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Review Article

MAP kinase phosphatase-1, a critical negative regulator of the innate immune response

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Abstract: Mitogen-activated protein (MAP) kinase cascades are crucial signal transduction pathways in the regulation of the host inflammatory response to infection. MAP kinase phosphatase (MKP)-1, an archetypal member of the MKP family, plays a pivotal role in the deactivation of p38 and JNK. *In vitro* studies using cultured macrophages have provided compelling evidence for a central role of MKP-1 in the restraint of pro-inflammatory cytokine biosynthesis. Studies using MKP-1 knockout mice have strengthened the findings from *in vitro* studies and defined the critical importance of MKP-1 in the regulation of pro-inflammatory cytokine synthesis *in vivo* during the host response to bacterial cell wall components. Upon challenge with TolI-like receptor ligands MKP-1 knockout mice produced dramatically greater amounts of inflammatory cytokines, developed severe hypotension and multi-organ failure, and exhibited a remarkable increase in mortality. More recent investigations using intact bacteria confirmed these observations and further revealed novel functions of MKP-1 in host defense against bacterial infection. These studies demonstrate that MKP-1 is an essential feedback regulator of the innate immune response, and that it plays a critical role in preventing septic shock and multi-organ dysfunction during pathogenic infection. In this review, we will summarize the studies on the function of MKP-1 in innate immune responses and discuss the regulation of this novel protein phosphatase.

Key words: MAP kinase, MKP-1, negative regulator, innate immune response, cytokines, inflammation

Introduction

Sepsis represents a serious challenge to public health. Each year in the United States, sepsis accounts for approximately 750,000 hospitalizations and 215,000 deaths, costing nearly $17 billion [1, 2]. Despite advances in disease prevention and treatment, the incidence of sepsis is rising at an astonishing rate [2, 3]. The Centers for Disease Control estimated that the incidence of sepsis increased from 73.6 per 100,000 people in 1979 to 175.9 per 100,000 in 1989 [4]. The overall mortality rate is approximately 30-50% for all adults [1]. Sepsis and septic shock are an equally important cause of morbidity and mortality in children and neonates. Epidemiologic studies of these populations have documented an incidence ranging from 20 to 50 per 100,000 in children aged 1-15 years, to over 500 per 100,000 in neonates and infants less than 1 year of age [5, 6]. Sepsis-related mortality averages 10% in children and increases to 17% in pediatric patients with severe sepsis or septic shock [5, 6]. Sepsis is likely an even more severe threat to the general population in developing countries than in industrial countries, due to vast population, lower standards of living, and limited access to health care. The epidemiology of severe sepsis in these developing regions is largely unknown. Limited available data suggests that outcomes of sepsis are catastrophic. It has been reported

that mortality rates from severe sepsis in several developing countries including Pakistan, Turkey, and Thailand are as high as 80-90% [7-9]. A recent epidemiological study conducted with patients in intensive care units at ten university hospitals in the People's Republic of China has reported a rate of 8.7% of severe sepsis in critically ill patients. Total hospital mortality was reported to be 49% for patients with severe sepsis [10]. It has been estimated that sepsis occurs in neonates at a rate between 12-21% in China [11]. These studies were conducted at hospitals in large metropolitan areas, where health care facilities are more accessible and standards of living are generally higher than in vast rural areas. As such, the actual sepsis incidences in the country are likely to be considerably higher.

The term sepsis indicates a complex and potentially self-destructive clinical syndrome resulting from excessive and dysregulated host inflammatory responses to microbial infection. In sepsis patients, serum levels of both pro- and anti-inflammatory cytokines are elevated and expression often occurs in waves. In fact, the condition has often been referred to as a "cytokine storm" [12, 13]. Clinically, septic shock is characterized by abnormal coagulation, profound and unresponsive hypotension, vasodilatory shock, and multi-organ failure secondary to the excessive production of pro-inflammatory cytokines, such as TNF-α and IL-1β [14, 15]. These pro-inflammatory cytokines in turn trigger secondary inflammatory cascades, resulting in the production of additional cytokines and chemokines, lipid mediators, and reactive oxygen species, as well as the up-regulation of cell adhesion molecules to facilitate migration of inflammatory cells into tissues. Pro-inflammatory cytokines also promote the expression of inducible nitric oxide synthase (iNOS) and augment nitric oxide (NO) production, thus decreasing systemic vascular resistance. The resultant profound hypotension is the clinical hall-mark in adult septic shock (~90% of cases). In contrast, pediatric patients afflicted with septic shock have a hemodynamic profile most often characterized by decreased cardiac output (~80% of cases) with either normal or even elevated systemic vascular resistance [16]. Over-production of NO and reactive oxygen species appears to be a critical contributor of myocardial dysfunction in sepsis [17]. In addition to these important cardiovascular effects, proinflammatory cytokines also initiate systemic coagulation, leading to impairment of microvascular circulation. Hypotension further exacerbates organ ischemia caused by microvascular occlusion, ultimately leading to end-organ failure and the development of multi-organ dysfunction syndrome [18].

The innate immune system serves as the frontline defense against invading pathogenic organisms. While dysregulated cytokine production is associated with such devastating pathologies as septic shock, adequate production of proinflammatory cytokines in response to invasion by pathogenic microorganisms is critical for competent host defense against microbial infections [19, 20]. Cytokines and other inflammatory mediators are required for the recruitment of leukocytes to the site of infection, which is necessary for the containment and ultimate eradication of the invading pathogen. These cytokines are also important for the initiation of the acute phase response and for the production of reactive oxygen and nitrogen species which possess potent microbicidal activities. Microbial pathogens are recognized and contained by both complement and phagocytosis mediated by macrophages and neutrophils [21]. Interaction of innate immune cells with microbial components leads to the activation of multiple signaling cascades, including the family of interleukin-1 receptor associated kinases (IRAK) [22-27], and phosphotidylinositol-3-phosphate (PI3) kinase [28], ultimately leading to the activation of MAP kinase pathways and multiple transcription factors. A critical transcription factor activated in innate immune cells is nuclear factor (NF)-κB. NF-κB binds to the promoter regions of a large number of cytokine and chemokine genes and activates their transcription [19, 29]. MAP kinases, including extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38, also play crucial roles in this process [30]. These kinases not only participate in the transcription of many pro-inflammatory cytokine genes by phosphorylation of certain transcription factors and promoting chromatin remodeling, but also participate in the transport, stabilization, and translation of cytokine mRNA transcripts. As a result, the host promptly adopts a highly pro-inflammatory phenotype and produces a myriad of pro-inflammatory cytokines, anti-

microbial peptides, and other effector proteins which participate in the containment and killing of microbial pathogens.

In mammalian cells, MAP kinases are primarily deactivated by a group of dual specificity protein phosphatases through dephosphorylation of tyrosine and threonine residues critical for MAP kinase activation [31]. Thus, this group of protein phosphatases may serve as negative regulators in the innate immune response during microbial infection and thus, play a significant role in the prevention and resolution of sepsis pathophysiology. Supporting this idea, a number of recent studies using knockout mice have demonstrated that MAP kinase phosphatase (MKP)-1 plays an essential role in the protection of the host against endotoxic shock [32-35]. Moreover, several well-defined regulators of the innate immune response, including IRAK-M and certain cytokines, can modulate the expression of MKP-1. In this review, we summarize recent progress in our understanding of the function and regulation of MKP-1 in the innate immune response to microbial infection. We will discuss the role of MKP-1 in the mechanism of action of corticosteroids as well as some pro- and anti-inflammatory cytokines.

The function and regulation of MAP kinases

MAP kinases are a group of serine/threonine protein kinases highly conserved across eukaryotic species. There are three well-defined MAP kinase subfamilies: ERK, JNK, and p38 [36]. The MAP kinase pathway is activated through a cascade of sequential phosphorylation events, beginning with the activation of MAP kinase kinase kinase. MAP kinase kinase kinase activates MAP kinase kinase by phosphorylating two serine residues. MAP kinase kinase in turn activates MAP kinase by phosphorylating the MAP kinase at the adjacent threonine and tyrosine residues in a conserved TXY motif in a regulatory loop between the kinase subdomains VII and VIII [37]. Once activated, the MAP kinase can phosphorylate a wide array of downstream targets, including protein kinases and transcription factors which facilitate the transcription of MAP kinase-regulated genes [36]. MAP kinases also regulate gene expression through facilitation of chromatin remodeling and activation of numerous transcription factors, including activating protein (AP)-1 [36]. Therefore, MAP kinases play a pivotal role in a variety of cellular processes including cell proliferation, differentiation, stress response, apoptosis, and host immune defense.

In innate immune cells, MAP kinases are crucial for the syntheses of numerous cytokines, chemokines, and other inflammatory mediators which are necessary for the immune system to combat pathogenic infections [30]. In addition to controlling the transcription of a variety of pro-inflammatory mediators, MAP kinases also regulate protein expression by altering the stability, transport, and translation of mRNA transcripts containing AU-rich elements (ARE), AUUUA [30]. It has been demonstrated that tristetraprolin (TTP) binds to the AREs of many cytokine transcripts and promotes deadenylation and destabilization of these ARE-containing mRNAs [38]. The p38 MAP kinases, particularly p38α and β, can inhibit the activity of TTP. p38 activates MAP kinase-activated protein kinase (MK)-2 through phosphorylation. MK-2, in turn, inactivates TTP by phosphorylation [39, 40], thereby inhibiting TTP-mediated degradation of ARE-containing transcripts (**Figure 1**). Many pro-inflammatory cytokine transcripts, including TNF-α, IL-1β, IL-6, granulocyte-macrophage colony stimulating factor (GM-CSF), and IL-2, contain ARE(s) in their mRNA and are targets of TTP-mediated mRNA decay [41]. In addition to the regulation of the expression of inflammatory mediators, MAP kinases are also implicated in the regulation of reactive oxygen and nitrogen species, which are critical for the killing of microbes engulfed by phagocytes. In adaptive immune cells, MAP kinases serve as critical regulators in the maturation of T-lymphocytes and clonal expansion of effector T- and B-lymphocytes through modulation of cytokine production, cell proliferation, and survival [30].

MAP kinase pathways are activated through phosphorylation, thus dephosphorylation of MAP kinases by phosphatases is likely the most efficient mode of negative regulation. A number of protein phosphatases are known to deactivate MAP kinases, including tyrosine, serine/threonine, and dual specificity phosphatases [31]. In mammalian cells, a group of dual specificity protein phosphatases are the primary phosphatases responsible for dephosphorylation/deactivation of MAP kinases [31]. These dual specificity protein

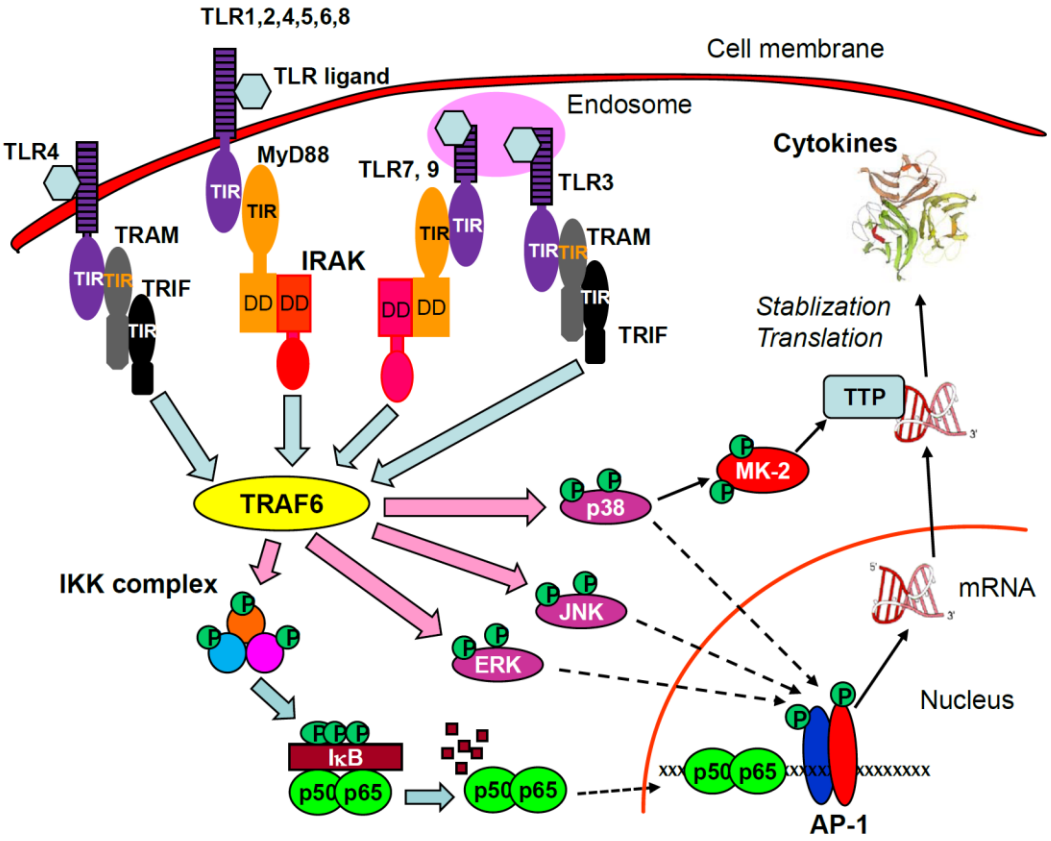


Figure 1. Diagram of the signal transduction pathways initiated at TLRs by microbial components. Binding of microbial components (ligands) to TLRs triggers conformational changes that lead to the recruitment of IRAK and TRAF-6 mediated by adaptor proteins MyD88 and TRIF. TRAF-6 can activate both the NF-κB and MAP kinase pathways. NF-κB is critical for the transcription of inflammatory response genes, including genes of various cytokines and chemokines. MAP kinases, including ERK, JNK, and p38, also regulate the expression of many inflammatory genes. MAP kinases can activate AP-1 transcription factor, thus enhancing gene transcription. MAP kinases, p38 in particular, also enhance cytokine production through post-transcriptional mechanisms. p38 phosphorylates/activates MK-2, which in turn phosphorylates TTP, leading to both enhanced cytokine mRNA stability and accelerated cytokine mRNA translation. DD, death domain.

phosphatases are often referred to as MAP kinase phosphatases (MKPs). To date, at least 10 MKPs have been identified in mammalian cells [31].

MAP kinase phosphatase (MKP)-1

The mouse MKP-1 cDNA was initially identified in the early 1980s as an immediate-early gene induced by mitogens through differential hybridization screening of a BALB/c 3T3 cDNA library [42]. The cDNA clone was initially referred to as 3CH134, which encodes a protein of ~40 kDa. The DNA sequence of 3CH134 was first published in early 1992 [43], and shortly after, a human homolog (CL100) was identified as a tyrosine phosphatase gene strongly induced by hydrogen peroxide [44]. Structurally, both 3CH134 and its human homologue CL100 contain a (I/V)HCXAGXXR(S/T)AG signature motif characteristic of the catalytic domain of tyrosine phosphatases (**Figure 2**). They also share considerable homology with the dual specificity phosphatase of vaccinia virus, VH1, especially at the catalytic sites. However, compared to VH1, both 3CH134 and CL100 are substantially larger due to a novel N-terminal domain. The 3CH134 protein and its human homologue exhibit relatively high

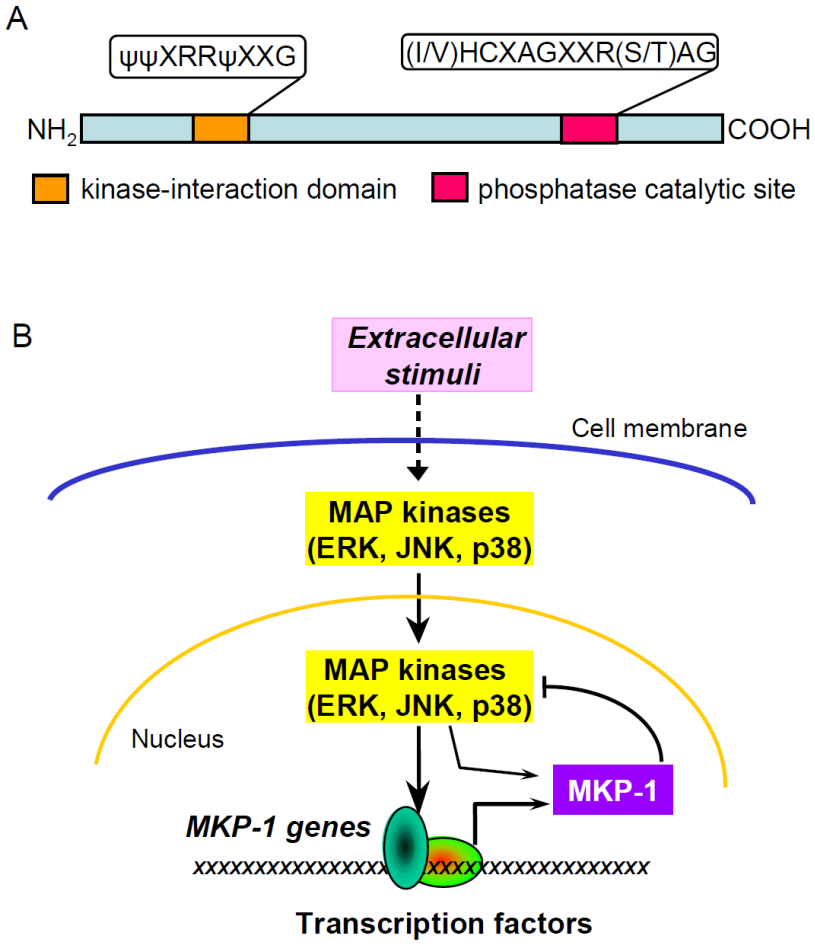


Figure 2. Diagram of the structure and function of MKP-1. (A) The primary structure of MKP-1. MKP-1 has an amino terminal domain responsible for interaction with MAP kinases. The catalytic domain is located at the carboxyl terminus. (**B**) Feedback control of MAP kinases by MKP-1. Extracellular stimulation triggers the activation of MAP kinases. Upon activation, MAP kinases translocate to the nucleus where they phosphorylate and activate transcription factors, leading to altered gene transcription. Among the genes activated by MAP kinases is MKP-1. MKP-1 protein can dephosphorylate MAP kinases, thus terminating MAP kinase-regulated gene transcription. By phosphorylating MKP-1 protein, MAP kinases can regulate the stability of MKP-1 protein.

selectivity towards the ERK MAP kinases, both *in vitro* and in cultured cells [45-48]. As it was the first protein phosphatase found to be specific for the MAP kinases, selectively targeting their phosphotyrosine and phosphothreonine residues, it was designated as MAP kinase phosphatase (MKP)-1 [48]. Since MKP-1 is robustly induced by mitogenic stimulation which also activates ERK MAP kinases, it was proposed that MKP-1 is an important feedback control mechanism governing the ERK pathway (**Figure 2**). Although MKP-1 was initially thought to be an ERK-specific phosphatase, subsequent studies provide compelling evidence that MKP-1 also efficiently, and perhaps preferentially, inactivates the stress-activated JNK and p38 MAP kinases [49, 50]. We found that MKP-1 was robustly induced by genotoxic stress which potently activates JNK but has little effect on ERK, suggesting that MKP-1 may play an important role in the feedback control of the stress-activated MAP kinase subfamilies [49]. To address the substrate preference of MKP-1, Franklin and Kraft established a U937 cell line which conditionally expresses MKP-1.

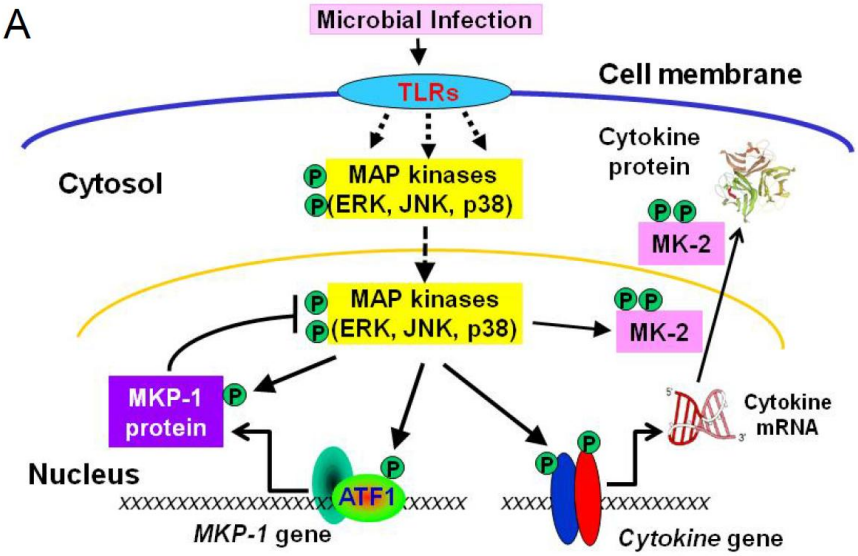
By titrating the expression level of MKP-1, they demonstrated that p38 and JNK were much more sensitive than ERK to dephosphorylation by MKP-1 [51]. Recently, several additional studies conducted using MKP-1 knockout cells further support the conclusion that p38 and JNK, but not ERK, are the preferred substrates of MKP-1 [32, 33, 35]. However, these studies do not exclude the possibility that MKP-1 may also participate in the inactivation of ERK, particularly when MKP-1 is expressed in high levels. In fact, in mouse lung, where MKP-1 expression levels are high [43], knockout of MKP-1 gene results a substantial increase in ERK activity in (Zhao and Liu, unpublished observations).

The activity of MKP-1 can be regulated at many levels. First, MKP-1 expression can be robustly induced by growth factors and stress [31]. Moreover, the induction of MKP-1 by extracellular stimuli occurs in a manner independent of *de novo* protein synthesis [42]. In response to extracellular stimulation, MKP-1 mRNA levels are often increased by 10-100 fold within 15-60 minutes. Since the stability of MKP-1 mRNA does not appear to change significantly [52], it is likely that the induction of MKP-1 expression is primarily mediated by a transcriptional mechanism. Second, the stability of MKP-1 can be altered by phosphorylation. MKP-1 protein is degraded by the ubiquitin-directed proteasome complex [53]. MAP kinases have been shown to phosphorylate MKP-1, and this alters its stability. MKP-1 can be phosphorylated by both ERK and JNK [53, 54]. Phosphorylation by ERK inhibits ubiquitin-mediated degradation, thus enhancing MKP-1 stability [53]. On the contrary, while JNK also phosphorylates MKP-1, such phosphorylation actually stimulates the degradation of MKP-1 [54]. The underlying mechanism for JNK-mediated degradation of MKP-1 is still unclear. At least *in vitro,* p38 efficiently phosphorylates MKP-1 (Chen and Liu, unpublished findings). Whether such phosphorylation is biologically significant awaits further examination. Finally, in addition to transcriptional induction and increased protein stability, the catalytic activity of MKP-1 protein can be enhanced by interaction with its substrate MAP kinases [55, 56]. We have shown that inclusion of any of the three MAP kinases, ERK, JNK, or p38, increases the catalytic activity of MKP-1 protein by 6-8 fold in an *in vitro* biochemical assay [55]. Analysis of the crystal structure of a related phosphatase, MKP-3, has suggested that interaction between MKP-3 and its substrate ERK MAP kinase enables the phosphatase to adopt a more efficient conformation at the catalytic site [57]. Subsequent studies have demonstrated that the interaction between MAP kinases and the MKP family is dependent on the kinase-interaction domain at the amino terminus of the phosphatase and the acidic domain located at the carboxyl terminus of the kinase. The kinase-interaction domain of all MKPs has the consensus sequence of ψψXRRψXXG (where ψ represents a hydrophobic residue and X is any amino acid), which is flanked by two Cdc25-homology domains [31]. The fact that MKP-1 catalytic activity is enhanced by MAP kinases suggests that conformational change upon binding to its substrates is also an important mechanism regulating MKP-1 activity.

The role of MKP-1 in the regulation of host inflammatory response to pathogens

Due to the critical role of MAP kinases in the regulation of innate immune responses, it was long suspected that MKP-1 may be important in innate immune regulation. This notion was supported by the observation that upon exposure to Gram-positive *Listeria monocytogenes*, immortalized murine macrophages underwent a robust MKP-1 induction [58]. Over-expression of MKP-1 in immortalized macrophages significantly attenuated the phagocytosis of *L. monocytogenes*, suggesting that MKP-1 may inhibit innate immune function [59]. Using macrophages derived from mouse bone marrow, Valledor *et al.* demonstrated that MKP-1 was potently induced by bacterial lipopolysaccharide (LPS) through a transcriptional mechanism mediated by protein kinase Cε and a tyrosine kinase(s) [60]. They also found that MKP-1 induction coincided with ERK inactivation, and suggested that MKP-1 may be responsible for modulating ERK MAP kinases in this system [60]. To understand the negative regulation of cytokine expression in innate immune cells during bacterial infection, we studied the role of MKP-1 using RAW 264.7 macrophages and LPS as a model system. We found that stimulation of RAW264.7 macrophages with LPS resulted in a spike in the activity of both JNK and p38 [61]. The activities of these MAP kinases reached peak levels within 15 min,

A



B

Dynamics of the inflammation process

Inflammatory phase	MAPK activity	MKP-1 Level	Cytokine	
			level	synthesis
before infection	very low	very low	very low	minimal
early phase	high	very low	low	fast
intermediate phase	intermediate	intermediate	intermediate	intermediate
late phase	low	high	high	slow
refractory phase	low	high	low	slow

Figure 3. Restraint of pro-inflammatory cytokine biosynthesis by MKP-1. (A). Diagram illustrating the role of MKP-1 in the regulation of the innate immune response. In response to microbial infection, the TLRs initiate a series of signal transduction pathways, including NF-κB and MAP kinase cascades, leading to production of pro-inflammatory cytokines. Simultaneously, the signals initiated at the TLRs also induce MKP-1 gene transcription. MAP kinases regulate MKP-1 expression by two mechanisms. MAP kinases enhance MKP-1 gene transcription. ERK MAP kinase also increases MKP-1 protein stability by phosphorylating MKP-1 and slowing its degradation. The MKP-1 protein in turn dephosphorylates MAP kinases, particularly JNK and p38, thus stopping the perpetuation of the inflammatory cascades and terminating cytokine production. (B) Dynamic shifting of the inflammatory signaling events during the innate immune response. In macrophages upon stimulation with bacterial products, such as LPS, MAP kinases are maximally activated within minutes (early phase). By 30 min (intermediate phase), MKP-1 protein becomes detectable while MAP kinase activity starts to decline. By 60-120 min (later phase), MKP-1 protein reaches peak levels whereas MAP kinase activity returns to nearly basal levels. This is followed by a period of endotoxin non-responsiveness or endotoxin tolerance (refractory phase), which can last for a few days. During this period, MAP kinase activation is inhibited due to the relatively high MKP-1 levels. As a result, cells produce little cytokines.

and returned to nearly basal levels within 60 min, while MKP-1 protein levels were increased dramatically from undetectable basal levels. The kinetics of p38 and JNK deactivation correlated closely with the accumulation of MKP-1 protein such that the increasing level of MKP-1 was temporally associated with the diminution of p38 and

JNK. Unlike JNK and p38, ERK was potently activated in response to LPS stimulation, and its activity did not change significantly with the accumulation of MKP-1 protein. As with LPS, stimulation of RAW264.7 macrophages with peptidoglycan also elicited a transient activation of JNK and p38. The deactivation of these kinases also occurred concomitantly with MKP-1 induction [62]. The importance of MKP-1 in the deactivation of p38 and JNK was demonstrated by blocking of MKP-1 expression pharmacologically with triptolide, a diterpenoid triepoxide. Blockade of MKP-1 induction by the non-specific compound triptolide in LPS-stimulated macrophages prolonged p38 and JNK activation, but had little effect on ERK activity [61, 62]. These results illustrated the importance of MKP-1 in the deactivation of p38 and JNK in these cells. A modest increase in MKP-1 expression in RAW264.7 cells shortened the window of p38 and JNK activation in LPS-stimulated cells, and substantially inhibited the production of both TNF-α and IL-6 [61-63]. These studies established the concept that MKP-1 is a pivotal negative regulator of the innate immune response. The very low basal level of MKP-1 in quiescent innate immune cells permits a narrow window of robust inflammatory response necessary for cytokine production. Yet the rapid induction of MKP-1 following stimulation allows the system to tune down the inflammatory response, preventing the harmful consequences of overzealous inflammation. Thus, by modulating the activities of both p38 and JNK, MKP-1 limits the strength and duration of the important signals controlling the production of inflammatory cytokines. In other words, MKP-1 serves as an internal restraining mechanism to prevent overreaction of the innate immune system (**Figure 3**).

To delineate the physiological function of MKP-1 in microbial infection, several laboratories, including our own, have studied the effects of MKP-1 deficiency on host immune responses using mice as model system [32-35, 63]. MKP-1 knockout mice do not exhibit any phenotype under normal housing conditions [64]. The functions of MKP-1 in the innate immune response were studied using primary macrophages isolated from these mice. Compared to primary macrophages isolated from wild type mice, macrophages originating from MKP-1 knockout mice exhibited prolonged p38 and JNK activation after stimulation with LPS [32]. The kinetics of ERK activation were not altered by MKP-1 deficiency. Similar findings were found with peptidoglycan and lipoteichoic acid, two important cell wall components of Gram-positive bacteria (**Figure 4**). These results confirmed the observation made in immortalized macrophages [62], and firmly established MKP-1 as a primary phosphatase for p38 and JNK in innate immune cells. Compared to wild type macrophages, macrophages isolated from MKP-1-deficient mice produced substantially larger quantities of pro-inflammatory cytokines, including TNF-α and IL-6. Reflecting a profound exaggeration in host inflammatory responses, MKP-1 knockout macrophages also synthesized considerably higher levels of chemokines, including macrophage inflammatory protein (MIP)-1α, MIP-1β, and MIP-2, than wild type macrophages [32-35]. It is important to note that in addition to augmented production of pro-inflammatory cytokines and chemokines, deletion of MKP-1 gene also profoundly enhanced the synthesis of a potent anti-inflammatory cytokine, IL-10. The production of TNF-α, IL-6, and IL-10 was dramatically enhanced in macrophages, splenocytes, and bone marrow-derived dendritic cells derived from MKP-1 knockout mice [32, 35]. The augmented inflammatory responses in MKP-1-deficient innate immune cells are not restricted to the responses to LPS, but are seen in cells exposed to other microbial components, including ligands for TLR2, TLR3, TLR5, TLR7 and TLR9 [35, 62, 65]. However, the expression of two classic T_H-1 cytokines, IL-12 and interferon (IFN)-γ, was decreased in MKP-1-deficient splenocytes and dendritic cells [32], suggesting a shift in cytokine production profiles. To understand the function of MKP-1 in the regulation of inflammation systemically, Hammer *et al*. stimulated wild type and MKP-1 knockout mice with endotoxin and analyzed gene expression profiles in the spleens of the two strains of mice using microarray [33]. They found that in the spleens of wild type mice approximately 160 genes exhibited >2-fold increase in expression levels while in the spleens of the MKP-1 knockout mice approximately 3 times as many genes exhibited >2-fold increase.

Consistent with the notion that MKP-1 knockout results in hyper-inflammation in response to LPS challenge, upon LPS injection MKP-1-deficient mice produced substantially

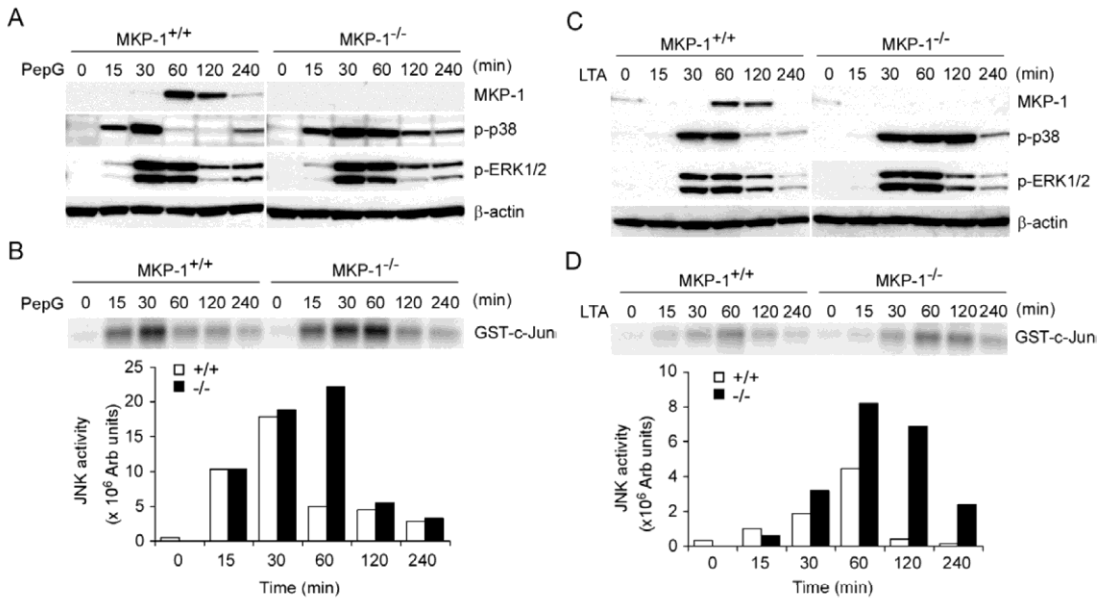


Figure 4. MKP-1 knockout results in prolonged p38 and JNK activation in primary macrophages stimulated with cell wall components of Gram-positive bacteria, peptidoglycan (PepG) and lipoteichoic acid (LTA). Peritoneal macrophages isolated from MKP-$1^{+/+}$ and MKP-$1^{-/-}$ mice were stimulated with 10 μg/ml PepG or 10 μg/ml LTA for the indicated time, and harvested. The activities of ERK and p38 were assessed by Western blot analysis using phosphor-ERK or phosphor-p38 antibodies (**A, C**). JNK activity was analyzed by immune complex kinase assays, using [γ-^{32}P] ATP and recombinant GST-c-Jun as substrates (**B, D**).

greater amounts of TNF-α, IL-1β, monocyte chemoattractant protein (MCP)-1/CCL2, GM-CSF, IL-6, and IL-10, than did wild type mice [32-35]. The excessive production of the pro-inflammatory mediators was associated with a marked increase in LPS sensitivity. Compared to wild type mice, mice deficient in MKP-1 more readily succumbed to LPS challenge, as indicated by injury and dysfunction in multiple organs and a higher rate of mortality [32-35]. The lungs of the LPS-challenged MKP-1 knockout mice exhibited more severe lung edema associated with massive neutrophil infiltration [32]. Higher blood alanine aminotransferase activities were detected in these mice, [32]. Moreover, a marked increase in leukocyte infiltration in the vicinity of the bile ducts occurred in LPS-challenged MKP-1 knockout mice, but not in similarly treated wild type mice. Kidney function was also compromised in the MKP-1 knockout mice upon challenge with modest dose of LPS, whereas similarly treated wild type mice exhibited normal kidney function [32]. Severe hypotension is a clinical characteristic of sepsis and plays a direct role in the development of shock and multi-organ dysfunction syndrome [15]. MKP-1 knockout mice exhibited impaired cardiovascular responses after LPS challenge [32]. While LPS challenge at a dose of 1.5 mg/kg did not significantly affect the systemic blood pressure in wild type mice, the same challenge caused a substantial and long-lasting decrease in systemic blood pressure in MKP-1 knockout mice. Underlying the severe decrease in blood pressure in MKP-1 knockout mice, a marked increase in circulating NO was detected in these mice [32]. Analysis of the lungs and livers of these mice have indicated that iNOS expression levels were substantially greater in MKP-1 knockout mice than in similarly treated wild type mice (Zhao and Liu, unpublished observations). Additionally, in response to either cell wall components isolated from *Staphylococcus aureus* or heat-killed *S. aureus*, a clinically relevant Gram-positive bacterial pathogen, MKP-1 knockout mice exhibited more severe injury and greater mortality than did similarly treated wild type mice [65]. These observations strongly support the conclusion that MKP-1 functions as a

critical negative regulator during both Gram-positive and Gram-negative bacterial infection. By limiting the strength and duration of the inflammatory signals, MKP-1 serves to constrain the host inflammatory responses and prevents septic shock (**Figure 3**).

In addition to the function of MKP-1 in the regulation of cytokines, MKP-1 also plays an important role in the regulation of cyclooxygenase (COX)-2 and mucin during microbial infection. We have recently found that MKP-1 knockout mice express dramatically greater levels of COX-2 in response to Gram-negative bacteria, *E. coli* (Wang and Li, unpublished observations). The products of COX-2 have been implicated in a variety of physiological processes including metabolism and phagocytosis [66, 67]. It is plausible that many of the metabolic processes and antimicrobial functions of the immune system are regulated by MKP-1. This speculation is supported by experiments studying the effects of MKP-1 on the physiology and immune function during *E. coli* infection. We found that mice deficient in MKP-1 exhibited marked abnormalities in lipid metabolism and bacterial clearance (Frazier, Wang, and Liu, unpublished findings).

Mucosal epithelial cells in the respiratory tract act as the first line of host innate defense against inhaled microbes by producing a range of molecules for clearance, including mucins. Epithelial mucins facilitate the mucociliary clearance by physically trapping inhaled microbes, and increased mucin production thus represents an important host innate defense mechanism against invading microbes. However, excessive mucin production overwhelms the mucociliary clearance, and therefore is detrimental for mucosal defenses. Thus, tight regulation of mucin production is critical for maintaining an appropriate balance between beneficial and detrimental effects. Ha *et al.* have recently demonstrated that the PAK4-JNK signaling pathway acts as a negative regulator for *Streptococcus pneumoniae* pneumolysin-induced MUC5AC mucin transcription [68]. Moreover pneumolysin selectively induced expression of MKP-1 *via* a TLR4-dependent MyD88-TRAF6-ERK signaling pathway. Their studies indicate that by inhibiting the PAK4-JNK signaling pathway MKP-1 up-regulates mucin production, thereby facilitating effective mucosal protection against *S. pneumoniae* infection.

The regulation of MKP-1 during innate Immune responses

During the innate immune response, the activity of MKP-1 is regulated at multiple levels, including transcriptional induction, protein stabilization, and catalytic activation. Recently, MKP-1 has also been reported to undergo acetylation, and its acetylation enhances the interaction with p38, thus stimulating its catalytic activity. We will discuss each of these aspects in detail.

Transcriptional regulation of MKP-1

Transcriptional induction of MKP-1 gene is a major contributing factor to the increases in MKP-1 protein during the innate immune response. MKP-1 mRNA can be detected within 15 minutes after exposure of macrophages to bacterial components, with maximal mRNA levels > 100-fold above basal levels reached within 1 hour. Valledor *et al.* demonstrated that MKP-1 was potently induced in bone marrow-derived macrophages by LPS through a transcriptional mechanism mediated by protein kinase Cε and a tyrosine kinase (or kinases) [60]. In RAW264.7 macrophages, the transcriptional induction of MKP-1 by LPS was substantially inhibited by the MEK1/2 inhibitor U0126, suggesting that ERK plays an important role in the induction of MKP-1 transcription. Since the ERK pathway is regulated, at least in part, by the protein kinase Cε and a tyrosine kinase(s) [69], it is tempting to speculate that at least some of the effects of these upstream regulators on MKP-1 are mediated by ERK MAP kinases. However, U0126 did not completely block MKP-1 induction, suggesting that other pathways also contribute to MKP-1 induction [61]. Sanchez-Tillo *et al.* demonstrated recently that JNK1 is required for the induction of MKP-1 in macrophages in response to LPS [70]. Moreover, we found that p38 also plays a significant role in the induction of MKP-1 in RAW264.7 macrophages. Thus, MKP-1 induction during the innate immune response is likely regulated by multiple signaling pathways.

Although it has been recognized for more than 20 years that MKP-1 gene transcription is

potently induced by a variety of extracellular stimuli in many cell types [42, 44, 71, 72], the transcription factors involved remain poorly understood. Several putative transcription factor-binding elements were identified in the promoter region of MKP-1 gene, including two cAMP-responsive elements, three SP-1 sites, one AP-1 site, two AP-2 sites, and one NF-1 element within a ~400-bp region upstream of the transcription start site in the human MKP-1 promoter [72]. A major reason for the slow progress in the understanding of transcriptional regulation is the seemingly constitutive activity of the MKP-1 promoter in transient transfection assays [73]. The endogenous gene is expressed at a very low basal level for most cell types and tissues, except the lung [43]. In response to extracellular stimulation, MKP-1 transcription undergoes dramatic induction, with mRNA increases of tens or hundreds fold within a short period [31, 74]. Unlike the endogenous gene, the MKP-1 reporter exhibits a very high basal activity, and little or no increase is observed with extracellular stimulation [73]. However, a substantial increase in reporter activity was observed after extracellular stimulation when the reporter construct was integrated into the genome, suggesting that chromatin remodeling is involved in the transcriptional induction of MKP-1 gene [73]. Very recently, significant inroads have been made in the understanding of MKP-1 induction. Lu *et al*. have shown that retinoic acid induces MKP-1 expression through a transcriptional mechanism mediated by cAMP-response element binding protein (CREB) and upstream transcription factor 1 (USF1) in human immunodeficiency virus-infected podocytes. The important role of CREB in MKP-1 induction in macrophages after stimulation with TLR ligands has also been demonstrated convincingly by Ananieva *et al.* in an elegantly designed experiment [75]. Ananieva *et al.* showed that ligands for TLR2, 4, and 9 activate ERK and p38 MAP kinases, leading to activation of downstream kinases referred to as mitogen- and stress-activated protein kinase (MSK) 1 and MSK2. MSK1/2 potently phosphorylates transcription factors that bind CRE sequences in the MKP-1 promoter, including CREB and ATF1. The importance of MSK1 and 2 in the induction of MKP-1 and IL-10 mediated by CREB and/or ATF1 are demonstrated by chromatinimmunoprecipitation assays. CREB is phosphorylated by MSK1/2 on serine-133. Since mutation of serine-133 to alanine in CREB attenuated IL-10 but not MKP-1 induction, Ananieva *et al.* speculated that MKP-1 induction in response to TLR ligands is likely mediated by ATF1 [75]. Further supporting the critical role of MSK-mediated upregulation of MKP-1 and IL-10, mice deficient in MSK1 and MSK2 exhibited enhanced inflammatory responses and increased mortality relative to wild type mice after LPS challenge. These studies filled a critical gap in the understanding of MKP-1 regulation, and significantly advanced our understanding of the negative regulation of the innate immune response.

To determine which mediators involved in TLR signaling are responsible for the induction of MKP-1, Chi *et al.* examined the expression of MKP-1 in primary macrophages lacking either MyD88 or TRIF [35]. It is well established that LPS initiates innate immune responses through two pathways: a cascade mediated by MyD88 and a signaling pathway mediated by TRIF. The induction of MKP-1 by LPS was reduced in both the MyD88- and TRIF-deficient cells, as compared with wild type cells, indicating that both MyD88 and TRIF contribute to optimal MKP-1 induction. However, in response to ligands of TLR9 and TLR2, which only signal through MyD88, MKP-1 induction was completely ablated in $MyD88^{-/-}$ macrophages, but was normal in TRIF-deficient cells. Conversely, loss of TRIF, but not of MyD88 function, eliminated MKP-1 expression induced by poly(I-C), a TLR3 ligand that signals only through TRIF. Together, these results demonstrate that MKP-1 is induced through MyD88 and TRIF-dependent pathways in response to various TLR ligands.

Stabilization of MKP-1 protein during the innate immune response

MKP-1 protein becomes markedly more stable upon LPS stimulation, with a four-fold increase in half-life [61]. The increase in MKP-1 stability in response to LPS is abolished by a pharmacological inhibitor of the ERK pathway, supporting an additional role of ERK in the regulation of MKP-1. Perhaps it is not a surprise that ERK-mediated stabilization of MKP-1 protein in response to LPS was largely abolished by deletion of 53 amino acids from the carboxyl terminus of MKP-1 [61]. This carboxyl terminal region contains an ERK-docking site [76] and two serine residues phosphorylated by ERK [53]. Previously,

Brondello *et al.* showed that phosphorylation of MKP-1 by ERK attenuates MKP-1 degradation, a process mediated by the ubiquitin-directed proteasome complex [53]. Additionally, IRAK-M contributes to LPS-mediated MKP-1 stabilization. In contrast to wild type cells, IRAK-M-deficient macrophages exhibit a marked decrease in MKP-1 levels following LPS challenge [26]. Similar to MKP-1 deficient cells, IRAK-M deficient macrophages demonstrate sustained activation of p38 and JNK upon LPS challenge [26, 77]. In addition, the expression levels of IL-6 and GM-CSF in IRAK-M deficient cells following LPS stimulation were significantly elevated compared with those in wild type cells [26]. During the later phase of septic shock, humans or experimental animals often undergo significant changes in immune cell function. Characteristics of the state of immunological deactivation in late sepsis include decreased expression of cell surface markers of activation (e.g. the MHC-II molecule, HLA-DR), decreased antigen presentation, and decreased capacity for production of cytokines following stimulation with a variety of agonists [23, 78-80]. This may serve as a compensatory mechanism to down-modulate excessive inflammation. Intriguingly, IRAK-M levels are induced during experimental sepsis in animal models and humans with septic shock, potentially contributing to the endotoxin-tolerant phenotype [81-83]. Further studies are warranted to examine the cross-talk between IRAK-M and MKP-1 and its physiological implications during the inflammatory processes accompanying septic shock. The molecular mechanism underlying IRAK-M-mediated MKP-1 stabilization also remains to be delineated.

Acetylation of MKP-1 enhances its association with p38 MAP kinase and potentiates its regulatory capacity during innate immune responses

In addition to stabilization of MKP-1 protein by phosphorylation, acetylation of MKP-1 protein has recently emerged as another mode of post-translational modification [84]. Cao *et al.* have demonstrated that MKP-1 protein is acetylated on lysine-57 residue in macrophages stimulated with LPS, and pretreatment with trichostatin A (a histone deacetylase inhibitor) enhanced MKP-1 acetylation. Lysine-57 is located near the kinase-interaction motif in the MKP-1 molecule [85]. Cao *et al.* found that acetylation of MKP-1 neither affects its protein stability nor alters its intrinsic phosphatase activity. Biochemical analysis demonstrated that MKP-1 interacts with p300, a transcriptional coactivator with acetyltransferase activity. Moreover, both p300 and PCAF, another transcriptional coactivator with acetyltransferase activity, can catalyze the acetylation of MKP-1 *in vitro*. This group also demonstrated that acetylation of MKP-1 potentiates its interaction with p38. Because of the increased affinity between the two proteins, MKP-1 undergoes more efficient catalytic activation in the presence of p38 protein, thus providing a mechanistic explanation for the inhibition of p38 and macrophage responses by trichostatin A. In wild type mice, trichostatin A substantially inhibited the innate immune responses to LPS, and prevented mortality. However, the protective effects of trichostatin A were substantially compromised in MKP-1-deficient mice, illustrating the importance of MKP-1 acetylation in the regulation of innate immune responses. Future studies are required to confirm these findings in other laboratories, and to understand whether MKP-1 acetylation is involved in the regulation of the immune responses to other TLR ligands.

MKP-1 and immunomodulatory agents

The fact that MKP-1 acts as a critical negative regulator of the inflammatory response raises an intriguing question of whether MKP-1 plays a significant role in the mechanism of action of immunomodulatory agents. We examined the effects of a panel of commonly used anti-inflammatory drugs on the expression of MKP-1. We found that MKP-1 is significantly induced by dexamethasone, an anti-inflammatory glucocorticoid, in RAW264.7 macrophages [61]. Such induction provided a mechanistic explanation to an earlier observation by Swantek *et al*. that dexamethasone inhibited LPS-induced JNK activation [86]. To understand the mechanism underlying the inhibitory effect of dexamethasone on COX-2 expression, Lasa *et al.* studied the effect of dexamethasone on MKP-1 expression in HeLa cells [87, 88]. They demonstrated that dexamethasone induced MKP-1 expression in HeLa cells, and that this induction was responsible for the inhibition of p38 and decreased expression of COX-2 [89]. An earlier investigation by Kassel *et al.* indicated that dexamethasone induced MKP-1

in mast cells and that such an induction was responsible for the inhibitor effects of glucocorticoids on ERK activity [90]. To delineate the role of MKP-1 in the anti-inflammatory function of glucocorticoids, our laboratory compared a group of synthetic corticosteroids with different anti-inflammatory potencies with regard to their capacity to induce MKP-1 expression. We found that in RAW264.7 macrophages the abilities of these synthetic glucocorticoids to induce MKP-1 expression were closely associated with their relative anti-inflammatory potencies [63]. Very recently, using macrophages isolated from MKP-1 knockout mice, Abraham *et al.* demonstrated that p38 and JNK activation, in response to LPS stimulation, was no longer inhibited by dexamethasone in MKP-1-deficient cells [91]. Accordingly, many of the inflammatory genes, including certain cytokines, are less sensitive to the suppressive effects of dexamethasone in MKP-1-deficient cells. Moreover, we found that dexamethasone was unable to prevent endotoxic shock in MKP-1 knockout mice while it effectively protects wild type mice from endotoxin-induced mortality [92]. It is important to note that MKP-1 is not only involved in the anti-inflammatory action of exogenously administered synthetic glucocorticoids, but also mediates the immunosuppressive effects of the endogenous stress hormone cortisol [93]. These studies suggest that MKP-1 induction constitutes an important part of the anti-inflammatory mechanism of glucocorticoids. Since glucocorticoids are immunosuppressive substances released endogenously upon exposure to stress, MKP-1 induction by corticosteroids may represent a potential mechanism underlying the immunosuppressant property of stress. It should be pointed out that, while MKP-1 is required for the optimal anti-inflammatory activity of glucocorticoids, corticosteroids inhibit inflammatory responses through multiple mechanisms. For example, in addition to MAP kinases, glucocorticoids potently inhibit the transcription factor NF-κB [94]. We found that in the MKP-1-deficient macrophages and MKP-1 knockout mice, dexamethasone still exhibited a potent inhibitory effect on TNF-α production [92]. Our results are consistent with the observation of Meier *et al.* who demonstrated TNF-α production in MKP-1-deficient mast cells is still sensitive to glucocorticoid inhibition [95].

IL-10 also enhances MKP-1 activity induced by LPS, although IL-10 alone does not increase MKP-1 expression [96]. Hammer *et al*. performed a systematic analysis of genes whose expression was altered in response to IL-10 and LPS exposure, and found that several MKP genes were induced in macrophages by LPS [96]. Interestingly, they found that MKP-1 expression was transiently up-regulated after stimulation with LPS alone, and MKP-1 expression was enhanced and prolonged when cells were stimulated with both IL-10 and LPS. IL-10 also synergized with dexamethasone in the induction of MKP-1 and in the inhibition of IL-6 and IL-12 production. Up-regulation of MKP-1 by IL-10 in LPS-stimulated macrophages was correlated with a faster p38deactivation, suggesting that induction of MKP-1 may constitute an important part of the anti-inflammatory mechanism of IL-10 [96].

Since MKP-1 acts to restrain inflammatory responses, it is not surprising that cytokines known to boost inflammation can inhibit MKP-1 expression. IFN-γ is a T_H-1 cytokine which enhances the antimicrobial activity of macrophages. It has been shown that priming resident peritoneal macrophages with IFN-γ dramatically increases the production of NO and TNF-α upon stimulation with LPS [97, 98]. We found that priming of peritoneal macrophages with IFN-γ significantly attenuates the MKP-1 expression induced by LPS, which is associated with prolonged activation of p38 and JNK [32]. Interestingly, while LPS does not significantly induce iNOS expression in wild type resident macrophages without IFN-γ priming, LPS in the absence of IFN-γ induces a substantial iNOS expression in MKP-1-deficient resident macrophages (Zhao and Liu, unpublished observations). Recently Vallendor *et al.* have shown that inhibition of MKP-1 by IFN-γ is responsible for the prolonged MAP kinase activation and underlies the growth inhibitory effects of IFN-γ on M-CSF-stimulated macrophages [99, 100]. These observations suggest that inhibition of MKP-1 by IFN-γ may be an important part of the mechanism underlying the immunomodulatory properties of IFN-γ.

Macrophage migration inhibitory factor (MIF) is a potent pro-inflammatory cytokine which enhances the expression of other pro-inflammatory cytokines in macrophages. MIF is

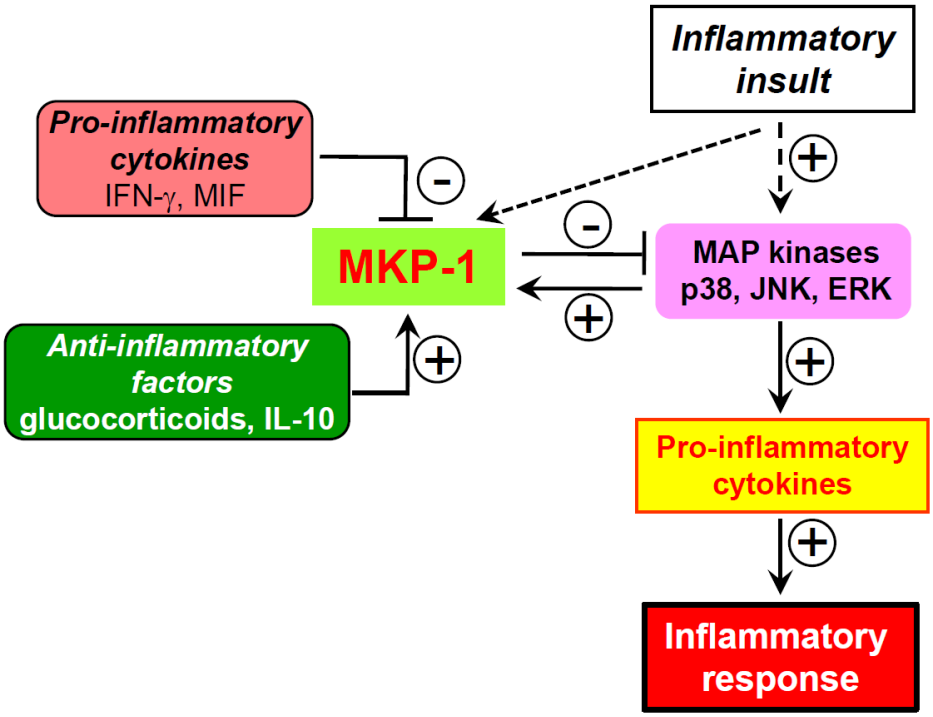


Figure 5. Regulation of MKP-1 expression by immunomodulatory agents. Anti-inflammatory/ immunosuppressive agents, such as glucocorticoids and IL-10, induce/augment MKP-1 expression, leading to inhibition of the p38 and JNK cascades and attenuation of inflammatory response. In contrast, pro-inflammatory cytokines, such as IFN-γ and MIF, inhibit MKP-1 expression, thereby perpetuating the p38 and JNK signalling pathways and enhancing the inflammatory response.

tightly associated with mortality caused by bacterial sepsis in experimental models. Either knockout of the MIF gene or depletion of MIF protein protects animals from septic shock [101, 102]. MIF is considered as counter-regulator of the immunosuppressive effects of glucocorticoids [103]. Recently, Roger *et al*. reported that MKP-1 is a critical mediator in the MIF-glucocorticoid crosstalk [104]. They demonstrated that recombinant MIF antagonized the dexamethasone effect in activated macrophages. They also found that MIF inhibited the induction of MKP-1 by LPS and dexamethasone, and prevented the inhibition of TNF-α and IL-8 production caused by dexamethasone in macrophages. In contrast, blockade of MIF expression augmented MKP-1 induction by dexamethasone, leading to decreased TNF-α production. Independently, Aeberli *et al*. also found that endogenous MIF modulates glucocorticoid sensitivity in macrophages *via* inhabiting MKP-1 [105]. These studies demonstrate that MIF acts through attenuating MKP-1 expression to override inhibition by glucocorticoids of cytokine production in innate immune effector cells. Taken together, it appears that a number of immunomodulatory agents influence the MAP kinase-mediated inflammatory responses, at least in part, through regulating MKP-1 expression (**Figure 5**).

Closing Remarks

The balance between activation and subsequent deactivation of the inflammatory response is critical during the host immune responses against microbial infection. While initiation of the signal transduction cascades is pivotal for mounting an aggressive immune response to invading pathogens, deactivation of the signaling pathways limits the potentially harmful effects of excessive inflammation on

the host, thus preventing collateral damage. Moreover, deactivation of the inflammatory cascades also "resets" the regulatory circuits, allowing the immune system to react to subsequent pathogenic challenges. Through millions of years, evolution has put in place a regulatory mechanism of staggering sophistication to control the immune responses. To respond to the vast array of pathogenic challenges, a number of negative regulators operate at almost every step in the critical signal transduction pathways to moderate immunological responses. These negative regulators act to restrain the strength and duration of the transduced signals, thereby modulating the production of inflammatory cytokines and determine the course of the adaptive immune responses [106]. It has been shown that TLR4 is briefly down-regulated upon exposure to endotoxin [107]. In addition to negative regulation at the receptor level, a number of anti-inflammatory proteins are induced with the expression of effector pro-inflammatory cytokines. These anti-inflammatory proteins include IRAK-M, suppressor of cytokine-signaling-1, IκB, MKP-1, anti-inflammatory cytokines, such as IL-10, and cytokine receptor decoys such as IL-1Ra [106]. These inhibitory proteins turn off downstream signaling events, thus not only stopping the propagation of the inflammatory signals, but also restoring the homeostasis in the innate immune cells. Therefore, a timely termination of the signaling events is crucial, as it not only prevents the over-production of the potentially harmful cytokines, but also prepares the cells for responding to subsequent infections. The discovery of MKP-1 as a crucial negative regulator of the innate immune response, both *in vivo* and *in vitro*, places it in the center of the complex negative regulatory mechanism. The fact that many known immunomodulatory agents exert their immuno-regulatory actions at least partially through adjusting MKP-1 activity highlights the potential of MKP-1 as a therapeutic target in the treatment of immunological disorders. Thus, small molecule chemicals capable of enhancing or inhibiting MKP-1 activity could be novel drug candidates for treatment of certain human diseases including septic shock, arthritis, and cancer.

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Abbreviations used: MKP, MAP Kinase Phosphatase; MAP, mitogen-activated protein; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; MK-2, MAP kinase-activated protein kinase-2; MSK, mitogen- and stress-activated protein kinase; IKK, IκB kinase; IRAK, IL-1 receptor-associated kinase; LPS, lipopolysaccharides; TNF, tumor necrosis factor; IL, interleukin; IFN, interferon; GM-CSF, granulocyte macrophage colony stimulating factor; MIF, macrophage migration inhibitory factor; MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein; NO, nitric oxide; iNOS, inducible nitric oxide synthase; COX, cyclooxygenase; NF-κB; nuclear factor-κB; I-κB; inhibitor-κB; AP-1, activating protein-1; AP-2, activating protein-2; SP-1, stimulating protein-1; CRE, camp-response element; CREB, camp-response element-binding protein; NF-1, nuclear factor-1; ATF1, activating transcription factor 1; ARE, AU-rich element; TTP, tristetraprolin; TLR, toll-like receptor; MyD88, myeloid differentiation factor 88; TIR, TLR/IL-1 receptor domain; TRIF, Toll-IL-1 receptor domain containing adaptor inducing IFN-β; TRAM, TRIF-related adaptor molecule; TRAF; TNF receptor-associated factor; MHC, major histocompatibility complex; HLA, human lymphocyte antigen.

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