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p100, a precursor of NF-κB2, inhibits c-Rel and reduces the expression of IL-23 in dendritic cells

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ABSTRACT

Nuclear factor κB regulates various genes involved in the immune response, inflammation, cell survival, and development. NF-κB activation is controlled by proteins possessing ankyrin repeats, such as IκBs. A precursor of the NF-κB2 (p52) subunit, p100, contains ankyrin repeats in its C-terminal portion and has been found to act as a cytoplasmic inhibitor of RelA in the canonical pathway of NF-κB activation. Here, we demonstrate that p100 also suppresses c-Rel function in dendritic cells. Expression of the p19 and p40 subunits of IL-23, a c-Rel-dependent cytokine, was enhanced in p100-deficient cells, although expression of a RelA-dependent cytokine, TNF-α, was reduced. Nuclear translocation of c-Rel was enhanced in p100-deficient cells. p100, and not the processed p52 form, associated with c-Rel in the steady state and dissociated immediately after lipopolysaccharide stimulation in wild-type dendritic cells. Four hours after the stimulation, p100 was newly synthesized and associated with c-Rel again. In cells expressing both c-Rel and RelA, c-Rel is preferentially suppressed by p100.

1. Introduction

Nuclear factor κB (NF-κB) is an important transcription factor that regulates expression of various genes involved in the immune response, inflammation, cell survival, and development. Mammalian NF-κB is composed of five members: RelA, c-Rel, RelB, p50 (NF-κB1), and p52 (NF-κB2) [1]. p50 and p52 are synthesized as the large precursor proteins p105 and p100, respectively. NF-κB is activated by two different pathways: the canonical pathway, which activates heterodimers of RelA/p50 and c-Rel/p50, and the noncanonical pathway, which activates RelB/p52 heterodimers. Each pathway can be activated by distinct receptor–ligand complexes. The tumor necrosis factor (TNF)-α receptor and Toll-like receptors (TLRs) activate the canonical pathway, and receptor of B cell-activating factor, a member of TNF family, activates the noncanonical pathway. The lymphotoxin-β receptor and receptor activator of nuclear factor κ-B activate both pathways [2]. In the steady state, the NF-κB proteins are sequestered in the cytoplasm by a family of inhibitors possessing ankyrin repeats. RelA/p50 and c-Rel/p50 heterodimers are sequestered by IκB-α, IκB-β, and IκB-ε [3]. Once activated by canonical pathway ligands, IκBs are degraded, and the NF-κB complexes translocate into the nucleus. In the noncanonical pathway, RelB associates with p100, which contains IκB-like ankyrin repeats in its C-terminal portion. After ligation of receptors activating the noncanonical pathway, p100 is processed into p52, and the RelB/p52 heterodimer translocates into the nucleus [2]. NF-κB heterodimers bind to κB sites in the promoters and enhancers of a variety of genes and induce or reduce transcription.

The canonical and noncanonical pathways are thought to cross-talk each other, but the molecular mechanism has just begun to be elucidated [4]. For example, TRAF3, a negative regulator of the noncanonical pathway, also negatively regulates the canonical pathway [5]. RelB is regulated by IκB-α and IκB-ε via the canonical pathway [6]. p100 has classically been considered to be a negative regulator in the noncanonical pathway, but it has been reported to negatively regulate RelA function [7–9]. In fact, cells deficient in IκB-α,-β, and -ε, were used to show that p100 is also a cytoplasmic inhibitor of RelA [10,11]. In dendritic cells and macrophages, c-Rel, whose expression is restricted to immune cells, regulates the expression of a distinct set of genes from those regulated by RelA [12]. In RelA-deficient cells, the expression of TNF-α and IL-6 is markedly decreased [12]. In contrast, expression levels of the p40

Abbreviations: BMDC, bone marrow-derived dendritic cells; HPRT, hypoxanthine–guanine phosphoribosyltransferase; IFN, interferon; LPS, lipopolysaccharide; NF-κB, nuclear factor κB; PLA, proximity ligation assay; TLR, Toll-like receptor; TNF, tumor necrosis factor.

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subunit of IL-12 and IL-23, and of the IL-23 p19 subunit are decreased in c-Rel-deficient cells [13–15]. Both RelA-dependent and c-Rel-dependent genes are classified into two types, early-induced genes, which begin to be expressed 30 or 60 min after induction, and late-induced genes, whose expression peaks 6–8 h after induction [16]. Late-induced genes, such as the p40 subunit of IL-12 and IL-23 and the p35 subunit of IL-12, are required for chromatin remodeling [16–18]. Previously, we demonstrated that expression of the p19, p35, and p40 subunits is enhanced in NF-κB2-deficient ($p100^{-/-}p52^{-/-}$) dendritic cells [15]. To elucidate the role of p100 as a negative regulator in the canonical pathway, we examined cytokine expression in $p100^{-/-}p52^{-/-}$ dendritic cells. p100 deficiency enhanced c-Rel-dependent cytokine expression, but not RelA-dependent expression. We have also demonstrated that p100 associates with c-Rel and inhibits c-Rel function in dendritic cells.

2. Materials and methods

2.1. Mice and cells

NF-κB2-deficient ($p100^{-/-}p52^{-/-}$) mice were supplied by Bristol-Myers Squibb Company (New York, NY, USA). These mice were produced by targeted disruption of the NF-κB2 locus by introduction of the NEO cassette into exon 4 [19].

$p100^{-/-}$ mice were provided by Dr. Falk Weih (Leibniz Institute for Age Research, Fritz Lipmann Institute, Jena, Germany). The mice were produced by insertion of a termination codon at a position corresponding to amino acid 451 [20], which is positioned between the glycine-rich region and the first ankyrin motif. The mice produce the processed form of p52, but not p100.

aly/*aly* and *aly*/+ mice were purchased from CLEA Japan Inc.

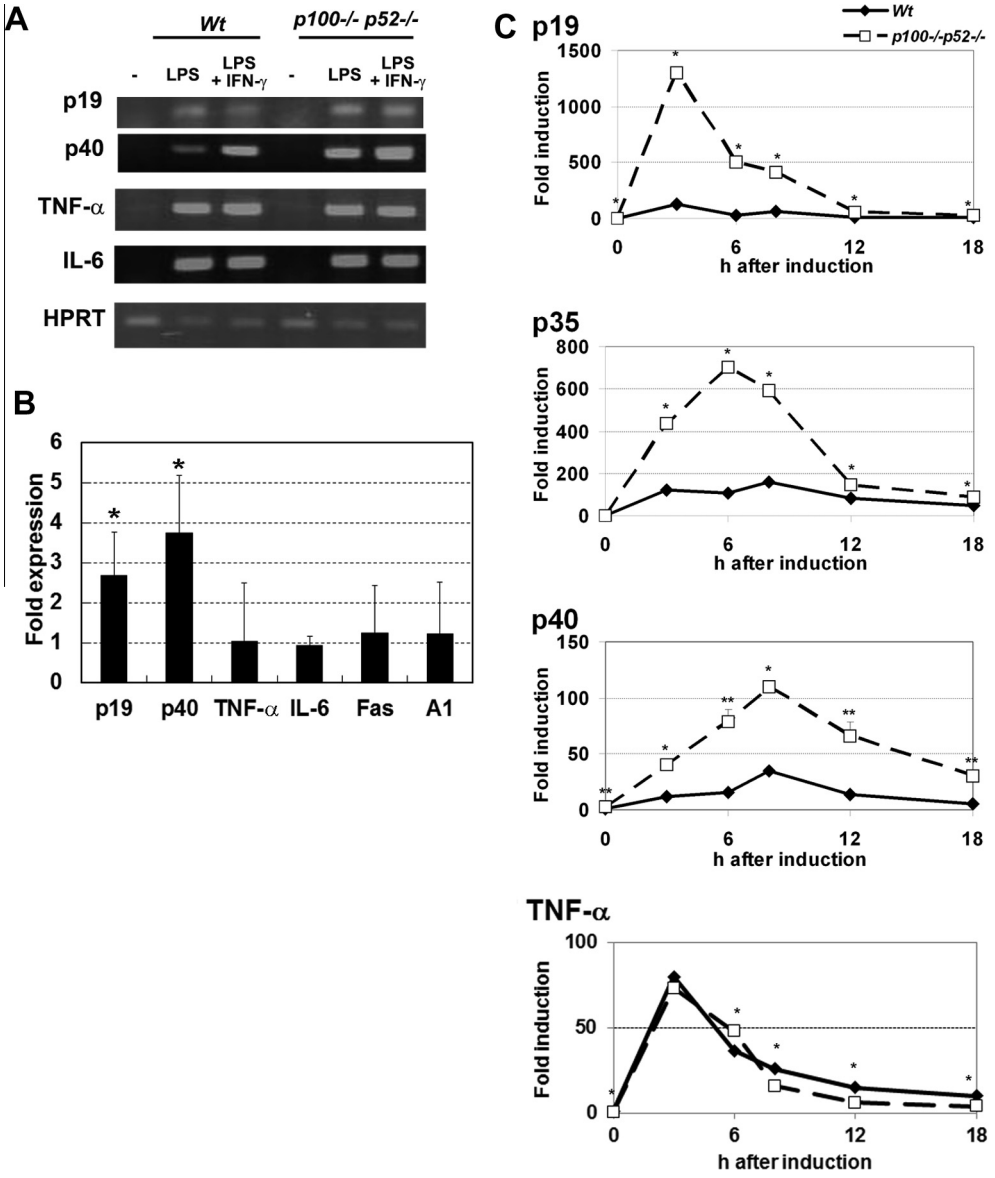

Fig. 1. Cytokine expression in $p100^{-/-}p52^{-/-}$ BMDC. (A) Expression of p19, p40, TNF-α, and IL-6 in $p100^{-/-}p52^{-/-}$ and wild-type (*Wt*) BMDC was analyzed by using semi-quantitative PCR 6 h after stimulation with 1 μg/ml LPS with or without 1 ng/ml IFN-γ. Expression of HPRT was measured as an internal control. The data are representative of more than 5 experiments. (B) Quantification of the levels of cytokine mRNA by use of real-time PCR 6 h after LPS stimulation. The expression levels are indicated as fold expression compared with those of *Wt* BMDC. The values are means ± SD of results from 5 different mice. $^{*}p < 0.01$ versus *Wt*. (C) Time courses of p19, p35, p40, and TNF-α mRNA expression were examined in $p100^{-/-}p52^{-/-}$ and *Wt* $CD11c^{+}$ cells by using real-time PCR. The values are presented as fold induction of LPS-stimulated cells compared with non-stimulated *Wt* cells. Enhanced expression of p19, p35, and p40 was observed in $p100^{-/-}p52^{-/-}$ $CD11c^{+}$ cells. The data presented are representative of 3 independent experiments. $^{*}p < 0.01$; $^{**}p < 0.05$.

Bone marrow-derived dendritic cells (BMDC) were grown for 7 days in RPMI-1640 containing 20% fetal bovine serum, 10 mM HEPES, 1 mM sodium pyruvate, and 5×10^{-5} M 2-mercaptoethanol, supplemented with 10 ng/mL granulocyte–macrophage colony stimulating factor (WAKO Pure Chemical Industries Ltd., Japan). Loosely attached cells were suspended by vigorous pipetting, and CD11c$^+$ cells were purified by using CD11c (N418) microbeads and LS columns (Miltenyi Biotec, Germany).

2.2. Analysis of mRNA

Cells were stimulated with 1 μg/mL lipopolysaccharide (LPS; 055:B5, Sigma–Aldrich Co., USA). Recombinant mouse interferon (IFN)-γ was purchased from e-Bioscience (San Diego, USA). Total RNA was prepared, and quantitative PCR was performed as described previously [15]. The primers used for cytokine expression were as follows:

hypoxanthine–guanine phosphoribosyltransferase (HPRT),	sense: CTTTGCTGACCTGCTGGATT and antisense: TATGTCCCCCGTTGACTGAT
p19,	sense: CCAGCGGGACATATGAATCTAC and antisense: CCTTGAGTCCTTGTGGGTCA;
p35,	sense: GCCAGGTGTCTTAGCCAGTC and antisense: TGATCGATGTCTTCAGCAGTG;
p40,	sense: TTGGAAGCACGGCAGCAGAA and antisense: CAGCTGACCTCCACCTGTGA;
TNF-α	sense: CAAATGGCCTCCCTCTCAT and antisense: CACTTGGTGGTTTGCTACGA;
IL-6,	sense: GTTCTCTGGGAAATCGTGGA and antisense: TTCTGCAAGTGCATCATCGT;
Fas,	sense: TGCTGATAAATGCAGAAGATGC and antisense: TATTCTGGGTCAGGGTGCAG;
A1	sense: GCCCTGGATGTATGTGCTTAC and antisense: GATCTGTCCTGTCATCTGCAG.

2.3. Transfection and luciferase activity

293T cells were transfected with pcDNA-flag-c-Rel with or without pcDNA-myc-p100 by using the calcium phosphate transfection method. The luciferase activity of the co-transfected p19-937 luciferase reporter gene [15] and β-galactosidase activity were measured 2 days after transfection as described previously [15].

2.4. Western blotting and immunoprecipitation

Nuclear and cytoplasmic extraction was performed by using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, USA) in accordance with the manufacturer's instructions. Western blotting was performed as described previously [21] by using anti-c-Rel (Santa Cruz, USA) and anti-NF-κB2 p100/p52 (Cell Signaling Technology Inc., USA) polyclonal antibodies, and anti-lamin B1 (ZYMED Laboratories, USA), anti-myc (9E1, Roche, Switzerland), anti-DYKDDDDK tag (1E6, WAKO) monoclonal antibodies. Cell lysates obtained with 1% NP-40 were immunoprecipitated with protein A-Sepharose CL-4B (GE Healthcare, UK) previously incubated with an anti-c-Rel antibody.

2.5. In situ proximity ligation assay

In the proximity ligation assay (PLA), CD11c$^+$ cells were plated on a Lab-Tek II chamber slide (Nunc, Denmark). After fixation with 4% paraformaldehyde and permeabilization with phosphate buffered saline containing 0.1% Triton X-100, cells were blocked with the blocking solution of the DuoLink detection kit (Olink, Sweden) and stained with a mouse anti-c-Rel monoclonal antibody (1E7, Thermo Scientific) and rabbit polyclonal anti-NF-κB2 p100/p52 antibody. The proximity ligation probes were developed with the DuoLink detection kit (Olink) in accordance with the manufacturer's instructions. The cells were observed by using a laser scanning microscope (LSM 710, Carl Zeiss, Germany).

2.6. Statistics

Statistical significance was analyzed by using Student's *t* test.

3. Results and discussion

3.1. c-Rel-dependent cytokine expression is enhanced in p100-deficient dendritic cells

As reported previously [15], the expression of the p19, p35, and p40 subunits of IL-12 and IL-23 is significantly enhanced in $p100^{-/-}p52^{-/-}$ BMDC (Fig. 1). To understand the mechanism by which the expression of these cytokines is enhanced in $p100^{-/-}p52^{-/-}$ BMDC, we examined the expression of other NF-κB-dependent genes. The expression levels of TNF-α and IL-6 were similar in wild-type and $p100^{-/-}p52^{-/-}$BMDC (Fig. 1A and B). The expression of Fas and A1, which are NF-κB RelA-dependent genes, was also intact. We noticed that the cytokines whose expression was enhanced in $p100^{-/-}p52^{-/-}$ BMDC (p19, p35, and p40) mainly depend on c-Rel function [13–15]; in contrast, TNF-α, IL-6, Fas, and A1 are RelA-dependent [12,22]. To more precisely understand the effect of NF-κB2 deficiency on cytokine expression, we observed a time course of cytokine expression using purified CD11c$^+$ dendritic cells. Like TNF-α [16], p19 is an early-induced gene; its mRNA was induced quickly after LPS stimulation and began to decline by 6 h after the stimulation (Fig. 1C). The expression of p19 in $p100^{-/-}p52^{-/-}$ CD11c$^+$ cells was markedly enhanced compared with expression in wild-type cells, but the kinetics of the expression were similar. Similarly, the expression of p35 and p40 was higher in $p100^{-/-}p52^{-/-}$ CD11c$^+$ cells than in wild-type cells, but the kinetics were similar, with expression peaking 6–8 h after stimulation [16]. TNF-α expression was comparable between wild-type and knockout cells.

3.2. p100, but not p52, downregulates the expression of IL-12 and IL-23 without involving the noncanonical pathway

To explore which protein, p100 or its processed form p52 is responsible for the suppression of c-Rel-dependent gene expression, we examined cytokine expression by using $p100^{-/-}$ mice, which have a deletion of the C-terminal region of p100. Although they constitutively express of p52, $p100^{-/-}$ CD11c$^+$ cells expressed

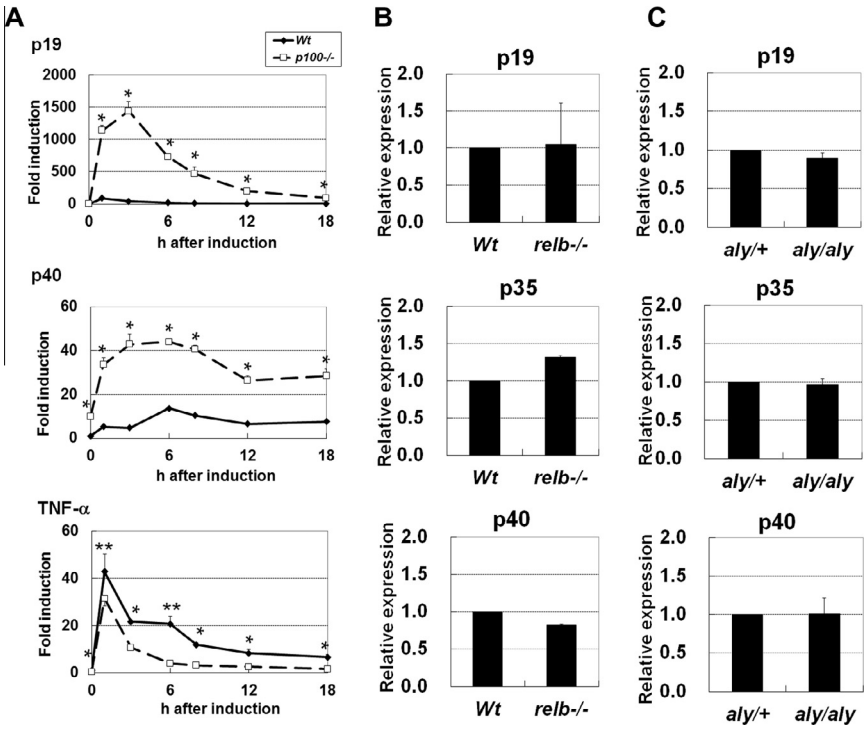

Fig. 2. Cytokine expression in $p100^{-/-}$ CD11c$^+$ cells (A) and $relb^{-/-}$ and *aly*/*aly* BMDC (B and C). (A) The time courses of p19, p40, and TNF-α mRNA expression were examined in $p100^{-/-}$ and wild-type (*Wt*) CD11c$^+$ cells. The data were normalized by the values of HPRT. The values are presented as fold induction of LPS-stimulated cells compared with non-stimulated *Wt* cells. The expression of p19 and p40 was markedly enhanced in $p100^{-/-}$ cells, whereas the expression of TNF-α was significantly lower in $p100^{-/-}$ cells than in *Wt* cells. The data presented are representative of 3 independent experiments. $^*p < 0.01$; $^{**}p < 0.05$. (B and C) The expression of the p19, p35, and p40 subunits was not affected by deficiency of the noncanonical pathway of NF-κB. The cytokine expression in $relb^{-/-}$ (B) or *aly*/*aly* (C) BMDC was compared with that in *Wt* cells and *aly*/+ cells, respectively. The values are means ± SD of results from 3 different mice.

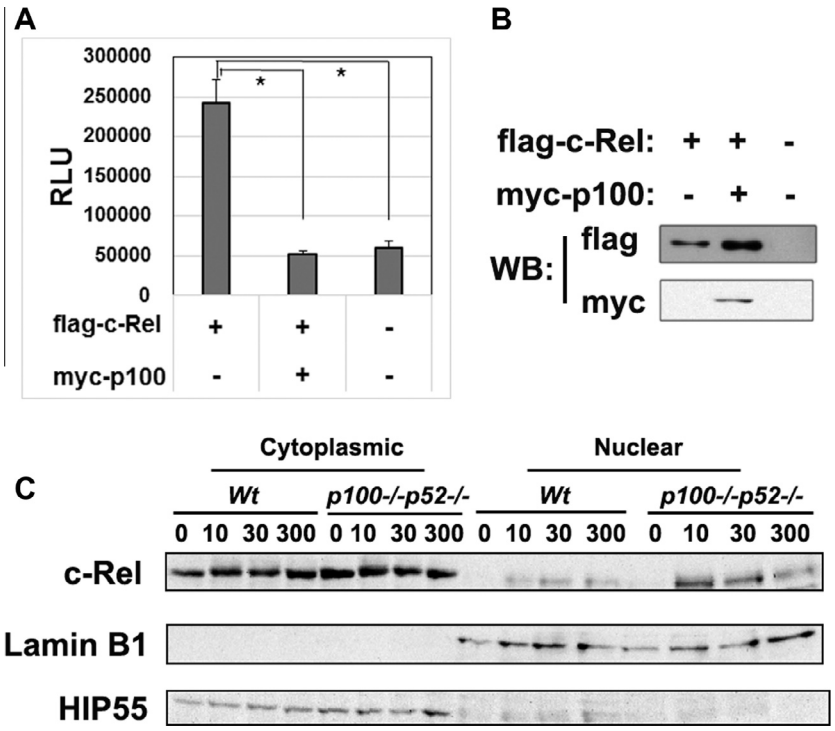

Fig. 3. p100 inhibits c-Rel function. (A) pcDNA-flag-c-Rel was transfected into 293T cells with or without pcDNA-myc-p100. c-Rel expression enhanced p19 reporter activity, and co-expression of p100 prevented it. (B) Western blot of total lysates of the transfected cells. (C) Nuclear translocation of c-Rel is enhanced in $p100^{-/-}p52^{-/-}$ CD11c$^+$ cells. Cells were stimulated with LPS for the indicated times (in minutes), and then cytoplasmic and nuclear fractions were separated. Western blotting revealed that the translocation of c-Rel into the nucleus was enhanced in $p100^{-/-}p52^{-/-}$ cells. LaminB1 and HIP55 are a nuclear and a cytoplasmic marker, respectively. The data are representative of 3 experiments.

higher levels of p19 and p40, but not TNF-α, compared with wild-type cells (Fig. 2A). Thus, the deficiency of p100, not p52, was responsible for the upregulation of c-Rel-dependent cytokine expression. The constitutive expression of p52 in *p100*$^{-/-}$ mice also leads to the constitutive activation of the noncanonical pathway of NF-κB [23]. If the noncanonical pathway enhances the expression of IL-12 and IL-23, its loss would be expected to diminish the expression of these cytokines. But this is not case: expression of IL-12 and IL-23 subunits was not affected in either *relb*$^{-/-}$ cells or *aly*/*aly* mice, which have a natural loss-of-function mutation in the gene encoding *Nik* (NF-κB-inducing kinase) [24] (Fig. 2B and C). These data suggest that noncanonical pathway activation may not be involved in the expression of these subunits.

3.3. p100 inhibits c-Rel function

To explore the possibility that p100 directly inhibits c-Rel function, we used a reporter gene possessing the promoter region of the p19 gene from nt −937 to nt 16 [15]. When c-Rel was overexpressed in 293T cells, p19 promoter activity was markedly enhanced, but p100 expression prevented the enhancement (Fig. 3A and B), suggesting that p100 directly inhibits c-Rel function. Then we compared the nuclear translocation of c-Rel between wild-type and *p100*$^{-/-}$*p52*$^{-/-}$ CD11c^{+} cells after LPS stimulation. We observed enhanced nuclear translocation of c-Rel in *p100*$^{-/-}$*p52*$^{-/-}$ CD11c^{+} cells (Fig. 3C). However, the expression levels of c-Rel and p50 in *p100*$^{-/-}$*p52*$^{-/-}$ CD11c^{+} cells were not different from those in wild-type cells (Fig. S1). These data indicated that p100 inhibits c-Rel function.

3.4. p100 associates with c-Rel to suppress its function

To explore whether p100 directly inhibits c-Rel function, we observed the association of c-Rel and p100 by using immunoprecipitation and *in situ* PLA. The c-Rel antibody co-immunoprecipitated p100 in the cells before LPS stimulation (Fig. 4A). The coprecipitated p100 was barely detectable 30 min after the stimulation, but 4 h after LPS stimulation, p100 expression was upregulated and the amount of p100 associated with c-Rel had increased. Then we detected the association of p100 and c-Rel with *in situ* PLA. In wild-type cells, the association was detected in the cytoplasm, but not in the nucleus, in the steady state (Fig. 4B). The *in situ* PLA signal was specific because neither *p100*$^{-/-}$*p52*$^{-/-}$ cells nor c-Rel-deficient cells possessed the signal (Fig. S2A and B). The signals were also not detected in cells deficient in p100 but expressing p52 (Fig. S2C and D), demonstrating that p100, not p52, associates with c-Rel and suggesting that the ankyrin repeat of p100 is responsible for the association. In accordance with the immunoprecipitation result, the association decreased 30 min after stimulation with LPS (Fig. 4C) and then increased 4 h after the stimulation (Fig. 4D). Analysis of the number of dots per cell indicated that this difference was statistically significant (Fig. 4E).

Our study indicates that the absence of p100 enhances the nuclear translocation of c-Rel, resulting in enhanced c-Rel-dependent gene expression. p100 directly suppressed c-Rel function, because the association of p100 with c-Rel was detected in the cytoplasm of the cells, and not in the nucleus, both in the steady state and 4 h after LPS stimulation.

We have provided evidence that p100 suppresses c-Rel function in the canonical pathway. Several studies have demonstrated that p100 associates with RelA and inhibits RelA function [10,11,25–27]. However, the inhibition of RelA by p100 was observed under somewhat non-physiological conditions, such as in cells deficient in all other IκBs (IκB-α, -β, and -ε [10,11] and in cells expressing a mutant p100 that cannot be processed [26]. Ligands that solely activate the canonical pathway do not induce the dissociation of RelA from p100 [25]. Our study demonstrates that p100 suppresses c-Rel function in the steady state and dissociates from c-Rel after LPS stimulation. The enhanced nuclear localization of c-Rel was also observed in *p100*$^{-/-}$ thymocytes, although the molecular mechanism has not been elucidated [28]. Consistent with the reports of others [10,29], LPS stimulation did not induce the processing of p100 to p52 in our experiments (data not shown). We do not know how p100 dissociates from c-Rel after the stimulation, but protein modifications of c-Rel and/or p100 may be responsible. Four hours after the stimulation, we observed the enhanced expression of p100 and the re-association of p100 with c-Rel. An enhanced association has also been observed between p100 and RelA at a similar time point after stimulation [11]. The association may be responsible for shutting down the activation.

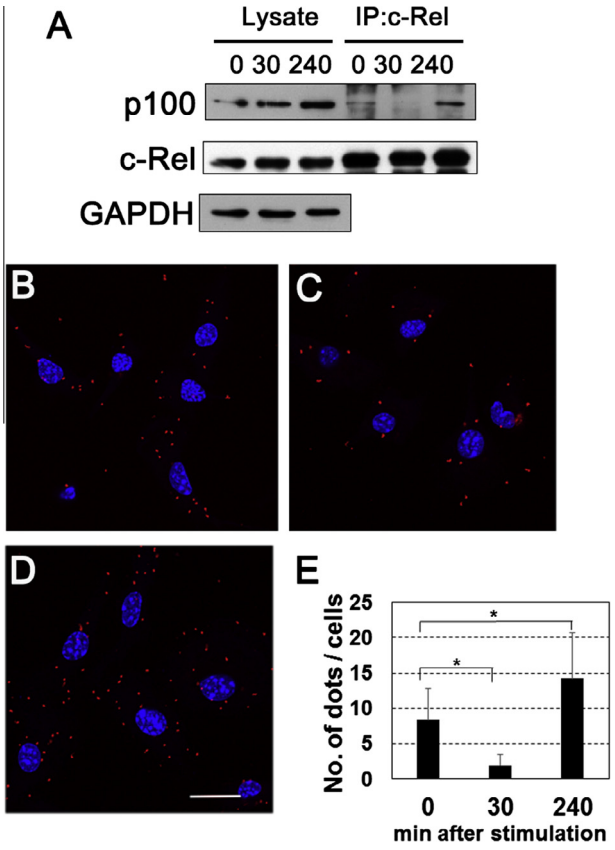

Fig. 4. p100 binds to c-Rel in the steady state and dissociates after stimulation. (A) Immunoprecipitation was performed at the indicated times (in minutes) after LPS stimulation by using an anti-c-Rel antibody. The anti-c-Rel antibody coprecipitated p100 before the stimulation. Thirty minutes after the stimulation, the amount of p100 associated with c-Rel decreased. Four hours after the stimulation, the total amount of p100 in the cell lysate and the amount of p100 associated with c-Rel increased again. (B–D) *In situ* PLA was performed before LPS stimulation (B) and 30 min (C) and 4 h (D) after LPS stimulation. Interactions between p100 and c-Rel were detected as orange dots. Cells were stained with DAPI to detect nuclei. (E) Dots per cell were counted at the indicated times after the stimulation. The data presented are representative of 3 independent experiments. *$p < 0.01$.

Homodimers of p50 or p52, which lack transactivation domains, act as inhibitors of NF-κB activation [30]. In our study, homodimers of p52 were not responsible for the inhibition of c-Rel, because the expression of p19 and p40 was enhanced in the *p100*$^{-/-}$ cells expressing p52. RelA and c-Rel may be present in high-molecular-weight heterogeneous complexes in dendritic cells [31]. We are continuing to study the molecular modifications and dynamics of p100 and c-Rel by using dendritic cells to evaluate the ability of IκBs and p100 to inhibit c-Rel activation.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.09.143.

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