Inhibitor of growth-4 promotes IκB promoter activation to suppress NF-κB signaling and innate immunity

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Ing4 is a member of the inhibitor of growth (ING) family of chromatin-modifying proteins. Biochemical experiments indicate that Ing4 is a subunit of the HB01-JADE-hEAF6 histone acetyltransferase complex responsible for most nucleosomal histone H4 acetylation in eukaryotes, and transfection studies suggest that Ing4 may regulate a wide variety of cellular processes, including DNA repair, apoptosis, cell-cycle regulation, metastasis, angiogenesis, and tumor suppression. However, in vivo evidence for a physiological role for Ing4 in cell-growth regulation is lacking. We have generated Ing4-deficient mice to explore the role of Ing4 in development, tumorigenesis, and in NF-κB signaling. Ing4-null mice develop normally and are viable. Although mice deficient for Ing4 fail to form spontaneous tumors, they are hypersensitive to LPS treatment and display elevated cytokine responses. Macrophages isolated from Ing4-null mice have increased levels of nuclear p65/RelA protein, resulting in increased RelA binding to NF-κB target promoters and up-regulation of cytokine gene expression. However, increased promoter occupancy by RelA in LPS-stimulated, Ing4-null cells does not always correlate with increased NF-κB target-gene expression, as RelA activation of a subset of cytokine promoters also requires Ing4 for proper histone H4 acetylation. Furthermore, activation of the IκBα promoter by RelA is also Ing4-dependent, and LPS-stimulated, Ing4-null cells have reduced levels of IκBα promoter H4 acetylation and IκB gene expression. Thus, Ing4 negatively regulates the cytokine-mediated inflammatory response in mice by facilitating NF-κB activation of IκB promoters, thereby suppressing nuclear RelA levels and the activation of select NF-κB target cytokines.

RelA | macrophage | inflammation | tumorigenesis | mice

Innate immunity, the initial response a host mounts against a pathogen, involves the production and secretion of proinflammatory cytokines in response to microbial products (1). This response is orchestrated by NF-κB, a dimeric complex of Rel proteins that can activate, and in some cases repress, transcription of cytokine genes (2). Macrophages are the primary mediators of inflammatory responses and contain Toll-like pattern-recognition receptors (TLR) on their surface that recognize LPS or other components of microbial pathogens (3, 4). Binding of LPS to the TLR4 receptors activates the most common NF-κB heterodimer (p65-RelA and p50) in macrophages, resulting in increased expression of NF-κB -responsive cytokine genes in these cells.

NF-κB activity is tightly regulated in macrophages by sequestration of the NF-κB heterodimer in the cytoplasm, thereby inhibiting nuclear NF-κB–mediated transcriptional activation. This cytoplasmic sequestration is governed by NF-κB–inhibitory, IκB proteins. Following pathogen stimulation of TLR on the surface of responsive cells, cytosolic IκB proteins are phosphorylated by IκB kinases (IKK1 and IKK2) and subsequently degraded. Degradation of IκB permits the NF-κB complex to translocate into the nucleus, where it binds to κB sequence elements in the promoters of NF-κB–responsive genes. This binding activates transcription of the NF-κB–responsive genes, including a number of proinflammatory cytokine genes. How NF-κB transcriptional activation of responsive promoters is further regulated or attenuated is not well understood (5, 6). However, recent transfection experiments in glioma cell lines have indicated that NF-κB activation of responsive promoters may require the histone acetyltransferase-associated ING4 protein (7).

ING4 is a member of the inhibitor of growth (ING) family, which is comprised of five evolutionarily conserved proteins (ING1–ING5) characterized by a conserved carboxyl-terminal plant homeodomain-like zinc finger (8, 9). These nuclear proteins have been proposed to play roles in numerous biological functions by interacting with different acetylation and deacetylation complexes involved in chromatin remodeling and gene expression (10, 11). In addition, biochemical analysis has indicated that ING4 interacts with methylated histone H3 (12–14) and with the HB01-JADE-hEAF6 histone acetyltransferase complex (11). This latter complex is responsible for most nucleosomal histone H4 acetylation in eukaryotes, and knockdown experiments indicated that Ing4-HB01 association is required for cells to progress properly through the DNA synthesis (S) phase of the cell cycle (15, 16).

ING4 has been proposed to be a tumor suppressor, as ING4 expression is reduced in human cancers and the *Ing4* gene mutated in various cancer-cell lines (9). In addition, transfection experiments have revealed that ING4 can complex with the p53 tumor suppressor, and exogenous ING4 diminishes cell colony formation, decreases S-phase in cycling cells, and induces apoptosis of transfected RKO cells in a p53-dependent manner (17). ING4 may also alter NF-κB signaling in tumor cells. In addition, forced overexpression and coimmunoprecipitation experiments performed in the U87MG glioblastoma cells revealed that ING4 physically interacts with RelA, the large subunit of the nuclear factor NF-κB (7). Inhibition of ING4 in these cells by antisense RNA promoted tumor vascularization in transplanted SCID mice and down-regulated the expression of several NF-κB target genes involved in angiogenesis. In addition, ING4-RelA binding decreased activation of a canonical NF-κB-responsive promoter in transfection assays. Thus, ING4 was proposed to inhibit cell survival and angiogenesis by complexing with and inhibiting RelA (7). More recently, transfection and knock-down experiments performed in glioma cell lines have indicated that ING4 may regulate NF-κB activity by binding simultaneously with RelA to NF-κB promoters and altering the levels of promoter histone acetylation (18). However, the precise role of ING4 regulation of

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NF-κB activity in cancer is unclear, and definitive proof that ING4 regulates tumorigenesis is lacking.

In this study, we generated and characterized Ing4-deficient mice to explore the physiologic role of Ing4 in development, tumor suppression, and NF-κB signaling. Surprisingly, Ing4-null mice are fully viable and do not form spontaneous tumors upon aging. However, mice deleted for Ing4 are highly sensitive to LPS treatment, revealing a role for Ing4 in regulation of innate immunity. Analysis of serum and peritoneal macrophages isolated from Ing4-deficient mice reveals that Ing4 suppresses the production of some (but not all) cytokines in LPS-stimulated mice. Furthermore, Ing4 is required for robust activation of the IκBα promoter, and IκB levels are reduced and nuclear RelA levels and NF-κB promoter binding increased in stimulated Ing4-null cells. However, activation of cytokine genes in stimulated Ing4-null mice is selective, and depends upon the promoter requirement for Ing4-mediated acetylation of histone H4. These experiments reveal that physiologic levels of Ing4 govern innate immunity in mice by regulating the levels of IκB and NF-κB proteins and the activation of select cytokine promoters.

Results

Generation of Ing4-Deficient Mice. To examine the physiological role of Ing4 in the mouse, we first determined the expression pattern of Ing4 in a panel of WT C57BL/6 mouse tissues by real-time quantitative RT-PCR. The *Ing4* transcript is ubiquitiously expressed in the adult mouse (Fig. S1*A*). In contrast to the multiple spliced isoforms of ING4 present in humans, only one *Ing4* transcript is observed in mice (10, 19, 20). An *Ing4*-gene trapped ES cell clone (Fig. S1*B*) was used in standard blastocyst microinjection experiments, and germ-line transmission of the mutant *Ing4* allele was obtained by breeding the resulting chimeric mice (Fig. S1*C*).

Intercrosses of Ing4-heterozygous, F1 generation mice (C57BL/6 × 129S9) were performed to generate Ing4-null homozygous mice. Ing4-heterozygous mice and Ing4-homozygous null mice were recovered in the expected Mendelian ratios from these crosses and appeared phenotypically indistinguishable from their WT littermates. Loss of *Ing4* gene expression in *Ing4*-null cells was confirmed by performing RT-PCR on RNA isolated from mouse embryonic fibroblasts using PCR primers spanning exons 4 to 8 of the *Ing4* gene or using primers contained within exon 8 of the *Ing4* gene (Fig. S1 *D* and *E*). These data confirm that the retroviral trap insertion in the *Ing4* locus generates an *Ing4* message-minus allele, and that cells and mice homozygous for the modified *Ing4* allele are *Ing4*-null as they lack *Ing4* transcripts ($Ing4^{-/-}$). Thus, Ing4 is not required for proper development in mice.

Cohorts of $Ing4^{-/-}$ mice ($n = 45$) and WT mice ($n = 50$) were established and aged for 20 mo. No spontaneous tumors formed in these mice. Upon necropsy, 3 of the 45 $Ing4^{-/-}$ mice presented with enlarged spleens, but this was determined by histopathology to be extramedullary hematopoiesis and not cancer. Therefore, Ing4 does not play a role in suppressing spontaneous tumorigenesis in mice because *Ing4*-null mice were tumor-free. Additionally, *Ing4* mice were of normal size and body weight, and did not exhibit any other overt signs of spontaneous inflammatory diseases.

Suppression of Innate Immunity by Ing4. To determine if NF-κB activity was altered in mice lacking Ing4, we examined the sensitivity of $Ing4^{-/-}$ mice to LPS challenge. LPS is known to activate NF-κB signaling via the TLR4 receptor, leading to inflammatory cytokine production and lethal shock. Twelve WT or $Ing4^{-/-}$ mice were i.p. injected with either 0.25 mg (solid lines) or 0.75 mg (dashed lines) of LPS and monitored over the course of 1 wk (Fig. 1*A*). Nearly one half (5 of 12) of the $Ing4^{-/-}$ mice exhibited morbidity at the lower LPS dose, whereas only 1 of 12 of the WT mice became morbid by 6 d postinjection. An even greater difference was seen at the higher dose. All 12 of the $Ing4^{-/-}$ mice died within 24 h of LPS injection, whereas only one third (4 of 12) of the injected WT mice died at 24 h, and one-third of the WT mice failed to exhibit any illness throughout the study period. These results indicate that Ing4-deficient mice are hypersensitive to LPS-induced lethal shock.

We examined cytokine levels in the serum, spleen, liver, and lung of mice after LPS treatment. WT mice ($n = 8$) and $Ing4^{-/-}$ mice ($n = 8$) were either mock-injected or injected i.p. with 0.5 mg LPS, and serum and organs were collected at 6 h postinjection. ELISAs were conducted to determine the levels of IL-6, monocyte chemoattractant protein-1 (MCP-1), and RANTES (regulated upon activation, normal T-cell expressed and secreted) in these samples. Expression of these cytokines is activated in response to LPS mostly through MyD88-dependent NF-κB signaling, although up-regulation of the IRF-3 transcription factor and, to some extent, NF-κB, may also occur via a MyD88-independent pathway (21). The levels of IL-6, MCP-1, and RANTES were elevated in the serum of $Ing4^{-/-}$ mice relative to WT levels following LPS injection (Fig. 1*B*). In addition, ELISAs revealed that IL-6 levels were also elevated in the spleen, liver, and lung of $Ing4^{-/-}$ mice after LPS injection (Fig. S2). In addition, LPS-injection also elevated liver and lung MCP-1 levels and spleen and lung RANTES levels to higher levels in $Ing4^{-/-}$ mice than in WT mice (Fig. S2). The increase in cytokine levels in $Ing4^{-/-}$ mice after LPS treatment suggests that NF-κB activity is up-regulated in these mice.

Ing4-Null Macrophages Display Elevated Expression of NF-κB-Responsive Genes. Macrophages respond to bacterial infection by secreting proinflammatory cytokines and chemokines (22) and are essential components of innate immunity. To further examine the hypersensitivity of $Ing4^{-/-}$ mice to LPS, we generated peritoneal macrophages by injecting WT or $Ing4^{-/-}$ mice with 4% thioglycollate and collecting peritoneal exudate cells by lavage at 4 d postinduction. Macrophages were plated at a density of 10^6 cells per well in a 24-well plate and either mock-treated or treated with either LPS or heat-attenuated *Escherichia coli.* Macrophage supernatants were collected 24 h after treatment and used in ELISAs to determine the levels of IL-6 and MCP-1. Similar to what was observed in vivo, IL-6 and MCP-1 cytokine expression was elevated in $Ing4^{-/-}$ macrophages relative to WT macrophages after exposure of cells to LPS (Fig. 1*C*). Similar results were obtained in $Ing4^{-/-}$ cells exposed to heat-killed bacteria (Fig. S3). The enhanced cytokine response of $Ing4^{-/-}$ macrophages to LPS was most pronounced at low doses of LPS, with an approximate 8-fold increase in IL-6 and a 4-fold increase in MCP-1, relative to WT control cells.

We next determined whether deletion of Ing4 altered the levels of cytokine gene expression. Quantitative real-time PCR (qPCR) was performed on RNA isolated from nonstimulated macrophages to examine the basal expression levels of various cytokine genes. No significant difference was detected between WT and $Ing4^{-/-}$ macrophages in the expression levels of IL-6, MCP-1, IP-10, or TNF–α (Fig. S4). In contrast, LPS stimulation of $Ing4^{-/-}$ macrophages resulted in large differences in the expression of various cytokine genes known to be activated by NF-κB signaling. Expression of IL-6 and IP-10 was much greater in $Ing4^{-/-}$ cells than in WT cells treated with 1 μg/mL LPS for 1, 2, or 6 h, and IFN-β expression levels were also dramatically higher in $Ing4^{-/-}$ cells at 1 or 2 h after LPS treatment (Fig. 1*D*). However, increased cytokine gene activation in $Ing4^{-/-}$ cells was not consistent, as activation of TNF–α was reduced in $Ing4^{-/-}$ cells relative to WT cells following LPS treatment (Fig. 1*D*). These data indicate that Ing4 negatively regulates the expression of a subset of NF-κB-responsive genes following exposure of macrophages to LPS.

Ing4 Inhibits Nuclear NF-κB Levels and Promoter Binding. To explore the molecular basis of altered NF-κB activity in $Ing4^{-/-}$ cells, we examined the RelA (p65) levels in the nuclear compartment of

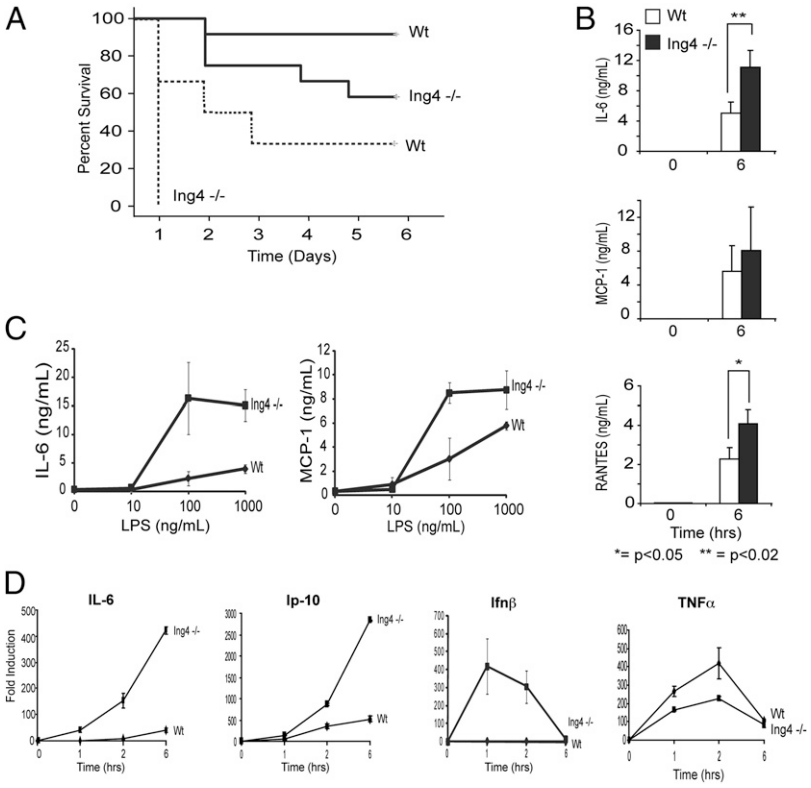

Fig. 1. Ing4-null mice are hypersensitive to LPS challenge and display elevated response of select cytokines. (*A*) Age-matched WT ($n = 12$) or Ing4-null ($n = 12$) mice were injected with either 0.25 mg (solid lines) or 0.75 mg (dashed lines) LPS and monitored for morbidity over the course of 1 wk. Graphs show Kaplan-Meier survival analysis. At both doses there was a significant survival difference between WT and Ing4-null animals ($P \leq 0.005$). (*B*) ELISAs for IL-6, MCP-1, or RANTES were performed on serum from WT ($n = 8$) or Ing4-null ($n = 8$) mice at 0 or 6 h postinjection with 0.5 mg LPS. Graphs show geometric mean with SE. Asterisks represent *P* values, as shown above. (*C*) Ing4-null macrophages show elevated cytokine levels. Macrophages from WT ($n = 5$) or Ing4-null ($n = 5$) mice were either mock-treated or treated with increasing amounts of LPS. ELISAs were performed on the supernatants 24 h posttreatment. (*D*) Expression of IL-6, Ip-10, IFN-β, and TNF-α in macrophages following treatment with LPS. Macrophages from WT ($n = 4$) or Ing4-null ($n = 4$) mice were treated with LPS and qRT-PCR was done in triplicate for each sample. All graphs show average with SD.

macrophages. Ing4$^{-/-}$ macrophages and WT macrophages were untreated (0 min) or treated with 1 μg/mL LPS and harvested at 30 or 60 min posttreatment. Nuclear extracts were prepared and analyzed via Western blot for translocated RelA (Fig. 2*A*). As determined by densitometry on the Western blots, there was a 3.1-fold elevation in nuclear RelA levels in nonstimulated macrophages lacking Ing4. EMSA were performed with these nuclear extracts using a canonical RelA- NF-κB-binding DNA-sequence motif (23). The results revealed increased RelA binding in untreated Ing4$^{-/-}$ macrophage nuclear extracts relative to untreated WT extracts, and increased RelA DNA binding at 30 or 60 min after LPS treatment relative to WT cells (Fig. S5). These results suggest that Ing4 may inhibit nuclear RelA levels and thus inhibit NF-κB binding to responsive promoters. In keeping with this hypothesis, cotransfection of an Ing4 expression vector into HEK293 cells suppressed the expression of a luciferase reporter gene placed under transcriptional control of a canonical NF-κB–responsive promoter (Fig. 2*B*).

To confirm that increased RelA activity in the nuclear compartment of Ing4$^{-/-}$ macrophages could alter endogenous NF-κB promoter responses, we performed ChIP experiments to examine binding of RelA to the IL-6 promoter. Ing4$^{-/-}$ or WT macrophages were either mock-treated or treated with LPS for 1 or 3 h. Immunoprecipitation of crosslinked DNA was performed using either a RelA (anti-p65) antibody or an antibody to acetylated histone H4, and a qPCR to IL-6 promoter sequences was performed. Greater RelA occupancy was observed at the IL-6 promoter in the Ing4$^{-/-}$ macrophage at 0 and 1 h (Fig. 2*C*), whereas equivalent amounts of RelA was observed on the IL-6 promoter after 3 h post-LPS treatment of WT and Ing4$^{-/-}$ cells. Furthermore, the large difference seen in p65 binding to the IL-6 promoter correlated with increased levels of promoter histone H4 acetylation, indicating that binding of NF-κB induced IL-6 promoter activation (Fig. 2*C*). These data are in agreement with our qPCR results indicating up-regulation of IL-6 expression in Ing4$^{-/-}$ cells (Fig. 1*D*), and further reveals that H4 acetylation of the IL-6 promoter does not require Ing4. In addition, ChIP assays were performed on the TNF-α promoter and KC cytokine promoter for RelA and acetylated H4. Similar to what was seen for the IL-6 promoter, the TNF-α and KC promoters displayed greater RelA occupancy shortly after LPS stimulation in Ing4$^{-/-}$ macrophages (Fig. 2 *D* and *E*), reflecting the increased levels of nuclear RelA in these cells. However, in contrast to the IL-6 promoter, the TNF-α and KC promoters have far less H4 acetylation in stimulated Ing4-null cells than in stimulated WT cells. These results are in agreement with the reduced activation TNF-α expression observed in Ing4-null cells after LPS treatment (Fig. 1*D*), and indicates that a subset of NF-κB-responsive cytokine promoters require Ing4 for proper promoter activation following RelA binding.

Expression of IκB Is Regulated by Ing4. Our results indicate that nuclear RelA levels and RelA-DNA binding was increased in nonstimulated and LPS-stimulated, Ing4$^{-/-}$ macrophages (Fig. 2 *A*–*C*). Because nuclear levels of RelA are tightly regulated by IκB, and IκB expression is a target for RelA activation (2, 24, 25), we

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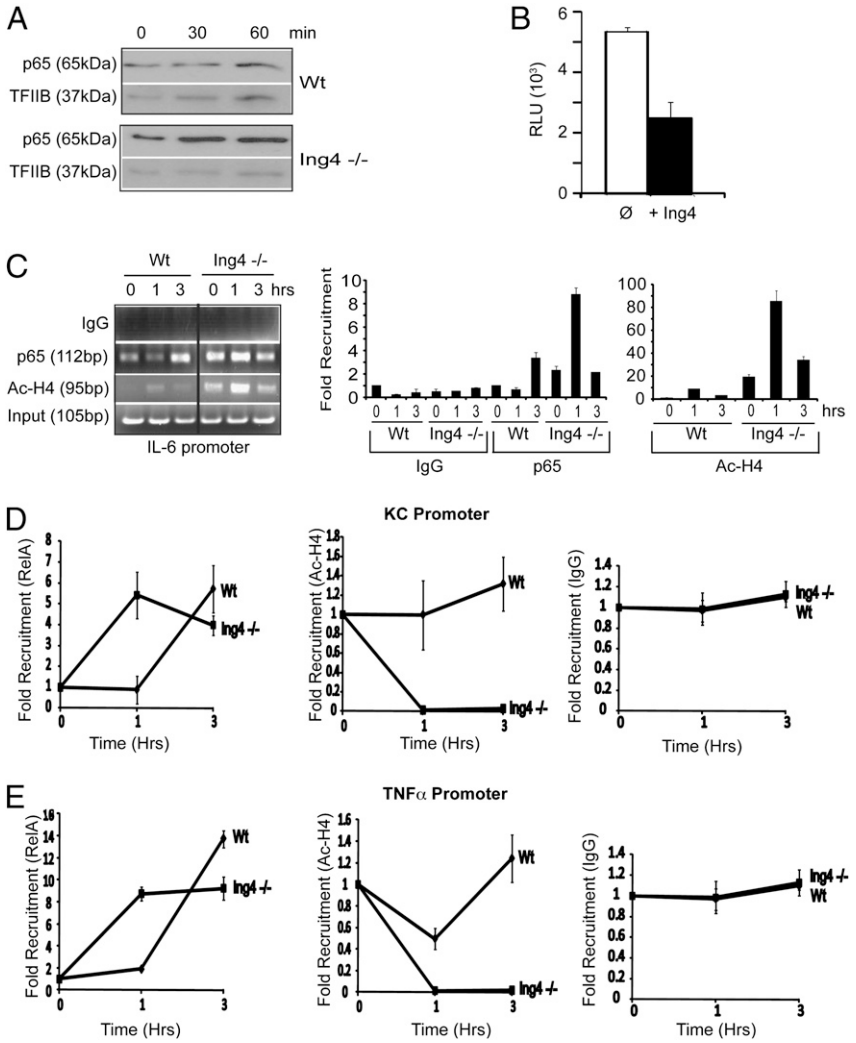

Fig. 2. Increased levels and binding of nuclear NF-κB. (*A*) Western blot for p65 was performed on nuclear extracts from WT or Ing4-null macrophages treated with LPS. The nuclear transcription factor TFIIB was used as a loading control. Western blot was performed on a single gel, and the picture cut for presentation purposes. Densitometry indicates a 3.1-fold increase in RelA/p65 levels in nonstimulated (0 time point) Ing4$^{-/-}$ cells, as adjusted for TFIIB levels. (*B*) Overexpression of Ing4 inhibits the expression of luciferase from a NF-κB reporter. HEK293T cells were transfected with plasmids containing the *Renilla* luciferase gene, an NF-κB luciferase reporter construct, and the RelA cDNA. Graphs show the average and SD values of three independent experiments. (*C*) ChIP experiments show increased p65 recruitment to IL-6 promoter and increased Ac-H4. Anti-IgG antibodies were used as a control for specificity. Real-time qPCR was performed in triplicate. Shown are a representative gel picture of the qPCR products and graph, average and SD, quantifying the results. (*D*) ChIP experiments performed before (time 0) and after LPS treatment of WT and Ing4$^{-/-}$ macrophages using antibodies against RelA (*Left*), acetylated H4 (*Center*), or a control IgG antibody (*Right*). PCR primer pairs to the KC cytokine promoter. Time-point values represent average of three samples, with SD. (*E*) ChIP experiments performed as in *D*, using PCR primer pairs to the TNF-α promoter.

examined endogenous IκB levels in the presence or absence of Ing4. Although there were increased levels of RelA protein in untreated extracts, this did not correspond with an increase in IκBα protein in untreated, whole-cell extracts of Ing4$^{-/-}$ macrophages (Fig. 3*A*). To confirm this observation, Western blots for IκBα were performed using cytoplasmic fractions isolated from LPS-treated WT and Ing4$^{-/-}$ macrophages. In WT cells, the level of IκBα is greatly reduced 15 min after LPS treatment, and begins to recover by 60 min posttreatment (Fig. 3*B*). Although Ing4$^{-/-}$ cells also display reduced IκBα levels 15 min after LPS treatment, the kinetics of IκBα resynthesis were much delayed in these cells relative to WT cells, and IκBα levels remained much lower in Ing4$^{-/-}$ cells even at 24 h poststimulation. To determine if the reduced amount of IκBα protein in Ing4$^{-/-}$ cells reflects a decrease in the expression of IκB genes, we performed qPCR on RNA isolated from LPS-treated WT and Ing4$^{-/-}$ macrophages (Fig. 3*C*). Expression of IκBα, IκBβ, and IκBNS was reduced in stimulated Ing4$^{-/-}$ cells relative to the levels observed in WT cells.

Although reduced IκB expression levels in stimulated Ing4-null cells correlates with the reduced IκB protein levels seen in these cells, this finding is surprising because IκB promoters are known targets of RelA activation (2). To examine further the effects of Ing4 loss on activation of IκB expression, we performed ChIP analysis on the IκBa promoter in LPS-treated WT and Ing4$^{-/-}$ macrophages. Similar to what was seen for other NF-κB target promoters, there was increased RelA occupancy of the IκBα promoter in Ing4$^{-/-}$ cells shortly after stimulation (Fig. 3*D*). However, there was little promoter activation, as H4 acetylation of the IκBα promoter was greatly reduced in stimulated Ing4$^{-/-}$ cells. Therefore, although increased RelA binding to the IκBα promoter is observed in stimulated cells lacking Ing4, activation and increased expression of IκBα requires Ing4 for

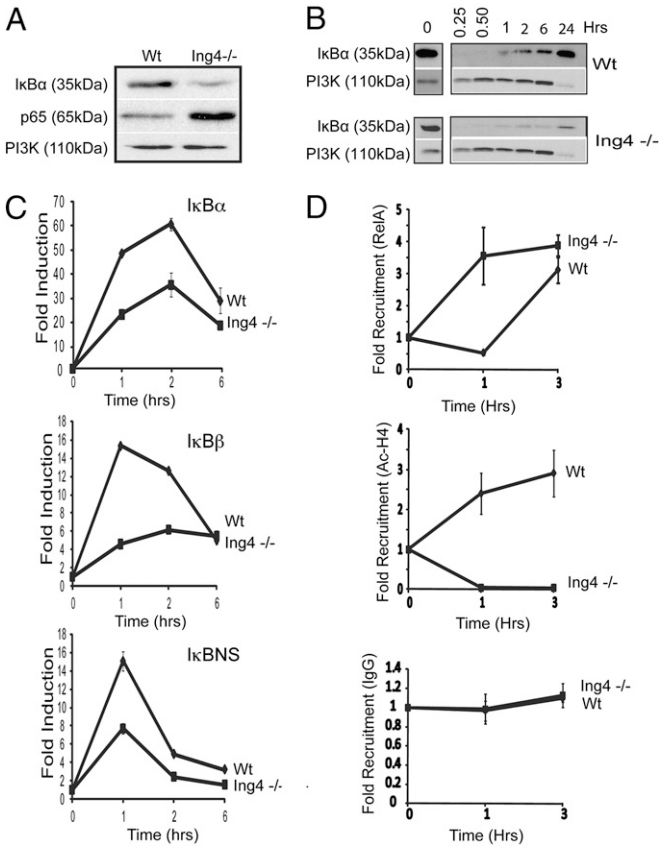

Fig. 3. Ing4 regulates *IκB* transcript levels. (*A*) Whole-cell extracts from WT and Ing4-null macrophages (nonstimulated) were run on a 10% SDS/PAGE gel and probed with antibodies to p65 or IκBα. PI3K was used as a loading control. Densitometry indicates a 55% increase in RelA/p65 levels as adjusted for PI3K levels. (*B*) Western blots for IκBα were performed on cytoplasmic extracts from WT or Ing4$^{-/-}$ macrophages either mock- or LPS-treated for the indicated times. PI-3-kinase was used as a loading control. (*C*) Real-time qRT-PCR assays using primer pairs to IκBα, IκBβ, and IκBNS were performed on WT or Ing4$^{-/-}$ macrophages treated with LPS (1 μg/mL) for the indicated time. All samples were done in triplicate and shown are averages and SD. (*D*) ChIP experiment performed before (time 0) and after LPS treatment of WT and Ing4$^{-/-}$ macrophages using antibodies against RelA (*Top*), acetylated H4 (*Middle*), or a control IgG antibody (*Bottom*). A PCR primer pair for the IκBα promoter was used. Time-point values represent average of three samples, with SD.

promoter H4 acetylation. To further confirm that IκBα expression is positively regulated by Ing4, we transfected an Ing4 expression vector into HEK-293T cells and measured the level of IκBα expression by qPCR. IκBα expression was elevated in cells transduced with Ing4 in the presence and absence of LPS stimulation (Fig. S6).

Discussion

We generated Ing4-deficient mice and primary cells to explore the role of Ing4 in development, tumorigenesis, and innate immunity. Our results reveal that Ing4 is not required during embryogenesis and does not suppress spontaneous tumorigenesis. ING4 has been recently proposed to complex with the large subunit of NF-κB and to inhibit NF-κB-mediated transactivation of gene expression (7). As NF-κB plays a role in multiple biological processes, including angiogenesis, apoptosis, and the innate and adaptive immune response (26, 27), regulation of NF-κB by Ing4 might also impact tumor formation. To examine the ability of Ing4 to regulate NF-κB activity in vivo, we examined the innate immune response of *Ing4*$^{-/-}$ mice. *Ing4*-null mice were highly sensitive to LPS treatment, and contained elevated levels of IL-6, MCP-1, and other cytokines in serum and in various organs, indicating that Ing4 suppresses NF-κB signaling in mice. Peritoneal-derived Ing4$^{-/-}$macrophages displayed elevated levels of nuclear RelA/p65 and increased binding of RelA/p65 to relevant NF-κB promoters. Although the increase in basal levels of nuclear RelA do not result in altered basal (nonstimulated) levels of most cytokines in Ing4$^{-/-}$ cells, LPS induction of NF-κB signaling in Ing4$^{-/-}$ cells increases the level of promoter H4 acetylation and activation of certain cytokines such as IL-6. However, increased promoter occupancy by RelA in Ing4$^{-/-}$ cells does not result in increased activation of all cytokines following LPS treatment, as Ing4 is required for proper H4 acetylation of select NF-κB responsive promoters, such as TNF-α and KC. This finding is in agreement with a recently published report suggesting that ING4-containing HBO1 complexes are responsible for acetylation of H4 and possibly transcription elongation (18).

In addition, Ing4 was also necessary for activation of the RelA-responsive, IκBα promoter, and expression of IκBα and other IκBα genes was reduced in stimulated Ing4-null macrophages, even though nuclear RelA were increased in these cells. Therefore, we propose that up-regulation of RelA levels and NF-κB activity in stimulated Ing4$^{-/-}$ mice and cells reflects the decreased expression of RelA inhibitors such as IκBα, IκBβ, and IκBNS, as these genes require Ing4 for proper promoter H4 acetylation and activation (Fig. 4).

Recently, transfection studies indicated that overexpression of ING4 can inhibit the expression of NF-κB target genes in glioblastoma cell lines by binding simultaneously with NF-κB at responsive promoters and attenuating NF-kB activity (28). Thus, it is possible that Ing4 is inhibiting the activation of certain cytokine promoters (such as IL-6) to suppress innate immunity, and that loss of Ing4 would lead to increased activation of these suppressed promoters. However, Ing4 is clearly required in macrophages for robust activation of other NF-kB–responsive promoters, such as TNF-α. Furthermore, our in vivo studies indicate that endogenous levels of Ing4 facilitates IκB promoter

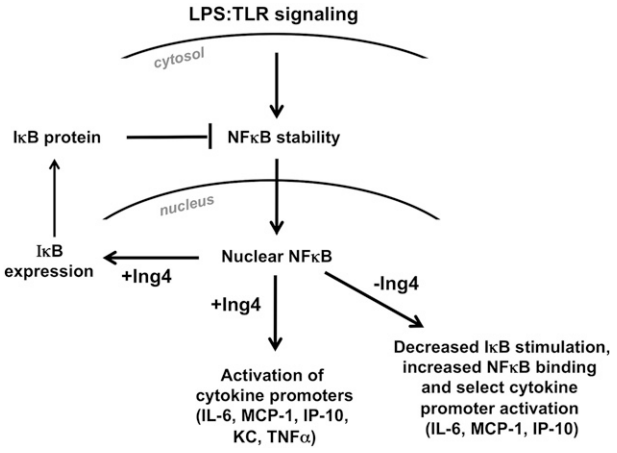

Fig. 4. Schematic diagram for role of Ing4 in regulation of IκB expression and NF-κB activity. Up-regulation of nuclear NF-κB by LPS treatment leads to increased RelA-responsive promoter activation and cytokine production. However, Ing4 is not required for promoter H4 acetylation of certain cytokines (such as IL-6), which can be activated by increased RelA activity even in the absence of Ing4. Thus, loss of Ing4 in LPS-treated macrophages decreases NF-κB-induced IκB expression, thereby further up-regulating NF-κB activation of select cytokine promoters (such as IL-6) that do not require Ing4 for promoter H4 acetyltation, resulting in a hyper-inflammatory response in Ing4$^{-/-}$ mice.

activation, resulting in increased IκB expression, lower nuclear NF-κB levels, and reduced RelA target-promoter occupancy, which would also explain Ing4-inhibition of various cytokine genes. Further experiments involving an Ing4-ChIP grade antibody will likely be needed to address the precise role of Ing4 at various NF-κB-responsive promoters. Interestingly, not all NF-κB promoters are dependent upon Ing4 for H4 acetylation. Whether other ING proteins, such as Ing5, regulate H4 acetylation of the IL-6 or other cytokine promoters in Ing4-null cells remains to be determined. However, the results of our present study reveal that Ing4 is an important regulator of the inflammatory response of mice to bacterial components, such as LPS, through its ability to suppress NF-κB activation of select cytokine genes in stimulated macrophages. Collectively, these results establish a previously unexplored role for Ing4 in regulating NF-κB–mediated innate immunity.

Materials and Methods

Generation of Ing4 Knockout Mice and LPS Challenge. Mouse (129 strain) ES cells (BayGenomics clone XG370) containing a retroviral promoter-gene trap inserted into one allele of Ing4 were used to generate Ing4-null mice. Mice were genotyped by PCR analysis of genomic DNA using the following primer sequences: ING4g1439F: 5′-CGGGCGGATTTCTAAGTTCG-3′, ING4g2118R: 5′-AAAAGACAAAACAAGAGGGGGC-3′, ING4c8015F: 5′-AAGGGAATAAGGGCG-ACACG-3′. Mice were injected i.p. with LPS (*E. coli* O111:B4; Sigma-Aldrich) and monitored for 1 wk. All mice were maintained and used in accordance with federal guidelines and those established by the University of Massachusetts Animal Care and Use Committee.

Macrophage Preparation, ELISAs, Real-Time RT-PCR, and Luciferase Assay. Macrophages were generated as previously described (29). Peritoneal exudates cells were plated in RPMI1640 with 10% heat-inactivated FCS and incubated at 37 °C and 5% CO_2. ELISAs were performed according to the manufacturer's instructions (BD Pharmingen). Relative levels of mRNA expression were analyzed by qRT-PCR, as previously described (30, 31). Primer sequences used in the PCR reactions provided upon request. Luciferase assays were done on HEK293T cells transfected with a consensus NF-κB luciferase reporter construct, a RelA plasmid, a *Renilla*-expression plasmid, and either a plasmid containing Ing4 cDNA or plasmid backbone using Gene Juice transfection reagent. Twenty-four hours posttransfection the cells were stimulated with 1 μg/mL LPS for 4 to 6 h. Both firefly and *Renilla* luciferase were assayed using the dual luciferase reporter-assay system (Promega). Samples were read on a Glomax.

Antibodies, Protein Extracts, Western Blots, and ChIPs. Antibodies for Western blots recognized IκB (1:1,000; Cell Signaling), p65 (1:4,000; Santa Cruz), tubulin (1:2,500; Sigma), TFIIB (1:250, Santa Cruz), and PI3K (1:4,000; Santa Cruz); ChIPs were tetra-acetylated-H4 (Upstate), p65 (Abcam), and IgG (Abcam). ChIPs were performed according to the manufacturer's instructions (USB) and real-time PCR was performed on the immunoprecipitated DNA. The primer sequences for the κB binding site in the IL-6 promoter were: 5′-AGCTACAGACATCCCCA-GTCTC-3′ and 5′-TGTGTGTCGTCTGTCATGCG-3′. Primer sequences for the IκBα promoter were 5′-AAATCTCCAGATGCTACCCGAGAG-3′ and 5′-ATAATGTCA-GACGCTGGCCTCCAA-3′. Primer sequences for the TNF–α promoter were 5′-CCCCAGATTGCCACAGAATC-3′ and 5′-CCAGTGAGTGAAAGGGACAG-3′. Primer sequences for the KC promoter were 5′-GTCACTCCCTCCCCTTTG-3′ and 5′-CAGAGAAGCGAGCGGGTG-3′. The antibodies used were p65/RelA (Abcam), acetylated-H4 (Upstate), and IgG (Abcam).

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