**FLN29, a Novel Interferon- and LPS-inducible Gene Acting as a Negative Regulator of Toll-like Receptor Signaling**

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**Lipopolysaccharide (LPS) activates macrophages through toll-like receptor (TLR) 4.** Although the mechanism of the TLR signaling pathway has been well documented, the mechanism of the negative regulation in response to LPS, particularly LPS tolerance, is still poorly understood. In this study we identified and characterized a novel interferon- and LPS-inducible gene, FLN29, which contains a TRAF6-related zinc finger motif and TRAF family member-associated NF-κB activator-related sequences. The induction of FLN29 was dependent on STAT1. The forced expression of FLN29 in macrophage-like RAW cells resulted in the suppression of TLR-mediated NF-κB and mitogen-activated protein kinase activation, while a reduced expression of FLN29 by small interfering RNA partly cancelled the down-regulation of LPS signaling. Furthermore, we demonstrated that NF-κB activation induced by TRAF6 and TAB2 was impaired by co-expression of FLN29, suggesting FLN29 may regulate the downstream of TRAF6. Taken together, FLN29 is a new negative feedback regulator of TLR signaling.

Lipopolysaccharide (LPS) induces inflammatory responses through toll-like receptor (TLR) 4 in macrophages (1, 2). Among these responses, the production of inflammatory mediators such as tumor necrosis factor α (TNFα) and interleukin (IL)-6, as well as reactive oxygen species such as nitric oxide (NO), play important roles in protecting the host against bacterial infection. TLR-mediated signaling enhances the ability of dendritic cells to present antigen to T cells, thus leading to the activation of adaptive immune responses (3). Furthermore, LPS also causes B cells to accelerate their expansion and enhances the expression of co-stimulatory molecules such as CD80 as well as CD86 (4).

LPS-mediated TLR signaling requires the binding of LPS to TLR4-MD2 complex in the presence of CD14 as costactors (5). The binding of ligand to TLRs results in the recruitment of adaptor proteins such as myeloid differentiation 88 (MyD88) (6–8) and Toll/IL-1 receptor (TIR) domain-containing adaptor-inducing interferon β (TRIF) (9, 10) through the TIR domain in the receptor. Subsequently, MyD88 signals through association with IL-1R-associated kinase (IRAK) via the death domain in these proteins (11). Among four species of the known IRAK family, IRAK-1, IRAK-2, IRAK-4, and IRAK-M, only IRAK-1 and IRAK-4 have kinase activity; IRAK-M functions as a negative regulator acting as a dominant negative version of IRAK-1 (reviewed in Ref. 12).

IRAK-1 binds to TNF-receptor-associated factor (TRAF) 6 through P-X-E-X-X-(Ar/As) motifs in IRAK and the coiled-coil domain of TRAF6 (13), thus leading to the oligomerization of TRAF6 followed by the ubiquitination of TRAF6 for the activation of TGF-β-activating kinase 1 (TAK1). TRAF6 contains the RING finger domain, a zinc finger domain having E3 ligase activity. Upon stimulation, TRAF6 ubiquitinates itself in the presence of E1, Mms2, and Ubc13 in a lysine 63-linked manner (14). The polymerized ubiquitin on TRAF6 is regarded as a binding target of cuel1-homologous domain and the zinc finger domain of TGF-β-activating kinase 1-binding protein 2 (TAB2), which induces the formation of a complex with TGF-β-activating kinase 1 (TAK1) and TGF-β-activating kinase 1-binding protein (TAB1) (15). Subsequently, TGF-β-activating kinase 1 activates IKKβ by phosphorylating serine 181; IKKβ is a kinase that forms a complex with IKKε and NF-κB essential modulator. Next, the activated IKKβ phosphorylates the inhibitor of NF-κB α (IκBα) at serines 32 and 36 (16). The phosphorylated IκBα undergoes degradation by a ubiquitin-mediated process (2).

Finally, NF-κB, a transcription factor comprising p65 and p50, translocates from the cytoplasm to the nucleus to initiate the transcription of such proinflammatory genes as TNFα, IL-1β, and inducible NO synthase in macrophages.

The activation of macrophages must be strictly regulated by a variety of negative regulators, because the hyperactivation of macrophages is usually harmful, even sometimes inducing fatal septic shock. For example, LPS tolerance, a transient hyporesponsiveness of bodies as well as macrophages to the second LPS challenge after first LPS treatment, has been well described; however, the mechanism of tolerance is still poorly understood (17, 18). Recent studies on the molecular mechanism imply that multiple molecules may be involved in this event. For example, at the receptor level, single immunoglobulin IL-1R-related molecule (19) and ST2 (20) act as the dominant-negative form of TLR4 to modulate externally mediated signaling from pathogens. The short form of MyD88 blocks NF-κB signaling by competitively binding to TLR4 instead of to MyD88 that makes signals weaker (8). IRAK-M, a homolog of IRAK that is induced by LPS after 24 h, alternatively interacts with MyD88 to negatively regulate the signaling to TRAF6 (21). The suppressor of cytokine signaling 1 (SOCS1) and SHIP, which contain a phos...
phorylated tyrosine residue-interacting domain, namely Src homology 2 domain, have also been shown to be involved in LPS tolerance (22). IL-10, a well known anti-inflammatory cytokine, also suppresses the TLR signaling; however, the precise molecular mechanism has not yet been clarified. Despite the accumulation of negatively regulating molecules in TLR signaling acting in the membranous region, the cytosolic factor(s) that act as negative regulators remain uncertain.

We herein report a novel interferon- and LPS-inducible gene, FLN29, which attenuates TLR-mediated NF-κB signaling by interacting with TRAF6 in macrophages. When FLN29 is stably overexpressed in macrophage-like RAW cells, we found the NF-κB activation and the production of TNFα and NO to be significantly impaired. In contrast, LPS-induced tolerance in vitro was significantly disrupted in FLN29 siRNA cells. Biochemical assays, including luciferase and protein interaction assays, revealed that FLN29 binds to TRAF6 and attenuates NF-κB signaling.

**EXPERIMENTAL PROCEDURES**

Reagents and Antibodies—LPS from *Escherichia coli* (L-4005, O55: B5), poly(I:C), zymosan (Z-4250), and anti-FLAG (M2) antibody were purchased from Sigma. IFNγ, IL-1β, and TNFα were purchased from Peprotech (Rocky Hill, NJ). CpG-DNA has been previously described (22). Anti-phospho-ERK (9106), anti-phospho-c-Jun NH2-terminal kinase (JNK) (9255), anti-phospho-p38 (9216), anti-JNK (9252), anti-p38 (9212), anti-phospho-IkBα (9246), anti-IκBα (9242) antibodies were from Cell Signaling Technology (Beverly, MA). Anti-ERK (C-14), anti-TRAF6 (H-274), anti-NF-κB p65 (C-20), and anti-STAT5 (C-17) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-labeled antibodies for rabbit IgG and mouse IgG were from Jackson ImmunoResearch Laboratory (Bar Harbor, ME).

Microarray Analysis—The RNA samples from monocytic M1 cells treated with or without IFNγ + LPS were subjected to microarray analysis as described (23).

Cell Culture—RAW and 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin in humidified 5% CO2 at 37 °C in the presence and absence of non-essential amino acids, respectively. 293T cells stably expressing TLR4, MD2, and CD14 were prepared into primary RAW cells for mFLN29 and were treated with or without IFNγ/H9251. The full-length FLN29 and mFLN29 were amplified by PCR using cDNA from 293T and were sequenced to verify the sequences. The following hairpin inserts for psiRNA expression vector pGEX-4T3 (Amersham Biosciences) and transformed into E. coli strain BL21. GST and GST fusion proteins were expressed and purified by affinity chromatography. Anti-FLN29 antibody was raised against rabbits by immunizing a full-length protein of bacterially expressed recombinant hFLN29-GST. The protocol was essentially the same as that previously reported (27).

Microscopy—Immunofluorescence microscopy was performed as described previously (26). In brief, cells were fixed with 3.7% formaldehyde, followed by staining with primary antibody (1:100) for 1 h at room temperature. In addition, secondary antibodies with fluorescent dye Alexa488 and Alexa546 were reacted for 1 h at room temperature. Images were recorded by immunofluorescence microscopy (AxioPlan 2; Zeiss).

**RESULTS**

Isolation of a Novel TRAF6- and TANK-related Gene—To identify the LPS-inducible gene that potentially modulates the cellular function of monocytes/macrophages, we performed a microarray analysis in monocytic M1 cells. Among the many genes induced by LPS, we noticed the Fln29 gene (Fig. 1A) because its protein product contains a TRAF6-related zinc finger domain and a TANK-related domain (Fig. 1B). Human and mouse (m) FLN29 proteins contain 582 and 576 amino acids, respectively (Fig. 1A). The zinc finger domain of mFLN29 (residues 13–107) has a 27% identity and 52% similarity of the amino acid sequence to that of mouse TRAF6 (residues 134–232) (Fig. 1B).

STAT1-dependent Induction of FLN29—We further investigated the regulation of FLN29 protein expression using a specific antibody raised against recombinant hFLN29 that was fused to GST. We found that the treatment with not only LPS but also IFNβ and IFNγ increased the
FIGURE 1. Amino acid sequence of FLN29 and its expression profile. A, amino acid sequences of human and mouse FLN29 proteins. B, schematic representation of the similarity of mouse FLN29 to mouse TRAF6 and mouse TANK in amino acids. C, accumulation of FLN29 protein in the RAW cells induced by IFNγ (10 ng/ml), LPS (100 ng/ml), IFNγ (10 ng/ml) + LPS (100 ng/ml), IFNγ (10 ng/ml) + LPS (100 ng/ml), IFNγ (10 ng/ml) + LPS (100 ng/ml), IFNγ (10 ng/ml), and leukemia-inducible factor (LIF) (10 ng/ml). The cells were treated with the above cytokines for the indicated periods of time, and the accumulation of FLN29 protein and of phosphorylated STAT1, STAT1, phosphorylated STAT3, and STAT3 was examined. Rabbit polyclonal antibody against human FLN29 protein was used. D, the accumulation of FLN29 protein in the mouse peritoneal macrophages induced by IFNγ (10 ng/ml), LPS (100 ng/ml), IFNγ (10 ng/ml) + LPS (100 ng/ml), IFNγ (10 ng/ml), and LPS (100 ng/ml). The peritoneal macrophages were obtained after induction with thioglycolate for 3 days. The cells were treated with the above cytokines for the indicated periods of time, and the accumulation of FLN29 protein and of phosphorylated STAT1, STAT1, phosphorylated STAT3, and STAT3 was examined. E, the STAT1-dependent induction of FLN29 protein in bone marrow-derived macrophages. Bone marrow-derived macrophages from STAT1 knock-out (STAT1−/−) mice were stimulated with 100 ng/ml LPS (top) and 10 ng/ml IFNγ (bottom), and the changes in FLN29 protein were examined by Western blotting. F, accumulation of FLN29 mRNA in RAW cells induced by 10 ng/ml IFNγ, 100 ng/ml LPS, and 10 nM phorbol 12-myristate 13-acetate by Northern blot analysis. G, distribution of FLN29 mRNA determined by Northern blot analysis in mice tissue.
protein levels of FLN29 in both RAW cells (Fig. 1C) and mouse peritoneal macrophages (Fig. 1D). Leukemia-inducible factor did not induce the production of FLN29 protein. Among them, IFNβ was the most potent and rapid inducer of FLN29. Cytokines such as LPS, IFNγ as well as IFNβ induced the accumulation of phosphorylated STAT1, whereas leukemia-inducible factor induced phosphorylated STAT3 without accumulating any phosphorylated STAT1. In addition, we examined the effect of anti-inflammatory cytokines such as IL-4 as well as IL-10 on the expression of FLN29 protein. Neither of these cytokines induced the accumulation of FLN29 protein in RAW cells, whereas these cytokines did induce the phosphorylation of STAT6 and STAT3, respectively. Furthermore, LPS did not induce the production of FLN29 in the presence of IL-4 or IL-10, probably because of the suppression of STAT1 activation by these cytokines (data not shown). Therefore, we confirmed the role of STAT1 in FLN29 induction using bone marrow-derived macrophages (BMDM) from STAT1-deficient mice. As shown in Fig. 1E, the accumulation of FLN29 protein in STAT1-deficient BMDM was severely impaired in response to LPS and IFNβ. In addition, the accumulation of mRNA in mouse embryonic fibroblasts (MEFs) was abolished in STAT1-deficient MEFs, thus clearly demonstrating that FLN29 expression entirely depends on STAT1 (data not shown). Because the LPS-induced STAT1 activation is shown to be dependent on IFNβ, which is induced via the TRIF-IRF3 pathway, FLN29 induced by LPS is probably induced by STAT1 activated by IFNβ. We next examined the mRNA levels of FLN29 gene in macrophage-like RAW cells in response to various stimulations (Fig. 1F). A marked accumulation of the mRNA of FLN29 was observed in response to LPS as well as IFNγ, whereas there was no induction in mRNA by phorbol 12-myristate 13-acetate stimulation. Regarding tissue distribution, FLN29 was widely expressed in the tissue from mice (Fig. 1G) and human cell lines (data not shown); however, the expression levels in these tissues were relatively low compared with that in IFN-activated RAW cells. These data suggest that interferon- and LPS-inducible FLN29 may have a potential role in modulating the inflammatory function of macrophages.

**FIGURE 2.** Examination of the cellular function of FLN29 in LPS-mediated signaling. A, establishment of stable FLN29 transformants that express FLAG-tagged FLN29. B, the transformant of FLN29 RAW cells significantly impaired the accumulation of phosphorylated IκBα in response to 100 ng/ml LPS. C, changes in the accumulation of NO2− in response to 100 ng/ml LPS from parental RAW cells (open circles) and FLN29 transformant (closed circles) cells in response to LPS for 24 h. E, the dose-dependent accumulation of TNFα from the parental (open circles) and FLN29 transformant (closed circles) cells in response to LPS for 24 h. F, accumulation of NO2− from parental RAW (open box) and FLN29 transformant (closed box) cells in response to 10 ng/ml TNFα or 10 ng/ml IFNγ for 24 h. G, the accumulation of IP10 mRNA in response to 100 ng/ml LPS by RT-PCR in parental (left) and FLN29 transformant RAW (right) cells.
Effect of Overexpression of FLN29 on LPS Signaling—To address this possibility, we established RAW cell clones in which the FLN29 gene has been stably transformed (Fig. 2A). For this purpose, the expression pCMV vector carrying FLAG-tagged FLN29 was transfected into RAW cells and stable clones were selected. In transformants stably expressing FLN29, the accumulation of phosphorylated IkBα as well as phosphorylated ERK appears to be less than that in parental cells over 60 min after stimulation (Fig. 2B), indicating that FLN29 acts as a suppressor of TLR signaling. We further examined the biological effect of FLN29 using stable transformants. The accumulation of NO2- in response to LPS increased up to 24 h in parental cells, whereas such accumulation was impaired in FLN29 transformant cells (Fig. 2C). As shown in Fig. 2D, we found a significant suppression of NO2- in the range of 10 ng/ml-10 μg/ml LPS. Similarly, the amount of produced TNFα from FLN29 transformants was significantly attenuated over the 10 ng/ml-10 μg/ml range of LPS. In contrast to LPS signaling, the stimulation of these cells with other macrophage activators such as IFNγ produced an equal secretion of NO2- demonstrating that FLN29 transformants have the same capacity to produce NO2- in comparison with parental cells (Fig. 2F). To further examine whether FLN29 specifically attenuates the LPS-induced NF-κB pathway, we investigated the accumulation of IP10 mRNA (Fig. 2G). It has been shown that IP10 is induced through the MyD88-independent, IRF3-dependent IFNβ pathway (28). There was no difference in the accumulation of IP10 mRNA between the two cell lines, thus suggesting that FLN29 does not impair the IRF3-mediated chemokine production in response to LPS.

Effect of FLN29 Knockdown on LPS Tolerance—Next, we established FLN29 siRNA cells (Fig. 3A). After treating the control cells with 100 ng/ml LPS for 8 h, a significant amount of FLN29 protein was accumulated, whereas no such accumulation was observed in FLN29 siRNA-1 cells in which effective FLN29 siRNA was transfected (Fig. 3A, left). However, there was no suppression of FLN29 protein levels after LPS stimulation in ineffective siRNA transformants (Fig. 3A, right, FLN29 siRNA-2).
There were no significant changes in the accumulation of phosphorylated IkBa and phosphorylated ERK over 30 min in parental RAW cells and FLN29 siRNA-1 cells (Fig. 3B, pretreatment (−)). This result was expected because FLN29 protein levels were very low in the early stage of LPS stimulation in RAW cells. Then we examined the effect of siRNA on multiple LPS stimulation. The pretreatment of LPS is known to cause cells to be less responsive to second challenge of LPS, known as LPS tolerance. As shown in Fig. 3B, second LPS stimulation induced much less phosphorylation of IkBa in parental RAW cells pretreated with LPS for 8 h. However, a significant accumulation of phosphorylated IkBa was observed in FLN29 siRNA-1 cells even by the second LPS treatment (Fig. 3B, LPS 8h). These data suggest that endogenously produced FLN29 protein suppressed LPS-induced NF-κB activation. We further examined the changes in NO2− and TNFα from these cells as a readout of NF-κB activation. As expected, the production of NO2− and TNFα from control cells was significantly impaired by the pretreatment with LPS (Fig. 3, C and D, closed squares) in comparison to non-treated cells (open squares). In sharp contrast, significant levels of NO2− and TNFα were produced from LPS-pretreated FLN29 siRNA-1 cells (Fig. 3, E and F, closed squares). Such a break of tolerance was not obvious in ineffective FLN29 siRNA-2 (Fig. 3G), suggesting that knock down of FLN29 siRNA was specifically involved in this phenomenon. Although parental RAW cells and FLN29 siRNA cells behaved quite differently in LPS tolerance, these cells produced an equal amount of NO2− in response to 10 ng/ml IFNγ (Fig. 3H). The accumulation of IP10 mRNA was also similar in control and FLN29 siRNA-1 cells, suggesting that FLN29 specifically attenuates the Myd88-dependent NF-κB activation pathway (Fig. 3I). These data confirmed that FLN29 plays an important regulatory role in NF-κB activation during TLR tolerance.

Role of FLN29 in Signal Transduction by Other TLR Ligands—We asked whether the signaling through other TLR ligands might be similarly inhibited by FLN29. A significant decrease in the phosphorylation of IkBa, ERK, JNK, and p38 was observed in the FLN29 stable transfectants (data not shown). Then we examined the effect of LPS pretreatment on other TLR ligand stimulation. As expected, both in control and in FLN29 siRNA cells, there was an accumulation of phosphorylated IkBa and phosphorylated ERK in response to these stimuli. However, pretreatment of 100 ng/ml LPS for 8 h with control cells resulted in no accumulation of phosphorylated IkBa or phosphorylated ERK (Fig. 4A, bottom right). However, such down-regulation was not obvious in the FLN29 siRNA-1 cells (Fig. 4A, top right).

We further investigated the accumulation of TNFα from FLN29 siRNA-1 cells as a measure of NF-κB activation in response to different TLR ligands. In control cells, pretreatment of 100 ng/ml LPS for 8 h dramatically attenuated the production of TNFα in response to the second stimulation with LPS as well as CpG and poly(I:C) (Fig. 4B). In contrast, the amount of formed TNFα (Fig. 4C) from FLN29 siRNA-1 cells was significantly increased when FLN29 siRNA-1 cells were first treated with LPS for 8 h followed by restimulation with LPS as well as CpG and poly(I:C) for 24 h. We concluded that FLN29 acts as a negative regulator of NF-κB signaling in response to LPS as well as poly(I:C) and CpG in macrophages.

FLN29 Inhibits TLR Signaling by Interacting with TRAF6—To define the molecular mechanism of FLN29-mediated suppression of TLR signaling, we performed a transient NF-κB reporter assay in 293T cells. The NF-κB reporter gene was activated in response to LPS in 293 cells stably expressing TLR4, MD2, and CD14, and this NF-κB activation was suppressed by the expression of FLN29 (Fig. 5A). We next examined the effect of FLN29 on NF-κB activation by the TLR signaling components. Although FLN29 significantly attenuated NF-κB activation induced by TRAF6 + TAB2, FLN29 had little effect on the NF-κB activation induced by TGF-β-activating kinase 1 + TAB1 as well as IKKβ, thus suggesting that FLN29 acts between TRAF6 and TAB2. In addition to the classical NF-κB activation pathway involving MyD88, the TRIF-dependent NF-κB activation pathway that uses TRAF6 as an adaptor has been recently revealed (29). As expected, the expression of FLN29 suppressed TRIF-mediated NF-κB activation (Fig. 5A). FLN29 also suppressed IL-1β-induced NF-κB activation (data not shown). These data further suggest that FLN29 attenuates NF-κB activation through TRAF6.

The association of FLN29 and FLAG-tagged TRAF6 transfected to 293T cells was observed by immunoprecipitation experiment (Fig. 5B). Using FLN29 overexpressing RAW cells, we were also able to detect FLN29 protein associated with endogenous TRAF6, indicating that FLN29 interacted with TRAF6 in these cells (Fig. 5C). These data suggest that FLN29 inhibits TRAF6 activity by a direct binding to TRAF6.

Identification of the Domains of FLN29 Responsible for the Interaction with TRAF6—To examine the relevant region in FLN29 for the inhibition of TRAF6-induced NF-κB activation, we created a series of deletion mutants of FLN29 and then assessed their ability for inhibition (Fig. 6A). The C-terminal deletion mutant FLN29-(1–210) had no inhibitory effect on the TRAF6-induced NF-κB activation. However, FLN29-(1–270) mutant could suppress NF-κB activation, thus suggesting that FLN29 amino acids 210–270 could be one of the inhibitory region(s). This was further supported by N-terminal deletion constructs such as FLN29-(173–576). In N-terminal deletion mutants, we also found that
FLN29-(301–576) partially suppressed the TRAF6-mediated NF-κB activation, whereas FLN29-(391–576) failed to suppress NF-κB activation at all. However, the effect of NF-κB inhibition by the FLN29 C-terminal region (301–576) was relatively weak in comparison to that in the N-terminal region (1–270). These data suggest that there are two inhibitory regions, (210–247) and (301–390), for TRAF6-mediated NF-κB activation, although the C-terminal part has a weaker effect compared with the N-terminal part. Because FLN29 contains a TANK-related region in FLN29-(249–304) as indicated in Fig. 1B, we also created a mutant lacking this region, FLN29-(Δ247–304), and performed a reporter assay to test whether the TANK-related region might inhibit TRAF6-induced NF-κB activation. However, the mutant lacking the TANK-related region also attenuated NF-κB activation, clearly demonstrating that this region is irrelevant for inhibition. The central part of FLN29 containing amino acids 173–390 therefore clearly suppressed the TRAF6-induced NF-κB activation.

Given that the central part of FLN29 might be the relevant region that causes such inhibition, we hypothesized that FLN29-(173–390) might bind to TRAF6. To examine this possibility, we performed a GST pull-down experiment by reacting GST-tagged TRAF6 and FLAG-tagged FLN29 in 293T cells. As expected from an NF-κB reporter assay (Fig. 6A), FLN29-(173–390) bound to TRAF6 (Fig. 6B). In contrast, we could not detect the interaction of TRAF6 with FLN29-(391–576) and -(1–210), thus supporting the notion that the central part of FLN29 that inhibits NF-κB activation is physically bound to TRAF6. To explore the binding region in TRAF6 with FLN29, the reaction of His-tagged FLN29 and GST-tagged TRAF6 and its deletion mutants was examined. We found that only the C-terminal region con-
containing the coiled-coil domain of TRAF6 was co-precipitated with FLN29 (Fig. 6C). We concluded that the central region of FLN29 that interacts with the C-terminal region of TRAF6 attenuates TRAF6-induced NF-κB activation.

**DISCUSSION**

We have herein described the cellular and molecular function of FLN29, a novel interferon- and LPS-inducible gene that acts as a negative regulator in TLR signaling. It has been well recognized that the pretreatment of macrophages with LPS causes endotoxin tolerance, which makes macrophage less responsive to a second challenge of LPS (17, 18). We herein propose that FLN29 functions as an LPS-inducible negative regulator in innate immunity while FLN29 may thus play an important role in endotoxin tolerance.

LPS tolerance is a transient hyporesponsive state to second LPS stimulation (17, 18). Under pathophysiological conditions, it has been well recognized that inflammatory cytokines such as TNFα and IL-1 reach maximal levels after 1–3 h of LPS injection in serum (30). It has been well documented that LPS regulates the expression of multiple genes. They are shown to be involved in LPS tolerance at different times after stimulation. For example, LPS induces SOCS-1 gene expression after 1–3 h of stimulation (22). In contrast, the induction of IRAK-M, which blocks the signaling at IRAK, occurs after 24 h of LPS stimulation (21).

As demonstrated in this study, FLN29 is induced ∼8 h after LPS stimulation and the physiological role of FLN29 could be attributed to a negative regulator of TLR signaling. Although the production of cytokines through the transcription of genes still occurs, many studies have shown the amount of the activated form of upstream signaling molecules, including phosphorylated kinases, reaches the initial levels within several hours. In theory, it is possible that LPS can restimulate macrophages once the dephosphorylation of activated IκBα and mitogen-activated protein kinases occurs. However, because of LPS tolerance, such a case does not normally occur because LPS-inducible protein(s) such as FLN29 block LPS signaling by suppressing the function of adapter proteins like TRAF6. Alternatively, FLN29 might be involved in the termination of LPS signaling. Further studies are underway to define the physiological relevance of FLN29 in response to LPS in animal models.

Because the induction of FLN29 is dependent on STAT1, we speculate that FLN29 induction by LPS or other TLR ligands is a result of IFNβ that is rapidly induced by TLR stimulation through IRF3 or some other unknown mechanisms. In fact, IFNβ is the strongest inducer of FLN29 among IFNβ, IFNγ, and LPS within examined. FLN29 is therefore not like A20 or IκBα, which are directly induced by NF-κB. FLN29 could thus be a relatively late phase negative regulator. On the other hand, in some cases IFNs suppress TLR signaling to abrogate the harmful hyperactivation of macrophages (21, 31). Usually, IFNγ and LPS...
synergistically activate macrophages. However, SOCS1 induced by TLR ligands suppresses IFNγ signaling. FLN29 could be a mechanism of IFN-α-mediated suppression of the TLR signaling pathway. SOCS1-deficient mice die within 3 weeks after birth with severe inflammation because of the infiltration of macrophages into most organs. As a result, in a manner similar to SOCS1, FLN29 may inhibit an overactivation of macrophages, and it is therefore necessary to control inflammation. FLN29 knock out mice can therefore help to clarify the physiological function of FLN29 in vivo.

FLN29 contains a zinc finger motif (residues 13–107) that is similar to that in TRAF6 protein with 27% of homology. We assumed that the zinc finger moiety of FLN29 might be related to the regulation of NF-κB signaling. However, our biochemical study using an NF-κB reporter assay and a protein interaction assay revealed that the central part of FLN29, but not N- or C-terminal regions, bound to the C-terminal region containing the coiled-coil region of TRAF6 and this region was sufficient to attenuate TRAF6-mediated NF-κB activation (Fig. 6).

The activation of TRAF6 by TLR signaling has been well studied: the mechanism includes the binding of TRAF6 with IRAK, the oligomerization of TRAF6 followed by self-ubiquitination, and activation of TGF-β-activating kinase 1. Among many possibilities, we first thought that FLN29 might prevent the binding of TRAF6 with upstream molecules such as IRAK, because previous studies have reported that the proteins that inhibit NF-κB signaling through TRAF6 normally contain the P-X-E-X-X-(Ar/Ac) motif in the sequences to disturb interaction between IRAK and TRAF6 (13). Actually, mFLN29 contains a TRAF6 binding sequence. We therefore created an EA mutant of mFLN29 in which glutamate at 294 has been replaced by alanine. However, such a mutant also attenuated TRAF6-induced NF-κB activation as FLN29 wild type did (data not shown). Furthermore, FLN29 overexpression did not affect the binding between IRAK and TRAF6 (data not shown). Second, we speculated that attenuation of ubiquitination of TRAF6 by FLN29 contributes to the impairment of TLR-mediated NF-κB signaling, because a RING finger domain and zinc finger domains of TRAF6 protein in its N-terminal catalyze the self-ubiquitination of TRAF6 in the presence of E1, Mms2, and Ubc13 (14). However, so far the ubiquitination reaction of TRAF6 has not been affected by the FLN29 overexpression (data not shown). Therefore, we currently hypothesize that FLN29 might inhibit the downstream of TRAF6. Further study is underway to define the precise molecular mechanism of the suppression of the TRAF6-mediated NF-κB signaling by FLN29.

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