

Role of the COP9 Signalosome and IKK complexes in Atherosclerosis

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ABBREVIATIONS

LDL	low density lipoprotein
ApoB	apolipoprotein B
HOCl	hypochlorous acid
ECs	endothelial cells
CCL	chemokine (C-C motif) ligand
MIF	macrophage migration inhibitory factor
ICAM-1	intercellular adhesion molecule-1
VCAM-1	vascular cell adhesion molecule-1
M-CSF	macrophage colony-stimulating factor
SR	scavenger receptor
CD	cluster of differentiation
mLDL	modified LDL
DCs	dendritic cells
T cells	thymus cells
NKT	natural killer T
SMCs	smooth muscle cells
MI	myocardial infarction
apoE	apolipoprotein E
LDLR	low density lipoprotein receptor
GJ	gap junctions
IL	interleukin
MIP	macrophage inflammatory protein
NF- κ B	nuclear factor-kappa B
ERK	extracellular signal regulated kinase
MAPK	mitogen-activated protein kinase
Src	sarcoma
CCR	chemokine (C-C motif) receptor
CXCL1	chemokine (C-X-C motif) ligand
CX ₃ CL	chemokine (C-X ₃ -C motif) ligand
CX ₃ CR	chemokine (C-X ₃ -C motif) receptor
PPRs	pattern recognition receptors
MHC	major histocompatibility complex
TLRs	toll-like receptors

AP-1	activator protein-1
TH	helper T cells
PPAR	peroxisome proliferator-activated receptor
S1P	sphingosine 1-phosphate
SCID	severe combined immunodeficiency
DNA	deoxyribonucleic acid
RHD	rel homology domain
NLS	nuclear localization sequence
TAD	transactivation domain
I κ B	inhibitor of kappa B
BCL	B-cell lymphoma encoded protein
NEMO	NF- κ B essential modulator
IKK	inhibitor of kappa B Kinase
NES	nuclear export signal
TNF	tumor necrosis factor
TCR	T-cell receptor
LPS	lipopolysaccharide
TNFR	tumor necrosis factor receptor
BAFFR	B cell activating factor receptor
LT β R	lymphotoxin β -receptor
RANK	receptor activator for nuclear factor κ B
TRAF	tumor necrosis factor receptor-associated factor
SCF	Skp1-culin-Roc1/Rbx1/Hrt-1-F-box
IFN	interferon
CREB	cAMP response element binding protein
CBP	CREB-binding protein
HDAC-1	histone deacetylase 1
kDa	kilodalton
TRADD	TNFR type 1-associated DEATH domain protein
JNK	c-Jun N-terminal kinase
PGI ₂	prostacyclin
GPCR	G protein-coupled receptor
PGI ₂	prostaglandin I ₂
eNOS	endothelial nitric oxide synthase

MLCK	myosin-light-chain kinase
WPBs	weibel-palade bodies
PAF	platelet activating factor
MMP	metalloprotease
CRL	cullin ring ubiquitin ligase
COP9	constitutive photomorphogenesis 9
CSN	COP9 signalosome
RNA	ribonucleic acid
JAB1	jun-activation-domain-binding protein
MPN	MPR1–PAD1–N-terminal domain
Nedd8	neural precursor cell-expressed developmentally downregulated-8
JAMM	JAB1/MPN domain metalloenzyme
CAND1	cullin-associated and neddylation-dissociated 1
DEN1	deneddylase 1
COS7	african green monkey SV40-transformed kidney fibroblast cell line
LFA-1	lymphocyte function-associated antigen-1
UPP	ubiquitin proteasome pathway
HDL	high-density lipoprotein
HUVECs	human umbilical vein endothelial cells
siRNA	small interfering RNA
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
BSA	bovine serum albumin
ELISA	enzyme-linked immunosorbent assay
HRP	horseradish peroxidase
PCR	polymer chain reaction
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
cDNA	copy DNA
EDTA	ethylenediaminetetraacetic acid
FACS	fluorescent activated cell sorting
HBSS	Hank's balanced salt solution
MFI	mean fluorescence intensity

FCS

fetal calf serum

RPM

Roswell park memorial institute

1 INTRODUCTION

1.1 Atherosclerosis

Atherosclerosis is a progressive chronic inflammatory disease of the blood vessels characterized by the thickening of the arterial wall that becomes clinically evident when there is plaque rupture and thrombus formation¹. It is the primary cause of cardiovascular diseases with enhanced mortality rate in the Western world². Intense immunological activity resulting from the interactions between modified lipids, cells of the immune system and of the vascular wall ultimately leads to the formation of lesions that protrude into the arterial lumen³. The normal human coronary artery is made up of endothelial cells that are exposed to blood in the arterial lumen and rest on a basement membrane. The tunica intima contains few smooth muscle cells scattered within the intimal extracellular matrix. The internal elastic lamina forms the barrier between the tunica intima and the underlying tunica media. The media consists of multiple layers of smooth muscle cells, much more tightly packed than in the diffusely thickened intima, and embedded in a matrix rich in elastin as well as collagen⁴ (Figure 1).

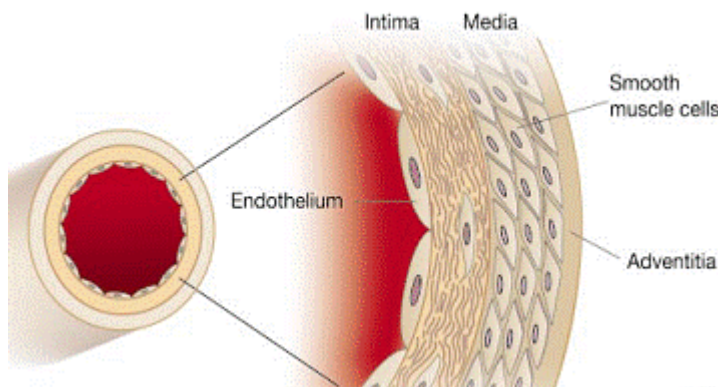


Figure 1 A typical trilaminar structure of the human coronary artery. The endothelial barrier is exposed to blood flowing in the arterial lumen. An inner tunica intima with few smooth muscle cells and a tunica media with tightly packed smooth muscle cells separate the endothelium from the adventitia (Taken from Libby *et al.*, Nature 2002).

Atherosclerosis is mediated by the transport of cholesterol in the blood by LDL which contains a core of esterified cholesterol and triglycerides surrounded by a shell of phospholipids, free cholesterol and ApoB100. Particles of LDL can accumulate in the intima where ApoB100 binds to the proteoglycan of the extracellular matrix through

ionic interactions retaining the LDL particles in the subendothelial space^{5, 6}. Several enzymes such as myeloperoxidases and lipoxygenases⁷ and some reactive oxygen species like HOCl and phenol radical intermediates¹ have been reported to be involved in the modification of LDL. This initial retention of low-density lipoprotein in the subendothelial space and its subsequent modification triggers the activation of endothelial cells (ECs), which secrete chemokines such as CCL2 and macrophage migration inhibitory factor (MIF), and upregulate the expression of adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and P-selectin, to recruit inflammatory cells, such as monocytes, granulocytes and T-cells, into the intima^{8, 9}. In the intima, macrophage colony-stimulating factor (M-CSF) produced by activated endothelial cells drives the differentiation of monocytes to macrophages which is necessary for development of atherosclerosis¹⁰. A hallmark of the transition from monocytes to macrophages is the increased expression of two major scavenger receptors: scavenger receptor class A (SR-A) and CD36¹¹. These receptors bind different forms of LDL and mediate the uptake of modified lipids from the vessel wall. The accumulation of cholesterol eventually turns these macrophages to foam cells that are characteristics of early atherosclerotic lesion which is followed by inflammatory responses. In later stages of atherosclerosis there is death of large lipid-laden cells that causes the formation of a necrotic core which determines the stability of the atherosclerotic plaque and therefore is considered to be clinically important. The advanced stage of atherosclerosis is marked by the migration of smooth muscle cells to the subendothelial space where they proliferate and form connective tissues which lead to fibrotic cap formation (Figure 2)¹².

The atherosclerotic plaque is mainly composed of lipids, infiltrated immunocytes such as macrophages, T cells and mast cells, and a fibrous cap composed mostly of collagen that is formed by vascular smooth muscle cells. Fatty streak, which is an early lesion, consists of lipids deposited at the subendothelial space, macrophage foam cells loaded with cholesterol and T cells (Figure 3)¹. Early lesions which are prevalent in young people do not cause symptoms and can either progress into mature atherosclerotic plaque or disappear with time¹³.

Adesion molecules

(VCAM1, ICAM1, P-selectin)

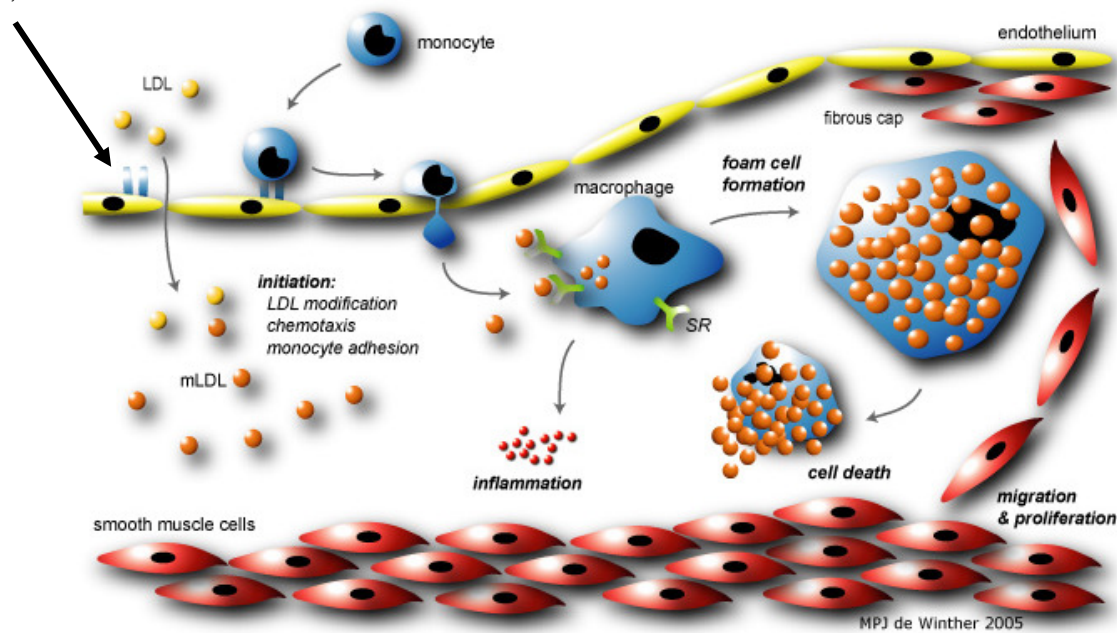


Figure 2 Schematic representation of atherogenesis. Initiation of atherosclerosis is characterized by the activation of endothelial cells by components of modified LDL (mLDL) and possibly also by turbulent blood flow at arterial branching points. This induces the expression of adhesion molecules and secretion of chemokines. Monocytes swimming in the blood stream bind to the adhesion molecules and infiltrate into the subendothelial space where they are differentiated into macrophages. Macrophages with the help of their scavenger receptors scavenge mLDL in the vessel wall resulting in foam cell formation which is accompanied by inflammatory responses. Later stages involve fibrotic cap formation through migration and proliferation of smooth muscle cells and cell death resulting in necrotic core (Adapted from de Winther *et al.*, ATVB 2005).

A mature plaque that develops with time has a more complex structure than the early lesion. In the centre of a matured plaque, foam cells and extracellular lipid droplets form a core region that is surrounded by a fibrous cap of smooth muscle cells and a collagen-rich covered matrix. Other cell types present in plaques include dendritic cells (DCs), mast cells, a few numbers of B cells and natural killer T (NKT) cells. The shoulder region of a mature plaque, which is where it grows, has particularly abundant accumulation of macrophages and T cells, both of which are activated, leading to the production of a cascade of cytokines that maintain the inflammatory state. Concomitantly, activated smooth muscle cells (SMCs) are responsible for plaque growth and stability through their continued synthesis of fibrous tissue¹⁴.

Over time, these lesions mature and gain new characteristics which leads to the clinical complications of atherosclerosis⁴.

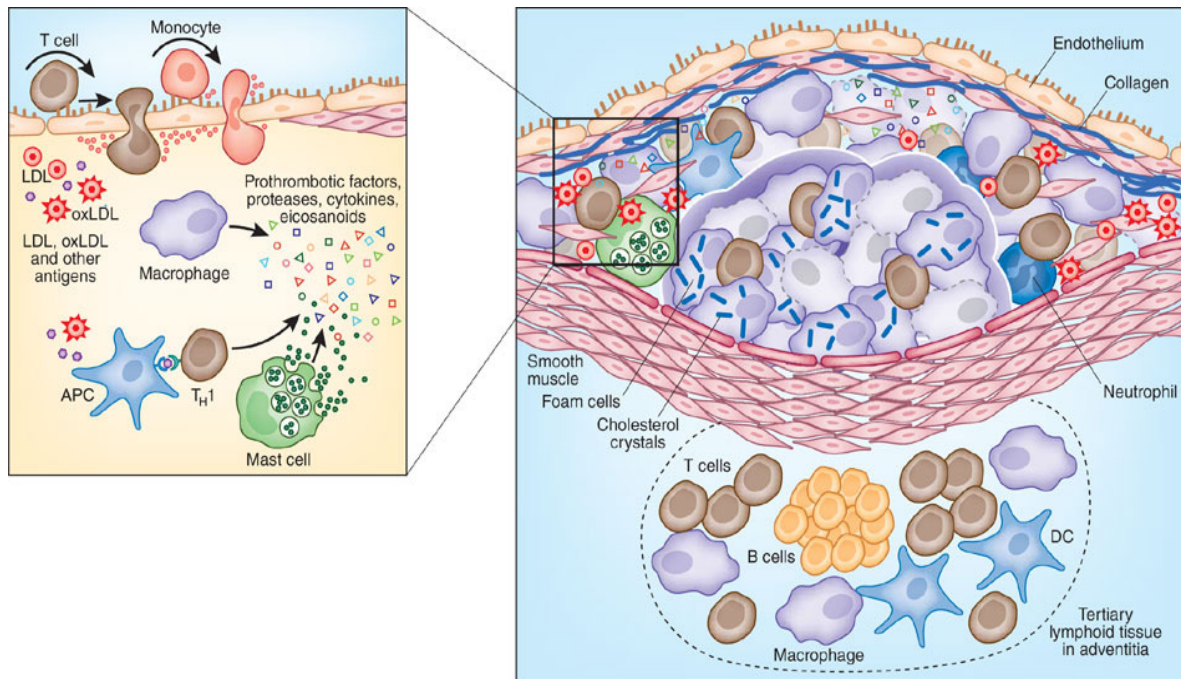


Figure 3 Immune components of the atherosclerotic plaque. The atheroma has a core of lipids, including cholesterol crystals, living and apoptotic cells and a fibrous cap with smooth muscle cells and collagen. Plasma lipoproteins accumulate in the subendothelial region. Several types of cells of the immune response are present throughout the atheroma including macrophages, T cells, mast cells and DCs. The atheroma builds up in the intima, the innermost layer of the artery. Outside the intima, the media contains smooth muscle cells that regulate blood pressure and regional perfusion, and further abuminally, the adventitia continues into the surrounding connective tissue. Here, cells of the immune response accumulate outside advanced atheroma and may develop into tertiary lymphoid structures with germinal centers (Taken from Hansson *et al.*, Nat. Immunol. 2011).

Although these complications can already arise from stenotic lesions causing limited flow of blood through the arterial lumen, the most severe clinical events follow plaque rupture due to fibrous cap disruption, which exposes the prothrombotic material in the plaque to the blood and causes sudden thrombotic occlusion of the artery at the site of disruption. In the heart, atherosclerosis can lead to myocardial infarction (MI) and heart failure; whereas in the brain it can cause ischemic stroke and transient ischemic attacks. If atherosclerosis affects other arterial branches, it can result in renal impairment, hypertension, abdominal aortic aneurysms and critical limb ischemia.

Genome-wide association studies are being used to identify genes that are significantly associated with atherosclerotic disease and its risk factors¹⁵⁻¹⁷. However, the systematic investigation of the mechanisms underlying atherosclerosis development relies on animal models that show substantial overlap with the human disease. To generate murine models for atherosclerosis, homologous recombination in embryonic stem cells technique has been utilized to produce strains of mice lacking apolipoprotein E (*Apoe*^{-/-}), which plays a role in lipoprotein metabolism. These mice developed spontaneous hypercholesterolemia and atherosclerotic plaque which was exacerbated by an atherogenic diet^{18, 19}. The same technology was used to inactivate the gene for low-density-lipoprotein receptor (LDLR). LDLR deficient mice (*Ldlr*^{-/-}) had lower hypercholesterolemia levels when compared to the *Apoe*^{-/-} mice and only developed atherosclerotic plaque when fed an atherogenic diet²⁰. These experimental animal models have been of great help in showing that signs of inflammation appear hand-in-hand with incipient lipid accumulation in the arterial wall and contribute to each stage of atherosclerosis development. The recruitment of immune cells is essential for plaque formation and progression. In the plaque, about 40% of the cells express macrophage markers and about 10% are CD3+ T cells, which together with the small populations of mast cells, B cells and DCs, traffic between the blood, the damaged arterial wall and the regional lymph nodes¹⁴.

1.1.1 Cellular mechanisms in atherosclerosis

The walls of the blood vessels are composed of many constituents in addition to the endothelium such as matrix basement, smooth muscle cells, and pericytes. These components form a coherent tissue that requires, for proper function, a constant exchange of information via electrical and chemical signals²¹. A well coordinated cell-to-cell communication is pivotal in determining whether these cells will work within the limits of homeostasis or induce pathogenesis of the organ in which they reside.

Endothelial cell dysfunction and activation often induced by hypercholesterolemia and other cardiovascular risk factors, is part of the initial stages of atherosclerosis (Figure 4)²². The alteration of blood flow from laminar to disturbed shear stress at vessel branch points, bifurcations and regions of high curvature, or the accumulation of oxidized lipids causes ECs activation.

This induces a change in the expression of adhesion molecules notably, ICAM-1, VCAM-1, E- and P-selectin, and the synthesis of critical chemokines like CCL2, CXCL8, CCL5 and CCL3 that influence leukocyte recruitment^{23, 24}. The modulation of the endothelial phenotype by disturbed flow requires the integration of numerous mechanotransduction pathways²⁵⁻²⁷. Shear-stress is transmitted from the apical surface of the EC through the cytoskeleton to points of attachment at cell-cell and cell-matrix adhesions²⁸. Although these interactions mediate unique down-stream signaling, integrin and junctional signals activate common effectors that maintain a complex cross-talk, such as NF- κ B, ERK and p38 MAPKs, Akt and Src kinases²⁵. Under high shear conditions and on early atherosclerotic lesions, already adherent leukocytes mediate the capture of other leukocytes from the free flow. Platelets adhered to the vessel wall at sites of EC activation contribute largely to this secondary capture by releasing adhesive ligands, such as P-selectin, microparticles, inflammatory and mitogenic mediators into the local microenvironment, thereby altering the chemotactic and adhesive properties of ECs²⁹.

One of the initial responses to cholesterol accumulation in the intima is the expression of VCAM-1 by endothelial cells³⁰ and its patchy distribution corresponds to the position at which fatty streaks will subsequently be formed³¹. Again, these regions overlap areas where endothelial cells experience disturbed flow resulting in impaired atheroprotective mechanisms³². The activation of ECs induces the recruitment of mainly monocytes and T cells^{3, 8, 33}, but also platelets³⁴. Platelet rolling is initiated by activation-dependent selectins present on endothelial and platelet surfaces³⁵. MCSF produced by ECs and SMCs stimulates the differentiation of monocytes into macrophages and T cells become activated^{10, 36}.

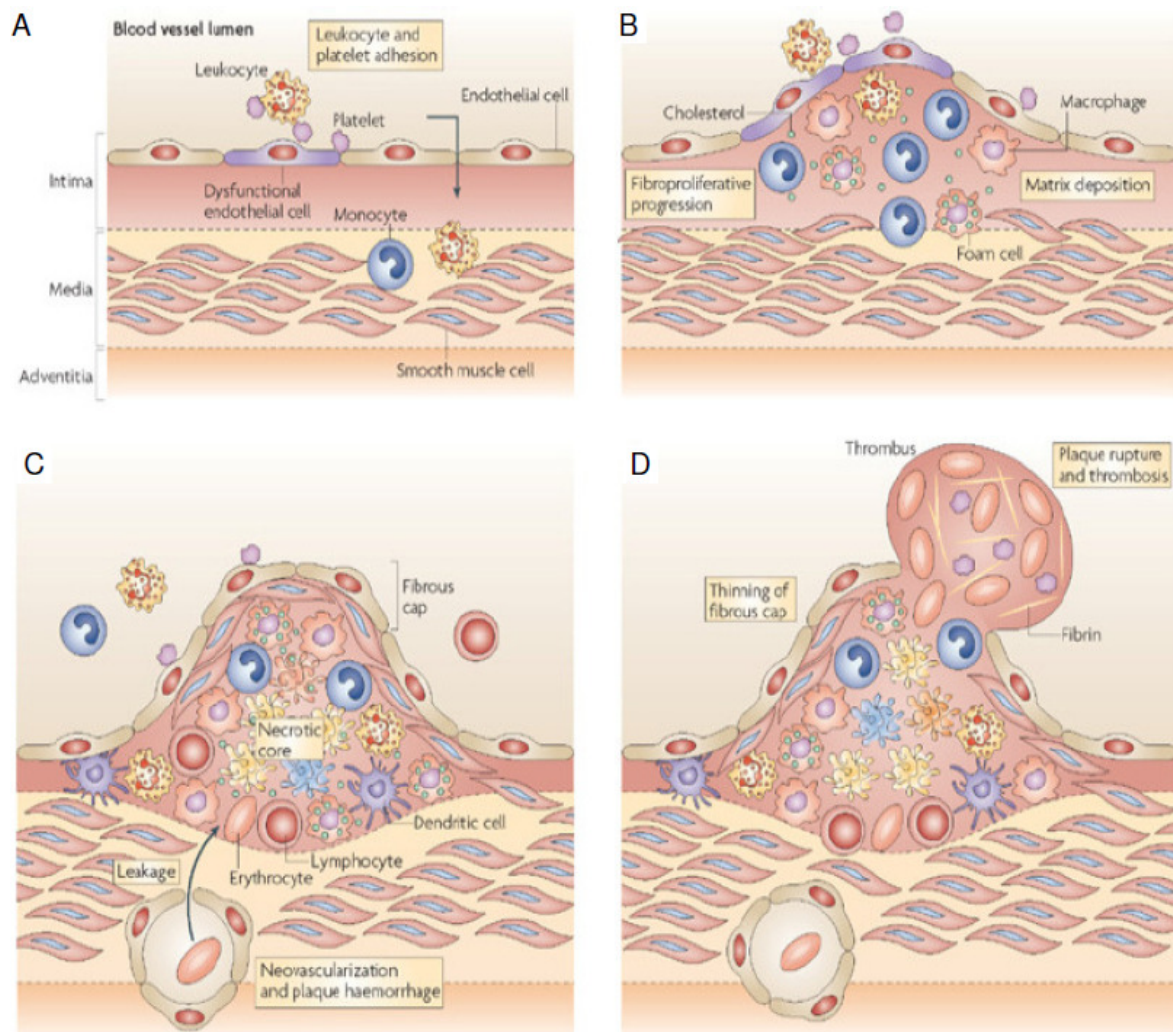


Figure 4 Development of atherosclerosis. A. Hyperlipidaemia and turbulent blood flow at arterial branching points cause endothelial-cell dysfunction and activation leading to expression of adhesion molecules and subsequent adhesion of platelet and leukocyte. B. Monocytes that are recruited to the intima and subintima differentiate into macrophages that accumulate lipids leading to the formation of fatty streaks. Matrix components are deposited and smooth muscle cells are recruited giving rise to the fibroproliferative progression of the plaque. C. Death of lipid-laden cells results in necrotic core and fibrous cap formation. Neovascularization can occur within the plaque and from the adventitia, and leakage of fragile vessels can lead to plaque haemorrhage. D. In unstable plaques, the fibrous cap can rupture exposing thrombogenic material leading to platelet aggregation and thrombus formation which is a risk for myocardial infarction (Taken from Weber *et al.*, Nat. Immunol. 2008)

Chemokines produced by vascular cells that guide the recruitment of leukocytes include CCL2 and its receptor CCR2³⁷, CCL8, CXCL1, CCL3, CCL4, CCL5, CXCL10 and CXCL11³⁸. The mast cell attractant, CCL11³⁹, CXCL16 which can function as scavenger receptor or chemokine⁴⁰ and CX₃CL1 which is a transmembrane protein preferentially expressed by SMCs⁴¹ are crucial in recruiting leukocytes.

Monocyte extravasation is a key step in the development of atherosclerosis. Different subsets of monocytes are recruited into the atheromatous lesion by means of differentially employed CCR2, CCR5, CXCR2 and CX3CR1 chemokines⁴². Of note, circulating monocytes in the mouse exist as two equally abundant major subsets with differing cell surface markers and chemokine receptor expression patterns. There is the $\text{Ly6C}^{\text{high}}\text{CCR2}^+\text{CX3CR1}^{\text{low}}$ and the $\text{Ly6C}^{\text{low}}\text{CCR2}^-\text{CX3CR1}^{\text{high}}$ monocytes subsets⁴³. $\text{Ly6C}^{\text{high}}$ monocytes are short-lived in the circulation and preferentially recruited to the inflamed tissue such as in myocardial infarction⁴⁴ and in early atherosclerotic plaque^{42, 45}. By contrast, Ly6C^{low} monocytes persist longer in the circulation, where they engage in so-called patrolling behavior, interacting with the endothelium without extravasation⁴⁶. Ly6C^{low} monocytes show delayed incorporation into inflamed and damage tissues like the infarcted myocardium⁴⁴. Recruitment of Ly6C^{low} monocytes into atherosclerotic plaques depends on CX3CL1 rather than CCL2, and their mobilization into the circulation depends on CCR5^{42, 47}. Monocytes swimming in the peripheral blood adhere to the surface of ECs which triggers monocytes gene expression programs to upregulate mandatory extravasation-specific genes⁴⁸. This initiates the differentiation of monocytes towards inflammatory macrophages that express a complete array of pattern recognition receptors (PRRs), such as scavenger receptors, CD36, CD68, CXCL16, LOX-1, SR-A and SR-BI^{49, 50}. These receptors mediate the internalization of modified lipoproteins with subsequent cholesterol accumulation and MHC-class-II-restricted antigen presentation, and expression of TLRs that mediate the inflammatory response by activating NF- κ B, and MAPK/AP1 signaling pathways^{51, 52}.

Monocytes can differentiate into two main types of macrophages: classically activated (M1) macrophages that promote inflammation, and M2 macrophages that promote resolution of inflammation⁵³. The precursors of M1 macrophages, $\text{Ly6C}^{\text{high}}$ monocytes, increase in the blood during hypercholesterolemia in mice, partly due to their impaired conversion into less inflammatory Ly6C^{low} monocytes⁴⁵. Models of inflammation have depicted the participation of $\text{Ly6C}^{\text{high}}$ monocytes in the acute response to inflammation, whereas Ly6C^{low} monocytes have been associated with inflammation resolution⁵⁴. An imbalance in the M1/M2 ratio in the advanced stage of atherosclerosis therefore, may partly reflect inflammation resolution⁵⁵. It is however worth noting that the inflammatory process in atherosclerosis is a chronic one and

maximal suppression of atherogenesis might require genetic manipulations that prevent entry of both subsets⁴². Among the factors that can shift the balance in favor of M2 macrophage differentiation are an increase in T helper 2 (TH2) cell-secreted molecules, such as interleukin-4 (IL-4)⁵³, the transcription factors peroxisome proliferator-activated receptor- γ (PPAR γ) and PPAR δ ^{56, 57} and the bioactive lipid sphingosine 1-phosphate (S1P)⁵⁸.

Nonresolving inflammation is a key driver of atherosclerosis, and monocytes continue to infiltrate into subendothelial space where they differentiate into macrophages as atherosclerotic lesions progress. These macrophages contribute to size-independent changes in plaque morphology, notably formation of the necrotic core and thinning of the fibrous cap, which are characteristic features of a vulnerable plaque (Figure 5)⁵⁹. Lesion complication often culminates in rupture of the fibrous cap overlying the lipid core. The integrity of the fibrous cap, and thus its resistance to rupture, depends critically on the collagenous extracellular matrix of the plaque's fibrous cap. This aspect of plaque structure in turn depends upon the balance between synthesis and degradation of the macromolecules that comprise the extracellular matrix of the cap, principally interstitial forms of collagen derived from arterial smooth muscle cells. Collagen breakdown, however, appears to depend critically on macrophages. Plaque macrophages express a variety of matrix-degrading enzymes that can contribute to the weakening of the fibrous cap (Figure 5). In this way, macrophages can critically influence aspects of the biology of human atheroma related to lesion stability⁶⁰.

Another sign of chronic inflammatory state in advanced human plaques is the infiltration of T cells which often accumulate at the site of atheroma rupture¹⁴. Though not as abundant as macrophages, T cells are recruited in parallel with macrophages by a similar mechanism involving adhesion molecules and chemokines¹. It has been suggested that a first round of activation with “athero-antigens” might occur in regional lymph nodes after antigen presentation by DCs migrating from the plaque to the lymph node⁶¹. After entering the blood, previously activated memory/effector T cells bind to plaque-activated ECs and transmigrate into the plaque, where macrophages further activate them by antigen presentation^{3, 61}.

Depletion of CD4⁺ and CD8⁺ T cells has been shown to reduce fatty-streak formation in C57BL/6 mice⁶². In contrast, transfer of CD4⁺ T cells from *Apoe*^{-/-} mice to the severe combined immunodeficient (SCID)/ *Apoe*^{-/-} mice increased the lesion area⁶³.

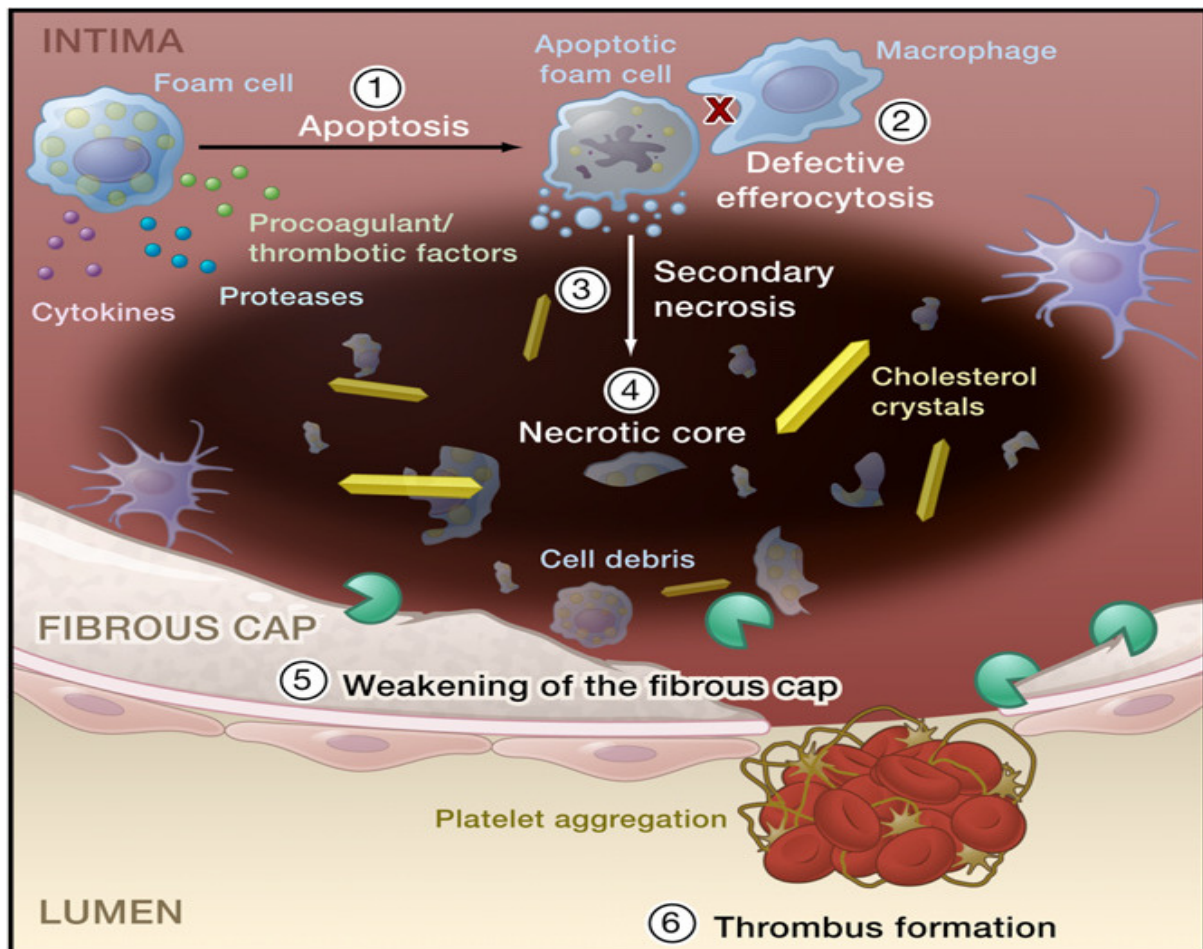


Figure 5 Macrophages in advanced atherosclerotic lesions. (1) Monocytes that are transformed into macrophages in the intima and subintima take up modified lipids leading to foam cell formation and secretion of proteases, cytokines and thrombotic factors. (2) These foam cells are not effectively cleared by active macrophages due to defective efferocytosis (3) leading to secondary cellular necrosis. (4) Secondary necrosis, over time, contributes to the formation of a necrotic core. (5) The fibrous cap formed by smooth muscle cells and matrix deposition weakens when proteases degrade the matrix and smooth muscle cells die. (6) This exposes the thrombogenic material in the lesion causing platelet aggregation and thrombus formation (Taken from Moore *et al.*, Cell 2011).

As depicted in Figure 3, the atherosclerotic plaque is composed of several other immunocytes like B cells, DCs and mast cells. Neutrophils have also been shown and described to initiate innate immunity in atherosclerosis³³. However, the detailed role of these cells in lesion initiation and progression will not be described in the context of this thesis.

Atherosclerosis as already described and also shown in Figure 5 is a chronic inflammatory disease of the vessel wall. Monocyte-derived macrophage foam cells and T-cells in early fatty streak lesions contribute to sustained inflammation, driving a gradual progression towards more complex and eventually rupture-prone lesions^{1, 22}. A key regulator of the inflammatory and immune processes associated with atherogenesis is the transcription factor nuclear factor- κ B (NF- κ B), which controls the expression of pro- and anti-inflammatory genes, cell proliferation and apoptosis¹².

1.2 Nuclear factor-kappa B (NF-κB) transcription factors: regulators of complex biological processes

In 1986, three proteins that showed variable nucleo-cytoplasmic subcellular localization were reported in a prestigious journal. These transcription factors, NF-κB, v-Rel and Dorsal, were shown to bind to a site of murine kappa light chain enhancer (κB site) stimulating its transcription in B lymphocytes⁶⁴⁻⁶⁸. These proteins were later demonstrated to be members of the same family of proteins. The protean nature of NF-κB became evident over time when it was demonstrated to be composed of two subfamilies: NF-κB proteins and the Rel proteins⁶⁹. There is a highly conserved DNA-binding and dimerization immunoglobulin-like domain called Rel Homology Domain (RHD) which is the N-terminal region of the sequence in all three Rel proteins and in both NF-κB proteins. In NF-κB proteins, the dimerization domain is located in the C-terminal region of the sequence, whereas the N-terminal part of the sequence contains the DNA-binding domain. Close to the C-terminal end of the RHD is the nuclear localization signal (NLS) which is pivotal for the transport of active NF-κB complexes into the nucleus. Of note, the NF-κB proteins lack transactivation domain. The Rel subfamily includes c-Rel, RelB, RelA (p65), and Dorsal and Dif in *Drosophila*. These are characterized by a C-terminal Transcription Activation Domain (TAD) that is essential for positive regulation of gene expression, and which is often not conserved at sequence level across species. Posttranslational modification of both RHD and TAD eventually affect the transcriptional activity and the DNA binding of NF-κB. Members of the NF-κB subfamily which are distinguished by a long C-terminal domain that lacks TAD but compensated for by multiples of ankyrin repeats include p105, p100 and *Drosophila* Relish. The NF-κB proteins are processed to shorter and active DNA-binding proteins, p105 to p50 and p100 to p52, after limited proteolysis or arrested translation. Unless associated with members of Rel subfamily, these processed NF-κB proteins may repress transcription due to the lack of TAD. Constitutive binding of p50 or p52 homodimers to κB sites on NF-κB responsive promoters may therefore act to check NF-κB transactivation until competed off by transcriptionally competent dimers⁷⁰ (Figure 6).

In steady-state conditions, NF-κB is sequestered in the cytoplasm through association with one of the typical inhibitor of kappa B (IκB) proteins: IκBα, IκBβ, IκBε and *Drosophila* Cactus. There are two inducibly expressed and atypical IκB proteins,

BCL-3 and I κ B ξ , and an alternative transcript of the NF- κ B1 gene that in mouse encodes I κ B γ , whose biological function is still elusive. A characteristic feature of I κ B proteins is the presence of multiple ankyrin repeat domains which have different affinities for individual NF- κ B dimers (Figure 6). In mammalian cells, the three principal I κ B proteins, I κ B α , I κ B β and I κ B ϵ are regulated by phosphorylation and proteolysis and show differences in their tissue -specific expression patterns.

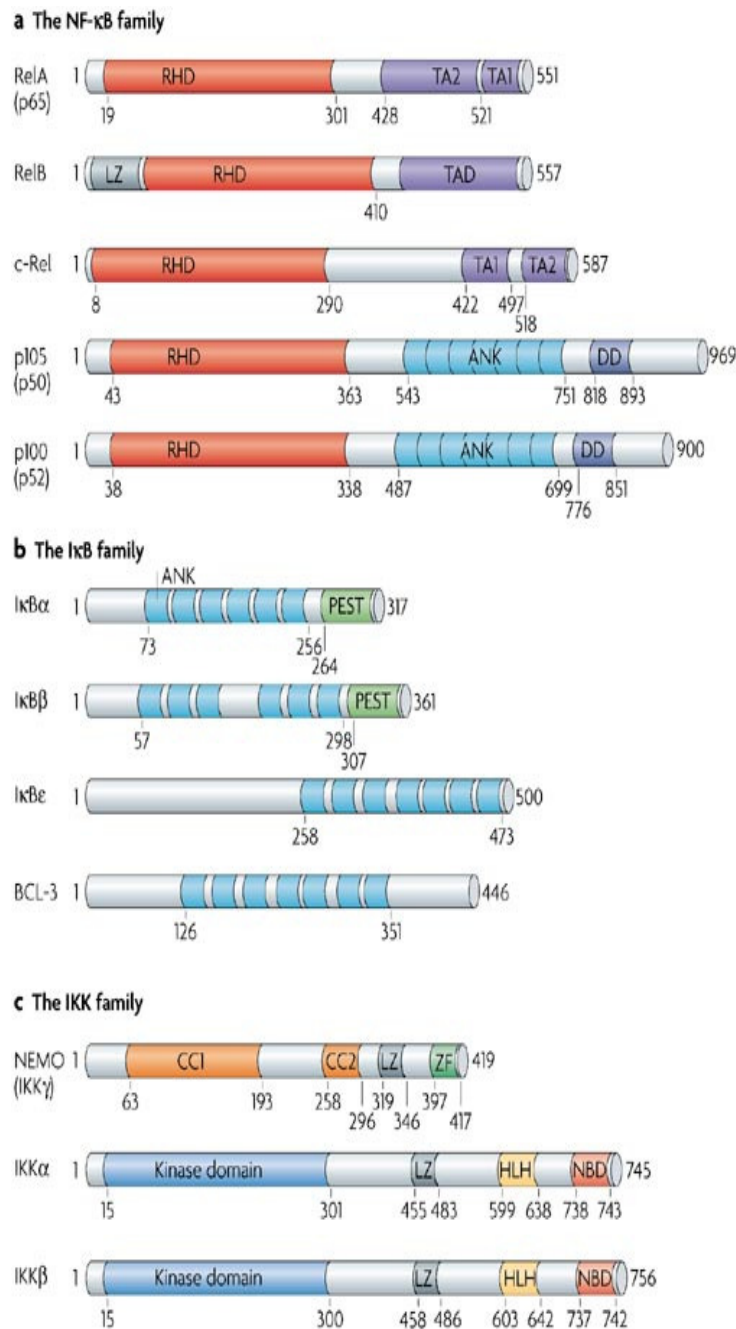


Figure 6 The members of NF- κ B, I κ B α and IKK mammalian family of proteins. Panel A shows five NF- κ B family members: RelA (p65), RelB, c-Rel, p50/p105 (NF- κ B1) and p52/p100 (NF- κ B2). p50 and p52 (not shown) are derived from the longer precursor proteins p105 and p100, respectively in mammalian cells. All NF- κ B family members contain an N-terminal Rel homology domain (RHD) that mediates binding and dimerization of DNA and a nuclear-localization domain. The Rel subfamily, RelA, RelB and c-Rel, contain C-terminal transcriptional activation domains (TADs). TA1 and TA2 are subdomains of the RelA transactivation domain. Panel B shows the inhibitor of NF- κ B (I κ B) family members consisting of I κ B α , I κ B β , I κ B ϵ and BCL-3. Like p105 and p100, the I κ B proteins contain ankyrin-repeat motifs (ANK) in their C termini. Panel C shows the three core subunits of the I κ B kinase (IKK) complex: the catalytic subunits IKK α and IKK β and the regulatory subunit called the NF- κ B essential modifier (NEMO, also known as IKK γ). The principal structural motifs of each protein are shown, together with amino-acid numbers corresponding to the human proteins, although some definitions of where a domain begins and ends might differ between publications. CC, coiled-coil; DD, region with homology to a death domain; HLH, helix-loop-helix; LZ, RelB-transactivation-domain containing a putative leucine-zipper-like motif; NBD, NEMO-binding domain; PEST, domain rich in proline (P), glutamate (E), serine (S) and threonine (T); ZF, zinc-finger domain (Perkins ND, Nat Rev Mol Cell Biol. 2007).

The classical model of I κ B function depicts that I κ B α , the most studied member, retains NF- κ B, mainly p65/p50 dimers, in an inactive conformation in the cytoplasm preventing their nuclear translocation and subsequent DNA binding. Biochemical and structural studies have determined that I κ B α makes multiple contacts with NF- κ B. These associations mask the nuclear localization sequence (NLS) located in the RHD of p65 retaining the I κ B α /NF- κ B complex in the cytoplasm. The crystal structure of I κ B α bound to p65/p50 heterodimer reveals that I κ B α only masks the NLS of p65 whereas that of p50 remains exposed. The exposed NLS of p50 acts in synergy with the nuclear export sequence (NES) in both I κ B α and p65 to constantly shuttle I κ B α /NF- κ B complexes between the nucleus and the cytoplasm, despite steady-state localization that appears almost exclusively cytosolic⁷¹. The atypical I κ B proteins, BCL3 and I κ B ξ , regulate NF- κ B in a different but non-opposing manner. BCL3 has been shown to associate with p50- and p52-containing dimers. BCL3 contains a TAD that may mediate the release of transcriptional repression by removing p50 homodimers from κ B sites. Alternatively, BCL3 may also stabilize repressive p50 homodimers and inhibit NF- κ B activation by preventing its access to κ B sites⁷². I κ B ξ has weak homology to other I κ Bs and is more similar to BCL3. I κ B ξ is upregulated in response to IL-1 and TLR4 ligands but not TNF α . Upon expression I κ B ξ remains localized in the nucleus associated primarily with p50 homodimers and is required for the regulation of a subset of LPS- or IL-1-induced target genes⁷³.

A wide variety of pro-inflammatory signals trigger NF- κ B activation through differential signaling pathways, the canonical and the non-canonical pathways, which all converge on the activation of the I κ B kinase (IKK) complex, consisting of two catalytically active components (IKK1/IKK α and IKK2/IKK β) and one regulatory component (IKK γ /NEMO)⁷⁴.

1.2.1 Differential NF- κ B activation signaling cascade

The activation of NF- κ B signaling has been described for two well distinct signaling pathways: the canonical or the classical and the non-canonical or alternative pathways. The canonical pathway is activated by a wide range of inflammatory stimuli including pro-inflammatory cytokines such as tumor necrosis factor- α (TNF α) and interleukin-1 (IL-1), engagement of T-cell receptor (TCR) or exposure to bacterial

products such lipopolysaccharide (LPS)⁷⁵ (Figure 7). These extracellular stimuli can activate cells through their respective receptors, which will induce the activation of a couple of signal transduction pathways which will all eventually converge on the activation of the IKK complex. This complex consists of two homologous subunits with kinase activity, IKK1/IKK α and IKK2/IKK β , and a regulatory subunit IKK γ /NEMO. The subunits with kinase activity share 52% overall sequence identity with a greater degree of homology in the catalytic domain. The regulatory subunit is not related to the catalytic subunits and contains a C-terminal Zn finger-like domain, a leucine zipper and coiled-coil domains at the N and C terminus⁷⁶. Although generally found in heteromeric kinase complex, IKK α and IKK β are selectively required for specific NF- κ B signaling cascades. In most canonical NF- κ B signaling, IKK β is necessary and sufficient for phosphorylation of I κ B α on serine residues 32 and 36, and of I κ B β on serine residues 19 and 23. This phosphorylation induces ubiquitination which is followed by proteasomal degradation. While not always required for I κ B α phosphorylation and degradation in canonical NF- κ B signaling, IKK α can mediate I κ B α phosphorylation and might play a critical role in certain canonical NF- κ B-dependent transcriptional responses⁷⁷. The inducible degradation of NF- κ B inhibitory proteins, particularly I κ B α , results in a subsequent nuclear translocation of NF- κ B transcription factors of which p50/p65 dimers are the predominant⁷⁸.

The non-canonical NF- κ B pathway is triggered by several extracellular stimuli that signal through specific set of receptors including TNFR superfamily members like B-cell-activating factor belonging to TNF family receptor BAFFR^{79, 80}, CD40⁸¹, lymphotoxin β -receptor (LT β R)⁸² and receptor activator for nuclear factor κ B (RANK)⁸³. Ligand binding to these receptors results in signaling that leads to specific biological roles depending on the kind of ligand-receptor mediating the signaling. A characteristic feature of these receptors is the presence of a TRAF-binding motif which recruits members of the TRAF family of proteins to the receptor complex during ligand ligation⁸⁴. The TRAFs recruited to the signaling receptor are targeted for degradation by cellular inhibitor of apoptosis 1 (cIAP1)-mediated ubiquitination triggering the release and stabilization of NF- κ B-inducing kinase (NIK)⁸⁵. NIK, a central mediator of the activation of the non-canonical NF- κ B pathway, is constitutively degraded preventing the basal activation of this pathway.

However upon stabilization, NIK accumulates and activates IKK α which triggers the phosphorylation and subsequent processing of p100 resulting in the activation of the non-canonical NF- κ B pathway⁸⁶(Figure 7). Activation of the non-canonical NF- κ B pathway regulates specific biological processes such as lymphoid organ development, B cell maturation and bone metabolism⁸⁷.

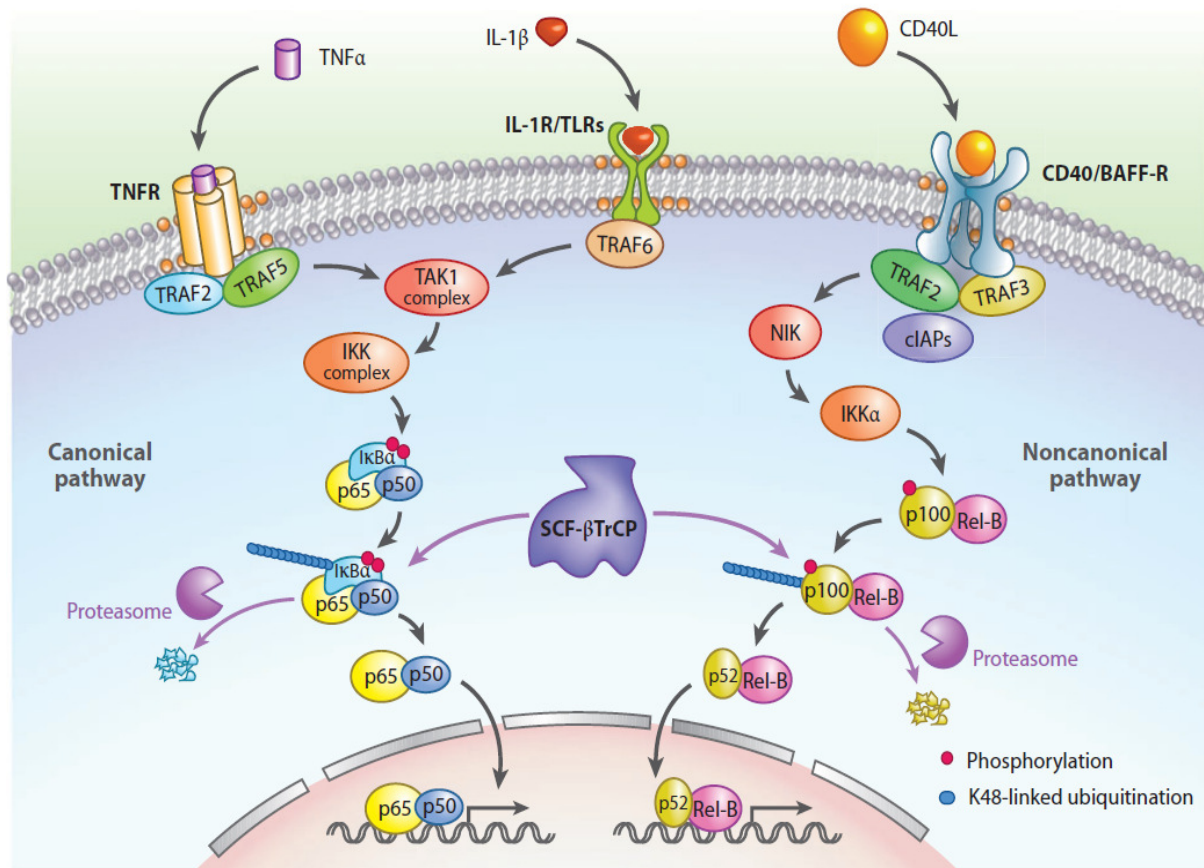


Figure 7 Canonical and non-canonical pathways of NF- κ B activation. The engagement of TNFR, IL-1R, and TLRs by their specific ligands leads to the recruitment of TRAFs to the signaling receptor and subsequent activation of TAK1 in the canonical pathway or NIK in the non-canonical pathway. In the canonical pathway, TAK1 phosphorylates IKK in its activation loop leading to the activation of IKK which then phosphorylates I κ B α . Phosphorylated I κ B α is targeted by specific SCF- β TrCP E3 ligase complex inducing the proteasomal degradation of I κ B α resulting in rapid and transient nuclear translocation of NF- κ B. The non-canonical pathway relies on phosphorylation-induced processing of p100 which is triggered by NIK-induced activation of IKK α . Phosphorylation of p100 by IKK α targets p100 for ubiquitination by SCF- β TrCP E3 ligase complex and sequential processing of p100 to p52. Dimers of p52/RelB translocate into the nucleus to participate in gene transcription (Taken from Skaug *et al.*, Annu. Rev. Biochem. 2009).

1.2.2 Some key players in NF- κ B signal transduction pathway

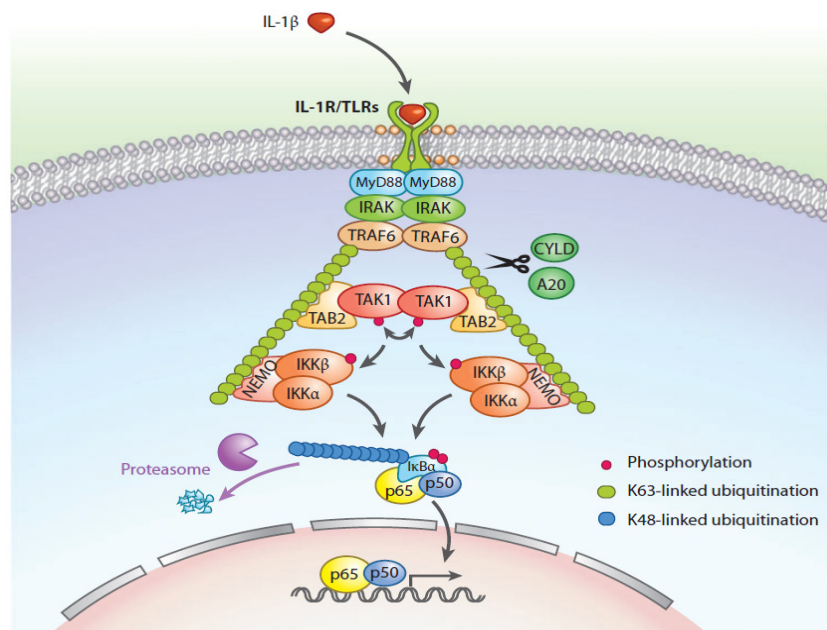
Tumor necrosis factor (TNF) receptor-associated factors (TRAFs) represent important signal transducers for the TNF/IL-1/Toll-like receptor (TLR) superfamily and with the exception of the DNA-damage response, are required for all NF- κ B signaling pathways⁸⁸. All TRAF proteins share the C-terminal TRAF domain consisting of a coiled-coil domain that mediates protein-protein interactions. TRAFs 2-7 have N-terminal RING finger domains that may function as E3 ubiquitin ligases catalyzing the transfer of ubiquitin to target proteins. This E3 ubiquitin ligase activity plays a central role in the activation of the IKK complex leading to overall activation of both the canonical and non-canonical NF- κ B signaling cascade⁸⁹. In particular, TRAF6 has been shown to be an E3 ubiquitin ligase that catalyzes the synthesis of a unique polyubiquitin chain required for IKK activation⁹⁰. TRAF6-catalyzed polyubiquitination leads to activation of TAK1 which in turn phosphorylates IKK in the activation loop leading to the activation of IKK⁹¹ (Figure 8A). In the non-canonical pathway, TRAF3 regulates the activity of NIK through constitutive association which results in constitutive ubiquitination and rapid turnover of NIK. Receptor-induced degradation or sequestration of TRAF3 precedes processing of p100 due to an increase in the steady-state amount of NIK when TRAF3 becomes a bystander⁹². Accumulated NIK activates IKK α which drives the activation of the non-canonical NF- κ B pathway. TRAF2 has been proven to recruit the IKK complex to the activated TNF receptor 1 (TNFR1), through interaction with the LZ motifs of IKK α and IKK β ⁹³. Recruitment and subsequent activation of IKK is mediated by other key molecules of the NF- κ B signal transduction pathway, RIPs (receptor interacting proteins), which act with TRAF proteins upstream of IKK⁹⁴ (Figure 8B).

RIPs are characterized by their conserved serine/threonine kinase domains and are implicated in most canonical NF- κ B-TRAF-dependent and -independent pathways⁹⁵.⁹⁶ Among the family of RIP proteins, only RIP1 has been shown to be required for TNFR-dependent NF- κ B activation. In TNFR1 signaling, RIP1 binds to NEMO upon receptor stimulation and mediates IKK recruitment to the TNF receptor⁹⁷. In the absence of RIP1, recruitment of IKK occurs through TRAF2 but does not result in activation of IKK⁹³ suggesting a role for the kinase activity of RIP1 in activation of IKK. However, the kinase activity of RIP1 has been shown to be dispensable for the activation of NF- κ B⁹⁸.

There are speculations that because RIP1 is ubiquitinated during TNFR1 activation and TRAF2 seems to be involved in this process, TRAF2-dependent ubiquitination of RIP1 might be involved in the formation of a stable supramolecular complex between TRAF2, RIP1, and IKK. This complex will then induce the activation of IKK through oligomerization of NEMO and subsequent autophosphorylation of IKK⁹⁹. In antigen receptor signaling, RIP2 is known to be required for BCL10-mediated NF- κ B signaling¹⁰⁰. RIP2 binds to NEMO and acts with TAK1 and TRAFs to induce NEMO-ubiquitination and down-stream signaling^{101, 102}. Several kinases are involved in the signaling to IKK downstream of RIPs and TRAFs. In the canonical NF- κ B pathway, TAK1 serves as the direct upstream kinase of IKK β whereas in the non-canonical pathway NIK phosphorylates IKK α resulting in its activation.

Activation of IKK in the canonical pathway is initiated by K63-linked polyubiquitination of IKK γ /NEMO which functions as a binding site for other signaling molecules required for activation of IKK β ¹⁰³⁻¹⁰⁵ (Figure 8A). Depending on the upstream signaling, different NEMO ubiquitin E3 ligases are activated causing ubiquitination at different lysine residues in NEMO. In TCR signaling, activation of T-cell receptor results in BCL10/MALT-1/Carma1-mediated TRAF6-dependent K63-linked ubiquitination of NEMO at lysine 399¹⁰⁶. In response to TNF α and IL-1, TRAF6, 2 and 5 mediate the polyubiquitination of NEMO whereas bacterial infection leads to RIP2-dependent K63-linked polyubiquitination of IKK γ /NEMO¹⁰¹. Ubiquitin-based modifications serve to oligomerize and activate members of the IKK signaling pathway leading to recruitment of IKK activating kinases to the complex which will phosphorylate IKKs for maximal activation¹⁰⁵.

A



B

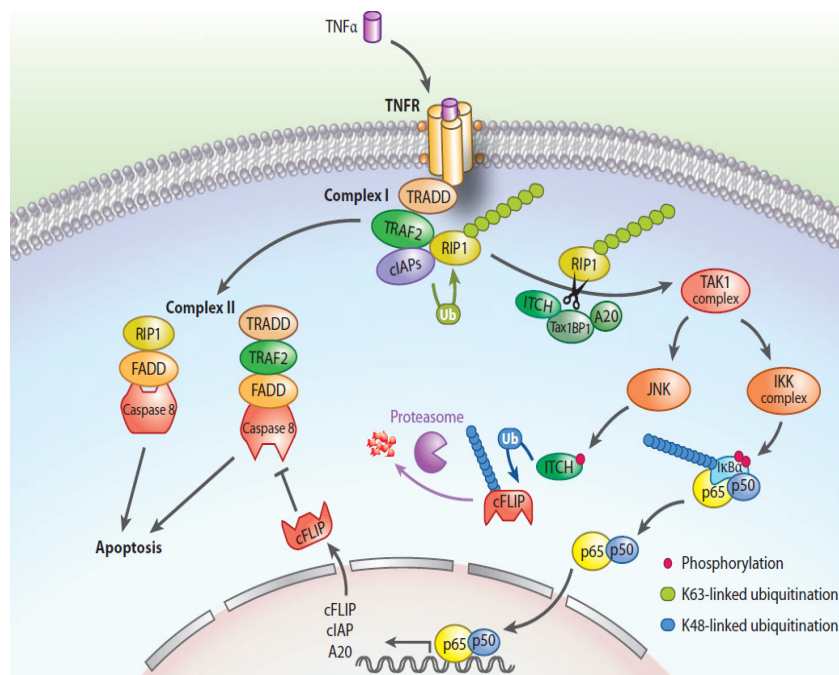


Figure 8 Role of ubiquitination in the canonical NF- κ B signaling cascade. A. Ligation of IL-1R and TLRs to their specific ligands induces the recruitment of MyD88, IRAK, and TRAF6 to the receptor. TRAF6 catalyzes the synthesis of K63-linked polyubiquitin chains which serve as scaffold to recruit TAK1 and IKK complexes thereby inducing autophosphorylation of TAK1. TAK1 in turn phosphorylates IKK β leading to phosphorylation and subsequent degradation of I κ B α . B. Binding of TNF α to TNFR, however, leads to the recruitment of TRADD, TRAF2, cIAP1 and cIAP2, and RIP1. TRAF2 and cIAPs mediate K63 polyubiquitination of RIP1, which recruits and activates the TAK1 kinase complex. TAK1 phosphorylates IKK resulting in the activation of NF- κ B. (Taken from Skaug *et al.*, Annu. Rev. Biochem. 2009).

In the non-canonical pathway, TRAF3 induces ubiquitination and subsequent degradation of NIK but TRAF3 undergoes receptor-induced sequestration upon stimulation resulting in the accumulation and activation of NIK⁹². NIK then phosphorylates its direct downstream target, IKK α , at serine residues 176 and 180 within its activation loop leading to activation of IKK α . The activation of IKK is a transient event that requires negative feedback regulation. Evidence exists for an intrinsic negative feedback loop of IKK through phosphorylation of the C-terminal NEMO binding domain (NBD) of IKK. Phosphorylation of NEMO at serine 68 and IKK NBD induces structural changes that may allow subsequent dephosphorylation of the activation loop serines in IKK as well as of N-terminal phosphorylation sites in NEMO by protein phosphatase 2A (PP2A) or PP2C β ¹⁰⁷⁻¹⁰⁹.

1.2.3 Activation and regulation of the transcription factor NF- κ B

The activation of the transcription factor NF- κ B in the canonical pathway is driven by IKK β -mediated phosphorylation of conserved serine residues in the three major cytoplasmic inhibitors of NF- κ B. These inhibitory proteins, I κ B α , β and ϵ , are then polyubiquitinated in a K48-linked manner by β TrCP containing Skp1-culin-Roc1/Rbx1/Hrt-1-F-box (SCF) E3 ubiquitin ligase complexes (SCF ^{β TrCP}) which is followed by proteasomal degradation¹¹⁰. Degradation of I κ B α occurs rapidly in cells whereas that of I κ B β and ϵ is delayed or does not occur¹¹¹. In response to stimuli such as hypoxia, hydrogen peroxide and tyrosine phosphatase inhibitor pervanadate, I κ B α is phosphorylated by tyrosine kinases. This phosphorylation of I κ B α at tyrosine-42 by putative kinases like c-Src leads to dissociation and subsequent degradation of I κ B α ¹¹⁰. Regulation of NF- κ B inhibitory proteins represents a crucial mode of controlling the activation of NF- κ B. Thus, these mechanisms described for I κ B α degradation depict a way of fine-tuning NF- κ B activation in the canonical pathway. Interestingly, there is a rapid transcription of I κ B α upon NF- κ B activation that serves as a negative feedback loop and terminates the response of NF- κ B¹¹². In the non-canonical pathway, phosphorylation of p100 within its C-terminal domain⁷⁴ creates an SCF ^{β TrCP} binding site, similar to those found in I κ Bs. This triggers ubiquitination and partial proteasomal degradation of p100 with p52 as the end product^{113, 114}. This mechanism for generating p52 contrasts that for p50 in the canonical pathway as p50 is co-translationally generated in a constitutive manner. Although IKK β -mediated inducible processing of p105, the precursor for p50, can occur, it often leads to

complete degradation of the protein without generation of p50. However, IKK β can also induce SCF ^{β TrCP}-independent processing of p105 leading to the formation of p50^{115, 116}. This mechanism though dependent on the ubiquitin system does not involve the ubiquitination of p105¹¹⁶.

Another posttranslational modification described to affect the activity of RelA/p65 is acetylation¹¹⁷. The coactivators p300 and CBP can acetylate p65 at lysine residues 218, 221 and 310. Acetylation of p65 at lysines 218 and 221 enhances its DNA binding and inhibits its association with newly synthesized I κ B α whereas acetylation of lysine-310 activates p65 without interfering with its ability to bind DNA or I κ B¹¹⁸. This way of activating p65 can be abrogated through co-repressor-mediated deacetylation of the acetylated residues¹¹⁹. In contrast, lysine residues 122 and 123 of p65 can also be acetylated and if they do, results in decreased affinity of p65 for DNA binding. Phosphorylation of p65 can result in increased p300 binding to p65 that may lead to enhance acetylation of lysine residues in p65^{119, 120}. Termination of NF- κ B transcriptional response has also been shown by phosphorylation of p65 at serine-536 by IKK α that might results in the degradation of p65¹²¹. Finally, cross-talk between NF- κ B and other transcription factors such as PPARs^{122, 123}, enhancer binding proteins- β ^{124, 125}, AP-1¹²⁶ and Janus family of kinase (Jak)-STAT¹²⁷ can also affect the NF- κ B signaling pathway. These complicated regulatory mechanisms contribute to the complex cellular responses of NF- κ B activation.

1.3 The NF- κ B pathway in physiology and inflammation

More than two decades of research in NF- κ B signaling has resulted in extensive evidence that shows a pivotal role for this family of transcription factors in the immune system, cancer biology and atherogenesis. The role of NF- κ B in the immune response is broad and expanding. It has been shown to be involved in the development of lymphoid organs and hematopoiesis. This extends to the elaboration of immediate antimicrobial responses mounted by pattern recognition receptors (PRRs) such as TLRs after they have recognized pathogens. There is a subsequent progression of inflammatory response until the elaboration of adaptive immune responses that can further develop into cancer¹²⁸⁻¹³⁰. Several studies using gene targeting and inhibitors have suggested a causative role of the transcription factor NF- κ B in the inflammatory response.

Importantly, NF- κ B is responsible for the transcription of genes encoding many of the pro-inflammatory cytokines and chemokines that orchestrate the recruitment of leukocytes. Expression of adhesion molecules that mediate the extravasation of leukocytes to the site of infection are also induced by activation of NF- κ B. Numerous genes that regulate cellular differentiation, survival and proliferation of vascular and immune cells involved in inflammatory response are target of NF- κ B¹². NF- κ B signaling mediates the effects of important pro-inflammatory cytokines such as TNF α or IL-1. There is a cross-talk between IKK/ NF- κ B and interferon pathways that results in the release of dominant cytokines in chronic inflammatory states¹³¹. NF- κ B is therefore crucial in the propagation of the inflammatory response and also the resolution that might follow. Ablation of p65 and TNFR1 in mice showed increased susceptibility to bacterial infection demonstrating the dependence of the early innate response on the canonical NF- κ B pathway¹³².

In a similar vein, IKK β ^{-/-}/TNFR1^{-/-} mice showed a pronounced effect in innate responses and succumbed to infection even more rapidly than p65^{-/-}/TNFR1^{-/-} mice, corroborating the role of the canonical NF- κ B pathway in early innate response¹³³⁻¹³⁵. Inactivation of RelB in mice resulted in a complex inflammatory phenotype including infiltration of T cells in multiple organs and pronounced defects in hematopoiesis^{136, 137}. The combined deficiency of RelB and p50 exacerbates the severity of organ inflammation observed in RelB knockout mice¹³⁸. In a model of chronic liver disease, the absence of p50 homodimers in NF- κ B1 knockout mice resulted in increased susceptibility to hepatic inflammation and fibrosis. This demonstrates the role of p50-containing dimers in limiting inflammatory response through modulation of pro-inflammatory gene expression such as TNF α ¹³⁹. The pro-survival role of NF- κ B for hepatocytes has also been reported in NEMO-deficient hepatocytes¹⁴⁰. Studies in models of liver disease have demonstrated NF- κ B to constitute a link between chronic inflammation and cancer^{141, 142}. A transgenic model of IKK2 overexpression in pancreas identified the IKK/NF- κ B axis in acinar cells as being key to the development of experimental pancreatitis and the major factor inducing the inflammatory response typical of this disease¹⁴³.

The transcription factor NF- κ B also has an active role in the resolution of inflammation and tissue repair¹⁴⁴. Cells such as macrophages can become resistant

to repeated pro-inflammatory stimuli such as LPS. LPS tolerance involves the activity of BCL3 that is induced late in LPS stimulation. BCL3 acts in synergy with p50 dimers to selectively affect chromatin remodeling of pro-inflammatory genes and also facilitate the expression of anti-inflammatory gene IL-10¹⁴⁵. Of note, expression of IFN γ and proliferation of NK cells are negatively regulated by p50¹⁴⁶. Importantly, it has been shown that inhibition of NF- κ B during the resolution phase may prolong the inflammatory process and prevent tissue repair¹⁴⁷. In models of local and systemic inflammation, genetic deletion of IKK α in macrophages resulted in increased production of pro-inflammatory cytokines and chemokines indicating an increased inflammatory response. These studies suggested that IKK α negatively regulates expression of these target genes through mediating the degradation of p65 and c-Rel following activation of macrophages by LPS^{121, 148}. A recent study where IKK β was selectively deleted in the myeloid lineage, suggested an anti-inflammatory role for IKK β by inhibiting activation of macrophages through cross-talk with the Stat-1 pathway¹³¹. This was described to prevent the overactivation of macrophages during infection and thereby contributing to resolution of inflammation. In an acute inflammatory state, activation of NF- κ B mainly involves p50/p65 heterodimers which induces transcription through the recruitment of coactivators such as CBP/p300. During the resolution phase of inflammation, p50 homodimers mediate transcription of anti-inflammatory cytokines such as IL-10. Homodimers of p50 can also displace p50/p65 heterodimers on the promoters of pro-inflammatory genes, and recruit corepressors such as HDAC-1 leading to repression of transcription. These concerted events ensure that inflammation does not persist though a more complex mechanism in which signaling cross-talks might have a fine-tuning modulation of the inflammatory response has been postulated^{131, 149-151}.

1.3.1 TNF as a promoter of the inflammatory response

The potent pro-inflammatory cytokine TNF) is a member of the TNFR superfamily which consists of a number of receptors and ligands that exhibit a remarkable diversity in tissue distribution and physiology¹⁵². TNF is primarily produced as a type II transmembrane protein arranged in stable homotrimers^{153, 154}. From this membrane-integrated form, the soluble homotrimeric cytokine (sTNF) is released via proteolytic cleavage by the metalloprotease TNF alpha converting enzyme

(TACE)¹⁵⁵. The soluble 51 kDa trimeric sTNF tends to dissociate at concentrations below the nanomolar range, thereby losing its bioactivity¹⁵².

The 17 kDa TNF protomers are composed of two antiparallel β -pleated sheets with antiparallel β -strands, forming a 'jelly roll' β -structure, typical for the TNF ligand family, but also found in viral capsid proteins¹⁵⁶. The members of the TNF ligand family exert their biological functions via interaction with their cognate membrane receptors, comprising the TNF receptor (TNF-R) family¹⁵⁷. TNF family of receptors lack intrinsic enzymatic activity, signaling is therefore achieved by recruitment of intracellular adapter molecules that associate with the cytoplasmic tail of the receptor in a signal-dependent manner. Two receptors, TNFR1 and TNFR2, bind membrane integrated TNF as well as soluble TNF, but also the secreted homotrimeric molecule lymphotoxin- α (LT α)^{158, 159}. TNFR1 is constitutively expressed in most tissues but the expression of TNFR2 is highly regulated and is restricted to immunocytes. Despite the variety of biological responses, TNF signaling converges on activation of NF- κ B and AP-1. The recruitment of TNFR1 to membrane microdomains (lipid rafts) with subsequent assembly of a complex is necessary for signaling to NF- κ B¹⁶⁰. TNF α binds to TNFR1 inducing aggregation of the receptor which allows binding of TNFR-associated death domain protein (TRADD). TRADD subsequently recruits adapter proteins that are necessary for the activation of IKK, p38 and JNK¹⁶¹ (Figure 8B and 9). TNFR1 signaling can induce both cell death and survival, and this involves participation of both TNF transcription factors, NF- κ B and AP-1 (Figure 9). Blocking NF- κ B activation results in TNF becoming a potent inducer of apoptosis as indicated by either p65/TNFR1¹³² or IKK β /TNFR1¹³³ double knockout mice. TNF transiently activates both JNK and p38 with prolonged activation under apoptotic conditions¹⁶². TNFR2 signaling is mediated by the recruitment of TRAF adaptor proteins mainly TRAF1 and TRAF2 which results in the activation of kinases like IKK, MKK3 or JNK. This triggers the activation of TNF transcription factors that induce the expression of target genes important for cell survival¹⁶³ (Figure 9).

Although TNF receptors are differentially expressed on a wide range of cells and tissues, many of the proinflammatory effects of TNF can be explained on the basis of TNF's effect on vascular endothelium and endothelial/leukocyte interactions. In response to TNF, endothelial cells promote inflammation by displaying, in a distinct

temporal, spatial and anatomical pattern, different combinations of adhesion molecules for leukocytes¹⁶⁴⁻¹⁶⁶.

These include E-selectin, ICAM-1 and VCAM-1 which act in synergy with chemokines like CCL2 and CXCL8 to recruit different leukocyte populations to the intima independent of antigen recognition¹⁶⁷⁻¹⁶⁹. Many of the classical features of inflammation can be produced by local effects of TNF on endothelial cells.

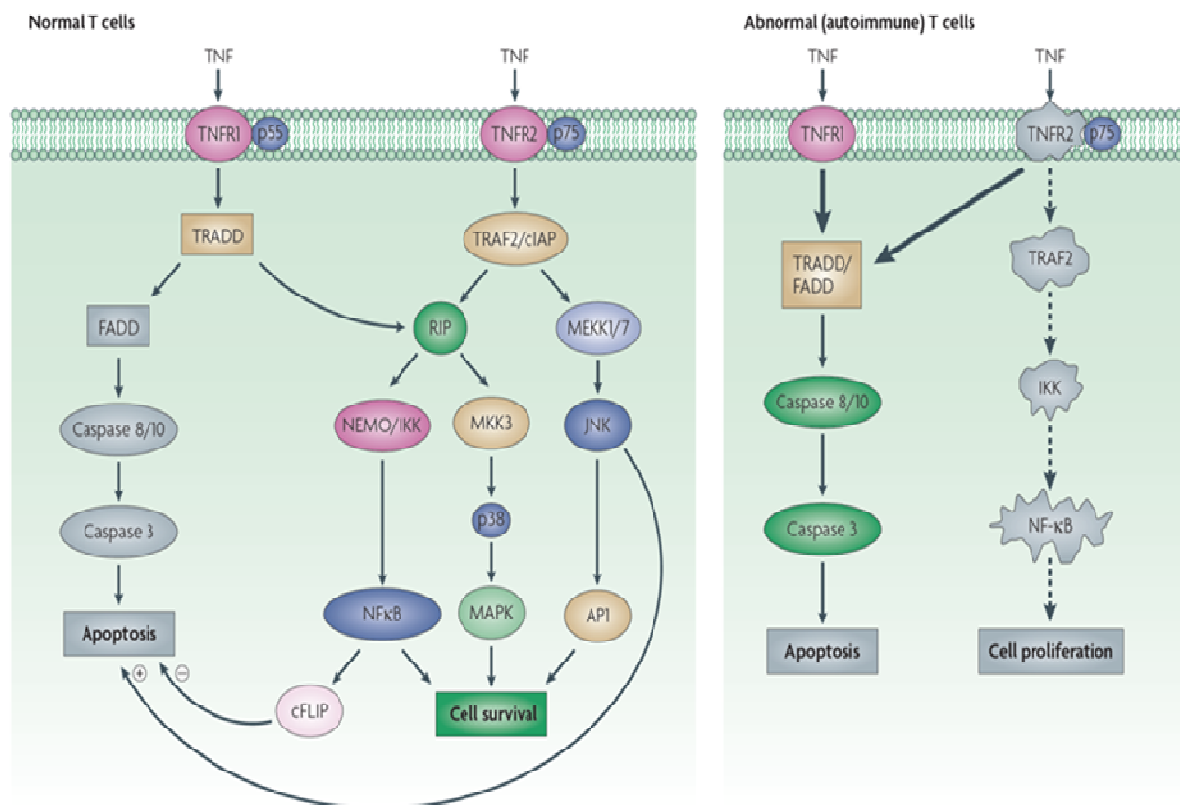


Figure 9 TNF signaling through TNFR1 and TNFR2 in T cells. TNF binds to its receptors, TNFR1 and TNFR2, triggering two separate intracellular signaling cascade. In normal T cells, binding of TNF to TNFR1 leads to the activation of signaling cascade that can result in apoptosis. This is not only cell type specific but also dependent on the state of activation of the cell and the cell cycle. In contrast, engagement of TNFR2 by TNF drives cell survival pathways that can lead to cell proliferation. In autoimmune T cells, there are defects in TNFR2 signaling pathway at different levels along the signaling cascade thereby dampening cell survival and proliferation effects. This forces autoimmune T cells to preferentially use TNFR1 signaling and the outcome is apoptosis. (Taken from Faustman *et al.*, Nat Rev Drug Discov. 2010).

TNF-induced expression of cyclooxygenase 2 can increase endothelial cells production of vasodilatory PGI₂ resulting in vasodilatation causing 'rubor' and 'calor' through increased local blood flow¹⁷⁰. Tumor can result from TNF-mediated increased vascular permeability, allowing the increased trans-endothelial passage of fluid and macromolecules to create edema. In addition, TNF-induced expression of pro-coagulant proteins, such as tissue factor, and down-regulation of anticoagulant proteins, such as thrombomodulin TNF, can cause intravascular thrombosis¹⁷¹.

1.4 The Vascular endothelium in inflammation

The vascular endothelium is central to the cellular and molecular events that take place during the inflammatory response. It is the continuous non-thrombogenic single-cell lining of the cardiovascular system that forms an interface between the blood and its components on one side and the tissues and organs on the other¹⁷². Vascular endothelial cells (ECs) possess an innate heterogeneity, expressed by differences in their structure and function according to the vessel they are lining or tissues in which they reside. As such, major qualitative differences exist between macro- and micro-vascular endothelial cells¹⁷³. Vascular ECs constitute a selective semi-permeable barrier endowed with a machinery of caveolae, coated-pits, and vesicles to actively sort and gate permeant molecules. ECs react to physical and chemical stimuli occurring in plasma and interstitial fluid, regulating the hemostasis, vasomotor tone, and the immune response^{172, 174}. Endothelial cells support all the various phases of the inflammatory process by changing their phenotypes concomitantly as the inflammatory response evolves¹⁷⁵. Three main components of ECs activation underlie the onset and progression of inflammation: an increase in local blood flow, accounting for the red color (rubor) and warmth (calor) of inflamed tissues; a localized leakage of plasma protein-rich-fluid into the tissue, accounting for the swelling (tumor) of inflamed tissues; and a localized recruitment and activation of circulating leukocytes. Sustained activation of ECs nevertheless leads to deleterious functions that contribute to the development of immune/inflammatory-mediated disorder diseases, such as rheumatoid arthritis, inflammatory bowel disease or atherosclerosis¹⁷³.

Acute inflammation is a result of endothelial cell activation which can be divided into rapid responses that are independent of new gene expression, type-I activation, and slower responses that depend on new gene expression, type-II activation. Whereas type-I activation is typically mediated by ligands that bind to G protein-coupled receptors (GPCRs) such as histamine H1 receptors, type-II activation is mediated by TNF α , IL-1, and other inflammatory cytokines derived from leukocytes. Signaling through GPCRs leads to transient intracellular elevations of Ca²⁺ and activation of the Ras homolog (RHO) pathway, which combine to mediate the type-I activation response. Increased blood flow results from Ca²⁺-mediated release of arachidonic acid from membrane phosphatidylcholine and its rapid conversion to prostaglandin I₂ (PGI₂), which is a muscle vascular tone. In addition, the cytosolic Ca²⁺-calmodulin complex activates endothelial nitric oxide synthase (eNOS) to produce NO, the main EC vasodilator that acts in concert with PGI₂¹⁷⁵. Vascular leakiness results from activation of myosin-light-chain kinase (MLCK) and this mediates the contraction of actin filaments that are attached to tight and adherens junction. This results in the opening of gaps between adjacent endothelial cells leading to the leakage of plasma proteins from the blood into the tissues. In the tissues, plasma proteins assemble into a provisional matrix that supports the attachment, survival and migration of leukocytes¹⁷⁶.

A fundamental event in inflammatory response is the recruitment of leukocytes from the blood to the site of injury or infection¹⁷⁷. Leukocyte recruitment is a sequential cascade of events involving leukocyte and endothelial cell adhesion molecules that support leukocyte tethering and rolling, firm adhesion, and transendothelial migration under laminar shear stress¹⁷⁸. Cell adhesion molecules synergize with chemokines to guide this process, and their combination specifies the recruitment of the appropriate leukocytes to the site of inflammation¹⁷⁹. Additionally, the activation of myosin-light-chain (MLC) causes the release of P-selectin from specialized secretory vessels, known as Weibel-Palade bodies (WPBs). Concomitantly, an endothelial-cell-derived acyl form of platelet activating factor (PAF) is generated which acts with P-selectin to initiate extravasation of neutrophils. Firm adhesion of leukocytes on the surface of ECs via endothelial adhesion molecules and leukocyte integrins is a key step that initiates intracellular signaling events that facilitate leukocyte recruitment¹⁸⁰.

GPCR signaling lasts for some minutes after which the receptors become desensitized preventing a further stimulatory response and limiting the degree of neutrophils extravasation. A more sustained inflammatory response therefore requires a more persistent stimulation that is provided by type-II activation in response to inflammatory cytokines TNF α and/or IL-1, and activation of NF- κ B. Like type-I activation, type-II response leads to increased blood flow, increased vascular leakage of plasma proteins and increased leukocytes recruitment at the site of inflammation. These effects are enhanced and more effective in type-II activation due to the expression of new genes. The recruitment of leukocytes is sustained due to prolonged synthesis of chemokines and the display of adhesion molecules on the surface of ECs that enhances neutrophils extravasation. Of note, ECs have the ability to present chemokines, synthesized by themselves or by other cells, on their surface through binding to proteoglycans¹⁸¹⁻¹⁸³. More recent evidence suggests that chemokine-triggered arrest via integrin activation requires close localization among apical chemokines, their cell receptors and integrin adhesion molecules¹⁸⁴. Failure of acute inflammation to eradicate the triggering stimulus, and especially if an adaptive immune response is activated by persistent stimulation, results in the inflammatory process evolving to a chronic form in which specialized effector cells are involved. ECs participate in this process by presenting antigens to circulating effector and memory T cells¹⁸⁵. The vascular endothelium is therefore indeed central to the cellular and molecular events that take place during inflammatory response.

1.5 Inflammatory mechanisms drive atherosclerosis

Inflammatory mechanisms play a crucial role in all stages of atherosclerosis: from early endothelial activation by modified lipids to the eventual rupture of the atherosclerotic plaque^{1, 4}. The activation of the canonical NF- κ B signal transduction pathway, mediated by the phosphorylation and degradation of its inhibitory protein I κ B α by the I κ B-Kinase (IKK) complex as previously described, is a central step in pro-inflammatory target gene expression such as that of chemokines or adhesion molecules, which have been strongly implicated in atherosclerosis¹⁸⁶. Knockout and transgenic models of the NF- κ B signal transduction pathway have provided much evidence on the role of NF- κ B in atherogenic inflammation. Several years after demonstrating the presence of activated NF- κ B in human atherosclerotic lesions using an antibody specifically recognizing phosphorylated p65 in smooth muscle

cells, macrophages, and endothelial cells¹⁸⁷, Kanters and colleagues surprisingly showed that, the inhibition of NF- κ B activation in macrophages increases atherosclerosis in LDL-receptor-deficient mice¹⁸⁸. This they showed by transplantation of bone marrow from mice that specifically lack IKK β in macrophages into LDLR^{-/-} mice and observed an increased in the size of atherosclerotic lesion with enhanced inflammation in the lesion. The authors attributed the increase in atherosclerotic lesion to the reduced levels of IL-10 observed when IKK β was deleted in macrophages because absence of IL-10 accelerates atherosclerosis¹⁸⁹. Studies using bone marrow from p50-deficient mice transplanted into an LDLR^{-/-} mice showed a reduction in lesion size with a distinct plaque phenotype, characterized by reduced foam and immune cells¹⁹⁰. Coherently, they observed an increased IL-10 levels in the p50-depleted macrophages which may account for the small lesion size. There is therefore a complex role of the canonical NF- κ B pathway in atherogenic inflammation which seems to be cell type-specific (endothelial cells versus macrophages) leading to regulation of different genes.

Several *in vitro* studies have confirmed the role of the canonical NF- κ B pathway in atherogenic inflammation by regulating the expression of different target genes that have been strongly implicated in the events in atherogenesis. NF- κ B not only regulates a number of enzymes demonstrated to be crucial in the modification of LDL and the formation of inflammatory lipid mediators such as 5-lipoxygenase, 12-lipoxygenase⁷ and COX2¹⁹¹ but also, chemokines like CCL2¹⁹² and adhesion molecules like ICAM-1, VCAM-1, P-selectin and E-selectin¹⁹³⁻¹⁹⁵. Activation of NF- κ B also mediates the transcription of genes like tissue factor and MMPs that have plaque thrombotic potential¹⁹⁶. Evidence exists for the critical role of NF- κ B in smooth muscle cell proliferation and viability by regulating genes with survival functions. Using a balloon injury model in the rat carotid artery, low levels of constitutively activated p50, p65 and c-Rel were shown in normal vessels, a reduction in the levels of I κ B α and I κ B β and a subsequent expression of VCAM-1 and CCL2 were observed. An antisense oligo against p65 inhibited SMC proliferation and intimal formation in rat carotid arteries. More recently, I κ B α -expressing adenovirus applied in a rabbit iliac artery restenosis model, showed a reduction of ICAM-1 and CCL2 expression, impaired recruitment of macrophages and a narrowing of the lumen without affecting intimal thickness¹⁹⁷⁻¹⁹⁹.

Taken together, data from different models are inconsistent, indicating that the underlying mechanisms by which NF- κ B modulates the complex events in atherogenic inflammation needs further investigation.

The transcription factor NF- κ B is subjected to a good number of control mechanisms as previously described. Briefly, there is, among other regulatory mechanisms, an auto-feedback regulation of NF- κ B by its inhibitor I κ B α , which gets rapidly transcribed upon binding of NF- κ B to DNA, deactivating the activated transcription factor¹¹². The inhibitory protein, I κ B α , is also regulated by a SCF-type cullin-RING E3 ligase (CRL)-driven ubiquitination process, which in turn is controlled by the COP9 signalosome complex (CSN)²⁰⁰.

1.6 The COP9 signalosome (CSN) complex

The constitutive photomorphogenesis 9 (COP9) signalosome (CSN) complex was first identified in *Arabidopsis* as an essential regulator of photomorphogenesis²⁰¹. This conclusion was drawn when researchers were screening the plant, *Arabidopsis thaliana* for mutants that exhibit light-grown seedling characteristics. They realized that the CSN represses photomorphogenesis leading to dark-grown growth patterns and that light signals transduced from photoreceptors lead to a release of this repression (Figure 8).

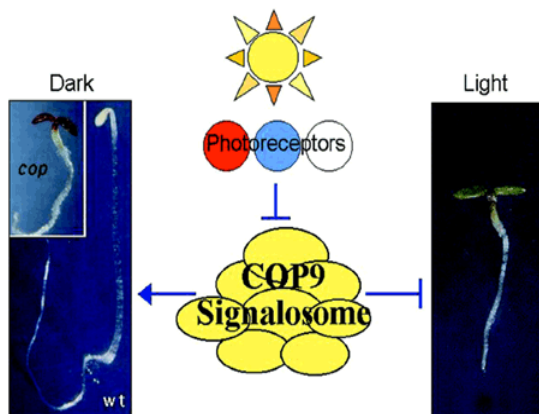


Figure 10 Model of COP9 signalosome (CSN) function in photomorphogenesis. In the absence of light signals, the CSN represses photomorphogenesis (light) leading to dark-grown (dark) growth patterns (wt, left) (Taken from Chamovitz *et al.*, EMBO Rep. 2001).

This protein complex was rediscovered some few years later during the purification of proteasomes from mammalian red blood cells²⁰² as well as purified from the spleen of pigs²⁰³. This indicated that the function of the CSN was not limited to control of plant photomorphogenesis. CSN fractionates as a 450–550-kDa complex in gel filtration columns and further work revealed eight subunits of the complex. These subunits were designated as CSN1 to CSN8 depending on their sizes, from the largest, CSN1, to the smallest, CSN8²⁰⁴. The first architecture of the CSN was revealed by electron microscopy (Figure 9) and subunit-subunit interaction studies. Each subunit was linked to a corresponding subunit in the 19S lid subcomplex (Figure 9) of the 26S proteasome due to sequence homology²⁰⁵. Since its discovery in plants and mammals, the COP9 signalosome complex has been identified in a variety of different eukaryotic organisms.

This includes *Saccharomyces cerevisiae*^{206, 207}, *Schizosaccharomyces pombe*²⁰⁸, *Aspergillus nidulans*²⁰⁹, *Neurospora crassa*²¹⁰, *Caenorhabditis elegans*²¹¹, and *Drosophila melanogaster*²¹². The range of biological responses in which the CSN is involved is extensive, from embryonic development, the cell cycle, checkpoint control, T-cell development, signal transduction, oocyte maturation, to autophagy and circadian rhythm, reflecting the multifunctionality of the complex²¹³.

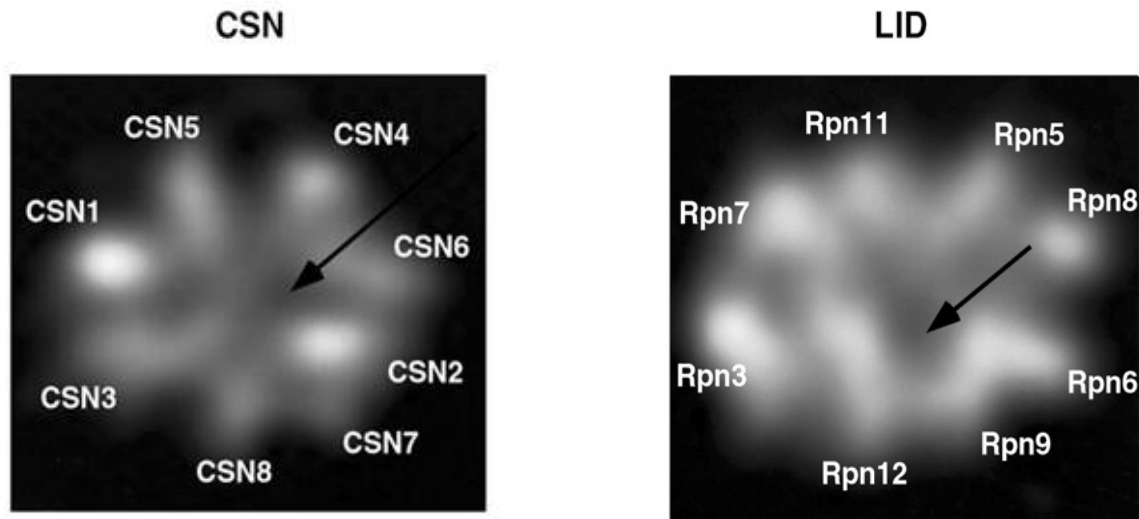


Figure 11 2D electron microscopic images of the CSN and the 19S LID subcomplex of the 26S proteasome. The putative subunit arrangement of the CSN on the basis of the subunit-subunit interaction studies²⁰⁵ is indicated. LID subunit-subunit interactions were deduced from CSN data by arranging homologous subunits. The central groove is marked by black arrow. (Taken from Bech-Otschir *et al.*, J Cell Sci. 2002)

A characteristic feature of the CSN is the presence of two signature domains: a PCI domain that resides at the C-terminus and which is present in six subunits and an MPN domain that is found at the N-terminus and is present in two subunits, CSN5 and CSN6²¹⁴. The PCI domain has been shown to be composed of two subdomains: a winged-helix subdomain at the C-terminus, and a helix bundle subdomain at the N-terminus (Figure 10)²¹⁵⁻²¹⁷. Proteins that bind DNA/RNA have a winged-helix domain which has triggered speculations that the CSN might even bind nucleic acids²¹⁷.

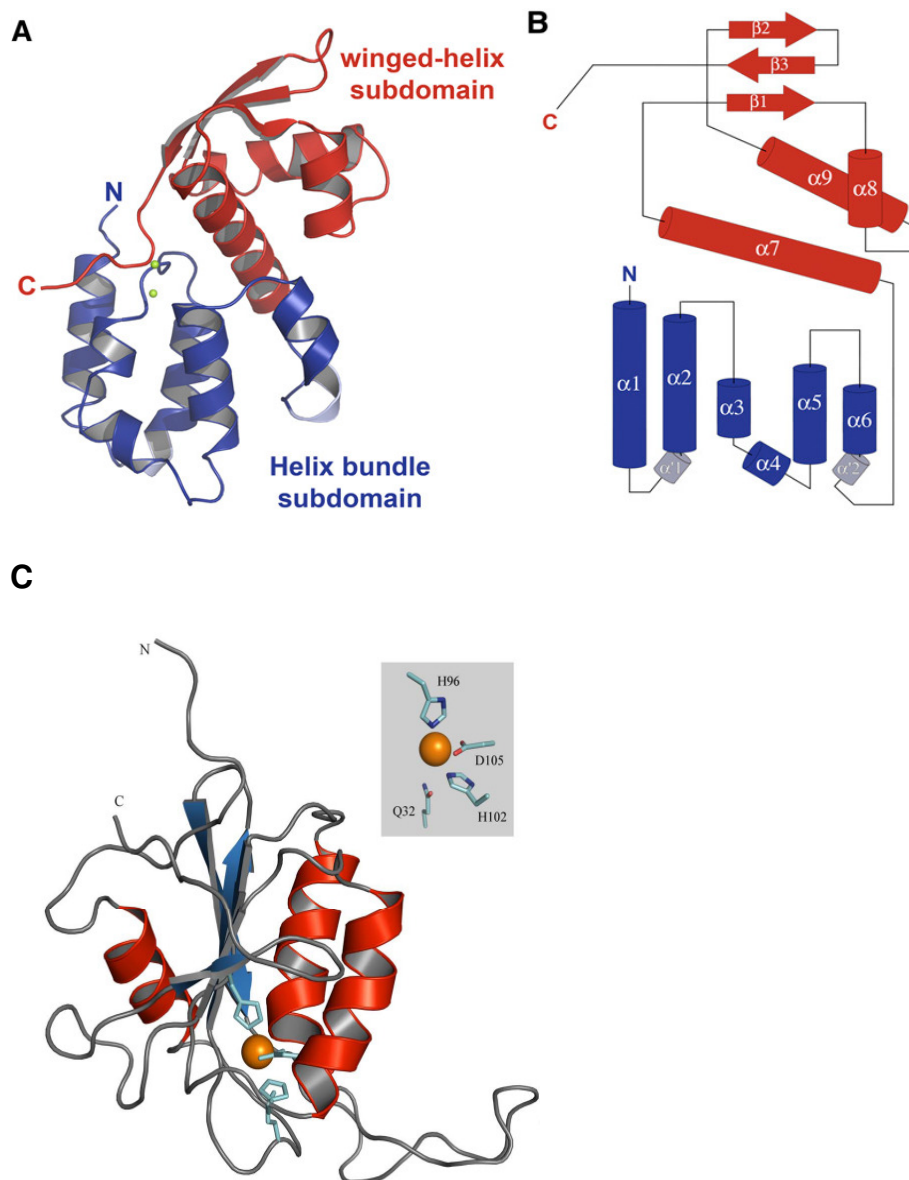


Figure 12 Structures of the CSN7 PCI Domain and the MPN Domain of CSN. (A) and (B) Cartoon (A) and topology depictions (B) of the CSN7 PCI domain. The 1.5 Å resolution structure of CSN7's PCI domain reveals two subdomains: the HB subdomain (blue) and the WH subdomain of CSN (red). α , α' , and β denote α -helix, 3_{10} helix, and β strand, respectively. (C) Shows the MPN domain with its Zn atom (Taken from Dessau *et al.*, The Plant Cell 2008).

The subunits of the CSN were identified to either interact with each other (Figure 11) or independently with several signaling molecules though not all of these interactions have been verified by several techniques (Table 1). The fifth mammalian subunit, CSN5, was first identified as Jun-activation-domain-binding protein (JAB1) because of its interaction with the activation domain of c-Jun. JAB1/CSN5 was found to stabilize complexes of the transcription factors c-Jun or JunD at their specific AP-1

transcription factor binding sites, increasing the specificity of target gene activation²¹⁸.

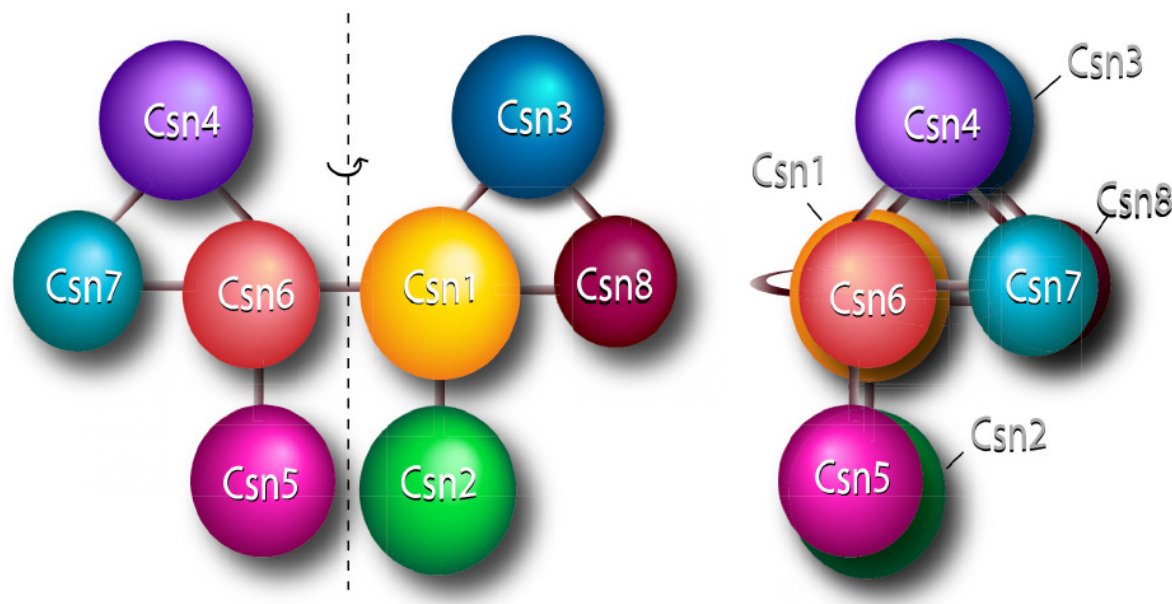


Figure 13 Subunit-subunit interaction of the human CSN complex. These interactions were generated through integration of mass spectrometry (MS) and tandem MS data using the algorithm SUMMIT. Depicted on the left side are two subunit clusters: CSN1,2,3,8 and CSN4,5,6,7; interaction between CSN1 and 6 links the two clusters. Shown on the right side are the subunit-subunit interactions (Taken from Sharon *et al.*, Structure 2009).

Table 1 Binding partners of the CSN subunits

<i>CSN subunit</i>	<i>Binding partner</i>	<i>Method used in Analysis</i>	<i>Reference</i>
CSN1	Inositol 1,3,4-triphosphate 5/6 kinase	rip, eip	Sun <i>et al.</i> 2002
	eIF3c/NIP1, subunit of translation initiation factor 3	Y2H	Karniol <i>et al.</i> 1998
	RPN6 (AtS9), proteasome lid non-ATPase subunit	Y2H	Kwok <i>et al.</i> 1999
CSN2	ICSBP, interferon consensus sequence binding protein	pd, rip	Cohen <i>et al.</i> 2000
	Cullin 1 (CUL1), SCF complex core component	ms, Y2H	Lyapina <i>et al.</i> 2001,
	IκBα	rip	Schwechheimer <i>et al.</i> 2001, Schweitzer, <i>et al.</i> 2007
	Cullin 2 (CUL2)	Y2H, rip	Yang <i>et al.</i> 2002
	DAX-1, COUP-TF1, ecdysone receptor, (nuclear receptors)	Y2H, pd, rip	Dressel <i>et al.</i> 1999, Altincicek <i>et al.</i> 2000
	TR, thyroid hormones receptor	Y2H, pd	Lee <i>et al.</i> 1995

CSN3	IKK γ , IKK complex	Y2H, FRET, rip, eip	Hong <i>et al.</i> 2001, Orel <i>et al.</i> 2010
	COP10, ubiquitin-conjugating enzyme variant	Y2H	Suzuki <i>et al.</i> 2002
	Int-6/eIF3e, subunit of translation initiation factor 3, CK2, PKD	rip, eip	H. Alves <i>et al.</i> 2002, Uhle <i>et al.</i> 2003
CSN4	COP10, ubiquitin-conjugating enzyme variant	Y2H	Suzuki <i>et al.</i> 2002
CSN5	c-Jun transcription activation domain, JunD	Y2H	Claret <i>et al.</i> 1996
	p27 ^{Kip1} , cyclin-dependent kinase inhibitor	Y2H, pd, rip	Tomoda <i>et al.</i> 1999
	Bcl3, member of the I κ B multigene family	Y2H, pd, rip	Dechend <i>et al.</i> 1999
	LFA-1, integrin adhesion receptor	Y2H, pd, rip, eip	Bianchi <i>et al.</i> 2000
	rLHR, lutropin receptor precursor	Y2H, pd, rip	Li <i>et al.</i> 2000
	MIF, chemokine-like function chemokines	Y2H, pd, rip, eip	Kleemann <i>et al.</i> 2000
	PR, progesterone receptor and SRC-1	Y2H, pd	Chauchereau <i>et al.</i> 2000
	p53, tumor suppressor	pd	Bech-Otschir <i>et al.</i> 2001
	HIF-1 α , hypoxia-inducible factor-1	Y2H, pd, eip	Bae <i>et al.</i> 2002
	Smad4, TGF- β signal transducer	Y2H, pd, rip, eip	Wan <i>et al.</i> 2002
	HPO, hepatopoietin	Y2H, pd, rip, eip	Lu <i>et al.</i> 2002
	TRC8, RING-finger membrane protein	pd, rip	Gemmill <i>et al.</i> 2002
	PGP9.5, member of ubiquitin C-terminal hydrolase	Y2H, eip	Caballero <i>et al.</i> 2002
	GLHs, germ-line RNA helicases	Y2H, pd	Smith <i>et al.</i> 2002
	Smad5, a major intracellular mediator of BMP Signaling	Y2H, rip	Aigner and Haag, 2006
	Smad 7, TGF- β signal transducer	Y2H, pd, rip	Kim <i>et al.</i> 2004
	IKK complex	Y2H, FRET, rip, eip	Orel <i>et al.</i> 2010
	Vpr, HIV-1 protein	Y2H, pd	Mahalingam <i>et al.</i> 1998
	Rbx1/Roc1/Hrt1, SCF RING protein component	ms, Y2H	Lyapina <i>et al.</i> 2001,
			Schwechheimer <i>et al.</i> 2001
CSN6	Int-6/eIF3e, subunit of translation initiation factor 3	rip, eip	H. Alves <i>et al.</i> 2002
CSN7	Int-6/eIF3e, subunit of translation initiation factor 3	rip, eip	H. Alves <i>et al.</i> 2002,
	IKK complex	Y2H, FRET, rip, eip	Yahalom <i>et al.</i> 2001, Orel <i>et al.</i> 2010
	PMF-1, polyamine-modulated factor	Y2H	Y. Wang <i>et al.</i> 2002
CSN8	eIF3c/NIP1, subunit of translation initiation factor 3	Y2H	Karniol <i>et al.</i> 1998
	COP10, ubiquitin-conjugating enzyme variant	Y2H	Suzuki <i>et al.</i> 2002

Y2H, yeast two hybrid; pd, *in vitro* pull down assay; rip, coimmunoprecipitation with overexpressed constructs; eip, endogenous coimmunoprecipitation; FRET, fluorescence resonance energy transfer; ms, mass spectrometry

Later, JAB1/CSN5 was repeatedly found to be an interactor of various intracellular regulators (Table 1). Another common theme of possible significance is the frequent occurrence of interactions between CSN5 and its partner proteins in the context of multi-protein complexes, and the apparent connection of these interactions to ubiquitin-dependent protein degradation pathways. The N-terminus of CSN5 contains an MPN domain which has an intrinsic JAMM (JAB1 MPN domain metalloenzyme) motif that serves as the catalytic center for CSN isopeptidase activity. This JAMM motif can coordinate a Zn^{2+} atom, which is a cofactor critical for enzymatic activity^{219, 220}. A well-described enzymatic activity of the CSN is the cleavage of ubiquitin-like Nedd8 conjugates from the cullin subunit of CRLs, including SCF-type CRLs²⁰⁰.

1.6.1 CSN as a cullin deneddylase

Among the different ubiquitin ligases, CRL form the largest family and the cullin subunit in CRLs are post-translationally modified by covalent conjugation with the ubiquitin-like modifier Nedd8/Rub1²²¹. This modification is crucial for the enzymatic activity of CRLs. CSN catalyzes the reaction to remove Nedd8 conjugates from cullin a process termed deneddylation, and subsequently regulates the activity of CRLs. This deneddylation activity is catalyzed by the JAMM motif of CSN5, which is the only CSN subunit with known catalytic activity; however this enzymatic activity requires the entire CSN holocomplex²⁰⁰. The alternate conjugation and removal of Nedd8 facilitates the dynamic assembly and disassembly of the SCF core complex with a substrate-binding module containing adaptor protein and substrate receptor (Figure 12)^{222, 223}. This may regulate the availability of SCFs for ubiquitination-induced proteasomal degradation of different substrates, as p27^{224, 225} and IκBα²²⁶, depending on the cellular needs. Whereas neddylation is required for an optimal ubiquitin ligase activity of SCFs²²⁷⁻²³⁰, CSN-mediated deneddylation has been linked with the dissociation of the substrate-binding module from the SCF complex²²³ and with the inhibition of SCF-mediated substrate ubiquitination *in vitro*^{231, 232}. However, as SCFs trigger their self-inactivation through auto-ubiquitination²²³, dynamic dissociation and reassembly of the SCF with non-ubiquitinated components is a prerequisite for an efficient function of the SCF, which explains the requirement of the CSN for sustained SCF activity *in vivo*²³³⁻²³⁸.

The association of the CSN complex with ubiquitin depolymerization activity through its interaction with the deubiquitylase enzyme Ubp12p/USP15 in yeast²³⁹ and mammalian cells^{240, 241}, respectively, has been shown to contribute to prevent excessive auto-ubiquitination and degradation of SCF subunits^{239, 241}.

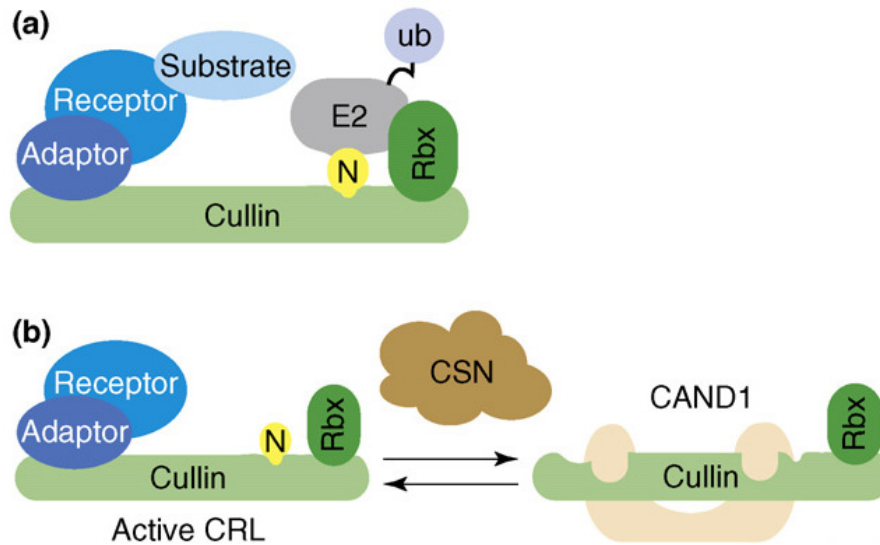


Figure 14 The CRL family of ubiquitin ligases and its regulation by the CSN. (a) The CRL superfamily of ubiquitin ligases is characterized by an enzymatic core that contains a cullin family member (light green) and RBX1 (dark green), a RING protein. Specific substrates (light blue) are recruited to the core by a substrate-binding module consisting of an adaptor protein (or domains; blue) and various substrate receptors (turquoise). (b) Cullin neddylation enables the assembly of an active CRL, whereas deneddylation by CSN (light brown) facilitates the dissociation of the substrate-binding module and the association of CAND1 (beige) with the core complex. Color code: E2, gray; ubiquitin (Ub), sky blue; Nedd8 (N), yellow (Taken from Wei, N. *et al.*, Trends Biochem. Sci. 2008).

Similarly, I κ B α ²⁴² and BCL10²⁴³, important signaling molecules in the antigen receptor signaling pathway to IKK/NF- κ B, are stabilized through CSN-mediated deubiquitination in response to tumor necrosis factor (TNF)- α administration and T-cell receptor stimulation, respectively.

In addition to the CSN, it has been shown that deneddylase 1 (DEN1) exhibits deneddylating activity^{244, 245}. However, DEN1 and the CSN seem to have different roles. Whereas DEN1 seems to function predominantly as a Nedd8 C-terminal hydrolase, the CSN has been proven to be the key factor that determines the rate of deneddylation of cullins^{233, 244, 246-248}.

1.6.2 CSN5 as a protean subunit of the CSN complex

The gene that encodes for CSN5/JAB1 is highly conserved and has been extensively studied in mice. Whereas the murine *Csn5* gene is located on chromosome 2, the human *CSN5* gene is located on chromosome 8 (Figure 13)²⁴⁹. The human CSN5 protein consists of 334 amino acids and has a molecular mass of 38 kDa (Figure 11A, B). While there are two Arabidopsis CSN5 homologue genes, AJH1 and AJH2, there is only one known isoform in humans²⁵⁰. CSN5 is evolutionarily conserved in humans, mice, fission yeast, and plants, providing evidence that CSN5 is critical for cell survival and proliferation^{202, 203, 251}.

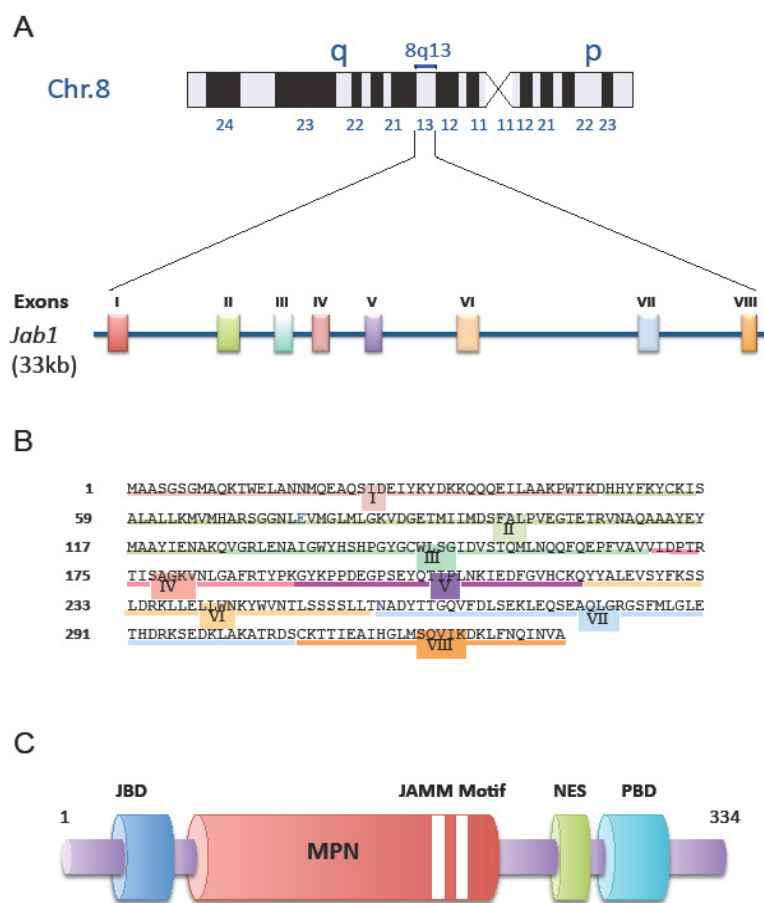


Figure 15 Structure of the *CSN5* gene and domains of the CSN5 protein. (A) Schematic of the CSN5 gene which is 33 kb long and localized at chromosome 8Q13.2. (B) The 8 exons within the 334 amino acid sequence are shown. (C) Schematic of CSN5 protein which consists of 334 amino acids with a JAB1/MPN domain metalloenzyme (JAMM)-containing Mpr1-Pad1-N-terminal (MPN) domain and a nuclear export signal (NES) domain close to the p27 binding domain (PBD) at the C-terminal end (Modified after Shackelford *et al.*, Cell Div. 2010).

Although all the eight subunits of the CSN complex have important roles in the overall function of the complex, CSN5 is unique. CSN5 does not only harbor the catalytic center for CSN isopeptidase activity as previously described but can also exist stably and independently of the CSN holocomplex²¹³. Of note, several reports indicate that, depleting cellular CSN5 levels reduces monomeric CSN5 levels more dramatically than the CSN holocomplex. Inversely, transient overexpression of CSN5 in cultured cells usually leads to an increase in the levels of CSN5 monomers with little effect on CSN holocomplex^{238, 252, 253}. Whereas CSN-associated CSN5 is located primarily in the nucleus, monomeric CSN5 can be both cytoplasmic and nuclear^{254, 255}. It is now clear that CSN5 actively participates in crucial biological processes, both as part of the CSN holocomplex and independently of the CSN. Interestingly, recent reports indicate that the JAMM domain of CSN5 also has deubiquitinase activity, which regulates ubiquitinated protein sorting into exosomes when CSN5 is associated with the CSN²⁵⁶. This links the CSN with the regulation of ubiquitinated proteins that are transferred by exosomes, a process that impacts the flow of information from neighboring or distant cells.

CSN5 has numerous binding partners which include important cellular regulators (Table 1). Depending on the specific target, CSN5 either induces degradation or enhances stability of these target proteins^{238, 253, 257}. CSN5 directly interacts with p27 thereby controlling the cell cycle. Binding of p27 to CSN5 leads to cell cycle progression and the concomitant export of p27 from the nucleus and subsequent degradation²⁵⁸. The nuclear to cytoplasmic shuttling of p27, induced by CSN5, plays an important role in the regulation and function of p27. Accumulation of cytoplasmic p27 results in increased cell motility and migration through its interaction with RhoA, a GTPase that regulates cell motility by reorganization of actin filaments^{259, 260}. Therefore the role of CSN5 in p27 cellular localization interrupts the inhibitory effect of p27 on cellular proliferation and also puts it in position to interact with RhoA to mediate cell motility and migration which has an overall tumorigenic effect on the cell. This effect involves the nuclear-cytoplasmic translocation of CSN5. The subcellular redistribution of CSN5 is a recurring theme, as CSN5 also appears to function at the plasma membrane²⁶¹. In normal COS7 cells, transiently expressed CSN5 maintains a diffuse distribution. In experiments in which these cells also transiently express the adhesion receptor LFA-1, a fraction of CSN5 was enriched in the same peripheral

membrane regions as this ectopically expressed integrin, consistent with an integrin–CSN5 interaction that had been found using the yeast two-hybrid assay²⁶¹. As the CSN has never been shown to localize to peripheral membranes, it can be assumed that this form of CSN5, is CSN-independent²⁶².

CSN5, initially identified as a coactivator of c-Jun, can specifically stabilize the protein-DNA complexes on the off-rate of c-Jun and JunD with its cognate AP-1 DNA binding sites and potentiates c-Jun transactivation without affecting JunB or v-Jun binding. This offers a mechanism of specificity in the binding of c-Jun to target genes²⁴⁹. CSN5, through its interaction with c-Jun, is involved in c-Jun-induced AP-1-mediated transcriptional activation and also affect the interaction between c-Jun and other interacting proteins. CSN5 affects other proteins and their interaction with c-Jun by binding directly to and changing their subcellular localization. This is true for the following proteins and in general lead to activation of AP-1: LFA-1^{261, 263}, MIF²⁵⁸, hepatopoietin²⁶⁴, and hepatitis B virus X (HBX)²⁶⁵. In addition to c-Jun, CSN5 has been shown to be a transcriptional coactivator and potential specificity factor for several other molecules like NF-κB. CSN5 has been shown to interact with the proto-oncogene BCL3, a member of the IκB family of inhibitory proteins and is present predominantly in the nucleus. Most members of the IκB family of inhibitory proteins act to inhibit NF-κB; however, BCL3 can activate the transcription of NF-κB. Binding of CSN5 to BCL3 leads to enhanced NF-κB, p50, and DNA complex formation and may link NF-κB and AP-1 gene transcription²⁶⁶. CSN5 also bridges coactivators and receptors in the progesterone receptor-steroid receptor coactivator complex (Table 1). These studies provide evidence of the role of CSN5 as specificity factor with less defined mechanisms by which it acts.

1.6.3 CSN-associated protein kinase activity and deubiquitylation activity

Purification of the CSN complex from red blood cells led to the finding that the CSN has an associated serine/threonine kinase activity. The CSN preparation was found to phosphorylate c-Jun (Ser63 and Ser73), IκBα, and the NF-κB precursor, p105, as well as tumor suppressor p53 (Ser149, Thr 150, and Thr 155) *in vitro*^{202, 267}. This CSN-associated kinase activity was however shown to be inhibited by curcumin, a dietary pigment with known pleiotropic properties of antioxidation, anti-inflammation, and anti-carcinogenesis.

Whereas CSN-mediated phosphorylation targets p53 for degradation by the ubiquitin-26S proteasome system²⁶⁷, that of c-Jun is believed to stabilize the molecule and contribute to the JNK-independent activation of AP-1²⁶⁸. In both cases, CSN5, which binds to amino acids 31–57 of c-Jun²¹⁸ and 1–154 of p53²⁶⁷, might serve as a substrate docking site. Another CSN-associated kinase found is the inositol 1, 3, 4-trisphosphate 5/6-kinase (5/6 kinase). The 5/6 kinase phosphorylates inositol 1,3,4-trisphosphate [Ins(1,3,4)P3] to form Ins(1,3,4,5)P4 and Ins(1,3,4,6)P4. The 5/6 kinase is also conserved from plants to humans. This enzyme co-purifies with the entire CSN complex from calf brain and directly interacts with the CSN1 subunit. The 5/6 kinase displays substrate spectrum and characteristics similar to the described CSN-associated kinase purified from red blood cells. It phosphorylates c-Jun, IκBα, ATF-2, and p53, but not c-Jun (1-79), and it is inhibited by curcumin^{269, 270}. CSN itself is a target of phosphorylation, since a number of its subunits have been shown to be phosphorylated^{214, 271}.

Another biochemical activity that has been reported for CSN is the CSN-associated deubiquitinylation activity. This activity can be classified as either deconjugation of ubiquitin from mono-ubiquitinated substrates or depolymerization of polyubiquitin chains²⁴⁰. Whereas deconjugation of ubiquitin from mono-ubiquitinated substrates requires the JAMM motif in CSN5, depolymerization is associated with the CSN holocomplex. Collectively, these indicate that CSN has both deneddylation and deubiquitinylation activities either by possessing these activities on their own or by selectively recruiting different enzymes based on cellular needs.

1.6.4 CSN's cellular functions

The biochemical activities reported for CSN alongside its association with the 26S proteasome, places CSN in a position which renders it capable of determining the fate of proteins to which it binds. Based on this notion, the CSN would either sensitize or desensitize proteins towards the ubiquitin-proteasome pathway (UPP). The CSN has been shown to negatively regulate G1-S phase cell cycle progression by desensitizing p27 towards the UPP. This effect of the CSN was linked to the deneddylase activity that resides in the JAMM motif at the N-terminus of CSN5, which is only functional in the context of the CSN holocomplex, as anti-CSN2 antibodies nullified this CSN-dependent p27 stabilization and G1-S blockade²³².

In a rat model of intimal hyperplasia, an adenovirus expressing a dominant-negative form of I κ B α inhibited intimal hyperplasia *in vivo* and the proliferation of smooth muscle cells *in vitro*. This *in vitro* retardation of SMC proliferation was linked to the observation that the cyclin-dependent kinase inhibitor p27 was upregulated²⁷². The proliferation of SMC in atherosclerosis, may mediate cap formation which is critical in plaque-stabilizing process. Hence, any effort to drive the proliferation of SMC will be a step in the right direction in stable plaque formation, and the interplay between p27 and CSN might be of interest for further investigation. CSN also works as a positive regulator of AP-1 activity by stabilizing c-Jun through the phosphorylation of c-Jun by CSN-associated kinases like CK2 and PKD. While CSN-dependent phosphorylation appears to desensitize c-Jun, an opposite effect is exerted on the tumor suppressor p53 resulting in its degradation. This indicates a dynamic and multifunctional role of the CSN because depending on its target protein it either induces its degradation or stabilizes it.

The CSN inhibits the activation of NF- κ B by stabilizing I κ B α and this has a negative effect on apoptosis in epithelial cells²⁷¹. In the activation and proliferation of mature T cells, NF- κ B has been implicated in regulating survival signals at many stages of T-cell development and maturation²⁷³. Using a conditional knockout of *Csn5* in thymocytes, Panattoni *et al* showed that there is a defective S phase progression and massive apoptosis of thymocytes. This was associated to a tremendous increase in p53 and its target genes and a downregulation of I κ B α - and NF- κ B-regulated pro-survival genes²⁷⁴. *Csn8*-deficient peripheral T cells also exhibited a delayed progression from S phase to G2 and M phase²⁷⁵. Of note, CSN has been shown to control the ubiquitylation and deubiquitylation of the ATP-binding cassette protein A1 (ABCA1)²⁷⁶. ABCA1 mediates the transfer of cellular free cholesterol and phospholipids to apolipoprotein A-I (apoA-I), an extracellular acceptor in plasma, to form high-density lipoprotein (HDL). ABCA1 synergizes with ABCG1 to mediate macrophage cholesterol efflux and protect against macrophage foam cell formation, infiltration of inflammatory cells, apoptosis and atherosclerosis²⁷⁷. Desensitization of ABCA1 towards the UPP which might result in effective cholesterol efflux represents a way of decreasing circulating monocytes and subsequent decrease in atherosclerosis.

The autophagy-lysosome pathway represents a second important route for the clearance of eukaryotic intracellular proteins. Autophagy regulates homeostasis in the cell in a nonselective or selective manner. During starvation, nonselective autophagy helps the cell to survive an energy crisis temporarily by self-eating portion of its cytoplasm. Selective autophagy serves as a quality control mechanism in the cell by removing aged/damaged organelles and aggregated proteins²⁷⁸. Again, the CSN has been shown to have a crucial role in autophagosome maturation through regulating the expression of Rab7. Impaired autophagosome maturation after ablation of *Csn8* in a perinatal cardiomyocytes-restricted model drove cardiomyocytes into necrosis²⁷⁹. Taken together, the CSN seems to be a key player in regulation of cellular functions which are implicated in several diseases including perhaps cardiovascular disease.

1.7 Aim of the study

Inflammatory processes play a crucial role in all stages of atherogenesis. As a key trigger, the activation of the NF- κ B signaling pathway in endothelial cells and macrophages drives the expression of pro-inflammatory and pro-atherosclerotic genes. The COP9 signalosome (CSN), a multi-functional protein complex involved in the regulation of Cullin-RING-E3 ubiquitin ligases (CRLs), has emerged as a regulator of NF- κ B signaling. This function has been assigned to the deneddylase activity of its subunit CSN5. The CSN has been suggested to exhibit an important pathophysiological role in cancerogenesis, but its contribution to inflammation has been less well studied. Although the CSN has been implicated in β 2-integrin signaling, a process critical in inflammatory and atherogenic leukocyte recruitment, the role of the CSN in atherosclerosis has been unknown. Using test tube approach and animal models, the aims of the study were threefold:

1. To probe the role of CSN5 in IKK/NF- κ B signaling and atherogenic processes in the endothelium.
2. To translate the *in vitro* findings to mouse atherosclerosis models by generating endothelial and macrophage-specific *Csn5* knockout mice using the Cre/loxP recombination system.
3. To follow CSN5 and CSN expression levels over the course of atherosclerosis pathogenesis in humans and mouse models.

2 MATERIALS

2.1 Chemicals and reagents

Substance	Manufacturer
DNA Ladder (1kb)	Fermentas, St.Leon-Rot
Acrylamide (30%)	Biorad, München
Ampicillin (100mg/ml)	Sigma, München
APS	Sigma, München
DTT	Sigma, München
EDTA	Merck, Darmstadt
Glycerol	Sigma, München
NuPAGE LDS sample buffer	Invitrogen, Karlsruhe
NuPAGE LDS running buffer	Invitrogen, Karlsruhe
NuPAGE LDS transfer buffer	Invitrogen, Karlsruhe
Penicillin/Streptomycin	Invitrogen, Eggenstein
Protease Inhibitor cocktail	Roche, Mannheim
TEMED	Biorad, München
Methanol	Roth, Karlsruhe
Protein G Sepharose	NEB, Frankfurt am Main
Protein Marker – Multi Mark™	Invitrogen, Karlsruhe
RPMI 1640	Invitrogen, Eggenstein
Super Signal West Dura	Pierce, USA
Trypsin	Invitrogen, Eggenstein
Tween 20	Fluka, Switzerland
β-Mercaptoethanol	Sigma, München
DMSO	Sigma, München
Endothelial Cell Growth Medium	Promocell, Heidelberg
Ethanol	Carl Roth, Karlsruhe
FCS	Gibco, Eggenstein
Ficoll-Paque plus	GE Healthcare Europe GmbH, Freiburg
Hank's Balanced Salt Solution (HBSS)	Biochrom. AG, Berlin

Substance	Manufacturer
Accutase	PAA, Pasching
Acetonitril	Sigma, München
Calcein-AM	Calbiochem, USA
Isopropanol	KMF Laborchemie, Lohmar
Calcium chloride	Calbiochem, USA
Potassium chloride	Merck, Darmstadt
Magnesium chloride	Calbiochem, USA
Sodium chloride	Merck, Darmstadt
Sodium dodecyl sulfate	Merck, Darmstadt
MG132 (proteasome inhibitor)	Merck, Darmstadt
Triton X-100	Sigma-Aldrich, Steinheim
dNTP-Mix	Fermentas, France
Albumin Fraction V (BSA)	Carl Roth, Karlsruhe
Dulbecco's PBS	Sigma, München
Dynabeads Protein A	Invitrogen, Oslo
Gentamycine	Invitrogen, Karlsruhe
Collagen G	Biochrom. AG, Berlin
TNF- α	Peprotech, USA
Trypan blue	Invitrogen, Karlsruhe

2.2 Enzymes and buffers for the enzymes

Description	Manufacturer
Green GoTaq Flexi Buffer	Promega, USA
GoTaq polymerase	Promega, USA
Proteinase K	Fermentas, St. Leon-Rot
M-MLV Reverse Transcriptase	Promega, USA
M-MLV 5x Buffer	Promega, USA

2.3 Primer pairs for qPCR

Description	Sequence
CSN5	5'- CGCAAATTGCTTGAGCTGTTGTGGAA-3' 5'-CCAGCTGGGCTTCTGACTGC-3'
CCL2	5'-GATCTCAGTGCAGAGGCTCG-3' 5'- TGCTTGTCCAGGTGGTCCAT-3'
VCAM1	5'-TGTGCCCCACAGTAAGGCAGGC-3' 5'- AGCTGGTAGACCCTCGCTGGA-3'
ICAM1	5'-GCTCCTGCCTGGGAACAACCG-3' 5'-GGGGAGGGGTGCCAGTTCCA-3'
GAPDH	5'- GCCTCAAGATCATCAGC-3' 5'-ACCACTGACACGTTGGC-3'

All primers above were manufactured by Sigma-Aldrich, Germany.

2.4 Strains of bacteria and plasmids

2.4.1 Strains of bacteria

Strain	Source
XL-10	Invitrogen
DH5 α	Invitrogen

2.4.2 Plasmids

Plasmid	Resistance	Source/Reference
pGFP	Kanamycin	AG Bernhagen, RWTH
pCDNA5/CTAP	Ampicillin	AG Lüscher, RWTH
pCDNA5/CTAP-CSN5	Ampicillin	AG Bernhagen, RWTH
plgk3ConALuc	Ampicillin	AG Baens, K.U. Leuven
pEQ176- β Gal	Kanamycin	AG Bernhagen, RWTH
Flag-CSN2	Ampicillin	AG Schmid, M.U. Vienna

2.5 Antibodies

2.5.1 Primary antibodies

Description	Use	Species	Manufacturer
Anti-CSN5	IP/WB/IHC	rabbit	Santa Cruz
Anti-CSN3	IP/WB	mouse	Santa Cruz
Anti-CSN1	IHC	rabbit	Enzo Life Science GmbH
Anti-CSN8	IHC	rabbit	Enzo Life Science GmbH
Anti-phospho-IKK1/2	WB	rabbit	Cell Signaling
Anti-IkB α	WB	rabbit	Cell Signaling
Anti-IKK1	IP/WB	rabbit	Cell Signaling
Anti-IKK2	IP/WB	rabbit	Cell Signaling
Anti-Cullin1	WB	rabbit	Abcam
Anti- β -actin	WB	mouse	MP Biomedicals
Anti-ICAM-1	FACS	mouse	Donation (AG Koenen)
Anti-P-selectin-PE	FACS	mouse	BD Bioscience
Anti-CD31	IHC	goat	Santa Cruz
Anti-IgG-PE	FACS	mouse	BD Bioscience

Detailed description of antibodies and their specific usage are noted in the methods and figure legends.

2.5.2 Secondary antibodies

Description	Use	Species	Manufacturer
Anti-rabbit HRP	WB	goat	Pierce
Anti-mouse HRP	WB	goat	Amersham
Anti-mouse-Cy3	FACS	donkey	Jackson ImmunoResearch
Anti-rabbit-FITC	IHC	donkey	Jackson ImmunoResearch
Anti-goat-Cy3	IHC	donkey	Jackson ImmunoResearch

2.6 Media, buffers and solutions

2.6.1 Media for cell culture

Endothelial cell growth medium supplemented with FCS and supplement mix was purchased from Promocell, Heidelberg. RPMI medium was obtained from Gibco and was supplemented with 10% FCS and 1% penicillin-streptomycin.

2.6.2 Media for culture of bacteria

Description	Concentration
Luria Bertani (LB) Medium	1% (w/v) NaCl 1% (w/v) Bacto tryptone 2% (w/v) Yeast extract 1 mg/ml Ampicillin or Kanamycin *
LB-Agar	1.5% (w/v) Bacto-Agar 1 mg/ml Ampicillin or Kanamycin * Dissolved in LB Medium

* This depends on the antibiotic resistance gene in the vector.

2.6.3 Buffers and solutions

Distilled water was added to all buffers unless otherwise stated.

Description	Concentration
FACS staining buffer*	100 µl normal mouse serum (2% final) 100 µl normal rabbit serum (2% final) 100 µl normal human serum (2% final) 333 µl 30% BSA (2% final) 5 ml PBS
HANKS Complete*	500 ml Hank's balanced salt solution (HBSS) 1 ml 30% BSA (0.06% final) 1.5 ml 100 mM EDTA (0.30 mM final)

* For antibody stainings during flow cytometry experiments, these buffers were used in a ratio of 1:1.

Description	Concentration
CoIP buffer	50 mM Tris-HCl 100-150 mM NaCl 15 mM EGTA 0.1 % Triton X-100 1 mM NaAzid 10 % Glycerin 0.5 mM Na ₃ VO ₄ 1 mM DTT 25 mM NaF Protease inhibitor cocktail (1:100)
1x LDS buffer	500 µL 4x NuPage LDS 250 µL 1M DTT 1250 µL distilled H ₂ O
Nuclear extract buffer 1 (NEB1)	10 mM Hepes 10 mM KCl 0.1 mM EDTA 5 mM Na ₄ P ₂ O ₇ 10 mM NaF Adjusted to pH 7.9
Nuclear extract buffer 2 (NEB2)	20 mM Hepes 0.4 mM NaCl 1 mM EDTA Adjusted to pH 7.9
PBS	137 mM NaCl 2.7 mM KCl 1.5 mM KH ₂ PO ₄ 8.1 mM Na ₂ HPO ₄

Description	Concentration
TBS (pH 7.2)	20 mM Tris-HCl 150 mM NaCl
TE buffer (pH 8)	10 mM Tris 1 mM EDTA
TBE	8.9 mM Tris-HCl 8.9 mM Boric acid 0.2 mM EDTA
Ethidium bromide solution	10 mg/ml
10x DNA gel loading buffer	0.2% Bromphenol Blue 0.2% Xylene xylano 0.2% Orange G 60% Saccharose
SDS PAGE Running buffer	5% 20x MES-running buffer
Transfer buffer	0.5% 20x Transfer buffer 25 ml Methanol
Washing buffer	0.05% Tween dissolved in TBS buffer
Blocking buffer	1-5% BSA or 5% non-fat dry milk
Western blot strip buffer	50 mM Tris (pH 6.8) 100 mM β -mercaptoethanol 2% SDS

Description	Concentration
Resolving gel	12% (w/v) acrylamide/Bis 375 mM Tris-HCl, pH 8.8 0.1% (w/v) SDS 0.1% (w/v) ammonium persulfate 0.1% (v/v) TEMED
Stacking gel	5% (w/v) acrylamide/Bis 125 mM Tris-HCl, pH 6.8 0.1% (w/v) SDS (sodium-dodecylsulfate) 0.1% (w/v) ammonium persulfate 0.1% (v/v) TEMED

2.7 Multi-component systems

Description	Manufacturer
Plasmid Maxi Kit	Qiagen, Hilden
RNeasy Mini Kit	Qiagen, Hilden
QIAshredder	Qiagen, Hilden
Express SybrGreenER qPCR Supermix	Invitrogen, Karlsruhe
HUVEC Nucleofector Kit	Lonza, Cologne
Bright-Glo TM Luciferase Assay System	Promega, USA
β-Galactosidase Enzyme Assay System	Promega, USA
TransAM NF-κB Transcription factor ELISA Kit	Active Motif, USA

2.8 Equipment and consumables

2.8.1 Equipment

Equipment	Manufacturer
CO ₂ incubator	Sanyo, München
Electrophoresis Power Supply	Pharmacia Biotech, Freiburg
SDS-PAGE NuPAGE/ XCell SureLock	Invitrogen, Karlsruhe
Gel Electrophoresis-Apparatus Horizon 11.14	Invitrogen, Eggenstein

Equipment

Beckmann J2-21 Centrifuge
Microscope Olympus CK40
Heatblock Thermomixer Comfort
LAS 3000 CCD Camera
Centrifuge 5417 R Eppendorf
Dynal Magnetic Particle-Concentrator-S
Nucleofector Device
Fluorescence Microscope IX50
Fluorescence Microscope Leica DMLB
FACSCanto™ II Flow Cytometer
Micro Centrifuge
Sonifier Cell Disruptor B15
VICTOR² 1420 Multilabel Counter
Flow Adhesion Chamber
Thermal Cycler 7900HT
T3000 Thermocycler
PCR-machine Primus 96
Neubauer Cell Chamber
PHM82 Standard pH-Meter
AF 100 Flake ice maschine
High temperature dry block heater
MS2 Mini shaker
Shaker Rocky
Spectrophotometer Nanodrop ND-1.000
Water bath Typ 1004
Centrifuge EBA12
Centrifuge Z383K
Centrifuge Z400K
Tissue culture hood
-152 °C cryogenic freezer
-80 °C ultra low freezer
-20 °C freezer
4 °C refrigerator

Manufacturer

Beckmann, USA
Olympus Co., Hamburg
Eppendorf, Hamburg
Fujifilm, Düsseldorf
Eppendorf, Hamburg
Invitrogen, Karlsruhe
Lonza, Cologne
Olympus, Hamburg
Leica Microsystems, Wetzlar
BD Bioscience, Heidelberg
Carl Roth, Karlsruhe
Branson, USA
PerkinElmer, USA
IMCAR, RWTH Aachen
Applied Biosystems, Darmstadt
Biometra, Göttingen
MWG AG Biotech, Ebersberg
Paul Marienfeld, Lauda-Königshofen
Radiometer, Denmark
Scotsman, Italy
Grant Instruments, UK
IKA, Staufen
CTF, Wasserburg
PeqLab Biotech. GmbH, Erlangen
GFL, Burgwedel
Hettich, Tuttlingen
Hermle, Wehingen
Hermle, Wehingen
KOJAIR Tech Oy, Finland
Sanyo Electric Biomedical, Japan
Sanyo Electric Biomedical, Japan
Bosch, Stuttgart
Liebherr, Ochsenhausen

2.8.2 Consumables

Description	Manufacturer
Cell scraper	Nunc, USA
ELISA-Microplate (96 wells) white	Nunc, USA
FACS tube (5 ml)	Falcon, Heidelberg
Filter 0.2 µm	Whatmann GmbH, Dassel
Gel blotting paper	Schleicher & Schuell Bioscience, Dassel
Needle (25 G, 23 G)	BD Microlance, Heidelberg
Cryogenic vials	Nalgene, USA
NuPage/ Bis-Tris 4-12 % Gel	Invitrogen, Karlsruhe
NuPage gel cassette	Invitrogen, Karlsruhe
Glass slide	Langenbrinck, Emmendingen
Glass slide folder	Carl Roth, Karlsruhe
Petri dish	Greiner Bio-One GmbH, Frickenhausen
Pipette tips blue (1000 µl)	Brand, Wertheim
Pipette tips yellow (200 µl)	Brand, Wertheim
Pipette tips white (20 µl)	Sarstedt, Nümbrecht
Nitrocellulose transfer membrane	Whatman GmbH, Dassel
Syringe	BD Microlance, Heidelberg
Centrifuge tube	Greiner Bio-One GmbH, Frickenhausen

2.9 Cell lines used

Description	Origin/Phenotype	Source
HUVECs	Human Umbilical Vein Endothelial Cells	PromoCell, Heidelberg
THP-1	Human Acute Monocytic Leukemia Cells	IMCAR, RWTH Aachen

2.10 Mice

Description	Source
<i>LysMCre-Csn5</i> -KO mice	AG Gessner, Universitätsklinikum Erlangen
<i>Apoe</i> ^{-/-} mice	AG Schober, RWTH Aachen

3 METHODS

3.1 Cell culture, cell counting, transfection, and gene silencing

Culturing of mammalian cells was done under sterile conditions in a laminar flow hood and maintained in a CO₂ incubator at 37°C and a humidified 5% CO₂ atmosphere. Human Umbilical Vein Endothelial Cells (HUVECs) were purchased from PromoCell, plated on cell culture dishes coated with collagen and cultured in endothelial cell growth medium, according to the manufacturer's recommendations. These primary cells were used at passages ranging from 3 to 5 for all experiments. Cells were counted using the Neubauer counting chamber under a light microscope. The number of cells per milliliter of medium was calculated using the formula: number of cells/ml = $n/4 \times 10^4$. Transfection of HUVECs with small interfering RNA (siRNA) or with plasmid DNA was performed by electroporation using the HUVEC Nucleofector Kit following the manufacturer's instructions. The concentration of siRNA duplexes was maintained at 3 µg/ml throughout the experiment whereas that of plasmid DNA varied depending on the experiment. In the overexpression experiments, GFP was cotransfected to follow the transfection efficiency. After electroporation, cells were allowed to recuperate for 48 h (in overexpression experiments) or 96 h (in gene silencing experiments) before being used in experiment. Where indicated, HUVECs were stimulated with 20 ng/ml human TNF-α for the indicated time periods. For coimmunoprecipitation, HUVECs were pretreated for 1 h with 50 µmol/L MG132 to block the activity of the proteasome, prior to TNF-α treatment. The open reading frame of CSN5 was cloned into the pCDNA5/FRT/TO/CTAP vector by means of molecular cloning techniques to create the CTAP-CSN5 construct. The following predesigned siRNAs (MWG Biotech AG) were used for gene knockdown experiments: CSN5: 5'-GCUCAGAGUAUCGAUGAAAdTdT-3'²⁸⁰; CSN2: 5'-GCACUGAAACAAUGAUUATT-3'²⁴²; scrambled: 5'-AACAGUCGCGUUUGCGACUTT-3'.

3.2 Freezing and thawing of mammalian cells

Frozen stocks of cell lines were stored in 1 ml cryovials in cryogenic freezer. Cells were first harvested and then resuspended at a concentration of 10⁶ cells/ml complete medium containing 5% (v/v) dimethylsulfoxide (DMSO).

The cryovials were placed overnight at -80°C in a cryocontainer to ensure slow cooling and then transferred to -152°C cryogenic freezer for long-term storage. For thawing of cells, cryovials were rapidly thawed in a 37°C water bath. Cells were slowly added to 4 ml warm growth medium, centrifuged and resuspended in growth medium.

3.3 Cell lysis, coimmunoprecipitation, western blot and immunodetection

3.3.1 Cell lysis

For total cell lysates, HUVECs were washed with cold PBS and lysed with 1x NuPAGE-LDS-sample buffer containing 1 mmol/L DTT. Cells were further treated with sonifier cell disruptor to ensure complete lysis. For coimmunoprecipitation, the cells were lysed in coimmunoprecipitation (CoIP) buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 15 mmol/L EGTA, 1% Triton X-100, 1 mmol/L DTT, 25 mmol/L NaF, 1% Proteinase Inhibitor Cocktail).

3.3.2 Coimmunoprecipitation

Cell lysates were made by homogenization. After centrifugation for 5 min at 14000 rpm, the supernatant was transferred to new Eppendorf tubes. The supernatants were incubated overnight with 2 µg/ml specific antibodies or an isotype control, followed by incubation with dynabeads protein A for 2 h. The Eppendorf tubes were centrifuged for 2 min at 4°C at 5000 rpm. The supernatants were discarded and the beads washed three times with 500 µl CoIP buffer. Immunoprecipitated proteins were eluted with 1x NuPAGE-LDS-sample buffer at 70°C for 10 min. Immunoprecipitated proteins were directly separated by SDS-PAGE or stored at -20°C for later use.

3.3.3 Western blot and immunodetection

Total cell lysates or immunoprecipitated proteins were directly separated by SDS-PAGE at 190V in NuPAGE/XCell SureLock-chamber with NuPAGE MES-running buffer. Protein samples were first concentrated in a stacking gel before migrating into the resolving gel. Proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane XCell II Blot module from Invitrogen. The blot was run at 33 V for 1.5 h under cold conditions after which it was incubated in BSA at RT for 1 h to

prevent non-specific binding. After blocking, primary antibodies were incubated either at room temperature for 1 h or at 4 °C overnight depending on the affinity of the antibody for the antigen being pursued. The concentration of BSA used for these incubations varied from 1% to 3% BSA depending on the specificity of the antibody used. In all experiments, secondary antibodies were incubated at room temperature for 1 h in 1% BSA. Primary anti-CSN5 (FL-337) and anti-CSN3 (RR12) antibodies were purchased from Santa Cruz Biotechnology. Anti-phospho-IKK1/2 (Ser 176/180, #2687), anti-IkB α (#9242), anti-IKK1 (#2682) and anti-IKK2 (#2678) antibodies were bought from Cell Signaling Technology. Anti-Cullin1 (ab2964) and anti- β -actin (#08691001) antibodies were purchased from Abcam and MP Biomedicals, respectively. As secondary antibodies, HRP-conjugated anti-mouse or anti-rabbit antibodies (Pierce) were used and blots were developed with Super Signal West Dura Extended Duration ECL reagent (Pierce/KMF). Protein bands were visualized with a LAS 3000 Imager and quantified using the Aida Image Analyzer Software (Fuji/Raytest).

3.4 Cellular fractionation: preparation of nuclear and cytoplasmic extract

To separate the nuclear fraction from the cytoplasmic fraction, HUVECs, pretreated depending on the experiment, were lysed in nuclear extract buffer 1 (NEB1) containing freshly added 1 mM Na₃VO₄. Cells were transferred into eppendorf tubes and incubated on ice for 15 min. 0.5% NP-40 was added and the tubes were vortexed for 10 sec followed by centrifugation at 13000 rpm for 30 sec at 4 °C. The supernatant was collected as the cytoplasmic fraction and the pellet resuspended in nuclear extract buffer 2 (NEB 2) and incubated on ice for 30 min with vortexing in between the incubation period. The tubes were centrifuged at 13000 rpm for 5 min at 4 °C after which the supernatant was collected as the nuclear extract.

3.5 NF- κ B DNA binding activity

The binding of NF- κ B to DNA was monitored using the TransAM NF- κ B Transcription factor ELISA Kit from Active Motif, USA. TransAM NF- κ B family kit designed specifically for the study of NF- κ B pathways, contain a 96-well plate to which oligonucleotides containing NF- κ B consensus binding site have been immobilized.

The activated NF- κ B contained in nuclear extracts specifically binds to this oligonucleotide. Nuclear extracts were prepared as indicated in 3.4 and incubated in the 96-well plate to which oligonucleotides containing NF- κ B consensus binding site have been immobilized. An antibody directed against p65 was used to detect the NF- κ B complex bound to the oligonucleotide. A secondary antibody conjugated to HRP was incubated to aid a sensitive colorimetric readout that was quantified by VICTOR² 1420 multilabel counter spectrophotometer from PerkinElmer, USA.

3.6 NF- κ B luciferase reporter assay

Reporter gene assay relies on protein translation and measures the regulatory potential of DNA-sequence. This can be achieved by linking a promoter sequence to an easily detectable reporter gene such as that encoding for the firefly luciferase. This was done for NF- κ B to monitor its regulation in pretreated HUVECs. The NF- κ B luciferase reporter construct plgk3ConALuc (1 μ g) and the β -galactosidase expression construct pEQ176 (1 μ g) were cotransfected with either siRNA duplexes or plasmid DNA, depending on the experiment, into HUVECs. After 48 h (overexpression experiments) or 96 h (gene silencing experiments), cells were harvested using reporter lysis buffer and assayed for luciferase and β -galactosidase activities using Bright-GloTM Luciferase Assay System and β -Galactosidase Enzyme Assay System according to manufacturer's protocol. The colorimetric readout was quantified by a plate reader. Luciferase values were normalized for β -galactosidase values to correct for differences in transfection efficiency (plotted as luciferase activity/ β -galactosidase activity).

3.7 Quantitative real-time PCR (RT-PCR)

The real time-PCR technique is based on the ability of the fluorescent dye SYBR Green to bind to double-stranded DNA; the increase in double-stranded DNA can therefore be directly detected. Thus, the increase of fluorescence over the reaction time can be linked to the mRNA or total RNA concentration used in the experiment. Based on this knowledge, RNA was isolated from HUVECs using the RNeasy Mini Kit and the amount determined using Spectrophotometer Nanodrop ND-1.000. A maximum of 1 μ g RNA was reverse-transcribed using oligo-dT primers.

RT-PCR analysis was performed using Express SybrGreenER qPCR Supermix in a thermal cycler 7900HT with specific human primer pairs for CSN5, CCL2, VCAM-1, ICAM-1 and GAPDH as reference gene.

cDNA synthesized as follows:

- 1 µg template RNA
- 2 µl 10 µM oligo-dT primer
- H₂O to a final volume of 17.5 µl
- Incubation at 70°C for 5 min
- 5 µl 5x M-MLV reverse transcription buffer
- 1.5 µl dNTP mix (10 mM each)
- Incubation at 37°C for 5 min
- 1 µl M-MLV reverse transcriptase
- Incubation at 42°C for 60 min

RT-PCR mix was performed as:

- 12.5 µl 2x Express SybrGreenER qPCR Supermix
- forward primer to a final concentration of 0.2 µM
- reverse primer to a final concentration of 0.2 µM
- 2 µl cDNA or H₂O
- H₂O to a final volume of 25 µl

RT- PCR Program:

Initial activation	95°C, 20 sec	
Denaturation	95°C, 3 sec	} 40 x
Annealing	60°C, 30 sec	
Melting curve	60°C to 95°C; read every 0.5°C, hold 1 sec between reads	

The relative mRNA expression was estimated as:

$$\Delta Ct = Ct (\text{target}) - Ct (\text{reference})$$

$$\Delta\Delta Ct = \Delta Ct - \Delta Ct (\text{target control})$$

$$\text{relative mRNA expression: } 2^{(-\Delta\Delta Ct)}$$

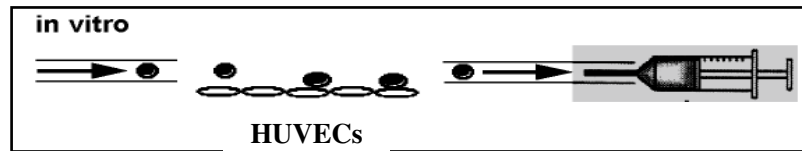
Ct: Cycle number at which the fluorescence intensity exceeds a determined threshold.

3.8 Flow cytometry

Flow cytometry is used to analyze cells by their size, granularity and surface expression of proteins using fluorescently labeled detection antibodies or to sort cell populations (FACS =Fluorescent activated cell sorting). In a buffer stream, one cell at a time passes by an argon laser, which excites fluorescently labeled cells. Measurements of the size (forward scatter) and granularity (sideward scatter) are independent of the fluorescence signal. Measurement of fluorescence intensity using fluorescently labeled antibodies was done to examine the expression of chemokines and adhesion molecules on the surface of HUVECs. To do this, pretreated HUVECs were washed with Hank's Balanced Salt Solution (HBSS) containing 0.3 mmol/L EDTA and 0.1% BSA. Cells were labeled with specific antibodies: a primary anti-ICAM1 antibody and a secondary Cy3-conjugated antibody; anti-P-selectin-PE antibody or the appropriate isotype controls. After careful washing, cell suspensions were measured on a FACSCanto TM II (BD Bioscience) and analyzed using FlowJo software (Tree Star). The results were displayed as histograms showing the logarithmic distribution of the fluorescence intensity or as diagrams demonstrating the change in mean fluorescence intensity (MFI).

3.9 Laminar flow adhesion assay

A key step in atherogenesis is the expression of adhesion molecules and secretion of chemokines upon activation of endothelial cells which trigger the binding and arrest of monocytes on the activated endothelium. This can be pursued in an *in vitro* setting which mimics the *in vivo* situation as depicted in Figure 14.



Adhered monocytes on EC surface

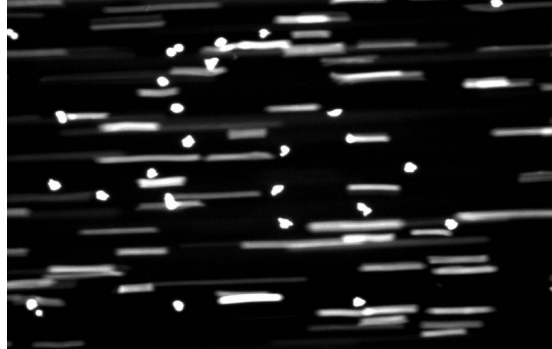


Figure 16 Setup of flow adhesion assay. Monocytes prestained with calcein were perfused over HUVECs monolayer for 4 min. Monocytes adhered depending on expressed adhesion molecules on the surface of HUVECs were counted.

Adhesion of calcein-AM (Molecular Probes)-labeled THP1 cells on confluent HUVEC monolayers, pretreated with 20 ng/mL human TNF- α as indicated, was quantified in parallel wall chambers under flow conditions (1.5 dynes/cm², 4 min)⁹.

3.10 Immunohistochemistry and immunofluorescence on human lesions

Collection of histologically normal human carotid artery specimens from autopsied individuals and early and advanced human carotid artery lesions from patients undergoing endarterectomy was approved by an institutional review committee and with the patients' informed consent, in accordance with institutional guidelines (EK287/11). For immunohistochemistry, an antibody against CSN5 (FL-337, Santa Cruz Biotechnology) or an appropriate isotype control antibody was used and visualized by the immunohistochemical avidin-biotin complex method with alkaline phosphatase enzyme development (Vector Laboratories, Ontario, Canada). Cells were counterstained by Mayer's hemalaun. Double immunofluorescence staining was performed with antibodies against CSN5 (FL-337, Santa Cruz Biotechnology), CSN1 (PW8285, Enzo Life Science GmbH) and CSN8 (PW8290, Enzo Life Science GmbH) in combination with anti-CD31 (M-20 sc-1506, Santa Cruz Biotechnology), and proteins were visualized with FITC- (for CSN) or Cy3-conjugated (for CD31) secondary antibodies (Jackson ImmunoResearch). Nuclei were counterstained by 4',

6-diamidino-2-phenylindole (DAPI). Images were recorded with a Leica DMLB fluorescence microscope and charge-coupled device camera, and image analysis was performed with Diskus Software (Hilgers).

3.11 Animal experiments

3.11.1 Generation of macrophage-specific *Csn5* KO mice

LysMCre-Csn5-KO mice (provided by AG Gessner, Universitätsklinikum Erlangen) were obtained by crossing male mice with *loxP*-flanked *Csn5* with female mice expressing Cre under control of the lysozyme M gene (The Jackson Laboratory). Heterozygous mice were then bred to obtain homozygous KO mice which were further crossed with *Apoe*^{-/-} provided by AG Schober, IMCAR RWTH Aachen) to obtain macrophage-specific deletion of *Csn5* on *Apoe*^{-/-} background. Heterozygous and wild-type littermates were used for experiments. Genomic DNA was amplified with specific primers as below.

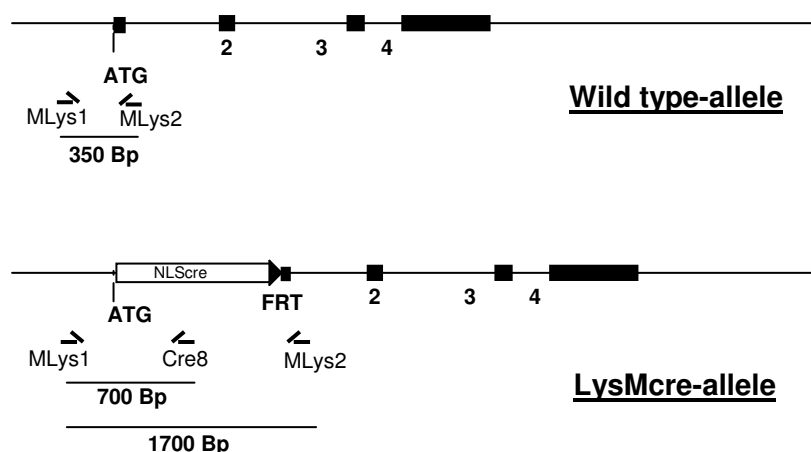
PCR distinguishes wild-type, heterozygous and homozygous mice as described by AG Gessner.

Primers:	Cre 8:	5'-CCC AGA AAT GCC AGA TTA CG-3'
	Mlys1:	5'-CTT GGG CTG CCA GAA TTT CTC-3'
	Mlys2:	5'-TTA CAG TCG GCC AGG CTG AC-3'

WT band is 350 bp, LysMcre: 700 bp and 1.7 kb (see below)

Program:	94 °C 5 min	
	94 °C 30 sec	} 35 x
	58 °C 30 sec	
	72 °C 50 sec	
	72 °C 5 min	

(1.7 kb band for the LysMcre allele was never obtained)



PCR genotyping for *Csn5-loxP* mice

PCR distinguishes wt, heterozygous and homozygous mice

Primers: InII1for: 5'-GGT CAG AAA GCT AGG CCT AAG AAG G-3'

ExII1rev: 5'-GGC ATG CAT CAC CAT TTT CAG TAG-3'

Bands: WT/WT - 397 bp; WT/Flox - 397 bp and 484 bp; Flox/Flox - 484 bp.

Program:

1. 94 °C	60 s	
2. 94 °C	30 s	} 40 x
3. 55 °C	30 s	
4. 72 °C	40 s	
5. 72 °C	5 min	

3.11.2 Genotyping of Mice

Efficient pursuit of animal research and reduction in the number of animals involved in a research project is achieved through proper identification of genetically engineered animals. DNA extracted from tissues of young rodents is mostly used in determining the genotype of these animals. DNA for PCR analysis can be obtained from ear punches, hair or fecal samples, oral or rectal swabs. Depending on the requirements of the study, investigators are urged to consider these noninvasive alternatives. Larger amounts of DNA are required for Southern Blot determination of the genotype. Obtaining tissue from a mouse for DNA analysis via tail biopsy is a safe, effective and humane procedure that causes minimal or transient pain and distress when performed properly. DNA prepared from tail biopsies is suitable for analysis by either Southern Blot or PCR. To check the genotype of mice used in this study, a tail biopsy was carried out in order to get enough DNA for PCR analysis while at the same time minimizing the stress on the mice. After taking biopsies from the tail, 500 µl tail buffer (100 mM Tris HCl (pH 8.5), 5 mM EDTA, 200 mM NaCl,

0.2% SDS) containing 10 µl Proteinase K was added to each tail in 1.5 ml eppendorf tubes. The tails were either incubated in 56°C water bath overnight or in a thermomixer at 56°C for 2-3 h with vigorous shaking and vortexing. The tubes were centrifuge at 14000 rpm for 5 min at RT, after which the supernatant was transferred into new tubes. 500 µl isopropanol was added to the supernatant and shook until white fibers were visible indicating DNA precipitation. The tubes were again centrifuged at 14000 rpm for 5 min at 4°C and the supernatant discarded. 500µl of 70% ethanol was added to the resulting pellet followed by centrifugation at 14000 rpm for 5 min at 4°C. The supernatant which is primarily ethanol was removed and the pellet dried at 37°C on a thermomixer. The dried pellet which is basically DNA was dissolved in 200µl distilled water and vortexed several times to ensure complete solvation. The concentration and purity of the resulting DNA was measured with Nanodrop ND-1000 Spectrophotometer and used directly for PCR analysis or stored at -20 °C.

3.11.3 Isolation of bone marrow cells and generation of bone marrow-derived macrophages

Mice were sacrificed by cervical dislocation and the abdomen and hind legs were sterilized with 70% ethanol. An incision in the midline of the abdomen was made and was clipped outward to expose the hind legs. The femur was separated from the tibia by cutting the knee joints followed by removal of muscle tissue from the bones. The bones were then cut at both ends and flushed with RPMI medium containing 1% penicillin-streptomycin and 10% FCS using 5 ml syringe and 25-gauge needle. Single-cell suspensions were made by pipetting the bone marrow cells up and down. The cells were passed through 40 µM cell strainer and the strainer washed with another 5 ml medium. Cells were incubated with erythrocytes lysis buffer for 3 min and the reaction stopped with RPMI medium followed by additional washing with RPMI medium. Centrifugations were done at 1250 rpm (option: slow) for 10 min. Single-cells were counted and incubated in medium containing 25 ng/ml M-CSF for differentiation. Cells were washed with PBS every 2-3 days and incubated in fresh M-CSF containing medium. Progenitors of macrophages adhered to the cell dish and were not washed away. Macrophages were fully differentiated at day 6-7 and were used for experiments.

3.11.4 Mouse model of atherosclerotic disease progression

LysMCre-Csn5-KO and their wild-type littermates, all on *Apoe*^{-/-} background, were fed an atherogenic diet containing 21% fat (Altromin) for 12 weeks. Thereafter, the mice were anesthetized with 10% ketamine mixed with 2% xylazine and isotonic NaCl solution intraperitoneally. Cardiac puncture was performed with 10% Na⁺-citrate to draw blood and the thorax was opened to facilitate drawing of any remaining blood. Part of the blood was stored in EDTA tubes for FACS analysis and the remaining stored in serum tubes. Supernatants from the blood in serum tubes were collected after centrifugation at 3000 rpm for 15 min and flash-frozen in liquid nitrogen and stored at -80 °C for further analysis like secretion of cytokines. The heart and its vasculature were rinsed with PBS until the liver was uncolored. Lymph nodes, spleen, femur and tibia were harvested, flash-frozen and stored at -80 °C for further use. After harvesting these organs, the heart and its vasculature were again rinsed with 4% PFA solution containing sucrose and EDTA. The heart, aortic arch and the descending aorta were harvested by in situ perfusion fixation with 4% PFA. The heart was sectioned with a microtome to get access to the aortic root.

The extent of atherosclerosis was assessed in aortic roots and in the descending aorta by either staining of 5 µm transversal sections, 5 sections per mouse, or the prepared aorta respectively for lipid deposition with Oil-red-O. After staining with Oil-red-O, tissue sections were counterstained with hemalaun and then washed with PBS until the buffer remained clear. Images were recorded with a Leica DMLB fluorescence microscope with CCD camera and quantification done by computerized image analysis (Diskus software, Hilgers, Aachen, Germany). All animal experiments were conducted according to the protocol approved by local authorities and complied with German animal protection law.

3.12 Bacteria culture and transformation

3.12.1 Bacteria culture

Strains of Bacteria were plated on agar selection plates and incubated overnight at 37 °C. Colonies on the plate were then selected and inoculated in LB medium containing the antibiotic of interest.

The inoculum was then incubated overnight at 37°C with agitation. After centrifugation, the pellets were used for the purpose of the experiment or stored at -20 °C for further use.

3.12.2 Transformation of bacteria

Competent cells were thawed on ice for about 15 min. The plasmid DNA was mixed with the competent cells in a tube and incubated on ice for 30 min. The tube was transferred to a 42°C water bath for 90 sec (heat shock). This sudden change in temperature caused the pores to open up to larger sizes, allowing DNA molecules to enter. The tube was then quickly transferred on ice for 2 min causing the pores to close up again trapping the DNA inside. 1 ml of LB medium was added to the cells and incubated at 37°C for 1h with agitation. After centrifugation at 4000 rpm for 5 min, the pellets were resuspended in about 100 µl LB medium and plated on agar selection plate containing preferred antibiotic. The plates were incubated overnight at 37°C.

3.13 Plasmid preparation

The isolation of plasmid DNA from competent cells was achieved through Maxi- or Mini- preparations using QIAprep Spin Miniprep Kit and Plasmid Maxi Kit from QIAGEN. For Minipreps, the manufacturer's protocol was closely followed to obtain purified DNA. For Maxipreps, 200 – 300 ml inoculum incubated overnight at 37°C with agitation was centrifuged at 6000 rpm for 15 min using the Beckmann J2-21 Centrifuge. Resulting pellet was weighed and resuspended in buffer P1; for every 2 g pellet, 10 ml of buffer P1 was added. Equal volume of buffer P2 was added to the suspension and incubated for 5 min at room temperature. Pre-chilled buffer P3 was also added at the same volume as buffer P1 followed by incubation on ice for 20 min. The suspension was centrifuged at 17000 rpm for 30 min using the Beckmann J2-21 Centrifuge. The supernatant was run through a column equilibrated with 10 ml equilibration buffer and the bound DNA was washed three times with washing buffer. The washed DNA was eluted with 15 ml elution buffer followed by addition of 10.5 ml isopropanol. The eluted DNA was pelleted by centrifugation at 17000 rpm for 30 min and the pellet was subsequently dissolved in TE buffer followed by the addition of pre-chilled absolute ethanol and stored at -80 °C for 30 min.

The DNA was again pelleted by centrifugation at 13200 rpm for 30 min and then dissolved in TE buffer. The concentration and purity of DNA was determined and stored at -20 °C for further use.

3.14 Measurement of DNA concentration

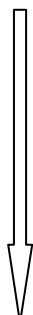
The concentration and purity of DNA was determined using the Nanodrop ND-1000 Spectrophotometer. The purity was determined from the ratio of the absorption at 260 and 280 nm which the spectrophotometer measures and generates by itself.

3.15 Digestion of DNA with restriction enzymes

DNA digestion was carried out with 3-4 U endonucleases per µg of DNA and 10% (v/v) reaction buffer. The reaction was incubated at 37 °C for 2 h.

3.16 Polymerase chain reaction (PCR)

The amplification of DNA using polymerase chain reaction was carried out in PCR Primus 96 Plus Thermocycler. Listed below are the components of the PCR, their respective concentrations and the programme used.

Components	Concentration	PCR - programme			
		Step	T (°C)	t (min)	
DNA template	1 µg	Initialization	95	2	
Primer- forward	10 pM	Denaturation	95	1	
Primer- reverse	10 pM	Annealing	58- 63	1	
Taq polymerase buffer	10x	Elongation	72	3	
MgCl ₂	25 mM	Final Elongation	72	5	
dNTP	10 mM	Final hold	8	∞	
ddH ₂ O	18.5 µl				
Taq polymerase	3 U				

3.17 Separation of DNA fragments on agarose gel

The fragments of DNA were separated on 1% agarose gel with 1x TBE buffer using the Horizon® 11.14 gel electrophoresis apparatus from Biometra. 10x DNA loading buffer was added to the DNA sample in a ratio of 1:5 loading buffer to DNA volume. DNA samples were then loaded on the gel and ran at 120V for 35-40 min. After running, the DNA band was visualized with UV light after incubation in ethidium bromide for 10-15 min.

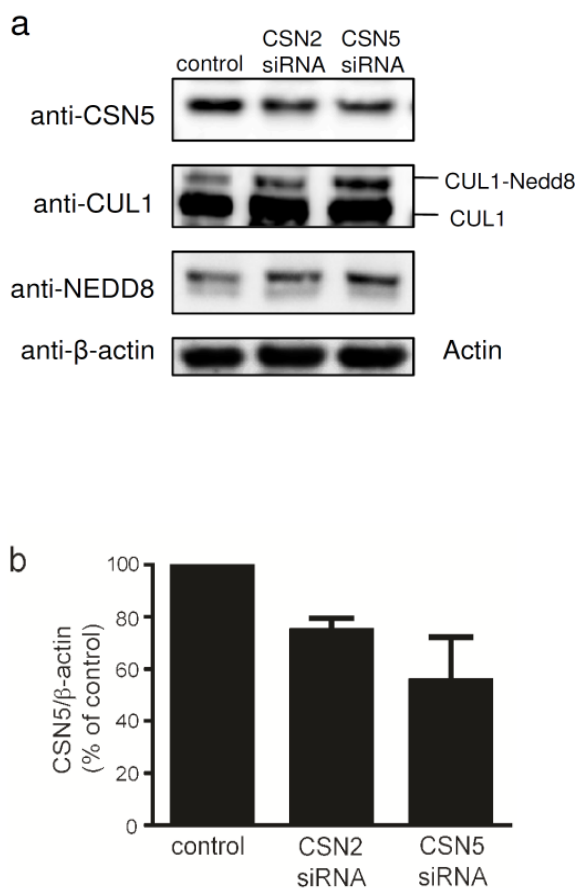
3.18 Statistical analysis

Numerical data represent mean for 2-6 independent experiments depending on experiment as in figure legends, with range/2 (for n =2) and SEM (for n=3) and were analyzed by two-tailed t-test with or without Welch's correction, one- or two-way ANOVA with Newman-Keuls or Bonferroni post test, as appropriate (Graphpad Prism 5.0 software). 8-10 mice per group were used for *in vivo* atherosclerosis studies. Observed differences with $p < 0.05$ were considered to be statistically significant.

4 RESULTS

4.1 CSN5 controls the deneddylation of cullins in endothelial cells

To begin to investigate whether CSN5, the only subunit of the CSN with known catalytic activity, has a functional role in the atherogenic endothelium, *CSN5* expression was transiently silenced in HUVECs using siRNA technology (Figure 17a-b). The effect on CUL1 neddylation was also examined. As shown in Figure 17a and c, reduction of CSN5 protein led to enhanced CUL1 neddylation, as evidenced by an increase in the density of the slower migrating CUL1 band in SDS-PAGE gels which was confirmed by anti-nedd8 antibody. Knock-down of *CSN2* also promoted CUL1 neddylation, although to a smaller degree, and also led to a decrease in CSN5 protein levels (Figure 17a-b), confirming previous reports that suppressing CSN2 destabilizes the CSN complex and reduces the levels of other CSN subunits^{237, 242} as well as the deneddylation activity of the CSN complex. Thus, these data indicate that in HUVECs, CSN5 and CSN2, and hence the CSN complex, are actively involved in the deneddylation of cullins.



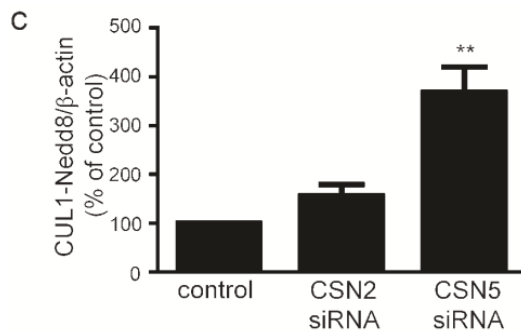
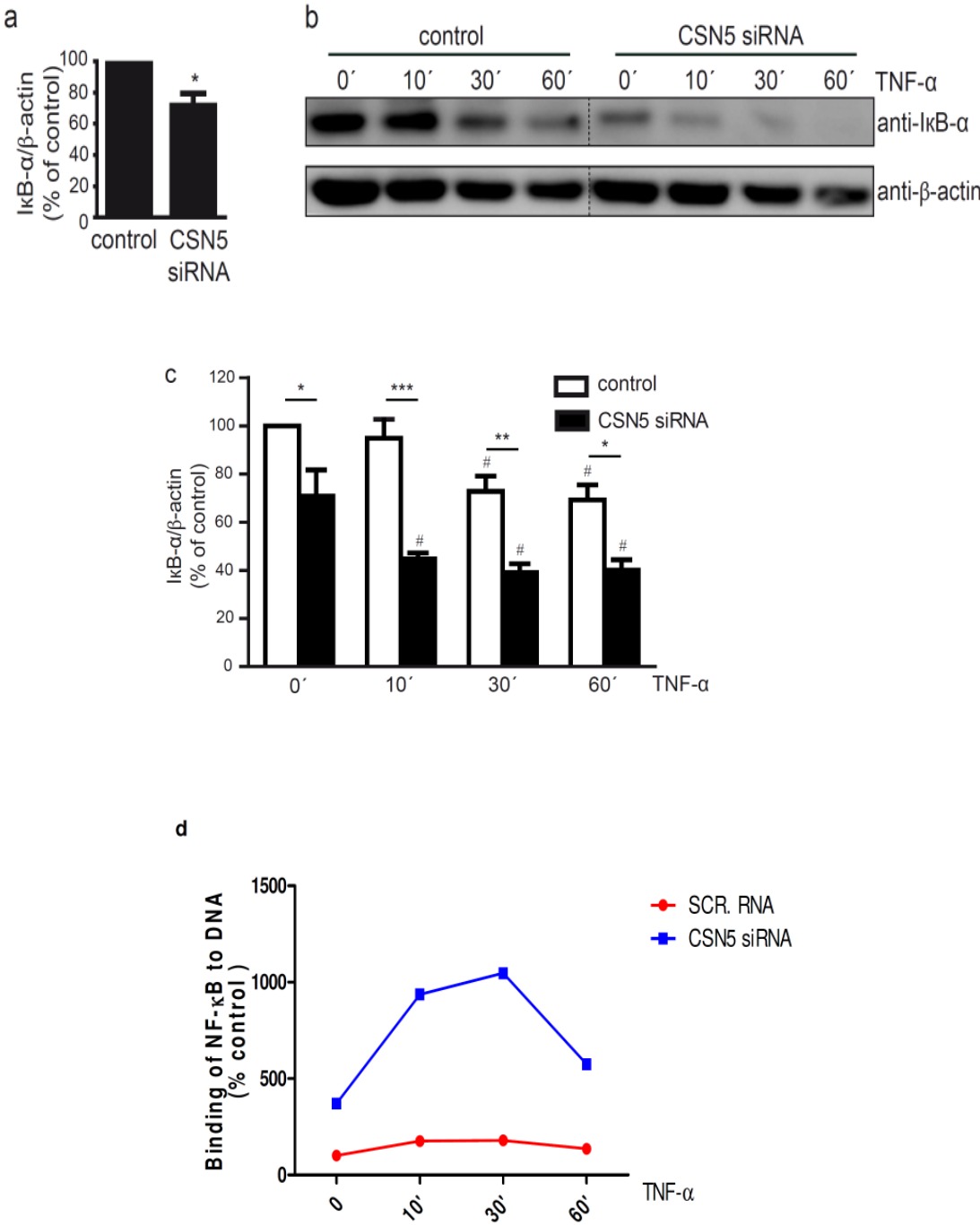


Figure 17 CSN5 triggers the deneddylation of CUL1 in HUVECs. HUVECs were transiently transfected with *CSN5* siRNA or scrambled control siRNA (control). 96 h post transfection, cells were harvested for Western blot analysis. Different lanes of the same blot (same gel and exposure) were combined as indicated by the dotted lines, after excluding irrelevant samples. (a) Immunodetection of CSN5, CUL1, Nedd8 and β-actin using corresponding specific antibodies. Shown is a representative immunoblot. (b, c) Densitometric quantification of CSN5 (b) and CUL1-Nedd8 (c) in immunoblots as in (a), after normalization to β-actin. The graph represents means of 2-3 independent experiments, with the range/2 (for n=2) or SEM (for n=3). One-way ANOVA with Newman-Keuls post-test was applied for comparison versus control: **p<0.01.

4.2 CSN inhibits the NF-κB pathway in TNF-α-stimulated endothelial cells

Contrasting findings have been observed for the effect of the CSN complex on IκB-α stability in different cell types. Whereas the CSN has been shown to destabilize basal IκB-α in a cervical adenocarcinoma cell line²⁴², *CSN5* knock-down markedly reduced steady-state IκB-α levels in thymocytes²⁷⁴. This study specifically focused on the role of CSN5 in HUVECs and demonstrated a significant decrease in basal IκB-α levels upon *CSN5* knock-down (Figure 18a). Similarly, silencing of *CSN2* reduced IκB-α protein amount (Figure 18f). Furthermore, the inducible degradation of IκB-α upon TNF-α challenge, i.e. mimicking inflammatory or atherogenic stimulation, was significantly enhanced by *CSN5* reduction (Figure 18b-c). This coincided with a markedly increased activation of NF-κB as read out by NF-κB binding to DNA activity (Figure 18d) and a luciferase reporter gene assay (Figure 18e). Inversely, transient overexpression of CSN5 using a CTAP-CSN5 fusion construct increased steady-state IκB-α levels (Figure 19a-b) and also delayed IκB-α degradation upon TNF-α stimulation (Figure 19c). In line with this, NF-κB activity was significantly reduced in both unstimulated and TNF-α-stimulated HUVECs (Figure 19d). Thus, these data

clearly show that CSN5 impairs basal and TNF α -triggered activation of NF- κ B by stabilization of the NF- κ B inhibitor I κ B- α .



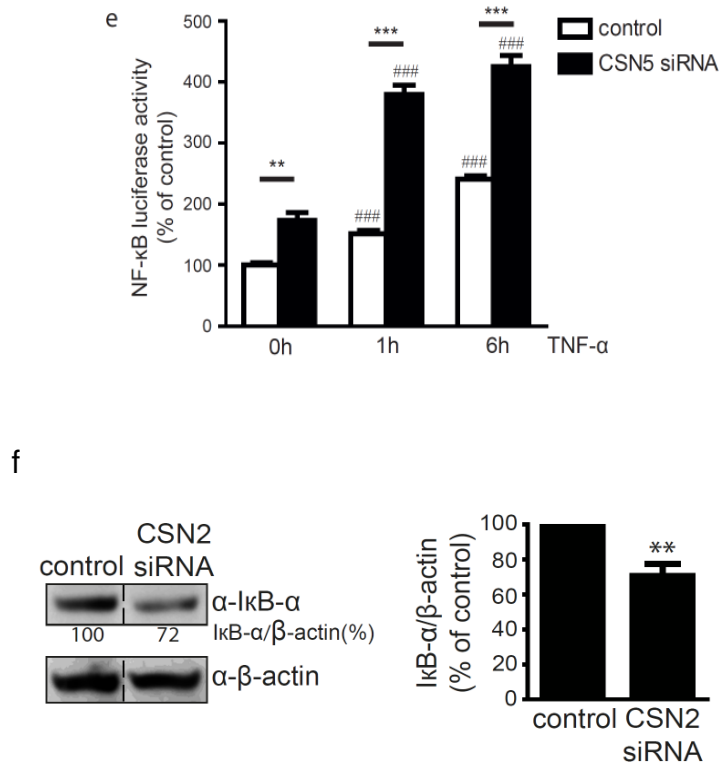


Figure 18 CSN modulates the NF-κB pathway in TNF-α-stimulated endothelial cells. HUVECs were transiently transfected with siRNA that targets *CSN2*, *CSN5* or scrambled control RNA. After 96 h, cells were either left untreated or stimulated with TNF-α for the indicated time periods as indicated, and prepared for Western blot analysis. The *CSN5* knockdown rate was similar to that in Figure 1. (a) Quantification of basal IκB-α normalized to β-actin; these data represent means±SEM of 4 independent experiments. Two-tailed t-test: * $p < 0.05$. (b) Immunodetection of IκB-α, CSN5, and β-actin in cell lysates of HUVECs treated with TNF-α for the indicated time points. Different lanes of the same blot (same gel and exposure) were combined as indicated by the dotted lines, after excluding irrelevant samples (c) Quantification of IκB-α, normalized to β-actin. The data represent means±SEM of 3 independent experiments. (d) Binding of NF-κB to NF-κB consensus sequence immobilized on a plate. (e) NF-κB-dependent luciferase activity after normalization to β-galactosidase activity, relative to the untreated control. Data represent means±SEM of one representative experiment out of 4, performed in triplicate each. (c,e) Two-way ANOVA with Bonferoni post-test for comparison of control versus *CSN5* siRNA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. One-way ANOVA with Newman-Keuls post-test for comparison versus unstimulated cells: # $p < 0.05$, ### $p < 0.001$. (f) Left panel: Representative immunodetection of IκBα and β-actin, after silencing the gene expression for CSN2, using the corresponding specific antibodies. Right panel: Quantification of IκBα, normalized to β-actin. The graph represents means±SEM of 4 independent experiments. Two-tailed t-test: ** $p < 0.01$.

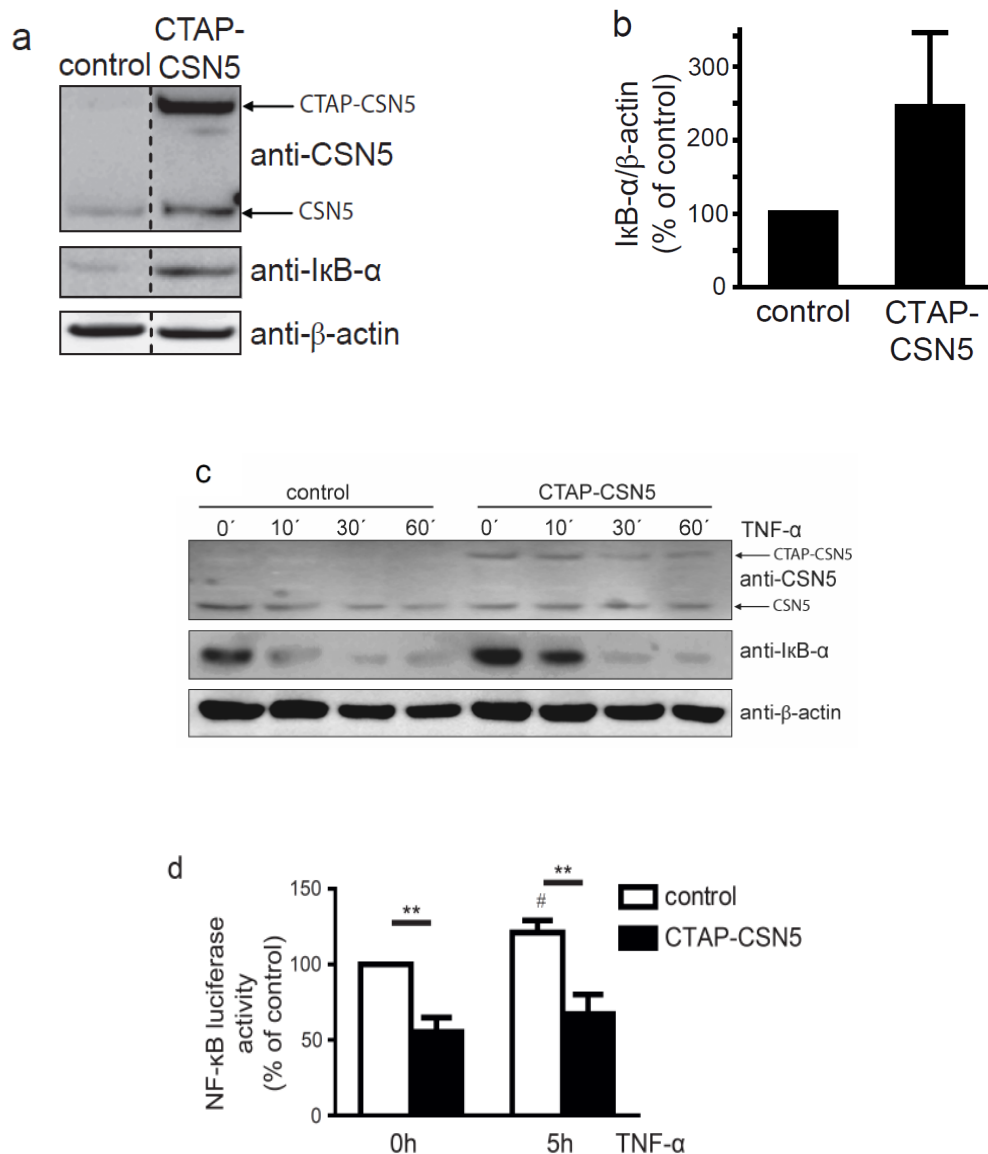
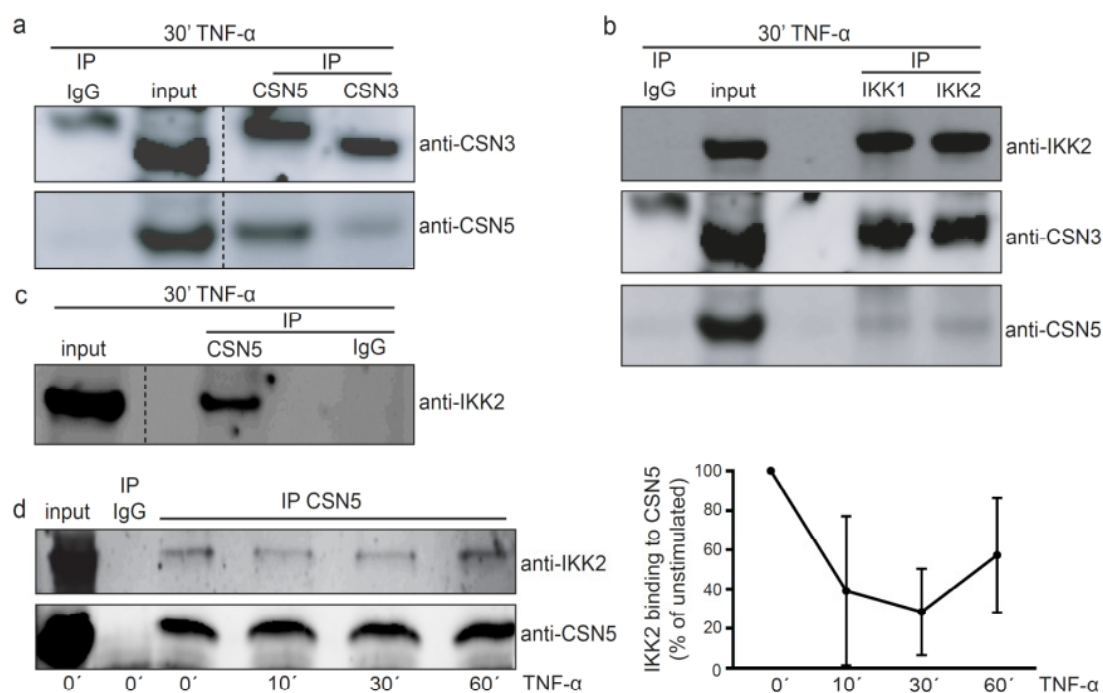


Figure 19 CSN5 stabilizes IκB-α and impairs TNFα-induced NF-κB activation in ECs. HUVECs were transiently transfected with a CTAP-CSN5 fusion construct or empty vector as control. After 48 h, cells were either left untreated or stimulated with TNF-α for the indicated time periods. (a) Immunodetection of basal IκB-α, CSN5, and β-actin using the corresponding specific antibodies. (b) Quantification of IκB-α, normalized to β-actin. The graph represents means±range/2 of 2 independent experiments (c) Immunodetection of IκB-α, CSN5, and β-actin in cell lysates of HUVECs treated with TNF-α for the indicated time points. (d) NF-κB-dependent luciferase activity after normalization to β-galactosidase activity, relative to the untreated control. Data represent means±SEM of 4 independent experiments. Two-way ANOVA with Bonferoni post-test for comparison of control versus CTAP-CSN5: **p<0.01. Two-tailed t-test for comparison versus unstimulated cells: #p<0.05.

4.3 Endogenous interaction between the CSN and the IKK complex

The observation that CSN5 inhibits NF- κ B activation in TNF α -stimulated ECs, set the stage to pursue a possible interaction between the CSN and the IKK complex, which is immediately upstream of I κ B- α . The presence of a CSN complex in HUVECs was examined by coimmunoprecipitation (CoIP) of CSN3 and CSN5, confirming their interaction at the endogenous level (Figure 20a). Furthermore, immunoprecipitation of endogenous IKK1 and IKK2 from TNF α -stimulated HUVECs resulted in a coprecipitation of endogenous CSN3 and CSN5 (Figure 20b). Inversely, endogenous IKK2 was found to coprecipitate with CSN5 (Figure 20c). In addition, the interaction between the CSN and the IKK complex was revealed to be sensitive to TNF- α stimulation. Coprecipitation of IKK2 and CSN5 was maximal in unstimulated HUVECs and decreased upon TNF- α exposure in a time-dependent manner (Figure 20d). In addition, a semi-endogenous immunoprecipitation revealed interaction of CSN2 with the regulatory subunit of IKK, IKK γ (NEMO) (Figure 20e)



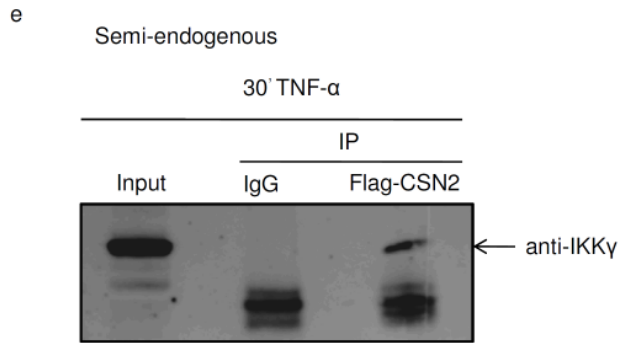


Figure 20 Interaction between CSN and IKK complexes in HUVECs. (a) Immunoprecipitation of endogenous CSN5 and CSN3 from TNF- α -stimulated HUVECs (30 min), followed by immunodetection for CSN3 and CSN5 in immunoprecipitates (IP) and input as indicated. (b) Immunoprecipitation of endogenous IKK1 and IKK2 from TNF- α -stimulated HUVECs (30 min), followed by immunodetection for IKK2, CSN3 and CSN5 in immunoprecipitates (IP) and input as indicated. (c) Immunoprecipitation of endogenous CSN5 from TNF- α -stimulated HUVECs (30 min), followed by immunodetection for IKK2 in immunoprecipitate (IP) and input as indicated. (d) HUVECs were treated with TNF- α for different time periods, followed by immunoprecipitation of endogenous CSN5 and immunoblotting for IKK2 and CSN5. A representative immunoblot is shown (left). The graph (right) represents the quantification of immunoprecipitated IKK2 after normalization to immunoprecipitated CSN5. The graph represents means of 2-3 independent experiments, and the range/2 (for $n=2$) or SEM (for $n=3$). (e) HUVECs were transiently transfected with Flag-CSN2 expression construct and treated with TNF- α for 30 min followed by immunoprecipitation of Flag-CSN2 and immunodetection of IKK γ /NEMO using their specific antibodies. (a-e) Immunoprecipitation with an isotype antibody (IgG) was used as negative control.

4.4 CSN attenuates the activation of IKK in TNF- α -stimulated endothelial cells

Interaction of proteins often leads to one protein exerting its effect on the binding partner which subsequently reflects on the biological functions in which they participate. The urge to pursue the effect of CSN on the activation of IKK was induced by the observation that CSN interacts with IKK (Figure 20) and that, silencing the gene expression for *CSN5* leads to enhanced NF- κ B activation (Figure 18e). It was therefore speculated that, inactivation of CSN might influence the activity of IKK. To verify this hypothesis, CSN was depleted from HUVECs through siRNA technology that targeted either *CSN2* or *CSN5*. Knockdown efficiency here was similar to that in Figure 1. Enhanced phosphorylation of IKK was observed after challenging the cells with TNF- α (Figure 21). Importantly, this enhanced phosphorylation stayed up to 60 min in *CSN5* knockdown cells (Figure 21) indicating

prolonged activation when CSN5 levels in the cells were reduced. This is in line with enhanced degradation of I κ B- α when CSN5 levels is depleted (Figure 18 b-c).

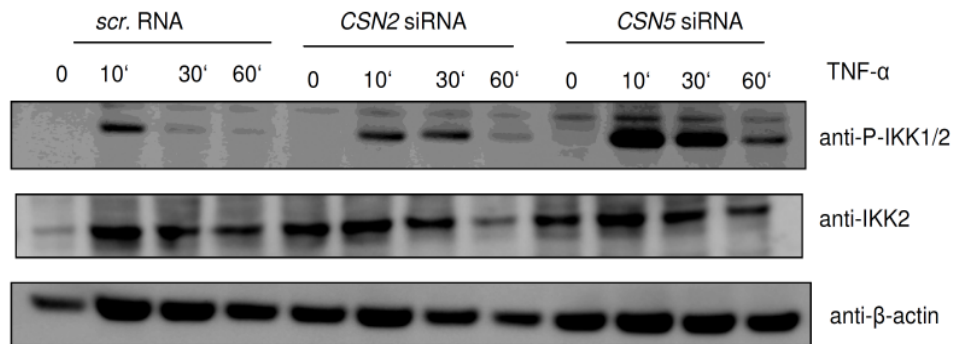


Figure 21 TNF α -induced phosphorylation of IKK is prolonged by *CSN2* and *CSN5* siRNA. HUVECs were transiently transfected with *CSN2* or *CSN5* siRNA and a scrambled RNA was used as a control. 96 h after transfection, cells were stimulated for different time intervals and harvested for Western blot analysis. Upper, middle and lower panels show immunodetection of P-IKK1/2, IKK2, and β -actin levels respectively using corresponding specific antibodies.

4.5 CSN5 depletion triggers the expression of key atherogenic chemokines and adhesion molecules in inflamed endothelial cells

In response to inflammatory and atherogenic stimuli, including TNF- α , the transcription factor NF- κ B has been shown to up-regulate the expression of chemokines such as CCL2 and adhesion molecules such as ICAM-1, VCAM-1, and P-selectin, all strongly implicated in atherogenesis^{192-195, 281}. The observation that *CSN5* knock-down results in an enhanced activation of NF- κ B (Figure 18d), prompted us to examine its effect on NF- κ B-dependent atherogenic gene expression. Although *CSN5* silencing in HUVECs did not significantly affect the basal levels of NF- κ B-dependent target genes (Figure 22a), TNF α -triggered transcription of *CCL2*, *ICAM-1* and *VCAM-1* was markedly enhanced upon *CSN5* knock-down (Figure 22b-d). Also the cell surface protein expression of ICAM-1 and P-selectin was markedly increased in TNF α -stimulated HUVECs upon *CSN5* depletion (Figure 22e-f). Together, these data indicate that *CSN5* knock-down upregulates the expression of atherogenic, NF- κ B-dependent genes in inflamed endothelium.

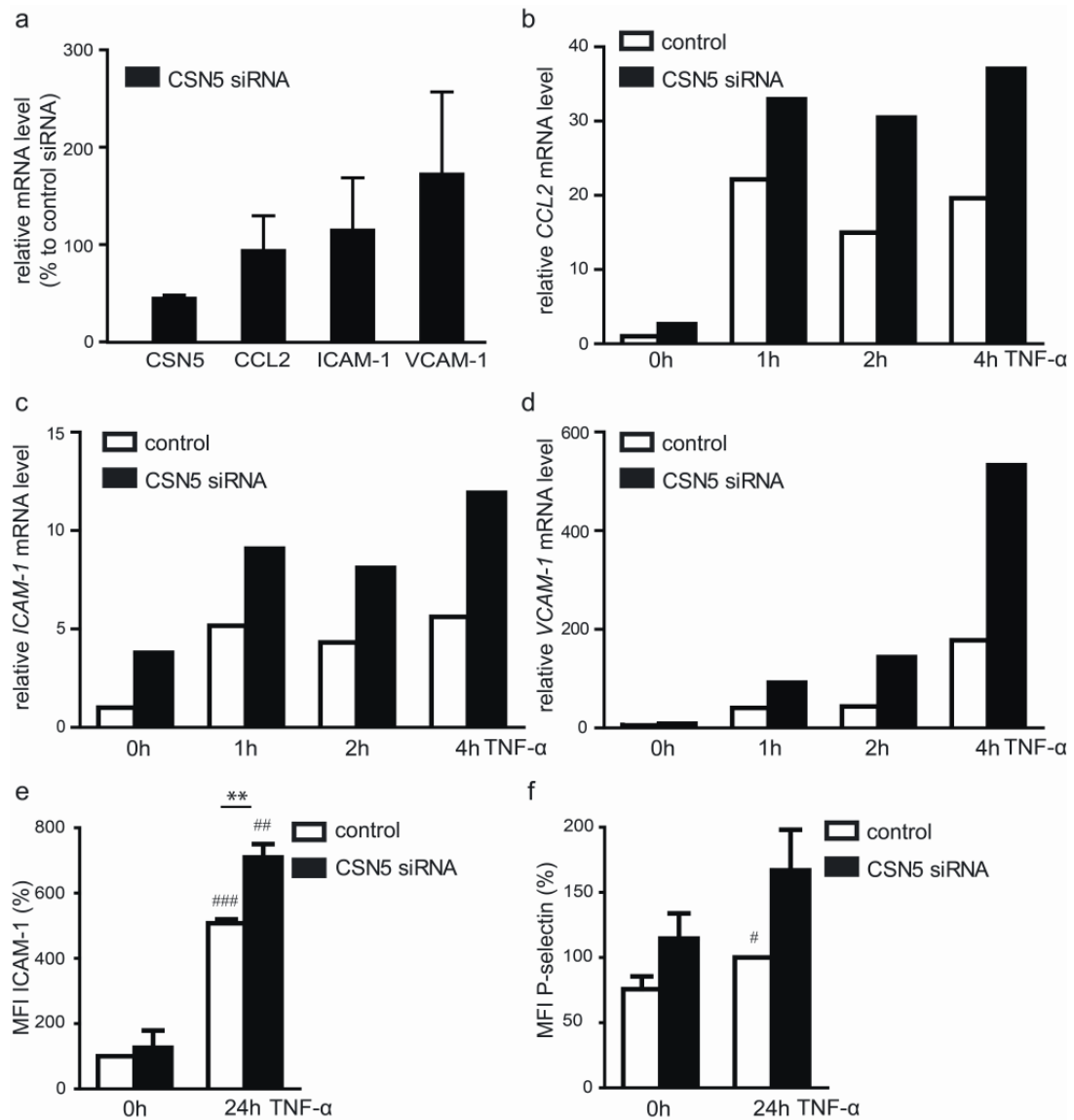


Figure 22 *CSN5* depletion in ECs upregulates the expression of NF-κB-dependent atherogenic chemokines and adhesion molecules upon TNF-α stimulation. HUVECs were transiently transfected with *CSN5* siRNA or scrambled control siRNA. After 72-96 h, cells were stimulated with TNF-α for indicated time periods. (a-d) Real time RT-PCR analysis of *CSN5*, *CCL2*, *ICAM-1*, and *VCAM-1* mRNA levels after normalization to *GAPDH*. Shown is (a) the means±SEM of 5-6 independent experiments and (b-d) one representative experiment of three performed. (e-f) Flow cytometric analysis of ICAM-1 and P-selectin. MFI, mean fluorescence intensity. Data represent means±SEM of 3-4 independent experiments. Two-way ANOVA with Bonferoni post-test for comparison of control versus *CSN5* siRNA: **p<0.01. Two-tailed t-test for comparison versus unstimulated cells: #p<0.05; ##p<0.01; ###p<0.001.

4.6 Endothelial CSN5 impairs monocyte arrest on TNF- α -stimulated endothelial cells

Adhesion molecules act in synergy with chemokines to trigger the binding and arrest of monocytes on activated endothelium, a key step in atherogenesis³³. To corroborate the finding that *CSN5* knock-down increases TNF- α -induced adhesion molecule expression (Figure 22) and more importantly, to ask whether an endothelial knockdown of *CSN5* would lead to altered monocyte adhesion rates, laminar flow adhesion assays were performed to investigate the arrest of perfused THP1 monocytes on resting and TNF- α -stimulated HUVECs. As expected, TNF- α administration significantly enhanced THP1 adhesion to HUVECs. Of note, this effect was significantly increased upon *CSN5* knock-down (Figure 23), while resting HUVECs only showed a small, non-significant increase in THP1 adhesion when *CSN5* was silenced (Figure 23). These results show that an endothelial *CSN5* knock-down functionally translates into an increased monocyte arrest rate on inflamed endothelium, indicating that endothelial *CSN5* counteracts atherogenic leukocyte recruitment processes.

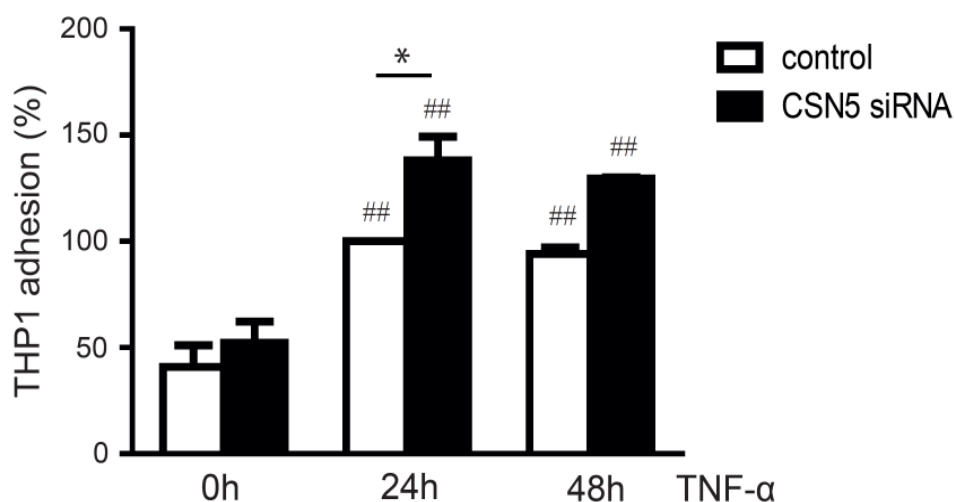


Figure 23 TNF- α -triggered mononuclear cell arrest on HUVECs is enhanced by the depletion of *CSN5*. HUVECs were transiently transfected with *CSN5* siRNA or scrambled control siRNA. After 72 h, cells were stimulated with TNF- α for indicated time periods and perfused with calcein-AM-labeled THP1 monocytic cells. THP1 adhesion was quantified as previously established⁹ and is expressed as % of the 24 h TNF- α -stimulated control. Data represent means of 2-3 independent experiments, with the range/2 (for n=2; 48 h) or SEM (for n=3, 24 h). Two-way ANOVA with Bonferoni post-test for comparison of control versus CSN5 siRNA: *p<0.05. One-way ANOVA with Newman-Keuls post-test for comparison versus unstimulated cells: ##p<0.01.

4.7 CSN5 is upregulated in human atherosclerotic lesions

Data in Figure 23 indicate that endothelial CSN5 can impair monocyte adhesion to ECs under pro-atherogenic conditions. This is the first functional piece of evidence suggesting that CSN5 could play a role in atherogenesis. Thus, CSN5 expression in human healthy and atherosclerotic carotid arteries was examined. As shown in Figure 24a, only a very weak CSN5 staining was visible in the endothelial cell layer of healthy carotid arteries. However, a clear CSN5 expression was detected in diseased specimens, and increased in the endothelium as well as in macrophage foam cells upon atheroprogession. Immunofluorescent colocalization of CSN5 with the endothelial cell marker CD31 further confirms endothelial expression in advanced plaques (Figure 24b, upper panel). Interestingly, also the CSN subunits CSN8 and CSN1 were found to be markedly expressed in atherogenic vessels and to colocalize with CD31 (Figure 24b, middle and lower panel). Together, these results suggest an active role for CSN5 and the entire CSN complex in the endothelial cell layer of atherosclerotic lesions.

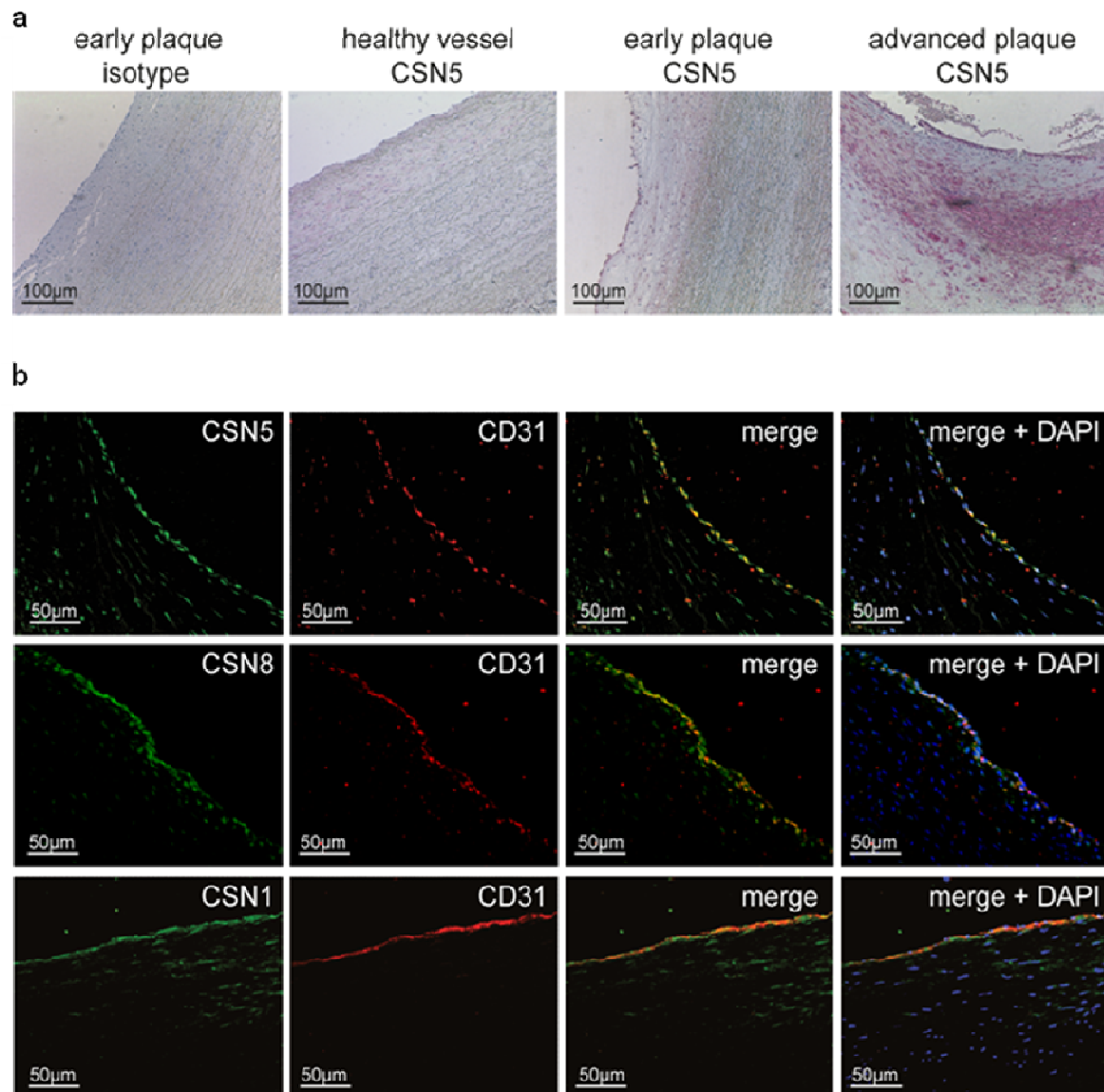
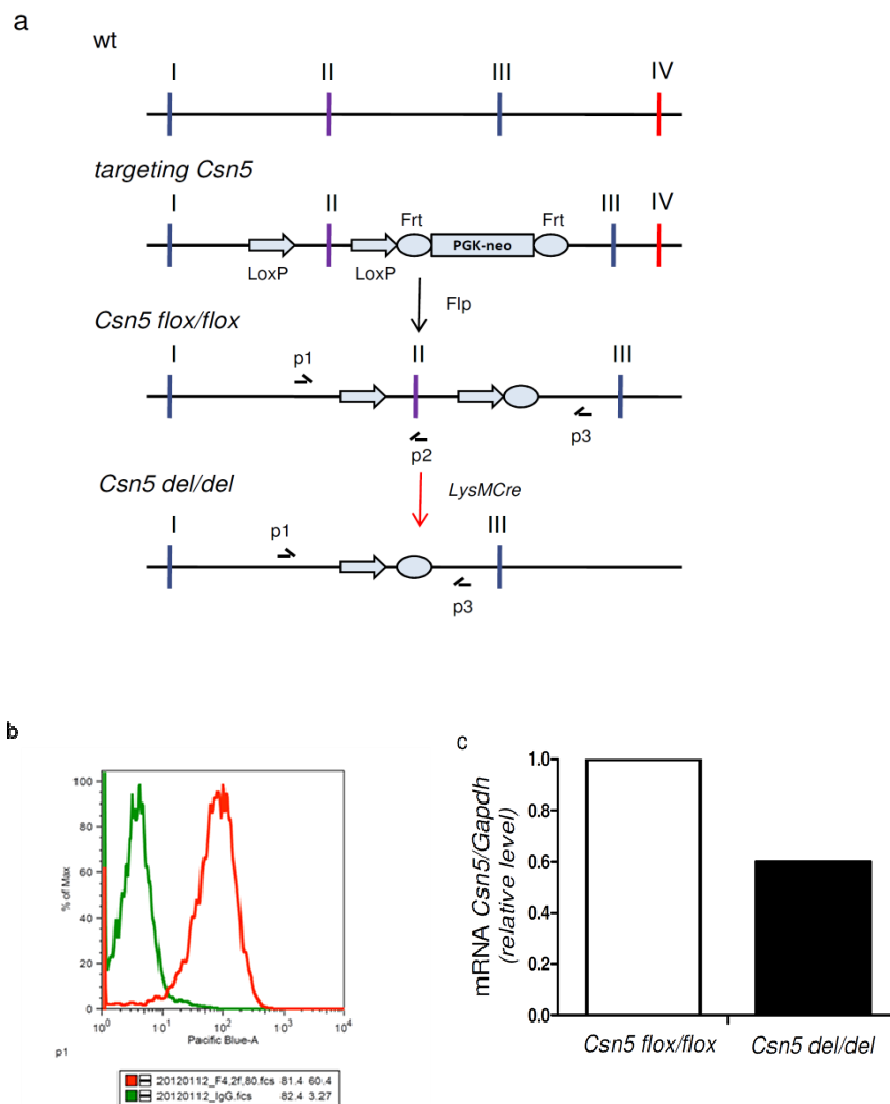


Figure 24 Expression of CSN in human carotid artery atherosclerotic lesions. (a) Immunohistochemical staining with alkaline phosphatase enzyme development of CSN5 in human healthy vessels or early and advanced atherosclerotic plaques. Specificity is shown by isotype negative control staining. Cell nuclei were counterstained by Mayer's hemalaun; scale bars 100 µm. (b) Double-immunofluorescent colocalization staining of CSN5 (upper panel, green), CSN8 (middle panel, green) and CSN1 (lower panel, green) with CD31 (red) in advanced human plaque tissue specimens. Cell nuclei were stained with DAPI. Scale bars: 50 µm.

4.8 Macrophage-specific deletion of *Csn5* via the Cre/loxP recombination system

To corroborate the findings that CSN5 has an active role in atherogenesis, a macrophage-specific deletion of *Csn5* in mice was generated with the help of the Cre/loxP system (Figure 25a). These mice were cross-bred with *Apoe*^{-/-} mice and cells isolated from the bone marrow. The efficiency of *in vitro* differentiation of committed myeloid progenitor cells into macrophage lineage was assessed by F4/80 surface antigen (Figure 25b). Analysis of mRNA (Figure 25c), genomic DNA (Figure 25d) and protein levels (Figure 25e) of *Csn5* in bone marrow-derived macrophages confirmed the deletion of *Csn5*.



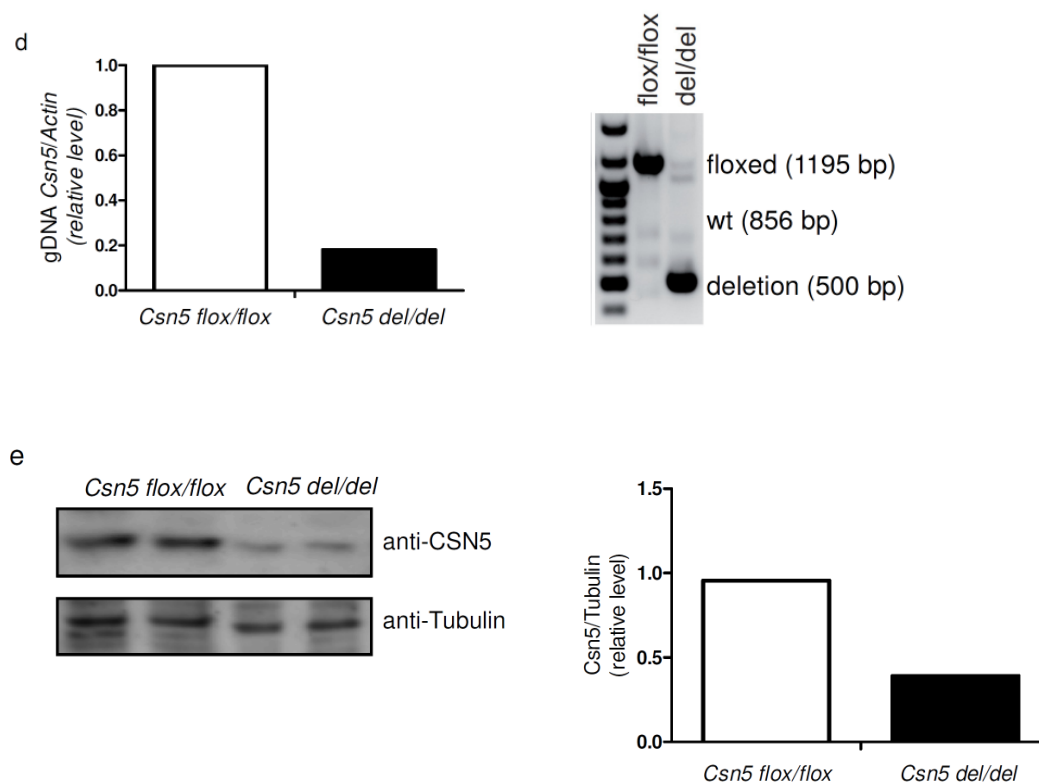
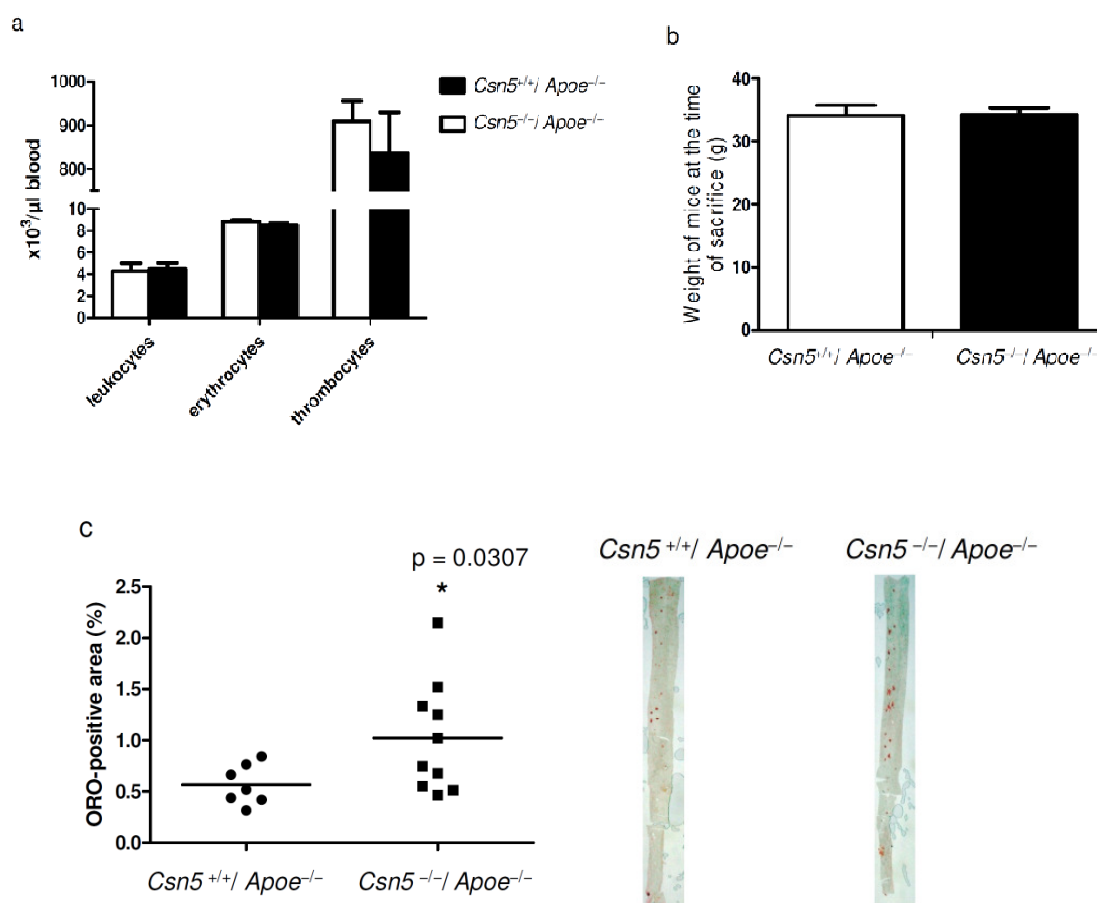


Figure 25 Targeted deletion of the *Csn5* gene in murine macrophages. (a) Strategy for targeting of exon II of the mouse *Csn5* locus with loxP sites. The targeting vector was designed to generate, after homologous recombination, the targeted *Csn5* allele with floxed (LoxP) exon II and the Phosphoglucokinase gene (PGK)-neomycin (PGK-neo) cassette flanked by FLP recognition target (Frt) sites. Generation of the floxed allele was achieved by removing the neo cassette through crossing with FLPe deleter mice. *LysMCre*-mediated deletion of the floxed allele led to out-of-frame rejoining and resulted in the deleted allele. Indicated are the positions and orientation of primers used to detect various alleles. (b) Bone marrow cells were isolated and differentiated into macrophages. The histogram shows flow cytometric analysis of the surface antigen F4/80 (red) and IgG control (green). (c-e) Genomic DNA and RNA were isolated from bone marrow-derived macrophages. The graphs represent real time RT-PCR analysis of RNA (c), genomic DNA (d) and protein levels of Csn5. Shown in the right panel of (d) are the PCR products of genomic DNA from *Csn5* floxed (flox/flox), *Csn5* deleted (del/del) mice, run on a gel. Indicated is the size of the wild type (wt) gene.

4.9 *Csn5* deletion in macrophages exacerbates atherosclerosis

CSN5 triggers an enhanced adhesion of monocytes to ECs under pro-atherogenic conditions (Figure 23) and is highly expressed in human atherosclerotic carotid arteries. This was observed particularly in the endothelium as well as in macrophage foam cells upon atheroprogession (Figure 24a-b), prompting us to speculate that CSN5 might have a role in atherosclerosis. To test the hypothesis that *Csn5* has a functional role in murine atherosclerosis *in vivo*, *Csn5*^{-/-}/*Apoe*^{-/-} and *Csn5*^{+/+}/*Apoe*^{-/-} littermates were fed a high fat diet (HFD) for 12 weeks. All mice appeared healthy, reproduced according to Mendelian ratios, and had a normal life span without any obvious abnormalities. There was no difference in the leukocyte, erythrocyte, thrombocyte counts and weight of the mouse groups used for the study (Figure 26a-b). Plaque analysis revealed that *Csn5*-deficient mice had significantly larger atherosclerotic plaques in the thoracoabdominal aorta and also in the aortic roots (Figure 26 c-d) when compared with control mice on HFD. This finding clearly confirmed the anti-atherogenic property of CSN5 as indicated by my *in vitro* data (see Figures 17-24).



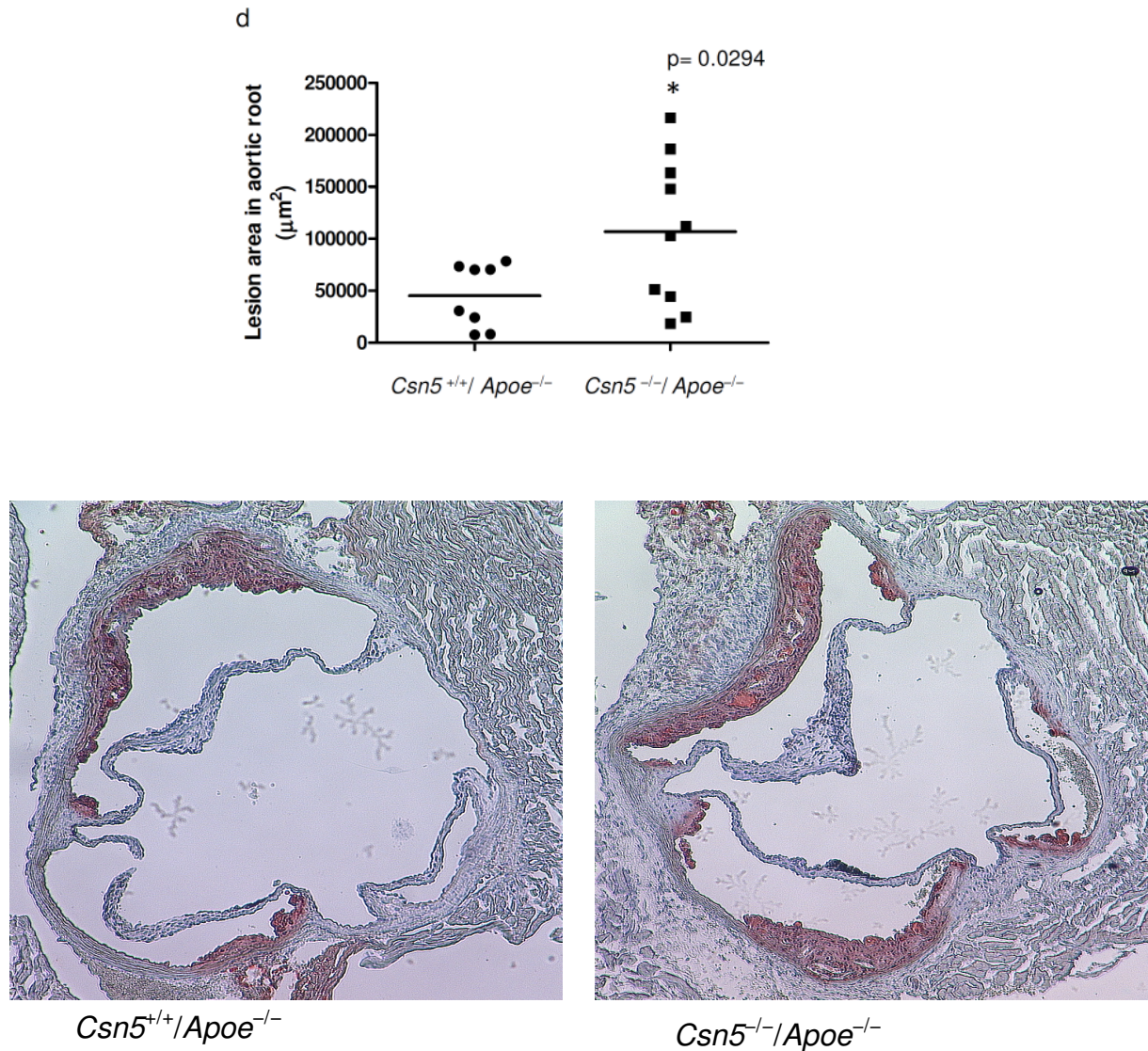


Figure 26 Macrophage-specific deletion of *Csn5* accelerates murine atherosclerosis. (a) Differential blood analysis revealed that, leukocyte, erythrocyte and thrombocyte population did not differ between *Csn5*-deficient and control mice. (b) Weight of mouse groups taken at the time of sacrifice. (c-d) *Csn5*^{-/-}/*Apoe*^{-/-} and *Csn5*^{+/+}/*Apoe*^{-/-} mice consumed HFD for 12 weeks and oil red O (ORO)-stained intimal lesion size in thoracoabdominal aorta (c) as well as aortic root (d) were quantified. Individual data points represent the average of plaque areas per mouse aortic root or thoracoabdominal aorta; horizontal bars denote mean. Representative images of the aortic root are shown. Two-tailed unpaired t-test with Welch's corrections for comparison versus control mice: **p*<0.05.

5 DISCUSSION

5.1 CSN5 controls the deneddylation of cullins in endothelial cells

The CSN complex has an established role in regulating the activity of CRL E3 ubiquitin ligases by deneddylation of the cullin subunit. This activity has been associated with the dissociation of CRLs, maintaining a dynamic activation cycle through transient disassembly and assembly of CRL subunits^{222, 223}. Being the only component with known catalytic activity, cullin deneddylation has been assigned to the JAMM motif of CSN5, which harbors an intrinsic metallo-isopeptidase activity²⁰⁰. In line with this, this study demonstrates that silencing of *CSN5* in ECs increases the CUL1 neddylation level (Figure 17), confirming earlier findings in other cell types^{237, 238, 242, 274}. Also, *CSN2* knock-down enhanced CUL1 neddylation concomitant with a decrease in CSN5 expression. The reduction in CSN5 levels upon *CSN2* silencing can be explained by the destabilization of the CSN holo-complex upon depletion of CSN subunits, resulting in decreased levels of all other CSN members^{242, 282, 283}. However, CSN5 can also stably exist independently of the holo-complex, i.e. as monomer or in small complexes, and performs important biological functions as such²²². It was shown that *CSN5* silencing can completely abolish CSN5 monomers²³⁷, while in most reports only moderately influencing the stability of the other CSN subunits^{236, 238, 240} and the CSN holo-complex^{238, 282} indicating that the CSN complex-bound form of CSN5 might be more stable. Inversely, transiently overexpressed CSN5 was reported to mostly exist as a monomer or in small complexes, although tagged CSN5 fusion proteins were demonstrated to be incorporable into the CSN complex^{252, 284, 285}. Taken together, these observations indicate that it might be difficult to attribute effects of modulating CSN5 expression clearly to monomeric CSN5 or the complexes to which it binds. It is expected that CSN5 equilibrates dynamically between these different forms and that the gene suppression of CSN5 may also lead to reduced levels of the CSN complex at least after prolonged periods of time.

5.2 CSN5 inhibits NF- κ B pathway in TNF- α -stimulated endothelial cells

I κ B- α is a known substrate of the SCF ^{β TrCP}-CRL, and its ubiquitination and proteasomal degradation triggered by a multitude of inflammatory stimuli is required to release the NF- κ B dimer for gene transcription²²⁶. This study shows that *CSN5* depletion enhances I κ B- α degradation upon TNF α stimulation of ECs (Figure 18b-c), whereas transient overexpression of *CSN5* delays I κ B- α turnover (Figure 19c). Steady-state levels of I κ B- α were similarly affected, showing a reduced or increased I κ B- α protein level upon *CSN5* silencing or overexpression, respectively, supporting a role for *CSN5* in I κ B- α stabilization. Comparable results were obtained in *CSN5*-deficient thymocytes, demonstrating markedly decreased steady-state levels of I κ B- α compared to wild-type cells²⁷⁴. And although *CSN2*- and *CSN5* knock-down increased basal I κ B- α protein concentrations in HeLa cells, the reduced re-accumulation of I κ B- α upon TNF α stimulation was similarly associated with a stabilizing function of the CSN towards *de novo*-synthesized I κ B- α ²⁴². Whereas Nedd8 conjugation is required for an optimal ubiquitin ligase activity of protein-destabilizing CRLs²²⁷⁻²³⁰, CUL1 deneddylation has been linked with CRL dissociation²²³ and with reduced CRL activity *in vitro*^{231, 232}. On the other hand, CUL deneddylation protects CRL components from self-destruction triggered by excess auto-ubiquitination-induced degradation^{223, 234-237}, as it supports a dynamic disassembly of old, ubiquitinated CRL complexes and the reassembly with fresh subunits^{222, 223}. This explains the counter-intuitive observation that, complete CSN inactivation decreases CRL activity *in vivo*²³³⁻²³⁸ despite enhanced CUL neddylation^{223, 233, 237}. In general, *CSN5* and the CSN complex might regulate I κ B- α by several mechanisms: first, the deneddylase activity of CSN might reduce the stability and activity of the SCF complex promoting polyubiquitination of I κ B- α . A reduction of *CSN5* would thereby lead to a destabilization of I κ B- α (Figure 18). Second, there might be an alternative mechanism of I κ B- α protection by CSN, involving the CSN- associated deubiquitinase USP15, as shown by Schweitzer and colleagues²⁴². In 293 cells, stable siRNA-knockdown of *CSN5* protected the cells from apoptosis induced by TNF- α or serum starvation. This implies that the enhanced NF- κ B activity achieved after silencing *CSN5* gene induces the expression of target genes that improve cell survival under stress conditions²⁷¹.

5.3 CSN endogenously interacts with IKKs and attenuates IKK phosphorylation

The IKK complex has previously been shown to bind CSN5, and TNF α -triggered phosphorylation of CSN5 by IKK2 preceded their dissociation, suggested to be required for a rapid ubiquitination and degradation of I κ B- α ²⁷¹. This transient interaction of IKK2 and CSN5 was confirmed in ECs, reaching a minimum upon 30 min of TNF- α administration (Figure 20). This interaction between the CSN and IKK complexes in endothelial cells was found to favor the stability of I κ B- α as silencing *CSN5* and also *CSN2* led to enhanced and prolonged phosphorylation of IKK α / β at serine 176/180 and therefore activation of IKK complex (Figure 21). An active IKK would phosphorylate I κ B- α thereby mediating the degradation of I κ B- α . This observation is in line with the finding that silencing *CSN5* or *CSN2* enhances the degradation of I κ B- α (Figure 18). Inactivation of the IKK complex by CSN5 could thus provide an additional mechanism underlying the observed I κ B- α destabilization upon *CSN5* silencing. To investigate the relative importance of this mechanism, the effect of an IKK phosphorylation-defective CSN5 mutant on TNF α -triggered I κ B- α degradation could be examined, but this first requires the identification of the phospho-acceptor sites in CSN5. Additionally, the activation of IKK in the canonical pathway is initiated by K63-linked polyubiquitination of IKK γ /NEMO which functions as a binding site for other signaling molecules like TAK1 required for activation of IKK β ¹⁰³⁻¹⁰⁵. The observation that CSN5 and CSN2, and hence the CSN complex controls CRL E3 ubiquitin ligases by deneddylation of the cullin subunit, places CSN5 in a position to control the polyubiquitination of IKK γ /NEMO by a possible depolymerization of the polyubiquitin chains. This will impair the activation of TAK1 and affect the activity of IKK which may explain the observation that silencing *CSN5* or *CSN2* results in an increased activity of IKK.

5.4 CSN5 inhibits the expression of key atherogenic chemokines and adhesion molecules and impairs monocyte arrest on TNF α -stimulated ECs

In accordance with the observed effects on basal and TNF- α -induced I κ B- α levels, transient overexpression of CSN5 significantly reduced NF- κ B-dependent transcription (Figure 19d), while *CSN5*-silenced ECs displayed a marked increase in NF- κ B transcriptional activity (Figure 18d). The latter corresponded with an increased

expression of NF- κ B-dependent target genes, both at the RNA as well as protein level (Figure 21). Furthermore, the enhanced cell surface presentation of the adhesion molecules ICAM-1 and P-selectin on TNF- α -stimulated ECs upon *CSN5* silencing was associated with a significantly increased ability to trigger the arrest of perfused THP1 monocytes (Figure 22). Although knock-down of *CSN5* also markedly enhanced the expression of these adhesion molecules in steady-state, their basal levels remained well below the concentrations reached in conditions of TNF α administration, which can explain the absence of a significant effect on steady-state THP1 binding by ECs upon silencing of *CSN5*. Mononuclear cell adhesion to inflamed endothelium is a crucial step in atherogenesis, as it precedes transendothelial migration and accumulation of inflammatory cells in the intima of atherosclerotic lesions. Thus, the observation that *CSN5* acts as a negative regulator of mononuclear cell adhesion to inflamed endothelium by keeping endothelial NF- κ B activation in check, suggests that endothelial *CSN5* exhibits anti-atherogenic functions.

5.5 *CSN5* is upregulated in human atherosclerotic lesions and reduces murine atherosclerosis

Immunohistochemistry experiments verified that *CSN5* is expressed in the endothelial cell layer of human early atherosclerotic plaques. Importantly, we found that endothelial *CSN5* expression was further increased upon atheroprogession. Advanced lesions also displayed intense staining in macrophage foam cells, confirming earlier findings²⁸⁶. However, whether *CSN5* plays a similar role in NF- κ B attenuation in macrophages remains to be investigated. In addition, NF- κ B inhibition in macrophages may well have an outcome on atherogenesis that is different from the outcome of the endothelial NF- κ B blockade. This is indicated by the observation that deletion of IKK γ or overexpression of a dominant-negative I κ B- α protein specifically in ECs, reduced atherosclerotic plaque formation in *Apoe*^{-/-} mice²⁸⁷, while a macrophage-specific deficiency of *IKK2* resulted in increased atherosclerosis in *Ldlr*^{-/-} mice, predominantly by a reduction in anti-inflammatory gene expression and cell survival¹⁸⁸. Although deletion of *CSN5* in macrophages led to an increased atherosclerotic plaque, whether NF- κ B/*CSN* axis is involved remains elusive. A link between *CSN5* silencing and enhanced cell survival, likely through increased NF- κ B

activation, has been observed in stressed 293 cells²⁷¹. In contrast, *CSN5*-deficient thymocytes show defective cell cycle progression and substantial apoptosis at specific developmental stages, which was linked to reduced steady-state NF- κ B activation combined with increased p53 signaling, shifting the balance from anti- to pro-apoptotic gene transcription²⁷⁴. This emphasizes the importance to analyze the effect of CSN5 on NF- κ B activation in a cell type-specific manner. On the other hand, it might be possible that complete deletion of CSN5 has drastically different effects than knocking down to low basal expression level. It is conceivable, that a reduction of the CSN5 level results in increased NF- κ B activation and expression of anti-apoptotic genes, while complete elimination of CSN5 has the opposite effect (e.g. by eliminating the function of CRLs due to a block in the disassembly/re-assembly activation cycle).

In addition to regulating NF- κ B activation, CSN5 promotes the stabilization (e.g. as for HIF-1 α) or destabilization (e.g. p27, p53) of several critical cellular regulators²²². Furthermore, CSN5 has been shown to act as a transcriptional co-activator for AP-1²¹⁸ and MYC²⁸⁸, and also binds MIF^{258, 286}, a non-canonical chemokine with important pro-atherogenic properties^{9, 289}. Whereas MIF impairs CSN5-mediated JNK/AP-1 transcriptional activity²⁵⁸, CSN5 counter-regulates MIF secretion²⁹⁰, also supporting an atheroprotective role for CSN5. Thus, it can be speculated that the overall outcome of CSN5 on atherosclerosis will be the result of interplay of diverse cellular pathways, with both impaired NF- κ B activation and reduced MIF secretion pointing towards an atheroprotective behavior of CSN5 in ECs. The results presented in this study identify endothelial CSN5 to reduce inflammatory responses and mononuclear cell arrest by impairing NF- κ B activation. This argues that CSN5 confers atheroprotection in the endothelium. Importantly, this atheroprotective role of CSN5 was also observed in a mouse model of atherosclerosis when *Csn5* was ablated in macrophages. The acceleration of atherosclerosis observed in *Csn5*-KO mice when compared with littermate control confirms the atheroprotective role of CSN5. The exact mechanism behind this effect needs to be addressed specifically for macrophages with careful extrapolation of the effect seen for CSN5 on ECs to macrophages.

Besides modulating the activity of NF- κ B, CSN5 has also been shown to interact with the cytoplasmic domain of LFA-1²⁶¹, a critical factor in atherogenic leukocyte recruitment. Since CSN5 either sensitizes or desensitizes its target proteins towards the UPP²³⁸, it could be speculated that CSN5 might interfere with the firm adhesion and transmigration of leukocytes, a process critical for atherogenesis and controlled partly by LFA-1. Intuitively, CSN5 would destabilize LFA-1 leading to impaired leukocyte recruitment or block inside out signaling; consequently, deletion of *Csn5* in a murine model of atherosclerosis may reverse this process leading to accelerated atherosclerosis. Also, CSN5 has been shown to interact with ABCA1²⁷⁷, a mediator of HDL formation, which acts in concert with ABCG1 to mediate macrophage cholesterol efflux. This mechanism protects macrophages against foam cell formation and atherosclerosis. Desensitization of ABCA1 towards the UPP by CSN5 might result in effective cholesterol efflux and could represent a way of decreasing circulating monocytes and a subsequent decrease in atherosclerosis. This is in line with exacerbation of atherosclerosis in *LysMCre-Csn5/ Apoe*^{-/-} mice when compared with wild-type littermates. These interplays might contribute to the overall effect of CSN5 on atherosclerosis which again might be different for ECs, macrophages and SMCs.

6 SUMMARY

The COP9 signalosome (CSN), a multi-functional protein complex involved in the regulation of E3 ubiquitin ligases, has been described as a regulator of NF- κ B signaling, a function assigned to the metalloisopeptidase activity of its subunit CSN5. However, its effect on NF- κ B activation and pro-atherogenic responses in endothelial cells and macrophages and in atherogenesis *in vivo* remains unknown. This study sought to identify a role of the CSN complex and in particular CSN5 in IKK/NF- κ B signaling in atherogenic endothelial cells *in vitro* and in murine atherosclerosis *in vivo*. Endothelial cells (ECs) are not just barrier bystanders, but actually determine whether immune cells will work within the limits of homeostasis or go overboard into autoimmunity and inflammation. As the vascular endothelium plays a pivotal role in the inflammatory reactions driving atherosclerosis, the first part of this study focused on the role of CSN5 in modulating inflammatory and atherogenic processes in ECs through the regulation of NF- κ B. By using ECs, this study for the first time demonstrates endothelial CSN5 to reduce inflammatory responses and mononuclear cell arrest by impairing NF- κ B activation, proposing that CSN5 confers atheroprotective effects in the endothelium. This was mechanistically shown by co-immunoprecipitation in human umbilical vein endothelial cells (HUVECs) that revealed the presence of a supercomplex between IKK and the CSN, which dissociates upon TNF α administration. The CSN was found to attenuate the phosphorylation of IKK when cells were treated with siRNAs targeting CSN subunits 2 and 5. Furthermore, transient overexpression of CSN5 protected I κ B- α from degradation and reduced NF- κ B activation upon TNF α stimulation, whereas silencing RNA-mediated CSN5 depletion enhanced TNF α -induced I κ B- α degradation and the concomitant NF- κ B activity in NF- κ B binding and luciferase reporter assays. Sequentially, NF- κ B-dependent expression of chemokines and adhesion molecules was upregulated, as measured by real-time PCR and flow cytometry, which translated into an increased arrest of perfused monocytes on TNF α -stimulated, CSN5-depleted HUVECs. Importantly, double-immunostaining for CSN and CD31 confirmed the expression of CSN subunits in the endothelial layer of human atherosclerotic lesions, and revealed an increased expression of CSN5 upon atheroprogession.

The second part of this work intended to elucidate the functional role of CSN5 in murine atherosclerosis *in vivo*. Macrophages are key cells not only in acute inflammation but also in chronic inflammation as they release a number of cytokines and other factors upon activation. Chronic inflammatory processes underlie atherosclerosis and macrophages have been shown to be key cells in atherosclerotic processes. Employing a macrophage-specific deletion of *Csn5* in a murine atherosclerosis model, this study identifies *Csn5* as a novel player in atherosclerosis. Ablation of *Csn5* was found to exacerbate murine atherosclerosis, an observation that confirms the atheroprotective role of CSN5 demonstrated for the first time in this study. The evidence presented in this thesis sets the pace to pursue the functional role of CSN5 and the entire CSN complex in atherosclerosis in a broader manner including EC- and SMC-specific knockout of *Csn5 in vivo* by conditional deletion. This could also be extended to other *Csn* subunits and the various proatherogenic mouse models.

7 Zusammenfassung

Das COP9 Signalosom (CSN) ist ein multifunktionaler Proteinkomplex, der an der Regulation der E3-Ubiquitinligasen beteiligt ist und dem eine regulatorische Rolle bei der NF- κ B-Signaltransduktion zugeschrieben wird, der die Metallopeptidase-Aktivität seiner Untereinheit CSN5 zugrunde liegt. Dennoch ist der Einfluss des COP9-Signalosoms auf die NF- κ B-Aktivierung und proatherogene Vorgänge sowohl in Endothelzellen und Makrophagen als auch in der Atherogenese *in vivo* unbekannt. Das Ziel dieser Arbeit war, die Rolle des CSN-Komplexes insbesondere CSN5 zu untersuchen und seine Beteiligung an der IKK/NF- κ B-Signalweg in atherogenen Endothelzellen *in vitro* und in der murinen Atherosklerose *in vivo* zu identifizieren. Endothelzellen (ECs) sind hierbei nicht nur als „Randspieler“ zu betrachten, sondern sie bestimmen, ob sich die Aktivität der Immunzellen innerhalb der homöostatischen Grenzen bewegt oder ob Autoimmunität und Entzündungsprozesse eingeleitet werden. Da das Gefäßendothel eine zentrale Rolle bei den inflammatorischen Reaktionen spielt, die die Atheroskleroseentwicklung vorantreiben, liegt der Fokus des ersten Teils dieser Studie auf der Rolle der CSN5-Untereinheit bei der Modulation von entzündlichen und atherogenen Prozessen in ECs durch die NF- κ B-Regulierung. Durch diese Studie konnte zum ersten Mal gezeigt werden, dass endothelstämmiges CSN5 zu Entzündungsreaktionen und zum Arrest mononukleärer Zellen durch die Hemmung der NF- κ B-Aktivierung in ECs reduziert, was den Hinweis auf atheroprotektive Effekte im Endothel liefert. Diese Entdeckung konnte ebenfalls durch Co-Immunpräzipitationsversuche in menschlichen Nabelvenen-Endothelzellen (HUVECs) unterstützt werden, die auf das Vorhandensein eines Superkomplexes zwischen IKK und CSN hinweisen, dessen Dissoziation durch TNF α reguliert wird. Ebenso konnte gezeigt werden, dass die Phosphorylierung von IKK herabgesetzt wird, wenn Zellen mit CSN2- und CSN5-siRNA behandelt wurden. Transiente Überexpression von CSN5 verhinderte der Abbau von I κ B- α und eine Reduktion der NF- κ B Aktivierung nach TNF α -Stimulation, hingegen CSN5 siRNA förderte der Abbau von I κ B- α und die damit einhergehende NF- κ B-Aktivität in NF- κ B-Bindungs- und Luciferase-Reporter-Assays. Demzufolge wird die NF- κ B-abhängige Expression von Chemokinen und Adhäsionsmolekülen hochreguliert, wie auch mittels *real-time*-PCR und Durchflusszytometrie gezeigt werden konnte, wobei ein verstärkter Zellarrest perfundierter Monozyten auf TNF α -stimulierten und CSN5-armen HUVECs festgestellt werden konnte.

Die Immundoppelfärbung von CSN und CD31 bestätigte darüber hinaus sowohl die Expression der CSN-Untereinheiten in athero-sklerotischen Läsionen der humanen Endothelschicht als auch eine erhöhte Expression von CSN5 während der Atheroprogession.

Der zweite Teil dieser Arbeit beschäftigt sich damit, die funktionelle Rolle der CSN5 in der murinen Atherosklerose *in vivo* aufzuklären. Makrophagen spielen nicht nur bei der akuten Immunabwehr eine wichtige Rolle, aber auch bei chronischen Entzündungen, da sie an der Freisetzung einer Reihe von Cytokinen und anderen Faktoren beteiligt sind. Sie gelten als Schlüsselzellen in atherosklerotischen Prozessen, denen chronische Inflammation zugrunde liegt. In dieser Studie konnte mithilfe einer Makrophagen-spezifischen Deletion im *Csn5*-Gen das CSN5 als neuer Spieler im murinen Artherosklerosemodell identifiziert werden. Durch die Beobachtung, dass das Fehlen von CSN5 die murine Artherosklerose verschärft, konnte die atheroprotektive Funktion des Proteins in dieser Studie zum ersten Mal bestätigt werden. Die im Rahmen dieser Arbeit erbrachten Beweise setzen den ersten Schritt, die funktionelle Rolle von CSN5 und im weiteren Sinne des gesamten CSN-Komplexes in Atherosklerose zu erfassen. Die in diesem Zusammenhang eingesetzten EC- und SMC-spezifischen *Csn5-knockouts in vivo* mithilfe von konditionaler Deletion könnten auch auf andere CSN-Untereinheiten und verschiedene proatherogenic Mausmodellen ausgeweitet werden.

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Positions

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Publications

Kanzler I, Tuchscheerer N, Steffens G, Simsekylmaz S, Konschalla S, Kroh A, Simons D, **Asare Y**, Schober A, Bucala R, Weber C, Liehn, E.A, Bernhagen J (2012) Pivotal role of MIF in endothelial progenitor cell-induced angiogenesis (*in revision*)

Asare Y, Shagdarsuren E, Schmid J.A, Grommes J, Bounkari O.EL, Weber C, de Winther M.P.J, Noels H, Bernhagen J (2012) Endothelial CSN5 Impairs NF-κB Activation and Monocyte Adhesion to Endothelial Cells and is Highly Expressed in Human Atherosclerotic Lesions (*in revision*)

Declaration

I hereby declare that, this thesis is my own original work done under the supervision of Univ.-Prof. Dr. Jürgen Bernhagen, Prof. Dr. Menno de Winther and Dr. Heidi Noels. That, no part of this work has been submitted in the past, or is being submitted for a degree in any University. All materials, help of others and references required for this work are stated.

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