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ABIN-3: a Molecular Basis for Species Divergence in Interleukin-10-Induced Anti-Inflammatory Actions[▽]

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Whereas interleukin-10 (IL-10) is an anti-inflammatory cytokine known to regulate macrophage activation, its full mechanism of action remains incompletely defined. In a screen to identify novel IL-10-induced genes, we cloned the mouse ortholog of human ABIN-3 (also termed LIND). ABIN-3 expression was induced selectively by IL-10 in both mouse and human mononuclear phagocytes coordinately undergoing proinflammatory responses. In contrast to the previously characterized ABINs, mouse ABIN-3 was incapable of inhibiting NF-κB activation by proinflammatory stimuli. Generation and analysis of ABIN-3-null mice demonstrated that ABIN-3 is unnecessary for the anti-inflammatory effects of IL-10 as well as for proper negative regulation of NF-κB. Conversely, human ABIN-3 was capable of inhibiting NF-κB activation in response to signaling from Toll-like receptor, IL-1, and tumor necrosis factor. Enforced expression of human ABIN-3 in human monocytic cells suppressed the cytoplasmic degradation of IκBα, the activation of NF-κB, and the induction of proinflammatory genes. Comparative sequence analyses revealed that mouse ABIN-3 lacks a complete ABIN homology domain, which was required for the functional activity of human ABIN-3. ABIN-3 is, thus, an IL-10-induced gene product capable of attenuating NF-κB in human macrophages yet is inoperative in mice and represents a basis for species-specific differences in IL-10 actions.

The proinflammatory functions of monocytes and macrophages represent an important component of host responses to infection. These responses occur, in part, as a consequence of the interaction between pathogen-associated molecular patterns (PAMPs) expressed by infectious agents and cellular pathogen recognition receptors (PRRs) expressed by the host. PRRs are a large and diverse group of microbial sensors that include the membrane-associated Toll-like receptors (TLRs) and the cytoplasmic NOD-like receptors and RIG-like helicases (1, 38, 46). In macrophages, engagement of a PRR by its PAMP ligand, e.g., bacterial lipopolysaccharide (LPS), peptidoglycan (PGN), or viral double-stranded RNA, leads to triggering of mitogen-activated protein kinase (MAPK) signaling cascades and activation of transcription factors belonging to the NF-κB, the AP-1, and the IRF families (1, 40, 58). These signaling processes effect the induction of genes that promote the inflammatory response including cytokines, chemokines, and enzymes responsible for producing reactive oxygen and nitrogen species.

The cytokine interleukin-10 (IL-10) plays a critical role in limiting the magnitude and duration of inflammatory responses (13, 39). This conclusion is supported by the finding that mice lacking an IL-10 response display exacerbated inflammatory responses and develop a chronic inflammatory bowel disease clinically related to Crohn's disease (28, 56, 59). Despite the clearly established physiologic relevance of IL-10's anti-inflammatory properties, its mechanism of action remains incompletely understood. This subject is particularly complex because IL-10 has been reported to exert its anti-inflammatory functions through multiple mechanisms that include inhibition of gene transcription, destabilization of mRNA, and suppression of protein translation (2, 8, 10, 12, 16, 26, 27, 41, 67). Some of these effects have been reported to reflect the ability of IL-10 to antagonize PRR-induced signaling and thereby inhibit the activation of NF-κB and p38 MAPK (17, 27, 53, 62). However, these mechanisms appear insufficient to account for the breadth of IL-10's biological effects. Additionally, the extent to which IL-10 mediates its effects via cell- or species-specific mechanisms of action remains unclear.

It is now generally accepted that IL-10 manifests its anti-inflammatory effects by binding to a specific heterodimeric cell surface receptor (consisting of the IL-10R1 and IL-10R2 polypeptides), resulting in the activation of the receptor-associated tyrosine kinases JAK1 and Tyk2 and the latent transcription factor STAT3 (13, 39). STAT3 activation was shown to be obligatory for IL-10's anti-inflammatory effects on both mouse and human mononuclear phagocytes (51, 59, 65). Mice lacking STAT3 specifically in macrophages and neutrophils display an overexuberant inflammatory response phenotype similar to that of mice lacking either IL-10 or the IL-10R2 receptor subunit (59, 64). Based on the findings that STAT3 and new protein synthesis are required for IL-10 function, several groups performed large-scale gene microarray analyses of human and mouse myeloid cells to identify IL-10-regulated genes (25, 31, 47, 66). At present, relatively few of the IL-10-induced genes identified thus far have been examined for their role in mediating IL-10 action (16, 42, 67). The genes that have been proposed to be functionally important appear to act selectively in specific IL-10-dependent anti-inflammatory functions, and as a result, no single IL-10-induced gene acts as a master regulator of the cytokine's broad effects.

Using a representational difference analysis (RDA) approach, we cloned a novel gene product that was induced in

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mouse macrophages by the combination of IL-10 and LPS and was homologous in sequence to human LIND (*Listeria* INDuced). LIND had been previously identified as a gene product of unknown function expressed in human monocytes during *Listeria monocytogenes* infection and, based on sequence homology, was also termed the third member of the ABIN (*A*20-*b*inding *i*nhibitor of *N*F-κB activation) family of proteins, i.e., ABIN-3 (21, 57). Because previously characterized members of the ABIN family are capable of inhibiting NF-κB activation in response to particular stimuli in vitro (20, 24, 35, 61), we addressed the question of whether the IL-10-inducible ABIN-3 protein we identified in mouse macrophages possessed an anti-inflammatory effector function. Herein we show that IL-10, in combination with proinflammatory stimuli, such as LPS, leads to optimal induction of ABIN-3 expression in both mouse and human mononuclear phagocytes. Although enforced expression of mouse ABIN-3 in cells had no effect on the activation and function of the NF-κB pathway, expression of the human ortholog indeed suppressed both NF-κB activation and induction of proinflammatory gene expression. This functional disparity was due, in part, to the absence of an intact ABIN homology domain in mouse ABIN-3. These results identify a novel divergence between IL-10's actions on human and mouse cells, which may have significant mechanistic or functional consequences for anti-inflammatory function between different species.

MATERIALS AND METHODS

Reagents. Recombinant cytokines were obtained from the following sources: mouse IL-10 (Schering-Plough), human IL-10 (eBioscience), mouse IL-6 and human IL-1β (R&D Systems), mouse IL-1β (UCB/Celltech), human alpha interferon A/D (IFN-αA/D; Hoffman-LaRoche), and mouse and human tumor necrosis factor alpha (TNF-α) (Genentech). The IL-10 receptor-neutralizing antibody (αIL-10R1 monoclonal antibody [MAb], clone 1B13A) was provided by Schering-Plough. LPS (from *Escherichia coli* 0127:B8), PGN (from *Staphylococcus aureus*), and poly(I:C) were obtained from Sigma. Phosphorothioate-stabilized CpG oligodeoxynucleotide (5′-TCC ATG ACG TTC CTG ACG TT) (19) and non-CpG oligodeoxynucleotide (5′-CCA TGG CCA TGG CCA TGG) (15) were obtained from IDT.

Cell culture and mice. The mouse monocyte/macrophage RAW264.7 and the human epithelial HEK 293T cell lines were cultured in complete Dulbecco's minimum essential medium with 10% fetal calf serum. The human monocytic cell lines THP-1 and U937 were cultured in complete RPMI 1640 medium supplemented with 10% fetal calf serum and 50 μM 2-mercaptoethanol. Mouse bone marrow-derived macrophages (BMM) were prepared as described previously (9). IL-10-null mice were obtained from the Jackson Laboratory and bred alongside wild-type mice in a specific-pathogen-free facility in accordance with American Association of Laboratory Animal Science guidelines.

Cloning of mouse ABIN-3 and ABIN-3β. RAW264.7 cells were stimulated with either LPS (50 ng/ml) alone or LPS plus IL-10 (100 ng/ml) for 2 h, and RDA was performed as described previously (23, 43). After three sequential rounds of subtraction using tester/driver ratios of 1:50, 1:400, and 1:800, final PCR products were cloned into pBluescript II SK. A λ phage cDNA library was constructed (Uni-ZAP-XR; Stratagene) from LPS plus IL-10-stimulated RAW264.7 and screened with an RDA-derived probe, yielding full-length mouse ABIN-3 and ABIN-3β cDNA.

Plasmid constructs and antibodies. The cDNAs for mouse ABIN-3Long (GenBank accession no. AK033534), ABIN-1, ABIN-2, Toll-like receptor 2 (TLR2), TLR3, TLR4, MD2, and CD14 were amplified by reverse transcription (RT)-PCR from BMM-derived total RNA using SuperScript RT (Invitrogen) and *Pfu* Turbo (Stratagene). Similarly, the cDNAs for human ABIN-3, ABIN-3β, RIP1, TRAF6, IKKγ, IKKβ, and RelA were subcloned from human leukocyte-derived cDNA (BD Clontech). The mouse A20 cDNA was kindly provided by David Goeddel (Amgen, San Francisco, CA). The human ABIN-3 mutants ΔAHD1 (amino acids [aa] 1 to 151 fused to aa 173 to 325), ΔAHD2 (aa 1 to 198 fused to aa 222 to 325), and ΔAHD1/2 (aa 1 to 151 fused to aa 222 to 325) were constructed by PCR and blunt-end ligation. For protein expression, cDNAs were subcloned into pcDNA3 (Invitrogen) or the retroviral green fluorescent protein (GFP)-RV vector (44, 50). For antibody generation, glutathione *S*-transferase fusion proteins containing sequences specific for mouse ABIN-3 (aa 141 to 208) or mouse ABIN-3β (aa 141 to 168) or common to both isoforms (aa 17 to 89) were bacterially expressed, and hamster MAbs against ABIN-3β (T1C1H10) and ABIN-3 (Tgr21A5) were produced.

Gene targeting. ABIN-3 (encoded by the gene *Tnip3*; official symbol 9030611K07Rik) genomic clones were isolated from a 129/Sv genomic library (Lambda FIX II; Stratagene). The 5′ homology arm represented a 1.6-kb NcoI-NcoI fragment located upstream of exon 1 and was subcloned into the NotI-linearized TNLOX1-3 gene-targeting vector (49). The 3′ homology arm represented a 6.0-kb SacII-SalI fragment located downstream of exon 5 and was subcloned into the AscI-PmeI site of the vector. The resulting vector was linearized and electroporated into the MC-50 embryonic stem (ES) cell line (55). Two hundred and sixty-seven ES clones doubly resistant to G418 and ganciclovir were screened for homologous recombination by PCR and Southern blotting with 5′ and 3′ external probes. Three correctly targeted clones were identified and, following in vitro Cre-mediated excision of the neomycin resistance gene, two clones transmitted the mutant allele to produce independent lines of ABIN-3-null mice (129/Sv background).

Retroviral vector transduction. For retrovirus packaging, HEK 293T cells were transfected with 2.5 μg GFP-RV vector (empty or containing an N-terminal Myc epitope-tagged human ABIN-3 ortholog [hABIN-3]), 2.0 μg pUMVC3-gag-pol, and 0.5 μg VSVg expression plasmids. THP-1 cells were infected in retroviral supernatant supplemented with 4 μg/ml Polybrene (Sigma). Transduced cells were enriched for GFP expression (under an internal ribosome entry site) by multiple rounds of cell sorting using a Vantage SE model fluorescence-activated cell sorter (BD Biosciences) and maintained at a purity of >97% as measured by flow cytometry.

mRNA analysis. For Northern blotting, 15 μg of total RNA (RNA-Bee; Tel-Test, Inc.) was fractionated in agarose-formaldehyde gels, transferred to nylon membranes (Zeta Probe; Bio-Rad), and hybridized using PerfectHyb Plus (Sigma). For quantitative real-time RT-PCR (qRT-PCR), 1 μg of DNase I-treated total RNA was used to make random- or oligo(dT)-primed first-strand cDNA using SuperScript RT (Invitrogen). Expression was measured using SYBR Green (Applied Biosystems) and an ABI Prism 7000 (Applied Biosystems). Primers were designed using either MacVector (Accelrys Software) or PrimerBank (63) and are available upon request.

NF-κB-dependent reporter gene assay. HEK 293T cells were plated in either duplicate (in 24-well plates) or triplicate (in 96-well plates) and 24 h later were transfected with 1 μg or 0.33 μg total DNA using Lipofectamine 2000 (Invitrogen), respectively. Each transfection contained the indicated expression plasmids, with 120 ng of 2κB-Luc reporter plasmid (34) and 30 ng of control pRL-TK plasmid (*Renilla* luciferase; Promega). Twenty-four hours posttransfection, cells were stimulated for 5 h and lysed in Passive lysis buffer (Promega), and luciferase was measured using a dual-luciferase reporter assay system (Promega) and a Monolight 3096 luminometer (BD Pharmingen).

Electrophoretic mobility shift assay. Ten micrograms of nuclear extract was incubated with IRDye700-labeled DNA probes (LI-COR) for NF-κB (Ig κ promoter) or for STAT1/3 (M67) (52). Each reaction included an IRDye800-labeled Y-box (Eα promoter) probe to normalize loading to the constitutive NF-Y complex. Protein-DNA complexes were resolved on 5% gels and imaged with an Odyssey infrared imaging system (LI-COR).

Immunoprecipitation and Western blotting. For immunoprecipitation, cell lysates were prepared by freeze-thaw in isotonic buffer containing 0.1% NP-40 and were incubated with anti-Myc-agarose beads (Santa Cruz Biotechnology). For Western blotting, whole-cell lysates were prepared in boiling sodium dodecyl sulfate (SDS) lysis buffer (50 mM Tris [pH 7.6], 0.5% SDS, and 2 mM dithiothreitol). For Western blots coupled to luciferase assays, after preparation of luciferase lysates, the pellets were solubilized in boiling SDS lysis buffer. Cell equivalents of soluble and insoluble extracts were acetone precipitated, fractionated, and blotted. Western blots were visualized with Alexa 680-conjugated (Molecular Probes) or IRDye800-conjugated (Rockland) secondary antibodies and an Odyssey infrared imaging system (LI-COR). The Myc (9E10), IKKγ (FL-419), IκBα (FL), glycemldehyde-3-phosphate dehydrogenase (GAPDH) (V-18), and Lamin B1 (C-20) antibodies were obtained from Santa Cruz Biotechnology.

Nucleotide sequence accession number. The nucleotide sequences of mouse ABIN-3, mouse ABIN-3β, and human ABIN-3β have been submitted to GenBank under accession numbers EF585494, EF585495, and EF585496, respectively.

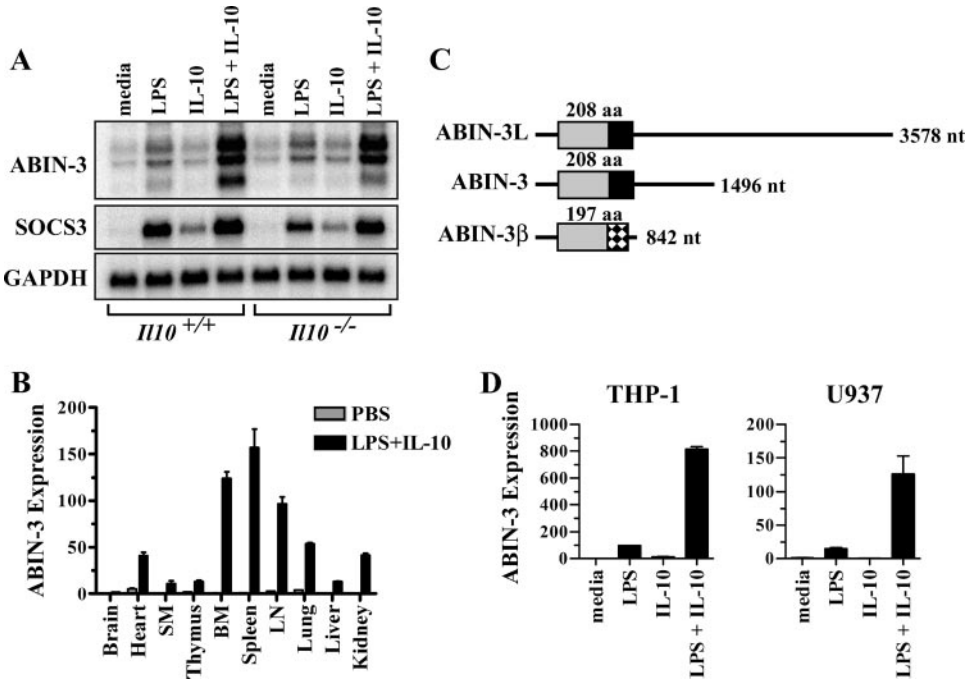

FIG. 1. IL-10 induces ABIN-3 gene expression in LPS-stimulated mononuclear phagocytes. (A) BMM from wild-type (*Il10*$^{+/+}$) or IL-10-null (*Il10*$^{-/-}$) mice were cultured in the absence (media) or presence of LPS (100 ng/ml), IL-10 (20 ng/ml), or LPS plus IL-10 for 2 h. Total RNA was prepared and analyzed by Northern blotting. (B) 129/Sv mice were injected intraperitoneally with phosphate-buffered saline (PBS) or LPS (250 μg) plus IL-10 (5 μg). After 2 h, tissue total RNA was prepared and analyzed by qRT-PCR in duplicate, and values normalized to 18S rRNA are presented as means ± standard deviations (SD). SM, skeletal muscle; BM, bone marrow; LN, lymph nodes. (C) Schematic of the mouse ABIN-3 transcripts. The ABIN-3 and ABIN-3L mRNAs are derived by alternative polyadenylation and encode the identical protein, while the ABIN-3β mRNA is derived by alternative splicing and encodes a protein with a distinct C terminus (diamond pattern versus black, respectively) and 3′ untranslated region. (D) THP-1 and U937 cells were cultured in the absence (media) or presence of LPS (100 ng/ml), IL-10 (20 ng/ml), or LPS plus IL-10 for 2 h. Total RNA was analyzed by qRT-PCR and values normalized to GAPDH are presented as means ± SD.

RESULTS

ABIN-3 expression is induced by IL-10 in LPS-stimulated macrophages. In order to identify novel IL-10-induced genes in LPS-stimulated macrophages, we performed RDA using the mouse monocyte/macrophage RAW264.7 cell line stimulated with IL-10 plus LPS or with LPS alone. After screening approximately 500 enriched cDNA fragments, we identified a novel 200-base pair gene product that was preferentially induced in IL-10 plus LPS-stimulated cells. Using this gene fragment to screen a cDNA library, we isolated full-length clones that, upon sequence analysis, were found to represent the mouse ortholog of human LIND, a gene discovered previously whose expression was induced in human monocytes infected with *Listeria monocytogenes* (57). Based on sequence homology, LIND has also been termed human ABIN-3, the third member of the ABIN family of proteins (21).

We confirmed that the combination of IL-10 plus LPS induced the expression of ABIN-3 in mouse macrophages and tissues. Three ABIN-3-related transcripts were slightly induced in BMM following LPS stimulation (as detected by Northern blotting), not induced by IL-10 alone, but were robustly induced by the combination of IL-10 and LPS (Fig. 1A). This expression pattern was distinct from that of SOCS3, which was induced by either LPS or IL-10 alone and showed a modestly enhanced induction by the combined stimuli. Similar results were obtained using RAW264.7 cells or thioglycolate-elicited peritoneal exudate macrophages (data not shown). The modest induction of ABIN-3 by LPS stimulation alone was still observed when BMM from the IL-10-null mouse were tested, thereby documenting that LPS alone could indeed induce a low level of expression in the absence of the autocrine action of IL-10. In vivo, as detected by qRT-PCR, ABIN-3 transcripts displayed low or undetectable basal expression in tissues from naive mice but were induced, particularly in myeloid-rich tissues, after mice were injected with the combination of IL-10 and LPS (Fig. 1B).

A schematic of the three mouse ABIN-3 transcripts, indicating their open reading frames, is presented in Fig. 1C. The three mRNA species derive from a single gene in mice, *Tnip3*, and are generated by way of either alternative splicing (in the case of the smallest form, referred to hereafter as ABIN-3β) or alternative polyadenylation (in the case of the largest form, referred to hereafter as ABIN-3Long or ABIN-3L). The mouse ABIN-3 and ABIN-3L transcripts have identical open reading frames and encode the 208-aa ABIN-3 protein isoform. In contrast, the ABIN-3β splice variant encodes a 197-aa protein that is identical to mouse ABIN-3 from residues 1 to 140 but has a unique 57-aa C terminus and 3′ untranslated region.

To determine whether ABIN-3 gene expression was also regulated by IL-10 in humans, the human monocytic cell lines THP-1 and U937 were stimulated with LPS, IL-10, or the combination of LPS plus IL-10, and ABIN-3 mRNA levels were measured by

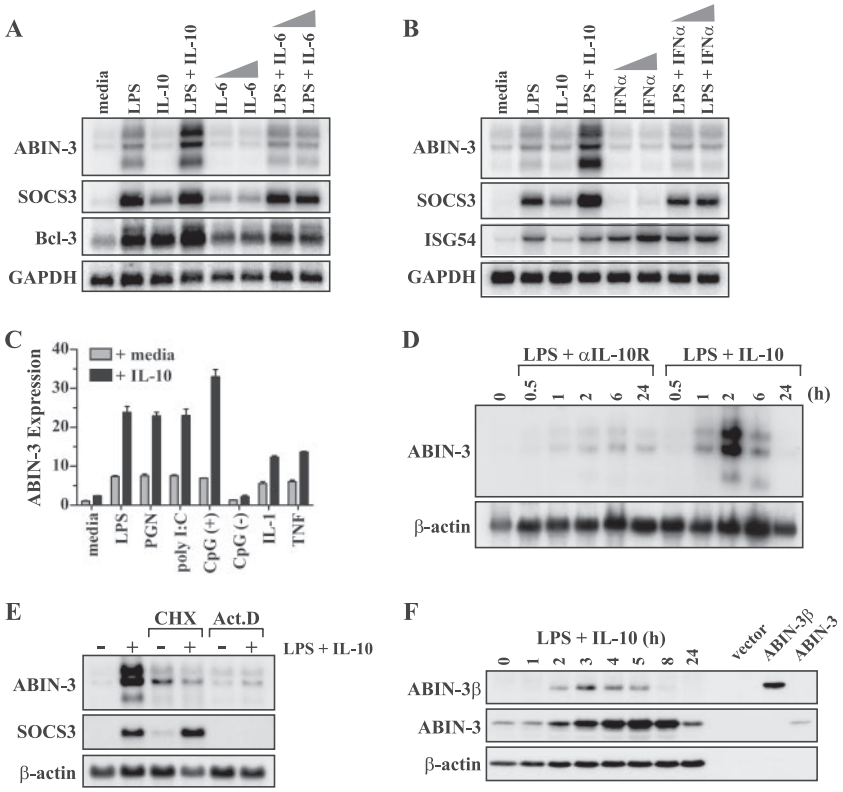

FIG. 2. ABIN-3 is a delayed-response gene induced selectively by IL-10 in the presence of proinflammatory stimuli. (A) BMM from *Il10*$^{-/-}$ mice were cultured in the absence (media) or presence of LPS (100 ng/ml), IL-10 (10 ng/ml), IL-6 (10 ng/ml or 100 ng/ml) alone or in combination as indicated for 2 h and analyzed by Northern blotting. (B) Similar to that shown in panel A, except that IFN-α was substituted for IL-6. (C) BMM from wild-type 129/Sv mice were cultured in the absence (media) or presence of IL-10 (20 ng/ml) alone or in combination with LPS (100 ng/ml), PGN (20 μg/ml), poly(I:C) (150 μg/ml), CpG DNA [CpG (+)] (10 μg/ml), non-CpG DNA control [CpG (−)] (10 μg/ml), IL-1β (10 ng/ml), or TNF-α (10 ng/ml) for 2 h and analyzed by qRT-PCR in duplicate. Expression was normalized to 18S rRNA, and values are presented as means ± standard deviations (SD). (D) BMM were cultured in the presence of LPS (100 ng/ml) together with αIL-10R1-neutralizing MAb (10 μg/ml) or with IL-10 (20 ng/ml) and analyzed by Northern blotting. (E) BMM were cultured with LPS (100 ng/ml) plus IL-10 (20 ng/ml) for 2 h, in either the absence or the presence of cycloheximide (CHX; 10 μg/ml) or actinomycin D (Act.D; 10 μg/ml), and analyzed by Northern blotting. (F) BMM were cultured for the indicated times in the presence of LPS (100 ng/ml) plus IL-10 (20 ng/ml), and total cell lysates were analyzed by Western blotting. Equivalent amounts of in vitro-translated proteins (empty vector, ABIN-3β, or ABIN-3) were loaded as controls.

qRT-PCR (Fig. 1D). As was the case with mouse, human ABIN-3 transcripts were induced strongly by IL-10 in the presence of LPS.

Characteristics of ABIN-3 induction: agonist specificity and requirement for de novo protein synthesis. We assessed the selectivity of ABIN-3 induction by IL-10 and LPS with mouse macrophages. First, we determined whether ABIN-3 could be induced by proinflammatory cytokines that also activate STAT3 such as IL-6 and type I IFN (IFN-α/β). Stimulation of BMM with IL-6 did not lead to the induction of ABIN-3, either when the cytokine was added alone or in the presence of LPS (Fig. 2A). In contrast, IL-6 induced the expression of the SOCS3 and Bcl-3 control genes. Likewise, stimulation of BMM with IFN-α, either alone or in combination with LPS, failed to induce significant ABIN-3 expression, although IFN-α indeed induced expression of the ISG54 control gene (Fig. 2B). Thus, ABIN-3 expression is induced selectively in response to IL-10, relative to proinflammatory cytokines that activate JAK-STAT pathways including STAT3.

We next tested whether ABIN-3 expression could be induced by IL-10 in the presence of proinflammatory agonists other than LPS. For this purpose, BMM were stimulated with IL-10 in the presence of LPS, PGN, double-stranded RNA [poly(I:C)], unmethylated CpG DNA, IL-1, or TNF. As was the case for LPS, ABIN-3 expression was induced by IL-10 in the presence of each of these proinflammatory stimuli (Fig. 2C). Thus, ABIN-3 is an IL-10-regulated gene product that is induced in macrophages undergoing proinflammatory responses, i.e., that are being stimulated by TLR, IL-1R, and TNF receptor agonists.

To characterize the kinetics of ABIN-3 induction, a time course of stimulation was analyzed by Northern blotting. In response to IL-10 and LPS, ABIN-3 transcripts were induced at 1 h, reached maximal levels at 2 h, and began to decline by 6 h (Fig. 2D). ABIN-3 expression was not induced when macrophages were cultured in the presence of the translation inhibitor cycloheximide or the transcription inhibitor actinomycin D, indicating that ABIN-3 induction depends on both de novo protein synthesis and gene transcription (Fig. 2E). This characteristic is in contrast to the induction of SOCS3, which occurred independently of de novo protein synthesis. Both of the ABIN-3 protein isoforms could be detected in stimulated mouse BMM and displayed induction kinetics that reflected

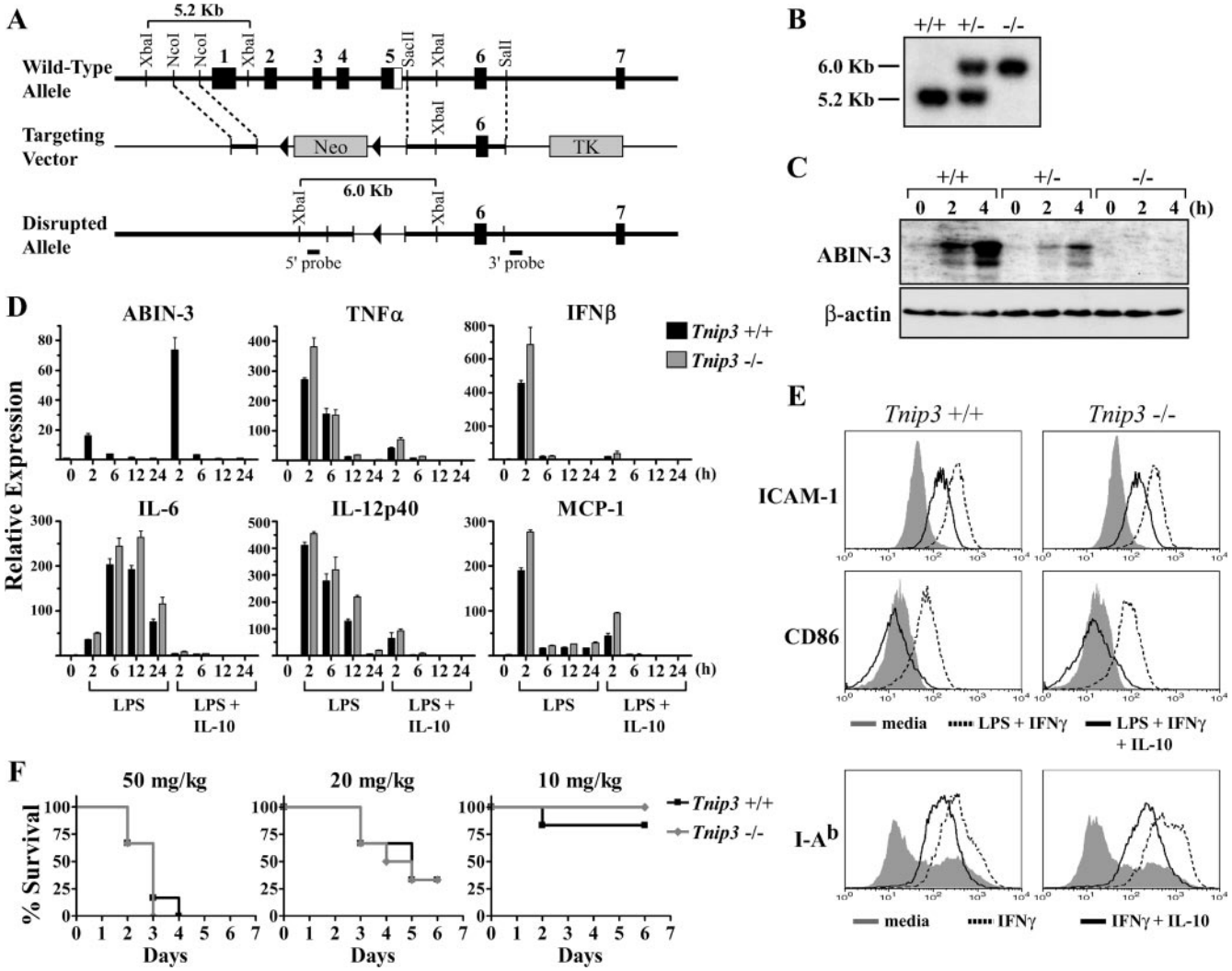

FIG. 3. ABIN-3-null macrophages and mice display normal responses to IL-10 and LPS. (A) Schematic of the mouse ABIN-3 locus (*Tnip3*), the gene targeting vector, and the disrupted allele with relevant features indicated, including the neomycin resistance (Neo) cassette flanked by *loxP* sites (black triangles), the thymidine kinase (TK) cassette, and the external Southern blotting probes. (B) Southern blotting of XbaI-digested genomic DNA from wild-type (+/+) mice and mice heterozygous (+/−) or homozygous (−/−) for the mutant *Tnip3* allele, using a 5′ external probe. (C) Western blotting with ABIN-3-specific MAb demonstrating a lack of protein expression in $Tnip3^{-/-}$ BMM stimulated with LPS (100 ng/ml) and IL-10 (20 ng/ml). (D) Wild-type ($Tnip3^{+/+}$) or ABIN-3-null ($Tnip3^{-/-}$) BMM were stimulated with LPS (100 ng/ml) alone or in the presence of IL-10 (10 ng/ml) for the indicated times, and total RNA was analyzed by qRT-PCR in duplicate. Values are normalized to 18S rRNA and presented as means ± standard deviations. (E) BMM were mock stimulated (gray histograms) or stimulated with IFN-γ (10 ng/ml) or IFN-γ plus LPS (100 ng/ml) (dashed lines) in the absence or presence of IL-10 (10 ng/ml) (solid lines). Surface levels of ICAM-1, $I\text{-}A^b$, or CD86 were measured by flow cytometry. (F) $Tnip3^{+/+}$ ($n = 6$) and $Tnip3^{-/-}$ ($n = 6$) mice were injected intraperitoneally with LPS as indicated and monitored daily.

that of their mRNAs. The cellular ABIN-3β protein disappeared more rapidly than the ABIN-3 isoform, suggesting that the former was less stable (Fig. 2F). These data collectively characterize ABIN-3 as a delayed response gene downstream of the combined IL-10 and TLR/IL-1/TNF stimulation in macrophages.

ABIN-3 is not required for IL-10's anti-inflammatory actions or NF-κB regulation in mice. In view of the selective regulation of ABIN-3 by IL-10 and the fact that other ABIN family members (namely, ABIN-1 and ABIN-2) were characterized as NF-κB inhibitors in overexpression studies, we tested whether mouse ABIN-3 was required for IL-10's anti-inflammatory actions by generating ABIN-3 knockout mice (Fig. 3). The gene that encodes ABIN-3 (*Tnip3*) is located on mouse chromosome 6 and comprises 8 exons. Our gene-targeting strategy deleted exons 1 to 5, which includes all of the N-terminal sequence shared between ABIN-3 and ABIN-3β, as well as the C-terminal sequence specific to ABIN-3β (Fig. 3A). Wild-type (+/+), heterozygous (+/−), and homozygous (−/−) ABIN-3 knockout mice were identified by restriction fragment length polymorphism/Southern blotting (Fig. 3B). ABIN-3-null mice were born in the expected Mendelian distribution (70 wild type:140 heterozygous:91 knockout) and were fertile. BMM from ABIN-3-null mice expressed neither

ABIN-3 transcripts nor proteins when tested before or after stimulation (Fig. 3C and D and data not shown).

In contrast to mice that lack IL-10 responses, ABIN-3-null mice displayed no clinical phenotype. IL-10-null mice develop spontaneous enterocolitis within the first few months of age, with a disease severity that is dependent on the genetic background of the gene-targeted mouse (129/Sv and BALB/c mice display greater severity than C57BL/6 mice) (3, 28, 56, 59). Associated with the inflammatory disease, the peripheral myeloid and lymphoid cells from IL-10-null mice display a hyperactive phenotype compared to control cells (28, 59). In contrast, our ABIN-3-null mice, generated and maintained on the susceptible 129/Sv genetic background, did not develop spontaneous inflammatory or autoimmune disease. ABIN-3-null mice developed normally, exhibited normal tissue and organ architecture, and showed no signs of inflammatory disease even when monitored up to 18 months of age (data not shown). Furthermore, ABIN-3-null mice displayed normal levels and activation status of peripheral lymphoid and myeloid cells.

To directly test whether mouse ABIN-3 was required for the anti-inflammatory effects of IL-10 on macrophages in vitro, we compared proinflammatory gene induction in BMM from mice with an intact versus a disrupted ABIN-3 gene. IL-10 produced the same suppressive effects on LPS-induced proinflammatory gene expression in macrophages from ABIN-3-null mice as it did from wild-type mice (Fig. 3D). ABIN-3-null macrophages did not exhibit quantitatively or kinetically enhanced responses to LPS signaling in the absence or presence of IL-10 compared to that of wild-type macrophages. Moreover, IL-10 responses in ABIN-3-null macrophages were equivalent to those of wild-type macrophages throughout a wide dose range of cytokine and LPS (data not shown). Additionally, no differences were observed for either LPS- or TNF-α-dependent activation of NF-κB or for the induction of classical NF-κB IκBα and A20 target genes (data not shown). Last, IL-10-mediated suppression of LPS and/or IFN-γ-induced surface expression of major histocompatibility class II ($I\text{-}A^b$), B7-2 (CD86), and intercellular adhesion molecule (ICAM)-1 was also unaltered in ABIN-3-null macrophages (Fig. 3E). Similar results were obtained when elicited peritoneal exudate macrophages were examined (data not shown).

To test whether ABIN-3 deficiency led to discernible effects on IL-10 responses in vivo, we assessed the susceptibility of ABIN-3-null mice in three disease models for which IL-10 is known to play a critical role. Compared to wild-type mice, IL-10-null mice exhibit increased sensitivity to endotoxin shock (4), increased susceptibility to experimental colitis induced by oral administration of dextran sulfate sodium (60), and increased resistance to infection with microbial pathogens including *Listeria monocytogenes* (11, 39). However, when they were tested in these same models, no differences could be detected between ABIN-3-null mice and wild-type mice (Fig. 3F and data not shown). Taken together, these results indicate that ABIN-3 is not an essential mediator of IL-10's broad anti-inflammatory actions in mice and, moreover, that it does not appear to play an obligatory role in the negative regulation of NF-κB or other proinflammatory signaling pathways.

Functional divergence in mouse and human ABIN-3 for NF-κB inhibition. The aforementioned experiments were complemented by testing whether enforced expression of ABIN-3 was sufficient to effect NF-κB inhibition akin to that of ABIN-1 and ABIN-2. Using a reporter gene assay, HEK 293T cells transfected with an NF-κB-dependent luciferase gene expressed luciferase in response to TNF stimulation (Fig. 4A). This induction was completely inhibited by the coexpression of mouse A20 protein and partially inhibited by coexpression of mouse ABIN-1 or mouse ABIN-2. In contrast, enforced expression of mouse ABIN-3 (mABIN-3) or mABIN-3β, singly or in combination, had no effect on NF-κB activation (Fig. 4A and data not shown). The lack of activity of mouse ABIN-3 could not be explained simply by a deficit of protein expression since the Myc epitope-tagged mouse ABIN-3 was present at levels comparable to or higher than those of the functional Myc-tagged ABIN family members (Fig. 4A, right panel). Mouse ABIN-3β, which also did not inhibit NF-κB, was not significantly expressed in the transfected cells, confirming its relative instability. In contrast, enforced expression of hABIN-3 inhibited NF-κB activation in a manner similar to that of ABIN-1 and ABIN-2 (Fig. 4A). The inhibitory activity of human ABIN-3 was dose dependent (i.e., dependent on protein expression level) and was generalizable to IL-1-induced NF-κB activation as well (Fig. 4B).

We next addressed whether enforced expression of human ABIN-3 was sufficient to inhibit TLR-induced NF-κB activation. As TLRs diverge in their usage of specific TIR-containing adaptor proteins for signaling (1), we tested the effect of human ABIN-3 against a representative group of receptors which included TLR2, TLR3, and TLR4. Notably, human ABIN-3 inhibited LPS-induced NF-κB activation in HEK 293T cells engineered to express the TLR4 receptor complex (Fig. 4C). Likewise, enforced expression of human ABIN-3 inhibited TLR2- and TLR3-induced NF-κB activation. In contrast, mouse ABIN-3 or ABIN-3β failed to inhibit TLR-induced NF-κB activation. Collectively, these results demonstrate that although ABIN-3 represents an IL-10-inducible gene in both humans and mice, only the human protein is capable of suppressing proinflammatory signaling pathways leading to canonical NF-κB activation.

Functional divergence correlates with genetic divergence: mouse ABIN-3 lacks the ABIN homology domain. Human ABIN proteins share only a short stretch of protein sequence homology, referred to as the ABIN homology domain (AHD). The AHD consists of two homologous stretches of approximately 20 amino acids each, separated by a stretch of approximately 30 nonhomologous amino acids (21, 61). The two homologous subregions within the AHD have been designated AHD1 and AHD2 (21). Deletion of the entire AHD from ABIN-1 or ABIN-2 or site-directed mutagenesis within the AHD2 subdomain of ABIN-1 renders the proteins incapable of inhibiting NF-κB (21, 36). These results point to a critical role for the AHD and specifically the AHD2 sequence in mediating the ability of ABINs to inhibit NF-κB. The AHD1 subdomain, on the other hand, plays a functional role in mediating the ability of ABINs to interact with A20. Thus, the AHD1 and AHD2 subdomains appear to mediate distinct ABIN activities.

Sequence alignment of the functionally divergent human and mouse ABIN-3 proteins revealed that the mouse protein lacks 92 amino acids present within the C terminus of human ABIN-3. Consequently, mouse ABIN-3 is devoid of all but the

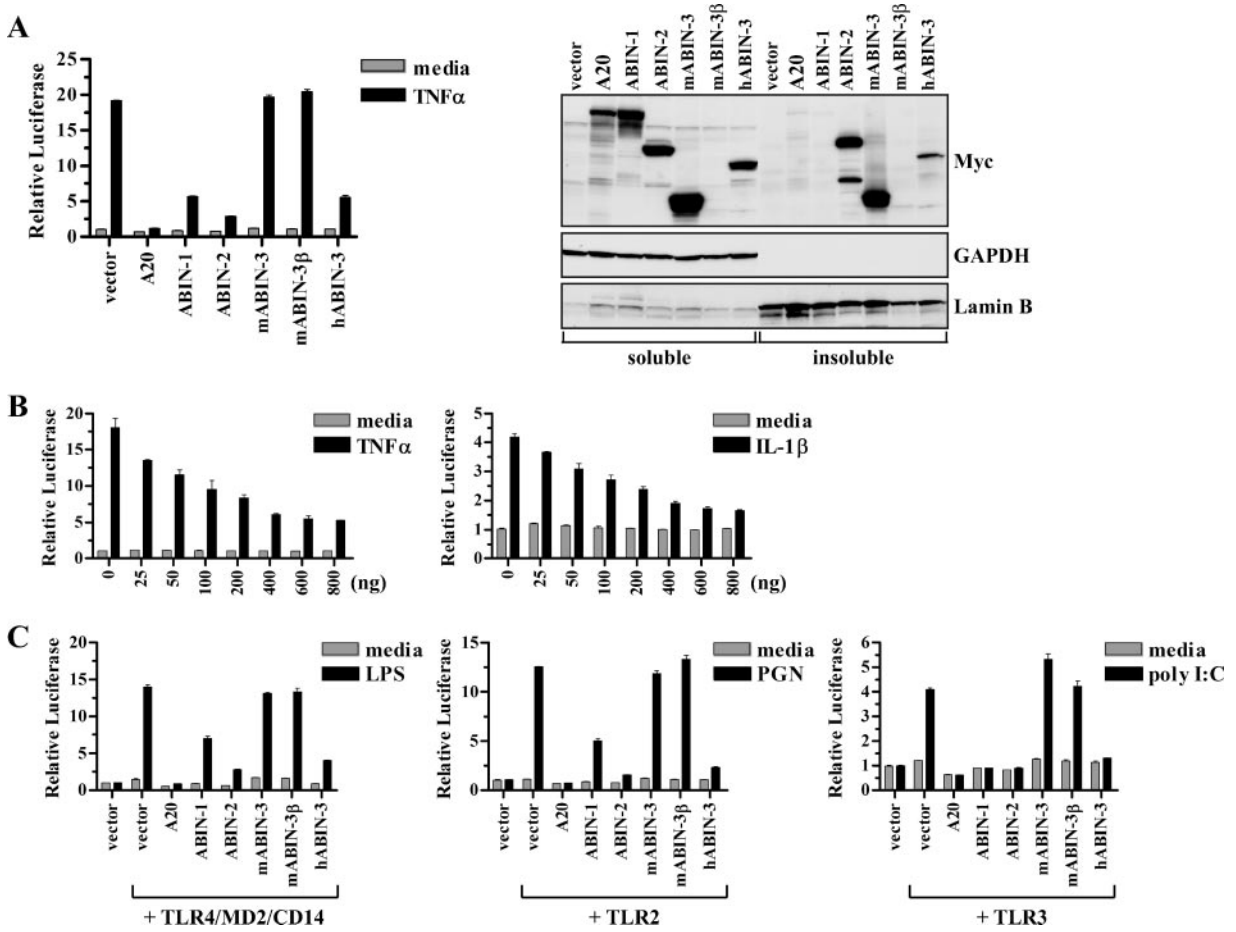

FIG. 4. Expression of human ABIN-3, but not mouse ABIN-3, inhibits NF-κB activation in response to TNF, IL-1, and TLR signaling. (A) NF-κB-dependent luciferase assay with HEK 293T cells cotransfected in duplicate with the indicated expression plasmids and cultured in the absence (media) or presence of TNF-α (20 ng/ml) for 5 h. (Left panel) Normalized luciferase activity is presented as means ± standard deviations. (Right panel) Western blotting monitoring transfected protein expression. (B) Similar to panel A, except that cells were transfected with a dose range of hABIN-3 expression plasmid and cultured in the absence (media) or presence of TNF-α (20 ng/ml) or IL-1β (20 ng/ml). (C) Similar to panel A, except that cells were cotransfected with plasmids for the TLR4 receptor complex, TLR2, or TLR3 and cultured in the absence (media) or presence of LPS (500 ng/ml), PGN (40 μg/ml), or poly(I:C) (150 μg/ml) for 5 h.

five most N-terminal residues of the AHD2 subdomain (Fig. 5A). In humans, this specific sequence is derived from three genomic exons that are not present in the mouse gene (Fig. 5C). Alternatively, both the mouse and human ABIN-3β splice variants display even greater deficits in the ABIN homology domain because both lack approximately half of AHD1 and all of the intervening and AHD2 sequences. In both species, ABIN-3β arises as a consequence of analogous alternative splicing of the ABIN-3 gene. Despite this equivalent processing, the encoded C-termini of the human and mouse ABIN-3β proteins are distinct due to a lack of sequence conservation within the corresponding region of exon 5 between the human and mouse genes (Fig. 5B and C).

We have not been able to identify, through either bioinformatic or experimental means, a mouse ABIN-3 transcript that contains a human-like insertion sequence containing the AHD2, nor have we been able to identify a human ABIN-3 splice variant that lacks all or part of the 92-amino-acid insertion sequence and AHD2 analogous to the mouse ortholog. An examination of publicly available genome sequences revealed that the rat ABIN-3 ortholog was similar to the mouse (i.e., it lacks the human-like AHD2-containing sequence), while ABIN-3 from chimpanzee, cow, dog, and chicken were similar to the human protein (Fig. 5D).

The ABIN homology domain is required but not sufficient for human ABIN-3 function. We performed structure-function analyses to address whether the functional divergence between human and mouse ABIN-3 was due to the presence or absence of the intact AHD. Deletion of AHD1, AHD2, or the entire AHD from human ABIN-3 had no deleterious effect on the expression or stability of the protein (Fig. 6A). Nonetheless, the ability of human ABIN-3 to inhibit NF-κB activity was partially impaired by deletion of AHD1 and completely abrogated by deletion of either AHD2 or the entire AHD. These latter two human mutants functionally resembled mouse ABIN-3, which naturally lacks the AHD2 sequence (Fig. 6B). Thus, the presence of the intact AHD is required for the anti-inflammatory effects of human ABIN-3 and is sufficient to account for the functional disparity between the human and mouse proteins.

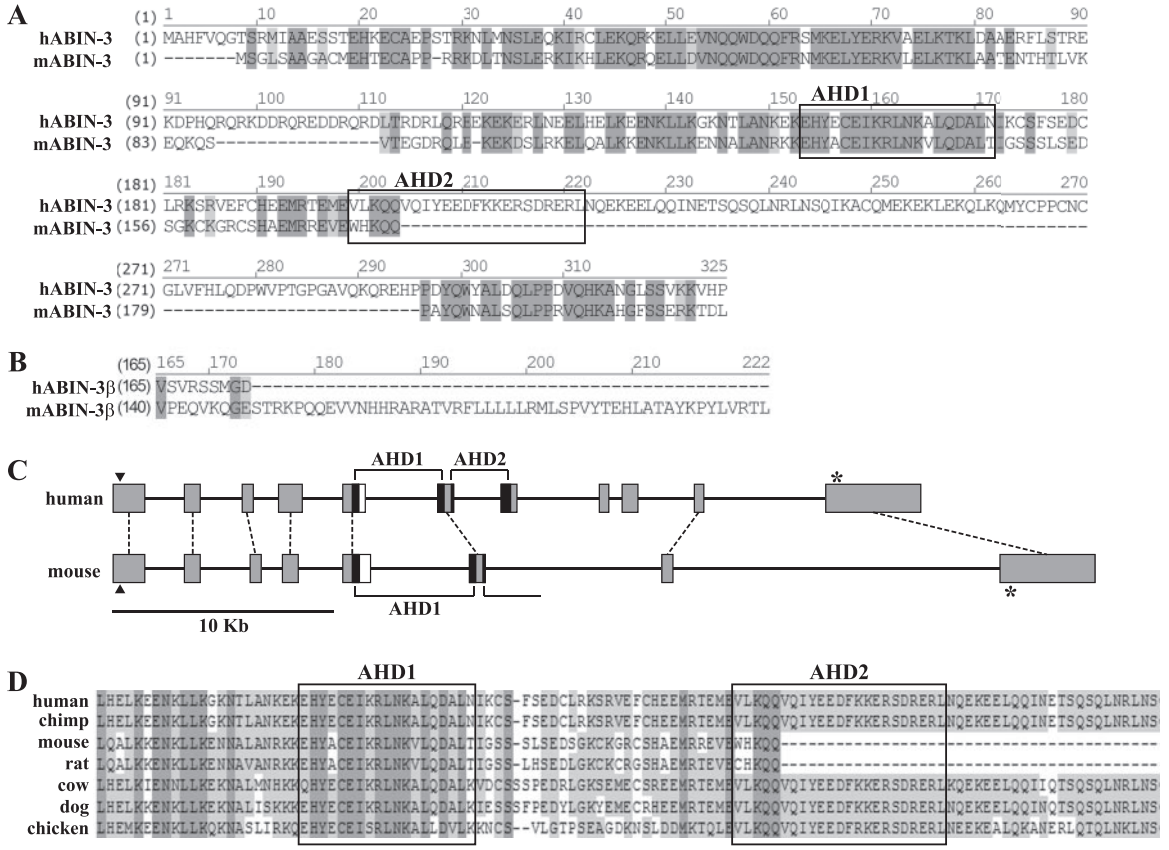

FIG. 5. Mouse ABIN-3 lacks a complete ABIN homology domain. (A) Protein sequence alignment of human and mouse ABIN-3. AHD1 and AHD2 subdomains of human ABIN-3 are shown. (B) Protein sequence alignment of human and mouse ABIN-3β, focusing on their unique β splice variant-specific C-terminal sequences starting at aa 165 of human and aa 140 of mouse. (C) Schematic alignment of the human and mouse ABIN-3 genes. Gene and intron lengths are drawn to scale, while exons (boxes) are shown enlarged but relative in size to one another. Arrowheads and asterisks indicate the translation initiation and termination sequences, respectively. Dotted lines indicate homologous exons. Exon regions that give rise to AHD1 and AHD2 are shaded black, while exon 5 regions specific to the ABIN-3β splice variants are white. (D) Protein sequence alignment of ABIN-3 from multiple species corresponding to the region surrounding the AHD.

We next addressed whether mouse ABIN-3 could be converted into a functional protein by generating a mouse-human-mouse chimera containing the 92-amino-acid insertion sequence of human ABIN-3 that includes AHD2 (Fig. 5A and Fig. 6C). Although this chimeric protein was expressed at sufficient levels relative to that of human ABIN-3, it was still incapable of inhibiting NF-κB activation as measured by the reporter gene assay (Fig. 6C and D). In this chimeric protein, the first 5 residues of AHD2 (WHKQQ) were derived from a mouse sequence while the more C-terminal 18 residues were derived from human (Fig. 5A and Fig. 6C). We considered the possibility that the inactivity of the chimeric protein was due to a species-specific incompatibility in the AHD2. Therefore, using site-directed mutagenesis, we generated another chimera with an entirely humanized AHD2. However, this second chimeric protein (chimera WH>VL) was yet again not capable of inhibiting NF-κB (Fig. 6C and D). Collectively, these data demonstrate that whereas the presence of the AHD2 sequence is required for human ABIN-3 to inhibit NF-κB activation, its incorporation into the mouse protein is not sufficient to impart human ABIN-3-like activity. Theses results suggest that additional nonhomologous sequences between human and mouse contribute to ABIN-3 function.

Enforced expression of human ABIN-3 suppresses proinflammatory gene responses. To extend our findings using the reporter gene assay, we constitutively expressed human ABIN-3 in the human THP-1 monocytic cell line and examined proinflammatory gene responses. Enforced expression of Myc epitope-tagged human ABIN-3 was confirmed by Western blotting and qRT-PCR, and its expression level was found to be comparable to that of the endogenous gene induced by LPS and IL-10 (data not shown). Compared to that of control cells, THP-1 cells stably expressing human ABIN-3 displayed partially impaired induction of several proinflammatory NF-κB-dependent genes in response to LPS or PGN (Fig. 7). The mRNA levels for IL-8, TNF-α, IL-12p40, and IL-6 were suppressed at multiple time points after stimulation (Fig. 7A), or when various doses of ligand were employed (Fig. 7B). The magnitude of the suppression of proinflammatory gene induction in human ABIN-3-expressing THP-1 cells was comparable to that of NF-κB inhibition observed for the reporter gene assays (Fig. 4). The effect of human ABIN-3 was both gene- and stimulus-specific as there was no suppression of IFN-γ-induced IRF-1 or IL-10-induced SOCS3 expression (Fig. 7C). These data support the notion that human ABIN-3 expression

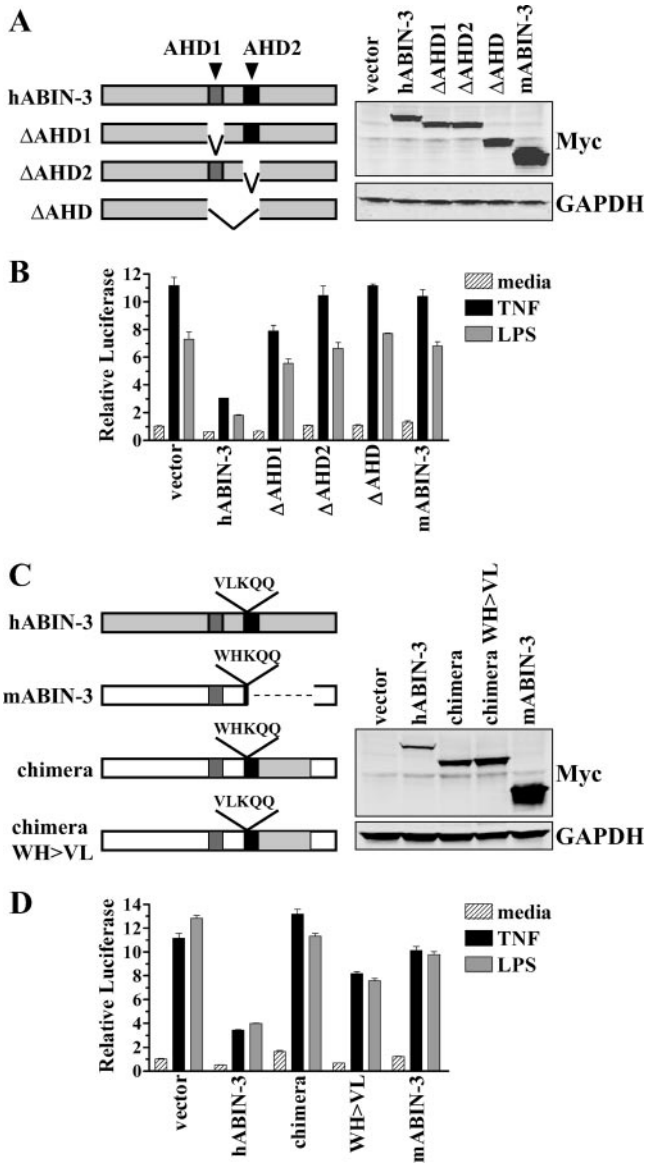

FIG. 6. The ABIN homology domain is required for human ABIN-3 to inhibit NF-κB. (A) Left panel shows a diagram of human ABIN-3 deletion mutants. ΔAHD1 lacks aa 152 to 172, ΔAHD2 lacks aa 199 to 221, and ΔAHD lacks aa 152 to 221. Right panel shows Western blotting of the mutants expressed in HEK 293T cells. (B) NF-κB-dependent luciferase assay with HEK 293T cells cotransfected in duplicate with plasmids for the TLR4 complex and the indicated ABIN-3 expression plasmids. Cells were cultured in the absence (media) or presence of TNF-α (20 ng/ml) or LPS (500 ng/ml) for 5 h. Normalized luciferase activity is presented as means ± standard deviations. (C) Left panel shows a schematic of mouse-human-mouse ABIN-3 chimera constructs. The chimera has aa 204 to 295 of hABIN-3 inserted between aa 178 and 179 of mABIN-3. Chimera WH>VL has had the W174/H175 aa of mouse changed to match the V199/L200 aa of human ABIN-3. Right panel shows Western blotting of the chimeras expressed with HEK 293T cells. (D) NF-κB-dependent luciferase assay as described in panel B.

suppresses NF-κB-dependent proinflammatory gene induction.

Human ABIN-3 acts upstream of IKKβ activation to inhibit NF-κB signaling. To explore the mechanism by which human ABIN-3 expression inhibits NF-κB activity, we first examined whether it had an effect on NF-κB nuclear translocation and/or DNA binding. THP-1 cells stably expressing human ABIN-3 displayed reduced levels of nuclear NF-κB DNA-binding activity when stimulated with LPS or PGN as measured by electrophoretic mobility shift assay (EMSA) (Fig. 8A, upper panel). This effect correlated with reduced degradation of cytoplasmic IκBα as measured by Western blotting (Fig. 8A, lower panel). The partially impaired IκBα degradation and NF-κB activation led to a reduction in the NF-κB-dependent resynthesis of IκBα at the later time points. A similar partial reduction in nuclear NF-κB DNA-binding activity and IκBα degradation was observed when measured at a fixed time point using a dose range of the TLR agonists (Fig. 8B). In agreement with our finding that human ABIN-3 had no effect on IFN-γ- or IL-10-induced gene expression (Fig. 7C), we observed no inhibitory effect on the activation of STAT1 or STAT3 (Fig. 8C).

These results indicate that human ABIN-3 acts in the cytoplasm to suppress IκBα degradation. This notion is consistent with both the cytoplasmic localization of the ABIN-3 protein and the mechanisms of action reported for ABIN-1 and ABIN-2 (20, 61 and data not shown). In order to confirm this concept for human ABIN-3, additional epistatic experiments were carried out in which NF-κB was activated in a ligand-independent manner by overexpressing individual pathway components (Fig. 9A). Overexpression of TLR4, RIP1, or TRAF6 in HEK 293T cells led to the activation of NF-κB-dependent luciferase, which could be inhibited by human ABIN-3 coexpression in an AHD2-dependent manner. In contrast, human ABIN-3 was unable to inhibit NF-κB activation in response to the overexpression of either IKKβ or RelA. These results indicate that human ABIN-3 acts upstream of the IKKβ kinase. The regulatory subunit of the IKK complex, IKKγ (also termed NEMO), is an attractive candidate for the target of human ABIN-3's actions since it is shared among all pathways leading to IKKβ and canonical NF-κB activation (6, 18). In agreement with this reasoning, ABIN-2 (and more recently ABIN-1) has been shown to physically interact with IKKγ (36, 37). To determine whether human ABIN-3 could likewise interact with IKKγ, Myc epitope-tagged versions of human ABIN-3, mouse ABIN-3, or mouse ABIN-2 were coexpressed with human IKKγ in cells and protein-protein interaction was assessed by coimmunoprecipitation (Fig. 9B). The results confirmed that human ABIN-3 associates with IKKγ in a stimulus-independent manner and to a similar extent as that of the mouse ABIN-2 control. Collectively, these data suggest that expression of human ABIN-3 acts to inhibit activation of the IKK complex through an interaction with IKKγ. However, interaction with IKKγ is not sufficient to fully account for the inhibitory action of human ABIN-3 since the noninhibitory mouse ABIN-3 also coimmunoprecipitated IKKγ (Fig. 9B). Thus, the precise mechanism by which an ABIN-IKKγ interaction might productively lead to suppression of IκB kinase activation remains to be elucidated.

DISCUSSION

In this study, we identified ABIN-3 as a gene induced by IL-10 in mononuclear phagocytes coordinately responding to proinflammatory stimuli. Previously, the two other known

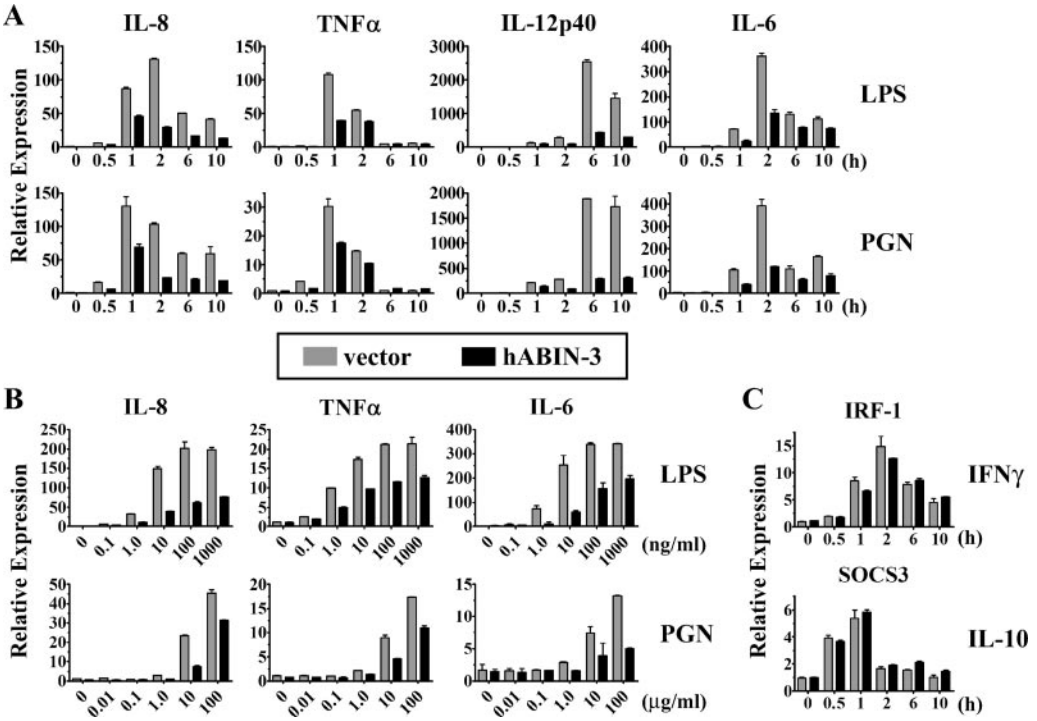

FIG. 7. Enforced expression of human ABIN-3 in human monocytic cells suppresses proinflammatory gene expression. (A) THP-1 cells transduced with empty vector or those stably expressing hABIN-3 were cultured in the presence of LPS (100 ng/ml) or PGN (10 μg/ml) for the indicated time, and total RNA was analyzed by qRT-PCR in duplicate. Values are normalized to GAPDH and are presented as means ± standard deviations. (B) Similar to panel A, except that cells were treated for 45 min with the indicated doses of LPS or PGN. (C) Similar to panel A, except that cells were treated with IFN-γ (25 ng/ml) or IL-10 (25 ng/ml).

ABIN family members, ABIN-1 and ABIN-2, were characterized as a consequence of their interaction with the NF-κB negative feedback regulator, A20, and, in vitro, were shown to inhibit NF-κB activation in their own right (20, 61). Although the role of A20 in negative regulation of NF-κB has been firmly established (7, 33), the significance of its interaction with ABIN family members and the physiologic roles of the ABINs in regulating NF-κB are presently unclear. In contrast to A20, the expression of ABIN-1 and ABIN-2 are not under the control of NF-κB and so they do not readily fit into an analogous model of negative feedback. Moreover, whereas ABIN-1-null mice have not yet been reported, studies with ABIN-2-null mice and cells have failed to demonstrate a role for ABIN-2 in the negative regulation of NF-κB. Instead, ABIN-2 appears to serve a positive signaling role in TLR-induced activation of the Tpl2-ERK pathway (32, 45). Therefore, our discovery that ABIN-3 was unique among ABINs in its induction by IL-10 prompted our pursuit of its potential role as a mediator of IL-10's anti-inflammatory actions.

Through the generation and analysis of ABIN-3-null mice and macrophages, we conclude that mouse ABIN-3 does not play an obligatory role in mediating the broad anti-inflammatory effects of IL-10. Furthermore, mouse ABIN-3 does not play a major role in the normal negative regulation of NF-κB or other proinflammatory signaling pathways. ABIN-3-null mouse macrophages responded by producing proinflammatory molecules in a manner that was indistinguishable from wild-type macrophages. ABIN-3 deficiency did not lead to an alteration in the magnitude or kinetics of NF-κB activation or NF-κB-dependent gene expression in mice. Importantly, ABIN-3-null macrophages responded normally to IL-10, and ABIN-3-null mice did not develop the autoimmune and inflammatory phenotype observed with mice that lack IL-10 responses.

In parallel with our studies investigating the requirement for ABIN-3 in promoting IL-10's actions or in regulating NF-κB signaling in mice, we questioned whether ABIN-3 expression was sufficient to mediate IL-10 or ABIN-like anti-inflammatory effects. Consistent with our analysis of ABIN-3-null mice, enforced expression of either isoform of mouse ABIN-3 (i.e., ABIN-3 or ABIN-3β) was not sufficient to inhibit NF-κB transactivation. In striking contrast, enforced expression of the human ABIN-3 ortholog in cells inhibited NF-κB activation and proinflammatory gene expression in response to TNF, IL-1, and TLR ligands. As the manuscript was being prepared, a report from Wullaert et al. appeared in press showing that human ABIN-3 was an LPS-inducible gene and that its enforced expression could inhibit NF-κB activation (68). Our study confirms and significantly extends the concurrent work of Wullaert et al. by demonstrating the important role of IL-10 in regulating ABIN-3 gene expression, characterizing the species-specific differences that exist for this gene, and unequivocally documenting the lack of anti-inflammatory function for mouse ABIN-3 in vivo.

The functional differences that we document herein between the mouse and the human proteins reflect the structural differences between the two. Human ABIN-3 contains a complete ABIN homology domain, while the mouse ortholog does

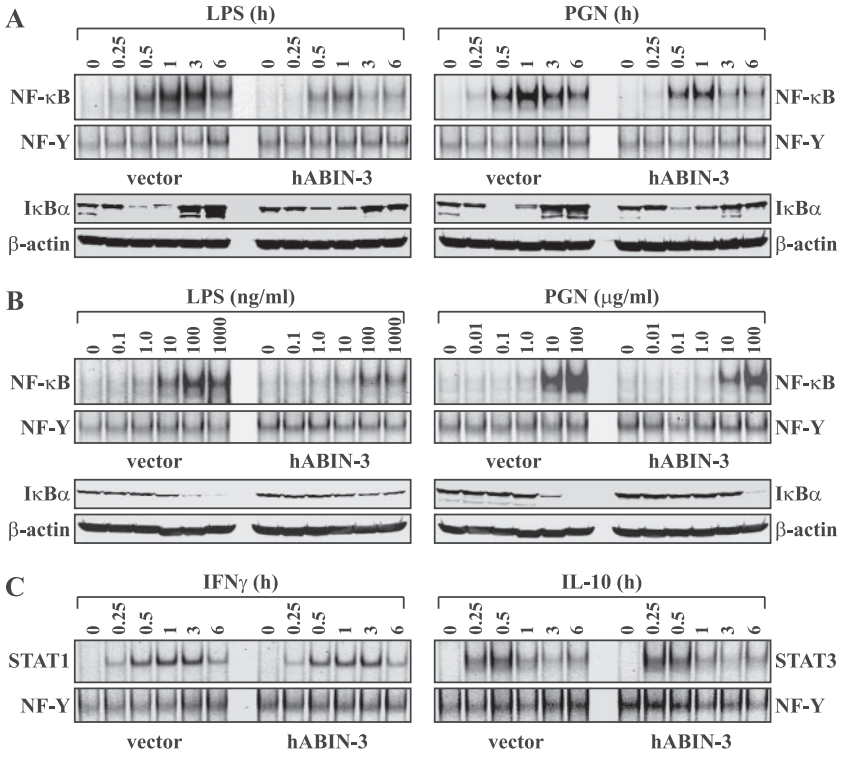

FIG. 8. Enforced expression of human ABIN-3 in human monocytic cells suppresses NF-κB activation. (A) THP-1 cells transduced with empty vector or stably expressing human ABIN-3 were cultured in the presence of LPS (100 ng/ml) or PGN (10 μg/ml). Nuclear and cytoplasmic extracts were prepared at the indicated time and analyzed by EMSA for NF-κB activity (upper panels) or by Western blotting for IκBα degradation (lower panels). NF-Y and β-actin served as loading controls. (B) Similar to panel A, except that cells were treated for 30 min with the indicated doses of LPS or PGN. (C) Similar to panel A, except that cells were treated with IFN-γ (25 ng/ml) or IL-10 (25 ng/ml), and nuclear extracts were analyzed by EMSA for STAT1 or STAT3 activation, respectively.

not. Deletion of the AHD2 subdomain of human ABIN-3 renders it incapable of inhibiting NF-κB. Therefore, the absence of the AHD2 in mouse ABIN-3 is sufficient to explain its functional inactivity. However, introducing the AHD2 sequence of human ABIN-3 into the mouse ortholog was not sufficient to impart NF-κB inhibitory capacity to mouse ABIN-3. These results suggest that additional ABIN-3 sequences may have functional importance in inhibiting NF-κB. It is noteworthy that the genetic and structural differences between mouse and human ABIN-3 are reflected in other species. Rat ABIN-3 resembles the mouse ortholog in that it also lacks an intact AHD. In contrast, ABIN-3 of chicken, dog, cow, and chimpanzee contain an intact AHD similar to the human ortholog. This species divergence in ABIN-3 may ultimately impact the ways in which different host species respond to inflammatory signals.

Although the mechanisms by which IL-10 manifests its anti-inflammatory effects remain incompletely understood, there is generalized agreement that STAT3 is integral in mediating IL-10 function. In contrast, the question of whether IL-10 acts to inhibit NF-κB activation has been a matter of debate (16, 67). Part of the disagreement may stem from the use of varied experimental conditions or could reflect a true heterogeneity of effector mechanisms downstream of IL-10-induced STAT3 activation that differ between cell types and/or species. Reports suggesting a role for IL-10 in inhibiting NF-κB have pointed to multiple and distinct mechanisms of action. IL-10 has been shown to inhibit IKK activation in the cellular cytoplasm while also inhibiting NF-κB transactivation potential in the nucleus (14, 53, 54). Evidence for the former stems primarily from work using human mononuclear phagocytes (53, 54, 62), whereas evidence for the latter mechanism stems from studies using both human and mouse cells (14, 30, 53).

Herein, we provide evidence that IL-10-induced human ABIN-3 can mediate anti-inflammatory effects in the cytoplasm by inhibiting the activation of the IKK kinase, thus preventing the degradation of IκBα. The IKK regulatory subunit IKKγ may be a target of human ABIN-3 since it is required for IKK activation and could associate with human ABIN-3 when coexpressed in cells. In this regard, IL-10 induction of ABIN-3 in inflammatory human macrophages might terminate NF-κB signaling at a point upstream of IKK activation in a manner comparable to the negative feedback actions of A20. Moreover, since our results show that human, but not mouse, ABIN-3 is structurally competent to exert this activity, they establish a novel mechanism that could underlie species-specific differences in the mechanism of action of IL-10.

Research by many groups has led to an emerging picture wherein IL-10 mediates its broad effects through the induction of numerous target genes. Some of these genes play essential roles in mediating highly selective IL-10-induced effects. For example, the IL-10-induced IκB proteins Bcl-3 and IκBNS appear to act as selective transcriptional corepressors at the level of individual NF-κB target gene promoters. At least in

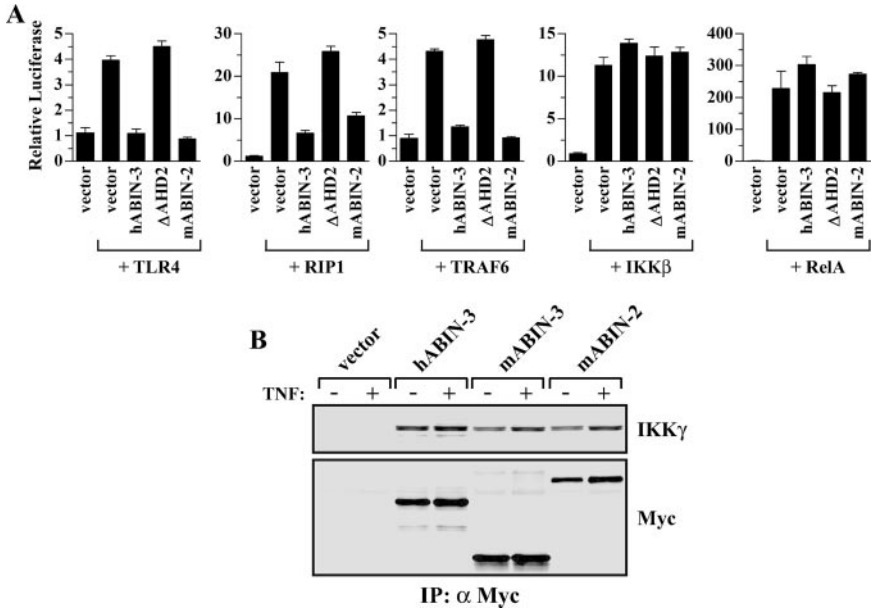

FIG. 9. Enforced expression of human ABIN-3 inhibits NF-κB activation upstream of IKKβ activation. (A) NF-κB-dependent luciferase assay with HEK 293T cells cotransfected in triplicate with plasmids encoding TLR4, RIP1, TRAF6, IKKβ, or RelA to induce ligand-independent activation of NF-κB. Luciferase was measured 36 h posttransfection, and normalized values are presented as means ± standard deviations. (B) Association of ABIN-3 with IKKγ. HEK 293T cells were transfected with empty vector or expression plasmids for Myc-tagged versions of human ABIN-3, mouse ABIN-3, or mouse ABIN-2 in combination with a plasmid encoding human IKKγ. After 72 h, cells were treated with TNF-α (50 ng/ml) for 10 min, and cell lysates were subjected to immunoprecipitation with αMyc followed by Western blotting.

mice, Bcl-3 participates in IL-10-mediated suppression of TLR-induced TNF-α synthesis, whereas IκBNS appears to modulate TLR-induced IL-6 and IL-12p40 transcription (22, 29, 30). In addition to antagonizing NF-κB, IL-10 mediates yet broader effects including destabilization and translational inhibition of specific cytokine and chemokine mRNAs. Several other IL-10-induced genes have been shown or suggested to be important for select IL-10-mediated effects, but to date, ablation of any gene in isolation has not led to an abrogation of all of IL-10's anti-inflammatory effects (5, 16, 48, 67). These data lend support to two models of IL-10 action subsequent to its activation of STAT3: one involving the induction of heterogeneous effector pathways with distinct proinflammatory target gene specificities and a second involving the induction of redundant mechanisms for the suppression of particular target genes.

Our data support the concept that IL-10 mediates its anti-inflammatory effects on mononuclear phagocytes through one or more of its induced gene products and that one of the cytokine's effects might be the attenuation of NF-κB signaling. We raise the possibility that this latter aspect is species specific due to structural differences in the IL-10-induced ABIN-3 gene. Future studies should address whether ABIN-3 plays an obligatory role in IL-10-mediated attenuation of NF-κB signaling in human mononuclear phagocytes and, if so, whether its ablation leads to impairment of the anti-inflammatory effects of IL-10. Our current attempts to ablate ABIN-3 expression in human mononuclear phagocytes using RNA interference (RNAi), employing both small interfering RNAs transiently and short hairpin RNAs stably, have not yet yielded sufficient reduction in gene expression to answer these questions in an unequivocal manner. Nevertheless, the work described herein identifies human ABIN-3 as a potential effector of IL-10's anti-inflammatory effects in humans.

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