Role of Coagulation Factor XII in Atherosclerosis

Rolle des Koagulationsfaktors XII in der Atherosklerose

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For my parents

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1 Summary/Zusammenfassung

1.1 Summary

Atherosclerosis is considered a chronic inflammatory disease of the arterial vessel wall which is not only modulated by innate and adaptive immune responses but also by factors of the blood coagulation system.

In general hypercoagulability seems to increase the development and progression of experimental atherosclerosis in mice on an atherogenic background. In addition, the great majority of coagulation proteins including coagulation factor XII (FXII) have been detected in early and advanced human atherosclerotic lesions supporting the cross-link between the coagulation system and atherosclerosis. Moreover, FXII has been detected in close proximity to macrophages, foam cells and smooth muscle cells in these lesions and has been demonstrated to be functionally active in human plaques. Although these data indicate that factor XII may play a role in atherogenesis a direct contribution of FXII to atherogenesis has not been addressed experimentally to date. Furthermore, clinical studies examining the function of FXII in vascular disease have yielded conflicting results.

Hence, in order to investigate the function of coagulation factor XII in atherosclerosis apolipoprotein E and FXII-deficient (*F12*$^{-/-}$*apoE*$^{-/-}$) mice were employed. Compared to *F12*$^{+/+}$*apoE*$^{-/-}$ controls, atherosclerotic lesion formation was reduced in *F12*$^{-/-}$*apoE*$^{-/-}$ mice, associated with diminished systemic T-cell activation and Th1-cell polarization after 12 weeks of high fat diet. Moreover, a significant decrease in plasma levels of complement factor C5a was evidenced in *F12*$^{-/-}$*apoE*$^{-/-}$ mice. Interestingly, C5a increased the production of interleukin-12 (IL-12) in dendritic cells (DCs) and enhanced their capacity to trigger antigen-specific interferon-gamma (IFNγ) production in OTII CD4^{+} T cells *in vitro*. Importantly, a reduction in frequencies of IL-12 expressing splenic DCs from atherosclerotic *F12*$^{-/-}$*apoE*$^{-/-}$ *versus* *F12*$^{+/+}$*apoE*$^{-/-}$ mice was observed *in vivo*, accompanied by a diminished splenic *Il12* transcript expression and significantly reduced IL-12 serum levels.

Consequently, these data reveal FXII to play an important role in atherosclerotic lesion formation and to promote DC-induced and systemic IL-12 expression as well as pro-inflammatory T-cell responses likely at least in part via the activation of the complement system.

1.2 Zusammenfassung

Die Arteriosklerose wird als eine chronisch entzündliche Erkrankung der arteriellen Gefäßwand angesehen, welche nicht nur durch Antworten des angeborenen und erworbenen Immunsystems, sondern auch durch Faktoren des Blutgerinnungssystems beeinflusst wird.

Aus Tierstudien weiß man, dass Hyperkoagulabilität im Allgemeinen die Entwicklung und das Fortschreiten der Arteriosklerose in Mäusen mit atherogenem Hintergrund steigert. Ferner wurde die Mehrheit der Blutgerinnungsproteine inklusive des Koagulationsfaktors XII (FXII) in frühen und fortgeschrittenen arteriosklerotischen Läsionen in Menschen nachgewiesen, was eine mögliche Verbindung zwischen Koagulationssystem und Arteriosklerose untermauert. Darüber hinaus wurde der FXII in diesen Läsionen im unmittelbaren Umfeld von Makrophagen, Schaumzellen und glatten Muskelzellen nachgewiesen und dessen funktionelle Aktivität in humanen Plaques aufgezeigt. Obwohl diese Daten eine Funktion von FXII in der Arteriosklerose nahelegen, wurde eine direkte Beteiligung von FXII an der Atherogenese bislang experimentell noch nicht untersucht. Ferner erzielten klinische Studien, welche die Funktion von FXII in Gefäßerkrankungen untersuchten, widersprüchliche Resultate.

Um die Funktion des Koagulationsfaktors XII in der Atherosklerose zu untersuchen, wurden daher Apolipoprotein E und FXII-defiziente (*F12*$^{-/-}$*apoE*$^{-/-}$) Mäuse eingesetzt. Die Ausbildung arteriosklerotischer Läsionen war in *F12*$^{-/-}$*apoE*$^{-/-}$ Mäusen im Vergleich zu *F12*$^{+/+}$*apoE*$^{-/-}$ Kontrollen reduziert. Dies ging mit einer verringerten systemischen T-Zell Aktivierung und Th1-Zell Polarisierung nach 12-wöchiger atherogener Diät einher. Ferner konnte eine signifikante Reduktion der C5a-Plasmaspiegel in *F12*$^{-/-}$*apoE*$^{-/-}$ Mäusen nachgewiesen werden. Interessanterweise konnte C5a die Interleukin-12 (IL-12) Produktion in dendritischen Zellen (DCs) steigern, sowie deren Fähigkeit erhöhen, eine antigen-spezifische Interferon-gamma (IFNγ) Produktion *in vitro,* in OTII CD4^{+} T-Zellen zu induzieren. Insbesondere konnte *in vivo* eine Reduktion IL-12-exprimierender DCs in Milzen arteriosklerotischer

F12$^{-/-}$*apoE*$^{-/-}$ im Vergleich zu *F12*$^{+/+}$*apoE*$^{-/-}$ Mäusen beobachtet werden, welche mit einer verminderten Expression an *Il12* Transkripten in der Milz und signifikant reduzierten IL-12 Serumspiegeln einherging.

Zusammenfassend zeigen diese Daten, dass FXII eine wichtige Rolle in der Bildung arteriosklerotischer Läsionen spielt und die DC-vermittelte und systemische IL-12 Expression, sowie pro-inflammatorische T-Zell Antworten, vermutlich teilweise über eine Aktivierung des Komplementsystems, fördert.

Index of Abbreviations

α	Alpha
β	Beta
β-FXIIa	Hageman factor fragment
γ	Gamma
°C	Degree Celsius
µg	Microgram
µl	Microliter
µm	Micrometer
µM	Micromolar
a	Activated
Ab	Antibody
ACE	Angiotensin converting enzyme
ACS	Acute coronary syndrome
Ag	Antigen
AMI	Acute myocardial infarction
APC	Allophycocyanin/Antigen presenting cell/ Activated protein C
ApoE	Apolipoprotein E
aPPT	Activated partial thromboplastin time
ARG1	Arginase-1
BK	Bradykinin
BM	Bone marrow
BMDC	Bone marrow derived dendritic cell
BMM	Bone marrow derived macrophage
BMT	Bone marrow transplantation
bp	Base pair
BR	Bradykinin receptor
BSA	Bovine serum albumin

C	Complement component
C1INH	C1 esterase inhibitor
C1qR	Complement C1q receptor
CAD	Coronary artery disease
CCR	Chemokine receptor
CD	Cluster of differentiation
cDC	Conventional dendritic cell
cDNA	Complementary DNA
CFSE	Carboxyfluorescein succinimidyl ester
CK-1	Cytokeratin-1
CMKLR1	Chemokine receptor-like 1
CO_2	Carbon dioxide
CLTA-4	Cytotoxic T-lymphocyte antigen 4
Ctrl	Control
CXCR	Chemokine receptor
Cy3	Cyanine 3
Cy5.5	Cyanine 5.5
Cy7	Cyanine 7
DAPI	4',6-Diamidino-2-phenylindole
DC	Dendritic cell
DDW	Double distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
D-PBS	Dulbecco’s phosphate buffered saline
DS	Dermatane sulfate
EAL	Early atherosclerotic lesion
EC	Endothelial cell
ECM	Extracellular matrix
e.g.	For example
EDTA	Ethylenediaminetetraacetic acid

EGFR	Endothelial growth factor receptor
ELISA	Enzyme linked immunosorbent assay
EMMPRIN	Extracellular matrix metalloproteinase inducer
F	Factor
FACS	Fluorescence activated cell sorting
FBS/FCS	Fetal bovine serum/Fetal calf serum
FcγRI	Fc gamma receptor 1
FH	Complement component factor H
Fig.	Figure
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead box P3
FSC	Forward scatter
Fwd primer	Forward primer
FXII	Factor XII
g	Relative centrifugal force/ G force
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage colony stimulating factor
GP	Glycoprotein
GPCR	G protein-coupled receptor
gr	Gram
h	Hour
hBcl-2	human B-cell lymphoma 2
hi	High
HMWK/HK	High molecular weight kininogen
H&E	Haematoxylin and Eosin
HBSS	Hank’s balanced salt solution
HCl	Hydrochloric acid
HDL	High density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hf	Hageman factor fragment

HFD	High fat diet
HMW	High molecular weight
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HRG	Histidine-rich glycoprotein
HRP	Horse radish peroxidase
IBM	Inflammatory bowel disease
ICAM-1	Intercellular adhesion molecule-1
IFNγ	Interferon gamma
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor-1
IL	Interleukin
iNOS	Inducible nitric oxide synthase
kb	Kilobase
KKS	Kallikrein-kinin system
K_m	Michaelis konstant
KO	Knockout
l	Liter
LDL	Low density lipoprotein
LDLR	LDL receptor
LFA-1	Lymphocyte function-associated antigen 1
LN	Lymph node
Lo	Low
LPS	Lipopolysaccharide
M	Molarity
mAb	Monoclonal Antibody
Mac	Macrophage
MACS	Magnetic activated cell sorting
MAPK	Mitogen-activated protein kinases
MCP-1	Monocyte chemotactic protein-1
M-CSF	Macrophage colony stimulating factor

MFI	Mean fluorescence intensity
mg	Milligram
$MgCl_2$	Magnesium chloride
MHC	Major histocompatibility complex
MIF	Macrophage migration inhibitory factor
min	Minutes
ml	Milliliter
mm	Millimeter
mM	Millimolar
MMP	Matrix metalloproteinase
MRC-1	C-type mannose receptor-1
mRNA	Messenger RNA
ng	Nanogram
nm	Nanometer
nM	Nanomolar
N_2	Nitrogen
Na_2HPO_4	Disodium phosphate
NaCl	Sodium chloride
NOS2	Nitric oxide synthase 2
nt	Nucleotide
o/n	Overnight
OCT	Optimal cutting temperature
oligo(dT)	Oligo desoxythymidine
OSM	Oncostatin M
OVA	Ovalbumin
oxLDL	Oxidized low density lipoprotein
PAI-I	Plasminogen activator inhibitor I
PAR	Protease activated receptor
pAb	Polyclonal antibody
PAMP	Pathogen associated molecular pattern

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PECAM-1	Platelet endothelial cell adhesion molecule 1
PerCP	Peridinin chlorophyll
PFA	Paraformaldehyde
PK	Prekallikrein
PLA	Platelet-leucocyte aggregate
PLT	Platelet
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear leucocyte
PRCP	Prolylcarboxypeptidase
PRR	Pattern recognition receptor
PSGL-1	P-Selectin glycoprotein ligand 1
P/S	Penicillin and Streptomycin
q.s.	Quantity sufficient
qPCR	Quantitative real time PCR
RA	Rheumatoid arthritis
RBC	Red blood cell
Rev primer	Reverse primer
RNA	Ribonucleic acid
RORγt	RAR related orphan receptor γt
ROS	Reactive oxygen species
rpm	Rotations per minute
RPMI	Roswell park memorial institute
RT	Room temperature
RT-PCR	Reverse transcription PCR
SAAL	Stable advanced atherosclerotic lesion
SD	Standard deviation
SDS	Sodium dodecyl sulfate

SEM	Standard error of mean
SMA	Smooth muscle cell actin
SMC	Smooth muscle cell
SN	Supernatant
SSC	Sideward scatter
TAFI	Thrombin-activated fibrinolytic inhibitor
T-bet	T-box expressed in T cells
TMCAO	Transient middle cerebral artery occlusion
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TGFβ	Transforming growth factor beta
Th	Helper T cell
TLR	Toll-like receptor
TMB	3,3’,5,5’-Tetramethylbenzidine
TNFα	Tumour necrosis factor alpha
t-Pa	Tissue plasminogen activator
Treg	Regulatory T cell
TRIS	Tris(hydroxymethyl)aminomethane
u-Pa	Urokinase plasminogen activator
uPAR	Urokinase plasminogen activator receptor
UT	Untreated
UV	Ultraviolet
VCAM-1	Vascular cell adhesion molecule 1
VLDL	Very low density lipoprotein
VSMC	Vascular smooth muscle cell
WBC	White blood cell
WT	Wildtype

2. Introduction

2.1 Atherosclerosis

Atherosclerosis is considered a chronic inflammatory disorder of the arterial vessel wall[1-3] and represents the main cause of morbidity and mortality in the western world. Progression of atherosclerosis is characterized by a gradual reduction in vessel lumen, accompanied by an increased risk for plaque rupture, and is the underlying cause of coronary artery disease (CAD) or cerebrovascular disease with complications such as acute coronary syndrome (ACS), myocardial infarction or stroke[4,5].

Although hyperlipidemia is still considered to be the major risk factor for the development of atherosclerosis[6], due to extensive studies in the field of atherogenesis it is also quite clear that disease initiation and progression are far more complex and involve a multifaceted network of cellular and molecular responses[1]. Moreover, numerous individual risk factors, such as age, smoking, hypertension, hypercholesterolemia, diabetes, obesity, genetic disorders or physical inactivity all promote endothelial damage and dysfunction[7].

It is well accepted by the scientific community that the initiation of atherosclerosis that precedes lesion formation is caused by endothelial activation and dysfunction altering the homeostatic functions of the endothelium including an increase in endothelial permeability and the up-regulation of adhesion molecules **(Figure 2.1)** such as vascular cell-adhesion molecule-1 (VCAM-1)[1,8,9]. There is strong experimental and clinical evidence that high blood lipid and cholesterol levels promote atherosclerosis. In the blood cholesterol is transported by low-density-lipoproteins (LDL). LDL particles consist of esterified cholesterol, triglycerides, phospholipids, free cholesterol and apolipoprotein B100 (ApoB100) and accumulate in the arterial intima where they bind to proteoglycans of the extracellular matrix (ECM) via ApoB100, which makes them an important factor in the initiation of vascular inflammation and atherosclerosis[2,4,10,11].

LDL particles are retained in the intima where they accumulate and undergo oxidative modifications resulting in the activation of endothelial cells and macrophages and the initiation of inflammatory responses. LDL and oxLDL, the latter covering a broad spectrum of chemical modifications[2,12], promote the activation of the endothelium and result in the up-regulation of adhesion molecules and the migration of leucocytes from the circulation into the intima, promoting fatty streak formation[2,12]. Fatty streaks are early lesions consisting of sub-endothelial lipid depositions, T cells and lipid-laden monocytes and macrophages (foam cells). This progression of lesion formation is accompanied by T-cell activation, smooth muscle cell (SMC) migration from the media into the intima, increased adherence of platelets to the dysfunctional endothelium, exposed collagen or macrophages, as well as continuous recruitment of leucocytes contributing to inflammation and thus promoting the formation of advanced lesions[1] **(Figure 2.1).**

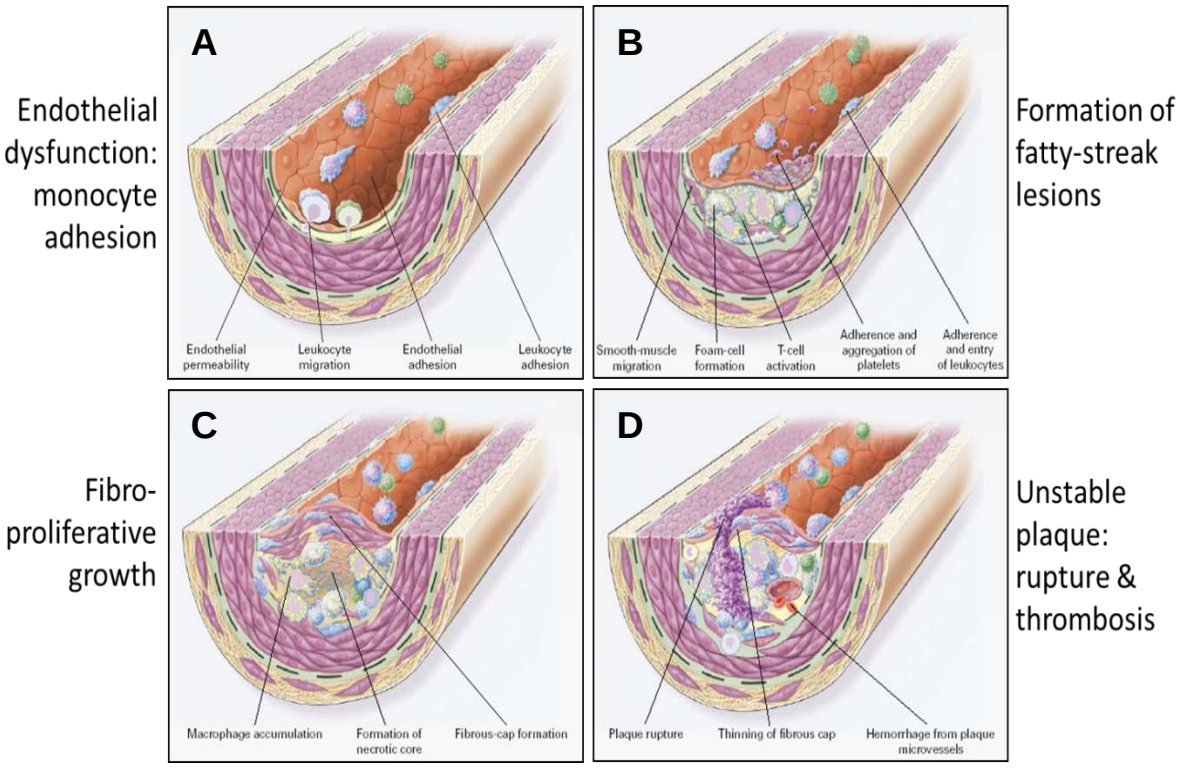

Figure 2.1 The progression of atherosclerotic plaques. Source: Adapted from Ross R. 1999[1].

Advanced atheromas (mature plaques) are characterized by the presence of a lipid rich necrotic core, containing leucocytes, lipids, cholesterol crystals, apoptotic cells and cell debris and are covered by a fibrous cap that prevents the contact of pro-thrombotic plaque material with the blood and consists mainly of smooth muscle cells and collagen fibers[13]. Interferon-gamma (IFNγ) from activated Th1 cells in the plaque has been to inhibit the production of interstitial collagens by vascular smooth muscle cells (VSMCs) and to cause apoptosis of SMCs. In addition CD40 ligation was demonstrated to increase the expression of matrix-degrading proteases such as matrix metalloproteinases (MMPs) by macrophages[13]. This results in a thinning of the fibrous cap and gives rise to the formation of unstable rupture-prone fibrous plaques[13,14]**(Figure 2.1)**. Upon plaque rupture, pro-thrombotic plaque content is exposed to the blood triggering thrombus formation which may ultimately result in vascular occlusion causing severe clinical complications such as myocardial infarction or ischemic stroke[2,13,15].

2.1.1 Atherosclerosis - An immune disease

Although various non-immune cells are involved in atherosclerosis including endothelial cells (ECs), vascular smooth muscle cells and platelets, it is nowadays well established that atherosclerosis is a chronic inflammatory disease, with both innate and adaptive immunity playing major roles in disease initiation and progression[13,15,16].

2.1.2 The innate immune system in atherosclerosis

2.1.2.1 Monocytes

Already at the early stage of endothelial activation, monocytes, dendritic cells (DCs) and T cells get recruited to the intima in response to chemokines that are released from endothelial cells upon endothelial activation. Monocytes, as part of the innate immune system, are among the first cells to enter the intima[17], due to their rapid mobilization from the blood under

inflammatory conditions. They are recruited to the sites of endothelial dysfunction by chemokines and adhesions molecules and transmigrate through the endothelium[18] where they promote a pro-inflammatory environment.

In mouse models, an increased influx and accumulation of the inflammatory Ly6C^{hi} monocyte subset was observed[19]. Ly6C^{lo} monocytes also enter atherosclerotic lesions, however less efficiently[20]. Whereas Ly6C^{hi} monocytes are thought to promote inflammation, the Ly6C^{lo} monocyte subset is considered to resolve inflammation[17]. The importance of monocyte recruitment in atherogenesis has been experimentally proven since inhibition of monocyte migration by either blocking attracting chemokines or the cognate chemokine receptors on the monocytes results in a reduction of atherosclerosis[21,22].

In addition, Ly6C^{hi} monocytes within the lesion were demonstrated to differentiate into macrophages induced by EC- or SMC-derived macrophage-colony stimulating factor (M-CSF)[19,23]. However, under inflammatory conditions monocytes can also develop into monocyte-derived DCs, which possess many of the conventional DC features[24]. Moreover, under certain inflammatory conditions monocytes may express the pan DC marker CD11c, along with MHCII and co-stimulatory molecules, acquiring a DC phenotype but maintaining functional characteristics of monocytes[25]. Also, in *CD11c*$^{-/-}$*apoE*$^{-/-}$ mice under hypercholesterolemic conditions, deficiency of CD11c results in diminished monocyte/macrophage accumulation in the lesions and attenuated atherosclerosis possibly due to a lack of CD11c^{+} “foamy” monocyte adhesion to ECs[26].

2.1.2.2 Macrophages

Macrophages represent not only the predominant cell type in atherosclerotic lesions[2,27] but also promote lesion development and progression. It has been demonstrated that besides monocyte-derived macrophages also resident aortic macrophages, local proliferation of macrophages as well as apoptosis of macrophages contribute to disease

progression[17,28]. The importance of macrophages in plaque development is also emphasized by the report of a direct correlation between the number of plaque-infiltrating macrophages and lesion size[29].

Several studies demonstrated the presence of classically activated, inflammatory M1 and alternatively activated, reparative M2 macrophage subsets in human and murine atherosclerotic plaques[27,29-31]. A recent study moreover described an initial predominance of reparative Arginase-I positive ($ArgI^{+}$) M2 macrophages in *apoE*$^{-/-}$ mice, whereas with disease progression a phenotype switch with prevalence of M1 over M2 macrophages occurs, which may possibly be triggered by a predominance of IFNγ-producing Th1 cells in advanced plaques[29]. However, the effects of M1 or M2 released cytokines, chemokines and other factors have not been assessed so far *in vivo* in atherosclerosis, making it possible to only refer to the existing *in vitro* data.

M2 macrophages are considered to be wound-healing macrophages and the release of anti-inflammatory cytokines IL-4, IL-10 and IL-13 from M2 macrophages may not only dampen EC and SMC activation via a suppression of macrophage and T-cell activation but may also induce SMC proliferation and formation of anti-inflammatory Th2 cells and Tregs in atherosclerosis[27,32]. Since M2 macrophages are very poor phagocytes, the inefficient oxLDL uptake by M2 macrophages makes them less prone to foam cell formation. In addition, M2 macrophages were demonstrated to have a high efferocytosis potential[18,27] which may promote clearance of apoptotic cells in early lesions. However, the production of various MMPs (e.g. MMP2, 9, 12 and 13) may result in plaque destabilization promoting plaque rupture in progressed plaques[27].

M1 macrophages on the other hand may aggravate atherosclerosis through their release of the pro-inflammatory cytokines TNFα, IL-1β and IL-6 which were shown to induce EC-activation and dysfunction as well as SMC activation[27]. The pronounced phagocytic potential together with the low efferocytosis of M1 macrophages observed *in vitro* may promote foam cell formation, necrotic core formation and plaque instability[17,18,27,33]. M1 macrophages have been shown to induce T and B cell chemotaxis by the release of various chemokines like CCL5 and CCL19. In addition, this

macrophage subtype may drive polarization of T cells to the Th1 and Th17 types by the release of cytokines such as IL-6, IL-1β, IL-12 and IL-23[27,33]. Also, the production of the matrix metalloproteinases MMP1, MMP3 and MMP9 in M1 macrophages may facilitate matrix remodeling as well as a subsequent thinning of the fibrous cap which may ultimately lead to plaque rupture[27].

Monocyte-derived macrophages possess a large variety of toll-like receptors (TLRs) and other pattern recognition receptors (PRRs). TLRs have been shown to possess pro-atherosclerotic functions, since deficiency of myeloid differentiation primary response gene 88 (MyD88) which is a key adaptor for signaling of the majority of TLRs, or a deletion of certain TLRs such as TLR4, which has been demonstrated to also recognize oxLDL[34], results in reduced atherosclerosis[35].

Macrophages accumulating in the atherosclerotic lipid rich lesions have the capacity to phagocytose native or modified LDL, the latter via scavenger receptors (SRs) such as scavenger receptor A (SR-A) as well as CD36 as a member of the B family ultimately resulting in foam cell formation[17,36]. Recent studies however suggest that additional mechanisms of uptake also play a role[17]. Phagocytosis and receptor-activation trigger enhanced release of pro-inflammatory cytokines and chemokines, which are known to promote increased recruitment and activation of other disease promoting cell types[37].

While in early lesions apoptotic cells and inflammatory debris are efficiently cleared by other macrophages via efferocytosis limiting lesion progression, in advanced lesions this removal of apoptotic macrophages is impaired with consequences such as secondary necrosis and necrotic core formation[17]. This in turn triggers further lesional inflammation as well as enhanced leucocyte recruitment. The great importance of macrophages in disease development was moreover demonstrated by studies showing that mice deficient in M-CSF possess reduced macrophage numbers resulting in atheroprotection[38].

2.1.2.3 Dendritic cells

DC subtypes and DC functions

Another very important group of innate immune cells with respect to atherosclerosis are dendritic cells (DCs), which represent together with macrophages, as professional antigen-presenting cells (APCs), an important link between the innate and adaptive immune system[39,40].

DCs are an extremely heterogeneous cell population which can be divided into several groups according to their origin (lymphoid DCs, myeloid DCs, monocyte-derived DCs), location in the body (dermal DCs, Langerhans cells, splenic DCs and vascular DCs) or function (steady-state DCs, inflammatory DCs)[41,42]. There are four suggested major categories of DCs, namely conventional DC (cDC), Langerhans cells (LCs), plasmacytoid DCs (pDCs) and monocyte-derived DCs[24,43].

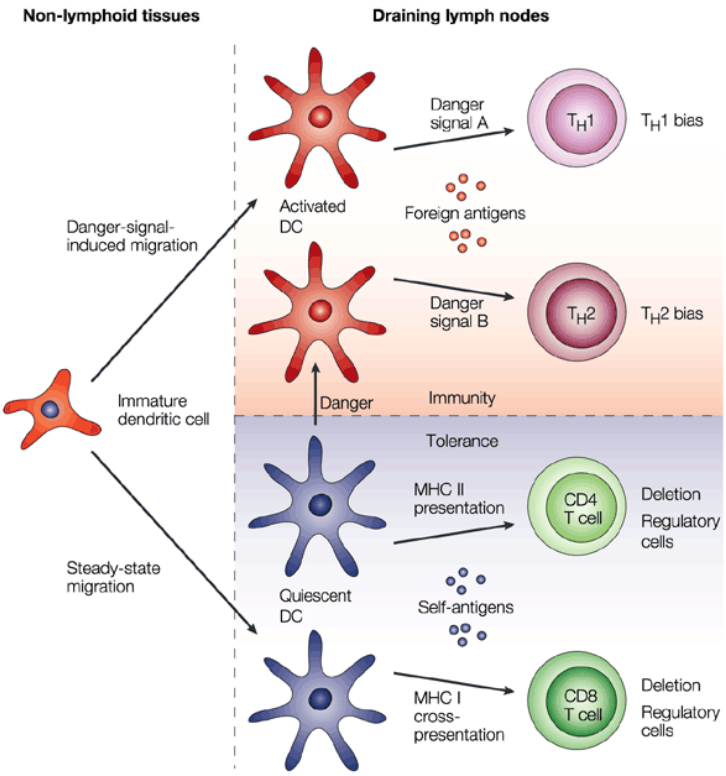

Figure 2.2 Dendritic cell mediated immunoregulation of T cells. Dependent on the activation status of DCs and the antigens they encounter they may either promote immunity or tolerance. Source: Shortman K. & Liu Y.J. 2002[44].

Immature DCs, characterized by a high phagocytic potential and low levels of the antigen-presenting molecules MHC class I and II[45], patrol through the tissue where they capture, process and present foreign or self-antigens as MHC-peptide complexes on their surface. Exogenous antigens are presented via MHC class II whereas endogenous or self-antigens are presented via MHC class I[46]. Maturation of DCs can be induced by various factors including pro-inflammatory cytokines (e.g. type I and II interferons, TNFα) or pathogenic stimuli (CpG-motifs, bacterial DNA, LPS, HSPs, viral dsRNA), recognized via PPRs, TLRs and cytokine receptors[44,46,47]. Mature DCs are characterized by reduced phagocytic capacity, up-regulation of MHC class I or II molecules, co-stimulatory molecules (e.g. CD80, CD86) and specific adhesion receptors (e.g. ICAM-1, LTA-3)[45,48] Following antigen exposure primed DCs home to the T cell areas of secondary lymphoid organs such as spleen and lymph nodes where they efficiently present the antigen-peptide complexes to naïve $CD4^+$ or cytotoxic $CD8^+$ T cells in order to evoke adaptive immune responses [49].

DCs however are not only involved in initiating immunity but also in the induction of central and peripheral T-cell tolerance. Central tolerance is mediated by intrathymic DCs which initiate the apoptotic death of self-reactive cells in the thymus[44,50,51]. Peripheral tolerance however may be maintained by different mechanisms. While there is some evidence of a specialized subtype of regulatory DCs[52], it is also possible that the maturation state of DCs defines their role in immunity and tolerance. The latter concept suggests that under steady-state conditions immature tissue-resident DCs or mature but quiescent DCs can induce T-cell anergy or apoptosis thereby maintaining tolerance, whereas immunity is induced only by mature and fully activated DCs[53,54] **(Figure 2.2)**. Apart from inducing T-cell anergy, DCs promote peripheral tolerance by induction of Tregs and suppression of naïve T-cell activation[51,55,56 57].

DCs in atherosclerosis

Similar to the observation of an accumulation of $CD11c^+$ DCs in the normal arterial intima in mice in areas which are predisposed to atherosclerosis[58], a network of DCs can already be detected in the arterial intima of healthy young individuals[59]. DCs are also present in atherosclerotic lesions of humans and mice[60-62]. With progression of atherosclerosis the number of $CD11c^+$ cells increases, a process that at least in part depends on the fractalkine receptor CX_3CR1 and the vascular cell adhesion molecule 1 (VCAM-1), since genetic deletion of the genes encoding for these molecules results in decreased DC frequencies[58,63]. Apart from the recruitment of DCs into vascular lesions, DCs in the intima can also derive from circulating $Ly6c^{high}$ and $Ly6c^{low}$ monocytes[64], from GM-CSF mediated intimal proliferation of lesional DCs[65] or from an impaired egress of monocyte-derived DCs as a result of hyperlipidemia in advanced plaques[66,67].

Several lines of evidence point to a modulatory role of DCs in atherosclerosis. A reduced DC accumulation due to genetic deficiency of CX_3CR1 or CCR5, for example, results in diminished atherosclerotic plaque formation due to impaired DC migration[21,63,67]. Furthermore, a lack of co-stimulatory molecules or molecules crucial for antigen presentation resulted in reduced T-cell responses and decreased atherosclerosis[68,69], supporting an important role of DCs and other APCs in atherogenesis. The close contact between mature DCs and T cells detected in atherosclerotic plaques[60] as well as the presence of clonally expanded T cells[70] moreover support a local interaction between DCs and T cells within atherosclerotic lesions[2], which may ultimately yield in antigen-specific T-cell activation or re-stimulation in the arterial vessel wall[6771]. Furthermore, there is proof that residential intimal DCs accumulate lipids and differentiate into foam cells initiating nascent foam cell lesion formation in early stages of atherosclerosis[72].

DCs contribute to plaque development by different mechanisms. On the one hand they can produce various pro-inflammatory cytokines such as TNFα, IL-6 and IL-12[67,73]. The pro-atherogenic role of IL-12 has been confirmed in several studies showing that blockade or deficiency of IL-12 in atherosclerotic mouse models results in reduced lesion size[74,75] whereas administration of

recombinant IL-12 shows the opposite effect[76]. Even though the release of IL-12 from DCs seems to play an essential role in Th1 induction, DCs may also modulate Th1 and Th17 polarization via IL-23 and IL-27 release[77-81]. CCL17-expressing DCs, however, were demonstrated to restrain Treg hemostasis which results in aggravated atherosclerosis in *apoE*$^{-/-}$ mice[82]. Yet, a depletion of CD11c^{+}CD11b^{-}CD103^{+} DCs due to Flt3 deficiency aggravated atherosclerotic lesion formation in *Ldlr*$^{-/-}$ mice. The increase in lesion burden was accompanied by a severe reduction in aortic Treg numbers and increased levels in the pro-inflammatory cytokines TNFα and IFNγ, demonstrating that Flt3-signaling dependent CD11c^{+}CD103^{+} DCs function as tolerogenic DCs protecting against atherosclerosis[27,83]. While TNFα seems to exert exclusively pro-atherogenic functions, since deficiency reduces lesion size [84], IL-6 seems to play a dual role in atherogenesis, acting as a potent pro-inflammatory mediator but also exerting anti-inflammatory effects[85,86]. On the other hand, DCs may also contribute to atherogenesis by the recruitment of T cells, monocytes and DCs into lesions via the release of chemokines such as CCL17 (TARC), CCL22 (MDC), CCL2 (MCP-1) and CCL4 (MIP-1β)[67,87,88].

Apart from their role in cell recruitment, T-cell activation and differentiation in atherogenesis, DCs have been demonstrated to be implicated in the control of cholesterol homeostasis and lipid content. Depletion of resident intimal CD11c^{+} DCs under hypercholesterolemic conditions results in a significant reduction in lipid accumulation and foam cell formation in very early stages of disease[72]. However, short-term depletion of CD11c^{+} DCs leads to cholesterol elevation under high fat diet[89]. Equally an increase in DC life-span by DC-specific overexpression of hBcl-2 results in lowering of cholesterol levels and a reduction in lesion size[89].

Importantly, it is not only DCs modulating cholesterol and lipid homeostasis, but conversely elevated levels of LDL and oxLDL in hypercholesterolemia are capable of altering DC-phenotype and function; a process strongly dependent on the extent and nature of lipoprotein modifications. While native LDL and oxLDL upregulate co-stimulatory molecule expression in DCs, and oxLDL induces expression of CCR7 and scavenger receptors, IL-6 release as well as DC-induced T-cell proliferation;

high concentrations of highly oxidized LDL result in apoptosis[90,91]. In addition, it has been demonstrated that resident intimal DCs are not only capable of accumulating lipids but also to induce foam cell lesion formation[72].

Hence, the overall extent of dyslipidemia seems to differentially modulate DC functions. While low or moderate dyslipidemia may aggravate local inflammation due to peripheral sequestration of activated DCs, resulting from an impaired migration to the lymph nodes[90,92], data addressing the impact of excessive dyslipidemia on DC function is inconsistent. While one study demonstrates that DCs remain functional antigen-presenting cells which maintain their ability to prime $CD4^+$ T cells even loaded with excess cholesterol[93], another study suggests that myeloid $CD8\alpha^-$ DCs become hypo-responsive in severe dyslipidemia and fail to induce solid Th1 responses, due to impaired expression of co-stimulatory molecules and IL-12 production[90,94].

2.1.3 The adaptive immune system in atherosclerosis

Although the innate immune systems plays an important role in atherosclerosis, cells of the adaptive immune system such as T and B lymphocytes are also present in atherosclerotic lesions, however to a lesser extent. $CD3^+$ T cells, which comprise approximately 10% of the cells in atherosclerotic lesions[13] are considered to be key players in atherogenesis.

The majority of the $CD3^+$ T cells found in lesions are of the αβ type, with a $CD4^+$ to $CD8^+$ T-cell ratio in advanced lesions comparable to that in the blood, even though TCRγδ T cells are present as well[13]. After activation of lymphatic T cells upon presentation of athero-specific antigens such as oxLDL from migrated APCs, $CD4^+$ T cells turn into T helper cells **(Figure 2.3)** whereas $CD8^+$ T cells form cytotoxic T lymphocytes (CTLs), which then are recruited to the lesion site directed by adhesion molecules and chemokines. In addition, T cells have also been demonstrated to undergo clonal expansion implying antigen-specific reactions within the lesions[70,95].

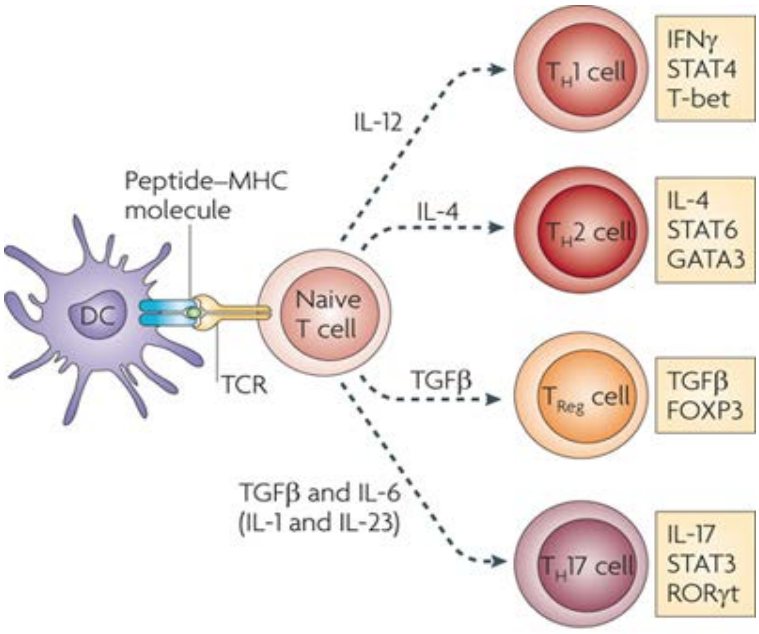

Figure 2.3 Polarization of naïve T cells to T helper cell subsets with upregulation of subset specific transcriptions factors (STAT3, STAT4, STAT6, FOXP3, GATA-3, T-bet or RORγt) and signature cytokines (IFNγ, IL-4, TGFß, IL-17) by DCs dependent on the cytokine milieu. Source: Zou W. & Restifo N.P. 2010[96].

2.1.3.1 Th1 cells

Atherosclerosis is considered to be a T helper type 1 (Th1) driven disease[13,97,98], with Th1 cells representing the majority of T cells in atherosclerotic lesions[99]. This concept is supported by the fact that T cells reactive to oxLDL, the major antigen in plaques, have been demonstrated to be of the Th1 subtype[100]. A hallmark of Th1 cells is the production and release of IFNγ, a pro-inflammatory cytokine, promoting disease progression.

IFNγ is a cytokine with pleiotropic functions depending on the cell type in the plaque. It has been shown to activate macrophages promoting the production of pro-inflammatory cytokines, pro-thrombotic mediators, as well as nitric oxide. In endothelial cells (ECs), it inhibits proliferation, whereas in smooth muscle cells (SMCs), it restricts not only their proliferation and differentiation but also limits collagen production thereby promoting inflammation, ECM degradation and plaque destabilization[13].

The pro-atherogenic potential of Th1 cells has been experimentally confirmed employing atherosclerotic mouse models with deficiencies in Th1-related genes. A significant reduction in atherosclerotic lesion burden was observed in mice deficient in *Ifng* or the *Ifng*-receptor underlining the pro-atherogenic role of Th1 cells[101-103]. A similar decrease was detected in mice lacking *Il18* or *Il12* which encode for important Th1 inducing cytokines or with deletion of *Tbx21* encoding for the main Th1 differentiation transcription factor T-bet[74,104,105]. Supporting these data, treatment of mice with IL-12, IL-18 or IFNγ has been demonstrated to promote lesion formation[76,101,106,107].

2.1.3.2 Th2 cells

The potential roles of Th2 and Th17 cells have also been investigated in the context of atherosclerosis, however yielding conflicting results. The typical Th2 related cytokines are IL-4, IL-5 and IL-13 [108] and this T helper cell subset is known to mainly interact with B cells. The role of Th2 cells in atherosclerosis however is not so easy to delineate. While some studies report a pro-atherogenic role for IL-4[74,109] another study failed to detect changes[110]. Moreover, IL-13 and IL-5 have been demonstrated to confer atheroprotection[111,112].

It is also important to note that Th2 cells very likely play only a subordinate role in atherosclerosis, since only few T cells in human plaques were producing the prime Th2 cytokines IL-4 and IL-5, while the majority of cells present in the plaque were shown to express IL-2, IFNγ, IL-12, IL-18 and TNFα, all of them being involved in Th1 responses[99,113].

2.1.3.3 Th17 cells

The main cytokine produced by Th17 cells is IL-17 which has been shown to be involved in the pathogenesis of a number of autoimmune diseases including rheumatoid arthritis (RA), inflammatory bowel disease (IBD) and asthma[114,115]. IL-17 expressing T cells have been detected in

atherosclerotic lesions of mice and humans[116,117], nonetheless the data regarding the role of Th17 cells in atherogenesis is contradictory.

While a number of studies support a pro-atherogenic role for IL-17, with antibody blockade of IL-17A, overexpression of a soluble IL-17 decoy receptor, reconstitution of lethally irradiated *Ldlr*$^{-/-}$ mice with *Il17ra*$^{-/-}$ bone marrow and deficiency of IL17RA or IL-17A in *apoE*$^{-/-}$ mice leading to a reduction in atherosclerosis[114,118-123]; other studies have failed to detect changes in plaque burden[124,125] or even evidenced atheroprotective effects[126,127].

2.1.3.4 Regulatory T cells (Tregs)

Another important subset of CD4^{+} T cells involved in atherosclerosis are the so-called regulatory T cells (Tregs), which maintain immunological tolerance to self-antigens and terminate excessive T-cell responses. Tregs are characterized by the expression of the transcription factor FoxP3 and the IL-2 receptor α-subunit CD25. Tregs are capable of suppressing the antigen-presenting potential of DCs by downregulation of CD80/CD86 co-stimulatory molecule expression in an LFA-1 and CTLA-4 dependent manner[128]. Several studies have demonstrated an anti-atherogenic role of Tregs in atherosclerosis, since a reduction or depletion of Tregs has been shown to be associated with an increase in lesion burden[129,130] and low numbers of Foxp3^{+} Tregs were detected in all stages of human atherosclerotic lesions[131]. Moreover, there is experimental proof that IL-10 and TGFβ, being key cytokines of Tregs, exert atheroprotective functions[129,132]. Another line of evidence supporting the atheroprotective role of Tregs is provided by a study demonstrating a reduction in lesion burden upon adoptive transfer of Tregs[132].

2.1.4 The haemostatic system in atherosclerosis

Increasing data implies a role of the coagulation system not only in atherothrombosis[133] but also in the onset and progression of atherosclerosis[134,135]. Besides their interaction with other coagulation zymogens, many coagulation proteases may also modulate inflammation by signaling via a family of G-protein coupled receptors (GPCRs) called protease activated receptors (PARs)[136]. In general, hypercoagulability in mice shows a tendency to increased atherosclerosis and *vice versa*[135]. Moreover, a broad range of functionally active coagulation factors were detected in early and advanced human atherosclerotic lesions; early atherosclerotic lesions showing a more pro-coagulant phenotype compared to stable advanced lesions[137]. Besides the demonstrated co-localization of coagulation factors, mainly with SMCs and macrophages in atherosclerotic plaques, a local synthesis of certain coagulation factors within atherosclerotic vessels was suggested[137].

2.1.4.1 Tissue factor (TF)

Several coagulation factors have been implicated in atherogenesis, including tissue factor (TF), the main physiological trigger of blood coagulation[138], which binds to the active coagulation factor VIIa (FVIIa) and mediates signaling via the PAR-2 receptor[139,140]. Within atherosclerotic lesions, TF has been shown to be present in the relatively acellular lipid-rich core and to be expressed by different cell types including macrophages, endothelial cells, VSMCs and foam cells[141-145]. However, in murine models of atherosclerosis diminished TF expression did not result in reduced atherosclerotic lesion formation[146]. On the other hand, a number of human studies indicate a role of TF in vascular disease[144,147 148].

2.1.4.2 Factor X (FX)

Other studies suggest a modulatory role of downstream mediators of the extrinsic and intrinsic coagulation pathway such as FXa and thrombin in atherosclerosis[134]. Activated FX can act alone or in complex with TF-FVIIa through PAR-1 and -2, which are expressed in various cell types including ECs, VSMCs, DCs and leucocytes[127]. It not only promotes the production of pro-inflammatory cytokines and chemokines such as IL-6 and CCL2, growth factors and cell-adhesion molecules but also increases TF expression and induces the proliferation of VSMCs[134,149,150]. Moreover, although not altering plaque progression, the specific FXa inhibitor, rivaroxaban was shown to dampen inflammatory responses and to promote lesion stability in the *apoE*$^{-/-}$ mouse model[151].

2.1.4.3 Factor XI (FXI)

A possible modulatory role of FXIa, which was shown to be activated by FXIIa but also FXII-independently by thrombin[152-155] is supported by clinical studies demonstrating an association of inhibitory complexes of FXIa as well as FIXa with acute myocardial infarction (AMI)[156].

2.1.4.4 Factor VIII (FVIII)

While various studies explore the role of the extrinsic or the common pathway of coagulation, hardly any studies focus on the impact of the intrinsic pathway of coagulation in atherogenesis. The intrinsic pathway of coagulation in atherosclerosis so far was assessed in hemophilia A mouse models that were deficient in coagulation factor VIII (FVIII). In *apoE*$^{-/-}$ mice, deficiency in FVIII resulted in significantly reduced lesion formation mainly at early stages of atherogenesis, which was accompanied by diminished fibrin(ogen) deposits and absence of platelets in the lesions compared to controls, despite increased hyperlipidemia[157]. However, this effect was not perceived in the *Ldlr*$^{-/-}$ mouse model[158]. Again, clinical studies focusing on the role of hypocoagulability (FVIII or FIX deficiency) on atherogenesis reveal conflicting

data[128]. The majority of these studies, however, indicate that hemophilia does not protect against atherosclerosis[135,159].

2.1.4.5 Thrombin

Thrombin (FIIa) generated from prothrombin via the prothrombinase complex in the course of coagulation, can act via PAR-1, 3 and 4[136] and fulfills a large variety of functions apart from representing the central coagulation protease[160-162]. These include the induction of pro-inflammatory molecules such as IL-6, MCP-1, IL-8 or MIF as well as of cell adhesion molecules, thereby enhancing the recruitment of monocytes into the vessel wall[162]. Furthermore, thrombin triggers endothelial dysfunction induces ROS-formation and promotes VSMC migration and proliferation as well as angiogenesis[162]. In addition, thrombin may drive atherogenesis through the activation of platelets, which have been shown to participate in all stages of atherosclerosis[163-165]. Possibly as a negative feedback mechanism during coagulation, it binds to thrombomodulin, supporting the activation of protein C (APC formation) which exerts anti-coagulant as well as anti-inflammatory functions. Besides, thrombin itself holds anti-inflammatory functions since it dampens immune reactions by suppressing IL-12 and promoting IL-10 release[134]. However, thrombin may finally promote atherosclerosis progression when endothelial expression of thrombomodulin and the endothelial cell protein C receptor are diminished due to endothelial dysfunction in the process of disease[134,162,166]. The pro-atherogenic potential of thrombin has been validated in several animal studies[162], demonstrating that specific thrombin inhibition results for example in a reduction of restenosis in rabbits[167].

In addition, direct thrombin inhibitors such as melagatran have been verified to attenuate atherosclerotic lesion formation, promote plaque stability and diminish MMP-9 synthesis in *apoE*$^{-/-}$ mice[168]. Antithrombin and heparin cofactor II (HCII) share their ability to inactivate thrombin, FXa and FIXa[134]. While clinical studies reveal conflicting data concerning the role of HCII in atherosclerosis and CAD either suggesting it to be anti-atherogenic or non-predictive[169,170], mouse studies clearly indicate an anti-atherogenic function of

this factor, since deficiency in HCII results in enhanced neointima formation and atherogenesis[171,172].

2.1.4.6 Platelets

Besides their established role in hemostasis and thrombosis, platelets have been demonstrated to be important mediators in pro-inflammatory diseases, including atherosclerosis[173,174]. Extensive studies in recent years revealed that platelets not only mediate atherothrombosis upon plaque rupture but also are important modulators in disease onset and progression.

Adherence of platelets to atherosclerosis-prone sites has been demonstrated by intravital microscopy to occur before lesions are even detectable[175,176]. In experimental mouse models of atherosclerosis it has moreover been shown that platelet specific glycoproteins such as the integrins GPIbα and GPIIb/IIIa (αIIbβ3) as well as glycoprotein VI (GPVI) contribute to atheroprogression. Deletion of the α-subunit in GPIIb/IIIa reduces lesion size in the carotid arteries and the aortic arch of *apoE*$^{-/-}$ mice due to a reduction of platelet adhesion to dysfunctional ECs and extracellular matrix after endothelial denudation[177]. Likewise, deletion of platelet GPIbα in *apoE*$^{-/-}$ mice resulted in reduced intimal leucocyte accumulation and diminished lesion formation, supporting the importance of platelet adhesion to the endothelium for the initiation of lesion formation[176]. Furthermore, GPVI which binds to the activated atherosclerotic endothelium already at early stages of atherosclerosis (possbily via fibronectin) seems to play a pro-atherogenic role, since soluble GPVI-Fc or selective inhibition of GPVI using anti-GPVI antibodies resulted in a reduction of transient platelet adhesion, improved endothelial function accompanied by attenuated lesion formation in *apoE*$^{-/-}$ mice and cholesterol-fed rabbits[178]. GPVI however may also affect disease progression by modulation of platelet-leucocyte interactions, since it has been shown to increase monocyte recruitment to the vascular wall via EMMPRIN (CD147)-GPVI interactions[179].

P-Selectin is expressed on the surface of platelets and ECs upon activation and subsequent release from its intracellular stores (α-granules,

Weibel-Palade bodies)[174]. The importance of platelet P-Selectin in atherosclerosis has been experimentally proven since P-Selectin deficiency protects from advanced atherosclerosis[180]. Moreover, repetitive injections of activated P-Selectin sufficient platelets into *apoE*$^{-/-}$ mice were shown to exacerbate atherosclerosis while injection of P-Selectin deficient platelets reduced lesion formation[174,181]. P-Selectin is not only important for platelet-endothelium but also for platelet-leucocyte interactions. Via P-Selectin platelets bind P-Selectin glycoprotein ligand 1 (PSGL-1) on leucocytes thereby forming platelet-leucocyte aggregates (PLAs), which promote activation of ECs and transmigration of leucocytes[174]. This interaction moreover induces the release of pro-inflammatory chemokines (CCL2, CCL5) and cytokines (e.g. IL-1β), as well as augmented P-Selectin expression by ECs, thereby promoting leucocyte rolling[174,181,182].

Additionally, CD40-ligand (CD40L) expressed by platelets also contributes to thrombotic and inflammatory processes in atherosclerosis, as shown by administration of CD40L-deficient platelets. These failed to induce aggravation of atherosclerosis as observed with CD40L-sufficient platelets[183]. Moreover, CD40L-sufficient platelets have been revealed to transiently increase pro-atherogenic effector T cells while reducing Treg numbers which very likely contributes to disease progression[183].

Furthermore, soluble factors released from platelets upon activation are crucial for disease promotion. It has been shown that e.g. RANTES (CCL5) deposition by platelets induces monocyte arrest on inflamed atherosclerotic endothelium[184]. Likewise, the platelet chemokine PF4 (CXCL4), detected in fatty streaks and lesions in humans[185], correlates with disease progression and a lack of PF4 displayed diminished lesion formation[174,186].

2.1.4.7 Tissue factor pathway inhibitor (TFPI)

The importance of the coagulation system in atherogenesis is further underscored by animal studies that delineate the role of tissue factor pathway inhibitor (TFPI) not only as main inhibitor of the TF-FVIIa complex[187] but also for the TF-mediated pro-atherogenic signaling events[134]. Animal models have

shown that TFPI is a potent inhibitor of MMPs and hence a decrease in TFPI expression results in an upregulation of MMPs yielding vulnerable unstable plaques. TFPI moreover suppresses endothelial migration, monocyte recruitment, angiogenesis and the release of pro-atherogenic factors such as CCL2 or MMP-2 thereby restricting neointima formation and restenosis[188-191]. In line with these data, mice bearing a partial deficiency in TFPI displayed significantly increased lesion formation[192]. Additionally, a vascular-directed overexpression of TFPI has been demonstrated to reduce plasma cholesterol levels and atherosclerotic lesion formation[193]

2.1.4.8 Hypercoagulability models

Studies with mice on atherogenic background displaying a hypercoagulable phenotype including Factor V (FV) Leiden mutation mice, mice with a heterozygous protein C deficiency or mice with a thrombomodulin Pro/Pro mutation showed increased atherosclerotic lesion burden[194-196]. However, FV Leiden mutation mice and TM Pro/Pro mice both displaying a hypercoagulable phenotype due to increased thrombin formation also showed an enhanced plaque stability, indicating that hypercoagulability may support a more stable plaque phenotype[194].

2.1.4.9 Fibrinolysis

Since coagulation results in active thrombin generation and the conversion of fibrinogen to fibrin is a key function of thrombin[197], impaired fibrin removal due to reduced fibrinolysis results in extensive fibrin deposition in the course of atherosclerosis[198]. Both, fibrinogen and fibrin exert pro-inflammatory functions by inducing the expression of TNFα and IL-1β in monocytes/macrophages as well as chemokines (e.g IL-8 and MCP-1) in ECs and fibroblasts[199] and may further drive atherosclerosis. Accumulation of fibrin due to impaired fibrinolysis may promote atherosclerosis progression by contribution to the intimal mass.

2.2 Coagulation factor XII (Hageman Factor, FXII)

2.2.1 Blood coagulation pathways

Blood coagulation represents a part of the haemostatic response to vessel injuries culminating in thrombin generation and fibrin formation (secondary hemostasis) in order to maintain vascular integrity. One can distinguish between two pathways of blood coagulation - the extrinsic pathway (tissue factor pathway) and the intrinsic pathway (contact activation pathway).

In the extrinsic pathway, TF is exposed to plasma components in the course of vessel injury. TF-FVII(a) complexes (extrinsic factor Xase) are formed which activate FIX and FX. FVII bound to TF can get activated by FVIIa, FIXa and FXa resulting in feedback amplification. FXa binds and activates FV forming the so-called pro-thrombinase complex that converts prothrombin (FII) to thrombin (FIIa). Thrombin feedback results in partial activation of platelets, the activation of additional FV well as of the intrinsic coagulation factors FXI and FVIII thereby amplifying the reaction. FIXa forms the intrinsic factor Xase complex with its co-factor VIIIa and is capable to activate FX at a up to 100-fold higher rate than the TF-FVII(a) complex. Thrombin then cleaves fibrinogen resulting in fibrin formation. Fibrin polymers finally get covalently cross-linked, mediated by thrombin-activated FXIIIa forming a stable fibrin clot[200-207] **(Figure 2.4)**.

The intrinsic coagulation pathway however is initiated when Factor XII gets auto-activated upon (FXII, Hageman factor) exposure to negatively charged (anionic) surfaces. FXIIa subsequently activates FXI which leads to the activation of FIX. FIXa forms the so called intrinsic Xase complex with FVIIIa which can then also convert FX into its activated form **(Figure 2.4)**. At that step, both coagulation pathways merge into the common pathway resulting in fibrin generation[200-204]. However, although FXII has been shown to contribute to fibrin formation *in vitro*, it seems to be dispensable for hemostasis *in vivo* since FXII-deficiency in humans or mice does not result in extended bleeding despite a prolongation of the activated partial thromboplastin time (aPTT)[208,209].

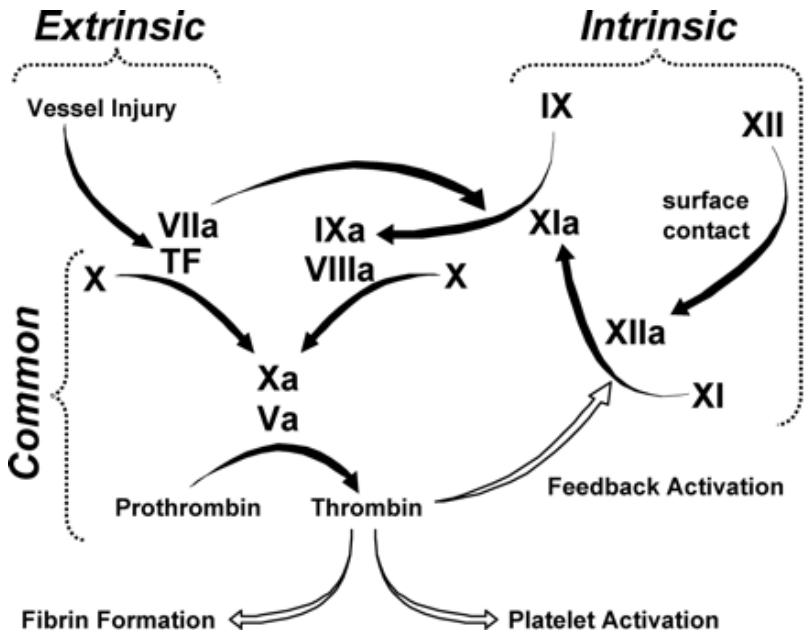

Figure 2.4 Coagulation cascade with intrinsic, extrinsic and common pathway. Source: Schumacher W.A. et al. 2010[206].

2.2.2 Factor XII (Hageman factor)

FXII is part of the so-called plasma contact system which consists of the plasma proteins factor XII (FXII), factor XI (FXI), plasma prekallikrein (PK) and the non-enzymatic cofactor high molecular weight kininogen (HK, HMWK)[210,211]. FXII being the zymogen of the serine protease FXIIa is produced and secreted primarily in the liver[212,213]. However, FXII mRNA expression in humans has also been detected in the placenta[213] and FXII protein expression has recently been demonstrated in human lung fibroblasts upon TGFβ1 stimulation[214]. Since there is only very limited literature regarding the structure or function of FXII in mice, the literature discussed in the following will mainly be related to FXII in humans.

2.2.2.1 Activation of FXII

Contact activation of FXII

Factor XII can be activated upon exposure to negatively charged surfaces or by kallikrein. Besides various non-physiological activators such as glass, kaolin, elagic acid or high molecular weight (HMW) dextrane-sulfate[210], also several potential physiological *in vivo* activators have been recently identified including heparin from mast cells[215], extracellular RNA released from damaged cells[216], collagen[217], polyphosphates released from dense granules of activated platelets[218,219], very-low density lipoprotein (VLDL) phospholipids[220] or misfolded proteins[221]. Misfolded protein aggregates were shown to selectively trigger FXII mediated activation of the kallikrein-kinin system without initiating coagulation[221]. The mechanisms for such a selective activation are still elusive, but it is speculated that higher local concentrations of prekallikrein (PK) compared to FXI or a preference towards β-FXIIa (FXII fragment, Hageman factor fragment Hf) formation as a result of differences in FXII binding may be responsible for the shift towards kallikrein activation[210].

Activation of FXII on endothelial cell surfaces

Apart from contact activation, FXII can also be activated on endothelial cells. FXII, HMWK and PK can bind to endothelial cells in a Zn^{2+} dependent manner via a multiprotein assembly consisting of endothelial cell urokinase plasminogen activator receptor (uPAR), Cytokeratin-1 (CK-1) and the first complement component receptor gC1qR[222]. It has been shown that FXII and HMWK compete for the same binding sites on the endothelial cell surface[223]. Moreover, lower concentrations of Zn^{2+} are sufficient for the binding of the HMWK/PK complex when compared to FXII. In addition, in absence of FXII in the plasma, activation of PK on endothelial cell surfaces was unaltered compared to normal plasma, indicating that PK when assembled on HK is initially activated independent of FXII on endothelial cell surfaces[224].

This suggests that on endothelial cells FXII activation is secondary to PK activation and rather contributes to the extent of measured enzymatic

activity[225]. Moreover, a PK activator identified to be the serine protease prolylcaboxypeptidase (PRCP) was purified from endothelial cells[226,227]. The preferential activation of PK by PRCP over the activation by FXIIa-α on endothelial cells *in vivo* may result from the very low Michaelis constant (K_m) for PRCP activation of PK when compared to that of FXIIa-α on negatively charged surfaces[228].

2.2.2.2 *In vivo* inhibitors of FXII activity

The main physiological inhibitor of FXIIa in plasma is considered to be C1 esterase inhibitor (C1INH)[229,230]. It binds irreversibly to α-FXIIa and β-FXIIa inhibiting more than 90% of FXIIa in plasma[230]. C1INH however has additional substrates such as Kallikrein, FXIa and the complement proteases C1r and C1s. Plasminogen activator inhibitor-1 (PAI-1), antithrombin III, α2-antiplasmin and α2-macroglobulin have also been described to reduce FXII activity, however less effectively[229,231,232]. Recently it has been reported that histidine-rich glycoprotein (HRG) is also capable to inhibit FXII autoactivation and FXIIa-mediated FXI activation[233].

2.2.3 Functions of FXII

2.2.3.1 Coagulation and Kallikrein-Kinin-Pathway

Upon activation, FXIIa (FXIIa-α) initiates the intrinsic pathway of coagulation via the activation of FXI triggering thrombin generation and fibrin formation **(Figure 2.5)**. As part of the kallikrein-kinin system (KKS) FXIIa leads to the formation of active plasma kallikrein by proteolytic cleavage of prekallikrein. Plasma kallikrein then reciprocally not only activates more FXII to FXIIa but also cleaves HMWK to liberate the pro-inflammatory, pro-angiogenic and vasoactive mediator bradykinin **(Figure 2.5)** and a biologically active two-chain kininogen fragment (HKa)[211,234,235 228].

2.2.3.2 Fibrinolysis

FXII has also been shown to possess fibrinolytic properties. Fibrinolysis is the dissolution of fibrin clots into fibrin degradation products. FXII structurally resembles the fibrinolytic proteins tissue-type plasminogen activator (t-Pa) and urokinase-plasminogen activator (u-Pa) and has been reported to activate plasminogen either directly or via FXIa or kallikrein yielding plasmin formation[236-240]. The direct contribution of FXIIa as a plasminogen activator has been shown to be more than 20% in normal plasma[239,241].

Kallikrein can mediate fibrinolysis by direct conversion of pro-urokinase to uPA[242] or through liberation of bradykinin which induces the release of tPA from endothelial cells[243]. An additional mechanism by which FXII may enhance fibrinolysis is the inactivation of PAI-1 which has been demonstrated in *in vitro* assays[244]. FXIIa may also negatively affect fibrinolysis through activation of FXI, since FXIa indirectly drives anti-fibrinolytic functions by promoting thrombin mediated thrombin-activatable fibrinolysis inhibitor (TAFI) activation[245,246]. In conclusion, it can be stated that FXII is directly and indirectly involved in pro- as well anti-fibrinolytic pathways. However, the net-effect of contact mediated fibrinolysis *in vivo* seems not clear.

2.2.3.3 FXII as a growth factor

FXIIa as well as its zymogen FXII exert mitogenic effects EGF-responsive cell lines such as HepG2 hepatocytes, fetal alveolar type II cells, aortic smooth muscle cells and EC-12 endothelial cells and epithelioid carcinoma cells *in vitro*[247-249]

2.2.3.4 FXII in angiogenesis

It has also been shown that FXII and FXIIa are involved in angiogenic responses. FXII conducts direct angiogenic effects via binding of uPAR, EGFR and integrins[250] already at very low concentrations. Moreover, FXII has been demonstrated to induce aortic sprouting in mice[250] *in vitro*. Nonetheless, FXII

is dispensable for angiogenesis during development, implicating a role of FXII mediated angiogenesis mainly in tissue repair in response to injury or in inflammation[250].

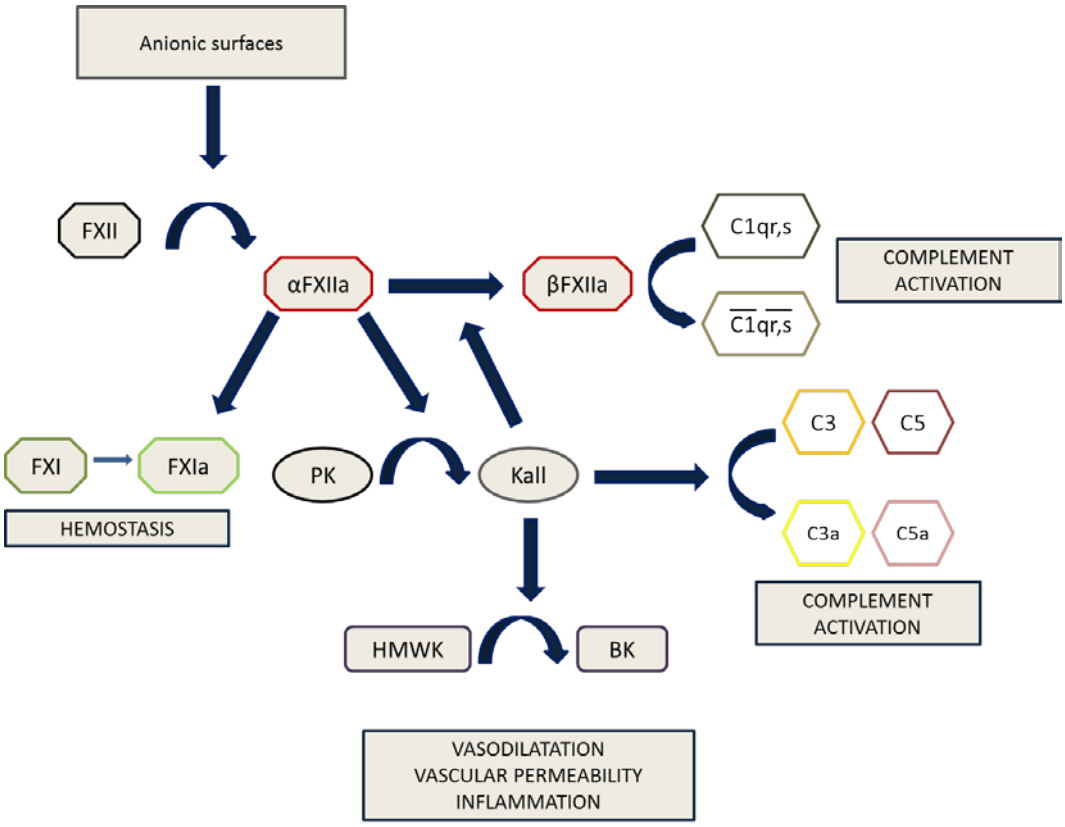

Figure 2.5 FXII functions upon activation on anionic surfaces. Source: partially adapted from Schmaier A.H. 2008[228].

2.2.3.5 FXII in hemostasis and thrombosis

Various experimental data indicate that FXII-mediated thrombin formation is not essential for hemostasis. First of all, it has been shown that humans or mice with severe FXII-deficiency do not display a relevant bleeding phenotype despite a prolonged aPTT[202211]. Furthermore, it was demonstrated that FXI can get activated by thrombin itself and that this is the preferential mechanism of FXIa formation *in vivo*[153,154,251]. In addition, FXIa mediated thrombin formation seems only to be essential when the extrinsic pathway is

impaired, e.g. in presence of low TF levels[252,253]. TF/FXII deficient mice however are viable and were not altered from low TF mice which confirms that FXII is non-essential for hemostasis[230,254].

Although being dispensable for hemostasis *in vivo*, FXII seems to play a pivotal role in pathological thrombus formation since FXII-deficient mice were shown to display defective thrombus formation and stabilization using different experimental thrombosis models[255,256]. While the initiation of thrombus formation on the vessel wall is not impaired, FXII and also FXI are involved in the stabilization of occlusive blood clots. Hence, FXII and FXI deficiency result in unstable thrombi that fail to induce vessel occlusion[256-258]. Likewise, employing the Rose Bengal/laser injury carotid artery thrombosis model, a role of FXIIa mediated FXI activation in thrombus formation was demonstrated *in vivo*[255].

Also, FXII deficiency is protective in cerebral ischemia due to impaired FXII-dependent fibrin formation/deposition, resulting in a significantly reduced infarct volume when employing the transient middle cerebral artery occlusion (tMCAO) model[259]. Pharmacological targeting of FXII activity using PCK (H-D-Pro-Phe-Arg-chloromethylketone) or recombinant human albumin Infestin-4 also mediated protection from cerebral ischemia without affecting normal hemostasis[259,260]

Since FXI deficient mice are also protected from thrombotic vessel occlusion[258], and specific inhibition of FXIIa-mediated FXI activation impaired thrombus formation in baboons[255] it is very likely that the FXII mediated pathological thrombus formation is mainly exerted via the intrinsic pathway of coagulation. This hypothesis is further supported by data demonstrating that a combined FXI and FXII deficiency in mice does not result in an additional thromboprotection in a pulmonary embolism model when compared to mice with single deficiencies[219,261]

Based on the results gained from FXII deficient mouse models or inhibitors of FXII activity one would expect FXII-deficient human patients to be protected from thrombosis[211]. Clinical studies however revealed opposing data, some suggesting that FXII deficiency predisposes for thromboembolic

diseases[262,263] while others failed to correlate FXII deficiency with increased thrombotic risk[211,264-266].

2.2.4 FXII in inflammation – and overview about how FXII may influence atherosclerosis

2.2.4.1 FXII in inflammatory diseases

Besides the important function of FXII in pathological thrombus formation[252249], FXII is also implicated in the regulation of inflammatory processes. This is in line with the contribution of increased FXII activity and other factors of the contact system to hereditary angioedema[267,268] and the described activation of the contact system in other pro-inflammatory diseases such as mast cell driven edema formation, arthritis, sepsis, infection and allergy[215,261,269,270]. Moreover, a pro-inflammatory role of the contact system in experimental arthritis, enterocholitis and sepsis has been described[271]. In addition, FXIIa was shown to directly interact with different types of immune cells potentially modulating their functions.

2.2.4.2 Interactions of FXII with immune cells

Already in the 1990s it has been shown that the essential contact-phase proteins HK, FXI, FXII and PK can assemble on the surface of human neutrophils suggesting a potential role for local kinin production at sites of inflammation[272]. Similarly, macrophages may fulfill an analogous role, since FXII can not only bind to the U-937 monocyte/macrophage cell line through Mac-1 and gC1qR, but the assembly of HK, FXII and PK on macrophages results in the production and release of high amounts of active kinins[273]. However, also in absence of FXII significant amounts of kinins were formed, suggesting an additional FXII-independent mechanism of PK activation on macrophages[273]. Neutrophils (PMNs) can bind FXIIa and fibrin protofibrils resulting in increased FXIIa-dependent thrombin generation and platelet-

independent fibrin formation through elastase/cathepsinG mediated pathways[267]. In addition, FXIIa was demonstrated to activate neutrophils inducing human neutrophil aggregation and degranulation[274]. Moreover, FXIIa, probably via a domain in its heavy chain, reduces the expression Fc gamma recpeptor I (FcγRI), which represents the binding site for monomeric immunoglobulin G (IgG) on monocytes or macrophages[275].

FXIIa is also capable of triggering the activation of the chemo-attractant chemerin[276]. Activated chemerin may then recruit pDCs, monocyte-derived DCs and macrophages or tissue macrophages via chemokine-like receptor 1 (CMKLR1)-mediated chemotaxis[276]. FXIIa may also directly affect cytokine secretion from innate immune cells, since FXIIa was shown to be a potent inducer of IL1β release from human monocytes *in vitro,* accompanied by an increase in IL-1-induced IL-6 expression[277].

2.2.4.3 FXII and the complement system

Besides its function to activate the pro-inflammatory kallikrein-kinin system yielding kallikrein activation and bradykinin formation, FXII has been demonstrated to also be involved in the activation of the complement system. Hageman factor fragment (β-FXIIa; Hf), cleaved from α-FXIIa by kallikrein, is able to directly activate the C1-complex ($C1qr_2s_2$) to C1r and C1s, triggering the classical cascade of the complement system[278,279 228], which finally can result in the generation of anaphylatoxins like C3a and C5a, both of which exert a variety of pro-inflammatory functions. In addition, plasma kallikrein being a downstream mediator of activated FXII has been shown to activate C3 in humans[280] and to induce a C5a-like molecule in rabbits[281] **(Figure 2.5)**.

Besides its role in fighting bacterial, viral and parasital infections by means like chemoattraction, opsonization and lysis, clearance of apoptotic and necrotic cells by phagocytosis[282-284], the complement system has also been associated with increased inflammation in and progression of atherosclerosis[285-287]. The anaphylatoxins C3a and C5a have moreover been demonstrated to modulate adaptive immune responses triggering induction of

different effector T cell subsets, besides their classical functions in innate immunity and host defense[288].

A potential role of the complement system in atherosclerosis is further supported by studies demonstrating the augmented presence of complement components such as C3, C5 and C5b-9[289-291], the local synthesis of complement components in the plaque[292,293] as well as the expression of anaphylatoxin receptors C3aR and C5aR in human lesions[294].

2.2.4.4 Inflammatory functions of downstream mediators of FXII

Mediators downstream of FXIIa such as FXIa, bradykinin and plasma kallikrein themselves possess a variety of mechanisms to modulate inflammatory processes.

FXIa is capable of inhibiting IL-8- or fMLP-triggered chemotaxis of polymorphonuclear leucocytes[295]. In addition, specific inhibiton of FXI activation in an abdominal sepsis model as well as FXI-deficiency in a listeriosis model resulted in decreased coagulopathy and attenuated inflammation[296,297].

Plasma kallikrein can induce neutrophil activation[298] and elastase release[299]. However, the most important mechanism by which kallikrein modulates inflammation is the cleavage of HMWK to release bradykinin, which acts through the two G-protein coupled receptors B1 (B1R) and B2 (B2R)[271].

Bradykinin increases vascular permeability and promotes the release of nitric oxide (NO) and prostacyclin from ECs thereby inducing vasodilation, inhibiting platelet function and the proliferation of VSMCs[271]. Upon vessel injury however, bradykinin can enhance the proliferation of VSMCs stimulating vascular repair[271]. Bradykinin has also been reported to induce IL-12 release from DCs via the B2-receptor mediating Th1 responses[300,301]. Yet, in the context of atherosclerosis, selective deficiency of the inflammatory B1R in *apoE*$^{-/-}$ mice was shown to augment atherosclerotic lesion formation and to predispose for the development of aortic aneurysms under hypercholesterolemia[302]. Angiotensin conversion enzyme (ACE)-inhibitors

have been shown to be cardioprotective[303,304] and to even have beneficial effects on endothelial dysfunction at different stages of atherosclerosis[305], due to the prevention of bradykinin degradation and angiotensin II formation. While a number of animal studies suggest a positive impact of ACE inhibitors on regression of plaque burden, clinical studies revealed conflicting results[305]. Besides, there are studies demonstrating that a genetic kininogen deficiency may predispose for aortic aneurysm development but does not affect atherosclerosis[306].

2.2.4.5 FXII modulated fibrinolytic factors in inflammation

FXII and its downstream factors may also alter inflammatory processes via their fibrinolytic activities which were already mentioned. Fibrinolytic factors, although being beneficial with respect to prevention of thromboembolic complications, also possess pro-inflammatory functions, some of which may even promote atherogenesis.

Plasmin

Plasmin, besides its function as a key enzyme in fibrinolysis, is also involved in inflammation. In addition to fibrin, plasmin is able to cleave various other substrates including coagulation factors V and VIII, metalloproteinases, extracellular matrix proteins and the complement components C3 and C5[307-309]. Furthermore, this protease directly affects a number of different cell types including monocytes, macrophages, DCs, ECs, SMCs and platelets. In monocytes, it induces e.g. the expression of pro-inflammatory cytokines, tissue factor and MMP-1, leukotriene release and chemotaxis. In DCs, plasmin can not only induce a chemotactic response but also trigger IL-12 release, driving Th1 responses[308,310].

Plasmin and also plasma kallikrein, as a downsteam mediator of activated FXII have been shown to reduce HDL-induced cholesterol efflux from foam cells *in vitro*[311]*.* This indicates a pro-atherogenic function of these two proteases by impairment of cholesterol efflux from macrophage foam cells

either directly by cleavage of HDL, reducing or abolishing its capability to accept cholesterol from foam cells or indirectly by activation of MMPs[311]. Moreover, plasmin was detected in close proximity to DCs in atherosclerotic lesions implying a possible role of plasmin in DC recruitment to the vessel wall and the induction of adaptive immune responses which may exacerbate disease progression[308,310]. In endothelial cells, plasmin induces prostacyclin synthesis and MCP-1 release, inhibits thrombin mediated PAR-1 signaling, at high concentrations it may even induce apoptosis. In addition, plasmin triggers the proliferation of SMCs[301].

The potential role of plasmin in atherogenesis has been assessed in atherosclerotic mouse models showing conflicting results[308]. While plasminogen-deficient mice were shown to display reduced transplant atherosclerosis (graft arterial disease)[312], plasminogen deficiency in *apoE*$^{-/-}$ mice enhanced lesion formation under hypercholesterolemic conditions[313]. The latter study is contradictory to a study from 2008 which demonstrated that plasminogen deficiency in presence or absence of uPA overexpression results in diminished aortic root lesions[314], supporting a pro-atherogenic role of plasmin(ogen).

PAI-1

The role of the anti-fibrinolytic factor plasminogen activator inhibitor 1 (PAI-1) in atherosclerosis is also quite contradictory and inconclusive since animal studies suggest PAI-1 to be either atheroprotective, pro-atherogenic or to play no role in lesion progression[315-317].

uPA

Besides its pivotal role in plasmin generation, uPA is involved in a number of other processes including cell adhesion, migration, differentiation and proliferation as well as the degradation of extracellular matrix (ECM)[318]. Macrophages and SMCs within human atherosclerotic lesions are able to secrete uPA and also express uPAR on their surface[318].

The uPA/uPAR system has been implicated to play a role in atherogenesis, since uPA was not only shown to be expressed by

macrophages and SMCs in human atherosclerotic lesions, but local uPA expression also correlates with lesion severity[318,319]. In addition, overexpression of uPA in arteries seems to result in arterial constriction and lumen loss and accelerates intimal growth in rabbits[320]. A different study however has shown that deficiency of uPA promotes the progression and instability of atherosclerotic lesions in *apoE*$^{-/-}$ mice, increases fibrin content and decreases cellularity and collagen content resulting in a more vulnerable plaque phenotype[321].

Selective overexpression of uPA in macrophages leads to accelerated atherosclerosis and coronary artery occlusion in *apoE*$^{-/-}$ mice[322]. In line with these data, bone marrow transfer (BMT) experiments with bone marrow from macrophage targeted uPA-deficient mice into *apoE*$^{-/-}$ mice showed a significant reduction in lesion size when compared to recipients of bone marrow from uPA-WT mice. However, no changes in aortic root intimal area between the two groups were detected[323]. An additional study confirms former results that macrophage-expressed uPA accelerates atherosclerotic lesion progression and promotes aortic dilation. Moreover, it shows that most of the effects are largely uPAR independent[324]. In addition, an upregulation of S100A8/A9 gene expression was detected in macrophage-targeted uPA overexpressing mice. These genes encode for calgranulin A and B which have been demonstrated to be atherogenic in mice[324,325]. Furthermore, it was demonstrated that macrophage-expressed uPA accelerates plaque growth and induces aortic root dilation in a plasminogen-dependent manner[314]. A recent study has shown that uPA promotes the differentiation of monocytes into macrophages and also attenuates oxLDL-induced apoptosis of macrophages *in vitro* possibly favoring atherosclerosis progression[326].

2.2.5 FXII in atherosclerosis

Only limited data exists concerning the role of FXII in atherosclerosis, the majority of it being derived from clinical studies evaluating the role of FXII in vascular disease.

However, contradictory findings on the function of FXII in vascular disease in humans have been described. In several clinical studies an association between plasma triglyceride levels and partially also plasma cholesterol levels with increased plasma concentrations of FXIIa in coronary heart disease has been demonstrated[327-331]. Taken together with data showing that VLDL phospholipids can trigger activation of the contact activation pathway in platelet-rich plasma (PRP) and whole blood[220], these studies may indicate that increased plasma lipoprotein levels as they occur in hyperlipidemia, may be responsible for the positive associations between plasma levels of triglyceride-rich lipoproteins and FXIIa levels.

Elevated levels of activated FXII were positively associated with cholesterol levels and other conventional risk factors for coronary heart disease, suggesting FXII to serve as a marker of atherosclerotic vascular damage[327,329] . In line with these findings, increased levels of activated FXII were demonstrated to be associated with an elevated risk of coronary heart disease[332,333], coronary artery calcification[327], coronary atherosclerosis[328] as well as with the extent of coronary stenosis and a past history of myocardial infarction[330]. While several studies indicate an association between high circulating FXIIa levels and disease severity or the risk of coronary heart disesase[322,330,325,326,334], other studies concluded that low levels of activated FXII neither protect from coronary heart disease[327,332,335] nor serve as a predictor for thrombotic pathologies[265,266]. Various studies suggest FXIIa rather to be a risk marker for atherogenicity rather than exerting direct atherogenic functions[327,329].

Yet again, it was also shown that low FXII activity or lower plasma FXII or FXIIa may constitute a risk factor for coronary heart disease[262,336], myocardial infarction[337] or myocardial re-infarction in patients with ischemic heart disease[338,339].

An increase in factor XII levels upon hypercholesterolemic conditions was also seen in mouse studies with *Ldlr*$^{-/-}$ mice, though levels of activated FXII were not investigated in this study[340]. In addition, the presence of functionally active FXII in early and advanced human atherosclerotic lesions was demonstrated by Borissoff *et al.*[137]*.* However, they observed that most coagulation factors detected, including FXII, exert higher activities in early atherosclerotic lesions (EAL) in comparison to stable advanced atherosclerotic lesions (SAAL), indicating a more pro-coagulant state in EAL throughout all vessel wall layers[137].

Moreover, FXII mRNA was detected in the arterial vessel wall, suggesting a local expression of FXII in human atherosclerotic lesions. By immunohistochemistry, Borissoff *et al.* demonstrated a focal endothelial distribution and a scarce and focal co-localization of FXII with macrophages and foam cells and intimal SMCs[137]. Medial SMCs were found to be highly positive for FXII, and even in the adventitia, the vasa vasorum and fibroblasts, FXII was present, indicating possible cell-directed effects of FXII[137].

Aim of this project:

It has become clear that atherogenesis is not only modulated by the innate and adaptive immune system but also by the blood coagulation system. It is evident that the haemostatic system besides being involved in the late stages of atherosclerosis, where it triggers atherothrombosis upon plaque rupture, also seems to impact the development of atherosclerosis. So far, the vast majority of studies however focused on the role of the extrinsic coagulation system on disease progression, while the influence of the intrinsic coagulation system herein mainly still remains elusive. Moreover, most studies predominantly emphasize the effect of single coagulation factors on plaque development and progression, while often insufficiently linking coagulation and the pro-inflammatory immune phenotype, observed in atherosclerosis.

Coagulation factor XII, as part of the contact activation pathway, upon activation not only triggers the intrinsic coagulation cascade but also is involved in activation of the kallikrein-kinin system, the complement system and the fibrinolytic system. Up until now, human studies evaluating the role of FXII in the context of cardiovascular disease revealed quite conflicting data. Therefore, it appears crucial to clarify the role of this factor in the context of atherogenesis and disease progression.

Hence, the aim of this thesis was to address the role of FXII in atherosclerotic lesion formation, employing apolipoprotein E-deficient (*apoE*$^{-/-}$) mice deficient in FXII.

3. Material and Methods

3.1. Material

3.1.1 Consumables and technical instruments

3.1.1.1 Consumables

10 /20 /200 /1000 µl filter tips	Starlab (Hamburg, Germany)
10 /200 /1000 µl pipette tips	Sarstedt (Nümbrecht, Germany)
15 ml/ 50 ml tubes	Sarstedt (Nümbrecht, Germany)
384-well plates	Life Technologies (Darmstadt, Germany)
6-/ 24-well plates	Sarstedt (Nümbrecht, Germany)
70 µm cell strainer	BD (Heidelberg, Germany)
96-well plates, round bottom	Sarstedt (Nümbrecht, Germany)
Bottle filter top	Sarstedt (Nümbrecht, Germany)
Coverslips	VWR (Ismaning, Germany)
EDTA-coated tubes	Sarstedt (Nümbrecht, Germany)
FACS tubes	BD (Heidelberg, Germany)
Hemocytometer	Labor optic (Friedrichsdorf, Germany)
High fat diet (21% milk fat, 0.15% cholesterols, 19.5% casein)	Altromin (Lage, Germany)
Injection needles (26 gauge)	Dispomed (Gelnhausen, Germany)
LS-columns	Miltenyi Biotec (Bergisch Gladbach, Germany)
MS-columns	
Pre-separation filters	
Nunc Maxisorp ELISA plates	Thermo Scientific (St. Leon-Rot, Germany)
PAP pen for immunostaining	Sigma Aldrich (Taufkirchen, Germany)
Paraffin	Vogel (Giessen, Germany)
PCR-tubes	Biozym (Vienna, Austria)

Poly-L-lysin glass slides	Menzel GmbH (Braunschweig, Germany)
Serum gel tubes	Sarstedt (Nümbrecht, Germany)
Starfrost Slides	KNITTEL (Braunschweig, Germany)
Stericup filters 0.2 µm	Sarstedt (Nürnbrecht, Germany)
Superfrost Plus Slides	Thermo Scientific/Menzel (Braunschweig, Germany)
T175 flasks	Sarstedt (Nümbrecht, Germany)
T25 flasks	
Tissue culture dishes (100x20 mm)	
0.2 µm filter unit	Sartorius (Göttingen, Germany)
1.5 ml reaction tubes	Sarstedt (Nümbrecht, Germany)
1 / 10 / 20 ml syringes	BD (Heidelberg, Germany)
5/ 10/ 25 ml serological pipettes	Sarstedt (Nümbrecht, Germany)

3.1.1.2 Technical instruments

Automated vacuum tissue processor ASP 200S	Leica (Wetzlar, Germany)
Balance CP225D	Sartorius (Göttingen, Germany)
Balance TE3102S	
Cell culture incubators	Binder (Tuttlingen, Germany)
Centrifuge Heraeus Multifuge 3XR	Thermo Scientific (St. Leon-Rot, Germany)
Cryostat CM3050 S	Leica (Wetzlar, Germany)
Dissection tools	Fine Science Tools (Heidelberg, Germany)
Eppendorf 5430R centrifuge	Eppendorf (Hamburg, Germany)
FACS Canto II	BD (Heidelberg, Germany)
FLUOstar OPTIMA	BMG Labtech (Ortenberg, Germany)
Freezer and refrigerators	Liebherr (Bulle, Switzerland)

Gel electrophoresis chamber Sub-cell GT	Bio-Rad (Munich, Germany)
Heraeus Dry-air oven	Thermo (St. Leon-Rot, Germany)
Heraeus Fresco 17 centrifuge	
Heraeus HERAfreeze, -80°C freezer	
Leica upright microscope DM4000 B	Leica (Wetzlar, Germany)
Microwave oven	Privileg (Stuttgart, Germany)
Multipette Xstream	Eppendorf (Hamburg, Germany)
Multiskan EX plate reader	Thermo Scientific (St. Leon-Rot, Germany)
Nanodrop ND-2000	
Pipettes (1/ 10/ 20/ 100/ 1000 µl)	VWR (Ismaning, Germany) Eppendorf (Hamburg, Germany)
pipetus, electronic pipette controller)	Hirschmann Laborgeräte (Eberstadt, Germany)
Power Supplies for electrophoresis PowerPac basic	Bio-Rad (Munich, Germany)
RAININ Multichannel pipettes (8- /12- channel)	Mettler-Toledo (Giessen, Germany)
Rotary microtome RM 2255	Leica (Wetzlar, Germany)
Stereomicroscope SZX10	Olympus (Hamburg, Germany)
Sterile benches for cell culture SK-1200	BDK Luft- und Reinraumtechnik (Sonnenbühl, Germany)
Stretching table H1220	Leica (Wetzlar, Germany)
Sysmex KX-21N	Sysmex (Norderstedt, Germany)
Thermal PCR Cycler S1000/C1000	Bio-Rad (Munich, Germany)
Thermomixer comfort/standard	Eppendorf (Hamburg, Germany)
Tissue embedding station EG1160	Leica (Wetzlar, Germany)
Tissue floating bath 1052	GFL (Burgwedel, Germany)
Vortex mixer VV3	VWR (Ismaning, Germany)
Water bath	P-D Industriegesellschaft mbH (Dresden, Germany)

3.1.2 Kits and reagents

3.1.2.1 Kits

Amplex red cholesterol assay kit	Life Technologies (Darmstadt, Germany)
CD4+CD62L+ T cell isolation kit II	Miltenyi (Bergisch Gladbach, Germany)
CD11b microbeads	
CD11c microbeads	
Cytofix/Cytoperm staining kit	BD (Heidelberg, Germany)
EnzyChrom triglyceride assay kit	Biotrend (Cologne, Germany)
First strand cDNA synthesis kit	Thermo Scientific (St. Leon-Rot, Germany)
FoxP3 transcription buffer staining set	eBioscience (Frankfurt a. Main, Germany)
Murine bradykinin ELISA Kit	Hölzel-Diagnostica (Köln, Germany)
Murine IL-12 mini ELISA development kit	Peprotech (Hamburg, Germany)
Nucleo spin RNA II	Macherey-Nagel (Düren, Germany)
QIA shredder kit	Qiagen (Hilden, Germany)
RNeasy micro kit	
RNeasy mini kit	

3.1.2.2 Cell culture reagents

Bovine serum albumin (BSA)	Sigma-Aldrich (Taufkirchen, Germany)
Fetal calf serum (FCS)	PAA (Pasching, Austria)
Hank`s balanced salt solution (HBSS)	Life Technologies (Darmstadt, Germany)
Penicillin/Streptomycin	
Dulbecco´s Phosphate buffered saline (DPBS) w/o calcium/magnesium	
RPMI-1640 (GlutaMAX)	
β-Mercaptoethanol	
0.05% Trypsin-EDTA	
HEPES	

3.1.2.3 Molecular biology reagents

ABTS liquid substrate solution	Sigma-Aldrich (Taufkirchen, Germany)
Brefeldin A	Sigma-Aldrich (Taufkirchen, Germany)
Collagenase D	Roche (Mannheim, Germany)
Cytofix solution	BD (Heidelberg, Germany)
Futhan (FUT-175)	Sigma-Aldrich (Taufkirchen, Germany)
Gene ruler 1kb plus DNA ladder	Thermo Scientific (St. Leon-Rot, Germany)
Hanks balanced salt solution (HBSS)	Life Technologies (Darmstadt, Germany)
Ionomycin	Sigma-Aldrich (Taufkirchen, Germany)
Liberase blendzyme TL (#3)	Roche (Mannheim, Germany)
mouse serum	Sigma-Aldrich (Taufkirchen, Germany)
Normal horse serum	VectorLaboratories (Buringam, Canada)
Normal mouse serum	
Normal rabbit serum	
$OVA^{323-339}$ (aa-sequence: NH-Ile-Ser-Gln-Ala-Val-His-Ala-Ala-His-Ala-Glu-Ile-Asn-Glu-Ala-Gly-Arg-OH)	AnaSpec (Seraing,Belgium)
PCR Master Mix	Thermo Scientific (St. Leon-Rot, Germany)
Perm/Wash buffer	BD (Heidelberg, Germany)
Phorbol-12-myristat-13-acetat (PMA)	Sigma-Aldrich (Taufkirchen, Germany)
Proteinase K	Thermo Scientific (St. Leon-Rot, Germany)
rabbit serum	Sigma-Aldrich (Taufkirchen, Germany)
Streptavidin-HRP	BD (Heidelberg, Germany)
SYBR green Mix (2x)	Thermo Scientific (St. Leon-Rot, Germany)
Tissue Tek OCT compound	Sakura Finetek (Staufen, Germany)
TMB substrate solution	Sigma-Aldrich (Taufkirchen, Germany)
Vectashield with DAPI	VectorLaboratories (Burlingam, Canada)
Vitro-Clud	R. Langenbrinck (Emmendingen, Germany)

3.1.3 Chemicals

Absolute ethanol p.A.	AppliChem (Darmstadt, Germany)
Acetic acid	Sigma-Aldrich (Taufkirchen, Germany)
Acid aldehyde	Sigma-Aldrich (Taufkirchen, Germany)
Ammoniumchloride (NH_4Cl)	Carl Roth (Karlsruhe, Germany)
Basic fuchsin	Sigma-Aldrich (Taufkirchen, Germany)
Citric acid	Carl Roth (Karlsruhe, Germany)
Concentrated hydrochloric acid	AppliChem (Darmstadt, Germany)
Disodium carbonate (Na_2CO_3)	Sigma-Aldrich (Taufkirchen, Germany)
Disodium phosphate (Na_2HPO_4)	Carl Roth (Kalsruhe, Germany)
Ethanol 100%, denatured	Carl Roth (Kalsruhe, Germany)
Ethanol 70%, denatured	Carl Roth (Kalsruhe, Germany)
Ethylendiaminetetraacetic acid (EDTA)	Sigma-Aldrich (Taufkirchen, Germany)
Gelatin (from bovine skin –type B)	Sigma-Aldrich (Taufkirchen, Germany)
Hemalm	Carl Roth (Kalsruhe, Germany)
Hydrochloric acid (HCl)	Carl Roth (Karlsruhe, Germany)
Isopropanol	Carl Roth (Kalsruhe, Germany)
Lidocain-HCl	Sigma-Aldrich (Taufkirchen, Germany)
Monopotassium phosphate (KH_2PO_4)	Carl Roth (Karlsruhe, Germany)
Mowiol 4-88	Carl Roth (Karlsruhe, Germany)
Oil-Red-O	Sigma-Aldrich (Taufkirchen, Germany)
Paraformaldehyde	Carl Roth (Karlsruhe, Germany)
Potassium chloride (KCl)	Carl Roth (Karlsruhe, Germany)
Potassiumhydrogen carbonate ($KHCO_3$)	Carl Roth (Karlsruhe, Germany)

Saturated aqueous picric acid	Sigma-Aldrich (Taufkirchen, Germany)
Sirius red	Sigma-Aldrich (Taufkirchen, Germany)
Sodium citrate (NaCl)	Carl Roth (Karlsruhe, Germany)
Sodium dodecyl sulfate (SDS)	Carl Roth (Karlsruhe, Germany)
Sodium acetate (NaOAc)	Carl Roth (Karlsruhe, Germany
Sodium hydroxide (NaOH)	AppliChem (Darmstadt, Germany)
Sodiumhydogen carbonate ($NaHCO_3$)	Sigma-Aldrich (Taufkirchen, Germany)
Sucrose	Sigma-Aldrich (Taufkirchen, Germany)
Sulfuric acid (H_2SO_4)	AppliChem (Darmstadt, Germany)
TRIS	Carl Roth (Karlsruhe, Germany)
Triton-X100	Carl Roth (Karlsruhe, Germany)
Tween-20	AppliChem (Darmstadt, Germany)
Xylene	Carl Roth (Karlsruhe, Germany)

3.1.4 Cytokines and Growth-factors

rmC5a	R&D Systems (Wiesbaden-Nordenstadt, Germany)
rmGM-CSF	Peprotech (Hamburg, Germany)
rmTNFα	Peprotech (Hamburg, Germany)

3.1.5 Antibodies

3.1.5.1 Conjugated antibodies

Epitope	Fluorophore	Clone	Manufacturer
B220	FITC	RA3-6B2	eBioscience (Frankfurt, Germany)
CD115	PE	AFS98	eBioscience (Frankfurt, Germany)
CD11b	FITC	M1/70	eBioscience (Frankfurt, Germany)
CD11b	PerCP-Cy5.5	M1/70	eBioscience (Frankfurt, Germany)
CD11b	V500	M1/70	eBioscience (Frankfurt, Germany)
CD11c	Alexa Fluor 488	N418	eBioscience (Frankfurt, Germany)
CD11c	APC	N418	eBioscience (Frankfurt, Germany)
CD11c	FITC	N418	eBioscience (Frankfurt, Germany)
CD19	APC	eBio1D3	eBioscience (Frankfurt, Germany)
CD19	FITC	1D3	BD (Heidelberg, Germany)
CD25	APC	PC61.5	eBioscience (Frankfurt, Germany)
CD3e	FITC	145-2C11	eBioscience (Frankfurt, Germany)
CD3e	PerCP Cy5.5	145-2C11	BD (Heidelberg, Germany)
CD3e	V450	500A2	BD (Heidelberg, Germany)
CD3e	V500	500A2	BD (Heidelberg, Germany)
CD4	APC	RM4-5	eBioscience (Frankfurt, Germany)
CD4	FITC	GK1.5	eBioscience (Frankfurt, Germany)
CD4	PE	RM4-5	eBioscience (Frankfurt, Germany)
CD4	PE-Cy7	GK1.5	eBioscience (Frankfurt, Germany)
CD40	APC	1C10	eBioscience (Frankfurt, Germany)
CD44	PerCP-Cy5.5	IM7	eBioscience (Frankfurt, Germany)
CD45	APC-Cy7	30-F11	BD (Heidelberg, Germany)
CD62L	PE-Cy7	MEL-14	eBioscience (Frankfurt, Germany)
CD80	APC	16-10A1	eBioscience (Frankfurt, Germany)

CD86	PE	GL1	eBioscience (Frankfurt, Germany)
CD86	PE	PO3.1	eBioscience (Frankfurt, Germany)
CD88	Alexa Fluor 647	10/92	AbD Serotec (Düssel-dorf, Germany)
CD8a	PE	53-6.7	eBioscience (Frankfurt, Germany)
CD8a	PE-Cy7	53-6.7	eBioscience (Frankfurt, Germany)
CD8a	V500	53-6.7	BD (Heidelberg, Germany)
F4/80	APC	BM8	eBioscience (Frankfurt, Germany)
F4/80	V450	BM8	eBioscience (Frankfurt, Germany)
FoxP3	PE	FJK-16s	eBioscience (Frankfurt, Germany)
Gr1	PerCP-Cy5.5	RB6-8C5	BD (Heidelberg, Germany)
IFNg	APC	XMG1.2	BD (Heidelberg, Germany)
IL-12	PE	C15.6	BD (Heidelberg, Germany)
IL-17a	PE	eBio17B7	eBioscience (Frankfurt, Germany)
MHCII	APC	M5/114.15.2	eBioscience (Frankfurt, Germany)
MHCII	eFluor 450	M5/114.15.2	eBioscience (Frankfurt, Germany)
MHCII	FITC	2G9	BD (Heidelberg, Germany)
SMA (smooth muscle actin)	Cy3	1A4	Sigma-Aldrich (Taufkirchen, Germany)
TCRb	PerCP-Cy5.5	H57-597	eBioscience (Frankfurt, Germany)
TCRb	V450	H57-597	BD (Heidelberg, Germany)
TCRgd	APC	eBioGL3	eBioscience (Frankfurt, Germany)
TCRgd	FITC	GL3	eBioscience (Frankfurt, Germany)

3.1.5.2 Unconjugated primary antibodies

Epitope	Clone	Manufacturer
CD3	CD3-12	AbD Serotec (Düsseldorf,Germany)
Isotype control for CD3 staining	IgG2a	AbD Serotec (Düsseldorf,Germany)
Mac-2	M3/38	Cedarlane (Burlington, Canada)
Isotype control for Macrophage staining	SC-2026	Santa-Cruz (Dallas, USA)
C5a	I52-1486	BD (Heidelberg, Germany)
CD3 AF/LE	145-2C11	BD (Heidelberg, Germany)
CD28 AF/LE	37.51	BD (Heidelberg, Germany)

3.1.5.3 Conjugated secondary antibodies

Epitope	Fluorophore	Clone	Manufacturer
Anti-Rat	Alexa 488	Polyclonal goat anti-rat	Life Technologies (Darmstadt,Germany)
C5a	Biotin	I52-278	BD (Heidelberg, Germany**)**

3.1.6 Primer

3.1.6.1 Primer for qPCR

Primer below were purchased from Eurofins MWG Operon (Ebersberg, Germany) unless indicated otherwise.

mRNA	Name	Sequence
mArg1	mARG1 for	5'-GGAAAGCCAATGAAGAGCTG-3'
	mARG1 rev	5'-GATGCTTCCAACTGCCAGAC-3'
mCd80	mCD80 for	5'-TCGTCTTTCACAAGTGTCTTCAG-3'
	mCD80 rev	5'-TTGCCAGTAGATTCGGTCTTC-3'
mCd86	mCD86 for	5'-GAAGCCGAATCAGCCTAGC-3'
	mCD86 rev	5'-CAGCGTTACTATCCCGCTCT-3'
mCd74	mCD74 for	5'-CACCGAGGCTCCACCTAA-3'
	mCD74 rev	5'-GCAGGGATGTGGCTGACT-3'

mHprt	mHPRT for mHPRT rev	5’-TCCTCCTCAGACCGCTTTT-3’ 5’-CCTGGTTCATCATCGCTAATC-3’
mIcam1	mICAM1 for mICAM1 rev	5’-TGGCCTGGGGGATGCACACT-3’ 5’-GGCTGTAGGTGGGTCCGGGT-3’
mIgf1	mIGF1 for mIGF1 rev	5’-TCGGCCTCATAGTACCCACT-3’ 5’-ACGACATGATGTGTATCTTTATTGC-3’
mIl4	mIL4 for mIL4 rev	5’-CAACGAAGAACACCACAAGAG-3’ 5’-ATGAATCCAGGCATCGAAAAGC-3’
mIl6	mIL6 for mIL6 rev	5’-GTGGCTAAGGACCAAGACCA-3’ 5’-ACCACAGTGAGGAATGTCCA-3’
mIl10	mIL10 for mIL10 rev	5’-TGCACTACCAAAGCCACAAGG-3’ 5’-TGGGAAGTGGGTGCAGTTATTG-3’
mIl12a		mIL-12a Quantitect primer assay QT01048334 Qiagen (Hilden, *Germany)*
mMrc1	mMRC1 for mMRC1 rev	5’-CACTCATCCATTACAACCAAAGC-3’ 5’-CAGGAGGACCACGGTGAC-3’
mNos2	mNOS2 for mNOS2 rev	5’-GTTCTCAGCCCAACAATACAAGA-3’ 5’-GTGGACGGGTCGATGTCAC-3’
mPecam1	mPECAM1 for mPECAM1 rev	5’-GGAACGAGAGCCACAGAGACGG-3’ 5’-GCTTTCGGTGGGGACAGGCTC-3’
mPselectin	mPselectin for mPselectin rev	5’-CTGCCCCATGCTCTGTTGGGC-3’ 5’-CCCCAACCACCTGCCTCCGT-3’
mTgfb	mTGFβ for mTGFβ rev	5’-GACGTCACTGGAGTTGTACGG-3’ 5’-GGTTCATGTCATGGATGGTGC-3’
mTnfa	mTNFα for mTNFα rev	5’-CTGTAGCCCACGTCGTAGC-3’ 5’-GGTTGTCTTTGAGATCCATGC-3’
mVcam1	mVCAM1 for mVCAM1 rev	5’-TGATTGGGAGAGACAAAGCA-3’ 5’-AACAACCGAATCCCCAACTT-3’

3.1.6.2 Primer for genotyping PCR

Gene	Name	Sequence
ApoE	IMRO 180	5‘–GCCTAGCCGAGGGAGAGCCG–3‘
	IMRO 181	5‘–TGTGACTTGGGAGCTCTGCAGC–3‘
	IMRO 182	5‘–GCCGCCCCGACTGCATCT–3‘
F12	WT-forward	5‘–GGCCTCTTGTATTGACTGATGA–3‘
	WT-reverse	5‘–AACTGCCATCATAACGTTAGCC–3‘
	KO-forward	5‘–GCAGAGGTTACGGCAGTTTGTCTCTCC–3‘
OTII	oIMR 1825	5‘–GCTGCTGCACAGACCTACT–3‘
	oIMR 1826	5‘–CAGCTCACCTAACACGAGGA–3‘
	oIMR 1880	5‘–AAAGGGAGAAAAAGCTCTCC–3‘
	oIMR 1881	5’–ACACAGCAGGTTCTGGGTTC–3’

3.1.7 Animals

C57BL6/J (Bl6)	Jackson laboratories (Maine, USA)
B6.129P2-ApoE^{tm1Unc}/J (ApoE$^{-/-}$)	
B6.Cg-Tg(TcraTcrb)425Cbn/J (OT-II)	
B6. 129P2-ApoE^{tm1Unc}/J (ApoE$^{-/-}$) *F12* $^{-/-}$	Kindly provided by Prof. Dr. Bernhard Nieswandt, Würzburg

3.1.8 Media, buffers and solutions

3.1.8.1 Media

DC medium

- RPMI-1640 media with 2 mM L-glutamine
- 10 % (v/v) FCS
- 100 U/ml penicillin/streptomycin
- 50 µM β-mercaptoethanol

L929 growth medium

- RPMI-1640 medium with 2 mM L-glutamine
- 10 % FCS
- 100 U/ml penicillin/streptomycin
- 25 mM HEPES

L929 conditioned medium

- Cultured L929 cells in L929 growth medium for 10 days
- Collected spent medium at day 10
- Sterile filtration of SN through a 0.2 µm filter

IL-17a/IFNγ stimulation medium (for intracellular staining)

- DC medium
- 50 ng/ml PMA
- 750 ng/ml ionomycin
- 2.5 µg/ml brefeldin A

IL-12 stimulation medium (for intracellular staining)

- DC medium
- 2.5 µg/ml brefeldin A
- 1 µg/ml LPS

3.1.8.2 Buffers

Phosphate buffered saline solution (PBS)

- 137 mM NaCl
- 2.7 mM KCl
- 18.61 mM Na_2HPO_4
- 1.8 mM KH_2PO_4
- DDW
- pH was adjusted to 7.4

Red blood cell lysis buffer (RBC buffer)

- 155 mM NH_4Cl
- 10 mM $KHCO_3$
- 0.1mM EDTA
- DDW

Hank`s Complete

- 1x HBSS
- 0.6 % BSA
- 300 µM EDTA, pH 8.0

FACS staining buffer

- 1x PBS
- 2 % BSA
- 2 % mouse serum
- 2% rabbit serum

MACS buffer

- 0.5 % w/v BSA
- 2 mM EDTA, pH 8.0
- add PBS, sterile filtration

FACS staining buffer (Stock)

- PBS
- 2 % BSA
- 2 % mouse serum
- 2% rabbit serum

FACS staining buffer (Working solution)

- 1 part HBSS
- 1 part FACS staining buffer stock

Tail lysis buffer

- 10 mM Tris, pH 8.0
- 10 mM EDTA, pH 8.0
- 10 mM NaCl
- 0.5 % SDS
- DDW

TAE buffer

- 40 mM Tris
- 20 mM acetic acid
- 1 mM EDTA
- DDW

Sodium Carbonate buffer

- 4.2 g $NaHCO_3$
- 1.78 g Na_2CO_3
- q.s. to 500 ml with DDW
- pH was adjusted to 9.5

Blocking/Staining buffer for CD3 T cell staining

- 0.1% BSA in PBS

Blocking/Staining buffer for Mac-2 macrophage staining

/α-SMC actin smooth muscle cell staining

- 1x PBS
- 1 % BSA
- 2 % normal mouse serum
- 2 % normal rabbit serum
- 2 % normal horse serum
- 0.1 % Triton X-100

3.1.8.3 Solutions

Antigen retrieval stock solution A

- 2.10 % citric acid
- DDW

Antigen retrieval stock solution B

- 2.94 % sodium citrate
- DDW

Antigen retrieval working solution

- 0.18 parts stock solution A
- 0.82 parts stock solution B
- 9 parts DDW

Aldehyde-Fuchsin solution

stock solution

- 70 % (v/v) Ethanol
- 0.5 % (w/v) aldehyde fuchsin

working solution

- 50 ml aldehyde fuchsin stock solution
- 2.5 ml acid aldehyde
- 1 ml concentrated hydrochloric acid
- solution kept at RT o/n and filtered prior to use

Kaiser's glycerin jelly

Stock solution:

- 4 g gelatin
- 21 ml DDW
- 25 ml glycerin

Working solution:

- 3 parts Kaiser´s glycerin jelly stock solution
- 7 parts DDW

Lidocaine-EDTA

- 1x PBS
- 10 mM EDTA pH 8.0
- 0.4 % w/v Lidocaine-HCl

Liberase Blendzyme

Stock solution:

70 mg Liberase Blendzyme TL was dissolved in 2 ml DDW
(storage at -20°C)

Working solution:

1:20 dilution of Liberase Blendzyme Stock solution in RPMI-1640

Mowiol solution

- 2.4 g Mowiol 4-88
- 6 g glycerol
- 6 ml DDW

Stir to mix at RT for several hours. 12 ml of 0.2 M Tris (pH 8.5) was added and heated to 50 °C for 10 min with mixing in between. After the Mowiol powder was dissolved, solution was cleared by centrifugation at 5000 g for 15 minutes. Solution was stored in aliquots at 20°C.

Oil-Red-O (ORO) Lipid Staining Solution

Stock solution:

- isopropanol
- 0.5 % (w/v) Oil-Red-O

Working solution:

- 180 ml stock solution
- 120 ml DDW
- incubation for 1h at RT, followed by filtration

Picrosirius Red solution

- 0.1% (w/v) picrosirius red
- saturated aqueous picric acid
- solution was mixed thoroughly and filtered prior to use

3.2 Methods

3.2.1 Animal work

3.2.1.1 Mice and breeding

C57BL/6J (*B6*), *B6.129P2-ApoE^{tm1Unc}/J* (*apoE$^{-/-}$*), *C57BL/6-Tg(TcraTcrb)^{425Cbn}/J* (OT-II) mice were obtained from The Jackson Laboratory (Maine, USA). *F12$^{-/-}$* mice and *F12$^{-/-}$apoE$^{-/-}$* mice were kindly provided by Bernhard Nieswandt (RVZ, Würzburg). *ApoE$^{-/-}$* mice were crossed with *B6.F12$^{-/-}$* to obtain *F12$^{-/-}$apoE$^{-/-}$* mice. All mice were bred and maintained in the Zentrum für Experimentelle Molekulare Medizin (ZEMM), Zinklesweg 10, 97078 Würzburg (B6) or in the animal facility of the Rudolf-Virchow-Zentrum (RVZ), Josef-Schneider-Str. 2, 97080 Würzburg (*OTII*, *apoE$^{-/-}$, F12$^{-/-}$*, *F12$^{-/-}$ apoE$^{-/-}$*).

For induction of atherosclerosis at the age of 8 weeks, age-matched *F12$^{-/-}$apoE$^{-/-}$* and a*poE$^{-/-}$* controls (stated from now on as *F12$^{+/+}$apoE$^{-/-}$*) were placed on atherogenic diet (21 % milk fat, 0.15 % cholesterol, 19.5 % casein, Altromin) for 12 weeks. All experiments conducted were approved by local authorities.

3.2.1.2 Sacrifice of mice and organ harvest

Mice were sacrificed by cervical dislocation. Blood was obtained from the retro-orbital plexus or by cardiac puncture and collected in EDTA-containing tubes or serum-separation tubes. Mice were then slowly flushed with PBS by trans-cardiac perfusion prior to harvesting organs for FACS analysis. Harvesting of organs for histology was performed after an additional perfusion step with 4 % PFA.

3.2.1.3 Organ processing for flow cytometry

For preparation of single cell suspensions, spleens and lymph nodes (brachial, axillary, inguinal and para-aortic) were dissected and passed through 70 µm cell strainers. Single cell suspensions from the bone marrow (BM) of the femurs of mice were obtained by flushing the bone marrow cavities with Hank`s complete and passing the cells through a 70 µm cell strainer. Cell suspensions were washed once in Hank`s complete.

Splenocytes and BM cells were subjected to red blood cell lysis by a 5 min incubation with 3 ml RBC lysis buffer at RT. Lysis was terminated by addition of 7-10 ml Hank`s complete followed by centrifugation (350 g, 5 min at 4°C). Cells were resuspended in Hank`s complete for further use.

Aortas were cleaned from extraneous fat, dissected and cut into small pieces into a reaction tube containing 200 µl Blendzyme working solution. Aortic digestion was performed for 30 min at 37°C on a thermo-shaker adjusted to 1400 rpm. Every 10 min samples were pipetted up and down. Following digestion samples were passed through a 70 µm cell strainer, washed once with Hank`s complete and adjusted with Hank`s for further use.

Peripheral blood for FACS analysis was obtained from the retro-orbital plexus and collected in EDTA-containing tubes. 150 µl blood was lysed in 3 ml RBC lysis buffer for 5 min at RT. Lysis was stopped with Hank`s complete and resuspended in Hank`s complete after centrifugation.

3.1.2.4 Preparation of serum and plasma samples

Blood was collected in serum gel tubes by cardiac puncture, allowed to clot at RT for 30 min and centrifuged at 10 000 g for 5 minutes at 20°C to obtain serum. Serum samples were stored in aliquots at –80°C.

Plasma samples for measurement of active complement components were obtained using EDTA/futhan treated tubes (1 mg/ml EDTA; 0.1 mg/ml FUT-175)[341], since futhan prevents *in vitro* complement activation. Plasma samples were stored at –80°C.

3.2.2 Assays

3.2.2.1 Blood cholesterol and triglycerid measurements

Triglycerid levels in serum were measured using the Amplex Red cholesterol assay kit or the EnzyChrom triglyceride assay kit, respectively, according to the manufacturer´s instructions using a FLUOstar OPTIMA plate reader.

3.2.2.2 ELISA

IL-12 ELISA

IL-12 protein levels were quantified in serum using the murine IL-12 mini ELISA development kit (Peprotech) according to the manufacturer's instructions.

Bradykinin ELISA

Bradykinin levels were quantified in serum using a bradykinin ELISA Kit (Uscn, Wuhan (China)) according to the manufacturer's instructions.

C5a ELISA

C5a levels were determined in plasma samples, obtained from blood collected in EDTA/futhan-containing tubes[341] to inhibit *in vitro* activation of complement. 96-well plates for ELISAs were pre-coated with purified rat anti-mouse C5a antibody (not cross-reacting o/n with C5, clone I52-1486) diluted in sodium carbonate coating buffer (pH 9.5) o at 4°C. Plate was washed 5 times in PBS containing 0.05 % Tween-20. Non-specific binding was blocked with 10 % FCS for 1h at RT. Subsequently, plate was washed 5 times with PBS/0.05 % Tween-20 and incubated with plasma samples and standards for 2 h at RT. After 5 washes which were performed as described before, plate was incubated with a biotin-labeled rat anti-mouse C5a detection antibody (clone I52-278, BD Biosciences) for 1 h at RT followed by additional 5 washing steps prior to incubation with the streptavidin-HRP conjugate (1:1000 dilution) for 30 minutes at RT. Before adding the TMB substrate solution for up to 30 min at RT in the dark plate was washed again 7 times in

PBS/Tween. The reaction was stopped using 2N H_2SO_4 and plate was read within 30 min at 450 nm (adapted from Pavlovski *et al*.)[342].

3.2.3 Flow cytometry

3.2.3.1 Surface staining

Single cell suspensions from organs or blood were washed once in Hank`s complete, distributed into 96-well round bottom plates, followed by resuspension in 50 µl of a 1:1 mixture of FACS staining buffer and Hank`s complete. Antibodies for surface staining were added in a 1:300 dilution and incubated for 30 minutes at 4°C in the dark. Following staining, cells were washed twice with 200 µl Hank`s complete per well and measured immediately or fixed with Cytofix solution (diluted 1:2 with PBS) and stored at 4°C till measurement.

3.2.3.2 Intracellular Staining

IFNγ and IL-17a staining

For intracellular staining of IFNγ and IL-17a cell suspensions were subjected to a 4 hour stimulation with DC media supplemented with 50 ng/ml PMA, 750 ng/ml Ionomycin and 2.5 µg/ml Brefeldin A at 37°C, 5 % CO_2.

Following stimulation cells were washed once with Hank`s complete. Surface staining was performed as described in 3.2.3.1, however surface antibodies were diluted in a 1:2 dilution of BD Stain buffer and incubated 30 minutes at 4°C, followed by two washes with 200 µl/well diluted BD Stain buffer. After pulse vortexing, cells were incubated for 20 min at 4°C with 100 µl/well Cytofix/Cytoperm buffer (BD). Plate was washed twice with 200 µl 1x Perm/Wash buffer (BD) followed by a 30 min incubation at 4°C with intracellular antibodies (anti-mouse IFNγ and anti-mouse IL-17A) in a 1:100 dilution in 1x Perm/Wash buffer. After staining, cells were washed twice with

1x Perm/Wash buffer and either resuspended in Hank`s complete or fixed using Cytofix solution (1:2 diluted in PBS).

FOXP3 staining

Surface staining was performed as described in 3.2.3.1. Intracellular staining was performed using the eBioscience FoxP3 transcription buffer staining kit. After pulse vortexing, cells were incubated for at least 30 min at 4°C with 100 µl/well of fixation buffer 1:4 diluted with fixation diluent in the dark. After two washes in 200 µl/well permabilization buffer cells were stained with anti-mouse FOXP3 antibody in a final dilution of 1:100 in 50 µl/well permeabilization buffer. Samples were stained for 30 min at 4°C followed by two washes with 200 µl/well permeabilization buffer. Cells were respuspended in Hank`s complete for flow cytometric analyses.

IL-12 staining

Prior to staining, splenocytes were stimulated with 100 ng/ml rmTNF-α and 5 µg/ml Brefeldin A for 4 h at 37°C , 5 % CO_2. After surface staining, intracellular staining using conjugated anti-mouse IL-12 antibodies was performed as described in 3.2.3.2.

3.2.4 Histology

3.2.4.1 En face preparation of aortas

Mice were sacrified and blood was drawn for serum by cardiac puncture. Mice were flushed first with PBS followed by 4 % PFA in PBS via trans-cardiac reperfusion. Aortas were dissected, and mounted on rubberized glass slides after removal of excessive fat. Aortas were incubated in 4 % PFA in PBS o/n. After removal of adventitial tissue, staining for lipid depositions was performed using Oil-Red-O and aortas were mounted using Kaiser´s glycerin jelly.

3.2.4.2 Preparation of aortic roots

Hearts were dissected after fixation by reperfusion with 4 % PFA in PBS. After fixation in 4 % PFA o/n, hearts were processed using an automated vacuum tissue processor according to the following protocol

Reagent	Step	Duration	Temperature
70 % ethanol	1	1 hour	
70 % ethanol	2	1 hour	
96 % ethanol	3	1 hour	
96 % ethanol	4	1 hour	
100 % ethanol	5	1 hour	
100 % ethanol	6	1 hour	
100 % ethanol	7	1 hour	
Xylene	8	1 hour	45 °C
Xylene	9	1 hour	45 °C
Xylene	10	1 hour	45 °C
Paraffin	11	1 hour	62 °C
Paraffin	12	1 hour	62 °C
Paraffin	13	over night	62 °C

After embedding in paraffin blocks, 5 µm serial transversal sections through the aortic root were cut. The cuts were allowed to stretch in a tissue floating bath or stretching plate adjusted to 40°C. Slides were dried o/n at 56°C and stored at RT for further analyses.

Alternatively for cryo-sections, hearts were fixed in 4 % PFA in PBS as described. For cyro-protection of the tissue, fixing solution was then exchanged against 30 % Sucrose solution in PBS for 24 h before freezing the heart in 1:1 OCT/PBS mixture compound and prior to cutting 5 µm serial sections through the aortic root.

3.2.4.3 Deparaffinization and Rehydration

Paraffin sections were deparaffinzed according to the following protocol:

Reagent	Step	Duration
Xylene	1	15 minutes
Xylene	2	15 minutes
Xylene	3	15 minutes
100 % ethanol	4	1 minute
100 % ethanol	5	1 minute
90 % ethanol	6	1 minute
70 % ethanol	7	1 minute
70 % ethanol	8	10 minutes
ddH_2O	9	10 minutes

After deparaffinization of paraffin sections, either Oil-Red-O staining for quantification of lesion formation or antigen retrieval prior to subsequent immune-histochemical stainings was performed.

3.2.4.4 Antigen retrieval

For antigen retrieval, working solution was pre-warmed in the microwave prior to placing the slides. De-paraffinized sections were then heated in a microwave for 10 min before half of the solution was discarded and replaced with fresh antigen retrieval solution. After additional 10 min of heating, the slides were allowed to cool down for 30 min at RT in the antigen retrieval solution. Slides were then washed twice for 5 min in PBS prior to immune-histochemical stainings. Cryosections did not require antigen retrieval and were only washed twice for 5 min in PBS before performing immune-histochemical staining.

3.2.4.5 Oil-Red-O lipid staining for aortas and aortic roots

Samples were washed in PBS for 5 min, followed by ten short dips in 60 % isopropanol. After 15 minutes incubation in Oil-Red-O working solution, samples were dipped ten more times in 60 % isopropanol and mounted with Kaiser´s glycerin jelly (aortas), Mowiol (cryosections of aortic roots) or Vitro-Clud (paraffin sections of aortic roots).

3.2.4.6 Collagen/Elastin staining of aortic root sections

After deparaffinization, sections were rehydrated in 70 % ethanol, followed by staining in aldehyde fuchsin working solution for 15 min at RT. Subsequently, slides were dipped five times in 70 % ethanol and placed in deionized water for 5 minutes. Samples were stained for 90 min in sirius red solution at RT followed by a 1 min incubation in 0.01 M hydrochloric acid at RT. After dehydration via an ascending alcohol series to xylene slides were dried and mounted with Vitro-Clud.

3.2.4.7 T cell staining of aortic root sections

After deparaffinization and antigen-retrieval, aortic root sections were air-dried and borders of the roots were circled using a pap pen. The following incubation steps were performed in a humidified incubation chamber. After blocking in 50 µl blocking buffer for 30 min, buffer was removed and slides were incubated with a final concentration of 20 µg/ml primary α-human CD3 antibody or isotype control in staining buffer o/n at 4°C followed by a 15 minute wash in PBS on a shaker. Samples were washed two more times in PBS for 5 min prior to incubation with the Alexa-488 conjugated secondary antibody at a final concentration of 4 µg/ml in staining buffer for 1-2 h at RT. After secondary antibody incubation, sections were washed in PBS as mentioned before. After the last wash step, excessive liquid was removed and slides were mounted using Vectashield containing DAPI for counterstain of the nuclei. Borders were sealed with nail polish.

3.2.4.8 Macrophage (MAC) and smooth muscle cell (SMC) staining of aortic root sections

After deparaffinization and antigen-retrieval as described, aortic root sections were air-dried and borders of the roots were circled using a pap pen. The following incubation steps were performed in a humidified incubation chamber. After blocking in 50 µl blocking/staining buffer for 30 min at RT, buffer was removed and sections were incubated with 50 µl antibody cocktail over night at 4°C. Antibody cocktail contained α-mouse Mac2 and Cy3-conjugated α-mouse SMA antibodies in final concentrations of 1.67 µg/ml and 5 µg/ml respectively in blocking/staining buffer. After washing the slides for 15 min and 2 times 5 min at RT, PBS was removed and α-rat Alexa 488 antibody was added at a final concentration of 4 µg/ml in 1 % BSA for 1 h at RT in the dark. After secondary antibody incubation, sections were washed in PBS as mentioned before. After the last wash step, excessive liquid was removed and slides were mounted using Vectashield with DAPI for counterstain of the nuclei. Borders were sealed with nail polish.

3.2.4.9 Analysis of plaque area in the aorta and aortic root

Quantification of the atherosclerotic lesion area in aortas was performed by marking the total aortic area and the plaque area using Diskus image analysis software.

Plaque area in the aortic root was analyzed in 3 sections per animal. The total area of the aortic roots as well as the plaque area was marked using Diskus image analysis software.

3.2.4.10 Analysis of necrotic core area, SMC, MAC, T cell and collagen content in the aortic root plaque

For each staining, two sections per mouse (close to the sections chosen for plaque quantification) were analyzed.

Necrotic core area was analyzed in the same slides that were used for MAC/SMC staining. Necrotic core area was quantified by marking the acellular necrotic core area in the plaque as well as the total plaque area using Diskus image analysis software.

For SMC analysis; total plaque area, SMC area, number of SMCs, number of non-smooth muscle cells as well as the total number of plaque cells were analyzed using Diskus image analysis software. For macrophage analysis the same analysis was performed.

T cell analysis was performed by analyzing the total number of T cells in the plaque, the number of non T cells and the total number of cells in the plaque using Diskus image analysis software.

Collagen content analysis was performed by analyzing polarized images of the collagen-positive area per valve and the total plaque area per valve using Image J software.

3.2.5 Cell culture methods

3.2.5.1 Bone marrow-derived dendritic cell (BMDC) culture

Femurs were removed from donor mice and cleaned from excessive tissue. The bones were prepared under aseptic conditions. After a short wash in 70 % ethanol followed by a wash in PBS, marrow cavities were flushed with PBS and bone marrow was passed through a 70 µm cell strainer to obtain single cell suspensions followed by a wash in PBS. After centrifugation for

5 min at 350 g, cells were resuspended in DC medium, counted and seeded at a density of $2x10^6$ cells/ml in DC medium supplemented with 50 ng/ml GM-CSF in 10 cm dishes. Culture medium supplemented with GM-CSF was replaced after 3 days and then every second day for a total of 7-8 days; cells were maintained at a density of $2 \cdot 10^6$ cells/ml throughout the culture[82]. After a differentiation period of 7-8 days cells were used for further experiments.

3.2.5.2 Generation of L929-conditioned media

Macrophage-colony stimulating factor (M-CSF, CSF-1) is highly expressed by the L929 mouse cell line[343] and L929 cell-conditioned medium was used as a source of M-CSF in our BMM cultures.

L929 cells were thawed from liquid nitrogen by adding warm media. Cells were then resuspended gently and washed twice in a 20x volume of media to remove DMSO. After the last wash, cell pellet was resuspended in 5 ml L929 medium and seeded in T25 flasks. In order to expand the cells, cultures were split when confluent at a ratio of > 1:3 into T175 flasks. Media was changed at least twice per week. To obtain conditioned media, five confluent T175 flasks were split to 26 T175 flasks. Each flask was supplemented with 55 ml of L929 medium. The cells were kept for 10 days at 37°C, 5 % CO_2 in the incubator. For collection of the media, it was centrifuged at 350 g for 5 min and supernatant was sterile filtered using bottle top vacuum filters, aliquoted into 50 ml portions and stored at -80°C for further use.

3.2.5.3 Bone marrow-derived macrophage (BMM) culture

Single cell suspensions from BM were obtained as described in 3.2.5.1 and cultured at a density of $2x10^6$ cells/ml in DC medium supplemented with 15 % L929 conditioned medium for 7 days to generate BMMs[344]. Media was replaced every 2 days till day 7 by aspirating the supernatant, a careful wash of the plate with PBS and replacement with fresh DC media supplemented with 15 % L929-conditioned medium.

To harvest the BMMs, media was aspirated and detached adding approximately 5 ml Lidocaine-EDTA solution to the plate. Plate was tapped and mixed well with a pipette followed by 2-5 min incubation at 37°C. Lidocaine-EDTA solution containing detached cells was transferred into a 50 ml reaction tube containing DC media. These steps were repeated 2-4 times till the majority of cells were detached. After centrifugation, cells were adjusted to $1x10^6$ cells/ml in DC medium supplemented with 15 % L929-conditioned medium and allowed to attach at least for one day before performing further experiments.

3.2.5.4 Antigen-specific T-cell proliferation and polarization

Naïve CD4 T cells were isolated from spleen and lymph nodes of transgenic OT-II mice using the $CD4^+CD62L^+$ T Cell isolation kit II according to the manufacturer's instructions. Isolated cells were adjusted to $20x10^6$ cells/ml and incubated with 5 µM CFSE in DC medium for 10 min at 37 °C for antigen-dependent proliferation or left untreated for antigen-dependent polarization. Cells were washed once with DC medium and $1.5x10^5$ naïve T cells were seeded with $0.5x10^5$ OVA pulsed BMDCs in DC medium. For OVA loading, BMDCs were adjusted to $1x10^6$ cells/ml and incubated with 0.25 µg/ml $OVA^{323–339}$ for 45 min at 37°C. OVA pulsed DCs were washed once in DC media before adding to the T cells. Cells were incubated in 96-well round bottom plates for 48 h at 37°C before staining for flow cytometry. As negative controls for proliferation assays, non-OVA pulsed BMDCs were cultured with CFSE labeled T cells. For *in vitro* T-cell polarization, no CFSE staining of $CD4^+CD62L^+$ T cells was performed. Antigen-dependent polarizations were performed for 3 days at 37°C in presence of 5 ng/ml and 20 ng/ml rmC5a, human FXIIaα or left untreated.

3.2.5.5 Antigen independent T-cell polarization

Naïve $CD4^+$ T cells isolated from C57Bl6/J mice were stimulated with anti-mouse CD3 (2.5 µg/ml, BD Biosciences)/anti-mouse CD28 antibodies (1 µg/ml, BD Biosciences) in presence of rmC5a (5 ng/ml or 20 ng/ml) or human FXIIaα (100 ng/ml) for 3 days in DC medium. T-cell polarization was analyzed by flow cytometry.

3.2.6 Nucleic acid preparation and visualization

3.2.6.1 Preparation of tissues for RNA isolation

Cultured cells were pelleted for 10 min at 350 g, flash frozen in liquid nitrogen and stored at -80 °C for RNA isolation. Tissues (spleen, lymph nodes or aortas) were isolated from sacrificed mice after trans-cardiac reperfusion with PBS, flash frozen in liquid nitrogen and stored at -80 °C till processed.

Frozen tissues were ground to powder in presence of liquid nitrogen using mortar and pistil. Ground tissue was transferred into 1.5 ml reaction tubes and RNA isolation was performed according to the manufacturer´s instructions.

3.2.6.2 RNA isolation

Total RNA was isolated using RNeasy mini, micro (for aortas) or NucleoSpin RNA II kit according to the manufacturer´s instructions. RNA concentration and purity was determined using a Nanodrop 2000 spectrometer.

3.2.6.3 Reverse transcription (RT) and quantitative Real-Time PCR (qPCR)

cDNA synthesis was performed with the first strand cDNA synthesis kit using oligo dT primers according to the manufacturer´s instructions.

Quantitative PCR (qPCR) was performed using 20 ng cDNA per reaction and gene specific primer sets at a final concentration of 200-500 nM in a 10 µl reaction mixture including maxima SYBR green/ROX qPCR master mix. Primer standard curves with serial dilutions of cDNA were included to verify primer efficiencies. Moreover, non-template controls were included to exclude buffer or primer contaminations.

The qPCR included an initial activation step of 15 min at 95˚C, followed by 45 cycles of 15 sec denaturation at 95˚C, 30 sec hybridisation at 60˚C or 55°C, dependent on the gene specific primer sets used, and 30 sec elongation at 72˚C. For melting curve analysis, subsequent steps of 15 sec at 95˚C, 15 sec at 60˚C and again 15 sec at 95˚C were performed. Results were analyzed using the ΔΔCt method[345].

3.2.6.4 Isolation of genomic tail DNA

Mouse tails were incubated in 190 µl tail lysis buffer supplemented with 10 µl Proteinase K (1 mg/ml) for 5-6 h or o/n in a thermo block adjusted to 56°C and 1400 rpm. After digestion, samples were centrifuged for 10 min at 20,000 g and SN was transferred into a fresh 1.5 ml reaction tube. Genomic DNA was precipitated by adding 300 µl tail lysis buffer, 50 µl 3M NaOAc and 450 µl isopropanol. Genomic DNA was pelleted by centrifugation at 20,000 g for 30 min. SN was discarded and genomic DNA was washed by adding 500 µl 70 % EtOH. After another centrifugation step at 20,000 g for 20 min, SN was discarded and pellet was air-dried. DNA was resolved in 75 µl 10 mM Tris, pH 8.0. DNA concentration and purity was measured, DNA was adjusted to 150 ng/µl and stored at 4°C for consecutive genotyping.

3.2.6.5 Genotyping PCR

For genotyping, reactions were set up in PCR-tube strips in a final volume of 25 µl including 2x PCR master mix, 0.5 µM of each primer and 3 µl genomic DNA. The reactions were filled up to 25 µl with nuclease-free water. The PCR programs for the OTII, apoE and F12 genotyping and the expected band sizes were as follows:

OTII

step	temperature	duration	cycle
1	94°C	3‘	no
2	94°C	30‘‘	35x
3	56°C	1‘	
4	72°C	1‘	
5	72°C	1‘	no
6	10°C	∞	no

TCRα: approximately 160 bp

TCRβ: approximately 500 bp

ApoE

step	temperature	duration	cycle
1	94°C	3‘	no
2	94°C	30‘‘	35x
3	68°C	30‘‘	
4	72°C	40‘‘	
5	72°C	4‘	no
6	10°C	∞	no

$ApoE^{+/+}$: approximately 155 bp
$ApoE^{-/-}$: approximately 248 bp

F12

step	temperature	duration	cycle
1	96°C	4‘	No
2	94°C	30‘‘	35x
3	58°C	30‘‘	
4	72°C	45‘‘	
5	72°C	5‘	No
6	10°C	∞	No

$F12^{+/+}$: approximately 842 bp
$F12^{-/-}$: approximately 492 bp

PCR products were analyzed on 1.5 % agarose gels containing 13 µl Midori Green/ 250 ml gel volume. Gels were run at 140 V for approximately 70-100 min depending on the expected band sizes. Bands were visualized under UV-light using a gel documentation system.

3.2.7 Data analysis

Flow cytometry data was analyzed in Treestar FlowJo 10.0.4 (Ashland, USA). PCR data was analyzed in Life Technologies' Sequence Detection System (SDS) 2.4.1 (Darmstadt, Germany). Quantification of atherosclerotic lesion area and immunohistochemical analyses were performed using the image analysis software Diskus (Königswinter, Germany).

Statistical analyses were performed in GraphPad Prism 5.02 (La Jolla, USA). Data represent mean ± SEM and were analyzed by Student's t-test, ANOVA with Tukey´s or Dunn`s multiple comparison test, non-parametric Mann-Whitney test or Kruskal-Wallis test with Dunn's post-hoc test, as appropriate using GraphPad Prism 5.02 software (La Jolla USA). Differences with $p<0.05$ were considered to be statistically significant.

4. Results

4.1. Analysis of atherosclerotic lesion progression in *apoE*$^{-/-}$ mice deficient in FXII

4.1.1 General characterization

To investigate the contribution of coagulation factor XII in atherosclerotic lesion development, age and sex matched *F12*$^{-/-}$*apoE*$^{-/-}$ and *F12*$^{+/+}$*apoE*$^{-/-}$ mice were placed on atherogenic diet for 12 weeks as described in the material methods part. When comparing spleen or lymph node numbers between the two groups, no differences were observed **(Figure 4.1 a,b)**, showing that lymphoid organs were not altered in size.

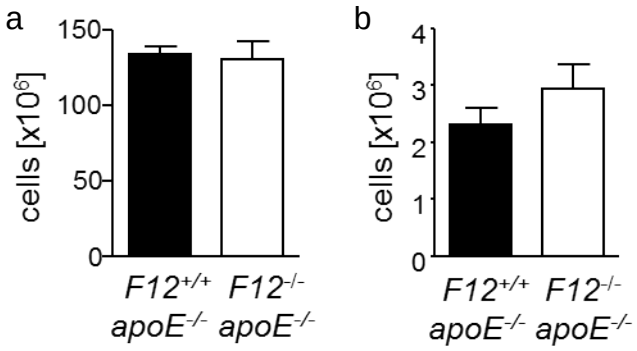

Figure 4.1. Size of secondary lymphoid organs in *F12*$^{+/+}$*apoE*$^{-/-}$ and *F12*$^{-/-}$*apoE*$^{-/-}$ mice after 12 weeks of high fat diet.
Spleen (a) and lymph node (b) size, assessed by cell counts of spleen and para-aortic lymph nodes from atherosclerotic *F12*$^{+/+}$*apoE*$^{-/-}$ and *F12*$^{-/-}$*apoE*$^{-/-}$ mice after 12 weeks of high fat diet. Bars represent mean ± SEM (n=6-10).

Since diet-induced atherosclerosis is accompanied by hyperlipidemia, total cholesterol and triglyceride levels were determined in serum samples of *F12*$^{-/-}$*apoE*$^{-/-}$ and *F12*$^{+/+}$*apoE*$^{-/-}$ mice revealing no difference between groups. Body weight was also unaltered **(Table 4.1)**.

Table 4.1 Lipid levels and body weight

	F12$^{+/+}$*apoE*$^{-/-}$	*F12*$^{-/-}$*apoE*$^{-/-}$
Serum cholesterol [µg/ml]	7033.0 ± 513.9	6810.0 ± 637.1
Serum triglycerides [µmol/L]	817.4 ± 77.43	988.6 ± 133.4
Body weight [g]	22.5 ± 0.5	24.4 ± 0.9

Total serum cholesterol, serum triglycerides and body weight of atherosclerotic *F12*$^{+/+}$*apoE*$^{-/-}$ and *F12*$^{-/-}$*apoE*$^{-/-}$ mice after 12 weeks of high fat diet. Values are expressed as mean ± SEM.

4.1.2. Plaque characterization

The extent of lesion formation was assessed by analyzing lesion area in serial aortic root sections as well as in *en face* aortas. Compared to *F12*$^{+/+}$*apoE*$^{-/-}$ controls, *F12*$^{-/-}$*apoE*$^{-/-}$ mice displayed a significant reduction in the total plaque area as well as the percent plaque area in the aortic root **(Figure 4.2a, data not shown)**. Atherosclerotic lesion area (as percent of total aortic surface area) was also observed to be significantly decreased in the aortas of *F12*$^{-/-}$*apoE*$^{-/-}$ mice **(Figure 4.2b)**, clearly demonstrating that FXII promotes atherosclerotic lesion formation.

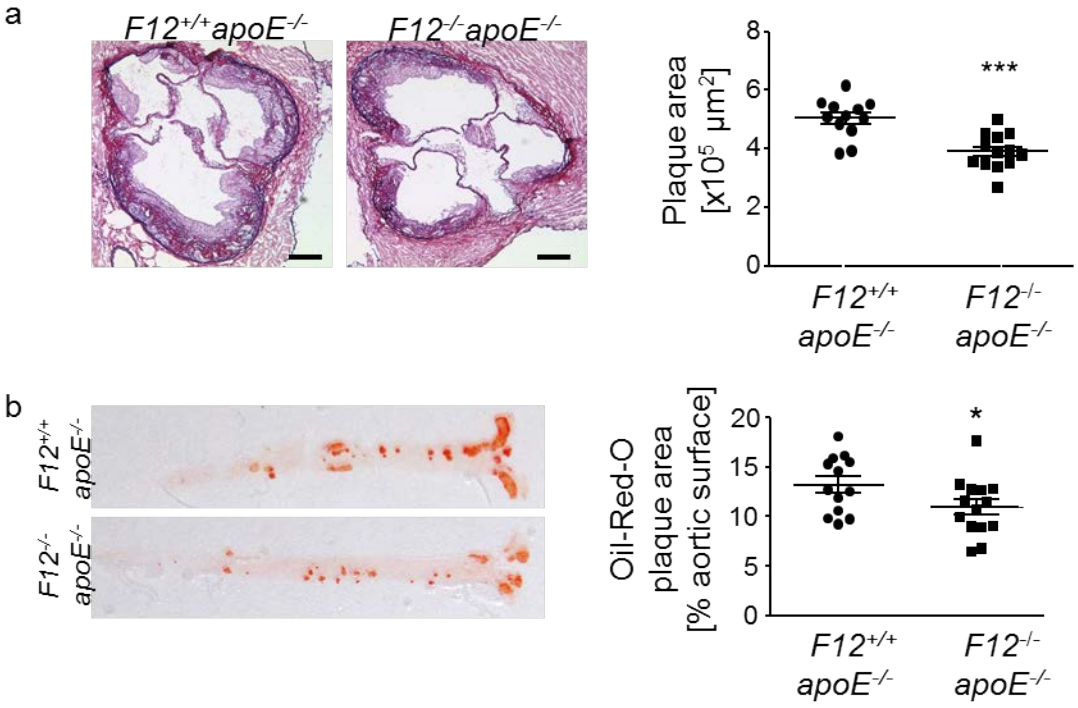

Figure 4.2. Deficiency of FXII in *apoE*$^{-/-}$ mice reduces atherosclerotic lesion formation. Atherosclerotic lesion formation was analyzed in *F12*$^{+/+}$*apoE*$^{-/-}$ and *F12*$^{-/-}$*apoE*$^{-/-}$ mice fed a high fat diet for 12 weeks. (a) Quantification of plaque area in Aldehyde-Fuchsin stained aortic root sections (representative sections are shown, scale bars, 200 µm) and (b) in Oil-Red-O stained aortas (representative aortas are shown). Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001

When analyzing potential differences in cellular plaque composition by quantitative immuno-staining, no changes in relative Mac-2^{+} macrophage area, relative α-actin^{+} smooth muscle cell area, CD3^{+} T cell numbers, relative collagen area or acellular necrotic core area in aortic root sections were detected in *F12*$^{-/-}$*apoE*$^{-/-}$ mice compared to *F12*$^{+/+}$*apoE*$^{-/-}$ controls **(Figure 4.3c-g)**, indicating an overall unaltered plaque phenotype.

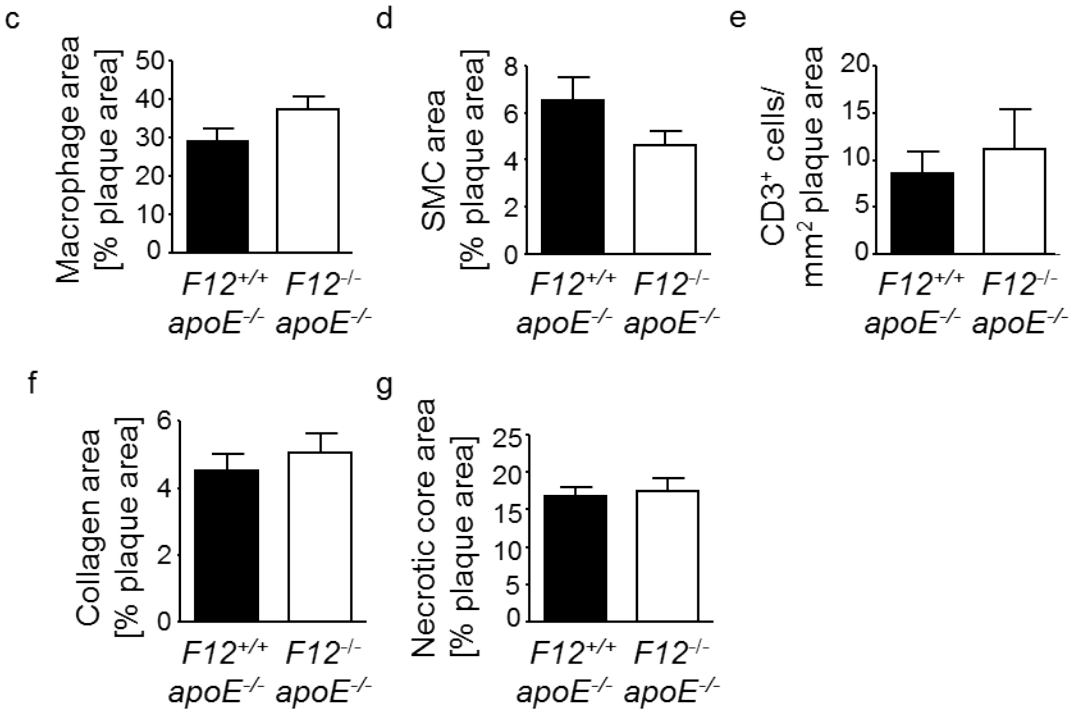

Figure 4.3. Deficiency of FXII in *apoE*$^{-/-}$ mice reduces atherosclerotic lesion formation. (c) Quantification of the Mac-2^{+} macrophage area, (d) α-smooth muscle actin^{+} smooth muscle cell area, (e) CD3^{+} T cells numbers, (f) Sirius-red^{+} collagen area and (g) acellular necrotic core area in aortic root sections. Data represent mean ± SEM.

It is well known that vascular adhesion molecules become up-regulated upon endothelial activation in the onset of atherosclerosis. Since vascular adhesion molecules play an important role in the recruitment of immune cells to atherosclerotic lesion sites and hence in the development and progression of atherosclerosis[346-348], mRNA expression of the typical vascular adhesion molecules *Vcam1*, *Icam1*, *Pecam1* and *Pselectin* was analyzed in the aortas of *F12*$^{-/-}$*apoE*$^{-/-}$ and corresponding controls, exposing no alterations between groups **(Figure 4.4)** which is in line with the observed absence of an altered plaque phenotype.

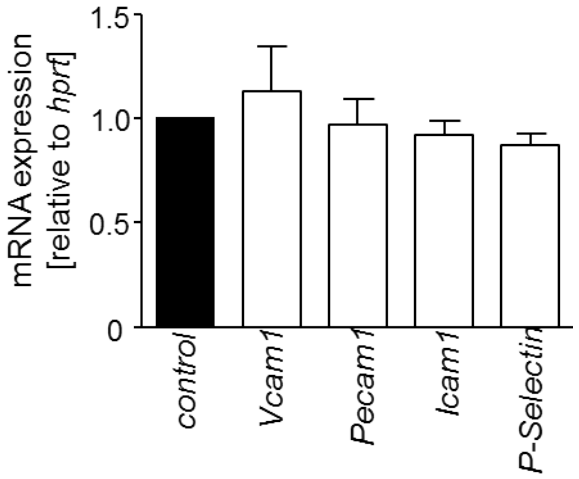

Figure 4.4. Deficiency of FXII in *apoE*$^{-/-}$ mice does not alter adhesion molecule expression in aortas.
mRNA expression of cell adhesion molecules *Vcam1*, *Pecam1*, *Icam1* and *Pselectin* in aortas of *F12*$^{-/-}$*apoE*$^{-/-}$ mice (white bars) compared to *F12*$^{+/+}$*apoE*$^{-/-}$ controls (black bar) after 12 weeks of high fat diet (normalized to *hprt* and expressed relative to control, data represent mean ± SEM, n=4-5

4.1.3 Analysis of the immunological phenotype in *apoE*$^{-/-}$ mice deficient in FXII

To characterize the immunological phenotype of these mice in the context of atherosclerosis, flow cytometric analyses of various immune cell types including cells from the innate (monocytes, neutrophils) as well as the adaptive (B cells, T cells) immune system were performed in blood and secondary lymphoid organs.

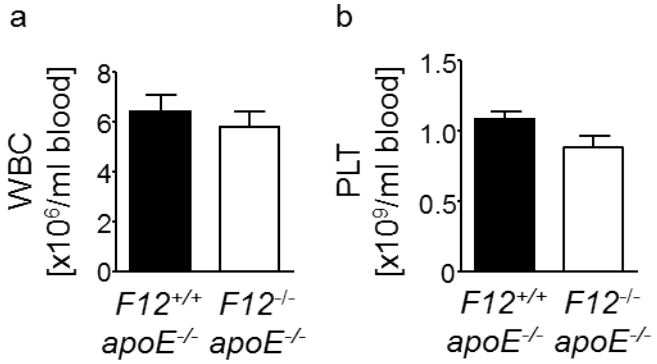

Figure 4.5. *ApoE*$^{-/-}$ mice deficient in FXII show no alteration in circulating leucocyte or platelet count.
(a) White blood cell (WBC) and (b) platelet (PLT) counts in whole blood, assessed by Sysmex measurement. Data represent mean ± SEM, n=6-8

No changes in circulating leukocyte or platelet counts were observed **(Figure 4.5a,b)** and frequencies of circulating $CD115^{+}CD11b^{+}$ monocytes, $CD115^{-}CD11b^{+}Gr\text{-}1^{high}$ neutrophils or $B220^{+}$ B cells did not differ between groups **(Figure 4.6a-d)**.

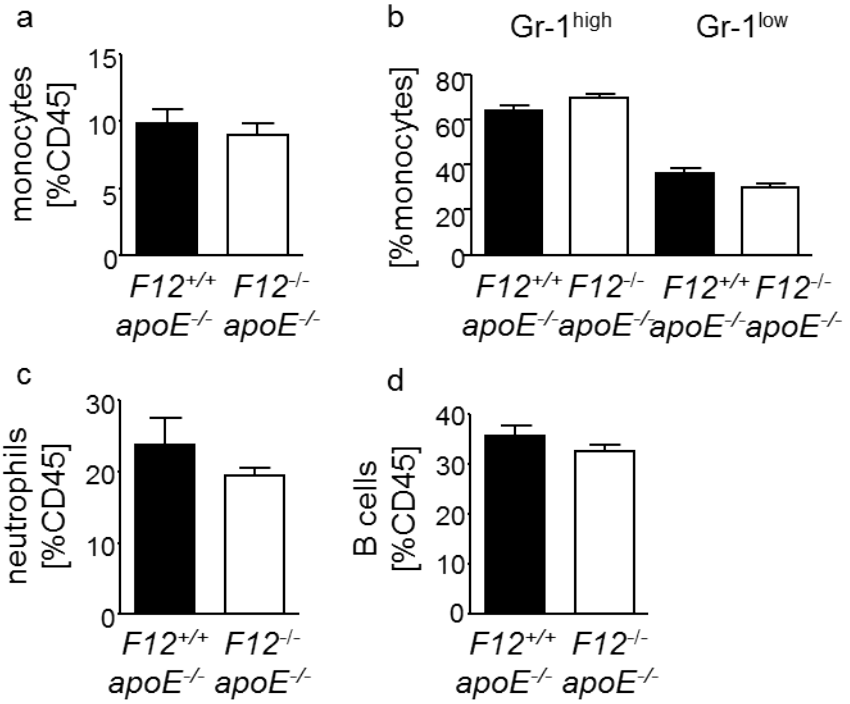

Figure 4.6. *ApoE*$^{-/-}$ mice deficient in FXII show no alteration in frequencies of circulating monocytes, neutrophils or B cells.

Flow cytometric analyses in blood (a-d) of atherosclerotic *F12*$^{+/+}$ *apoE*$^{-/-}$ (n=9) and *F12*$^{-/-}$ *apoE*$^{-/-}$ (n=6) mice fed a high fat diet for 12 weeks. Frequencies of $CD11b^{+}CD115^{+}$ monocytes (a); $Gr\text{-}1^{high}$ and $Gr\text{-}1^{low}$ subsets among $CD11b^{+}CD115^{+}$ monocytes (b); $CD11b^{+}CD115^{-}$ neutrophils (c) and $B220^{+}$ B cells (d) were quantitated. Data represent mean ± SEM.

The analysis of T-cell distribution and phenotype in organs of *F12*$^{-/-}$*apoE*$^{-/-}$ mice compared to *F12*$^{+/+}$*apoE*$^{-/-}$ controls in spleen and blood revealed no substantial alterations in frequencies or numbers of CD3^{+} T cells **(Figure 4.7a,d)** or CD4^{+} and CD8^{+} T cell frequencies among CD3^{+} T cells **(Figure 4.8b,e)**, whereas in lymph nodes a small but significant increase in CD3^{+} T cell frequencies **(Figure 4.7h)** was evidenced comprising an increase in CD4^{+} as well as a decrease in CD8^{+} T cells **(Figure 4.7i)**.

In addition, T-cell activation and polarization were included in the analysis, since both of them were shown to promote atherogenesis[2]. In terms of T-cell activation all organs analyzed displayed a significant decrease in frequencies of CD44^{hi}CD62L^{lo} memory effector T cells as well as an increase in CD62L^{hi}CD44^{lo} naïve T cell frequencies among CD4^{+} T cells **(Figure 4.7c,f,g,j,k)**.

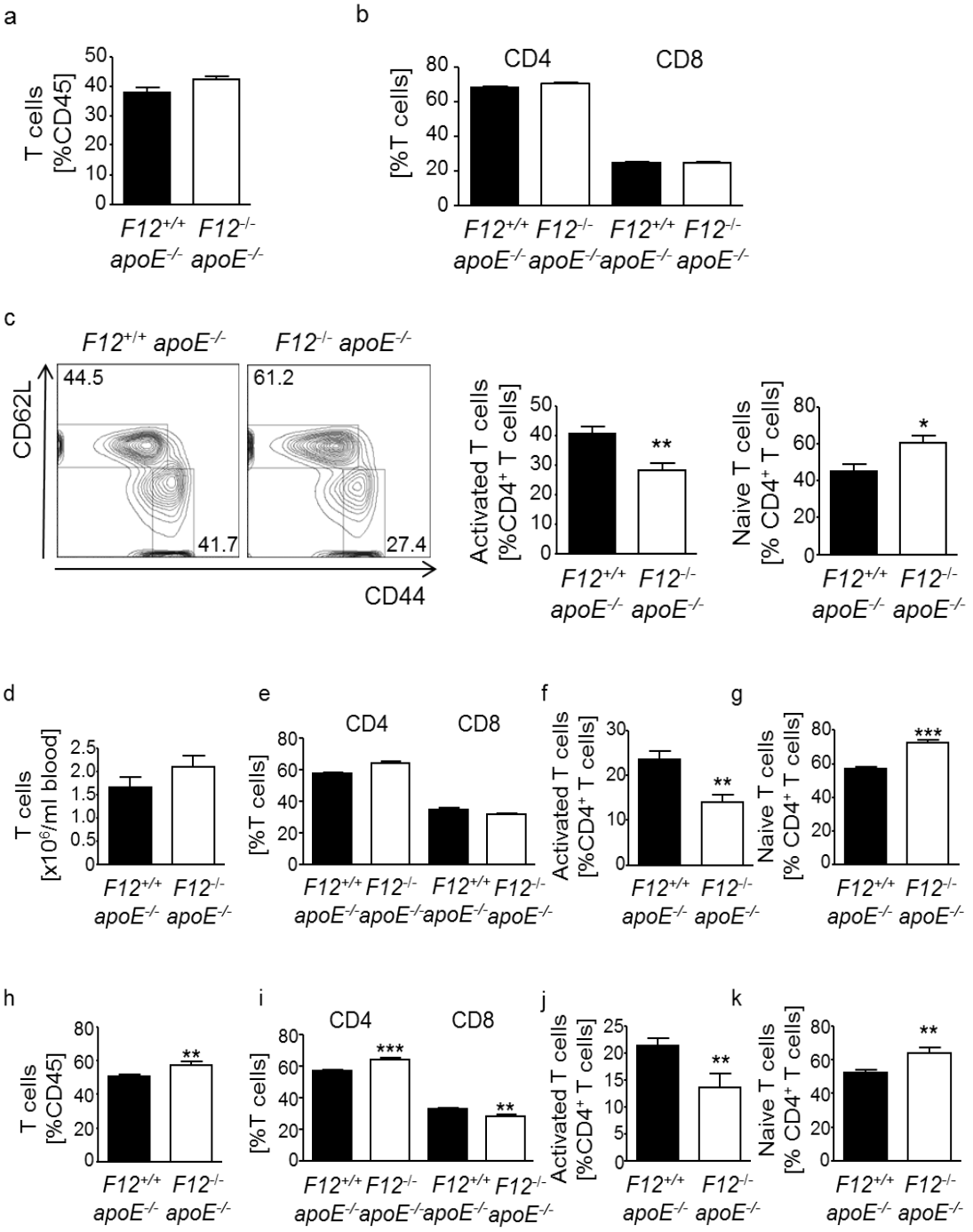

Figure 4.7. *F12*$^{-/-}$*apoE*$^{-/-}$ mice display reduced T-cell activation
Flow cytometric analyses of T cell distributions in spleens (a-c), blood (d-g) and lymph nodes (h-k) of atherosclerotic *F12*$^{+/+}$*apoE*$^{-/-}$ (n=9-10) and *F12*$^{-/-}$*apoE*$^{-/-}$ mice (n=6) after 12 weeks of high fat diet feeding. Frequencies or numbers of (a,d,h) CD3^{+} T cells among CD45^{+} leukocytes, (b,e,i) CD4^{+} and CD8^{+} T cells among CD3^{+} T cells, (c,f,j) activated CD44^{high}CD62L^{low} and (c,g,k) naïve CD62L^{high}CD44^{low} CD4^{+} T cells (representative dot plots are shown). Data represent mean ± SEM; *p<0.05, **p<0.01, ***p<0.001.

Furthermore, T helper cell subsets were assessed by intracellular staining revealing a substantial reduction in IFNγ^{+} Th1 cells among the CD4^{+} population in spleen, blood and lymph nodes of *F12*$^{-/-}$*apoE*$^{-/-}$ mice when compared *F12*$^{+/+}$*apoE*$^{-/-}$ controls **(Figure 4.8a,d,g)**. Frequencies of IL-17^{+} Th17 cells tended to be decreased without reaching statistical significance **(Figure 4.8a,c,f)**, and no alterations in Foxp3^{+}CD25^{+} regulatory T cells were observed between groups **(Figure 4.8b,e,h)**. These data demonstrate that protracted atherosclerotic lesion formation in *F12*$^{-/-}$*apoE*$^{-/-}$ mice is associated with diminished T-cell activation and Th1 cell polarization.

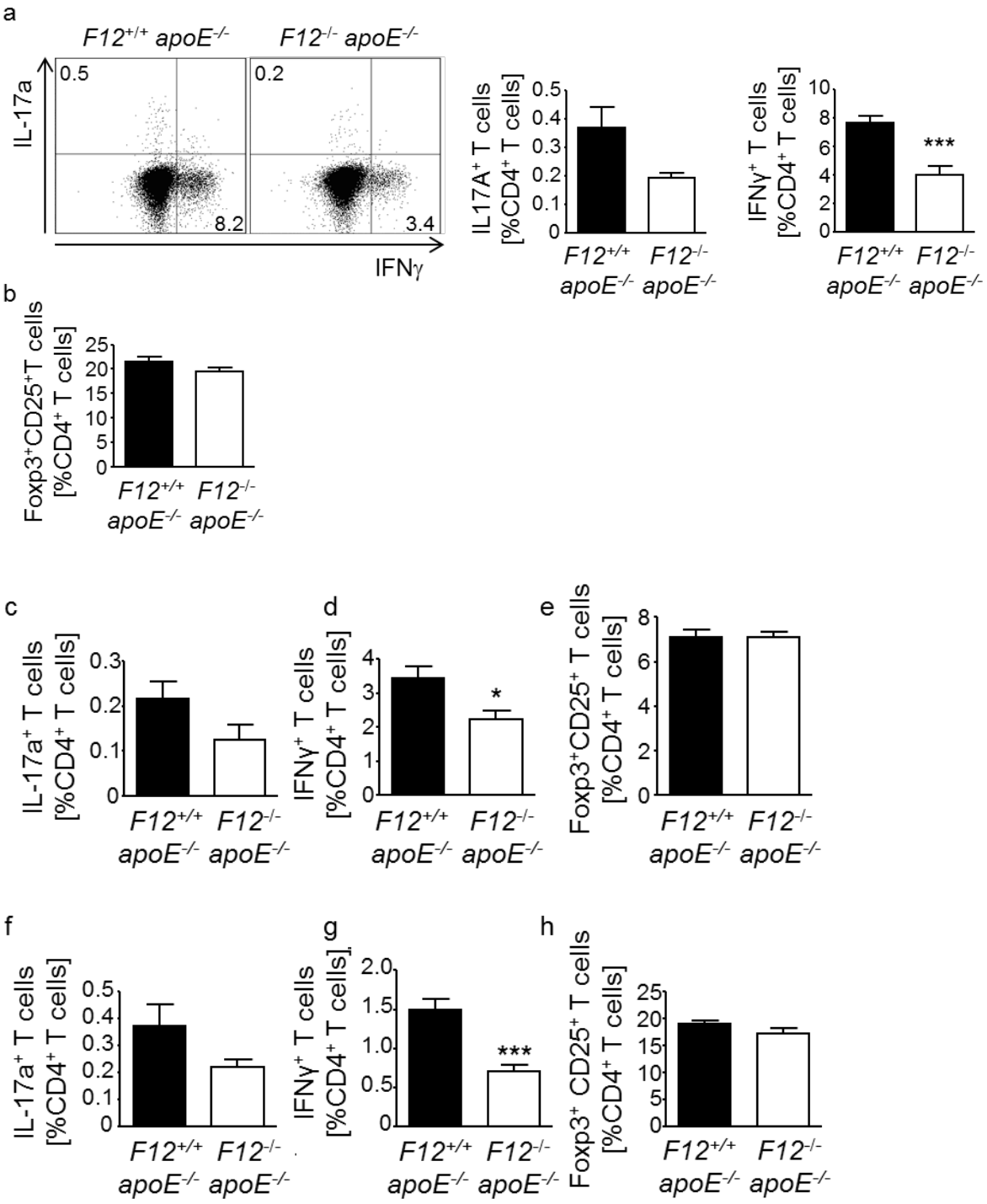

Figure 4.8. *F12*$^{-/-}$*apoE*$^{-/-}$ mice display reduced Th1-polarization.
Flow cytometric analyses of T cell distributions. Frequencies of (a,d,g) IFNγ^{+} and (a,c,f) IL-17a^{+} CD4^{+} T cells (representative dot plots are shown), and of (b,e,h) Foxp3^{+}CD25^{+} CD4^{+} T cells in spleens (a-b), blood (c-e) and lymph nodes (f-h) of atherosclerotic *F12*$^{+/+}$*apoE*$^{-/-}$ (n=8-10) and *F12*$^{-/-}$*apoE*$^{-/-}$ mice (n=6) after 12 weeks of high fat diet feeding. Data represent mean ± SEM; *p<0.05, **p<0.01, ***p<0.001.

4.2 Analysis of mice deficient in FXII under non-inflammatory homeostatic conditions

4.2.1 General characterization

The marked reduction in T-cell activation and polarization seen in *F12*$^{-/-}$*apoE*$^{-/-}$ mice under atherosclerotic conditions raised the question whether FXII may already influence the T cell phenotype in a non-inflammatory homeostatic setting. Therefore, healthy *F12*$^{-/-}$ mice on normal chow were compared to *B6* controls to analyze the mice under physiological conditions.

Analogous to pro-atherogenic conditions, neither changes in circulating leucocyte or platelet counts nor alterations in the size of lymphoid organs between groups were detected **(Figure 4.9)**.

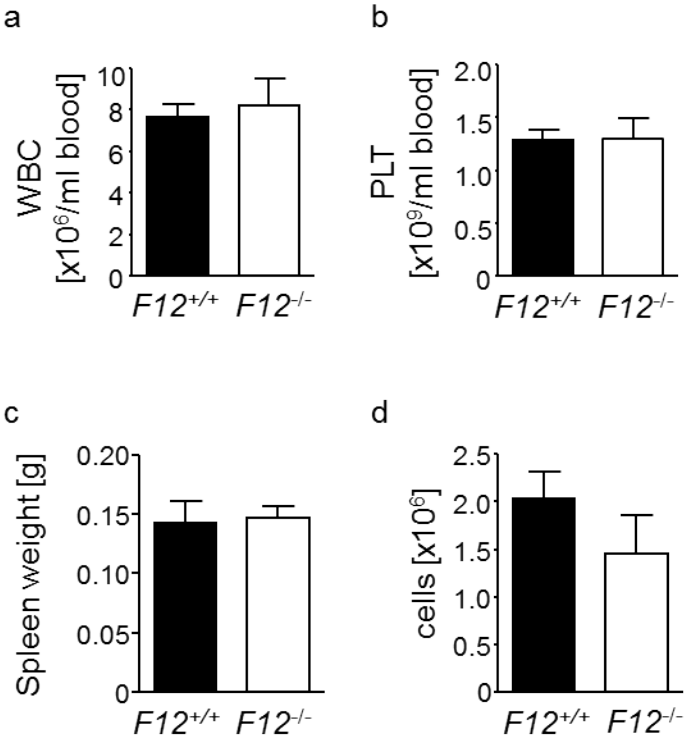

Figure 4.9. Circulating leucocyte and platelet counts and size of secondary lymphoid organs in *F12*$^{+/+}$ and *F12*$^{-/-}$ mice on normal chow
(a) White blood cell (WBC) and (b) platelet (PLT) counts in whole blood, assessed by Sysmex measurement. Spleen (c) and lymph node (d) size, assessed by spleen weight and absolute counts of para-aortic lymph nodes from healthy *F12*$^{+/+}$ and *F12*$^{-/-}$ mice on normal chow. Bars represent mean ± SEM (n=4-5).

In line with findings under atherosclerotic conditions in mice on *apoE*$^{-/-}$ background, the frequencies of circulating CD115^{+}CD11b^{+} monocytes, CD115^{-} CD11b^{+}Gr-1^{high} neutrophils or CD19^{+} B cells did not differ between groups **(Figure 4.10)**.

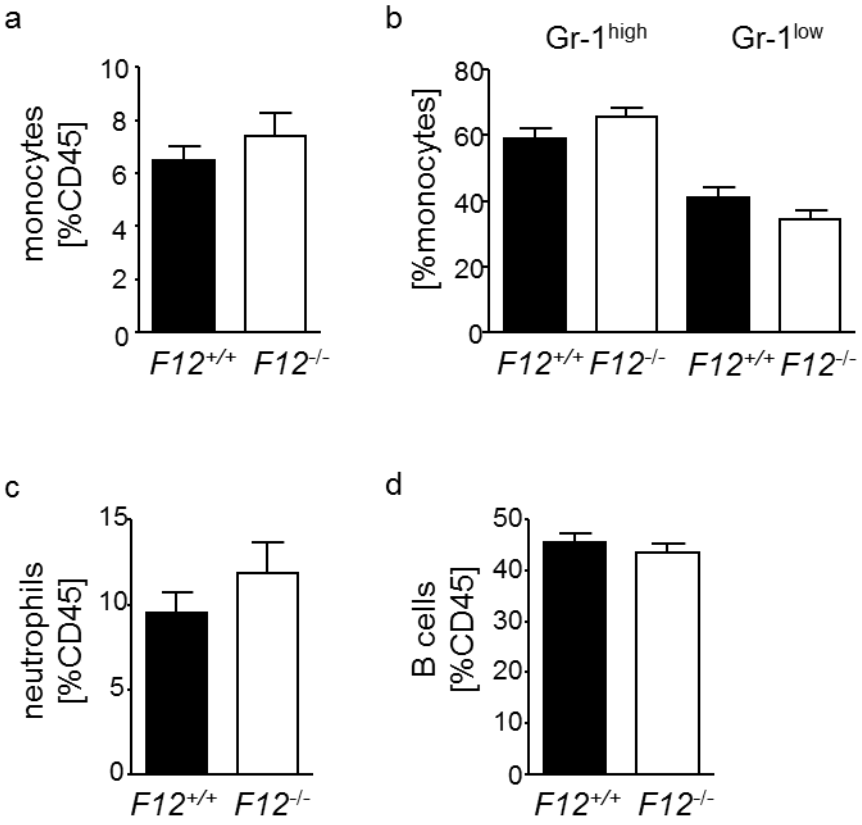

Figure 4.10. Mice deficient in FXII show no alterations in frequencies of circulating monocytes, neutrophils or B cells.

Flow cytometric analyses in blood (a-d) of healthy *F12*$^{+/+}$ (n=5) and *F12*$^{-/-}$ (n=5) mice on normal chow. Frequencies of CD11b^{+}CD115^{+} monocytes (a); Gr-1^{high} and Gr-1^{low} subsets among CD11b^{+}CD115^{+} monocytes (b); CD11b^{+}CD115^{-} neutrophils (c) and B220^{+} B cells (d) were quantitated. Data represent mean ± SEM.

4.2.2 Analysis of the immunological phenotype of mice deficient in FXII

Again, the T-cell compartment itself was not altered with respect to $CD3^+$ T cell frequencies or numbers between the two groups in all organs analyzed. Also, the $CD4^+$ and $CD8^+$ frequencies among $CD3^+$ T cells were unchanged, with exception to a small but significant decrease in $CD8^+$ T cell frequencies in the lymph nodes of *F12*$^{-/-}$ mice when compared to *B6* controls **(Figure 4.11)**.

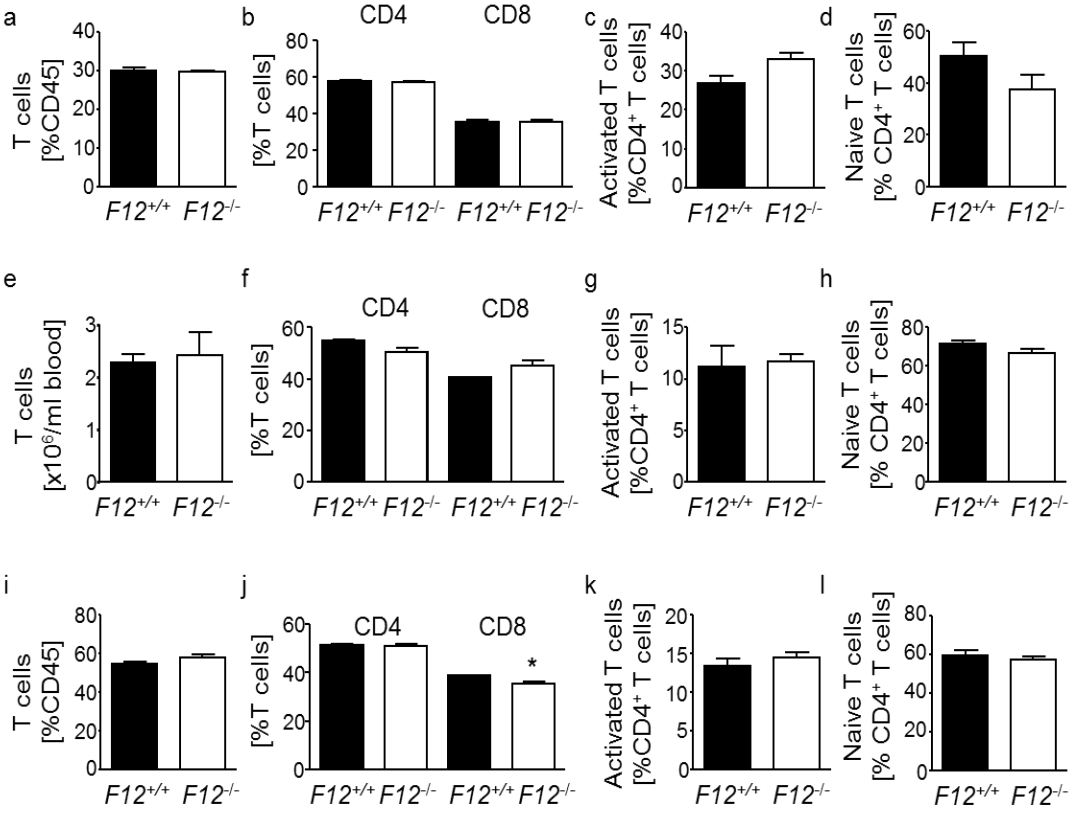

Figure 4.11. Deficiency of FXII does not alter T-cell activation under homeostatic conditions.
Flow cytometric analyses of T cell distributions in spleens (a-d), blood (e-h) and lymph nodes (i-l) of healthy *F12*$^{+/+}$ and *F12*$^{-/-}$ *mice* (n=4-5 each). Frequencies or numbers of $CD3^+$ T cells among $CD45^+$ cells (a,e,i), $CD4^+$ and $CD8^+$ T cells among $CD3^+$ T cells (b,f,j), memory effector $CD44^{high}$ $CD62L^{low}$ (c,g,k) and naïve $CD62L^{high}$ $CD44^{low}$ (d,h,l) $CD4^+$ T cells. Data represent mean ± SEM. *p<0.05

Remarkably however, no substantial changes were observed in the T-cell compartment with respect to T-cell activation **(Figure 4.11)** or polarization **(Figure 4.12)** in spleen, blood and lymph nodes when comparing healthy, chow-fed *F12*$^{-/-}$ mice versus *F12*$^{+/+}$ controls, indicating that atherosclerosis-related inflammation triggers FXII-dependent modulation of T-cell activation and polarization.

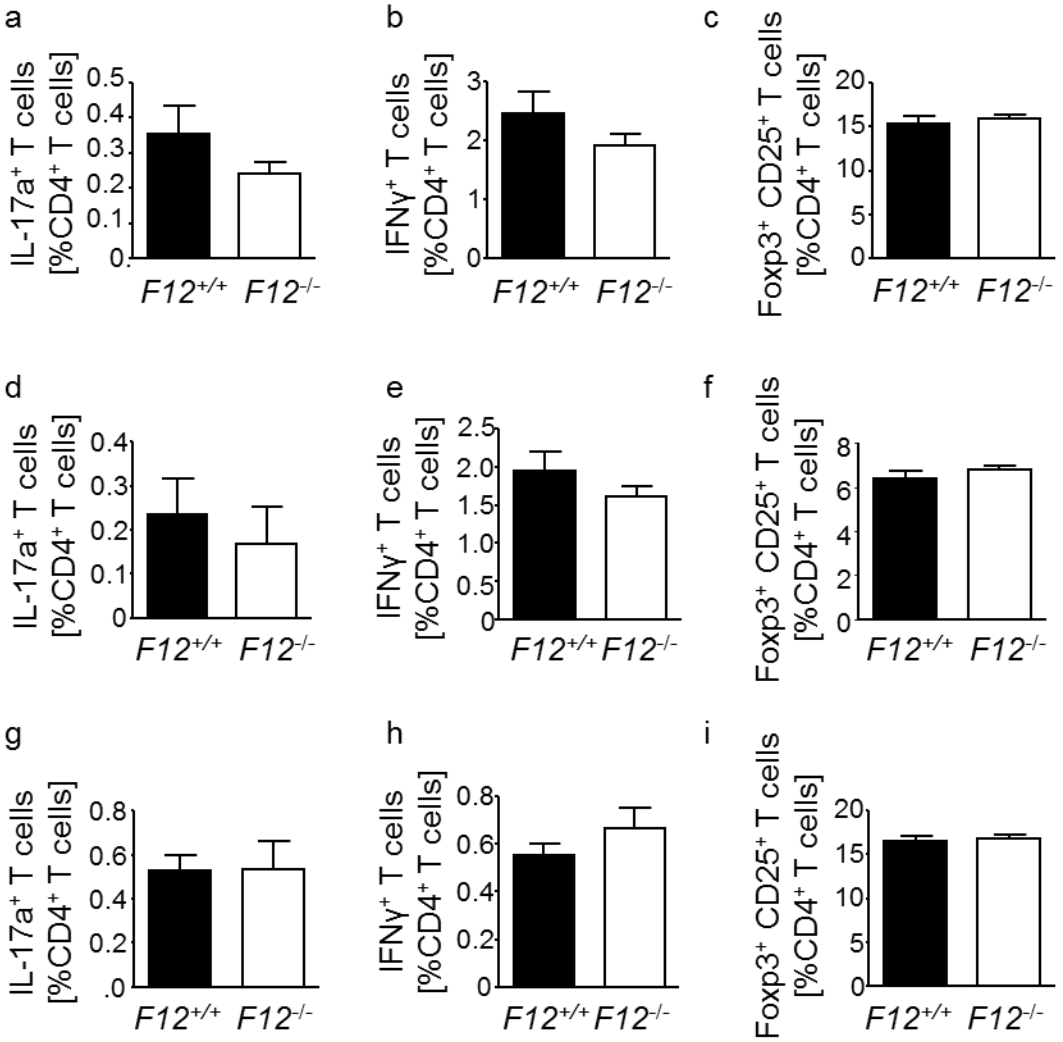

Figure 4.12. Deficiency of FXII does not alter T-cell polarization under homeostatic conditions.
Flow cytometric analyses of IL-17a^{+} CD4^{+} (a,d,g), CD4^{+} IFNγ^{+} (b,e,h) and of Foxp3^{+}CD25^{+} CD4^{+} T cells (c,f,i) in spleen (a-c), blood (d-f) and lymph nodes (g-i) of healthy *F12*$^{+/+}$ and *F12*$^{-/-}$ mice (n=4-5 each). Data represent mean ± SEM.

4.3 Analysis of direct FXII influences on DCs, T cells or macrophages

To gain insight into the mechanisms by which FXII may trigger T-cell activation, Th1 polarization as well as the aggravation in atherosclerotic lesion formation, different pathways in which FXII has been shown to play a role were further investigated.

Efficient T-cell activation and T helper cell polarization are shaped by co-stimulatory molecule engagement and exposure to a specific cytokine milieu, with the expansion of Th1 T cells critically depending on IL-12 secretion from DCs[349,350].

Since FXII was demonstrated to be capable of binding various cell types *in vitro* including VSMCs as well as macrophage-like cell lines, the first approach was to investigate whether FXII may directly affect DC activation or T-cell polarization. BMDCs generated from *C57Bl/6J* mice were therefore stimulated with different concentrations of human FXIIaα, ranging from 10 ng/ml to 3 µg/ml for 6 hours. Human FXII has been shown before to function in mice[259]. However, no changes were detected in the *Cd74* (MHCII) expression or the expression of the co-stimulatory molecules *Cd80, Cd86* and *Cd40* on mRNA level **(Figure 4.13a, data not shown)** when compared to untreated controls.

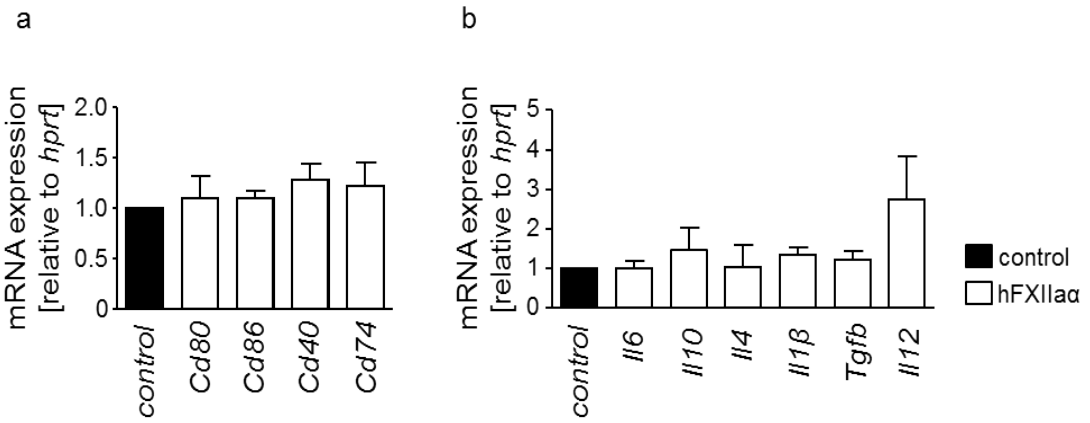

Figure 4.13. FXII does not alter DC activation or phenotype
(a) mRNA expression of co-stimulatory molecules *Cd80*, *Cd86*, *Cd40* and *Cd74* (MHCII) and (b) expression of cytokines *Il6*, *Il10*, *Il4*, *Il1b*, *Tgfb* or *Il12* in BMDCs left untreated (control) or after treatment with human FXIIaα (100 ng/ml) for 6 h (normalized to *hprt* and expressed relative to untreated controls, n=4 each). Data represent mean ± SEM.

Moreover, with the exception of *Il12,* no alterations in cytokine expression on mRNA level could be observed **(Figure 4.13b, data not shown)**. While with increasing concentration of human FXIIaα *Il12* mRNA levels were upregulated **(Figure 4.14a)**, this increase was not reflected by increased IL-12 protein expression as confirmed by ELISA from the supernatants of FXIIaα treated DCs **(Figure 4.14b)**.

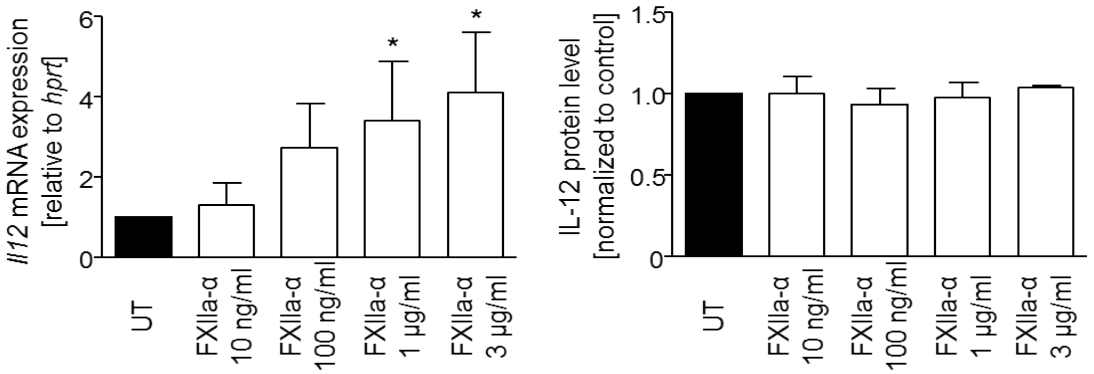

Figure 4.14. FXII does not consistently alter DC activation or phenotype
(a) *Il12* mRNA and (b) IL-12 protein expression of untreated (UT) DCs or after treatment with increasing concentrations of human FXIIaα (normalized to *hprt* and expressed relative to untreated controls, n=4 each). Data represent mean ± SEM. *p<0.05

In addition, treatment of anti-CD3/CD28-antibody stimulated naïve T cells with human FXIIaα did not reveal changes in Th1 or Th17 cell polarization **(Figure 4.15)** when compared to untreated controls.

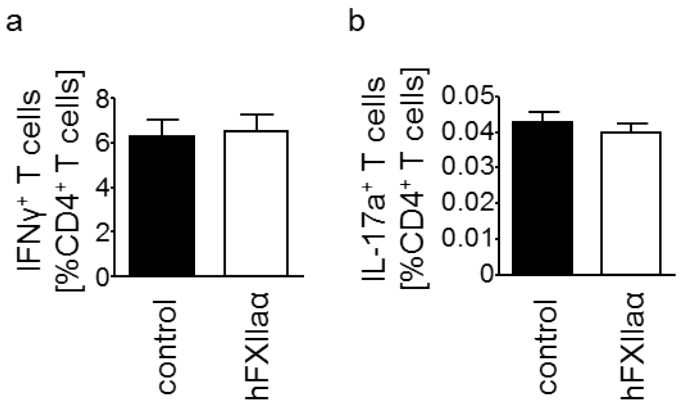

Figure 4.15. FXII does not alter T-cell polarization *in vitro*
Flow cytometric analyses of (a) IFNγ$^+$ T cells and (b) IL17a$^+$ T cells frequencies among the CD4$^+$ T cell population in anti-CD3/CD28-antibody stimulated CD4$^+$ T cells treated with hFXIIaα (100 ng/ml) or left untreated (control). Data represent mean ± SEM; n=4

Treatment of *B6* macrophages with hFXIIaα also did not result in changes in macrophage polarization, as demonstrated by unchanged mRNA levels of the M2 markers *Il10*, *Arg1*, *Mrc1* and *Igf1* as well as the M1 marker *Il6*. mRNA levels of the M1 marker *Il12* were undetectable in both groups **(Figure 4.16)**. It seems thus very unlikely that direct effects of FXII mediate altered immune cell activation.

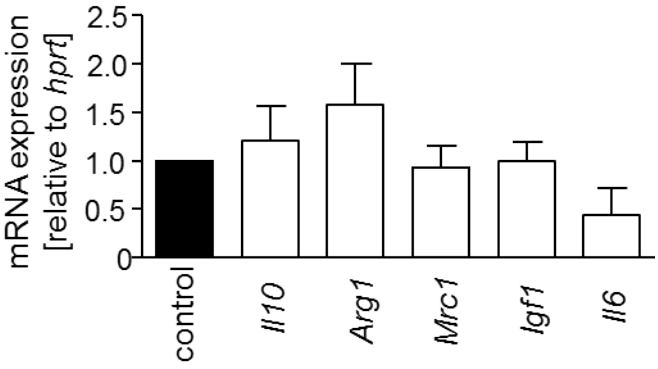

Figure 4.16. FXII does not alter macrophage polarization
mRNA expression of M2 and M1 macrophage polarization markers *Il10*, *Arg1*, *Mrc1*, *Igf1*, *Il6* in hFXIIaα (100 ng/ml) treated *B6* macrophages (white bars) compared to untreated controls (black bar) (normalized to *hprt* and expressed relative to control. Data represent mean ± SEM; n=3

4.4 Analysis of changes in FXII modulated pathways

Since FXII however is involved in several pathways besides the intrinsic pathway of coagulation, including the activation of the complement system as well as the initiation of the kallikrein-kinin system[211,228,230,351], it was crucial to explore whether FXII deficiency results in alterations of these pathways in *apoE*$^{-/-}$ mice on atherogenic diet.

Therefore, changes in the kallikrein-kinin system were assessed by quantification of bradykinin serum levels. However, no reduction in bradykinin levels in *F12*$^{-/-}$*apoE*$^{-/-}$ mice when compared to *F12*$^{+/+}$*apoE*$^{-/-}$ controls could be observed **(data not shown)**. Evaluating a possible role of FXII in complement activation, C5a plasma levels were quantitated as a readout for complement activation, revealing a significant decrease in plasma levels of complement factor C5a in *F12*$^{-/-}$*apoE*$^{-/-}$ mice compared *F12*$^{+/+}$*apoE*$^{-/-}$ controls **(Figure 4.17)**.

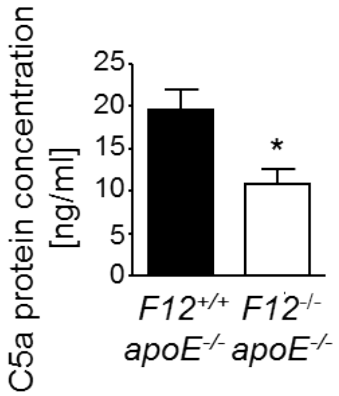

Figure 4.17. Reduced plasma levels of C5a in *F12*$^{-/-}$*apoE*$^{-/-}$ mice
C5a protein levels in plasma of *F12*$^{+/+}$*apoE*$^{-/-}$ (n=10) and *F12*$^{-/-}$*apoE*$^{-/-}$ mice (n=9) after 12 weeks of high fat diet feeding, as quantified by ELISA. Data represent mean ± SEM; *p<0.05.

4.5 Investigating the role of C5a in *F12*$^{-/-}$*apoE*$^{-/-}$ mice and in DC-mediated T cell responses

Given that complement factor C5a was previously shown to modulate immune responses[288], we verified the capacity of C5a to modulate DC-driven T-cell responses. The induction of antigen-specific IFNγ^{+} but not IL-17^{+} CD4^{+} OTII T cells was significantly enhanced in the presence of recombinant murine C5a (rmC5a) in a concentration-dependent manner, when compared to T cells exposed to OVA-loaded but otherwise untreated BMDCs **(Figure 4.18, data not shown)**, corroborating that C5a can drive Th1 T-cell polarization. Notably, these changes in T cell phenotype mirrored the marked decrease in frequencies of Th1 cells in atherosclerotic *F12*$^{-/-}$*apoE*$^{-/-}$ mice.

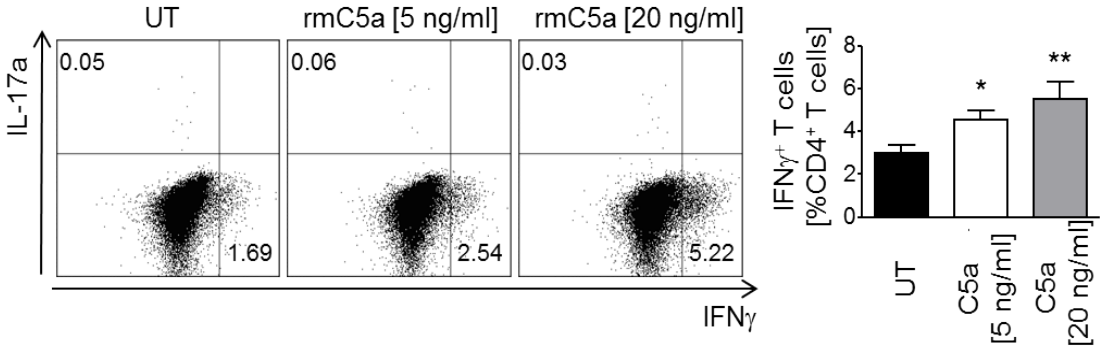

Figure 4.18. C5a functions in DC - T cell interaction.
OVA$^{323-339}$ peptide-pulsed BMDCs were co-cultured with naïve CD4^{+} OTII T cells for 3 days in the presence or absence of rmC5a at indicated concentrations. Representative dot plots of intracellular staining for IFNγ and IL-17 in CD4^{+} T cells are shown; quantification of IFNγ^{+} T cells (n=3 independent experiments performed in triplicates). Data represent mean ± SEM; *p<0.05, **p<0.01.

To address whether C5a can directly affect T-cell activation, isolated naïve T cells were stimulated with rmC5a and anti-mouse CD3/CD28 antibodies. No changes in frequencies of IFNγ^{+} Th1 or IL-17^{+} Th17 cells were detected **(Figure 4.19)**, which is in line with absence of the C5a receptor CD88 on T cells[352].

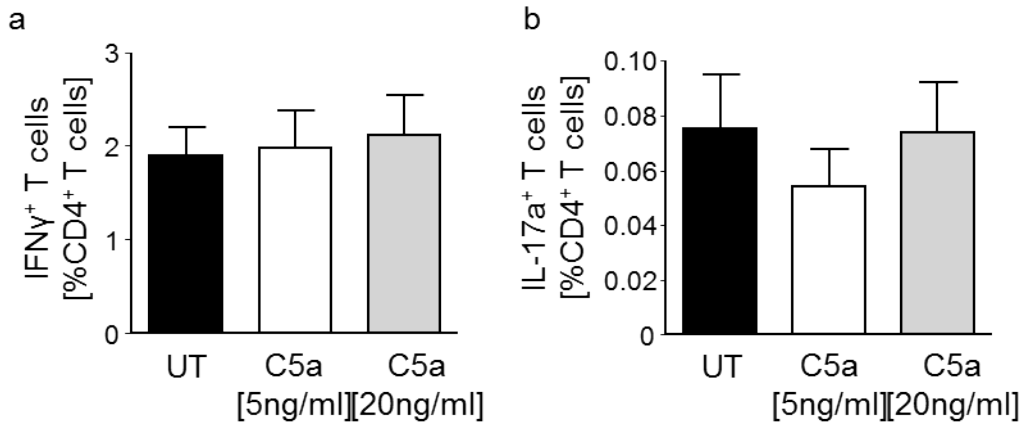

Figure 4.19. rmC5a does not directly affect T-cell polarization in absence of DCs.
Isolated naïve $CD4^+$ T cells stimulated with anti-mouse CD3/CD28 antibodies were co-cultured for 3 days in the presence or absence of rmC5a at indicated concentrations. Quantification of $IFN\gamma^+$ $CD4^+$ T cells (a) and $IL17a^+$ $CD4^+$ T cells (b), assessed by flow cytometry. Data represent mean ± SEM; n=3

Importantly, no changes in the mRNA expression of the complement C5a receptor (*Cd88*) were observed in spleen, lymph nodes or aortas between groups **(Figure 4.20)**.

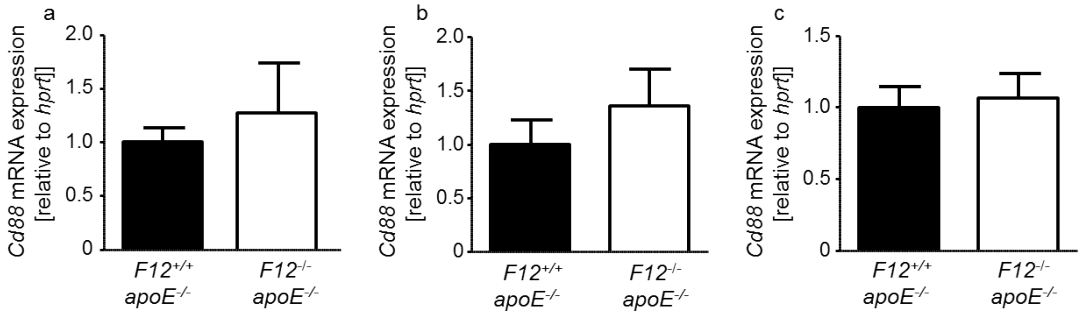

Figure 4.20. FXII deficiency does not alter *Cd88* expression
mRNA expression of *Cd88* in (a) lymph node, (b) spleen and (c) aorta of $F12^{-/-}apoE^{-/-}$ mice and $F12^{+/+}apoE^{-/-}$ controls. (normalized to *hprt*). Data represent mean ± SEM; n=4-5

However when comparing *Cd88* mRNA levels in the aortas of both groups to those of age matched wild-type *C57Bl/6J* mice a significant increase in *Cd88* gene expression compared to $F12^{-/-}apoE^{-/-}$ and $F12^{+/+}apoE^{-/-}$ on 12 weeks high fat diet was observed **(Figure 4.21)**, which is consistent with previous reports[353].

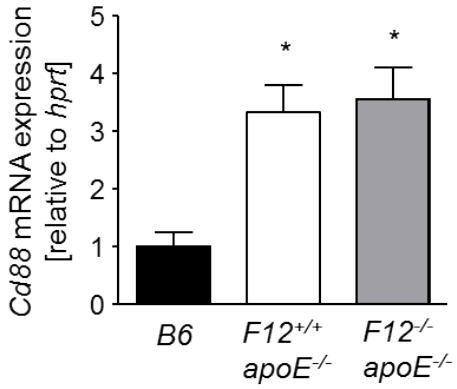

Figure 4.21. *Cd88* expression is enhanced under atherosclerotic conditions
mRNA expression of *Cd88* in aorta of age matched *B6*, *F12*$^{+/+}$*apoE*$^{-/-}$ and *F12*$^{-/-}$*apoE*$^{-/-}$ mice. (normalized to *hprt* and normalized to *B6* control) n=4-5, data represent mean ± SEM.

Efficient T-cell activation and T helper cell polarization were demonstrated to be shaped by co-stimulatory molecule engagement and exposure to a specific cytokine milieu, with the expansion of Th1 T cells critically depending on IL-12 secreted from DCs[350]. Moreover, several studies have illustrated a role for C5a and other anaphylatoxins in the modulation of adaptive immune responses, demonstrating a direct link between C5a modulated DC functions and altered T helper cell responses[288,354] .

Therefore it was investigated whether exogenous C5a stimulation may modify DC functions. Treatment of BMDCs with increasing concentrations of rmC5a did not affect co-stimulatory molecule expression by DCs, as evidenced by unaltered mRNA **(Figure 4.22a-c)** and protein **(Figure 4.22d-f)** expression of CD80, CD86 and CD40.

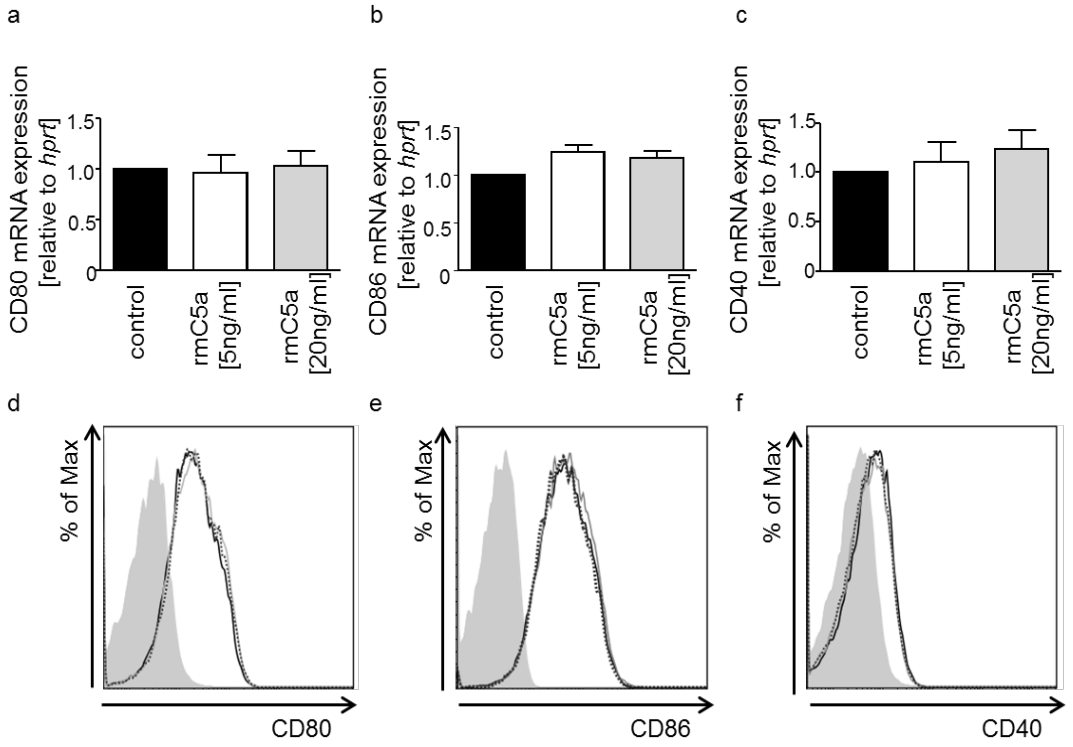

Figure 4.22. rmC5a does not alter co-stimulatory molecule expression by DCs
(a-c) mRNA expression and protein expression (d-f) of co-stimulatory molecules CD80 (a,d), CD86 (b,e), and CD40 (c,f), in BMDCs from *B6* mice left untreated (control) or after treatment with rmC5a at indicated concentrations for 12 h, analyzed by qPCR (n=5) or flow cytometry. mRNA expression was normalized to *hprt* and presented relative to untreated controls. Representative histograms for CD80, CD86 and CD40 mean fluorescence intensity (MFI) are shown (solid black line – UT, dotted dark grey line - CD80, CD86 or CD40 respectively with 5 ng/ml C5a treatment, filled dark grey line – CD80, CD86 or CD40 respectively with 20 ng/ml C5a treatment, filled grey line - fluorescence minus one control (FMO). Data represent mean ± SEM.

In line with these data, no changes in co-stimulatory molecule expression by splenic DCs were observed in atherogenic *F12*$^{-/-}$*apoE*$^{-/-}$ mice when compared to *F12*$^{+/+}$*apoE*$^{-/-}$ controls as assessed by flow cytometric measurement of CD80, CD86 and CD40 **(Figure 4.23)**.

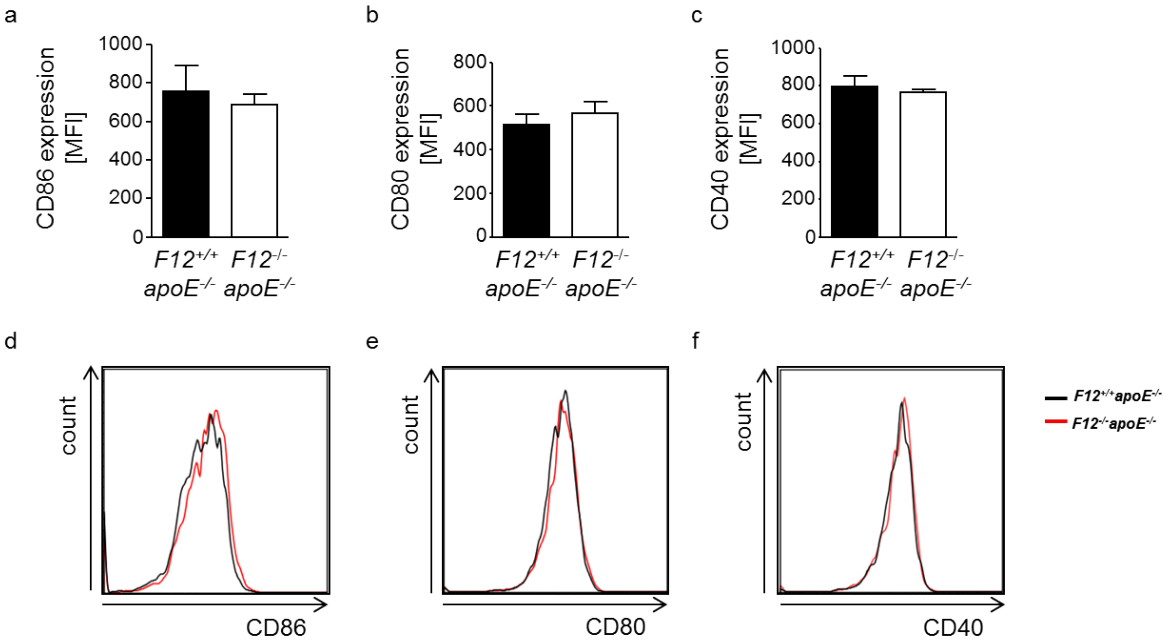

Figure 4.23. Deficiency of FXII in *apoE*$^{-/-}$ mice does not alter co-stimulatory molecule expression by DCs
Protein expression of co-stimulatory molecules CD86 (a,d), CD80 (b,e), and CD40 (c,f), in CD11c^{+}MHCII^{hi} DCs from *F12*$^{-/-}$*apoE*$^{-/-}$ mice and *F12*$^{+/+}$*apoE*$^{-/-}$ controls after 12 weeks of high fat diet assessed by flow cytometry; data represent mean ± SEM (n=3-5). Representative histograms for CD86, CD80 and CD40 mean fluorescence intensity (MFI) are shown (solid black line – *F12*$^{+/+}$*apoE*$^{-/-}$ DCs, solid red line - *F12*$^{-/-}$*apoE*$^{-/-}$.

Real time-PCR analyses of BMDCs treated with increasing concentrations of C5a however displayed a trend towards elevated mRNA expression of *Il12* (**Figure 4.24a**) and a significant increase in IL-12 protein levels in supernatants compared to untreated controls (**Figure 4.24b**), supporting the co-culture data and demonstrating that C5a induces secretion of IL-12 by DCs.

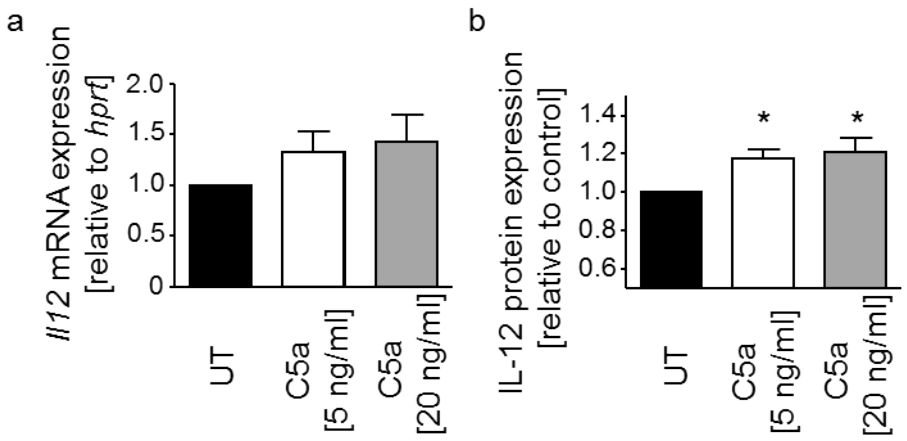

Figure 4.24. Increase in IL-12 levels in BMDCs upon C5a stimulation
(a) *Il12* mRNA expression in BMDCs left untreated (controls) or stimulated with C5a for 12 h (n=4 each), normalized to *Hprt* and expressed relative to controls. (b) IL-12 protein expression in supernatants of untreated (UT) or C5a-stimulated BMDCs after 12 h, normalized to untreated controls. Data represent mean ± SEM; *p<0.05; n=5

4.6 Analysis of IL-12 levels and other selected cytokines in *apoE*$^{-/-}$-mice deficient in FXII

Notably, a significant reduction in IL-12 expressing splenic CD11c^{+}MHCII^{+} DCs from atherosclerotic *F12*$^{-/-}$*apoE*$^{-/-}$ mice compared to *F12*$^{+/+}$*apoE*$^{-/-}$ mice fed a high fat diet for 12 weeks was detected (**Figure 4.25**). No alterations in the frequencies of CD11c^{+}MHCII^{hi} DCs among CD45^{+} cells between the groups were observed **(data not shown)**.

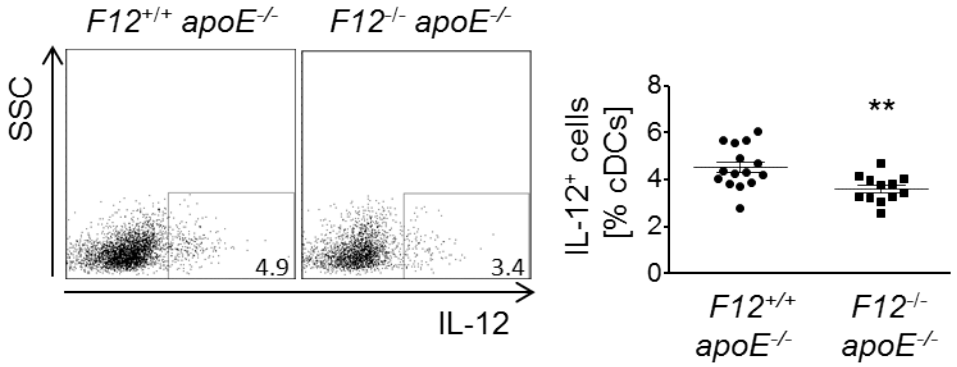

Figure 4.25. Deficiency in FXII in *apoE*$^{-/-}$ mice entails a reduction in IL-12 expression
(a) Frequencies of IL12^{+} cDCs in the spleen of *F12*$^{+/+}$*apoE*$^{-/-}$ (n=15) and *F12*$^{-/-}$*apoE*$^{-/-}$ mice (n=12) after 12 weeks of high fat diet feeding, as assessed by flow cytometry; representative dot plots are shown. Data represent mean ± SEM; **p<0.01

The decrease in IL-12^{+} DCs was paralleled by a clear decline in *Il12* mRNA expression in spleens of *F12*$^{-/-}$*apoE*$^{-/-}$ compared to *F12*$^{+/+}$*apoE*$^{-/-}$ mice (**Figure 4.26a**), together with a marked reduction in IL-12 serum levels (**Figure 4.26b**).

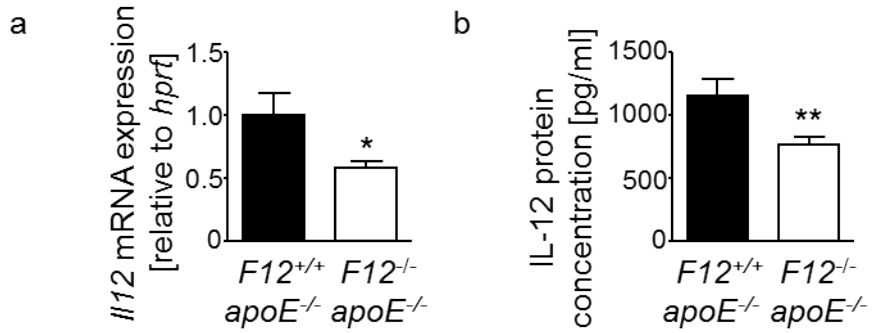

Figure 4.26 Deficiency in FXII in *apoE*$^{-/-}$ mice entails a reduction in IL-12 expression.
Il12 mRNA expression in spleens of *F12*$^{+/+}$*apoE*$^{-/-}$ and *F12*$^{-/-}$*apoE*$^{-/-}$ mice (normalized to *hprt* and expressed relative to *F12*$^{+/+}$*apoE*$^{-/-}$ controls, n=8-10 mice); (b) IL-12 protein levels in serum of *F12*$^{+/+}$*apoE*$^{-/-}$ and *F12*$^{-/-}$*apoE*$^{-/-}$ mice after 12 weeks of high fat diet feeding, as quantified by ELISA (n=5 mice per group). Data represent mean ± SEM; *p<0.05, **p<0.01

Analysis of additional cytokines known to be released by DCs besides *Il12* revealed a significant elevation in *Il6* mRNA levels upon C5a treatment. *Tgfb* levels showed a rather small but significant increase. Moreover, a trend towards higher *Il1b* levels compared to untreated controls was observed, which did not reach statistical significance **(Figure 4.27)**.

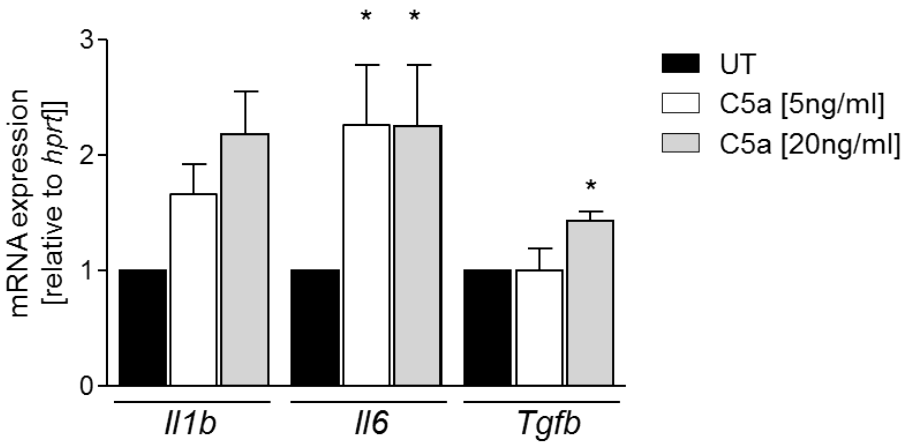

Figure 4.27. Alterations of *Il1b*, *Il6* and *Tgfb* levels in BMDCs upon C5a stimulation
Il1b, Il6 and Tgfb mRNA expression in BMDCs left untreated (UT) or stimulated with C5a for 12 h, normalized to *hprt* and expressed relative to untreated controls. Data represent mean ± SEM; *p<0.05. n=4 each

Analysis of the same cytokine panel in spleens from *F12*$^{+/+}$*apoE*$^{-/-}$ and *F12*$^{-/-}$*apoE*$^{-/-}$ mice after a 12 week high fat diet revealed a significant decrease in *Il6* expression together with an elevation in *Tgfb* expression in FXII deficient mice compared to controls, which possibly contributes to the anti-inflammatory phenotype observed in *F12*$^{-/-}$*apoE*$^{-/-}$ mice. Interestingly *Il1b* levels were unaltered between groups **(Figure 28)**.

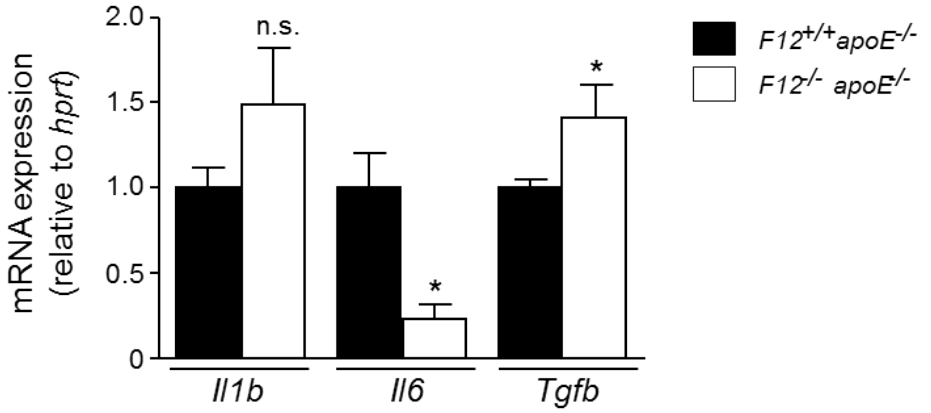

Figure 4.28. Alterations in *Il6* and *Tgfb* mRNA levels in spleens of FXII
Il1b, Il6 and Tgfb mRNA expression in spleens from *F12*$^{+/+}$*apoE*$^{-/-}$ and *F12*$^{-/-}$*apoE*$^{-/-}$ mice after 12 weeks of high fat diet. Data represent mean ± SEM; *p<0.05

4.7 Analysis of the macrophage polarization state in *apoE*$^{-/-}$ mice deficient in FXII

To rule out potential influences of macrophages to the observed phenotype, enriched splenic *F12*$^{-/-}$*apoE*$^{-/-}$ CD11c^{-}C11b^{+}F4/80^{+} macrophages and the corresponding controls were evaluated with respect to the expression of M1 and M2 macrophage polarization markers. Though, no changes in the M1 markers *Nos2*, *Il6* and *Il12* or the M2 markers *Il10*, *Arg1*, *Mrc1* and *Igf1* could be detected between groups **(Figure 4.29)**, indicating an unaltered macrophage phenotype in FXII deficient mice under atherogenic conditions.

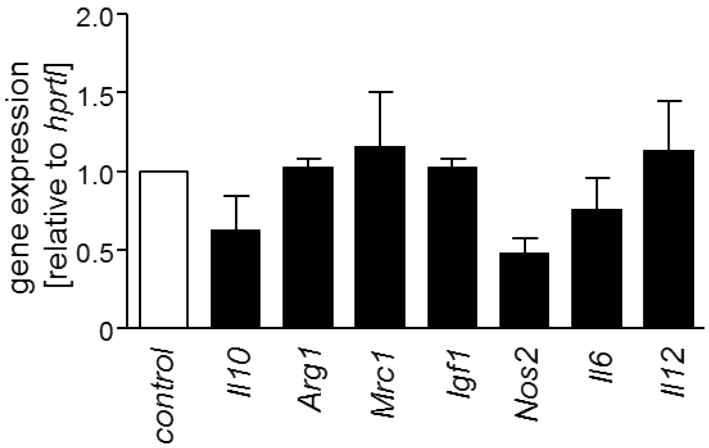

Figure 4.29. Deficiency in FXII in *apoE*$^{-/-}$ mice does not alter macrophage polarization mRNA expression of M2 macrophage markers *Il10*, *Arg1*, *Mrc1*, *Igf1* and M1 macrophage markers *Nos2*, *Il6* and *Il12* were analyzed in enriched CD11c^{-} C11b^{+}F4/80^{+} macrophages from *F12*$^{+/+}$*apoE*$^{-/-}$ and *F12*$^{-/-}$*apoE*$^{-/-}$ mice after 12 weeks of high fat diet (normalized to *hprt* and expressed relative to *F12*$^{+/+}$*apoE*$^{-/-}$ controls), data represent mean ± SEM. n=4 mice per group.

These data demonstrate that FXII, at least partially via C5a activation, is required for efficient IL-12 production by DCs and the establishment of a systemic IL-12 environment, known to promote a pro-atherogenic Th1 phenotype and atherosclerotic lesion formation[2,350].

Hence, scrutinizing the role of coagulation factor XII in atherosclerosis employing FXII-deficient mice crossed with atherosclerosis-prone a*poe*$^{-/-}$ mice, we were able to establish FXII as being a critical mediator in driving atherosclerotic lesion formation, complement activation and supporting systemic pro-inflammatory T-cell responses.

5. Discussion

Atherosclerosis as a chronic inflammatory disease has been previously demonstrated to be influenced not only by the innate and adaptive immune system[4,15,355,356] but also by the haemostatic system comprising platelets and coagulation factors[134,135]. The majority of experimental mouse studies investigating hypercoagulability, induced by genetic modifications such as FV Leiden mutation, partial protein C deficiency, thrombomodulin mutation or partial TFPI deficiency, on atherogenic background support a mainly pro-atherogenic role of a procoagulant phenotype in the development and progression of the disease[135,192,194-196].

To date, the majority of studies examining the impact of the haemostatic system on atherosclerosis however focus on the role of the so-called extrinsic pathway of coagulation or the common pathway and the coagulation factors involved as well as other parts of hemostasis including platelets or anti-coagulant factors (e.g. TFPI, protein C, thrombomodulin, heparin co-factor II)[127,128].

While FXII has been demonstrated to be present and functionally active in human atherosclerotic lesions[137] and increased FXII levels were observed in early atherosclerosis in low density lipoprotein receptor-deficient mice[340], its function in vascular disease remains controversial. In clinical studies FXIIa has been shown to correlate with conventional risk factors such as hyperlipidemia and cholesterolemia[327-331] and to the risk of coronary heart disease or to disease severity[322,330,325,326,334]. Other studies, however, have concluded that low FXII activity or lower plasma FXII or FXIIa may even increase the risk for CHD[255,329] and myocardial infarction[337]. Since earlier studies have been inconclusive and contradicting, this project investigates the role of FXII in atherosclerosis.

To investigate the role of FXII deficiency in atherosclerosis *F12*$^{-/-}$*apoE*$^{-/-}$ mice and corresponding *F12*$^{+/+}$*apoE*$^{-/-}$ controls were placed on a 12 week high-fat diet, revealing no alterations in body weight, triglyceride or cholesterol levels between groups, indicating that FXII does not have an impact on lipid

metabolism. These data also exclude changes in lipid levels as an underlying cause for any differences observed in lesion size.

In comparison to *F12*$^{+/+}$*apoE*$^{-/-}$ controls, *F12*$^{-/-}$*apoE*$^{-/-}$ mice displayed a significant reduction in plaque size in aortic roots as well as aortas, demonstrating FXII to be important for driving atherosclerotic lesion formation. This data is consistent with a study from Khallou-Laschet *et al.* which assessed the role of the intrinsic coagulation pathway in atherogenesis in hemophilic *FVIII*$^{-/-}$*apoE*$^{-/-}$ mice[157]. FVIIIa is a co-factor for activated FIXa in the intrinsic pathway of coagulation downstream of FXIIa and FXIa[357,358]. This study reported a pro-atherogenic role of FVIII in early-stage lesion formation, which was accompanied by reduced fibrinogen deposits and diminished platelet attachment[157]. While the reduction in lesion size observed in *FVIII*$^{-/-}$ *apoE*$^{-/-}$ mice is in line with our data, it is important to note that with the experimental setup employed by Khallou-Laschet *et al.* it is not possible to ascribe the effects they detected to an activation of the intrinsic coagulation pathway by FXIIa, since FXI being directly downstream of FXII has been shown in several studies to be also activated by thrombin independently of FXIIa, at least *in vitro*[152-155]. Moreover, thrombin generation also results from activation of the extrinsic pathway via TF activation. Importantly, FIX can be directly activated via the extrinsic TF-FVIIa complex independently from the intrinsic pathway[140] and FVIII activation is not only mediated by thrombin but also by FXa[359]. Besides employing a F12 deletion model, not only the intrinsic pathway of coagulation is suppressed but also all other pathways that FXII is involved in.

The assessment of cellular plaque composition showed an overall unaltered plaque phenotype revealing no changes in relative macrophage, smooth muscle cell or collagen content as well as unchanged T cell numbers and necrotic core size. However, it was rather unexpected to detect no changes in relative SMC or macrophage content since immune-histochemical analysis of human atherosclerotic lesions has shown FXII in close proximity to macrophages and vascular smooth muscle cells[137]. Likewise, there is *in vitro* evidence of FXII assembly on a macrophage-like cell line[273] and VSMC[249] suggesting possible cell-directed functions of FXII. Indeed, it has been

demonstrated that FXII is capable of inducing proliferation of aortic SMCs *in vitro*[248]. Yet, given the unaltered SMC content, it seems unlikely that FXII has a profound effect on SMCs in atherosclerosis.

In this project the effect of FXII deficiency on macrophage content has been investigated immune-histochemically in the plaque revealing no differences. Moreover, quantitative real-time analyses for macrophage polarization markers in enriched $CD11c^{-}CD11b^{+}F4/80^{+}$ macrophages from the spleens of these mice were performed. Analysis of typical M1 and M2 macrophage polarization markers however exposed no differences between groups. This is in line with the unaltered mRNA expression data obtained from FXIIaα treated bone marrow-derived macrophages when compared to untreated controls. Together these data indicate that deficiency of FXII alters neither the macrophage content in the plaque nor the phenotype of macrophages under atherosclerotic conditions.

When analyzing secondary lymphoid organs and blood, no changes in organ size or absolute leucocyte counts were detected between *F12*$^{-/-}$*apoE*$^{-/-}$ mice and *F12*$^{+/+}$*apoE*$^{-/-}$ controls. Moreover, no differences in circulating leucocytes, frequencies of monocytes, neutrophils, B- or T cells could be observed, which indicates an unaltered recruitment of these cell types into the periphery or atherosclerotic lesions. This is supported by an unaltered gene expression of vascular cell adhesion molecules such as *Vcam1, Pecam1, Icam1* and *Pselectin* in the aorta of both groups.

F12$^{-/-}$*apoE*$^{-/-}$ mice on HFD were not only shown to display decreased $CD4^{+}$ T-cell activation but also to have significantly lower frequencies of IFNγ-producing T cells among the $CD4^{+}$ T cell population, suggesting FXII to trigger T-cell activation and to elicit a Th1 response under atherogenic conditions. This data is very remarkable since atherosclerosis has been proven to be a Th1 driven disease[13,97,98], its signature cytokine IFNγ provoking pro-inflammatory and pro-atherogenic responses[101,103,107]. Hence, the decline in IFNγ-producing Th1 cells in FXII-deficient mice results in the decrease in atherosclerosis.

Although a clear trend towards reduced Th17 frequencies was observed, the overall percentage of IL-17a producing $CD4^+$ T cells is rather low so that the differences in Th17 frequencies observed between both groups are probably negligible compared to the impact of the detected Th1 changes. Nevertheless, it cannot be excluded that the decrease in Th17 frequencies witnessed in FXII-deficient mice may possibly promote[108,111-116] or diminish[119,120] the anti-atherogenic effect of FXII deficiency in the *apoE*$^{-/-}$ mouse model. Yet, it is clear that the strongly distinctive Th1-mediated effects on atherosclerosis outweigh any such possible effects derived from Th17 related changes.

As no alterations in systemic T-cell activation and polarization could be observed under physiological homeostatic conditions in *Bl6* mice deficient in FXII, atherosclerosis-related inflammatory processes seem to trigger FXII-dependent modulation of immune responses. This may be possibly associated with an increased burden of *in vivo* contact activators of FXII, such as extracellular RNA[216], collagen[217], polyphosphates released from activated platelets[218,219], misfolded proteins[221], or very-low-density lipoprotein (VLDL) phospholipids[220] under these conditions. Most of these triggers may be enhanced under atherosclerotic conditions. In line, increased FXII plasma levels have been observed in early atherosclerosis in hyperlipidemic low-density lipoprotein receptor-deficient mice[340] and FXIIa levels have also been shown to correlate with conventional risk factors such as hyperlipidemia and cholesterolemia in humans[320,322]. FXII has moreover been co-localized to SMCs and focally to macrophages in human atherosclerotic lesions, which may provide an additional, local source for FXII in atherosclerosis[137].

Since T-cell activation and polarization are strongly dependent on antigen-presentation by and cytokine release from DCs and an expansion of Th1 T cells was verified to critically depend on IL-12 secreted from DCs[349,350], it was obvious to initially investigate direct effects of activated FXII on DCs and T cells. However, despite the strong effects on DCs and T-cell phenotype observed in mice deficient in FXII, such changes could not be recapitulated in these cell types by the addition of FXIIa *in vitro*, suggesting that effectors down-stream of FXII affect immune cell responses.

A reduction in bradykinin levels in the serum of *F12*$^{-/-}$*apoE*$^{-/-}$ mice; bradykinin being a part of the kallikrein-kinin system; could not be observed, even though this would have been expected, as an earlier study demonstrated reduced levels of bradykinin in FXII-deficient mice[360]. These conflicting data however may reflect a predominance of FXII-independent prekallikrein activation[224,226] in *F12*$^{-/-}$*apoE*$^{-/-}$ mice or diminished levels of bradykinin-cleaving plasma proteases such as neutral endopeptidase (NEP) secondary to the reduction in C5a levels[361]. An additional possibility might be reduced levels of the bradykinin-cleaving protease angiotensin converting enzyme (ACE) in *F12*$^{-/-}$*apoE*$^{-/-}$ mice compared to *F12*$^{+/+}$*apoE*$^{-/-}$ controls, as a secondary effect to the diminished Th1 phenotype in these mice. ACE was demonstrated to accumulate in human atherosclerotic lesions[362-364] and to play a role in the modulation of atherosclerosis[365]. Furthermore, there is experimental evidence that type II interferons such as IFNγ can upregulate ACE-expression in myeloid cells[366]. Thus, reduced IFNγ levels as consequence of a diminished Th1 response in *F12*$^{-/-}$*apoE*$^{-/-}$ mice may lead to a reduction in ACE expression limiting bradykinin degradation. It has to be emphasized that the possible explanations for the absence of reduced bradykinin levels in *F12*$^{-/-}$*apoE*$^{-/-}$ mice mentioned above are so far plain hypothetical assumptions lacking experimental evidence.

Besides its essential function in triggering the intrinsic coagulation pathway which was not addressed in this thesis, FXII has also been shown to initiate complement activation[228,351]. Indeed, markedly decreased C5a plasma levels in *F12*$^{-/-}$*apoE*$^{-/-}$ mice support an important role of FXII in complement activation under inflammatory conditions in atherosclerosis. These data support and extend previous findings showing reduced complement activation upon FXII neutralization under inflammatory conditions in septic baboons[367].

FXII may trigger complement activation either directly via the classical pathway through Hageman factor fragment (Hf)[278,279], indirectly via plasma kallikrein, cleaving C3 to C3a or C5 to a C5-like peptide[280,281], or by factors downstream of FXII, such as thrombin, FXIa, plasmin, FXa and FIXa, which are capable of cleaving C3 and C5 to C3a and C5a, respectively[307,368]. Also, various coagulation factors from the extrinsic pathway (TF, FVIIa, FVII) have

failed to generate C3 or C5 cleavage activity[307], strengthening the role of the intrinsic coagulation pathway with respect to complement activation.

Additionally, plasma kallikrein activated by FXIIa on dermatan sulfate (DS) has been shown to cleave complement component factor H (FH), which is a negative regulator of the alternative pathway of complement, accelerating the decay of C3 and C5 convertases, thereby limiting C3a and C5a production[369]. Thus, a cleavage of FH may result in increased levels of the anaphylatoxins C3a and C5a. This could indeed represent an additional mechanism for FXIIa-mediated complement modulation, since atherosclerosis progression was demonstrated to be accompanied by increased levels of certain proteoglycans, including DS-containing proteoglycans in atherosclerotic lesions[370]. An overview about these potential FXII-mediated pathways for C5a induction are illustrated in **Figure 5.1**.

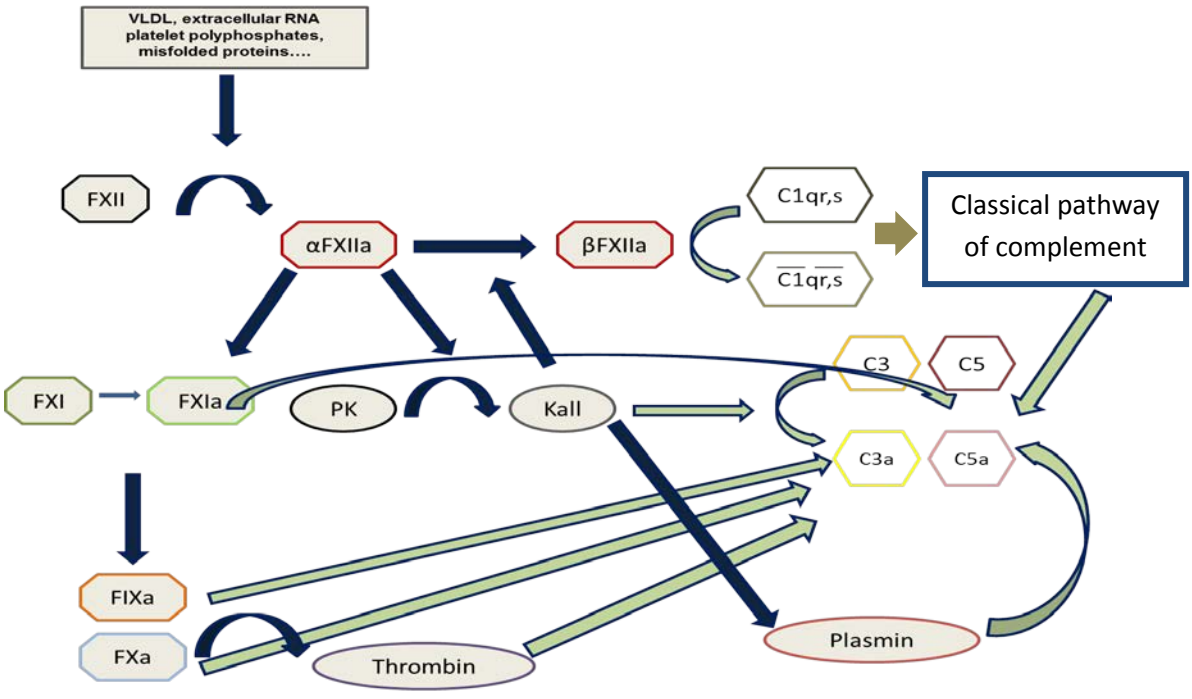

Figure 5.1 Potential FXIIa-mediated mechanism for C5a induction
Blue arrows indicate the actions of the illustrated factors upon activation; green arrows illustrate the complement activating actions of factors downstream of FXII activation as described in literature. FXIa, FIXa, FXa, Thrombin and Plasmin result in C3 and C5 activation. Ultimately also the classical pathway of complement activated via FXIIaβ can result in formation of the anaphylatoxins C3a and C5a.

Importantly, C5a has been shown to not only be present in human unstable coronary lesions[291] but also to serve as a predictor of cardiovascular events in advanced atherosclerosis[371]. Likewise, increased C5a levels caused by a polymorphism in the C5 gene represent a risk factor for adverse outcome in patients with atherosclerosis[372].

While *C5*$^{-/-}$*apoE*$^{-/-}$ mice do not display any alterations in plaque size in the aortic root[373], pharmacological blockade of the C5a receptor (C5aR) has been shown to reduce atherosclerotic burden[353] as well as neointimal hyperplasia after carotid denudation injury in atherosclerotic mice[374], suggesting pro-atherogenic effects of C5aR activation. The inconsistencies between experimental data on C5 in the context of atherosclerosis may be due to different experimental approaches in these studies. The majority of studies scrutinizing the role of C5a in atherosclerosis however suggest a pro-atherogenic role.

C5a contributes to and modulates inflammation in various ways, as it has been shown to be chemotactic for leucocytes (e.g. neutrophils, T cells, monocytes), to increase vascular permeability, to cause contraction of SMCs, to trigger histamine release from mast cells and an oxidative burst in neutrophils with formation of reactive oxygen species (ROS)[282,375].

Moreover, C5a induces the expression of adhesion molecules[376,377] and tissue factor[378] on ECs and promotes the release of inflammatory mediators such as IL-1β[379], IL-6[380,381], TNF-α[382], IL-8[383,384] and OSM[385] but also of cytokines considered to be anti-inflammatory such as TGFβ[380]. In line, a concentration-dependent increase of *Il6* and *Il1b* transcript levels as well as a small but significant increase in *Tgfb* levels was observed upon C5a-stimulation of BMDCs. These *in vitro* data were partially recapitulated *in vivo* where a significant decline in *Il6* levels in spleens from *F12*$^{-/-}$*apoE*$^{-/-}$ was seen when compared to controls.

Also, reduced *Il6* levels in *F12*$^{-/-}$*apoE*$^{-/-}$ may explain the reduction in Th17 frequencies, since IL-6 has been demonstrated to be crucial for Th17 induction[386,387]. Yet, the elevation in *Tgfb* and unaltered *Il1b* transcript levels *in vivo* do not match the *in vitro* data, suggesting other factors and mediators

in *F12*$^{-/-}$*apoE*$^{-/-}$ mice to also play a role in defining the cytokine environment in these mice. Furthermore, it has to be noted that the *in vivo* data was generated from total splenocytes and not from isolated DCs making it impossible to directly compare both data sets. Nevertheless, the increase in *Tgfb* and the reduction in *Il6* transcript levels *in vivo* probably further contribute to a less inflammatory phenotype.

An excessive activation of the complement system and high C5a levels can be observed in several inflammatory diseases such as sepsis and rheumatoid arthritis[388,389]. Apart from their classical modulatory role in inflammation, the anaphylatoxins C3a and C5a were recently also demonstrated to play an important role in DC and T-cell mediated diseases modulating adaptive immune responses[288].

Abrogation of C5aR signaling in DCs was reported to result in Th17 and Treg expansion[390]. Conversely, in studies employing different experimental models such as EAE and auto-immune arthritis, pharmacological C5a blockade or general C5aR deficiency caused impaired Th17 differentiation[391,392]. Also, C5a was demonstrated to promote IL-17 production from human T cells dependent on B7, IL-1β and IL-6 expression from monocytes[393].

While no alterations in Treg frequencies were observed, a clear trend towards diminished Th17 levels was detected in *F12*$^{-/-}$*apoE*$^{-/-}$ mice in this study. *In vitro* assays stimulating BMDCs with C5a however did not alter Th17 frequencies. Discrepancies between these results and literature may be ascribed to an incomplete abolishment of C5a signaling in our model. Instead, C5a levels in *F12*$^{-/-}$*apoE*$^{-/-}$ mice are markedly reduced still enabling a certain amount of signaling, which could be a possible explanation. In addition, differences in the *in vitro* data in this project such as lack of modulations in co-stimulatory molecule expression and unaltered Th17 frequencies following C5a stimulation presented in this thesis may be explained by differences in the experimental setup, including variances in C5a concentrations or stimulation time compared to previously published studies. The decrease in Th17 frequencies in *F12*$^{-/-}$*apoE*$^{-/-}$ mice compared to *F12*$^{+/+}$*apoE*$^{-/-}$ controls after HFD however may be attributed partially to the reduced *Il6* levels in these mice,

since the IL-6-gp130-Stat3 pathway is crucial for RORγt expression in T cells and Th17 development[394].

C5a-C5aR interactions were also shown to positively regulate DC activation and DC-induced T-cell proliferation and IFNγ production[395,396]. Murine splenic APCs and BMDCs from C5- or C5aR-deficient mice or C5aR-antagonist treated BMDCs employed in different experimental models elicited impaired T-cell responses and displayed diminished MHC and co-stimulatory molecule expression. Moreover the DCs in these models showed reduced IL-12 production and impaired Th1 responses[396-398]. Conversely, C5a stimulation performed in some of these studies up-regulated DC activation, IL-12 expression[396,397] and DC-mediated Th1 polarization[396].Conflicting literature demonstrating C5a to up-regulate or down-regulate IL-12 expression by antigen-presenting cells (APCs)[396-402] could be attributed to different experimental designs as demonstrated by Lalli *et al.*[397].

It has also been demonstrated that C5a can induce IL-12 production and induction of Th1 responses in macrophages[288,397] which also represent a local source of complement proteins[403]. However, in this project no alterations in transcript levels of M1 or M2 markers between *F12*$^{-/-}$*apoE*$^{-/-}$ mice and controls on atherogenic diet were noticed *in vivo*. Yet, a contribution of macrophages to the observed Th1 phenotype cannot be totally excluded based on transcript levels only.

While no changes were detected in co-stimulatory molecule expression in BMDCs treated with different concentrations of C5a or in DCs from atherosclerotic *apoE*$^{-/-}$ *versus F12*$^{-/-}$*apoE*$^{-/-}$ mice *in vivo,* an augmented IL-12 secretion in C5a-treated BMDCs could be observed. Although C5a may cause these changes by affecting cells other than T cells and DCs, the lack of C5aR on T cells[352], an unaltered T-cell activation in the presence of C5a, together with the C5a-triggered enhancement in naïve T-cell differentiation towards IFNγ-producing Th1 cells in co-cultures with C5a-stimulated BMDCs strongly argues for a direct effect of C5a on DCs. Notably, these changes in T-cell phenotype recapitulated the marked decrease in frequencies of CD44^{hi}CD62L^{lo} effector/memory T cells and Th1 cells in atherosclerotic *F12*$^{-/-}$*apoE*$^{-/-}$ mice.

By employing a C5aR1/GFP knock-in mouse model to track C5aR expression, it was shown that C5aR is expressed on monocytes/inflammation-induced macrophages and cultured BMDCs, whereas myeloid DCs in the spleen did not express this receptor[352]. Together with changes in the systemic T cell phenotype in atherosclerotic *F12*$^{-/-}$*apoE*$^{-/-}$ mice but not in healthy *F12*$^{-/-}$ mice it may be conceivable that C5aR-responsive DCs in our study are derived from circulating monocytes, as previously demonstrated in atherosclerosis[83].

IL-12 production by DCs was previously shown to be linked to an increased capacity to stimulate T-cell proliferation and IFNγ secretion[349,350]. Similarly, reduced frequencies of IL-12 expressing CD11c^{+}MHCII^{+} DCs, together with a strong reduction in *Il12* mRNA expression in the spleen and decreased IL-12 serum levels are very likely to underlie limited T-cell activation and Th1 polarization, and in turn retarded atherosclerotic lesion formation in atherosclerotic *F12*$^{-/-}$*apoE*$^{-/-}$ mice.

A hypothetical model, illustrating potential mechanisms by which FXII may promote a pro-atherogenic Th1 response aggravating atherosclerosis, combining data from this thesis and literature is depicted in **Figure 5.2**.

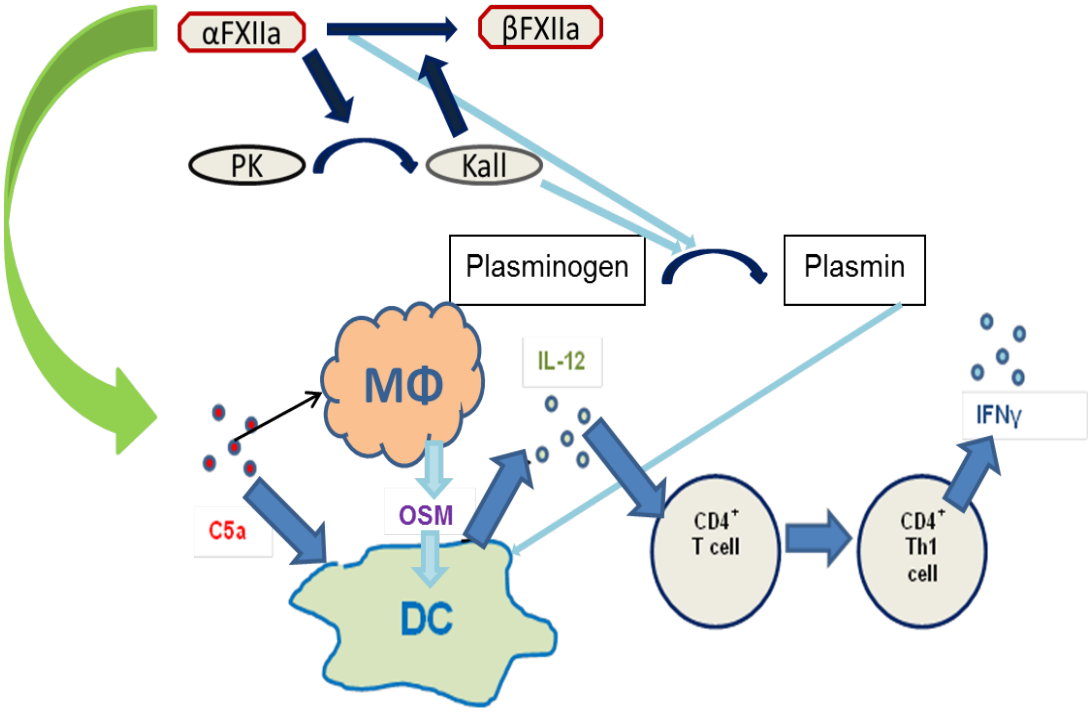

Figure 5.2. Hypothetical model for potential FXII-mediated regulations of a pro-atherogenic Th1 response under atherosclerotic conditions.

FXII leads to an upregulation of C5a under atherosclerotic conditions in the *apoE*$^{-/-}$ mouse model as indicated by the green arrow. Potential mechanism for the observed C5a upregulation are depicted in Figure 5.1. The upregulation of C5a induces IL-12 expression and release from DCs. IL-12 polarizes naïve T cell towards a Th1 phenotype (dark blue arrows). This model of C5a induced DC activation and T cell polarization was confirmed in this thesis *in vitro.* Apart from this potential FXII-mediated modulation of a pro-atherogenic Th1 response, FXII may also result in an activation of plasmin, which was demonstrated to induce IL-12 production in monocyted-derived DCs thereby promoting Th1 responses (light blue arrows, thin). Furthermore the upregulation of C5a may influence other cell types apart from DCs, since macrophages were shown to release OSM upon C5a treatment and OSM is able to induce IL-12 expression in DCs supporting Th1 responses (light blue arrows, thick)

This model however also points out the limitations of this thesis. While reduced C5a-levels can be considered to at least partially contribute to the phenotype observed in *F12*$^{-/-}$*apoE*$^{-/-}$ mice under atherosclerotic conditions, the participation of other factors modulated by FXIIa under atherogenic conditions cannot be ruled out. The serine protease plasmin, activated either directly by FXIIa or indirectly via kallikrein or FXIa[236-240] for instance could also promote pro-atherogenic Th1 responses, since plasmin may not only promote atherosclerosis through suppression of HDL-induced cholesterol efflux from foam cells[311], but also through induction of monocyte-derived DC chemotaxis as well as IL-12 production triggering Th1 polarization[310]. Furthermore, C5a may not only directly act on DCs eliciting IL-12 production and release,

thereby promoting Th1 responses but also indirect ways are conceivable. C5a has been shown for example to drive oncostatin M (OSM) expression in human macrophages via AP-1 activation[385] and OSM again was confirmed to stimulate IL-12 expression in DCs supporting Th1 immune responses[404].

Likewise, it should be contemplated that FXII may modulate atherosclerosis through additional mechanisms apart from the C5a-mediated promotion of a pro-atherogenic Th1 phenotype delineated in this thesis.

Although a direct influence of activated FXII on macrophages seems unlikely according to the *in vitro* data in this project, FXIIa may modulate other innate immune cells including neutrophils or monocytes as FXIIa was demonstrated to be a potent inducer of IL-1β and IL-6 release from human monocytes[277] and to mediate neutrophil aggregation and degranulation as well as fibrin deposition[274,405]*,* both of which could contribute to disease aggravation. Plasma kallikrein and plasmin, being downstream mediators of FXII, were described to impair HDL-induced cholesterol efflux from foam cells *in vitro*, suggesting a pro-atherogenic function of these two proteases[311]. FXII may further result in an increase of pro-fibrinolytic (↓PAI-1, ↑uPA, ↑plasmin)[236-240,242-244] as well as anti-fibrinolytic (↑TAFI)[245,246] functions. Depending on the net-effect of these changes *in vivo,* FXII deficiency may either aggravate atherosclerosis due to impaired fibrinolysis possibly promoting thrombotic events in late stages of atherosclerosis[406] or *vice versa*. FXII mediated increase in u-PA levels could influence atherosclerosis moreover in additional ways besides its function as plasminogen activator yielding plasmin formation[228-232,310,311], since local uPA expression itself was shown to correlate with lesion severity in human plaques[318,319] and overexpression of uPA in macrophages resulted in accelerated lesion formation in several experimental studies[314,322,324]. Also, although this thesis clearly shows an influence of FXII on C5 activation, an *in vivo* modulation of other complement components such as C1[278,279] and C3[280,307] as implicated by literature has to be considered as well. Such changes may potentially contribute to the phenotype observed under FXII deficiency, since C3a similar to C5a has been demonstrated to induce BMDC-mediated Th1 responses in different experimental models[407,408]. Further experiments however would need to be pursued in *F12*$^{-/-}$*apoE*$^{-/-}$ mice

on atherogenic diet to investigate additional FXII mediated mechanisms that may contribute to the phenotype of these mice.

In summary, this thesis for the first time demonstrates that the deficiency of FXII inhibits atherosclerotic lesion formation in the *apoE*$^{-/-}$ mouse model by modulating adaptive immune responses, extending the notion that a cross-talk between coagulation, complement activation and immunity is critical in inflammation and atherosclerosis. FXII-deficiency results in a lowered systemic C5 complement activation, diminished frequencies of IL-12 expressing DCs and a systemic reduction in IL-12 levels, together with an attenuation of T-cell activation and Th1 polarization. These data demonstrate that FXII, by promoting a pro-atherogenic DC/T cell phenotype, drives atherosclerotic lesion development. FXII may thus not only serve as a marker of atherosclerotic vascular damage, but functions to directly contribute to atherosclerotic lesion formation.

6. References

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8. Publications

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Koch M., Manthey H.D., Busch M., Chaudhari S.M., Thielman I, Stegner S., Nieswandt B[*]; Zernecke A[*]: Coagulation factor XII promotes atherosclerosis and pro-inflammatory T cell responses in mice. *submitted*

Sweena M.C.[*], Judith C.S.[*], **Koch M.**,Manthey H.D., Busch M., Daemen M.J., Caballero-Franco C., Kissler S., Hermanns H.M., Zernecke A.: HIF1alpha in dendritic cells restrains inflammation and atherosclerosis in mice. *submitted*

Cochain C[*]; Chaudhari S.M.[*], **Koch M**., Wiendl H, Eckstein HH, Zernecke A.: PD-1 deficiency exacerbates T-cell activation and atherogenesis despite expansion of regulatory T cells in atherosclerosis-prone mice. *submitted*

Manthey H.D.[*], Cochain C.[*], Barnsteiner S.[*], Karshovska E., Pelisek J., **Koch M.**, Chaudhari S.M., Busch M., Eckstein H.H., Weber C., Koenen R.R., Zernecke A.: Role of the chemokine receptor Ccr6 in monocyte recruitment and atherosclerosis in mice. *Thrombosis Haemostasis, 2013; 110(6)*

Weber C[*]; Meiler S[*], Döring Y.[*], **Koch M.**, Drechsler M, Megens R.T.A., Rowinska Z., Bidzhekov K, Fecher C., Ribechini E, Zandvoort M.A.M.J, Binder C.J., Jelinek I, Hristov M, Boon L, Jung S, Korn T., Lutz M.B., Förster I, Zenke M, Hieronymus T, Junt T., Zernecke A.: CCL17-expressing dendritic cells drive atherosclerosis by restraining regulatory T cell homeostasis in mice. *Jounal of Clinical Investigation, 2011; 121(7):2898-2910*

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10. Affidavit/Eidesstattliche Erklärung

AFFIDAVIT

I hereby confirm that my thesis titled “Role of Coagulation Factor XII (FXII) in Atherosclerosis” is the result of my own work. I did not receive any help or support from commercial consultants. All sources and/or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not been submitted as part of another examination process neither in identical nor in a similar form.

Würzburg,

EIDESSTATTLICHE ERKLÄRUNG

Hiermit erkläre ich an Eides statt, die Dissertation „Rolle des Koagulationsfaktors XII (FXII) in der Atherosklerose“ eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters angefertigt, und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Würzburg,