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The Tumor Necrosis Family Member 4-1BBL Sustains Inflammation by Interacting with Mediators of Toll-Like Receptor Signaling

Jianhui Ma[1,*], **Bo-Ram Bang**[1,*], **Jiawei Lu**[1,2], **So-Young Eun**[3], **Motoyuki Otsuka**[4], **Michael Croft**[3], **Peter Tobias**[1], **Jiahuai Han**[2], **Osamu Takeuchi**[5], **Shizuo Akira**[6], **Michael Karin**[7], **Hideo Yagita**[8], and **Young Jun Kang**[1,†,*]

[1]Department of Immunology and Microbial Science, The Scripps Research Institute, La Jolla, CA 92037, USA

[2]State Key Laboratory of Cellular Stress Biology and School of Life Sciences, Xiamen University, Xiamen, Fujian, China

[3]Division of Immune Regulation, La Jolla Institute for Allergy and Immunology, La Jolla, CA 92037, USA

[4]Department of Gastroenterology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

[5]Laboratory of Infection and Prevention, Institute for Virus Research, Kyoto University, Kyoto, Japan

[6]Laboratory of Host Defense, World Premier International Immunology Frontier Research Center, Osaka University, Osaka, Japan

[7]Laboratory of Gene Regulation and Signal Transduction, Department of Pharmacology, School of Medicine, University of California, San Diego, La Jolla, CA 92093, USA

[8]Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan

Abstract

Activation of Toll-like receptor (TLR)-dependent signaling leads to the expression of genes that encode pro-inflammatory factors, such as tumor necrosis factor α (TNF-α), and this process is sustained for the duration of the inflammatory response. TLR-mediated inflammation, which occurs in two phases, depends on the TNF family member 4-1BB ligand (4-1BBL) to sustain TNF-α production during late-phase signaling. Here, we showed that Toll–interleukin-1 receptor (TIR) domain–containing adaptor protein (TIRAP) and IL-1R–associated kinase 2 (IRAK2) were required to mediate the late phase of the TLR4 response through their interaction with 4-1BBL.

†Corresponding author: ykang@scripps.edu.
*These authors contributed equally to this work.

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Expression of *4-1bbl* was dependent on early TLR signaling that also induced the expression of *Tnf*, and the resulting 4-1BBL protein translocated to the plasma membrane, where it physically interacted with TLRs to mediate late-phase, secondary TLR signaling. TLR4–4-1BBL–mediated signaling depended on TIRAP and IRAK2, as well as a downstream complex consisting of the E3 ubiquitin ligase TRAF6, the kinase TAK1, and the adaptor protein TAB1, leading to the stimulation of mitogen-activated protein kinases. Whereas early TLR4 signaling involved the formation of a signaling complex consisting of TLR4, the adaptor proteins MyD88 and TIRAP, and IRAK2, formation of a secondary complex consisting of TLR4, 4-1BBL, TIRAP, and IRAK2 enabled late-phase signaling. Inhibition of this late-phase pathway reduced the extent of TNF-α production by mouse macrophages exposed to the TLR4 ligand lipopolysaccharide (LPS), and ameliorated LPS-induced sepsis in mice. Together, these data suggest that TIRAP and IRAK2 are critical for the sustained inflammatory response that is mediated by late-phase signaling by the TLR–4-1BBL complex.

INTRODUCTION

The innate immune response is the first line of defense against microbial infection, and it plays a critical role in inflammation. The production of proinflammatory cytokines, such as tumor necrosis factor α (TNF-α) and interleukin-1 (IL-1) is rapidly stimulated upon exposure of cells to microbial products, and it is sustained for the duration of the inflammatory response. Recognition of microbial products by Toll-like receptors (TLRs) stimulates receptor-proximal signaling cascades that promote inflammatory responses in macrophages (1). TLR-associated adaptor proteins, such as myeloid differentiation marker 88 (MyD88), Toll-interleukin 1 receptor (TIR) domain–containing adaptor-inducing interferon-β (TRIF), Toll-interacting protein (Tollip), and TIR domain–containing adaptor protein (TIRAP, also known as Mal), are selectively recruited to specific TLRs (2). MyD88 is a common adaptor protein that mediates the activation of all TLRs, except TLR3, and TRIF mediates TLR3- and TLR4-dependent signaling. Tollip limits TLR-mediated inflammation by inhibiting activation of the transcription factor nuclear factor κB (NF-κB) by TLR2 and TLR4 (3, 4). TIRAP is required for TLR2- and TLR4-dependent signaling, and it is degraded as a result of the activation of Bruton's tyrosine kinase (Btk) or IL-1R–associated kinase 1 (IRAK1) or IRAK4 (5–7), members of a family of serine and threonine kinases that play regulatory roles in TLR-mediated signaling (8). IRAK4 is essential for TLR-mediated cytokine production (9–12). IRAK1 is also involved in TLR signaling; however, its activity can be compensated for by those of other IRAKs (13, 14). IRAK2 is critical for late-phase TLR responses, whereas IRAK1 and IRAK2 function redundantly during early TLR-dependent signaling (15). Downstream of TLRs, the E3 ubiquitin ligase TNF receptor–associated factor 6 (TRAF6), transforming growth factor β–activated kinase 1 (TAK1), and TAK-binding protein 1 (TAB1) and TAB2 activate NF-κB and mitogen-activated protein kinase (MAPK) pathways to induce the expression of genes encoding pro-inflammatory cytokines (16–19).

4-1BB ligand (4-1BBL) is a member of the TNF superfamily and it plays an essential role in regulating adaptive immune responses (20, 21). Previously, we showed that 4-1BBL is required for the sustained production of TNF-α during innate immune responses (22). We

found that the microbial product lipopolysaccharide (LPS), a ligand of TLR4, stimulates TNF-α production to similar extents by macrophages from wild-type and *4-1bbl*-deficient mice early after stimulation; however, TNF-α production is not sustained in *4-1bbl*-deficient macrophages at later time points. Two different TLR4-containing complexes sequentially form and selectively regulate the early and late phases of TNF-α production in macrophages: a TLR4-MyD88 complex mediates the early phase, whereas a TLR4–4-1BBL complex mediates the late phase.

Here, we investigated the signaling mechanism that regulates the 4-1BBL–dependent production of TNF-α by macrophages. We demonstrated that the intracellular domains of TLR and 4-1BBL were required for their physical association, which was needed to stimulate sustained TNF-α production. The TLR2- and TLR4-specific adaptor protein TIRAP and the TLR-proximal kinase IRAK2 mediated the 4-1BBL–dependent production of TNF-α by forming a complex that included TLR and 4-1BBL at the late stage of macrophage activation. Furthermore, TRAF6 was recruited to this complex to interact with TAK1 and TAB1, which was required for MAPK-dependent, NF-κB–independent production of TNF-α by macrophages. Consistent with these findings, inhibition of signaling by the TLR4–4-1BBL–TIRAP complex reduced the extent of sustained inflammation in mice and ameliorated LPS-induced septic shock. Our results suggest that TIRAP and IRAK2 are critical for the 4-1BBL–dependent late-phase signaling required to sustain TNF-α production by macrophages, and that therapeutic targeting of 4-1BBL–dependent signaling might be effective in inhibiting inflammation.

RESULTS

The TNF superfamily member 4-1BBL physically interacts with TLR4

TLRs are indispensable for the sustained production of TNF-α by macrophages through their interaction with 4-1BBL, and the cytoplasmic domains of TLRs and 4-1BBL are essential for their physical association with each other (22). First, we tested whether the cytoplasmic domain of TLR4 was essential for the activation of 4-1BBL–mediated signaling. We performed luciferase reporter assays with human embryonic kidney (HEK) 293 cells transfected with control plasmid or plasmids encoding full-length TLR4 or truncated mutants of TLR4, and found that full-length TLR4, increased the activity of reporters responsive to the transcription factors cAMP response element-binding protein (CREB) and CCAAT/enhancer binding protein (C/EBP)-α, as well as of a reporter driven by the *Tnf* promoter (Fig. 1A). CREB and C/EBP-α are required for the 4-1BBL–dependent expression of *Tnf*. A mutant TLR4 protein lacking the extracellular domain still supported reporter activation, whereas a TLR4 mutant lacking the cytoplasmic domain did not (Fig. 1A). Additionally, cotransfection of cells with plasmids encoding 4-1BBL and TLR4 enhanced the reporter activity, whereas expression of 4-1BBL alone did not, suggesting that the presence of both TLR4 and 4-1BBL was essential for activation of the 4-1BBL signaling pathway (Fig. 1A). Similarly, in cells expressing 4-1BBL, concomitant expression of a TLR4 mutant lacking the extracellular domain somewhat stimulated reporter activity, whereas expression of a mutant TLR4 lacking its cytoplasmic domain did not (Fig. 1A),

suggesting that the cytoplasmic domain of TLR4 was essential to activate signaling downstream of 4-1BBL.

Next, we investigated the domains of 4-1BBL that were essential for its interaction with TLR4. The cytoplasmic domain of 4-1BBL contains a consensus sequence for phosphorylation by casein kinase I (CKI), which has been implicated in signaling by other TNF superfamily members (23, 24). We generated truncated mutants of 4-1BBL by deleting some amino acid residues in the cytoplasmic domain, and that we tested their ability to associate with TLR4 and to activate the CREB-dependent reporter (Fig. 1, B and C). We found that full-length 4-1BBL interacted with TLR4 and induced CREB activation (Fig. 1, C and D). The cytoplasmic domain of 4-1BBL was required for its functional association with TLR4 provided that the transmembrane domain of 4-1BBL was also present; however, the isolated cytoplasmic domain of 4-1BBL did not interact with TLR4 (Fig. 1, C and D). Mutant 4-1BBL proteins from which had been removed the first nine (1 to 9, CytΔ1) or 18 amino acid residues (1 to 18, CytΔ2, resulting in loss of the CKI phosphorylation site) still physically associated with TLR4; however, the extent of activation of CREB was substantially reduced by deletion of the CKI phosphorylation site, suggesting that this site in the cytoplasmic domain is necessary for the activation of 4-1BBL signaling. These results suggest that the cytoplasmic domain of 4-1BBL is essential for activation of the downstream signaling events required for TNF-α production, and that the transmembrane domain of 4-1BBL is indispensable for its association with TLR4.

TRAF6, TAK1, and TAB1 are essential for the activation of 4-1BBL–mediated signaling

TRAF6 functions as a mediator of TLR and IL-1R signaling. By linking the receptor activation to downstream signaling events, such as the activation of the inhibitor of NF-κB (IκB) kinases (IKKs) and MAPKs, TRAF6 is indispensable for innate immune responses. Activation of TRAF6 signaling involves TAK1, TAB1, and TAB2. To test the involvement of TRAF6 in 4-1BBL–mediated signaling, we transiently transfected a mouse macrophage cell line RAW264.7 with small interfering RNAs (siRNAs) specific for *Traf2* or *Traf6* (Fig. 2A). Cells in which either TRAF6 or TRAF2 was knocked down were treated with 4-1BB-Fc (recopmbinant protein of 4-1BB, a receptor of 4-1BBL, fused with Ig Fc) and anti-Fc antibody to crosslink 4-1BBL and stimulate TNF-α production. In previous studies, macrophages from wild-type mice or various strains of knockout mice were successfully infected with a *4-1bbl*-encoding adenovirus (Adv-4-1BBL) to induce the expression of *4-1bbl* and stimulate TNF-α production, but some cell types, such as *Myd88*-deficient mouse peritoneal macrophages or siRNA-transfected macrophage cell lines cannot be infected. Alternatively, these cells can be treated with 4-1BB-Fc and anti-Fc antibody to induce 4-1BBL crosslinking to activate the downstream signaling. Production of TNF-α by wild-type macrophages and *Trif*-deficient macrophages in response to 4-1BBL crosslinking by 4-1BB-Fc plus anti-Fc antibody were comparable; however, the amount of TNF-α produced by *Tlr4*-deficient cells was reduced (fig. S1), which is consistent with our previous experiments with Adv-4-1BBL (22)., Because *Traf2*- or *Traf6*-deficient cells, or other cells such as *Ikkb*-, *Irak2*-, or *Irak4*-deficient macrophages (shown below) were not infected with Adv-4-1BBL, TNF production in those cells was induced by aggregation of 4-1BBL. The amount of TNF-α produced by *Traf2*-knockdown macrophages was comparable to that

produced by wild-type macrophages (Fig. 2A); however, knockdown of *Traf6* resulted in a substantial reduction in the amount of TNF-α produced (Fig. 2A), which suggested that TRAF6, but not TRAF2, was involved in 4-1BBL–dependent production of TNF-α. Experiments in which we knocked down TRAF6 with an additional siRNA that targeted a different sequence of *Traf6* further confirmed that TRAF6 was required for 4-1BBL–dependent TNF-α production (fig. S2A). Moreover, TRAF6 was co-immunoprecipitated with 4-1BBL in transfected HEK 293T cells, which supported the involvement of TRAF6 in 4-1BBL signaling (fig. S2B).

We further tested the roles of TAK1, TAB1, and TAB2 in 4-1BBL–dependent signaling. We infected immortalized mouse macrophages with lentiviruses expressing a control short hairpin RNA (shRNA) or shRNAs specific for *Tak1*, *Tab1*, or *Tab2* (Fig. 2B). Cells were then further infected with Adv-4-1BBL to induce cell-surface expression of 4-1BBL and TNF-α production. We found that the amount of TNF-α produced by *Tak1* or *Tab1* knockdown cells was substantially reduced compared to that produced by control cells; however, the amount of TNF-α produced by *Tab2* knockdown cells was not affected (Fig. 2C). As TAK1, TAB1, and TAB2 are required to mediate TLR4-dependent signaling (16-19), LPS-induced TNF-α production was reduced in their respective knockdown cells (Fig. 2C). However, TNF-α production in response to the C-type lectin receptor (CLR) ligand curdlan was not affected by knockdown of TAK1, TAB1, or TAB2 (Fig. 2C), consistent with a previous report that found that CLR signaling is not mediated by any of these proteins (25), indicating the specific knockdown of the target proteins by shRNAs. These results suggest that the TRAF6-TAK1-TAB1 signaling pathway plays a regulatory role in the 4-1BBL-mediated late phase of TNF-α production in macrophages.

Protein kinase pathways are involved in the 4-1BBL–mediated late phase of TNF-α production

Activation of TRAF6 and its interacting signaling components plays an important role in regulating the activation of signaling pathways downstream of TLRs, such as those mediated by NF-κB and MAPKs. We next tested the involvement of IKKβ and the MAPK p38α in 4-1BBL–dependent TNF-α production. We found that the amounts of TNF-α produced by wild-type and *Ikkb*-deficient mouse bone marrow-derived macrophages (BMDMs) in response to 4-1BBL crosslinking were comparable, whereas the amount of TNF-α produced by *p38α*-deficient macrophages was reduced compared to that produced by control cells (Fig. 3A). Adenoviral-mediated expression of an IκBα "super-repressor" (which inhibits NF-κB signaling) in cells in which TAK1, TAB1, or TAB2 was knocked down confirmed that 4-1BBL–mediated TNF-α production was independent of NF-κB activation (fig. S3), and that TAK1-TAB1 signaling did not activate NF-κB to induce 4-1BBL–dependent TNF-α production (fig. S3). Therefore, we concluded that 4-1BBL–dependent TNF-α production is regulated by the activation of p38α MAPK, but not NF-κB.

Studies have demonstrated that activation of protein kinase C (PKC), protein kinase A (PKA), and phosphatidylinositol 3-kinase (PI3K) by LPS increases the production of inflammatory cytokines (26–31); Thus, we tested whether 4-1BBL stimulated the activation of these protein kinases. We previously showed that infection of cells with Adv-4-1BBL

stimulated the phosphorylation (and activation) of the MAPKs p38α, extracellular signal–regulated kinase (ERK), and c-Jun N-terminal kinase (JNK), but did not affect NF-κB signaling (22). We infected macrophages with Adv-4-1BBL and prepared cell lysates to test for the phosphorylation of PKA and PKC. The serine and threonine kinase Akt is downstream of PI3K activation, and we examined the phosphorylation status of Akt as a proxy for PI3K activity because of the limited availability of reliable antibodies specific for phosphorylated PI3K. Overexpression of *4-1bbl* induced the phosphorylation of PKC, PKA, and Akt in macrophages (Fig. 3B). In addition, TNF-α production by Adv-4-1BBL–infected macrophages was reduced by a PKC inhibitor, whereas PKA or PI3K inhibitors reduced TNF-α production when used at higher concentrations (fig. S4). These results implied that PKC, PKA, and PI3K signaling pathways were involved in late-phase, 4-1BBL–dependent signaling.

Next, we tested whether 4-1BBL was required for the late-phase, LPS-dependent activation of protein kinases in macrophages. We treated wild-type and *4-1bbl*-deficient macrophages with LPS and monitored the phosphorylation of PKA, PKC, and Akt over time. We detected 4-1BBL protein in wild-type macrophages 4 hours after they were treated with LPS, at which point, we also observed the phosphorylation of PKA, PKC, and Akt (Fig. 3C). However, we did not detect any substantial increase in the phosphorylation states of these kinases in *4-1bbl*-deficient macrophages, which suggests that stimulation of TLR4 signaling first induced *4-1bbl* expression, which was then followed by the phosphorylation of these protein kinases at the late phase of macrophage activation (Fig. 3C). Together, these data suggest that the 4-1BBL-production of TNF-α is mediated by the activation of p38α, PKA, PKC, and Akt, but not by NF-κB.

TIRAP and IRAK2 mediate the 4-1BBL–dependent sustained production of TNF-α

Although late-phase 4-1BBL–dependent production of TNF-α requires TLRs, the adaptor proteins MyD88 and TRIF are not involved (22). Thus, we tested whether the TLR-proximal adaptors Tollip and TIRAP mediated 4-1BBL–dependent signaling. We found that Adv-4-1BBL-infected wild-type and *Tollip*-knockdown macrophages produced comparable amounts of TNF-α, suggesting that Tollip does not play a role in regulating 4-1BBL–mediated signaling (fig. S5A); however, TNF-α production by Adv-4-1BBL-infected *Tirap*-deficient cells was substantially impaired compared to that of wild-type cells (Fig. 4A). We found that TIRAP co-immunoprecipitated with 4-1BBL in transfected HEK 293T cells (fig. S5B). Together, these results suggest that TIRAP is critical for the late-phase, 4-1BBL–mediated inflammatory response.

We further tested the involvement of TIRAP in the late phase of macrophage activation in experiments with a TIRAP inhibitory peptide (TIRAP inhibitor) that contains a protein transduction (PTD) sequence that enables it to enter cells. Because *Tirap*-deficient macrophages showed impaired TNF-α production during the early phase of LPS-induced activation (Fig. 4A), we could not test the involvement of TIRAP in the late phase response with these cells. Similar to the impairment of TNF-α production by LPS-stimulated *Tirap*-deficient macrophages, pretreatment of wild-type macrophages with the TIRAP inhibitor reduced TNF-α production in response to LPS without affecting the amounts of TNF-α

produced by these cells in response to the TLR3 ligand poly(I:C) or the TLR9 ligand CpG DNA (Fig. 4B), consistent with TIRAP being a specific adaptor protein for TLR2 and TLR4 signaling (32).

To assess late-phase signaling, we treated macrophages with the TIRAP inhibitor after the early phase of the TLR response was complete; that is, 4 hours after the initiation of TLR signaling. Unlike cells treated with the control peptide, cells treated with the TIRAP inhibitor 4 hours after stimulation by the TLR ligands LPS, poly(I;C), or CpG DNA did not exhibit sustained production of TNF-α (Fig. 4B). The amounts of TNF-α produced did not substantially differ for the first 8 hours, but were reduced after 12 hours (Fig. 4B). Co-administration of the TIRAP inhibitor with TLR ligands also reduced the extent of TNF-α production after the initial phase of activation of macrophages. To confirm that the TIRAP inhibitor specifically blocked the interaction of 4-1BBL with TIRAP at the late phase of activation, we tested whether the TIRAP inhibitor affected the late-phase production of TNF-α by *4-1bbl*-deficient macrophages (fig. S6). As previously reported (22), LPS-induced TNF-α production was not sustained in *4-1bbl*-defiecient macrophages (fig. S6); however, it was intact in wild-type macrophages. Treatment of wild-type cells with the TIRAP inhibitor 4 hours after the cells were stimulated with LPS blocked the sustained production of TNF-α. When *4-1bbl* KO macrophages were treated with either control peptide or TIRAP inhibitor peptide 4 hours after they were treated with LPS, they exhibited no differences in TNF-α production (fig. S6), which suggested that the TIRAP inhibitor specifically blocked the interaction of 4-1BBL and TIRAP during the late phase. Collectively, our results suggest that TIRAP participates not only in the initial phase but also in the 4-1BBL–dependent late phase of TLR signaling to sustain TNF-α production by macrophages.

To further determine whether the inhibition of late-phase TIRAP activity blocked sustained inflammatory responses, we tested the specific inhibition of 4-1BBL-mediated late phase signaling. Consistent with previous findings (22), we observed that inhibition of TLR4–4-1BBL aggregation by a neutralizing anti-4-1BBL antibody resulted in reduced TNF-α production by macrophages (fig. S7A). We found that TNF-α production was sustained in macrophages treated with LPS or CpG DNA together with isotype control antibodies, whereas the addition of anti–4-1BBL antibodies 4 hours after TLR4 or TLR9 stimulation reduced the extent of TNF-α production (fig. S7, B & C), indicating that specific inhibition of late-phase 4-1BBL-mediated signaling reduced the sustained inflammatory responses of macrophages.

There are two different signaling complexes that are sequentially formed during LPS-induced macrophage activation. First, TLR4 and the adaptor MyD88 associate to form a complex during the early phase to induce the expression of inflammatory genes, such as *Tnf* and *4-1bbl*. This is followed by the formation of a MyD88- and TRIF-independent complex containing TLR4 and 4-1BBL during the late phase of the response that is required to sustain TNF-α production (22). We found that TIRAP physically interacted with 4-1BBL when overexpressed in transfected HEK 293T cells (fig. S5B), which indicates that TIRAP and 4-1BBL are capable of forming a complex at the late phase of macrophage activation as 4-1BBL is expressed and interacts with TLRs only at the late phase of macrophages

activation. Because TLR4–4-1BBL complex formation was demonstrated during sustained macrophage activation (22), we speculated about the formation of a TLR4–4-1BBL–TIRAP complex during the late phase of TLR signaling; thus, we further examined potential interactions between the 4-1BBL–TIRAP and TRAF6-TAK1-TAB1 complexes. In overexpression experiments in 293T cells, we found that TRAF6 co-immunoprecipitated with 4-1BBL and TIRAP, and that TAK1 and TAB1 coimmunoprecipitated with 4-1BBL and TIRAP when TRAF6 was present (fig. S8). TAB1 coimmunoprecipitated with 4-1BBL and TRAF6 in cells expressing TAK1, which suggested that TAK1 was essential for the recruitment of TAB1 to the 4-1BBL signaling complex (fig. S8). We conclude that a physical interaction between 4-1BBL and TIRAP is required for signaling downstream of the association between TLR4 and 4-1BBL through the formation of a complex containing TRAF6, TAK1, and TAB1 during the late phase of macrophage activation.

We next determined the roles of IRAKs in the 4-1BBL-mediated signaling. In experiments with transfected HEK 293T cells, we found that 4-1BBL coimmunoprecipitated with IRAK2, but not with IRAK1 or IRAK4 (fig. S9). We next treated wild-type, *Irak2*-, and *Irak4*-deficient BMDMs with 4-1BB-Fc and anti-Fc antibody to compare their abilities to produce TNF-α. We found that *Irak2*-deficient macrophages produced less TNF-α than did wild-type cells, whereas loss of *Irak4* had no effect on TNF-α production (Fig. 4C). IRAK4 is required for LPS-dependent activation of IRAK2 and for TLR-induced proinflammatory cytokine production (15). To examine the role of IRAK4 in the activation of IRAK2 during late-phase 4-1BBL signaling, we transfected HEK 293T cells with plasmids encoding 4-1BBL, IRAK2, or IRAK4, and examined the physical associations between these proteins (fig. S10). In studies of cells expressing either *4-1bbl* and *Irak2* or *4-1bbl* and *Irak4*, we found that IRAK2, but not IRAK4, coimmunoprecipitated with 4-1BBL. However, in HEK 293T cells expressing *4-1bbl*, *Irak2*, and *Irak4*, IRAK2 and IRAK4 both coimmunoprecipitated with 4-1BBL, suggesting that IRAK4 physically interacted with IRAK2 that was already associated with 4-1BBL. These data suggest that IRAK4 mediates late-phase TLR signaling by interacting with IRAK2, but not by directly associating with 4-1BBL. Therefore, we conclude that late-phase signaling is mediated by a TLR4–4-1BBL–TIRAP–IRAK2 complex, and that IRAK4 interacts with IRAK2, but not 4-1BBL, to activate downstream signaling.

TLR-induced expression of *Tnf* and *4-1bbl* shares a common pathway

Because TNF-α and 4-1BBL are members of the TNF superfamily and their production is induced by TLR signaling, we investigated whether they were regulated in a similar manner. The induction of 4-1BBL protein in response to LPS was reduced in *Tirap*-deficient macrophages compared to that in wild-type macrophages (Fig. 5A). The LPS-dependent expression of *4-1bbl* and *Tnf* mRNAs was impaired in *Irak2*-deficient BMDMs compared to that in wild-type BMDMs (Fig. 5B). We next investigated the roles of TAK1, TAB1, and TAB2 in TNF-α and 4-1BBL production with the knockdown cells that we generated earlier (Fig. 2B). We found that the expression of *4-1bbl* and *Tnf* were both rapidly induced by LPS in wild-type cells, but were substantially reduced in *Tak1*-, *Tab1*-, or *Tab2*-knockdown cells (Fig. 5C), which indicated that the LPS-dependent induction of both 4-1BBL and TNF-α was mediated by TAK1-TAB1-TAB2 signaling. IKKβ and p38α are the most important

downstream signaling components that induce *Tnf* expression in response to TLR activation. We found that deletion of *Ikkb* or *p38α* resulted in a reduction in LPS-induced *4-1bbl* expression compared to that in wild-type cells (Fig. 5D). Together, these results suggest that TLR4-dependent *4-1bbl* expression is regulated by the same pathway that controls the TLR4-dependent expression of *Tnf* in macrophages.

Inhibition of 4-1BBL-mediated signaling ameliorates LPS-induced septic shock

Mice lacking *4-1bbl* survive longer than do wild-type mice upon challenge with LPS because of their inability to maintain sustained TNF-α production, and LPS-induced lethality in wild-type mice is attenuated by administration of a neutralizing anti–4-1BBL antibody (22). These findings suggest that 4-1BBL is important for sustained TNF-α production in vivo and that blocking of 4-1BBL oligomerization reduces the sustained inflammatory response. Thus, we examined whether 4-1BBL–mediated late-phase signaling could be targeted in mice as part of an anti-inflammatory strategy.

We showed that inhibition of late-phase TIRAP activity by the TIRAP inhibitor reduced sustained TNF-α production by macrophages in vitro (Fig. 4B). Therefore, we tested whether inhibition of late-phase TIRAP activity could ameliorate LPS-induced septic shock in mice (Fig. 6). Because we found that the timing of injection of the control peptide did not affect LPS-induced septic shock, we always injected the mice with control peptide before they were injected with LPS. Pre-administration of the TIRAP inhibitor peptide to mice provided them with more protection against a subsequent injection of LPS than did pretreatment with the control peptide (Fig. 6B), which is consistent with the resistance of *Tirap*-deficient mice to septic shock (33). Co-administration of the TIRAP inhibitor with LPS resulted in the extended survival of the mice compared with that of mice that were injected with both control peptide and LPS simultaneously (Fig. 6B), whereas administration of the TIRAP inhibitor peptide to the mice after they were injected with LPS still provided some protection from septic shock (Fig. 6B). These results suggested that the TIRAP inhibitor blocked the activation of inflammatory responses during sepsis by inhibiting TLR4 signaling not only the early phase, but also the late phase, and that inhibition of the late-phase interaction between TLR4, TIRAP, and 4-1BBL alleviated LPS-induced sepsis. The serum concentrations of TNF-α in mice that were injected with LPS after or at the same time that they were injected with the TIRAP inhibitor peptide were reduced compared to those of mice injected with the control peptide.

Administration of the TIRAP inhibitor peptide to mice 1 hour after they were injected with LPS reduced their serum concentrations of TNF-α 2 and 4 hours later, which suggests that the inhibitor peptide blocked late-phase 4-1BBL–mediated signaling by inhibiting TIRAP activity (Fig. 6C). In contrast, administration of an MyD88 inhibitor to mice after they were injected with LPS resulted in serum TNF-α concentrations that were comparable to those of mice injected with the control peptide, whereas pretreatment of mice with the MyD88 inhibitor substantially reduced the serum TNF-α concentrations in mice subsequently injected with LPS (fig. S11). These findings are consistent with a role for MyD88 as an adaptor protein that mediates early TLR responses. This result supports the hypothesis that TIRAP, but not MyD88, is also involved in late-phase 4-1BBL–mediated inflammation.

Collectively, these data suggest that the involvement of TIRAP during late-phase TLR signaling is necessary for the 4-1BBL–dependent sustained production of TNF-α, because inhibition of late-phase TIRAP activity ameliorated LPS-induced sepsis by reducing the extent of TNF-α production in vivo.

In conclusion, we suggest that 4-1BBL is generated in response to TLR4-dependent signaling at the early phase of the response, and that it interacts with TLR4 to form a secondary signaling complex consisting of TLR4, 4-1BBL, TIRAP, IRAK2 during the late phase of the response, which stimulates downstream signaling to sustain TNF-α production by macrophages (Fig. 7). Furthermore, our in vivo data also suggest that targeting late-phase 4-1BBL–mediated signaling might be used as an anti-inflammatory strategy.

DISCUSSION

Here, we investigated the intracellular signaling mechanism by which 4-1BBL sustains TNF-α production by macrophages during inflammatory responses. Inflammation is initiated to protect hosts from microbial infection and tissue damage, and it is prolonged for a period of time. We have previously suggested that 4-1BBL-mediated signaling is important for the progression of inflammatory responses in macrophages (22), but the underlying mechanism was not well-identified. Here, we have found that TIRAP and IRAK2 mediated the late-phase production of TNF-α, and that inhibition of late-phase signaling blocked the sustained inflammatory response by macrophages.

4-1BBL–mediated signaling requires a physical interaction between 4-1BBL and TLRs. The intracellular domains of TLRs and 4-1BBL are essential for their physical association and the activation of downstream signaling events. A consensus sequence for phosphorylation by CKI in the cytoplasmic domain of 4-1BBL was essential not only for its interaction with TLR4, but also for the activation of downstream signaling. Whereas the cytoplasmic domain of 4-1BBL was required for signaling, the transmembrane domain was indispensible. We previously showed that newly synthesized 4-1BBL translocates to the cell-surface of macrophages, where it interacts with resident TLRs to sustain TNF-α production, because blocking of its translocation inhibits sustained TNF-α production by LPS-treated cells (22). Therefore, the location of 4-1BBL on the cell surface is critical for its interaction with TLRs and to sustain late-phase inflammatory signaling.

We found that 4-1BBL–mediated late-phase signaling was a MyD88- and TRIF-independent, but TIRAP- and IRAK2-dependent process, whereas the early phase of TLR signaling is mediated by MyD88, TRIF, TIRAP, and IRAKs, depending on type of TLR involved. Although 4-1BBL interacts with TLRs during the late-phase response, the proximal event of this secondary signaling complex is not the same as that of the initial TLR signaling pathway. Late-phase 4-1BBL signaling was further linked to the recruitment of TRAF6 to form a complex with TAK1 and TAB1, but not TAB2. Of note, TAK1 is essential for the activation of NF-κB and MAPK, whereas TAB1 stimulates the activation of MAPK, but not NF-κB, signaling (34, 35). TAB2 contributes to the activation of NF-κB by facilitating the ubiquitylation of TRAF6 (36). In 4-1BBL-mediated signaling, a TRAF6-TAK1-TAB1 complex was key to the activation of MAPKs required for TNF-α production,

without activating the NF-κB pathway. We found that TRAF6 was essential for the 4-1BBL–dependent production of TNF-α by macrophages, and 4-1BB, the receptor for 4-1BBL, stimulates the activation of a TRAF2-mediated signaling cascade that activates both p38α and NF-κB in T cells (37).

Given that TIRAP is important for early TLR2 and TLR4 signaling (33, 38), and that 4-1BBL–dependent TNF-α production was reduced in *Tirap*-deficient macrophages, we confirmed the involvement of TIRAP in late-phase signaling in experiments with a TIRAP-specific inhibitory peptide. Pre-treatment of cells with the TIRAP inhibitor reduced the extent of TNF-α production at early time points in response to LPS, but had no effect in cells treated with ligands for endosomal TLRs. Administration of the TIRAP inhibitor to mice after the early signaling events were completed, such as the association of TIRAP with TLR2 or TLR4 and then its subsequent degradation after ligand treatment, completely blocked sustained TNF-α production by macrophages. That TIRAP is rapidly degraded in response to ligand stimulation of TLR2 or TLR4 and then is resynthesized (6, 39) is relevant to our finding that TIRAP was involved in both the early and late phases of TLR signaling. Similar to the phenotype of LPS-injected *Tirap*-deficient mice (33), prior or simultaneous administration of the TIRAP inhibitor extended the survival of mice injected with LPS because of a reduction in the amount of TNF-α produced.

Although the extent to which survival was extended was not as substantial as that provided by pre-treatment with the TIRAP inhibitor, administration of the inhibitor after the mice were injected with LPS also enhanced their survival. A study proposed that genetic variations in the human gene encoding TIRAP may provide protection from sepsis (40). Inhibition of sustained TNF-α production and amelioration of LPS-induced septic shock by administration of the TIRAP inhibitor after treatment with LPS was a result of blocking TLR4–4-1BBL–TIRAP signaling during the late phase of macrophage activation. TLRs and TIRAP were essential for 4-1BBL–dependent signaling, because 4-1BBL-induced TNF-α production by *Tlr*- or *Tirap*-deficient macrophages was substantially reduced compared to that by wild-type macrophages. In addition, 4-1BBL formed a complex with TLRs and TIRAP, and formation of a TLR–4-1BBL–TIRAP complex was critical for late-phase signaling. Although TIRAP is required for TLR2- and TLR4-mediated, MyD88-dependent signaling at the early phase of macrophage activation, we excluded the possible inhibitory effect of the TIRAP inhibitor on the interaction between MyD88 and TIRAP for a number of reasons. First, the association of MyD88 and TIRAP is involved only in the early phase of the TLR response. Second, MyD88 is not involved in late-phase 4-1BBL–mediated signaling (22). Third, our data showed that administration of the MyD88 inhibitory peptide to mice 1 hour after they were injected with LPS did not alter their serum concentrations of TNF-α, whereas pre-treatment of mice with the MyD88 inhibitor substantially reduced the subsequent LPS-induced production of TNF-α.

Given that IRAK2 was required for 4-1BBL–dependent TNF-α production, and that it also plays a critical role in early and late-phase TLR-dependent inflammatory responses, it is likely that IRAK2 functions in both early and late-phase TLR signaling events. During this process, IRAK4 is required for IRAK2 activation, and the kinase activity of IRAK2 is essential for the TLR response (15). We found that 4-1BBL–dependent TNF-α production

was impaired in *Irak2*-deficient macrophages, but not in *Irak4*-deficient cells, and that both IRAK2 and IRAK4 coimmunoprepitated with 4-1BBL, whereas IRAK2, but not IRAK4, was detected in 4-1BBL immunoprecipitates. Therefore, despite the fact that IRAK4 did not directly associate with 4-1BBL in macrophages, it seems that IRAK4 interacted with IRAK2 transiently to stimulate the activation of 4-1BBL-mediated, late-phase signaling.

We found that the expression of *4-1bbl* is mediated by the same pathway that induces *Tnf* expression, and that 4-1BBL interacted with the resident TLRs to form a secondary signaling complex consisting of TLR and 4-1BBL to initiate late-phase signaling. TIRAP and IRAK2 were then recruited to this late-phase complex to stimulate the MAPK-dependent, NF-κB–independent downstream signaling required to sustain TNF-α production by macrophages. Thus, blocking this 4-1BBL–dependent sustained signaling pathway may be beneficial for the treatment of inflammatory diseases. TNF-α is the most potent inflammatory cytokine and it is a major cause of many inflammatory disorders. As a result of extensive efforts to block TNF-α activity to reduce tissue damage during inflammation, some anti–TNF-α pharmaceuticals, including anti–TNF-α antibodies and chimeric TNFR-immunoglobulin G (IgG)-Fc molecules were successfully generated and are being used in the treatment of some inflammatory diseases. Because inhibition of late-phase TLR–4-1BBL signaling by an anti–4-1BBL antibody or by chemical inhibitors that specifically block signaling, such as the TIRAP inhibitory peptide, reduces the production of TNF-α by macrophages, a strategy targeting 4-1BBL–dependent signaling might also used as an anti-inflammatory treatment. Unlike current approaches that block the activity of secreted TNF-α, inhibition of late-phase 4-1BBL signaling would have the advantage of reducing the amount of TNF-α produced by macrophages. Therefore, the synergistic anti-inflammatory effects of targeting TNF-α activity and TLR–4-1BBL-mediated late-phase signaling may be more beneficial for the treatment of inflammatory diseases.

MATERIALS AND METHODS

Mice

C57Bl/6 background wild-type, *4-1bbl*-, *Tirap*-, *Irak2*-, *Irak4*-deficient, macrophage-specific *p38α*-deficient (LysMCre-$p38\alpha^{\Delta/\Delta}$), and IFN-inducible promoter-driven Cre-*Ikk-β* (Mx1Cre-*Ikk-β*) mice were described previously (15, 33, 41, 42). Protocols for the use of animals were approved by the Institutional Animal Care and Use Committee.

Reagents

IL-1β, recombinant mouse 4-1BB-Fc and anti–4-1BBL (AF1246) were purchased from R&D Systems; anti-GFP antibody (8372-2) was obtained from Clontech; antibodies against HA (Y-11), myc (A-14), and phosphorylated PKAα (sc-32968) were purchased from Santa Cruz Biotechnology; anti-GAPDH antibody (MAB374) was from Chemicon; anti-FLAG antibody (F3165) and LPS from *Escherichia coli* O111:B4 for in vivo experiments were obtained from Sigma-Aldrich; LPS from *E. coli* O111:B4 for in vitro experiments was purchased from List Biological Laboratories; anti-pPKCα/β (#9375) and anti-pAkt (Ser^{473}, #9271) were from Cell Signaling; phosphorothioate-stabilzed CpG DNA, poly(I:C), and

curdlan were purchased from Invivogen; and control peptide and TIRAP and MyD88 inhibitory peptides were obtained from Imgenex.

Cells

HEK 293T cells and RAW264.7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Peritoneal macrophages were obtained from the peritoneal cavities of mice after they received intraperitoneal (i.p.) injections of 4% thioglychollate. Bone marrow–derived macrophages (BMDMs) were prepared and cultured in BMDM culture medium (DMEM containing 30% L929 culture supernatant and 20% FBS) to differentiate into macrophages. Immortalized macrophage cell lines were generated by infecting BMDMs with retrovirus encoding *v-raf* and *v-myc* (43, 44). The TKS-1 hybridoma was cultured to obtain anti–4-1BBL antibody (45).

Plasmids and siRNAs

Mammalian expression plasmids encoding full-length or truncated mutants of 4-1BBL and TLR4 were previously described (22). Complementary DNAs (cDNAs) encoding human TRAF2, TRAF6, TAK1, TAB1, TIRAP, IRAK2, and IRAK4 were generated by polymerase chain reaction (PCR) assays and were cloned into mammalian expression plasmids. The shRNA constructs specific for *Tak1*, *Tab1*, or *Tab2* were designed based on the single oligonucleotide RNA interference technology, and lentiviral vectors expressing these shRNAs were generated according to the manufacturer's instructions (Biosettia). The sequences of the oligonucleotides used to generate there shRNAs are as follows: *Tak1*: 5′-AAAAGGACATTGCTTCTACAAATTTGGATCCAAATTTGTAGAAGCAATGTCC-3′; *Tab1*: 5′-AAAAGGTGAGGAACTTTGGCTATTTGGATCCAAATAGCCAAAGTTCCTCACC-3′; *Tab2*: 5′-AAAAGCACATGTGGATAGAATAATTGGATCCAATTATTCTATCCACATGTGC-3′; A negative control sequence of 5′-AAAAGCTACACTATCGAGCAATTTTGGATCCAAAATTGCTCGATAGTGTAGC-3′ was also used. Predesigned siRNAs specific for mouse *Traf2*, *Traf6*, or *Tollip* were purchased from Ambion. For real-time PCR analysis, 1 to 2 μg of total RNA were used to prepare cDNA. The SYBR green PCR Master Mix kit (Applied Biosystems) was used for the real-time PCR analysis. Values corresponding to the genes of interest were normalized to the abundance of mRNA of the house-keeping gene *gapdh*, and relative expression was calculated according to the $\Delta\Delta C_T$ method.

Adenovirus and lentivirus

Adenovirus expressing mouse *4-1bbl* was obtained from T. Watts (University of Toronto) and adenovirus encoding the IkB super repressor was provided by J. Han (The Scripps Research Institute). Recombinant lentiviruses were packaged in HEK 293T cells by transfecting the cells with the shRNA-encoding plasmid, together with the helper plasmids pRSV-REV, pMDLg, and pVSV-G according to the manufacturer's instructions (Biosettia).

Reporter assays

HEK 293T cells were transfected with plasmids, a CREB-dependent luciferase reporter, a C/EBP-dependent luciferase reporter (obtained from Lisa Choy, UCSF), or a *Tnf* promotor-driven reporter, and with the *pTK-RL* control reporter (Promega). Luciferase activity was measured with the DUAL Luciferase Assay System (Promega). *Renilla* luciferase activity was measured as a normalization control.

Immunoprecipitations and Western blotting analysis

HEK 293T cells were transfected with plasmids encoding 4-1BBL and TLR4, and cell lysates were prepared and incubated with the appropriate primary antibodies before being incubated with Protein G-agarose. The immunoprecipitates were then resolved by SDS-PAGE and analyzed by Western blotting according to standard protocols. To examine physical interactions between endogenous proteins, we treated mouse peritoneal macrophages with LPS, subjected cell lysates to immunoprecipitation with anti-TLR4 or anti-TIRAP antibodies, and then analyzed samples by Western blotting, which was performed with chemiluminescence substrate, and bands corresponding to proteins of interest were visualized with x-ray film or with the Odyssey scanner (LI-COR Bioscience, Lincoln, NE).

Measurement of cytokine production

The concentrations of TNF-α or IL-6 in culture supernatants or blood sera were measured by ELISA with specific kits (eBioscience).

Statistical analysis

Data were analyzed with the Student's *t*-test. Kaplan-Meier plots were constructed and a log-rank test was used to determine the differences in the survival of mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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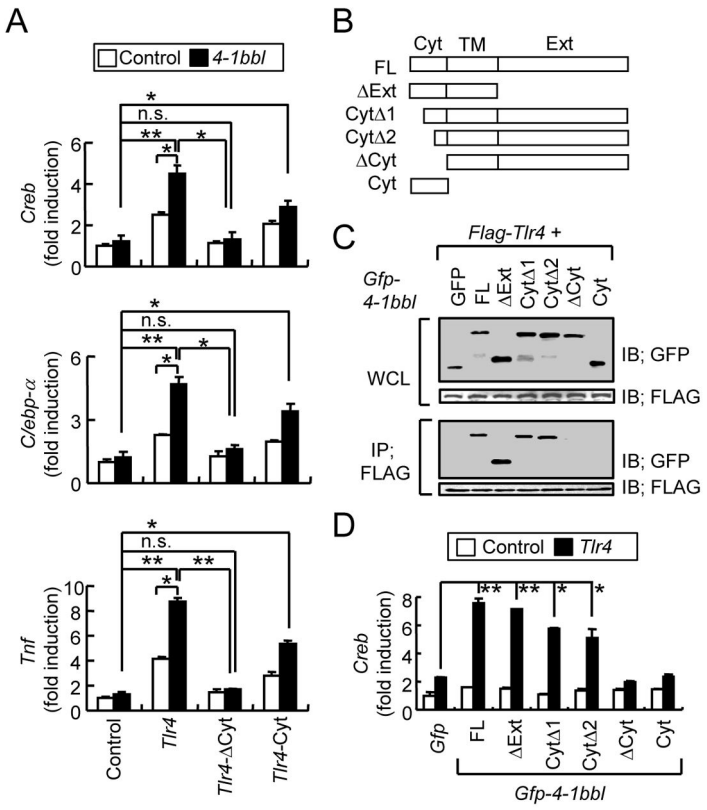

Fig. 1. Characterization of the physical interaction between TLR4 and 4-1BBL

(**A**) HEK 293T cells were cotransfected with empty control plasmid or with plasmid encoding full-length 4-1BBL, together with control plasmid or plasmid encoding full-length TLR4, a mutant TLR4 lacking the cytoplasmic domain (TLR4-ΔCyt), or a mutant TLR4 lacking its extracellular region (TLR4-Cyt). In addition, the cells were cotransfected with the *Creb*-luc, *C/ebp*-luc, or *Tnf*-promoter-luc reporter plasmids. xxx hours after transfection, cells were analyzed by luciferase assay to measure reporter activities. Data are mean fold-increases in luciferase activity ± SD relative to the luciferase activity of cells transfected with control plasmids, and are from three independent experiments. **P* < 0.05; ***P* < 0.01; n.s.; not significant. (**B** – **D**) Characterization of the domains of 4-1BBL that interact with TLR4. (B) Scheme showing full-length (FL) 4-1BBL, 4-1BBL lacking its extracellular domain (ΔExt), truncated mutants of 4-1BBL lacking different amounts of its cytoplasmic domain (CytΔ1 and CytΔ2), a 4-1BBL mutant lacking the full cytoplasmic domain (ΔCyt), and a truncation mutant of 4-1BBL consisting only of the entire cytoplasmic domain (Cyt). (C) HEK 293T cells were cotransfected with plasmid encoding FLAG-tagged TLR4 (FLAG-TLR4) together with plasmid encoding green fluorescent protein (GFP) or plasmid encoding a fusion protein of GFP and the indicated 4-1BBL proteins. Top: Whole-cell lysates (WCLs) were analyzed by Western blotting (IB) with antibodies against the indicated targets. Bottom: WCLs were subjected to immunoprecipitation (IP) with an anti-FLAG antibody and then were analyzed by Western blotting with antibodies against the indicated targets. Western blots are representative to two or three independent experiments. (**D**) HEK 293T cells were cotransfected with the pCREB-luc and pRl-tk-luc reporter plasmids, as well as with empty plasmid or plasmid encoding TLR4 together with plasmids encoding the indicated GFP-tagged 4-1BBL proteins. Cells were then analyzed by luciferase assay to determine the extent of CREB reporter activity. Data are mean fold-increases in luciferase activity ± SD relative to the luciferase activity of control cells and are from three independent experiments. **P* < 0.05; ***P* < 0.01.

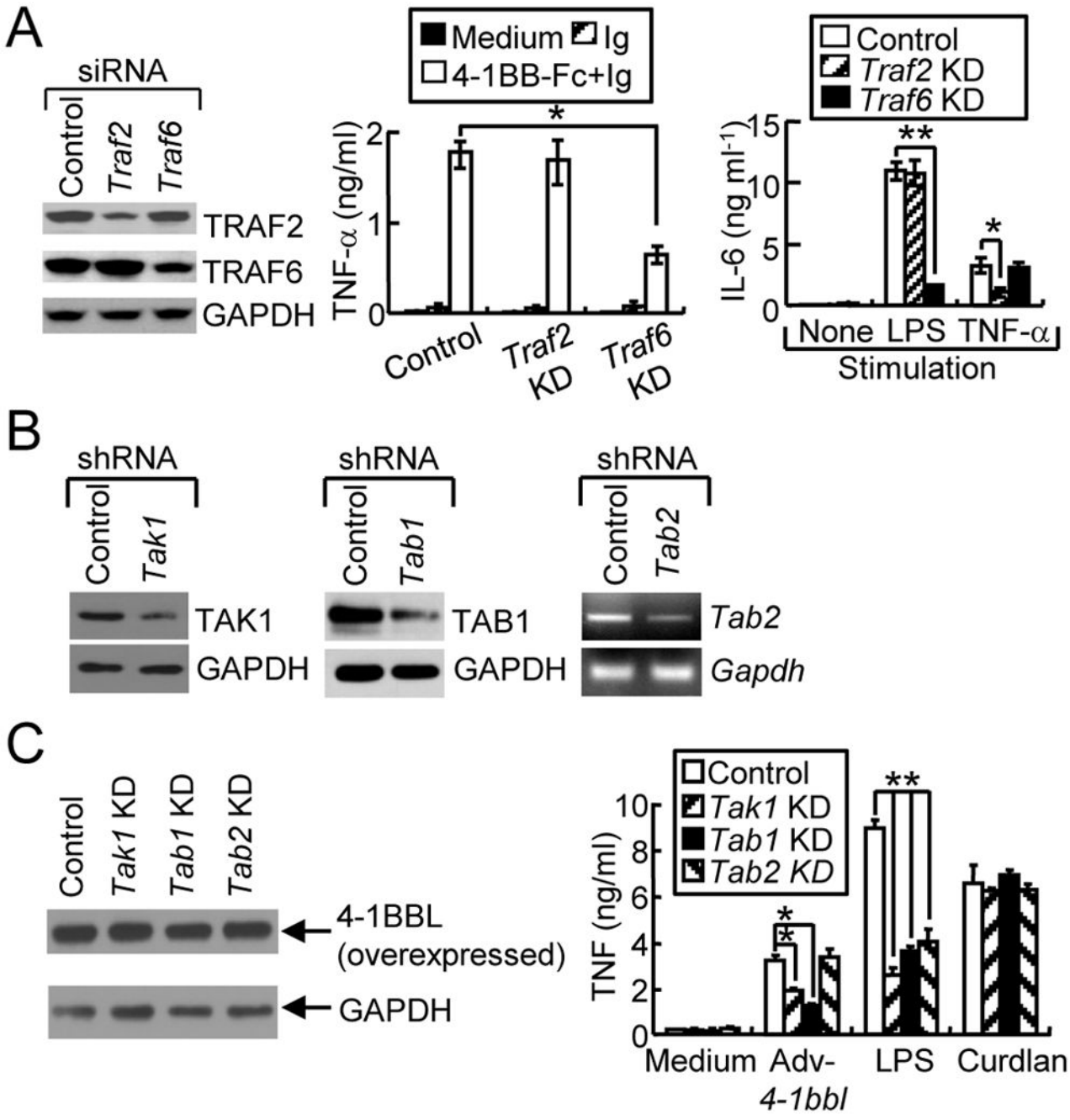

Fig. 2. TRAF6, TAK1, and TAB1 mediate the 4-1BBL–dependent production of TNF-α

(**A**) TRAF6 is required for 4-1BBL–mediated TNF-α production by macrophages. Left: RAW264.7 cells were transfected with control siRNA or with *Traf2*- or *Traf6*-specific siRNAs, and forty-eight hours later, the extent of knockdown (KD) of these targets was assessed by Western blotting analysis with antibodies against the indicated proteins. GAPDH was used as a loading control. Middle: Control and TRAF6 KD cells were treated with medium, anti-Fc antibody (Ig, 2.5 μg/ml), or mouse 4-1BB-Fc (5 μg/ml) and anti-Fc antibody (4-1BB-Fc + Ig) (middle), or treated with medium, LPS or TNF-α (right). Twenty-four hours later, the amounts of TNF-α or IL-6 in the culture media were determined by ELISA. Data are means ± SD of at least three independent experiments. **P* < 0.05; ***P* < 0.01. (**B** and **C**) TAK1 and TAB1, but not TAB2 mediate 4-1BBL–dependent

signaling. (B) Immortalized mouse macrophages were infected with lentivirus expressing control shRNA or with lentiviruses expressing *Tak1*-, *Tab1*-, or *Tab2*-specific shRNAs. The extent of knockdown of these targets was assessed by Western blotting (left and middle) or semi-quantitative PCR analysis (right). (C) Macrophage cell lines in which TAK1, TAB1, or TAB2 were knocked down as described in (B) were treated with medium (negative control) or were infected with Adv-*4-1bbl* (10^4 pfu/10^6 cells), treated with LPS (0.1 μg/ml), or treated with curdlan (20 μg/ml). Left: Twenty-four hours later, the abundance of exogenous 4-1BBL was determined by Western blotting analysis, with GAPDH used as a loading control. Right: The concentrations of TNF-α in the culture media of Adv-*4-1bbl*–infected cells were determined by ELISA. Data are means ± SD of at least three independent experiments. **P* < 0.05; ***P* < 0.01.

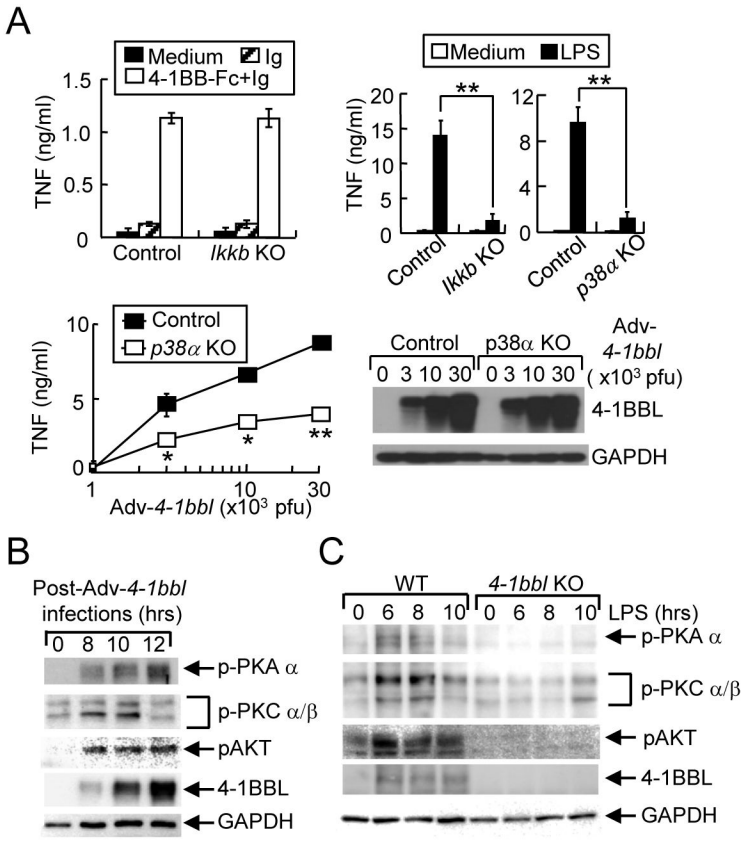

Fig. 3. 4-1BBL–dependent TNF-α production is mediated by the activation of protein kinases, but not by NF-κB

(**A**) Top left: Control or *Ikkb*-deficient (*Ikkb* KO) BMDMs were treated with medium or were treated with anti-Fc antibody (Ig, 2.5 μg/ml) in the presence or absence of 4-1BB-Fc (5 μg/ml). Twenty-four hours later, the concentrations of TNF-α in the culture media were determined by ELISA. Top right: Wild-type, *Ikkb* KO, or *p38α* KO mouse peritoneal macrophages were treated with medium or LPS (0.1 μg/ml) for 24 hours. The concentrations of TNF-α in the culture media were determined by ELISA. Data are means ± SD from 3–4 experiments. Bottom left: Wild-type and *p38a* KO mouse peritoneal macrophages were left uninfected or were infected with the indicated amounts of Adv-4-1BBL (per 10^6 cells). Twenty-four hours later, the amounts of TNF-α in the culture media were measured by ELISA. Data are means ± SD from three independent experiments. Bottom right: The same uninfected and infected cells were analyzed by Western blotting with an anti–4-1BBL antibody. GAPDH was used as a loading control. Western blots are representative of 3–4 independent experiments. (**B** and **C**) Analysis of protein kinase activation. (B) Wild-type mouse peritoneal macrophages were left uninfected or were infected with Adv-4-1BBL for the indicated times. (C) Peritoneal macrophages from wild-type (WT) or *4-1bbl* KO mice were left untreated or were treated with LPS for the indicated times. (B and C) Whole-cell lysates were analyzed by Western blotting with antibodies specific for the indicated proteins. GAPDH was used as a loading control. Western blots are representative of 2–3 independent experiments.

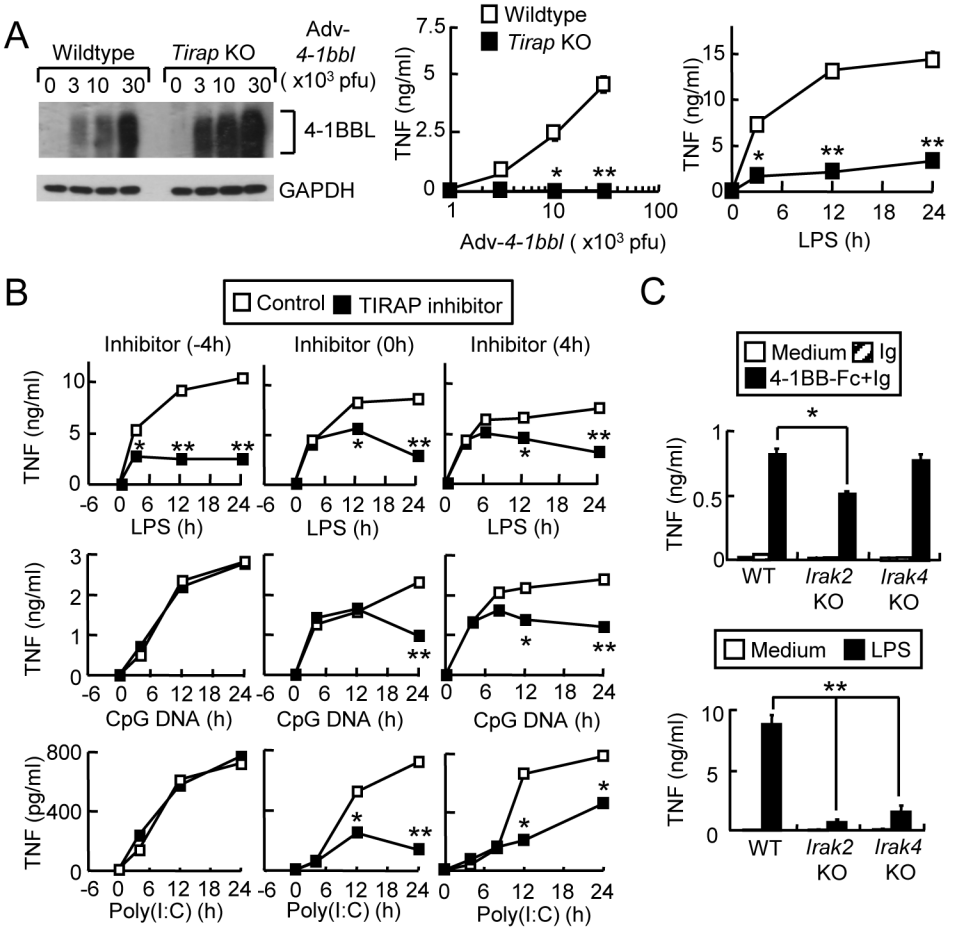

Fig. 4. TIRAP and IRAK2 mediate late-phase, 4-1BBL–dependent production of TNF-α

(**A**) Macrophages from WT or *Tirap* KO mice were left uninfected or were infected with the indicated amounts of Adv-*4-1bbl* or were left untreated or were treated with LPS for the indicated times. Left: Whole-cell lysates of the indicated uninfected and infected cells were analyzed by Western blotting to determine the abundance of 4-1BBL protein. Western blots are representative of xxx experiments. Middle and right: The concentrations of TNF-α in the culture media of infected or LPS-treated cells were determined by ELISA. Data are means ± SD of three independent experiments. **P* < 0.05; ***P* < 0.01. (**B**) TIRAP mediates late-phase TLR signaling in macrophages. Macrophages were treated with 100 μM control peptide (empty squares) or 100 μM TIRAP inhibitor peptide (filled squares) 4 hours before (left column), simultaneously with (middle column), or 4 hours after (right column) they were treated with the indicated TLR ligands, and cells were cultured for a further 24 hours after TLR stimulation. Samples of culture media taken at the indicated times were analyzed by ELISA to determine the concentrations of TNF-α. Data are means ± SD from three independent experiments. **P* < 0.05; ***P* < 0.01. (**C**) IRAK2, but not IRAK4, is required for 4-1BBL–dependent production of TNF-α. Top: BMDMs from WT, *Irak2*-deficient, or *Irak4*-deficient mice were treated with medium or were treated with anti-Fc antibody (Ig) in the absence or presence of 4-1BB-Fc. Twenty-four hours later, the concentrations of TNF-α in the culture media were determined by ELISA. Bottom: As a control, the same cells were treated with LPS to confirm the effects of loss of *Irak2* and *Irak4* on TNF-α production. Data are means ± SD from three independent experiments. **P* < 0.05; ***P* < 0.01.

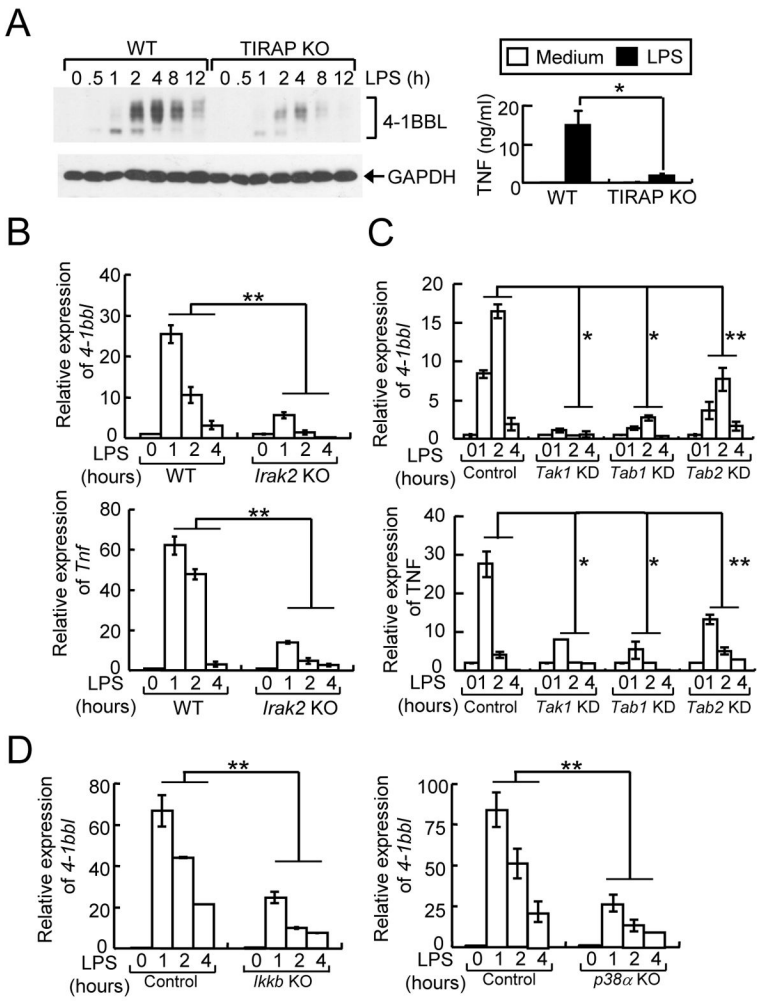

Fig. 5. Analysis of the signaling molecules required for the TLR4-dependent expression of *4-1bbl* and *Tnf*

(**A**) Expression of *4-1bbl*. Macrophages from WT or *Tirap* KO mice were treated with LPS for the indicated times. Left: 4-1BBL production was analyzed by Western blotting. Right: The concentrations of TNF-α in the culture media of the indicated cells were determined by ELISA. Data are means ± SD from at least three independent experiments. **P* < 0.01. (**B** to **D**) Real-time PCR analysis of *4-1bbl* and *Tnf* mRNA abundances. (B) WT or *Irak2*-deficient BMDMs, (C) WT, *Tak1*-, *Tab1*-, or *Tab2*-knockdown cells, or (D) WT, *Ikkβ* KO, or *p38α* KO macrophages were treated with LPS for the indicated times. The relative abundances of *4-1bbl* and *Tnf* mRNAs were measured by real-time PCR analysis and are expressed as the fold-increase relative to those of each unstimulated cells. Data are means ± SD from three independent experiments. **P* < 0.01; ***P* < 0.05.

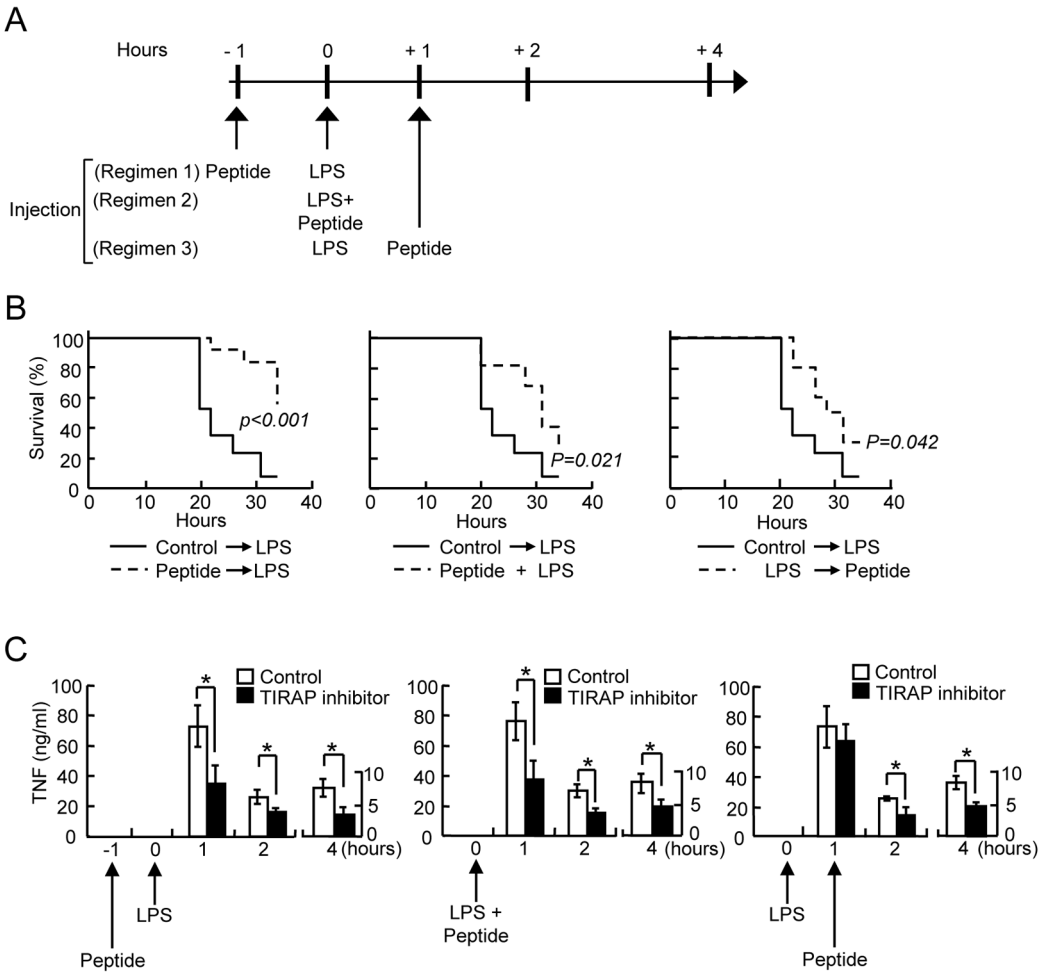

Fig. 6. Inhibiting late-phase TIRAP activity ameliorates LPS-induced septic shock in mice

(**A**) Scheme of the treatment of mice with LPS and either control peptide or the TIRAP inhibitor peptide. In all experiments, control WT mice were injected intraperitoneally (i.p.) with control peptide before being injected i.p. with LPS. In the case of the TIRAP inhibitor peptide, the mice were treated by one of three different regimens. The mice were injected with TIRAP inhibitor peptide 1 hour before, simultaneously with (time 0), or 1 hour after injection with LPS. (**B**) Effect of TIRAP inhibition on the survival of LPS-treated mice. Mice were injected (i.p.) with control peptide (250 μg/mouse) before being injected with LPS (350 μg/mouse, n = 16 mice). Mice were injected with the TIRAP inhibitory peptide (250 μg/mouse) and LPS according to the three regimens described in (A) (n = 12 mice for each condition). The survival of the mice was monitored over time. *P* values are shown. (**C**) Effect of TIRAP inhibition on the serum concentrations of TNF-α in LPS-treated mice. WT mice were injected with control peptide and LPS or with TIRAP inhibitor peptide and LPS according to the three regimens described in (A). Arrows indicate the times of injection of LPS and peptides. Serum concentrations of TNF-α were measured by ELISA at the indicated times after injection with LPS. Data are means ± SD from ten mice. **P* < 0.05.

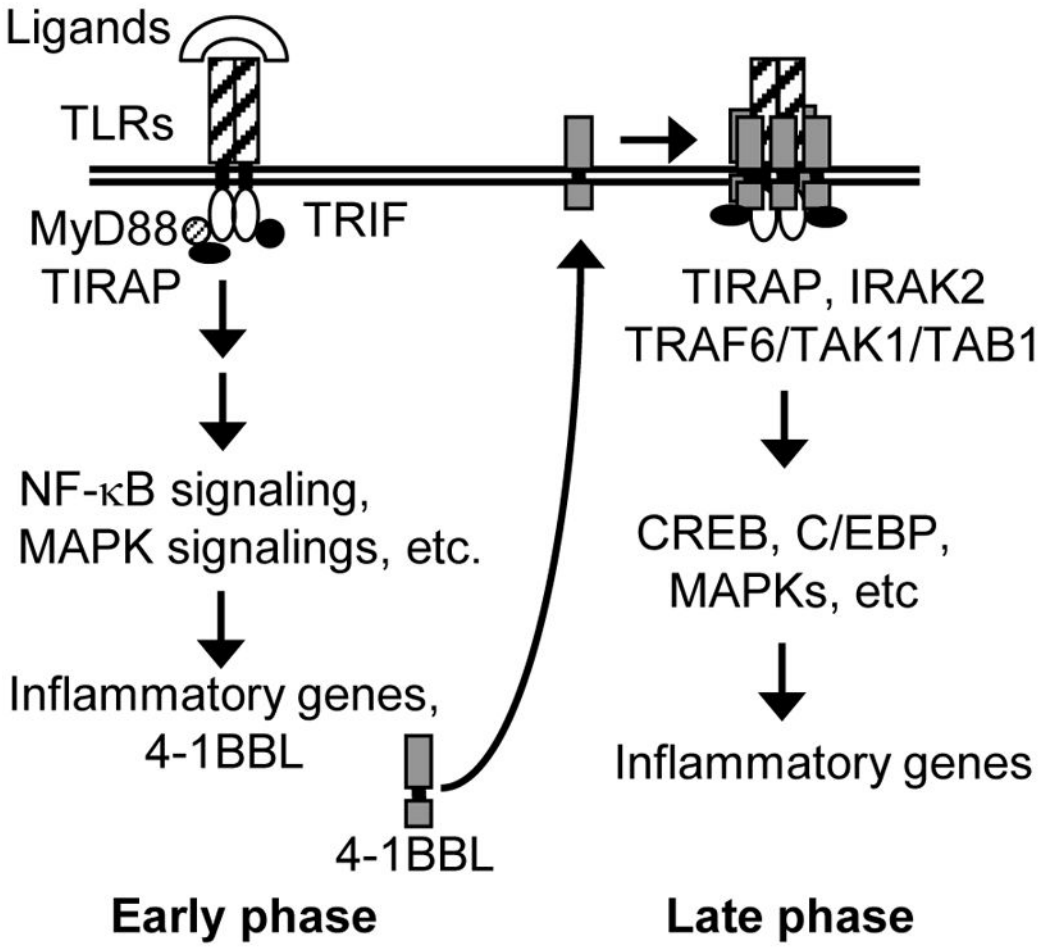

Fig. 7. Proposed model of the sequential nature of TLR signaling in macrophages

Upon binding by their specific ligands, cell-surface TLRs stimulate early-phase signaling pathways dependent on the adaptor proteins MyD88, TRIF, or both, which lead to the activation of NF-κB and MAPK signaling pathways and the expression of genes, such as *Tnf* and *4-1bbl*, encoding proinflammatory factors. In response to this early signaling, 4-1BBL is produced and translocates to the cell surface, where it physically interacts with resident TLRs. The resulting 4-1BBL- and TLR-containing complexes initiate late-phase activation of TIRAP- and IRAK2-dependent signaling by forming a secondary signaling complex consisting of TLR, 4-1BBL, TIRAP, and IRAK2. Signaling downstream of this complex involves the transcription factors CREB and C/EBP, and results in the sustained expression of genes encoding inflammatory factors, such as TNF-α.