The role of the mitogen-activated protein kinases (MAPKs) in endotoxin tolerance

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Preface

This thesis at the end of my study in biomedical life sciences, is the perfect moment to look back and speak out my appreciation.

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My family, mama and Freddy, for giving me every opportunity and supporting me during the last four years. And last but not least, Bert, for just being their for me and listening when I needed someone to talk to, to stress out or just to be happy.

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List of abbreviations

AB	Antibody
ADP	Adenosine 5'-diphosphate
A-mulV	Abselon Leukaemia Virus
AP	Activating protein
ASK	Apoptosis signal-regulating kinase
ATP	Adenosine 5'-triphosphate
BMK	Big MAPK
BSA	Bovine serum albumin
CD	Cluster of differentiation
CREB	cAMP response element-binding
DC	Dendritic cells
D-domain	Docking domain
DLK	Dual leucine zipper-bearing kinase
DMEM	Dulbecco's modified Eagl's medium
DNA	Deoxyribonucleic acid
DSP	Dual-specificity phosphatases
dsRNA	Double stranded RNA
ECL	Enhanced chemiluminescence
EDA	Extra domain A
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immuno sorbent assay
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
GPI	Glycosylphosphatidylinositol
HRP	Horseradish peroxidase
Hsp	Heat-shock protein
IEG	Immediate early genes
IgG	Immunoglobulin G
IL	Interleukin
INF	Interferon
IRAK	Interleukin-1 receptor associated kinase
IκB	Inhibitor of kappa light chain gene enhancer in B-cells
JNK	c-jun N-terminal kinase
LBP	Lipopolysaccharid binding protein
LPS	Lipopolysaccharid
MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MEK	Mitogen-activated protein kinase kinase
MKP	Mitogen-activated protein kinase phosphatase
mRNA	Messenger ribonucleic acid

MyD88	Myeloid differentiation factor 88
NF-κB	Nuclear transcription factor
NIK	NF-κB inducing kinase
PBS	Phosphate buffered saline
PKC	Protein kinase C
PMSF	Phenylmethanesulphonylfluoride
Rel	Reticuloendotheliosis oncogene
RISC	Ribonucleic acid induced silencing complex
RNAi	Ribonucleic acid interference
RPMI	Roswell Park Memorial Institute
RSK	Ribosomal S6 kinases
SAPK	Stress-activated protein kinase
SDS	Sodium dodecyl sulfate
Ser	Serine
shRNA	Small hairpin ribonucleic acid
siRNA	Short interfering ribonucleic acid
STAT	Signal transducer and activator of transcription
TBS-T	Tris buffered salt-tween
TGF	Transforming growth factor
Th	T helper
Thr	Threonine
TIR	Toll-interleukin 1 receptor
TLR	Toll-like receptors
TMB	Tetramethylbenzidine
TNF	Tumor necrosis factor
TRAF	TNF-α receptor associated factor
Tyr	Tyrosine
UV	Ultraviolet

Samenvatting

Het aangeboren immuunsysteem maakt gebruik van patroon herkennings receptoren zoals TLRs voor het herkennen van evolutionair bewaarde patronen op pathogenen. Stimulatie van deze TLRs induceert verschillende signaal transductie pathways, waaronder de NF-κB en de MAKP signalerings pathways. Activatie van deze pathways is noodzakelijk om de productie van pro-inflammatoire cytokines te induceren en het verworven immuunsysteem te activeren. Bij de mens bestaan er drie welbeschreven MAPK pathways, namelijk de ERK1/2, de p38 en de JNK1/2 pathway. Deze worden actief wanneer ze gefosforyleerd worden op tyrosine en threonine residues. Aangezien overproductie van pro-inflammatoire cytokines septische shock en zelfs de dood kan veroorzaken, is het beëindigen van de activatie van deze pathways erg belangrijk. MAPK phosphatases (MKPs) zijn verantwoordelijk voor het defosforyleren en daarbij inactiveren van MAPKs. Wanneer cellen echter voorbehandeld worden met endotoxines zoals LPS, kunnen ze tolerant worden voor een tweede blootstelling aan hetzelfde endotoxine. Dit fenomeen staat bekend als endotoxine tolerantie en werd eerder reeds waargenomen bij septische shock patiënten. Tolerante cellen slagen er niet in pro-inflammatoire cytokines te produceren om het pathogeen, dat de endotoxine stimulus veroorzaakte, te elimineren. Hierdoor worden patiënten opmerkelijk gevoeliger voor secundaire infecties die ernstige schade kunnen veroorzaken. Om de moleculaire mechanismen van endotoxine tolerantie, en meer precies de rol van MAPKs en MKPs, te onderzoeken, werd er een *in vitro* LPS tolerant model opgestart in murine macrofagen (RAW 264.7) en humane monocyt-verworven dendritische cellen (DCs). Deze cellen werden, zoals in de literatuur, éénmaal (voor de controle condities) of tweemaal (om tolerantie te induceren) gestimuleerd met LPS. Het TNF-α cytokine productie profiel werd gebruikt als readout omdat geweten is dat tolerante cellen dit cytokine niet produceren. DCs en macrofagen vertoonden inderdaad een dramatisch verminderde productie TNF-α na een tweede stimulatie, maar niet na de eerste stimulatie. Bovendien werden in deze tolerante cellen lagere fosforylatie niveaus van ERK1/2 en p38 waargenomen in vergelijking met niet tolerante cellen. Zoals eerder ook vermeld in de literatuur, werd er ook een stabilisatie van IκB na een tweede LPS stimulatie waargenomen, terwijl na één enkele stimulatie een degradatie zichtbaar was na 30 minuten. Tenslotte zijn we erin geslaagd een downregulatie van MKP-1 te bekomen na transfectie met siRNA specifiek tegen MKP-1 in RAW 264.7 cellen. Het kon echter niet geconcludeerd worden dat MKP-1 betrokken is in LPS-geïnduceerde tolerantie bij RAW 264.7 macrofagen.

Abstract

The innate immune response uses pattern recognition receptors, such as TLRs, to recognize highly conserved structures on pathogens. Stimulation of these TLRs induces several signal transduction pathways like NF-κB and MAPK signaling pathways. Activation of these pathways is important to induce the production of pro-inflammatory cytokines and helps to induce the adaptive immune response. In human, there are three well known MAPKs pathways: ERK1/2, p38 and JNK1/2. They become activated upon phosphorylation on their threonine and tyrosine residues. Termination of these pathways is important to prevent overproduction of pro-inflammatory cytokines which can cause septic shock and even death. MAPK phosphatases (MKPs) are responsible for dephosphorylating and thereby inactivating MAPKs. However, pretreatment with LPS for example, can render immune cells tolerant to a second exposure with the same endotoxin. This phenomenon is known as endotoxin tolerance and has also been observed in septic shock patients. Tolerant cells fail to produce pro-inflammatory cytokines to eliminate the pathogen which caused the endotoxin stimulus; this makes patients more susceptible to a secondary infection which has severe detrimental effects. To investigate the molecular mechanisms of endotoxin tolerance and more precisely the role of the MAPKs and MKPs, we started setting up a murine and human *in vitro* LPS tolerance model using murine macrophages (RAW 264.7) and human monocyte-derived dendritic cells (DCs). The cells were stimulated once for the control condition or twice to induce the tolerance state as was reported in the literature. TNF-α cytokine production profile was used as readout because it is well known that tolerant cells fail to produce this cytokine. Indeed, we observed dramatic decreases of TNF-α secretion when the macrophage or DCs were challenged twice with LPS but not when these cells were activated once. In the tolerant cells, a lower ERK1/2 and p38 phosphorylation was observed compared to non tolerant cells. As previously reported in the literature, we also observed a stabilization of IkB upon two successive stimulations with LPS while a nice degradation of this protein was induced 30 min after a single LPS stimulation. Finally, we were able to downregulate by almost 50% MKP-1 after transfection with specific siRNA against MKP-1 in RAW 264.7 cells. However, we were not able to conclude if MKP-1 is involved in the LPS-induced tolerance in RAW 264.7 macrophages.

1 Introduction

Mammalian organisms, including humans, have two lines of defense against the entry of bacteria, viruses or other pathogens (1). These are called the innate and adaptive immune responses.

The first line of defense is the innate immune system (1, 2), involving leukocytes such as neutrophils and macrophages (1). Macrophages have a limited number of different receptors that recognize certain patterns on pathogens, known as pattern recognition receptors (3). Although the number of different receptors is limited, they are able to recognize a broad spectrum of pathogens because they recognize highly conserved structures on pathogens that are not found in higher eukaryotes and are not subject to high mutation rates (3, 4). Stimulation of the innate immune system gives rise to the synthesis of a wide range of inflammatory mediators and cytokines (3, 4) and helps to induce the second line of defense, the adaptive immune system, by presenting opsonized bacteria as antigens, and providing costimulatory signals necessary for lymphocyte activation (1, 5). The adaptive immune system consists of a more diverse range of receptors (B-cell and T-cell receptors) that are more specific (1). These receptors have also the ability to remember previous attacks of specific pathogens and thereby respond quickly upon reencounter. Because the adaptive immune response is slow, the innate immune system is very important to protect against pathogens during the first and most critical hours of infection (5).

1.1 Toll like receptors

Many of the mammalian cell-surface pattern recognition receptors are members of the Toll-like receptor (TLR) family (6). They are named after a protein found in Drosophila (Toll) which is involved in resistance against fungal infections. In humans at least eleven TLRs have been reported and are activated by different ligands (6, 7). Stimulation of TLR usually results in the induction of transcription of genes encoding pro-inflammatory cytokines and also helps to induce the adaptive immune response. Not only humans have been reported to use TLR against pathogens, in other mammals, including mice, TLR have also been indicated in the recognition of microbial targets (3).

TLR2 can be activated by an enormous array of molecules, including bacterial lipopeptides, peptidoglycan, zymosan and several structural variants of lipopolysaccharid (LPS) (3). TLR2 has broad ligand specificity because TLR2 has to dimerize with other TLRs (heterodimerization) to detect ligands and induce signaling (3).

TLR5 and TLR9 can be activated by only a few microbial targets compared to other TLRs. TLR5 is activated by bacterial flagellin and TLR9 is activated by unmethylated CpG motifs, these motifs are more abundant in bacterial DNA than they are in mammalian DNA (3). Furthermore TLR9 seems to be involved in the recognition of viral DNA (herpes simplex virus type-1) (8).

One of the best characterized members of the TLR-family is TLR4, which is responsible for LPS recognition (3, 4, 6, 7). LPS is an endotoxin that resides in the outer membrane of the cell wall of Gram-negative bacteria (7). LPS is not the only activator of TLR4, Taxol, heat shock protein 60 (hsp-60) and the extra domain A (EDA) region of fibronectin are also potent activators (3). In murine macrophages the sensitivity to LPS is controlled by the modulation of TLR4 biosynthesis or activity. This can be achieved by altering the expression of TLR4 or by changing the sensitivity of the signal initiated by TLR4 (7).

TLR4 forms a complex with CD14 and MD-2. For the detection of LPS both MD-2 and CD14 are necessary (figure 1) (3, 4). CD14 is a protein residing in the outer membrane of innate immune cells and is linked to glycosylphosphatidylinositol (GPI). LPS is detected by CD14 and thereby activates the TLR4 complex (3), eventually this leads to production of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL) -1 and IL-6 (6). TLRs have an extracellular domain which is responsible for their ligand specificity and an intracellular domain (TIR) which is homologues to the IL-1 receptor (IL-1R) (6). Activation of a TLR causes the TIR domain to bind to the adaptor protein myeloid differentiation factor 88 (MyD88). This protein activates a series of intracellular molecules, for example IL-1 receptor associated kinase (IRAK) which results in activation of several signaling pathways (figure 1) (3, 6).

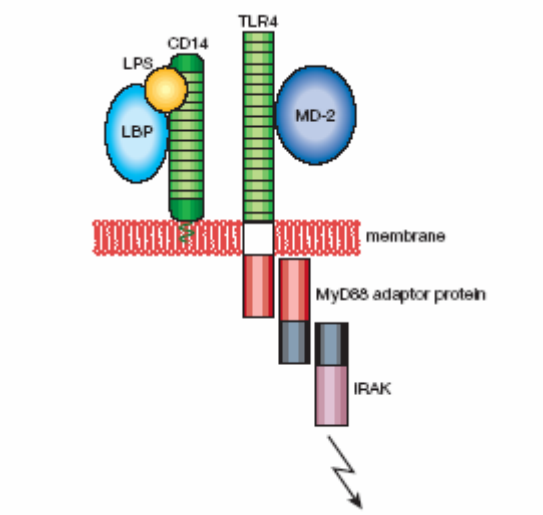

Figure 1: Toll-like receptor-4 and its associated proteins *The LPS/LBP complex is recognized by CD14 on the surface of the macrophage. The ternary LPS-LBP-CD14 complex activates the TLR4/MD-2 complex which signals through the adaptor protein MyD88 and the serine kinase IRAK (4).*

1.2 Signaling pathways

One of the best known activators of cellular signaling pathways is TNF-α. This cytokine is able to induce different signaling pathways which can induce proliferation, differentiation, inflammation or apoptosis. Examples of signaling pathways that can be activated by TNF-α are the nuclear transcription factor (NF-κB) and the mitogen-activated protein kinases (MAPK) signaling pathways (9).

TNF-α binds on its receptor, this activates TNF-α receptor associated factors (TRAFs) which on their turn activates c-jun N-terminal kinase (JNK) or NF-κB inducing kinase (NIK) (9, 10). NIK activates IκB kinases which phosphorylate and inactivate IκB, this is necessary for NF-κB to translocate to the nucleus and therefore transcription of pro-inflammatory cytokines. On the other hand the activation of JNK by TNF-α is found to be required for TNF-α induced apoptosis. JNK is a member of the MAPK family (9). Thus, the TNF-α receptors can induce different downstream molecules that can influence either the NF-κB or the JNK activation (anti-apoptotic or apoptotic signals) (9).

1.2.1 The NF-κB pathway

The NF-κB pathway is a common pathway via which different TLRs signal to the nucleus (6). NF-κB is a dimeric transcription factor, composed of different members of the Rel family, such as p65, p50 and p52. Inactivated NF-κB forms a complex with inhibitor of kappa light chain gene enhancer in B-cells (IκB) and resides in the cytoplasm of unstimulated cells.

Stimulating signals like bacterial endotoxins (for example LPS), TNF-α, IL, UV light, oxidative stress and other stress factors, induce phosphorylation of IκB which causes it to undergo ubiquitinilation and proteolytic degradation (9, 10). This causes the active form of NF-κB to translocate to the nucleus where it interacts with other transcription factors to regulate the transcription of several effectors genes (9, 10). This transcriptionally active form of NF-κB has a DNA-binding domain and a transactivation domain (9). The effectors genes are genes involved in cell stress reaction, inflammation and apoptosis (9, 10). To which effectors genes NF-κB will bind is determined by the composition of the subunits (9).

1.2.2 The MAPK pathway

Besides activating the NF-κB pathway, the activation of TLRs, for example by the binding of LPS to TLR4 at the surface of the cell, leads also to activation of a signal transduction pathway which triggers activation of serine/threonine (Ser/Thr) protein kinases that belong to the MAPK subfamilies. Since activated MAPKs phosphorylate and activate transcription factors, such as NF-κB, the activation status of these kinases determines the production of pro-inflammatory cytokines and the initiation of cellular processes such as proliferation, differentiation and development (2, 11-13).

The MAPK signaling cascades are ancient and evolutionary conserved pathways that play also an essential role in both innate and adaptive immune responses (2, 11). In mammals, the MAPK super-family consists of four members. Each family-member has its own activators, inhibitors and substrates which causes signal specificity although some “cross-talk” appears to exist (2, 12). These four distinct MAPK signaling pathways currently known are (i) the extracellular signal-regulated kinase (ERK or p44/p42) pathway, (ii) stress-activated protein kinase (SAPK)/JNK pathway, (iii) the p38 pathway and (iv) the big MAPK1/ERK5 (BMK1) pathway (figure 2). Whereas ERK is mainly involved in the transduction of mitogenic signals and JNK and p38 in the transduction of stress signals, BMK is activated both by mitogens and stress stimuli (2). Together these MAPK pathways transmit extracellular signals to intracellular targets. Ultimately they alter gene expression profiles and regulate cell function. Therefore they are also involved in cellular processes such as proliferation, differentiation and development (figure 2) (2, 12).

The activation of the MAPK pathways is regulated by a triple kinase cascade: MAP–KKK, MAP-KK and MAP-K. MAP-KKK activates MAP-KK by phosphorylating their Ser/Thr residues, MAP-KK on its turn activates MAP-K by phosphorylation of its Thr and tyrosine (Tyr) residues (2, 14) (figure 2). Phosphorylation of MAPKs occurs in their activation loop,

more in particular in the TxY motif of the kinase subdomain VIII. X is variable for the different subclasses of MAPK, for ERK1/2 x is defined by glutamate, for JNK by a proline and for p38 MAPK by a glycine (15-17). Interactions of MAPKKs with MAPKs occur by binding of a docking domain (D-domain) on MAPKKs to the docking groove on MAPKs (Figure 3) (18, 19). MAPKKK are very diverse in structure and can therefore be activated by a variety of upstream stimuli. Upon stimulation, MAPKKKs selectively regulate their MAPKKs. MAPKKs are highly specific for their substrates and therefore there's only minimal variation for the MAPKK-MAPK part of the cascade possible (2, 12).

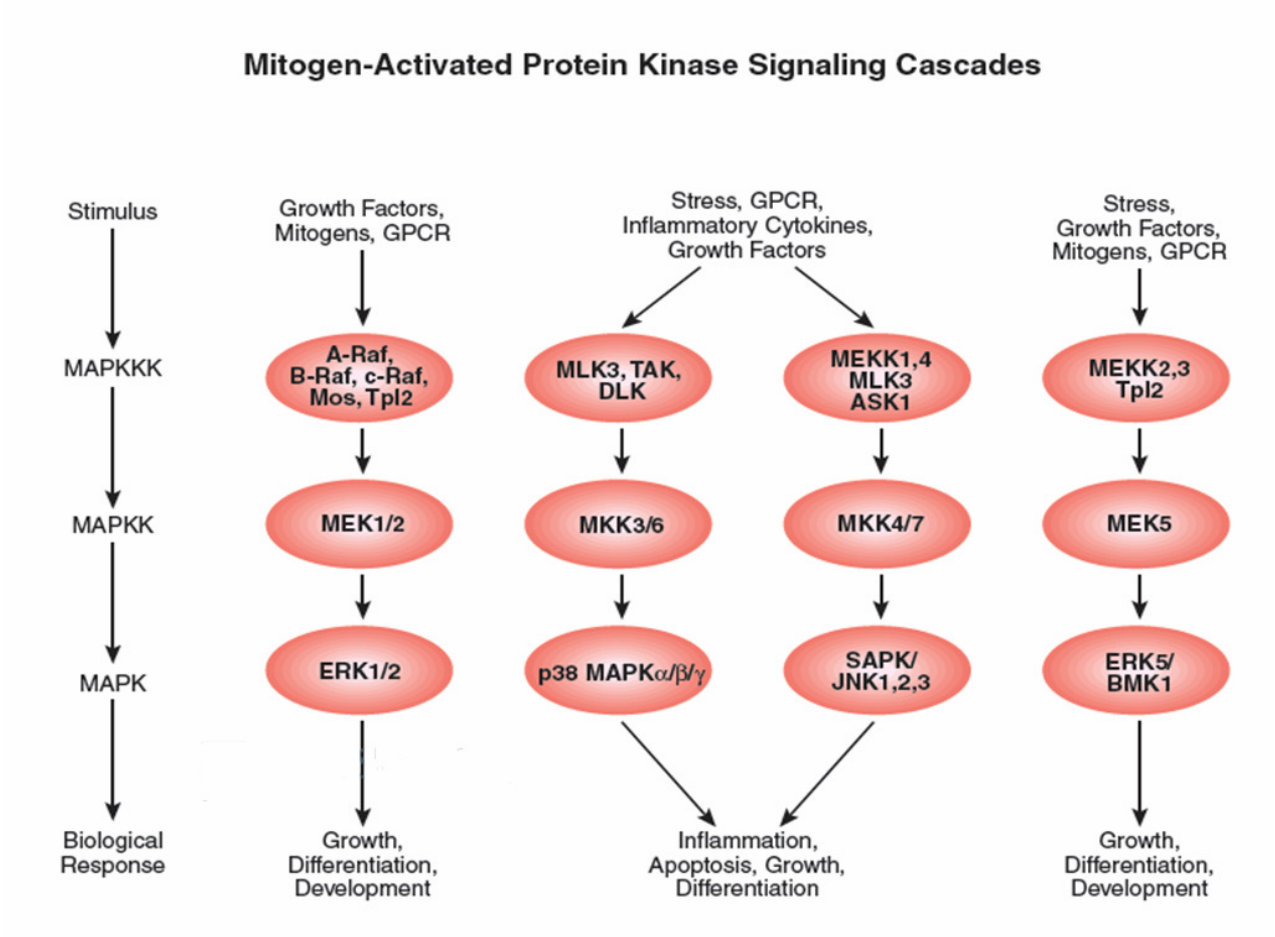

Figure 2: Schematic overview of the MAPK signaling pathway *In response to a stimulus, for example cell stress due to LPS stimulation, MAPKKK gets activated, which on his turn activates MAPKK and this activates MAPK, which finally leads to a biological response, usually transcription of pro-inflammatory cytokines. There are four MAPK-pathways: the ERK1/2 , the p38 MAPK , the SAPK/JNK and the ERK5/BMK1 pathway (20).*

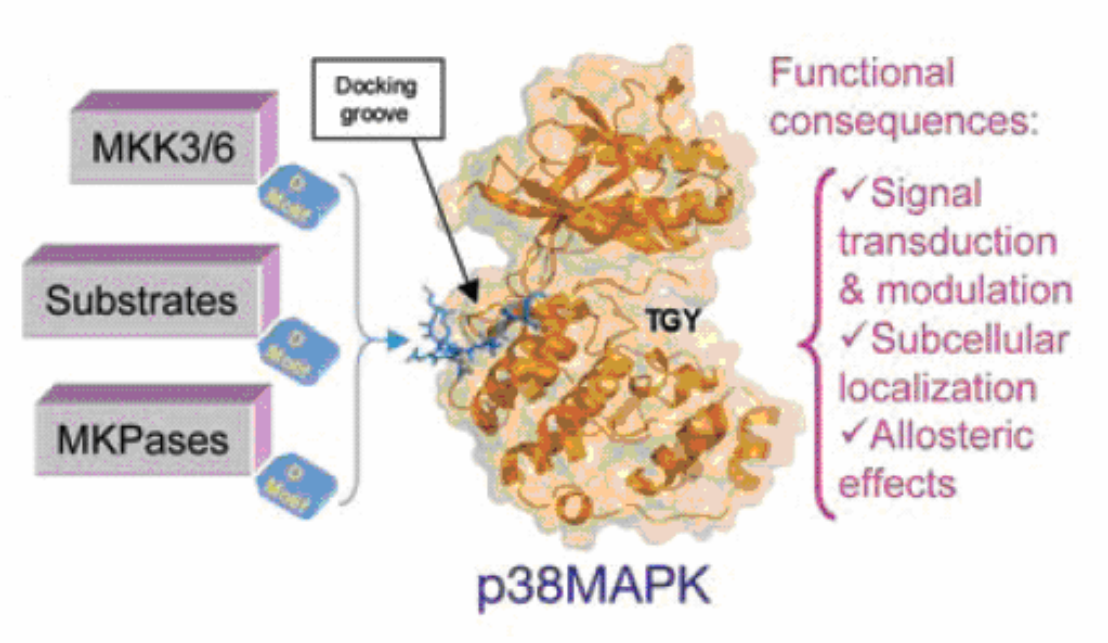

Figure 3: Schematic representation of the structure of p38 MAPK *This schematic representation of p38 shows the docking groove, located near the activation domain of MAPKs, for p38 this is TGY. This is the site where MAPKKs bind to phosphorylate MAPKs, where activated MAPKs bind their substrates and where negative regulators such as MKPs are able to bind. All these interactions occur through association with a D-domain and the docking groove. And through this interactions the activation loop is either phosphorylated or dephosphorylated, which has consequences on the functional outcome. This is not only true for p38MAPK but also for other MAPKs (18).*

Of the four MAPK signaling pathways currently known, ERK1/2, p38 MAPK and SAPK/JNK are best understood and the most described in the literature.

1.2.2.1 P38 MAPK

Cellular stress factors (UV radiation, osmotic shock, LPS,...) and certain cytokines like TNF-α induce the activation of p38 MAPK pathway by activating MKK3/6 which phosphorylates and activates p38 MAPK (figure 3) (14, 21, 22). MKK3/6 is activated by MAPKKK such as MKKK1-4, apoptosis signal-regulating kinase-1 (ASK-1) and dual leucine zipper-bearing kinase (DLK) (18). Downstream targets of the p38 MAPK pathway include transcription factors such as p53, activator protein-1 (AP-1), cAMP response element-binding (CREB), signal transducer and activator of transcription (STAT), NF-κB and others (18, 21, 23).These transcription factors orchestrate events leading to cell cycle arrest, DNA repair and apoptosis (21). For the immune system this means that the p38 MAPK pathway plays an essential role in inducing apoptosis by a caspase-dependent mechanism, mainly in $CD8^+$ cells. In $CD4^+$ cells p38 MAPK is a positive regulator of INF-γ production and Th1 differentiation, it is

necessary for T-cell activation and IL-12 production. In the innate immune response p38 MAPK is important for the production of IL-12 (2).

1.2.2.2 SAPK/JNK

Like p38 MAPK, SAPK/JNK gets activated in response to environmental stresses and inflammatory cytokines which activates MAPKK4/7. Upon phosphorylation by MAPKK4/7 JNK gets activated (13, 14). LPS or inflammatory cytokines such as TNF or IL-1 are able to activate this pathway which is necessary for cytokine production leading to the activation of the innate immune response. In the adaptive immune response SAPK/JNK is mainly involved in $CD4^+$ cell proliferation and effector T-cell function. JNK2 is responsible for INF-γ production and JNK1 is more involved in regulating the Th2 response. Taken together JNK is responsible for T-cell differentiation into a Th1 response, activation of $CD4^+$ T-cells and positively regulating $CD8^+$ T-cell effector function (2). JNK1/2 phosphorylation is also involved in programmed cell death (13).

1.2.2.3 ERK1/2

Growth factors bound to their receptors are able to activate the small GTPase Ras which is associated with the membrane and located downstream of the tyrosine kinase of the growth factor receptor. Ras together with protein kinase C (PKC) activates Raf-1 by hyperphosphorylating it (12). Activated Raf-1 phosphorylates and activates mitogen-activated protein kinase 1/2 (MEK1/2), which in turn leads to the phosphorylation and activation of ERK1/2 (12, 19). ERK phosphorylates ribosomal S6 kinases (RSKs). Both ERK1/2 and RSKs are able to translocate to the nucleus where they can phosphorylate important transcription factors together with nuclear RSKs. Examples of regulated transcription factors are Elk-1, CREB and histone H3. These transcription factors are able to transcribe immediate early genes (IEG) which are involved in regulating the cell cycle and cell survival (12, 19). Other substrates for ERK include factor receptor genes, Raf-1 and MEK, which suggests that ERK serves as a feedback mechanism for its upstream components that are responsible for its activation. ERK has additional substrates such as cytoskeletal elements, for example MAP-1, MAP-2, MAP-4, Tau and others, and RSK. Taken together, all these substrates play an important role in controlling cellular processes that occur in response to mitogenic stimulation, thus ERK is important in controlling events that lead to a transition of cells from the G0 to the G1 phase of the cell cycle (12). Therefore ERK is involved in cellular growth and differentiation, the adaptive immune response, T-cell activation and Th2 differentiation.

In the innate immune response ERK is mainly responsible for TNF-α regulation and especially the transport of TNF-α mRNA from the nucleus to the cytoplasm (2).

Xu et al demonstrated that treating murine macrophages with LPS results in the activation of the MAPK pathways that can be totally inhibited after silencing (7).

1.3 MAPK-phosphatase

The magnitude and duration of the activation of MAPKs are important processes for regulation of the immune responses (2). When MAPKs remain active for to long they give rise to an overproduction of pro-inflammatory cytokines, for example TNF-α. These cytokines are able to produce a secondary inflammation cascade. These events can cause severe hypotension and occlusion of the microvasculature which can ultimately cause multiple organ failure. This phenomenon is known as septic shock (24). Effective termination of pro-inflammatory cytokine production during innate and adaptive immune responses is therefore essential to prevent potentially detrimental systemic effects, such as septic shock and auto-immunity (11, 24). Termination during the innate immune response is regulated by a negative controlling mechanism, responsible for inactivating the MAPKs (2, 24). It is well known that activation of the pro-inflammatory MAPK signaling cascade also triggers negative feedback mechanisms that are responsible for termination of MAPK signaling (11). A new protein family, the MAP kinase phosphatases (MKPs) has been defined as negative regulators of MAPKs. MKPs are members of the protein tyrosine phosphatase superfamily and in particular are a part of the dual-specificity phosphatases (DSPs) subfamily because they are able to dephosphorylate both threonine and tyrosine residues in the activation loop of MAPKs (2, 16, 25-27). DSPs share the same catalytic structure, located in the carboxyl-terminal half of the protein. This motif contains residues which are crucial for the two-step catalytical reaction by which dephosphorylation occurs (15, 16). MKP genes become rapidly transcribed and translated by various stimuli like growth factors, cytokines, cellular stress and oncogenes (16). Multiple MKPs exist with variation concerning their preferences for target MAPKs. MKP-1, for example, preferentially dephosphorylates activated p38 MAPK and JNK and in a lesser extent ERK (figure 4), MKP-3 however seems to prefer ERK as a substrate (16, 26). MKPs bind their substrates by their amino-terminus which causes the inactive catalytical domain of MKPs to become activated and capable of inactivating their target MAPKs by dephosphorylation or preventing phosphorylation (16). It seems that MKP activation is in part also regulated by MAPKs themselves, suggesting a negative feedback system (16, 28).

MKP-1 was one of the first known MKPs and therefore one of the best studied. Expression of MKP-1 is induced by a variety of stimuli, including cellular stress, LPS and pro-inflammatory cytokines (27). ERK1/2 stabilizes the MKP-1 protein by phosphorylating it on 2 C-terminal serine residues (figure 4). Although MKP-1 is a substrate for ERK1/2 it is also capable of dephosphorylating ERK1/2 (28). Since MKP-1 mediates inhibition of MAPK signaling and pro-inflammatory gene expression it is an important negative regulator of many aspects of the inflammatory responses (27).

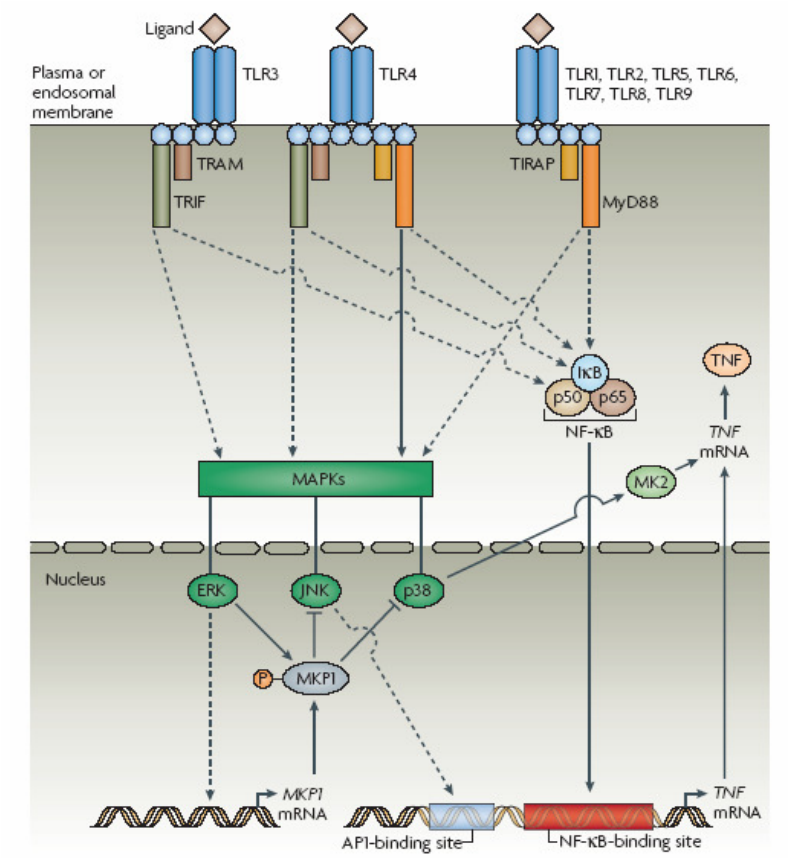

Figure 4: The function and regulation of MKP-1 *Stimulation of TLRs activates several signaling pathways like the MAPK or the NF-κB pathway. Activation of the MAPK pathway however, also induces a negative feedback because activated ERK is capable of stabilizing MKP-1 mRNA and phosphorylating MKP-1 by which the latter becomes activated. Activated MKP-1 inhibits MAPKs (mainly JNK and p38 and to a lesser extend ERK) by dephosphorylating them. Because of this negative feedback system pro-inflammatory cytokine production (for example TNF-α) is stopped (29).*

1.4 Endotoxin tolerance

Stimulation with endotoxins like LPS causes an increase in pro-inflammatory cytokines, leading to the activation of the immune system and therefore eliminating the pathogens. However high cytokine production can be auto destructive and be responsible for tissue damage, septic shock and even death (30-34). Pretreatment, both in vitro and in vivo, with sublethal doses of LPS can cause a tolerance to a subsequent challenge with high concentrations. This insensitive state is termed "endotoxin tolerance" and is transient (35-37). It seems to be a protective mechanism to limit the inflammatory damage due to excessive activation of immune cells by LPS and therefore prevent septic shock but on the other hand it also causes a downregulation in the immune response against other microbial invasions which increase the susceptibility of the host to a secondary infection (35, 36). Greisman noted that endotoxin tolerance is a dual phase process. The first stage is a non-specific state in which antibody formation is not involved. This stage was termed non-specific because tolerance extends to endotoxins unrelated to the one used for desensitization. The second phase is more specific and depends on formation of endotoxin-specific-antibodies (38-40). Several mechanisms are thought to be responsible for endotoxin tolerance: (i) a blockade of intracellular signaling events that are required for gene expression, (ii) the overproduction of anti-inflammatory cytokines such as IL-10 and TGF-β and (iii) endotoxin tolerance secondary to a downregulation of LPS receptors at the surface of immune cells. However, the endotoxin tolerant phenotype is dependant upon the model that is used, therefore it is likely that all the above mentioned mechanisms contribute to the tolerant phenotype but that one or the other is more pronounced in one model versus another (36). For example in macrophages it was shown that TNF production is decreased in an endotoxin tolerant state (41-43) and that this decrease was already visible at mRNA levels, therefore it is believed that endotoxin tolerance suppresses signaling cascades prior to transcription. This was concluded from both in vivo and in vitro studies (44, 45).

An endotoxin tolerant phenotype shows increased cell surface CD14 expression, impaired NF-κB activation, downregulation of IRAK and a decrease in phosphorylation of ERK1/2, JNK and p38 in response to stimulation with LPS. The condition of tolerance is accompanied by an increase in IL-1 and a decrease in TNF-α levels (37, 46-49). Endotoxin tolerance, however, is not specific to the action of LPS. Therefore it is likely that cross-reactivity with other exogenous stimuli occurs. Various cytokines such as TNF and IL-10 can mimic the

effects of endotoxin both in vivo and in vitro (37, 50). Furthermore the tolerant state is transient and will disappear after a certain time (37).

As mentioned before signal transduction alterations can be an important mechanism underlying endotoxin tolerance (37, 44, 45). It seems likely that the characteristics of endotoxin tolerance arise secondary to alterations in signal transduction pathways. Some possible alterations are: (i) an alteration in the phosphatase activity; (ii) a change in the subunit composition of NF-κB which leads to a shift towards p50 homodimers instead of p50/p65 heterodimers, which in turns leads to a decrease in the capacity to transcribe mRNA of pro-inflammatory cytokines; and (iii) a suppressed degradation of Iκ-Bα en Iκ-Bβ, which attenuates nuclear translocation of NF-κB and therefore would inhibit gene transcription, together with an impaired LPS stimulated activation of the transcription factors NF-κB and AP-1. However, this last alteration doesn't seem to cause inhibition of gene expression because of elevated levels of p65 in the cytoplasm, and Iκ-B binds preferentially to p65 instead of NF-κB (37).

Hu et al just recently found that LPS tolerance is, at least in part, mediated by p38-induced MKP-1 expression (51). More evidence for the possible involvement of MKP-1 in endotoxin tolerance was delivered by Zhao et al, who found that *MKP-1*$^{-/-}$ cells show a prolonged activation of p38 and JNK upon stimulation with LPS and a higher production of TNF-α and IL-6. Furthermore they report that *MKP-1* knock-out mice showed severe hypotension, multiple organ failure and a higher rate of mortality upon treatment with LPS (24).

1.5 Objectives

The overall objective of this work is the establishment of a simple *in vitro* endotoxin tolerance model that will allow us to investigate the role of different MAPKs and MAPK phosphatases in endotoxin tolerance.

1.5.1 Specific aims

1- To set up the LPS tolerance model in the murine macrophage cell line RAW 264.7 as well as in human dendritic cells as reported in the literature. As readout, TNF-α secretion will be evaluated as reported in the literature.

2- To investigate the phosphorylation status of the MAPKs ERK and p38 in LPS tolerance conditions.

3- To investigate the IkB level after single or double stimulation with LPS.

4- To investigate the role of the MAPK phosphatase MKP-1 in endotoxin tolerance.

2 Materials and methods

For the setting of the endotoxin tolerance model, the following materials and techniques were used.

2.1 Cells and media

The mouse monocyte/macrophage cell line RAW 264.7 was used for the setting of the endotoxin tolerance model. The cell line is originated from ascites of a tumor induced in a male mouse by intraperitoneal injection of Abelson Leukemia virus (A-MulV). The cells were maintained in Dulbecco's modified Eagl's medium (DMEM) supplemented with 10% (v/v) of heat-inactivated fetal bovine serum (FBS), 30 U/ml penicillin, 30 µg/ml streptomycin, 1% (v/v) of sodium pyruvate (100mM) and 1% (v/v) of non-essential amino acids (100x) all from GIBCO (GIBCO-Invitrogen, Carlsbad, USA).

Dendritic cells (DCs) were differentiated from the human monocytes derived from Peripheral Blood Mononuclear Cells (PBMCs). PBMC were isolated from healthy human buffy coat obtained from the Liège University Blood bank. Buffy coat is the non coagulating blood fraction obtained after centrifugation and is highly enriched of white blood cells. To prepare the PBMC, the human blood was diluted twice in RPMI 1640 (GIBCO-Invitrogen, Carlsbad, USA) without serum and added on the top of 15 ml of lymphoprep (Axis-Shield, Oslo, Norway). The falcon tubes were then centrifuged at 1430 rpm during 20min to separate the different cells fractions. The erythrocytes and granulocytes have a higher density compared to lymphoprep while leukocytes and platelets have a density lower then lymphoprep. Thus, the two later form a ring at the interface between lymphoprep and plasma. After centrifugation, the PBMCs were collected, washed twice in RPMI medium to remove residual lymphoprep and platelets. The cells were then counted and incubated during 2h at 37°C under 5% of CO_2/95% humid atmosphere. The non adherent cells were then removed. The remaining cells were washed with 37°C pre-warmed RPMI medium and cultivated during 6 to 7 days in RPMI complemented with heat-inactivated 10% (v/v) fetal bovine serum (FBS), 1% (v/v) sodium pyruvate (100mM), 1% (v/v) non-essential amino acids (100x), 50U/ml penicillin, 50µg/ml streptomycin and 1% (v/v) β-mercaptoethanol together with IL-4 (100U/ml) and GM-CSF (800U/ml) to differentiate the adherents monocytes to DCs. After the 7 days culture, the DC maturation was analyzed by flow cytometry based on there size and granulosity as

well as on the known surface markers. Indeed, differentiated DCs express CD1a, are $CD86^{low}$, $CD80^{low}$, $CD14^{low}$ and $HLA\text{-}DR^{low}$.

2.1.1 Test for mycoplasma infection

To exclude mycoplasma cell culture contamination, which may induces LPS non specific cell response and may cause an endotoxin independent tolerance, cells were tested for mycoplasma contamination every 2 weeks using the Mycoalert® mycoplasma detection kit (Cambrex, Rockland, USA). Mycoplasm enzymes catalyze the conversion of ADP to ATP. Therefore the test measures the amount of ATP in the supernatant of cells. Luminescence was measured after adding reagents (reading A) to measure the amount of ATP already present in the medium, and after adding substrate, which lyses the viable mycoplasma and therefore, liberate the enzymes responsible for ATP production (reading B).

2.2 Endotoxin tolerance induction

RAW 264.7 macrophages were cultured in DMEM and were plated in 24 wells plates or plastic petri-dishes with respectively 100 000 and 3 000 000 cells (counted by using a hemacytometer). 24h after plating, the cells were initially exposed to 1µg/ml, 0.1µg/ml or 0.01µg/ml LPS (E. Coli 0111:B4) (Sigma-Aldrich, St-Louis, USA) for 24 hr. Non-tolerant cells received an equal volume of DMEM. The cells were serum starved for 24h to prevent serum induced MAPK activation. After the first 24h activation with LPS, the cells were washed 3 times and re-challenged with 1µg/ml of LPS for the second time. After the appropriate incubation time the supernatants were collected and frozen at -20°C to perform TNF-α ELISA. The cells were washed with sterile PBS and mechanically removed from the surface using a rubber spatula. The cells were collected in sterile PBS and centrifuged for 5 minutes at 2000 rpm. The cell pellets were then collected and lysed.

DCs were also plated as explained for RAW 264.7 and initially exposed to 1µg/ml, 0.1µg/ml or 0.01µg/ml LPS for 24h. However dendritic cells were not serum starved, nor washed after the first treatment with LPS (to avoid discarding cells when washing them). The second LPS stimulus (1µg/ml) was just added to the previous. Supernatant and cells were collected in a similar manner as RAW 264.7 cells

2.3 Cell lysis, protein extraction and immunoblotting

Cells were lysed in 20mM Tris-HCl at pH7.5, 150mM NaCl, 5mM EDTA containing 1% NP-40, 1mM Na_3VO_4, 10µg/ml aproptinin and leupeptin, 100µg/ml soybean trypsin inhibitor and

1mM phenylmethylsulfhonyl fluoride (PMSF), incubated on ice for 20min then centrifuged at 20 000g for 20min. The proteins concentration was then determined using the Bradford method. Bovine serum albumin (BSA) was used as a reference (52). The proteins were then boiled at 95°C for 5min in the buffer containing TrisHCl 50nM (pH 6.8), 10% glycerin, 0.05% bromophenol blue, 5%β-mercapto-ethanol and 1% SDS.

40µg of proteins were loaded on a SDS-polyacrylamide gel electrophoresis (SDS-PAGE), proteins were then transferred to a nitrocellulose membrane. The membranes were washed twice with TBS-T buffer then incubated at room temperature in TBS-T containing 5% of nonfat dry milk for 1h to block the non specific binding of antibodies. The primary antibodies were diluted (see table 1) in fresh TBS-T/5% nonfat dry milk or TBS-T/3% BSA (for the phospho-specific antibodies), added to the nitrocellulose membranes and incubated at room temperature for 1h or overnight at 4°C with agitation. The nitrocellulose membranes were then washed 3 times (15min each) in TBS-T buffer. The HRP-conjugated secondary antibody reagent of choice (see table 1) was added for 1 hour at room temperature with agitation. The nitrocellulose membrane was washed 3 times 15 minutes each with TBS-T. The proteins were detected using the enhanced chemiluminescence (ECL) method (ECL and ECL-plus, Amersham Biosciences, Buckinghamshire, UK).

2.4 Antibodies and reagents

The antibodies used for flow cytometry are CD1a-FITC, CD14-APC and HLA-DR PE, purchased from Dako (Glostrup, Denmark); and CD86-PE and CD80-APC purchased from BD (BD biosciences, Franklin Lakes, USA).

All the primary antibodies that were used for Western blotting are mentioned in table 1. Antibodies directed against phosphorylated proteins were diluted in TBS-T + 3% BSA + 0.01% azide to prevent the interaction with milk-components. Other antibodies were diluted in TBS-T + 5% milk powder + 0.01% azide.

The detection of the non-phosphorylated proteins was done as a loading control and performed on the same membrane used for the detection of the phosphorylated form. This was done after stripping the nitrocellulose membrane during 20 min at 70°C after the revelation of the phosphorylated proteins. Afterwards, the membrane was blocked again and re-probed with the antibody against the unphosphorylated form of the protein.

Table 1: List of antibodies. *List of the antibodies used.*

AB directed against	Molecular weight of target protein	Dilution	Produced in	Source
IκBα	37kDa	1/1000	Rabbit	Santa Cruz Biotechnology
MEK 1/2	45kDa	1/1000	Rabbit	Cell Signaling Technology
P-MEK 1/2	45kDa	1/1000	Rabbit	Cell Signaling Technology
P38 MAPK	43kDa	1/1000	Rabbit	Cell Signaling Technology
P-p38 MAPK	43kDa	1/1000	Rabbit	Cell Signaling Technology
p44/42 MAPK	42/44kDa	1/1000	Rabbit	Cell Signaling Technology
P-p44/42 MAPK	42/44kDa	1/2000	Mouse	Cell Signaling Technology
MKP-1	39kDa	1/1000	Rabbit	Sigma
HRP-conjugated anti-mouse	--	1/3000	Sheep	Amersham Biosciences
HRP-conjugated anti-Rabbit	--	1/3000	Donkey	Amersham Biosciences

2.5 TNF-α ELISA

Murine TNF-α in RAW macrophage culture supernatants was detected with a murine TNF-α ELISA ready-set-go detection kit (eBioscience, San Diego, USA). Cells were activated with LPS (double or single stimulation) and the supernatants were collected as described above. To measure the TNF-α, a 96-wells plate was first coated with the capture antibody (clone TN3.19) according to the protocol delivered with the detection kit. After incubation and washing of the standards and the samples supernatant, the biotin-conjugated detection antibody was added. After removing the unbound antibodies by washing the plate, avidin-HRP was added and the excess was washed away. Finally the substrate, tetramethylbenzidine

(TMB), was added. The reaction was stopped by adding 2N of H_2SO_4. The absorption was measured at 450nm. The obtained data for the standard were used for the standard curve, which was used to calculate the concentration of TNF-α in the samples.
Human DCs received also a single or double stimulation with LPS and the supernatants were collected as described above. To measure human TNF-α produced by DCs cells a human TNF-α cytoset^{TM} ELISA kit was used (Biosource, Camarillo, USA). A 96-wells plate was coated with capture antibody against human TNF-α according to the manufacturer's protocol. After incubating the standard and samples and washing the plate, a biotin-conjugated antibody was added. After removing unbound antibodies by washing the plate, strepavidin-HRP was added. After washing away the excess, the substrate, TMB, was added. The reaction was stopped with 2N H_2SO_4 and the absorption measured at 450nm. The standard curve served to calculate the concentration of TNF-α present in the samples.

2.6 Small interfering RNA and transfection

RNAi was first observed as a natural phenomenon in plants, C. elegans and D. melanogaster (53-55). These organisms express anti-sense RNA complementary to an expressed message. The two strands anneal to generate long double-stranded RNA (dsRNA). An enzyme known as Dicer digests long dsRNA into short (< 30 nucleotide) RNA duplexes having 3-prime two-nucleotide overhangs and 5-prime phosphates. These molecules are known as small interfering RNA (siRNA). A complex of proteins known as the RNA Induced Silencing Complex (RISC) then unwinds siRNA and uses one strand to identify other copies of the original message by the annealing of identical sequences. RISC cuts the mRNA in the middle of the shared sequence leaving the message susceptible to degradation by exonucleases thereby silencing the expression of the corresponding gene.

RNAi also occurs in the more commonly used model system of mammalian cells, which contain a conserved machinery (56). Experimental methods take advantage of the endogenous mechanism to suppress the expression of genes of interest by the exogenous introduction of nucleic acid. Some model systems accept antisense RNA or even dsRNA. Experimental RNAi in the mammalian system involves the introduction of siRNA, typically 21 to 23 base pair duplexes, or small hairpin RNA (shRNA). We have chosen to use the RNAi duplexes because shRNA is time consuming (requires prior cloning). To extinguish MKP-1, the ON-TARGET plus SMARTpool of 4 different target sequences against MKP-1 mRNA was used (Dharmacon, Lafayette, USA). The different RNAi duplexes pooled sequences were as folow:

duplex 1 sense : CCAAUUGUCCCAACCAUUUUU - duplex 1 anti-sense : 5'-PA AAUGGUUGGGACAAUUGGUU. Duplex 2 sense : GCAUAACUGCCUUGAUCAAUU - duplex 2 anti-sense: 5'-PUUGAUCAAGGCAGUUAUGUU. Duplex 3 sense: GCGCAA GUCUUCUUCCUCAUU - duplex 3 anti-sense: 5'PUGAGGAAGAAGACUUGCGCUU. Duplex 4 sense: GAAGGGUGUUUGUCCACUGUU – Duplex 4 anti-sense : 5'-PCAGUGGACAAACACCCUUCUU. RAW 264.7 cells were plated in a six-wells plate in complete medium without antibiotics, after 24 hours they were transfected using oligofectamine reagent (Invitrogen, Carlsbad, USA) and SMART pool siRNA for MKP-1 (200nM) mixed in DMEM medium. Cells treated only with oligofectamine were used in parallel as a control. 4 hours after transfection, DMEM complete medium was added to the cells. 48 hours after transfection, the cells supernatants were collected for TNF-α measurement, the cells were lysed and the expression of MKP-1 was verified by western blotting to test the efficiency of the RNAi tested.

2.7 Buffers and solutions

PBS: 0.137M NaCl, 2.7mM KCl, 0.01M Na_2HPO_4, 1.4mM KH_2PO_4 and desionized water.

PBS-T: PBS auditioned with 0.05% Tween.

Lysis buffer: 20mM Tris-HCl at pH7.5, 150mM NaCl, 5mM EDTA containing 1% NP-40, 1mM Na_3VO_4, 10µg/ml aproptinin and leupeptin, 100µg/ml soybean trypsin inhibitor and 1mM phenylmethylsulfhonyl fluoride.

Denaturation buffer: TrisHCl 50nM (pH 6.8), 10% glycerin, 0.05% bromophenol blue, 5%β-mercapto-ethanol and 1% SDS.

SDS running buffer : 0.05M Tris, 0.384M glycin and 0.1% SDS in desionized water.

Transfer buffer : 0.025M Tris, 0.192M glycin and 10% methanol in desionized water.

TBS-T: 0.02M Tris, 0.137M NaCl, and 0.1% Tween at 20%.

Stripping buffer: 2% SDS, 50mM Tris-HCl at pH6.8 and 100mM β-mercapto-ethanol in desionized water.

3 Results

To study the MAPKs activation in an endotoxin tolerance model, two different cell types were used, the murine macrophage cell line, RAW 264.7, and primary human DCs differentiated from peripheral blood monocytes. The first part of the results will describe the murine macrophage model while the second part will describe the results obtained using the human DCs.

3.1 Murine macrophages endotoxin tolerance model

This part of the results will describe the observations made in the murine macrophages endotoxin tolerance model.

3.1.1 Cytokine production profile

To set up the LPS endotoxin tolerance model on RAW 264.7 murine macrophages, we decided to measure first the TNF-α secretion after LPS treatment as readout. Indeed, it is well known that these cells, when challenged twice with LPS, do not produce TNF-α (48).

The cells were seeded in 24-wells plates prior to stimulation. After 24h, cells were washed twice with DMEM serum free medium then incubated in DMEM serum free medium with or without LPS at three different concentrations (0.01µg/ml; 0.1µg/ml or 1µg/ml). After 24h in culture, all cells were (re)stimulated with a fixed concentration of LPS (1µg/ml). The supernatant of the cell culture was removed and the amount of TNF-α produced by the cells was measured by ELISA.

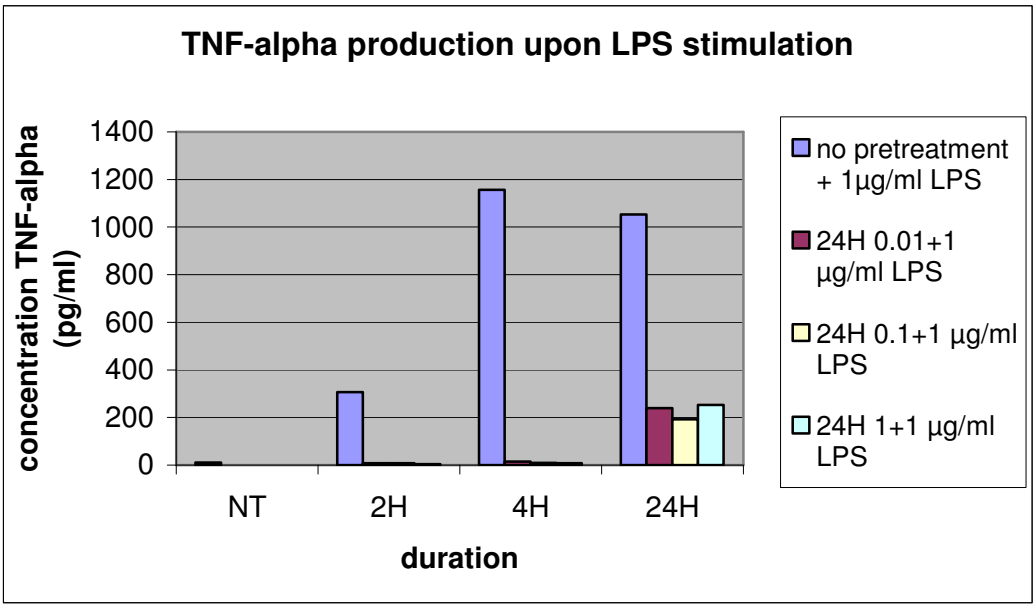

Figure 5: TNF-α production of RAW 264.7 cells upon stimulation with LPS. *One stimulation with 1µg/ml LPS induces TNF-α production. Cells that were restimulated with a fixed concentration of LPS (1µg/ml) after LPS pretreatment at different concentrations (0.01µg/ml; 0.1µg/ml or 1µg/ml) show a lower TNF-α production of TNF-α. As a negative control untreated cells were used (NT).*

As shown in figure 5 stimulation with LPS induces the production of the pro-inflammatory cytokine TNF-α. This secretion increases gradually after LPS single stimulation to reach the maximum of 1200 ng/ml after 4h of treatment and stabilizes at 24h. However, upon a second stimulation with LPS, cytokine production diminishes to become almost undetectable. This tendency diminishes after 24 hours although the level of TNF-α produced upon second stimulus is still remarkably lower than after one single stimulus with LPS.

3.1.2 Phosphorylation pattern of MAPKs

It has been previously demonstrated that endotoxin tolerance is associated with inhibition of several MAPKs such as ERK and MEK (46, 47). To validate our model, the activation of the MAPKs MEK, ERK and p38 was investigated after a double or single stimulation with LPS. Since MAPKs are known to be activated by serum, all the experiments described below were performed in serum free medium to avoid LPS non specific MAPKs activation. RAW 264.7 cells were cultivated in petri-dishes in DMEM serum free medium for 24 hours with or without 1µg/ml LPS. Afterwards cells were washed 3 times in 37°C pre-warmed PBS then (re)stimulated with 1µg/ml LPS for several time points. Cells were then lysed in TNE buffer. 40µg of proteins obtained from these cells were separated on a SDS-page gel and blotted on a nitrocellulose membrane then immunoblotted with antibodies directed against both phosphorylated and unphosphorylated forms of MEK, ERK and p38 MAPKs.

Proteins of unstimulated cells served as a negative control. The cells were tested for mycoplasma to avoid LPS independent MAPKs activation.

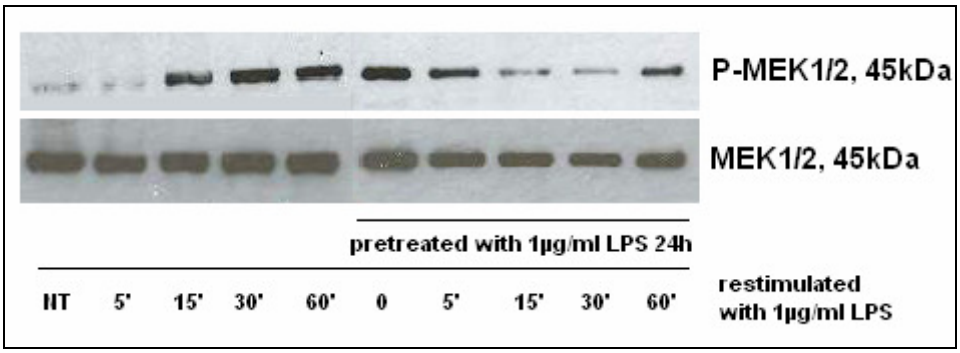

Figure 6: Phosphorylation status of MEK (MAPKK) after single or double stimulation with 1µg/ml LPS. *RAW 264.7 cells were pretreated or not with 1µg/ml LPS for 24 hours in a serum free medium. After 24 hours, cells were restimulated with 1µg/ml LPS for indicated time points. Non treated (NT) cells were used as as a negative control (first lane). A blot for P-MEK (upper panel) shows the phosphorylation status of the MEK protein whereas the blot for MEK (lower panel) serves as a loading control.*

Figure 6 shows an increase in phosphorylation of MEK upon treatment with LPS, however, cells that received a second stimulus with LPS show a decrease in phosphorylation compared to the cells that were stimulated once. After 60 minutes this decrease is less pronounced but still present.

To see whether these results could also be obtained for ERK and p38, the phosphorylation status of these two kinases was evaluated using anti-phospho ERK and anti-phospho-p38 antibodies. Figure 7 shows the phosphorylation patterns of p38 and ERK1/2 (p44/p42) in RAW 264.7 cells together with their loading control (respectively p38 and ERK1/2). Upon a single LPS treatment (1µg/ml) the phosphorylation of p38 increases slightly 5min after the initiation of the activation and reaches the maximum at 15min. This phosphorylation decreases at 30min to become almost undetectable 1 hour after the addition of LPS to the culture medium. At 24h after the first activation with LPS, a high phosphorylation of p38 MAPK is observed. After the second LPS addition (1µg/ml), the phosphorylation of p38 increases further until 30min and stabilizes till 2h.

The results are similar regarding the phosphorylation status of ERK1/2 (p42/p44). Indeed, ERK1/2 phosphorylation increases after the first LPS activation to reach the maximum at 30min but no phosphorylation was detected 30 min later. ERK1/2 becomes phosphorylated again 24h after the initiation of the LPS activation. Similarly to the results observed for p38,

upon the second LPS activation, the phosphorylation of ERK1/2 increases and reaches a plateau after 30 min till 2h.

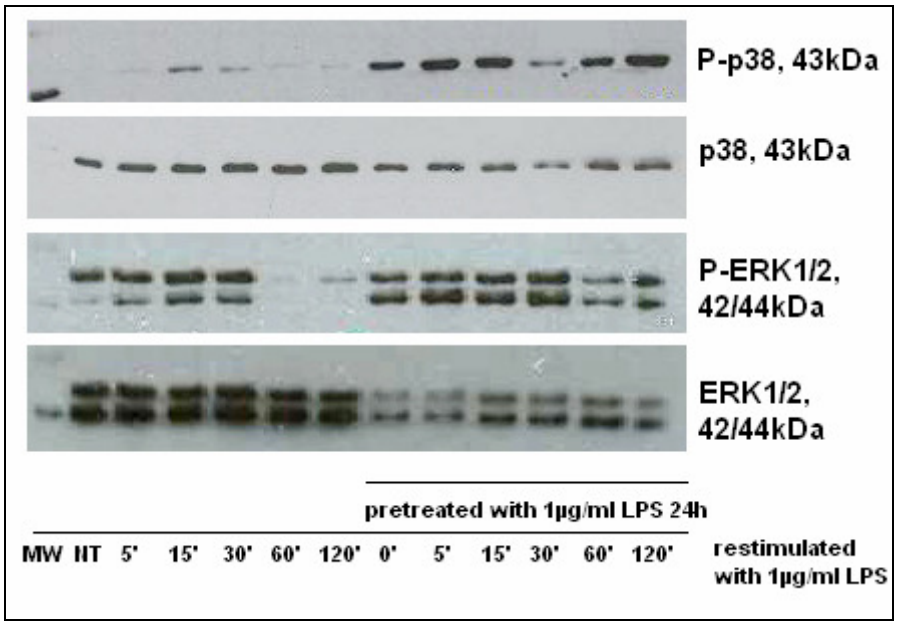

Figure 7: The phosphorylation pattern of the MAPKs p38 and ERK1/2 (p42/p44) in RAW 264.7 cells upon LPS stimulation. *The phosphorylation pattern of p38 MAPK and ERK1/2 upon single and double LPS (1µg/ml) stimulation. P38 and ERK1/2 serve as the loading control. Not treated (NT) cells are a negative control. These results are representative of 3 independents experiments. MW: molecular weight.*

3.1.3 Degradation of IκB

Endotoxin tolerance is also associated with a suppressed degradation of IκBα and IκBβ which attenuates nuclear translocation of NF-κB and therefore would inhibit gene transcription (46, 47). To validate our model, the IκBα degradation after single or double stimulation with LPS was investigated. The cell lysates prepared as described above were immunobloted with an antibody directed against IκBα. As shown in figure 8, there is an important decrease of IκBα 15 minutes after LPS stimulation. 30 min after LPS activation, no IκBα is detected. After this time-point IκBα reappears and sustains till 2h. However, when the cells are challenged twice with LPS, IκBα is not degraded.

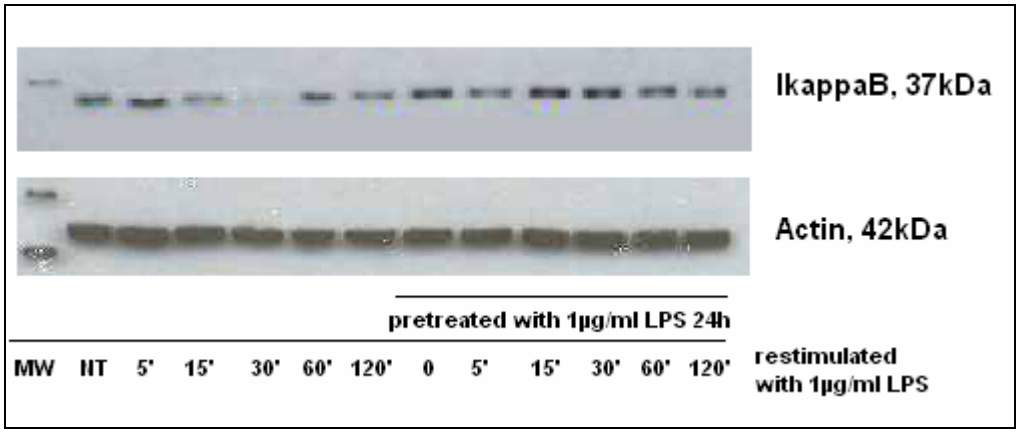

Figure 8: Degradation pattern of IκBα after LPS stimulation. *RAW 264.7 cells were either pretreated or not with 1µg/ml LPS for 24 hours. Cells were then restimulated with 1µg/ml LPS for indicated time points. Non treated (NT) cells serve as a negative control. A blot for IκBα shows the degradation at 15 and 30 min after a single LPS stimulation. No degradation of IkB is observed when cells are treated twice with LPS (upper panel). Actin serves as a loading control (lower panel).MW: molecular weight.*

3.2 The role of MKP-1 in endotoxin tolerance

It has been recently described that knockout of *MKP-1* sensitizes mice to LPS induced endotoxic shock (57). Zhao Q and his coworkers demonstrated that upon LPS challenge, MKP-$1^{-/-}$ cells exhibit prolonged p38 and JNK activation as well as enhanced TNF-α IL-6 production compared with wild-type cells. Thus, MKP-1 seems to be a feedback control regulator of the innate immune response and plays a critical role in suppressing endotoxic shock. To evaluate the role of this MAPK phosphatase in our in vitro LPS tolerance model, the MKP-1 gene was silenced using the RNA interference technology. RAW 264.7 cells were transfected with siRNA for MKP-1 or with oligofectamine as control. 48h after transfection, the cells were lysed and an MKP-1 blot was performed to determine the efficiency of the MKP-1 silencing. Figure 9 shows that cells transfected with siRNA for MKP-1 express less MKP-1 compared to Mock cells (oligofectamine alone). The densitometric ratio (MKP1/loading control) shows 50% decease of MKP-1 expression in siRNA MKP-1 compared to control (figure 10).

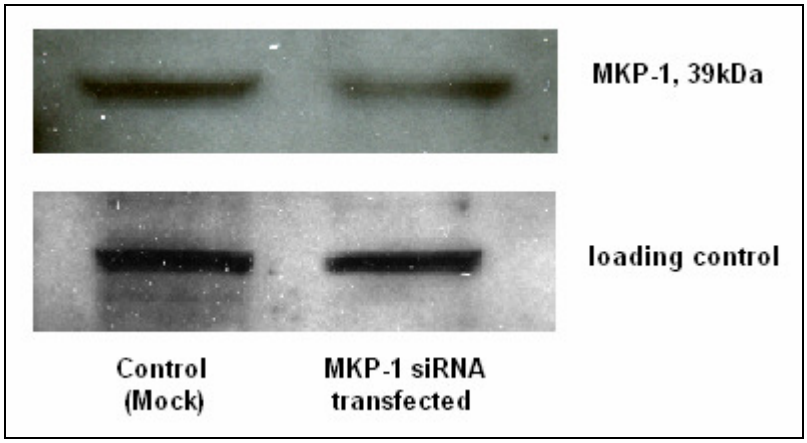

Figure 9: MKP-1 expression in RAW 264.7 cells after MKP-1 siRNA transfection. *MKP-1 levels are compared between Mock cells, treated with oligofectamine, and MKP-1 siRNA transfected cells. Actin was used as a loading control.*

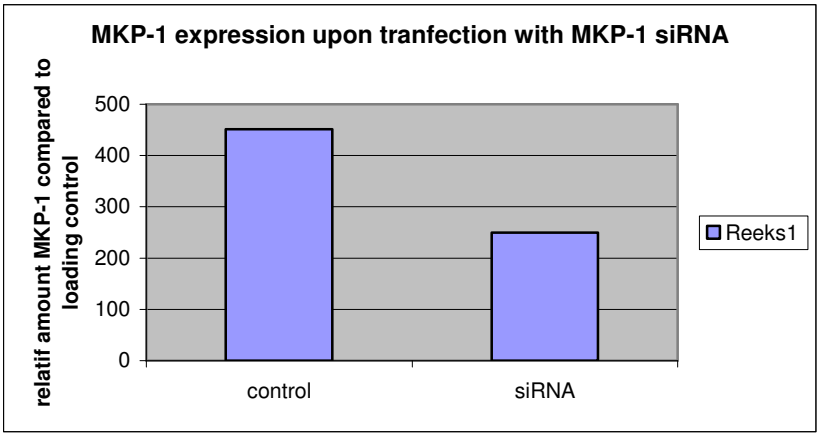

Figure 10: Densitometric analysis for MKP-1 expression in MKP-1 siRNA transfected cells and actin. *The relative amount of MKP-1 expression is obtained by comparing the ration between MKP-1versus actin densities.*

3.2.1 TNF-α secretion after MKP-1 silencing

To evaluate the role of MKP-1 in our LPS tolerance model, RAW 264.7 cells were transfected with siRNA for MKP-1 or Mock. 24h after transfection, the cells were activated (or not) with LPS at different concentrations (0.01, 0.1 and 1µg/ml) for an additional 24h. The cells were then washed twice and re-stimulated for different time points with LPS (1µg/ml). The supernatants of the cells were removed to perform an ELISA for TNF-α. Figure 11 shows that TNF-α production at time point 0 (non LPS treated cells) for both siMKP-1 and Mock cells were abnormally high (500 pg/ml) compared with the results obtained previously. The LPS activation did not increase the TNF-α secretion remarkably. All these abnormalities make the interpretation of these results very difficult.

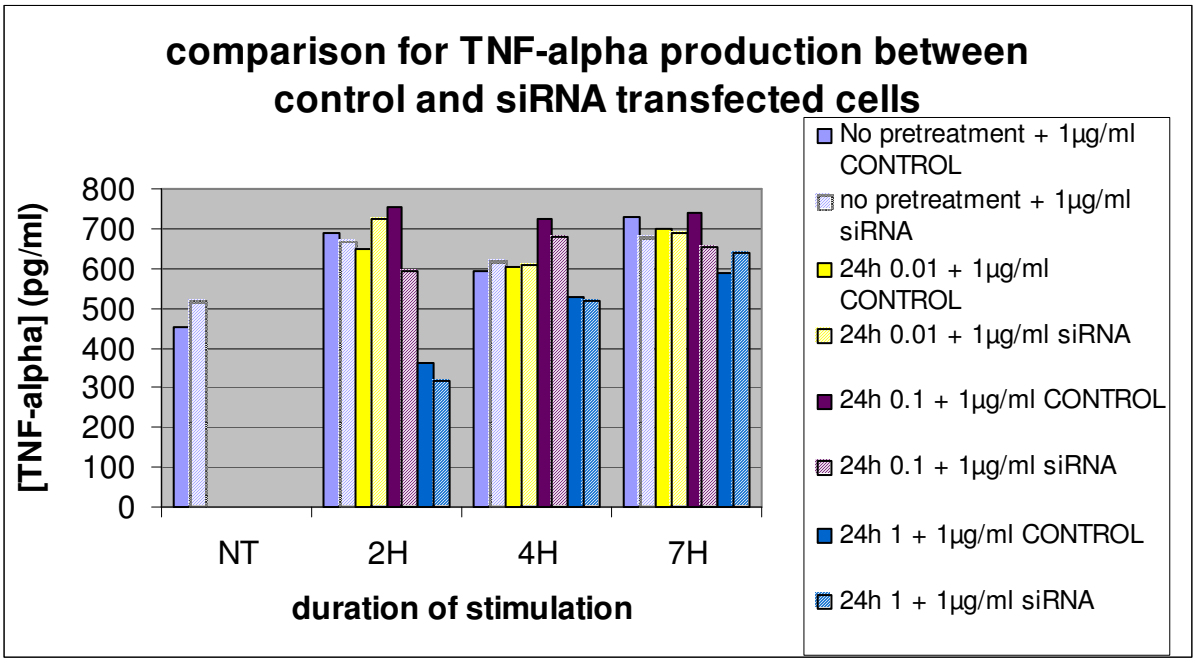

Figure 11: TNF-α production of RAW cells compared between control and MKP-1 siRNA transfected cells upon LPS stimulation. *Mock cells treated with oligofectamine (control, full bars) or transfected with MKP-1 siRNA (striped bars) were pretreated with 0, 0.01, 0.1 or 1µg/ml LPS for 24h, cells were restimulated with 1µg/ml LPS. Control and transfected cells, not treated (NT) with LPS, serve as negative controls.*

3.3 Human dendritic cell endotoxin tolerance model

In parallel with setting up an endotoxin tolerance in murine macrophages, the setting of an endotoxin tolerant model for primary human DCs was started.

3.3.1 Preparation of human dendritic cells

DCs were prepared as described in the materials and methods. Briefly, monocytes prepared from fresh human PBL were differentiated during 7 days to dendritic cells using IL-4 and GM-CSF. The quality of the differentiated cells was evaluated by flow cytometry upon immunostaining with anti-CD1a, anti-CD14, anti-CD80, anti-CD86 and anti-HLA antibodies and based on the cell size and granulosity. As shown in figure 12, DCs show a high FSC and a high SSC (panel A). DCs show a shift in CD1a and are $CD14^{low}$, which are maturation markers (panel B). They are highly positive for HLA, an activation marker (panel C); furthermore there is a high level of CD80 positive and some CD86 positive DCs, both activation markers (respectively panel D and E).

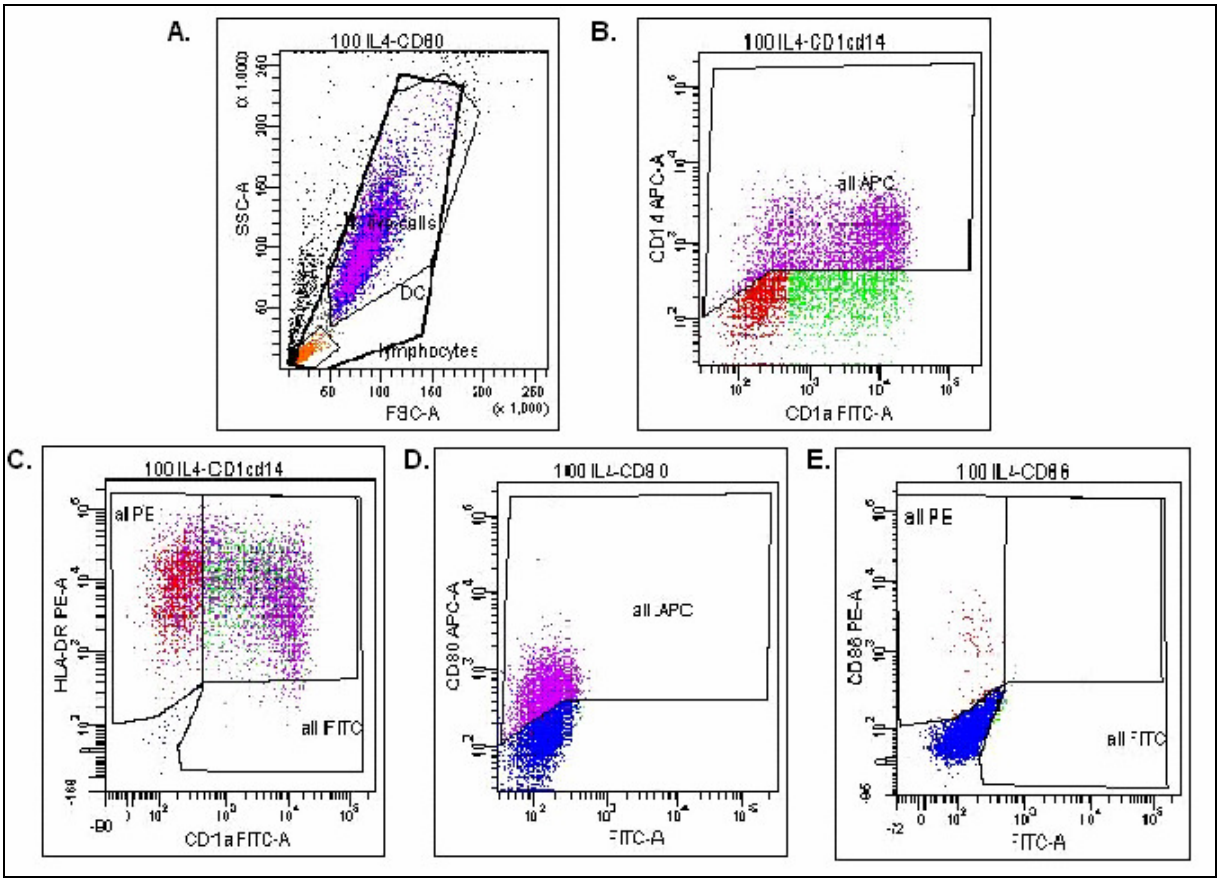

Figure 12: Immunophenotyping of the differenciated DCs. *A) a dot plot of in vitro differentiated DCs. X-axis represent the FCS and Y-axis SSC. The DCs gate was set at high FSC and high SSC. DCs gated on A show a shift in CD1a and are low in CD14 (B), are highly positive for HLA-DR (C), 50% of the gated DCs are CD80$^+$ (D) and are mostly negative for CD86 (E).*

3.3.2 Cytokine production profile

DCs prepared as described above were plated 24 hours before starting LPS treatments in 24-wells plates in complete RPMI. LPS was then added at concentration of 0.01, 0.1 and 1µg/ml. 24h later, cells were (re)stimulated with a fixed LPS concentration (1µg/ml) for 2, 4 or 7 hours. Supernatants were removed and TNF-α secretion was determined using the ELISA method. The obtained results are shown in figure 13. Cells that were not pretreated with LPS and received just once LPS (1µg/ml) show an increase in TNF-α production after 2 hours. The longer cells are stimulated the more TNF-α is produced to reach its maximum 7 hours after addition of LPS to the culture. However, when the DCs have been pretreated with LPS for 24h with either 0.01, 0.1 or 1µg/ml LPS and were restimulated with a fixed concentration LPS (1µg) for 2, 4 or 7 hours, these cells were not able to produce TNF-α.

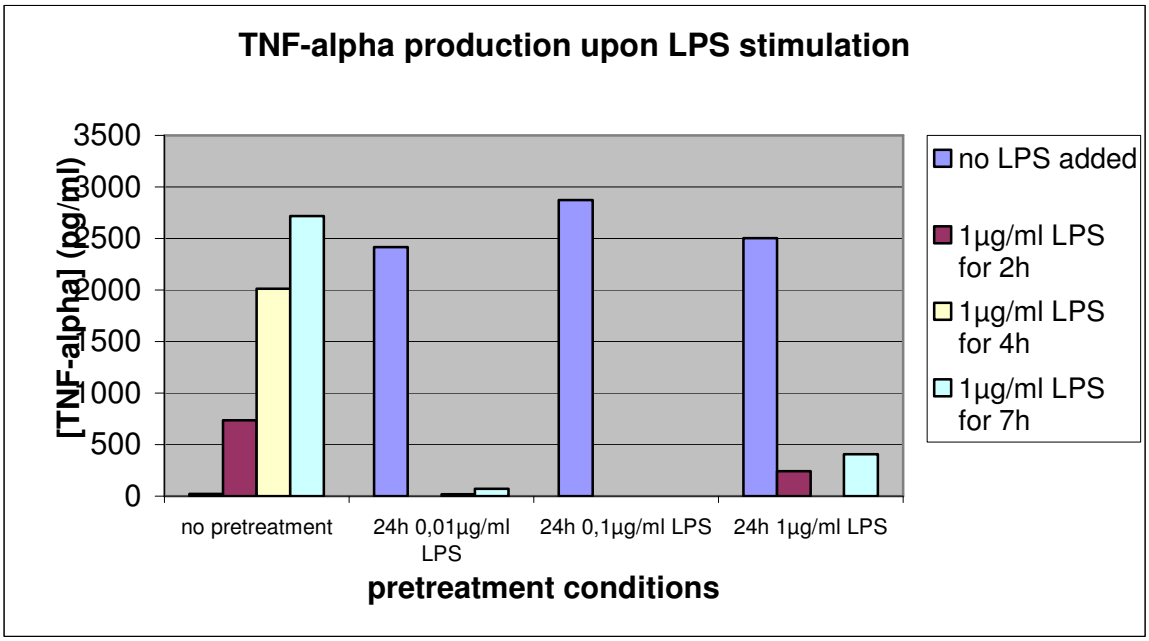

Figure 13: TNF-α production of human DCs upon stimulation with LPS. *Cells that were not pretreated show an increase in TNF-α production upon restimulation. Cells that were pretreated with different concentrations of LPS (respectively 0.01;0.1 and 1µg/ml) show a strong decrease in TNF-α production upon restimulation with a fixed LPS concentration (1µg/ml).*

3.3.3 Phosphorylation pattern of MAPKs

To see whether a difference in the phosphorylation pattern of MAPKs could also be observed in the LPS tolerized DCs, these cells were cultivated in petri-dishes, serum starved for 24 hours and either pretreated or not with 1µg/ml LPS for 24 hours. Afterwards cells were (re)stimulated with 1µg/ml LPS for several time points. 40µg of proteins obtained from these cells were separated on a SDS-page gel and transferred to a nitrocellulose membrane then immunoblotted with antibodies directed against both phosphorylated and unphosphorylated forms of MAPKs ERK and p38. Proteins of unstimulated cells were used as a negative control. Mycoplasma test was performed before using the cells to make sure that cells were not activated prior to LPS stimulation. The obtained phosphorylation patterns for ERK1/2 and p38 are shown in figure 14. Densitometric analysis on these results is shown in figures 15 and 16.

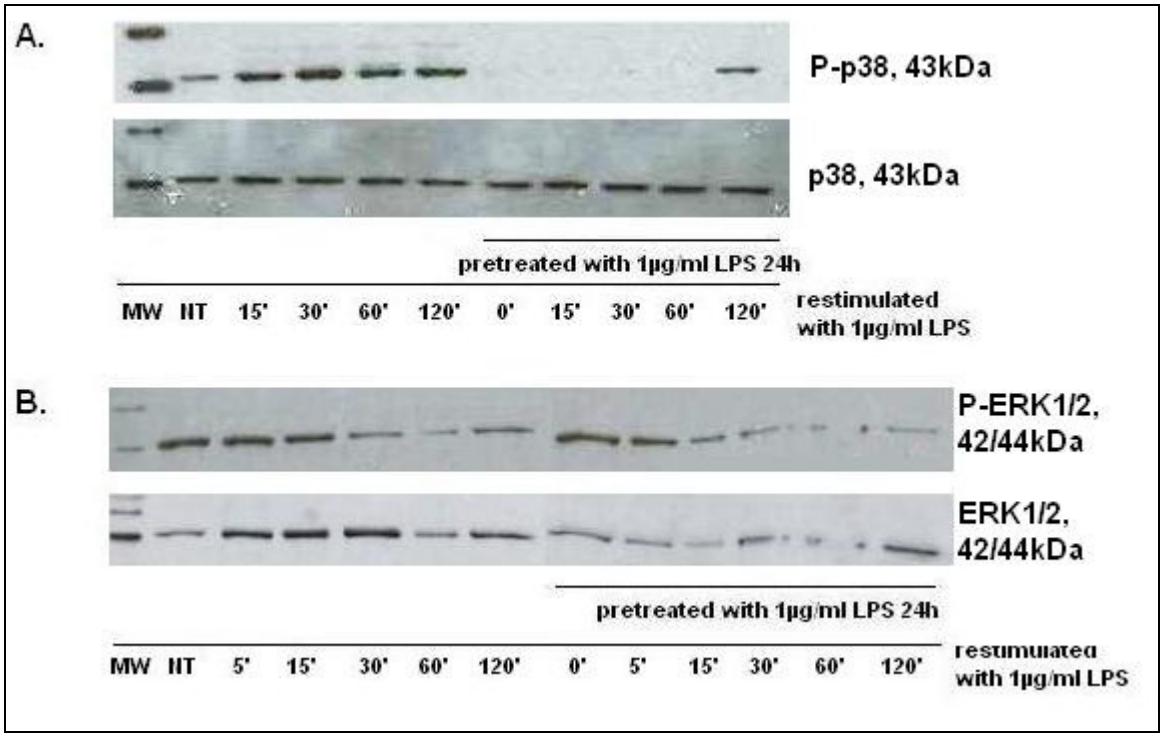

Figure 14: The phosphorylation pattern of MAPKs in DCs upon LPS stimulation. *A)The phosphorylation pattern of p38 MAPK upon single and double LPS (1µg/ml) stimulation. P38 serves as the loading control. B)The phosphorylation pattern of ERK1/2 upon single and double LPS (1µg/ml) stimulation. ERK1/2 serves as the loading control. Non treated (NT) cells are shown as a basal level for p38 and ERK1/2 activation. MW: molecular weight.*

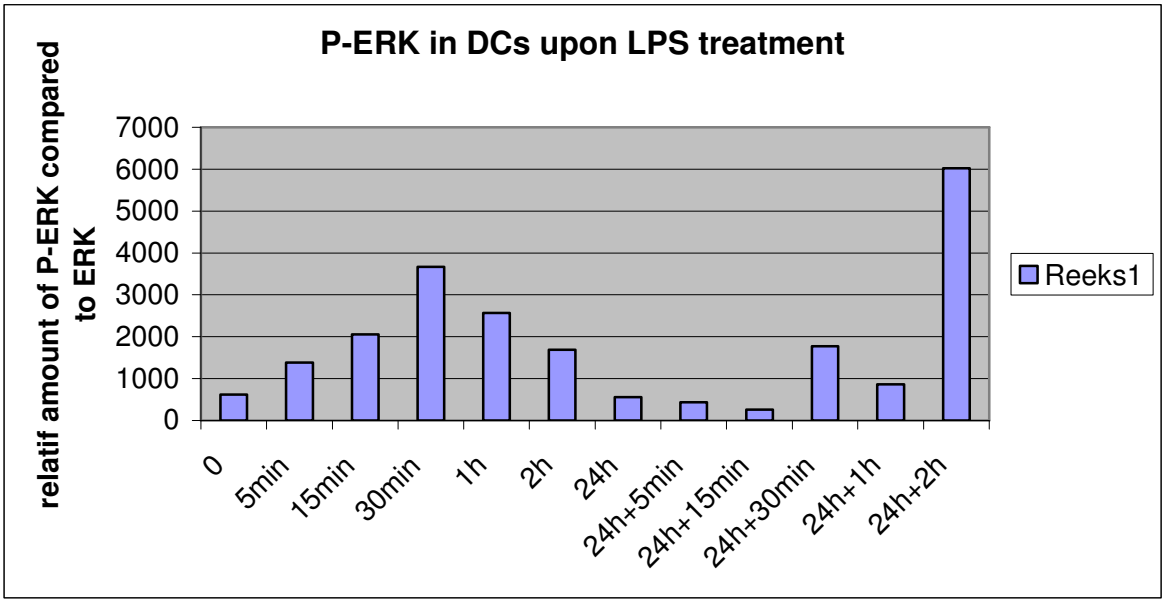

Figure 15: Densitometric analysis for the phosphorylation pattern of ERK1/2. *Phosphorylation levels of ERK1/2 are shown as a ratio between phospho-ERK1/2 and ERK1/2. Cells were either non stimulated (point 0), stimulated once (for 5min, 15min, 30min, 1h, 2h and 24h) or twice (24h+5min, 24h+15min, 24h+30min, 24h+1h and 24h+2h) with LPS (1µg/ml).*

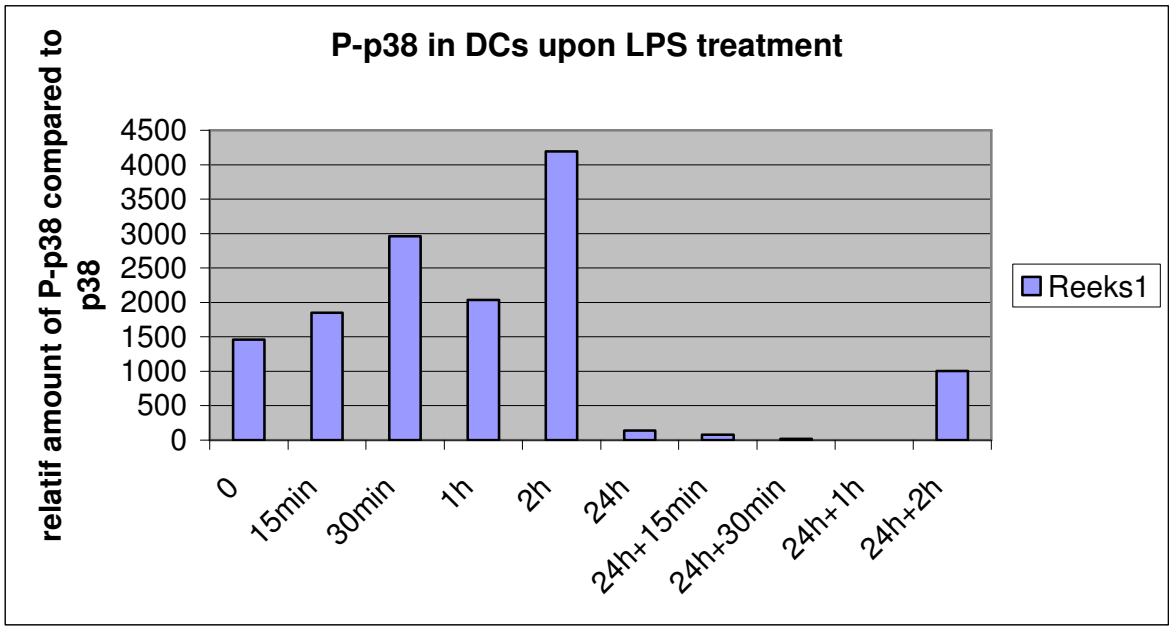

Figure 16: Densitometric analysis for the phosphorylation pattern of p38. *Phosphorylation levels of p38 are shown as a ratio between phospho-p38 and p38. Cells were either non stimulated (point 0), stimulated once (for 5min, 15min, 30min, 1h, 2h and 24h) or twice (24h+5min, 24h+15min, 24h+30min, 24h+1h and 24h+2h) with LPS (1µg/ml).*

Figures 14, 15 and 16 show a gradual increase in phosphorylation of both p38 and ERK1/2 upon a single stimulation with LPS. ERK1/2 reaches the maximum of its phosphorylation 30min after the initiation of the LPS activation and decreases gradually to reach the basal level 24 hours later. Restimulation with LPS induces a very discrete phosphorylation of ERK1/2 peaking 2h after the addition of LPS. As for p38, we did not observe any phosphorylation of this MAPK after restimulation of the DCs with LPS at early time points (15min, 30min and 1h) while an increase in phosphorylation was detected 2h after the initiation of the LPS reactivation.

3.3.4 Degradation of IκB

An immunoblot for IκBα degradation was also performed on cell lysates of human DCs that were stimulated once or twice with 1µg/ml LPS. Results can be seen in figure 17. As for the RAW 264.7 murine macrophages, an immunoblot for IκBα was performed after single or double LPS stimulation of human DCs. Figure 17 shows an important decrease of IκBα 15 minutes after LPS stimulation. 30 min after LPS activation, no IκBα is detected. After this time-point IκBα reappears and still sustains till 2h. However, when the cells are challenged twice with LPS, IκBα is not degraded and still present in non treated cells.

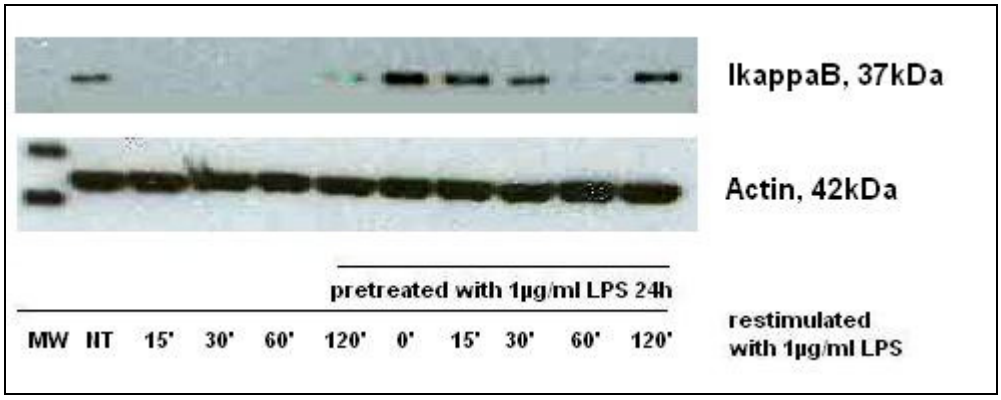

Figure 17: Degradation pattern of IκBα after LPS stimulation. *DCs were either not pretreated or pretreated with 1µg/ml LPS. After 24h all cells were restimulated with 1µg/ml LPS for the indicated time points. Non treated (NT) cells serve as a negative control. A blot for IκBα shows the degradation after 15 min with a single LPS stimulation. No degradation of IκB is observed when cells are treated twice with LPS (upper panel). Actin serves as a loading control (lower panel). MW: molecular weight.*

4 Discussion and conclusion

Endotoxin tolerance was described for the first time by Favorite and Morgan in 1942 (58). Decades later, macrophages were identified as pivotal cellular participants in the acquisition of the tolerant phenotype (59). Endotoxin tolerance is not limited to the potent TLR4 agonist, enterobacterial LPS, but also targets signaling initialized by agonists that are recognized by other members of the Toll/IL-1R family (37, 50). The major signaling components leading to NF-κB translocation have been reported to be perturbed in LPS-tolerized macrophages (37) as well as the MAPKs pathways (46, 47). In recent years, evidence has emerged that, similar to MAPKs, MKPs play a pivotal role in the regulation of immune responses and particularly in the innate immune response. However, except for the MKP-1, the role of the different MKPs in the endotoxin tolerance is still largely unknown. Our laboratory is particularly interested in the investigation of the role or several MKPs in the LPS-induced tolerance. Thus, it was necessary to establish a simple in vitro endotoxin tolerance model using a macrophage cell line.

RAW 264.7 murine macrophages cell line was chosen to set up such a model because these cells are easy to cultivate and are easy to transfect. LPS was used as TLR4 agonist and TNF-α cytokine secretion was used as readout for the tolerance state. In the last part of this work human DCs were also used to investigate if the tolerant conditions in the murine macrophages cell line can be applied to human DCs.

It has been observed that TNF-α production profile for RAW 264.7 murine macrophages shows a clear increase in TNF-α production upon one stimulus with LPS. When stimulating a second time, cells produce remarkably less TNF-α. The phosphorylation of MEK1/2 in these cells increases upon stimulating once with LPS and after a second treatment the phosphorylation decreases. p38 doesn't get highly phosphorylated until 24hours after a first LPS treatment and upon a second stimulus there was only a small decrease that was still high compared with basal conditions. ERK1/2 gets phosphorylated after one treatment LPS but 1 and 2 hours after the first stimulus the phosphorylation level decreases and ERK1/2 returns phosphorylated after 24 hours with a single treatment. Upon a second addition of LPS, P-ERK1/2 decreases only slightly after 1 hour. However, IκB gets degraded upon a single stimulus but remains intact upon a second treatment with LPS. The MKP-1 siRNA tranfection performed had an efficiency of 50% but there is no difference visible for the TNF-α production profile after LPS stimulation when comparing Mock cells and siRNA transfected cells, also the basal level of TNF-α was already high in both conditions.

DCs show a pronounced phosphorylation pattern, both for p38 and for ERK1/2 there is an increase in phosphorylation upon one LPS treatment and a decrease after a second treatment. IκB degrades after the first LPS stimulus and remains intact upon a secondary treatment.

The results from the RAW 264.7 TNF-α cytokine production profile were similar to those obtained by Cagiola et al who used also a variable concentration of LPS for pretreatment and a fixed concentration to restimulate the cells (42).Other authors also showed hat upon a single LPS stimulus the production of pro-inflammatory cytokines increases and that upon inducing a secondary stimulus with LPS a decrease in pro-inflammatory cytokine production, especially TNF-α, occurs (41-43). Bowling, Virca and Medvedev also shown this in RAW 264.7 cells (46, 48, 49). Because this cytokine production profile is characteristic for endotoxin tolerance (37, 41-43, 46, 48, 49), it can be concluded that we were able to induce tolerance in RAW 264.7 murine macrophages according to the TNF-α production profile.

According to the literature, the endotoxin tolerant phenotype is not only characterized by a decrease in pro-inflammatory cytokine production, but also by a decrease in phosphorylation of MAPKs (37, 46-49). Our RAW 264.7 murine macrophage model shows a similar phosphorylation profile of MEK1/2 as seen in literature. For ERK and p38 this pattern was less pronounced as the one seen in literature (46, 47) The increase in phosphorylation of p38 is most clear after 30 minutes stimulation with one single dose LPS, which is consistent with the results of Medvedev et al, although Medvedev did not show any increase after 24hours with one treatment (46). Furthermore, we were only able to show a very small decrease in phosphorylation after a secondary LPS treatment of 30 minutes, whereas Medvedev et al observed a decrease at all time points (46). The increase in phosphorylated ERK is already high after 5 minutes of LPS stimulation but decreases after 1 and 2 hours stimulation. A decrease in phosphorylation can also be seen after 1 hour with a second stimulus of LPS. In the article of Medvedev et al it is found that cells treated with one stimulus of LPS also show a lower extend in phosphorylation of ERK1/2 after 1 hour compared to the phosphorylation after 30 min. However upon restimulation they show a strong decrease in phosphorylation already after 5 minutes (46). Clearly our results are not similar to the results obtained by Medvedev et al, in RAW 264.7 murine macrophages (46). Tominaga et al reported the same result (as Medvedev) using primary peritoneal murine macrophages. Additional experiments with different concentration of LPS need to be done. In addition, these experiments certainly

need to be performed in primary macrophages. Although, the TNF-α readout was similar in our culture conditions compared to experiment of the two different groups cited.
Tominaga et al and Medvedev et al further have shown that endotoxin tolerance is also accompanied by an impaired activation of NF-κB (46, 47). This was shown by the degradation of IκB. When IκB is degradated NF-κB becomes active (9, 10). In RAW 264.7 cells a degradation of IκB and thus an activation of NF-κB is obtained after one stimulus with LPS for 15 and 30 minutes. A small increase in IκB and thus a decrease in NF-κB activation is visible after secondary LPS treatment for 15 and 30 minutes. These results are consistent with the results obtained by other authors (46, 47) and by results of experiments done on human THP-1 cells done in collaboration with another group in our host institution (Sadzot C, personal communication). All together, these results suggest that we have obtained endotoxin tolerance in RAW 264.7 murine macrophages.

As for MKP1 silencing, transfected cells show only 50% decrease in MKP-1 compared with control cells. This is probably due to difficulties in tranfecting RAW 264.7 cells. Further experiments need to be performed to improve the transfection efficiency and thus, better silencing for this phosphatase. However, this low silencing efficiency does not explain by its own the results obtained for TNF-α production between transfected and not transfected cells. According to literature, MKP-1 knock-out is accompanied by an enhanced TNF-α production compared to control conditions (26). However, in our preliminary experiments, no increase of TNF-α was observed, furthermore, in this specific experiment, an inhibition of TNF-α secretion is not even seen in the control conditions (LPS-LPS treated non transfected cells) as obtained in all the experiments performed earlier. The high basal level of TNF-α secretion suggests that the cells were activated. However, the Mycoalert® mycoplasma test was performed prior to the transfection and turned negative. A more sensitive mycoplasma test such as PCR test should be performed to definitely exclude any contamination.

In DCs, similar results concerning the TNF-α cytokine production were obtained as for the RAW 264.7 cells, namely a decrease in TNF-α production upon restimulation with LPS. This is consistent with what has been described in literature (60). Thus, based on the TNF-α production profile in DCs, it can be concluded that endotoxin tolerance was achieved in this model.

The model based on DCs also shows a more pronounced phosphorylation pattern as the model based on RAW 264.7 cells, both for p38 and for ERK1/2, which is more consistent with literature (46, 47). There is a strong increase in phosphorylation after one LPS stimulus for both p38 and ERK1/2. Upon secondary treatment P-ERK1/2 and P-p38 decrease strongly. However, the MAPKs studied were phosphorylated in non stimulated conditions. These results are in agreement with the flow cytometry analysis showing a slight increase in CD80 and CD86 in the cells used in this experiment. This suggests that these cells were somehow activated. Furthermore cells were not serum starved before treating them with LPS to avoid the loss of non-adherant cells. Indeed, serum is a known activator for MAPKs. In the future this problem will be solved by performing negative cell sorting and optimizing the differentiation and maturation conditions of DCs.

For the degradation pattern of IκB in DCs, a similar, although more pronounced pattern of IκB degradation was observed in DCs compared to RAW 264.7 cells. Again the pattern observed is consistent with the literature (46, 47).

Some general conclusions that can be drawn from these ELISA readouts and blots is that we have indeed obtained an endotoxin tolerant model that is charactarized by a decrease in pro-inflammatory cytokine production, a decrease in phosphorylation of MAPKs and an impaired activation of NF-κB. Furthermore it is clear that our model also shows that tolerance is a transient state (34-36) because TNF-α production increases again after 24hours restimulating in RAW 264.7 cells. Further experiments need to be performed both for DCs and RAW macrophages regarding the DCs purification, differentiation and maturation, better transfection conditions for RAW 264.7 cells and a more sensitive mycoplasma assay such as PCR. The LPS tolerisation experiments should also be extended to primary murine peritoneal macrophages.

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