Enhanced TLR-mediated NF-IL6–dependent gene expression by Trib1 deficiency

Masahiro Yamamoto,1,3 Satoshi Uematsu,1 Toru Okamoto,2 Yoshiharu Matsuura,2 Shintaro Sato,4 Himanshu Kumar,1 Takashi Satoh,1,4 Tatsuya Saitoh,1 Kiyoshi Takeda,3,2 Ken J. Ishii,4 Osamu Takeuchi,1,4 Taro Kawai,1,4 and Shizuo Akira1,4

1Department of Host Defense and 2Department of Molecular Virology, Research Institute for Microbial Diseases and 3Department of Microbiology and Immunology, Graduate School of Medicine, Osaka University, Suita, Osaka 565-0871, Japan
4Exploratory Research for Advanced Technology, Japan Science and Technology Corporation, Suita, Osaka, 565-0871, Japan

Toll-like receptors (TLRs) recognize a variety of microbial components and mediate downstream signal transduction pathways that culminate in the activation of nuclear factor κB (NF-κB) and mitogen-activated protein (MAP) kinases. Trib1 is reportedly involved in the regulation of NF-κB and MAP kinases, as well as gene expression in vitro. To clarify the physiological function of Trib1 in TLR-mediated responses, we generated Trib1-deficient mice by gene targeting. Microarray analysis showed that Trib1-deficient macrophages exhibited a dysregulated expression pattern of lipopolysaccharide-inducible genes, whereas TLR-mediated activation of MAP kinases and NF-κB was normal. Trib1 was found to associate with NF-IL6 (also known as CCAAT/enhancer-binding protein β). NF-IL6–deficient cells showed opposite phenotypes to those in Trib1-deficient cells in terms of TLR-mediated responses. Moreover, overexpression of Trib1 inhibited NF-IL6–dependent gene expression by down-regulating NF-IL6 protein expression. In contrast, Trib1–deficient cells exhibited augmented NF-IL6 DNA–binding activities with increased amounts of NF-IL6 proteins. These results demonstrate that Trib1 is a negative regulator of NF-IL6 protein expression and modulates NF-IL6–dependent gene expression in TLR-mediated signaling.
TLR ligands/IL-1 (12). However, whether Trib family members regulate TLR-mediated signaling pathways under physiological conditions is still unknown.

In this study, we generated Trib1-deficient mice by gene targeting and analyzed TLR-mediated responses. Although the activation of NF-κB and MAP kinases in response to LPS was comparable between wild-type and Trib1-deficient cells, microarray analysis revealed that a subset of LPS-inducible genes was dysregulated in Trib1-deficient cells. Subsequent yeast two-hybrid analysis identified the CCAAT/enhancer-binding protein (C/EBP) family member NF-IL6 (also known as C/EBPβ) as a binding partner of Trib1, and phenotypes found in NF-IL6-deficient cells were opposite to those observed in Trib1-deficient cells. Moreover, overexpression of Trib1 inhibited NF-IL6-mediated gene expression and reduced amounts of NF-IL6 proteins. Inversely, NF-IL6 DNA-binding activity and LPS-inducible NF-IL6-target gene expression were up-regulated in Trib1-deficient cells, in which amounts of NF-IL6 proteins were increased. These results demonstrate that Trib1 plays an important role in NF-IL6-dependent gene expression in the TLR-mediated signaling pathways.

RESULTS

Comprehensive gene expression analysis in Trib1-deficient macrophages

To assess the physiological function of Trib1 in TLR-mediated immune responses, we performed a microarray analysis to compare gene expression profiles between wild-type and Trib1-deficient macrophages in response to LPS (Fig. 1 A and Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20070183/DC1). Out of 45,102 transcripts, we first defined the genes induced more than twofold after LPS stimulation in wild-type cells as “LPS-inducible genes” and identified 790 of them (Table S1). We next compared the LPS-inducible genes in wild-type and Trib1-deficient macrophages after LPS stimulation and found 59, 703, and 28 genes as up-regulated, similarly expressed, and down-regulated in Trib1-deficient cells, respectively (Table S1).

Among the up-regulated genes, several were subsequently tested by Northern blotting to confirm the accuracy. LPS-induced expression of prostaglandin E synthase (mPGES), lipocalin-2 (24p3), arginase type II, and plasminogen activator inhibitor type II, which were highly up-regulated in the microarray analysis (Table S1), was indeed enhanced in Trib1-deficient macrophages (Fig. 1 B). Furthermore, in contrast to proinflammatory cytokines such as TNF-α and IL-6, which were similarly expressed between wild-type and Trib1-deficient cells in response not only to LPS but also to other TLR ligands, IL-12 p40 was down-regulated in Trib1-deficient cells compared with wild-type cells (Fig. 1 C; Fig. S2, A–C, available at http://www.jem.org/cgi/content/full/jem.20070183/DC1; and Table S1). Thus, the comprehensive microarray analysis revealed that a subset of LPS-inducible genes is dysregulated in Trib1-deficient cells.

Previous in vitro studies demonstrate that human Trib family members modulate activation of MAP kinases and NF-κB (7–12). Both wild-type and Trib1-deficient cells showed similar levels and time courses of phosphorylation of p38, Jnk and extracellular signal-regulated kinase, and IκBα degradation (Fig. S2 D), indicating that the dysregulated NF-κB (7–12). Both wild-type and Trib1-deficient cells showed similar levels and time courses of phosphorylation of p38, Jnk and extracellular signal-regulated kinase, and IκBα degradation (Fig. S2 D), indicating that the dysregulated
expression of LPS-inducible genes in Trib1-deficient cells might be the independent of activation of NF-kB and MAP kinases.

**Interaction of Trib1 with NF-IL6**

To explore signaling aspects of Trib1 deficiency other than NF-kB and MAP kinases, we performed a yeast-two-hybrid screen with the full length of human Trib1 as bait to identify a binding partner of Trib1 and identified several clones as being positive. Sequence analysis subsequently revealed that three clones encoded the N-terminal portion of a member of the C/EBP NF-IL6 (unpublished data). We initially tested the interaction of Trib1 and NF-IL6 in yeasts. AH109 cells were transformed with a plasmid encoding the full length of Trib1 together with a plasmid encoding the N-terminal portion of NF-IL6 obtained by the screening (Fig. 2 A). We next examined the interaction in mammalian cells using immunoprecipitation experiments. HEK293 cells were transiently transfected with a plasmid encoding the full length of mouse Trib1 together with a plasmid encoding the full length of mouse NF-IL6. Myc-tagged NF-IL6 was coimmunoprecipitated with Flag-Trib1 (Fig. 2 B), showing the interaction of Trib1 and NF-IL6 in mammalian cells.

**TLR-mediated immune responses in NF-IL6-deficient macrophages**

An in vitro study showing the interaction of Trib1 and NF-IL6 prompted us to examine the TLR-mediated immune responses in NF-IL6-deficient cells, because LPS-induced expression of mPGES is shown to depend on NF-IL6 (13). We initially analyzed the expression pattern of genes affected by the loss of Trib1 in NF-IL6-deficient macrophages by Northern blotting. LPS-induced expression of 24p3, plasminogen activator inhibitor type II, and arginase type II, as well as mPGES, was profoundly defective in NF-IL6-deficient cells (Fig. 2 C). We next tested IL-12 p40 production by ELISA. As previously reported, IL-12 p40 production by LPS stimulation was increased in a dose-dependent fashion in NF-IL6-deficient cells compared with control cells (Fig. 2 D) (14). In addition, the production in response to bacterial lipoprotein (BLP), macrophage-activating lipopeptide-2 (MALP-2), or CpG DNA was also augmented in

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**Figure 2.** Association of Trib1 with NF-IL6 and TLR-mediated responses in NF-IL6-deficient macrophages. (A) Plasmids expressing human Trib1 fused to the GAL4 DNA-binding domain or an empty vector were cotransfected with a plasmid expressing NF-IL6 fused to GAL4 transactivation domain or an empty vector. Interactions were detected by the ability of cells to grow on medium lacking tryptophan, leucin, and histidine (L-W-H). The growth of cells on a plate lacking tryptophan and leucine (L-W) is indicative of the efficiency of the transfection. (B) Lysates of HEK293 cells transiently cotransfected with 2 μg of Flag-tagged Trib1 and/or 2 μg Myc-tagged NF-IL6 expression vectors were immunoprecipitated with the indicated antibodies. (C) Peritoneal macrophages from wild-type or NF-IL6-deficient mice were stimulated with 10 ng/ml LPS for the indicated periods. Total RNA (10 μg) was extracted and subjected to Northern blot analysis for expression of the indicated probes. (D and E) Peritoneal macrophages from wild-type or NF-IL6-deficient mice were cultured with the indicated concentrations of LPS (D) or with 100 ng/ml BLP, 30 ng/ml MALP-2, or 1 μM, CpG DNA (E) in the presence of 30 ng/ml IFN-γ for 24 h. Concentrations of IL-12 p40 in the culture supernatants were measured by ELISA. Indicated values are means ± SD of triplicates. Data are representative of three (B) and two (C–E) separate experiments. N.D., not detected.
NF-IL6–deficient cells (Fig. 2E). Together, compared with Trib1-deficient cells, converse phenotypes in terms of TLR-mediated immune responses are observed in NF-IL6–deficient cells.

**Inhibition of NF-IL6 by Trib1 overexpression**

To test whether Trib1 down-regulates NF-IL6–dependent activation, HEK293 cells were transfected with an NF-IL6–dependent luciferase reporter plasmid together with NF-IL6 and various amounts of Trib1 expression vectors (Fig. 3A). NF-IL6–mediated luciferase activity was diminished by coexpression of Trib1 in a dose-dependent manner. Moreover, RAW264.7 macrophage cells overexpressing Trib1 exhibited reduced expression of mPGES and 24p3 in response to LPS (Fig. S3A, available at http://www.jem.org/cgi/content/full/jem.20070183/DC1). We next tested NF-IL6 DNA-binding activity by EMSA and observed less NF-IL6 DNA-binding activity in HEK293 cells coexpressing NF-IL6 and Trib1 than in ones transfected with the NF-IL6 vector alone (Fig. 3B), presumably accounting for the down-regulation of the NF-IL6–dependent gene expression by Trib1. We then examined the effect of Trib1 on the amounts of NF-IL6 proteins by Western blotting. Although the diminution of NF-IL6 by Trib1 was marginal when excess amounts of NF-IL6 were expressed, we found that the transient expression of lower levels of NF-IL6, together with Trib1, resulted in a reduction of NF-IL6 in HEK293 cells (Fig. 3C). Also, endogenous levels of NF-IL6 proteins in RAW264.7 cells overexpressing Trib1 were markedly less than those in control cells (Fig. 3D). These results demonstrated that overproduction of Trib1 might negatively regulate NF-IL6 activity in vitro.

**Up-regulation of NF-IL6 in Trib1–deficient cells**

We next attempted to check the in vivo status of NF-IL6 in Trib1–deficient cells by comparing the NF-IL6 DNA-binding activity in Trib1–deficient macrophages with that in wild-type cells by EMSA. Although LPS-induced NF-kB–DNA complex formation in Trib1–deficient cells was similarly observed, Trib1–deficient cells exhibited elevated levels of C/EBP–DNA complex formation compared with wild-type cells (Fig. 4A). We further examined whether the C/EBP–DNA complex in Trib1–deficient cells contained NF-IL6 by supershift assay. Addition of anti–NF-IL6 antibody into the C/EBP–DNA complex yielded more supershifted bands in Trib1–deficient cells than in wild-type cells (Fig. 4B). In addition, the C/EBP–DNA complex was not shifted by the addition of anti-C/EBPβ (also known as NF-IL6β) antibody (Fig. S4A, available at http://www.jem.org/cgi/content/full/jem.20070183/DC1), suggesting that NF-IL6 DNA-binding activity is augmented in Trib1–deficient cells. We then examined the amounts of NF-IL6 proteins by Western blotting (Fig. 4C). Compared with wild-type cells, Trib1–deficient cells showed increased levels of NF-IL6 proteins. Finally, we examined NF-IL6 mRNA levels by Northern blotting and observed enhanced expression of NF-IL6 mRNA in Trib1–deficient cells (Fig. 4D), which is consistent with the autocrine induction of NF-IL6 mRNA.
Especially regarding IL-12 p40, although the microarray data showed an almost twofold reduction of the mRNA in Trib1-deficient cells (Table S1), the production was three to four times lower than that in wild-type cells (Fig. 1C), suggesting translational control of IL-12 p40 by Trib1 in addition to the transcriptional regulation. Moreover, the transcription of the IL-12 p40 gene itself may be affected by not only the amount of NF-IL6 proteins but also the phosphorylation or the isoforms such as liver-enriched activator protein and liver-enriched inhibitory protein (16–18). The molecular mechanisms of how Trib1 deficiency affects IL-12 p40 production on the transcriptional or translational levels through NF-IL6 regulation need to be carefully studied in the future.

The name Trib is originally derived from the Drosophila mutant strain tribbles, in which the Drosophila tribbles protein negatively regulates the level of Drosophila C/EBP slbo protein and C/EBP-dependent developmental responses such as border cell migration in larvae (19–22). It is also of interest that Trib1-deficient female mice and Drosophila in adulthood are both infertile (unpublished data) (18). In mammals, other Trib family members such as Trib2 and Trib3 have recently been shown to be involved in C/EBP-dependent responses (23, 24). Mice transferred with bone marrow cells, in which Trib2 is retrovirally overexpressed, display acute myelogenous leukemia–like disease with reduced activities and amounts of C/EBP\alpha (23). In addition, ectopic expression of Trib3 inhibits C/EBP-homologous protein–induced ER stress–mediated apoptosis (24). Thus, the function of tribbles to inhibit C/EBP activities by controlling the amounts appears to be conserved throughout evolution.

Given the up-regulation of the mRNA in Trib1-deficient cells (Fig. 4D), the reduction of NF-IL6 in Trib1-overexpressing cells (Fig. 3C), the auto-regulation of NF-IL6 by itself (15), and the degradation of C/EBP\alpha by Trib2 (23) and slbo by tribbles (22), the loss of Trib1 might primarily result in impaired degradation of NF-IL6 and, subsequently, in excessive accumulation of NF-IL6 via the autoregulation in Trib1-deficient cells.

In this study, we focused on the involvement of Trib1 in TLR-mediated NF-IL6–dependent gene expression. However, given that the levels of NF-IL6 proteins were increased in Trib1-deficient cells, it is reasonable to propose that other non–TLR-related NF-IL6–dependent responses might be enhanced in Trib1-deficient mice. Moreover, Trib3 is also shown to be involved in insulin-mediated Akt/PKB activation in the liver by mechanisms apparently unrelated to C/EBP, suggesting that Trib family members possibly function in a C/EBP-independent fashion (25–27). Future studies using mice lacking other Trib family members, as well as Trib1, may help to unravel the nature of mammalian tribbles in wider points of view.

DISCUSSION

In this study, we demonstrate by microarray analysis and biochemical studies that Trib1 is associated with NF-IL6 and negates NF-IL6–dependent gene expression by reducing the amounts of NF-IL6 proteins in the context of TLR-mediated responses.

MATERIALS AND METHODS

Generation of Trib1-deficient mice. A genomic DNA containing the Trib1 gene was isolated from the 129/SV mouse genomic library and characterized by restriction enzyme mapping and sequencing analysis. The gene encoding mouse Trib1 consists of three exons. The targeting vector was constructed by replacing a 0.4-kb fragment encoding the second exon of the
Western blot analysis and immunoprecipitation. Peritoneal macrophages were stimulated with the indicated ligands for the indicated periods, as shown in the figures. The cells were lysed in a lysis buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM Tris-Cl [pH 7.5], 5 mM EDTA) and a protease inhibitor cocktail (Roche). The cell lysates were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. For immunoprecipitation, cell lysates were precleared with protein G–sepharose (GE Healthcare) for 2 h and incubated with protein G–sepharose containing 1 μg of the antibodies indicated in the figures for 12 h, with rotation at 4°C. The immunoprecipitants were washed four times with lysis buffer, eluted by boiling with Laemmli sample buffer, and subjected to Western blot analysis using the indicated antibodies, as previously described (28).

EMSAs and supershift assay. 2 × 10^6 peritoneal macrophages were stimulated with the indicated ligands for the indicated periods, as shown in the figures. 2 × 10^6 HEK293 cells were transfected with 0.1 μg Myc–NF-IL6 and/or 4 μg Flag–Trib1 expression vectors. Nuclear extracts were purified from cells and incubated with a probe containing a consensus C/EBP-DNA-binding sequence (5′-TGCAGATTGGCACAATCTGCA-3′; Fig. 4, A and B) or mouse C/EBP-DNA binding sequence (sense, 5′-CCCTCTGTTGCT-CAACCCTTGA-3′; antisense, 5′-TGAAGGTTGACCAACAGGAAG-3′; Fig. 3 B), electrophoresed, and visualized by autoradiography, as previously described (28, 30). When the supershift assay was performed, nuclear extracts were mixed with the supershift-grade antibodies indicated in the figures before the incubation with the probes for 1 h on ice.

Online supplemental material. Fig. S1 showed our strategy for the targeted disruption of the mouse Trib1 gene. Fig. S2 showed the status of proinflammatory cytokine production in response to various TLR ligands and LPS–induced activation of MAP kinases and IkB degradation. Fig. S3 showed decreased expression of NF-IL6–dependent gene in Trib1-overexpressing cells. Fig. S4 showed that the C/EBP-DNA complex in Trib1–deficient cells contained NF-IL6, but not C/EBP. Table S1 provides a complete list of the LPS-inducible genes studied. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20070183/DC1.

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