MyD88$_S$, a splice variant of MyD88, differentially modulates NF-κB- and AP-1-dependent gene expression

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Abstract MyD88 is an adapter protein that is involved in Toll-like receptor (TLR)- and interleukin-1 receptor (IL-1R)-induced activation of nuclear factor-κB (NF-κB) and c-Jun N-terminal kinase (JNK). By directly binding IL-1R-associated kinase (IRAK)-1 and IRAK-4, MyD88 serves as a bridging protein, enabling IRAK-4-induced IRAK-1 phosphorylation. We previously identified a lipopolysaccharide-inducible splice variant of MyD88, MyD88$_S$, which specifically prevents the recruitment of IRAK-4 into the IL-1R complex and thus inhibits IRAK-4-mediated IRAK-1 phosphorylation. MyD88$_S$ is not able to activate NF-κB, and in contrast functions as a dominant negative inhibitor of TLR/IL-1R-induced NF-κB activation. Unexpectedly, we here demonstrate that MyD88$_S$ still allows JNK phosphorylation and activator protein (AP)-1-dependent reporter gene induction upon overexpression in HEK293T cells. These observations indicate that NF-κB and JNK activation pathways can already diverge at the level of MyD88. Moreover, the regulated expression of a MyD88 splice variant which specifically interferes with NF-κB- but not AP-1-dependent gene expression implies an important role for alternative splicing in the fine-tuning of TLR/IL-1R responses.

Key words: MyD88; c-Jun N-terminal kinase; Interleukin-1 receptor-associated kinase; Nuclear factor-κB; Activator protein-1; Toll/interleukin-1 receptor

1. Introduction

MyD88 is a universal adapter protein for members of the Toll-like receptor/interleukin-1 receptor (TLR/IL-1R) superfamily (reviewed in [1]). TLR/IL-1Rs are a group of evolutionarily conserved receptors that coordinate the innate immune response and are characterized by the presence of a so-called Toll/IL-1R (TIR) motif in their intracellular domain. Whereas IL-1R signaling seems to be specifically mediated by MyD88 [2], several TLRs also require other adapter molecules, Mal (also named TIRAP) or TRIF (also named TICAM-1) [3–6], for downstream signaling. MyD88 or MyD88-like adapters couple to several members of the IL-1R-associated kinase (IRAK) family (reviewed in [7]). Through its C-terminal TIR domain and its N-terminal death domain, MyD88 links receptors of the TLR/IL-1R family with IRAK-1 [8]. Via its intermediate domain (ID), MyD88 also recruits IRAK-4 and in this way mediates close association of the two IRAK family members [9]. This enables IRAK-4 to phosphorylate IRAK-1, which is probably the initial trigger to activate IRAK-1′s own kinase activity and IRAK-1 hyperphosphorylation [10]. Phosphorylated IRAK-1 then interacts with tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6), which further transmits a signal leading to activation of the IκB kinase complex and c-Jun N-terminal kinase (JNK) [11]. This eventually results in enhanced transcriptional activity of nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) transcription factors. It is generally accepted that activation of NF-κB- and AP-1-dependent gene transcription upon TLR/IL-1R triggering strongly contributes to the development of the innate immune response.

We previously identified a lipopolysaccharide (LPS)-inducible splice variant of MyD88, MyD88$_S$, which lacks the ID [12]. MyD88$_S$ prevents recruitment of IRAK-4, but not IRAK-1, into the receptor complex and therefore does not allow IRAK-4-mediated IRAK-1 phosphorylation [9]. As a result, MyD88$_S$ exerts a dominant negative effect on LPS- and IL-1-induced NF-κB activation, whereas TNF-induced NF-κB activation is unaffected [12]. In the present study, we show that MyD88$_S$ still induces JNK activation and AP-1-dependent gene expression, suggesting an important role for alternative splicing in the fine-tuning of TLR/IL-1R signaling.

2. Materials and methods

2.1. Cell culture and biological reagents

HEK293T cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 50 μM 2-mercaptoethanol. HEK293-mycIL1RI cells, stably expressing Myc-IL1RI, were grown in DMEM/NutMixF12 (with pyridoxine and glutamax), supplemented with 2% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Recombinant murine IL-1β was produced by *Escherichia coli* and purified to at least 99% homogeneity. The IL-1β preparation used had a specific biological activity of 3.65×10^8 IU/mg purified protein, as determined with the international standard (code 93/668; National Institute for Biological Standards and Control, Potters Bar, UK).

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Abbreviations: AP, activator protein; ID, intermediate domain; IL-1R, interleukin-1 receptor; IRAK, IL-1R-associated kinase; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; TIR, Toll/interleukin-1 receptor; TLR, Toll-like receptor; TRAF, tumor necrosis factor receptor-associated factor

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2.2. Expression vectors

pCAGGS-E-MyD88$_L$, pCAGGS-E-MyD88$_S$ and pCRIII-MyD88-lpr (MyD88 with point mutation F56N) have been described previously [9,12]. pCDNA3-AU1-MyD88-TIR(152–296) was a generous gift of Dr. M. Muzio (Mario Negri Institute, Milan, Italy) and has been described elsewhere [13]. pNFconluc, containing the luciferase reporter gene driven by a minimal NF-κB responsive promoter, was a gift of Dr. A. Israel (Institut Pasteur, Paris, France). pactβgal, containing the β-galactosidase gene after the β-actin promoter, was obtained from Dr. Inoue (Institute of Medical Sciences, Tokyo, Japan). An AP-1-dependent luciferase reporter construct, pAP-1luc, was purchased from Stratagene (PathDetect System). pJNK1-Flag was obtained from Christian Widmann (Denver, CO, USA).

2.3. Transient transfection and NF-κB and AP-1 reporter gene assays

HEK293T and HEK293-IL1RI cells were transiently transfected by the DNA calcium phosphate precipitation method with 100 ng pNFconluc or 100 ng pAP-1luc, 100 ng pactβgal and different concentrations of specific MyD88 expression plasmids. The total amount of DNA was kept constant by adding empty vector up to 1 μg DNA per well in a six-well plate. NF-κB and AP-1 activity was determined by measuring the luciferase activity present in cell extracts. Luciferase values were normalized for differences in transfection efficiency on the basis of β-galactosidase activity in the same extracts, and expressed as fold induction values relative to the unstimulated empty vector control. In parallel, cell extracts of the same transfections were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and immunoblotted to assess expression levels of the transfected proteins.

2.4. Western blotting

Protein lysates were prepared in RIPA buffer (25 mM Tris, pH 8.2, 50 mM NaCl, 0.5% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 10 μM iodoacetate) supplemented with protease and phosphatase inhibitors. Lysates were analyzed by 10% SDS–PAGE and Western blotting. Antibodies used were anti-MyD88 (Immucor, Roedermark, Germany), anti-Flag/M2 (Sigma, St. Louis, MO, USA), anti-phosphoJNK (Cell Signaling, Beverly, MA, USA), anti-JNK (Cell Signaling) and anti-AU1 (Eurogentec, Seraing, Belgium).

3. Results

3.1. MyD88$_S$ differentially affects AP-1- and NF-κB-dependent gene expression

We previously demonstrated that MyD88$_S$ is no longer able to activate NF-κB-dependent gene expression [12]. In the present study, we analyzed the ability of MyD88$_S$ to activate AP-1-dependent gene expression. Therefore, different concentrations of MyD88$_L$ and MyD88$_S$ expression plasmids were cotransfected with an AP-1-dependent luciferase reporter gene construct in HEK293T cells. An earlier described mutant of MyD88, MyD88-TIR, which only encodes the TIR domain and activates neither JNK nor NF-κB [8,14], was also tested as a control. Proper expression of the different MyD88 constructs was evaluated on Western blot (Fig. 1A,B, lower panels). In line with previous data [14], overexpression of MyD88$_L$ induced AP-1 reporter gene expression (Fig. 1A). Interestingly, MyD88$_S$ also induced AP-1 activation in a dose-dependent way, whereas MyD88-TIR did not. In contrast, and as previously described [12], MyD88$_S$ was unable to activate NF-κB-dependent gene expression (Fig. 1B). These results demonstrate that in contrast to MyD88$_L$, MyD88$_S$ specifically induces AP-1-dependent gene expression, suggesting that in HEK293T cells NF-κB and AP-1 activation pathways can diverge early in the TLR/IL-1R signaling pathway.

3.2. MyD88$_S$ and MyD88$_L$ induce JNK phosphorylation

Since AP-1 is regulated by JNK, the above observations suggest that MyD88$_S$ still activates the JNK pathway. Therefore, we investigated if MyD88$_S$ was still able to activate JNK phosphorylation. We cotransfected a Flag-tagged expression vector for JNK1 with expression plasmids for MyD88$_L$, MyD88$_S$ or MyD88-TIR, and analyzed JNK phosphorylation by immunoblotting with phospho-JNK-specific antibodies. We were unable to detect phosphorylation of endogenous JNK (data not shown). Both in the presence of MyD88$_L$ and in the presence of MyD88$_S$, a marked induction of phosphorylated JNK could be observed, although MyD88$_L$ proved to be a more potent activator (Fig. 2A, upper panel). In contrast, MyD88-TIR did not induce JNK phosphorylation.

In line with these observations, IL-1-induced JNK phosphorylation was not affected by co-expression of MyD88$_S$ or MyD88$_L$ (Fig. 2B). In contrast, co-expression of MyD88-TIR or MyD88-lpr (a F56N mutant that corresponds to the site that is mutated in the Fas-lpr receptor and disrupts the proper folding of the death domain [14]) strongly interfered with IL-1-induced JNK phosphorylation. Thus, in contrast to its dominant negative effect on IL-1-induced NF-κB activa-

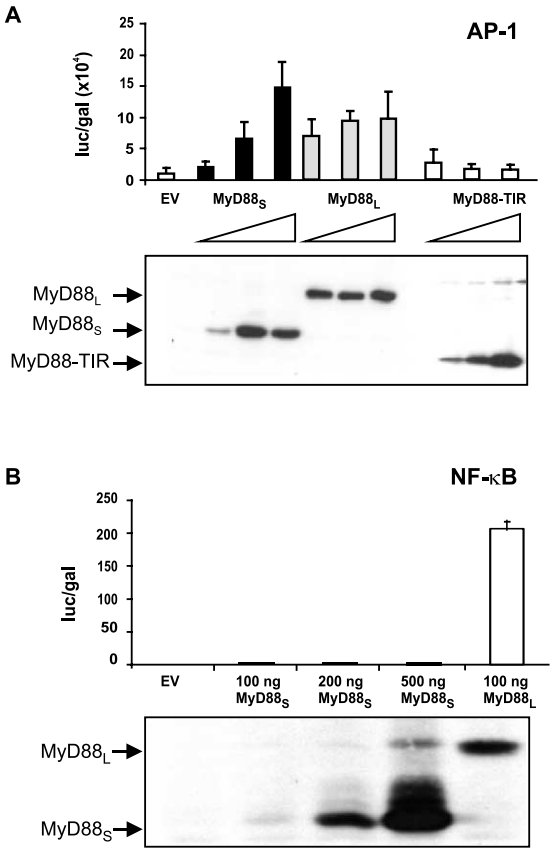

Fig. 1. Effect of MyD88$_S$ and MyD88$_L$ on AP-1- and NF-κB-dependent gene expression. HEK293T cells were transiently transfected with an AP-1-dependent luciferase reporter plasmid (A) or an NF-κB-dependent luciferase reporter plasmid (B) in the absence or presence of increasing concentrations (100–500 ng) of MyD88$_S$, MyD88$_L$, MyD88-TIR or empty vector (EV) as indicated. Luciferase activity in cell extracts was normalized for differences in transfection efficiency on the basis of β-galactosidase activity, resulting from the co-expression of the β-galactosidase reporter plasmid pactβgal. Values are the mean (±S.E.M.) of three different transfections within a single experiment, and are expressed as fold induction relative to the EV control. In parallel, cell extracts were assayed for proper expression of the different MyD88 constructs by Western blotting (lower panel of A and B).

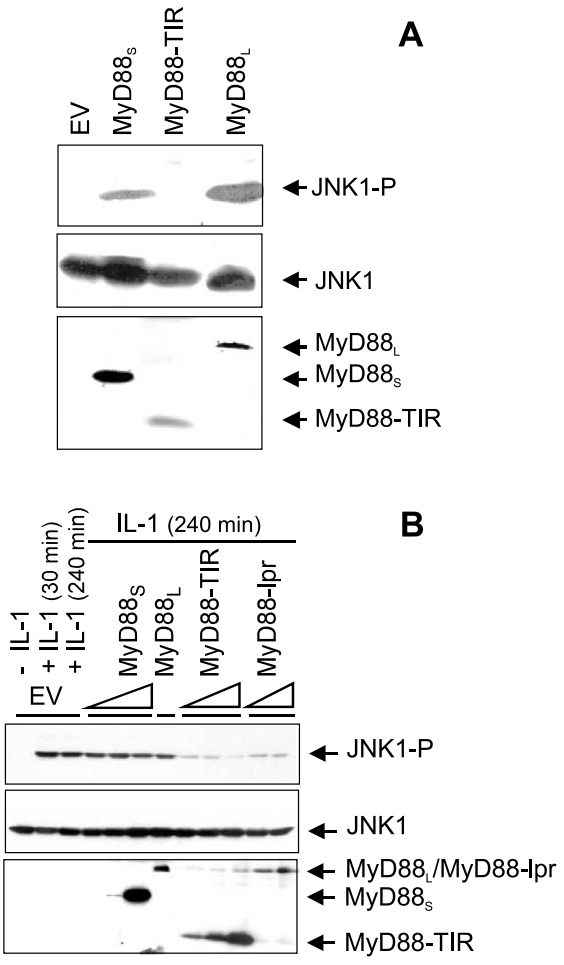

Fig. 2. Effect of $MyD88_S$ and $MyD88_L$ on JNK phosphorylation. HEK293T (A) or HEK293-IL1RI (B) cells were transfected with a vector encoding Flag-JNK1 together with expression plasmids for $MyD88_S$ (100 ng in A; 100, 200 or 500 ng in B), $MyD88_L$ (100 ng in A and B), MyD88-TIR (100 ng in A; 100, 200 or 500 ng in B), MyD88-Ipr (100 or 200 ng in B), or empty vector (EV; 100 ng in A; 500 ng in B) as indicated. In B, cells were treated 48 h after transfection with 100 ng/ml IL-1β for the times indicated. Cell extracts were assayed for JNK phosphorylation by Western blotting with phospho-JNK-specific antibodies (upper panels). In parallel, blots were probed for total JNK and MyD88 variants (lower panels).

tion [12], $MyD88_S$ has no effect on IL-1-induced JNK activation and AP-1-dependent gene expression.

4. Discussion

In the present study, we demonstrated that $MyD88_S$, a splice variant of MyD88, still induces JNK phosphorylation and AP-1-dependent reporter gene expression upon overexpression, while being unable to activate NF-κB-dependent reporter gene expression. In line with these data, $MyD88_S$ did not interfere with IL-1-induced JNK phosphorylation. In this way, $MyD88_S$ behaves very differently from MyD88-TIR, an earlier described dominant negative mutant of MyD88, which abrogates both IL-1-induced NF-κB and JNK activation [8]. $MyD88_S$ is not constitutively expressed, but can be induced by LPS treatment in monocytes [12]. Therefore, our present observations suggest that the regulated expression of $MyD88_S$ enables a cell to fine-tune TLR/IL-1R-dependent signaling, interfering specifically with one of the two major downstream signaling cascades, while leaving the other intact. Furthermore, they also suggest that deletion of the ID in MyD88, and thus interference with IRAK-1 phosphorylation [9,12], does not abolish the capacity of MyD88 and IRAK-1 to signal to JNK. This is in agreement with earlier observations of Li et al. [15], who demonstrated by means of reconstitution experiments in IRAK-1-deficient cells that phosphorylation of IRAK-1, and thus binding to TRAF6, is essential for activation of NF-κB, whereas it is dispensable for IL-1-induced JNK activation. These data are quite unexpected in view of the previous observation that TRAF6-deficient MEF cells are completely defective in IL-1-induced JNK and NF-κB activation [16]. The latter observation suggested that JNK and NF-κB pathways diverge downstream of TRAF6, most likely at the level of the TRAF6 binding proteins ECSIT and TAB [17,18]. However, we speculate that TRAF6 might be recruited to the IL-1R in an alternative way, which involves its N-terminal domain and which is sufficient for activation of MAP3Ks and JNK, but not NF-κB (Fig. 3). This is also supported by the fact that different domains of TRAF6 are needed for IL-1-induced JNK and IL-1-induced NF-κB activation, respectively, as revealed by restoration experiments with TRAF6 deletion mutants in TRAF6-deficient MEF cells [19]. How IRAK-1 and IRAK-4 are implicated in this alternative mechanism of TRAF6 recruitment and activation is not yet known, but IRAK-1 might be needed for stabilization of the complex, or might indirectly mediate TRAF6 recruitment. It is important to note that gene disruption of IRAK-4 abrogated both the IL-1-induced NF-κB and JNK signaling [20], suggesting an essential function of IRAK-4-mediated phosphorylation in both pathways. However, one cannot rule out the existence of other IRAK-4 targets that are essential for JNK activation, but which do not require the binding of IRAK-4 to MyD88.

We previously hypothesized a role for $MyD88_S$ expression in the development of endotoxin tolerance [12]. This is further supported by our present finding that $MyD88_S$ differentially regulates NF-κB and mitogen-activated protein kinase (MAPK) activation, since a recent report showed that monocytes from trauma patients have an impaired NF-κB response to *E. coli* LPS, while their p38 MAPK response is fully intact [21]. In contrast, however, several other studies have shown that NF-κB as well as MAPK signaling pathways are blunted in endotoxin-tolerant cells (for review see [22]), suggesting that other molecules than $MyD88_S$ can also contribute to the development of endotoxin tolerance.

It has recently been proposed that the balance between JNK and NF-κB activation is critical for the decision between life and death, i.e. NF-κB activation provides a strong anti-apoptotic signal due to the activation of cell survival genes and to the inhibition of the JNK signaling pathway. Inhibition of the NF-κB pathway was shown to result in a prolonged JNK activation and the induction of apoptosis upon stimulation with TNF [23,24]. In view of the specific inhibition of IL-1-induced NF-κB activation by $MyD88_S$, it could be expected that $MyD88_S$ overexpression sensitizes cells to IL-1-induced apoptosis. However, we were unable to demonstrate a pro-apoptotic effect of IL-1 in cells overexpressing $MyD88_S$ (data not shown). In fact, this is in agreement with the finding of Tang and colleagues, who demonstrated that

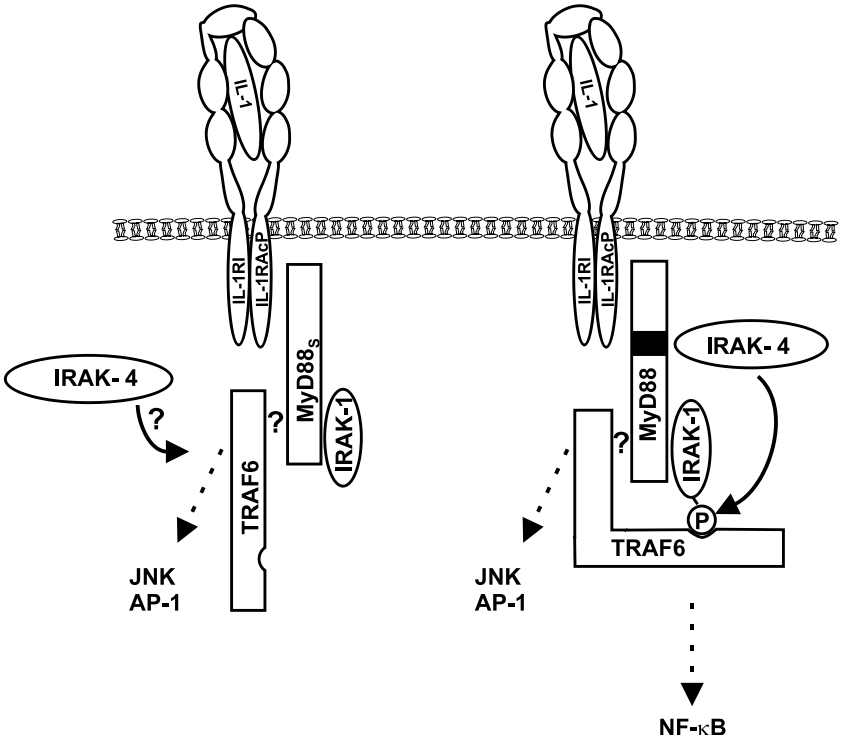

Fig. 3. Model for the differential modulation of JNK and NF-κB signaling pathways by MyD88. Right panel: In the presence of MyD88, IRAK-4 is recruited via the ID (indicated in black) of MyD88 to the IL-1R complex and then phosphorylates IRAK-1, thereby creating a docking site for TRAF6. Phosphorylation of IRAK-1 and IRAK-1/TRAF6 interaction are required for IL-1-induced NF-κB activation. Left panel: We propose that in the presence of $MyD88_S$, TRAF6 can still be recruited via an alternative mechanism to the IL-1R, which involves the N-terminal domain of TRAF6, and which is sufficient for activation of JNK and AP-1. If and how IRAK-1 and IRAK-4 are implicated in this recruitment is not yet known.

the cross-talk between NF-κB and JNK is specific for the TNF response, and does not hold for the response to IL-1 [24].

In conclusion, although the underlying mechanism and the physiological relevance for the differential modulation of NF-κB and JNK activation by $MyD88_S$ remains unclear, our observations suggest that the regulated expression of $MyD88_S$ allows a cell to modulate its transcriptional response to specific environmental stimuli.

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