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This information is current as of January 17, 2018.

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*J Immunol* 2004; 172:6820-6827; ;  
doi: 10.4049/jimmunol.172.11.6820  
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The American Association of Immunologists, Inc.,  
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



# Toll-Like Receptor 9 Signaling Activates NF- $\kappa$ B through IFN Regulatory Factor-8/IFN Consensus Sequence Binding Protein in Dendritic Cells<sup>1</sup>

Hideki Tsujimura,<sup>2\*</sup> Tomohiko Tamura,\* Hee Jeong Kong,\* Akira Nishiyama,\* Ken J. Ishii,<sup>†</sup> Dennis M. Klinman,<sup>†</sup> and Keiko Ozato<sup>3\*</sup>

Unmethylated CpG DNA binds to the Toll-like receptor 9 (TLR9) and activates NF- $\kappa$ B to induce cytokine genes in dendritic cells (DCs). IFN regulatory factor (IRF)-8/IFN consensus sequence binding protein is a transcription factor important for development and activation of DCs. We found that DCs from IRF-8<sup>-/-</sup> mice were unresponsive to CpG and failed to induce TNF- $\alpha$  and IL-6, targets of NF- $\kappa$ B. Revealing a signaling defect selective for CpG, these cytokines were robustly induced in IRF-8<sup>-/-</sup> DCs in response to LPS that signals through TLR4. IRF-8<sup>-/-</sup> DCs expressed TLR9, adaptor myeloid differentiation factor 88, and other signaling molecules, but CpG failed to activate NF- $\kappa$ B in  $-/-$  cells. This was due to the selective inability of  $-/-$  DCs to activate I- $\kappa$ B kinase  $\alpha\beta$ , the kinases required for NF- $\kappa$ B in response to CpG. IRF-8 reintroduction fully restored CpG activation of NF- $\kappa$ B and cytokine induction in  $-/-$  DCs. Together, TLR signals that activate NF- $\kappa$ B are diverse among different TLRs, and TLR9 signaling uniquely depends on IRF-8 in DCs. *The Journal of Immunology*, 2004, 172: 6820–6827.

Bacterially derived or synthetic DNA that contains unmethylated CpG is highly immunostimulatory (1). CpG DNA triggers production of proinflammatory cytokines and promotes dendritic cell (DC)<sup>4</sup> maturation (2, 3). It also activates B cells and enhances macrophage functions (4). With these broad activities, CpG can confer protection against infectious pathogens (5). CpG binds to the Toll-like receptor (TLR) 9 that resides in the endoplasmic reticulum and recruits MyD88 to initiate signaling (6–13). Although signaling by TLR9 is dependent on myeloid differentiation factor 88 (MyD88) (14), TLR4 signaling by LPS involves an additional adaptor Toll-IL-1R domain-containing adaptor protein/Mal (15, 16). Adaptor recruitment is followed by the recruitment and phosphorylation of members of the IL-1R-associated kinase (IRAK) family, leading to further recruitment of TNFR-associated factor 6 (TRAF6) (11, 12). This leads to the activation of NF- $\kappa$ B, the major endpoint of the TLR signaling, a feature conserved in all TLR signaling throughout evolution (12, 17). NF- $\kappa$ B is a key regulator of innate immunity, and has a critical role in establishing adaptive immunity (18–20). NF- $\kappa$ B activation

is dependent on phosphorylation and degradation of the inhibitory subunit, I- $\kappa$ B $\alpha$  or I- $\kappa$ B $\beta$ , an event mediated by the phosphorylation of I- $\kappa$ B kinase (IKK)  $\beta$  of the IKK complex (21, 22). NF- $\kappa$ B activation is also dependent on IKK $\alpha$  phosphorylation (23, 24). All NF- $\kappa$ B-activating signals lead to the activation of the IKK complex, an obligatory step for NF- $\kappa$ B activation.

IFN regulatory factor (IRF)-8, also called IFN consensus sequence binding protein, is a transcription factor of the IRF family expressed only in the immune system (25). It resides primarily in the nucleus, although it may temporarily be in the cytoplasm (26). IRF-8 interacts with proteins of the Ets family, such as PU.1 and TEL, and regulates the development of myeloid cells (25, 27). It is important for induction of IL-12p40 expression in macrophages, thereby contributing to host defense (25, 28). Recently, DCs have emerged as the cell type critical for host defense (29). DCs are heterogeneous population of cells and are classified into distinct DC subsets (30). We previously noted that IRF-8<sup>-/-</sup> mice have much fewer plasmacytoid DCs (pDCs) and CD8 $\alpha^+$  DCs than normal mice, indicating that this factor is essential for the development of these DC subtypes (31–34). Nevertheless, IRF-8<sup>-/-</sup> mice have other DCs that express CD11c and CD11b with or without the CD4 marker. Both pDCs and B220<sup>-</sup> non-pDCs express TLR9 among other TLRs, although at a lower level than pDCs (32). These cells can be stimulated by CpG to induce a series of proinflammatory cytokines, including TNF- $\alpha$  and IL-6 similar to other cell types (2, 6, 9).

We found that IRF-8<sup>-/-</sup> DCs, mostly non-pDCs, were unresponsive to CpG and failed to induce TNF- $\alpha$  and IL-6, despite that these  $-/-$  cells expressed TLR9 and TLR signaling molecules. The unresponsiveness to CpG contrasted with the robust response to LPS and induction of the cytokines in IRF-8<sup>-/-</sup> DCs. The TNF- $\alpha$  and IL-6 genes carry  $\kappa$ B sites in the promoter, and are directly activated by NF- $\kappa$ B (35, 36). In IRF-8<sup>-/-</sup> DCs, NF- $\kappa$ B was not activated in response to CpG, while it was strongly activated in response to LPS, indicating that the TLR9 signaling pathway is selectively disrupted in IRF-8<sup>-/-</sup> DCs. Further analysis found that phosphorylation of IKK $\alpha\beta$ , required for NF- $\kappa$ B activation, was selectively aborted in IRF-8<sup>-/-</sup> cells in response to CpG.

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Received for publication October 21, 2003. Accepted for publication March 23, 2004.

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<sup>1</sup> H.T. and A.N. were supported in part by the Japan Society for Promotion of Science.

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<sup>4</sup> Abbreviations used in this paper: DC, dendritic cell; BM, bone marrow; BMDC, BM-derived DC; DBD, DNA binding domain; Flt3L, Flt3 ligand; IAD, IRF association domain; IKK, I- $\kappa$ B kinase; IRAK, IL-1R-associated kinase; IRF, IFN regulatory factor; MAPK, mitogen-activated protein kinase; MyD88, myeloid differentiation factor 88; pDC, plasmacytoid DC; TLR, Toll-like receptor; TRAF, TNFR-associated factor.

Introduction of IRF-8 restored TLR9 signaling, allowing CpG to activate NF- $\kappa$ B, thereby restoring cytokine production. Taken together, TLR-mediated NF- $\kappa$ B activation pathways are diverse in DCs and modulated by IRF-8. The participation of IRF-8 in the TLR signaling pathways may add multiplicity to the mechanism of NF- $\kappa$ B activation in DCs, enabling cell type- and signal-specific activation of target genes.

## Materials and Methods

### DC preparation and retroviral transduction

Preparation of splenic DCs and in vitro generation of bone marrow-derived DCs (BMDCs) with Flt3 ligand (Flt3L) as well as magnetic beads-based separation of B220<sup>+</sup> pDCs and B220<sup>-</sup>, CD11b<sup>+</sup> non-pDCs were described (32, 33). To activate DCs, cells were stimulated with 200 ng/ml LPS (*Salmonella* Minnesota; Sigma-Aldrich, St. Louis, MO) or 1  $\mu$ g/ml synthetic CpG oligomer 1668 (8) or control CpG DNA 1668-GC (3) for 6 h. For the CpG uptake study, FITC- or Cy3-conjugated CpG DNA (37) was used.

### ELISA and real-time RT-PCR

Supernatants from DCs were analyzed for TNF- $\alpha$  using an ELISA kit (BD PharMingen, San Diego, CA). Semiquantitative RT-PCR was performed, as described (33). For real-time PCR, amplification of sample cDNA was monitored with the fluorescent DNA-binding dye SYBR Green (SYBR Green PCR master kit; Applied Biosystems, Foster City, CA) in combination with the ABI Prism 7000 Sequence Detection System (Applied Biosystems), according to the manufacturer's instructions. Transcript levels were normalized by GAPDH levels. Primer sequences used for PCR are available on request.

### Flow cytometry

Abs used for surface marker analysis were purchased from BD PharMingen. For intracellular phospho-p38 mitogen-activated protein kinase (MAPK) detection, cells were fixed with 1% paraformaldehyde, permeabilized with 90% methanol, and stained with rabbit anti-phospho-p38 (Cell Signaling, Beverly, MA), followed by FITC anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Stained cells were collected on FACSCalibur (BD Biosciences, San Jose, CA), and data were analyzed by the FlowJo software (Tree Star, San Jose, CA).

### Immunoblot

Whole cell extracts were subjected to the standard immunoblot procedure using anti-IKK $\alpha$ , IKK $\beta$ , phospho-IKK $\alpha$  $\beta$  (Cell Signaling), or  $\alpha$ -tubulin (Sigma-Aldrich) Abs combined with HRP-conjugated donkey anti-rabbit or sheep anti-mouse IgG (Amersham, Arlington Heights, IL). Gels were visualized by Chemiluminescent Reagent Plus (NEN, Boston, MA).

### EMSA

Nuclear extracts were prepared from BMDCs that had been stimulated with LPS (200 ng/ml) or CpG (1  $\mu$ g/ml) for indicated periods of time. Extracts from 10<sup>5</sup> cells were incubated with <sup>32</sup>P-labeled, double-stranded oligomer containing two tandemly positioned NF- $\kappa$ B binding sites (5'-ATCAGG GACTTCCGCTGGGGACTTCCG-3' and 5'-GGCGAAAGTCCCGA GCGGAAAGTCCCTGAT-3') (38) in binding buffer containing 50 mM KCl, 20 mM HEPES (pH 7.8), 0.1 mM EDTA, 2.5 mM DTT, 10% glycerol, and 2  $\mu$ g of poly(dIdC) for 30 min at room temperature. For competition assay, a 100-fold excess of unlabeled probe was added to nuclear extracts for 10 min before addition of labeled probe. Reaction mixtures were separated in 5% polyacrylamide gel electrophoresis and visualized by autoradiography.

### Immunofluorescent staining

DCs were placed on cytospin slides and fixed with 4% paraformaldehyde, permeabilized with 100% methanol or 0.2% Triton X-100, and blocked by 3% BSA. Cells were then incubated with Abs for p65/RelA, c-Rel, or RelB (Santa Cruz Biotechnology, Santa Cruz, CA), followed by biotinylated anti-rabbit Ab (Amersham) and streptavidin-Cy2 (Amersham), and counterstained with Hoechst 33342. Stained cells were viewed on a confocal microscope (Leica, Deerfield, IL; model TCS SP2).

## Results

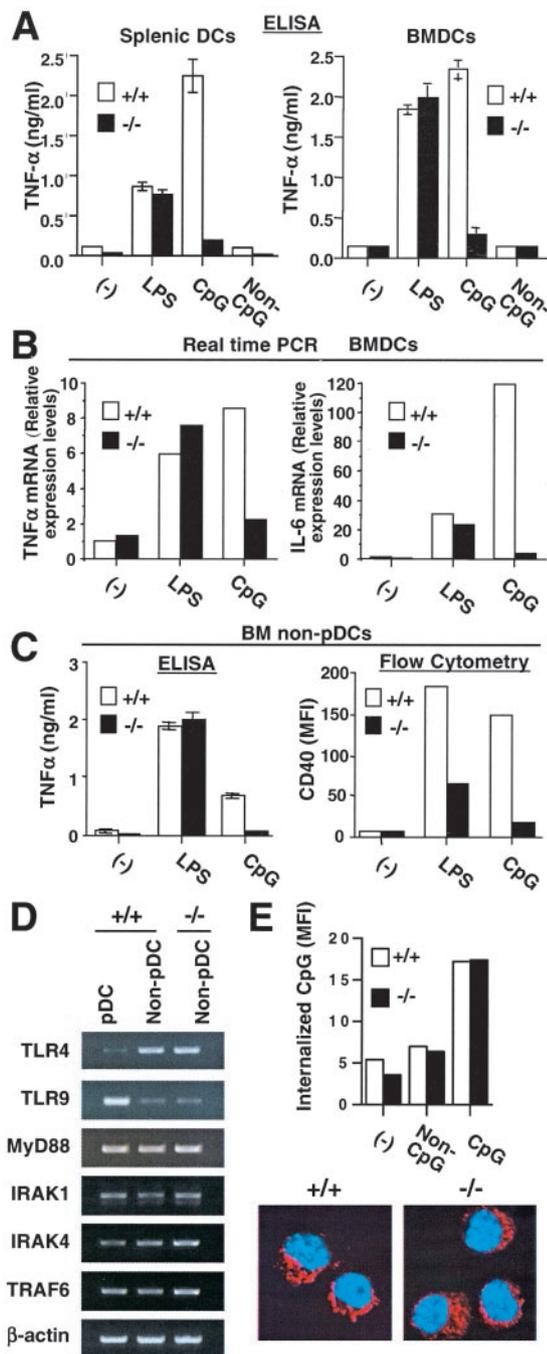
### CpG induction of TNF- $\alpha$ and IL-6 is impaired in IRF-8<sup>-/-</sup> DCs

Although IRF-8<sup>-/-</sup> mice do not have pDCs and CD8 $\alpha$ <sup>+</sup> DCs, they have normal levels of other DCs (34). To determine whether IRF-

8<sup>-/-</sup> DCs are functionally intact, we first examined IRF-8<sup>-/-</sup> splenic DCs for induction of TNF- $\alpha$  following stimulation with LPS or CpG for 6 h. ELISA in Fig. 1A (*left*) showed that LPS stimulated TNF- $\alpha$  production both in  $+/+$  and  $-/-$  DCs at similar levels, indicating that TLR4 signaling by LPS is intact in  $-/-$  cells. In contrast, IRF8<sup>-/-</sup> DCs did not produce a significant amount of TNF- $\alpha$  in response to CpG, despite that the same CpG led abundant TNF- $\alpha$  production in  $+/+$  DCs. Confirming specificity of response, control DNA without CpG motif did not induce the cytokine in  $+/+$  DCs. To further establish the disparate responses to LPS and CpG in IRF-8<sup>-/-</sup> DCs, we tested BMDCs cultured in vitro in the presence of Flt3L (33). Cells from  $+/+$  mice gave rise to mixed DC populations comprising  $\sim$ 70% non-pDCs and  $\sim$ 30% pDCs. As expected, most, if not all DCs generated from  $-/-$  bone marrow (BM) cells were non-pDCs. Paralleling the results with freshly harvested DCs,  $-/-$  BMDCs produced TNF- $\alpha$  only in response to LPS, but not to CpG, while  $+/+$  DCs produced the cytokine in response to both ligands (Fig. 1A, *right*). In Fig. 1B (*left*), TNF- $\alpha$  mRNA levels in BMDCs were quantified by real-time PCR. Consistent with the ELISA data, CpG stimulated TNF- $\alpha$  transcripts only in  $+/+$  DCs, but not in  $-/-$  DCs, whereas LPS stimulated the transcripts in both cells. The TNF- $\alpha$  promoter has several NF- $\kappa$ B sites and is dependent on NF- $\kappa$ B for induction (35, 36). We next tested IL-6, another target of NF- $\kappa$ B (36, 39) (Fig. 1B, *right*). Similar to the results with TNF- $\alpha$ ,  $-/-$  DCs did not induce IL-6 transcripts following CpG, while they did following LPS. As expected,  $+/+$  DCs induced the transcripts following both LPS and CpG stimulation.

To exclude the possibility that the absence of CpG response in  $-/-$  DCs was due to the lack of pDCs, BMDCs from  $+/+$  and  $-/-$  DCs were separated into B220<sup>+</sup> pDCs and B220<sup>-</sup> non-pDCs, and were individually tested for TNF- $\alpha$  production. Data with purified non-pDCs paralleled those with the whole DCs (Fig. 1C, *left*), in that  $+/+$  DCs produced the cytokine in response to both CpG and LPS, while  $-/-$  DCs did only to LPS. We also examined whether  $-/-$  DCs were impaired in their capacity to enhance surface marker expression following CpG stimulation (Fig. 1C, *right*). CD40 expression was enhanced both by LPS and CpG in  $+/+$  cells, while the enhancement was seen only by LPS in  $-/-$  cells. In addition, CpG enhancement of MHC class II and CD80 in  $-/-$  DCs was less pronounced than  $+/+$  non-pDCs (data not shown). These results indicate that IRF-8<sup>-/-</sup> DCs have a selective defect in the responsiveness to CpG.

CpG is recognized by TLR9 (6, 8–10). Semiquantitative RT-PCR was conducted to test TLR9 expression in IRF-8<sup>-/-</sup> DCs. Data in Fig. 1D showed that TLR9 was expressed in  $-/-$  pDCs at levels comparable to  $+/+$  non-pDCs, although lower than that in  $+/+$  pDCs, excluding the possibility that the failure of  $-/-$  DCs to respond to CpG is due to the absence or low expression of TLR9. TLR9 resides in the endoplasmic reticulum. Upon CpG binding, the ligand-receptor complex moves to endosomes and then to a lysosomal compartment to form aggregates (9, 13, 37). To assess whether TLR9 in  $-/-$  DCs was capable of processing CpG and forming aggregates,  $+/+$  and  $-/-$  DCs were incubated with FITC-labeled CpG DNA and uptake was quantified by flow cytometry. Results in Fig. 1E (*top*) showed that  $+/+$  and  $-/-$  cells incorporated similar amounts of CpG DNA. Incorporation of control DNA without CpG motif appeared less than CpG DNA. Ligand binding and aggregate formation were then visualized by confocal microscopy with cells incubated with Cy3-conjugated CpG for 2 h (Fig. 1E, *bottom*). CpG was found in the cytoplasm as small aggregates in both  $+/+$  and  $-/-$  DCs in the same manner. These data indicate that  $+/+$  and  $-/-$  DCs express functional TLR9 and that the failure of  $-/-$  DCs to respond to CpG is not

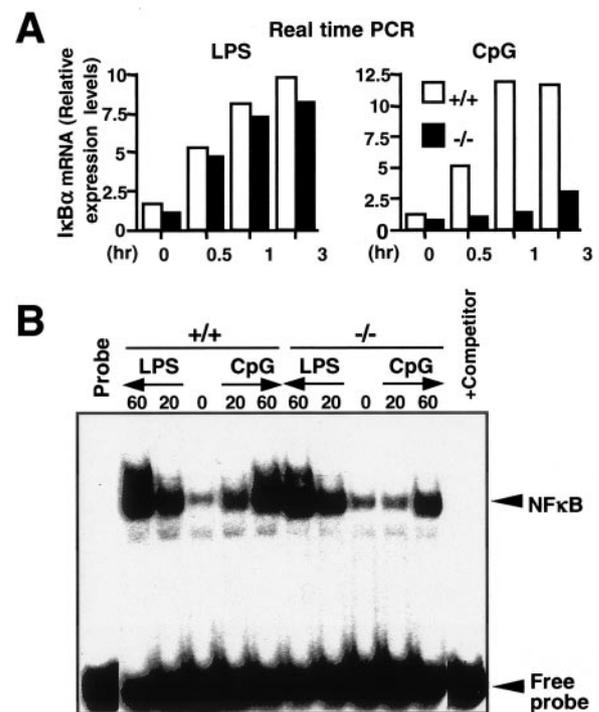


**FIGURE 1.** Selective absence of cytokine production by CpG in IRF-8<sup>-/-</sup> DCs. *A, Left*, Splenic DCs from IRF-8<sup>+/+</sup> and IRF-8<sup>-/-</sup> mice were stimulated with LPS (200 ng/ml), CpG, or control oligomer (1 μg/ml) for 6 h. Production of TNF-α in supernatants was measured by ELISA. Values represent the average of three assays ± SD. *Right*, BMDCs generated in the presence of Flt3L were stimulated with LPS, or CpG and TNF-α in supernatants were measured by ELISA. *B*, Real-time PCR analysis of TNF-α and IL-6 mRNA expression in +/+ and -/- BMDCs stimulated with LPS or CpG, as above. *C, Left*, BM non-pDCs separated from the whole DC population were stimulated as above and analyzed for TNF-α by ELISA. *Right*, BM pDCs and non-pDCs from +/+ and -/- mice were stimulated as above and analyzed for CD40 expression by flow cytometry. Bars represent the mean fluorescence intensity (MFI). *D*, BM pDCs and non-pDCs were separately tested for expression of indicated mRNA by semiquantitative RT-PCR. *E, Top*, +/+ and -/- BMDCs were incubated with FITC-conjugated CpG (2 μg/ml) for 2 h. Surface-bound FITC was quenched using trypan blue. Internalized FITC was measured by flow cytometry. Data represent the MFI. *Bottom*, Cells were incubated with Cy3-conjugated CpG (red) for 2 h, fixed, counterstained for DNA (blue), and visualized by confocal microscopy.

due to a defect in CpG-TLR9 binding, nor intracellular movement of the ligand/receptor complex, but to a defect downstream of CpG movement. To assess the expression of the molecules involved in TLR signaling, we examined transcripts for the adaptor MyD88, exclusively used in CpG signaling (14), as well as additional downstream factors, IRAK1, IRAK4, and TRAF6 (Fig. 1D). Expression of these transcripts was comparable in +/+ and -/- DCs.

#### CpG fails to activate NF-κB in IRF-8<sup>-/-</sup> DCs

Activation of NF-κB is a final event in TLR signaling that globally affects gene expression patterns in DCs and promotes their terminal maturation (10, 36, 40). Given that IRF-8<sup>-/-</sup> DCs failed to induce TNF-α and IL-6, NF-κB's direct targets, we next investigated whether -/- DCs were capable of activating NF-κB in response to CpG by testing induction of I-κBα. I-κBα is an inhibitory subunit of NF-κB that is degraded upon activation. This sets off its re-expression as a feedback mechanism to restore steady state NF-κB (36, 41). Thus, I-κBα induction signifies functional activation of NF-κB. Real-time PCR measurement of I-κBα in Fig. 2A showed that I-κBα transcripts were induced by LPS both in +/+ and -/- DCs within 30 min and the levels increased steadily for up to 3 h. CpG induced similar amounts of transcripts in +/+ DCs. In contrast, CpG induction of I-κBα in -/- DCs was markedly attenuated in -/- DCs, reaching <20% of +/+ cells. These results indicate that -/- DCs have a defect in activating NF-κB in response to CpG, while maintaining the capacity to induce it in response to LPS. Because this defect was selective for CpG, it is unlikely to be due to the absence/mutation of molecules involved in all TLR signaling, pointing to a mechanistic defect specific for TLR9 signaling.



**FIGURE 2.** The lack of NF-κB activation in IRF-8<sup>-/-</sup> DCs after stimulation with CpG. *A*, IRF-8<sup>+/+</sup> and IRF-8<sup>-/-</sup> DCs were incubated with LPS or CpG for indicated times, and expression of I-κBα was analyzed by real-time PCR. *B*, Nuclear extracts from +/+ and -/- BMDCs stimulated with LPS or CpG for indicated time (min) were tested for NF-κB-binding activity by EMSA.

It was of importance to determine whether IRF-8<sup>-/-</sup> DCs were defective in inducing NF- $\kappa$ B-binding activity in response to CpG. To this end, EMSA was performed with a  $\kappa$ B probe for nuclear extracts from +/+ and -/- BMDCs stimulated with CpG or LPS. As shown in Fig. 2B, NF- $\kappa$ B-binding activity was strongly induced in +/+ cells 20 min following CpG addition, with a further ~10-fold increase in binding activity at 60 min. In contrast, NF- $\kappa$ B binding was not induced in -/- DCs 20 min after CpG addition, although a slight induction in binding was noted 60 min after stimulation. Importantly, NF- $\kappa$ B-binding activity was induced in -/- cells both 20 and 60 min after LPS stimulation at levels comparable to +/+ cells. These results indicate that -/- DCs have a defect in activating NF- $\kappa$ B in response to TLR9 signaling. A weak band seen in unstimulated samples (time 0) from +/+ and -/- cells may in part represent binding activity artificially induced during cell/extract preparations.

To verify that the lack of NF- $\kappa$ B-binding activity in CpG-stimulated -/- cells observed in EMSA actually represents an event in vivo, we next examined translocation of NF- $\kappa$ B from the cytoplasm to nucleus (18–20). In Fig. 3A, +/+ and -/- DCs were stimulated with LPS or CpG, fixed, permeabilized, and stained for p65/RelA, the major activating component of NF- $\kappa$ B. Before stimulation, p65/RelA was found mostly in the cytoplasm in both +/+ and -/- DCs. The intensity of p65/RelA staining was similar in

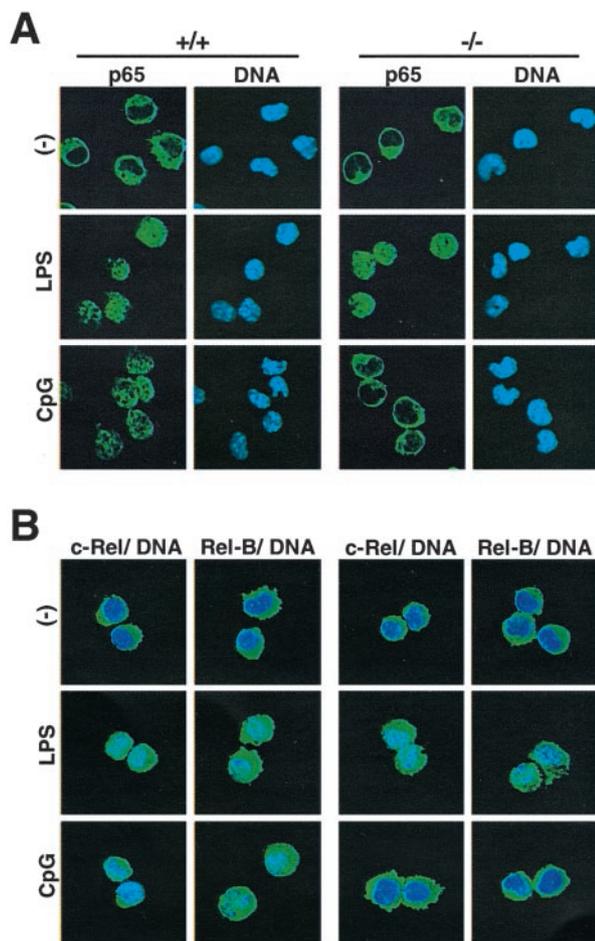
+/+ and -/- DCs. LPS stimulation led to nuclear translocation of the majority of fluorescence both in +/+ and -/- DCs, indicating that p65/RelA was activated and moved to the nucleus in response to LPS in both cells. In contrast, CpG stimulation led to p65/RelA nuclear translocation only in +/+ DCs. In -/- DCs, the majority of fluorescence remained in the cytoplasm. Essentially all +/+ DCs showed nuclear translocation of p65/RelA, indicating that nuclear translocation took place in both pDCs and non-pDCs for +/+ cells. In Fig. 3B (lower panel), we tested translocation of other NF- $\kappa$ B family members, RelB and c-Rel, that also carry the activation domain. A modified permeabilization procedure was used to enhance the sensitivity of detection, because they are expressed at lower levels than p65/RelA in most cells (19). Similar to p65/RelA, CpG stimulated nuclear translocation of both RelB and c-Rel in +/+ DCs, but not in -/- DCs, whereas LPS stimulated nuclear translocation of these proteins in both cells. These results are in agreement with EMSA data above and show that IRF-8<sup>-/-</sup> DCs fail to activate NF- $\kappa$ B in response to CpG.

#### CpG fails to phosphorylate IKK $\alpha$ and p38 MAPK in IRF-8<sup>-/-</sup> DCs

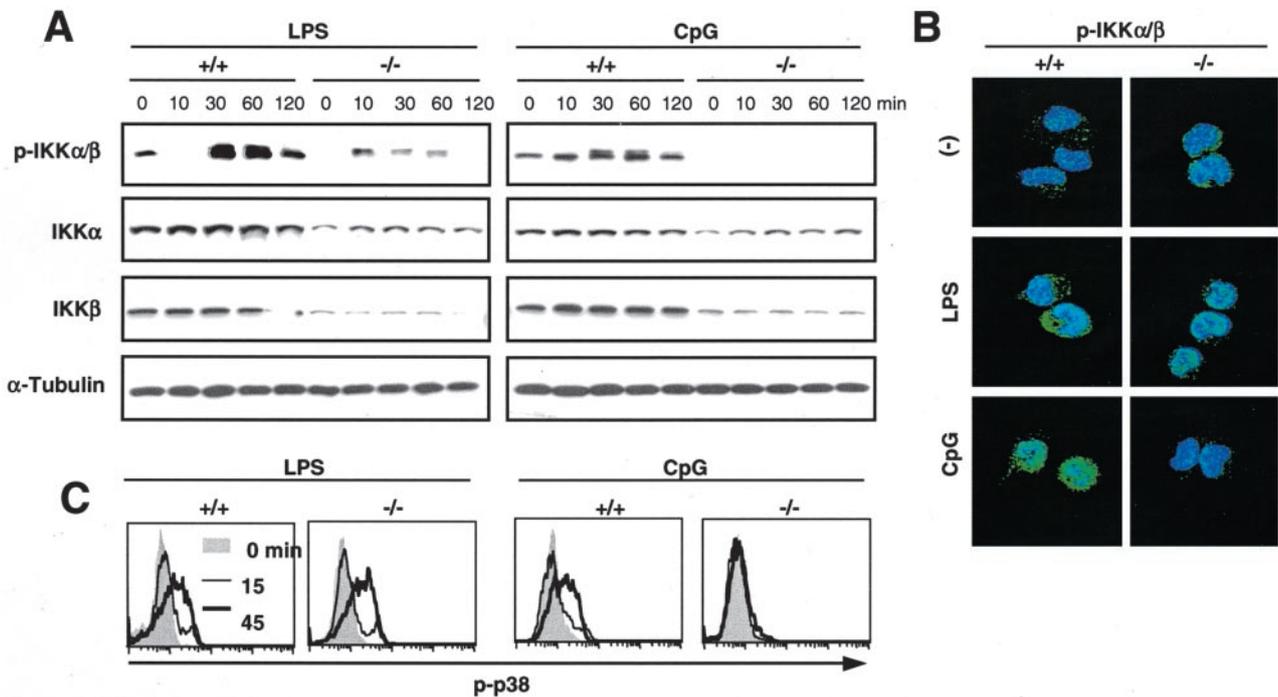
The above data indicate that TLR9 signaling is aborted before NF- $\kappa$ B activation in -/- DCs. It has been shown that signals that activate NF- $\kappa$ B converge on the phosphorylation of IKK $\alpha$  and IKK $\beta$  in the IKK complex, a process essential for NF- $\kappa$ B activation (20, 42). We next studied whether CpG can phosphorylate IKK $\alpha$  and IKK $\beta$  in IRF-8<sup>-/-</sup> DCs. Immunoblot analysis in Fig. 4A (top row) showed that phospho-IKK $\alpha$  and phospho-IKK $\beta$  (lower and upper band, respectively, p-IKK $\alpha$  $\beta$ ) were markedly increased after LPS or CpG addition in +/+ cells. Phospho-IKK $\alpha$  $\beta$  was detected after LPS stimulation in -/- DCs, albeit to a significantly reduced level relative to +/+ DCs. Significantly, no phospho-IKK $\alpha$  $\beta$  was observed after CpG stimulation in -/- DCs, indicating that CpG failed to induce IKK phosphorylation in these cells. Because LPS-induced IKK $\alpha$  $\beta$  phosphorylation appeared to be lower in -/- DCs than +/+ cells, we examined the total levels of IKK $\alpha$  and IKK $\beta$  in these cells by immunoblot, using Abs for unphosphorylated IKKs. As shown in Fig. 4A (second and third rows), -/- DCs expressed IKK $\alpha$  and IKK $\beta$ , but their levels were significantly lower than those in +/+ DCs, suggesting that expression of IKK $\alpha$  and IKK $\beta$  is regulated by IRF-8. It is of note that despite the reduced expression of IKK $\alpha$  and IKK $\beta$ , IRF-8<sup>-/-</sup> DCs responded to LPS, indicating that their levels are sufficient to activate NF- $\kappa$ B when stimulated by LPS.

To gain additional evidence for the absence of IKK phosphorylation by CpG in -/- DCs, immunostaining was performed with the anti-phospho-IKK $\alpha$  $\beta$  Ab. As seen in Fig. 4B, unstimulated +/+ and -/- DCs showed little staining for phospho-IKK $\alpha$  $\beta$ , as expected. In most +/+ DCs, LPS and CpG dramatically increased phospho-IKK $\alpha$  $\beta$  staining, which was detected as small discrete dots over the cytoplasm and nucleus. In -/- DCs, LPS induced similar, intense dot-like phospho-IKK $\alpha$  $\beta$  staining. In contrast, little staining over background was observed following CpG stimulation. The paucity of phospho-IKK $\alpha$  $\beta$  staining in -/- DCs was confirmed by inspection of samples from three separate experiments. These results strengthen immunoblot data in Fig. 4A, and show that IRF-8<sup>-/-</sup> DCs are defective in the activation of IKK $\alpha$  $\beta$  in response to CpG.

Several lines of evidence indicate the existence of cross talk between the NF- $\kappa$ B pathway and the MAPK pathway, including that involving p38 (39, 40, 43). Because CpG is known to activate p38 that contributes to cytokine induction (44), we assessed the status of p38 activation in IRF-8<sup>-/-</sup> DCs after CpG stimulation. IRF-8<sup>+/+</sup> and IRF-8<sup>-/-</sup> DCs were stimulated with LPS or CpG,



**FIGURE 3.** The lack of NF- $\kappa$ B nuclear translocation in IRF-8<sup>-/-</sup> DCs after CpG stimulation. *A*, DCs stimulated with LPS or CpG for 6 h were fixed, permeabilized with methanol, stained with anti-p65/RelA Ab, followed by biotinylated second Ab and Cy2-conjugated third Ab (green), and counterstained for DNA (blue). *B*, Cells were fixed, permeabilized with Triton X-100, and stained with anti-c-Rel or RelB Ab, as above.



**FIGURE 4.** Failure of CpG to phosphorylate IKK $\alpha\beta$  and p38 in IRF-8 $^{-/-}$  DCs. **A**, BMDCs were stimulated with LPS or CpG for indicated time, and phosphorylated IKK $\alpha\beta$  (top row), unphosphorylated IKK  $\alpha$  and  $\beta$  (second and third rows), and  $\alpha$ -tubulin (bottom) were detected by immunoblot. **B**, BMDCs stimulated with LPS or CpG for 45 min were processed as in Fig. 3A and stained for phospho-IKK $\alpha\beta$ , as above. **C**, BMDCs were stimulated with LPS or CpG, fixed, permeabilized, stained with anti-phospho-p38 Ab, followed by FITC-conjugated second Ab, and analyzed by flow cytometry. The gray pattern represents background fluorescence.

immunostained for phospho-p38, and analyzed by flow cytometry (Fig. 4C). Phospho-p38 was not detected before stimulation both in +/+ and -/- cells, but became detectable after LPS stimulation in both cells at comparable levels. Similarly, CpG led to the appearance of phospho-p38 in +/+ DCs with a profile similar to that by LPS. In contrast, phospho-p38 was not detectable in -/- DCs following CpG stimulation. Thus, in addition to the block in the IKK $\alpha\beta$  phosphorylation, CpG-induced p38 phosphorylation appears selectively blocked in IRF-8 $^{-/-}$  DCs.

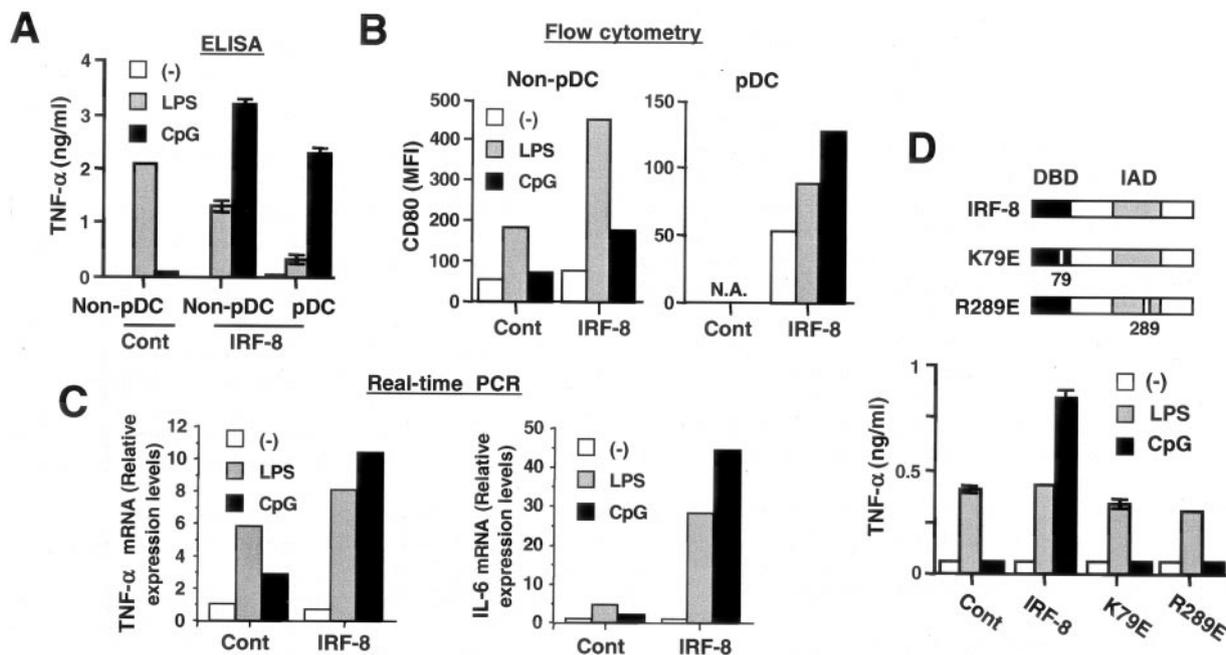
#### IRF-8 gene transfer restores CpG-induced cytokine expression

We next studied whether reintroduction of IRF-8 can rescue CpG responses in -/- DCs. It is possible that IRF-8 has only an indirect role in mounting CpG responses, for example, being required transiently during DC development. In such a case, artificial re-expression of IRF-8 may not reconstitute the pathway for CpG response. However, if IRF-8 has a direct role and acts as part of the CpG signaling pathway, IRF-8 re-expression might be sufficient to enable cells to respond to CpG. IRF-8 $^{-/-}$  BM progenitor cells were transduced with an IRF-8 vector and allowed to develop in vitro in the presence of Flt3L for 9 days. As expected, IRF-8 transduction led to the generation of both pDCs and non-pDCs, while control vector transduction produced only non-pDCs (32). pDCs and non-pDC were separately tested for TNF- $\alpha$  induction (Fig. 5A). Both non-pDCs and pDCs transduced with IRF-8 regained the ability to produce TNF- $\alpha$  in response to CpG, whereas cells transduced with control vector remained unresponsive to CpG and did not produce TNF- $\alpha$  at a detectable level. To substantiate the restoration of CpG response by IRF-8, real-time PCR analysis was performed to measure transcript levels for TNF- $\alpha$  and IL-6 (Fig. 5C). IRF-8 transduction led to induction of both transcripts after CpG stimulation, whereas control vector did not. In addition, IRF-8-transduced cells regained CpG induction of surface marker CD80 expression in both non-pDCs and pDCs (Fig. 5B).

Transcriptional activity of IRF-8 depends on the intact DNA binding domain (DBD) and the IRF association domain (IAD) required for interaction with partner proteins (25) (diagram in Fig. 5D, left). To validate the ability of IRF-8 to rescue CpG response, two IRF-8 mutants, K79E and R289E, were tested for restoration of CpG-induced cytokine expression. The mutants each had a point mutation in the DBD or IAD, respectively. Although the mutants were expressed in their nucleus at a level similar to the wild-type IRF-8 (33) (data not shown), only the wild-type IRF-8, but neither mutant rescued CpG induction of TNF- $\alpha$ . As expected, cells transduced with these mutants responded to LPS to induce the cytokine at comparable levels. These data indicate that both the intact DBD and IAD are required for rescuing CpG response.

#### IRF-8 restores CpG-mediated activation of NF- $\kappa$ B

It was important to ascertain whether the restoration of CpG response correlates with that of NF- $\kappa$ B activation. Real-time PCR analysis of I- $\kappa$ B $\alpha$  in Fig. 6A showed that when transduced with the wild-type IRF-8 vector, -/- cells regained the ability to induce I- $\kappa$ B $\alpha$  transcripts in response to CpG, but not when transduced with the mutants K79E, R289A, nor control vector, although all transduced cells induced I- $\kappa$ B $\alpha$  after LPS stimulation. These results indicate that IRF-8 confers the ability to activate NF- $\kappa$ B in response to CpG upon -/- cells. To confirm restoration of NF- $\kappa$ B activation following IRF-8 transduction, immunofluorescent staining was conducted to visualize NF- $\kappa$ B nuclear translocation (Fig. 6B). In control vector-transduced cells, the bulk of p65/RelA remained in the cytoplasm after CpG stimulation, as was found in untransduced cells. In IRF-8-transduced cells, by contrast, much of p65/RelA translocated to the nucleus after CpG in a manner similar to that seen after LPS. The same outcome was observed in virtually all cells in the fields and in separate preparations of samples. These results show that IRF-8 rescues CpG activation of NF- $\kappa$ B in -/- DCs.



**FIGURE 5.** Restoration of cytokine induction after IRF-8 transduction. *A*, IRF-8<sup>-/-</sup> BMDCs generated following transduction with control or IRF-8 vector were separated into pDCs and non-pDCs. They were then stimulated with LPS or CpG. Production of TNF- $\alpha$  in supernatants was measured by ELISA. *B*, Above cells were tested for expression of CD80 by flow cytometry. *C*, Real-time RT-PCR analysis of TNF- $\alpha$  and IL-6 transcript expression in  $-/-$  BMDCs transduced with control or IRF-8 vector. *D*, *Top*, Diagram of IRF-8 mutants. K79E and R289E carry a point mutation in the DBD and IAD, respectively, and are transcriptionally inactive. *Bottom*, TNF- $\alpha$  production in  $-/-$  BMDCs transduced with control, wild-type IRF-8 or mutants was analyzed by ELISA.

## Discussion

### Multiplicity of NF- $\kappa$ B activation pathways

In IRF-8<sup>-/-</sup> DCs, signaling by TLR9 and that by TLR4 displayed a remarkable disparity, in that CpG induction of TNF- $\alpha$  and IL-6 by CpG was completely eliminated, although induction by LPS was not impaired. In IRF-8<sup>-/-</sup> DCs, NF- $\kappa$ B activation did not take place following CpG stimulation. This was striking, as it is generally thought that all TLR signaling converges on a common event that leads to NF- $\kappa$ B activation (11, 12). The dichotomy of CpG and LPS responses observed with IRF-8<sup>-/-</sup> cells indicates that the pathway that leads to NF- $\kappa$ B activation in DCs is not uniform and involves multiple mechanisms, and that IRF-8 contributes to a previously unappreciated pathway necessary for NF- $\kappa$ B activation by TLR9. The absence of CpG responses in IRF-8<sup>-/-</sup> DCs raises a question of whether signaling by other TLRs is likewise impaired in  $-/-$  DCs. In this regard, we found that cytokine induction by imidazoquinoline, and to a lesser degree, poly(I/C), the ligand for TLR7 and TLR3, respectively, was compromised in IRF-8<sup>-/-</sup> DCs, although the induction was not completely abolished (data not shown), suggesting that IRF-8 plays a role in signaling by other TLRs as well.

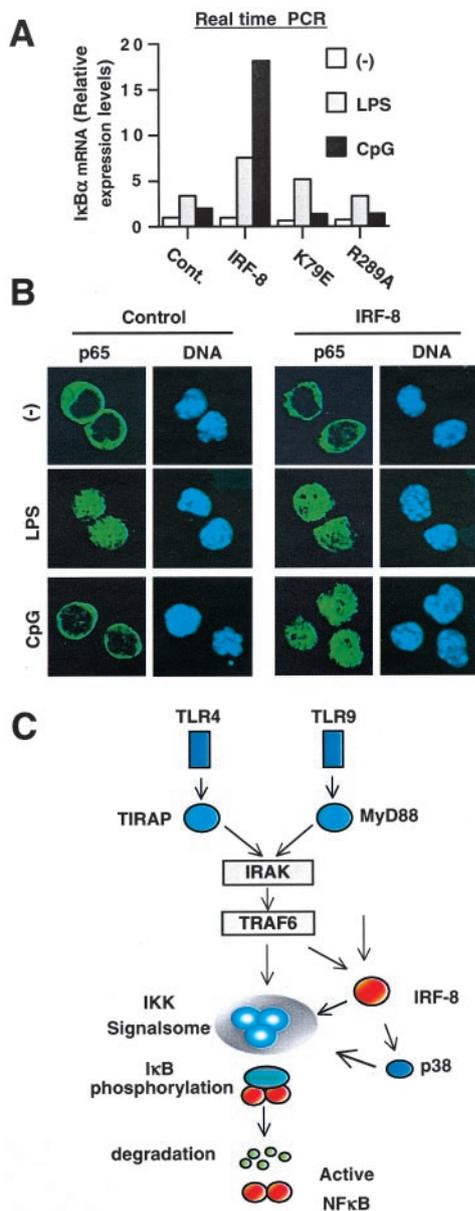
### The site of IRF-8 action

In light of the fact that TLR9 and signaling molecules were expressed in IRF-8<sup>-/-</sup> DCs, and LPS signaling was intact in IRF-8<sup>-/-</sup> DCs, it is unlikely that the defect seen in CpG response is due to the general absence/failure of signal transducer activities in  $-/-$  DCs. Furthermore, given that IRF-8 predominantly resides in the nucleus, moving only temporarily to the cytoplasm if at all, it seems unlikely that IRF-8 influences TLR signaling occurring in the cytoplasm, including recruitment of IRAK and activation of TRAF6, events before IKK activation (11, 12, 45). A plausible possibility may be that IRF-8 acts downstream of TRAF6, at a step

leading to IKK phosphorylation (21, 42). Recent evidence indicates that IKK action is complex and involves events in the nucleus and nuclear-cytoplasmic shuttling (23, 24, 46), supporting potential physical proximity between IRF-8 and IKK or other nuclear proteins that affect IKK activity. IKK $\alpha$  and IKK $\beta$  both carry kinase domains, and are themselves phosphorylated upon activation. Previously, it was shown that phosphorylation of IKK $\beta$ , but not IKK $\alpha$ , is important for I- $\kappa$ B degradation and NF- $\kappa$ B activation (20). Recent reports indicate that IKK $\alpha$  also plays a role in NF- $\kappa$ B-activated gene expression by phosphorylating histone H3, thereby increasing accessibility of NF- $\kappa$ B to the promoters (23, 24). IKKs are shown to form a large multiprotein complex called the IKK signalsome (12, 47). The signalsome complex may be heterogeneous in composition and activity. Given that relatively little is known about the individual components of IKK signalsomes and the pathways that target the IKK complex (20), it might be envisaged that IRF-8 acts within or in the vicinity of the IKK signalsomes to contribute to their activation process (a model, Fig. 6C). We noted that IKK $\alpha$  and IKK $\beta$  levels were lower in  $-/-$  DCs than  $+/+$  cells before and following stimulation, indicating that IRF-8 not only contributes to IKK $\alpha$  $\beta$  phosphorylation, but regulates their expression levels as well. However, considering that LPS can fully activate NF- $\kappa$ B in IRF-8<sup>-/-</sup> DCs, the reduced IKK $\alpha$  $\beta$  expression alone does not explain the total absence of NF- $\kappa$ B activation by CpG in  $-/-$  DCs.

### Cross talk with MAPK p38

p38 activation is an important part of CpG signaling (44). A series of evidence supports cross talk between NF- $\kappa$ B and p38. In myocytes, IKK $\beta$  is activated by p38 (39). Furthermore, Akt and IL-1 $\beta$  are shown to activate p38 in an IKK-dependent manner, and the activated p38 stimulates NF- $\kappa$ B by a mechanism involving histone



**FIGURE 6.** Restoration of CpG induced NF- $\kappa$ B activation by IRF-8 re-expression. **A**, IRF-8<sup>-/-</sup> DCs were transduced with indicated vector, and I- $\kappa$ B $\alpha$  mRNA expression was analyzed by real-time PCR. **B**, -/- DCs transduced with control or IRF-8 vector were stained for p65/RelA (green) and Hoechst (blue). **C**, A model for IRF-8 action. IRF-8 is required for TLR9-mediated activation/phosphorylation of IKK $\alpha\beta$  and p38 as well as the ensuing NF- $\kappa$ B activation. However, TLR4 signaling does not require this pathway, and can activate NF- $\kappa$ B in the absence of IRF-8. The model illustrates the plurality of NF- $\kappa$ B activation mechanisms in DCs.

acetylase p300/CREB-binding protein (43). In addition, it is reported that p38 is involved in the phosphorylation of histone H3 at Ser<sup>10</sup>, enhancing the activity of subsets of NF- $\kappa$ B target promoters (40). The present work indicates that CpG regulation of p38 phosphorylation is under control of IRF-8. In view of this evidence, IRF-8 may regulate not only NF- $\kappa$ B activation, but p38-NF- $\kappa$ B cross talk, influencing the functionality of NF- $\kappa$ B. Whether p38 is directly involved in CpG-dependent phosphorylation of IKK $\alpha\beta$  in DCs is not known at present.

### Role for IRF-8 conferring signal-dependent, cell type-specific mechanisms of NF- $\kappa$ B activation upon DCs

It should be borne in mind that IRF-8 is an immune system-specific transcription factor expressed mostly in APCs, such as DCs, macrophages, and B cells (25). Thus, IRF-8-dependent responses to CpG are expected to be operational only in these cell types, but not in other cell types, including fibroblasts, the cell type extensively used for studying NF- $\kappa$ B activation. It has been shown that transfection of human or murine TLR9 allows CpG to activate NF- $\kappa$ B in HEK 293 cells, in which IRF-8 expression is absent or low (8–10). It is of note that, in these studies, NF- $\kappa$ B activation was measured mostly with transiently transfected NF- $\kappa$ B reporters, and CpG did not cause induction of many endogenous target genes. Nevertheless, in these artificial systems, NF- $\kappa$ B activation most likely took place in the absence of IRF-8. These reports and our observation in this work suggest that in DCs, NF- $\kappa$ B activation follows more complex, multifaceted processes, in which factors not expressed in fibroblasts are involved. The complexity of the NF- $\kappa$ B activation processes may provide immune cells with a mechanism by which to afford cell type- and signal-specific activation of many NF- $\kappa$ B target genes. This complexity may also be important for the modulation of the levels and kinetics of target gene activation. The role for IRF-8 in NF- $\kappa$ B activation may, therefore, be to ensure fine tuning of NF- $\kappa$ B activation in DCs necessary for establishing proper innate and adaptive immunity.

### Acknowledgment

We thank T. Kaisho (Osaka University, Osaka, Japan) for advice on DC stimulation.

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