EMSA

The cells were stimulated with 100 ng/ml LPS for 30 or 60 min. Then, nuclear proteins were extracted and incubated with an end-labeled, double-stranded oligonucleotide containing a NF-κB binding site on the IL-6 promoter in 25 μl of binding buffer (10 mM HEPES-KOH, (pH 7.8), 50 mM KCl, 1 mM EDTA, (pH 8.0), 5 mM $MgCl_2$, and 10% glycerol) for 20 min at room temperature and loaded onto a native 5% polyacrylamide gel. The DNA-protein complexes were visualized by autoradiography. The specificities of the shifted bands were determined by adding Abs specific for p65 and p50 (Santa Cruz Biotechnology).

Luciferase assay

RAW264.7 cells (1×10^5) were transiently transfected with a total 0.5 μg of expression vector, and 100 ng of IL-6 promoter-luciferase construct (21) or TNF-α promoter-luciferase construct (22) using a Superfect transfection reagent (Qiagen). After 24 h, cells were treated with or without 10 ng/ml LPS for 6 h. The luciferase activity was measured using the dual-luciferase reporter assay system (Promega). The Renilla-luciferase reporter gene (20 ng) was used as an internal control.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed essentially with a described protocol (Upstate Biotechnology). In brief, RAW cells were stimulated with 100 ng/ml LPS for 1 or 2 h, and then fixed with formaldehyde for 10 min. The cells were lysed, sheared by sonication, and incubated overnight with specific Ab followed by incubation with protein A-agarose saturated with salmon sperm DNA (Upstate Biotechnology). Precipitated DNA was analyzed by quantitative PCR (35 cycles) using primers 5′-ACTAGCCAGGAGGGAGAACAGAAACTC-3′ and 5′-CA CAAGCAGGAATGAGAAGAGGCTGAG-3′ for the TNF-α promoter and 5′-TAGCAGCAGGTCCAACTGTGCTATCTG-3′ and 5′-AAGC CTCCGACTTGTGAAGTGGTATAG-3′ for the IL-6 promoter.

In another experiment, peritoneal macrophages from wild-type mice and Stat3 mutant mice were pretreated with 10 ng/ml IL-10 for 18 h, then stimulated with 100 ng/ml LPS for 1 h, and used for ChIP assay.

RNA interference

RAW cells (4×10^6) were transfected with 500 pmol of dsRNA using Nucleofector (Amaxa). The target small interference RNA (siRNA), 5′-GUGCAGAUGUUACUGCAAAA-3′, was designed and produced by Dharmacon. The control siRNA was purchased from Dharmacon (catalog no. D-001206-08-05).

Results

Characterization of CD11b-positive cells in the colonic lamina propria

To analyze the function of colonic macrophages, we first isolated CLPMϕ according to procedures described in *Materials and Methods*. Flow cytometric analysis showed that 30–40% of CLPMϕ also expressed CD11c, indicating the presence of both macrophage-lineage cells and dendritic cell-lineage cells in the population. Using highly purified (over 97% purity) CLPMϕ, we analyzed their response to TLR ligands such as LPS and CpG DNA. We first stimulated CD11b-positive cells from the spleen and colonic lamina propria with LPS or CpG DNA, and analyzed for inflammatory cytokine production (Fig. 1*A*). CD11b-positive cells from the spleen produced significant amounts of TNF-α, IL-6, IL-12p40, and IL-10 in response to LPS or CpG DNA. However, TLR ligand-induced increase in production of TNF-α, IL-6, and IL-12p40 was not observed in CLPMϕ, although IL-6 was produced in the absence of stimulation. In addition, IL-10 production was constitutively observed in CLPMϕ. We next analyzed TLR ligand-induced augmentation of surface molecules such as CD40, CD80, CD86, and MHC class II (Fig. 1*B*). These surface molecules were not up-regulated in response to LPS or CpG DNA in CLPMϕ.

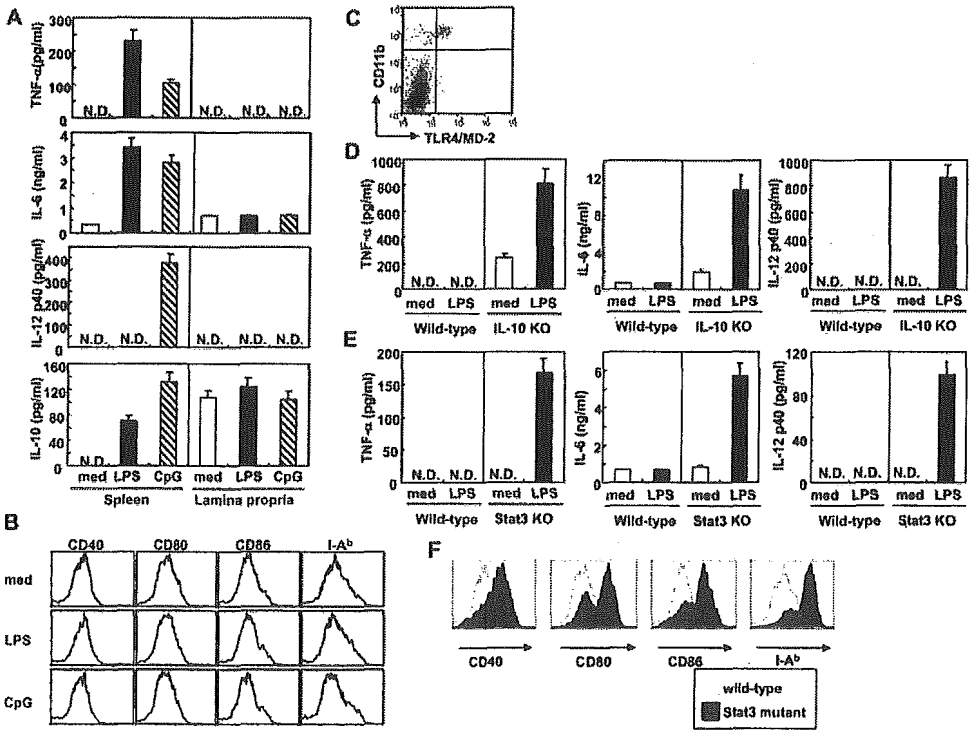

FIGURE 1. Characterization of CD11b-positive cells in the colonic lamina propria (CLPMϕ). *A*, CD11b-positive cells were isolated from the spleen and the colonic lamina propria, then stimulated with 100 ng/ml LPS or 10 nM CpG DNA for 24 h. Concentrations of TNF-α, IL-6, IL-12p40, and IL-10 in the culture supernatants were measured by ELISA. N.D., Not detected. *B*, The cells were also analyzed for surface expression of CD40, CD80, CD86, and MHC class II by flow cytometry. *C*, Colonic lamina proprial cells were stained with anti-CD11b and anti-TLR4/MD-2 Abs. *D*, CLPMϕ were isolated from IL-10-deficient mice, in which chronic colitis was already developed, and were analyzed for LPS-induced production of TNF-α, IL-6, and IL-12p40 by ELISA. *E*, CLPMϕ were isolated from 4- to 5-wk-old Stat3 mutant mice, in which chronic colitis was not developed yet, and analyzed for LPS-induced production of TNF-α, IL-6, and IL-12p40 by ELISA. *F*, CLPMϕ isolated from 4- to 5-wk-old wild-type and Stat3 mutant mice were analyzed for surface expression of CD40, CD80, CD86, and MHC class II by flow cytometry.

Thus, CLPMϕ were refractory to TLR ligands in terms of inflammatory cytokine production and costimulatory molecule expression. Surface expression of TLR4-MD-2 complex on CLPMϕ was observed (Fig. 1*C*). Therefore, the hyporesponsiveness to TLR ligands was not due to the lack of TLR4 expression in CLPMϕ (Fig. 1*C*).

We next isolated CLPMϕ from IL-10-deficient mice, in which chronic colitis has already developed, and analyzed for inflammatory cytokine production in response to TLR ligands (Fig. 1*D*). Although CLPMϕ from wild-type mice did not show LPS-induced production of TNF-α and IL-6, CLPMϕ from IL-10-deficient mice produced small amounts of TNF-α and IL-6 even when cultured with media alone, and the production was robustly enhanced in response to LPS. CLPMϕ from mice lacking Stat3 in macrophage (Stat3 mutant mice) also showed increased TNF-α and IL-6 production in response to LPS (data not shown). Even in CLPMϕ from young (4- to 5-wk-old) IL-10-deficient or Stat3 mutant mice, which have not developed colitis yet, LPS stimulation resulted in increased production of TNF-α and IL-6, indicating that enhanced production of inflammatory cytokines was not due to environmental effects, but intrinsic to CLPMϕ themselves (Fig. 1*E*). Surface expression of CD40, CD80, CD86, and MHC class II was upregulated in CLPMϕ from young Stat3 mutant mice (Fig. 1*F*). Thus, CLPMϕ from IL-10-deficient or Stat3 mutant mice showed enhanced inflammatory response even before colitis was developed. These findings suggest that under normal conditions, CLPMϕ become tolerant to TLR ligand stimulation, and failure to establish tolerance correlates with the development of chronic colitis.

Identification of genes that are specifically expressed in CLPMϕ

In the next set of experiments, we tried to reveal the mechanisms for differential responses to TLR ligands seen in CLPMϕ of wild-type and IL-10-deficient mice. DNA microarray analysis using mRNA purified from CLPMϕ of wild-type mice and IL-10-deficient mice led to identification of several genes that are selectively expressed in wild-type CLPMϕ, but not in IL-10-deficient CLPMϕ (data not shown). These genes include IκBNS, Bcl-3, macrophage scavenger receptor 2, and CD163. RT-PCR analysis confirmed that these genes were expressed in wild-type CLPMϕ, but not in IL-10-deficient CLPMϕ or wild-type peritoneal CD11b-positive cells (Fig. 2*A*). CD163 is a member of the scavenger receptor cysteine-rich superfamily, and was shown to be an IL-10-inducible gene in monocytes/macrophages (23–25). Bcl-3 has been shown to be induced by IL-10 in macrophages and is responsible for suppression of LPS-induced TNF-α production (22). In addition to Bcl-3, IκBNS was selectively expressed in wild-type CLPMϕ. Like Bcl-3, IκBNS is a member of the nucleus-localized IκB family proteins bearing ankyrin-repeats (26). We analyzed whether IκBNS expression is induced by IL-10 in the RAW macrophage cell line and peritoneal macrophages. Real-time RT-PCR analysis showed that IκBNS mRNA was induced within 1 h of IL-10 treatment in both RAW cells and peritoneal macrophages, indicating that like Bcl-3, IκBNS is an IL-10-inducible gene in these cells (Fig. 2, *B* and *C*). Because Bcl-3 was shown to inhibit LPS-induced TNF-α production and IκBNS is structurally related to Bcl-3, we decided to analyze the role of IκBNS in macrophages.

IκBNS inhibits IL-6 production in macrophages

To analyze the role of IκBNS in macrophages, we introduced IκBNS together with GFP into RAW264.7 cells using a lentiviral vector system (22, 27). A lentiviral vector containing GFP alone was used as control in all experiments. RAW264.7 cells were infected with lentivirus, and after 2 days of culture, GFP-positive cells were isolated by FACS sorting. Following stimulation with LPS, the production of TNF-α and IL-6 in the culture supernatants was analyzed (Fig. 3*A*). RAW cells expressing GFP alone secreted significant amounts of IL-6 in response to LPS. However, LPS-induced secretion of IL-6 was severely reduced in cells expressing IκBNS/GFP. Similar amounts of TNF-α were produced by RAW cells expressing GFP alone and IκBNS/GFP in response to LPS. We also analyzed the LPS-induced mRNA expression of IL-6, TNF-α, and IL-1β (Fig. 3*B*). Introduction of IκBNS/GFP resulted in severely impaired LPS-induced IL-6 mRNA expression. However, even in cells expressing IκBNS/GFP, LPS-induced mRNA expression of TNF-α and IL-1β was not impaired. Thus, lentiviral expression of IκBNS in macrophages resulted in specific inhibition of LPS-induced IL-6 production.

IκBNS associates with NF-κB p50 and enhances its DNA-binding activity

Because Bcl-3 has a regulatory function on NF-κB activity, we next examined LPS-induced NF-κB activation in cells expressing IκBNS/GFP. We first analyzed LPS-induced degradation of IκBα by Western blot analysis (Fig. 4*A*). LPS stimulation induced degradation of IκBα in cells expressing IκBNS/GFP as well as cells expressing GFP alone. LPS-induced phosphorylation of ERK1/2 and p38 was not impaired in cells expressing IκBNS/GFP, indicating that expression of IκBNS did not affect LPS signaling pathway in the cytoplasmic compartment (Fig. 4*B*). We next analyzed whether the DNA-binding activity of NF-κB was altered in cells expressing IκBNS (Fig. 4*C*). LPS stimulation predominantly enhanced DNA-binding activity of p50/p65 heterodimers in cells transduced with GFP alone. In cells expressing IκBNS/GFP, the DNA-binding activity of p50/p50 homodimers became evident even before LPS stimulation, and the DNA-binding activity of

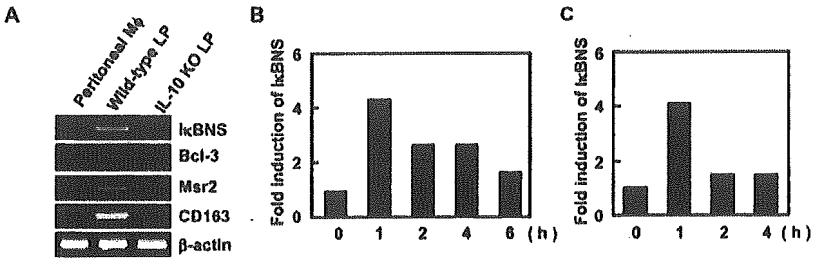

FIGURE 2. Identification of genes that are selectively expressed in CLPMϕ. *A*, Total RNA was purified from CLPMϕ of wild-type or IL-10-deficient mice, and peritoneal CD11b-positive cells (Mϕ), and then analyzed for expression of IκBNS, IκBζ, Bcl-3, macrophage scavenger receptor 2 (Msr2), and CD163 by RT-PCR. *B* and *C*, RAW264.7 cells (*B*) and peritoneal macrophages (*C*) were treated with 10 ng/ml IL-10 for the indicated periods. IκBNS and EF1-α mRNA were measured by quantitative real-time PCR. Expression of IκBNS was normalized to housekeeping gene EF1-α. Data were expressed as relative fold induction of IκBNS compared with nontreated condition.

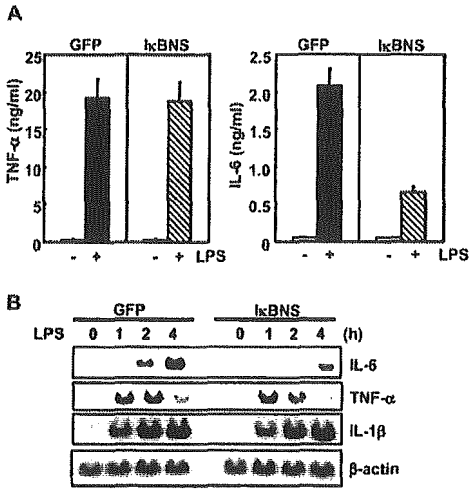

FIGURE 3. Lentiviral introduction of IκBNS results in impaired LPS-induced IL-6 production in macrophages. *A*, RAW cells were infected with lentivirus expressing IκBNS/GFP or GFP alone. After infection for 24 h, RAW cells were washed and additionally incubated for 2 days. Then, GFP-positive cells were purified by FACS sorting, and stimulated with 100 ng/ml LPS for 24 h. Concentrations of TNF-α and IL-6 in the culture supernatants were determined by ELISA. *B*, GFP-positive cells (GFP alone and IκBNS/GFP) were purified, stimulated with LPS (100 ng/ml) for the indicated period, and then total RNA extracts were analyzed for the mRNA expression of IL-6, TNF-α, and IL-1β. Hybridization with the β-actin probe confirmed even loading of RNA in each lane.

p50/p50 homodimers remained dominant even after LPS stimulation. The specificity of the bands was confirmed by supershifts using anti-p50 and anti-p65 Abs. Thus, cells expressing IκBNS showed altered DNA-binding activity of NF-κB. Previous studies showed that Bcl-3 preferentially interacted with p50 subunit of NF-κB (28). Therefore, we next analyzed whether IκBNS associates with NF-κB. RAW cells were lentivirally introduced with Flag-tagged IκBNS and subjected to coimmunoprecipitation analysis using Abs that detect endogenous p50 or p65 subunit (Fig. 4*D*). Flag-tagged IκBNS that coimmunoprecipitated with p50, but not p65, was detected by anti-Flag Ab (Fig. 4*D*, *left*). Conversely, p50, but not p65, coimmunoprecipitated with IκBNS (Fig. 4*D*, *right*). Thus, these findings indicate that like Bcl-3, IκBNS specifically associates with p50 subunit of NF-κB in macrophages.

IκBNS inhibits LPS-induced activation of the IL-6 promoter

We next analyzed the mechanism by which IκBNS specifically inhibits IL-6 production in macrophages. We first examined the effect of transient overexpression of IκBNS on LPS-induced activation of the IL-6 and TNF-α promoters using a reporter gene assay. Ectopic expression of IκBNS suppressed LPS-induced transcriptional activity of the IL-6 promoter in RAW cells in a dose-dependent manner (Fig. 5*A*). In contrast, IκBNS expression had no effect on LPS-induced transactivation of the TNF-α promoter (Fig. 5*B*). Thus, IκBNS has an inhibitory effect on the LPS-induced activation of the IL-6 promoter, but not the TNF-α promoter.

We next performed ChIP assays to further investigate how IκBNS specifically regulates IL-6 promoter activity. RAW cells constitutively expressing Flag-IκBNS were stimulated with LPS for 1 or 2 h, and ChIP assays were performed using Abs that detect Flag or endogenous p50 and p65 (Fig. 5*C*). In RAW cells expressing IκBNS, both IκBNS and p50 were recruited to the IL-6 promoter before LPS stimulation. The recruitment of IκBNS and p50 to the IL-6 promoter was stably observed even after LPS stimulation. Furthermore, LPS-induced recruitment of p65 to the IL-6 promoter was reduced in RAW cells expressing IκBNS. In contrast, IκBNS was not recruited to the TNF-α promoter, and LPS-induced recruitment of p50 and p65 was normally observed at the TNF-α promoter, indicating that IκBNS expression did not have any effect on the TNF-α promoter. Taken together, these results suggest that IκBNS suppresses the IL-6 promoter activity by selective recruitment to the IL-6 promoter with NF-κB p50.

We addressed whether the altered recruitment of p50 and p65 to the IL-6 promoter was observed in IL-10-pretreated primary macrophages, in which IκBNS expression was induced. Peritoneal macrophages from wild-type mice were pretreated with IL-10 for 18 h, then stimulated with LPS for 1 h, and analyzed by ChIP assay (Fig. 6). In nonpretreated macrophages, recruitment of p50 and p65 to the IL-6 promoter was observed only after LPS stimulation. However, in IL-10 pretreated cells, p50 was recruited to the IL-6 promoter even before LPS stimulation and LPS-induced recruitment of p65 was severely impaired. In addition, IL-10-mediated alteration of NF-κB recruitment to the IL-6 promoter was not observed in Stat3-deficient macrophages, in which IL-10 signaling was abolished. Thus, IκBNS-mediated alteration of NF-κB recruitment to the IL-6 promoter correlates with changes mediated by IL-10 in macrophages, indicating that IκBNS mediates IL-10-induced inhibition of IL-6 production.

Inhibition of IκBNS expression results in increased IL-6 production in macrophages

To further clarify the involvement of IκBNS in suppression of LPS-induced IL-6 production in macrophages, we used siRNA to block expression of IκBNS in RAW cells. RAW cells were transfected with control (nonspecific) siRNA or IκBNS siRNA. After transfection, the cells were stimulated with LPS and analyzed for expression of IκBNS, IL-6, and TNF-α. IκBNS mRNA expression was induced by LPS as well as IL-10 in RAW cells (Fig. 7*A*). Introduction of IκBNS siRNA resulted in reduced IκBNS mRNA expression to ~30% (Fig. 7*A*). In these cells, LPS-induced TNF-α mRNA expression was not significantly altered. However, LPS-induced IL-6 mRNA expression was increased to ~200% of the level found in control cells. LPS-induced production of TNF-α and IL-6 was also analyzed by ELISA (Fig. 7*B*). IκBNS knockdown in RAW cells had no effect on LPS-induced TNF-α production. However, in cells transfected with IκBNS siRNA, LPS-induced IL-6 production was increased by about 2-fold compared with cells transfected with control siRNA. We also analyzed activity of the IL-6 promoter in cells transfected with IκBNS siRNA (Fig. 7*C*). LPS-induced activation of the IL-6 promoter, but not the TNF-α promoter, was increased in cells with reduced IκBNS expression. Thus, siRNA-mediated reduction of IκBNS expression in macrophages enhanced LPS-induced activation of the IL-6 promoter and production of IL-6. Taken together, these data demonstrate that IκBNS negatively regulates LPS-induced IL-6 production in macrophages.

Discussion

In this study, we first characterized CLPMϕ. Several studies in animal models of IBD and human IBD patients indicate that cells of macrophage lineage play an important role in intestinal mucosal immune responses. Aberrant activation of macrophages due to the absence of Stat3 led to the development of chronic colitis (12). Increased CD40L-induced production of IL-12 in mucosal dendritic cells was also demonstrated in mice with colitis (29). In humans, mucosal macrophages from IBD patients showed higher expression of several surface molecules such as CD14, CD16, and HLA-DR (30–32). Furthermore, mucosal macrophages from IBD patients showed enhanced activity, such as the release of oxygen

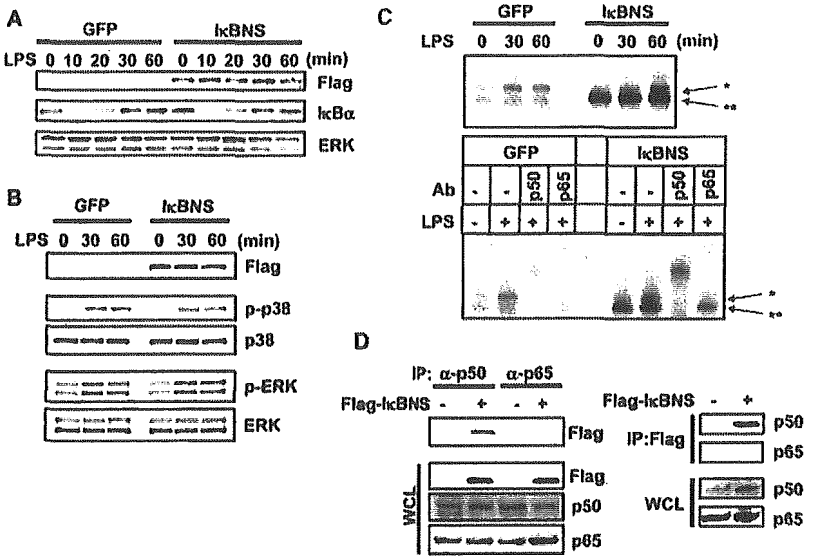

FIGURE 4. Impaired NF-κB activity in IκBNS-introduced macrophages. *A*, GFP-positive cells (GFP alone and IκBNS/GFP) were purified, stimulated with LPS (100 ng/ml) for the indicated period (min), and the total cell lysates were analyzed for expression of Flag-IκBNS, IκBα, and ERK by Western blot analysis. *B*, Western blots showing the extent of phosphorylation of p38 and ERK MAPKs at the indicated time period after LPS stimulation in RAW cells expressing GFP alone or IκBNS/GFP. *C*, RAW cells introduced with GFP alone or IκBNS/GFP were stimulated with 100 ng/ml LPS for the indicated time period. Nuclear extracts were subjected to EMSA using the NF-κB binding site of the IL-6 promoter as a probe. The specificities of the shifted bands were determined by adding specific Abs to p50 and p65. Two types of NF-κB bindings to the probe, p50/p65 heterodimer (*) and p50/p50 homodimer (**), are indicated. *D*, RAW cells introduced with GFP alone or IκBNS/GFP were lysed and immunoprecipitated (IP) with anti-p50 or anti-p65 Abs (*left*). The immunoprecipitated lysates were subsequently immunoblotted with anti-Flag Ab. The same lysates were immunoprecipitated with anti-Flag Ab, and blotted with anti-p50 or anti-p65 Abs (*right*).

radicals and mature IL-1β, and presentation of Ag (33–35). Thus, enhanced activity of mucosal macrophages is associated with the pathogenesis of colitis.

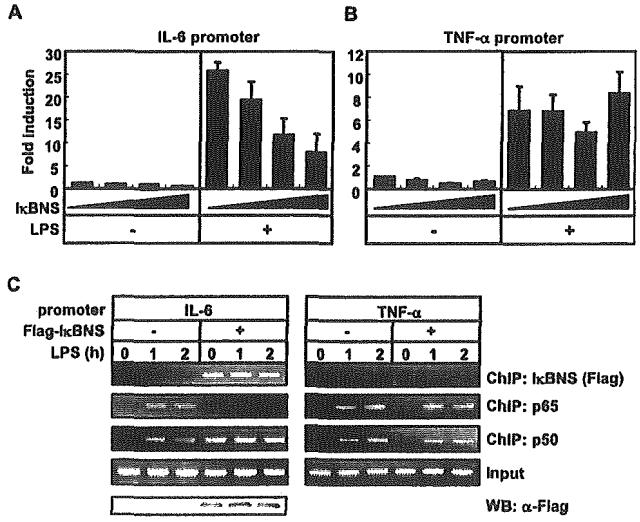

FIGURE 5. IκBNS is specifically recruited to the IL-6 promoter. *A* and *B*, RAW cells were transiently cotransfected with the IL-6 promoter-luciferase or TNF-α promoter-luciferase construct (10 ng), together with an IκBNS expression vector (0, 0.05, 0.25, or 0.5 μg) as indicated. After 24 h of transfection, cells were treated with or without 10 ng/ml LPS for 6 h and then the luciferase activities were measured. The data are representative of three independent experiments yielding similar results. Data are expressed as relative fold activation compared with the nonstimulated (−) set. *C*, RAW cells that constitutively express Flag-IκBNS were stimulated with 100 ng/ml LPS for 1 or 2 h, and ChIP assays were performed with anti-Flag, anti-p50, or anti-p65 Abs. The immunoprecipitated IL-6 promoter (*left*) or TNF-α promoter (*right*) was detected by PCR with promoter-specific primers. Data are representative of three independent experiments. The expression of Flag-IκBNS by Western blot analysis is shown at *bottom*.

Recently, TLRs have been shown to be essential for activation of innate immune cells, such as macrophages and dendritic cells, through the recognition of microbial components (8–11). Involvement of TLR-dependent responses in the pathogenesis of IBD has further been postulated because chronic colitis was greatly reduced in mice lacking Stat3 and TLR4 (15). In the present study, we demonstrated that CLPMϕ in normal mice, unlike CD11b-positive cells from the spleen, are refractory to TLR stimulation in terms of inflammatory cytokine production and costimulatory molecule expression. Furthermore, CLPMϕ from IL-10-deficient or Stat3 mutant mice, even in the absence of colitis, showed significant responses to TLR stimulation, indicating that the responsiveness of CLPMϕ to TLR ligands correlates with the pathogenesis of chronic colitis in these mutant mice.

We further analyzed the molecular basis for the hyporesponsiveness of CLPMϕ by comparing gene expression profiles of CLPMϕ from wild-type mice and IL-10-deficient mice. DNA microarray analysis led to the identification of several genes that are selectively expressed in CLPMϕ of wild-type mice, but not in peritoneal macrophages or CLPMϕ of IL-10-deficient mice. Among these genes, CD163 and Bcl-3 were reported to be induced by IL-10 in macrophages or monocytes (22, 23, 36). In addition, we found that IκBNS is selectively expressed in CLPMϕ of wild-type mice, and further is induced by IL-10 in peritoneal macrophages and RAW cells. IκBNS was originally identified as a gene that is induced upon TCR stimulation and affects NF-κB activity in T cells (26). Although IκBNS was suggested to be involved in negative selection of thymocytes, the role of IκBNS in macrophages remained unclear. In addition, although IκBNS was shown to be localized in the nucleus and preferentially associates with p50 subunit of NF-κB, the mechanism by which IκBNS inhibits NF-κB activity remains unknown. Because IκBNS is structurally related to Bcl-3, we postulated that IκBNS has a regulatory role in macrophages, and analyzed the effect of IκBNS expression in the RAW macrophage cell line. Lentiviral expression and siRNA-mediated knockdown of IκBNS in RAW cells demonstrated

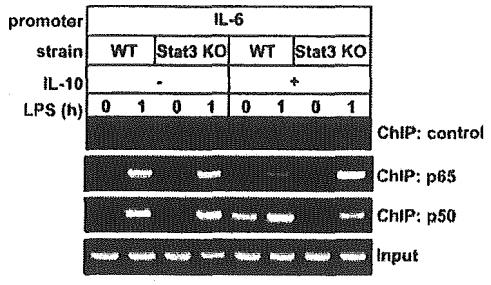

FIGURE 6. IL-10-induced alteration in recruitment of p50 and p65 to the IL-6 promoter. Peritoneal macrophages from wild-type or Stat3 mutant mice were cultured for 18 h in the presence or absence of 10 ng/ml IL-10, then stimulated with 100 ng/ml LPS for 1 h, and ChIP assays were performed with anti-p50, anti-p65 Abs, or control rabbit Ig. The immunoprecipitated IL-6 promoter was detected by PCR. Representative of three independent experiments.

that this molecule suppresses LPS-induced IL-6 production. Similar to the case of Bcl-3, which is specifically recruited to the TNF-α promoter together with the p50 subunit of NF-κB thereby inhibiting LPS-induced TNF-α production (22), IκBNS suppressed LPS-induced activation of the IL-6 promoter through constitutive recruitment to the promoter together with p50. Thus, nuclear IκB proteins Bcl-3 and IκBNS differentially modulate TLR-mediated gene induction in macrophages.

IκBNS and Bcl-3 are differentially recruited to specific promoters through association with p50 homodimers. But, it remains unclear how the specific recruitment of these nuclear IκB proteins are induced. Nuclear IκB proteins do not seem to have the ability to directly bind the promoters. Therefore, association with p50 may cause modulation of the DNA binding specificity through unknown mechanisms. Similar specific recruitment was also demonstrated in IκBζ, which is structurally related to IκBNS and Bcl-3 (21, 37–39). In the case of IκBζ, the specific recruitment to the IL-6 promoter led to activation of the promoter (21, 37). Thus, several lines of evidence demonstrate that nuclear IκB proteins specifically regulate expression of NF-κB target genes.

In normal condition, both IκBNS and IκBζ have regulatory roles in the production of IL-6 in macrophages. IκBNS suppresses LPS-induced IL-6 production, whereas IκBζ promotes IL-6 production (21). Thus, although the ankyrin-repeats of both molecules are highly conserved, they have opposite functions. IκBNS consists of 327 amino acids and the ankyrin-repeat covers almost the entire protein. In contrast, IκBζ is a protein with 629 amino acids and possesses an N-terminal region of ~300 amino acids in addition to the C-terminal ankyrin-repeat. The full-length IκBζ has no inhibitory effect on NF-κB activity, but introduction of the C-terminal ankyrin-repeat of IκBζ resulted in impaired NF-κB activation (37). Thus, the ankyrin-repeat of IκBζ has an inhibitory effect on NF-κB activity, but the N-terminal region may activate NF-κB with unknown mechanisms.

IκBNS and Bcl-3 are expressed in CLPMϕ of wild-type mice, but not the CLPMϕ of IL-10-deificient mice. In addition, expression of IκBNS and Bcl-3 is induced by IL-10 treatment in peritoneal macrophages and RAW cells. Therefore, expression of IκBNS and Bcl-3 in CLPMϕ of wild-type mice might be induced by IL-10. Indeed, constitutive production of IL-10 was observed in CLPMϕ of wild-type mice. Additionally, IL-10 might be provided from other types of cells such as IL-10-producing type 1 regulatory T cells or epithelial cells. There might be other explanations of the characteristics of CLPMϕ of wild-type mice. IκBNS and Bcl-3 were also found to be induced by LPS stimulation, but their expression was not observed in CLPMϕ of IL-10-deficient or Stat3 mutant mice. Therefore, CLPMϕ of wild-type mice might be a distinct cell population from macrophages residing in other tissues, and IL-10 might be involved in the development of these unique CLPMϕ or in the recruitment of CLPMϕ to the colonic lamina propria. Constitutive expression of both IκBNS and Bcl-3 in CLPMϕ may suppress exaggerated inflammatory responses in the intestinal mucosal surface. A more precise characterization of

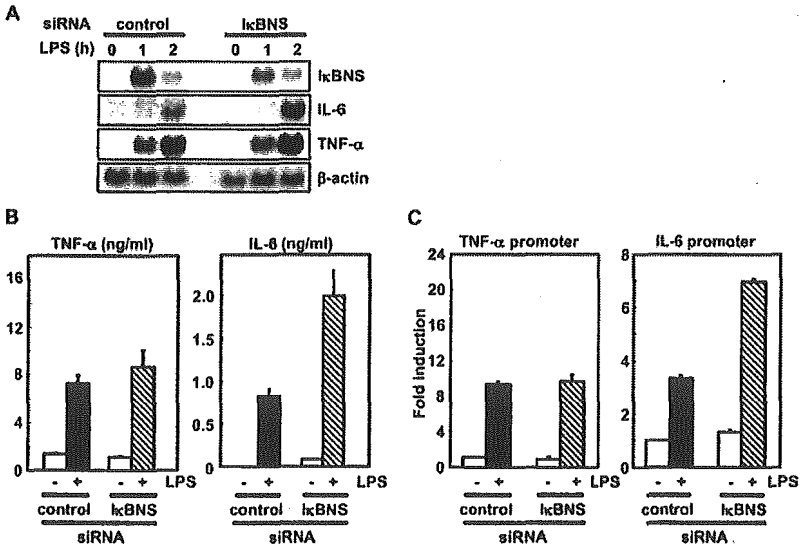

FIGURE 7. IκBNS is required for suppression of LPS-induced IL-6 expression. *A*, RAW cells were transfected with 500 pmol IκBNS siRNA or control siRNA as indicated. Three hours later, the cells were stimulated with 100 ng/ml LPS for 1 or 2 h. Total RNA was prepared and analyzed for expression of IκBNS, IL-6, TNF-α, and β-actin by Northern blotting. Data are representative of three independent experiments. *B*, After transfection of IκBNS siRNA or control siRNA, RAW cells were stimulated with 100 ng/ml LPS for 24 h. Concentrations of TNF-α and IL-6 in the culture supernatants were determined by ELISA. *C*, RAW cells were transfected with 500 pmol IκBNS siRNA or control siRNA together with 100 ng of IL-6 or TNF-α promoter reporter plasmid. Three hours after transfection, the cells were stimulated with 100 ng/ml LPS for 6 h, and the luciferase activities were measured. Representative of three independent experiments yielding similar results. Data are expressed as relative fold activation compared with the nonstimulated (−) set.

CLPMϕ of wild-type mice will be required to answer to this question.

CLPMϕ are tolerant to TLR stimulation and show no inflammatory cytokine production. However, Bcl-3 and IκBNS are likely to be involved in suppression of the specific cytokine such as TNF-α and IL-6, respectively. Therefore, there might be other mechanisms that suppress TLR responses. NOD2, a member of the NOD family of proteins, might be involved in one of such mechanisms. NOD2 is implicated in the negative regulation of TLR2-mediated NF-κB activation (40). Mutations in NOD2 have been shown to be associated with Crohn's diseases (16, 17).

In this study, we showed that nuclear IκB proteins, such as Bcl-3 and IκBNS, differentially modulate LPS-induced inflammatory cytokine production in macrophages. CLPMϕ in wild-type mice are tolerant to TLR stimulation, which may contribute to prevention of chronic intestinal inflammation. Several mechanisms are involved in the hyporesponsiveness of CLPMϕ, and selective inhibition of LPS responses by nuclear IκB proteins may explain a part of these mechanisms. Further studies will be required to understand regulations of CLPMϕ activity, which will definitely be useful for the development of an effective cure for IBD.

Acknowledgments

We thank H. Miyoshi for providing us a lentivirus vector, N. Okita and N. Kitagaki for technical assistance, and M. Hashimoto and M. Kurata for secretarial assistance.

Disclosures

The authors have no financial conflict of interest.

References

1. Podolsky, D. K. 1991. Inflammatory bowel disease. *N. Engl. J. Med. 325:928.*
2. Strober, W., I. J. Fuss, and R. S. Blumberg. 2002. The immunology of mucosal models of inflammation. *Annu. Rev. Immunol. 20:495.*
3. Bouma, G., and W. Strober. 2003. The immunological and genetic basis of inflammatory bowel disease. *Nat. Rev. Immunol. 3:521.*
4. Neurath, M. F., S. Finotto, and L. H. Glimcher. The role of Th1/Th2 polarization in mucosal immunity. *Nat. Med. 6:567.*
5. Maloy, K. J., and F. Powrie. 2001. Regulatory T cells in the control of immune pathology. *Nat. Immunol. 9:816.*
6. Mottet, C., H. H. Uhlig, and F. Powrie. 2003. Cutting edge: cure of colitis by $CD4^+CD25^+$ regulatory T cells. *J. Immunol. 170:3939.*
7. Mizoguchi, A., E. Mizoguchi, H. Takedatsu, R. S. Blumberg, and A. K. Bhan. 2002. Chronic intestinal inflammatory condition generates IL-10-producing regulatory B cell subset characterized by CD1d upregulation. *Immunity 16:219.*
8. Janeway, C. A. Jr., and R. Medzhitov. 2002. Innate immune recognition. *Annu. Rev. Immunol. 20:197.*
9. Akira. S., K. Takeda, and T. Kaisho. 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat. Immunol. 2:675.*
10. Takeda, K., T. Kaisho, and S. Akira. 2003. Toll-like receptors. *Annu. Rev. Immunol. 21:335.*
11. Akira, S., and K. Takeda. 2004. Toll-like receptor signaling. *Nat. Rev. Immunol. 4:499.*
12. Takeda, K., B. Clausen, T. Kaisho, T. Tsujimura, N. Terada, I. Förster, and S. Akira. 1999. Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. *Immunity 10:39.*
13. Kuhn, R., J. Lohler, D. Rennick, K. Rajewsky, and W. Muller. 1993. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell 75:263.*
14. Berg, D. M., N. Davidson, R. Kühn, W. Müller, S. Menon, G. Holland, L. Thompson-Snipes, M. W. Leach, and D. Rennick. 1996. Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and $CD4^+$ TH1-like responses. *J. Clin. Invest. 98:1010.*
15. Kobayashi, M., M. Kweon, H. Kuwata, H. Kiyono, K. Takeda, and S. Akira. 2003. Toll-like receptor-dependent IL-12p40 production causes chronic enterocolitis in myeloid cell-specific Stat3-deficient mice. *J. Clin. Invest. 111:1297.*
16. Ogura, Y., D. K. Bonen, N. Inohara, D. L. Nicolae, F. F. Chen, R. Ramos, H. Britton, T. Moran, R. Karaliuskas, R. H. Duerr, et al. 2001. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature 411:603.*
17. Hugot, J. P., M. Chamaillard, H. Zouali, S. Lesage, J. P. Cezard, J. Belaiche, S. Almer, C. Tysk, C. A. O'Morain, M. Gassull, et al. 2001. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature 411:599.*
18. Inohara, N., and G. Nunez. 2003. NODs: intracellular proteins involved in inflammation and apoptosis. *Nat. Rev. Immunol. 3:371.*
19. Mizoguchi, E., R. J. Xavier, H. C. Reinecker, H. Uchin, A. K. Bhan, D. K. Podolsky, and A. Mizoguchi. 2003. Colonic epithelial functional phenotype varies with type and phase of experimental colitis. *Gastroenterology 125:148.*
20. Matsumoto, M., D. Einhaus, E. S. Gold, and A. Aderem. 2004. Simvastatin augments lipopolysaccharide-induced proinflammatory responses in macrophages by differential regulation of the c-Fos and c-Jun transcription factors. *J. Immunol. 172:7377.*
21. Yamamoto, M., S. Yamazaki, S. Uematsu, S. Sato, H. Hemmi, K. Hoshino, T. Kaisho, H. Kuwata, O. Takeuchi, K. Takeshige, et al. 2004. Regulation of Toll/IL-1-receptor-mediated gene expression by the inducible nuclear protein IκBζ. *Nature 430:218.*
22. Kuwata, H., Y. Watanabe, H. Miyoshi, M. Yamamoto, T. Kaisho, K. Takeda, and S. Akira. 2003. IL-10-inducible Bcl-3 negatively regulates LPS-induced TNF-α production in macrophages. *Blood 102:4123.*
23. Williams, L., G. Jarai, A. Smith, and P. Finan. 2002. IL-10 expression profiling in human monocytes. *J. Leukocyte Biol. 72:800.*
24. Sulahian, T. H., P. Hogger, A. E. Wahner, K. Wardwell, N. J. Goulding, C. Sorg, A. Droste, M. Stehling, P. K. Wallace, P. M. Morganelli, and P. M. Guyre. 2000. Human monocytes express CD163, which is upregulated by IL-10 and identical to p155. *Cytokine 12:1312.*
25. Buechler, C., M. Ritter, E. Orso, T. Langmann, J. Klucken, and G. Schmitz. 2000. Regulation of scavenger receptor CD163 expression in human monocytes and macrophages by pro- and antiinflammatory stimuli. *J. Leukocyte Biol. 67:97.*
26. Fiorini, E., I. Schmitz, W. E. Marissen, S. L. Osborn, M. Touma, T. Sasada, P. A. Reche, E. V. Tibaldi, R. E. Hussey, A. M. Kruisbeek, et al. 2002. Peptide-induced negative selection of thymocytes activates transcription of an NF-κB inhibitor. *Mol. Cell. 9:637.*
27. Miyoshi, H., U. Blomer, M. Takahashi, F. H. Gage, and I. M. Verma. 1998. Development of a self-inactivating lentivirus vector. *J. Virol. 72:8150.*
28. Nolan, G. P., T. Fujita, K. K. Bhatia, C. Huppi, H. C. Liou, M. L. Scott, and D. Baltimore. 1993. The Bcl-3 proto-oncogene encodes a nuclear IκB-like molecule that preferentially interacts with NF-κB p50 and p52 in a phosphorylation-dependent manner. *Mol. Cell. Biol. 13:3557.*
29. Krajina, T., F. Leithauser, P. Moller, Z. Trobonjaca, and J. Reimann. 2003. Colonic lamina propria dendritic cells in mice with $CD4^+$ T cell-induced colitis. *Eur. J. Immunol. 33:1073.*
30. Rogler, G., T. Andus, E. Aschenbrenner, D. Vogl, W. Falk, J. Scholmerich, and V. Gross. 1997. Alterations of the phenotype of colonic macrophages in inflammatory bowel disease. *Eur. J. Gastroenterol. Hepatol. 9:893.*
31. Allison, M. C., S. Cornwall, L. W. Poulter, A. P. Dhillon, and R. E. Pounder. 1998. Macrophage heterogeneity in normal colonic mucosa and in inflammatory bowel disease. *Gut 29:1531.*
32. Mahida, Y. R., S. Patel, P. Gionchetti, D. Vaux, and D. P. Jewell. 1989. Macrophage subpopulations in lamina propria of normal and inflamed colon and terminal ileum. *Gut 30:826.*
33. Mahida, Y. R., K. C. Wu, and D. P. Jewell. 1988. Characterization of antigen-presenting activity of intestinal mononuclear cells isolated from normal and inflammatory bowel disease colon and ileum. *Immunology 65:543.*
34. Rugtveit, J., G. Haraldsen, A. K. Hogasen, A. Bakka, P. Brandtzaeg, and H. Scott. 1995. Respiratory burst of intestinal macrophages in inflammatory bowel disease is mainly caused by $CD14^+L1^+$ monocyte derived cells. *Gut 37:367.*
35. McAlindon, M. E., A. Galvin, B. McKaig, T. Gray, H. F. Sewell, and Y. R. Mahida. 1999. Investigation of the expression of IL-1β converting enzyme and apoptosis in normal and inflammatory bowel disease (IBD) mucosal macrophages. *Clin. Exp. Immunol. 116:251.*
36. Lang, R., D. Patel, J. J. Morris, R. L. Rutschman, and P. Murray. 2002. Shaping gene expression in activated and resting primary macrophages by IL-10. *J. Immunol. 169:2253.*
37. Yamazaki, S., T. Muta, and K. Takeshige. 2001. A novel IκB protein, IκB-ζ, induced by proinflammatory stimuli, negatively regulates nuclear factor-κB in the nuclei. *J. Biol. Chem. 276:27657.*
38. Kitamura, H., K. Kanehira, K. Okita, M. Morimatsu, and M. Saito. 2000. MAIL, a novel nuclear IκB protein that potentiates LPS-induced IL-6 production. *FEBS Lett. 485:53.*
39. Haruta, H., A. Kato, and K. Todokoro. 2001. Isolation of a novel interleukin-1-inducible nuclear protein bearing ankyrin-repeat motifs. *J. Biol. Chem. 276:12485.*
40. Watanabe, T., A. Kitani, P. J. Murray, and W. Strober. 2004. NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses. *Nat. Immunol. 5:800.*

29. Inohara, N. & Nunez, G. NODs: intracellular proteins involved in inflammation and apoptosis. *Nature Rev. Immunol.* **3**, 371–382 (2003).
30. Ruefli-Brasse, A. A., Lee, W. P., Hurst, S. & Dixit, V. M. Rip2 participates in Bcl10 signaling and T-cell receptor-mediated NF-κB activation. *J. Biol. Chem.* **279**, 1570–1574 (2004).

Supplementary Information accompanies the paper on **www.nature.com/nature**.

Acknowledgements We thank D. Dornan and other members of the Dixit laboratory for discussions, E. Humke for help with illustrations, and K. O'Rourke, D. Wadley, Z. Gu, C. Olsson, M. Bauer, L. Tom, J. Kloss, M. Fuentes, M. Osborn, C. Tan, J. Hongo, T. Wong and A. Chuntharapai for technical assistance.

Competing interests statement The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to V.M.D. (dixit@gene.com).

Regulation of Toll/IL-1-receptor-mediated gene expression by the inducible nuclear protein IκBζ

Masahiro Yamamoto[1], Soh Yamazaki[3], Satoshi Uematsu[1], Shintaro Sato[1,2], Hiroaki Hemmi[1], Katsuaki Hoshino[6], Tsuneyasu Kaisho[6], Hirotaka Kuwata[1], Osamu Takeuchi[1,2], Koichiro Takeshige[3], Tatsuya Saitoh[7], Shoji Yamaoka[7], Naoki Yamamoto[7], Shunsuke Yamamoto[8], Tatsushi Muta[3,4], Kiyoshi Takeda[5] & Shizuo Akira[1,2]

[1]*Department of Host Defense, Research Institute for Microbial Diseases, Osaka University,* [2]*ERATO, Japan Science and Technology Agency, 3-1 Yamada-oka, Suita Osaka 565-0871, Japan*
[3]*Department of Molecular and Cellular Biochemistry, Graduate School of Medical Sciences,* [4]*PRESTO, Japan Science and Technology Agency,* [5]*Department of Embryonic and Genetic Engineering, Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan*
[6]*RIKEN Research Center for Allergy and Immunology, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan*
[7]*Department of Molecular Virology, Graduate School of Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan*
[8]*Department of Food and Nutrition, Beppu University, Kita-ishigaki, Beppu, Oita 874-0851, Japan*

Toll-like receptors (TLRs) recognize microbial components and trigger the inflammatory and immune responses against pathogens. IκBζ (also known as MAIL and INAP) is an ankyrin-repeat-containing nuclear protein that is highly homologous to the IκB family member Bcl-3 (refs 1–6). Transcription of IκBζ is rapidly induced by stimulation with TLR ligands and interleukin-1 (IL-1). Here we show that IκBζ is indispensable for the expression of a subset of genes activated in TLR/IL-1R signalling pathways. IκBζ-deficient cells show severe impairment of IL-6 production in response to a variety of TLR ligands as well as IL-1, but not in response to tumour-necrosis factor-α. Endogenous IκBζ specifically associates with the p50 subunit of NF-κB, and is recruited to the NF-κB binding site of the IL-6 promoter on stimulation. Moreover, NF-κB1/p50-deficient mice show responses to TLR/IL-1R ligands similar to those of IκBζ-deficient mice. Endotoxin-induced expression of other genes such as *Il12b* and *Csf2* is also abrogated in IκBζ-deficient macrophages. Given that the lipopolysaccharide-induced transcription of IκBζ occurs earlier than transcription of these genes, some TLR/IL-1R-mediated responses may be regulated in a gene expression process of at least two steps that requires inducible IκBζ.

IκBζ is thought to be induced in response to IL-1 or lipopolysaccharide (LPS) (TLR4 ligand)[4–6]. In addition to IL-1 and LPS, IκBζ messenger RNA was strongly upregulated on stimulation with peptidoglycan (PGN) (TLR2 ligand), bacterial lipoprotein (BLP) (TLR1/TLR2), flagellin (TLR5), MALP-2 (TLR6/TLR2), R-848 (TLR7) and CpG DNA (TLR9), but not with tumour-necrosis factor-α (TNF-α) (Fig. 1a). In contrast, other IκB family members such as IκBα and Bcl-3 were induced in response to TNF-α as well as the TLR ligands and IL-1. Thus, IκBζ is induced in the TLR/IL-1R signalling pathway but not the TNF signalling pathway. Furthermore, IL-1- or LPS-induced expression of IκBζ was completely abolished in *MyD88*$^{-/-}$ embryonic fibroblasts (MEFs; Fig. 1b), showing that IκBζ is inducible in the MyD88-dependent part of the TLR/IL-1R signalling pathway[7–15].

To elucidate the physiological role of IκBζ in the TLR/IL-1R response, we generated *IκBζ*$^{-/-}$ mice by targeted gene disruption (see Supplementary Discussion 1 and Supplementary Fig. 1a–d). *IκBζ*$^{-/-}$ splenocytes showed defective proliferation in response to LPS but not to anti-CD40, IL-4 and anti-IgM (Supplementary Fig. 1e, f), suggesting that the TLR4 response is impaired in *IκBζ*$^{-/-}$ cells. Moreover, although *IκBζ*$^{-/-}$ mice grew normally after birth, some of them started to develop atopic dermatitis-like skin lesions with acanthosis and lichenoid changes at the age of 4–5 weeks (Supplementary Fig. 2a, b). All *IκBζ*$^{-/-}$ mice developed the disease by the age of 10 weeks. Histological analysis of 5-week-old *IκBζ*$^{-/-}$ mice showed pathological changes in the conjunctiva, including a heavy lymphocyte infiltration into the submucosa and loss of goblet cells in the conjunctival epithelium (Supplementary Fig. 2c–f).

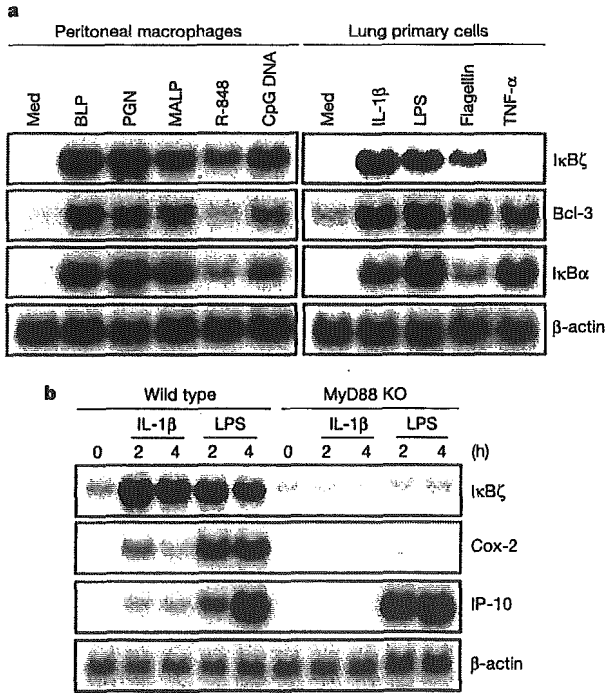

Figure 1 Specific induction of IκBζ on stimulation by TLR/IL-1R ligands. **a**, The indicated cells were stimulated with 100 ng ml^{-1} BLP, 10 μg ml^{-1} PGN, 30 ng ml^{-1} MALP-2, 100 nM R-848, 3 μM CpG DNA, 10 ng ml^{-1} IL-1β, 10 μg ml^{-1} LPS, 100 ng ml^{-1} flagellin and 10 ng ml^{-1} TNF-α for 2 h. Total RNA (10 μg) was extracted and subjected to northern blot analysis for expression of IκBζ, Bcl-3, IκBα and β-actin. Med, medium. **b**, *MyD88*$^{+/+}$ (wild type) and *MyD88*$^{-/-}$ (KO) MEFs were stimulated with 10 ng ml^{-1} IL-1β and 10 μg ml^{-1} LPS for the indicated periods. Total RNA (10 μg) was extracted and subjected to northern blot analysis for expression of IκBζ, Cox-2, IP-10 and β-actin.

We analysed the LPS-induced production of inflammatory mediators in macrophages. *IκBζ*$^{+/+}$ macrophages produced TNF-α, IL-6 and nitric oxide (NO) in response to LPS (Fig. 2a). Although LPS-induced production of TNF-α and NO was normal, production of IL-6 was severely impaired in *IκBζ*$^{-/-}$ macrophages. In addition, production of IL-6 via stimulation by various TLR ligands was also profoundly inhibited in *IκBζ*$^{-/-}$ cells (Fig. 2b–d). Moreover, *IκBζ*$^{-/-}$ cells exhibited defective IL-1-induced IL-6 production; however, IL-1-induced activation of NF-κB and mitogen-activated protein kinases was not impaired in these cells, indicating that there is no defect in the intracellular signalling pathways (Supplementary Fig. 3a, b). On the other hand, TNF-α-induced IL-6 production was not impaired in *IκBζ*$^{-/-}$ cells (Fig. 2e). The impaired production of IL-6 in response to LPS correlated well with the reduced induction of IL-6 mRNA in *IκBζ*$^{-/-}$ cells (Fig. 2f).

When full-length or a deletion mutant form of IκBζ was transfected into MEFs, full-length IκBζ, but not the deletion mutant, rescued the defective production of IL-6 on stimulation with IL-1 in *IκBζ*$^{-/-}$ cells (Fig. 2g), suggesting that expression of IκBζ is required for TLR/IL-1-mediated production of IL-6. As the genes for IκBζ and IL-6 are inducible in response to TLR ligands and IL-1 (refs 4–6, 16, 17), we compared the time course of mRNA expression in macrophages. On stimulation with LPS, induction of IκBζ expression was observed at 30 min and reached maximal levels after 120 min. On the other hand, induction of IL-6 mRNA occurred at later time points compared with IκBζ or TNF-α (Fig. 2h). Taken together, these results indicate that the TLR/IL-1R-mediated expression of the IL-6 gene (*Il6*) requires the preceding induction of IκBζ. Given that IκBζ is also an inducible protein in TLR/IL-1R-mediated signalling pathways, the TLR/IL-1R-mediated production of pro-inflammatory IL-6 may be controlled in at least a two-step fashion.

Our data and those of a previous study[5] indicate the positive role of IκBζ in the TLR/IL-1R-mediated expression of IL-6. To test

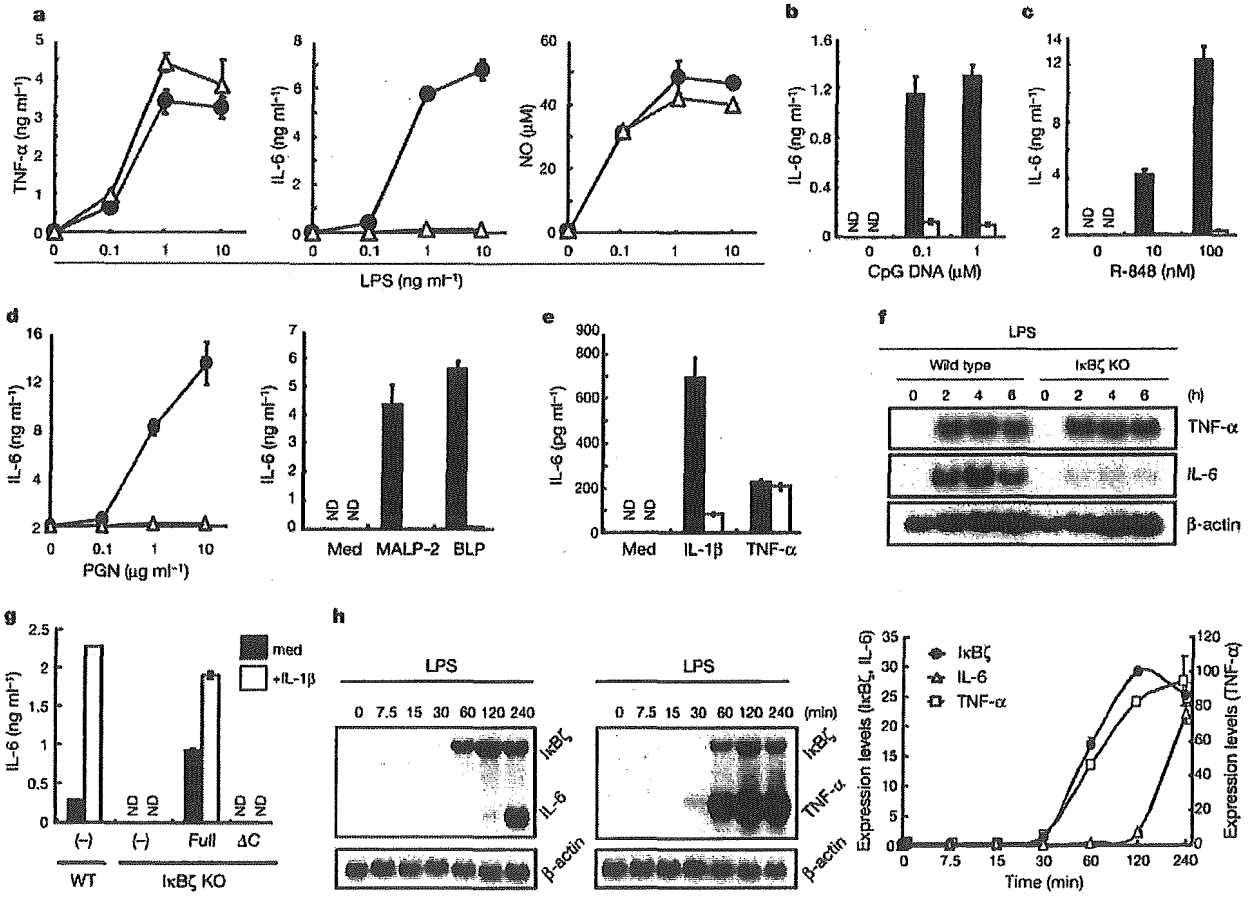

Figure 2 Immune responses in *IκBζ*$^{-/-}$ cells and kinetics of IκBζ induction. **a–d**, *IκBζ*$^{+/+}$ (filled symbols/columns) and *IκBζ*$^{-/-}$ (open symbols/columns) peritoneal macrophages were cultured with 10 ng ml^{-1} LPS, 100 ng ml^{-1} BLP, 30 ng ml^{-1} MALP-2, and the indicated concentrations of PGN, R-848 and CpG DNA in the presence of 30 ng ml^{-1} IFN-γ for 24 h. Values are means ± s.d. of triplicate experiments. ND, not detected. **e**, *IκBζ*$^{+/+}$ (filled columns) and *IκBζ*$^{-/-}$ (open columns) MEFs were stimulated with 10 ng ml^{-1} IL-1β and 10 ng ml^{-1} TNF-α. Values are means ± s.d. of triplicate experiments. **f**, Peritoneal macrophages were stimulated with 10 ng ml^{-1} LPS for the indicated periods. Total RNA (5 μg) was extracted and subjected to northern blot analysis for expression of IL-6, TNF-α and β-actin. **g**, Rescue of IL-1 responsiveness in *IκBζ*$^{-/-}$ MEFs by retroviral transfection with full-length (Full), but not deletion mutant (ΔC), IκBζ. Indicated values are means ± s.d. of triplicate experiments. **h**, Double RNA products indicative of IκBζ, IL-6 and TNF-α mRNA transcripts after LPS simulation of wild-type peritoneal macrophages resolved by electrophoresis. Two independent experiments with independently derived wild-type cells were quantified by Phospholmager (left and middle panels), and mRNA abundance (right panel) is shown for the indicated genes in arbitrary units (left axis, IκBζ and IL-6; right axis, TNF-α) relative to β-actin. Indicated values are means ± s.d. of duplicate experiments.

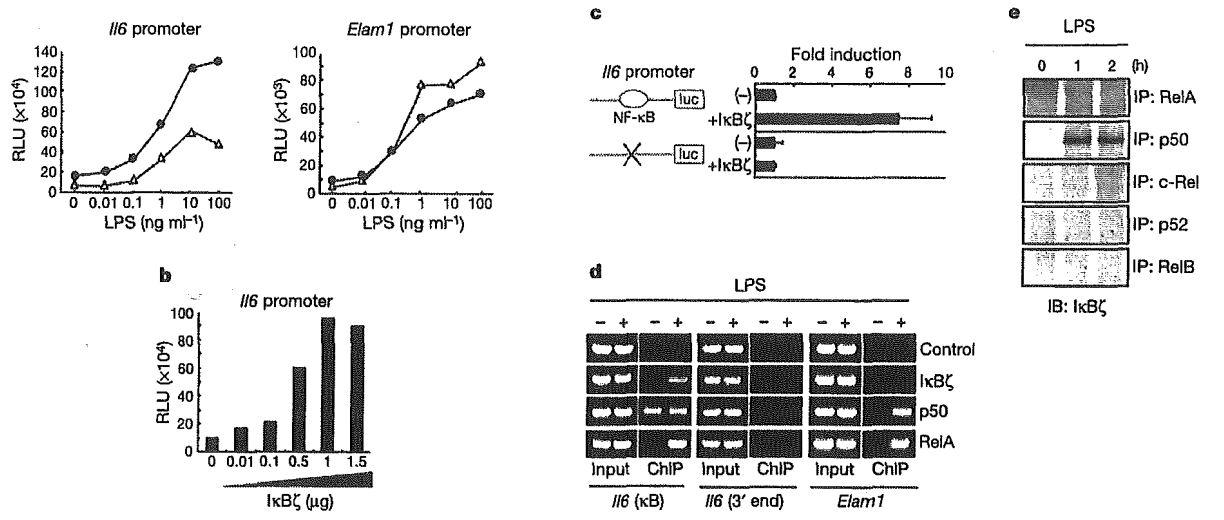

Figure 3 *In vitro* analysis of IκBζ on the *Il6* promoter. **a**, RAW 264.7 cells were transiently transfected with luciferase reporter constructs of either the murine *Il6* promoter or the *Elam1* promoter together with either control (open symbols) or the IκBζ expression plasmid (filled symbols). Luciferase activities are expressed as fold-increase values over the background shown by lysates prepared from untransfected cells. Data are representative of three separate experiments. Cells were stimulated with the indicated concentrations of LPS. RLU, relative luciferase units. **b**, Untreated RAW 264.7 cells were transiently transfected with the IκBζ expression vector together with constant amounts of the *Il6* reporter plasmid. Data are representative of three separate experiments. **c**, P19 cells were transiently transfected with either wild-type or mutant *Il6* promoter reporter constructs together with either control or the IκBζ expression plasmid. Luciferase activities were normalized in each case by dividing the fold-increase values of IκBζ-expressed cells over the background values of lysates with that of mock-expressed cells. Values are means ± s.d. of triplicate experiments. **d**, Chromatin from untreated (−) or LPS-treated (+) (1 μg ml^{-1} for 3 h) RAW 264.7 cells was used for ChIP assays with the indicated antibodies. Precipitated DNA for the *Il6* κB site (left), the 3′ region of the *Il6* gene (centre), or the *Elam1* promoter (right) was assayed by PCR (ChIP). Data are representative of two independent experiments. **e**, Unstimulated or LPS-stimulated (10 ng ml^{-1}) peritoneal macrophages were immunoprecipitated with the indicated antibodies. The immunoprecipitated (IP) lysates were subsequently immunoblotted (IB) with anti-IκBζ.

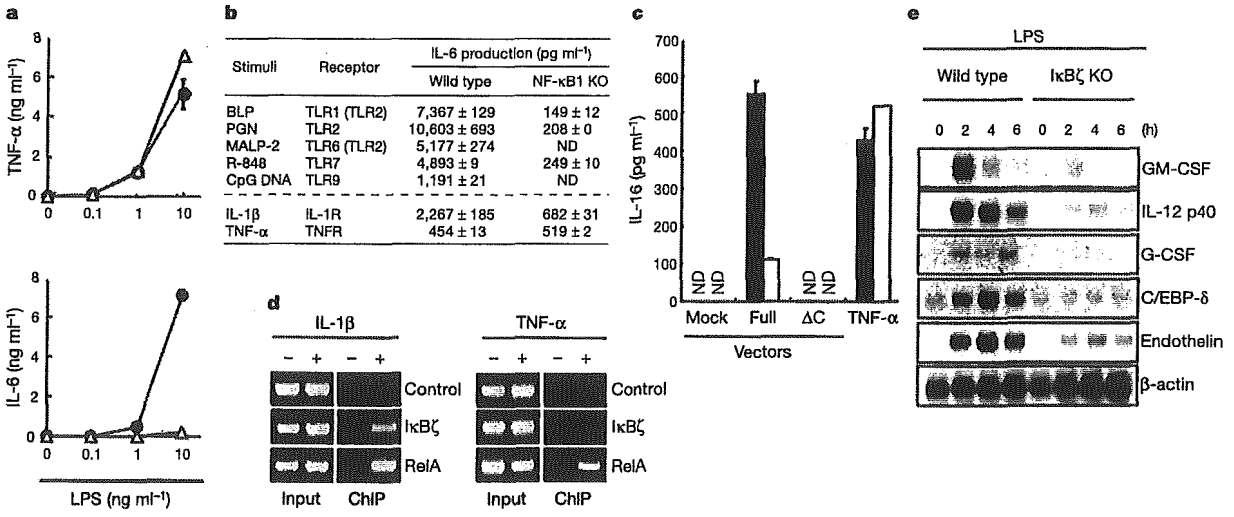

Figure 4 The TLR/IL-1R responses in NF-κB1/p50-deficient cells and microarray analysis of *IκBζ*$^{-/-}$ cells **a**, *NF-κB1*$^{+/+}$ (filled symbols) and *NF-κB1*$^{-/-}$ (open symbols) peritoneal macrophages were cultured with 10 ng ml^{-1} LPS in the presence of 30 ng ml^{-1} IFN-γ for 24 h. Values are means ± s.d. of triplicate experiments. **b**, *NF-κB1*$^{+/+}$ and *NF-κB1*$^{-/-}$ peritoneal macrophages and MEFs were cultured with 100 ng ml^{-1} BLP, 10 μg ml^{-1} PGN, 30 ng ml^{-1} MALP-2, 100 nM R-848, 3 μM CpG DNA, 10 ng ml^{-1} IL-1β or 10 ng ml^{-1} TNF-α in the presence of 30 ng ml^{-1} IFN-γ for 24 h. IL-1β- and TNF-α-induced IL-6 production were analysed by MEFs. Values are means ± s.d. of triplicate experiments. **c**, *NF-κB1*$^{+/+}$ (filled columns) and *NF-κB1*$^{-/-}$ (open columns) MEFs were retrovirally transfected with either the full-length (Full) or the deletion mutant (ΔC) of IκBζ. Furthermore, the same lines of untransfected cells were stimulated with 10 ng ml^{-1} TNF-α. Values are means ± s.d. of duplicate experiments. **d**, Chromatin from untreated (−), IL-1β-treated (left panel (+); 10 ng ml^{-1} for 3 h) or TNF-α-treated (right panel (+); 10 ng ml^{-1} for 3 h) wild-type MEFs were used for ChIP assays with the indicated antibodies. Precipitated DNA for the input (left) or the *Il6* κB site (right) was assayed by PCR. **e**, *IκBζ*$^{+/+}$ and *IκBζ*$^{-/-}$ peritoneal macrophages were stimulated with 10 ng ml^{-1} LPS for the indicated periods. Total RNA (5 μg) was extracted and subjected to northern blot analysis for expression of the indicated probes. GM-CSF, granulocyte-macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor.

whether IκBζ promotes the ligand-induced activation of the *Il6* promoter, we introduced reporter plasmids containing the promoter of either *Il6* or *Elam1* (also known as *Sele*) into RAW 264.7 cells together with control or IκBζ expression vectors. LPS stimulation activated both the *Il6* and *Elam1* promoters in a dose-dependent manner. Under conditions of IκBζ overexpression, we found that the promoter activities of *Il6*, but not *Elam1*, were further enhanced (Fig. 3a). Ectopic expression of IκBζ alone also upregulated the activity of the *Il6* promoter in unstimulated cells (Fig. 3b). As an NF-κB binding site in the *Il6* promoter has been shown to have an important function in its activation, we transfected P19 cells with either a wild-type reporter or a mutant reporter in which the NF-κB binding site was disrupted[18]. IκBζ overexpression activated the wild-type reporter, but not the mutant NF-κB reporter (Fig. 3c). To probe directly the specific involvement of IκBζ in the κB site of the *Il6* promoter, we performed a chromatin immunoprecipitation assay (ChIP) to investigate the proteins bound to the region. In unstimulated cells, the NF-κB p50 subunit was readily detected in the *Il6* κB site as described previously[19]. On stimulation with LPS, RelA as well as p50 bound to the κB sites of the *Il6* and the *Elam1* promoter, but not the 3′ end of the *Il6* gene. In contrast, we found that IκBζ only bound to the *Il6* κB site, but not the other sites tested, in LPS-stimulated cells (Fig. 3d), demonstrating a specificity of IκBζ for the κB site in the *Il6* promoter. Finally, we addressed association of IκBζ with NF-κB family members. Immunoprecipitation analysis showed that IκBζ proteins interacted with p50, but not with RelA, RelB, c-Rel or the p52 subunit (Fig. 3e). These findings indicate that the positive effects by IκBζ may be exerted through association with the p50 subunit.

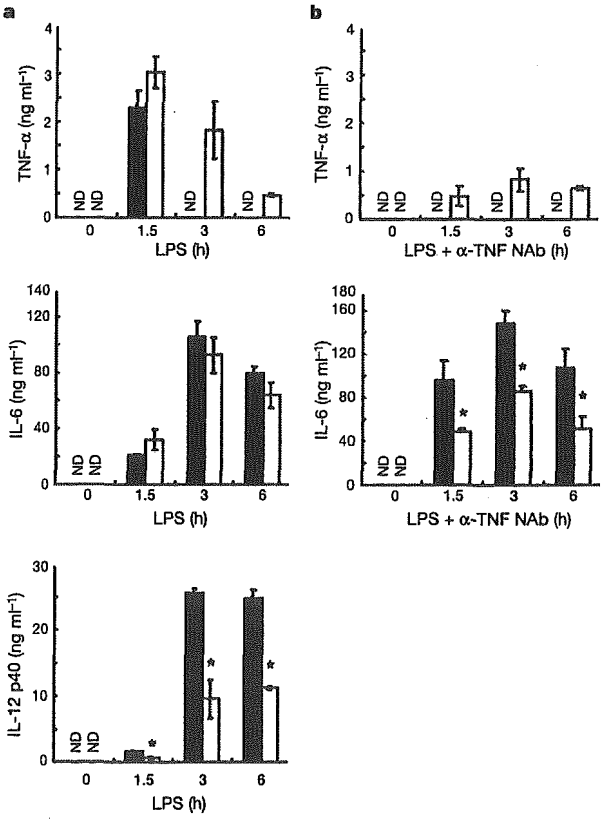

Figure 5 *In vivo* cytokine production in $I\kappa B\zeta^{-/-}$ mice. **a**, **b**, Age-matched $I\kappa B\zeta^{-/-}$ (open columns; $n = 3$ in **a** and 2 in **b**) and $I\kappa B\zeta^{+/+}$ (filled columns; $n = 3$ in **a** and **b**) mice were intraperitoneally injected with 1.5 mg LPS only (**a**), or with 10 μg anti-TNF NAb 1 h before the LPS injection (**b**). Sera were collected at the indicated times. Values are means ± s.d. of sera samples at the indicated times. Asterisk, statistical significance ($P < 0.05$) in a two-tailed Student's *t*-test comparing $I\kappa B\zeta^{+/+}$ and $I\kappa B\zeta^{-/-}$ mice.

The aforementioned results prompted us to study NF-κB1/p50-deficient cells. As previously reported, whereas LPS-induced TNF-α production in *NF-κB1*$^{-/-}$ macrophages is normal, *NF-κB1*$^{-/-}$ macrophages show defective LPS-induced production of IL-6 (refs 20, 21; see also Fig. 4a). Additionally, production of IL-6 in response to stimulation by IL-1 and other TLR ligands was severely impaired in *NF-κB1*$^{-/-}$ cells (Fig. 4b). Thus, the TLR/IL-1R-mediated responses in *NF-κB1*$^{-/-}$ mice are similar to those in $I\kappa B\zeta^{-/-}$ mice. We tested further whether IκBζ overexpression induced IL-6 production in *NF-κB1*$^{-/-}$ cells. Compared with *NF-κB1*$^{+/+}$ cells, IκBζ-mediated production of IL-6 was markedly reduced in *NF-κB1*$^{-/-}$ MEFs, indicating that NF-κB1 is critical for the effect of IκBζ (Fig. 4c). Both IL-1 and TNF-α activate similar sets of signalling molecules such as NF-κB and mitogen-activated protein kinases, and culminate in IL-6 expression. However, signalling mediated by IL-1/TLR ligands, but not TNF-α, specifically recruited IκBζ to the *Il6* promoter (Fig. 4d), presumably accounting for the responsiveness difference between TLR/IL-1R and TNFR. Next, we searched for other LPS-inducible genes regulated by IκBζ using microarray analysis. Dozens of LPS-inducible genes were significantly affected by the IκBζ deficiency (Supplementary Fig. 4a). Several of them were subsequently tested by northern blot analysis for confirmation of accuracy. Among them, LPS-induced expression of granulocyte-macrophage colony-stimulating factor (*Csf2*), IL-12 p40 (*Il12b*), granulocyte colony-stimulating factor (*Csf3*), C/EBP-δ (*Cebpd*) and endothelin 1 (*Edn1*) was compromised in $I\kappa B\zeta^{-/-}$ macrophages (Fig. 4e). Indeed, time course northern blot analysis showed that the kinetics of LPS induction of these genes were similar to that for IL-6 rather than TNF-α (Fig. 2h, Supplementary Fig. 4b, and data not shown), further supporting the model of a two-step regulation of these genes in TLR/IL-1R signalling pathways. Regarding the relationship between the κB sequences of the genes tested and IκBζ requirement, the promoters of IκBζ-regulated genes contained distinct κB sequences. Therefore, it may be difficult to determine whether an arbitrary LPS-inducible gene is IκBζ-dependent through simple sequence comparison of the κB sites (ref. 22; see also Supplementary discussion 2 and Supplementary Fig. 4c–e).

Finally, we examined *in vivo* cytokine production after LPS injection. Although LPS-induced IL-12 p40 production was impaired, IL-6 production was almost normal in $I\kappa B\zeta^{-/-}$ mice. Surprisingly, $I\kappa B\zeta^{-/-}$ mice exhibited more prolonged TNF-α production than $I\kappa B\zeta^{+/+}$ mice (Fig. 5a). As TNF-α is a major IL-6 producer under conditions of endotoxin-induced shock, we next attempted to negate biological activities of TNF-α by prior treatment with anti-TNF-α neutralizing antibodies (anti-TNF NAbs) (ref. 23). In anti-TNF NAb-treated $I\kappa B\zeta^{-/-}$ mice the serum concentration of LPS-induced IL-6 was significantly reduced compared with anti-TNF NAb-treated $I\kappa B\zeta^{+/+}$ mice (Fig. 5b), demonstrating that the prolonged TNF-α production might compensate for impaired IL-6 production in $I\kappa B\zeta^{-/-}$ mice. The prolonged TNF-α production might be secondary to the loss of IκBζ-regulated factors that negatively modulate TNF-α production. Alternatively, IκBζ might act directly as a negative regulator for TNF-α production in certain cells. Although the molecular mechanism of the prolonged TNF-α production remains unknown, such prolonged TNF-α production might lead to development of the skin lesion in aged $I\kappa B\zeta^{-/-}$ mice as demonstrated in TNF-α-mediated inflammatory diseases occurring in other mouse models[24,25].

We provide genetic evidence that IκBζ is essential for TLR/IL-1R-mediated IL-6 production. As IκBζ itself is an inducible protein, TLR/IL-1R-mediated IL-6 expression may be regulated in a two-

step mechanism. Moreover, microarray analysis showed that IκBζ might control LPS-inducible genes other than *Il6*. Further analysis that clarifies the molecular basis of IκBζ-dependent gene expression will provide new insight into the TLR/IL-1R-mediated MyD88-dependent immune responses. □

Methods

Generation of *IκBζ*$^{-/-}$ mice

Genomic DNA containing the *IκBζ* gene was isolated, as described previously[26]. We constructed the targeting vector by replacing a 2.0-kilobase (kb) fragment encoding the central portion of IκBζ with a *neo*^{R} cassette that was transfected into embryonic stem cells (E14.1). G418 and gancyclovir doubly resistant colonies were screened by polymerase chain reaction (PCR) and Southern blotting. We micro-injected two independent homologous recombinants into C57BL/6 blastocysts and intercrossed heterozygous F_1 progenies to obtain *IκBζ*$^{-/-}$ mice. Mice from these independent clones displayed identical phenotypes. *IκBζ*$^{-/-}$ mice and their wild-type littermates at the age of 6–12 weeks were used for the current studies. All animal experiments were conducted with the approval of the Animal Research Committee of the Research Institute for Microbial Diseases (Osaka University, Osaka, Japan).

Reagents and mice

LPS, PGN, MALP-2, flagellin, R-848 and CpG oligodeoxynucleotides were prepared as described previously[26]. Polyclonal antibody against IκBζ was obtained by immunizing rabbit with the C-terminal portion of the murine IκBζ protein. We used antibodies against phosphorylated ERK and JNK (Cell signalling), antibodies against ERK, JNK, p38, RelA, p50, c-Rel RelB, p52 (Santa Cruz), antibodies against RelA in ChIP assays (Biomol) and antibodies against murine TNF-α for neutralization (R&D). Recombinant TNF-α and IL-1β were obtained from Genzyme. NF-κB1/p50-deficient mice were as described previously[20].

Measurement of pro-inflammatory cytokine and NO

Thioglycollate-elicited peritoneal macrophages or MEFs were cultured as described previously[26]. Concentrations of TNF-α (Genzyme), IL-6 (R&D) and IL-12 p40 (Genzyme) in the culture supernatant were measured by an enzyme-linked immunosorbent assay according to the manufacturer's instructions. Concentrations of NO were measured by the Griess method according to the manufacturers' instructions (DOJINDO). To measure *in vivo* cytokine concentrations, sera were taken from *IκBζ*$^{+/+}$ or *IκBζ*$^{-/-}$ mice. Anti-TNF NAbs were reconstituted in PBS to 20 μg ml^{-1}, and 10 μg (500 μl) of the reagent was intraperitoneally injected 1 h before LPS injection.

Electrophoretic mobility shift assay

This assay was performed as described previously[26].

Plasmids and retroviral transfection

The reporter plasmids consisted of the 5′ flanking region (−1240/+40) of the murine *Il6* gene, and were used in Fig. 3a, b. The reporter plasmids used in Fig. 3c were as described previously[18,27]. The full-length or the deletion mutant IκBζ, which lacks the C-terminal portion (ΔC; ref. 4), was cloned into pMRX retroviral vector, and the transfection was performed as described previously[28].

Luciferase reporter assay

Reporter plasmids were transiently co-transfected into RAW 264.7 and P19 cells with either the control or IκBζ expression vectors using SUPERFECT transfection reagent (Qiagen). Luciferase activities of total cell lysates were measured using the Dual-luciferase reporter assay system (Promega) as described previously[29].

Chromatin immunoprecipitation assay

The ChIP assay was performed essentially with a described protocol (Upstate Biotechnology). 2×10^6 RAW 264.7 cells or 5×10^5 MEFs were stimulated with LPS (1 μg ml^{-1} for 3 h), IL-1β (10 ng ml^{-1} for 3 h) or TNF-α (10 ng ml^{-1} for 3 h), respectively. Precipitated DNAs were analysed by quantitative PCR (35–40 cycles) using primers 5′-CGATGCTAAACGACGTCACATTGTGCA-3′ and 5′-CTCCAGAGCAGAAT GAGCTACAGACAT-3′ for the κB site in the *Il6* promoter, 5′-GCAGATGGACTTAGC TCGTCTCATTCA-3′ and 5′-CCACTCCTTCTGTGACTCCAGCTTATC-3′ for a 3′ gene segment in the *Il6* promoter and 5′-GATGCAGTTGAGAATTTCCTCTTAGCC-3′ and 5′-TGGAATAGTTGTTCTGGCGTTGGATCC-3′ for the κB site in the *Elam1* promoter.

Western blot analysis and immunoprecipitation

Western blot was performed as described previously[26].

Gene chip analysis

Miroarray analysis (Affimetrix) using *IκBζ*$^{+/+}$ and *IκBζ*$^{-/-}$ peritoneal macrophages was performed as described previously[30]. The colour image for gene expression was generated by GeneSpring6.0 (Silicon Genetics) software.

Histological analysis

Tissues were fixed in 10% phosphate-buffered formalin, and paraffin-embedded tissue sections were stained with haematoxylin and eosin or PAS staining using standard techniques.

Received 1 April; accepted 7 June 2004; doi:10.1038/nature02738.

1. Takeda, K., Kaisho, T. & Akira, S. Toll-like receptors. *Annu. Rev. Immunol.* **21**, 335–376 (2003).
2. Janeway, C. A. Jr & Medzhitov, R. Innate immune recognition. *Annu. Rev. Immunol.* **20**, 197–216 (2002).
3. O'Neill, L. A. Therapeutic targeting of Toll-like receptors for inflammatory and infectious diseases. *Curr. Opin. Pharmacol.* **3**, 396–403 (2003).
4. Yamazaki, S., Muta, T. & Takeshige, K. A novel IκB protein, IκB-ζ, induced by proinflammatory stimuli, negatively regulates nuclear factor-κB in the nuclei. *J. Biol. Chem.* **276**, 27657–27662 (2001).
5. Kitamura, H., Kanehira, K., Okita, K., Morimatsu, M. & Saito, M. MAIL, a novel nuclear IκB protein that potentiates LPS-induced IL-6 production. *FEBS Lett.* **485**, 53–56 (2000).
6. Haruta, H., Kato, A. & Todokoro, K. Isolation of a novel interleukin-1-inducible nuclear protein bearing ankyrin-repeat motifs. *J. Biol. Chem.* **276**, 12485–12488 (2001).
7. Muzio, M., Ni, J., Feng, P. & Dixit, V. M. IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. *Science* **278**, 1612–1615 (1997).
8. Wesche, H., Henzel, W. J., Shillinglaw, W., Li, S. & Cao, Z. MyD88: an adapter that recruits IRAK to the IL-1 receptor complex. *Immunity* **7**, 837–847 (1997).
9. Medzhitov, R. *et al.* MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. *Mol. Cell* **2**, 253–258 (1998).
10. Alexopoulou, L., Holt, A. C., Medzhitov, R. & Flavell, R. A. Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3. *Nature* **413**, 732–738 (2001).
11. Kawai, T. *et al.* Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *J. Immunol.* **167**, 5887–5894 (2001).
12. Doyle, S. *et al.* IRF3 mediates a TLR3/TLR4-specific antiviral gene program. *Immunity* **17**, 251–263 (2002).
13. Ghosh, S. & Karin, M. Missing pieces in the NF-κB puzzle. *Cell* **109** (suppl.), S81–S96 (2002).
14. Zhang, G. & Ghosh, S. Molecular mechanisms of NF-κB activation induced by bacterial lipopolysaccharide through Toll-like receptors. *J. Endotoxin Res.* **6**, 453–457 (2000).
15. Janssens, S. & Beyaert, R. Functional diversity and regulation of different interleukin-1 receptor-associated kinase (IRAK) family members. *Mol. Cell* **11**, 293–302 (2003).
16. Eto, A., Muta, T., Yamazaki, S. & Takeshige, K. Essential roles for NF-κB and a Toll/IL-1 receptor domain-specific signal(s) in the induction of IκB-ζ. *Biochem. Biophys. Res. Commun.* **301**, 495–501 (2003).
17. Akira, S., Taga, T. & Kishimoto, T. Interleukin-6 in biology and medicine. *Adv. Immunol.* **54**, 1–78 (1993).
18. Matsusaka, T. *et al.* Transcription factors NF-IL6 and NF-κB synergistically activate transcription of the inflammatory cytokines, interleukin 6 and interleukin 8. *Proc. Natl Acad. Sci. USA* **90**, 10193–10197 (1993).
19. Zhong, H., May, M. J., Jimi, E. & Ghosh, S. The phosphorylation status of nuclear NF-κB determines its association with CBP/p300 or HDAC-1. *Mol. Cell* **9**, 625–636 (2002).
20. Sha, W. C., Liou, H. C., Tuomanen, E. I. & Baltimore, D. Targeted disruption of the p50 subunit of NF-κB leads to multifocal defects in immune responses. *Cell* **80**, 321–330 (1995).
21. Sanjabi, S., Hoffmann, A., Liou, H. C., Baltimore, D. & Smale, S. T. Selective requirement for c-Rel during IL-12 P40 gene induction in macrophages. *Proc. Natl Acad. Sci. USA* **97**, 12705–12710 (2000).
22. Hoffmann, A., Leung, T. H. & Baltimore, D. Genetic analysis of NF-κB/Rel transcription factors defines functional specificities. *EMBO J.* **22**, 5530–5539 (2003).
23. Hill, M. R. & McCallum, R. E. Identification of tumor necrosis factor as a transcriptional regulator of the phosphoenolpyruvate carboxykinase gene following endotoxin treatment of mice. *Infect. Immun.* **60**, 4040–4050 (1992).
24. Pasparakis, M. *et al.* TNF-mediated inflammatory skin disease in mice with epidermis-specific deletion of IKK2. *Nature* **417**, 861–866 (2002).
25. Douni, E. *et al.* Transgenic and knockout analyses of the role of TNF in immune regulation and disease pathogenesis. *J. Inflamm.* **47**, 27–38 (1995).
26. Yamamoto, M. *et al.* Role of adaptor TRIF in the MyD88-independent Toll-Like Receptor signaling pathway. *Science* **301**, 640–643 (2003).
27. Kinoshita, S., Akira, S. & Kishimoto, T. A member of the C/EBP family, NF-IL6 beta, forms a heterodimer and transcriptionally synergizes with NF-IL6. *Proc. Natl Acad. Sci. USA* **89**, 1473–1476 (1992).
28. Saitoh, T. *et al.* TWEAK induces NF-κB2 p100 processing and long lasting NF-κB activation. *J. Biol. Chem.* **278**, 36005–36012 (2003).
29. Toshchakov, V. *et al.* TLR4, but not TLR2, mediates IFN-β-induced STAT1α/β-dependent gene expression in macrophages. *Nature Immunol.* **3**, 392–398 (2002).
30. Kuwata, H. *et al.* IL-10-inducible Bcl-3 negatively regulates LPS-induced TNF-α production in macrophages. *Blood* **102**, 4123–4129 (2003).

Supplementary Information accompanies the paper on **www.nature.com/nature.**

Acknowledgements We thank T. Kitamura, A. Aderem, D. Golenbock and H. Tomizawa for providing Plat-E packaging cell lines, flagellin, the ELAM1 reporter plasmid, and R-848, respectively. We also thank T. Kawai and K. Ishii for discussions; M. Hashimoto for secretarial assistance; and N. Okita and N. Iwami for technical assistance. This work was supported by grants from Special Coordination Funds, the Ministry of Education, Culture, Sports, Science and Technology, Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists, The Uehara Memorial Foundation, The Naito Foundation, and The Junior Research Associate from RIKEN.

Competing interests statement The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to S.A (sakira@biken.osaka-u.ac.jp).

Oncogene (2004) 23, 340–349

www.nature.com/onc

Estrogen-independent activation of erbBs signaling and estrogen receptor α in the mouse vagina exposed neonatally to diethylstilbestrol

Shinichi Miyagawa[1,2,3], Yoshinao Katsu[2,3], Hajime Watanabe[2,3] and Taisen Iguchi*[,1,2,3]

[1]*Department of Molecular Biomechanics, School of Life Science, The Graduate University for Advanced Studies, Okazaki 444-8585, Japan;* [2]*Center for Integrative Bioscience, Okazaki National Research Institutes, Okazaki 444-8585, Japan;* [3]*Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Corporation, Kawaguchi 332-0012, Japan*

Growth factors and estrogen receptor (ER) signaling cooperate to play essential roles in cell proliferation, differentiation and tumor progression in mouse reproductive organs. Treatment of neonatal mice with diethylstilbestrol (DES) induces an estrogen-independent persistent proliferation and cornification of the vaginal epithelium, which results in cancerous lesions later in life. However, the mechanisms of the estrogen-dependent and -independent pathways essentially remain unknown. We characterized the expression of epidermal growth factor (EGF)-like growth factors (EGF, transforming growth factor α (TGF-α), heparin-binding EGF-like growth factor (HB-EGF), betacellulin (BTC), amphiregulin (APR), epiregulin (EPR) and neuregulin (NRG) 1) and erbB receptors (EGF receptor (EGFR), erbB2/neu, erbB3 and erbB4) in the vaginae of mice treated either neonatally (0–4 day) or as adults (55–59 day) with estrogens. EGFR and erbB2 were activated in the vaginal epithelium of mice by estrogen treatment. This activation was also encountered in vaginae from neonatally DES-exposed mice, along with the expression of EGF, TGF-α, HB-EGF, BTC, APR, EPR and NRG1. Immunohistochemical analysis indicated that erbB2 was primarily expressed in vaginal epithelium. Finally, we found that serine 118 and 167 located in the AF-1 domain of ERα were phosphorylated in these vaginae. AG825, AG1478 or ICI 182,780 administration blocked proliferation of vaginal epithelium induced by neonatal DES exposure. Thus, signal transduction via EGFR and erbB2 could be related to the estrogen-induced vaginal changes and persistent erbBs phosphorylation and sustained expression of EGF-like growth factors, leading to ERα activation that may result in cancerous lesions in vaginae from neonatally DES-exposed mice later in life.
Oncogene (2004) **23**, 340–349. doi:10.1038/sj.onc1207207

Keywords: mouse; diethylstilbestrol; vagina; erbBs signaling; estrogen receptor

Introduction

The proliferation and differentiation of mouse vaginal epithelial cells are strongly regulated by ovarian estrogens, such as 17β-estradiol (E_2). The vaginae of ovariectomized (OVX) mice show an atrophied epithelium of 2–3 cell layers, but estrogen administration rapidly induces epithelial cell proliferation, stratification and cornification. In rodents, crosstalk between growth factor signaling and estrogen receptor α (ERα) contribute to estrogen action in uterus and vagina. Epidermal growth factor (EGF) has mitogenic effects on the mouse uterus similar to estrogens (Ignar-Trowbridge *et al.*, 1992) in that administration of EGF or transforming growth factor α (TGF-α) to OVX adult mice induces cell proliferation and differentiation in the female reproductive tracts (Nelson *et al.*, 1991, 1992). However, in ERα-deficient mice, both estrogen and EGF stimulation of uterine growth was disrupted (Curtis *et al.*, 1996). EGF-like growth factors are composed of EGF, TGF-α, heparin-binding EGF-like growth factor (HB-EGF), betacellulin (BTC), amphiregulin (APR), epiregulin (EPR) and neuregulins (NRGs) and interact with the erbB receptor tyrosine kinases: EGF receptor (EGFR)/erbB1, erbB2/neu, erbB3 and erbB4. The members of the erbB receptor family interact as homo- or heterodimers upon ligand binding, which leads to the crossactivation of the receptors. ErbBs have the potential to recruit and activate interacting proteins, thereby initiating signaling cascades that culminate in distinct cellular responses, such as cell proliferation, differentiation and morphogenesis. Overexpression of erbBs is associated with carcinogenesis in the reproductive organs (Dickson and Lippman, 1995) and erbB2 is frequently amplified and overexpressed in cancer cells (Hynes and Stern, 1994).

Epidemiological and laboratory studies have shown that estrogens are important for the normal proliferation that occurs in reproductive organs. Moreover, long-term estrogenic stimulation is a well-known risk factor for carcinogenesis in human reproductive organs (Marselos and Tomatis, 1992a). Beginning in the 1940s, a synthetic estrogen, diethylstilbestrol (DES) was routinely prescribed to pregnant women for the prevention of miscarriages. To date, it is well known that *in utero* exposure to DES induces vaginal clear-cell adenocarci-

*Correspondence: T Iguchi, Center for Integrative Bioscience, Okazaki National Research Institutes, 5-1 Higashiyama, Myodaiji, Okazaki, Aichi 444-8585, Japan; E-mail: taisen@nibb.ac.jp
Received 29 May 2003; revised 10 September 2003; accepted 12 September 2003

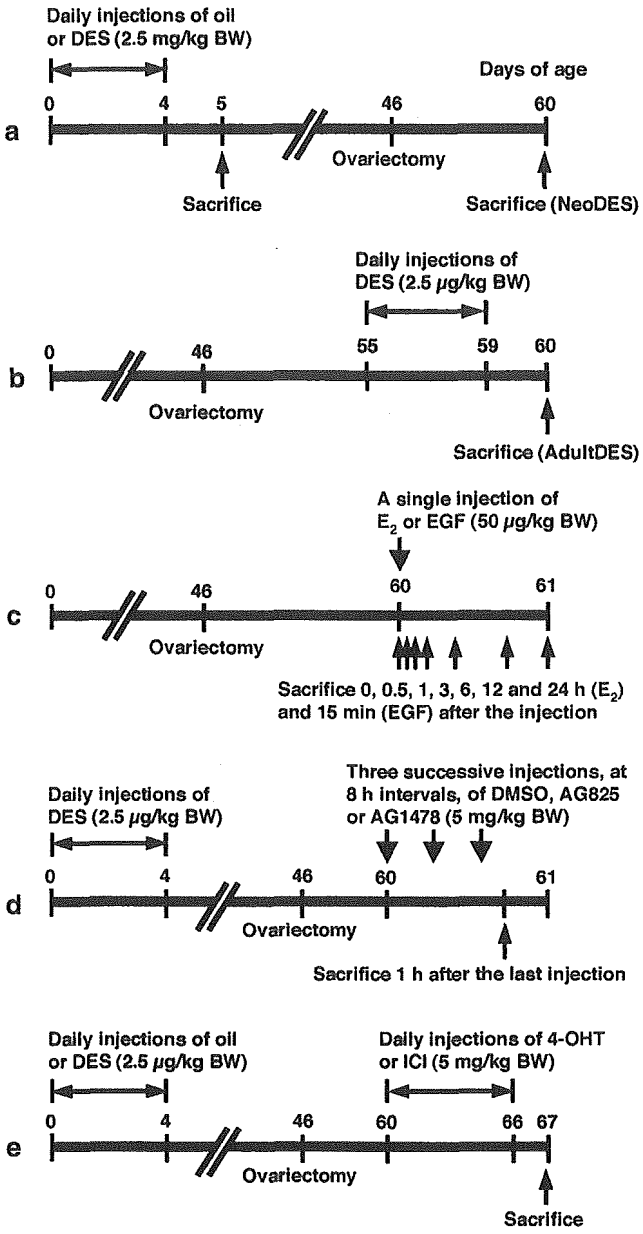

Figure 1 Treatment timelines. (**a**) Female newborn mice were given five daily injections of 2.5 mg DES/kg BW or the vehicle alone from day 0 (the day of birth). They were OVX on day 46 and killed on day 60. (**b**) To examine the effects of DES on adult mouse vagina, intact mice were OVX on day 46 and given five daily injections of 2.5 μg DES/kg BW from day 55, then killed 24 h after the last injection. (**c**) Effects of E_2 or EGF on phosphorylation of erbB2, EGFR and ERα were examined in OVX adult mice 0.5, 1, 3, 6, 12 and 24 h (E_2) or 15 min (EGF) after a single injection of 50 μg E_2 or 50 μg EGF/kg BW. (**d**) Three injections of AG825 or AG1478 (5 mg/kg BW) were administered every 8 h. BrdU was injected at the last injections of AG825 or AG1478, and killed 1 h later. (**e**) 4-OHT and ICI (5 mg/kg BW/day) were administered to mice that had been OVX on day 46 for 7 days from days 60 to 66, and then killed 24 h after the last injection

noma in young women (Herbst *et al.*, 1971) and various malformation in the reproductive tracts (Marselos and Tomatis, 1992a; Herbst, 2000). In males, *in utero* DES exposure is associated with an increased risk of testicular cancer and epididymal cysts (Marselos and Tomatis, 1992a; Herbst, 2000). Like humans, perinatal female mice exposed to natural or synthetic estrogens develop estrogen-independent persistent cell proliferation, stratification and cornification of the vaginal epithelium, resulting in hyperplastic lesions and vaginal cancer later in life (Takasugi *et al.*, 1962; Dunn and Green, 1963; Forsberg, 1979; McLachlan *et al.*, 1980; Iguchi, 1992). This rodent model, which simulates the effects of developmental DES exposure in humans, has been characterized, yet the underlying mechanisms remain poorly understood. This irreversible proliferation and differentiation of the vaginal epithelium may be attributable to estrogen-independent, persistent activation of downstream growth factor expression. Indeed, high levels of EGF and TGF-α are expressed in the vaginae of DES treated mice, even after ovariectomy (Nelson *et al.*, 1994; Sato *et al.*, 1996a). However, the role of crosstalk between erbB signaling and ER in estrogen-independent effects induced by neonatal DES treatment has not yet been elucidated.

Cell proliferation in reproductive organs is tightly regulated by hormones, particularly estrogens. Tumors of reproductive organs can exhibit 'hormone-independent cell proliferation'. Thus, the vaginae of mice exposed neonatally to estrogens can be used as a model system to understand the mechanisms leading to the change from normal to aberrant cell proliferation and differentiation, and tumor formation *in vivo*. The importance of growth factor induction of ER transcriptional activity in normal physiology and in tumor progression is essentially unknown. In this report, we characterized estrogen-independent vaginal epithelial cell proliferation and differentiation by focusing on the crosstalk between erbBs signaling and ERα. We found that neonatal DES treatment led to increased phosphorylation of EGFR, erbB2 and ERα persistently. These phosphorylations are each capable of activating the receptors in a ligand-independent manner, suggesting a possible mechanism whereby neonatal DES treatment leads to persistent, estrogen-independent proliferation of the vaginal epithelium.

Results

Expression of EGF-like growth factors

We quantified the time-course mRNA expression of growth factors (EGF, TGF-α, HB-EGF, BTC, APR, EPR, insulin-like growth factor (IGF)-I, IGF-II and keratinocyte growth factor (KGF)) in 60-day-old OVX mice after a single injection of E_2 by quantitative real-time RT–PCR (Q-PCR) (see Figure 1 for experimental design). Although it has been reported that estrogen administration induces EGF and TGF-α mRNA expression in mouse uterus (DiAugustine *et al.*, 1988; Nelson *et al.*, 1992), a single injection of E_2 did not induce significant changes in the expression of growth factor genes in vagina. APR mRNA was the highest

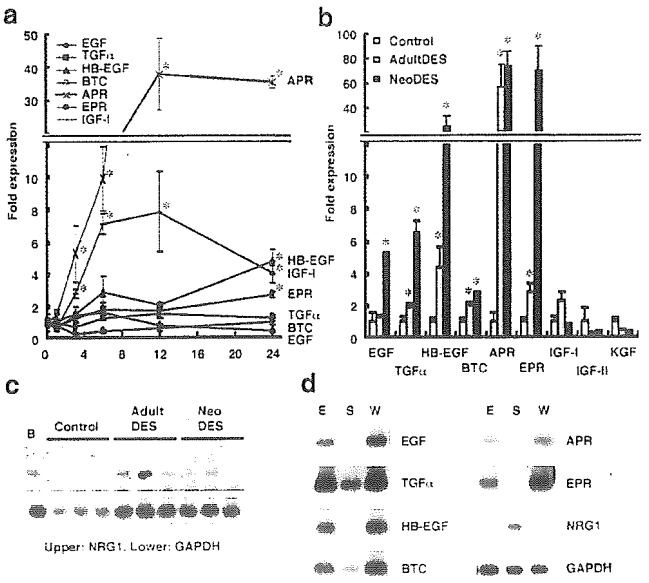

Figure 2 Expression of growth factor mRNA in mouse vagina. (**a**) Time-course analysis of growth factor mRNA expression. The samples were from vaginae of mice 0 (control), 1, 3, 6, 12 and 24 h after a single injection of 50 μg E_2/kg BW. (**b**) Expression profiles of growth factor mRNA in vaginae of 60-day-old OVX mice treated with oil (control), adultDES and neoDES. (**c**) Expression of NRG1 was examined by RT–PCR following electrophoresis. B; brain cDNA used as positive control. (**d**) Tissue distribution of growth factor mRNA expression in neoDES vaginae. E; epithelium, S; stroma, W; whole tissue

among E_2-responsive growth factors in this study (Figure 2a).

We also quantified the expression level of mRNAs encoding ligands for erbB receptors in vaginae of oil-treated OVX control, 60-day-old OVX mice after five daily injections of DES (adultDES) and 60-day-old OVX mice treated neonatally with DES for 5 days (neoDES mice). In neoDES vaginae, all ligands for erbB receptors were highly expressed (Figure 2b). Successive DES treatments induced the expression of erbBs in adultDES vaginae that closely resembled that seen in neoDES vaginae, but the magnitude of the induction was lower (Figure 2b). The NRGs primarily bind to erbB3 and erbB4 and have been shown to be a complex gene family with many alternatively spliced forms. Therefore, we examined NRG1 transcript expression by RT–PCR and gel electrophoresis to allow detection of splicing variants. NRG1 transcripts were expressed in both adultDES and neoDES vaginae, with adultDES tissue showing higher expression levels (Figure 2c).

IGF-I signaling is involved in E_2-induced responses, such as epithelial cell proliferation in rodent uterus (Klotz *et al.*, 2000); therefore, we also examined IGF-I mRNA expression. In mouse vagina, a single injection of E_2 induced IGF-I mRNA expression in a time-dependent manner (Figure 2a). In adultDES and neoDES vaginae, IGF-I mRNA levels were not altered when compared to controls. Similarly, other growth factors, IGF-II and KGF did not show changes in mRNA expression in vaginae from adultDES nor neoDES mice (Figure 2b) and OVX mice given a single injection of E_2 (data not shown). Although we do not exclude a possible role of IGF-I in vaginal responses, persistent vaginal epithelial cell proliferation and differentiation is mainly contributed by ligands of erbB receptors in neoDES vagina.

To study the tissue distribution of growth factors, epithelium and stroma were separated by incubating in a trypsin solution, and followed by RT–PCR analysis. Excluding NRG1, which was expressed in the stroma only, the other ligands were primarily expressed in the epithelium. In particular, APR and EPR were only detected in the epithelium (Figure 2d).

ErbB mRNA and protein expression in vagina

We confirmed the expression of mRNAs encoding all four erbB types in both adult and neonatal mouse vagina by RT–PCR (data not shown) and the erbB proteins by immunoblot analysis (Figure 3a). Q-PCR was employed to quantitate the response of the erbB mRNAs to DES treatment. In vaginae of adultDES, there was no significant decrease in the expression of erbB mRNAs (Figure 3b). In contrast, EGFR and erbB2 mRNA expression was significantly decreased in neoDES vaginae (Figure 3b). Reduction of EGFR expression following neonatal DES exposure has been reported previously (Iguchi *et al.*, 1993). However, we have now observed that erbB2 expression was also significantly decreased in neoDES vagina. ErbB3 and erbB4 mRNA levels also appeared to decrease but these changes were not statistically significant. We also assessed the expression of erbB mRNAs in vaginae

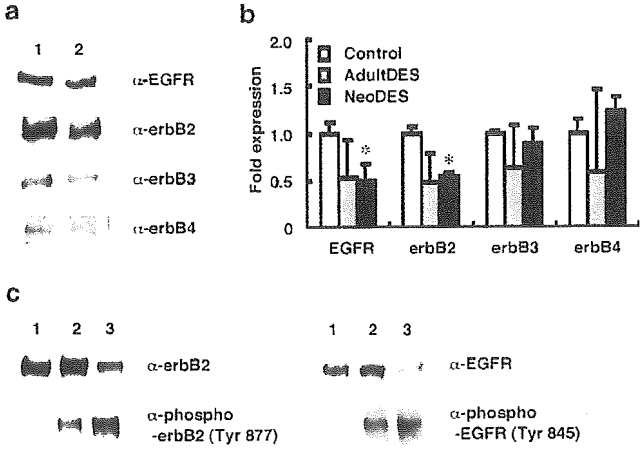

Figure 3 ErbB mRNA and protein expression pattern in vaginae of OVX control, adultDES and neoDES mice. (**a**) Immunoblots of erbBs in vaginae of oil control (lane 1) and neoDES (lane 2). Each contained the same amount of protein. (**b**) Quantification of gene expression in vaginae of 60-day-old mice of oil (control), adultDES and neoDES using Q-PCR. The expression of each receptor mRNA in vaginae of the oil-treated control mice was regarded as the basal level (1.0). (**c**) Phosphorylation of erbB2 and EGFR was detected by antiphospho-erbB2 and EGFR antibodies. The samples are from vaginae of OVX mice treated with oil (lane 1), adultDES (lane 2) and neoDES (lane 3)

from 5-day-old mice and found that successive DES treatment from the day of birth suppressed the expression of erbB2 mRNA (data not shown). The expression of EGFR and erbB4 mRNAs was slightly, but not significantly decreased (Figure 3b). ErbB2 protein expression was slightly reduced in neoDES vaginae (Figure 3a). Thus, erbB2 downregulation appears to be correlated with vaginal tissue morphology.

Phosphorylation of erbB2 and EGFR in vagina

The phosphorylation status of erbB2 in vaginae from adultDES and neoDES mice was examined using antiphospho-erbB2 antibody, which recognizes phosphotyrosine 877 from human protein sequences. Phospho-erbB2 was detected in vaginae from both adultDES and neoDES mice but not in vaginae from controls, although erbB2 protein was expressed in controls (Figure 3c). Tyrosine 845 of EGFR was also phosphorylated in vaginae of neoDES and adultDES mice (Figure 3c). Taken together, these data suggest that DES treatment induces phosphorylation of erbB2 and EGFR, which would be expected to upregulate the downstream signaling pathways under their control.

Considering the correlation between DES treatment and erbB2 expression, we next investigated the distribution of erbB2 mRNA in mouse vagina using immunohistochemistry. Intense membrane staining for erbB2 was observed in epithelial cells of vaginae from 60-day-old OVX mice. Apical cells were more strongly stained than basal cells whereas stromal cells beneath the basement membrane were weakly stained (Figure 4a).

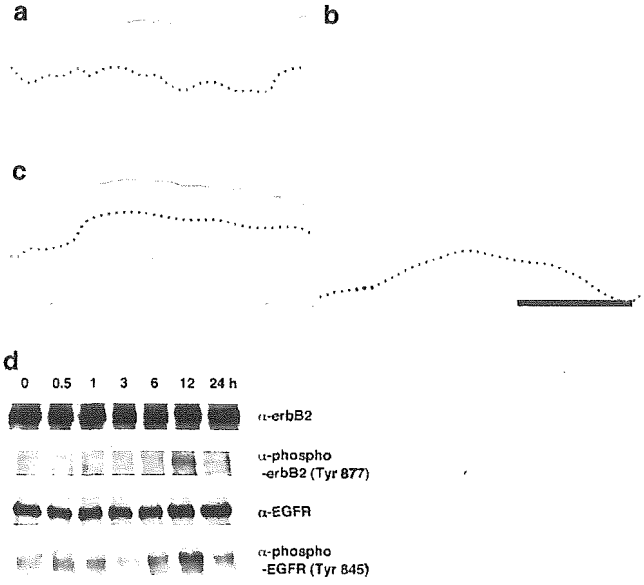

Figure 4 ErbB2 activation status and distribution in the mouse vagina. (**a–c**) Immunohistochemical detection of erbB2 in vaginae of 60-day-old mice from control (**a**) and neoDES (**b**). No immunostaining was observed with normal rabbit serum (**c**). The boundary between epithelium and stroma is indicated by a dotted line. Scale bar = 50 μm. (**d**) Time-course analysis of erbB2 and EGFR phosphorylation. The samples are from vaginae of mice 0 (control), 0.5, 1, 3, 6, 12 and 24 h after a single injection of 50 μg E_2/kg BW

In neoDES vaginae, erbB2 immunostaining was found in basal epithelial cells, whereas the differentiating cells that migrated toward the surface of the epithelium exhibited decreased erbB2 expression (Figure 4b). Stromal cells were not stained (Figure 4b). Immunoreactivities were abolished by incubation with control rabbit IgG demonstrating the specificity of the erbB2 staining (Figure 4c).

The time course of erbB2 phosphorylation was examined in vaginae from 60-day-old OVX mice after a single injection of E_2. Phospho-erbB2 was transiently detected in vaginae 12 h after E_2 injection (Figure 4d). The phosphorylation pattern of EGFR was similar to that observed for erbB2 (Figure 4d). These data suggested the existence of a crosslink between erbB2 phosphorylation and estrogen action in the mouse vagina.

Phosphorylation of ERα in vagina

To investigate the downstream of erbB2 and EGFR phosphorylation further, we tested whether increased erbB signaling would affect downstream target genes. ErbBs activation derived from administration of EGF, the most well-known ligands for erbBs, leads to vaginal epithelial cell proliferation and differentiation in OVX mice (Nelson *et al*., 1991). It is known that erbBs can stimulate the transcriptional activity of ERα by phosphorylating serine 118 and 167 of this receptor (located within the AF-1 region) through mitogen-activated protein kinase (MAPK) and/or Akt cascades (Lannigan, 2003). Actually, EGF administration led to phosphorylation of ERα with erbB2 and EGFR activation in vagina (Figure 5a).

ERK1/2 exhibited a high basal level of phosphorylation even in OVX mice, and phosphorylation of ERK1/2 above basal levels did not occur in adultDES and neoDES vaginae as previously reported using uterine tissue from mice (Klotz *et al*., 2002). However, p90 ribosomal S6 kinase (RSK), which is one of the substrates for ERK1/2 and can phosphorylate ERα (Joel *et al*., 1998), was activated in neoDES and adultDES vaginae (Figure 5b). Furthermore, both serine residues of ERα were phosphorylated in neoDES and adultDES vaginae (Figure 5c), confirming that erbBs phosphorylation could lead to the activation of ERα independently of the continuing presence of estrogens in vaginal epithelium. Detectable levels of ERα are present in both stromal and epithelial compartments of the vaginae obtained from neonatally DES-treated mice (Sato *et al*., 1996b). After tissue separation by trypsin, we weakly detected total and phosphorylated ERα protein in vaginal epithelial tissue, but in stromal tissue, we could not detect even total ERα protein due to the long trypsin incubation (data not shown).

We examined AF-1 function of ERα in mouse vagina using 4-hydroxytamoxifen (4-OHT), which acts as an agonist for AF-1, but as an antagonist for AF-2 (Berry *et al*., 1990; McDonnell *et al*., 1995; Metzger *et al*., 1995). Administration of 4-OHT to intact OVX mice induced vaginal epithelial stratification and cornification

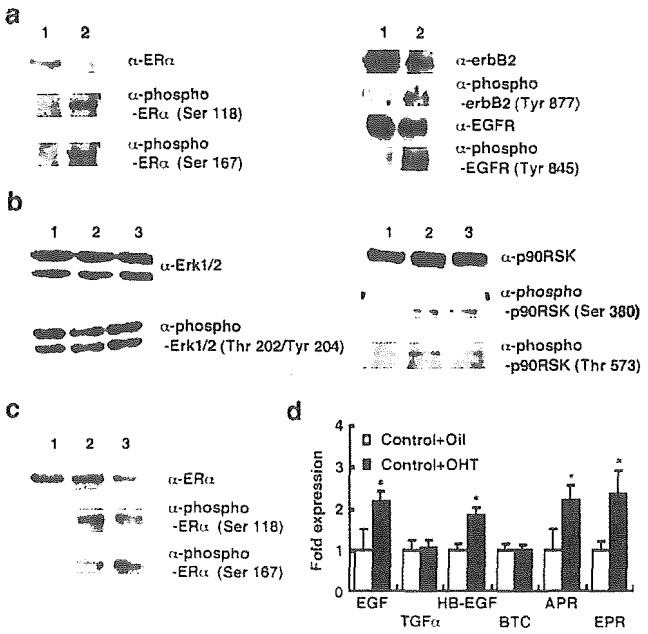

Figure 5 Activation status of erbB downstream signaling in the mouse vagina. (**a**) ERα, EGFR and erbB2 phosphorylation status in vaginae of mice treated with saline (lane 1) or EGF (lane 2). (**b**) Erk1/2 and p90RSK activation status was detected by antiphospho-Erk1/2 and p90 RSK antibodies. The samples were from vaginae of OVX mice treated with oil (lane 1), adultDES (lane 2) and neoDES (lane 3) (**a** and **b**). (**c**) ERα activation status was detected by antiphospho-ERα antibodies. (**d**) Expression profiles of growth factor mRNA in vaginae of intact OVX mice treated with oil control or 4-OHT

(data not shown), accompanied with sustained mRNA expression of EGF-like growth factors, such as EGF, HB-EGF, APR and EPR (Figure 5d). These data indicated that only AF-1 activation of ERα induced ligands for erbB receptors. Further, ERα and erbB signaling formed on activation loop without E_2, which initially must regulate this signaling pathway.

This hypothesis was supported by the experiment using inhibitors of EGFR (AG1478) and erbB2 (AG825). The number of BrdU-incorporated cells was slightly and significantly reduced in neoDES vagina treated with AG825 and AG1478, respectively (Figure 6a–c). The level of phosphorylation of ERα, in particular Ser 118, was also reduced in these vaginae treated with the inhibitors (Figure 6d).

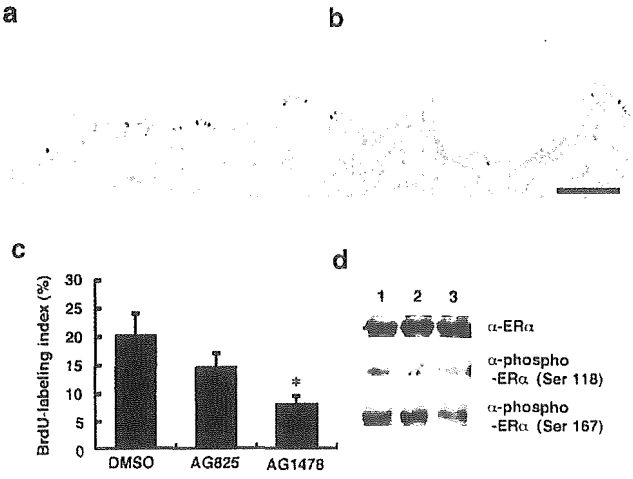

Figure 6 Administration of inhibitors of erbBs blocked BrdU incorporation and ERα phosphorylation. (**a** and **b**) BrdU-labeling cells in neoDES vagina treated with DMSO (**a**) and AG1478 (**b**). Sections were counterstained with methyl green. Scale bar = 100 μm. (**c**) BrdU-labeling index. Index is presented using percent values based on the number of BrdU-incorporated cells per 100 cells in the epithelial basal layer. The bars indicate standard error ($n = 5$–6). *, $P < 0.05$ *vs* control (DMSO). (**d**) ERα phosphorylation status after injections of erbB inhibitors. The samples are from neoDES vagina treated with DMSO (lane 1), AG825 (lane 2) and AG1478 (lane 3)

Effects of ICI 182,780 in NeoDES vagina

We next tested whether ICI 182,780 (ICI), an antagonist for both AF-1 and AF-2 of the ERα, could block the irreversible effects in neoDES vaginae. As shown in Table 1 and Figure 7, ICI administration partially blocked epithelial differentiation, accompanied by low expression of growth factors, erbB receptors and their phosphorylation levels in neoDES vagina killed 24 h after the last ICI injection. These data confirm that deregulation of AF-1 leads to estrogen-independent ERα action. Administration of ICI to intact OVX mice did not induce vaginal epithelial stratification (data not shown).

Discussion

Integrated hormonal signaling networks modulate the reproductive systems in animals. E_2 is responsible for

Table 1 Effects of ICI on neoDES mice

Treatment (No. of mice)	*No. of mice showing vaginal epithelial*		*Thickness of vaginal epithelium (μm)*[a]
	Cornification	*Stratification*	
neoDES + oil (9)	9	9	133 ± 6.0
neoDES + ICI (11)	3[b,c]	11	55 ± 9.4[d]

[a]Mean thickness of vaginal epithelium was estimated by measuring the epithelial cell layers in vaginae of three random regions (mean ± s.e.). [b]These mice showed cornified epithelium in partial region. [c]Statistical difference between neoDES + Oil and neoDES + ICI group by Fisher's exact probability test ($P < 0.05$). [d]Statistical difference between neoDES + Oil and neoDES + ICI group by Student's *t*-test or Welch's *t*-test followed by F-test ($P < 0.05$)

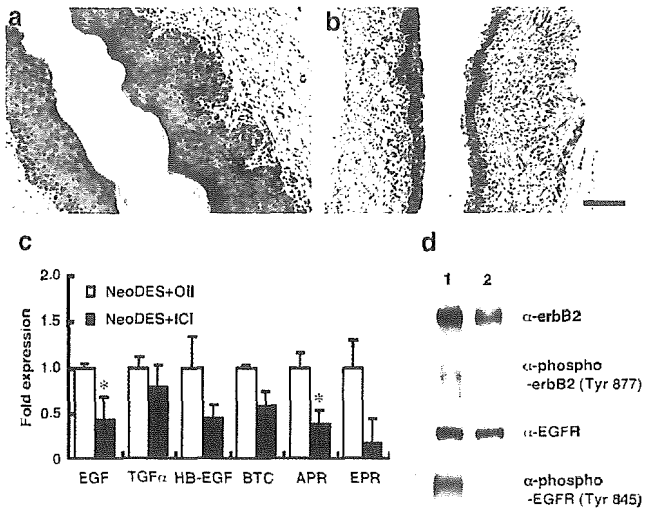

Figure 7 Administration of ICI blocked proliferation of vaginal epithelial cells in neoDES mice. (**a** and **b**) Histology of vaginae from 60-day-old neoDES (**a**) and neoDES + ICI (**b**) mice. Sections were stained with hematoxylin and eosin. Scale bar = 100 μm. (**c**) Expression profiles of growth factor mRNA in vaginae of neoDES mice treated with oil control or ICI. (**d**) ErbB2 and EGFR phosphorylation status in neoDES vaginae of mice treated with oil (lane 1) or ICI (lane 2)

promoting estrus, influences the development and maintenance of female sex characteristics, including the induction of behavioral and physiological processes in a variety of organ systems. Like most hormones, E_2 exhibits acute and transient actions in its target organs. For example, E_2 administration increases organ weight and promotes cell proliferation and differentiation in the adult female reproductive tracts (Iguchi *et al.*, 1986). E_2 withdrawal induces rapid involution of uteri and vaginae, resulting in atrophy (Sato *et al.*, 2003). This reversible and specific effect of E_2 is important in maintaining homeostasis and is required for normal health and reproduction. In contrast, long-term exposure to estrogens induces an imbalance in cell proliferation and increases the risk of cancer of the reproductive organs in rodents and humans (Marselos and Tomatis, 1992a, b). It was reported that *in utero* exposure to DES caused vaginal clear-cell adenocarcinoma in a subset of exposed females (Herbst *et al.*, 1971). To help understand this phenomenon, a laboratory rodent model has been characterized; mice treated neonatally with DES develop estrogen-independent proliferation and cornification in the vaginal epithelium and tumors later in life (Takasugi, 1976; Forsberg, 1979; Iguchi, 1992). Since the differentiative response of the vaginal epithelium is complex and involves the generation and differentiation of multiple suprabasal layers, an appropriate *in vitro* system for studying vaginal epithelium has not been established. For example, isolated vaginal epithelium *in vitro* fails to stratify or cornify in response to E_2 (Iguchi *et al.*, 1983; Tsai *et al.*, 1991). Therefore, to elucidate mechanisms of estrogen-dependent and -independent effects, which involve the induction of tumors, we used neonatal DES-treated mice as a model system. The present study was focused on the signaling of ER and erbBs and their ligands, the EGF-like growth factors in mouse vagina, since many cancers of estrogen-target organs can be related to these factors (Kurokawa *et al.*, 2000; Atanaskova *et al.*, 2002).

Although many growth factors have been reported to be related to reproductive organ growth, EGF-like growth factors were hypothesized as major factors involved in estrogen-dependent and -independent growth. It is notable that HB-EGF, APR and EPR were expressed at higher levels than EGF or TGF-α, which are considered to be powerful mitogens in the reproductive tracts of adult mouse (Nelson *et al.*, 1991). Interestingly, it was reported that APR was induced by progesterone but not by estrogens in rodent uterus (Das *et al.*, 1995). On the contrary, APR contributed to the autocrine growth of keratinocytes and tumor progression (Normanno *et al.*, 1994; Piepkorn *et al.*, 1994). This is the first report that APR is an estrogen-inducible growth factor and may be involved in vaginal epithelial cell growth, such as stratification and cornification. The sustained expression of other growth factors may be induced by APR, as EGF-like growth factors are capable of inducing other EGF-like growth factors, the so called crossinduction (Hashimoto *et al.*, 1994).

In addition to increased EGF-like growth factors, neonatal DES treatment led to increases in phosphorylation of EGFR and erbB2 even after ovariectomy. The erbB family has been implicated in numerous physiological processes and is generally regarded as a major contributor to cell proliferation and tumor progression. Amplification or overexpression of erbB2 has been reported in various cancer cells (Hynes and Stern, 1994), thus it is expected that erbB mRNAs would be increased in vaginae from neoDES mice. Instead, we observed a reduction of EGFR and erbB2 mRNA and protein expression in vaginae from both neoDES and adultDES mice, accompanied by an increase in phosphorylations of these receptor proteins. Phosphorylation of erbB2 is probably due to ligand binding and ultimately results in downregulation of the receptor, a property commonly known to receptor tyrosine kinases (Pastan and Willingham, 1983). ErbB expression in neoDES vaginae was quite similar to that in adultDES vaginae, even in the absence of stimulation by estrogens. It should be noted that although erbB expression differs between neoDES vaginae and vaginal tumors, persistent phosphorylation and activation of erbB2 have been suggested to be an early stage in tumor formation. Although all erbBs were expressed in mouse vagina and have the potential as mediators of estrogen action, EGFR likely interacts with erbB2 since we found that both EGFR and erbB2 were phosphorylated in neoDES vaginae, erbB2 is expressed in the vaginal epithelium and it was previously reported that EGFR was expressed in mouse vaginal epithelium (Falck and Forsberg, 1996).

Finally, we found that ERα is phosphorylated at serine 118 and 167 within the AF-1 domain in neoDES vaginae. Phosphorylation of these residues regulates estrogen-independent transactivation by the receptor. The activity of the ERα AF-1 domain is ligand

independent and constitutive, whereas the function of AF-2 in the ligand-binding domain is dependent on ligand binding. When E_2 dissociates from the receptor, ERα becomes transcriptionally inactive because the potential function of the AF-1 is suppressed by the unliganded ligand-binding domain (Kato *et al.*, 2000). Phosphorylation of the AF-1 domain in mouse ERα also affects the transcriptional function of AF-2 (Lahooti *et al.*, 1995). It is well established that growth factors can activate ERα in the absence of its ligand through phosphorylation in AF-1 (Kato *et al.*, 1998; Yee and Lee, 2000). Although participation of other growth factor signaling pathways cannot be ignored, erbBs can activate AF-1 of ERα by MAPK and Akt cascades (Lannigan, 2003). In our study, activated erbB signal induced ERα phosphorylation via at least p90RSK, which has the potential to phosphorylate serine 167 of ERα (Joel *et al.*, 1998), while phosphorylated ERα induces EGF-like growth factors, which activates erbB receptors. Thus, estrogen-independent actions in the vagina were characterized by the formation of an activation loop between ERα and erbB signaling.

Normal E_2-induced epithelial proliferation in the vagina is mediated through stromal ERα (Buchanan *et al.*, 1998), and the alterations in stromal–epithelial interactions may lead to the onset and/or progression of carcinogenesis. In our study, epithelial cells in neoDES vagina seem to exhibit direct mitogenic and differentiation responses in epithelial cells, and they lose their normal stromal association. Actually, tissue recombinant experiments showed that the reciprocal recombination of neonatally estrogenized vaginal epithelium and untreated vaginal stroma exhibited ovary-independent hyperplasia (Cunha *et al.*, 1977). This is the first report that ligand-independent ER action induces a precancerous status in estrogen-target organ *in vivo*.

It has not been determined whether a major contributor to estrogen-independent effects in neoDES vaginae are the erbBs or the ER. Our study demonstrated that, at least, ERα might be critical for estrogen-independent vaginal changes in neoDES mice, even in the absence of estrogen. AF-1 activation following the administration of 4-OHT induced expression of ligands for erbB receptors in vaginae. Furthermore, the administration of ICI to neoDES mice blocked phosphorylation of EGFR and erbB2, resulting in a reduction in the number of vaginal epithelial cell layers. As to erbBs, the administration of EGF rapidly induced the phosphorylation of ERα in the present study. The administration of EGF leads to vaginal cell proliferation and differentiation (Nelson *et al.*, 1991). Thus, erbBs play roles in the phosphorylation of ERα and mediation of ER action in vaginal cells. We also demonstrated that inhibitors of erbBs blocked or slightly reduced BrdU incorporation and ERα phosphorylation of serine 118 in neoDES mice. These data suggests that ERα and erbBs make a crosstalk and form activation loop, then it leads to estogen-independent activation in neoDES vagina.

The mechanisms driving the constitutive activation of ERα that occurs only in neoDES mice remain unknown. DES activates both ERα and erbBs in the vagina of normal adult mice in the similar manner to that observed in the neoDES mice. However, the activation loop has not been established in the normal adult mice and the effect of estrogen is always reversible. Additional factors, such as dysregulation of phosphatase or signals through other receptor tyrosine kinases may be involved in the persistent proliferation and differentiation of vaginal epithelial cells.

Estrogen signals acting through ERα cause both proliferation and differentiation of the vaginal epithelial cells. While hyperproliferation leads to cancer, the superactivation of differentiation signal may prevent cancer development. The balance of these two signaling cascades seems to be an important factor in estrogen-induced cancers. The constitutive activation of the ERα-erbB signaling loop seems to induce both proliferation and differentiation. Hence, an alternation in this activation loop may shift the tissue response toward more proliferation than differentiation during carcinogenesis. Therefore, further analyses are needed in order to clarify the activation status of ERα and erbB family members in the precancerous and cancer stages.

Despite decades of research, the mechanisms by which developmental exposure to estrogens results in persistent alteration of growth and differentiation of reproductive tracts in humans and rodents remain unknown. As generation of women exposed to DES becomes older, with the possibility of a second rise in DES-associated vaginal clear-cell adenocarcinoma (Herbst, 2000). Various studies to date have shown that DES-like effects can be induced following exposure to xenoestrogens in laboratory animals and wildlife (McLachlan, 2001). Therefore, further characterization of the DES model is needed to advance our knowledge of the potential risk of the carcinogenetic effects of estrogens, including developmental effects of xenoestrogens. In conclusion, we demonstrated that estrogen-independent pathway required for ERα, and activation of crosstalk between erbBs signaling and ER leads to ligand-independent activation of ERα which, in turn, leads to persistent, estrogen-independent vaginal changes and may lead to cancer in the mouse vagina later in life.

Materials and methods

Animals and treatments

Female C57BL/6J mice were maintained under 12 h light/12 h dark at 23–25°C and fed laboratory chow (CA-1, CLEA, Tokyo, Japan) and tap water *ad libitum*. All procedures and protocols were approved by the Institutional Animal Care and Use Committee at the National Institute for Basic Biology, Okazaki National Research Institutes.

Treatment time lines are shown in Figure 1. Female newborn mice were given a daily subcutaneous (s.c.) injection of 2.5 mg DES (Sigma, St Louis, MO, USA)/kg body weight (BW)/day dissolved in sesame oil or the vehicle alone beginning from days 0 (the day of birth) to 4. Some mice were killed 24 h after the last injection, and others were OVX at day 46 and killed at day 60 (referred to as neoDES mouse) (Figure 1a). Subsets of these mice were administered three injections of AG825 or AG1478 (Tocris, Ellisville, MO, USA)

(5 mg/kg BW) dissolved in dimethyl sulfoxide (DMSO), at 8 h intervals. BrdU (50 mg/kg BW) (Sigma) was injected at the last injection of the inhibitors and killed 1 h later (Figure 1d). The other subset of mice were administered with daily 4-OHT (Sigma) or ICI 182,780 (ICI, Tocris) (5 mg/kg BW/day) dissolved in sesame oil for a week and killed 24 h after the last injection (Figure 1e). To examine the acute and reversible effects of estrogens on the vagina, untreated mice were OVX at day 46 and treated with a daily injection of 2.5 μg DES/kg BW/day for 5 days, then killed 24 h after the last injection (referred to adultDES mouse) (Figure 1b). This treatment was sufficient to induce stratified and keratinized vaginal epithelium in OVX mice. For examining direct effects of estrogen and EGF, a single injection of 50 μg E_2 (Sigma) or 50 μg EGF (Biomedical, Stoughton, MA, USA)/kg BW was given to 60-day-old mice, which were OVX on day 46, and killed 0.5, 1, 3, 6, 12 and 24 h (E_2) or 15 min (EGF) after the injection (Figure 1c).

For tissue separation, neoDES vagina were cut into small pieces, placed into 1% trypsin (Difco, Kansas, MO, USA) in Hanks' balanced salt solution (Invitrogen, Carlsbad, CA, USA) and digested at 4°C for 90 min. The vaginal epithelium and stroma were then separated mechanically using fine surgical forceps.

Immunohistochemistry and BrdU immunostaining

Tissues were fixed in neutral buffered 10% formalin, embedded in paraffin and sectioned at 6 μm. Deparaffinized sections were incubated in 0.3% H_2O_2 in methanol for 15 min to eliminate endogenous peroxidases. After washing with PBS, the sections were stained with Histofine (Nichirei, Tokyo, Japan) according to the manufacturer-supplied protocol. Anti-erbB2 antibody was obtained from Novocastra (Newcastle, UK). The sections were incubated at a 1 : 500 dilution in PBS containing 1% BSA (Sigma) for 60 min at room temperature. For negative controls, normal rabbit immunoglobulin fraction (Dako, Carpinteria, CA, USA) at the same dilution of each antibody was used.

For BrdU-immunostaining, deparaffinized, sections were incubated in 3% H_2O_2 in methanol for 30 min and immersed in 2 N HCl for 20 min in order to denature the genomic DNA. After washing with PBS, the sections were incubated with anti-BrdU antibody (Boehringer Mannheim, Mannheim, Germany) diluted 1 : 10 in PBS containing 1% BSA for 60 min at room temperature. The sections were subsequently incubated with 3,3-diaminobenzidine tetrahydrochloride containing hydrogen peroxide. BrdU-labeling index was estimated by counting the number of BrdU-incorporated cells in the vaginal epithelial basal layer. Statistical analysis was performed using Student's *t*-test or Welch's *t*-test followed by F-test.

RT–PCR

Changes in gene expression were confirmed and quantified using Q-PCR with the ABI Prism 5700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Total RNA (2.5 μg), isolated with an RNeasy kit (QIAGEN, Chatsworth, CA, USA) from each groups, was used in RT–PCR reactions carried out with SuperScript II reverse transcriptase (Invitrogen) and SYBR Green Master Mix (Applied Biosystems) according to the manufacturer's instructions. PCR conditions were as follows: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min in 15 μl volumes. Sequences of gene primer sets are given in Table 2. Relative RNA equivalents for each sample were obtained by standardization of ribosomal protein L8 (L8) levels. More than three pools of samples per group were run in triplicate to determine sample reproducibility, and the average relative RNA equivalents per sample was used for further analysis. Error bars represent the standard error, with all values represented as fold change compared to the control treatment group normalized to an average of 1.0. Statistical analysis was performed using Student's *t*-test or Welch's *t*-test followed by F-test; differences with $P<0.05$ were considered significant.

The expression of NRG1 was examined using RT–PCR following electrophoresis. The annealing temperature was 60°C and 31 cycles for NRG1 and 24 cycles for glyceraldehyde-3-phosphate dehydrogenase.

Protein preparation and immunoblotting

Vaginae removed from mice were immediately homogenized in buffer A (20 mM HEPES, 2 mM EDTA, 2 mM EGTA, 250 mM sucrose, 100 mM β-glycerophosphate, 2 mM Na_3VO_4 and protease inhibitor cocktail (Complete Mini; Boehringer Mannheim, Germany), pH 7.5). The homogenates were centrifuged at 900*g* for 15 min at 4°C. The pellets were suspended in buffer B (20 mM HEPES, 2 mM EDTA, 2 mM EGTA, 250 mM NaCl, 1% Triton X-100, 100 mM

Table 2 Sequences of gene primer sets for RT–PCR

	Primer sequences (5′-3′)	
Gene	*Forward*	*Reverse*
EGFR	ATTCATGCGAAGACGTCACATT	GTTCCACGAGCTCTCTCTCTTGA
erbB2	GCTGCCCGAAACGTGCTA	CCGTGCCAGCCCGAA
erbB3	AGGCTTGTCTGGATTCTGTGGTT	GGGATCGGGTGCAGAGAGA
erbB4	GGAGGCTGCTCAGGACCAA	ACGCACGCTCCACTGTCAT
EGF	TTCACAGAGCACACCTCAAAGGT	GAATGTAAGCGTGGCTTCC
TGF-α	CCAGATTCCCACACTCAGT	GGAGGTCTGCATGCTCACA
HB-EGF	CAAGGTTCCCAGACAGGATCTC	GGAGGACAGCGAGGTTCCA
BTC	AGATGCCGCTTCGTGGTG	CGAGCCCCAAAGTAGCCTT
APR	CCGGTGGAACCAATGAGAACT	CCTAAGACCAGCAGCAACAGC
EPR	GCTGCACCGAGAAAGAAGGA	GGGAACCTAGACAAAGCAGCG
IGF-I	TTCAGTTCGTGTGTGGACCGAG	TCCACAATGCCTGTCTGAGGTG
IGF-II	TTCGCCTTGTGCTGCATC	TCAACAAGCTCCCCTCCG
KGF	GAAAGGGACCCAGGAGATGAA	TGATTGCCACAATTCCAACTG
L8	ACAGAGCCGTTGTTGGTGTTG	CAGCAGTTCCTCTTTGCCTTGT
GAPDH	AACGACCCCTTCATTGACCTC	CCTTGACTGTGCCGTTGAATT
NRG-1	TGAAAGACCTTTCAAACCCCTC	CTCTTCTGGTACAGCTCCTCCG

β-glycerophosphate, 2 mM Na_3VO_4 and protease inhibitor, pH 7.5) and used as nuclear samples. Supernatant fluids were recentrifuged at 105 000 *g* for 60 min and the resulting pellets were suspended in buffer B. Protein contents were determined using the Bradford Assay (Protein Assay Reagent, BioRad, Hercules, CA, USA).

The samples mixed with Laemmli sample buffer were boiled then electrophoresed on SDS–polyacrylamide gels; proteins were transferred onto a nitrocellulose membrane. The membranes were preincubated with 3% BSA in TBS contained 0.1% Tween-20 over night at 4°C. Incubations with each antibody were performed at a dilution of 1 : 1000 at room temperature for 2 h in TBS contained 0.1% Tween-20 with or without 1% BSA. Anti-erbB2, anti-erbB3, anti-ERα and anti-p90RSK-1 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-erbB4 was obtained from NeoMarkers, anti-EGFR, anti-Erk1/2 and antiphospho-EGFR (Tyr 845 of human sequence corresponding to Tyr 847 of mouse sequence), -erbB2 (Tyr 877; mouse sequences corresponding to human Tyr 877 is not known because N-terminal sequence is not determined), -Erk1/2 (Thr 202/Tyr 204 to Thr 203/Tyr 205 of mouse sequence), -p90RSK (Thr 574 to Thr 611 of mouse sequence) and -ERα (Ser 118 and Ser 167 to Ser 122 and Ser 171 of mouse sequences) antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). The numbering of amino-acid residues in this paper is according to the human protein sequences as the manufacturer's product name and specificity of these phosphospecific antibodies against mouse proteins is described in the manufacturer's instruction. Signals were detected with the ECL kit (Amersham, Arlington Heights, IL, USA).

Acknowledgements
We are grateful to Dr Raphael Guzman, Cancer Research Laboratory and Department of Molecular Cell Biology, University of California at Berkeley, Dr Bruce Blumberg, Department of Developmental and Cell Biology, University of California at Irvine and Dr Louis Guillette Jr, Department of Zoology, University of Florida, for their critical readings of the manuscript. This work was supported in part by a grant-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and Health Sciences Research Grant from the Ministry of Health, Labour and Welfare, Japan.

References

Atanaskova N, Keshamouni VG, Krueger JS, Schwartz JA, Miller F and Reddy KB. (2002). *Oncogene,* **21,** 4000–4008.

Berry M, Metzger D and Chambon P. (1990). *EMBO J.*, **9,** 2811–2818.

Buchanan DL, Kurita T, Taylor JA, Lubahn DB, Cunha GR and Cooke PS. (1998). *Endocrinology,* **139,** 4345–4352.

Cunha GR, Lung B and Kato K. (1977). *Dev. Biol.*, **56,** 52–67.

Curtis SW, Washburn T, Sewall C, DiAugustine R, Lindzey J, Couse JF and Korach KS. (1996). *Proc. Natl. Acad. Sci. USA,* **93,** 12626–12630.

Das SK, Chakraborty I, Paria BC, Wang XN, Plowman G and Dey SK. (1995). *Mol. Endocrinol.*, **9,** 691–705.

DiAugustine RP, Petrusz P, Bell GI, Brown CF, Korach KS, McLachlan JA and Teng CT. (1988). *Endocrinology,* **122,** 2355–2363.

Dickson RB and Lippman ME. (1995). *Endocr. Rev.*, **16,** 559–589.

Dunn TB and Green AW. (1963). *J. Natl. Cancer Inst.*, **31,** 425–455.

Falck L and Forsberg JG. (1996). *Anat. Rec.*, **245,** 459–471.

Forsberg JG. (1979). *Natl. Cancer Inst. Monogr.*, 41–56.

Hashimoto K, Higashiyama S, Asada H, Hashimura E, Kobayashi T, Sudo K, Nakagawa T, Damm D, Yoshikawa K and Taniguchi N. (1994). *J. Biol. Chem.*, **269,** 20060–20066.

Herbst AL. (2000). *Gynecol. Oncol.*, **76,** 147–156.

Herbst AL, Ulfelder H and Poskanzer DC. (1971). *N. Engl. J. Med.*, **284,** 878–881.

Hynes NE and Stern DF. (1994). *Biochim. Biophys. Acta,* **1198,** 165–184.

Ignar-Trowbridge DM, Nelson KG, Bidwell MC, Curtis SW, Washburn TF, McLachlan JA and Korach KS. (1992). *Proc. Natl. Acad. Sci. USA,* **89,** 4658–4662.

Iguchi T. (1992). *Int. Rev. Cytol.*, **139,** 1–57.

Iguchi T, Edery M, Tasi PS, Ozawa S, Sato T and Bern HA. (1993). *Proc. Soc. Exp. Biol. Med.*, **204,** 110–116.

Iguchi T, Takei T, Takase M and Takasugi N. (1986). *Acta Anat. (Basel),* **127,** 110–114.

Iguchi T, Uchima FD, Ostrander PL and Bern HA. (1983). *Proc. Natl. Acad. Sci. USA,* **80,** 3743–3747.

Joel PB, Smith J, Sturgill TW, Fisher TL, Blenis J and Lannigan DA. (1998). *Mol. Cell. Biol.*, **18,** 1978–1984.

Kato S, Kitamoto T, Masuhiro Y and Yanagisawa J. (1998). *Oncology,* **55** (Suppl. 1), 5–10.

Kato S, Masuhiro Y, Watanabe M, Kobayashi Y, Takeyama KI, Endoh H and Yanagisawa J. (2000). *Genes Cells,* **5,** 593–601.

Klotz DM, Hewitt SC, Ciana P, Raviscioni M, Lindzey JK, Foley J, Maggi A, DiAugustine RP and Korach KS. (2002). *J. Biol. Chem.*, **277,** 8531–8537.

Klotz DM, Hewitt SC, Korach KS and Diaugustine RP. (2000). *Endocrinology,* **141,** 3430–3439.

Kurokawa H, Lenferink AE, Simpson JF, Pisacane PI, Sliwkowski MX, Forbes JT and Arteaga CL. (2000). *Cancer Res.*, **60,** 5887–5894.

Lahooti H, White R, Hoare SA, Rahman D, Pappin DJ and Parker MG. (1995). *J. Steroid Biochem. Mol. Biol.*, **55,** 305–313.

Lannigan DA. (2003). *Steroids,* **68,** 1–9.

Marselos M and Tomatis L. (1992a). *Eur. J. Cancer,* **28A,** 1182–1189.

Marselos M and Tomatis L. (1992b). *Eur. J. Cancer,* **29A,** 149–155.

McDonnell DP, Clemm DL, Hermann T, Goldman ME and Pike JW. (1995). *Mol. Endocrinol.*, **9,** 659–669.

McLachlan JA. (2001). *Endocr. Rev.*, **22,** 319–341.

McLachlan JA, Newbold RR and Bullock BC. (1980). *Cancer Res.*, **40,** 3988–3999.

Metzger D, Berry M, Ali S and Chambon P. (1995). *Mol. Endocrinol.*, **9,** 579–591.

Nelson KG, Sakai Y, Eitzman B, Steed T and McLachlan J. (1994). *Cell Growth Differ.*, **5,** 595–606.

Nelson KG, Takahashi T, Bossert NL, Walmer DK and McLachlan JA. (1991). *Proc. Natl. Acad. Sci. USA,* **88,** 21–25.