Our previous studies have revealed that the signaling protein BCL10 plays a major role in adaptive immunity by mediating NF-κB activation in the LPS/TLR4 pathway. In this study, we show that IRAK-1 acts as the essential upstream adaptor that recruits BCL10 to the TLR4 signaling complex and mediates signaling to NF-κB through the BCL10-MALT1-TRAF6-TAK1 cascade. Following dissociation from IRAK-1, BCL10 is translocated into the cytosol along with TRAF6 and TAK1, in a process bridged by a direct BCL10-Pellino2 interaction. RNA interference against MALT1 markedly reduced the level of NF-κB activation stimulated by lipopolysaccharide (LPS) in macrophages, which suggests that MALT1 plays a major role in the LPS/TLR4 pathway. MALT1 interacted with BCL10 and TRAF6 to facilitate TRAF6 self-ubiquitination in the cytosol, which was strictly dependent on the dissociation of BCL10 from IRAK-1. We show that BCL10 oligomerization is a prerequisite for BCL10 function in LPS signaling to NF-κB and that IRAK-1 dimerization is an important event in this process.

BCL10 and MALT1 are intracellular NF-κB activators that are involved in MALT lymphoma translocations (1, 2). These translocations are associated with MALT tumorigenesis via constitutive NF-κB activation, which provides both anti-apoptotic and proliferative signals by up-regulating the transcription of specific targets (3). BCL10 contains the caspase recruitment domain (CARD), which is involved in protein oligomerization and protein-protein associations (4), as well as the MALT1-binding sequence containing 16 amino acids.

BCL10 proteins can form homo-oligomers through CARD-CARD interaction under suitable conditions. In TCR signaling, BCL10 binds to its downstream adaptor MALT1 and co-operatively activates NF-κB (4–6). Recent studies have shown that BCL10 and MALT1 mediate NF-κB activation by facilitating Lys-63 polyubiquitination of NEMO, which is an essential regulatory subunit of the IKK complex (7). In this process, tumor necrosis factor receptor-associated factor 6 (TRAF6), which is a polyubiquitin ligase, fills the gap between BCL10-MALT1 and NEMO in TCR signaling (8). MALT1 binds to TRAF6 through two putative C-terminal TRAF6-binding motifs. In vitro experiments using purified MALT1, TRAF6, TGF-β-activated kinase (TAK1), and ubiquitination enzymes, which include Ubc13/ Uev1A, allowed reconstitution of the pathway from BCL10 to IKK activation. As reported previously, BCL10 and MALT1 form oligomers that bind to TRAF6, induce TRAF6 oligomerization, and activate the ligase activity of TRAF6 to promote self-ubiquitination and polyubiquitination of NEMO. This cascade model is predicted to be responsible for TCR signaling and NF-κB activation in T lymphocytes (8).

Toll-like receptors (TLRs) play essential roles in innate immune recognition in mammalian species. Upon engagement of their respective ligands, TLRs stimulate the transcription of effector genes through activation of common transcription factors, which include nuclear factor-κB (NF-κB), activating protein 1, and activating transcription factor. Of these transcription factors, NF-κB plays a central role in many biological processes of both the innate and adaptive immune systems. Stimuli that activate NF-κB include mitogens for antigen receptors, proinflammatory cytokines, and the bacterial product lipopolysaccharide (LPS). LPS elicits a multitude of effects on the immune system and is a ligand recognized by TLR4 (9, 10). LPS ligation to TLR4 results in signaling activation via downstream signaling factors, which include the adaptor MyD88, IL-1RI-associated protein kinases (IRAKs), TRAF6, and TAK1. Upon LPS stimulation, IRAK-1, a serine-threonine kinase, is phosphorylated within receptor complexes that include Tollip, MyD88, and TRAF6. Phosphorylation of IRAK-1 presumably triggers IRAK-1 to dissociate from the receptor complexes and translocate along with TRAF6 to the membrane-bound pre-associated TAK1-TAB1-TAB2 complex. IRAK-1 is required for IL-1-induced phosphorylation of TAK1, TAB1, and TAB2 in the membrane-bound complexes (11, 12). Phosphorylated IRAK-1 is eventually ubiquitinated and degraded. TAK1-TAB1-TAB2-TRAF6 then dissociates from phosphorylated IRAK-1 and translocates to the cytosol, in which phosphorylated TAK1 is activated (12), in a process that is at least partially

The IRAK-1-BCL10-MALT1-TRAF6-TAK1 Cascade Mediates Signaling to NF-κB from Toll-like Receptor 4*

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dependent on TRAF6 oligomerization and self-ubiquitination (8, 13).

Similar to most TRAF proteins, TRAF6 is composed of a highly conserved C-terminal TRAF domain and a more variable N-terminal domain that contains a RING finger and several zinc fingers (14, 15). C-terminal TRAF domains mediate homotypic and heterotypic TRAF oligomerization, as well as receptor docking. TRAF6 has been shown to act as a RING finger-dependent ubiquitin-protein isopeptide ligase ubiquitin ligase and to ubiquitinate itself through Lys-63-Ub conjugation in conjunction with Ubc13 and Uev1A. The NF-κB-dependent ubiquitin-protein isopeptide ligase ubiquitin ligase activates TRAF6 polyubiquitinates NEMO and activates TAK1, which in turn phosphorylates IKK-β and leads to IKK activation. In these processes, homotypic TRAF6 oligomerization is a prerequisite for TRAF6 ubiquitin ligase activation and self-ubiquitination (8–13).

BCL10 is a well known signaling protein in adaptive immunity, and our previous studies have shown that it plays a major role in mediating NF-κB activation during LPS/TLR4 signaling. In a mouse macrophage cell line treated with LPS, BCL10 was rendered these mouse macrophages unable to activate recruited to the TLR4 signaling complexes. RNAi against BCL10 in innate immunity, has been shown to impair signaling by knocking off mice found that LPS signaling to NF-κB (16). On the other hand, a recent study using BCL10 signaling protein that is involved in innate immunity, has been shown to impair signaling by knocking off mice found that LPS signaling to NF-κB (16).

BCL10 is a well known signaling protein in adaptive immunity, and our previous studies have shown that it plays a major role in mediating NF-κB activation during LPS/TLR4 signaling. In a mouse macrophage cell line treated with LPS, BCL10 was recruited to the TLR4 signaling complexes. RNAi against BCL10 in our previous studies have shown that it plays a major role in mediating NF-κB activation during LPS/TLR4 signaling.

The following primer sequences were used to generate mouse IRAK-1 cDNA by PCR from a universal mouse cDNA library as template (PTGLab): forward mutagenic primer, 5’-cttctacacagctgacgccc-3’; reverse mutagenic primer, 5’-atacgctgaactcatcagc-3’. The following primer sequences were used to generate mouse IRAK-1 cDNA: forward mutagenic primer 1, 5’-gccgtacatcagccggagcc-3’; reverse mutagenic primer 2, 5’-gtaggagttctcctgggtca-3’.

The following primer sequences were used to generate mouse IRAK-1 cDNA: forward mutagenic primer 1, 5’-gccgtacatcagccggagcc-3’; reverse mutagenic primer 2, 5’-gtaggagttctcctgggtca-3’. The following primer sequences were used to generate mouse IRAK-1 cDNA: forward mutagenic primer 3, 5’-gtgctggtgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctg}
The following primer sequences were used to generate truncated forms of human BCL10. For BCL10 (residues 1–103) forward mutagenic primer, 5'-gggct-3' was used; this primer was used to introduce an EcoRi site before Met-1 in the primary sequence; for reverse mutagenic primer, 5'-ggctgccgactcattcgtgtaac-3' was used; this primer was used to introduce a Sall site after the Val-103 in the primary sequence. For BCL10 (residues 1–119), forward mutagenic primer, 5'-ggaattcagcaggggagggccc-3' was used; this primer was used to introduce an EcoRi site before Met-1 in the primary sequence; for reverse mutagenic primer, 5'-cgtctgaggggagcccgaggtggggtgctgctgcagcttctctt-3' was used; this primer was used to introduce a Sall site after the Cys-119 in the primary sequence. For BCL10 (residues 104–233), forward mutagenic primer, 5'-gaattcagcaggggagggccc-3' was used; this primer was used to introduce an EcoRi site before Met-1 in the primary sequence; for reverse mutagenic primer, 5'-cggtcgactcagctctgaaattca-3' was used; this primer was used to introduce a SalI site after the Ser-233 in the primary IRAK-1 sequence. For IRAK-1 (dUD), pair 1 was used to amplify fragment 2 (nt 1855–2139) by PCR from primary IRAK-1 sequence, for forward mutagenic primer 1, 5'-cgtctgaggggagcccgaggtggggtgctgctgcagcttctctt-3' was used; this primer was used to introduce an EcoRi site before Met-1 in the primary IRAK-1 sequence; for reverse mutagenic primer, 5'-cggtcgactcagctctgaaattca-3' was used; this primer was used to introduce a Sall site after the Ala-618 in the primary IRAK-1 sequence.

His-tagged BCL10 proteins were expressed in bacteria and purified by nickel columns (Qiagen) according to the manufacturer’s instructions. Recombinant IRAK-1, IRAK-1b and IRAK-1 (T66E) were expressed in the SF-9 cells using the Bac-to-Bac baculovirus expression system (Invitrogen).

Cell Culturing, Stable Transfection, and Treatment—The murine macrophage cell line RAW 264.7 (American Type Culture Collection) was maintained in Dulbecco’s modified Eagle’s medium (Sigma) that was supplemented with 10% fetal bovine serum (Sigma). Approximately $6 \times 10^5$ cells/well were seeded into 6-well plates and transfected 24 h later with $10 \mu g$ of each vector using Lipofectin 2000 (Invitrogen) according to the manufacturer’s instructions. The transfectants were cultured in the presence of 0.8 mg/ml G418 (Sigma) until the clones appeared. Every isolated clone was picked and seeded in 96-well plates individually. All the RAW lines stably transfected are clonal. In all experiments, the cells were stimulated with 100 ng/ml LPS or 20 ng/ml recombinant human TNF-α (final concentrations).

NF-κB Assay—Cells were transfected with 900 ng of the pNF-κB luciferase reporter (Stratagene) and 100 ng of the pRL-TK Renilla reporter (Promega) in combination with the indicated expression plasmids. Cells were harvested after the indicated periods of time, and the firefly luciferase levels were determined using the dual-luciferase reporter assay system (Promega). The level of Renilla luciferase activity was used to normalize the transfection efficiencies.

RNA Interference—The RNAi pSuper system (OligoEngine) was used. The different pSuper recombinant vectors were constructed by inserting 64-mer synthetic DNA oligonucleotides that encode two 19-nucleotide (nt) reverse complements with homology to a portion of the target gene. For the mIRAK-1 siRNA construct, the insert was prepared by annealing two 64-mer synthetic DNA oligonucleotides that encode two 19-nt reverse complements derived from the mouse IRAK-1 cDNA sequence (nt 192–210). For the two mMALT1 siRNA constructs, the insert was prepared by annealing two 64-mer syn-
thetin DNA oligonucleotides that encode two 19-nt reverse complements derived from the mouse MALT1 cDNA sequence, thereby generating clone RAWdeMa (nt 768–786) and clone RAWdeMb (nt 333–351), respectively. For the EGFP siRNA construct as scrambled RNAi, the insert was prepared by annealing two 64-mer synthetic DNA oligonucleotides that encode two 19-nt reverse complements derived from the EGFP cDNA sequence (nt 158–176).

The following sequences of oligonucleotides were used for RNAi experiments. For pSUPER-IRAK-1: forward, 5′-gatcccccatcatacagctgccatccatcaggtctgccagtgttgattttttggaaa-3′, and reverse, 5′-agctttctacaaatcatcatacagctgctctcttgagtgactctctctctttttttggaaa-3′, were used. For pSUPER-MALT1a (nt 768–786): forward, 5′-gatcccgcttggtgctgttgaagttcaggaactcctacagtctcttgaactgtaggaagtcgctca-3′, and reverse, 5′-agctttctacaaatcatcatacagctgctctctctttttttggaaa-3′, were used. For pSUPER-MALT1b (nt 333–351): forward, 5′-gatccccactgcagccgctctctctcaggtctgccagtgttgattttttggaaa-3′, and reverse, 5′-agctttctacaaatcatcatacagctgctctctctttttttggaaa-3′, were used. For pSUPER-EGFP: forward, 5′-gatcccgcttggtgctgttgaagttcaggaactcctacagtctcttgaactgtaggaagtcgctca-3′, and reverse, 5′-agctttctacaaatcatcatacagctgctctctctttttttggaaa-3′, were used.

After each insert was prepared, the pSUPER plasmid was digested with BglII and HindIII and purified. Ligation was carried out by mixing 10 μl of pSUPER vector and 1 μl of annealed insert in a ligation reaction, which was incubated at 16 °C for 8 h. The ligation reaction was then incubated at 70 °C for 10 min and subjected to BglII digestion in a 100-μl volume for 3–4 h at 37 °C. The bacteria were transformed with insert-ligated pSUPER vectors. Plasmid DNA isolated from the bacteria was digested with EcoRI and HindIII. Positive clones were about 350 bp in size.

All the RAW lines are clonal, including RAWdeI, RAWdeMa, and RAWdeMb. Briefly, 10 μg of the pSUPER vector or recombinant pSUPER vectors with ligated inserts were stably transfected into ~6 × 10^5 RAW 264.7 cells. The transformants were cultured in the presence of 0.8 mg/ml G418 (Sigma) until the clones appeared. Every isolated clone was picked and subjected to BglII digestion in a 100-μl volume for 3–4 h at 37 °C. The bacteria were transformed in insert-ligated pSUPER vectors. Plasmid DNA isolated from the bacteria was digested with EcoRI and HindIII. Positive clones were about 350 bp in size.

**RESULTS**

**IRAK-1 Interacts with BCL10 in Response to LPS Stimulation**

In a previous study, we observed that BCL10 is recruited into TLR4 signaling complexes in response to LPS stimulation, which raised the question as to how BCL10 is recruited to the receptor. To identify the molecule(s) that act as the adaptor for BCL10 recruitment to the TLR4 signaling complexes, we screened for BCL10-associated proteins using the T7Select phage display system (Novagen). A peptide identical to the coding sequence of human interleukin-1 receptor-associated kinase-1 (residues 502–642) was found to interact with BCL10 (Fig. 1). This interesting finding encouraged us to examine the potential in vivo association between BCL10 and IRAK-1. Extracts of RAW 264.7 cells treated with or without LPS were immunoprecipitated with anti-IRAK-1 antibody, followed by Western blot analysis with the anti-BCL10 antibody. BCL10 interacted with IRAK-1 only in the presence of LPS stimulation. These results clearly indicate that BCL10 interacts with IRAK-1 in an LPS signal-dependent manner in vivo, and suggest that IRAK-1 links BCL10 to TLR4 complexes following LPS stimulation (Fig. 2).

**Glycerol Gradient Ultracentrifugation**—Purified wild-type IRAK-1 and IRAK-1 mutants (5 μg) expressed from the Bac-to-Bac system were incubated with 5 μg of BCL10, and the mixtures were applied to a 10–50% glycerol gradient in 50 mM Tris-HCl (pH 7.5) and 150 mM NaCl. After centrifugation at 250,000 × g for 3 h in a TLS-55 rotor (Beckman Coulter) at 4 °C, the 200-μl fractions were analyzed by immunoblotting with antibody against GST or BCL10.

**In Vitro Transcription-Translation**—Coupled in vitro transcription-translation reactions with IRAK-1 and mutants thereof were performed with TNT-T7 system (Promega). GST Resin Pulldown Assay—Twenty microliters of glutathione-Sepharose beads coated with GST fusion proteins were mixed with equal amounts of in vitro translated IRAK-1 or bacterially expressed BCL10 and rocked at 4 °C for 1 h in modified GBT buffer (10% glycerol, 50 mM HEPES-NaOH (pH 7.5), 175 mM KCl, 7.5 mM MgCl2, 0.1 mM EDTA, 0.1 mM dithiothreitol, 1% Triton X-100) that contained 1% bovine serum albumin. The beads were collected by low speed centrifugation and washed extensively in modified GBT buffer. Bound proteins were eluted in sample buffer and analyzed by 10% SDS-PAGE and Western blotting.
The peptide sequence in IRAK-1 that potentially mediates BCL10 interaction is located at the fringe of the KD and C1 regions. To map the IRAK-1 domain(s) required for the interaction with BCL10 in more detail, we generated various deletion mutant constructs and examined their associations with BCL10 in pulldown experiments. Deletions of UD, DD, KD, and C2 had no effect on the interaction with BCL10, whereas mutants that lacked the C1 domain lost the ability to bind BCL10 (Fig. 3A). Recently, it has been shown that an alternative splice variant of IRAK-1 (IRAK-1b) that lacks exon 12 (residues 514–543) has widespread tissue expression and is evolutionarily conserved in the mouse and human (19). Interestingly, the missing exon 12 (residues 514–543) is located in the 502–642-residue region that we screened as a BCL10-interacting peptide. We could not detect any association between BCL10 and IRAK-1b in co-precipitation assays, which suggests that exon 12 (residues 514–543) is essential for the BCL10-IRAK-1 interaction.

As mentioned above, human BCL10 has the N-terminal CARD-containing domain (residues 1–106), which functions as an oligomerization region, the MALT1-binding domain (residues 104–119), and the C-terminal Ser/Thr-rich domain (residues 120–233), which may be regulated by phosphorylation (4). To investigate the region of BCL10 that mediates the IRAK-1-BCL10 interaction, we constructed several human BCL10 mutants, including a BCL10 CARD domain deletion mutant and a C-terminal domain deletion mutant. The results of the pulldown assays indicate that BCL10 binding to IRAK-1 requires both the MALT1-binding domain and C-terminal Ser/Thr-rich domain (Fig. 3B).

IRAK-1 Recruits BCL10 into the TLR4 Complex—To determine whether the recruitment of BCL10 to the receptor occurs through IRAK-1, we generated the IRAK-1-silent cell line RAWδel by stably transfecting RAW 264.7 cells with the RNAi vector pSUPER-IRAK-1 (Fig. 4A). Extracts of the wild-type RAW 264.7 and RAWδel cells either untreated or treated with LPS were immunoprecipitated with the anti-TLR4 antibody, followed by Western blot analyses with antibodies against BCL10 and TLR4. Although BCL10 was recruited to the receptor in the wild-type cells, it failed to interact with the receptor in the IRAK-1-deficient RAWδel cells (Fig. 4B). However, we also observed that overexpression of the human kinase-dead IRAK-1 mutant K239S ensured the inclusion of BCL10 in TLR4 complexes in response to LPS stimulation, which further indicates that IRAK-1 acts mainly as a proximal adaptor in BCL10-mediated signal transduction independent of its kinase activity (Fig. 4B).

To examine whether BCL10 plays a role in transducing signals directly downstream of IRAK-1, we examined the NF-κB activation status of BCL10-deficient RAW 264.7 cells in response to IRAK-1 overexpression. We found that BCL10 expression knockdown could to some extent impair NF-κB activation mediated by mouse IRAK-1 overexpression. In con-
contrast, NF-κB activation mediated by mouse IRAK-1b overexpression was normal in BCL10-deficient RAW 264.7 cells (Fig. 5), which indicates that BCL10 takes no part in IRAK-1b signaling to NF-κB and that the IRAK-1 group proteins, which include IRAK-1 and IRAK-1b, may participate in different sub-pathways. Although IRAK-1b is biologically distinct from IRAK-1, it can activate NF-κB. Because the splice variant between IRAK-1 and IRAK-1b is evolutionarily conserved in mammals, we speculate that IRAK-1b represents another regulatory model for LPS signaling to NF-κB.
BCL10 Oligomerization Is Essential for LPS Signaling to NF-κB—Recent studies have provided evidence that in TCR signaling to NF-κB, BCL10 interacts with MALT1 and that the oligomerization of BCL10 induces TRAF6 oligomerization, so as to activate the ligase activity of TRAF6 to polyubiquitinate NEMO (8). To verify that BCL10 oligomerization is essential for LPS signaling to NF-κB, we generated a RAW 264.7 cell line that stably expresses a form of mouse BCL10 with two mutated residues (L41Q and G78R) (Fig. 6A). The L41Q and G78R mutations resulted in the inhibition of BCL10 oligomerization mediated by CARD-CARD interaction (20). In this cell line, LPS-induced NF-κB activation was attenuated in comparison with that in wild-type RAW 264.7 cells (Fig. 6B), whereas the BCL10 (L41Q and G78R)-IRAK-1 interaction was normal (Fig. 6C), which indicates that BCL10 (L41Q and G78R) competes for IRAK-1 binding to the wild-type BCL10 and that BCL10

**FIGURE 7. IRAK-1 oligomerization is an optimal factor for BCL10 oligomerization.** A, purified human BCL10, GST-tagged human IRAK-1, GST-tagged human IRAK-1b, and GST-tagged human IRAK-1 (T66E) proteins were fractionated by glycerol gradient ultracentrifugation (10–50%; the density of the glycerol gradient increased from the top to the bottom). The various fractions were collected and immunoblotted with the anti-BCL10 or anti-GST antibody. B, wild-type RAW 264.7 cells were transiently transfected with pCMV-FLAG, pCMV-FLAG-hIRAK-1b, or pCMV-FLAG-hIRAK-1 (T66E). At 36 h post-transfection, the cells were stimulated with LPS. Anti-FLAG antibody immunoprecipitates (IP) were analyzed by Western blotting with the anti-BCL10 antibody. Lysates were also subjected to immunoblotting to verify FLAG-hIRAK-1b or FLAG-hIRAK-1 (T66E) expression. Vector denotes the RAW 264.7 cells transfected with the empty expression vector pCMV-FLAG.

**FIGURE 8. MALT1 is essential for optimal LPS-induced NF-κB activation.** A, wild-type (WT) RAW 264.7 cells were stably infected with pSUPER-MALT1 or the control vector pSUPER-EGFP. Cell lysates were prepared and probed with the anti-MALT1 antibody. B, wild-type or MALT1-deficient RAW 264.7 cells were transfected with 900 ng of pNF-κB luciferase reporter and 100 ng of pRL-TK Renilla reporter. At 36 h post-transfection, the cells were stimulated with LPS for 30 min, followed by luciferase reporter assays. Under the same conditions and at 30 h post-transfection, the cells were incubated with TNF-α for 6 h and subjected to luciferase reporter assays to ensure specificity. The fold induction values are relative to those of the wild-type and MALT1-deficient cells transfected with 1000 ng of reporter DNA and incubated in the absence of LPS or TNF-α. C, wild-type RAW 264.7 cells were transfected with 900 ng of pNF-κB luciferase reporter and 100 ng of pRL-TK Renilla reporter. At 36 h post-transfection, the cells were stimulated with 50, 100, 150, 200, or 250 ng/ml LPS (final concentration) for 30 min, followed by luciferase reporter assays. The fold induction values are relative to those of the wild-type, BCL10-deficient, and MALT1-deficient cells transfected with 1000 ng of reporter DNA and incubated in the absence of LPS.
The BCL10-MALT1 Cascade Is Critical for Optimal LPS Signaling to NF-κB—In the TCR pathway, MALT1 is a critical signaling protein directly downstream of BCL10 (5). To explore whether MALT1 participates in LPS/TLR4 signaling that culminates in NF-κB activation, we stably transfected RAW 264.7 cells with the RNAi vector pSUPER-MALT1. MALT1 expression was almost completely knocked down in these cells (RAWdeMa) (Fig. 8A). LPS-induced NF-κB activation was obviously attenuated in RAWdeMb cells, which suggests that MALT1 is required for optimal LPS signaling to NF-κB (Fig. 8B). Moreover, we found that LPS treatment effectively induced the BCL10-MALT1 and MALT1-TRAF6 interactions, which indicates that the BCL10-MALT1 cascade is also an essential element for LPS signaling to NF-κB and innate immunity (Fig. 8C).

Recently, several groups have described inconsistent results regarding the phenotypes of BCL10 and MALT1 knock-out mice in response to LPS stimulation. Interestingly, in the absence of intact BCL10 or MALT1, LPS signaling to NF-κB has been variably reported as being normal or impaired. Our data for the BCL10-deficient RAW 264.7 cells and RAWdeMa cells obviously contradict the previous finding that the lymphocytes from BCL10 or MALT1 knock-out mice have the normal proliferative responses to LPS. To preclude concerns that our findings are the consequences of a single dose-response study and that the phenotypes of the BCL10-deficient RAW 264.7 cells and RAWdeMa cells reflect a relative shift in LPS sensitivity, we carried out dose-dependent studies on the changing LPS sensitivities of these cells. The BCL10-deficient RAW 264.7 cells (RAWdeB) (16) and RAWdeMb cells were exposed to 50, 100, 150, 200, or 250 ng/ml LPS (final concentration) for 30 min. In the wild-type RAW 264.7 cells, NF-κB activation was triggered efficiently by LPS in a dose-dependent manner. However, for the BCL10-deficient RAW 264.7 cells (RAWdeB), in which BCL10 expression was knocked down about 80% (16), LPS stimulated NF-κB activation in a partially dose-dependent manner and to levels that were far lower than those seen for the wild-type RAW 264.7 cells (Fig. 8D). For the RAWdeMb cells, in which MALT1 expression was almost completely inhibited, the NF-κB activations were constantly at a low level. Nevertheless, we observed that the curve for NF-κB activation was still changed in a dose-dependent manner, which demonstrates that the levels of NF-κB activation were markedly higher than those seen for the wild-type RAW 264.7 cells (Fig. 8D). For the RAWdeMb cells, in which MALT1 expression was knocked down by about 50% (Fig. 8D), is a cell clone derived from RAW 264.7 cells by pSUPER-MALT1 stable transfection. We found that for RAWdeMb cells stimulated with different LPS doses, the levels of NF-κB activation were markedly higher than those in RAWdeMa cells, which suggests that the changes in NF-κB activation are related to MALT1 expression levels (Fig. 8D). Taken together, these data indicate that although macrophages treated with LPS are able to use pathways that are independent of BCL10 and MALT1, the BCL10-MALT1 cascade is a critical element that transduces optimal LPS signaling to NF-κB from TLR4.

It should be noted that under conditions of BCL10 knock-down by RNAi, the LPS-induced interactions between MALT1
BCL10 was detected in both the membrane-bound and cytosolic complexes or the TLR4 complexes (Fig. 10A). However, we could not find any MALT1 molecules in either the membrane-bound TAK1 complex or the cytosolic TAK1 complex. We could detect IRAK-1 in the cytosolic TAK1 complex, despite the fact that IRAK-1 was reliably detected in both the membrane-bound TRAF6 fraction and TAK1 complexes (Fig. 10C). These data indicate that IRAK-1 interacts exclusively with TRAF6 and TAK1 in the membrane-bound complex. Importantly, we were unable to detect IRAK-1 in the cytosolic BCL10 complex fraction (Fig. 10C), which demonstrates that IRAK-1 dissociates from BCL10 before BCL10 translocates to the cytosol. These results support our hypothesis that BCL10 dissociation from IRAK-1 is a prerequisite for the BCL10-MALT1 interaction. Indeed, our previous data suggest that in BCL10 both the MALT1-binding domain and the C-terminal Ser/Thr-rich domain are required for interaction with IRAK-1. We think that IRAK-1 binding to BCL10 would mask the MALT1-binding site to preclude subsequent MALT1-BCL10 interaction until dissociation between IRAK-1 and BCL10. For consistency, this viewpoint is supported by the observation that the MALT1 proteins could not be detected in both IRAK-1 and TLR4 complexes.

A proposed model is that an IRAK-1-TRAF6 complex is initially formed on the receptor and is subsequently released from the receptor to interact with the pre-associated TAK1 complex on the membrane. The TRAF6-TAK1 complex then dissociates from IRAK-1 and translocates to the cytosol, leaving the phosphorylated IRAK-1 to be degraded (12). Consistent with previous studies, we could not find IRAK-1 in either the cytosolic TRAF6 fraction or the cytosolic TAK1 complex. From these data, we propose that an IRAK-1-TRAF6 complex is initially formed on the receptor and is subsequently released from the receptor to interact with the pre-associated TAK1 complex on the membrane. The TRAF6-TAK1 complex then dissociates from IRAK-1 and translocates to the cytosol, leaving the phosphorylated IRAK-1 to be degraded (12). Consistent with previous studies, we could not find IRAK-1 in either the cytosolic TRAF6 fraction or the cytosolic TAK1 complex. Importantly, we were unable to detect IRAK-1 in the cytosolic BCL10 complex fraction (Fig. 10C), which...
is interesting to study whether Pellino proteins participate in mediating BCL10 translocation following dissociation from IRAK-1. Following LPS stimulation, Pellino2 was detected in both the membrane-bound and cytosolic TAK1 complexes in conjunction with BCL10 (Fig. 11A). However, the use of RNAi against Pellino2 in RAW 264.7 cells efficiently inhibited the recruitment of BCL10 to the cytosolic TAK1 complexes following LPS stimulation (Fig. 11A). Consistent with this result, LPS-induced MALT1-TRAF6 interactions were effectively attenuated by RNAi against Pellino2 (Fig. 11B). These data indicate that Pellino proteins bridge BCL10-TRAF6 interactions after dissociation from IRAK-1.

**DISCUSSION**

In this study, we identified IRAK-1 as an essential upstream adaptor that recruits BCL10 to the TLR4 signaling complex and that mediates signals transduced from the receptor through the BCL10-MALT1-TRAF6-TAK1 cascade. In support of our previous findings, we provide more detailed evidence that both BCL10 and MALT1 play major roles in LPS/TLR4 signaling to NF-κB, as the use of RNAi against these two factors markedly reduced the levels of NF-κB activation induced by LPS in macrophage cells. Following dissociation from IRAK-1, BCL10 is translocated into the cytosol along with TRAF6 and TAK1, bridged by a direct BCL10-Pellino2 interaction. MALT1 interacts with BCL10 and TRAF6 to facilitate TRAF6 self-ubiquitination in the cytosol, in a process that is strictly dependent on the dissociation of BCL10 from IRAK-1. We show that BCL10 oligomerization is a prerequisite for BCL10 fulfilling its role in LPS signaling to NF-κB and that IRAK-1 dimerization is an optimal factor in this process (Fig. 12).

IRAK-1 appears to be a platform from which different regulatory signaling proteins are integrated. Many studies have revealed that different domains in IRAK-1 carry out different functions, including subpathway selection and signaling complex translocation. Our studies also show that deletion of the C1 domain of IRAK-1 completely abolishes the BCL10-IRAK-1 interaction. As described previously, two putative TRAF6-binding sites are located in the C1 domain and one in the C2 domain (15). Because deletion of either the C1 or C2 region does not affect IL-1-dependent binding to TRAF6, whereas deletion of the entire C-terminal region abolishes this binding, we believe that TRAF6 binding to IRAK-1 occurs in a random manner with comparatively low intrinsic affinity. Although the question remains as to whether one TRAF6 molecule can bind to one IRAK-1 molecule at all three putative TRAF6-binding sites, it is known that one consensus TRAF6-binding motif is sufficient to introduce one-hit-touched TRAF6 binding (11, 12). Thus, it is conceivable that under certain conditions, a cluster of at least three TRAF6 molecules binds to one IRAK-1 molecule. This may be the TRAF6 oligomerization-optimal condition for the formation of an abundant TRAF6 pool. However, these IRAK-1-TRAF6 interactions may be too random to guarantee that every TRAF6 molecule is oligomerized. Therefore, to achieve more effective activation, LPS signaling may program TIFA in membrane-bound phosphorylated IRAK-1 complexes (25, 26), MALT1 in the cytosol (8), and some other undiscovered factors to facilitate the oligomerization of non-oligomerized TRAF6 in the TRAF6 pool.

In studying the molecular basis for the BCL10-IRAK-1 inter-
action, we have discovered that IRAK-1b, which is an alternative splice variant of IRAK-1, loses the critical BCL10-binding sites as the result of naturally splicing (19). We propose that BCL10 selectively and specifically interacts with IRAK-1 but not with its splice variant. It has been reported that IRAK-1b is distinct both biologically and functionally from IRAK-1. Our findings that IRAK-1b mRNA was co-expressed with IRAK-1 mRNA and that the ratios of the cDNA products derived from both mRNAs were similar in all the tissues tested indicate that the failure to detect the BCL10-IRAK-1b interaction reflects the evolutionary complexity of innate immunity regulation.

The different features of BCL10 binding to IRAK-1 isoforms may represent a meaningful regulation model that should be studied further.

Recently, some supplementary signaling proteins have been proposed as components of the TLR signaling cascades, which include several known common adaptors and kinases such as MyD88, IRAK-1, IRAK-4, TRAF6, and Tak1 complexes (TAK1-TAB1-TAB2). These signaling proteins fulfill regulatory and non-redundant functions that lead to optimal TLR pathway activation. The Pellino proteins (23, 24, 27), RICK/Rip2 (27, 28), and TIFA (25, 26) may also belong to this group. It is striking that BCL10 and MALT1, which are critical signaling adaptors of the TCR pathway and adaptive immune system, are implicated in the TLR4 pathway and innate immune system. Several recent studies describing the phenotypes of BCL10 and MALT1 knock-out mice have reported inconsistent results in terms of responses to LPS stimulation (18). LPS signaling to NF-κB in the absence of intact BCL10 or MALT1 has been variously reported as normal or impaired (17, 29–31). This discrepancy may be because of the different mouse strains and different cell specimens used. Because the macrophage is a cell type that relies on LPS signaling for activation, we used macrophage RAW 264.7 cells, in which LPS signaling is extraordinarily active, to examine the role of BCL10 in LPS signaling to NF-κB. In these studies, we have confirmed the following: 1) the positive role of BCL10 in regulating optimal LPS-induced NF-κB activation; 2) that BCL10 is recruited to TLR4 complexes; and 3) that it co-operates with its downstream adapter, Pellino2, in transducing signals (16). In this study, we show that BCL10 is not only a regulatory element for optimal LPS signaling to NF-κB but also an important supplementary component of the classic TLR4 cascade, which includes IRAK-1, TRAF6, and Tak1. Moreover, in this study, we verify that MALT1 is a regulator of LPS/TLR4 signaling to NF-κB. Currently, we are unable to confirm that the BCL10-MALT1 cascade plays a central role, because TIFA, which is another mediator of the IRAK-1-TRAF6 interaction and activator of TRAF6 self-ubiquitination, fulfills a function that is similar to that of BCL10-MALT1 (25, 26). However, it is clear that the BCL10-MALT1 cascade represents a meaningful regulatory model. Data from recent studies indicate that TLR4 signaling is not a linear cascade but includes some essential components, including MyD88, IRAK-1, IRAK-4, TRAF6, Tak1, Tab1, and Tab2. IRAK-1-TRAF6, which is just one component of this cascade, is the regulatory target for so-called supplementary signaling proteins, which include Pellino proteins, RICK/Rip2, and TIFA. For TLR4 signaling, a particular physiological outcome would presumably require special regulators, which may differ in terms of expression pattern, sub-pathway direction or selection (i.e. the Pellino proteins), and regulatory function (i.e. TIFA as an activator of TRAF6 ubiquitination and RICK as an adaptor or kinase). Our findings on BCL10 and MALT1 reflect these complexities.

The implication of the BCL10-MALT1 cascade in innate immunity may increase the significance of the cross-talk between the adaptive and innate immune systems. This type of cross-talk may represent a long process of interdependent evolution. Until now, it has been reported that a series of signaling proteins, which include TRAF6, Tak1, Rick, and BCL10, participate in different pathways of the adaptive and innate immune systems (8, 16–18, 27–28, 32). MyD88 has been implicated in Th1 responses (33), and IRAK-1 plays an essential role in T cell priming and the development of autoimmunity (34), which suggests that the initiation of adaptive immune responses is controlled by innate immune recognition. Some traditionally accepted viewpoints propose that innate immunity represents a more primitive or antecedent system, whereas the adaptive immune system may have evolved in part from the innate immunity system. It is clear that during the process of evolution, some of the critical signaling components in innate immunity have become integrated into the adaptive immune system. However, BLAST searches do not reveal parallel isoforms of BCL10 and MALT1 in nonmammalian species. Thus, it is possible that the evolution of the adaptive and innate immune systems does not simply track in one direction. Insight into the precise regulatory systems underlying the BCL10-MALT1 cascade in adaptive and innate immunity will provide a better understanding of these processes.
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