrounds a core of necrotic debris, proteoglycans and extracellular lipids, and it protects against plaque rupture. In vulnerable plaques, proinflammatory cytokines suppress SMC proliferation and collagen production, while vascular cell-secreted matrix metalloproteinases and ROS slowly degrade collagen fibres, rendering the plaque prone to rupture. Rupture and endothelial erosion are the ultimate complications of atherosclerosis, which lead to exposure of the thrombogenic material to the circulation, thrombus formation, and life-threatening clinical events (**Figure 4**).[179,180]

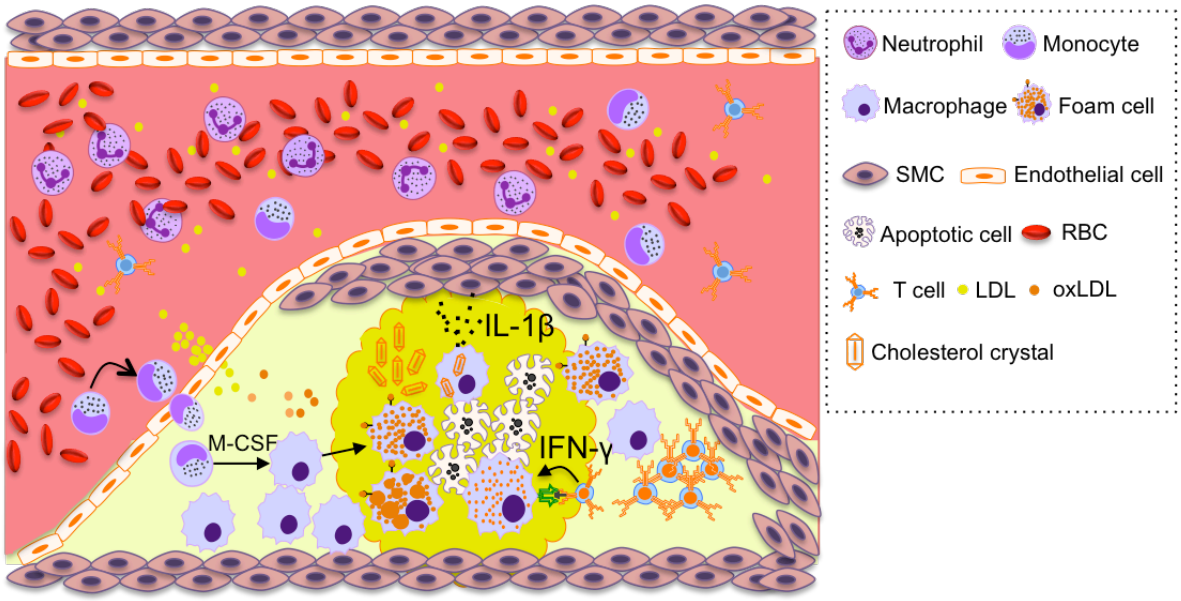

Figure 4. Pathogenesis of atherosclerosis. The advanced atherosclerotic plaque has a necrotic core consisting of lipid-engorged macrophages, apoptotic cells, extracellular lipids, and proteoglycans. The necrotic core is surrounded by a fibrous cap consisting of proliferating SMCs and collagen. The stability of the fibrous cap can be compromised by cell-derived proteases and ROS, which can lead to plaque rupture and thrombosis. Plaque formation is initiated by the accumulation and retention of LDL particles in the intima, where LDL undergoes various modifications. OxLDL activates endothelial cells, which express adhesion molecules on their luminal surface and attract leukocytes into the intima. Monocytes transmigrate into the intima and differentiate into macrophages, which accumulate oxLDL in their cytoplasm and become foam cells. These cells secrete various proinflammatory molecules in response to oxidized phospholipids, microbial products, heat-shock proteins and cholesterol crystals. The latter activate the NLRP3 inflammasome. In the shoulder region of the plaque, T cells recognize plaque antigens, proliferate, and secrete proinflammatory and plaque-destabilizing cytokines, such as IFN-γ.

6.1 INNATE IMMUNITY IN ATHEROSCLEROSIS

The importance of innate immunity in atherosclerosis is underscored by the predominance of macrophages in atherosclerotic plaques and by the observation that monocyte depletion

attenuates atherosclerosis in hyperlipidaemic mice.[181,182] In mice, hypercholesterolemia results in the expansion of peripheral inflammatory Ly6C$^+$ monocytes that home to inflamed arterial segments, where they differentiate into macrophages that take up oxLDL, become activated and transform into foam cells.[183] Upon activation, these cells upregulate proinflammatory genes and produce cytokines and other effector molecules, such as NO, ROS, and matrix-degrading enzymes. Of note, a number of macrophage-secreted cytokines, including TNF, macrophage migration inhibitory factor (MIF), IL-1, IL-18, IL-12 and M-CSF, have been shown to promote atherosclerotic cardiovascular disease.[184] In addition, the inducible isoform of nitric oxide synthase and certain NADPH oxidases have been implicated in the progression of atherosclerosis.[185,186]

Plaque macrophages constitute a heterogeneous population of cells with distinct phenotype and functions, but with a plasticity that allows them to adapt to the lesion microenvironment. Classically differentiated M1 macrophages have been associated with proinflammatory functions and plaque vulnerability, whereas M2 macrophages have been suggested to mediate anti-inflammatory functions and tissue-repair. In addition to the M1 and M2 macrophages representing, respectively, 40% and 20% of macrophages in advanced mouse atheromata, a distinct subset of Mox macrophages has been identified. Mox macrophages are induced by oxidized phospholipids, are characterized by Nrf2-dependent expression of redox-regulated genes, such as haem oxygenase 1 (HO-1), express the M1-related molecules IL-1β and COX-2, possess limited phagocytic and chemotactic capacity, and comprise approximately 34% of macrophages in advanced mouse plaques.[187] In human atherosclerosis, the picture becomes even more complicated by the description of additional macrophage subsets, including M(Hb), Mhem and M4 macrophages.[38,39]

As mentioned earlier, innate immune cells recognize PAMPs and DAMPs via pattern recognition receptors, including TLRs, NLRs and SRs. A number of these receptors are abundantly expressed in mouse and human atheromata and have been implicated in atherogenesis.[188] Our group has previously shown that NOD2, an NLR activated by bacterial peptidoglycan, is expressed in plaque macrophages and endothelial cells, induces the PGE2 pathway and promotes the formation of necrotic lipid cores.[189,190] Experimental studies on genetically engineered mice have suggested an atherogenic role for TLR2 and TLR4, which suggests that the presence of endogenous TLR ligands may promote disease development.[191-194] In contrast to the aforementioned surface TLRs, TLR7 and TLR9, endosomal TLRs respectively recognizing single-stranded RNA and double-stranded DNA with hypomethylated CpG motifs, have been shown to exhibit a beneficial role in experimental

atherosclerosis.[195,196] TLR3, an endosomal TLR that senses double-stranded RNA, presents a more complex situation, as whole body TLR3 is atheroprotective in apoE$^{-/-}$ mice, whereas bone marrow cell TLR3 expression promotes atherosclerosis in LDLR$^{-/-}$ mice.[197,198] Deficiency of myeloid differentiation primary response 88 (MyD88), a transducer in the signalling pathways of all TLRs apart from TLR3 (but also in IL-1/IL-18 receptor signalling) ameliorates atherosclerosis in apoE$^{-/-}$ mice.[191]

The identification of PAMPs and DAMPs promoting atherosclerosis has been a challenge in cardiovascular research. A hypothesis was that chronic infection with certain microbes generates PAMPs or DAMPs that promote atherosclerosis. However, there is lack of evidence pointing to infection as the primary inducer of atherogenic innate immune responses. An alternative hypothesis is that endogenous DAMPs are responsible for the atherogenic innate immune responses. Among a number of potential candidates, oxLDL and cholesterol crystals have assumed much interest over the past decade.

A growing body of evidence suggests that oxidation-specific epitopes (OSEs) on oxLDL and apoptotic cells are DAMPs that engage inflammatory innate immune responses.[199] For instance, oxidized cholesterol esters and oxidized phospholipids induce TLR4-dependent activation of macrophages and endothelial cells, respectively.[200,201] OxLDL triggers the assembly of a TLR4-TLR6 heterodimer, a process that is regulated by CD36, thus inducing activation of the NF-κB pathway and cytokine secretion in macrophages.[202] Interestingly, conditions associated with deranged cell cholesterol efflux, such as ABCA1 deficiency in patients with Tangier disease or low HDL plasma levels, increase plasma membrane lipid rafts and potentiate MyD88-dependent TLR signalling.[203] In contrast to oxLDL, minimally oxidized LDL cannot activate the NF-κB pathway in macrophages. However, it induces cytokine expression by activating the extracellular-signal-regulated kinase (ERK) 1/2 pathway in a TLR4-dependent manner and the PI3K pathway in a TLR4-independent manner.[204] OSEs are recognized by natural IgM antibodies, which exert an atheroprotective function possibly by blocking the uptake of oxLDL by macrophage SRs and promoting efferocytosis.[205-207] Interestingly, OSEs present on oxLDL or apoptotic cells show molecular mimicry with antigens in the cell wall of certain bacteria, particularly pneumococci. Indeed, vaccination of atherosclerosis-prone mice with *Streptococcus pneumoniae* increases oxLDL-specific IgM antibodies and ameliorates atherosclerosis.[208]

As lipid-engorged macrophages accumulate cholesterol, cholesterol crystals form in the cytosol and activate the NLRP3 inflammasome, thus triggering caspase-1−dependent IL-1β secretion.[22] The deleterious role of IL-1β in atherosclerosis has been shown in apoE$^{-/-}$ mice,

which develop less disease upon IL-1β deletion.[209] With some exceptions, the vast majority of experimental data support a proatherogenic role of inflammasome activation.[22,210] More importantly, the recently published CANTOS trial showed that neutralization of IL-1β with monoclonal antibodies decreases major cardiovascular events.[26]

6.2 T CELLS IN ATHEROSCLEROSIS

Adaptive immunity has been implicated in atherogenesis by the presence of T cells in atheromata, the increased concentration of antibodies to LDL in the circulation of patients with vascular disease, and the arteriopathy observed in heart transplants of patients without conventional cardiovascular risk factors.[211] The importance of adaptive immune cells in atherosclerosis has been confirmed in experimental studies using gene-targeted mice. For instance, mice deficient for both T and B cells present less atherosclerosis than immunocompetent mice in the context of hypercholesterolemia.[212-215]

Immunohistochemical staining has shown that T cells account for 7-20% of infiltrating cells in human atherosclerotic plaques, with a predominance of $CD4^+$ T cells.[175] A substantial proportion of T cells in the plaque are in an activated state, as indicated by the expression of human leukocyte antigen (HLA)-DR and the CD25 subunit of the IL-2 receptor.[216,217] These observations suggest that cell-mediated immune responses are taking place in the vascular wall. Indeed, T cells that are reactive to self-antigens, such as oxLDL and HSP60, have been isolated from human plaques.[174,218] This supports the notion that local autoimmune responses occur in the vascular wall and influence the development of atherosclerosis.

The net effect of $CD4^+$ T_H cells is proatherogenic, as indicated by the reduction of atherosclerosis in mice after depletion of these cells.[219,220] Moreover, adoptive transfer of $CD4^+$ T cells into immunodeficient mice increases atherosclerotic disease, which is associated with elevated systemic levels of IFN-γ.[214] In addition to cytokine secretion, the expression of stimulatory surface proteins is another crucial effector function of $CD4^+$ T cells. One such molecule expressed on activated T_H cells is CD40L, which binds to its receptor CD40 on APCs and B cells.[29] Abrogation of this interaction either by use of antibodies or genetic ablation of CD40L in hyperlipidaemic mice reduces lesion size and promotes a more stable plaque phenotype.[221,222] In contrast to T_H cells, $CD8^+$ T cells have been studied to a lesser extent. Although genetic deletion of $CD8^+$ T cells has no effect on atherosclerosis,[223] antibody-mediated depletion of this cell population reduces the extent of disease.[224] Despite the aforementioned data on the proatherogenic role of T cells, there are also studies showing a protective effect of cell-mediated immunity against the disease.[223,225-228]

6.3 T_H CELL SUBSETS IN ATHEROGENESIS

The balance between different T cell subsets may influence atherogenesis. In the plaque, there is a predominance of T_H1 cells that secrete the proinflammatory cytokine IFN-γ, which induces the expression of MHC molecules on APCs, increases the efficiency of antigen presentation, inhibits smooth muscle cell proliferation and collagen production, and activates macrophages to release cytokines, chemokines, ROS and proteolytic enzymes.[229,230] Importantly, deletion of the signature T_H1 transcription factor T-bet or the IFN-γ receptor ameliorates atherosclerosis.[231,232] The T_H1-inducing cytokines IL-12 and IL-18 are also deleterious in atherosclerosis.[169]

Despite accumulating data on the proinflammatory function of T_H1 responses, the role of T_H2 cells is not clear. The T_H2 cytokine IL-4 has been shown to promote macrophage proliferation in plaques.[165] Genetic ablation of IL-4 in apoE$^{-/-}$ mice decreases atherosclerosis.[233] Consistent with this observation, King and co-workers showed that IL-4 deficiency in bone marrow-derived cells could ameliorate disease in the LDLR$^{-/-}$ mouse model, suggesting a proatherogenic role of this signature T_H2 cytokine.[234] The authors, however, later showed that genetic deficiency or exogenous administration of IL-4 did not affect atherosclerosis development in both apoE$^{-/-}$ and LDLR$^{-/-}$ mice as well as in a model of angiotensin II-induced atherosclerosis.[235] At odds with these studies, exogenous IL-4 administration was found to be protective in mildly hyperlipidaemic mice and to lead to a reduction of atherosclerosis.[220] IL-5, another T_H2 cytokine that is also produced by mast cells and is important for B cell differentiation, probably has a beneficial role in atherogenesis, as suggested by the aggravated disease in IL-5$^{-/-}$ mice.[236]

In addition to T_H1 and T_H2 cells, IL-17−producing T_H17 cells have also been described in the context of atherosclerosis with mixed results. IL-17 is known to induce the expression of proinflammatory cytokines and chemokines, such as IL-6, IL-1β, TNF, MCP-1 and CXCL1.[237] Most experimental studies support a proatherogenic effect of IL-17 signalling.[237-240] Using IL-17A$^{-/-}$ apoE$^{-/-}$ mice, Madhur and co-workers showed that IL-17A increases high-fat diet−induced IFN-γ production by splenocytes, plaque macrophage content and ROS production, but it does not increase the atherosclerotic plaque size.[241] In contrast, T_H17 cells have recently been suggested to promote plaque stabilization through IL-17A−mediated collagen production by vascular SMCs.[242]

While T cells, as a whole, induce inflammation and atherosclerosis, regulatory T cells secrete the anti-inflammatory cytokines IL-10 and TGF-β, suppress effector T cells, and exert atheroprotective functions. IL-10 deficiency,[243,244] abrogation of T cell-specific TGF-β

signalling by a dominant negative mutation in TGF-β receptor II,[245] or inhibition of TGF-β signalling by neutralizing antibodies aggravate atherosclerotic disease in mouse models.[246] In addition, depletion of T_{reg} cells leads to an increase in the extent of disease in both $apoE^{-/-}$ and $LDLR^{-/-}$ mice.[247,248] Another immunoregulatory T cell subset, the IL-10−producing Tr1 cells, inhibits T_H1 responses, increases plaque IL-10, and ameliorates atherosclerosis in adoptive transfer experiments to $apoE^{-/-}$ mice.[249]

6.4 B CELLS IN ATHEROSCLEROSIS

Although B lymphocytes are rarely detected in the intima, nodular aggregations of B cells—also known as tertiary lymphoid organs— are found in the adventitia of advanced lesions.[250] Moreover, antibodies against plaque antigens are present in the circulation of humans and mice, which suggests that B cell responses may influence the progression of the disease.[77] Intravenous immunoglobulin (IgG) was found to ameliorate atherosclerosis in $apoE^{-/-}$ mice, an effect that was later suggested to be mediated by the binding of the Fc portion to the inhibitory FcγRIIb.[251-253] Moreover, induction of IgG antibodies to OSEs via immunization ameliorates experimental atherosclerosis.[254] Initial studies suggested that B cells provide protection against atherosclerosis. Caligiuri and co-workers showed that splenectomy in $apoE^{-/-}$ mice accelerates atherosclerosis, an effect that is reversed by adoptive transfer of B cells.[255] Consistent with this observation, $LDLR^{-/-}$ mice reconstituted with bone marrow from B cell-deficient (μMT) mice have significantly increased atherosclerotic disease compared with recipients of wild type bone marrow.[256]

The beneficial role of B cells, especially the IgM-producing B1 cell population, was strengthened by Lewis and co-workers, who showed that lack of serum IgM ($sIgM^{-/-}$) aggravates atherosclerosis in $LDLR^{-/-}$ mice.[257] B1 cell-derived natural IgM antibodies against OSEs have been shown to inhibit oxLDL uptake by macrophages.[254] In the same line of evidence, Kyaw and co-workers showed that splenectomy in $apoE^{-/-}$ mice results in a substantial reduction of peritoneal B1a cells and aggravation of atherosclerosis. Interestingly, B1a, but not B2, cell transfer into splenectomized mice restores this cell population and protects against atherosclerosis, an effect that is dependent on IgM secretion.[258]

The apparent protection conferred by B cells was challenged by two independent studies showing that B cell depletion by monoclonal anti-CD20 antibodies reduces the extent of atherosclerosis in $apoE^{-/-}$ and $LDLR^{-/-}$ mice.[259,260] The explanation for this discrepancy probably relies on differential effects of B cell subsets. In fact, anti-CD20 treatment preferentially depletes conventional B2 cells and peritoneal B1b cells, whereas the peritoneal

B1a cell population is spared. B2 cells most likely promote atherosclerosis, as indicated by the increased plaque burden caused by adoptive transfer of B2 cells in apoE$^{-/-}$ mice lacking lymphocytes (Rag2$^{-/-}$ γ-chain$^{-/-}$) or B cells.[259] The proatherogenic function of B2 cells was further supported by studies on the interaction between the B cell activating factor (BAFF) and BAFF receptor (BAFFR), which is crucial for the survival of B2 (but not B1a) cells. Genetic ablation of BAFFR in atherosclerotic mouse models reduces the plaque burden.[261,262] At odds with these studies, Doran and co-workers found that adoptive transfer of B2 cells from apoE$^{-/-}$ to B cell deficient apoE$^{-/-}$ mice attenuates the development of atherosclerosis.[263] In summary, peritoneal B1a cells are thought to exert an atheroprotective role, whereas the majority of studies support a proatherogenic role for conventional B2 cells.

6.5 ADAPTIVE IMMUNE RESPONSE TO LDL

Experimental studies have illuminated the role of adaptive immunity, a necessary component of autoimmune responses, in the development of atherosclerosis. Hyperlipidaemic apoE$^{-/-}$ mice with severe combined immunodeficiency (*scid/scid*) develop much less atherosclerosis than immunocompetent controls.[214] Moreover, transfer of CD4^{+} from old apoE$^{-/-}$ mice into immunodeficient controls aggravates atherosclerosis,[214] while bone marrow transplantation from mice lacking B cells into LDLR$^{-/-}$ mice also leads to deteriorated disease.[256] These observations support T cell-mediated responses rather than an autoantibody response as a potential driving force in atherogenesis.

The most studied autoantigens in the field of atherosclerosis are HSP60 and modified LDL. HSP60 is a phylogenetically highly conserved mitochondrial protein that is found in both prokaryotic and eukaryotic cells. T cells reactive to HSP60 have been isolated from human atherosclerotic plaques. In addition, immunization of experimental animals with the mycobacterial homologue HSP65 aggravates atherosclerosis, a finding that provides indirect evidence of autoimmunity, according to the criteria described by Rose and Bona.[264-266] However, HSP60 is not specific for atherosclerosis, as it has also been described for other inflammatory disorders such as arthritis. Moreover, later studies focusing on later stages of atherosclerosis have shown opposite results, as HSP65 immunization reduces atherosclerosis in apoE$^{-/-}$ mice.[267]

A more specific autoantigen for atherosclerosis, modified LDL, has also been implicated in atherogenesis.[268] A number of peptide and lipid epitopes are present in the LDL particle and circulating autoantibodies against these epitopes are found in both animals and humans. T cells recognizing different forms of LDL have been isolated from human atherosclerotic

plaques. Importantly, adoptive transfer of $CD4^+$ T cells reactive to malondialdehyde (MDA)-oxidized LDL aggravates atherosclerosis in $apoE^{-/-}$ *scid/scid* mice.[269] Although this study provided evidence for an autoimmune component in atherogenesis, it was limited by the fact that it used immunodeficient *scid/scid* mice.

The aforementioned proatherogenic responses of $CD4^+$ T cells were observed in *scid/scid* mice lacking T and B cells. Therefore, possible T cell-dependent B cell responses could not be evaluated in this model. Several studies support an atheroprotective role for humoral responses to oxLDL and native LDL (nLDL). In 1959, Gero and co-workers demonstrated an atheroprotective effect of immunization with β-lipoprotein in animals fed a high-cholesterol diet.[270] Thirty-six years later, Palinski and co-workers showed that immunization of $LDLR^{-/-}$ rabbits with homologous MDA-modified LDL increases IgG antibodies against MDA-LDL and confers atheroprotection.[271] The same group showed that immunization of $LDLR^{-/-}$ mice with either MDA-modified or native LDL confers a similar degree of atheroprotection, similar modest decreases in plasma cholesterol, but large differences in the levels of antibodies against oxidization-specific epitopes.[272] Ameli and co-workers also found that immunization with homologous copper-oxLDL or nLDL increases anti-oxLDL IgG antibodies and ameliorates diet-induced atherosclerosis in rabbits. Interestingly, immunization with nLDL was more effective in reducing plaque size in this study.[273] These findings were supported by additional studies in hyperlipidaemic rabbits and mice.[272,274-276]

In summary, LDL has been considered the predominant autoantigen involved in atherogenesis. T cell-mediated immunity to modified LDL appears to be proatherogenic in experimental studies. In 2010, Hermansson and co-workers created apoB100-reactive $CD4^+$ T cell hybridomas that express a particular TCR variable β (TRBV) chain, TRBV31. Interestingly, immunization of atherosclerosis-prone mice with a TRBV31-derived peptide induced antibodies that could block the recognition of apoB100 by $TRBV31^+$ T cells, which was associated with a reduction in experimental atherosclerosis.[277] In contrast, the majority of data on humoral anti-LDL responses suggest an atheroprotective role of antibodies to oxidized or native LDL. Different mechanisms for this atheroprotection have been suggested, including antibody-mediated LDL clearance from the circulation, Fc-receptor-mediated oxLDL clearance from plaques, IgG-mediated stimulation of inhibitory Fc receptors, generation of T_{reg} cells or unknown T cell-mediated immune responses.

7 AIMS

The general aim of the present thesis was to increase understanding of the reciprocal relationship between immunity and metabolism in atherosclerosis and to explore novel immunomodulatory therapeutic strategies against atherosclerotic CVD.

The specific aims of each article were as follows:

I. To investigate the role of T cell-mediated responses to LDL in atherosclerosis.

II. To evaluate the potential of triggering the T_{reg} cell − IDO1 axis to promote vascular tolerance mechanisms and treat atherosclerosis.

III. To explore the role of IDO1-mediated tryptophan metabolism in atherosclerosis.

IV. To explore the atheroprotective molecular mechanisms of the tryptophan metabolite 3-HAA and to assess the potential effects of increasing endogenous 3-HAA levels.

8 METHODOLOGICAL CONSIDERATIONS

8.1 MOUSE MODELS OF ATHEROSCLEROSIS

Mice are resistant to atherosclerosis development when fed a normal diet. Their plasma cholesterol is below 100 mg/ml and is mainly found within the HDL fraction. Only high-cholesterol high-fat diets that also include cholic acid can induce small lesions (fatty steaks) in the region of valve leaflets of certain atherosclerosis-prone mouse strains, such as the C57BL/6 strain.

A widely used mouse model of atherosclerosis is the apolipoprotein E knockout mouse.[278,279] These mice lack apoE, an apolipoprotein that mediates the clearance of circulating lipoprotein particles, show high levels of plasma cholesterol even on low-fat, low-cholesterol diets, and develop atherosclerosis spontaneously. Importantly, plasma cholesterol is contained mainly in chylomicrons, VLDL and IDL particles, a profile similar to that observed in human type III hyperlipoproteinemia. The atherosclerotic lesions of these mice pass through all stages of human plaque development (fatty streak, fibrous plaque, complex plaque) and develop at atherosclerosis-prone vascular sites, such as the lesser curvature of the aorta, branch points and proximal parts of aorta branches. A disadvantage of this mouse model is that it is not optimal for bone marrow transplantation studies, since macrophage-derived apoE normalizes cholesterol levels and prevents lesion development.[280] Moreover, apoE has been shown to influence the proliferation of bone marrow haematopoietic stem cells and myeloid progenitors of monocytes and neutrophils.[281]

Another common mouse model of atherosclerosis is the LDLR knockout mouse.[282] This mouse shows approximately twice as high plasma cholesterol levels in comparison to wild type controls but does not develop atherosclerotic lesions on a low-fat, low-cholesterol diet. Importantly, cholesterol is mainly contained in the IDL and LDL fractions. A reason why LDLR deficient mice on chow diet are resistant to atherosclerosis is that mouse liver produces a high proportion of apoB48-containing lipoprotein particles, which can be taken up by receptors other than LDLR, such as LRP1. When fed a high-fat, high-cholesterol diet, these mice show pronounced plasma cholesterol levels and develop lesions that are mostly of the fatty streak variety. This mouse model is widely used to study the role of proteins expressed by bone marrow-derived cells during atherosclerosis development.

Transgenic mice expressing human apoB have also been used to study atherosclerosis. These mice have 1,5-2 times higher plasma cholesterol levels (contained in the LDL and HDL fraction) but are resistant to atherosclerosis on a low-fat, low-cholesterol diet.[283,284] However,

when these mice are crossed with LDLR knockout mice, they show pronounced plasma cholesterol levels (contained in the LDL fraction) and develop complex atherosclerotic plaques on a chow diet.[285] While mouse models have undoubtedly contributed to our current understanding of the pathogenesis of atherosclerosis, they are limited by the typical absence of plaque rupture and thrombosis, which are common features of human coronary atherosclerosis.

In **Papers I and II**, we employed *Human APOB100-tg Ldlr^{tm1Her}* (*HuBL*) mice backcrossed to C57BL/6J for 10 generations. *HuBL* mice express a truncated nonfunctional LDLR and are transgenic for human apoB, in which leucine is substituted for glutamine-2153 to prevent apoB100 mRNA editing and formation of apoB48.[155,286] In **Paper I**, three mouse strains transgenic for three different TCRs were generated by microinjection of hCD2-VA expression vectors containing specific TCR α and TCR β constructs into C57BL/6J embryos. These vectors direct the expression of the transgenes on the surface of T lymphocytes.[287] The three transgenic strains were designated *BT1* (TRAV12, TRBV31), *BT2* (TRAV4, TRBV31) and *BT3* (TRAV14, TRBV31). The chosen TCRs were cloned from previously described T cell hybridomas that respond to native LDL and apoB100.[277] The different BT strains were crossed with *HuBL* mice, or with a reporter mouse expressing green fluorescent protein (GFP) from the Nr4a1 (Nur77) locus, which is activated upon TCR stimulation.[288] In **Papers III and IV**, we used the apoE$^{-/-}$ and LDLR$^{-/-}$ mouse model, respectively. All animal experiments were conducted in accordance with institutional guidelines and Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and were approved by the regional board for animal ethics in Stockholm.

8.2 DENDRITIC CELL-BASED IMMUNOTHERAPY

Dendritic cells were generated from bone marrow cells obtained from femurs and tibias of *HuBL* mice, depleted of red blood cells, and cultured at 37°C and 7.5% CO_2 for 8 days in FBS containing DMEM with 10 ng/ml GM-CSF and 10 ng/ml IL-4.[289] Isolation of DCs was performed using a CD11c magnetic cell-sorting kit. DCs were characterized as CD11c$^+$ CD11b$^+$ I-A^{b+} CD80$^+$ CD86$^+$ CD205$^+$ cells, and more than 90% of the cells were viable. Next, the cells were transferred into cell culture dishes, where they were incubated with 5 ng/mL TGF-β_2 with or without 25 μg/mL apoB100 in serum-free DMEM at 37°C and 5% CO_2. After 4 hours, 0,1 ng/ml LPS was added to the medium, and the cells were incubated for an additional 14 hours. Low-dose LPS was used to induce minimum maturation of DCs and to ensure apoB100 could be presented by MHC-II. Finally, DCs were washed with DMEM, kept on ice and injected intravenously into eleven-week-old male *HuBL* mice within 1 hour.

Five days later, the mice were placed on a Western diet (corn starch, cocoa butter, casein, glucose, sucrose, cellulose flour, minerals, and vitamins; 0,15% cholesterol, 21% fat, 17% protein, 43% carbohydrates, 10% H_2O, and 3.9% cellulose fibres; R638 Lantmännen, Kimstad, Sweden) and maintained for 10 weeks. In parallel to the cell injection preparations, DCs, prepared as mentioned above, were used in *in vitro* experiments assessing cytokine secretion and in T_{reg} cell conversion assays.

8.3 LDL ISOLATION FROM HUMAN PLASMA

LDL was freshly prepared from the pooled plasma of 2-3 healthy donors using a two-step ultracentrifugation process.[290] Briefly, plasma was ultracentrifuged in a Beckman Optima™ L-90K ultracentrifuge equipped with a SW40Ti swinging-bucket rotor at 40,000 rpm for 20-24 hours (4°C). After centrifugation, the upper layer containing chylomicrons, VLDL, IDL and a small fraction of LDL was carefully removed with a glass Pasteur pipette. Beneath the upper layer was a colourless region that was also discarded to a large extent. The lowest part of this colourless region as well as the underlying yellow layer was collected in a volumetric flask for density adjustment. The density was adjusted to 1.060 using potassium bromide. The density-adjusted plasma was then again ultracentrifuged as previously described, and the uppermost LDL fraction was carefully collected. Potassium bromide was removed from LDL using a PD-10 desalting column (GE Healthcare Life Sciences, Uppsala, Sweden). Ethylenediaminetetraacetic acid (EDTA) was added to the LDL preparation (1 mg/ml protein concentration) at a final concentration of 1 mM. For detection of antibodies against native LDL, both EDTA and 20 μM butylated hydroxytoluene (BHT) were added to the plasma before ultracentrifugation.

8.4 LDL OXIDATION

OxLDL was prepared using copper sulphate ($CuSO_4$). The LDL samples were first diluted with phosphate-buffered saline (PBS) to a final protein concentration of 1 mg/ml and then incubated with 20 μM $CuSO_4$ for 18 hours at 37°C. $CuSO_4$ was then removed using a PD-10 desalting column, and oxidation was halted by addition of 1 mM EDTA.

8.5 APOLIPOPROTEIN B PREPARATION

ApoB100 was prepared from isolated human LDL using a chloroform/methanol extraction protocol.[291] Four parts methanol, 1 part chloroform and 3 parts distilled water were added to 1 part LDL solution. The mixture was vortexed vigorously and then centrifuged at 9000 g for 10 minutes. The apoB100 protein was precipitated at the interface, isolated, washed with PBS, and dissolved in sodium dodecyl sulphate (SDS) buffer. Excess SDS was removed

using a PD-10 desalting column. Soluble apoB100 was isolated by size-exclusion chromatography using a Superdex200 column equilibrated with Tris-buffered saline (TBS) at pH 7.6 (0.5 ml/min).

8.6 LDL-IGG IMMUNE COMPLEX AND APOB CLEARANCE EXPERIMENTS

LDL was labelled with FITC as previously described. Briefly, LDL (2 mg/ml in terms of protein) was dialysed overnight against 0.5 M $NaHCO_3$ at pH 9.5. Next, 100 μg FITC dissolved in DMSO was added for every 1 mg of LDL protein, and the mixture was incubated for 2 hours at room temperature. Then, the conjugates were separated from free fluorochrome using a PD-10 column and PBS as elution buffer. Protein concentrations were measured using the Bradford assay. Human FITC-labelled LDL (100 μg protein) was mixed with plasma from *HuBL* mice that had received either BT3 $CD4^+$ T cells or B6 $CD4^+$ T cells. The mixture was then injected in the tail vein of *HuBL* mice, and blood was collected at several time intervals. Fluorescence was measured in plasma samples diluted 1:25 in PBS using a Perkin Elmer Wallac 1420 Victor^2 plate reader. Fluorescence was normalized to the signal obtained in plasma collected 1 minute after injection.

IgG antibodies were purified from the plasma of *HuBL* or *BT3xHuBL* mice using Protein G resin columns (GE Healthcare Life Sciences, Uppsala, Sweden), according to the manufacturer's instructions. After purification, IgG solutions were extensively dialysed against PBS, and their protein concentration was measured with a Nanodrop 1000 spectrophotometer. Two-hundred micrograms of purified IgG preparations were injected intravenously into *HuBL* recipient mice, and blood samples were collected at several time intervals for quantification of the plasma apoB concentrations.

8.7 IMMUNE COMPLEXES AND ANTIBODIES TO LDL, OXLDL, APOB100

Antibodies against LDL, oxLDL and apoB100 and corresponding immune complexes were determined using the enzyme-linked immunosorbent assay (ELISA). For determination of antibody titres, LDL, oxLDL or apoB100 (10 μg/ml, 30 μl/well) was coated onto Corning™ Costar™ 96-well microplates and incubated overnight at 4°C. For immune complex detection, anti-apoB100 antibodies were used to coat the plates instead of lipoprotein or apoB100 solutions. Coated plates were washed twice, and "blocked" with 1% gelatine in PBS for 1 hour at room temperature. The plates were washed twice with PBS, and mouse plasma diluted in TBS with 0.1% gelatine was added to the wells (30 μl/well) for 2 hours. The plates were washed three times with PBS containing 0.05% Tween® 20. Biotinylated anti-mouse IgG or anti-mouse IgM antibodies, diluted in TBS with 0.1% gelatine, were then added to the

wells for 1 hour. After washing, horseradish peroxidase (HRP)-conjugated streptavidin, diluted in TBS with 0.1% gelatine, was added for 30 minutes. The plates were washed again, and HRP activity was detected using TMB (3,3',5,5'-tetramethylbenzidine) substrate followed by the addition of sulphuric acid stop solution to yield a yellow colour, the absorbance of which was measured in a Versamax™ microplate reader.

In competition ELISA assays, purified IgG (10 μg/ml) from either *HuBL* or *BT3xHuBL* mice was preincubated with increasing concentrations of native LDL, oxLDL and apoB100 in glass tubes at 4°C overnight. The mixtures were then used in the ELISA assays to detect IgG antibodies against LDL and oxLDL, as described above.

8.8 EVALUATION OF ATHEROSCLEROSIS

The extent of atherosclerosis development was evaluated using two different methods: *en face* staining of thoracic aortas with Sudan IV and staining of transverse sections of the proximal 800 micrometers of the aortic root (starting from the level where the aortic leaflets become visible in the microscope) with Oil Red O (ORO). Sudan IV and ORO stain lipids and fatty substances in cells and tissues with a red colour. The term *en face* refers to longitudinally opened aortas that have been removed from the thoracic cavity of animals and pinned on a plastic surface. This configuration allows quantification of the intima area stained with Sudan IV. Lesion size was quantified in a blinded fashion using ImageJ software.

8.9 STATISTICS

When a Gaussian distribution could not be assumed, the following non-parametric statistical tests were used: the Mann-Whitney U test for comparisons of mean values between 2 groups, and Kruskal-Wallis one-way analysis of variance (ANOVA) followed by Dunn's multiple comparison post hoc test for comparisons between more than 2 groups. Spearman's rank correlation coefficient was used to assess correlations between 2 variables. In **Paper I**, if normality was suggested by the Shapiro-Wilk test, the following parametric tests were used: Student's t-test for comparison of means between 2 groups, one-way ANOVA with Dunnett's multiple comparison test for comparison of means between more than 2 groups, two-way ANOVA with Bonferroni's post-test for comparisons of mean values between groups that have been split on two independent variables, and Pearson correlation coefficient for linear correlations between continuous variables. Continuous variables are presented in various graph types (e.g., column bar graphs, scatter plots) as the mean ± standard error of the mean (SEM). The alpha (α) level denoting the type I error rate or statistical significance was set at

0.05 (5%). Statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc., CA, USA).

9 RESULTS AND DISCUSSION

9.1 LDL-REACTIVE T CELLS LOWER PLASMA CHOLESTEROL AND PROTECT AGAINST ATHEROSCLEROSIS (PAPER I)

To investigate the definitive the role of apoB100-reactive T cells in atherosclerosis, we generated 3 transgenic mouse strains in which the vast majority of $CD4^+$ T_H cells recognize apoB100. The strains, termed *BT1*, *BT2* and *BT3*, expressed the transgenic TCR variable β chain TRBV31 and one of the following variable α chains: TRAV12 (*BT1*), TRAV4 (*BT2*) or TRAV14 (*BT3*), respectively. The majority of $TRBV31^+$ T_H cells in BT strains were naïve cells, while a minority of cells comprised T_{reg} cells (<5%) and T_H1 (<3%) cells. Transgenic T cells could be strongly activated *in vitro* by human LDL and to a lower extent by oxLDL, confirming previous findings.[277] The ability of $TRBV31^+$ T cells to respond to human LDL was also verified in vivo through LDL injections in transgenic mice crossed with the Nur77-GFP reporter mouse.

The effects of cellular immunity to LDL on atherosclerosis were assessed by two different experimental setups: adoptive transfer of LDL-reactive T cells from transgenic mice into *HuBL* mice (*BT1*, *BT2* or *BT3−>HuBL*), and crossbreeding of transgenic strains with *HuBL* mice. Both approaches showed that a significant proportion of LDL-reactive T cells differentiated into T_{FH} cells, which helped B cells form strong germinal center reactions, differentiate into plasma cells and produce anti-LDL IgG antibodies. These antibodies were reactive to native LDL, oxLDL and apoB100 and formed immune complexes with circulating LDL. This antibody response led to increased lipoprotein clearance, which reduced plasma cholesterol levels and halted the development of atherosclerosis. The IgG antibody-induced lipoprotein clearance was suggested by experiments in *HuBL* mice showing enhanced clearance of injected FITC-labeled LDL that had previously been incubated with plasma from *BT3−>HuBL* mice, as well as a reduction in plasma apoB after injection of purified IgG antibodies from *BT3xHuBL* mice.

An interesting observation is that although the *BT3−>HuBL* mice presented higher absolute numbers of T_{FH} cells and higher levels of anti-LDL antibodies than *BT1−>HuBL* mice, the magnitude of reduction in plasma cholesterol and atherosclerosis in these two groups was similar. Potential explanations for the similar phenotypes are a saturation of the antibody-mediated LDL clearance, differences in cholesterol synthesis between the groups of mice, or blunting of the antibody-mediated atheroprotection by stronger T_H1 responses in the *BT3−>HuBL* group.

In the cell transfer experiments, flow cytometry of spleens suggested that apoB100-reactive T cells differentiated into T_{FH} and T_H1 cells, while T_{reg} cell differentiation was negligible. Similar results were observed in the *BT1xHuBL* and *BT3xHuBL* cross. These crosses provide a humanized mouse model of cellular immunity to LDL, where the autoantigen is already present from birth. In this context, our study illustrates the importance of different tolerance mechanisms. Negative selection took place in the thymus, as shown by the elimination of $TRBV31^{bright}$ cells. Nevertheless, a substantial proportion of self-reactive T cells escaped central deletion and survived as $TRBV31^{dim}$ cells. Another tolerance mechanism, T cell anergy, took place in the periphery, as suggested by the lack of proliferation of splenocytes from *BT1xHuBL* mice in response to LDL stimulation *in vitro*. Interestingly, T_{reg} cells did not seem to have played an important role in our models.

Based on previous findings by Hermansson and co-workers,[277] the initial hypothesis of our study was that cellular immunity to LDL would promote atherosclerosis due to increased proinflammatory T_H1 responses in the vascular wall. However, our models showed a predominant role of T_{FH} cell differentiation and humoral anti-LDL responses, which probably shadowed the potential deleterious effects of T_H1 LDL-reactive T cells. Notably, our mouse models allowed the evaluation of chronic atherosclerosis development that does not capture acute events, where the role of vascular T_H1 cells could be particularly important.

Our results are in line with previous studies suggesting an atheroprotective role of B cell antibody responses to LDL.[255] Epidemiological studies have shown that low levels of IgG antibodies to specific apoB100 peptide epitopes are associated with higher risk for cardiovascular events or mortality.[292-294] Moreover, a number of immunization studies using different LDL/apoB100 preparations have shown that antibody responses to LDL, oxLDL or apoB protect from atherosclerosis.[270-276] Although some of these studies could detect reductions in plasma cholesterol,[270,271] the role of LDL-specific antibodies in lipoprotein clearance has been underappreciated. Thus, the exact mechanisms mediating the effects of these antibodies have never been fully elucidated. In our study, the accumulation of IgG in the liver and the increased cholesterol content in the feces of *BT3xHuBL* mice point towards a possible hepatic route of LDL-IgG immune complex clearance. Indeed, it is known that IgG immune complexes can be cleared by Fcγ receptors of Kupffer cells and sinusoidal endothelial cells in the liver as well as by galactose receptors in hepatic parenchymal cells.[295-297] Of note, a potential unwanted side effect of immune complexes is their deposition in glomeruli, which may cause glomerulonephritis.[298] Although no differences in serum

creatinine were detected in our experiments, potential adverse effects of IgG-LDL immune complexes warrant further investigation.

Another possible atheroprotective mechanism of anti-LDL antibodies is the clearance of modified LDL from the intima,[299] which can inhibit endothelial cell activation, monocyte accumulation and macrophage activation in the vascular wall. In our study, decreased vascular inflammation was suggested by lower expression of vascular cell adhesion protein 1 (VCAM-1) in plaques and lower IL-6 mRNA expression in para-aortic lymph nodes of *BT1xHuBL* mice. Moreover, IgG antibodies may confer atheroprotection through binding to inhibitory Fcγ receptors.[253]

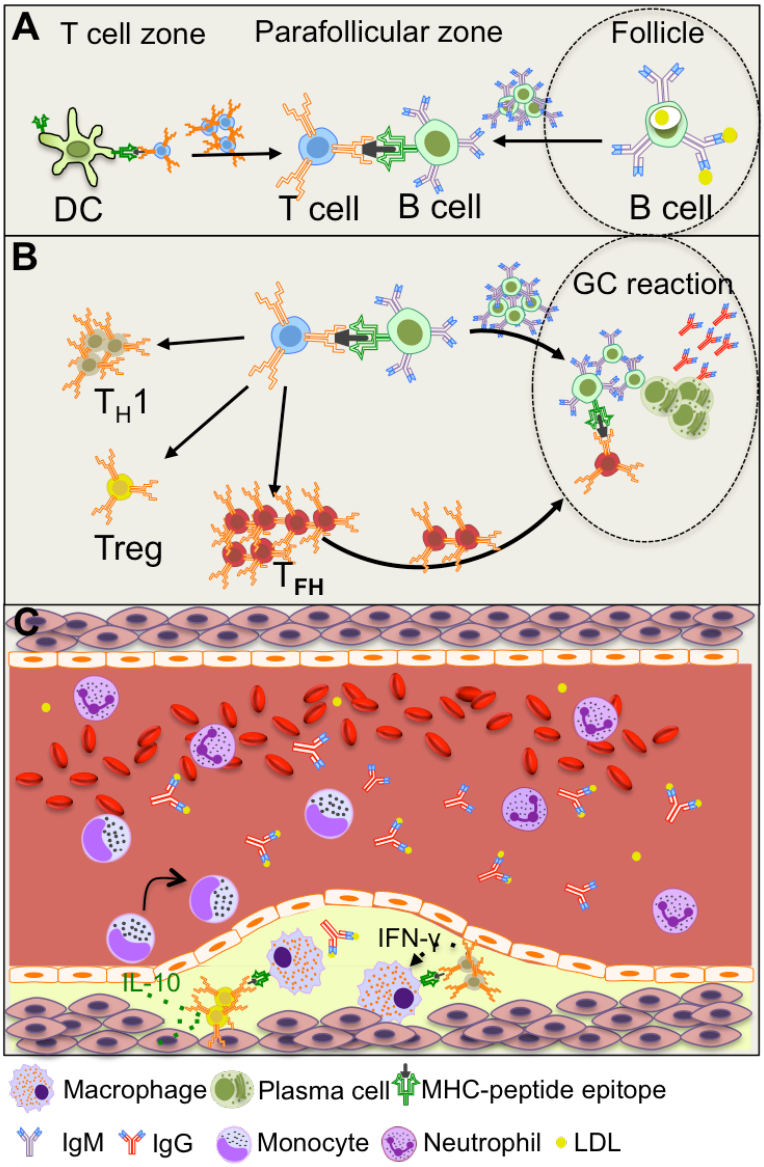

Figure 5. Summary of Paper I. A) An LDL-reactive T cell in the T cell zone of peripheral lymphoid organs recognizes a peptide epitope on apoB100, gets activated and proliferates. In parallel, B cells recognize oxidation specific epitopes on LDL, get activated and proliferate. The activated T cells and B cells migrate towards one another. Different B cell clones now act as APCs and present the apoB100 peptide epitope to apoB100-specific T cells. **B)** In parafollicular areas, the T-B cell interaction promotes an expansion of T_{FH} cells that migrate together with activated B cells to the follicle. There, T_{FH} cells help B cells form germinal center (GC) reactions and differentiate into plasma cells secreting IgG antibodies to various LDL epitopes. **C)** IgG antibodies bind to various LDL epitopes, clear LDL from the circulation and ameliorate atherosclerosis.

In conclusion, **Paper I** indicates that cellular immunity against LDL protects from atherosclerosis via induction of humoral anti-LDL responses that clear lipoproteins from the circulation and reduce plasma cholesterol (**Figure 5**). Hence, our data reinforces the concept

that immunization with an LDL preparation is a promising therapeutic strategy for the prevention of atherosclerosis. Our findings may form the basis for further studies focusing on the identification of specific apoB100 peptide epitopes, elucidation of the exact mechanisms behind antibody-mediated atheroprotection, and identification of potential adverse effects.

9.2 ACTIVATION OF THE TREG−IDO1 AXIS IN THE VASCULAR WALL REDUCES ATHEROSCLEROSIS (PAPER II)

In **Paper II**, we used DC-based immunotherapy in order to induce two important peripheral tolerance mechanisms in the vascular wall, namely expansion of apoB-specific T_{reg} cells and induction of the immunoregulatory enzyme IDO1. Regulatory T cells promote immunological tolerance via secretion of the anti-inflammatory cytokines IL-10 and TGF-β, cell−cell contact-mediated induction of T cell anergy, and CTLA-4−mediated induction of IDO1 in dendritic cells.[123] In turn, IDO1, which is known to suppress proinflammatory T cell responses and ameliorate various experimental autoimmune diseases, can induce T_{reg} cell differentiation.[121,122] In this study, we hypothesized that activation of this T_{reg}−IDO1−T_{reg} loop in the vascular wall could inhibit the development of atherosclerosis.

To promote the accumulation of T_{reg} cells in developing plaques, we employed immunotherapy with tolerogenic DCs loaded with an atherosclerosis-relevant antigen, apoB100. DCs were generated from bone marrow-derived progenitor cells and rendered tolerogenic through treatment with TGF-β_2 during loading with apoB100. The tolerogenic properties of these DCs were suggested by *in vitro* experiments showing an anti-inflammatory cytokine secretion profile, reduced expression of MHC-II and CD86, increased IDO1 expression, and the ability to induce T_{reg} cell differentiation.

Intravenous injection of tolerogenic TGF-β_2−treated, apoB100-loaded DCs into *HuBL* mice resulted in an accumulation of T_{reg} cells in the aortic root after 10 weeks of high-fat diet. In line with this finding, mRNA levels of CTLA-4, which is constitutively expressed on T_{reg} cells, were upregulated in the para-aortic lymph nodes of these mice. CTLA-4 binding to CD80/CD86 is known to regulate IDO1 activity in DCs.[123,300] Indeed, the increase in T_{reg} cells in the aortic root of experimental mice was associated with increased IDO1 expression. Immunofluoresence staining revelead that IDO1 was expressed in vascular smooth muscle cells, macrophages and endothelial cells. Increased IDO1 expression was accompanied by increased IDO1 activity, as shown by the increased L-kynurenine staining in plaques of mice receiving TGF-β_2−treated, apoB100-loaded DCs. Of note, L-kynurenine is an endogenous AHR agonist that can induce differentiation of naïve T cells to T_{reg} cells.[141] Most importantly,

the induction of the T_{reg} cell−IDO1 axis in the vascular wall was associated with reduced macrophage infiltration and reduced atherosclerosis.

Previous studies suggest that T_{reg} cells must be specific to disease-relevant antigens in order to exert an immunosuppressive effect *in vivo*.[87,301,302] ApoB100 contains multiple T cell epitopes and is abundantly present in atherosclerotic lesions, hence its use in our study.[303] Notably, induction of the T_{reg} cell−IDO1 axis and amelioration of atherosclerosis required treatment of DCs with both TGF-β_2 and apoB100, as the cytokine or the antigen alone had no effect.

In **Paper I**, apoB100-specific cellular immune responses induced anti-LDL antibody responses, thereby lowering plasma cholesterol. In **Paper II**, the groups of mice receiving apoB100-pulsed DCs (irrespective of TGF-β_2 treatment) showed higher levels of IgG antibodies to apoB100 (unpublished data). Although no differences in plasma cholesterol were observed in **Paper II**, we can not exclude that anti-LDL antibodies contributed to atheroprotection via other mechanisms.

Consistent with our findings, positive correlations between IDO1 expression and T_{reg} cell numbers have been shown in human studies as well as in experimental models of cancer, infection, transplantation and chronic inflammation.[304-306] In the context of atherosclerosis, Yun and co-workers demonstrated that T_{reg} cells and IDO1 gene expression in the vascular wall increase linearly with plaque size, and IDO1 deficiency in myeloid cells leads to reduced T_{reg} cells in the aorta of $LDLR^{-/-}$ mice.[307] In keeping with this finding, advanced human atherosclerotic plaques are characterized by upregulation of Foxp3, CTLA-4 and IDO1 compared to non-atherosclerotic vessels.[308] In our study, T_{reg} cells and IDO1 expression were associated with smaller atherosclerotic plaques, suggesting that triggering the T_{reg} cell−IDO1 axis during early atherosclerosis can change the natural course of disease.

The CTLA-4–CD80/CD86 interaction is known to induce IDO1 in DCs.[123] Using the fusion protein CTLA-4-Ig in human primary cell cultures, we showed that CTLA-4 regulates IDO1 expression and activity in aortic SMCs, human umbilical vein endothelial cells (HUVECs) and monocyte-derived macrophages. Interestingly, CTLA-4-Ig induced IDO1 only in cells that had previously been exposed to IFN-γ. At the mRNA level, IFN-γ upregulated CD80 but not CD86, suggesting that IDO1 induction in vascular cells is mediated by the CTLA-4–CD80 interaction.[309] Of note, Grohmann and co-workers showed that the CTLA-4-Ig–mediated IDO1 induction in DCs requires IFN-γ and STAT-1.[300]

Autologous dendritic cell "vaccination" (also known as immunotherapy) is currently evaluated in clinical trials for the treatment of a number of cancers, including melanoma, prostate and breast cancer.[310] In atherosclerosis, DC immunotherapy is still at the experimental level.[87,311] In **Paper II**, TGF-β_2 was chosen, because it can modulate the function of APCs rendering them capable of inhibiting T_H1 responses and promoting T_H2 and T_{reg} cell responses.[312,313] Notably, DC treatment with TGF-β_2 was superior to IL-10 in T_{reg} cell differentiation *in vitro*. In line, immunotherapy with TGF-β_2–induced tolerogenic DCs resulted in accumulation of T_{reg} cells in atherosclerotic plaques, which was not the case with a previous IL-10–based approach.[87]

In summary, **Paper II** indicates that activation of the T_{reg} cell–IDO1 axis in the vascular wall is an attractive immunomodulatory approach against atherosclerotic cardiovascular disease (**Figure 6**). Our findings suggest that CTLA-4-Ig, an approved biologic therapy (abatacept, ORENCIA®) for the treatment of rheumatoid arthritis, psoriatic arthritis and juvenile idiopathic arthritis, may induce IDO1-mediated tryptophan catabolism in the vascular wall, which might have clinical implications in this patient population characterized by chronic inflammation and increased cardiovascular risk.

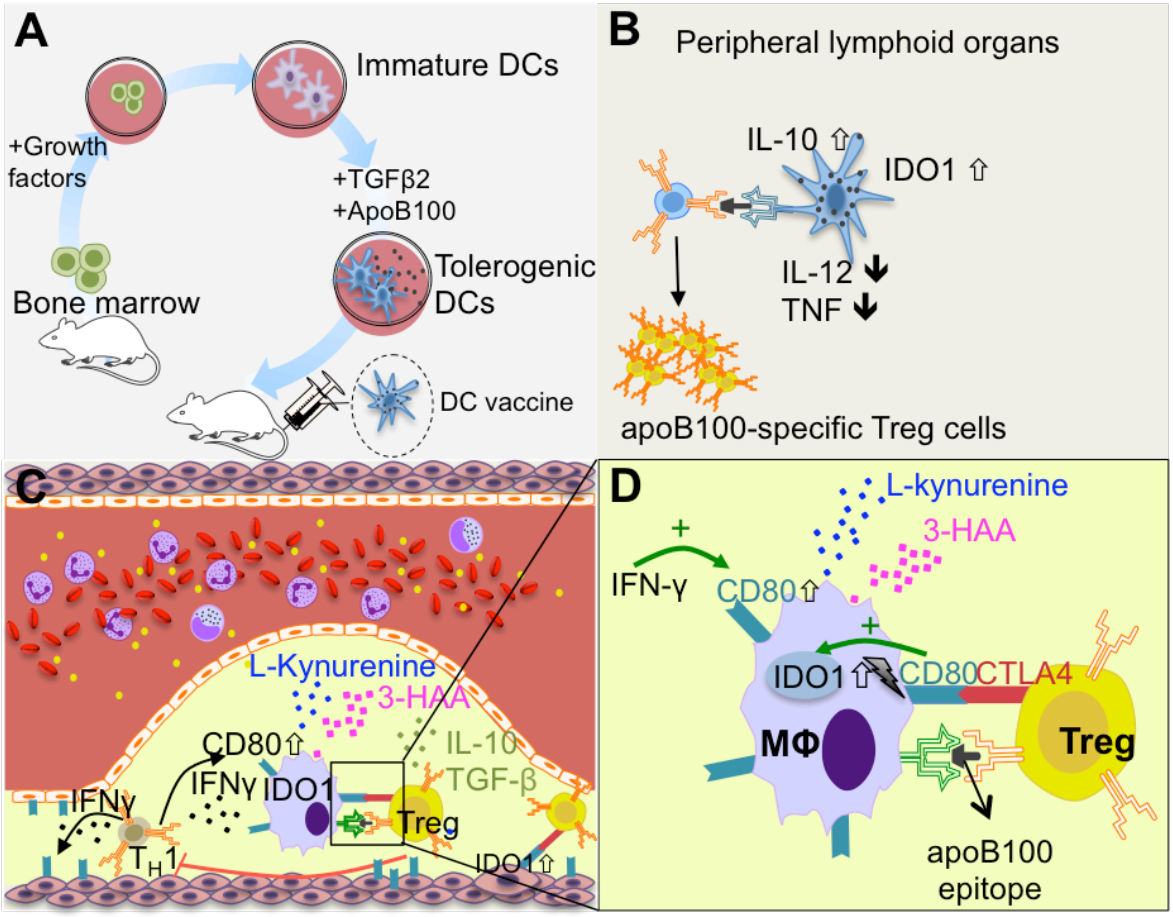

Figure 6. Summary of Paper II. A) Bone marrow cells were taken from *HuBL* mice and cultured with GM-CSF and IL-4 to generate immature DCs. DCs were then treated with TGF-β_2, pulsed with apoB100, and stimulated with LPS. Matured DCs were then injected into *HuBL* recipient mice. **B)** In peripheral lymphoid organs, tolerogenic DCs presented apoB100 epitopes to apoB-specific naïve T cells, and skewed T cell effector responses towards T_{reg} cells. **C-D)** ApoB100-specific T_{reg} cells accumulated in the developing plaques, which was associated with increased IDO1-mediated tryptophan metabolism. *In vitro* experiments suggested that the T_{reg} cell molecule CTLA-4 could induce IDO1 in macrophages, SMCs and endothelial cells through an interaction with the costimulatory molecule CD80. **D)** The inset depicts, in magnification, the CTLA-4−CD80 interaction between a T_{reg} cell and a macrophage.

9.3 IDO1-MEDIATED TRYPTOPHAN METABOLISM REGULATES VASCULAR INFLAMMATION AND ATHEROGENESIS (PAPER III)

Systemic IDO1 activity predicts future cardiovascular events in both healthy individuals and patients with coronary heart disease.[314,315] In experimental atherosclerosis, IDO1 is expressed in macrophages, SMCs and endothelial cells.[316,317] In **Paper III**, we used a pharmacological approach to study the role of IDO1-mediated tryptophan metabolism in atherosclerosis. Treatment of high-fat diet fed apoE$^{-/-}$ mice with the IDO1 inhibitor 1-MT promoted macrophage accumulation in the intima and aggravated atherosclerosis. Gene expression analysis showed upregulation of the inflammatory markers VCAM-1, MCP-1 and TNF in the aortas of these mice. Interestingly, immunohistochemical staining of the aortic roots of 1-MT−treated mice revealed strikingly increased expression of the adhesion molecule and NF-κB activation marker VCAM-1 in the media, which correlated with atherosclerotic plaque size.

Systemic inhibition of tryptophan catabolism by 1-MT was confirmed by the decreased kynurenine/tryptophan ratio in the spleen and the duodenum, two organs with relatively high IDO1 expression.[113] The DL stereoisomer was chosen because it had a superior inhibitory effect on LPS-stimulated PBMCs and it had been used extensively in experimental studies.[125-127] The inhibitor was provided in the drinking water at a concentration based on an experimental cancer study that paved the way for clinical trials.[131] Our data may have direct implications in clinical practice, since the D stereoisomer of 1-MT is currently used in cancer clinical trials. Notably, we found that treatment of apoE$^{-/-}$ mice with D-1-MT also aggravates atherosclerosis, while L-1-MT exerts no effect (unpublished data).

Several studies have shown that IDO1 regulates T cell responses. In our study, 1-MT treatment increased the absolute plaque area stained for macrophages but did not increase the number of lesional T cells. Gene expression of T cell transcription factors in spleens and aortas as well as splenocyte cultures did not yield any differences either. However, there was

a trend towards increased lymphocytes in the peripheral blood of 1-MT−treated mice (unpublished data). These results do not exclude functional consequences or the possibility that 1-MT influenced T cells in earlier stages of the disease. Actually, a later study using IDO1$^{-/-}$ apoE$^{-/-}$ mice showed that IDO1 deficiency promoted the accumulation of lesional T cells at 15 weeks but there was difference at 20 weeks of age.[318] In our study, mice were sacrificed at 20 weeks.

3-HAA has been shown to reduce total plasma cholesterol and triglycerides, increase HDL cholesterol, and ameliorate atherosclerosis.[319] In **Paper III**, reduced tryptophan metabolism resulted in a non-significant increase in total plasma cholesterol (due to slightly increased VLDL and LDL cholesterol) and a significant decrease in HDL. This suggests that reduced levels of 3-HAA could be an important regulator of lipoprotein metabolism.

In a rescue experiment, exogenous administration of 3-HAA to 18-week-old apoE$^{-/-}$ mice for 4 weeks halted the 1-MT−induced vascular inflammation and progression of atherosclerosis. Of note, 3-HAA is a known inhibitor of the NF-κB pathway and can inhibit TNF-induced VCAM-1 expression in HUVECs.[320] Because of the striking effects on medial VCAM-1 observed in our experiments, we studied the role of IDO1 in the regulation of VCAM-1 in human primary vascular SMCs. Consistent with our *in vivo* findings, inhibition of IDO1-mediated tryptophan catabolism in coronary SMCs induced VCAM-1 expression, an effect that was abrogated by 3-HAA.

The role of VCAM-1 as adhesion molecule in endothelial cells is well known. This molecule is also expressed in plaque macrophages and SMCs as well as in medial SMCs,[321,322] and its expression correlates with intimal leukocyte content.[323] The exact role of VCAM-1 in vascular SMCs is unknown. It has been suggested that this molecule is not merely a marker of NF-κB activation but exerts biological functions driving atherosclerosis.[324] VCAM-1 on intimal SMCs may contribute to the retention of monocytes and macrophages in the developing plaque.[325] Electron microscopy studies showing direct contact between intimal SMCs and macrophages support this hypothesis. Moreover, VCAM-1 on SMCs can protect lesional monocytes from apoptosis via induction of the PI3K−Akt pathway, and promote foam cell formation via induction of ERK1/2−CD36.[326] Finally, early VCAM-1 expression in medial SMCs was observed in atherosclerosis-prone arterial segments just prior to or coincident with leukocyte infiltration in both apoE$^{-/-}$ and LDLR$^{-/-}$ mice.[327]

In our study, inhibition of IDO1-mediated tryptophan metabolism increased medial inflammation substantially. Whether medial inflammation contributed to atherogenesis or

was secondary to intima hyperplasia/inflammation remains unknown. An interesting hypothesis that was not evaluated in our study is that IDO1 regulates SMC proliferation, migration and transdifferentiation into macrophages.[177,178] The hypothesis that the effects of 1-MT on medial SMCs could have driven atherosclerosis is supported by a number of previous observations proposing a central role of IDO1 in SMCs: the inflammatory cytokines IFN-γ and TNF induce IDO1 expression and activity in human vascular SMCs to a much larger extent than in monocytes and endothelial cells.[328] It has also been proposed that IDO1 induction in the media under inflammatory conditions spares this layer from T cell infiltration ("medial immunoprivilege").[329] IFN-γ, the most potent inducer of IDO1, is crucial for the prevention of chronic viral inflammation/vasculitis in the arterial wall. Interestingly, IFN-$\gamma^{-/-}$ mice developed vasculitis in atherosclerosis-prone arterial segments.[330]

A few months after the publication of **Paper III**, two studies evaluating genetic IDO1 deletion in $apoE^{-/-}$ and $LDLR^{-/-}$ mice showed contradictory results. Metghalchi and co-workers showed that IDO1 inhibits IL-10 release by bone marrow-derived cells and exacerbates atherosclerosis in $LDLR^{-/-}$ mice. In this study, IDO1-deficient bone marrow-derived cells showed increased secretion of IL-10 upon *in vitro* stimulation with LPS and IFN-γ.[316] Conversely, Cole and co-workers showed that IDO1 deficiency in $apoE^{-/-}$ mice fed a normal chow diet reduces IL-10−secreting B cells and aggravates atherosclerosis. The authors showed direct *in vivo* effects on IL-10, as plasma IL-10 concentration was reduced in $IDO1^{-/-}$ $apoE^{-/-}$ compared to $apoE^{-/-}$ controls.[318] This latter study shows many similarities with our study: it used $apoE^{-/-}$ mice, showed that IDO1 is atheroprotective, and did not detect differences in plaque $CD4^+$ T cell numbers at later disease stages.

Potential explanations for the conflicting results on the role of IDO1 in atherosclerosis are study differences in mouse strains ($LDLR^{-/-}$ versus $apoE^{-/-}$), diet (high-fat versus normal chow), and gut microbiome. A difference between the genetic IDO1 studies and our study is that our pharmacological approach allows the evaluation of enzyme activity, whereas a genetic approach evaluates both enzyme activity and potential other unknown biological functions. For instance, it has been shown that IDO1 has an intracellular signalling function that is crucial for the TGF-β−induced immunoregulatory phenotype of plasmacytoid DCs.[331]

In summary, **Paper III** shows that IDO1-mediated tryptophan metabolism along the kynurenine pathway regulates vascular inflammation and exerts atheroprotective functions (**Figure 7**). The observed effects of IDO1 could be mediated by tryptophan depletion, the generation of kynurenines, or both. The ability of 3-HAA to inhibit the 1-MT−induced atheroprogression suggests that this metabolite is a key mediator of atheroprotective

mechanisms. Importantly, 3-HAA can positively influence both plasma lipids and vascular inflammation, which makes it an attractive candidate for drug development.

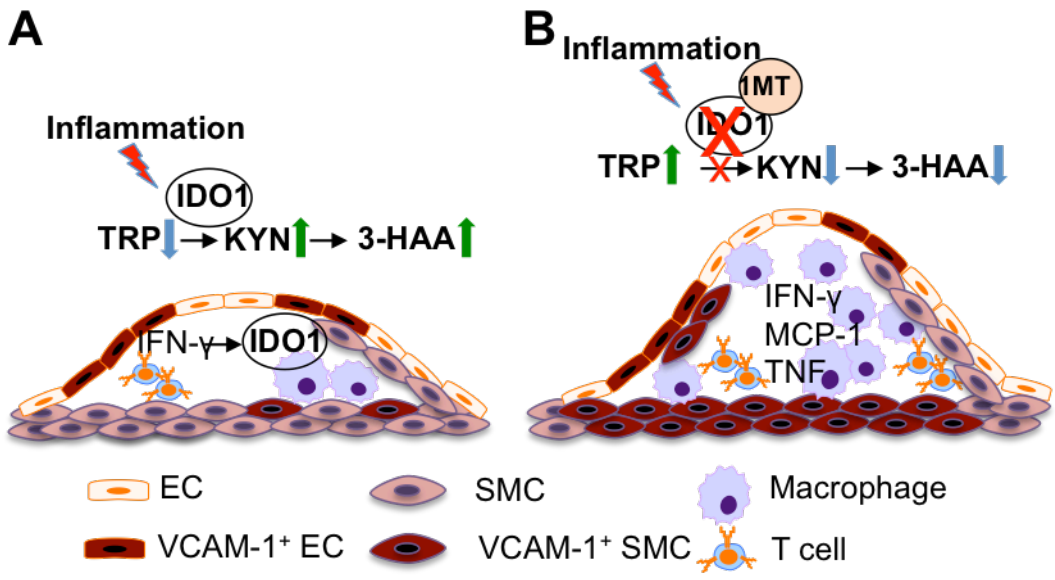

Figure 7. Summary of Paper III. A) During atherogenesis, endothelial cells get activated, express the adhesion molecule VCAM-1, and attract blood-borne monocytes, which translocate into the intima and differentiate into macrophages. The resulting chronic inflammatory process induces the enzyme IDO1 in smooth muscle cells, macrophages and endothelial cells. IDO1 activation results in the metabolism of tryptophan along the kynurenine pathway, which generates various metabolites with biological properties, such as L-kynurenine and 3-HAA. Paper III suggests that IDO-1−mediated tryptophan metabolism has an anti-inflammatory and atheroprotective function. **B)** Systemic treatment of apoE knockout mice with the IDO1 inhibitor 1-MT inhibited degradation of tryptophan along the kynurenine pathway, which resulted in increased expression of proinflammatory mediators (TNF, MCP-1), increased expression of VCAM-1 in the media, and increased macrophage accumulation in developing plaques. These effects were abrogated, when 3-HAA was administrated exogenously.

9.4 THE ATHEROPROTECTIVE TRYPTOPHAN METABOLITE 3-HAA DOWNREGULATES HEPATIC SREBP-2 AND INHIBITS THE NLRP3 INFLAMMASOME (PAPER IV)

In **Paper IV**, we explored at the molecular level the potential mechanisms behind the lipid-lowering and anti-inflammatory effects of 3-HAA on hepatocytes and macrophages, respectively.[319,332] Moreover, we investigated whether elevating endogenous 3-HAA levels through inhibition of the 3-HAA−metabolizing enzyme HAAO affects lipoprotein metabolism and atherosclerosis.

In vitro experiments on human HepG2 cells showed that 3-HAA decreased nuclear SREBP-2 (nSREBP-2) as well as SREBP-2 gene expression. SREBP-2 regulates its own transcription, which may explain the reduced SREBP-2 gene expression in 3-HAA−treated cells.[333] These

results imply that 3-HAA acts via a different mechanism than statins, which inhibit cholesterol synthesis, thereby inducing SREBP-2 and LDLR-mediated uptake of apoB-containing lipoproteins. Moreover, 3-HAA treatment resulted in lower levels of apoB in the supernatants of HepG2 cells, indicating decreased lipoprotein secretion. It has been suggested that apoB secretion is affected by the cellular triglyceride content.[334] A possible scenario is that 3-HAA reduces nSREBP-2, thereby leading to decreased cholesterol and triglyceride synthesis and hence decreased secretion of apoB-containing lipoproteins.

The SREBP-2 pathway can be inhibited by different mechanisms, including increased SCAP-INSIG binding, inhibition of S1P or S2P or increased degradation of nSREBP-2. A possible mechanism mediating the 3-HAA effects on nSREBP-2 is inhibition of the PI3K−Akt pathway,[335] as 3-HAA has been shown to inhibit PDK1, a signalling molecule that phosphorylates Akt.[143] Actually, inactivation of Akt has been shown to inhibit the transport of SCAP from ER to Golgi, thus reducing nSREBP-2.[335]

Paper IV also explored the capacity of 3-HAA to inhibit the NLRP3 inflammasome in mouse bone marrow-derived macrophages *in vitro*. 3-HAA is a known inhibitor of the NF-κB pathway, which primes the NLRP3 inflammasome by upregulating pro-IL-1β and NLRP3. As expected, 3-HAA treatment of macrophages during inflammasome priming with LPS reduced pro-caspase-1, active caspase-1 and IL-1β secretion. Interestingly, 3-HAA inhibited not only the priming but also the activation of the inflammasome, since 3-HAA treatment after LPS priming and immediately prior to ATP stimulation decreased the secretion of cleaved caspase-1 and IL-1β without affecting the levels of pro-caspase-1.

The effects of increased endogenous 3-HAA were then evaluated *in vivo* by treating $LDLR^{-/-}$ mice with the HAAO inhibitor 4,6-di-bromo-3-hydroxyanthranilic acid (NCR-631), which has been shown to increase endogenous levels of 3-HAA.[336] Consistent with our *in vitro* results, NCR-631 decreased hepatic mRNA levels of SREBP-2 and its target gene HMG-CoA reductase. More importantly, NCR-631−treated mice had lower plasma cholesterol and triglyceride levels and reduced atherosclerosis. Moreover, these mice presented reduced steatosis and inflammation in the liver compared to control mice.

In conclusion, **Paper IV** provides mechanistic insights on the lipid-lowering and anti-inflammatory functions of 3-HAA and identifies HAAO as a potential target for drug development against atherosclerotic cardiovascular disease (**Figure 8**).

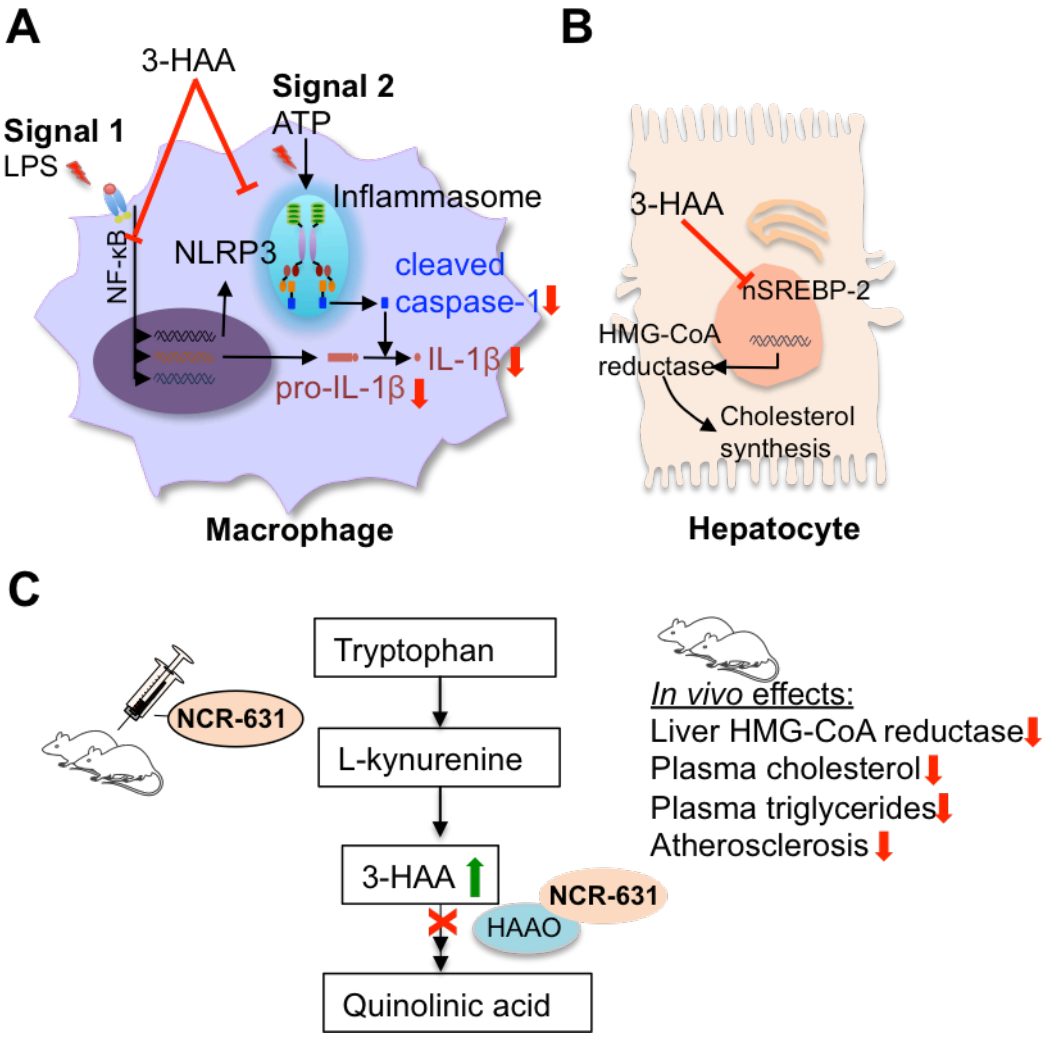

Figure 8. Summary of Paper IV. A) *In vitro* experiments in mouse bone marrow-derived macrophages showed that 3-HAA inhibits not only the priming but also the activation of the NLRP3 inflammasome. **B)** Treatment of HepG2 cells with 3-HAA resulted in reduced nuclear SREBP-2, and hence reduced gene expression of HMG-CoA reductase. **C)** Treatment of $LDLR^{-/-}$ mice with NCR-631, a molecule known to inhibit HAAO and increase endogenous 3-HAA levels, reduced plasma lipids and ameliorated atherosclerosis.

10 CONCLUDING REMARKS

Despite the currently available treatment options against atherosclerotic cardiovascular disease, substantial residual inflammatory risk remains. This underscores the need for development of immunomodulatory drugs that directly target vascular inflammation. Harnessing the immune system will open up completely new therapeutic opportunities in the management and prevention of CVDs. The studies of the present thesis illustrate the intricate interplay between metabolism, particularly metabolism of plasma lipoproteins and the amino acid tryptophan, and immunity/inflammation in atherosclerosis, and investigate the therapeutic potential of novel immunomodulatory treatment strategies.

Paper I shows that cellular immunity to apolipoprotein B-100 can induce T follicular helper cells, which initiate humoral anti-LDL antibody responses that clear plasma lipoproteins and reduce atherosclerosis. Hence, vaccination with apoB epitopes, which could induce similar responses, is a promising therapeutic strategy against cardiovascular disease. **Paper II** illustrates the capacity of regulatory T cells to activate tryptophan metabolism in atheroma-associated cells, and the potential of tolerogenic dendritic cell-based immunotherapy to induce anti-inflammatory mechanisms in the vascular wall. **Paper III** shows that IDO1-mediated tryptophan metabolism along the kynurenine pathway regulates vascular inflammation and atherosclerosis. This study strengthens previous data on the atheroprotective role of the tryptophan metabolite 3-HAA, which exhibits both lipid-lowering and anti-inflammatory properties. Finally, **Paper IV** shows that 3-HAA can modulate the SREBP-2−mediated lipid homeostasis and inhibit the activation of NLRP3 inflammasome. This study also identifies the enzyme HAAO as an attractive target for development of drugs with pleiotropic, lipid-lowering and anti-inflammatory, properties.

Paper I and **Paper II** exploited adaptive immune responses to the atherosclerosis-related antigen apoB100, whereas **Paper III** and **Paper IV** targeted tryptophan metabolism to modulate lipid metabolism, immunity and atherosclerosis in an antigen-independent manner. The theoretical disadvantage of targeting innate immunity is that it may compromise antimicrobial defense. This was evident in the CANTOS trial in which treatment with monoclonal antibodies to IL-1β increased fatal infections.[26] Nevertheless, anti-inflammatory therapies could be particularly effective in more selected patient groups, such as patients with acute cardiovascular events.[337] On the other hand, targeting adaptive immunity has the potential to affect only relevant lymphocyte clones without compromising the rest of the immune system. Hence, LDL vaccination represents a promising future therapeutic strategy

against atherosclerosis. Here, the major challenge would be to translate experimental data into humans, who exhibit large variability in HLA genes.

As a final conclusion, my thesis illustrates the crosstalk between metabolism and immunity/inflammation in atherogenesis. It is my firm belief that the knowledge gained from our studies will contribute to the development of novel immunomodulatory strategies for the prevention and treatment of atherosclerotic cardiovascular disease.

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