Mal connects TLR2 to PI3Kinase activation and phagocyte polarization

Sandra Santos Sierra¹, Sachin D Deshmukh¹,
Julia Kalnitski¹, Peter Küenzi²,
Matthias P Wymann³, Douglas T Golenbock⁴
and Philipp Henneke^{1,*}

¹Center for Pediatrics and Adolescent Medicine and Centre of Chronic Immunodeficiency, University Medical Center Freiburg, Freiburg, Germany, ²Institute of Pharmaceutical Biology, University of Basel, Basel, Switzerland, ³Institute of Biochemistry and Genetics, Department of Biomedicine, University of Basel, Basel, Switzerland and ⁴Department of Medicine, University of Massachusetts Medical School, Worcester, MA, USA

The recognition of bacterial lipoproteins by toll-like receptor (TLR) 2 is pivotal for inflammation initiation and control in many bacterial infections. TLR2-dependent signalling is currently believed to essentially require both adaptor proteins MyD88 (myeloid differentiation primary response gene 88) and Mal/TIRAP (MyD88-adaptor-like/TIR-domain-containing adaptor protein). TLR2-dependent, but MyD88-independent responses have not been described yet. We report here on a novel-signalling pathway downstream of TLR2, which does not adhere to the established model. On stimulation of the TLR2/6 heterodimer with diacylated bacterial lipoproteins, Mal directly interacts with the regulatory subunit of phosphoinositide 3-kinase (PI3K), p85 α , in an inducible fashion. The Mal–p85 α interaction drives PI3K-dependent phosphorylation of Akt, phosphatidylinositol(3,4,5)P₃ (PIP₃) generation and macrophage polarization. MyD88 is not essential for PI3K activation and Akt phosphorylation; however, cooperates with Mal for PIP₃ formation and accumulation at the leading edge. In contrast to TLR2/6, TLR2/1 does not require Mal or MyD88 for Akt phosphorylation. Hence, Mal specifically connects TLR2/6 to PI3K activation, PIP₃ generation and macrophage polarization.

The EMBO Journal (2009) 28, 2018–2027. doi:10.1038/emboj.2009.158; Published online 2 July 2009

Subject Categories: signal transduction

Keywords: bacterial lipopeptides; cytokines; macrophages; molecular biology; signal transduction

Introduction

The currently favoured model of toll-like receptor (TLR)-mediated initiation of signalling involves ligand-induced conformational changes in pre-assembled, low-affinity TLR

dimers (Latz *et al*, 2007). The best established TLR heteromers comprise TLR2 and either TLR6 or TLR1 (Ishii *et al*, 2008). As shown by structural studies, triacylated lipopeptides, which resemble lipoproteins from Gram-negative rods, are ligands for TLR1/2, that is they directly bind to the extracellular domain of these receptors (Jin *et al*, 2007). A similar mode of interaction can be assumed to occur between the TLR2/6 dimer and its agonists, diacylated lipoproteins from mycoplasmatocae and Gram-positive organisms (Lien *et al*, 1999; Henneke *et al*, 2008). It seems that ligand binding induces a conformational change of the dimer, which brings the TIR domains of the two TLRs into closer proximity, although this has yet to be confirmed by co-crystallization of the involved molecules (O'Neill and Bowie, 2007). In the case of TLR4-containing dimers, this creates a platform for homophilic interactions with the TIR-domain-containing adaptor proteins MyD88 (myeloid differentiation primary response gene 88) and Mal (MyD88-adaptor-like) (Nunez *et al*, 2007). Ample opportunity of TLR-dependent-signal regulation exists in the case of bacterial lipoproteins, as two distinct TLR heteromers interact with two adaptors, which will ultimately lead to discrete-activation patterns of specific transcription factors. However, currently available studies assign a fairly narrow gene-activation pattern in response to TLR2 stimulation irrespective of the engaged dimer (Farhat *et al*, 2008). This relative invariability of the TLR2 response suits well to the notion that Mal and MyD88 are not independent TLR2 adaptors, but that Mal is primarily a subordinate of MyD88. It is its primary function to localize MyD88 to the plasma membrane and facilitate the formation of a TLR-adaptor interphase (Kagan and Medzhitov, 2006). There is little evidence that Mal has its own signalling capabilities. As an example, Mal interacts with TRAF6 through a specific-binding motif, which is not present in MyD88 (Mansell *et al*, 2004). However, neither this nor other Mal interactions with further downstream molecules have been evaluated in the context of MyD88 deficiency, that is a contribution of Mal to cellular functions independently of MyD88 has not been described. Accordingly, it is currently unclear whether the model of Mal and MyD88 as two components of one functional TLR2 adaptor is sufficiently developed to justify the evolutionary necessity of the complex TLR2-6-1-adaptor multimer. Furthermore, conclusive models, which integrate cytoplasmic proteins without a TIR domain into the TLR adapter, have not been developed.

Earlier, it was shown that TLR2 agonists activate the lipid-kinase phosphoinositide 3-kinase (PI3K) and that the regulatory subunit 5 α interacts with the TLR2-TIR-associated complex (Arbibe *et al*, 2000; Henneke *et al*, 2005). This finding was intriguing, as PI3K exerts many biologically important functions, which are not classical effects of TLR ligation, such as cytoskeletal remodelling and trafficking of intracellular organelles (Hazeki *et al*, 2007). However, the relationship of PI3K to MyD88 and Mal in the context of TLR2 had not been

*Corresponding author. Centre for Paediatrics and Adolescent Medicine and Centre of Chronic Immunodeficiency, University Medical Centre Freiburg, Mathildenstrasse 1, Freiburg 79106, Germany.
Tel.: +49 761270 4526; Fax: +49 761270 4378;
E-mail: philipp.henneke@uniklinik-freiburg.de

Received: 5 November 2008; accepted: 22 May 2009; published online: 2 July 2009

addressed. Furthermore, the biological implications of TLR2-mediated PI3K activation beyond NF κ B activation were not resolved. Accordingly, this study was designed to clarify the relative position for PI3K in the TLR2-adaptor complex and the functional role in response to TLR2 ligation.

We found that TLR2-dependent PI3K activation by diacylated lipopeptides was dependent on Mal, whereas substantial cellular activation by triacylated lipopeptides could be observed in Mal-deficient macrophages. Mal interacted with p85 α in an inducible fashion. PI3K activation through Mal was sufficient to induce Akt phosphorylation and, together with MyD88, essentially mediates phosphatidylinositol(3,4,5)P₃ (PIP₃) generation at the leading edge of macrophages.

Results

Mal is essential for TLR2/6, but not TLR2/1-dependent activation of NF κ B

Earlier studies have shown that TLR2 and TLR4 are the sole TLRs that engage both MyD88 and Mal for signal transduction (Fitzgerald *et al*, 2001; Horng *et al*, 2001; Yamamoto *et al*, 2002). Here, we analysed whether the nature of the TLR2 heteromer influences the contribution of Mal to the signalling response. In full accordance with the proposed model that Mal and MyD88 are both required for TLR2-dependent cytokine formation, the formation of TNF- α was severely impaired in MyD88-, Mal- and TLR2-deficient mouse macrophages on stimulation with the diacylated lipopeptide MALP2 (Figure 1A). In contrast, the TNF- α response to the triacylated TLR2 ligand Pam₃CSK₄ was largely independent of Mal, although the response was somewhat reduced in Mal-deficient cells at low Pam₃CSK₄ concentrations (Figure 1B). Notably, MyD88- and Mal-deficient cells exhibited an impaired response to LPS from *Escherichia coli*, but responded normally to PolyI:C stimulation (Figure 1C and data not shown), in full accordance with the published phenotype (Yamamoto *et al*, 2002; Kenzel *et al*, 2009). These data indicate that, in contrast to the universal role of MyD88, Mal has a more subtle function in the TNF- α production depending on the degree of acylation of the lipopeptide ligand and probably the nature of the TLR2-containing heteromer.

Mal is not sufficient to drive NF κ B activation in the absence of MyD88

To further resolve whether Mal and MyD88 occupy discrete positions in NF κ B activation, we transfected wild-type (WT), MyD88 and Mal knock-out macrophages with plasmids encoding for these adaptor proteins or parental plasmid. As shown in Figure 2A, expression of the adaptors MyD88 or Mal in WT macrophages led to a strong, ligand-independent induction of an NF κ B-dependent luciferase reporter gene, which was comparable to that obtained by MALP2 stimulation. Similarly, expression of both adaptors potently induced NF κ B activation in Mal-deficient macrophages (Figure 2C). This indicated that MyD88 is located downstream of Mal, as it does not require Mal for transcriptional activation. In contrast, expression of Mal in MyD88 knock-out macrophages did not activate the NF κ B-dependent reporter (Figure 2B). These observations were fully compatible with a model, in which Mal is located upstream of MyD88 and requires the presence of MyD88 for the induction of NF κ B. The relative

Figure 1 Mal is essential for the macrophage TNF- α response to the TLR2/6 ligand MALP2. WT, MyD88^{-/-}, Mal^{-/-} and TLR2^{-/-} mouse macrophages were stimulated overnight with increasing concentrations of MALP2 (A), Pam₃CSK₄ (B) or with PolyI:C (50 μ g/ml) (C). Then, TNF- α concentration in the culture supernatant was measured by ELISA ($n=2 \pm$ s.d.). Data shown are representative of at least three independent experiments.

contribution of Mal and MyD88 was not cell-type specific, as embryonic fibroblasts from WT-, MyD88- and Mal-deficient mice exhibited the same phenotype (data not shown).

The PI3K activity in response to TLR2/6 agonists is delayed in MyD88-deficient cells

Several studies have underlined the importance of PI3K function in NF κ B activation downstream of IL-1R and various TLRs (O'Neill *et al*, 1997; Arbibe *et al*, 2000; Ojaniemi *et al*, 2003). However, although TLR2 was the first TLR, which was reported to activate PI3K, the relationship of PI3K to the TLR2-adaptor proteins had not been clarified. First, we analysed, whether PI3K is located up- or downstream of

Figure 2 Mal requires MyD88 for the activation of NFκB. WT (A), MyD88^{-/-} (B) and Mal^{-/-} (C) mouse macrophages were transfected with the NFκB-dependent reporter Elam.luc-pCDNA, plus a Renilla luciferase reporter, plus MyD88, Mal or parental plasmid and stimulated, where indicated, with MALP2 (10 ng/ml) or vehicle. After 5 h luciferase activity was measured. Data were normalized to the Renilla luciferase values and are shown as the mean ± s.d. of triplicate wells. The experiment shown is representative of at least three independent experiments.

Mal and MyD88. We used Akt phosphorylation as a read out of PI3K activity. Two principal pathways, which connect PI3K to phosphorylation of Akt on residues Thr308 and Ser473, have been described. First, PI3K catalyses the phosphorylation of phosphatidylinositol(4,5)P₂ (PIP₂) to PIP₃, which, in turn, binds to and phosphorylates Akt through the phosphatidylinositol-dependent kinases (PDK1 and PDK2) (Cantley, 2002). More recently, it has been shown that mammalian TOR (mTOR) directly phosphorylates Akt on Ser473 *in vitro* and facilitates Thr308 phosphorylation by PDK1 (Sarbasov *et al*, 2005).

As depicted in Figure 3A, MALP2 induced phosphorylation of Akt in WT macrophages with a maximum between 10 and 20 min after stimulation. MALP2-induced Akt phosphorylation was PI3K-dependent, as it was abrogated by preincubation of the cells with the PI3K inhibitor LY294002. As compared with WT cells, Akt phosphorylation by MALP2 was severely impaired in Mal-deficient macrophages (Figure 3C), as well as Mal-deficient mouse embryonic fibroblasts (MEF) (Figure 3D). In contrast, Akt phosphorylation was induced by MALP2 in MyD88-deficient macrophages (Figure 3E). Notably, the peak of Akt phosphorylation was retarded when compared with WT cells (maximal phosphor-

ylation at 60 min after stimulation). These findings indicated that Mal, but not MyD88, essentially contributed to TLR2/6-induced PI3K activation, although MyD88 accelerated the kinetics of this process. Intriguingly, the TLR2/1 agonist Pam₃CSK₄ induced PI3K activity independently of Mal and of MyD88 (Figure 3B, C and E). Accordingly, TLR2/6 and TLR2/1 induced PI3K activation through distinct pathways, which differ in the usage of Mal and MyD88.

Mal requires PI3K for efficient NFκB activation

We have shown that Mal is essential for PI3K activation. As PI3K has been implicated in the transactivation of the NFκB subunit p65 in response to TLR2 stimulation (Strassheim *et al*, 2004), we wondered whether PI3K was involved in ligand-independent NFκB activation on heterologous expression of Mal. To clarify this subject, we transfected HEK293 cells with TLR2 and an NFκB-dependent ELAM-luciferase reporter plasmid. As shown in Figure 4A, NFκB activation by the diacylated lipopeptide FSL-1 was inhibited by increasing concentrations of LY294002 in a dose-dependent fashion. This finding confirmed earlier reports on an important role of PI3K in the response to different TLR2 agonists (Arbibe *et al*, 2000; Henneke *et al*, 2005). Next, we transfected HEK293 cells with Mal and ELAM-luciferase reporter plasmids and inhibited PI3K with LY294002. As shown in Figure 4B, Mal transfection in the presence of endogenous MyD88 in these cells was sufficient to potently induce the ELAM-luciferase reporter and this activation was inhibited by LY294002 in a dose-dependent fashion. NFκB transcriptional activity relies on two mechanisms: degradation of the repressor IκB allows its translocation to the nucleus and phosphorylation of the p65 subunit on residue Ser536 further enhances gene expression (Perkins, 1997; Jefferies *et al*, 2001). To better understand the specific role of PI3K in TLR2-dependent NFκB activation, we made use of a p65 reporter system, which is activated independently of IκB degradation (Vanden Berghe *et al*, 1998). As shown in Figure 4C, incubation of the cells with increasing concentrations of LY294002 decreased the p65-Gal4/Gal-luciferase reporter response to heterologous Mal expression. Hence, PI3K activation downstream of Mal is essential for the transactivation of the NFκB subunit p65.

Mal interacts with p85α independently of TLR2 and MyD88

As outlined above, PI3K activation occurred downstream of Mal. Next, we analysed whether Mal and p85α, the regulatory subunit of PI3K, interacted physically with each other. First, RAW264.7 macrophages were stimulated with MALP2 for the indicated time periods (Figure 5A). Then cells were lysed and cell lysates were subjected to immunoprecipitation and western blot analysis. Under these conditions, we found that endogenous p85α and Mal interacted in an inducible fashion with a maximal effect 5–10 min after stimulation. The same result was obtained in immortalized bone-marrow macrophages from C57BI/6 mice (data not shown). In the case of another TLR, namely TLR5, it has been postulated that the adaptor MyD88 bridges the interaction between the TIR domain of the receptor and PI3K (Rhee *et al*, 2006). Therefore, we wondered whether MyD88 was involved in the association of Mal with p85α. To address this question, we created the cell line HEK-MyD88si, in which MyD88 expression is effectively silenced by shRNA as confirmed by

Figure 3 Mal is essential for TLR2-induced Akt phosphorylation (Ser473). As indicated, 8×10^5 WT, MyD88^{-/-} and Mal^{-/-} macrophages (A–C, E) and 3×10^5 MEFs (D) were stimulated with MALP2 (10 ng/ml) or Pam₃CSK₄ (100 ng/ml) for 10–120 min or with PolyI:C (50 µg/ml; 30 min). Where indicated, the cells were pretreated during 40 min with the PI3K inhibitor LY294002 (75 µM) and stimulated for 20 min. Then, cells were lysed and cell lysates were subjected to western blot analysis for phospho-Ser473 Akt and after membrane stripping for total Akt.

immunoblot and functional analysis (abrogated NFκB response to IL-1β, Figure 5B and C). Importantly, the response to polyI:C, which stimulates NFκB in an MyD88-independent fashion, was preserved in these cells. HEK293 and HEK293-MyD88si cells were co-transfected with Mal and p85α, respectively. Immunoprecipitation and western blot analysis of these constructs revealed that MyD88 expression was not a prerequisite for the interaction of Mal and p85α (Figure 5B). Furthermore, the only established transmembrane partners of Mal, TLR2 and 4 were not essential in this context, as the used HEK293 cells did not express either of these TLRs (data not shown). In full accordance with this finding, endogenous Mal and p85α interacted in response to TLR2 stimulation in an inducible fashion in MyD88-deficient macrophages (Figure 5D). Notably, the colocalization of both proteins was retarded in MyD88 deficient as compared with WT macrophages. Accordingly, although MyD88 is not required for TLR2-dependent PI3Kinase activation, it does influence the kinetics of both the physical Mal–p85α interaction and Akt phosphorylation (Figures 3E and 5D).

Next, we assessed the spatial distribution of the Mal–p85α complex. To this end, we exploited the observation that heterologous expression leads to ligand-independent interaction of both proteins (Figure 5B). Therefore, we stably expressed GFP-p85α in HEK293 cells and transiently transfected the resulting clonal HEK-GFP-p85 cell line with the fluorescent fusion construct CFP-Mal. As shown in

Figure 5E, CFP-Mal is predominantly localized at the plasma membrane and in internal vesicle-like structures, whereas p85α seemed equally distributed throughout the cytoplasm in resting cells, in accordance with earlier reports (Gillham *et al*, 1999; Kagan and Medzhitov, 2006). When both proteins were co-expressed in HEK-GFP-p85 cells, GFP-p85α co-localized with CFP-Mal at the plasma membrane.

The p85α regulatory subunit contains an amino-terminal and a carboxi-terminal Src homology domain 2 (SH2), both of which mediate the association with proteins containing phosphotyrosines in the context of YxxM/W sequence motifs. The so-called inter-SH2 domain binds to p110, the catalytic subunit of PI3K. Furthermore, an amino-terminal SH3 domain mediates the interaction of p85α with proteins containing PxxP motifs. As the amino-terminus of Mal comprises various PxxP motifs, we analysed whether the SH3 domain of p85α was critical for the interaction with Mal. We engineered a vector encoding for GFP-SH3 and expressed it together with Mal in HEK293 cells. In contrast to full-length p85α, the GFP-tagged SH3 domain of p85α did not colocalize with Mal (Figure 5E). The confocal-microscopy data were confirmed by immunoprecipitation analysis, in which GFP-SH3 was found not to be associated with Mal under conditions in which full-length p85α constitutively interacted with Mal (Figure 5F). Hence, the SH3 domain of p85α was structurally not sufficient for the interaction with Mal. Next we assessed, whether a change in the residue Pro125 of Mal

Figure 4 PI3K mediates Mal-induced transactivation of the NFκB subunit p65. (A) HEK293 cells were transfected with Elam.luc-pCDNA-luciferase reporter and TLR2-Flag and stimulated with the diacylated lipopeptide FSL-1 (TLR2/6 agonist, 5 ng/ml) after incubation with increasing concentrations of LY294002 or vehicle (DMSO). (B) HEK293 cells were transfected with Elam.luc-pCDNA or p65-Gal4 and Gal4-luciferase reporter plasmid (C) and Mal and incubated with increasing concentrations of LY294002 or with DMSO. After 6 h, luciferase activity was measured. Data were normalized to the Renilla luciferase values and are shown as the mean ± s.d. of triplicate wells. The results shown are representative of at least three independent experiments for A and B and two independent experiments for C.

disrupted the Mal-p85α association. Mal/Pro125 is an essential residue involved in Mal-Btk (Bruton's tyrosine kinase) interaction and subsequent Mal phosphorylation on Tyr residues (Piao *et al*, 2008). However, exchange of this residue by histidine (MalP125H; Fitzgerald *et al*, 2001) did not affect the interaction of Mal with p85α. Hence, the SH3 domain of p85α was not sufficient and the Pro125 in Mal was not necessary for the formation of the Mal-p85α complex.

Mal mediates PIP₃ generation and membrane ruffling at the leading edge

The presented data show that Mal is required for proper PI3K activation on TLR2 stimulation. PI3K has earlier been shown

to mediate PIP₃ generation and membrane ruffling at the leading edge of macrophages (Evans *et al*, 2006; Lasunskia *et al*, 2006). Accordingly, we wondered whether TLR2 ligands induced these morphological changes and whether Mal was a critical intermediate in this process.

First, we used a quantitative HPLC analysis to evaluate PIP₃ accumulation. WT-, Mal- and MyD88-deficient macrophages were chased with ³²P and stimulated with FSL-1 for 90 min in the presence or absence of LY294002. Subsequently, the cells were lysed and, after lipid extraction and HPLC separation (Figure 6A and B), the levels of PIP₃ were quantified. We found, in full accordance with the data presented above, that engagement of TLR2/6 induced the specific formation of PIP₃ as compared with PIP₂, and that both PI3K and Mal were essential in this process. However, as opposed to our data on Akt phosphorylation under these conditions, MyD88 was necessary for PIP₃ formation. Hence, although Mal, but not MyD88, is required for PI3K activation by diacylated lipoproteins, both are necessary for PIP₃ formation.

Next, we analysed whether engagement of TLR2 by lipoproteins led to macrophage polarization and assessed the roles of Mal and MyD88 in this context. For that reason, we transfected RAW264.7 macrophages with a plasmid construct, which encodes for the pleckstrin homology (PH) domain of Akt fused to GFP (GFP-PH). This domain recognizes and translocates to PIP₃-rich domains in the plasma membrane (Downward, 1998). As shown in Figure 7A, GFP-PH was homogeneously distributed throughout the cytoplasm in resting macrophages. In contrast, stimulation of TLR2 with diacylated lipoproteins (FSL-1, 90 min) led to progressive macrophage polarization, membrane ruffling and localization of GFP-PH at the plasma membrane of the leading edge. Notably, when RAW264.7 or WT macrophages were transfected with Mal (Figure 7A and data not shown), we observed a similar pattern of GFP-PH localization at the leading edge structure, which indicated that ligand-independent activation of Mal drives PIP₃ formation and macrophage polarization. Furthermore, treatment of Mal-transfected macrophages with LY29400 inhibited cell polarization, which confirmed the involvement of PI3K in these processes. However, when Mal or MyD88-deficient macrophages were transfected with GFP-PH and were stimulated with FSL-1, accumulation of GFP-PH at the leading edge was abrogated (Figure 7B). Hence, Mal and MyD88 are both required for TLR2/6-mediated PI3K-dependent macrophage polarization and PIP₃ formation, whereas only Mal is essential for Akt phosphorylation.

Discussion

In the normal host, resident phagocytes destroy invading bacteria without a measurable systemic inflammatory response (Mancuso *et al*, 2004). To ensure this, phagocytes need to be primed for the rapid mounting of antimicrobial forces by spurious concentrations of microbial products, before bacteria and phagocytes physically meet. The network of TLRs represents an attractive solution for the highly sensitive recognition of foreign molecules. In the context of discrete-signal regulation that converts recognition of bacterial components by TLR2 into important morphological changes in macrophages and accumulation of lipid mediators

at the leading edge, this study provides three key novel findings. First, downstream of TLR2/6, Mal directly interacts with the PI3K subunit p85 α . This interaction is sufficient to drive PI3K-dependent Akt phosphorylation. Second, MyD88 is not essential for PI3K activation (as indicated by MyD88-independent Akt phosphorylation), but cooperates with Mal in PIP₃ generation and polar shape changes of the macrophage. Third, Mal is a specific regulator for the TLR2/6 heterodimer, but not for TLR2 in general.

One of the most compelling findings in the history of TLR research has been the absolute requirement of MyD88 in the

context of TLR2 function, whenever its role was assessed. Early on, the principal difference to TLR4 was appreciated, as TLR4 engaged MyD88 dependent an independent pathways. The quest for the alternative TLR4 adaptor led to the discovery of Mal, also denominated TIRAP, which was originally believed to serve this function (Fitzgerald *et al*, 2001; Horng *et al*, 2002). However, one unexpected feature of the Mal^{-/-} mouse phenotype was the striking deficiency in TLR2 signalling (Horng *et al*, 2002; Yamamoto *et al*, 2002). As these observations were published, Mal and MyD88 have been regarded as part of an inextricably linked adaptor complex, that is Mal and MyD88 deficiency resulted in the same phenotype with respect to TLR2 activation. Compelling recent evidence led to further elaboration of a model for MyD88–Mal interaction, albeit in the context of TLR4 (Kagan and Medzhitov, 2006). Recruitment of MyD88 to the plasma membrane is now regarded as the principal function of Mal and this is achieved through the amino-terminal-binding domain for PIP₂. In accordance with this concept, Mal has a bridging function between MyD88 and TLR4 because the TIR-domain surfaces of MyD88 and TLR4 are electro-positive, whereas that of Mal is predominantly electro-negative.

Our study supports a model, in which Mal is located upstream of MyD88 in the TLR2/6-signalling pathway: MyD88 expression drives NF κ B activation in the absence of Mal, whereas Mal cannot do the same in the absence of MyD88. Hence, Mal is the adapter protein in utmost proximity to TLR2. Our data indicate that TLR2 signalling and PI3K activation converge at the level of Mal and, therefore, very upstream in the signalling pathway. On stimulation of TLR2/6, Mal and p85 α colocalize. This process is predominantly localized at the plasma membrane and occurs in the absence of MyD88. The interaction of Mal with p85 α is specific and does not essentially involve TLR2 as an intermediate, as ligand-independent colocalization of Mal and p85 α occurs in the absence of TLR2. Our experiments further suggest that

Figure 5 TLR2/6 activation induces colocalization of Mal and p85 α . (A) RAW264.7 macrophages were stimulated with MALP2 (10 ng/ml) for the indicated time periods. Lysates of these cells were subjected to immunoprecipitation with anti-Mal-coated protein A/G sepharose and western blot analysis with p85 α - and Mal-specific antibodies. The intensity of the p85 α bands was quantified as relative to the bands resulting from Mal immunoprecipitation and western blot. (B) HEK293 cells or HEK293-MyD88si, which stably express shRNA for MyD88 were transfected with p85 α and Mal. Mal was immunoprecipitated with anti-CFP antibody followed by western blot with anti-p85 α . Input lysates were analysed for the expression levels of endogenous MyD88. Total Akt served as a loading control. (C) HEK293-MyD88si or the parental cell line HEK293 were transfected with an NF κ B reporter and Renilla luciferase reporter plasmids and stimulated with IL-1 β (25 ng/ml) or PolyI:C (50 μ g/ml). Luciferase activity was measured as indicated in the methods section. (D) MyD88^{-/-} macrophages were stimulated and lysed as in (A). Then, cell lysates were immunoprecipitated with Mal antibody, and the intensity of p85 α bands was quantified as relative to the bands resulting from Mal immunoprecipitation. (E) HEK293 cells were transfected with GFP-p85 α or GFP-SH3 plus CFP-Mal. The intracellular localization of Mal and p85 α was evaluated by confocal microscopy. Bar 20 μ m. (F) HEK293 cells were transfected as indicated and cell lysates were immunoprecipitated with anti-GFP. Subsequently, Mal-HA, MalP125H-HA and p110-HA were detected by immunoblot with HA antibody. Expression levels of Mal in the cell lysates were determined by immunoblot.

p85 α domains other than the SH3 domain mediate the interaction. It seems intriguing that Mal binds to the PI3K substrate PIP₂ through a specific amino-terminal domain (Kagan and Medzhitov, 2006). Accordingly, we propose a novel role

for Mal: Mal recruits PI3K to membranous sites of high substrate (PIP₂) availability.

Moreover, although Mal was absolutely required for TLR2/6-dependent cytokine formation and PI3K activation, TLR2 dependent, but TLR6-independent, signalling was largely independent of Mal (Figure 1). Hence, Mal has distinct functional roles depending on the composition of the TLR heteromer. It is tempting to speculate on a yet to be identified functional Mal equivalent in TLR6-independent TLR2 signalling, which is partially TLR1 dependent (Figure 8). The complexity and variability in the interaction of PI3K with individual TLRs is further exemplified by a study on TLR5, in which MyD88 is involved in the interaction (Rhee *et al*, 2006). Importantly, Mal has not been implicated as part of the TLR5-signalling complex.

The observation that Mal induces a specific sequence of signalling events is in line with reports on its ability to recruit TRAF6 through a TRAF6-binding domain, which distinguishes it from MyD88 (Mansell *et al*, 2004). Furthermore, Mal is modified by Btk (Gray *et al*, 2006; Piao *et al*, 2008) and processed by caspase-1 (Miggin *et al*, 2007), which leads to transactivation of NF κ B in both cases. However, the interaction of Mal with PI3K is privileged over that between Mal and TRAF6, as Mal-dependent activation of NF κ B, but not Akt, is dependent on MyD88 (Figure 2). Activation of PI3K regulates many biological processes through the generation of the potent second messenger PI(3,4,5)P₃ from PI(4,5)P₂ in the plasma membrane. PIP₃ attracts a number of PH domain-containing proteins, such as Akt, Rac and the cdc42 nucleotide exchange factor Vav (Lemmon and Ferguson, 2000). Termination of this pathway is ensured mainly by the two phosphatases SHIP and PTEN, which break down PIP₃ to PI(3,4)P₂ and PI(4,5)P₂ (Sly *et al*, 2003). The formation of an actively ruffling leading edge with local accumulation of PIP₃ is a key cellular event that always precedes migration of macrophages (Evans *et al*, 2006). PI3K, Ca²⁺ flux and F-actin polymerization are the three major mechanisms that underlie leading edge activity and stability. Therefore, a discrete-signalling branch in close proximity to TLR2 specifically links recognition of bacterial toxins to cellular polarization and preparation for migration.

Earlier studies indicated that the regulatory subunit of PI3K, p85 α , may interact directly with the TIR domain of

Figure 6 TLR2/6 ligand-induced PIP₃ generation is abrogated in Mal^{-/-} and MyD88^{-/-} macrophages. **(A)** WT, Mal^{-/-} and MyD88^{-/-} macrophages were grown to 80% confluence and were pretreated with LY294002 or vehicle and stimulated with FSL-1 during 90 min. Then, lipids were extracted and separated by HPLC and the ratio of PI(3,4,5)P₃ to the precursor PI(4,5)P₂ was calculated ($n=3 \pm$ s.d.). **(B)** The lipid preparation as described in (A) was resolved by HPLC. The retention time of PI(3,4,5)P₃ is indicated.

Figure 7 Mal and MyD88 are essential mediators of macrophage polarization. **(A)** RAW264.7 macrophages were transfected with the PH domain of Akt (GFP-PH) and Mal or control vector and stimulated with FSL-1 (bottom left panel) or incubated with LY294002 (150 μ M, bottom right panel). **(B)** WT, Mal and MyD88-deficient macrophages were transfected with GFP-PH and stimulated with FSL-1 (10 ng/ml). In all cases, micrographs were taken at 90 min. Bar indicates 20 μ m.

Figure 8 Model for the role of Mal connecting PI3K to TLR2/6. NFκB activation in response to di- and triacylated TLR2 ligands occurs in an MyD88-dependent fashion. The cytoplasmic TLR2 adaptor comprises Mal upstream of MyD88 in the TLR2/6-dependent NFκB pathway. Mal binds to PIP₂-rich domains in the plasma membrane through its specific PIP₂-binding domain. Mal furthermore binds directly to p85α, the regulatory subunit of PI3K, which uses PIP₂ as its substrate for PIP₃ generation. In addition, PI3K activates downstream events like Akt phosphorylation and macrophage polarization. In the case of TLR2/1 ligands (Pam₃CSK₄) another adaptor may mediate PI3K activation, as this process occurs in the absence of Mal and MyD88.

TLR2, as mutations in putative-binding motives for SH2 domains (YxxM) abrogated downstream signalling (Arbibe *et al*, 2000; Henneke *et al*, 2005). A role of YxxM in PI3K activation was confirmed for TLR3; however, not for TLR5 (Sarkar *et al*, 2003, 2004, 2007; Rhee *et al*, 2006). At this stage, we cannot discard the possibility that, next to Mal, TLR2 or TLR6 contain binding sites for p85α. However, in case direct TLR2/6–p85α interactions occur, they are not sufficient in this context, as MALP2 does not drive Akt phosphorylation in Mal-deficient cells (Figure 3C and D). It is conceivable that the exchange of tyrosine residues in the cytoplasmic domain of TLR2 interferes with the formation of the entire TLR2-adaptor complex, rather than specifically with recruitment of p85α to TLRs.

It seems intriguing that activation of TLR2/6 leads to two PI3Kinase-dependent pathways. The first pathway is only Mal dependent and absolutely required for Akt phosphorylation, whereas the second is Mal and MyD88 dependent and is required for PIP₃ generation and macrophage polarization. Moreover, we observed enhanced kinetics of both TLR2-dependent Akt phosphorylation and Mal–p85α colocalization in MyD88 competent as opposed to MyD88-deficient cells (Figures 3 and 5). It is tempting to speculate that MyD88 mediates the rapid formation of a signalling platform that increases the affinity of docking sites for other signalling molecules. Whether differential engagement of the mTOR complex accounts for distinct but overlapping roles of Mal and MyD88 in PI3K activation (PIP₃ turn over) and Akt phosphorylation remains to be established (Sarbasov *et al*, 2005).

The discovery of discrete roles for Mal and MyD88 in TLR2-dependent PI3K activation leads to a relevant modification of the TLR2-adaptor model. Mal is not only aiding in the localization of MyD88, but also contributes to the specific profile of the cellular response to bacterial lipoproteins.

A particular role of Mal in infection biology is backed up by studies that associate molecular variations of Mal with a reduced susceptibility to a range of infectious diseases, such as Malaria, tuberculosis and pneumonia (Hawn *et al*, 2006; Khor *et al*, 2008). As of yet, single nucleotide polymorphisms of MyD88 have not been linked to increased susceptibility or resistance of disease. However, autosomal recessive MyD88 deficiency results in a relatively specific infection profile, that is increased susceptibility to invasive streptococcal and staphylococcal disease (von Bernuth *et al*, 2008). Whether these associations relate to distinct roles of Mal and MyD88 in innate immunity remains speculative at this stage.

In summary, we herewith report on a novel pathway, which induces morphological changes in macrophages exposed to diacylated lipoproteins and functionally discriminates Mal from MyD88.

Materials and methods

Reagents

The synthetic diacylated lipopeptides MALP2 (Macrophage-activating Lipopeptide-2, Axxora, San Diego, USA) and FSL-1 (Pam₂CDDPKHSPKSF, InvivoGen, San Diego, USA) are of equimolar potency (TNF-α response in primary mouse macrophages) and both essentially require TLR2 and 6 for cytokine formation. PolyI:C (polyinosine-polycytidylic acid) was purchased from InvivoGen, Pam₃CSK₄ (N-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-(R)-cysteine) from EMC microcollections (Tübingen, Germany). Sources for antibodies were as follows: polyclonal and monoclonal antibodies against total Akt and phospho-Akt (Ser-473)—Cell Signaling Technology (Danvers, USA); polyclonal anti-Mal—Axxora; polyclonal anti-MyD88—eBioscience (San Diego, USA); monoclonal anti-p85α—Assay Designs (MI, USA); monoclonal anti-HA—InvivoGen; monoclonal and polyclonal anti-GFP—BD Biosciences (CA, USA) and Invitrogen (CA, USA); HRP conjugated anti-rabbit and anti-mouse—Amersham Biosciences (NJ, USA). Plasmids encoding for Mal-HA-pCDNA, Mal-CFP-pCDNA and MyD88-YFP-pCDNA were constructed as described (Latz *et al*, 2002). WJ Gullick (Kent) kindly provided us with GFP-p85α. P85-myc and Akt-PH domain were kind gifts of LC Cantley (Boston) and J Downward (London). p110-HA was obtained from Addgene (Cambridge), p65-Gal4 and Gal-luciferase reporter system was a present from KA Fitzgerald. Plasmid preparations were done using EndoFree plasmid purification kit (Qiagen, Düsseldorf) and GenElute HP endotoxin-free kit (Sigma, St Louis). The plasmid encoding for the SH3 domain of p85α was engineered with the Quickchange site directed mutagenesis system (Promega, Madison) and the oligonucleotides 5'-CCTCCACACCATAGCCCCGGCCACCT-3' and 5'-AGGTGGCCGGGGCTATGGTGTGGGAGG-3' and GFP-p85α as a template. TNF-α was quantified using commercially available ELISA kits (R&D Systems), according to the manufacturer's instructions.

Western blot analysis

Macrophages were grown overnight in 12-well plates. After stimulation, the cells were washed with PBS and then lysed with lysis buffer (200 mM NaCl, 1% Triton, 0.05% CHAPS, 1 mM NaF, 1 mM Na₃VO₄, 50 mM Tris-HCl, pH 7.5) and a protease inhibitor mix (complete-mini from Roche Applied Science). The lysates were cleared by centrifugation at 8000g for 15 min (4°C) and equal amounts of the supernatant were separated by electrophoresis on SDS 12% polyacrylamide gels (NuSep, Sydney, Australia) and transferred to a PVDF membrane (BioRad). The membrane was blocked for 1 h in 5% milk in TBST (20 mM Tris-HCl, pH 7.6, 0.15 M NaCl and 0.1% Tween20), then it was incubated overnight at 4°C with primary antibody and subsequently with peroxidase-conjugated secondary antibody for 2 h. Immunoreactive proteins were detected by using ECL detection reagents (Amersham Pharmacia) and the ChemiDoc analyzer followed by quantification with the Quantity One software (BioRad).

Immunoprecipitation assay

Cell lysates were prepared as indicated above. Protein concentrations were quantified with the Bradford assay (BioRad) and loaded onto ProteinA/G sepharose beads (Calbiochem), which had been pretreated with a specific antibody or IgG control (2 h, 4°C), and incubated for 4 h at 4°C. After washing (3 × in lysis buffer), proteins were eluted (2 × SDS-loading buffer) and analysed by western blot.

Luciferase reporter assay

HEK293 cells were seeded into 96-well dishes at a density of 4×10^4 per well. Next day, cells were transiently transfected with the indicated plasmids using Trans-IT LT1 transfection reagent (Mirus). The reporter plasmids Elam.luc-pCDNA and Gal-luciferase were transfected to assess NFκB activity and the transfection efficacy was normalized by cotransfection of the constitutively active Renilla luciferase reporter plasmid (Promega). The following day, cells were stimulated for 5 h as indicated and lysed in reporter lysis buffer (Promega). Reporter gene activity was measured with a plate-reader luminometer (MicroLumat Plus, Berthold Detection Systems).

Transfection of macrophages

Macrophages (2×10^6 per well) were transfected with the indicated plasmids using the Nucleofector (Amaxa Biosystems) and nucleofection buffer (Mouse Macrophage Nucleofector Kit) with 2 µg of DNA according to the manufacturer instructions. Then, cells were seeded in 24-well plates. Luciferase assay was performed after 11 h. NFκB transcriptional activity was normalized by activity of the Renilla reporter.

Cell lines

HEK293 cells stably expressing GFP-p85α. HEK293 cells were transfected with the GFP-p85α vector and stable transfectants were selected with Zeocin (InvivoGen). Single clones were isolated by FACS sorting and GFP-p85α protein expression levels were determined by FACS and western blot.

HEK293 cells with silenced MyD88. HEK293 cells were transfected with a vector expressing shRNA targeting human MyD88 (InvivoGen). Stable transfectants were selected with Zeocin. Monoclonal cell lines were isolated by limiting dilution (96-well plates) and tested for MyD88 protein expression levels by western blot. WT-, Mal- and MyD88-deficient mice were obtained from S Akira (Adachi *et al*, 1998; Yamamoto *et al*, 2002). Mouse embryonic fibroblasts were a kind gift of O Takeuchi (Takeuchi *et al*, 2001). Knock-out macrophages were provided by DT Golenbock. The immortalized cell lines were generated with a J2 recombinant retrovirus (carrying

v-myc and v-raf(mil) oncogenes) from primary bone-marrow cells as described (Hornung *et al*, 2008). The macrophage phenotype was verified by surface expression of the markers CD11b (M1/70, BD Pharmingen) and F4/80 (BM8, eBiosciences) as well as a range of functional parameters, including responsiveness to TLR ligands and bacterial uptake.

Confocal-microscopy/life imaging

Macrophages were transfected, grown overnight on glass coverslips, stimulated with FSL-1 (10 ng/ml) for 90 min and fixed with 3% paraformaldehyde at room temperature for 15 min. Nuclei staining was done with Hoechst (1 ng/ml in PBS, Molecular Probes). Images were collected using a Zeiss ApoTome/Axioplan2 microscope.

For life imaging cells were grown in an eight-well chamber (IBIDI) overnight and transfected with Mal-CFP, GFP-SH3 or parental plasmid. After overnight incubation, the images were captured with a microscope LSM-iNLO2, axiovert 200 M. The temperature was maintained at 37°C using an IBIDI heating stage. GFP was excited with a 488-nm laser line and detected at 505 nm. CFP was excited with a 458-nm laser line and detected at 480–520 nm followed by spectral unmixing from GFP fluorescence using the LSM Image software.

Phosphatidylinositol(3,4,5)-trisphosphate measurement

Levels of cellular phosphatidylinositides were determined essentially as described (Laffargue *et al*, 2002). Briefly, macrophages were incubated for 60 min in phosphate-free DMEM containing 0.5% FBS and 100 µM LY294002 where applicable, before addition of 1 mCi/ml [³²Pi]-orthophosphate (carrier-free; Perkin Elmer, Switzerland) and 20 ng/ml FSL-1 for 90 min. After removal of non-incorporated [³²Pi], lipids were extracted, deacylated and separated on HPLC as described (Arcaro and Wymann, 1993).

Acknowledgements

We are grateful to Bernhard Kremer and Björn Corleis for outstanding technical assistance. This work was supported by grants from the Bundesministerium für Bildung und Forschung (BMBF 01 EO 0803), the Deutsche Forschungsgemeinschaft (He 3127/2-3 and 3-1 to PH), from the Swiss National Science Foundation (3100A0-109718 to MPW) and from the National Institutes of Health (RO1 AI052455-06A1 to DTG).

Conflict of interest

The authors declare that they have no conflict of interest.

References

- Adachi O, Kawai T, Takeda K, Matsumoto M, Tsutsui H, Sakagami M, Nakanishi K, Akira S (1998) Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity* **9**: 143–150
- Arbibe L, Mira JP, Teusch N, Kline L, Guha M, Mackman N, Godowski PJ, Ulevitch RJ, Knaus UG (2000) Toll-like receptor 2-mediated NF-κB activation requires a Rac1-dependent pathway. *Nat Immunol* **1**: 533–540
- Arcaro A, Wymann MP (1993) Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses. *Biochem J* **296** (Part 2): 297–301
- Cantley LC (2002) The phosphoinositide 3-kinase pathway. *Science* **296**: 1655–1657
- Downward J (1998) Mechanisms and consequences of activation of protein kinase B/Akt. *Curr Opin Cell Biol* **10**: 262–267
- Evans JH, Murray D, Leslie CC, Falke JJ (2006) Specific translocation of protein kinase Cα to the plasma membrane requires both Ca²⁺ and PIP2 recognition by its C2 domain. *Mol Biol Cell* **17**: 56–66
- Farhat K, Riekenberg S, Heine H, Debarry J, Lang R, Mages J, Buwitt-Beckmann U, Roschmann K, Jung G, Wiesmuller KH, Ulmer AJ (2008) Heterodimerization of TLR2 with TLR1 or TLR6 expands the ligand spectrum but does not lead to differential signaling. *J Leukoc Biol* **83**: 692–701
- Fitzgerald KA, Palsson-McDermott EM, Bowie AG, Jefferies CA, Mansell AS, Brady G, Brint E, Dunne A, Gray P, Harte MT, McMurray D, Smith DE, Sims JE, Bird TA, O'Neill LAJ (2001) Mal (MyD88-adaptor-like) is required for Toll-like receptor-4 signal transduction. *Nature* **413**: 78–83
- Gillham H, Golding MC, Pepperkok R, Gullick WJ (1999) Intracellular movement of green fluorescent protein-tagged phosphatidylinositol 3-kinase in response to growth factor receptor signaling. *J Cell Biol* **146**: 869–880
- Gray P, Dunne A, Brikos C, Jefferies CA, Doyle SL, O'Neill LA (2006) MyD88 adapter-like (Mal) is phosphorylated by Bruton's tyrosine kinase during TLR2 and TLR4 signal transduction. *J Biol Chem* **281**: 10489–10495
- Hawn TR, Dunstan SJ, Thwaites GE, Simmons CP, Thuong NT, Lan NT, Quy HT, Chau TT, Hieu NT, Rodrigues S, Janer M, Zhao LP, Hien TT, Farrar JJ, Aderem A (2006) A polymorphism in Toll-interleukin 1 receptor domain containing adaptor protein is associated with susceptibility to meningeal tuberculosis. *J Infect Dis* **194**: 1127–1134
- Hazeki K, Nigorioka K, Hazeki O (2007) Role of phosphoinositide 3-kinase in innate immunity. *Biol Pharm Bull* **30**: 1617–1623

- Henneke P, Dramsi S, Mancuso G, Chraibi K, Pellegrini E, Theilacker C, Hubner J, Santos-Sierra S, Teti G, Golenbock DT, Poyart C, Trieu-Cuot P (2008) Lipoproteins are critical TLR2 activating toxins in group B streptococcal sepsis. *J Immunol* **180**: 6149–6158
- Henneke P, Morath S, Uematsu S, Weichert S, Pfitzenmaier M, Takeuchi O, Muller A, Poyart C, Akira S, Berner R, Teti G, Geyer A, Hartung T, Trieu-Cuot P, Kasper DL, Golenbock DT (2005) Role of lipoteichoic acid in the phagocyte response to group B Streptococcus. *J Immunol* **174**: 6449–6455
- Hornig T, Barton GM, Flavell RA, Medzhitov R (2002) The adaptor molecule TIRAP provides signalling specificity for Toll-like receptors. *Nature* **420**: 329–333
- Hornig T, Barton GM, Medzhitov R (2001) TIRAP: an adapter molecule in the Toll signaling pathway. *Nat Immunol* **2**: 835–841
- Hornung V, Bauernfeind F, Halle A, Samstad EO, Kono H, Rock KL, Fitzgerald KA, Latz E (2008) Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nat Immunol* **9**: 847–856
- Ishii KJ, Koyama S, Nakagawa A, Coban C, Akira S (2008) Host innate immune receptors and beyond: making sense of microbial infections. *Cell Host Microbe* **3**: 352–363
- Jefferies C, Bowie A, Brady G, Cooke EL, Li XX, O'Neill LAJ (2001) Transactivation by the p65 subunit of NF-kappa B in response to interleukin-1 (IL-1) involves MyD88, IL-1 receptor-associated kinase 1, TRAF-6, and Rac1. *Mol Cell Biol* **21**: 4544–4552
- Jin MS, Kim SE, Heo JY, Lee ME, Kim HM, Paik SG, Lee H, Lee JO (2007) Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. *Cell* **130**: 1071–1082
- Kagan JC, Medzhitov R (2006) Phosphoinositide-mediated adaptor recruitment controls toll-like receptor signaling. *Cell* **125**: 943–955
- Kenzel S, Santos-Sierra S, Deshmukh SD, Moeller I, Ergin B, Fitzgerald KA, Lien E, Akira S, Golenbock DT, Henneke P (2009) Role of P38 and Egr-1 in the macrophage response to group B streptococcus. *Infect Immun* **77**: 2474–2481
- Khor CC, Chapman SJ, Vannberg FO, Hill AV, O'Neill LA (2008) Analysis of association of the TIRAP (MAL) S180L variant and tuberculosis in three populations—reply. *Nat Genet* **40**: 262–263
- Laffargue M, Calvez R, Finan P, Trifileff A, Barbier M, Altruda F, Hirsch E, Wymann MP (2002) Phosphoinositide 3-kinase gamma is an essential amplifier of mast cell function. *Immunity* **16**: 441–451
- Lasunskiaia EB, Campos MN, de Andrade MR, Damatta RA, Kipnis TL, Einicker-Lamas M, Da Silva WD (2006) Mycobacteria directly induce cytoskeletal rearrangements for macrophage spreading and polarization through TLR2-dependent PI3K signaling. *J Leukoc Biol* **80**: 1480–1490
- Latz E, Verma A, Visintin A, Gong M, Sirois CM, Klein DC, Monks BG, McKnight CJ, Lamphier MS, Duprex WP, Espevik T, Golenbock DT (2007) Ligand-induced conformational changes allosterically activate Toll-like receptor 9. *Nat Immunol* **8**: 772–779
- Latz E, Visintin A, Lien E, Fitzgerald KA, Monks BG, Kurt-Jones EA, Golenbock DT, Espevik T (2002) Lipopolysaccharide rapidly traffics to and from the Golgi apparatus with the toll-like receptor 4-MD-2-CD14 complex in a process that is distinct from the initiation of signal transduction. *J Biol Chem* **277**: 47834–47843
- Lemmon MA, Ferguson KM (2000) Signal-dependent membrane targeting by pleckstrin homology (PH) domains. *Biochem J* **350**: 1–18
- Lien E, Sellati TJ, Yoshimura A, Flo TH, Rawadi G, Finberg RW, Carroll JD, Espevik T, Ingalls RR, Radolf JD, Golenbock DT (1999) Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products. *J Biol Chem* **274**: 33419–33425
- Mancuso G, Midiri A, Beninati C, Biondo C, Galbo R, Akira S, Henneke P, Golenbock D, Teti G (2004) Dual role of TLR2 and myeloid differentiation factor 88 in a mouse model of invasive group B streptococcal disease. *J Immunol* **172**: 6324–6329
- Mansell A, Brint E, Gould JA, O'Neill LA, Hertzog PJ (2004) Mal interacts with tumor necrosis factor receptor-associated factor (TRAF)-6 to mediate NF-kappa B activation by Toll-like receptor (TLR)-2 and TLR4. *J Biol Chem* **279**: 37227–37230
- Miggin SM, Palssson-McDermott E, Dunne A, Jefferies C, Pinteaux E, Banahan K, Murphy C, Moynagh P, Yamamoto M, Akira S, Rothwell N, Golenbock D, Fitzgerald KA, O'Neill LAJ (2007) NF-kappa B activation by the Toll-IL-1 receptor domain protein MyD88 adapter-like is regulated by caspase-1. *Proc Natl Acad Sci USA* **104**: 3372–3377
- Nunez MR, Wong J, Westoll JF, Brooks HJ, O'Neill LA, Gay NJ, Bryant CE, Monie TP (2007) A dimer of the Toll-like receptor 4 cytoplasmic domain provides a specific scaffold for the recruitment of signalling adaptor proteins. *PLoS ONE* **2**: e788
- O'Neill LAJ, Bowie AG (2007) The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nat Rev Immunol* **7**: 353–364
- O'Neill LAJ, Matthews JS, Palsson E, Jeffries C, Brennan P, Cantrell DA (1997) Interleukin 1 signalling: synergy with RAC1 and PI3 kinase. *Biochem Soc Trans* **25**: S578
- Ojaniemi M, Glumoff V, Harju K, Liljeroos M, Vuori K, Hallman M (2003) Phosphatidylinositol 3-kinase is involved in Toll-like receptor 4-mediated cytokine expression in mouse macrophages. *Eur J Immunol* **33**: 597–605
- Perkins ND (1997) Achieving transcriptional specificity with NF-kappa B. *Int J Biochem Cell Biol* **29**: 1433–1448
- Piao W, Song C, Chen H, Wahl LM, Fitzgerald KA, O'Neill LA, Medvedev AE (2008) Tyrosine phosphorylation of MyD88 adapter-like (Mal) is critical for signal transduction and blocked in endotoxin tolerance. *J Biol Chem* **283**: 3109–3119
- Rhee SH, Kim H, Moyer MP, Pothoulakis C (2006) Role of MyD88 in phosphatidylinositol 3-kinase activation by flagellin/toll-like receptor 5 engagement in colonic epithelial cells. *J Biol Chem* **281**: 18560–18568
- Sarbasov DD, Guertin DA, Ali SM, Sabatini DM (2005) Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* **307**: 1098–1101
- Sarkar SN, Elco CP, Peters KL, Chattopadhyay S, Sen GC (2007) Two tyrosine residues of Toll-like receptor 3 trigger different steps of NF-kappa B activation. *J Biol Chem* **282**: 3423–3427
- Sarkar SN, Peters KL, Elco CP, Sakamoto S, Pal S, Sen GC (2004) Novel roles of TLR3 tyrosine phosphorylation and PI3 kinase in double-stranded RNA signaling. *Nat Struct Mol Biol* **11**: 1060–1067
- Sarkar SN, Smith HL, Rowe TM, Sen GC (2003) Double-stranded RNA signaling by Toll-like receptor 3 requires specific tyrosine residues in its cytoplasmic domain. *J Biol Chem* **278**: 4393–4396
- Sly LM, Rauh MJ, Kalesnikoff J, Blchse T, Krystal G (2003) SHIP, SHIP2, and PTEN activities are regulated *in vivo* by modulation of their protein levels: SHIP is up-regulated in macrophages and mast cells by lipopolysaccharide. *Exp Hematol* **31**: 1170–1181
- Strassheim D, Asehnoune K, Park JS, Kim JY, He QB, Richter D, Kuhn K, Mitra S, Abraham E (2004) Phosphoinositide 3-kinase and Akt occupy central roles in inflammatory responses of toll-like receptor 2-stimulated neutrophils. *J Immunol* **172**: 5727–5733
- Takeuchi O, Kawai T, Muhlradt PF, Morr M, Radolf JD, Zychlinsky A, Takeda K, Akira S (2001) Discrimination of bacterial lipoproteins by Toll-like receptor 6. *Int Immunol* **13**: 933–940
- Vanden Berghe W, Plaisance S, Boone E, De Bosscher K, Schmitz ML, Fiers W, Haegeman G (1998) p38 and extracellular signal-regulated kinase mitogen-activated protein kinase pathways are required for nuclear factor-kappaB p65 transactivation mediated by tumor necrosis factor. *J Biol Chem* **273**: 3285–3290
- von Bernuth H, Picard C, Jin Z, Pankla R, Xiao H, Ku CL, Chrabieh M, Mustapha IB, Ghandil P, Camcioglu Y, Vasconcelos J, Sirvent N, Guedes M, Vitor AB, Herrero-Mata MJ, Arostegui JJ, Rodrigo C, Alsina L, Ruiz-Ortiz E, Juan M *et al* (2008) Pyogenic bacterial infections in humans with MyD88 deficiency. *Science* **321**: 691–696
- Yamamoto M, Sato S, Hemmi H, Sanjo H, Uematsu S, Kaisho T, Hoshino K, Takeuchi O, Kobayashi M, Fujita T, Takeda K, Akira S (2002) Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4. *Nature* **420**: 324–329