Caveolin-1 and CD40L-CD40-TRAF interactions in vascular and metabolic disease

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Caveolin-1 and CD40L-CD40-TRAF interactions in vascular and metabolic disease

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Caveolin-1 and CD40L-CD40-TRAF interactions in vascular and metabolic disease

PROEFSCHRIFT

Ter verkrijging van de graad van doctor aan de Universiteit Maastricht,
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Chapter 1

General Introduction

Nowadays, it is widely accepted that atherosclerosis as well as obesity are chronic inflammatory diseases. Both disorders are characterized by an ongoing accumulation of immune cells in the arterial wall or the adipose tissue, respectively. Besides that, both diseases are accompanied by changes in chemokine, cytokine, and adhesion molecule expression, further propagating disease progression. During the last decades, it has become obvious that the metabolic syndrome characterized by features such as obesity, type 2 diabetes, hyperinsulinemia, and insulin resistance, is a major risk factor for the development of cardiovascular diseases.

The different criteria used to diagnose the metabolic syndrome, as well as age, ethnic group, prevalence of obesity, and environmental factors influencing the studied population, make it difficult to give exact rates of the prevalence of the metabolic syndrome ¹. Nevertheless, it is estimated that approximately 35% of the US population suffers from the metabolic syndrome ². A minimal gender difference can be observed with 0.1% more women being classified as suffering from the metabolic syndrome ³. On a worldwide basis, prevalence rates were found to be similar although different individuals were included using different criteria ⁴. The definition of the metabolic syndrome has been under debate for several decades since the first description as 'Syndrome X' by Reaven in 1988 ⁵. The metabolic syndrome consists of a plethora of risk factors for developing diabetes and cardiovascular disease but its exact etiology remains to be elucidated ^{6, 7}. Experts argue whether the metabolic syndrome has to be considered as a syndrome of diverse risk factors, obesity, or insulin resistance. Furthermore, it is not yet established whether the risk factors are unrelated or rather traits of a common mechanism ⁸⁻¹⁰. Although several open questions remain regarding the nature of the metabolic syndrome, there is no doubt that insulin resistance and visceral obesity are two of its main features.

Upon high levels of blood glucose, insulin is released from pancreatic β cells leading to glucose uptake into muscle and adipose tissue while suppressing gluconeogenesis. The glucose transport into the cells is mediated by the glucose transporter GLUT4 ¹¹. Under conditions of type 2 diabetes and obesity, GLUT4 levels in adipocytes are reduced, and obese humans have dysfunctional GLUT4 in their skeletal muscle ^{12,13}. These factors lead to a slight insulin resistance and postprandial hyperglycemia ¹⁴. Hence, the second-phase

insulin response is exaggerated leading to hyperinsulinemia and, finally, to complete insulin resistance¹⁵.

Obesity is independently associated with, and plays an important role in insulin resistance¹. Adipose tissue has been shown to be an endocrine organ that secretes many metabolically active and inflammatory substances such as leptin, adiponectin, lipoprotein lipase, insulin like growth factor-1, interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF α)^{16, 17}. In addition, obesity contributes to a large extent to the metabolic syndrome by increasing nonesterified fatty acids thereby promoting insulin resistance, dyslipidemia, and hypertension.

The Framingham Heart Study, as well as several other studies have shown that the degree of cardiovascular disease is strongly correlated with the severity of the metabolic syndrome¹⁸⁻²¹. Considering atherosclerosis, at least three additional risk factors arise that commonly accompany obesity. First, patients suffering from the metabolic syndrome experience atherogenic dyslipidemia characterized by high triglyceride levels, small low density lipoprotein (LDL) particles, and low high density lipoprotein (HDL) levels, a lipid profile which has been shown to be pro-atherogenic²²⁻²⁴. Second, several hypotheses have suggested insulin resistance and its companion, hyperinsulinemia, which are common in the metabolic syndrome, to be a risk factor for cardiovascular diseases²⁵⁻²⁷. The third risk factor for cardiovascular diseases arising from the metabolic disarrangements is the typical pro-inflammatory state found in these patients. This state is defined by elevated levels of high-sensitivity C-reactive protein as well as high levels of pro-inflammatory cytokines such as TNF α and IL-6 which are believed to be able to convert otherwise stable plaques into vulnerable plaques leading to clinical complications²⁸⁻³⁰.

Atherosclerosis

Atherosclerosis is the main underlying cause of cardiovascular disease such as coronary heart disease, peripheral arterial disease, and stroke. Atherosclerosis and its co-morbidities accounted for approximately one third of all deaths in the United States in 2007 and it is believed that this number is going to increase in the US as well as worldwide³¹. In addition, several underlying factors, which promote atherosclerosis progression, such as genetic

predisposition, hypertension, smoking, type 2 diabetes and obesity, have been identified. Due to these facts, there has been a tremendous interest in investigating the nature of atherosclerosis initiation and progression and to elucidate its underlying mechanisms. Today, atherosclerosis is seen as a chronic inflammatory disease affecting the vessel wall of large- and medium-sized arteries ³². The first signs of atherosclerosis can already be observed during childhood when so-called fatty streaks appear ^{33, 34}. Over the years, it has become evident that atherosclerotic plaques only develop at certain arterial sites that are characterized by a disturbed flow pattern. These sites are typically represented by arterial bifurcations with local rheological factors, such as low and oscillatory blood-to-wall shear stress ^{35, 36}. As a consequence, endothelial cells (ECs) get activated, leading to a modification of gene expression as well as endothelial permeability, finally predisposing the arterial wall to LDL influx and monocyte adhesion. The initiation of atherosclerosis is characterized by an accumulation of these lipids, followed by the infiltration of macrophages into the sub-endothelial space in order to clear the accumulated lipids ³⁷. During the progression of atherosclerosis, from lipid and foam cell rich fatty streaks towards mature plaques, an ongoing accumulation of lipids and the recruitment and proliferation of various cell types including monocytes/macrophages, leukocytes and smooth muscle cells (SMCs) can be observed ³⁸. The activated macrophages and dying foam cells are the origin of enhanced cytokine and chemokine production, thereby triggering further leukocyte recruitment to the plaque ³⁹. T cells have been shown to secrete both pro- and anti-inflammatory cytokines, and are thus believed to govern the inflammatory reactions in the plaque itself ^{40, 41}. Advanced atherosclerotic plaques contain a necrotic core and a plethora of modified lipids, leukocytes and foam cells which are covered by a SMC and collagen-rich fibrous cap ⁴². These atheromas are regarded as the clinical preferable, stable plaque phenotype. Eventual weakening of the fibrous cap, due to the action of matrix metalloproteinases (MMPs) and ongoing apoptosis inside the plaque, can cause plaque rupture upon which the pro-thrombotic content of the necrotic core comes into contact with the blood stream, which results in thrombus formation leading to clinical complications such as myocardial infarction and stroke ⁴³⁻⁴⁵.

Today, it is widely accepted that atherosclerosis represents an inflammatory disease and due to this fact, the immune system has been subject to intensive research. The innate immune system is regarded as the first line of defense against pathogens. Upon encounter with a pathogen, the innate immune system is immediately activated and executed by cells of the myeloid lineage. The innate immune system recognizes so called pathogen associated molecular patterns (PAMPs) via toll-like receptors (TLRs), the primary innate immune receptors, which subsequently leads to the production of pro-inflammatory cytokines and the up-regulation of co-stimulatory molecules ⁴⁶. During the last years, substantial evidence has been found for the involvement of cholesterol crystals and LDL particles in innate immune activation and due to that, the innate immune system has been shown to be a key player in atherosclerosis ⁴⁷⁻⁴⁹. In addition, genetic deletion of several TLRs and their downstream signaling intermediates has been shown to have significant effects on atherosclerosis ⁵⁰⁻⁵².

The most prominent cell of the innate immune system in atherosclerosis is the monocyte. The adhesion and extravasation of monocytes during atherosclerosis is a crucial step. It is well defined that monocytes first roll on inflamed endothelium in a P-selectin-dependent manner. Later, firm adhesion to the endothelium is conferred by vascular cell adhesion molecule 1 (VCAM-1) and it has been shown that genetic loss of VCAM-1 leads to a reduction in atherosclerotic lesion formation ⁵³⁻⁵⁵. In addition, several reports have illustrated the involvement of chemokine/chemokine receptor pairs in the regulation of leukocyte recruitment during atherosclerosis ⁵⁶. As monocytes give rise to macrophages, which, during the progression of atherosclerosis accumulate lipids and become macrophage foam cells, the macrophage is the most important cell within atherosclerotic lesions ⁴⁵. Furthermore, it has been shown that lack of macrophages leads to decreased atherosclerosis in *Apoe*^{-/-} mice, underlining the importance of macrophages during atherosclerosis development ⁵⁷.

Macrophages are extremely plastic cells and are divided into classically activated (M1) and alternatively activated (M2) macrophages. Macrophages polarize towards the M1 phenotype under the action of lipopolysaccharides (LPS) and interferon gamma (IFN γ), which results in the secretion of pro-inflammatory cytokines such as IL-12, IL-6, and TNF α , amongst others.

Therefore, M1 macrophages are seen as the pro-inflammatory macrophage subset. On the other hand, M2 macrophages develop under the influence of IL-4, IL-13, and IL-1. This differentiation leads to M2 macrophages which secrete high amounts of the anti-inflammatory cytokine IL-10 rendering the M2 macrophage the anti-inflammatory macrophage subset ⁵⁸. Interesting observations have been made by Caligiuri et al. ⁵⁹, who found that during early stages of atherosclerosis the M2 phenotype is the most prominent. During later stages of atherosclerosis they found M1 macrophages to be more abundant than M2 macrophages. They argue that this could be due to the fact that during early atherosclerosis, the IL-4 secreted by neutrophils and natural killer T cells (NKT) primes the macrophages towards an M2 phenotype, whereas during late atherosclerosis the cytokine environment had changed. They documented an increase in IFN γ on the expense of IL-4, thus favoring the M1 phenotype. In addition, the authors proposed that, although they cannot rule out that macrophages recruited during late stages of atherosclerosis develop into M1 macrophages, the enrichment in M1 macrophages could be due to a phenotypic conversion of M2 macrophages.

In addition to macrophages, there are other cell types of the innate immune system with important functions in atherosclerosis, namely mast cells and neutrophils. Despite the fact that mast cells are rare, they have been found in atherosclerotic plaques ⁶⁰. Because mast cells contain high amounts of proteolytic enzymes and secrete a broad spectrum of pro-inflammatory cytokines, they have been implied in atherosclerotic plaque destabilization ⁶¹. Indeed, mast cells have been found to be localized in the shoulder region and at sites of plaque rupture ⁶². Furthermore, mast cell-deficient mice experience reduced inflammation, a thicker fibrous cap, and increased collagen content in their atherosclerotic plaques, highlighting the involvement of mast cells in atherosclerosis ⁶¹.

Next to mast cells, neutrophils are emerging as important cells in atherosclerosis. Neutrophils are characterized by their ability to secrete biologically active substances such as myeloperoxidase (MPO) and proteinases ⁶³. In addition, they have been proven to exert fundamental functions in atherosclerosis. Blockage of the cytokine C-X-C motif receptor 4 (CXCR4), a cytokine important for both the egress of neutrophils from the bone marrow and the recruitment of neutrophils to the atherosclerotic lesion, was

found to result in neutrophilia with an increased number of neutrophils in the atherosclerotic plaques, accompanied by a pro-inflammatory plaque phenotype ⁶⁴. As a consequence, neutrophils have been attributed a pro-inflammatory role in atherosclerosis.

Another important cell of the innate immune system is the dendritic cell (DC). The most essential function of DCs are priming of innate and adaptive immune responses and involvement in maintaining the immune tolerance to self antigens. It has been shown that DCs co-localize with CD40L⁺ T cells in the shoulder region of rupture prone plaques and produce chemokine C-C motif ligand 19 (CCL19) and CCL21, which might lead to an enhanced recruitment of leukocytes ⁶⁵. Furthermore, DCs can be IFN γ ⁺ and might contribute to CD4⁺ T cell apoptosis ⁶⁶. Lichtman et al. showed that CD11c⁺ DCs remain functional during dyslipidemia ⁶⁷. Antigen processing and presentation capabilities were unaltered and DCs were still able to prime CD4⁺ T cells. These results clearly highlight the function of DCs in inducing pathogenic as well as protective T cell responses. Another study by Cybulsy et al. demonstrated an important role for CD11c⁺ DCs in early atherosclerosis ⁶⁸. Upon depletion of CD11c⁺ cells in *CD11c-DTR/Ldlr^{-/-}* mice a 55% decrease in intimal lipid area was observed. In addition, lesions of DC-depleted mice had only a few foam cells, linking DCs to foam cell formation. Nevertheless, the exact function of DCs in atherosclerosis still needs to be further elucidated.

Besides the innate immune system, the adaptive immune system with its diverse leukocyte subsets has been shown to have fundamental effects on atherosclerosis ⁶⁹. The first studies have focused on the so called CD4⁺ T helper-1 (Th1) and Th2 cells. The typical factors involved in Th1 responses include IFN γ and interleukins which promote IFN γ secretion ⁷⁰. It was shown that deficiency of IFN γ reduced the atherosclerotic burden and enhanced collagen deposition, whereas administration of IFN γ enhanced atherosclerosis ^{71, 72}. Furthermore, Th1 development is known to be promoted by CD40L-CD40 interactions, a co-stimulatory dyad known to exert pro-atherogenic functions ⁷³. In summary, these data provide profound evidence for the pro-atherogenic nature of Th1 responses.

Th2 responses on the other hand have been implied to exert anti-atherogenic functions and are regarded as antagonists to the pro-atherogenic Th1 responses. Nevertheless, several studies have brought up controversial

results. IL-4, a cytokine being associated with pro-atherogenic functions, is secreted by Th2 cells ⁷⁴. Indeed, mice deficient for IL-4 showed reduced, site specific plaque formation underlining the pro-atherogenic capacity of IL-4 ⁷⁵. Surprisingly, the pro-atherogenic function could not be proven in another study, in which IL-4 did not influence the atherosclerotic burden in a mouse model of diet and angiotensin-II induced atherosclerosis ⁷⁶. Next, IL-10 derived from Th2 cells regulates the balance between Th1 and Th2 responses ⁶³. It has been shown that IL-10 deficiency resulted in plaques rich in T cells, low in collagen, and elevated levels of IFN γ ⁷⁷. Additional studies were able to prove the atheroprotective function of IL-10 when *IL10*^{-/-}*Apoe*^{-/-} mice presented with increased levels of atherosclerosis, thrombosis, and rupture prone plaques ⁷⁸. These studies clearly demonstrate the versatile functions of Th2 responses and suggest that the stage of the disease as well as the experimental model may affect the Th2 pathways in the setting of atherosclerosis.

Later, the regulatory T cell (Treg) moved into the research focus and was attributed to have a protective role in atherosclerosis. Two different subtypes of Tregs, natural and induced Tregs have been identified. Natural regulatory T cells develop in the thymus and are defined by the expression of CD4, CD25, and FoxP3. In contrast, induced Tregs develop in the periphery during an ongoing immune response. While natural Tregs are important for the prevention of auto-immunity, induced Tregs and naive CD4⁺ CD25⁻ T cells, which can be converted under the influence of transforming growth factor beta (TGF β) or IL-10 into induced Tregs, are atheroprotective due to their direct effect on T cells and antigen-presenting cells (APCs) ^{69, 79}. Mallat et al. have elegantly shown that loss of natural Tregs increased atherosclerosis in their mouse model and by that implied fundamental functions for Tregs in atherosclerosis ⁸⁰.

Recently, a third lineage of T helper cells, the IL-17 secreting Th17 cells, has been identified. Besides their physiological role in the defense against bacteria and fungi, Th17 cells have been implicated in numerous auto-immune diseases such as inflammatory bowel disease, rheumatoid arthritis, and psoriasis ⁸¹. Interleukin-17 was shown to elicit the production of IL-6 in synoviocytes of patients with rheumatoid arthritis ⁸². Furthermore, the production of IL-17, TNF, and IL-1 has been proven to be predictive of joint destruction in rheumatoid arthritis ⁸³. In psoriasis, the main T cell population

extractable from skin lesions has a Th17 phenotype which goes hand in hand with the accumulation of inflammatory cells in the tissue ⁸⁴. These studies suggest a strong pro-inflammatory role of Th17 cells in chronic inflammatory disease. On the other hand, McGeachy et al. have shown that the induction of Th17 cells under the influence of anti-inflammatory cytokines such as TGF β can have protective effects ⁸⁵. The function and contribution of Th17 cells in atherosclerosis remains mainly unknown to date. Two studies have shown that IL-17 deficiency reduces atherosclerosis when *Ldlr*^{-/-} mice were transplanted with IL-17 deficient bone marrow, and *Apoe*^{-/-} mice were treated with an IL-17 neutralizing antibody ^{86, 87}. Opposing results were published from a human study, in which patients suffering from cardiovascular disease did not show elevated levels of circulating IL-17 ⁸⁸. These data, together with the fact that IL-17 activates nuclear factor kappa B (NF κ B), render Th17 responses to be mainly pro-inflammatory, whereas also anti-inflammatory effects may occur under specific conditions ^{88, 89}. Unraveling the exact mechanisms of Th17 responses in general, as well as in atherosclerosis, remains a topic of further investigation.

In addition to T cells, B cells are the second prominent member of the adaptive immune system. Their general function is to produce antibodies against certain antigens or function as APCs. Eventually, they can become memory B cells. In atherosclerosis, B cells were first discovered in the adventitia ⁹⁰. During the last years, several studies have proven the substantial contribution of B cells to atherosclerosis. For example, the transfer of B cell deficient bone marrow into *Ldlr*^{-/-} mice resulted in a 40% increase in atherosclerosis ⁹¹. Another study showed that the transfer of B cells from atherosclerotic *Apoe*^{-/-} mice into young *Apoe*^{-/-} mice reduced atherosclerosis ⁹². From these results it seems likely that atheroprotective immunity develops during the progression of atherosclerosis and that B cells mediate these protective functions. In contrast to these results, a more recent study claimed B cell deficiency to be atheroprotective ⁹³. B cell depletion by administration of an anti-CD20 monoclonal antibody reduced atherosclerosis in *Ldlr*^{-/-} and *Apoe*^{-/-} mice. In addition, B cell depleted mice exhibited less DC and T cell activation, with less T cells present in atherosclerotic lesions and higher levels of the anti-inflammatory cytokine IL-17. These results identify B cell modulation as an interesting target in the treatment of atherosclerosis.

Despite the fact that enormous investigations have been undertaken to understand the nature of atherosclerosis, its underlying mechanisms have not been completely elucidated to date. The advances in technical equipment, atherosclerosis imaging, and the availability of refined animal models of atherosclerosis are promising strategies to further understand and design treatment for this chronic inflammatory disease.

Obesity

It is evident that obesity is rapidly increasing in the US. Data from a large health survey reveal that approximately 32% of American adults are obese. When these data were compared to data from 1999, a significant increase in obesity was observed. Furthermore, it was predicted that by 2030 more than 50% of American adults will suffer from obesity ^{94, 95}. Regarding obesity statistics on a worldwide basis, nearly 10% of the world population is obese and it was estimated that the numbers will rise from 396 million people in 2005 to 579 million people in 2030 ⁹⁶. Besides the fact that obesity is associated with a whole plethora of co-morbidities ⁹⁷ and thus also with a higher mortality ^{98, 99}, obesity also imposes a significant economic load. In 2008, 9.1% (\$147 billion dollars) of all medical expenditures in the US were related to obesity and the costs are expected to rise up to \$860 - \$950 billion (15% - 17.5% of the total health costs) in 2030 ^{94, 100}. These facts underline the importance of obesity research and management and imply that a lot of effort needs to be undertaken to be able to deal with the pandemic of obesity.

During the last decade, it has become evident that inflammation plays a pivotal role in the setting of obesity and the development of metabolic diseases ¹⁰¹. Obese adipose tissue has been shown to be infiltrated by immune cells, a hallmark of chronic inflammation which locally develops in adipose tissue and is thought to be involved in the onset of insulin resistance and type 2 diabetes ¹⁰²⁻¹⁰⁴. It has become evident, that T cell and macrophage subsets, as well as other immune cells, such as mast cells, are involved in the chronic low grade inflammation during obesity ¹⁰⁵⁻¹¹³. These immune cells, their mutual interaction and their interaction with adipocytes increase the expression of chemokines, cytokines, and adhesion molecules in adipose tissue, thereby aggravating the inflammatory response ¹¹⁴⁻¹¹⁶. Until now, there are only limited data on the exact pathways that trigger inflammation in obesity. Nevertheless,

some prominent mediators of inflammation in obesity have been identified in the past years. The most prominent ones might be the adipokines. Twenty four adipokines have been identified and their circulating levels were found to be elevated in the obese. Most of these adipokines were identified as inflammatory proteins such as IL-8, IL-6, IL-18, PAI-1, MCP-1, and TNF α ¹¹⁷. Another expected mediator of inflammation in obesity is TLR-4. TLR-4 recognizes bacterial lipopolysaccharides, has an important function in innate immunity, and was shown to be involved in obesity ¹¹⁸⁻¹²⁰. Arising from the fact that obese adipose tissue is poorly oxygenated, hypoxia seems to be an interesting inflammatory mediator as well ^{121, 122}. Already in 2004 this hypothesis was proposed by Trayhurn et al. ¹²³, but the mechanisms remain mainly unknown, although the paradigm that hypoxia inducible factor 1 alpha (HIF1 α) activates NF κ B, thus leading to elevated levels of inflammatory adipokines is widely accepted.

Caveolin-1

Besides the well-documented involvement of diverse leukocyte subsets and their products in inflammatory disease, compartmentalization and transport molecules have a profound effect on inflammation as well.

Caveolae have first been described as non clathrin coated, plasmalemmal vesicles by Palade in 1953 ¹²⁴. In the following decades, much attention was drawn towards the characterization of caveolin-1 (Cav-1) and caveolae. It has become evident that caveolin-1, a 22-kDa trans-membrane protein, is inserted into the plasma membrane by a 33 amino acid region, while the N- and C-terminus face the cytoplasm ^{125, 126}. Two membrane attachment domains organize Cav-1 oligomerization with itself and Cav-2, a process by which caveolae, flask or Ω -shaped 50-100nm cell surface plasma membrane invaginations are formed. A 20 amino acid scaffolding domain is responsible for the interaction between Cav-1 and several Cav-1 associated proteins ^{127, 128}. Hence, caveolin-1 is the main structural component of caveolae and besides the invaginations, tubes and vesicle like structures can be observed ^{129, 130}. Cav-1 is found in various cell types such as macrophages, smooth muscle cells, fibroblasts, adipocytes, and, with the highest prevalence of 5000-10000 invaginations per cell, in endothelial cells ¹³¹.

Chapter 1

During the last years, Cav-1 has been shown to exert various functions which can be divided into three major categories. First, Cav-1 is essential for the formation, assembly, composition, and function of caveolae¹²⁸. Fra et al. proved that lymphocytes, usually lacking caveolae, start to form de novo caveolae when expressing VIP21-caveolin¹³². In addition, it has been shown that Cav-1 deficient mice show a lack of caveolae in all non-muscle tissues¹³³⁻¹³⁵. Hence, these findings confirm the necessity of this protein for proper formation of caveolae. Second, Cav-1 is involved in macromolecule transcytosis¹³⁶⁻¹³⁸, maintenance of cellular cholesterol homeostasis^{137, 139} and diapedesis¹⁴⁰. In addition to plasma membrane and trans-golgi-network association^{126, 141, 142}, Cav-1 is also associated with endosomes, and caveosomes¹⁴³⁻¹⁴⁷. Finally, Cav-1 acts as an inhibitor of several enzymes e.g. endothelial nitric oxide synthase (eNOS), and various signaling molecules¹⁴⁸⁻¹⁵⁰, and functions as a signaling platform in general^{149, 151-153}. There is emerging evidence, that signaling systems are compartmentalized and that 'molecular zip codes' determine the targeting of proteins to different cell compartments¹⁵². As such, caveolae are elegant ways to segregate or integrate different molecular signaling pathways.

The three main functions discussed above led to the implication of caveolae in several diseases, amongst them atherosclerosis and obesity. Lisanti et al. have shown in several experiments that caveolae have a great impact on atherosclerosis, mainly attributable to the function of caveolae in cholesterol homeostasis. They have shown that Cav-1 deficient mice exhibit a tremendous reduction in plaque formation and size, and that the uptake of LDL is greatly diminished in caveolin-1 deficient mouse embryonic fibroblast and peritoneal macrophages^{134, 154}. Recently, the group of Sessa elegantly demonstrated that endothelial caveolin-1 plays a critical role during the progression of atherosclerosis. *Cav-1^{-/-}Apoe^{-/-}* mice were genetically modified to re-express Cav-1 solely in endothelial cells. These transgenic mice exhibited identical levels of atherosclerosis when compared to *Cav-1^{+/+}Apoe^{-/-}* mice¹⁵⁵. In addition, the same group showed that endothelial over-expression of Cav-1 accelerates atherosclerosis, providing further evidence for the importance of endothelial Cav-1¹⁵⁶.

Cav-1 has been shown to play a role in obesity as well. Cav-1 deficient mice were found to be lean and resistant to diet induced obesity, which was

mainly attributed to their impairment in converting triglycerides in lipoprotein form into triglycerides in lipid droplet form ¹⁵⁷. More recently it was shown that the expression of Cav-1 is upregulated during human obesity. In addition to Cav-1 up-regulation, a positive correlation between Cav-1 mRNA levels and the mRNA levels of inflammatory markers was observed ¹⁵⁸.

The results mentioned above profoundly demonstrate the versatile functions of Cav-1 and make its interactions an interesting target in chronic inflammatory disease research.

Co-stimulation

In order to give rise to an effective immune response, the activation of leukocytes is mainly accompanied by co-stimulatory signals. These signals usually involve a receptor-ligand pair, which delivers a second signal from an APC to a T cell. Through this interaction, activated T cells are given the ability to survive and to promote the induction of additional T cells.

Generally, a two step process is required for optimal T cell activation; first the recognition of an antigen, and second a co-stimulatory signal ¹⁵⁹. The antigen-elicited signal is transduced via the T cell receptor which binds to the MHC molecule presented on the membrane of an APC. The co-stimulatory signal is antigen non-specific. It is supplied by the interaction of co-stimulatory molecules which are present on the cell surface of T cells and APCs. Co-stimulation has been shown to be essential for T cell survival, proliferation, and differentiation, and non-existing co-stimulation presumably results in anergy ^{160, 161}. There are two major families of co-stimulatory molecules, namely the B7 and the TNF family members, as outlined in **Chapter 3** ^{162, 163}. The next paragraphs will deal in more depth with two distinct members of the TNF family.

CD40L-CD40-TRAF

CD40 belongs to the TNF receptor super family and is the classic receptor for CD40L ¹⁶⁴. Due to the fact that CD40 lacks intrinsic signaling activity, special adaptor proteins, the tumor necrosis factor receptor associated factors (TRAFs), need to be recruited to elicit proper CD40 signaling. CD40 can bind four out of the seven existing TRAF molecules and upon binding, several pro-inflammatory transcription factors are activated, leading to the production of cytokines and chemokines ¹⁶⁵. During the last decade, CD40 signaling has been shown to be involved in immunity ¹⁶⁴ as well as in the initiation and progression of various inflammatory disease ¹⁶⁶⁻¹⁷¹. A detailed overview of the actions of the CD40L-CD40-TRAF axis in atherosclerosis is given in **Chapter 3** of this thesis.

Recently, the pro-inflammatory receptor ligand pair, CD40-CD40L, has been implicated in playing a fundamental role during obesity-associated inflammation, as sCD40L levels have been shown to be elevated in obese as well as type 2 diabetic patients ¹⁷²⁻¹⁷⁵. In addition, Poggi et al. have shown that CD40 is highly expressed by adipocytes and that stimulation of human adipocytes by activated T cells or recombinant sCD40L evoked the production of adipokines and decreased the amount of insulin signaling molecules ¹⁷⁶. These data make the CD40-CD40L dyad one of the most interesting and promising targets in obesity research.

Hypothesis and outline of this thesis

The inflammatory nature of atherosclerosis and obesity is the subject of this thesis. During the last decades, it has become obvious that various mediators are involved during the progression of inflammation. Cholesterol transporting molecules have been implicated in atherosclerosis and co-stimulatory molecules have been proven to exert fundamental functions during the progression of inflammatory diseases in general.

Therefore, we hypothesize that caveolin-1 deficiency as well as blockage of co-stimulation on various levels of the signal transduction cascade fundamentally influence the initiation and progression of atherosclerosis and/or obesity.

In **chapter 2**, we investigate the effect of caveolin-1 deficiency on leukocyte recruitment to the atherosclerotic plaque and changes in T cell

subsets. The co-stimulatory dyad CD40L-CD40, together with their adaptor molecules, the so-called TRAFs, has been shown to have multifunctional cell-type-specific signaling capacities in atherosclerosis. Furthermore, a whole plethora of other co-stimulatory molecules have been implicated in contributing to atherosclerosis and other chronic inflammatory diseases such as obesity. In **chapter 3**, we review the signal transduction cascades and the cell-type-specific actions of CD40L-CD40 and discuss the therapeutic potentials. **Chapter 4** identifies which CD40 signaling cascades are important in atherosclerosis formation and we investigate which of the different CD40-dependent TRAF signaling cascades are involved. Recent research has linked the CD40L-CD40 dyad to obesity as well. CD40 was shown to be highly expressed on adipocytes and to cause a reduction in insulin signaling molecules. Therefore, in **chapter 5** we review the impact of CD40L-CD40 interactions on pancreatic, systemic, and vascular inflammation in type 2 diabetes and its complications. In **chapter 6**, we investigate the effect of genetic CD40L deficiency as well as CD40L blocking antibody administration in a mouse model of diet-induced obesity. In **chapter 7**, we further examine the effect of genetic CD40 deficiency in a setting of diet induced obesity, and elucidate on the interplay between different CD40-TRAF signaling cascades herein. Finally, in **chapter 8**, the experimental results obtained in this thesis are discussed, and future perspectives are given.

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Chapter 2

Caveolin-1 deficiency decreases atherosclerosis by hampering leukocyte influx into the arterial wall and generating a regulatory T cell response

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Abstract

Objective: Caveolin-1 plays a crucial role in atherosclerosis, which is mainly attributed to its effects on low-density-lipoprotein (LDL) transcytosis. However, caveolin-1 has also been implicated in the regulation of inflammation. We investigated the effects of caveolin-1 deficiency in atherosclerosis with its accompanying changes in plaque- and lymphoid-related immunology and inflammation.

Methods and Results: *Cav1^{-/-}Apoe^{-/-}* mice exhibited a 15-fold reduction in plaque size with plaques containing fewer macrophages, T cells, and neutrophils. Intravital microscopy revealed 83% less leukocyte adhesion to the vessel wall in *Cav1^{-/-}Apoe^{-/-}* mice, which could be attributed to reduced endothelial chemokine ligand-2 (CCL-2/MCP-1) and vascular cell adhesion molecule-1 (VCAM-1) expression. Caveolin-1 deficiency resulted in a 57% increase in regulatory T cells and a 4% decrease in CD4⁺ effector T cells in lymphoid organs. Bone marrow transplantations revealed that *Cav1^{-/-}Apoe^{-/-}* mice receiving *Cav1^{+/+}Apoe^{-/-}* or *Cav1^{-/-}Apoe^{-/-}* bone marrow presented 4-4.5 fold smaller plaques with no additional phenotypic changes. In contrast, atherosclerosis was not affected in *Cav1^{+/+}Apoe^{-/-}* recipients receiving *Cav1^{-/-}Apoe^{-/-}* or *Cav1^{+/+}Apoe^{-/-}* bone marrow. However, the presence of *Cav1^{-/-}Apoe^{-/-}* bone marrow was associated with an anti-inflammatory T cell profile.

Conclusions: Our study reveals that non-hematopoietic caveolin-1 determines plaque size whereas hematopoietic caveolin-1 regulates lymphoid immune-modulation. However, both are required for phenotypic modulation of plaques.

Introduction

Caveolae are 50-100 nm cell surface plasma membrane invaginations that are present in the majority of terminally differentiated cell types ^{1, 2}. Caveolae are present in many cell types such as endothelial cells (ECs), smooth muscle cells (SMCs), adipocytes, fibroblasts and macrophages (MΦ). Studies using modulation of caveolin-1, the most important coat protein of caveolae, have elucidated many of its cellular functions ^{3,4}.

In ECs, caveolin-1 mediates transcytosis of LDL ⁵, supports CD36-mediated transcytosis ⁶, and negatively regulates endothelial nitric oxide synthase (eNOS) activity ^{7, 8}. While it regulates the formation, storage and mobilization of lipid stores in adipocytes ⁹, caveolin-1 impairs SMC migration by inhibiting signaling via the mitogen-activated protein kinase (MAPK) pathway ¹⁰.

Interestingly, in immune cells, caveolae appear present only in cells of the myeloid lineage, but not in cells of the lymphoid lineage, with the exception of particular T cell leukemia cell lines and bovine lymphocytes ^{11, 12}. In MΦ, caveolin-1 was reported to exert anti-inflammatory effects upon inflammatory stimuli, with strong reductions in tumor necrosis factor α (TNF α), interleukin 6 (IL-6), and increased production of IL-10 ¹³. Caveolin-1 also appears to be an early marker of macrophage apoptosis ¹⁴. In neutrophils, deficiency of caveolin-1 decreases their oxidant production, their adhesion and transendothelial migration ¹⁵.

In recent years, caveolin-1 has been ascribed a causative role in several diseases, such as cancer ¹⁶, type 2 diabetes ¹⁷, obesity ⁹, hypertrophic cardiomyopathy ¹⁸, pulmonary hypertension and fibrosis ^{19, 20}, hypercalciuria, urolithiasis, retinal degenerative diseases and rheumatoid arthritis ^{1, 4}, but also in vascular pathologies such as neointima formation and atherosclerosis ²¹⁻²⁵.

The role of caveolin-1 in atherosclerosis is paradoxical. Although caveolin-1 is expressed in the majority of vascular related cell types (vascular smooth muscle cells (VSMCs), ECs, MΦ), its expression in human and rabbit atherosclerotic plaques decreases with disease severity ^{22, 26-28}. Moreover, loss of caveolin-1 in human plaques correlates with plaque vulnerability and provides a prognostic value for cardiovascular events ²². A similar phenotype was observed in models of neointima formation, in which loss of caveolin-1 accelerated lesion formation ²¹. However, in atherosclerotic mouse models,

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deficiency of caveolin-1 appeared atheroprotective as *Cav1^{-/-}Apoe^{-/-}* mice exhibited a 70% decrease in plaque area, despite the presence of hypercholesterolemia²³⁻²⁵. This phenotype was mainly attributed to defective transendothelial migration of LDL, which was confirmed in atherosclerotic mouse models with EC-specific over-expression of caveolin-1²³⁻²⁵.

Since caveolin-1 also plays a crucial role in the internalization of receptors important in inflammation, and thus, in their signal transduction²⁹, we investigated whether the immune system itself is affected by caveolin-1 deficiency and therefore affects atherosclerosis. Moreover, we aimed to investigate how plaque phenotype, inflammatory status, and leukocyte adhesion are changed during early and late stages of the disease and how cell type-specific loss of caveolin-1 impacts atherosclerosis. We show that total caveolin-1 deficiency decreases atherosclerosis and plaque inflammation. This is mediated by a reduced influx of leukocytes, and a systemic anti-inflammatory T lymphocyte profile. In addition, we show that especially the non-hematopoietic caveolin-1-expressing cells (ECs, VSMCs) determine plaque size, while hematopoietic caveolin-1-expressing cells mediate changes of the systemic and plaque-restricted immunological profile.

Methods

Animals

All animal procedures were approved by the animal experimental committee and performed in accordance with institutional guidelines. To generate *Cav1^{+/+}Apoe^{-/-}* and *Cav1^{-/-}Apoe^{-/-}* mice, *Cav1^{-/-}* mice (C57Bl6 background, Radu Stan, Dartmouth Medical School, Hanover, NH, USA) were backcrossed seven generations to *Apoe^{-/-}* mice (C57Bl6 background, Charles River, France) All mice were fed a standard fat diet (Cat# V1535, ssniff Spezialdiäten GmbH, Soest, Germany), had *ad libitum* access to food and water, and were housed under a 12 hour light-dark cycle. At the age of 17 or 26 weeks, male/female mice were sacrificed (n=15 for each genotype and time point) by an overdose of pentobarbital. Blood was subsequently obtained from the right ventricle and plasma was used for cholesterol measurements and lipoprotein profiles. Thereafter, the arterial tree was perfused through the left

ventricle with 10ml phosphate buffered saline (PBS, containing 0.1 mg/ml sodium nitroprusside) to achieve vessel relaxation and 10 ml 1% paraformaldehyde (PFA) for tissue fixation. The aortic arch and its main branch points (brachiocephalic trunk, left common carotid artery and left subclavian artery) were taken out and fixed overnight in a 1% PFA solution. Two additional groups of mice (male n=6, female n=5-6) were sacrificed for flow cytometric analysis of blood, spleen, and lymph nodes (pool of mesenteric, salivary and axil lymph nodes) at the age of 17 weeks. Mice were kept on the same protocol as mentioned above.

Histology and morphometry of atherosclerotic plaques

The aortic arch was embedded longitudinally in paraffin and cut into 40 consecutive 4µm sections (n=15 for each phenotype and time point). Twenty slides that best represented the three-dimensional structure of the arch were chosen for the additional immunohistochemical analysis. Four sections of the aortic arch (20µm apart) were stained with hematoxylin/eosin (HE) and the atherosclerotic lesions were classified as initial or advanced according to the Virmani classification³⁰. This procedure enabled us to calculate the individual plaque area per type (initial or advanced). Furthermore, we quantified the number of cells per lesion and the number of plaques per arch, by calculating the mean plaque number of the four HE stained sections, which can lead to a plaque number per arch <1. For each plaque, area and lipid core content was determined using a microscope coupled to a computerized morphometry system (Qwin 3.5, Leica, Wetzlar, Germany). Morphometric parameters were determined as previously described³¹.

Immunohistochemistry

Immunohistochemistry was performed on paraffin sections of the aortic arch (n=15 for each phenotype and time point): α-smooth muscle actin monoclonal antibody (1:500 dilution; Dako, Glostrup, Denmark) as a marker for SMC; CD3 polyclonal antibody (1:200 dilution, Dako, Glostrup, Denmark) to detect T lymphocytes; CD45 polyclonal antibody (1:5000 dilution, BD Bioscience, Bedford, MA, USA) to detect total leukocytes; Ly6G monoclonal antibody (1:200 dilution, BD Bioscience, Bedford, MA, USA) to detect Ly6G

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positive neutrophils; Mac3 monoclonal antibody (1:30 dilution, BD Bioscience, Bedford, MA, USA) to detect macrophages; Foxp3 monoclonal antibody (1:50 dilution, Ebioscience, San Diego, CA, USA) to detect regulatory T cells; Perl's iron staining (1:1 solution of 2g potassium hexacyanoferrate dissolved in 100ml distilled water and 54ml 37% concentrated hydrochloric acid in 1000ml distilled water) to detect bleeding; anti-von Willebrand factor (1:500 dilution, Dako, Glostrup, Denmark) to detect disrupted endothelium, and finally Caspase-3 polyclonal antibody (1:20 dilution, Cell Signaling, Danvers, MA, USA) to detect apoptotic cells. To determine the relative amount of T cells, leukocytes, macrophages, and regulatory T cells, the number of positive cells per lesion was divided by the total number of cells per lesion, as determined by number of nuclei per lesion. The number of Ly6G⁺ neutrophils was expressed as positive cells per mm² of plaque. The staining for SMCs and collagen was evaluated quantitatively with Qwin 3.5. Anti-caveolin-1 polyclonal antibody (1:800 dilution, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was used to localize caveolin-1 in the plaque and to prove the complete absence of caveolin-1 in *Cav1*^{-/-}*Apoe*^{-/-} mice. The results of the caspase-3 staining were divided into positive (staining visible) and negative (no staining at all) cases. The VCAM-1 staining was performed on frozen sections from mouse aortic roots, using a rat-anti-mouse monoclonal antibody (1:200 dilution, BD Bioscience, Bedford, MA, USA).

Bone marrow transplantation

Female *Cav1*^{+/+}*Apoe*^{-/-} and *Cav1*^{-/-}*Apoe*^{-/-} deficient mice (8-9 weeks old) were transferred to filter top cages and administered water containing antibiotics (polymyxine B sulphate 60000 U/l, neomycin 100mg/l) one week before the bone marrow transplantation (BMT). One day prior to the BMT, the mice were lethally irradiated (10Gy, 0.5Gy/min, Philips MU15F/225kV, Hamburg, Germany). The next day, the irradiated mice were intravenously injected with 10⁷ bone marrow cells of male *Cav1*^{+/+}*Apoe*^{-/-} and *Cav1*^{-/-}*Apoe*^{-/-} mice, respectively, generating four different groups (n=5 to 8 per group). Mice were maintained on the antibiotic water for an additional four weeks to restore bone marrow function. Following this recovery time, the mice were administered a western-type diet (0.21% cholesterol, Cat# 4621.06, Hope Farms, Woerden, The Netherlands). After 10 weeks of western-type diet, the

mice were sacrificed and aortic arches were processed and analyzed as described above. Additionally blood, spleen, and lymph nodes were taken for flow cytometric analysis.

Flow cytometry and blood count

Blood, spleen, and lymph nodes were collected, processed, and stained with fluorescent antibodies against CD3, CD4, CD8, CD25, Foxp3, B220, CD11b, Ly6C, Ly6G (all eBiosciences, San Diego, CA, USA), and analyzed as described previously ³² (n=5-8 for each group). Briefly, spleen and lymph nodes were homogenized, filtered through a 70µm mesh, and subjected to red blood cell lysis. All cells were stained in FACS buffer (1xPBS, 0.5% BSA, 0.01% NaN₃). Staining for blood count analyses was conducted using antibodies to CD45, CD115, Gr1, CD19, and CD3 (all eBiosciences, San Diego, CA, USA) in HBSS with 0.3 mM EDTA and 0.1 % BSA. Blood was subjected to red blood cell lysis. Cell counts were estimated utilizing CountBright™ absolute counting beads (Invitrogen, Carlsbad, USA). Disposables were purchased from Bio Greiner One. All flow cytometry analysis was performed on a BD Canto II (BD Bioscience, Bedford, MA, USA).

Cholesterol measurement and lipoprotein profiles

Plasma cholesterol levels were measured using a colorimetric assay (CHOD-PAP, Roche, Mannheim, Germany) (n=15 for each genotype and time point). Lipoprotein profiles were determined on pooled plasma samples using an AKTA Basic chromatography system with a Superose 6PC 3.2/30 column (Amersham Biosciences, Roosendaal, The Netherlands). Blood samples of mice were collected and the plasma of three mice (20µl each) with the same genotype was pooled. Samples were loaded onto the column. The plasma was passed over the columns at a flow rate of 0.5ml/min and 36 fractions were collected.

Quantitative polymerase chain reaction

RNA was isolated from aortic arches by homogenizing the tissue in trizol, followed by a purification step (nucleo spin RNAII, Marcherey & Nagel, Düren, Germany) and quantified using a Nanodrop 1000 (Thermo Fisher Scientific, Wilmington, DE, USA) (n=7 for each genotype). Total RNA (0.2 µg)

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was reverse transcribed to generate cDNA employing the SuperScript® VILO™ cDNA Synthesis Kit according to the instructions of the manufacturer (Invitrogen, Carlsbad, USA). Real-time PCR employing EXPRESS SYBR® GreenER™ qPCR SuperMix (Invitrogen, Carlsbad, USA) and primers as indicated in **Table 1** was carried out on an ABI 7900 HT Fast RT-PCR system (Applied Biosystems, Foster City, CA, USA). The reactions were performed in duplicate utilizing cDNA corresponding to 5ng RNA. Cycling conditions were as follows: 95°C for 20s followed by 40 cycles of 95°C for 5s and 60°C for 20s. Specificity was confirmed by appearance of a single peak in a final dissociation step (60°C - 95°C, 2% ramp rate) Data were analyzed on the basis of the relative expression method with the formula $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = \Delta C_T (\text{sample}) - \Delta C_T (\text{calibrator})$, and ΔC_T is the C_T of the housekeeping gene (glyceraldehyde-3-phosphate dehydrogenase, GAPDH) subtracted from the C_T of the target gene.

Table 1: Primers for quantitative real-time PCR		
Gene	Forward	Reverse
Ccl2	GCT GGA GAG CTA CAA GAG GAT CA	TCT CTC TTG AGC TTG GTG ACA AAA
Ccl3	GAC TAT TTT GAA ACC AGC AGC CTT T	GAT CTG CCG GTT TCT CTT AGT CA
Ccl4	GAA GCT TTG TGA TGG ATT ACT ATG AGA	GTC TGC CTC TTT TGG TCA GGA A
Ccl5	GGA GTA TTT CTA CAC CAG CAG CAA	GCG GTT CCT TCG AGT GAC A
Ccr1	CAG AAA CAA AGT CTG TGT GGA CAA A	TGT GAA ATC TGA AAT CTC CAT CCT T
Ccr2	CAG GTG ACA GAG ACT CTT GGA ATG	GAA CTT CTC TCC AAC AAA GGC ATA A
Ccr3	TTG AAG TGA GGT CTG AGC ATC AA	AAC GCA TCA CAG TTA CAA CAT AAT TCT
Ccr5	TCC TGA AAG GCG GCT GTA AA	GCA GTC AGG CAC ATC CAT AGA C
Icam1	GGA CCA CGG AGC CAA TTT C-	CTC GGA GAC ATT AGA GAA CAA TGC
Vcam1	TGCCGAGCTAAATTACACATTG	CCT TGT GGA GGG ATG TAC AGA
p-selectin	GGT ATC CGA AAG ATC AAC AAT AAG TG	GTT ACT CTT GAT GTA GAT CTC CAC ACA
Gapdh	ATT GTC AGC AAT GCA TCC TG	ATG GAC TGT GGT CAT GAG CC

ELISA

CCL-2 levels in sera were analyzed by using a commercial enzyme-linked immunosorbent assay (ELISA) according to the protocol provided by the manufacturer (R&D Systems, Minneapolis, MN, USA).

Intravital microscopy

Intravital microscopy of the left carotid artery was performed as described previously^{33, 34}. Leukocyte adhesion was visualized by i.v. injection of rhodamine 6G. For luminal detection of chemokines presented on the endothelium, 50µl of Protein G Fluoresbrite® YG Microspheres (Polysciences Inc., Warrington, PA, USA) were coupled to 50µg of polyclonal antibodies to CCL2 (n=6 mice/genotype for leukocyte adhesion and n=7 mice/genotype for anti CCL-2 directed beat adhesion). Beads and antibodies were incubated for 30 min at room temperature, washed twice, and subsequently injected i.v. after exposure of the external carotid. Antibody/bead complexes were allowed to circulate for 15 min and immobilized complexes were detected by intravital microscopy using an Olympus BX51 microscope equipped with a Hamamatsu 9100-02 EMCCD camera (Hamamatsu Photonics, Hamamatsu City, Japan) and a 10x saline-immersion objective. For image acquisition and analysis, Olympus cell^r software (Olympus, Hamburg, Germany) was used.

Statistical Analysis

All data are expressed as means ± SEM. The non-parametric Mann-Whitney U-test was used to analyze all mouse data (*Cav1*^{+/+}*Apoe*^{-/-} mice were compared to *Cav1*^{-/-}*Apoe*^{-/-} mice) and quantitative real time PCR results. Fisher's exact test was used to analyze the caspase-3 staining. Data were considered statistically significant at $P < 0.05$.

Results

Caveolin-1 expression in atherosclerosis

Caveolin-1 was expressed in all stages of murine atherosclerosis, ranging from intimal xanthomas to fibrous cap atheromata. Interestingly, as reported for human plaques, the expression of caveolin-1 decreased with lesion progression, suggesting that loss of caveolin-1 indicates plaque progression (Fig. 1A, B). Most of the caveolin-1 protein was present in ECs, but also in SMCs and MΦ.

Figure 1. Caveolin-1 expression decreases with plaque progression.

A) Caveolin-1 is highly expressed in intimal xanthomas. B) Caveolin expression decreases during plaque progression and its expression is minimal in fibrous cap atheromas. C) Plasma cholesterol levels and D) lipoprotein profiles of *Cav1*^{+/+} *Apoe*^{-/-} and *Cav1*^{-/-} *Apoe*^{-/-} mice. Scale bars = 100 μ m. Values are means $\pm$ SEM. * $P < 0.05$; *** $P < 0.001$.

Caveolin-1 deficiency increases lipid levels but dramatically decelerates plaque progression

Plasma cholesterol levels were substantially increased in the *Cav1^{-/-}Apoe^{-/-}* mice after 17 and 26 weeks on a normal chow diet, corroborating previous studies (**Fig. 1C**)^{23, 24}. Gel filtration chromatography revealed that cholesterol content was especially increased in the very low density lipoprotein- (VLDL) and LDL-sized fractions. These findings are remarkable as it has been reported that *Cav1^{-/-}* mice display an impaired VLDL production³⁵. Nevertheless, a disturbed degradation of the VLDL particles might cause the elevated VLDL levels. The involvement of caveolin-1 in insulin signaling could be part of the mechanism behind this phenomenon. No changes were observed in the high density lipoprotein (HDL) fraction (**Fig. 1D**).

To analyze the effect of caveolin-1 deficiency on atherosclerotic plaque initiation and progression, we examined two experimental groups sacrificed after 17 and 26 weeks on normal chow diet. At 17 weeks of age, aortic arches of the mice exhibited early signs of atherosclerosis with plaques predominantly composed of MΦ foam cells (**Fig. 2A, B**). At 26 weeks of age, atherosclerosis had progressed towards an advanced stage with the majority of plaques containing a lipid core and/or a fibrous cap (**Fig. 2C, D**). The lower number of initial plaques in the aortic arch of 26 week old mice can be explained by the observed atherosclerosis progression and the fact that different initial plaques can fuse over time. Despite the dramatic increase in plasma cholesterol in the VLDL and LDL fraction (**Fig. 1C, D**), lack of caveolin-1 reduced total plaque area in the aortic arch 6.9- to 15-fold (**Fig. 2E**). This was paralleled by a decrease in the number of plaques per aortic arch in *Cav1^{-/-}Apoe^{-/-}* mice (**Fig. 2F**) and by a dramatic decrease in plaque area of individual advanced plaques (**Fig. 2G**), indicating that not only plaque initiation, but also progression, is inhibited in the absence of caveolin-1.

Similar results were obtained using female mice and no differences compared to male mice with respect to the above mentioned parameters were observed (Supplemental Table 1a).

Figure 2. *Cav1^{-/-}Apoe^{-/-}* mice exhibit elevated plasma cholesterol levels but are protected from atherosclerosis.

A-D) HE stained sections of atherosclerotic plaques in the aortic arch of *Cav1^{+/+}Apoe^{-/-}* and *Cav1^{-/-}Apoe^{-/-}* mice. Panels A, B represent intimal xanthomas of 17-wk-old mice, whereas panels C, D display fibrous cap atheromas of 26-wk-old mice, showing that these plaques are smaller in *Cav1^{-/-}Apoe^{-/-}* mice. All mice were fed a normal chow diet. E) Total plaque area in the aortic arch of *Cav1^{+/+}Apoe^{-/-}* and *Cav1^{-/-}Apoe^{-/-}* mice. Values represent mean plaque area for each mouse. F) Mean number of plaques in the aortic arch of each mouse. G) Mean size of initial and advanced plaques in the aortic arch of *Cav1^{+/+}Apoe^{-/-}* and *Cav1^{-/-}Apoe^{-/-}* mice (n=12-15 animals/group). Scale bars = 100 μm . ini, initial plaques (intimal xanthomas and pathological intimal thickenings); adv, advanced plaques (fibrous cap atheromas). Values are means $\pm$ SEM. ** P < 0.01; *** P < 0.001.

Caveolin-1 deficiency impairs leukocyte adherence to the endothelium and abolishes plaque inflammation

An initial step in atherosclerosis development is the adhesion and extravasation of leukocytes through the EC layer. To investigate whether leukocyte adhesion to the endothelium differs in *Cav1^{+/+}Apoe^{-/-}* and *Cav1^{-/-}Apoe^{-/-}* mice, we performed intravital microscopy on the carotid artery

bifurcation in rhodamine 6G-injected mice. Although there was no effect on leukocyte rolling (*Cav1^{+/+}Apoe^{-/-}* 29.8 ± 9.0 vs. *Cav1^{-/-}Apoe^{-/-}* 33.0 ± 14.8 cells/field), significantly fewer leukocytes adhered to the endothelium of *Cav1^{-/-}Apoe^{-/-}* mice compared to their wild type controls (**Fig. 3A**).

Injection of antibody-coated beads directed against CCL-2 demonstrated that *Cav1^{-/-}Apoe^{-/-}* mice had a decreased presentation of CCL-2 at the endothelial-monocyte interface (**Fig. 3B**). Interestingly, CCL-2 mRNA levels within the plaque (*Cav1^{+/+}Apoe^{-/-}* 1.4 ± 0.3 vs. *Cav1^{-/-}Apoe^{-/-}* 0.9 ± 0.2 , **Table 2**) or plasma CCL-2 levels were not different (*Cav1^{+/+}Apoe^{-/-}* 169.2 ± 28.2 vs. *Cav1^{-/-}Apoe^{-/-}* 101.6 ± 33.9 , Supplemental Table 1b). In addition, VCAM-1 was almost absent on the endothelium of *Cav1^{-/-}Apoe^{-/-}* mice (**Fig. 3C**), whereas inter-cellular adhesion molecule-1 (ICAM-1) expression was not different (**Table 2**).

Our results clearly show that caveolin-1 plays a crucial role in leukocyte adhesion and extravasation into the arterial wall, particularly by affecting endothelial VCAM-1 expression and CCL-2 presentation at the leukocyte-endothelial interface. The decreased plaque progression was accompanied by changes in plaque composition. In advanced plaques of 26 week old *Cav1^{-/-}Apoe^{-/-}* mice, we observed a significant 43% decrease in relative MΦ content compared to *Cav1^{+/+}Apoe^{-/-}* animals (*Cav1^{+/+}Apoe^{-/-}* 47.5 ± 10.1 vs. *Cav1^{-/-}Apoe^{-/-}* 27.3 ± 10.3 , $p < 0.05$) (**Fig. 3D**). The absolute number of CD3⁺ T cells was lower in plaques of *Cav1^{-/-}Apoe^{-/-}* mice after 26 weeks (*Cav1^{+/+}Apoe^{-/-}* 4.5 ± 0.7 vs. *Cav1^{-/-}Apoe^{-/-}* 2.7 ± 0.7 , $p < 0.05$) of normal chow diet (**Fig. 3E**). In addition, plaque infiltration by neutrophils was impaired in young and old *Cav1^{-/-}Apoe^{-/-}* mice fed a normal chow diet (17 weeks: *Cav1^{+/+}Apoe^{-/-}* 70.3 ± 10.5 vs. *Cav1^{-/-}Apoe^{-/-}* 56.5 ± 10.9 , $p < 0.05$; 26 weeks: *Cav1^{+/+}Apoe^{-/-}* 21.6 ± 3.3 vs. *Cav1^{-/-}Apoe^{-/-}* 1.2 ± 0.3 , $p < 0.05$) with far less neutrophils present in plaques of old mice (**Fig. 3F**). Interestingly, *Cav1^{-/-}Apoe^{-/-}* mice displayed less caspase-3 positive cells in their plaques after 17 and 26 weeks of normal chow diet (17 weeks: *Cav1^{+/+}Apoe^{-/-}* 13 cases vs. *Cav1^{-/-}Apoe^{-/-}* 2 cases, $p < 0.001$; 26 weeks: *Cav1^{+/+}Apoe^{-/-}* 14 cases vs. *Cav1^{-/-}Apoe^{-/-}* 1 case, $p < 0.001$), suggesting that caveolin-1 deficiency prevents apoptosis. Additional stainings for collagen (Sirius red), α -smooth muscle actin (ASMA), regulatory T cells, disrupted epithelium (von Willebrand factor) and iron deposition (Perl's iron) revealed no

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differences between both groups (Supplemental Table 2). These data show that caveolin-1 deficiency prevents plaque inflammation and apoptosis.

Figure 3. Caveolin-1 loss impairs leukocyte adhesion and results in less inflamed plaques.

A, B Left panels: quantitative analysis of intra vital microscopy of leukocyte adherence (**A**) and CCL-2 antibody coated beads adherence (**B**) to the endothelium in the carotid artery bifurcation of *Cav1^{+/+}Apoe^{-/-}* and *Cav1^{-/-}Apoe^{-/-}* mice, showing that less leukocytes as well as beads adhere to the endothelium of *Cav1^{-/-}Apoe^{-/-}* mice. Right panels: representative micrographs (n=5-6 animals/group). **C** Aortic root cross sections stained for VCAM-1 with abundant expression in *Cav1^{+/+}Apoe^{-/-}* but not in *Cav1^{-/-}Apoe^{-/-}* mice. **D-F** Quantitative analysis (left panels) and representative images (right panels) of macrophage content (**D**), CD3⁺ T cell content (**E**), and Ly6G positive neutrophil content (**F**) in advanced plaques of 26-wk-old *Cav1^{+/+}Apoe^{-/-}* and *Cav1^{-/-}Apoe^{-/-}* mice (n=12-15 animals/group). Scale=100µm. ini, initial plaques (intimal xanthomas and pathological intimal thickenings); adv, advanced plaques (fibrous cap atheromas). Values are means ± SEM. * P < 0.05; **P < 0.01.

Table 2: Results of the quantitative real time PCR

Gene	<i>Cav1^{+/+}Apoe^{-/-}</i>	<i>Cav1^{-/-}Apoe^{-/-}</i>	P
<i>Ccl2</i>	1.37 ± 0.28	0.93 ± 0.24	NS
<i>Ccl3</i>	1.82 ± 0.47	0.72 ± 0.13	< 0.05
<i>Ccl4</i>	2.11 ± 0.81	0.66 ± 0.10	< 0.05
<i>Ccl5</i>	1.35 ± 0.32	0.85 ± 0.07	NS
<i>Ccr1</i>	1.07 ± 0.13	0.97 ± 0.04	NS
<i>Ccr2</i>	0.90 ± 0.08	1.22 ± 0.19	NS
<i>Ccr3</i>	0.90 ± 0.10	1.17 ± 0.12	NS
<i>Ccr5</i>	1.31 ± 0.19	0.82 ± 0.10	< 0.05
<i>Icam1</i>	1.07 ± 0.12	0.97 ± 0.06	NS
<i>Vcam1</i>	1.02 ± 0.15	1.06 ± 0.12	NS
<i>P-selectin</i>	0.88 ± 0.10	1.22 ± 0.12	NS

All values are means ± SEM of relative gene expression normalized to the housekeeping gene *GAPDH*. NS, not significant.

Caveolin-1 deficiency increases the regulatory T cell population

To assess the mechanism by which caveolin-1 affects immune cell numbers or subset distribution, we analyzed blood, spleen, and lymph nodes of *Cav1^{+/+}Apoe^{-/-}* and *Cav1^{-/-}Apoe^{-/-}* mice using flow cytometry. Blood counts for T cells, B cells, neutrophils, and monocytes revealed no differences between the two genotypes (**Fig. 4**). Lymph nodes of *Cav1^{-/-}Apoe^{-/-}* mice showed a relative increase in their CD3⁺ T cells (+17%; $p < 0.05$), CD8⁺ cytotoxic T cells (+6%; $p < 0.05$) and CD4⁺CD25⁺Foxp3⁺ regulatory T cells (+37%; $p < 0.01$), but a reduction in their CD4⁺ effector T cells (-4%; $p < 0.05$; **Fig. 5A-D**), indicating that caveolin-1 deficiency changes T cell homeostasis towards an anti-inflammatory, regulatory T cell profile. Analysis of blood and spleen samples revealed similar results. No differences could be found in blood, spleen, and lymph node samples concerning B cells, monocyte subsets (Ly6C^{high/low}) and granulocytes. These data show that, since no regulatory T cells could be detected in the atherosclerotic plaques, caveolin-1 deficiency induces a regulatory T cell profile, which, probably together with regulatory T cells in the adventitia, contributes to the observed anti-inflammatory plaque phenotype of *Cav1^{-/-}Apoe^{-/-}* mice.

Figure 4. Blood counts do not differ between *Cav1*^{+/+}*Apoe*^{-/-} and *Cav1*^{-/-}*Apoe*^{-/-} mice.

Graphs show the amount of T and B cells, neutrophils, monocytes as well as monocyte subsets, in blood of *Cav1*^{+/+}*Apoe*^{-/-} and *Cav1*^{-/-}*Apoe*^{-/-} mice (n=6/group).

Figure 5. Caveolin-1 loss leads to an anti-inflammatory lymphocyte profile.

Quantitative analysis of the percentages of CD3⁺ T cells (A), CD4⁺ helper T cells (B), CD8⁺ cytotoxic T cells (C) and CD4⁺/CD25⁺/Foxp3⁺ regulatory T cells (D) of 17-wk-old *Cav1*^{+/+}*Apoe*^{-/-} and *Cav1*^{-/-}*Apoe*^{-/-} mice. Lymph node samples were analyzed using flow cytometry (n=6/group). Values are means ± SEM. * P < 0.05; ** P < 0.01.

The intricate interplay of hematopoietic and non-hematopoietic caveolin-1 in atherosclerosis

As our results showed that total loss of caveolin-1 leads to athero-protection and changes in immune cell subsets we aimed to investigate whether loss of caveolin-1 in hematopoietic or non-hematopoietic cells is responsible for the observed phenotype in *Cav1^{-/-}Apoe^{-/-}* mice. To this end, we created bone marrow chimeras by reciprocal BMT. *Cav1^{-/-}Apoe^{-/-}* recipients that received either *Cav1^{+/+}Apoe^{-/-}* or *Cav1^{-/-}Apoe^{-/-}* bone marrow showed a 1.3 to 1.6 fold increase in plasma cholesterol levels compared to the *Cav1^{+/+}Apoe^{-/-}* recipients (**Fig. 6A**), while *Cav1^{+/+}Apoe^{-/-}* recipients did not show elevated cholesterol levels. This suggests that caveolin-1 in non-hematopoietic cells is responsible for cholesterol homeostasis.

Similar to the *Cav1^{-/-}Apoe^{-/-}* mice, all *Cav1^{-/-}Apoe^{-/-}* recipients developed fewer and remarkably smaller plaques compared to *Cav1^{+/+}Apoe^{-/-}* recipients, regardless of whether they received *Cav1^{+/+}Apoe^{-/-}* or *Cav1^{-/-}Apoe^{-/-}* bone marrow ($p < 0.01$, 0.05 respectively; **Fig. 6B, C, Table 3**). Flow cytometric analysis of blood, spleen, and lymph nodes showed that reconstitution of *Cav1^{-/-}Apoe^{-/-}* recipients with *Cav1^{+/+}Apoe^{-/-}* bone marrow increased the number of CD4⁺ effector T cells compared to reconstitution with *Cav1^{-/-}Apoe^{-/-}* bone marrow (*Cav1^{+/+}Apoe^{-/-}* reconstituted 64.0 ± 1.28 vs. *Cav1^{-/-}Apoe^{-/-}* reconstituted 61.0 ± 0.63), and slightly decreased the number of regulatory T cells (**Fig. 7**).

These data suggest that the non-hematopoietic caveolin-1 compartment is responsible for plaque growth and trans-endothelial migration of macrophages, while (the combination of non-hematopoietic and) hematopoietic caveolin-1 appears to be required for immune modulation in lymphoid organs and atherosclerotic plaques.

Caveolin-1 deficiency decreases atherosclerosis

Figure 6. Non-hematopoietic caveolin-1 loss confers protection against atherosclerosis.

A) Plasma cholesterol levels of *Cav1^{+/+}Apoe^{-/-}* and *Cav1^{-/-}Apoe^{-/-}* mice, which received either *Cav1^{+/+}Apoe^{-/-}* or *Cav1^{-/-}Apoe^{-/-}* bone marrow. B-C) Number (B) and quantification (C) of plaques in the aortic arch of bone marrow transplanted mice (n=6-8 animals/group). ini, initial plaques (intimal xanthomas and pathological intimal thickenings); adv, advanced plaques (fibrous cap atheromas). Values are means $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$.

Figure 7. Non-hematopoietic caveolin-1 loss rather than higher regulatory T cell numbers protect against atherosclerosis in *Cav1*^{-/-}*Apoe*^{-/-} mice.

Quantitative analysis of the percentages of CD3⁺ T cells (A), CD4⁺ helper T cells (B), CD8⁺ cytotoxic T cells (C) and CD4⁺CD25⁺FoxP3⁺ regulatory T cells (D) of the bone marrow transplanted *Cav1*^{+/+}*Apoe*^{-/-} and *Cav1*^{-/-}*Apoe*^{-/-} mice. Lymph node samples were analyzed using flow cytometry (n=5-8 animals/group). *Cav1*^{+/+}*Apoe*^{-/-}, transplanted with *Cav1*^{+/+}*Apoe*^{-/-} bone marrow; *Cav1*^{-/-}*Apoe*^{-/-}, transplanted with *Cav1*^{-/-}*Apoe*^{-/-} bone marrow. Values are means ± SEM. * P < 0.05.

Table 3: Characteristics of bone marrow chimeras (*p<0.05, **p<0.01; *Cav1*^{+/+→+/-} vs *Cav1*^{+/+→-/-}, *Cav1*^{+/+→+/-} vs. *Cav1*^{-/-→-/-} and *Cav1*^{-/-→+/-} vs. *Cav1*^{-/-→-/-})

	<i>Cav1</i> ^{+/+→+/-}	<i>Cav1</i> ^{-/-→+/-}	<i>Cav1</i> ^{+/+→-/-}	<i>Cav1</i> ^{-/-→-/-}
plasma cholesterol (mmol/l)	27.7 ± 11.3	22.5 ± 9.2	34.7 ± 12.3 *	35.7 ± 13.5 *
plaque size (μm ² x 10 ⁵)	5.3 ± 1	4.7 ± 0.8	1.0 ± 0.4 **	1.0 ± 0.3 **
initial plaque size (μm ² x 10 ⁵)	0.2 ± 0.1	0.3 ± 0.1	0.1 ± 0.02	0.1 ± 0.02 *
advanced plaque size (μm ² x 10 ⁵)	1.5 ± 0.3	1.5 ± 0.2	0.5 ± 0.1 **	0.8 ± 0.3
plaque number / aortic arch	4.3 ± 1.7	4.1 ± 1.7	2.7 ± 0.9 *	2.1 ± 0.8 *
lipid core area (% of total)	25.8 ± 4.6	33 ± 3.7	20.3 ± 3.1	18.2 ± 6.6 *
CD45 ⁺ cells (% of total)	19.3 ± 4	6.4 ± 1.3	7 ± 1.5	25.2 ± 6.1 **
CD3 ⁺ cells(% of total)	11.6 ± 2.2	7 ± 1.4	9 ± 1.9	20.6 ± 5.3 **
Haemorrhage	yes	no	no	no
Chondrocytes	yes	no	yes	yes

All values are means ± SEM. * P < 0.05; ** P < 0.01. All values were compared to *Cav1*^{+/+→+/-} mice.

Discussion

In recent years, caveolae have been subject to intensive research in cardiovascular science, and the analysis of caveolin-1 deficient mice has revealed many of its functions. Besides mediating vascular homeostasis (e.g. inhibition of eNOS ⁷, regulation of cellular Ca²⁺ entrance ³⁶, short- and long-term mechanotransduction ³⁷, regulation of microvascular permeability ³⁸), caveolin-1 also has profound effects in atherosclerosis.

The groups of Lisanti and Sessa were the first to show that deficiency of caveolin-1 decreased atherosclerosis, despite elevating plasma lipid levels. Recent studies especially stressed the importance of endothelial-derived caveolin-1 in the pathogenesis of atherosclerosis. *Cav1*^{-/-}*Apoe*^{-/-} mice that were reconstituted with a transgene containing canine caveolin-1 under a preproendothelin-1 promotor have similar atherosclerosis levels and plaque features compared to control *Apoe*^{-/-} mice ²⁴. Furthermore, *Apoe*^{-/-} mice containing the respective transgene, exhibit features of accelerated atherosclerosis compared to control *Apoe*^{-/-} mice ²⁵. Mechanistically, loss of (endothelial cell) caveolin-1 resulted in impaired transendothelial LDL transport ^{5, 24, 25}, promoted NO production ^{7, 39, 40}, and reduced the expression of adhesion molecules ⁴¹⁻⁴³.

However, the effect of caveolin-1 on immune cells in atherosclerosis has not been addressed before. In the present paper, we show for the first time that caveolin-1 has two divergent functions in modulating atherosclerosis. The first function is clearly dependent on endothelial-cell caveolin-1, where caveolin-1 mediates the expression of VCAM, and affects deposition of CCL-2 at the endothelial cell/monocyte interface. This results in decreased leukocyte adhesion to the arterial wall in *Cav1^{-/-}Apoe^{-/-}* mice, and consequently, in smaller atherosclerotic plaques.

The second function of caveolin-1 in atherosclerosis is related to the hematopoietic compartment. Plaques of *Cav1^{-/-}Apoe^{-/-}* mice contain less macrophages, neutrophils and T cells. This can be caused by the reduced expression of CCL-3, CCL-4 and CCR-5 on immune cells, which will consequently impair their chemotactic capacity and immune reactivity. Surprisingly, CCL-2 mRNA levels were not affected, whereas the deposition of CCL-2 on endothelial cells was decreased in *Cav1^{-/-}Apoe^{-/-}* mice. This can be explained by the dependency of leukocyte adhesion molecule expression on caveolae and the association of caveolin-1 with CCL-2 presentation and distribution⁴². Our results are in line with these findings and underline the CCL-2/CCR-5 dependent contributions to neutrophil infiltration into early atherosclerotic plaques reported by Drechsler et al.³⁴.

Interestingly, *Cav1^{-/-}Apoe^{-/-}* mice exhibit enhanced numbers of regulatory T cells in blood, spleen, and lymph nodes, as well as reduced numbers of CD4⁺ effector T cells, an immunological phenotype associated with protection against atherosclerosis⁴¹. In human plaques, however, loss of caveolin-1 expression is associated with atherosclerotic plaque vulnerability, and with plaques containing elevated levels of inflammatory cells, IL-6, IL-8, and matrix metalloproteinase-9 (MMP-9)²². This discrepancy might be explained by the fact that the cellular composition of plaques changes during plaque progression, that certain cell types have different numbers of caveolae, and that caveolin-1 exerts divergent actions in different cell types.

Our reciprocal bone marrow transplantations show that both endothelial and hematopoietic caveolin-1 is important in the pathogenesis of atherosclerosis. Non-hematopoietic caveolin-1 critically determines plaque mass, whereas hematopoietic caveolin-1 especially mediates plaque phenotype and affects the systemic immune system.

Until now, only scattered and paradoxical information has existed on the function of caveolin-1 in inflammation and in the immune system. Caveolin-1 is involved in sequestering P42/44 MAPK members, signaling proteins involved in cytokine production, thereby preventing inflammation⁴⁴. Likewise, caveolin-1 can bind toll-like receptor 4 (TLR-4) and prevent the release of TNF α and IL-6⁴⁵. Furthermore, cyclooxygenase-2 (Cox-2) binds to caveolin-1 at the endoplasmic reticulum leading to its rapid degradation⁴⁶. In M Φ , caveolin-1 acts as an immune modulator upon stimulation with lipopolysaccharide (LPS), thereby suppressing the release of TNF α and IL-6, and inducing the anti-inflammatory cytokine IL-10^{13, 47}. On the other hand, caveolin-1 is also involved in pro-inflammatory actions. Caveolin-1 is reported to enhance the oxidant production, adhesion capacities, and transendothelial migration of neutrophils¹⁵, to increase apoptosis in M Φ ¹⁴, and to induce antigen-specific T cell proliferation and activation via interaction with the co-stimulatory molecule CD26⁴⁸. Caveolin-1 plays an important role during bacterial infections, in which it promotes the entrance of bacteria into host cells causing alterations in innate immunity and activating inflammatory responses⁴⁹.

In our study, we could elucidate the function of hematopoietic caveolin-1 and showed that in atherosclerosis caveolin-1 deficiency leads to an anti-inflammatory state. Our results highlight the important role for caveolae in atherosclerosis. Although the major role for caveolin-1 in atherosclerosis was heretofore considered to be the transporting of LDL into the vascular wall, our study reveals a clear function of caveolin-1 in mediating inflammatory and immunological actions in vascular disease. These findings may allow for the development and investigation of new drugs that target caveolin-1. Nonetheless, the precise actions of caveolin-1 in immune cells have been unknown to date and should still be subject to further investigation.

Supplemental Tables

Supplemental Table 1a :Characteristics of male and female *Cav1^{+/+}Apoe^{-/-}* and *Cav1^{-/-}Apoe^{-/-}* mice

	<i>Cav1^{+/+}Apoe^{-/-}</i>	<i>Cav1^{-/-}Apoe^{-/-}</i>	<i>Cav1^{+/+}Apoe^{-/-}</i>	<i>Cav1^{-/-}Apoe^{-/-}</i>
	Male		Female	
plaque size ($\mu\text{m}^2 \times 10^5$)	0.47 $\pm$ 0.12	0.07 $\pm$ 0.02	0.53 $\pm$ 0.19	0.17 $\pm$ 0.08
individual plaque area ($\mu\text{m}^2 \times 10^5$)	0.15 $\pm$ 0.04	0.04 $\pm$ 0.01	0.12 $\pm$ 0.04	0.07 $\pm$ 0.02
plaque number / aortic arch	2.10 $\pm$ 0.21	1.30 $\pm$ 0.14	2.10 $\pm$ 0.26	2.00 $\pm$ 0.41
CD3 ⁺ cells (% of total cells)	4.15 $\pm$ 0.81	3.96 $\pm$ 1.57	7.26 $\pm$ 2.57	7.88 $\pm$ 4.36
Mac3 ⁺ cells (% total cells)	49.7 $\pm$ 3.85	47.8 $\pm$ 6.64	56.7 $\pm$ 5.97	31.9 $\pm$ 11.4
Plasma cholesterol content (mmol/l)	12.4 $\pm$ 0.79	16.7 $\pm$ 1.44	11.2 $\pm$ 1.84	13.5 $\pm$ 4.54
All values are mean $\pm$ SEM. No significant differences have been observed between male and female mice.				

Supplemental Table 1b: Raw data of plasma CCL-2 levels

sample	<i>Cav1^{+/+}Apoe^{-/-}</i>	<i>Cav1^{-/-}Apoe^{-/-}</i>
1	84.00	98.43
2	63.57	87.43
3	68.43	53.00
4	87.29	17.29
5	55.71	392.43
6	56.14	57.14
7		86.29
8		83.29
9		39.14
mean $\pm$ SEM	69.19 $\pm$ 28.24	101.60 $\pm$ 33.87

Supplemental Table 2: Plaque phenotypic characteristics of 17 and 26 week old mice (All values are mean $\pm$ SEM. * $p < 0.05$; * $p < 0.001$ compared to *Cav1^{+/+}Apoe^{-/-}*)**

	17 weeks		26 weeks	
	<i>Cav1^{+/+}Apoe^{-/-}</i>	<i>Cav1^{-/-}Apoe^{-/-}</i>	<i>Cav1^{+/+}Apoe^{-/-}</i>	<i>Cav1^{-/-}Apoe^{-/-}</i>
CD45 (% of total cells)	5.6 $\pm$ 1.1	7.2 $\pm$ 2	5 $\pm$ 0.7	4.1 $\pm$ 1.5
CD3 (% of total cells)	4.2 $\pm$ 0.9	4.1 $\pm$ 1.6	4.5 $\pm$ 0.6	2.7 $\pm$ 1.2*
Mac3 (% of total cells)	65 $\pm$ 13.4	52 $\pm$ 7.6	48.2 $\pm$ 4.3	41.1 $\pm$ 5.7
Mac3 (% of total cell/ini plaque)	-	-	43.2 $\pm$ 3.8	53.2 $\pm$ 7.5
Mac3 (% of total cells/adv plaque)	-	-	47.5 $\pm$ 4.6	27.3 $\pm$ 5*
LC (% of total area)	8.5 $\pm$ 4.8	6.7 $\pm$ 2.4	15.7 $\pm$ 4.2	4.5 $\pm$ 2.9
Caspase-3	13 cases	2 cases***	14 cases	1 case***
SR (% of total plaque area)	24.7 $\pm$ 10.1	19.4 $\pm$ 11.2	42.5 $\pm$ 9.8	50.8 $\pm$ 5.6
α SMA (% of total plaque area)	9.3 $\pm$ 4.2	26.44 $\pm$ 26.44	11 $\pm$ 2.9	12.9 $\pm$ 5.7
Tregs	no positive cells	no positive cells	no positive cells	no positive cells
FVIII	no disruption	no disruption	no disruption	no disruption
Perl's iron	no staining	no staining	no staining	no staining

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Caveolin-1 deficiency decreases atherosclerosis

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Chapter 3

The immunobiology of CD154–CD40– TRAF interactions in atherosclerosis

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Abstract

Atherosclerosis is a chronic disease of the large arteries that is responsible for the majority of cardiovascular events. In its pathogenesis, the immune system plays a pivotal role. The effectuation of the immune response through interactions between immune cells, which is mediated by co-stimulatory molecules, determine atherosclerosis severity. This review will highlight the role of one of the most powerful co-stimulatory dyads, the CD154 also known as CD40 ligand (CD40L)-CD40 dyad, in atherosclerosis. Its cell type specific actions, signal transduction cascades and its therapeutic potential will be discussed.

Introduction

Atherosclerosis is the most common underlying pathology of cardiovascular diseases like myocardial infarction, stroke and peripheral arterial disease ¹. In histology, atherosclerosis is characterized by the accumulation of lipids, calcifications, extracellular matrix and immune cells in the arterial wall, the so-called 'plaque'. Atherosclerotic plaques predominantly exist in large- and medium-sized arteries ². Atherosclerosis is considered as normal 'wear and tear' of the arteries, and becomes symptomatic when plaques that contain a high amount of inflammatory cells and have low levels of extracellular matrix, the 'thin fibrous cap atheromas', have formed. This plaque-stage is prone to 'plaque rupture'. Rupture of an atherosclerotic plaque exposes its thrombogenic components to the blood and results in thrombosis and often in acute occlusion of the respective artery ².

Although retention and modification of low-density lipoprotein in the arterial wall form the basis of the onset of atherosclerosis, the subsequent activation of the immune system has been proven to play a pivotal role in its pathogenesis. All cells of the innate and adaptive immune system, such as macrophages, mast cells, natural killer cells, T and B lymphocytes and dendritic cells, have been detected in the atherosclerotic plaque, and can exert pro- or anti-atherogenic functions ^{1,3,4}.

Especially the interactions between different immune cells and their bidirectional signaling define the inflammatory activity within the atherosclerotic plaque. These interactions also mediate the differentiation of different T lymphocyte subsets, such as Th1 (TNF α , IL-1, IFN γ , IL-12), Th2 (IL-4, IL-5, IL-10), Tregs (regulatory T cells) (TGF β , IL-10) and Th17 (IL-17, IL-6, IL-23), which have been shown to minutely regulate immune responses in the plaque. The Th1 and Th17 subsets are generally pro-atherogenic, while the Th2 and Treg subsets exert anti-atherogenic functions ⁵. Polarization of naive T cells into effector T cells is regulated by interaction with antigen presenting cells (APCs: DCs, macrophages, B lymphocytes), both in the plaque and in lymphoid organs. Upon interaction with antigen presenting cells, naive T cells undergo clonal expansion and differentiate into effector T cells. Moreover, the APCs undergo maturation, and start producing pro-atherogenic cytokines, such as IL-12.

Antigen dependent activation of naive T cells, and their differentiation into the pro-atherogenic effector T cell are enhanced by co-stimulation or regulated through co-inhibition. Not surprisingly, most of the characterized co-stimulatory and co-inhibitory receptor-ligand pairs are expressed in atherosclerotic lesions ^{6, 7} and have a divergent role in atherosclerotic plaque formation ⁸ (Table 1).

Table 1. Co-stimulatory and co-inhibitory molecules in atherosclerosis

	Ref.	Experiment	Plaque inflammation	Plaque fibrosis	Plaque area
<i>B7/CD28 family</i>					
B7-1/2	[13]	B7-1/2 ^{-/-} LDLR ^{-/-} mice	–	–	Decreased
	[14]	BMT B7-1/2 ^{-/-} > LDLR ^{-/-} mice	?	?	Increased
CD28	[14]	BMT CD28 ^{-/-} > LDLR ^{-/-} mice	–	+	Increased
ICOS	[15]	Immunized ApoE ^{-/-} mice with ICOS-Ig	+	?	Increased
	[16]	BMT ICOS ^{-/-} > LDLR ^{-/-} mice	+++	+	Increased
PD1/PD2-L	[17]	PD-L1/2 ^{-/-} LDLR ^{-/-} mice	+++	+	Increased
<i>TNF/TNFR family</i>					
Ox40L	[18]	Ox40L ^{-/-} in C3H/He mice	?	?	Decreased
	[18]	Ox40L tg in C3H/He mice	?	?	Increased
	[19]	Inhibiting mAb in ApoE ^{-/-} mice	–	?	Decreased
	[20]	Agonistic mAb in ApoE ^{-/-} mice	++	–	Increased
CD137	[26]	CD40L ^{-/-} ApoE ^{-/-} mice	– – –	+++	Decreased
CD40L	[27]	Antagonistic mAb in ApoE ^{-/-} mice	– – –	+++	No effect
	[25,28]	Antagonistic mAb in LDLR ^{-/-} mice	– – –	+++	Decreased
	[29,30]	BMT CD40L ^{-/-} > LDLR ^{-/-} mice	No effect	No effect	No effect
CD40	[38]	CD40 ^{-/-} LDLR ^{-/-} mice	No effect	No effect	No effect
	[39]	CD40 ^{-/-} mice with neointima	– –	++	Decreased
CD40–TRAF	[39]	CD40–TRAF-2/3/5 ^{-/-} mice with neointima	No effect	No effect	No effect
	[39]	CD40–TRAF-6 ^{-/-} with neointima	– –	++	Decreased

Overview of the effects of co-stimulatory and co-inhibitory molecules of the B7 and TNF-family in atherosclerosis, with special focus on the plaque area and plaque phenotype. BMT: bone marrow transplantation; ... > LDLR^{-/-}: transplantation of respective bone marrow into lethally irradiated LDLR^{-/-} recipients; –: decrease; ++: stronger decrease; +++: strong decrease; +: increase; ++: stronger increase; +++: strong increase; ?: unknown, data not available; mAb: monoclonal antibody.

Co-stimulatory molecules in atherosclerosis

Two major families of co-stimulatory molecules include the B7 and tumor necrosis factor (TNF) families⁹⁻¹². These molecules bind to receptors of the CD28 and TNF-receptor family. In the B7/CD28 family, genetic deficiency or inhibition of B7-1, B7-2, ICOS and PD-L1/2 affected atherosclerosis⁸. Deficiency of B7-1 and B7-2 in *Ldlr*^{-/-} mice was shown to inhibit early atherosclerotic lesion development, and reduced the amount of MHCII expression in atherosclerotic plaques, and their CD4⁺ T cells produced less IFN γ ¹³. However, different results were obtained when B7-1/B7-2^{-/-} or CD28^{-/-} bone marrow was given to irradiated *Ldlr*^{-/-} mice. These chimeric mice developed more atherosclerosis and this was attributed to their impaired Treg development¹⁴. Similar contradictory results were obtained by studying inhibition of ICOS, a positive co-stimulatory molecule for CD4⁺ cells. Instead of the expected reduction in atherosclerosis, both immunization with ICOS as well as bone marrow transplantation of ICOS^{-/-} bone marrow into *Ldlr*^{-/-} mice showed an aggravation of atherosclerosis, which was also due to an impaired Treg function^{15, 16}. Moreover, deficiency of PD-PD-L1/2 interactions, a co-inhibitory dyad, aggravated atherosclerosis, and induced a pro-inflammatory plaque phenotype¹⁷.

These studies with sometimes opposing results illustrate the complexity of co-stimulatory and co-inhibitory pathways which can influence functions of both pro-inflammatory effector T cells and Treg suppression. For the TNF and TNF-R family members, the results are more consistent. Inhibition of Ox40-Ox40L signaling results in an impaired atherosclerosis development, while mice over expressing Ox40L have accelerated atherosclerosis^{18, 19}. The same is true for CD137-CD137L (4-1BB/4-1BBL), where treatment with an agonistic CD137 antibody results in accelerated atherosclerosis and the development of an inflammatory, vulnerable plaque phenotype²⁰.

One of the most elaborately studied co-stimulatory molecules in atherosclerosis is the CD154-CD40 dyad. Inhibition of CD154-CD40 signaling results in a reduction of atherosclerotic plaque size, but also in the induction of a plaque that is extremely low in inflammation and high in fibrosis (**Fig. 1**). This makes this intervention one of the most powerful plaque stabilizing

therapeutics in a laboratory setting, and therefore a potential therapeutic target for the treatment of human atherosclerosis²¹.

In this review, we will highlight the mechanisms how CD154-CD40 interactions affect atherosclerosis different stages of its pathogenesis. Moreover, we will emphasize the cell type specific effects of the CD154-CD40 signal transduction cascade. Finally, we will discuss the potential of the CD154-CD40 system as a therapeutic target in atherosclerosis.

Figure 1. Inhibition of CD40L reduces atherosclerosis and induces a stable plaque phenotype.

The upper panel shows a normal advanced atherosclerotic plaque. The atherosclerotic plaque consists of macrophage foam cells, a necrotic core, VSMCs in the fibrous cap and is covered by endothelial cells. In the atherosclerotic plaque, continuous leukocyte recruitment takes place. The lower panel shows an atherosclerotic plaque in a mouse treated with an anti-CD40L antibody. Inhibition of CD40L reduces atherosclerosis and confers a stable plaque phenotype that contains few inflammatory cells and a high extracellular matrix (ECM) content, the equivalent of a stable atherosclerotic plaque.

CD154-CD40 in atherosclerosis

The CD154-CD40 pathway is a special co-stimulatory dyad. It is not strictly a mediator of T cell co-stimulation, but functions mainly to activate APCs by the T cell. Then, CD40-signaling in the APC induces the expression of other co-stimulatory molecules and thus also activates T cells²². Interestingly, the expression of CD154 is not restricted to T cells and the expression of CD40 is not confined to the APC, suggesting other functions of the CD154-CD40 dyad than pure co-stimulation²¹.

In atherosclerotic lesions both CD154 and CD40 are expressed on the vast majority of immune cells (T lymphocytes, B lymphocytes, macrophages, dendritic cells, neutrophils and mast cells) and non-immune cells (e.g. endothelial cells and vascular smooth muscle cells) present in the plaque, as well as on monocytes and platelets in the circulation^{23, 24}. Both CD154 and CD40 are already expressed in early stages of atherosclerosis, in fatty streak lesions. Their expression increases with plaque progression and is highest in thin fibrous cap atheromas and ruptured plaques²⁴.

In the late 90s, the importance of CD154-CD40 interactions was established. In 1998, Mach et al. showed that hyperlipidemic *Ldlr*^{-/-} mice treated with an anti-CD154 antibody significantly reduced the size and lipid content of aortic atherosclerotic lesions²⁵. In 1999, we showed that *CD154*^{-/-}*Apoe*^{-/-} mice exhibited a 5.5 fold decrease in plaque area²⁶. Moreover, we also found that plaques of *CD154*^{-/-}*Apoe*^{-/-} animals displayed a remarkable phenotype. Advanced atherosclerotic lesions contained increased amounts of collagen and smooth muscle cells, while plaque lipid levels and the number of inflammatory cells were strongly reduced²⁶. These plaques are the equivalent of clinically favorable, stable plaques in humans. To test whether antagonizing CD154 would be feasible, we used an anti-CD154 antibody (MR-1). Anti-CD154 antibody was administered in *Apoe*^{-/-} mice on normal chow diet, either at the onset of atherosclerosis or when established atherosclerotic lesions were present, as the equivalent for the situation in patients. In both treatment groups, anti-CD154 antibody treatment did not result in a decrease in plaque area but resulted in the development of lipid-poor, collagen-rich, stable plaques²⁷. Schonbeck et al. showed similar results with a different anti-CD154 antibody (M158, Immunex) in *Ldlr*^{-/-} mice that were on a high fat diet²⁸.

Transplantation of CD154^{-/-} bone marrow into *Ldlr*^{-/-} mice did not significantly alter atherosclerosis^{29, 30}. However, bone marrow transplantation may not be a good tool to study CD154 on hematopoietic cells since CD154 on both progenitor cells and stroma seems important in homing.

Besides the membrane-associated form, CD154 also exists in a truncated soluble form, sCD154 (sCD40L). sCD154 is cleaved from the CD154 protein upon activation, especially in platelets³¹. Whether this sCD154 is biologically active, is still under investigation. However, sCD154 has been proven to be a useful biomarker for disease severity. Elevated levels of sCD154 have been associated with hypercholesterolemia, diabetes, ischemic stroke and acute coronary syndromes and predict increased restenosis after percutaneous coronary and carotid interventions³²⁻³⁷.

For CD40, the receptor for CD154, the results are contradictory. Zirlik et al. reported that *CD40*^{-/-}*Ldlr*^{-/-} mice do not have any changes in atherosclerosis and claims that CD40 is not the only receptor for CD154, but that CD154 can interact with the integrin Mac-1³⁸. Interestingly, in a model for neointima formation, we found that absence of CD40 decreased neointimal areas, while absence of CD154 had no effect³⁹. The mechanisms and pathways, as well as the interacting cell-types that modulate CD154-CD40-signaling in vascular biology are still under investigation, but many *in vitro* studies have provided insights how CD154-CD40 interactions may affect the different stages of atherosclerosis.

Leukocyte recruitment

As stated in the introduction, atherosclerosis starts with the retention of (modified) LDL in the arterial wall, the subsequent induction of chemokines and expression of leukocyte adhesion molecules. Consequently, monocytes, neutrophils and T-lymphocytes are sequestered to the endothelium, tether, adhere, and enter the arterial intima via diapedesis⁴. Part of this process is mediated by CD154-CD40 interactions.

CD154-CD40 interactions stimulate the expression of adhesion molecules like vascular adhesion molecule-1 (VCAM), inter-cellular adhesion molecule-1 (ICAM) and E-selectin on the endothelium, and cause more

leukocytes to adhere to and enter the arterial wall ⁴⁰. Once monocytes have differentiated into macrophages, CD40 stimulation induces them to express a plethora of chemokines like monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein α and β (MIP-1 α , MIP-1 β), and RANTES, thereby recruiting additional leukocytes into the arterial wall ⁴¹.

Another mechanism, by which monocytes are sequestered to the arterial wall, is via platelets. Also here, CD154-CD40 interactions play a crucial role. Henn et al. proved that platelets, through CD154-CD40 interactions, are capable to initiate various inflammatory responses on endothelial cells such as the expression of inflammatory adhesion receptors (e.g. E-selectin, VCAM-1, ICAM-1) and the production of chemokines (e.g. MCP-1, and interleukin-8) ⁴².

Not only can platelet CD154 interact with endothelial CD40, which promotes leukocytes to enter via endothelial platelet scaffolds, platelet CD154 is also able to interact with CD40 on leukocytes. Platelet-leukocyte aggregates hereby stimulate monocytes to differentiate into a pro-adhesion and pro-migratory phenotype and promote leukocyte recruitment across the endothelium ⁴³. Interestingly, Li et al. reported that platelet CD154 upregulates Mac-1 on neutrophils via a P-selectin mediated pathway, and that platelet-neutrophil complexes adhere to the injured arterial wall, thereby facilitating neutrophil influx ⁴⁴.

Plaque progression and destabilization

During atherosclerosis progression, inflammatory cells start to accumulate in the lesion. Macrophages phagocytose lipids and become foam cells, and T-lymphocytes and neutrophils start entering the plaque. In the center of the plaque, cells die from necrosis or apoptosis and are not cleared properly. Cholesterol crystals and calcium are deposited, and a necrotic core forms. Moreover, medial smooth muscle cells are triggered to migrate into the plaque, form collagen and extracellular matrix, and create a fibrous cap. In most of these steps CD154 and CD40 are involved ^{1, 3, 4}.

Upon stimulation of CD40, plaque macrophages are able to release a wide range of cytokines (e.g. IL-1 β , IL-2, IL-6, IL-12, TNF- α , IFN- γ) that aggravate inflammation and therefore atherosclerosis ⁴⁵. Moreover,

macrophages and DCs in the plaque express MHCII and antigen dependent T cell activation is initiated, in which CD40-CD40L co-stimulation drives T cell proliferation and skews them towards Th1 differentiation. Th1 cells are considered pro-atherogenic and produce pro-atherogenic cytokines like $\text{TNF}\alpha$, $\text{IFN}\gamma$, IL-1 and IL-18¹.

Besides catalyzing inflammation, CD40-activated macrophages are able to synthesize and secrete matrix metalloproteinases (e.g. MMP-1, MMP-2, MMP-3, MMP-9), which degrade extracellular matrix. Extracellular matrix degradation strongly weakens the fibrous cap, thereby creating a plaque that is prone to rupture⁴¹.

After rupture, the CD154-CD40 system is also involved in subsequent thrombus formation. The CD154–CD40 system induces VSMCs, macrophages and endothelial cells to secrete tissue factor via CD154 responsive elements in the tissue factor promoter^{46, 47}. Moreover, CD154 is a platelet agonist, because it promotes the stability of platelet aggregates signaling via $\alpha_{\text{IIb}}\beta_3$ ⁴⁸.

Inhibition of CD40: a potential treatment for atherosclerosis

In principle, these findings render targeting of CD154 or CD40 a promising strategy to reduce atherosclerosis and to stabilize atherosclerotic plaques. Unfortunately, clinical trials using an anti-CD154 antibody have been omitted due to thromboembolic complications, particularly because CD154 is present on platelets and can interact with the integrin $\alpha_{\text{IIb}}\beta_3$ ^{48, 49}.

Conceivably, more targeted intervention strategies using antagonists of CD40 or antagonists of atherosclerosis-associated CD40-signaling intermediates will be less accompanied by such deleterious side effects, and would be an alternative approach for treating human atherosclerosis.

However, CD40 signal transduction cascades are multiple, complex and depend on the cell type and pathology involved.

CD40-signaling intermediates: TNF-receptor Associated Factors (TRAFs)

Like the other TNF-receptor family members, CD40 lacks intrinsic signaling activity, and needs to recruit adaptor molecules, the TRAFs, upon activation. TRAF proteins bind to the cytoplasmic tail of CD40 and subsequently mediate the activation of multiple signaling pathways including NF κ B, C-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein (MAP) kinase, which in turn phosphorylate and activate downstream transcription factors. The TRAF-protein family is composed of six members and is characterized by a conserved 180 amino acid fold, the TRAF domain (TD), which supports the interaction between the TNF-receptor and its downstream signaling intermediates. The C-terminus of the TRAF-domain mediates homo- and hetero-dimerization with other TRAF-proteins and the receptor that recruits them⁵⁰.

The cytoplasmic domain of CD40 has a proximal TRAF6 binding site and a more distal TRAF 2/3/5 binding site. The TRAF2/3/5 binding site is most often occupied by TRAF2 and TRAF3. TRAF1 only binds to CD40 when CD40-signaling is already active. It can bind to the TRAF 2/3/5 binding site and replaces or hetero-dimerizes with TRAF2 and acts as a regulator rather than an activator of CD40 signaling⁵¹. Whether TRAF5 directly or indirectly binds to the TRAF2/3/5 binding domain is still under debate. TRAF5 was found to bind CD40 directly in a yeast 2 hybrid study⁵². However, more recent studies state that TRAF5 is only able to bind to CD40 via TRAF3/TRAF5 heterodimers⁵⁰. Recently, a second TRAF2 binding site on CD40 was identified, that appeared functional in mediating B cell activation, proliferation and differentiation⁵³. Until now, TRAF6 is the only protein able to bind the TRAF6 binding site of CD40⁵⁰. When TRAF proteins bind to the cytoplasmic tail of CD40, multiple signaling cascades including NF κ B, C-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein (MAP) kinase pathways can be activated⁵⁰. Which one that will be, depends on the TRAF family member that binds, on the cell type that is activated and on the conditions that are present.

For example, in B-lymphocytes, CD40-TRAF-6 interactions are required for CD40-mediated IgM production, IL-6 secretion and isotype switching, whereas the TRAF-2/3/5 binding site is required for CD40-mediated upregulation of B7 and protection from B cell antigen-receptor mediated

growth arrest⁵⁴. Moreover, both binding domains also seem to exert cell type specific actions.

CD40-TRAF signaling in atherosclerosis

This is also true for the cell types present in atherosclerotic plaques. In monocytes and macrophages, the interaction between CD40 and TRAF6 is crucial for the activation of Src/ERK1/2 and IKK/NFkB pro-inflammatory pathways, while CD40-TRAF2/3/5 interactions are not required for induction of inflammation⁵⁴. However, in endothelial cells and smooth muscle cells, TRAF-2 mediates the activation of pro-inflammatory pathways⁵⁵. Interestingly, upon shear stress, TRAF3 inhibits CD40-signaling, thereby preventing recruitment of inflammatory cells into the arterial wall⁵⁶.

To obtain insights which CD40-TRAF interactions are required in vascular biology, we used mice carrying a CD40 transgene with targeted mutations at the CD40-TRAF2/3/5, CD40-TRAF6, or CD40-TRAF2/3/5/6 recognition site. In a model of carotid neointima formation, we found that deficiency in CD40-TRAF6 and CD40-TRAF2/3/5/6, but not CD40-TRAF2/3/5 resulted in a strong decrease in neointima formation, indicating that CD40-TRAF6 signaling is specifically required for arterial neointima formation³⁹. CD40-TRAF6 signaling was required for inflammatory cell infiltration and collagen turnover in the neointima. These data unveil a clear bifurcation of CD40-signaling in vascular biology with a role for CD40-TRAF6 but not CD40-TRAF2/3/5 interactions in vascular biology and establish that targeting specific components of the CD154-CD40 pathway *in vivo* harbors the potential to achieve therapeutic effects in atherosclerosis.

Concluding Remarks

The role of CD154-CD40L signaling in atherosclerosis is complex. Activation of CD40-signaling promotes the initiation, progression and destabilization of atherosclerotic plaques. Inhibition of CD154 and CD40 in mouse models limits atherosclerosis and establishes the equivalent of a clinically favorable, stable atherosclerotic plaque phenotype. However, although intervening in this co-stimulatory pathway yields interesting opportunities for treatment of atherosclerosis, caution should be applied and unexpected, potential pro-inflammatory side effects should be monitored carefully.

The recent discovery of the relevant CD40-signaling pathways in atherosclerosis heralds a new area in the development of therapeutic agents with only limited inflammatory side effects. We have shown that particularly CD40-TRAF6 interactions in leukocytes play a key role in vascular biology. Inhibition of the CD40-TRAF6 axis, while leaving the CD40-TRAF2/3/5 axis intact by antagonizing antibodies that modify the CD40-TRAF6 interaction site or by small molecules, is a promising strategy in the treatment of human atherosclerosis.

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Chapter 3

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Chapter 4

Deficient CD40-TRAF6 signaling in leukocytes prevents atherosclerosis by skewing the immune response towards an anti-inflammatory profile

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Abstract

The CD40-CD40 ligand (CD40L) signaling axis plays an important role in immunological pathways. Consequently, this dyad is involved in chronic inflammatory diseases, including atherosclerosis. Inhibition of CD40L in apolipoprotein E (*ApoE*)-deficient (*ApoE*^{-/-}) mice not only reduced atherosclerosis but also conferred a clinically favorable plaque phenotype that was low in inflammation and high in fibrosis. Blockade of CD40L may not be therapeutically feasible, as long-term inhibition will compromise systemic immune responses. Conceivably, more targeted intervention strategies in CD40 signaling will have less deleterious side effects. We report that deficiency in hematopoietic CD40 reduces atherosclerosis and induces features of plaque stability. To elucidate the role of CD40-tumor necrosis factor receptor-associated factor (TRAF) signaling in atherosclerosis, we examined disease progression in mice deficient in CD40 and its signaling intermediates. Absence of CD40-tumor necrosis factor-receptor associated factor-6 (TRAF6) but not CD40-TRAF2/3/5 signaling abolishes atherosclerosis and confers plaque fibrosis in *ApoE*^{-/-} mice. Mice with a defective CD40-TRAF6 signaling display a reduced blood count of Ly6C^{high} monocytes, an impaired recruitment of Ly6C⁺ monocytes to the arterial wall, and polarization of macrophages towards an anti-inflammatory regulatory M2 signature. These data unveil a role for CD40-TRAF6, but not CD40-TRAF2/3/5, interactions in atherosclerosis and establish that targeting specific components of the CD40-CD40L pathway harbors the potential to achieve therapeutic effects in atherosclerosis.

Introduction

Atherosclerosis is a chronic inflammatory disease of the large arteries that involves multiple immunological processes^{1, 2}. During the progression of atherosclerosis, ongoing activation of the immune system causes continuous recruitment of inflammatory cells into the plaque and degradation of its extracellular matrix. This creates a so-called vulnerable plaque, which is prone to rupture and therefore likely to cause acute complications such as myocardial infarction or stroke³.

The CD40-CD40L dyad, a co-stimulatory receptor-ligand pair, plays a crucial role in enhancing immune responses and inflammation and contributes to a plethora of chronic inflammatory diseases, for example, colitis, arthritis, allergic encephalitis and multiple sclerosis⁴⁻⁶. Disruption of the *CD40L* gene in *Apoe*^{-/-} mice abrogated atherosclerosis and caused a plaque phenotype with only few inflammatory cells and a high percentage of extra-cellular matrix⁷⁻¹⁰ which is reminiscent of a clinically favorable stable atherosclerotic plaque in humans³. This phenotype was copied when *Apoe*^{-/-} mice with initial plaques or established atheromata were treated with a blocking anti-CD40L antibody^{8,10}.

In principle, these findings render targeting of CD40L a promising strategy to reduce atherosclerosis and to stabilize atherosclerotic plaques. Unfortunately, clinical trials using an anti-CD40L antibody have been omitted as a result of thromboembolic complications, particularly because CD40L is present on platelets and can interact with the integrin $\alpha_{IIb}\beta_3$ in platelets^{11,12}.

Antagonizing CD40, the receptor for CD40L, or its signaling intermediates would be an alternative approach for treating human atherosclerosis. However, information on the effects of CD40 and CD40-associated signal transduction pathways in atherosclerosis is scarce. It has been shown that low-density lipoprotein-receptor-deficient (*Ldlr*^{-/-}) mice lacking the *CD40* gene do not develop smaller atherosclerotic lesions than control *Ldlr*^{-/-} mice¹³, although a pro-atherogenic role for endothelial CD40-TNF receptor associated factor (TRAF) 1, 2, 3, 5 and 6 signaling in atherosclerosis has been claimed¹⁴. Using mice carrying a CD40 transgene with targeted mutations at the CD40-TRAF6 recognition site, we found that CD40-TRAF6 signaling is specifically required for arterial neointima formation¹⁵.

In the present study, we tried to elucidate the role of CD40 and its associated signaling intermediates, the TRAFs, in primary atherosclerosis *in vivo* in detail. We found that *CD40*^{-/-}*Apoe*^{-/-} mice had a reduction in atherosclerosis and developed a stable atherosclerotic plaque phenotype. Bone marrow transplantation of *CD40*^{-/-} bone marrow into *Ldlr*^{-/-} mice revealed that hematopoietic CD40 was responsible for the observed phenotype. Surprisingly, when CD40-TRAF6, but not CD40-TRAF2/3/5, interactions were defective, atherosclerosis was completely abrogated.

Methods

Animals

CD40^{-/-}, *CD40-Twt*, *CD40-T2/3/5^{-/-}*, *CD40-T6^{-/-}* and *CD40-T2/3/5/6^{-/-}* mice (all on a C57Bl6 background) ¹⁶ were backcrossed for 7 generations to *Apoe^{-/-}* mice. *Ldlr^{-/-}* mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All study protocols involving animal experiments were approved by the Animal Care and Use committee of the University of Maastricht and were performed according to official rules formulated in the Dutch law on care and use of experimental animals, which are highly similar to those of the National Institutes of Health.

Primary atherosclerosis

CD40^{-/-}Apoe^{-/-} (n=16), *CD40^{+/+}Apoe^{-/-}* (n=19), *CD40-Twt* (n=14), *CD40-T2/3/5^{-/-}* (n=17), *CD40-T6^{-/-}* (n=26) and *CD40-T2/3/5/6^{-/-}* (n=14) mice were fed a normal chow diet throughout the experiment.

Bone marrow transplantation

Ldlr^{-/-} mice (n=44) were maintained in filter-top cages and given water containing 60.000U/liter polymyxin B sulfate (Invitrogen) and 100 mg/liter neomycin (Invitrogen) from week 1 before bone marrow transplantation until 4 weeks thereafter. Mice were lethally irradiated (10 Gy, 0.5Gy/min, MU 15F/225 kV; Philips) and intravenously injected with 10⁷ bone marrow cells from *CD40^{-/-}* mice or *CD40^{+/+}* mice. 4 weeks after transplantation, mice were fed a 1.25% cholesterol diet for 24 weeks.

Atherosclerosis experiments

Mice were sacrificed and the arterial tree was perfused. The aortic arch and its main branch points were excised, fixed over night, and embedded in paraffin. The rest of the arterial tree was fixed, opened, pinned, and stained with Oil-red-O for *en-face* analysis. Longitudinal sections of the aortic arch were analyzed for plaque extent and phenotype as previously described ^{3, 17}. For phenotypic parameters, immunohistochemistry was performed for CD3 (Dako), CD45 (BD), mac-3 (BD), FVIII (DAKO), α -smooth muscle actin (Sigma-Aldrich), caspase 3 (Cell signaling Technology), FoxP3 (eBioscience), collagen

type I (Abcam), collagen type III (Abcam), MMP-2 (Cell signaling Technology), and MMP-9 (Santa Cruz Biotechnology Inc.). In addition, Perl's iron staining and a Sirius red staining were performed as previously described¹⁷. Morphometric analyses were performed using a Quantimet (Leica) with Qwin3 software (Leica). Plasma cholesterol levels were measured enzymatically (Roche), organs were analyzed by HE staining, and no abnormalities were observed. Serum sCD40L levels were measured by ELISA (eBioscience).

***In-vitro* macrophage culture**

Bone-marrow cells were isolated from n=6 mice/group and cultured in RPMI supplemented with L929 conditioned medium to generate bone marrow-derived macrophages as previously described¹⁸. Cells were pretreated with 100 U IFN γ for 24 hrs to induce MHCII expression and then treated for 3 hrs with 100 ng/ml FGK45, a CD40-stimulating antibody. Cytokine levels in the medium were measured by FACS using a cytometric bead assay (BD), and quantitative gene expression of cytokines, M1 and M2 macrophage markers were analyzed as previously described¹⁹. Disposables were purchased from Bio Greiner One.

Macrophage migration assay

Macrophage migration was assessed using 24-well Transwell migration chambers (Costar, Corning) with a pore size of 6 μ m. Bone marrow-derived macrophages (0.5×10^6 cells) of the respective genotypes, suspended in serum free medium, were added to each chamber. Complete medium including 100 ng/mL CCL2 (R&D systems), was added to the lower chambers and migration was performed at 37°C for 4h. Non-migrated cells were removed from the membranes, and migrated cells within the membrane were fixed with methanol and stained with toluidine blue. Membranes were cut out of inserts and mounted onto slides in immersion oil. The number of migrated cells was counted on five randomly chosen fields of each membrane.

Gelatinase assay

Gelatinase activity in the supernatant of FGK45-treated macrophages was determined using the EnzCheck gelatinase/collagenase assay kit (Invitrogen). Substrate hydrolysis was analyzed after 24h at room temperature and fluorescence was detected using a fluorescence microplate reader.

Intravital microscopy

Leukocyte endothelial interactions were analyzed by intravital microscopy of the left carotid artery (n=8 mice/group)²⁰. 6h after i.p. injection of 1.0 µg of mouse TNF (PeproTech), mice were anaesthetized with ketamine/xylazine and the left carotid artery was exposed. Circulating leukocytes were labelled by i.v. injection of rhodamine 6G. Ly6C⁺ inflammatory monocytes were labeled with fluorescent latex beads as previously described²¹. The antibody to IL10 (clone JES5.2A) was injected twice i.p. (500 µg/mouse), 24 and 2h before intravital microscopy. Recordings were made using a microscope (BX51, Olympus) equipped with a saline-immersion 20x objective.

Statistics

Data are presented as mean ± SEM. Data were analyzed by a nonparametric Mann-Whitney U test or Welch corrected *t*-test, as appropriate, using Prism 4 software (GraphPad Software, Inc.). *P* values < 0.05 were considered significant.

Results

CD40 deficiency reduces atherosclerosis

We generated *CD40^{-/-}ApoE^{-/-}* mice and analyzed the extent and phenotype of atherosclerosis at 26 wks of age on a normal chow diet. Body weight, as well as cholesterol content or plasma sCD40L levels did not differ between the groups (**Table 1**). As in *CD40L^{-/-}ApoE^{-/-}* mice, deficiency of CD40 reduced the atherosclerotic plaque area in the aortic arch and its branch points, as well as in the thoraco-abdominal aorta (**Fig. 1a**). This was associated with a less inflammatory and more fibrotic plaque quality, as reflected by the low amount of thin fibrous cap atheromata³ that developed in absence of CD40 (Supplemental Figure 1a). The absence of CD40 reduced the lipid core size (*ApoE^{-/-}*, 35.4% vs. *CD40^{-/-}ApoE^{-/-}*, 23.3%; $P < 0.05$), the number of plaque macrophages, and the content of CD45⁺ cells and CD3⁺ T lymphocytes (**Fig. 1b, c**). The numbers of FoxP3⁺ regulatory T cells in plaque or adventitia was unaffected (Supplemental Figure 1c, d). The numbers of cleaved caspase-3⁺ (apoptotic) cells (Supplemental Figure 1b), as well as the degree of iron or fibrin deposition in the plaque (not depicted) were unaffected.

Besides the decrease in inflammatory cell content, plaques of *CD40^{-/-}ApoE^{-/-}* mice had a more fibrotic appearance. The content of α -smooth muscle actin (α SMA⁺ cells; **Fig. 2a**) and collagen (**Fig. 2b**) was significantly increased. Bright-field polarization microscopy revealed that in the absence of CD40, the color distribution of the collagen deviated towards the red spectrum, indicating larger collagen fibrils (**Fig. 2c**)²². Concordant with these findings, we found an increase in the amount of collagen type I and III in the plaque (**Fig. 2d, e**). Recent studies have identified the involvement of various matrix metalloproteinases in atherosclerotic lesion formation and their contribution to fibrous cap thinning by degrading extracellular matrix²³. Interestingly, MMP-2 and MMP-9 expression was decreased in the plaques of *CD40^{-/-}ApoE^{-/-}* mice (Supplemental Figure 2a, b). To confirm an impairment of proteolytic activity and effects of tissue inhibitor of metalloproteinase (TIMP)-1, we measured gelatinase activity and TIMP-1 mRNA levels in macrophages stimulated with the CD40-clustering antibody FGK45. We found that gelatinase activity was decreased (Supplemental Figure 2c), whereas TIMP-1 levels were increased in CD40-deficient macrophages (Supplemental Figure 2d).

Table 1. Body weights, plasma cholesterol levels, and sCD40L serum levels did not differ between the genotypes (P > 0.05)

Genotype	Body weight	Cholesterol	sCD40L
	<i>g</i>	<i>mg/dl</i>	<i>ng/ml</i>
<i>Apoe</i> ^{-/-}	28.7 ± 0.8	275 ± 16	4.0 ± 0.8
<i>CD40</i> ^{-/-} <i>Apoe</i> ^{-/-}	29.2 ± 0.8	299 ± 24	5.6 ± 1.6
<i>CD40-Twt</i>	26.1 ± 0.8	328 ± 26	3.3 ± 1.0
<i>CD40-T2/3/5</i> ^{-/-}	24.9 ± 0.7	297 ± 33	5.0 ± 2.6
<i>CD40-T6</i> ^{-/-}	24.8 ± 0.8	283 ± 12	2.9 ± 0.5
<i>CD40-T2/3/5/6</i> ^{-/-}	28.9 ± 0.7	330 ± 36	5.3 ± 1.9

Figure 1. Role of CD40 in atherosclerosis.

(a-c) *Apoe*^{-/-} mice (n=19) were fed a normal chow diet for 26 wks and were compared to *CD40*^{-/-}*Apoe*^{-/-} (n=16). Sections of the aortic arch and its main branch points (brachiocephalic artery, right subclavian artery, right carotid artery, left carotid artery and left subclavian artery) were stained with haematoxylin and eosin (HE, representative sections in a, bottom) to analyze the extent of atherosclerosis (plaque area; a, top left: plaque area aortic arch and branchpoints; a, top right: en face staining thoraco-abdominal aorta; bars, 2 mm; horizontal bars represent mean) and plaque phenotype (b-c). (b) Macrophage infiltration was expressed as the absolute number of Mac3⁺ cells per plaque (bars, 100 μm). (c) CD45 and CD3 content were expressed as the percentage of CD3⁺ or CD45⁺ cells of all plaque cells (arrows indicate CD45⁺ cells; bars, 50 μm). Error bars represent mean $\pm$ SEM. *, $P < 0.05$

Figure 2. Deficiency of CD40 induces plaque fibrosis.

Sections of the aortic arch and its main branch points (brachiocephalic artery, right and left subclavian artery, and right and left carotid artery) of $Apoe^{-/-}$ mice (n=19) and $CD40^{-/-}Apoe^{-/-}$ (n=16) mice fed a normal chow diet for 26 wk were stained for α SMA to detect the content of SMCs (a, representative sections on the left; bars, 50 μ m), which was quantified as the percentage of α SMA-positive plaque area (a, right). (b and c) Consecutive sections were stained with sirius red to assess the content of collagen in the plaque by light (b) and polarized light (c) microscopy (representative sections on the left; bars, 50 μ m). In addition, the content of collagen was expressed as the percentage of α SMA-positive plaque area (b, right) and the color spectrum obtained using polarization microscopy was analyzed (c, right). (d and e) The content of collagen type I (d) and collagen type III (e) was assessed by immunohistochemistry (representative sections on the left; bars, 100 μ m) and expressed as the percentage of positively stained plaque area (right). Error bars represent mean $\pm$ SEM. *, $P < 0.05$

Leukocyte CD40 deficiency reduces atherosclerosis and impairs macrophage migration

To unequivocally establish the involvement of leukocyte versus non-leukocyte CD40 in atherosclerosis, we reconstituted lethally irradiated *Ldlr*^{-/-} mice with *CD40*^{-/-} or WT BM and fed the mice with a high-fat diet for 24 wk. Body weights and plasma cholesterol levels did not differ between the groups (body weight: 20.1 vs 19.7 g in *Ldlr*^{-/-} mice; *P* > 0.05; cholesterol, 368 vs 357 mg/dL in *Ldlr*^{-/-} mice). Chimeric *Ldlr*^{-/-} mice with *CD40*^{-/-} BM exhibited markedly reduced atherosclerosis in the aortic arch including its branch points and in the thoraco-abdominal aorta (**Fig. 3a**). Similar to *CD40*^{-/-}*Apoe*^{-/-} mice, atherosclerotic plaques of chimeric *CD40*^{-/-}*Ldlr*^{-/-} mice contained less Mac3⁺ macrophages (**Fig. 3b**). In culture, *CD40*^{-/-} macrophages showed an impaired migration towards CCL2 (**Fig. 3c**). Moreover, the percentage of CD45⁺ cells and CD3⁺ T lymphocytes in the plaque was decreased (**Fig. 3e**).

In accordance with this anti-inflammatory profile in the plaque, absence of CD40 strongly decreased the expression of I κ B α in FGK45-stimulated macrophages (**Fig. 3d**), suggesting that the phenotypic effects are mediated by NF κ B signaling. Total collagen content (**Fig. 3f**), and smooth muscle cell (SMC) content (**Fig. 3f**) were increased in the plaque in absence of CD40. These data reveal a crucial role for leukocyte-dependent CD40-signaling in atherosclerosis.

Deficiency of CD40 polarizes macrophages towards an M2 phenotype

Stimulation of CD40 with the clustering antibody FGK45 in BM-derived macrophages resulted in a significant induction of the pro-inflammatory cytokines IL-12, iNOS (**Fig. 4a**) and CCL-2 (not depicted) and a significant reduction of IL-10 (**Fig. 4c**) WT but not in *CD40*^{-/-} macrophages, indicating that absence of CD40 polarizes macrophages towards an M2 phenotype²⁴.

To further differentiate the M2 subset into 'wound-healing' and 'regulatory' macrophages²⁵, real-time PCR analysis for respective markers was performed in FGK45-stimulated BM-derived macrophages. We observed that CD40-deficiency significantly reduced the inflammatory M1 markers iNOS and IL-12 (**Fig. 4a**), only slightly reduced the expression of the 'wound healing' macrophage markers YM-1 and RELM α , and did not affect the expression of

arginase-I, IGF-1, or DCIR (**Fig. 4b**), suggesting that CD40-signaling is essential for polarization of macrophages towards the M1 subtype, but not towards 'wound-healing' macrophages. Moreover, absence of CD40 did not result in an increase in collagen 1A1 α production in these macrophages (**Fig. 4b**). In contrast, deficiency of CD40 strongly induced the 'regulatory' macrophage marker IL-10, whereas SPKH1 expression was not affected (**Fig. 4c**), indicating that CD40 deletion shifts the macrophage phenotype to an anti-inflammatory regulatory signature.

Figure 3. Role of hematopoietic cell-derived CD40 in diet-induced atherosclerosis.

(a-f) *WT*→*Ldlr*^{-/-} chimeras (n=19) were fed a normal chow diet for 26 wk and were compared with *CD40*^{-/-}→*Ldlr*^{-/-} BM chimeras (n=19). Sections of the aortic arch and its main branch points (brachiocephalic artery, right and left subclavian artery, and right and left carotid artery) were stained with haematoxylin and eosin and the thoraco-abdominal aorta was stained with Sudan IV (representative examples in a, right) to analyze the extent of atherosclerosis (a, left: plaque area in the arch; a, middle: plaque area in the thoraco-abdominal aorta; horizontal bars represent mean) and phenotype (b-f). (b) Macrophage infiltration was expressed as the absolute number of Mac3⁺ cells per plaque (bars, 100 μm). Migration of BM-derived macrophages towards CCL2 for 4h was analyzed in a Transwell migration assay (n=6 per group, three independent experiments). (d). Levels of IkB α in FGK45-stimulated macrophages were determined by real-time PCR (n=6). (e) CD45 and CD3 content were expressed as the percentage of CD3⁺ or CD45⁺ cells of all plaque cells (arrows indicate CD45⁺ cells; bars, 50 μm). (f) The content of collagen (representative sections on the right; bars, 100 μm) and α SMA was expressed as the percentage of positively stained plaque area. Error bars represent mean $\pm$ SEM. *, $P < 0.05$

Figure 4. Deficiency in CD40 induces polarization of macrophages towards a regulatory M2 macrophage phenotype.

BM-derived macrophages of WT and CD40^{-/-} mice were cultured, stimulated with 1μg/ml of the CD40-clustering antibody FGK45, and analyzed for expression of different M1 (a) and M2 (b, wound-healing macrophages; c, regulatory macrophages) subset markers by real-time PCR. n=6 per group of two independent experiments. Error bars represent mean ± SEM. *, P < 0.05

CD40 deficiency affects T cell and DC phenotype

To elaborate on the immune phenotyping, we performed flow cytometry analysis of blood, spleen and lymph node cells (Supplemental Figure 3). Consistent with the atherosclerotic phenotype, analysis of the different T-lymphocyte and DC subsets revealed an anti-inflammatory profile in *CD40*^{-/-}*Apoe*^{-/-} versus *Apoe*^{-/-} mice. Whereas effector memory (CD44^{high}CD62L^{low}) CD4⁺ and CD8⁺ T-lymphocytes were decreased, naive (CD44^{low}CD62L^{high}) CD4⁺ and CD8⁺ T-lymphocytes were increased (Supplemental Figure 3a). Moreover, CD11c⁺CD8⁻CD4⁺ DCs, a population associated with anti-inflammatory responses, was more prominent, whereas CD11c⁺CD4⁻CD8⁻ and plasmacytoid DCs, populations associated with pro-inflammatory responses, were reduced (Supplemental Figure 3b)²⁶.

CD40-TRAF signaling in atherosclerosis

We next examined which of the CD40-associated signal transduction pathways was responsible for the phenotype observed in *CD40*^{-/-}*Apoe*^{-/-} mice. CD40 has no intrinsic signaling ability, but requires adaptor molecules, TRAFs, to confer signaling. The cytoplasmic tail of CD40 contains three binding domains: a proximal domain that binds TRAF6 and two distal domains that bind TRAF1, TRAF2, TRAF3, and, indirectly, TRAF5²⁷⁻²⁹. The separate TRAF binding sites are coupled to divergent signaling pathways involving different CD40 downstream modulators and effectors^{27, 30, 31}, depending on the target cell types involved. To unravel the contribution of CD40-TRAF2/3/5 versus CD40-TRAF6 signaling in MHCII⁺ cells to atherosclerosis, we used *CD40*^{-/-} mice expressing a chimeric CD40 transgene with mutations at the TRAF6 and/or TRAF2/3/5 binding site under control of the MHCII promoter (*CD40-T2/3/5*^{-/-}, *CD40-T6*^{-/-}, and *CD40-T2/3/5/6*^{-/-} mice, respectively) or *CD40*^{-/-} mice carrying the transgene without mutations (*CD40-Twt* mice)^{15, 16}, and backcrossed them with *Apoe*^{-/-} mice. At 26 wk of age on a normal chow, atherosclerosis was analyzed in the aortic arch, including its main branch points and the thoraco-abdominal aorta, including the iliac artery bifurcation (unpublished data). Body weight, plasma cholesterol level, and serum sCD40L level did not differ between the genotypes (Table 1).

CD40-TRAF2/3/5 signaling does not affect atherosclerosis

The atherosclerotic plaque area, the plaque phenotype³, the plaque quality, as evident by the content of the lipid core, Mac-3⁺ cells, CD45⁺ cells, CD3⁺ T cells, collagen (type I and III), SMCs, cleaved caspase 3, and macrophage polarization did not differ between *CD40-T2/3/5^{-/-}* and *CD40-Twt* mice, indicating that deficiency in CD40-TRAF2/3/5 signaling does not affect atherosclerosis (**Fig. 5, 6**). In fact, *CD40-T2/3/5^{-/-}* mice displayed even higher numbers of CD4⁺ cells (Supplemental Figure 4a), and CD8⁺CD44^{high} CD62L^{low} effector memory T-lymphocytes in blood, spleen and/or lymph nodes than *CD40-Twt* mice and elevated CD8⁺ resident DCs (Supplemental Figures 3c, d), a subtype which is characterized by increased IL-12 production. This apparently pro-inflammatory profile was counter-balanced by elevated numbers of CD4⁺CD25⁺FoxP3⁺ regulatory T-lymphocytes in blood, spleen, and lymph nodes of *CD40-T2/3/5^{-/-}* mice (Supplemental Figure 4b). Regulatory T cell function had not changed (Supplemental Figure 4c), and because of the balanced effector T cell and regulatory T cell ratio, systemic levels of IFN γ , IL-6, TNF α , IL-12, or IL-10 were unaltered (not depicted). Notably, in *CD40-T2/3/5^{-/-}* mice the content of CD4⁺CD25⁺FoxP3⁺ regulatory T lymphocytes was also increased in the adventitia underlying the plaques, but not in the plaque itself (Supplemental Figure 4d).

Figure 5. Role of CD40-TRAF signaling in atherosclerosis.

CD40-Twt (n=12), CD40-T2/3/5^{-/-} (n=16), CD40-T6^{-/-} (n=19) and CD40-T2/3/5/6^{-/-} (n=14) were backcrossed to *Apoe*^{-/-} mice and fed a normal chow diet for 26 wks. (a-d) Sections of the aortic arch and its main branch points (a; brachiocephalic artery, right subclavian artery, right carotid artery, left carotid artery, and left subclavian artery) were stained with HE to analyze the extent of atherosclerosis (plaque area) and plaque phenotype. (a) Plaque area and distribution of morphological plaque phenotypes according to the classification by Virmani et al. (top; horizontal bars represent mean). Representative HE stained sections in the middle and on the bottom reveal only limited atherosclerosis in absence of CD40-T6 signaling (bars:[middle] 2 mm, [bottom] 100μm). (b-d) CD40-T6^{-/-} mice have small atherosclerotic lesions that contain few macrophages (b), CD45⁺ cells, and CD3⁺ T cells (c). The level of cleaved caspase 3 was determined by grading plaque from 0 (no expression) to 3 (high expression; d). Error bars represent mean ± SEM. *, P < 0.05

Figure 6. Role of CD40-TRAF signaling in plaque fibrosis.

(a-c) Sirius red-stained sections showed low levels of collagen in the initial plaques of *CD40-T6*^{-/-} mice and increased levels in plaques of *CD40-T2/3/5/6*^{-/-} mice (bars, 50 μ m). Collagen content was assessed by light (a) and polarized light (b) microscopy, and expressed as the percentage of sirius red-stained plaque area (right). In addition, the color spectrum obtained under polarization microscopy was analyzed (c). (d and e) The content of collagen type I (d) and collagen type III (e) was assessed by immunohistochemistry (representative sections on the left; bars, 100 μ m) and expressed as the percentage of positively stained plaque area (right). Error bars represent mean $\pm$ SEM. *, $P < 0.05$

Inhibition of CD40-TRAF6 signaling abolishes atherosclerosis

In a remarkable contrast, deficiency of CD40-TRAF6 signaling almost completely abolished the development of atherosclerosis, as only few intimal xanthomas or fatty-streak lesions (classified according to Virmani *et al*³) were detectable in the aortic arch and its branch points (**Fig. 5a**), and no plaques were present in the thoraco-abdominal aorta of *CD40-T6^{-/-}* mice (**Fig. 5a**). This reduction of atherosclerosis was even more pronounced than in *CD40^{-/-}Apoe^{-/-}* mice, likely due to effects of residual CD40-TRAF2/3/5 signaling on regulatory T lymphocytes. Concomitantly, macrophage, CD45⁺ cell, and CD3⁺ T lymphocyte infiltration, but also collagen (type I and III), α SMA, and MMP-2 and MMP-9 content, were substantially reduced in the hardly evolved lesions of *CD40-T6^{-/-}* mice (**Fig. 5b, c, Fig. 6**, Supplemental Figure 5).

The population of peripheral blood Ly6C^{high} monocytes was significantly declined (**Fig. 7a**). Moreover, *CD40-T6^{-/-}* macrophages had an impaired capacity to migrate towards CCL2 (**Fig. 7b**). Intravital microscopy showed that the adhesion of circulating leukocytes and, in particular, that of Ly6C⁺ monocytes to carotid arteries was significantly impaired in *CD40-T6^{-/-}* mice (**Fig. 7c, d**).

Figure 7. CD40-TRAF signaling affects monocyte homeostasis and recruitment.

(a) The percentage of Ly6C^{high} monocytes is reduced in the peripheral blood of *CD40-T6^{-/-}* mice. Horizontal bars represent the mean. (b) Macrophages deficient in CD40-TRAF6 signaling have an impaired capacity to migrate towards CCL2 in a transwell migration assay. (c and d) Accordingly, rhodamine-labeled leukocytes (c) and Ly6C⁺ monocytes (d) display an impaired luminal adhesion to the wall of carotid arteries in absence of CD40-T6 interactions *in vivo*, which is reversed by pretreatment with a blocking antibody to IL-10 (d). Error bars represent mean $\pm$ SEM. *, $P < 0.05$

CD40-TRAF6 deficiency polarizes macrophages towards a regulatory M2 phenotype

BM derived macrophages of *CD40-T6^{-/-}* mice stimulated with FGK45 produced less IL-12 and iNOS, but increased amounts of IL-10, (**Fig. 8**), indicating that CD40-TRAF6 deficiency skews macrophage polarization towards an anti-inflammatory M2-phenotype. Further subtyping of the M2 macrophages into wound-healing and regulatory macrophages²⁵ showed that CD40-TRAF6 interactions did not affect the wound-healing macrophage markers YM-1, RELM α , arginase I, IGF1, or DCIR but markedly dampened the M1 polarization response and, rather, induced a regulatory macrophage subtype that produces high amounts of IL-10. Importantly, the process of monocyte recruitment driven by CD40-TRAF6 was mediated by IL-10, as treatment of *CD40-T6^{-/-}* mice with a blocking antibody to IL-10 reversed the protective effect on Ly6C⁺ cell numbers and adhesion (**Fig. 7d**).

The phenotype that occurs in *CD40-T6^{-/-}* mice is likely associated with an impaired NF κ B signaling, because I κ B α levels in macrophages were markedly decreased (**Fig. 8d**). Consistent with findings in CD40-deficient macrophages, *CD40-T6^{-/-}* macrophages displayed a reduced gelatinolytic activity and increased expression of TIMP-1 (Supplemental Figure 5c, d). Flow cytometry analysis further revealed reduced numbers of CD4⁺CD44^{high}CD62L^{low} pro-migratory effector memory T lymphocytes and plasmacytoid DCs (Supplemental Figures 3c, d).

Figure 8. Deficiency in CD40-TRAF6 interactions induces polarization of macrophages towards a regulatory M2 macrophage phenotype.

(a-c) BM-derived macrophages of *CD40-Twt*, *CD40-T2/3/5^{-/-}*, *CD40-T6^{-/-}*, and *CD40-T2/3/5/6^{-/-}* mice were cultured, stimulated with 1 µg/ml of the CD40-clustering antibody FGK45, and analyzed for expression of different M1 (a) and M2 (b, wound-healing macrophages; c, regulatory macrophages) subset markers by real-time PCR. n=6 per group of two independent experiments. (d) The levels of IκBα in FGK45-stimulated macrophages were decreased in BM-derived macrophages of *CD40-T6^{-/-}* and *CD40-T2/3/5/6^{-/-}* mice (n=6). Error bars represent mean ± SEM. *, P < 0.05

Deficiency of CD40-TRAF2/3/5/6 interactions induces plaque fibrosis

It is of note that mice with a combined deficiency in CD40-TRAF2/3/5/6 binding were characterized by an intermediate phenotype, further supporting counter-curing effects of CD40-TRAF2/3/5 and CD40-TRAF6 signaling in atherosclerosis. *CD40-T2/3/5/6^{-/-}* mice showed only a slight reduction in atherosclerosis (**Fig. 5a**), but displayed features of atherosclerotic plaque stability (**Fig. 5a**), with an increased number of fibrocalcific plaques containing smaller lipid cores and reduced CD45⁺ cell and CD3⁺ T lymphocyte infiltrations.

Plaques of *CD40-T2/3/5/6^{-/-}* mice exhibited high levels of collagen (**Fig. 6a-c**) and α SMA-positive SMCs (3.1 ± 0.7 vs 5.1 ± 0.9 ; $P < 0.05$). Polarized light microscopy on Sirius-Red-stained sections further showed that the majority of this collagen consisted of large fibrils, as evident by the high percentage of the red fraction (**Fig. 8b, c**). In concordance with this finding, plaque levels of collagen type I were significantly increased, whereas collagen type III levels were unaffected (**Fig. 6d, e**). Moreover, plaque MMP-2 and MMP-9 levels were significantly reduced in *CD40-T2/3/5/6^{-/-}* mice (Supplemental Figure 5a, b), indicating a decreased proteolytic activity in these plaques. Accordingly, BM-derived macrophages of *CD40-T2/3/5/6^{-/-}* mice treated with CD40-clustering antibody FGK45 displayed reduced gelatinolytic activity and elevated levels of TIMP-1 mRNA (Supplemental Figure 5c, d).

As in *CD40-T6^{-/-}* mice, macrophages of *CD40-T2/3/5/6^{-/-}* mice stimulated with FGK45 exhibited an M2 macrophage profile with decreased IL-12 production and increased production of IL-10, whereas Ly6C^{high} monocyte numbers and adhesive function were unaffected (**Fig. 7, 8**). *CD40-T2/3/5/6^{-/-}* mice shared increased CD8⁺ effector-memory T lymphocytes with *CD40-T2/3/5^{-/-}* mice and decreased plasmacytoid DC numbers with *CD40-T6^{-/-}* mice (Supplemental Figure 3c, d). This indicates that some features, namely reduced inflammatory monocyte arrest and macrophage polarization are exclusive to *CD40-T6^{-/-}* mice and are not compensated by deficiency of both TRAF binding sites.

Discussion

In this study, we found a clearly divergent action of the two TRAF binding sites of CD40 in atherosclerosis. Deficiency of CD40-TRAF6 interactions in MHCII⁺ cells nearly abrogated atherosclerosis, an effect more marked than CD40 deletion itself, whereas deficiency of CD40-TRAF2/3/5 interactions did not affect atherosclerosis but, rather, showed a tendency to aggravate atherosclerosis. The CD40-TRAF6 interactions appeared to be specifically required for the proatherogenic activity exerted by cells of the myeloid lineage. Deficiency of CD40-TRAF6 interactions limits the subset of Ly6C^{high} inflammatory monocytes and attenuates their adhesion to and infiltration into the arterial wall, thereby abolishing atherosclerotic plaque formation. The few monocytes that succeed in infiltrating the arterial wall, rather, differentiate into macrophages with an anti-inflammatory, regulatory M2-signature, thereby preventing plaque inflammation and progression.

In this paper, we provide conclusive evidence for an important role of CD40-TRAF6 interactions in the recruitment and fate of monocytes/macrophages. Diversity and plasticity are a hallmark of the monocyte/macrophage lineage, and their phenotype strongly depends on their microenvironment and the inflammatory signals they perceive ^{25, 32, 33}. We found that CD40-TRAF6 signaling affects the homeostasis of both monocyte and macrophage polarization. *CD40-T6*^{-/-} mice showed reduced numbers and reduced arterial recruitment of Ly6C^{high} monocytes, the inflammatory monocyte subset that dominates in hyperlipidemia ^{34, 35}, can easily enter the arterial wall, and can differentiate into intimal macrophages. In parallel, *CD40-T6*^{-/-} mice showed a reduction in the classically activated M1 macrophage subset producing iNOS and IL-12, which is involved in clearing intracellular parasites or tumors and eliciting tissue disruption ^{25, 32}. Macrophages of *CD40-T6*^{-/-} mice were skewed towards the M2 phenotype and particularly towards the IL-10-producing regulatory subtype ²⁵. Interestingly, the reduced recruitment of Ly6C^{high} monocytes in the carotid artery of *CD40-T6*^{-/-} mice could be reversed by blocking IL-10, indicating that there could be a link between the IL-10-producing macrophage phenotype and effects on subsequent monocyte recruitment. Surprisingly, markers of the eponymously termed wound-healing macrophages, which contribute to the healing response

after tissue destruction, were unaltered in absence of CD40-TRAF6 interactions, although high levels of plaque fibrosis were observed in *CD40-T2/3/5/6^{-/-}* mice. However, the relevance of these data has to be further substantiated and validated, since the functional role of different monocyte and macrophage subsets in atherosclerosis remains to be fully elucidated^{36, 37}. For now, our data clearly reveal that a deficiency of CD40-T6 interactions confers an immune-regulatory phenotype in the monocyte/macrophage lineage, which reduces atherosclerosis and is capable of inducing plaque stability.

In vascular pathobiology, only few data are available on the role CD40-TRAF6 interactions. Recently, CD40-TRAF6 interactions in MHCII⁺ cells have also been implicated in neointima formation in a mouse ligation model¹⁵. Moreover, it has been observed that stimulation with CD40L induces CD40-TRAF6 interactions in endothelial cells, but that their disruption surprisingly results in increased expression of CCL2¹⁴. More evidence has been gathered regarding the role of CD40-TRAF6 interactions in hematopoietic cell types including cells of the myeloid lineage. In B cells, effects of CD40-TRAF6 signaling comprise germinal center formation, NFκB activation, IL-6 production and up-regulation of co-stimulatory molecules^{16, 38, 39}. In monocytes/macrophages, CD40-TRAF6 interactions induce NFκB and ERK activation, as well as the production of TNFα and IL-6⁴⁰. In DCs, CD40-TRAF6 signaling is required for MHCII surface expression, IL-6 and IL-12 production⁴¹. Differences in the effector functions of CD40-TRAF6 signaling between the various cell types may be a result of context-specific embedding of downstream pathways.

Our data further show that CD40-TRAF2/3/5 interactions on MHCII⁺ cells appear to be required for maintaining T cell homeostasis in atherosclerosis. Absence of CD40-TRAF2/3/5 interactions increased the numbers of CD4⁺ T cells and pro-migratory CD8⁺CD44^{high}CD62L^{low} effector memory T lymphocytes, which by itself would likely be sufficient to aggravate atherosclerosis. However, the increase in T cells was compensated by an increase in CD4⁺CD25⁺FoxP3⁺ regulatory T lymphocytes, both systemically and in the vasculature. This expansion of regulatory T cells as an atheroprotective T cell subtype⁴² may protect *CD40-T2/3/5^{-/-}* mice against the T cell-induced exacerbation of atherosclerosis.

Data on the role of CD40-T2/3/5 signaling in vascular biology are scarce and not uniform, as the induction of CD40-TRAF2/3/5 signaling yields different outcomes depending on the cell types and pathologies involved. Consistent with the present study, we did not find an effect on neointima formation after carotid artery ligation in *CD40-T2/3/5^{-/-}* mice¹⁵. However, *TRAF2^{-/-}* and *TRAF5^{-/-}* endothelial cells failed to produce IL-6 upon stimulation with CD40L, whereas CCL2 induction was unaffected¹⁴. In areas with high shear stress, TRAF3 expression in endothelial cells covering human atherosclerotic plaques is up-regulated and may act as a feedback mechanism limiting endothelial activation, as its overexpression prevented CD40-induced pro-inflammatory cytokine expression⁴³.

A clearer picture for the function of CD40-TRAF2/3/5 interactions has emerged in B cell biology. CD40-TRAF2 signaling is crucial for germinal center formation, induces JNK activation, and, in particular, NFκB activation^{39, 44, 45}. This is in accordance with our findings that IκBα mRNA expression was not significantly decreased in *CD40-T2/3/5^{-/-}* macrophages. Together with CD40-TRAF5 interactions, which are also required for Ig production, CD40-TRAF2 signaling up-regulates co-stimulatory molecules⁴⁵. In contrast, TRAF3 acts as an inhibitor, reducing co-stimulatory molecule expression and IgM production⁴⁶. In cell types and organs other than B cells or lymph nodes, however, most of these functions are irrelevant. Consistent with our data, the overall effect of CD40-TRAF2/3/5 signaling with regards to atherogenesis appears to be balanced.

Our findings may harbor new and interesting therapeutic possibilities. The complete inhibition of CD40-CD40L signaling in atherosclerosis is not therapeutically feasible because long-term treatment will compromise systemic immune responses and also entails thromboembolic complications. Therefore, inhibition of the TRAF6 binding site on CD40, using small molecules or an antagonizing CD40 antibody that changes the conformation of the CD40-TRAF binding sites, may be a suitable alternative. It is conceivable that the protection by CD40-TRAF6 blockade is conferred by skewing the immune response to a more anti-inflammatory profile, namely through increased production of IL-10 and its effects on mitigating the relative propensity, recruitment, and differentiation of pro-inflammatory monocytes. Not only could inhibition of the CD40-TRAF6 binding site reduce atherosclerosis more

effectively than a complete inhibition of CD40 or CD40L by avoiding an interference with potentially protective effects by remaining CD40-signaling pathways, it may also leave the normal functions of these pathways unaffected, and would therefore be expected to only cause limited side-effects. However, although our results in the mouse model are promising, caution should be applied when extrapolating these experimental data to the human situation. The effects of such selective targeting strategies will have to be meticulously scrutinized before being translated into a clinical setting.

Supplemental Figures

Supplemental Figure 1. Additional phenotypic features of the atherosclerotic plaques of *CD40*^{-/-}*ApoE*^{-/-} mice.

(a) Morphological plaque phenotypes according to Virmani et al. show that deficiency of CD40 results in an increased number of intimal xanthomas and a decrease in the number of fibrous cap atheromata. (b-d) Moreover, absence of CD40 decreases the amount of vulnerable thin fibrous cap atheromata. No difference could be observed in the amount of cleaved caspase 3 in the plaque (b) or the number of FoxP3⁺ regulatory T lymphocytes in plaque (c) or adventitia (d). Error bars represent mean ± SEM

Supplemental Figure 2. Deficiency of CD40 reduces proteolysis.

(a and b) The content of MMP-2 and MMP-9, expressed as absolute positively stained area per plaque, was reduced in *CD40*^{-/-}*ApoE*^{-/-} mice. (c) Consistent with these data, gelatinase activity was reduced in BM-derived FGK45-treated macrophages of *CD40*^{-/-} mice. (d) The levels of TIMP-1 were increased in *CD40*^{-/-} macrophages. Error bars represent mean ± SEM. *, P < 0.05

Supplemental Figure 3. Immunophenotyping of blood, spleen and lymph node cells.

Depicted are the results of FACS experiments of splenic cells from *Apoe*^{-/-} and *CD40*^{-/-}*Apoe*^{-/-} (a and b) and *CD40-Twt*, *CD40-T2/3/5*^{-/-}, *CD40-T6*^{-/-}, and *CD40-T2/3/5/6*^{-/-} mice (c and d). (a) CD40 deficiency reduces the fraction of CD4⁺ and CD8⁺ CD44^{high}CD62L^{low} effector memory T cells and increases the fraction of naive T cells. (b) CD40 deficiency increases the amount of CD8⁺CD4⁺ DCs and CD8⁺CD4⁻ DCs and decreases the fraction of plasmacytoid (CD3⁺CD19⁺CD11c^{low}B220^{high}Ly6C^{high}) DCs. (c) Deficiency in CD40-T6 signaling reduces the fraction of CD4⁺CD44^{high}CD62L^{low} effector memory T cells, whereas deficiency in CD40-T2/3/5 signaling increases the amount of CD8⁺CD44^{high}CD62L^{low} effector memory T cells. (d) Deficiency of CD40-T2/3/5 and CD40-T2/3/5/6 signaling reduces the fraction of CD8⁺CD4⁺ DCs and increases the fraction of CD8⁺CD4⁻ DCs, whereas the fraction of plasmacytoid DCs decreases in absence of CD40-T6 and CD40-T2/3/5/6 signaling. Horizontal bars represent the mean. * P < 0.05.

Supplemental Figure 4. The balanced effector T cell phenotype in absence of CD40-TRAF2/3/5 interactions.

(a) Deficiency of CD40-TRAF2 signaling results in an increase in CD3⁺CD4⁺ T lymphocytes in spleen, blood and lymph nodes (not depicted). (b-d) This increase in effector T lymphocytes is compensated by an increase in CD4⁺CD25⁺FoxP3⁺ regulatory T lymphocytes in spleen (b), blood, and lymph nodes (not depicted) and in the adventitia underlying the plaque (c), whereas regulatory T cell function had only significantly changed in the CD40-T2/3/5/6^{-/-} mouse (d). Horizontal bars represent the mean (a, b). Error bars represent mean $\pm$ SEM (c, d). *, $P < 0.05$.

Supplemental Figure 5. Deficiency of CD40 reduces proteolysis.

(a and b) The content of MMP-2 and MMP-9, expressed as absolute positively stained area per plaque, was reduced in *CD40-T6^{-/-}* and *CD40-T2/3/5/6^{-/-}* mice. (c) Consistent with these data, gelatinase activity was reduced in BM-derived FGK45-treated macrophages of *CD40-T6^{-/-}* and *CD40-T2/3/5/6^{-/-}* mice. (d) The levels of TIMP-1 were increased in macrophages of all *CD40-TRAF* mouse models. Error bars represent mean ± SEM. *, P < 0.05.

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Chapter 5

CD40-CD40L: linking pancreatic, adipose tissue and vascular inflammation in type 2 diabetes and its complications

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In press

Abstract

Numerous epidemiological studies have consistently demonstrated the strong association between type 2 diabetes mellitus (T2DM) and an increased risk to develop cardiovascular disease. The pathogenesis of T2DM and its complications is characterized by pancreatic, adipose tissue and vascular inflammation. CD40 and CD40L, members of the TNF(R) family, are well known for their role in immunity and inflammation. Here, we give an overview on the role of CD40-CD40L interactions in the pathogenesis of T2DM with a special focus on pancreatic, adipose tissue and vascular inflammation. In addition, we explore the role of sCD40L as a potential biomarker for the development of cardiovascular disease in T2DM subjects. Finally, the therapeutic potential of CD40-CD40L inhibition in T2DM is highlighted.

Introduction

Numerous epidemiological studies have consistently demonstrated the strong association between type 2 diabetes mellitus (T2DM) and cardiovascular disease. T2DM patients have a 200% increased risk of cardiovascular death compared to the non-diabetic population, and vascular complications, such as coronary artery disease and peripheral artery disease, are the major cause of morbidity and mortality in T2DM ^{1, 2}. The increasing global prevalence of diabetes makes the prevention and treatment of its vascular complications a public health priority ³. Hence, identification of those individuals at risk for vascular complications within the heterogeneous population of T2DM patients is essential to prevent and reduce vascular morbidity and mortality ⁴.

In T2DM, two types of vascular disease are distinguished: microvascular diseases such as retinopathy, neuropathy and nephropathy, and macrovascular disease such as coronary artery disease, cerebrovascular disease, and peripheral artery disease ⁵. The pathophysiology of T2DM that eventually results in the development of microvascular and/or macrovascular complications is complex, and remains incompletely understood.

The key pathophysiological characteristics of T2DM include insulin resistance, glucose intolerance, hyperinsulinemia, hyperglycemia, and diabetic dyslipidemia ⁵. In addition, T2DM is commonly complicated by the presence of co-morbidities including obesity, metabolic syndrome and hypertension, among others. Together these factors promote a chronic, systemic, low-grade inflammation, eventually resulting in the progression of T2DM and the development of vascular disease ⁵. Increasing evidence suggests that the Tumor Necrosis Factor (Receptor) family members CD40 and CD40L contribute to the T2DM-associated inflammation and subsequent development of vascular complications.

CD40-CD40L

The co-stimulatory molecule CD40 and its ligand CD40L (CD154, GP139) are expressed on immune cells, including B cells, T cells, dendritic cells, and monocytes, but also on non-immune cells, including platelets, endothelial cells, fibroblasts, pancreatic islet β -cells, and pancreatic ductal cells⁶⁻⁹. Besides a membrane-bound form, CD40L also exists as a soluble molecule; soluble CD40L (sCD40L), mainly derived from activated platelets and T cells¹⁰. Although the exact biological function of sCD40L remains elusive, increased levels of sCD40L have been associated with the presence of cardiovascular disease (CVD)¹¹. CD40-CD40L interactions are required for many immune processes, e.g. chemokine and cytokine production, B cell activation, co-stimulation, immunoglobulin isotype switching, and memory cell formation^{6, 10}. In addition, CD40-CD40L signaling is involved in the pathophysiology of numerous inflammatory diseases, including atherosclerosis, inflammatory bowel disease, systemic lupus erythematosus, rheumatoid arthritis, type 1 diabetes mellitus and allograft rejection¹²⁻¹⁵. For example, in atherosclerosis, CD40-CD40L interactions critically contribute to the development of atherosclerotic plaques in the vessel wall. Inhibition of the CD40-CD40L axis in murine models of atherosclerosis results in the formation of smaller atherosclerotic plaques, characterized by a clinically favorable stable phenotype which is low in inflammatory cell numbers and high in fibrosis^{13, 14}. Until now, inhibition of CD40-CD40L is one of the most powerful plaque stabilizing strategies in laboratory settings.

In recent years, experimental and clinical studies have demonstrated the involvement of the CD40-CD40L axis in the development and progression of T2DM and its vascular complications. Plasma levels of sCD40L are elevated in sub-optimally treated, hyperglycemic and dyslipidemic T2DM patients and sCD40L levels may also predict the occurrence of cardiovascular events in these patients¹⁶⁻¹⁸. Experimental studies suggest that inhibition of CD40-CD40L may reduce the systemic inflammatory response responsible for the progression of T2DM and the development of CVD, as discussed below^{8, 9, 19}.

CD40-CD40L interactions in the pathogenesis of pancreatic inflammation in T2DM

Chronic, low-grade inflammation is a hallmark of T2DM and it manifests in the pancreas, adipose tissue, liver, vasculature, and in the circulation ⁵. The inflammatory responses are characterized by increased leukocyte numbers and elevated expression of pro-inflammatory cytokines, such as interleukin (IL)-1 β and IL-6 ²⁰, which are induced and maintained by oxidative stress, lipotoxicity, glucotoxicity, and ectopic lipid deposition ⁵.

The co-stimulatory molecule CD40 is expressed on both human and mouse pancreatic islet β -cells and pancreatic duct cells, but not on α -cells ⁹. The expression of CD40 on pancreatic islet and ductal cells is increased upon exposure to pro-inflammatory cytokines, including tumor necrosis factor (TNF)- α , IL-1 β , and interferon (IFN)- γ , all abundantly present in the diabetic pancreas ^{8, 9}. Binding of CD40L to CD40 on β -cells results in the activation of nuclear factor kappa B (NF κ B), and subsequently induces the expression of cytokines, including IL-6, IL-8, and chemokines, such as monocyte chemoattractant protein (MCP)-1, and macrophage inflammatory protein (MIP)-1 β ^{8, 9}. This further enhances pancreatic inflammation, and impairs β -cell insulin release or production. Although *in vitro* experiments suggested that insulin metabolism is not affected by CD40L signaling, Poggi et al. recently demonstrated that obese *CD40L*^{-/-} mice have preserved insulin sensitivity and low plasma insulin levels compared to obese *CD40L*^{+/+} mice ^{8, 19}. However, *CD40L*^{-/-} mice were not more glucose tolerant, probably as a result of decreased plasma insulin levels ¹⁹. The effects reported in *CD40L*^{-/-} mice could be mimicked by antibody-mediated inhibition of CD40L in obese *CD40L*^{+/+} mice ¹⁹. Interestingly, the improvement of insulin sensitivity and decreased plasma insulin concentrations in mice treated with an anti-CD40L antibody was independent from body weight ¹⁹, suggesting that CD40L may have direct effects on insulin metabolism, at least in a mouse model of diet-induced obesity.

CD40-CD40L interactions may thus induce or exacerbate pancreatic inflammation in T2DM, thereby indirectly impairing insulin metabolism. Also, initial studies suggest a direct role for CD40-CD40L in insulin production or

release. Future studies are required to explore the full effects of the CD40-signaling pathway on insulin metabolism.

CD40-CD40L promotes adipose tissue inflammation in T2DM

Systemic inflammation is a key feature of T2DM and its co-morbidities such as obesity and the metabolic syndrome ^{21, 22}. Systemic inflammation aggravates the progression of T2DM and the development of vascular complications. In recent years, it has been demonstrated that adipose tissue is a major endocrine organ that contributes to the development of systemic inflammation in various inflammatory diseases, such as obesity, atherosclerosis, and T2DM ^{22, 23}.

CD40 mRNA levels in adipose tissue and plasma levels of sCD40L positively correlate with body mass index, and CD40 is expressed on adipocytes and stromal adipose tissue, including immune cells ²⁴. CD40L signaling in adipose tissue increases the expression of pro-inflammatory mediators, including TNF α , IL-6, and MCP-1 ²⁴. Furthermore, CD40L stimulation enhances lipid droplet accumulation and adipogenesis in 3T3-L1 cells ²⁵. Interestingly, CD40L exposure reduces insulin-mediated glucose uptake by adipocytes, as a result of reduced expression of IRS-1 and GLUT-4, whereas IRS-2 and GLUT-1 levels remain unaffected ^{19, 25}. These findings could be reproduced by co-culture of adipocytes and T cells, the main source of membrane-bound CD40L ¹⁹. Thus, *in vitro* experiments suggest that CD40L⁺ T cells may directly induce adipocyte inflammation and impair adipose tissue insulin sensitivity, thereby contributing to systemic inflammation and insulin resistance (IR).

IR results in increased plasma glucose levels and compensatory hyperinsulinemia ²⁶. Both hyperglycemia and chronic hyperinsulinemia increase the expression of membrane-bound CD40L on circulating platelets. Platelet CD40L subsequently promotes the formation of platelet-leukocyte aggregates and leukocyte-endothelium interactions, thereby enhancing vascular inflammation ^{27, 28}. In contrast to chronic hyperinsulinemia in T2DM, short-term hyperinsulinemia decreased plasma sCD40L levels ²⁹. Although the underlying mechanisms were not elucidated, one can speculate that short-

term hyperinsulinemia mimics the physiological response to transient fluctuations in glucose metabolism and does not reflect inflammatory conditions, whereas chronic hyperinsulinemia reflects a pathological condition²⁹.

In conclusion, CD40-CD40L signaling plays a major role in adipose tissue inflammation, adipogenesis, and insulin resistance. Together, these factors promote systemic inflammation, thereby stimulating the progression of T2DM and its complications. However, current evidence is mainly based on correlation studies and *in vitro* studies, emphasizing the need for additional *in vivo* studies to unravel the exact role of CD40-CD40L in adipose tissue inflammation in T2DM.

The key role of CD40-CD40L in vascular inflammation

Atherosclerosis, the pathological substrate of macrovascular disease in T2DM, is the result of chronic inflammation of the large and mid-sized arteries³⁰. The immune system plays an important role in the development of atherosclerotic plaques. Plaque rupture subsequently results in thrombosis and vascular occlusion, thereby causing clinical symptoms, such as peripheral occlusive artery disease and myocardial infarction³⁰. In the last decade, we and others have demonstrated a critical role of the CD40/CD40L dyad in the development of atherosclerosis^{6, 12-14}. Both, genetic and pharmacologic inhibition of CD40(L) signaling reduced plaque size and induced a plaque phenotype characterized by high levels of collagen and low numbers of inflammatory cells^{12, 13, 31}. Interestingly, even when anti-CD40L treatment was started after plaques had developed, these plaques transformed into this beneficial phenotype.

Both, CD40 and CD40L are expressed on immune cells and non-immune cells present on cells in the atherosclerotic plaque, as discussed above. In initial atherosclerotic plaques, CD40-CD40L interactions promote leukocyte recruitment by at least three mechanisms. First, activation of CD40 on endothelial cells results in the expression of adhesion molecules, including vascular cell adhesion molecule (VCAM)-1, intercellular cell adhesion molecule (ICAM)-1, and E-selectin³². Second, macrophages, present in initial plaques,

secrete pro-inflammatory chemokines upon CD40L exposure, such as MCP-1, MIP-1 α , MIP-1 β , and RANTES^{33, 34}. Third, platelet CD40L contributes to the formation of platelet-leukocyte aggregates, and promotes leukocyte adhesion to the activated endothelium²⁸.

In advanced plaques, CD40-CD40L interactions further aggravate inflammation by stimulating macrophage cytokine production (IL-1 β , IL-2, IL-6, TNF α). Also, CD40-induced production of matrix metalloproteinases contributes to destabilization of the atherosclerotic plaque, thereby promoting plaque rupture and subsequent arterial occlusion³³⁻³⁵.

Unfortunately, antibody-mediated blockage of CD40L was complicated by thromboembolism when applied to patients, as a result of disrupted CD40L- $\alpha_{IIb}\beta_3$ interactions in arterial thrombi³⁶. Therefore, clinical trials using the anti-CD40L antibody were stopped. Further investigation of CD40L-induced signaling pathways is required to identify therapeutic targets without these side effects. Our group recently demonstrated that disruption of CD40L-CD40-TRAF6 interactions in leukocytes prevents atherosclerosis, without affecting thrombus stability¹², thereby rendering inhibition of CD40-TRAF6 interactions a promising therapeutic target for atherosclerosis.

Do sCD40L levels reflect the inflammatory status in T2DM patients?

Glycated hemoglobin A1c (HbA1c) is used as a marker for glycemic control of diabetes. Current guidelines state that the treatment of T2DM should aim to reduce HbA1c levels to 7% of total hemoglobin, or even lower ³⁷. However, an increasing amount of evidence indicates that HbA1c may not adequately predict the development of micro- and macrovascular complications ^{2, 38-42}. Thus, additional biomarkers that reflect systemic inflammation may be required to identify the T2DM patients who are at risk to develop macrovascular complications.

sCD40L levels may predict the risk of major cardiovascular events, including acute myocardial infarction, sudden cardiac death, and recurrent angina in patients suffering from coronary artery disease ⁴³⁻⁴⁵. For example, Lobbes et al. demonstrated that sCD40L levels predict the occurrence of myocardial infarction and cardiovascular death in a population free from CVD and in a population suffering from CVD ¹¹. Interestingly, T2DM is independently associated with elevated sCD40L levels in patients suffering from acute coronary syndromes and myocardial infarction ⁴⁶.

As discussed above, IR, hyperinsulemia, hyperglycemia, and obesity are all associated with increased sCD40L levels ⁴⁷. Correlative studies have demonstrated that treatment of these factors may reduce plasma levels of sCD40L. For example, thiazolidinediones reduced sCD40L levels by 27% in recent onset T2DM, while in long-standing disease sCD40L was reduced by 29% in patients without macrovascular complications and by 34% in patients suffering from these complications ⁴⁸. Furthermore, treatment of diabetic dyslipidemia using atorvastatin reduced sCD40L levels in T2DM patient that suffered from coronary artery disease ⁴⁹. However, conflicting study outcomes are reported ⁵⁰⁻⁵³, and we suggest that this is the result of heterogeneity in study protocols. At least three factors may contribute to this heterogeneity. As mentioned, activated platelets are the main source of sCD40L. Hence, the levels of the protein differ between plasma and serum samples. The highest levels have been reported in serum samples, and sCD40L levels in clotting plasma increased with time ^{54, 55}. Since low temperatures inhibit the increase in serum samples, it is likely that the increase is the result of *ex vivo* release of sCD40L from activated platelets ⁵⁴⁻⁵⁶. In addition, Dominguez-Rodriguez et al.

observed a circadian rhythm in sCD40L levels in patients suffering from myocardial infarction ⁵⁷. Samples drawn at 9 PM contained 41.5% higher sCD40L levels than samples obtained at 2 AM. Although the exact mechanisms were not investigated, it was hypothesized that circadian differences in proteinase levels may be involved ⁵⁷. Finally, the use of anti-platelet drugs, such as cyclooxygenase inhibitors or adenosine diphosphate receptor inhibitors, may affect the release of sCD40L from platelets ⁵⁸. Hence, the use of this type of drugs should be carefully monitored in clinical studies.

As a result, both experimental and clinical studies suggest that sCD40L may reflect the inflammatory status of T2DM patients, and may be used as a biomarker to identify those who are at risk to develop CVD. However, standardization of study protocols is of great importance to evaluate the full potential of sCD40L as a novel biomarker.

CD40-CD40L as a therapeutic target in T2DM

Commonly used anti-diabetic drugs may reduce inflammation in T2DM. For example, metformin and thiazolidinediones have been shown to reduce C-reactive protein (CRP) levels ⁵⁹. These effects are not merely due to glucose lowering, because the reductions in inflammatory markers are greater than the reduction seen after similar glucose lowering induced by other anti-hyperglycemic approaches ^{59, 60}. Nevertheless, these drugs do not prevent the occurrence of CVD in a major number of T2DM patients, emphasizing the need for additional anti-inflammatory therapeutic strategies. For example, the blockade of the pro-inflammatory cytokine IL-1 β improved endocrine pancreas function, and reduced hyperglycemia and systemic inflammation in T2DM patients ^{61, 62}.

The therapeutic potential of CD40 and CD40L has been investigated in multiple inflammatory diseases, including atherosclerosis, inflammatory bowel disease, psoriasis, systemic lupus erythematosus (SLE), rheumatoid arthritis, allograft rejection, and type I diabetes ¹⁵. The anti-CD40 monoclonal antibody (mAb) ch5D12 reduced disease severity in Crohn's disease, and Ruplizimab, an anti-CD40L mAb, was successfully used in the treatment of SLE and inflammatory bowel disease ¹⁰. In addition, Poggi et al. demonstrated that anti-

CD40L mAb (MR-1) treatment improved insulin sensitivity in a mouse model of diet-induced obesity¹⁹. As discussed above, specific inhibition of CD40-TRAF6 interactions in a murine model for atherosclerosis prevents the formation of atherosclerotic plaques¹². Thus, specific inhibition of CD40-CD40L signaling may become a promising strategy to improve adipose tissue and vascular inflammation, thereby reducing the occurrence of vascular complications of T2DM.

Concluding remarks and future directions

The CD40-CD40L dyad has a complex and not fully elucidated role in the pathogenesis of T2DM. However, both experimental and clinical studies show that CD40-CD40L interactions promote pancreatic, adipose tissue, and vascular inflammation, and induce a chronic, systemic inflammatory response that eventually results in the formation of atherosclerosis (**Fig. 1**). Numerous clinical studies have demonstrated that sCD40L levels are elevated in patients suffering from diabetes and its complications. Although the exact inflammatory mechanisms that are responsible for this increase are not completely understood, it is clear that sCD40L reflects the inflammatory status in T2DM. Future studies should explore the potential of sCD40L as a biomarker to identify those patients who are at risk of developing vascular complications within the heterogeneous T2DM population. In order to do so, it is essential to standardize sCD40L measurement protocols. Finally, elucidation of CD40-CD40L signaling pathways involved in diabetic inflammation may identify novel therapeutic targets for T2DM and its complications.

Key messages

- CD40-CD40L interactions promote pancreatic, adipose tissue and vascular inflammation in type 2 diabetes mellitus.
- sCD40L may become a biomarker for the development of cardiovascular disease in patients with type 2 diabetes mellitus.
- Specific inhibition of CD40-CD40L signaling may reduce the occurrence of vascular complications of type 2 diabetes mellitus.

Figure 1. CD40L binding to CD40 results in the up-regulation of pro-inflammatory cytokines and chemokines by pancreatic islet β cells, macrophages, and adipocytes.

The expression of adhesion molecules on endothelial cells is increased. Insulin-mediated glucose uptake is impaired in adipocytes as a result of decreased IRS-1 and GLUT-4 expression. Together, CD40-CD40L interactions induce a systemic inflammatory response that promotes not only the development of cardiovascular disease and the metabolic syndrome, but also further increases the expression of CD40L and elevates sCD40L levels.

TNFα, Tumor Necrosis Factor α; *IL-1β*, interleukin 1β; *IFN-γ*, interferon γ; *MCP-1*, monocyte chemoattractant protein 1; *VCAM-1*, vascular cell adhesion molecule 1; *ICAM-1*, intercellular adhesion molecule 1; *IL-6*, interleukin 6; *MIP-1α*, macrophage inflammatory protein 1α; *MMP-9*, matrix metalloproteinases 9; *IRS-1* insulin receptor substrate 1; *GLUT-4*, glucose transporter type 4.

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Chapter 6

CD40L deficiency ameliorates adipose tissue inflammation and metabolic manifestations of obesity in mice

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Abstract

Objective: Obese adipose tissue shows hallmarks of chronic inflammation, which promotes the development of metabolic disorders. The mechanisms by which immune cells interact with each other or with metabolism-associated cell types, and the players involved are still unclear. The CD40/CD40L co-stimulatory dyad plays a pivotal role in immune responses and in diseases like atherosclerosis, and may therefore be a mediator of obesity. Here we investigated whether CD40L is involved in adipose tissue inflammation and its associated metabolic changes.

Methods and Results: To assess a putative role of CD40L in obesity *in vivo*, we evaluated metabolic and inflammatory consequences of 18 weeks high-fat feeding in *CD40L*^{+/+} and *CD40L*^{-/-} mice. In addition, C57Bl6 mice were injected with neutralizing anti-CD40L (α CD40L) antibody for 12 weeks while being fed a high-fat diet. Genetic deficiency of CD40L attenuated the development of diet-induced obesity, hepatic steatosis and increased systemic insulin sensitivity. In adipose tissue, it impaired obesity-induced immune cell infiltration and the associated deterioration of glucose and lipid metabolism. Accordingly, α CD40L treatment improved systemic insulin sensitivity, glucose tolerance and CD4⁺ T cell infiltration in adipose tissue with limited effects on adipose tissue weight.

Conclusion: CD40L plays a crucial role in the development of obesity-induced inflammation and metabolic complications.

Introduction

In recent years, it has become clear that obesity is associated with a chronic, low-grade inflammatory state, considered responsible for the development of insulin resistance, type 2 diabetes, and atherosclerosis ^{1, 2}. Obese adipose tissue shows hallmarks of chronic inflammation, with the involvement of T cell subsets such as Th1 ³, Th2 ⁴, Th17 ⁵, regulatory T cells ⁶⁻⁸ and macrophage subsets ^{9, 10}, but also of other immune cells such as mast cells.¹¹ These immune cells, their cell-cell interactions, and their interactions with adipocytes result in an increased expression of leukocyte adhesion molecules ¹², chemokines, and cytokines in adipose tissue ¹³, thereby enhancing the inflammatory response and affecting insulin signaling ¹⁴. Although extensive research is dedicated to the effects of inflammation on obesity-related complications, little is known about the immunologic pathways and factors that initiate, regulate, and amplify the inflammatory cascade during obesity.

The CD40-CD40L dyad, known as a co-stimulatory receptor-ligand pair, plays an important role in enhancing and regulating immune responses, as well as inflammation, and contributes to a plethora of chronic inflammatory diseases, such as colitis, Crohn's disease, allergic encephalitis, and multiple sclerosis ¹⁵. Disruption of the *CD40L* gene, or inhibition of CD40L with different antibodies in *Apoe*^{-/-} and *Ldlr*^{-/-} mice, abrogates atherosclerosis and results in plaques that are extremely low in inflammation and high in fibrosis, the equivalent of a clinically preferable, stable atherosclerotic plaque in humans ¹⁶⁻¹⁸. CD40L is predominantly expressed by CD4⁺ T cells and activated platelets, and displays a variable expression in several other cell types, such as macrophages, mast cells, endothelial cells, and vascular smooth muscle cells ¹⁹.

Interestingly, two studies have shown that the CD40-CD40L dyad is involved in obesity-associated inflammatory processes ^{20, 21}. We have previously reported that CD40 mRNA levels in adipose tissue were correlated with body mass index and that CD40 was not only expressed in the stromal adipose fraction containing immune cells, but also on the adipocyte itself ²⁰. Stimulation of human adipocytes by recombinant sCD40L or activated T lymphocytes elicited the production of pro-inflammatory adipokines and, in addition, decreased the expression of insulin signaling molecules like GLUT4

and IRS-1²⁰. This phenomenon required activation of mitogen-activated protein kinase (MAPK) and I κ B/NF- κ B pathways¹⁸. The study by Missou et al confirmed these results and showed that supernatants of CD40L-stimulated adipocytes induced the activation of endothelial cells²¹. Interestingly, patients afflicted by obesity²², type 2 diabetes^{23, 24} and the metabolic syndrome²⁵ exhibit elevated serum sCD40L levels which can be modulated by antidiabetic treatments²⁶.

Considering the important role of CD40-CD40L in inflammation, its effects on chronic inflammatory diseases, and our previous data showing involvement of CD40L in adipocyte stimulation, we hypothesize that CD40L plays a pivotal role in initiating and amplifying the chronic low-grade inflammatory status in adipose tissue, thereby inducing obesity-related metabolic complications. Our study reveals that genetic CD40L deficiency and neutralizing α CD40L antibody treatment impair the development of inflammation in adipose tissue and obesity-induced metabolic disturbances, indicating a role for CD40L in the pathogenesis of metabolic disorders.

Methods

Animals

CD40L^{-/-}, *CD40L*^{+/-} mice (100% C57Bl6J background) and C57Bl6J mice were fed a standard-fat diet (SFD, 70% kcal carbohydrate, 10% kcal fat, 20% kcal protein, 3.68 kcal/g, SDS Special Diets Services, Witham, UK) or high-fat diet (HFD, 35% kcal carbohydrate, 45% kcal fat, 20% kcal protein, 4.54 kcal/g, SDS Special Diets Services). Two experimental studies were conducted: **1)** *CD40L*^{-/-} and *CD40L*^{+/-} mice fed with the SFD or HFD for 18 weeks starting at 6-12 weeks of age, and **2)** C57Bl6 mice intraperitoneally (ip) injected with neutralizing anti-CD40L (α CD40L, clone: MR-1) or control IgG antibodies (200 μ g twice per week) and fed with the HFD for 12 weeks over the same period beginning at 8 weeks of age. All mice were maintained under a 12h light-dark cycle and were allowed free access to food and water. The food intake was measured weekly. After the experimental period, overnight-fasted animals were euthanized, blood was collected and organs [subcutaneous (scAT) and epididymal white adipose tissue (epiAT), brown adipose tissue

(BAT), liver, spleen] were dissected. Studies were approved by the animal experimental commission of the University of Maastricht and executed according to the institutional guidelines.

Biochemical measurements and glucose/insulin tolerance tests

A glucose tolerance test (GTT) and insulin tolerance test (ITT) was performed, and fasting insulin levels were measured. For the GTT, overnight-fasted conscious mice were injected i.p. with glucose (1mg/g) For the ITT, 5h fasted conscious mice were injected i.p. with insulin (0,75mU/g, Actrapid, Novonordisk, Bagsvaerd, Denmark). Glucose levels were measured from whole blood using a glucometer (Roche Diagnostics, Basel, Switzerland) at times indicated in the figures. Fasting insulin (Mercodia, Uppsala, Sweden), IP-10 (RD systems, Minneapolis, MN, USA), IL-6 (Invitrogen, Breda, The Netherlands) and leptin levels were measured in plasma by enzyme-linked immunoabsorbent assay (Chrysal chem., Downers Grove, IL USA). The homeostasis model assessment of insulin resistance (HOMA-IR) was estimated using the following formula: fasting insulin (μ international unit/ml) x glucose/22.5. Cholesterol levels were measured using a colorimetric assay (CHOD-PAP, Roche, Mannheim, Germany) and triglycerides by enzymatic assay (Wako, Neuss, Germany).

Flow Cytometric Analysis

Stromal vascular cells (SVC) were isolated from epiAT using collagenase (Sigma-Aldrich, Zwijndrecht, The Netherlands). The samples were incubated at 37°C with shaking until complete digestion, passed through a 100 μ m cell strainer (Falcon, distributed by BD biosciences, Breda, The Netherlands), washed and centrifuged to obtain the final SVC pellet. Spleens were pressed through a 70 μ m cell strainer and washed after erythrocyte lysis. Fc-blocking (CD16/32 antibody) was performed prior to cell labeling. FACS for CD3, CD4, CD8, CD25, FoxP3, Ly6G, B220, CD11c and CD19 was performed on SVC and splenocytes^{27, 28}. All antibodies were purchased from e-Biosciences (San Diego, CA, USA) or BD Pharmingen (distributed by BD Biosciences). Disposables were purchased from Bio Greiner One.

Morphometry and Immunohistochemistry (IHC)

Immunohistochemistry for CD3 and macrophages was performed on 4µm thin sections of epiAT and scAT. Liver sections were stained with haematoxylin-eosin, sirius red and Oil red O and used to determine the degree of steatosis and non-alcoholic steato-hepatitis as indicated by the NASH clinical research network ²⁹. All morphometric parameters were determined using a microscope coupled to a computerized morphometry system (Qwin, Leica, Rijswijk, The Netherlands). From each animal, images of 6-7 fields were acquired. Subsequently, the diameters of all adipocytes and the number of crown-like structures (CLS) and positive stained cells were determined in each field by an observer blinded to the conditions using Qwin3 software.

Real-Time PCR

Total RNA was extracted using the Trizol method (Invitrogen). cDNA was synthesized using i-Script cDNA synthesis kit (BIO-RAD). PCRs were performed with a Bio-Rad instrument and software under standard conditions. The relative amounts of the different mRNAs were quantified by using the second derivate maximum method. The relative quantification for mRNA was corrected to 36B4 mRNA values used as an invariant control. Results were expressed relative to the control group (WT SFD), which was assigned a value of 1.

Statistical analysis

Results are means $\pm$ SEM. A Mann–Whitney *U* test or a 2-way ANOVA was used. Significance was set at $P < 0.05$.

Results

***CD40L*^{-/-} mice are protected from weight gain**

On the standard-fat diet, a slight but significant decrease in body weight gain was observed in *CD40L*^{-/-} mice (**Fig. 1A**). However, scAT, epiAT, brown adipose tissue, and liver weights were not affected by CD40L deficiency (**Table 1**). Food intake was also not different, but feeding efficiency was significantly less in *CD40L*^{-/-} mice, suggesting a higher energy expenditure in absence of CD40L signaling.

In HFD-fed obese *CD40L*^{-/-} mice, body weight gain, as well as food intake and feeding efficiency, was strongly reduced compared with obese wild-type (WT) mice (**Fig. 1A, Table 1**). In addition, scAT and liver weights tended to decrease (**Table 1**). This indicates that during obesity, absence of CD40L reduces food intake and feeding efficiency, and that fat pads and other fat-accumulating tissues, such as liver, tend to weigh less, thereby contributing to the reduction in total body weight.

Surprisingly, adipocyte size of epiAT, but not that of scAT, increased in obese *CD40L*^{-/-} mice. In brown adipose tissue, mRNA levels of uncoupling protein-1 were increased (Supplemental Figure I), indicating that deficiency of CD40L increases thermogenesis, thereby preventing body weight gain. Moreover, plasma leptin levels decreased in *CD40L*^{-/-} mice (**Table 1**). This seems inconsistent with the reduction in food intake, but considering its role in regulating energy intake and expenditure, this is in accordance with the lower body weights in *CD40L*^{-/-} mice.

CD40L deficiency improves insulin resistance

In lean mice, deficiency of CD40L did not affect fasting blood glucose, plasma insulin levels, or insulin sensitivity as measured by the homeostasis model assessment of insulin resistance (**Table 1**). However, in obese *CD40L*^{-/-} mice, both plasma insulin levels and homeostasis model assessment of insulin resistance were reduced (**Table 1**). Likewise, the insulin tolerance test displayed higher insulin sensitivity in *CD40L*^{-/-} than in WT animals, as indicated by the lower levels of blood glucose after insulin injection (**Fig. 1B**). However, *CD40L*^{-/-} mice were not more glucose tolerant according to the glucose tolerance test (**Fig. 1C**), which could be attributed to their lower baseline

insulin level (**Table 1**). Absence of CD40L did not affect plasma cholesterol levels (**Table 1**). These results reveal that obese *CD40L*^{-/-} mice are partially protected against the impaired insulin sensitivity associated with obesity.

CD40L deficiency attenuates obesity-induced hepatic steatosis

After the induction of diet-induced obesity, liver weights increase because of steatosis³⁰. Surprisingly, hepatic steatosis was only infrequently present in *CD40L*^{-/-} mice (**Table 1**). Histological analysis revealed that 75% of *WT* mice developed NASH, whereas only 25% of *CD40L*^{-/-} mice displayed some signs of NASH. Moreover, livers of *CD40L*^{-/-} mice were protected against steatosis, foamy degeneration, ballooning, and lobular inflammation (**Fig. 1D, E**).

Altered signaling in a number of biological pathways, including glycolysis, fatty acid synthesis, and fat uptake, is associated with liver steatosis³¹. Indeed, expression of 2 major glycolytic genes, glucokinase (GK) and liver specific pyruvate kinase (LPK), was reduced in livers of obese *CD40L*^{-/-} mice (**Fig. 1F**). Furthermore, livers of *CD40L*^{-/-} mice tended to have a reduced expression of a number of genes involved in *de novo* lipid synthesis, such as the genes for ATP-citrate lyase (Acl), acetyl-coenzyme A carboxylase (ACC1), and stearyl-coenzyme A desaturase (SCD-1) (**Fig. 1G**) and had a significantly reduced expression of the mRNA levels of the fatty acid transporter CD36 (**Fig. 1H**). Consistent with decreased steatosis, the lipogenic transcription factor peroxisome proliferator-activated receptor- γ (PPAR γ) and sterol regulatory element-binding protein-1c (SREBP1c) were reduced in livers of *CD40L*^{-/-} mice (**Fig. 1I**). These data indicate that CD40L deficiency attenuates obesity-induced deterioration of glucose and lipid homeostasis, as well as hepatic steatosis.

Figure 1. Metabolic profiles of *CD40L*^{-/-} mice.

A) Body weight gain of WT (line, diamonds) and *CD40L*^{-/-} mice (dotted line, circles) on standard fat (SFD; open symbol) or high fat diet (HFD; filled symbol) for 18 weeks. B) Insulin tolerance test in 5 hours-fasted and C) glucose tolerance test in overnight-fasted WT (line, diamond) and *CD40L*^{-/-} (dotted line, circle) mice fed with a SFD (open symbols) or HFD (filled symbols) for 16 and 17 weeks. Representative pictures of (D) HE- and (E) Oil red O-stained liver sections from obese WT and *CD40L*^{-/-} mice (x20). mRNA levels in WT (white bars) and *CD40L*^{-/-} liver (black bars) of genes involved in lipid metabolism: F) glucokinase (GK) and liver specific pyruvate kinase (LPK), G) ATP-citrate lyase (Acly), acetyl-coA carboxylase 1 (ACC1), fatty acid synthase (FAS), elongase of long chain fatty acid family 6 (ELOVL6), stearyl-coA desaturase (SCD-1); H) CD36, I) sterol regulatory element-binding protein (SREBP1c) and peroxisome proliferators activated receptor γ (PPAR γ). * $P < 0.05$, *** $P < 0.001$ for comparison between obese and lean mice within each genotype. † $P < 0.05$, for comparison between genotypes after the same diet. $n=7$ and 8 respectively for SFD and HFD-fed WT mice, $n=5$ for *CD40L*^{-/-} mice.

Table 1. Characteristics of *WT* and *CD40L*^{-/-} mice submitted to a standard-fat diet (SFD) or a high-fat diet (HFD) for 18 weeks. Data are means ± SEM. ND not determined. * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001 for comparison between obese and lean mice within each genotype. † *P* < 0.05, †† *P* < 0.01, ††† *P* < 0.001 for comparison between genotypes after the same diet. *n*=7 and 8 respectively for SFD and HFD-fed *WT* mice, *n*=5-6 for *CD40L*^{-/-} mice.

	<i>WT</i> -SFD	<i>CD40L</i> ^{-/-} -SFD	<i>WT</i> -HFD	<i>CD40L</i> ^{-/-} -HFD
Body weight (g)	30.17±0.26	29.97±1.71	45.10±1.90***	38.73±0.76*†
Food intake (g/week)	18.22±0.26	18.69±0.31	27.36±1.4***	18.76±1.24†††
Feeding efficiency (g/100kcal)	0.65±0.03	0.28±0.05†††	1.21±0.14***	1.05±0.07***†††
Liver weight (g)	1.09±0.03	1.00±0.15	1.50±0.12*	1.14±0.06
AT characteristics				
Epididymal adipose tissue (g)	0.72±0.06	0.75±0.10	2.11±0.12***	2.18±0.05*
Epididymal adipocyte diameter (μm)	40.26±2.55	38.38±1.7	53.53±1.40***	60.13±1.13*††
Subcutaneous adipose tissue (g)	0.45±0.05	0.45±0.09	1.99±0.25***	1.47±0.16*
Subcutaneous adipocyte diameter (μm)	29.35±1.84	31.96±3.07	47.59±1.2.6**	49.84±5.72*
Brown adipose tissue (g)	0.12±0.01	0.12±0.007	0.27±0.04	0.22±0.04
Blood characteristics				
Glucose (mg/dL)	83.3±2.4	68.0±7.0	112.0±4.9**	103.8±9.4*
Insulin (μg/dL)	0.16±0.03	0.19±0.03	1.51±0.15***	0.43±0.15††
HOMA-IR	0.58±0.08	0.61±0.14	7.39±0.76***	2.06±0.74†††
Cholesterol (mg/dL)	91.6±8.4	104.1±10.9	152.8±7.2*	106.7±22.1
Triglycerides (mg/dL)	53.7±5.6	47.4±7.5	71.3±16.6	52.3±3.9
Leptin (ng/mL)	5.41±0.98	3.83±0.46†	43.17±7.8***	22.41±2.1*†
IL-6 (pg/mL)	ND	ND	7.43±2.6	0.41±2.6
IP-10 (pg/mL)	ND	ND	72.0±10.0	44.0±5.6†

CD40L deficiency alters obesity-associated inflammation

Surprisingly, the obesity associated increase in immune cells was reduced in *CD40L*^{-/-} mice compared to *WT* mice. CD3⁺ T cell numbers, as well as the number of T cell crown-like structures (CLSs), were lower in epiAT of obese *CD40L*^{-/-} mice than in obese *WT* mice (**Fig. 2A-C**), as were the number of macrophages and the number of macrophage CLSs (**Fig. 2D-F**). In contrast to the epiAT, scAT contained fewer immune cells, which were more dispersed and rarely seen in clusters. However, a similar reduction in T cell and macrophage content was also detected in scAT of obese *CD40L*^{-/-} mice (CD3⁺ T cell infiltration in *WT* vs *CD40L*^{-/-}: 20.7 ± 3.9 vs. 12.9 ± 2.0 CD3⁺ per 100 adipocytes, macrophage infiltration in *WT* vs. *CD40L*^{-/-}: 12.0 ± 2.9 vs. 3.9 ± 1.9 per 100 adipocytes).

FACS analysis of the stromal vascular fraction revealed that in epiAT, induction of obesity resulted in an increase of the CD3⁺ T cell fraction (**Fig. 2G**). Within this CD3⁺ T cell fraction, clear populations of CD8⁺, CD4⁺ and CD4⁻CD8⁻ T

cells could be identified. Upon induction of obesity, the fraction of CD8⁺ cells significantly increased in *WT* epiAT, whereas CD40L deficiency prevented this increase (**Fig. 2G-H**). As reported before⁶⁻⁸, we detected a greater subfraction of CD4⁺CD25⁺FoxP3⁺ regulatory T cells in epiAT compared to lymphoid organs, such as spleen and lymph nodes (Supplemental Figure II). Interestingly, the amount of regulatory T cells was even higher in epiAT of CD40L deficient mice (**Fig. 2J**). This suggests that deficiency of CD40L induces immune modulation in obese adipose tissue. Likewise, conventional dendritic cells (CD11c^{high} B220[−] gated on non-CD3⁺CD19⁺ cells) were abundantly present in adipose tissue of obese *WT* mice, but not in obese *CD40L*^{−/−} mice (**Fig. 2K**).

In concordance with the flow cytometry data and immunohistochemistry data, epiAT of *CD40L*^{−/−} mice showed a reduced expression of inflammatory mediators, such as monocyte chemoattractant protein-1 (MCP-1) and IL-6 (**Fig. 3A**). In addition, CD40L deficiency reduced the expression of the Th1 cytokines interferon-γ (IFNγ), regulated on activation normal T cell expressed and secreted (RANTES), tumor necrosis factor-α (TNFα), and IL12. It also reduced the expression of the macrophage markers F4/80, CD68, and CSFR-1; and induced the expression of the anti-inflammatory M2 macrophage marker CD163 (**Fig. 3A, B**).

Concomitant with an increased inflammatory state, obesity decreased the expression of genes required for lipid metabolism and insulin signaling in *WT* mice. This decrease was prevented in *CD40L*^{−/−} mice, where mRNA levels of genes involved in adipocyte function, such as *lipe* (hormone-sensitive lipase) and *gpam* (glycerol-3-phosphate acyltransferase, mitochondria), were higher than in *WT* adipose tissue (**Fig. 3C**). Also, adiponectin mRNA levels were higher in obese adipose tissue of *CD40L*^{−/−} compared with that of *WT* mice (**Fig. 3C**). A similar but less pronounced effect of CD40L deficiency was observed in scAT for genes involved in lipid metabolism, adiponectin, and other inflammatory cytokines, such as IL-6, TNFα, and the macrophage marker CD68 (Supplemental Figure III). In addition to local inflammation in AT, obesity also increased plasma levels of leptin and the inflammatory cytokines IP-10 and IL-6. These levels were reduced in *CD40L*^{−/−} mice (**Table 1**). Overall, these data show that *CD40L*^{−/−} mice are protected against obesity-induced immune cell accumulation and activation in adipose tissues and in the circulation.

Figure 2. Immune cell accumulation in epididymal adipose tissue.

A-F) Immunohistochemical analysis of CD3⁺ T cells (arrows) and macrophages (arrows) in epiAT from lean and obese WT and CD40L^{-/-} mice after 18 weeks of diet. Graphs B, C, E and F represent the number of positive cells/100 adipocytes and the numbers of crown-like structures/field in each group. * P < 0.05, ** P < 0.01 for comparison between obese and lean mice within each genotype. † P < 0.05 for comparison between genotypes after the same diet. G-K) FACS analysis of the stromal vascular fraction from the epiAT of WT (white bars) and CD40L^{-/-} mice (black bars) fed with a SFD or HFD for 18 weeks. The fraction of CD3⁺, CD8⁺, CD4⁺ T cells, Tregs (CD4⁺CD25⁺FoxP3⁺) and conventional dendritic cells (CD11c^{high}B220⁻ gated on non-CD3⁺CD19⁺ cells) was determined in the immune cell gate of SVF. n=7 and 8 respectively for SFD and HFD-fed WT mice, n=5 for CD40L^{-/-} mice.

Figure 3. Epididymal adipose tissue gene expression.

mRNA levels of inflammatory molecules (A), macrophage markers (B) and genes involved in metabolism (C) in WT (white bars) and CD40L^{-/-} epiAT (black bars) after 18 weeks of HFD. * P < 0.05, ** P < 0.01. n=7 and 8 respectively for SFD and HFD-fed WT mice, n=5-6 for CD40L^{-/-} mice.

Anti-CD40L (α CD40L) treatment prevents obesity-associated anomalies

To examine the potential of therapeutic inhibition of CD40L during obesity, we examined the effects of a CD40L inhibiting antibody on the inflammatory and metabolic responses in our diet-induced obesity model. Antibody treatment was continued for only 12 weeks to prevent the formation of auto reactive antibodies. To confirm the effectiveness of α CD40L treatment, immune cell activation and composition of the spleen was analyzed by FACS. After 12 weeks of antibody treatment, we were able to show that $CD3^+$, $CD4^+$, $CD4^+FoxP3^+$ and $CD11c^{high}B220^-$ cell contents were reduced (**Fig. 4**). After the 12-week treatment period, mice had developed intermediate obesity compared with the first study (**Fig. 5A**). As in the $CD40L^{-/-}$ mouse study, total body weight and scat, epiAT, and liver weights tended to be lower in α CD40L-treated mice (**Fig. 5A-D**). As in CD40L-deficient mice, food intake was reduced upon inhibition of CD40L (**Fig. 5E**).

In intermediate obesity, treatment with the α CD40L antibody significantly increased glucose tolerance, as well as insulin sensitivity, and lowered plasma insulin levels (**Fig. 5F-H**). In addition, IL-6 and leptin plasma levels tended to be diminished (IL-6, IgG-treated vs. α CD40L-treated mice, 8.7 ± 3.7 vs. 2.8 ± 0.9 , leptin, IgG-treated vs. α CD40L-treated mice, 14.6 ± 4.2 vs. 10.2 ± 2.1).

Quantitative immunohistochemistry provided a tendency similar to that observed in $CD40L^{-/-}$ mice. The number of $CD3^+$ cells and macrophages, as well as the number of CLSs, were reduced in α CD40L treated mice (number of $CD3^+$ T cells per 100 adipocytes: 30.0 ± 6.0 vs. 19.7 ± 3.6 , $P = 0.09$, number of $CD3^+$ CLSs/field: 0.5 ± 0.2 vs. 0.1 ± 0.04 , $P < 0.05$, number of macrophages per 100 adipocytes: 18.3 ± 4.2 vs. 10.6 ± 2.7 , number of macrophages CLSs/field: 0.5 ± 0.2 vs. 0.1 ± 0.0 , $P < 0.05$ control vs. α CD40L-treated mice).

FACS analysis in epiAT of α CD40L-treated mice showed an intermediate activation state of the immune system compared to the $CD40L^{-/-}$ mouse study. First of all, the $CD3^+$ T cell fraction of the stromal vascular fraction was not altered, nor was the $CD8^+$ T cell fraction within the $CD3^+$ gate. However, the $CD4^+$ T cell fraction had decreased, without affecting the regulatory T cell fraction, suggesting that α CD40L treatment during intermediate obesity reduces the number of T-effector cells (**Fig. 5I-M**). Furthermore, the expression of major histocompatibility complex II (MHCII) on

antigen-presenting cells was reduced upon treatment (**Fig. 5L**), suggesting fewer T cell receptor-MHCII interactions and thereby less activation of the immune system. As shown in our first experiment, immune regulation by regulatory T cells seems to be an event that occurs in more severe stages of obesity, whereas reduced immune cell activation occurs in moderate obesity.

Figure 4. Anti-CD40L treatment: immune cell composition of the spleen.

FACS analysis of the spleen cells of mice fed with HFD and treated with IgG or αCD40L antibody for 12 weeks. CD3⁺ (A), CD4⁺ (B), CD8⁺ (C), Tregs (CD4⁺CD25⁺FoxP3⁺) (D), MHCII expression (E) and conventional DC (CD11c^{high} gated non CD3⁺CD19⁺ cells) (G) were analysed. * P < 0.05, ** p < 0.01, *** P < 0.001. n=9 for IgG treated mice and n=11 for αCD40L antibody treated mice.

Figure 5. Anti-CD40L antibody treatment affects metabolism and adipose tissue inflammation.

A-F) Body weight, scAT, epiAT and liver weights, food intake, plasma insulin levels of mice fed with HFD and treated with IgG control (white bars) or anti-CD40L (black bars) for 12 weeks. G-H) Glucose tolerance test in overnight-fasted and insulin tolerance test in 5h-fasted mice fed with HFD and treated with IgG control (line, filled circles) or anti-CD40L (dotted line, open circles). I-M) FACS analysis of the stromal vascular fraction from the epiAT of mice fed with HFD and treated with IgG control or antiCD40L for 12 weeks. CD3⁺, CD4⁺, CD8⁺ cells, Tregs (CD4⁺CD25⁺FoxP3⁺), and MHCII MFI were analyzed in the immune cells gated in the SVF. * P < 0.05, ** P < 0.01, *** P < 0.001. n=9 for IgG treated mice and n=11 for α CD40L antibody treated mice, except for fig F-G n=6 in each group.

Discussion

The communication between cells of the innate ³² and adaptive ^{6,7} immune system is key in the pathogenesis of obesity, a disease in which the immune system not only mediates obesity induced (tissue) inflammation, but also affects metabolic processes. The main communicators of the immune system are co-stimulatory molecules, implying a profound role for this family in the pathogenesis of obesity ^{28, 33}. In the present study, we identified the costimulatory molecule CD40L as an important player in intermediate and severe forms of diet-induced obesity. In general, deficiency of CD40L reduced the amount of immune cell infiltration in adipose tissue. In a model of intermediate obesity (12 weeks of HFD), a clear reduction in the CD4⁺ effector T cell fraction, associated with an improved insulin resistance ⁶, as well as a decrease in the antigen presenting capacity of the dendritic cells were observed after inhibition of CD40L. In a severe obesity model (18 weeks of HFD), deficiency of CD40L decreased the CD8⁺ T cell fraction, the T cell subtype associated with adipose tissue inflammation and activation of adipose tissue macrophages ⁶, and strongly increased the fraction of regulatory T cells. We can therefore conclude that, during the progression of obesity, the effect of CD40L inhibition switches from mere down-regulation of T cell activation to a mode of strong immune modulation.

Besides mediating (AT) inflammation, T cells and macrophages have recently been shown to contribute to metabolic dysfunction, such as insulin resistance and fat distribution, such as occurs in diet-induced obesity. Genetic and immunological depletion of CD8⁺ T cells improves insulin resistance in obesity, whereas CD8⁺ T cell adoptive transfer aggravates metabolic dysfunction ⁶. The balance between CD4⁺ effector T cells and regulatory T cells in AT affects metabolic functions, with a clear beneficial role for regulatory T cells ⁷. Interestingly, a reduction of macrophages and CLSs in AT, as well as a reduction in classically activated macrophages, not only reduces AT inflammation, but also ameliorates hepatic steatosis and improves glucose homeostasis and insulin sensitivity ^{10, 34}. Consistent with these studies, the attenuated CD8⁺ T cell response and the increase in regulatory T cells in our *CD40L*^{-/-} mice with severe obesity, the decrease in CD4⁺ effector T cells in AT of α CD40L antibody-treated mice with intermediate obesity, the reduction in

macrophage accumulation, and the switch to alternatively activated macrophages coincided with a reduction in weight gain, decreased insulin plasma levels, and an improved systemic insulin resistance.

Steatosis and NASH are strongly associated with obesity and insulin resistance and are now recognized as being part of the metabolic syndrome^{35, 36}. We found that in *CD40L*^{-/-} mice, liver steatosis and NASH were strongly reduced, as were the expression levels of genes involved in glycolysis, fatty acid synthesis, and fat uptake. Contrary to our results, Villeneuve et al³⁷ found that CD40L was protective against the development of steatosis after administration of olive oil. However, the different dietary stresses that are used in the two models may be accountable for this difference.

Both, genetic deficiency of CD40L and inhibition of CD40L by antibody treatment reduce food intake, thereby preventing body weight gain. Remarkably, leptin levels were lower. Because decreased leptin levels are expected to decrease satiety and increase food intake, other mechanisms may play a role³⁸. It is very possible that the absence of CD40L protects against the development of leptin resistance, which is usually associated with obesity³⁹. On the other hand, leptin is not the only factor associated with the regulation of appetite and energy expenditure. Reduced food intake could for example be caused by alterations of neuropeptide Y or proopiomelanocortin levels in the central nervous system⁴⁰.

Another function of leptin is the activation of immune cells. For example, leptin polarizes T cells towards a Th1 phenotype, thereby inducing proatherogenic cytokine production^{41, 42}. Moreover, in murine dendritic cells, leptin has been shown to induce CD40 expression and drive T cell proliferation⁴³. In our study, the reduced leptin levels may have contributed to the observed anti-inflammatory phenotype.

Although it is difficult to distinguish the primary effects of CD40L deficiency on metabolic parameters from secondary effects, such as reduced food intake and impaired body weight gain, we believe that CD40L directly affects metabolism, independent of the reduction in weight gain. For example, despite the strong reduction in body weight in *CD40L*^{-/-} mice, their epiAT weight was virtually similar. Epididymal adipocyte size was even higher in *CD40L*^{-/-} mice. Interestingly, expression of genes involved in lipid metabolism such as *lipo* and *gpam* stayed elevated, whereas they are known to be

downregulated in obese adipose tissue^{44, 45}. This indicates that, in *CD40L*^{-/-} mice, lipogenesis and lipolysis do not shut down during obesity, which may be responsible for their protection against weight gain and fat accumulation in other regions of the body, such as the liver. Indeed, in obesity, counterintuitive lipogenesis can represent a decreased capacity to store the excess circulating triglycerides and induce ectopic fat accumulation⁴⁴. A high level of UCP1, with its unique expression in brown adipose tissue, could further explain the protection against body-weight gain in *CD40L*^{-/-} mice. Brown adipose tissue is specialized for thermogenic energy expenditure and provides a natural defense against cold and obesity⁴⁶. Moreover, the finding that neutralizing anti-CD40L antibody treatment did not affect body weight but improved systemic insulin action indicates an important metabolic role for the CD40L protein independent of the weight. These data suggest a direct effect of CD40L on the insulin pathway independent of inflammatory reaction.

Collectively, we have identified a crucial role for the co-stimulatory molecule CD40L in obesity, obesity-induced activation of the immune system, and metabolic parameters such as insulin sensitivity *in vivo*. Our data increase the knowledge about the mechanisms underlying the inflammatory and inflammatory induced metabolic changes in obese adipose tissue and raise the potential to identify new therapeutic targets in the CD40L pathway that may prevent or ameliorate obesity and obesity-related vascular co-morbidities.

Supplemental Figures

Supplemental Figure I. mRNA levels of UCP-1 in brown adipose tissue of WT (white bars) and *CD40L*^{-/-} brown adipose tissue (black bars) after a SFD or HFD. n=7 and 8 respectively for SFD and HFD-fed WT mice; n=5 for *CD40L*^{-/-} mice.

Supplemental Figure II. FACS analysis of the spleen cells of mice fed with SFD or HFD for 18 weeks. CD3⁺ (A), CD8⁺ (B), CD4⁺ (C) and Tregs (CD4⁺CD25⁺FoxP3⁺) (D) were analyzed. Values are means ± SEM. * P < 0.05, *** P < 0.001 for comparison between obese and lean mice within each genotype. † P < 0.05 for comparison between genotypes after the same diet. n=7 and 8 respectively for SFD and HFD fed WT mice, n=5 for *CD40L*^{-/-} mice.

Supplemental Figure III. mRNA levels of inflammatory molecules (A), macrophage markers (B) and genes involved in metabolism (C) in scAT of WT (white bars) and CD40L^{-/-} mice (black bars) after 18 weeks of HFD. * P < 0.05. ** P < 0.01. n=7 and 8 respectively for SFD and HFD fed WT mice, n=5 for CD40L^{-/-} mice.

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Chapter 7

CD40 signaling via the CD40-TNF-Receptor associated factor (TRAF) 2/3/5 axis confers protection against adipose tissue inflammation and metabolic complications in diet induced obesity

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In preparation

Abstract

Objective: We recently showed that absence of CD40L alleviates adipose tissue inflammation and metabolic manifestations of obesity. Here we hypothesize that a specific CD40-TRAF axis mediates obesity.

Methods and Results: *CD40^{+/+}*, *CD40^{-/-}*, or *CD40^{-/-}* mice bearing a CD40 transgene under an MHCII promotor with or without mutations at the CD40-TRAF2/3/5, CD40-TRAF6 or both binding sites were fed a SFD or HFD for 20 weeks and metabolic and inflammatory parameters were evaluated.

Surprisingly, *CD40^{-/-}* mice were more glucose intolerant than *CD40^{+/+}* and *CD40L^{-/-}* mice and displayed excessive adipose tissue (AT) inflammation with high numbers of CD3⁺, CD8⁺ T cells and low numbers of Tregs. Compared to *CD40-Twt* mice, HFD fed *CD40-T2/3/5^{-/-}* and *CD40-T2/3/5/6^{-/-}* mice had a strong increase in body weight gain in the first 8 weeks of diet feeding, while *CD40-T6^{-/-}* mice gained less weight, although having the highest food intake. This increase in body weight was predominantly due to the increase in liver weight and subcutaneous adipose tissue. Livers of *CD40-T2/3/5/(6)^{-/-}* mice displayed aggravated NASH, hepatic steatosis, increased triglyceride levels, and altered lipogenesis. All mice displayed clear signs of glucose intolerance and insulin resistance after a HFD with *CD40-T2/3/5/6^{-/-}* mice exhibiting the severest phenotype. FACS of the epididymal AT revealed that *CD40-T2/3/5(6)^{-/-}* mice had a pro-inflammatory T cell profile characterized by a high number of CD3⁺, CD8⁺ T cells and low numbers of CD4⁺ helper T cells and Tregs whereas *CD40-T6^{-/-}* mice had a similar T cell profile as *CD40-Twt* mice.

Conclusion: CD40 deficiency, in contrast to CD40L deficiency, leads to aggravated inflammation in AT and promotes metabolic manifestations of obesity. Genetic ablation of CD40-TRAF2/3/5 and CD40-TRAF2/3/5/6 signaling, but not CD40-TRAF6 signaling, aggravates adipose tissue inflammation and metabolic manifestations of obesity. We therefore conclude that CD40 is crucial in maintaining glucose homeostasis and adipose tissue inflammation via the CD40-TRAF2/3/5, but not the CD40-TRAF6 axis.

Introduction

Today, obesity is considered an inflammatory disease. Obese adipose tissue shows hallmarks of chronic inflammation, which is believed to facilitate the development of metabolic disorders^{1, 2}. Several studies have shown the involvement of T cells as well as macrophages and other immune cells in adipose tissue inflammation³⁻⁸. The actions carried out by these immune cells via cell-cell contact or crosstalk with adipocytes increases the expression of pro-inflammatory molecules such as chemokines and cytokines⁹. These effects are believed to enhance leukocyte recruitment to the adipose tissue, thereby further enhancing inflammation.

The co-stimulatory receptor ligand pair CD40-CD40L plays a crucial role during the initiation and progression of inflammatory diseases during which it promotes immune responses and enhances inflammation. Amongst others, the CD40-CD40L dyad has been shown to contribute to colitis, arthritis, and also to atherosclerosis¹⁰⁻¹⁴. Since permanent CD40L inhibition results in thromboembolic complications¹⁵, targeting of the CD40 associated signaling intermediates, the TRAFs, has become an interesting opportunity to target inflammatory diseases. Recently, we and others have shown diverse functions of different TRAF molecules in atherosclerosis. It was shown that TRAF-1, -2, -3, -5 and -6 are present in atherosclerotic plaques¹⁶, that TRAF5 deficiency accelerates atherogenesis¹⁷, and that TRAF6 is not associated with atherosclerosis¹⁸. We showed that a specific deficiency in CD40-TRAF6 signaling in leukocytes prevents atherosclerosis and leads to an anti-inflammatory immune profile¹⁹. These findings render the different CD40-TRAF axes an interesting molecular target for the treatment of inflammatory disorders.

Interestingly, it turned out that CD40-CD40L interactions are also involved in obesity-related inflammation. We have previously shown that CD40 is expressed on adipocytes and that the stimulation of these adipocytes resulted in a decrease of insulin signaling molecules and the induction of adipokines²⁰. These results were validated by a study of Missiou et al. in which they discovered that ECs got activated when incubated with the supernatant of CD40L-stimulated adipocytes²¹. We recently discovered that genetic ablation or pharmacological inhibition of CD40L ameliorated adipose tissue inflammation as well as its co-morbidities, such as insulin resistance and

hepatic steatosis, in a mouse model of diet induced obesity²². A recent study confirmed our results and, in addition, established a role for CD40L and the production of pathogenic IgG-antibodies in diet-induced adipose tissue inflammation²³.

In the present study, we attempted to investigate the effect of genetic CD40 deficiency on diet-induced obesity. Surprisingly, we found that CD40 deficiency, in contrast to CD40L deficiency, was not protective but aggravated adipose tissue inflammation. Furthermore, we explored the involvement of different CD40-TRAF signaling cascades. We could show that loss of CD40-TRAF2/3/5 signaling, in contrast to CD40-TRAF6 deficiency, mimicked the phenotype of CD40 deficient mice. Hence, CD40 seems to be important in adipose tissue inflammation by signaling via the CD40-TRAF2/3/5 axis.

Methods

Animals

CD40^{+/+}, *CD40*^{-/-} mice (100% C57Bl6J background) were fed a standard-fat diet (SFD, 70% kcal carbohydrate, 10% kcal fat, 20% kcal protein, 3.68 kcal/g, SDS Special Diets Services, Witham, UK) or high-fat diet (HFD, 35% kcal carbohydrate, 45% kcal fat, 20% kcal protein, 4.54 kcal/g, SDS Special Diets Services) for 18 weeks. All mice were maintained under a 12h light-dark cycle and were allowed free access to food and water. Food intake and body weight were measured weekly. After the experimental period, overnight-fasted animals were euthanized, blood was collected and organs [subcutaneous (scAT) and epididymal white adipose tissue (epiAT), brown adipose tissue (BAT), liver, spleen] were dissected. Studies were approved by the animal experimental commission of the University of Maastricht and executed according to the institutional guidelines.

CD40-TRAF signaling

With regard to our findings in CD40 deficient mice, which show aggravated adipose tissue inflammation when consuming a HFD, we examined which CD40 downstream signaling pathways were responsible for the *CD40*^{-/-} phenotype. As CD40 lacks intrinsic signaling capacity, adaptor molecules, the

so-called tumor necrosis factor receptor associated factors (TRAF), are required to elicit proper signaling. These TRAF molecules bind to the cytoplasmic tail of CD40 and it has been shown that there are two distinct binding sites. The first one binds TRAF6 and the second one binds TRAF1, TRAF2, TRAF3, and, indirectly, TRAF5²⁴⁻²⁶. Each of these TRAF binding sites is coupled to a unique signaling pathway²⁶⁻²⁸. To unravel the contribution of CD40-TRAF2/3/5 versus CD40-TRAF6 signaling in MHCII⁺ cells to obesity, we used *CD40*^{-/-} mice expressing a chimeric CD40 transgene with mutations at the TRAF6 and/or TRAF2/3/5 binding site under control of the MHCII promotor (*CD40-T2/3/5*^{-/-}, *CD40-T6*^{-/-}, and *CD40-T2/3/5/6*^{-/-} mice, respectively) or *CD40*^{-/-} mice carrying the transgene without mutations (*CD40-Twt* mice)^{29, 30}. After 20 weeks on a SFD or HFD respectively, the mice were sacrificed and metabolic as well as immunological parameters were assessed.

Biochemical measurements and Glucose/Insulin tolerance test

A glucose tolerance test (GTT) and insulin tolerance test (ITT) was performed, and fasting insulin levels were measured. For the GTT, overnight-fasted conscious mice were injected i.p. with glucose (1mg/g). For the ITT, 5h fasted conscious mice were injected i.p. with insulin (0.75mU/g, Actrapid, Novonordisk, Bagsvaerd, Denmark). Glucose levels were measured from whole blood using a glucometer (Roche Diagnostics, Basel, Switzerland). Fasting insulin (Mercodia, Uppsala, Sweden), and leptin (Chrysal chem., Downers Grove, IL USA) levels were measured in plasma by enzyme-linked immunoabsorbent assay. Cholesterol levels were measured using a colorimetric assay (CHOD-PAP, Roche, Mannheim, Germany) and triglycerides by enzymatic assay (Wako, Neuss, Germany)

Flow cytometric analysis

Stromal vascular cells (SVC) were isolated from epiAT using collagenase (Sigma-Aldrich, Zwijndrecht, The Netherlands). The samples were incubated at 37°C with shaking until complete digestion, passed through a 100µm cell strainer (Falcon, distributed by BD biosciences, Breda, The Netherlands), washed and centrifuged to obtain the final SVC pellet. Spleens were pressed through a 70µm cell strainer and washed after erythrocyte lysis. Fc-blocking (CD16/32 antibody) was performed prior to cell labeling. FACS for

CD3, CD4, CD8, CD25, FoxP3, Ly6G, Ly6C, MHCII, B220, CD11c, CD11b, F4/80, CD44, CD62L, and CD19 was performed on SVC and splenocytes^{19, 31}. All antibodies were purchased from e-Biosciences (San Diego, CA, USA) or BD Pharmingen (distributed by BD Biosciences). Disposables were purchased from Bio Greiner One.

Morphometry and immunohistochemistry

Immunohistochemistry for CD3 and macrophages was performed on 4µm thin sections of epiAT and scAT. Liver sections were stained with haematoxylin-eosin, sirius red and Oil red O and used to determine the degree of steatosis and non-alcoholic steato-hepatitis as indicated by the NASH clinical research network³². All morphometric parameters were determined using a microscope coupled to a computerized morphometry system (Qwin, Leica, Rijswijk, The Netherlands). From each animal, images of 8 fields within the AT were acquired. Subsequently, the diameters of all adipocytes and the number of crown-like structures (CLS) and positive stained cells were determined in each field by an observer blinded to the conditions using Qwin3 software.

Real time PCR

Total RNA was extracted using Trizol (Invitrogen). cDNA was synthesized using i-Script cDNA synthesis kit (BIO-RAD). PCRs were performed with a Bio-Rad instrument and software under standard conditions. The relative amounts of the different mRNAs were quantified by using the second derivate maximum method. The relative quantification for mRNA was corrected to 36B4 mRNA values used as an invariant control. Results were expressed relative to the control group (CD40^{+/+} SFD), which was assigned a value of 1. In addition, a TaqMan Mouse Immune Array from Applied Biosystems was used (Carlsbad, CA, USA). Analysis was performed according to the manufacture's protocol. GAPDH was used as a housekeeping gene.

Statistical analysis

Results are indicated as mean ± SEM. Data were tested for normal distribution. Accordingly, a Student's T-Test or a Mann–Whitney *U* test was applied. The GTT, ITT and body weight gain results were analyzed by a 2-way ANOVA. Significance was set at $P < 0.05$.

Results

Total body weight is not affected by CD40 deficiency

CD40 deficient mice displayed no significant differences in body weight compared to their wild type counterparts when fed a standard or high fat diet. Remarkably, CD40 deficient mice had a higher food intake on a HFD (Fig. 1A, B). In addition to total body weight, we investigated whether adipose tissue, and other fat accumulating tissues, such as the liver differed in their weight. Except from the epiAT of CD40 deficient mice fed a SFD no differences have been found regarding liver, epiAT, scAT, and brown adipose tissue weights (Fig. 1C-F).

Figure 1. Body and organ weights are not affected by CD40 deficiency.

A) Total body weight of $CD40^{+/+}$ (WT) and $CD40^{-/-}$ mice on a SFD or HFD. B) Food intake. C-F) Liver (C), epididymal adipose tissue (epiAT) (D), sub-cutaneous adipose tissue (scAT) (E), and brown adipose tissue weights (BAT) (F) of WT and $CD40^{-/-}$ mice on a SFD or HFD. G) UCP-1 mRNA levels in BAT. Values are mean $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$.

Obese $CD40$ deficient mice are glucose intolerant

Plasma analysis of lean $CD40^{+/+}$ and $CD40^{-/-}$ mice displayed no differences in fasting blood glucose, insulin, cholesterol, and triglycerides. On a HFD, the results remained the same, except for fasting blood glucose (Fig. 2A-D). $CD40$ deficient mice exhibited a strong increase in blood glucose levels compared to their wild type counterparts, whereas insulin levels remained equal (Fig. 2A, B). These findings were confirmed in a glucose tolerance test. Obese $CD40^{-/-}$ mice displayed clear signs of glucose intolerance whereas they did not differ in their response to insulin when compared to $CD40^{+/+}$ animals (Fig. 2E, F). Hence, we can conclude that $CD40^{-/-}$ mice are more glucose intolerant.

Figure 2. $CD40$ deficiency results in glucose intolerance.

A-D) Fasting blood glucose (A), insulin (B), plasma cholesterol (C) and plasma triglyceride concentrations (D) of WT and $CD40^{-/-}$ mice on a SFD or HFD showing an increased glucose concentration in the blood of $CD40^{-/-}$ mice fed a HFD. E) GTT displaying glucose intolerance of $CD40^{-/-}$ mice fed a HFD and F) ITT. Values are mean $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$.

Absence of CD40 leads to an aggravated adipose tissue inflammation

To our surprise, we found that CD40 deficient mice displayed more CD3⁺ T cells in their epiAT (**Fig. 3A**). In addition, the occurrence of T cell crown like structures (CLSs) was more than 2-fold increased in these mice (**Fig. 3B**). The adipose tissue phenotype was accompanied by a third alteration, namely the enlargement of the adipocytes of CD40 deficient mice, a well-known effect of obesity (**Fig. 3C**). Macrophage numbers were not found to be different in epiAT or scAT of obese *CD40*^{-/-} mice, although MCP-1 levels had decreased in epiAT of *CD40*^{-/-} mice (**Fig. 3D-F**). No differences in immune cell infiltration could be observed in scAT of *CD40*^{+/+} and *CD40*^{-/-} mice.

FACS analysis of epiAT confirmed the immunohistochemical data, and revealed that CD40 deficiency induces the influx of CD8⁺ T cells, while reducing the CD4⁺ T cell population (**Fig. 4A-D**). Interestingly, analysis of the immune cell composition in spleen did not reveal major differences, except for a profound decrease in regulatory T cells which is characteristic for lymphoid organs of *CD40*^{-/-} mice (Supplemental Figure 1) ³³. These findings clearly demonstrate a prominent immune-regulatory function for CD40 in diet-induced obesity.

Figure 3. CD40 deficiency leads to a massive accumulation of T cells in epiAT.

A-B) Number of T cells (A), and T cell crown like structures (CLSs) (B) in the epiAT of WT and *CD40*^{-/-} mice on a SFD or HFD displaying more inflammation in *CD40*^{-/-} mice. C) Adipocytes in the epiAT are enlarged in *CD40*^{-/-} mice fed a SFD or HFD. D-E) Number of macrophages (D), and macrophage crown like structures (CLSs) in the epiAT of WT and *CD40*^{-/-} mice on a SFD or HFD. F) MCP-1 mRNA levels in epiAT. Values are mean ± SEM. * P < 0.05; ** P < 0.01.

Figure 4. CD40 deficiency reduces CD4⁺ and increases the amount of CD8⁺ T cells in epiAT.

Quantitative analysis of the percentages of CD3⁺ T cells (A), CD4⁺ helper T cells (B), CD8⁺ cytotoxic T cells (C) and CD4⁺CD25⁺Foxp3⁺ regulatory T cells (D) of WT and *CD40*^{-/-} mice on a SFD or HFD. epiAT was analyzed using flow cytometry. Values are mean ± SEM. * P < 0.05.

CD40-signaling in MHCII⁺ cells does not regulate glucose tolerance but mediates adipose tissue inflammation

To address the function of CD40-signaling in MHCII⁺ cells in obesity, we compared *CD40*^{+/+}, *CD40*^{-/-} and *CD40-Twt* mice, which are CD40-deficient, but have functional CD40 signaling in their MHCII⁺ cells (see methods). Although these mice had the same phenotype as the *CD40*^{-/-} mice regarding glucose tolerance and insulin sensitivity (**Fig. 5A, B**), their adipocyte size and T cell infiltration into the adipose tissue was the same as in *CD40* WT mice, indicating less advanced obesity and less inflammation in the epididymal adipose tissue (**Fig. 5C-G**). These data indicate that CD40-signaling on MHCII⁺ cells is of utmost importance for the modulation of adipose tissue inflammation.

Figure 5. Inflammation in AT is mediated by CD40 signaling in MHCII⁺ cells.

A) Glucose tolerance test (GTT) displaying glucose intolerance of *CD40*^{-/-} and *CD40-Twt* mice. B) Insulin tolerance test (ITT). C) Assessment of the number of T cells in the epiAT, showing an increased abundance of T cells in the epiAT of *CD40*^{-/-} mice. D) Mean area of the epiAT adipocytes, revealing an increased adipocytes size in *CD40*^{-/-} mice. E-G) Adipose tissue sections stained for CD3⁺ T cells (in brown), displaying T cell abundance, crown-like structures, and adipocytes size. Scale bars = 100μm. Values are mean ± SEM. * P < 0.05; *** P < 0.001.

CD40-TRAF signaling

As CD40 lacks intrinsic signaling capacity, adaptor molecules, the so-called TRAFs are required to elicit proper signaling. To identify which CD40-TRAF signaling pathways in MHCII⁺ cells are involved in metabolic regulation and adipose tissue inflammation, we used a special mouse model¹⁹. CD40 deficient mice were given back a CD40 transgene, under the control of the MHCII promoter, which lacked different CD40-TRAF binding sites. For a detailed description revisit the materials and method section.

Deficiency of CD40-TRAF2/3/5 signaling induces obesity

CD40-T2/3/5^{-/-} mice gained more weight when compared to their *CD40-Twt* controls. Interestingly, an even stronger phenotype was observed in *CD40-T2/3/5/6*^{-/-} mice. The increase in body weight was highest during the first weeks of HFD feeding (**Fig. 6A**). Obese *CD40-T2/3/5/6*^{-/-} mice showed significant body weight gain compared to *CD40-Twt* mice despite a decreased food intake, which might be due to a higher feeding efficiency. *CD40-T6*^{-/-} animals experienced the mildest weight gain, although they had the highest food intake (**Fig. 6A, B**).

The epiAT weight and the adipocyte size were significantly decreased in *CD40-T2/3/5/6*^{-/-} mice, whereas scAT weight slightly increased compared to their *CD40-Twt* controls (**Fig. 6C, D**). Consequently, we found more adipocytes/mm² in *CD40-T2/3/5/6*^{-/-} mice (**Fig. 6E**). *CD40-T6*^{-/-} mice displayed no alterations compared to *CD40-Twt* mice. In addition, plasma cholesterol (*CD40-T2/3/5*^{-/-} and *CD40-T2/3/5/6*^{-/-} vs. *CD40-Twt*; $p < 0.01$) and plasma triglyceride levels (*CD40-T2/3/5/6*^{-/-} vs. *CD40-Twt*; $p < 0.001$) were elevated upon HFD feeding (**Fig. 6F, G**). CD40-TRAF6 deficiency did not result in aberrations in plasma cholesterol and triglycerides.

Thus, our data strongly suggest that CD40-TRAF2/3/5 signaling mediates obesity associated alterations, such as weight gain and adipose tissue phenotype, and that the additional loss of CD40-TRAF6 signaling further propagates the disease.

Figure 6. Obesity is induced upon loss of CD40-TRAF2/3/5 signaling.

A) Total body weight gain of *CD40-Twt*, *CD40-T2/3/5*, *CD40-T6*, and *CD40-T2/3/5/6* mice on a HFD. Body weight gain was highest during the first weeks with *CD40-T2/3/5*, and *CD40-T2/3/5/6* mice gaining the most weight. B) Food intake displaying the highest intake in *CD40-T6* mice although they gained less weight. C-D) epiAT and scAT weight. E) Number of adipocytes in the AT. F-G) Plasma cholesterol and plasma triglyceride levels. Values are mean $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

***CD40-T2/3/5/6^{-/-}* mice are glucose intolerant and insulin resistant**

All four genotypes showed clear signs of glucose intolerance after HFD feeding, with *CD40-T2/3/5/6^{-/-}* mice displaying the highest glucose levels during the GTT (**Fig. 7A**). *CD40-T2/3/5^{-/-}* and *CD40-T6^{-/-}* mice performed slightly better during the GTT compared to *CD40-Twt* mice, which in the case of *CD40-T6^{-/-}* mice might be due to their significantly lower fasted glucose levels (**Fig. 7B**). *CD40-T2/3/5^{-/-}* and *CD40-T6^{-/-}* mice showed clear signs of insulin resistance, and *CD40-TRAF2/3/5/6* deficiency resulted in complete insulin resistance (**Fig. 7C**). Insulin levels did not differ between the four genotypes (**Fig. 7D**). Muscle mRNA analysis revealed significantly lower levels of carnitine palmitoyltransferase 1 (CPT1), an enzyme important for long chain fatty acid transport into

mitochondria, in *CD40-T6^{-/-}* and *CD40-T2/3/5/6^{-/-}* mice. Consequently, these mice exhibited higher plasma cholesterol and triglyceride levels. Additional genes like glucose transporter type 4 (GLUT4), insulin receptor substrate 1 (IRS-1), and glycogen synthase 1 (GS-1) were not found to be altered in muscle tissue. As the loss of CD40-TRAF2/3/5 and CD40-TRAF2/3/5/6 signaling reflects the phenotype of *CD40^{-/-}* mice we propose that CD40 signaling via the CD40-TRAF2/3/5 axis is important to maintain glucose homeostasis in diet induced obesity.

Figure 7. CD40-TRAF2/3/5/6 deficiency causes glucose intolerance and insulin resistance.

Glucose tolerance test (GTT) (A), and insulin tolerance test (ITT) (C) of HFD fed mice. B and D) Overnight-fasted blood glucose levels (B) and plasma insulin levels (D) of HFD fed mice. Values are means $\pm$ SEM. ** $P < 0.01$; *** $P < 0.001$.

CD40-T2/3/5^{-/-} and CD40-T2/3/5/6^{-/-} mice develop extensive NASH

CD40-T2/3/5^{-/-} and CD40-T2/3/5/6^{-/-} mice had a remarkable increase in liver weight after 18 wks of HFD feeding ($p < 0.0001$), with an elevated level of liver triglycerides and strong fat accumulation ($p < 0.05$) (**Fig. 8A-F**). Histological analysis revealed that all genotypes developed steatosis, with the most severe phenotypes in the *CD40-T2/3/5^{-/-} and CD40-T2/3/5/6^{-/-}* mice, where 87.5% and 100% of the mice developed grade 3 steatosis, compared to only 62.5% of the *CD40-Twt* mice (Supplemental Figure 2A). Thus, deficiency of CD40-TRAF6 interactions resulted in a milder form of NASH. *CD40-T6^{-/-}* mice mostly developed grade 1 or 2 steatosis, which was localized at the central vein, and showing only limited expansion (Supplemental Figure 2A). Steatosis extended from the central vein to the periportal vein in 87.5% and 100% of the *CD40-T2/3/5^{-/-} and CD40-T2/3/5/6^{-/-}* mice, whereas in *CD40-Twt* mice this was the case in only 37.5% of the mice (Supplemental Figure 2B). All genotypes predominantly developed macrovesicular steatosis, with additional microvesicular steatosis in the *CD40-T2/3/5^{-/-} and CD40-T2/3/5/6^{-/-}* mice (Supplemental Figure 2C). The liver parenchyma showed grade 1 lobular inflammation in all genotypes, although 37.5% of the *CD40-T6^{-/-}* mice developed <1 inflammation (Supplemental Figure 2D). Portal inflammation, microgranulomas or lipogranulomas were not observed. Ballooning of hepatocytes was a frequent observation in all genotypes, although *CD40-T6^{-/-}* mice seemed to be protected.

In line with these results, we found fundamental metabolism associated genes to be altered in *CD40-T2/3/5/6^{-/-}* mice. Glucose transporter 2 (GLUT-2), liver glucokinase (GK), and liver pyruvate kinase (LPK), all genes involved in glycolysis, were significantly upregulated (**Fig. 9A-C**). In addition, genes involved in lipogenesis such as acetyl-CoA carboxylase (ACC1), fatty acid synthase (FASN), and stearoyl-CoA desaturase (SCD1) have been found to be elevated as well (**Fig. 9D-F**). Furthermore, we detected higher mRNA levels of the fatty acid transporter CD36 and of the transcription factors sterol regulatory element-binding protein 1c (SREBP1c) and carbohydrate responsive element-binding protein 1c (chREBP1c), which positively regulate glycolysis and lipogenesis (**Fig. 9G-I**).

These data, together with the elevated TG levels and liver weight, strongly imply a function for CD40-TRAF2/3/5 signaling in liver metabolism under obese circumstances. Additionally, loss of CD40-TRAF6 interactions aggravates the phenotype significantly.

Figure 8. CD40-TRAF2/3/5 and CD40-TRAF2/3/5/6 deficiency result in liver weight increase and promotes fat accumulation.

A) Liver weight and B) Triglyceride content in the liver. C-F) Oil red O stained frozen liver sections of *CD40-Twt*, *CD40-T2/3/5^{-/-}*, *CD40-T6^{-/-}*, and *CD40-T2/3/5/6^{-/-}* mice. Scale bars = 100µm. Values are mean ± SEM. * P < 0.05, *** P < 0.001.

Figure 9. CD40-TRAF2/3/5/6 deficiency affects the gene expression profile of the liver.

Liver mRNA levels of genes involved in glycolysis (GLUT2 (A), GK (B), and LPK (C)), lipogenesis (ACC1 (D), FASN (E), and SCD-1 (F)), and of the fatty acid transporter CD36 (G) and of transcription factors which positively regulate glycolysis and lipogenesis (SREBP1c (H), ChREBP1c (I)). Values are mean $\pm$ SEM. * P < 0.05; ** P < 0.01.

CD40-TRAF2/3/5 and CD40-TRAF2/3/5/6 deficiency alter adipose tissue inflammation in diet-induced obesity

FACS analysis on the stromal vascular fraction of the epiAT revealed evident pro-inflammatory changes in the number of T cells and macrophages (**Fig. 10, 11**). The percentage of CD3⁺ T cells was elevated in *CD40-T2/3/5*^{-/-} and *CD40-T2/3/5/6*^{-/-} mice while the percentage of CD3⁺ T cells in *CD40-T6*^{-/-} mice equaled *CD40-Twt* levels. Remarkably, the increase in the percentage of CD3⁺ T cells was accompanied by an increase in the CD8⁺ T cell fraction in adipose tissue in these mice (**Fig. 10A-C**). In both genotypes, the fraction of regulatory T cells decreased substantially (**Fig. 10D**). Both the CD4⁺ and CD8⁺ T cell fraction in *CD40-T2/3/5*^{-/-} and *CD40-T2/3/5/6*^{-/-} mice contained an increase in CD44^{high}CD62L^{low} effector memory cells, with a decrease in CD44^{low}CD62L^{high} naive helper T cells (**Fig. 10E-I**). This T cell profile is characterized by a more vigorous (CD8) T cell response, and an increased migration potential, resulting in an aggravation of adipose tissue inflammation.

Besides T cells, DC subsets and macrophages were investigated. We found that the number of Ly6C^{high} B220^{high} plasmacytoid DC was not altered, whereas the CD11b⁺ resident DC subset was markedly decreased in *CD40-T2/3/5/6*^{-/-} mice. In addition, *CD40-T2/3/5*^{-/-} and *CD40-T2/3/5/6*^{-/-} mice exhibited increased percentages of F4/80^{high}CD11b⁺ macrophages in their epiAT (**Fig. 11A-C**). Additional qPCR analysis of the epiAT for M2 markers revealed that CD40-TRAF2/3/5 deficiency led to a significant increase in the M2 marker Arg1, while YM1 and CD206 were slightly affected. *CD40-T2/3/5/6*^{-/-} mice exhibited elevated levels of ARG1 and CD206 as well (Supplemental Figure 3). The M1 marker IL12 was significantly down regulated in *CD40-T2/3/5*^{-/-}, *CD40-T6*^{-/-}, and *CD40-T2/3/5/6*^{-/-} mice, suggesting a compensatory M2 reaction in these animals in response to the inflammation in AT.

Figure 10. CD40-TRAF2/3/5 and CD40-TRAF2/3/5/6 deficiency affect the lymphocyte subsets leading to more adipose tissue inflammation.

Quantitative analysis of the percentages of CD3⁺ T cells (A), CD4⁺ helper T cells (B), CD8⁺ cytotoxic T cells (C), CD4⁺CD25⁺Foxp3⁺ regulatory T cells (D), CD4⁺CD44^{low}CD62L^{high} naive helper T cells (E), CD4⁺CD44^{high}CD62L^{low} effector memory T cells (F), CD8⁺CD44^{low}CD62L^{high} naive T cells (G), CD8⁺CD44^{high}CD62L^{high} central memory T cells (H), and CD8⁺CD44^{high}CD62L^{low} effector T cells (I). epiAT was analyzed using flow cytometry. Values are mean $\pm$ SEM. * P < 0.05; ** P < 0.01; *** P < 0.001.

Figure 11. CD40-TRAF2/3/5 and CD40-TRAF2/3/5/6 deficiency decrease CD11c⁺ resident DC's and increase macrophage content in adipose tissue.

Quantitative analysis of the percentages of Ly6C^{high}B220^{high} plasmacytoid DC (A), CD11c⁺ resident DC (B), and F4/80^{high} macrophages (C). epiAT was analyzed using flow cytometry. Values are mean $\pm$ SEM. * P < 0.05; *** P < 0.001.

CD40-TRAF2/3/5 and CD40-TRAF2/3/5/6 deficiency change inflammatory gene expression in diet induced obesity

Analysis of gene expression in the epiAT of obese mice revealed a pro-inflammatory chemokine expression in *CD40-T2/3/5^{-/-}* and *CD40-T2/3/5/6^{-/-}* mice. Genes important in recruiting T cells and macrophages, and genes important in activating T and B cells, namely chemokine C-C motif ligand 3 (CCL3), CCL5, chemokine C-C motif receptor 7 (CCR7), and chemokine C-X-C motif receptor 3 (CXCR3) were found to be more expressed in *CD40-T2/3/5^{-/-}* and *CD40-T2/3/5/6^{-/-}* mice compared to their *CD40-Twt* counterparts (Fig. 12A-D). In addition, mRNA levels of CD3 and CD68 were found to be elevated in *CD40-T2/3/5^{-/-}* and *CD40-T2/3/5/6^{-/-}* mice, which is also reflected by the higher number of T cells and macrophages in the AT of these mice (Fig. 12E, F). Furthermore, we observed an increased mRNA expression of the pro-inflammatory cytokines IL1 α and IL2 and of the inflammation marker tumor necrosis factor (TNF) in *CD40-T2/3/5^{-/-}* mice (Fig. 12G-I). We also found that the apoptosis promoting Bcl2-associated X protein (BAX) was elevated in *CD40-T2/3/5^{-/-}* and *CD40-T2/3/5/6^{-/-}* mice as well (Fig. 12J). These results reveal a clear shift towards a pro-inflammatory AT gene profile, particularly in mice

that are deficient for CD40-TRAF2/3/5, but also for mice deficient in CD40-TRAF2/3/5/6 signaling. These results are representative for a typical pro-inflammatory profile and show that CD40-TRAF2/3/5 signaling maintains the inflammatory balance in adipose tissue during obesity.

Figure 12. CD40-TRAF2/3/5 and CD40-TRAF2/3/5/6 deficiency result in a pro-inflammatory gene profile in the epiAT.

epiAT mRNA levels of genes involved in lymphocyte recruitment (CCL3 (A), CCL5 (B), CCR7 (C) and CXCR3 (D)), T cell and macrophage markers (CD3 (E), and CD68 (F)), pro-inflammatory cytokines (IL1 α (G), and IL2 (H)), the inflammation marker TNF (I), and the apoptosis promoting BAX protein (J)). Values are mean $\pm$ SEM. * P < 0.05; ** P < 0.01; *** P < 0.001.

Discussion

In the present study we investigated the effect of CD40 deficiency in the setting of diet induced obesity. Furthermore, we elucidated the contribution of CD40 signaling in MHCII⁺ cells and established the importance of different CD40-TRAF signaling pathways in diet induced obesity.

Recently, we reported a protective function for CD40L deletion in a mouse model of diet-induced obesity²². *CD40L*^{-/-} mice were protected against weight gain, insulin resistance, and obesity associated liver pathologies. Furthermore, CD40L deficiency resulted in lower inflammation of the adipose tissue and provoked a strong immune modulation, leading to low CD8⁺ and high regulatory T cell numbers.

In contrast, in the present study we found that genetic loss of CD40 aggravates adipose tissue inflammation and metabolic manifestations of obesity. The increased CD8⁺ T cell fraction, associated with adipose tissue inflammation and activation of adipose tissue macrophages³⁴, and the severely reduced regulatory T cell fraction, associated with a beneficial role in obesity³⁵, might be the driving force behind the aggravated adipose tissue inflammation in *CD40*^{-/-} mice. T cells as well as macrophages have been implicated in metabolic dysfunction such as insulin resistance and fat distribution. Loss of CD8⁺ T cells was shown to improve insulin resistance, while adoptive transfer of CD8⁺ T cells aggravates metabolic dysfunction³⁴. In line with these findings, our *CD40*^{-/-} mice were glucose intolerant and presented with enlarged adipocytes, a clear sign for advanced obesity. Consistent with these studies, the enhanced CD8⁺ T cell response, the decrease in regulatory T cells, and the increased immune cell accumulation in AT of *CD40*^{-/-} mice coincides with the non altered body weight levels, glucose intolerance and adipocyte enlargement.

A possible explanation to our opposing results concerning CD40L and CD40 deletion, might be the fact that CD40L can bind to other receptors than CD40. Indeed, it was reported that CD40 deletion in an atherosclerotic mouse model did not result in smaller lesions, but that CD40L binds to MAC-1, inducing MAC-1 dependent adhesion and migration of leukocytes³⁶. However, administration of an anti-Mac-1 antibody in obese mice also induced aggravation of obesity and a remarkable adipose tissue inflammation, indicating that CD40L-Mac-1 interactions are not involved (data not shown). On the other hand, we found that CD40 deficiency decreases neointima formation

and that this protection was mediated by deficient CD40-TRAF6 signaling³⁰. These results clearly demonstrate the versatile actions of the CD40L-CD40 axis and its adaptor molecules. Next to a possibly undiscovered receptor of CD40L, the contradictory results could also be due to the different diseases investigated. We believe, that a minutely examination of the TRAF adaptor proteins might be the key to unravel the exact contribution of CD40L-CD40 interactions in diverse diseases.

Interestingly, our data show that CD40 signaling in MHCII⁺ cells executes protective functions during the progression of diet induced obesity. As expected, CD40 signaling in MHCII⁺ cells was responsible for the inflammatory changes, but had no effect on metabolic parameters. We believe, that this result is rooted in the co-stimulatory capacity of CD40 signaling between leukocytes and APCs. Although this might sound contradictory as the CD40L-CD40 signaling axis is known for its pro-inflammatory potential, studies in atherosclerosis prone mice have shown that blockage of distinct CD40 downstream molecules results in plaque fibrosis, while plaque size remained unaltered¹⁹. Hence, we believe that blocking of co-stimulation is not necessarily leading to less inflammation, but is rather cell-, tissue- and disease-specific.

To further assess the contribution of CD40 signaling intermediates in obesity, and to unravel the regulation of metabolic abnormalities and AT inflammation, we made use of our MHCII⁺ cell type-specific CD40-TRAF mouse model. We observed that CD40-TRAF2/3/5 deficiency induced obesity, especially in the first weeks of diet feeding, and that CD40-TRAF2/3/5 as well as CD40-TRAF2/3/5/6 deficiency altered adipose tissue inflammation and the inflammatory gene profile. Once full blown obesity had developed, fewer differences in insulin resistance and glucose tolerance are observed. Most likely, an earlier assessment of these parameters would have revealed greater differences and could rule out that the insulin resistance was just a consequence of the elevated weight gain. In addition, CD40-TRAF2/3/5(6) loss in MHCII⁺ cells led to severe liver abnormalities. These abnormalities are most likely a consequence of the elevated blood glucose, triglyceride and cholesterol levels which result from the insulin resistance and obesity. As a result, more glucose, cholesterol, and triglycerides are taken up by the liver, and genes

involved in glycolysis and lipogenesis are up regulated, which ultimately lead to the fatty degeneration and ballooning of the liver in *CD40-T2/3/5/6*^{-/-} mice.

The knowledge on TRAF interactions in diet induced obesity is sparse until now. Olefsky et al. showed that hematopoietic TLR4 deficiency leads to a decrease in adipose tissue inflammation and changes in pro-inflammatory gene expression³⁷. TLR4 activation by fatty acids has been shown to result in the activation of IKK/NFkB and JNK1 pathways, with enhanced secretion of pro-inflammatory chemokines and cytokines³⁸⁻⁴⁰. The same pathways are known to be elicited upon CD40-TRAF signaling²⁴. Hence, although our mice do express TLR4, these findings are interesting and do link the outcome of both studies as the identical pathways are activated. The fact that CD40-TRAF2/3/5/6 loss leads to severe liver abnormalities raises some controversy. It is known that hepatic TRAF2 deletion does not alter liver inflammation⁴¹. Generally, this is in accordance with our findings, since we also do not observe any ameliorative effect on liver pathologies due to CD40-TRAF2/3/5 deletion. Nevertheless, Rui et al. report a positive effect of hepatic TRAF2 deletion on glucose metabolism which we were not able to find back in our study. Most likely, this is due to the fact that we made use of a mouse model in which only CD40-TRAF2 interactions were deleted in MHCII⁺ cells, whereas Riu et al. generated mice with a hepatocyte-specific TRAF2 deletion.

Another important point to be mentioned is the fact that *CD40-T6*^{-/-} mice resembled the *CD40-Twt* phenotype. *CD40-T6*^{-/-} mice displayed a remarkable high food intake, while their body weight gain remained moderate. These findings are surprising, but could be explained by a strong effect of the BAT, which is important in regulating energy expenditure and temperature homeostasis. Indeed, it has been shown that alternatively activated macrophages from the white adipose tissue activate fatty acid mobilization and energy expenditure in BAT⁴². In addition, we previously showed that CD40-TRAF6 signaling affects the phenotype of macrophages in an atherosclerotic setting¹⁹. Macrophages of *CD40-T6*^{-/-} mice were skewed towards an anti-inflammatory M2 phenotype. On the basis of these findings, we suggest that CD40-TRAF6 deficiency alone can balance the deteriorating effects of obesity due to a positively regulated BAT function. In conjunction with CD40-TRAF2/3/5 deficiency, the ameliorative effects of CD40-TRAF6 deficiency get lost, probably as a result of the extreme obesity of the *CD40-T2/3/5/6*^{-/-} mice, which

ultimately leads to an aggravated diet induced phenotype. An additional explanation to the observed aggravated phenotype of *CD40-T2/3/5/6^{-/-}* mice, in comparison to *CD40-T2/3/5^{-/-}* mice, was revealed by the analysis of liver genes. We found genes important for triglyceride synthesis (*ACC1*) and glucose uptake (*IRS1*) to be elevated in *CD40-T6^{-/-}* mice. Furthermore, *SREBP1c*, a transcription factor regulating glycolysis and lipogenesis, was elevated in *CD40-T6^{-/-}* mice. While the expected negative effects of such alterations, such as elevated TG levels in liver and blood, were not observed in *CD40-T6^{-/-}* mice, probably due to their increased BAT function, *CD40-T2/3/5^{-/-}* mice display these alterations, and additional deletion of CD40-TRAF6 even further aggravated the phenotype. Although we could reveal several functions of TRAF adaptor molecules in diet induced obesity, the exact mechanism of CD40-TRAF signaling still remain to be elucidated.

Nevertheless, several publications describe the versatile actions of TRAF adaptor molecules^{16, 17, 25, 27, 29, 30, 43}. Our group found that CD40-TRAF6 deletion in leukocytes was protective against atherosclerosis, whereas Zirlik et al. did not report any involvement for total TRAF6 deficiency in atherosclerosis^{18, 19}. Additionally, it has been reported that CD40L can also bind to other receptors than CD40, e.g. MAC1, and thereby elicit signaling³⁶. Consequently, CD40-TRAF inhibition does obviously not abolish pro-inflammatory signaling in general. The above mentioned results clearly indicate the complicated and versatile action of CD40-TRAF signaling.

In conclusion, we believe that our results significantly contribute to the understanding of how different CD40-dependent signaling pathways affect obesity and its co-morbidities. Nevertheless, further research is definitely needed to fully elucidate CD40-TRAF interactions in diet-induced obesity. It is widely accepted that signaling through any pro-inflammatory pathway activates a broad range of intracellular cascades. As a consequence, these cascades activate a number of interconnected and overlapping pathways⁴⁴. With regards to these findings, Osborn and Olefsky have recently suggested that therapeutic strategies should aim at more proximal or common steps in these pathways⁴⁴. Generally, we believe that this opinion is correct as such interference would target the pathways in a broader way. On the other hand, total abolishment of pro-inflammatory signaling might also lead to unwanted immune suppression. We believe that a more specific intervention also has the

potential to lead to effective treatment strategies and might additionally leave necessary signaling pathways unaffected. Obviously, a precise control of the effects of such treatment strategies is fundamental.

Supplemental Figures

Supplemental Figure 1. CD40 deficiency does not alter immune cell composition in the spleen except for a profound decrease in regulatory T cells.

Quantitative analysis of the percentages of CD3⁺ T cells (A), CD4⁺ helper T cells (B), CD8⁺ cytotoxic T cells (C) and CD4⁺CD25⁺Foxp3⁺ regulatory T cells (D) of WT and CD40^{-/-} mice on a SFD or HFD. Spleen samples were analyzed using flow cytometry. Values are mean $\pm$ SEM. * $P < 0.05$.

CD40-TRAF2/3/5 signaling protects against obesity and its complications

Supplemental Figure 2. CD40-TRAF2/3/5 and CD40-TRAF2/3/5/6 deficiency results in extensive NASH.

HE stained liver sections were analyzed. *A*) Hepatic steatosis was graded from 0 (mild) to 3 (severe). *B*) Steatosis extension was quantified as minimal steatosis, steatosis in the central vein area (c), extension of steatosis from the central vein to mid-portal steatosis (c-m), and steatosis extending from the central vein area to periportal area (c-p). *C*) Macro- and microvesicular steatosis was assessed (ma, macrovesicular steatosis; ma>mi, more macrovesicular than microvesicular steatosis; ma=mi, macrovesicular same as microvesicular steatosis). *D*) The percentage of mice exhibiting lobular inflammation was counted.

Supplemental Figure 3. CD40-TRAF2/3/5 deficiency skews macrophages towards an M2 phenotype.

epiAT mRNA levels of the macrophage marker F4/80 (A), and the M2 macrophage markers YM1 (B), Arg-1 (C), and CD206 (D). Values are mean ± SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

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Chapter 8

General Discussion

During the last decades, atherosclerosis as well as obesity have been classified as chronic inflammatory diseases ^{1, 2}. Besides this common trade, obesity has been shown to be an independent risk factor for the development of cardiovascular disease in general. Today, it is known that both diseases have a high prevalence in western society and substantially affect the health care system as well as the economy ^{3, 4}. In addition, it is expected that the number of people being affected by either atherosclerosis, obesity, or both will dramatically rise during the next ten to twenty years. Due to these facts, and due to the involvement of innate and adaptive immunity and co-stimulatory molecules in both diseases, this thesis aimed to elucidate the effects of hematopoietic and non-hematopoietic transport mechanisms and cell-type specific CD40L-CD40-TRAF signaling pathways in atherosclerosis and obesity. The main findings of this thesis are that:

- I. Caveolin-1 deficiency reduces atherosclerosis, abolishes leukocyte infiltration, and generates a regulatory T cell response.
- II. Deficient CD40-TRAF6 signaling in leukocytes prevents atherosclerosis by hampering inflammatory monocyte adhesion and extravasation.
- III. CD40L deficiency improves adipose tissue inflammation and metabolic manifestations in a mouse model of diet induced obesity.
- IV. CD40 deficiency aggravates adipose tissue inflammation and metabolic manifestations in a mouse model of diet-induced obesity. This effect is mediated via the CD40-T2/3/5, but not the CD40-T6 axis.

Caveolin-1 deficiency protects against atherosclerosis

In this thesis we found that genetic deletion of caveolin-1, the main structural component of caveolae, fundamentally protects against atherosclerosis initiation and progression. We established that the protective effect of caveolin-1 deletion is mediated by the impaired adhesion and trans-endothelial migration of leukocytes. We were able to prove that caveolin-1 deficiency affected endothelial VCAM-1 and CCL-2 expression at the leukocyte-endothelial interface. In addition, we found that the abundance of anti-

inflammatory regulatory T cells was increased by 37% in caveolin-1 deficient animals. By generating bone marrow chimeras, we could further show that caveolin-1 in non-hematopoietic cells is responsible for cholesterol homeostasis, plaque growth, and trans-endothelial migration of macrophages, while hematopoietic caveolin-1 is required for immune modulation.

Our results fundamentally contribute to the understanding of caveolar biology. Whereas, until now, the main function of caveolae in atherosclerosis was considered to be the transport of LDL into the vascular wall, we now shed new light on the caveolae-mediated effects on adhesion molecule expression and T cell subset distribution. We clearly show that caveolin-1 deficiency leads to an anti-inflammatory state, and have established a clear function for caveolae in mediating inflammatory and immunological actions in vascular disease. We believe that due to the versatile actions carried out by caveolae, additional research should be dedicated towards caveolar biology. Besides the generally well-studied caveolar cholesterol transport in endothelial cells, additional focus should be on the effects caveolin-1 has on the expression and distribution of adhesion molecules. The exact determination of the signaling and transport pathways involved, could lead to a more targeted intervention against e.g. CCL-2 and VCAM-1 deposition on endothelial cells. Furthermore, this strategy might abolish the negative side effects which are usually accompanied by total caveolin-1 deficiency⁵⁻⁷. Next to endothelial cell caveolae, also immune cell caveolae harbor therapeutically interesting potential. Elucidating the exact mechanism by which caveolin-1 deficiency affects T cell subsets, leading to an anti-inflammatory profile, could make it possible to intervene in this process as well. In general, we believe that a more targeted intervention strategy should be the goal in treating atherosclerosis in a caveolin-1 dependent manner. A combination of abrogated endothelial LDL delivery, as well as immune system manipulation toward an anti-inflammatory state is the most promising idea. Nevertheless, we have to keep in mind that caveolae exert important functions during homeostatic cell biology and that any kind of intervention should be minutely monitored.

CD40 signaling in atherosclerosis

During the last years, our laboratory has intensively studied CD40L-CD40 interactions in atherosclerosis (reviewed in **chapter 3**). CD40L is necessary for atherosclerosis progression and CD40L antibody treatment induces a stable plaque phenotype^{8,9}. In addition, we could show that genetic loss of CD40 resulted in smaller neointima formation¹⁰. As it is known that CD40 has no intrinsic signaling capacity, but rather needs to recruit adaptor molecules, the so called TRAFs, to elicit proper signaling, we investigated these downstream intermediate signaling molecules and aimed to elucidate their individual contribution to atherosclerosis.

CD40 deficiency reduces atherosclerosis, leads to a stable plaque phenotype, and is leukocyte dependent

In **chapter 4**, we show that CD40 deficiency reduces atherosclerosis in mice. The atherosclerotic plaques of CD40 deficient mice were smaller, less infiltrated by leukocytes, and characterized as clinically favorable stable plaques. Furthermore, we have been able to reveal that this protection is mediated by CD40 deficient leukocytes as CD40^{-/-} bone marrow transplanted mice exhibited the same plaque phenotype as CD40^{-/-} mice. In addition, CD40 deficient macrophages were polarized towards the M2 anti-inflammatory phenotype. These results clearly demonstrate a fundamental role for leukocyte CD40 in the progression of atherosclerosis.

The TRAF6 axis is the determinant signaling pathway for CD40 in atherosclerosis

As we have been able to demonstrate the effect of leukocyte CD40 in atherosclerosis, we further investigated the downstream signaling intermediates in this cell type. It turned out that CD40-T6 deficient mice were extremely protected against atherosclerosis and experienced an even more pronounced phenotype than mice that were completely CD40 deficient. Furthermore, we could show that CD40-T6 deficient mice display a shift in their macrophage populations towards the anti-inflammatory M2 phenotype as well. Altogether, our data clearly demonstrate the fundamental role for leukocyte CD40-TRAF6 signaling in atherosclerosis. Furthermore, our studies reveal how the non-feasible anti-CD40L therapy could be modulated for the

clinical practice. Instead of blocking the complete CD40L-CD40-TRAF pathway, it might be much more promising to abolish individual signaling pathways in diverse diseases. Small molecules that target the TRAF6 binding site on CD40 or the region on TRAF6 that is crucial for CD40 binding may allow selective modulation of the signaling pathway, and could therefore improve anti-CD40 related therapies.

CD40L deficiency ameliorates diet-induced obesity

In **chapter 6**, we proved the positive effect of CD40L deficiency in a mouse model of diet-induced obesity. *CD40L*^{-/-} mice were protected against diet induced obesity, displayed less inflamed adipose tissue and were additionally protected against metabolic manifestations such as hepatic steatosis and insulin resistance. Furthermore, we could establish a positive effect for an anti-CD40L antibody treatment. Our data clearly imply a strong relationship between CD40L and obesity initiation and progression. Whereas in our intermediate obesity model we could observe a down regulation of CD4⁺ effector T cells, we observed a strong immune modulatory effect in our severe obesity model. The strong increase in regulatory T cells and the decrease in CD8⁺ effector T cells in severe obesity renders CD40L to be a potential target for pharmacological interventions. However, systemic inhibition of CD40L is generally not feasible as clinical trials had to be terminated due to thrombo-embolic complications. Therefore, targeted inhibition of CD40L, solely in the adipose tissue, might be a good strategy to circumvent the problems related to systemic inhibition of CD40L. Next to the direct effect of CD40L deficiency on inflammatory parameters, we observed additional effects of CD40L deficiency such as altered gene expression profiles, improved liver histology and increased insulin sensitivity. To further study these effects and to unravel the molecular mechanism behind them might bring up even more intervention possibilities to treat obesity and its co-morbidities.

The CD40-T2/3/5 axis is crucial in maintaining glucose homeostasis and adipose tissue inflammation

In **chapter 7**, we studied the effects of different CD40-TRAF deletions on obesity. We could show that deficiency of CD40-T2/3/5 signaling induces obesity, whereas CD40-TRAF6 deletion mimicked the phenotype of *CD40-Twt* mice. Interestingly, *CD40-T2/3/5/6^{-/-}* mice were glucose intolerant, insulin resistant, and showed severe liver abnormalities. *CD40-T2/3/5^{-/-}* mice as well as *CD40-T2/3/5/6^{-/-}* mice exhibited a pronounced adipose tissue inflammation compared to *CD40-Twt* mice. Hence, different as well as additional TRAF deletions result in a variety of phenotypical changes. These results clearly illustrate the complicated and tightly regulated action of CD40 signaling in obesity. We believe that CD40 signaling is important in maintaining glucose homeostasis and adipose tissue inflammation and suggest the CD40-TRAF2/3/5 axis as the main responsible signaling pathway. In the same way as it holds true for atherosclerosis, also obesity might be treatable by targeted intervention with the CD40 signaling pathway. Moreover, due to the fact that the main signaling pathways differ in atherosclerosis and obesity, treatment of one of the diseases might not interfere with the necessary signaling of CD40 in general. The generation of small molecules that lead to a persistent CD40-TRAF2/3/5 signaling or even stimulate this pathway could also be a promising treatment strategy in obesity.

Concluding remarks and future perspectives

In this thesis we aimed to elucidate the contributions of caveolin-1 and the co-stimulatory molecule CD40 in two different chronic inflammatory diseases namely atherosclerosis and obesity.

We could prove that the globally expressed caveolae exert fundamental functions in atherosclerosis initiation and progression. The main novel finding is that, besides the well-established transport of LDL into the sub-endothelial space, caveolae loss additionally affects the immune system and leads to an anti-inflammatory T cell profile. Furthermore, the adhesion and extravasation of leukocytes, a main step in atherosclerosis progression, is impaired by caveolin-1 deletion due to an impaired presentation of CCL-2 and VCAM-1 on the endothelial cell surface. These new insights harbor the

potential to develop new treatment strategies to combat atherosclerosis in a caveolin-1 dependent manner.

In this thesis we have shown that CD40, together with its ligand (CD40L) and the associated signaling adaptor molecules (TRAFs) exert fundamental functions in atherosclerosis, as well as in obesity. We could attribute a positive effect for CD40 deletion in atherosclerosis, which was evident by identifying less and smaller atherosclerotic plaques, which were rich in collagen. More remarkably we could attribute the positive effect to TRAF6 as being the main constituent of CD40 signaling in atherosclerosis.

With regard to obesity, we could show that CD40L deficiency, in contrast to CD40 deficiency, ameliorated adipose tissue inflammation and metabolic manifestations of obesity. Driven by the fact that two interventions in the same signaling pathway led to such different results we investigated the downstream signaling intermediates as well. Remarkably, we were able to acknowledge the positive effect of interfering with the CD40 signaling pathway to TRAF2/3/5.

Our results clearly demonstrate how versatile different chronic inflammatory diseases are functioning. In addition, it becomes obvious how different a single signaling pathway can affect different diseases in different ways. We believe that our results shed new light on atherosclerosis and obesity and that we provide novel potential therapeutic targets to treat these chronic inflammatory diseases.

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Summary

Summary

Atherosclerosis and obesity are chronic inflammatory diseases. As such, they share characteristic features, which include the permanent accumulation of immune cells, dramatic changes in chemokine and cytokine levels and an elevated level of adhesion molecules. All of these features have been shown to be involved at the onset of the disease as well as in disease progression. The prevalence of atherosclerosis and obesity has increased over the last decades and has become a major health and economic problem. Furthermore, it has been shown that obesity, besides the classical risk factors like hypercholesterolemia, smoking, and high blood pressure amongst others, is an additional risk factor for cardiovascular disease. Due to the close relationship between atherosclerosis and obesity we focused on elucidating the molecular mechanisms behind the disease initiation and progression by using specialized mouse models.

Transport molecules, such as caveolin-1, and co-stimulatory molecules, such as the CD40L-CD40 dyad, have been shown to execute fundamental functions in atherosclerosis and in obesity. The loss of caveolin-1 leads to a tremendous decrease in atherosclerotic lesions in mice although elevated cholesterol levels have been reported. The CD40L-CD40 dyad has been shown to efficiently modulate atherosclerotic plaque formation and stability. Regarding obesity, it has become evident that sCD40L levels are up-regulated during obesity and that adipocytes express high levels of CD40. These facts render the CD40L-CD40 signaling cascade to be an interesting target in obesity research.

Transport mechanisms and co-stimulatory molecules are two of the main features involved in atherosclerosis and obesity. Consequently, this thesis focuses on elucidating the contributions of transport and co-stimulatory mechanisms in atherosclerosis and obesity in general and on a cell type specific level.

The genetic deletion of caveolin-1 in atherosclerotic prone mice has been shown to be atheroprotective. Besides elevated plasma cholesterol levels *Cav-1^{-/-}/ApoE^{-/-}* mice presented with significantly smaller atherosclerotic lesions compared to their wild type counterparts. In **chapter 2**, we identify that non-hematopoietic caveolin-1 deficiency is responsible for the diminished abundance and size of atherosclerotic plaques and constrains the leukocyte influx into atherosclerotic lesions. Furthermore, we show that caveolin-1 loss in

hematopoietic cells results in an anti-inflammatory T cell response which is characterized by elevated levels of regulatory T cells.

In **chapter 3**, we describe the functions of co-stimulatory molecules in atherosclerosis with a special focus on the CD40L-CD40-TRAF axis. In this chapter, we give an overview of the current relevant knowledge on the impact of co-stimulatory molecules on leukocyte recruitment, plaque progression and destabilization. Furthermore, we discuss the therapeutic possibilities that may arise from targeted interventions in the CD40L-CD40-TRAF axis.

Inhibition of CD40L-CD40 signaling has been shown to be atheroprotective, but clinical trials were terminated due to thrombo-embolic complications. Therefore, we investigated if more specified intervention strategies in CD40 signaling harbor therapeutic potential. In **chapter 4**, we report that CD40 deficiency in the hematopoietic compartment leads to reduced atherosclerosis and results in a stable plaque phenotype. Moreover, abrogation of CD40-TRAF6, but not CD40-TRAF2/3/5 signaling in MHCII⁺ cells prevents atherosclerosis indicating that CD40-TRAF6 mediates the pro-atherogenic pathways. CD40-TRAF6 deficiency leads to a reduced number of inflammatory monocytes, less recruitment of these cells to the arterial wall, and skews macrophages toward the anti-inflammatory M2 phenotype. Our data unravel the effects of specific interference with the CD40L-CD40-TRAF signaling cascade and establish that target specific intervention strategies hold the potential to achieve therapeutic effects in atherosclerosis.

In **chapter 5**, we review the current knowledge of CD40L-CD40 interactions in type 2 diabetes and how this co-stimulatory dyad links pancreatic, adipose tissue, and vascular inflammation. Furthermore, we elucidate the role of sCD40L in patients with type 2 diabetes and discuss its potential as biomarker for cardiovascular disease. In addition, we highlight the therapeutic potential of CD40L-CD40 blockage in type 2 diabetes.

Regarding the potent role of CD40L-CD40 interactions in type 2 diabetes and its complications, as reviewed in chapter 5, we examined the role of CD40L in a mouse model of diet induced obesity. In **chapter 6**, we show that genetic deficiency of CD40L diminishes adipose tissue inflammation, attenuates the development of diet-induced obesity, and increases systemic insulin sensitivity. Consequently, treatment with an anti-CD40L blocking antibody improved systemic insulin sensitivity, glucose tolerance and CD4⁺ T cell

Summary

infiltration in adipose tissue. Our data harbor the potential to identify novel therapeutic targets to prevent or diminish obesity and its co-morbidities.

In line with chapter 4, we investigated the impact of CD40 downstream signaling intermediates in diet-induced obesity as well. In **chapter 7**, we investigated the effects that are caused by different CD40-TRAF deletions and assessed their impact on obesity. Our results show that CD40-TRAF2/3/5 deletion aggravated obesity and its co-morbidities in a mouse model of diet induced obesity, whereas *CD40-TRAF6*^{-/-} mice generally resembled the wild type phenotype. From our findings we conclude that the CD40-TRAF2/3/5 signaling axis is crucial in maintaining glucose homeostasis and adipose tissue inflammation in obesity. In addition, our research opens the window towards the discovery of new potential therapeutic targets in obesity.

Finally, **chapter 8** summarizes and discusses the findings of this thesis and future directions are given. In conclusion, this thesis provides novel insights into the biology of caveolae and co-stimulatory molecules in chronic inflammatory diseases. Especially for CD40L-CD40-TRAF interactions, cell type specific contributions are evaluated and potential therapeutic targets for new treatment strategies for atherosclerosis, obesity and the metabolic syndrome are highlighted.

Samenvatting

Samenvatting

Atherosclerose, ook bekend onder de naam aderverkalking, en obesitas, beter bekend als vetzucht, zijn chronische inflammatoire ziekten, die karakteristieke kenmerken delen, zoals een permanente accumulatie van immuuncellen, sterke veranderingen in chemokine en cytokine expressie en een toename in adhesiemoleculen. Al deze factoren zijn zowel betrokken bij de initiatie van deze ziekten, als ook bij de verdere ontwikkeling hiervan. De prevalentie van atherosclerose en obesitas is in de laatste decennia sterk toegenomen en vormt een steeds groter wordend gezondheidsprobleem met groeiende economische gevolgen. Verder is aangetoond dat obesitas, onafhankelijk van de klassieke risicofactoren zoals hypercholesterolemie, roken en hoge bloeddruk, een risicofactor is voor cardiovasculaire aandoeningen. Vanwege de sterke relatie tussen atherosclerose en obesitas, hebben we ons gericht op het ontrafelen van de moleculaire mechanismen die van belang zijn bij initiatie en verder ontwikkeling van deze ziekten. Hierbij hebben we gebruik gemaakt van specifieke muismodellen.

Uit onderzoek is gebleken dat transportmoleculen, zoals caveoline-1, en co-stimulatoire moleculen, zoals het CD40-CD40L receptor-ligand paar, een fundamentele rol spelen in atherosclerose en obesitas. Muizen met caveoline-1 deficiëntie vertonen een sterke afname van het aantal atherosclerotische plaques en in de grootte hiervan, hoewel het cholesterolniveau in het bloed toeneemt. Het CD40-CD40L receptor-ligand paar kan op efficiënte wijze zorgen voor modulatie van de vorming en stabiliteit van atherosclerotische plaques. Op het gebied van obesitas is bekend dat sCD40L in het bloed verhoogd is en dat adipocyten CD40 verhoogd tot expressie brengen. Dit gegeven maakt de signaleringscascade van het CD40-CD40L receptor-ligand paar tot een interessant doelwit in het onderzoek naar de mechanismen van obesitas.

Transportmechanismen en co-stimulatoire moleculen zijn dus van groot belang in atherosclerose en obesitas. Vandaar dat dit proefschrift zich richt op het ontrafelen van de bijdrage die transportmechanismen en co-stimulatie leveren aan atherosclerose en obesitas, zowel op systemisch als op een celtype-specifiek niveau.

In de literatuur is reeds beschreven dat deletie van caveoline-1 in atherosclerotische muizen beschermt tegen de ontwikkeling van atherosclerose. Naast een stijging in plasma cholesterol niveau, hadden *Cav1^{-/-}/Apoe^{-/-}* muizen significant kleinere atherosclerotische lesies in

vergelijking met wildtype controles. In **hoofdstuk 2** hebben we aangetoond dat niet-hematopoietische caveoline-1 deficiëntie verantwoordelijk is voor de afname in het aantal atherosclerotische plaques, als ook voor een afname in plaque grootte. Verder beperkt niet-hematopoietische caveoline-1 deficiëntie de influx van leukocyten in de plaque. Daarnaast hebben we aangetoond dat afwezigheid van caveolin-1 in hematopoietische cellen zorgt voor een anti-inflammatoire T-cel respons, die gekenmerkt wordt door een toename in regulatoire T-cellen.

In **hoofdstuk 3** beschrijven we de functies van co-stimulatoire moleculen in atherosclerose, met in het bijzonder de CD40L-CD40-TRAF as. In dit hoofdstuk wordt een overzicht gegeven van de huidige kennis over de rol die co-stimulatoire moleculen spelen bij rekrutering van leukocyten, plaque ontwikkeling en plaque destabilisatie. Verder worden potentiële therapeutische opties op basis van interventies in de CD40L-CD40-TRAF as bediscussieerd.

Het is reeds aangetoond dat inhibitie van CD40L-CD40 signalering beschermt tegen atherosclerose. Helaas zijn de klinische trials op dit vlak stopgezet zijn in verband met trombo-embolische complicaties. Daarom hebben we onderzocht of meer specifieke interventies in CD40-signalering therapeutisch opties zouden kunnen bieden. In **hoofdstuk 4** tonen we aan dat CD40-deficiëntie in hematopoietische cellen resulteert in een afname van atherosclerose en zorgt voor een stabiel plaque fenotype. Bovendien verhindert inhibitie van CD40-TRAF6 signalering, maar niet CD40-TRAF2/3/5 signalering in MHCII⁺ cellen, de ontwikkeling van atherosclerose. Dit geeft aan dat CD40-TRAF6 een belangrijke pro-atherogene rol speelt. CD40-TRAF6 deficiëntie leidt tot een vermindering in het aantal inflammatoire monocysten, een vermindering in rekrutering van deze cellen naar de arteriële vaatwand, en differentiatie van macrofagen naar een anti-inflammatoir M2 fenotype. Onze data tonen aan dat specifiek kan worden ingegrepen op de CD40L-CD40-TRAF as en dat gerichte interventies potentieel bruikbaar zijn voor klinische behandeling van atherosclerose.

Hoofdstuk 5 levert een overzicht over de huidige kennis van CD40L-CD40 interacties in type 2 diabetes, en over hoe dit co-stimulatoire systeem betrokken is bij ontsteking van de alvleesklier, het vetweefsel en de arteriën in type 2 diabetes. Verder lichten wij in dit hoofdstuk de rol van sCD40L in

patiënten met type 2 diabetes toe en wordt de potentie van sCD40L als biomarker voor cardiovasculaire ziekten bediscussieerd. Aansluitend wordt er nog aandacht besteed aan de therapeutische mogelijkheden van CD40L-CD40 inhibitie in type 2 diabetes.

Met zicht op de potentiële rol van CD40L-CD40 interacties in type 2 diabetes en de complicaties hiervan, hebben we de rol van CD40L in een muismodel voor dieet-geïnduceerde obesitas onderzocht. In **hoofdstuk 6** tonen we aan dat genetische CD40L-deficiëntie een afname veroorzaakt in ontsteking van het vetweefsel. Bovendien remt CD40L-deficiëntie de ontwikkeling van dieet-geïnduceerde obesitas en zorgt het voor een toename van insuline-gevoeligheid. Inhibitie van CD40L met een blokkerend antilichaam leidde tot een verbetering van systemische insuline-gevoeligheid, glucose-tolerantie en CD4⁺ T-cel infiltratie in het vetweefsel.

In lijn met hoofdstuk 4, is ook de rol van signaal-transductiemoleculen van CD40 in dieet-geïnduceerde obesitas onderzocht. In **hoofdstuk 7** is gekeken naar de effecten die deletie van specifieke CD40-TRAF moleculen heeft op obesitas. Hieruit is gebleken dat deletie van CD40-TRAF2/3/5 leidt tot een verergering van obesitas en hieraan gerelateerde co-morbiditeit in muizen met dieet-geïnduceerde obesitas. Daarentegen toonden *CD40-TRAF6*^{-/-} muizen een fenotype dat overeenkomt met het fenotype in wildtype muizen. Hieruit concluderen wij dat de CD40-TRAF2/3/5 signaaltransductie van cruciaal belang is voor het behoud van de normale glucose homeostase en voor onderdrukking van inflammatie in vetweefsel. Aansluitend toont dit onderzoek potentiële opties voor nieuwe therapeutische mogelijkheden in het behandelen van obesitas.

Tot slot worden in **hoofdstuk 8** de bevindingen samengevat en bediscussieerd, en worden opties voor verder onderzoek besproken. Concluderend geeft dit proefschrift nieuwe inzichten in de biologie van caveolae en co-stimulatoire moleculen in chronische ontstekingsziekten. In het bijzonder worden celtype-specifieke bijdragen van de CD40L-CD40-TRAF as bestudeerd en worden potentiële therapeutische doelen voor de behandeling van atherosclerose, obesitas en het metabool syndroom belicht.

Zusammenfassung

Zusammenfassung

Atherosklerose (Arterienverkalkung) und Adipositas (Fettleibigkeit) gehören zu den chronischen Entzündungserkrankungen. Als solche teilen sie charakteristische Merkmale wie etwa die permanente Anhäufung von Immunzellen, dramatische Veränderungen der Chemokin- und Cytokin-zusammensetzung sowie eine erhöhte Expression an Adhäsionsmolekülen. Jedes dieser Merkmale ist essentieller Bestandteil der jeweiligen Krankheit, sowohl bei Beginn als auch während fortgeschrittener Erkrankungsphasen. Die Häufigkeit des Auftretens von Atherosklerose und Adipositas ist in den letzten Jahrzehnten stark angestiegen und stellt heute ein erhebliches Gesundheits- und Wirtschaftsproblem dar. Darüber hinaus stellt die Adipositas - neben den klassischen Risikofaktoren wie etwa einem erhöhten Cholesterinwert, Rauchen oder Bluthochdruck - einen zusätzlichen Risikofaktor für Herz-Kreislauf-Erkrankungen dar. Aufgrund des engen Zusammenhangs zwischen Atherosklerose und Adipositas befasst sich diese Arbeit mit den molekularen Mechanismen, die sich hinter der Entstehung und dem Fortschreiten oben genannter Krankheiten verbergen und versucht diese aufzuklären. Zu diesem Zweck wurde für diese Arbeit von speziell entwickelten Mausmodellen Gebrauch gemacht.

Transportmoleküle (z.B. Caveolin-1) und kostimulatorische Moleküle (z.B. CD40 und CD40-Ligand (CD40L)) sind dafür bekannt, fundamentale Funktionen in Atherosklerose und Adipositas auszuüben. Der Verlust von Caveolin-1 führt zu einer erheblichen Reduktion der Atherosklerose bei Mäusen, obwohl erhöhte Cholesterinwerte gemessen wurden. Für die kostimulatorischen Moleküle CD40/CD40L wurde gezeigt, dass sie effizient den Aufbau und die Stabilität von atherosklerotischen Plaques verändern können. Im Hinblick auf die Adipositas ist heute bekannt, dass die Menge an sCD40L stark erhöht ist und dass Fettzellen (Adipozyten) CD40 in hohem Maße exprimieren. Aufgrund dieser Fakten stellt die CD40L-CD40-Signalkaskade ein interessantes molekulares Ziel für die Adipositas-Forschung dar.

Transportmechanismen und kostimulatorische Moleküle sind zwei der Hauptmerkmale von Atherosklerose und Adipositas, weshalb sich diese Arbeit mit der Erforschung von Transport- und Kostimulationsmechanismen bei der Atherosklerose und Adipositas befasst und tut dies sowohl im Hinblick auf generelle Prozesse als auch auf Zell spezifischer Ebene.

Es ist bekannt, dass der genetische Verlust von Caveolin-1 in atherosklerotisch prädestinierten Mäusen einen positiven Effekt hat. Trotz erhöhter Plasma-Cholesterinwerte haben Apolipoprotein- und Caveolin-1-defiziente (*Apoe^{-/-}Cav1^{-/-}*) Mäuse kleinere atherosklerotische Plaques verglichen mit *Apoe^{-/-}* Kontrollmäusen. In **Kapitel 2** dokumentieren wir, dass die nicht-hämatopoietische Caveolin-1-Defizienz für die Verringerung der Anzahl und Größe der Plaques verantwortlich ist. Zudem verhindert die nicht-hämatopoietische Caveolin-1-Defizienz das Einwandern von Leukozyten in die atherosklerotischen Plaques. Des Weiteren fanden wir heraus, dass eine hämatopoietische Defizienz von Caveolin-1 ein anti-entzündliches T-Zell-Profil hervorruft, welches durch eine erhöhte Anzahl an regulatorischen T-Zellen charakterisiert ist.

In **Kapitel 3** beschreiben wir die Funktionen von kostimulatorischen Molekülen, insbesondere der CD40L-CD40-TRAF-Achse und ihren Einfluss auf die Atherosklerose. In diesem Kapitel geben wir einen Überblick über den aktuellen Wissensstand der Auswirkungen von kostimulatorischen Molekülen auf die Rekrutierung von Leukozyten und der Plaqueprogression bzw. -destabilisierung. Zusätzlich diskutieren wir mögliche therapeutische Strategien, die aus der gezielten Intervention mit der CD40L-CD40-TRAF-Achse hervorgehen könnten.

Obwohl die Hemmung von CD40L-CD40 vor experimenteller Atherosklerose schützt, mussten klinische Studien aufgrund von thrombo-embolischen Komplikationen abgebrochen werden. Aus diesem Grund haben wir erforscht, ob eine gezieltere Interventionsstrategie potentielle Therapien in sich birgt. In **Kapitel 4** konstatieren wir, dass das Fehlen von CD40 in hämatopoietischen Zellen zu einer Reduzierung der Atherosklerose führt und einen stabilen Plaquephenotyp bedingt. Des Weiteren konnten wir zeigen, dass die Außerkraftsetzung von CD40-TRAF6-Interaktionen, nicht aber von CD40-TRAF2/3/5-Interaktionen, in MHCII⁺-Zellen die Atherosklerose unterdrückt. Daraus kann geschlossen werden, dass die CD40-TRAF6-Achse ein entzündungsfördernder Signalweg ist. Die CD40-TRAF6-Defizienz führt zu einer geringeren Anzahl von entzündlichen Monozyten im Peripherblut und einer verminderten Ansammlung dieser Zellen in der Gefäßwand. Zudem wiesen die Makrophagen eine anti-entzündliche M2-Signatur auf. Unsere Daten beschreiben die Effekte einer gezielten Intervention mit der CD40L-CD40-

Zusammenfassung

TRAF-Signalkaskade und legen dar, dass diese spezifischen Interventionen das Potential beinhalten, therapeutische Effekte in der Atherosklerosebehandlung zu erzielen.

In **Kapitel 5** geben wir eine Literaturübersicht über den aktuellen Forschungsstand von CD40L-CD40-Interaktionen und ihren Auswirkungen auf Typ 2 Diabetes mellitus. Erläutert wird dabei der Zusammenhang zwischen pankreatischer, adipöser und vaskulärer Endzündung. Des Weiteren explizieren wir die Bedeutung von sCD40L als einen möglichen Biomarker zur Bestimmung des kardiovaskulären Risikos von Typ 2 Diabetikern und unterstreichen die potentiellen therapeutischen Möglichkeiten einer CD40L-CD40-Intervention bei Typ 2 Diabetes mellitus.

Aufgrund der fundamentalen Rolle von CD40L-CD40-Interaktionen bei Typ 2 Diabetes mellitus untersuchten wir den Effekt einer CD40L-Defizienz in einem Diät-induzierten Adipositas-Mausmodell. In **Kapitel 6** zeigen wir, dass der genetische Verlust von CD40L die Entzündung im Fettgewebe verringert, die Entstehung der Adipositas vermindert und die systemische Insulinsensitivität erhöht. Folgerichtig resultierte auch die Injektion eines CD40L-blockierenden Antikörpers in einer erhöhten Insulinsensitivität, einer verbesserten Glukosetoleranz und einer verminderten Infiltration des Fettgewebes durch CD4⁺ T-Zellen. Unsere Daten bieten das Potential zur Identifizierung neuer therapeutischer Ansätze zur Behandlung von Adipositas und der damit einhergehenden Folgeerkrankungen.

Sowie bereits in Kapitel 4 für Atherosklerose beschrieben, untersuchten wir den Einfluss der CD40-Signalkaskade auch bei Adipositas. In **Kapitel 7** beschreiben wir die Auswirkungen von gezielten CD40-TRAF-Defizienzen und untersuchten deren Einfluss auf die Adipositas. Wir haben festgestellt, dass der Verlust der CD40-TRAF2/3/5-Achse die Adipositas und deren Begleiterscheinungen verschlimmert wohingegen der Verlust der CD40-TRAF6-Achse zu einem Wildtyp-ähnlichen Phenotyp führt. Aus unseren Resultaten schließen wir, dass die CD40-TRAF2/3/5-Achse einen bedeutenden regulatorischen Einfluss auf die Glukosehomeostase und die Entzündung des Fettgewebes hat. Des Weiteren eröffnen unsere Untersuchungen die Erforschung und Entwicklung neuer potentieller therapeutischer Strategien zur Behandlung der Adipositas.

In **Kapitel 8** werden die Ergebnisse dieser Arbeit zusammengefasst und diskutiert. Zudem geben wir einen Ausblick auf mögliche zukünftige Forschungsschwerpunkte. Zusammenfassend lässt sich sagen, dass diese Arbeit neue Einblicke in die biologische Relevanz von Caveolin-1 und kostimulatorischen Molekülen und ihren Einfluss auf den Verlauf von chronischen Entzündungserkrankungen gibt. Besonders für CD40L-CD40-TRAF-Interaktionen werden zelltypspezifische Einflüsse bewertet und mögliche neue therapeutische Ansätze diskutiert.

Curriculum Vitae

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International Research

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Grants and prizes

2012, Noordwijkerhout, NL	Best session presentation at the CVC, Title: "Interplay between CD40- TRAF2/3/5 and CD40-TRAF6 interactions in diet induced obesity"
2010, Stockholm, SE	Travel grant (3 months) Boehringer Ingelheim Foundation
2009, Maastricht, NL	Best presentation at Pathology Science Day, Title: "Protection against atherosclerosis in Cav-1 ^{-/-} mice is due to Cav-1 ^{-/-} in-non-hematopoietic cells"

Oral Presentations

2012, Noordwijkerhout, NL
 2010, Hamburg, D
 2010, Biesenmortel, NL
 2009, Ede, NL

Cardiovascular Conference
 European Atherosclerosis Society
 DEBS/MiVaB Meeting
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Poster Presentations

2012, Noordwijkerhout, NL
 2011, Orlando, US

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 2011, Ede, NL
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 2009, Papendal, NL

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Cardiovascular Conference
 American Heart Association: Scientific Sessions 2011
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 Keystone Symposia: Advances in Molecular Mechanisms in Atherosclerosis
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Other Conferences attended

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 2009, Noordwijkerhout, NL
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NVVI Course: Guiding the Action of the Immune System
 NVVI Winter School
 Dutch Atherosclerosis Society meeting
 Annual Meeting of the Society for Microcirculation and Vascular Biology
 4th EVGN Summer School

Additional scientific activities

2011, Reviewer
 2011, Reviewer

Regulatory Peptides
 Thrombosis and Hemostasis

Publications

Engel D , Poggi M , Kusters P, Beckers L, Wijnands E, Winkels H, Biessen E.A.L., Driessen A, Seijkens T, Gerdes N, Lutgens E.: CD40 signaling via the CD40-TNF-Receptor associated factor (TRAF) 2/3/5 axis confers protection against adipose tissue inflammation and metabolic complications in diet induced obesity. *In preparation*.

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List of Abbreviations

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ACC1	acetyl-coenzyme A carboxylase
Acly	ATP-citrate lyase
APC	antigen presenting cell
ApoE/Apoe	apolipoprotein E
ASMA	α smooth muscle actin
AT	adipose tissue
BAT	brown adipose tissue
BM	bone marrow
BMT	bone marrow transplantation
Cav-1	caveolin-1
CCL	CC chemokine ligand
CCR	CC chemokines receptor
CD	cluster of differentiation
CD40L	CD40 ligand
cDNA	complementary deoxyribonucleic acid
CLS	crown like structure
Cox-2	cyclooxygenase-2
CRP	C-reactive protein
CSFR1	colony stimulating factor 1 receptor
CVD	cardiovascular disease
CXCR	chemokine (C-X-C motif) receptor
DC	dendritic cell
DTR	diphtheria toxin receptor
EC	endothelial cell
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
Elovl6	elongase of long chain fatty acid family 6
eNOS	endothelial nitric oxide synthase
epiAT	epididymal adipose tissue
ERK	extracellular signal-regulated kinases
FACS	fluorescence activated cell sorting
FAS	fatty acid synthase
FGK45	CD40 stimulating antibody
FoxP3	forkhead box P3
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GK	glucokinase
GLUT	glucose transporter
GPAM	glycerol-3- phosphate acyltransferase mitochondria
GTT	glucose tolerance test
HbA1c	glycated hemoglobin A1c
HDL	high density lipoprotein
HE	haematoxylin and eosin
HFD	high fat diet

HIF1 α	hypoxia inducible factor 1 α
ICAM-1	intercellular adhesion molecule-1
ICOS	inducible T cell costimulator
Ig	immunoglobulin
IGF-1	insulin-like growth factor 1
IHC	immunohistochemical
I κ B α	nuclear factor of κ light polypeptide gene enhancer in B cells inhibitor α
IL	Interleukin
IFN γ	interferon γ
iNOS	inducible nitric oxide synthase
ip	intraperitoneal
IP-10	interferon gamma-induced protein 10
ITT	insulin tolerance test
JNK	janus kinase
IRS	insulin receptor substrate
LC	lipid core
LDL	low density lipoprotein
LDLR	low density lipoprotein receptor
LIPE	hormone-sensitive lipase
LPK	liver specific pyruvate kinase
LPS	lipopolisaccharide
M1	classically activated macrophage
M2	alternatively activated macrophage
M Φ	macrophage
Mac-1	macrophage 1 antigen
MAPK	mitogen-activate protein kinase
MCP-1	monocyte chemotactic protein-1
MHC	major histocompatibility complex
MIP-1 α	macrophage inflammatory protein-1 α
MIP-1 β	macrophage inflammatory protein-1 β
MMP	matrixmetalloproteinase
MPO	myeloperoxidase
MR-1	anti-CD40L antibody
mRNA	messenger ribonucleic acid
NASH	non-alcoholic steato-hepatitis
NF κ B	nuclear factor κ B
NK cell	natural killer cell
NKT cell	natural killer T cell
NO	nitric oxide
PAI	plasminogen activator inhibitor
PAMPs	pathogen associated molecular patterns
PBS	phosphate buffered saline
PCR	polymerase chain reaction

List of Abbreviations

PFA	paraformaldehyde
PPAR γ	peroxisome proliferator-activated receptor- γ
RANTES	regulated upon activation normal T cell expressed and secreted
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
scAT	subcutaneous adipose tissue
SCD1	stearyl-coenzyme A desaturase
sCD40L	soluble CD40 ligand
SEM	standard error of mean
SFD	standard fat diet
SMC	smooth muscle cell
SR	sirius red
SREBP1c	sterol regulatory element-binding protein-1c
SVC	stromal vascular cells
T2DM	type 2 diabetes mellitus
TBS	tris buffered saline
TD	TRAF domain
TGF β	transforming growth factor β
Th cell	helper T lymphocyte
TIMP	tissue inhibitor of matrixmetalloproteinase
TLR	toll like receptor
TNF	tumor necrosis factor
TRAF	tumor necrosis factor receptor associated factor
Tregs	regulatory T cell
UCP-1	uncoupling protein 1
VCAM-1	vascular cell adhesion molecule-1
VLDL	very low density lipoprotein
vSMC	vascular smooth muscle cell
WT	wild type

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Dear Prof. dr. Esther Lutgens, dear Chef 1, beste Esther. Already some time ago you had an interview with a guy from Germany applying for a PhD position within your CD40 group. What that guy did until then, was as far away from atherosclerosis research as the moon. He was namely dealing with some strange fish species which live in even stranger rivers that lose their water during the summer months. So a real typical river!!! Nevertheless, you took the risk in hiring him and I can say, that from the first day I have been in Maastricht, I had the feeling that you believed that I'll be able to make it. Your confidence in me, the enthusiasm with which you promote your group, the scientific excellence and the perfect supervision during the last four and a half years are the hallmarks of my PhD period in your lab. Additionally, there was not only work in the lab but also a lot of meetings and trips that we made. A summer school in Krakau that I was allowed to go to, a fantastic trip to the Keystone meeting (with some nice skiing!!!), innumerable meetings in the Netherlands and Germany on which I was able to train my presentation abilities and a really nice week that we spent in the Sunshine State at the AHA meeting. Next to that, I will not forget to mention the 3 month that I was able to stay in the Hansson lab in Sweden, which was also possible because you really supported my idea of going there. Most likely, I have forgotten tons of things that would be noteworthy, but finally I am running out of pages. Please stay as you are and keep on running your group in the special "Fascinating Esther Style". Thanks for everything that you did for me in the last years. I'll never forget it and the CD40-Team will always be part of my scientific career.

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I think I spider, bis Denver!!!

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FACSFather, Chef 4, beste Erwin. Es kommt mir vor wie gestern, als ich zum ersten Mal im Labor gearbeitet habe. Du hast mir gezeigt, wie man mouse tissues zurecht schneidet, um sie später gut in Paraffin einbetten zu können. In den darauf folgenden Jahren habe ich noch mindestens 1000 andere wichtige Dinge von dir gelernt. Zum einen natürlich flow cytometry, aber für immer im

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Chère Marjorie. This is already everything that I can remember from my French class at school, sorry ;-). It was a great pleasure to work with you in the CD40-Team and we had very fruitful co-operations. I am looking forward to see our obesity TRAF story published and I have to thank you a lot for teaching me all this obesity-metabolic syndrome-liver inflammation stuff.

The only MD in the CD40 group, next to Esther of course, is you Tom. And one could already tell by the outer appearance!!! Styled hair, shaved like visiting his grandmother, a well ironed shirt underneath a very proper pullover, flawless jeans, exquisite shoes (how many do you have???) and of course, most characteristic, a lab coat with short sleeves. But that outer appearance, usually linked to a very serious person, is by far not giving the right impression of what you really are. Next of being a brilliant scientist, in my eyes you are one of the

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Dear Sylvia. Thanks for all the nice chats, interesting discussions, help with designing my DEC's and for teaching me how to classify human atherosclerotic plaques.

Hey Jack. Thanks a lot for providing me with all these difficult qwin-obesity-atherosclerosis-cell-counting-collagen-measuring-programms. Without you life would be much more difficult when it comes to slide analysis. Your expertise is marvelous.

The blackest sheep of all is definitely...? Lieve!!! At least when it comes to dressing up for Carnival. Besides that it has always been lovely to be together in the centrifuge with you. Probably we can repeat that one day ;-). With regard to everything, you are really an enrichment to the entire group. I wish you all the best for the last month to go before your family enlarges for the second time.

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Liebe Heidi, Lieber Drops. Ich weiß, dass es noch nicht die Habilitation ist, die Heidi ja schon vor vielen Jahren prophezeit hat. Da man aber nie genau wissen kann, ob es dafür reicht, freue ich mich sehr, dass ihr zu meiner Promotion gekommen seid. Wenn es klappt, werdet ihr sicherlich nochmals eingeladen ;-).

Lieber Opa. Vielen Dank für dein stetiges Interesse an dem, was ich mache und für die zahlreichen netten Fußballabende mit Bratkartoffeln und Spiegelei bei dir zu Hause. Es war toll, so lange in deiner Heimatstadt einen Steinwurf von dir entfernt zu wohnen.

Liebe Daggi, lieber Mike, liebe Pauline und liebe Maja. In den letzten Jahren haben wir nahe beieinander gewohnt und es war immer toll, jederzeit bei euch vorbeikommen zu können. Zum Essen, aber auch oft genug für eine umkämpfte Partie „Risiko“. In Zukunft werden wir uns leider seltener sehen, aber ich komme ganz bestimmt dann und wann mal vorbei. Danke für all die schönen Abende, die wir hatten.

Liebe Mama, Lieber Papa. Ich denke, dass ich an dieser Stelle wirklich nicht viel schreiben muss. Euch gebührt der größte Dank, für alles was ihr in den letzten 32 Jahren für mich getan habt. Ich hoffe ihr seid stolz, auf das was ihr da produziert habt. Ich freu mich schon auf die nächsten 32 Jahre. Euer Sputnik.