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ROLE OF IMMUNE MEDIATORS IN METABOLIC SYNDROME AND ATHEROSCLEROSIS

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CONTENTS

LIST OF ABBREVIATIONS

Ab	antibody
ABCA	ATP-binding cassette transporter sub-family A member 1
ABCG1	ATP-binding cassette sub-family G member 1
ACS	acute coronary symptom
Ag	antigen
AKT	serine/threonine kinase (also known as protein kinase B PKB)
AP-1	activator protein 1
APC	antigen presenting cell
apo	apolipoprotein
BAT	brown adipose tissue
Bcl6	B-cell lymphoma 6 protein
BCR	B cell receptor
βGlcCer	β-D-glucopyranosylceramide
BMI	body mass index
BP	blood pressure
CAD	coronary artery disease
cAMP	cyclic adenosine monophosphate
CCR	C-C chemokine receptor type (CCR)7
CD	cluster of differentiation
CETP	cholesteryl ester transfer protein
CM	chylomicron
CpG DNA	double-stranded deoxyribo-nucleic acid with repeated unmethylated CpG
CT	computer tomography
CTL	cytotoxic T lymphocyte
CVD	cardiovascular diseases
DALYs	disability-adjusted life years
DAMP	damage- or danger-associated molecular pattern
DC	dendritic cell
DEREG	depletion of regulatory T cells
DP	double positive
dsRNA	double-stranded ribonucleic acid
DT	diphtheria toxin
eGFP	enhanced green fluorescent protein
EGR	early growth response protein
ER	Endoplasmic reticulum
ERK	extracellular-signal-regulated kinase
FA	fatty acid
FDG	[^{18}F]2-fluoro-D-deoxyglucose
FFA	free fatty acid
FIZZ1	found in inflammatory zone 1
FOX	forkhead box

Foxo1	forkhead box protein O1
FoxP3	forkhead box P3
FPLC	Fast protein liquid chromatographic
GARP	Glycoprotein A repetitions predominant
GATA3	Trans-acting T-cell-specific transcription factor GATA3
Gck	glucokinase
GLUT	Glucose transporter
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GSK 3	glycogen synthase kinase 3
HDL	high-density lipoprotein
hDTR	human diphtheria toxin diphtheria toxin receptor
HELIOS	member of the Ikaros transcription factor family
HFD	High-fat diet
HIF1α	hypoxia-inducible factor 1α
HMGCoA	3-hydroxy-3-methyl-glutaryl-CoA
HOMA-IR	homeostatic model assessment of insulin resistance
HSL	Hormone-sensitive lipase
HSPG	heparan sulfate proteoglycans
IDF	International Diabetes Federation
IDL	Intermediate-density lipoprotein
IFG	impaired fasting glycaemia
IFN	interferon
Ig	Immunoglobulin
IGF1R	insulin-growth factor-1 receptor
IGT	impaired glucose tolerance
IL	interleukin
iNKT cell	invariant natural killer T cell
IRAK	IL-1R-associated kinase
IRF3	interferon regulatory factor 3
IRS	insulin receptor substrate
ISPAD	International Society for Pediatric and Adolescent Diabetes
ITAMS	immune receptor tyrosine-based activation motif
JNK	c-Jun N-terminal kinase
KLF4	Krüppel-like factor 4
LCAT	Lecithin-cholesterol acyltransferase
LD	lipid droplet
LDL	low-density lipoprotein
LDLR	LDL-receptor
LN	lymph node
LPL	lipoprotein lipase
LPS	lipopolysaccharide
LRP	LDL receptor-related protein
LTα	Lymphotoxin-alpha

LXR	liver X receptor
LY6C	lymphocyte antigen 6C
lysoPE	lysophosphatidylethanolamine
M	mitochondria
MAG	2 monoacylglycerol
MAPK	mitogen-activated protein kinases
MBL	mannose-binding lectin
MCP-1	monocyte chemotactic protein-1
MDA-5	melanoma differentiation-associated protein 5
mDC	myeloid dendritic cell
MHC I (or II)	major histocompatibility complex class I (or II)
MI	myocardial infarction
mLDL	modified low-density lipoprotein
mRNA	messenger ribonucleic acid
mTORC1	mammalian target of rapamycin complex 1
MTTP	microsomal TG transfer protein
Myd88	myeloid differentiation primary response gene (88)
NF-κB	nuclear transcription factor kappa beta
NK	Natural killer cell
NLRP3	NOD-like receptor family, pyrin domain containing 3
NO	nitric oxide
NOD	nucleotide-binding oligomerization domain
O^-	superoxide
OGTT	Oral glucose tolerance test
oxLDL	oxidized low-density lipoprotein
PAMP	pathogen-associated molecular pattern
pDC	Plasmacytoid dendritic cell
PEPCK	phosphoenolpyruvate carboxykinase
PET	positron emission tomography
PI3K	phosphoinositide 3-kinase
PIP_3	phosphatidylinositol (3,4,5)-triphosphate
PLTP	phospholipid transfer protein
PLZF	promyelocytic leukaemia zinc finger
PPARγ	peroxisome proliferator-activated receptor
PPR	pattern recognition receptor
rER	rough Endoplasmic reticulum
RIG-1	retinoic acid-inducible gene 1
RIP1	receptor-interacting protein 1
rRNA	ribosomal RNA
SNAP-25	Synaptosomal-associated protein 25
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SR-BI	Scavenger receptor class B member 1
SREBP	Sterol regulatory element-binding protein
SRP	signal recognition particles

ssRNA	single-stranded ribonucleic acid
STAT6	signal transducer and activator of transcription 6
TAK1	transforming-growth-factor -β activated kinase 1
T-bet	T-box transcription factor expressed in T cells
TCR	T cell receptor
TFH	follicular helper T cells
TG	triglyceride
Th cells	T helper cell
TIR	Toll-IL-1R-resistance
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptor
TNF	tumor necrosis factor
TRAF	TNF receptor associated factor
TRAM	TRIF–related adaptor molecule
Treg	regulatory T cell
TRIF	TIR-domain-containing adapter-inducing IFNβ
VAMP-2	vesicle-associated membrane protein 2 or synaptobrevin
WAT	white adipose tissue
VCAM-1	vascular cell adhesion protein 1
WHO	World health organization
VLDL	very low-density lipoprotein
α-GalCer	α-Galactosylceramide

1 INTRODUCTION

1.1 OBESITY

According to World health organization (WHO) more than 1.4 billion adults (data from 2008) and more than 40 million children under the age of five (2011) were overweight (i.e. body mass index (BMI, kg/m^2) over 25). Of these, 500 million adults were obese (BMI > 30). In fact, overweight and obesity are globally the fifth leading risk factor of death and are linked to more deaths worldwide than underweight (1). Although obesity itself is a considerable risk factor of shorter lifespan and reduced life quality, it is usually accompanied by other health problems such as metabolic syndrome, type 2 diabetes, cardiovascular diseases (CVD), certain types of cancer, non-alcoholic fatty liver disease and psychological problems. The increasing number of overweight and obese adults and especially children are likely due to lifestyle changes and changes in eating behavior. Urbanization and a more sedentary lifestyle are two reasons, among others, that lead to reduced daily physical activity. *How many times have you used the elevator today?* At the same time processed food is often energy dense, meaning highly caloric. Furthermore, the cheaper alternative to fresh unprocessed food, is most of the time high in sugar and high in fat, but low in quality, reflected by low vitamin content due to food processing. Unfortunately this food is available, roughly speaking, at every corner for a low price. This might be one reason for the increasing number of obesity, especially in low-income countries (1, 2).

1.1.1 Does it matter where we are fat? Abdominal/visceral obesity vs. subcutaneous adipose tissue

To put it very simple, the body of people can be shaped as an apple or in a pear form. This mirrors the distribution of white adipose tissue (WAT) in the body. The pear shape is common in women where the fat is mostly located on the lower part of the body, the hips and the gluteal region. Men have mostly the apple shape with fat around the belly. The latter is also associated with increased visceral fat in the abdominal cavity. Beside the location, visceral and subcutaneous WAT differ in their metabolic activity. Gene expression analysis of subcutaneous and visceral WAT showed a different gene expression patterns between these regions. An increased expression of genes related to insulin resistance, such as leptin and peroxisome proliferator-activated receptor (PPAR)γ, was found in visceral WAT (3). Additionally, visceral WAT has also increased lipolytic activity and a therefore increased release of free fatty acids (FFA) that

potentially cause metabolic disturbances (4). In contrast to subcutaneous WAT, visceral fat is highly vascularized resulting in increased blood supply. In line with that, it is characterized by increased infiltration of inflammatory immune cells (5). Among these cells macrophages (6, 7), T cells (8-15) and even B cells (16) participate in WAT inflammation. Together with adipocytes these cells secrete adipokines/cytokines that propagate an inflammatory milieu locally as well as systemically.

Although the BMI is widely used as a measure of body weight in relation to body height, it does not reflect the body composition or fat distribution which strongly influences metabolic disturbances and CVD (17-21). Another method of characterization is the waist-to-hip-ratio. Combining both measurements is probably the best way to estimate the health risk.

To get back to the question above: *Yes, it matters where we accumulate the fat! Visceral WAT is worse than subcutaneous fat. However, it is not possible to influence where we gain or lose the fat.*

1.2 METABOLIC SYNDROME

The definition of the metabolic syndrome is not standardized yet, but the International Diabetes Federation (IDF) characterized it as a group of metabolic risk factors including diabetes and pre-diabetes, abdominal obesity, elevated plasma cholesterol and high blood pressure (22) (Table 1).

Table 1: Definition of metabolic syndrome

Central obesity	waist circumference ≥ 94cm for European men and ≥ 80cm for European women, with ethnicity-specific values for other groups
Plus any two of the following four factors:	
• Raised triglyceride (TG) level	≥ 1.7 mmol/l (150 mg/dl), or specific treatment for this lipid abnormality
• Reduced high-density lipoprotein (HDL) cholesterol	< 1.03 mmol/l (40 mg/dl) in males and < 1.29 mmol/l (50 mg/dl) in females, or specific treatment for this lipid abnormality
• Raised blood pressure (BP)	systolic BP ≥ 130 or diastolic BP ≥ 85 mm Hg, or treatment of previously diagnosed hypertension
• Raised fasting plasma glucose	≥ 5.6 mmol/l (100 mg/dl), or previously diagnosed type 2 diabetes

Adapted from IDF; (22)

1.3 TYPE 2 DIABETES MELLITUS

Type 2 diabetes is a progressively developing heterogeneous disease characterized by inefficient response of the body to insulin, insufficient insulin production in the β cells of the pancreas, or the combination of both (23). Aside from manifest type 2 diabetes, there exists a preform of diabetes characterized by Impaired Glucose Tolerance (IGT) and Impaired Fasting Glycaemia (IFG). Table 2 summarizes the diagnostic criteria compiled by the International Society for Pediatric and Adolescent Diabetes (ISPAD) and the IDF (24). Type 2 diabetes, if left untreated, can lead to damage of organs such as kidneys, eyes, nerves, and blood vessels which may precipitate clinical complications such as neuropathy (foot ulcer, amputation), diabetic retinopathy (blindness), kidney failure, and myocardial infarction (MI).

Table 2. Criteria for the diagnosis of diabetes mellitus and Pre-diabetes. The latter includes IGT and IFG

Symptoms of diabetes plus casual* plasma glucose concentration	≥ 11.1 mmol/l (200 mg/dl)**
Fasting plasma glucose or	≥ 7.0 mmol/l (≥ 126 mg/dl)
2 hour plasma glucose†	11.1 mmol/l (≥ 200 mg/dl) during an OGTT
Criteria for the diagnosis of IGT and IFG	
IGT: 2 hour postload plasma glucose†	7.8-11.1 mmol/l (140-199 mg/dl)
IFG: plasma glucose	5.6-6.9 mmol/l (100-125 mg/dl)

adapted from ISPAD and IDF (24)

* Casual is defined as any time of day without regard to time since last meal.

**Corresponding values are ≥ 10.0 mmol/l for venous whole blood and ≥ 11.1 mmol/l for capillary whole blood

† 75 g glucose dissolved in water

1.4 CARDIOVASCULAR DISEASES - ATHEROSCLEROSIS

Obesity, particularly the accumulation of abdominal fat, is strongly associated with the risk of CVD – diseases affecting the heart and the blood vessels. Among others, ischemic heart disease and ischemic stroke are the most common causes of CVD and account for 5,2% and 1,6% of the global disability-adjusted life years (DALYs) (25). The underlying pathology of most CVDs is termed atherosclerosis and is a chronic inflammatory condition of the arterial wall (26). Atherosclerosis affects the large- and medium-sized

arteries. Lesion development starts during youth and develops over decades before it causes clinical complications.

A healthy artery consists of the *tunica adventitia* (the outer layer of the vessel) with connective tissue, fibroblasts and few macrophages and other immune cells, the *tunica media* with layers of smooth muscle cells, and the *tunica intima* which contains smooth muscle and a layer of endothelial cells. The *tunica intima* (intima) is in direct contact with the blood flow. Lesions are asymmetric thickenings of the arterial intima and characterized by inflammation, lipid accumulation, cell death, and fibrosis. These lesions are preferentially located in areas of disturbed blood flow close to the branching sites.

The development of a so-called fatty streak is the first step in atherogenesis and is an accumulation of lipid-containing cells under the endothelial cell layer, which might later result in atheroma formation (26, 27). The atheroma is composed of a core and a shoulder region surrounded by a cap of smooth muscle cells and collagen-rich matrix (Figure 1) (28). The core region of the atherosclerotic lesion contains extracellular lipids, including cholesterol crystals, apoptotic cells, and lipid-laden foam cells. In contrast, the shoulder region of an advanced plaque is highly immunologically active characterized by infiltrating immune cells such as macrophages, T cells, mast cells, and dendritic cells (DC).

The cholesterol-rich low-density lipoprotein (LDL) is believed to play a major role in initiating atherosclerotic plaque formation when its apolipoprotein (apo) B100 binds to proteoglycans in the sub-endothelial extracellular matrix (29). This process leads to an inflammatory response through activation of the endothelial cells by components of modified LDL (mLDL) particles (e.g., oxidized (ox) LDL). The inflammatory response is facilitated by enhanced expression of adhesion molecules (e.g. vascular cell adhesion protein 1 (VCAM-1)) on the endothelium, mediating leucocyte attachment. Chemokine release enables leucocyte migration into the sub-endothelial space (26). Monocytes migrating into the nascent lesion, differentiate to macrophages, up-regulate pattern-recognition receptors (PRRs) and may become foam cells by ingesting mLDL (30, 31).

Atherosclerotic plaques are immunologically highly active, as indicated by the presence of antigen presenting cells (APCs) (macrophages, DCs) and T cells of different subtypes. Of note, in a recently published study (not included in this thesis) we show that also native LDL can trigger immune response from major histocompatibility complex class II (MHCII)-restricted $CD4^+$ T cells (32). Following activation, T cells produce pro-inflammatory cytokines (e.g., interferon (IFN) γ) that can activate other cells including macrophages, endothelial, and smooth muscle cells. By producing IFNγ, T helper cells

of the sub-type 1 (Th1) cells can stimulate macrophages to release vasoactive mediators, proteolytic enzymes, and pro-inflammatory cytokines such as tumor necrosis factor (TNF) α and interleukin (IL)-1β. Beside the pro-inflammatory Th1 cell subtype, atherosclerotic plaques also harbor so-called regulatory T cells (Tregs), which are considered to exhibit anti-inflammatory properties (28).

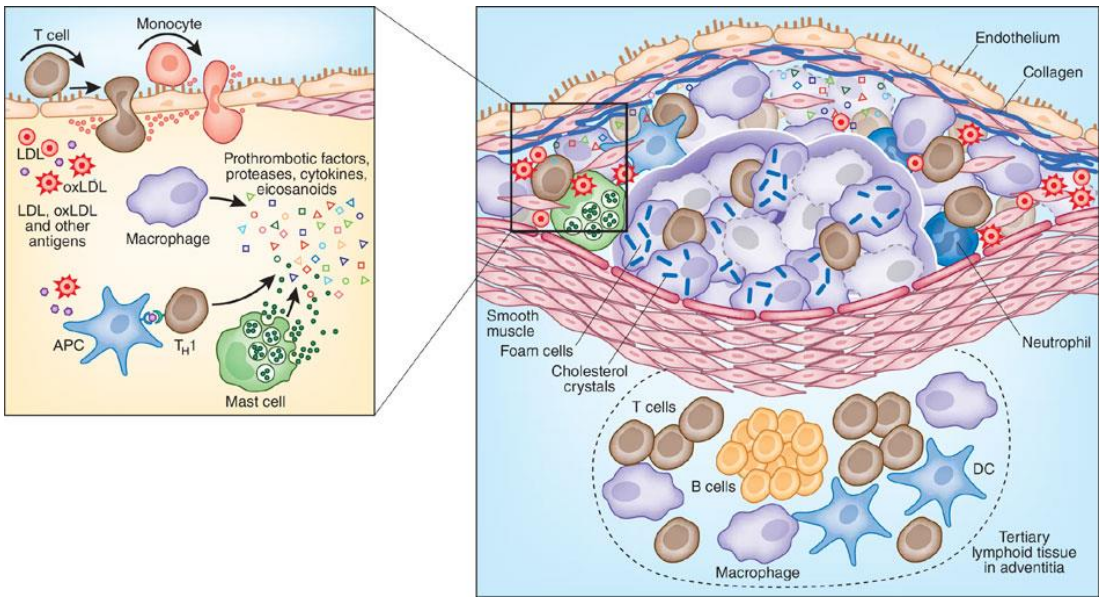

Figure 1: Immune components of the atherosclerotic plaque. The atherosclerotic lesion has a core of lipids, including cholesterol crystals, living and apoptotic cells and a fibrous cap with smooth muscle cells and collagen. Plasma lipoproteins accumulate in the sub-endothelial region. Several types of cells involved in the immune response are present throughout the atheroma including macrophages, T cells, mast cells and DCs. The atheroma builds up in the intima, the innermost layer of the artery. Outside the intima, the media contains smooth muscle cells that regulate blood pressure and regional perfusion, and and the outermost layer of the vessel, the adventitia continues into the surrounding connective tissue. Here, cells of the immune response accumulate outside an advanced atheroma and may develop into tertiary lymphoid structures with germinal centers. *Reprinted by permission from Macmillan Publishers Ltd: Nature Immunology, Hansson GK et al. Mar;12(3):204-12. copyright 2011,* (28).

Plaque development can progress over years without causing any symptoms, but it can rapidly change to a life-threatening situation with acute coronary symptoms (ACS) such as MI, or, in the brain, ischemic stroke. The clinical outcome of atherogenesis largely depends on the plaque structure. The artery can compensate the enlargement of the plaque, by outward remodeling, until a certain level before occlusion occurs (33). However, sudden disruption of the plaque is the main cause of deaths and is still challenging to predict. Plaques that are prone to rupture are frequently characterized by a thin fibrous cap, a large lipid core, increased inflammatory activity and calcification (34).

1.4.1 Atherosclerosis and Diabetes

Epidemiological studies reviewed by Beckman *et al* (35) indicate a 2- to 4-fold increased risk for coronary artery disease (CAD) (36) and a worse outcome of ACS in patients suffering from type 2 diabetes. Additional studies suggest that increased plasma levels of FFA, hyperglycemia and insulin resistance, which are established in diabetic patients, promote vascular smooth muscle- and endothelial dysfunction and likely cause increased risk for vaso-constriction, increased inflammation and thrombosis. In fact these conditions may lead to initiation of atherogenesis and / or instability of established plaques (35).

1.5 ADIPOSE TISSUE

Adipose tissue falls roughly into two categories based on their structure and function in the body: brown adipose tissue (BAT) and WAT. The view on BAT has changed dramatically in recent years and new advanced imaging techniques have made it possible to visualize BAT. These advanced imaging techniques include hybrid positron emission tomography (PET)/computed tomography (CT) scan (PET/CT scan) and [^{18}F]2-fluoro-D-deoxyglucose (FDG)-PET scanning. Since this thesis focuses on investigations around WAT only a few facts and differences between WAT and BAT shall be mentioned here.

1.5.1 Brown adipose tissue

BAT differs in the structure as it is highly vascularized and the adipocytes contain, in contrast to white adipocytes, abundant mitochondria (Figure 2). BAT can be found in infants and young children (under 10 years old) between the shoulder blades and around the neck. In infants it has thermoregulatory functions to maintain the body temperature. Recent studies demonstrated that even in adults a significant amount of BAT can be found. Heaton *et al* demonstrated the existence of BAT in autopsies 1972 (37). Several recent studies, performed under cold-exposure, expand the knowledge about the location (38-41). However, the physiological relevance and function of BAT in adults is still unclear.

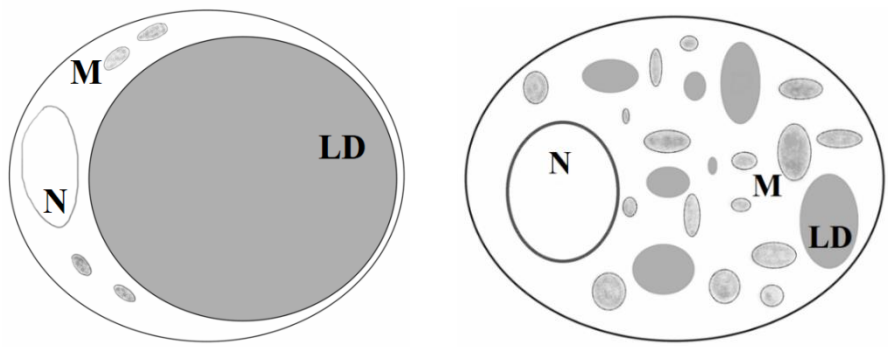

Figure 2: Structure of white (left) and brown adipocyte (right). Adipocytes from WAT display a big lipid droplet (LD) and few mitochondria (M). In contrast, adipocytes from BAT have many small LDs and numerous mitochondria giving them a rather dark appearance. N nucleus.

1.5.2 White adipose tissue

WAT is not a regulator of body temperature, although it also functions as heat insulation. Instead it stores energy in forms of TG, but it also causes metabolic and inflammatory responses to excess nutrients (42). WAT consist of lipid-filled adipocytes, stromal cells and blood vessels. Although WAT is distributed throughout the body, the two main locations are under the skin (subcutaneous) and in the abdominal cavity (visceral) (Figure 3) (43). As mentioned earlier, subcutaneous and visceral WAT do not only differ in location within the body, but also with regard to immunological activity.

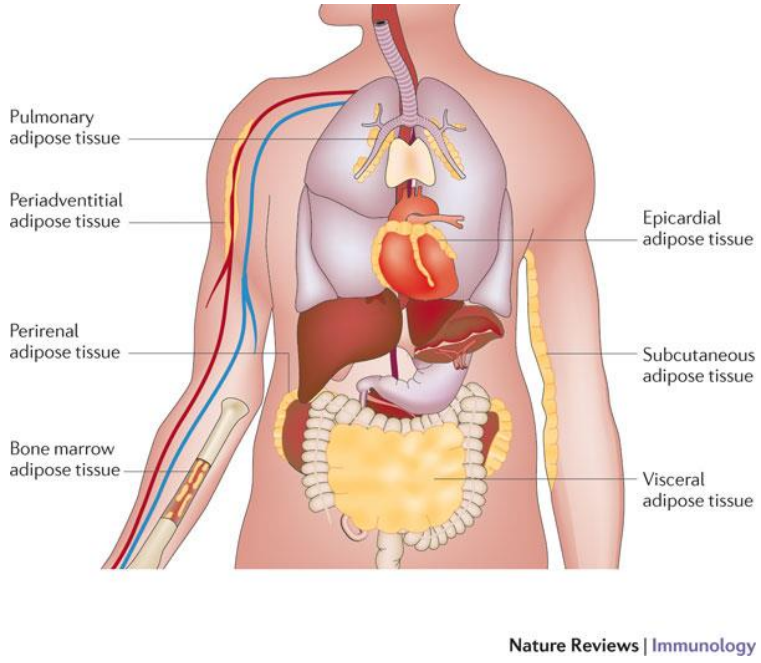

Figure 3: Distribution of WAT. WAT is mainly found in subcutaneous and visceral depots. Under conditions of obesity, WAT expands in these and other depots throughout the body. Common sites of WAT accumulation include the heart, the kidneys and the adventitia of blood vessels. Differential adipokine secretion by various WAT depots may

selectively affect organ function and systemic metabolism. *Reprinted by permission from Macmillan Publishers Ltd: Nature Review Immunology, Ouchi N et al. Feb;11(2):85-97. copyright 2011* (43).

Whereas the size of adipocytes varies enormously, the number of adipocytes is predetermined early in life. The total WAT mass is a result of TG storage and removal. During an estimated lifetime of 10 years per adipocyte, TGs are renewed up to 6 times. Interestingly obesity is associated with a decreased lipid removal and increased lipid storage in humans, possibly leading to accumulation of fat tissue (44). WAT contains a considerable number of immune cells. Visceral WAT in particular is infiltrated by macrophages (6, 7), T cells (8-13, 15, 45, 46), mast cells (47), B cells (16), neutrophils (48), and eosinophils (49) which may all contribute to metabolic disturbances. Beside hormones and enzymes, WAT secretes so-called adipokines (cytokines secreted by adipocytes), such as leptin, adiponectin, and resistin. Furthermore they secrete classical cytokines TNFα, IL-1β, IL-6, IL-18, and monocyte chemotactic protein-1 (MCP-1). All these factors contribute to the metabolic milieu by recruiting immune cells, manipulating signaling cascade and modifying lipid metabolism. The specific roles of WAT-resident immune cells and cytokines in metabolic disturbances, such as insulin resistance, will be discussed separately.

Taken together, contrasting to a long-lasting assumption, WAT serves not only as energy storage but is rather an organ with substantial immunological activity paralleling the findings in atherosclerotic lesions.

1.6 LIVER

Together with WAT, muscle, and pancreas, the liver is an important organ involved in metabolic regulation and function. As a multifunctional organ it facilitates carbohydrate and lipid metabolism through controlling of gluconeogenesis, storage of glycogen, lipogenesis, and control of cholesterol synthesis and secretion (see also the section 1.9.7. and 1.9.8). The role of the liver and its immune cells was a focus of part of the studies in **paper II-IV**. We show that hampered clearance of very low-density lipoprotein (VLDL) and chylomicron remnants from the blood contribute to atherosclerosis development (**paper II**). Similar to WAT, the liver is immunologically active as it contains macrophage-like Kupffer cells (~20% of non-parenchymal cells), endothelial cells (~50%), stellate cells (less than 1%), DCs (less than 1%) and lymphocytes (~25%). Immune cells residing in the liver contribute to the inflammatory and metabolic environment locally, but also in distant peripheral tissue, such as WAT (**paper III**).

In addition the liver regulates amino acid metabolism by using amino acids for protein synthesis (e.g., albumin, fibrinogen, prothrombin, transferrin). It also facilitates detoxification and vitamin storage (vitamin A, D, E, K, and B 12) (42, 50).

1.7 MUSCLE

The musculature can be divided into 3 main groups: cardiac muscle, skeletal muscles, and smooth muscles (reviewed in (51)). These differ by composition, function, and structure. While WAT and liver are heavily involved in lipid storage and lipid disposal, **skeletal muscle** is the main organ for glucose uptake in the body. As reflected by the name they are connected to the skeleton and, in contrast to cardiac and smooth muscle, their contractions are controlled voluntary via motor neurons in the spinal cord and the neurotransmitter acetylcholine. The voluntary-controlled muscle contraction is Ca^{2+}-dependent and increases the translocation of the glucose transporter (GLUT) 4 to the membrane facilitating uptake of glucose.

The **cardiac muscle** contains numerous myoglobin and mitochondria to accomplish high levels of energy needed. TGs (65%), glucose (30%) and proteins or ketone bodies (5%) are primarily used as energy source and the contraction of cardiac muscles is facilitated by so-called pacemaker cells, delivering rhythmical impulses, and cardiac muscle fibers that are connected to the autonomous nervous system. The **smooth muscle** is less structured and its activity is facilitated by interaction of actin- and myosin filaments. The contraction of this muscle can be initiated by nerve impulses, hormone signals and stretching of the muscle. The smooth muscles are part of the digestive-, respiratory-, urinary-, and reproductive tract, blood- and lymph vessels, and muscles in the skin and the eyes.

1.8 PANCREAS

The pancreas has the capability of regulating glucose metabolism and it is part of the digestive system. Research on the pancreas can be tracked back until a defined anatomical description was made in 1642 (Johann Wirsung) and the first secretion studies performed by Regnier de Graaf in 1664. The first milestones in the diabetes research included the discovery of islets of Langerhans (Paul Langerhans, 1869), the discovery of the hormone secretin and the introduction of the "concept of hormones" (Ernest Starling and William Bayliss (1902/1905)). Furthermore, the isolation of insulin (Frederic Banting and John Macleod, Nobel prize 1923) followed by determination of the molecular structure of insulin (Frederick Sanger, Nobel prize 1958) and the development

of the first radioimmunoassay for insulin (Rosalyn Yalow, Nobel prize 1977) were crucial for the diabetes research (52). Sub-divided into head, body and tail region (Figure 4), the pancreas exerts exocrine functions by producing and secreting digestive enzymes (e.g. trypsinogen, chymotrypsinogen, pro-elastase, procarboxy peptidase) and endocrine functions by hormone secreting cells in the islets of Langerhans (Figure 5). The exocrine compartment of the pancreas contains so-called acinar cells, which are pyramidal shaped epithelial cells that synthesize, store, and secrete digestive enzymes in their inactive perform, preventing self-digestion of the pancreas (23). The islets of Langerhans contain the hormone-producing α (glucagon), β (insulin), δ (somatostatin) (Figure 5), ε (grehlin) and pp (polypeptide) cells. The insulin secretion process from β cells was studied in **paper IV**. There we analyzed the role of Toll-like receptor (TLR)-3 on glucose homeostasis and β cell insulin secretion. The process of insulin secretion from β cells will be discussed later in section *Glucose-induced insulin secretion and glucose metabolism (1.10.2).* In brief, this process involves the uptake of glucose into the islet via GLUT2, followed by depolarization of the β cell membrane and a Ca^{2+} influx into the cell. The increased Ca^{2+} level triggers the fusion of insulin containing vesicles with the membrane and a release of the content.

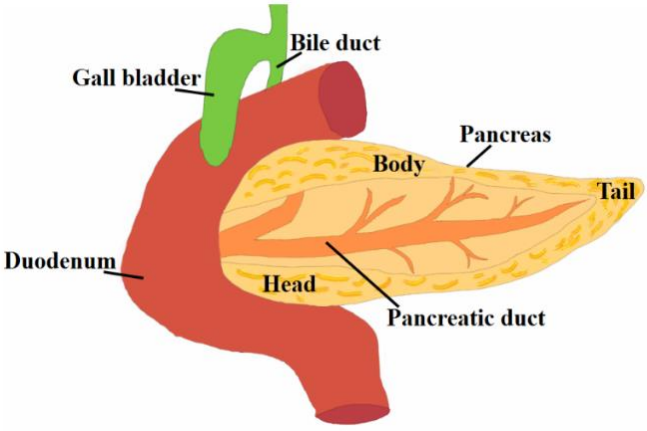

Figure 4: The anatomy of the pancreas.
The pancreas is located close to the duodenum and can be divided into a head, a body, and a tail region. *Adapted from Bardeesy N. et al, Nature reviews Cancer. 2002.* (53)

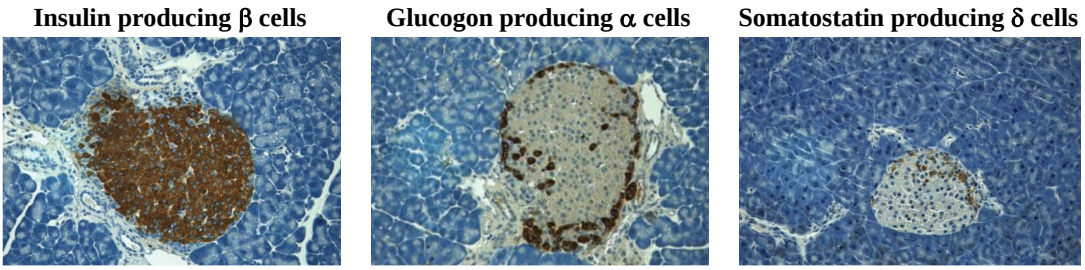

Figure 5: Pancreatic islet morphology of C57/B6 mouse. Immunohistochemical staining for insulin (left), glucagon (middle), and somatostatin (right). Taken from **paper IV**.

1.9 LIPID METABOLISM

1.9.1 General

Dyslipidemia (raised TG- and reduced HDL levels) is one feature of the metabolic syndrome. According to the Swedish national food agency the total energy intake should derive from 10-20% proteins, 50-60 % carbohydrates, and 25-35% fat (54). Fat, carbohydrates, and proteins are metabolized in different ways in the body. Since lipid- and glucose metabolism are the major topics of this thesis and they will be discussed here more deeply. Certainly a profound understanding of the complex molecular machinery of lipid- and glucose metabolism is essential to explore novel and improve already existing, therapeutic approaches for obesity and its consequences.

1.9.2 Lipoproteins

Lipoproteins enable the transport of fat throughout the body and differ in size and density. They are classified as: chylomicrons, VLDL, LDL, and HDL. The size of the lipoproteins range from about 1000nm (chylomicrons) to 30-80nm (VLDL) and to the smallest particles LDL (25nm) and HDL (10nm) (55). A summary of the following description of lipoprotein metabolism is shown in Figure 6.

1.9.3 Chylomicrons

Fat absorption after a meal takes place in the intestines. The subsequent lipolyzed TG are taken up by the enterocytes in the form of FFA and 2 monoacylglycerol (MAG) molecules and re-packaged to TG. The nascent chylomicron particle consists of ~85% TG plus phospholipids, cholesterol, cholesterol ester, proteins, and apoB48. These large chylomicron particles enter the blood stream after passing the thoracic duct (56). The nascent chylomicrons have a short half-life time of 5-15 minutes after fat intake (57). Once entering the blood stream, an exchange of apolipoproteins from HDL particles to

nascent chylomicrons takes place. As a result, the mature chylomicron contains, in addition to apoB48, apoC2 and apoE. The binding of apoC2 to lipoprotein lipase (LPL) on the endothelial surface in capillaries of peripheral tissue (e.g., WAT) facilitates the lipolysis of TG into FFA and diacylglcyerol. In a last step, chylomicron remnants which lack apoC, are taken up by the liver (58). This step is facilitated in several ways: through binding of apoE to heparan sulfate proteoglycans, to LDL receptor-related protein (LRP) and/or binding to LDL-receptor (LDLR). Once in the liver the TG are used for VLDL synthesis (59) (Figure 6).

1.9.4 VLDL

Similar to chylomicrons, VLDL-particles are rich in TG but are synthesized in the liver. With a TG content of ~50-75% VLDL particle are still relatively lipid-rich. However, in contrast to the chylomicrons, they contain more cholesterol (~15%) and they have apoB100 integrated into the membrane. The mature VLDL particle is the product of two steps in the endoplasmic reticulum (ER) forming the pre-VLDL particle and secondly in the Golgi apparatus forming the mature VLDL-1 or -2 (60). VLDL-1 is, in contrast to VLDL-2, rich in TG and it is associated with metabolic disturbances and atherosclerosis/CVD (61-64). Lipid transfer to apoB through microsomal TG transfer protein (MTTP) in the lumen of the ER and the fusion of these particles with lipid droplets are essential steps in VLDL synthesis. The mature VLDL particle contains apoB100, apoC, and apoE where the latter two lipoproteins are transferred from HDL particles. The stimulation of activation of LPL on cells of the peripheral tissues via apoC on the VLDL particle promotes the lipolysis of TG to FFA and glycerol. VLDL particles are either taken up by the liver via apoE-LDLR binding or remain on the peripheral tissue for further TG breakdown. Sortilin-1 is a sorting receptor localized in the Golgi apparatus and in the plasma membrane in the liver. It facilitates the trafficking of ligands to the lysosome, and it was involved in both regulation of VLDL secretion and uptake of VLDL particles into the liver (65, 66). As discussed below, insulin partly impacts on sortilin-1 expression. The continuous lipolysis of TG from VLDL particles on the peripheral tissue yields intermediate density lipoprotein (IDL)-particles, which can be refilled with TG to rebuild VLDL particles in the liver. Alternatively, IDL particles can undergo further lipolysis in peripheral tissues, leading to their conversion into LDL particles.

1.9.5 HDL

HDL is the smallest lipoprotein (~10nm) and has, in contrast to the other lipoproteins, apoA-1 integrated into the membrane (reviewed in (55, 67)). The main function of HDL is the transport of cholesterol from peripheral tissues to the liver, also called reverse cholesterol transport. A pre-form of HDL is synthesized in cells from the liver and intestine or is a product of chylomicron- or VLDL-degradation. This pre-form does not contain lipids. The uptake of cholesterol into the HDL particle from different cells is mediated by ATP-binding cassette transporters (ABCA1, ABCG1). Cholesterol is then converted to cholesterylester by an enzyme called lecithin-cholesterol acyltransferase (LCAT). Depending on the amount of cholesterol and TG, HDL particles can be distinguished between HDL_3 and HDL_2-particles with the latter being richer in cholesterol and TGs. As mentioned before, the mature HDL particle can transfer the stored TG to VLDL- or chylomicron particles via the cholesteryl ester transfer protein (CETP)-system or it can bind to apoA-receptors on hepatocytes and release the cholesterol. Once in the liver cholesterol will be further transformed to bile acid and transported to the gall bladder.

1.9.6 LDL

LDL particles are produced in the liver or in the circulation as a result of VLDL-degradation (see VLDL-section). In contrast to VLDL, LDL particles contain mostly cholesterol and cholesteryl ester. The uptake of LDL into a cell is mediated via the LDLR or in case of LDL-modification (e.g, through oxidization), through scavenger receptors. In the section 1.9.8 the role of LDL will be discussed in more detail.

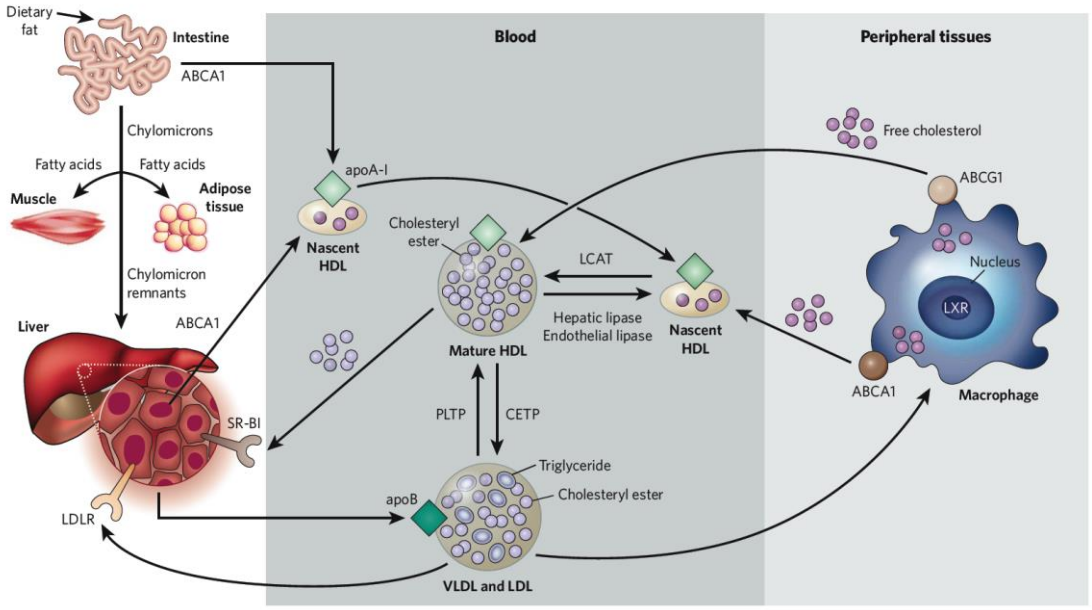

Figure 6: Lipoprotein metabolism has a key role in atherogenesis. It involves the transport of lipids, particularly cholesterol and TG in the blood. The intestine absorbs dietary fat and packages it into chylomicrons (large TG-rich lipoproteins), which are transported to peripheral tissues through the blood. In muscle and WAT, the enzyme LPL breaks down TG of chylomicrons, and fatty acid (FA) enter these tissues. The chylomicron remnants are subsequently taken up by the liver. The liver loads lipids onto apoB and secretes VLDL, which undergoes lipolysis by LPL to form LDL. LDL is then taken up by the liver through binding to LDLR, as well as through other pathways. By contrast, HDL is generated by the intestine and the liver through the secretion of lipid-free apoA-I. ApoA-I then recruits cholesterol from these organs through the actions of the transporter ABCA1, forming nascent HDLs, and this protects apoA-I from being rapidly degraded in the kidneys. In the peripheral tissues, nascent HDLs promote the efflux of cholesterol from tissues, including from macrophages, through the actions of ABCA1. Mature HDLs also promote this efflux but through the actions of ABCG1. (In macrophages, the nuclear liver X receptor (LXR) upregulates the production of both ABCA1 and ABCG1.) The free (unesterified) cholesterol in nascent HDLs is esterified to cholesteryl ester by the enzyme LCAT, creating mature HDLs. The cholesterol in HDLs is returned to the liver both directly, through uptake by the Scavenger receptor class B member 1 (SR-BI), and indirectly, by transfer to LDLs and VLDLs through CETP. The lipid content of HDLs is altered by the enzymes hepatic lipase and endothelial lipase and by the transfer proteins CETP and phospholipid transfer protein (PLTP), affecting HDL catabolism. *Reprinted by permission from Macmillan Publishers Ltd: Nature, Rader and Daugherty. 2008 Feb 21;451(7181):904-13. copyright 2008* (67)

1.9.7 Dysregulation of lipid metabolism in metabolic syndrome

As mentioned earlier, one characteristic of the metabolic syndrome is the disturbed lipid profile characterized by increased levels of TG in the blood and reduced number of HDL particles. Modification of lipid metabolism in metabolic disorders (e.g., obesity and type 2 diabetes) and CVD (atherosclerosis) involves a number of molecules, such as hormones

and lipases. In the following, only a few that play a central role in my thesis projects are discussed in more detail.

1.9.7.1 LPL, Insulin and VLDL

LPL, localized on the surface of the capillary endothelium (68), facilitates lipolysis of TG in chylomicrons and VLDL particles. It is highly expressed in organs with immense requirement of energy such as skeletal and cardiac muscle (69, 70). The regulation of LPL activity is very complex and takes place at different levels. LPL activity is regulated by hormones, fat-rich diet, exercise etc. (70). The activity of LPL is also stimulated by insulin, one of the most important LPL regulators, on the transcriptional (71), posttranscriptional, and posttranslational (72) levels.

1.9.7.2 The role of insulin in lipid metabolism

Secreted by the β cells in the pancreas in response to glucose, insulin regulates in several metabolic processes. While synthesis and production of insulin will be discussed in a separate section, its impact on lipid metabolism is described here. Insulin modulates VLDL assembly and secretion (73, 74) and it facilitates the degradation of apoB via the mammalian target of rapamycin complex 1 (mTORC1) pathway and by increasing the activity of phosphoinositide 3-kinase (PI3K) (75, 76). In other words, insulin activates mTORC1 that further activates the sterol regulatory element-binding protein (SREBP)-1c. Increased signaling through SREBP-1c leads to enhanced FA synthesis and finally to increased TG synthesis (lipogenesis). Activation of mTORC1 may lead to enhanced or decreased apoB secretion, depending on the pathway. mTORC1 may block apoB formation resulting in decreased apoB secretion. Alternatively, insulin signaling via mTORC1 can suppress sortilin-1, which impairs apoB secretion. By suppressing sortilin-1 via mTORC1, apoB secretion is enhanced (Figure 7).

As apoB is the major lipoprotein integrated into the VLDL particle, increased apoB degradation and decreased secretion is accompanied by diminished VLDL secretion. A reversed effect, displayed by increased apoB-VLDL secretion, is seen in conditions of insulin resistance (77). Furthermore, insulin interferes in the process of lipid transfer to apoB during VLDL synthesis, which is mediated by MTTP (Figure 7). Insulin hereby impacts on AKT which leads to the inhibition of forkhead box protein O1 (FoxO1), causing an inhibition of MTTP expression (78, 79). Moreover, insulin modifies apoB

clearance from the circulation through LDLR, LRP1, and heparan sulfate proteoglycans (HSPG)(74).

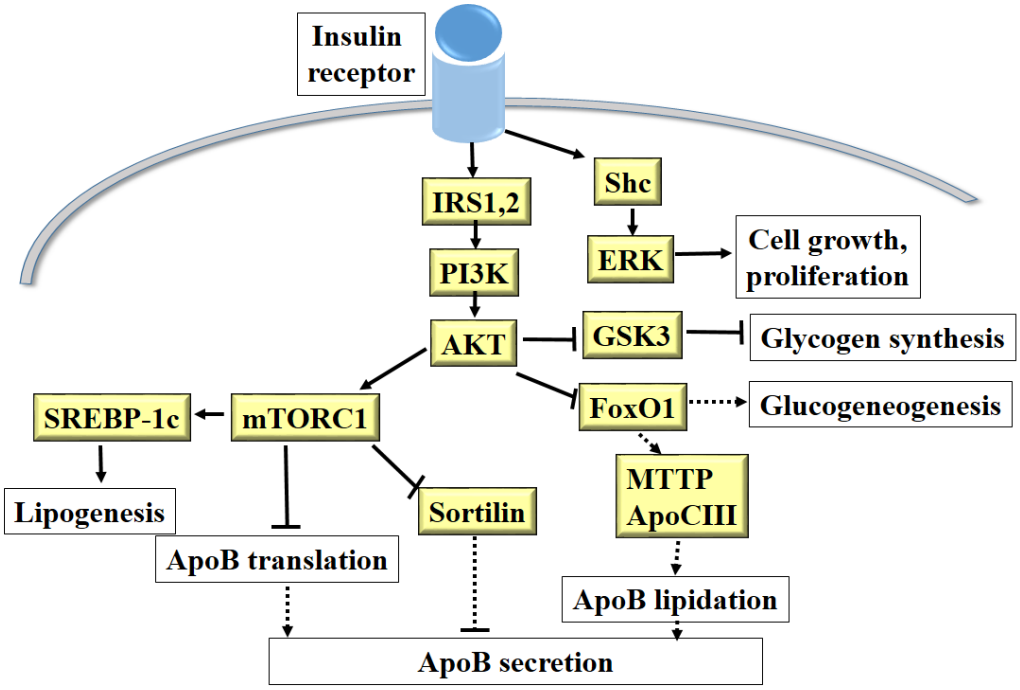

Figure 7: Insulin signaling cascade and its impact on lipoprotein metabolism. For further description see text. In brief, insulin can activate different pathways, leading to increased apoB secretion via the mTORC1-sortilin pathway or decreased apoB secretion directly via mTORC1. Furthermore insulin signaling can decrease apoB secretion by inhibiting MTTP mediated lipid transfer to apoB during VLDL synthesis. Adapted from *Haas ME et al. Trends Endocrinol Metab. 2013* (74)

1.9.8 Dysregulation of lipid metabolism in CVD

High cholesterol levels, hypertension, smoking, diabetes, and a family history of CVD are major risk factors of developing atherosclerosis. Additionally, research from the last decades strongly suggest inflammation as an important risk factor.

Lipoproteins play a crucial role in the initiation and progression of atherosclerosis, especially small apoB-containing lipoproteins such as LDL. The process by which LDL particles bind to matrix molecules on the surface of cells in the sub-endothelial space of the intima is referred to as lipoprotein retention (29, 80). Retained lipoproteins are susceptible to modifications which can initiate an inflammatory responses in the intima of the artery (28). The latter will be discussed in more detail in section 1.11.3.5.

The accumulation of cholesterol-rich LDL particles in the intima of the artery wall, promotes the initiation and progression of atherosclerosis. In contrast to the relatively small LDL particles, chylomicrons can only enter the arterial wall as remnant particles

(81, 82). LPL is an enzyme that facilitates the binding of LDL-particle to proteoglycans (29). Thus it has a dual role in lipid metabolism and atherosclerosis with pro-atherogenic (enabling LDL-proteoglycan binding) and anti-atherogenic properties (clearance of potentially atherogenic lipoproteins from the plasma). Enormous efforts have been made to investigate the basic molecular mechanisms behind atherosclerosis since such knowledge could lead to therapeutic approaches. The most common drugs are statins (hydroxymethyl glutaryl coenzyme A reductase) which interfere rather early in this cascade. In brief, statins interfere in the cholesterol metabolism by inhibiting 3-hydroxy-3-methyl-glutaryl-CoA (HMGCoA-) reductase. This leads to upregulation of LDLR on hepatocytes, clearance of LDL from the blood, and a reduction of circulating cholesterol (83, 84).

1.10 GLUCOSE METABOLISM

The liver, WAT, skeletal muscle and pancreas all play crucial roles in glucose metabolism and homeostasis. They facilitate both glucose production (gluconeogenesis in the liver) and its uptake and utilization (skeletal muscle, WAT). Additionally, cells in the pancreas produce hormones that are involved in glucose metabolism (mainly insulin and glucagon of the pancreas)(23).

1.10.1 Insulin regulates glucose homeostasis

Mature insulin consists of 51 amino acids and organized as A and B chains and a C-peptide (85) and its synthesis starts in the rough ER (rER) with the 110 amino acid long preproinsulin. The interaction of the hydrophobic N-terminal with the cytosolic ribonucleoprotein signal recognition particles (SRP) enables the translocation of preproinsulin from the rER membrane to the lumen and the cleavage of the peptide to proinsulin (86, 87). The final step in insulin maturation includes the folding of proinsulin by chaperone and the translocation from the ER to the Golgi apparatus where it is cleaved to mature insulin and C-peptide.

Besides facilitating the correct folding of insulin, recent studies suggest additional functions of C-peptide. A role of C-peptide in intracellular signaling in kidney (88-92), fibroblasts and lungs (93, 94) were proposed and subsequently a potential role in inflammation, renal, nervous, and vascular function have been discussed (95). Supplementation with C-peptide inhibited endothelial cell apoptosis (96) and positively influenced renal microvasculature in an animal model for type 1 diabetes (97). However,

treatment of atherosclerosis-prone mice with C-peptide was associated with increased monocyte infiltration and lipid deposition in the plaque (98).

Glucagon and **somatostatin** are antagonists of insulin. Somatostatin is a hormone produced by δ cells in the pancreas and it inhibits the secretion of insulin and glucagon (99). Glucagon is a hormone secreted by the α cells of the pancreas and display the antagonist to insulin as it is released when glucose levels in the blood are low (Figure 8).

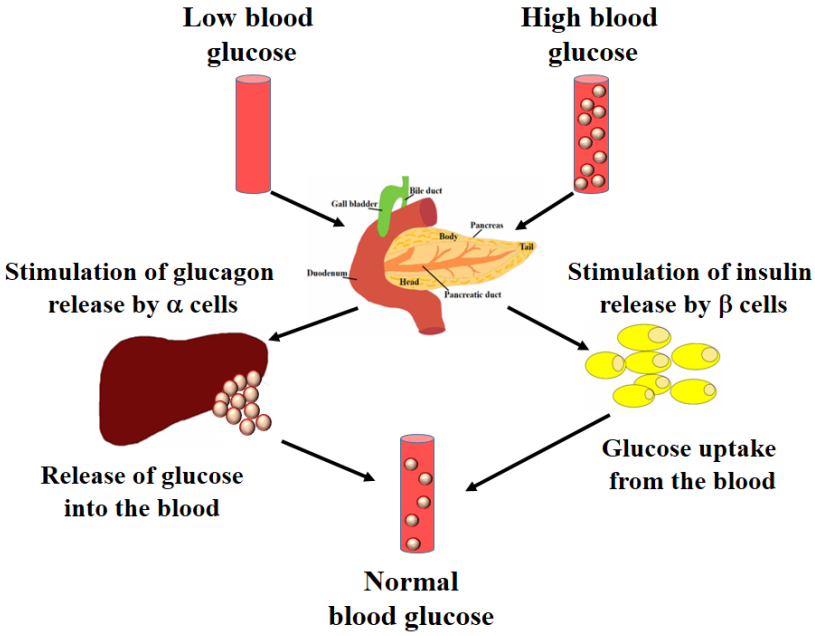

Figure 8: Interaction of insulin and glucagon. Fasting causes reduced blood glucose and the stimulation of glucagon release from the cells in the pancreas. This in turn leads to gluconeogenesis in the liver and a release of glucose into the blood stream. Insulin is the antagonist as it leads to increased glucose uptake from the peripheral tissue (skeletal muscle and WAT). Insulin release from the pancreatic β cells is triggered by high circulating blood glucose. Not shown in this figure is somatostatin, that regulates insulin and glucagon release. The interaction of somatostatin, insulin and glucagon lead to a normal blood glucose.

1.10.2 Glucose-induced insulin secretion and glucose metabolism

Being one of the most important sources of energy, glucose is both used and metabolized by numerous cell types and tissues. Liver, muscle, WAT, pancreas, and brain are the most crucial organs influencing glucose homeostasis.

The intake of carbohydrate-rich food leads to increased levels of glucose in the blood and a subsequent release of insulin to facilitate glucose uptake from peripheral tissues. This process is initiated by glucose uptake in the β cells of the pancreas. Glucose enters the

cell via GLUT proteins. Fourteen isoforms of GLUT proteins are known today and they are classified as class I (GLUT 1-4) glucose transporters, class II (GLUT 5, 7, 9) fructose transporter, and class III (GLUT 6, 8, 10, 12 and 13) with atypical structures and not yet fully characterized (100, 101). GLUTs are anchored in the membrane through 12 highly glycosylated transmembrane helices (102) (Figure 9).

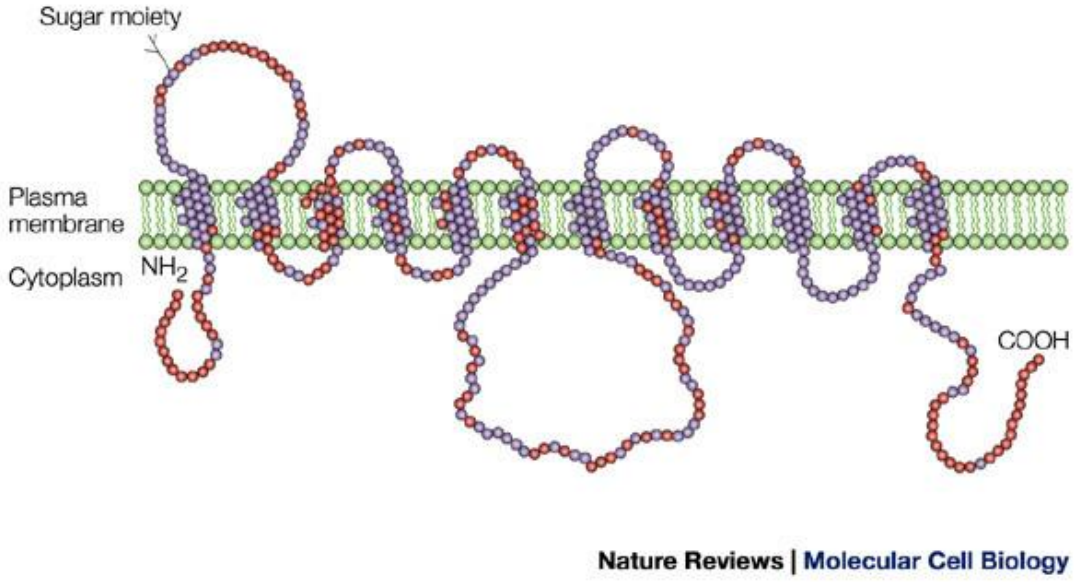

Figure 9: Glut 4 structure as an example of the GLUT family. The GLUT family of proteins is predicted to span the membrane 12 times with both amino- and carboxyl-termini located in the cytosol. On the basis of sequence homology and structural similarity, three subclasses of sugar transporters have been defined: Class I (GLUTs 1-4) are glucose transporters; Class II (GLUTs 5, 7, 9 and 11) are fructose transporters; and Class III (GLUTs 6, 8, 10, 12, and 13) are structurally atypical members of the GLUT family, which are poorly defined at present. The diagram shows a homology plot between GLUT1 and GLUT4. Residues that are unique to GLUT4 are shown in red. *Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology, Bryant NJ et al. Apr;3(4):267-77. Copyright 2002 (101)*

While GLUT 1 is expressed in erythrocytes, endothelial cells and epithelial barriers of the brain, eye, peripheral nerves, and placenta (103), the translocation of GLUT 4 is the rate-limiting step in insulin-stimulated glucose uptake in skeletal- and cardiac muscle and WAT (100). GLUT 2 is expressed in liver, kidney, intestine (104) and β cells (105). After entering the β cell via GLUT2, glucose is first metabolized by the enzyme glucokinase (Gck) to glucose-6-phosphate that is the rate-limiting step in glucose metabolism. This is followed by the subsequent glycolysis to pyruvate and its metabolism to acetyl-CoA, which is then further oxidized in the tricarboxylic acid cycle. These processes take place in the mitochondria and result in increased production of

ATP, which further initiates membrane depolarization by inhibition of ATP sensitive K^+ channel. The ensuring influx of Ca^{2+} into the cell via voltage-dependent Ca^{2+} channels and the resulting increase of intracellular Ca^{2+} concentration eventually leads to the fusion of insulin-containing granules with the membrane. Insulin secretion is mediated in two cycles. The first immediate release is mediated by so-called rapidly released granules with already stored insulin. With continuous stimulation, the synthesis and release of insulin from reserved pools is initiated (the second phase of insulin secretion) (23, 87, 106).

The fusion of insulin-filled granules with the membrane is facilitated by SNARE proteins (soluble N-ethylmaleimide-sensitive factor attachment protein receptor). They have a central coiled-coil 60-70 amino acid cytosolic domain that facilitates the recognition and interaction of SNARE proteins. This priming process is triggered by voltage-dependent Ca^{2+} channels and the subsequently increased intracellular Ca^{2+} concentration leads to a transformation of the SNARE complex from its *trans* to *cis* configuration. This process brings the vesicle membrane (from insulin filled granules) with the SNARE protein VAMP-2 (vesicle-associated membrane protein 2 or synaptobrevin) closer to the target SNARES (synaptosomal-associated protein 25 (SNAP-25) and syntaxin-1A) in the membrane of β cells (107, 108). The importance of the SNARE complex in insulin secretion is emphasized by studies on diabetic rodents and humans that displayed decreased SNARE protein levels (109, 110).

1.10.3 Insulin receptor and insulin signaling in peripheral tissue

The insulin receptor belongs to a subfamily of receptor tyrosine kinases and consists of two α chains and two β chains that are linked by disulfide bonds and anchored in the plasma membrane. Binding of insulin to its receptor mediates a conformational change and a subsequent phosphorylation and of tyrosine residues on the β subunit. An additional conformational change via auto-phosphorylation initiates the signaling cascade by activation of receptor protein kinase activity (111).

The insulin signaling cascade is very complex and includes numerous molecules. The following will give a very simplified overview of the insulin signaling cascade with the key proteins and pathways involved in regulation of insulin response.

Besides binding to insulin-growth factor-1 receptor (IGF1R), insulin binds mainly to its receptor in the plasma membrane following activation of insulin receptor substrate **(IRS) proteins** via tyrosine phosphorylation. The activation of IRS proteins can lead to either activation of the PI3K-pathway or activation of Ras- mitogen-activated protein kinases

(MAPK) pathway. Because insulin mediates numerous important metabolic processes in the tissue/body the signaling is tightly regulated. The negative regulation of IRS protein is mediated by protein tyrosine phosphatase, serine phosphorylation and ligand-induced down regulation.

Numerous processes including stimulation of glucose transport, glycogen- and lipid synthesis, and adipocyte differentiation are mediated via the **PI3K pathway**. PI3K consists of a regulatory and catalytic subunit that facilitates the formation of phosphatidylinositol (3,4,5)-triphosphate (PIP_3) which in turn can activate the AKT/ protein kinase B (PKB) pathway. The phosphorylation of PI3K by its own catalytic subunit and the following decrease of PI3K enzyme activity is a way to regulate its own function. The activation of the **AKT/PKB pathway** by PI3K leads to (i) increased glycogen synthesis via glycogen synthase kinase 3 (GSK3) activation, (ii) regulation of glucose uptake by phosphorylation and inhibition of Rab-GTPase activating protein AS160, (iii) regulation of protein synthesis via activation of mTOR pathway, (iiii) regulation of expression of gluconeogenic and lipogenic enzymes by controlling forkhead box (FOX) transcription factors. The **Ras-MAPK pathway** activated by insulin via IRS proteins leads to activation and phosphorylation of several kinases (MAPK and extracellular-signal-regulated kinases (ERKs)) via Ras and Raf proteins. Activation of this pathway mediates cell growth and cell differentiation (112).

1.10.4 Glucose metabolism and its dysregulation in metabolic syndrome and CVD

1.10.4.1 Impaired insulin signaling affects several organs in the body

Under certain circumstances cells and tissue that are involved in lipid- and glucose metabolism develop a disturbed response to insulin. Risk factors such as obesity and sedentary lifestyle, family history of type 2 diabetes, age, but also ethnical background are discussed to promote insulin resistance. Of note, the same risk factors correlate to type 2 diabetes as dysregulated insulin metabolism is a hallmark of type 2 diabetes.

Nicely illustrated in a review by *Rask-Madsen and Kahn*, insulin resistance affects not only the obvious tissues, such as WAT, muscle and liver. Disturbed insulin response may also cause metabolic changes in the brain, and tissues related to CVD such as vessels and the myocardium (113).

1.10.4.2 WAT, liver and muscle and their contribution to insulin resistance

WAT, depending on its distribution within the body (subcutaneous vs. visceral), differs in its metabolic activity. In addition, the size of the adipocytes plays a role in insulin resistance (114). Excess TG storage and the accompanied enlargement of adipocytes (hypertrophy) have been shown to be associated with insulin resistance (115, 116). As a consequence of excess TG storage, FFA, a product of TG-lipolysis, are increased in the circulation and impact on insulin signaling in liver and muscle by increased serine phosphorylation of IRS-1 and inhibiting the AKT/PKB pathway downstream of the signaling cascade (117). Changes in insulin signaling by elevated FFA in the liver eventually lead to increased gluconeogenesis promoting hyperglycaemia and further aggravation of lipid disturbances by increased TG synthesis (114). In addition to enhanced release of FFA, increased body fat is accompanied by increased cytokine release, promoting further infiltration of immune cells into the WAT. The release of pro-inflammatory mediators further aggravates inflammation. All in all, these metabolic changes eventually modulate insulin signaling and processes that are closely regulated by insulin. In the **liver** gluconeogenesis is, amongst others, regulated by FFA and insulin. Insulin suppresses phosphoenolpyruvate carboxykinase (PEPCK) expression (118), a key enzyme involved in gluconeogenesis. As mentioned above in the context of lipid metabolism, insulin modulates VLDL assembly and secretion, and LDL and VLDL clearance in the liver. A modified lipid metabolism with increased VLDL secretion or decreased LDLR and accompanied defects in LDL clearance, was observed in animal models and type 2 diabetic patients (119-121).

Skeletal muscle requires glucose as energy source. In conditions of disturbed insulin sensitivity due to impaired insulin signaling through IRS-1 and AKT (122-125), glucose uptake is diminished due to decreased GLUT4 translocation (126). These impairments are reversible by changes of lifestyle such as exercise, moderate diet, or combination of both (127-129).

1.11 THE IMMUNE SYSTEM IN VIEW OF CVD AND METABOLIC COMPLICATIONS

1.11.1 General

Both, atherosclerosis and obesity-related diseases, were not considered to be immunologically active processes decades ago. Atherosclerosis was characterized as accumulation of lipid in the vessel wall and WAT was believed to be a source of energy in form of stored TG only. This picture has changed dramatically with the discovery that immune cells actively infiltrate both atherosclerotic plaques (130) and WAT (6, 13).
Before going deeper into the role of the immune system in atherosclerosis and (obese) WAT, the following section will provide a short overview about the innate and adaptive immune system and its immune cells.

1.11.2 The Innate Immune system

The immune system is composed of the innate and the adaptive part (summarized from (131)). The innate immune system reflects the early defense of the body against invading microbes. Its immediate response takes place in the first 4 hours after the microbe has entered the body via the skin, the gut, the eyes, the nose, or the oral cavity. When mechanical (epithelial cells), chemical (e.g., FA, low pH, enzymes), and microbial (normal microbiota) barriers fail to keep pathogens from invading the body, soluble molecules such as antimicrobial peptides, antimicrobial enzymes, and a system of plasma proteins are the first line of response and kill the pathogen immediately or weaken its effect. When the pathogen is not immediately eliminated, a so-called early induced immune response, that last up to 96 hours, is initiated. The recognition of the pathogen by PPRs on the immune cells leads to their activation and further recruitment of immune cells to the place of infection. If these two phases do not succeed in clearance of the pathogen from the body, the adaptive immune response will be activated by transporting the antigens (AG) to the lymphoid organs. Here the APCs (macrophages, DCs, B cells) present components of the pathogen to naïve T lymphocytes and activate them, leading to clonal expansion and possibly elimination of the pathogen.

1.11.2.1 Receptors of the innate immune system and their role in WAT and atherosclerosis

PRR recognize highly conserved structures on microbes (bacteria, fungi, viruses, ect), but also molecules from cell debris of dying or damaged cells. These structures are termed pathogen-associated molecular patterns (PAMPs) for pathogen-related structures and damage- or danger-associated molecular patterns (DAMPs) for cell debris-related structures. The receptors of the innate immune system are encoded in the germ line and can be classified into 3 main groups: (1) freely circulating receptors in the serum (e.g., mannose-binding lectin (MBL)), (2) membrane bound phagocytic and signaling receptors (e.g., scavenger receptors, TLRs), (3) cytoplasmic signaling receptors (e.g., nucleotide-binding oligomerization domain (NOD)-like receptor, retinoic acid-inducible gene 1 (RIG-1), melanoma differentiation-associated protein 5 (MDA-5)). Once bound to its ligand, PRRs initiate a signaling cascade eventually leading to inflammatory response accompanied by the recruitment and activation of additional immune cells or the direct elimination of viruses.

1.11.2.1.1 Toll-like receptors

TLRs were firstly discovered in the fruit fly *Drosophila melanogaster* as part of the embryonic development system (dorsal-ventral axis). The ability of these receptors to defend against microbes in the adult fly was discovered later and today it is well accepted that TLRs play a crucial role in pathogen recognition. Eleven TLRs in human and 13 TLRs in mice are known. However, not all of them are well characterized yet. These receptors are homologous to the fly protein Toll and they are located intracellularly (TLR-3, -7, -8, -9, -13) or integrated in the plasma membrane on the cell surface (TLR-1, -2, -4, -5, -11, -12) of numerous cells throughout the body (Figure 10) (131-133).

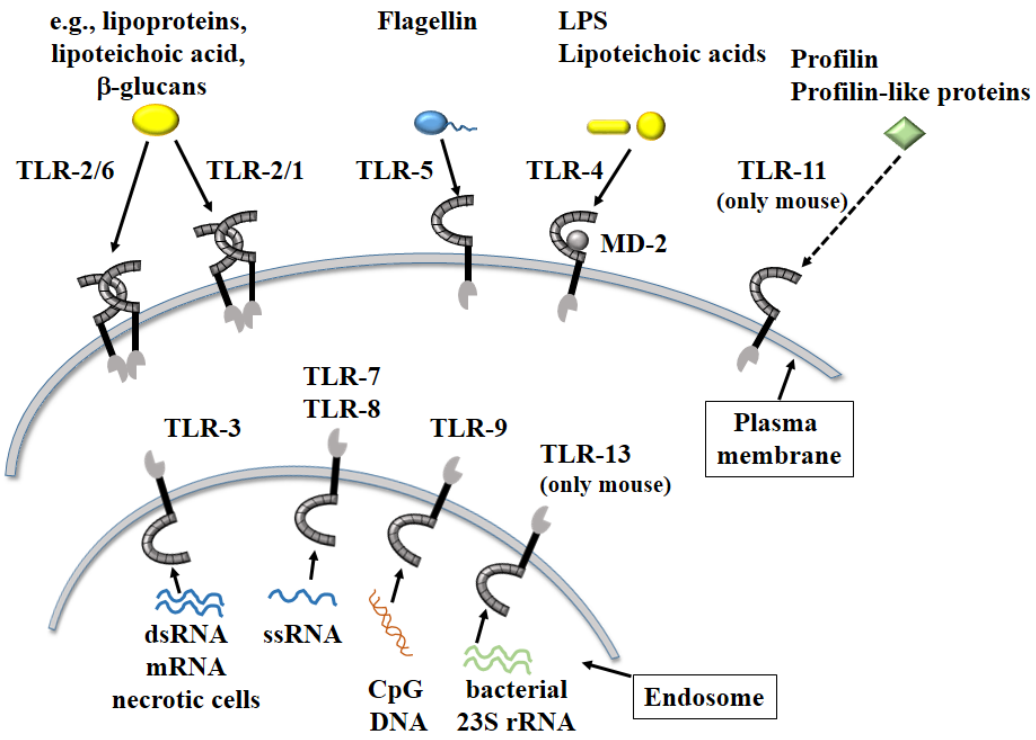

Figure 10: TLRs and selected ligands. TLRs are located in the plasma membrane on the cell surface or intracellularly. The function and role of TLR-10 and TLR-12 need more investigation and are therefore not included in the figure. *Adapted from Murphys Janeway's Immunobiology. 8th ed.; 2012 (131, 133).*

TLRs consist of 18-25 copies of an extracellular leucine-rich repeat (LRR) forming a horseshoe-shaped protein (131). The binding of PAMPs (e.g., LPS, flagellin, dsRNA, CpG DNA) or DAMPs (e.g., FA, heat shock protein, necrotic cells, mLDL) to these receptors initiates dimerization of two TLRs, bringing their TIR (Toll-IL-1R-resistance) domains closer together in the cytoplasm (Figure 10). TLR-signaling is mediated by the adapter molecules; myeloid differentiation primary response gene (Myd88) (88), TIR domain-containing adaptor protein (TIRAP), TIR-domain-containing adapter-inducing IFNβ (TRIF), and/or TRIF–related adaptor molecule (TRAM) (Figure 11: example of signaling). Eventually, the induced signaling cascade leads to activation of genes encoding for pro-inflammatory cytokines and / or antiviral type 1 IFNs (131).

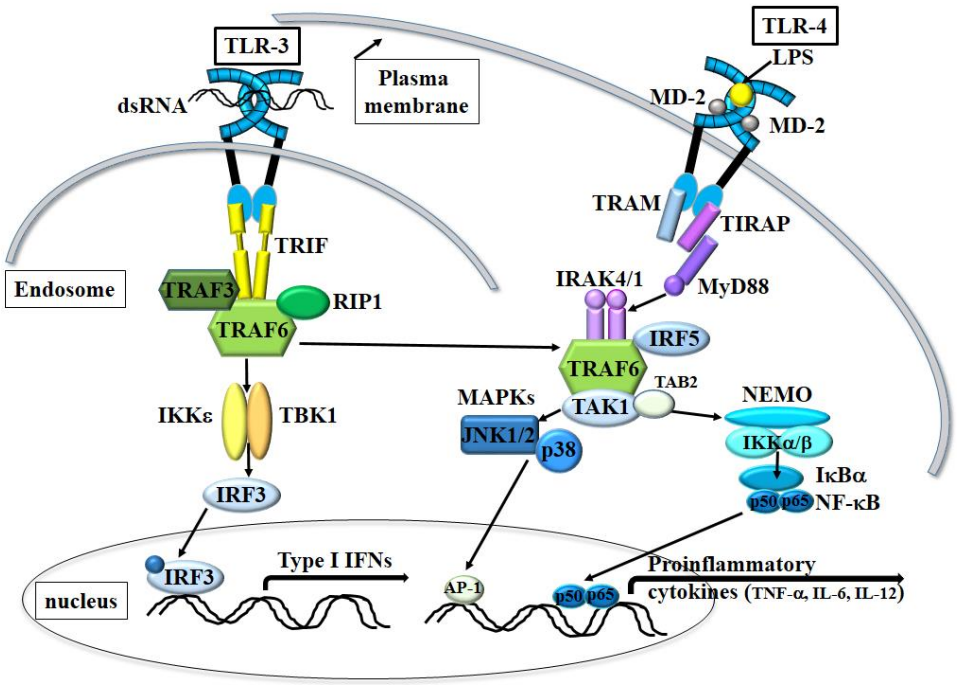

Figure 11: Example of TLR signaling. The intracellular TLR-3 uses the adapter molecule TRIF (in contrast to TLR-4 that, together with its associate protein MD-2, uses the adapter protein combination Myd88/TIRAP or TRIF/TRAM). The signaling via TLR-3/TRIF and the subsequent activation of IRF3 leads to activation of genes encoding for type I IFNs. In addition, signaling through TLR-3/TRAF6 and TLR-4/TRAF6 leads to activation of genes encoding for pro-inflammatory cytokines. *Adapted from Yang et al, Front Physiol. 2012 May 22; 3:138.(134).*

1.11.2.1.2 TLRs in obesity and atherosclerosis

TLRs recognize DAMPs such as FA, necrotic cells and mLDL. Therefore, they are probably able to impact on metabolic pathways in obesity and atherosclerosis, by influencing the inflammatory milieu in the afflicted tissue (WAT, liver, muscle, and artery wall). Thus, the lack of TLR-2 (135-137) or TLR4 (138-140) lead to improved insulin sensitivity and diminished inflammation in WAT, liver, and muscle (141). As shown in **paper IV**, TLR-3 is also involved in regulation of insulin secretion and thereby glucose metabolism. As discussed in section *1.11.2.2.3* macrophages are present in large numbers in obese WAT where they play a crucial role in manipulating the inflammatory milieu via cytokine secretion and cross-talk with other immune cells, specifically T cells. Data suggest that TLR-2 (135) and TLR-4 (142) signaling modulates macrophage infiltration into the WAT and a shift of macrophage phenotype is at least partly mediated by TLR-4 (142). Moreover TLR-2 and -4 are suggested to impact on β cell function and insulin secretion (143-145) in the pancreas.

Altogether these data implicate TLRs in modulation of the inflammatory milieu in WAT and other insulin target tissue together with cells of the innate, but also the adaptive immune system.

Similar to their role in insulin target tissue, TLRs impact also on vascular inflammation (141). Almost all TLRs (1, 2, 3, 4, 5, 7 and 9) are present in the endothelium of the artery (28, 146). A deficiency in TLR-2 (147), TLR-4 or the adapter molecule MyD88 was shown to reduce atherosclerosis (148, 149), and the involvement of TLR-2 (150) and TLR-4 (150, 151) in foam cell formation was suggested.
The role of TLR-3 in atherosclerosis appears to be controversial, as studies have shown both protective (152) and a pro-atherosclerotic (153) roles. In addition studies suggest the involvement of TLR-3 in endothelial dysfunction (154) as well as a role in collagen degradation (155). In addition to the more studied TLRs -2, -3, and -4, TLR-7 was described to have a protective role in atherosclerosis, as depletion of this receptor leads to increased lipid deposition and macrophage infiltration resulting in enlargement of the core region (132, 156).
To summarize, TLRs play a crucial role in obesity associated diseases as well as in atherosclerosis. It is challenging to unravel the impact of TLR-expression and function on these complex diseases as they bind numerous ligands from different sources and have a complex signaling net leading to an abundant expression of cytokines.

1.11.2.2 Cells of the innate immune system and their role in WAT and atherosclerosis

1.11.2.2.1 Neutrophils in WAT and atherosclerosis

Together with eosinophils and basophils, neutrophils belong to the granulocytes, a cell type with special shaped nuclei and cytoplasmic granules.
Neutrophils are one of the first cells at sites of infection or inflammation. Besides their phagocytic function and their ability to recruit more macrophages to the site of inflammation, they function as effector cells initiating the adaptive immune response (157-159).
The infiltration of neutrophils into WAT and their interaction with adipocytes was demonstrated to occur shortly after high-fat diet-induced obesity in a mouse model (160). Furthermore, neutrophils may aggravate metabolic dysregulation in obesity, indicated by

impaired insulin signaling in liver and increased insulin resistance in hepatocytes stimulated with the neutrophil protease elastase. The deletion of neutrophil elastase in a mouse model lead to an improved glucose response and increased insulin sensitivity (48). Likewise, neutrophils may play a pro-atherosclerotic role in early lesion development (161, 162). The infiltration of neutrophils was confirmed in human plaques (163-165).

1.11.2.2.2 Monocytes and macrophages

Monocytes are predominantly circulating in the blood stream, until they migrate into the tissue where they differentiate into macrophages (summarized from (131)).

Macrophages belong to the phagocytic cells and their role is first and foremost to eliminate microorganisms by engulfing and killing them. Equally important, macrophages are crucial scavenger cells (clearance of dead cells and debris) and a source of pro-inflammatory cytokines which attract other immune cells to the inflamed tissue. Besides scavenger receptors and TLRs, macrophages express other PRRs such as mannose-, complement-, and lipid receptor, and dectin-1 (β-glucan receptor) enabling them to detect PAMPs on pathogens. Macrophages do not always succeed in killing pathogens directly. In these cases, macrophages present peptides from the pathogen on MHC molecules to cells of the adaptive immunity - T cells, and thereby serving as a bridge between innate and adaptive immunity. Two signals from the Th1 are necessary to activate the macrophage to fully perform its antimicrobial properties. One signal derives from the secreted cytokine IFNγ and the other one derives from the binding of the CD40 ligand on the Th1 cell to CD40 on the macrophage. The activation of the macrophage leads to increased expression of CD40 and the TNF receptor. The latter serves as a target for autocrine stimulation via TNFα. The activation of macrophages eventually leads to increased anti-microbial properties by induction of nitric oxide (NO) and superoxide (O^-) production, increased expression of co-stimulatory molecule B7 and MHC class II molecules which further promote CD4 T cell activation.

Many studies have described different subsets of macrophages and the fact that macrophages adapt very fast to their microenvironment makes it difficult to characterize and group them. Aside from the classical M1/M2-model (Table 3) other sub-types of macrophages are discussed, such as lymphocyte AG 6C (LY6C)^{hi} monocytes, LY6C^{low} monocytes, and Mox macrophages (166). LY6C^{hi} and LY6C^{low} monocytes are suggested to be precursor forms of M1 and M2 macrophages respectively (Table 3) (166). Another

macrophage phenotype was proposed to be induced by oxidized phospholipid treatment (167).

Table 3: Characterization of M1 and M2 macrophages and their suggested role in WAT and atherosclerosis.

	M1	M2
Activation by	Classical by LPS or other TLR ligands	Alternative by IL-4, IL-13
Secretion/production of	IL-1β, IL-12, TNFα, NO^- synthases, NO,	Anti-inflammatory cytokines (e.g. IL-10, IL-1 receptor)
Transcription factor	NFκB, AP-1, HIF1α	KLF4, PPAR-γ, STAT6
Receptor	MHC class II molecules, co-stimulatory molecules CD80 & CD86	CD163, mannose receptor 1, FIZZ1
Suggested role in inflammation	Pro-inflammatory	Anti-inflammatory
Suggested role in WAT	Promote insulin resistance through TNFα and IFNγ secretion	Maintain insulin sensitivity through IL-4 and IL-10 secretion
	Increased type of M1 macrophages in obese WAT	Predominantly M2 in lean WAT
Suggested role in atherosclerosis	Enriched in progressing plaques	Enriched in regressing plaques
		Promotion of tissue repair by arginase 1 & collagen expression/secretion

Adapted from *Moore et al, Nature reviews Immunology. 2013 (166)* and *Tateya et al, Frontiers in endocrinology. 2013* (168).

1.11.2.2.3 Macrophages in WAT

A positive correlation of macrophages in WAT with increasing body weight was first demonstrated by *Xu et al* (169) and then *Weisberg et al* (6). Furthermore, the infiltration of macrophages into the WAT was linked to increased adipocyte death (170). This seems particularly prominent in the early morphologic changes of WAT that occur due to increased obesity. The infiltrating macrophages are localized preferably around dead adipocytes, forming crown-like structures (171). As summarized in table 3, macrophages in WAT can have pro- and anti-inflammatory properties depending on the nutritional status (obese vs. lean WAT) and cytokine released from other cell types. In contrast to

M2 macrophages, M1 macrophages (residing predominantly in obese WAT) are suggested to promote insulin resistance (168). In concert with other immune cells and their release of inflammatory mediators these macrophages modulate WAT environment and impact on systemic metabolic disturbances (Figure 15).

T cells of the adaptive immune system are also present in WAT inflammation. While T cells were reported to attract macrophages into developing obese WAT (9, 172), a direct crosstalk between these two cell types in obese WAT was recently revealed by *Morris et al*., demonstrating that macrophages in obese WAT function as APCs regulating $CD4^+$ T cell proliferation (173). The crosstalk between AG-presenting macrophages and $CD4^+$ T cells in high-fat diet-induced obese WAT is suggested to be mediated by enhanced expression of MHCII and co-stimulatory molecules (CD40 and CD80) on $F4/80^+CD11b^+$ macrophages. Blocking MHCII signaling resulted in a reduced number of $CD4^+$ T cells in WAT (173).

1.11.2.2.4 Macrophages in atherosclerosis

As described in section *1.4*, initiation of inflammation in the artery by binding of LDL to the endothelial layer in the intima of the vessel and the subsequent activation causes an inflammatory response which eventually leads to migration of inflammatory cells into the vessel. Monocytes are among the first immune cells that migrate and differentiate to macrophages (174). These macrophages are able to take up oxLDL in the forming lesion via scavenger receptors, leading to intracellular accumulation of cholesterol in a process called foam cell formation. The increased expansion of foam cells eventually leads to cell death and accumulation of apoptotic bodies and cell debris. Together with released lipids, these structures form the necrotic core of the plaque (28, 166). The process of lesion formation by increased macrophage accumulation was suggested to be reversible (175), possibly after cholesterol efflux to HDL (176), which is believed to have anti-atherogenic properties (177). Pro-inflammatory characteristics of macrophages are promoted by the ability of cholesterol crystals (178, 179) to activate the NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome in the cell. The NLRP3 activation is possibly assisted by the PRR CD36, which can deliver LDL-derived cholesterol for crystal formation, in this way potentiating the activation of NLRP3 in the macrophage (180). The activation of NLRP3 leads secretion of IL-1β and increased inflammation in the atherosclerotic plaque.

1.11.2.2.5 Dendritic cells and their role in WAT and atherosclerosis

By engulfing and presenting AGs to T cells, DCs are an important bridge between innate and adaptive immunity. DCs develop from $CD34^+$ hematopoietic stem cells in the bone marrow or from monocytes in response to granulocyte-macrophage colony-stimulating factor (GM-CSF) and cytokines such as IL-4, IFNα, TNFα, or IL-15 (181). They can be subdivided into conventional **myeloid DCs** (mDC) and **plasmacytoid DCs** (pDC). These cells circle in peripheral tissue (mDCs) or in the blood (pDCs) where they recognize and take up AGs. AG-presentation to T cells takes place in lymphoid tissue (131). **Follicular DCs** are specialized as they are restricted to lymphoid follicles where they present AGs to B cells (182).

Increased accumulation of DCs were observed in obese WAT (183, 184) and a positive correlation of DCs with BMI, homeostatic model assessment of insulin resistance (HOMA-IR), and Th17 cell was found (183). However, the role of DCs in obesity-induced inflammation is not clear yet.

The development of atherosclerosis is accompanied by increased numbers of DCs preferably located in the shoulder region of the plaque (185) and these are most numerous in vulnerable plaques (186, 187) in close contact to T cells (186, 188). DCs in the artery may originate from circulating monocytes, migrating into the vessel and differentiating under GM-CSF influence to DCs. Deletion of GM-CSF in an atherosclerotic mouse model leads to reduced T cell number, decreased DC numbers and reduced plaque size (189, 190). Treatment with tolerogenic apoB-100-loaded DCs attenuates atherosclerosis in mice (191).

1.11.3 The adaptive immune system

1.11.3.1 General

The innate immune system acts very rapidly by recognizing foreign pathogens or structures, eliminating pathogens, and/or recruiting more immune cells to the infection site. Two types of immune response can be initiated when the innate immune system fails to eliminate pathogens: I) humoral immunity and II) cellular immunity (131).

1.11.3.2 Humoral immunity

Humoral immunity is driven by antibody (Ab) producing B cells. After binding of an Ag to the B cell receptor (BCR), these cells proliferate and differentiate into plasma B cells

that secrete Abs. Five main Ab-isoforms (Immunoglobulin (Ig)M, IgD, IgG, IgA, IgE) and several sub-types exist, each of them with different functions and distribution. Abs neutralize, opsonize (mark pathogen for ingestion by phagocytes), or activate the complement system and by that render the pathogen harmless, prevent pathogen from entering the tissue, or make it visible for phagocytes (131).

1.11.3.3 Cell mediated immunity

The cell mediated immunity is carried out by T cells. Naïve T cells that have not yet met an Ag, travel in the circulation and in peripheral lymphoid tissue where they eventually meet "their" Ag presented by DCs.

Lymphoid tissues include spleen, lymph node (LN), mucosal tissue, thymus, and bone marrow. Presentation and activation of naïve T cells in lymphoid tissue leads to proliferation and differentiation into effector T cells. Ags are presented on MHC molecules: MHCI binds cytosolic peptides such as fragments of viral proteins; MHCII binds peptides from intracellular vesicles. The activation of T cells requires the interaction of T cell receptor (TCR) with the peptide-MHC complex and a co-stimulatory signal from the APC. When the latter is missing the T cell will become anergic (inactive). Two hallmarks of the adaptive immune system are the specificity and the ability to memorize, causing fast and effective response to known pathogens.

As response to a known pathogen, effector memory T cells transform into IFNγ, IL-4 and IL-5 secreting cells that express β_1 and β_2 integrins, facilitating entry into inflamed tissue. In contrast to effector memory T cells, central memory T cells express C-C chemokine receptor (CCR) type 7, which enables recirculation into lymphoid tissue (131). Several effector responses are triggered upon activation of T cells such as cytotoxicity, production of inflammatory and immune regulatory cytokines, and the help to initiate Ab production.

1.11.3.3.1 Cells facilitating the cell mediated immunity

Cells of the adaptive immune system include T and B cells (summarized from (131)). Although both lymphocytes develop from hematopoietic cells in the bone marrow, T cells travel to the thymus where they undergo several maturation steps. The T cell maturation includes the assembly of the TCR, the positive and negative selection of T

cells in the thymus and activation and proliferation of T cells in the periphery. The components of the TCR complex are shown in Figure 12.

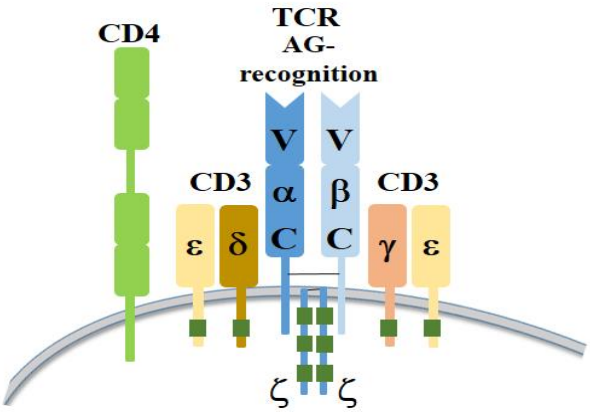

Figure 12: TCR complex consisting of TCR with the α and β transmembrane glycoprotein composed of the variable (V) Ag recognition region and the constant (C) region. CD3, including the δ, γ, and the two ε chains, and a co-stimulatory molecule (here CD4) complete the TCR complex with its associated ζ chains and the immune receptor tyrosine-based activation motif (ITAMS, green) which facilitate the signaling into the cell. Modified from *Murphy's Janeway's Immunobiology 2012* (131)

T cells undergo several development steps. Eventually the T cell maturation in the thymus results in $CD3^+CD4^+CD8^-$ and $CD3^+CD4^-CD8^+$ T cells that travel into the periphery where they can differentiate into several subpopulations (Figure 13).

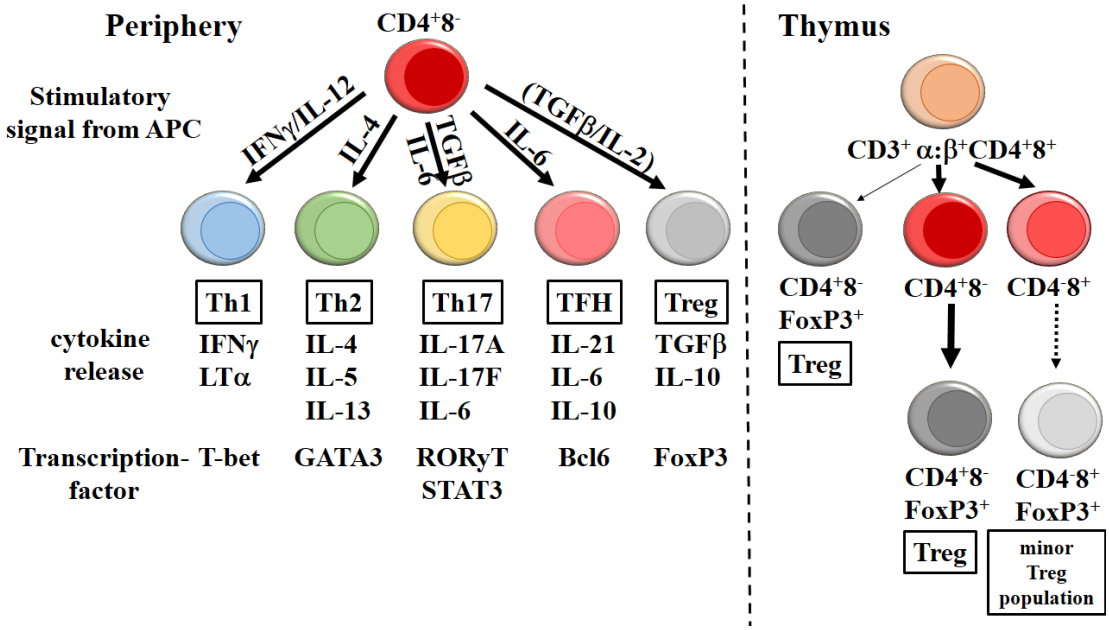

Figure 13: Differentiation of effector T cells maturation in the periphery and thymus. Adapted from *Murphy's Janeway's Immunobiology 2012, Feuerer et al Nat Immunol. 2009* (192) and *Benoist et al Cold Spring Harb Perspect Biol. 2012* (193)

1.11.3.3.2 CD4 T cells

$CD3^+CD4^+$ T cells become activated upon peptide:MHCII binding, mediated by APC. The binding of peptide:MHCII to the TCR, the co-stimulation of CD4 and the release of cytokines from the APC leads to differentiation of T cell subtypes (Figure 13).

Treg cells

The crucial function of Treg cells is the maintenance of tolerance, prevention of autoimmunity, and the regulation of inflammation. Overall these cells impact on the immune homeostasis and function mainly as suppressor cells (131, 194). Tregs are a heterogeneous group of T cells and the picture given here might rather be simplified as the characterization and classification of Treg cells by different markers and the functionality of different subtypes is still controversial. However, there seems to be a consensus about the expression of the transcription factor forkhead box P3 (FoxP3) that distinguish these cells from other regulatory T cells, such as Tr1 and Th3 regulatory T cells. These cells execute their (suppressive) regulatory function mainly via cytokines such as IL-10 and TGFβ respectively. There are two main $FoxP3^+$ Tregs *in vivo;* (I) Tregs originated from the thymus and (II) peripheral Tregs. It is suggested that Tregs in the thymus develop directly from double positive (DP) precursor ($CD3^+CD4^+CD8^+$) T cells and partly from single positive T cells (Figure 13). However, the main population is $CD4^+$, whereas $CD8^+$ Tregs are rare (192). Additionally, Tregs can be induced in the periphery from naïve $CD4^+$ T cells. However, there are difficulties to distinguish Tregs that have been induced in the periphery from Tregs that originate from the thymus, migrate into the tissue and proliferate there in response to an Ag. It is suggested that Treg cells develop in two steps: (I) $FoxP3^-CD25^{high}$ and (II) the influence of IL-2 leads to the conversion into $FoxP3^{high}CD25^{high}$ Treg cells (195).

The development of $CD4^+$ T cells into Treg is suggested to be dependent on TCR:MHC interactions and a co-stimulatory signal from CD28 (193, 196-198). Tregs facilitate their effector function directly, for example by killing Ag-carrying DCs, or indirectly, mediated by inhibitory cytokines (IL-10, TGFβ, and IL-35) or by cAMP- or adenosine-mediated inhibition. Target cells of Tregs include DCs, natural killer (NK)-, B and T cells (193). Helios, GARP and neurophilin-1 are suggested markers to distinguish Tregs originated from the thymus or from the periphery (194). However, the study results are controversial. Helios was a proposed marker of thymus originated Tregs (199), however

its expression was also found in peripheral Tregs (193, 200). Taken together, initially described as $CD4^+CD25^+$ Tregs (201), the majority of studies suggest FoxP3 as an additional marker for Tregs. The expression of additional markers is dependent on the origin of the Treg and the environment (exposure to cytokines and contact to other immune cells).

NKT cells

NKT cells are leukocytes that combine characteristics from both natural killer cells and T lymphocytes (202, 203). In contrast to MHC I and MHC II–restricted T cells, NKT cells recognize lipids. Two main types of NKT cells can be distinguished based on their TCRs. Both express the αβTCR, however, type I NKT cells express the invariant TCR chain (Vα14-Vα18 [mouse], Vα24-Vα18 [human]) while type II NKT cells are a diverse population and they have a TCR repertoire that varies in their Vα chains. The focus here will be on type I NKT cells, as **paper III** is based on this type. Invariant NKT (iNKT) cells develop in the thymus from DP T cells (204-206) following four developing steps. Based on the surface molecules iNKT cells develop through stage (0) with $CD24^+CD44^-NK1.1^-$, stage (1) with $CD24^-CD44^-NK1.1^-$, stage (2) with $CD24^-CD44^+NK1.1^-$, and stage (3) with $CD24^-CD44^+NK1.1^+$ iNKT cells. The final expression of the major transcription factor promyelocytic leukaemia zinc finger (PLZF)(207, 208) is a result of a selection process that requires a strong TCR signal (209), followed by the elevated expression of early growth response protein (EGR) 1 and 2 (203, 210).

In contrast to conventional T cells, iNKT cells are activated by lipids presented by the transmembrane molecule CD1d. The Ag-binding site consists of two main helices (α1 and α2) that form a so-called pocket in which the lipid is embedded and presented to iNKT cells (203, 211). Lipid-Ag can be ceramide-based glycolipids or glycerol-based lipids, which can be self-Ags or origin from microorganism. One of the most used lipids in experimental iNKT cell research is α-Galactosylceramide (α-GalCer) or KRN7000 (212). Several lipids are thought to activate iNKT cells, but the exact mechanism behind the activation is not clear yet. Plasmalogen lysophosphatidylethanolamine (plasmalogen lysoPE) is one of the suggested self-Ags potentially important for iNKT cell development that activate iNKT cells in humans, but not in mice (213, 214). On the other hand, some of the lipids (e.g., β-D-glucopyranosylceramide (βGlcCer)) do not activate iNKT cells if the APC has not previously been stimulated by TLRs (215).

Similar to the control of Treg activation, the activation of iNKT cells is tightly controlled. The activation of iNKT cells requires always two signals; (I) from the interaction with CD1d:lipid and TCR and (II) a cytokine signal (e.g. IL-12, IL-18, IL-23,IL-25) (Figure 14). Eventually the activation of iNKT cells leads to secretion of large amounts of cytokines and the activation of other effector T cells; depending on the secreted cytokines Th1 or Th2 T cells (203). iNKT cells are localized in vast amounts in the liver (20-30%) (216), spleen, but also bone marrow, skin, lung, lymph nodes (203). In these tissues, iNKTs interact with other cells of the innate and the adaptive immune system via cytokines and/or direct cell:cell interaction. Figure 14 adapted from Brennan *et al* summarized these interactions.

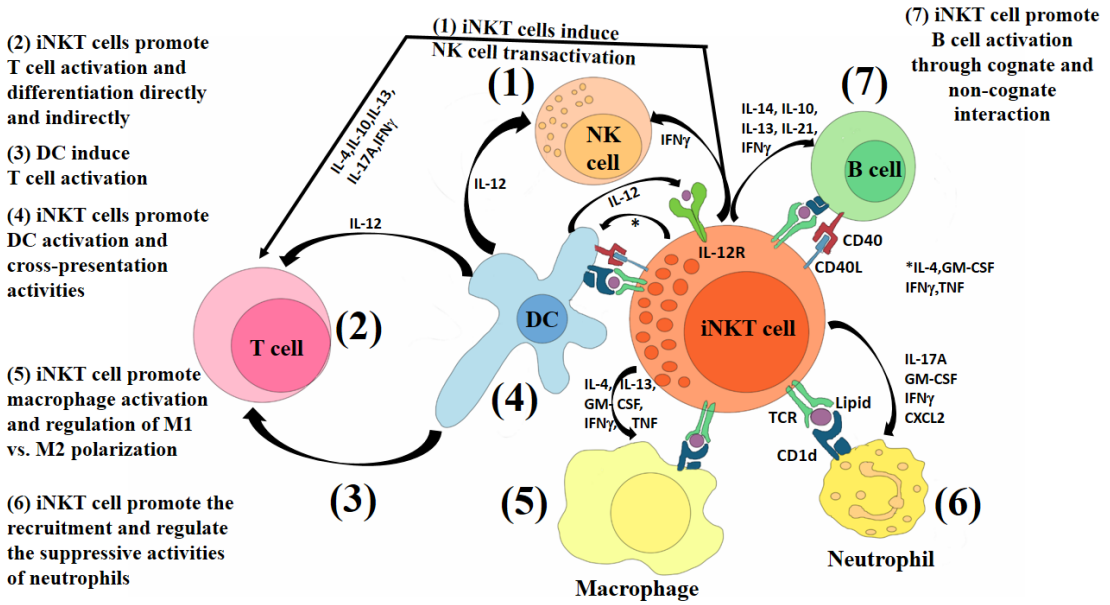

Figure 14: Interaction of iNKT cells with other cells of the immune system. Adapted from *Brennan et al Nat Rev Immunol. 2013* (203)

1.11.3.3.3 CD8 T cells

$CD8^+$ T cells develop parallel to $CD4^+$ T cells from DP cells. These cells recognize peptides presented on MHCI molecules. CD8 T cells are also called cytotoxic T lymphocytes (CTL) as they contain vesicles with cytotoxic granules (e.g., granzyme and perforin) that can be released upon activation. The activation of CD8 T cells is dependent on a strong signal that may come from other CD4 T cells that recognize peptides presented by MHCII. The activation eventually triggers the polarization of CD8 T cells

to facilitate the release of cytotoxic proteins, which induce apoptosis of the target cell. Furthermore, cytokines released from CD8 T cells (e.g., IFNγ, TNFα, LTα) are able to induce cytokine-mediated apoptosis (131).

1.11.3.4 Cells of the adaptive immune system and their role in WAT

Beside cells of the innate immune system, WAT harbors an orchestra of powerful adaptive immune cells (T and B cells) that have been shown to contribute to metabolic changes in obesity. Several studies, including ours, have highlighted the role of T cells in WAT and described the main T cell sub-types in lean as well as obese WAT (8-13, 15, 46). The picture arising from these studies is complex and not yet complete.

However, strong data show that lean WAT is characterized by infiltration of Th2 T cells and FoxP3$^+$ Treg cells in contrast to obese WAT in which pro-inflammatory Th1 and CD8$^+$ T cells are predominant (10-12) (Figure 15). In line with the mouse data, analysis of human WAT confirm the shift from 6:1 (Th1:Treg) in lean to 12:1 in obese WAT (11). Feuerer *et al* demonstrated that up to 50% of the CD3$^+$CD4$^+$ T cells express FoxP3$^+$ (12). Furthermore, Tregs could be induced in WAT. The induction of Tregs was accompanied by reduced inflammation and an ameliorated metabolic phenotype, indicated by lower blood glucose, hepatic fat accumulation, and reduced cholesterol (217). Together the secretion of anti-inflammatory and suppressive (IL-4, IL-13, IL-10) cytokines from Treg as well as Th2 cells contribute to maintaining the anti-inflammatory milieu in lean WAT. (168).

The expansion of WAT during the cause of obesity leads to a change from anti- to pro-inflammatory milieu. Th1, CD8$^+$ T cells, and cells of the innate immune system, are the predominant contributors to the so-called low-grade inflammation. Macrophages have been detected in WAT before T cells were described there (6, 7, 169). However, previous studies suggest that CD8$^+$ T cells in particular infiltrate first into obese WAT and recruit macrophages (9, 10). The recruitment and the polarization of macrophages towards the M1 phenotype contribute to ameliorated inflammation in obese WAT. T cells and macrophages have been shown to interact directly via MHCII molecules. Furthermore, co-stimulatory molecules such as CD40 and CD80 on macrophages, stimulate T cell proliferation, and IFNγ production (173). Eventually, expanding adipocytes, T cells, macrophages, but also mast cells and B cells contribute to the maintenance of WAT inflammation.

iNKT cells are also proposed to modulate inflammation in WAT. However, data are controversial and will be discussed separately in the "Discussion" section of this thesis. TNFα, IL-6, MCP-1, IL-1β, and IFNγ are the prominent cytokines in obese WAT. They have been shown to participate in the development of metabolic deregulation such as insulin resistance. TNFα (218, 219), secreted by macrophages and adipocytes, impact on adipocyte metabolism, glucose and FA metabolism and hormone signaling in WAT (220-222). IFNγ secreted by Th1 cells (8), but also from macrophages and adipocytes, promote M1 macrophage polarization and the release of pro-inflammatory cytokines (IL-6 and TNFα) by the latter (223).

In summary, studies suggest that lean WAT is characterized by Th2 cells, resident M2 macrophages, Tregs and cytokines (e.g., IL-10, IL-4, IL-13), that contribute to the anti-inflammatory environment. Obesity is associated with systemic changes in the gut (microbiota) (224, 225), the liver (226), and other organs leading to an increased pro-inflammatory status eventually affecting WAT homeostasis (42). *Deng et al* recently proposed a model by which excessive nutrients accompanied by hyperglycemia and increased FFA lead to modified cytokine release from the adipocytes (227). Furthermore they show that adipocytes express increased levels of MHCII shortly after high-fat diet induction, leading to activation, further recruitment, differentiation and proliferation of T cells into WAT (227). In line with Kintscher *et al* demonstrating the infiltration of T cells before the onset of macrophage infiltration (9), $CD8^+$ T cells have been shown to recruit macrophages into WAT (10). The direct crosstalk between macrophages and T cells via MHCII (173), but also the increased infiltration of other immune cells (neutrophils (160), mast cells (47), B cells (16)) and their secretion of inflammatory mediators contribute substantially to the inflamed status of obese WAT (227).

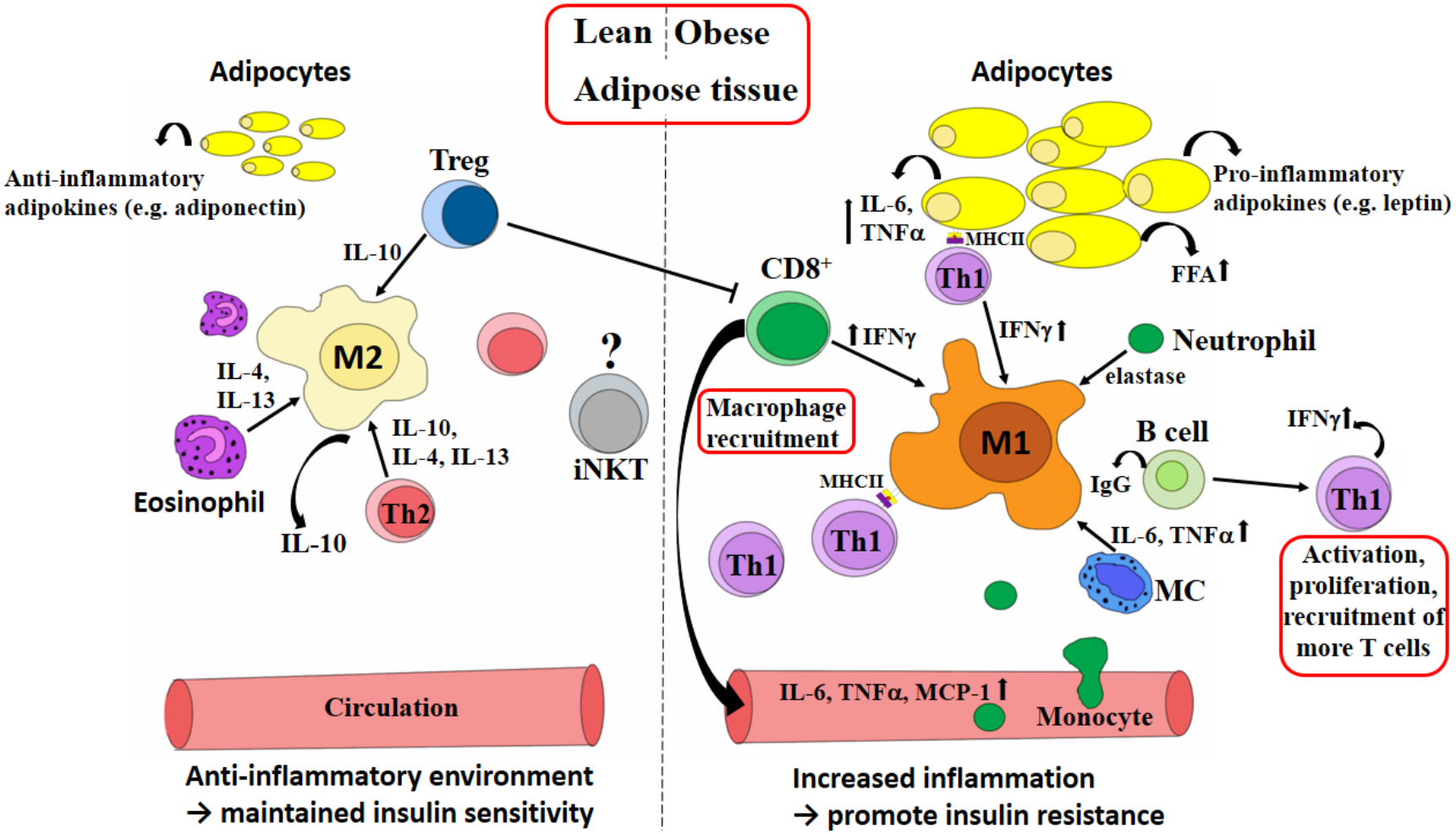

Figure 15: Changes of the cellular composition in lean vs. obese WAT. Modified from *Cildir G. et al, Trends Mol Med. 2013* (228)

1.11.3.5 Cells of the adaptive immune system and their role in atherosclerosis

B cells are found in the adventitial layer (229) rather than in the plaque, in contrast to T cells which actively infiltrate into the growing plaque, firstly demonstrated by *Jonasson and Hansson* (130, 230, 231). In particular the role of B cells in atherosclerosis development seem to be less clear, as some studies suggest a protective role of B cells (232, 233) in lesion development and others describe a pro-atherogenic role of B cells (234, 235). As with T cells, B cells consist of several sub-types and it is suggested that the different outcomes of these studies are due to the different B cell subtypes which have been studied (236). The picture for T cells in lesion development is more clearly compared to B cells. Comparable to inflammation in obese WAT, Th1 T cells are suggested to exacerbate the inflammatory milieu in atherosclerotic plaque. Amongst others, by secreting cytokines such as IFNγ (28) which was shown to impact on smooth muscle cell differentiation, MHCII expression, and activation of macrophages with ensuing production of pro-inflammatory cytokines (28, 237, 238). Targeting Th1 $CD4^+$ T cells in experimental models stress their pro-atherogenic role (239-241). Likewise, NKT cells have been suggested to mediate inflammation in the plaque leading to accelerated lesions (242, 243). On the other hand, the anti-atherogenic properties of $FoxP3^+$ Tregs and Tr1 have been shown by several studies, including ours (244-246). The role of Th2 cells or their secreted cytokines respectively is less clear and controversial discussed as data show pro-atherogenic (247, 248) as well as protective properties (28, 249).

2 AIMS

The studies included in this thesis aimed to investigate the role of molecular mediators in metabolic syndrome and atherosclerosis.

More specifically the aim of the studies was to:

- Investigate the effect of the combination of immune inflammation and hyperlipidemia on adipose inflammation and insulin resistance (**paper I)**
- Define the role of FoxP3-expressing Tregs in atherosclerosis. (**paper II**)
- Examine the impact of iNKT cells on glucose and lipid metabolism in liver and WAT. (**paper III**)
- Examine the function of TLR-3 in glucose and lipid metabolism. (**paper IV**)

3 RESULTS AND DISCUSSION

3.1 T CELLS INFILTRATE WAT. HOWEVER, IMMUNE INFLAMMATION AND HYPERLIPIDEMIA DOES NOT *PER SE* LEAD TO INSULIN RESISTANCE. (PAPER I)

With the recognition of macrophage infiltration into WAT and the secretion of pro-inflammatory cytokines from immune cells and adipocytes, it was appreciated that obesity is associated with a chronic low-grade inflammatory condition in WAT (2). First, data demonstrated the infiltration of T cells into obese WAT, however, their role was not clear (13, 250). Data suggested an increased prevalence of metabolic syndrome during inflammatory chronic diseases, such as psoriasis (251), Crohn's disease (252), and rheumatoid arthritis (253) independently of any obesity. Therefore lean but inflamed *Apoe*$^{-/-}$xCD4dnTbR mice were used to dissect the role of inflammation alone in WAT metabolism.

3.1.1 Mouse model used in paper I

The *Apoe*$^{-/-}$ mouse is a commonly model used in atherosclerosis research. ApoE mediates the uptake of VLDL and chylomicron from the liver. Lack of ApoE therefore leads to accumulation of VLDL and chylomicron particles in the circulation (254). This causes spontaneous atherosclerosis in mice.

The CD4dnTbR mouse carries a dominant-negative TGFβ receptor II construct under the CD4 promoter, leading to loss of TGFβ dependent inhibition of T cell activation and, as a consequence, aggravated T cell-dependent inflammation (255).

The *Apoe*$^{-/-}$xCD4dnTbR mouse combines aggravated inflammation, hyperlipidemia, and atherosclerosis.

The o*b/ob* mouse has a mutation in the gene *Lep**ob*** leading to leptin deficiency. These mice are characterized by increased obesity accompanied by type 2 diabetes symptoms including hyperglycaemia, glucose intolerance, and elevated insulin levels (256).

C57BL/6 is a widely used inbred strain and serves as a control group in **paper I, III,** and **IV**. C57BL/6 mice are susceptible to diet-induced obesity and therefore commonly used in studies investigating obesity related symptoms (**paper III** and **IV**).

We speculated that the combination of immune inflammation and hyperlipidemia may cause adipose inflammation that leads to insulin resistance.

3.1.2 CD4+ T cells infiltrate WAT

In line with previous data, we showed an infiltration of macrophages and T cells into WAT. T cells were mostly of the $CD4^+$ sub-type and an increased infiltration was seen into WAT of hyper-inflamed but lean *Apoe*$^{-/-}$xCD4dnTbR. Interestingly, $CD4^+$ T cells were also detected in WAT of obese diabetes prone *ob/ob* mice (Figure 16). $CD4^+$ T cells formed aggregates suggesting a clonal activation of T cells. The increased inflammation was reflected in elevated levels of CD3, TNFα, MCP-1 and IFNγ both in the lean *Apoe*$^{-/-}$ xCD4dnTbR and the obese *ob/ob* mouse (Figure 17).

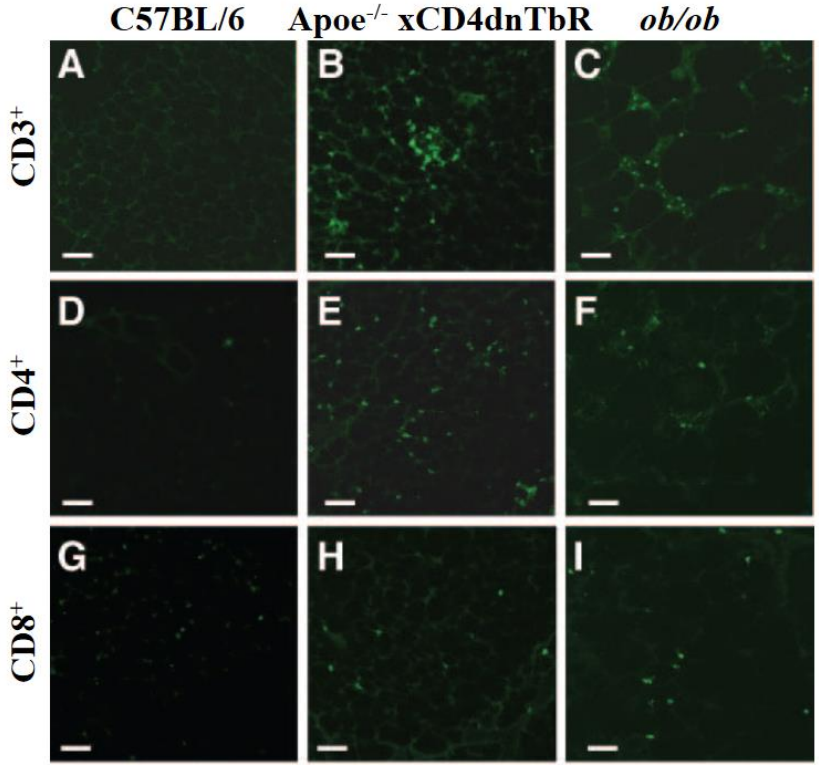

Figure 16: T cell infiltration into WAT of C57BL/6 (left), *Apoe*$^{-/-}$ xCD4dnTbR (middle), *ob/ob* (right) mice. Scale bars = 20 µm. Representative micrographs from 1 of 6 experiments.

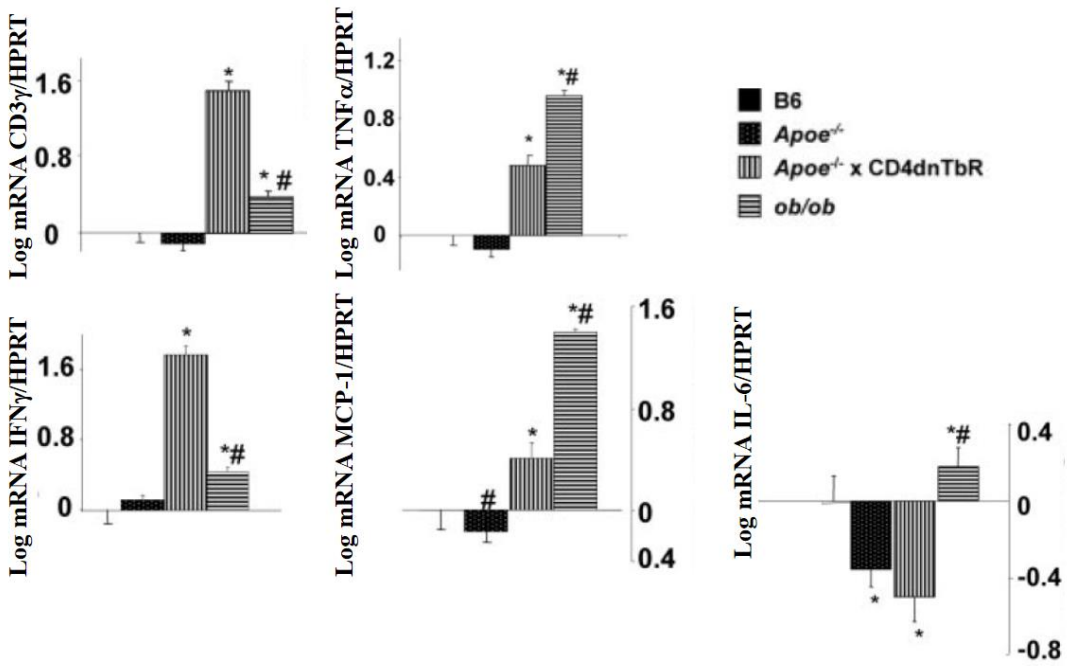

Figure 17: Inflammatory gene expression in WAT of different mouse strains. Data show relative mRNA levels measured by real-time RT-PCR of total WAT RNA. Data represent means ±SEM (n = 6 mice per group). *P < 0.05 vs B6 (C57BL/6), #P < 0.05 vs *Apoe*$^{-/-}$xCD4dnTbR.

3.1.3 Hyper-inflammation and hyperlipidemia was accompanied by lack of IL-6, but not associated with impaired insulin signaling

IL-6 is a cytokine that has diverse effects on insulin sensitivity depending on the tissue and the model used. Insulin signaling was hampered in IL-6-treated 3T3-L1 adipocytes (257). Likewise, chronic exposure to IL-6 causes hepatic insulin resistance (258) and fat cells from insulin resistant subjects express increased levels of IL-6 (257). In contrast, lack of IL-6 was associated with obesity (259) and local production of IL-6 was suggested to delay the onset of diabetes (260).

We demonstrate that inflamed WAT in obese and lean mice differ in their IL-6 expression (Figure 17). Lean hyper-inflamed *Apoe*$^{-/-}$xCD4dnTbR mice have significantly reduced IL-6 mRNA in contrast to obese WAT from *ob/ob* mice.

We analyzed the effect of IL-6 treatment in hyper-inflamed *Apoe*$^{-/-}$ xCD4dnTbR mice with regard to insulin sensitivity. Adiponectin levels are inversely correlated with the degree of insulin resistance (261). Indeed, *Apoe*$^{-/-}$ xCD4dnTbR treated with IL-6 secreted significantly lower adiponectin from WAT, mirrored by decreased serum adiponectin. Furthermore, treatment with IL-6 caused decreased insulin-dependent lipogenesis in WAT. Taken together, treatment with recombinant IL-6 caused changes in adipokine production and lipogenesis in WAT compatible with reduced insulin sensitivity in

inflamed *Apoe*$^{-/-}$ xCD4dnTbR mice. However, we observed no significant effects on AKT and ERK1/2 phosphorylation in WAT.

In summary, **paper I** shows that inflammation of WAT can occur even in the absence of obesity. T cell driven WAT inflammation as well as that caused by obesity is characterized by increased macrophage and T cell infiltration and expression of pro-inflammatory cytokines. Interestingly, IL-6 expression differ in the 2 forms of WAT.

Using female mice to study WAT insulin sensitivity might be a limitation of this study. As summarized by Shi *et al* (262), there is a sex difference in terms of fat distribution and insulin sensitivity. In contrast to men, premenopausal women have more subcutaneous WAT, which is known to be less inflamed. Furthermore, sex hormones such as estrogen, influence body weight homeostasis and insulin sensitivity. The changes of estrogen levels with the menopause in women are associated with increased visceral obesity and increased insulin resistance. Likewise, female mice display an increased insulin sensitivity compared to male mice (262).

Although the decreased level of IL-6 in *Apoe*$^{-/-}$ xCD4dnTbR mice might contribute to the improved insulin sensitivity, we cannot rule out that hormonal changes are, at least partly, involved.

Secondly, although *Apoe*$^{-/-}$ mice have been used to study the effect of insulin (263, 264) and C-peptide (98) on the development of atherosclerosis, *Apoe*$^{-/-}$ xCD4dnTbR mice display an altered lipid profile and a distinctive inflammatory phenotype. Thus, it represents an extreme case of metabolic dysregulation and immune activation.

To get closer to the human pathophysiology of metabolic disturbances and to avoid confounding results by altered hormone status, we used mostly C57BL/6 male mice if not otherwise stated for **paper III** and **IV**.

3.2 INKT CELLS MEDIATE METABOLIC INTERACTION BETWEEN LIVER AND WAT (PAPER III)

The finding of infiltrating T cells in WAT drove us to investigate the impact of T cell subtypes on lipid and glucose metabolism. iNKT cells combine characteristics from both natural killer cells and T lymphocytes. In contrast to MHC I and MHC II–restricted T cells, they recognize lipids presented by CD1d. Because of their unique characteristic ability to recognize lipids, the effect of iNKT cells on lipid and glucose metabolism is of interest in a diet-induced obese model.

3.2.1 Mouse model used in paper III

In **paper III** we used C57BL/6 wild-type mice and a mouse model that has a non-functional Vα14Jα18 T cell receptor gene and therefore lacks iNKT cells. In the following text I will refer to them as *Jα18*$^{-/-}$ mice.

In contrast to CD4^{+} and CD8^{+} T cells, hardly any iNKT cells could be detected in WAT. As expected, a large population of such cells was identified in the liver, but not in WAT, of wild-type mice. Again, these cells were not present in *Jα18*$^{-/-}$ mice (Figure 18).

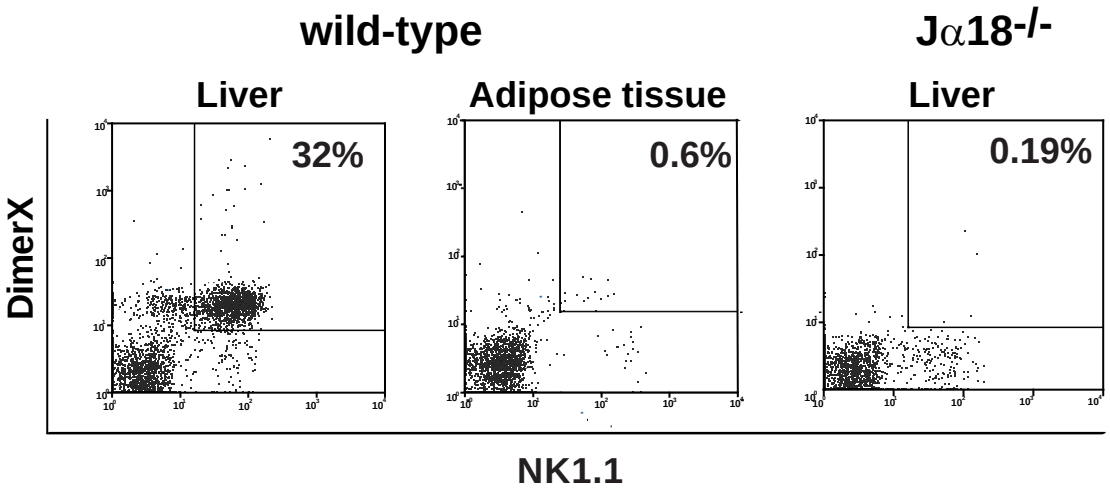

Figure 18: Most iNKT cells are present in the liver. Flow cytometric analysis of iNKT cells in liver and WAT from wild-type (left and middle) and *Jα18*$^{-/-}$ mice (right) fed high-fat diet (HFD). Cells in the gate are defined as percentage of single, live CD19^{-}, CD3^{+}, CD4^{+}, DimerX^{+}, NK1.1^{+}lymphocytes.

In contrast to other studies (265-267), we do not observe a significant infiltration of iNKT cells in WAT. This discrepancy might reflect differences in models or experimental protocols. iNKT cells in **paper III** are characterized as percentage of single, live, CD19^{-}, CD3^{+}, CD4^{+}, DimerX^{+}, NK1.1^{+} lymphocytes. To further confirm the data obtained by multicolor flow cytometry, we analyzed the level of Vα14 mRNA in WAT. Indeed, the Ct value for Vα14 mRNA in wild-type mice was on the detection limit (Ct ~37), confirming the low number of iNKT cells in WAT of wild-type mice.

Although iNKT cells in WAT might not be present in large numbers, they reside in the liver. The results of **paper III** clearly show that lack of iNKT cells does influence WAT homeostasis, indicated by decreased adipocyte volume and increased lipogenesis. The latter is likely counterbalanced by increased hormone-sensitive lipase (HSL) and LPL mRNA and increased lipolysis (Figure 19).

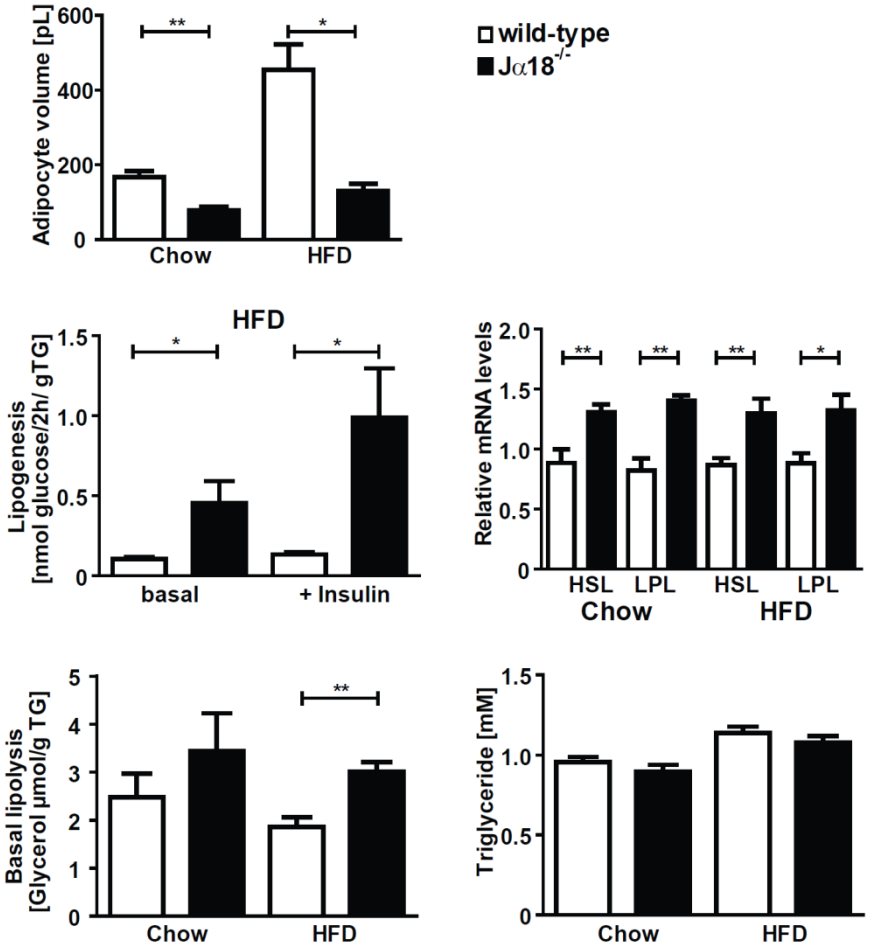

Figure 19: Altered lipid metabolism in WAT of mice lacking iNKT cells indicated by smaller adipocytes, increased lipogenesis, HSL- and LPL mRNA level, and increased basal lipolysis. Overall no changes of circulating TG were observed. Means ± SEM. *P<0.05, **P<0.01

Although the exact mechanism by which liver-residing iNKT cells regulate WAT homeostasis is not clear yet, it is tempting to speculate that changes observed are mediated by cytokines. Indeed, it has been shown that LPL and HSL expression and activity can be regulated by TNFα and IFNγ (268-271). TNFα was shown to inhibit the activity of LPL and HSL in adipocytes *in vitro* (269). TNFα was shown to regulate the binding of proteins in the promoter region of LPL gene (270). Additionally, IFNγ, a cytokine secreted by numerous immune cells including iNKT cells and macrophages, was shown to inhibit LPL activity (271). Indeed in **paper III** we show a significant decrease of TNFα mRNA in WAT and liver, and reduced IFNγ mRNA in liver of *Jα18*$^{-/-}$ mice. Furthermore, the composition of immune cells in liver and WAT was altered in mice lacking iNKT cells. In fact, flow cytometry analysis displayed a marked decrease of infiltrated CD4^{+} T cells and F4/80^{+} macrophages in WAT and liver. The

changes in cell composition possibly contribute to the decreased IFNγ and TNFα mRNA in *Jα18*$^{-/-}$ mice. Thus, decreased IFNγ and TNFα mRNA level might lead to reduced inhibition of HSL and LPL in *Jα18*$^{-/-}$ mice. HSL is also the key enzyme initiating adipocyte lipolysis (272, 273) and we could demonstrate that the increased HSL expression in *Jα18*$^{-/-}$ mice is accompanied by increased (basal) lipolysis.

We speculated that the increased lipolysis potentially promotes release of FFA that is counterbalanced by improved lipogenesis, resulting in plasma TG concentrations that are similar to that of wild-type mice. However, that was not the case in *Jα18*$^{-/-}$ mice, which had decreased rather than increased FFA levels. We can only speculate that this might be a consequence of enhanced catabolic processes that use circulating FFA. Furthermore increased insulin sensitivity in *Jα18*$^{-/-}$ mice, as suggested by reduced HOMA-IR, might also contribute to this finding, as it was shown that insulin may influence FA clearance (274).

In summary, using the appropriate mouse model (*Jα18*$^{-/-}$ mice), that specifically lack iNKT cells, we demonstrated a strong metabolic interaction between liver and WAT. We show that depleting iNKT cells, which largely reside in the liver, modulate WAT homeostasis and lipid metabolism.

3.3 TLR-3 INFLUENCES GLUCOSE HOMEOSTASIS VIA β CELL INSULIN SECRETION AND INFLUENCES VLDL-TG BIOSYNTHESIS (PAPER IV)

In **paper I-III** we analyzed the impact of adaptive immune cells on obesity related dysfunction and atherosclerotic lesion development, respectively. We demonstrated an infiltration of T cells into WAT and investigated the impact of iNKT cells on WAT homeostasis. Certainly, T cells can be activated directly by APCs, which mostly belong to the innate immune system. TLRs, as part of the innate immune system, recognize endogenous molecules, such as certain lipids and inflammatory mediators that are elevated during tissue stress and cell death in chronic inflammatory diseases (275). Certain TLRs have been implicated in glucose metabolism and type 2 diabetes. Both TLR-2 and TLR-4 deficient mice are protected against diet-induced obesity and insulin resistance (135-137, 145, 276, 277). TLR-3, which recognizes viral dsRNA and mRNA from dying cells, has very high expression levels in the pancreas (278), possibly suggesting a regulatory function in metabolism.

3.3.1 Mouse model used in paper IV

Tlr3$^{-/-}$ mice

We used a diet-induced obesity model deficient in TLR-3 to investigate the function of this receptor in metabolic disturbances. *Tlr3*$^{-/-}$ mice were fully backcrossed (N14) onto C57BL/6. The latter served as a wild-type control.

3.3.2 TLR-3 impacts on glucose metabolism by influencing insulin secretion, possibly via Gck and VAMP-2 expression

Our data demonstrate an enhanced glycaemic control upon glucose challenge in *Tlr3*$^{-/-}$ mice (Figure 20 A), which may be attributed to increased circulating levels of insulin. This, in turn, was caused by amplified insulin secretion from β cells in *Tlr3*$^{-/-}$ mice (Figure 20 B), accelerating the response to glucose. Increased insulin secretion was demonstrated both *in vivo* (hyperglycaemic clamp) and *in vitro* (perifusion of islets) (Figure 20 B and C). We suggest that this was partly due to improved glucose metabolism locally in pancreatic β cells, indicated by the increase of the rate limiting enzyme for glycolysis Gck. Furthermore we provide evidence that this is also caused by changes in the secretory machinery with an increased expression of the v-SNARE protein VAMP-2 in *Tlr3*$^{-/-}$ islets.

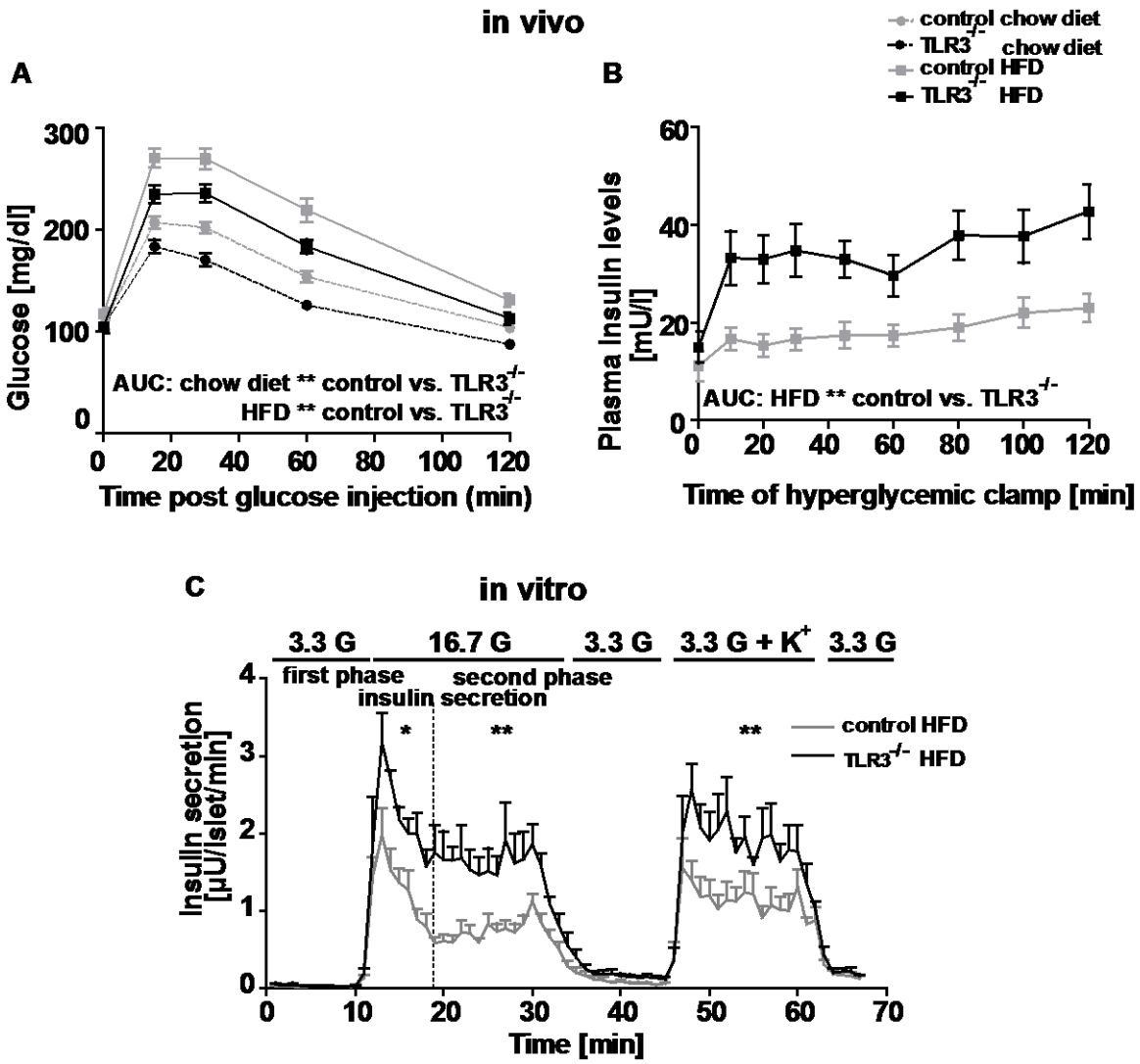

Figure 20: Lack of TLR-3 improves response to glucose metabolism and increases insulin secretion. (A) ipGTT was performed by injecting glucose (1g/kg; i.p.) and glucose concentration was analyzed in blood from the tail vein. (B) Hyperglycaemic clamps were performed on mice fed a HFD and insulin was measured by ELISA. (C) Islets from control and *Tlr3*$^{-/-}$ fed HFD were perifused with 3.3 mmol/l glucose in HFD-fed mice, followed by 16.7 mM glucose and 20 mmol/l (K^+). Samples of the perifused islets were collected every minute, and levels of secreted insulin were analyzed by RIA. AUC was calculated for first and second phase of insulin secretion after glucose stimulation (indicated by dashed line), and after K^+ stimulation. Mean values from 5 mice per group are shown. Means ± SEM. *P<0.05, **P<0.01

3.3.3 Absence of TLR-3 signaling leads to reduced VLDL-TG caused by reduced VLDL biosynthesis

In **paper IV** we demonstrate that lack of TLR-3 leads to changes in lipid metabolism, indicated by reduced circulating TG levels. Analysis of the lipid profile revealed a reduction in VLDL-TG (Figure 21 A and B). Indeed, blocking of LPL-activity in the liver caused a significant reduction of newly synthesized TG in *Tlr3*$^{-/-}$ mice, suggesting a potential role of TLR-3 in TG-biosynthesis from the liver (Figure 21 C).

Sortilin-1 is involved both in regulating VLDL secretion and in re-uptake of VLDL particles (65, 66). We speculate therefore that changes in sortilin-1 expression may account for at least some of the effects on VLDL observed in our study (**paper IV**, Supplemental figure 3).

Interestingly, TLR-3 impacts on insulin secretion, even under physiological conditions, as demonstrated by the increased insulin secretion in islets from chow diet fed *Tlr3*$^{-/-}$ mice. The endogenous TLR-3 ligand that mediates the effects observed in our study, in the absence of viral infection or exogenous stimulation, is unknown. TLR-3 is activated by mRNA from damaged cells, but we could not detect any cell death in the islets. Interestingly, pancreatic tissue contains and secretes large amounts of RNases; thus, it would be reasonable that a control system that involves TLR-3, an innate receptor for RNA, could detect and respond to abnormalities in this system, even in the absence of obesity.

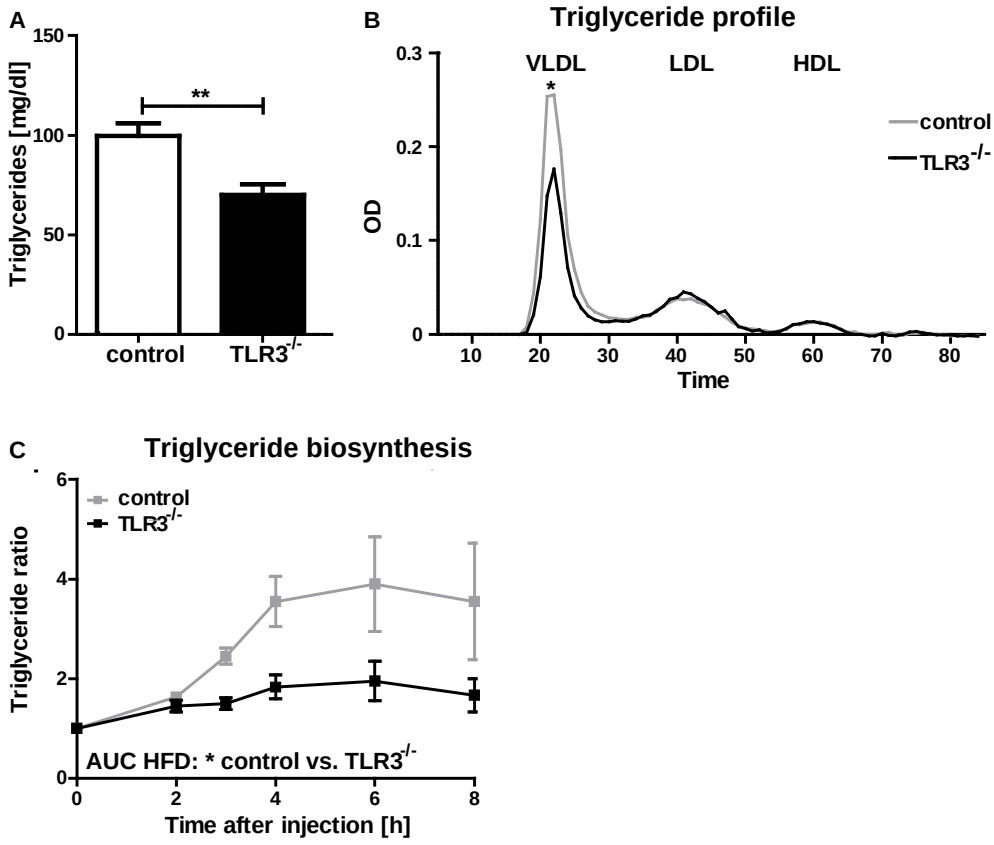

Figure 21: *Tlr3*$^{-/-}$ mice have reduced TG in VLDL due to decreased TG-biosynthesis. (A) Control and *Tlr3*$^{-/-}$ mice fed HFD were analyzed for plasma levels of TG. Size analysis of lipoprotein profile was performed on plasma and the concentration of (B) TG was measured in each fraction and plotted against retention fraction number. 2-3 plasma samples were pooled per genotype and a total of 5 pools per group were analyzed. Mean profiles are shown. AUC was calculated for each fraction of VLDL, LDL, and HDL. (C) Newly synthesized VLDL-TG was assessed in HFD-fed mice by injecting Tyloxapol (i.v.). Graph is shown as TG ratio normalized to baseline TG. N = 6 each genotype. Means ± SEM. *P<0.05, **P<0.01.

3.4 FOXP3$^+$ REGULATORY T CELLS INFLUENCE LIPID METABOLISM AND ATHEROGENESIS (PAPER II)

As demonstrated in **paper I, III** and **IV** immune cells and their receptors impact on obesity related metabolic disturbances. In **paper II** we investigated the role of FoxP3$^+$ Tregs on atherosclerosis. Previous studies suggested that Treg cells inhibit atherosclerosis. However, the mechanisms behind it were not clear. Tregs are a heterogeneous group of T cells. In **paper II**, they were described as CD3$^+$CD4$^+$ FoxP3$^+$ T cells.

3.4.1 Mouse model used in paper II

To study the effect of Tregs in atherosclerosis, we used:

The DEREG (depletion of regulatory T cells) mouse model expresses a fusion protein of the human diphtheria toxin (DT) receptor (hDTR) and enhanced green fluorescent protein (eGFP) under control of the FoxP3 promoter. Therefore, administration of DT enables selective depletion of eGFP$^+$ FoxP3$^+$ T cells. Wild-type mice do not express the hDTR receptor and are thus insensitive to DT.

Ldlr$^{-/-}$ mice lack the receptor for LDL leading to impaired uptake of this lipoprotein particle (LDL) from the circulation. When put on a diet rich in fat and cholesterol these mice exhibit a disturbed lipid profile with high circulating cholesterol promoting atherosclerosis lesion development.

In order to study the role of Tregs in atherosclerosis, we used a bone marrow chimeric model. This involves irradiation of recipient mice followed by transplantation of bone marrow derived cells from donor mice. The transplantation scheme is shown in Figure 22.

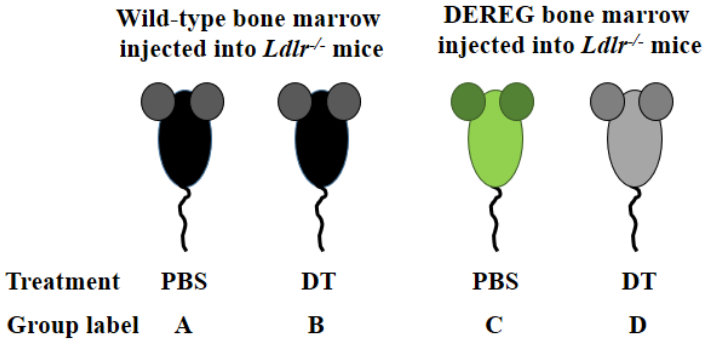

Figure 22: Groups of mice used in paper II.
Group A: Wild-type bone marrow injected into *Ldlr*$^{-/-}$ mice treated for 8 weeks with PBS.
Group B: Wild-type bone marrow injected into *Ldlr*$^{-/-}$ mice treated for 8 weeks with DT.
Group C: DEREG bone marrow injected into *Ldlr*$^{-/-}$ mice treated for 8 weeks with PBS.
Group D: DEREG bone marrow injected into *Ldlr*$^{-/-}$ mice treated for 8 weeks with DT.

eGFP^{+} FoxP3^{+} T cells were depleted after 4 weeks DT treatment in DEREG x *Ldlr*$^{-/-}$ mice. This treatment became less effective over time as indicated by the increased percentage of eGFP^{+} FoxP3^{+} T cells after 8 weeks treatment.

Due to a significant population of eGFP^{-} FoxP3^{+} cells in DT treated DEREG x *Ldlr*$^{-/-}$ mice, the total number of FoxP3^{+} Tregs did not differ between DT and PBS treated mice. Previous studies have shown that Tregs are less sensitive to irradiation and may survive or partly recover from the radiation protocol (279). Indeed, eGFP^{-} FoxP3^{+} cells derived from *Ldlr*$^{-/-}$ recipient mice, demonstrated by transplantation of DEREG bone marrow from CD45.2^{+} mice into irradiated CD45.1^{+} *Ldlr*$^{-/-}$ mice. The performed proliferation assay together with the data from Baru *et al* (280) suggest that eGFP^{-} FoxP3^{+} cells are rather not fully functional and thus, might not contribute to the phenotype in DEREG x *Ldlr*$^{-/-}$ mice.

3.4.2 Tregs are involved inhibition of atherosclerosis in chimeric *DEREGxLdlr*$^{-/-}$ mice

Treatment of DEREG *x Ldlr*$^{-/-}$ mice with DT led to increased atherosclerotic lesion size (Figure 23). In contrast, DT-treatment of wild-type x *Ldlr*$^{-/-}$ mice did not change lesion size, indicating, that DT alone does not account for the increased lesion size in DEREG *x Ldlr*$^{-/-}$ mice. The abrogation of FoxP3^{+} Tregs causes increased atherosclerotic lesion size. However, **paper II** provides the first direct evidence for athero-protective properties of FoxP3^{+} Tregs and is in line with previous work showing increased atherosclerotic lesion in mice with reduced Treg activity (244, 281).

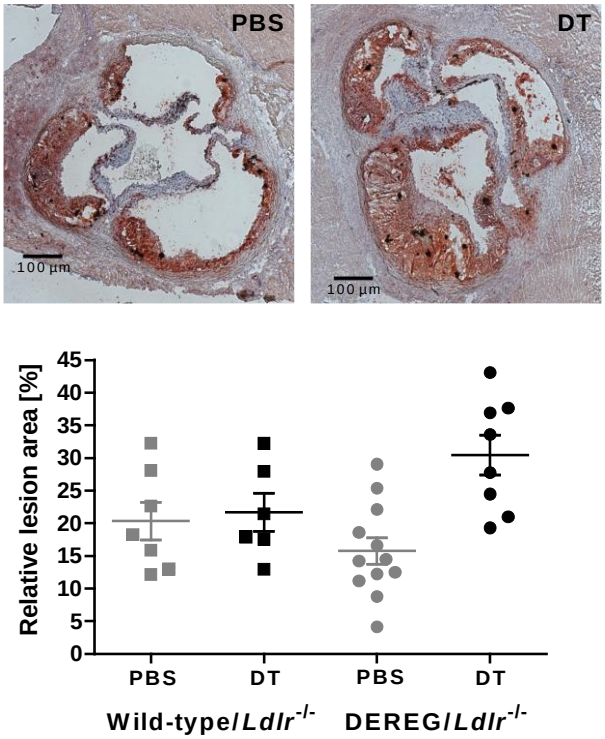

Figure 23: Depletion of transgenic Tregs aggravates atherosclerosis.
(upper figure) Representative photomicrographs showing Oil Red O- and H&E-stained sections from the proximal aorta of chimeric DEREG *x Ldlr*$^{-/-}$ mice treated for 8 weeks with PBS or DT. (lower figure) Relative lesion area (lesion area/area inside external elastic lamina × 100) calculated from 4 sections per mouse (300–600 μm) for wild-type/*Ldlr*$^{-/-}$ and DEREG *x Ldlr*$^{-/-}$ mice treated for 8 weeks with PBS or DT, respectively. *P < 0.05; **P < 0.01. Scale bars: 100 μm.

3.4.3 FoxP3^{+} Tregs regulate lipoprotein metabolism

As shown in **paper III** T cell subsets modulate the inflammatory milieu and also impact on lipid metabolism. In **paper II** we demonstrate that depletion of FoxP3^{+} Tregs leads to dramatic changes in lipoprotein metabolism, indicated by increased cholesterol levels (Figure 24). The changes in cholesterol occur mainly in the VLDL fraction. The increase in cholesterol levels occur early (4 weeks) after treatment and correlates positively with lesion size. The increased circulating cholesterol level could either be due to reduced VLDL-biosynthesis, as we show in *Tlr3*$^{-/-}$ mice (**paper IV**), or due to impaired clearance.

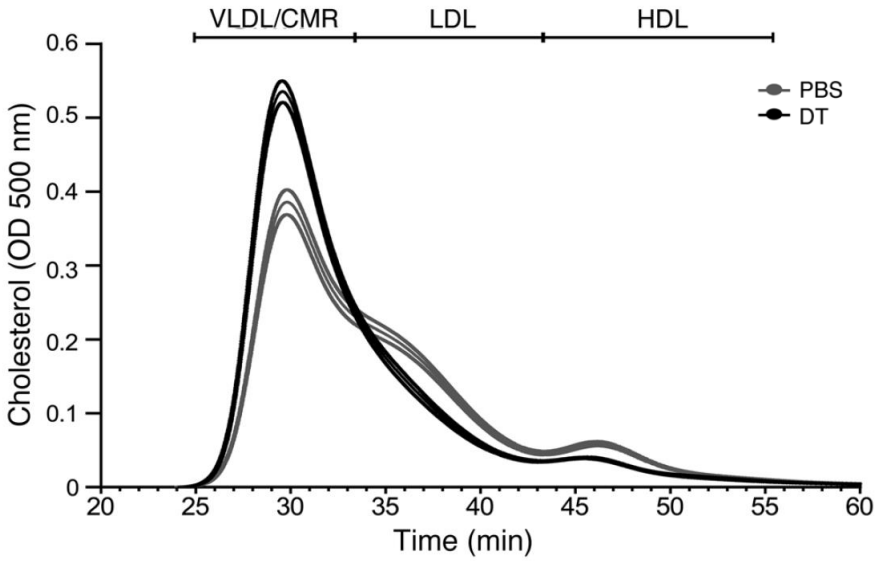

Figure 24: DT-induced depletion of transgenic Tregs promotes hyper-cholesterolemia. (B) Fast protein liquid chromatographic (FPLC) analysis of plasma lipoprotein profiles from DEREG *x Ldlr*$^{-/-}$ mice treated for 8 weeks with PBS (gray line) or DT (black line). The cholesterol concentration in each fraction (y axis) is plotted against retention time (x axis), with the corresponding lipoprotein fractions (identified by human plasma standards) indicated at the top. Mean (thick line) and SEM (fine lines) are shown; n = 5 per group.

VLDL catabolism was assessed by injecting FITC-labeled VLDL particles. Plasma analysis revealed a delayed clearance of VLDL/chylomicron particles in DT treated DEREG *x Ldlr*$^{-/-}$ mice. A more detailed analysis demonstrated a delayed clearance of chylomicrons in DT treated DEREG *x Ldlr*$^{-/-}$ mice accompanied by reduced chylomicrons in the liver (Figure 25).

Gene expression analysis displayed increased LPL and hepatic lipase (Lipc) mRNA level, both involved in TG hydrolysis. The increased TG hydrolysis might partly explain the accumulation of cholesterol rich, TG-poor VLDL/CRM particle (Figure 25).

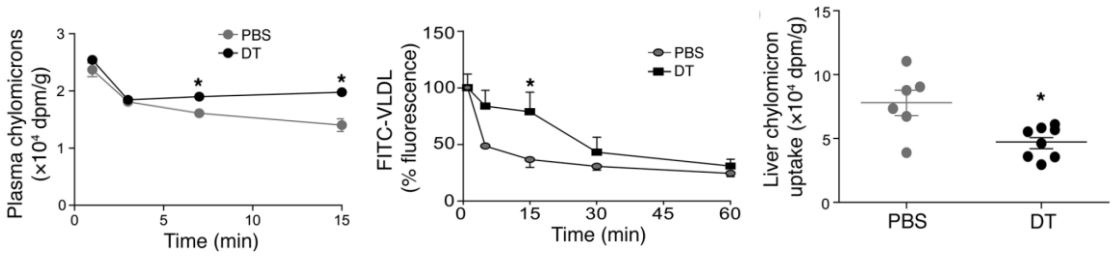

Figure 25: VLDL/CMR lipoprotein catabolism is impaired in Treg-depleted mice. (left) *In vivo* turnover of CM particles injected into chimeric *DEREG/Ldlr*$^{-/-}$ mice treated for 8 weeks with DT or PBS. Data show kinetics of the CM [^{14}C] retinol core particle clearance from blood and are expressed as radiolabeled moieties corrected for weight; n = 5 (PBS); n = 7 (DT). (middle) Clearance of injected FITC-VLDL in chimeric *DEREG/Ldlr*$^{-/-}$ mice treated for 8 weeks with DT or PBS. FITC-derived fluorescence was analyzed in plasma samples at the indicated time points. Data for each individual were normalized to the fluorescence of plasma taken 1 minute after injection; n = 4 per group. (right) CM [^{14}C] retinol uptake in the liver. Data expressed as radiolabeled moieties corrected for weight; n = 5 (PBS); n = 7 (DT). *P < 0.05.

The impaired clearance of VLDL and chylomicrons was likely mediated by reduced sortilin-1 in the liver, in combination with increased plasma enzyme activity of LPL, hepatic lipase, and PLTP.

Previous work has shown that Tregs, characterized as CD4$^+$CD25$^+$, inhibit lesion development (244). Treatment with anti-CD25 led to increased lesion size (244). CD25 was long considered as a marker for Tregs. However, it is expressed on several immune cells including B cells and recently activated T effector cells. Therefore, treatment with anti-CD25 could possibly target other cells than Tregs and cause unwanted side-effects. To avoid confounding results, we used a mouse model in which FoxP3$^+$ Tregs can specifically be depleted. However, there is a possibility that the increase of atherosclerotic lesion size is partly due to the changes in lipid metabolism. In contrast to the expected increase in inflammation in lesion, we did not see changes of macrophage, DC, or CD3$^+$ T cell numbers in the lesion. Therefore, changes in lipid metabolism may be more important for Treg effects on atherosclerosis than effects modulating vascular inflammation, at least in this model.

4 CONCLUDING REMARKS

Almost everybody has someone in the family or in the circle of friends that has CVD and/or is continuously fighting against overweight or obesity.

When working with obesity and atherosclerosis you will, at one point, be confronted with questions like: *Do we have any good medication against obesity?* The answer would spontaneously be: *Yes, the answer is simple! Just eat less, and be more active!* Obviously it is not that simple! As I described in the introduction, food is available, at least in the industrialized countries, at every corner every time. The food that leads to health problems is high in sugar and fat and it is usually cheaper than the healthy alternative. This is a problem especially in low income countries, where obesity is increasing enormously. In addition we live a sedentary lifestyle and industry designs campaigns to sell food and sweets, especially targeted for children.

But it is more complicated than that! As I wrote in the introduction, insulin influences numerous processes in the body. Among others, it impacts on the brain where it mediates hormonal signals and hunger signals. In very obese people these signals might be deranged. But also behavioral eating patterns are difficult to break!

The projects in my thesis did not aim to find a way to treat obesity in the first place. The results from my thesis rather contribute to understanding processes in organs that are important for metabolism like liver, WAT, and pancreas. We demonstrate that immune cells do infiltrate into WAT (**paper I**). The increased inflammation in obese WAT may also lead to systemic changes, triggered, among others, by cytokine release and increased FFA release. We further show the impact of liver-residing inflammatory iNKT cells on lipid metabolism, controlling metabolic processes distally in WAT (**paper III**). We also demonstrated the impact of the innate receptor TLR-3 on insulin secretion and lipid metabolism (**paper IV**). These basic findings contribute to the understanding of metabolic regulations, and might be useful in the future when discovering new therapeutic approaches.

Obesity is a risk factor for atherosclerosis. Atherosclerotic lesions develop silently without symptoms over years. But this can change dramatically when a plaque raptures. This might lead to thrombotic occlusion of the artery and obstructing the blood flow. Depending on the area of the clot this might lead to stroke, MI, or other life-threatening events. It is accepted that changes in lipid metabolism and inflammation contribute to lesion development. Efforts are made to find treatment to prevent, stop or slow down lesion development. Different strategies are conceivable including immune-modulation

and even vaccination. The project in my thesis that displays the impact of FoxP3 Tregs on lipid metabolism and atherosclerosis (**paper II**) encourages such work.

Altogether, the findings in my thesis are based on *in vitro* and *in vivo* models of obesity and atherosclerosis, diseases that can promote each other's development. We broke down the complex processes to study the involvement of single cell types (iNKT, $FoxP3^{+}$ Tregs), receptors (TLR-3), and cytokines (IL-6). Together these approaches contribute to the understanding of the molecular mechanisms driving these diseases and will hopefully contribute to new therapeutic approaches.

5 ACKNOWLEDGEMENTS

Ta det lugnt, det ordnar sig! (Take it easy, it will be alright!)
A phrase that I have heard a thousand times during my stay in Sweden, but especially while I was writing and preparing my thesis defense. This is probably the most important lesson I have learnt here in Sweden and during my time as PhD student. Having a bit of self-criticism in my luggage and doubt if I will manage what I was dreaming about, I learned that I can accomplish much more than I thought. Finally, here I am! The book is printed and the BIG day is coming soon. Personally I have achieved a big step and learned to be a bit more relaxed. Maybe it is true and everything will be alright in the end ;-)
I met wonderful people who believed in me, who pushed me to my limits, who challenged me, and/or who were just there for me during the last years.

I would like to thank in particular:

Göran Hansson, my supervisor, for accepting me as PhD student in your group, for your enthusiasm for science and your impressive knowledge about music and world politics. Sitting next to you at a table is really inspiring! I appreciate that you gave me the freedom to find my own way to survive in the (sometimes) tough scientific world. Vielen Dank!

Norbert Gerdes, my co-supervisor and a bit more than that, for being there for me from the very first phone call until the last steps of my PhD. Although your time management gave me one or two stressful moments the last years ;-), you were always there for me and my family when it was critical. You tried to teach me to believe in research and, most importantly, in myself. I would like to thank you for your never ending optimism!

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