LIND/ABIN-3 Is a Novel Lipopolysaccharide-inducible Inhibitor of NF-κB Activation*§

Received for publication, August 7, 2006. Published, JBC Papers in Press, November 6, 2006, DOI 10.1074/jbc.M607481100

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Recognition of lipopolysaccharide (LPS) by Toll-like receptor (TLR)4 initiates an intracellular signaling pathway leading to the activation of nuclear factor-κB (NF-κB). Although LPS-induced activation of NF-κB is critical to the induction of an efficient immune response, excessive or prolonged signaling from TLR4 can be harmful to the host. Therefore, the NF-κB signal transduction pathway demands tight regulation. In the present study, we describe the human protein Listeria INDuced (LIND) as a novel A20-binding inhibitor of NF-κB activation (ABIN) that is related to ABIN-1 and -2 and, therefore, is further referred to as ABIN-3. Similar to the other ABINs, ABIN-3 binds to A20 and inhibits NF-κB activation induced by tumor necrosis factor, interleukin-1, and 12-O-tetradecanoylphorbol-13-acetate. However, unlike the other ABINs, constitutive expression of ABIN-3 could not be detected in different human cells. Treatment of human monocytic cells with LPS strongly induced ABIN-3 mRNA and protein expression, suggesting a role for ABIN-3 in the LPS/TLR4 pathway. Indeed, ABIN-3 overexpression was found to inhibit NF-κB-dependent gene expression in response to LPS/TLR4 at a level downstream of TRAF6 and upstream of IKKβ. NF-κB inhibition was mediated by the ABIN-homology domain 2 and was independent of A20 binding. Moreover, in vivo adenoviral gene transfer of ABIN-3 in mice reduced LPS-induced NF-κB activity in the liver, thereby partially protecting mice against LPS/o-(-)-galactosamine-induced mortality. Taken together, these results implicate ABIN-3 as a novel negative feedback regulator of LPS-induced NF-κB activation.

The innate immune response to microbial pathogens begins when pathogen-associated molecular patterns meet their cognate Toll-like receptors (TLRs) on effector cells of the immune system, such as monocytes and macrophages (1). Lipopolysaccharide (LPS), an integral cell wall component of Gram-negative bacteria and one of the most potent stimulators in innate immunity, is recognized by the TLR4-MD2 receptor complex (2). In the past years, much progress has been made in understanding the intracellular signaling cascades that are initiated when LPS stimulates TLR4 (reviewed in Refs. 3 and 4). Ligation of the TLR4-MD2 complex by LPS initially results in the recruitment of myeloid differentiation factor (MyD)88 and MyD88-adaptor like (Mal), also called TIRAP, to the receptor cytoplasmic domain. MyD88 then facilitates recruitment of the serine/threonine kinases IL-1R-associated kinase (IRAK)-1 and -4, thus enabling IRAK4 to phosphorylate IRAK1. The latter subsequently dissociates from the receptor complex and associates with tumor necrosis factor (TNF) receptor-associated factor (TRAF6), constituting a cytoplasmic signaling complex. Upon ubiquitination, TRAF6 activates transforming growth factor-β-activated kinase 1, which in turn activates the inhibitor of κB kinase (IKK) complex that consists of the regulatory subunit IKKγ (also known as NEMO) and the kinases IKKα and IKKβ. The latter eventually phosphorylates the inhibitory IκB proteins, resulting in their ubiquitination and degradation. This allows the transcription factor NF-κB to translocate to the nucleus and initiate transcription of inflammatory cytokines, such as TNF, which contribute to mounting an inflammatory response. Apart from this MyD88-dependent signaling pathway, TLR4 also initiates a MyD88-independent signaling pathway that is mediated by the adapter proteins Toll/IL-1 receptor domain-containing adaptor-inducing interferon-β (TRIF; also known as TICAM-1) and TRIF-related adaptor molecule (also known as TICAM-2). Although

* This work was supported in part by grants from the Fonds voor Wetenschappelijk Onderzoek-Vlaanderen (FWO), the Interuniversitaire Attractiepelen, the Emmanuel Vanderschueren stichting, and the Geconcerteerde Onderzoeksacties. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

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* The abbreviations used are: TLR, Toll-like receptor; ABIN, A20-binding inhibitor of NF-κB activation; AHD, ABIN homology domain; GalN, D-(-)-galactosamine; GFP, green fluorescent protein; IκB, inhibitory protein of κB; IKK, IκB kinase; IL, interleukin; IL-1R, interleukin-1 receptor; IRAK, IL-1R-associated kinase; LPS, lipopolysaccharide; Luc, luciferase; Mal, MyD88-adaptor like; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor-κB; PBMC, peripheral blood mononuclear cells; RIP, receptor interacting protein; TNF, tumor necrosis factor; TRAF, TNF-receptor-associated factor; TRIF, Toll/IL-1 receptor domain-containing adaptor inducing interferon-β; LIND, Listeria INDuced; CMV, cytomegalovirus; E1, ubiquitin-activating enzyme; E3, ubiquitin-protein isopeptide ligase; pfu, plaque-forming unit(s); TPA, 12-O-tetradecanoylphorbol-13-acetate; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
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the TRIF/TRIF-related TLR4 adapter molecule pathway may contribute to delayed NF-κB activation, it is mainly responsible for interferon regulatory factor 3 transcription factor activation via IKKε and TANK-binding kinase 1.

Although the LPS-induced inflammatory response is indispensable for controlling the growth of pathogenic microorganisms (5), excessive cytokine production can be harmful to the host and may even contribute to a life-threatening condition termed septic shock (6). In addition, TLR4-initiated signaling pathways have recently been implicated in the pathogenesis of various autoimmune and chronic inflammatory diseases. For instance, activation of TLR4 has been shown to contribute to experimental models of autoimmune encephalomyelitis, asthma, and atherosclerosis (7–9). This universal and inherently dangerous role of TLR4 in inflammation emphasizes the need for tight regulation of TLR4-initiated signaling pathways.

As such, it is not surprising that the host acquired several proteins that can hold LPS-induced NF-κB activation in check (reviewed in Ref. 10). One of these negative feedback regulators is the zinc finger protein A20. This protein was originally identified (reviewed in Ref. 10). One of these negative feedback regulators was identified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mmglutamine, 4 mm sodium pyruvate, and antibiotics. The human THP-1 myelomonocytic cell line was obtained from the American Type Culture Collection and was grown in RPMI 1640 supplemented with 10% fetal bovine serum, 0.4 mm sodium pyruvate, 2 mmL-glutamine, 4 μmβ-mercaptoethanol, and antibiotics. The murine RAW264.7 macrophage cell line was obtained from the ATCC (Manassas, VA) and was cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum.

Recombinant human TNF and recombinant murine IL-1β were produced in Escherichia coli in our laboratory and were purified to at least 99% homogeneity. TNF had a specific biological activity of 2.3 × 10^11 IU/mg of purified protein, as determined with the international standard code 87/650 (National Institute for Biological Standards and Control, Potters Bar, UK). IL-1β had a specific activity of 3.65 × 10^8 IU/mg of purified protein, as determined with the international standard code 93/668. LPS from Salmonella abortus equi was obtained from Sigma.

Cloning of ABIN-3—TBLASTN searches with the region of homology between ABIN-1 and ABIN-2 were conducted using the NCBI online service, in the non-redundant and expressed sequence tag data base. A “full insert sequence” of a clone of human coronary artery smooth muscle cells (accession number AK024815) was identified as a potential homologue. 5’ Rapid amplification of cDNA ends (RACE) was performed on HeLa mRNA, using the SMART RACE kit (Clontech Laboratories, Palo alto, CA) according to the instructions from the manufacturer. Primers for first round and nested PCR were 5’-cgttctctttccttctccgtgc-3’ and 5’-cctggctccttgctgcttccc-3’, respectively. Full-length cDNA was amplified using forward (5’-ggagatctggctcctggctatgctgcttccc-3’) and reverse (5’-gaagacctctgctgcttccc-3’) primers. The open reading frame of ABIN-3 was cloned in-frame with an N-terminal E tag into the mammalian expression vector pCAGGS. The cloned fragment was sequenced on both strands with a cycle sequencer (Applied Biosystems, Foster City, CA). Deletion mutants were generated by PCR and cloned in pCAGGS using the following primers: 5’-tgccggagaaaaacttgatcttttccttgttcgcaagag-3’ and 5’-gaaatgacactctgctgcttcccgaagag-3’ for ABIN-3 ΔADH1 and 5’-gagacagaattctgctgcttcccgaagagag-3’ and 5’-ctctgctgcttcccgaagagagagag-3’ for ABIN-3 ΔADH2.

Plasmids and Adenoviruses—Plasmids coding for GFP and FLAG-tagged A20 have been described previously (16, 17). The plasmid encoding TLR4 was a kind gift from Dr. M. Muzio (Dept. of Immunology and Cell Biology, Mario Negri Institute, Milano, Italy) (18), and plasmids encoding MyD88, IRAK1, and TRAF6 were kind gifts from Dr. J. Tschopp (Institute of Biochemistry, University of Lausanne, Switzerland) (19, 20). The plasmid pNFconluc (21), encoding the luciferase (Luc) gene driven by a minimal NF-κB-responsive promoter, was a gift from Dr. A. Israel (Institut Pasteur, Paris, France). The plasmid pUT651, encoding β-galactosidase, was supplied by Eurogentec (Seraing, Belgium). For the production of a recombinant ABIN-3 adenovirus, the ABIN-3 cDNA, N-terminally fused to an E tag, was cloned into the pACpLpA.CMV shuttle vector and cotransfected with the rescue plasmid pJM17 (which encodes the adenovirus dl309 genome, lacking E1 and E3 functions) into HEK293 cells using calcium phosphate coprecipitation (22). Recombinant plaques were isolated and expression of ABIN-3 from the ubiquitously active cytomegalovirus (CMV) promoter was confirmed by Western blotting. Control viruses without transgene (AdR5) or expressing the β-galactosidase gene (AdLacZ) were generated with the same pJM17 adenoviral backbone vector. A virus expressing an NF-κB luciferase reporter gene (AdNFkBLuc) (23) was obtained from Dr. B. McGarvey (University of Iowa College of Medicine, Iowa City, IA). High titer virus stocks were prepared in HEK293 cells and

**MATERIALS AND METHODS**

Cell Lines and Reagents—Human embryonic kidney cells (HEK293T) were a kind gift from Dr. M. Hall (University of Birmingham, Birmingham, UK) and were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mmL-glutamine, 0.4 mm sodium pyruvate, and antibiotics. The human THP-1 myelomonocytic cell line was obtained from the American Type Culture Collection and was grown in RPMI 1640 supplemented with 10% fetal bovine serum, 0.4 mm sodium pyruvate, 2 mmL-glutamine, 4 μmβ-mercaptoethanol, and antibiotics. The murine RAW264.7 macrophage cell line was obtained from the ATCC (Manassas, VA) and was cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum.
purified by single CsCl banding. Titers were determined by plaque assay in HEK293 cells and calculated as plaque forming units (pfu) per millilitre of virus stock.

**Isolation and Culture of Primary Monocytes—**Peripheral blood mononuclear cells (PBMCs) were prepared from fresh blood samples of healthy donors drawn on citrate/phosphate/dextrose (Etablissement Français du Sang, Paris, France). Blood was diluted 1:2 in RPMI 1640 Glutamax medium (BioWhittaker, Verviers, Belgium) and centrifuged over Ficoll (MSL, Eurobio, Les Ulis, France) for 20 min at 15 °C and 600 × g. Human monocytes were selected from PBMCs by adherence. PBMCs were plated at 6 × 10⁶ cells/ml and allowed to adhere for 1 h at 37 °C in a 5% CO₂ air incubator in a humidified atmosphere. Non-adherent cells were removed; adherent cells were washed with RPMI and cultured in RPMI supplemented with antibiotics (100 IU/ml penicillin and 100 g/ml streptomycin) and 0.2% normal human serum (BioWhittaker). Monocytes were stimulated with 10 ng/ml LPS from E. coli (Alexis, San Diego, CA).

**Transfection, Coimmunoprecipitation, and Western Blotting—**2 × 10⁶ HEK293T cells were seeded in 10-cm Petri dishes and transfected with a total of 5 μg of DNA per plate using the DNA calcium phosphate coprecipitation method, as described (24). After 24 h, the cells were lysed in 500 μl of lysis buffer (50 mM Hepes, pH 7.6, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40), supplemented with protease and phosphatase inhibitors. Immunoprecipitation was performed with a monoclonal anti-FLAG M2 antibody (Sigma), and immunocomplexes were bound to protein A–trisacryl beads (Pierce). Beads were washed twice with lysis buffer, twice with the same buffer containing 1 mM NaCl, and again twice with lysis buffer. Binding proteins were eluted with Laemmli buffer and analyzed by 12.5% SDS-PAGE and Western blotting. Detection of co-precipitating and transfected proteins was achieved with a monoclonal anti-E tag (Amersham Biosciences) or anti-FLAG tag (Sigma) antibody, each of which was coupled to horseradish peroxidase. Immunoreactivity was revealed with a Renaissance-enhanced chemiluminescence system (PerkinElmer Life Sciences).

**Reporter Gene Assays for NF-κB—**2 × 10⁶ HEK293T cells were grown in 6-well plates and transiently transfected by DNA calcium phosphate coprecipitation with a total of 1 μg of DNA. The DNA mixture comprised 100 ng of pUT651, 100 ng of pNFconluc, and 800 ng of specific expression plasmids. After 24 h, the cells were transfected using Lipofectamine 2000 and Opti-MEM (Invitrogen) and 500 μl of lysis buffer (50 mM Hepes, pH 7.6, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40), supplemented with protease and phosphatase inhibitors. Immunoprecipitation was performed with a monoclonal anti-FLAG M2 antibody (Sigma), and immunocomplexes were bound to protein A–trisacryl beads (Pierce). Beads were washed twice with lysis buffer, twice with the same buffer containing 1 mM NaCl, and again twice with lysis buffer. Binding proteins were eluted with Laemmli buffer and analyzed by 12.5% SDS-PAGE and Western blotting. Detection of co-precipitating and transfected proteins was achieved with a monoclonal anti-E tag (Amersham Biosciences) or anti-FLAG tag (Sigma) antibody, each of which was coupled to horseradish peroxidase. Immunoreactivity was revealed with a Renaissance-enhanced chemiluminescence system (PerkinElmer Life Sciences).

**IL-8 Determination—**IL-8 levels in cell supernatants were determined via specific enzyme-linked immunosorbent assay (BD Pharmingen) according to the manufacturer’s instructions. **ABIN-3 mRNA and Protein Expression Analysis—**For multiple tissue RT-PCR, a human “Fast Scan™ cDNA panel” was purchased from Origene Technologies (Rockville, MD). PCR was performed with gene-specific primers (5’-actggacgccccgg-agagat-3’ and 5’-tgccggaagtgctgcaag-3’) that amplify a 691-bp fragment of the ABIN-3 open reading frame. For RT-PCR on THP-1 monocytes, 5 × 10⁶ cells were seeded in 10-cm Petri dishes and allowed to grow for 48 h. At the end of this period the cells were either left untreated or stimulated with LPS (1 μg/ml) or TNF (1000 IU/ml). Total RNA of THP-1 cells was isolated by the guanidium isothiocyanate-phenoI-chloroform method (25), and first strand cDNA was synthesized using the SuperScript™ first-strand synthesis system for RT-PCR (Invitrogen). For RT-PCR on PBMC, total RNA was prepared using the RNeasy Mini Kit (Qiagen) and reverse-transcribed with Superscript II RNase H (Invitrogen) according to the manufacturer’s protocol. cDNA samples were amplified by PCR with gene-specific primers (5’-ggagctctggcctcgtagctgctggag-3’ and 5’-ggagatctcggctgctggag-3’) that amplify the complete open reading frame of ABIN-3. As a control for cDNA integrity, either RT-PCR for a β-actin fragment was performed using 5’-gcttcgctcttgc-3’ and 5’-ctggctggagtggctggag-3’ primers, or RT-PCR for GAPDH was performed using 5’-gtaaggctggctggagtggctggag-3’ and 5’-ctggctggagtggctggag-3’ primers. For ABIN-3 protein expression analysis in THP-1 monocytes, 5 × 10⁶ cells were seeded in 10-cm Petri dishes and were either left untreated or stimulated with LPS (1 μg/ml) or TNF (1000 IU/ml) for various time periods. Subsequently, cell lysates were prepared and immunoblotted with a rabbit polyclonal ABIN-3 antibody raised against an ABIN-3–specific peptide (NH₂-CDVHKANGLSSVKKH-COOH) coupled to keyhole limpet hemocyanin.

**For real-time quantitative PCR, total RNA of PBMC was prepared using the RNeasy Mini Kit (Qiagen), Purified RNA was reverse-transcribed with Superscript II RNase H (Invitrogen) **

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according to the manufacturer’s protocol. The expression levels of ABIN-3 and GAPDH were determined by real-time quantitative PCR, using a LightCycler FastStart DNA Master SYBR Green I kit (Roche Applied Science). Forward and reverse primers for human ABIN-3 were, respectively, 5′-caagagaaagatcag-3′ and 5′-tgtcttagctctcttctc-3′. Primers for GAPDH were the RT2 PCR primer set from SuperArray (Frederick, MD). The cDNA copy number of each gene was determined using a six-point standard curve. Standard curves were run with each set of samples, the correlation coefficients (r²) for the standard curves being >0.98. All results were normalized with respect to the expression of GAPDH. To confirm the specificity of the PCR products, the melting profile of each sample was determined using the LightCycler, and by heating the samples from 60 °C to 95 °C at a linear rate of 0.10 °C/s while measuring the fluorescence emitted. Analysis of the melting curve demonstrated that each pair of primers amplified a single product. In all cases, the PCR products were checked for size by agarose gel separation and ethidium bromide staining to confirm that a single product of the predicted size was amplified. For ABIN-3, each run consisted of an initial denaturation time of 5 min at 95 °C and 40 cycles at 95 °C for 8 s, 56 °C for 8 s, and 72 °C for 15 s. For GAPDH, the run consisted of 40 cycles at 95 °C for 15 s, 58 °C for 15 s, and 72 °C for 25 s.

Animal Treatments—Female C57BL/6 mice (8–12 weeks old) were purchased from Charles River (Sulzfeld, Germany). All animals were maintained under standard specific pathogen-free conditions and received humane care in accordance with the National Institutes of Health guidelines and with the legal requirements in Belgium. All animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Research Advisory Committee. For adenovirus infection, mice were intravenously injected with a total of 5 × 10⁹ pfu of virus diluted in pyrogen-free phosphate-buffered saline. In preliminary experiments, we found that adenoviral transgene expression in the liver is maximal 3 days after infection. Therefore, mice were challenged with LPS/GalN 3 days after infection. In the LPS/GalN-induced model of acute lethal hepatitis, mice were injected intraperitoneally with 200 ng of LPS in combination with 20 mg of GalN (Sigma), corresponding to the LD₅₀/₅ determined in preliminary studies.

Statistics—All data represent at least three independent experiments and are expressed as mean values ± S.D. Survival curve was compared using a log rank χ² test, and the level of probability was noted (*, p < 0.05; **, p < 0.01; and ***, p < 0.0001).

RESULTS

Identification of LIND as an ABIN—The protein sequences of the A20-binding inhibitors of NF-κB ABIN-1 and ABIN-2 show significant homology over a region of ~70 amino acids. It is in this region that the previously described ABIN homology domain 1 (AHD1) and AHD2 are located (26) (Fig. 1). Using this homologous region in BLAST searches, we identified LIND, a protein that is induced in human mononuclear phagocytes infected with Listeria (13), as a potential ABIN protein. Comparison of the full-length protein sequence of LIND with the sequences of ABIN-1 and ABIN-2 revealed that LIND was much more homologous to ABIN-1 than to ABIN-2. Besides the AHD1 and AHD2 regions of homology, LIND and ABIN-1 also share a third region of strong homology, indicated as AHD3, which is not present in ABIN-2 (Fig. 1). Because of this strong sequence homology with ABIN-1 and ABIN-2, we will henceforth refer to LIND as ABIN-3 (=TNIP3).

To test if ABIN-3, besides sequence homology, also shows functional homology with ABIN-1 and ABIN-2, we investigated whether ABIN-3 could interact with the zinc finger protein A20. Therefore, expression plasmids for E-tagged ABIN-3 and FLAG-tagged A20 were cotransfected in HEK293T cells, followed by immunoprecipitation with an anti-FLAG tag antibody. Immunoblotting with anti-E tag revealed that ABIN-3 indeed immunoprecipitated with A20, indicating that both proteins can associate with each other in mammalian cells (Fig. 2A). In addition to interacting with A20, ABIN-1 and ABIN-2 are also characterized by the ability to inhibit the activation of NF-κB in response to TNF, IL-1β, and TPA (14, 15). To test if ABIN-3 shares this NF-κB inhibiting activity, we coexpressed ABIN-3 with an NF-κB-dependent luciferase reporter gene in HEK293T cells. The effects of the NF-κB inhibitor A20 and the irrelevant protein GFP were used as positive and negative controls, respectively. ABIN-3 was indeed able to inhibit NF-κB-dependent luciferase expression induced by TNF, IL-1β, or TPA (Fig. 2B). Taken together, this strong sequence and functional homology with ABIN-1 and ABIN-2 identifies LIND as a novel A20-binding inhibitor of NF-κB activation, named ABIN-3.

ABIN-3 Is an LPS-inducible Protein—Tissue distribution of ABIN-3 mRNA was investigated by PCR amplification of a cDNA panel containing first strand cDNA samples from 24 different human tissues. No ABIN-3 mRNA could be detected in heart, salivary gland, adrenal gland, pancreas, ovary, or fetal brain. High levels of ABIN-3 mRNA were detected in most of the other tissues, except for kidney and bone marrow, both of which showed only a low expression level of ABIN-3 mRNA (Fig. 3A).

We subsequently analyzed ABIN-3 mRNA expression by semi-quantitative RT-PCR on mRNA isolated from various human cell lines such as THP-1, HEK293, and HepG2. Constitutive expression of ABIN-3 mRNA could not be detected in any of the cell lines (data not shown). However, a clear induction of ABIN-3 mRNA could be observed in THP-1 monocytes after stimulation for 3 h with LPS (Fig. 3B). In contrast, stimulation of THP-1 cells with TNF only led to a slight induction of ABIN-3 mRNA. To investigate if the observed induction of ABIN-3 mRNA in THP-1 cells was also reflected at the protein level, polyclonal antibodies against ABIN-3 were generated in rabbits, and used to analyze the expression of ABIN-3 protein in THP-1 cells treated with LPS or TNF. Consistent with the RT-PCR data, ABIN-3 protein could not be detected in unstimulated cells. However, after 6-h LPS treatment ABIN-3 protein was clearly visible (Fig. 3C). In contrast, stimulation of THP-1 cells with TNF did not lead to detectable expression levels of ABIN-3 protein (data not shown). We also evaluated LPS-inducible expression of ABIN-3 in primary human monocytes, which were selected by adherence from peripheral blood mononuclear cells of healthy donors. These monocytes were stimulated either with vehicle or with LPS for 2 or 20 h. Total RNA was isolated, and expression of ABIN-3 mRNA was ana-
lyzed by semi-quantitative RT-PCR (Fig. 3D) as well as by real-time quantitative PCR (Fig. 3E). In both cases, expression of ABIN-3 mRNA was induced already slightly after 2-h treatment with LPS, and was more pronounced after 20 h. All together, these data demonstrate that LPS is a potent inducer of ABIN-3 expression in monocytic cells.

**ABIN-3 Inhibits LPS/TLR4-induced NF-κB Activation**

The above results show that expression of ABIN-3 is inducible by LPS and thus suggest a role for ABIN-3 in the LPS/TLR4-induced pathway to NF-κB. To investigate whether ABIN-3 prevents LPS/TLR4-induced NF-κB activity, we first tested the effect of ABIN-3 overexpression on NF-κB-dependent luciferase reporter gene expression in response to transient TLR4 overexpression in HEK293T cells, which as such is already sufficient to activate NF-κB. The upper panel of Fig. 4A illustrates that ABIN-3 expression significantly reduced TLR4-induced NF-κB-dependent luciferase gene expression. To evaluate whether ABIN-3 also inhibits the expression of an endogenous NF-κB target gene, we also analyzed the TLR4-induced production of IL-8 in the same experiment. IL-8 levels in the HEK293T cell supernatant were increased 6-fold upon TLR4 expression (Fig. 4A, lower panel). Consistent with the NF-κB inhibitory effect of ABIN-3 in the luciferase reporter assay and the fact that IL-8 expression is known to be at least partially NF-κB-dependent (18, 27), coexpression of ABIN-3 significantly reduced the expression of IL-8 in response to TLR4. To investigate the effect of ABIN-3 in a more physiologically relevant cell line, we next investigated whether ABIN-3 also inhibits LPS/TLR4-induced expression of an NF-κB-dependent luciferase reporter gene in the RAW264.7 macrophage cell line. As can be seen in Fig. 4B, ABIN-3 expression indeed significantly reduced LPS-induced luciferase expression in RAW264.7 macrophages, further establishing the function of ABIN-3 as an inhibitor of LPS/TLR4-induced NF-κB-dependent gene expression.

For the previously described NF-κB inhibitors ABIN-1 and ABIN-2, it was shown that their AHD2 region is essential for NF-κB inhibition (26). In addition, ABIN-1 and ABIN-2 have been shown to bind A20 through the more upstream AHD1. To elucidate whether AHD1 and AHD2 of ABIN-3 have a similar function,
we made deletion mutants of ABIN-3 that either lacked AHD1 (ABIN-3 ΔAHD1) or AHD2 (ABIN-3 ΔAHD2) and evaluated the binding of these mutants to A20 as well as their NF-κB-inhibiting potential. Transient overexpression of FLAG-tagged A20 together with E-tagged ABIN-3 WT, ABIN-3 ΔAHD1, or ABIN-3 ΔAHD2, followed by immunoprecipitation of A20 with an anti-FLAG antibody, clearly demonstrated that ABIN-3 ΔAHD2 and ABIN-3 WT bind equally well to A20. In contrast, ABIN-3 ΔAHD1 did not coprecipitate with A20, showing that the binding of ABIN-3 to A20 requires AHD1 (Fig. 5A). Similar results were obtained when binding was studied via yeast two-hybrid experiments (data not shown). The NF-κB-inhibiting effect of the ABIN-3 deletion mutants was analyzed by means of an NF-κB-dependent luciferase reporter gene, ABIN-3 ΔAHD2 had no effect any more (Fig. 5B). This indicates that AHD2 is essential for the NF-κB inhibiting function of ABIN-3, whereas AHD1 is not. Because AHD1 is essential for ABIN-3/A20 binding, these results also indicate that ABIN-3 does not need to bind A20 to prevent NF-κB activation.

We next investigated the level in the NF-κB signaling pathway at which ABIN-3 interferes with LPS/TLR4-induced NF-κB activation. Therefore, we analyzed the effect of ABIN-3 coexpression on NF-κB activation induced by overexpression of the TLR4 signaling proteins MyD88, IRAK1, and TRAF6, as well as by IKKβ, which is acting more downstream in the pathway and mediates NF-κB activation by all stimuli that activate the "classic" NF-κB pathway. As shown in Fig. 6, ABIN-3 inhibited NF-κB activation induced by MyD88, IRAK1, and TRAF6 but not that induced by IKKβ. This suggests that ABIN-3 interferes with LPS/TLR4-induced NF-κB activation at a level downstream of TRAF6 but upstream of IKKβ.

**ABIN-3 Inhibits LPS-induced NF-κB Activation in the Liver and Protects Mice against LPS/GalN-induced Mortality**—To validate the NF-κB inhibitory function of ABIN-3 in vivo, we also tested the effect of ABIN-3 on LPS-induced NF-κB activation in mouse liver. For this purpose, mice were infected with an adenovirus expressing an NF-κB-dependent luciferase reporter gene, together with an adenovirus expressing either an ABIN-3 transgene (AdABIN-3), or no transgene (AdRR5) as a control. Three days after AdABIN-3 infection, ABIN-3 transgene expression was clearly detectable in total liver cell extracts by Western blotting (Fig. 7A, upper part). LPS injection of AdRR5-infected mice resulted in a 13-fold increase in NF-κB-dependent luciferase activity in the liver (Fig. 7A, lower part). However, consistent with the NF-κB inhibitory effect of ABIN-3 in vitro, LPS-induced NF-κB activity was substantially lower in the liver of AdABIN-3-infected mice.

Because these data indicate that ABIN-3 can inhibit LPS-induced NF-κB activity in the liver, we investigated the effect of adenoviral gene transfer of ABIN-3 in the murine model of LPS/GalN-induced acute liver failure. Therefore, C57BL/6 mice were injected intravenously with 5 × 10⁹ pfu of AdRR5 or AdABIN-3. Three days later, mice were challenged intraperitoneally with a lethal dose of LPS/GalN. In the control group, all mice died within 10 h after LPS/GalN injection. In contrast, AdABIN-3-infected mice were significantly (p = 0.0038) protected against LPS/GalN-induced mortality, as one-third of them survived the LPS/GalN challenge (Fig. 7B). These observations clearly demonstrate that ABIN-3-mediated NF-κB inhibition in the liver is associated with a protective effect against LPS/GalN-induced liver failure.

**DISCUSSION**

Although essential to combat bacterial infections, LPS-induced activation of NF-κB acts as a double-edged sword. Inappropriate or prolonged activation of NF-κB can lead to an exaggerated immune response, which might be harmful to the host. Therefore, to prevent excessive immune responses to LPS, the host may acquire mechanisms that dampen the response to LPS or even confer unresponsiveness to successive triggers with LPS, a phenomenon named LPS tolerance (28). Down-regulating LPS-induced responses can at least partially be accomplished by the LPS-induced production of NF-κB inhibitory proteins, which...
FIGURE 3. Expression profiling of ABIN-3. A, PCR was performed on cDNA from different human tissues to amplify a 691-bp fragment of the ABIN-3 open reading frame (upper panel). PCR for β-actin served as a control (lower panel). B, expression of ABIN-3 mRNA in THP-1 monocytes, either untreated or stimulated for 3 h with 1000 IU/ml TNF or 1 μg/ml LPS. First strand cDNA was prepared, and PCR was performed to amplify the complete open reading frame of ABIN-3 (upper panel). PCR for β-actin served as a control (lower panel). C, expression of ABIN-3 protein in THP-1 cells after treatment with LPS for various time periods as indicated. Total cell lysates were prepared and Western blotting was performed with anti-ABIN-3 antibodies. As loading controls, two nonspecific bands (*) are shown. D, expression of ABIN-3 mRNA in human primary monocytes stimulated for 2 or 20 h with 100 ng/ml LPS. One representative RT-PCR experiment out of three performed on different donors is shown. E, mRNA expression of ABIN-3 in human primary monocytes that were stimulated for 2 or 20 h with LPS was analyzed by real-time quantitative PCR. The figure represents the mean ± S.D. of three independent experiments performed with different donors. Expression of ABIN-3 was normalized to that of GAPDH.

then provide a negative feedback loop (reviewed in Refs. 10 and 29). For example, LPS-inducible alternative splicing of MyD88 can shut down LPS-induced NF-κB activation by preventing the recruitment of IRAK4 to MyD88 (30, 31). Several other LPS-inducible proteins were shown to inhibit NF-κB activation by targeting different steps in the TLR4 signaling pathway and include, among others, A20 (12), SOCS1 (32, 33), IRAK-M (34), and ST2 (35). Here we identify human ABIN-3 as a novel protein that fulfills two essential criteria to be implicated in the negative feedback regulation of LPS-induced NF-κB activation. First, ABIN-3 expression is induced by LPS in mononuclear cells. Second, we could show that expression of ABIN-3 inhibits NF-κB-dependent gene expression in response to LPS, both in vitro as well as in vivo. Human ABIN-3 shows partial sequence homology with ABIN-1 and ABIN-2 and shares with these proteins the ability to bind A20 and to inhibit TNF-α, IL-1-β, and LPS-induced NF-κB activation upon overexpression in HEK293T cells (14, 15).9 These overlapping activities suggest that the function of ABIN-1, -2, and -3 might be at least partially redundant. The fact that ABIN-2-deficient mice are normal and do not show any defect in NF-κB activation in response to different stimuli might also reflect such redundancy (36). However, we cannot exclude cell type- or stimulus-specific effects of distinct ABINs on NF-κB signaling. The more restricted expression of ABIN-3 in specific tissues, as well as its inducibility by LPS, suggests that ABIN-3 might indeed have a unique function. In this respect, it is worth mentioning that coimmunoprecipitation experiments have shown that ABIN-3 does not compete with the other ABINs for binding to A20 (data not shown). Moreover, our ongoing yeast two-hybrid experiments demonstrate different protein-protein interactions for each ABIN family member.

It is still unclear how ABINs interfere with NF-κB signaling. Our finding that ABIN-3 still prevents TRAF6-induced NF-κB activation, but no longer IKKβ-induced NF-κB activation, indicates that ABIN-3 interferes with LPS/TLR4 signaling at the level of or downstream of TRAF6 but upstream of IKKβ. A similar effect on proximal signaling was previously shown for ABIN-1 and -2, which inhibit TNF-induced NF-κB activation downstream of TRAF2 and upstream of IKKβ. The binding of ABIN-3 to A20 suggests that its NF-κB inhibitory effect might be mediated by A20. A20 was recently proposed to inhibit NF-κB activation by de-ubiquitinating several proteins, including TRAF6, RIP, and IKKγ (12, 37–39). In fact, while this report was prepared, ABIN-1 was described to physically link A20 to IKKγ by directly binding IKKγ, thus facilitating A20-mediated de-ubiquitination of IKKγ (39). However, our data demonstrate that the NF-κB inhibitory potential of ABIN-3 does not correlate with ABIN-3/A20 binding, as an AHD1-deletion mutant of ABIN-3, which can no longer bind A20, is still fully capable of inhibiting NF-κB activation. Although we cannot exclude that ABINs somehow regulate or modulate the function of A20, these findings make it unlikely that the NF-κB inhibitory effect of ABIN-3 is exclusively mediated by A20.

Another model that could explain the NF-κB inhibitory effect of ABIN-3 implicates the possibility that ABIN-3 prevents the formation of specific protein-protein interactions in the cell. Deletion analysis of ABIN-3 showed that, like in ABIN-1 and -2 (26), the AHD2 region is necessary for its NF-κB inhibiting function. In this context it is worth mentioning that AHD2 shows strong sequence homology with a region in IKKγ that was recently shown to mediate the binding of IKKγ to polyubiquitin chains (26, 40, 41). This allows IKKγ to bind polyubiquitinated receptor interacting protein (RIP) 1 in the TNF signaling pathway, which is essential for TNF-induced NF-κB activation. Although IKKγ/RIP1 binding most likely
also involves other surrounding amino acids that provide further specificity, similar ubiquitin-dependent protein-protein interactions might be mediated by ABINs via their AHD2. In this way, ABIN-3 might also compete with IKK or other signaling proteins to form crucial protein-protein interactions in response to TLR4 triggering. Because RIP1 is not involved in the LPS/TLR4-induced MyD88-dependent signaling pathway that is inhibited by ABIN-3 (42), ubiquitin-dependent protein-
that it encodes a smaller protein that does not contain the complete AHD2. Moreover, as overexpression of this murine ABIN-3-like protein did not inhibit NF-κB activation (data not shown), it does not qualify as a true ABIN. Although a functional murine ABIN-3 gene might not exist, it is worth mentioning that expression of human ABIN-3 is able to prevent LPS-induced NF-κB activation in murine cells as reflected by our experiments with murine RAW264.7 macrophages as well as our in vivo mouse experiments.

Multiple stimuli can activate NF-κB by partially overlapping signaling pathways. Therefore, ABIN-3 might also affect the activation of NF-κB by other stimuli than the ones tested in this study (TNF, IL-1, TPA, and LPS). In this respect, it is worth mentioning that ABIN-3 has previously been described as LIND (Listeria Induced), a protein that is induced in mononuclear phagocytes infected with Listeria (13). Because Listeria is not a Gram-negative bacterium and thus has no LPS, a TLR agonist other than LPS must be responsible for inducing ABIN-3 expression, raising the possibility that ABIN-3 acts as a negative regulator of inflammatory responses initiated by a wide range of TLRs. In addition, it cannot be excluded that ABIN-3 also regulates pathways different from NF-κB, such as the activation of interferon regulatory factor 3 and AP-1. In contrast to its ability to inhibit TPA-induced NF-κB activation (Fig. 2B), ABIN-3 did not prevent TPA-induced AP-1 activation in the same cells (data not shown), demonstrating that ABIN-3 does not act in a non-specific way. Further studies will be needed to reveal the complex interplay between ABIN-3 and other signaling proteins in the regulation of different signaling pathways.

In addition to the NF-κB inhibitory potential of ABIN-3 in cultured cells, we were able to show that adenoviral gene transfer of ABIN-3 inhibits LPS-induced expression of an NF-κB-dependent luciferase reporter gene in the liver, and partially protects mice against LPS/GalN-induced mortality. In this model of acute liver failure, LPS induces the production and release of several cytokines, including TNF, IL-1, and IL-6, whose production is known to be NF-κB-dependent. These cytokines subsequently contribute to the pathogenesis of hepatic liver failure (43). As studies with NF-κB decoy oligonucleotides have previously been shown to prevent LPS-induced fatal liver failure (44), the NF-κB inhibitory effect of ABIN-3 is most likely responsible for the observed protection against LPS/GalN-induced mortality. This suggestion is reinforced by our observation that human ABIN-3 also inhibits NF-κB activation in murine macrophages, which are the predominant cytokine-producing cells after LPS challenge (45). On the other hand, other NF-κB-independent effects of ABIN-3 might also account for the protection of mice against LPS/GalN, as the closely homologous ABIN-1 protein was recently shown to possess an anti-apoptotic effect in hepatocytes, enabling it to protect mice against TNF/GalN-induced mortality (46). However, we were unable to show a similar anti-apoptotic effect for ABIN-3 (data not shown).

In conclusion, we identified ABIN-3 as a novel player in the negative feedback regulation of LPS-induced NF-κB activation. Because NF-κB has an important role in the development and progression of septic shock and different autoimmune and chronic inflammatory diseases, strategies that increase the expression or the activity of ABIN-3 might have an important therapeutic potential.
Negative Regulation of TLR4-induced NF-κB Activation

Acknowledgments—We thank Drs. B. De Geest, B. McCoy, M. Klinkenberg, J. Tschopp, and A. Israel for providing adenoviruses and plasmids. A. Meeuws is thanked for technical assistance.

REFERENCES

1. Medzhitov, R., and Janeway, C., Jr. (2000) *Immunol. Rev.* 173, 89–97
2. Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B., and Beutler, B. (1998) *Science* 282, 2085–2088
3. O’Neill, L. A. (2006) *Curr. Opin. Immunol.* 18, 3–9
4. Fitzgerald, K. A., Rowe, D. C., Golenbock, D. T. (2004) *Microbes Infect.* 6, 1361–1367
5. O’Neill, L. A., and Dinarello, C. A. (2000) *Immunol. Today* 21, 206–209
6. Danner, R. L., Elin, R. J., Hosseini, J. M., Wesley, R. A., Reilly, J. M., and Parillo, J. E. (1991) *Chest* 99, 169–175
7. Kerfoot, S. M., Long, E. M., Hickey, M. J., Andonegui, G., Lapointe, B. M., Zanardo, R. C., Bondet, C., James, W. G., Robbins, S. M., and Kubes, P. (2004) *J. Immunol.* 173, 7070–7077
8. Eisenbarth, S. C., Piggott, D. A., Huleatt, J. W., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B., and Beutler, B. (1998) *Science* 282, 2085–2088
9. Klinkenberg, M., Van Huffel, C., Heyninck, K., Wullaert, A., and Beyaert, R. (2003) *Cell* 112, 36560–36566
10. Heyninck, K., De Valck, D., Fitting, C., and Adib-Conquy, M. (2000) *J. Biol. Chem.* 275, 22123–22130
11. Wu, C. J., Conze, D. B., Li, T., Srinivasula, S. M., and Ashwell, J. D. (2006) *J. Biol. Chem.* 281, 18482–18488
12. Cusson-Hermance, N., Khurana, S., Lee, T. H., Fitzgerald, K. A., and Kellis, M. A. (2005) *J. Biol. Chem.* 280, 36560–36566
13. Heyninck, K., Wullaert, A., and Beyaert, R. (2003) *Chem. Biol.* 10, 245–257
14. Wu, C. J., Conze, D. B., Li, T., Srinivasula, S. M., and Ashwell, J. D. (2006) *Nat. Cell Biol.* 8, 398–406
15. Cusson-Hermance, N., Khurana, S., Lee, T. H., Fitzgerald, K. A., and Kellis, M. A. (2005) *J. Biol. Chem.* 280, 36560–36566
16. Heyninck, G., Delaey, C., Heyninck, K., De Valck, D., and Beyaert, R. (2001) *FEBS Lett.* 498, 93–98
17. Sanioglu, S., Williams, C. M., Samavati, L., Butler, N. S., Wang, G., McCray, P. B., Jr., Ritchie, T. C., Hunninghake, G. W., Zandi, E., and Engelhardt, J. F. (2001) *J. Biol. Chem.* 276, 30188–30198
18. O’Mahoney, J. V., and Adams, T. E. (1994) *DNA Cell Biol.* 13, 1227–1232
19. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* 162, 156–159
20. Heyninck, K., Kreike, M. M., and Beyaert, R. (2003) *FEBS Lett.* 536, 135–140
21. Hoffmann, E., Dittrich-Breiholz, O., Holtmann, H., and Kracht, M. (2002) *J. Leukoc. Biol.* 72, 847–855
22. Cavaillon, J. M., Adrie, C., Fitting, C., and Adib-Conquy, M. (2003) *J. Endotoxin Res.* 9, 101–107
23. Fan, H., and Cook, J. A. (2004) *J. Endotoxin Res.* 10, 71–84
24. Janssens, S., Burns, K., Tschopp, J., and Beyaert, R. (2002) *Curr. Biol.* 12, 467–471
25. Brint, E. K., Xu, D., Liu, H., Dunne, A., McKenzie, A. N., O’Neill, L. A., and Liew, F. Y. (2004) *Nat. Immunol.* 5, 373–379
26. Papoutsopoulou, S., Symons, A., Tharmalingham, T., Belich, M. P., Kaiser, T., Ma, A., Koonin, E. V., and Dixit, V. M. (2004) *Nature* 430, 694–699
27. Heyninck, K., and Beyaert, R. (2005) *Trends Biochem. Sci.* 30, 1–4
28. Wertz, I. E., O’Rourke, K. M., Zhou, H., Eby, M., Avrind, L., Seshagiri, S., Wu, P., Wiesmann, C., Baker, R., Boone, D. L., Ma, A., Koonin, E. V., and Dixit, V. M. (2004) *Nature* 430, 694–699
29. Mauro, C., Pacifico, F., Mallone, S., Iannetti, A., Acquaviva, R., Formisano, S., Vito, P., and Leonard, A. (2006) *J. Biol. Chem.* 281, 18482–18488