TUIJA HÖMMÖ

Mitogen-activated Protein Kinase Phosphatases in Inflammation and as Anti-inflammatory Drug Targets

ACADEMIC DISSERTATION
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UNIVERSITY OF TAMPERE

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II Korhonen R, Turpeinen T, Taimi V, Nieminen R, Goulas A and Moilanen E (2011) Attenuation of the acute inflammatory response by dual specificity phosphatase 1 by inhibition of p38 MAP kinase. Molecular Immunology 48:2059-2068.

III Turpeinen T, Nieminen R, Taimi V, Heittola T, Sareila O, Clark AR, Moilanen E and Korhonen R (2011) Dual specificity phosphatase 1 regulates human inducible nitric oxide synthase expression by p38 MAP kinase. Mediators of Inflammation Article ID 127587, 1-15.

IV Hömmö T, Pesu M, Moilanen E, Korhonen R (2014) Regulation of inflammatory cytokine production by MKP-5 in macrophages. Submitted for publication.

V Korhonen R, Hömmö T, Keränen T, Laavola M, Hämäläinen M, Vuolteenaho K, Lehtimäki L, Kankaanranta H, Moilanen E. (2013) Attenuation of TNF production and experimentally-induced inflammation by PDE4 inhibitor rolipram is mediated by MAPK phosphatase-1. British Journal of Pharmacology. 169:1525-36

ABBREVIATIONS

APO	apolipoprotein
AC	adenylate cyclase
AKAP	achieved by A Kinase Anchoring Protein
AP	activator protein
ASK1	apoptosis signal-regulating kinase 1
ATP	adenosine triphosphate
CAAT	controlled amino acid treatment
cAMP	cyclic adenosine 3'5'monophosphate
CUS	chronic unpredictable stress
cGMP	cyclic guanosine monophosphate
CIA	collagen-induced arthritis
CNG	cyclic nucleotide gated ion channel
COPD	chronic obstructive pulmonary disease
COX-2	cyclooxygenase-2
CRE	cAMP-responsive element
CREB	cAMP responsive element binding protein
DUSP	dual specificity phosphatase
EAE	experimental autoimmune encephalomyelitis
Epac	targeting exchange protein directly activated by cAMP
ERK	extracellular signal regulated kinase
EyA	eyes absent
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GM CSF	granulocyte macrophage colony stimulating factor
GPCR	G-protein-coupled receptor
GRE	glucocorticoid responsive elements
HAD	Haloacid dehalogenase
HuR	Hu antigen R
IFN	interferon
IL	interleukin
iNOS	inducible nitric oxide synthase
JNK	c-Jun N-terminal kinase
KIM	kinase interaction motif
KO	knock-out
LPS	lipopolysaccharide

LR	linker region
MAPK	mitogen-activated protein kinase
miR	micro-RNA
MK2	MAPKAP kinase 2
MKK	mitogen-activated protein kinase
MKKK	mitogen-activated protein kinase kinase
MKP	mitogen-activated protein kinase phosphatases
MMP	matrix metalloproteinase
MSK	mitogen- and stress-activated protein kinase
NF-κB	nuclear factor-kappaB
NFAT	nuclear factor of activated T cells
NO	nitric oxide
ox-LDL	oxidized LDL
PDE	phosphodiesterase
PKA	protein kinase A
PKC	protein kinase C
PLA2	phospholipase A2
PTP	protein tyrosine phosphatase
SP	specificity protein
TAK1	TGFβ -activated protein kinase 1
TLR	toll like receptor
TNF	tumor necrosis factor
UCR	upstream conserved
VDRE	vitamin D receptor element
WT	wild-type

ABSTRACT

The immune system is a defense mechanism against pathogens, and it is essential for the survival of the host. In addition to professional immune cells, the immune system can be considered to include physical barriers (skin, epithelial lining), non-specific chemical factors (lower pH values in skin and in the secretions of the stomach), non-specific anti-microbial peptides, and inflammatory responses of tissue cells. Furthermore, immune and other cells produce factors (e.g. cytokines and lipid mediators) that regulate the functions and responses of inflammatory as well as other cells, e.g. epithelial cells and cells of the tissue parenchyma. Inflammation is a crucial contributor to the pathophysiological processes in many diseases, although it is often an important part of the recovery and regenerative processes in tissues. However, in certain diseases, the immune system is disturbed leading to an uncontrolled and prolonged inflammatory response resulting in tissue destruction and permanent pathological changes in the organs.

Mitogen-activated protein kinases phosphatases (MKPs) are members of the subgroup of dual specificity phosphatases (DUSPs). At the moment, 11 different MKPs have been recognized, and they differ from each other in terms of their substrate specificities, subcellular localizations, tissue distributions and expressional patterns. MKPs regulate the activity of mitogen-activated protein kinases (MAPKs) by dephosphorylating their threonine and tyrosine residues in the activation motif of the kinase. MAPKs are signaling pathways that regulate several important cell functions, including differentiation, proliferation, apoptosis and inflammation. MKP-1 is an interesting member of the MKP family, it is widely expressed in diverse cell types and tissues, and its expression is increased by various factors, e.g. inflammatory mediators, growth factors and cellular stress. The anti-inflammatory glucocorticoids and the anti-rheumatic gold compound, aurothiomalate as well as vitamin D all increase MKP-1 levels in cells. MKP-1 has been associated not only with inflammatory diseases but also with other diseases including cancer, atherosclerosis, and diabetes. Another interesting and less well known phosphatase is MKP-5. MKP-5 is expressed in a wide variety of cell types and it has also been associated with inflammation.

The aim of the present study was to investigate the role of MKP-1 and MKP-5 in the regulation of inflammatory gene expression and inflammation. Another aim

was to investigate the role of MKP-1 in the anti-inflammatory effects of the phosphodiesterase 4 (PDE4) inhibitor rolipram.

It was found that MKP-1 could regulate the phosphorylation of p38 MAPK and JNK in lung epithelial cells and the phosphorylation of p38 MAPK in macrophages. MKP-5 seemed to dephosphorylate principally p38 MAPK. MKP-1 suppressed inflammatory gene expression and inflammation *in vivo* in mice. In cell models, MKP-5 inhibited the release of inflammatory factors TNF and IL-6. The PDE4 inhibitor, rolipram, increased MKP-1 expression and decreased the production of TNF in macrophages and attenuated carrageenan-induced acute inflammation *in vivo* in mice. Interestingly, those anti-inflammatory effects of rolipram were attenuated in MKP-1 KO mice and in macrophages harvested from these animals. This indicates that the anti-inflammatory effects of rolipram are mediated, at last partly, through increased MKP-1 expression. This is a novel anti-inflammatory mechanism of action of PDE4 inhibitors and emphasizes the role of MKP-1 as an anti-inflammatory drug target. This study extends our understanding of the regulation of inflammatory gene expression and the inflammatory response, particularly the roles of MKP-1 and MKP-5 in this process and as anti-inflammatory drug targets. The result may be useful in the development of novel drugs for the treatment of inflammatory diseases.

TIIVISTELMÄ

Immuunijärjestelmä on elintärkeä suojamekanismi ulkoisia uhkatekijöitä, kuten taudinaiheuttajia, vastaan. Immuunijärjestelmän solut sekä monet muut elimistön solut osallistuvat immuniteetin muodostamiseen tuottamalla monia tekijöitä, jotka säätelevät sekä immuunijärjestelmän että kudosten solujen toimintaa. Tulehdusreaktio liittyy monien sairauksien kulkuun ja usein on välttämätön sairauden paranemisessa ja syntyneiden elinvaurioiden korjaamisessa. Toisinaan tulehdusreaktio voi kuitenkin pitkittyä tai reaktio voi virheellisesti kohdistua elimistön omia kudoksia vastaan. Tällöin pitkittyneeseen ja väärin kohdistuneeseen tulehdusreaktioon liittyy kudostuhon eteneminen, joka usein johtaa pysyvien elinvaurioiden kehittymiseen.

Mitogeeniaktivoituvat proteiinikinaasifosfataasit (engl. mitogen-activated protein kinase phosphatases, MKPs) kuuluvat laajempaan kaksois-spesifisten fosfataasien (engl. dual specificity phosphatases, DUSPs) perheeseen. Tällä hetkellä tunnetaan 11 eri MKP-perheen fosfataasia, jotka eroavat toisistaan substraattispesifisyyden, solunsisäisen sijainnin ja kudosjakauman perusteella. MKP:t säätelevät mitogeeniaktivoituvien proteiinikinaasien (engl. mitogen-activated protein kinases, MAPKs) aktiivisuutta defosforyloimalle niiden treoniini- ja tyrosiini-tähteet kinaasin aktivaatioon liittyvässä rakenteessa. MAPK:t ovat solun sisäinen signalointijärjestelmä, jotka säätelevät monia tärkeitä toimintoja, kuten solun jakautumista, stressivastetta, apoptoottista solukuolemaa ja tulehdusta. MKP-1 on kiinnostava fosfataasi. Se ilmentyy laajasti eri solutyypeissä ja kudoksissa, ja sen ilmentyminen lisääntyy monien tekijöiden, kuten tulehdustekijöiden, solustressin ja kasvutekijöiden vaikutuksesta. Hiljattain on todettu, että lääkeaineista glukokortikoidit ja reumalääkkeenä käytettävä aurotiomalaatti sekä D-vitamiini lisäävät MKP-1:n ilmentymistä, ja tämä fosfataasi osin välittää näiden yhdisteiden tulehdusta rauhoittavan vaikutuksen. MKP-1 on yhdistetty tulehdussairauksien lisäksi mm. syöpään, aterosklerosiin ja diabetekseen. Toinen tulehdusreaktion kannalta mielenkiintoinen fosfataasi on MKP-5, mutta sen säätely ja vaikutukset tunnetaan huonosti.

Väitöskirjassa tutkittiin MKP-1:n ja MKP-5:n merkitystä tulehdusgeenien ilmentymisen ja tulehdusreaktion säätelijöinä. Lisäksi tutkittiin MKP-1:n osuutta fosfodiesteraasi (PDE)4-estäjä rolipraamin anti-inflammatoristen vaikutusten välittäjänä.

Tutkimuksessa havaittiin, että MKP-1 sääteli p38 MAPK:n ja JNK:n fosforylaatiota keuhkoepiteelin soluissa ja p38 MAPK:n fosforylaatiota makrofageissa. RNA-interferenssi -menetelmällä ja MKP-1 -poistogeenisiä eläimiä hyödyntävillä tutkimusmenetelmillä havaittiin, että MKP-1 vaimentaa tulehdustekijöiden tuottoa ja akuuttia tulehdusreaktiota. Solumalleissa tehdyissä kokeissa todettiin, että MKP-5 sääteli p38 MAPK:n fosforylaatiota ja tulehdustekijöiden TNF:n ja IL-6:n tuottoa makrofageissa. PDE4:n estäjä rolipraami lisäsi MKP-1:n ilmentymistä ja toisaalta vähensi TNF:n tuottoa makrofageissa ja tämä anti-inflammatorinen vaste oli vähäisempi MKP-1 -poistogeenisten hiirten makrofageissa. Rolipraami hillitsi myös karrageenin aiheuttamaa akuuttia tulehdusta kontrolli hiirissä, mutta ei MKP-1 -poistogeenisissä hiirissä. Näyttäsi että MKP-1 välittää PDE4-estäjä rolipraamin anti-inflammatorista vaikutusta, joka on uusi vaikutusmekanismi näille estäjille. Havainto edelleen alleviivaa MKP-1:n merkitystä tulehdusreaktiota vaimentavana ja anti-inflammatorisia lääkevasteita välittävänä tekijänä.

Tämä väitöskirja laajensi tietämystä tulehdusgeenien säätelystä ja tulehdusvasteesta sekä solunsisäiseen signaalinvälitykseen liittyvien tekijöiden, erityisesti MKP-perheen kahden fosfataasin MKP-1:n ja MKP-5:n merkityksestä inflammaatiossa ja anti-inflammatorisissa lääkevasteissa. Väitöskirjan tuloksia voidaan hyödyntää uusien tulehdusta vaimentavien lääkkeiden kehitystyössä.

1. INTRODUCTION

The main function of immune system is to detect pathogens and to elicit a response aimed to eliminate the pathogens and to protect the host from the harmful effects of the pathogen (Medzhitov and Janeway, 2000). The first lines of defense in the immune system are physical barriers and reflexes (e.g. skin, epithelial lining in the gut and airways, and cough reflex), chemical factors (such as lower pH in the skin, acidic fluids in the stomach) and the presence of anti-microbial enzymes in the body secretions (e.g. enzymes in tears, mucus and skin) that prevent potentially harmful factors from entering the body. If pathogens succeed to penetrate these barriers, immune system is activated in order to isolate and eradicate the invading pathogens. The immune system can be divided into two components, innate immunity and adaptive immunity. Innate immunity is rapidly activated and consists of different cell types, including dendritic cells, macrophages and monocytes, polymorphonuclear cells and natural killer cells. In addition, several tissue cells possess certain features of the innate immune cells. Innate immune cells and other cells express so called pathogen recognition receptors (PRRs). These receptors recognize structures typically present in pathogens, also known as pathogen-associated molecular patterns (PAMPs), including bacterial lipopolysaccharide (LPS), peptidoglycan, lipoteichoic acids, mannans, bacterial DNA, double-stranded RNA and glucans. The activation of these receptors leads to the production of inflammatory mediators, including cytokines, chemokines, nitric oxide, eicosanoids and reactive oxygen species. These factors serve as regulators and effectors in the innate immunity and its translation to adaptive immunity.

Adaptive immunity is a highly specific and effective host defense mechanism consisting particularly of two cell types, T-lymphocytes and B-lymphocytes. All B- and T-lymphocytes are unique with respect to the receptors that recognize non-host structures, i.e. antigens. Once activated by their specific antigen, T and B cells start to proliferate to constitute an antigen-specific lymphocyte population (aka a clone) and to produce antibodies, cytokines and other inflammatory mediators. These cells can function also as memory cells and upon re-encountering their antigen, they become rapidly activated. The onset and development of the adaptive immune response takes several days or weeks to develop. Despite their obvious differences, the innate and adaptive immunity systems interact with each other closely and in a synergistic manner. The activation and development of a proper immune response requires the presence of an inflammatory environment having been created by the cytokines released from innate immune cells. (Kawai and Akira, 2007; Medzhitov and Janeway, 2000)

Toll-like receptors (TLR) are key PRRs in the inflammatory response. There are more than ten different mammalian TLRs differing from each other in terms of their ligand specificities and subcellular localizations. For example, TLR4 activation by LPS leads to the activation of Toll/Interleukin-1 Receptor (TIR), and its interaction with different factors, for example myeloid differentiation factor 88 (MyD88) or TIR-domain-containing adapter-inducing interferon-β (TRIF). These activate downstream intracellular signaling pathways, such as mitogen-activated protein kinases (MAPK), and inflammatory transcription factors nuclear factor (NF) - κB and activator protein 1 (AP-1). (Akira and Takeda, 2004; Akira et al., 2006; Kawai and Akira, 2007)

Usually the immune system protects the body from harmful pathogens and is critical for survival. However, if the immune system is inefficient, overactive or inappropriately regulated, it may result to or allow the development of inflammatory

diseases. Overactive immune responses may cause chronic inflammation and autoimmunity diseases, where the immune response attacks the tissues of the host.

Although there are many drug available for the treatment of chronic inflammatory diseases, a considerable proportion of patients are still lacking effective anti-inflammatory treatment or the side-effects of the drugs limit their use. One approach to the drug treatment of inflammatory diseases is to target the intracellular signaling cascades. The protein kinases are key regulators in several major pathways, and they regulate a variety of important cell functions, including cell division, cell death, growth, differentiation and cellular memory as well as the production and secretion of inflammatory mediators. Because protein kinases are activated in many inflammatory diseases, they may well become important drug targets. There are several different kinase inhibitors at different stages of development, e.g. molecules targeting mitogen-activating protein kinases (MAPKs), spleen tyrosine kinase (Syk), Janus kinases (JAKs) and Bruton's tyrosine kinase (Btk) (Kyttaris, 2012). Many of these have demonstrated efficacy to decrease the production of inflammatory factors and to suppress inflammatory response in cell and animal models.

2. REVIEW OF LITERATURE

2.1 Mitogen-activated protein kinases

The mitogen-activated protein kinases (MAPKs), namely p38 MAPK, extracellular signal-regulated kinase (ERK) and Jun N-terminal kinase (JNK), are a group of serine/threonine protein kinases. MAPKs modulate several physiological processes, including cell growth, differentiation and stress response, and, importantly, they regulate inflammation. MAPKs are activated in response to a number of extracellular signals, such as cytokines, growth factors and bacterial substances via the phosphorylation by their upstream kinase cascade. MAPK pathways form a three-tier signaling cascade, that includes mitogen-activated protein kinase kinase kinases (MKKKs), mitogen-activated protein kinase kinase (MKKs) and finally MAPK (Fig. 1). Activated MAPKs can regulate their substrates both in the cytosol and nucleus. For example. MAPKs affect gene expression by modulating chromatin structure, by regulating activity of transcription factors [e.g. activator protein (AP-1), cAMP responsive element (CREB), serum response factor (SRF) and CCAAT-enhancer-binding proteins (c/EBPβ)] or by enhancing the stability of mRNAs containing AU-rich elements (AREs). AREs also regulate the stability of many proteins. (Cuadrado and Nebreda, 2010; Davis, 1995; Dong et al., 2002; Johnson and Lapadat, 2002)

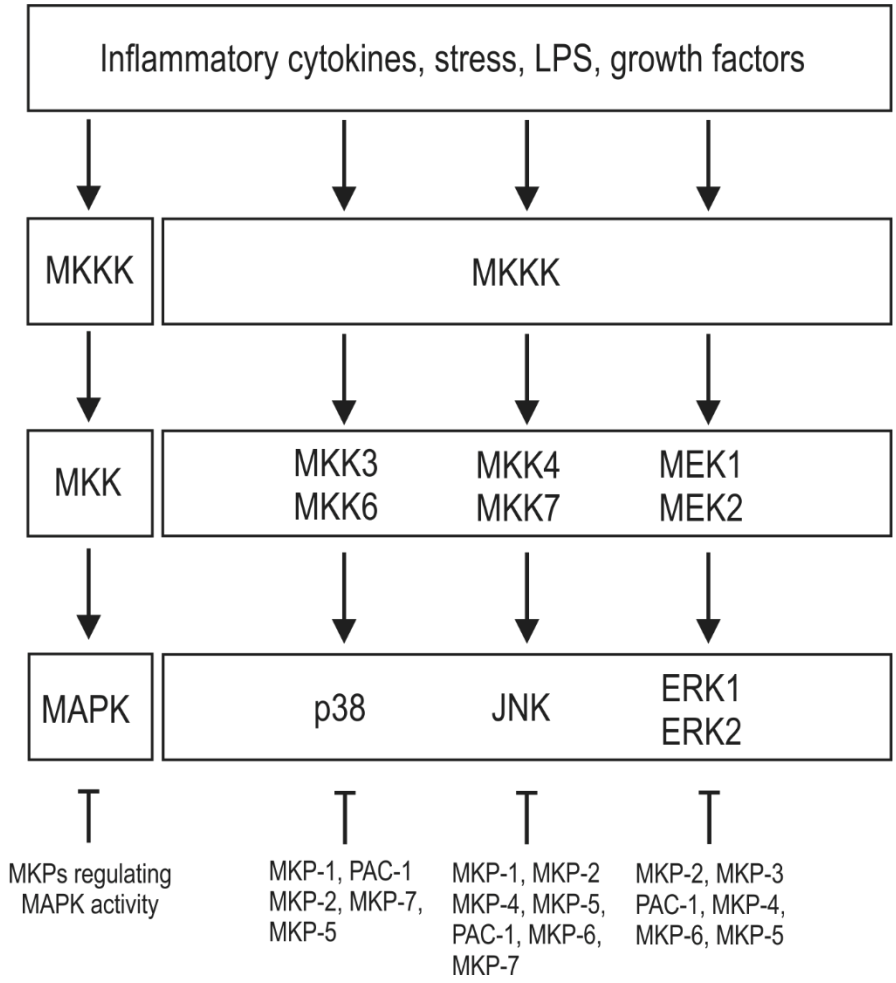

Figure 1. MAPK pathways. MAPK pathways are three-tier signaling cascades which are activated in response to e.g. inflammatory stimuli, cell stress conditions or growth factors. Activation of cell surface receptors leads to the phosphorylation-dependent activation of mitogen-activated protein kinase kinase kinases (MKKK). This kinase subsequently activates mitogen-activated protein kinase kinases (MKK), which then phosphorylates MAPKs on specific tyrosine and threonine residues. The activity of MAPKs is regulated by MAPK phosphatases (MKPs). MKPs are able to dephosphorylate both tyrosine and threonine residues in MAPKs, and this leads to the inactivation of MAPK activity and abrogation of MAPK signaling.

2.1.1 p38 MAPK pathway

p38 MAPK has four isoforms p38α, p38β, p38γ and p38δ. These isoforms are approximately 60 % identical, but they are encoded by distinct genes (Ashwell, 2006; Thornton and Rincon, 2009). They are differentiated from each other also by their tissue expression patterns and sensitivities to pharmalogical inhibitors. There are also differences in their substrate specificities. For p38γ and p38δ MAPK, the microtubule associated protein tau is a more common substrate, while MAPKAP kinase-2 (MK2) is a substrate for p38α and p38β MAPK. p38α and p38β MAPK substrates include transcription factors, other protein kinases that in turn may phosphorylate transcription factors, cytoskeletal proteins, components of translation machinery, and enzymes (Chung, 2011). Typical p38γ and p38δ MAPK targets include activating transcription factor-2, and serum response factor accessory protein 1. p38α MAPK is important during fetal development, since in the mouse models its deficiency is lethal due to defective placenta development (Mudgett et al., 2000; Tamura et al., 2000). In contrast, other p38 MAPK isoforms deficient mice or double p38γ and p38δ KO mice are fertile and viable (Beardmore et al., 2005; Sabio et al., 2005).

The p38 MAPK pathway is activated in response to a variety of stimuli, including bacterial substances, cytokines and cellular stress, (Ashwell, 2006; Thornton and Rincon, 2009). MKKK4 is the best characterized member of the MKKKs in the p38 MAPK pathway. Other factors at the MKKK level for the p38 MAPK pathway are apoptosis signal-regulating kinase 1 (ASK1) and TGFβ -activated protein kinase 1 (TAK1). TAK1 seems to be an important factor in the immune and inflammatory responses (Clark and Dean, 2012). MKK3 and MKK6 are the upstream activators of p38 MAPK. Activated p38 MAPKs phosphorylates its substrates, such as MK2, MK3 and mitogen- and stress-activated protein kinase 1/2 (MSK1/2). The downstream effectors of p38 MAPK include several transcription factors (e.g. ATF-2, STAT1 and CREB) regulating the expression of pro-inflammatory genes (Clark

and Dean, 2012; Delghandi et al., 2005). p38 MAPK regulates the inflammatory response also at the post-transcriptional level, by regulating the mRNA stability of many cytokines (Clark et al., 2003).

The p38 MAPK pathway is involved in different pathological conditions. The effect of the inhibition of the p38 MAPK pathway has been studied in some chronic inflammatory diseases and also in neurological diseases. In rheumatoid arthritis, p38α MAPK signaling appears to predominate but also in some cells, p38β MAPK and p38γ MAPK are activated (Korb et al., 2006). There are reports that MK2 KO mice (Hegen et al., 2006; Inoue et al., 2006) as well as MKK3 KO (Inoue et al., 2006) and MKK6 KO (Yoshizawa et al., 2009) mice are protected from experimental arthritis, and all these factors are components of the p38 MAPK pathway. p38 MAPK activity was increased in macrophages and other alveolar cells from patients with chronic obstructive pulmonary disease (COPD) when compared to healthy subjects (Renda et al., 2008). The p38 MAPK activity also correlated with the severity of alveolar wall inflammation in these patients. The p38 MAPK inhibitor, SD282, has been reported to attenuate allergen-induced bronchial hyperresponsiveness in a mouse model (Nath et al., 2006). Recent studies in experimental autoimmune encephalitis in the mouse (EAE, a mouse model of multiple sclerosis) have revealed that p38 MAPK pathway is an important immunopathogenic signaling pathway also in this disease model (Krementsov et al., 2013).

In clinical trials for the treatment of arthritis in man, p38 MAPK inhibitors have not been as effective as would have been expected on the basis of the results from inflammatory animal models. In two studies conducted in rheumatoid arthritis patients, the p38 MAPK inhibitor, SCIO-469, did not achieve any statistically significant effect over placebo (Genovese et al., 2011). However, a more recent p38 MAPK inhibitor, PH-797804, has shown promising results in clinical trials in the treatment of chronic obstructive pulmonary disease (MacNee et al., 2013). Patients with moderate to severe COPD treated with short-acting bronchodilators

supplemented with PH-797404 experienced improvements in lung function and dyspnea scores.

2.1.2 Extracellular signal-regulated kinases (ERK) 1/2 pathway

ERK1 and ERK2 are 83 % identical and they are expressed in all tissues (Shaul and Seger, 2007). Since, all known inhibitors affect to some extent both ERK1 and ERK2. Individual genetically-manipulated animals e.g. ERK1 KO and ERK2 KO mice, have proven useful models in clarifying the effects of the kinases. ERK1 KO mice are viable, fertile and in general, they develop normally (Pages et al., 1999). ERK1 KO mice have, however, abnormalities in thymocyte development and neuronal synaptic plasticity (enhancement of striatum-dependent long-term memory) (Mazzucchelli et al., 2002). ERK1 deficiency affects also adipocytes, and ERK1 KO mice are protected from a high-fat diet induced obesity and protected against insulin resistance (Bost et al., 2005). The ERK2 deficiency is embryonically lethal, proving that ERK1 and ERK2 have different functions (Hatano et al., 2003). It seems that ERK2 is essential for placental development and a lack of ERK2 leads to impairment of mesoderm differentiation. Although there is increased activation of ERK1 in embryonic stem cells in the ERK2 KO mice, this cannot compensate for the ERK2 deficiency (Yao et al., 2003).

ERK pathway can be activated by different extracellular stimuli, including growth factors, insulin, cytokines and osmotic stress (Roskoski, 2012). The first step in the pathway is the receptor-mediated activation of GTPase Ras, which then typically activates MKKK kinase Raf (Shaul and Seger, 2007). Other possible MKKKs in ERK pathway are c-Mos, proto-oncogene TPL2 and MEKK1. Activated MKKK phosphorylates MEK1/2, which is able to phosphorylate ERK1/2. In turn ERK1/2 phosphorylates many substrates in all cellular compartments. The ERK1/2 substrates include transcription factors and their regulators. There are also several

nuclear substrates for example Elk1 and c-Fos and the cytoplasmic substrates include ribosomal s6 kinase family (Roskoski, 2012).

The role of ERK1/2 pathway in cellular proliferation and apoptosis is important and has been more extensively studied than the role of ERK1/2 in regulating inflammatory responses. During inflammation, ERK1/2 is an essential signal transduction molecule mediating antigen-specific immune responses, since it regulates T cell activation as well as antibody production in B cells (Thiel et al., 2007). The ERK1/2 pathway enhances the production of cytokines, such as interleukin-1 (IL-1), TNF, and IL-6. ERK1/2 pathway also regulates the production of matrix metalloproteinases in certain cell types (Brauchle et al., 2000). It was reported that the ERK1/2 inhibitor, FR180204, could suppress both T and B cell activation and attenuated the severity of collagen-induced arthritis (CIA) in a mouse model (Ohori et al., 2007). Many studies demonstrating that MEK1/2 (which is an upstream kinase of ERK1/2) can enhance the production of inflammatory mediators (Ajizian et al., 1999; Martel-Pelletier et al., 1999; van der Bruggen et al., 1999). In agreement, MEK inhibitors PD198306, PD98059 and U0126, which inhibit ERK1/2 activation, have been reported to ameliorate experimental osteoarthritis in rabbits (Pelletier et al., 2003). Importantly, the ERK1/2 pathway, as well as two other MAPK pathways, is activated in synovium of rheumatoid arthritis patents (Schett et al., 2000) indicating that the ERK1/2 pathway is a potential drug target in the treatment of inflammatory diseases such as rheumatoid arthritis. ERK5 is novel and less well known member of the MAPK family (Nithianandarajah-Jones et al., 2012). It is believed to be involved in the development of the cardiovascular system, and in the pathophysiology of chronic pain and cancer (Nithianandarajah-Jones et al., 2012).

2.1.3 c-Jun N-terminal kinases (JNK) pathway

JNKs are encoded by three genes, namely JNK1, JNK2, and JNK3, and they all have splice variants. JNK1 and JNK2 are ubiquitously expressed while JNK3 is expressed

only in the brain, heart and testes (Bogoyevitch, 2006). These isoforms differ from each other in their substrate specificities, and they are also differentially activated. JNK1 KO mice are fertile and have no obvious defects. They have improved insulin signaling (Hirosumi et al., 2002), and reduced lipid accumulation in the liver in hepatic steatosis model (Schattenberg et al., 2006). JNK1 KO animals also display hypoproliferative T cell responses (Dong et al., 1998). JNK2 KO mice are viable and fertile (Sabapathy et al., 1999; Yang et al., 1998). These animals have defects in T cell activation and apoptosis in response to antigen stimulation (Yang et al., 1997). In addition, the differentiation of mature naïve CD4+ T cells into the Th1 effector cells is impaired due to reduced IFNγ production during the early stages of differentiation (Yang et al., 1997). It has been also shown that a JNK2 deficiency can protect non-obese diabetic mice from destructive insulitis and they exhibit a reduced disease progression in type 1 diabetes, while JNK1 is more involved in type 2 diabetes (Jaeschke et al., 2005). JNK3 is expressed mainly in the brain, and thus its deletion has been claimed to be involved in neuronal cell death and possibly in defects in brain development (Yang et al., 1997).

There are fourteen different MKKKs regulating the JNK pathway activation (Johnson and Nakamura, 2007). This provides the possibility for diverse activation of the JNK pathways. JNK pathways can be activated by several external stimuli including stresses, cytokines or growth factors (Bogoyevitch, 2006). JNK becomes activated by MKK4 or MKK7. JNK regulates the inflammatory response, stress-induced and developmentally programmed apoptosis, actin reorganization and cell transformation (Aouadi et al., 2006). In turn JNK can regulate the activities of its many substrates including transcription factors c-Jun, JunD and ATF2, FOXO4, and nuclear hormone receptors, e.g. peroxisome proliferator-activated receptor-γ, the glucocorticoid receptor and the retinoic acid receptor (Bogoyevitch, 2006).

JNK inhibitors usually bind to the ATP-binding site of the enzyme (Bogoyevitch et al., 2010). JNK isoforms display considerable similarities in the ATP-binding sites

making it difficult to develop specific inhibitors for the distinct JNK isoforms. JNK3 inhibitors have been studied for the treatment of neurodegenerative disorders and inhibitors of the other JNK isoforms have been examined for the treatment of diabetes or prevention of pathological enlargement of the heart in a cardiac pressure overload model (Asano et al., 2008). Moreover, JNK2 has been reported to be involved in the pathogenesis of rheumatoid arthritis since MKK4 and MKK7 levels are known to be elevated in rheumatoid arthritis synovium (Han et al., 1999; Sundarrajan et al., 2003).

2.1.4 MAPKs in the immune response

The MAPK pathways are involved in both innate and adaptive immune responses. MAPKs are important regulators of many events in the immune response, e.g. cellular proliferation, differentiation, survival and apoptosis, chemoattraction and the production of inflammatory factors (Chang and Karin, 2001; Dong et al., 2002; Johnson and Lapadat, 2002).

The p38 MAPK pathway plays an important role in a variety of immune cells. In CD4+ T cells, p38 MAPK regulates the differentiation of naïve CD4+ T cells into effector cells and also promotes interferon (IFN)-γ production and Th1 differentiation. In CD8+ T cells, p38 MAPK is needed for the production of IFNγ and it has a major role in Fas-induced cell death. There is report that the p38 MAPK pathway is less important for Th2 differentiation (Rincon et al., 1998), but it is involved in Th17 differentiation (Huang et al., 2012b). In macrophages, the p38 MAPK pathway plays a major role in regulating TLR activated production of inflammatory mediators (Bode et al., 2012).

In naïve CD4+ and CD8+ T cells, there are very low basal levels of JNK1 and JNK2 but the kinase levels are up-regulated after cell activation (Weiss et al., 2000). Because the expression of JNK is low in naïve CD4+ cells, it is though unlikely that

JNK would have a significant effect on the production of early stage cytokines such as IL-2 (Dong et al., 1998; Yang et al., 1998). There are different functions in JNK1 and JNK2 (Yang et al., 1998). In JNK1 KO mice, Th2 differentiation is enhanced (Dong et al., 1998). In CD8+ T cells, JNK1 deficiency evoked T cell hypoproliferation and lower production of IL-2 *in vitro* and therefore decreased expansion of antigen-specific CD8+ T cells, while the lack of JNK2 caused hyperproliferation and increased the production of IL-2 and enhanced the expansion of antigen-specific CD8+ T cells (Arbour et al., 2002). Thus, JNK1 seems to be involved in the development of Th1 response whereas JNK2 controls the CD8+ T cell expansion.

ERK1/2 is associated with cell growth and differentiation, but the ERK1/2 pathway is also required to develop an immune response and inflammation. ERK pathway, especially ERK1 is important for thymocyte maturation (Pages et al., 1999) and the ERK1/2 pathway is also essential to Th2 differentiation (Yamashita et al., 1999).

MAPKs regulate the production of many inflammatory factors. For example both, p38 MAPK and JNK have been reported to mediate the production of TNF, IL-1β, IL-6, COX-2 and iNOS (Ashwell, 2006; Dong et al., 2002; Korhonen et al., 2007; Nieminen et al., 2005; Rincon and Davis, 2009). In contrast, the inhibition of p38 MAPK has been claimed to increase the production of IL-12 (Korhonen et al., 2012).

All three MAPKs have been reported to be involved in the development of arthritis at least in animal models (Thalhamer et al., 2008). In the CIA mouse model, JNK2 KO mice are partially protected from the development of joint destruction (Han et al., 2002) and JNK inhibitors, to some extent, could attenuate paw swelling in rat adjuvant-induced arthritis model (Han et al., 2001). The p38 MAPK pathway has been associated with arthritis, for example p38 MAPK is known to mediate the production of TNF, which is one of the important inflammatory mediators in

arthritis (Kumar et al., 2003). The MAPKs, especially p38 MAPK, have also been associated with osteoarthritis. The adipokine adiponectin is known to mediate cartilage destruction in osteoarthritis. Interestingly, adiponectin activated MAPKs, and the adiponectin-induced production of NO, IL-6, MMP-1 and MMP-3 was inhibited by MAPK inhibitors, especially by p38 MAPK inhibitor (Koskinen et al., 2011).

2.2 Dual specificity phosphatases (DUSPs)

Phosphorylation of tyrosine residues in proteins is an important mechanism in the regulation of protein function and it is utilized in multicellular eukaryote species (Alonso et al., 2004). Defective or aberrant protein tyrosine phosphorylation has been associated with many human diseases, including many cancer types and immune deficiencies. The phosphorylation of tyrosine residues in proteins is reversed by protein tyrosine phosphatases (PTPs). PTPs play an important role in the regulation of many physiological functions and in counterbalancing protein tyrosine phosphorylation. The PTPs have been categorized into four groups based on the amino acid sequences of their catalytic domains, namely class I cysteine-based PTPs, class II cysteine-based PTPs, rhodanese-related class III cysteine-based thyrosine/threonine-specific PTPs and aspartic acid-based PTPs (Table 1). Class I PTPs are further divided into two subgroups; classical tyrosine-specific PTPs and the heterogeneous dual specificity phosphatases (DUSPs). A common feature of DUSPs is their ability to dephosphorylate both tyrosine and serine/threonine residues within one substrate (Patterson et al., 2009). DUSPs are further classified into 7 groups (listed in Table 1).

Table 1. Classification of PTPs

PTPs			
Class I Cys-based	**Class II Cys-based**	**Class III Cys-based**	**Aspartic acid-based**
Classical tyrosine-specific	Low molecular mass	CDC25 proteins	Eyes absent
1.Trans-membrane			Haloacid dehalogenase
2. Non-receptor			
Dual-specificity			
1.MKPs			
2.Atypical			
3.Slingshots			
4.PRLs			
5.CDC14s			
6.PTENs			
7.Myotubularins			

protein tyrosine phosphatases; PTP, MAPK phosphatases; MKPs, phosphatases of Regenerating Liver; PRL, phosphatase and tensin homologs; PTEN, cell division cycle 14 phosphatases; CDC14

MAPK phosphatases (MKPs) are one of the subgroups of DUSPs. They can dephosphorylate both threonine and tyrosine residues on the MAPK activation motif Thr-Xaa-Tyr. MKPs differ from each other in their substrate specificities, subcellular localizations, tissue distributions and expressional patterns (Table 2.). The protein structure of MKPs contains Cdc25-like domain and C-terminal catalytic domain. Cdc25-like domain in the N-terminal half has a kinase interaction motif (KIM), which regulates the specificity of the MAPK-MKP interaction. The C-terminal catalytic domain shares a strong homology with other MKPs. (Caunt and Keyse, 2013; Kondoh and Nishida, 2007; Owens and Keyse, 2007)

MKPs can be divided into three classes based on their gene structures, sequence similarities, substrate selectivities and subcellular localizations (Owens and Keyse, 2007; Patterson et al., 2009; Theodosiou and Ashworth, 2002). The first class

consists of the inducible nuclear phosphatases (DUSP1/MKP-1, DUSP2/PAC-1, DUSP4/MKP-2 and DUSP5), the second class includes ERK-specific cytoplasmic phosphatases (DUSP6/MKP-3, DUSP7/MKP-X and DUSP9/MKP-4) and the third class contains the phosphatases found in both nuclear and cytosolic compartments and have substrate selectivity towards p38 MAPK and JNK (DUSP8, DUSP10/MKP-5 and DUSP16/MKP-7).

Table 2. MKPs

DUSP	Subcellular localization	Substrate specificity	Phenotype of KO mice	Reference
DUSP1 **MKP-1**, CL100, hVH1, 3CH134	Nuclear	p38, JNK	↑ cytokines, innate immune response, sepsis, CIA, sensitivity to bacterial infection ↓ IL-12, adaptive immune response, EAE, diet-induced obesity	(Chi et al., 2006; Frazier et al., 2009; Hammer et al., 2006; Hammer et al., 2010; Salojin et al., 2006; Shipp et al., 2010; Vattakuzhi et al., 2012; Wang et al., 2007; Wu et al., 2006; Zhang et al., 2009; Zhao et al., 2006)
DUSP2 PAC-1	Nuclear	ERK, p38	↓ cytokines, survival of mast cells, CIA	(Jeffrey et al., 2006)
DUSP4 **MKP-2,** hVH2, TYP1	Nuclear	ERK, JNK, p38	↑ susceptibility to L. mexicana, IL-2 signaling, CD4+ T cell proliferation ↓ sensitivity to LPS and infection in vivo	(Al-Mutairi et al., 2010; Huang et al., 2012a)
DUSP5 vVH3, B23	Nuclear	ERK	Abnormal T cell maturation in thymus ↑ autoimmunity	(Kovanen et al., 2008)
DUSP6 **MKP-3,** Pyst1, rVH6	Cytosolic	ERK	Dominant postnatal lethality, skeletal dwarfism, coronal craniosynostosis and hearing loss, FGFR-like syndrome	(Li et al., 2007)
DUSP7 MKP-X	Cytosolic	ERK	ND	(Orlev et al., 2004)
DUSP8 hVH5	Nuclear and cytosolic	p38, JNK	ND	(Muda et al., 1996)
DUSP9 **MKP-4**	Nuclear and cytosolic	ERK	Placental defects →embryonic death	(Christie et al., 2005)
DUSP10 **MKP-5**	Nuclear and cytosolic	p38, JNK	↑ cytokine and ROS in macrophages and T cells, mortality to lymphocytic choriomeningitis virus ↓ T cell proliferation, EAE	(Qian et al., 2009; Qian et al., 2012; Zhang et al., 2004)
DUSP14 **MKP-6**	Nuclear and cytosolic	ERK, JNK	ND	(Marti et al., 2001)
DUSP16 **MKP-7**	Cytosolic	JNK	↑ IFNγ (CD4+ and CD8+ T cells)	(Kumabe et al., 2010; Musikacharoen et al., 2011)
DUSP18	Nuclear and cytosolic	JNK	ND	(Kim et al., 2012)

↑ increased; ↓ decreased, knock-out; KO, collagen-induced arthritis; CIA, experimental autoimmune encephalomyelitis; EAE, no data; ND

2.2.1 MKP-1

MKP-1 (also called as DUSP1, hVH1, CL100, 3CH134, Erp) is an archetype of the MKP family (Wancket et al., 2012). MKP-1 is widely expressed in many cell types (Lang et al., 2006). First it was thought that MKP-1 was involved in regulating the ERK phosphorylation (Sun et al., 1993), but later studies in MKP-1 KO mice have revealed that MKP-1 is more p38 MAPK and JNK specific than it is ERK specific (Chi et al., 2006; Zhao et al., 2006). MKP-1 expression is increased by various factors, including several inflammatory factors, cell stress, growth factors, cAMP and by at least two anti-inflammatory drugs dexamethasone, and aurothiomalate (Abraham et al., 2006; Brion et al., 2011; Lang et al., 2006; Lee et al., 2012b; Nieminen et al., 2010; Zhang et al., 2008). Because MKP-1 is a nuclear immediate-early gene whose expression is elevated by the same signals that activate MAPK pathways, one can assume that MKP-1 is an important factor in attenuating gene expression through inhibition of MAPK pathways (Sun et al., 1993).

Regulation of MKP-1 expression

MKP-1 expression and function is tightly regulated at multiple levels, including gene expression, protein stability and phosphatase activity (Wancket et al., 2012). There are four exons and three introns in the human MKP-1 gene (Kwak et al., 1994). MKP-1 promoter contains binding sites for many transcription factors, including activator protein (AP)-1 and 2, nuclear factor (NF)- κB, specificity protein (SP)-1 and CAAT (controlled amino acid treatment)-binding transcription factor/nuclear factor 1, and also cAMP responsive element (CREB), E-box, glucocorticoid responsive element (GRE) and vitamin D receptor element (VDRE) (Korhonen and Moilanen, 2014). Many factors and signaling pathways that regulate MKP-1 transcription are listed in Table 3.

MKP-1 expression is increased also by stabilization of mRNA. MKP-1 mRNA contains ARE in the 3'untranslated region, and mRNAs containing these kind of

sequences are usually highly labile. Hu antigen R (HuR) and nuclear factor 90 (NF90) have been shown to bind to 3' untranslated region of MKP-1 mRNA leading to more stable MKP-1 mRNA and increased MKP-1 protein expression (Kuwano et al., 2008).

There are several signaling pathways that regulate MKP-1 expression. ERK pathway increases the MKP-1 transcription (Brondello et al., 1997) and ERK also enhances MKP-1 protein levels by increasing the stability of MKP-1 protein by preventing its degradation via the proteosomal pathway (Brondello et al., 1999). JNK1 has also been reported to regulate MKP-1 induction in LPS-activated macrophages (Sanchez-Tillo et al., 2007). Under stress conditions, p38 MAPK increases MKP-1 levels, and the phosphorylation and acetylation of histone H3 and chromatin remodeling in MKP-1 gene locus is required for MKP-1 induction (Li et al., 2001). In addition to the MAPK pathways, protein kinase C (PKC) epsilon and intracellular calcium ions have been reported to regulate MKP-1 expression (Brion et al., 2011; Lee et al., 2012c; Ryser et al., 2001; Scimeca et al., 1997; Valledor et al., 2000; Zhang et al., 2008). In LPS stimulated macrophages, micro-RNA (miR)-101 has been shown to decrease MKP-1 expression via the PI3K/Akt pathway (Zhu et al., 2010).

Table 3. Factors and signaling pathways regulating MKP-1 expression

Type	*Factor of pathway*	*Reference*
Transcription	AP-1 ↑ CREB ↑ GR ↑ NF-κB ↑ USF ↑ VRD ↑	(Casals-Casas et al., 2009; Fujita et al., 2007; Shipp et al., 2010; Sommer et al., 2000; Wang et al., 2008; Zhang et al., 2012)
mRNA stability	HuR ↑ NF90 ↑	(Kuwano et al., 2008; Lee et al., 2012c)
Signaling pathways/ factors, others	MAPK ↑ Intracellular Ca^{2+} ↑ PKCε ↑ miR-101 ↓	(Brion et al., 2011; Brondello et al., 1997; Ryser et al., 2001; Sanchez-Tillo et al., 2007; Scimeca et al., 1997; Zhu et al., 2010)

AP-1, activator protein; CREB, cAMP responsive elements; GR, glucocorticoid receptor; NF-κB, nuclear factor-kappaB; USF, upstream stimulatory factor; VRD, vitamin D receptor; HuR, Hu antigen R; NF90, nuclear factor 90; PKC, protein kinase C; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; miR, microRNA; ↑ increase MKP-1 expression; ↓ decrease MKP-1 expression

Role of MKP-1 in inflammation and immune responses

Studies with MKP-1 KO mice have revealed a diverse and important role of MKP-1 in immune responses (Chi et al., 2006; Hammer et al., 2006; Salojin et al., 2006; Zhao et al., 2006). In innate immunity and inflammation, TLR4 activation leads to increased activity in the MAPK pathway resulting in elevated release of cytokines and other inflammatory mediators (Brown et al., 2011). MKP-1 is an important negative regulator of p38 MAPK and JNK activity and therefore controls the production of many inflammatory factors. Down-regulation of MKP-1 resulted in enhanced production of pro-inflammatory cytokines (TNF, IL-6, and IL-1β), anti-inflammatory cytokine IL-10 and chemokines (MCP-1, MIP-1α, MIP-1β, MIP-2α) (Chen et al., 2002; Chi et al., 2006; Hammer et al., 2006; Korhonen and Moilanen, 2014; Shepherd et al., 2004; Wang et al., 2007; Zhao et al., 2006). Reciprocally, the overexpression of MKP-1 has been shown to reduce IL-6, IL-10 and TNF mRNA levels (Yu et al., 2011).

MKP-1 knock-out mice suffer from more severe endotoxic shock than the corresponding wild-type animals (Chi et al., 2006; Hammer et al., 2006; Salojin et al., 2006; Zhao et al., 2006). After an LPS challenge, MKP-1 knock-out mice develop severe acute inflammation with increased infiltration of leukocytes into inflamed tissues, microvascular damage, failure of kidney, hepatic and pulmonary function and mortality. In the periodontitis mouse model, local LPS delivery caused more severe bone loss and inflammation as compared to the wild-type animal (Sartori et al., 2009). The role of MKP-1 in bacterial infections may be dependent on the bacterial species. Infection by gram-negative bacteria triggered an increased inflammatory response and cytokine production and, unexpectedly, reduced bacterial clearance in MKP-1 KO mouse as compared to the wild-type mice (Frazier et al., 2009). However, there were no differences between MKP-1 KO and wild-type mice in survival and bacterial tissue burden in Gram-positive *Staphylococcus aureus* sepsis model (Wang et al., 2007).

MKP-1 is also an important regulator of adaptive immune responses. Despite the fact that MKP-1 is expressed in the thymus (Tanzola and Kersh, 2006), MKP-1 KO mice show a normal lymphocyte maturation (Zhang et al., 2009). MKP-1 KO mice and wild-type mice have no differences in thymocyte development or in CD4+ / CD8+ ratio (Tanzola and Kersh, 2006; Zhang et al., 2009). MKP-1 controls also T cell activation and functions (Zhang et al., 2009). MKP-1 negatively regulates the phosphorylation of JNK in T cells (Zhang et al., 2009). MKP-1 expression is high in naïve CD4+ T cells, but is substantially reduced after activation. IL-2 production and T cell proliferation were attenuated in cells from MKP-1 KO mice due to reduced nuclear factor of activated T cells (NFAT) activity (Zhang et al., 2009). MKP-1 is present in all effector Th cells (Th1, Th2 and Th17) but it seems to be important only to Th1 and Th17 differentiation and function (Zhang et al., 2009). Furthermore, deficiency of MKP-1 leads to enhanced T cell-dependent B cell response and antibody production (Zhang et al., 2009). MKP-1 KO mice suffer more severe influenza A virus infection, due to impaired clearance of the influenza virus and defective CD4+ and CD8+ T cell responses (Zhang et al., 2009).

Interestingly, MKP-1 KO mice are resistant to EAE (Zhang et al., 2009), while they develop more severe disease in the mouse CIA model and the incidence of the disease is also increased (Salojin et al., 2006).

Other roles of MKP-1

In addition to its role in immune responses and inflammation, there is evidence that MKP-1 is involved also in energy metabolism, skeletal muscle metabolism, cancer development, CNS disorders and atherosclerosis. In high-fat diet and in genetic models of obesity in mice, increased JNK activity has been observed (Hirosumi et al., 2002). Results obtained in animal models have revealed that, JNK1 is involved in the obesity-induced insulin resistance *in vivo*. There are reports that p38 MAPK activity is increased in diet-induced obesity in skeletal muscle, liver and heart (Leng et al., 2004; Li et al., 2005). ERK1 is involved in adipose tissue development and thus ERK1 KO mice on high-fat diet display reduced weight gain and do not develop insulin resistance (Bost et al., 2005). Taking these all together, one may assume that MKP-1 is involved also in the energy metabolism by regulating MAPK activity and MKP-1 KO mice show that activity of all MAPKs is enhanced in insulin-responsive tissues in obesity (Wu et al., 2006).

MKP-1 also controls skeletal muscle repair and regeneration by controlling satellite cell proliferation and differentiation in the damaged areas (Shi et al., 2010). Up-regulation of MKP-1 may provide improved neuroprotective outcomes in Huntington's disease by inhibiting the JNK and p38 pathways (Taylor et al., 2013). In addition, it has been speculated that MKP-1 could be neuroprotective factor also in cerebral infarction, by suppressing neuronal death by deactivating JNK pathway (Koga et al., 2012).

MKP-1 may also be involved in the pathogenesis of atherosclerosis, depression and cancer. MKP-1 is expressed in atherosclerotic lesions and an MKP-1 deficiency leads to decrease in atherosclerotic lesion size in apolipoprotein (Apo)E-null mice

(Reddy et al., 2004; Shen et al., 2010). MKP-1 expression also correlates with hypercholesterolemia and the development of atherosclerosis in mouse models of atherosclerosis (Reddy et al., 2004). Furthermore, MKP-1 may be involved in the regulation of mood and behavior, MKP-1 mRNA levels were increased in certain areas of the brain (dental gyrus and Cornu Ammonis 1) in patients with a major depressive disorder as compared to the healthy subjects, and MKP-1 KO mice did not develop depressive-like behavior under experimental conditions (Duric et al., 2010). In the rat chronic unpredictable stress (CUS) model, MKP-1 expression was increased. MKP-1 KO mice were resistant to CUS-induced behavioral deficits (Duric et al., 2010). In addition, MAPKs regulate cell proliferation and differentiation, so one may assume that dysregulated MAPK signaling due to aberrant MKP-1 expression may be involved in the cancer development. Altered MKP-1 levels have been reported, for example, in lung cancer and prostate cancer (Rauhala et al., 2005; Vicent et al., 2004).

MKP-1 in mediating drug effects

MAPKs are important mediators in the regulation of the inflammatory response and therefore MKP-1 is an interesting and potential anti-inflammatory drug target. It has been reported that glucocorticoids increase the expression of MKP-1 in many cell types, including macrophages, airway epithelial and smooth muscle cells, chondrocytes and endothelial cells (Abraham et al., 2006; Bhavsar et al., 2008; Furst et al., 2007; Issa et al., 2007). Studies with MKP-1 KO mice have revealed that the synthetic glucocorticoid derivative, dexamethasone, can inhibit the phosphorylation of p38 MAPK and JNK in bone marrow-derived macrophages in wild-type mice, while dexamethasone had impaired effect on p38 MAPK and JNK phosphorylation in cells from MKP-1 KO mice (Abraham et al., 2006). Mapracorat, which is a novel selective glucocorticoid receptor agonist, enhanced the expression of MKP-1 in a similar manner and potency as dexamethasone in macrophages (Vollmer et al., 2012). Mapracorat also inhibited p38 MAPK activity after LPS stimulation. Down-

regulation of MKP-1 reduced the effect of mapracorat on p38 MAPK and its direct substrate, MK2, phosphorylation suggesting that the anti-inflammatory effect of mapracorat is at least partly mediated by MKP-1.

The MKP-1 promoter contains a cAMP-responsive element (CRE) (Kwak et al., 1994), and CRE binds activated CREB, a transcription factor regulated by cAMP. The expression of MKP-1 has been reported to be increased in response to elevated cAMP levels (Brion et al., 2011). β_2-receptors are G-protein-coupled receptor (GPCRs), and they activate adenylyl cyclase leading to increased cAMP levels and cAMP-mediated signaling in cells. The expression of MKP-1 is increased also by β_2-agonists (Kaur et al., 2008; Keränen et al., 2014; Manetsch et al., 2012b). The long-acting β_2-agonist, formoterol, was recently reported to enhance MKP-1 mRNA and protein expression in a cAMP-mediated manner via the β_2-adrenergic receptor-PKA pathway (Manetsch et al., 2012b).

MKP-1 has been shown to mediate anti-inflammatory effect of the anti-rheumatic gold compound, aurothiomalate. Aurothiomalate increased the expression of MKP-1 and decreased the phosphorylation of p38 MAPK. In chondrocytes and in the cartilage from rheumatoid arthritis patients, auratiomalate could suppress IL-1β induced COX-2, IL-6 and MMP-3 production. Both the inhibition of the phosphorylation of p38 MAPK and the expression of COX-2, MMP-3, and IL-6 by aurothiomalate were impaired in cells where MKP-1 had been silenced by siRNA or in cartilage from MKP-1 KO mice demonstrating that MKP-1 mediated anti-inflammatory effects of aurothiomalate. (Nieminen et al., 2010)

L-glutamine is a common amino acid present in plasma. In addition to its role in cell metabolism, glutamine has also anti-inflammatory properties (Ayush et al., 2013; Kim et al., 2006; Ko et al., 2009; Singleton et al., 2005). Interestingly, there are reports that glutamine can inhibit LPS-induced p38 MAPK activity by increasing the expression of MKP-1 and attenuating cytokine release in alveolar macrophages in lung tissue (Ko et al., 2009; Singleton et al., 2005) and in 2,4-dinitro-l-fluorobenzene

(DNFB)-induced contact dermatitis model (Ayush et al., 2013). Glutamine also suppressed airway neutrophilia inhibition of p38 MAPK phosphorylation by increasing expression of MKP-1 (Lee et al., 2012a).

Vitamin D is known to regulate calcium homeostasis and bone metabolism, but it also participates in the immune response and inflammation (Querfeld, 2013). Also, reduced serum vitamin D levels have been associated with more severe asthma, poor prognosis of the disease and impaired response to inhaled glucocorticoid treatment (Sutherland et al., 2010). Interestingly, this may be related to the MAPK/MKP-1 pathway because it has been demonstrated that vitamin D could increase MKP-1 expression in human monocytes and murine bone marrow-derived macrophages and also decreased the phosphorylation of p38 MAPK (Sutherland et al., 2010; Zhang et al., 2012). Furthermore, the inhibitory effects of vitamin D on LPS-induced p38 MAPK phosphorylation and IL-6 and TNF production were reduced in MKP-1 KO mice as compared to wild-type mice (Zhang et al., 2012).

MKP-1 also seems to be involved in the regulation of mood or in the pathogenesis of depression. MKP-1 KO mice did not develop depressive behavior in experimental mouse models of depression, and in an autopsy study, patients with depression had increased MKP-1 levels in the brain (Duric et al., 2010). Thus, one could speculate that modulators of MKP-1 pathway may be effective in certain psychiatric disorders or alternatively their use for other indications may be limited by their adverse effects on the central nervous system.

2.2.2 MKP-5

MKP-5 (DUSP10) is a less well studied member of the MKP family. Originally, MKP-5 was cloned and characterized in 1999 (Tanoue et al., 1999). MKP-5 shares a common structure with the other MKPs, including the Cdc25-like domain and a C-terminal phosphatase motif. However, in contrast to other MKPs, MKP-5 has an additional long N-terminal region, although its role is still unclear (Tanoue et al.,

1999). Another characteristic feature of MKP-5 is that MAPK docking motif is located N-terminally, while this motif is located C-terminally in all other MKPs (Theodosiou et al., 1999). MKP-5 is found both in nucleus and cytoplasm (Tanoue et al., 1999; Theodosiou et al., 1999).

MKP-5 displays substrate specificity towards p38 MAPK and JNK (Qian et al., 2012; Shi et al., 2013; Tanoue et al., 1999; Theodosiou et al., 1999). With high MKP-5 expression levels in transfection models, MKP-5 dephosphorylated all MAPKs in fibroblasts (Tanoue et al., 1999; Theodosiou et al., 1999). In T cells, it seems that MKP-5 regulates primarily JNK activity (Zhang et al., 2004), whereas in neutrophils, MKP-5 regulates mainly p38 MAPK activity (Qian et al., 2009). In bone marrow derived macrophages from MKP-5 KO mice, it seems that also ERK phosphorylation is increased after stimulation with high a LPS dose, especially in later time points as compared to the wild-type mice (Qian et al., 2012). MKP-5 may regulate ERK phosphorylation, either by modulating cross-talk between the p38 MAPK and ERK pathways (Finch et al., 2012) or by preventing the nuclear translocation of phosphorylated ERK and by inhibiting the activated ERK from phosphorylating its substrates (Nomura et al., 2012).

MKP-5 is predominantly expressed in human liver and skeletal muscle, but it is found also in other tissues, such as retina, macrophages, colon, infant brain, lung, kidney, pregnant uterus and ovarian tumors (Tanoue et al., 1999; Theodosiou et al., 1999). MKP-5 has a high basal activity in some cell types and its expression has been reported to be increased by LPS, anisomycin, osmotic stress and vitamin D in epithelial cells, mouse macrophages and pancreas cells (Jeong et al., 2006; Nonn et al., 2006; Tanoue et al., 1999; Zhang et al., 2004). In CD4+ T cells, MKP-5 is constitutively expressed (Zhang et al., 2004). MKP-5 deficient mice have no obvious phenotype and lymphoid and myeloid cells developed normally.

Role of MKP-5 in inflammation and immune responses

MKP-5 has been shown to negatively regulate AP-1 mediated transcription in RAW macrophages and Jurkat T cells (Zhang et al., 2004). A deficiency of MKP-5 was reported to lead to increased levels of IL-6, TNF and MIP-2 after LPS stimulation or infection with Gram-positive bacterium *Listeria monocytogenes* in macrophages (Qian et al., 2012; Zhang et al., 2004). Also, the expression of inducible nitric oxide synthase (iNOS) and the production of nitric oxide (NO) were also higher in macrophages from MKP-5 KO mice than wild-type mice (Qian et al., 2012).

MKP-5 negatively regulated neutrophil NADPH oxidase and thereby production of superoxide (Qian et al., 2009). CD4+ T cells showed reduced proliferation after anti-CD3/anti-CD28 stimulation, evidence that MKP-5 is essential for proper T cell expansion. Production of IFN-γ and IL-4 was enhanced in Th1 and Th2 cells, respectively, but the production of IFN-γ and TNF was reduced in effector CD8+ T cells from MKP-5 KO mice in comparison to the wild-type mice.

In the EAE mouse model, MKP-5 KO mice experienced reduced disease severity and this was associated with reduced number of CD4+ T cells in the brain. In the mouse model of acute LPS-induced lung injury, MKP-5 KO mice suffered from more severe tissue damage and increased edema (Qian et al., 2012). Accordingly, in a model of local Shwartzman reaction, MKP-5 KO mice had increased erythrocyte extravasation, microcapillary thrombus formation and neutrophil accumulation at the site of the injury, while in wild-type mice, no macroscopic changes were observed (Qian et al., 2009).

In summary, MKP-5 seems to be an important regulator of the inflammatory response. In addition, MKP-5 seems to also have different effects in different diseases depending on the type of inflammation involved in the pathology.

Other roles of MKP-5

There are some data on the role of MKP-5 in inflammation and the immune response, but its other functions are far less well understood. Curcumin, a plant alkaloid, which may have preventive or therapeutic effects in cancer, up-regulated MKP-5 expression in human prostatic epithelial cells (Nonn et al., 2007). MKP-5 has been shown to be a direct target of miR-181 (Song et al., 2013). miR-181 is often dysregulated in human hepatocellular carcinoma and the levels of miR-181s and MKP-5 mRNA were shown to be inversely correlated in cancerous hepatocytes.

MKP-5 has also been linked to obesity and atherosclerosis. For example, MKP-5 is involved in the differentiation of brown adipocytes (Choi et al., 2013) and MKP-5 expression is elevated in response to oxidized LDL (ox-LDL) (Luo et al., 2012). Ox-LDL is important in the formation of foam cells and thus in the development of atherosclerosis. MKP-5 positively regulates the ox-LDL induced NF-κB signaling pathway and increases the formation of foam cells.

2.3 Phosphodiesterase 4 (PDE4)

3',5'-Cyclic adenosine monophosphate (cAMP) is an important second messenger in many cellular functions. cAMP signaling pathway is targeted by many drugs, for instance by compounds affecting cAMP-linked GPCRs or by inhibitors of phosphodiesterases (PDEs) (Beavo and Brunton, 2002). Studies over the last years have shown that PDEs are important regulators of cAMP signaling in cells and inhibitors of certain PDE isoforms have proved to be important drug targets in human diseases (Houslay et al., 2005; Page and Spina, 2012).

2.3.1 cAMP signaling and effectors

cAMP is the archetypal second messenger (Beavo and Brunton, 2002) and the first second messenger to be identified (Sutherland.E.W and Rall, 1958). cAMP is synthesized from adenosine triphosphate (ATP) by adenylate cyclases (AC) in response to activation of GPCRs (Grandoch et al., 2010) (Fig. 2). The regulation of cAMP signaling is complex and there is also cross-talk with other signaling pathways. In the cell, cAMP concentrations regulated in balance between ACs and PDEs. cAMP is converted/inactivated to 5'AMP by PDE -catalyzed hydrolysis, and this terminates cAMP signaling inside the cell.

cAMP has an important role in signaling in a number of cellular responses, such as cell metabolism, regulation of intracellular calcium concentration, cardiac and smooth muscle contraction, secretion, ion channel conductance, learning and memory, cell growth and differentiation, apoptosis, and inflammation. cAMP may regulate inflammatory responses in many ways. cAMP influences on inflammatory response by modulating inflammatory signaling pathways, such as the PKA-CREB pathway, the NF-κB pathway and the Jak/STAT pathway (David et al., 1996; Gerlo et al., 2011). Low cAMP concentrations coincide with increased expression of pro-inflammatory mediators and rise in cAMP levels reduces the activation of macrophages and in that way it decreases the expression of inflammatory cytokines (Shirshev, 2011).

Effects of cAMP are mediated via three principal mechanisms: activation of protein kinase A (PKA)/ cAMP response element binding protein (CREB) pathway, activation of cAMP-gated ion channels (CNGs) and activation of exchange proteins directly activated by cAMP (Epacs) (Grandoch et al., 2010). PKA was discovered in the 1960s at laboratory of Edwin G. Krebs. Protein kinase A (PKA) is a serine/threonine kinase that regulates a number of processes important for cell metabolism and also for inflammation. PKA consists of two catalytic domains bound to two regulatory subunits forming an inactive PKA complex (Taylor et al.,

2008). When cAMP is produced, it binds to the PKA regulatory subunits, whereupon they disengage from the catalytic subunits. The catalytic subunits are then activated and they phosphorylate their substrates (Fig. 2). PKA substrates include ion channels, metabolic enzymes and transcription factors. Specificity of PKA is achieved by A Kinase Anchoring Proteins (AKAPs) (Wong and Scott, 2004). AKAPs anchor PKAs to specific locations near to their substrates within the cells (e.g. ion channel, a co-transporter) or near organelles (e.g. mitochondria or Golgi). AKAPs may provide an interesting way to selectively interfere PKA signaling.

CREB is one of the classical PKA substrates (Mayr and Montminy, 2001). PKA phosphorylates CREB on its Ser 133 residue and this activates CREB, which then translocates to the nucleus. CREB binds to the cAMP-responsive elements (CRE) and activates gene transcription. CREB is important mediating the immune response, for example, it inhibits NF-κB activity and increases IL-10 production and the generation of regulatory T-cells (Wen et al., 2010). Other cAMP-responsive transcriptional activators are cAMP-responsive element modulator (CREM) and cAMP-dependent transcription factor (ATF)-1 (Hummler et al., 1994).

Ion channels directly gated by cyclic nucleotides are activated by binding either cAMP or cGMP. Ion channel activation by cAMP/cGMP binding opens the channels leading to calcium and sometimes sodium or potassium influx. Changes in free cytosolic calcium modify many cell processes, such as cell metabolism, secretion, and muscle contraction. Initially, these channels were discovered in sensory cell types, but nowadays they have been found in almost every cell type. (Hofer, 2012; Kraus-Friedmann, 2000)

Epacs were discovered in 1998 by Rooij and colleagues (de Rooij et al., 1998). There are two Epac isoforms, Epac1 and Epac2 (Schmidt et al., 2013; Shirshev, 2011). Epac2 has a limited distribution pattern and it is expressed in adrenals, brain and pancreatic β cells. Epac1 is widely expressed in several cell types including monocytes, macrophages, B cells, T cells, eosinophils, neutrophils, platelets and

hematopoetic CD34+ cells. Epac1 activates its substrates Rap 1 and Rap 2 and R-Ras. The epac1 seems to be involved in inflammatory diseases and there is reduced expression is in the lung tissue in patients with COPD (Oldenburger et al., 2012). Epac1 has also been associated with Alzheimer's disease, diabetes, and atherosclerosis. Interestingly, Epac2 expression is reduced in Alzheimer's disease while Epac1 expression is increased. It is not known whether the elevation of Epac1 levels or the decrease of Epac2 levels is important in the pathogenesis of Alzheimer's disease (McPhee et al., 2005).

In summary, cAMP is an important factor in mediating cell responses to inflammatory and related signals. The intracellular concentration of cAMP depends on the production of cAMP by AC, as well as degradation of cAMP by PDEs. The effects of cAMP are mediated through interactions of cAMP with PKA, Epac or ion channels.

2.3.2 Introduction to phosphodiesterases (PDEs)

PDEs are a part of the cellular machinery that degrades cyclic nucleotides and in that respect they are involved in terminating cyclic nucleotide signaling. There are 11 different PDE genes and a total of 21 different isoforms with multiple splicing variants, which increases the repertoire of different PDEs (Francis et al., 2011). PDEs differ from each other by their substrate specificities and cyclic nucleotide affinities, regulatory mechanisms and tissue distribution. PDEs can be dividing into three groups based on their substrate specificities. PDE 1, 2, 3, 10 and 11 can hydrolyze both cAMP and cGMP; PDE 4, 7 and 8 are cAMP specific; and PDE 5, 6 and 9 are specific for cGMP.

PDE inhibitors have been studied in many diseases. As will be discussed below, PDE4 inhibitors can suppress the inflammatory response and the first selective PDE4 inhibitor, roflumilast, has been approved for treatment of COPD as an anti-

inflammatory drug. In immune cells, PDE4 inhibition has been shown to suppress the inflammatory response in basophils, eosinophils, lymphocytes, monocytes/macrophages and neutrophils. Also inhibitors of PDE3 and PDE5 have been studied in the treatment of inflammatory disorders. However, despite the expression of the other PDEs in the immune cells, their inhibition has little or no independent anti-inflammatory efficacy (Essayan, 1999). PDEs have several splice variants and they have distinct tissue distributions and expressional patterns, which provides the possibility to develop selective PDE inhibitors to different diseases. One limitation for the use of PDE inhibitors may a rise from the fact that PDEs are widely expressed in a wide range of cells and tissues. This may result in the appearance of many and perhaps unexpected side-effects (DeNinno, 2012).

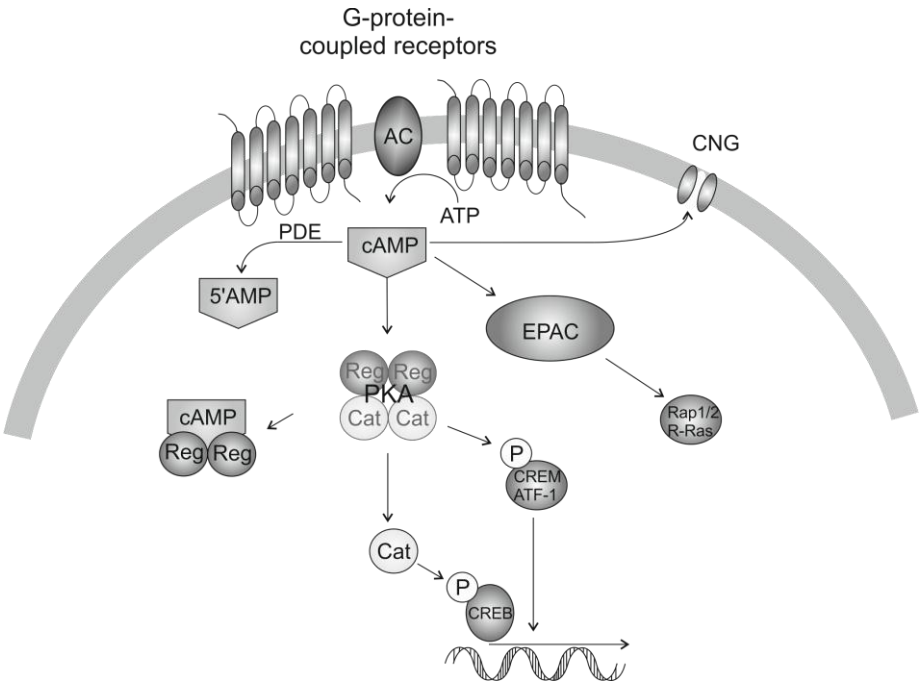

Figure 2. cAMP-PKA signaling pathway. Activation of G-protein-coupled-receptors leads to the production of cAMP from adenosine triphosphate (ATP) by adenylate cyclases (AC). The effects of cAMP are mediated via activation of protein kinase A (PKA)/cyclic AMP response element binding protein (CREB) pathway or via other PKA targets. cAMP binds to PKA regulatory subunits (Reg), whereupon catalytic subunits (Cat) are released from the inactive PKA-complex. Catalytic subunits translocate to the nucleus where they phosphorylate CREB. CREB regulates the expression of many genes and this way alters the activation state of the cell. Effects of cAMP are mediated also via cAMP-gated ion channels (CNGs) and through exchange proteins directly activated by cAMP (Epac). Phosphodiesterases (PDEs) convert cAMP to inactive 5'AMP leading to the attenuation of cAMP signaling.

2.3.3 PDE4: genes and structure

PDE4 is a cAMP-specific phosphodiesterase and different forms of PDE4 are expressed in the brain, inflammatory cells, cardiovascular tissues and smooth muscles. PDE4 is the largest PDE family (Lugnier, 2006). There are four PDE4 genes, namely PDE4A, PDE4B, PDE4C and PDE4D. In addition, there are alternative mRNA splice variants of different PDE4 isoenzymes, meaning that there

are at least 35 different PDE4 proteins. PDE4 isoforms can be divided into three categories: long, short and super-short. All PDE4s isoforms have a catalytic domain, which are 20-45 % identical in all PDEs (Fig. 3). PDE4s have an N-terminal region, called upstream conserved region 1 and 2 (UCR1 and UCR2). Both UCR regions are found in longer PDE4 isoforms, while short isoforms have only UCR2. Linker region 1 (LR1) is between two UCR regions and LR2 connects UCR2 to the catalytic domain (Richter and Conti, 2004). PKA phosphorylation site, phosphatic acid binding sites and dimerization sites are located in the N-terminal region. ERK2 phosphorylation site is located in the C-terminal region.

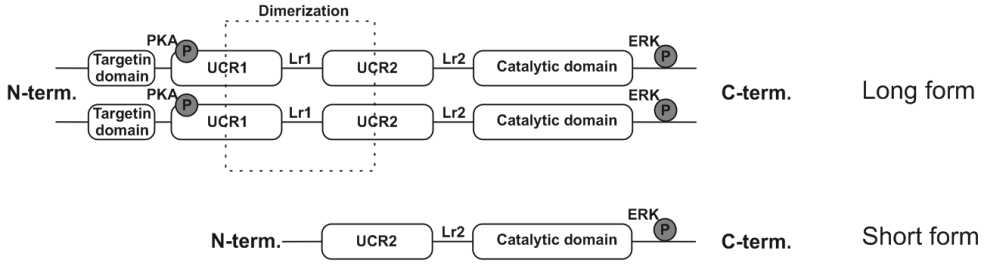

Figure 3. PDE4 protein structure. Linker region, LR; upstream conserved region, UCR. (Modified from Richter and Conti, 2004)

2.3.4 Effects of PDE4 and PDE4 inhibitors in inflammation

Since PDE4s turn off the biological activity of cAMP which is known to suppress the activity of inflammatory cells, PDE4 inhibitors were hypothesized to have anti-inflammatory potential (DeNinno, 2012). Studies with PDE4 KO mice have revealed different functions for distinct PDE4 genes. PDE4D KO mice have delayed growth, reduced viability and female fertility and, interestingly, antidepressant-like behavior profile (Jin et al., 1999). A deficiency of PDE4D reduces α_2-adrenoceptor-mediated anesthesia and may also cause emesis (Robichaud et al., 2002). PDE4B is important for TNF release, since in leukocytes from PDE4B KO mice, LPS-induced TNF production was severely reduced (Jin and Conti, 2002). Both PDE4B and PDE4D are important for neutrophil recruitment during lung

inflammation. Both PDE4B and PDE4D KO mice displayed reduced accumulation of neutrophils in acute LPS-induced lung injury models and this seemed to be related to reduce neutrophil migration (Ariga et al., 2004). PDE4B, but not PDE4D, has important and non-redundant functions in the development of Th2 immune responses and airway hyperresponsiveness due to allergen sensitization. PDE4B KO mice did not develop airway hyperresponsiveness in the ovalbumin-induced lung inflammation model and T cells from those mice produced less IL-4, IL-5 and IL-13 after ovalbumin stimulation *in vitro*. (Jin et al., 2010)

PDE4 inhibitors have clear anti-inflammatory effects *in vitro*. PDE4 inhibitor roflumilast inhibited the production of NO, TNF and IL-1β in mouse macrophages after LPS stimulation (Kwak et al., 2005). Another PDE4 inhibitor ASP3258 inhibited the production of TNF in rat whole blood cells and also in rat alveolar macrophages (Kobayashi et al., 2011). The classical PDE4 inhibitor, rolipram, inhibited TNF production also in human peripheral blood mononuclear cells (Link et al., 2008). The PDE4 inhibitor, GSK25606, inhibited the production of TNF in human peripheral blood monocytes in response to LPS (Tralau-Stewart et al., 2011). Functional PDE4 seems to be important for the production of inflammatory factors also in the rheumatoid synovium. Apremilast and two other PDE4 inhibitors, INH 0061 and INH 0062, inhibited the production of TNF, MCP-1, MIP-1α, MIP-1β, IL-1β and RANTES in synovial membranes from rheumatoid arthritis patients (Crilly et al., 2011).

PDE4 inhibitors have shown efficacy also in *in vivo* models. In chronic subcutaneous inflammation model, rolipram inhibited the production of TNF and MCP-1 in subcutaneously placed tissue implants (Mendes et al., 2009). In SCID mice which were transplanted with human skin xenocrafts from patients with psoriasis, the PDE4 inhibitor apremilast attenuated the formation and the severity of psoriasiform lesions in skin xenocrafts (Schafer et al., 2010). In ovalbumin-sensitized mice, re-exposure to ovalbumin increased the lung inflammation and the

accumulation of eosinophils, lymphocytes and neutrophils into the lung, and rolipram inhibited those dose-dependently (Kanehiro et al., 2001). Rolipram also decreased the production of IL-4 and IL-5 in response to ovalbumin (Kanehiro et al., 2001). PDE4 inhibitor GSK256066 reduced LPS-induced pulmonary neutrophilia and exhaled nitric oxide levels in the mouse, and decreased ovalbumin-induced pulmonary eosinophilia in rats (Nials et al., 2011).

Rolipram is a classical non-selective PDE4 inhibitor used in many *in vitro* and *in vivo* disease models (Christensen et al., 1998; Lugnier, 2006), and currently it is tested in the clinical studies for the treatment of major depressive disorders, Huntington disease and multiple sclerosis (see Clinical Trials Registry, 15.4.2014). At the moment, there are few other PDE4 inhibitors in clinical use or under clinical development as well. One such inhibitor, roflumilast, is already in clinical use for the treatment of COPD as an anti-inflammatory remedy to prevent the exacerbations of the disease. Roflumilast improved lung function and also health-related quality of life in patients with COPD (Calverley et al., 2007; Calverley et al., 2009; Fabbri et al., 2009; Rabe et al., 2005). In addition to COPD, there is evidence that roflumilast can attenuate the symptoms of asthmatic patients (Bateman et al., 2006; Gauvreau et al., 2011). Roflumilast has been reported to suppress allergen-induced airway inflammation in patients with mild atopic stable asthma (Gauvreau et al., 2011). Roflumilast also improved lung function in asthma patients (Bateman et al., 2006). Cilomilast is another PDE4 inhibitor that is in the clinical trials. Cilomilast has been studied for the treatment of COPD, where it improved lung functions (Compton et al., 2001; Rennard et al., 2006).

Apremilast is another PDE4 inhibitor that is approved for the clinical use. It has been reported to be effective in the treatment of psoriasis and psoriatic arthritis (Papp et al., 2012; Schett et al., 2012), and apremilast has been approved by the FDA for the treatment of psoriatic arthritis. There are some data suggesting that it also possesses efficacy in the treatment of ankylosing spondylitis; apremilast had positive

effects on the biomarkers that reflect the activity of disease and it was well tolerated in those patients (Pathan et al., 2013). The PDE4 inhibitor GSK256066 is undergoing clinical trials for the treatment of COPD. There were promising results in inflammatory markers, but its efficacy has not yet been proved (Watz et al., 2013).

3. AIMS OF THE STUDY

Cells respond to extracellular inflammatory signals by producing cytokines and other inflammatory mediators that elicit and govern the course of the inflammatory response. MAPKs are important components of the intracellular signaling pathways that integrate and convey these exogenous inflammatory signals within cells enabling appropriate inflammatory cell responses. MKPs are phosphatases that regulate MAPK pathways. In the present study, the role of MKPs in the regulation of inflammatory gene expression and inflammatory response was investigated. In addition, the role of MKPs in the anti-inflammatory effects of the PDE4 inhibitor, rolipram, was studied.

The following hypotheses were tested:

1. MKP-1 and/or MKP-5 inactivate (dephosphorylate) MAPKs and down-regulate the expression of inflammatory genes.
2. PDE4 inhibitor, rolipram, increases MKP-1 levels in macrophages possibly through cAMP signaling pathway
3. Anti-inflammatory effects of a PDE4 inhibitor rolipram are mediated, at least partly, by MKP-1

4. MATERIALS AND METHODS

4.1 Reagents

4.1.1 Chemicals

Gibco, Wien, Austria

Fetal bovine serum (FBS)
Penicillin
Streptomycin,
Amphotericin B

R&D Systems Inc., Minneapolis, MA, USA:

Recombinant human TNFα, IFNγ and IL-1β
Recombinant mouse macrophage colony-stimulating factor (M-CSF)

Sigma-Aldrich Inc., St. Louis, MO, USA:

Lipopolysaccharide (LPS) from *E. coli* strain 0111:B4
IBMX (1-Methyl-3-isobutylxanthine)
8-Br-cAMP (8-Bromoadenosine 3',5'-cyclic monophosphate)
ethylenediaminetetraacetic acid (EDTA)
dimethyl sulfoxide (DMSO)

Tocris Biosciences, Bristol, UK:

SB 202190 (4-[4-(4-Fluorophenyl)-5-(4-pyridinyl)-1H-imidazol-2-yl] phenol)

AxonMedChem, Groningen, the Netherlands

BIRB 796 (1-(5-tertbutyl-2-p-tolyl-2H-pyrazol-3-yl)-3(4-(2-morpholin-4-yl-ethoxy)naphthalen-1-yl)urea)
rolipram [4-(3-Cyclopentyloxy-4-methoxy-phenyl)-pyrrolidin-2-one

Calbiochem, Merck Chemicals, Darmstadt, Germany:

JNK inhibitor VIII (N-(4-Amino-5-cyano-6-ethoxypyridin-2-yl)-2-(2,5-dimethoxyphenyl)acetamide)

Orion Oyj, Espoo, Finland

medetomidine (Domitor® 1 mg/ml)

Pfizer Oy Animal Health, Helsinki, Finland

ketamine (Ketalar® 10 mg/ml)

4.1.2 Antibodies

Cell Signaling Technology Inc., Beverly, MA, USA:

Phospho-p38 antibody (#9211)
p38 antibody (#9212),
phospho-JNK antibody (#9251),
phospho-MK2 antibody (27B7) (#3007),
MK2 antibody (#3042)
phospho-ERK antibody (#9101)
ERK antibody (#9102)

Abcam plc, Cambridge, UK:

p38 antibody (ab27986)

Sigma-Aldrich Inc., St. Louis, MO, USA

MKP-1 antibody (SAB2500331)

Santa Cruz Biotechnology, Santa Cruz, CA, USA:

Actin antibody (sc-1615)
polyclonal anti-rabbit antibody (sc-2004)
polyclonal anti-goat antibody (sc-2020)
COX-2 antibody (sc-1745)
actin antibody (sc-1616-R)
MKP-1 antibody (sc-1102)
phospho-c-Jun antibody (sc-822)
c-Jun antibody (sc-45)
JNK antibody (sc-571)
Lamin A/C antibody (sc-20681)

Upstate, Lake Placed, NY, USA

PABP1 antibody (05-847)

Pierce Biotechnology, Rockford, IL, USA

polyclonal anti-mouse antibody (32430)

Reagents for cell culture, ELISA, Western blotting, RNA extraction and quantitative transcriptase polymerase chain reaction (qRT-PCR) are discussed in the next chapters.

4.2 Cell culture

A549 human lung epithelial cells (ATCC, Manassas, VA, USA) were cultured at 37°C in 5 % CO_2 atmosphere in Ham's F12K (Kaighn's modification) medium (Invitrogen, Paisley, UK) containing 5 % heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B. For cytokine measurements, Western blot and quantitative RT-PCR, cells were seeded on a 24-well plate at the density of 4 x 10^5 cells/well. Cell monolayers were grown for 48 h before the experiments were started.

J774 macrophages (ATCC, Manassas, VA, USA) were cultured at 37°C in 5 % CO_2 atmosphere in Dulbecco's modified Eagle's medium (Lonza, Verviers Sprl, Verviers, Belgium) with Ultraglutamine 1 supplemented with 10 % heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B. In the experiments, cells were seeded on 24-well plates at the density of 2 × 10^5 cells/well. Cell monolayers were grown for 72 h before the experiments were started.

THP-1 human monocytes (ATCC, Manassas, VA, USA) were cultured at 37ºC in 5 % CO_2 atmosphere in RPMI 1640 medium (Lonza Verviers SPRL, Belgium) containing 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 mg/ml glucose, and 1.5 mg/ml bicarbonate and supplemented with 10 % heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B, and 0.05 mM 2-mercaptoethanol. Cells were seeded on a 24-well plate at the density

of 3 × 10^5 cells/well, and differentiated to macrophages by adding 100 nM phorbol ester 12-O-tetradecanoylphorbol-13-acetate for 72 h before the experiments were started.

Rolipram, IBMX, SB 202190, BIRB 796 and JNK inhibitor VIII were dissolved in dimethyl sulfoxide (DMSO, v/v 0,1 %), and 8-Br-cAMP was dissolved in Hank's Balanced Salt Solution and added to the cells in fresh culture medium at concentrations indicated 30 min (SB 202190, BIRB 796 and JNK inhibitor VIII) or 1 h (rolipram and IBMX) prior to the stimulation with cytokines or LPS. Cells were further incubated for the time indicated.

4.2.1 Animals

Inbred C57BL/6 MKP-1(–/–) mice were originally generated by the R. Bravo laboratory at Bristol-Myers Squibb Pharmaceutical Research Institute (Dorfman et al., 1996), and bred at the University of Tampere School of Medicine animal facilities. MKP-1 knock-out (KO) mice and their wild-type (WT) littermates were housed under conditions of optimum light, temperature and humidity (12:12 h light: dark cycle, 22±1°C, 50-60 %) with food and water provided *ad libitum*.

Permission for the use of experimental animals and tissues and cells from those animals for experiments were given by the Animal Care and Use committee of University of Tampere and the respective provincial committee for animal experiments (II and III) and National Animal Experiment Board (V).

Isolation of peritoneal macrophages, and cell culture

Mice were killed by suffocation with CO_2 followed by an immediate cervical dislocation. Peritoneal macrophages (PMs) were obtained by intraperitoneal lavage with sterile phosphate buffered saline (PBS) supplemented with 0.2 mM ethylenediaminetetraacetic acid (EDTA). Cells were washed, resuspended in RPMI

1640 medium supplemented with 2 % heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin, and seeded on 24-well plates (5×10^5 cells/well). The cells were incubated overnight and washed with PBS to remove non-adherent cells before the experiments. Pharmacological compounds or the solvent (DMSO, v/v 0.1 %) were added to the cells in fresh culture medium containing 2 % heat-inactivated FBS and the antibiotics, and cells were stimulated with LPS (100 ng/ml). Cells were incubated for the time indicated.

Isolation of the bone-marrow-derived macrophages and cell culture

Bone marrow-derived macrophages (BMMs) were isolated from femur and tibia of the hind legs of mice aged 10-12 weeks. Mice were anesthetized by intraperitoneal injection of 0.05 mg/100 g body weight of medetomidine (Domitor® 10 mg/ml, Orion Corp., Espoo, Finland) and 7.5 mg/100 g body weight of ketamine (Ketalar® 10 mg/ml, Pfizer Oy Animal Health, Helsinki, Finland), and mice were terminated by suffocation with CO_2 followed by an immediate cervical dislocation. BMMs were differentiated from bone marrow hematopoietic stem cells by incubating the cells for 5-7 days in RPMI 1640 medium supplemented with 10 % heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 10 ng/ml M-CSF. Thereafter, BMMs (1 x 10^6 cells/well) were seeded on 24-well plates, cultured overnight in complete culture medium, and followed by serum starvation overnight. At the beginning of the experiment, LPS and the compound of interest were added to the cells in fresh culture medium containing 10 % FBS and antibiotics, and BMMs were incubated for the time indicated.

4.2.2 Cell viability

The effect of used compounds or siRNA transfection on the cell viability was investigated by XTT assay using Cell Proliferation Kit II (Roche Diagnostics, Mannheim, Germany) and by visual assessment of the cells with a microscope after

the experiments in a routine manner. Viable cells metabolize sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) to formazan by mitochondrial dehydrogenase. The cells were incubated with the tested compounds and LPS for 20 h before addition of XTT (final concentration 0.3 mg/ml) and N-methyl dibenzopyrazine methyl sulfate (1.25 mM). Cells were incubated for another 4 h and the amount of formazan accumulated into the growth medium being assessed spectrophotometrically. Triton-X treated cells were used as a positive control for cell death. Conditions were considered toxic if the cells' ability to metabolize XTT to formazan was lowered by more than 20 % as compared to cells exposed to LPS only (Korhonen et al., 2001, Hämäläinen et al., 2009). LPS, other tested compounds, or siRNA transfection were not observed to cause cytotoxicity.

4.3 Enzyme linked immunosorbent assay (ELISA)

Culture medium samples were kept at -20°C until assayed. The concentrations of human IL-6 (PeliPair® ELISA, Sanquin, Amsterdam, the Netherlands), mouse IL-6 (DuoSet® ELISA, R&D Systems Europe Ltd., Abindgon, U.K.), human IL-8 (BD OptEIATM Set, BD Biosciences, San Diego, USA) and mouse and human TNF (DuoSet® ELISA, R&D Systems Europe Ltd., Abindgon, U.K.) were determined by ELISA according to the manufacturer's instructions.

4.4 Nitrite assay

Nitric oxide (NO) production was estimated by measuring the concentrations of nitrite (the stable metabolite of NO in aqueous solution) in the culture medium by Griess reaction (Green et al., 1982). Equal volumes (50 µl) of culture medium and Griess reagent (0,1 % naphthylethylenediamine dihydrochloride, 1 % sulfanilamine,

2,4 % H_3PO_4) were incubated together and the absorbance measured at 540 nm. The concentration of nitrite was calculated using sodium nitrite as the standard.

4.5 Preparation of cell lysates and Western blotting

At the indicated time points, the culture medium was removed from the cells. Cells were rapidly washed with ice-cold PBS and solubilized in cold lysis buffer containing 10 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl, 1% Triton-X-100, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 20 μg/ml leupeptin, 50 μg/ml aprotinin, 5 mM sodium fluoride, 2 mM sodium pyrophosphate and 10 μM *n*-octyl-β-D-glucopyranoside. After incubation for 15 min on ice, lysates were centrifuged, supernatants were collected and mixed in a ratio of 1:4 with SDS loading buffer (62.5 mM Tris-HCl, pH 6.8, 10 % glycerol, 2 % SDS, 0,025 % bromophenol blue, and 5 % β-mercaptoethanol) and stored at -20°C until analyzed. The protein concentrations of the extracts were measured by the Coomassie blue method (Bradford, 1976).

Prior to Western blot analysis, the samples were boiled for 10 min. Equal aliquots of protein (20 μg) were loaded on a 10 % SDS-polyacrylamide gel and separated by electrophoresis. Proteins were transferred to Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham, Buckinghamshire, U.K.) by semidry electroblotting. After transfer, the membrane was blocked in TBS/T (20 mM Tris-base pH 7.6, 150 mM NaCl, 0.1 % Tween-20) containing 5 % nonfat milk or bovine serum albumin (BSA) for 1 h at room temperature. Membranes were incubated overnight at 4ºC with the primary antibody, and for 1 h at room temperature with the secondary antibody, and the chemiluminescent signal was detected either by FluorChem 8800 imaging system (Alpha Innotech Corporation, San Leandro, CA, USA) or ImageQuant™ LAS 4000 mini (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The chemiluminescent signal was quantified with FluorChem software version 3.1 or ImageQuant TL 7.0 Image Analysis Software.

4.6 RNA extraction and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Primers and probes for qRT-PCR were obtained from Metabion International AG (Martinsried, Germany). At the indicated time points, culture medium was removed and total RNA extraction was carried out with GenElute™ Mammalian Total RNA Miniprep Kit (Sigma Aldrich, St Louis, MO, USA) according to the manufacturer's instructions. The amount of RNA was measured spectrophotometrically and purity was confirmed via the absorbance ratio at A_{260}/A_{280}.

Total RNA was reverse-transcribed to cDNA using TaqMan Reverse Transcription reagents and random hexamers (Applied Biosystems, Foster City, CA, USA). cDNA obtained from the RT-reaction was diluted 1:20 with RNAse-free water and was subjected to quantitative PCR using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and ABI PRISM 7000 Sequence detection system. The primer and probe sequences are listed in Table 4. The primer and probe sequences and concentrations were optimized according to the manufacturer's guidelines in TaqMan Universal PCR Master Mix Protocol part number 4304449 revision C. Expression of explored genes mRNA were measured using TagMan® Gene Expression Assays (Applied Biosystems, Foster City, Ca, USA). PCR reaction parameters were as follows: incubation at 50ºC for 2 min, incubation at 95ºC for 10 min, and thereafter 40 cycles of denaturation at 95ºC for 15 s and annealing and extension at 60ºC for 1 min. A standard curve method was used to determine the relative mRNA levels as described in the Applied Biosystems User Bulletin: A standard curve for each gene was created using total RNA isolated from stimulated cells. Isolated RNA was reverse transcribed and dilution series of cDNA ranging from 1 pg to 10 ng were subjected to PCR. The obtained threshold cycle values were plotted against the dilution factor to create a standard curve. Relative mRNA levels in the test samples were then calculated from the standard curve. mRNA levels were normalized against GAPDH.

Table 4. Primer and probe sequences

Human/mouse	Gene	Oligonucleotide	Sequence 5' → 3'
Human	hCOX-2	forward	CAACTCTATATTGCTGGAACATGGA
	hCOX-2	reverse	TGGAAGCCTGTGATACTTTCTGTACT
	hCOX-2	probe	TCCTACCACCAGCAACCCTGCCA
	hDUSP1	forward	ACGAGGCCATTGACTTCATAGAC
	hDUSP1	reverse	TCGATTAGTCCTCATAAGGTAAGCAA
	hDUSP1	probe	CCACTGCCAGGCAGGCATTTCC
	hGAPDH	forward	AAGGTCGGAGTCAACGGATTT
	hGAPDH	reverse	GCAACAATATCCACTTTACCAGAGTTAA
	hGAPDH	probe	CGCCTGGTCACCAGGGCTGC
	hiNOS	forward	GCAGGTCGAGGACTATTTCTTTCA
	hiNOS	reverse	TCCTTCTTCGCCTCGTAAGGA
	hiNOS	probe	TCAAGAGCCAGAAGCGCTATCACGAAGATA
	hIL-6	TagMan gene expression assay (Applied Biosystems)	
	hIL-8	TagMan gene expression assay (Applied Biosystems)	
Mouse	mCOX-2	forward	AAGCGAGGACCTGGGTTCA
	mCOX-2	reverse	ACCTGATATTTCAATTTTCCATCCTT
	mCOX-2	probe	TGTGATTTAAGTCCACTCCATGGCCCA
	mGAPDH	forward	GCATGGCCTTCCGTGTTC
	mGAPDH	reverse	GATGTCATCATACTTGGCAGGTTT
	mGAPDH	probe	TCGTGGATCTGACGTGCCGCC
	mIL-6	forward	TCGGAGGCTTAATTACACATGTTC
	mIL-6	reverse	CAAGTGCATCATCGTTGTTCATAC
	mIL-6	probe	CAGAATTGCCATTGCACAACTCTTTTCTCA
	miNOS	forward	CCTGGTACGGGCATTGCT
	miNOS	reverse	GCTCATGCGGCCTCCTT
	miNOS	probe	CAGCAGCGGCTCCATGACTCCC
	mMKP1	forward	CTCCTGGTTCAACGAGGCTATT
	mMKP1	reverse	TGCCGGCCTGGCAAT
	mMKP1	probe	CCATCAAGGATGCTGGAGGGAGAGTGTT
	mTNF	forward	GACCCTCACACTCAGATCATCTTCT
	mTNF	reverse	CCTCCACTTGGTGGTTTGCT
	mTNF	probe	AAAATTCGAGTGACAAGCCTGTAGCCCA
	mMKP-5	TagMan gene expression assay (Applied Biosystems)	

4.7 Down-regulation of MKP-1 or MKP-5 by siRNA

A549 cells were transfected with siRNA (Table 5.) using HiPerFect transfection Reagent (QIAGEN, Valencia, Ca, USA) according to the manufacturer's instructions. Briefly, A549 cells were seeded at the density of $1.25x10^5$ cells/well on a 24-well plate in 500 µl of medium with 5 % FBS without antibiotics followed by transfection with non-targeting negative control siRNA (control siRNA), Lamin A/C siRNA, MKP-1 siRNA 1 or MKP-1 siRNA 2. For one well, 3 µl of siRNA stock solution (2 µM) was mixed with 1.5 µl of transfection reagent in a final volume of 100 µl of medium. Cells were further incubated for 48 h. Fresh culture medium was changed and cytokines were added into the culture medium. Cells were further incubated for the time indicated and then the gene expression was analyzed. All the experiments were done in triplicates.

J774 cells were transfected with siRNA (Table 5.) using Dharmafect 4 transfection reagent (Dharmacon, Lafayette, CO, USA) according to the manufacturer's instructions. Briefly, cells (1×10^5 cells/well) were seeded on a 24-well plate in 500 µl of medium with 5 % or 10 % FBS without antibiotics, and incubated overnight. For one well, the final transfection medium applied to the cells contained 25 µl (MKP-1 siRNA) or 3 µl (MKP-5 siRNA) of siRNA stock solution (2 µM) mixed with 1 µl of transfection reagent in final volume of 500 µl of medium. The cells were further incubated for 48 h. Fresh culture medium was changed and LPS was added. Cells were further incubated for the time indicated and gene expression was analyzed.

Transfection efficacy was monitored with green fluorescent siRNA oligos (siGLO green transfection indicator, Cat. number D-001630-01, Dharmacon, Lafayette, CO, USA) using Nikon Eclipse TS100 microscope (Nikon, Tokyo, Japan). Approximately 90 % of the cells emitted green fluorescence signal when transfected with siGLO and HiPerFect or siGLO and Dharmfect 4. Less than 5 % of the cells

emitted signal, when they were incubated with siGLO oligos in the absence of the transfection reagent.

Table 5. siRNA oligos

Human/mouse	siRNA	Cat. Number	Manufacture
Human	MKP-1 siRNA 1	J-003484-09-0005	Dharmacon, Lafayette, CO, USA
	MKP-1 siRNA 2	J-003484-10-0005	Dharmacon, Lafayette, CO, USA
	hLamin A/C	1022050	QIAGEN, Valencia, Ca, USA
	non-targeting control siRNA	1022076	QIAGEN, Valencia, Ca, USA
Mouse	MKP-1 siRNA	L-040753-00-0005	Dharmacon, Lafayette, CO, USA
	non-targeting siRNA	D-001210-01	Dharmacon, Lafayette, CO, USA
	MKP-5 siRNA	SI04922883	QIAGEN, Valencia, Ca, USA

4.8 Carrageenan-induced inflammation in mice

C57BL/6 mice (20-25 g) were divided into groups of six mice. The groups were treated with 200 µl of PBS or rolipram (100 mg/kg in PBS) by an intraperitoneal injection (i.p.) 2 hours before applying carrageenan. Prior to the administration of carrageenan, the mice were anesthetized by intraperitoneal injection of 0.5 mg/kg of medetomidine (Domitor® 1 mg/ml. Orion Oyj, Espoo, Finland) and 75 mg/kg of ketamine (Ketalar® 10 mg/ml, Pfizer Oy Animal Health, Helsinki, Finland). The mice received a 30 µl injection into one hind paw of normal saline containing carrageenan (1.5 %). The contralateral paw received 30 µl of saline and it was used as a control. Paw volume was measured before and three hours after carrageenan injection by the use of a plethysmometer (Ugo Basile, Comerio, Italy). After the experiments, the anesthetized animals were killed by cervical dislocation. The results are reported in accordance with the ARRIVE guidelines (McGrath et al., 2010).

4.9 Statistics

The results are expressed as mean ± standard error of mean (S.E.M.). When indicated, statistical significance was calculated by one-way or two-way analysis of variance with Dunnett's or Bonferroni's post test or by unpaired Student's t-test using InStat version 3.05 for Windows 95/NT or GraphPad Prism 5 for Windows version 5.04 (GraphPad Software, Inc., San Diego, CA). Differences were considered significant at *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$.

5. RESULTS

5.1 p38 MAPK inhibitors SB 201290 and BIRB 796 regulate the expression of inflammatory factors (I-III)

MAPK pathways are important signaling routes in inflammation and they regulate the production of several inflammatory factors. First, the effects on p38 MAPK inhibitors SB 201290 and BIRB 796 and JNK inhibitor JNK VIII on the production of IL-6, TNF, COX-2, and iNOS were investigated.

Phosphorylation of p38 MAPK was detected at the time point of 30 minutes in response to cytokines (TNF, IFNγ and IL-1β 10 ng/ml each) in human A549 pulmonary epithelial cells or in response to LPS (10ng/ml) in mouse macrophages. In A549 cells, the phosphorylation of p38 MAPK increased again at the time point 2 h and soon returned to the basal level. Phosphorylation of JNK was observed at 30 minutes after the stimulation with cytokines or LPS and it was restored to the basal level at 1 h.

Two structurally distinct p38 MAPK inhibitors SB 202190 and BIRB 796 were used. They have been reported to inhibit effectively p38 MAPK at 1 μM (SB 202190) and 100 nM (BIRB 796) concentrations (Bain et al., 2007). The phosphorylation of p38 MAPK substrate MK2 was measured in stimulated J774 and A549 cells to find the suitable inhibitor concentration for SB 202190 and BIRB 796. SB 202190 and BIRB 796 inhibited MK2 phosphorylation at 1 μM and 100 nM concentrations, respectively, this being in line with previously published results. The effect of JNK inhibitor VIII on the phosphorylation of JNK substrate c-Jun was tested in A549

cells. JNK inhibitor VIII inhibited c-Jun phosphorylation submaximally at 1 μM drug concentration and completely at 10 μM drug concentration.

Next, the effects of the inhibitors of p38 MAPK or JNK on the expression of pro-inflammatory factors were investigated. Cells were pre-incubated with the inhibitors for 30 min and stimulated with a cytokine mixture or LPS for 3 h and 24 h prior to mRNA and protein measurements, respectively. In addition, iNOS mRNA was measured at 6 h. Unstimulated cells or cells pre-treated with SB 202190, BIRB 796 or JNK inhibitor VIII alone did not express inflammatory factors. In A549 cells, the cytokine mixture induced the production of IL-6 and IL-8 this effect was inhibited in a dose-dependent manner by SB 202190 and BIRB 796 (Fig. 4A and 4B). Furthermore, the expressions of iNOS and COX-2 were induced by cytokines and again this could be inhibited in a dose-dependent manner by SB 202190 and BIRB 796. In contrast, JNK inhibitor VIII did not inhibit the production of IL-6, IL-8 or COX-2 in A549 cells. In primary peritoneal macrophages (PM), p38 MAPK inhibitor decreased the production of TNF and COX-2 (Fig. 4C and 4B), while the JNK inhibitor had no effect on these inflammatory genes. Similarly to the situation in PM cells, LPS induced the expression of IL-6, TNF, COX-2 and iNOS as well as NO production in J774 macrophages. The production of these inflammatory factors was inhibited by the p38 MAPK inhibitors.

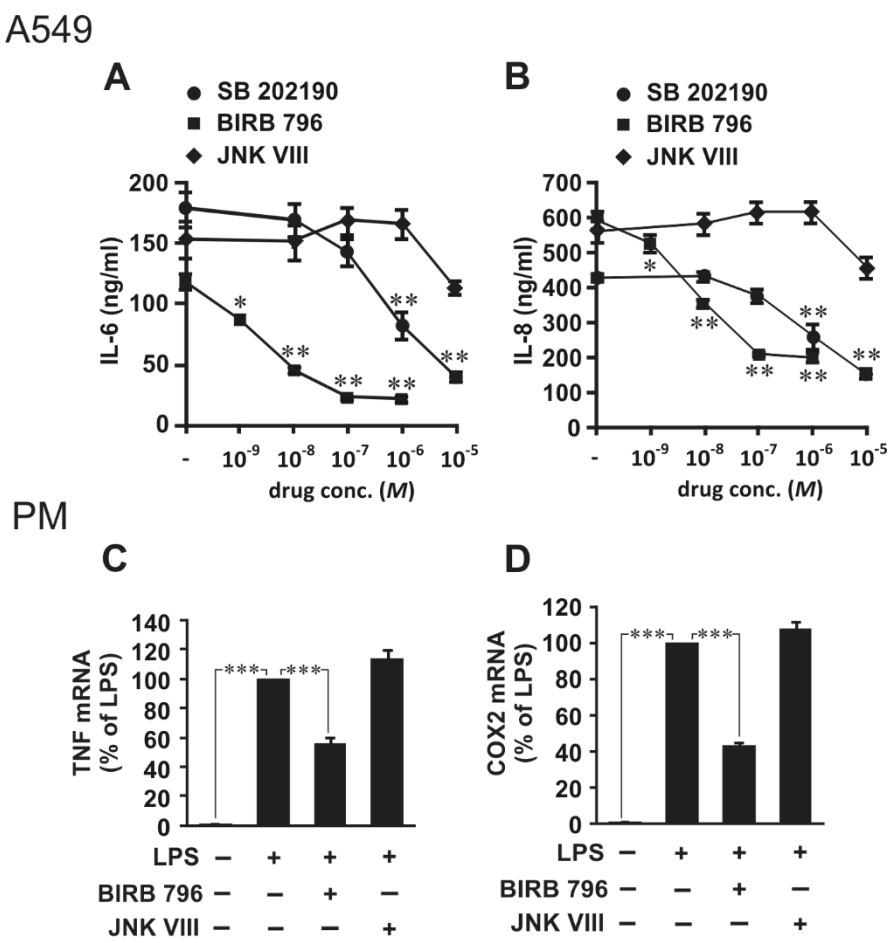

Figure 4. **The effects of p38 MAPK inhibitors SB 202190 and BIRB 796, and JNK inhibitor VIII on the production of inflammatory factors in A549 cells and peritoneal macrophages (PM).** (A and B) A549 cells were pre-incubated with SB 202190, BIRB 796 or JNK inhibitor VIII at concentrations indicated for 30 min and stimulated with cytokine mixture (TNF, INFγ and IL-1β, 10 ng/ml each) for 24 h. IL-6 and IL-8 concentration were determined by ELISA in the culture medium. Results are expressed as mean ± S.E.M., n=6. One-way ANOVA with Dunnett's post test was performed and statistical significance was indicated as *$p < 0.05$ and **$p < 0.01$ as compared cells treated with CM. (C and D) PMs were preincubated with BIRB 796 (100 nM) or JNK inhibitor VIII (1 μM) for 30 min and stimulated with LPS (100 ng/ml) for 3 h in the presence of the inhibitors. Total RNA was extracted and TNF and COX-2 mRNA expression levels were analyzed by qRT-PCR. mRNA expression levels are expressed in arbitrary units, LPS-stimulated cells set as 100 %, and the other values are related to that value. The results are expressed as mean ± S.E.M., n=4. One-way ANOVA with Bonferroni's post test was performed, and statistical significance is indicated as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

5.2 MKP-1 regulates the phosphorylation of p38 MAPK and JNK (I-III)

Unstimulated cells showed low basal MKP-1 protein levels. MKP-1 mRNA and protein levels were increased in response to LPS or cytokines at 1 h and returned to near to the basal level at 2 h and 3 h in epithelial cells and macrophages.

In an attempt to determine whether MKP-1 regulated the phosphorylation of p38 MAPK or JNK, MKP-1 expression was silenced by siRNA. MKP-1 silencing resulted in increased p38 MAPK phosphorylation in A549 and J774 (Fig. 5A and 5B). In A549 cells, silencing of MKP-1 by siRNA also increased JNK phosphorylation. However, in J774 cells, the down-regulation of MKP-1 did not affect JNK phosphorylation.

To confirm that MKP-1 regulated the phosphorylation of p38 MAPK, the phosphorylation of p38 MAPK was investigated in macrophages from MKP-1 KO mice. LPS increased p38 MAPK phosphorylation in bone marrow-derived macrophages (BMM), and it was further enhanced in BMMs from MKP-1 KO mice (Fig. 5C). These results indicated that MKP-1 catalyzed the dephosphorylation of p38 MAPK and thereby inactivated p38 MAPK.

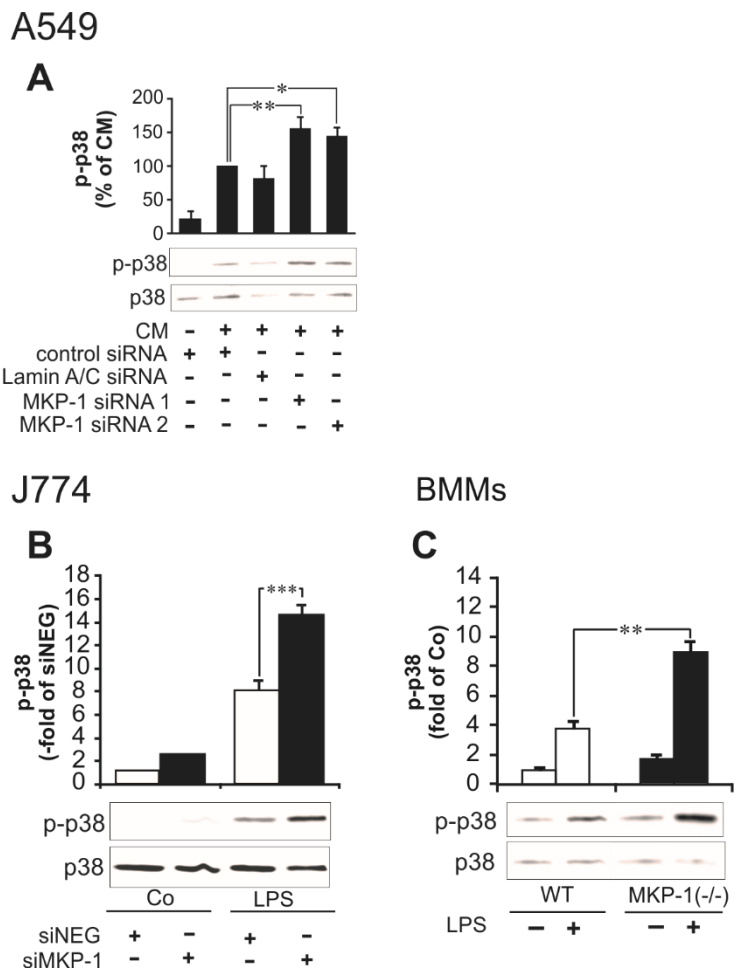

Figure 5. Effect of MKP-1 on p38 MAPK phosphorylation in A549 cells, J774 cells and bone marrow-derived macrophages (BMM). (A) A549 cells were transfected with MKP-1-specific siRNA 1 or 2 (MKP-1 siRNA 1 or 2), non-targeting control siRNA (control siRNA) or Lamin A/C-specific siRNA (Lamin A/C siRNA). (B) J774 cells were transfected with MKP-1 specific siRNA (siMKP-1) or non-targeting siRNA (siNEG). A549 or J774 cells were stimulated with cytokines (CM: TNF, IFNγ and IL-1β, 10 ng/ml each) or LPS (10 ng/ml), respectively for 1 h. p38 MAPK phosphorylation were determined by Western blot. The chemiluminescent signal was quantified and phosphorylated p38 MAPK was normalized against total p38 MAPK. Results are expressed as a percentage of CM or LPS and as mean ± S.E.M., n=6. One-way ANOVA with Bonferroni's post-test was performed, and statistical significance is indicated with * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$. (C) BMMs were stimulated with LPS (10 ng/ml) for 30 min, and p38 MAPK phosphorylation was detected by Western blot. The chemiluminescent signal was quantified and phosphorylated p38 MAPK was normalized against total p38 MAPK. Protein phosphorylation levels are expressed in arbitrary units, unstimulated wild-type cells set as 1, and the other values are related to that value. The results are expressed as mean ± S.E.M., n=3. Unpaired Student's t-test was performed, and statistical significance is indicated as **$p < 0.01$.

5.3 MKP-1 regulates the expression of inflammatory gene and acute inflammation *in vivo* (I-III)

The effect of MKP-1 on the production of pro-inflammatory factors was investigated. In these experiments, MKP-1 was silenced by siRNA. Cells were stimulated with the cytokine mixture for 3 h for mRNA and 24 h for NO and protein production. In cells transfected with MKP-1 siRNA, the production of IL-6 and IL-8 was increased as compared to that present in cells transfected with control siRNA (Fig. 6A and 6B). As expected, the expression of iNOS and COX-2 were also increased in cells transfected with MKP-1 specific siRNA when compared to the control cells. Lamin A/C-specific siRNA did not affect mRNA or protein levels of these genes revealing that the increased expression of inflammatory genes by MKP-1 siRNA was not due to a general activation of RISC pathway. Similarly, down-regulation of MKP-1 by siRNA increased COX-2 and iNOS protein expression as well as production of TNF, IL-6 and NO in J774 cells.

Inflammatory gene expression was also investigated in bone marrow macrophages (BMMs) from MKP-1 KO and wild-type mice. LPS-induced production of IL-6 and TNF was enhanced in BMMs from MKP-1 KO animals as compared to those from wild-type mice (Fig. 6C and 6D). The COX-2 and iNOS levels were also increased in BMMs from MKP-1 KO mice when compared to the wild-type mice. These results show that MKP-1 negatively regulates the expression of pro-inflammatory factors following LPS stimulation.

The effect of MKP-1 on acute inflammation was investigated in the carrageenan-induced paw inflammation model. In MKP-1 KO mice, carrageenan-induced inflammatory paw edema was increased by 36 % as compared to that seen in wild-type mice. These results indicate that MKP-1 is also a suppressive factor during acute inflammatory process *in vivo*.

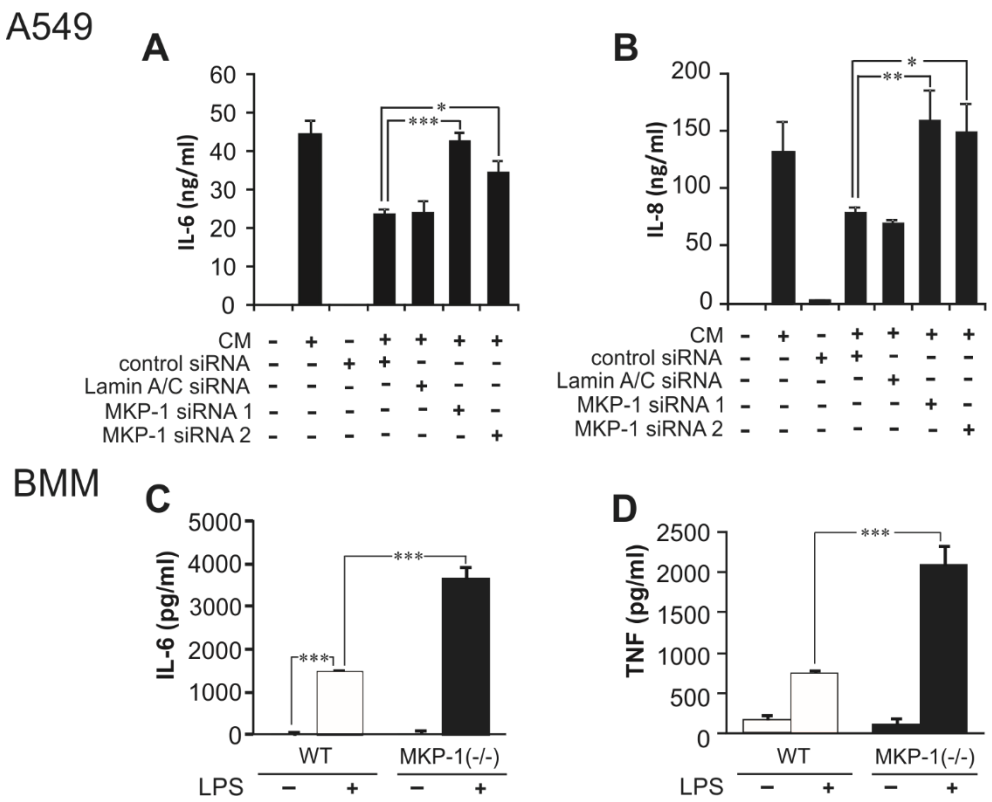

Figure 6. Effect of MKP-1 on IL-6, IL-8 and TNF production in A549 cells and BMMs. (A and B) A549 cells were transfected with MKP-1-specific siRNA 1 or 2 (MKP-1 siRNA 1 or 2), non-targeting control siRNA (control siRNA) or Lamin A/C-specific siRNA. Cells were stimulated with cytokines (CM: TNF, IFNγ and IL-1β, 10 ng/ml each) for 24 h, and IL-6 andIL-8 production was determined by ELISA. Results are expressed as a percentage of CM-stimulated cells transfected with non-targeting control siRNA, mean ± S.E.M, n=6. One-way ANOVA with Bonferroni's post test was performed, and statistical significance is indicated with *p < 0.05, **p < 0.01, and ***p < 0.001. (C and D) BMMs were incubated with LPS (10 ng/ml) for 24 h. IL-6 and TNF production was measured by ELISA. The results are expressed as mean ± S.E.M., n=3. One-way ANOVA with Bonferroni's post test was performed, and statistical significance is indicated as ***p < 0.001.

5.4 MKP-5 inhibits p38 MAPK phosphorylation and suppresses TNF and IL-6 release in macrophages (IV)

The effect of MKP-5 on the production of pro-inflammatory genes IL-6 and TNF in mouse macrophages was investigated. MKP-5 mRNA levels were not changed by LPS (10 ng/ml) in mouse J774 macrophages up to 4 h follow-up. To investigate the role of MKP-5, the expression of MKP-5 was silenced by siRNA. MKP-5 siRNA

down-regulated MKP-5 expression approximately 60 % when compared to the control cells, indicating that siRNA was functional.

MKP-5 silencing by siRNA resulted in increased production of IL-6 and TNF as compared to the control cells (Fig. 7). The levels of phosphorylated p38 MAPK were also increased (Fig. 8A). MKP-5 had no significant effects on the phosphorylation of ERK or JNK (Fig. 8B and 8C). Treatment with the MKP-5 inhibitor AS077234-4 (1 μM) was used as another method to investigate the function of MKP-5. This MKP-5 inhibitor also increased the phosphorylation of p38 MAPK and the production of IL-6 and TNF.

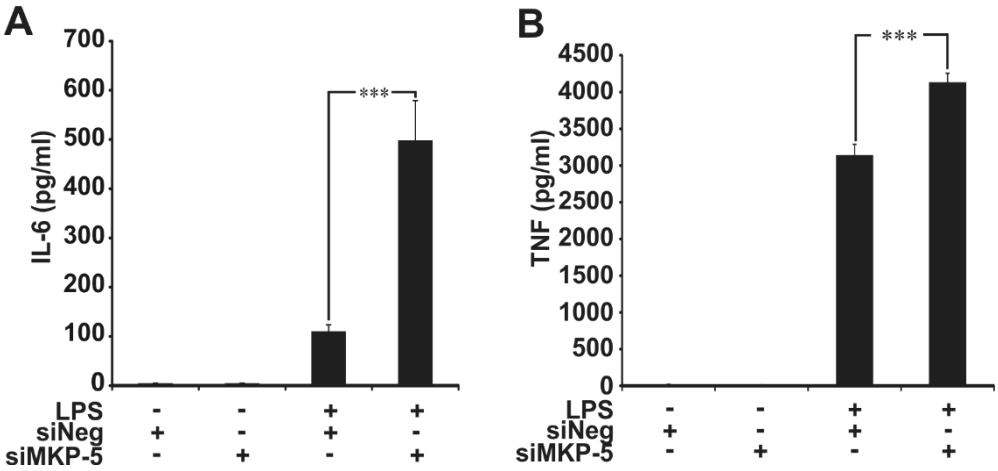

Figure 7. Effect of MKP-5 on IL-6 and TNF production in J774 cells. J774 cells were transfected with MKP-5 siRNA (siMKP-5) or control siRNA (siNeg) and stimulated with LPS (10 ng/ml) for 4h. (A) IL-6 and (B) TNF protein levels were determined by ELISA. The results are expressed as mean ± S.E.M., n=8. Two-way ANOVA with Bonferroni's post-test was performed, and statistical significance is indicated as * $p < 0.05$ and *** $p < 0.001$

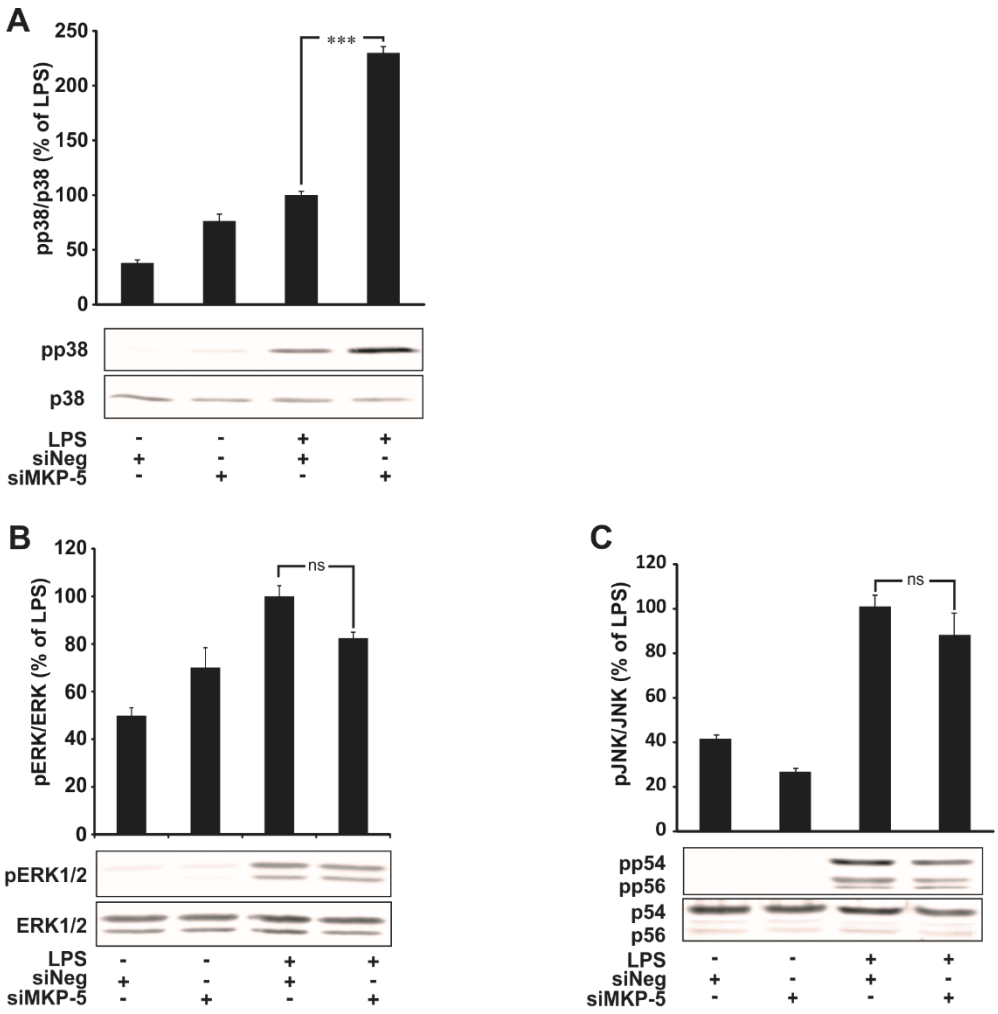

Figure 8. Effect of MKP-5 on MAPK phosphorylation in J774 cells. Cells were transfected with MKP-5 siRNA (siMKP-5) or control siRNA (siNeg) and stimulated with LPS (10 ng/ml) for 30 min. (A) p38 MAPK, (B) ERK and (C) JNK phosphorylation were determined by Western Blot. Chemiluminescent signal was quantified and phosphorylated MAPK levels were normalized to total MAPK. Results are expressed as a percentage of LPS and as mean ± SEM, n=8. Two-way ANOVA with Bonferroni's post-test was performed, and statistical significance is indicated as ns = non-significant and *** $p < 0.001$.

5.5 MKP-1 mediates the anti-inflammatory effects of PDE4 inhibitor rolipram *in vitro* and *in vivo* (V)

There are two copies of cAMP responsive elements in MKP-1 promoter and expression of MKP-1 has been reported to be increased by cAMP (Brion et al., 2011; Kwak et al., 1994). In addition, it has been reported that CREB is involved in the transcription of the MKP-1 gene (Zhang et al., 2008). PDE4 inhibitors increase cAMP signaling in cells by preventing the cAMP degradation (Houslay et al., 2005). Therefore the effects of PDE4 inhibition on MKP-1 levels and on inflammation related factors were determined. First, the expressions of PDE4 isoforms (PDE4A, B, C and D) were investigated. Only PDE4B mRNA was expressed in mouse PM cells from wild-type and MKP-1 KO mice. LPS stimulation increased the amounts of PDE4B mRNA at the time point 3h. Other isoforms were undetected or expressed at very low mRNA levels. In J774 macrophages, PDE4A was the most dominant isoform, but also PDE4B and PDE4D mRNA and protein were detected. Stimulation of LPS did not enhance the expression of these isoforms in these cells.

Rolipram, a selective PDE4 inhibitor (Christensen et al., 1998), was used in the studies. Rolipram increased LPS-induced expression of MKP-1 in PM cells and J774 cells (Fig. 9). In addition, the cAMP analog, 8-Br-cAMP, and a non-selective PDE inhibitor, IBMX, also increased MKP-1 mRNA levels in LPS stimulated J774 cells (Fig. 9A). PKA inhibitor 6-22 amide (PKAi) reversed the effect of rolipram on the expression of MKP-1. Phosphorylation of CREB was increased in the presence of rolipram and also this effect was reversed by the PKA inhibitor.

The effect of rolipram on the levels of phosphorylated p38 MAPK was investigated. p38 MAPK phosphorylation was decreased by rolipram and the PKA inhibitor antagonized that effect of rolipram in J774 cells.

Rolipram decreased LPS-induced TNF production in PM cells from wild-type mice (Fig. 10A). In addition, a non-selective PDE inhibitor IBMX and cAMP analog

8-Br-cAMP decreased TNF production in J774 macrophages. Interestingly, rolipram did not significantly reduce TNF production in PMs from MKP-1 KO mice (Fig. 10A). Furthermore, in carrageenan-induced paw inflammation rolipram reduced paw edema in wild-type mice but did not have effect in MKP-1 KO mice (Fig. 10B). These results indicate that MKP-1 mediates the anti-inflammatory effects of the PDE4 inhibitor, rolipram.

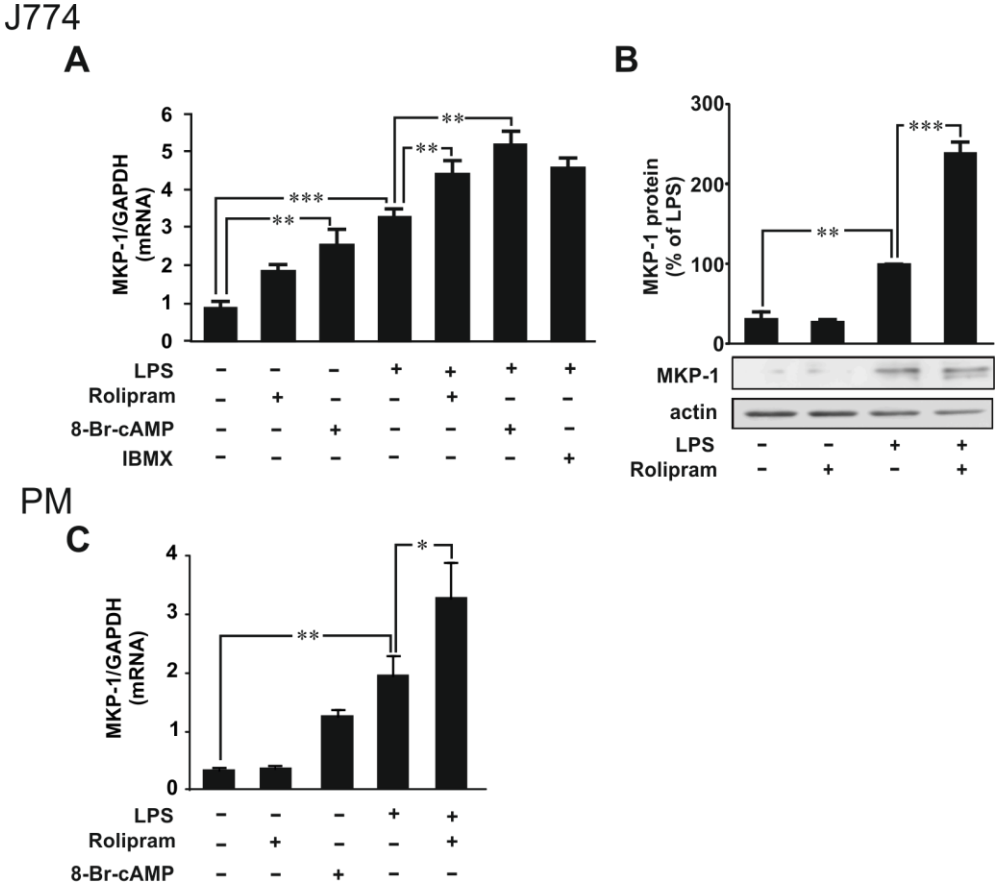

Figure 9. Effects of rolipram, IBMX and 8-Br-cAMP on MKP-1 expression in J774 cells and peritoneal macrophages (PM). J774 cells were stimulated with LPS (10 ng/ml) in the presence or in the absence of rolipram (2 μM), IBMX (100 μM) or 8-Br-cAMP (100 μM) for 1 h. (A) MKP-1 mRNA was detected by quantitative RT-PCR, and MKP-1 mRNA expression levels were normalized against GAPDH mRNA levels. The results are expressed as mean ± S.E.M., n=6. (B) MKP-1 protein was detected by Western blot. The chemiluminescent signal was quantified, and the amount of MKP-1 was normalized against actin. LPS-simulated cells were set as 100 %, and the other values were related to that value. The results are expressed as mean ± S.E.M., n=4. (C) Peritoneal macrophages were treated with LPS (100 ng/ml) in the presence or in the absence of rolipram (2 μM) or with 8-Br-cAMP (100 μM) alone for 1 h. MKP-1 mRNA levels were measured by qRT-PCR and normalized against GAPDH mRNA levels. The results are expressed as mean ± S.E.M., n=4. One-way ANOVA with Bonferroni's post test was performed, and statistical significance is indicated as * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

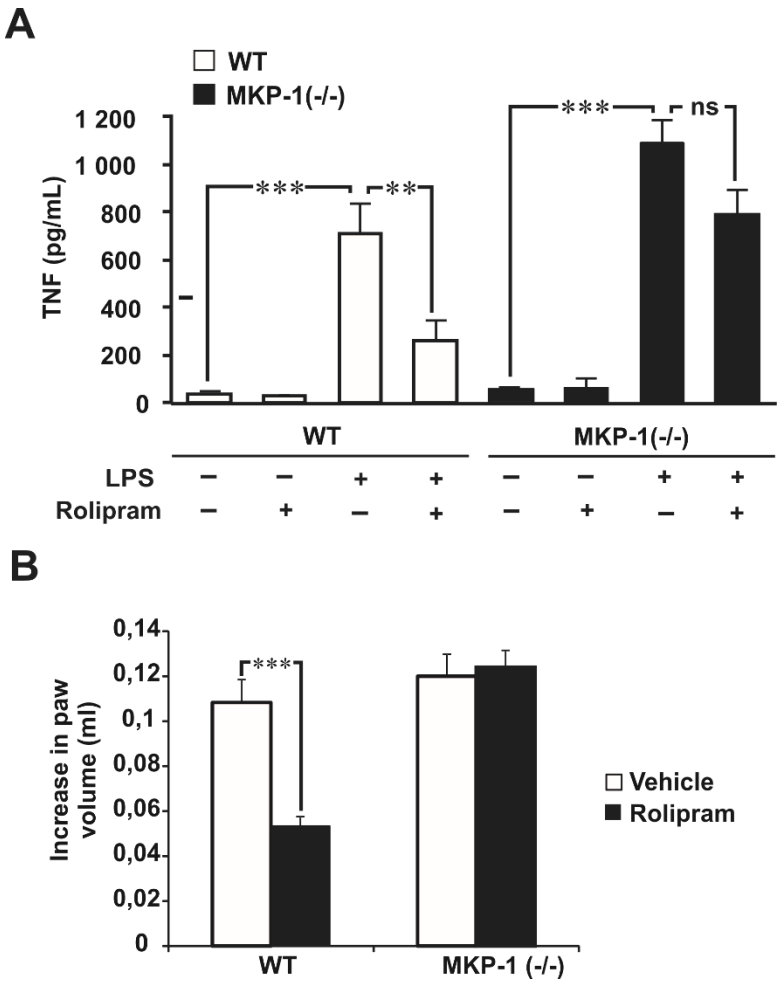

Figure 10. Effect of MKP-1 on the inhibition of TNF production by rolipram in primary mouse peritoneal macrophages and on carrageenan-induced acute paw inflammation. (A) Peritoneal macrophages were incubated with LPS (100 ng/ml) for 24 h. TNF protein levels were determined by ELISA. The results are expressed as mean ± S.E.M., n=8. Two-way ANOVA with Bonferroni's post-test was performed, and statistical significance is indicated as ns = non-significant, ** $p < 0.01$ and *** $p < 0.001$. (B) Rolipram (100 mg/kg, i.p.) was administered 2 h prior to the experiment. At beginning of the experiment (0 h), hind paw volumes were measured with a plethysmometer. Carrageenan or vehicle (control) was injected intradermally, and paw volumes were measured after 3 h with a plethysmometer. Edema was estimated as the difference between the volume changes of the carrageenan treated paw and the control paw, mean ± S.E.M., n=6. Two-way ANOVA with Bonferroni's post test was performed, and statistical significance is indicated as ns = non-significant and *** $p < 0.001$.

6. DISCUSSION

6.1 Methodology

In this Thesis study, many of the experiments were carried out in cell lines, because they provide an easy and reproducible model for experiments. The key experiments were also repeated in mouse primary macrophages. It is considered that the phenotype of primary cells when grown *ex vivo* is closer to that of cells *in vivo*, and thereby the responses of primary cells reflect the *in vivo* situation. In contrast, cell lines are immortalized cells, and the transformation may have affected their responses. This is one reason, why the key findings were clarified in primary cells. Result obtained from cell lines and primary cells were consistent, proving that the cell lines used were suitable for this study. For practical and ethical reasons it was regarded not reasonable only to use primary cells.

Macrophages were used here as the main cell model because they are an important cell type in inflammatory responses. The major functions of macrophages in inflammation are antigen presentation, phagocytosis and immunomodulation (production of cytokines and growth factors). Epithelial cells are vital in the first line defense against pathogens and other foreign material. As these cells also produce several inflammatory and regulatory factors and thus they also are important for inflammation and host defense responses.

siRNA is a practical tool to investigate gene function in cells. In this method, small complementary RNA oligonucleotides are transfected into cells to induce the degradation of their target mRNAs. This leads to the reduced production (and

further, reduced cellular levels) of a given protein, and this phenomenon is called gene silencing. This is a relatively simple and effective method to specifically investigate the functions of a selected gene/protein. Nonetheless, transfection of siRNA oligos inevitably causes some off-target effects. These non-specific effects were controlled by using non-targeting siRNA. The use of two different siRNA oligos in the same experiment also reduces the possibility that the observed changes in the read-outs would be due to off-target effects. In some experiments, Lamin A/C-specific siRNA was used as a positive control, because it is known that this agent has no direct effect on inflammatory gene expression. And in fact, these control siRNAs did not have effects on the tested parameters, i.e. the observed effects were due to down-regulation of the target gene and not due to non-specific effects of siRNA oligos or general activation of cellular RNA interference machinery.

In this study, MKP-1 KO mice were an important not only in clarifying the functions of MKP-1 but more importantly to confirm the biological significance of the results obtained from the cell culture experiments. Results from KO mice or from cells isolated from those mice were in line with the results seen in the cell lines. In MKP-5 experiments, MKP-5 specific inhibitor and MKP-5 siRNA were used. Also, these data were consistent. The IC_{50} value of the MKP-5 inhibitor AS077234-4 is 710 nM (Gobert et al., 2009). AS077234-4 was used at 1 μM concentration, which was considered to be effective but still specific for MKP-5.

Two structurally different p38 MAPK inhibitors SB 202190 and BIRB 796 were used to inhibit p38 MAPK signaling. The use of chemical inhibitors is often complicated by their non-specific effects. SB 202190 is a potent inhibitor of p38α and p38β MAPKs, but it also inhibits cyclin G-associated kinase (GAK) and casein kinase 1 (CK1) (Bain et al., 2007). The IC_{50} value for SB 202190 to inhibit p38 MAPK is approximately 100 nM. In the present experiments, SB 202190 significantly inhibited the phosphorylation of the p38 MAPK substrate MK2 at a concentration of 1 μM. The other p38 MAPK inhibitor BIRB 796 has been reported

to be a more potent inhibitor of p38α and p38β MAPKs that SB 202190 (Bain et al., 2007). BIRB 796 does not inhibit GAK or CK1, but it has been shown to inhibit p38γ and p38δ MAPK and JNK2 at 1 μM concentration. In the present study, BIRB 796 did not inhibit the phosphorylation of the JNK substrate c-Jun at a concentration of 1 μM and thus it was used at a 100 nM concentration, which inhibited significantly the phosphorylation of the p38 MAPK substrate MK-2. In addition, the two p38 MAPK inhibitors (SB 202190 and BIRB 796) provided similar results indicating that those results were most probably due to p38 MAPK inhibition and not due to other effects of the inhibitors. JNK inhibitor VIII has been shown to have 1000-fold selectivity for JNK over the other MAPKs (Szczepankiewicz et al., 2006). In the present experiments, 1 μM concentration of JNK inhibitor VIII inhibited the phosphorylation of the JNK substrate c-Jun completely, but did not inhibit inflammatory gene expression in A549 cells indicating that JNK is not involved in the regulation of those genes in A549 cells.

Carrageenan-induced paw edema is a widely used model for the acute inflammatory response. Carrageenan induces the production of many inflammatory factors, including COX-2, TNF, IL-6 and IL-1 as well as bradykinin, histamine, prostaglandins, and reactive nitrogen and oxygen species (Kalokasidis et al., 2009; Loram et al., 2007; Morris, 2003). Inflammatory cells also accumulate into the tissue in response to carrageenan. This model offers a useful tool to investigate the effects of various interventions on acute inflammation.

6.2 MKP-1 and MKP-5 in the regulation of the MAPK pathways

Activities of MAPKs are regulated by phosphorylation/dephosphorylation. MKP-1 showed specificity towards p38 MAPK and JNK. MKP-1 dephosphorylated p38 MAPK in A549 human bronchial epithelial cells and mouse macrophages. In

bronchial epithelial cells, MKP-1 also regulated JNK. Increased p38 MAPK phosphorylation was observed in macrophages from MKP-1 KO mice as compared to cells from wild-type mice. It was also found that MKP-5 regulated the levels of phosphorylated p38 MAPK, but not those of JNK or ERK, in J774 macrophages.

At first it was thought that MKP-1 regulated mainly ERK phosphorylation, but later it has been revealed that MKP-1 dephosphorylated mainly p38 MAPK and JNK (Franklin and Kraft, 1997; Franklin et al., 1998). It has been shown that in macrophages from MKP-1 KO mice, the phosphorylation of p38 MAPK and JNK is enhanced in response to LPS when compared to the wild-type mice (Salojin et al., 2006). MKP-1 has been reported to regulate p38 MAPK and JNK phosphorylation also in endothelial cells (Zakkar et al., 2008). In chondrocytes, MKP-1 was claimed to regulate p38 MAPK phosphorylation (Nieminen et al., 2010). It is possible that the differences in substrate specificity between the cell types are related to the MKP-1 protein levels in the cells. Intense cell stimulation, for example with high LPS concentration, may lead to excessive MKP-1 expression. In such cases, it is possible that MKP-1 may start to dephosphorylate also JNKs and ERKs, which are not regulated by MKP-1 under more physiological conditions. This may also explain why MKP-1 dephosphorylates ERK in experiments using artificial MKP-1 expression systems (Groom et al., 1996; Liu et al., 1995; Sun et al., 1993). In the present study, MKP-1 expression was silenced (or was absent in cells from MKP-1 knock-out animals), this is a clear advantage with this approach since non-specific effects related to the excessive MKP-1 expression can be avoided.

MKP-5 has been shown to have substrate specificity towards p38 MAPK and JNK, although this depends on the cell type. In fibroblasts which have high phosphatase levels, MKP-5 has been shown to dephosphorylate all three MAPKs. Lower MKP-5 levels seem to result only in p38 MAPK and JNK dephosphorylation (Tanoue et al., 1999; Theodosiou et al., 1999). In T cells, MKP-5 was reported to regulate JNK phosphorylation only (Zhang et al., 2004), whereas in neutrophils

MKP-5 dephosphorylated only p38 MAPK (Qian et al., 2009). In macrophages, MKP-5 has been reported to regulate also ERK phosphorylation especially at later (60-120min) time points in response to strong LPS stimulation (Qian et al., 2012). However, in the present study, MKP-5 did not alter either ERK or JNK phosphorylation in J774 macrophages. The differences in the dephosphorylation between distinct MAPKs may be related to the level of MKP-5 expression or to the balance between different MAPK phosphatases.

p38 MAPK is an important factor in the regulation of many inflammatory factors including pro-inflammatory cytokines (TNF, IL-6, IL-8), chemokines (MCP-1 and MIP-1) degradative enzymes (MMP1 and MMP3), growth factors (GM-CSF and VEGF), adhesion molecules (VCAM-1 and ICAM-1) and many other inflammatory factors (such as COX-2, iNOS, IL-10) (Arthur and Ley, 2013; Ashwell, 2006; Cuenda and Rousseau, 2007; Zhang et al., 2007). In present study, p38 MAPK inhibitors SB 202190 and BIRB 796 were found to inhibit the production of IL-6, IL-8 and COX-2 in a dose-dependent manner in human epithelial cells, and the production of IL-6, TNF, iNOS and NO in mouse macrophages, these findings are in line with previously published results.

In conclusion, p38 MAPK was found to be a significant target of MKP-1 and MKP-5, and it was also an important signaling pathway in the production of inflammatory factors in macrophages and lung epithelial cells. In these cells, JNK and ERK signaling pathways seemed to be less important targets of MKP-1 and MKP-5.

6.3 MKP-1 and MKP-5 in the regulation of the inflammatory response

The effects of MKP-1 and MKP-5 on the production of inflammatory factors was an important aspect of the present study. The down-regulation of MKP-1

increased the production of IL-6, IL-8, TNF, COX-2, iNOS and NO in macrophages and in lung epithelial cells in response to inflammatory stimulus. MKP-5 also regulated the production of IL-6 and TNF in mouse macrophages. Another interesting finding was that carrageenan induced a more severe paw edema in MKP-1 KO mice than in their wild-type counterparts.

MKP-1 has been shown to regulate both innate and adaptive immunity. Studies with MKP-1 KO mice have shown that MKP-1 controls the production of many inflammatory factors and the acute inflammatory response *in vivo* (Chi et al., 2006; Hammer et al., 2006; Salojin et al., 2006; Zhao et al., 2006). Intraperitoneal administration of LPS resulted in increased production of IL-6 and TNF in macrophages and also in increased mortality rates in MKP-1 KO mice as compared to wild-type animals (Hammer et al., 2006). Interestingly, MKP-1 also negatively regulated the production of IL-10 (Chi et al., 2006), which is a known anti-inflammatory cytokine. MKP-1 KO mice have an impaired microbial control (Frazier et al., 2009; Hammer et al., 2010). One possibility is that increased IL-10 production is responsible for the impaired microbial clearance in MKP-1 KO mice. Another possibility is that the impaired antimicrobial response to due to reduced production of IL-12 in these MKP-1 KO mice (Huang et al., 2011a; Korhonen et al., 2012).

MKP-1 has been also associated with chronic inflammation. In the collagen induced arthritis (CIA) mouse model, MKP-1 KO mice were reported to have an increased disease incidence and they developed more severe and destructive arthritis (Salojin et al., 2006; Vattakuzhi et al., 2012). This has been also related to the increased osteoclast activity and bone erosious (Vattakuzhi et al., 2012). In addition to experimental chronic arthritis, suppressed MKP-1 signaling has been associated with the pathogenesis of psoriasis. MKP-1 mRNA levels were lower in skin biopsies from psoriatic lesions as compared to healthy skin (Kjellerup et al., 2013) suggesting that defective MKP-1 signaling may contribute to the disease progression. In

addition to arthritis and psoriasis, MKP-1 has also been linked with the pathogenesis of asthma. Thus, MKP-1 suppressed the inflammatory gene expression in primary human airway smooth muscle cells (Manetsch et al., 2012a). In the present study, it was also observed that MKP-1 suppressed the production of inflammatory factors in lung epithelial cells. Inhaled glucocorticoids are the cornerstone of the anti-inflammatory treatment in asthma, and glucocorticoids are known to enhance MKP-1 expression (Abraham et al., 2006; Bhavsar et al., 2008; Furst et al., 2007; Issa et al., 2007). Interestingly, glucocorticoid-insensitive asthmatics display a lower induction of MKP-1 in response to glucocorticoids in alveolar macrophages and peripheral blood mononuclear cells when compared to those cells from glucocorticoid-sensitive asthmatics (Bhavsar et al., 2008; Sutherland et al., 2008).

MKP-5 has been also shown to inhibit the expression of inflammatory genes (Qian et al., 2009; Qian et al., 2012; Zhang et al., 2004). In MKP-5 KO mice, the productions of IL-6, TNF and MIP-2 in response to LPS or gram-positive bacteria were enhanced as compared to the wild-type mice (Qian et al., 2012; Zhang et al., 2004). In the present study, the down-regulation of MKP-5 by siRNA or MKP-5 inhibitor also increased production of TNF and IL-6.

In summary, MKP-1 and MKP-5 regulated the production of inflammatory mediators by decreasing the activation of p38 MAPK (Fig. 11) and in this respect MKP-1 and MKP-5 can be considered as important endogenous suppressors of inflammation.

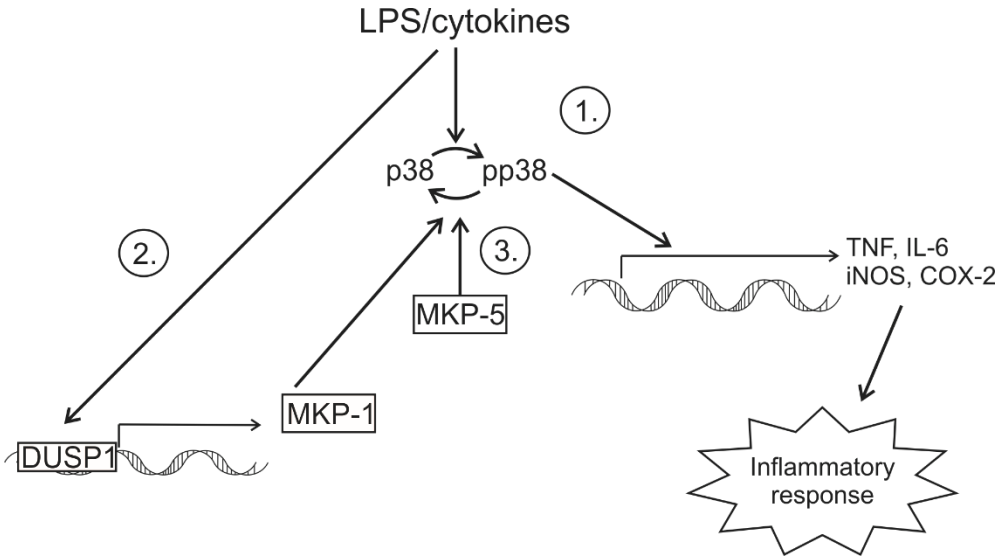

Figure 11. Summary of p38 MAPK pathway regulating inflammatory response. 1. LPS activates p38 MAPK pathway, which increases the production of inflammatory factors and inflammation. 2. LPS also increases DUSP1 gene transcription and MKP-1 protein expression. MKP-1 dephosphorylates and thereby inactivates p38 MAPK and attenuates inflammatory response. 3. MKP-5 also dephosphorylates p38 MAPK, and suppresses inflammatory gene expression and the severity of inflammation.

6.4 The role of MKP-1 in mediating the anti-inflammatory effects of the PDE4 inhibitor rolipram in vitro and in vivo

In this study, the PDE4 inhibitor, rolipram, was found to increase the expression of MKP-1 and decrease p38 MAPK phosphorylation in J774 macrophages by a mechanism likely dependent on the cAMP-PKA-CREB pathway (Fig. 12). Rolipram also inhibited TNF production and this effect was clearly attenuated in macrophages obtained from MKP-1 KO mice. Moreover, rolipram effectively inhibited carrageenan-induced paw edema in wild-type mice, but failed to inhibit the response in MKP-1 KO mice.

Monocytes are known to express PDE4A, PDE4B and PDE4D isoforms of PDE4 (MacKenzie and Houslay, 2000). The differentiation of monocytes into

macrophages results in the down-regulation of PDE4A and PDE4D, and in macrophages, PDE4B is the most important PDE4 isoform (Jin and Conti, 2002; Jin et al., 2005). In this study, J774 macrophages were found to express PDE4A, PDE4B and PDE4D isoforms while in peritoneal macrophages only the PDE4B isoform was detectable. This may reflect the different phenotype of the cells. J774 macrophages are a monocytic cell line, while peritoneal macrophages have been differentiated from monocytes to macrophages *in vivo*.

PDE4B is the most important PDE4 isoform in inflammation. PDE4B KO mice display a suppressed innate immune response (Jin and Conti, 2002; Jin et al., 2005). These animals have also better survival after systemic LPS administration and have decreased TNF production in macrophages when compared to wild-type mice. In addition, PDE4B KO mice have suppressed ovalbumin-specific T cell responses and decreased lung inflammation to inhaled ovalbumin and ameliorated airway hyper-responsiveness to methacholine (Jin et al., 2010).

PDE4 inhibitors elevate cAMP levels and suppress cytokine production and reduced the inflammatory response (Page and Spina, 2012). There are two CRE elements in the MKP-1 promoter (Kwak et al., 1994). Increased cAMP levels have been reported to up-regulate the expression of MKP-1 (Brion et al., 2011; Lee et al., 2012c; Zhang et al., 2008), and that was found in this study, also. The expression of MKP-1 can be increased also by β_2-agonists (Kaur et al., 2008; Keränen et al., 2014; Manetsch et al., 2012b). Manetsch et al. have shown that the long-acting β2-agonist, formoterol, enhanced MKP-1 mRNA and protein expression in a cAMP-mediated manner via the β_2-adrenergic receptor-PKA pathway (Manetsch et al., 2012b).

The present results demonstrated that the PDE4 inhibitor, rolipram, increased MKP-1 expression and decreased TNF production in activated macrophages, and that MKP-1 mediated at least partly the anti-inflammatory effects of rolipram in macrophages. These results also suggest that rolipram increased MKP-1 expression through the cAMP-PKA-CREB pathway. (Fig. 12)

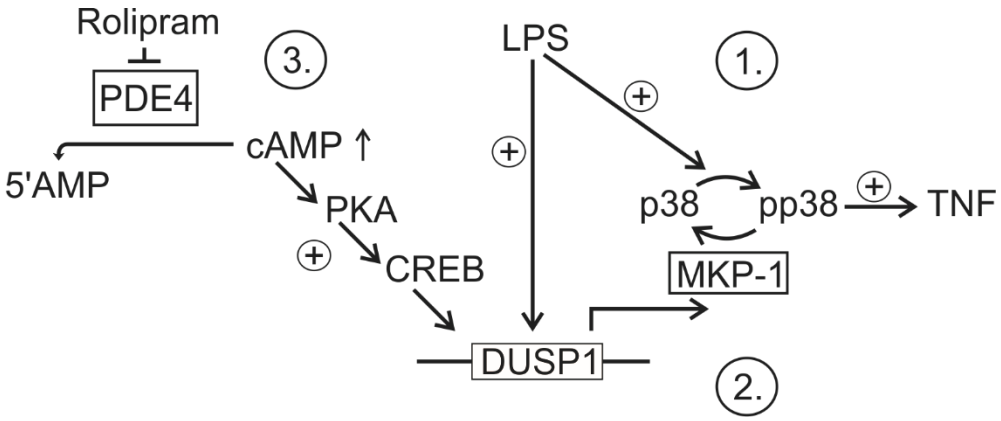

Figure 12. PDE4 inhibitor rolipram inhibits TNF production by elevating MKP-1 through cAMP-PKA-CREB pathway. 1. LPS activates p38 MAPK pathway and induces TNF production. 2. LPS also increases DUSP1 gene transcription and MKP-1 protein expression. MKP-1 dephosphorylates p38 MAPK, and this suppresses the release of TNF. 3. PDE inhibitor rolipram increases cAMP levels by preventing the hydrolysis of cAMP. Enhanced cAMP signaling increases DUSP1 gene transcription via cAMP-PKA-CREB pathway, and increases MKP-1 expression which mediates the anti-inflammatory effects of the PDE4 inhibitor rolipram on TNF production.

6.5 MAPKs and MKPs as drug targets

In chronic autoimmune diseases, excessive production of cytokines and other inflammatory factors underlies the pathophysiology of these diseases. As discussed above, p38 MAPK, MKP-1 and MKP-5 all participate in the regulation of the inflammatory response. Therefore, the p38 MAPK pathway, MKP-1 and MKP-5 may represent potent targets for anti-inflammatory drug development.

6.5.1 p38 MAPK as a drug target

p38 MAPK inhibitors have been investigated for the treatment of inflammatory diseases. For example, Badger et al. reported that the p38 MAPK inhibitor SB 203580 had beneficial effects in arthritis, in endotoxin shock and in immune function in the mouse models (Badger et al., 1996). There are many other cell or animals models, in which p38 MAPK inhibitors have shown clear anti-inflammatory effects (Branger et al., 2002; Burnette et al., 2009). At the moment, there are, however, no

p38 MAPK inhibitors approved for clinical use and their clinical efficacy seems to be dependent on the type and phase of inflammation. For instance treatment with the p38 MAPK inhibitor, VX-702, reduced the number of tender and swollen joints in patients with rheumatoid arthritis as early as 2 weeks following the initiation of the therapy and also reduced biomarkers, but those effects did not differ from placebo at week 12 anymore (Damjanov et al., 2009). In addition, the p38 MAPK inhibitor BIRB 796 decreased CRP levels in a dose dependent manner in the first week of an 8-week clinical trial in Crohn's disease patients, but CRP levels returned to the elevated baseline levels later on (Schreiber et al., 2006). Pamapimod did not show improvement over placebo in rheumatoid arthritis (Alten et al., 2010). The limited efficacy of p38 MAPK inhibitors in rheumatoid arthritis trials may be due to pharmacokinetic problems or too low dose of the drug. Another reason behind the failures in the clinical trials may have been inappropriate selection of the patients. In general, p38 MAPK inhibitors have been relatively well-tolerated in the clinical trials. Most of the adverse events being mild, i.e. many of them were common adverse events in the clinical drug trials, for example skin disorders, gastrointestinal disorders and dizziness. Other commonly reported side effects were pyrexia, diarrhea, elevated neutrophil, protein in urine, arthralgia and back pain, headache, nausea or skin rash. In some cases, there were more infections such as gastroenteritis, respiratory tract infection, upper respiratory tract infection, and acute bronchitis. (Alten et al., 2010; Damjanov et al., 2009; Lomas et al., 2012; MacNee et al., 2013; Schreiber et al., 2006). p38 MAPK inhibitors suppress inflammatory response, so it is not surprising that these drugs would cause, at least to some extent, increased infection rates in clinical trials.

On the other hand, in COPD patients, treatment with the p38 MAPK inhibitor, losmapimod, has been shown to reduce fibrinogen as well as IL-6, IL-8 and CRP levels in the circulation (Lomas et al., 2012). Another interesting p38 MAPK inhibitor is PH-797804 (MacNee et al., 2013). This inhibitor showed improvements in lung function and dyspnea scores in patients with moderate to severe COPD.

Interestingly, by combination of a p38 MAPK inhibition with PDE4 inhibition gave promising results in an arthritis model in the rat (Koch et al., 2013). This dual-inhibitor, CBS-3595, effectively reduced paw volume when compared to the placebo group. This inhibitor also reduced allodynia and IL-6 production and increased the production of an anti-inflammatory cytokine IL-10 even better than the p38 MAPK inhibitor ML-3403. There appears to be place for the development of more effective p38 MAPK inhibitors, and also the indication selection for p38 MAPK inhibitors in the clinical trials seems to be one crucial factor when testing their efficacy in the treatment of human diseases.

6.5.2 MKP-1 and MKP-5 as drug targets

The p38 MAPK pathway mediates many inflammatory responses, and therefore drugs affecting this pathway have potential as anti-inflammatory agents. MKP-1 is an important factor controlling the activity of the p38 MAPK pathway *in vivo* (Chi et al., 2006; Zhao et al., 2005). This makes MKP-1 as an interesting drug target. It is known that parts of the anti-inflammatory effects of glucocorticoids are mediated by MKP-1 (Abraham et al., 2006). Aurothiomalate was found to suppress the production of COX-2, IL-6 and MMP-3 in chondrocytes and cartilage and interestingly, and to increase the expression of MKP-1 (Nieminen et al., 2010). Nieminen et al. have also shown that MKP-1 mediates these anti-inflammatory effects of aurothiomalate (Nieminen et al., 2010). In the present study, PDE inhibitor rolipram was found to inhibit the production of TNF in mouse macrophages and to attenuate carrageenan-induced inflammation; and MKP-1 mediated those anti-inflammatory effects at least in part. It would be interesting to investigate, whether the anti-inflammatory effects of the dual-inhibitor CBS-3595 were mediated by MKP-1, because MKP-1 is linked with both PDE4 and p38 MAPK pathways.

There are very limited data on MKP-5 and on the mechanisms involved in the expressional regulation of MKP-5. MKP-5, like MKP-1, has been reported to suppress inflammatory responses and MKP-5 KO mice have more robust inflammatory response than wild-type mice (Zhang et al., 2004). Therefore compound which would increase MKP-5 expression and/or activity would most likely have anti-inflammatory properties.

The phenotype of MKP-1 or MKP-5 KO mouse does not reveal any obvious defects. Thereby one may assume that these MKPs are not vital and drugs that affect the expression or function of these MKPs may be well tolerated. MKP-1 KO mice display a more robust innate immune response and increased production of inflammatory factors, including TNF, IL-6 and IL-10 than the corresponding WT mice (Chi et al., 2006). MKP-1 deficient mice have also increased acute inflammatory reaction and increased mortality in response to systemic LPS administration (Hammer et al., 2006; Zhao et al., 2006). These findings suggest that MKP-1 is an important endogenous factor in the control of the inflammatory response. Expression of MKP-1 seems to play an important role also in the effective antimicrobial responses. MKP-1 regulates the production of IL-12 by dendritic cells and macrophages, and IL-12 is an important factor driving antimicrobial responses (Huang et al., 2011b; Korhonen et al., 2012). It seems that MKP-1 suppresses excessive inflammation, but supports microbial defense. This is interesting because one typical adverse effect of potent anti-inflammatory and immunosuppressive drugs is the increased risk for severe infections.

In summary, MKP-1 and MKP-5 hold potential as anti-inflammatory treatment targets. Up-regulation of MKP-1 or MKP-5 suppresses inflammatory gene expression and attenuates the severity of the inflammatory response. In addition, PDE4 inhibitor rolipram was found to increase MKP-1 expression, and MKP-1 mediated its anti-inflammatory effects. According to the results, MKP-1 and/or

MKP-5 are important factors that suppress inflammation and importantly, are potential targets for anti-inflammatory drugs.

SUMMARY AND CONCLUSIONS

The aim of the present thesis was to investigate the role of MKP-1 and MKP-5 in inflammation and in mediating the effects of anti-inflammatory drugs. The major findings and conclusions are as follows:

1. Impaired MKP-1 and MKP-5 increased the levels of phosphorylated p38 MAPK in cells suggesting that they dephosphorylate and thereby inactivate p38 MAPK.

2. MKP-1 and MKP-5 were found to be negative regulators of inflammatory gene expression. MKP-1 also suppressed the acute inflammatory response.

3. PDE4 inhibitor rolipram increased the expression of MKP-1, and this was likely dependent on cAMP-PKA-CREB pathway. Rolipram decreased the production of TNF in macrophages and suppressed the carrageenan-induced paw inflammation, and those anti-inflammatory effects of rolipram were attenuated in the absence of MKP-1. These result strongly indicate that MKP-1, at least in part, mediates the anti-inflammatory effects of rolipram.

Uncontrolled inflammation is the main cause of the symptoms and pathological findings in many inflammatory diseases. This study extends our understanding of the regulation of inflammatory gene expression and inflammatory response in general and on the role of MAPKs and MKPs in these processes. The present study demonstrated that, p38 MAPK mediated the production of many key inflammatory factors, including TNF and IL-6 in human epithelial cells and in mouse macrophages. MKP-1 and MKP-5 suppressed the phosphorylation and thereby the

activity of p38 MAPK, and inhibited the release of inflammatory factors. Enhancement of MKP-1 and/or MKP-5 expression and/or activity may provide an alternative way to regulate inflammation dependent on p38 MAPK activity. Thereby, MKP-1 and MKP-5 pathways are potential targets for the development of novel anti-inflammatory drugs.

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Tuija Hömmö

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Mitogen-Activated Protein Kinase Phosphatase-1 Negatively Regulates the Expression of Interleukin-6, Interleukin-8, and Cyclooxygenase-2 in A549 Human Lung Epithelial Cells

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ABSTRACT

Mitogen-activated protein kinase phosphatase (MKP)-1 is a protein phosphatase that regulates the activity of p38 mitogen-activated protein (MAP) kinase and c-Jun NH_2-terminal kinase (JNK) and, to lesser extent, p42/44 extracellular signal-regulated kinase. Studies with MKP-1(−/−) mice show that MKP-1 is a regulating factor suppressing excessive cytokine production and inflammatory response. The data on the role of MKP-1 in the regulation of inflammatory gene expression in human cells are much more limited. In the present study, we investigated the effect of MKP-1 on the expression of interleukin (IL)-6, IL-8 and cyclooxygenase (COX)-2 in response to stimulation with cytokines (tumor necrosis factor, IL-1β, and interferon-γ; 10 ng/ml each) in A549 human lung epithelial cells. Cytokines enhanced p38 and JNK phosphorylation and MKP-1 expression. p38 MAP kinase inhibitors 4-[4-(4-fluorophenyl)-5-(4-pyridinyl)-1*H*-imidazol-2-yl] phenol (SB202190) and 1-(5-*tert*-butyl-2-*p*-tolyl-2*H*-pyrazol-3-yl)-3(4-(2-morpholin-4-yl-ethoxy)naphthalen-1-yl)urea (BIRB 796) inhibited cytokine-induced phosphorylation of p38 substrate MAP kinase-activated protein kinase 2 and expression of IL-6, IL-8, and COX-2. An aminopyridine-based JNK inhibitor, *N*-(4-amino-5-cyano-6-ethoxypyridin-2-yl)-2-(2,5-dimethoxyphenyl)acetamide (JNK inhibitor VIII), inhibited phosphorylation of a JNK substrate c-Jun but did not have any effect on IL-6, IL-8, or COX-2 expression. Down-regulation of MKP-1 with small interfering RNA enhanced p38 and JNK phosphorylation and increased IL-6, IL-8, and COX-2 expression in A549 cells. In conclusion, cytokine-induced MKP-1 expression was found to negatively regulate p38 phosphorylation and the expression of IL-6, IL-8, and COX-2 in human pulmonary epithelial cells. Our results suggest that MKP-1 is an important negative regulator of inflammatory gene expression in human pulmonary epithelial cells, and compounds that enhance MKP-1 may have anti-inflammatory effects and control inflammatory response in the human lung.

Mitogen-activated protein (MAP) kinases include extracellular signal-regulated protein kinases (ERKs) and stress-activated protein kinases c-Jun NH_2-terminal kinase (JNK) and p38. ERKs are activated in response to various physiological and pathophysiological stimuli, and they regulate cellular proliferation and differentiation. p38 and JNK are activated in response to a wide range of stimuli, including proinflammatory cytokines, growth hormones, G protein-coupled receptors, and cellular stress (Johnson and Lapadat, 2002; Rincón and Davis, 2009). The family of p38 MAP kinases consists of four isoforms, namely, p38α, p38β, p38γ, and p38δ. p38α and p38β are widely expressed, whereas p38γ is expressed in skeletal muscle and p38δ in testes, pancreas, small intestine and in $CD4^+$ T cells. The targets of p38 include transcription factors and a wide range of other cellular regulatory proteins. p38 regulates cellular growth and differentiation and, importantly, immune response (Ashwell, 2006; Rincón and Davis, 2009). The JNK family of MAP

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ABBREVIATIONS: MAP, mitogen-activated protein; ERK, extracellular signal-regulated-kinase; TNF, tumor necrosis factor; IL, interleukin; COX, cyclooxygenase; Th, T helper; IFN, interferon; MKP, mitogen-activated protein kinase phosphatase; DUSP, dual-specificity phosphatase; LPS, lipopolysaccharide; GRO-α, growth-related oncogene protein-α; SB202190, 4-[4-(4-fluorophenyl)-5-(4-pyridinyl)-1*H*-imidazol-2-yl] phenol; BIRB 796, 1-(5-*tert*-butyl-2-*p*-tolyl-2*H*-pyrazol-3-yl)-3(4-(2-morpholin-4-yl-ethoxy)naphthalen-1-yl)urea; JNK, c-Jun NH_2-terminal kinase; JNK inhibitor VIII, *N*-(4-amino-5-cyano-6-ethoxypyridin-2-yl)-2-(2,5-dimethoxyphenyl)acetamide; RT-PCR, reverse transcription-polymerase chain reaction; MK2, mitogen-activated protein kinase-activated protein kinase 2; FAM, 5-carboxyfluorescein; TAMRA, 5-carboxytetramethylrhodamine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; siRNA, small interfering RNA; ELISA, enzyme-linked immunosorbent assay; ANOVA, analysis of variance; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)*IH*-imidazole.

kinases consists of three JNK isoforms, namely, JNK1, JNK2, and JNK3. JNK1 and JNK2 are ubiquitously expressed, whereas the expression of JNK3 is restricted to testes, heart, and brain. JNK is involved in the regulation of various cellular functions, such as cellular growth, differentiation, and apoptosis (Johnson and Lapadat, 2002; Rincón and Davis, 2009).

Both p38 and JNK regulate immune responses. p38 and JNK have been shown to regulate the expression of cytokines and inflammatory factors, including tumor necrosis factor (TNF); interleukin (IL)-1β, IL-2, IL-6, and IL-8 (also known as CXCL8); cyclooxygenase (COX)-2; and inducible nitric-oxide synthase (Chen et al., 2000; Clark et al., 2003; Korhonen et al., 2007). In addition, p38 and JNK are both involved in the activation of T helper (Th) cells, Th1 differentiation, and the production of Th1 cytokines, such as interferon (IFN)-γ, by Th1 cells. p38 and JNK also regulate cytokine production and other inflammatory cellular responses of macrophages and other cells in immune response (Ashwell, 2006; Rincón and Davis, 2009).

MAP kinase phosphatases (MKPs) are dual-specific phosphatases (DUSPs) that dephosphorylate tyrosine and threonine residues in MAP kinases and thereby inactivate them (Liu et al., 2007; Boutros et al., 2008). In mammalian cells, 11 MKPs have been described, and they differ from each other by expressional pattern, tissue distribution, cellular location, and substrate specificity. MKP-1 (also termed DUSP1) is a nuclear phosphatase that is expressed in various cell types and tissues. MKP-1 expression is induced in response to a range of stimuli, such as cellular stress, cytokines, LPS, and glucocorticoids, in several inflammatory (such as macrophages) and noninflammatory cells. Although originally MKP-1 was described to dephosphorylate p42/44 ERK (Sun et al., 1993), MKP-1 has been shown to have specificity toward p38 and JNK over p42/44 ERK (Franklin and Kraft, 1997; Liu et al., 2007; Boutros et al., 2008). Data from knockout mice suggest that MKP-1 negatively regulates innate immune response. In macrophages, defects in MKP-1 function results in increased and prolonged p38 activation (Zhao et al., 2005; Hammer et al., 2006; Salojin et al., 2006), and MKP-1 knockout mice have increased expression of TNF, IL-6, IL-10, COX-2, and macrophage inflammatory protein-1α in response to in vivo LPS challenge (Salojin et al., 2006). Exposure of MKP-1(−/−) mice to *Staphylococcus aureus* or Gram-positive bacterial products resulted in elevated cytokine production and inducible nitric-oxide synthase expression, and these mice had increased mortality rate, increased neutrophil infiltration in lungs, and they suffered from more severe organ damage (Wang et al., 2007). MKP-1 is involved in the anti-inflammatory effects of glucocorticoids. The inhibition of COX-2 and E-selectin expression by glucocorticoids has been reported to be mediated by MKP-1 (Abraham et al., 2006; Fürst et al., 2007). In addition, the inhibition of growth-related oncogene protein-α (GRO-α) expression by glucocorticoids has been shown to be mediated by MKP-1 in human airway smooth muscle cells (Issa et al., 2007).

Chronic inflammatory airway diseases, such as asthma and chronic obstructive pulmonary disease, are characterized by increased infiltration of inflammatory cells and production of proinflammatory cytokines in the lungs (Barnes, 2008). Human pulmonary epithelial cells have been reported to produce IL-6 and IL-8 in a p38-dependent mechanism in response to stimulation with IL-17 (Laan et al., 2001). Because the production of cytokines and the activation of MAP kinases are interrelated, we investigated the effect of MKP-1 on the p38 and JNK activation and on the regulation of cytokine production in A549 human lung epithelial cells.

Materials and Methods

Materials. Reagents were obtained as follows. Recombinant human TNF, IFN-γ and IL-1β were purchased from R&D Systems (Minneapolis, MA). SB202190 (Tocris Biosciences, Bristol, UK), BIRB 796 (Axon MedChem, Groningen, The Netherlands), and *N*-(4-amino-5-cyano-6-ethoxypyridin-2-yl)-2-(2,5-dimethoxyphenyl)acetamide (JNK inhibitor VIII; Calbiochem, Merck Chemicals, Darmstadt, Germany) were purchased as indicated. All other reagents were from Sigma-Aldrich (St. Louis, MO) unless otherwise stated below.

Cell Culture. A549 human lung epithelial cells (American Type Culture Collection, Manassas, VA) were cultured at 37°C in 5% CO_2 atmosphere in Ham's F-12K (Kaighn's modification) medium containing 5% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B (all from Invitrogen, Paisley, UK). For cytokine measurements, Western blot and quantitative RT-PCR, cells were seeded on a 24-well plate at density of 4×10^5 cells/well. Cell monolayers were grown for 48 h before the experiments were started. SB202190 or JNK inhibitor VIII at concentrations indicated were added to the cells in fresh culture medium containing 5% fetal calf serum and antibiotics 30 min before the stimulation with cytokines (TNF, IFN-γ, and IL-1β; 10 ng/ml each). Cells were further incubated for the time indicated.

Preparation of Cell Lysates for Western Blot Analysis. At the indicated time points, culture medium was removed. Cells were rapidly washed with ice-cold phosphate-buffered saline and solubilized in ice-cold lysis buffer containing 10 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl, 1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 20 μg/ml leupeptin, 50 μg/ml aprotinin, 5 mM sodium fluoride, 2 mM sodium pyrophosphate, and 10 μM *n*-octyl-β-D-glucopyranoside. After incubation for 20 min on ice, lysates were centrifuged, and supernatants were collected and mixed in a ratio of 1:4 with SDS loading buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.025% bromphenol blue, and 5% β-mercaptoethanol) and stored at −20°C until analyzed.

Western Blot Analysis. COX-2, actin, MKP-1, phospho-c-Jun, c-Jun, JNK, lamin A/C, polyclonal anti-rabbit, and polyclonal anti-goat antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Phospho-p38, p38, phospho-JNK, phospho-MAP kinase-activated protein kinase 2 (MK2), MK2 antibodies were from Cell Signaling Technology Inc. (Danvers, MA). Poly(A)-binding protein 1 antibody was from Millipore (Billerica, MA). Polyclonal anti-mouse antibody was from Pierce Chemical (Rockford, IL).

Before Western blot analysis, the samples were boiled for 10 min. Equal aliquots of protein (10–20 μg) were loaded on a 10% SDS-polyacrylamide electrophoresis gel and separated by electrophoresis. Proteins were transferred to Hybond enhanced chemiluminescence nitrocellulose membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK) by semidry electroblotting. After transfer, the membrane was blocked in 20 mM Tris base, pH 7.6, 150 mM NaCl, and 0.1% Tween 20 containing 5% nonfat milk for 1 h at room temperature. For detection of phosphoproteins, membranes were blocked in 20 mM Tris base, pH 7.6, 150 mM NaCl, and 0.1% Tween 20 containing 5% bovine serum albumin. Membranes were incubated overnight at 4°C with primary antibody, and for 1 h with secondary antibody, and the chemiluminescent signal was detected by FluorChem 8800 imaging system (Alpha Innotech, San Leandro, CA) and ImageQuant LAS 4000 mini (GE Healthcare). The chemilumi-

nescent signal was quantified with FluorChem software version 3.1 and ImageQuant TL 7.0 image analysis software.

RNA Extraction and Real-Time RT-PCR. At the indicated time points, culture medium was removed and total RNA extraction was carried out with GenElute Mammalian Total RNA Miniprep kit (Sigma-Aldrich) according to the manufacturer's instructions. Total RNA (100 ng) was reverse-transcribed to cDNA using TaqMan Reverse Transcription reagents and random hexamers (Applied Biosystems, Foster City, CA). cDNA obtained from the reverse transcription reaction was diluted 1:20 with RNase-free water and was subjected to quantitative PCR using TaqMan Universal PCR Master Mix and ABI Prism 7000 sequence detection system (Applied Biosystems). The primer and probe sequences and concentrations were optimized according to manufacturer's guidelines in TaqMan Universal PCR Master Mix Protocol part 4304449 revision C and were as follows: 5′-GCCAGGGCTGAACTTCGAA-3′ (human COX-2 forward primer; 300 nM), 5′-CAACTCTATATTGCTGGAACATGGA-3′ (human COX-2 reverse primer; 300 nM), 5′-TGGAAGCCTGTGATACTTTCTGTACT-3′ (human COX-2 probe; 150 nM, containing 6-FAM as 5′-reporter dye and TAMRA as 3′-quencher), 5′-TCCTACCACCAGCAACCCTGCCA-3′ [human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward primer; 300 nM)], 5′-GCAACAATATCCACTTTACCAGAGTTAA-3′ (human GAPDH reverse primer; 300 nM), and 5′-CGCCTGGTCACCAGGGCTGC-3′ (human GAPDH probe; 150 nM, containing 6-FAM as 5′-reporter dye and TAMRA as 3′-quencher). Expression of human IL-6 and human IL-8 mRNA was measured using TaqMan gene expression assays (Applied Biosystems). PCR reaction parameters were as follows: incubation at 50°C for 2 min, incubation at 95°C for 10 min, and thereafter 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. Each sample was determined in duplicate. A standard curve method was used to determine the relative mRNA levels as described in the Applied Biosystems User Bulletin: A standard curve for each gene was created using RNA isolated from A549 cells stimulated with cytokines (TNF, IL-1β, and IFN-γ; 10 ng/ml each). Isolated RNA was reverse-transcribed, and a dilution series of cDNA ranging from 1 pg to 10 ng were subjected to real-time PCR. The obtained threshold cycle values were plotted against the dilution factor to create a standard curve. Relative mRNA levels in test samples were then calculated from the standard curve. When calculating the results, IL-6, IL-8, and COX-2 mRNA levels were normalized against GAPDH.

Down-Regulation of MKP-1 by siRNA. MKP-1 siRNA (5′-CCAAUUGUCCCAACCAUUU-3′) and MKP-1 siRNA 1 (5′-GCAUAACUGCCUUGAUCAA-3′) were purchased from Dharmacon RNA Technologies (Lafayette, CO). Lamin A/C siRNA (5′-AACUGGACUUCCAGAAGAACA-3′) and nontargeting control siRNA (5′-AAUUCUCCGAACGUGUCACGU-3′) were purchased from QIAGEN (Valencia, CA). A549 cells were transfected with siRNA using HiPerFect transfection reagent (QIAGEN) according to the manufacturer's instructions. In brief, cells were seeded at density of 1.25×10^5 cells/well on a 24-well plate in 500 μl of medium with 5% fetal calf serum without antibiotics followed by transfection with nontargeting control siRNA (control siRNA), lamin A/C siRNA, MKP-1 siRNA 1, or MKP-1 siRNA 2. Cells were further incubated for 48 h. Fresh culture medium was changed, and cytokines were added into the culture medium. Cells were further incubated for the time indicated, and gene expression was analyzed. All the experiments were done in triplicates. Transfection efficacy was monitored with green fluorescent siRNA oligonucleotides (siGLO green indicator; Dharmacon RNA Technologies). Approximately 90% of the cells emitted green fluorescence signal when transfected with siGLO and HiPerFect. <5% of the cells emitted signal when cells were incubated siGLO oligonucleotides without transfection reagent.

Enzyme-Linked Immunosorbent Assay. Culture medium samples were kept at −20°C until assayed. The concentrations of human IL-6 (PeliPair ELISA; Sanquin, Amsterdam, The Netherlands) and human IL-8 (BD OptEIA set; BD Biosciences, San Diego, CA) were determined by ELISA according to the manufacturer's instructions.

Statistics. Results are expressed as the mean ± S.E.M. When appropriate, one-way ANOVA with Dunnett's or Bonferroni's post-test was performed using InStat version 3.05 for Windows 95/NT (GraphPad Software, Inc., San Diego, CA). Differences were considered significant at *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$.

Results

p38 and JNK Were Phosphorylated in Response to Cytokines in A549 Pulmonary Epithelial Cells. A549 pulmonary epithelial cells were stimulated with a cytokine mixture (TNF, IFN-γ, and IL-1β; 10 ng/ml each), and cells were harvested for protein extraction at time points indicated (Fig. 1). Phosphorylation of p38 MAPK was detected in response to stimulation with a cytokine mixture at time point of 30 min, and it was reverted near the basal level at 1 h. Another submaximal increase in p38 phosphorylation was observed at 2 h after the stimulation with a cytokine mixture, which was reversed near the basal level at 3 h and remained at that level up to the 5-h follow-up (Fig. 1A). Marked phosphorylations of JNK p54 and p46 were observed at 30 min

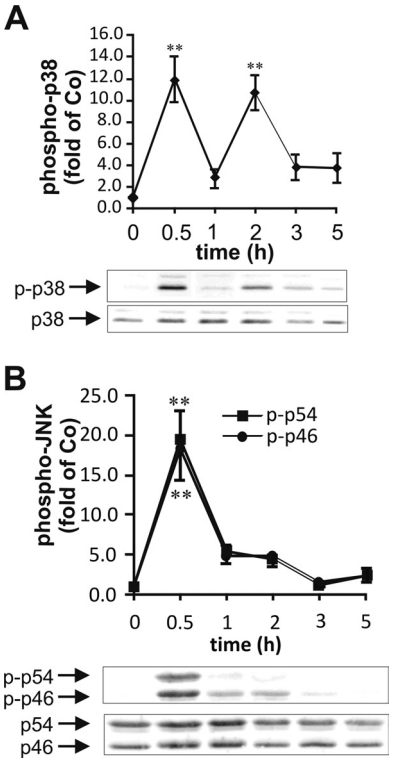

Fig. 1. Phosphorylation of p38 and JNK in response to cytokine mixture in A549 cells. Cells were stimulated with cytokine mixture (CM: TNF, IFN-γ, and IL-1β; 10 ng/ml each) for the time indicated, and cells were then harvested for protein extraction. Phosphorylation of p38 (A) and JNK (B) in response to cytokines was determined by Western blot. The gel is a representative of six separate experiments with similar results. Chemiluminescent signal was quantified and phospho-p38, phospho-p46, and phospho-p54 expression was normalized against p38, p46, and p54, respectively. Phosphorylation levels are expressed in arbitrary units, unstimulated cells set as 1, and the other values related to that. Results are expressed as mean ± S.E.M.; $n = 6$. One-way ANOVA with Dunnett's post-test was performed, and statistical significance was indicated with **, $p < 0.01$ compared with unstimulated cells.

after cytokine treatment, and it was reverted near the basal level at 1 h (Fig. 1B).

p38 Inhibitors SB202190 and BIRB 796 and JNK Inhibitor VIII Inhibited the Phosphorylation of MK2 and c-Jun, Respectively, in A549 Cells. p38 inhibitors SB202190 and BIRB 796 have been reported to effectively inhibit p38 at 1 μM and 100 nM concentrations, respectively (Bain et al., 2007). The effect of SB202190 and BIRB 796 on the phosphorylation of p38 substrate MAP kinase-activated protein kinase 2 (MK2) in A549 cells was investigated. A549 cells were preincubated with p38 MAPK inhibitors SB202190 or BIRB 796 for 30 min and stimulated then with the cytokine mixture (TNF, IFN-γ and IL-1β; 10 ng/ml each) for 30 min. The phosphorylation of MK2 was detected by Western blot. SB202190 and BIRB 796 inhibited the MK2 phosphorylation at concentrations of 1 μM and 100 nM, respectively (Fig. 2, A and B).

The IC_{50} values of JNK inhibitor VIII for JNK1 and JNK2 in kinase assay have been reported to be 2 and 4 nM, respectively, and the EC_{50} value for c-Jun phosphorylation in HepG2 cells has been reported to be 920 nM (Szczepankiewicz et al., 2006). The effect of JNK inhibitor VIII on the phosphorylation of JNK substrate c-Jun was tested in A549 cells. Cells were preincubated with JNK inhibitor VIII for 30 min and stimulated then with the cytokine mixture for 30 min. The phosphorylation of c-Jun was detected by Western blot. JNK inhibitor VIII inhibited c-Jun phosphorylation clearly at 1 μM drug concentration and completely at 10 μM drug concentration (Fig. 2C).

p38 MAPK Inhibitors SB202190 and BIRB 796 Regulated IL-6, IL-8, and COX-2 Expression in Response to Cytokines in A549 Cells. The effects of p38 MAPK inhibitors SB202190 and BIRB 796 or JNK inhibitor VIII on the expression of IL-6, IL-8 and COX-2 in A549 cells were investigated. A549 cells were preincubated with SB202190, BIRB 796 or JNK inhibitor VIII for 30 min and stimulated with a cytokine mixture (TNF, IFNγ and IL-1β, 10 ng/ml each) for 24 h. Supernatants were collected and total cellular proteins were extracted, and IL-6, IL-8, and COX-2 expression was determined. Unstimulated cells did not express measurable levels of IL-6, IL-8, or COX-2 and pretreatment with SB202190, BIRB 796, or JNK inhibitor VIII alone did not induce IL-6, IL-8, or COX-2. Cytokine mixture induced the production of IL-6 and IL-8, and their synthesis was inhibited in a dose-dependent manner by SB202190 and BIRB 796. In contrast, JNK inhibitor VIII did not inhibit the production of IL-6 or IL-8 (Fig. 3, A and B). COX-2 expression was also induced in response to cytokines. BIRB 796 dose-dependently inhibited COX-2 expression and SB202190, at 1 μM concentration, inhibited cytokine-induced COX-2 expression. JNK inhibitor VIII (1 μM) did not have effect on COX-2 expression (Fig. 3C).

The expression of IL-6, IL-8, and COX-2 mRNAs over time was investigated. A549 cells were stimulated with the cytokine mixture (TNF, IFN-γ, and IL-1β; 10 ng/ml each) for 0 to 4 h, and IL-6, IL-8, and COX-2 mRNA levels were measured. Cells were preincubated with SB202190 and BIRB 796 for 30 min and stimulated with cytokines. IL-6 mRNA expression was increased in response to cytokines up to 4 h, and IL-8 and COX-2 mRNA expression reach maximal levels at 3 h (Fig. 4). The effect of SB202190 and BIRB 796 on mRNA levels of IL-6, IL-8, and COX-2 were investigated at a time point of 3 h, because all IL-6, IL-8, and COX-2 expressed maximal or submaximal mRNA levels at 3 h. SB202190 and BIRB 796 markedly inhibited the expression of IL-6, IL-8, and COX-2 mRNAs (Fig. 5).

MKP-1 Regulated the Phosphorylation of p38 MAPK and JNK in A549 Pulmonary Epithelial Cells. At first, we investigated the expression of MKP-1 in A549 cells. Cells were stimulated with the cytokine mixture (TNF, IFN-γ, and IL-1β; 10 ng/ml each), and cells were harvested for protein extraction at time points indicated (Fig. 6A). Unstimulated cells showed low-level basal MKP-1 protein levels, and MKP-1 protein expression was markedly increases in response to cytokines at 1 h. MKP-1 expression was returned to basal level at 2 h and remained at that level up to 5 h (Fig. 6A). To investigate whether MKP-1 regulated the phosphorylation of p38 or JNK in A549 cells, we used siRNA to down-regulate MKP-1 expression. In cells transfected with MKP-1-specific siRNA (MKP-1 siRNA 1 and 2), the protein levels of MKP-1 were reduced compared with the cells transfected with a nontargeting control siRNA (control siRNA) showing that siRNA effectively down-regulated MKP-1 in A549 cells (Fig. 6B). We proceeded to investigate the effect of the down-regulation of MKP-1 on the phosphorylation of p38 and JNK. The down-regulation of MKP-1 by siRNA resulted in an increased p38 and JNK phosphorylation in response to cytokine stimulation at 1 h (Fig. 7A). Lamin A/C-specific siRNA reduced lamin A/C protein expression (100 ± 4.8 versus 45.3 ± 4.0% in cells transfected with nontargeting control siRNA and lamin A/C-specific siRNA, respectively; $n = 3$) but did not affect on p38 or JNK phosphorylation in A549 cells (Fig. 7). These data show that the reduction of MKP-1 by siRNA was functional at the level of p38 phosphorylation and that MKP-1 regulated the phosphorylation of both p38 and JNK in A549 cells.

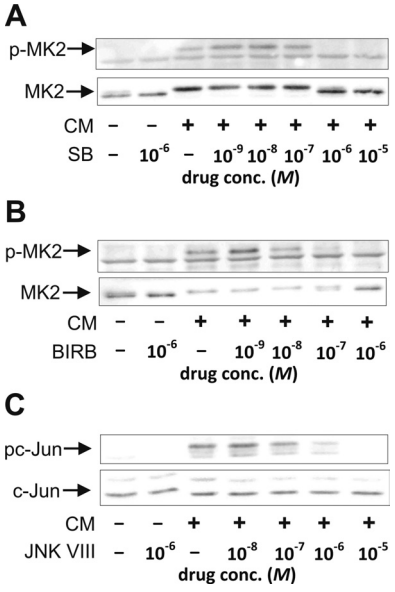

Fig. 2. Effect of SB202190 and BIRB 796 and JNK inhibitor VIII on the phosphorylation of MK2 and c-Jun in response to cytokine mixture in A549 cells. A549 cells were preincubated with SB202190 (A), BIRB 796 (B), or JNK inhibitor VIII (C) at concentrations indicated for 30 min and stimulated with cytokine mixture (CM: TNF, IFN-γ, and IL-1β; 10 ng/ml each) for 30 min, and the phosphorylation of MK2 (A and B) or c-Jun (C) was detected by Western blot. The gels are representatives of six separate experiments with similar results.

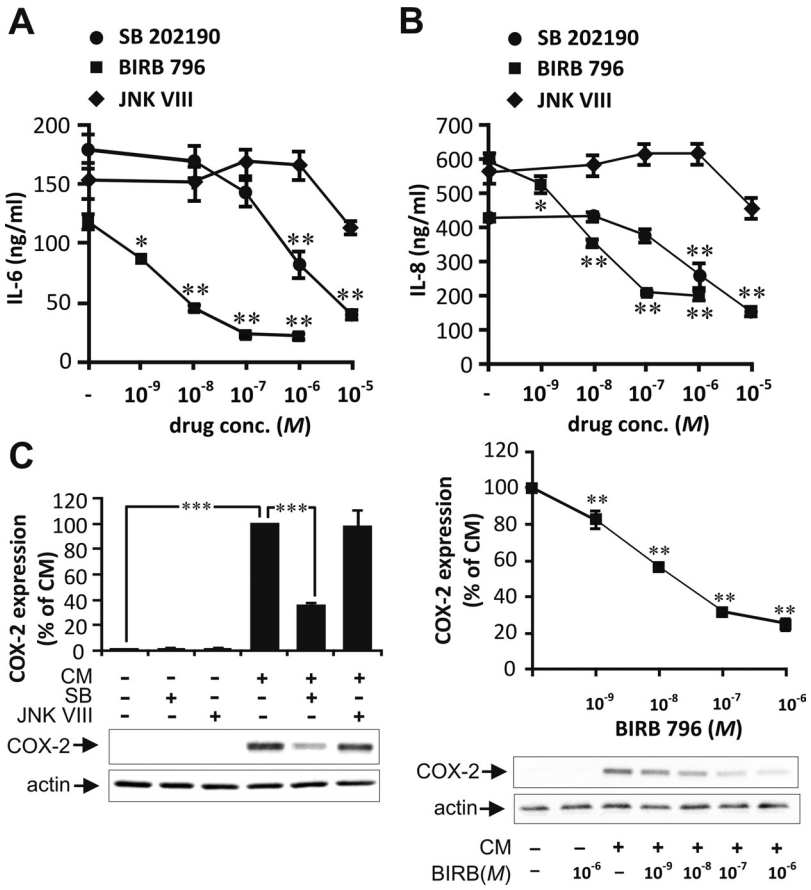

Fig. 3. Effect of SB202190, BIRB 796, and JNK inhibitor VIII on IL-6 and IL-8 production and COX-2 expression in response to cytokine mixture in A549 cells. A549 cells were preincubated with SB202190, BIRB 796, or JNK inhibitor VIII at the concentrations indicated for 30 min and then stimulated with cytokine mixture (CM: TNF, IFN-γ, and IL-1β; 10 ng/ml each) for 24 h. A and B, IL-6 (A) and IL-8 (B) concentrations were determined by ELISA in the culture medium. Results are expressed as mean ± S.E.M.; $n = 6$. One-way ANOVA with Dunnett's post-test was performed and statistical significance was indicated as *, $p < 0.05$ and **, $p < 0.01$ compared with cells treated with CM. C, to investigate the effect of SB202190, BIRB 796, and JNK inhibitor VIII on COX-2 expression, A549 cells were preincubated with SB202190 (SB; 1 μM), BIRB 796 (BIRB; at concentrations indicated), or JNK inhibitor VIII (JNK VIII; 1 μM) for 30 min and stimulated with cytokine mixture (CM) for 24 h, and COX-2 expression was determined by Western blot. The gels are representatives of six separate experiments with similar results. Chemiluminescent signal was quantified and COX-2 expression was normalized to actin. Results are expressed as a percentage of CM, mean ± S.E.M. ($n = 6$). For SB and JNK inhibitor VIII, one-way ANOVA with Bonferroni's post-test was performed, and statistical significance is indicated by ***, $p < 0.001$. For BIRB 796, one-way ANOVA with Dunnett's post-test was performed, and statistical significance is indicated by **, $p < 0.01$ compared with cells treated with CM.

MKP-1 Regulated the Expression of IL-6, IL-8, and COX-2 in A549 Cells. The effect of MKP-1 on the expression of IL-6, IL-8, and COX-2 in response to stimulation with cytokine mixture (TNF, IFN-γ, and IL-1β; 10 ng/ml each) in A549 cells was investigated with siRNA. Cells were transfected with MKP-1-specific siRNA 1 and 2 to down-regulate MKP-1. Cells were stimulated with the cytokine mixture for 3 and 24 h for mRNA and protein analyses, respectively. In cells transfected with MKP-1 siRNA 1 and 2, the expression of IL-6, IL-8, and COX-2 mRNA was increased compared with the cells transfected with either nontargeting control siRNA or lamin A/C-specific siRNA (Fig. 8). Down-regulation of MKP-1 by siRNA resulted in increased IL-6, IL-8, and COX-2 protein expression (Fig. 9). Lamin A/C-specific siRNA did not affect mRNA or protein levels of IL-6, IL-8, or COX-2, showing that the increased expression of IL-6, IL-8, and COX-2 observed with MKP-1 siRNA 1 and 2 was not due to a general activation of RNA-induced silencing complex pathway.

Discussion

In the present study, we investigated the effect of MKP-1 on the expression of IL-6 and IL-8, and COX-2 in response to stimulation with the combination of cytokines TNF, IL-1β, and IFN-γ in human A549 lung epithelial cells. IL-6, IL-8, and COX-2 mRNA and protein expression were inhibited by p38 inhibitors SB202190 and BIRB 796, but not with the JNK inhibitor VIII. Stimulation of A549 cells with cytokines induced also the expression of MKP-1. Down-regulation of MKP-1 by siRNA resulted in enhanced p38 and JNK phosphorylation and increased IL-6, IL-8, and COX-2 mRNA and protein expression.

Two structurally distinct p38 inhibitors SB202190 and BIRB 796 inhibited IL-6, IL-8, and COX-2 expression in A549 cells. This is in line with previous reports, in which p38 inhibitors SB202190 and SB203580, when used at higher concentrations (10–30 μM), have been shown to inhibit IL-6, IL-8, and COX-2 expression in A549 cell (Huang and Zhang, 2003; Kuwahara et al., 2006; Chen et al., 2007). The IC_{50} value for SB202190 in p38 inhibition has been reported to be approximately 100 nM and effective p38 inhibition takes place at 100 to 300 nM drug concentrations (Bain et al., 2007). In our study, SB202190 inhibited IL-6 and IL-8 mRNA and protein expression clearly at the 1 μM concentration, but the reduction in IL-6 and IL-8 protein expression with 100 nM SB202190 was statistically not significant. Therefore, we tested another structurally unrelated p38 inhibitor BIRB 796, which has been reported to effectively inhibit p38 at the 100 nM concentration (Bain et al., 2007). BIRB 796 inhibited IL-6 and IL-8 production in a dose-dependent manner, and the maximal inhibition of IL-6 and IL-8 mRNA and protein expression was observed at the 100 nM drug concentration. To further investigate whether the SB202190 and BIRB 796 inhibited p38, the effect of these compounds on the phosphorylation of a p38 substrate MK2 was investigated. SB202190 and BIRB 796 abrogated the phosphorylation of MK2 at concentrations of 1 μM and 100 nM, respectively, showing that these compounds were p38 inhibitors at the concentration used. These data suggest that the reduction of IL-6 and IL-8 expression by SB202190 and BIRB 796 was due to p38

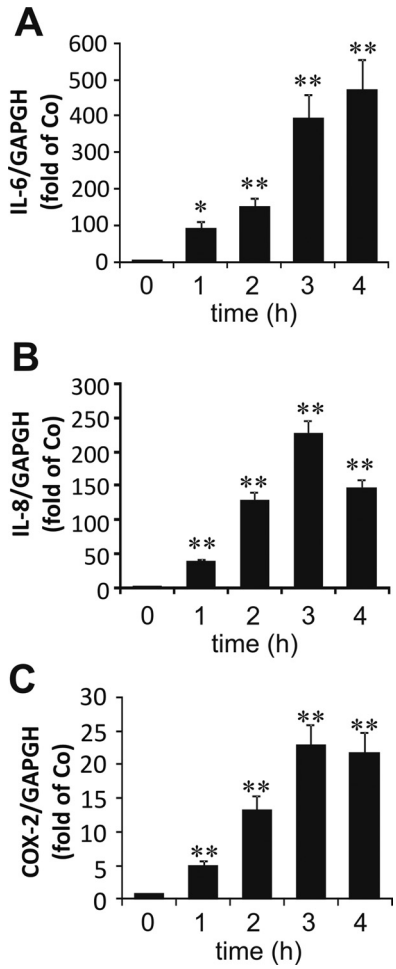

Fig. 4. Expression of IL-6 mRNA, IL-8 mRNA, and COX-2 mRNA over time in response to cytokine mixture in A549 cells. Cells were stimulated with cytokine mixture (CM: TNF, IFN-γ, and IL-1β; 10 ng/ml each) for the time indicated, and cells were harvested for total RNA extraction. The expression of IL-6 mRNA (A), IL-8 mRNA (B), and COX-2 mRNA (C) was determined by quantitative real-time RT-PCR, and mRNA expression was normalized against GAPDH mRNA. Results are expressed as mean ± S.E.M. ($n = 3$), unstimulated cells (0 h) set as 1, and other values were related to that. One-way ANOVA with Dunnett's post-test was performed, and statistical significance is indicated with *, $p < 0.05$ and **, $p < 0.01$ compared with unstimulated cells.

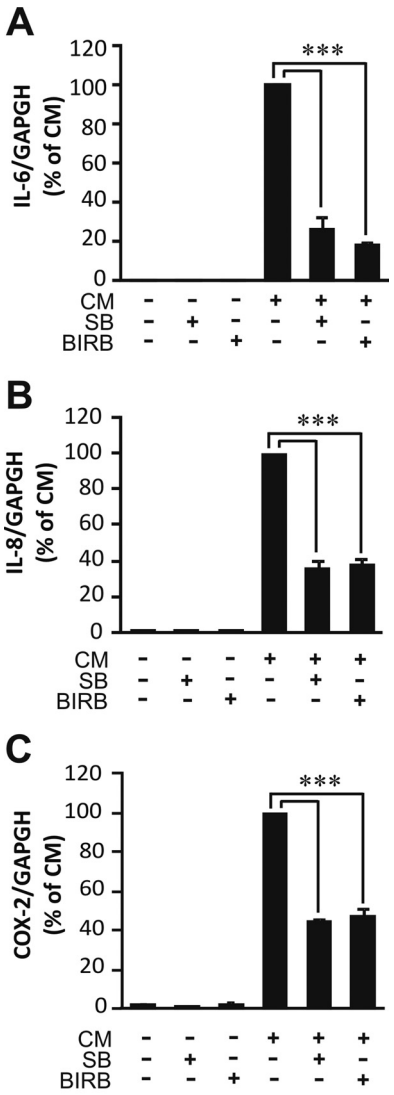

Fig. 5. Effect of SB202190 and BIRB 796 on IL-6 mRNA, IL-8 mRNA, and COX-2 mRNA expression in response to cytokine mixture in A549 cells. Cells were preincubated with SB202190 (1 μM) or BIRB 796 (100 nM) for 30 min and stimulated with cytokine mixture (CM: TNF, IFN-γ, and IL-1β; 10 ng/ml each) for 3 h, and then cells were harvested for total RNA extraction. The expression of IL-6 mRNA (A), IL-8 mRNA (B), and COX-2 mRNA (C) was determined by quantitative real-time RT-PCR, and mRNA expression was normalized against GAPDH mRNA. Results are expressed as a percentage of CM, mean ± S.E.M. ($n = 3$). One-way ANOVA with Bonferroni's post-test was performed, and statistical significance is indicated with ***, $p < 0.001$.

MAPK inhibition and not due to nonspecific effects of these compounds in A549 cells.

In A549 cells, SB203580, a p38 inhibitor structurally related to SB202190, has been reported to inhibit COX-2 expression at 1 to 10 μM concentrations (Newton et al., 2000), and SB203580 has been shown to partially inhibit hsp27 phosphorylation (a p38 MAPK substrate) at the 1 μM concentration and completely at the 10 μM concentration (King et al., 2009b). In our experiments, SB202190 inhibited COX-2 mRNA and protein expression at 1 μM concentration, which also abrogated the phosphorylation of MK2. In addition, BIRB 796 inhibited the expression of COX-2 in a dose-dependent manner, with maximal inhibition seen with the 100 nM drug concentration. These data suggest that, in our study, the reduction in COX-2 expression observed with SB202190 and BIRB 796 was probably due to the inhibition of p38 and that p38 regulates COX-2 expression in A549 cells. Taken together, these results suggest that p38 MAPK regulates the expression of IL-6, IL-8, and COX-2 in A549 pulmonary epithelial cells.

We also investigated the effect of JNK pathway on the expression of IL-6, IL-8, and COX-2 in response to stimulation with the proinflammatory cytokines. In these experiments, we used a recently described aminopyridine-based compound JNK inhibitor VIII (Szczepankiewicz et al., 2006). This inhibitor has been shown to have >1000-fold selectivity for JNK over other MAP kinases. JNK inhibitor VIII inhibited the phosphorylation of c-Jun, a direct substrate of JNK, and the submaximal inhibition in phosphorylation was seen at the 1 μM drug concentration. With this inhibitor, we did not see a reduction in IL-6 and IL-8 production or COX-2 expression in response to the cytokines in A549 cells. Using

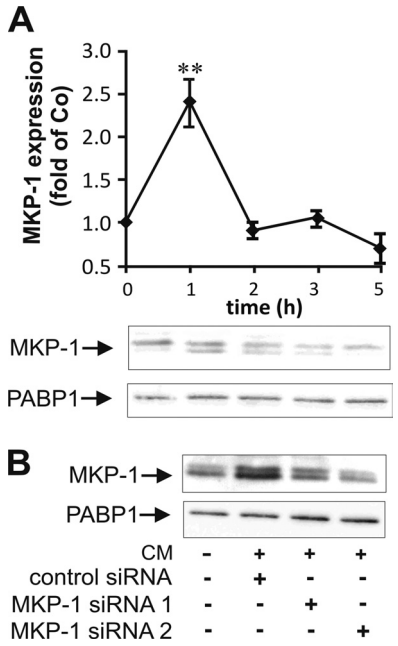

Fig. 6. Expression of MKP-1 in response to cytokine mixture, and the effect of MKP-1-specific siRNA on MKP-1 expression in A549 cells. A, cells were stimulated with cytokines (CM: TNF, IFN-γ, and IL-1β; 10 ng/ml each) for the time indicated, and MKP-1 expression was determined by Western blot. Chemiluminescent signal was quantified and MKP-1 expression was normalized against poly(A)binding protein 1 (PABP1). MKP-1 protein levels are expressed in arbitrary units, MKP-1 expression in unstimulated cells (0 h) set as 1, and the other values are related to that (mean ± S.E.M.; $n = 6$). One-way ANOVA with Dunnett's post-test was performed, and statistical significance is indicated with **, $p < 0.01$ compared with unstimulated cells. The gel is a representative of six separate experiments with similar results. B, cells were transfected with MKP-1-specific siRNA 1 or 2 (MKP-1 siRNA 1 or 2) or nontargeting control siRNA (control siRNA) and incubated for 48 h. Cells were then stimulated with CM for 1 h, and MKP-1 expression was determined by Western blot. The gel is a representative of three separate experiments with similar results.

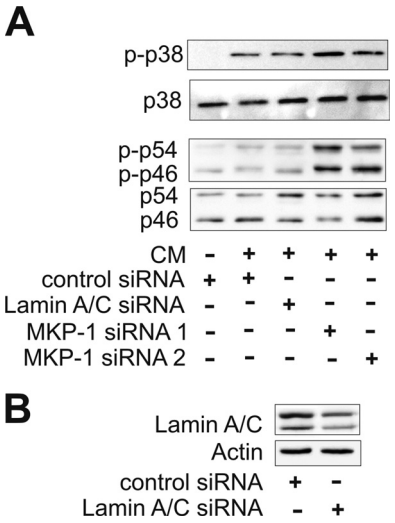

Fig. 7. Effect of MKP-1-specific siRNA on the phosphorylation of p38 and JNK in A549 cells. A, cells were transfected with MKP-1-specific siRNA 1 or 2 (MKP-1 siRNA 1 or 2), nontargeting control siRNA (control siRNA) and incubated for 48 h. Cells were then stimulated with cytokines (CM: TNF, IFN-γ, and IL-1β; 10 ng/ml each) for 1 h, and the phosphorylation of p38 and JNK was determined by Western blot. The gels are representatives of five separate experiments with similar results. B, cells were transfected with lamin A/C-specific siRNA (lamin A/C siRNA) or nontargeting control siRNA (control siRNA) and incubated for 48 h, and lamin A/C protein expression was detected with Western blot. The gel is a representative of three separate experiments with similar results.

overexpression of dominant-negative JNK and antisense method in human keratinocytes, JNK has been shown to regulate IL-6 and IL-8 production (Krause et al., 1998). Stretch-induced IL-8 mRNA expression has been reported to be inhibited with a JNK inhibitor SP600125 at a concentration of 5 μM (Li et al., 2003). SP600125 has also been reported to inhibit COX-2 expression in response to inflammatory stimulation (Nieminen et al., 2006; Chen et al., 2007). The results obtained with the inhibitor SP600125 may be complicated by the fact that SP600125 has been shown to inhibit other kinases also (Bain et al., 2007). Cell type-specific differences in the role of JNK on the expression of inflammatory genes seem also to exist. In vivo administration of SP600125 did not inhibit IL-6 mRNA expression in mice with ozone-induced airway inflammation (Williams et al., 2007), and yet in human primary eosinophils, SP600125 inhibited IL-25-induced IL-6 and IL-8 production (Wong et al., 2005). Taken together, our results with a novel compound JNK inhibitor VIII suggest that JNK does not regulate the expression of IL-6, IL-8, and COX-2 in A549 pulmonary epithelial cells.

We used siRNA to investigate the effect of MKP-1 on p38 and JNK phosphorylation and expression of IL-6, IL-8, and COX-2. Down-regulation of MKP-1 with two different siRNAs reduced cytokine-induced MKP-1 expression in A549 cells. In addition, the down-regulation of MKP-1 with two different siRNAs increased p38 phosphorylation at 1 h after cytokine stimulation, showing that the reduction of MKP-1 expression by siRNA was functional at the level of MAP kinase activation. The substrate specificity of MKP-1 has been reported to vary between cell types. MKP-1 was found to regulate p38 in macrophages (Salojin et al., 2006), JNK in airway smooth muscle cells (Issa et al., 2007), and both p38 and JNK in endothelial cells (Zakkar et al., 2008). We found an increase in both p38 and JNK phosphorylation in A549 pulmonary epithelial cells with MKP-1-specific siRNA 1 and 2. This suggests that MKP-1 regulates the phosphorylation of both p38 and JNK in A549 cells. Lamin A/C-specific siRNA was used as a positive control in siRNA experiments. Lamin A/C-specific siRNA down-regulated lamin A/C protein expression approximately by 50%, showing that lamin A/C siRNA was functional. Lamin A/C-specific siRNA did not have effect on p38 and JNK phosphorylation or IL-6, IL-8, or COX-2 expression, showing that the effects seen with MKP-1-specific siRNA were probably due to specific down-regulation of MKP-1 and not due to general activation of RNA-induced silencing complex pathway per se.

IL-8 is an important chemokine in the regulation of neutrophil migration (Donnelly and Barnes, 2006). Prominent neutrophil infiltration, along with other inflammatory cells, characterizes both chronic obstructive pulmonary disease and severe asthma (Barnes, 2008). We found that MKP-1 negatively regulated the expression of IL-8, which is in line with the reports showing that p38 positively regulates IL-8 expression (Kuwahara et al., 2006). In a recent report using overexpression of MKP-1, MKP-1 has been shown to negatively regulate IL-8 mRNA expression in human BEAS-2B bronchial epithelial cells (King et al., 2009a). After intraperitoneal administration of bacterial products from Gram-positive bacteria, MKP-1(−/−) mice showed increased neutro-

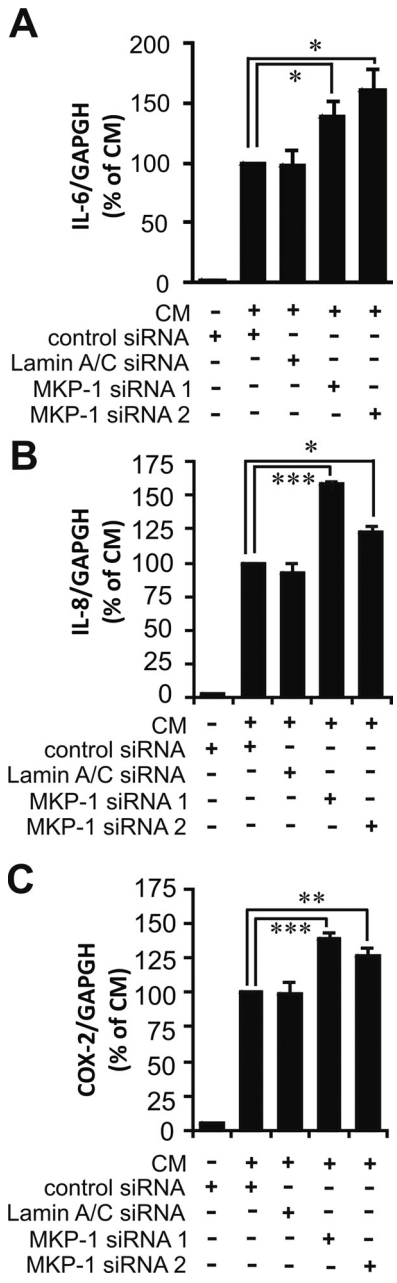

Fig. 8. Effect of MKP-1-specific siRNA on IL-6 mRNA, IL-8 mRNA and COX-2 mRNA expression in A549 cells. Cells were transfected with MKP-1-specific siRNA 1 or 2 (MKP-1 siRNA 1 or 2), nontargeting control siRNA (control siRNA), or lamin A/C-specific siRNA and incubated for 48 h. Cells were then stimulated with cytokines (CM: TNF, IFN-γ, and IL-1β; 10 ng/ml each) for 3 h, and IL-6 mRNA (A), IL-8 mRNA (B), and COX-2 mRNA (C) expression was determined by quantitative real-time RT-PCR. mRNA expression was normalized against GAPDH mRNA. Results are expressed as a percentage of CM-stimulated cells transfected with nontargeting control siRNA (mean ± S.E.M.; $n = 5$). One-way ANOVA with Bonferroni's post-test was performed, and statistical significance is indicated with *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

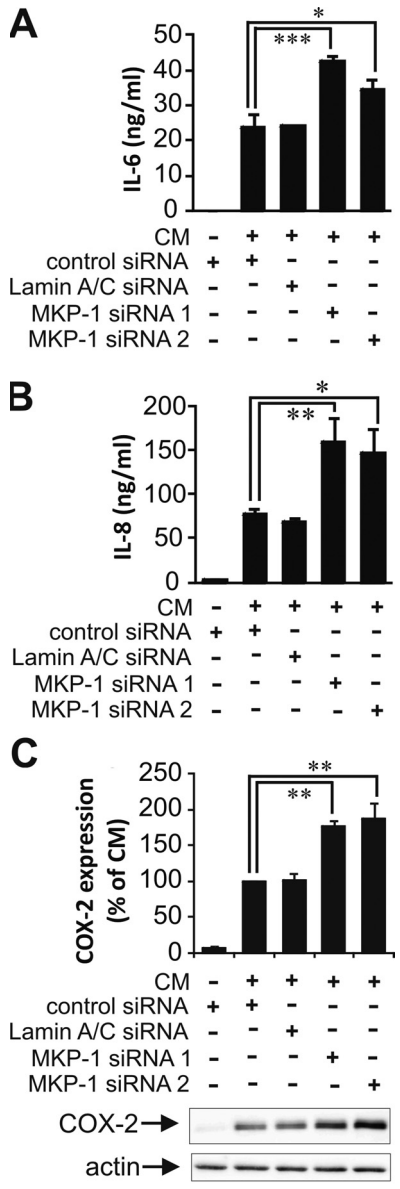

Fig. 9. Effect of MKP-1-specific siRNA on IL-6 and IL-8 production and COX-2 protein expression in A549 cells. Cells were transfected with MKP-1-specific siRNA 1 or 2 (MKP-1 siRNA 1 or 2), nontargeting control siRNA (control siRNA), or lamin A/C-specific siRNA and incubated for 48 h. Cells were then stimulated with cytokines (CM: TNF, IFN-γ, and IL-1β; 10 ng/ml each) for 24 h, and IL-6 (A) and IL-8 (B) production was determined by ELISA, and COX-2 protein expression (C) was determined by Western blot (chemiluminescent signal was quantified and COX-2 was normalized against actin). Results are expressed as a percentage of CM-stimulated cells transfected with nontargeting control siRNA (mean ± S.E.M.; $n = 6$). One-way ANOVA with Bonferroni's post-test was performed, and statistical significance is indicated with *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$. The gel is representative of six separate experiments with similar results.

philic infiltration in the lung (Wang et al., 2007). IL-17 has been found to be present in eosinophils of asthmatic subjects and has been shown to induce the expression of IL-6, IL-8, and GRO-α in bronchial epithelial cells and fibroblasts (Molet et al., 2001). In addition to its effect on IL-8 found in the present study, MKP-1 has been reported to regulate the expression of GRO-α, another neutrophil chemokine, in human pulmonary smooth muscle cells (Issa et al., 2007), suggesting that MKP-1 is an important negative factor in the regulation of inflammatory cell migration. MKP-1 is possibly an important regulatory factor in the pathogenesis of chronic obstructive pulmonary disease or neutrophilic airway inflammation related to severe asthma, and increased MKP-1 expression may provide a rationale drug target for the treatment of airway inflammation.

p38 MAPK has been reported to regulate the expression of both IL-6 and COX-2 (De Cesaris et al., 1998; Dean et al.,

1999). In our experiments, IL-6 production and COX-2 expression were inhibited by p38 inhibitors SB202190 and BIRB 796 in A549 cells. IL-6 and COX-2 expression have also been shown to be increased in LPS-stimulated macrophages from MKP-1(−/−) mice (Abraham et al., 2006; Hammer et al., 2006; Wang et al., 2007). In our experiments, down-regulation of MKP-1 by siRNA resulted in increased expression of IL-6 and COX-2 in A549 cells, which is in line with the previous data from mouse macrophages.

In conclusion, our results suggest that the inhibition of p38 regulates the expression of IL-6, IL-8, and COX-2 in response to cytokines in A549 human lung epithelial cells. MKP-1 negatively regulated the phosphorylation of p38 and JNK. Results also suggest that MKP-1 negatively regulates the expression of IL-6, IL-8, and COX-2 in response to cytokine stimulation by inhibiting the p38 MAPK phosphorylation. Our results suggest that MKP-1 is an important negative regulator of inflammatory gene expression in human lung epithelial cells, and compounds that enhance MKP-1 may have anti-inflammatory effects and control inflammatory response in the human lung.

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Attenuation of the acute inflammatory response by dual specificity phosphatase 1 by inhibition of p38 MAP kinase

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ABSTRACT

Dual specificity phosphatase 1 (DUSP1) dephosphorylates and, hence, regulates the activity of MAP kinases. The present study investigated the effect of DUSP1 on inflammatory gene expression and on the development of carrageenan-induced inflammation. It was found that DUSP1 expression was increased by LPS, and the down-regulation of DUSP1 by siRNA enhanced the phosphorylation of p38 MAPK, while JNK phosphorylation was not affected in murine macrophages. LPS-induced interleukin (IL)-6, tumor-necrosis factor (TNF) and cyclooxygenase-2 (COX2) expression were enhanced in bone marrow-derived macrophages (BMMs) from DUSP1(−/−) mice as compared to those from wild-type mice. In addition, down-regulation of DUSP1 by siRNA enhanced IL-6, TNF and COX2 expression in J774 macrophages, while p38 MAPK inhibitors SB202190 and BIRB 796 inhibited the expression of those inflammatory factors. *In vivo*, the intensity of the carrageenan-induced paw edema reaction was increased in DUSP1(−/−) mice as compared to the wild-type animals. In conclusion, DUSP1 is an important negative regulator of the acute inflammatory response by limiting p38 MAPK, and compounds which enhance DUSP1 expression or activity may hold a promise as anti-inflammatory drugs.

1. Introduction

There are several mitogen-activated protein kinases (MAPKs) including stress-activated protein kinases p38 MAPK and Jun N-terminal kinase (JNK) and extracellular-signal regulated protein kinase (ERK). MAPKs are activated in response to various extracellular stimuli, such as growth factors and cellular stress through G-protein coupled receptor activation. The substrates of MAP kinases include transcription factors, such as Jun, Myc, Fos, activating transcription factor 2, and several other regulatory proteins, such as mitogen-activated protein kinase-activated protein kinases (MAPKAPKs) (Johnson and Lapadat, 2002).

There are four p38 MAPK isoforms, namely p38α, p38β, p38γ and p38δ. p38α and p38β MAPKs are widely expressed, while p38γ expression is restricted to skeletal muscle and that of p38δ to testes, pancreas, small intestine, and $CD4^+$ T cells. p38 MAPK is known to regulate cellular growth and differentiation and it has been shown to participate in the regulation of immune response (Rincón and Davis, 2009).

Inflammatory stimulation leads to the activation of p38 MAPK pathway, and the expression of inflammatory genes, such as those coding for tumor necrosis factor (TNF) and interleukins (IL), is regulated by p38 MAPK (Ono and Han, 2000; Mahtani et al., 2001; Turpeinen et al., 2010). Genetic deficiencies in mitogen-activated protein kinase kinase (MAP2K) 3 and MAP2K6, upstream activators of p38 MAPK, inhibits the production of IL-12 by macrophages and T helper (Th)1 differentiation and interferon-γ (IFNγ) production (Lu et al., 1999). Mice lacking MAPKAPK2, a direct substrate for p38 MAPK, exhibit an impaired response to lipopolysaccharide (LPS) and cells isolated from their spleens produce markedly lower amounts of TNF, IL-6, IL-10 and IFNγ (Kotlyarov et al., 1999). p38 MAPK inhibitors have been shown to inhibit the disease progression in a rodent arthritis models and to suppress the inflammatory response during endotoxemia in humans (Branger et al., 2002; Burnette et al., 2009).

Dual specificity phosphatases (DUSPs) are a large group of protein phosphatases that are able to dephosphorylate phosphotyrosine and phosphoserine/phosphothreonine residues in their

Abbreviations: BMMs, bone marrow-derived macrophages; COX2, cyclooxygenase-2; DUSP, dual specificity phosphatase; ERK, extracellular-signal-regulated-kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GM-CSF, granulocyte/macrophage-colony stimulating factor; IFNγ, interferon-γ; IL, interleukin; JNK, Jun N-terminal kinase; LPS, lipopolysaccharide; M-CSF, macrophage colony-stimulating factor; MAPK, mitogen-activated protein kinase; MAPKAPK, mitogen-activated protein kinase-activated protein kinase; siRNA, small interfering RNA; TNF, tumor necrosis factor.

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target proteins (Patterson et al., 2009). There is one subgroup of DUSPs that target MAP kinases; these are also known as MAP kinase phosphatases (MKPs). This subgroup of DUSPs includes more than ten members that differ from each other in their expressional pattern, cellular location and substrate specificity (Liu et al., 2007; Boutros et al., 2008). DUSP1 belongs to the MKP subgroup of DUSPs. It is a nuclear phosphatase widely expressed in various cell types and tissues, and its expression is induced by a range of stimuli, including cellular stress, cytokines and LPS. DUSP1 displays specificity towards p38 and JNK over p42/44 ERK (Franklin and Kraft, 1997; Liu et al., 2007; Boutros et al., 2008). Macrophages from DUSP1(−/−) mice display increased and prolonged activation of p38 in response to LPS, and LPS-induced inflammatory responses are amplified in DUSP1(−/−) mice as compared to wild-type animals (Zhao et al., 2005; Hammer et al., 2006; Salojin et al., 2006).

In the present study, we investigated the effect of DUSP1 on the expression of inflammatory genes TNF, IL-6 and COX2 in activated macrophages and on the development *in vivo* of an acute carrageenan-induced edema response. DUSP1 acted as a negative regulator of the expression of inflammatory genes and acute inflammatory response by limiting p38 MAPK.

2. Materials and methods

2.1. Materials

Reagents were obtained as follows: LPS from *Escherichia coli* strain 0111:B4 (Sigma–Aldrich Inc., St. Louis, MO, USA), SB202190 (Tocris Biosciences, Bristol, UK), BIRB 796 (Axon Medchem, Groningen, The Netherlands), JNK inhibitor VIII (Calbiochem, Merck Chemicals, Darmstadt, Germany) and macrophage colony-stimulating factor (M-CSF) (RD Systems Inc., Minneapolis, MA, USA) were purchased as indicated. All other reagents were from Sigma Aldrich Inc. unless otherwise stated below.

2.2. Cell culture

J774 murine macrophages (ATCC, Rockville Pike, MD, USA) were cultured at 37 °C in 5% CO_2 atmosphere in Dulbeccoïs modified Eagle's medium supplemented with glutamax-1 containing 5% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B (all from Invitrogen, Paisley, UK). For cytokine measurements and Western blot experiments, cells were seeded on 24-well plates at density of 2×10^5 cells/well. Cell monolayers were grown for 72 h before the experiments were started. BIRB 796 and SB202190 were dissolved in DMSO. BIRB 796 and SB202190 at concentrations indicated or DMSO (0.1%, v/v) were added to the cells in fresh culture medium containing 5% FBS and the above antibiotics 30 min prior to the stimulation with LPS (10 ng/ml). Cells were further incubated for the time indicated. The effect of LPS and the tested chemicals on cell viability was evaluated by visual assessment in a microscope and by the XTT test using Cell Proliferation Kit II (Roche Diagnostics, Mannheim, Germany) (Korhonen et al., 2001; Hämäläinen et al., 2009). Neither LPS nor the other chemical used in the experiments were found to evoke cytotoxicity.

2.3. Isolation of the bone-marrow-derived and peritoneal macrophages and cell culture

The study was approved by the Animal Care and Use Committee of the University of Tampere and the respective public provincial authority committee for animal experiments. Inbred C57BL/6 DUSP1(−/−) mice were originally generated by the R. Bravo laboratory at Bristol–Myers Squibb Pharmaceutical Research Institute (Dorfman et al., 1996). Bone marrow-derived macrophages (BMMs) were isolated from femur and tibia of the hind legs of mice aged 10–12 weeks. Briefly, mice were anesthesized by intraperitoneal injection of 0.05 mg/100 g body weight of medetomidine (Domitor® 1 mg/ml, Orion Corp., Espoo, Finland) and 7.5 mg/100 g body weight of ketamine (Ketalar® 10 mg/ml, Pfizer Oy Animal Health, Helsinki, Finland), and mice were terminated by cervical dislocation. BMMs were generated from bone marrow hematopoietic stem cells with 5–7 days of incubation in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin and 10 ng/ml M-CSF. Thereafter, BMMs (1×10^6 cells/well) were seeded on a 24-well plates, cultured overnight in complete culture medium followed by serum starvation overnight. At the beginning of the experiment, LPS was added to the cells along with the culture medium containing 10% FCS and antibiotics, and BMMs were incubated for the time indicated. Primary mouse peritoneal macrophages were obtained by peritoneal lavage with sterile PBS supplemented with 0.2 mM EDTA. Cells were washed, resuspended in RPMI 1640 medium supplemented with 2% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and seeded on 24-well plates (5×10^5 cells/well). The cells were incubated overnight and washed with PBS to remove non-adherent cells before the experiments.

2.4. Carrageenan induced paw edema

The study was approved by the Animal Care and Use Committee of the University of Tampere and the respective provincial committee for animal experiments. Male C57BL/6 mice (20–25 g) were used with six animals per group. Prior to the administration of carrageenan, the mice were anesthesized by intraperitoneal injection of 0.05 mg/100 g of medetomidine (Domitor® 1 mg/ml. Orion Oyj, Espoo, Finland) and 7.5 mg/100 g of ketamine (Ketalar® 10 mg/ml, Pfizer Oy Animal Health, Helsinki, Finland). The mice received a 30 μl intradermal injection in one hind paw of carrageenan (1.5%) dissolved in normal saline. The contralateral paw received 30 μl of saline and it was used as a control. Paw volume was measured before and 3 h after the carrageenan injection with a plethysmometer (Ugo Basile, Comerio, Italy).

2.5. Preparation of cell lysates and Western blot analysis

At the indicated time points, the culture medium was removed. Cells were rapidly washed with ice-cold PBS and solubilized in cold lysis buffer containing 10 mM Tris–HCl, 5 mM EDTA, 50 mM NaCl, 1% Triton-X-100, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM sodiumorthovanadate, 20 μg/ml leupeptin, 50 μg/ml aprotinin, 5 mM sodium fluoride, 2 mM sodium pyrophosphate and 10 μM *n*-octyl-β-D-glucopyranoside. After incubation for 20 min on ice, lysates were centrifuged, supernatants were collected and mixed in a ratio of 1:4 with SDS loading buffer (62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 0.025% bromophenol blue, and 5% β-mercaptoethanol) and stored at −20 °C until analyzed.

COX2, actin, DUSP1, JNK, phospho-c-Jun, c-Jun, polyclonal anti-rabbit and polyclonal anti-goat antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phospho-p38, p38, phospho-JNK and JNK antibodies were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA).

Prior to Western blot analysis, the samples were boiled for 10 min. Equal aliquots of protein (10–20 μg) were loaded on a 10% SDS-polyacrylamide gel and separated by electrophoresis. Proteins were transferred to Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham, Buckinghamshire, UK) by semidry electroblotting. After transfer, the membrane was blocked in TBS/T (20 mM Tris–base pH 7.6, 150 mM NaCl, 0.1% Tween-20) containing 5% nonfat milk for 1 h at room temperature. For detection of phospho-proteins, membranes were blocked in TBS/T

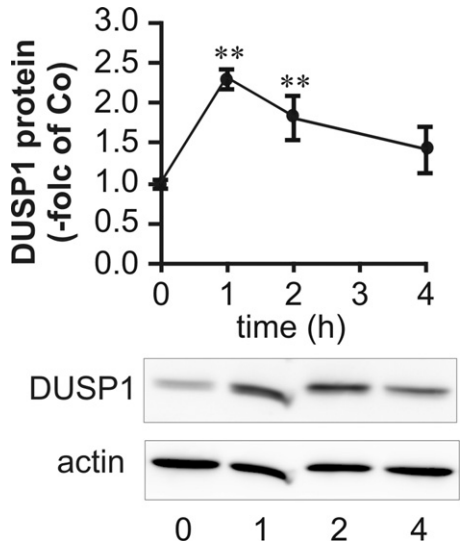

Fig. 1. The expression of DUSP1 in J774 mouse macrophages. J774 cells were stimulated with LPS (10 ng/ml) for the time indicated and the expression of DUSP1 was detected by Western blot. Chemiluminescent signal was quantified and DUSP1 expression was normalized against actin. Protein expression levels are given in arbitrary units, unstimulated cells set as 1, and the other values are related to that value. The results are expressed as mean ± S.E.M.; $n = 6$. One-way ANOVA with Dunnett's post test was performed, and statistical significance was indicated as $^{**}p < 0.01$ compared to unstimulated cells.

containing 5% bovine serum albumin. Membranes were incubated overnight at 4 °C with primary antibody, and for 1 h with secondary antibody, and the chemiluminescent signal was detected by FluorChem 8800 imaging system (Alpha Innotech Corporation, San Leandro, CA, USA) or ImageQuant™ LAS 4000 mini (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The chemiluminescent signal was quantified with FluorChem software version 3.1. and ImageQuant TL 7.0 Image Analysis Software.

2.6. RNA extraction and real-time RT-PCR

At the indicated time points, the culture medium was removed, and cell homogenization and RNA extraction were carried out using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma, Saint Louis, MO, USA) according to the manufacturer's instruction. Reverse transcription of RNA to cDNA was performed by TaqMan® Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA) according to the supplier's instructions. The primer and probe sequences and concentrations were optimized according to the manufacturer's guidelines in TaqMan® Universal PCR Master Mix Protocol part number 4304449 revision C (Applied Biosystems, Foster City, CA, USA) and were as follows: 5′-CAAGGATGCTGGAGGGAGAGT-3′ (forward, 300 nM), 5′-TGAGGTAAGCAAGGCAGATGGT-3′ (reverse, 300 nM), 5′-TTTGTTCATTGCCAGGCCGGCAT-3′ (probe containing 6-FAM as 5′-reporter dye and TAMRA as 3′-quencher, 150 nM) for mouse DUSP1, 5′-AATGGCCTCCCTCTCATCAGTT-3′ (forward, 300 nM), 5′-TCCTCCACTTGGTGGTTTGC-3′ (reverse, 300 nM), 5′-CTCAAAATTCGAGTGACAAGCCTGTAGCCC-3′ (probe containing 6-FAM as 5′-reporter dye and TAMRA as 3′-quencher, 150 nM) for mouse TNF, 5′-AAGCGAGGACCTGGGTTCA-3′ (forward, 300 nM), 5′-ACCTGATATTTCAATTTTCCATCCTT-3′ (reverse, 300 nM), 5′-TGTGATTTAAGTCCACTCCATGGCCCA-3′ (probe containing 6-FAM as 5′-reporter dye and TAMRA as 3′-quencher, 150 nM) for mouse COX2 and 5′-GCATGGCCGGCCGTGTTC-3′ (forward, 300 nM), 5′-GATGTCATCATACTTGGCAGGTTT-3′ (reverse, 300 nM) and 5′-TCGTGGATCTGACGTGCCGCC-3′ (probe containing 6-FAM as 5′-reporter dye and TAMRA as 3′-quencher, 150 nM) for mouse GAPDH. Primers and probes were obtained from Metabion (Martinsried, Germany). The PCR reaction parameters were as follows: incubation at 50 °C for 2 min, incubation at 95 °C for 10 min, and thereafter 40 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. Each sample was determined in duplicate. A standard curve method was used to estimate the relative mRNA levels. When calculating the results, DUSP1 mRNA levels were first normalized against GAPDH.

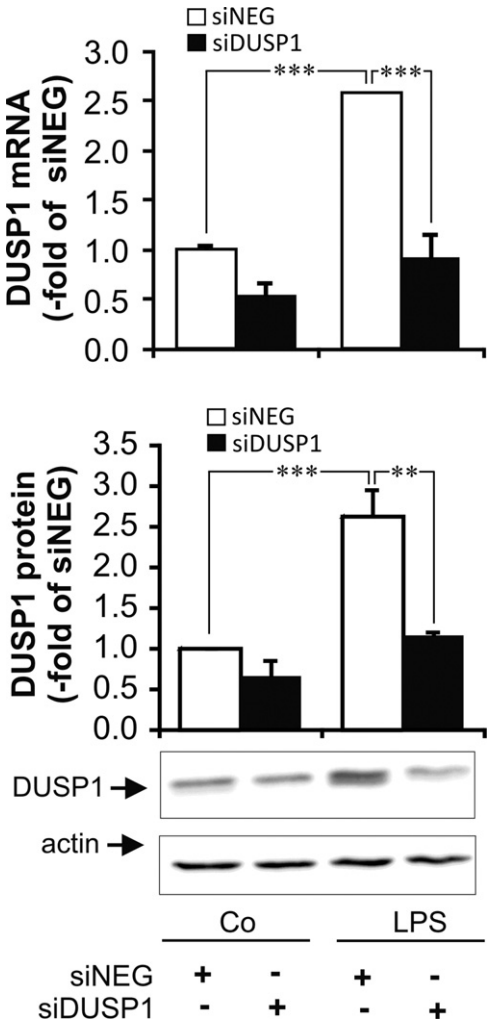

Fig. 2. Down-regulation of DUSP1 by siRNA in J774 mouse macrophages. J774 cells were transfected with DUSP1 specific siRNA (siDUSP1) or non-targeting siRNA (siNEG). Cells were stimulated with LPS (10 ng/ml) for 1 h. DUSP1 mRNA and protein levels were determined by quantitative RT-PCR, and Western blot, respectively. mRNA and protein expression levels are expressed in arbitrary units, unstimulated cells transfected with siNEG set as 1, and the other values are related to that value. The results are expressed as mean ± S.E.M., $n = 6$ for DUSP1 mRNA and $n = 3$ in DUSP1 protein. One-way ANOVA with Bonferroni's post test was performed, and statistical significance is indicated as $^{**}p < 0.01$ and $^{***}p < 0.001$.

2.7. Down-regulation of DUSP1 by siRNA

All oligos and transfection reagents were from Dharmacon Research (Dharmacon Research, Lafayette, CO, USA). J774 cells were transfected with DUSP1 specific (siDUSP1) ON-TARGET*plus* SMARTpool or with non-targeting (siNEG) siCONTROL® siRNA using DharmaFECT 4 transfection reagent according to the manufacturer's instructions. Briefly, J774 macrophages were seeded at a density of 1.0×10^5 cells/well on a 24-well plate in 500 μl of medium with 5% FCS without antibiotics. Cells were incubated

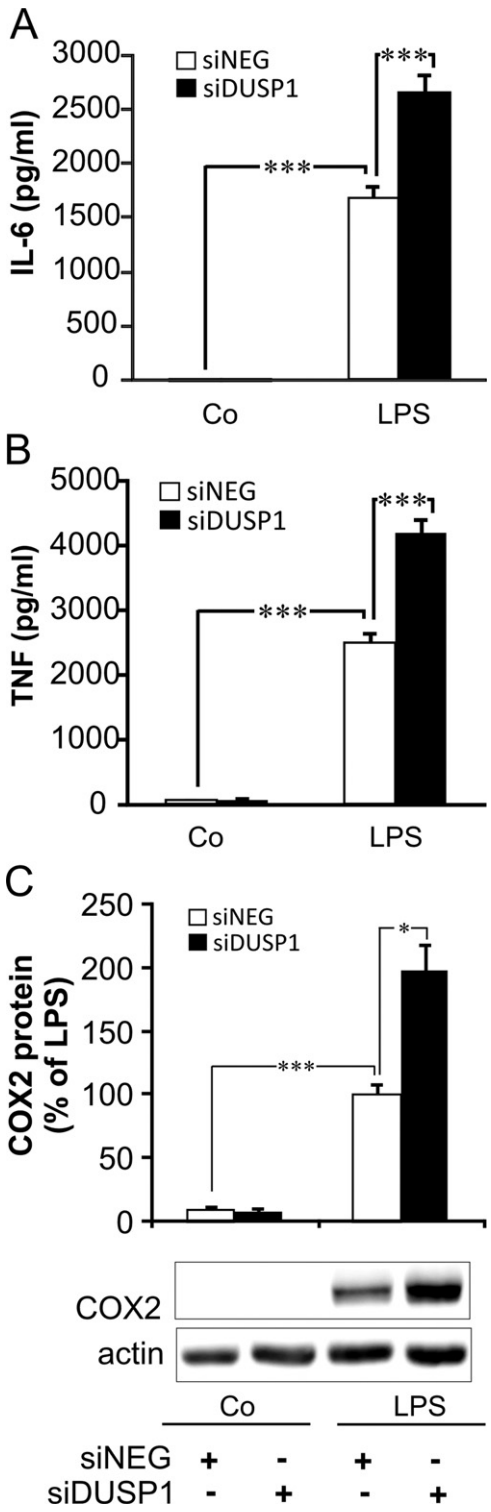

Fig. 3. The effect of DUSP1 siRNA on IL-6 and TNF production and COX2 expression in J774 mouse macrophages. J774 cells were transfected either with DUSP1 siRNA (siDUSP1) or non-targeting siRNA (siNEG) and stimulated with LPS (10 ng/ml) for 24 h. (A) IL-6 and (B) TNF production was measured by ELISA. (C) COX2 expression was detected by Western blot, and chemiluminescent signal was quantified and normalized to actin. COX2 levels are expressed as percentage, LPS-induced cells transfected with siNEG set as 100%, and the other values are related to that value. The results are expressed as mean ± S.E.M., $n = 6$. One-way ANOVA with Bonferroni's post test was performed, and statistical significance is indicated as $^{*}p < 0.05$ and $^{***}p < 0.001$.

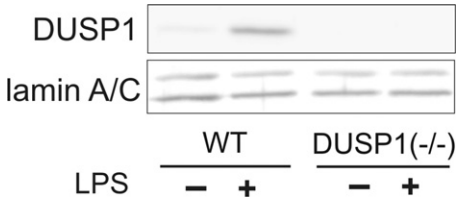

Fig. 4. The expression of DUSP1 protein in response to LPS in BMMs from wild-type and DUSP1 deficient mice. BMMs isolated from wild-type and DUSP1 deficient mice were stimulated with LPS (10 ng/ml) for 1 h, and DUSP1 expression was detected by Western blot using lamin A/C as a loading control. The experiment was repeated three times with similar results.

for 24 h and transfected then with DUSP1 siRNA or non-targeting siRNA followed by 48 h incubation. Thereafter fresh culture medium was changed, and cells were further incubated with or without LPS for the time indicated, and gene expression was analyzed. All the experiments were done in triplicate. Transfection efficacy was monitored with siGLO Green Transfection Indicator. More than 95% of the cells incubated with siGLO and DharmaFECT 4 emitted green fluorescence signal, while less than 5% of the cells incubated with siGLO oligos alone emitted green fluorescence signal after 6 h of incubation.

2.8. Enzyme-linked immunosorbent assay (ELISA)

Culture medium samples were kept at −20 °C until assayed. The concentrations of TNF and IL-6 (DuoSet® ELISA, R&D Systems Europe Ltd., Abindgon, UK) were determined by ELISA according to the manufacturer's instructions.

2.9. Statistics

Results are expressed as the mean ± S.E.M. Data presented as either fold-change or as percentages were log-transformed before statistical analyses. When appropriate, unpaired *t*-test, or one-way ANOVA with Dunnett's or Bonferroni's post test was performed using GraphPad InStat version 3.05 for Windows 95/NT (GraphPad Software, San Diego, CA, USA). Differences were considered significant at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

3. Results

3.1. Regulation of inflammatory gene expression by DUSP1 in mouse macrophages

Low-level basal DUSP1 expression was observed in unstimulated J774 macrophages. LPS (10 ng/ml) increased DUSP1 protein expression at time point of 1 h, and it gradually declined during 4 h follow-up (Fig. 1). The effect of DUSP1 on inflammatory gene expression was investigated by siRNA. DUSP1 mRNA and protein expression were effectively down-regulated by siRNA in J774 macrophages (Fig. 2). Down-regulation of DUSP1 by siRNA resulted in increased production of IL-6 and TNF as well as enhanced expression of COX2 in response to LPS in J774 macrophages (Fig. 3).

The effect of DUSP1 on inflammatory gene expression was also studied in primary mouse bone marrow-derived macrophages (BMMs) from wild-type and DUSP1(−/−) mice. First, the expression of DUSP1 in BMMs was investigated. DUSP1 protein was expressed in LPS-stimulated BMMs from wild-type animals, and, as expected, DUSP1 could not be detected in BMMs from DUSP1(−/−) mice (Fig. 4). LPS induced the production of IL-6 and TNF as well as the expression of COX2, and those were enhanced in BMMs from DUSP1(−/−) animals as compared to BMMs from wild-type mice

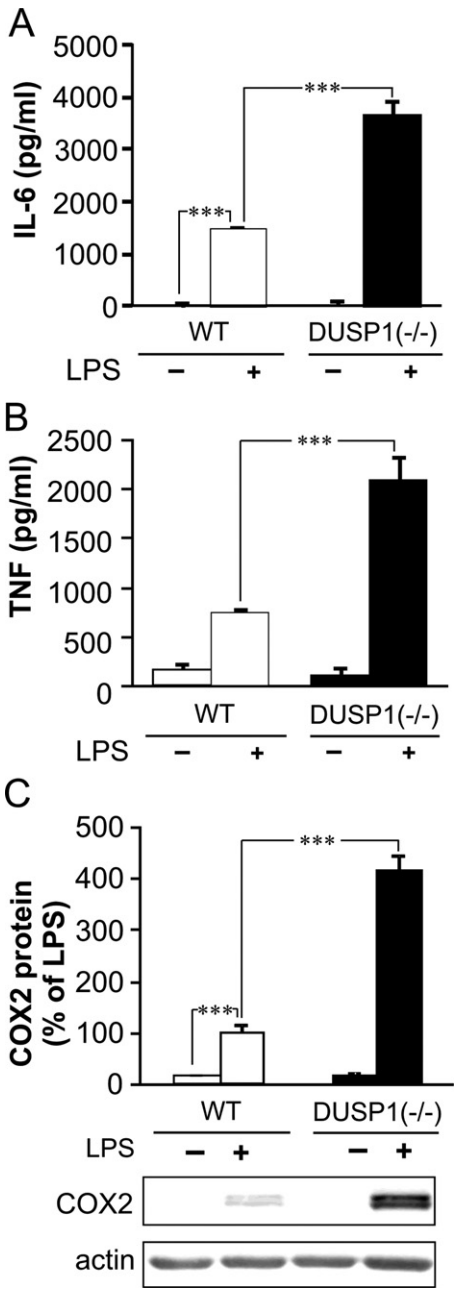

Fig. 5. The effect of DUSP1 on IL-6 and TNF production and COX2 expression in primary mouse macrophages. Bone marrow-derived macrophages from wild type (DUSP1(+/+)) and DUSP1 deficient (DUSP1(−/−)) mice were incubated with LPS (10 ng/ml) for 24 h. (A) IL-6 and (B) TNF production was measured by ELISA. (C) COX2 expression was detected by Western blot. Chemiluminescent signal was quantified and COX2 expression was normalized to actin. COX2 levels are expressed as percentages, LPS-induced cells transfected with siNEG set as 100%, and the other values are related to that value. The results are expressed as mean ± S.E.M., $n = 3$. One-way ANOVA with Bonferroni's post test was performed, and statistical significance is indicated as $^{***}p < 0.001$.

(Fig. 5). These results reveal that DUSP1 could negatively regulate the expression of proinflammatory factors in macrophages following LPS stimulation.

3.2. The effect of DUSP1 on carrageenan-induced paw inflammation in mice

The effect of DUSP1 on the development of acute inflammatory response *in vivo* was investigated by measuring the severity

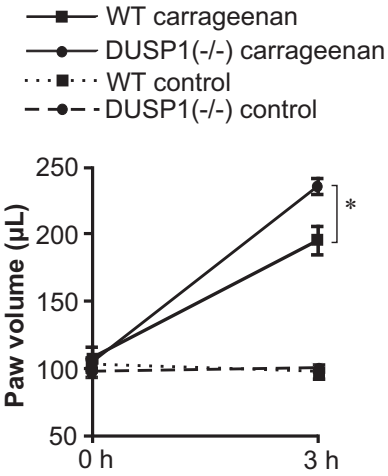

Fig. 6. The effect of DUSP1 on carrageenan-induced acute paw inflammation. In the beginning of the experiment (0 h), hind paw volumes were measured with a plethysmometer. Carrageenan or vehicle was injected intradermally, and paw volumes were measured after 3 h with a plethysmometer. The results are expressed as mean ± S.E.M., $n = 6$. Unpaired Students' t-test was performed, $^{*}p < 0.05$.

of carrageenan–induced paw inflammation in DUSP1(−/−) and wild-type mice. In DUSP1(−/−) mice, carrageenan-induced inflammatory paw edema was increased by 36% as compared to that seen in wild-type mice (Fig. 6). These results indicate that DUSP1 also was a suppressive factor during the acute inflammatory process *in vivo*.

3.3. The effect of DUSP1 on the phosphorylation of p38 MAPK and JNK in mouse macrophages

DUSP1 has been reported to dephosphorylate and thereby, inhibit the activity of p38 MAPK and, in some cells, JNK (Zhao et al., 2005; Chi et al., 2006; Hammer et al., 2006; Turpeinen et al., 2010). Therefore, the effect of DUSP1 on the phosphorylation of p38 MAPK and JNK in response to LPS was investigated in J774 macrophages and BMMs. The cells were stimulated with LPS for 0–4 h, and the levels of phosphorylated p38 and JNK were estimated by Western blot. The phosphorylation of p38 MAPK and JNK p54 and p46 isoforms was increased at 30 min after LPS stimulation, and returned near the basal level at 1 h and remained at that level during the 4 h follow-up in J774 cells (Fig. 7). In BMMs, the phosphorylation of JNK p54 and p46 peaked at 30 min and returned near the basal level, although it was still elevated, at 1 h. A statistically significant increase in the phosphorylation of p46 isoform was observed at 2 h, also.

Next, the effect of DUSP1 on the phosphorylation of p38 MAPK and JNK in J774 macrophages was investigated by siRNA. Down-regulation of DUSP1 by siRNA resulted in enhanced p38 MAPK phosphorylation in response to LPS, but the phosphorylation of JNK isoforms p46 and p54 was not altered in J774 cells (Fig. 8A). The effect of DUSP1 on p38 MAPK and JNK phosphorylation was also investigated in BMMs from wild-type and DUSP1(−/−) mice, also. LPS increased p38 MAPK phosphorylation in BMM from wild-type mice, and it was further enhanced in BMMs from DUSP1(−/−) mice (Fig. 8B). The level of LPS-induced phosphorylation of JNK p46 was not altered in BMMs from DUSP1(−/−) mice as compared to that detected in BMMs from wild-type mice. The phosphorylation of JNK p54 in response to LPS was found to be lower/reduced in BMMs from DUSP1(−/−) as compared to that in BMMs from wild-type mice (Fig. 8B).

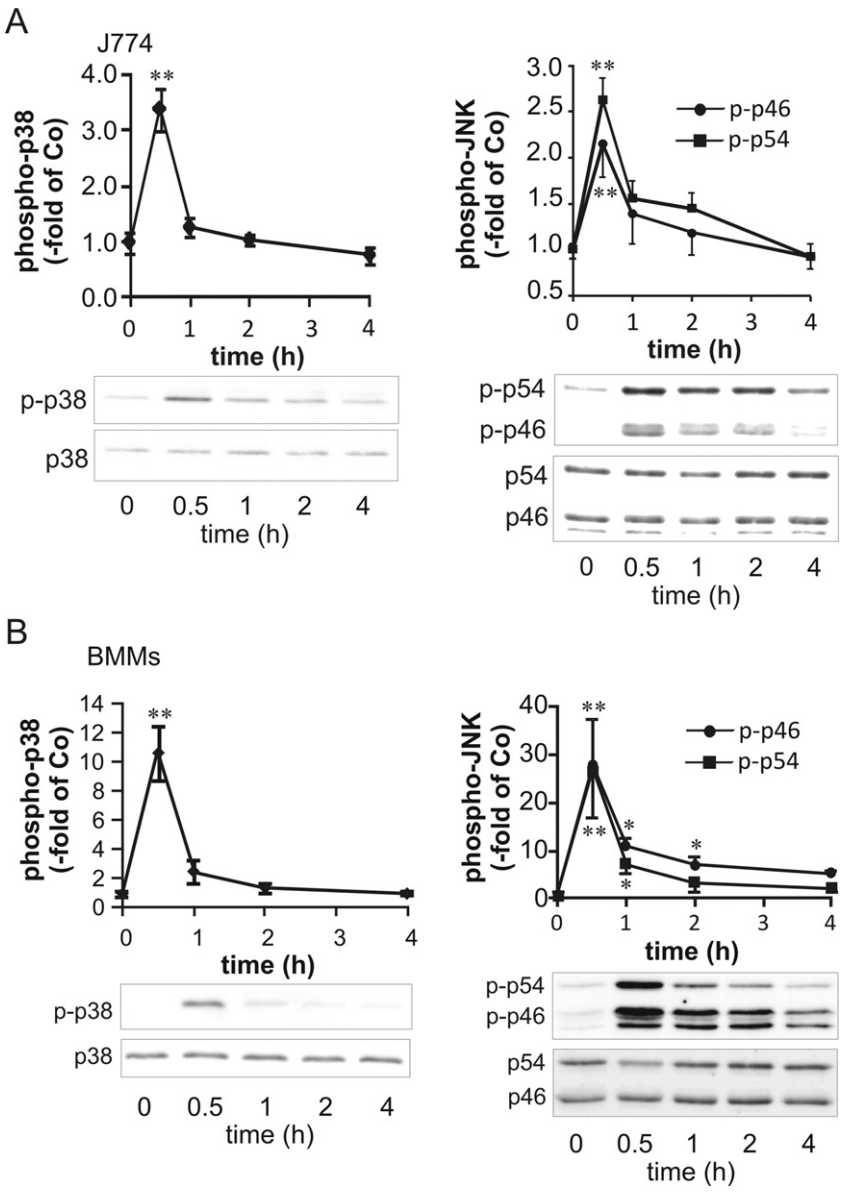

Fig. 7. Phosphorylation of p38 MAPK and JNK in response to LPS in mouse macrophages. J774 cells and bone marrow-derived macrophages (BMMs) were stimulated with LPS (10 ng/ml) for the time indicated, and cells were harvested for protein extraction. The phosphorylation of p38 MAPK and JNK was detected by Western blot in (A) J774 cells and (B) BMMs. The chemiluminescent signal was quantified and the amount of phosphorylated p38 MAPK or JNK was normalized against total p38 MAPK or JNK, respectively. Protein phosphorylation levels are expressed in arbitrary units, unstimulated cells set as 1, and the other values are related to that value. The results are expressed as mean ± S.E.M.; $n = 6$ in J774 cells and $n = 3$ in BMMs. One-way ANOVA with Dunnett's post test was performed, and statistical significance was indicated as $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to unstimulated cells.

3.4. Inhibition of LPS-induced expression of IL-6, TNF and COX2 by p38 MAPK inhibitors in mouse macrophages

p38 MAPK is known to regulate inflammatory gene expression (Burnette et al., 2009; Hope et al., 2009). In our experiments, DUSP1 deficiency increased p38 MAPK phosphorylation and inflammatory gene expression. p38 MAPK inhibitors SB202190 and BIRB 796 have been shown to effectively inhibit p38 MAPK function at drug concentrations of 1 μM and 100 nM, respectively (Bain et al., 2007; Turpeinen et al., 2010). Therefore, we used BIRB 796 and SB 202190 at these concentrations to clarify the role of p38 MAPK on IL-6, TNF and COX2 expression in J774 macrophages and primary mouse peritoneal macrophages isolated from wild type animals. Two p38 MAPK inhibitors, BIRB 796 and SB202109, markedly inhibited LPS-induced IL-6 production and COX2 expression in J774 macrophages (Fig. 9). BIRB 796 inhibited LPS-induced expression of TNF and COX2 mRNA in primary mouse peritoneal macrophages isolated from wild type mice, also. Since JNK p54 phosphorylation was inhibited in BMMs from DUSP1(−/−) mice in comparison to that from wild-type mice, we investigated the effect of JNK inhibitor VIII on the expression of TNF and COX2 mRNA in primary mouse peritoneal macrophages. JNK inhibitor VIII (1 μM) effectively inhibited the phosphorylation of a direct JNK substrate c-Jun (Supplemental Fig. 1) (Szczepankiewicz et al., 2006; Turpeinen et al., 2010). A JNK VIII inhibitor did not inhibit the expression of TNF or COX2 mRNA in primary mouse peritoneal macrophages in response to LPS (Fig. 10). These results indicate that DUSP1 had negatively regulated inflammatory gene expression by limiting p38 MAPK.

4. Discussion

The present study investigated the effect of DUSP1 on acute inflammatory. Here, we report that DUSP1 inhibited the phosphorylation of p38 MAPK, suppressed inflammatory responses in

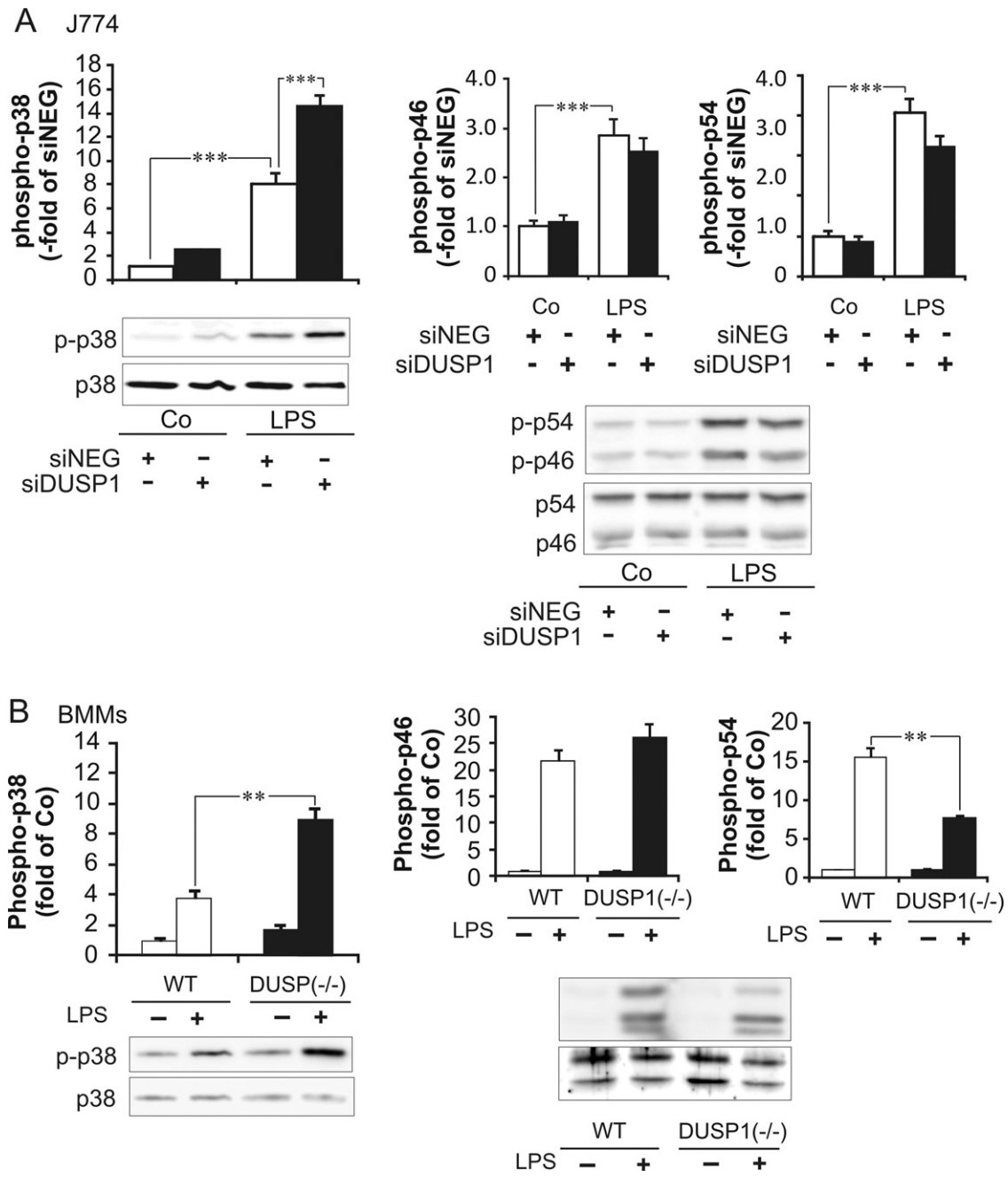

Fig. 8. The effect of DUSP1 on LPS-induced phosphorylation of p38 MAPK and JNK in mouse macrophages. (A) J774 cells were transfected either with DUSP1 siRNA (siDUSP1) or non-targeting siRNA (siNEG) and stimulated with LPS (10 ng/ml) for 1 h. p38 MAPK and JNK phosphorylation was detected by Western blot. The chemiluminescent signal was quantified and the amount of phosphorylated p38 MAPK or JNK was normalized against total p38 MAPK or JNK, respectively. Protein phosphorylation levels are expressed in arbitrary units, unstimulated cells set as 1, and the other values are related to that value. The results are expressed as mean ± S.E.M., $n = 6$. One-way ANOVA with Bonferroni's post test was performed, and statistical significance is indicated with $^{**}p < 0.01$ and $^{***}p < 0.001$. (B) Bone marrow-derived macrophages (BMMs) were stimulated with LPS (10 ng/ml) for 30 min, and p38 MAPK and JNK phosphorylation was detected by Western blot. The chemiluminescent signal was quantified and phosphorylated p38 MAPK or JNK was normalized against total p38 MAPK or JNK, respectively. Protein phosphorylation levels are expressed in arbitrary units, unstimulated wild-type cells set as 1, and the other values are related to that value. The results are expressed as mean ± S.E.M., $n = 3$. Unpaired Students' t-test was performed, and statistical significance is indicated as $^{**}p < 0.01$.

macrophages, and reduced the severity of carrageenan-induced paw inflammation.

DUSP1 is an inducible nuclear tyrosine/threonine phosphatase that has been shown to primarily regulate the phosphorylation of p38 MAPK and, in some cells, JNK (Zhao et al., 2005; Chi et al., 2006; Hammer et al., 2006; Turpeinen et al., 2010). In our experiments, p38 MAPK phosphorylation was enhanced in macrophages from DUSP1(−/−) mice and in J774 macrophages, in which DUSP1 expression had been silenced with siRNA. JNK phosphorylation was not increased by DUSP1 siRNA in J774 macrophages or in BMMs from DUSP1(−/−) mice. Unexpectedly, the phosphorylation of the JNK p54 (but not the p46) isoform in response to LPS was lower in DUSP1(−/−) than in wild type BMMs. This may be due to a compensatory increase in the expression of the other DUSP(s) that

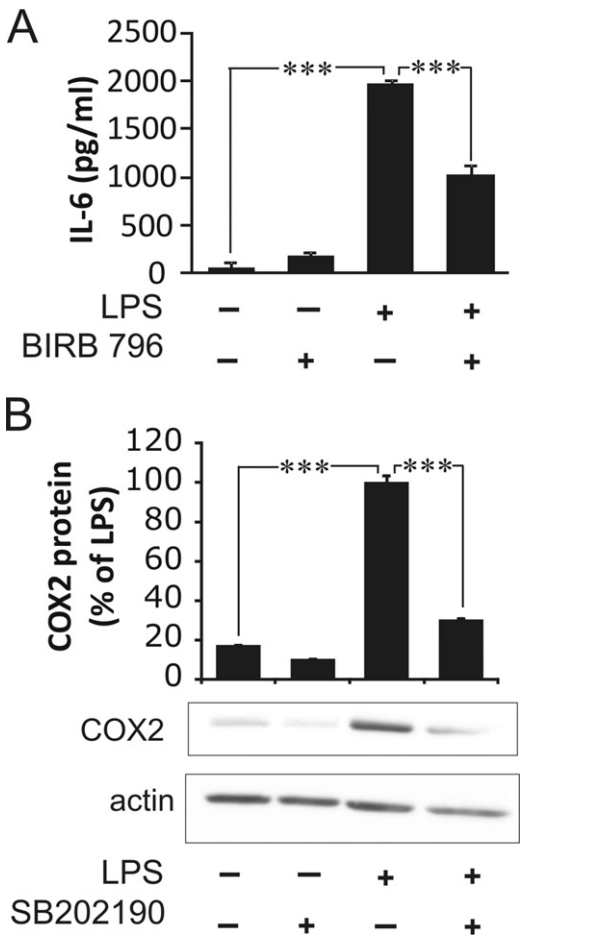

Fig. 9. The effect of p38 MAPK inhibitors BIRB 796 and SB202190 on IL-6 production and COX2 expression in response to LPS in J774 mouse macrophages. J774 cells were preincubated with BIRB 796 (100 nM) or SB202190 (1 μM) for 30 min and stimulated with LPS (10 ng/ml) for 24 h in the presence of the p38 inhibitors. (A) IL-6 production was measured with ELISA and the results are expressed as mean ± S.E.M., $n = 6$. (B) COX2 protein expression was detected with Western blot. The chemiluminescent signal was quantified and COX2 expression was normalized to that of actin. The results are expressed as a percentage of LPS, mean ± S.E.M., $n = 6$. One-way ANOVA with Bonferroni's post test was performed, and statistical significance is indicated as $^{***}p < 0.001$ compared to LPS-treated cells.

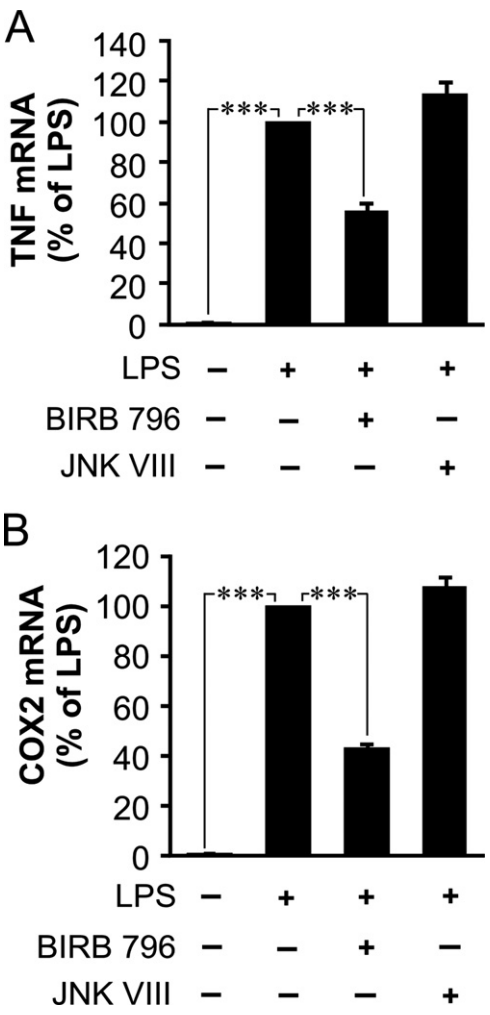

Fig. 10. The effect of p38 MAPK inhibitor BIRB 796 and JNK inhibitor VIII on TNF and COX2 mRNA expression in response to LPS in primary mouse peritoneal macrophages. Peritoneal macrophages were preincubated with BIRB 796 (100 nM) or JNK inhibitor VIII (1 μM) for 30 min and stimulated with LPS (100 ng/ml) for 3 h in the presence of the inhibitors. Total RNA was extracted and (A) TNF and (B) COX2 mRNA expression was analyzed by quantitative RT-PCR. mRNA expression levels are expressed in arbitrary units, LPS-stimulated cells set as 100%, and the other values are related to that value. The results are expressed as mean ± S.E.M., $n = 4$. One-way ANOVA with Bonferroni's post test was performed, and statistical significance is indicated as $^{***}p < 0.001$.

regulate JNK phosphorylation. The DUSPs that primarily regulate JNK phosphorylation include the subtypes DUSP4, DUSP8, DUSP10 and DUSP16 (Boutros et al., 2008).

p38 MAPK inhibitors have been shown to inhibit inflammatory gene expression in macrophages (Branger et al., 2002; Burnette et al., 2009; Hope et al., 2009), and this was also confirmed in the present study. We also found that a deficiency in DUSP1 function increased the production of inflammatory genes in macrophages *in vitro* and enhanced acute carrageenan-induced paw edema reaction *in vivo*. Carrageenan-induced paw edema is an acute inflammatory response model. In tissues, carrageenan causes an accumulation of inflammatory cells into the tissue, and evokes the release of many inflammatory mediators, *e.g.* bradykinin, histamine, prostaglandins, reactive nitrogen and oxygen species, as well as inducing the expression COX2, TNF, IL-6 and IL-1β (Morris, 2003; Rocha et al., 2006; Loram et al., 2007). In our experiments, the severity of the carrageenan-induced paw edema was greater in DUSP1(−/−) mice than encountered in wild-type mice, and therefore, DUSP1 seems to attenuate the acute carrageenan-induced inflammatory response. Previously, carrageenan and carrageenan-induced inflammatory factors have been shown to cause endothelial barrier dysfunction (Bucci et al., 2005). Interestingly, DUSP1 has been shown to limit the increase in vascular permeability and the development of TNF-induced endothelial barrier dysfunction through a mechanism related to the inhibition of p38 MAPK (Kiemer et al., 2002; Yang et al., 2010). Although LPS-induced phosphorylation of JNK p54 was reduced in DUSP1(−/−) BMMs, a JNK inhibitor VIII, at an effective concentration, did not alter the expression of TNF or COX2 mRNA in primary mouse peritoneal macrophages. This indicates that the altered JNK p54 phosphorylation/activity was not responsible for the enhanced expression of IL-6, TNF and COX2 in BMMs from DUSP1(−/−) mice or for the increased carrageenan-induced paw inflammation response in the DUSP1(−/−) mice.

It has been reported that p38 MAPK inhibitors can reduce the inflammatory response to LPS in humans, and that the suppression of inflammatory response correlates with the inhibition of p38 MAPK activity (Branger et al., 2002; Burnette et al., 2009; Hope et al., 2009). p38 MAPK inhibitors have also been shown to attenuate inflammation and impair the development of joint destruction and bone erosion in murine models of arthritis (Burnette et al., 2009; Hope et al., 2009) as well as attenuating carrageenan-induced

inflammation and TNF and prostaglandin production (Nishikori et al., 2002).

DUSP1 is expressed in response to LPS or inflammatory cytokines, and it serves as an endogenous mechanism to limit MAPK activity and the inflammatory response (Zhao et al., 2005; Chi et al., 2006; Hammer et al., 2006). DUSP1 deficient mice respond to LPS by increased production of inflammatory factors and show higher mortality rates following systemic LPS administration (Chi et al., 2006; Hammer et al., 2006; Zhao et al., 2006). Despite the enhanced inflammatory response, DUSP1 deficient mice have an increased bacterial burden in septic peritonitis (Hammer et al., 2010) and *Chlamydophila pneumonia* lung infection (Rodriguez et al., 2010). Thus, DUSP1 seems to augment the clearance of pathogenic microbes while suppressing excessive inflammatory response. However, reduced expression of DUSP1 may be beneficial in protozoan parasite infection, since in experimental visceral leishmaniasis, increased macrophage activity and a reduced organ parasite burden were associated with suppressed DUSP1 expression (Kar et al., 2010).

In chronic inflammatory diseases, the modulation of DUSP1 expression or its function may provide an alternative way to modulate and/or suppress the inflammatory process. There is already some evidence in support of this kind of treatment modality. Anti-rheumatic gold compounds were recently found to increase DUSP1 expression in chondrocytes and cartilage, and DUSP1 has been shown to be involved in mediating the anti-inflammatory effects of the gold compounds (Nieminen et al., 2010). Furthermore, glutamine administration has been reported to protect mice from lethal endotoxic shock, and this was probably mediated by expression and function of DUSP1 (Ko et al., 2009). DUSP1 gene transfer has recently been shown to suppress osteoclastogenesis in macrophages, to reduce the accumulation of inflammatory cells in bone and to attenuate the inflammatory bone loss in response to LPS (Yu et al., 2010). Glucocorticoids, a group of potent and widely used anti-inflammatory drugs, are known to enhance DUSP1 expression (Kassel et al., 2001; Shipp et al., 2010), and the anti-inflammatory effects of glucocorticoids have been shown to be mediated, at least in part, by glucocorticoid-induced expression of DUSP1 *in vitro* and *in vivo* (Abraham et al., 2006; Wang et al., 2008). Interestingly, in certain clinical states, impaired anti-inflammatory effect of glucocorticoids seems to be associated with reduced DUSP1 expression. Alveolar macrophages from patients with asthma resistant to inhaled steroids show reduced DUSP1 expression and increased p38 MAPK activity in response to glucocorticoids (Bhavsar et al., 2008). In addition, there is evidence that obesity may reduce the responsiveness to inhaled steroids in the treatment of asthma (Peters-Golden et al., 2006), and peripheral blood mononuclear cells from obese/overweight asthmatics displayed a reduced DUSP1 expression in response to dexamethasone (Sutherland et al., 2008).

5. Conclusions

In conclusion, our results show that DUSP1 limits p38 MAPK activity and reduces inflammatory gene expression in macrophages as well as suppressing the acute inflammatory response *in vivo*. DUSP1 thus functions as an endogenous mechanism limiting the inflammatory response. Strategies aimed at increasing DUSP1 expression or enhancing its function may represent potential targets for the development of novel anti-inflammatory therapeutic interventions.

Conflict of interest

The authors declare that they have no competing financial interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molimm.2011.06.439.

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Research Article

Dual Specificity Phosphatase 1 Regulates Human Inducible Nitric Oxide Synthase Expression by p38 MAP Kinase

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The role of dual specificity phosphatase 1 (DUSP1) in inducible nitric oxide synthase (iNOS) expression in A549 human pulmonary epithelial cells, J774 mouse macrophages and primary mouse bone marrow-derived macrophages (BMMs) was investigated. iNOS expression was induced by a cytokine mixture (TNF, IFNγ and IL-1β) in A549 cells and by LPS in J774 cells, and it was inhibited by p38 MAPK inhibitors SB202190 and BIRB 796. Stimulation with cytokine mixture or LPS enhanced also DUSP1 expression. Down-regulation of DUSP1 by siRNA increased p38 MAPK phosphorylation and iNOS expression in A549 and J774 cells. In addition, LPS-induced iNOS expression was enhanced in BMMs from DUSP1$^{(-/-)}$ mice as compared to that in BMMs from wild-type mice. The results indicate that DUSP1 suppresses iNOS expression by limiting p38 MAPK activity in human and mouse cells. Compounds that enhance DUSP1 expression or modulate its function may be beneficial in diseases complicated with increased iNOS-mediated NO production.

1. Introduction

Nitric oxide (NO) is a gaseous signaling molecule that regulates various physiological and pathophysiological processes in many tissues and organ systems. NO is synthesized from L-arginine in a reaction catalyzed by nitric oxide synthase (NOS) enzyme. Three NOS enzyme isoforms exist: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). nNOS and eNOS are constitutively expressed, and, in general, they produce relatively small amounts of NO in the context of physiological regulation of cellular and tissue functions. The expression of iNOS is induced by a number inflammatory and other stimuli, such as inflammatory cytokines, bacterial products, and hypoxia. NO is an important effector molecule in microbicidal host defense, and it serves as a regulatory and proinflammatory molecule in acute and chronic inflammatory responses [1–4].

The expression of iNOS is regulated at transcriptional and posttranscriptional levels. There are considerable differences in the transcriptional regulation of mouse and human iNOS expression. *Mouse* iNOS promoter activity is substantially induced by interferon (IFN)γ and bacteria-derived substances, such as lipopolysaccharide (LPS). iNOS promoter contains two regions responsive to LPS and IFNs. The proximal region is located between -48 and -209 bp upstream of transcriptional start site and contains binding site for nuclear factor κB (NK-κB) and is essential for NF-κB-dependent inducible iNOS promoter activity. The distal region, at position -913 to -1029 bp, contains NF-kB binding site, gamma-activated site (GAS) and two copies of interferon-stimulated response element (ISRE) [5]. Interferon-stimulated gene factor 3 (ISGF3; a heterotrimer of signal transducer and activator of transcription (STAT)1, STAT2, and interferon regulatory factor (IRF)9) bound to the distal responsive element and NF-κB bound to the proximal responsive element have been shown to cooperate to induce iNOS expression [6]. Several other transcription factors have been shown to regulate mouse iNOS transcription including

IRF-1, Octamer factor (Oct-1), activating protein-1 (AP-1), and high-mobility group protein HMG-I(Y) [2, 7].

Transcriptional regulation of *human* iNOS expression shows complexity. Human iNOS promoter shows basal promoter activity, and regulatory elements involved in the cytokine-induced human iNOS transcription are located between −3.8 and −16 kb upstream of the transcriptional start site [7, 8]. A number of transcription factors contribute to human iNOS transcription. NF-κB and STAT1 are considered to be the key transcription factors regulating human iNOS transcription [9, 10]. AP-1 has been reported to have positive and negative effects on human iNOS promoter activity [11, 12]. Several other transcription factors have been shown to be involved in human iNOS transcription including Oct-1, cAMP-responsive element-binding protein, CCAAT-enhancer box-binding protein, STAT3, NF-IL6, and hypoxia-induced factor-1 [7].

Mitogen-activated protein kinases (MAPKs) have been shown to regulate iNOS expression, especially by posttranscriptional mechanisms. iNOS mRNA stability has been shown to be regulated by p38 MAPK and Jun N-terminal kinase (JNK) [13–15]. Other factors involved in the regulation of iNOS expression at posttranscriptional level include transforming growth factor β, glucocorticoids, and inhibitors of calcineurin [16–18]. Proteins that bind to the 3′ untranslated region of iNOS mRNA and regulate iNOS expression at posttranscriptional level include embryonic lethal abnormal visual RNA-binding protein HuR, tristetraprolin, KH-type splicing regulatory protein, and heterogeneous nuclear ribonucleoprotein D and I [13, 19–22].

MAPKs are a group of serine/threonine protein kinases involved in the cellular signal transduction, and the members of this signalling pathway group include p38 MAPK, JNK and p42/44 ERK. They are activated via phosphorylation of specific tyrosine and threonine residues by the upstream kinases. MAPKs regulate various physiological processes, including cell growth, differentiation, and stress responses, and p38 and JNK are associated with the regulation of inflammatory and immune responses [23–25]. There are four p38 MAPK isoforms (p38α, p38β, p38γ, and p38δ), all encoded by separate genes. Especially p38α and p38β have been found to regulate immune response [24–26]. Many different stimuli, including LPS, cytokines and growth factors, activate p38 MAPK pathway [27–31]. The activation of p38 MAPK is involved in the expression of several inflammatory genes, such as tumor necrosis factor (TNF), interleukin(IL)-1, IL-6, IL-8, cyclooxygenase-2 (COX-2) and iNOS [13, 26, 27, 32–35]. p38 MAPK inhibitors have been shown to suppress the expression of inflammatory cytokines, progression of arthritis, and pulmonary fibrosis in animal models and attenuate inflammatory response during endotoxemia in humans [36–38].

Dual specificity phosphatases (DUSPs) are a group of protein phosphatases that dephosphorylate phosphotyrosine and phosphoserine/threonine residues in their target proteins and regulate several intracellular signaling pathways. DUSPs associated with MAPK pathways (at least ten members) differ from each other by substrate specificity, tissue distribution, cellular localization, and expressional pattern [39, 40]. DUSP1 dephosphorylates tyrosine and threonine residues in MAPK Thr-Xaa-Tyr activation motif and thereby inactivates MAPK. DUSP1 has substrate specificity towards p38 and JNK over ERK [41–44]. DUSP1 deficient mice produce elevated levels of inflammatory cytokines and develop more severe NO-mediated hypotensive response and organ failure after administration of LPS or peptidoglycan and lipoteichoic acid [41, 43, 45, 46].

We have previously reported that DUSP1 negatively regulates IL-6, IL-8 and COX-2 expression in A549 human epithelial cells [47]. In addition, we have recently shown that the suppression of the expression of COX-2, matrix metalloproteinase 3 (MMP-3), and IL-6 by antirheumatic drug aurothiomalate in mouse and human chondrocytes and cartilage is mediated by DUSP1 [48]. In the present study, we investigated the effect of DUSP1 on the expression of iNOS in human and murine cells. The main finding was that DUSP1 suppresses iNOS expression by limiting p38 signaling in human cells, which is a novel finding, and this was observed in mouse macrophages also.

2. Materials and Methods

2.1. Materials. Reagents were obtained as follows. BIRB 796 (1-(5-tertbutyl-2-p-tolyl-2H-pyrazol-3-yl)-3(4-(2-morpholin-4-yl-ethoxy)naphthalen-1-yl)urea, Axon MedChem, Groningen, The Netherlands), SB202190 (4-[4-(4-Fluorophenyl)-5-(4-pyridinyl)-1H-imidazol-2-yl] phenol, Tocris Bioscience, Bristol, UK), recombinant human TNF, recombinant human IFNγ, recombinant human IL-1β, recombinant mouse macrophage colony-stimulating factor (M-CSF) (R&D Systems Inc., Minneapolis, Mass, USA), medetomidine (Domitor 1 mg/mL, Orion Oyj, Espoo, Finland), and ketamine (Ketalar 10 mg/mL, Pfizer Oy Animal Health, Helsinki, Finland) were obtained as indicated. All other reagents were purchased from Sigma Chemicals Co. (Saint Louis, Mo, USA) unless otherwise stated below.

2.2. Cell Culture. A549 human lung epithelial cells (ATCC, Manassas, Va, USA) were cultured at 37°C in 5% CO_2 atmosphere in Ham's F12K (Kaighn's modification) medium supplemented with 5% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B (all from Invitrogen, Paisley, UK). J774 macrophages (ATCC, Manassas, Va, USA) were cultured at 37°C in 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium with Ultraglutamine 1 (Lonza, Verviers Sprl, Verviers, Belgium) supplemented with 5% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B.

For experiments, A549 cells (4×10^5 cells/well) were seeded on a 24-well plate and grown for 48 h prior to the experiments. J774 cells (2×10^5 cells/well) were seeded on a 24-well plate and grown for 72 h prior to the experiments. BIRB 796 and SB202190 were dissolved in DMSO. BIRB 796, SB202190 at concentrations indicated, or DMSO (v/v 0.1%) were added to the cells in fresh culture medium containing 5% FBS and antibiotics 30 min prior to the stimulation with a cytokine mixture containing TNF, IFNγ, and IL-1β

(10 ng/mL each) or LPS (10 ng/mL). Cells were further incubated for the time indicated.

2.3. Animals and Isolation and Culture of Bone Marrow Macrophages. Murine bone marrow macrophages (BMMs) were obtained from wild-type and DUSP1$^{(-/-)}$ C57BL/6 mice. Inbred C57BL/6 DUSP1$^{(-/-)}$ mice were originally generated by the R. Bravo laboratory at Bristol-Myers Squibb Pharmaceutical Research Institute [49], and the wild-type mice originated from the same strain. The study was approved by the Animal Care and Use Committee of the University of Tampere and the respective provincial committee for animal experiments. Female mice aged 10–12 weeks were used in the study. The mice were anesthetized by intraperitoneal injection of 0.05 mg/100 g body weight of medetomidine and 7.5 mg/100 g body weight of ketamine. Finally, mice were euthanized by cervical dislocation. Bone marrow cells were obtained by aspiration with sterile syringe needle from femur and fibia. BMMs were generated from bone marrow cells with 5–7 days of incubation in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/mL penicillin, 100 μg/mL streptomycin and 10 ng/mL M-CSF. BMMs (1×10^6 cells/well) were then seeded on a 24-well plate and cultured overnight in complete culture medium. BMMs were then serum-starved overnight. In the beginning of the experiment, LPS was added to the cells along with the culture medium containing 10% FCS and antibiotics, and BMMs were incubated for the time indicated.

2.4. Preparation of Cell Lysates for Western Blot Analysis. At the indicated time points, culture medium was removed. Cells were rapidly washed with ice cold PBS and solubilized in cold lysis buffer containing 10 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl, 1% Triton-X-100, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM sodiumorthovanadate, 20 μg/mL leupeptin, 50 μg/mL aprotinin, 5 mM sodium fluoride, 2 mM sodium pyrophosphate, and 10 μM *n*-octyl-D-glucopyranoside. After incubation for 20 min on ice, lysates were centrifuged (12 000 g, 10 min) and supernatants were collected, mixed in a ratio of 1 : 4 with SDS loading buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0,025% bromophenol blue, and 5% mercaptoethanol) and stored at −20°C until analyzed. Protein concentrations in the samples were measured by the Coomassie blue method.

2.5. Western Blotting. Actin (sc-1616-R), DUSP1 (M-18, sc-1102), lamin A/C (sc-20681), and polyclonal antirabbit (sc-2004) and polyclonal antigoat (sc-2020) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif, USA). Phospho-p38 MAPK (Cat. no. 9218), p38 MAPK (Cat. no. 9212), mitogen-activated protein kinase-activated protein kinase 2 (MK2) (Cat. no. 3042) and phospho-MK2 (27B7, Cat. no. 3007) antibodies (Cell Signaling Technology Inc, Beverly, Mass, USA) were obtained as indicated. Prior to Western blot analysis, the protein samples were boiled for 10 min. Equal aliquots of protein (10–20 μg) were loaded on a 10% SDS-polyacrylamide electrophoresis gel and separated by electrophoresis. Proteins were transferred to Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham, Buckinghamshire, UK) by semidry electroblotting. After transfer, the membrane was blocked in TBS/T (20 mM Tris-base pH 7.6, 150 mM NaCl, and 0.1% Tween-20) containing 5% nonfat milk for 1 h at room temperature. For detection of phospho-proteins, membranes were blocked in TBS/T containing 5% bovine serum albumin. Membranes were incubated overnight at 4°C with the primary antibody and for 1 h with the secondary antibody in room temperature, and the chemiluminescent signal was detected by ImageQuant LAS 4000 mini (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The chemiluminescent signal was quantified with ImageQuant TL 7.0 Image Analysis Software.

2.6. NO Measurement. Cells were incubated with compounds of interest for 24 h. Culture medium was then collected, and nitrite (a stable metabolite of NO in aqueous conditions) levels were measured by the Griess reaction.

2.7. RNA Extraction and Quantitative RT-PCR. Primers and probes for quantitative RT-PCR were obtained from Metabion International AG (Martinsried, Germany). At the indicated time points, culture medium was removed and total RNA extraction was carried out with GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St Louis, Mo, USA) according to the manufacturer's instructions. Total RNA was reverse-transcribed to cDNA using TaqMan Reverse Transcription reagents and random hexamers (Applied Biosystems, Foster City, Calif, USA). cDNA obtained from the RT-reaction was diluted 1: 20 with RNAse-free water and was subjected to quantitative PCR using TaqMan Universal PCR Master Mix and ABI PRISM 7000 Sequence detection system (Applied Biosystems, Foster City, Calif, USA). The primer and probe sequences and concentrations (Table 1) were optimized according to manufacturer's guidelines in TaqMan Universal PCR Master Mix Protocol part number 4304449 revision C. Expression of human Lamin A/C mRNA and human DUSP1 mRNA were measured using TagMan Gene Expression Assays (Applied Biosystems, Foster City, Calif, USA). PCR reaction parameters were as follows: incubation at 50°C for 2 min, incubation at 95°C for 10 min, and thereafter 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. Each sample was determined in duplicate. A standard curve method was used to determine the relative mRNA levels as described in the Applied Biosystems User Bulletin: A standard curve for each gene was created using RNA isolated from A549 cells stimulated with cytokines (TNF, IL-1β, and IFNγ; 10 ng/mL each) and J774 cells stimulated with LPS (10 ng/mL). Isolated RNA was reverse-transcribed, and dilution series of cDNA ranging from 1 pg to 10 ng were subjected to real-time PCR. The obtained threshold cycle values were plotted against the dilution factor to create a standard curve. Relative mRNA levels in test samples were then calculated from the standard curve.

2.8. Downregulation of DUSP1 by siRNA. Human DUSP1 siRNA 1 (Cat. no. J-003484-09-0005) and human DUSP1

Table 1: Primer and probe sequences for quantitative RT-PCR.

Gene	Oligonucleotide	Sequence	Conc. (nM)
Human GAPDH	Forward primer	TCCTACCACCAGCAACCCTGCCA	300
	Reverse primer	GCAACAATATCCACTTTACCAGAGTTAA	300
	Probe	CGCCTGGTCACCAGGGCTGC	150
Human iNOS	Forward primer	GCAGGTCGACTATTTCTTTCA	300
	Reverse primer	TCCTCCTCCGCCTCGTAAGGA	300
	Probe	TCAAGAGCCAGAAGCGCTATCACGAAGATA	150
Mouse GAPDH	Forward primer	GCATGGCCTTCCGTGTTC	300
	Reverse primer	GATGTCATCATACTTGGCAGGTTT	300
	Probe	TCGTGGATCTGACGTGCCGCC	150
Mouse iNOS	Forward primer	CCTGGTACGGGCATTGCT	300
	Reverse primer	GCTCATGCGGCCTCCTT	300
	Probe	CAGCAGCGGCTCCATGACTCCC	150
Mouse DUSP1	Forward primer	CTCCTGGTTCAACGAGGCTATT	300
	Reverse primer	TGCCGGCCTGGCAAT	300
	Probe	CCATCAAGGATGCTGGAGGGAGAGTGTT	150

siRNA 2 (Cat. no. J-003484-10-0005) were purchased from Dharmacon (Dharmacon, Lafayette, Colo, USA). Lamin A/C siRNA (Cat. no. 1022050,) and nontargeting control siRNA (Cat. no. 1022076) were purchased from QIAGEN (QIAGEN, Valencia, Calif, USA). Mouse DUSP1 was silenced using ON-TARGET SMART pool (Dharmacon, Cat. no. L-040753-00-0005). siCONTROL nontargeting siRNA #1 (Dharmacon, Cat. no. D-001210-01) was used as a negative control siRNA in J774 cells.

A549 cells were transfected with siRNA using HiPerFect transfection Reagent (QIAGEN) according to the manufacturer's instructions. Briefly, cells (1.25×10^5 cells/well) were seeded on a 24-well plate in 500 μL of medium with 5% FBS without antibiotics. For one well, 3 μL of siRNA stock solution (2 μM) was mixed with 1.5 μL of transfection reagent in final volume of 100 μL of medium, incubated for 5 min in room temperature, and applied over the cells. Cells were further incubated for 48 h. Fresh culture medium was changed and cytokines were added into the culture medium. Cells were further incubated for the time indicated, and gene expression was analyzed.

J774 cells were transfected with siRNA using DharmaFECT 4 transfection reagent (Dharmacon, Lafayette, Colo, USA) according to the manufacturer's instructions. Briefly, cells (1×10^5 cells/well) were seeded on a 24-well plate in 500 μL of medium with 5% FBS without antibiotics and incubated overnight. For one well, the final transfection medium applied to the cells contained 25 μL of siRNA stock solution (2 μM) mixed with 1 μL of transfection reagent in final volume of 500 μL of medium. Cells were further incubated for 48 h. Fresh culture medium was changed, and LPS was added into the culture medium. Cells were further incubated for the time indicated, and gene expression was analyzed.

Transfection efficacy was monitored with green fluorescent siRNA oligos (siGLO green transfection indicator, Cat. no. D-001630-01, Dharmacon, Lafayette, Colo, USA) using Nikon Eclipse TS100 microscope (Nikon, Tokyo, Japan). Approximately 90% of the cells emitted green fluorescence signal when transfected with siGLO and HiPerFect (A549 cells) or siGLO and DharmaFECT 4 (J774 cells). Less than 5% of the cells emitted signal when cells were incubated siGLO oligos without transfection reagent.

2.9. Statistics. Results are expressed as the mean ± S.E.M. When appropriate, one-way ANOVA with Dunnett's or Bonferroni's post test was performed using GraphPad InStat version 3.05 for Windows 95/NT (GraphPad Software, San Diego, Calif, USA). Differences were considered significant at $^*P < .05$, $^{**}P < .01$, and $^{***}P < .001$.

3. Results

3.1. p38 MAPK Inhibitors SB202190 and BIRB 796 Down-regulated iNOS Expression and NO Production in Response to Inflammatory Stimuli in A549 Cells and J774 Cells. A549 pulmonary epithelial cells and J774 macrophages were stimulated with a cytokine mixture (TNF, IFNγ, and IL-1β; 10 ng/mL each) and LPS (10 ng/mL), respectively, for the time indicated. Cells were then harvested for protein extraction, and the phosphorylation of p38 MAPK was detected by Western blot. p38 MAPK phosphorylation was increased in response to stimulation at time point of 30 min, and it was returned to the basal level in 1 h (Figure 1(a)). p38 MAPK inhibitors SB202190 and BIRB 796 have been reported to effectively inhibit p38 MAPK at 1 μM and 100 nM concentrations, respectively [50]. To confirm the inhibiting effect of SB202190 and BIRB 796 on p38 MAPK activity in the current experimental condition, their effect on the phosphorylation of p38 MAPK substrate MK2 (47 kDa) was investigated in A549 cells and J774 cells. Cells were preincubated with p38 MAPK inhibitors SB202190 or BIRB 796 for 30 min and stimulated with the cytokine mixture or LPS for 30 min. The phosphorylation of MK2 was detected

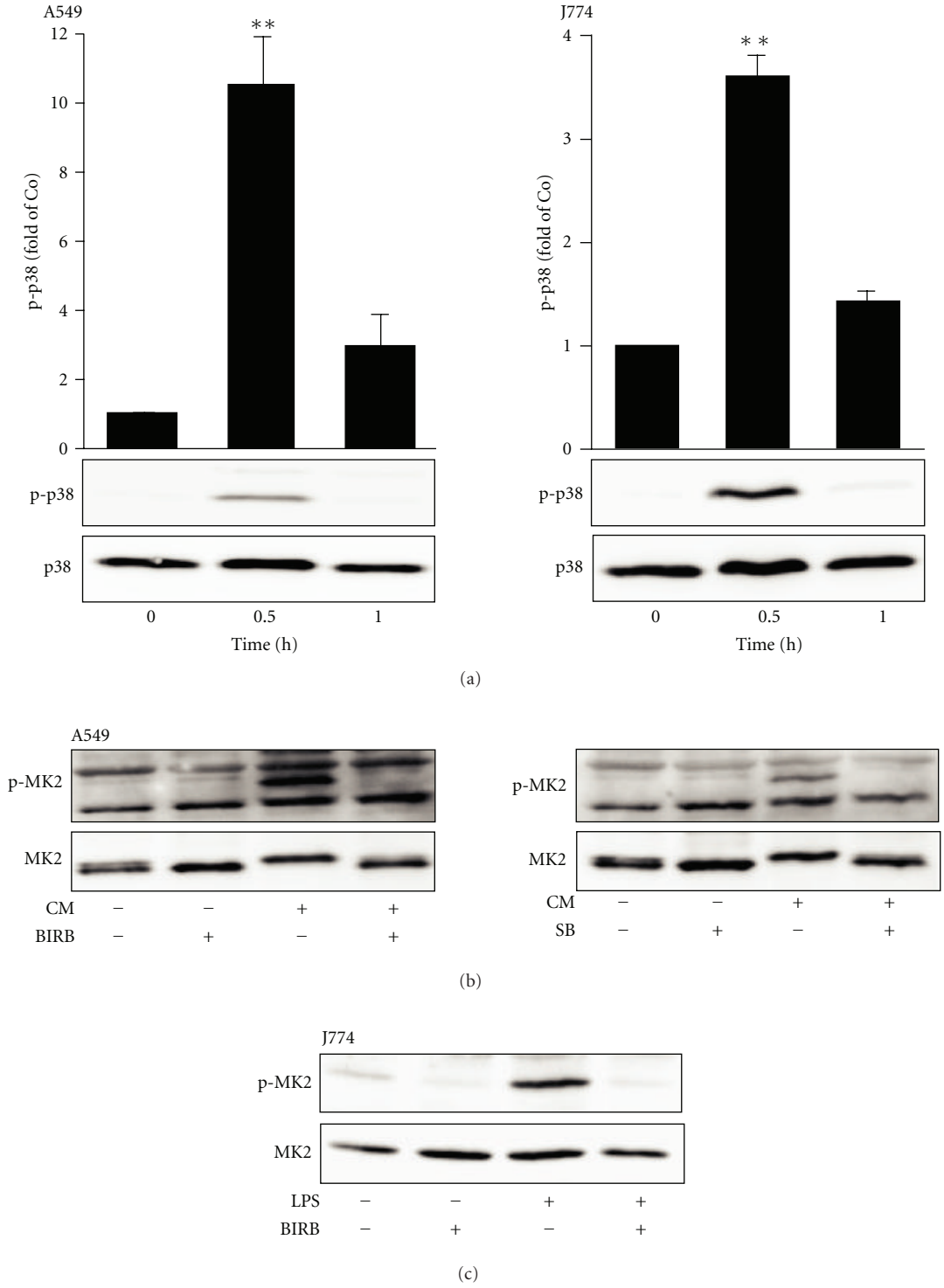


Figure 1: Phosphorylation of p38 MAPK and its substrate MK2 in response to stimulation with cytokine mixture or LPS in A549 and J774 cells. (a) A549 and J774 cells were stimulated with the cytokine mixture (CM: TNF, IFNγ, and IL-1β; 10 ng/mL each) or LPS (10 ng/mL), respectively for the time indicated. Cells were then harvested for protein extraction, and phosphorylation of p38 MAPK was detected by Western blot. The gel is a representative of six separate experiments with similar results. Chemiluminescent signal was quantified, and phosphorylated p38 MAPK was normalized against total p38 MAPK. Phosphorylation levels are expressed in arbitrary units, unstimulated cells set as 1, and the other values are related to that. Results are expressed as mean ± S.E.M.; $n = 6$. One-way ANOVA with Dunnett's posttest was performed, and statistical significance was indicated with $^{**}P < .01$ compared with unstimulated cells. ((b) and (c)) The effect of SB202190 and BIRB 796 on the phosphorylation of MK2 in response to cytokine mixture in A549 and J774 cells. Cells were preincubated with SB202190 (1 μM) or BIRB 796 (100 nM) for 30 min and stimulated with cytokine mixture (A549 cells) or LPS (J774 cells) for 30 min, and the phosphorylation of MK2 was detected by Western blot. The gels are representatives of six separate experiments with similar results.

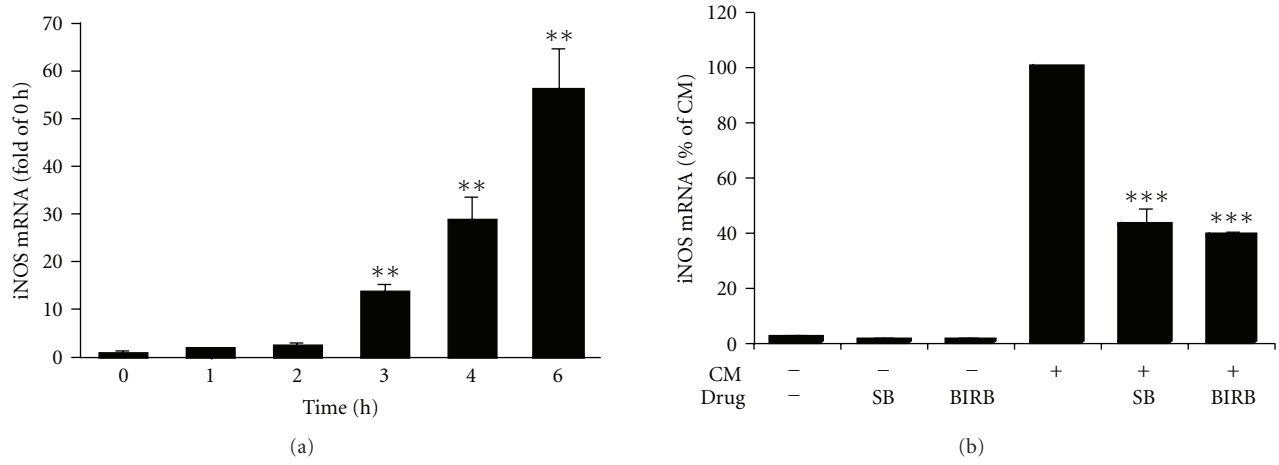


Figure 2: Expression of iNOS mRNA over time and effects of SB202190 and BIRB 796 on the expression of iNOS mRNA in response to cytokine mixture in A549 cells. (a) Cells were stimulated with cytokine mixture (CM: TNF, IFNγ, and IL-1β; 10 ng/mL each) for the time indicated, and total RNA was extracted. The expression of iNOS mRNA was determined by quantitative real-time RT-PCR, and mRNA expression was normalized against GAPDH mRNA. Unstimulated cells (0 h) were set as 1, and other values were related to that. Results are expressed as mean ± S.E.M., $n = 3$. One-way ANOVA with Dunnett's posttest was performed, and statistical significance is indicated with $^{**}P < .01$ as compared to unstimulated cells. (b) Cells were preincubated with SB202190 (1 μM) or BIRB 796 (100 nM) for 30 min, stimulated with cytokine mixture for 6 h, and harvested for total RNA extraction. The expression of iNOS mRNA was determined by quantitative real-time RT-PCR, and mRNA expression was normalized against GAPDH mRNA. Results are expressed as a percentage of CM, mean ± S.E.M., $n = 6$. One-way ANOVA with Bonferroni's posttest was performed, and statistical significance is indicated with $^{***}P < .001$ as compared to cells treated with CM.

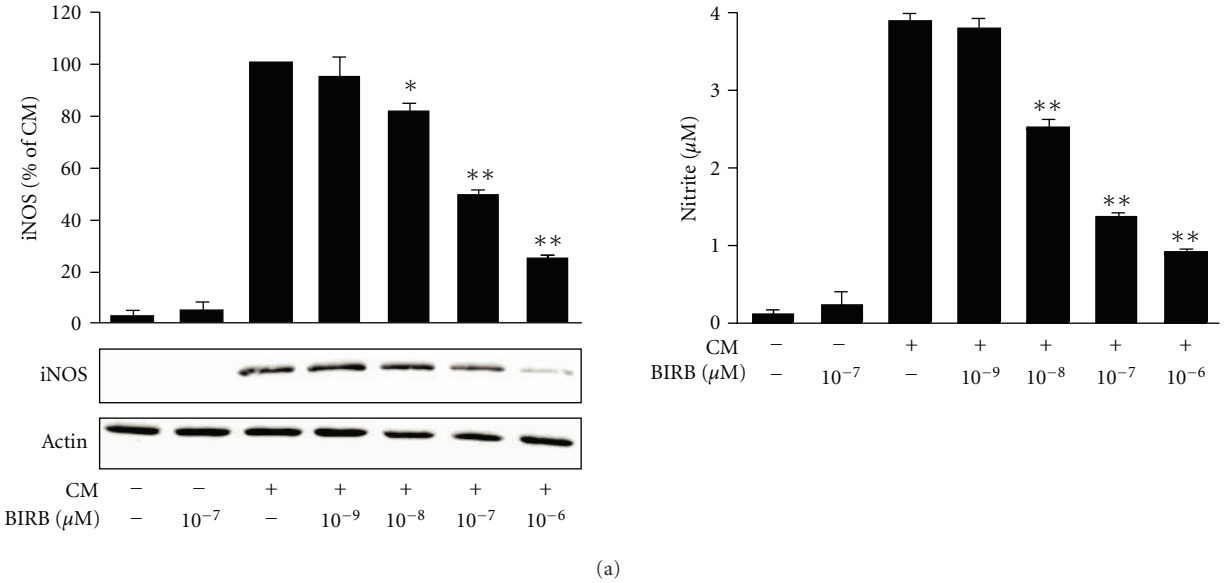
by Western blot. SB202190 and BIRB 796 inhibited MK2 phosphorylation at concentrations of 1 μM and 100 nM, respectively (Figures 1(b) and 1(c)). These results confirmed that SB202190 and BIRB 796, at concentrations used, inhibited p38 MAPK function.

The expression of iNOS mRNA over time was investigated in A549 cells. Cells were stimulated with the cytokine mixture for 0–6 h, and iNOS mRNA levels were measured. iNOS mRNA expression was increased in response to the stimulation with cytokine mixture up to 6 h (Figure 2(a)). The effect of p38 MAPK inhibitors on iNOS mRNA expression was investigated. Cells were preincubated with SB202190 (1 μM) and BIRB 796 (100 nM) for 30 min and stimulated for 6 h. SB202190 and BIRB 796 inhibited the expression of iNOS mRNA in A549 cells (Figure 2(b)).

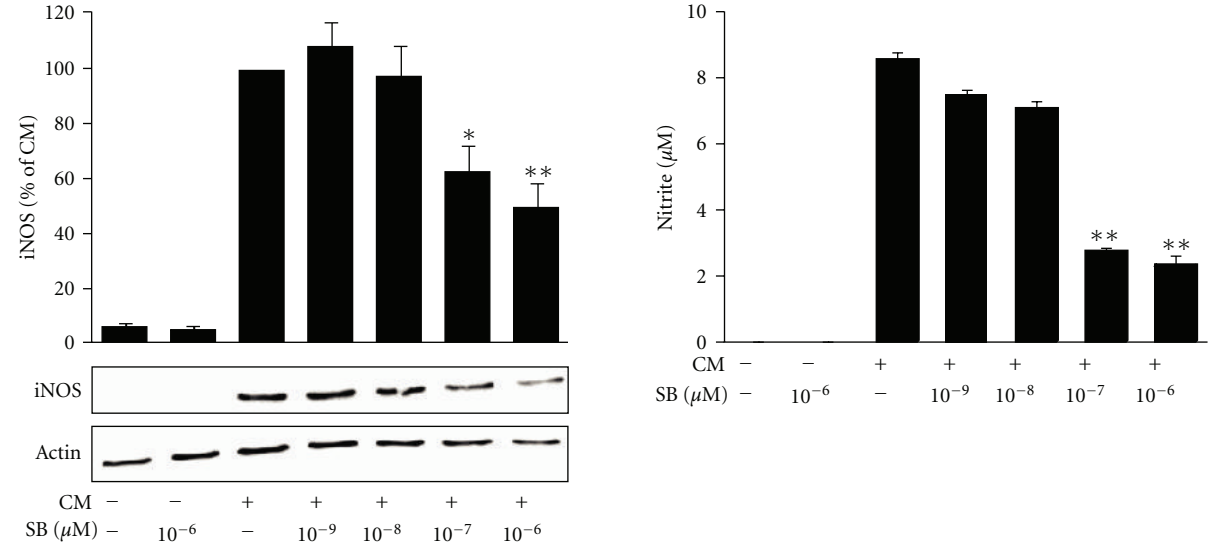
The effects of p38 MAPK inhibitors SB202190 and BIRB 796 on iNOS protein expression and NO production were investigated. Cells were preincubated with SB202190 or BIRB 796 for 30 min and stimulated with the cytokine mixture (A549 cells) or LPS (J774 cells) for 24 h. Supernatants were collected and total cellular proteins were extracted for determination of nitrite production (a stable metabolite of NO in aqueous solution) and iNOS protein expression, respectively. Unstimulated cells did not express detectable levels of iNOS or produce NO, and pretreatment with SB202190 or BIRB 796 alone did not induce iNOS expression or NO production. Stimulation with the cytokine mixture or LPS induced iNOS protein expression and NO production, and SB202190 and BIRB 796 inhibited iNOS expression and NO production in A549 cells (Figure 3) and J774 cells (Figure 4) in a dose-dependent manner.

3.2. DUSP1 Negatively Regulated the Phosphorylation of p38 MAPK. The expression of DUSP1 was investigated in A549 and J774 cells. A549 and J774 cells were stimulated with the cytokine mixture (TNF, IFNγ, and IL-1β) and LPS, respectively, and cells were then harvested for total RNA or protein extraction at the time points indicated. Unstimulated cells showed low-level basal DUSP1 protein (40 kDa) expression. DUSP1 mRNA and protein expression was enhanced by the cytokine mixture (A549 cells) or LPS (J774 cells). The maximal mRNA and protein expression was observed at 1 h after stimulation (Figure 5). DUSP1 mRNA expression was returned to basal level in 2 h in both cell types. DUSP1 protein expression was reduced near the basal level at 2 h and 3 h in A549 and J774 cells, respectively (Figure 5). In DUSP1 Western blots in A549 cells, an immunoreactive band of higher molecular weight was observed. The manufacturer's data sheet suggests this to be DUSP4. The immunoreactive band of higher molecular weight was not reduced by DUSP1 siRNA indicating that it is a molecule different from DUSP1 (Figure 6(a)).

To investigate the effect of DUSP1 on the phosphorylation of p38 MAPK, we used siRNA to downregulate DUSP1 expression. In A549 cells transfected with two DUSP1-specific siRNAs, the protein and mRNA levels of DUSP1 were reduced as compared to the cells transfected with a nontargeting control siRNA showing that siRNA effectively downregulated DUSP1 (Figure 6(a)). The downregulation of DUSP1 by siRNA resulted in an increased p38 MAPK phosphorylation in response to stimulation at 1 h in A549 cells (Figure 6(a)). Lamin A/C siRNA, used as a positive control, downregulated lamin A/C mRNA by approximately



(a)



(b)

Figure 3: The effect of p38 MAPK inhibitors BIRB 796 and SB202190 on iNOS expression and NO production in response to cytokine mixture in A549 cells. A549 cells were preincubated with increasing concentrations of (a) BIRB 796 or (b) SB202190 for 30 min and stimulated with a cytokine mixture (CM: TNF, IFNγ, and IL-1β; 10 ng/mL each) for 24 h. iNOS expression was detected by Western blot. The gels are representatives of six separate experiments with similar results. Chemiluminescent signal was quantified, and iNOS (iNOS) expression was normalized to actin. NO production was measured as nitrite accumulated in the culture medium by Griess reaction. The results are expressed as mean ± S.E.M., $n = 6$. One-way ANOVA with Dunnett's posttest was performed, and statistical significance is indicated with $^{*}P < .05$ and $^{**}P < .01$ compared to cells treated with CM.

67% ($n = 3$, data not shown), but it did not affect DUSP1 expression or p38 MAPK phosphorylation in A549 cells (Figure 6(a)). The effect of DUSP1 siRNA on DUSP1 expression and p38 MAPK phosphorylation was investigated in J774 cells also. DUSP1 siRNA inhibited DUSP1 mRNA and protein expression and enhanced p38 MAPK phosphorylation in J774 cells (Figure 6(b)). These results show that DUSP1 catalyzed the dephosphorylation of p38 MAPK and thereby inactivated p38 MAPK in A549 and J774 cells.

3.3. DUSP1 Negatively Regulated iNOS Expression and NO Production. The effect of down-regulation of DUSP1 on iNOS expression and NO production in response to the cytokine mixture (TNF, IFNγ, and IL-1β; A549 cells) or LPS (J774 cells) was investigated. A549 cells were transfected with two DUSP1-specific siRNA. Cells were then stimulated with the cytokine mixture for 6 h (iNOS mRNA analysis) and 24 h (iNOS protein analysis and NO production). Silencing of DUSP1 by siRNA resulted in increased iNOS mRNA and

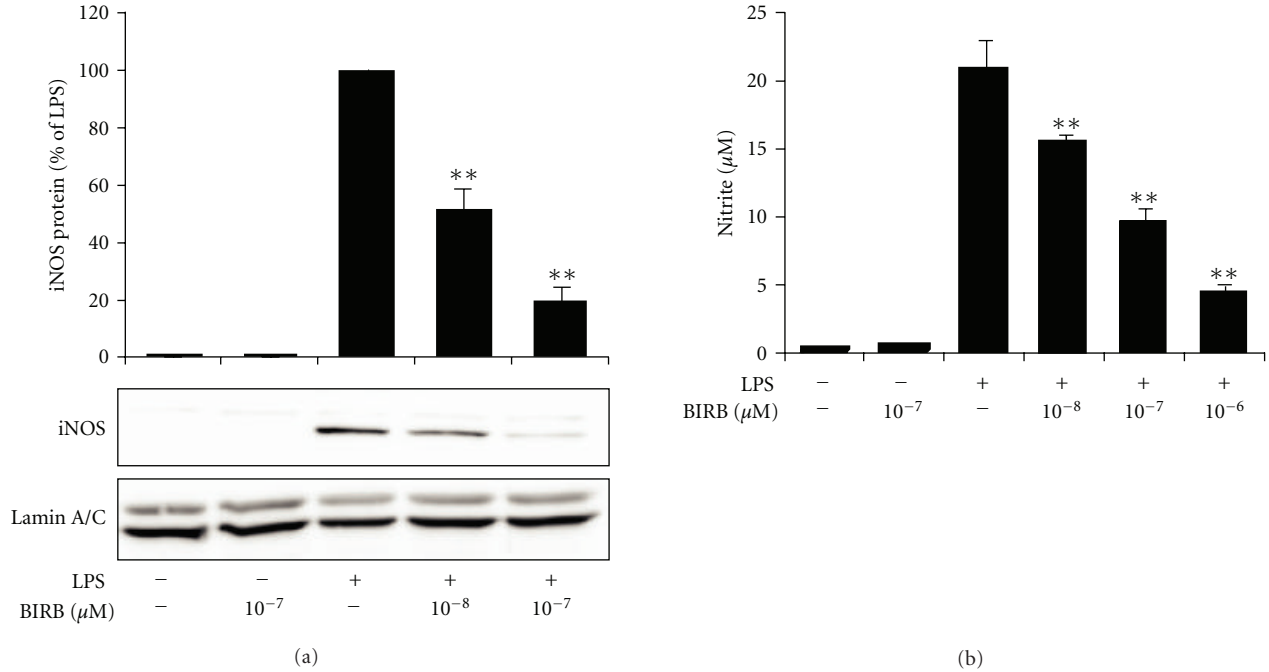


Figure 4: The effect of p38 MAPK inhibitor BIRB 796 on mouse iNOS protein expression and NO production in response to LPS in J774 cells. J774 cells were preincubated with increasing concentrations of BIRB 796 for 30 min and stimulated with LPS (10 ng/mL) for 24 h. iNOS protein expression was detected with Western blot. Chemiluminescent signal was quantified, and iNOS expression was normalized to lamin. The gels are representatives of six separate experiments with similar results. Results are expressed as a percentage of LPS, mean ± S.E.M., $n = 6$. NO production was measured as nitrite accumulated in the culture medium by Griess reaction, and the results are expressed as mean ± S.E.M., $n = 6$. One-way ANOVA with Dunnett's posttest was performed, and statistical significance is indicated with $^{**}P < .01$ compared to LPS-treated cells.

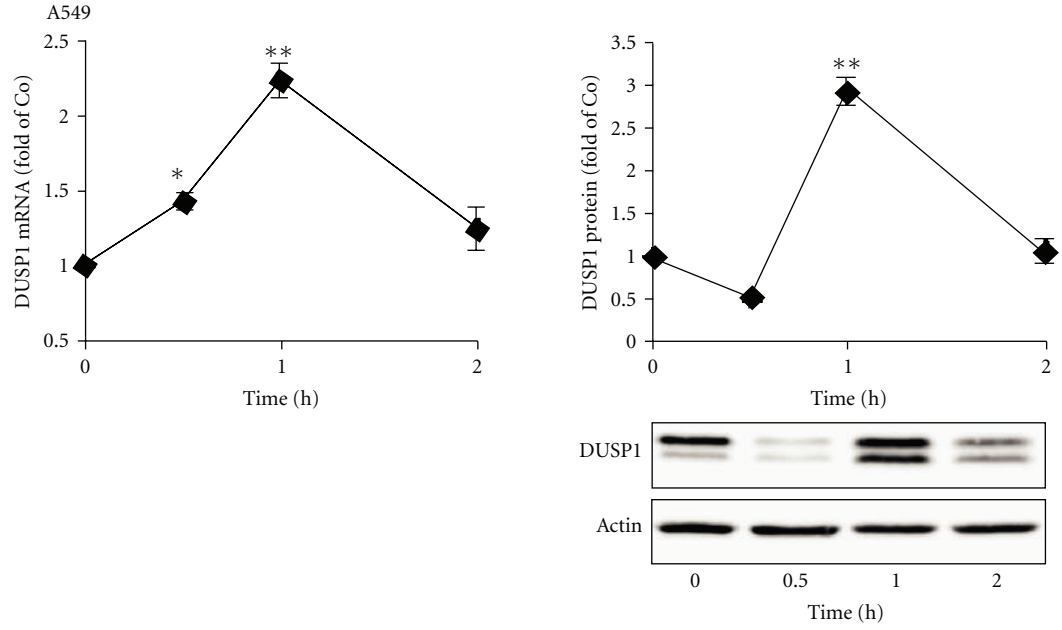
protein expression and NO production in A549 cells (Figures 7(a) and 7(b)). Human iNOS mRNA expression induced by cytokine mixture in untransfected cells was comparable to that seen in transfected cells (Figure 7(a)). Untransfected cells expressed human iNOS protein at somewhat higher level and produced slightly more NO as compared to the transfected cells (Figure 7(b)). Similarly, down-regulation of DUSP1 by siRNA increased iNOS protein expression in J774 cells (Figure 7(c)). In A549 cells, Lamin A/C-specific siRNA did not affect iNOS expression or NO production (Figures 7(a) and 7(b)) although it downregulated lamin A/C expression by about 70%. This strongly suggests that the increased iNOS expression and NO production in A549 cells caused by DUSP1-specific siRNA (Figures 7(a) and 7(b)) were due to the down-regulation of DUSP1 and not to nonspecific effects of siRNA, or general activation of RNA-induced silencing complex (RISC) pathway.

To further confirm the effect of DUSP1 on iNOS expression, the induction of iNOS expression by LPS was investigated in bone marrow macrophages (BMMs) from DUSP1$^{(-/-)}$ and wild-type mice. BMMs were stimulated with LPS (10 ng/mL) for 24 h. Cells were harvested for protein extraction, and iNOS expression was analyzed by Western blot. Unstimulated cells from wild-type and DUSP1$^{(-/-)}$ mice did not express detectable amounts of iNOS. LPS enhanced iNOS expression in BMMs, and LPS-induced iNOS expression was markedly enhanced in BMMs isolated from DUSP1$^{(-/-)}$ mice as compared to cells from wild-type mice (Figure 8).

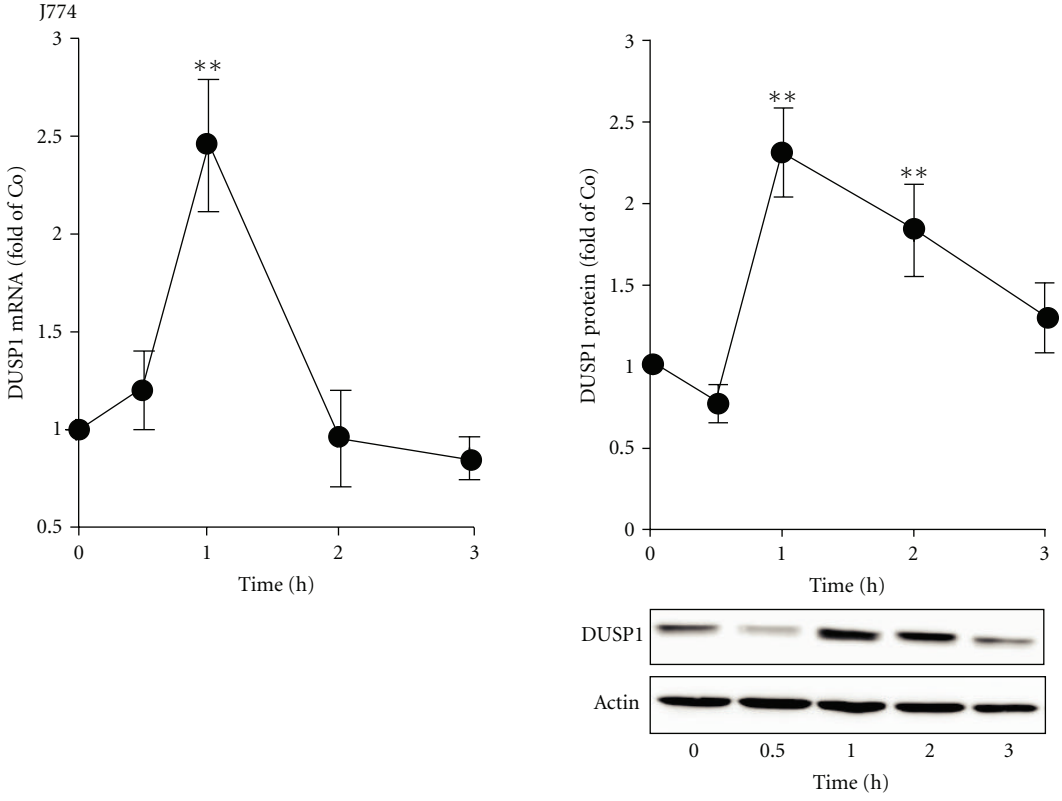
4. Discussion

In the present study, we investigated the effect of DUSP1 on the expression of iNOS and production of NO in response to stimulation with cytokines (TNF, IFNγ, and IL-1β) in human A549 lung epithelial cells and with LPS in murine J774 macrophages and primary mouse BMMs. The main finding of this study was that DUSP1 negatively regulates iNOS expression and NO production by inhibiting the p38 MAPK phosphorylation both in mouse and human cells. This is the first study showing that DUSP1 regulates iNOS expression in human cells.

Structurally distinct p38 MAPK inhibitors SB202190 and BIRB 796 have been reported to inhibit p38 MAPK at concentration range of 100 nM to 1 μM in kinase assays [50]. We have previously reported that SB202190 and BIRB 796 inhibit phosphorylation of MK2 (a p38 MAPK substrate) in a dose-dependent manner with maximal inhibition at 1 μM and 100 nM concentrations, respectively [47]. In the present study, SB202190 and BIRB 796 inhibited MK2 phosphorylation at these concentrations showing that both these inhibitors effectively inhibited p38 MAPK function in A549 and J774 cells. iNOS mRNA expression was reduced by both p38 MAPK inhibitors at these concentrations in A549 cells.



(a)



(b)

Figure 5: Expression of DUSP1 in response to cytokine mixture or LPS in A549 and J774 cells. Cells were stimulated with (a) cytokines (CM: TNF, IFNγ, and IL-1β; 10 ng/mL each) or (b) LPS (10 ng/mL) for the time indicated, and DUSP1 mRNA and protein expression was determined by quantitative real-time RT-PCR and Western blot, respectively. DUSP1 mRNA expression was normalized against GAPDH mRNA. In Western blots, chemiluminescent signal was quantified and DUSP1 protein expression was normalized against actin. The gels are representatives of six separate experiments with similar results. DUSP1 protein and mRNA levels are expressed in arbitrary units, DUSP1 expression in unstimulated cells (0 h) is set as 1, and the other values are related to that (mean ± S.E.M.; $n = 6$). One-way ANOVA with Dunnett's posttest was performed, and statistical significance is indicated with $^{*}P < .05$ and $^{**}P < .01$ compared with unstimulated cells.

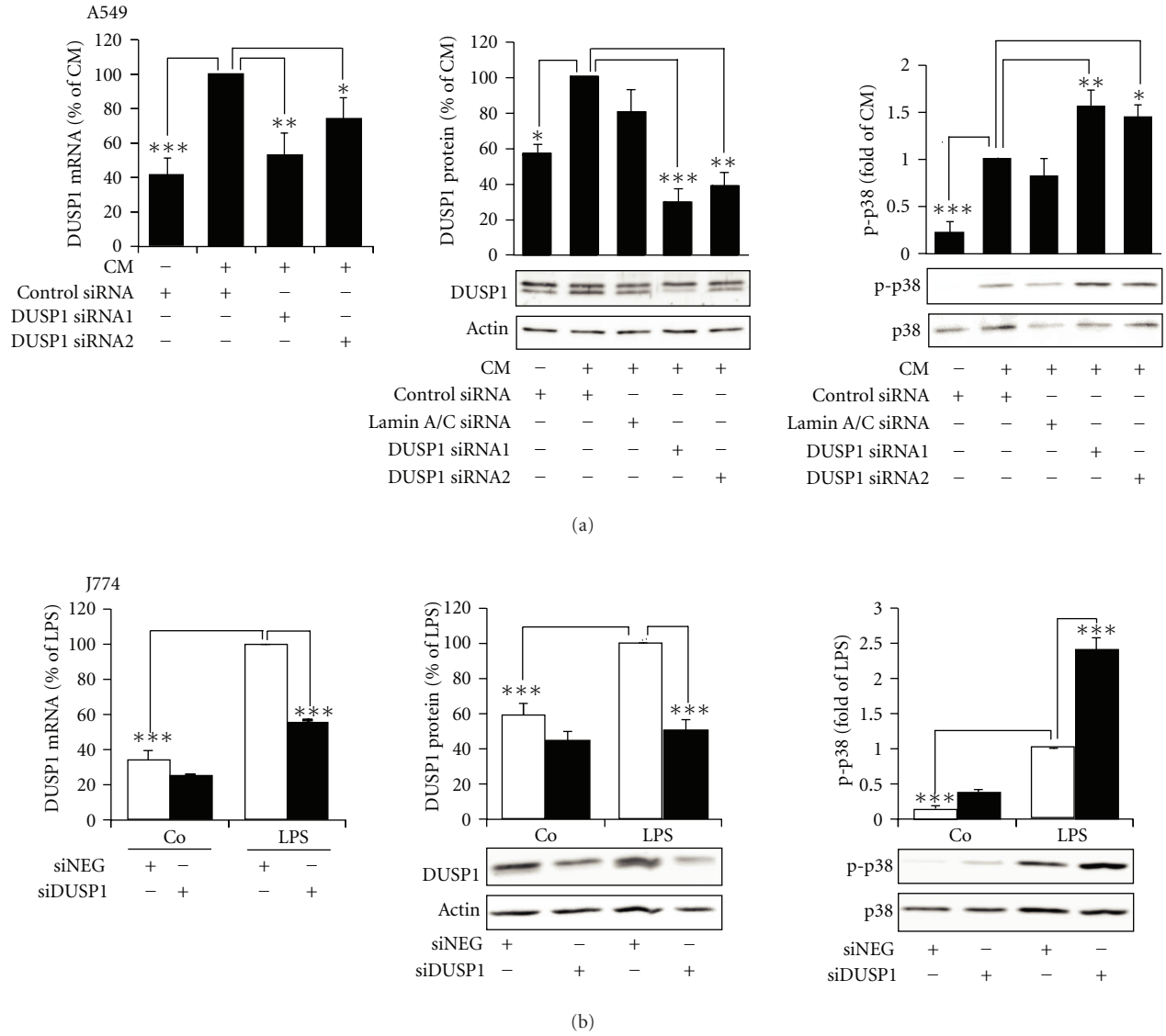


Figure 6: The effect of DUSP1 siRNA on DUSP1 expression and p38 MAPK phosphorylation in A549 and J774 cells. (a) A549 cells were transfected with DUSP1-specific siRNA 1 or 2 (DUSP1 siRNA 1 or 2), nontargeting control siRNA (control siRNA), or Lamin A/C-specific siRNA (Lamin A/C siRNA). (b) J774 cells were transfected with DUSP1-specific siRNA (siMKP-1) or nontargeting siRNA (siNEG). Cells were stimulated with cytokines (CM: TNF, IFNγ, and IL-1β; 10 ng/mL each) or LPS (10 ng/mL) for 1 h. DUSP1 mRNA expression was determined by quantitative real-time RT-PCR and normalized against GAPDH mRNA. DUSP1 protein expression and p38 MAPK phosphorylation were determined by Western blot. Chemiluminescent signal was quantified and DUSP1 expression was normalized against actin, and phosphorylated p38 MAPK was normalized against total p38 MAPK. Results are expressed as a percentage of CM or LPS. One-way ANOVA with Bonferroni's posttest was performed, and statistical significance is indicated with $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$, $n = 6$, except for DUSP1 mRNA in J774 cells, $n = 3$.

Also, iNOS protein expression and NO production were reduced by SB202190 and BIRB 796 in a dose-dependent manner in both A549 and J774 cells. iNOS expression has been reported to be inhibited by a p38 MAPK inhibitor SB203580, a compound structurally related to SB202190, at corresponding concentrations in human cells [13], and by SB203580 and SB202190 in J774 cells [35].

DUSPs are protein phosphatases capable to dephosphorylate tyrosine and threonine/serine residues and, hence, regulate the activity of their target proteins. A subgroup of DUSPs target MAPKs and dephosphorylate tyrosine and threonine residues in MAPKs. Currently, at least ten MAPK-associated DUSPs have been identified and they differ from each other by substrate specificity, tissue distribution, cellular localization, and expressional pattern [40, 44]. DUSP1 is a nuclear phosphatase inducible by LPS and cytokines, and it has substrate specificity towards p38 MAPK and JNK. DUSP1 has been shown to regulate the phosphorylation of p38 MAPK and JNK in primary mouse macrophages [42, 43, 51] and endothelial cells [52]. We have recently

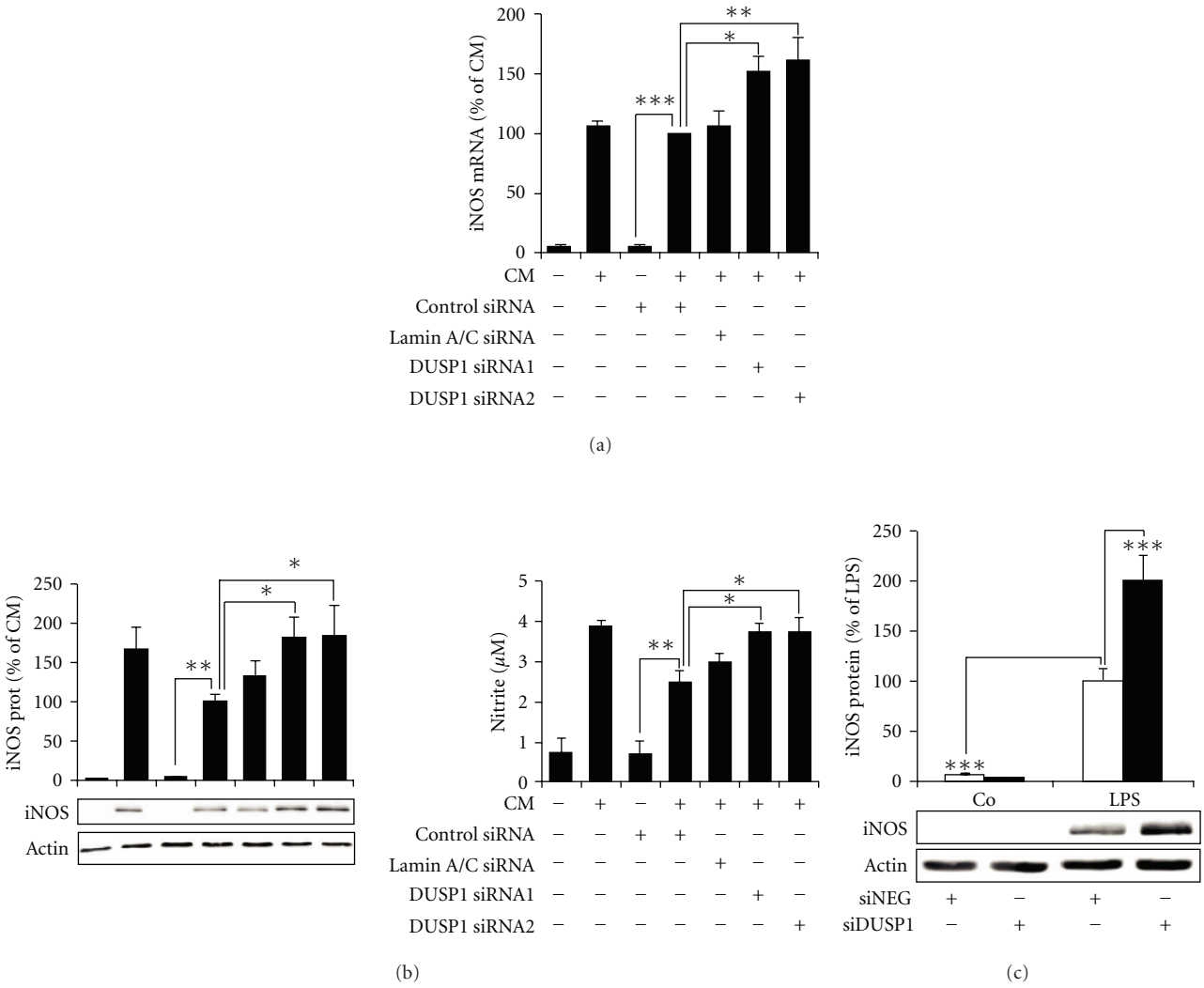


Figure 7: The effect of DUSP1 siRNA on iNOS mRNA and protein expression and NO production in A549 and J774 cells. A549 cells were transfected with DUSP1-specific siRNA 1 or 2 (DUSP1 siRNA 1 or 2), nontargeting control siRNA (control siRNA) or Lamin A/C-specific siRNA (Lamin A/C siRNA), and untransfected cells were used as controls. (a) Cells were stimulated with the cytokine mixture (CM: TNF, IFNγ, and IL-1β; 10 ng/mL each) for 6 h, and iNOS mRNA was determined with quantitative real-time PCR and normalized against GAPDH mRNA. (b) Cells were stimulated with CM for 24 h, and iNOS protein expression was detected by Western blot and NO production as nitrite by Griess reaction. (c) J774 cells were transfected either with DUSP1-specific siRNA (siDUSP1) or nontargeting siRNA (siNEG). Cells were stimulated with LPS (10 ng/mL) for 24 h, and iNOS protein expression was detected by Western blot. In Western blots, chemiluminescent signal was quantified and iNOS expression was normalized against actin. Results are expressed as related to cells treated with CM or LPS. One-way ANOVA with Bonferroni's posttest was performed, and statistical significance is indicated with $^{*}P < .05$; $^{**}P < .01$ and $^{***}P < .001$, $n = 6$.

reported that DUSP1 regulates the phosphorylation of both p38 MAPK and JNK in A549 human pulmonary epithelial cells [47] and p38 MAPK in chondrocytes [48]. In the present study, stimulation with cytokines or LPS enhanced the expression of DUSP1 in A549 and J774 cells. Transfection of DUSP1-specific siRNA decreased DUSP1 protein and mRNA expression and resulted in enhanced p38 MAPK phosphorylation, iNOS expression, and NO production in A549 cells. This is the first report showing that DUSP1 regulates human iNOS expression.

Down-regulation of DUSP1 by siRNA increased iNOS expression also in murine macrophages. Accordingly, LPS-induced iNOS expression was enhanced in BMMs isolated from DUSP1-deficient mice, which confirmed our results with cells in which DUSP1 had been silenced with siRNA. These results are also in line with the previous reports showing that iNOS expression is increased in DUSP1-deficient mice in response to low-dose LPS administration or septicemia due to Gram-positive bacteria *in vivo* [45, 46]. Interestingly, *E. coli* infection in DUSP1-deficient mice was

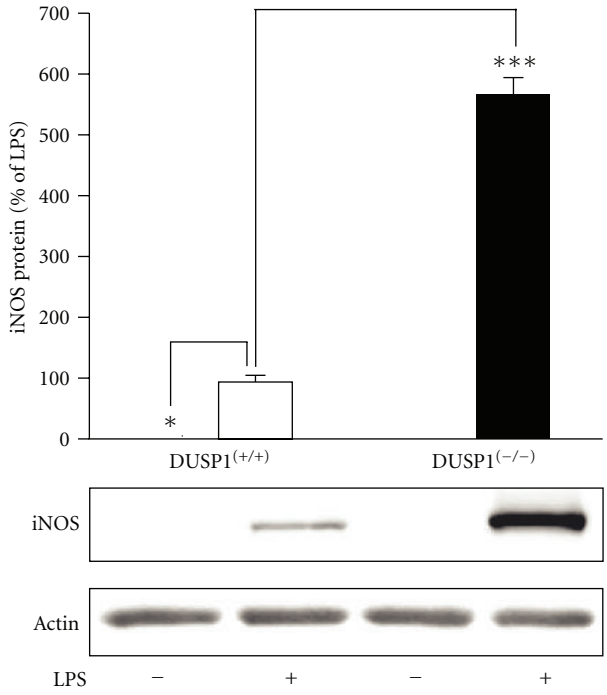


Figure 8: iNOS protein expression in bone marrow macrophages from wild-type and DUSP1-deficient mice. Bone marrow-derived macrophages differentiated from wild-type ($DUSP1^{+/+}$ and DUSP1-deficient ($DUSP1^{-/-}$) mice were incubated with LPS (10 ng/mL) for 24 h, and iNOS protein was measured by Western blot. The gels are representatives of three separate experiments with similar results. Chemiluminescent signal was quantified, and iNOS expression was normalized to actin. Results are expressed as a percentage of LPS, mean ± S.E.M. ($n = 3$). One-way ANOVA with Bonferroni's posttest was performed, and statistical significance is indicated with $^{*}P < .05$ and $^{***}P < .001$.

reported to result in reduced serum nitrate levels and lower iNOS expression in liver as compared to wild-type mice [53]. These conflicting findings may reflect the differences in innate immune response to purified cell wall component (LPS) of Gram-negative bacteria and viable bacteria.

Translation of the results describing the mechanisms of mouse iNOS expression to that of human is not always straightforward. The size of the iNOS promoter differs greatly between mouse (~1.5 kb) and human (up to 16 kb). LPS and IFNγ induce mouse iNOS promoter activity 50–100-fold [5]. Human iNOS promoter shows low basal activity in the absence of measurable mRNA [19, 54]. Cytokine stimulation increases human iNOS promoter activity only approximately 7-to-10 fold, while mRNA expression increases more than 100-fold [8, 19]. This demonstrates the differences in transcriptional but also in posttranscriptional regulation of iNOS expression between mouse and human cells. From a therapeutic point of view, the mechanisms regulating iNOS expression ought to be applicable for human iNOS expression. In this study, we demonstrated the negative regulation of human iNOS expression by DUSP1, which has not previously been reported. Also, DUSP1 regulated mouse iNOS expression. According to our results, p38 MAPK/DUSP1 pathway seems to be a conserved signaling/regulatory mechanism for iNOS expression across species despite the considerable other differences in the regulation of iNOS expression between mouse and human. p38 MAPK/DUSP1 pathway may regulate iNOS expression both at transcriptional and posttranscriptional levels. Human iNOS has been shown to be regulated by p38 MAPK by a mechanism dependent on the interaction between human iNOS mRNA 3′ untranslated region and tristetraprolin, an mRNA-binding protein. p38 MAPK positively regulates tristetraprolin, which stabilizes iNOS mRNA and leads to increased iNOS expression [13]. In addition, human and rodent iNOS promoter activity has also been reported to be regulated by p38 MAPK [11, 55, 56].

Therapeutic approaches targeted to enhance DUSP1 expression or its function may provide a novel mechanism for anti-inflammatory treatment. Glucocorticoids enhance DUSP1 expression, and some of the anti-inflammatory effects of glucocorticoids, such as the inhibition of cytokine and chemokine expression, are mediated by DUSP1 [57, 58]. In alveolar macrophages from patients with glucocorticoid-resistant asthma, the expression of DUSP1 in response to glucocorticoids was reported to be reduced and, correspondingly, p38 MAPK phosphorylation was increased [59]. Also, disease-modifying antirheumatic drug aurothiomalate was recently found to enhance DUSP1 expression in chondrocytes and cartilage, and DUSP1 mediated its inhibitory effect on COX-2, IL-6, and matrix metalloproteinase 3 expression [48]. Interestingly, DUSP4, another MAPK-associated DUSP, has been reported to regulate inflammatory response via ERK and DUSP1. DUSP4-deficient mice were protected from the excessive inflammatory response during septic infection. These animals showed increased ERK phosphorylation due to DUSP4 deficiency. Increased ERK activity resulted in enhanced DUSP1 expression and, in turn, reduced TNF and IL-6 production by macrophages [60].

In addition to inflammatory conditions, increased iNOS-derived NO production has been shown to be present in various solid tumors. Myeloid-derived suppressor cells (MDSCs) are a group of myeloid progenitor cells, immature macrophages, granulocytes, and dendritic cells capable of suppressing functions of T cells. In malignancies, they infiltrate in solid tumors and promote tumor growth. Tumor-associated monocytic MDSCs express iNOS at high level. iNOS-derived NO by MDSCs targets tumor infiltrating T cells and suppresses their functions by inhibiting T cell receptor signaling and Jak/STAT pathway activation and inducing T cell apoptosis. NO production is one of the central mechanisms by which MDSCs promote tumor growth [61]. IL-6 and granulocyte/monocyte colony-stimulating factor (GM-CSF) have been reported to induce the differentiation of MDSCs from peripheral blood mononuclear cells [62]. Interestingly, DUSP1 suppresses IL-6 and GM-CSF, expression [41, 47, 63]. DUSP1 may limit the differentiation and functions on MDSCs by suppressing the expression of IL-6, GM-CSF and iNOS. DUSP1 has been shown to be upregulated in early phases of epithelial carcinogenesis in bladder, colon, and prostate cancers with progressive loss on expression with higher histological grades and in metastasis

[64]. In lung cancer, DUSP1 predicted improved survival [65]. *In vitro*, DUSP1 overexpression induced apoptosis at colon cancer cells [66]. These findings imply that DUSP1 may have antitumor activity. However, the antitumor effects of DUSP1 may well be related to the cancer type and the stage of the disease. It is noteworthy that DUSP1 has been recently linked to the depressive behavior in animal experiments [67], and if this appears to be the case also in humans, it may limit the therapeutic potential of DUSP1 in inflammatory and other conditions.

5. Conclusions

In conclusion, our results show that DUSP1 negatively regulated iNOS expression and NO production induced by inflammatory stimuli by inhibiting p38 MAPK phosphorylation in murine and human cells. This study extends our understanding on the role of DUSP1 in inflammation and on the mechanisms that regulate iNOS expression especially in human cells. This may give new insights in the development of novel drug treatments for diseases complicated with increased iNOS-mediated NO production.

Abbreviations

AP-1: Activating protein-1
BMMs: bone marrow macrophages
COX-2: Cyclooxygenase-2
DUSP: Dual specificity phosphatase
ERK: Extracellular signal-regulated kinase
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
GM-CSF: Granulocyte/monocyte colony-stimulating factor
iNOS Inducible nitric oxide synthase
IFNγ: Interferon-γ
IRF: Interferon-regulatory factor
IL: Interleukin
JNK: Jun N-terminal kinase
LPS: Lipopolysaccharide
M-CSF: Macrophage colony-stimulating factor
miRNA: MicroRNA
MK2: Mitogen-activated protein kinase-activated protein kinase 2
MAPK: Mitogen-activated protein kinase
MDSC: Myeloid-derived suppressor cell
NO: Nitric oxide
NOS: Nitric oxide synthase
Oct-1: Octamer factor
siRNA: Small interfering RNA
TNF: Tumor necrosis factor.

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Regulation of inflammatory cytokine production by MKP-5 in macrophages

Running title: MKP-5 and cytokine release

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Abstract

Mitogen-activated protein kinases (MAPK) include p38 MAPK, Jun N-terminal kinase (JNK) and extracellular-regulated kinase (ERK), and they regulate many cell processes, such as cell division, differentiation and release of inflammatory mediators. MAPK activity is controlled by mitogen-activated protein kinase phosphatases (MKPs), a phosphatase family with eleven members. MKP-1 is the most studied member of MKP family, and it is one of the anti-inflammatory factors induced by glucocorticoids. Less is known about the other MAPK phosphatases although they hold a promise as anti-inflammatory drug targets. In the present study, we investigated the effect of MKP-5 on MAPK phosphorylation and cytokine production in J774 mouse macrophages. We used MKP-5 siRNA and an MKP-5 inhibitor (AS077234-4) to modulate MKP-5 function. We found that MKP-5 controlled p38 MAPK, but not JNK or ERK, phosphorylation. In addition, the production of IL-6 and TNF was suppressed by MKP-5 in macrophages. Our results introduce a novel concept that compounds able to enhance MKP-5 expression and/or activity hold anti-inflammatory potential, because MKP-5 down-regulates the release of inflammatory mediators by controlling p38 MAPK activity.

Key words: MKP-5, DUSP10, TNF, IL-6, p38 MAPK, cytokine

Introduction

Protein tyrosine phosphatases are a large superfamily of phosphatases that catalyze tyrosine dephosphorylation and thereby regulate protein functions. The dual-specificity phosphatases (DUSPs) belong to the protein tyrosine phosphatases, and their characteristic feature is their ability to dephosphorylate both tyrosine and threonine residues in their target proteins [1, 2]. Mitogen-activated protein kinase phosphatases (MKPs) are a subgroup of DUSPs. Mitogen-activated protein kinases (MAPKs) are activated when their Thr-X-Tyr motif in MAPK activation loop is phosphorylated by upstream kinases. MKPs dephosphorylate Thr-X-Tyr motif and thereby restrict the magnitude and duration of MAPK activation. There are 11 different MKPs and they differ from each other by substrate specificity, tissue distribution, cellular localization and expressional pattern [3, 4]. MKPs have been shown to regulate inflammatory response and they are involved in the development of certain cancer types, also. MKP-1 is the most investigated phosphatase among MKPs. MKP-1 suppresses p38 MAPK and JNK activity and it is a suppressor of inflammation *in vivo* [5-8]. Interestingly, enhanced MKP-1 expression has been reported to mediate certain anti-inflammatory effects of glucocorticoids, anti-rheumatic cold compounds and a PDE4 inhibitor rolipram [9-11].

MKP-5, also known as DUSP10, is a less known member of MKP family. MKP-5 is expressed in lung, liver, skeletal muscle, retina, spleen and macrophages. It is found both in cytoplasm and nucleus, and it has a high basal activity. LPS, vitamin D, anisomycin and osmotic stress have been reported to increase its expression [12-15]

This study investigated the effect of MKP-5 on MAPK phosphorylation and cytokine production in J774 mouse macrophages. By using siRNA and MKP-5 inhibitor, we found that MKP-5 regulated p38

MAPK phosphorylation and cytokine production. These results suggest that MKP-5 is an important phosphatase in the control of p38 MAPK activity and cytokine production in macrophages.

Materials and methods

Materials

Reagents were obtained as follows: Lipopolysaccharide (LPS) from *E. coli* strain 0111:B4 (Sigma-Aldrich Inc., St. Louis, MO, USA), phospho-p38 MAPK (#9211; Cell Signaling Technology Inc., Beverly, MA, USA), p38 MAPK antibody (ab27986; Abcam plc., Cambridge, UK), phospho-JNK (#9251; Cell Signaling Technology Inc., Beverly, MA, USA), JNK (sc-571; Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-ERK (#9101; Cell Signaling Technology Inc., Beverly, MA, USA), ERK (#9102; Cell Signaling Technology Inc., Beverly, MA, USA), polyclonal anti-rabbit antibody (sc-2004; Santa Cruz Biotechnology, Santa Cruz, CA, USA). An MKP-5 inhibitor AS-077234-4 was obtained from Merck Serono AS (Geneva, Switzerland). All other reagents were from Sigma Aldrich Inc. unless otherwise stated below.

Cell culture

J774 macrophages (ATCC, Manassas, VA, USA) were cultured at 37°C in 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium with Ultraglutamine 1 (Sigma-Aldrich Inc., St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B.

J774 cells (2 x 10^5 cells/well) were seeded on a 24-well plate and grown for 72 h prior to the experiments. An MKP-5 inhibitor AS077234-4 (1 µM) [16] was added to the cells in fresh culture medium 60 min prior to the stimulation with LPS (10 ng/ml). Cells were further incubated for the time

indicated. For siRNA experiments, J774 cells (1 x 10^5 cells/well) were seeded on a 24-well plate and cultured with 10% FBS without antibiotics overnight. After that, cells were transfected with siRNA and further incubated for 48 h before experiments.

Preparation of Cell Lysates for Western Blot Analysis

At the indicated time points, culture medium was removed. Cells were rapidly washed with ice cold phosphate buffered saline (PBS) and lysed in cold lysis buffer containing 10 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl, 1% Triton-X-100, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM sodiumorthovanadate, 20 µg/ml leupeptin, 50 µg/ml aprotinin, 5 mM sodium fluoride, 2 mM sodium pyrophosphate and 10 µM *n*-octyl-β-D-glucopyranoside. After incubation for 20 min on ice, lysates were centrifuged (12 000 g, 10 min) supernatants were collected and mixed in a ratio of 1:4 with SDS loading buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0,025% bromophenol blue, and 5% β-mercaptoethanol) and stored at -20°C until analyzed. Protein concentrations in the samples were measured by the Coomassie blue method [17].

Western blotting

Prior to Western blot analysis, the protein samples were boiled for 10 min. Equal aliquots of protein (10-20 µg) were loaded on a 10% SDS-polyacrylamide electrophoresis gel and separated by electrophoresis. Proteins were transferred to nitrocellulose membrane by iBlot® dry blotting system (Life Technologies, Carlsbad, California). After transfer, the membrane was blocked in TBS/T (20 mM Tris-base pH 7.6, 150 mM NaCl, 0.1% Tween-20) containing 5% bovine serum albumin for phospho-proteins and 5% nonfat milk for others at 1 h at room temperature. Membranes were incubated

overnight at 4ºC with primary antibody, and for 1 h with the secondary antibody at room temperature, and the chemiluminescent signal was detected by ImageQuant™ LAS 4000 mini (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The chemiluminescent signal was quantified with ImageQuant TL 7.0 Image Analysis Software.

Enzyme-linked immunosorbent assay (ELISA)

Culture medium samples were collected and stores at -20°C until assayed. The concentrations of mouse TNF and mouse IL-6 (DuoSet® ELISA, R&D Systems Europe Ltd., Abindgon, U.K.) were determined by ELISA according to the manufacturer's instructions.

RNA Extraction and Real-Time RT-PCR

At the indicated time points, the culture medium was removed, and cell homogenization and RNA extractions were carried out using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma, Saint Louis, MO, USA) according to the manufacturer's instruction. Reverse transcription of RNA to cDNA was performed by TaqMan® Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA) according to the supplier's instructions. The primer and probe sequences and concentrations were optimized according to the manufacturer's guidelines in TaqMan® Universal PCR Master Mix Protocol part number 4304449 revision C (Applied Biosystems, Foster City, CA, USA) and were as follows: 5'-GACCCTCACACTCAGATCATCTTCT-3' (mouse TNF forward 900 nM), 5'-CCTCCACTTGGTGGTTTGCT-3' (mouse TNF reverse, 300 nM), 5'-AAAATTCGAGTGACAAGCCTGTAGCCCA-3' (mouse TNF probe containing 6-FAM as 5'-reporter dye and TAMRA as 3'-quencher, 200 nM), 5'-TCGGAGGCTTAATTACACATGTTC-3'

(mouse IL-6 forward 900 nM), 5'-CAAGTGCATCATCGTTGTTCATAC-3'(mouse IL-6 reverse, 300 nM), 5'-CAGAATTGCCATTGCACAACTCTTTTCTCA-3'(mouse IL-6 probe containing 6-FAM as 5'-reporter dye and TAMRA as 3'-quencher, 200 nM), 5'-GCATGGCCGGCCGTGTTC-3'(mouse GAPDH forward, 300 nM), 5'-GATGTCATCATACTTGGCAGGTTT-3' (mouse GAPDH reverse, 300 nM) and 5'-TCGTGGATCTGACGTGCCGCC-3' (mouse GAPDH probe containing 6-FAM as 5'-reporter dye and TAMRA as 3'-quencher, 150 nM). Expression of mouse MKP-5 mRNA was measured using TagMan® Gene Expression Assays (Mn00517678_m1; Applied Biosystems, Foster City, Ca, USA).

Primers and probes were obtained from Metabion (Martinsried, Germany). The PCR reaction parameters were as follows: incubation at 50ºC for 2 min, incubation at 95ºC for 10 min, and thereafter 40 cycles of denaturation at 95ºC for 15 s and annealing and extension at 60ºC for 1 min. A standard curve method was used to estimate the relative mRNA levels. When calculating the results, MKP-5, TNF and IL-6 mRNA levels were first normalized against GAPDH.

Down-regulation of MKP-5 by siRNA

J774 cells were transfected with siRNA using DharmaFECT 4 transfection reagent (Dharmacon, Lafayette, CO, USA) according to the manufacturer's instructions. Briefly, cells were seeded on a 24-well plate in 500 µl of medium with 10% FBS without antibiotics, and incubated overnight. For one well, the final transfection medium applied to the cells contained 12.5 nmol of siRNA solution mixed with 1 µl of transfection reagent in final volume of 500 µl of medium. Cells were further incubated for 48 h. Fresh culture medium was changed and LPS was added into the culture medium. Cells were further incubated for the time indicated and cell samples were then subjected to further analyses.

Transfection efficacy was monitored with green fluorescent siRNA oligos (siGLO green transfection indicator, Cat. number D-001630-01, Dharmacon, Lafayette, CO, USA) using Nikon Eclipse TS100 microscope (Nikon, Tokyo, Japan). Approximately 90 % of the cells emitted green fluorescence signal when transfected with siGLO and HiPerFect or siGLO and Dharmfect 4. Less than 5% of the cells emitted signal, when cells were incubated siGLO oligos without transfection reagent.

Statistics

Results are expressed as the mean ± SEM. When appropriate, One-way ANOVA with Bonferroni's or Dunnett's post test or Two-way ANOVA with Bonferroni's post test was performed using GraphPad InStat3 (GraphPad Software Inc., La Jolla, CA, USA). Differences were considered significant at $*p < 0.05$, $**p < 0.01$, or $***p < 0.001$.

Results

MKP-5 regulated IL-6 and TNF production in J774 macrophages

At first, we investigated MKP-5 expression in J774 cells. In unstimulated cells, we were able to detect MKP-5 mRNA at moderate levels. When the cells were stimulated with LPS (10 ng/ml), MKP-5 mRNA levels did not change in response to LPS over the 4 h follow-up (Fig. 1).

We then proceeded to investigate the effect of MKP-5 on cytokine expression in macrophages by using siRNA to silence MKP-5. In cells transfected with MKP-5 siRNA, MKP-5 mRNA levels were reduced by approximately 60% as compared to the cells transfected with a non-targeting control siRNA (siNeg), showing that siRNA effectively down-regulated MKP-5 expression (Fig 2A). In cell transfected with MKP-5 siRNA, IL-6 (Fig 2 B and C) and TNF (Fig. 2 D and E) mRNA and protein levels were increased as compared to the cells transfected with control siRNA. These results suggest that MKP-5 is present and functional in J774 macrophages and it suppresses the production of IL-6 and TNF.

MKP-5 regulated the phosphorylation of p38 MAPK in J774 macrophages

Because p38 MAPK and c-Jun N-terminal kinases (JNK) are important regulators of the production of many inflammatory cytokines and it seems that MKP-5 regulates IL-6 and TNF production, we further investigated if MKP-5 would affect p38 MAPK or JNK phosphorylation. In these experiments, MKP-5 was silenced by siRNA. The down-regulation of MKP-5 increased the amount of phosphorylated p38 MAPK, but did not affect the phosphorylation of JNK in response to LPS stimulation (Fig 3). The

phosphorylation of extracellular-regulated kinase (ERK) was also investigated, and the silencing of MKP-5 did not have any effect of ERK phosphorylation. These data indicate that MKP-5 exclusively regulated p38 MAPK, but not JNK or ERK, activity in J774 macrophages.

MKP-5 inhibitor AS077234-4 enhanced IL-6 and TNF expression in response to LPS in J774 cells.

To further investigated the effect of MKP-5 on the expression of IL-6 and TNF in macrophages, we used an MKP-5 specific inhibitor AS077234-4 [16]. AS077234-4 have been shown to be a potent MKP-5 inhibitor at 1 μM concentration (IC_{50} 710 nM) [16]. J774 cells were pre-incubated with AS077234-4 (1 μM) for 1 h and stimulated then with LPS for 4 h for mRNA and 24 h for protein measurements. LPS increased IL-6 and TNF mRNA and protein levels and those were further enhanced in the presence of an MKP-5 inhibitor AS077234-4 (Fig 4).

Discussion

In this study, we investigated the effects of MKP-5 on MAPK phosphorylation and cytokine production in J774 mouse macrophages. By using gene silencing and an MKP-5 inhibitor, we found that MKP-5 regulated the production of IL-6 and TNF through p38 MAPK in mouse macrophages.

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine protein kinases and they include p38 MAPKs, ERKs and JNKs [18, 19]. MAPKs convey extracellular signals to intracellular effector molecules, enabling the onset of appropriate cellular responses to changes in microcellular environment. Cell functions regulated by MAPK include cell growth, differentiation, cell survival as well as inflammation and immune response [20, 21]. In general, MAPK pathways consist of a module of three kinases. Typically, kinase-linked or G-protein-coupled receptors trigger the MAPK kinase kinase (MKKK) - MAPK kinase (MKK) - MAPK signaling cascade. MAPK activation requires the phosphorylation of threonine and tyrosine residues of a conserved T-X-Y motif within the activation loop of the kinase. MAPK then passes the activation signal on by phosphorylating serine or threonine residues in their target proteins. Many MAPK substrates are transcription factors, kinases and other regulatory proteins [20, 21].

The phosphorylation status and thereby the activity of MAP kinases are regulated by MKPs. MKPs are able to dephosphorylate both tyrosine and threonine residues in the activation motif of MAPKs [22]. MKPs are separated to distinct groups by their cellular localization and substrate specificity: The first group is formed by nuclear phosphatases MKP-1/DUSP1, PAC-1/DUSP2, MKP-2/DUSP4 and DUSP5. The second group includes MKP-3/DUSP6, MKP-X/DUSP7 and MKP-4/DUSP9, and these are ERK-specific cytosolic phosphatases. The third group contains DUSP8, MKP-5/DUSP10 and

MKP-7/DUSP16 and these are p38 MAPK / JNK specific, and they are found both in nuclear and cytosolic compartments in cells [23, 24].

The molecular weight of MKP-5 is approximately 52 kDa [14, 25], and it has considerable sequence and structural similarities with other MKPs. MKP-5 contains two Cdc25-like domains and a C-terminal phosphatase motif, both domains common for most MKPs. MKP-5 has a unique N-terminal extension of 150 amino acids, although the function of this segment is unknown [14, 26]. Another specific feature for MKP-5 is N-terminally located MAPK docking motif, whereas in all other MKPs MAPK docking motif is found at the C-terminal end of the protein [1, 23].

MKP-5 expression is considered to be inducible by inflammatory and stress signals. MKP-5 mRNA levels have been reported to be increased by stress stimuli and LPS in HeLa and BK cells [14] and in RAW 364.7 macrophages [13]. In the present study, LPS stimulation did not change MKP-5 mRNA levels in macrophages. Accordingly, it has been published that MKP-5 levels were not changed in neutrophils after inflammatory stimulation [27]. So, it seems that the inducibility of MKP-5 expression may vary depending on the cell type.

MKP-5 has been reported to display substrate specificity towards p38 MAPK and JNK. In transfection experiments with high MKP-5 expression plasmid concentration, MKP-5 showed phosphatase activity towards all three MAPKs in fibroblasts. With lower MKP-5 plasmid concentrations, there was a clear selectivity for p38 MAPK and JNK [14, 25]. There seems to be cell type-specific differences in MKP-5 substrate specificity, also. Studies with MKP-5(-/-) mice have shown that MKP-5 regulated only JNK activity in T cells, (Zhang et al., 2004) whereas in neutrophils it regulated mainly p38 MAPK phosphorylation [27]. In the present study, MKP-5 seemed to regulate only p38 MAPK activity in macrophages. Using siRNA to silence MKP-5, we found that only p38 MAPK, but not JNK or ERK

phosphorylation was affected by MKP-5. In the previous transfection studies [14, 25], MKP-5 function was investigated by using artificial MKP-5 expression systems. This technique may increase the amount of MKP-5 protein in cells to such levels that its substrate specificity changes. Interestingly, bone marrow macrophages from MKP-5(-/-) mice showed increased p38 MAPK and JNK, as well as ERK phosphorylation especially at later (60-120 min) time points in response to LPS [15]. This suggests that MKP-5 may regulate ERK activity, also, although we did not observe this in our experiments. It has also been speculated that MKP-5 may regulate ERK activity, either by modulating cross-talk between p38 MAPK and ERK pathways [28] or by preventing the nuclear translocation of phosphorylated ERK and preventing the activated ERK to phosphorylate its substrates [29].

p38 MAPK pathway is an important regulator of inflammation. p38 MAPK is involved in the expression of many inflammatory cytokines, chemokines and enzymes responsible for the production of inflammatory mediators [5, 30-32]. p38 MAPK is also involved in the pathogenesis of many chronic inflammatory diseases, such as rheumatoid arthritis [33-35] and chronic obstructive pulmonary disease [36]. Endogenous mechanisms that limit p38 MAPK phosphorylation may therefore offer new possibilities for drug treatments to suppress inflammation. MKP-1, the expression of which is increased by LPS or cytokines, is a feedback mechanism in cells to limit excessive p38 MAPK activity and inflammatory response [5, 23, 37, 38], and it has been shown to mediate certain anti-inflammatory drug effects, such as inhibition of cytokine production and *in vivo* inflammatory response of glucocorticoids, anti-rheumatic gold compounds and a PDE4 inhibitor rolipram [9-11]. MKP-5 seems to be another important negative regulator of inflammatory response by regulating p38 MAPK activity, and endogenous or exogenous factors modulating MKP-5 function may provide possibilities for the development of immunomodulatory drug treatments.

In conclusion, we found that interruption of MKP-5 function either by siRNA of pharmacological inhibitor AS077234-4 increased IL-6 and TNF production by enhancing p38 MAPK phosphorylation in macrophages. Thus, MKP-5 is a negative regulator of p38 MAPK activity and cytokine expression in macrophages and holds a promise as an anti-inflammatory drug target.

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Conflict of Interest: None

Reference

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Figure 1. MKP-5 expression in J774 cells

Cells were stimulated with LPS (10 ng/ml) for time indicated, and MKP-5 mRNA levels were determined by qRT-PCR. MKP-5 mRNA levels were normalized to GAPDH mRNA. mRNA levels are expressed in arbitrary units, MKP-5 mRNA levels in unstimulated cells (0 h) set as 1, and other values are related to that. The results are expressed as mean ± SEM, number of repeats=6. One-way ANOVA with Dunnett's post-test was performed and no differences were found as compared to the basal value.

Figure 2. Effects on MKP-5 on IL-6 and TNF production in J774 macrophages

Cells were transfected with MKP-5 siRNA (siMKP-5) or control siRNA (siNeg) for 48 h. In the experiments, cells were stimulated with LPS (10 ng/ml) for 4 h. (A) MKP-5 mRNA levels were measured by qRT-PCR. IL-6 and TNF mRNA (B and D) and protein levels (C and E) were determined by qRT-PCR and ELISA, respectively. MKP-5, IL-6 and TNF mRNA levels were normalized to GAPDH mRNA levels. The results are expressed as mean ± SEM, number of repeats=4 for mRNA and number of repeats=8 for ELISA. Two-way ANOVA with Bonferroni's post-test was performed, and statistical significance is indicated as * $p < 0.05$ and *** $p < 0.001$

Figure 3. The effect of MKP-5 siRNA on MAPK phosphorylation in J774 macrophages.

Cells were transfected with MKP-5 siRNA (siMKP-5) or control siRNA (siNeg) for 48 h. Cells were then stimulated with LPS (10 ng/ml) for 30 min. p38 MAPK (A), ERK (B) and JNK (C) phosphorylation were determined by Western Blot. Chemiluminescent signal was quantified and the amounts of phosphorylated MAPKs were normalized to the amounts of total MAPKs. LPS-stimulated cells were set as 100% and the other values were related to that value. Results are expressed as mean ±

SEM, number of repeats=8. Two-way ANOVA with Bonferroni's post-test was performed, and statistical significance is indicated as *** $p < 0.001$.

Figure 4. The effect of MKP-5 inhibitor AS077234-4 on IL-6 and TNF production on J774 macrophages.

Cells were pre-incubated with AS077234-4 (1µM) for 1 h and stimulated with LPS (10 ng/ml) for 4 h and 24 h for the determination of mRNA and protein levels, respectively. (A) IL-6 mRNA and (C) TNF mRNA were determined by qRT-PCR, and mRNA levels was normalized against GAPDH mRNA levels. (B) IL-6 and (D) TNF protein levels were measurement by ELISA. The results are expressed as mean ± SEM, numbers of repeats=4 for mRNA and number of repeats=8 for ELISA. One-way ANOVA with Bonferroni's post-test was performed, and statistical significance is indicated as * $p < 0.05$ and ** $p < 0.01$.

Figure 1.

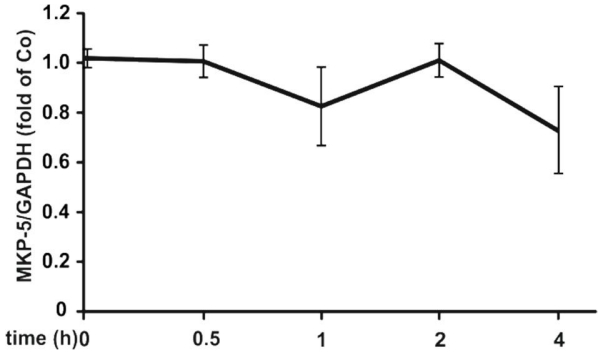

Figure 2.

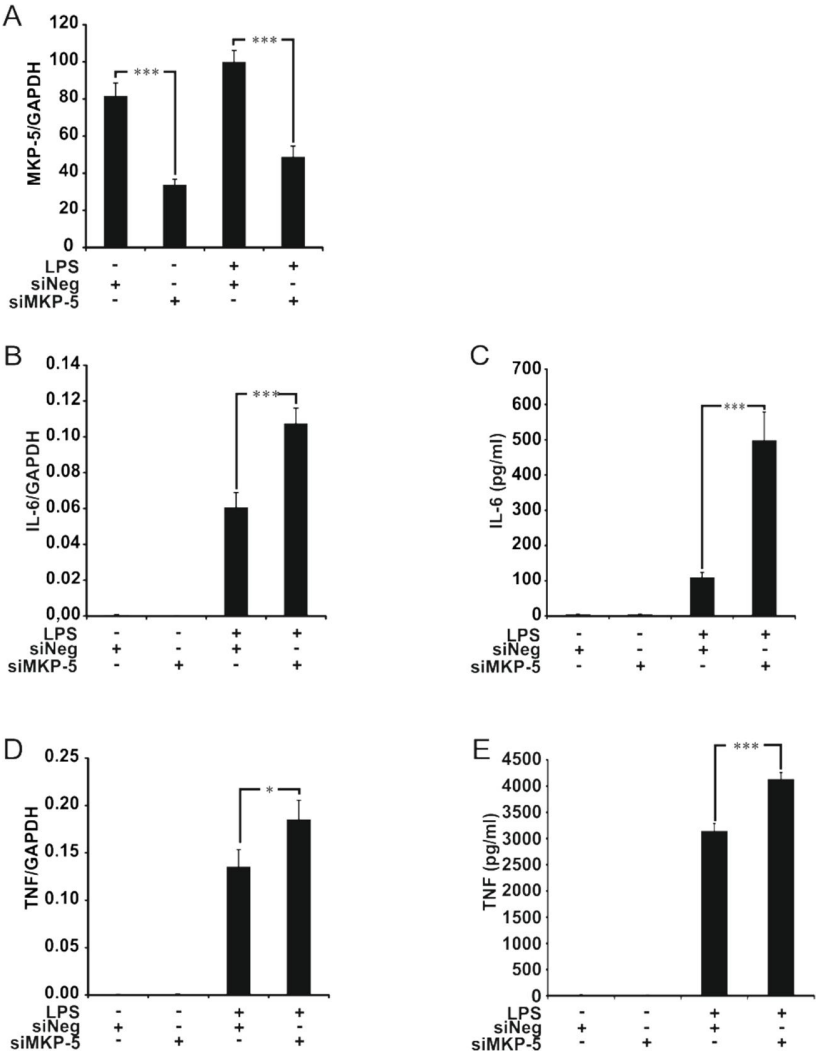
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Figure 3.

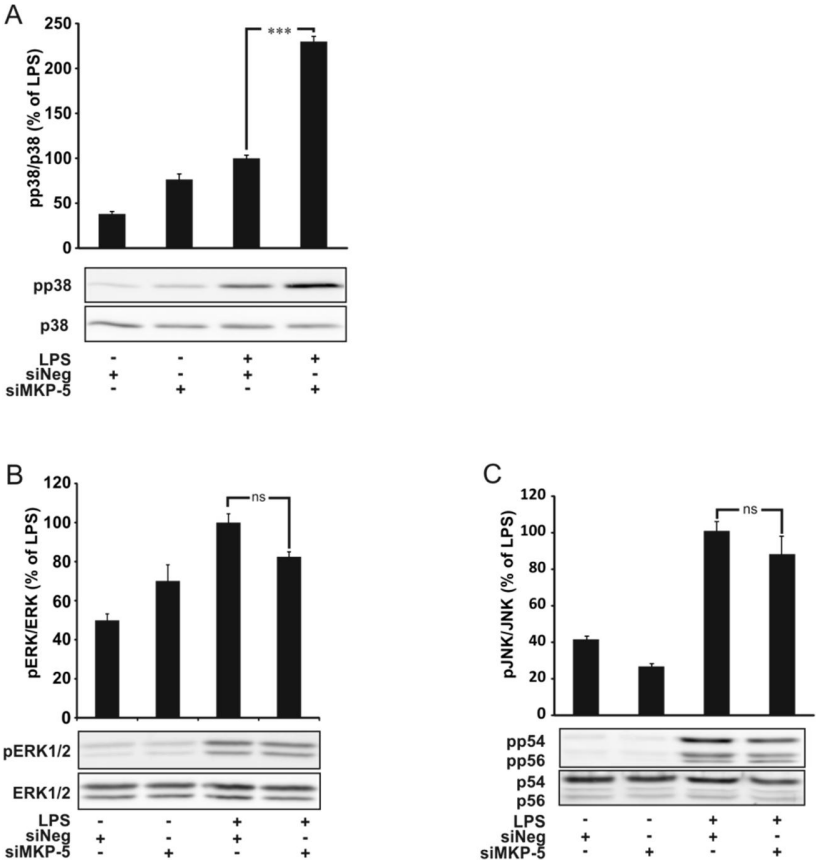
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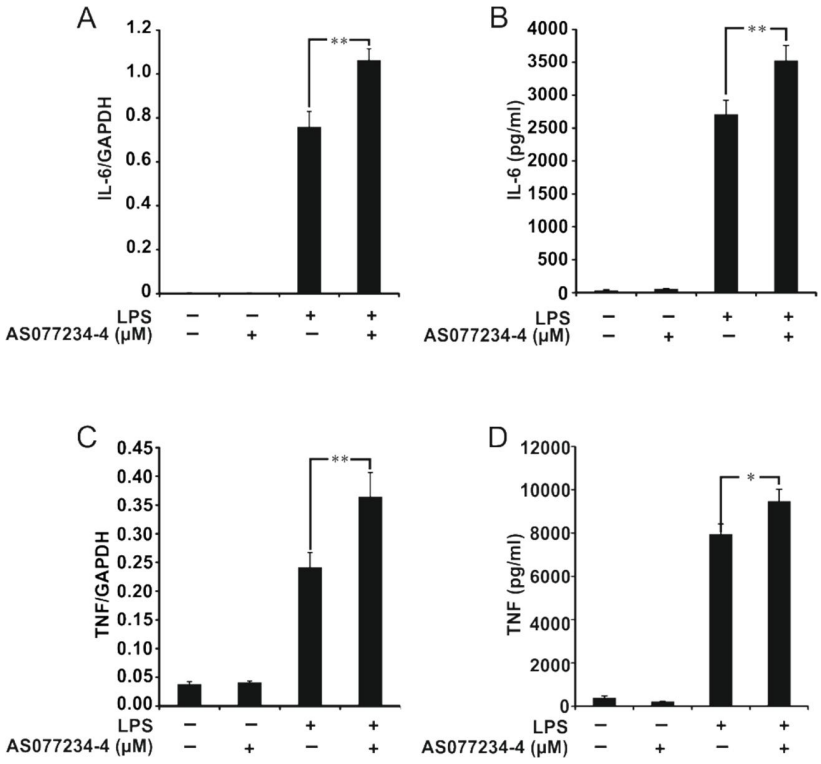
Figure 4.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RESEARCH PAPER

Attenuation of TNF production and experimentally induced inflammation by PDE4 inhibitor rolipram is mediated by MAPK phosphatase-1

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BACKGROUND AND PURPOSE

3′,5′-Cyclic nucleotide PDE4 is expressed in several inflammatory and immune cells, and PDE4 catalyses the hydrolysis of cAMP to 5′AMP, down-regulating cAMP signalling in cells. MAPK phosphatase-1 (MKP-1) is an endogenous p38 MAPK signalling suppressor and limits inflammatory gene expression and inflammation. In the present study, we investigated the effect of a PDE4 inhibitor rolipram on MKP-1 expression and whether MKP-1 is involved in the anti-inflammatory effects of rolipram.

EXPERIMENTAL APPROACH

The effect of rolipram on TNF production was investigated in J774 mouse macrophage cell line and in primary mouse peritoneal macrophages (PM) from wild-type (WT) and MKP-1(–/–) mice. We also investigated the effect of rolipram on carrageenan-induced paw inflammation in WT and MKP-1(–/–) mice.

KEY RESULTS

MKP-1 expression was enhanced by rolipram, by a non-selective PDE inhibitor IBMX and by a cAMP analogue 8-Br-cAMP in J774 cells and in PM. Enhanced MKP-1 mRNA expression by rolipram was reversed by a PKA inhibitor. Rolipram, IBMX and 8-Br-cAMP also inhibited TNF production in activated macrophages. Accordingly, rolipram inhibited TNF production in PMs from WT mice but, interestingly, not in PMs from MKP-1(–/–) mice. Furthermore, rolipram attenuated carrageenan-induced paw inflammation in WT but not in MKP-1(–/–) mice.

CONCLUSIONS AND IMPLICATIONS

PDE4 inhibitor rolipram was found to enhance the expression of MKP-1, and MKP-1 mediated, at least partly, the anti-inflammatory effects of PDE4 inhibition. The results suggest that compounds that enhance MKP-1 expression and/or MKP-1 activity hold potential as novel anti-inflammatory drugs.

Abbreviations

8-Br-cAMP, 8-bromoadenosine 3′,5′-cyclic monophosphate; CRE, cAMP-responsive element; CREB, cAMP-responsive element binding protein; DMSO, dimethyl sulfoxide; MK2, MAPK-activated protein kinase 2; MKP-1, MAPK phosphatase-1; PKAi, PKA inhibitor 6–22 amide; PM, peritoneal macrophages

Introduction

cAMP is a fundamental second-messenger molecule produced in essentially all mammalian cells. 3′5′-Cyclic nucleotide PDEs are the enzymatic machinery that is responsible for the degradation of cAMP in cells by catalysing the hydrolysis of cAMP to 5′AMP. There are 11 known families (PDE1–11) in PDE superfamily, and, currently, at least 24 PDE isoforms have been characterized. Compounds that selectively inhibit PDE4 have prominent anti-inflammatory effects, and selective PDE4 inhibitors have recently entered to the clinics for the treatment of chronic obstructive pulmonary disease, and they have been proven effective in the treatment of plaque psoriasis and psoriatic arthritis as well (Chong *et al.*, 2011; Page and Spina, 2012; Papp *et al.*, 2012; Schett *et al.*, 2012).

PDE4 family members specifically degrade cAMP, and they are expressed essentially in all immune cells including macrophages, neutrophils, eosinophils and lymphocytes, and in many other cell types, including epithelial cells, endothelium and fibroblasts. PDE4 isoforms differ from one another in structure, substrate specificity, tissue and cell distribution, intracellular localization, and regulation by other proteins. PDE4 family contains four genes, namely *PDE4A*, *PDE4B*, *PDE4C* and *PDE4D*, and they give rise to more than 20 alternative splice variants (Bender and Beavo, 2006; Spina, 2008; Keravis and Lugnier, 2012; Page and Spina, 2012). PDE4 isoforms interact with various scaffold proteins, such as β-arrestin, disrupted in schizophrenia 1, receptor for activated C-kinase 1, A-kinase-anchoring proteins, p75 neurotrophin receptor and aryl-hydrocarbon receptor interacting protein. Interactions with specific scaffold proteins evoke sequestered localization of specific PDE4 isoforms in cells. By virtue of such sequestered intracellular localization, PDE4 isoforms create cAMP gradients in cells that elicit temporally and spatially coordinated, also called a compartmentalized, cAMP signalling. PDE4 activity is regulated also by PKA and ERK1/2, and the regulatory outcome on the PDE4 activity by these kinases varies between isoforms and splice variant (Houslay and Adams, 2003; Houslay *et al.*, 2005; Houslay, 2010).

PDE4 plays an important role in immune cell functions. PDE4B(–/–) mice displayed improved tolerance to the development of shock in endotoxemia, and it was associated with the reduced TNF production by macrophages (Jin *et al.*, 2005). Moreover, in ovalbumin-sensitized lung inflammation model, PDE4B-deficient mice had markedly attenuated airway constriction, reduced lung eosinophilia and suppressed production of T helper 2 cytokines after ovalbumin inhalation challenge (Jin *et al.*, 2010). Rolipram, a classic PDE4-specific inhibitor that elevates cAMP levels, inhibited TNF production in monocytes and macrophages (Kelly *et al.*, 1996; Gantner *et al.*, 1997; Wang *et al.*, 1997). Oral or intratracheal administration of PDE4-specific inhibitors have been shown to reduce lung inflammation and airway hyperresponsiveness, attenuate leukocyte infiltration and inhibit cytokine production in the lung (Dastidar *et al.*, 2009; Kobayashi *et al.*, 2011; Nials *et al.*, 2011).

MAPKs include ERK1/2 and ERK5, and stress-activated protein kinases p38 MAPK and JNK. They regulate many cellular responses such as mitosis, differentiation, cellular growth and inflammatory response. MAPKs are activated by phosphorylation of regulatory tyrosine and threonine residues within the activation loop by upstream kinases in response to extracellular stimuli such as cellular stress, bacterial products, growth factors, cytokines or chemokines through activation of GPCRs or kinase-linked receptors (Plotnikov *et al.*, 2011).

MAPK phosphatases (MKPs) are a subgroup of a larger family of dual specificity phosphatases that dephosphorylate phosphotyrosine and phosphoserine/phosphothreonine residues in regulatory and signalling proteins. MKPs have a distinct MAPK binding domain, which enables them to interact with MAPK, and hence, they function as endogenous inhibitors of MAPK pathways (Patterson *et al.*, 2009). MKPs include more than 10 phosphatases and they have differences in expressional pattern, tissue distribution, intracellular location and substrate specificity. MKP-1 is a nuclear phosphatase present in most cell types and tissues. The expression of MKP-1 is up-regulated by factors such as cellular stress, cytokines and LPS (Boutros *et al.*, 2008). MKP-1 mainly inhibits p38 MAPK and, in some cell types, JNK signalling, and it suppresses the expression of inflammatory genes, including TNF (Chi *et al.*, 2006; Zhao *et al.*, 2006; Turpeinen *et al.*, 2010; Korhonen *et al.*, 2011). MKP-1(–/–) mice display more severe inflammatory response and increased mortality to LPS *in vivo*, and they have reduced IL-12 production and impaired antimicrobial clearance (Chi *et al.*, 2006; Zhao *et al.*, 2006; Huang *et al.*, 2011; Korhonen *et al.*, 2012).

Glucocorticoids and anti-rheumatic gold compounds increase the expression of MKP-1, and their anti-inflammatory effects have been shown to be mediated, at least in part, by MKP-1 (Abraham *et al.*, 2006; Nieminen *et al.*, 2010). The promoter of MKP-1 contains two cAMP-responsive elements (CRE) (Kwak *et al.*, 1994), which bind a transcription factor cAMP-responsive element binding protein (CREB), and MKP-1 expression has been reported to be regulated by cAMP-PKA-CREB pathway (Zhang *et al.*, 2008; Brion *et al.*, 2011; Lee *et al.*, 2012). Therefore, we hypothesized that MKP-1 expression may be regulated by PDE4 inhibitors. In the present study, we investigated the effects of PDE4 inhibitor rolipram on MKP-1 expression and on TNF production in activated macrophages and on the development of an acute carrageenan-induced oedema response *in vivo*. Interestingly, rolipram increased MKP-1 expression, inhibited TNF production in macrophages and suppressed carrageenan-induced inflammation in mice, and these effects were mediated by MKP-1.

Methods

Materials

Reagents were obtained as follows: LPS from *Escherichia coli* strain 0111:B4 (Sigma-Aldrich Inc., St. Louis, MO, USA), rolipram [4-(3-cyclopentyloxy-4-methoxy-phenyl)-pyrrolidin-2-one; Axon MedChem, Groningen, The Netherlands], PKA inhibitor 6–22 amide (PKAi; Millipore, Merck Chemicals Ltd., Nottingham, UK) and BIRB 796 [1-(5-*tert*-butyl-2-*p*-tolyl-2*H*-pyrazol-3-yl)-3(4-(2-morpholin-4-yl-ethoxy)naphthalen-1-yl)urea; Axon MedChem] were purchased as indicated. IBMX and 8-Br-cAMP (8-bromoadenosine 3′,5′-cyclic monophos-

phate) were obtained from Sigma-Aldrich Inc. Antibodies for phospho-CREB (#9196), CREB (#9197), phospho-p38 MAPK (#9211), MAPK-activated protein kinase 2 (MK2) (#3042) and phospho-MK2 (27B7, #3007) were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). p38 MAPK antibody (ab27986) and PDE4A antibody (ab14607) were purchased from Abcam plc. (Cambridge, UK) and MKP-1 antibody (SAB2500331) was obtained from Sigma-Aldrich Inc. Actin antibody (sc-1615), PDE4B antibody (sc-25812), PDE4D antibody (sc-25814), polyclonal anti-rabbit antibody (sc-2004) and polyclonal anti-goat antibody (sc-2020) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Lipofectamine 2000 (Life Technologies Europe BV, Espoo, Finland) and Luciferase Assay System (Promega, Madison, WI, USA) were obtained as indicated. CRE reporter [pCRE(luc)neo] was a kind gift from Professor Hartmut Kleinert (Johannes Gutenberg University, Mainz, Germany). All other reagents were from Sigma-Aldrich Inc., unless otherwise stated.

Cell culture

J774 murine macrophages (ATCC, Rockville Pike, MD, USA) were cultured at 37°C in 5% CO_2 atmosphere in DMEM supplemented with glutamax-1 containing 10% heat-inactivated FBS, 100 U·mL^{-1} penicillin, 100 μg·mL^{-1} streptomycin and 250 ng·mL^{-1} amphotericin B (all from Invitrogen, Paisley, UK). For experiments, cells were seeded on 24-well plates at a density of 2×10^5 cells per well. Cell monolayers were grown for 72 h before the experiments were started. Rolipram, IBMX and BIRB 796 were dissolved in dimethyl sulfoxide (DMSO), and 8-Br-cAMP in HBSS. LPS (10 ng·mL^{-1}) or the compounds of interest at concentrations indicated or the solvent (DMSO, 0.1% v/v) were added to the cells in fresh culture medium containing 10% FBS and the supplements. Cells were further incubated for the time indicated.

The effect of LPS and the tested chemicals on cell viability was evaluated by Cell Proliferation Kit II (XTT) (Roche Diagnostics, Mannheim, Germany) (Korhonen *et al.*, 2001; Hämäläinen *et al.*, 2009). Neither LPS nor the other chemicals used in the experiments were observed to evoke cytotoxicity.

Animals

Inbred C57BL/6 MKP-1(–/–) mice were originally generated by the R. Bravo laboratory at Bristol-Myers Squibb Pharmaceutical Research Institute (Dorfman *et al.*, 1996) and bred at the University of Tampere School of Medicine animal facilities. MKP-1(–/–) mice and their wild-type (WT) littermates were housed under conditions of optimum light, temperature and humidity (12:12 h light–dark cycle, 22 ± 1°C, 50–60%) with food and water provided *ad libitum*.

Isolation of peritoneal macrophages and cell culture

For the collection of peritoneal macrophages (PMs), mice were killed according to the regulations of the University of Tampere School of Medicine Animal Unit. Mice were killed by suffocation with CO_2, followed by an immediate cervical dislocation. Primary mouse PMs were obtained by i.p. lavage with sterile PBS supplemented with 0.2 mM EDTA. Cells were washed, resuspended in RPMI 1640 medium supplemented with 2% heat-inactivated FBS, 100 U·mL^{-1} penicillin, 100 μg·mL^{-1} streptomycin, and seeded on 24-well plates (5×10^5 cells per well). The cells were incubated overnight and washed with PBS to remove non-adherent cells before the experiments. Pharmacological compounds or the solvent (DMSO, 0.1% v/v) were added to the cells in fresh culture medium containing 2% heat-inactivated FBS and the antibiotics (see previous discussion), and cells were stimulated with LPS (100 ng·mL^{-1}). Cells were further incubated for the time indicated. The results are reported in accordance with the ARRIVE guidelines (McGrath *et al.*, 2010).

Carrageenan-induced paw oedema

The study was approved by the National Animal Experiment Board. C57BL/6 mice (20–25 g) were divided into groups of six mice and treated with 200 μL of PBS or rolipram (100 mg·kg^{-1} in PBS) by an i.p. injection 2 h before applying carrageenan. Before the administration of carrageenan, the mice were anaesthetized by i.p. injection of 0.5 mg·kg^{-1} of medetomidine (Domitor® 1 mg·mL^{-1}; Orion Oyj, Espoo, Finland) and 75 mg·kg^{-1} of ketamine (Ketalar® 10 mg·mL^{-1}; Pfizer Oy Animal Health, Helsinki, Finland). The mice received a 30 μL i.d. injection of carrageenan (1.5%, dissolved in normal saline) in one hind paw. The contralateral paw received 30 μL of saline and it was used as a control. Paw volume was measured before and 3 h after the carrageenan injection with a plethysmometer (Ugo Basile, Comerio, Italy). Oedema is expressed as a change in paw volume over time. After the experiments, the anaesthetized animals were killed by cervical dislocation. The results are reported in accordance with the ARRIVE guidelines (McGrath *et al.*, 2010).

Preparation of cell lysates and Western blot analysis

At the indicated time points, the culture medium was removed from the cells. Cells were rapidly washed with ice-cold PBS and solubilized in cold lysis buffer containing 10 mM Tris–HCl, 5 mM EDTA, 50 mM NaCl, 1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 20 μg·mL^{-1} leupeptin, 50 μg·mL^{-1} aprotinin, 5 mM sodium fluoride, 2 mM sodium pyrophosphate and 10 μM *n*-octyl-β-D-glucopyranoside. After incubation for 15 min on ice, lysates were centrifuged, and the supernatants were collected and mixed in a ratio of 1:4, with SDS loading buffer (62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 0.025% bromophenol blue and 5% β-mercaptoethanol), and stored at –20°C until analysed.

Prior to Western blot analysis, the samples were boiled for 10 min. Equal aliquots of protein (20 μg) were loaded on a 10% SDS-polyacrylamide gel and separated by electrophoresis. Proteins were transferred to Hybond-enhanced chemiluminescence nitrocellulose membrane (Amersham, Buckinghamshire, UK) by semi-dry electroblotting. After transfer, the membrane was blocked in TBS/T [20 mM Tris-base (pH 7.6), 150 mM NaCl, 0.1% Tween-20] containing 5% non-fat milk for 1 h at room temperature. For detection of phosphorylated proteins, membranes were blocked in TBS/T containing 5% BSA. Membranes were incubated overnight at 4°C with primary antibody and for 1 h with secondary antibody, and the chemiluminescent signal was detected by ImageQuant™ LAS 4000 mini (GE Healthcare Bio-Sciences

AB, Uppsala, Sweden). The chemiluminescent signal was quantified with ImageQuant TL 7.0 Image Analysis Software (GE Healthcare Bio-Sciences AB).

RNA extraction and real-time RT-PCR

At the indicated time points, the culture medium was removed, and cell homogenization and RNA extraction were carried out using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich Inc.) according to the manufacturer's instruction. Reverse transcription of RNA to cDNA was performed by TaqMan® reverse transcription reagents (Applied Biosystems, Foster City, CA, USA), according to the supplier's instructions. The primer and probe sequences and concentrations were optimized according to the manufacturer's guidelines in TaqMan Universal PCR Master Mix Protocol part number 4304449 revision C (Applied Biosystems) and were as follows: 5′-CAAGGATGCTGGAGGGAGAGT-3′ (forward, 300 nM), 5′-TGAGGTAAGCAAGGCAGATGGT-3′ (reverse, 300 nM), 5′-TTTGTTCATTGCCAGGCCGGCAT-3′ (probe containing 6-FAM as 5′-reporter dye and TAMRA as 3′-quencher, 150 nM) for mouse MKP-1, 5′-AATGGCCTCCCTCTCATCAG TT-3′ (forward, 300 nM), 5′-TCCTCCACTTGGTGGTTTGC-3′ (reverse, 300 nM), 5′-CTCAAAATTCGAGTGACAAGCCTGT AGCCC-3′ (probe containing 6-FAM as 5′-reporter dye and TAMRA as 3′-quencher, 150 nM) for mouse TNF, 5′-GCATG GCCGGCCGTGTTC-3′ (forward, 300 nM), 5′-GATGTCAT CATACTTGGCAGGTTT-3′ (reverse, 300 nM) and 5′-TCGTG GATCTGACGTGCCGCC-3′ (probe containing 6-FAM as 5′-reporter dye and TAMRA as 3′-quencher, 150 nM) for mouse GAPDH. Primers and probes were obtained from Metabion (Martinsried, Germany). TaqMan Gene Expression assays for mouse PDE4A (Mm01147149_m1), mouse PDE4B (Mm00456879_m1), mouse PDE4C (Mm01333237_m1) and mouse PDE4D (Mm01253862_m1) were purchased from Life Technologies (Life Technologies Europe BV). The PCR parameters were as follows: incubation at 50°C for 2 min, incubation at 95°C for 10 min, and thereafter, 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. Each sample was determined in duplicate. A standard curve method was used to estimate the relative mRNA levels. When calculating the results, MKP-1 and TNF mRNA levels were first normalized against GAPDH.

ELISA

Culture medium samples were collected and stored at –20°C until assayed. The concentrations of mouse TNF (DuoSet® ELISA, R&D Systems Europe Ltd., Abingdon, UK) were determined by ELISA according to the manufacturer's instructions. The concentrations of acetylated cAMP in J774 cell lysates were determined using cAMP ELISA Kit (Cell Biolabs Inc., San Diego, CA, USA) according to the manufacturer's instructions.

Statistics

Results are expressed as mean ± SEM. When appropriate, one-way ANOVA with Dunnett's or Bonferroni's post-test, two-way ANOVA with Bonferroni's post-test or two-way repeated measures ANOVA with Bonferroni's post-test was performed using GraphPad Prism 5 for Windows version 5.04 (GraphPad Software Inc., La Jolla, CA, USA). Differences were considered significant at $^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$.

Results

PDE4 inhibitor rolipram enhanced MKP-1 expression in activated macrophages

PDE4 isoforms are widely expressed in different cell types, including inflammatory cells (Bender and Beavo, 2006). First, we investigated the expression of PDE4 in J774 macrophages and PM. PDE4 isoforms A, B and D have been reported to be present in monocytes and PDE4B is expressed in macrophages (Shepherd *et al.*, 2004). Both mRNA and protein of PDE4A, PDE4B and PDE4D were found to be present in J774 cells. LPS stimulation for 1 h did not change PDE4A, PDE4B or PDE4D levels in J774 cells (Supporting Information Figure S1). PDE4A mRNA was the most abundant isoform detected by qRT-PCR in J774 cells. Only PDE4B mRNA was detected in mouse primary PM from WT and MKP-1(–/–) mice. Three hour stimulation with LPS increased PDE4B mRNA in PM (Supporting Information Figure S1). mRNA levels of PDE4A and PDE4D were very low, and PDE4C was not detected in PMs from WT or MKP-1(–/–) mice (Supporting Information Figure S1).

Rolipram is a selective PDE4 inhibitor that inhibits all PDE4 isoforms A, B, C and D (Wang *et al.*, 1997). Rolipram increased cAMP levels in J774 macrophages, as expected (Supporting Information Table S1). MKP-1 promoter contains two copies of cAMP responsive (Kwak *et al.*, 1994), and CREB has been reported to regulate the transcription of MKP-1 gene (Zhang *et al.*, 2008). Therefore, we investigated the effect of a PDE4 inhibitor rolipram on MKP-1 expression in J774 mouse macrophages. MKP-1 mRNA and protein expression was increased by LPS and it was further enhanced in the presence of rolipram in J774 cells (Figure 1A, B). MKP-1 mRNA expression was increased also by a cAMP analogue 8-Br-cAMP and a non-selective PDE inhibitor IBMX in J774 cells (Figure 1A).

Because MKP-1 is an endogenous inhibitor of p38 MAPK in macrophages (Chi *et al.*, 2006; Zhao *et al.*, 2006; Korhonen *et al.*, 2011), we investigated the effect of rolipram on p38 MAPK pathway. Phosphorylation of p38 MAPK and its direct substrate MK2 was increased in response to LPS and they were reduced by rolipram (Figure 1C, D). p38 MAPK inhibitor BIRB 796, used as a control compound, inhibited MK2 phosphorylation, as expected (Figure 1D). LPS-stimulated MKP-1 expression was increased by rolipram in PM also (Figure 2).

Increased MKP-1 expression by rolipram was inhibited by a PKA inhibitor

MKP-1 expression has been shown to be increased in response to cAMP (Brion *et al.*, 2011), and PDE4B has recently been reported to inhibit MKP-1 expression in mucosa by a mechanism dependent on cAMP and PKA (Lee *et al.*, 2012). Therefore, we continued by investigating the effect of PKA inhibitor on MKP-1 expression in J774 macrophages. PKA inhibitor 6–22 amide (PKAi), alone or in combination with LPS, did not affect MKP-1 levels in J774 cells. Rolipram increased LPS-induced MKP-1 mRNA expression,

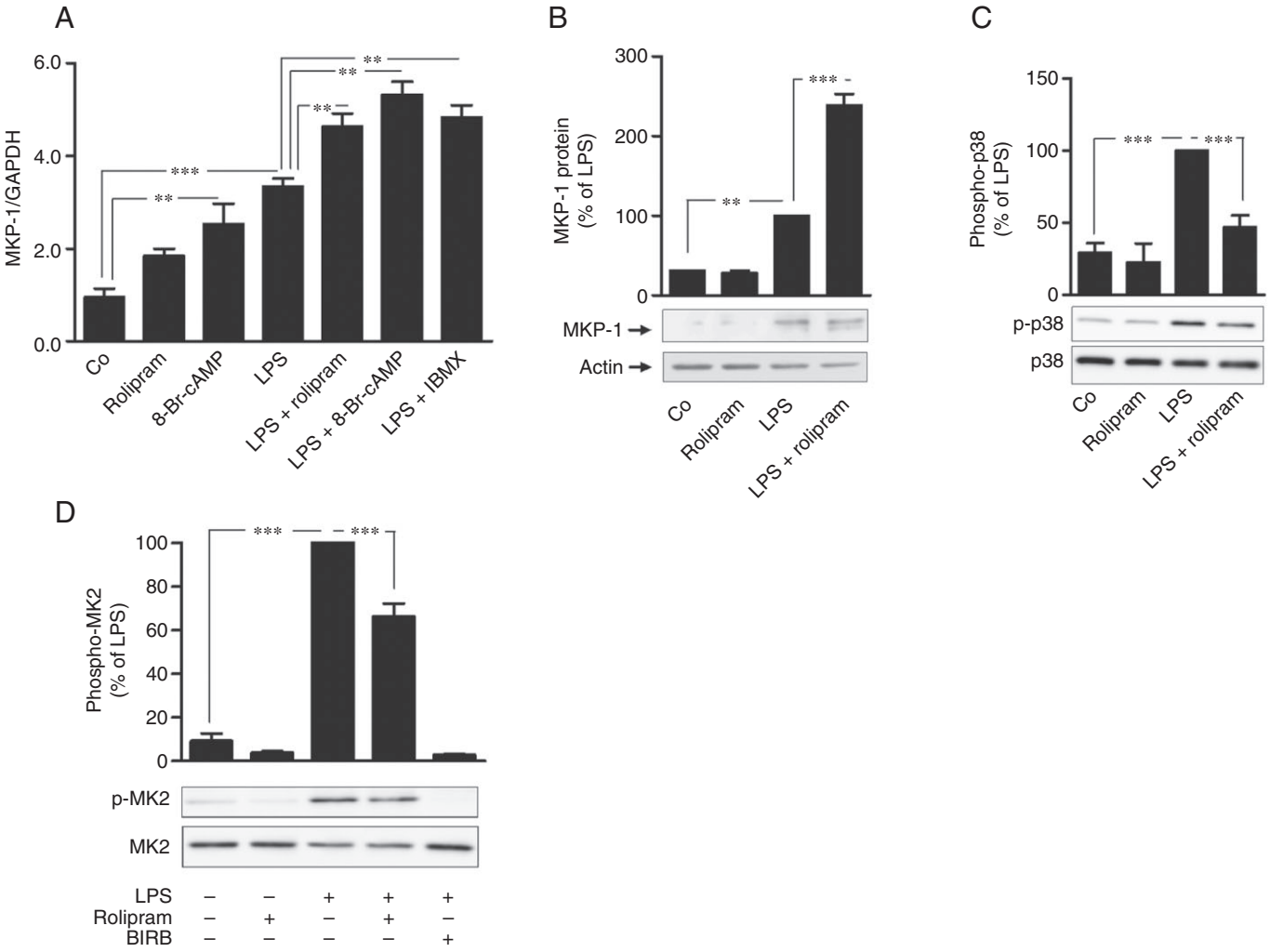

Figure 1

Effects of rolipram, IBMX and 8-Br-cAMP on MKP-1 expression and p38 MAPK phosphorylation in activated J774 macrophages. (A) J774 cells were stimulated with LPS (10 ng·mL^{-1}) in the presence or absence of rolipram (2 μM), IBMX (100 μM) or 8-Br-cAMP (100 μM) for 1 h. MKP-1 mRNA was detected by quantitative RT-PCR, and MKP-1 mRNA expression levels were normalized against GAPDH mRNA levels. The results are expressed as mean ± SEM, number of repeats = 6. J774 cells were stimulated with LPS (10 ng·mL^{-1}) in the presence or absence of rolipram (2 μM) or BIRB 796 (100 nM) and (B) MKP-1 protein (at 1 h), (C) phosphorylated p38 MAPK (at 30 min) and (D) phosphorylated MK2 (at 30 min) were detected by Western blot. The chemiluminescent signal was quantified and the amounts of MKP-1 and phosphorylated p38 MAPK or MK2 were normalized against actin and total p38 MAPK or MK2 respectively. Phosphorylated p38 MAPK and MK2 levels are expressed in arbitrary units, LPS-simulated cells were set as 100%, and the other values were related to that value. The results are expressed as mean ± SEM; number of repeats = 4 for MKP-1 and MK2 and number of repeats = 6 for p38 MAPK. One-way ANOVA with Bonferroni's post-test was performed and statistical significance is indicated as $^{**}P < 0.01$ and $^{***}P < 0.001$.

but that effect was not seen in the presence of PKAi (Figure 3A). Rolipram also increased CREB phosphorylation in LPS-treated cells, and this effect was also reverted by PKAi in J774 cells (Figure 3B). Furthermore, rolipram increased luciferase activity in A549 cells transfected with a CRE reporter plasmid, and the increased luciferase activity was reversed by the PKAi (Supporting Information Figure S2). These results suggest that rolipram increased MKP-1 expression by a mechanism dependent on cAMP-PKA-CREB pathway. In addition, reduction in LPS-induced p38 MAPK phosphorylation by rolipram was also reversed in the presence of PKAi (Figure 3C), supporting the hypothesis that PDE4 inhibition leads to the increased MKP-1 expression and this has functional significance to the activity of p38 MAPK.

PDE4 inhibitor rolipram inhibited TNF production in activated mouse macrophages

TNF is a cytokine whose expression is known to be regulated by MKP-1 and p38 MAPK. Therefore, we continued by investigating the effect of rolipram on TNF production in macrophages. Rolipram inhibited LPS-induced TNF production in

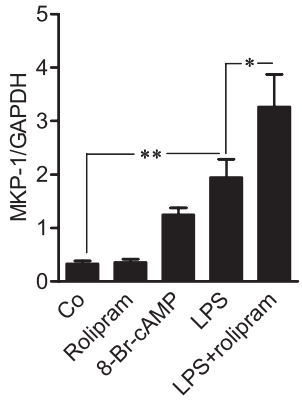

Figure 2

Effect of rolipram on MKP-1 mRNA levels in primary mouse peritoneal macrophages. Peritoneal macrophages were treated with LPS (100 ng·mL^{-1}) in the presence or in the absence of rolipram (2 μM) or with 8-Br-cAMP (100 μM) alone for 1 h. MKP-1 mRNA levels were measured by quantitative RT-PCR and normalized against GAPDH mRNA levels. The results are expressed as mean ± SEM, number of repeats = 4. One-way ANOVA with Bonferroni's post-test was performed and statistical significance is indicated as *P < 0.05 and **P < 0.01.

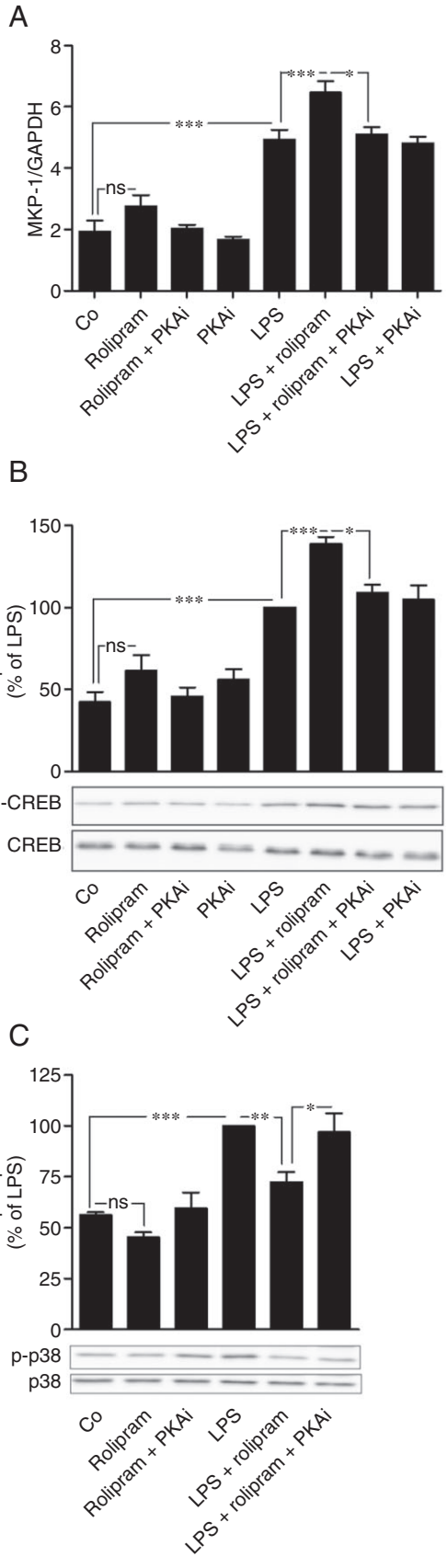

Figure 3

Effects of rolipram and PKA inhibitor on the expression of MKP-1 mRNA and phosphorylation of CREB and p38 MAPK in activated J774 macrophages. (A) J774 cells were pre-incubated with rolipram (2 μM) or PKA inhibitor 6–22 amide (PKAi, 5 μM) for 1 h and stimulated then with LPS (10 ng·mL^{-1}) for 1 h. MKP-1 mRNA was detected by quantitative RT-PCR and normalized against GAPDH mRNA levels. The results are expressed as mean ± SEM, number of repeats = 8. (B, C) J774 cells were pre-incubated with rolipram (2 μM) or PKA inhibitor 6–22 amide (PKAi, 5 μM) for 1 h and stimulated with LPS (10 ng·mL^{-1}) for 30 min and phophorylated CREB and phosphorylated p38 MAPK were detected by Western blot. The chemiluminescent signal was quantified, and the phosphorylated CREB and p38 MAPK were normalized against total CREB and total p38 MAPK respectively. Phosphorylated CREB and p38 MAPK levels are expressed in arbitrary units, LPS-simulated cells were set as 100%, and the other values were related to that value. The results are expressed as mean ± SEM, number of repeats = 8. One-way ANOVA with Bonferroni's post-test was performed and statistical significance is indicated as *P < 0.05, **P < 0.01 and ***P < 0.001.

a dose-dependent manner (IC_{50} 25.9 nM), and maximal/submaximal inhibition was observed with 2 μM drug concentration in J774 cells (Figure 4A). Rolipram also reduced LPS-induced TNF mRNA levels in J774 macrophages (Figure 4C). TNF production was also inhibited by a non-selective PDE inhibitor IBMX and by a cAMP analogue 8-Br-cAMP (Figure 4B).

In addition to PDE4, monocytes and macrophages have been reported to express PDE3B, which degrades both cAMP and cGMP in a manner that cGMP inhibits the degradation of cAMP (Bender and Beavo, 2006). A PDE3 inhibitor milrinone at 10 μM concentration (Sudo *et al.*, 2000) did not inhibit TNF production in J774 cells (Figure 4B), suggesting that the

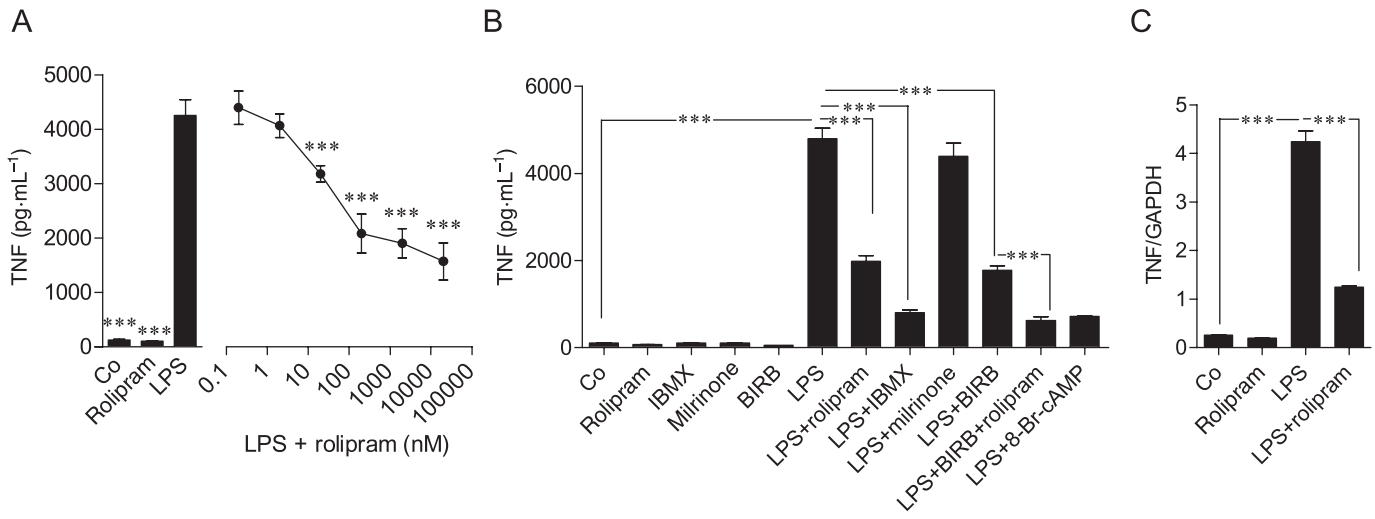

Figure 4

Effects of rolipram, IBMX and 8-Br-cAMP on TNF expression in J774 mouse macrophages. (A) J774 cells were incubated with rolipram alone (2 μM) or in the presence of increasing concentrations of rolipram for 1 h and stimulated then with LPS (10 ng·mL^{-1}) for 24 h, and TNF accumulated into the culture medium was measured by ELISA. (B, C) J774 cells were pre-incubated by rolipram (2 μM), IBMX (100 μM), milrinone (10 μM), BIRB 796 (100 nM) and stimulated with LPS (10 ng·mL^{-1}) or with the combination of LPS and 8-Br-cAMP (100 μM) for 24 h (TNF protein) or 3 h (TNF mRNA). TNF accumulated into the culture medium was measured by ELISA. TNF mRNA levels were measured by quantitative RT-PCR and normalized against GAPDH mRNA levels. The results are expressed as mean ± SEM, number of repeats = 6 for TNF protein and number of repeats = 3 for TNF mRNA. One-way ANOVA with Dunnett's post-test (A) or Bonferroni's post-test (B, C) was performed and statistical significance is indicated as ***P < 0.001.

PDE4 activity is selectively involved in the regulation of TNF production in J774 macrophages. A p38 inhibitor BIRB 796 also inhibited TNF production as expected and it further enhanced the effect of rolipram (Figure 4B). TNF production was inhibited by the p38 MAPK inhibitor BIRB 796 in PMs also (Figure 5).

The inhibition of TNF expression and carrageenan-induced paw inflammation by a PDE4 inhibitor rolipram was mediated by MKP-1

Because rolipram increased MKP-1 expression and inhibited TNF production, we hypothesized that MKP-1 could mediate the anti-inflammatory effects of rolipram. To test the hypothesis, we first investigated the effect of rolipram on LPS-induced TNF production in PM from WT and MKP-1(–/–) mice. TNF mRNA and protein expression was induced by LPS in PM from WT mice, and that was clearly (by 74 and 63% for TNF mRNA and TNF protein, respectively) inhibited by rolipram. LPS-induced TNF production was enhanced in PM from MKP-1(–/–) mice as compared to that in PM from WT mice, which is in line with the published results (Chi *et al.*, 2006; Zhao *et al.*, 2006; Korhonen *et al.*, 2011). Interestingly, the inhibition of TNF mRNA and protein expression by rolipram was markedly attenuated in PM from MKP-1(–/–) mice and did not reach statistical significance (Figure 6).

To investigate whether MKP-1 could mediate the anti-inflammatory effects of rolipram also *in vivo*, we tested the effect of rolipram on the severity of carrageenan-induced paw inflammation in WT and MKP-1(–/–) mice. Carrageenan-induced paw oedema was markedly attenuated by rolipram in

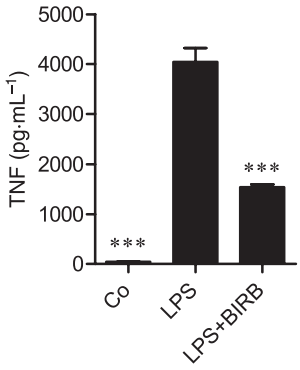

Figure 5

Effect of p38 MAPK inhibitor BIRB 796 on TNF production in primary mouse peritoneal macrophages. Peritoneal macrophages were pre-incubated with a p38 MAPK inhibitor BIRB 796 (100 nM) for 30 min and stimulated then with LPS (100 ng·mL^{-1}) for 24 h. TNF accumulated into the culture medium was measured by ELISA. The results are expressed as mean ± SEM, number of repeats = 4. One-way ANOVA with Bonferroni's post-test was performed and statistical significance is indicated as ***P < 0.001.

WT mice, while rolipram did not have any effect on paw oedema in MKP-1(–/–) mice (Figure 7).

Discussion and conclusions

In the present study, we investigated the effects of a PDE4 inhibitor rolipram on TNF expression in macrophages and on

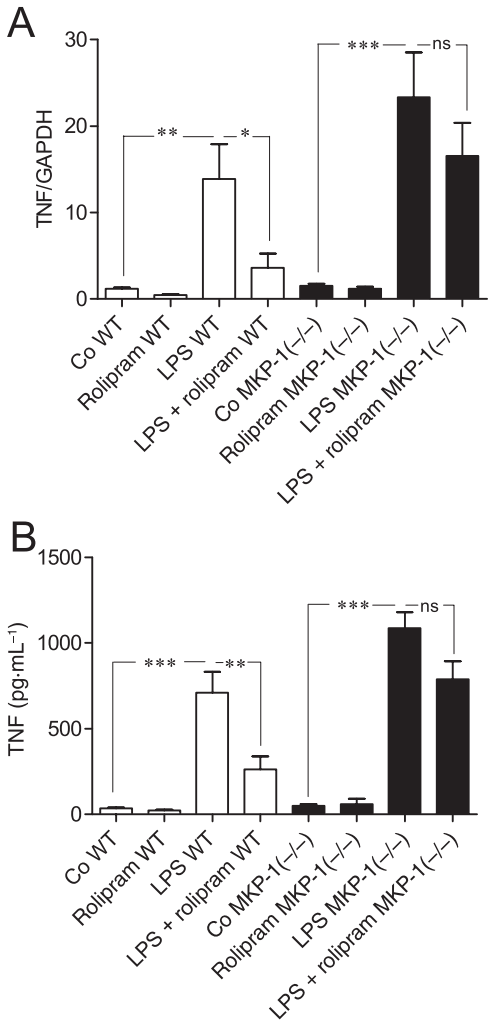

Figure 6

Effect of MKP-1 on the inhibition of TNF production by rolipram in primary mouse peritoneal macrophages. Peritoneal macrophages from wild-type (WT, open bars) and MKP-1(–/–) (black bars) mice were incubated with LPS (100 ng·mL^{-1}) for (A) 3 h and (B) 24 h for TNF mRNA and protein determinations respectively. TNF mRNA levels were measured by quantitative RT-PCR and normalized against GAPDH mRNA levels. TNF protein accumulated into the culture medium was measured by ELISA. The results are expressed as mean ± SEM, number of repeats = 6 for TNF mRNA and number of repeats = 8 for TNF protein. Two-way ANOVA with Bonferroni's post-test was performed and statistical significance is indicated as *ns*, non-significant; *P < 0.05, **P < 0.01 and ***P < 0.001.

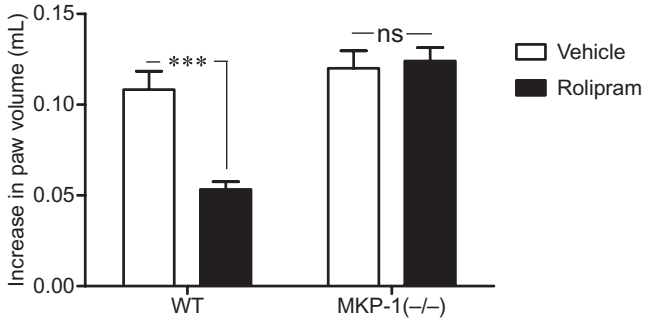

Figure 7

Effect of rolipram and MKP-1 on carrageenan-induced acute paw inflammation. Rolipram (100 mg·kg^{-1}, i.p.) was administered 2 h prior to the experiment. In the beginning of the experiment (0 h), hind paw volumes were measured with a plethysmometer. Carrageenan or vehicle (control) was i.d. injected, and paw volumes were measured after 3 h with a plethysmometer. Oedema is expressed as the difference between the volume changes of the carrageenan treated paw and the control paw, mean ± SEM, number of repeats = 6. Two-way repeated measures ANOVA with Bonferroni's post-test was performed and statistical significance is indicated as ns (non-significant) and ***P < 0.001.

carrageenan-induced acute paw inflammation *in vivo*, and the role of MKP-1 in mediating those effects of rolipram. The results show that rolipram increased the expression of MKP-1, inhibited the production of TNF in activated macrophages and reduced the severity of carrageenan-induced paw inflammation. Furthermore, using MKP-1(–/–) mice and macrophages from these animals, we showed that those effects of rolipram were mediated by MKP-1.

Studies with different PDE4 isoform-deficient mice and with selective PDE4 inhibitors have shown that PDE4 is a potential target for anti-inflammatory drug treatments. PDE4A, PDE4B and PDE4D are present in human and mouse monocytes and macrophages, and the expression of PDE4B is increased after *in vivo* LPS challenge (Verghese *et al.*, 1995; Jin and Conti, 2002). Earlier studies have shown that in U937 human monocytes, certain splice variants of PDE4A and PDE4D predominates and when these cells are differentiated to macrophages, PDE4B2 activity becomes predominating (Shepherd *et al.*, 2004). In this study, PDE4A, PDE4B and PDE4D were found to be present in J774 cells, while in PM, which differentiate from monocytes to macrophages *in vivo*, only PDE4B expression was observed.

Of the PDE4 isoforms present in inflammatory cells, PDE4B, and PDE4D to some extent, seems to be an important regulator of inflammation and immune response. PDE4B-deficient mice display suppressed innate immune response and increased survival after *in vivo* LPS administration, and macrophages from PDE4B(–/–) mice, but not from PDE4A(–/–) or PDE4D(–/–) mice, produce less TNF in response to LPS as compared to WT macrophages (Jin and Conti, 2002; Jin *et al.*, 2005). Furthermore, PDE4B(–/–) mice have attenuated ovalbumin-specific T-cell responses, and this was associated with reduced lung inflammation evoked by inhaled ovalbumin and ameliorated airway hyper-responsiveness induced by methacholine (Jin *et al.*, 2010). Adhesion of neutrophils to endothelium and their chemotaxis is impaired in PDE4B(–/–) and PDE4D(–/–) mice, showing that both PDE4B and PDE4D are intrinsic and non-redundant regulators of neutrophil functions (Ariga *et al.*, 2004). PDE4 selective inhibitor rolipram has been shown to inhibit airway hyper-responsiveness, leukocytes accumulation and cytokine release in the lung in ovalbumin-asthma model in mice (Ikemura *et al.*, 2000; Kanehiro *et al.*, 2001). In humans, selective PDE4 inhibitor roflumilast has been approved for the treatment of chronic obstructive pulmonary disease, and it improves lung functions and reduces the incidence of disease

exacerbations (Chong *et al.*, 2011). Furthermore, roflumilast has been reported to attenuate lung inflammation in patients with mild asthma after allergen challenge (Gauvreau *et al.*, 2011). Another selective PDE4 inhibitor apremilast has been recently proven effective in the treatment of plaque psoriasis and psoriatic arthritis (Papp *et al.*, 2012; Schett *et al.*, 2012).

MKP-1 is a nuclear tyrosine/threonine phosphatase that primarily regulates the phosphorylation of p38 MAPK and JNK, and MKP-1(–/–) mice display excessive inflammatory response and increased mortality to LPS challenge (Chi *et al.*, 2006; Zhao *et al.*, 2006; Korhonen *et al.*, 2011) as well as exacerbated chronic inflammation in arthritis models (Salojin *et al.*, 2006; Vattakuzhi *et al.*, 2012). MKP-1 promoter contains two CRE (Kwak *et al.*, 1994), and cAMP-PKA-CREB pathways have been reported to regulate MKP-1 expression (Zhang *et al.*, 2008; Brion *et al.*, 2011; Lee *et al.*, 2012). In this study, we found that rolipram increased cAMP levels and enhanced CREB phosphorylation and MKP-1 expression in J774 cells and CREB-driven transcription in A549 cells, and those effects were decreased by a PKA inhibitor. In addition, cAMP analogue 8-Br-cAMP increased MKP-1 levels in J774 cells. Moreover, rolipram inhibited p38 MAPK phosphorylation and that effect was removed by the PKA inhibitor. These results suggest that the increased MKP-1 expression by rolipram was due to activation of cAMP-PKA-CREB pathway. Our results also show that the increased MKP-1 expression by rolipram via cAMP-PKA-CREB pathway had functional effects on p38 MAPK pathway, as evidenced by reduced phosphorylation of MK2, which is a direct down-stream target of p38 MAPK. This was further supported by the findings that rolipram did not inhibit TNF expression in PMs from MKP-1(–/–) mice, and that carrageenan-induced paw inflammation was inhibited by rolipram in WT mice, but not in MKP-1(–/–) mice. These findings strongly suggest that the anti-inflammatory effects of rolipram on TNF expression and on carrageenan-induced inflammation *in vivo* are mediated by MKP-1, which is a novel finding and a new anti-inflammatory mechanism of action of PDE4 inhibitors.

PDE3B is expressed in macrophages, and it degrades both cAMP and cGMP. PDE3 has been suggested to participate in insulin, insulin-like growth factor 1 and leptin signalling (Barber *et al.*, 2004; Bender and Beavo, 2006). In our experiments, selective PDE3 inhibitor milrinone (Sudo *et al.*, 2000) did not inhibit TNF production, which is in line with a previous study reporting that pharmacological inhibition of PDE3 does not affect TNF production in macrophages (Gantner *et al.*, 1997).

MKP-1 has also been linked to the pathogenesis of chronic inflammatory diseases and anti-inflammatory drug effects. MKP-1(–/–) mice display increased disease penetrance and exacerbated inflammation and osteolysis in experimental arthritis models (Salojin *et al.*, 2006; Vattakuzhi *et al.*, 2012). This is interesting because PDE4 inhibitors may hold therapeutic potential in the treatment of chronic inflammation other than inflammatory lung diseases. PDE4 inhibitors ameliorate the development of arthritis in murine models, and roflumilast and another selective PDE4 inhibitor apremilast reduce inflammatory cytokine release from human rheumatoid synovial cells (Ross *et al.*, 1997; Yamaki *et al.*, 2007; McCann *et al.*, 2010; Crilly *et al.*, 2011). Recently, apremilast has been reported to be effective in the treatment of plaque psoriasis and psoriatic arthritis (Papp *et al.*, 2012; Schett *et al.*, 2012). Apremilast also reduced the epidermal thickening, keratinocyte proliferation and the development of psoriform-like histopathological features in human skin xenograft/severe combined immunodeficiency mouse model of psoriasis (Schafer *et al.*, 2010). It is possible that enhanced MKP-1 expression, at least in part, mediates the anti-inflammatory effects of PDE4 inhibitors in chronic inflammation.

There is evidence showing that MKP-1 is associated with effects of other anti-inflammatory drugs. MKP-1 expression is increased by glucocorticoids (Kassel *et al.*, 2001; Shipp *et al.*, 2010), and the macrophages from MKP-1(–/–) mice are less sensitive to the inhibition of inflammatory gene expression by glucocorticoids (Abraham *et al.*, 2006). Furthermore, the attenuation of the acetylcholine-induced bronchial contraction by glucocorticoids was reduced in MKP-1(–/–) mice (Li *et al.*, 2011). We have recently reported that anti-rheumatic gold compounds increase the expression of MKP-1 in chondrocytes and cartilage from rheumatoid arthritis patients; and using siRNA and MKP-1(–/–) mice, we showed that the inhibition of inflammatory gene expression by gold compounds was mediated by MKP-1 (Nieminen *et al.*, 2010). In addition, MKP-1 gene transfer suppressed osteoclastogenesis in macrophages, reduced the accumulation of inflammatory cells into the bone and attenuated the inflammatory bone loss evoked by LPS (Yu *et al.*, 2010). Based on these findings, it is possible that some anti-inflammatory drug effects are mediated by MKP-1 also in patients. Importantly, MKP-1 expression in dendritic cells has been shown to be important for development of effective antimicrobial responses (Huang *et al.*, 2011). Also, MKP-1 enhances the expression of interferon regulatory factor 1 and IL-12 (Korhonen *et al.*, 2012), both important factors for the development of cell-mediated immune response and effective antimicrobial defence. Hence, the present study and the published reports support the idea that MKP-1 holds potential as a novel anti-inflammatory drug target. MKP-1 is an endogenous factor that suppresses inflammatory response and the development of pathological changes in tissues without compromising anti-microbial defence.

In conclusion, the present study shows that a PDE4 inhibitor rolipram enhanced MKP-1 expression through cAMP-PKA-CERB pathway and inhibited TNF production in activated macrophages and attenuated carrageenan-induced paw inflammation in mice. Importantly, rolipram failed to inhibit TNF production in macrophages from MKP-1(–/–) mice and to attenuate carrageenan-induced paw inflammation in MKP-1-deficient animals. We propose that the anti-inflammatory effects of PDE4 inhibitors are, at least partly, mediated by enhanced MKP-1 expression, which is a novel finding and a new mechanism of action for the anti-inflammatory effects of PDE4 inhibitors. The results also support the idea that compounds that enhance MKP-1 expression or its function hold potential as novel anti-inflammatory drugs.

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Conflicts of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1 The expression of PDE4A, PDE4B, PDE4C and PDE4D in J774 macrophages and mouse primary peritoneal macrophages (PM) from WT and MKP-1(–/–) mice.

Figure S2 cAMP-responsive element-driven transcription was increased by rolipram and inhibited PKA inhibitor 6-22 amide (PKAi) in A549 human bronchial epithelial cells.

Table S1 The effect of rolipram on cAMP levels in J774 macrophages.