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CUTTING EDGE

Cutting Edge: NF-κB2 Is a Negative Regulator of Dendritic Cell Function[1]

Kendra Speirs, Linda Lieberman,* Jorge Caamano,† Christopher A. Hunter,* and Phillip Scott[2]**

RelB, a member of the NF-κB family of transcription factors, is essential for dendritic cell (DC) maturation. Recent findings indicate that RelB is exclusively regulated through its interaction with cytoplasmic NF-κB2/p100. The studies presented in this report show that DCs lacking NF-κB2 have dramatically enhanced RelB activity, associated with increased MHC class II and costimulatory molecule expression and an enhanced ability to induce CD4$^+$ T cell responses. These studies identify a novel role for NF-κB2 in the negative regulation of RelB-induced DC maturation, with critical consequences for the regulation of adaptive immune responses. The Journal of Immunology,* 2003, 171: 752–756.

Dendritic cells (DCs)[3] have the unique capacity to initiate primary T cell responses and host defense against infection. Upon maturation, DCs up-regulate MHC class II and costimulatory molecule expression and produce high levels of the proinflammatory cytokines TNF-α, IL-1, IL-6, and IL-12. However, the intracellular signaling mechanisms that regulate DC maturation remain poorly defined. Recently, activation of the NF-κB family was shown to correlate with DC maturation in both mice and humans (1, 2). Indeed, DC Ag presentation could be blocked using adenoviral transfer of the endogenous inhibitor of NF-κB, IκBα, suggesting an absolute requirement for this transcription factor in DC function (2, 3).

There are five known NF-κB family members, NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p100), RelA, RelB, and c-Rel, which are maintained in the cytoplasm as dimers bound to one of several inhibitory proteins of the IκB family or the precursor proteins p105 and p100 (4). Phosphorylation of the NF-κB inhibitor protein targets it for degradation, thus enabling NF-κB dimers to translocate to the nucleus and activate gene transcription (4). Analysis of mice deficient in individual NF-κB components has revealed unique roles for each family member in cellular function. The combined absence of NF-κB1 and RelA, for example, results in dramatic defects in DC development, whereas the absence of NF-κB1 and c-Rel leads to impaired DC survival and IL-12 production (5). Requirements for RelB are implicated in the development of splenic CD11c$^+$CD8$^-$ DCs, and in the maturation and Ag presentation of bone marrow-derived DCs (6, 7).

In contrast to other NF-κB components, whose activity is primarily regulated by association with IκB, a recent report finds that RelB is exclusively repressed by NF-κB2/p100 in HeLa cells (8). Considering the importance of RelB in DC maturation, we asked whether p100 also represses RelB activity in DCs. We show that RelB is highly active in NF-κB2/p100 knockout (KO) DCs and correlate dysregulated RelB activity with alterations in DC function. In the absence of NF-κB2, DCs are hyperactivated, showing increased MHC class II and costimulatory molecule expression both constitutively and in response to stimulation. As a consequence, NF-κB2 KO DCs are dramatically more efficient than wild-type (WT) DCs at inducing CD4$^+$ T cell activation. Our report provides the first demonstration of a biological requirement for NF-κB2/p100 in the negative regulation of DC maturation and development of adaptive immune responses.

Materials and Methods

Mice

C57BL/6 and C57BL/6 Thy1.1 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). OT-II mice were described previously (9). NF-κB2 KO mice on a C57BL/6 (eight generations) background were described previously (10).

Bone marrow DC generation

DCs were generated as previously described (11). Briefly, bone marrow monocytes from NF-κB2 KO and WT B/6 mice were seeded in petri dishes at 2×10^5 cells/ml in 10 ml of medium. At day 3, 10 ml of medium containing 20 ng/ml GM-CSF was added. At days 6 and 8, 10 ml of culture supernatant was removed and replaced with 10 ml of fresh medium containing 20 ng/ml GM-CSF. For generation of immature DCs, supernatant was removed at day 10 and replaced with only 5 ng/ml GM-CSF. Cells were harvested at day 11 and were 95% enriched for DCs (CD11c$^+$).

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[3] Abbreviations used in this paper: DC, dendritic cell; KO, knockout; WT, wild type; BCL-3, B cell lymphoma 3.

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Western blot

NF-κB2 KO and WT DCs (day 11) were rested overnight and stimulated for 60 min with 100 ng/ml LPS (L6143; Sigma-Aldrich, St. Louis, MO). Cytoplasmic and nuclear extracts were isolated using an iso-osmotic/Nonidet P-40 lysis protocol as previously described (12). Nuclear translocation was assessed by SDS-PAGE and transferred to nitrocellulose. The membrane was blotted for RelB (C-19; Santa Cruz Biotechnology, Santa Cruz, CA), washed, and incubated with donkey anti-rabbit-HRP (Amersham Pharmacia, Arlington Heights, IL). The signal was detected using ECL chemiluminescence reagent (Amersham Pharmacia).

EMSA

NF-κB2 KO and WT DCs (day 11) were incubated for 1 h with 100 ng/ml LPS. Nuclear extracts were prepared as previously described (13). The double-stranded oligodeoxynucleotides corresponding to the palindromic B site (5′-GGGAATTCCC-3′) were used in this assay. EMSAs were performed as described previously (14). Supershift was conducted using anti-RelB (SC 226X) from Santa Cruz Biotechnology. Equivalent free probe was detected in all wells, confirming that probe was added in excess to each sample. Gel was exposed for 2 h without supershift and 12 h following the addition of anti-RelB.

DC cytokine production and expression of cell surface proteins

NF-κB2 KO and WT DCs were primed for 4 h with 10 U/ml recombinant murine IFN-γ and stimulated in the presence of 100 ng/ml LPS or 10 μg/ml anti-CD40 (clone 323; BD PharMingen, San Diego, CA). Supernatants were harvested after 72 h, and IL-12 p40, IL-6, TNF-α, and IL-1β levels were measured by ELISA, as previously described (15). Expression of DC surface molecules was quantified by flow cytometry using CD11c-FITC and MHC class II ($I\text{-}A^b$), CD80, or CD86-PE-conjugated Abs (BD PharMingen). Samples were analyzed using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences, San Jose, CA).

LPS injection and splenic DC analysis

LPS (L4641; Sigma-Aldrich) was i.p. injected into NF-κB2 KO and WT mice. After 24 h, spleens were removed and digested with 400 U/ml liberase (Roche Applied Science, Indianapolis, IN) for 30 min at 37°C. Single-cell suspensions were prepared and stained for CD11c-FITC and MHC class II ($I\text{-}A^b$), CD80, or CD86-PE-conjugated Ab (BD PharMingen). Samples were analyzed by flow cytometry.

DC priming ability in vitro

NF-κB2 KO and WT DCs were stimulated with 1 or 10 μg endotoxin-free OVA (Worthington, Lakewood, NJ) in the presence or absence of 100 ng/ml LPS. $CD3^+$ T cells were purified from OT-II spleens using T cell enrichment columns (R&D Systems, Minneapolis, MN). T cell proliferation was analyzed by labeling the cells with CFSE (Molecular Probes, Eugene, OR) and culturing them with the DCs at a 1:10 DC:T cell ratio (16). After 72 h, the T cells were harvested, and intracellular staining for IFN-γ was performed, as previously described (17). Cells were washed and analyzed by flow cytometry.

DC priming ability in vivo

$CD3^+$ T cells from OT-II mice were purified and CFSE labeled. A total of 5×10^6 cells was transferred by i.v. injection into C57BL/6 Thy 1.1 mice. Twenty-four hours later, PBS or OVA- and LPS-treated NF-κB2 KO or WT DCs were i.p. injected into the T cell-recipient mice. After 72 h, spleens were removed from the T cell- and DC-coinjected mice, and single-cell suspensions were prepared. Cells were surface stained with anti-CD4-CyChrome and anti-Thy1.2-PE (BD PharMingen), and cell division was analyzed by flow cytometry.

Results

RelB is highly activated in NF-κB2 KO DCs

RelB is the primary NF-κB family member required for the maturation of myeloid DCs (7, 18). Because NF-κB2/p100 is exclusively responsible for the inhibition of RelB activity in HeLa cells, we predicted that RelB would be more active in NF-κB2 KO DCs (8). To test this, NF-κB2 KO and WT DCs were stimulated with LPS, and cytoplasmic and nuclear extracts were harvested to measure RelB activation. High levels of RelB were present in the nuclei of unstimulated NF-κB2 KO vs WT DCs and were further elevated in response to LPS treatment (Fig. 1*a*).

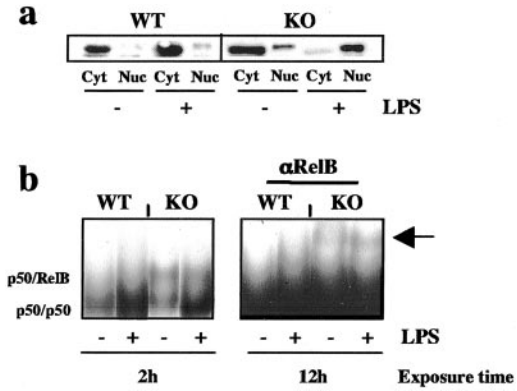

FIGURE 1. RelB is activated in resting and mature NF-κB2 KO DCs. Bone marrow DCs were activated for 1 h with LPS. *a*, Nuclear and cytoplasmic extracts were isolated, and nuclear translocation was assessed by immunoblotting with a RelB-specific mAb. *b*, Nuclear extracts were incubated with a κB oligonucleotide, and the DNA binding ability of RelB was analyzed by EMSA. Gel was exposed for 2 h without supershift and 12 h following the addition of anti-RelB. Arrow indicates the RelB supershift. Data shown are representative of two experiments.

To determine whether RelB was active and able to bind DNA, nuclear extracts were incubated with a κB oligonucleotide and analyzed by gel shift. As shown in Fig. 1*b*, constitutive κB binding activity was enhanced following LPS treatment, with NF-κB2 KO and WT DCs showing a similar increase in a fast-migrating complex composed of p50/p50 homodimers (5). Nuclear extracts from NF-κB2 KO DCs revealed an additional slow-migrating band that was likely composed of p50/RelB heterodimers (Fig. 1*b*). Prolonged exposure of the gel and addition of specific antisera for RelB confirmed the presence of this transcription factor in NF-κB2 KO but not WT DCs (Fig. 1*b*, arrow). This is particularly noteworthy given that RelB is often difficult to detect by gel shift analysis (14, 19). Addition of antisera for NF-κB1, RelA, and c-Rel revealed no differences in the activation of these transcription factors in NF-κB2 KO and WT DCs (data not shown). These data illustrate that NF-κB2/p100 specifically inhibits RelB activation in DCs, and raise the question of how DC function may be altered in the absence of this control mechanism.

NF-κB2 KO DCs show enhanced expression of activation markers but produce normal levels of cytokines

Because RelB is required for the maturation of bone marrow DCs, we asked whether dysregulated RelB activity alters this process. To test this, we compared the phenotype of NF-κB2 KO and WT DCs. Ten days after generation with GM-CSF, NF-κB2 KO and WT cultures revealed a large number of cells with characteristic DC morphology and a comparable percentage of $CD11c^+$ cells, suggesting that DCs develop normally in the absence of NF-κB2 (data not shown). To assess the maturation phenotype of NF-κB2 KO DCs, we quantitated the surface expression of MHC class II, CD86, and CD80 after stimulation with anti-CD40 or LPS. NF-κB2 KO DCs had a higher baseline expression of class II and CD86 than WT DCs, and were more responsive to both stimuli (Fig. 2*a*). In contrast, NF-κB2 KO DCs produced normal levels of IL-12 p40, IL-6, TNF-α, and IL-1β, suggesting that NF-κB2 is dispensable for DC cytokine production (Fig. 2*b*). These observations suggest

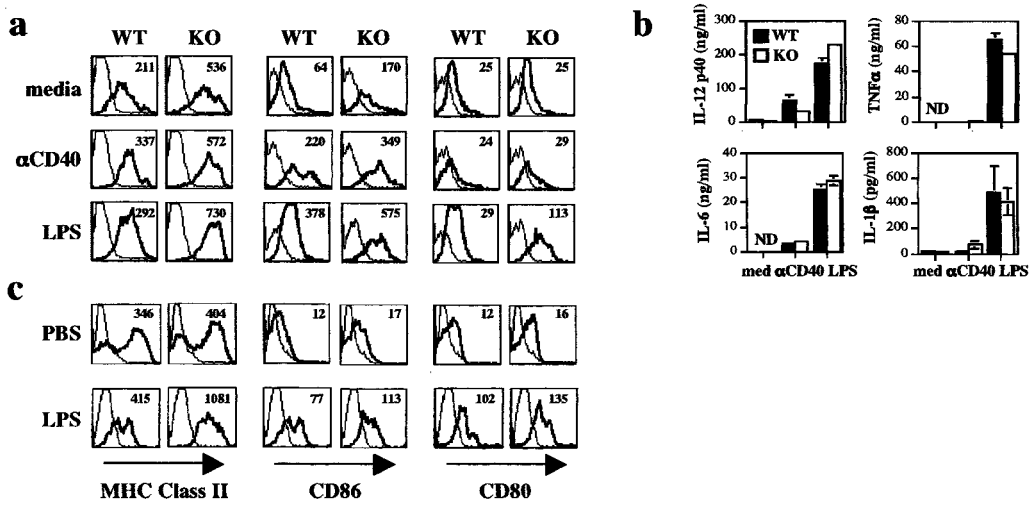

FIGURE 2. NF-κB2 regulates bone marrow and splenic DC maturation. *a*, Bone marrow DCs were stimulated with anti-CD40 or LPS. After 72 h, DCs were surface stained for CD11c, MHC class II, CD86, and CD80, and analyzed by flow cytometry. Plots are gated on $CD11c^+$ cells, and values represent the mean fluorescence intensity of the experimental groups (bold). Also shown are the isotype controls for each surface marker (fine). *b*, Supernatents were collected and IL-12 p40, IL-6, TNF-α, and IL-1β production was quantitated by ELISA. Data are means ± SD of three mice and are representative from one of five experiments. *c*, Splenocytes were harvested 24 h following in vivo stimulation with LPS. Cells were surface stained for CD11c, MHC class II, CD86, and CD80, and analyzed by flow cytometry. Plots are gated on $CD11c^+$ cells, and values represent the mean fluorescence intensity of the experimental groups (bold). Also shown are the isotype controls for each surface marker (fine). Data are representative of three experiments.

that NF-κB2/p100 represses the expression of class II and costimulatory molecules, and that the two measures of DC maturation, activation marker up-regulation and proinflammatory cytokine production, are differentially controlled by NF-κB signaling.

To determine whether NF-κB2 performs a similar function in endogenous DC populations, we investigated the in vivo effects of LPS on splenic DCs from NF-κB2 KO and WT mice. Indeed, NF-κB2 KO splenic DCs were more sensitive to LPS than WT DCs, as evidenced by enhanced activation marker expression (Fig. 2*c*). Our data show that NF-κB2 acts as a negative regulator of DC maturation in two distinct DC populations, and support an important role for this transcription factor in mediating immune responses.

OVA-pulsed NF-κB2 KO DCs exhibit an increased capacity to stimulate $CD4^+$ T cells in vitro

To determine whether the enhanced maturation status of NF-κB2 KO DCs has functional relevance, we analyzed how effectively these cells activate Ag-specific T cell responses. NF-κB2 KO and WT DCs were pulsed with OVA (1 or 10 μg) in the presence or absence of LPS, and cultured with CFSE-labeled OT-II T cells in vitro. After 72 h, the T cells were harvested and analyzed for their ability to proliferate and produce IFN-γ. WT DCs pulsed with 10 μg of OVA failed to promote T cell activation; however, OVA-pulsed NF-κB2 KO DCs induced strong T cell proliferation and IFN-γ production (Fig. 3*a*). These data correlate with the increased baseline expression levels of class II and CD86 and suggest that NF-κB2 KO DCs can present Ag even in the absence of microbial stimulation. OVA- and LPS-primed NF-κB2 KO DCs also revealed an enhanced capacity to activate T cell responses, as illustrated by the high levels of IFN-γ produced by the responding T cells (Fig. 3*b*). No differences were observed in the survival of NF-κB2 KO and WT DCs (data not shown). These results demonstrate that DCs induce a stronger Ag-specific T cell response in the absence of NF-κB2.

OVA-pulsed NF-κB2 KO DCs exhibit an increased capacity to stimulate $CD4^+$ T cells in vivo

To determine whether NF-κB2 KO DCs can also promote enhanced T cell responses in vivo, we transferred fluorescently labeled OT-II T cells into congenic Thy1.1 mice. Twenty-four

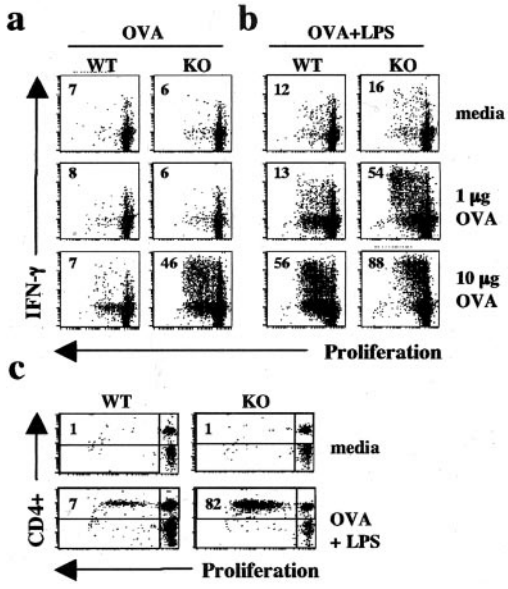

FIGURE 3. NF-κB2 KO DCs reveal an enhanced capacity to prime T cells. *a* and *b*, Bone marrow DCs were pulsed overnight with OVA alone (1 or 10 μg) (*a*) or OVA plus LPS (*b*), and cultured with CFSE-labeled OT-II T cells. After 72 h, T cells were stained for CD4 and intracellular IFN-γ and analyzed by flow cytometry. Shown are the percentages of proliferating $CD4^+$ T cells producing IFN-γ. Data are representative of three experiments. *c*, CFSE-labeled OT-II T cells were transferred i.v. into Thy 1.1 congenic mice. After 24 h, the same animals were injected i.p. with OVA- and LPS-pulsed DCs. After 72 h, spleens were removed from the recipient mice, stained for Thy1.2 and CD4, and analyzed by flow cytometry. Shown is the percentage of $Thy1.2^+CD4^+$ T cells that proliferated in vivo. Data are representative of three experiments.

hours later, these mice were injected with unactivated or OVA- and LPS-activated NF-κB2 KO or WT DCs. The splenocytes were recovered after 72 h and surface stained for Thy1.2 and CD4 expression. T cells from mice receiving untreated DCs were unable to promote T cell proliferation in vivo (Fig. 3*c*). Importantly, T cells from mice receiving activated NF-κB2 KO DCs proliferated 10 times more effectively than T cells from mice receiving activated WT DCs (Fig. 3*c*). Together, these experiments provide functional relevance for the enhanced maturation status of NF-κB2 KO DCs and define a role for NF-κB2/p100 in the control of RelB activation and Ag presentation.

Discussion

In this report, we define a novel role for NF-κB2 in the negative regulation of DC maturation. Our results show for the first time that NF-κB2/p100 represses RelB in DCs, and suggest that this regulatory pathway may function to prevent DC hyperactivation. Indeed, nuclear RelB levels are elevated in both resting and activated NF-κB2 KO DCs, a result that correlates with increased expression of the activation markers, MHC class II, CD86, and CD80. We show that the enhanced maturation status of NF-κB2 KO DCs increases their ability to induce Ag specific $CD4^+$ T cell responses. This work illustrates that NF-κB2 is essential for maintaining DCs in an immature state, a finding that is critical to our understanding of DC biology.

Despite the close association between RelB activation and DC maturation, there may be alternative explanations for the enhanced maturation status of NF-κB2 KO DCs. In addition to RelB, p100 was recently shown to associate with the death-inducing signaling complex and aid in promoting Fas-induced apoptosis (20). Although these findings are yet to be extended in vivo, it might be predicted that immature DCs lacking p100 are less sensitive to apoptosis and readily available to mature. However, this possibility is unlikely, because NF-κB2 and WT DCs have similar rates of survival (data not shown). Another factor known to interact with NF-κB2 is B cell lymphoma 3 (BCL-3). Although it is a member of the IκB family, BCL-3 has the unique ability to form a transactivating complex with p52/p52 homodimers (21). The defects in splenic microarchitecture observed in BCL-3-deficient mice are similar to those associated with the absence of NF-κB2 and suggest that the relationship between these factors has in vivo relevance (22, 23). BCL-3 is highly expressed in maturing DCs, although the specific genes induced by p52/p52/BCL-3 complexes, and their potential role in preventing DC maturation, remain to be defined (1).

We hypothesize that the hyperactivation of NF-κB2 KO DCs is accounted for by dysregulated RelB activity. Elevated RelB levels are similarly associated with the enhanced maturation status of DCs found in nonobese diabetic mice (24). In addition, hyperactive DCs may contribute to cutaneous T cell lymphomas that are shown to result from mutations in NF-κB2/p100 (25, 26). Indeed, mice deficient in the p100 portion of NF-κB2 fail to regulate lymphocyte activation, a phenotype that manifests itself as splenomegaly and gastric hyperplasia, and results in early postnatal death (27). The lymphoproliferation observed is associated with an increase in p52, p50, and RelB-containing complexes in nuclear extracts from several tissues. The maturation status of p100-deficient DCs and their contribution to T cell hyperactivation and lymphoma formation remains to be determined. However, there is evidence that the clonal proliferation of T cells in cutaneous lymphomas occurs in response to specific antigenic stimulation from DCs (28).

Finally, our finding that NF-κB2 is a critical negative regulator of DC function may have important clinical implications. On the one hand, inhibitors of NF-κB2 could enhance DC Ag-presenting function in vaccination therapies designed to boost T cell responses to cancer or infection (29). Alternatively, drugs might be designed to prevent the degradation of p100 and subsequent activation of RelB, thus promoting tolerance and preventing the hyperactive DC phenotypes associated with certain autoimmune diseases.

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