Dyslipidemia, metabolism and autophagy : antigen-independent modulation of T cells in atherosclerosis

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CHAPTER 7

General discussion and perspectives

BACKGROUND

Atherosclerosis is the main underlying pathology of cardiovascular disease (CVD), which is the most common cause of death worldwide [1]. Atherosclerosis is a lipid-driven autoimmune-like disease in which stenotic lesions develop as a result of local accumulation of native and modified lipoproteins. These lipoproteins elicit a chronic inflammatory response which is largely T cell-mediated [2–5]. Hence, dyslipidemia, in the form of hypercholesterolemia and/or hypertriglyceridemia, is a major risk factor of developing atherosclerosis and subsequent acute cardiovascular events such as a myocardial infarction or stroke. Surgical treatments to restore blood flow after such an ischemic episode include bypass surgery, endarterectomy or percutaneous coronary intervention to circumvent or remove stenotic lesions. However, surgical treatment of perfusion defects to prevent mortality is invasive and often associated with recurrent CVD. Patients at risk of developing CVD, such as familial hypercholesterolemia (FH) patients, or with a history of CVD are often treated with lipid lowering therapies. The current standard of lipid lowering therapy is based on statins which have proven to effectively lower the amount of circulating low density lipoprotein (LDL) cholesterol and thereby reduce the risk of developing CVD [6]. Unfortunately, statins only lower the risk of CVD by 25-30% and patients with a history of acute coronary events have a 20% higher chance of a recurrent cardiovascular event, despite treatment [7]. Novel therapeutic approaches are required to treat atherosclerosis and prevent acute cardiovascular events. Given the contribution of immune cells to the pathophysiology of atherosclerosis [8], experimental research has focused on developing immunomodulatory therapies to treat atherosclerosis. Immunological research in atherosclerosis has shown that dyslipidemia drives T cell-mediated immunity in atherosclerosis by increasing the abundance of antigens derived from native and modified lipoproteins [9]. Long-lasting immunomodulation to treat atherosclerosis through vaccination is successful in mice using peptide sequences from ApoB100, the core-protein of LDL and very low density lipoprotein [10,11]. Thus, the antigen-dependent contribution of dyslipidemia to T cell mediated-autoimmunity in atherosclerosis and the immunomodulatory potential thereof have been explored extensively. The antigen-independent immunomodulatory effects of dyslipidemia on T cells is relatively unexplored but has recently gained more interest [12]. In part, this is due to recent advances in the field of immunometabolism and its link with autophagy which have shown that bioenergetic and biosynthetic processes in T cells are crucial in the different phases that a T cell undergoes during an immune response, including cell growth, clonal expansion, differentiation and migration [13]. Whether lipid-associated metabolic processes in specific subsets of T cells are altered by dyslipidemia and whether these metabolic processes contribute to the pathophysiology of atherosclerosis or can be targeted for anti-atherosclerotic therapies is unknown.

THIS THESIS

In this thesis, we used an experimental approach to identify antigen-independent immunomodulatory effects that diet-induced dyslipidemia can have on the cellular metabolism and autophagy in $CD4^+$ T cells. By using this approach we aimed to identify novel and confirm known pathogenic mechanisms involving cellular metabolism and autophagy in T cell-mediated inflammation during atherosclerosis development (fig. 1). Moreover, we aimed to contribute to knowledge on the immunomodulatory effects of clinically available modulators of cellular metabolism and autophagy and their feasibility to dampen T cell-mediated inflammation and atherosclerosis.
We aimed to elucidate the antigen-independent effect of dyslipidemia on T cells by:

1) conceptualizing how metabolic disease affects T cell metabolism,
2) examining how dyslipidemia-induced metabolic adaptations in Treg cells are linked to their impaired phenotypic integrity in atherosclerosis,
3) examining how dyslipidemia-induced priming of $CD4^+$ naïve T (Tn) cells alters their effector phenotype,
4) examining the link between dyslipidemia and autophagy in T cells,
5) examining the effect of lipocalin-2 (Lcn2) deficiency on different metabolic parameters associated with atherosclerosis which could affect T cell metabolism.

The first study was literature-based and in **chapter 2** we described the main metabolic pathways in T cells, discussed the most important modulators of these metabolic pathways and summarized the role of autophagy in T cells and its connection with cellular metabolism. Also, we described different therapeutic approaches based on T cell metabolism and autophagy to ameliorate diseases hallmarked by both metabolic disorder and T cell-mediated autoimmunity. We postulated different mechanisms through which a metabolic disease-associated aberrant microenvironment can affect T cell-mediated immunity and progress disease. As described in chapter 2, we proposed five ways through which this could occur: 1) through increased substrate abundance, 2) through increases in intracellular substrate reservoirs, 3) through skewing of substrate dependence, which could alter the activity of bifunctional enzymes or, 4) skew differentiation of Tn cells into T helper cells, Treg cells and affect their differentiation into memory T cells and lastly, 5) through selective metabolic restriction.
We discuss the five mechanisms in type 1 diabetes mellitus (DM) and hyperglycemia as an example but these are also applicable to atherosclerosis and dyslipidemia as was illustrated by our results reported in chapter 3 and 4. Below, we contemplate where the possibilities and challenges lie in the modulation of T cell metabolism for immunomodulatory therapy atherosclerosis-induced CVD.

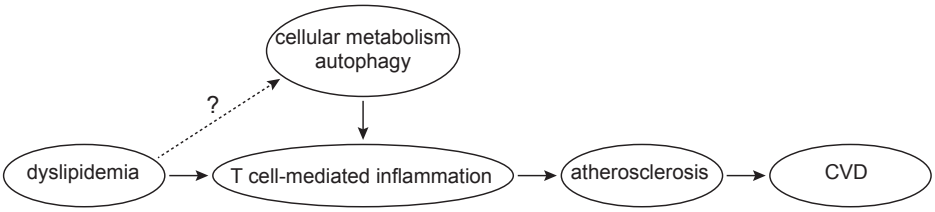

Figure 1 Simplified scheme of the aim of this thesis. Current knowledge of T cell-mediated inflammation in atherosclerosis, and in the development of cardiovascular disease (CVD), is mainly focused on the antigen-dependent effects of dyslipidemia on T cells. Cellular metabolism and autophagy are crucial determining factors in T cell-mediated inflammation. This thesis aimed to examine the antigen-independent effects of dyslipidemia on cellular metabolism and autophagy in T cells (dotted arrow) and how this may affect T cell-mediated inflammation.

One of the translational challenges, which arises from research on cellular metabolism in T cells is the difficulty of targeting T cells in an antigen-independent manner. This can be overcome by applying *ex vivo* cell therapy in which peripheral blood T cells are isolated, treated *ex vivo* to modulate metabolism and then adoptively transferred into the same individual. An *ex vivo* cellular engineering approach is used in T cell-based immunotherapy to successfully treat some forms of cancer [14]. In patients at risk of developing CVD, *ex vivo* modulation of the metabolism of peripheral blood T cells to expand Treg cells may be feasible as their immunosuppressive capacity can be exploited in an antigen-independent manner. *In vivo,* therapeutics which modulate metabolism, such as rapamycin, can be easily and specifically delivered to antigen presenting cells using nanoparticles such as liposomes [15]. Interestingly, antibody-liposome conjugates might be used to target T cells in a similar manner as suggested by a report describing that Burkitt's lymphoma cells can be targeted using anionic liposomes which contain rapamycin and were conjugated to anti-CD19 antibodies [16].

Targeting specific T cells to modulate their cellular metabolism is feasible only when it has been identified which unique membrane proteins correspond to which subset of T cells. In humans this identification has been performed to a certain extent in the Human Immuno Phenotyping project [17]. For example, naïve Treg cells are identified using flow cytometry as $CD3^{+}CD4^{+}CCR4^{+}CD25^{+}CD127^{low}CD45RO^{-}$ cells [17]. Naturally, targeting cells with such a complex 'membrane signature' is difficult and future research is required to assess the feasibility of targeting the metabolism of specific subsets of human T cells *in vivo* using antibodies. In chapter 2, the theoretical usage of drug-cytokine conjugates to deliver specific compounds to T cells with high expression of cytokine receptors (such as CD127) was discussed. A prerequisite for the feasibility of this approach is that drug-cytokine-cytokine receptor complexes are internalized through clathrin-mediated or clathrin-independent endocytosis [18] for the conjugated drug to enter T cells and have the anticipated effect. This approach remains highly speculative but, given the thera-

peutic potential of metabolism modulation in T cells, warrants additional research to the feasibility of antigen-independent T cell targeting.

Another approach to modulate metabolism of specific T cell populations *in vivo* is by targeting the metabolic pathway, which distinguishes the population of interest from other cell types. For example, murine Treg cells and memory T cells have been described to rely more on fatty acid (FA) oxidation fueled oxidative phosphorylation than on glycolysis for their bioenergetic demand as compared to effector T cells [19–21]. Of note, a recent publication has suggested that some of the reported FA oxidation-dependent processes in T cell differentiation were actually caused by off-target effects of the FA oxidation inhibitor etomoxir on mitochondrial complex 1 [22]. T helper (Th) cells have higher membrane expression of glucose transporter 1 (Glut1) than Tn cells and Treg cells [23] suggesting that $CD4^+$ T cell-specific inhibition of Glut1 to inhibit effector function in atherosclerosis predominantly impacts Th cells. Further stratification of the different metabolic signatures of subsets of T cell populations is required to predict the impact of this proposed therapeutic approach. In support of this, even subsets of Treg cells, e.g. thymus-derived and induced Treg cells, have distinct metabolic pathways which function to meet the required bioenergetic and biosynthetic demand [24]. Elaborate characterization of the metabolic signature on a single cell level of atherosclerotic lesion-derived immune cells is required to assess the feasibility of metabolism-based T cell targeting. Naturally, potential side effects of the long term use of systemically administered metabolism modulating compounds, such as rapamycin, need to be considered [25,26]. Nevertheless, research in the field of angiogenesis has proven that at optimal dosage, a small molecule inhibitor of PFKFB3, a glycolytic enzyme, can be used for therapeutic purposes to induce vessel normalization whereas at high dosage the inhibitor causes toxicity [27–29]. This suggests that systemic low dosage administration of metabolism modulators can fine-tune dyslipidemia-induced alterations in the metabolism of T cells for therapeutic purposes with minimal side effects.

Targeting of T cell metabolism for therapeutic purposes is particularly interesting as it can be exploited to modulate the adaptive arm of immunity in diseases of affluence without knowing the disease-associated antigen. Moreover, the problem of antigenic diversity in these diseases of affluence due to varying human leukocyte antigen molecules [30] in the patient population of interest is irrelevant.

An appealing therapeutic approach to inhibit T cell mediated inflammation in atherosclerosis can be based on the modulation of T cell metabolism during vaccination. Vaccination against atherosclerosis with oxidized LDL [31], heat-shock protein 60 [32] and specific ApoB100 peptides [10] reduces atherosclerosis by inducing Treg cells. As both rapamycin and metformin have been shown to expand Treg cells [19,33,34], and are clinically available, short-term treatment with these Treg inducing compounds during vaccination can boost the induction of tolerance. As vaccination can induce immunological memory

and memory Treg cells exert antigen-specific immunosuppressive function upon secondary exposure [35], memory Treg cells represent an interesting subset of Treg cells in the context of vaccination against atherosclerosis. Moreover, rapamycin also induces tolerogenic dendritic cells (DC) which also promote Treg cell expansion [36]. However, Treg cell induction has been shown to depend on mammalian target of rapamycin complex 1 activity [37] which suggests that systemic administration of rapamycin to drive the expansion of Treg cells during vaccination against atherosclerosis could be a double-edged sword. A final consideration in the targeting of T cell metabolism for immunotherapy is the proposedly limited permeability of atherosclerotic lesions, which will cause some systemically administered compounds to affect T cells in the blood, lymphatic vessels and lymphoid organs but not T cells inside the lesions.

Our second approach, as described in **chapter 3**, to study the antigen-independent effects of dyslipidemia on T cell-mediated autoimmunity was based on examining how dyslipidemia-induced metabolic adaptations in Treg cells is linked to their impaired phenotypic integrity in advanced atherosclerosis. We showed that feeding LDL-receptor deficient mice (*Ldlr*$^{-/-}$) a Western-type diet to induce dyslipidemia and advanced atherosclerosis skewed the cellular metabolism of Treg cells to less glycolysis and more FA oxidation. We showed that these diet-induced metabolic alterations in Treg cells were mediated by cholesterol-induced mTORC1 inhibition and the activation of peroxisome proliferator activated receptor delta (PPARδ) by various polyunsaturated FA (PUFA), eicosanoids and eicosanoid-metabolites. Moreover, we showed that Treg cell migration was skewed away from lymph node homing in an mTORC2-dependent manner and that the metabolic phenotype induced by dyslipidemia actually enhanced their capacity to migrate towards sites of inflammation. Despite this effect of dyslipidemia on the metabolic and migratory phenotype of Treg cells, advanced atherosclerotic lesions in humans and mice contain low numbers of Treg cells and is associated with diminished immunosuppression [38]. In mice, the low abundance has been suggested to be caused by impaired migration of Treg cells towards atherosclerotic lesions [39] but our data suggests this is not the case. Dyslipidemia has been linked to Treg cell apoptosis inside atherosclerotic lesions [39,40] and may also induce the loss of FoxP3 expression and differentiation into T helper cell subsets, including Th1 cells [41] and T follicular helper (Tfh) cells inside atherosclerotic lesions [42]. Apparently, the unique microenvironment within atherosclerotic lesions impairs the functional integrity of Treg cells. Part of what makes the microenvironment in atherosclerotic lesions unique is the vast amounts of cholesterol in the form of native LDL and oxLDL particles. Interestingly, intracellular cholesterol content is a decisive factor in the conversion of Treg cells to Tfh cells inside atherosclerotic lesions as the administration of ApoA1, a protein which extracts cholesterol from cells via cholesterol efflux transporters, prevents their conversion inside lesions [42]. Another factor which makes the atherosclerotic microenvironment aberrant from the

blood and lymphoid tissues is that atherosclerotic plaques contain hypoxic regions [43] and macrophage-rich regions which can be low in glucose in humans and mice [44,45]. Sufficient FoxP3 expression is a prerequisite for Treg cells to adapt to a metabolically challenging environment with low-glucose levels [46]. Human carotid atherosclerotic lesions with high-risk histological characteristics have a distinct metabolic profile, which is characterized by increased glycolysis and amino acid utilization, as compared to low-risk lesions [47] suggesting that the local metabolic environment in atherosclerotic lesions is a key factor in the stability of atherosclerosis.

An important question with respect to the data reported in chapter 3 is whether dyslipidemia in humans also induces metabolic and migratory changes in Treg cells and whether the human atherosclerotic lesions impair the functional and phenotypic integrity of Treg cells. LDL-cholesterol levels in CVD patients and FH patients are controlled with lipid-lowering therapies. Consequently, LDL-cholesterol levels >400 mg/dL are rare in the clinic although FH homozygotes can have cholesterol levels of 650-1000 mg/dL when untreated [48]. In fact, dyslipidemia in mice is quite distinct from dyslipidemia in humans, and non-human primates would presumably be a better model to study the reported effects of dyslipidemia on T cells [49]. Compared to dyslipidemic *Ldlr*$^{-/-}$ mice which have total serum cholesterol levels > 1000mg/dL which is mostly present in the VLDL fraction, dyslipidemia in humans is generally much less severe [49]. Therefore, future research in patients with FH or obesity should be performed to identify whether mTORC1 signaling and PPARδ activity is altered human Treg cells as a consequence of dyslipidemia. A confounding factor in future research on this effect of dyslipidemia is that FH patients and (obese) CVD patients are often treated with statins which has metabolic implications for Treg cells [50].

Interestingly, a recent study by Christensen et al. reported that children with FH have elevated levels of circulating PPARδ ligands or their precursors, including omega-6 FA, omage-3 FA, docosahexaenoic acid (DHA), independent of statin use [51]. In the same study, statin use in FH children was associated with lower amounts of PUFAs which can act as PPARδ ligands, as compared to non-statin treated FH children. This suggests that dyslipidemia in FH patients can lead to PPARδ activation in Treg cells and alter their metabolic phenotype and that statins can counteract this. Certain PCSK9 inhibitors may be preferred over statins to control dyslipidemia in FH children as the PCSK9 inhibitor RG7652 reduced serum cholesterol without affecting the circulating levels of inflammation-related eicosanoids [52]. Based on our research in chapter 3, dietary intake of specific PUFAs may increase the serum abundance of PPARδ ligands which can have beneficial effects on atherosclerosis and CVD as they promote the migratory capacity of Treg cells. However, it is important to realize that certain PUFAs can act as ligands for other PPARs and can be metabolized to render them ineffective as PPARδ ligands. Nevertheless, clinical observations in the past have suggested that high levels of n-3 PUFA (such as

docosahexaenoic acid) in blood, for example by eating fish regularly, reduce the risk of coronary artery disease [53,54]. A large study following 44,895 men without CVD showed however that simply increasing the intake of n-3 polyunsaturated FAs by increasing the weekly intake of fish from 1-2 servings to 5-6 serving does not substantially protect against CVD [55].

Other studies have tried to unravel whether the increased intake of specific PUFA, instead of a dietary regime which prescribes a specific type of food, can reduce the risk of CVD. A recent study showed that 15-18 months of dietary supplementation with n-3 PUFA (docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA)) did not decrease the progression of carotid-intima-media thickness [56]. Another study showed that daily intake of 1.86 grams of EPA and 1.5 grams of DHA significantly reduces the progression of fibrous coronary plaques in patients with a low-intensity statin treatment [57]. In line, serum abundance of EPA and DHA is negatively correlated with atherosclerotic plaque progression in DM patients which had undergone percutaneous coronary intervention of vessels with non-culprit lesions [58]. Other studies have shown that dietary supplementation of PUFA can have immunomodulatory effects in humans [59,60], as well as in mice [61]. In our study, many known PPARδ ligands were from the hydroxyeicosatetraenoic acid (HETE) subclass which represents a diverse subclass of lipid metabolites generated from EPA, arachidonic acid and dihomo-γ-linolenic acid. A study by Mallat et al. shows that atherosclerotic plaques which were removed from symptomatic patients through endarterectomy contain more HETEs than plaques from asymptomatic patients [62].

Altogether, further experimental research is required to elucidate the immunomodulatory effect of PPARδ in Treg cells, for example using Treg cell-specific PPARδ-deficient mice, in atherosclerosis. Next, a prospective study which uses extensive genomics and lipidomics profiling to fully characterize the lipidome of dyslipidemia patients and subsequently correlates this to the development of symptomatic CVD, cardiac events or cardiac death should lead to more effective dietary regimes to prevent CVD. It is unlikely that the protective effects of n-3 PUFA on CVD which have been reported are solely Treg cell-mediated and whether these effects are PPARδ-dependent remains elusive.

Nevertheless, the notion that dietary lipids can instruct Treg cell metabolism and migratory capacity is interesting and future research should elaborate whether this has any clinical relevance.

In chapter 4, we studied the antigen-independent effect of dyslipidemia on T cell-mediated autoimmunity by examining how dyslipidemia-induced priming of $CD4^+$ Tn cells alters their effector phenotype. Priming of $CD4^+$ Tn cells was performed by pre-incubating $CD4^+$ Tn cells with excess lipoproteins or dyslipidemic serum from WTD-fed *Ldlr*$^{-/-}$ mice in the presence or absence of compounds which modulate lipid metabolism. Subsequently, the cells were activated by suboptimal antibody stimulation or antigens presented on MHC molecules by bone marrow derived dendritic cells. These data

showed that the specific method to induce lipid accumulation and the activating stimulus which was used, determined whether priming affected the effector phenotype. The use of lipoproteins to induce lipid accumulation had the advantage that the observed effects of priming were lipid-specific. A disadvantage was that lipid accumulation occurred without the context of blood-borne inflammatory factors (such as cytokines), which did occur during dyslipidemic serum-induced lipid accumulation. Arguing against the use of serum-induced lipid accumulation is that serum induces confounding factors in the form of the aforementioned blood-borne inflammatory factors. Whether lipoprotein-induced priming of $CD4^+$ Tn cells altered the effector phenotype was most likely dependent on the TCR-stimulating signal (antibody/antigen), which suggests that the amount of antigen present determines whether priming of $CD4^+$ Tn cells with lipids affects their effector function. The lack of an effect on the effector phenotype after antigen-stimulation of primed $CD4^+$ Tn cells isolated from OT-II cells could also be explained by the fact that OT-II T cells have a functional LDL-receptor whereas *Ldlr*$^{-/-}$ T cells do not. This suggested that priming of *Ldlr*$^{-/-}$ during dyslipidemia has a larger effect as compared to wildtype T cells as the LDL receptor-mediated uptake of native lipoproteins is absent. These findings were relevant as mutations in the *Ldlr* gene is a frequent cause of FH [63].

Future research should focus on whether lipid accumulation occurs in human Tn cells residing in lymphoid tissues and how priming of human $CD4^+$ Tn cells contributes to inflammation when DCs and other APCs are also affected by priming. Regarding the latter, DCs loaded with oxLDL have enhanced antigen presentation and are capable of stronger induction of T cell proliferation [64,65], indicating that this is an additional factor in dyslipidemia-induced antigen-independent effects on T cell activation.

An interesting finding from chapter 4 is that modulation of lipid metabolism with both an LXR agonist as well as a lysosomal inhibitor had mild anti-inflammatory effects on the effector phenotype of $CD4^+$ T cells specifically under dyslipidemia-like priming conditions. From a translational point of view, this suggests that *ex vivo* modulation of peripheral $CD4^+$ Tn cells from patients with dyslipidemia using compounds which modulate lipid metabolism might have distinct effects from those observed in healthy, normolipidemic individuals. As mentioned above, patients at risk of developing CVD are often treated with lipid lowering therapies. Lipid lowering therapy in FH patients has already been shown to affect the degree of lipid accumulation in circulating monocytes and their inflammatory phenotype [66], which adds to the complexity of translating our findings to the human situation.

Dyslipidemia was not associated with increased autophagy in $CD4^+$ T cells, in contrast to acetylated-LDL which does induce autophagy in foam cells [67]. Our findings on autophagy reported in chapter 4 suggested that lipid accumulation does not require an autophagy-dependent response to prevent lipotoxicity in the total $CD4^+$ T cell population. However, it is possible that Treg cells, which have been described to affect systemic

lipoprotein metabolism [68] and are more sensitive to environmental lipid perturbations, do require enhanced autophagy-mediated degradation of lipid droplets to prevent lipotoxicity. Our results in chapter 4 support the hypothesis that modulation of autophagy might be feasible as a therapy to dampen T cell-mediated immunity and ameliorate atherosclerosis [69]. Naturally, this approach requires more research but seems feasible as chloroquine treatment of $CD4^+$ Tn cells during priming with dyslipidemia serum had anti-inflammatory effects on their effector phenotype. In addition, chloroquine has been described to induce tolerogenic DCs [70]. Chloroquine is safe when used in small dosage and is already applied in the treatment of other autoimmune diseases, such as rheumatoid arthritis, to inhibit the immune system and ameliorate disease.

Our fourth approach was based on studying the effect of dyslipidemia on autophagy in T cells and the effect of pharmacological and genetic blockade of autophagy on T cell-mediated inflammation in atherosclerosis. Here we studied the effects of pharmacological autophagy inhibition during priming phase of $CD4^+$ Tn cells, as described above. The effect of genetic autophagy blockade in T cells on the T cell-mediated contribution to atherosclerosis was described in **chapter 5**. We generated a model of genetic blockade of autophagy in T cells by generating mice with T cell specific deletion of autophagy related protein 7 (Atg7), as whole body deficiency of Atg7 is lethal in mice within 24 hours after birth [71]. We used a recombinant adeno-associated virus which induced overexpression of murine PCSK9 (rAAV2/8-D377Y-mPCSK9) to mimic the *Ldlr*$^{-/-}$ phenotype in mice [72] with wild type Atg7 or a T cell-specific deficiency of Atg7. To our surprise, T cell-specific Atg7 deficiency impaired the development of hepatic steatosis. Hepatic steatosis, also known as non-alcoholic fatty liver disease, is characterized by excessive hepatocellular lipid accumulation. Hepatic steatosis can be caused by metabolic diseases, such as dyslipidemia and hyperglycemia, and is thus associated with obesity and diabetes mellitus [73]. When steatosis persists it elicits an inflammatory response, thus leading to steatohepatitis which can subsequently lead to hepatic fibrosis [74]. Research has primarily focused on the contribution of T cells in the inflammatory response which progresses steatosis to steatohepatitis and hepatic fibrosis. Interestingly, it has been suggested that inflammation precedes the development of steatosis [75]. High fat diet-fed C57BL/6 mice without hepatic steatosis which are injected with casein, to induce inflammation, develop more severe hepatic steatosis than PBS-injected mice by disrupting fatty acid synthesis and oxidation [76]. A recent report described how nutrient excess in the liver induces DNA damage and promotes T helper 17 (Th17) cell-mediated inflammation of white adipose tissue (WAT), which subsequently increased insulin resistance and FFA release in the circulation. This increase in circulating FFAs increased the amount of esterified FFAs (triglycerides) in the liver, thereby promoting hepatic steatosis development [75]. Interestingly, systemic administration of chloroquine has been described to exacerbate hepatic steatosis development in a high-fat diet-induced hepatic steatosis

model [77], suggesting that chloroquine treatment to modulate the immune system in patients with dyslipidemia and hepatic steatosis may be troublesome. One of the limitations in the studies presented in chapter 5 is the lack of a T cell subset-specific knock-out of Atg7. Indeed, it would be interesting to generate mice which have Atg7 deficiency, specifically in Th1 cells or Th17 cells. A limitation in the generation of these mice is that activated $CD8^+$ T cells also express T-bet, which is the characteristic transcription factor for Th1 cells. A recent paper has shown that this limitation can be overcome through the use of two orthogonal recombination systems in which Cre recombinase is expressed only in cells where Dre recombinase is also expressed [78]. The paper by Pu et al. reports this approach in endothelial cells as an example to prove that this system works, but theoretically it could be applied to immune cells as well. Hence, a *CD4-Dre Tbx21-CreER Atg7$^{f/f}$* mouse would allow for temporal deletion of Atg7 more specifically in Th1 cells, although Tbx21 would also be deleted through recombination in double-positive T cells in the thymus. This approach would also be interesting to further unravel the role of Th2 and Th17 cells in different stages of experimental atherosclerosis as their contributions to inflammation in atherosclerosis remain unclear.

Finally, **chapter 6** described our studies in which we aimed to unravel some of the antigen-independent effect of dyslipidemia on T cell-mediated immunity. This chapter was specifically based on the effect of lipocalin-2 (Lcn2) deficiency on different metabolic parameters associated with atherosclerosis which could affect T cell metabolism. Lcn2 is an inflammatory mediator, which is produced by macrophages, neutrophils and epithelial cells in the gut, lungs and kidneys. It is associated with severity of atherosclerosis and CVD in humans and has been shown in mice to promote obesity, hepatic steatosis and the development of insulin resistance [79]. Obesity is associated with increased levels of the adipokine leptin, a hormone which is primarily secreted by adipocytes and regulates the sensation of satiety in the hypothalamus and can thereby regulate body weight. Moreover, leptin regulates glucose metabolism in activated T cells [80], mTOR signaling in Treg cells [81] and can regulate autophagy in conventional T cells after TCR stimulation [82]. Additionally, Lcn2 can affect macrophage phenotype as well as neutrophil recruitment and function [79]. Therefore, we hypothesized that Lcn2 is involved in atherosclerosis by impacting T cell-mediated inflammation through its effect on systemic lipid metabolism and the inflammatory environment.

Lcn2 deficiency in *Ldlr$^{-/-}$* mice did not affect the metabolic parameters which we examined, including body weight, inguinal white adipose tissue weight, serum cholesterol levels, serum triglyceride levels and fasting glucose levels. Additionally, we measured mRNA expression of *Lep*, the gene encoding leptin, in WAT from *Ldlr$^{-/-}$* and *Ldlr$^{-/-}$Lcn2$^{-/-}$* mice but this was also unaltered between both genotypes. Flow cytometry analysis of T cell activation status and T cell subset percentages also showed no differences between *Ldlr$^{-/-}$* and *Ldlr$^{-/-}$Lcn2$^{-/-}$* mice, suggesting that Lcn2 deficiency had no major T cell-mediated

effects in our studies. Interestingly, Treg cells express *24p3r*, the Lcn2 receptor, suggesting that Lcn2, which is usually generally considered to affect innate immune cells, might modulate Treg cells (data on the *Lep* expression in WAT, effects of Lcn2 deficiency and T cells and Lcn2 receptor expression in Treg cells were not included in chapter 6). Lcn2 deficiency did impact the size and morphological composition of atherosclerotic lesions as Lcn2 deficiency promoted lesion growth in initial stages of plaque development and decreased the necrotic core size in advanced lesions. Hence, we did not focus on T cells in this chapter but aimed to elaborate how Lcn2 deficiency affected the aforementioned lesion characteristics. We reported that Lcn2 deficiency most likely increased the size of moderately progressed lesions by increasing the recruitment of monocytes to atherosclerotic lesions as Lcn2 interacts with various inflammatory factors involved in atherosclerosis, including leukotriene B4 [83]. We indeed observed more inflammatory monocytes in the circulation. Further research is however required to identify what the exact effects are of the interactions between Lcn2 and certain inflammatory factors, such as leukotriene B4, on their functionality. A report by Fernandez-García et al. which responded to our publication suggested that the growth in moderate lesion size was smooth muscle cell (SMC)-mediated [84]. While we disagree that the lesion promoting effect of Lcn2 deficiency is exclusively SMC-mediated [85], Fernandez-García et al. had a valid point that further identification of the cells expressing Lcn2 in atherosclerotic lesion, and their dynamics in diet-induced atherosclerosis, would be relevant in order to elucidate the mechanism by which Lcn2 affects atherosclerosis in different stages. This is especially relevant as not only inflammatory cells but also SMCs and endothelial cells express Lcn2 in atherosclerotic lesions [86,87].

In advanced stages of atherosclerosis, Lcn2 deficiency was associated with decreased intraplaque matrix metalloproteinase (MMP) activity and decreased necrotic core size suggesting that Lcn2 affects atherosclerotic lesion stability by increasing MMP-induced cell death. These findings were in line with clinical observations that serum Lcn2 is a predictor of major adverse cardiac events [88,89] and serum Lcn2 levels are higher in patients with symptomatic carotid atherosclerosis as compared to patients with asymptomatic carotid atherosclerosis [90]. Moreover, serum Lcn2/MMP9 complexes are associated with major adverse cardiac events [91]. Active MMP9 degrades matrix proteins in the fibrous cap of atherosclerotic lesions [92,93] and Lcn2 stabilizes MMP9 by preventing its inhibition by tissue inhibitor of matrix metalloproteinase 1 [94]. This suggests that Lcn2 could be used as a biomarker to identify patients at risk of an acute coronary event which could be followed by additional treatment of these patients with MMP inhibitors to stabilize atherosclerotic lesions. Pharmacological inhibition of Lcn2 itself can compromise an individual's resistance to bacterial infection as Lcn2 is a crucial bacteriostatic agent in the acute phase of infection with *E. Coli* [95]. Therefore, we expect future research into Lcn2

in atherosclerosis and CVD will aim to improve its use as a biomarker for CVD rather than as a target for therapy.

In conclusion, the research in this thesis has provided novel antigen-independent pathophysiological mechanisms through which diet-induced dyslipidemia can affect T cell-mediated immunity in atherosclerosis. Moreover, it supports the notion that modulation of cellular metabolism and autophagy in T cells is feasible to dampen inflammation and inhibit atherosclerotic lesion development, potentially preventing CVD. Future research is required to further elaborate the effect of dyslipidemia on cellular metabolism and autophagy in specific T cell subsets in a temporal and site-specific fashion and to identify whether similar pathophysiological mechanisms are present in humans.

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