

Atheroprotective role of the C5aR2 deficiency in apolipoprotein E deficient mice

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Abbreviations

°C	degree Celsius
µg	microgram
µl	microliter
µm	micrometer
µM	micromole
ABCA1	ATP-binding cassette transporter A1
ABCG1	ATP-binding cassette transporter G1
AKT	protein kinase B
APCs	antigen presenting cells
APOE	apolipoprotein E
α-SMA	alpha smooth muscle actin
ASP	acylation stimulating protein
BMDM	bone marrow-derived macrophage
B _{reg}	B regulatory cells
BSA	bovine serum albumin
C5aR1	complement component 5a receptor 1
C5aR2	C5a receptor-like 2
CD4 ⁺	T helper cells
CD8 ⁺	cytotoxic T-cells
cDNA	complementary DNA
CLP	common lymphoid progenitor cells
CMP	common myeloid progenitor cells
CVD	cardiovascular diseases
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cells
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EC	endothelial cell

Abbreviations

ECA	external carotid artery
EDTA	ethylenediaminetetraacetic acid
EEL	external elastic lamina
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal–regulated kinase
FACS	fluorescence-activated cell sorting
FCS	forward scatter
FITA	acid incorporation into adipose triglyceride
g	gram
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GPCR	G protein-coupled receptor
h	hour
H ₂ O	water
HCL	hydrochloric acid
HFD	high fat diet
HMGB1	high mobility group box 1
HRP	horseradish peroxidase
HSC	hematopoietic stems cells
HSL	hormone-sensitive lipase
i.p.	intraperitoneal
ICA	internal carotid artery
IEL	internal elastic lamina
IFN	interferon
IL	interleukin
iNOS	inducible nitric oxide synthase
kDa	kilo Dalton
kg	kilogram
KO	knock-out
l	liter
LCA	left carotid artery

LCM	L929 conditioned medium
LDL	low-density lipoprotein
LPS	lipopolysaccharide
m	meter
mm	millimeter
M	mole
M1	classically activated macrophages
M2	alternatively activated macrophages
MAC	membrane attack complex
MASP-2	mannan-binding lectin serine protease 2
MAPK	mitogen-activated protein kinase
MCP 1	chemokine monocyte chemoattractant protein-1
M CSF	macrophage colony stimulating factor
MFI	mean fluorescence intensity
mg	milligram
min	minute
ml	milliliter
mRNA	messenger RNA
NaCl	sodium chloride
NET	neutrophil extracellular traps
ng	nanogram
NK cells	natural killer cells
NO	nitric oxide
oxLDL	oxidized low-density lipoprotein
PAMPs	pathogen associated molecule patterns
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PRRs	pattern recognition receptors
q-PCR	quantitative PCR

Abbreviations

RANTES	CCL5, C-C motif chemokine ligand 5
RNA	ribonucleic acid
RNAse	ribonuclease
RPAR	reverse passive arthus reaction
rpm	revolutions per minute
RPMI	roswell park memorial Institute medium
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
sec	second
SEM	standard error of the mean
SMC	smooth muscle cell
SSC	sideward scatter
TLR	toll-like receptor
TNF- α	tumor necrosis factor-alpha
Treg	T regulatory cell
TUNEL	terminal-desoxyribosyl-transferase-mediated-dUTP-nick-end-labeling
U	unit
VCAM 1	vascular cell adhesion molecule-1
WHO	World Health Organization
WT	wild-type

1 Introduction

1.1 Immune system

In all cellular organisms, a complex system of cells and molecules has evolved to protect its integrity from harmful causes. The so called immune system is a network of various mechanisms to protect the host system from pathogens by discriminating between self and foreign origin [1]. The first evidence of a basic immune system can already be found in invertebrates. It comprises mechanisms such as phagocytosis, release of antimicrobial peptides or complement activation which developed in and was maintained from primitive immune systems to current immune systems in vertebrates [2]. In vertebrates the immune system consists of white blood cells or leucocytes which originate from the hematopoietic system. Hematopoietic stems cells (HSC) located in the bone marrow differentiate into common myeloid progenitor cells (CMP) and common lymphoid progenitor cells (CLP) which gives rise to the rich diversity of immune effector cells (see: Figure 1) [3].

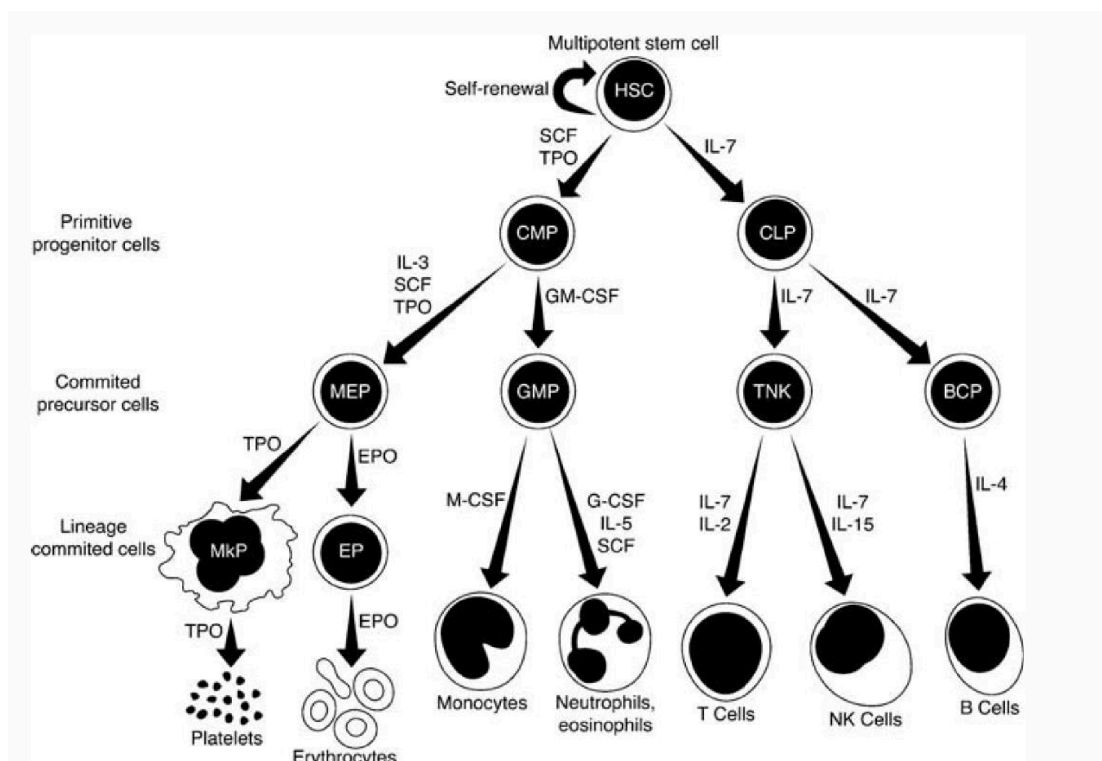


Figure 1: Hematopoiesis, showing the development of the different kinds of immune cells from hematopoietic stem cells. HSC in the bone marrow give rise to the two legs of hematopoietic lineage: the common myeloid progenitor cells (CMP) and common lymphoid progenitor cells (CLP). CMP cells give rise to monocytes, neutrophils and eosinophils, whereas, CLP cells give rise to T-cells, B-cells and natural killer (NK) cells (Adapted from Robb, 2007 [4]).

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Cells from the myeloid lineage are the precursors for macrophages, granulocytes and other leukocytes which are the main players in the innate immune system [5]. The cells from the lymphoid lineage differentiate into B-cells, T-cells and natural killer cells (NK) and eventually into plasma cells [6]. Both legs of the HSC can give rise to dendritic cells [5, 7]. The hematopoiesis is depicted in Figure 1: Hematopoiesis. The typical immune system in vertebrates is divided into innate immunity and adaptive immunity [8] (see: Figure 2).

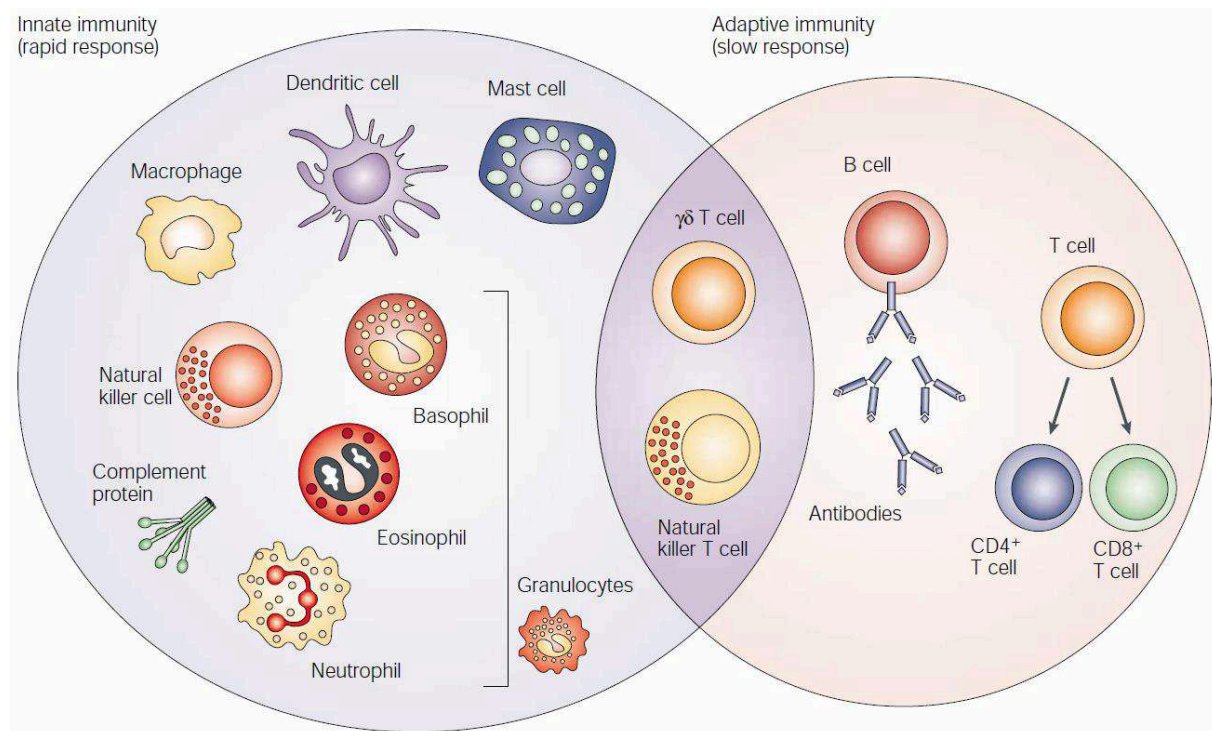


Figure 2: Cells in innate and adaptive immunity. Cells involved in innate immune response are mainly monocytes/macrophages, mast cells, dendritic cells and granulocytes. In adaptive immunity, B-cells are responsible for antibody production and CD8⁺ cytotoxic T-cells fight infected cells. CD4⁺ helper T-cells are the main player in orchestrating adaptive immune response. Natural killer cells are involved in both types of immune responses (Adapted from Dranoff, 2004 [9]).

The innate immune system is antigen-independent and functions as a first line of defense. Upon facing a pathogen or other harmful stimuli it reacts fast, within minutes to hours. In vertebrates the innate immunity is comprised of phagocytic cells, mast cells, eosinophils, basophiles, NK cells [8]. The phagocyte based system derived from the myeloid lineage, constitutes an ancient immune system inherited from the invertebrates. [10]. They ingest and kill microbes to remove them from the body by producing and utilizing degradative substances. Phagocytes recognize pathogens via specific molecules which are only present in or on the pathogen but not in host cells.

These molecules are called pathogen-associated molecule patterns (PAMPs) which are recognized by pattern recognition receptors (PRRs) found on phagocytes. These receptors are the primary mode of action in innate immune responses [11] for recognizing repeating patterns of carbohydrates or lipid motifs specific for microbial surfaces [10]. Activation of these receptors after encountering pathogens causes the cells of the innate immune system to start secreting molecules which are involved in recruiting and activating leukocytes and are partly responsible for the activation of antigen-presenting cells (APCs) [12, 13]. A major player of these PRRs is to the family of toll-like receptors (TLRs). They are responsible for transmitting such signals which activate the host defense and additionally initiate the adaptive immune system [11]. Cells in injured or inflamed tissue eventually undergo necrosis leading to the release of cellular content. That cellular content and molecules from pathogen can active the innate immune system via these TLRs. Activation of TLRs increases pro-inflammatory cytokine and chemokine production, leading to the release of Type I interferon (IFN), interleukin-6 (IL-6), IL-8, tumor necrosis factor-alpha (TNF- α), chemokine monocyte chemoattractant protein-1 (MCP-1). These cytokines and chemokines trigger the activation and recruitment of phagocytes, inducing the infiltration of neutrophils and monocytes, as well as neighboring tissue like macrophages, leading to a clearance of necrotic cell debris, eradicating the invading pathogen and thereby facilitating tissue regeneration [14].

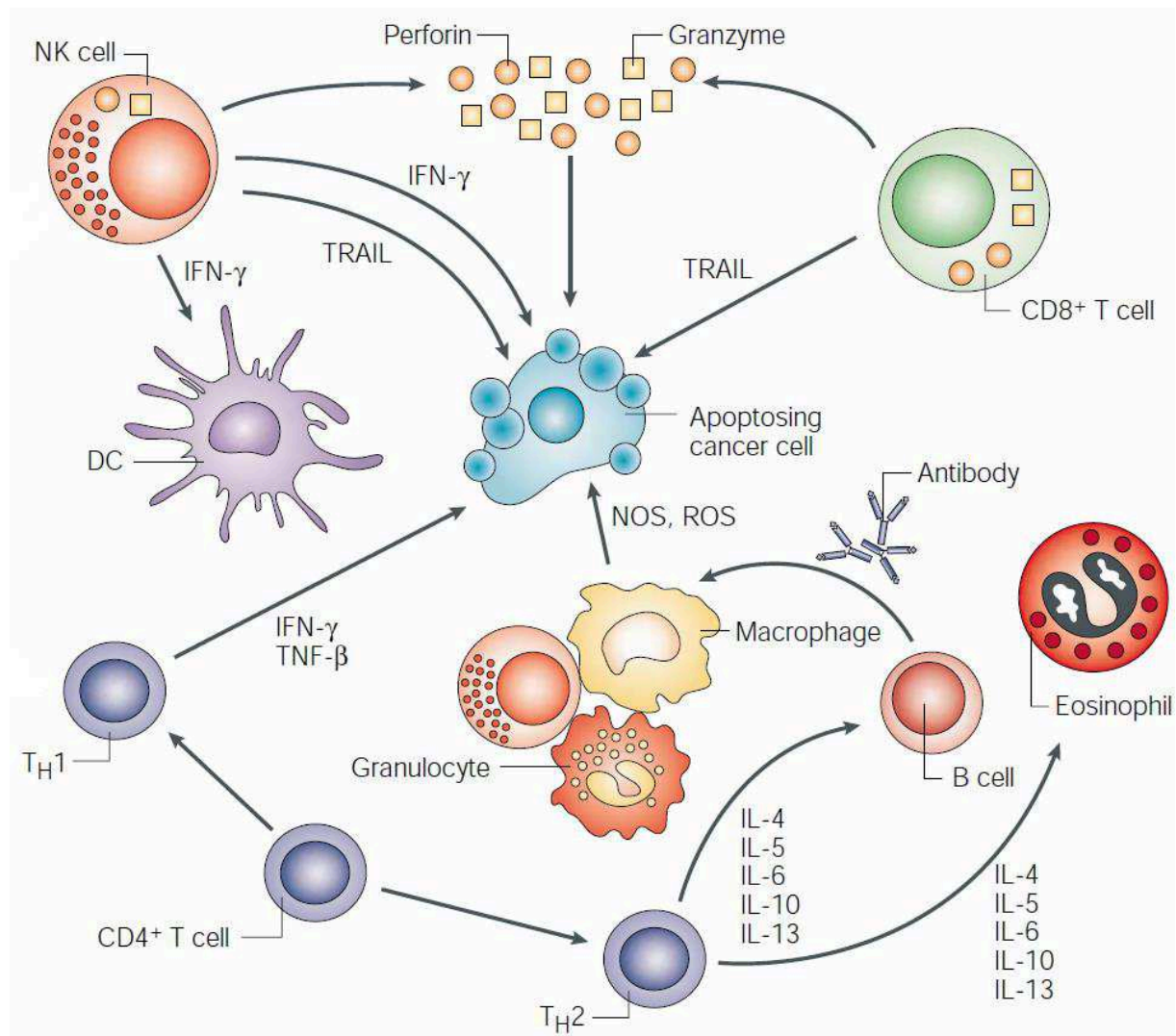


Figure 3: Network of immune cells in immune response. Cells from the innate immune system as well from the adaptive immunity harbor a complex network to fight infected cells (Adapted from Dranoff, 2004 [9]).

The adaptive immune system is an antigen-specific immune response which evolved in early vertebrates and was essentially built on top of the innate immune system [15]. It acts as a second line of defense and comes into play only if pathogens are not sufficiently cleared by the innate immune response. During an ongoing infection, cells from the adaptive immune system start producing antibodies specifically targeted at a pathogens antigen. It can take days to respond after the encounter of a new pathogen. Nevertheless, memory cells make the adaptive immune system response faster after a second re-encounter of the same pathogen [16]. Cells of the adaptive immune system are lymphocytes, namely B-cells and T-cells. It can be divided into two major branches: cell-mediated immunity and humoral immunity (see. Figure 3).

The cell-mediated immune response involves the activation of macrophages, natural killer cells, the production of antigen-specific cytotoxic T-cells and the release of numerous cytokines. Naive T-cells circulating lymphatic tissue and the blood are activated by binding to a specific antigen presented by antigen presenting cells via the TLRs. There are different subsets of T-cells depending on their role in adaptive immune response. The T-helper cells (CD4⁺) are the central mediators and regulators of initiation and activation of the immune response. Upon activation by a specific antigen, CD4⁺ T-cells start secreting cytokines aiding phagocytosis and antibody production by B-cells or in activating other T-cells. Activated (CD8⁺) cytotoxic T-cells recognize cells presenting pathogen antigens and induce apoptosis. Memory T-cells maintain quick and effective response upon re-encounter of pathogens [8, 10, 17].

The humoral immune response is mediated by B-cells and relies on the production of antibodies specifically targeted at a pathogen antigen. Antibodies are proteins, which are generated by somatic rearrangement in B-cells and function as opsonins and markers for targeted destruction by phagocytes or clustering of pathogens. B-cells are co-activated by T-helper cells. Upon encounter of the pathogen, they retreat into lymphoid tissue where they rapidly divide and transform into plasma cells or memory B-cells. Plasma cells produce high amounts of antibodies, which enter circulation to mark pathogens. Pathogens covered with antibody are rapidly eliminated by phagocytes and can activate the T-cell response or the complement system [18, 19].

The immune system is an effective mechanism to protect the host organism from intruding pathogens and maintain its integrity. Innate immunity acts as a first line of defense generally clearing pathogens by phagocytosis and effector molecules from the complement system. On the other hand, adaptive immunity is targeted at a specific antigen present on a pathogen, which are recognized and marked by antibodies produced by B-cells. Cytotoxic T-cells recognize infected cells in which apoptosis is induced [8].

1.2 Atherosclerosis

1.2.1 Cardiovascular diseases

Cardiovascular diseases (CVDs) are the main cause of death in the industrialized western world [20]. According to the World Health Organization (WHO), more people die annually from CVDs than from any other disease. CVDs still show rising incidence, also in developing countries [21]. Future projection indicated that CVD will hold the position as the single leading cause of death [22]. CVDs is a term for a collection of various diseases including diseases of the vascular system responsible for blood supply to the heart, brain and other organs, damages to the heart muscle and the heart valves, malformations of the heart structure and diseases such as deep vein thrombosis [23]. CVD risk factors are physical inactivity, an unhealthy diet and the abuse of alcohol and tobacco. These factors, together with obesity and overweight, may lead to raised blood pressure, blood glucose and blood lipids. They are responsible for a higher risk to develop heart attack, stroke, heart failure and other complications such as deep vein thrombosis or pulmonary embolism [24].

Atherosclerosis is the underlying pathophysiological cause for CVDs [25]. It causes myocardial infarction, angina pectoris, sudden cardiac death and stroke [26]. Atherosclerosis is an inflammatory disease of the arterial vessel wall and evolves mainly at sites of disturbed blood flow as seen at bifurcations [27]. Fatty depositions in the vessel wall cause a diminished artery lumen, eventually leading to blockage of blood flow and thrombus formation [28]. Blocked blood flow can lead to stroke and myocardial infarction [29] (see: Figure 5).

The tremendous impact on society and the economic burden make it necessary to further investigate the underlying processes of atherosclerosis leading to CVDs. Identifying mechanisms in disease formation and progression can give new strategies at hand to develop new therapeutic treatments and boost drug development to overcome the burden of CVDs.

1.2.2 Pathogenesis of atherosclerosis

Atherosclerotic lesions form in vessel walls of big arteries, preferentially at sites with a turbulent blood flow. Large arteries are anatomically and morphologically constructed in three layers (tunica): The tunica intima, the tunica media and the tunica adventitia (see: Figure 4). The tunica intima is the innermost layer facing the lumen separated from blood flow by a monolayer of endothelial cells (EC). It consists of a matrix of

extracellular connective tissue, mainly collagens and proteoglycans. An embedded sheet of elastic fibers forms the internal elastic lamina (IEL). The tunica media is the middle layer comprised of smooth muscle cells (SMC). Bordering the surrounding tissue is the tunica adventitia, which consists of connective tissues with interspersed SMCs and fibroblasts. Within the tunica adventitia the external elastic lamina (EEL) is situated [30].

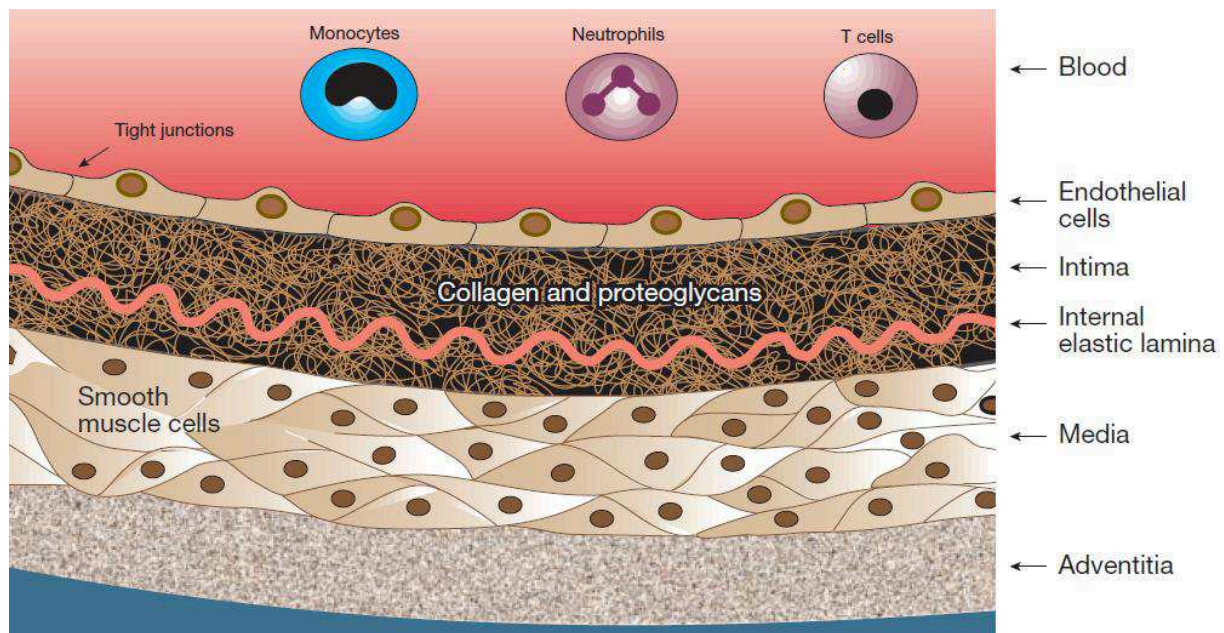


Figure 4: Anatomy of a large artery. Large arteries are built up of three layers: tunica intima, tunica media and tunica adventitia. The inner layer, the tunica intima borders the blood flow with a monolayer of ECs and the internal elastic lamina within it. The tunica media is the middle layer composed of SMCs. The tunica adventitia is the outer layer and consists of connective tissues with interspersed SMCs and fibroblasts (Adapted from Lusis, 2000 [30]).

The initial step in atherosclerosis is thought to be a damage of the endothelium causing a dysfunction and activation of ECs [31]. Cardiovascular risk factors promoting endothelial dysfunction typically are hyperlipidemia, hypertension, smoking and oxidative stress [32]. In addition, a turbulent or disturbed blood flow in regions of arterial branching or curvature facilitates dysfunction and increases permeability to macromolecules such as low-density lipoprotein (LDL) [33]. In these regions ECs have polygonal shapes with no particular orientation which makes it a preferential site for atherosclerotic lesion formation [34, 35].

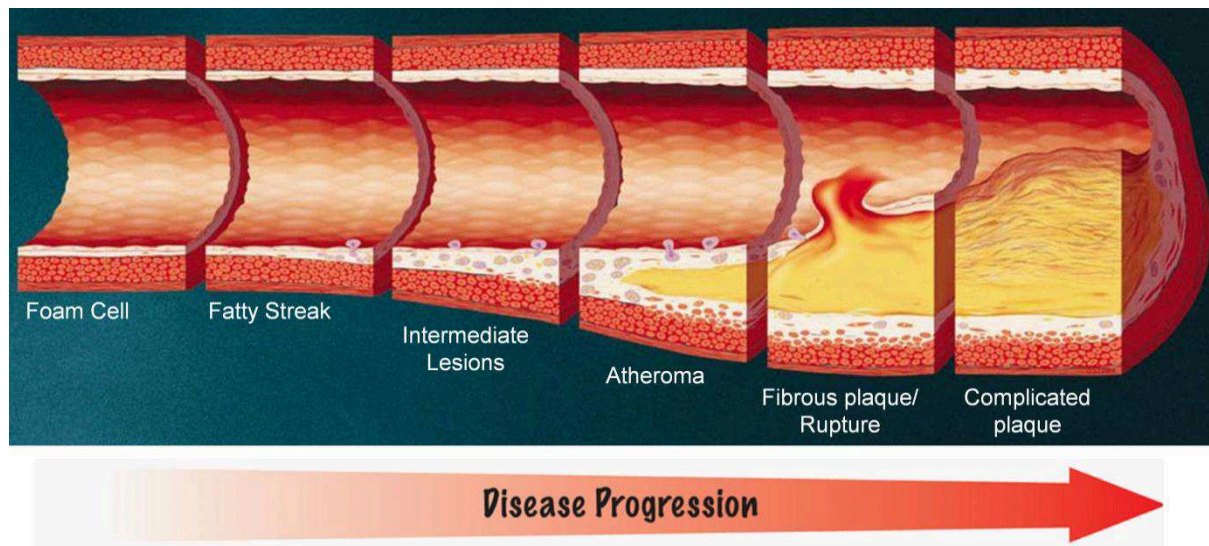


Figure 5: Progression of atherosclerosis. Depicted are the different stages of atherosclerotic disease progression. Macrophage influx accompanied with oxLDL uptake is leading to the formation of foam cells and the fatty streak. Ongoing inflammatory processes and cell necrosis eventually lead to the formation of a plaque with a fibrous cap. Late stage atherosclerotic plaques are prone to rupture, leading to thrombus formation and vessel occlusion (Adapted from Pepine, 1998 [36]).

A dysfunctional and activated endothelium facilitates the influx of LDL and the transmigration of inflammatory cells [37]. LDL accumulates subendothelial where it undergoes various modifications and oxidation by reactive oxygen species released by inflammatory cells. Oxidized LDL (oxLDL), in turn, further activates the endothelium and enhances expression of adhesive molecules and the secretion of chemokines. In addition, oxLDL can also inhibit the production of nitric oxide (NO), a chemical mediator with numerous anti-atherogenic properties. Nitric oxide acts as a vasorelaxant and thereby its inhibition leads to raised blood pressure, which can contribute to enhanced atherosclerosis [38]. Activated ECs start expressing adhesive molecules like vascular cell adhesion molecule-1 (VCAM-1), E-selectin and P-selectin. The secretion of the chemokine monocyte chemoattractant protein-1 (MCP-1) is promoting the attraction of immune cells like blood monocytes, neutrophils or T-cells. Monocytes are the first cells to transmigrate into the subendothelial space. These cells start rolling and attaching themselves to the endothelium and eventually transmigrate through the endothelial layer into the intima [32]. Once migrated, monocytes start to proliferate and subsequently differentiate into macrophages [39]. Macrophages are seen as the crucial players in atherosclerotic lesion formation and atherogenesis [40]. Taken together, the initiation of atherosclerosis can be seen as a response to injury, which is characterized by lipoproteins or other risk factors as the injurious agents and the influx of immune cells [41, 42].

There are two leading causes of atherosclerotic lesion initiation. First, atherosclerosis can arise over a long time driven by a cholesterol overload and second, as an acute response to injury of the endothelium. The underlying pathophysiological mechanisms differ in acute injury-induced and chronic diet-induced atherosclerosis [43, 44].

The chronic atherosclerosis is characterized by an influx and accumulation of lipids in the tunica intima and a recruitment of inflammatory cells [31, 45]. The early atherosclerotic stages are characterized by a thickening of the tunica intima, migration of SMCs, an accumulation of intracellular lipids and foam cell formation [31, 45]. Advanced atherosclerotic lesions are characterized by enhanced inflammation, extensive intra and extra cellular lipid depositions and the formation of a necrotic core covered by a fibrous cap [43, 44].

Acute atherosclerosis is an acute inflammatory process occurring after surgical vascular intervention, like stent implantation. These inflammatory processes can lead to neointima formation causing re-narrowing of the vessel lumen, which is called restenosis [46]. Neointima formation is characterized by a migration and proliferation of SMCs from the tunica media into the tunica intima [47]. Removal of the endothelium results in a burst of SMC migration and proliferation, which subsides when the endothelium regenerates [34]. This restenosis is to restore vessel wall integrity and function [47, 48]. Here, SMCs make up the majority of cells in neointimal lesion formation.

The initiation and progression of atherosclerosis is primarily driven by macrophages which are seen as the crucial players in atherosclerotic lesion formation and atherogenesis [40]. Macrophages have a set of specific scavenger receptors to take up oxLDL, namely the SR-A and CD36 [39, 49]. It has been demonstrated that LDL needs to undergo modification beforehand, as native LDL is barely taken up by macrophages [50]. Upon accumulation of oxLDL within the macrophages, they start secreting pro-inflammatory cytokines, further promoting local inflammation. Ongoing uptake of oxLDL ultimately causes macrophages to transform into lipid rich foam cells [42]. Early atherosclerotic lesion marked by an accumulation of foam cells and ongoing inflammation is called a fatty streak [51]. In the course of atherogenesis lipid-laden foam cells become apoptotic, releasing lipids in the subendothelial space. The accumulation of extracellular and intracellular lipids combined with the migrated immune cells form a necrotic core, a hallmark of atherosclerotic progression. Necrotic

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core content even enhances and nurses ongoing local inflammation. Alongside, vascular SMCs migrate from the adventitia and media to the intima where they proliferate and start building up a collagen rich fibrous cap covering the atherosclerotic lesion, which is thus called the atherosclerotic plaque. A thick fibrous cap can stabilize the plaque but on the other hand protrudes into the lumen of the artery narrowing the effective diameter and diminishing blood flow [52]. Progression of atherosclerotic lesions can lead to a more complex plaque with a high number of inflammatory cells and intimal calcification [53]. Proteases secreted by inflammatory cells thin the fibrous cap resulting in a more unstable plaque. An unstable plaque is prone to rupture exposing thrombotic elements to the blood stream. Thrombus formation can occlude the vessels and depending on the anatomic location lead for example to myocardial infarction or stroke [45, 54] (see: Figure 6).

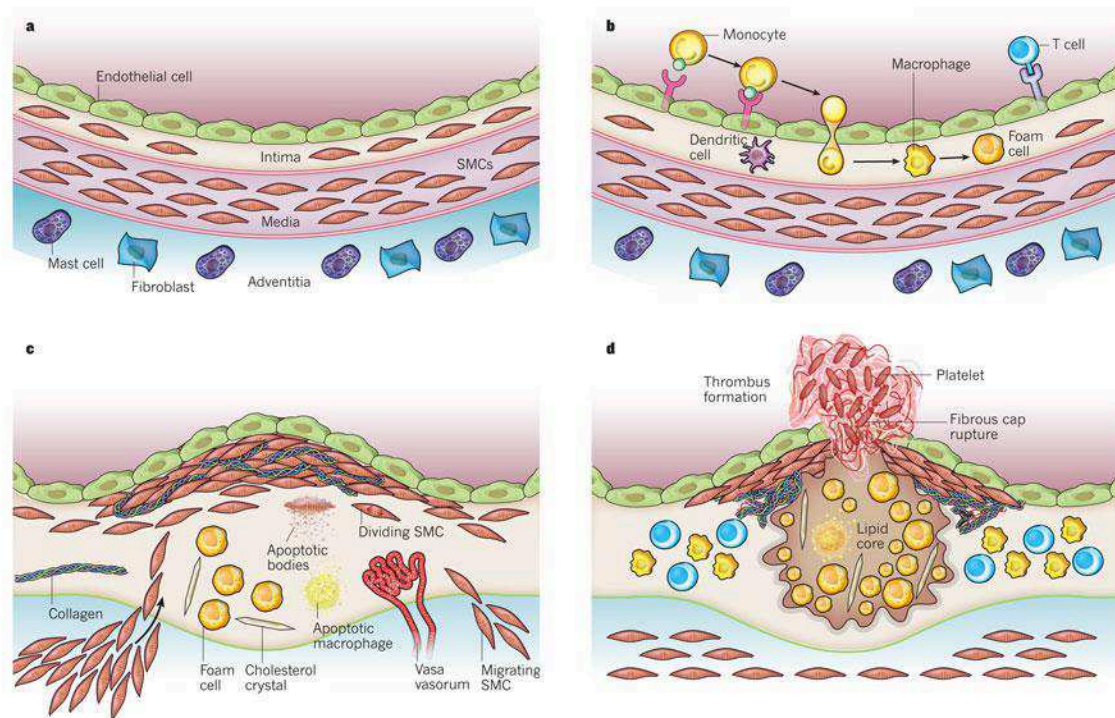


Figure 6: Atherosclerotic stages in lesion progression. (A) A healthy artery is composed of three layers. The inner layer is the tunica intima with the lamina elastic interna and a monolayer of ECs bordering the blood flow. The tunica media is the middle layer made of SMCs. The outer layer bordering surrounding tissue is the tunica adventitia. It is made up of connective tissue with interspersed SMCs and fibroblasts. (B) Damage or pro-inflammatory stimuli cause activation of endothelium. Activation and dysfunction leads to the expression of adhesive molecule on ECs and the influx of LDL. Monocytes transmigrate into the tunica intima and differentiate into macrophages and take up modified LDL. Macrophages start secreting various pro-inflammatory cytokines and eventually transform into foam cells. (C) Foam cells undergo apoptosis causing enhanced inflammatory stimuli. A necrotic core is built with apoptotic debris and lipids. SMCs migrate from the tunica adventitia to the tunica intima and form a fibrous cap covering the atherosclerotic plaque. (D) Ongoing inflammation weakens the fibrous cap from proteases released by macrophages and foam cells undergoing apoptosis. This can lead to plaque rupture causing blood clotting and thrombus formation. Blood clots can occlude vessels and stop blood flow causing stroke or myocardial infarction (Adapted from Libby, 2011 [55]).

1.2.3 Cells in atherosclerosis

As described above different immune cells play a crucial role in atherosclerotic lesion initiation and progression. A central player in these processes is occupied by macrophages. In early atherosclerotic lesions, activated endothelium attracts circulating monocytes, which in turn differentiate into macrophages. This differentiation is driven by the macrophage colony-stimulation factor (M-CSF) [56]. Interferon type 1 (IFN-1) has been shown to cause enhanced endothelial adhesion of macrophages. And specifically IFN- β has been shown to facilitate lesion formation by recruiting more monocytes and lymphocytes to the lesion [57]. Lesional macrophages take up modified LDL via the scavenger receptor SR-A and CD36 [58] or by pinocytosis and phagocytosis [59, 60]. An excess of modified LDL within macrophages can lead to foam cell formation, which subsequently leads to apoptosis, and the formation of a necrotic core. It has also been demonstrated, that macrophages can be protected from foam cell formation through a mechanism called cholesterol efflux via the receptors ATP-binding cassette transporter A1 (ABCA1) and ATP-binding cassette transporter G1 (ABCG1). Sometimes an early lesion regression can be observed which is thought to be due to these cholesterol efflux receptors [61]. Macrophages are reported to have versatile functions in immune responses and disease progression, which in part are contradictory. Therefore, a theory of macrophage polarization was formed (see: Figure 7). On the one hand, there are the classical activated macrophages (M1) and on the other hand, the alternatively activated macrophages (M2) [62]. M1 macrophages possess a more pro-inflammatory phenotype, releasing cytokines like TNF- α , IL-1 β and IL-6. However, M2 are involved in tissue remodeling, repair and in angiogenesis [63]. In atherosclerosis, M1 macrophages are found in the plaque shoulders, sites known to be prone for plaque rupture [64]. By releasing TNF- α , IL-1 β and IL-6 macrophages can further activate ECs leading to aggravated atherosclerotic progression [65]. In contrast, M2 macrophages may inhibit EC activation due to their production of anti-inflammatory cytokines, such as IL-4, IL-13 and IL-10 [65]. M2 are found more frequently in stable plaque areas [66]. It has also been shown that M2 can facilitate atheroprotective regulatory T-cell differentiation [67]. It can be observed, that during classical M1 activation, there is upregulation of the inducible nitric oxide synthase (iNOS), whereas arginase-1 is upregulated during the alternative M2 activation. These enzymes compete for the substrate arginine, suggesting an important regulation of nitric oxide production, interfering with the killing power of

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classically activated and atheroprotective properties of alternatively activated macrophages [68].

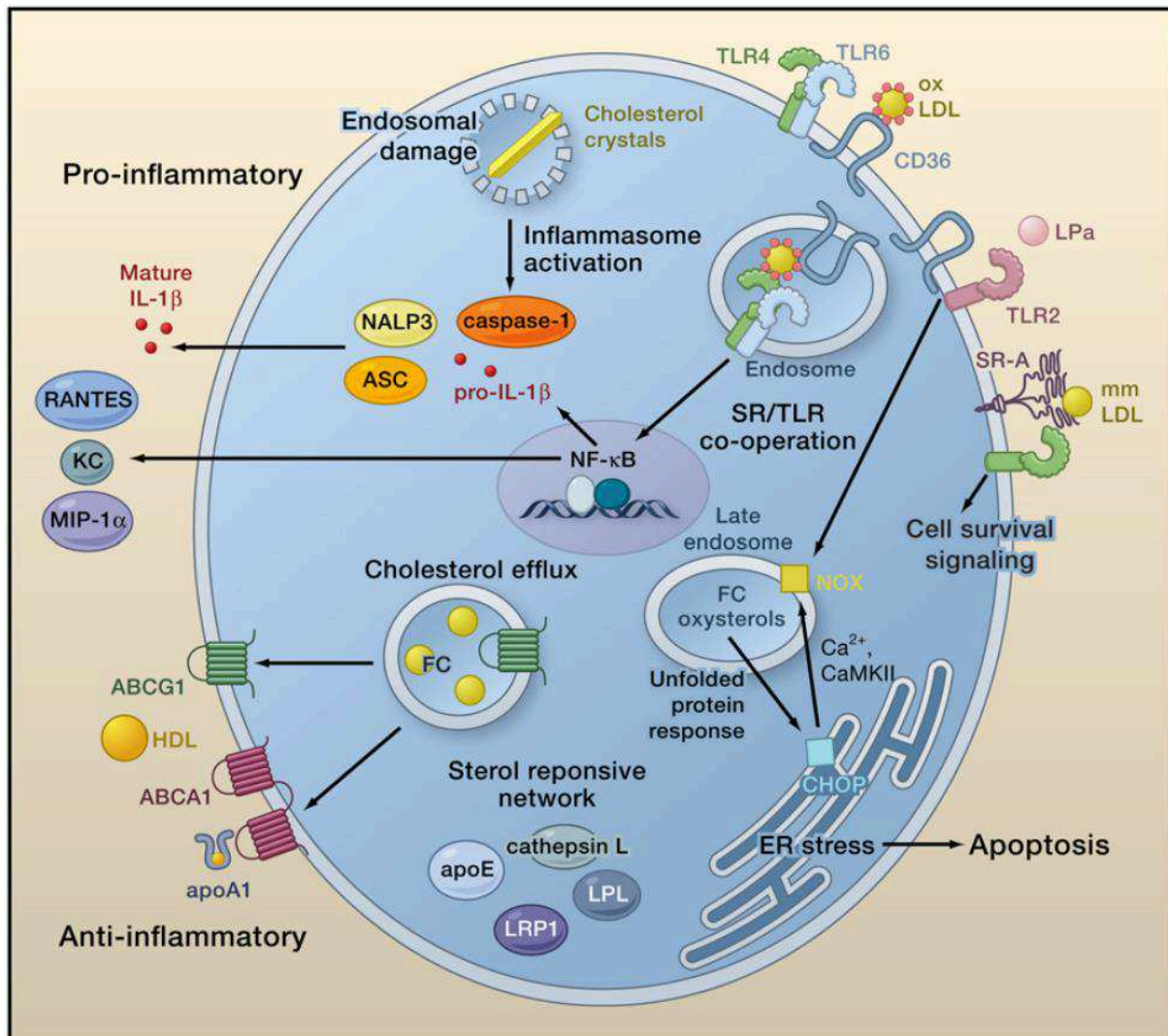


Figure 7: Macrophage phenotypes. Macrophages can acquire different phenotypes. They can show pro-inflammatory properties by the release of pro-inflammatory cytokines like IL-1 β , IL-8 (KC) or MIP-1 α . This can be initiated by receptor activation. TLRs or the scavenger receptor CD36 for oxidized LDL harbor a crucial role in the pro-inflammatory immune response. Excess of modified LDL can cause macrophage apoptosis, which facilitates atherosclerotic lesion progression. By cholesterol efflux macrophages can prevent themselves from foam cell formation and thus have anti-atherogenic properties (Adapted from Moore, 2011 [69]).

Besides macrophages, neutrophils play a role in the initiation and progression of atherosclerosis [40]. In hyperlipidemia the number of circulating neutrophils is increased, which in turn infiltrate the lesion in elevated counts [70]. It was shown that, neutrophils can attract monocytes to the lesion [71]. An important feature of neutrophils in immune responses is their ability to produce neutrophil extracellular traps (NETs) which can promote disease progression and thrombus stabilization [72, 73].

In low numbers T-cells are present in atherosclerotic lesions. IFN- γ released by Th1 helper cells can inhibit SMC proliferation and collagen production leading to an unstable plaque which is more prone to rupture [74]. The Th17 T-cell subset releases the pro-inflammatory cytokine IL-17 that has pro-atherogenic properties [75]. T regulatory (T_{reg}) cells have been shown to have atheroprotective properties by controlling and modulating Th1 and Th2 activity [76]. T_{reg} express TRAIL, which is responsible for the removal of plaque-resident cells which were shown to enhance plaque vulnerability [77].

Monocytes also can differentiate into dendritic cells (DCs). It has been demonstrated, that DCs play a crucial role in atherogenesis and can even be detected in the intima before lesion development [78]. Continued accumulation in lesion prone regions correlates with atherosclerotic progression and inflammation [79]. DC producing IFN- γ have been shown to be atherogenic by recruiting and activating macrophages to the site of lesions [80, 81].

Different studies have indicated that B-cells also play a role in all stages of atherosclerosis. Depending on the different sub-class, B-cells can show atheroprotective or atherogenic properties. A subset of B regulatory cells (B_{reg}) cells is still under investigation whether they are atheroprotective or not [82, 83].

1.3 Complement system

The complement system is a part of the innate immune system and additionally has a major impact on antibody-mediated adaptive immunity. This builds an important connecting link between the innate and the adaptive immune system [84]. It is evolutionarily one of the oldest parts of the immune system and acts as a first line of defense, recognizing exogenous and endogenous threats and initializing the clearance of cell debris [85, 86]. It consists of at least 30 proteins that are mostly synthesized in the liver. These proteins circulate the blood system as inactive precursors and their enzymatic activity is regulated by a wide range of inhibitors [87]. Upon activation an enzymatic cascade is triggered, which ultimately leads to the formation of a membrane attack complex (MAC) and increased local inflammation causing elimination of pathogens and immune complexes [88]. Four independent activation pathways are known so far, which in the end all converge at the point of the cleavage of the effector molecule C3 into C3a and C3b [87, 89] (see: Figure 8). The complement system activation leads to the opsonization of pathogens, enhanced chemotaxis of

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inflammatory cells, the lysis of cells by rupturing the cell membrane and the agglutination of pathogens.

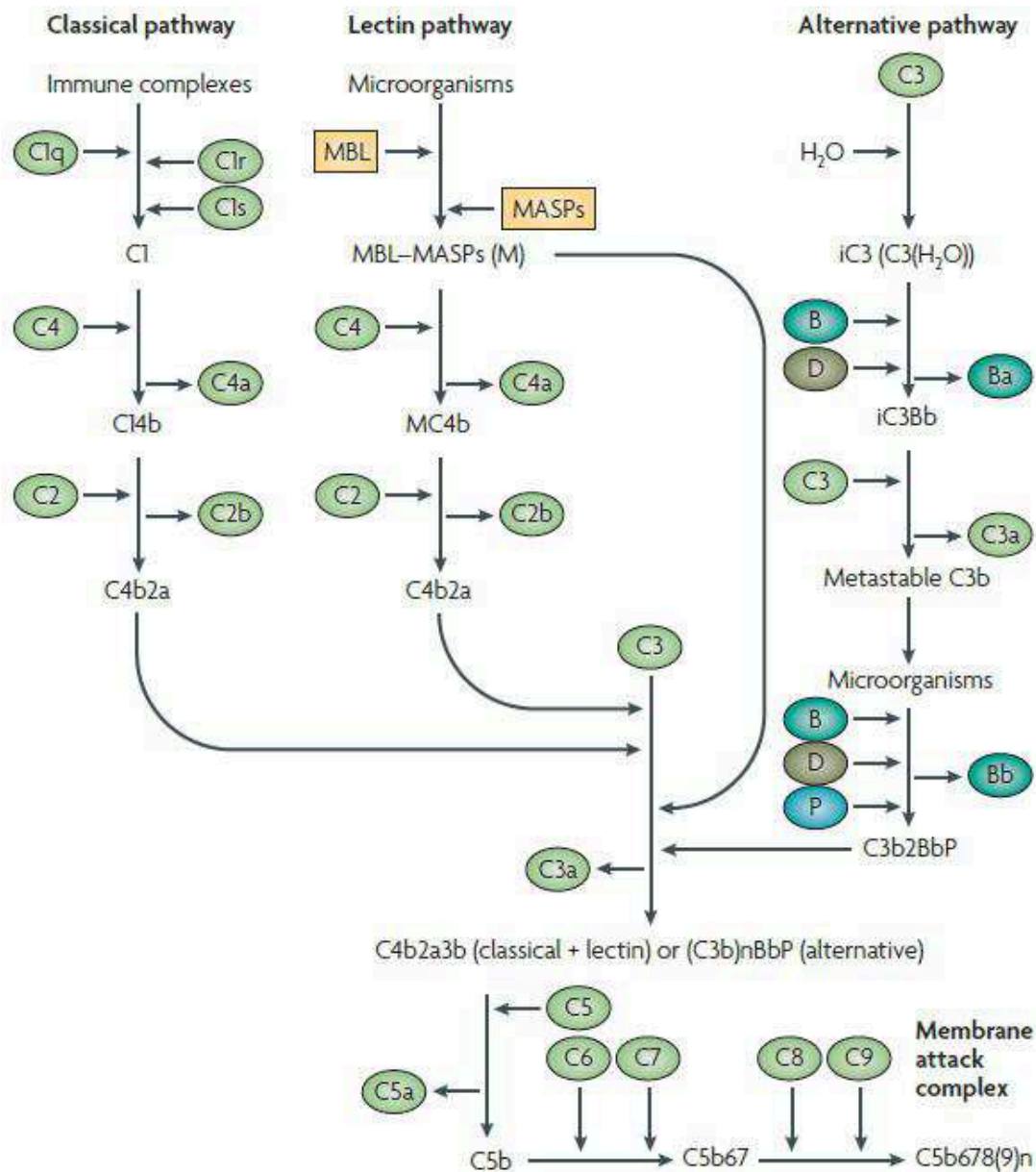


Figure 8: Complement system activation pathways. There are four different pathways of complement activation. Immune complexes trigger the classical pathway. Lectin pathway recognizes patterns of microbial surfaces by mannose binding lectins and ficolin binding receptors. Both these pathways end in the C4b2a3b C3-convertase. In the alternative pathway, spontaneous hydrolysis of C3 occurs, leading to the C3b2BbP C3-convertase. C3-convertases cleave C3 to C3a and C3b. C3b is an opsonin, which triggers phagocytosis and activates immune cells. The coagulation pathway activates the extrinsic pathways. All four pathways follow the same common terminal complement pathway. It finally ends with the formation of the membrane attack complex (MAC) and the release of pro-inflammatory mediators C3a and C5a (Adapted from Wagner, 2010 [90]).

The four pathways are activated differently but converge at the stage of the activation of a C3-convertase. Hereafter, they share a common terminal complement sequence leading to the cleavage products C3a, C5a and C5b-9 [91]. The four pathways are:

The classical pathway: The pathway is triggered by the binding of the complement component C1q directly to the pathogens' surface or by antibodies already bound to pathogens. Bound C1q then sequesters the components C1r and C1s to form a C4 convertase. C4 is cleaved into C4a and C4b. The first acts as a soluble mediator and the latter binds to the pathogens surface. C4b bound to pathogens in turn can bind C2, which then is cleaved by C1q to C2a, and C2b. C2b is released and C2a forms with C4b the C3-convertase C4b2a of the classical pathway. This convertase cleaves C3 to C3a (a pro-inflammatory mediator), and to C3b (opsonizing protein).

The lectin pathway: The lectin pathway is activated by the recognition of PAMPs by mannose binding lectins and ficolin binding receptors. Bound receptors sequester the mannan-binding lectin serine protease 2 (MASP-2) enzyme. Hereafter, it follows the same pattern as the classical pathway. MASP-2 forms a C4 convertase. C4 is cleaved into C4a and C4b. The first acts as a soluble mediator and the latter binds to the pathogens surface. C4b bound to pathogens in turn can bind C2, which then is cleaved by C1q to C2a, and C2b. C2b is released and C2a forms with C4b the C3-convertase.

The alternative pathway: The alternative pathway is activated by a spontaneous hydrolysis of C3 to C3(H₂O). Factor D and factor B can bind to C3(H₂O) allowing the binding of Properdin (factor P). The resulting complex C3bBb is the C3-convertase of the alternative pathway. This convertase is unstable and only in the presence of pathogen surfaces it can lead to a sufficient cleavage of C3 for opsonization.

The extrinsic protease pathway acts as a linkage between the complement and the coagulation system. The coagulation factor FXa can activate the complement system represented by the generation of C3a, C5a and the terminal complement complex. Generally, proteases from coagulation/fibrinolysis may act as natural C3 and C5-convertases, generating biologically active anaphylatoxins, linking both cascades [92].

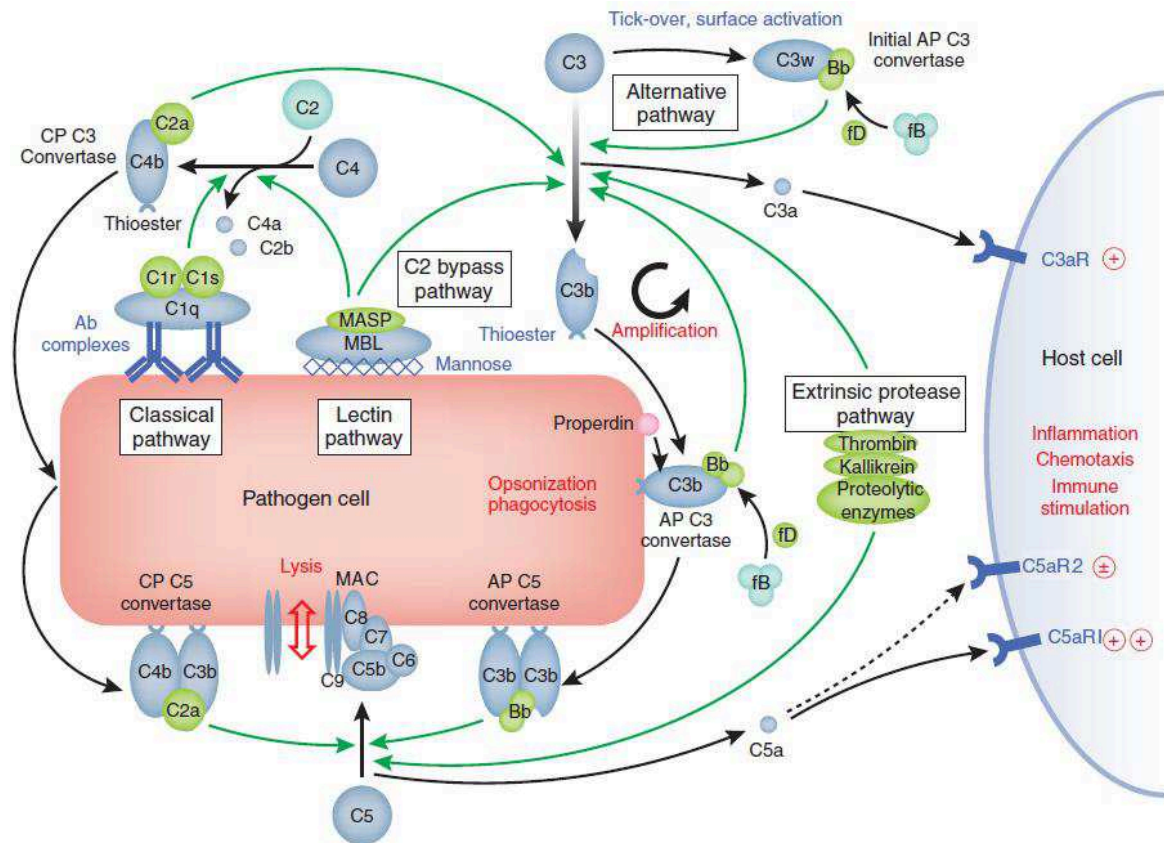


Figure 9: The complement system after activation by pathogens. All four pathways combined play a role in the elimination of pathogens. Depicted are the different ways of complement activation as well as their effector proteins and how they interplay in the clearance of foreign bodies (Adapted from Ricklin, 2007 [93]).

The cleavage of C3 to C3a and C3b is the point at which all pathways convert and follow a common terminal complement pathway. C3b covers the pathogens' surface triggering the engulfment of pathogens by phagocytes like macrophages. Additionally, C3b itself can bind to the C3-convertases C4b2a and C3bBb to form the C5-convertase C4b2a3b or C3bBb3b, respectively. The resulting anaphylatoxin C5a is released as a soluble mediator promoting local inflammation and recruitment of phagocytes to the site of infection. On the other hand, the C5b fragment initiates the formation of a membrane attack complex C5b-9. The MAC integrates in the pathogens' surface forming a pore ultimately leading to the lysis of the cell (for an overview see: Figure 9).

1.3.1 Complement component C5a

Complement component C5a is a 74 amino acid long polypeptide [94] resulting from the cleavage of C5 by the C5-convertases [84], proteolytic enzymes like kallikrein [95] or thrombin [87]. It belongs to the group of anaphylatoxins, together with C3a and C4a, acting as a strong pro-inflammatory mediator triggering histamine release from mast cells in acute allergic reactions [96]. C5a is usually present at sites of inflammation where it is increasing vascular permeability and induces the contraction of SMCs [94, 97]. It attracts circulating leukocytes and activates them to release reactive oxygen species [96]. It possesses strong chemoattractant properties towards macrophages and also constitutes the sole complement component showing chemotactic properties on leukocytes [98, 99]. In part, biological activity of C5a is dependent on the carboxyl-terminal arginyl residues. The activity of C5a is restricted by the anaphylatoxin inactivator carboxypeptidase H, which rapidly removes the arginyl residue and degrades C5a to C5a des Arg [100]. C5a has a higher binding affinity to its receptors than C5a des Arg [101]. It has been shown that C5a is involved in the pathogenesis of different inflammatory diseases such as rheumatoid arthritis, asthma, systemic lupus erythematosus, sepsis and psoriasis [102]. C5a also interacts with the coagulation pathway by directly inducing the expression of tissue factor in macrophages and ECs or indirectly inducing the expression of IL-8 facilitating the thrombus formation [103, 104]. Additionally, it interferes with the clearance of immune complexes. It stimulates the expression of FcγRIII, which is mainly responsible for the clearance of the immune complexes and as well suppressing the expression of the inhibitory FcγRII [105]. C5a signals and interacts mainly through its two receptors C5aR1 and C5aR2 (see: Figure 10) [106, 107]. They belong to a rhodopsin-like family and are seven transmembrane receptors. Although sharing structural similarities they show prominent differences [108] (see: Figure 11).

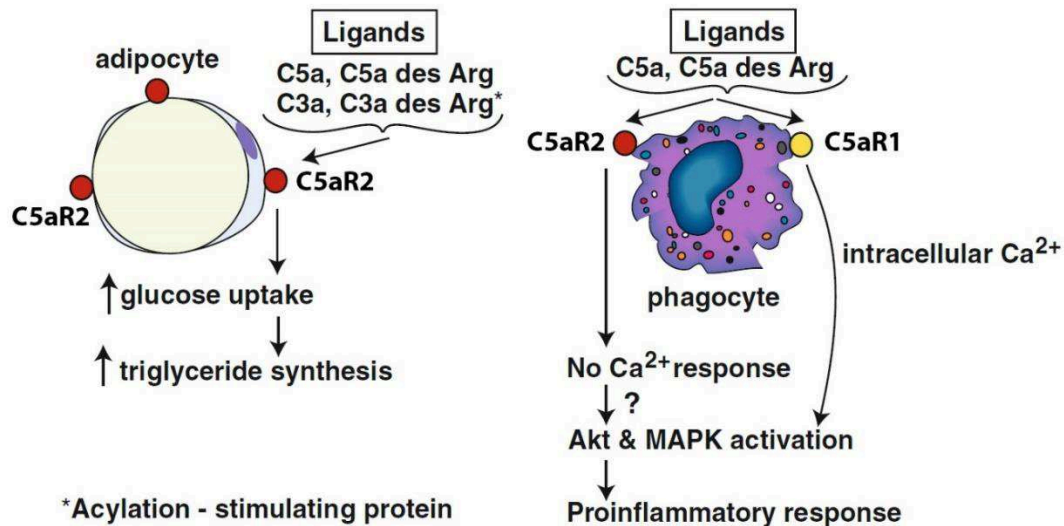


Figure 10: Functions of C5a receptors. The different functions of the two C5a receptors C5aR1 and C5aR2. The graph shows the clear pro-inflammatory response via intracellular Ca^{2+} increase and MAPK signaling after activation of C5aR1 and the hypotheses of pro-inflammatory response of C5aR2. Additionally, C5aR2 is involved in lipid metabolism by increasing glucose uptake and triglyceride synthesis (Adapted from Ward, 2009 [109]).

1.3.1.1 Complement receptor C5aR1

C5aR1 is a classical pro-inflammatory receptor, triggering pro-inflammatory cytokine release and migration of pro-inflammatory cells. C5aR1 is a G protein-coupled receptor (GPCR) for C5a [110] expressed on innate immune cells such as neutrophils, eosinophils, basophils, monocytes/macrophages, mast cells, dendritic cells or T cells and in a lesser amount on non-immune cells such as ECs, neuronal and in different tissues of the lung, kidney, liver, spleen, intestine, skin, and the heart [108, 111-113]. Binding of C5a with the C5aR1 activates MAPK signaling pathways resulting in a rapid increase of cytosolic Ca^{2+} ultimately mediating chemotaxis, respiratory bursts, release of toxic granules, and the regulation of cytokine expression [94, 114]. A two-site binding model for C5a-C5aR1 interaction is proposed with two distinct and independent sites for C5a binding [115]. C5a interacts with aspartic acid residues of the extracellular N-terminal domain sequence of C5aR1. This causes a conformational change in C5a allowing it to properly interact and activate C5aR1 [115, 116]. Upon binding of C5a the C5aR1 is phosphorylated and rapidly internalized [117] by clathrin-mediated endocytosis. They either get degraded in lysosomes or dephosphorylated and recycled to the cell membrane [118, 119]. Recycling of receptor is important for cellular migration in a C5a concentration gradient [120] but not for the clearance of C5a from the plasma [96]. Activation of C5aR1 leads to classic pro-inflammatory responses like cytokine production, chemotaxis and promoting phagocytic capacities [121].

1.3.1.2 Complement receptor C5aR2

C5a receptor-like C5a2 (C5aR2) is a second receptor for C5a [122] which is expressed on immune such as neutrophils and macrophages, as well other non-immune cells like adipocytes or skin fibroblast, however the C5aR2 is expressed at a much lower level compared to C5aR1 [123]. It is structurally highly similar to C5aR1 [108] with a high affinity to bind C5a, leading to strengthen the assumption that it is a second receptor for C5a [124]. Also highly similar to C5aR1 in structure, important differences can be detected in C5aR2 (see: Figure 11). C5aR2 has a similar pattern of tyrosine and acidic N-terminal residues to the C5aR1, which have been shown to be a major feature of extracellular binding of C5a. C5aR2 also shares similarities with the C5aR1 in the number of charged and hydrophobic residues in the loops and trans-membrane regions. Nonetheless, especially in conserved regions important for G-protein coupling major differences can be found. Important differences were identified in the DRY motif in the 3rd transmembrane domain, and the NPXXY motif in the 7th transmembrane domain, which have been demonstrated to be crucial for G protein signaling. Furthermore, C5aR2 was shown to harbor no G protein coupling as an arginine is replaced by a leucine in the DRY motif in the second intracellular domain [125].

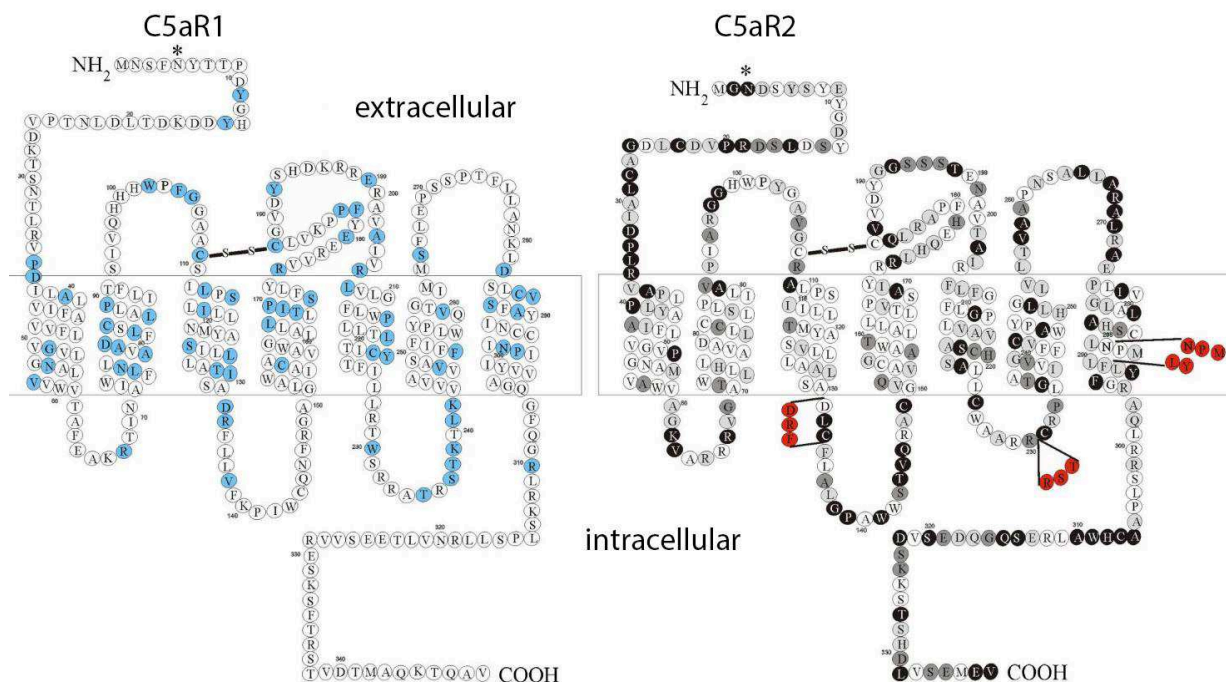


Figure 11: Sequences of the C5a receptors, C5aR1 and C5aR2, with potential glycosylation sites asterisked. Both receptors (42–45 kDa) also have characteristic arrays of Asp and Tyr residues at the N-termini; overall sequence homology is 35%. The degree of conservation for individual residues is shown by the depth of shading on the C5aR2. Residues identified in site-directed or random saturation mutagenesis studies as having an important role in ligand binding, and/or signaling by C5aR1 are shown in blue. Sequences critical for G protein coupling in C5aR1, which are changed in C5aR2, are shown in red (Adapted from Monk, 2007 [108]).

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Additionally, it binds C4a, C3 and C3desArg [108]. Although it belongs to the group of G protein-coupled receptor, none or only limited signaling via the MAPKs pathway can be observed [125]. Upon binding of C5a no rise in intracellular calcium concentration occurs [126]. However, cooperativity between the two receptors has been demonstrated. C5aR2 heterodimerizes with C5aR1 or C3aR and positively regulates signaling triggered by C5a or C3a [127]. These observations led to various controversial models of C5aR2 involvement in immune responses (see: Figure 12). On the one hand, an anti-inflammatory role of C5aR2 was postulated. As it is lacking G protein coupling it was stated that C5aR2 is merely acting as a decoy receptor sequestering C5a, thus preventing the pro-inflammatory interaction between C5a and C5aR1 [128]. According to the theory of decoy receptors, first postulated by Mantovani, the C5aR2 fulfills all the criteria to be classified as one [129]. C5aR2 showed repressed G protein-coupled signaling by various mechanisms and additionally lacked motifs of important G protein coupling motifs, namely the DRY motif in the 3rd transmembrane domain and the NPXXY motif in the 7th transmembrane domain. Also typical for G protein-coupled receptors is that they are expressed predominantly on the cell surface, whereas it was demonstrated that C5aR2 is primarily intracellularly expressed. Additionally, C5aR2 internalization is constitutive and independent from ligand binding. For many G protein-coupled receptors, however, ligand binding increases the rate of receptor internalization [130]. Furthermore, internalized C5aR2 is rapidly re-expressed, which is in line with the findings that decoy receptors recycle rapidly independent of ligand binding or ligand abundance [131]. Finally, ligands internalized by C5aR2 are retained and degraded within the cell and for C5aR2 this results in intracellular accumulation and degradation of C5a and C5a des Arg. Therefore, the C5aR2 was classified as a decoy receptor with anti-inflammatory properties modulating the immune response transmitted via the C5aR1.

Another model proposed that C5aR2 actually harbors signaling properties and acts as an intercellular receptor. This feature distinguishes C5aR2 from the sole decoy receptor theory. A decoy receptor solely modulates other receptors or sequesters ligands preventing them to signal through the main receptor. Here, C5aR2 actually is activated after C5a interaction. However, C5aR2 is only activated after ligand binding to C5aR1. Activated C5aR2 then interacts with β -arrestin. In addition, C5aR1 interacts with β -arrestin which activates ERK1/2 signaling, whereas interaction of β -arrestin and C5aR2 inhibits ERK1/2 signaling. Thus, C5aR2 regulates C5aR1 activity by inhibiting

or modulating β -arrestin pathway and signaling of C5aR1 via the MAPK pathway [132, 133]. Due to this, C5aR2 was classified as an anti-inflammatory receptor keeping C5aR1 activity in check. Supporting these theories, in many disease models an anti-inflammatory role of C5aR2 was found, furthermore in macrophages deficient for C5aR2 reduced ERK/AKT signaling can be found [127, 134].

On the other hand, it was shown that C5aR2 acts in a pro-inflammatory manner during acute sepsis and in anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV), leading to the idea that C5aR2 actually has signaling functions of its own [135, 136]. Activation leads to the enhanced expression of cytokines like IL-6 and TNF- α and the high mobility group box 1 (HMGB1) with the release of the latter only triggered by C5aR2 but not C5aR1 [137]. Activation of C5aR2 provokes HMGB1 release via MAPK (MEK1/2 and JNK1/2) and AKT pathways. Furthermore, it was shown that both lipopolysaccharide (LPS) and C5a triggers HMGB1 release. Here, LPS signals via TLR4 and C5a through C5aR2. LPS induced HMGB1 release was drastically attenuated in C5aR2 deficient mice, indicating the signaling depends on C5aR2 integrity and participation. In a model of acute sepsis both, C5aR1 and C5aR2, contribute synergistically to the harmful outcome. These data lead to the conclusion that C5aR2 has actual pro-inflammatory signaling functions which induces the release of the pro-inflammatory cytokine HMGB1 [137].

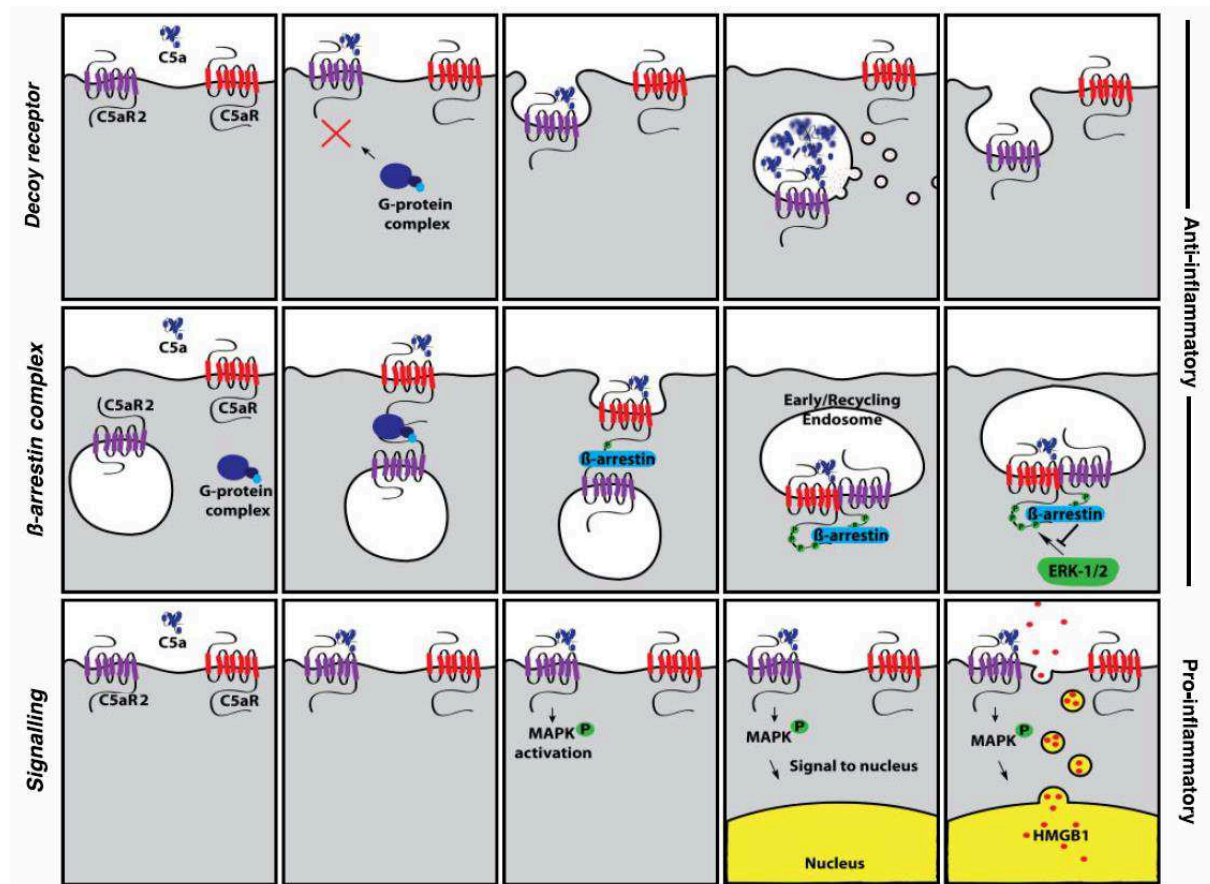


Figure 12: Different mechanisms of C5aR2 function. The role of the C5aR2 is still under debate. C5aR2 was shown to have anti-inflammatory properties. It may act as a decoy receptor sequestering C5a from signaling via C5aR1. Another hypothesis is that C5aR2 is interacting with β-arrestin thus inhibiting ERK1/2 activation by C5aR1. A pro-inflammatory response was verified as C5aR2 itself can trigger the release of the pro-inflammatory cytokine HMGB1 (Adapted from Li, 2013 [107]).

Furthermore, the acylation stimulating protein (ASP) was found as another ligand for C5aR2, later identified as C3a des Arg [138]. ASP only interacts with C5aR2, but neither with C3aR nor C5aR1. The C5aR2 has a distinct binding site for ASP from C5a. In adipocytes, ASP stimulates triglyceride synthesis in fat-storing cells [138, 139]. Here, ASP stimulates the fatty acid incorporation into adipose triglyceride (FIAT) and yet on the other hand increases glucose transport. Transport of glucose is enhanced through increased translocation of the glucose transporters Glut1, Glut3 and Glut 4 to the cell membrane.

A study by Van Harmelen *et al.* [140] demonstrated that ASP is increasing lipogenesis in adipocytes, but it also has the potential to inhibit hormone-sensitive lipase (HSL)-mediated lipolysis. Additionally, they showed that the effects of ASP on both FIAT and glucose transport are additive and independent from insulin [141-143], which is a well-known lipogenic hormone. Van Harmelen *et al.* further revealed, that in the presence of both insulin and ASP a reduction in net fatty acid output from the

adipocytes, with virtually complete re-esterification and storage of the available fatty acids [140]. Reduced adipose tissue triglyceride synthesis and increased muscle fatty acid oxidation in *C5ar2* knock-out (*C5ar2*^{-/-}) mice have been demonstrated [144]. In addition, APS mediates β -arrestin dependent C5aR2 internalization [145].

1.3.2 Complement in atherosclerosis

Over the past decade, the spotlight of research interest focused on the complement system in atherogenesis. In patients with late stage atherosclerosis C5a was abundantly found in serum blood samples and specimen of carotid arteries [146]. Further, high expression of complement components were detected in advanced human atherosclerotic plaques [147]. Complement components are typically retained from the plasma to lesion sites, however mRNA expression was also detected within the plaque indicating a local production [148]. Various activated complement factors and their receptors were detected in atherosclerotic lesions, but in contrast, in normal arteries no complement activation can be found [149-152]. C5a acts as a chemoattractant recruiting inflammatory cells and inducing expression of adhesive molecules on ECs [90]. In fatty streaks, an early stage in atherogenesis, complement factors can be found even before the arrival of inflammatory cells giving a hint to the importance of the complement system in the initiation of atherosclerosis [153]. After surgical intervention, coronary late lumen loss of drug eluting stents is associated with increased serum levels of the complement components C3a and C5a [154]. Furthermore, the level of circulating complement components increases after myocardial infarction [155]. In atherosclerotic lesions a range of different substances like modified LDL, immune complexes, cholesterol crystals or apoptotic cells can trigger the activation of complement. [149]. In atherosclerosis, complement is mainly activated by the classical and alternative pathway, so far only little is known about the lectin or the extrinsic pathway [156]. Immune complexes interacting with modified LDL have been shown to activate the classical and alternative pathway [156, 157]. A high abundance of MAC was detected in atherosclerotic plaques where the amount of MAC correlates to the extent of atherosclerosis [156, 158]. Inhibition of the alternative pathway factor B in LDL receptor deficient mice on a high fat diet (HFD) leads to a reduction in lesion size, indicating a pro-atherogenic role of the alternative pathway [159]. In general, the inhibition of the complement system reduces atherosclerotic lesion formation [160]. In contrary, the lectin pathway may be beneficial by clearing

debris in early lesions [161]. It has also been shown that the C5a plasma level predicts future cardiovascular events, and thus could be involved in plaque destabilization [155]. The complement system bridges innate and adaptive immune system by controlling the Fc γ -receptor and thereby triggering and modulating adaptive immune system response due to its involvement in the activation of dendritic cells, B- and T-cells [90, 156]. (see: Figure 13)

Various cytokines and pro-inflammatory mediators are described to contribute to atherogenesis and plaque stability. Furthermore, it has been reported, that macrophages can contribute to SMC death by the secretion of TNF- α [162]. It was also reported that plaque T-lymphocytes produce IFN- γ , which inhibits the proliferation of SMCs and their ability to synthesize collagen, weakening the fibrous cap and destabilizing the plaque [163, 164]. Thus, less TNF- α produced by macrophages would cause less apoptosis and reduced IFN- γ produced by T-lymphocytes would further contribute to the higher number of SMCs. Thus, a reduction in atherosclerosis in part is promoted by a reduced macrophage number accompanied by an increased number of SMCs [165]. Here, also the cytokine RANTES (CCL5) should be mentioned. It has been reported the deletion of CCR5, a receptor for RANTES, is reducing atherosclerosis [165] and *C5ar2* single knock-out show a general reduction of RANTES expression [166]. Cytotoxic T-cells release RANTES and in turn it was shown to activate T-lymphocytes and macrophages [167, 168]. HMGB1 is a pro-inflammatory mediator, which is solely released upon activation of C5aR2 [137]. It was demonstrated that HMGB1 has chemotactic and activating properties towards macrophages [169, 170].

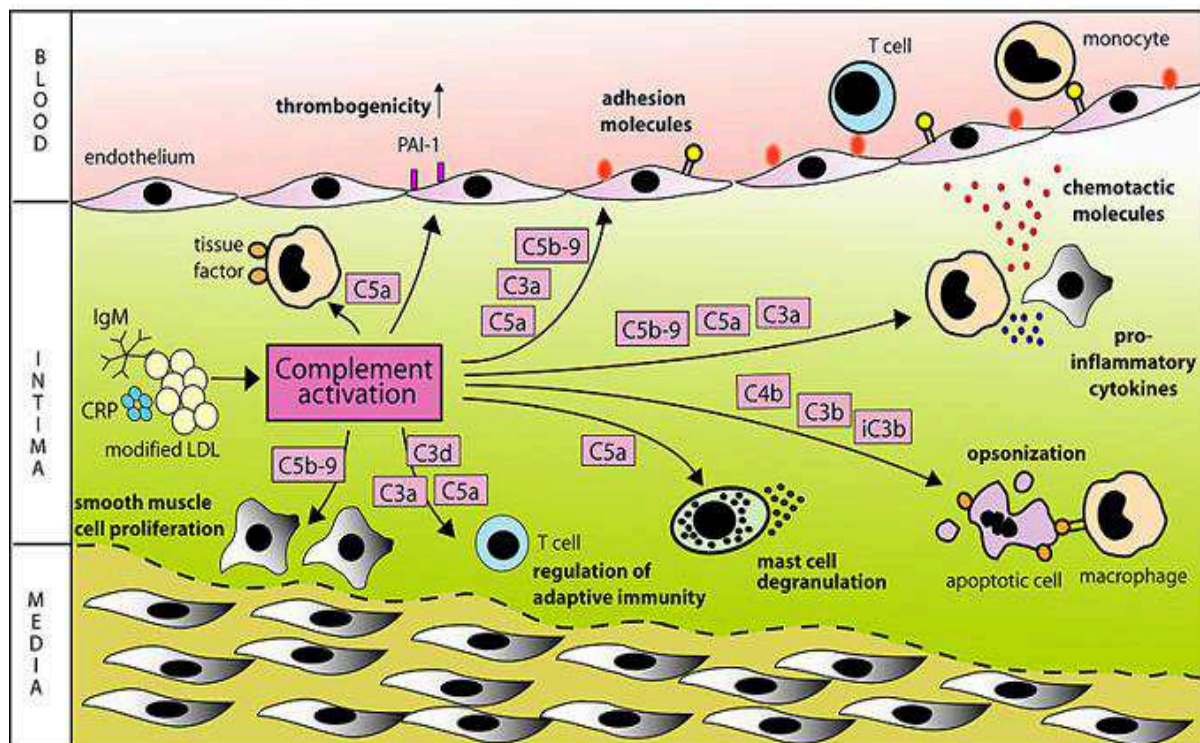


Figure 13: Biological effects of complement activation in atherosclerotic lesions. Complement activation in atherosclerotic lesions leads to the formation of the complement effector molecules: the anaphylatoxins C3a and C5a, the opsonins C3b and C4b and the membrane attack complex (C5b-9). They cause EC activation with expression of adhesion molecules and inflammatory cell migration into the atherosclerotic lesion. SMC proliferation is triggered as well as the degranulation of mast cells. The opsonins mark apoptotic cells to be cleared by macrophages. By triggering cytokine release, they support the pro-inflammatory milieu and additionally activate the adaptive immune response (Adapted from Oksjoki, 2007 [171]).

To study the molecular mechanism during atherogenesis the use of transgenic and gene-targeted mice revolutionized the understanding of the underlying processes [172]. The complex interplay studied in animals maintained under comparable environmental conditions shed light on numerous genetic loci that contribute to lipoprotein levels, body fat and other risk factors [173]. To study genetic and physiological conditions the most important and utilized mouse models are deficient in apolipoprotein E (APOE) or LDL receptor.

According to the American Heart Association, human atherosclerotic lesions are classified in stages 0-VI [174]. For better comparison, the plaque progression in these two mouse models also were classified stage 0-VI. According to their age and received diet, APOE and LDL deficient mice were assigned to one of these stages. Stages I-III are considered as early atherosclerotic lesions, which are mostly clinically asymptomatic [175]. These so-called fatty streaks occur in both, the *Apoe*^{-/-} and the *Ldlr*^{-/-} mouse models and morphologically resemble the early human atherosclerotic stages. They are characterized by a migration of SMCs, subendothelial lipid depositions and the

formation of foam cells, which results in a thickening of the lamina tunica. Further progression of the plaque leads to complex lesions of the stage IV-VI, which are characterized by a prominent necrotic core accompanied by extracellular lipid depositions and increased numbers of inflammatory cells. Pro-inflammatory cytokines secreted by these cells further aggravates atherosclerosis. Weakening of the fibrous cap by proteases leads to an unstable plaque phenotype. These unstable plaques are more prone to rupture, which can ultimately lead to thrombus formation and occlusion of vessels. Blocked blood circulation can cause myocardial infarction or strokes [45]. In contrast to the human atherosclerotic lesion in both mouse models a rupture of the plaque cannot be observed. Nevertheless, *Apoe*^{-/-} mice develop advanced atherosclerotic lesions of the stage IV and V in aortic roots and aortas after 14-20 weeks of HFD [176, 177]. In *Ldl*^{-/-} mice lesions of the stage IV can be observed, whereas stage V, which is characterized by the formation of the fibrous cap, cannot be found anymore [178]. Thus, in contrast to *Apoe*^{-/-} mice, LDLR deficient mice do not develop advanced atherosclerotic lesions.

Apoe^{-/-} mice show increased C5aR1 and reduced C5aR2 expression [179]. Furthermore, they develop a hyperlipidemia and atherosclerotic lesions. The increased C5aR1 expression is correlated with atherosclerotic lesion size [149, 180]. It was shown, that in mice receiving a normal chow diet the complement activation may be protective against atherosclerotic progression by clearing apoptotic cells and debris [181]. However, mice receiving a HFD show enhanced lesion formation [182, 183]. Blocking of C5aR1 after carotid denudation injury in *Apoe*^{-/-} with an antagonist or a blocking anti-C5aR1 monoclonal antibody reduces atherosclerotic lesions accompanied with a more stable plaque [151].

2 Aim

Atherosclerosis is a chronic inflammatory disease involving a wide range of mediators and cells from the innate and adaptive immune system including complement components. Short-term blockade of C5aR1 has a strong protective and anti-inflammatory effect and shows a reduced neointima formation after wire-induced arterial injury in mice. It is known, that C5aR2 is present in human atherosclerotic plaques and high expression of both C5a receptors, C5aR1 and C5aR2, correlates with high pro-inflammatory cytokines in atherosclerotic plaques, but up to date no clear data is present for the role of C5aR2 in atherosclerosis. We then hypothesized that C5aR2 might have a functional role in atherosclerosis. Therefore, the role of C5aR2 in atherosclerosis and vascular remodeling in two different animal models and isolated macrophages was investigated. For that reason, the aims of this thesis were:

1. To identify the contribution of C5aR2 in atherosclerosis and restenosis with regards to lesion size and plaque phenotype.

Therefore, *C5ar2^{-/-}/ApoE^{-/-}* mice were fed a HFD for 12 or 24 weeks. To study restenosis carotid arteries were injured applying the method of wire-induced endothelial denudation. After indicated time points atherosclerotic lesions were identified in whole aortas and in aortic roots or carotid arteries. Lesion size, cellular composition and lesion phenotype were examined by immunohistological and immunofluorescent staining and quantitative mRNA studies on cytokine expression.

2. To identify the underlying processes influenced by C5aR2 leading to the observed reduction in atherosclerosis and restenosis.

How is C5aR2 involved in cytokine production, the cellular composition of the plaque, the lipid metabolism and the phenotype of *in vitro* macrophages. The influence on these factors was studied by mRNA expression and blood serum analysis to identify in what way C5aR2 is affecting atherosclerotic plaque progression.

Aim

3. To investigate the cooperative impact of C5aR1 and C5aR2 on atherogenesis and their reciprocal regulation.

Therefore, the C5aR1 expression in *in vitro* macrophages and in different tissues *in vivo* was analyzed by immunohistological staining, quantification of mRNA expression, western-blot analyses and flow cytometry. To study the dual involvement of both receptors in atherosclerotic plaque progression a model was applied where C5aR1 was blocked in C5aR2 deficient mice.

3 Materials and Methods

3.1 Materials

The general chemicals were purchased from Sigma-Aldrich, Merck or Roth in analytical grade quality. All solutions were prepared with double distilled water (Heraeus Destamat, Heraeus, Germany), Millipore water (Milli-Q Plus ultrapure purification, Millipore, USA) or with Aqua ad injectabilia (Braun, Melsungen, Germany). The general equipment, consumables and kits are mentioned below.

3.1.1 General laboratory equipment

Table 1: List of laboratory equipment

Equipment	Name (Manufacturer)
Autoclave	Systec VX-150 (Systec, Wetzlar, Germany)
Centrifuge	Eppendorf 5417C (Eppendorf, Hamburg, Germany) Eppendorf 5425 (Eppendorf, Hamburg, Germany) Multifuge 3 S-R (Heraeus, Osterode, Germany)
Cryotome	Leica CM3050S (Leica, Wetzlar, Germany)
DNA Extractor	BioSprinter15 (Qiagen, Hilden, Germany)
ELISA plate reader	Infinite M200 (Tecan, Crailsheim, Germany)
Flow cytometer	FACSCanto-II (BD Biosciences, San Jose, USA)
Gel electrophoresis	Mini-sub cell GT (Bio-Rad, Hercules, CA, USA)
Homogenizer	TissueLyser LT (Qiagen, Hilden, Germany)
Incubator	Hera Cell 240 (Fisher Scientific, Schwerte, Germany)
Laminar flow	HeraSafe (Heraeus, Hanau, Germany)
Microscope	Leica DM2500 (Leica, Wetzlar, Germany) EvoS (Fisher Scientific, Schwerte, Germany)
Microtome	Leica RM2245 (Leica, Wetzlar, Germany)
PCR Thermocycler	MyCycler (Bio-Rad, Hercules, CA)
pH-meter	InoLab level 1 (WTW, Weilheim, Germany)
q-PCR Thermocycler	ViiA7 (Applied Biosystems, Foster City, USA)
Spectrophotometer	NanoDrop (Peglab, Erlangen, Germany) GeneQuant (GE Healthcare, Uppsala, Sweden)
TissueLyser	TissueLyserLT (Qiagen, Hilden, Germany)
Weighing balance	Analytical Plus (Ohaus, Pine Brook, USA) Sartorius CPA 64 (Sartorius, Göttingen, Germany)
Western Blot	Trans-Blot® Turbo™ Transfer System Mini-PROTEAN® Tetra Cell (Bio-Rad, Hercules, CA, USA)

3.1.2 General consumables

Table 2: List of general consumables

Consumable	Manufacturer
12-well plate	Greiner Bio-one, Frickenhausen, Germany
24-well plate	Greiner Bio-one, Frickenhausen, Germany
6-well plate	Greiner Bio-one, Frickenhausen, Germany
96-well plate	Greiner Bio-one, Frickenhausen, Germany
Cell filter (70/100 µm)	BD Biosciences, Franklin Lake, USA
Cell strainer	Miltenyi, Bergisch-Gladbach, Germany
Costar Transwell plate	Thermo Scientific, Braunschweig, Germany
Culture flasks (T25, T75, T175)	Greiner Bio-one, Frickenhausen, Germany
Culture slide (15 cm)	Greiner Bio-one, Frickenhausen, Germany
Disposable scalpel	Feather Safety Razor Co., Osaka, Japan
EDTA tubes	Sarstedt, Nümbrecht, Germany
FACS tubes	BD, Franklin Lakes, USA
Falcon tubes (15ml, 50ml)	Greiner Bio-one, Frickenhausen, Germany
Glassware	Schott, Mainz, Germany
Microscope slides	Thermo Scientific, Braunschweig, Germany
Needle 23/27 G	BD Microlance, Franklin Lake, USA
Reaction tubes	Greiner Bio-one, Frickenhausen, Germany
Serologic pipettes (5 ml, 10 ml, 25 ml)	Corning, New York, USA
Sterile filter tips	Starlab, Ahrensburg, Germany
Suture Seraflex, USP 7/0	Serag-Wiessner GmbH, Naila, Germany
Syringe (1 ml)	Braun, Melsungen, Germany
Syringe (2 ml, 5 ml, 10 ml, 20 ml)	Terumo, Leuven, Belgium

3.1.3 Miscellaneous reagents

Table 3: List of miscellaneous reagents

Reagent	Supplier
Accutase	PAA, Pasching, Austria
Bovine serum albumin (BSA)	Serva, Heidelberg, Germany
DAPI	Dako, Glostrup, Denmark
EDTA	Boehringer Mannheim, Indianapolis, USA
Eosin G-Solution 0,5%	Roth, Karlsruhe, Germany
Fetal calf serum (FCS)	PAA, Pasching, Austria
Gentamycin	Gibco, Darmstadt, Germany
HBSS	Gibco, Darmstadt, Germany
Hematoxylin	Applichem, Darmstadt, Germany
Hepes	Gibco, Darmstadt, Germany
Human serum	Innovative Research, Novi, USA
Isofluran	Abbott, Chicago, USA
Ketamine	CEVA, Düsseldorf, Germany
LDL	Calbiochem, Darmstadt, Germany
L-Glutamine	PAA, Pasching, Austria
Lyse Buffer 10 x	Cell Signaling, Cambridge, UK
Mouse serum	Sigma-Aldrich, St. Louis, USA
Oil-Red-O	Sigma-Aldrich, St. Louis, USA
Paraformaldehyde	Sigma-Aldrich, St. Louis, USA
PBS	PAA, Pasching, Austria
Penicillin	PAA, Pasching, Austria
Protease Inhibitor Cocktail	Roche, Basel, Switzerland
Rabbit serum	Sigma-Aldrich, St. Louis, USA
RNAlater	Ambion Life Technologies, Germany
RPMI 1640	Sigma-Aldrich, St. Louis, USA
Streptavidin	PAA, Pasching, Austria
Tissue Tek® O.C.T	Sakura Finetek Europe, Leiden, Netherland
Vectashield	Vector Laboratories, Inc, Burlingame., USA
Xylazine	Medistar, Hannover, Germany
β-mercaptoethanol	Sigma-Aldrich, St. Louis, USA

3.1.4 General buffers and solutions

Table 4: List of general buffer and solution

Buffer	Composition
0,01M HCl	1M HCl (stock) diluted 100x in aqua-dest
0,1% Sirius Red solution	0,1g Sirius Red; 100ml saturated picric acid
Blocking solution	PBS; 1% BSA; 2,5% horse serum
Citrate buffer	630 ml Millipore water; 12,6 ml solution A (21,01g citric acid in 1 l of aqua dest.); 57,4ml solution B (29,41g sodium-citrate in 1 l of aqua-dest.); 350 µl Tween20
FACS staining buffer	2% mouse serum; 2% rabbit serum; 2% human serum; 2% BSA; dissolved in PBS
Hank's complete buffer	1X HBSS; 0,3 mM EDTA; 0,1 % BSA
L929 culture medium	RPMI 1640 with L-Glutamine; 10% fetal calf serum; 5 mM Hepes; 1% penicillin
LCM	L929 conditioned medium (see: 3.2.2.4)
Normal medium	RPMI 1640; 10% fetal calf serum, 1% penicillin/streptavidin, 0,1% gentamycin, 1% Hepes, 15% LCM (add freshly)
Running gel buffer	Tris base 375 mM; 0,1 % SDS; pH=8,8
Stacking gel buffer	Tris base 125 mM; 0,1 % SDS; pH=6,8
Western blot Running buffer	Tris base 25 mM; glycine 192 mM; 0,1 % SDS

3.1.5 General kits

Table 5: List of general kits

Assay	Manufacturer
Cobas Enzymatic Assay Cholesterol + Triglyceride Kit	Roche, Basel, Switzerland
DNA digestion	Qiagen, Hilden, Germany
IL-10 ELISA	eBioscience, Frankfurt Germany
IL-6 ELISA	eBioscience, Frankfurt Germany
Cell Proliferation Assays - CyQUANT®	Thermo Scientific, Braunschweig, Germany
Maxima SYBR Green q-PCR Master Mix	Fermentas, St. Leon-Rot, Germany

Continuation of Table 5

Assay	Manufacturer
M-MLV Reverse Transcriptase cDNA kit	Promega, Madison, USA
RNeasy spin column kit	Qiagen, Hilden, Germany
TNF- α ELISA	eBioscience, Frankfurt Germany

3.1.6 Antibodies, Primers cytokines and inhibitors

Table 6: List of cytokines, endotoxins and inhibitors

Cytokine	Manufacturer
C5aR1 inhibitor JPE-1375	JPT Peptid Technologies, Berlin, Germany
LPS	Sigma-Aldrich, St. Louis, USA
Murine C5a	R&D Systems, Minneapolis, USA
Murine TNF- α	Peptotech, Rocky Hill, USA

All primers have been purchased at Eurofins, Ebersberg, Germany.

Table 7: List of Primer for genotyping

Primer	Sequence 5'-> 3'
<i>ApoE common</i>	5'-AGCCGGAGGTGACAGATCAG-3'
<i>ApoE wild-type</i>	5'-GGTAATCCCAGAAGCGGTTCA-3'
<i>ApoE knock-out</i>	5'-GCTCGACATTGGGTGGAAA-3'
<i>C5ar2 common</i>	5'-GGTCTCTCCCAGCATCATA-3'
<i>C5ar2 wild-type</i>	5'-GCCAGAGGCCACTTGTGTAG-3'
<i>C5ar2 knock-out</i>	5'-GGCAACGTAGCCAAGAAAAA-3'

Table 8: List of primers used for quantitative real time PCR

Primer	Sequence 5'-> 3'
<i>Arginase-1</i>	5'-TGGGCAACCTGTGTCCTTTCTCCT-3' 5'-TTCCCCAGGGTCTACGTCTCGCA-3'
<i>C5</i>	5'-AAACCTGTGAGGAGCGAGTG-3' 5'-TTGGACAGGTTTATGGGGGC-3'

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Continuation of Table 8

Primer	Sequence 5'→ 3'
<i>C5ar1</i>	5'-GGGATGTTGCAGCCCTTATCA-3' 5'-CGCCAGATTCAGAAACCAGATG-3'
<i>C5ar2</i>	5'-AGTGCGGTGACCAAGGTATCC-3' 5'-GTGCGATGGCCTAGGAAATC-3'
<i>GAPDH</i>	5'-ATTGTCAGCAATGCATCCTG-3' 5'-ATGGACTGTGGTCATGAGCC-3'
<i>HMGB1</i>	5'-AGAGCGGAGAGAGTGAGGAG-3' 5'-AGTTGACAGAAGCATCCGGG-3'
<i>IL-1β</i>	5'-CAACCAACAAGTGATATTCTCCATG-3' 5'-GATCCACACTCTCCAGCTGCA-3'
<i>IL-6</i>	5'-TCTGAAGGACTCTGGCTTTG-3' 5'-GATGGATGCTACCAAACCTGGA-3'
<i>IFN-γ</i>	5'-GCTGTTTCTGGCTGTTACTGC-3' 5'-TCACCATCCTTTTGCCAGTTCC-3'
<i>iNOS</i>	5'-TCATTGGGCCTGGTACGGGCA-3' 5'-ACACCAAGCTCATGCGGCCTC-3'
<i>TNF-α</i>	5'-CATCTTCTCAAATTCGAGTGACAA-3' 5'-TGGGAGTAGACAAGGTACAACCC-3'
<i>VCAM-1</i>	5'-TGCCGAGCTAAATTACACATTG-3' 5'-CCTTGTGGAGGGATGTACAGA-3'

Table 9: List of antibodies used for immunofluorescence and western blot

Epitope	Specificity	Conjugate	Use	Manufacturer
α-Sma	SMC	purified	IFS	Dako, Glostrup, Denmark
β-actin	Loading control	purified	WB	Abcam, Cambridge, UK
CD3	T-cells	purified	IFS	AbD Serotec, Raleigh, USA
CD88	C5a-Rezeptor1	-	WB, IFS	Abcam, Cambridge, UK
Goat IgG	Goat IgG	Cy3	IFS	Jackson ImmunoResearch, West Grove, USA
Ki-67	Proliferating cells	-	IFS	Dako, Glostrup, Denmark
Mac2	Macrophages	purified	IFS	Cedarlane, Burlington, Canada
Mouse IgG	Mouse IgG	Cy3	IFS	Jackson ImmunoResearch, West Grove, USA
Rat IgG	Rat IgG	FITC	IFS	Jackson ImmunoResearch, West Grove, USA
TNF-α	Tumor necrosis factor-alpha	-	IFS	Santa Cruz Biotechnology, Dallas, USA

3.1.7 List of used software

Table 10: List of software

Software	Manufacturer
Diskus	Hilgers, Königswinter, Germany
FACSDiva	BD Biosciences, San Jose, USA
FlowJo	Treestar, Ashland, USA
GraphPad Prism 6	GraphPad Software, La Jolla, USA
Image J	National Institutes of Health, USA

3.2 Methods

3.2.1 Animal experiments

All animal experiments were approved by local authorities in North Rhine-Westphalia (Landesamt für Natur, Umwelt und Verbraucherschutz (LANUV) Nordrhein-Westfalen, Germany) and complied with the German animal protection law. For experiments, 8 weeks old male or female mice were taken. All experiments and surgeries were performed under ketamine/xylazine anesthesia (10 mg/kg body weight xylazine, 100 mg/kg bodyweight ketamine, intraperitoneal (i.p.)). Blood was drawn by cardiac puncture and transferred either into EDTA tubes or into serum tubes.

3.2.1.1 Mouse models

To study the influence of C5a on the development and progression of atherosclerotic lesions and on restenosis *C5ar2^{-/-}/Apoe^{-/-}* mice were generated.

C5ar2-deficient mice (*C5ar2^{-/-}*), with a C57BL/6J background, were obtained from Prof. Dr. Andreas Klos from the Department for Medical Microbiology at the Hannover Medical School in Germany. Atherosclerosis-prone C57BL/6J Apolipoprotein E-deficient (*Apoe^{-/-}*) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) [184, 185]. *Apoe^{-/-}* deficient mice are an established mouse model to study atherosclerosis and restenosis [186]. To generate *C5ar2^{-/-}/Apoe^{-/-}* double knock-out mice, *C5ar2^{-/-}* were crossed with *Apoe^{-/-}* mice for more than 8 generations. As a control mouse strain, wild-type C57BL/6J from Jackson Laboratory were used.

3.2.1.2 Mice breeding and care taking

Mice breeding and caretaking was accomplished in the animal caretaking facility (Versuchstierkunde) of the RWTH University Hospital Aachen. Mice were kept under sterile conditions with a constant temperature of 21 degrees and day/night rhythm of 12 h each.

3.2.1.3 Organ isolation

For organ isolation, mice were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine. Blood was drawn via cardiac puncture and either stored in EDTA-buffer for whole blood count analyses or taken for blood serum analyses. Afterwards the mice were perfused with 10 ml PBS to flush out blood remains. Organs were taken out and stored accordingly for further experiments.

Organs and tissues used for protein or RNA isolation were taken out and quick frozen in liquid nitrogen and stored for further analyses at -80°C. Protein and RNA extraction was performed in carotids, cardiac muscle valves, aorta, liver, kidney, spleen and inguinal lymph nodes.

Analyses of atherosclerotic lesion formation was done in aortic roots, the aorta and in the carotid arteries. Therefore, aortic roots and carotid arteries were taken out and fixed in 4% PFA at 4°C overnight for histopathological analysis.

Carotid arteries, then, were dehydrated (see: Table 16) and embedded in paraffin. With a microtome serial transversal sections of 5 µm were made, starting at the bifurcation and spanning 500 µm of the carotid artery. For further analyses of the lesion size by Movat's pentachrome staining (see: 3.2.5.2.2) and the cellular composition by histochemical and immunofluorescent staining (see: 3.2.5) the sections were collected on glass microscopy slides.

The aortic roots, then, were embedded in TissueTek and 5 µm section were made with a cryotome and sections were collected on SuperFrost microscopy slides for further analyses of lipid depositions by Oil-Red-O (see: 3.2.5.2.1) and cellular composition (see: 3.2.5).

Lesion size in the aortas were visualized in an *en face* preparation. Therefore, the whole aorta was taken out and fixed in 4% PFA at room temperature overnight. The next day the adventitia was removed and the aorta was open longitudinal. Pinned on a rubber-coated slide with endothelium facing upwards the aorta was stained by Oil-Red-O for lipid deposition (see: 3.2.5.2.1).

For the preparation of bone marrow macrophages, the femur and tibia from both hind limbs were taken out and immediately put in ice cold PBS. Bone marrow was flushed out and differentiated to macrophages (see: 3.2.2.5).

3.2.1.4 C5aR1-antagonist application with osmotic mini-pumps

Up to date, there is no *C5ar1*^{-/-}/*C5ar2*^{-/-} double knock-out mouse model available. To study the effects of a total deficiency of both C5a receptors, the C5aR1-antagonist was applied to *C5ar2*^{-/-}/*Apoe*^{-/-} mice. The C5aR1-antagonist (C5aR1-A) JPE-1375 provided by JPT Peptid Technologies (Berlin, Germany) was used. The efficacy of JPE-1375 in blocking the C5aR1 was verified in a mouse model of the reverse passive arthus reaction (RPAR), which is an immune complex mediated disease. The progression of

this disease is also driven by activation of complement and is caused by a neutrophil influx into the mouse peritoneum. After a challenge with the chicken albumin, the number of neutrophils in the peritoneal lavage was determined. In this model of RPAR the JPE-1375 was shown to significantly inhibit the influx of neutrophils. Furthermore, it was shown *in vitro* that JPE-1375 was able to inhibit C5aR1 induced chemotaxis on J774A.1 cells, which is a murine monocyte/macrophage cell line. [187]. Therefore, male *Apoe*^{-/-} and *C5ar2*^{-/-}/*Apoe*^{-/-} mice were put 4 weeks before and up to 4 weeks after arterial injury on HFD. Starting three days before arterial injury mice were daily injected subcutaneous with C5aR1-antagonist (25 µg/day/mice). On the day of arterial injury an Alzet® mini-osmotic pumps with C5aR1-antagonist (25 µg/day/mice, flow rate 0,25 µl/h) was placed under the abdominal skin and kept there for 4 weeks. Therefore, mice were anesthetized by intraperitoneal injection of ketamine (100mg/kg body weight) and xylazine (10mg/kg body weight). Mice were shaved on the left abdomen and skin was disinfected. Via a small incision the mini-pump was implanted under the skin. Wounded skin was closed using wound clips. For pain relief mice were injected daily buprenorphine (10 mg/kg body weight) for 3 days following surgery. For two weeks after surgery, health and general condition of the mice was monitored daily, then twice a week until mice were sacrificed.

3.2.1.5 Model of wire-induced endothelial denudation of carotid arteries

To study the effect of endothelial injury in carotid arteries eight weeks old male wild-type (WT), *Apoe*^{-/-}, *C5ar2*^{-/-} and *C5ar2*^{-/-}/*Apoe*^{-/-} mice were put 4 weeks before and up to 4 weeks after arterial wire injury on HFD (21% total fat, 0,15% cholesterol and 19,5% casein (Altromin, Lage)). Four weeks after the injury mice were anesthetized and both, left and right, carotid artery were harvested for further analyses (see:3.2.1.3). Wire-induced endothelial denudation of carotid arteries is a mechanical method to remove the endothelium to mimic processes in restenosis. Therefore, mice were anesthetized by intraperitoneal injection of ketamine (100mg/kg body weight) and xylazine (10mg/kg body weight). The frontal neck was shaved and disinfected and a midline incision of the left ventral neck was made. Afterwards the left carotid artery including the bifurcation was exposed. Blood flow was stopped by the ligation of the left common carotid artery (LCA) and the internal carotid artery (ICA). The left external carotid artery (ECA) was ligated distally while a loose suture, not closing the artery, was places on the proximal ECA. Via an incision made between the two sutures on the

ECA a 0,6 mm flexible angioplasty guide wire was advanced by 1 cm into LCA. Complete and even endothelial denudation was achieved by 3 passes along the common carotid artery with a rotating motion. By closing the loose suture on the proximal ECA bleeding from the incision was prevented. To restore blood flow sutures on the LCA and the ECA were opened again ensuring sufficient blood supply through the ECA. Wounded skin was closed using wound clips. For pain relief mice were injected daily buprenorphine (10 mg/kg body weight) for 3 days following surgery. For two weeks after surgery, health and general condition of the mice was monitored daily, then twice a week until mice were sacrificed. Injured left and uninjured right arteries were excised 4 weeks after and embedded in paraffin for analysis of neointimal plaque size or flash frozen for analysis of the gene expression (see:3.2.1.3).

3.2.1.6 Diet-induced atherosclerosis

To study diet-induced atherosclerosis, 8 weeks old female wild-type (WT), *Apoe*^{-/-}, *C5ar2*^{-/-} and *C5ar2*^{-/-}/*Apoe*^{-/-} mice were put on HFD containing 21% total fat, 0,15% cholesterol and 19,5% casein (Altromin, Lage) for 12 or 24 weeks. Matching non-HFD control mice were also kept for 12 and 24 weeks. At indicated time points mice were anesthetized with 100 mg/kg Ketamine and 10 mg/kg Xylazine and organs were harvested for further analysis (see: 3.2.1.3).

3.2.2 Cell culture

3.2.2.1 General cell culture methods, Cell cultivation

Cells were cultured under sterile conditions in a laminar flow hood and kept in a humidified 5% CO₂ incubator at 37°C. If not stated differently, cells were cultured with corresponding normal cell culture medium for each kind of cell type.

3.2.2.2 Cryo-conservation and thawing of cells

For cryo-conservation cells were harvested from the flask with Accutase or trypsin solution. Then, cells were resuspended at a concentration of 10⁶ cells/ml in complete medium containing 5% (v/v) dimethylsulfoxide (DMSO) and transferred to 1 ml cryovials. For slow freezing the cryovials were placed at -80°C overnight in a cryocontainer and then transferred to -152°C liquid nitrogen cryogenic freezer for long-term storage. To thaw cells, cryovials were placed in a 37°C water bath. Cells were

transferred to growth medium, centrifuged and resuspended in appropriate growth medium.

3.2.2.3 Cell count determination

Under a light microscope, cells were counted with the Neubauer counting chamber. 10 µl of the cell suspension was stained with trypan blue and placed on the Neubauer chamber. Cells in four main quadrates were counted and the number of cells per milliliter of medium was calculated using the formula: number of $\frac{\text{cells}}{\text{mL}} = \frac{n}{4} * 10^4$.

3.2.2.4 Preparation of L929 conditioned medium

L929 cells (Sigma-Aldrich, St. Louis, USA) were thaw at 37°C (see: 3.2.2.2) and plated in L929 culture medium in a T-75 flask. Cell were split every 3 days until enough cells were available for 4 T-175. Flasks were filled up with L929 culture medium and left for 11 days at 37°C. After 11 days, the medium was pooled and sterile filtered. The cells were discarded. The filtered L929 conditioned medium (LCM) was aliquoted, flash-frozen and stored at -80°C.

3.2.2.5 Macrophage isolation

Bone marrow-derived macrophages (BMDMs) were isolated from wild-type (WT), *Apoe*^{-/-}, *C5ar2*^{-/-} and *C5ar2*^{-/-}/*Apoe*^{-/-} mice as previously described [188]. The macrophages were obtained from the bone marrow of the femur of 8-12 weeks old mice.

For disinfection, the femurs were first washed in 70% ethanol for 30 sec and afterwards in PBS. The joint heads were cut and the bone marrow was flushed out with 5 ml PBS from each side of the bone. Then the cells were filtered using a 70 µm cell strainer. The cells from each mouse was pooled and centrifuged at 300 rpm for 5 min. Supernatant was removed and cells were suspended in 17 ml macrophage culture medium. To differentiate the bone marrow cells to macrophages, 3 ml of L929 conditioned medium (see: 3.2.2.4) was added and cells were cultivated for in total 6-7 days in a humidified 5 % CO₂, 20 % O₂ air atmosphere at 37°C in topically treated 15 cm dishes. After 4 days macrophages medium was changed and fresh L929 conditioned medium was added.

For further experiments, macrophages were harvested by 1 time washing with PBS following an incubation with 3,5 ml Accutase for 15 min at 37°C. The collected macrophages were centrifuged at 300 rpm for 5 min. The supernatant was discarded.

Cells were counted (see: 3.2.2.3) and resuspended in 5-10 ml macrophages culture medium according to the desired cell count concentration.

3.2.2.6 Macrophage experiments

Macrophages were obtained and differentiated from the bone marrow (see: 3.2.2.5). To study mRNA expression level, in the morning, macrophages were harvested and seeded in 6-well plates in a concentration of 1×10^6 cells per well in macrophages culturing medium. In the evening, medium was exchanged with macrophage starving medium. The following day, the medium was changed to normal macrophage culturing medium again and cells were stimulated with either 100 ng/ μ l C5a, 100 ng/ μ l LPS, 50 ng/ μ l TNF- α or without stimulus as a control. After 6 h stimulation macrophages were harvested and RNA was isolated (see: 3.2.3.1) for analyses with quantitative real-time PCR (see: 3.2.3.5).

3.2.3 Molecular methods

3.2.3.1 DNA and RNA Isolation from cells and tissues

For genotyping, DNA was isolated from mouse-tails. Therefore, mouse-tail cuts were incubated in 600 μ l tail lysis buffer and 15 μ l Proteinase K over night at 50°C. The next day isolation was done using Bio Sprinter 96 from Qiagen (Hilden, Germany) following the manufacturer's protocol. DNA concentration was measured (see: 3.2.3.2) and amplified by PCR (see: 3.2.3.3). The genotype was determined by gel-electrophoresis. RNA was isolated from aortas, aortic roots, carotid arteries and liver tissue. The tissue was lysed in 350 μ l RLT buffer (Qiagen) and homogenized at 50 oscillation per second for 5 min using stainless-steel beads (Qiagen). Macrophages from cell culture were lysed in 350 μ l RLT buffer. The RNA isolation was performed using RNeasy spin column Kit (Qiagen) following manufactures' protocol. Briefly, tissue or cells were lysed in 700 μ l lysis buffer and transferred to a spin column and centrifuged for 15 sec at 10000 rpm. Flow-through was discarded and columns were washed 3 times with washing buffer. Flow-through was discarded. To elute total RNA from column, 50 μ l of RNase free water was added and the column was centrifuged for 1 min at 10000 rpm. RNA concentration was measured (see: 3.2.3.2) and analyzed by quantitative real-time PCR (see: 3.2.3.5).

3.2.3.2 Photometrical measurement of nucleic acid concentration

The concentration of RNA and DNA was determined photometrically by measuring the absorbance at a wavelength of 260 nm. The ratio of the absorbance at 260 nm/280 nm for pure DNA is 1.8 and for pure mRNA 2.0. A contamination with proteins lowers the ratio due to the absorbance of aromatic rings with a maximum at 280 nm. Remaining phenol from mRNA isolation procedure can decrease the 260 nm/280 nm ratio. 2 µl of mRNA or DNA was used in a Nanodrop system.

3.2.3.3 Genotyping of the knock-out mice

DNA for genotyping was isolated from mouse tails and analyzed by the polymerase chain reaction (PCR). The PCR is a method to amplify specific DNA regions. A thermo stable DNA polymerase is used to amplify the DNA. The amplified DNA region is defined with primers, which are small complementary DNA sequences flanking the wanted DNA sequence. The PCR is a process performed in three steps. In the first step, the DNA double strands are denaturized. In the second and third step, the annealing of the primers and the elongation of the DNA takes place, respectively.

For PCR the DNA concentration was adjusted to 100 ng/µl and a mastermix was prepared (see: Table 11).

Table 11: PCR mastermix for genotyping

Component	Volume	Final Concentration
5x Green GoTaq Flexi Puffer	5 µl	1 x
MgCL ₂	3 µl	3 mM
dNTPs	0,5 µl	0,2 mM
Primer 1	2,5 µl	0,2 µM
Primer 2	2,5 µl	0,2 µM
GoTaq DNA Polymerase	0,13 µl	-
H ₂ O	Fill up to 25 µl	-
DNA	2 µl	-
Final volume	25 µl	-

The PCR run was designed according to the parameters seen in Table 12. In each PCR run controls for wild-type, knock-out and heterozygote mice were included. Primers combination for detecting different knock-out are given in Table 7.

Table 12: Parameters for PCR

step	duration	temperature	number of cycles
initial denaturation	10 min	95 °C	1
denaturation	30 sec	95 °C	35
annealing	30 sec	see 2.1.10	
elongation	30 sec	72 °C	
final elongation	3 min	72°C	1

3.2.3.4 cDNA synthesis

The synthesis of complementary DNA (cDNA) was carried out with the First Strand cDNA Synthesis Kit from Fermentas. To transcribe the mRNA into cDNA the viral Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase and Oligo(dT)₁₈ primers were used. M-MuLV is an mRNA-dependent DNA polymerase, which also exhibits mRNAse H activity to cleave the mRNA strand from the newly synthesized DNA strand. To ensure mRNA specificity the Oligo(dT)₁₈ primers are directed against the Poly(A) tail of mRNA.

For the synthesis of the cDNA 0,5-1 µg RNA was mixed with 1 µl Oligo(dT)₁₈ Primer and filled up to 16,5 µl with RNase-free water. The mixture was incubated for 5 min at 70°C followed by 5 min on ice. For the reverse transcription 1 µl (40 U) reverse transcriptase, 2,5 µl dNTPs (10µM) und 5 µl 5 x Buffer were added. The sample was incubated for 1 h at 37°C. The reaction was stopped by incubating the sample for 10 min at 70°C.

3.2.3.5 Quantitative real-time PCR

The qPCR is a modified PCR, which allows a direct quantification of the DNA produced within the reaction. A DNA fluorescent dye whose fluorescence increases when it intercalates in double-stranded DNA was added to the PCR reaction to quantify the amount of DNA. To normalize the intensity of the fluorescent signal the reference dye ROXTM was used.

For each reaction mix for the qPCR 1µl H₂O, 1 µl per Primer (10 µM per primer) (see: Table 8), 5 µl Maxima SYBR Green q-PCR Master Mix and 1 µl cDNA were used. The parameters used for this qPCR are described in Table 13.

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Table 13: Parameters used for the quantitative real-time PCR

step	duration	temperature	number of cycles
initial denaturation	30 sec	95 °C	1
denaturation	30 sec	95 °C	40
annealing	30 sec	see 2.1.10	
elongation	30 sec	72 °C	
denaturation	2 min	95 °C	1
annealing	30 sec	see 2.1.10	
elongation	30 sec	72 °C	

To compensate differences in the efficiency of the PCR and differences in used cDNA amount the samples were normalized to the house-keeping gene Glycerinaldehyd-3-phosphat-Dehydrogenase (GAPDH).

Results were expressed as fold induction in reference to a pre-defined control group and were calculated with the $\Delta\Delta C_t$ method. The C_t values were normalized to the C_t values of the GAPDH. The fold induction was calculated with the normalized C_t values in reference to normalized C_t values of a control condition with the mathematical equation $2^{-\Delta\Delta C_t}$ ($\Delta\Delta C_t = \text{normalized } C_t \text{ values} / \text{normalized } C_t \text{ values of a control condition}$). The PCR reaction was carried out with the ViiA7 from Applied Biosystems.

3.2.4 Biochemical methods

3.2.4.1 Western Blot and SDS-PAGE

Western blot analysis is a method to identify specific proteins in a sample. Therefore, the protein in the samples were separated according to their size in a SDS polyacrylamide gel electrophoresis (SDS-PAGE). To detect a specific protein by immunostaining, the proteins were first transferred to a nitrocellulose membrane and then analyzed using specific antibodies (see: 3.2.4.2).

To separate the proteins according to their molecular weight, the discontinuous SDS-PAGE (Laemmli, 1970 [189]) was used. The sample buffer includes the reducing agent β -mercaptoethanol, which denaturizes proteins by disrupting the disulphide bonds. Further, it leads to a dissociation of multimeric proteins into monomeric subunits and to the disruption of the tertiary protein conformation. Non-covalent bonds form the secondary structure of proteins. The anionic detergent sodium dodecyl sulphate (SDS) binds to proteins in a ratio 3:2. This causes the disruption of the non-covalent bonds and a negative net charge of the SDS/protein complex.

The gel for separation consists of a stacking and a resolving gel. The pH of 6,8 in the stacking gel leads to a focusing of the protein sample. At the pH of 6,8 the glycine from the SDS-sample buffer is present as a zwitterion but as the isoelectric point is 6,06 the glycine ions have almost no charge. The protein samples stack between the slow migrating glycine ions and the fast migrating chloride ions from the hydrochloric acid in the stacking gel buffer. The resolving gel has a pH of 8.8. At this pH the glycine donates protons and get charged. The charged glycine ions now migrate faster than the protein samples and release them. In the absence of the stacking effect, the proteins are separated according to their molecular size.

For SDS-PAGE 60 - 100 μ g of each protein sample was mixed with 5 x SDS-sample buffer (Fermatas) and was incubated for 5 min at 95°C.

The separation gel was prepared according to Table 14. As a molecular weight marker, the Prestained Page Ruler™ Protein Ladder (Fermentas) was loaded onto a separate lane. The electrophoresis was performed in a Double-Twin electrophoreses chamber (Bio-Rad).

Table 14: Protocol for polyacrylamide gels

Chemicals	resolving gel		stacking gel
	10 %	12 %	5%
H ₂ O	11,9 ml	9,9 ml	10,2 ml
30% acrylamide	10 ml	12 ml	2,49 ml
TEMED	12 µl	12 µl	15 µl
10% APS	300 µl	300 µl	150 µl
10% SDS	300 µl	300 µl	150 µl
Resolving gel buffer	7,5 ml	7,5 ml	-
Stacking gel buffer	-	-	1,89 ml

3.2.4.2 Immune-detection of proteins

The proteins were transferred to a nitrocellulose membrane (Hybond ECL) for further analysis. They were transferred by semidry electro-blotting. The membrane and a set of filter papers were equilibrated in western blot transfer buffer. The blotting was carried out at a constant current of 150 V for 30 min. To check whether the transfer was successful the membrane was incubated with Ponceau-S. It stains all proteins on the membrane. Afterwards, the membrane was washed with PBS to remove the Ponceau-S. To block unspecific antibody binding sites the membrane was incubated in 5 % milk in PBS for 1 h at room temperature. Membranes were incubated with the respective primary antibodies (see: Table 9) at 4 °C over night. The primary antibodies were diluted in 5 % milk in PBS. Prior to the incubation with the secondary antibody, the membrane was washed three times with PBS for 10 min to remove unbound antibodies. The membrane was incubated for 1 h at room temperature with the secondary antibody (see: Table 9) which binds specifically to the Fc region of the primary antibody. To remove unbound antibodies, the membrane was washed with PBS three times again. The secondary antibodies are conjugated with horseradish peroxidase (HRP). The membrane was incubated in 10 ml ECL solution for 1 min to start the chemiluminescence reaction catalyzed by the HRP. The resulting chemiluminescence was detected by using the LAS3000 Imager from Fujifilm.

3.2.4.3 Flow cytometry

Flow cytometry is a method to analyze the morphological and biological properties of cells [190]. The cells pass a light source in a hydro dynamically focused fluid. The morphological properties can be studied with the forward scatter (FSC) and the sideward scatter (SSC). FCS and SSC are used to measure the size and the internal morphology of cells, like the shape of the nucleus or the granularity, respectively. The biological diversity is distinguished with fluorescence-labelled antibodies. Different biological properties can be studied simultaneously by antibodies labelled with different fluorescent dyes. With flow cytometry up to 10000 cells per second can be analyzed.

To prepare the cells for the flow cytometry analysis the medium was removed and the cells were washed twice with PBS. The traditionally used trypsin/EDTA was replaced with Accutase. With the use of Accutase, a better preservation of the epitopes on the cell surface was achieved. The cells were incubated with 300 µl Accutase for 10 min at 37°C to detach them from the culture dish. By adding 1 ml medium, the Accutase was inactivated. For analysis $0,1 \times 10^6$ cells were transferred to a reaction tube. The cells were washed in Hank's complete buffer and centrifuged at 1000 rpm, 4°C for 10 min. The cells were resuspended in 300 µl FACS staining buffer with C5aR1 (anti-mouse CD88-phycoerythrin rat IgG2a, Serotec, Oxford, UK) or anti-human CD88-FITC mouse IgG2a (Genway Biotech Inc, San Diego, USA) as isotype controls. The cell samples were protected from light and incubated for 30 min on ice. Afterwards the cells were washed with Hank's complete buffer and centrifuged at 1000 rpm, 4°C for 10 min. Subsequently they were analyzed in the FACSCanto™ II (BD Bioscience) and evaluated with FlowJo Software (Treestar, Ashland, USA). The results were displayed as diagrams demonstrating the change in mean fluorescence intensity (MFI).

3.2.4.4 ELISA

The enzyme-linked immunosorbent assay (ELISA) is a quantitative method to determine the concentration of for example proteins, cytokines or virus particles. The measurement is based on the specific capacity of antibody to bind to these proteins. An enzyme is coupled to the antibody catalyzing a reaction leading to a color change or chemiluminescence, which is then directly proportional to the concentration of the molecule of interest.

The concentration of the chemokines interleukin 6 (IL-6), tumor necrosis factor-alpha (TNF-α) and interleukin 10 (IL-10) was determined in serum samples of supernatants

of bone marrow-derived macrophages. The ELISA Ready-SET-Go! kits were acquired from eBioscience (Frankfurt) and were done following the manufactures' instruction. Briefly, 96-well plates were coated with 100 µl per well of capture antibody in 1X coating buffer overnight at 4 °C. The following day, wells were wash 3 times with 300 µl wash buffer and subsequently unspecific binding sites were blocked with 200 µl per well of 1X ELISA/ELISPOT at room temperature for 1 h. Standards were prepared in 1X ELISA/ELISPOT. 100 µl of the samples and of each standard concentration was added to the appropriate well. 96-well plate was incubate at room temperature for 2 h. Samples were washed 3 times with 300 µl wash buffer and incubate at room temperature for 1 h in 100 µl of detection antibody diluted in 1X ELISA/ELISPOT. Then, samples were washed 3 times with 300 µl wash buffer. 100 µl of avidin-HRP diluted in 1X ELISA/ELISPOT was added and samples were incubated at room temperature for 30 min. Samples were washed 5 times with 300 µl wash buffer, followed by an incubation of 100 µl of 1X TMB solution at room temperature for 15 min. Reaction was stopped with 50 µl of stop solution. Samples were analyzed in a 96-well plate reader at a wavelength of 450 nm.

3.2.4.5 Cholesterol and triglyceride analysis

The level of cholesterol and triglyceride in the blood serum were measured using Cobas enzymatic assays (Roche, Basel, Switzerland) following the manufacturer's protocol. Briefly, the standards and serum were diluted in 0,9 % NaCl. In a 96-well plate 5 µl of the serum and 200 µl of a chromogenic reagent were incubated for 30 minutes at room temperature. For cholesterol level the serum samples were diluted 1:5 in a 0,9 % NaCl solution beforehand. The absorbance in serum was measured at a wavelength of 505 nm by a 96-well plate reader.

3.2.5 Histological and immunohistological methods

Atherosclerotic lesion size was determined by histological staining. In carotid arteries, lesion size and neointima formation was analyzed with Movat's pentachrome staining. Aortic roots and the whole aorta were staining for lipid deposition with Oil-Red-O. In 10 consecutive sections of the carotid artery and 3 sections of the aortic root atherosclerotic lesion were analyzed for cellular composition. Cell numbers of macrophages and SMCs have been determined. Additionally, sections were stained for cells positive for C5aR1, TNF-α, IFN-γ, CD3 and for proliferating and apoptotic cells.

3.2.5.1 Deparaffinization and dehydration

For the staining the paraffin must be removed from the sections (Table 15). Additionally, the section used for immuno-histochemical staining also have to be dehydrated (Table 16). If not deparaffinized it is not possible to detect epitopes by antibodies.

Table 15: Protocol for Deparaffinization

Step	time
Xylol	2 x 15 min
100% Isopropanol	2 x 5 min
96% Isopropanol	5 min
70% Isopropanol	5 min
PBS	5 min

Table 16: Protocol for Dehydration

Step	time
70% Isopropanol	Quick wash
96% Isopropanol	Quick wash
100% Isopropanol	Quick wash
100% Isopropanol	Quick wash
Xylol	5 min

3.2.5.2 Histochemical staining

3.2.5.2.1 Oil Red-O staining

The Oil-Red-O staining was used to identify atherosclerotic lesion by lipid depositions in the aortic root and in the aorta. Therefore, 5 µm section of the aortic root and the longitudinal opened aorta was stained with a 0,5 % Oil-Red-O solution. First, the aortic roots were washed by shortly dipping into tap water and in case of the whole aorta, they were washed for 5 min in PBS. Next, roots and aorta were dipped 10 times in 60 % isopropanol followed by a 15 min incubation in 0,5 % Oil-Red-O solution. To prevent background-staining remains of Oil-Red-O were removed by dipping again into 60 % isopropanol afterwards and subsequently washing with water or PBS. Aortic roots were embedded with Vectashield mounting medium and whole aortas with glycerin gelantine on glass microscopy slides. Images were taken with a Leica DM2500 fluorescence microscope (Leica, Wetzlar, Germany). Atherosclerotic lesion size was

quantified using Diskus software (Hilgers, Königswinter, Germany) and the percentage of lipid deposition was calculated by dividing the stained area by the total aortic surface or the total aortic root area, respectively.

3.2.5.2.2 Movat's pentachrome staining

The Movat's pentachrome staining is a histochemical technique to visualize the atherosclerotic lesion size. Carotids were isolated and 5 µm paraffin-embedded section were made. For further analyzation, the sections needed to be deparaffinized first (see: Table 15). The deparaffinized carotid sections were incubated for 45 sec in hematoxylin followed by 7 min washing under flowing tap water. Tap water is used to increase the pH, thereby the color switches from red-brown to bluish violet. Afterwards, they were washed with aqua dest.. Afterwards, they were incubated in acidic orcein-verhoeff's hematoxylin for 30 min at 37 °C. Subsequent rinsing under flowing tap water for 3 min is followed by a short incubation in 2% iron (III)-chloride and thereafter in acidic alcohol (100 ml 95 % ethanol and 1 ml 25 % hydrochloric acid). For final dehydration the section were incubated in 100 % ethanol for 6 min and shortly in xylol. Sections were embedded with Vectashield mounting medium. To determine atherosclerotic lesion size, neointimal and medial areas were quantified in 6 serial sections per mouse within 250-500 µm of the bifurcation. The areas within external elastic lamina (EEL), internal elastic lamina (IEL) or lumen was measured using Diskus software (Hilgers, Königswinter, Germany). Media areas was calculated by dividing the measured area surrounded by internal elastic lamina through the area surrounded by external elastic lamina (media area=IEL/EEL). The neointima size was determined by dividing the area surrounded by the arteria lumen through the area of the internal elastic lamina (neointima=lumen/IEL).

3.2.5.3 Immunofluorescence

To detect a specific cell type in tissue samples the technique of immunofluorescence was used. The technique is based on the specific binding of antibodies directed against cell-type specific structures. Two different approaches are realized: the direct and the indirect detection. In the direct detection, the antibody directly is linked to a fluorochrom or an enzyme. In the indirect approach, the first antibody is not labeled and in turn is recognized by a second antibody, which is directed against the first antibody. Here, the

second antibody is linked to a fluorochrom or an enzyme. By that, the signal intensity can be increased.

Immunofluorescence detection was used to study the cellular composition of the atherosclerotic lesion and to specify cell types and numbers. Therefore, sections of carotid arteries and aortic roots were examined. To detect macrophages and SMCs primary antibody directed against Mac2 and α -SMA, respectively, were used. Further, cells positive for the complement receptor C5aR1 and TNF- α were detected. Proliferation and apoptosis was studied using antibody against the proliferation marker Ki-67. Finally, the T-cell count was determined as cells positive for CD3 (List of antibodies used in immunofluorescence: Table 9).

For immunofluorescence detection, the sections needed to be deparaffinized first (see: Table 15). In the process of formalin fixation and paraffinization the antigen epitopes are masked by cross-linking proteins. To unmask epitopes the sections were boiled in citrate buffer (see: Table 4) for 20 min in a microwave at 600 W. After cooling to room temperature, the sections were washed with PBS and the tissue on the microscopy slide was encircled with Pap-Pen. To reduce unspecific binding sections were incubated in a moist chamber with blocking solution (see: Table 4) for 30 min at room temperature. Blocking solution was shaken off and section were incubated with primary antibody over night at 4°C in a moist chamber. The next morning primary antibody was removed by washing section with PBS. The appropriate secondary antibody was used for incubation at room temperature for 1 h in a moist chamber. Secondary antibody incubation was followed by a washing step. Cell nuclei were counterstained with DAPI and sections were embedded on the microscopy slides with Vectashield mounting medium. Sections were kept dark and cold and were photographed the same day with a Leica DM2500 fluorescence microscope (Leica, Wetzlar, Germany). In all experiments, appropriate IgG isotype controls were used. Positive cells were expressed as percentage of whole plaque cell count determined using Diskus Software.

3.2.5.4 TUNEL-staining

Apoptotic cells were detected by terminal-Desoxyribosyl-Transferase-mediated-dUTP-nick-end-labeling (TUNEL) staining Kit, which is commercially available by Roche. Briefly, during apoptosis the DNA is fragmented by endonucleases. These fragments possess free hydroxyl groups, which get labeled with a fluorescent nucleotide by the enzyme terminal deoxynucleotidyl transferase (TdT) [191]. The fluorescence can be microscopically detected and later quantified. The staining was performed according to the manufacturer's protocol. Of note, if apoptotic cells in aortic root cryo-sections were analyzed the epitope unmasking was done by 2 min incubation on ice with freshly prepared 0,1 % TritonX-100 and 0,1 % sodium citrate (2:1) solution. Following steps were unchanged from the standard immunofluorescence staining protocol as described above. Sections needed to be photographed as quickly as possible as immunofluorescence here is very unstable.

3.2.6 Data illustration and statistical analysis

All data are represented as mean $\pm$ SEM, analyzed for normality by Kolmogorov-Smirnov test or D'Agostino & Pearson omnibus test. Then analyzed as appropriate either by 1-way ANOVA with Tukey's multiple comparison test or non-parametric Kruskal-Wallis test (Prism 6.0 software (GraphPad, CA)). P values < 0.05 was considered statistically significant.

4 Results

4.1 Identifying mice deficient for *C5ar2*^{-/-} and *ApoE*^{-/-}

To study the influence of the C5aR2 on the initiation and progression of atherosclerosis, *C5ar2*^{-/-} mice were crossed with hyperlipidemic *ApoE*^{-/-} mice to generate *C5ar2*^{-/-}/*ApoE*^{-/-} double knock-out mice. Mice, which are deficient for ApoE, develop under an HFD massive atherosclerotic lesions, showing strong similarities to human atherosclerosis. *ApoE*^{-/-} and *C5ar2*^{-/-} mice both were on C57BL/6J background. For breeding, 8-10 week old mice were used. To identify double knock-out mice a PCR based genotyping method was chosen (see: 3.2.3.3). Mice deficient for both *C5ar2*^{-/-} and *ApoE*^{-/-} were taken into experiments (see: Figure 14).

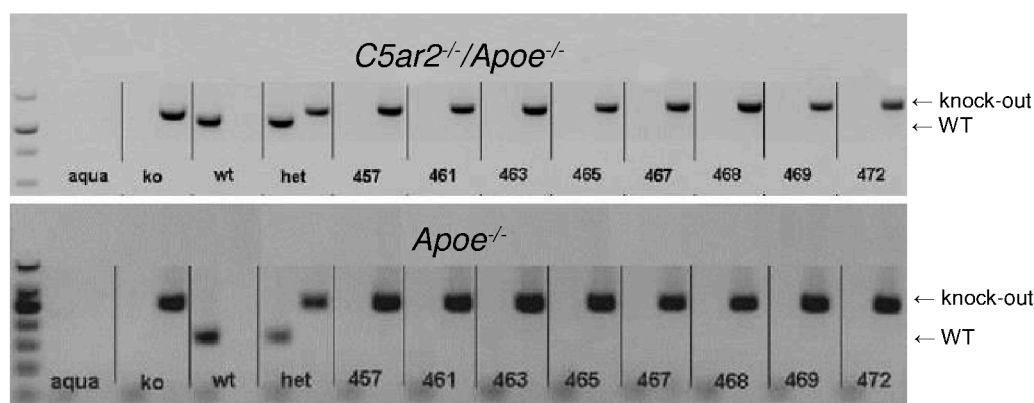


Figure 14: Results of the PCR based genotyping for double knock-out mice. Representative agarose gels of the genotyping PCR for *C5ar2*^{-/-}/*ApoE*^{-/-} deficient mice. As controls, samples from known genotype were taken including knock-out (ko), wild-type (wt) and heterozygote (het) mice as well as null control with water (aqua). Numbers indicate mice numbers used in breeding process. Each number resembles one mouse.

4.2 Effect of C5aR2 deficiency on diet-induced atherosclerosis

To study diet-induced atherosclerosis hyperlipidemic *C5ar2^{-/-}/Apoe^{-/-}* and *Apoe^{-/-}* mice 8 weeks old female mice were put for 12 or 24 weeks on a HFD containing 21 % total fat, 0,15 % cholesterol and 19,5 % casein. The time point of 12 weeks HFD was chosen to study intermediate atherosclerosis characterized by a thickening of the tunica intima, migration of SMCs, an accumulation of intracellular lipids and foam cell formation [31, 45]. In this stage, the atherosclerotic lesion in the mouse resembles the fatty streak found in human plaques. After 24 weeks of HFD properties of advanced atherosclerotic lesions can be observed in mice. This is accompanied by enhanced inflammation, extensive intra and extra cellular lipid depositions, the formation of the necrotic core with the covering fibrous cap [43, 44]. After indicated time points mice were sacrificed and aortic roots and whole aorta were analyzed to assess the extent of atherosclerotic lesion size and cellular composition. Additionally, change in gene expression of pro-inflammatory cytokines and adhesion molecules was also identified. First the whole blood count was analyzed to identify the impact of the deficiency on the peripheral blood cell count. Blood of *C5ar2^{-/-}/Apoe^{-/-}* and *Apoe^{-/-}* mice was analyzed for differences in whole blood count and drawn by cardiac puncture and transferred into serum tubes. Blood was then analyzed in an automated analysis aperture. The analysis revealed no significant difference in total leukocyte count after 12 or 24 weeks of HFD. However, monocytes were significantly reduced after 12 weeks, but not after 24 weeks of HFD. Leukocyte count was expressed as percentage of total cell count in the blood. Monocytes were expressed as percentage of leukocytes (see: Figure 15). Analysis of the whole blood count indicates that the C5aR2 deficiency influences peripheral blood monocyte count may result in an overall diminished monocyte and macrophage count.

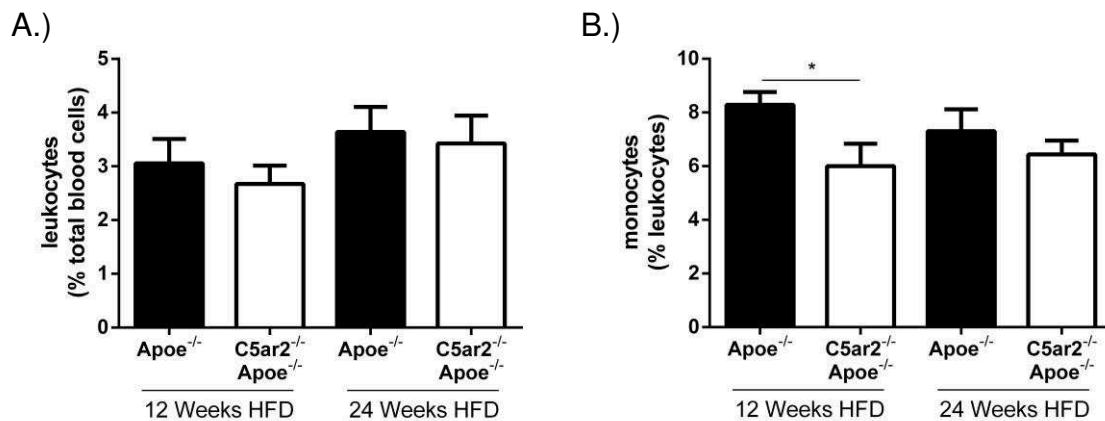


Figure 15: C5aR2 deficiency reduces monocyte count after 12 weeks of HFD. (A) Quantification of leukocyte count in whole blood samples from *Apoe*^{-/-} and *C5ar2*^{-/-}/*Apoe*^{-/-} mice. (B) Quantification of monocyte count in whole blood samples from *Apoe*^{-/-} and *C5ar2*^{-/-}/*Apoe*^{-/-} mice. Leukocytes are expressed as percentage of whole blood cell count, and monocytes as percentage of leukocytes, respectively. Graphs represent the mean $\pm$ SEM; n=15-20; 2-tailed t-test; *p<0,05.

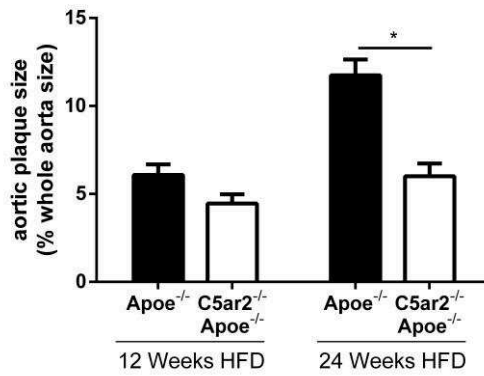
4.2.1 C5aR2 deficiency reduces atherosclerotic lesions in aortas and aortic roots

4.2.1.1 Atherosclerotic lesions in the whole aorta

Aortas were analyzed for atherosclerotic lesion size after 12 and 24 weeks of HFD. Atherosclerotic lesions normally form in the large arteries at sites of turbulent blood flow or bifurcations. Therefore, the aortas have been harvested and stained with Oil-Red-O to visualize lipid deposition (see: Figure 16 B). Atherosclerotic lesion size was determined and expressed as percentage of the whole aorta area. After 24, but not after 12 weeks of HFD significant smaller lesions were found in *C5ar2*^{-/-}/*Apoe*^{-/-} mice compared to the corresponding *Apoe*^{-/-} control animals (see: Figure 16 A). In whole aortas it can be seen, that the C5aR2 deficiency is damping advanced lesion progression which leads to a smaller atherosclerotic lesion.

Results

A.)



B.)

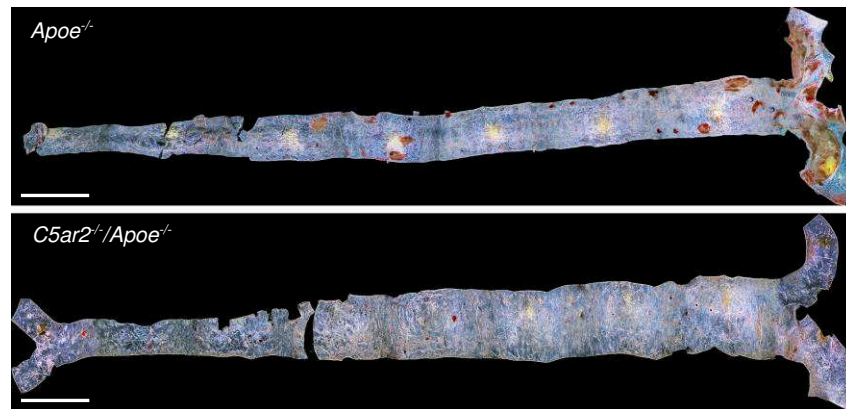


Figure 16: Reduced atherosclerotic lesion size in aortas of *C5ar2*^{-/-}/*Apoe*^{-/-} mice after 24 weeks of HFD. (A) Mice were kept on HFD for 12 or 24 weeks. Aortas were stained with Oil-Red-O for lipid depositions to determine atherosclerotic lesion size. After 24 weeks a significant reduction in lesion size in *C5ar2*^{-/-}/*Apoe*^{-/-} mice was observed, but not after 12 weeks. Aortic lesion size is expressed as percentage of whole aorta size. (B) Photographed aortas stained with Oil-Red-O for lipid depositions. Graphs represent the mean $\pm$ SEM; Scale bar = 5 mm; n=10-12; 2-tailed t-test; *p<0,05.

4.2.1.2 Atherosclerotic lesions in the aortic root

Atherosclerotic lesions preferentially develop at sites of turbulent blood flow and in mice at the heart valves. Thus, aortic roots were analyzed for atherosclerotic lesion size. The hearts of mice have been harvested and cryo-section were made of PFA fixated aortic roots. Aortic roots were stained with Oil-Red-O to highlight atherosclerotic lesions (see: Figure 17 B). According to the results found in aortas, only after 24, but not 12 weeks on HFD, the atherosclerotic lesion size was significantly reduced in aortic roots of *C5ar2*^{-/-}/*Apoe*^{-/-} mice compared to the corresponding *Apoe*^{-/-} control animals. Lesion size was determined and expressed as percentage of the whole aortic root area (see. Figure 17 A). In accordance with the results from whole aortas, in aortic roots, likewise, the C5aR2 deficiency reduces lesion size in advanced atherosclerosis. Thus, this effect is rather a general effect than being limit to a specific site or organ.

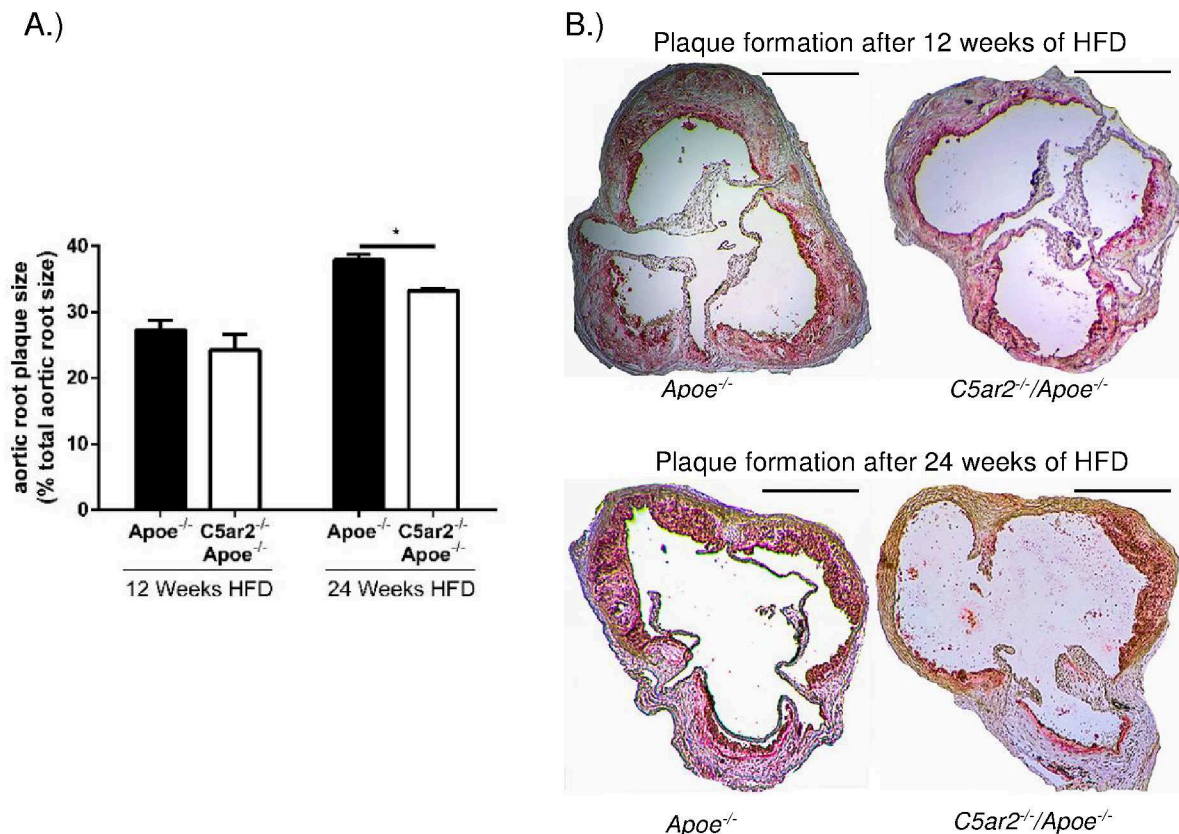


Figure 17: Reduced atherosclerotic lesion size in aortic roots of *C5ar2*^{-/-}/*Apoe*^{-/-} mice after 24 weeks of HFD. (A) Mice were kept on HFD for 12 or 24 weeks. Aortic roots were stained with Oil-Red-O and atherosclerotic lesion size was determined (red area). After 24 weeks a significant reduction in lesion size in *C5ar2*^{-/-}/*Apoe*^{-/-} mice was observed, but not after 12 weeks. Lesion size is expressed as percentage of whole aortic root area. (B) Photographed representative aortic roots stained with Oil-Red-O. Graphs represent the mean $\pm$ SEM; Scale bar = 100 μ m; n=10-12, 2-tailed t-test; *p<0,05.

4.2.2 Cellular composition of the plaque

To clarify the findings of the reduced lesion size in aortas and aortic roots the cells known to be involved in atherosclerosis were studied. Macrophages and SMCs play a central role in lesion progression and stability [55]. The amount and balance of these two cell types are influencing lesion progression and plaque stability. SMCs migrate into the tunica intima and form a fibrous cap, which is stabilizing the atherosclerotic lesion [192]. Macrophages, on the other hand, play a central role in the initiation and progression of atherosclerosis [40]. A hallmark in atherosclerotic progression is that macrophages take up modified lipids and eventually become foam cells, which further aggravates lesion progression. Therefore, we examined the changes in macrophages and SMCs, which were identified by immunofluorescent staining. Aortic roots were harvested and sections were stained for macrophages (Mac-2) and SMCs (α -SMA).

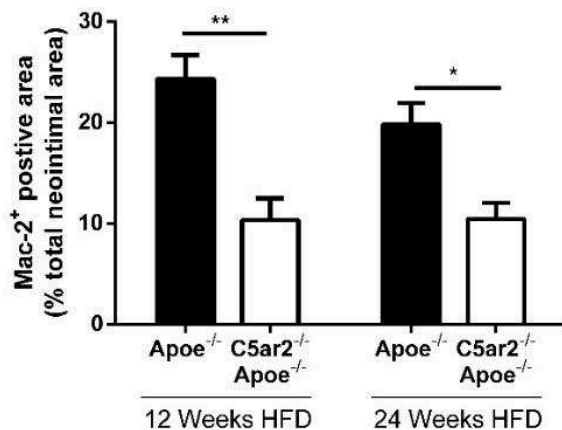
Results

The Mac-2 or α -SMA positive area was assessed and expressed as percentage of the whole lesion size.

A significant reduction in macrophages was observed after 12 weeks as well as after 24 weeks in aortic root lesions of *C5ar2*^{-/-}/*Apoe*^{-/-} mice compared to their corresponding *Apoe*^{-/-} control mice (see. Figure 18). In contrast, after both, 12 and 24 weeks, SMCs show a significant increase in *C5ar2*^{-/-}/*Apoe*^{-/-} mice compared to their corresponding *Apoe*^{-/-} control mice (see: Figure 19). Taking together, the results hint towards, that the C5aR2 deficiency promotes a more stable plaque in intermediate as well in advanced atherosclerotic lesions.

4.2.2.1 Smooth muscle cells and macrophages

A.)



B.)

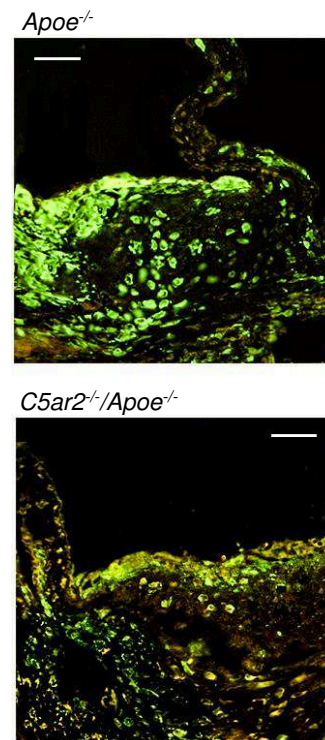


Figure 18: C5aR2 deficiency leads to decreased macrophages content in aortic roots. (A) Quantification of macrophage content in aortic roots of *Apoe*^{-/-} and *C5ar2*^{-/-}/*Apoe*^{-/-} mice by immunofluorescent staining for Mac-2. (B) Representative photographs of aortic roots of *C5ar2*^{-/-}/*Apoe*^{-/-} and *Apoe*^{-/-} mice. Green color represents Mac-2⁺ area. Quantifications are expressed as percentage of positive area to total plaque area. Graphs represent the mean $\pm$ SEM; Scale bars = 50 μ m; n=10-12; 2-tailed t-test; *p<0,05; **p<0,01.

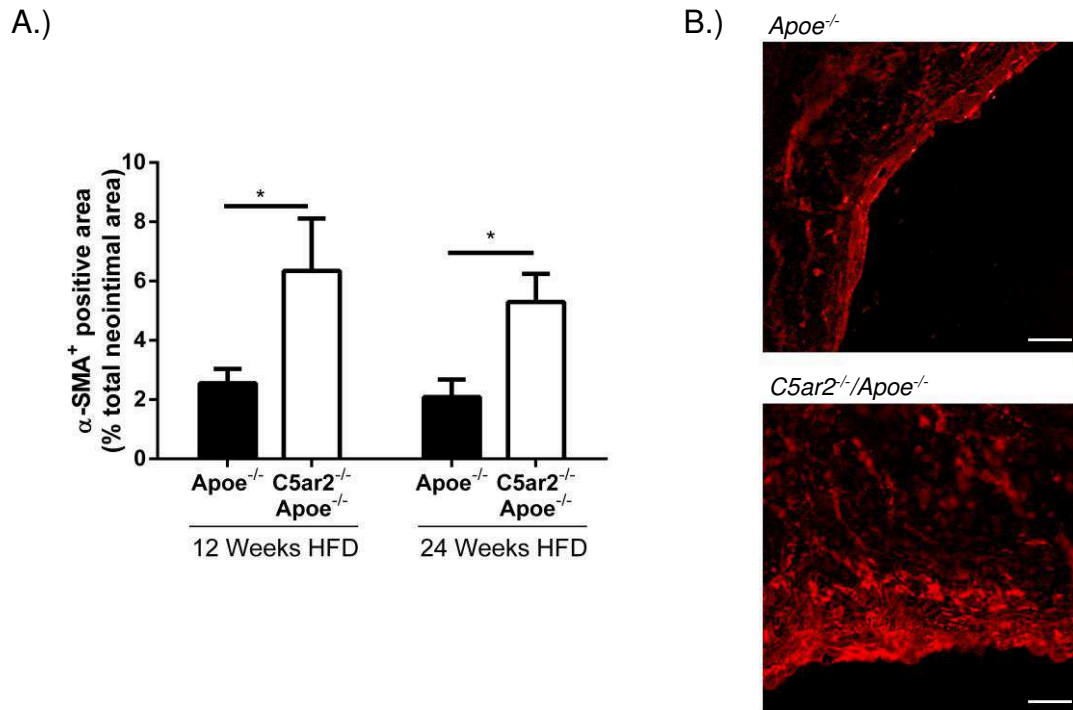


Figure 19: C5aR2 deficiency leads to increased SMC content in aortic roots. (A) Quantification of SMC content in aortic roots of *Apoe*^{-/-} and *C5ar2*^{-/-}/*Apoe*^{-/-} mice by immunofluorescent staining for α -SMA. (B) Representative photographs of aortic roots of *C5ar2*^{-/-}/*Apoe*^{-/-} and *Apoe*^{-/-} mice. Red color represents α -SMA⁺ area. Quantifications are expressed as percentage of positive area to total plaque area. Graphs represent the mean $\pm$ SEM; Scale bars = 50 μ m; n=10-12; 2-tailed t-test; *p<0,05.

The shown data indicate, that the C5aR2 deficiency is leading to a reduction in atherosclerotic lesion size in aortas and aortic roots of *C5ar2*^{-/-}/*Apoe*^{-/-} compared to *Apoe*^{-/-} control mice. This reduction in lesion size in aortic roots is accompanied by a reduction in macrophages and an increase in SMCs. Conclusively, the C5aR2 deficiency is favoring a smaller and more stable lesion.

4.2.3 Cytokine profile and adhesive molecules in diet-induced atherosclerosis

The observation of a reduced lesion size, which was accompanied by reduced macrophages and an increase in SMCs, lead to the question of whether it is caused by a change in cytokine and adhesive molecule expression. Macrophages play a central role in atherosclerotic lesion initiation and progression. They are the main source of pro-inflammatory cytokines and are responsible for ongoing inflammatory response in lesion sites [192]. To analyze these effects, the gene expression of pro-inflammatory cytokines and adhesion molecules was studied. Thus, aortas, aortic roots and livers were harvested and homogenized for total RNA isolation. After cDNA

Results

synthesis samples were used in quantitative real-time PCR to identify differences in gene expression between *C5ar2^{-/-}/Apoe^{-/-}* and *Apoe^{-/-}* mice. Gene expression was normalized to the house-keeping gene GAPDH. Furthermore, cytokine secretion was studied in serum blood samples of *C5ar2^{-/-}/Apoe^{-/-}* mice.

4.2.3.1 Analysis of gene expression

As two well-accepted pro-inflammatory cytokines, the gene expression of TNF- α and IL-6 was examined, as well as the expression of the adhesion molecule VCAM-1. It has been shown, that TNF- α can activate ECs and is partly responsible for smooth muscle death in atherosclerotic lesions [162]. IL-6, on the other hand, can activate macrophages and it has been shown to exacerbate atherogenesis [193]. As a pro-atherogenic molecule, VCAM-1 is critical for the initiation and progression of atherosclerosis by playing a significant role in the recruitment of circulating inflammatory cells to the vascular endothelium [194, 195]. It is expressed on vascular ECs, SMCs and tissue macrophages [196]. In atherosclerotic lesions, VCAM-1 was selectively upregulated in SMCs [197]. Blocking of VCAM-1 reduces monocytes adhesion to arteries of *Apoe^{-/-}* mice under shear stress conditions [198]. It was previously reported that VCAM-1 was upregulated in neointimal plaque of carotid arteries after vascular injury, which was reduced by treatment with C5aR1 antagonist [151]. Thus, these pro-inflammatory cytokines are taking part in plaque destabilization and lesion progression.

4.2.3.1.1 Gene expression in the aorta

First, as a reduction in lesion size in aortas was observed, the gene expression was therefore analyzed in the whole aortas. We observed a tendency to reduced expression of IL-6 and most notable, a very prominent significant reduction in VCAM-1 expression in *C5ar2^{-/-}/Apoe^{-/-}* aortas after 12 and 24 weeks of HFD (see: Figure 20). Thus, despite there being no significant change in pro-inflammatory IL-6 and TNF- α cytokine expression, the prominent reduction in VCAM-1 points to atheroprotective properties of the C5aR2 deficiency.

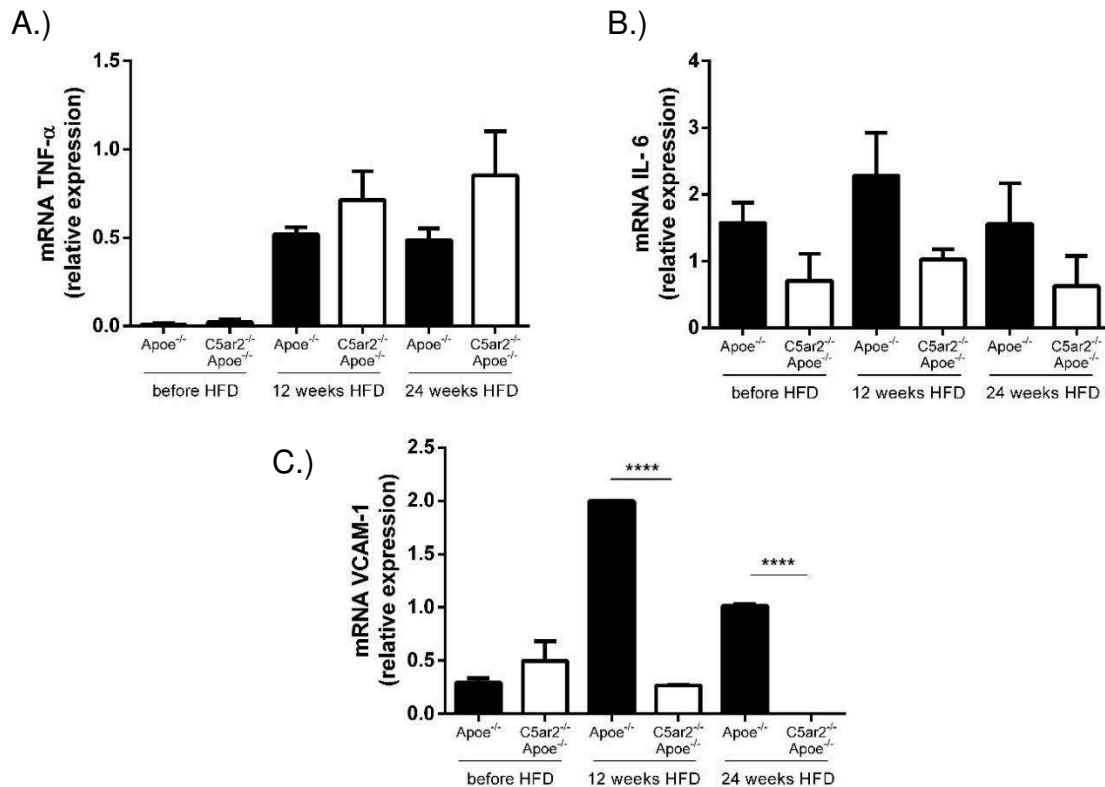


Figure 20: In aortas the *C5ar2*^{-/-}/*Apoe*^{-/-} reduces adhesion molecules. Quantification of (A) TNF-α, (B) IL-6 and (C) VCAM-1 by quantitative real-time PCR in *C5ar2*^{-/-}/*Apoe*^{-/-} and *Apoe*^{-/-} mice. Mice were fed a HFD for 12 or 24 weeks or control chow diet before HFD. Gene expression was normalized to the house-keeping gene GAPDH. Graphs represent the mean ± SEM; n=5-7; 2-tailed t-test; ****p<0,0001.

4.2.3.1.2 Gene expression in the aortic roots

Next, the gene expression in aortic roots was analyzed. After 24 weeks of HFD the expression of TNF-α and IL-6 was significantly reduced in *C5ar2*^{-/-}/*Apoe*^{-/-} mice compared to *Apoe*^{-/-} controls. We also saw a prominent decrease in VCAM-1 expression after 24 weeks of HFD. Under normal chow diet before HFD conditions, no change was seen neither for TNF-α and for IL-6 nor for VCAM-1 (see. Figure 21). In conclusion, the C5aR2 deficiency is promoting a reduced pro-inflammatory milieu in aortic roots with a general reduction in pro-inflammatory cytokines and adhesion molecules contributing to diminished atherogenesis.

Results

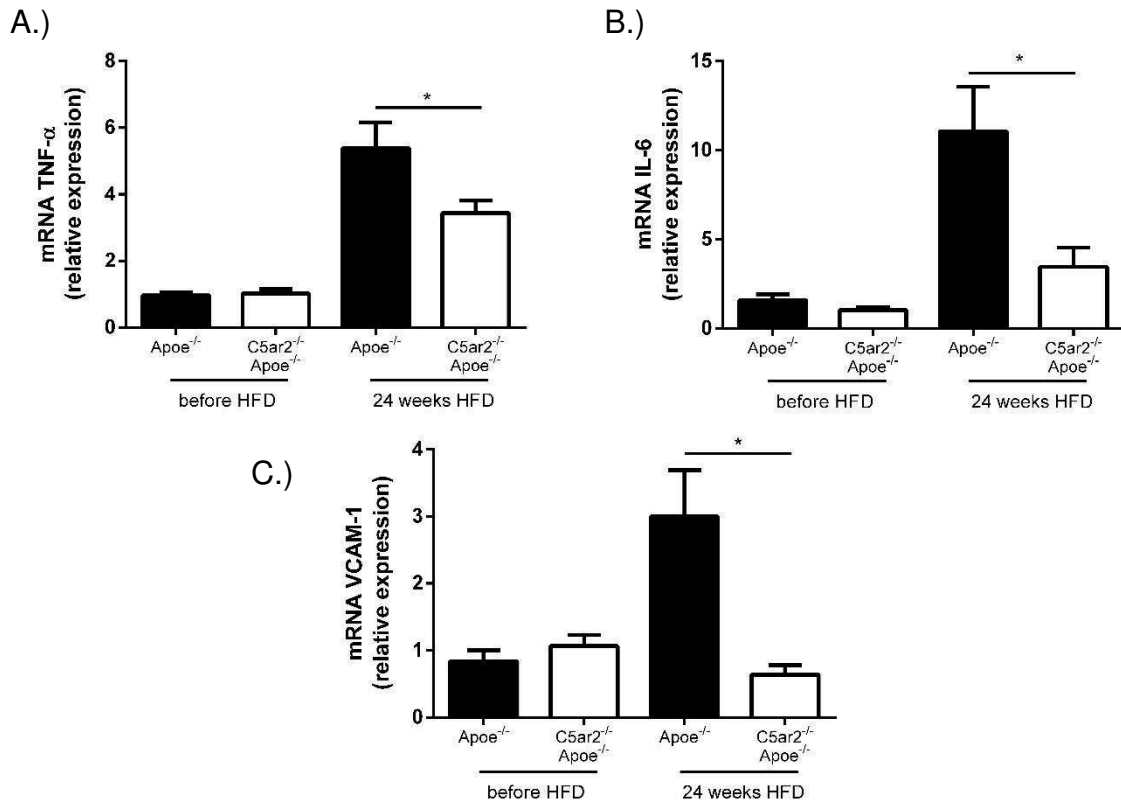


Figure 21: In aortic roots the *C5ar2*^{-/-}/*Apoe*^{-/-} reduces the gene expression of pro-inflammatory cytokines and adhesive molecules. Quantification of (A) TNF-α, (B) IL-6 and (C) VCAM-1 by quantitative real-time PCR in *C5ar2*^{-/-}/*Apoe*^{-/-} and *Apoe*^{-/-} mice. Mice were fed a HFD for 24 weeks or a control chow diet before HFD. Gene expression was normalized to the house-keeping gene GAPDH. Graphs represent the mean ± SEM; n=5-7; 2-tailed t-test; *p<0,05.

4.2.3.1.3 Gene expression in the liver

Finally, the gene expression of pro-inflammatory cytokines and of C5 was analyzed in livers. Due to the prominent role of the liver in the production of these complement components, it was thus of interest to examine the effect of C5aR2 deficiency on the expression of pro-inflammatory cytokines and C5 component. A significant increase of TNF-α expression after 24 weeks HFD was seen, which was still unchanged after 12 weeks. The IL-6 expression is also significantly increased after 12 and 24 weeks. For both, TNF- α and IL-6 no change could be found for mice on chow diet before HFD. C5 expression is unaltered after 12 and 24 weeks in *C5ar2*^{-/-}/*Apoe*^{-/-} compared to *Apoe*^{-/-} control mice, but increased under chow diet before HFD conditions (see: Figure 22). In summary, in the liver of *C5ar2*^{-/-}/*Apoe*^{-/-} mice an inflammatory milieu under long-term HFD conditions prevails. This inflammatory response is likely due to a stress response to HFD conditions rather than caused by the C5aR2 deficiency.

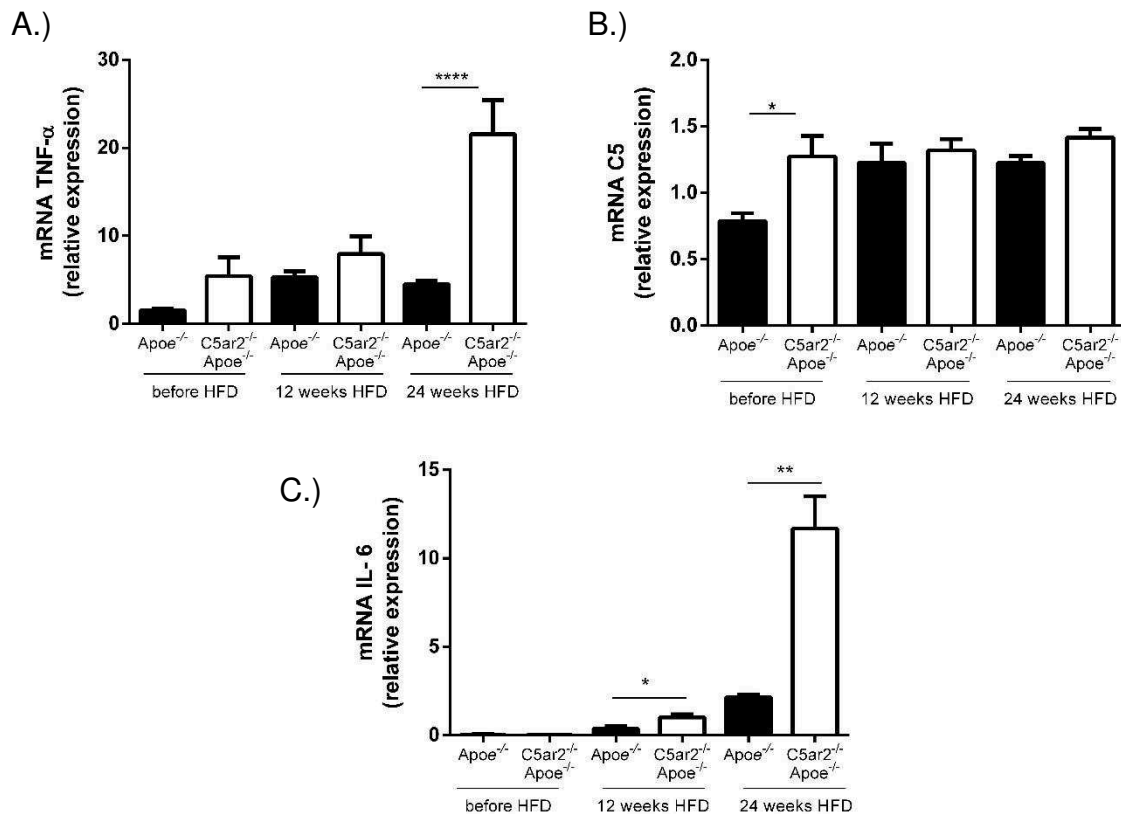


Figure 22: Changes in the gene expression of pro-inflammatory cytokines and complement component C5 in the liver of *C5ar2*^{-/-}/*Apoe*^{-/-} mice. Quantification of (A) TNF-α, (B) C5 and (C) IL-6 by quantitative real-time PCR in *C5ar2*^{-/-}/*Apoe*^{-/-} and *Apoe*^{-/-} mice. Mice were fed a HFD for 12 or 24 weeks or control chow diet before HFD. Gene expression was normalized to the house-keeping gene GAPDH. Graphs represent the mean ± SEM; n=5-7; 2-tailed t-test; *p<0,05; **p<0,01; ****p<0,0001.

4.2.3.2 Analysis of pro-inflammatory cytokines by ELISA

To figure out if the changes in gene expression reflects in systemic blood cytokine levels, ELISAs for the pro-inflammatory cytokine IL-6 were performed. Therefore, blood was drawn by cardiac puncture and blood serum was separated. The level of IL-6 was not altered under chow diet before HFD conditions but it was significantly elevated after 12 weeks of HFD in *C5ar2*^{-/-}/*Apoe*^{-/-} mice. A tendency for increased levels can be seen for 24 weeks HFD. (see: Figure 23). In conclusion, the elevated IL-6 serum concentration rather may be caused by the high IL-6 liver expression than by the C5aR2 deficiency.

Results

4.2.3.2.1 Analysis of IL-6 by ELISA

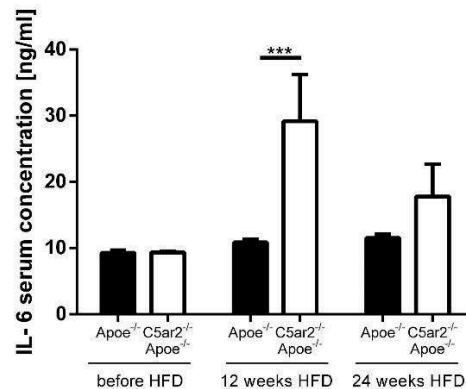


Figure 23: Blood serum concentration of IL-6 is increased after 12 weeks of HFD in *C5ar2*^{-/-}/*Apoe*^{-/-} mice. Analysis of blood serum concentration of IL-6 in *C5ar2*^{-/-}/*Apoe*^{-/-} and *Apoe*^{-/-} mice after 12 or 24 weeks HFD and und control chow diet before HFD condition. Graphs represent the mean $\pm$ SEM; n=5; 2-tailed t-test; ***p<0,001.

In conclusion, in diet-induced chronic atherosclerosis the C5aR2 shows pro-inflammatory properties. In *C5ar2*^{-/-}/*Apoe*^{-/-} mice, this manifests in a reduced lesion size in the aorta and in aortic roots. This is accompanied by a more stable plaque with reduced pro-inflammatory mediators and cells.

4.3 Effect of the C5aR2 deficiency on neointima formation after wire-induced arterial injury

To study acute atherosclerosis and neointima formation after arterial injury, hyperlipidemic 8 weeks old male *C5ar2^{-/-}/Apoe^{-/-}* and *Apoe^{-/-}* mice were fed a HFD containing 21 % total fat, 0,15 % cholesterol und 19,5 % casein. The model of restenosis is mimicking the acute inflammatory processes occurring after surgical vascular intervention, like stent implantation. These injury-induced inflammatory processes can lead to neointima formation causing re-narrowing of vessel lumen, which then is called restenosis. The underlying pathophysiological mechanisms differ in restenosis and chronic diet-induced atherosclerosis [43, 44]. An influx and accumulation of lipids in the tunica intima and a recruitment of inflammatory cells characterize the chronic atherosclerosis [31, 45]. In acute atherosclerosis, on the other hand, neointima formation is characterized by a proliferation and migration of SMCs from the tunica media into the tunica intima [47]. Removal of the endothelium results in a burst of SMC migration and proliferation, which subsides when the endothelium regenerates [34]. This restenosis therefore functions to restore vessel wall integrity and function [47, 48]. However, this restoring of the vessel wall integrity also leads to the narrowing of the vascular lumen, diminishing blood flow and oxygen supply.

Thus, after 4 weeks of HFD the left carotid artery was injured by the removal of the endothelium with a guide-wire (for wire injury see: 3.2.1.5). Four weeks after injury was inflicted, the mice were sacrificed and the left as well as the right carotid artery were collected and analyzed regarding atherosclerotic lesion size and cellular composition. Additionally, alterations in gene expression for pro-inflammatory cytokines and adhesions molecules were identified and right carotid arteries were taken as a control.

Initially, whole blood count was analyzed to identify changes in the recruitment of bone marrow cells. Blood of *C5ar2^{-/-}/Apoe^{-/-}* and *Apoe^{-/-}* mice was analyzed for differences in whole blood cell count. The blood was drawn by cardiac puncture and was transferred into serum tubes. Blood was analyzed in an automated analysis aperture. No significant difference in leukocytes or monocyte count was observed after wire-induced endothelial injury after HFD. Leukocyte count was expressed as a percentage of total cell count in the blood. Monocytes were expressed as a percentage of leukocytes (see: Figure 24). This observation indicates that the C5aR2 deficiency is

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not influencing the number of circulating monocytes, which could affect lesional macrophage numbers and lesion progression.

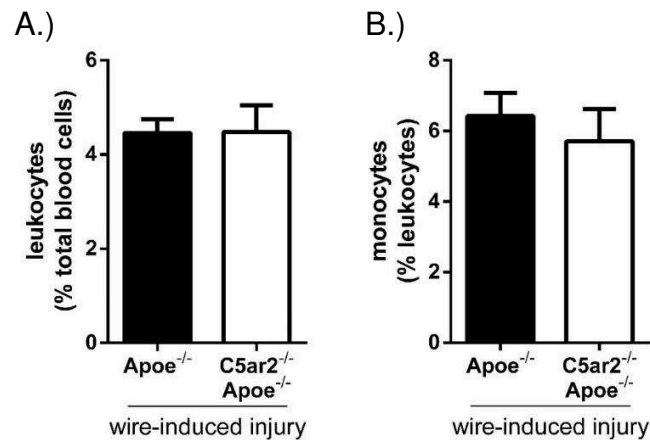


Figure 24: C5aR2 deficiency does not alter leukocyte or monocyte count after wire-induced injury. (A) Quantification of the leukocyte count in whole blood samples from *Apoe*^{-/-} and *C5ar2*^{-/-}*Apoe*^{-/-} mice. (B) Quantification of the monocyte count in whole blood samples from *Apoe*^{-/-} and *C5ar2*^{-/-}*Apoe*^{-/-} mice. Leukocytes are expressed as percentage of whole blood cell count, and monocytes as percentage of leukocytes, respectively. Graphs represent the mean ± SEM; n=15-20; 2-tailed t-test.

4.3.1 C5aR2 deficiency reduces neointima formation after endothelial denudation

As already demonstrated the C5aR2 deficiency resulted in a reduction in pro-inflammatory conditions which lead to a reduction in atherosclerotic plaque formation in the murine chronic diet-induced atherosclerosis model. The next step was to evaluate if *C5ar2*^{-/-}*Apoe*^{-/-} mice also may lead to reduced inflammatory processes following acute wire-induced neointima formation in restenosis. Restenosis is characterized by a migration of SMCs from the tunica media into the tunica intima [47]. This restenosis serves to restore vessel wall integrity and function [47, 48]. After wire-induced endothelial denudation, the carotid arteries were harvested and stained with Movat's pentachrome staining. Analysis of neointima formation in carotid arteries revealed a significant reduction in *C5ar2*^{-/-}*Apoe*^{-/-} compared to *Apoe*^{-/-} control mice (see: Figure 25). Thus, in acute atherosclerosis the C5aR2 harbors pro-inflammatory properties facilitating restenosis with subsequent re-narrowing of arterial lumen.

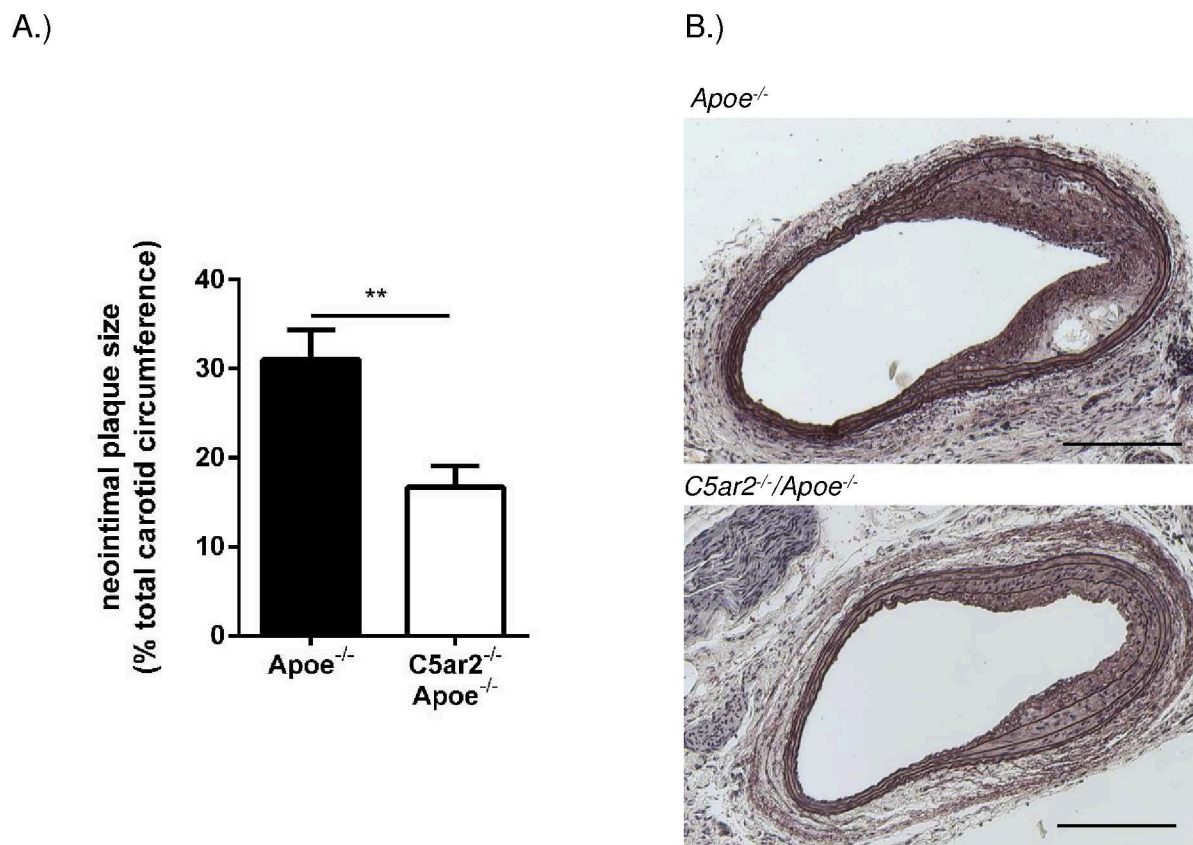


Figure 25: Reduced neointima formation in carotid arteries of *C5ar2*^{-/-}/*Apoe*^{-/-} deficient mice after wire-induced endothelial denudation. In *C5ar2*^{-/-}/*Apoe*^{-/-} and *Apoe*^{-/-} mice endothelium in the left carotid artery was removed with a guide-wire. Carotid arteries were collected and stained with Movat's pentachrome staining. (A) Quantification of neointima formation expressed as percentage of total artery circumference. (B) Photographed carotid arteries stained with Movat's pentachrome staining. Graphs represent the mean $\pm$ SEM; Scale bars = 100 μ m; n=10-12; 2-tailed t-test; **p<0,01.

4.3.2 Cellular composition of the plaque

To clarify the findings of the reduced lesion size in carotid arteries, the cells known to be involved in atherosclerosis were studied. Macrophages and SMCs play a central role in lesion progression and stability [55]. Furthermore, T-cell count (CD3), the number of proliferating cells (Ki-67), the influence on apoptosis (Tunel) and cells positive for IFN- γ , TNF- α and C5aR1 (CD88) were studied in detail to identify the effect of C5aR2 signaling in the context of neointima formation.

4.3.2.1 Smooth muscle cells and macrophages

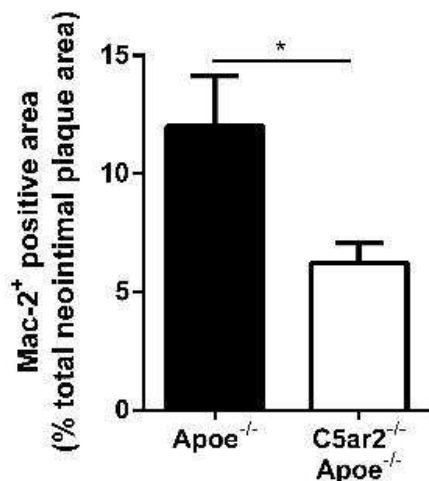
Macrophages and SMCs play a central role in neointima formation and atherosclerotic plaque stability. Therefore, changes in macrophages and SMCs were identified by immunofluorescent staining. Carotid arteries were harvested and paraffin embedded section were stained for macrophages (Mac-2) and SMCs (α -SMA). The Mac-2 or

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α -SMA positive area was assessed and expressed as percentage of the whole lesion size.

We observed a significant reduction in macrophages in the neointima of *C5ar2^{-/-}/Apoe^{-/-}* mice compared to their corresponding *Apoe^{-/-}* control mice (see. Figure 26). SMCs show a significant increase in *C5ar2^{-/-}/Apoe^{-/-}* mice compared to their corresponding *Apoe^{-/-}* control mice (see: Figure 27). The data suggest that the C5aR2 acts in a pro-inflammatory manner in neointima formation. Additionally, in *C5ar2^{-/-}/Apoe^{-/-}* mice neointimal plaques show features of a more stable plaque with reduced macrophages and increased SMCs forming a more prominent fibrous cap.

A.)



B.)

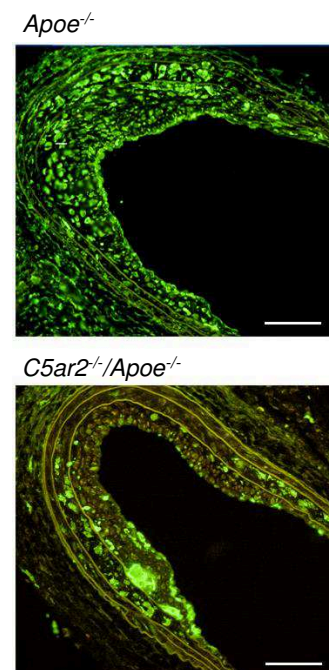
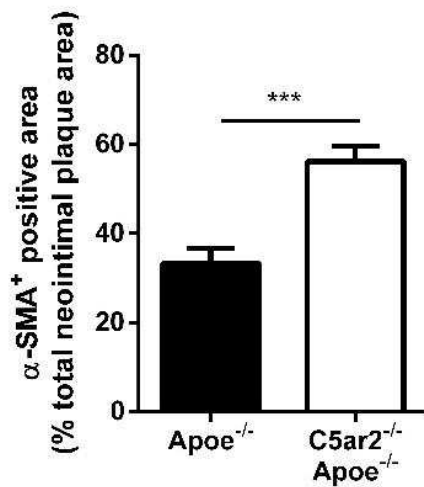


Figure 26: C5aR2 deficiency leads to decreased macrophage content in neointima in carotid arteries. (A) Quantification of macrophage content in neointima of *C5ar2^{-/-}/Apoe^{-/-}* and *Apoe^{-/-}* mice by immunofluorescent staining for Mac-2. (B) Representative pictures of Mac-2 stained carotid arteries. Green color represents Mac-2⁺ area. Quantifications are expressed as percentage of positive area to total neointima plaque area. Graphs represent the mean $\pm$ SEM; Scale bars = 50 μ m; n=12-15; 2-tailed t-test; *p<0,05.

A.)



B.)

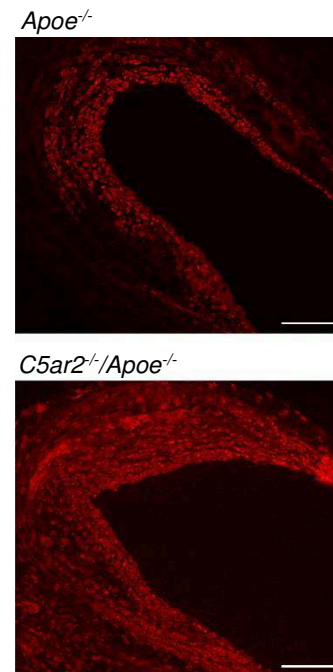


Figure 27: C5aR2 deficiency leads to increased SMC content in neointima in carotid arteries. (A) Quantification of SMC content in neointima of *C5ar2*^{-/-}/*Apoe*^{-/-} and *Apoe*^{-/-} mice by immunofluorescent staining for α-SMA. (B) Representative pictures of α-SMA stained carotid arteries. Red color represents α-SMA⁺ area. Quantifications are expressed as percentage of positive area to total neointima plaque area. Graphs represent the mean ± SEM; Scale bars = 50 μm; n=12-15; 2-tailed t-test; ***p<0,001.

4.3.2.2 Analysis of different cell types involved in neointima formation

Next to macrophages and SMCs various other cell types are present and involved in plaque homeostasis and in lesion progression. Thus, to further analyze the processes and cell types involved in neointima formation after wire-induced injury of the carotid artery T-cell count (CD3), the number of proliferating cells (Ki-67), the influence on apoptosis (Tunel) and the count of IFN-γ, TNF-α or C5aR1 (CD88) expressing cells were analyzed. This should provide us with a deeper insight into the observed reduction of the neointima formation in *C5ar2*^{-/-}/*Apoe*^{-/-} compared to *Apoe*^{-/-} mice. Here, positive cells are expressed as a percentage of total cell count within neointima formation. No neointima formation could be found in the right uninjured control carotid artery, thus no cell count could be determined.

In the neointima of *C5ar2*^{-/-}/*Apoe*^{-/-} mice, a significant reduction in T-cell count can be observed. Additionally, the number of C5aR1 (CD88), IFN-γ and TNF-α positive cells were significantly reduced in *C5ar2*^{-/-}/*Apoe*^{-/-} mice compared to *Apoe*^{-/-} control mice. Furthermore, apoptosis (Tunel) was reduced in *C5ar2*^{-/-}/*Apoe*^{-/-} mice. Finally, no

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change in proliferating cells (ki-67⁺) is present. (See: Figure 28). Taken together, Analysis of the different cell types residing in the neointimal plaque revealed a major reduction in inflammatory cells and markers. This points towards the explanation that C5aR2 acts as a pro-inflammatory receptor in atherosclerosis, which is involved in activating pro-inflammatory cells and facilitating neointima formation.

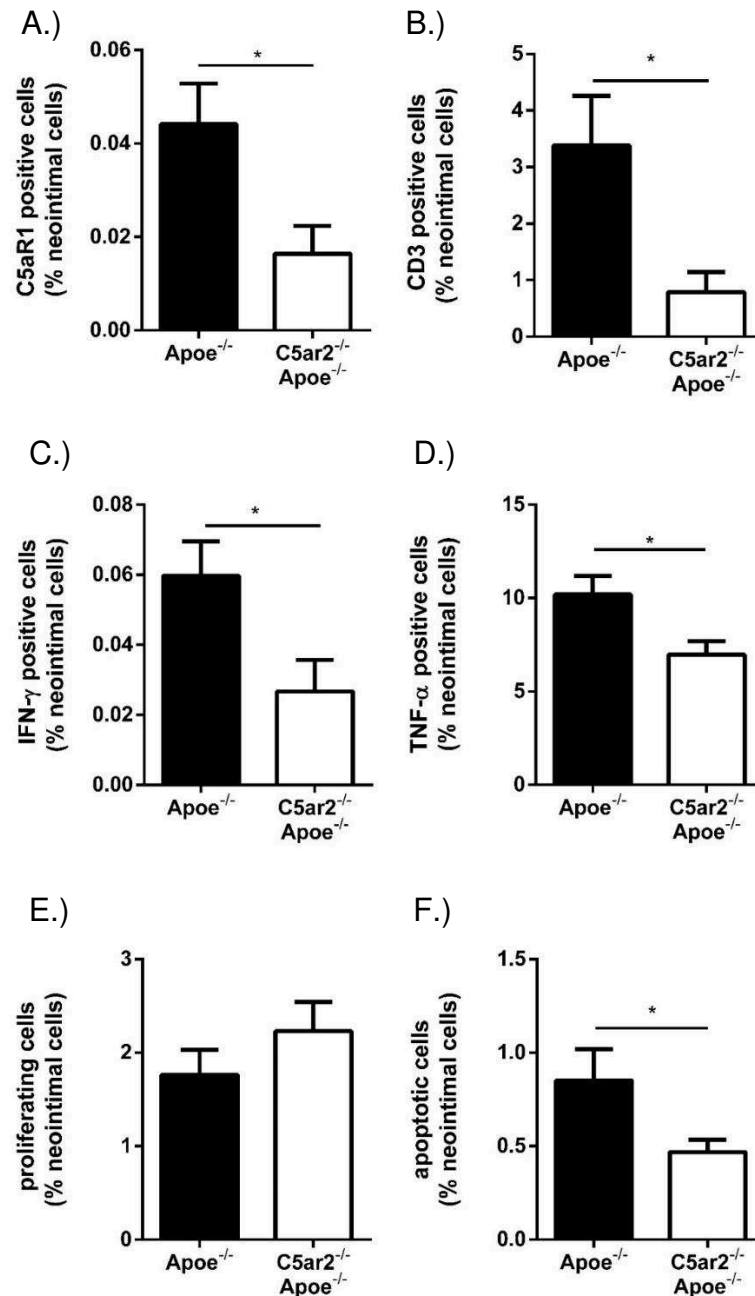


Figure 28: Analysis of different cell types present in neointima formation. Immunofluorescence staining of serial section of the left carotid artery after wire-induced injury in *C5ar2*^{-/-}/*Apoe*^{-/-} and *Apoe*^{-/-} control mice. Quantification of (A) C5aR1⁺, (B) CD3⁺, (C) IFN-γ⁺, (D) TNF-α⁺ and (E) proliferating cells as well as (F) apoptotic cells. Quantifications are expressed as percentage of total neointimal cells. Graphs represent the mean ± SEM; n=6-8; 2-tailed t-test; *p<0,05.

4.3.3 Changes in gene expression in carotid arteries

Next, the expression level of pro-inflammatory cytokines and adhesion molecules was determined. Differences in the expression might explain the differences in cell count in the neointima formation as these molecules are effecting the general inflammatory response. The main source of these cytokines are monocytes/macrophages and T-cells. [199]. So it was shown, that TNF- α and IL-1 α / β is primarily produced by monocytes and macrophages [200]. Additionally, the cytokine IFN- γ is expressed in atherosclerotic lesions by a set of different cells, mainly by T-cells and in a lesser amount by monocytes/macrophages and natural killer T-cells [201]. Furthermore, it was demonstrated that for optimal induction of pro-inflammatory cytokines a functionally working C5aR2 is necessary [147].

Therefore, the mRNA expression of TNF- α , IL-6, IFN- γ , RANTES, HMGB1 and VCAM-1 was evaluated. It has been shown that TNF- α can activate ECs and is partly responsible for smooth muscle death in atherosclerotic lesions [162]. IL-6, on the other hand, can activate macrophages and it was shown to exacerbate atherogenesis [193]. Rittirsch and colleagues demonstrating that the release of the pro-inflammatory cytokine HMGB1 depends on a functional C5aR2, but not C5aR1, thus it was of interest if the C5aR2 deficiency affects HMGB1 expression [137]. Additionally, it was also reported that the T-lymphocytes produce IFN- γ , which inhibits the proliferation of SMCs and their ability to synthesize collagen [163, 164]. Finally, RANTES was shown to aggravate atherosclerosis [165]. In general, a high VCAM-1 expression is correlated with an elevated recruitment of inflammatory cell into the lesion site. It was demonstrated to be expressed on macrophages, SMCs and vascular ECs [196]. It is critical for the initiation and progression of atherosclerosis by recruiting circulating inflammatory cells [194, 195] and it was shown to be upregulated in SMCs in atherosclerotic lesions [197]. Treatment with a C5aR1 antagonist reduced VCAM-1 in neointimal plaques after vascular injury [151].

After cDNA synthesis samples were used in quantitative real-time PCR to identify differences in gene expression between *C5ar2*^{-/-}/*Apoe*^{-/-} and *Apoe*^{-/-} control mice. Gene expression was normalized to the house-keeping gene GAPDH.

TNF- α , IL-6, RANTES and VCAM-1 expression was significantly increased in injured left carotid arteries compared to uninjured right carotid arteries in both, *Apoe*^{-/-} and *C5ar2*^{-/-}/*Apoe*^{-/-} mice. And importantly, expression of all these key inflammatory cytokines was significantly reduced in *C5ar2*^{-/-}/*Apoe*^{-/-} injured compared to *Apoe*^{-/-}

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injured carotid arteries. For the cytokines. IFN- γ and HMGB1 we also observed a significant increased expression in injured left carotid arteries compared to uninjured right carotid arteries in *Apoe*^{-/-} mice. But here in *C5ar2*^{-/-}/*Apoe*^{-/-} mice, IFN- γ expression is significantly reduced in injured arteries compared to uninjured arteries, whereas for. HMGB1 no difference can be found. However, also for IFN- γ and HMGB1 the expression was significantly reduced in *C5ar2*^{-/-}/*Apoe*^{-/-} injured compared to *Apoe*^{-/-} injured carotid arteries. (see: Figure 29). In summary, the C5aR2 deficiency is reducing pro-inflammatory cytokines in injury-induced restenosis, which contributes to the reduction in atherosclerotic lesion size. Thus, in restenosis C5aR2 acts in a pro-inflammatory way on cytokine production.

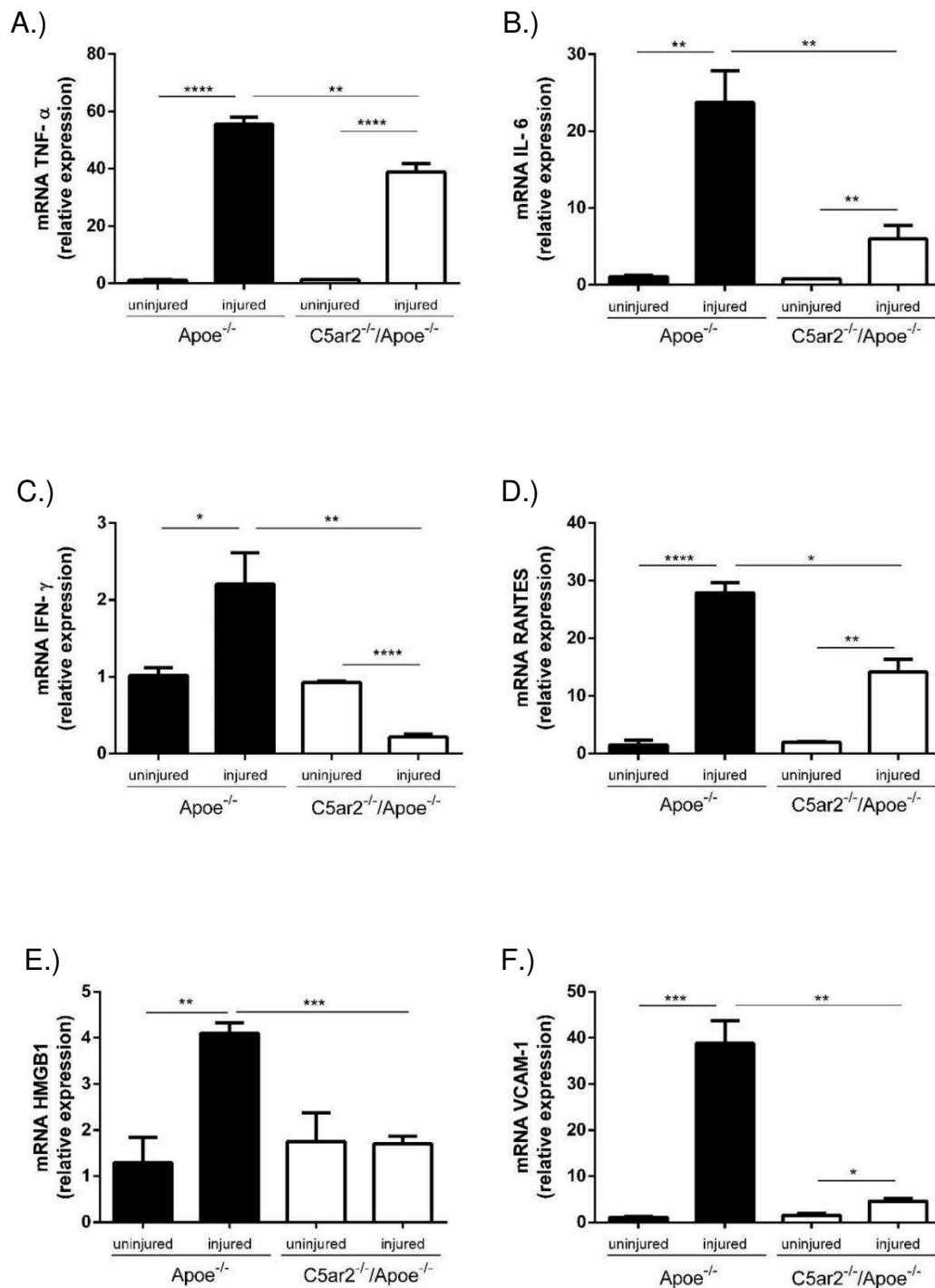


Figure 29: In carotid arteries the C5aR2 deficiency reduces the gene expression of pro-inflammatory cytokines and adhesion molecules after endothelial denudation. Quantification of (A) TNF- α , (B) IL-6, (C) IFN- γ , (D) RANTES, (E) HMGB1 and (F) VCAM-1 by quantitative real-time PCR in uninjured right and injured left carotid arteries of *Apoe*^{-/-} and *C5ar2*^{-/-}/*Apoe*^{-/-} mice. Mice were fed a HFD 4 weeks before and after endothelial denudation. Gene expression was normalized to the house-keeping gene GAPDH. Graphs represent the mean $\pm$ SEM; n=5-6; 2-tailed t-test; *p<0,05; **p<0,01; ***p<0,001; ****p<0,0001.

4.3.3.1 Neointima formation under non-hyperlipidemic conditions

To check whether the reduction in neointimal plaque size and cytokine production is driven by hypercholesterolemic background (apolipoprotein E deficiency), the above experiments were performed in *C5ar2*^{-/-} single knock-out and control WT mice. Less neointimal plaque size was detected in *C5ar2*^{-/-} single knock-out mice, however, these results achieved no statistical significance (see: Figure 30).

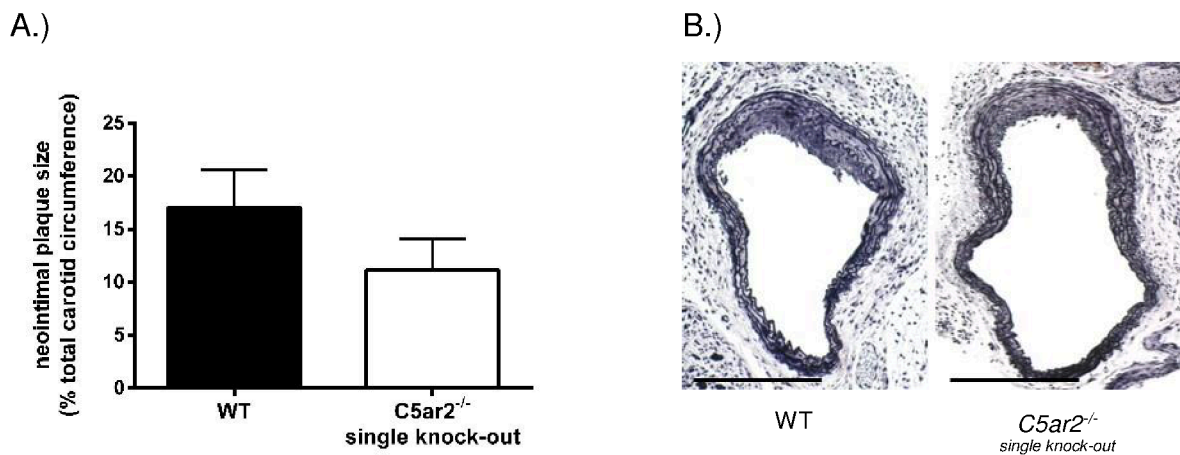


Figure 30: *C5ar2*^{-/-} single knock-out does not alter neointima formation in carotid arteries after wire-induced endothelial denudation. In *C5ar2*^{-/-} single knock-out and WT mice endothelium in the left carotid artery was removed with a guide-wire. Carotid arteries were collected and stained with Movat's pentachrome staining. (A) Quantification of neointima formation expressed as percentage of total artery lumen. (B) Photographed carotid arteries stained with Movat's pentachrome staining. Graphs represent the mean $\pm$ SEM; Scale bars = 200 μ m; n=5; 2-tailed t-test.

However, analysis of the cellular composition revealed significantly less lesional macrophages, whereas SMCs were unchanged within the neointima of *C5ar2*^{-/-} single knock-out carotid arteries compared to WT (see: Figure 31).

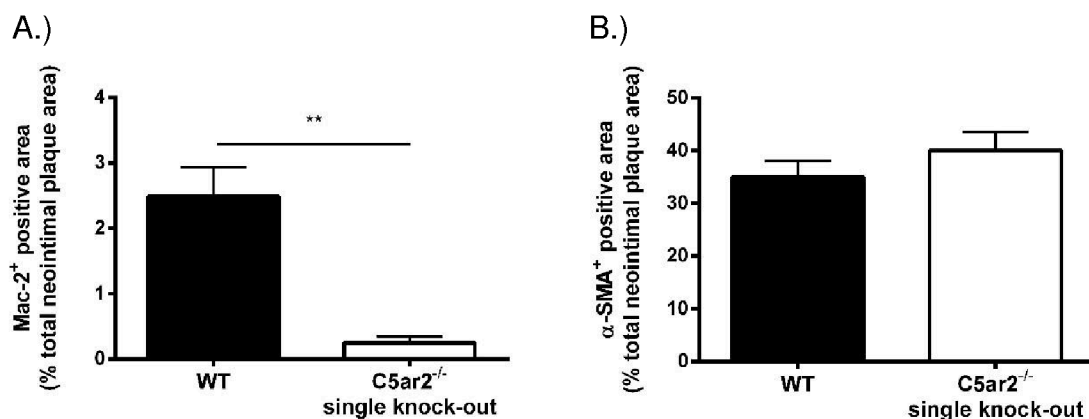


Figure 31: *C5ar2*^{-/-} single knock-out leads to decreased macrophages content in neointima in carotid arteries. (A) Quantification of macrophage content in neointima in WT and *C5ar2*^{-/-} single knock-out mice. (B) Quantification of SMC content in neointima in WT and *C5ar2*^{-/-} mice. Quantifications are expressed as percentage of positive area to total neointima plaque area. Graphs represent the mean $\pm$ SEM; n=5; 2-tailed t-test; **p<0,01.

Next, as no change in neointimal lesion size was observed, but a different macrophage content, the influence of the *C5ar2*^{-/-} single knock-out on pro-inflammatory cytokine production and adhesion molecules was analyzed. Here, a significant reduction in TNF- α expression in injured carotid arteries of *C5ar2*^{-/-} single knock-out mice compared to WT was observed. The expression of the adhesion molecule VCAM-1 was unaltered in injured arteries. Comparison of uninjured and injured WT carotid arteries, however, revealed a significant increase of VCAM-1 expression in injured carotid arteries (see: Figure 32).

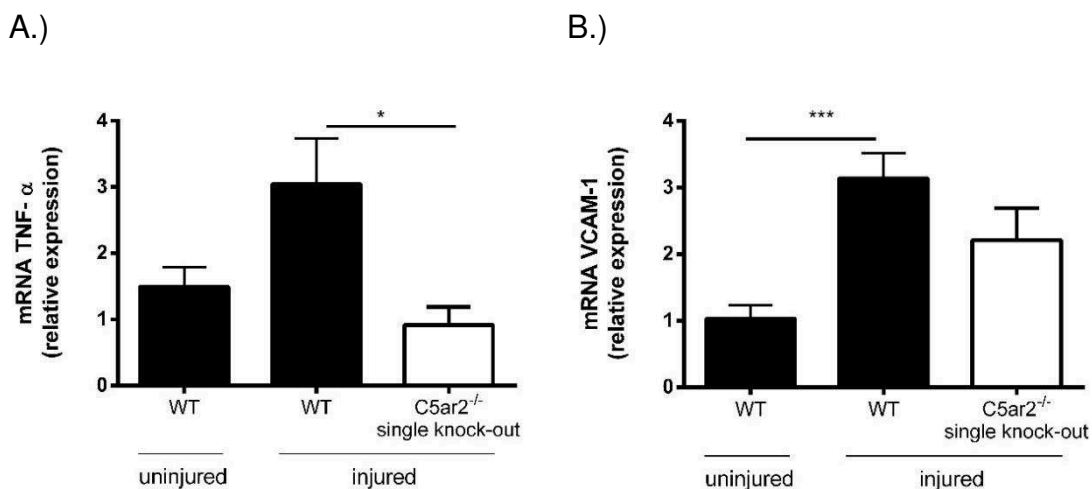


Figure 32: In carotid arteries the *C5ar2*^{-/-} single knock-out reduces the gene expression of pro-inflammatory cytokines. Quantification of (A) TNF- α , and (B) VCAM-1 by quantitative real-time PCR in carotid arteries of WT and *C5ar2*^{-/-} single knock-out mice. Mice were fed a HFD 4 weeks before and after endothelial denudation was performed. Gene expression was normalized to the house-keeping gene GAPDH. Graphs represent the mean $\pm$ SEM; n=5-6; 2-tailed t-test; *p<0,05; ***p<0,001.

The data obtained from *C5ar2*^{-/-} single knock-out and WT mice indicating that the effect of C5aR2 on neointima formation after arterial injury is most effective with a hypercholesterolemic background. This can provide an additional positive effect in handling atherosclerosis by manipulating C5aR2 activity, as hyperlipidemia is a major precondition in human atherosclerosis initiation.

Summarizing the model of acute atherosclerosis, the C5aR2 deficiency leads to a reduction in neointima formation after endothelial denudation accompanied with reduced macrophages and an increased amount of SMCs. Additionally, cells positive for pro-inflammatory cytokines were reduced within the plaque. Furthermore, apoptosis was reduced, whereas proliferation is not effected by the deficiency. Taken together, the deficiency reduces the gene expression of pro-inflammatory cytokines in injured carotid arteries, contributing to an anti-atherogenic milieu. These findings further strengthen the hypothesis of C5aR2 as a pro-inflammatory receptor.

4.4 Influence of the C5aR2 deficiency on lipid metabolism

Atherosclerosis is an inflammatory disease of the vessel wall of large arteries. One of the major events in lesion initiation and progression is the influx of cholesterol complexed with LDL into the tunica intima. Macrophages can take up modified LDL by scavenger receptors like CD36 [202, 203]. An excess of intracellular modified LDL can lead to foam cell formation. Foam cells in turn undergo apoptosis which can facilitate atherosclerotic lesion progression [42].

It has also been reported that the C5aR2 is involved in the lipid metabolism. C5aR2 is a receptor for the acylation-stimulating protein (ASP), which is identically to C3a des Arg [138]. Via ASP C5aR2 is involved in regulating the lipid uptake and metabolism in various cell types, like the adipocytes. Activation of C5aR2 by ASP leads to an increased triglyceride synthesis in adipocytes [138], a property which is unique and solely possessed by the C5aR2, but not by the C5aR1, as no ASP C5aR1 interaction was reported so far [204]. Furthermore, hypercholesterolemia is seen as an imbalance in intra- and extracellular cholesterol homeostatic mechanisms [205]. As hypercholesterolemia is a crucial prerequisite for atherogenesis, the role of the C5aR2 deficiency in these processes was studied in detail. Therefore, body weight, triglyceride and cholesterol level in blood samples were analyzed in *C5ar2^{-/-}/Apoe^{-/-}* mice compared to *Apoe^{-/-}* mice under chow diet and HFD conditions.

4.4.1 Analyses of the body weight

Body weight of *Apoe^{-/-}* and *C5ar2^{-/-}/Apoe^{-/-}* mice was measured before the experiments and after 12 and 24 weeks of HFD or chow diet (see: Table 17). All values are displayed as mean $\pm$ SEM. * $p < 0,05$; *** $p < 0,001$; *Apoe^{-/-}* vs. *C5ar2^{-/-}/Apoe^{-/-}* mice.

It has been found that *C5ar2^{-/-}/Apoe^{-/-}* mice have an unaltered body weight compared to *Apoe^{-/-}* before experiments. After receiving HFD for 12 or 24 weeks the *C5ar2^{-/-}/Apoe^{-/-}* mice showed a prominent increase in body weight. When receiving a chow diet *C5ar2^{-/-}/Apoe^{-/-}* mice also showed a slightly significant increase in body weight after 12 and 24 weeks (see: Table 17). Taking together, the C5aR2 deficiency is favoring an increased body weight under chow diet and HFD conditions.

Table 17: Body weight (in gram) of *C5ar2*^{-/-}/*Apoe*^{-/-} and *Apoe*^{-/-} mice

All values are displayed as mean $\pm$ SEM. n=12-15; 2-tailed t-test; * p<0,05; *** p<0,001; *Apoe*^{-/-} vs. *C5ar2*^{-/-}/*Apoe*^{-/-} mice.

	before experiment	12 weeks	24 weeks
HFD			
<i>Apoe</i> ^{-/-}	20,8 $\pm$ 0,9	24,7 $\pm$ 0,4	26,1 $\pm$ 0,8
<i>C5ar2</i> ^{-/-} / <i>Apoe</i> ^{-/-}	20,4 $\pm$ 0,9	27,0 $\pm$ 0,4***	28,7 $\pm$ 0,8*
Chow diet			
<i>Apoe</i> ^{-/-}	20,8 $\pm$ 0,9	27,2 $\pm$ 0,5	31,0 $\pm$ 0,5
<i>C5ar2</i> ^{-/-} / <i>Apoe</i> ^{-/-}	20,4 $\pm$ 0,9	30,7 $\pm$ 1,8*	32,5 $\pm$ 0,6*

4.4.2 Changes in lipid metabolism

Next, the blood lipid concentration of cholesterol and triglycerides was measured in serum samples of *Apoe*^{-/-} and *C5ar2*^{-/-}/*Apoe*^{-/-} mice. Differences in cholesterol and triglycerides might explain the differences in body weight observed before. Blood was drawn by cardiac puncture and transferred to serum tubes. Concentration was measure in blood of *Apoe*^{-/-} vs. *C5ar2*^{-/-}/*Apoe*^{-/-} mice before the experiments and after 12 and 24 weeks of HFD or chow diet. All values are displayed as mean $\pm$ SEM. *p<0,05; *** p<0,001; *Apoe*^{-/-} vs. *C5ar2*^{-/-}/*Apoe*^{-/-} mice.

The C5aR2 deficiency is resulting in reduced cholesterol serum levels under chow diet and HFD (see: Table 18). Triglyceride levels are significantly increased after 12 and 24 weeks of chow diet and HFD. (see: Table 19). Thus, changes in triglyceride and cholesterol serum levels can influence and promote atherogenesis indicating pro-atherogenic properties of the C5aR2.

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Table 18: Cholesterol blood concentration in *C5ar2^{-/-}/Apoe^{-/-}* and *Apoe^{-/-}* mice

All values are displayed as mean $\pm$ SEM. n=12-15; 2-tailed t-test; * p<0,05; *** p<0,001; *Apoe^{-/-}* vs. *C5ar2^{-/-}/Apoe^{-/-}* mice.

	before experiment	12 weeks	24 weeks
HFD			
<i>Apoe^{-/-}</i>	6,9 $\pm$ 0,4	15,2 $\pm$ 0,5	16,8 $\pm$ 0,8
<i>C5ar2^{-/-}/Apoe^{-/-}</i>	5,1 $\pm$ 0,5*	13,7 $\pm$ 1,4*	13,6 $\pm$ 0,6*
Chow diet			
<i>Apoe^{-/-}</i>	6,9 $\pm$ 0,4	14,2 $\pm$ 0,8	9,1 $\pm$ 1,0
<i>C5ar2^{-/-}/Apoe^{-/-}</i>	5,1 $\pm$ 0,5*	8,3 $\pm$ 1,1*	5,5 $\pm$ 0,9*

Table 19: Triglyceride blood concentration in *C5ar2^{-/-}/Apoe^{-/-}* and *Apoe^{-/-}* mice

All values are displayed as mean $\pm$ SEM. n=12-15; 2-tailed t-test; *** p<0,001; *Apoe^{-/-}* vs. *C5ar2^{-/-}/Apoe^{-/-}* mice.

	before experiment	12 weeks	24 weeks
HFD			
<i>Apoe^{-/-}</i>	0,9 $\pm$ 0,2	1,3 $\pm$ 0,1	1,1 $\pm$ 0,2
<i>C5ar2^{-/-}/Apoe^{-/-}</i>	1,1 $\pm$ 0,2	1,8 $\pm$ 0,2*	2,1 $\pm$ 0,1***
Chow diet			
<i>Apoe^{-/-}</i>	0,9 $\pm$ 0,2	0,8 $\pm$ 0,2	0,8 $\pm$ 0,1
<i>C5ar2^{-/-}/Apoe^{-/-}</i>	1,1 $\pm$ 0,2	1,5 $\pm$ 0,1*	1,8 $\pm$ 0,1***

Herein summary, it is shown that C5aR2 is involved in multiple mechanisms of lipid metabolism resulting in an impact on body weight and serum lipids. Thus, C5aR2 might contribute to atherogenesis via its modulation of lipid homeostasis.

4.5 In vitro mechanistic analysis of bone marrow-derived macrophages

Macrophages play an important role in atherogenesis. One of the major events in lesion initiation and progression is the influx of cholesterol and modified LDL, which can be taken up by macrophages through scavenger receptors like CD36 [202, 203]. An excess of intracellular modified LDL can lead to foam cell formation. Foam cells in turn undergo apoptosis, what can facilitate atherosclerotic lesion progression [42]. In contrast, macrophages that express the ABCA1 and ABCG1 receptors, which are responsible for cholesterol efflux; are thus anti-atherogenic [206, 207]. Furthermore, macrophages can acquire different polarization of their phenotype. On the one hand, there is the classical pro-inflammatory M1 phenotype and on the other hand the angiogenic M2 phenotype [62]. By identifying the impact of C5aR2 on macrophage phenotype, a deeper knowledge could be gained how macrophage contribute to atherogenesis. Additionally, C5aR2 is involved in the lipid metabolism via its interaction with ASP [138] and can stimulate triglyceride synthesis [204]. To gain a deeper understanding in the processes influenced by C5aR2 in macrophages and their impact on atherosclerotic initiation and progression, *in vitro* studies with macrophages were performed. Therefore, bone marrow cells were isolated from *C5ar2^{-/-}/Apoe^{-/-}* and *Apoe^{-/-}* mice and *in vitro* differentiated into bone marrow-derived macrophages. They were stimulated with C5a, TNF- α and LPS and analyzed regarding cytokine production, secretion and proliferation. Here, the C5a stimulation was chosen, to identify direct influences on cytokine production caused by a C5aR2-C5a interaction. TNF- α was shown to activate macrophages, thus a known inducer of cytokine production was taken as a stimulus. LPS in general is used as an immunostimulant to mimic an inflammatory milieu. By using this model, the impact of the C5aR2 deficiency on macrophages in an inflammatory environment could be studied. Thus, macrophages were homogenized and total RNA was isolated. After cDNA synthesis samples were used in quantitative real-time PCR to identify differences in gene expression between *C5ar2^{-/-}/Apoe^{-/-}* and *Apoe^{-/-}* control mice. Gene expression was normalized to the house-keeping gene GAPDH. Additionally, supernatants were collected to be analyze for cytokine production.

4.5.1 Gene expression of cytokines and adhesion molecules

To identify the impact of the C5aR2 deficiency on macrophages activation in an inflammatory environment bone marrow-derived macrophages from *C5ar2^{-/-}/Apoe^{-/-}*

Results

and *Apoe*^{-/-} mice were stimulated with LPS and the expression of pro-inflammatory cytokines and adhesion molecules was determined by quantitative real-time PCR. Macrophages have been shown to be a major source of pro-inflammatory cytokines and the integrity of C5aR2 is crucial to induce cytokine secretion. And in human advanced atherosclerotic lesions the amount of C5aR2 positive cells were proportional to the lesional stage [147]. In our study, TNF- α , IL-6 and VCAM-1 expression was significantly increased in LPS stimulated WT compared to non-stimulated WT macrophages. LPS stimulated *C5ar2*^{-/-}/*Apoe*^{-/-} macrophages show a reduced expression compared to LPS stimulated *Apoe*^{-/-}. Furthermore, VCAM-1 expression in LPS stimulated *C5ar2*^{-/-}/*Apoe*^{-/-} macrophages show a reduced expression compared to LPS stimulated WT, whereas this cannot be observed for TNF- α and IL-6 (see. Figure 33). Taken together, the data of cytokine expression indicate that the C5aR2 acts in a pro-inflammatory manner in bone marrow-derived macrophages.

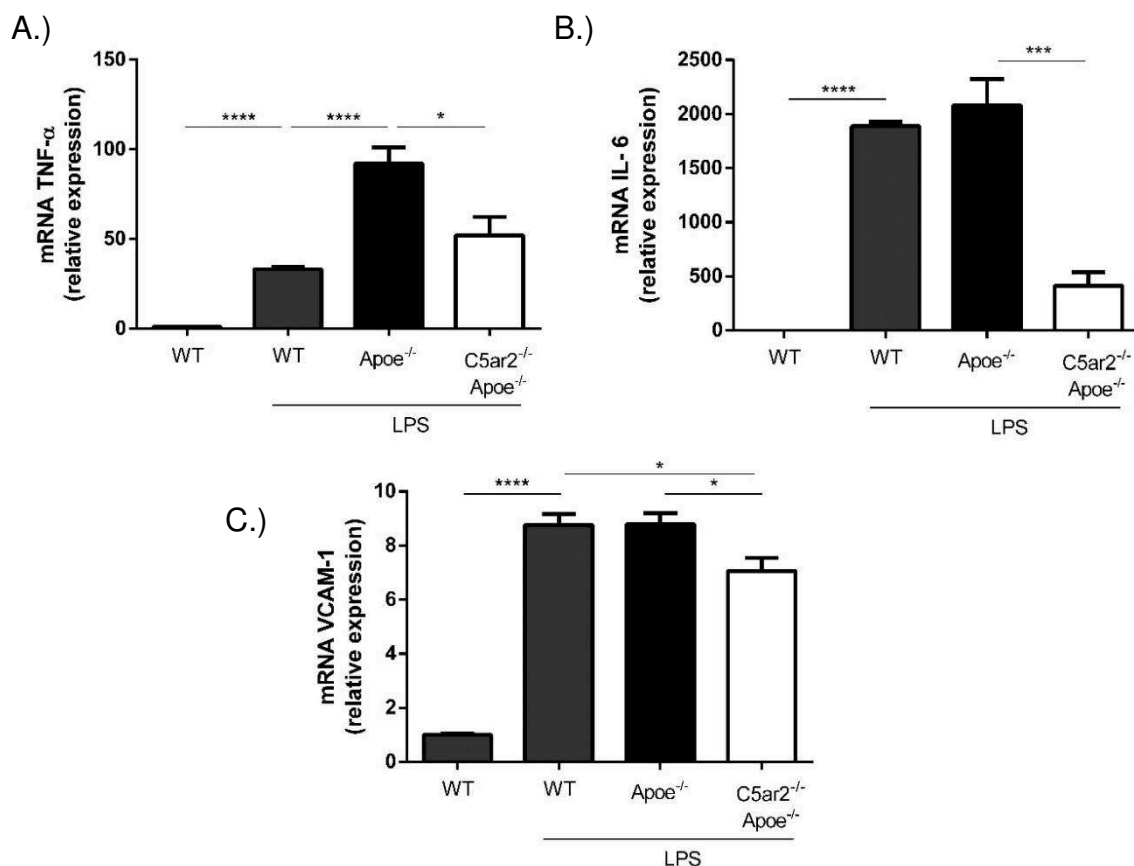


Figure 33: C5aR2 deficiency reduced expression of pro-inflammatory cytokines and adhesion molecules in bone marrow-derived macrophages. Quantification of (A) TNF- α and (B) IL-6 and (C) VCAM-1 by quantitative real-time PCR in WT, *C5ar2*^{-/-}/*Apoe*^{-/-} and *Apoe*^{-/-} macrophages. Macrophages were stimulated with LPS. Gene expression was normalized to the house-keeping gene GAPDH. Graphs represent the mean $\pm$ SEM; n=6; 2-tailed t-test; *p<0,05; ***p<0,001; ****p<0,0001.

4.5.2 Cytokine secretion of bone marrow-derived macrophages

As there were differences in mRNA expression in LPS stimulated bone marrow-derived macrophages, supernatants from these macrophages were examined by ELISA for differences in cytokine secretion. TNF- α secretion is not altered in non-stimulated and C5a stimulated macrophages. After TNF- α stimulation a reduced secretion can be found in *C5ar2*^{-/-}/*Apoe*^{-/-} compared to *Apoe*^{-/-}. LPS stimulation did not change secretion between *C5ar2*^{-/-}/*Apoe*^{-/-} and *Apoe*^{-/-}. IL-6 secretion is significantly higher in *C5ar2*^{-/-}/*Apoe*^{-/-} compared to *Apoe*^{-/-} macrophages after C5a and TNF- α stimulation. Non- and LPS stimulation did not lead to differences between the genotypes. Interestingly, the anti-inflammatory IL-10 secretion was not affected by stimulation or by genotype (see: Figure 34).

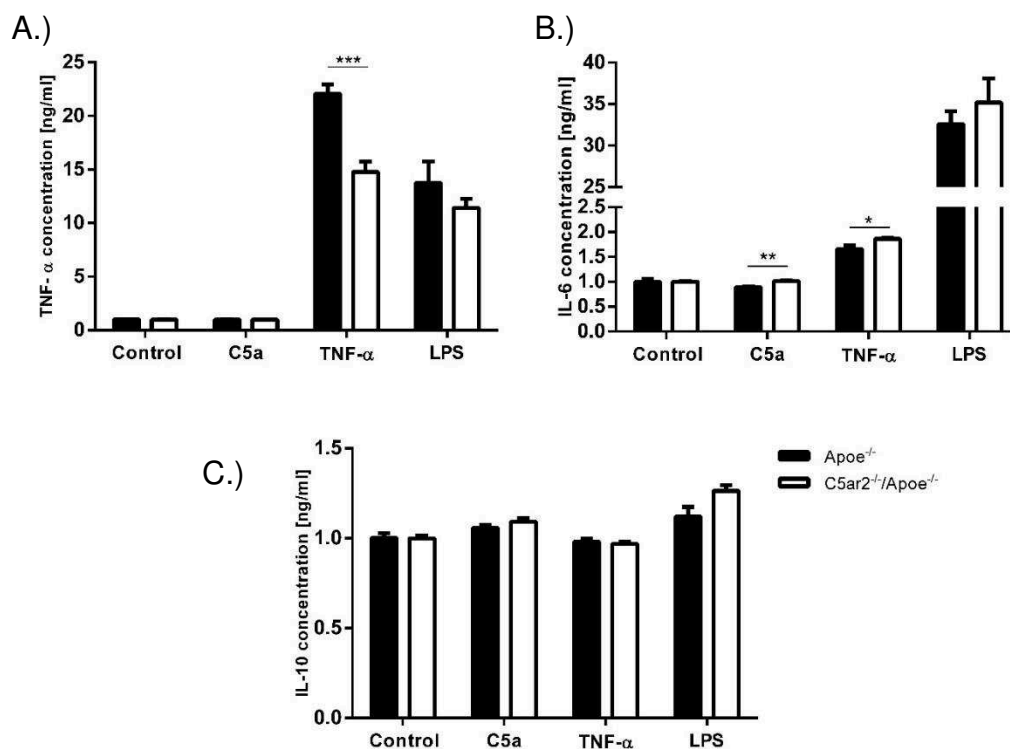


Figure 34: TNF- α and IL-6 secretion of bone marrow-derived macrophages is altered after stimulation with C5a, TNF- α or LPS. Quantification for secretion of (A) TNF- α and (B) IL-6 and (C) IL-10 by ELISA in WT, *C5ar2*^{-/-}/*Apoe*^{-/-} and *Apoe*^{-/-} macrophages. Macrophages were stimulated with C5a, TNF- α and LPS. Graphs represent the mean $\pm$ SEM; n=6; 2-tailed t-test; *p<0,05; **p<0,01; ***p<0,001.

4.5.3 Effects towards polarization and phenotype

To study the polarization of macrophages they were stimulated with LPS. The stimulation with LPS shifts the polarization to a M1 polarization. M1 polarization shows a rather inflammatory whereas M2 an angiogenic phenotype. A tool, which provides a first hint of the phenotype polarization, is to discriminate between these two polarizations by the study of the expression of the two genes Arginase-1 and the inducible nitric oxide synthases (iNOS). A relatively higher iNOS expression points toward an M1 polarization while a higher Arginase-1 expression is linked with a M2 phenotype [62].

A significant enhanced Arginase-1 expression in *C5ar2*^{-/-}/*Apoe*^{-/-} compared to *Apoe*^{-/-} macrophages is present. iNOS expression is significantly reduced in *C5ar2*^{-/-}/*Apoe*^{-/-} compared to *Apoe*^{-/-} macrophages (see: Figure 35). These data indicate that the C5aR2 deficiency may shift macrophage polarization to an anti-inflammatory M2 phenotype.

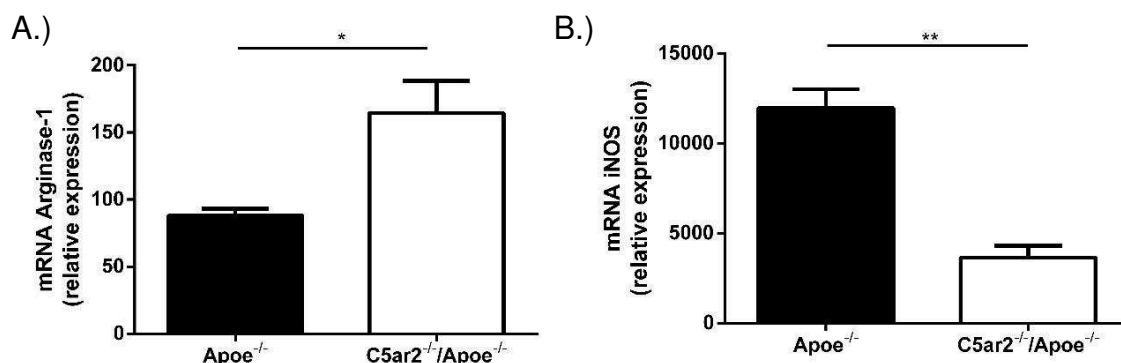


Figure 35: C5aR2 deficiency shifts bone marrow-derived macrophages towards a M2 polarization. Quantification of (A) Arginase-1 and (B) iNOS by quantitative real-time PCR in *C5ar2*^{-/-}/*Apoe*^{-/-} and *Apoe*^{-/-} macrophages. Macrophages were stimulated with LPS. Gene expression was normalized to the house-keeping gene GAPDH. Graphs represent the mean $\pm$ SEM; n=6; 2-tailed t-test; *p<0,05; **p<0,01.

The shown data indicates that the C5aR2 deficiency has an impact on macrophage polarization and the expression and secretion of pro-inflammatory cytokines and adhesion molecules, particularly with respect to the key pro-atherogenic factor TNF- α . This observation of a rather anti-inflammatory phenotype is further supported by the higher Arginase-1 expression, hinting to a M2 phenotype. Conclusively, the observed effects in *C5ar2*^{-/-}/*Apoe*^{-/-} macrophages point to a less inflammatory phenotype with a reduced ability to respond to classical immunostimulants.

4.6 Impact of the cooperative involvement of C5aR1 and C5aR2 in restenosis

It has already been reported that the deficiency of C5aR1 is leading to less prominent atherosclerotic lesions size and a reduced inflammatory response [151]. The previous data shown here, also demonstrate that the C5aR2 deficiency is leading to a similar reduction in lesion size and pro-inflammatory response. Which has led to the question of whether there is a reciprocal regulation of these two receptors. The complement component C5a is related to C5aR2, a ligand of the classical pro-inflammatory receptor C5aR1. For C5aR2, different mechanisms have been proposed on how it is functioning. It was suggested that C5aR2 is merely acting as a decoy receptor sequestering C5a, thus it cannot signal via the C5aR1 anymore [128]. In a different study, it was shown that C5aR2 interacts with β -arrestin and by this interfering in C5aR1 signaling [133]. Finally, it has been shown that C5aR2 itself harbors signaling function. Activation of C5aR2 by C5a leads to the secretion of the pro-inflammatory mediator HMGB1. For C5aR1 this observation is not possible, demonstrating that only the activation C5aR2 but not C5aR1 is causing HMGB1 release [137]. This highlights the complex interaction between these two receptors in regulation and modulation of each other's activity. To illuminate these interactions, *C5ar2^{-/-}/Apoe^{-/-}* mice, which underwent endothelial denudation, were additionally given a C5aR1 inhibitor. Further, the expression of C5aR2 and C5aR1 was studied *in vitro* by flow cytometry analysis and *in vivo* by measuring the gene expression levels in the aorta, aortic roots, the liver and in carotid arteries by quantitative real-time PCR.

4.6.1 Effects of the C5aR1 inhibition on wire-induced neointima formation in *C5ar2^{-/-}/Apoe^{-/-}* mice

At present there is no mouse model available which is deficient in APOE and both, C5aR2 and C5aR1. To overcome this problem, the use of osmotic mini-pumps equipped with a C5aR1 blocking peptide (C5aR1-A) were additionally implanted into *C5ar2^{-/-}/Apoe^{-/-}* mice undergoing wire-induced arterial denudation. As already described above, the C5aR2 deficiency reduces neointima formation in *C5ar2^{-/-}/Apoe^{-/-}* compared to *Apoe^{-/-}* mice. In *C5ar2^{-/-}/Apoe^{-/-}* mice treated with the C5aR1 inhibitor, the neointima formation is even further significantly reduced compared to *C5ar2^{-/-}/Apoe^{-/-}* and *Apoe^{-/-}* mice (see: Figure 36). These data demonstrate that blocking of the two receptors, C5aR1 and C5aR2, shows cooperative effects in the reduction in lesion size. Thus, inhibiting both receptors in restenosis could

Results

provide additional protection against lesion formation compared to solely blocking one at a time.

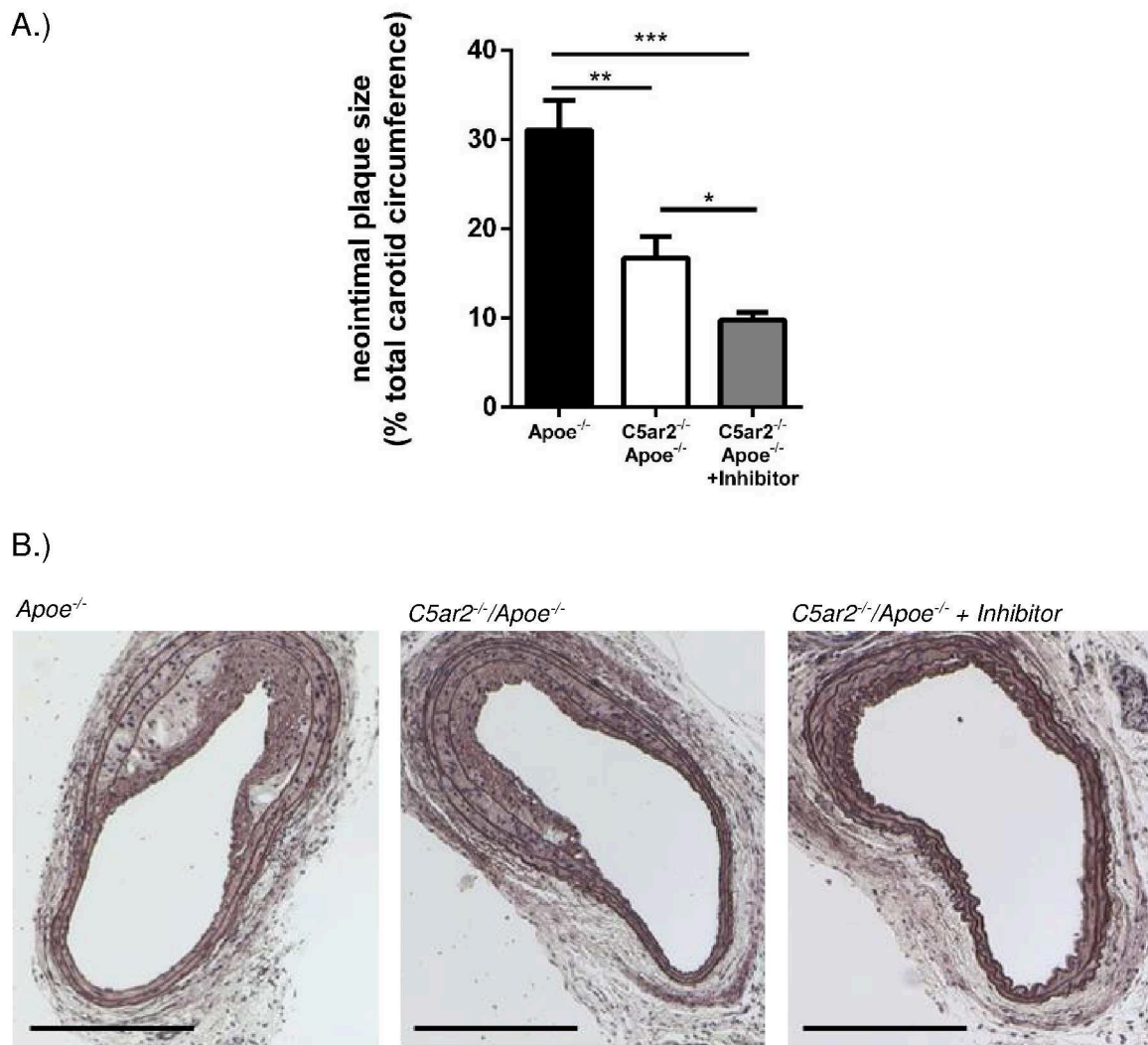


Figure 36: Inhibition of C5aR1 further reduces neointima formation in carotid arteries of C5ar2^{-/-}/Apoe^{-/-} mice after wire-induced endothelial denudation. In C5ar2^{-/-}/Apoe^{-/-} and Apoe^{-/-} mice endothelium in the left carotid artery was removed with a guide-wire. Left and right carotid arteries were collected and stained with Movat's pentachrome staining. In C5ar2^{-/-}/Apoe^{-/-} mice additionally received an implanted osmotic mini pump loaded with a C5aR1 inhibitor. (A) Quantification of neointima formation expressed as percentage of total artery lumen. (B) Photographed carotid arteries stained with Movat's pentachrome staining. Graphs represent the mean $\pm$ SEM; Scale bars = 200 μ m; n=5-12; 2-tailed t-test; *p<0,05; **p<0,01; ***p<0,001.

Next, macrophages and SMCs were determined in Apoe^{-/-}, C5ar2^{-/-}/Apoe^{-/-} and C5ar2^{-/-}/Apoe^{-/-}+C5aR1-A were analyzed to clarify if double blocking is favoring an even more stable plaque. Macrophages were significantly reduced in the neointima of C5ar2^{-/-}/Apoe^{-/-} compared to Apoe^{-/-}. Inhibition of C5aR1 reduced macrophages even more compared to Apoe^{-/-} and C5ar2^{-/-}/Apoe^{-/-} mice. SMCs are significantly increased

in the neointima of *C5ar2^{-/-}/Apoe^{-/-}*, as already described above. However, no difference was observed after additional inhibition of C5aR1 compared to *Apoe^{-/-}* or *C5ar2^{-/-}/Apoe^{-/-}* mice (see: Figure 37). Taken together, the double blocking of the receptors is leading to a further reduction of inflammation processes within the plaque.

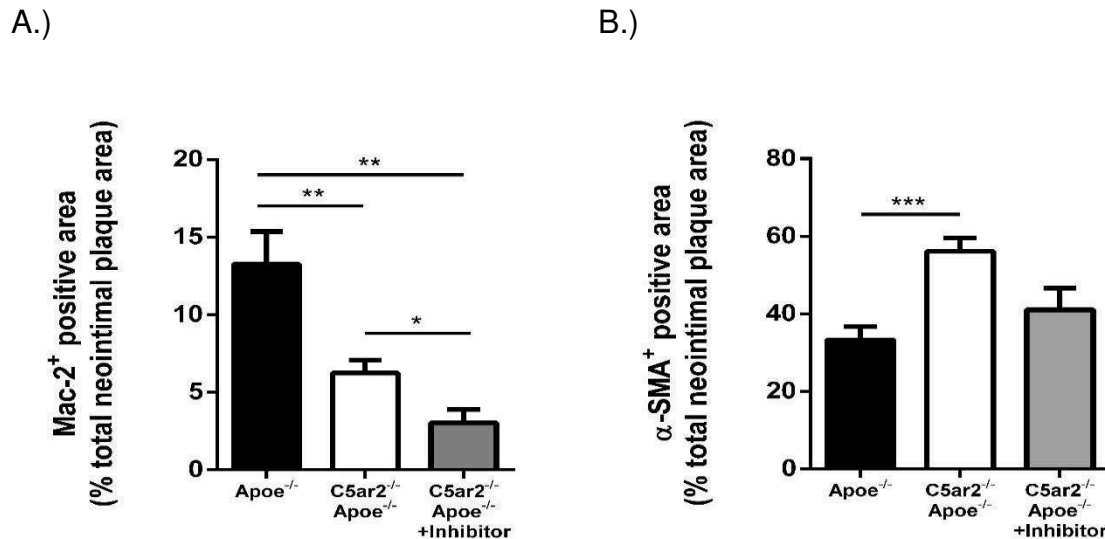


Figure 37: Inhibition of C5aR1 leads in *C5ar2^{-/-}/Apoe^{-/-}* mice to further decreased macrophage cell content in carotid artery neointima. (A) Quantification of macrophage content in neointima of *Apoe^{-/-}*, *C5ar2^{-/-}/Apoe^{-/-}* and *C5ar2^{-/-}/Apoe^{-/-} + C5aR1-A* mice by immunofluorescent staining for Mac-2. (C) Quantification of SMC content in neointima of *Apoe^{-/-}*, *C5ar2^{-/-}/Apoe^{-/-}* and *C5ar2^{-/-}/Apoe^{-/-} + C5aR1-A* mice by immunofluorescent staining for α-SMA. (B and C) representative pictures of Mac-2 and α-SMA stained carotid arteries. Quantifications are expressed as percentage of positive area to total neointima plaque area. Graphs represent the mean ± SEM; Scale bar = 100 μm; n=5-15; 2-tailed t-test; *p<0,05; **p<0,01; ***p<0,001.

Next, CD3⁺, TNF-α and C5aR1 positive cells were studied in *C5ar2^{-/-}/Apoe^{-/-} + C5aR1-A* carotid arteries. As it was already reported, blocking of C5aR1 alone already can reduce neointima formation [151] and the deficiency of C5aR2 is reducing the expression of C5aR1, it was of interest if double blocking of both receptor is reducing C5aR1 expression even further. T-lymphocytes were investigated, as they are known to be pro-inflammatory cells found in human and murine atherosclerotic lesions [201, 208]. TNF-α, on the other hand, is involved in plaque stability [162].

Cell which were positive for CD3⁺ were further decreased in *C5ar2^{-/-}/Apoe^{-/-} + C5aR1-A* compared to *C5ar2^{-/-}/Apoe^{-/-}* and *Apoe^{-/-}*. On the other hand, additional block of C5aR1 did not further reduce the number of C5aR1 and TNF-α positive cells in *C5ar2^{-/-}/Apoe^{-/-} + C5aR1-A* carotid arteries (see: Figure 38). In summary, by blocking

Results

both receptors an additional atheroprotective effect can be achieved by reducing inflammatory cells even further.

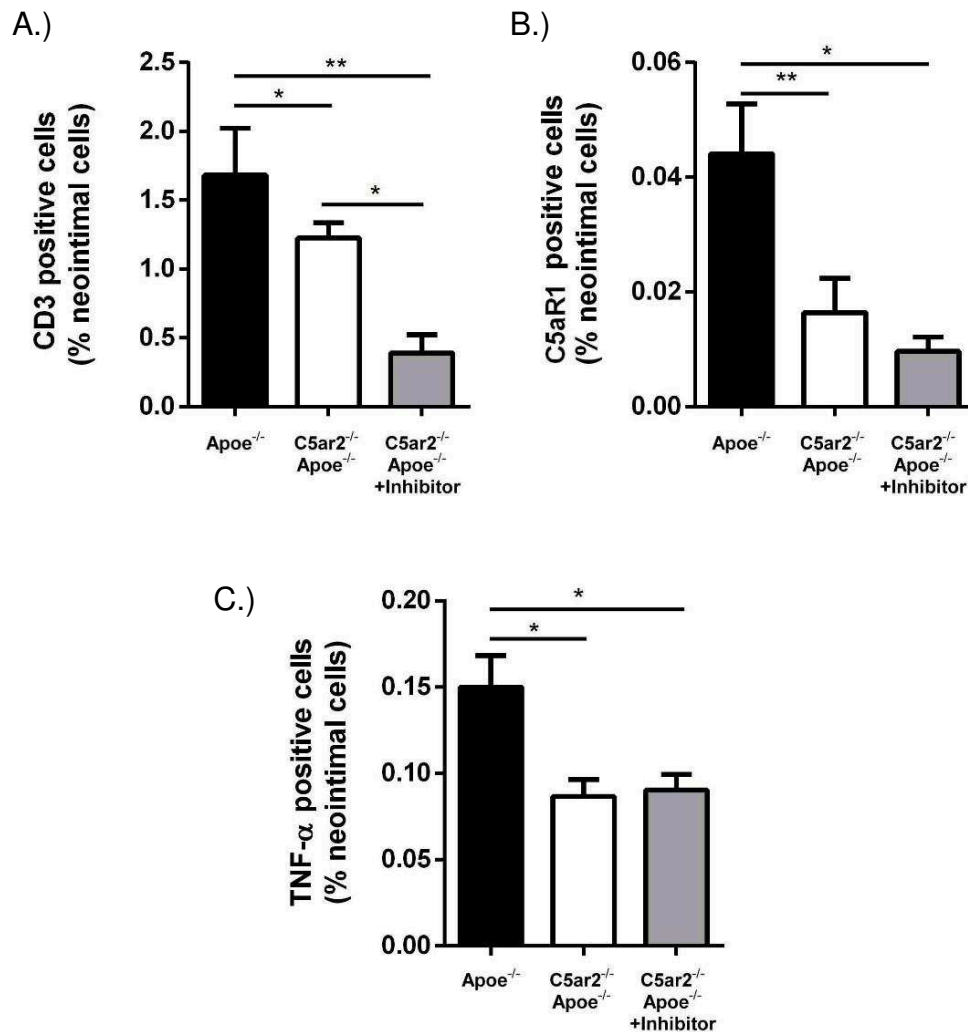


Figure 38: Inhibition of C5aR1 in *C5ar2*^{-/-}/*Apoe*^{-/-} mice leads to further decreased pro-inflammatory cell content in neointima in carotid arteries. (A) Quantification of T-cell (B) Quantification of C5aR1 positive cells. (C) Quantification of TNF-α positive cells. Quantifications are expressed as percentage of positive cells to total neointima plaque cells. Graphs represent the mean ± SEM; n=5; 2-tailed t-test; *p<0,05; **p<0,01.

4.6.2 C5aR2 deficiency reduces C5aR1 expression in bone marrow-derived macrophages

The correlation between C5aR1 and C5aR2 in the progression of atherosclerosis is not totally understood. In a recent publication it has only been demonstrated that the expression of C5aR2 in the aorta of *Apoe*^{-/-} mice was reduced when compared to WT mice [179]. But in human atherosclerotic plaques C5aR1 was detected in all stages of plaque progression, and is was shown to highly correlate with increasing plaque complexity. Additionally, it was demonstrated that C5aR2 expression in advanced

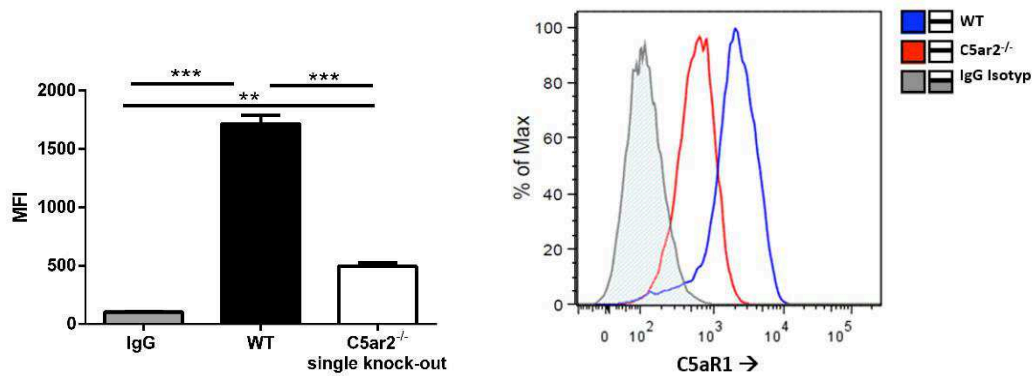
human plaques correlate with plaque complexity and levels of the pro-inflammatory cytokines, TNF- α and IL-1 β . Furthermore, in *in vitro* experiments in bone marrow-derived macrophages, the presence of both, C5aR1 and C5aR2, was necessary for optimal induction of the pro-inflammatory cytokines TNF- α and IL-1 β [147].

Thus, macrophages, as one of the main players in atherosclerotic progression, were studied, to reveal the impact of the C5aR2 deficiency on C5aR1 expression. WT, *Apoe*^{-/-}, *C5ar2*^{-/-} and *C5ar2*^{-/-}/*Apoe*^{-/-} macrophages were examined by flow cytometry. The C5aR1 protein amount was determined by western blot analysis. Therefore, macrophages were harvested and protein was isolated. Signal intensity for C5aR1 antibody binding was measured photometrically and normalized to β -actin.

Flow cytometry studies revealed a significant reduction in C5aR1 expression on *C5ar2*^{-/-} macrophages, which compare to the expression in WT. In *C5ar2*^{-/-}/*Apoe*^{-/-} macrophages, likewise, a significant reduction in C5aR1 expression was found when comparing to *Apoe*^{-/-} macrophages (see: Figure 39). These results confirmed the western blot analysis showing a reduction in *C5ar2*^{-/-}/*Apoe*^{-/-} compared to *Apoe*^{-/-} macrophages (see: Figure 40). This data also indicates an additional atheroprotective effect of the C5aR2 deficiency. The lack of the C5aR2 reduces the expression of the pro-inflammatory receptor C5aR1 thus indirectly further dampening the inflammatory response in the lesion.

Results

A.)



B.)

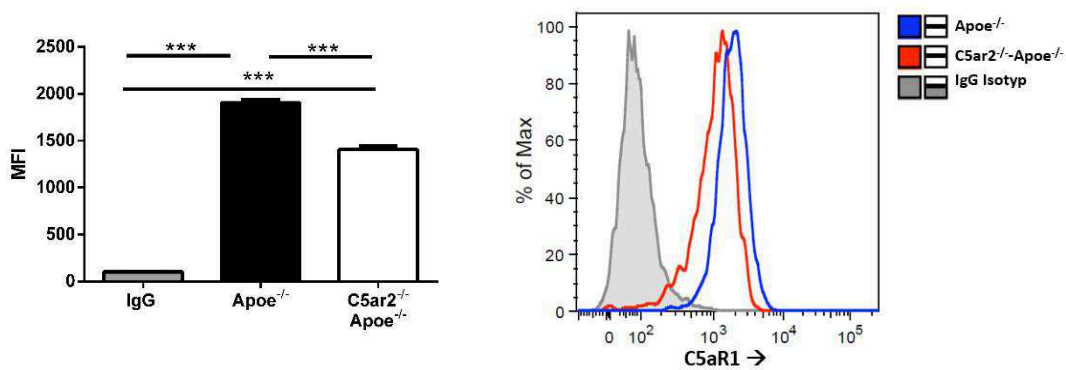


Figure 39: C5aR2 deficiency reduces C5aR1 expression on *C5ar2*^{-/-} and *C5ar2*^{-/-}/*Apoe*^{-/-} bone marrow-derived macrophages. (A) Flow cytometry analysis of the C5aR1 expression on WT and *C5ar2*^{-/-} macrophages. (B) Flow cytometry analysis of the C5aR1 expression on *Apoe*^{-/-} and *C5ar2*^{-/-}/*Apoe*^{-/-} macrophages. An appropriate antibody IgG isotype control was used. Graphs are expressed as mean fluorescence intensity (MFI) and represent the mean ± SEM. n=5; 2-tailed t-test; **p<0,01; ***p<0,001.

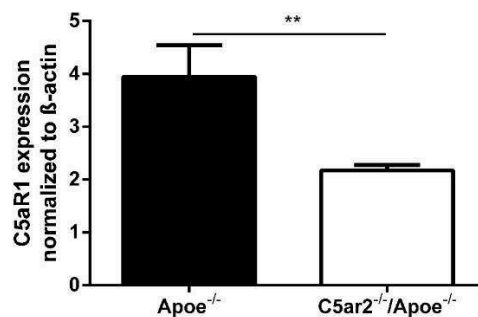


Figure 40: C5aR2 deficiency reduces C5aR1 expression in *C5ar2*^{-/-}/*Apoe*^{-/-} bone marrow-derived macrophages. Protein was isolated from *Apoe*^{-/-} and *C5ar2*^{-/-}/*Apoe*^{-/-} macrophages and C5aR1 expression was studied with western blot analysis. Values were normalized to β-actin. Graphs represent the mean ± SEM; n=4; 2-tailed t-test; **p<0,01.

4.6.3 Differential analysis of complement receptor expression *in vivo*

Next, to compare the results obtained from the *in vitro* experiments with macrophages, the mRNA expression level of C5aR2 and C5aR1 was analyzed *in vivo* by quantitative real-time PCR in the whole aorta, the aortic root, the carotid arteries and the liver of *C5ar2^{-/-}/Apoe^{-/-}* and *Apoe^{-/-}* mice. The organs of *C5ar2^{-/-}/Apoe^{-/-}* mice and *Apoe^{-/-}* control mice were collected. After cDNA synthesis samples were used in quantitative real-time PCR to identify differences in gene expression between *C5ar2^{-/-}/Apoe^{-/-}* and *Apoe^{-/-}* control mice. Gene expression was normalized to the house-keeping gene GAPDH. In *C5ar2^{-/-}* mice, no C5aR2 expression can be found, thus, here only the corresponding expression in ApoE deficient mice is shown.

4.6.3.1 Analyses of the gene expression in aortic roots

In aortic roots a significant reduction of C5aR1 expression in *C5ar2^{-/-}/Apoe^{-/-}* compared to *Apoe^{-/-}* can be seen under HFD conditions. Additionally, in *Apoe^{-/-}* there is a significant increase between mice on chow diet before HFD and HFD. The C5aR2 expression in *Apoe^{-/-}* increases when comparing before HFD and HFD (see: Figure 41). This data indicates that an additional atheroprotective effect of the C5aR2 deficiency is mediated via the reduction of the pro-inflammatory receptor C5aR1 thus indirectly damping the inflammatory response in the lesions. This finding is in line with the observed reduction in aortic root lesion size.

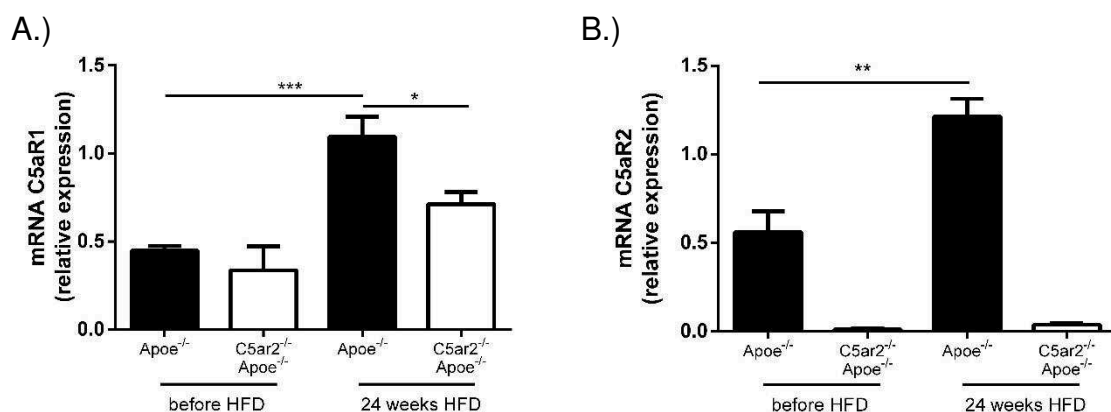


Figure 41: In aortic roots the C5aR2 deficiency reduces C5aR1 expression after 24 weeks HFD. Quantification of (A) C5aR1 and (B) C5aR2 expression by quantitative real-time PCR in *Apoe^{-/-}* and *C5ar2^{-/-}/Apoe^{-/-}* mice. Mice were on chow diet before HFD or HFD for 24 weeks. Gene expression was normalized to the house-keeping gene GAPDH. Graphs represent the mean $\pm$ SEM; n=5-7; 2-tailed t-test; *p<0,05; **p<0,01; ***p<0,001.

4.6.3.2 Analyses of the gene expression in the liver

Next, the expression in livers was analyzed. When comparing *C5ar2^{-/-}/Apoe^{-/-}* and *Apoe^{-/-}* control mice on chow diet before HFD no change in C5aR1 expression can be observed. A significant reduction of C5aR1 expression in *C5ar2^{-/-}/Apoe^{-/-}* could be seen after 12 and 24 weeks on HFD. C5aR2 expression is significantly increased after 12 and 24 weeks of HFD, however no difference can be found between 12 and 24 weeks HFD (see: Figure 42). Taken together, this data indicates that the liver inflammatory processes caused by HFD overrule the generally anti-inflammatory properties of the C5aR2 deficiency.

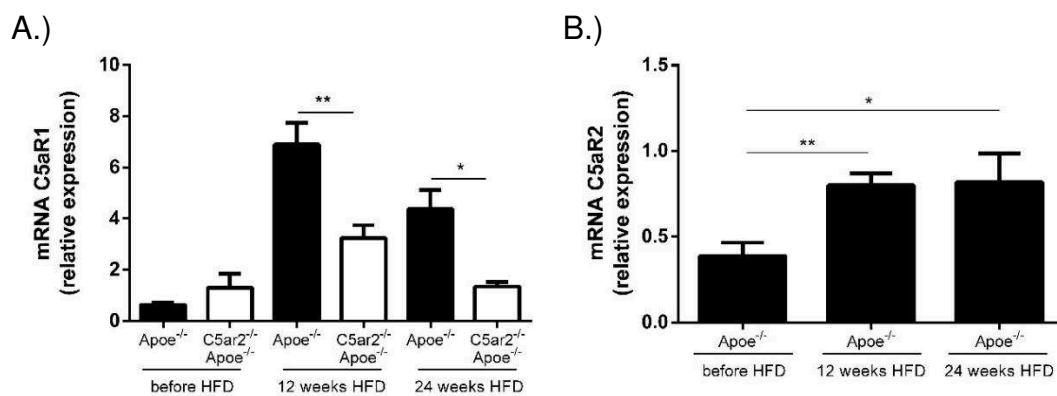


Figure 42: In the liver the C5aR2 deficiency increases C5aR1 expression after HFD. Quantification of (A) C5aR1 and (B) C5aR2 expression by quantitative real-time PCR in *Apoe^{-/-}* and *C5ar2^{-/-}/Apoe^{-/-}* mice. Mice were on chow diet before HFD or were fed a HFD for 12 or 24 weeks. Gene expression was normalized to the house-keeping gene GAPDH. Graphs represent the mean $\pm$ SEM; n=5-7; 2-tailed t-test; *p<0,05; **p<0,01; ***p<0,0001.

4.6.3.3 Analyses of the gene expression in whole aortas

Analysis of C5aR1 and C5aR2 expression in aortas of *C5ar2*^{-/-}/*Apoe*^{-/-} and *Apoe*^{-/-} mice did not reveal significant differences in expression (see: Figure 43).

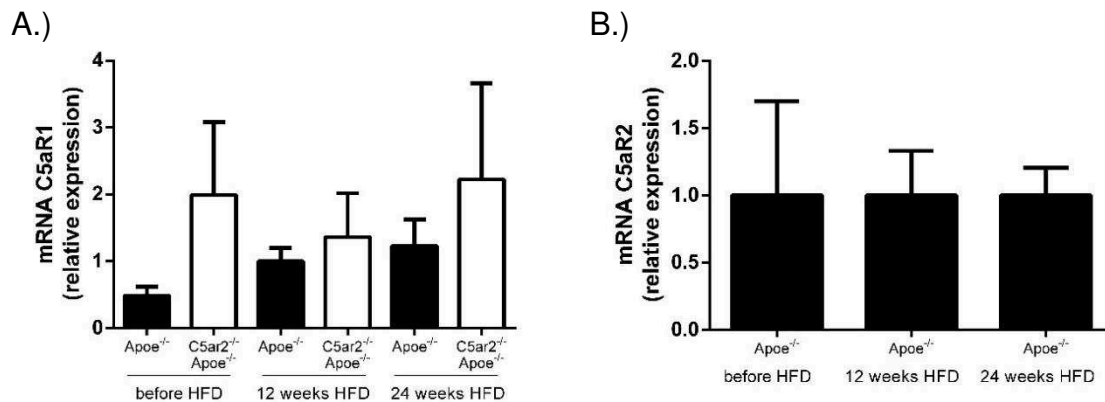


Figure 43: C5aR2 deficiency does not alter C5aR1 expression in the aorta. Analyses of (A) C5aR1 and (B) C5aR2 expression by quantitative real-time PCR in *Apoe*^{-/-} and *C5ar2*^{-/-}/*Apoe*^{-/-} mice. Mice were fed a chow diet before HFD or a HFD for 12 or 24 weeks. Gene expression was normalized to the house-keeping gene GAPDH. Graphs represent the mean $\pm$ SEM; n=5-7; 2-tailed t-test.

4.6.3.4 Analyses of the gene expression in carotid arteries

Comparing injured with uninjured arteries of *Apoe*^{-/-} mice, a strong significant increase in C5aR1 expression can be found. This can also be observed in *C5ar2*^{-/-}/*Apoe*^{-/-} mice, when comparing uninjured with injured carotid arteries. Most importantly, C5aR1 expression is drastically reduced in *C5ar2*^{-/-}/*Apoe*^{-/-} injured carotid arteries compared to *Apoe*^{-/-} injured carotid arteries.

C5aR2 expression in *Apoe*^{-/-} carotid arteries is significantly higher in injured compared to uninjured carotid arteries (see: Figure 44). This data indicates that an additional atheroprotective effect of the C5aR2 deficiency is produced via the reduction of the pro-inflammatory receptor C5aR1 thus indirectly dampening inflammatory response in the lesion. This finding is in line with the observed reduction in neointima formation in carotid arteries.

Results

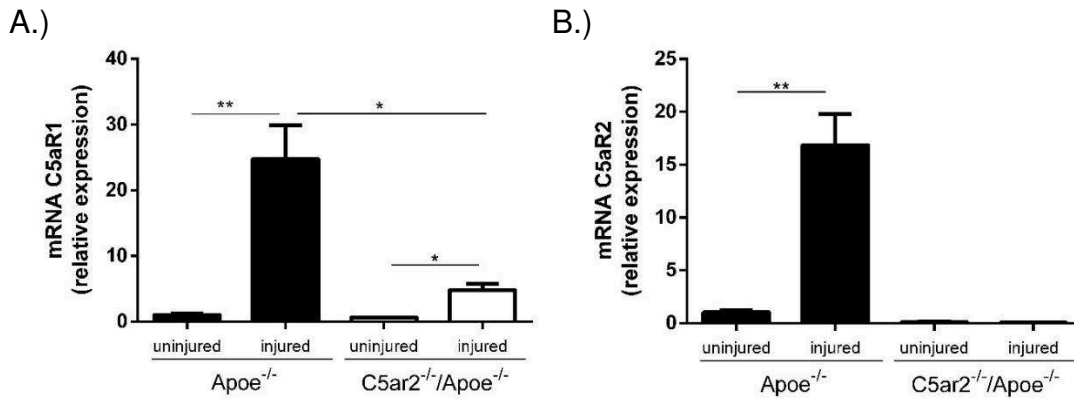


Figure 44: The C5aR2 deficiency decreases C5aR1 expression in injured carotid arteries. Analyses of (A) C5aR1 and (B) C5aR2 expression by quantitative real-time PCR in *Apoe*^{-/-} and *C5ar2*^{-/-}/*Apoe*^{-/-} mice. Mice were fed a HFD and left carotid artery was injured by endothelial denudation. Gene expression was normalized to the house-keeping gene GAPDH. Graphs represent the mean $\pm$ SEM; n=6; 2-tailed t-test; *p<0,05; **p<0,01.

In conclusion, the data show that inhibition of C5aR1 in C5aR2 deficient mice has an additive positive effect in the reduction of neointima formation, thus may be a strategy in handling atherosclerosis and restenosis.

5 Discussion

Atherosclerosis is a chronic inflammatory process in the arterial vessel wall, orchestrated by a variety of mediators of innate and adaptive immunity [40, 45]. Inflammatory cells and complement components play a crucial role in disease initiation and progression. The complement component C5a signals through its two receptors, the C5a receptor1 (CD88; C5aR1) and the relative newly identified C5a receptor-like 2 (C5L2; GPR77; C5aR2) [106, 107]. In many inflammation-driven diseases, a pro-inflammatory role of C5aR1 has been well demonstrated. In a model of acute sepsis it was proven to contribute to the harmful outcome and in a model of tubulointerstitial fibrosis interaction of C5a and C5aR1 mediates initiation and progression of renal fibrosis [137, 209]. Furthermore, it was reported that C5aR1 possesses a critical role in restenosis of vascular-damaged mice, as short-term blockade of C5aR1 limits neointimal plaque formation [151]. In contrast to the direct pro-inflammatory role of C5aR1, the role of C5aR2 is contradictory, as both pro-inflammatory and anti-inflammatory roles have been reported [133]. On the one hand, C5aR2 was stated as an anti-inflammatory receptor. As it is lacking G protein coupling it was stated that it is merely acting as a decoy receptor sequestering C5a preventing the pro-inflammatory interaction between C5a and C5aR1 [128]. On the other hand, it was shown that C5aR2 acts in a pro-inflammatory manner in acute sepsis and in anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV), where it aggravates disease progression [135, 136]. Thus, the role of C5aR2 stays highly controversial and especially the effects on atherosclerosis are mostly unknown. It is known, that in advanced human atherosclerotic plaques the C5aR2 expression level directly correlate with the stage of disease and the pro-inflammatory cytokine expression. Data obtained from in vitro experiments with macrophages revealed, that both C5aR1 and C5aR2 are necessary for an appropriate pro-inflammatory cytokine production [147]. Furthermore, it was demonstrated that the activation of MAPK and AKT are key downstream events induced by C5a [210, 211]. In wild-type neutrophils it was shown that C5a induces ERK1/2 activation, and slightly elevates phosphop38 levels. In *C5ar2^{-/-}* neutrophils only a weak induction of activated ERK1/2, JNK or p38 in response to C5a was present [127]. C5a is also known to have a critical role in regulating macrophage functions [212]. Chen and colleagues demonstrated that in *C5ar2^{-/-}* macrophages the induction of ERK1/2 and AKT was severely impaired after deletion of the C5aR2 [127]. This underpins the role of proper C5aR2 signaling for the

induction of inflammatory cytokines. Therefore, this study focuses on the contribution of C5aR2 in atherogenesis and the underlying processes leading to lesion formation. To study the function of C5aR2 in atherosclerosis and restenosis in detail, it was investigated in two models: one being a diet-induced atherosclerosis and the second an injury-induced restenosis model.

The presented results identify C5aR2 as a functional pro-inflammatory receptor in atherosclerosis. In two different models of atherosclerosis, deletion of C5aR2 led to a reduction of atherosclerotic plaque size with reduced inflammatory cells and pro-inflammatory cytokines in atherosclerosis-prone mice. An additional blockade of C5aR1 in *C5ar2^{-/-}/Apoe^{-/-}* mice using a C5aR1-antagonist was able to further reduce neointimal plaque size and inflammatory content after wire injury. This makes C5aR2 a suitable candidate in targeting atherosclerosis.

5.1 Reduced atherosclerotic lesion size and restenosis in *C5ar2^{-/-}/Apoe^{-/-}* mice

Many studies showed an influence of complement component C5a on atherogenesis. Blocking of C5aR1-C5a axis results in a reduction of neointima formation. In a model of wire-induced endothelial denudation of the carotid arteries in *Apoe^{-/-}* mice the blocking of the C5aR1 resulted in a smaller atherosclerotic lesion size accompanied by a less inflammatory milieu [151]. A recently identified second receptor C5aR2 is involved in various diseases revealing both pro- and anti-inflammatory properties [133]. To date, little is known about the role C5aR2 is playing in atherogenesis.

A model of diet-induced atherosclerosis is a representative model of chronic atherosclerosis, whereas a model of wire-induced injury and endothelial denudation of carotid arteries represents acute atherosclerosis and restenosis. The underlying pathophysiological mechanisms differ in restenosis and atherosclerosis [43, 44]. The chronic atherosclerosis is characterized by an influx and accumulation of lipids into the tunica intima and a recruitment of inflammatory cells [31, 45]. In acute atherosclerosis, on the other hand, the neointima formation is characterized by SMC proliferation and migration from the tunica media into the tunica intima [47]. This restenosis is to restore vessel wall integrity and function, thus restoring blood and oxygen supply [47, 48].

In the model for chronic atherosclerosis *C5ar2^{-/-}/Apoe^{-/-}* and *Apoe^{-/-}* mice were put on an HFD for 12 or 24 weeks. In this study, it could be shown that *C5ar2^{-/-}/Apoe^{-/-}* mice

have a significant reduction in atherosclerotic lesion size after 24 weeks of HFD in whole aortas and aortic roots. In both, no significant reduction can be seen after 12 weeks of HFD. Whole blood count revealed no change in total leukocyte count, but the percentage of monocytes was reduced in *C5ar2^{-/-}/Apoe^{-/-}* mice after 12 weeks of HFD. Monocyte transmigrate into the lesion and differentiate into macrophages aggravating atherogenesis. In a study by Swirski *et al.* it was shown that a hypercholesterolemia related monocytosis results in enhanced numbers of lesional macrophages [213]. Thus, a reduced monocyte count could be an explanation for reduced macrophages and lesion size. In our study, monocyte count is not changed after 24 weeks of HFD, whereas only after 24 weeks a reduction in lesion size is observed in whole aortas and aortic roots. As atherosclerosis is a chronic disease, the reduced monocyte count in the beginning could delay or diminish lesion progression in total, leading to the observed reduction in atherosclerotic lesions. Our analysis of the gene expression of the adhesion molecule VCAM-1 showed that its expression is strongly reduced in *C5ar2^{-/-}/Apoe^{-/-}* whole aortas after 12 and 24 weeks of HFD. This observation could only be seen in aortic roots at 24 weeks of HFD. As a pro-atherogenic molecule, VCAM-1 is critical for the initiation and progression of atherosclerosis by playing a significant role in the recruitment of circulating inflammatory cells to the vascular endothelium [194, 195]. In atherosclerotic lesions, VCAM-1 was selectively upregulated in vascular SMCs [197] and blocking VCAM-1 interactions reduced firm monocytes adhesion to arteries of *Apoe^{-/-}* mice under shear stress [198]. Furthermore, it was reported that VCAM-1 was upregulated in neointimal plaque of carotid arteries after vascular injury, which could be reduced by treatment with C5aR1-antagonist [151]. Thus, the reduced VCAM-1 expression in *C5ar2^{-/-}/Apoe^{-/-}* is in line with the reduction in lesion size. The reduction in lesion size may on the one hand be caused by the diminished monocyte count and yet on the other hand, the reduced VCAM-1-mediated transmigration of monocytes could contribute to reducing lesional macrophages. Furthermore, in our aortic roots after 24 weeks of HFD TNF- α and IL-6 was found to be significantly reduced in *C5ar2^{-/-}/Apoe^{-/-}* mice. We could not observe this reduction of TNF- α and IL-6 in whole aortas, which is probably due to comparable small lesion size in relation to the otherwise healthy aortic tissue. This is, however, in line with previous reports by other work groups, which demonstrate that high levels of TNF- α and IL-6 correlate with progression and severity of atherosclerotic lesion formation [162, 193]. Thus, the reduction in lesion formation indicate that C5aR2 is

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involved in the expression of pro-inflammatory cytokines and influences the migration of inflammatory cells into the lesion area.

In addition to the analysis of whole aorta and aortic roots, liver expression of C5 was measured. The impact on the liver of the C5aR2 deficiency was studied, as complement component production mostly takes place in the liver. In *C5ar2^{-/-}/ApoE^{-/-}* mice on chow diet before HFD an increased expression of C5 was found, whereas on HFD elevated C5a levels were measured but no difference was present comparing genotypes. Under normal, non-hyperlipidemic diet conditions this increase in C5 expression may be due to missing regulatory feedback signaling via C5aR2. Additionally, the overall increase under HFD could explain the enhanced TNF- α and IL-6 liver expression. The enhanced C5a levels in the blood could lead to a general state of inflammation in the liver causing pro-inflammatory cytokine production. In line with that, TNF- α and IL-6 expression was elevated and also serum cytokine analysis revealed elevated IL-6 serum levels. Our findings correspond with previous observations of Chen *et al.*, who showed that the C5aR2 deficiency resulted in enhanced LPS-induced IL-6 production *in vitro* [127]. Furthermore, it was demonstrated that septic *C5ar2^{-/-}* mice showed elevated plasma IL-6 levels [135], whereas Rittirsch and colleagues reported, in contrast to our findings, that TNF- α serum levels remained unchanged [137]. Apart from this, it has already been reported, that the APOE deficiency is already causing an inflammatory state of the liver [214]. Our findings of elevated liver IL-6 levels are in contrast with the otherwise reduced IL-6 levels, thus, the data suggest that in the liver the pro-inflammatory milieu is rather caused by the hyperlipidemia as a stress response, than it is attributed to the C5aR2 deficiency itself.

Taken together, in a model for diet-induced chronic atherosclerosis a reduction in lesion size can be found in whole aortas and aortic roots. These findings are in line with a reduced pro-inflammatory cytokine and adhesion molecule expression. However, especially with regard to liver TNF- α and IL-6 secretion further analysis are needed to clarify the role of the C5aR2 in these cytokine expressions.

In the model for acute atherosclerosis, it could be shown that *C5ar2^{-/-}/ApoE^{-/-}* mice have a significant reduction in neointimal lesion size after wire-induced endothelial injury. In contrast to the diet-induced atherosclerosis, the monocyte count was not altered. It was shown that a HFD could lead to increased numbers of monocytes circulating in

the blood stream [215]. Thus, the 8 weeks on HFD in the model of acute atherosclerosis may not be sufficient to result in a significant change in monocyte numbers. However, we observed a reduced expression of VCAM-1, giving a possible explanation for the reduced lesion size, as reduced VCAM-1 expression leads to less recruitment of monocytes into the lesion. In line with this, we found an overall reduction in pro-inflammatory cytokine expression in *C5ar2^{-/-}/Apoe^{-/-}* neointimal lesions. This leads to the conclusion that the diminished inflammatory milieu accompanied by a reduction of inflammatory cell infiltration, which is leading to the observed reduction in neointimal lesion size, is a result of the missing C5aR2 signaling. Of note, HMGB1 expression in *C5ar2^{-/-}/Apoe^{-/-}* carotid arteries is not changed at all, even comparing injured with uninjured carotid arteries. These results are conforming with a study by Rittirsch and colleagues demonstrating that HMGB1 release depends on a functional C5aR2, but not C5aR1, and even LPS stimulated HMGB1 release is impaired in C5aR2 deficient mice [137]. Thus, the C5aR2 deficiency results in less HMGB1 secretion, which is further contributing to the overall atheroprotective effect of the deficiency of C5aR2. Furthermore, analysis of neointima formation in mice without hypercholesterolemic background, meaning *C5ar2^{-/-}* single knock-out mice without an APOE deficiency, were performed. Despite a tendency, there is no significant difference between *C5ar2^{-/-}* single knock-out and WT carotid arteries in neointima size and also the numbers of SMCs were not altered. Of note, macrophage numbers were significantly reduced in *C5ar2^{-/-}* single knock-out carotid arteries. This is in line with a previous study, which demonstrated that *C5ar2^{-/-}* single knock-out 12 weeks on chow diet showed no change in SMCs but reduced macrophage numbers [216]. Interestingly, VCAM-1 expression was not changed, as the expression of cell surface VCAM-1 and transcripts has been previously demonstrated in both monocytes and bone marrow-derived macrophages [217]. Unchanged VCAM-1 expression stands in contrast with the reduction in macrophage numbers, as VCAM-1 is one of the major adhesion molecules responsible for monocyte recruitment into the lesion. Overall, here in our *C5ar2^{-/-}* single knock-out study the reduced inflammatory milieu could be responsible for less macrophage recruitment, apart from the unaltered VCAM-1-mediated recruitment. We further observed a reduced TNF- α expression in the lesion. The reduced TNF- α expression could be caused by the reduced macrophage numbers, as these cells normally are known to produce pro-inflammatory cytokines, including TNF- α [192]. Thus, the reduction in macrophages is responsible

for the diminished TNF- α expression levels. Nevertheless, insights gained from *C5ar2*^{-/-} single knock-out and WT mice indicated that the effect of C5aR2 on neointima formation after arterial injury requires a hypercholesterolemic background. This points to, that the modulation of C5aR2 is most effective in patients already suffering from high cardiovascular risk factors, like hyperlipidemia. In these patients, targeting C5aR2 as a means to prevent atherosclerotic lesion formation and restenosis is most rational and useful in fighting the harmful effects of CVDs.

Overall, the reduction of neointima formation in acute atherosclerosis accompanied by a reduced pro-inflammatory milieu, demonstrate that the C5aR2 has a functional role in initiation and progression of atherosclerosis. Taken together, the data provide evidence that the C5aR2 acts in a pro-inflammatory manner facilitating atherogenesis and restenosis.

5.2 Altered cellular composition in restenosis of *C5ar2*^{-/-}/*Apoe*^{-/-} mice

The observation of the reduction in lesion size encouraged us to identify the underlying processes how the C5aR2 is mediating the pro-atherogenic effect. One important step in atherogenesis is the recruitment and migration of inflammatory cells into the lesion site [45]. Moreover, it is known that C5aR2 itself can induce cytokine secretion via the MAPK and AKT pathways, which can lead to elevated secretion of cytokines such as IL-6 [127, 137]. In another report the inhibition of MEK1/2 (part of MAPK signaling) and AKT resulted in reduced HMGB1 release by monocytes [218]. Hence, the cellular composition within the lesion was analyzed with the focus on macrophages and SMCs as these are main players in atherosclerosis and plaque stability.

Especially diet-induced chronic atherosclerosis is characterized by a migration and proliferation of inflammatory cells in the arterial wall [43]. In our study we demonstrated that aortic lesion and aortic root lesion size is reduced after 24 weeks of HFD. In the blood of *C5ar2*^{-/-}/*Apoe*^{-/-} mice, a reduced number of circulating monocytes were found. First whole aortas were examined. Here, the RNA expression level examination revealed a significant reduction in VCAM-1 expression. Interestingly, the expression of TNF- α and IL-6 did not show changes. In other studies TNF- α was found to be pro-atherogenic by triggering smooth muscle death [162] and high levels of IL-6 were found to exacerbate atherogenesis in mice [193]. Thus, as the cytokines are not changed one might suggest that also the atherosclerotic lesion size is unaltered.

However, a possible explanation for this unaltered pro-inflammatory cytokines may be that the whole aorta and not only the lesion was analyzed, thus the effects could be overlaid by the analyzation of the mostly healthy aortic tissue. Overall, as diet-induced atherosclerosis is characterized by the migration and recruitment of inflammatory cells the reduced monocyte count and adhesion molecule expression may explain the diminished aortic lesion size.

Our analysis of the aortic roots revealed a reduction in macrophages after 12 and 24 weeks of HFD accompanied with an increase in SMCs at both time points. Adhesion molecule expression within aortic root tissue revealed a drastic reduction in VCAM-1 expression. VCAM-1 is highly implicated in the reduction of macrophages at aortic root lesions, as recruitment and migration are diminished. Since atherosclerosis is characterized as an inflammatory disease the expression of the typically pro-inflammatory cytokines TNF- α and IL-6 was examined. In our *C5ar2^{-/-}/Apoe^{-/-}* mice, significantly reduced TNF- α and IL-6 expression was detected. It has been reported that IL-6 has the potential to activate ECs with subsequent increased expression of adhesion molecules and secretion of chemoattractant factors [219, 220]. Besides, other reports have demonstrated that IL-6 can be directly induced by the activation of C5aR2 [147]. Therefore, reduced IL-6 expression in part is responsible for the reduced number of monocytes/macrophages within the lesion by reducing recruitment. Furthermore, it has been reported that IL-6 induces the differentiation of monocytes into macrophages [221]. Thus, IL-6 accounts in two ways on macrophage numbers in the plaque, firstly IL-6 influences migration as the cytokine readily induces the production of chemotactic factors, and secondly IL-6 also acts to encourage the differentiation of monocytes to macrophages. Furthermore, it was previously demonstrated that IL-6 affects the local expression of the scavenger receptor SR-A, which is involved in the uptake of modified LDL, by this promotes the formation of macrophage-derived foam cells [222]. Foam cells eventually undergo apoptosis releasing additional pro-inflammatory cell debris enhancing local inflammation. Thus, the observed reduced numbers of macrophages can be partly explained by the reduced IL-6 level, hence provides a causative mechanism which results in a reduced pro-inflammatory milieu within the lesion. The observation of increased SMCs is in line with the findings that TNF- α expression is reduced. It has been shown that macrophages are partly responsible for SMCs death via secretion of TNF- α [162]. Thus, the reduced macrophage numbers lead to reduced TNF- α levels within the

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lesion, which in turn causes increased SMC counts. Taken together, reduced macrophages and pro-inflammatory cytokines accompanied by enhanced SMC numbers are features of a stable plaque, less likely to rupture. Conclusively, in the model of diet-induced chronic atherosclerosis the missing C5aR2-induced cytokine secretion and inflammatory cell recruitment is atheroprotective leading to a diminished inflammatory milieu and a more stable plaque resulting in the observed smaller lesion size.

In acute injury-induced neointima formation the main mechanism to restore vessel integrity and function is the migration of smooth muscle into the neointima. Here, SMCs are mainly responsible for the formation of the plaque [47]. It has been shown that C5aR1 blockade with C5aR1-antagonist or antibodies leads to a diminished neointima formation, migration of macrophages and VCAM-1 expression [151]. In line with this, in our study it is shown that lesion formation is reduced in *C5ar2^{-/-}/Apoe^{-/-}* mice. The reduction in lesion size goes along with less macrophages found within the lesion. Differing from the diet-induced atherosclerosis, numbers of circulating monocytes were unaltered. Nevertheless, a drastic reduction in VCAM-1 expression is eminent causing less monocyte to migrate into the lesion and thus constitutes another factor which explains the reduced macrophages. In the *C5ar2^{-/-}/Apoe^{-/-}* mice, the other receptor C5aR1 is still present and it is known that the activation of the C5a-C5aR1 axis can lead to increased local inflammation, thus facilitating atherogenesis [223]. Therefore, cells within the lesion positive for C5aR1 expression were analyzed and revealed a reduction in *C5ar2^{-/-}/Apoe^{-/-}* mice. Together with the missing direct C5aR2-induced cytokine expression [127, 147], this reduction in C5aR1 as a pro-inflammatory receptor might be responsible for the general reduction in pro-inflammatory cytokines expression of TNF- α , IL-6, IFN- γ and RANTES in *C5ar2^{-/-}/Apoe^{-/-}* mice. Other than macrophages, pro-inflammatory cells such as T-lymphocytes can be found in human and murine atherosclerotic lesions [201, 208]. They were reported to be found at the site of plaque rupture in human coronary plaques after myocardial infarction, thus playing a role in homeostasis of plaque stability [224]. In the lesions of *C5ar2^{-/-}/Apoe^{-/-}* mice reduced numbers of T-lymphocytes were found, which also contributes to the observed reduced lesion size. Coherent with that, reduced numbers in IFN- γ positive cells within the lesion and a reduced IFN- γ mRNA expression can be found. IFN- γ is highly secreted by T-lymphocytes and can further activate cytotoxic T-cells and macrophages [225]. Thus, that reduction of T-lymphocytes and IFN- γ can evoke a less

inflammatory milieu with less T-lymphocytes and macrophages. Additionally, the reduced IFN- γ expression is in line with a study by Zhang *et al.* where they demonstrated that IFN- γ is reduced in *C5ar2^{-/-}* mice [226]. In agreement with the results of the diet-induced chronic atherosclerosis model IL-6 expression was reduced in acute atherosclerotic lesions. In addition to the influence on macrophages, as described already above, IL-6 can also promote migration and proliferation of T-lymphocytes and can directly be induced by C5aR2 [147, 227]. Here, reduced IL-6 expression contributes to a reduction of both, macrophages and T-lymphocytes, resulting in a more stable and less inflammatory lesion, which could be the underlying cause for the observed reduction in atherosclerotic lesions in *C5ar2^{-/-}/Apoe^{-/-}* mice. Nevertheless, the subsets of T-lymphocytes were not discriminated as it is reported that CD4 positive T-cells harbor pro-atherogenic properties in *Apoe^{-/-}* mice which is associated with a shift towards Th1 response [228, 229]. Furthermore, reduced numbers of T_{regs} were shown in various disease settings to pronounce disease symptoms [230]. Here, more experiments are needed to clarify the differential involvement of the subsets and the influence on atherosclerosis in the context of complement modulation.

Consistent with the concept of a more stable plaque are the increased SMC numbers in the lesion. As already mentioned, macrophages can contribute to SMC death by the secretion of TNF- α [162]. It was also reported that the plaque-activated T-lymphocytes produce IFN- γ , which inhibits the proliferation of SMCs and their ability to synthesize collagen, thereby weakening the fibrous cap and destabilizing the plaque [163, 164]. Thus, less TNF- α produced by macrophages causing less apoptosis and less IFN- γ produced by T-lymphocytes together contribute to the higher number of SMCs. This is in line with less IFN- γ and TNF- α positive lesion cells and reduced expression of IFN- γ and TNF- α in injured carotid arteries of *C5ar2^{-/-}/Apoe^{-/-}* mice. Additionally, reduced expression of the pro-inflammatory cytokine RANTES was found in *C5ar2^{-/-}/Apoe^{-/-}* mice. It has already been reported that the deletion of CCR5, a receptor for RANTES, is reducing atherosclerosis [165] and *C5ar2^{-/-}* single knock-out show a general reduction of RANTES expression [166]. Cytotoxic T-cells release RANTES and in turn activate T-lymphocytes and macrophages [167, 168]. Therefore, reduced RANTES expression can support the reduction in T-lymphocytes and macrophages and promote a more stable plaque phenotype. Importantly, the expression of HMGB1 was also reduced. HMGB1 is a pro-inflammatory mediator, which is solely released upon

activation of C5aR2 [137]. It was demonstrated that HMGB1 has chemotactic and activating properties towards macrophages [169, 170]. Thus, the reduced macrophage numbers can be accounted to the lower expression of HMGB1. Contrastingly, HMGB1 was found to stimulate migration and proliferation of SMCs in atherosclerosis [231, 232]. In that case, the observed higher numbers of SMCs are more likely to be caused by the general reduction in inflammation within the lesion than the lack of HMGB1 stimulation. At last, in *C5ar2^{-/-}/Apoe^{-/-}* mice apoptosis was reduced within the lesion. The group of Zheng et al. could demonstrate that apoptosis of foam cells can cause an increased necrotic core size, accompanied by the release of intracellular debris and pro-inflammatory mediators [233]. Thus, the reduced apoptosis is contributing to a reduced pro-inflammatory milieu within the lesion. On the other hand, proliferation was unaltered within the plaque. This is important, as it was shown that the numbers of macrophages within the plaque is not only influenced by the migration of circulating blood monocyte but also from proliferation in the plaque itself [234].

This data clearly reveals pro-inflammatory properties of the C5aR2 in chronic and acute atherosclerotic processes facilitating pro-inflammatory cell numbers and cytokine milieu. Here, the missing C5aR2 signaling causes a reduction in pro-inflammatory cytokine expression which subsequently leads to fewer inflammatory cells and the observed reduction in atherosclerotic lesion size.

5.3 The lipid metabolism is altered in *C5ar2^{-/-}/Apoe^{-/-}* mice

Atherosclerosis is characterized by an influx of inflammatory cells and lipids into the arterial wall. Lipids circulate as lipoproteins the bloodstream and can enter the vessel wall via a dysfunctional endothelium. The C5aR2 was shown to be involved in lipid metabolism via its capacity to bind ASP [138, 235]. ASP only interacts with C5aR2, but not with C3aR nor C5aR1. Furthermore, ASP has a distinct binding site from C5a. In adipocytes, ASP stimulates triglyceride synthesis in fat-storing cells [138, 139]. Reduced adipose tissue triglyceride synthesis and increased muscle fatty acid oxidation in *C5ar2^{-/-}* mice have been demonstrated [144]. This incited the interest to study the influence of the C5aR2 on lipid metabolism and the implications on atherogenesis. Therefore, the body weight, cholesterol and triglyceride serum levels were assessed.

First, we analyzed 8 weeks old mice before entering the experiments or receiving HFD, to identify the role of C5aR2 in the lipid metabolism without HFD conditions. We

observed lower cholesterol levels in *C5ar2^{-/-}/Apoe^{-/-}* compared to *Apoe^{-/-}* mice, whereas body weight as well as triglycerides were not altered. Also this is in contrast to the expected elevated triglyceride levels caused by the missing ASP stimulation, these findings are in line with a study by Paglialunga and colleagues which demonstrated that *C5ar2^{-/-}* mice have developed a compensatory mechanism of increased muscle fat oxidation to overcome high triglyceride levels [144]. This also may be responsible for the unchanged body weight, or the in general low calorie intake under chow diet conditions may not be sufficient to manifest in a different body weight. On the other hand, the reduced cholesterol levels may be due to a compensatory effect to the missing ASP stimulation. In facing higher triglyceride levels CD36, which is a scavenger receptor, is upregulated and is responsible for cholesterol uptake into the cell [236, 237]. And a study by Drover *et al.* showed that CD36 is important for clearing lipoproteins and the deficiency of CD36 in mice results in hyperlipidemia [238]. Thus, under chow diet conditions triglyceride levels are held in check by increased muscle fat oxidation, overruling the missing ASP-dependent clearance. Triglycerides are more likely to be metabolized than stored in adipose tissue, which also manifests in the unchanged body weights. Even so not being significant, the change in triglyceride metabolism could cause an increased CD36 expression, which results in reduced cholesterol levels.

This observation changes when mice receive a HFD. Administration of HFD 12 and 24 weeks revealed elevated body weight and triglycerides, whereas cholesterol levels are reduced. This observation is done, as well for chow diet mice as for the HFD. The findings of reduced cholesterol levels are confirming to a study by Zhukova in wild-type rats, which shows that cholesterol levels are reduced after HFD. They state that the reduced cholesterol level is due to a compensatory biosynthesis of fatty acids in the rat's liver as a response to the HFD induced inhibition of synthesis of apoproteins and lipoproteins [239]. Despite this results are in line with our findings, further experiments are needed to clarify the role of C5aR2 in cholesterol levels, as Zhukova chose another animal model and genetic background. As such, here reduced cholesterol levels may, like under chow diet conditions, be caused by a compensatory effect to the elevated triglyceride levels. A upregulation of CD36 in response to hypertriglyceridemia could cause the clearance of cholesterol from the blood, and probably also the increased body weights. Triglyceride levels in *C5ar2^{-/-}/Apoe^{-/-}* mice, are elevated and stay increased after HFD. Here, the missing ASP stimulation in *C5ar2^{-/-}/Apoe^{-/-}* mice may

be the reason for elevated and maintained high triglyceride levels, as they are not removed from the blood and stored in adipocytes. The describe increased muscle fat oxidation [144] to overcome high triglyceride levels is presumably overlaid by the high abundance in triglycerides caused by the HFD. However, as the lack of ASP-mediated triglyceride storage in adipocytes is lacking in *C5ar2^{-/-}/Apoe^{-/-}* mice this should manifest in a reduced body weight. As the intake of food rich in fat causes directly after digestion short-term high triglyceride and cholesterol blood levels, presumably other compensatory effects lead to a clearance and storage of triglyceride and cholesterol in fatty tissue independent from ASP, which leads to the observed increased body weights.

In this study only the overall body weight and effects on blood triglyceride and cholesterol level were analyzed, whereas a detailed investigation on the lipid metabolism within atherosclerotic lesions still needs to be the focus of further research. Here, the impact of the C5aR2 deficiency on the activity and expression of lipid transports and enzymes of the lipid homeostasis needs to be clarified, especially in the liver and in macrophages. The liver plays an important role in lipid homeostasis via triglyceride synthesis and lipoprotein particle production, whereas macrophage oxLDL uptake and foam cell formation is a crucial step in atherogenesis.

Taken together, the data shows that the C5aR2 is involved in the lipid metabolism and homeostasis. The observed triglyceride excess is caused by the missing C5aR2-ASP interaction. Despite ASP-dependent elevated triglyceride levels in mice fed either chow or high fat diet, *C5ar2^{-/-}/Apoe^{-/-}* mice showed reduced cholesterol level that may in part contribute to lower atherosclerotic lesions.

5.4 The macrophage phenotype shifts towards to an anti-inflammatory M2 in *C5ar2^{-/-}/Apoe^{-/-}* macrophages

Macrophages play a central role in the initiation and progression of atherosclerosis [40]. In macrophages it was demonstrated, that C5aR2 signals via MAPK and AKT leading to macrophage activation and cytokine release [137, 147]. A concept of polarization was formed by Mantovani. On the one hand, there are the classical activated (M1) and on the other hand, the alternatively activated macrophages (M2) [62]. M1 macrophages possess a more pro-inflammatory phenotype and release cytokines such as TNF- α , IL-1 β and IL-6, whereas M2 are involved in tissue remodeling and repair as well as angiogenesis [63]. During classical M1 activation, upregulation of the inducible nitric oxide synthase (iNOS) can be observed, whereas

arginase-1, an anti-inflammatory factor, increases during the alternative M2 activation. This gives a handy tool to discriminate between the polarizations of macrophages, although further phenotypic markers can be used to define a specific activation type within the full spectrum of macrophages activation. This prompted us to identify the impact of C5aR2 on macrophage phenotype and the consequences for atherosclerotic lesion progression and size.

It has been shown that *C3^{-/-}* in macrophages induces a shift towards M2 polarization. The cleavage product C3a is also acting as an anaphylatoxin and it shows interaction with the C5aR2, but in a weaker manner as C5a [240]. In line with this, in our *C5ar2^{-/-}/Apoe^{-/-}* macrophages a shift towards M2 was observed, as arginase-1 was increased and iNOS was reduced in *C5ar2^{-/-}/Apoe^{-/-}*. Accordingly, to the M2 polarization, in our study the expression of the pro-inflammatory cytokines TNF- α and IL-6, and the adhesion molecule VCAM-1 were reduced. The expression of cell surface VCAM-1 and transcripts has been previously demonstrated in both monocytes and bone marrow-derived macrophages [217]. Thus, less macrophages are recruited to the lesion resulting in a reduced number of lesional macrophages. TNF- α secreted by macrophages was shown to be involved in SMC death [162], whereas high levels of IL-6 was found to exacerbate atherogenesis in mice [193]. Furthermore, reduced expression of C5aR1 was found in *C5ar2^{-/-}/Apoe^{-/-}* macrophages. Activation of C5aR1 leads to classic pro-inflammatory responses like cytokine production and chemotaxis [121], thus reduced C5aR1 expression is dampening pro-inflammatory immune response. This general anti-inflammatory milieu in *C5ar2^{-/-}/Apoe^{-/-}* macrophages is in line with a study by Butcher et al. where it was demonstrated, that M2 macrophages are characterized by the production of anti-inflammatory cytokine, like IL-4, IL-13 and IL-10 [65]. In our study, cytokine production determined by ELISA confirmed a rather anti-inflammatory phenotype of the *C5ar2^{-/-}/Apoe^{-/-}* macrophages, as TNF- α was reduced and the secretion of the anti-inflammatory cytokine IL-10 was elevated. Interestingly, the secretion of IL-6 was increased. Generally, the concept of macrophage polarization is not a fix construct and macrophages can harbor a phenotype located in-between these two poles. So it was shown, that macrophages even within the same site and population can express different and constantly changing phenotypes, which was termed macrophage plasticity [241, 242] Apart from the fact, that the results were obtained from *in vitro* experiments in cultured bone

marrow-derived macrophages the concept of macrophage plasticity might explain the higher IL-6 secretion in an otherwise anti-inflammatory setting.

In conclusion, the results from *in vitro* macrophages confirm the data of the two *in vivo* models. Macrophages show a diminished pro-inflammatory phenotype, which is contributing to the reduction in lesion size and propagating a more stable plaque. The expression of pro-inflammatory cytokines is influenced by the C5aR2 which results in a shift towards a M2 phenotype. That, together with the reduction in cell numbers, contributes to the observed diminished atherosclerotic lesion size. The M2 phenotype and the reduction in lesion size is in line with the observation that M2 macrophages are found more frequently in stable plaque areas [66].

5.5 Differential analysis of C5aR1 and C5aR2 interaction

C5a signals through both C5aR1 and C5aR2. Cooperativity between the receptors of C5a in a model of acute sepsis has been demonstrated. It was shown that both receptors act synergistically on the harmful consequences in sepsis and only the combined blocking mediated the full beneficial effects. This not only manifests in a better outcome in sepsis but also shows a further reduced expression of pro-inflammatory cytokines like HMGB1 [137]. Thus, the combined blocking causes a generally less inflammatory milieu making targeting both receptors together a promising approach in handling diseases. Additionally, the expression of the receptors influences each other. Chen *et al.* proved that the surface expression of C5aR1 was reduced in *C5ar2^{-/-}* neutrophils [127], indicating that C5aR2 may be critical for upregulating cell surface expression of C5aR1. This could be one of the ways in which C5aR2 contributes to the improvement of C5a signals [127]. In addition, crosstalk between C5a receptors has been demonstrated in human neutrophils where C5aR2 was shown to fine tune C5aR1 response by modulating the β -arrestin pathway [133] and recently C5aR2 was found to form heterodimers with C5aR1 with subsequent AKT phosphorylation in macrophages and adipocytes [243, 244]. Of note, C5aR1 overall was shown to mediate both pro-inflammatory and pro-atherogenic mechanisms making it a suitable candidate for modulation and controlling inflammatory responses [108, 133, 151, 245]. In a setting of acute injury-induced atherosclerosis, an important protective role of C5aR1-inhibition with C5aR1-A (JPE1375) and anti-C5aR1 monoclonal antibody was demonstrated. Here, inhibition of C5aR1 leads to a drastic reduction in neointima formation after wire injury further pointing out the prominent role of C5aR1 in atherogenesis [151]. Additionally, long-term oral administration of the

C5aR1-antagonist PMX53 in a native atherosclerosis model resulted in a reduction in atherosclerotic lesion [179]. Due to all these facts, we therefore hypothesized that blockade of C5aR1 in *C5ar2^{-/-}/Apoe^{-/-}* mice may further reduce plaque formation. To investigate this, *C5ar2^{-/-}/Apoe^{-/-}* mice and control *Apoe^{-/-}* mice on HFD were treated with C5aR1-antagonist JPE1375 followed by wire injury of the carotid artery.

First, we checked if the C5aR2 deficiency influences the C5aR1 expression. A previous study demonstrated that in *C5ar2^{-/-}/Apoe^{-/-}* mice the C5aR1 expression in hyperlipidemic mice is reduced. Mice, which underwent endothelial denudation of the carotid artery, revealed a significant reduction in C5aR1 positive cells within the atherosclerosis lesion. This observation was also shown when bone marrow-derived macrophages were analyzed demonstrating that *C5ar2^{-/-}/Apoe^{-/-}* macrophages showed less C5aR1 surface expression than *Apoe^{-/-}* [216]. The findings in this study are in line with our results showing a strong reduction of C5aR1 expression in carotid arteries and aortic roots in mice receiving HFD. Furthermore, we observed a prominent reduction in C5aR1 surface expression comparing *C5ar2^{-/-}/Apoe^{-/-}* to *Apoe^{-/-}* bone marrow-derived macrophages, which was even more emphasized when comparing WT and *C5ar2^{-/-}* single knock-out macrophages. Of note, in whole aortas no difference could be seen which might be due to the largely healthy aortic tissue. The observation that C5aR1-positive cells are significantly reduced in neointimal plaques and a reduction of C5aR1 mRNA in carotid arteries in *C5ar2^{-/-}/Apoe^{-/-}* mice may be an additional mechanism underlining the atheroprotective role of C5aR2 deficiency. Interestingly, the C5aR2 expression in injured compared to uninjured carotid arteries of *Apoe^{-/-}* mice was significantly elevated. Uninjured arteries have only little to no atherosclerotic plaque formation in comparison to the injured. Thus the increase of C5aR2 expression is a response to the injury, as it has already been described, that C5aR2 expression correlates with plaque progression and complexity [147]. Additionally, C5aR2 did not only regulate the expression of C5aR1 in the arterial tissue and in macrophages but also in the liver. In the liver, there was significantly reduced C5aR1 mRNA between *C5ar2^{-/-}/Apoe^{-/-}* and *Apoe^{-/-}* mice after 24 weeks of HFD. C5aR2 expression in livers and aortic roots was increased in *Apoe^{-/-}* mice, which is in line with previous findings where C5aR2 expression correlates with human plaque complexity. A study in human atherosclerosis could demonstrate that the C5aR2 expression is rising with the stage of atherosclerosis and plaque complexity indicating a pro-inflammatory role of C5aR2 in human atherogenesis [147]. In conclusion, the

Discussion

C5aR1 expression is reduced in *C5ar2^{-/-}/Apoe^{-/-}* mice, especially under HFD conditions giving additional beneficial effects in targeting C5aR2.

To study the cooperative effect of blocking both receptors, we applied a model which gave us the opportunity to additionally block C5aR1 with an inhibitor (C5aR1-A). *C5ar2^{-/-}/Apoe^{-/-}* mice, which underwent endothelial denudation were additionally implanted with an osmotic mini-pump which contained the C5aR1-A. As expected, neointimal plaque size and inflammatory content were significantly reduced in C5aR1-A-treated *C5ar2^{-/-}/Apoe^{-/-}* mice compared to *C5ar2^{-/-}/Apoe^{-/-}* and *Apoe^{-/-}* mice. This is also in agreement with a previous report in which dual blockade of both C5a receptors was highly protective in a mouse model of sepsis where the two receptors work synergistically in mediating the harmful effect [137]. In addition, macrophage and CD3⁺ T-cell content was further decreased, whereas SMC content was comparable between C5aR1-A-treated and *C5ar2^{-/-}/Apoe^{-/-}* mice. The reduction in macrophages can explain the further reduction of neointimal lesion after dual absence of C5a signaling. Despite there being no further increase in SMC numbers, the plaque shows a more stable phenotype with a reduced inflammatory milieu. This is underlined by the further reduction in CD3⁺ T-cell content, as T-lymphocytes are known to be pro-inflammatory cells found in human and murine atherosclerotic lesions. In atherosclerotic lesions they were shown to influence plaque stability, and furthermore high numbers of T-cells correlate with a higher probability of plaque rupture [201, 208]. The content of C5aR1⁺ and TNF- α ⁺ cells were comparable between C5aR1-A-treated *C5ar2^{-/-}/Apoe^{-/-}* and *C5ar2^{-/-}/Apoe^{-/-}* mice, however, there was a significant decrease in both groups compared to *Apoe^{-/-}* mice. Even so, there is no further decrease in TNF- α , the observed low expression is atheroprotective as it was shown by Boyle and colleagues that TNF- α is involved in plaque stability and stimulating atherosclerosis [162].

This is in line with the previous study where C5aR1 was blocked with the antagonist JPE1375 in *Apoe^{-/-}* mice and demonstrated a significantly reduced neointimal plaque size [151]. Thus, the dual blockage is further facilitating a less inflamed atherosclerotic milieu, while targeting C5aR1 and C5aR2 alone already limits atherosclerosis [151], optimal protection may be achieved by blocking both receptors. Furthermore, our findings are corresponding to *in vitro* observations of Vijayan *et al.* which demonstrated that in bone marrow-derived macrophages C5aR2 alone can trigger TNF- α secretion,

but only the presence of both C5a receptors ensures maximal induction of pro-inflammatory cytokine secretion [147].

This data may explain why in human atherosclerotic plaques both C5aR1 and C5aR2 are simultaneously increased upon plaque progression in different stages of plaque development. [147]. Of note, the reduction of C5aR1 in *C5ar2^{-/-}/Apoe^{-/-}* mice may likely be a contributory mechanism to the overall atheroprotective effect of C5aR2 deficiency observed in this study and blocking both receptors may be more beneficial. The data presented here extends previous observation in human atherosclerotic plaques [147] and provides a direct role for C5aR2 in atherosclerosis. Thus, addressing both receptors may be an even more effective way in handling atherosclerosis.

5.6 Conclusion and perspective

This study focuses on role of the second receptor C5aR2 for the anaphylatoxin C5a in atherosclerosis. Here, it is shown that the C5aR2 has actual signaling function with pro-atherogenic properties, as demonstrated in two different mouse models of atherosclerosis. Inflammatory cytokine expression and inflammatory cells within the lesion of *C5ar2^{-/-}/Apoe^{-/-}* mice are reduced, which further contributes to a diminished atherosclerotic lesion formation.

We observed in both, a chronic diet-induced atherosclerosis model and in an acute injury-induced atherosclerosis model a reduction in atherosclerotic lesion size. This reduction is accompanied by a more stable plaque phenotype manifesting in a reduced number of macrophages and increased SMCs numbers. In line with this, an overall reduced pro-inflammatory milieu and less pro-inflammatory cells can be found within the lesion. Furthermore, the *in vitro* analysis of bone marrow-derived macrophages revealed an anti-inflammatory M2 polarization, but still, further studies are needed to clarify the detailed influence of the C5aR2 on macrophages polarization and then implication on atherosclerosis. In the model of chronic diet-induced atherosclerosis reduced numbers of circulating monocytes were found. Here, further experiments are also required, as it is important to gain more insight into how the C5aR2 effects recruitment of inflammatory cells and how that may possibly result in adverse effects. In our study we could show elevated triglyceride and reduced cholesterol level, as well as increased pro-inflammatory TNF- α and IL-6 gene expression in the liver. As the high triglyceride levels are in part caused by the missing ASP-C5aR2 interaction, a more detailed study is needed in respect to the role the C5aR2 plays in the liver. Especially

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if these findings can rather be explained by a stress response to the high triglyceride levels or if the effects are actually caused by the missing C5aR2 signaling. Furthermore, as already described by other workgroups, we reported that pro-inflammatory cytokine expression is reduced in *C5ar2^{-/-}/ApoE^{-/-}* mice, but still, the exact mechanism and involved signaling pathways need to be elucidated. The absence and blocking of both receptors, C5aR1 and C5aR2, revealed a cooperative relationship between the two receptors resulting in a pronounced anti-atherogenic phenotype in acute injury-induced atherosclerosis. Despite this promising approach in handling atherosclerosis by addressing both receptors, more studies need to be performed to identify possible interactions and how a modulation of them might affect the general immune response.

Taken together, this study demonstrates clear pro-atherogenic properties of the C5aR2 in two atherosclerosis models. This makes the C5aR2 an ideal target for synthetic drug application. Especially in injury-induced atherosclerosis, which occurs after vascular intervention and stent implantation, targeting C5aR2 could provide a useful tool to prevent restenosis and could make further interventions unnecessary. Making additional surgical intervention obsolete can greatly contribute to a better life expectancy, particularly for elderly people in general already suffering from a poor health condition.

6 Summary

In this work, the role of the C5a anaphylatoxin receptor C5aR2 in two atherosclerosis models was examined. Cardiovascular diseases (CVDs) are the main cause of death in the industrialized western world. Atherosclerosis is the underlying pathophysiological cause for CVDs [25]. It causes myocardial infarction, angina pectoris, sudden cardiac death and stroke [26]. Atherosclerosis is an inflammatory disease of the arterial vessel wall and evolves mainly at site of disturbed blood flow as seen at bifurcations [27]. The complement system is a part of the innate immune system and acts as a first line of defense against intruding pathogens. It has been shown to be involved in the initiation and progression of atherogenesis in humans and mice. Its activation triggers the cleavage of C5 into C5a and C5b. C5a is an anaphylatoxin, which signals through its two receptors C5aR1 and C5aR2. It was shown for C5aR1 to be implicated in atherosclerosis where it facilitates atherogenesis. On the other hand, it was demonstrated that expression of the C5aR2 in human correlates with the stage of atherogenesis. Nevertheless, the contribution of C5aR2 in atherosclerosis remains mainly unexplored. Thus, the aim of this study was to identify the role of C5aR2 in two independent mouse models of chronic diet-induced or acute injury-induced atherosclerosis and the underlying processes regulated and effected by C5aR2. For both approaches a *C5ar2^{-/-}/Apoe^{-/-}* mouse model was used, with a hyperlipidemic *Apoe^{-/-}* background.

To investigate the underlying processes and mechanisms and the C5aR2 contribution to atherogenesis, gene expression of pro-inflammatory cytokines and adhesion molecules, as well as the number of cells positive for pro-inflammatory markers were analyzed

In Both, the acute injury-induced atherosclerosis and the chronic diet-induced atherosclerosis, we found a reduced lesion size in *C5ar2^{-/-}/Apoe^{-/-}* compared to their corresponding *Apoe^{-/-}* control mice. This reduction was accompanied by a more stable plaque phenotype. Analysis of the cellular composition and gene expression within the lesion also revealed a reduction in inflammatory cells and cytokines.

Furthermore, *in vitro* analysis of bone marrow-derived macrophages indicate that in *C5ar2^{-/-}/Apoe^{-/-}* macrophages a shift in the polarization towards an anti-inflammatory M2 phenotype occurs, which results in a reduction in pro-inflammatory cytokine and adhesion molecule expression. On the other hand, systemic analysis demonstrated an increase in blood triglyceride and a reduction of cholesterol levels. Additionally, in the

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liver of *C5ar2^{-/-}/Apoe^{-/-}* mice elevated expression levels of pro-inflammatory cytokines was found which might be a stress response of the liver to the elevated systemic triglyceride levels.

Finally, to investigate the cooperative effect of blocking both C5a receptors, *C5ar2^{-/-}/Apoe^{-/-}* mice which underwent endothelial denudation additionally were implanted an osmotic mini-pump equipped with a C5aR1 blocking inhibitor (C5aR1-A). Analysis of the carotid arteries revealed a further reduction in lesion size in C5aR1-A-treated *C5ar2^{-/-}/Apoe^{-/-}* mice. This also manifests in a reduced macrophage number and pro-inflammatory cytokine and adhesion molecule expression, whereas SMC numbers stay unchanged. Taken together, these data indicate that manipulating both receptors for C5a could provide an additional beneficial effect in handling atherosclerosis and restenosis.

In summary, the shown data demonstrate that the C5aR2 is a functional receptor acting in a pro-atherogenic manner on the progression and initiation of atherosclerosis. C5aR2 induces pro-inflammatory cytokine expression and inflammatory cell recruitment, which may be the cause how C5aR2 is mediating the atherogenic effect. This makes C5aR2 a suitable candidate for future drug targeting in order to handle atherosclerosis and restenosis. Nevertheless, further experiments are needed to clarify the exact mechanism of how C5aR2 is mediating the pro-atherogenic effect.

7 Zusammenfassung

In dieser Arbeit wurde die Rolle des C5a Anaphylatoxin-Rezeptors C5aR2 in zwei Arteriosklerosemodellen untersucht. Kardiovaskuläre Erkrankungen sind die häufigste Todesursache in der westlichen industrialisierten Welt. Denen der kardiovaskulären Erkrankungen zugrundeliegenden pathophysiologischen Prozesse ist die Arteriosklerose. Diese ist unter anderem für Herzinfarkte, Angina Pectoris, plötzlicher Herzstillstand und Schlaganfälle verantwortlich. Die Arteriosklerose wird als eine entzündliche Krankheit der arteriellen Gefäßwände angesehen, welche bevorzugt an Stellen turbulenten Blutflusses, wie er an Bifurkationen auftritt, entsteht. Das Komplement System ist Teil des angeborenen Immunsystems und fungiert als eine erste Barriere gegen eindringende Pathogene. In der Atherogenese spielt es eine wichtige Rolle in der Initialisierung und dem Fortschreiten der Arteriosklerose. Die Aktivierung des Komplement Systems leitet die Spaltung von C5 in C5a und C5b ein. C5a ist ein Anaphylatoxin welches seine Wirkung über die beiden Rezeptoren C5aR1 und C5aR2 vermittelt. Für den C5aR1 Rezeptor konnte gezeigt werden, dass er in der Arteriosklerose involviert ist wobei er hier die Atherogenese beschleunigt. Zudem konnte für den C5aR2 Rezeptor gezeigt werden, dass seine Expression in Menschen mit den Arteriosklerosestadien korreliert. Nichtsdestotrotz ist der Einfluss des C5aR2 auf die Arteriosklerose weitgehend unbekannt. Aus diesem Grund war das Ziel dieser Arbeit die Rolle des C5aR2 Rezeptors in der Arteriosklerose und deren zugrundeliegenden Prozesse genauer zu bestimmen. Hierfür wurde zwei unabhängige Modelle herangezogen: Ein Modell der durch chronische Diät verursachten Arteriosklerose und ein Modell der durch akute Verletzung verursachten Arteriosklerose. In beiden Ansätzen wurden C5aR2 defiziente Mäuse herangezogen, welche einen hyperlipidämischen ApoE defizienten Hintergrund aufweisen.

Um die zugrundeliegende Prozesse und Mechanismen des C5aR2 Rezeptors in der Arteriosklerose zu untersuchen wurden die Expression von pro-inflammatorischen Zytokinen, Adhäsionsmolekülen und Anzahl an Zellen positiv auf pro-inflammatorischen Markern untersucht.

In beiden, dem akuten als auch dem chronischen Arteriosklerosemodell, zeigte sich eine Verringerung der Läsionsfläche in *C5ar2^{-/-}/ApoE^{-/-}* im Vergleich zu den entsprechenden *ApoE^{-/-}* Kontrollmäusen. Die beobachtete Verringerung war begleitet von einem stabileren Plaquephänotypen. Untersuchungen der läsionalen zellulären

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Zusammensetzung und der Genexpression von pro-inflammatorischen Zytokinen zeigten eine Verminderung an Zellen positiv für pro-inflammatorischen Markern und eine niedrigere Genexpression an pro-inflammatorischen Zytokinen.

Des Weiteren weisen in vitro Experimente in Knochenmarksmakrophagen darauf hin, dass die C5aR2 Defizienz eine Verschiebung der Makrophagenpolarisation hin zu einen anti-inflammatorischen M2 Phänotypen verursacht. Dies wird in einer Reduktion der Genexpression von pro-inflammatorischen Zytokinen und Adhäsionsmolekülen deutlich. Dem hingegen zeigen systemische Untersuchungen, dass es in C5aR2 defizienten Mäusen zu einer Verringerung der Cholesterinlevel und Erhöhung der Triglyzeridlevel kommt. Auch war in der Leber von *C5ar2^{-/-}/ApoE^{-/-}* Mäusen die Genexpression an pro-inflammatorischen Zytokinen erhöht. Dies könnte jedoch möglicherweise auf eine Stressreaktion der Leber auf die erhöhten Triglyzeridlevel zurückzuführen sein.

Zuletzt wurden die kooperativen Effekte einer Doppelblockade von beiden C5a Rezeptoren, dem C5aR1 und dem C5aR2, untersucht. Dafür wurden *C5ar2^{-/-}/ApoE^{-/-}* Mäusen, bei welchen eine endotheliale Denudation vorgenommen wurde, zusätzlich mit einer osmotischen Minipumpe versehen, welche einen C5aR1 Inhibitor (*C5ar2^{-/-}/ApoE^{-/-}*+C5aR1-A) enthielten. Untersuchungen der Karotiden zeigten eine weitere Verringerung der Läsionsfläche in *C5ar2^{-/-}/ApoE^{-/-}*+Inhibitor Mäusen zu den entsprechenden *C5ar2^{-/-}/ApoE^{-/-}* Mäusen auf. Diese Beobachtung konnte ebenfalls mit einer weiteren Verringerung der Makrophagenzahl gemacht werden, wohingegen die Zahl der glatten Muskelzellen im Vergleich zu den *C5ar2^{-/-}/ApoE^{-/-}* Mäusen unverändert blieb. Zudem waren ebenfalls die pro-inflammatorischen Zytokine und Adhäsionsmoleküle weiter verringert. Hier kann gesagt werden, dass die Blockade beider Rezeptoren zu einem positiven additivem Effekt in der Behandlung der Arteriosklerose und Restenose führt.

Abschließend kann zusammenfassend gesagt werden, dass die hier dargelegten Daten darauf hinweisen, dass der C5aR2 Rezeptor funktionale Eigenschaften hat und einen pro-atherogene Einfluss auf die Initialisierung und das Fortschreiten der Arteriosklerose hat. C5aR2 induziert die Expression von pro-inflammatorischen Zytokinen und stimuliert die Rekrutierung von inflammatorischen Zellen. Dies könnte die Ursache sein auf welche Weise der C5aR2 den atherogenen Effekt vermittelt. Diese Eigenschaft macht ihn zu einem geeigneten Kandidaten für zukünftige

Medikamentenentwicklung. Nichtsdestotrotz, sind noch weiterführende Experimente vonnöten, um die genauen Mechanismen aufzudecken auf welche Art und Weise der C5aR2 Rezeptor seine pro-atherogenen Effekte vermittelt

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11 Curriculum Vitae

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