The role of TAK1 in NF$_k$B signaling in macrophages

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II Abbreviations

ATF-2	Activating Transcription Factor-2
BMM	Bone Marrow derived Macrophage
BMP	Bone Morphogenetic Protein
CD40L	CD40 ligand
cDNA	complementary DNA
DEPC	DiEthyl PyroCarbonate
DNA	DeoxyriboNucleic Acid
dsRNA	double stranded RNA
EDTA	EthyleneDiamineTetracetic Acid
GAPdH	glyceraldehyde 3-phosphate dehydrogenase
HSP	Heat-Shock Protein
iFCS	(heat) inactivated Fetal Calf Serum
IKK	IκB kinase complex (consisting of IKKα, IKKβ and NEMO(IKKγ))
IκB	Inhibitor of NFκB
IL	Interleukin
JNK	C-jun N-terminal kinase
LDL	Low Density Lipoproteins
LPS	LipoPolySaccharide
MAPK	Mitogen-Activated Protein Kinase
MAPKK	Mitogen-Activated Protein Kinase Kinase
MAPKKK	Mitogen-Activated Protein Kinase Kinase Kinase
MAP3K7	Mitogen-Activated Protein Kinase Kinase Kinase 7 (TAK1)
miRNA	micro RNA
MKK	MAPKK
MKKK	MAPKKK
MBP	morphogenetic protein
MyD88	Myeloid Differentiation primary response gene 88
NFκB	Nuclear Factor kappa B
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
qPCR	Quantitative real time PCR Polymerase Chain Reaction
RIP	Receptor-Interacting Protein

RISC	RNA-induced silencing complex
RNA	RiboNucleic Acid
RNAi	RNA interference
RNase	RiboNuclease
s*iRNA*	small interfering RNA
TAB	TAK1-Binding protein
TAK1	Transforming growth factor β-Activated Kinase 1
TGF-β	Transforming growth factor-β
TIR domain	Toll–interleukin 1 receptor domain
TNF	Tumor Necrosis Factor
TNFR1	TNF receptor 1
TLR	Toll Like Receptor
TRADD	TNF receptor death domain
TRAF2	TNF receptor associated factor 2
UBD	Ub-Binding Domains
Ub	Ubiquitin
UTR	UnTranslated Region

III Preface

For six months I was a member of the Atherosclerosis group of the department molecular genetics in Maastricht. Here, I investigated the role of TAK1 in NFκB in signaling in macrophages which resulted in this paper. For me it would be impossible to complete this project without the help of some people, so I want to take this opportunity to say a word of thanks to these people.

First of all, I want to thank Daniëlle, my promoter, for giving me the chance to work on this interesting project. Her guidance and remarks were really helpful and taught me a lot. I really appreciated her trust in me that I would bring the task to a good end and giving me the responsibility to do a lot of independent work. I also would like to thank her for her fast and critical correction of this paper.

Next, I would like to thank Menno, my co-promoter, for accepting me in his group and reading my paper.

Further, I would like to thank al my co-workers of the Atherosclerosis group for the pleasant working environment. I am especially Monique very grateful for teaching me several techniques. She was very thorough in her explanation and always prepared to lend me a hand. Also, I would like to thank Inge for her help in the laboratory. Despite her own busy schedule she was always prepared to help me with a smile on her face.

Last but not least, I want to thank my family and in particular my parents for giving me the opportunity to attend college and participate in a study which I really liked. I also thank my girlfriend, she was always interested in my project and supported me throughout the entire period.

IV Abstract (EN)

Macrophages are white blood cells that play an important role in the immune system. They are characterized by their ability to phagocytize pathogens and other foreign material. Macrophages are recruited to infected tissues, where they produce immunological substances like cytokines and transcription factors to introduce an inflammatory response. One of the pathways by which macrophages activate the transcription of cytokines is the NFκB signaling pathway. The NFκB signaling pathway can be activated by several external stimuli, like LPS. Transforming growth factor activated kinase 1, TAK1, is one of the proteins involved in this pathway and regulates the activation of NFκB by activating the IKK complex. Activation of this complex leads to degradation of the IκB, which inhibits NFκB from going into the nucleus. The freed NFκB can now translocate to the nucleus for transcription of several genes like IL10, TNF-α, IL6 and IL12. These have all an effect on the inflammatory response. A second pathway that is influenced by TAK1, also known as MAP3K7, is the MAP kinase pathway and in particular the JNK and p38 kinase pathway. Activation of these pathways show pro-inflammatory effects.

In this project the emphasis lies on the role of TAK1 in NFκB signaling in bone marrow derived macrophages (BMMs). There are several methods for investigating gene expression: overexpression, knockout and silencing by RNA interference of the gene. Because TAK1 knockout is embryonic lethal, RNAi will be used in this project for knocking down the TAK1 gene. During this technique double stranded RNA is inserted into cells and is cleaved by the enzyme Dicer into small interfering RNA, siRNA. This siRNA can degrade the mRNA from the gene of interest.

The first goal of this project was to set up and optimize a technique for introducing TAK1 knockdown in BMMs by siRNA. In search of the optimal protocol for performing this technique, the different stadia of the RNAi protocol were observed and optimized. Transfection efficiency, the amount of siRNA added, the volume of transfection reagens used, the incubation period, are all factors influencing the RNAi protocol. This part lead to a protocol which is able to introduce a 35% knockdown of TAK1, but further optimization has still to occur. The second goal was to determine if the created knockdown had actually a biological effect. The applied knockdown seemed to introduce a more than 55% reduction in IL10 and around 30% reduction for TNF-α. IL6 and IL12 remained unchanged, but a biological effect has already been shown. With the developed technique the role of TAK1 in the NFκB signaling in BMMs can be further investigated.

V Abstract (NL)

Macrofagen zijn witte bloedcellen die een belangrijke rol vervullen in het immuunsysteem. Een van hun kenmerken is het fagocyteren van pathogenen en lichaamsvreemde stoffen. Wanneer er een infectie optreedt, worden ze aangetrokken naar het geïnfecteerde gebied, waar ze een ontstekingsreactie opwekken. Dit door allerlei stoffen, zoals cytokines en transcriptiefactoren te produceren. Een van de manieren waarop ze dit doen is via de NFκB pathway. Deze kan geactiveerd worden door verschillende extracellulaire stimuli, zoals LPS. TAK1, transforming growth factor activated kinase 1, is een van de eiwitten die betrokken is in de NFκB activatie pathway. Het activeert het IKK complex, dat op zijn beurt IκB activeert. IκB zal hierdoor gedegradeerd worden en het NFκB, dat geremd wordt door IκB, zal vrijkomen en de celkern ingaan. Hier zorgt het voor de transcriptie van onder andere IL10, TNF-α, IL6 en IL12, allemaal cytokines die een effect hebben op de ontstekingsreactie. Verder speelt TAK1, ook wel bekend als MAP3K7, een rol in de MAP kinase pathway en dan vooral in de JNK en p38 cascade. Activatie van deze cascades leidt tot een pro-inflammatoire response.

In dit project ligt de nadruk op de rol van TAK1 in the NFκB pathway in beenmerg macrophagen. Voor het onderzoeken van gen expressie zijn er verschillende technieken; knockout, overexpressie of knockdown door RNAi van een gen. TAK1 knockout leidt tot embryonale dood, daarom wordt in dit project RNA interference toegepast om TAK1 knockdown te creëren. Tijdens deze techniek wordt dubbelstrengs RNA in cellen gebracht, tot kleine stukjes geknipt(small interfering RNA siRNA), die kunnen leiden tot mRNA afbraak van het gewenste gen.

Het grootste deel van deze studie was het ontwikkelen en optimaliseren van een techniek: Het introduceren van TAK1 knockdown in primaire beenmerg macrofagen, met behulp van siRNA. Tijdens de optimalisatie werden alle variabele stappen van het RNAi protocol bekeken en waar nodig aangepast. Dit deel heeft geleid tot een protocol waarmee ongeveer 35% knockdown gecreëerd kon worden, echter dit protocol zal nog verder geoptimaliseerd dienen te worden. Het tweede deel van dit project was het bepalen of de gecreëerde knockdown ook daadwerkelijk een biologisch effect teweeg brengt. De 35% knockdown heeft tot een 55% vermindering van in IL10 en een 30% afname van TNF-α geleidt. De cytokines IL6 en IL12 lieten geen effect zien, maar een biologisch effect was reeds geconstateerd. Met de ontwikkeling van deze techniek kan de rol van TAK1 in de NFκB signalering in de toekomst verder onderzocht worden.

1. Introduction

The immune system protects organisms from infection by pathogens. It consists of two separated systems; the innate, non-specific immune system and the adaptive, specific immune system. These systems bear a great range of specialized cells. One of the cells that play an important role in both the adaptive and innate immune system is the macrophage.

1.1 Macrophages and the immune system

Macrophages are differentiated from specific white blood cells: the monocytes. These blood cells are derived from hematopoietic stem cells of the bone marrow and circulate through the organisms' blood and tissues. They play a role in the immune system by phagocytation intruded pathogens and cellular debris [1]. They can also act as antigen presenting cells to activate lymphocytes and start a T-cell response. Macrophages are recruited to affected tissues where they can produce immunological substances like cytokines and other transcription factors to introduce an inflammatory response. One of the key regulators of inflammation is nuclear factor kappa B (NFκB) which is regulated by the NFκB signaling pathway [2].

1.2 NFκB

Nuclear factor kappa B (NFκB) is a transcription factor which plays an important role in several processes, like the regulation of inflammatory and immune genes, apoptosis and cell proliferation [3].

1.2.1 The transcription factor NFκB

NFκB is in mammals a family name for the transcription factor consisting of 5 members: NFκB1(p50), NFκB2(p52), RelA(p65), RelB ad c-Rel. which can form a complex of either homo- or heterodimers. All five dimers share a Rel homology domain in their N-terminal, which mediates DNA binding, dimerization and interaction with inhibitor proteins. RelA, RelB and c-Rel contain next to the mentioned domain also a transcriptional activation domain which is present in their C-terminal domain [2]. This dimer is the most abundant one and most of the time, when referring to NFκB, p65/p50 is meant.

1.2.2 NFκB regulation/activation

Normally, the p65/p50 dimer is found in the cytosol, bound non-covalently to IκB, which is known as an inhibitor protein of this transcription factor [4]. In the normal condition NFκB can be activated by extracellular stimuli including immunological regulators, growth factors, stress, viral and bacterial products, like LPS. These stimuli can affect two activation pathways: the non-canonical activation pathway and the canonical activation pathway. Both activation pathways are well studied, but most of the information known about NFκB-regulation is based on the latter one. The non-canonical activation pathway, also referred to as the alternative activation pathway, is mediated through IκB kinase-α (IKKα). Activation by the non-canonical pathway results in the processing of p100, the precursor for p52 by IKKα. Eventually, p52 will form a dimer with RelB and translocate to the nucleus for transcription [2]. The canonical activation pathway, also referred to as the classical activation pathway, mediates NFκB through an IKK complex. The IKK complex consists of three units: IKKα, IKKβ and NEMO (IKKγ). When the IKK complex becomes activated by binding of an extracellular stimulus as mentioned above, it phosphorylates two conserved serines in the N-terminal domain of the IκBs. By doing so the IκBs are marked for ubiquitination, phosporylation and subsequent proteasomal degradation. After degradation, the p65/p50 complex detaches and can translocate to the nucleus where it can bind to specific promoter regions in the DNA, thereby regulating/inducing the transcription of several genes. Upon activation, NFκB can mediate the induction of more than 160 genes [6]. NFκB activates a variety of target genes relevant to the pathophysiology of the vessel wall, including cytokines, chemokines, and leukocyte adhesion molecules, as well as genes that regulate cell proliferation and mediate cell survival [3,6]. Some of the cytokines that are under regulation of the NFκB pathway are the pro-inflammatory cytokines TNFα, IL6 and IL12 and the anti-inflammatory cytokine IL10.

For NFκB activation there can be 3 major routes distinguished (figure 1.1). The different pathways start from different receptors in the cell membrane of the macrophages. By observing these receptors some classifications can be made. A first route is activated by binding of tumor necrosis factor (TNF) to the TNF receptor 1 (TNFR1) at the extracellular surface of the cell membrane. By binding to the receptor, TNF induces the recruitment of a large number of adaptor proteins that ultimately lead the activation of the IKK complex. First, TNF receptor associated factor 2 and 5 (TRAF2, TRAF5), NEMO and receptor-interacting protein (RIP) will become activated. Next, a protein called TNF receptor death domain (TRADD) is recruited and binds to TRAF2. TRAF2 has an NFκB-inducible kinase (NIK)

recruited [7]. When NIK forms a complex with MEKK1, a MAP kinase kinase, they can phosphorylate both the IKKα and the IKKβ. These two kinases can both independently phosphorylate IκB which will than be degradated and lead to the release of NFκB into the cytosol. NFκB will translocate to the nucleus and start gene transcription [8,9].

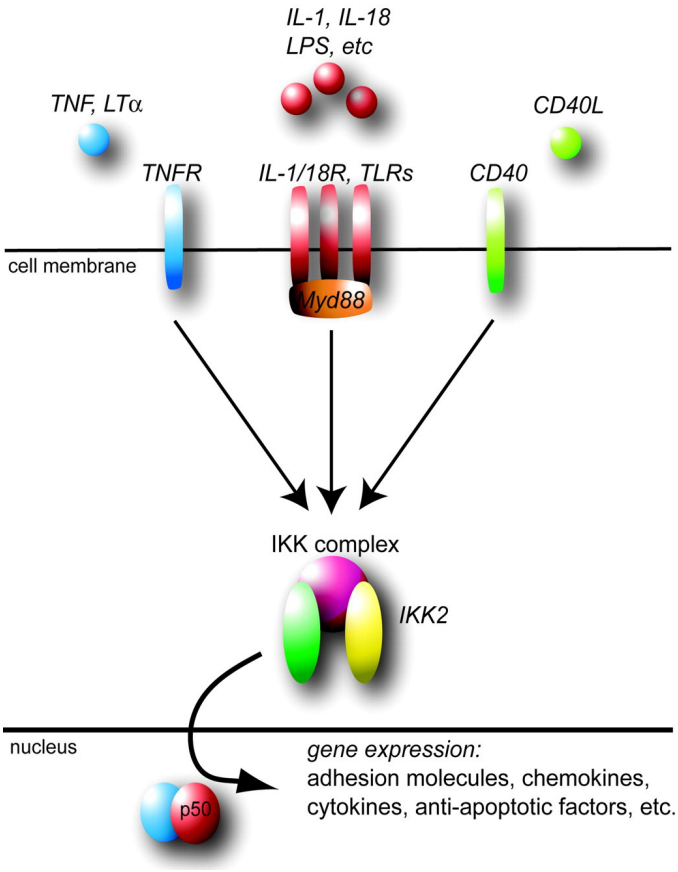

Figure 1.1: Schematic representation of the 3 major NF-κB signaling pathways. TNF, IL-1/IL-18/TLR, and CD40 signaling result in the activation of the IKK complex. IL-1/IL-18/TLR signaling is mediated through the adapter molecule Myd88. (adapted from DeWinther MPJ et al. 2005)

A second pathway for inducing NFκB activation involves a variety of toll like receptors (TLRs), especially TLR2 and TLR4, and IL1 and IL18 receptors. These receptors can be the target of several extracellular stimuli like peptidoglycan, chlamydia, viruses and lipopolysaccharide (LPS), which is present on the outer cell membrane of gram-negative bacteria [10]. Next to these exogenous stimuli there are also some endogenous ones like IL1, IL18, modified low density lipoproteins (LDL) and heat-shock proteins (HSP) for example HSP60. After activation by one of these substances the signal transduction pathways downstream these TLRs becomes activated. All TLRs have a common cytoplasmic signaling

domain, the Toll-interleukin 1 receptor domain (TIR domain). Eventually, this leads to the transcription of the genes targeted by the NFκB transcription factor [1].
A third pathway for inducing NFκB activation is by the CD40 receptor. This receptor is a member of the TNF receptor family and is activated by its ligand (CD40L) [11]. This activation pathway of NFκB signals through both the canonical and the non-canonical pathway.

1.3 TAK1

During the regulation of NFκB activation numerous proteins are involved. One of these proteins is transforming growth factor β-activated kinase 1 (TAK1). TAK1, also known as mitogen-activated protein kinase kinase kinase 7 (MAP3K7), is a serine/threonine kinase. TAK1 messenger RNA consists of 1.5 kb and the full length TAK1 cDNA encodes a protein of 579 amino acids [12]. It is present in most tissues and particular abundant in the thymus and the brain. From the TAK1 gene are four splice variants identified in humans, TAK1a till TAK1d. TAK1a has the highest homology with the gene encoding for TAK1 in the mouse (99%) and most research focused only on this variant. It has shown to be a mediator of TGF-β, bone morphogenetic protein (BMP) and is also involved in pro-inflammatory signaling pathways [12-16]. This inflammatory effect can be explained by the following pathways: the (mitogen activated protein) MAP kinase and the NFκB signaling pathway. The kinase activity from TAK1 is regulated by specific TAK1-binding proteins (TABs). These TABs consists of three members: TAB1, TAB2 and TAB3. It is shown that TAB1 binds to the NH_2-terminal catalytic domain of TAK1. TAB2 and TAB3 have shown to interact with the COOH-terminal region of TAK1 and function as adaptor proteins [17]. They harbor ubiquitin binding domains (UBD) and interact with the TAK1 protein. Before becoming active the UBDs on TAB2/TAB3 have to interact with ubiquitin.

1.3.1 TAK1 in the MAP kinase signaling

Figure 1.2 shows when the TAK1/TABs complex becomes activated it plays a role in MAP kinase pathways, p38 and C-Jun N-terminal kinases (JNK), and the NFκB signaling pathway.

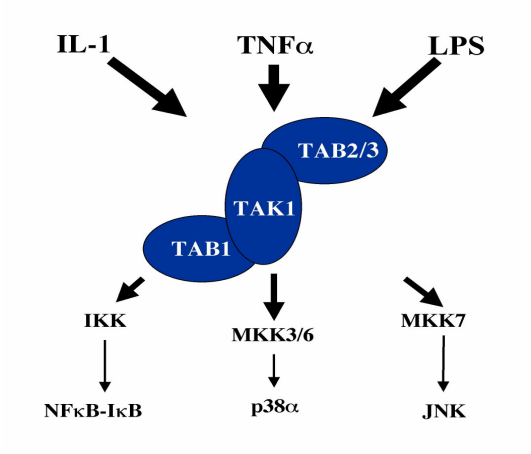

Figure 1.2: TAK1 forms a complex with the three TAB proteins after activation by one of the stimuli. This complex participates in several pathways including the JNK/p38-pathway and the IKK-NFκB signaling pathway. (adapted from)

During the JNK pathway TAK1, a MAPKKK, phosphorylates and activates MKK4 and MKK7, which in turn phosphorylate and activate JNK. In the p38 pathway MKK3 and MKK6 are phosphorylated by TAK1. JNK and p38 activation leads to the transcription of Elk-1, c-jun and ATF-2 (figure 1.3). These play an important role in the inflammatory response through their ability to contribute to the transcription of inflammatory cytokines [18].

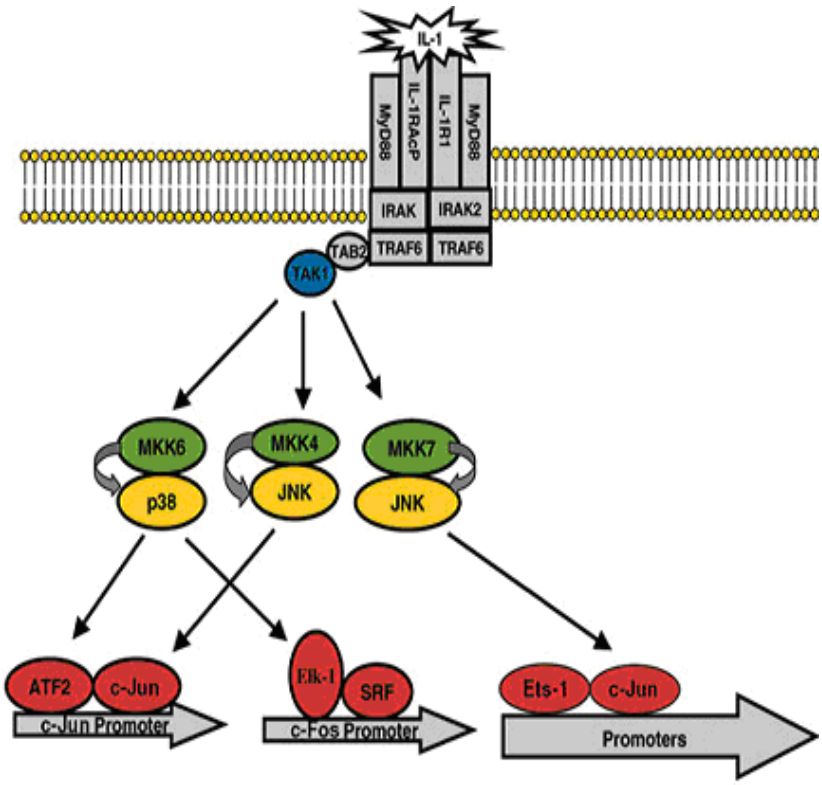

Figure 1.3: Activation of mitogen-activated protein kinase pathways by IL-1. Stimulation by IL-1 activates the MAPK kinase kinases, transforming-growth-factor-β-activated kinase-1 (TAK 1), which then phosphorylate and activate several MAPK kinases: MKK6, MKK4, MKK7. These MAPKKs in turn phosphorylate and activate the MAPKs, p38 and c-Jun N-terminal kinase (JNK), which translocate to the nucleus. There, these MAPKs phosphorylate and activate the transcription factors (activating transcription factor 2 (ATF2), c-Jun, Elk-1. (adapted from Vincenti et al. 2002)

1.3.2 TAK1 in the NFκB signaling

Figure 1.2 shows also a role for TAK1 in the NFκB activation pathway. When the TABs are activated by the TNFR1, they recruit TAK1 to TRAF2 and TRAF5. After the activation, oligomerization occurs of these TRAF proteins. Next to this oligomerization, polyubiquitination occurs of several proteins: receptor-interacting protein (RIP), NEMO and TRAF5, leading to their activation. These proteins play a role in the following cascade, which eventually leads to NFκB activation (figure 1.4) as already mentioned. Activation of this pathway by TLRs or IL1Rs leads to ubiquitin ligase activities of TRAF6, which recruits TAK1 and leads to NFκB activation. In this project the emphasis lies on the role of TAK1 in the NFκB signaling.

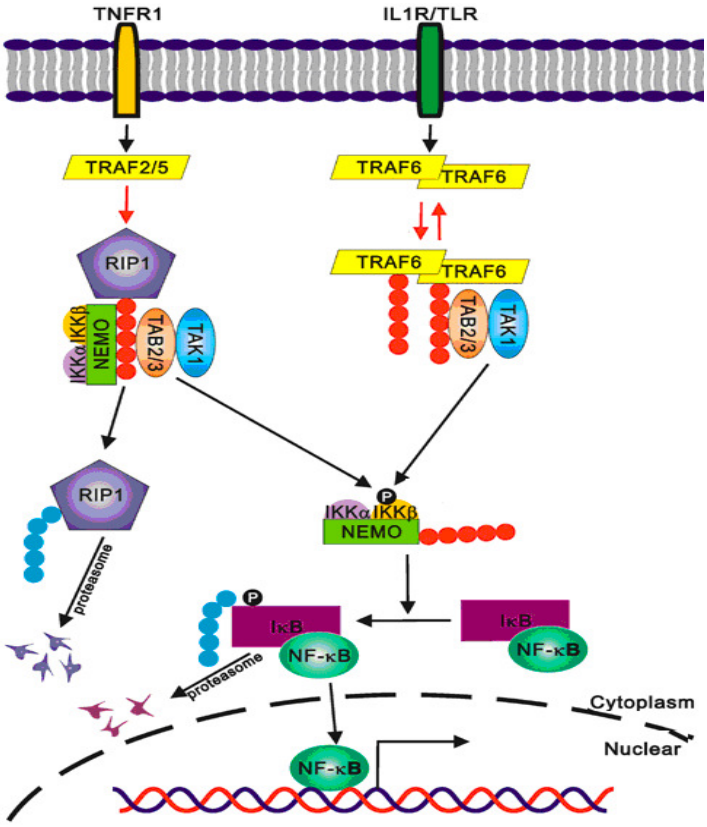

Figure 1.4: Stimulation of TNFR1 leads to the activation of ubiquitin ligase activities of TRAF2 and TRAF5, which then promotes activation of RIP1. RIP1 recruits the TAK1 kinase complex through the interaction between TAB2 and TAB3. The polyubiquitin chains also recruit IKK by binding to NEMO, thus allowing TAK1 to phosphorylate and activate IKK. IKK phosphorylates IκB and targets this inhibitor for degradation by the proteasome, allowing NFκB to enter the nucleus. Now it can turn on downstream target genes, which include mediators of immune and inflammatory responses. The IL1R and TLR signalling pathways activate IKK through TRAF6. The polyubiquitinated TRAF6 recruits TAK1 and IKK complexes to mediate the activation of these kinases. (adapted from Adhikari A et al. 2007)

1.3.3 Studying TAK1 expression

TAK1 seems to play a role in several chronic inflammatory diseases, for example rheumatoid arthritis [19]. It could be a potential target for therapeutic treatment and is therefore an interesting gene to look at. Studying gene expression can be performed with several techniques. One of the possibilities to observe gene expression is to create an overexpression of this particular gene [20,21]. A second possibility was performed by Akira et al. who investigated the expression of TAK1 using a conditional knockout [22]. A third technique, knocking out TAK1 was also done in mice, but lead unfortunately to embryonic death [23]. A last technique that is often used nowadays is gene silencing by RNA interference.

1.4 RNA interference

In 1998 Andrew Fire and Craig Mello discovered in a series of experiments in *Caenorhabditis elegans*, a free-living nematode (roundworm), that injection of sense or antisense RNAs led to almost no decrease of target RNA, whereas introduction of double-stranded RNA (dsRNA) resulted in effective and specific degradation of cytoplasmic mRNA. Furthermore, these silencing effects of dsRNA in *C elegans* were systemic and heritable. The sequence-specific gene silencing capacity of dsRNA is now known as RNA interference (RNAi) [25, 26].

RNAi is an intracellular mechanism for post-transcriptional gene silencing that most probably functions in the regulation of gene expression and defense against transposable DNA elements and viruses. Two types of small noncoding dsRNA can serve as effector molecules and trigger RNAi: small interfering RNA (siRNA) and micro RNA (miRNA) [27]. Long dsRNAs, introduced into the cell or generated as intermediates during viral infections are processed in the cytoplasm by a ribonuclease (RNase) III-like enzyme called Dicer. Only the antisense strand of the mature siRNA and/or miRNAs can be used in the active ribonucleoprotein effector complex: RNA-induced silencing complex (RISC) [28]. The antisense strand acts as a guide to target the homologous mRNA. SiRNAs are fully complementary and induce the cleavage and degradation of their target mRNA transcript. In contrast, miRNAs usually bind only with partial complementarity to the 3′ untranslated region (UTR) of their cognate mRNAs and lead to translational inhibition. Depending on the cell context and the degree of complementarity between the small RNA effector and the mRNA target, both siRNA and miRNA can induce posttranscriptional gene silencing through degradation or translational inhibition (figure 1.5).

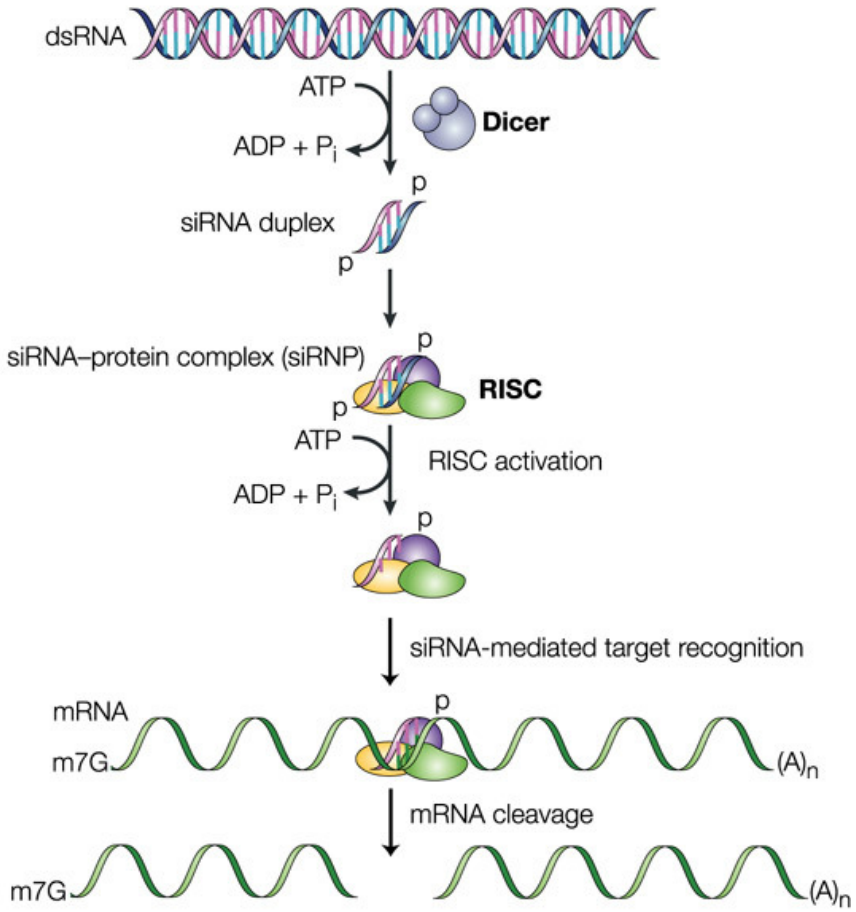

Figure 1.5: Long double-stranded RNA (dsRNA) is cleaved by the enzyme Dicer, into small interfering RNAs (siRNAs). These siRNAs are then incorporated into the RNA-induced silencing complex (RISC). Although the uptake of siRNAs by RISC is independent of ATP, the unwinding of the siRNA duplex requires ATP. Once unwound, the single-stranded antisense strand guides RISC to messenger RNA that has a complementary sequence, which results in the endonucleolytic cleavage of the target mRNA. (Adapted from Pulverer B et al. 2003)

The two common approaches for dsRNA delivery into the cells are lipid-mediated transfection and viral-mediated transduction. Determining which one of these approaches to use depends on the cell type being studied and whether transient or stable knockdown is desired. Transient transfection of unmodified or modified siRNAs, mediated by cationic lipids is most often used. This works in the following way. Positively charged lipids are formulated in aqueous solution with a neutral co-lipid to form unilamellar and multilamellar liposomes of about 100 to 400 nm in diameter. The positively charged cationic lipid reagents interact spontaneously with the negatively charged backbone of the added DNA or RNA to form complexes. These complexes interact with the negatively charged cell membrane, resulting in the delivery of the macromolecule into the cell. Once inside the cell, the DNA is transported

to the nucleus where it is transcribed [29]. For cell types not amenable to lipid-mediated transfection, viral vectors are often employed. Adenoviral vectors work well for transient delivery in many cell types; however, for some difficult cell lines, such as non-dividing cells and for stable RNAi expression, lentiviral vectors are the best delivery method [30].

1.5 Aims and objectives of the project

As mentioned, macrophages play several important roles during the immune response in organisms. One of these roles is the involvement of these cells in the inflammatory process. The NFκB pathway shows to play a great role in inflammation by the regulation of several inflammatory signals, like the pro-inflammatory cytokines TNFα, IL6 and IL12 and the anti-inflammatory cytokine IL-10. One of the proteins that play a role in the NFκB signaling pathway is TAK1. In this project the role of TAK1 in this signaling was investigated. Since the TAK1 mouse is embryonic lethal we chose to develop a different approach, using RNAi. Therefore, we aimed to develop RNAi for TAK1 in bone marrow derived macrophages (BMMs) and when succeeded determining the effects from this knockdown on the proteins that are regulated by activation of the NFκB signaling pathways. So the aims of this research were to;

1. **Set up and optimize TAK1 RNAi in BMMs**
2. **Determine the effects of TAK1 knockdown on the NFκB signaling pathways in BMMs**

During this project these aims were investigated according several approaches. First, optimal RNAi conditions were determined based on fluorescence microscopy. Transfection efficiency was analyzed using fluorescently labeled siRNA. Second, optimal gene silencing by the RNAi was determined based on qPCR. This was first done for lamin A, and later on for the gene of interest TAK1. During this set up and optimization of the technique, several variations of the standard protocol were performed. Finally, the biological effects of TAK1 knockdown were determined. These effects were observed after LPS stimulation of the BMMs and were also based on qPCR.

Thus, after completing this project an optimized protocol for RNAi in BMMs should be obtained. With this protocol it would than be possible to study the effect of TAK1 knockdown in signaling pathways in BMMs. A higher understanding in the role of this protein could lead to (a) potential target(s) for therapeutic treatment of several inflammatory diseases.

2. Materials and Methods

2.1 Bone marrow derived macrophage culture

Bone marrow derived macrophages (BMMs) were obtained from wild type C57Bl6 mice. First, the hind legs of the mice were removed and freed from surrounding tissue (see figure x). They were transferred into ice-cold phosphate buffered saline (PBS) without calcium and magnesium.

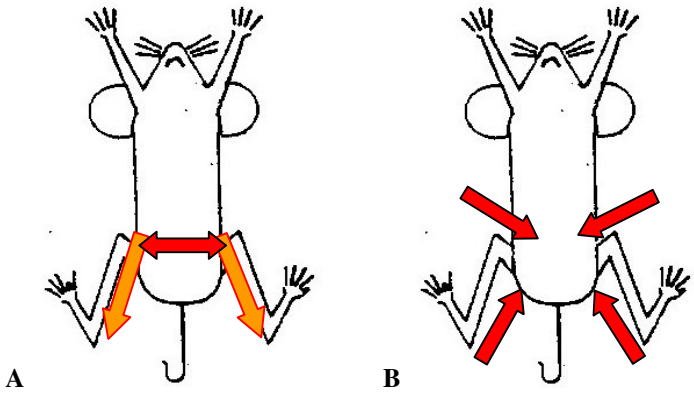

Figure x: Removing the hind legs and freeing them from surrounding tissue
First, an insertion was made in the abdomen of the mouse and in the skin of the hind legs until the toes are reached. (A) Next, the legs are bend to see where the hip joint is located. Now a cut was made in the pelvis above and around the hip joint. An angle of 45° was made to the point where the femur is attached to the hip. (B) Finally, the femurs and tibia were taken out of the mice by moving the leg (the hip part) into the direction of the toes, leading to removal of the skin.

Next, the femurs and tibia were rinsed in 70% ethanol and PBS and subsequently detached from each other and cut at both ends. Now it was possible to flush the bone marrow out of the bones, again with ice cold PBS without Ca and Mg, and to collect it in a Falcon tube. By transferring the bone marrow into new Falcon tubes through several syringes, first through a syringe with a 21 gauge diameter needle and next through one with a 25 gauge needle (BD Microlance 3), a single cell population was obtained. The cells were cultured in RPMI 1640 (GIBCO) containing 10%, 0.20 µM filtered, heat inactivated fetal calf serum (iFCS), 2mM L-glutamine, 10 mM HEPES, 100 U/ml penicillin and 100 µg/ml streptomycin (P/S) and cultured in 15 cm^2 suspension plates at 37ºC. The medium contained 15% L929 Conditioned Medium (LCM), a granulocyte-macrophage colony-stimulating factor(GMC-SF)-like solution, for differentiating the bone marrow cells into macrophages. During the following

week the non-macrophage like cells died, and the macrophage progenitor cells attached to the plastic and started differentiating. After this week of differentiation the cells were lifted from the plates using a pre-warmed (37ºC) PBS solution (0.20 µM filtered 4 mg/ml Lidocaine and 10mM EDTA) without Ca and Mg. Next, the cells were counted and diluted in medium containing 15% LCM to the appropriate concentrations for the experiments. Finally, the macrophages were plated in 96- and/or 48-well suspension plates at 30-50% confluency from Greiner depending on the experiment.

The cells were regularly monitored with a light microscope during the culture period and during the RNAi experiments. Each time the wells were scored for viability and the morphology of the macrophages was observed.

2.2 RNA interference

BMMs were plated in 96- and/or 48-well plates at 30-50% confluency. For a 96-well plate 25,000 BMMs were plated per well and for a 48-well plate the number of BMMs was 70,000 per well. Before incubating the BMMs with siRNAs, their medium was replaced by medium without P/S to prevent cell death due to transfection. Next, the dilutions of the small interfering RNAs (siRNAs) and the transfection reagens were prepared. The transfection reagens used was Lipofectamine™ 2000 from Invitrogen. Three siRNAs (Dharmacon) were used: one against TAK1, a fluorescently labeled one for Lamin A (siGLO) and a scrambled siRNA (siGenome) as a negative control. They were diluted in Opti-MEM® I Reduced Serum Medium (GIBCO). The dilutions and combinations used for each experiment are shown together with the results. The dilutions of the Lipofectamine were not allowed to stand for more than 25 minutes. The dilution of the lipofectamine and the siRNAs were mixed and incubated for 30 minutes to form complexes before adding it to the cells. The cells were incubated for at least 6 hours at 37ºC. Afterwards, the cells are put back on pre-warmed culture medium containing both 15% LCM and P/S. The siRNAs were now were incubated for 24-48 hours. Finally, all the medium was removed and the cells were lysed in TRI Reagent (Sigma) and stored at -80ºC until further processing.

Cells treated with siRNAs against lamin A were observed during the RNAi experiment with a fluorescent microscope. Because these were the only fluorescently labeled siRNAs they could be visually monitored for transfection into the BMMs.

LPS-stimulation

LPS stimulations with 100 ng/µl LPS for 3 hours were performed during the RNAi experiments to observe effects on the cytokines that are highly regulated by the NFκB signaling in macrophages, like TNF-α and IL-6. The cells were prepared and treated as mentioned above and stimulation occurred 27 hours after the siRNA treatment. After the stimulation period all medium was taken of the cells, they were lysed in TRI-reagent and stored at -80ºC until further processing.

2.3 RNA isolation

The plates from the RNAi experiments were allowed to stand at room temperature for about 10-15 minutes. During this period the wells are thawed out and the nucleoprotein complexes of the bone marrow macrophages were completely dissociated in the TRI reagent. Next, the solutions were transferred to eppendorf tubes and kept at 4ºC. 200µl chloroform per ml TRI reagent was added to the samples. They were vigorously shaken for about 15 seconds and allowed to stand at room temperature for 2-15 minutes. After this short incubation the samples are centrifuged for 15 minutes on 13,200 rpm at 4ºC and the mixtures are divided into three phases:

1. A red organic phase containing protein
2. A white interphase containing the DNA
3. A colorless upper aqueous phase containing the RNA

The aqueous phase was transferred to fresh tubes and 0.5ml isopropanol per ml TRI reagent was added to the samples. The RNA was now allowed to precipitate overnight at -20ºC. Next; an RNA pellet was obtained by centrifugation for 10 minutes on 13,200 rpm at 4ºC. The isopropanol was removed and the pellet was washed in 70% ethanol. After removing the ethanol the pellets were allowed to dry to the air for 10-15 minutes and eventually diluted in an appropriate volume of diethylpyrocarbonate(DEPC)-treated water. The concentration and quality of RNA was then measured using the NanoDrop® ND-1000 spectrophotometer and stored at -80ºC until further processing.

2.4 Complementary DNA (cDNA) synthesis

The iScript™ cDNA synthesis kit (BIORAD) was used for synthesizing the RNA into cDNA. It contains three components: nuclease free water, a 5x reaction mix and the enzyme RNase H^+ reverse transcriptase. The 5x reaction mix consists of an RNase inhibitor, a unique blend

of oligo(dT) and random primers. For each experiment, if possible, 500 ng RNA was converted to cDNA. Eventually the total volume of the mix had to be 20µl of which 1µl was the enzyme reverse transcriptase and 4µl was the 5x reaction mix. The remaining 15µl consisted of RNA sample and nuclease free water in a proportion that the right amount of RNA was added for each sample. After creating this mixture, the samples were incubated using the following protocol: First 5 minutes at 5ºC, then 30 minutes at 42ºC and finally 5 minutes at 85ºC. This protocol was performed using a PCR machine. Afterwards, the cDNA samples were kept overnight at 4ºC or for longer periods at -20ºC.

The cDNA samples were tested by performing a reverse transcription PCR for the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPdH). These samples were eventually loaded on a 2% agarose gel made of Tris/Borate/EDTA(TBE)-buffer. After running the gel it was checked for bands. As a negative control, RNA was included which had also undergone cDNA synthesis, but without adding the enzyme reverse transcriptase. Also included was a water control.

2.5 Quantitative Real Time Polymerase Chain Reaction (qPCR)

For measuring gene expression on the messenger level several qPCRs are performed on the obtained cDNA from the RNAi experiments. First, the cDNA samples were diluted in TE^{-4} until a concentration of 10 ng/µl. The standard curve consisted of a pool of the cDNA and had a starting concentration of 5 ng/µl and was 5 times diluted each step. Next, the 10 ng/µl cDNA samples were further diluted to a concentration of 2 ng/µl. Eventually, 5 µl of these 2 ng/µl samples are loaded on a qPCR plate (BIORAD) to become 10 ng of cDNA per well. As a negative control autoclaved milliQ was used. After loading the samples and the standard curve, the qPCR mix was created. The mix contained 0.4 µl reverse primer, 0.4µl forward primer, 4.2 µl autoclaved milliQ and 10µl of iQ™ SYBR Green supermix (BIORAD), per sample. This mix was added to each sample loaded on the qPCR plate before running it. Data collected from the qPCR had to be corrected for a housekeeping gene. The housekeeping genes used in this study were β2-Microglobulin, β-Actin, Cyclophilin A and GAPdH. For measuring knockdown the genes correlating with the used siRNAs, LaminA and TAK1 were observed. To look at the effects of the knockdown on the NFκB-pathway the genes IL-6, IL-10, IL-12 and TNFα were observed. The primers (Eurogentec) used can be observed in table 2.1. qPCR data were analyzed with the software program BIO-RAD-iQ5.

Table 2.1: Primersets for qPCR

Gene		Sequence 5'→ 3'
β2Microglobulin	FW	CTTTCTGGTGCTTGTCTCACTGA
	RV	GTATGTTCGGCTTCCCATTCTC
βactin	FW	GACAGGATGCAGAAGGAGATTACTG
	RV	CCACCGATCCACACAGAGTACTT
Cyclophilin A	FW	TTCCTCCTTTCACAGAATTATTCCA
	RV	CCGCCAGTGCCATTATGG
GAPdH	FW	CAACTCACTCAAGATTGTCAGCAA
	RV	TGGCAGTGATGGCATGGA
IL-6	FW	GCTACCAAACTGGATATAATCAGGAAA
	RV	CTTGTTATCTTTTAAGTTGTTCTTCATGTACTC
IL-10	FW	GGTTGCCAAGCCTTATCGGA
	RV	ACCTGCTCCACTGCCTTGCT
IL-12	FW	GGTGCAAAGAAACATGGACTTG
	RV	CACATGTCACTGCCCGAGAGT
Lamin A	FW	CCATAGCCCCCCTACTGACTT
	RV	TGCGCATGGCCACTTCT
TAK1	FW	AAATGGCACAGGAGTATATGAAAGTTC
	RV	TCCTGGTCCAATTCTGCAACTA
TNFα	FW	CATCTTCTCAAAATTCGAGTGACAA
	RV	TGGGAGTAGACAAGGTACAACCC

FW = forward primer
RV = reverse primer

2.6 Statistical analysis

The results in the graphs are presented as the mean values ± the standard error of the mean. All measurements were performed in duplo and/or in fourfold. Statistical analysis was performed using the student's t-test using GraphPad Prism 4 for windows. A P-value less than 0.05 was considered to be statistically significant.

3. Results

The major part of the project was a technique development and the optimization of this. The development and optimization occurred of a technique for introducing knockdown of TAK1 expression in BMMs using siRNAs.

3.1 Selection of optimal siRNA conditions based on transfection efficiency

First, transfection efficiency of the siRNAs was determined. In this experiment the siRNA against Lamin A, called siGLO, is used. Lamin A is one of the intermediate filament proteins that weave together to form a shell called the nuclear lamina. This nuclear lamina lines the inner surface of the nucleus of every eukaryotic cell and the effect of siGLO should be visual in every cell. It is fluorescently labeled which makes it possible to observe its transfection under a fluorescent microscope. It was chosen to use siGLO instead of fluorescently labeled TAK1, because siGLO is already commercially available and making another siRNA, like the one against TAK1, fluorescently labeled is expensive. Based on the protocol from Invitrogen cells had to be plated at 30-50% confluency at the time of transfection. For a 96-well plate 25,000 BMMs had to be plated per well and for a 48-well plate 70,000 BMMs per well. For the first experiment BMMs are plated in a 96-well plate and treated in duplo with the conditions mentioned in table 3.1. Controls included were untreated cells, cells incubated with only siGLO and cells incubated with only lipofectamine. The cells were incubated for 30 hours.

Table 3.1: Number of wells treated with the testconditions of siGLO and lipofectamine

		siGLO (pmol)			
		-	*2.5*	*5*	*12.5*
Lipofectamine (µl)	*-*	2	2	2	2
	0.125	2	2	2	2
	0.25	2	2	2	2
	0.5	2	2	2	2

3.1.1 Selection based on fluorescence

To determine the best condition for siRNA transfection based on fluorescence 6 hours after treatment the cells were analyzed. Photos were taken from each condition (figure 3.1) and were scored for fluorescence (table 3.2). First, morphology and cell viability of the

lipofectamine controls were examined and show no abnormalities (figure 3.1A, C, G and K). The different siGLO controls used during this experiment also showed no morphological differences in comparison to the control cells, so the siRNA itself has no effect on macrophages. Next was determined that siRNA actually enters the macrophages and not sticks on the outside of the cell wall. This occurred by comparing the control cells of 12.5 pmol siGLO with 0.5 µl lipofectamine (figure 3.1J) to the cells incubated with the same amount of siGLO, but without lipofectamine (figure 3.1B). The cells in figure 3.1J show high fluorescent signals compared to the ones in B, which only show some background fluorescence.

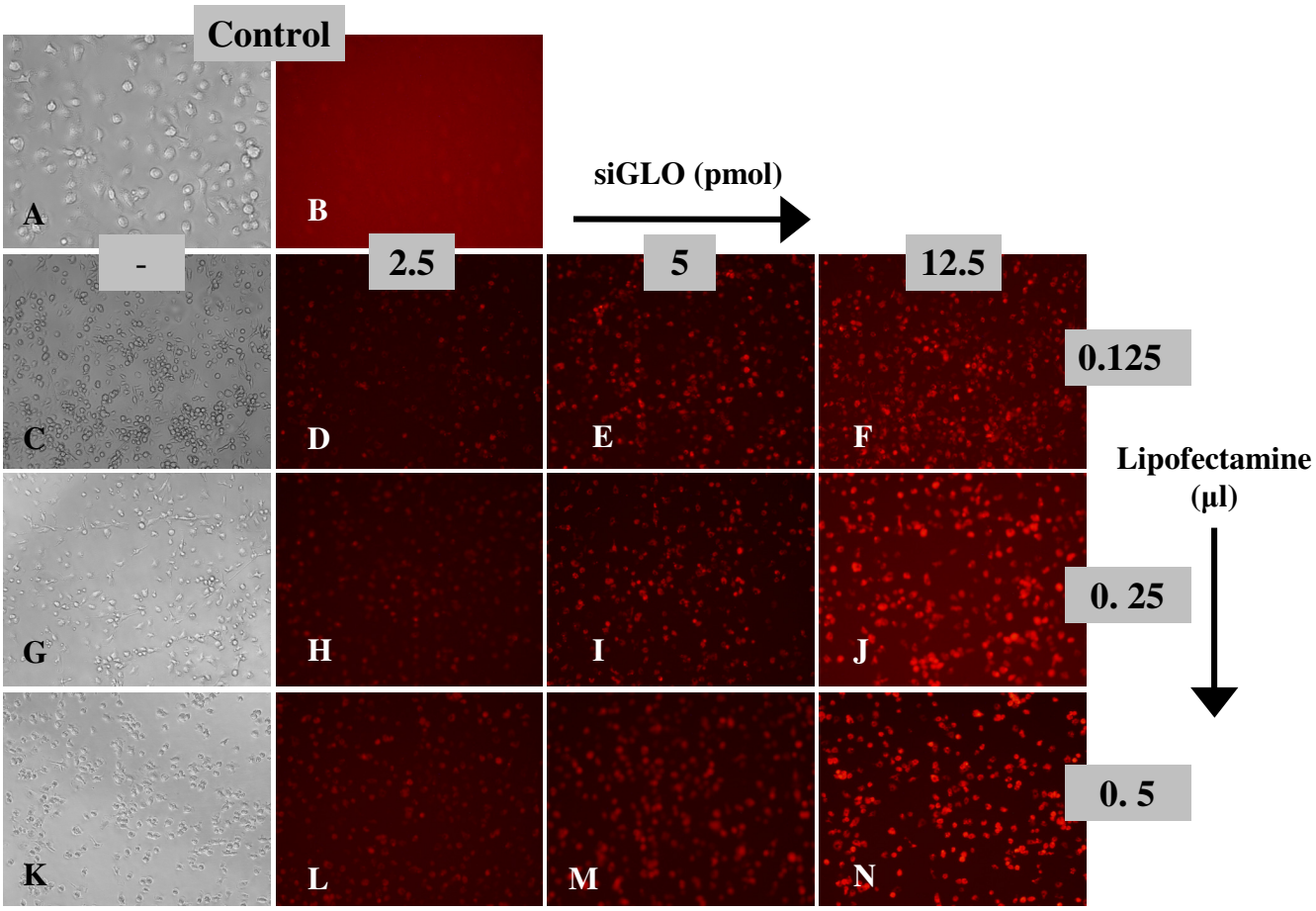

Figure 3.1: Microscopic analysis of the different siGLO conditions

The first two photos (A and B) are the control BMMs without lipofectamine, but with 12.5 pmol siGLO. The light microscopic photos (A, C, G and K) show no morphological abnormalities. The fluorescent photos (D-F, H-J and L-N) show that fluorescence increases when the concentration of siGLO is higher. The highest fluorescence is observed in the cells that were incubated with 12.5 pmol siGLO in combination with 0.25 µl of lipofectamine and in combination with 0.5 µl of the transfection reagens.

Scoring fluorescence for each condition was done by comparing it to the control cells. Fluorescence was scored using – for background fluorescence, -/+ for little fluorescence and + to +++ for increasing fluorescent intensities. The highest fluorescence is observed in the conditions 12.5 pmol siGLO with 0.25 µl lipofectamine and 12.5 pmol siGLO in combination with 0.5 µl lipofectamine. These conditions are highlighted in table 3.2.

Table 3.2: Scoring of the testconditions of siGLO and lipofectamine

		siGLO (pmol)			
		-	*2.5*	*5*	*12.5*
Lipofectamine (µl)	-	N/A	-	-	-
	0.125	N/A	-/+	+	++
	0.25	N/A	-/+	+	**+++**
	0.5	N/A	-/+	+	**+++**

N/A = Not Available, because no siGLO was used for these conditions

Based on the optimal fluorescence conditions, knockdown on the mRNA level should be investigated to check if the siRNAs that actually entered the cell had an effect on the gene expression. This was done using the highlighted 12.5 pmol siGLO conditions.

3.1.2 Selection based on RNA knockdown

RNA was isolated from the 96-well plate treated with the siGLO conditions mentioned in table 3.1. To obtain sufficient RNA the duplos were pooled. After preparation of the samples a qPCR was performed on lamin A and cyclophilin A. Cyclophilin A is a protein that binds to ciclosporin, an immunosuppressant. The cyclosporin A-cyclophilin A complex inhibits calcineurin, which is thought to suppress organ rejection. It is present in every cell and shows little regulation. Therefore, cyclophilin A was included in the experiment as a housekeeping gene and could be used as loading control for the experiment. All siGLO conditions are compared to the control well which is set at relative expression of 1 (figure 3.2). This procedure is used in most of the qPCR data analyzed and gives an overview of the relative expression compared to the control condition. The expression of lamin A should be corrected for a housekeeping gene. However, cyclophilin A seems to be regulated and can not be used for correting qPCR data (figure 3.2).

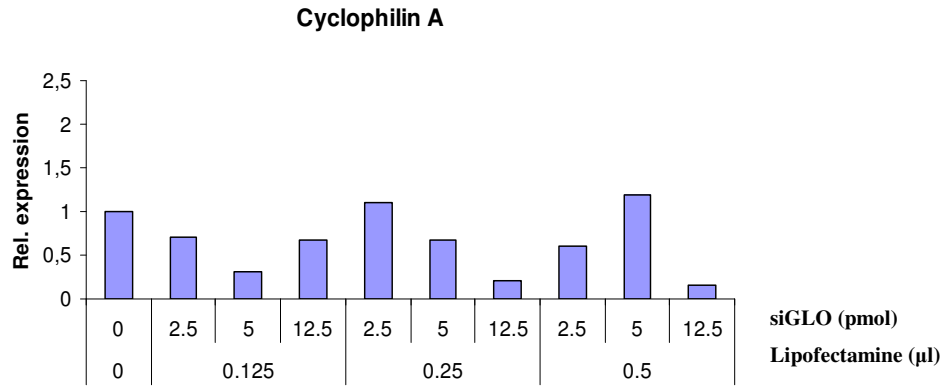

Figure 3.2: qPCR data showing regulation of the expression of cyclophilin A. On the first row of the x-axis the amount of added siGLO are presented in pmol. The second row represents the lipofectamine in µl. (n = 1)

Testing other genes that cyclophilin was necessary to find a good and stable housekeeping gene. The housekeeping genes β-Actin, GAPdH and β2-microglobulin were tested, converted to relative expression and compared to each other (figure 3.3). β-Actin is part of the cytoskeleton and present in all cells. Glyceraldehyde 3-phosphate dehydrogenase (GAPdH) plays an important role in the glycolysis and is also present in all the cells. β_2 microglobulin is a component of MHC class I molecules, which are present on almost all cell surfaces. β-Actin shows the less regulation, except for the condition 5 pmol siGLO in combination with 0.5 µl lipofectamine where it has the highest expression, but the expression of the other two genes show also a great increase for this condition.

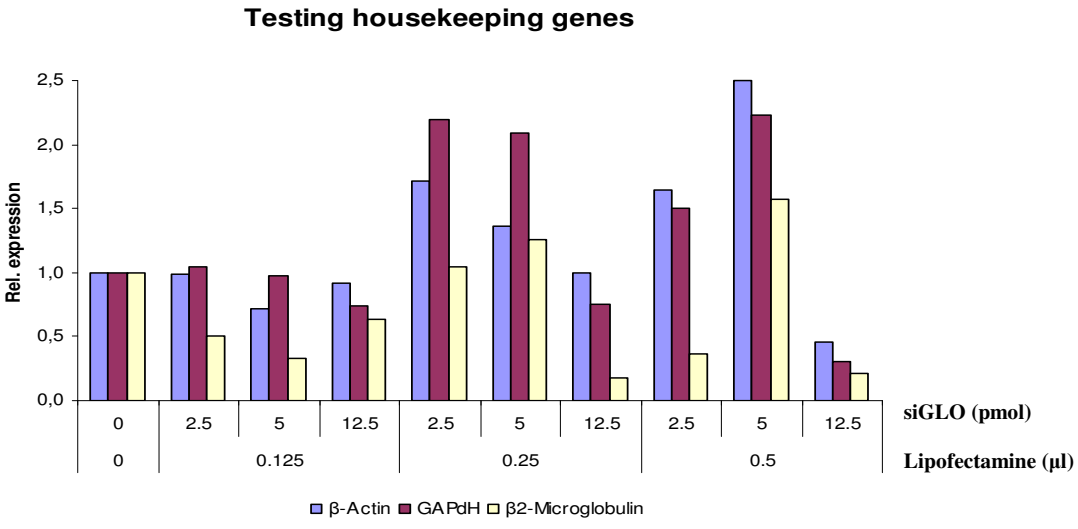

Figure 3.3: Testing housekeeping genes

Relative expression of the housekeeping genes βActin, GAPdH and β2-microglobulin in BMMs after siGLO treatment. βActin shows the less regulation and is the most stable housekeeping gene. Relative expression of β2-microglobulin is less than 1 for the majority, while GAPdH has conditions in which the relative expression lays above 1. The first row of the x-axis is the amounts of siGLO used in pmol. The second row represents the amount of lipofectamine in µl. (n = 1)

Overall, β-Actin was the most stable and unregulated housekeeping gene. This is also observed in later experiments where β-Actin and GAPdH were both included (data not shown). Therefore, β-Actin is used to correct qPCR data in this project.

The data for lamin A corrected for β-Actin showed approximately 65% knockdown for the combination of 12.5 pmol siGLO with 0.25 µl lipofectamine. The same result was shown for the combination of 2.5 pmol siGLO with 0.5µl lipofectamine. The optimal condition for creating lamin A knockdown using siGLO has shown to be somewhere around the conditions highlighted by the red square (figure 3.4). The two optimal conditions based on fluorescence, highlighted in table 3.2, are also present among the best conditions based on RNA knockdown.

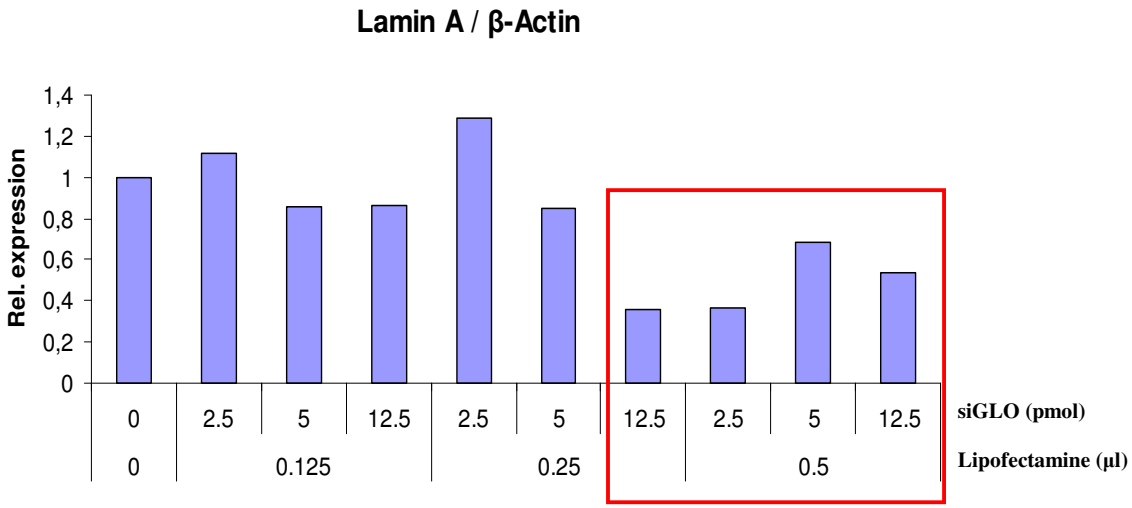

Figure 3.4: Relative expression of lamin A corrected for β-Actin. The first row of the x-axis shows the amounts of siGLO in pmol. The second row represents the amount of lipofectamine in µl. Correcting qPCR data for cyclophilin A can lead to a knockdown of around 65% can be observed for the combinations 12.5 pmol siRNA + 0.25 µl lipofectamine and 2.5 pmol siRNA + 0.5 µl lipofectamine. The optimal condition for creating knockdown lays somewhere between the conditions highlighted by the red square. (n = 1)

The combinations of siGLO and lipofectamine that gave the best results were extrapolated to a 48-well plate, because 96-well plates gave very little material for further processing.

3.1.3 Extrapolation of the optimal RNAi conditions to a 48-well plate

The four tested conditions of siGLO are presented in table 3.3. The combinations were put on the plate in fourfold, while the lipofectamine and siGLO controls were added in duplo.

Table 3.3: Number of wells with BMMs incubated with the conditions of siGLO and lipofectamine

		siGLO (pmol)			
		-	*5*	*10*	*20*
Lipofectamine (µl)	-	4	2	2	2
	0.5	2	X	X	4
	1	2	4	4	4

After transferring the cells to medium without P/S and incubating them with the combinations mentioned in table 3.3 for 6 hours, cells started dying and floating in their wells. Photos were taken 15 hours after incubation using a light microscope (figure 3.5) and cell viability was scored compared to the control cells (table 3.4).

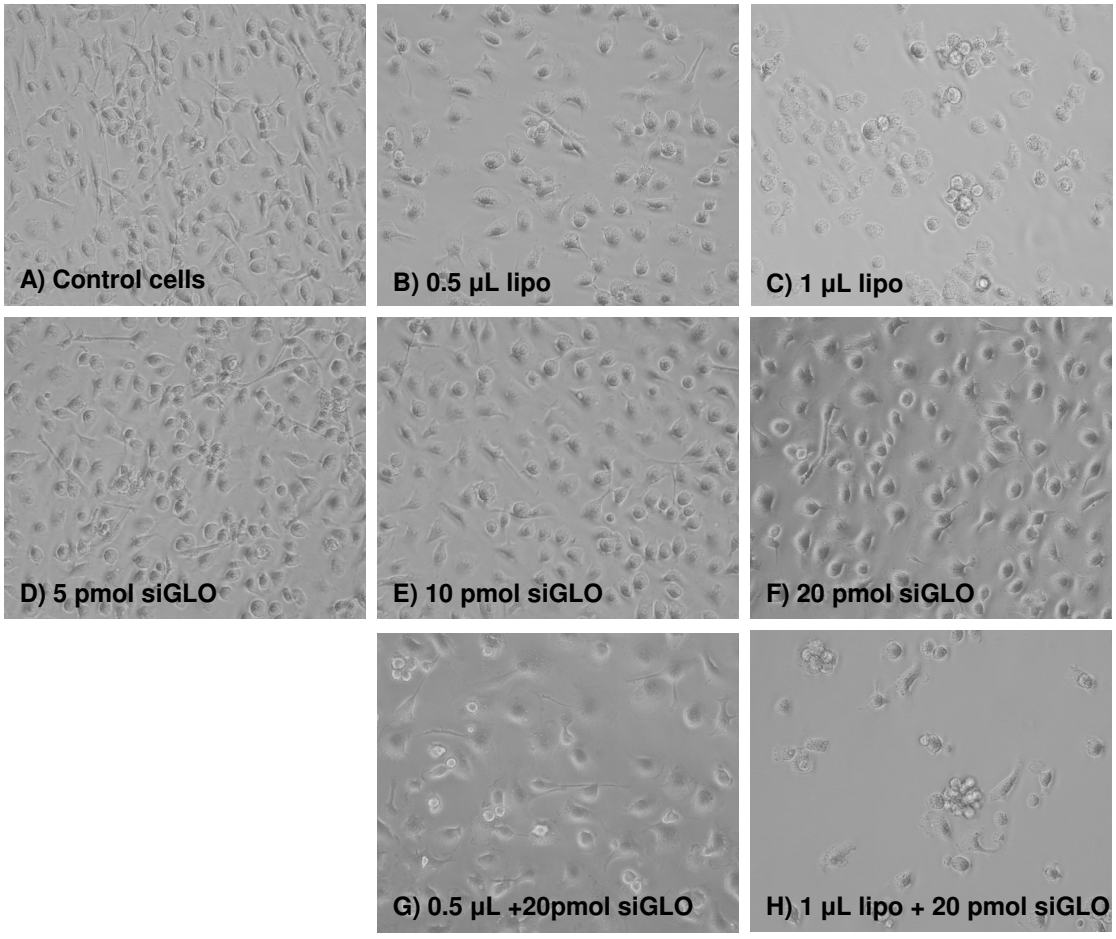

Figure 3.5: Cell death in the 48-well plate due to too high concentrations of lipofectamine
Microscopic photos of the BMMs during the siGLO experiment in a 48 well plate after 15 hours of incubation. Comparing the lipofectamine controls (B and C) with the control cells without any treatment (A) it can be observed that the BMMs start floating in their wells end dying with an amount of 0.5 µl lipofectamine. With 1 µl of the transfection reagens added, this devastating effect becomes even more clear. The siGLO controls (D-F) without any lipofectamine show no actual differences compared to the untreated control cells.

Untreated cells (figure 3.5A) and the siGLO controls (figure 3.5D-F) show no differences in cell viability (x). BMMs incubated with 0.5 µl lipofectamine with or without the presence of siRNA (figure 3.5B and 3.5G) show partial cell death (-), while cells incubated with 1 µl of lipofectamine (figure 3.5C and figure 3.5H) show massive death of BMMs (-/-).

Table 3.4: Scoring of cell viability

		TAK1 siRNA (pmol)			
		-	*5*	*10*	*20*
Lipofectamine (µl)	-	x	x	x	x
	0.5	-			-
	1	-/-	-/-	-/-	-/-

BMMs were plated in a new 48-well plate treated as mentioned in table 3.3, but the lipofectamine amounts were reduced to approximately 0.2 µl and 0.4 µl of the transfection reagens. Another difference was the use of the siRNA against TAK1 instead of siGLO. All siRNA conditions were plated in fourfold and the controls were included in duplo. The cells were observed under a light microscope and checked for viability and morphology 15 hours after treatment. BMMs appeared normal in all conditions and also no cell death was observed (data not shown).

Determining the optimal condition for creating knockdown of lamin A gave four conditions. These conditions, obtained from the RNA knockdown results, are used in the next step of the study for optimization knockdown for the gene of interest.

3.2 Optimization of TAK1 knockdown

The next step from this study was the optimization of creating TAK1 knockdown in BMMs using siRNAs. The 48-well plate already incubated with the siRNA against TAK1 with the conditions 20 pmol of siRNA in combination with 0.2 µl lipofectamine and 5, 10 and 20 pmol of siRNA in combination with 0.4 µl of lipofectamine, was used.

3.2.1 The four conditions of siRNA and lipofectamine obtained from the previous experiments show TAK1 knockdown

The 48-well plate was lysed after 30 hours of incubation. During RNA isolation two wells of the same condition were pooled to get an appropriate concentration of RNA. A qPCR was performed for TAK1 and the relative expression of TAK1 corrected for β-Actin is presented

in figure 3.6. An approximately 35% decrease in expression is observed for the combination of 10 pmol siRNA and 0.4 µl of lipofectamine, but is not statistically significant.

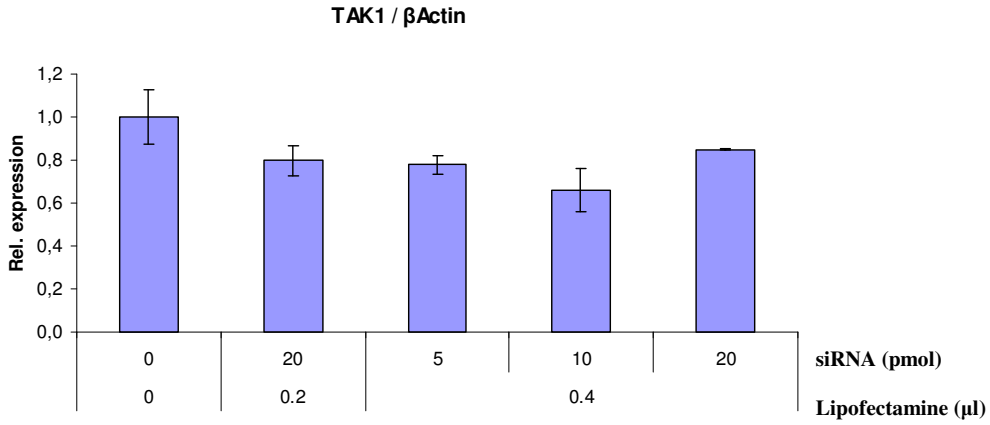

Figure 3.6: TAK1 knockdown

Relative knockdown of TAK1 for the several conditions in the RNAi experiment. The first row of the x-axis show the amount of siRNA against TAK1 in pmol. The second row represents the volume of lipofectamine in µl. A knockdown of around 35% can be observed for the combinations 10 pmol siRNA + 0.4 µl lipofectamine, but this is not statistically significant. (n = 2)

For determining the optimal condition of TAK1 knockdown some adjustments of the RNAi protocol can be made. The amounts of siRNA used can be increased, and the incubation period after treatment of the BMMs can be prolonged.

3.2.2 Optimization by increasing the incubation time after siRNA treatment

BMMs were plated in a 48-well plate using the conditions mentioned in table 3.3 with the adjusted lipofectamine volumes. The only variation is the prolonged incubation time of 48 hours instead of 30 hours after siRNA treatment. All wells were processed individually and all the conditions were measured in fourfold. Again, a qPCR was performed for TAK1 expression and the data were corrected for β-Actin. As can be seen in figure 3.7, almost no differences can be observed between the different incubation times. Both show the best condition for TAK1 knockdown around the 10 pmol siRNA + 0.4 µl lipofectamine. Both expressions are show a 30-35% decrease for that condition. All the conditions of the 48 hours incubation are statistically significant compared to the control cells.

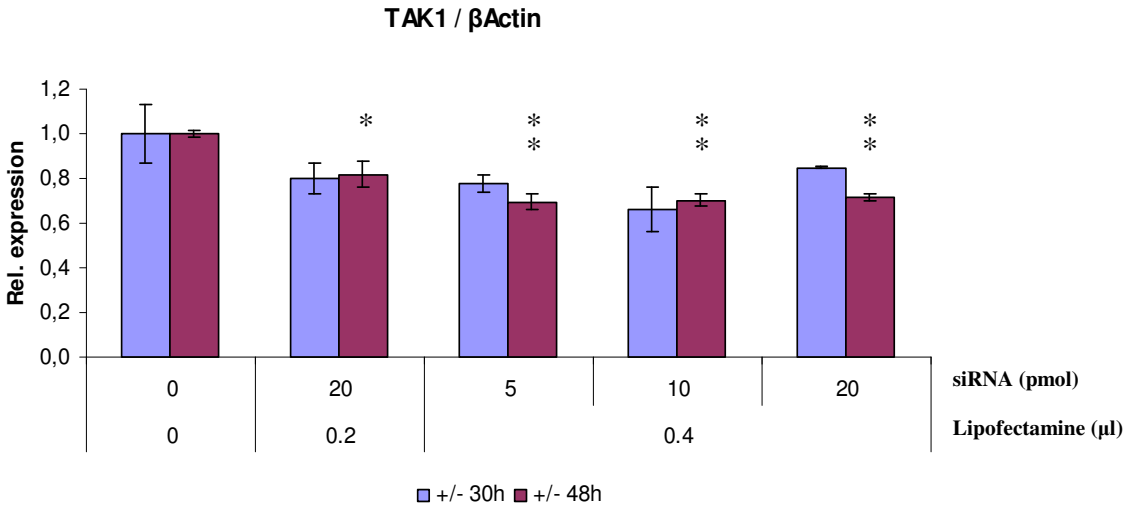

Figure 3.7: Relative knockdown of TAK1 corrected for β-Actin after different incubation periods
All conditions of BMMs treated with siRNAs against TAK1 and incubated for 48 hours show a significant difference for TAK1 expression compared to the control cells (n = 4, * = p-value < 0.05 and ** = p-value < 0.001). They show the highest decrease (± 32%) in TAK1 expression for the condition 10 pmol siRNA + 0.4 µl lipofectamine. The data from the shorter incubation period were not significantly different from the control cells (n = 2, p-values > 0.05), but show also the highest decrease in expression around this condition (± 35%). The first row of the x-axis show the amount of siRNA against TAK1 in pmol. The second row represents the volume of lipofectamine in µl.

3.2.3 Optimization by increasing the siRNA amounts

Another variation of the standard RNAi protocol that can be adjusted was the amount of siRNA added to the samples. Increasing the volume of lipofectamine was not possible, due to introducing cell death. In this experiment higher amounts of siRNA are included: 30pmol siRNA. In addition the lowest concentration of transfection reagens was increased from 0.2 µl to 0.3 µl leading to the following combinations (table 3.5).

Table 3.5: Number of wells treated with the conditions of siRNA against TAK1 and lipofectamine

		siRNA (pmol)			
		-	*10*	*20*	*30*
Lipofectamine (µl)	-	4	2	2	2
	0.3	2			4
	0.4	2	4	4	4

After processing the samples, again in fourfold, a qPCR was performed for TAK1 expression. The relative expression of TAK1 corrected for β-Actin, shows again the best result for the condition of 10 pmol siRNA + 0.4 µl lipofectamine. Comparing these results with the

experiment using lower amounts of siRNA, a new condition (30 pmol siRNA + 0.4 µl lipofectamine) is discovered wich gave approximately the same amount of knockdown (± 35%) as did the optimal condition with a lower amount of siRNA. These two conditions are statistically significant compared to the control cells (p-value < 0.05).

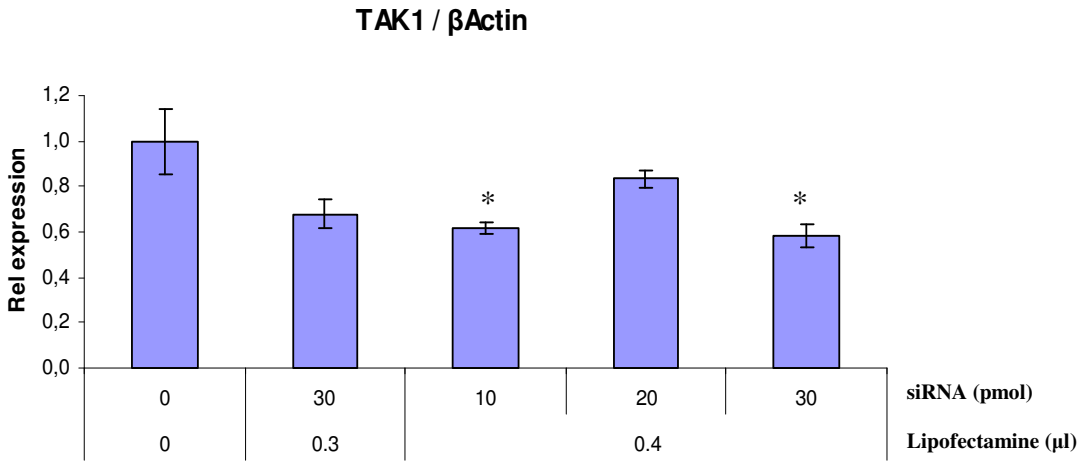

Figure 3.8: Relative expression of TAK1 corrected for βActin with higher amounts of siRNAs
The BMMs that were incubated with the conditions 10 pmol siRNA + 0.4 µl lipofectamine and 30 pmol siRNA + 0.4 µl lipofectamine showed the highest decrease of TAK1 expression (± 35%), which is statistically significant. The first row of the x-axis shows the amount of siRNA for TAK1 in pmol. The second row represents the volume of lipofectamine in µl. (n = 4, * = p-value < 0.05)

Changing the described parameters of the RNAi protocol did not lead to a more optimal condition for introducing TAK1 knockdown. All following experiments were performed using the protocol with unchanged parameters.

3.3 Validation of knockdown specificity by siGenome

The siRNAs used for the genes lamin A and TAK1 actual show some knockdown. To validate these results and test if it is not an aspecific effect the siRNA, a scrambled siRNA (siGenome), which should have no effect, was used as a negative control.

3.3.1 RNAi with siGenome had no significant effect on lamin A expression

A 48-well plate was incubated with the scrambled siRNA with the conditions 20 pmol of siRNA in combination with 0.2 µl lipofectamine and 5, 10 and 20 pmol of siRNA in combination with 0.4 µl of lipofectamine. used instead of the siRNA against TAK1. During

RNA isolation each well was individually processed and a qPCR was performed for lamin A. Analysis of the data obtained from this experiment shows no effect of siGenome on the expression of lamin A (figure 3.9). All conditions used during this experiment showed no significant difference compared to the control cells.

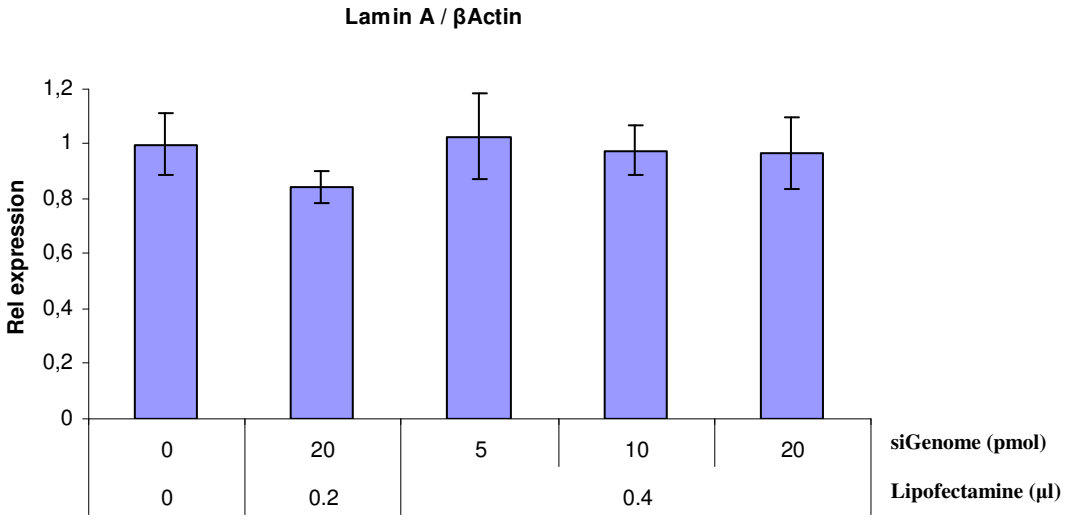

Figure 3.9: Relative expression of lamin A corrected for β-Actin

All conditions of BMMs incubated with siGenome show no significant difference for lamin A expression compared to the control cells. The first row of the x-axis shows the concentrations of siGenome in pmol. The second row represents the concentrations of lipofectamine in µl. (n = 4).

3.3.2 RNAi with the scrambled siRNA had also no significant effect on TAK1 expression

The same samples were also tested for the expression of TAK1. A second qPCR was performed on TAK1 expression and the data is shown in figure 3.10. All conditions of siGenome and lipofectamine show no significant difference compared to the control cells.

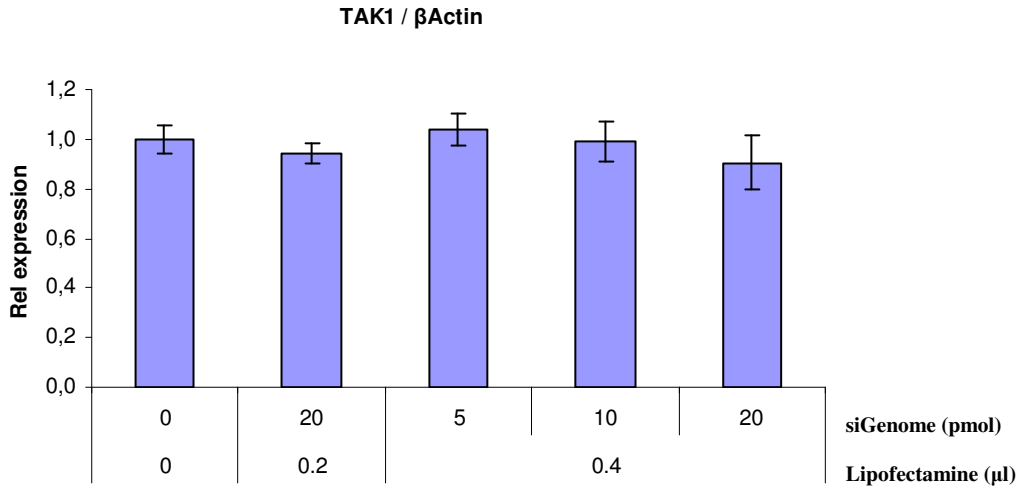

Figure 3.10: Relative expression of TAK1 corrected for β-Actin
All conditions of BMMs incubated with siGenome show no significant differences for TAK1 expression compared to the control cells. The first row of the x-axis shows the amounts of siGenome in pmol. The second row represents the volume of lipofectamine in µl. (n = 4)

siGenome showed no effect on gene expression of the genes lamin A and TAK1. Therefore, the results obtained from the experiments with the siGLO and the siRNA against TAK1 are validated.

Overall, the siRNA condition of 10 pmol in combination with 0.4 µl of lipofectamine, has shown to induce a maximum of 35% knockdown for a period of at least 48 hours. Therefore, this condition was used in the following experiments to test functional effects of TAK1 knockdown in BMMs.

3.4 Functional effects of TAK1 knockdown

To determine the functional effects of TAK1 knockdown on the NFκB signaling in macrophages, the expression of TNFα, IL6, IL10 and IL12 were analyzed after LPS stimulations of the BMMs, using qPCR.

3.4.1 LPS stimulation leads to a decrease in the expression of TAK1

BMMs were plated in a 48-well plate and were treated as mentioned in table 3.3 with the adjusted lipofectamine volumes. After 27 hours of incubation, two of the four wells from each siRNA condition and two control wells without any treatment were stimulated with LPS. All wells were individually processed and several qPCRs were performed for the following genes: TAK1, IL10, TNFα, IL6 and IL12. TAK1 expression was corrected for β-Actin. As

can be seen in figure 3.11, LPS stimulation causes a decrease of TAK1 expression. This decrease is only statistically significant for the control cells ($p < 0.05$). Samples without LPS stimulation gave again the highest decrease of TAK1 expression for the condition of 10 pmol siRNA + 0.4 µl lipofectamine. Around 30% knockdown is shown, but is not statistically significant ($p = 0.0573$). The samples with LPS stimulation show the highest decrease (± 35%) of TAK1 expression for the condition 20 pmol siRNA + 0.4 µl. This is a significant difference compared to the control cells ($p < 0.05$).

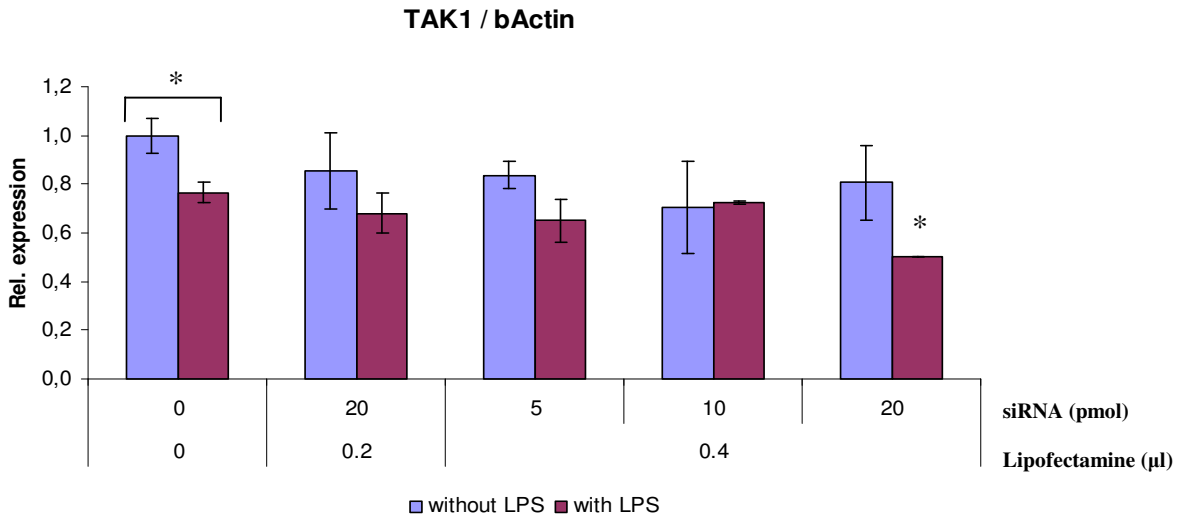

Figure 3.11: Relative knockdown of TAK1 corrected for β-Actin with and without LPS stimulation
The BMMs incubated with the siRNAs and LPS stimulation showed a decrease in TAK1 expression comparing them with the wells without LPS stimulation. The only significant difference was observed between the control wells. The wells without LPS stimulation show again the highest decrease of TAK1 expression (± 30%) for the condition of 10 pmol siRNA + 0.4µl lipofectamine. Comparing the LPS stimulated cells, the highest decrease in TAK1 expression was observed for the condition 20 pmol siRNA + 0.4 µl lipofectamine and is statistically significant. The first row of the x-axis shows the amount of siRNA against TAK1 in pmol. The second row represents the volume of lipofectamine in µl. (n = 2, * = p-value < 0.05)

3.4.2 TAK1 knockdown induces a decrease in LPS induced expression of IL-10 and TNF-α, but not for IL-6 and IL-12

The same samples with and without LPS stimulation were used to analyze the effects of TAK1 knockdown on the production of several cytokines by BMMs. The overall best condition for TAK1 knockdown was observed with the concentration of 10 pmol siRNA and 0.4 µl lipofectamine. Therefore, expression of the different cytokines was observed at this condition. The expression of the control cells for each gene were put at 100%. In figure 3.12

the percentage of expression from each gene compared to their own control are presented next to each other. A statistically significant decrease in expression of more than 55% is observed for IL-10, with a p-value < 0.05. TAK1 knockdown also shows a 30% decrease in LPS-induced TNF-α expression. TAK1 knockdown did not have any effect on IL-6 and IL-12 knockdown.

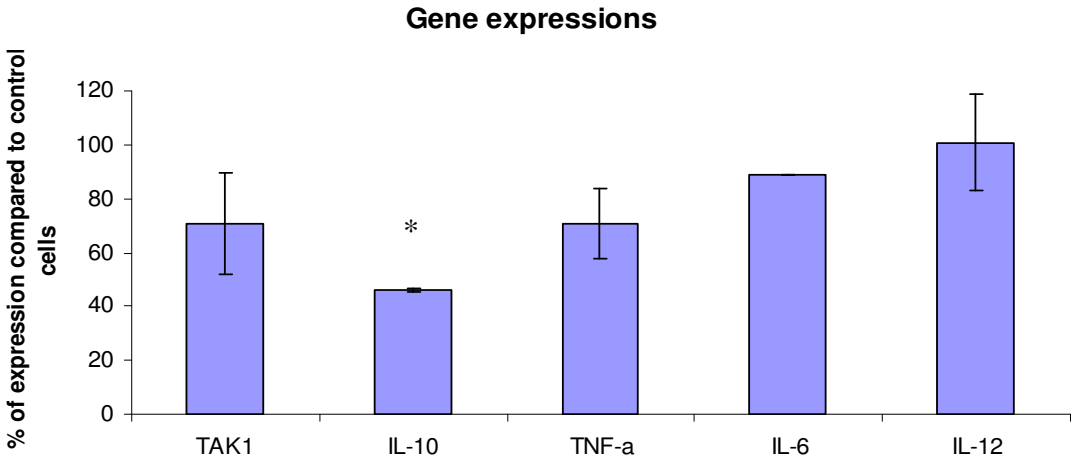

Figure 3.12: Percentages of the expression from TAK1, IL-10, TNF-α, IL-6 and IL-12 compared to their own control cells which was set at 100%.

BMMs show the highest reduction for TAK1 expression at the condition of 10 pmol siRNA + 0.4 µl lipofectamine. The only significant difference compared to the control wells was observed for IL-10, an approximately 55% reduction. TAK1 and TNF-α show a 30% reduction, but no effect is observed for IL-6 and IL-12 expression. The first row of the x-axis shows the amount of siRNA against TAK1 in pmol. The second row represents the volume of lipofectamine in µl. (n = 2, * = p-value < 0.05).

4. Discussion

The aims of the research during this project were clear; setting up and optimizing the protocol for TAK1 RNAi in BMMs and determining the biological effects of TAK1 knockdown on the NFκB signaling pathways in BMMs. The results obtained with regard to these aims are discussed below.

4.1 General

Macrophages are white blood cells that play an important role in the immune system. They are characterized by their ability to phagocytize pathogens and other foreign material. Macrophages are recruited to infected tissues, where they produce immunological substances like cytokines and transcription factors to introduce an inflammatory response. One of the pathways by which macrophages activate the transcription of cytokines is the NFκB signaling pathway. The NFκB signaling pathway can be activated by several external stimuli, like LPS. TAK1 is one of the proteins involved in this pathway and regulates the activation of NFκB by activating the IKK complex. Activation of this complex leads to degradation of the IκB which inhibits NFκB from going into the nucleus. The freed NFκB can now translocate to the nucleus for transcription of several genes, like IL10, TNF-α, IL6 and IL12. These have all an effect on the inflammatory response.

The major part of this project consisted of the setup and optimization of a technique for introducing TAK1 knockdown in BMMs by siRNA. In search of the optimal protocol for performing this technique, the different stadia of the RNAi protocol were observed and optimized. Transfection efficiency, the amount of siRNA added, the volume of transfection reagens used, the incubation period after siRNA treatment and the well size are all factors influencing the RNAi protocol. After setting up and optimization of the RNAi protocol has occurred the biological effect of TAK1 knockdown was analyzed.

4.2 Setup and optimization of the RNAi protocol

Tissue culture cells have provided a powerful system for studying signal transduction, cell differentiation and physiology. However, functional studies in cultured cells were almost not done in the past for the simple reason that there was no powerful method for measuring gene activity. Several technologies designed to knock down gene function, such as those based on

ribozymes, an RNA molecule with catalytic activity and antisense approaches, showed initial promise but ultimately failed to deliver good protocols. A turning point came with the discovery of RNAi [31]. RNA interference is an intracellular mechanism for post-transcriptional gene silencing functions in the regulation of gene expression and defense against unknown DNA elements and viruses [32]. Two types of small noncoding dsRNA can serve as effector molecules and trigger RNAi: small interfering RNA (siRNA) and micro RNA (miRNA). Today, the most commonly used approaches are based on, either synthetic siRNAs or vector-expressed short hairpin RNAs (shRNAs), small dsRNA constructs that are usually 22–29 nucleotides long and form a hairpin-like secondary structure. Around 75% of the siRNAs have shown to have some off-target effects [33, 34]. For studying the gene function of TAK1 in this project an RNAi protocol was setup and optimized, using synthetic siRNAs ordered from Dharmacon., that were actually pools of several siRNAs against the same target gene. This pool of siRNAs has shown to minimize the off-target effects [35]. Another problem observed using RNAi is that the use of long dsRNAs to trigger RNAi is initially hindered by the fact that these molecules simultaneously activate the interferon response, a primitive antiviral mechanism that triggers sequence-nonspecific degradation of mRNA and downregulation of cellular protein synthesis [36]. However, short dsRNAs can be designed to mimic small siRNAs, which were shown to perform a potent and specific RNAi response in cultured cells, without interferon-γ (INF-γ) activation [37]. During this project the INF-γ effect was not checked and when making conclusions it should be taken into account that the data could be influenced it. Future experiments should be checked for INF-γ activation.

4.2.1 Transfection efficiency based on fluorescence

The first step in the RNAi protocol is transfection of the BMMs with siRNA. For an optimal result of the siRNA, it is important that they actually enter the cells and not stick on the outside. For this experiment the fluorescently labeled siRNA against lamin A (siGLO) was used. Merkerova et al. showed in their experiment that primary cells (chronic myeloid leukemia) require a high efficiency of siRNA delivery into the cells for creating enough knockdown. They used, same as in this project, a fluorescent labeled RNAi to check where transfection efficiency is the highest. They used a variety of physical and chemical conditions to test which condition gave the highest fluorescent signal and thus was the most suitable transfer [38]. Further, they stated that commonly used transfection reagents show very low

delivery of siRNA into the cells. The reaction reagent used in this project, should be chosen carefully. Andreas R et al also mention this last statement. They tested several transfection reagents and showed the best results for lipofectamine 2000 [39]. Therefore this reagent is chosen in this project and the results show high fluorescent signals for some of the tested conditions compared to the control wells. From these results can be concluded that transfection efficiency was good, from which four conditions were obtained that showed the highest transfection efficiency. The next step in this experiment was to look if the transfection efficiency also correlated with knockdown/silencing efficiency.

4.2.2 Silencing efficiency based on gene expression measured by qPCR

qPCR data has to be corrected for a housekeeping gene. Thus, one of the first things to do during this step was to look for an unregulated housekeeping gene. Several studies using qPCR to analyze silencing efficiency of their siRNAs seemed to use different housekeeping genes. Klatt et al., for example, used GAPdH as housekeeping gene for correction in their study, while Han et al used cyclophilin A [40, 41]. Therefore, several potential housekeeping genes were tested and β-Actin was observed to be the less regulated house keeping gene. The use of housekeeping genes varies a lot among the studies and could be explained by the different cell types used in which the expression for these genes can differ. Now β-Actin has pointed out to be the best housekeeping gene qPCR data can be corrected for this gene, leading to a knockdown of approximately 85%. Merkerova et al. showed that transfection of siRNA in chronic myeloid leukemia primary cells showed low siRNA delivery and lead to less than 25% knockdown [38]. This is a big difference with the observation from this study, but can be explained by the different cell types used. Chemical transfection reagents commonly used to transfect siRNAs in CML cell lines show very low siRNA delivery in CML primary cells so the difference can again be explained by the difference in cell types used. CML cell lines seem harder to transfect than primary cells, like BMMs.

From the experiment with siRNA against lamin A, the four conditions that gave the highest silencing effect were also used in the experiment with siRNA against TAK1. The result was an approximately 35% knockdown, which is significantly lower than the knockdown observed for lamin A. Perhaps, the optimal conditions for lamin A were not the same for TAK1 and further optimization had to occur. Merkerova et al. showed also that transfecting their cells with electroporation, a significant increase in the electrical conductivity and permeability of the cell plasma membrane caused by an externally applied electrical field, achieved better results (suppression to 63%) but it was associated with high degree of cell

death, more than 60% [38]. Overall, electroporation shows higher silencing efficiency, than normal transfection in primary cells [42]. A better knockdown was obtained using nucleofector technology. The nucleofector technology is a novel transfection technology especially designed for the needs of primary cells and difficult-to-transfect cell lines. It is a non-viral method, which is based on a unique combination of electrical parameters and cell-type specific solutions [43]. With this transfection method Merkerova et al showed gene expression that ranged between 22–37% expression of that from original levels. The 35% knockdown observed in this project is very low compared to these results, but it must first be checked for biological functionality. This amount of knockdown can already show some biological effects. In the future it could be considered to transfect the BMMs with nucleofector technology, to check for higher knockdown. Now knockdown of TAK1 was created, optimization could occur by variations of the standard protocol.

4.2.3 Optimization of the RNAi protocol for TAK1 knockdown

A first variation was made in the incubation time of the BMMs with the RNAi conditions. Documented studies show a lot of variation in the incubation time of the RNAi [44, 45]. Different siRNAs have different optimal incubation times. In the literature these vary from 12 hours of incubation to 96 hours of incubation. The results obtained from this experiment show that there is no difference in TAK1 knockdown between 24- and 48-hour incubation. Both protocols gave a knockdown of approximately 30-35%. From these data can also be concluded that the knockdown stays for a minimal period of 48 hours after incubation. Perhaps, the periods are to close together to observe a difference and an incubation time of 72 hours or more should be investigated for higher or lower knockdown.

A second variation of the standard RNAi protocol was done by increasing the amount of siRNA From this data can be concluded that a new condition is observed with the same knockdown potential as the optimal condition seen until now. For this new condition is more siRNA necessary, but the effect stays the same. Therefore, the old optimal condition with 10 pmol of siRNA is favored above the new one. Overall, it can be concluded that the best results for TAK1 knockdown are observed after 30 hours of incubation with the condition of 10 pmol in combination with 0.4 µl lipofectamine.

4.2.4 Validation using siGenome

To validate the knockdown created by the siRNAs a scrambled siRNA, like siGenome can be used. This siRNA accepts a short DNA sequence, and returns a scrambled sequence. The scrambled sequence will have the same nucleotide composition as the input sequence, but will have no match with any mRNA and no known miRNA recognition sequence. Therefore, a scrambled siRNA has no effect on gene expression and can be used as a negative control. Xu et al. and Taishi et al. have also used a scrambled siRNA in their studies [46, 47] as a negative control. These scrambled siRNAs gave no effect and these are the same results as the scrambled siRNA in this project. Thus, the knockdown observed during the experiments is a true effect of the functional siRNA.

4.3 Biological effects of TAK 1 knockdown

During the regulation of NFκB activation numerous proteins are involved. One of these proteins is transforming growth factor β-activated kinase 1 (TAK1). TAK1, also known as mitogen-activated protein kinase kinase kinase 7 (MAP3K7), is involved in pro-inflammatory signaling pathways, the (mitogen activated protein) MAP kinase and the NFκB signaling pathway [48]. It can be activated by extracellular stimuli including immunological regulators, growth factors, stress, and viral and bacterial products, like LPS. Setting up and optimization of the RNAi technique has lead to optimal conditions which were used in the further experiments.

4.3.1 LPS stimulation leads to a decrease in TAK1 expression

The expression of TAK1 from the unstimulated wells show the same knockdown pattern (± 30%) as obtained from earlier results. From this experiment can be concluded that, when looking at the level of TAK1 expression between the LPS-stimulated and unstimulated wells, LPS stimulation causes a decrease of TAK1 expression. This effect is not mentioned in literature, but can be explained as follows. TAK1 is normally present in the cells as an unphosphorylated protein. When activated, by LPS for example, it is phosohporylated and will eventually activate NFκB. In this phosphorylated form it can not be detected by qPCR and explains the lower expression.

4.3.2 TAK1 knockdown reduces IL-10 and TNF-α expression, but has no effect on IL-6 and IL-12

The overall best condition for TAK1 knockdown was observed with the concentration of 10 pmol siRNA and 0.4 µl lipofectamine. Therefore, expression of the different cytokines was observed at this condition. TAK1 activation shows in literature several pro-inflammatory effects [49]. Knocking down this gene would lead to a decrease of inflammation by NFκB because of the impaired activation ok the IKK complex and the reduced degradation of IκB. Looking at TNF-α expression, a pro-inflammatory gene, a decrease can be seen as expected. TAK1 knockdown and TNF-α decrease were at the same level of around 30%. This experiment also showed a decrease of 55% for IL-10 expression, an anti-inflammatory gene. From this can be concluded that TAK1 also plays an anti-inflammatory role when activated. Perhaps there is some kind of equilibrium between pro- and anti-inflammatory signals induced by TAK1. No effect was seen on IL-6 and IL-12 expression. From this can be concluded that the effect of TAK1 on IL-6 and IL-12 is not very huge, and that the levels of these cytokines are kept at a steady state, probably by other pathways that buffer the expression of these pro-inflammatory genes, for example other MAPKKKs which can introduce JNK and p38 MAP linase pathways introduce pro-inflammatory signals through pathways without TAK1 contribution [50].

4.4 Conclusions and further recommendations

As an overall conclusion for the first and largest part of this study can be stated that the optimization of the technique for introducing knockdown in bone marrow derived macrophages using siRNAs is not entirely completed. It can be stated that in a 48-well plate an optimal condition for creating TAK1 knockout has been observed with the combination of 10 pmol siRNA and 0.4 µl lipofectamine, but still some conditions should be examined. Another conclusion that can be made from the data obtained of this optimization is that β-Actin was overall the most stable housekeeping gene and qPCR data should be corrected for this gene. Using higher amounts of siRNA (30 pmol) showed the same knockdown as for the 10 pmol siRNA, thus increasing the siRNA in the experiments is not necessary. An overall conclusion for the second part of this study is that TAK1 knockdown had a biological effect. It resulted in a decrease of the anti-inflammatory cytokine IL10 and a decrease in the pro-inflammatory cytokine TNF-a. No effect was seen on IL-6 and for IL-12 expression.

For future experiments, it would be interesting to investigate what the effect on knockdown will be when after siRNA treatment an incubation period of about 72 hours is performed. Will

the knockdown still be present or has it become even stronger? It will be also interesting to increase the amount of siRNA just above the 30 pmol used. Using the 30 pmol of siRNA showed an increase knockdown compared to the 20 pmol siRNA. By looking at the tendency of the graph a further increase of knockdown can be expected for a higher amount of siRNA and should be checked. Further attention should be given to check for knockdown on the protein level using Western blot. Knockdown on the messenger level does not have to be reflected on the protein level. Another interesting thing would be to extrapolate the optimal conditions of the 48-well plate to a 24-/12-/6-well plate for increasing the amount of material that can be obtained for further analysis. Changing the concentration of LPS used during the experiments for determining the functional effect, and/or changing the period of stimulation can also be of interest to determine the sensibility of the TAK1 knockdown.

Finally, some experiments performed during this project had a low number of samples, and showed no significant differences. All experiments should be repeated and checked for reproducibility and it should be checked if the data with more samples becomes (more) statistically significant.

Finally, this project has shown potential for the technique of RNAi for introducing TAK1 knockdown in BMMs. Further optimization is required, but a biological effect is already shown. In the future, when the RNAi protocol is fully optimized, it will be a handy tool for investigating the role of TAK1 in the NFκB signaling in macrophages. Maybe this could lead to a potential therapeutic target for the treatment of several inflammatory diseases.

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