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MAPPIT analysis of TLR adaptor complexes

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Abstract **Toll-like receptors (TLRs) are crucial components of the innate immune system, coupling pathogen recognition to a cellular response. We used the MAPPIT mammalian two-hybrid technique to investigate protein–protein interactions in the early steps in TLR signalling. A partial TLR-adaptor interaction map was constructed confirming several known but also documenting novel interactions. We show that the TLR adaptor Mal is critical for linking Myeloid Differentiation primary response protein 88 (MyD88) to TLR2 and TLR4. Analysis of the contributions of the different sub-domains of MyD88-adaptor-like protein (Mal) and MyD88 in adaptor homo- and hetero-dimerisation provides an initial mechanistic insight in this bridging function of Mal.**

Keywords: Toll-like receptor; MAPPIT; Signal transduction

1. Introduction

One of the first steps in pathogen clearance is the activation of signalling pathways by Toll-like receptors (TLRs). At present, 10 TLRs are identified in the human genome. Typical TLR ligands are bacterial lipopolysaccharide (LPS), recognised by TLR4 [1], bacterial lipopeptides (TLR2) [2], double stranded RNA (TLR3) [3], flagellin (TLR5) [4] and CpG motifs in DNA (TLR9) [5]. Next to ligand binding, adaptor molecules are recruited to the receptor through homotypic TIR–TIR (Toll/IL-1 receptor domain) domain interactions. MyD88 (Myeloid Differentiation primary response protein 88) was the first adaptor to be described [6], and is thought to be used by every TLR except TLR3. Subsequently, three more TIR-containing adaptors were found: Mal/TIRAP (MyD88-adaptor-like protein/TIR domain containing adaptor-like protein), which, based on knock-out studies, appears to be important in TLR2 and TLR4 signalling and exerts more or less the same function as MyD88 [7–10], Trif/Ticam-1 (TIR-domain containing adaptor inducing interferon-β/TIR containing adaptor molecule), an adaptor recruited by both TLR3 and TLR4 and responsible for activation of IRF3 [11,12] and Tram/Ticam-2 (Trif-related adaptor molecule/TIR containing adaptor molecule-2), known to act as a bridging adaptor between TLR4 and Trif [13,14]. Recently, Sarm (sterile alpha and HEAT-Armadillo motifs containing protein), a potential fifth TLR adaptor molecule, was shown to negatively regulate Trif-dependent Toll-Like receptor signalling [15]. The usage of different adaptors by different receptors partially provides a molecular basis for the specificity of the immune reaction against a given pathogen. However, the exact modalities of this specificity are not yet fully understood [16].

Further downstream signalling can be primarily divided into a MyD88-dependent branch, which ultimately leads to activation of NF-κB and mitogen-activated protein kinases (MAPK) like p38 and Jun N-terminal Kinase (JNK) and a MyD88-independent branch resulting in phosphorylation of Interferon Regulatory Factors (IRFs) and expression of interferon-β (IFN-β). The MyD88-dependent pathway is initiated by recruitment of IRAK-4 (IL-1 receptor associated kinase-4) to the activated receptor [17] through a homotypic interaction with the death domain of MyD88. Next, IRAK-4 phosphorylates IRAK-1 [18] and activated IRAK-1 associates together with TRAF-6 (TNF-α receptor-associated factor 6) [19] into a complex with TAB (TAK-1 binding) proteins and TAK-1 (TGF-β-activated kinase-1) [20]. This complex is a branching point for the activation of NF-κB and induction of the MAPK pathway. Activation of TLR3 and TLR4 also engages a MyD88-independent pathway. Recruitment of Trif to the activated receptor leads to activation of TBK1 (TANK-binding kinase-1) and IKKε, which are essential for IRF-3 phosphorylation and nuclear translocation and subsequent induction of IFN-β [21]. In this report we make use of the mammalian two-hybrid method MAPPIT (MAmmalian Protein–Protein Interaction Trap) [22] to study the modalities of TIR–TIR interactions involved in the early steps of TLR signalling.

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Abbreviations: IFN, interferon; LR, Leptin receptor; Mal, MyD88-adaptor-like protein; MyD88, Myeloid Differentiation primary response protein 88; Sarm, Sterile alpha and HEAT-Armadillo motifs containing protein; STAT, Signal Transducer and Activator of Transcription; TIR, Toll/IL-1 Receptor; TLR, Toll-like receptor; Tram, TRIF-related adaptor molecule; Trif, TIR-domain containing adaptor inducing IFNβ

2. Material and methods

2.1. Constructs

Generation of the pMG2 and pMG2-SVT prey vectors were described earlier [23]. Human full-length MyD88 was amplified using primers 1 and 2 on the pCDNA3-MyD88-AU1 plasmid (gift of Dr. Muzio). After *Eco*RI–*Xba*I digestion, the fragment was cloned in the pMG2 vector, resulting in pMG2-MyD88. The TIR domain of MyD88 was cloned in an analogous manner into the pMG2 vector

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using primers 3 and 4 and a *Bst*eII/*Xba*I digestion. The pMG2-Mal and pMG2-MalTIR plasmids were generated by amplification of Mal and the TIR domain of Mal from the pDC304–Mal vector (gift from Dr. O'Neill) with primers 5–6 and 7–8 respectively, and *Eco*RI/*Xba*I or *Bst*eII/*Xba*I digestion. The TIR domain of Sarm was amplified using primers 9–10 on HepG2 cDNA followed by *Eco*RI/*Xba*I digestion. This resulted in the pMG2-SarmTIR vector. The pMG2-Tram vector was generated by amplification of full-length Tram using primer 11–12 on Hek293 cDNA. After *Bst*eII/*Xho*I digestion, the fragment was cloned in the pMG2 vector.

Generation of the human LepR-bait constructs in the pcDNA5/FRT vector was described before [22], and this construct was named pCLL. The pCLL-MyD88 plasmid was generated by amplification of full-length MyD88 from the MyD88-AU1 vector using primer pairs 13–14. After *Bam*HI/*Not*I digestion, the fragments were cloned in the pCLL vector. The pCLL-Mal and pCLL-Tram plasmids were generated in an analogous manner using primers 15–16 and 17–18, respectively. The intracellular domains of Toll-Like receptor 2, 3, 4, 5, 7, 9 were amplified using primer sets 19–20, 21–22, 23–24, 25–26, 27–28, 29–30 on MRC5 cDNA (TLR2), the pFLAG CMV1 – TLR3 vector (gift from Dr. O'Neill), the pFLAG CMV1 – TLR4 vector (gift from Dr. O'Neill), and RZPD clones HU3_p983D0273D (TLR5), IRATp970B1256D (TLR7) IRATp970H1255D (TLR9) respectively. After *Bam*HI/*Not*I digestion, the fragments were cloned into the pCLL vector.

The MalP125H and MyD88P200H mutant vectors were generated by PCR based mutagenesis using primers 31–32 and 33–34, respectively (Quikchange™ site-directed mutagenesis method, Stratagene). The pcDNA5-TAP2-CIS vector was generated by amplification of TAP2-CIS using primers 35–36 on the pMET7-TAP2-flag vector, which was described earlier [24], followed by a *Kpn*I/*Not*I based ligation into the pCDNA5-FRT-TO vector (Invitrogen). Mal was amplified using primers 37–38 on the pDC304–Mal vector and *Eco*RI/*Not*I based ligation into the pcDNA5-TAP2-CIS vector resulted in the pcDNA5-Mal-flag plasmid.

The pXP2d2-rPAPI-luciferase reporter, originating from the rPAPI (rat pancreatitis associated protein I) promoter was used as previously described [22]. The pNFconluc reporter was a gift of Dr. A. Israel.

2.2. Cell culture, transfection, reporter assays and expression controls

Cell culture conditions, transfection procedures and luciferase assays for Hek293T cells were previously described [25]. For a typical luciferase experiment, 4×10^5 cells were seeded in 6-well plates 24 h before transfecting them overnight with the desired constructs together with the luciferase reporter gene. Cells were left untreated (negative control NC) or were stimulated overnight with 100 ng/ml leptin followed by measurement of luciferase activity in cell lysates by chemiluminescence.

Prey expression was examined by Western Blot using anti-FLAG mouse monoclonal antibody (Sigma), on lysates of transfected cells. All results are representative for at least three independent transfection experiments.

2.3. Co-immunoprecipitation

Approximately 2×10^6 Hek293T cells were transfected with different combinations of MAPPIT bait vector pCLL-TLR4ic, MyD88-prey plasmid, and an expression vector encoding Mal. Cleared lysates (modified RIPA lysis buffer: 200 mM NaCl, 50 mM Tris–HCl PH8, 0.05% SDS, 2 mM EDTA, 1% NP40, 0.5% DOC, Complete Protease Inhibitor Cocktail (Roche)) were incubated with a mixture of two rat anti-mouse LR monoclonal antibodies (4 μg/ml) [26] and protein G-sepharose (Amersham Biosciences). After immunoprecipitation, SDS–PAGE and Western Blotting, interactions were detected using anti-flag-tag antibody (Sigma), anti-HA (HA-11, CRPinc) and anti-mouse-HRP (horseradish peroxidase) (Amersham Biosciences).

2.4. Fluorescence microscopy and immunocytochemistry

Hek293T cells were fixed with 3% paraformaldehyde in phosphate-buffered saline (Invitrogen) for 20 min at room temperature. Next, cells were permeabilised in 0.1% Triton X-100, washed in phosphate-buffered saline, incubated with anti-flag antibody (Sigma) for 1 h at 37 °C and following several washes in phosphate buffered saline incubated with Alexafluor488-conjugated goat anti-mouse antibody (Molecular Probes) at room temperature. Nuclei were DAPI stained. Microscopic images were captured with a Zeiss Axiovert 200 epifluorescence microscope equipped with an Axiocam cooled CCD camera and processed using AXIOVISION software.

3. Results

3.1. MAPPIT concept

MAPPIT (MAmmalian Protein–Protein Interaction Trap), a mammalian two-hybrid method [22], was used to study protein–protein interactions in the TLRs pathway (Fig. 1A). In brief, we made a C-terminal fusion of a given 'bait' protein with a leptin receptor that is deficient in STAT3 recruitment. The 'prey' protein on the other hand is linked to a series of four functional STAT3 recruitment sites of the gp130 chain. Association of bait and prey and ligand stimulation leads to STAT3 activation and induction of a STAT3-responsive luciferase reporter (rPAPI-Luci). Expression of all the different preys used throughout this study was checked by Western blot analysis using an anti-FLAG antibody (Supplementary data). Bait expression was assayed by FACS analysis using an anti-LR antibody (Supplementary data) (see Table 1).

We cloned all TLR adaptors both as bait and as prey, and the intracellular part of selected TLRs as bait. An overview of all tested adaptor/adaptor and adaptor/TLR interactions can be found in Table 2. Well-documented interactions like homo- or hetero-oligomerisation of Mal and MyD88 or interaction of Tram with TLR4 were confirmed. In addition, new interactions were found, including the association of the TIR domain of Sarm with MyD88 and Tram. Other new findings will be discussed in more detail below. No MAPPIT signals were detected using Trif as bait or as prey. This cannot be explained by an a-specific effect of Trif on the MAPPIT readout since Trif over-expression did not influence an established interaction-dependent signal (data not shown). We also ruled out cytotoxic effects by the Trif-prey since its over-expression did not lead to increased annexin V binding, a marker for apoptosis (data not shown). We also constructed a Trif-prey with a C-terminal fusion of the gp130 tail to exclude interference of a N-terminal Trif fusion protein with protein–protein interactions, but again no positive signals could be observed (data not shown). Strikingly, the subcellular expression of the Trif-prey was limited to a perinuclear compartment, much in contrast to functional prey molecules (Fig. 1B). Since MAPPIT measures interactions in the sub-plasmamembranary space, this finding provides a likely explanation for the lack of Trif-dependent signals in MAPPIT experiments.

We also monitored the effect of bait expression on endogenous TLR signalling (Fig. 1C). The MyD88-bait and, to a lesser extent, the Mal-bait were able to activate the NF-κB pathway in a ligand independent manner. These data show that the MAPPIT setup forms functional signalling complexes at the cell membrane, underscoring its relevance to study TLR signalling.

3.2. Homo- and hetero-oligomerisation of Mal and MyD88

Mal and MyD88 both have a C-terminal TIR interaction domain (Toll/IL-1 receptor domain), a property shared with all TLRs and their adaptors. This TIR domain is thought to be the critical interaction domain for recruitment of TLR adaptors to their cognate receptors and for adaptor dimerisation. MyD88 bears an additional N-terminal Death Domain linked to the TIR domain by a short intermediate region.

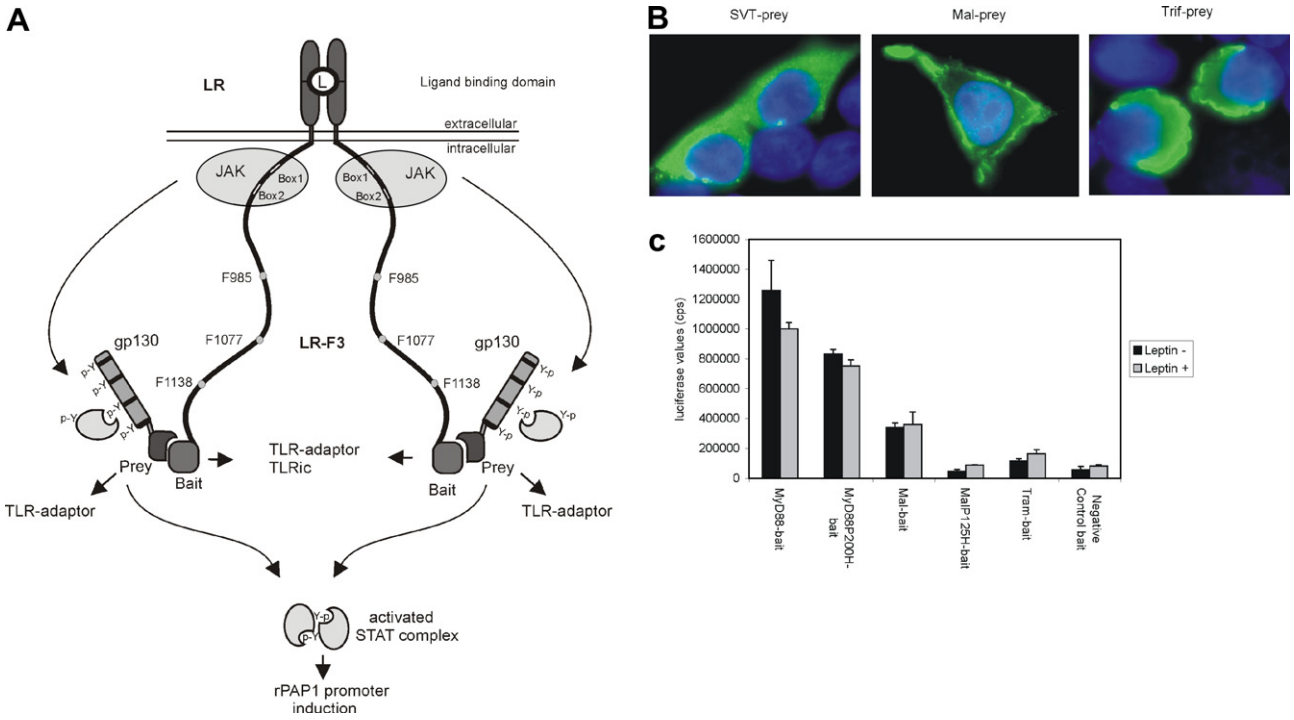

Fig. 1. (A) MAPPIT principle. MAPPIT, a cytokine receptor-based two hybrid method, makes use of a bait protein, C-terminally fused to a leptin receptor that is deficient in STAT3 recruitment by a Y1138F mutation, and a prey protein linked to a string of four functional STAT3 recruitment sites of the gp130 chain. Interaction of bait and prey leads to a ligand-dependent complementation of STAT3 activity that can be measured with a STAT3-responsive luciferase reporter (rPAPI-luci). TLR adaptors were used as bait or prey, while the intracellular parts of selected TLRs were used as bait. (B) Trif-prey localisation. Hek293T cells were transfected with either Trif-prey, Mal-prey or SVT-prey lacking its nuclear localisation sequence . Cells were fixed and incubated with anti-flag antibody. Prey expression was visualised with a fluorescent secondary antibody (anti-mouse IgG-alexa488). Nuclei were DAPI stained. (C) TLR adaptor bait expression activates NF-κB pathway. Hek293T cells were transfected with different TLR adaptor bait-constructs (1 μg) and a NF-κB responsive luciferase reporter (pNF-conluci). Twenty-four hours after transfection the transfected cells were stimulated with leptine (100 ng/ml) for another 24 h or left untreated (NS). Mean Luciferase activity + S.D. of triplicate measurements is plotted.

Mal on the other hand contains an N-terminal phosphatidylinositol 4,5-bisphosphate (PIP2) binding domain, which mediates Mal recruitment to the plasma membrane [27]. As seen in Table 2, the TLR adaptors Mal and MyD88 clearly can form homo- and hetero-dimers, a well-established early event in TLR signalling. To elucidate which domains of these adaptors are essential in this dimerisation process we mutated a proline to histidine in the TIR domain of Mal (P125H) and MyD88 (P200H), a mutation known to abrogate TLR signalling [1]. We also generated a prey construct only containing the C-terminal TIR domain of MyD88 and Mal.

Strikingly, when analysing the interaction properties of the Mal-bait (Fig. 2A), we observed a drastic effect of the P → H mutation. Mutation of the TIR domain of either bait or prey is sufficient to abrogate interaction. These data confirm that the TIR domain of Mal and MyD88 is necessary and sufficient for heterodimerisation. These properties were confirmed using MyD88 as bait (Fig. 2B).

Much in contrast, Mal homodimerisation depended on its N-terminal part in addition to TIR-TIR binding. No interaction was observed when using the Mal-bait combined with a MalTIR-prey only containing the C-terminal TIR region (Fig. 2A). However, this prey is still functional since clear association of the MyD88-bait and the MalTIR-prey was observed (Fig. 2B). Mutation of a single TIR domain leads to complete loss of homodimerisation (Fig. 2A), pointing again at the importance of this proline residue.

We also checked the effect of the P → H mutation on MyD88 homodimerisation (Fig. 2B). When using full-length MyD88 as bait and prey, mutation of both TIR domains did not affect interaction, whereas binding of the MyD88TIR-prey was completely lost when using MyD88P200H as a bait. These results indicate that the death domain of MyD88 is not only important in recruiting downstream signalling molecules, but also for MyD88 homodimerisation.

3.3. Mal bridges TLRs to MyD88

We next examined the TLR4 interaction profile using the intracellular part of TLR4 as bait (TLR4ic) and different TLR adaptors as prey (Fig. 3A). As expected, clear interaction was seen between TLR4ic and Tram, confirming its unique function in TLR4 signalling [13,14]. Mal/TIRAP also bound to TLR4ic, an interaction which was reported before [8]. Co-transfection of the TLR4ic-bait and the universal TLR adaptor MyD88-prey did not result in any luciferase induction although the role of MyD88 in TLR4 signalling is very well documented [28,29]. Therefore, we examined if the MyD88-TLR4 interaction could be indirect (Fig. 3B). Co-transfection of a Mal expression vector together with TLR4ic-bait and MyD88-prey resulted in a clear luciferase induction, indicating that Mal bridges MyD88 to TLR4. This interaction was completely lost when using the TIR disrupting Mal (MalP125H) or MyD88 (MyD88P200H) mutations, pointing at the importance of the different TIR domains in this interaction.

Table 1
Overview of primers used in this study

Primer 1	5′-GCGAATTCATGGCTGCAGGAGGTCCCGGCG-3′
Primer 2	5′-GCGCTCTAGATCAGGGCAGGGACAAGGCCTTGG-3′
Primer 3	5′-GCGGTAACCGTGCCGCCATGGACCCCCTGGGGCATATGCC-3′
Primer 4	5′-GCTCTAGACAGTCAGGGCAGGGACAAGGCC-3′
Primer 5	5′-GCGAATTCATGGCATCATCGACCTCC-3′
Primer 6	5′-GCGCTCTAGACTCAAAGTAGATCAGATAC-3′
Primer 7	5′-GCGGTAACCGTGCCGCCATGGGCAGTAGTCGCTGGAGC-3′
Primer 8	5′-GCTCTAGACAGTCAAAGTAGATCAGATACTGT-3′
Primer 9	5′-GCGCGAATTCACTCCAGATGTCTTCATCAGC-3′
Primer 10	5′-GGCCTCTAGATTAGCGGCCCTGCAGGAAGCGG-3′
Primer 11	5′-GCGGTAACCGCCGCCATGGATTACAAGGATGACGACGATAAGGGTATC GGAAGTCTAAAATAAATTCC-3′
Primer 12	5′-GCCTCGAGTCAGGCAATAAATTGTCTTTGTACC-3′
Primer 13	5′-GCGGATCCATGGCTGCAGGAGGTCCCGGC-3′
Primer 14	5′-GCGCGGCCGCTCAGGGCAGGGACAAGGCC-3′
Primer 15	5′-GCGAGCTCCGGATCCATGGCATCATCGACCTCC-3′
Primer 16	5′-ATAGTTTAGCGGCCGCTCACCGTAGATCAGATAC-3′
Primer 17	5′-GCCGGGATCCATGGGTATCGGGAAGTC-3′
Primer 18	5′-GCCGATGCGGCCGCTCAGGCAATAAATTGTC-3′
Primer 19	5′-GCGGATCCTGCCACCGTTTCCATGG-3′
Primer 20	5′-GCGCGGCCGCCTAGGACTTTATCG-3′
Primer 21	5′-GCGAGCTCGTCTTTTTATTGGAATG-3′
Primer 22	5′-GCGCGGCCGCTTAATGTACAGAGTTTTTGG-3′
Primer 23	5′-GCGGATCCTGCATAAAGTATGGTA-3′
Primer 24	5′-GCGCGGCCGCTCAGATAGATGTTGC-3′
Primer 25	5′-GCGGATCCACAAAGTTCCGGGGCTTCTG-3′
Primer 26	5′-CCGGCCGCGGCCGCCTTAGGAGATGGTTGCTAC-3′
Primer 27	5′-GCGGATCCCACCTCTATTTCTGGGATGT-3′
Primer 28	5′-CCGGCCGCGGCCGCCCTAGACCGTTTCCTTGAACACC-3′
Primer 29	5′-GCGCGCGGATCCTGGCGGGGGCGGCAAAGTGG-3′
Primer 30	5′-CCGGCCGCGGCCGCCCTATTCGGCCGTGGGTCCC-3′
Primer 31	5′-GCAACTCCGGGATGCAACCCACGGCGGCGCTATAGTGTCC-3′
Primer 32	5′-GGACACTATAGCGCCGCCGTGGGTTGCATCCCGGAGTTGC-3′
Primer 33	5′-GTCTGACCGCGATGTCCTCCATGGCACCTGTGTCTGGTC-3′
Primer 34	5′-GACCAGACACAGGTGCCATGGAGGACATCGCGGTCAGAC-3′
Primer 35	5′-GCGAGGTACCGCCACCATGGCCCAGCACGA-3′
Primer 36	5′-GCAGCGGCCGCTTAGAGTTGGAAGGGGTACTG-3′
Primer 37	5′-GCGCGAATTCATGGCATCATCGACCTCCC-3′
Primer 38	5′-GCCGCTGCGGCCGCTCAAAGTAGATCAGATACTGTAGC -3′

Table 2
Overview of tested adaptor/adaptor and adaptor/TLR interactions

	Mal	MalTIR	MyD88	MyD88TIR	Tram	SarmTIR	SVT
Prey							
Mal	**8.53**	2.54	**19.19**	**11.55**	2.3	4.57	1.68
MyD88	**23.77**	**44.84**	**19.99**	**45.14**	1.05	**12.15**	2.30
Tram	2.59	2.01	1.33	1.25	**18.66**	**11.66**	2.46
Bait							
TLR2ic	**8.71**	**8.18**	2.28	0.92	1.06	2.79	1.04
TLR3ic	4.01	3.07	1.01	0.99	1.16	4.43	1.22
TLR4ic	**26.69**	**56.65**	1.22	1.05	**16.40**	1.70	1.22

Hek293T cells were transiently co-transfected with the MAPPIT bait plasmid (0.5 μg), different TLR adaptor prey plasmids (0.5 μg) and the STAT3 responsive rPAPI-luci reporter (0.3 μg). Twenty-four hours after transfection the transfected cells were stimulated with leptin (100 ng/ml) for another 24 h or were left untreated (NS). Luciferase measurements were performed in triplicate. Data are expressed as mean fold induction (ratio Stimulated/NS). Positive interactions (fold induction >5) are highlighted in bold. SVT-prey was used as a negative control. Expression of all the different preys was checked by Western blot analysis using an anti-FLAG antibody.

These data were confirmed by co-immunoprecipitation (Fig. 3C). The extracellular portion of the leptin receptor was used to immunoprecipitate the MAPPIT TLR4ic-bait protein (using an anti-LR antibody) and only when Mal was co-expressed, the MyD88-prey protein could be co-immunoprecipitated.

In an analogous manner, the TLR2ic interaction profile was examined (Fig. 3D). In accordance with the TLR4ic interaction profile, no interaction between MyD88 and TLR2 could be observed. However, complementation with Mal gave rise to a clear luciferase induction, indicating that Mal also acts as bridging adaptor between MyD88 and TLR2.

4. Discussion

One of the first steps in pathogen recognition and clearance is the recognition of evolutionary conserved pathogen-associated molecular patterns whereby TLRs play a crucial role.

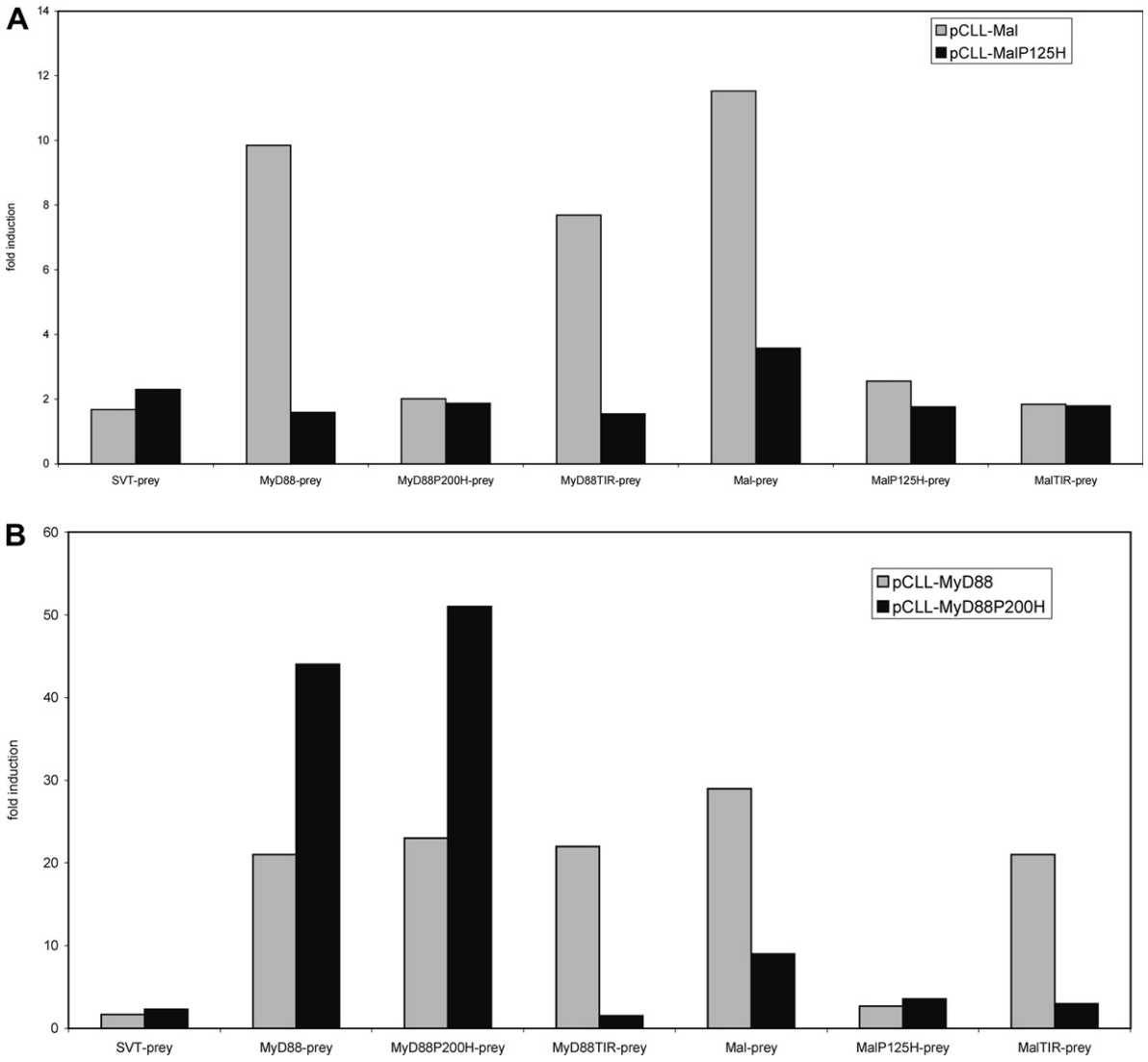

Fig. 2. Mal and MyD88 homo- and heterodimersiation. (A) Hek293T cells were transiently co-transfected with the MAPPIT bait plasmid pCLL-Mal or pCLL-MalP125H, different TLR adaptor prey plasmids and the STAT3 responsive rPAPI-luci reporter. Experimental setup was as in Table 2. (B) Interaction analysis of the MyD88 bait (pCLL-MyD88) or its mutant (pCLL-MyD88P200H). Experimental setup was as in Table 2.

Ligand binding to a given TLR activates complex signalling pathways, ultimately leading to pro-inflammatory responses. These responses vary between activated TLRs, which is in part explained by different adaptor usage. We here take a closer look at the mechanistics of TLR adaptor recruitment using MAPPIT, a mammalian two-hybrid strategy. We cloned all known TLR adaptors either as bait or as prey, and the intracellular part of selected TLRs as bait. As shown in Table 2, well-documented interactions like MyD88/Mal homo- and hetero-dimerisation, or recruitment of Tram to TLR4 could readily be detected. No interactions were observed using Trif as a bait or as a prey, probably due to its unique, non-cytoplasmic localisation that most likely interferes with the MAPPIT readout (Fig. 1B). Next to known interactions, some new observations were also found. Recently, Sarm was described as a negative regulator of Trif-dependent signalling pathways [15]. However, as seen in Table 2, we also observed a clear interaction with MyD88 or Tram when using the 'TIR' interaction domain of Sarm as a prey. These data suggest that Sarm, next to inhibition of Trif , also could be involved in regulating the TLR adaptors MyD88 and Tram.

MyD88 is commonly referred to as a "universal" adaptor, used by every TLR except TLR3. However, biochemical data providing a direct link between MyD88 and a TLR are scanty. In this report we could not detect an interaction between MyD88 and TLR2, TLR4 (Fig. 3). However, the TLR4-bait is fully functional since a clear signal was obtained for the well-documented interactions with the Mal- or Tram-preys [13,14]. This inability of MyD88 to bind TLR4 in MAPPIT is in line with a recent report showing no MyD88/TLR4 binding when analyzed via the yeast two-hybrid method [30]. Significantly, co-expression of the TLR adaptor Mal resulted in a clear MAPPIT signal for the TLR4-bait/MyD88-prey combination. Since Mal binds both TLR4 and MyD88, this observation demonstrates its bridging role between MyD88 and TLR4, analogous to the Tram/Trif branch of TLR4 signalling [31] (Fig. 3B). These data were confirmed using co-immunoprecipitation (Fig. 3C). While this manuscript was in

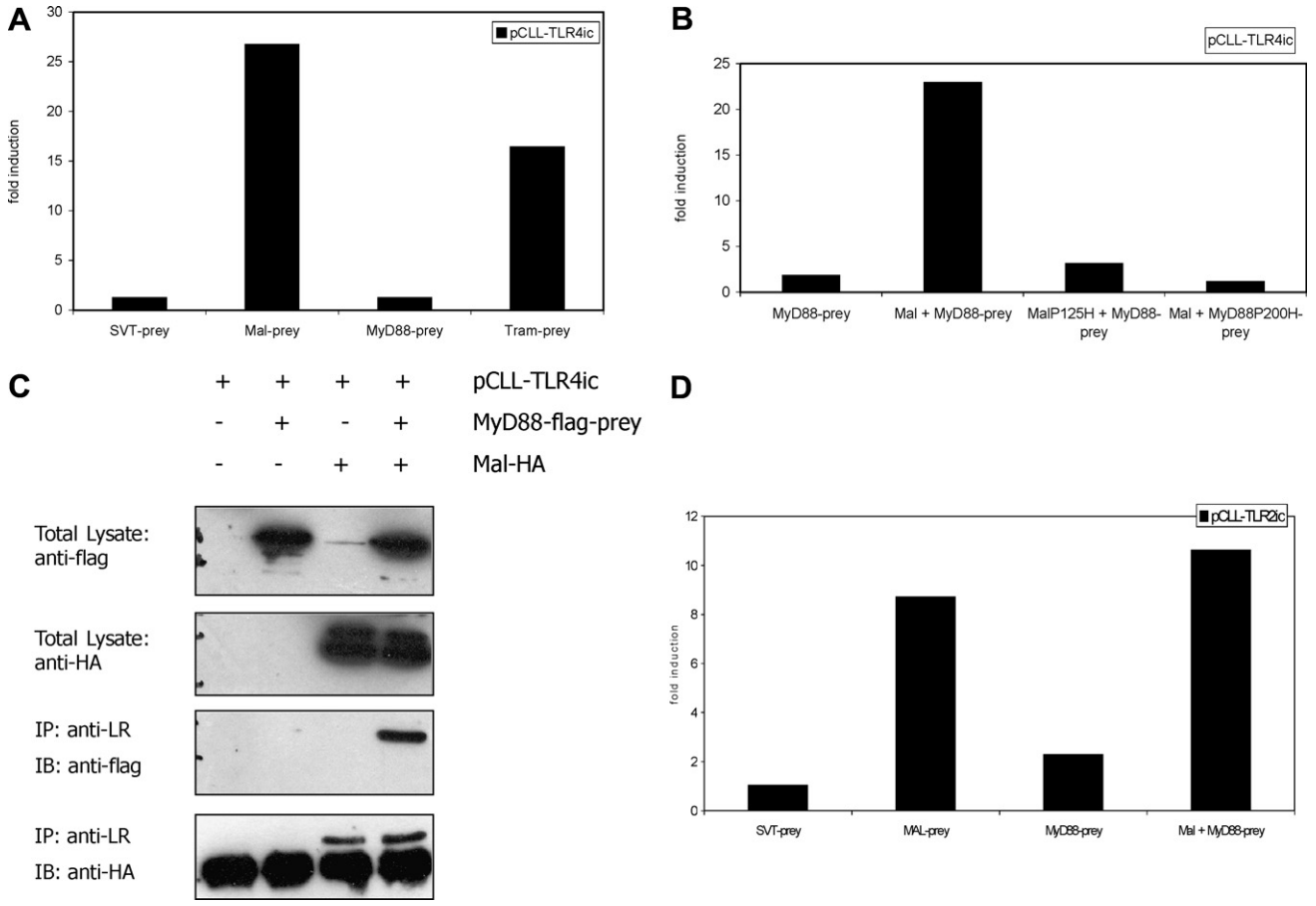

Fig. 3. TLR4ic interaction profile. (A) Hek293T cells were transiently co-transfected with the MAPPIT bait plasmid pCLL-TLR4ic, various TLR adaptor prey constructs (or a SVT-prey as negative control) and the rPAPI-luci reporter. Experimental setup was as in Table 2. (B) Effect of a Mal expression vector. Hek293T cells were transiently co-transfected with pCLL-TLR4ic, a MyD88-prey (pMG2-MyD88/pMG2-MyD88P200H), a Mal expression vector (pcDNA5-Mal/pcDNA5-MalP125H) and the rPAPI-luci reporter. Experimental setup was in Table 2. (C) Co-immunoprecipitation analysis. Hek293T cells were transfected with combinations of pCLL-TLR4ic, pMG2-MyD88, pDC304-Mal-HA. Cell lysates were immunoprecipitated with an a mixture of two rat monoclonal antibodies against the extracellular part of the leptin receptor (α-LR) and subsequently immunoblotted (IB) with anti-flag or anti-HA. (D) TLR2ic interaction profile. Hek293T cells were transiently co-transfected with the MAPPIT bait plasmid pCLL-TLR2ic, various TLR adaptor prey constructs, a Mal expression vector (or empty vector) and the rPAPI-luci reporter. Experimental setup was as in Table 2.

preparation, Kagan and Medzhitov showed that Mal contains a PIP2 binding domain that mediates Mal recruitment to membranes and that the primary function of Mal in TLR signalling is to facilitate MyD88 delivery to TLR4 via PIP2 binding [27]. These observations are also consistent with the phenotype of Mal-deficient mice, which is analogous to MyD88-deficient mice in terms of TLR2 and 4 signalling [7,10]. Similar findings were obtained supporting a TLR2/Mal/MyD88 bridging complex (Fig. 3D) in line with the need for Mal in TLR2 signalling. Of note, our findings are in contrast with a recent report [30], demonstrating direct TLR2/MyD88 binding with a yeast two-hybrid approach. The reason for this contradictory finding is unclear at present.

We also evaluated the interactions between the TIR domains of additional TLRs (TLR5, TLR7 and TLR 9) and MyD88 (data not shown). Most interestingly, in none of these cases could we observe any signal. Although we cannot rule out at present that our strategy fails to detect these interactions, we would like to point out that a control using a JAK2-binding-prey was clearly positive, indicating that the attached TIR domains did not interfere with the MAPPIT read out. Moreover, the yeast two-hybrid method also failed to detect direct interaction between TLR9 and MyD88 [30]. In addition, TLR7 and TLR9 signal from acidic endosomes. How MyD88 is recruited to those distinct cellular compartments remains unknown, and the use of accessory molecules, next to Mal and Tram, therefore cannot be excluded.

We next investigated the dimerisation properties of Mal and MyD88 in more detail. MyD88 and Mal both are two-domain proteins containing a C-terminal TIR domain preceded by a Death Domain and a N-terminal domain, respectively. Mutation of a single proline to histidine in the so called ''BB-loop'' of the TIR domain blocks TLR signalling. Various models of these TIR domains have been described, but the precise role of this conserved proline remains elusive [32,33]. Strikingly, mutation of the TIR domain of Mal (MalP125H) in either prey or bait, contrary to GST pull-down experiments [32], completely abolishes homodimerisation (Fig. 2A). These data support the model in which the BB loop of the TIR domain plays a crucial role

in TIR-TIR interactions [33] and provide a biochemical explanation for the drastic effect of this mutation on signalling. Moreover, in contrast with wild type Mal-bait, the MalP125H-bait is no longer capable of activating NF-κB signalling (Fig. 1C). This again reflects the importance of this proline residue in TLR signalling. In addition, we observed that the contribution of each interaction domain of Mal and MyD88 differs from homo- and hetero-dimerisation. For hetero-dimerisation, the isolated TIR domain of Mal or MyD88 is sufficient (Fig. 2). Mal homodimerisation on the other hand is dependent on the N-terminal domain of Mal, which is necessary to stabilise homodimer formation (Fig. 2A). This contrasts to MyD88 homodimerisation (Fig. 2B), where the isolated TIR domain is sufficient for interaction. Mutation of the conserved proline residue in the isolated TIR domain again abolished interaction. In contrast, full-size bait and prey homodimerisation is not affected by this P200H mutation nor does it eliminate NF-κB signalling (Fig. 1C). This implies that the MyD88 Death Domain not only interacts with downstream signalling molecules, as shown in several studies [17], but also can participate in MyD88 homodimer formation.

In brief, we examined the recruitment and dimerisation properties of the TLR adaptors Mal and MyD88 and provide a biochemical basis for the bridging function of Mal between an activated TLR and the TLR adaptor MyD88.

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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.febslet.2007.01.026.

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