c–Rel Regulates Interleukin 12 p70 Expression in CD8+ Dendritic Cells by Specifically Inducing *p35* Gene Transcription

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Abstract

Interleukin 12 (IL-12) is a 70-kD proinflammatory cytokine produced by antigen presenting cells that is essential for the induction of T helper type 1 development. It comprises 35-kD (p35) and 40-kD (p40) polypeptides encoded by separate genes that are induced by a range of stimuli that include lipopolysaccharide (LPS), DNA, and CD40 ligand. To date, the regulation of IL-12 expression at the transcriptional level has mainly been examined in macrophages and restricted almost exclusively to the *p40* gene. Here we show that in CD8+ dendritic cells, major producers of IL-12 p70, the Rel/nuclear factor (NF)-κB signaling pathway is necessary for the induction of IL-12 in response to microbial stimuli. In contrast to macrophages which require c–Rel for *p40* transcription, in CD8+ dendritic cells, the induced expression of *p35* rather than *p40* by inactivated *Staphylococcus aureus*, DNA, or LPS is c–Rel dependent and regulated directly by c–Rel complexes binding to the *p35* promoter. This data establishes the IL-12 p35 gene as a new target of c–Rel and shows that the regulation of IL-12 p70 expression at the transcriptional level by Rel/NF-κB is controlled through both the p35 and p40 genes in a cell type–specific fashion.

Key words: c–rel • IL-12 • dendritic cells • p35 gene • transcription

Introduction

IL-12 is a disulfide-linked 70-kD heterodimer composed of 35-kD (p35) and 40-kD (p40) subunits, each of which is encoded by a distinct gene (1). IL-12 p70 is important in the immune response to microorganisms and tumors, activating NK cells and T lymphocytes, which in turn initiates IFN-γ production and antigen-specific Th1 responses (1–3). Its role in promoting inflammation has also led to IL-12 being implicated in immunopathology associated with allergy and autoimmune diseases (1).

Although many different APCs produce IL-12 (1), dendritic cells (DCs)* have emerged as major producers of this cytokine both in culture and in vivo in response to stimuli of a microbial origin, or a T cell–derived signal such as CD40 ligand (4–7). The regulation of IL-12 production is complex, with evidence for the control of p35 and p40 expression at the transcriptional, posttranscriptional, and posttranslational levels (1). To date, the regulation of IL-12 expression has largely focused on p40. This is largely the result of early findings that showed the induction of IL-12 coincided with an upregulation of p40 mRNA levels, while *p35* gene expression was generally ubiquitous (1). Recent evidence, however, suggests that attributing the induced expression of IL-12 solely to an increase in p40 expression is an oversimplification. First, the level of p35 mRNA is rapidly elevated in monocytes and DCs by the same agents that induce p40 expression and IL-12 production (8, 9). Second, p40 is frequently produced in excess as a monomer or a homodimer (p40)2 (1, 10, 11). As p40 homodimers bind to the IL-12 receptor and antagonize IL-12–mediated immune function (12), the biological activity of IL-12 may be determined in part by the ratio of p70 to (p40)2, which in turn would indicate that p35 levels are

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Abbreviations used in this paper: DC, dendritic cell; EMSA, electrophoretic mobility shift assay; NF, nuclear factor; SAC, *Staphylococcus aureus*.

likely to be an important limiting factor in determining IL-12 production. This later point is consistent with the capacity of IL-4 to simultaneously increase p35 mRNA and IL-12 levels in DCs in response to microbial stimuli, while downregulating p40 mRNA and protein expression (13).

Among those transcription factors implicated in the control of IL-12 gene expression, Rel/nuclear factor (NF)-κB proteins, in particular c-Rel has been shown to be important for LPS-induced p40 transcription in macrophages (14, 15). Rel/NF-κB transcription factors are homodimeric and heterodimeric proteins composed of related polypeptides that are encoded by a multigene family (16). The mammalian subunits (c-Rel, RelA, RelB, NF-κB1, and NF-κB2) share a conserved NH$_2$-terminal domain that encompasses sequences required for DNA binding, dimerization, and nuclear localization (16). c-Rel, RelA, and RelB each possess COOH-terminal transcriptional transactivation domains. In contrast, the proteolytically processed 50- and 52-kD forms of NF-κB1 and NF-κB2, respectively, lack intrinsic transactivating properties and instead function as homodimeric repressors or modulators of the transactivating dimer partners (16). In most cells, Rel/NF-κB factors are retained in the cytoplasm as an inactive complex with inhibitor or IκB proteins (17). Diverse signals induce the nuclear translocation of Rel/NF-κB by activating an IκB kinase complex that phosphorylates the IκB proteins, targeting them for ubiquitin-dependent proteosome-mediated degradation (17, 18). Upon translocation to the nucleus, Rel/NF-κB proteins regulate gene expression by binding to specific sequences (κB elements) located within the transcriptional regulatory regions of cellular genes, particularly those encoding proteins involved in immune, acute phase, and inflammatory responses (16).

Although Rel/NF-κB regulation of IL-12 expression has been studied most extensively in macrophages and monocytic cell lines (1, 8, 14, 15, 19), the critical function DCs serve in initiating antigen–dependent T cell responses (2–6), prompted us to examine what role the Rel/NF-κB pathway has in controlling IL-12 expression in these APCs. Here we show using mice that lack various individual members of the Rel/NF-κB family, that in CD8$^+$ splenic DCs the rapid upregulation of IL-12 in response to microbial stimuli is dramatically reduced in the absence of c-Rel. In contrast to previous findings for *c-rel*$^{-/-}$ macrophages that show an inability to upregulate IL-12 coincided with a failure to induce p40 transcription (15), in CD8$^+$ DCs this defect is primarily due to impaired *p35* gene expression. Consistent with this finding, we also establish that the *p35* gene is a direct transcriptional target of c-Rel.

Materials and Methods

Mice. C57BL/6 mice were bred under specific pathogen free conditions in the animal facility of The Walter and Eliza Hall Institute. The generation of *c-rel*$^{-/-}$ (20), *nfkb1*$^{-/-}$ (21), and *rela*$^{-/-}$ mice (22) has been described previously. All mutant mouse strains have been backcrossed for eight or more generations with C57BL/6 mice.

Cytokines, Antibodies, and Reagents. Murine rGM-CSF and rIL-4 were gifts from Immunex Corp. Rat rIFN-γ (bioactive in mouse) was purchased from PeproTech. Murine rIL-12 p70 and murine rIL-12 (p40)$_2$ were purchased from R&D Systems. Flt-3 ligand (Flt-3L) was produced from the CHO-flk2 cell line provided by N. Nicola (The Walter and Eliza Hall Institute). Pansorbin (fixed and heat killed *Staphylococcus aureus* [SAC]) was purchased from Calbiochem-Novabiochem, and lipopolysaccharide was obtained from Difco. An oligonucleotide containing a CpG motif (CpG) was synthesized by GeneWorks according to a published sequence (CpG1668). The fluorescence-conjugated antibody used for selecting DC was FITC-conjugated anti-CD11c (N418). Monoclonal antibodies were purified and labeled as published elsewhere (23, 24).

Mouse DC Preparation. DCs were extracted from mouse spleens as described (23). In brief, organs were chopped, digested with collagenase, treated with EDTA, and light density cells collected by density centrifugation. Non-DC lineage cells were depleted by coating them with a mixture of monoclonal antibodies and then removing the coated cells with magnetic beads coupled to anti-rat-IgG (24). The DC enriched preparations were then stained with an anti–CD11c-FITC conjugated mAb. Propidium iodide (PI) was added in the final wash to label dead cells. For cytometric sorting, the cells were gated for DC characteristics, namely high forward and side scatter plus bright staining for CD11c, with PI-labeled cells excluded. The purity of the sorted DCs was typically >98%.

Stimulation of DCs in Culture for IL-12 Production. Sorted splenic mouse DCs (10^5) were cultured in 96-well round bottom plates in a final volume of 200 μl with an IL-12 stimulus (250 nM CpG DNA, 5 to 20 μg/ml of SAC, or 1 μg/ml of LPS) in the presence of IL-4 (100 U/ml or titrated), GM-CSF (200 U/ml or titrated), and IFN-γ (20 ng/ml or titrated). After 18 h of culture, the supernatant was collected, separated from cells by centrifugation, and stored at −70°C before analysis.

IL-12 Polypeptide Analysis by Western Blotting. Aliquots of DC culture supernatants were subjected to SDS-PAGE (9% acrylamide) under non-reducing conditions. The electrophoresed proteins were transferred onto Immobilon-P membrane (Millipore) according to the manufacturer's instructions. Membranes were blocked with 5% BSA in PBS overnight at 4°C. IL-12 polypeptides were detected by incubation with biotinylated C17.8 (anti–IL-12 p40) monoclonal antibody (0.5 μg/ml in 1% BSA, 0.05% Tween 20 in PBS) for 1 h at 4°C, followed by incubation with streptavidin-horseradish peroxidase conjugate (Amersham LifeScience) dilution in 1% BSA, 0.05% Tween 20 in PBS for 1 h at 4°C. Membranes were then developed with Supersignal West Pico Chemiluminescent Substrate (Pierce Chemical Co.), according to the manufacturer's instructions.

Analysis of Mouse IL-12 by ELISA. Aliquots of DC culture supernatants were assayed by two site ELISA. In brief, 96-well polyvinyl chloride microtiter plates (Dynatech Laboratories) were coated with the appropriate purified capture mAb, namely R2-9A5 (anti–mouse IL-12 p70; American Type Culture Collection) or C15.6 (anti–mouse IL-12 p40; BD PharMingen). Cytokine binding was then detected with an appropriate biotinylated detection mAb, namely R1-5D9 (anti–mouse IL-12 p40, American Type Culture Collection) or C17.8 (anti–mouse IL-12 p40, hybridoma provided by L. Schofield, The Walter and Eliza Hall Institute). The readout was obtained using streptavidin-horseradish peroxidase conjugate (Amersham Pharmacia Biotech) and a substrate solution containing 548 μg/ml ABTS (2,2′-Azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid)) (Sigma-Aldrich) and

0.001% hydrogen peroxide (Ajax Chemicals) in 0.1 M citric acid, pH 4.2, which was scanned at an optical density of 405–490 nm. As the mouse IL-12 p40 ELISA also detects mouse IL-12 p70, p40 and (p40)2 levels were determined by subtracting the amount of mouse IL-12 p70 obtained with the mouse IL-12 p70 ELISA from values obtained with the mouse IL-12 p40 ELISA.

Northern Analysis of IL-12 p40 and p35 mRNA Expression. For Northern blot analysis, groups of three mice were treated with Flt3-L (10 μg/day for 10 d) and the splenic DCs then isolated as described above. The administration of Flt3-L to C57BL/6 mice greatly enhances the number of splenic DCs (by >30-fold) without altering their IL-12 expression (25). Cultures of these DCs (13 × 10⁶ DCs/5 ml culture) were stimulated for 4 h with DNA (0.5 μM) or LPS (1 μg/ml) in the presence of IFN-γ (20 ng/ml) and IL-4 (50 U/ml). Total RNA isolated from approximately 2 × 10⁶ DCs using RNAagents (Promega) was fractionated on 1% formaldehyde-agarose gels and transferred onto Hybond-C membranes (Amersham LifeScience). Filters were baked, prehybridized in 50% formamide, 5× SCC, 0.02% Ficoll, 0.02% polyvinylpyrrolidine, 0.02% albumin, 500 μg/ml of denatured herring sperm DNA, and hybridized for 18 h at 42°C with radiolabeled probes at a concentration of 2 × 10⁶ cpm/ml. Filters were washed in 0.2× SCC, 0.1% SDS at 65°C and exposed to autoradiography at −70°C. For successive hybridizations, filters were first boiled in 10 mM EDTA, 0.1% SDS to remove bound probe. The probes used were a 1.0-kb EcoRI-HindIII murine p40 cDNA (13), a 0.66-kb EcoRI-KpnI fragment derived by PCR from the murine p35 cDNA (13), and a 1.1-kb PstI rat glyceraldehyde-3-phosphatedehydrogenase cDNA (26) insert. Probes were radiolabeled by random primer extension with [α-³²P]dATP to specific activities ranging between 5 × 10⁸ and 10⁹ cpm/μg.

Genomic Clones and Plasmid Constructs. A 62-bp genomic clone encompassing nucleotides −425 to +196 of the murine IL-12 *p35* gene (27) was isolated by PCR from C57BL6 liver genomic DNA and characterized by automated sequencing. This fragment was inserted into the promoterless reporter plasmid pA3luc (28) and designated p35κB-luc. The plasmid p35κB_m-luc is a derivative of p35κB-luc in which the Rel/NF-κB binding site (5′-GGGAATCCC-3′) at −63 to −54 (27) was altered by in vitro mutagenesis (29).

Transfections and Luciferase Assays. The J774 and A7L.13 cell line (30) were transiently transfected using Superfect (QIAGEN) as described previously (31). Equimolar amounts (1–2 μg) of the *p35* promoter reporter plasmids were transfected alone or with a threefold molar excess (10–12 μg) of the expression plasmids pDAMP56 or pDAMP56c-rel (32). Approximately 48 h later, transfected cells were harvested and luiferase assays performed on cell extracts that had been standardized for equivalent protein content. Transfections were performed five times, with a maximum variance of approximately 15% observed between replicate experiments.

Electrophoretic Mobility Shift Assays. The p35κB probe was prepared by end-labeling the double stranded oligonucleotide 5′-GTTACCCCACT<u>GGGAATCCC</u>TTCAGCCACC-3′ and electrophoretic mobility shift assay (EMSA) reactions performed with 1–2 μg of nuclear extract as described previously (33). For competition analysis, a 50-fold excess of unlabeled p35κB or p35κB_m (5′-GTTACCCCACT<u>GTCAATAAC</u>TTCAGCCA-CC-3′) competitor DNA was added to the reaction at room temperature for 15 min before addition of the radiolabeled probe. For supershift analysis, antibodies that specifically recognize NF-κB1 (sc1192; Santa Cruz Biotechnology, Inc.), c-Rel (33), RelA

(sc109), RelB (sc226), or NF-κB2 (sc298) were incubated with nuclear extracts on ice for 30 min. before adding radiolabeled probe. All EMSA reactions were incubated for 20 min at room temperature, 2 μl of Ficoll dye was added, and the reactions were fractionated on 5% nondenaturing polyacrylamide gels. Gels were then dried and exposed to autoradiography at −70°C.

Semiquantitative Reverse Transcription PCR. Total RNA was isolated using RNAgents (Promega) from the cell line A7L.13 (10⁶ cells) that were untreated or stimulated for 4 h with LPS (1 μg/ml) or CpG DNA (250 nM). cDNA synthesis on equivalent amounts of total RNA was performed essentially as described (31). For semiquantitative PCR, cDNA was added to a cocktail comprising 50 mM KCl, 2 mM MgCl₂, 10 mM Tris-HCl, pH 8.3, 0.01% (wt/vol) gelatin, 0.5 mM dNTPs, 1 U of Taq polymerase, and 1 μM of each oligonucleotide in a final volume of 50 μl. After an initial 5-min denaturation at 94°C, the cDNA was amplified for 25 cycles with each cycle programmed for denaturation at 94°C for 45 s, annealing at 58°C for 60 s, followed by elongation at 72°C for 90 s. Samples were then fractionated on a 1% agarose gel. The sequence of the oligonucleotides used for the amplification of murine *p35*, *p40*, and β-actin mRNA were: p35 (forward, ATGATGACCCTGTGCCTTGG; reverse, CCT-TTGGGGAGATGAGATGT; product size 448 bp), p40 (forward, AACCTCACCTGTACACGCC; reverse CAAGTC-CATGTTTCTTTGCACG; product size 309 bp), and β-actin (forward, CTGAAGTACCCATTGAACATGGC; reverse, CA-GAGCAGTAATCTCCTTCTGCAT; product size 726 bp).

Results

c-Rel Is Essential for Induced Expression of IL-12 by CD8⁺ DCs. To determine what role Rel/NF-κB transcription factors serve in regulating IL-12 expression by activated DCs, we used mutant mice that lacked either c-Rel, NF-κB1, or RelA. Equivalent numbers of purified splenic CD11c⁺ DCs isolated from *c-rel⁻/⁻* and *nfkb1⁻/⁻* mice, or lethally irradiated C57BL/6Ly5.1⁺ recipients engrafted with E13 *rela⁻/⁻* fetal liver hemopoietic progenitors were stimulated in culture for 18 h with SAC or unmethylated DNA containing CpG motifs in the presence of a cytokine cocktail (IL-4, IFN-γ, and GM-CSF) previously shown to enhance IL-12 expression (13). Levels of p40, (p40)₂, and IL-12 p70 secreted into culture supernatants were then measured by ELISA using anti–p40– and anti-p70–specific antibodies (Fig. 1). While the amount of IL-12 (Fig. 1 A) produced by activated *rela⁻/⁻* (Fig. 1 A, lanes 7 and 11) and *nfkb1⁻/⁻* (Fig. 1 A, lanes 8 and 12) DCs was only two and fourfold lower respectively than that secreted by wild-type cells (Fig. 1 A, lanes 5 and 9), IL-12 levels were reduced 30- to 50-fold in the *c-rel⁻/⁻* DC cultures (Fig. 1 A, lanes 6 and 10). As this reduction in IL-12 was observed for DNA and SAC stimulated *c-rel⁻/⁻* DCs, with the relative levels of IL-12 secreted by wild-type and *c-rel⁻/⁻* cells in the absence of cytokines remaining unchanged (results not shown), this indicated the defect was not associated with a specific microbial stimulus nor was it due to impaired cytokine responsiveness. In contrast to p70 expression, the combined level of p40 and (p40)₂ produced by wild-type and *c-rel⁻/⁻* DCs (Fig. 1 B) were equivalent upon SAC activation (Fig. 1 B, lanes 9 and 10) and 50% higher in the *c-rel⁻/⁻* cultures
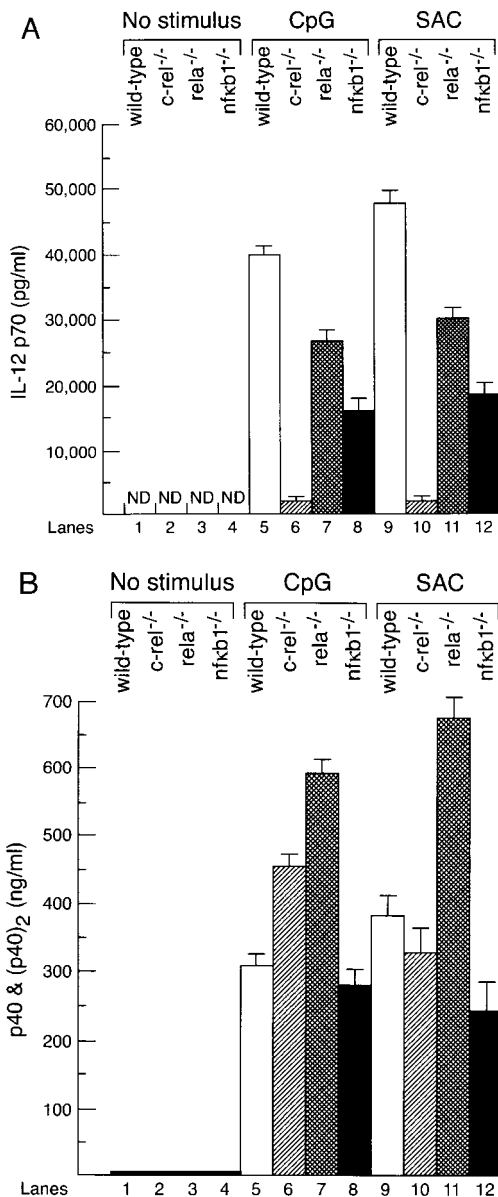
Figure 1. Production of IL-12 p70 but not p40 or (p40)$_2$ is markedly reduced in *c-rel*$^{-/-}$ splenic DCs. (A) IL-12 production by splenic DCs isolated from Rel/NF-κB mutant mice. Equivalent numbers of mouse splenic CD11c$^+$ DCs isolated from wild-type (lanes 1, 5, and 9), *c-rel*$^{-/-}$ (lanes 2, 6, and 10), and *nfkb1*$^{-/-}$ (lanes 4, 8, and 12) mice or lethally irradiated C57BL6Ly5.1$^+$ mice reconstituted with E13 *rela*$^{-/-}$ fetal liver cells (lanes 3, 7, and 11) were cultured in the absence of stimuli (lanes 1–4) or with either DNA containing CpG motifs (lanes 5–8) or inactivated SAC (lanes 9–12) in the presence of optimal concentrations of GM-CSF, IFN-γ, and IL-4 for 18 h. Supernatants were then assayed for IL-12 p70 content (pg/ml) using a p70-specific ELISA. The error bars represent the mean ± SD within a typical experiment, which was repeated four times with similar results. ND, refers to "not detectable" below a level of sensitivity of <15 pg/ml. (B) p40 and (p40)$_2$ production by activated CD11c$^+$ splenic DCs lacking different Rel/NF-κB proteins. p40 and (p40)$_2$ levels in culture supernatants described in panel A, were analyzed by p40-specific ELISA. The combined amount of p40 and (p40)$_2$ was determined by subtracting the IL-12 p70 reading obtained with the p70 ELISA from values obtained with the p40 ELISA. The combined level of p40 and (p40)$_2$ (ng/ml) is typical of four independent experiments. The error bars represent the variation within the experiment. The level of p40/(p40)$_2$ in unstimulated DCs (lanes 1–4) was ~5 ng/ml.

stimulated with DNA (Fig. 1 B, compare lanes 5 and 6). The combined p40 and (p40)$_2$ level was also consistently twofold higher than normal in *rela*$^{-/-}$ DC cultures (Fig. 1 B, lanes 5 and 7; 9 and 11).

Western blotting of culture supernatants fractionated on nonreducing gels and probed with anti–p40 antibodies (Fig. 2) confirmed the conclusions drawn from the ELISA assays. Both p40 and p40 homodimer levels secreted by *rela*$^{-/-}$ (Fig. 2, lane 3) and *c-rel*$^{-/-}$ (Fig. 2, lane 4) DCs were elevated slightly, but normal in *nfkb1*$^{-/-}$ cells (Fig. 2, lane 2), whereas IL-12 p70 levels were reduced in *nfkb1*$^{-/-}$ cultures (Fig. 2, lane 2) and barely detectable in *c-rel*$^{-/-}$ DC supernatants (Fig. 2, lane 4). The finding that p40 and (p40)$_2$ levels secreted by activated *c-rel*$^{-/-}$ DCs were similar to that produced by normal DCs indicated the reduction in IL-12 expression was not simply an indirect outcome of decreased *c-rel*$^{-/-}$ DC viability. This conclusion is consistent with our observation that CD11c$^+$ DC survival is normal in the absence of c-Rel (unpublished data).

As the CD8$^+$ population is the major source of IL-12 secreted by splenic CD11c$^+$ DCs (7), a reduction or absence of CD8$^+$ cells was one possible explanation for why IL-12 production by *c-rel*$^{-/-}$ splenic DCs was reduced. This was addressed by examining the expression of various surface markers on wild-type and *c-rel*$^{-/-}$ splenic DCs isolated from naive or FLT3 ligand treated mice. This data, summarized in Fig. 3, shows that the total number and phenotype of the CD8$^+$ DC populations in naive or FLT3 ligand treated wild-type and *c-rel*$^{-/-}$ mice were indistinguishable. p40, p70, and (p40)$_2$ production by stimulated FLT3 ligand treated wild-type and *c-rel*$^{-/-}$ DCs expressing intermediate (CD8^{int}) or high (CD8^{hi}) levels of CD8 were compared with the total CD11c$^+$ population by Western blot analysis (Fig. 4). As expected, IL-12 expression in normal splenic CD11c$^+$ DCs was accounted for by the CD8$^+$ population (Fig. 4, lanes 2–4). In the *c-rel*$^{-/-}$ CD8$^+$ DCs, IL-12 p70 was absent (Fig. 4, lanes 5 and 6), but p40 and (p40)$_2$ were present at normal levels (Fig. 4, compare lanes 2, 3, 5, and 6). Collectively these findings indicate that in CD8$^+$ DCs, c-Rel is critical for the expression of IL-12 p70, but not p40 or (p40)$_2$.

The comparatively normal induction of p40 and (p40)$_2$ expression in *c-rel*$^{-/-}$ DCs in response to SAC or DNA stimulation contrasted with recent reports showing c-Rel was essential for LPS-induced p40 expression in macrophages (15). To assess if this difference between *c-rel*$^{-/-}$
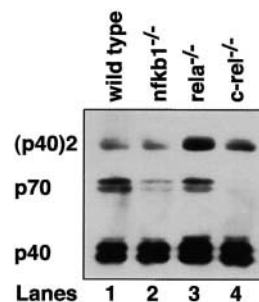

Figure 2. Western blotting of splenic DC supernatants. Supernatants from wild-type (lane 1), *nfkb1*$^{-/-}$ (lane 2), *rela*$^{-/-}$ (lane 3), and *c-rel*$^{-/-}$ (lane 4) CD11c$^+$ splenic DCs cultured for 18 h with SAC plus cytokines were subjected to nondenaturing electrophoresis and Western blotting using an anti–IL-12 p40-specific monoclonal antibody. p40, IL-12 p70, and (p40)$_2$ are indicated. This result is representative of that obtained from three independent experiments.
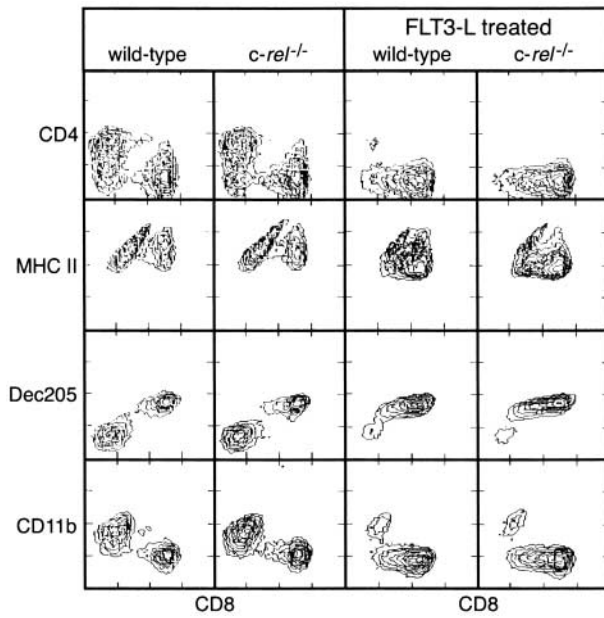
Figure 3. Splenic DC development in naive and FLT3 ligand treated *c-rel*^{−/−} mice is normal. Splenic DCs purified from untreated and FLT3 ligand treated wild-type or *c-rel*^{−/−} mice were stained with monoclonal antibodies specific for CD8 and CD4, MHC class II, DEC205, or CD11b and examined by flow cytometry. The data, displayed as contour plots, is representative of a typical experiment and was repeated three times.

APCs reflected cell type and/or stimulus specificity, p40/(p40)$_2$ and p70 IL-12 expression measured by ELISA was compared in wild-type and c-rel^{−/−} CD11c^{+} splenic DCs stimulated with LPS (Fig. 5). Consistent with previous reports, LPS is a comparatively weak inducer of p40/(p40)$_2$ and IL-12 in wild-type DCs (13), with cytokine levels being approximately 100-fold lower (Fig. 5, lanes 3 and 7) than that seen for SAC or DNA stimulated DCs (Fig. 1, A and B, lanes 5 and 9). Despite the weak induction of p40/(p40)$_2$ and p70 by LPS, the same pattern of expression was observed for LPS, SAC, and DNA stimulated *c-rel*^{−/−} DCs.
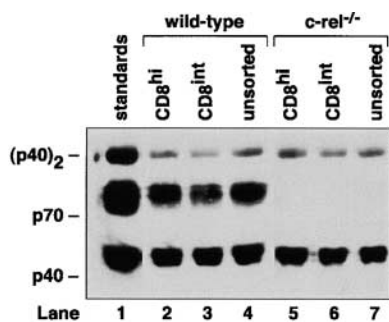

Figure 4. IL-12 production by purified CD8^{+} DCs. Supernatants harvested from equivalent numbers of wild-type (lanes 2–4) or *c-rel*^{−/−} (lanes 5–7) CD11c^{+} (lanes 4 and 7), CD8^{hi} (lanes 2 and 5), and CD8^{int} (lanes 3 and 6) splenic DCs stimulated in culture for 18 h, were fractionated on a non-denaturing acrylamide gel and proteins transferred to nitrocellulose membranes. p40, p70, and (p40)$_2$ were all detected using an anti-IL-12 p40 monoclonal antibody. Purified p40, p70, and (p40)$_2$ were used as standards (lane 1).
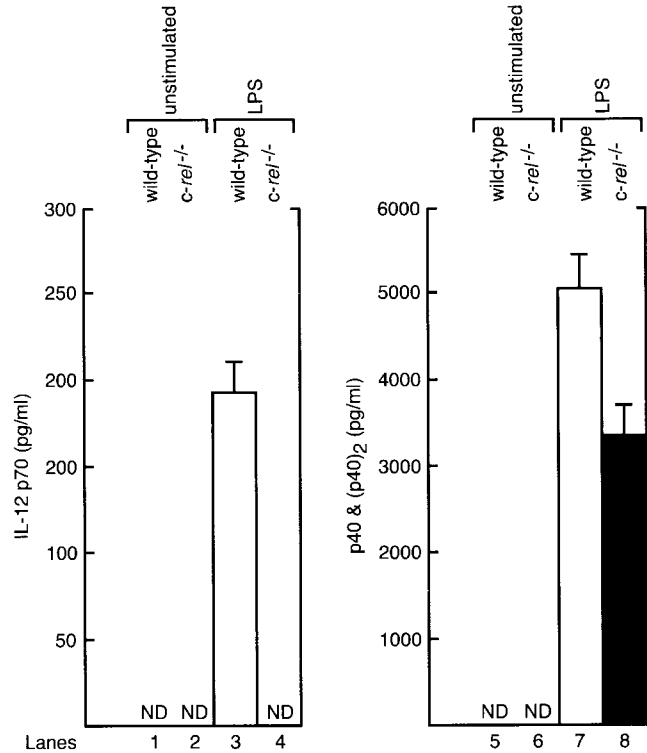

Figure 5. c-Rel is not essential for LPS induced p40 expression in CD8^{+} DCs. The level of IL-12 (lanes 1–4) and p40/(p40)$_2$ (lanes 5–8) production by wild-type (lanes 1, 3, 5, and 7) and c-rel^{−/−} (lanes 2, 4, 6, and 8) splenic DCs without activation (lanes 1, 2, 5, and 6) or upon stimulation with LPS for 18 h was assessed by ELISA. The error bars represent the mean ± SD within a typical experiment, which was repeated twice in triplicate with similar results. ND is an abbreviation for "not detectable," with the limit of sensitivity for the detection of IL-12 p70 at less than 15 pg/ml. p40/(p40)$_2$ levels in unstimulated cells were also below the limit of detection.

No IL-12 was detected in LPS stimulated *c-rel*^{−/−} DC cultures (Fig. 5, lane 4), whereas p40/(p40)$_2$ expression was evident, albeit reduced slightly (<twofold) compared with wild-type cells (Fig. 5, lanes 4 and 8). These findings indicate that the c-Rel–dependent regulation of p40/(p40)$_2$ expression appears to differ in macrophages and DCs.

In CD8^{+} DCs, c-Rel Is Required for the Induced Expression of p35 but Not p40. The reduced level of IL-12, but not p40 or (p40)$_2$ produced by c-rel^{−/−} CD8^{+} DC suggested that this defect may result from impaired p35 expression. This was assessed by comparing p35 and p40 gene expression in unstimulated and activated CD8^{+} DCs isolated from wild-type and Rel/NF-κB mutant mice. As IL-12 synthesized by activated CD8^{+} DCs can be detected within 2 h and reaches steady state levels within 10 h (13), RNA samples were taken 4 h after stimulation and subjected to Northern blot analysis (Fig. 6 A). Whereas p35 mRNA expression was strongly induced in wild-type cells upon activation (Fig. 6, lanes 1 and 2), it was barely upregulated in the stimulated c-rel^{−/−} DCs (Fig. 6, lanes 3 and 4). p35 expression was induced in rela^{−/−} (Fig. 6 A, lanes 5 and 6) and nfkb1^{−/−} (Fig. 6 A, lanes 7 and 8) DCs, albeit at
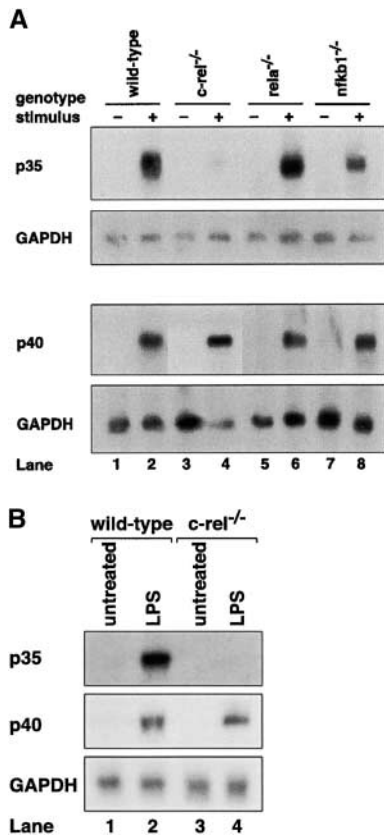
A

B

Figure 6. p35 but not p40 mRNA expression is markedly reduced in stimulated c-rel$^{-/-}$ CD8$^+$ DCs. (A) p35 and p40 mRNA expression in CD8$^+$ DCs from different Rel/NF-κB mutant mice. 5 μg samples of total RNA isolated from wild-type (lanes 1 and 2), c-rel$^{-/-}$ (lanes 3 and 4), rela$^{-/-}$ (lanes 5 and 6), or nfkb1$^{-/-}$ (lanes 7 and 8) CD8$^+$ splenic DCs that were unstimulated (lanes 1, 3, 5, and 7) or DNA treated (lanes 2, 4, 6, and 8) for 3 h, were analyzed by Northern blot hybridization. Filters were first hybridized with murine p35 or p40 cDNA probes, then rehybridized with a rat GAPDH probe. Filters were exposed to autoradiography for between 24 and 72 h. (B) LPS induced p35 and p40 mRNA expression in CD8$^+$ DCs. 5 μg samples of total RNA isolated from wild-type (lanes 1 and 2) and c-rel$^{-/-}$ (lanes 3 and 4) DCs that were untreated (lanes 1 and 3) or LPS stimulated (lanes 2 and 4) for 3 h, were analyzed by Northern blot hybridization as outlined in panel A. Filters were exposed to autoradiography for 24 h (GAPDH) and 72 h (p35 and p40).

somewhat reduced levels in cells lacking NF-κB1. In contrast, p40 mRNA levels were upregulated to the same extent in normal and c-rel$^{-/-}$ CD8$^+$ DCs (Fig. 6, lanes 2 and 4). A survey of p40 expression over an 18-h period confirmed that the level and induction kinetics of p40 expression were normal in activated c-rel$^{-/-}$ CD8$^+$ DC (data not shown). In the absence of RelA (Fig. 6 A, lanes 5 and 6) or NF-κB1 (Fig. 6 A, lanes 7 and 8), induced p40 mRNA expression also appeared normal. The LPS-induced expression of p35 and p40 mRNA was also compared in wild-type and c-rel$^{-/-}$ CD8$^+$ DCs (Fig. 6 B). While p35 mRNA levels were rapidly upregulated in response to LPS in wild-type DCs (Fig. 6, lane 2), it was undetectable in c-rel$^{-/-}$ cells (Fig. 6, lane 4). In contrast, LPS-induced p40 mRNA expression appeared normal in c-rel$^{-/-}$ DCs (Fig. 6, compare lanes 2 and 4). These findings indicate that the

impaired induction of IL-12 in activated c-rel$^{-/-}$ CD8$^+$ DCs results from an inability to upregulate p35 mRNA levels and that individually c-Rel, RelA, and NF-κB1 are all dispensable for SAC, LPS, or DNA induced p40 expression in CD8$^+$ DCs.

c-Rel Directly Induces Transcription of the Murine p35 Gene. To determine how c-Rel might regulate *p35* gene expression, we first examined the murine *p35* promoter. Although a putative κB element, 5'-GGGAATCCCT-3' was located 63 nucleotides upstream of the major transcription start site in the sequence determined by Tone et al. (27), this motif differed in the *p35* genomic sequence from an independent group (34). As both sequences were determined from C57BL6 mice, ruling out strain-specific polymorphisms, we decided to clone and sequence that region of the promoter encompassing the putative κB element and found it was identical to that reported by Tone et al. (27).

To establish if the κB-like motif was required for the c-Rel–dependent induction of p35 transcription, promoter reporter assays were performed. While macrophage cell lines such as J774 and W264 had been used successfully for p40 promoter studies (14, 15), the differences in c-Rel regulated p40 expression observed in macrophages and DCs prompted us to compare c-Rel–regulated p35 promoter function in both cell types. We chose to use J774 cells and ATL-13, a murine cell line with DC characteristics (30: unpublished results). The suitability of A7L.13 for this study was first assessed by examining endogenous p35 and p40 gene expression in response to LPS or DNA stimulation (Fig. 7 A). In the absence of stimuli, p35 and p40 mRNA levels in A7L.13 cells as measured by semiquantitative reverse transcription PCR were undetectable (Fig. 7 A, lanes 1 and 4), but were induced within 4 h by LPS (Fig. 7 A, lanes 2 and 5) or DNA (Fig. 7 A, lanes 3 and 6). As these findings showed p35 expression was upregulated in A7L.13 cells by c-Rel–dependent stimuli in a manner similar to that observed in primary CD8$^+$ DCs, this cell line was used for p35 promoter reporter transfections.

A region of the *p35* gene that encompassed the putative κB element, including the 5' untranslated region within exon 1 and extended 425 nucleotides upstream of the transcriptional start site (see Fig. 7 B) was inserted 5' of the luciferase gene in the promoterless reporter plasmid, pluc3 (28). This plasmid, designated p35κB-luc, was transiently transfected into J774 or A7L.13 cells in the absence or presence of an expression vector encoding c-Rel and the resultant data summarized in Fig. 7 C. The basal promoter activity (Fig. 7 C, lanes 3 and 9) of p35κB-luc was significantly higher than the parental vector (Fig. 7 C, lanes 1, 2, 7, and 8) in both cell lines and was further upregulated upon co-transfection with an expression vector for c-Rel (Fig. 7 C, lanes 4 and 10). The role of the putative κB site was assessed by mutating it to a version (5'-GT-CAATAACT-3') unable to bind Rel/NF-κB proteins. The plasmid, p35κBm-luc, which contained the mutant κB site within the context of the full length promoter, retained normal basal promoter activity in A7L.13 cells (Fig. 7 C, lane 11), but was reduced in J774 (Fig. 7 C, lane 5).
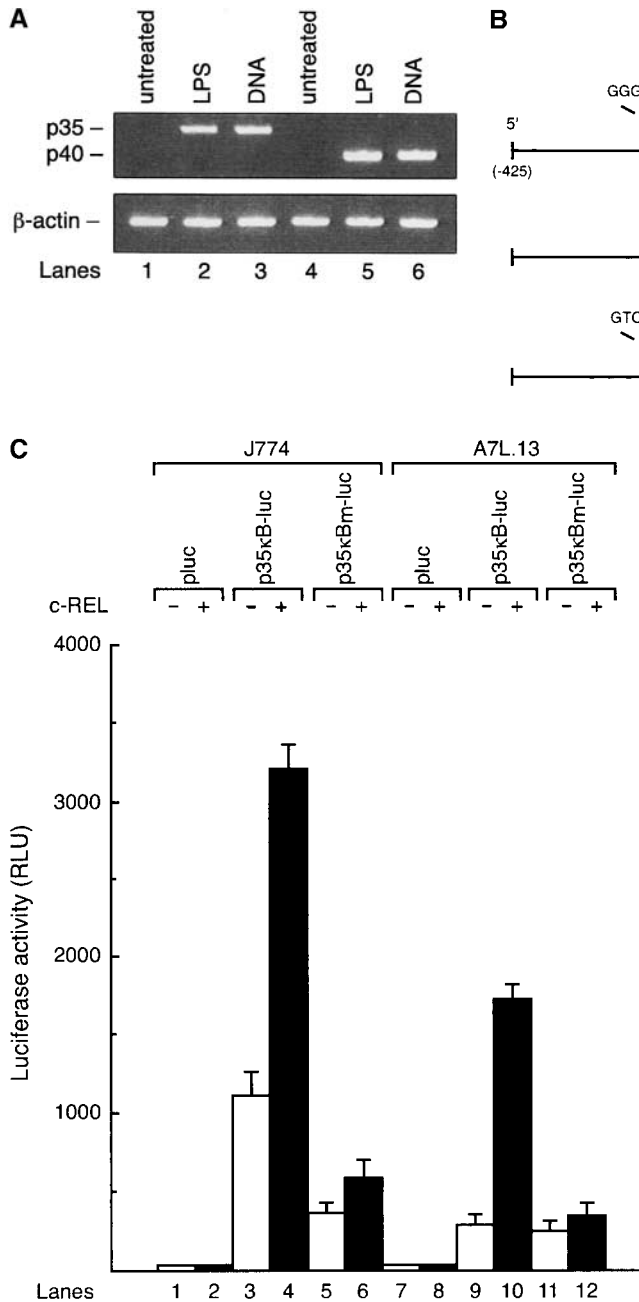
Figure 7. Functional analysis of the murine p35 promoter. (A) LPS and DNA induced expression of *p35* and *p40* in the mouse cell line A7L.13. Total RNA isolated from A7L.13 cells that were untreated (lanes 1 and 4) or stimulated with LPS (lanes 2 and 5) or DNA (lanes 3 and 6) for 4 h was subjected to semiquantitative reverse transcription PCR using *p35*, *p40*, and *β-actin*–specific primers. PCR products were fractionated on a 1.5% agarose gel. (B) Schematic representation of the p35 gene 5′ flanking region and luciferase reporter plasmids. The numbers in parenthesis were assigned according to the transcriptional start site as determined by Tone et al. (reference 27).The open box represents a putative Rel/NF-κB binding site (κB, 5′-GGGGAATCCCT-3′:−63 to −54), while the corresponding symbol with a cross represents the mutated version (κBm, 5′-GTCAATAACT-3′) of this element. The arrow at position 0 denotes the major transcriptional initiation site. E1 and E2 refer to exons 1 and 2 respectively of the murine p35 gene. The cross-hatched boxes represent 5′-untranslated sequence in exons 1 and 2, while the filled portion of exon 2 corresponds to coding sequence. The location of the p35 initiation codon in exon 2 is also shown. The open box designated "luc" refers to the firefly luciferase gene. Plasmid nomenclature is indicated to the right of each construct. (C) The κB motif in the murine p35 promoter is necessary for c-Rel–dependent transcription. The J774 and A7L.13 cell lines were each transiently transfected with 1–2 μg of the reporter plasmid pluc (lanes 1, 2, 7, and 8), p35κB-luc (lanes 3, 4, 9, and 10), or p35κBm-luc (lanes 5, 6, 11, and 12) plus an equimolar amount of (∼10–12 μg) of the expression plasmid DAMP56 with no insert (lanes 1, 3, 5, 7, 9, and 11) or c-Rel (lanes 2, 4, 6, 8, 10, and 12). The promoter activity measured in extracts of transfected cells is displayed as relative luciferase units. The results represent the mean luciferase activity ± SD obtained from five separate sets of transient transfections.

Moreover, p35κBm-luc activity was not upregulated by c-Rel (Fig. 7 C, lanes 6 and 12) in either cell line. The reduced κB-dependent basal promoter activity in J774 but not A7L.13 is consistent with constitutive nuclear Rel/NF-κB levels being higher in the J774 cells (results not shown). These results indicate that the κB element within the p35 promoter was necessary and sufficient for Rel/NF-κB–dependent transcription.

Nuclear c-Rel Complexes Induced in Activated CD8⁺ DCs Bind the κB Element in the p35 Promoter. The binding of c-Rel complexes to the p35κB site was examined using electrophoretic mobility shift assays (Fig. 8). Two major nuclear complexes in unstimulated wild-type (Fig. 8 A,

lane 1) and *c-rel*^{−/−} (Fig. 8 A, lane 3) CD8⁺ DCs, designated C1 and C2, bound a probe encompassing the p35κB motif. Within 2 h of DNA or SAC (results not shown) treatment, a novel nuclear complex (C3) present in wild-type (Fig. 8 A, lane 2) but not *c-rel*^{−/−} (Fig. 8 A, lane 4) CD8⁺ DCs, bound the probe, while C2 binding was reduced. To determine which complexes specifically bound to the κB site, a probe with the mutant κB motif (5′-GTCAATAACT-3′) was used. Whereas the mutant probe failed to bind the constitutive C1 (Fig. 8 A, lanes 5–8) and inducible C3 complexes seen in wild-type DCs (Fig. 8 A, lane 6), C2 binding was unaffected in cells of both genotypes (Fig. 8 A, lanes 5–8). These findings indi-

cated that only C1 and C3 bound the probe via the κB site.

The composition of C1 and C3 was examined by supershift analysis using antibodies specific for different Rel/NF-κB polypeptides (Fig. 8 B). The C3 complex was supershifted with antibodies specific for NF-κB1 (Fig. 8 B, lane 6) and c-Rel (Fig. 8 B, lane 7), but not RelA (Fig. 8 B, lane 8), NF-κB2, or Rel B (not shown), demonstrating that it mainly comprised a c-Rel/NF-κB1 heterodimer. Despite C1 binding being dependent on the p35κB element, the mobility of the complex was not typical of a Rel/NF-κB dimer, a conclusion supported by the inability of Rel/NF-κB–specific antibodies to inhibit its binding. This is consistent with our preliminary findings that indicate C1 is an HMG protein (unpublished data). Although C2 does not bind directly to the κB element, in some experiments C2 binding was reduced by preincubating with Rel/NF-κB–specific antisera. This result, however, was inconsistent between experiments (Fig. 8 B, compare lanes 6–8, 13–16). While the basis of this variability remains unclear, the predicted molecular weight of the C2 DNA binding protein as determined by cross-link-

Figure 8. Rel/NF-κB DNA binding activity in DCs. Nuclear extracts (1–2 μg) isolated from purified normal and c-$rel^{-/-}$ splenic CD8^{+} DCs that were unstimulated or activated in culture with DNA for 2 h were incubated with a ^{32}P-radiolabeled p35κB probe, resolved on 5% nondenaturing polyacrylamide gels, and exposed to autoradiography for 8–24 h at −70°C. (A) A nuclear complex induced in activated CD8^{+} DCs that binds the p35κB site is absent in c-$rel^{-/-}$ cells. Nuclear extracts from untreated (lanes 1, 3, 5, and 7) or activated (lanes 2, 4, 6, and 8) normal (lanes 1, 2, 5, and 6) and c-$rel^{-/-}$ (lanes 3, 4, 7, and 8) DCs were either incubated with a radiolabeled wild-type (lanes 1–4) or mutant (lanes 5–8) p35κB probe. C1 and C2 represent the fast and slower constitutive complexes respectively, while the inducible complex of slow mobility is designated C3. (B) The inducible C3 complex is a c-Rel/NF-κB1 heterodimer. Nuclear extracts from resting (lanes 1–4, 9–12) or activated (5–8, 13–16) wild-type (lanes 1–8) and c-$rel^{-/-}$ (lanes 9–16) DCs were incubated with preimmune (lanes 1, 5, 9, and 13), NF-κB1 (lanes 2, 6, 10, and 14), c-Rel (lanes 3, 7, 11, and 15), or RelA (lanes 4, 8, 12, and 16)-specific antisera before adding the radiolabeled p35 κB probe.

ing studies (unpublished data) indicates it is not a Rel/NF-κB family member.

Discussion

While a considerable amount of information has emerged on the control of IL-12 *p40* gene expression, little is known about the transcriptional regulation of the *p35* locus. Here we show the Rel/NF-κB signaling pathway is required for the induction of IL-12 in activated CD8^{+} splenic DCs and the impaired expression of IL-12, but not p40 or (p40)$_2$ in c-$rel^{-/-}$ CD8^{+} DCs is due to an inability to upregulate *p35* transcription which is c-Rel dependent.

Previous studies have established that c-Rel is not essential for lymphocyte or monocyte development; rather it is required for a variety of activation associated functions in mature cells from these lineages such proliferation and cytokine production (20, 35–37). The results presented here for DCs are consistent with these findings, namely the loss of c-Rel does not disrupt splenic DC development, but IL-12 expression by activated CD8^{+} DCs is impaired. Collectively, these data reinforce the notion that the indispensable roles served by c-Rel in the different hemopoietic lineages is strictly associated with the regulation of effector functions in mature cells. In contrast to c-Rel, an absence of RelB disrupts the differentiation of CD8^{+} myeloid DCs (38). The separate roles served by these different Rel/NF-κB proteins during the differentiation and function of DCs presumably reflects their regulation of distinct genes, a conclusion consistent with their different binding site specificity (39) and the inability of c-Rel and RelB to form heterodimers (40).

A direct association remains to be established between the impaired expression of IL-12 by c-$rel^{-/-}$ APCs and the immune defects displayed by c-$rel^{-/-}$ mice. This issue is complicated in part by the need to define what role particular APCs serve during immune responses. Whereas naive CD8^{+} DCs produce high amounts of IL-12 when activated (7, 13), macrophages isolated from unprimed mice produce very low amounts of IL-12 (19). Only in response to priming "in vivo" under inflammatory conditions or "in vitro" with GM-CSF, IFN-γ, and IL-4, are macrophages able to synthesize high levels of IL-12 (19, 41). Such findings may indicate that CD8^{+} DCs are the critical source of IL-12 during a primary immune response, while macrophages could be more important producers of IL-12 later in the response after adequate priming in the inflammatory milieu (2, 3, 42). Immune defects arising from the loss of c-Rel that might be explained in part by a reduction in IL-12 expression by APCs include the sensitivity of c-$rel^{-/-}$ mice to *Leishmania major* (36) and the resistance of these mutant mice to collagen-induced arthritis (43), a disease model in which IL-12 is known to be important (1).

The data outlined in this paper shows that c-Rel induces *p35* expression in CD8^{+} DCs activated by microbial agents. The rapid cRel-dependent induction of *p35* transcription triggered by LPS, SAC, or DNA is most likely
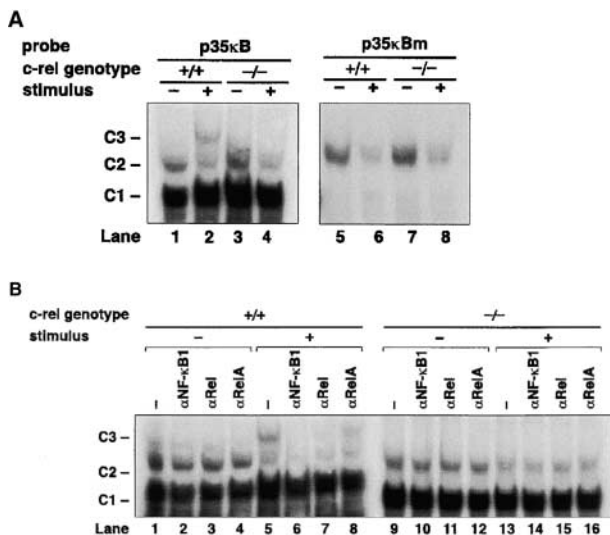
initiated through mammalian Toll-like receptors (TLRs), which represent an evolutionarily conserved component of immunity. For example, in *Drosophila*, innate immune responses to microbes are mediated by various antimicrobial peptides, the expression of which are induced through Toll signaling by Relish and Dif, the invertebrate counterparts of Rel/NF-κB, (44).

Both cRel and NF-κB1, but not RelA are required for maximal IL-12 expression by CD8$^+$ DCs, with their respective contributions to the induction of p70 reflecting the extent to which these transcription factors regulate *p35* expression. The absence of c-Rel resulted in a 30 to 50-fold decrease in IL-12 levels that coincided with a reduction of similar magnitude in *p35* mRNA. Without NF-κB1, a reproducible drop of three- to fourfold in both *p35* mRNA and IL-12 levels was observed. In contrast, the induced levels of *p40* mRNA, p40 monomer, and homodimer made by activated CD8$^+$ DCs lacking these transcription factors was unchanged. This shows that in CD8$^+$ DCs, the induction of p70 by Rel/NF-κB is regulated through *p35* rather than *p40* expression. Consistent with c-Rel and NF-κB1 both being necessary for optimal *p35* expression in CD8$^+$ DCs is the finding that NF-κB1/c-Rel is the major induced complex that binds the κB site in the *p35* promoter. c-Rel, however, is the most important of the dimer partners for *p35* expression. Similar findings for the relative contributions of these subunits in the regulation of *A1* and *IRF-4,* two genes rapidly induced in activated B cells (31, 45), reinforces the notion that NF-κB1 is mainly involved in modulating the c-Rel–dependent transcription of these genes. The *p35* κB element, 5′-GGGAATCCC-T-3′ is closely related to the sequence 5′-GGGATCC-3′, which is conserved within the three functional κB sites found in the *A1* and *IRF-4* promoters that bind NF-κB1/c-Rel and c-Rel homodimers (31, 45). The *p35* promoter data reinforces our previous proposal that the sequence 5′-GGGATCC-3′ is a signature for functional κB sites that preferentially bind these Rel/NF-κB dimers (31).

In CD8$^+$ DCs, IL-12 p70 expression coincides with a rapid induction of both *p35* and *p40* mRNAs in response to DNA, SAC, LPS, or CD40 (9) stimulation. Although the coordinated expression of both genes is consistent with a common mode of transcriptional regulation, *p35* but not *p40* transcription was found to depend on c-Rel and NF-κB1 in these cells. This result was unexpected given a previous report showing c-Rel was necessary for induced *p40* expression in macrophages (15). The most likely explanation for this difference is cell-type specific transcriptional regulation of *p40*. If this were the case, it is unlikely that *p40* transcription in CD8$^+$ DCs is dependent on another Rel/NF-κB family member such as RelA, an effective activator of *p40* promoter-reporters (15), as *p40* expression was slightly elevated rather than diminished in *rela$^{-/-}$* DCs. Despite c-Rel, RelA, and NF-κB1 all being individually dispensable for *p40* expression in CD8$^+$ DCs, it remains to be determined whether this truly reflects Rel/NF-κB–independent regulation of this gene or redundancy amongst these transcription factors (37). Cell-type–specific regula-

tion of inducible gene expression by c-Rel has been documented previously for GM-CSF. In T cells, c-Rel is required for the optimal induction of GM-CSF (35), whereas it is dispensable for its expression in elicited peritoneal macrophages, and functions as a repressor of *GM-CSF* transcription in resident peritoneal macrophages (36). Such differences seen for both *GM-CSF* and *p40* could be explained by selective interactions between c-Rel and other DNA binding proteins or coactivators in the various cell types. To date the most detailed molecular information on the role of Rel/NF-κB signaling in IL-12 regulation comes from the study *p40* transcription in macrophages (15). LPS induction of *p40* transcription in these cells is dependent upon c-Rel, which is activated through TRL-4 (46). While this requires nucleosome remodeling of the *p40* promoter (46), an event also regulated through this Toll-like receptor, it is independent of c-Rel and appears to involve a novel pathway. Ongoing studies aimed at comparing the role(s) c-Rel serves in determining the chromatin structure of the *p35* and *p40* promoters in various cell types in response to different stimuli, should increase our understanding of how IL-12 is regulated at the transcriptional level.

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