Toll-like receptor (TLR) pathways signal through microbial components stimulation to induce innate immune responses. Herein, we demonstrate that BCL10, a critical molecule that signals between the T cell receptor and IkB kinase complexes, is involved in the innate immune system and is required for appropriate TLR4 pathway and nuclear factor-κB (NF-κB) activation. In response to lipopolysaccharide (LPS) stimulation, BCL10 was recruited to TLR4 signaling complexes and associated with Pellino2, an essential component downstream of BCL10 in the TLR4 pathway. In a BCL10-deficient macrophage cell line, LPS-induced NF-κB activation was consistently defective, whereas activator protein-1 and Elk-1 signaling was intact. In addition, we found that BCL10 was targeted by SOCS3 for negative regulation in LPS signaling. The recruitment of BCL10 to TLR4 signaling complexes was attenuated by induced expression of SOCS3 in a feedback loop. Furthermore, ectopic SOCS3 expression blocked the interaction between BCL10 and Pellino2 together with BCL10-generated NF-κB activation and inducible nitric-oxide synthase expression. Together, these data define an important role of BCL10 in the innate immune system.

The innate immune response in vertebrates is the first line of defense against infectious pathogens. In the innate immune system, different phagocytes such as neutrophils, macrophages, and dendritic cells play crucial roles in discriminating between pathogens and self by utilizing signals from the Toll-like receptors (TLRs)1 (1, 2). TLRs are phylogenetically conserved mediators that are integral to both innate and adaptive immunity. All TLRs activate a common signaling pathway that culminates in the activation of nuclear factor-κB (NF-κB) transcription factors as well as the mitogen-activated protein kinases (MAPKs) extracellular signal-regulated kinase, p38, and c-Jun N-terminal kinase (2).

Bacterial lipopolysaccharide (LPS) can elicit a multitude of effects on the immune system and is a ligand recognized by TLR4. Ligation of LPS to TLR4 results in signaling activation via downstream signaling molecules, including the adapters MyD88, interleukin (IL)-1 receptor-associated protein kinases (IRAKs), tumor necrosis factor receptor-associated factor-6 (TRAF6), and transforming growth factor-β-activated kinase-1 (TAK1) (2). Pellino2 is newly identified signaling molecule in this process. A recent study revealed that ectopic expression of a mouse pellino2 antisense construct can inhibit LPS-induced activation of NF-κB (3). Additionally, Pellino3, a homolog with high sequence similarity to Pellino2, has been found to interact simultaneously with IRAK1, TRAF6, and TAK1 and to act as a scaffolding protein that may control signaling branch-point specificity leading to different activation pathways of NF-κB and MAPK (4). Although the precise function of Pellino proteins has remained largely undefined, it is still noteworthy and plausible that these proteins have an essential role in LPS and TLR4 signaling (3–5).

SOCS (suppressor of cytokine signaling) molecules are members of a growing family that were initially described as classical feedback inhibitors of cytokine signaling pathways and have an extensive role in the negative cross-talk between cytokines and other pro-inflammatory stimuli (6–9). SOCS3 can be induced by LPS stimulation in macrophages (10). Moreover, it has been found that constitutive SOCS3 expression inhibits LPS-induced pro-inflammatory responses in J774 cells and that SOCS3 is required for the IL-10-mediated deactivation of primary peritoneal macrophages treated with LPS (11). Thus, SOCS3 may participate in LPS signaling by acting as a negative feedback regulator.

BCL10 is an intracellular NF-κB activator that was originally found to be involved in t(1;14)/p22;q32) mucosa-associated lymphoid tissue (MALT) lymphoma translocation. This rearrangement results in the juxtaposition of the entire coding region of bcl10 with the chromosome under the control of the Ig enhancer element, which leads to deregulated expression of the BCL10 protein. BCL10 contains a caspase recruitment domain that is found exclusively in factors that mediate apoptotic signaling and NF-κB activation (12, 13). It has thus been hypothesized that BCL10 might induce MALT tumorigenesis via constitutive NF-κB activation, providing both anti-apoptotic and proliferative signals by up-regulating transcription of specific targets (14).

A previous study of knockout mice with a targeted disruption of bcl10 (bcl10−/−) revealed that the mice are markedly immuno-deficient due to defective NF-κB activation via antigen receptor complexes in both B and T cells (15). A panel of BCL10-associated proteins, including CARMA1, MALT, and Rip2/RICK, have been consistently implicated in T cell receptor

---

Asterisk to indicate this fact.

This paper is available online at http://www.jbc.org
BCL10 Mediates LPS/TLR4 Signaling

IP: anti-Bcl10  +   -   +   +
Control IgG  -   +   -   +
LPS  -   +   +   +

Input

Blot: Flag

FIG. 1. BCL10 interacts with Pellino2 in response to LPS stimulation. RAW264.7 cells were transiently transfected with pcMV-FLAG-mPellino2. At 48 h after transfection, cells were either stimulated with LPS for 30 min or left untreated. Cell lysates were prepared, and anti-BCL10 or control antibody immunoprecipitates (IP) were separated by SDS-PAGE, followed by immunoblot analysis with anti-FLAG antibody. Cell lysates were also probed with anti-FLAG or anti-BCL10 antibody. All experiments for all figures were repeated at least three times with reproducible results.

IP: anti-Flag  -   +   +
Control IgG  +   +   -

Input

Blot: anti-BCL10

FIG. 2. Region 169–233 in Pellino2 is essential for BCL10 association. RAW264.7 cells were transiently transfected with FLAG-tagged N-terminal deletion mutants of mouse Pellino2. At 48 h after transfection, cells were treated with LPS for 30 min, and cell lysates were prepared. The anti-FLAG antibody immunoprecipitates (IP) were probed with anti-BCL10 antibody. Cell lysates were also probed with anti-FLAG or anti-BCL10 antibody. The control IgG immunoprecipitate was from cells transfected with Pellino2(del168).

(TCR)-mediated NF-κB activation (16–19). In addition, recent reports demonstrate that BCL10 is coprecipitated in IκB kinase (IKK) signaling complexes (20). Thus, BCL10 takes part in regulating adaptive immune responses and is essential for the signal transduction from the TCR to IKK complexes (19, 21). However, it remains unclear whether BCL10 is involved in the signaling pathways of the innate immune system.

It has been reported recently that suppression of BCL10 expression renders marginal zone B cells unable to fully activate NF-κB in response to LPS stimulation and, moreover, that BCL10-deficient mice are very susceptible to bacterial pathogens Streptococcus pneumoniae challenge and thus fail to clear bacteria from their bloodstream or to survive infection (22), which hints that BCL10 might be a component of the innate immune system. In this case, BCL10 is postulated to act in a unique pathway specific for LPS signaling in marginal zone B cells.

Here, we report that BCL10 interacts with Pellino2 and mediates NF-κB activation in response to LPS stimulation. In a mouse macrophage cell line treated with LPS, BCL10 was recruited to TLR4 signaling complexes, and SOCS3 played a role in blocking this recruitment in a classic feedback manner. Moreover, ectopic SOCS3 expression blocked the interaction between BCL10 and Pellino2. Our results demonstrate for the first time that BCL10 is an important signaling molecule that mediates signaling in the TLR4 pathway in the innate immune system.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Anti-TLR4, anti-inducible nitric-oxide synthase, and horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG antibodies were from Santa Cruz Biotechnology. Anti-FLAG antibody was from Sigma. Anti-BCL10, anti-α-actin, and anti-SOCS3 antibodies were from Pgлаб Co. Anti-hemagglutinin antibody was from Roche Diagnostics. LPS and recombinant tumor necrosis factor-α (TNF-α) was from Sigma.

Mammalian Expression Plasmids—Mouse Pellino2, TAK1, and IKKγ were from Pgлаб Co. Mouse SOCS3 and mouse BCL10 were from American Type Culture Collection. The expression constructs pCMV-FLAG-mPellino2, pcDNA3.1(+)-mBCL10, pcDNA3.1(+)-mSOCS3, and pHM-mSOCS3 were cloned in our laboratory. The cDNAs encoding the dominant-negative mutants TAK1(K63W) and IKKγ(R44M) were constructed using a QuikChange site-directed mutagenesis kit (Stratagene) and subcloned into pCMV-FLAG. All mutations were verified by DNA sequence analysis. Mutant forms of mouse Pellino2 with N-terminal 168- and 233-amino acid truncations were subcloned into the expression vector pCMV-FLAG.

Cell Culture, Stable Transfection, and Treatment—The murine macrophage cell line RAW264.7 (obtained from American Type Culture Collection) was maintained in Dulbecco’s modified Eagle’s medium (Sigma) with 10% fetal bovine serum (Sigma) with 10% fetal bovine serum (Sigma). Approximately 6 × 10⁵ cells/well were seeded on 6-well plates and transfected 24 h later with each expression vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The positive transformants were selected and cultured in the presence of 0.8 mg/ml G418 (Sigma). In all experiments, cells were stimulated with 100 ng/ml LPS or 20 ng/ml recombinant human TNF-α (final concentrations).

Immunoblotting and Immunoprecipitation—Cells were lysed with MPER™ protein extraction reagent (Pierce), and equal amounts of samples were loaded for SDS-PAGE. Immunoblotting was carried out on nitrocellulose membranes (Amersham Biosciences) and detected with ECL reagents (Pierce). We carried out immunoprecipitation using an MPER™ immunoprecipitation kit (Pierce).

Northern Blotting—Total RNA was extracted using TRizol (Invitrogen), separated on a 2.4% formaldehyde-containing 1.0% agarose gel, and blotted onto a charged nylon membrane. The membrane was hybridized with digoxigenin-labeled riboprobes and visualized by alkaline phosphatase-labeled anti-digoxigenin antibody (Roche Diagnostics). The sense digoxigenin-labeled probe was synthesized using the T7Select Biopanning kit (Novagen). The antisense digoxigenin-labeled probe was synthesized using the T7Select Biopanning kit (Novagen) according to the manufacturer’s instructions. The hybridized membranes were exposed to Kodak XAR-5 films.

RNA Interference—For the mouse BCL10 small interfering RNA (siRNA) construct, the insert was prepared by annealing two oligonucleotides derived from mouse BCL10 cDNA sequence (40–58 bp). For the mouse Pellino2 siRNA construct, the insert was prepared by annealing two oligonucleotides derived from mouse Pellino2 cDNA sequence (207–225 bp). After transfection with siUPPER constructs, puromycin (1000 ng/ml)-resistant cell clones were isolated.

T7Select Biopanning—A premade T7Select lung cDNA library (Novagen catalog no. 70646-3) and a liver tumor cDNA library (catalog no. 70647-3) were screened with the T7Select Biopanning kit (Novagen) according to the manufacturer’s instructions. The bait was bacterially expressed human BCL10 (pET28 vector, Novagen) purified by affinity chromatography. After four rounds of selection, 10 randomly selected clones were picked from each library, sequenced, and searched for
RESULTS

Identification of Pellino2 and SOCS3 as BCL10-associated Proteins by the T7Select Phage Display System—In an effort to identify components in BCL10-related signaling pathways, we performed a screen for BCL10-associated proteins using the T7Select phage display system (Novagen). Six and eight positive clones (of a total of 20 randomly selected clones) encoding sequences identical to human Pellino2 and SOCS3 were identified in the lung and liver tumor cDNA libraries, respectively.

BCL10 Interacts with Pellino2 in Response to LPS Stimulation—Pellino2 has been shown to participate in the TLR4 signaling pathways through facilitating the release of phosphorylated IRAK from the receptor and is essential for LPS-induced NF-κB activation. To further verify the interactions, we performed immunoprecipitation and confirmed that ectopic FLAG-tagged Pellino2 could interact with endogenous BCL10, which was precipitated by anti-BCL10 antibody in LPS-treated RAW264.7 cells (Fig. 1). Therefore, the potential interaction between BCL10 and Pellino2 leads to the hypothesis that BCL10 may be functionally related to the TLR4 signaling pathway, which is traditionally classified in the innate immune system.

Pellino2 Associates with BCL10 through Region 169–233—In an attempt to map the region of Pellino2 required for BCL10 binding, we utilized two N-terminal deletion mutants, Pellino2(del168) and Pellino2(del233), with deletions of the N-terminal 168 and 233 amino acids, respectively. Because data base searches for conserved functional/structural domains in Pellino proteins did not reveal any significant matches, the construction strategy for truncated forms of Pellino2 is based on the analysis of predicted exon/exon boundaries, and the positions for truncation are two splice junctions in the pellino2 gene. In the subsequent immunoprecipitation assays, BCL10 could be coprecipitated with Pellino2(del168), whereas Pellino2(del233) did not exhibit any association with BCL10 (Fig. 2), indicating that region 169–233 of Pellino2 is essential for BCL10 interaction.

Mouse Pellino2 Is Involved in NF-κB Activation Triggered by LPS Treatment or Overexpressed BCL10—To further investigate the role of Pellino2 in BCL10-dependent signaling, we stably transfected the siRNA construct pSUPER-Pellino2 into RAW264.7 cells (Fig. 3A). In Pellino2-deficient RAW264.7 cells, LPS- or overexpressed BCL10-induced NF-κB activation was reduced to a certain level compared with that in wild-type cells (Fig. 3, B and C), further suggesting a potential role for BCL10 in mediating signals from the TLR through Pellino2. Recently, some controversy has arisen over the role of Pellino2 in NF-κB activation (3, 29, 30). We also could not confirm that expression of Pellino2 would result in NF-κB activation as reported (29). However, our data obtained with Pellino2-deficient RAW264.7 cells support the view that Pellino2 is required for optimal activation of NF-κB in response to LPS stimulation or BCL10 overexpression.

BCL10-induced NF-κB Activation Is Partially Dependent on the Signals Transmitted through Pellino2 and TAK1—As widely described, BCL10 has been implicated in the transmittance of signals from antigen receptors to the IKK complex in the adaptive immune system. In RAW264.7 cells, we found that BCL10-induced NF-κB activation was moderately attenuated by cotransfection of Pellino2(del168) or a dominant-negative form of TAK1 and severely impaired by dominant-negative IKKβ expression (Fig. 4A). We also studied their effects following TNF-α stimulation to ensure the specificity (Fig. 4A). The inhibitory function of Pellino2(del168) suggests that Pellino2 is
FIG. 4. Dominant-negative TAK1, dominant-negative IKKβ, and Pellino2(del168) impair BCL10-dependent NF-κB activation. A, RAW264.7 cells were transfected with 900 ng of pNF-κB luciferase reporter, 100 ng of pRL-TK Renilla reporter, and 1000 ng of pcDNA3.1(-mBCL10) in combination with increasing amounts of pCMV-FLAG-Pellino2(del168), pCMV-FLAG-DN-TAK1, or pCMV-FLAG-DN-IKKβ (500 and 1000 ng) and decreasing amounts of pCMV-FLAG (1000 and 500 ng). At 36 h after transfection, luciferase reporter assays were carried out. The -fold induction is relative to that in cells transfected with 1000 ng of reporter DNA, 1000 ng of pcDNA3.1(-mBCL10), and 1000 ng of pCMV-FLAG. Cell lysates were also probed with anti-FLAG antibody. As a control, RAW264.7 cells were transfected with 900 ng of pNF-κB luciferase reporter and 100 ng of pRL-TK Renilla reporter in combination with increasing amounts of pCMV-FLAG-Pellino2(del168), pCMV-FLAG-DN-TAK1, or pCMV-FLAG-DN-IKKβ (500 and 1000 ng) and decreasing amounts of pCMV-FLAG (1000 and 500 ng). At 30 h after transfection, the cells were incubated with TNF-α for 6 h, and luciferase reporter assays were carried out. The -fold induction is relative to that in cells transfected with 1000 ng of reporter DNA, 1000 ng of pcDNA3.1(-mBCL10), and 1000 ng of pCMV-FLAG.

FIG. 5. Defective NF-κB activation in RAW264.7 cells upon expression of BCL10 siRNA. A, lysates from RAW264.7 cells stably transfected with pSUPER-BCL10 or wild-type RAW264.7 cells were immunoblotted with anti-BCL10 antibody. B, 900 ng of pNF-κB luciferase reporter and 100 ng of pRL-TK Renilla reporter were transfected into wild-type (WT) or BCL10-deficient RAW264.7 cells. At 36 h after transfection, the cells were stimulated with LPS for 30 min, and luciferase reporter assays were carried out. Under the same conditions and at 30 h after transfection, the cells were incubated with TNF-α for 6 h, and luciferase reporter assays were performed to ensure specificity. The -fold induction is relative to that in cells (wild-type or BCL10-deficient) transfected with 1000 ng of reporter DNA without LPS or TNF-α treatment. C, 50 ng of pFA2-c-Jun or pFA2-Elk-1 and 500 ng of pFR-Luc were cotransfected into wild-type or BCL10-deficient RAW264.7 cells. The pRL-TK Renilla reporter (100 ng) was included to normalize the transfection efficiency. Thirty-six hours post-transfection, the cells were stimulated with LPS for 30 min, and luciferase reporter assays were carried out. The -fold induction is relative to that in cells (wild-type or BCL10-deficient) transfected with reporter DNA without LPS treatment.
BCL10 Mediates LPS/TLR4 Signaling

**Fig. 6.** BCL10 is recruited to the TLR4 signaling complex after LPS stimulation. Wild-type or Pellino2-deficient RAW264.7 cells were stimulated with LPS for 30 min. Cell lysates were immunoprecipitated (IP) with anti-BCL10 or control antibody and immunoblotted with anti-TLR4 antibody. Cell lysates were also probed with anti-BCL10 or anti-TLR4 antibody.

| Treatment | wild type | Pellino2 deficient |
|-----------|-----------|--------------------|
| IP: anti-BCL10 | + + - + + + | + + - + |
| Control IgG | - - + - - - | - - + - |
| LPS | - + + - + + | - + + + |

**Fig. 7.** BCL10 associates with SOCS3. RAW264.7 cells were transiently transfected with pHM-mSOCS3. At 36 h after transfection, cells were either left untreated or stimulated with LPS for 30 min. Anti-BCL10 and control antibody immunoprecipitates (IP) were separated by SDS-PAGE, followed by immunoblot analysis with anti-hemagglutinin (HA) antibody. Cell lysates were also probed with anti-hemagglutinin or anti-BCL10 antibody.

| Treatment | wild type | Pellino2 deficient |
|-----------|-----------|--------------------|
| IP: anti-BCL10 | + + - + | + + - + |
| Control IgG | - - + - | - - + - |
| LPS | - + + - | - + + + |

TLR4 signaling complex after LPS stimulation indicates that BCL10 also plays an essential role in NF-

**TLR4 Signaling**

- Not Involved in Activator Protein-1 (AP-1) and Elk-1 Signaling Pathways after LPS Stimulation—We used siRNA technology to verify the functional role of BCL10 in LPS signaling pathways. In the RAW264.7 mouse macrophage cell line stably transfected with the siRNA construct pSUPER-BCL10 (Fig. 4A), deficiency in BCL10 expression caused moderate reduction of NF-κB activation in response to LPS stimulation, whereas NF-κB activation triggered by TNF-α treatment was similar in both wild-type and BCL10-deficient RAW264.7 cells (Fig. 5B), which directly gives rise to the possibility that BCL10 might specifically signal downstream of TLR4. Pellino2 has been found to activate the AP-1 and Elk-1 pathways, and similarly, stimulation of TLRs by specific ligands also induces the activation of a set of MAPKs (29). However, we found that activation of the AP-1 and Elk-1 signaling pathways LPS stimulation was equivalent in both wild-type and BCL10-deficient RAW264.7 cells (Fig. 5C), indicating that the defect in BCL10 expression is specific for NF-κB signaling downstream of the TLR, but not for other parallel pathways initiated by LPS stimulation. Furthermore, even if Pellino2 is essential for activation of AP-1 and Elk-1, BCL10 seems to be an important adaptor that directs the signal specifically toward activation of NF-κB. In these processes, Pellino2 may act as a scaffolding protein that integrates the signal from BCL10 and ultimately controls signaling branch-point specificity.

**BCL10 Mediates LPS/TLR4 Signaling**

In the subsequent work to address the molecular mechanism underlying the role of BCL10 in TLR4 signaling, we investigated whether BCL10 is recruited to the TLR4 signaling complex in response to ligand stimulation. Intriguingly, we found that endogenous TLR4 was co-immunoprecipitated with BCL10 after LPS stimulation (Fig. 6), indicating that BCL10 may specifically participate in TLR4-mediated signaling. To examine whether Pellino2 is required for the recruitment of BCL10 to the TLR4 signaling complex, we analyzed endogenous TLR4 in the anti-BCL10 antibody-precipitated complex from Pellino2-deficient cells. The results demonstrated that defects in Pellino2 expression had no effect on the recruitment of BCL10 to the TLR4 signaling complex (Fig. 6). These data confirm the speculation that Pellino2 is an adapter downstream of BCL10 in the LPS signaling pathway. The detailed mechanism underlying recruitment of BCL10 to the TLR4 signaling complex should still be investigated.

**SOCS3 Interacts with BCL10 and Negatively Regulates BCL10-induced NF-κB Activation and Inducible Nitric-oxide Synthase Expression**—The SOCS family plays an important role in regulating the responses of immune cells to different cytokines. SOCS1 proteins have recently been recognized as having an extensive role in the negative cross-talk between cytokines and other pro-inflammatory stimuli (6–9). SOCS3 was found to block the signaling pathway in LPS-dependent...
NO synthesis and LPS-induced expression of IL-6 mRNA. To investigate the detailed mechanism underlying the function of SOCS3 in the LPS signaling pathway and to examine whether BCL10 may be a regulatory target of SOCS3, we carried out immunoprecipitation to study the in vivo binding activity of BCL10 with SOCS3. Consistent with the results obtained in the phage display screening, we observed that overexpressed SOCS3 could associate with endogenous BCL10 in RAW264.7 cells stimulated with LPS or left untreated (Fig. 7). To further confirm this, a RAW264.7 cell line stably expressing SOCS3 was constructed, and forced expression of SOCS3 was found to result in attenuation of BCL10-induced NF-κB activation and inducible nitric-oxide synthase expression (Fig. 8), indicating that BCL10 may be the targeted molecule of SOCS3 for negative regulation in LPS signaling.

Interaction between Pellino2 and BCL10 Is Blocked by SOCS3 Expression—To examine whether SOCS3 exerts a regulatory function on the interaction between BCL10 and Pellino2 and whether SOCS3 associates with Pellino2 directly or as mediated by BCL10, we transfected the FLAG-tagged Pellino2 expression construct into RAW264.7 cells stably expressing SOCS3. Upon forced expression of SOCS3, the association between BCL10 and Pellino2 was severely impaired, whereas BCL10 interacted with more SOCS3 proteins compared with wild-type RAW264.7 cells (Fig. 9A). However, Pellino2 was not detected in the anti-SOCS3 antibody-precipitated complex, and SOCS3 was not detected in the anti-FLAG antibody-precipitated complex (Fig. 9B), demonstrating that there is no association between SOCS3 and FLAG-tagged Pellino2. SOCS3 may negatively regulate BCL10 function by reducing its ability to interact with Pellino2.

Recruitment of BCL10 to the TLR4 Signaling Complex Is Inhibited by SOCS3 in a Feedback Loop—Because our data indicated that Pellino2 is a scaffold protein downstream of BCL10, it was necessary to investigate whether SOCS3 could also prevent BCL10 from receiving upstream signals by inhibiting the recruitment of BCL10 to the TLR4 signaling complex (Fig. 10). Intriguingly, forced expression of SOCS3 completely abolished the recruitment of BCL10 to the TLR4 signaling complex. Together, these data demonstrate that SOCS3 negatively regulates LPS signaling by facilitating seclusion of BCL10 to the receptor complex and subsequently blocks the signal transmitted through BCL10. It is very interesting that SOCS3 could also block the association between BCL10 and its downstream adaptor Pellino2, which suggests that BCL10 binding to SOCS3 may result in a complete loss of function in the LPS signaling pathway.

To examine whether the recruitment of endogenous BCL10 to the TLR4 signaling complex is time-dependent and negatively regulated by endogenous SOCS3, we stimulated RAW264.7 cells with LPS and used anti-BCL10 antibody to precipitate the BCL10 signaling complex. Endogenous TLR4 in the precipitated signaling complex was immunoblotted with anti-TLR4 antibody, and its amount peaked at 30 min after stimulation, decreased thereafter, and declined to the lowest point after 60 min. Time kinetics revealed that the association between SOCS3 and BCL10 emerged at 30 min, peaked at 120 min, and declined thereafter. Because LPS-induced expression of SOCS3 increased before 120 min after LPS stimulation, the association between SOCS3 and BCL10 was evidently LPS-stimulation-independent, but SOCS3 expression-dependent (Fig. 11).

**DISCUSSION**

Wang et al. (22) have indicated a role for TLRs in BCL10-dependent NF-κB activation in response to LPS stimulation in BCL10-deficient mice. They found that BCL10 seems to act in a unique pathway specific for LPS signaling in marginal zone B cells, but not in follicular B cells (22). It has been shown that LPS stimulates a strong proliferation response in marginal zone B cells through TLRs and a weak proliferation response in follicular B cells (22–24). Thus, it is plausible that the signaling mechanism underlying LPS stimulation in marginal zone B cells may be BCL10- and TLR-dependent.

We have shown that BCL10 appears to participate consistently in a TLR4-mediated NF-κB activation pathway in response to LPS stimulation. Our results indicate that the absence of BCL10 in macrophages would impinge on pathogen-associated molecular pattern-induced NF-κB activation, which implies an important role for BCL10 in the innate immune system. BCL10 is widely described as a caspase recruitment domain-containing NF-κB activator and has been implicated in the signaling from antigen receptors including the TCR to IKK complexes (12, 13, 19, 21). Our findings define for the first time a role for BCL10 in the innate immune system in addition to the adaptive immune system as described elsewhere (15, 19, 21). It is striking that Rip2/RICK, a caspase recruitment domain-containing serine/threonine kinase, has been found to associate with BCL10 following TCR engagement and to induce its phosphorylation (19). Rip2 has also been implicated in innate immune responses as a signal transducer recruited to TLR4 signaling complexes after LPS stimulation (25, 26). Parallel to these works, our findings may provide some new insight into their activities in the innate immune system. Because

**FIG. 8.** BCL10-generated NF-κB activation and inducible nitric-oxide synthase expression are inhibited by overexpression of SOCS3. RAW264.7 cells were stably transfected with pcDNA3.1(+)-mSOCS3 and screened for clones stably expressing ectopic SOCS3 in the presence of G418. Wild-type (WT) and SOCS3-overexpressing RAW264.7 cells were cotransfected with 900 ng of pNF-κB luciferase reporter, 100 ng of pRL-TK Renilla reporter, and increasing amounts of pHM-NBCL10 (500 and 1000 ng) and decreasing amounts of pHM6 (1000 and 500 ng). At 36 h after transfection, luciferase reporter assays were carried out. The fold induction is relative to that in cells transfected with 1000 ng of reporter DNA and 1000 ng of pHM6. Cell lysates were also probed with anti-hemagglutinin (HA) or anti-inducible nitric-oxide synthase (iNOS) antibody.
Rip2 associates with TRAF6 in innate immune responses and can be recruited to TLR4 signaling complexes after ligand stimulation (19, 27, 28), an appealing model suggests that BCL10 and Pellino2, which also bind TRAF6 (3–5), are collectively engaged in this receptor signaling complex. Alternatively, the signal may be transmitted from the receptor to TRAF6 and Pellino2 following the engagement of Rip2 and BCL10 in TLR4 signaling complexes. Further study is underway to define the relationship between BCL10 and Rip2 in the TLR4 signaling pathway.

The role of mammalian Pellino proteins, including Pellino1–3, which are homologs of the Pellino protein first identified in Drosophila, remains somewhat unclear (2–5). In general, Pellino proteins are presumed to play an important role in establishing and maintaining the specificity of TLR signaling subpathways (2). Despite remarkable degrees of conservation in human Pellino1–3, they exhibit distinct tissue distribution patterns, and pellino2 transcripts seem to be distributed during embryogenesis but in a more tissue-restricted manner in the adult (3). Moreover, although all three human Pellino proteins exert their functions by interacting with phosphorylated IRAK signaling complexes, they seem to distinctly direct the signaling subpathway components to different NF-κB activation levels (3–5). One representative opinion is that Pellino proteins may act as scaffold molecules by introducing a third dimension to the TLR signaling cascade to generate large multiprotein complexes, which permit particular signaling components such as BCL10 to regulate NF-κB signaling cascades (4). Strikingly, Pellino proteins can interact directly with TAK1 signaling complexes (4, 5). It has been shown that Pellino1 can activate NF-κB in IRAK-deficient cells and is suspected to interact with TAK1, which may act as a regulatory kinase of IKKs (5). Pellino3 can directly associate with TAK1, subsequently promoting the dissociation of active forms of IKKα and IKKβ from active TAK1 and leading to the activation of IKK complexes (4). It should be addressed that BCL10 was ever suspected to interact directly or indirectly with IKK complexes and to be critical in modulating its activity. Moreover,
BCL10 has been shown to be essential to appropriate TCR signaling through the IKK complexes. Thus, it is quite possible that BCL10 engages in an interaction pattern involving Pellino2 and TAK1 to regulate IKK activity. Although the detailed mechanism is still unclear, our results with dominant-negative mutants of Pellino2 and TAK1 at least define their crucial roles in BCL10-mediated NF-\(\kappa\)B activation.

At present, some controversy has arisen over the role of Pellino2 as a scaffolding protein that differentially supports the signaling subpathways. Although ectopic murine Pellino2 expression was shown not to result in NF-\(\kappa\)B-dependent reporter gene activation, reduction in murine Pellino2 expression leads to IL-1- or LPS-induced activation of NF-\(\kappa\)B-dependent IL-8 promoter activity (3). Moreover, human Pellino2 was observed to activate the MAPK pathway leading to AP-1 and Elk-1 activation (29). Thus, Pellino2 seems to direct and commit the signaling complex to facilitate the engagement of both the NF-\(\kappa\)B and MAPK pathways. However, on the other hand, suppression of endogenous human Pellino2 expression levels in human 293 and KB cells by the RNA interference approach was shown to have no significant effects on IL-1-induced IL-8 production or reporter gene activation (30). In addition, human Pellino2 has been suggested that overexpression of human Pellino2 has no effect on MAPK and NF-\(\kappa\)B activation, whereas Pellino2 overexpression increases the luciferase activity in most reporter assays, independent of specific transcription factor-binding sites (30). If the differences in the experimental procedures, species, and cell types are not taken into consideration, the observations in these studies are obviously contradictory, and it will be very difficult to explain these theoretical inconsistencies. The results from our ongoing study on murine Pellino2 indicate that at least murine Pellino2 is essential for LPS-induced NF-\(\kappa\)B activation and BCL10-dependent signaling in the TLR4 pathway in RAW264.7 cells. The different observations might be a result of cell type-specific functions of Pellino2. The murine pellino2 transcripts were shown to be abundant in the tissues that predominate in innate immune responses; and notably, these responses may be quite active in the macrophage, which is one of the most important components of the innate immune system.

Given that Pellino2 plays a crucial role in activation of the MAPK pathway, the findings in BCL10-deficient RAW264.7 cells further demonstrate that Pellino members might function as scaffold proteins that regulate signaling subpathway selection in TLR signaling cascades (4, 29). Because a defect in BCL10 expression resulted only in attenuation of NF-\(\kappa\)B activation, whereas AP-1 and Elk-1 activation was intact, it becomes more plausible that Pellino proteins might function in the TLR pathway as signal receivers and branch points that integrate specific signals carrying different information from upstream adaptors such as BCL10 for subpathway selection and separately transmit these signals to targeted ends in the NF-\(\kappa\)B or MAPK signaling cascades. Because of the complicated and delicate role of Pellino proteins, any deficiency in their expression might result in an alteration in the activation level of NF-\(\kappa\)B or MAPK. On the other hand, the increased expression of Pellino proteins would force the equilibrium toward complex formation even in the absence of an upstream signaling event. However, in both instances, especially in the latter one, downstream signaling activation is more dependent on the different expression patterns of components in Pellino-associated signaling complexes in specific cell types. These Pellino-associated molecules may function as the actual factors that limit the pace for subpathway activation, so forced expression of only Pellino2 hardly causes obvious increases in NF-\(\kappa\)B activation in cells expressing low level of these pace-limiting proteins. If these considerations are possible, they might reconcile some conflicts in the present results obtained with Pellino. There is a high degree of conservation among the three Pellino molecules (Pellino1–3) from human and mouse, so at least it is an abnormal phenomenon that they function in such distinct ways, especially human Pellino1 and Pellino2, which
share 81% identity and 90% homology (30). Our data indicate that defects in mouse Pellino2 expression in RAW264.7 cells would cause attenuation of NF-κB activation, which is consistent with the results on human Pellino1 deficiency (5). Because the studies on Pellino function were carried out basically at the forced expression (positive or negative) level, it is anticipated that studies with pellino knockout mice might accurately and minutely interpret the biological significance of Pellino proteins. Moreover, it would also be very interesting to investigate whether there is a molecularly redundant but physiologically reciprocal interaction between BCL10 and Pellino1, which has a distinct but somewhat reciprocal expression pattern in the different tissues compared with that of Pellino2.

The SOCS family members emerge as key physiological regulators of cytokine responses by inhibiting the JAK-STAT (signal transducer and activator of transcription) pathway. In recent years, SOCS proteins have subsequently been recognized as possessing an extensive role in the negative cross-talk between cytokines and other stimuli, including pro-inflammatory stimulation and TCR engagement (31). Recent studies indicate that SOCS3 expression could be induced by the exposure of cells to CpG DNA or LPS. Moreover, SOCS3 was shown to play an important role in the antagonistic effect of IL-10 on LPS-induced macrophage activation (11). Consistent with these findings, our data suggest that SOCS3 directly down-modulates LPS signaling through inhibition of both recruitment of BCL10 to TLR4 signaling complexes and interaction between BCL10 and Pellino2. Because of the increasing interaction between SOCS3 and BCL10 in response to LPS-attenuated SOCS3 expression, the amount of dissociated or free BCL10 molecules in the cellular protein pool might be greatly attenuated, which breaks the balance between formation and disso-

**REFERENCES**

1. Barton, G. M., and Medzhitov, R. (2003) Science 300, 1524–1525
2. Akira, S. (2003) J. Biol. Chem. 278, 38165–38168
3. Yu, K. Y., Kwon, H. J., Norman, D. A. M., Vig, E., Goebel, M. G., and Harrington, M. A. (2002) J. Immunol. 169, 4075–4078
4. Jensen, L. E., and Whitehead, A. S. (2002) J. Immunol. 171, 1500–1506
5. Jiang, Z., Johnson, H. J., Nie, H., Qin, J., Bird, T. A., and Li, X. (2003) J. Biol. Chem. 278, 10952–10956
6. Losman, J. A., Chen, X. P., Hilton, D. J., and Rothman, P. (1997) Nature 387, 917–921
7. Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsuji, K., Matsumoto, A., Tanizawa, S., Ohtsubo, M., Misawa, H., Miyazaki, T., Leonor, N., Tanguichi, T., Fujita, T., Kanakuya, Y., Konishi, Y., and Yoshimura, A. (1997) Nature 387, 921–924
8. Inohara, N., Fujikura, T., Vassalli, D., Haneke, H., Yokoyama, H., Tsuzuki, A., Yuki, K., and Akira, S. (1998) Immunity 17, 583–591
9. Zhuo, J., Zou, B., Tong, Y., Xin, Z., Lian, G., and Cheng, G. (2001) J. Biol. Chem. 276, 7082–7089
10. Steinberg, D., Kowarik, P., Cohnhey, S., Johnston, J. A., Steinlein, P., and Decker, T. (1999) J. Immunol. 163, 2640–2647
11. Berlato, C., Casattella, M. A., Kinjo, I., Gatto, L., Yoshimura, A., and Bazzoni, F. (2002) J. Immunol. 168, 6543–6541
12. Willis, T. G., Jadayel, D. M., Du, M. Q., Peng, H., Perry, A. R., Abdul, R. M., Price, H., Karrar, L., Majekodunmi, O., Wiodarska, I., Pan, L., Crook, T., Hamoudi, R., Isaacson, P. G., and Dyer, M. J. (1999) Cell 96, 35–45
13. Zhang, Q., Siebert, R., Yan, M., Hinzmann, B., Cui, X., Xue, L., Bakekast, K. M., Naeve, C. W., Beckmann, G., Weissenberg, D. D., Sanger, W. G., Nowatzy, H., Vosley, M., Callet-Bauchet, E., Sallos, G., Dixit, V. M., Rosenthal, A., Schlegelberger, B., and Morris, S. W. (1999) Nat. Genet. 22, 63–68
14. Cevoll, F., Isaacson, P. G., Gascayne, R. D., and Zucca, E. (2001) Hematology (N.Y.) 1, 241–258
15. Ruland, J., Duncan, G. S., Elia, A., del Barco Barrantes, I., Nguyen, L., Pyte, S., Miller, D. G., Bouchard, D., Wakeham, A., Ohashi, P. S., and Mak, T. W. (2001) Cell 104, 33–42
16. Bertin, J., Wang, L., Guo, Y., Jacobson, D. M., Poyet, J. L., Srinivasula, S. M., Merriam, S., DiStefano, P. S., and Alnemri, E. S. (2001) J. Biol. Chem. 276, 11877–11882
17. Thome, M., and Tschopp, J. (2003) Trends Immunol. 24, 419–424
18. Lucas, P. C., Yonezumi, M., Inohara, N., McAllister-Lucas, L. M., Abazaed, M. E., Chen, F. F., Yamaoka, S., Sato, M., and Nunez, G. (2001) J. Biol. Chem. 276, 19012–19019
19. Eozyi-Brassette, A. A., Lee, W. P., Hurst, S., and Dixit, V. M. (2004) J. Biol. Chem. 279, 1570–1574
20. Poyert, J. L., Srinivasula, S. M., and Alnemri, E. S. (2001) J. Biol. Chem. 276, 3183–3187
21. McAllister-Lucas, L. M., Inohara, N., Lucas, P. C., Ruland, J., Benito, A., Li, Q., Chen, S., Chen, F. F., Yamaoka, S., Verma, I. M., Mak, T. W., and Nunez, G. (2001) J. Biol. Chem. 276, 30589–30597
22. Xue, L., Morris, S. W., Ornahiela, C., Tuomanen, E., Cui, X., Wen, R., and Wang, T. (1999) J. Biol. Chem. 274, 22051–22058
23. Martin, F., and Kearney, J. F. (2002) Nat. Rev. Immunol. 2, 323–335
24. Chen, A. I., Dixit, V. M., Bruhn, K., Miller, J. F., Xu, Y., and Cheng, G. (2002) J. Biol. Chem. 276, 19012–19019
25. Kobayashi, K., Inohara, N., Hernandez, L. D., Galan, J. E., Nunez, G., Jane-
way, C. A., Medzhitov, R., and Flavell, R. A. (2002) Nature 416, 194–199
26. Lomaga, M. A., Yeh, W. C., Saroni, I., Duncan, G. S., Furlonger, C., Ho, A., Morony, S., Capparelli, C., Van, G., Kaufman, S., van der Heiden, A., Irie, A., Wakeham, A., Kho, W., Sasaki, T., Cao, Z., Penninger, J. M., Paige, C. J., Lacey, D. L., Dunstan, C. R., Boyle, W. J., Goeddel, D. V., and Mak, T. W. (1999) J. Biol. Chem. 274, 10151–10158
27. Mak, T. W., and Yeh, W. C. (2002) Nature 418, 835–836
28. Jensen, L. E., and Whitehead, A. S. (2003) FEBS Lett. 545, 199–202
29. Streit, A., Kellewe, C., and Nosse, H. (2003) FEBS Lett. 547, 157–161
30. Kubo, M., Hanada T., and Yoshimura, A. (2003) Nat. Immunol. 4, 1169–1176
31. Kamizono, S., Hanada, T., Yasukawa, H., Minoguchi, S., Kato, R., Minoguchi, M., Hattori, K., Morita, S., Kitamura, T., Kato, H., Nakayama, K., and Yoshimura, A. (2001) J. Biol. Chem. 276, 12530–12538
32. Wetherspoon, A. C., Dogan, A., and Du, M. Q. (2002) Curr. Opin. Hematol. 9, 50–55