The effect of IKK2 inhibition on NF-kappaB activation and macrophage function in atherosclerosis

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Eindverhandeling voorgedragen tot het bekomen van de graad
Master in de biomedische wetenschappen afstudeerrichting
klinische en moleculaire wetenschappen

Universiteit Maastricht

universiteit hasselt

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Abbreviations

ABCA1	ATP-binding cassette transporter-1 A
ABCG1	ATP-binding cassette transporter-1 G
acLDL	acetylated low-density lipoprotein
AGE	advanced glycation end product
APS	ammonium persulfate
ATP	adenosine triphosphate
BSA	bovine serum albumin
CD40L	CD40 ligand
cDNA	copy deoxyribonucleic acid
CHX	cycloheximide
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetracetic acid
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescent activated cell sorting
FADD	fas associated death domain
FCS	fetal calf serum
GFP	green fluorescent protein
HLH	helix-loop-helix
HRP	horseradish peroxidase
HSP60	heat-shock protein 60
IC_{50}	inhibitory concentration 50%
IFN-γ	interferon gamma
IKK	inhibitor of nuclear factor kappa B kinase
IKK2 KD	inhibitor of nuclear factor kappa B kinase 2 kinase dead
IL	interleukin
IRES	internal ribosomal entry site
IκB	inhibitor of nuclear factor kappa B
KBr	potassium bromide
KD	kinase domain

LDL	low-density lipoprotein
LPS	lipopolysaccharide
LTα	lymphotoxin alpha
LTR	long terminal repeat
LXRα	liver X receptor alpha
LZ	leucine zipper
mLDL	modified low-density lipoprotein
MyD88	myeloid differentiation factor 88
NaCl	sodium chloride
NF-κB	nuclear factor kappa B
NO	nitric oxide
OPD	ortho-phenylenediamine
oxLDL	oxidized low-density lipoprotein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI	propidium iodide
PMSF	phenylmethylsulphonylfluoride
PPARγ	peroxisome proliferator activated receptor-γ
RNA	ribonucleic acid
SDS PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulfate
SR-A	scavenger receptor type A
TEMED	tetramethylethylenediamine
TLR	toll like receptor
TNFα	tumor necrosis factor-α
TRADD	TNFR1 associated death domain protein
TRIS	tris(hydroxymethyl)aminomethane

Preface

This paper is the result of a traineeship within the department of molecular genetics to obtain my degree in biomedical sciences. From January 2006 until June 2006 I investigated the role of the transcription factor NF-κB in inflammatory signaling and lipid metabolism in macrophages by determining the effects of IKK2 inhibition in a monocyte/macrophage cell line on inflammation, apoptosis and foam cell formation, the three major processes involved in atherosclerosis.

Om te beginnen wil ik natuurlijk mijn promoter Daniëlle bedanken voor de fijne en goede begeleiding van mijn stage. Van je ervaring en directe aanpak heb ik veel geleerd. Je hebt me vooral inzicht gegeven in het efficiënt plannen en opzetten van experimenten. Hierdoor heb ik heel wat mooie resultaten bekomen. Ik wil je ook nog bedanken voor het snelle en grondig verbeteren van mijn thesis. Zonder jouw hulp was mijn thesis nooit zo mooi geworden.
Natuurlijk wil ik ook mijn tweede beoordelaar Menno bedanken. Ik weet zeker dat je mijn thesis met veel aandacht gaat lezen en er de gepaste beoordeling aan gaat geven.
Verder wil ik iedereen van de afdeling moleculaire genetica bedanken die me op de een af andere manier geholpen heeft. Vooral Inge wil ik nog extra bedanken. Je was altijd bereid om me te helpen met praktische dingen in het lab. Als ik ergens hulp bij nodig had, liet je meteen je eigen werk liggen om mij verder te helpen. Het plezier waarmee je me telkens geholpen hebt, apprecieer ik echt.
In het bijzonder wil ik mijn familie bedanken. Marga, voor het inscannen van de plaatjes voor in mijn thesis. Yannick, die altijd veel interesse vertoonde in mijn werk en me geholpen heeft bij het oplossen van al mijn computerproblemen en natuurlijk mijn ouders en grootouders die me stimuleerden om te studeren en me in alles onvoorwaardelijk steunden.

Abstract

Atherosclerosis is a chronic inflammatory disease, characterized by the accumulation of macrophage-derived foam cells in the vessel wall. One of the key regulators of inflammation is the transcription factor NF-κB, which controls the transcription of many genes with an established role in atherosclerosis. In resting cells, NF-κB is kept inactive by association with an inhibitory protein, IκB. NF-κB can be activated by a wide range of extracellular stimuli, resulting in activation of different signal transduction cascades, which eventually activate the IκB kinase (IKK) complex containing two catalytic subunits, IKK1 and IKK2 and a non-enzymatic regulatory component termed NEMO. IKK2 is the most important subunit for activation of the classical NF-κB pathway. Upon stimulation, the IKK complex phosphorylates IκB, inducing its ubiquitination and subsequent degradation. NF-κB is then free to translocate to the nucleus where it facilitates the transcription of many genes. In this study, we investigated the role of the transcription factor NF-κB in inflammatory signaling and lipid metabolism in macrophages by determining the effects of IKK2 inhibition in a monocyte/macrophage cell line on inflammation, apoptosis and foam cell formation, the three major processes involved in atherosclerosis. We used two methods to functionally inhibit IKK2. The first one was a retroviral approach to establish RAW 264.7 cells that stably express a dominant negative mutant of IKK2. A second method was the treatment of RAW 264.7 cells with the chemical IKK2 inhibitors SC-514 and SPC-839. This second method was used to validate the results obtained with the retroviral approach but also because these small molecules are very attractive agents for the use in future therapies. Our study provides some interesting observations. First of all, it was seen that the inhibition of IKK2 reduces the inflammatory response of macrophages. Second, the inhibition of IKK2 in macrophages increases the sensitivity of these cells to stimulus-induced cell death. Third, macrophages take up less modified LDL after functionally inhibiting IKK2, probably by down regulating the two main scavenger receptors, CD36 and SR-A, involved in lipid uptake. Overall, the effects reached with the retroviral approach were more pronounced than the ones induced with the chemical IKK2 inhibitors. This in vitro research can be important for future in vivo applications in atherosclerosis. Our finding that IKK2 inhibition reduces the inflammatory response and the uptake of lipids in macrophages is considered positive regarding atherosclerosis. The effect of IKK2 inhibition on apoptosis, on the other hand, can both be considered positive and negative. These data indicate that functionally inhibiting IKK2 might have opposing implications regarding atherosclerosis.

1. Introduction

1.1 Atherosclerosis

Atherosclerosis represents one of the major causes of morbidity and mortality in the western world. The causes of this vascular problem are manifold and different etiologies have been proposed [1].

An initiating event in atherosclerosis is the retention and subsequent modification of lipoproteins (LDL) in the vessel wall (figure 1.1). Cells in the vessel wall interpret this as a danger signal, and they call for reinforcements from the body's defense system [2]. In particular, the modified LDL stimulates endothelial cells to display adhesion molecules and to secrete chemokines. In this way, monocytes are attracted from the blood to the intima where they multiply and mature into active macrophages that take up the modified LDL by their scavenger receptors. The macrophages ultimately become so packed with fatty droplets that they become lipid-laden foam cells, which is a first hallmark of atherosclerotic plaque development [2]. These lipid-laden foam cells, which eventually die because they take up too much lipids, together with other immune cells like T-cells and neutrophils form the necrotic lipid core of the atherosclerotic plaque. Later on, smooth muscle cells become activated and involved. These cells promote the formation of a fibrous cap over the lipid core. The cap develops when smooth muscle cells of the media migrate to the top of the intima, multiply and produce a tough, fibrous matrix that glues the cells together. The cap adds to the size of the plaque but also walls it off safely from the blood. However, in some cases inflammatory substances secreted by foam cells can dangerously weaken the cap by digesting matrix molecules and damaging smooth muscle cells, which then fail to repair the cap. Meanwhile the foam cells may display tissue factor, a potent clot promoter. If the weakened plaque ruptures, tissue factor will interact with clot-promoting elements in the blood, causing a trombus to form which can locally stop the blood flow or be transported through the blood and cause occlusion elsewhere [2]. In this way, if the clot is big enough, it can halt the blood flow to the heart, brain, kidneys or lower extremities resulting in myocardial infarction, cerebral infarction or pheripheral vascular disease [3].

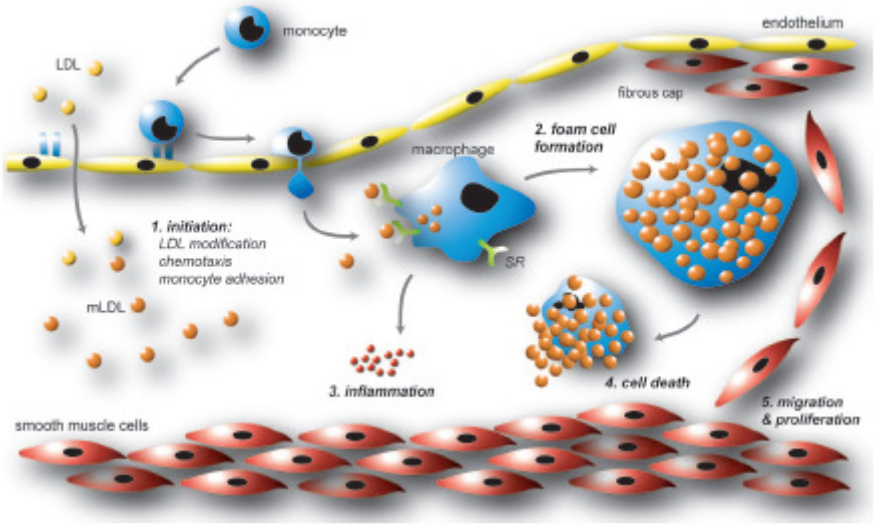

Figure 1.1. Schematic representation of atherogenesis. Indicated are the different cell types involved and the different steps in the atherosclerotic process. Initiation of atherosclerosis is characterized by the formation of modified LDL (mLDL), followed by the subsequent activation of endothelial cells and secretion of chemokines. On migration through the endothelial layer, the monocytes differentiate into macrophages and scavenge the modified LDL from the vessel wall, resulting in foam cell formation. The process is accompanied by inflammatory responses. Later stages involve the formation of a fibrotic cap through proliferation and migration of smooth muscle cells and cell death resulting in a necrotic core. (adapted from de Winther MP et al. 2005)

From the above, it becomes clear that inflammatory processes characterize all stages of atherosclerosis, from the early endothelial activation to the eventual rupture of the atherosclerotic plaque [4,5]. So, initially considered as a bland lipid storage condition, atherosclerosis is now generally accepted as a slowly progressing lipid-driven chronic inflammatory disease in which macrophages play an important role by regulating both inflammation and lipid metabolism [6,7].

1.2 Transcription factor NF-κB

One of the key regulators of inflammation, immune responses and cell survival is the transcription factor NF-κB (nuclear factor kappa B), which controls the transcription of many genes with an established role in atherosclerosis [8]. NF-κB is the general name for a family of transcription factors consisting of 5 members: RelA (p65), RelB, c-Rel, NF-κB1 (p50 and its precursor p105) and NF-κB2 (p52 and its precursor p100). All these members share a Rel homology domain which mediates DNA binding, dimerization and interaction with inhibitory proteins, whereas only the first 3 contain a transcriptional activation domain. These proteins can form different complexes of homodimers or heterodimers. The most abundant complex, often referred to as "NF-κB", is p65/p50. There are two NF-κB activation pathways known: a canonical (classical) activation pathway and a non-canonical (alternative) activation pathway. The non-canonical activation pathway is mediated through IKK1 and results in the processing

of p100 to p52, resulting in the nuclear transfer of the RelB-p52 dimer (figure 1.2) [9]. However, most of what is known about the regulation of NF-κB is based on the mechanism of activation of the p65/p50 dimer that forms the basis of the canonical activation pathway (figure 1.2). In this pathway, NF-κB is maintained in an inactive state via interaction with the inhibitory protein, inhibitor of nuclear factor kappa B (IκB). Association with this inhibitor masks the nuclear translocation sequence of NF-κB, thereby retaining it in the cytoplasm and preventing DNA binding. The activation of the NF-κB pathway can be initiated by the exposure of cells to a wide range of extracellular stimuli, including proinflammatory cytokines such as tumor necrosis factor-α (TNFα) and interleukin-1 (IL-1), but also viral products, bacterial components and yeast products. These agents can activate the cells through their respective receptors, resulting in the activation of different signal transduction cascades, which eventually activate the IκB kinase (IKK) complex [9]. Upon stimulation, the IKK complex phosphorylates two conserved serines in the N-terminal domain of IκB, inducing its ubiquitination and subsequent proteasomal degradation. NF-κB is then free to translocate to the nucleus where it facilitates the transcription of many genes, including proinflammatory cytokines, chemokines and anti-apoptotic factors [10].

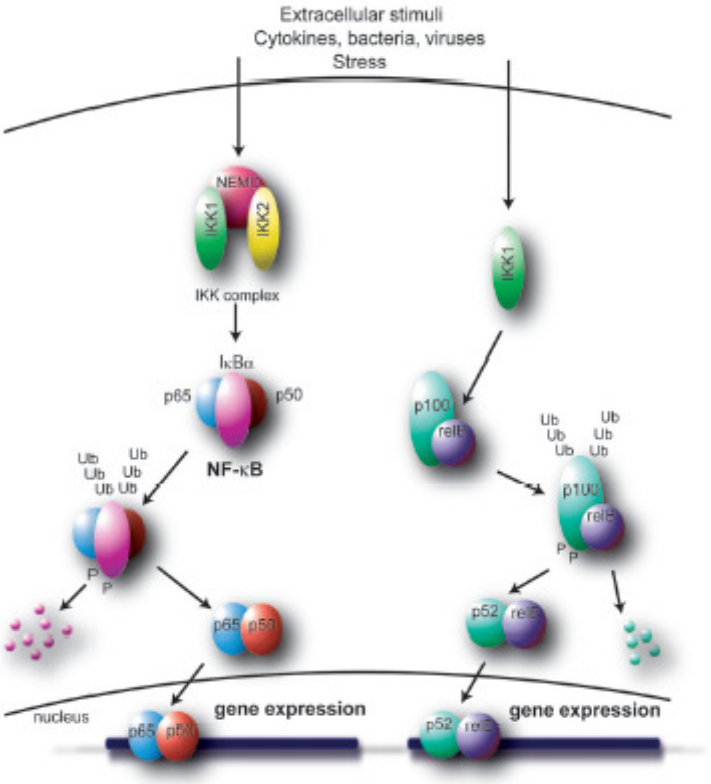

Figure 1.2. NF-κB activation. Two NF-κB activation cascades can be discriminated. The classical NF-κB activation pathway (left) involves the activation of the IKK complex with the subsequent degradation of IκBα and nuclear translocation of the NF-κB dimer. The alternative NF-κB activation cascade (right) is mediated through IKK1 and results in the processing of p100 to p52, resulting in the nuclear transfer of the RelB-p52 dimer. (adapted from de Winther MP et al. 2005)

1.3 The NF-κB pathways

As described above**,** a wide variety of extracellular stimuli can induce the activation of NF-κB through different signaling pathways that converge at the level of the IKK complex. Simplifying the activation routes to NF-κB, three major pathways can be distinguished [9,11] (figure 1.3).

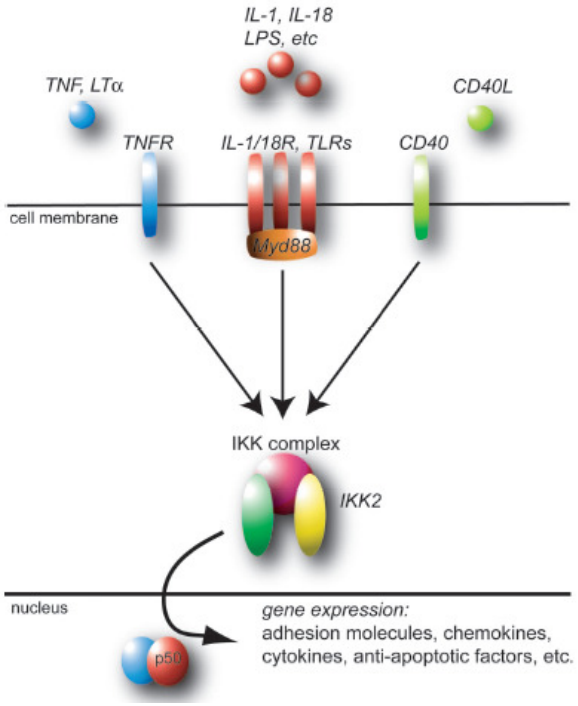

Figure 1.3. Schematic representation of the 3 major NF-κB signaling pathways that have been studied in atherogenesis. TNF, IL-1/IL-18/TLR, and CD40 signaling result in the activation of the IKK complex. IL-1/IL-18/TLR signaling is mediated through the adapter molecule Myd88. (adapted from de Winther MP et al. 2005)

The first pathway involves different receptors, including IL-1 and IL-18 receptors and toll like receptors (TLRs), that all share the use of the adaptor molecule myeloid differentiation factor 88 (MyD88). These receptors can bind many exogenous ligands like bacterial lipopolysaccharide (LPS), peptidoglycan, chlamydia and virusses but also many endogenous ligands like IL-1, IL-18, modified LDL and heat-shock proteins (HSP60) [12]. All these ligands are shown to induce NF-κB activation. Bacterial lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, is one of the most potent activators of the NF-κB pathway [13-15]. The primary target cells for LPS are monocytes and macrophages. Their response consists of massive production of proinflammatory cytokines, reactive oxygen-and nitrogen-intermediates, procoagulants and cell adhesion molecules. It was shown that LPS induces IKK activity in human monocytes and that a dominant negative mutant of IKK2 inhibited the LPS-induced NF-κB dependent gene expression in a dose dependent manner [13]. In another study were they studied the effect of LPS on monocytic

IKK activation and IκB proteolysis, it was seen that LPS predominantly stimulated IKK2 activity, which slowly increased and peaked at 30 minutes after LPS stimulation. A second peak was observed at a later time point following LPS stimulation, which consisted of both IKK1 and IKK2 activity [14]. These results provide evidence that the IKK complex constitutes the main intracellular target for LPS-induced NF-κB signaling to the nucleus to activate proinflammatory genes and that LPS induction of NF-κB-dependent gene expression requires the activation of IKK2. The effect of modified LDL on NF-κB-mediated inflammatory responses, in contrast with LPS, turned out to be dual faced. As described above, low density lipoproteins (LDL) become atherogenic after undergoing oxidation by vascular cells, which transform them into highly bioreactive oxidized lipoproteins (oxLDL). Oxidized LDL is involved in foam cell formation and triggers proatherogenic events such as overexpression of adhesion molecules, chemoattractant agents, growth factors and cytokines involved in the inflammatory process, cell proliferation and apoptosis. Moreover, this toxic effect of oxidized LDL plays probably a role in plaque erosion/rupture. Several biological effects of oxidized LDL are mediated through changes in the activity of transcription factors (NF-κB) and subsequently in gene expression. Depending on the exposure time and the amount of oxidation, LDL may up- or down-regulate the NF-κB activity. It was shown that short-term incubation or low concentrations of oxidized LDL enhance the activation of NF-κB in monocytes. However, longer incubations and more severely oxidized LDL have been shown to inhibit NF-κB-mediated inflammatory responses in macrophages [16].

A second signaling pathway leading to NF-κB activation involves the TNF signaling receptor, p55 TNFR. There are two known ligands for this receptor namely TNF (tumor necrosis factor) and LTα (lymphotoxin alpha). Upon binding of these ligands, a large number of adaptor proteins are recruited that ultimately lead to activation of the IKK complex and subsequently of NF-κB [11]. A clear role for this signaling pathway has not yet emerged. In different reports TNF was clearly shown to be a pro-atherogenic cytokine [17-20]. In other studies TNF signaling was found to have no effect on lesion development [21]. However, recent evidence suggests that TNF signaling in vascular cells can have anti-atherogenic consequences, but the mechanisms are poorly understood [22]. TNF is released by free cholesterol-loaded apoptotic macrophages and the clearance of these cells by phagocytic macrophages may help to limit plaque development. Namely, in this study it was shown that TNF signals primarily through NF-κB to induce the expression of ABCA1 in macrophages. In atherosclerotic plaques, this process may help phagocytic macrophages to efflux excess lipids derived from the ingestion of cholesterol-rich apoptotic corpses.

Finally, another member of the TNF receptor family that is expressed on different cell types in atherosclerotic plaques and that can activate NF-κB is CD40. CD40 is activated by CD40 ligand (CD40L) and signals to NF-κB, both through the classical and the alternative pathway. Different studies show that inhibition of CD40L results in smaller atherosclerotic lesions with a more stable plaque phenotype, characterized by a smaller lipid core and a thicker fibrous cap. These data show that the CD40-CD40L system is a proatherogenic signaling cascade that also highly affects plaque stability [23].

1.4 The IKK complex

As already mentioned, the phosphorylation of IκB by the IKK complex is an essential step in the activation of the canonical NF-κB activation cascade. The IKK complex is a high molecular weight kinase complex composed of two catalytic subunits named IKK1 (IKKα) and IKK2 (IKKβ) and a non-enzymatic regulatory component termed NEMO (IKKγ). IKK1 and IKK2 both have kinase activity and constitute the functional IKK complex. Of the two catalytic subunits, the most important for activation of the classical NF-κB signaling pathway is IKK2. Although NEMO does not have kinase activity, it is necessary for stimulus-mediated NF-κB activation [24,25].

Sequence analysis revealed that IKK1 and IKK2 are related protein serine kinases containing protein interaction motifs [26-29]. The two kinases show 52% identity at the amino acid level. The human IKK2 protein, that has 95% homology with the mouse IKK2 protein, is composed of 756 amino acids and it has a molecular weight of 87 kD (figure 1.4). It contains a NH_2-terminal protein kinase domain, a central leucine zipper motif and a helix-loop-helix domain in the COOH-terminal region. The protein kinase domain contains 286 amino acids. In this kinase domain there is a site of 9 amino acids and a site of 1 amino acid that both function as ATP binding sites. There is also one amino acid in this kinase domain, namely aspartic acid (D145) that functions as a proton acceptor and that is involved in the activity of the IKK2 enzyme. Also located in the kinase domain are two serines (S177, S188) that serve as the two main phosphorylation sites of the IKK2 protein. Activation of IKK2 depends on the phosphorylation of these two serines. They are the specific sites whose phosphorylation causes a conformational change that result in kinase activation. From previous studies it was reported that replacement of these two phospho-accepting serines with alanines interferes with IKK activation, while conversion of both serines to phospho-mimetic glutamate residues generates a constitutively active kinase IKK2 (EE) [29]. The central leucine zipper motif of

IKK2 is composed of 22 amino acids, its helix-loop-helix domain contains 40 amino acids and the nemo-binding site is composed of 6 amino acids (figure 1.4).

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      1          11          21          31          41          51
  1 MSWSPSLTTQ TCGAWEMKER LGTGGFGNVI RWHNQETGEQ IAIKQCRQEL SPRNRERWCL   60
 61 EIQIMRRLTH PNVVAARDVP EGMQNLAPND LPLLAMEYCQ GGDLRKYLNQ FENCCGLREG  120
121 AILTLLSDIA SALRYLHENR IIHRDLKPEN IVLQQGEQRL IHKIIDLGYA KELDQGSLCT  180
181 SFVGTLQYLA PELLEQQKYT VTVDYWSFGT LAFECITGFR PFLPNWQPVQ WHSKVRQKSE  240
241 VDIVVSEDLN GTVKFSSSLP YPNNLNSVLA ERLEKWLQLM LMWHPRQRGT DPTYGPNGCF  300
301 KALDDILNLK LVHILNMVTG TIHTYPVTED ESLQSLKARI QQDTGIPEED QELLQEAGLA  360
361 LIPDKPATQC ISDGKLNEGH TLDMDLVFLF DNSKITYETQ ISPRPQPESV SCILQEPKRN  420
421 LAFFQLRKVW GQVWHSIQTL KEDCNRLQQG QRAAMMNLLR NNSCLSKMKN SMASMSQQLK  480
481 AKLDFFKTSI QIDLEKYSEQ TEFGITSDKL LLAWREMEQA VELCGRENEV KLLVERMMAL  540
541 QTDIVDLQRS PMGRKQGGTL DDLEEQAREL YRRLREKPRD QRTEGDSQEM VRLLLQAIQS  600
601 FEKKVRVIYT QLSKTVVCKQ KALELLPKVE EVVSLMNEDE KTVVRLQEKR QKELWNLLKI  660
661 ACSKVRGPVS GSPDSMNASR LSQPGQLMSQ PSTASNSLPE PAKKSEELVA EAHNLCTLLE  720
721 NAIQDTVREQ DQSFTALDWS WLQTEEEEHS CLEQAS
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Figure 1.4. Amino acid sequence of the human IKK2 protein. The human IKK2 protein is composed of 756 amino acids. It contains a NH_2-terminal protein kinase domain, a central leucine zipper motif and a helix-loop-helix domain in the COOH-terminal region. The protein kinase domain contains 286 amino acids (grey). It has two ATP binding sites (blue and green). The aspartic acid (D145) in the kinase domain functions as a proton acceptor and is involved in the activity of the IKK2 enzyme (red). The two serines (S177, S188) in this domain serve as the two main phosphorylation sites of the IKK2 protein (bold). The central leucine zipper motif of IKK2 is composed of 22 amino acids (purple), it's helix-loop-helix domain contains 40 amino acids (orange) and the nemo-binding site is composed of 6 amino acids (light blue).

IKK2 is also the site for negative regulation of IKK activity [14,15]. IKK2 is rapidly activated through phosphorylation of serine 177 and serine 181 at its activation loop. This is followed by progressive autophosphorylation of a serine cluster located between the helix-loop-helix motif of IKK2 and its COOH-terminus, which decreases the catalytic activity of IKK2. There exists a certain model to explain this regulation of IKK activity (figure 1.5). This model states that the two catalytic subunits of the IKK complex (IKK1 and IKK2) associate into a heterodimer via their leucine zippers (LZ). In non-stimulated cells, the activation loop within the kinase domain (KD) is not phosphorylated, and the helix-loop-helix domain (HLH) interacts with the kinase domain. The C-terminal phosphorylation domain is not phosphorylated either. However, upon cell stimulation with for example TNFα or IL-1, the activation loop of IKK2 is phosphorylated and through trans-autophosphorylation of the second subunit IKK is activated. The activated IKK complexes then phosphorylate IκB subunits, triggering their ubiquitin-dependent degradation and the activation of NF-κB. Concurrently, the activated IKK undergoes progressive autophosphorylation at its C-terminal serine cluster. When at least nine of the C-terminal serines are phosphorylated, electrostatic

repulsion weakens the interaction between the activating HLH motif and the kinase domain such that the activity of IKK2 decreases.

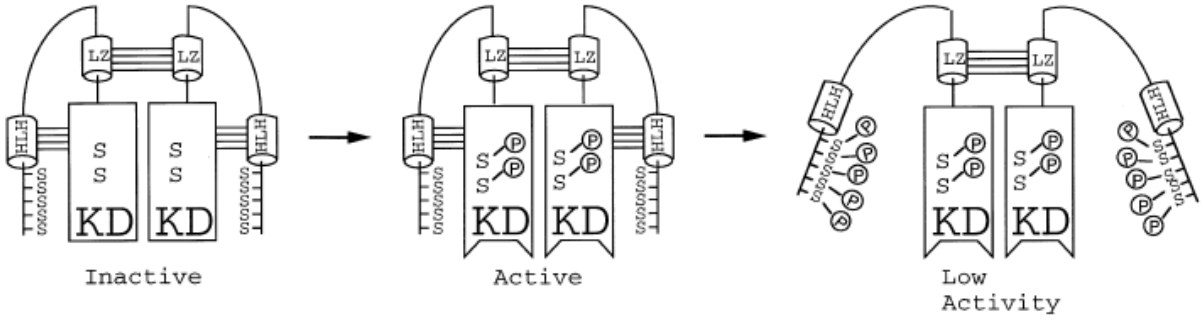

Figure 1.5. A three-step model for regulation of IKK activity by phosphorylation. The two catalytic subunits of IKK (IKK1 and IKK2) associate into a heterodimer via their leucine zippers (LZ). In non-stimulated cells, the activation loop within the kinase domain (KD) is not phosphorylated, and the helix-loop-helix (HLH) motif contacts the kinase domain. The C-terminal phosphorylation cluster is not phosphorylated either. Upon cell stimulation with for example TNFa or IL-1, the activation loop of IKK2 is phosphorylated and through trans-autophosphorylation of the second subunit (either IKK1 or IKK2, in the case of a homodimer) IKK is activated. Once activated, in addition to phosphorylation of IkBs, IKK undergoes progressive autophosphorylation at its C-terminal serine cluster. When at least nine of the C-terminal serines are phosphorylated, electrostatic repulsion alters the interaction between the activating HLH motif and the kinase domain such that the kinase reaches a low activity state. In this state IKK is likely to be more prone to inhibition by phosphatase action. (adapted from Karin M et al. 1999)

Because of the positive autoregulatory nature of the NF-κB signaling pathway and the potential toxicity and pathophysiology associated with its prolonged activation, it is important not only to rapidly activate this system but also to decrease its activity once the activating challenge disappears. Without negative autoregulation, a single bolus of a proinflammatory cytokine could result in prolonged IKK activation leading to prolonged NF-κB activation followed by increased production of both primary and secondary inflammatory mediators. As these mediators can cause further NF-κB activation, there is a genuine risk that in the absence of efficient negative feedback mechanisms even a minor proinflammatory insult would result in a major catastrophe, such as septic shock.

1.5 The role of NF-κB in macrophages in the processes of atherosclerosis

There is a lot of evidence suggesting a role for the NF-κB system in the process of atherosclerosis [9]. For example, activated NF-κB was demonstrated in human atherosclerotic lesions [30]. By the use of a specific antibody, NF-κB activation was shown in smooth muscle cells, macrophages and endothelial cells. Using immunofluorescence and immunohistochemical techniques, activated NF-κB was also detected in atherosclerotic lesions. Little or no activated NF-κB was detected in vessels lacking atherosclerosis. Moreover, it was also demonstrated that there is a colocalization of regions prone to develop atherosclerosis and increased levels of components of the NF-κB system [31].

Inflammation

There exists a lot of evidence that NF-κB plays an important role in the inflammatory process [9]. NF-κB regulates a lot of cytokines that are involved in the inflammatory process associated with atherosclerosis. The major NF-κB-regulated cytokines in atherosclerosis are: TNFα, IL-1β, IL-6, IL-10, IL-12 and IFN-γ. TNFα is multifunctional and one of the most important proinflammatory and immune modulatory cytokines [32]. Therefore, it is expected to have a major role in atherosclerosis. As discussed above it is not clear yet whether TNFα is a pro-atherogenic or an anti-atherogenic cytokine. IL-1β in contrast is a clear proinflammatory cytokine. It should be noted that that TNFα and IL-1 are regulated by NF-κB, but they are also activators of the NF-κB pathway. Hereby, these cytokines may effect atherosclerosis by regulating many other NF-κB dependent genes and amplify the inflammatory response. In line with the previous cytokine, IL-6 is often classified as proinflammatory. However, some observations indicated that IL-6 can also function as an anti-inflammatory cytokine required for controlling inflammation [33]. NF-κB activation may also mediate the expression of genes that are clearly anti-inflammatory. Some of these genes, like IL-10, have been linked to atherosclerosis. Recently it was shown that IL-10 can be induced in an NF-κB-dependent manner [34]. Moreover, deletion of IKK2 form macrophages was shown to strongly reduce IL-10 secretion after LPS stimulation [35]. These data show that NF-κB can drive factors that may protect against atherosclerosis but also factors that enhance atherosclerosis. It is assumed that NF-κB activation during the onset of inflammation is associated with pro-inflammatory gene expression and an increased influx of inflammatory cells, whereas NF-κB activation during the resolution of inflammation is associated with anti-inflammatory gene expression. These results indicate that NF-κB signaling is not per se pro-atherosclerotic. Depending on the time point of its activation, the duration of its action, and in

which specific cell type it is induced, NF-κB can confer protection or susceptibility to atherosclerosis [11].

Cell death

In later stages of atherosclerosis cell death becomes an important issue. Cell death is classified into two major types, apoptosis and necrosis, based on morphological and biochemical characteristics and the mechanism of cell killing [36]. Necrosis is a type of cell death in which cells swell and break open, release their contents and can damage neighboring cells and provoke inflammation. In contrast to necrosis, apoptosis (programmed cell death) is generally considered to be a silent event and not to evoke cellular activation, and is thereby important for maintaining homeostasis. The cells that are most sensitive to cell death in atherosclerosis are the lipid-laden macrophages. This cell death in atherosclerosis may be detrimental. Apoptotic cells need to be scavenged and cleared from the damaged site, but in the atherosclerotic lesion, scavenging of large lipid-filled cells may be inefficient which will lead to an augmented inflammatory response [9]. The first evidence that NF-κB might be involved in the regulation of apoptosis was obtained from mouse models in which different components of the NF-κB system were disrupted. These mice died in utero as a result of severe liver apoptosis [37]. These data show that NF-κB is an important cellular survival factor, and this is probably because of the fact that NF-κB induces the expression of several anti-apoptotic genes [36]. In a recent study were they used a macrophage-restricted deletion of IKK2 to investigate the role of NF-κB activation in macrophages in the development of atherosclerosis, it was shown that macrophage-specific deletion of IKK2 resulted in increased sensitivity to cell death after treatment with different stimuli [35]. These data show that in macrophages, NF-κB signaling is important for survival under certain circumstances. Inhibition of NF-κB in atherosclerosis might result in cells that are sensitive to cell death.

Foam cell formation

NF-κB may also be a crucial factor in the process of foam cell formation. Foam cell formation is described as the maturation of monocytes to active macrophages that take up modified LDL from the vessel wall. In foam cell formation, the differentiation of monocytes to macrophages is characterized by an increased expression of two major scavenger receptors on the surface of the cells, namely the type A scavenger receptor, SR-A and the type B scavenger receptor, CD36 [38]. Both receptors bind different forms of modified LDL and they mediate the scavenging of modified lipids from the vessel wall, thereby mediating the process of

atherosclerotic foam cell formation. Recent studies have addressed the relative importance of CD36 and SR-A in mediating the uptake of modified LDL. It was seen that CD36 and SR-A are responsible for the vast majority of modified LDL uptake in macrophages [39,40]. So, although oxLDL uptake is primarily mediated by CD36, SR-A is also involved. The regulation of these scavenger receptors is, in part, mediated by the transcription factor, peroxisome proliferator activated receptor-γ (PPARγ) [39,41]. It was shown that in the absence of p50, activated macrophages take up less modified LDL because of down-regulation of SR-A [42]. This could implicate that NF-κB has a role in the regulation of lipid uptake. On the other hand, the active efflux of cholesterol and phospholipids from macrophages is mediated by the ATP-binding cassette transporters-1, ABCA1 and ABCG1, that play an important role in cholesterol homeostasis and atherosclerosis [43]. The expression of ABCA1, like CD36, is also induced by PPARγ through a transcriptional cascade mediated by the nuclear receptor, liver X receptor alpha (LXRα). [44,45] Not much is known about whether the cholesterol efflux mechanisms are also affected by NF-κB activation, although LPS-induced repression of ABCA1 was shown to be reversed by NF-κB inhibitors [42]. It was also recently shown that TNFα induces ABCA1 expression in macrophages through NF-κB [22]. Moreover, the deletion of IKK2 in macrophages was shown to reduce the induction of ABCA1 by TNFα by 65 % [22].

1.6 Aim and objectives of this study

The above-mentioned data show that the transcription factor NF-κB has an important role in the different processes involved in atherosclerosis. In addition, macrophages are the most important inflammatory cells in the process of atherosclerosis and in the regulation of NF-κB in the control of inflammatory diseases. These characteristics make the study of NF-κB especially in macrophages very attractive for therapeutic interventions in a chronic lipid driven inflammatory disease like atherosclerosis. Atherosclerosis is an inflammatory disease but the mechanisms by which inflammatory cytokines affect atherogenesis are still poorly understood. Although in many studies the effects of decreased or increased levels of inflammatory cytokines on atherosclerosis were investigated, the exact inflammatory pathways are still unclear. Moreover, the role of NF-κB activation in macrophages during foam cell formation is largely unknown. Detailed analyses of the exact function of NF-κB in macrophages cholesterol influx are still lacking. Moreover, not much is known about whether the cholesterol efflux mechanisms are also affected by NF-κB.

For the above-mentioned reasons, the overall aim of this study is to investigate **the role of the transcription factor NF-κB in inflammatory signaling and lipid metabolism in macrophages**. The role of NF-κB in these processes of atherosclerosis is studied by inhibiting the transcription factor NF-κB. From the point of both efficiency and specificity, one of the most ideal targets for the inhibition of NF-κB appears to be IKK2. IKK2 is the most important subunit for activation of the classical NF-κB pathway. Namely, IKK2 is part of the IκB kinase complex that directly phosphorylates IκBα, a key regulatory signal for the ubiquitination-dependent proteolytic degradation of IκBα that results in liberation of NF-κB from the inactive cytoplasmic complex and its translocation to the nucleus.

The overall aim of this study will be addressed by the following three working hypotheses:

1) The inhibition of IKK2 in macrophages decreases the expression of both pro-inflammatory and anti-inflammatory cytokines.

2) The inhibition of IKK2 in macrophages decreases the expression of several anti-apoptotic genes which results in macrophages that are more sensitive to apoptosis.

3) The inhibition of IKK2 in macrophages decreases the process of foam cell formation by diminishing the lipid influx through down-regulation of the scavenger receptors CD36 and SR-A and by increasing the lipid efflux through up-regulation of ABCA1 and ABCG1.

To address the above mentioned research hypothesis, first the effects of IKK2 inhibition on inflammation and apoptosis are investigated. Second, it is investigated whether IKK2 inhibition has an effect on lipid loading and foam cell formation.

Two methods are used to functionally inhibit IKK2. The first method is a retroviral approach to establish RAW 264.7 cells that stably express a dominant negative mutant of IKK2 (IKK2 KD). A second method to functionally disrupt IKK2 is by the use of chemical compounds that disrupt the IKK2 activity. In our experiments two of these chemical IKK2 inhibitors, namely SPC-839 and SC-514 are used. The results achieved with these chemical IKK2 inhibitors are used to validate the results obtained with the retroviral approach. The reason why we used two methods to functionally inhibit IKK2 is because these methods might have different implications for future therapy. The chemical IKK2 inhibitors might be more attractive for the use in clinical applications because they are small molecules that are easy to administer. However, these chemical IKK2 inhibitors are not cell specific. That is why the retroviral approach might also be interesting because in this way it is possible to target a specific cell type. However, before we can speculate on future therapies, it is necessary to first thoroughly investigate the effects of IKK2 inhibition in in vitro and in vivo settings.

2. Materials and Methods

2.1 Cell culture

Monocyte/macrophage RAW 264.7 cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated, filtered (0.2 μm) fetal calf serum (FCS), 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO_2. Cells were routinely grown in 162 cm² culture flasks and passaged twice a week.

ΦNX producer cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4500 mg/l glucose, L-glutamine and pyruvate and supplemented with 10% heat-inactivated, filtered (0.2 μm) fetal calf serum (FCS), 50 U/ml penicillin and 50 μg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO_2. Cells were routinely grown in 75 cm² culture flasks.

Monocyte/macrophage RAW 264.7 cells, containing a luciferase NF-κB reporter construct with eGFP and a geneticin selection marker (clone 18), were cultured in RPMI 1640 supplemented with 10% heat-inactivated, filtered (0.2 μm) fetal calf serum (FCS), 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin and 100 μg/ml geneticin at 37°C in a humidified atmosphere with 5% CO_2. Cells were routinely grown in 162 cm² culture flasks and passaged twice a week.

2.2 Retroviral IKK2 KD (kinase dead) construct

In the pCFG5-IEGZ retroviral vector, a human IKK2 KD (kinase dead) plasmid was cloned.

The pCFG5-IEGZ retroviral vector (appendix 1) contains a polylinker upstream of an internal ribosomal entry site (IRES) driving an eGFP zeocin fusion protein cassette. In this way, the long terminal repeat-mediated expression of the inserted gene is coupled to the expression of a GFP-zeocin resistance fusion gene through an internal ribosome entry site. Green fluorescent protein (GFP) is a protein produced by a jellyfish Aequorea which fluoresces in the lower green portion of the visible spectrum (510 nm) after excitation with UV-light. This allows us to distinguish between infected and noninfected cells by fluorescence microscopy or by flow cytometric analysis of GFP expression.

In the IKK2 KD construct (figure 2.1), the aspartic acid (D145) located in the kinase domain and involved in the activity of IKK2, is substituted by asparagine (N145). In this way, IKK2 is not able anymore to perform its enzymatic function, namely the phosphorylation of IκB. This leads to an inhibition of the NFκB pathway.

```
      1          11          21          31          41          51
   1                  WEMKER LGTGGFGNVI RWHNQETGEQ IAIKQCRQEL SPRNRERWCL    60
  61 EIQIMRRLTH PNVVAARDVP EGMQNLAPND LPLLAMEYCQ GGDLRKYLNQ FENCCGLREG   120
 121 AILTLLSDIA SALRYLHENR IIHRDLKPEN IVLQQGEQRL IHKIIDLGYA KELDQGSLCT   180
 181 SFVGTLQYLA PELLEQQKYT VTVDYWSFGT LAFECITGFR PFLPNWQPVQ WHSKVRQKSE   240
 241 VDIVVSEDLN GTVKFSSSLP YPNNLNSVLA ERLEKWLQLM LMWHPRQRGT DPTYGPNGCF   300
```

Figure 2.1. Amino acid sequence of the kinase domain of the human IKK2 protein. Indicated in red is the amino acid aspartic acid (D145) that functions as a proton acceptor and that is involved in the activity of the IKK2 enzyme. In the IKK2 KD construct used to establish RAW 264.7 cells that stably express a dominant negative mutant of IKK2, this aspartic acid is substituted by asparagine (N145). In this way, IKK2 is not able anymore to perform its enzymatic function.

2.3 Transfection

ΦNX producer cells were transfected with the pCFG5-IEGZ vector containing the IKK2 KD construct (IKK2 KD). As a control, ΦNX producer cells were transfected with the empty pCFG5-IEGZ vector (IEGZ).

Eight hours before transfection, ΦNX producer cells were plated at a density of $1x10^6$ in a 10-cm plate. Transfection was performed using the FuGene 6 Transfection Reagent (Roche Diagnostics). The ratio Fugene/DNA we used was 3/1 (9 µl Fugene/3 µg DNA). Briefly, 9 µl FuGene was added to a total volume of 100 µl DMEM without FCS and incubated for 5 minutes at room temperature. Next, 3 µg plasmid DNA (IKK2 KD or IEGZ) was added to FuGene/DMEM and incubated for 15 minutes at room temperature. This DMEM/DNA/FuGene mixture was then added to the ΦNX producer cells and allowed to incubate overnight at 37°C. Twenty-four hours later, transfection efficiencies were determined by monitoring GFP expression by fluorescence microscopy. ΦNX cells were selected using 100 µg/ml of zeocin (Invitrogen). The cells were grown in the presence of this agent during 2 selection rounds of 48 hours with 24 hours interval. After selection more than 90% of the ΦNX cells were fluorescent. After the selection procedure, ΦNX cells were cultured in DMEM and the viral supernatants were harvested every 8 hours. Supernatants were filtered through a 0.45 µm filter, snap frozen and stored at -80°C until infection.

2.4 Retroviral infection

To establish RAW 264.7 cells that stably express dominant negative IKK2, RAW 264.7 cells were infected with the viral supernatant of the ΦNX cells transfected with the pCFG5-IEGZ vector containing the IKK2 KD construct. As a control, RAW 264.7 cells were infected with the viral supernatant of the ΦNX cells transfected with the empty pCFG5-IEGZ vector.

RAW 264.7 cells were seeded in a 6-well plate at a density of $3x10^5$ cells/well in 2 ml RPMI medium and incubated overnight with 0.2 μg/ml Tunicamycin. The next day, the viral supernatant was thawed out in a 37°C water bath and polybrene was added to this virus supernatant until a final concentration of 5 μg/ml. Then, the RPMI medium was removed from the cell cultures and replaced by 2 ml viral supernatant/polybrene. To optimize the contact between the viral supernatant and the RAW 264.7 cells, the 6-well plate was placed in the centrifuge and allowed to spin for 99 minutes at 2200 rpm at 37°C. The culture plate was then carefully placed back into the incubator. After 6 hours, the viral supernatant was diluted (1/2). The next day the viral supernatant was removed and the cells were replenished with fresh warm RPMI medium and placed back into the incubator for 48 hours. As an indication for the infection efficiency, the GFP positive cells were determined by fluorescence microscopy or analyzed by FACS. Forty-eight hours after infection, the cells were placed on antibiotic selection. Briefly, 500 μg/ml zeocine (Invitrogen) was added to the cells and incubated for 24 hours. Every 24 hours, the cells were replenished with fresh medium to remove dead cells and then placed back onto antibiotic selection. All experiments were performed when more than 90% of the cells were fluorescent and when the cells were at least 48 hours without selection.

2.5 Intracellular TNFα FACS

As a functional control for IKK2 inhibition in RAW 264.7 cells infected with the IKK2 KD construct, the LPS-induced intracellular TNFα expression was determined by FACS.

RAW 264.7 cells were seeded at a density of 350 000 cells per well in a 24-well plate and allowed to adhere overnight. The next day, protein transport inhibitor brefeldin A (2 μg/ml) and stimulant LPS (100 ng/ml) were added and allowed to incubate for 3 hours. Next, the cells were detached using PBS/EDTA and gentle scraping and collected by centrifugation. The cells were then washed in PBS and fixed for 10 minutes in ice-cold PBS containing 4% paraformaldehyde. After another wash in PBS, the cells were resuspended in 200 μl PBS containing 0.2% saponin, 2% fetal calf serum (FCS) and 5% normal mouse serum (NMS) and incubated for 15 minutes on ice to permeabilize the cells and to block nonspecific binding.

Next, the cells were incubated for 1 hour on ice with 1:300 anti-TNF-PE antibody in PBS containing 0.2% saponin and 2% fetal calf serum (FCS). Finally, the cells were washed three times in PBS containing 0.4% saponin and 2% fetal calf serum (FCS) and resuspended in PBS. Fluorescence was determined in the FL2 channel using a FACScalibur (Becton Dickinson). Quantification was performed using CellQuest software.

2.6 Chemical IKK2 inhibitors

To validate the results obtained with the above mentioned-retroviral approach, a second method to functionally disrupt IKK2 was used, namely the administration of chemical IKK2 inhibitors to RAW 264.7 cells. The chemical IKK2 inhibitors used in this study are SC-514 and SPC-839 (figure 2.2).

SC-514 (Merck) is a cell-permeable (thienothienyl)amino-acetamide compound that displays anti-inflammatory properties. This small molecule acts as a potent, reversible, ATP-competitive and highly selective inhibitor of IKK-2. It has a 20-fold selectivity for IKK2 (IC_{50}=3-12 μM) over IKK1 (IC_{50}>200 μM) [46].

SPC-839 (Signal Pharmaceuticals) is a potent, reversible, ATP-competitive inhibitor of IKK2. This quinazoline analogue inhibits IKK2 with nanomolar potency (IC_{50}=62 nM) and IKK1 with micromolar potency (IC_{50}=13 μM) [47,48]. It is already used in clinical practice for the treatment of rheumatoid arthritis. The effect of these chemical IKK2 inhibitors on cytokine gene expression may be a combination of inhibiting IκBα phosphorylation/degradation, affecting NF-κB nuclear import/export as well as the phosphorylation and transactivation of p65.

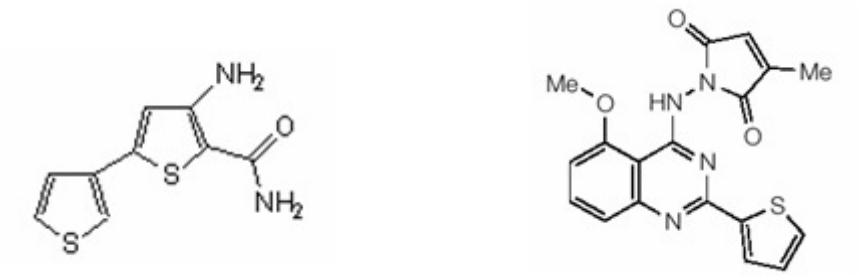

Figure 2.2. Chemical structures of the chemical IKK2 inhibitors SC-514 (left) and SPC-839 (right).

2.7 Luciferase reporter gene assay

Before performing any experiments with the chemical IKK2 inhibitors SC-514 and SPC-839, the optimal concentration and incubation time of these inhibitors had to be determined. This was done by looking at the LPS-induced NF-κB activity using a luciferase reporter gene assay after incubating RAW 264.7 cells containing a luciferase NF-κB reporter construct with different concentrations and for different time points with SC-514 or SPC- 839.

The luciferase reporter gene assay is based on the principle of bioluminescence (light emission catalysed by an enzyme). Firefly luciferase catalyses the release of light upon addition of luciferin and ATP. The luciferase activity is assayed and quantified by measurement of light production. Light is produced by converting the chemical energy of luciferin oxidation through an electron transition, forming the product molecule oxyluciferin. Firefly luciferase, a monomeric 61 kDa protein, catalyzes luciferin oxidation using ATP.Mg2+ as a cosubstrate (figure 2.3).

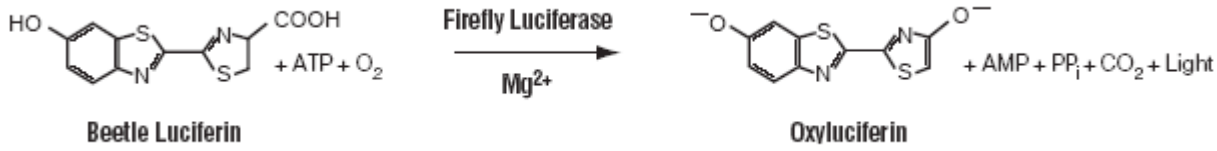

Figure 2.3: Bioluminescent reaction catalyzed by firefly luciferase.

RAW 264.7 cells, containing a luciferase NF-κB reporter construct, were seeded at a density of $1x10^5$ cells per well in a 96-well plate 4 hours before the assay. The cells were pre-incubated with different concentrations of the IKK2 inhibitor, SC-514 or SPC-839 for 1 hour, 24 hours or 48 hours. Then the cells were stimulated with 100 ng/ml LPS for 3 hours. The luciferase assay was performed using the luciferase assay system kit provided by Promega. Briefly, the cells were lysed in lysisbuffer (1x) and 10 µl of cell lysate was transferred to a luminometer plate containing the luciferase assay reagent (50 µl). The luciferase activity was measured by a luminometer (Victor 3, 1420 multilabel counter, Perkin Elmer, Wallac).

2.8 Trypan blue staining

To analyze the amount of cell death induced by treatment of RAW 264.7 cells with the chemical IKK2 inhibitor SPC-839, a trypan blue staining was performed. When trypan blue is added to the cells, viable cells exclude trypan blue, while dead cells stain blue do to trypan blue uptake.

RAW 264.7 cells were incubated for 24 hours or 48 hours with 250 nM, 400 nM or 500 nM SPC-839 and then stained with trypan blue. The percentage of dead cells was calculated by counting the amount of dead cells and dividing this number by the total amount of cells.

2.9 TNFα, IL-6 and IL-10 enzyme-linked immunosorbent assay (ELISA)

To study the effect of IKK2 inhibition on cytokine production, after exposure to LPS, an ELISA for TNFα, IL-6 and IL-10 was performed on the supernatants of RAW 264.7 cells preincubated with chemical IKK2 inhibitors or infected with the IKK2 KD construct.

RAW 264.7 cells were plated at a density of 350 000 cells per well in 500 µl medium in a 24-well plate. The next day, the cells used for the experiments with the chemical IKK2 inhibitors were pre-treated for 1 hour with different concentrations of SC-514 (10 µM, 25 µM or 50 µM) or for 24 hours with different concentrations of SPC-839 (50 nM, 250 nM, 500 nM) and then stimulated with 100 ng/ml LPS for 8 hours. The cells infected with the IKK2 KD construct or the empty pCFG5-IEGZ vector were stimulated with 100 ng/ml LPS for 8 hours at 37°C. After the indicated time point, supernatants were collected to measure secretion of TNFα, IL-6 and IL-10. To perform the ELISA, 96-well plates were coated overnight at 4°C with 100 µl of capture antibody diluted 1:800 in PBS. The next day, plates were washed with PBS containing 0.05% Tween (PBS-Tween). Subsequently, the wells were blocked with 200 µl of 0.5% bovine serum albumin (BSA) in PBS for 1.5 hours at room temperature. For the standard titration curve, TNFα (1ng/ml), IL-6 (1ng/ml) and IL-10 (0.5 ng/ml) were diluted in PBS containing 0.5% BSA and 5% FCS. After another wash with 0.05% PBS-Tween, plates were incubated with 100 µl of cell culture supernatants diluted in PBS containing 0.5% BSA and 5% FCS for 1 hour at room temperature. Next, the plates were washed three times with 0.05% PBS-Tween and incubated for 1 hour at room temperature with 100 µl of detection antibody diluted (1:5000 for TNFα, 1:10000 for IL-6 and 1:4000 for IL-10) in PBS containing 0.5% BSA. Plates were washed again three times with 0.05% PBS-Tween and filled with 100 µl Streptavidin-HRP diluted (1:2500 for TNFα, 1:5000 for IL-6 and 1:2500 for IL-10) in PBS containing 0.5% BSA. The plates were then incubated for 1 hour at room temperature, washed three times as described above, and incubated with OPD as the development agent for 10-30 minutes at room temperature in the dark. The reaction was stopped by adding 50 µl of 1.8 M H_2SO_4. Absorbance was measured at 490 nm using a microplate reader from BioRad.

2.10 NO assay

To determine the effect of IKK2 inhibition in macrophages on the production of NO after exposure to LPS, an NO assay was performed on the supernatants of RAW 264.7 cells infected with the IKK2 KD construct.

The NO assay is based on detection of nitrite in the medium of cultured cells. As NO forms nitrite in water based solvents, it can be used to measure NO content as well (figure 2.4).

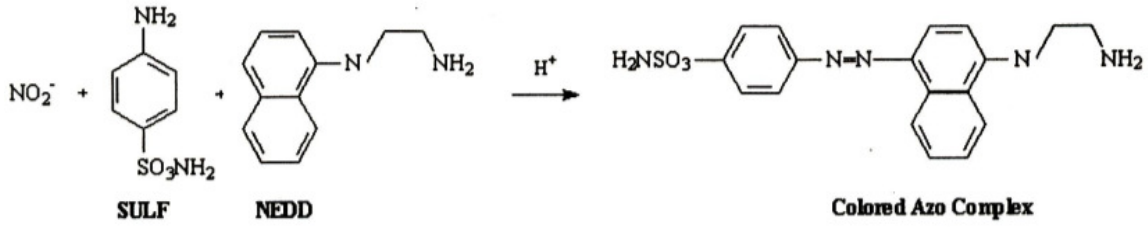

Figure 2.4. Principle of NO assay. Nitrite reacts with Greiss Reagent in acidic medium to form a colored adduct which absorbs at 540 nM. SULF=Sulfanilamide (color reagent 1), NEDD=N-Naphthylethylenediamine (color reagent 2), Neutral Greiss Reagent=NEDD + SULF.

RAW 264.7 cells infected with the IKK2 KD construct or the empty pCFG5-IEGZ vector were plated at a density of 350 000 cells per well in 500 µl medium in a 24-well plate. The next day, the cells were stimulated with LPS (100 ng/ml) for 24 hours. After the indicated time point, supernatants were collected to measure secretion of NO. Briefly, a standard range of sodium nitrite was made on a 96-well plate and the samples were placed on the plate. Next, Greiss reagent (Sigma) was added to the standard range and the samples. The nitrite reacts with this reagent in acidic medium to form a colored adduct which absorbs at 540 nM (figure 2.4).

2.11 Apoptosis assay

To study the effect of IKK2 inhibition on apoptosis, a propidium iodide assay was performed on RAW 264.7 cells infected with the IKK2 KD construct or the empty pCFG5-IEGZ vector. This assay is based on the fact that propidium iodide (PI) intercalates into the major groove of double-stranded DNA of viable cells. During cell death, the DNA of the cells falls apart so that the propidium iodide cannot intercalate between the DNA anymore. The amount of propidium iodide that is present in the cells is a measure for the viability of the cells and can be measured by flow cytometry. Propidium iodide produces a highly fluorescent adduct that can be excited at 488 nm with a broad emission centered around 600 nm.

RAW 264.7 cells were plated at a density of 300 000 cells per well in a 24-well plate and allowed to adhere overnight. Then the cells were replenished with fresh medium and either stimulated for 3 hours with LPS (100 ng/ml), overnight with LPS (100 ng/ml), overnight with cycloheximide (5 µg/ml) or overnight with cycloheximide (5 µg/ml) together with TNF (100 ng/ml). After the indicated incubation periods, the cells were detached by gentle scraping and then together with the supernatants of the cells collected by centrifugation. Next, the cells were washed in PBS, fixed in ice-cold 70% ethanol and stored overnight at 4°C. The next day, the cells were washed twice in PBS and permeabilized in PBS containing 0.1 % Nonidet P-40 (10 minutes on ice). After another wash in PBS, the cells were collected in 500 µl PBS containing 50 µg/ml propidium iodide and 25 µg/ml RNAse A to make sure that the propidium iodide does not intercalate between the RNA. After at least 30 minutes of incubation at room temperature, the cells were analyzed by measuring the propidium iodide staining in the FL3 channel using a FACScalibur (Becton Dickinson). Quantification was performed using CellQuest software.

2.12 LDL isolation and oxLDL, acLDL preparation

Human blood (50 ml) was centrifuged for 10 minutes at 4000 rpm to separate the serum from the rest of the blood. The upper layer of serum was collected and set to a density of 1.21 g/ml by adding an appropriate amount of KBr. Next a density gradient was created by adding three layers of NaCl to the serum with respectively densities of 1.063, 1.019 and 1.006 g/ml. After centrifuging the serum overnight at 32000 rpm at 4°C, the yellow layer between the two highest NaCl density layers that contains the low-density lipoprotein (LDL), was collected. The protein concentration of LDL was measured and one part of the LDL was oxidized to oxLDL and the other part was acetylated to acLDL. The oxidation was performed by incubating the LDL overnight in PBS in the presence of 10 µM $CuSO_4$ at 37°C. The next day, the oxidation was stopped by adding an excess of EDTA. The LDL was acetylated by adding first sodium acetate and then acetic anhydride. The acetic anhydride was added to the LDL on ice while gently stirring in portions of 2µl divided over 60 minutes. After adding the last amount of acetic anhydride, the LDL was incubated for another 30 minutes one ice while gently stirring. The oxidized LDL and acetylated LDL were then dialyzed overnight at 4°C against PBS in the presence of 10 µM EDTA. PBS was refreshed frequently. Next, the oxLDL and acLDL were filtered and a second protein concentration was performed. To test the oxidation level of the oxLDL and the acetylation level of the acLDL, the samples were run on a 0.8% agarosegel and stained with coomassieblue. Part of the oxLDL and acLDL were

diI-labeled by incubating them overnight with a small amount of diI while gently shaking. The next day, the diI-labeled oxLDL and acLDL were filtered and the protein concentration was determined. OxLDL, acLDL, diIoxLDL and diIacLDL were kept at 4°C until used.

2.13 DiI-labeled modified LDL uptake

To investigate whether IKK2 inhibition influences the uptake of oxLDL or acLDL, a DiI uptake experiment was performed on RAW 264.7 cells treated with the IKK2 inhibitor SC-514 and on RAW 264.7 cells infected with the IKK2 KD construct.

RAW 264.7 cells were seeded at a density of 350 000 cells per well in a 24-well plate. The next day, the cells were replenished with fresh medium. The cells used for the experiments with the chemical IKK2 inhibitor SC-514 were pre-treated with this inhibitor for 1 hour and then incubated for 3 hours with 25 µg/ml of DiIoxLDL or DiIacLDL. The infected RAW 264.7 cells were incubated for 3 hours with 25 µg/ml of DiIoxLDL or DiIacLDL. Next the cells were washed in cold PBS, detached by adding PBS/EDTA and gentle scraping and collected by centrifugation. The cells were then resuspended in PBS and the uptake of oxLDL and acLDL was measured by determining the fluorescence in the FL2 channel using a FACScalibur. Quantification was performed using CellQuest software.

2.14 RNA isolation

To study the effect of IKK2 inhibition on the gene expression of the scavenger receptors CD36 and SR-A, RAW 264.7 cells were incubated for 3 hours or 8 hours with oxidized LDL or acetylated LDL, RNA was isolated from the cells, reverse transcribed into cDNA and quantified with taqman real-time PCR.

RAW 264.7 cells were seeded at a density of 350 000 cells per well in a 24-well plate. The next day, the cells were replenished with fresh medium, pretreated with the chemical IKK2 inhibitor for 1 hour and then incubated for 3 hours at 37°C with either 25 µg/ml or 50 µg/ml of oxLDL or acLDL. The RAW 264.7 cells infected with the IKK2 KD construct or the empty vector were incubated for 8 hours with 25 µg/ml of oxLDL or acLDL at 37°C. After incubation, the cells were washed with cold PBS and directly lysed by adding Tri-reagent (Sigma). These homogenized cells were then transferred to eppendorf tubes and stored at -80°C until further isolation. RNA was extracted by first adding chloroform (200µl/ml Tri reagent used), vortexing and then centrifuging the cells for 15 minutes at 3200 rpm at 4°C. Centrifuging separates the mixture into three phases (bottom to top): a red organic phase (containing protein), an interphase (containing DNA) and a colorless aqueous phase

(containing RNA). The upper layer containing the RNA was collected and mixed with isopropanol to precipitate the RNA. After centrifuging the samples for 10 minutes at 3200 rpm at 4°C, the RNA pellets were washed in 70% ethanol and air-dried. Then, the RNA was resolved in miliQ/diethyl pyrocarbonate (DEPC) and stored at 4°C.

2.15 cDNA synthesis

Before subsequent cDNA synthesis, RNA purity was determined by the A260/A280 ratio and the RNA concentration was measured at 260 nm using NanoDrop® ND-1000. Approximately 1 µg of total RNA was reversed transcribed into cDNA by using the iScript™ cDNA synthesis kit (BioRad). Briefly, 1 µg of RNA was mixed with 4 µl 5x iScript reaction mix, 1 µl iScript reverse transciptase and nuclease-free water to a total volume of 20 µl. The conditions for PCR were as follows: 5 minutes at 25°C followed by 30 minutes at 42°C and 5 minutes at 85°C. The cDNA samples were stored at -20°C until analysis with Taqman RT-PCR.

2.16 Taqman Real-time quantitive PCR

The quantitive analysis of the gene expression was carried out according to the PCR protocol, briefly discussed as follows. The 25 µl reaction system contained 12.5 µl of Universal PCR Master Mix for SYBR Green (RT-SN2X-03+, Eurogentec), 0.5 µl forward primer, 0.5 µl reverse primer, 6.5 µl miliQ and 5 µl of cDNA template. A standard curve was generated for each gene. Quantification of the cDNA was carried out in an ABI Prism 7700 (Applied Biosystems). The conditions for PCR were as follows: 2 minutes at 50°C, 10 minutes at 95°C, 40 repeats of 15 seconds at 95°C and 1 minute at 60°C, another minute at 60°C and at the end 2 minutes at 95°C. Data were analysed with SDS 1.91 (Applied Biosystems). The final results were expressed as a relative expression by comparing the amount of target gene to an appropriate housekeeping gene.

2.17 Statistical analysis

Results are presented as mean values ± the standard deviation. Statistical analysis was performed using GraphPad Prism 4 for windows. To determine the significance of group differences, the Mann-Whitney U-test was used. P-values <0.05 were considered as statistically significant.

3. Results

3.1 RAW 264.7 cells were successfully infected with a retroviral vector containing a dominant negative mutant of IKK2

Infection efficiency of RAW 264.7 cells was determined by fluorescence microscopy (figure 3.1) and by flow cytometric analysis of GFP expression (figure 3.2). The initial fluorescence was 20 % but, as shown in figure 3.1 and 3.2, after 3 weeks of selection more than 90 % of the RAW 264.7 cells were fluorescent.

Figure 3.1 and figure 3.2 show that the infections of the RAW 264.7 cells with both the empty pCFG5-IEGZ vector and the pCFG5-IEGZ vector containing the IKK2 KD construct were successful.

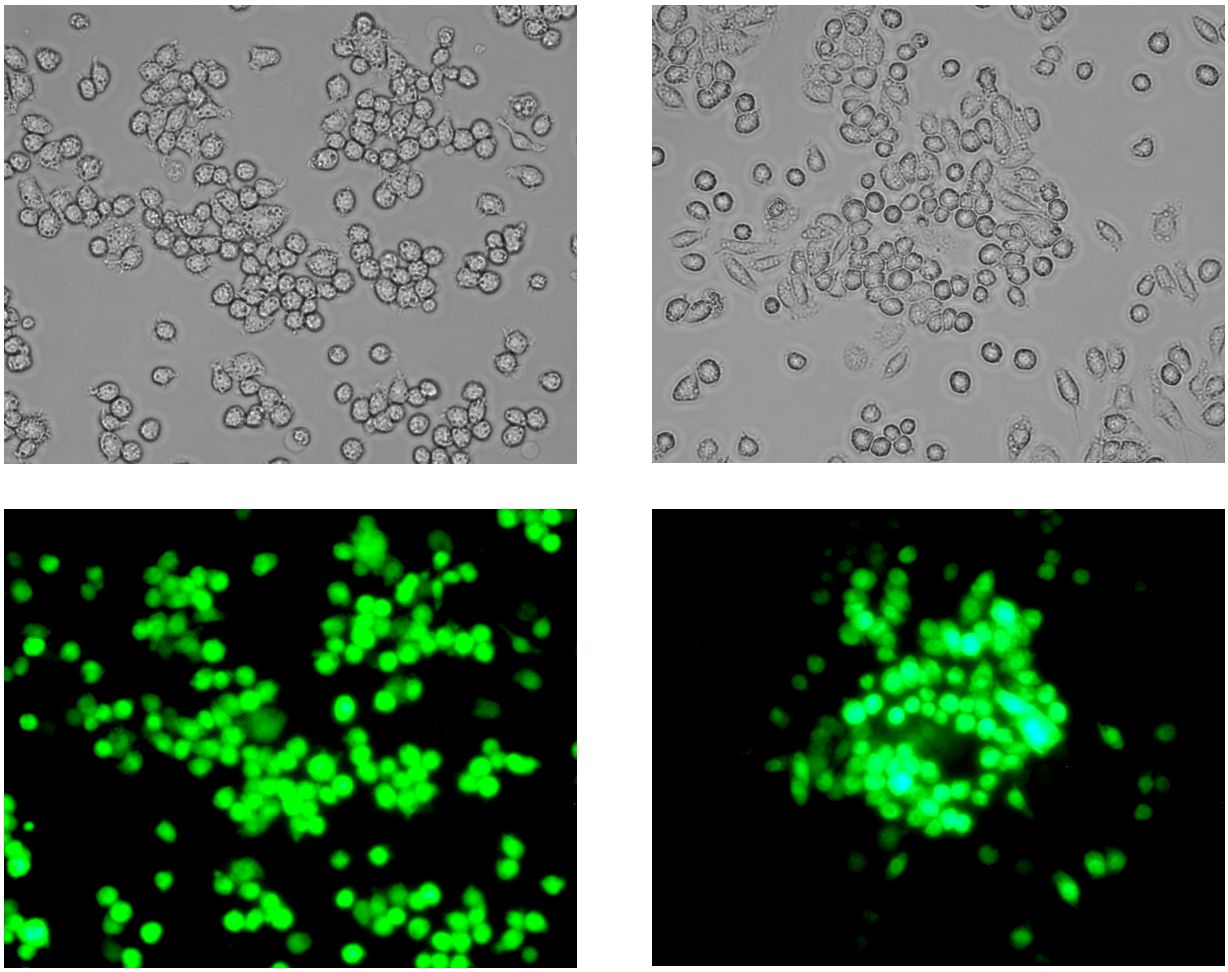

Figure 3.1. Microscopic images of RAW 264.7 cells infected with the empty pCFG5-IEGZ vector (left part) and RAW 264.7 cells infected with the pCFG5-IEGZ vector containing the IKK2 KD construct (right part). Infected cells light up green fluorescent (lower images) which is an indication for the infection efficiency.

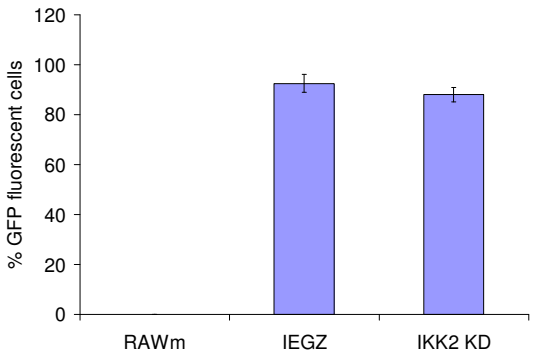

Figure 3.2. GFP expression levels in RAW 264.7 cells infected with either the pCFG5-IEGZ vector containing the IKK2 KD construct (IKK2 KD) or the empty pCFG5-IEGZ vector (IEGZ) compared to the non-infected RAW 264.7 cells (RAWm). The GFP expression levels were determined by FACS analysis.

3.2 RAW 264.7 cells that stably express a dominant negative mutant of IKK2 show a decreased intracellular TNFα protein expression

As a functional control for IKK2 inhibition and subsequent NF-κB activation, the LPS-induced intracellular TNFα protein expression was measured by FACS analysis in RAW 264.7 cells infected with the IKK2 KD construct.

As shown in figure 3.3, stimulation with LPS (100 ng/ml) for 3 hours increases the TNFα expression in the control cells. This TNFα response is statistically significant decreased for approximately 80 % in RAW 264.7 cells infected with the IKK2 KD construct ($P<0.0001$).

These results indicate that overexpression of the IKK2 KD construct indeed results in a down-regulation of NF-κB as measured by a decrease in TNFα protein expression.

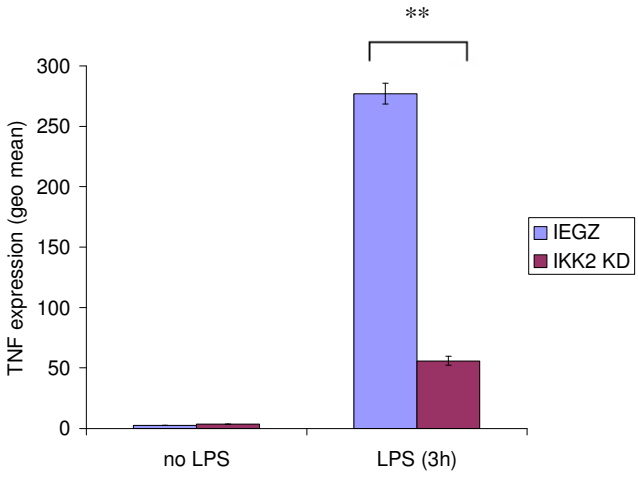

Figure 3.3. Intracellular TNFα protein expression in RAW 264.7 cells that stably express a dominant negative mutant of IKK2. RAW 264.7 cells infected with the IKK2 KD construct (IKK2 KD) or the empty pCFG5-IEGZ vector (IEGZ) were stimulated for 3 hours with LPS (100 ng/ml) or left unstimulated and then analyzed for intracellular TNFα protein expression by the use of FACS. (N=3, **$P<0.01$)

3.3 SC-514 is a fast- and short-working chemical IKK2 inhibitor

Before the use of SC-514 in experimental setups, the optimal concentration and incubation time of this chemical IKK2 inhibitor was determined.

RAW 264.7 cells containing a luciferase NF-κB reporter construct were pretreated for 1 hour with different concentrations of this inhibitor (10 µM, 100 µM and 300 µM) before subsequent LPS stimulation (100 ng/ml, 3 hours). As a control the RAW 264.7 cells were treated with different amounts of DMSO (0.5 %, 1 % and 3 %) for 3 hours. After the indicated time point, the NF-κB activity was analyzed using a luciferase reporter gene assay.

As shown in figure 3.4, the LPS-induced NF-κB activity is statistically significant inhibited in a concentration-dependent manner after treatment of the RAW 264.7 cells with the chemical IKK2 inhibitor SC-514 (R^2=0.9264, P<0.01). However, this LPS-induced NF-κB activity is also statistically significant inhibited after treating the cells with increasing amounts of DMSO, as seen in the control samples (R^2=0.954, P<0.01).

To minimize this inhibitory effect of DMSO on the NF-κB activity, the maximum concentration of DMSO in which the chemical IKK2 inhibitors are diluted was set to 0.1 % in further experiments. The control cells, not treated with the inhibitors, were incubated with a comparable amount of DMSO.

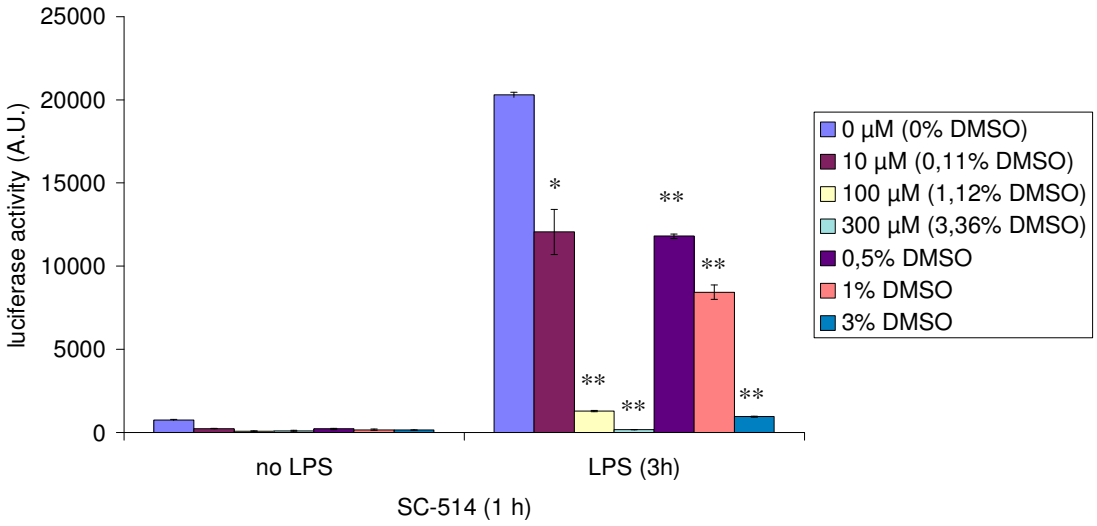

Figure 3.4. NF-κB activity in RAW 264.7 cells pretreated with the chemical IKK2 inhibitor SC-514. RAW 264.7 cells were pretreated for 1 hour with different concentrations of SC-514 (10 µM, 100 µM and 300µM) or DMSO (0.5 %, 1 % and 3 %) as a control and then stimulated with LPS (100 ng/ml) for 3 hours or left unstimulated. RAW 264.7 cells were then analyzed for NF-κB activity using a luciferase reporter gene assay. (N=2, **P<0.01, *P<0.05 compared to LPS treated control cells)

Because of the inhibitory effect of DMSO on the NF-κB activity seen in the previous experiment, the experiment was repeated with SC-514 diluted in a lower DMSO concentration (0.1 %).

To determine the effect of the chemical IKK2 inhibitor SC-514 on the NF-κB activity after a longer incubation period, in this experiment the RAW 264.7 cells, containing a luciferase NF-κB reporter construct were besides the pretreatment for 1 hour also pretreated for 24 hours with different concentrations of SC-514 (10 µM, 25 µM and 50 µM).

As indicated in figure 3.5, there is an increase in the NF-κB activity after stimulating the cells for 3 hours with LPS (100 ng/ml). This LPS-induced NF-κB activity is statistically significant inhibited for 55 % when the cells are pretreated for 1 hour with 50 µM of SC-514 ($P<0.05$). However, no significant inhibition is reached after treatment of the cells for 1 hour with 10 µM or 25 µM of SC-514 ($P>0.05$). Moreover, incubation of the cells for 24 hours with the same SC-514 concentrations has no inhibitory effect on the LPS-induced NF-κB activity.

These data indicate that pretreatment of RAW 264.7 cells for 1 hour with 50 µM of SC-514 are the optimal conditions to generate a significant inhibitory effect on the LPS-induced NF-κB activity. Lower concentrations of SC-514 or a longer incubation period cause no significant inhibition of NF-κB.

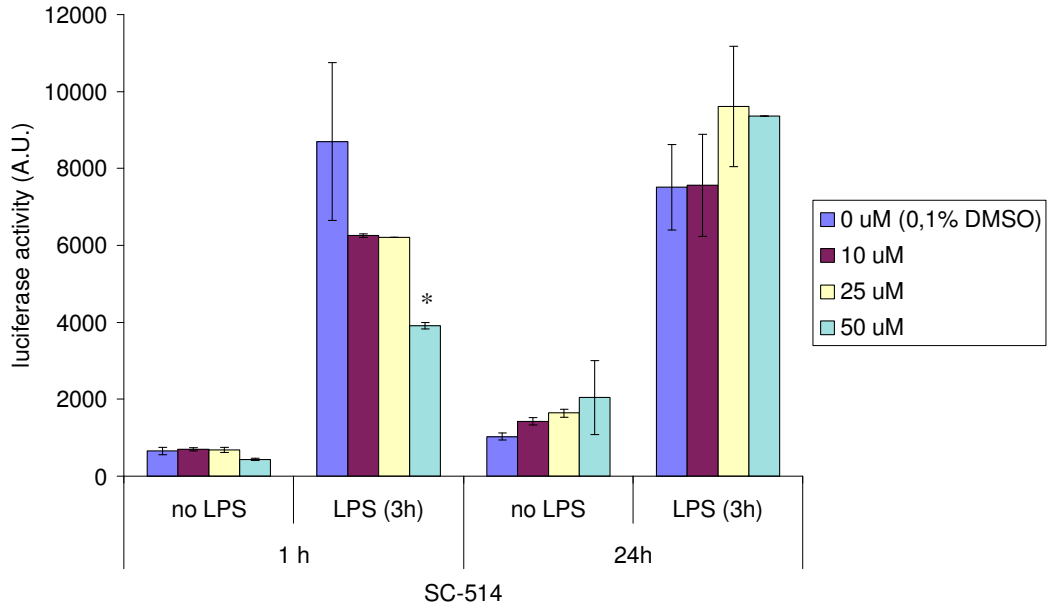

Figure 3.5. NF-κB activity in RAW 264.7 cells pretreated with the chemical IKK2 inhibitor SC-514. RAW 264.7 cells were pretreated for 1 hour or 24 hours with different concentrations of SC-514 (10 µM, 25 µM and 50 µM) and then stimulated with LPS (100 ng/ml) for 3 hours or left unstimulated. RAW 264.7 cells were then analyzed for NF-κB activity using a luciferase reporter gene assay. (N=2, *$P<0.05$ compared to LPS treated control cells)

3.4 SPC-839 is a slow- and long-working chemical IKK2 inhibitor

In a second experiment, the optimal concentration and incubation time of the other IKK2 inhibitor, SPC-839 was determined.

RAW 264.7 cells containing a luciferase NF-κB reporter construct were pretreated for 1 hour or 24 hours with different concentrations of this inhibitor (5 nM, 50 nM, 100 nM, 200 nM and 500 nM) and then stimulated for 3 hours with LPS (100 ng/ml). The NF-κB activity was analyzed by a luciferase reporter gene assay.

As shown in figure 3.6, pretreatment of the cells for 1 hour or 24 hours with the chemical IKK2 inhibitor SPC-839 significantly decreases the LPS-induced NF-κB activity ($P<0.05$). This decrease is not concentration-dependent. The highest inhibition is measured after treating the cells for 24 hours with 500 nM of SPC-839. After this incubation time and with this concentration the NF-κB activity is statistically significant inhibited for 39 % ($P=0.0055$).

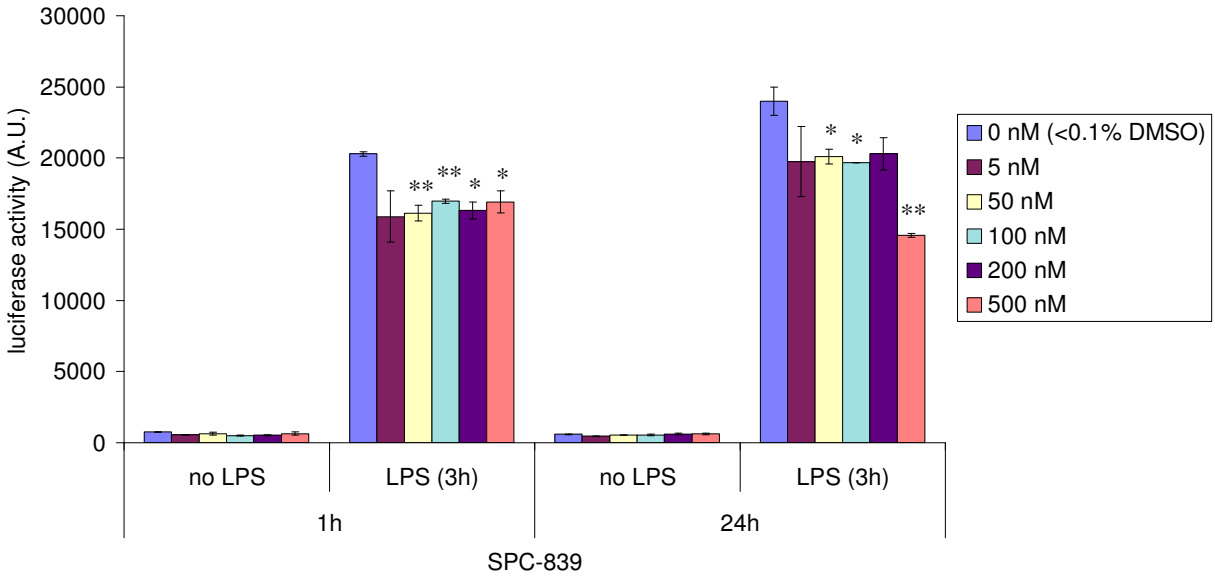

Figure 3.6. NF-κB activity in RAW 264.7 cells pretreated with SPC-839. RAW 264.7 cells were pretreated for 1 hour or 24 hours with different concentrations of SPC-839 (5 nM, 50 nM, 100 nM, 200 nM and 500 nM) and then stimulated with LPS (100 ng/ml) for 3 hours or left unstimulated. RAW 264.7 cells were then analyzed for NF-κB activity using a luciferase reporter gene assay. (N=2, **P<0.01, *P<0.05 compared to LPS treated control cells)

To analyze the effect of longer preincubations with the chemical IKK2 inhibitor SPC-839 on the NF-κB activity, RAW 264.7 cells containing a luciferase NF-κB reporter construct were pretreated for 48 hours with different concentrations of SPC-839 (50 nM, 100 nM, 250 nM, 400 nM and 500 nM) before subsequent LPS stimulation (100 ng/ml, 3 hours).

As shown in figure 3.7, there is an increase in the NF-κB activity after stimulating the cells for 3 hours with LPS. Pretreatment of the cells for 48 hours with SPC-839 significantly inhibits this LPS-induced NF-κB activity in a concentration-dependent manner (R^2=0.9121, P<0.05).

Taken together, these data indicate that pretreatment of RAW 264.7 cells for 24 hours with 500 nM SPC-839 are the optimal conditions to create a significant inhibitory effect on the LPS-induced NF-κB activity. Incubating the cells for 48 hours with 250 nM, 400 nM or 500 M also significantly inhibits the LPS-induced NF-κB activity. A shorter incubation period or a lower SPC-839 concentration causes less inhibition of NF-κB.

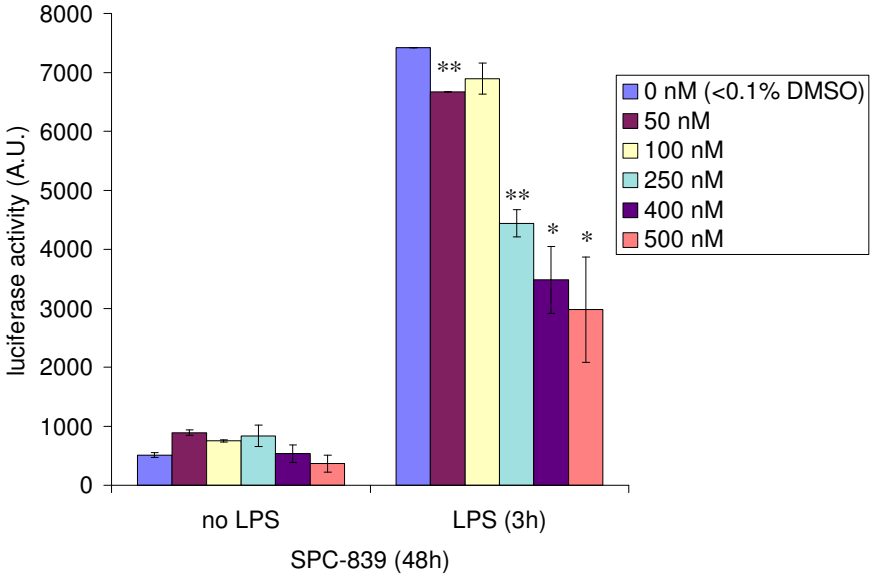

Figure 3.7. NF-κB activity of RAW 264.7 cells pretreated with SPC-839. RAW 264.7 cells were pretreated for 48 hours with different concentrations of SPC-839 (50 nM, 100 nM, 250 nM, 400 nM and 500 nM) and then stimulated with LPS (100 ng/ml) for 3 hours or left unstimulated. RAW 264.7 cells were then analyzed for NF-κB activity using a luciferase reporter gene assay. (N=2, **P<0.01, *P<0.05 compared to LPS treated control cells)

3.5 Treatment of RAW 264.7 cells with the chemical IKK2 inhibitors, SC-514 and SPC-839 induces cell death

In order to investigate the possible cytotoxic effects of the chemical IKK2 inhibitors, we determined the amount of cell death induced by SPC-839 by means of a trypan blue staining. The amount of cell death induced by SC-514 was estimated by studying the cells under the microscope.

As shown in table 3.1, treating the cells for 24 hours with 250 nM, 400 nM and 500 nM of SPC-839, leads to the induction of respectively 14.2 %, 16.2 % and 20.6 % of cell death. Treatment of these cells for 48 hours with the same concentrations of SPC-839 causes respectively 29.6 %, 72.4 % and 74.6 % of cell death.

When we looked at the cytotoxic effects of SC-514, there was no cell death visible after 1 hour of incubation. However, when the cells were incubated for 24 hours with 25 μM or 50 μM of this IKK2 inhibitor more than 50% of the cells were dead (data not shown).

These results indicate that the chemical IKK2 inhibitor SC-514 can not be used for experiments that take longer than 24 hours because then more than 50 % of the cells die. SPC-839, on the other hand, can still be used for experiments that take 24 hours. The amount of cell death we observe then lies still in the acceptable range in that the inhibition cannot merely be explained by cell death. However, the amount of cell death induced after 48 hours is not acceptable any more.

Table 3.1. Amount of cell death after treating RAW 264.7 cells with the chemical IKK2 inhibitor SPC-839. RAW 264.7 cells were treated for 24 hours or 48 hours with different concentrations of SPC-839 (250 nM, 400 nM and 500 nM). After the indicated time points, the amount of cell death was analyzed by a trypan blue staining. (N=1)

Concentration SPC-839		Amount of cell death
24 hours	250 nM	14.2 %
	400 nM	16.2 %
	500 nM	20.6 %
48 hours	250 nM	29.6 %
	400 nM	72.4 %
	500 nM	74.6 %

3.6 Inhibition of IKK2 in RAW 264.7 cells decreases the NO production and the secretion of inflammatory cytokines

First of all, we investigated whether the inhibition of IKK2 in macrophages has an effect on the inflammatory process. This was done by looking at the protein expression of several pro-inflammatory and anti-inflammatory cytokines that are regulated by NF-κB. In this way, the TNFα, IL-6 and IL-10 secretion after LPS stimulation of RAW 264.7 cells were investigated. The effect of IKK2 inhibition on the production of NO was also investigated because NO is an important component involved in macrophage activation and known to be regulated by NF-κB.

The results are depicted in figure 3.8 till 3.14. Stimulation of the control RAW 264.7 cells for 8 hours with LPS (100 ng/ml) induces the secretion of TNFα, IL-6 and NO but not of IL-10.

As shown in figure 3.8, the LPS-induced TNFα secretion in the IKK2 KD infected cells is statistically significant inhibited for approximately 30 % compared to the control cells ($P<0.0001$). The chemical IKK2 inhibitor SC-514 also significantly inhibits the LPS-induced TNFα secretion in a concentration-dependent manner ($R^2=0.9996$, $P<0.01$) (figure 3.9). The same effect was measured after pretreatment of the cells for 24 hours with different concentrations of SPC-839 ($R^2=0.8606$, $P<0.01$) (figure 3.10).

As shown in figure 3.11, the secretion of IL-6 by the IKK2 KD infected cells is statistically significant decreased for 7.5 % compared to the control cells ($P=0.0173$). Pretreatment of RAW 264.7 cells for 1 hour with the chemical IKK2 inhibitor SC-514 also significantly inhibits the LPS-induced IL-6 secretion in a concentration-dependent manner ($R^2=0.898$, $P<0.01$) (figure 3.12). The same is true for the cells that are pretreated for 24 hours with different concentrations of the chemical IKK2 inhibitor SPC-839 ($R^2=0.9414$, $P<0.01$) (figure 3.13).

In this experiment, there is no effect of IKK2 inhibition on the secretion of IL-10 measurable because stimulation of RAW 264.7 cells with LPS (100 ng/ml) for 8 hours does not induce IL-10 secretion (data not shown).

When the control and the IKK2 KD infected RAW 264.7 cells are compared for NO production, it is seen that there is a non-statistical significant trend towards an inhibition of NO production in RAW 264.7 cells that stably express a dominant negative mutant of IKK2 ($P=0.2071$) (figure 3.14).

Taken together, the data obtained with the retroviral approach and the chemical IKK2 inhibitors indicate that inhibition of IKK2 in macrophages significantly decreases the TNFα

and IL-6 secretion after LPS stimulation and that there is a trend towards inhibition of NO production.

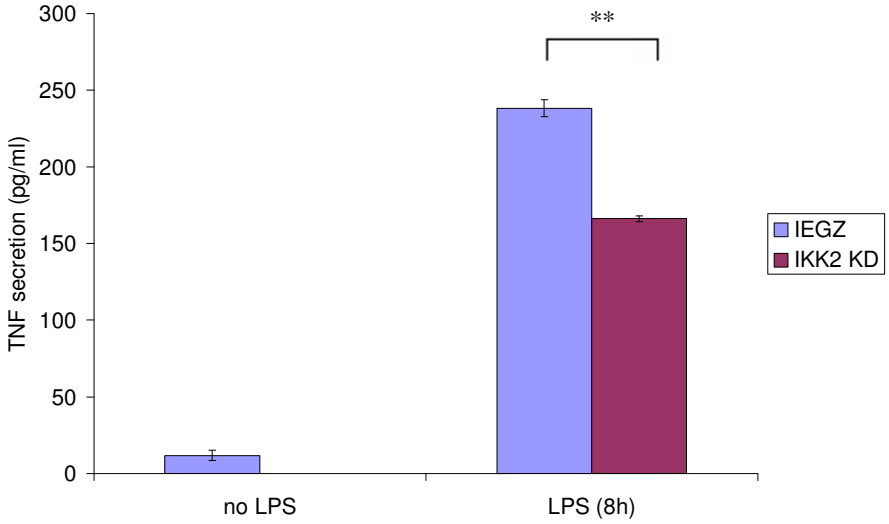

Figure 3.8. TNFα secretion by RAW 264.7 cells that stably express a dominant negative mutant of IKK2. RAW 264.7 cells infected with the IKK2 KD construct (IKK2 KD) or the empty pCFG5-IEGZ vector (IEGZ) were stimulated for 8 hours with LPS (100 ng/ml) or left unstimulated. The supernatants of the RAW 264.7 cells were then analyzed for TNFα secretion by ELISA. (N=3, **P<0.01)

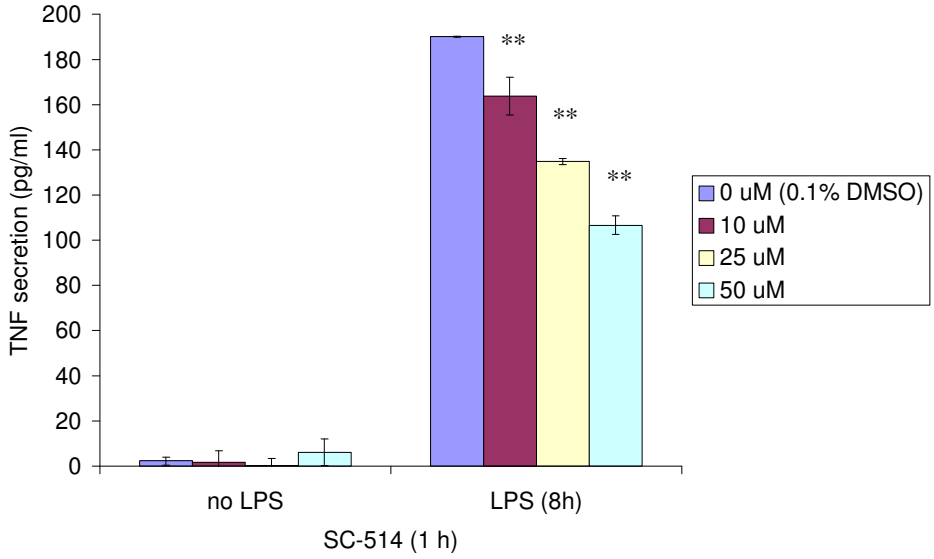

Figure 3.9. TNFα secretion by RAW 264.7 cells pretreated with the chemical IKK2 inhibitor SC-514. RAW 264.7 cells were pretreated for 1 hour with different concentrations of SC-514 (10 µM, 25 µM or 50 µM) and then stimulated with LPS (100 ng/ml) for 8 hours or left unstimulated. RAW 264.7 cells were then analyzed for TNFα secretion by ELISA. (N=3, **P<0.01 compared to LPS treated control cells)

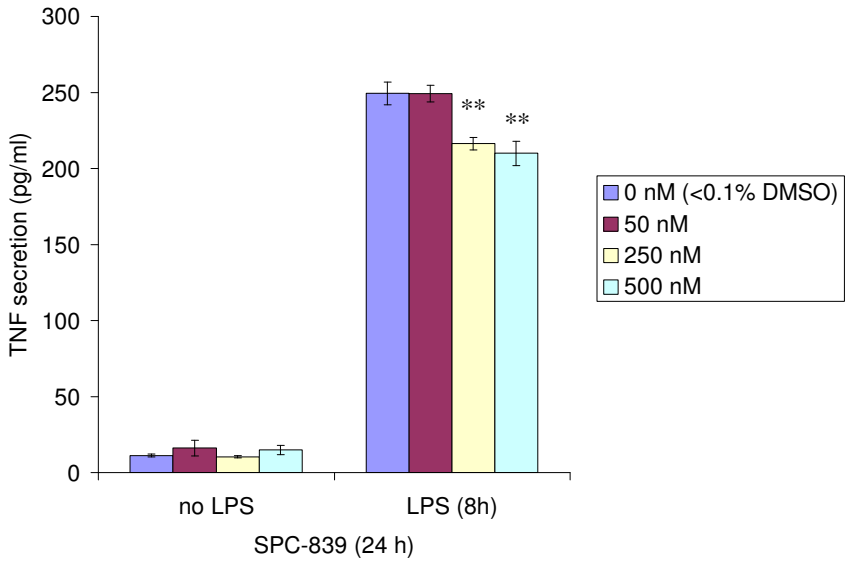

Figure 3.10. TNFα secretion by RAW 264.7 cells pretreated with the chemical IKK2 inhibitor SPC-839. RAW 264.7 cells were pretreated for 24 hours with different concentrations of SPC-839 (50 nM, 250 nM, 500 nM) and then stimulated with LPS (100 ng/ml) for 8 hours or left unstimulated. RAW 264.7 cells were then analyzed for TNFα secretion by ELISA. (N=3, **P<0.01 compared to LPS treated control cells)

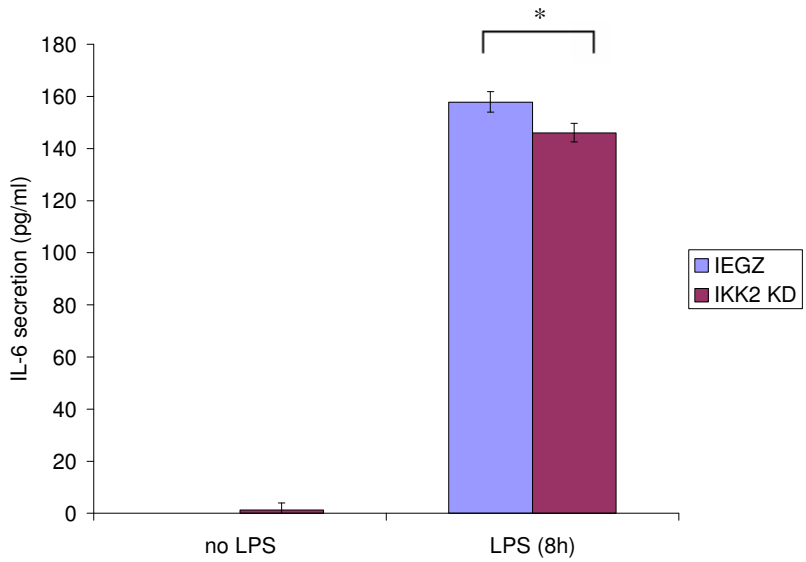

Figure 3.11. IL-6 secretion by RAW 264.7 cells that stably express a dominant negative mutant of IKK2. RAW 264.7 cells infected with the IKK2 KD construct (IKK2 KD) or the empty pCFG5-IEGZ vector (IEGZ) were stimulated for 8 hours with LPS (100 ng/ml) or left unstimulated. The supernatants of the RAW 264.7 cells were then analyzed for IL-6 secretion by ELISA. (N=3, *P<0.05)

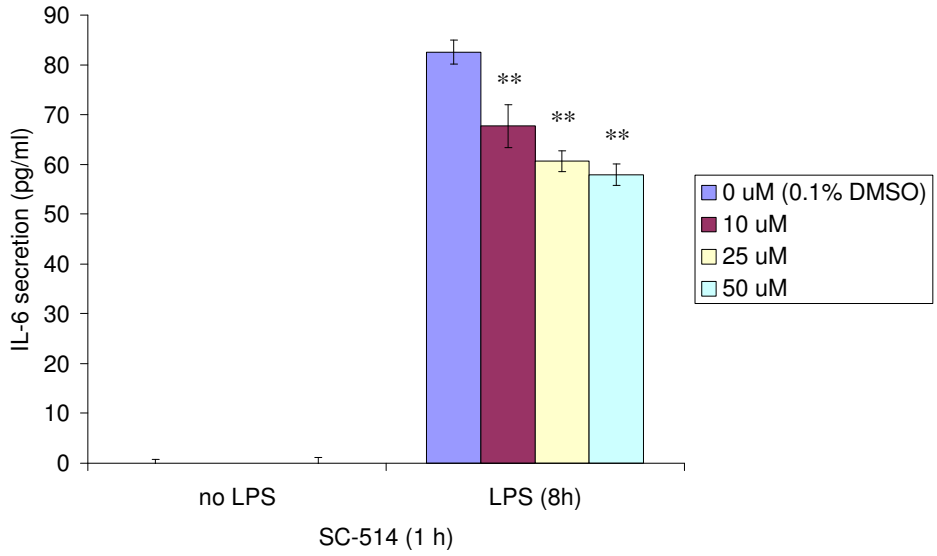

Figure 3.12. IL-6 secretion by RAW 264.7 cells pretreated with the chemical IKK2 inhibitor SC-514. RAW 264.7 cells were pretreated for 1 hour with different concentrations of SC-514 (10 µM, 25 µM or 50 µM) and then stimulated with LPS (100 ng/ml) for 8 hours or left unstimulated. RAW 264.7 cells were then analyzed for IL-6 secretion by ELISA. (N=3, **P<0.01 compared to LPS treated control cells)

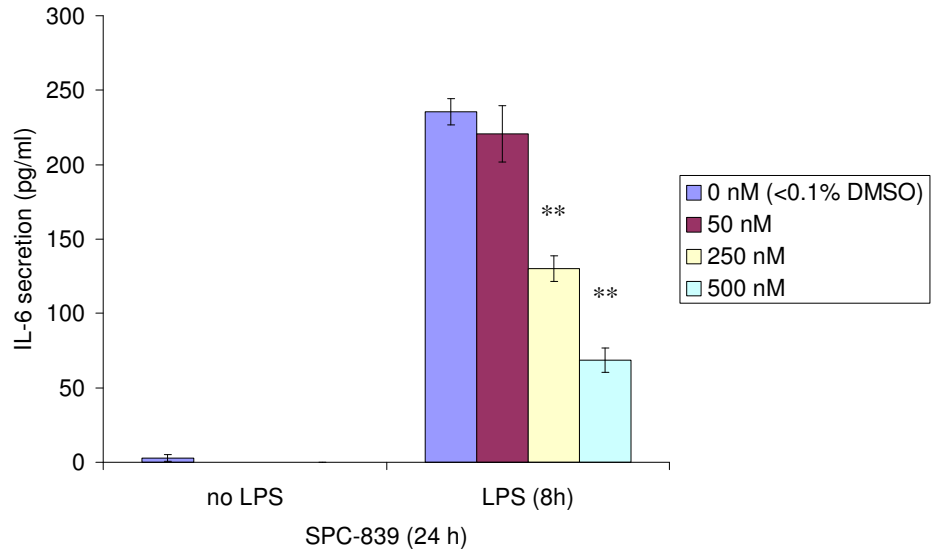

Figure 3.13. IL-6 secretion by RAW 264.7 cells pretreated with the chemical IKK2 inhibitor SPC-839. RAW 264.7 cells were pretreated for 24 hours with different concentrations of SPC-839 (50 nM, 250 nM, 500 nM) and then stimulated with LPS (100 ng/ml) for 8 hours or left unstimulated. RAW 264.7 cells were then analyzed for IL-6 secretion by ELISA. (N=3, **P<0.01compared to LPS treated control cells)

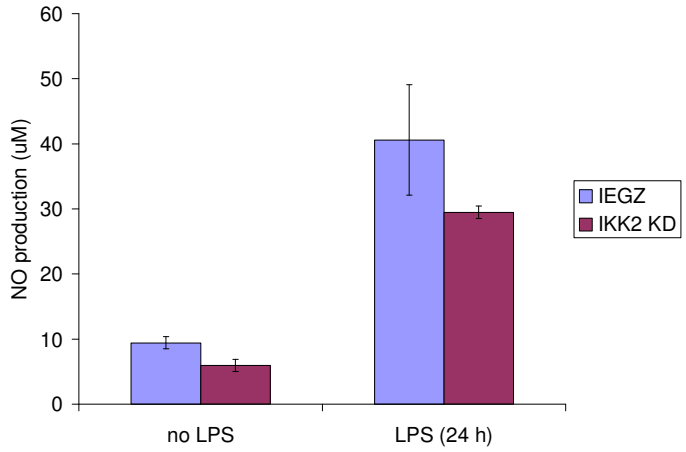

Figure 3.14. NO secretion by RAW 264.7 cells that stably express a dominant negative mutant of IKK2. RAW 264.7 cells infected with the IKK2 KD construct (IKK2 KD) or the empty pCFG5-IEGZ vector (IEGZ) were stimulated for 24 hours with LPS (100 ng/ml) or left unstimulated. The supernatants of the RAW 264.7 cells were then analyzed for NO secretion by an NO assay. (N=3)

3.7 Inhibition of IKK2 in RAW 264.7 cells significantly increases the amount of apoptosis

To study the effect of IKK2 inhibition in macrophages on apoptosis, we performed a propidium iodide assay on the viral infected RAW 264.7 cells in response to several cell death-inducing stimuli.

As indicated in figure 3.15, the basal level of apoptosis in the control cells is approximately 7.5%. Stimulating these cells with LPS (100 ng/ml) for 3 hours does not increase the amount of cell death. However, when these cells are incubated overnight with the same concentration of LPS the amount of apoptosis statistically significant increases to 15 % compared to the untreated cells (P=0.0008). Overnight incubation of these cells with cycloheximide (5µg/ml) significantly augments the amount of apoptosis to 27 %. When the cells are studied that were incubated overnight with cycloheximide (5 µg/ml) together with TNF (100 ng/ml), it is seen that TNF does not have an additional effect on the amount of apoptosis.

When these results are compared with the amount of cell death in the IKK2 KD infected cells, it is seen that inhibition of IKK2 slightly increases the basal level of apoptosis and the amount of apoptosis induced by 3 hours or overnight incubation with LPS. Moreover, when we compare the amount of cell death in the control cells, induced by cycloheximide or cycloheximide together with TNF, with the amount of cell death in the IKK2 KD infected cells, it is seen that inhibition of IKK2 statistically significant increases the amount of cell death to approximately 38 % (P=0.0042).

These results indicate that inhibition of IKK2 in macrophages significantly increases the amount of apoptosis.

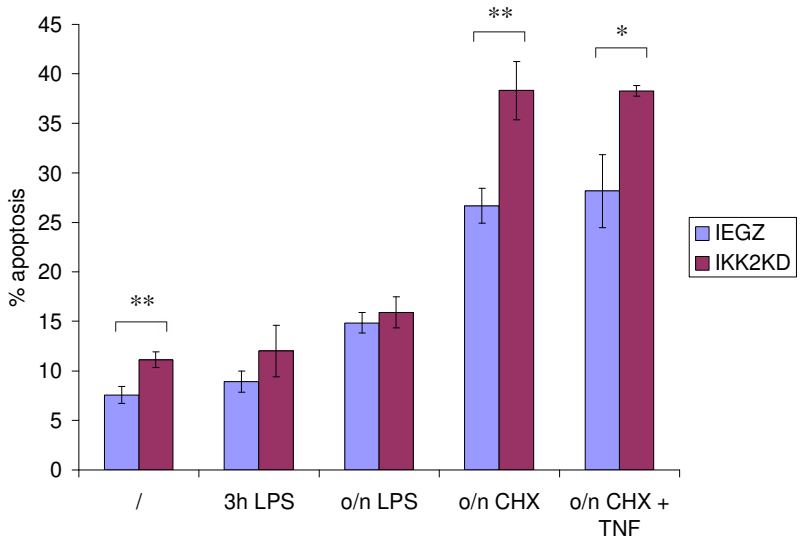

Figure 3.15. Apoptosis in RAW 264.7 cells that stably express a dominant negative mutant of IKK2. RAW 264.7 cells infected with the IKK2 KD construct (IKK2 KD) or the empty pCFG5-IEGZ vector (IEGZ) were stimulated for either 3 hours with LPS (100 ng/ml), overnight with LPS (100 ng/ml), overnight with cycloheximide (CHX) (5 µg/ml) or overnight with cycloheximide (5 µg/ml) together with TNF (100 ng/ml). After the indicated time points the amount of apoptosis was analyzed by measuring the propidium iodide intercalation with FACS. (N=3, **P<0.01, *P<0.05)

3.8 Inhibition of IKK2 in RAW 264.7 cells significantly decreases the uptake of modified LDL

To investigate the effect of IKK2 inhibition on the uptake of modified LDL, the uptake of diI-labeled oxidized LDL and diI-labeled acetylated LDL was analyzed by FACS after incubating the viral infected RAW 264.7 cells for 3 hours with this modified LDL (25 µg/ml).

As shown in figure 3.16, RAW 264.7 cells in which IKK2 is down regulated take up less oxidized and acetylated LDL compared to the control cells. The uptake of oxidized LDL is significantly inhibited for 47 % (P=0.0014) and the uptake of acetylated LDL for 29 % (P=0.0156).

These results indicate that inhibition of IKK2 in macrophages significantly decreases the uptake of modified LDL.

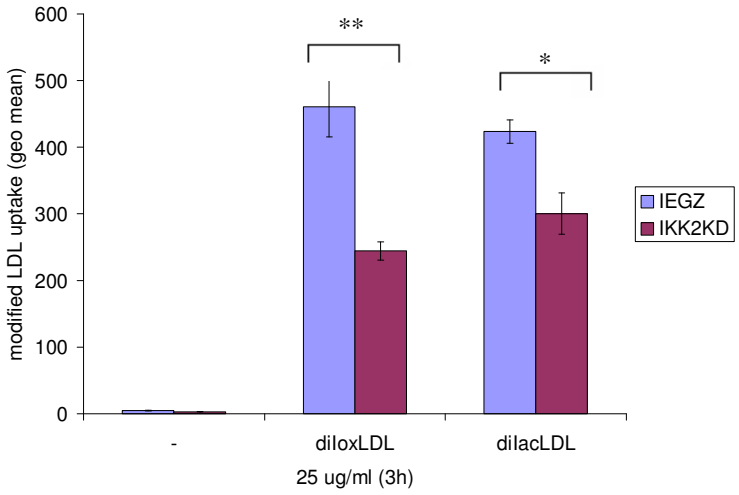

Figure 3.16. Uptake of modified LDL by RAW 264.7 cells that stably express a dominant negative mutant of IKK2. RAW 264.7 cells infected with the IKK2 KD construct (IKK2 KD) or with the empty pCFG5-IEGZ vector (IEGZ) were incubated for 3 hours with 25 µg/ml diI-labeled oxidized LDL or diI-labeled acetylated LDL. RAW 264.7 cells were then analyzed for modified LDL uptake by FACS. (N=3, **P<0.01, *P<0.05).

To validate the above-mentioned results obtained with the viral infected cells, the experiment was repeated on RAW 264.7 cells that were pretreated for 1 hour with the chemical IKK2 inhibitor SC-514 (50 µM) before subsequent incubation for 3 hours with diI-labeled oxidized LDL (25 µg/ml) or diI-labeled acetylated LDL (25 µg/ml).

As shown in figure 3.17, there is a non-statistical significant trend towards a decreased uptake of oxidized and acetylated LDL in RAW 264.7 cells pretreated for 1 hour with 50 µM of the chemical IKK2 inhibitor SC-514 (P>0.05).

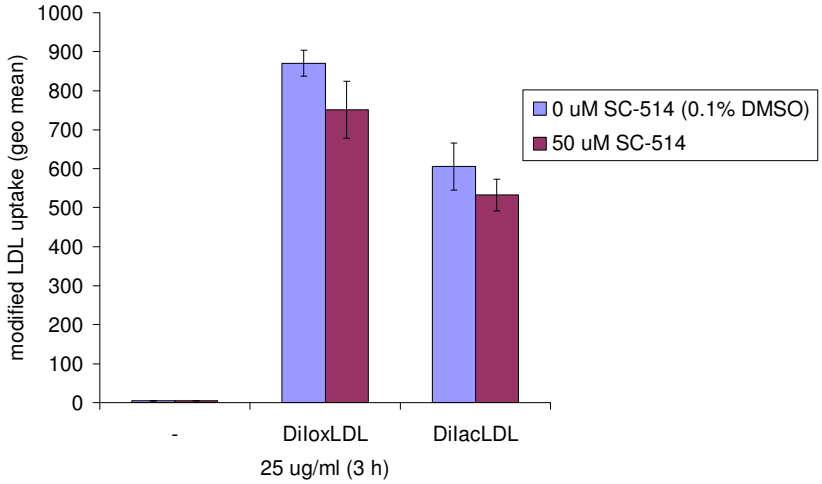

Figure 3.17. Uptake of modified LDL by RAW 264.7 cells pretreated with the chemical IKK2 inhibitor SC-514. RAW 264.7 cells were pretreated for 1 hour with 50 µM of SC-514 or left untreated and were then incubated with 25 µg/ml diI-labeled oxidized LDL or diI-labeled acetylated LDL for 3 hours. RAW 264.7 cells were then analyzed for modified LDL uptake by FACS. (N=3)

3.9 Inhibition of IKK2 in RAW 264.7 cells down-regulates the expression of the scavenger receptors, CD36 and SR-A

To investigate the involvement of the scavenger receptor expression in the down-regulation of modified LDL uptake, the gene expression of CD36 and SR-A was measured after incubating RAW 264.7 cells for 8 hours with oxidized or acetylated LDL.

Although this experiment was only performed once and has to be repeated, the preliminary results showed down-regulation of both CD36 and SR-A in RAW 264.7 cells that stably express a dominant negative mutant of IKK2 (data not shown).

4. Discussion

4.1 General

Atherosclerosis is a chronic inflammatory disease characterized by the accumulation of lipid-laden macrophages in the vessel wall. One of the key regulators of the inflammatory processes involved in atherosclerosis is the transcription factor NF-κB. The phosphorylation of IκB by the IKK complex is essential for the activation of NF-κB. In this complex, IKK2 is the most important catalytic subunit required for NF-κB activation upon proinflammtory stimuli. In addition, macrophages are the most important inflammatory cells in the process of atherosclerosis. They are involved in the uptake of modified lipids from the vessel wall and the regulation of NF-κB in the control of inflammatory processes. These characteristics make the study of NF-κB especially in macrophages very attractive for therapeutic interventions in a chronic lipid driven inflammatory disease like atherosclerosis. Only recently, the role of NF-κB activation specifically in macrophages has been addressed in two studies using mouse models [35,42].

In our study, we further addressed the role of IKK2-mediated NF-κB activation in macrophages by investigating the effect of IKK2 inhibition in macrophages on inflammation, apoptosis and foam cell formation, the three major processes involved in atherosclerosis. Our results provide some interesting observations. First of all, the inhibition of IKK2 reduces the inflammatory response of macrophages, characterized by a decrease in the secretion of the proinflammatory genes TNFα and IL-6. Second, the inhibition of IKK2 in macrophages increases the sensitivity of these cells to stimulus-induced cell death. Third, macrophages take up less modified LDL after functionally inhibiting IKK2, probably by down regulating the two main scavenger receptors, CD36 and SR-A, involved in lipid uptake.

4.2 Tools for functionally inhibiting IKK2

In this study, two methods were used to functionally inhibit IKK2. The first one was a retroviral approach to establish RAW 264.7 cells that stably express a dominant negative mutant of IKK2 (IKK2 KD). A second method used to functionally inhibit IKK2 was the treatment of RAW 264.7 cells with the chemical IKK2 inhibitors SC-514 and SPC-839. Overall, the retroviral approach showed a stronger inhibitory effect compared to the approach with the chemical IKK2 inhibitors.

Concerning the chemical IKK2 inhibitors, in the literature it is stated that the IC_{50} for SC-514 lies between 3 and 12 μM and that the IC_{50} for SPC-839 is localized around 62 nM

[46,47,48]. Because these values are obtained by studying other cell types than used in this study and because not much is mentioned about the incubation time in the literature, we performed our own optimizing experiments. This was done by looking at the NF-κB activity after treatment of RAW 264.7 cells containing a luciferase NF-κB reporter construct for different time points with different concentrations of the IKK2 inhibitors.

A first important result obtained while optimizing the concentration and incubation time of the IKK2 inhibitors is that the DMSO, in which these chemical IKK2 inhibitors are dissolved, has on its own an inhibitory effect on the NF-κB activity. It was seen that DMSO inhibits the NF-κB activity in a concentration-dependent manner. This effect of DMSO was also published in the literature. In a study were they determined the LPS response in murine macrophages after pretreatment with DMSO, it was shown that pretreatment with 1 or 2 % DMSO already reduced the LPS-induced protein levels of TNF between 4- and 20-fold [49]. This indicates that DMSO can modulate the LPS-induced cytokine production in macrophages through an NF-κB-mediated mechanism.

Based on our optimizing experiments, it was concluded that for SC-514 an incubation period of 1 hour with a concentration of 50 μM is most efficient to create an inhibition (55%) of NF-κB activity in RAW 264.7 cells. Longer incubations (24 hours) have no inhibitory effect and lead to unwanted cell death. The chemical IKK2 inhibitor SPC-839, in contrast, has only a minor inhibitory effect after 1 hour of incubation. The optimal condition to create an inhibitory effect (39%) with this IKK2 inhibitor is an incubation period for 24 hours with a concentration of 500 nM. Incubating the cells for 48 hours with 250 nM, 400 nM or 500 nM also inhibits the LPS-induced NF-κB activity. However, this inhibition of NF-κB seen after 48 hours is probably due to increased cell death instead of IKK2 inhibition, because after 48 hours, more than 70 % of the cells died as measured by trypan blue staining. The amount of cell death observed after 24 hours is still in the acceptable range in that the inhibition cannot merely be explained by cell death. Taken together these results indicate that SC-514 is a fast-but short-working chemical IKK2 inhibitor. It has already inhibitory properties after 1 hour of incubation but its inhibitory capacity decreases after 24 hours. SPC-839 on the other hand is a rather slow-but long-working chemical IKK2 inhibitor. It has only a minor effect after a short incubation period, it works at its best after 24 hours of incubation and it still does so after 48 hours. A drawback of both these chemical IKK2 inhibitors is the fact that they have cytotoxic effects after longer incubation periods (24 hours for SC-514 and 48 hours for SPC-839). From this it can be concluded that for short during experiments, SC-514 is the ideal IKK2 inhibitor. For longer during experiments, on the other hand, it is better to use SPC-839. However, none

of the inhibitors we used are useful for experiments that take longer than 24 hours. When the optimal concentrations needed to create a significant inhibition of NF-κB with these IKK2 inhibitors are compared with the ones mentioned in the literature, there are some discrepancies. For SC-514, the concentration that is needed in this study to reach an inhibition of 50% is five times as high as mentioned in the literature. For SPC-839 the needed concentration to reach an inhibition of 50% is even ten times as high as mentioned in the literature. This raises the question whether the inhibitors are still IKK2 specific with the high concentrations we used during our experiments. The answer is yes, because these inhibitors are highly selective for IKK2. For these inhibitors to have an effect on IKK1, the SC-514 concentration should be 20 times as high and for SPC-839 even 1000 times as high compared to the concentrations mentioned in the literature. The reason why we have to use higher concentrations of the inhibitors to reach an equal inhibitory effect as seen in other studies could be that we used another cell type but it can also be due to the fact that in our study the maximum concentration of DMSO was set to 0.1 %. If this was not the case in the experiments mentioned in the literature, they would logically see an inhibitory effect with a lower concentration of the inhibitor. The inhibitory effect in these studies could then be explained by a combined effect of the IKK2 inhibitor together with DMSO and not by a sole effect of the IKK2 inhibitor.

4.3 Inhibition of IKK2 reduces the inflammatory response of macrophages

A first important observation in our study was that the inhibition of IKK2 in macrophages decreases the secretion of the proinflammatory genes TNFα and IL-6. These data indicate that the inhibition of IKK2 reduces the inflammatory response of macrophages. From this experiment, not much could be concluded about the effect of IKK2 inhibition on the production of the anti-inflammatory cytokine IL-10 because stimulation of RAW 264.7 cells for 8 hours with LPS does not induce the production of IL-10. Moreover, it is assumed that NF-κB activation during the onset of inflammation is associated with pro-inflammatory gene expression, whereas NF-κB activation during the resolution of inflammation is associated with anti-inflammatory gene expression [11].

When we investigated the effect of IKK2 inhibition on the production of NO, it was seen that the NO production is inhibited in RAW 264.7 cells that stably express a dominant negative mutant of IKK2, as expected.

In our hypothesis about inflammation it was stated that the inhibition of IKK2 in macrophages diminishes the production of both proinflammatory and anti-inflammatory cytokines. This

hypothesis was based on the fact that LPS stimulation induces the production of proinflammatory and anti-inflammatory cytokines through NF-κB [9]. So, when NF-κB activation is diminished by functionally inhibiting IKK2 it is expected that this would lead to a diminished LPS-induced cytokine production. Although, we still have to investigate the effect of IKK2 inhibition on the production of anti-inflammatory cytokines, our data so far confirm the part of the hypothesis concerning the production of proinflammatory cytokines.

Our data are in line with data from another study were they showed that macrophage-restricted deletion of IKK2 lead to a strong reduction in total TNF production after LPS stimulation [35]. Moreover, the production of IL-6 was also reduced in IKK2 deleted macrophages after LPS stimulation for 3 hours. When they looked at the production of IL-10 after 24 hours of LPS simulation, a very strong and reproducible reduction of this anti-inflammatory cytokine was observed after IKK2 inhibition [35]. This same effect of IKK2 inhibition on inflammatory cytokine production was observed in many other studies. Alveolar macrophages expressing a defective IKK2 showed a reduction in TNFα, IL-6 and IL-8 production [50]. Treatment of human macrophages with a specific IKK2 blocking peptide resulted in a significantly inhibition of IL-1β-induced TNFα production and in a significantly reduction of TNFα-induced IL-6 production compared to untreated cells [51]. Fibroblast-like synoviocytes infected with a dominant negative mutant of IKK2 also showed a lack of IL-6 and IL-8 synthesis after IL-1 stimulation compared to control cells [52].

Taken together, our data and the data from other studies, indicate that IKK2 is essential for NF-κB mediated inflammatory gene expression in macrophages and other cell types.

4.4 Inhibition of IKK2 in macrophages results in increased susceptibility to stimulus-induced cell death

Because NF-κB is also involved in the regulation of apoptosis [37], the effect of IKK2 inhibition in macrophages on cell death was investigated.

First of all, we looked at the amount of cell death induced by several stimuli in the control cells. Because in our experiments we activated our cells predominantly with LPS, the amount of apoptosis after 3 or 24 hours incubation with LPS was investigated. It was seen that the amount of apoptosis in the control cells is only slightly augmented after 3 or 24 hours of LPS-stimulation. Because TNFα is believed to be a more potent inducer of cell death compared to LPS, we also examined the amount of cell death induced by TNFα in our control cells. In contrast to LPS, TNFα directly activates the apoptotic cascade. TNFα binds to the TNFR1 receptor which forms a signaling complex at the plasma membrane by recruiting the adaptor

protein TRADD (TNFR1 associated death domain protein) and the signaling proteins TRAF2, TRAF5 and RIP1. This complex leads to the activation of IKK2 [36]. In a second step, this complex dissociates from TNFR1, which can recruit FADD (Fas associated death domain) and caspase-8, triggering an apoptotic response [36]. Although TNFα is a potent inducer of apoptosis, on its own it is a rather poor inducer of apoptosis. It only triggers programmed cell death when new protein or RNA synthesis are inhibited or in NF-κB-deficient cells. [36]. For these reasons, we incubated some cells with cycloheximide, which is a potent inhibitor of protein synthesis, together with TNFα. As a control, cells were also incubated with cycloheximide alone. We observed a substantial increase in the amount of cell death after cycloheximide incubation. TNFα, however, did not show an additional effect. Normally, we would expect no or only minor cell death with cycloheximide alone. TNFα, on the other hand, is expected to increase the amount of cell death. The reason why we do see a high induction of cell death in our experiment after cycloheximide incubation can be explained by the high amount of cycloheximide we used. Probably, this high concentration is toxic for the cells and induces necrosis instead of apoptosis. The reason why we did not observe an additional effect of TNFα, on the other hand, can be due to the fact that the TNFα concentration we used was not high enough. The main purpose of this experiment however was to determine whether there is a difference in the amount of apoptosis between the IKK2 KD infected cells and the control cells. When the amount of cell death was compared, we observed a slight increase in the basal level of apoptosis and in the amount of apoptosis induced by 3 hours or overnight incubation with LPS in the IKK2 KD infected cells compared to the control cells. Moreover, the amount of cell death in the cells infected with the IKK2 KD construct induced by cycloheximide or cycloheximide together with TNFα was significantly increased compared to the control cells. These data indicate that inhibition of IKK2 results in macrophages that are more sensitive to apoptosis, especially when triggered by cell death-inducing stimuli.

In our hypothesis, we stated that inhibition of IKK2 in macrophages increases the amount of apoptosis. This hypothesis was based on data that show that NF-κB signaling is important for survival under certain circumstances [36,37]. The results on apoptosis obtained in this study confirm the above-mentioned hypothesis.

The data obtained in our study are in line with data from several other studies. In one study, it was shown that macrophage-specific deletion of IKK2 results in increased sensitivity to apoptosis after treatment with different stimuli [35]. Moreover, it was shown that IKK2-deficient mice die at mid-gestation from uncontrolled liver apoptosis, a phenotype remarkably similar to that of mice deficient in both RelA and NF-κB1 subunits of NF-κB [53]. It was also

observed that inhibition of NF-κB by overexpression of a dominant negative IKK2 in the common carotid artery of rats results in a nearly two-fold increase in apoptotic cells in the intima [54]. Moreover, it was shown that hepatocyte-specific deletion of IKK2 reduces the expression of several anti-apoptotic genes and that the administration of LPS substantially induces apoptosis in hepatocyt-specific IKK2 deleted cells [55].

Taken together, our data and the data from other studies, indicate that IKK2 is essential for evading apoptosis in all kinds of cell types upon several stimuli.

4.5 Inhibition of IKK2 reduces modified lipid uptake by macrophages

Because the effect of NF-κB activation in macrophages during foam cell formation is largely unknown, we studied the effects of IKK2 inhibition in macrophages on the uptake of modified LDL and on the gene expression of the scavenger receptors CD36 and SR-A.

A first important observation in our study was that macrophages expressing a dominant negative mutant of IKK2 take up less oxidized and acetylated LDL compared to the control cells. The same was seen in RAW 264.7 cells that were treated with the chemical IKK2 inhibitor SC-514. When we then investigated the involvement of the scavenger receptor expression in this decreased modified LDL uptake, the preliminary results indicate that the gene expression of CD36 and SR-A is down regulated after functionally inhibiting IKK2. Our data indicate that IKK2 is involved in the uptake of modified LDL by macrophages, probably through up-regulation of the scavenger receptors CD36 and SR-A.

It was hypothesized that the inhibition of IKK2 in macrophages decreases the process of foam cell formation by diminishing the lipid influx through down-regulation of the scavenger receptors CD36 and SR-A and by increasing the lipid efflux through up-regulation of ABCA1 and ABCG1. Although, we still have to investigate the effect of IKK2 inhibition on the lipid efflux by looking at the gene expression of ABCA1 and ABCG1, our data so far, confirm the part of the hypothesis concerning lipid uptake.

The role of NF-κB in the scavenging of modified lipids or foam cell formation has not been studied extensively and not much is known about whether the cholesterol efflux mechanisms are also affected by NF-κB activation. The little data that are known on this subject are somewhat contradictive.

Considering the uptake of modified LDL by the two main scavenger receptors the following data were found. In one study it was shown that deletion of IKK2 in bone marrow-derived macrophages does not affect the uptake of modified LDL [35]. This was also observed in bone marrow-derived macrophages lacking NF-κB1. However, on stimulation of the NF-κB

pathway with LPS, uptake was severely reduced in these macrophages because of down-regulation of SR-A [42]. However, no difference in CD36 expression was observed. The data of the second study indicate that NF-κB is involved in lipid uptake by increasing the expression of SR-A, but only in activated macrophages. In our study, on the other hand, we were able to show a decreased lipid uptake in non-activated macrophages after IKK2 deletion, probably due to a decrease in the expression of both CD36 and SR-A. The reason why we could observe an effect without first stimulating the cells with LPS can be explained by the fact that we used a cell line in contrast to the primary cells used in the other studies. The RAW 264.7 cells we used are more macrophage like (more activated), the bone marrow-derived macrophages in the other study are more monocyte like (less activated). In another study, it was seen that the oxLDL-induced NF-κB activation is attenuated in monocyt-derived macrophages from patients with CD36 deficiency compared to controls [56]. In addition macrophages from CD36-deficient take up less oxidized LDL [57]. Moreover, it was shown that AGEs (advanced glycation end products) increase both the expression of the major oxLDL receptor CD36 and the transcriptional activity of NF-κB [58]. This could indicate that the AGEs-induced enhancement of NF-κB might be involved in the increased expression of CD36. The above-mentioned studies deliver some evidence that the transcription factor NF-κB is necessary for the induction of both CD36 and SR-A upon stimulation with different stimuli and subsequent modified LDL uptake.

Concerning the expression of the transporter ABCA1 that is involved in the active efflux of lipids, there are some contradictive elements. One study shows that LPS down regulates the mRNA expression of ABCA1. This LPS-induced repression of ABCA1 was efficiently reversed by NF-κB inhibitors, suggesting a mechanism involving NF-κB [59]. In another study, on the other hand, it was shown that TNFα induces the expression of ABCA1 and that deletion of IKK2 in macrophages reduces this TNFα-induced ABCA1 expression [22]. These data indicate that NF-κB is also involved in the regulation of ABCA1. Whether NF-κB activation causes an up- or down-regulation of ABCA1 depends on the stimulus by which the transcription factor is activated.

Taken together, our data and the little data obtained from other studies, indicate that the scavenging of modified LDL by macrophages might be dependent on the activity of the transcription factor NF-κB.

4.6 Conclusions and further recommendations

As an overall conclusion, based on the results obtained in this study, it can be stated that IKK2 is an important protein involved in the regulation of many processes in macrophages. First of all, it can be concluded that IKK2 is essential for NF-κB mediated inflammatory gene expression in macrophages as seen by a decrease in the LPS-induced TNFα and IL-6 expression after functionally inhibiting IKK2. Second, it can be concluded that IKK2 is essential for evading apoptosis upon several stimuli because it was seen that inhibition of IKK2 in macrophages makes these cells more sensitive to stimulus-induced apoptosis. A third thing that can be concluded from the results obtained in this study, is that IKK2 is involved in the uptake of modified LDL by macrophages, probably by regulation of the scavenger receptors CD36 and SR-A.

For further experiments, it would certainly be interesting to investigate the effect of IKK2 inhibition on the LPS-induced expression of the anti-inflammatory cytokine IL-10 in macrophages. To investigate this, the RAW 264.7 cells have to be stimulated with LPS for at least 24 hours to induce the expression of IL-10 because it is assumed that NF-κB activation during the onset of inflammation is associated with pro-inflammatory gene expression, whereas NF-κB activation during the resolution of inflammation is associated with anti-inflammatory gene expression. It will also be interesting to take a closer look at the effect of IKK2 inhibition on the expression of several NF-κB regulated inflammatory genes upon stimulation with other stimuli than LPS like for example TNF or oxLDL.

Concerning the process of foam cell formation, it would be interesting to investigate the accumulation of cholesterol esters in RAW 264.7 cells by the use of HPTLC. The effect of IKK2 inhibition in RAW 264.7 cells on the efflux of lipids is also largely unknown. Because the balance in expression between the scavenger receptors that mediate the uptake of lipids and the transporters that are in charge for the active efflux of lipids determine the net accumulation of lipids, it would be interesting to determine whether NF-κB only has an effect on uptake or also on efflux. This can be done by investigating the effect of IKK2 inhibition on the gene expression of the two main transporters that mediate the active efflux of modified LDL, ABCA1 and ABCG1, maybe in combination with a radioactive cholesterol efflux assay. In this way, we can gain some more knowledge about the involvement of NF-κB in the formation of foam cells. Overall, still a lot of work has to been done to completely understand the function of NF-κB in atherosclerosis. For future experiments it is most interesting to focus on the involvement of NF-κB in the process of lipid accumulation in macrophages.

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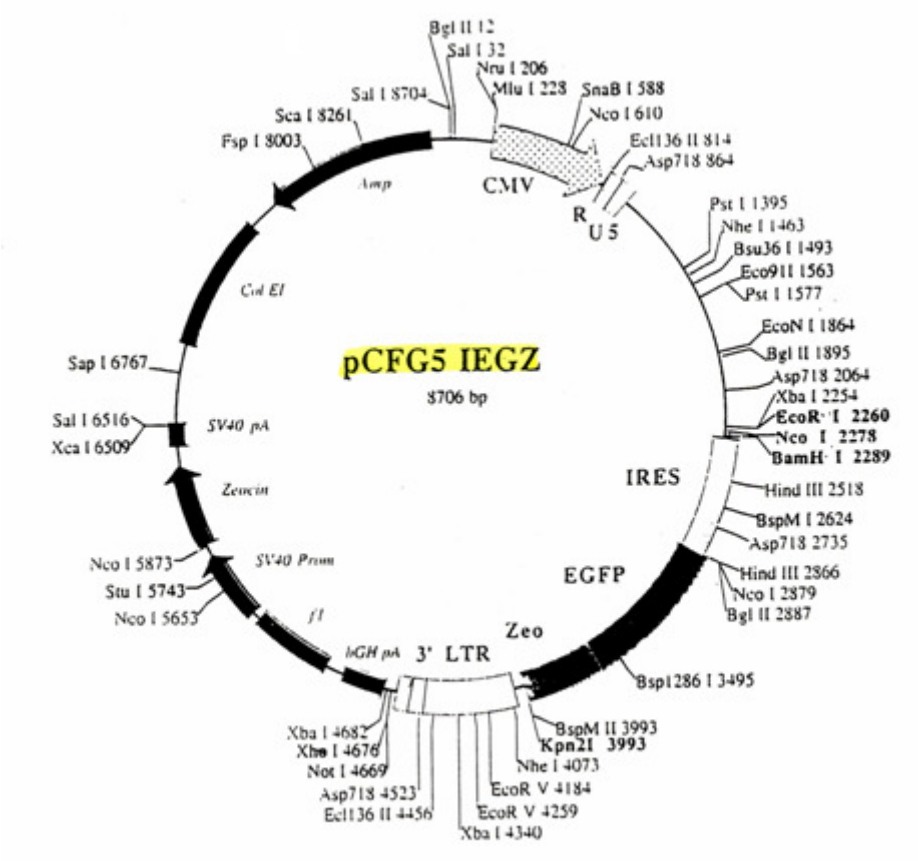
Appendix 1



pCFG5-IEGZ retroviral vector used for infection of RAW 264.7 cells. This vector contains a polylinker upstream of an internal ribosomal entry site (IRES) driving an eGFP zeocin fusion protein cassette. This allows us to distinguish between infected and noninfected cells by fluorescence microscopy.

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