Original article

Dexamethasone impairs the differentiation and maturation of murine dendritic cells by Toll-like receptor 4-nuclear factor-κB pathway

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Keywords: dexamethasone; dendritic cell; lipopolysaccharide; nuclear factor-kappa B

Background Recent studies have demonstrated that dexamethasone (DEX) interferes with immune responses by targeting key functions of dendritic cells (DCs) at the earliest stage. However, the cellular and molecular mechanisms are still incompletely understood. This study aimed to explore the possible mechanisms by investigating the roles of DEX on differentiation, maturation & function of murine DCs and the effects of DEX on DCs via Toll-like receptor 4 (TLR4)-nuclear factor (NF)-κB mediated signal pathway.

Methods Immature DCs (imDCs) were cultured from murine bone marrow (BM) cells. We added DEX into culture medium at different time. The expression of CD11c, CD86 and I-Aβ (mouse MHC class II molecule) was determined by flow cytometry. We determined the expression of NF-κB and its inhibitory protein I-κBα by electrophoretic mobility shift assay (EMSA) and Western blotting, respectively. The productions of interleukin (IL)-12p70 and IL-10 in cell culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA).

Results DEX impaired differentiation of DCs from murine bone marrow progenitors, and inhibited lipopolysaccharide (LPS) induced maturation of DCs. DEX significantly inhibited NF-κB expression of normal DCs, the higher the DEX concentration or the longer the DEX treatment time, the more obvious the effect. However, DEX had little effect on LPS-induced NF-κB activation, and partially impaired LPS-induced I-κBα degradation. DEX significantly decreased LPS induced IL-12p70 production by DCs. Interestingly, our results showed a synergistic effect between DEX and LPS on the production of IL-10 by DCs.

Conclusions DEX inhibits the differentiation and maturation of murine DCs involved in TLR4-I-κB-NF-κB pathway, and also indirectly impairs Th1 development and interferes with the Th1-Th2 balance through IL-12 and/or IL-10 secretion by DCs.
involved in DEX effect on DCs development and function remain incompletely defined. In this study, we investigated the influences of DEX on differentiation, maturation, cytokine production and signal transduction pathway of DCs, and explored the possible pharmacological mechanisms of DEX on DCs.

METHODS

Animals and materials
Male C57BL/6 (H2b) mice (6–8 weeks old) were purchased from the Animal Center of Capital Medical University (Beijing, China) and were housed in specific pathogen free condition. DEX and LPS were purchased from Sigma Chemical Co. (USA). Fluorescein isothiocyanate (FITC) anti-mouse CD11c and FITC Armenian hamster IgG isotype control, phycoerythrin (PE) anti-mouse CD86 and PE rat IgG2a isotype control, PE anti-mouse I-Aβ and PE rat IgG2b isotype control were purchased from eBioscience Inc. (USA). Interleukin (IL)-12p70 and IL-10 enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D Systems (USA). The LightShift Chemiluminescent electrophoretic mobility shift assay (EMSA) kit was purchased from Pierce Biotechnology (USA). Anti-IκBα monoclonal antibody (mAb) was purchased from Santa Cruz Biotechnology (USA). We used the following double stranded oligonucleotides: biotin-labeled NF-κB probe: 5′-AGTTGAGGGGACTTTCCCAGGC-3′; un-labeled activator protein 1 (AP-1) probe: 5′-CGCTTGATGAGT-TAGCTGGTGCCGC-3′ (Shanghai Sangon Biological Engineering Technology & Services Co. Ltd., China).

Generation of bone marrow-derived DCs (BMDCs)
We generated DCs derived from murine bone marrow as previously described. Briefly, bone marrow cells were flushed from femora and tibiae of C57BL/6 mice and cultured in 6-well culture plates (2.0×10^6 cells/well) with a volume of 2 ml of RPMI 1640 culture medium, which supplemented with 10% fetal calf serum (FCS), 10 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) and 2 ng/ml IL-4. One day thereafter, half of culture medium was changed. Non-adherent and loosely adherent cells were harvested on day seven and washed twice with RPMI 1640 medium for use in subsequent experiments.

Fluorescence-activated cell sorting (FACS) analysis
To investigate the effects of DEX on DCs differentiation, various concentrations of DEX (10^{-5}, 10^{-7}, 10^{-9}, or 10^{-11} mol/L) were added into culture medium from the beginning of cell culture. Cells without any treatment were used as the control. After seven days, cells were collected and stained with FITC conjugated anti-mouse CD11c mAb for 45 minutes at 4°C. The expression of CD11c was analyzed by a FACS caliber flow cytometer (BD Biosciences, USA).

For assessment of the influences of DEX on DCs maturation, BMDCs were cultured in RPMI 1640 complete medium and classified into four groups: (1) control DC: cells were cultured under normal condition for eight days; (2) LPS-DC: cells were treated with LPS (1 µg/ml) for 24 hours from day seven of cell culture to induce maturation; (3) DEX-DC: cells were treated with 10^{-7} mol/L DEX for 72 hours from day five of cell culture; (4) DEX + LPS-DC: cells were pretreated with 10^{-7} mol/L DEX for 48 hours from day five of cell culture, and then LPS (1 µg/ml) was added for an additional 24 hours in order to induce DCs maturation. Finally, DCs were collected and washed twice with RPMI 1640, and then double-stained with FITC anti-mouse CD11c & PE anti-mouse CD86, or FITC anti-mouse CD11c & PE anti-mouse I-Aβ mAbs for 45 minutes at 4°C. The percentages of cells double positive for CD11c & CD86 or CD11c & I-Aβ were determined by flow cytometry. Isotype-matched antibodies were used as negative controls.

Preparation of cytoplasmic and nuclear proteins
Nuclear protein extracts were obtained using a Nuclear Extract Kit (ActiveMotif, Carlsbad, USA), according to the manufacturer's protocol. Cytoplasmic fractions were also saved. All preparation procedures were carried out at 4°C. Total protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, USA).

EMSA
We investigated the dose-effect relationship of DEX on NF-κB activation in DCs after seven days of cell culture. DCs were divided into 10 groups and cultured in 10 cm culture dishes (the cell density adjusted to 1×10^5/ml, 20 ml/dish). The control group was cultured under normal condition; LPS treated group was stimulated with 0.1 µg/ml LPS for 1 hour; DEX treated groups were treated with 10^{-5}, 10^{-7}, 10^{-9}, or 10^{-11} mol/L DEX for 2 hours; DEX+LPS groups were pretreated with 10^{-5}, 10^{-7}, 10^{-9}, or 10^{-11} mol/L DEX for 1 hour, then 0.1 µg/ml of LPS was added for an additional hour.

To investigate the time-effect relationship of DEX on NF-κB activation of DCs, after five days of cell culture, DCs were assigned to eight groups and cultured in 10 cm culture dishes (1×10^6 cell/ml, 20 ml per group). The control group was cultured under normal condition for 48 hours; the LPS treated group was stimulated with 0.1 µg/ml LPS for 1 hour; the DEX treated groups were treated with 10^{-5}, 10^{-7}, 10^{-9}, or 10^{-11} mol/L DEX for 6, 24, or 48 hours; DEX+LPS groups were pretreated with 10^{-5} mol/L DEX for 5, 23, or 47 hours, 0.1 µg/ml of LPS was added for an additional hour.

After stimulation, nuclear proteins were extracted as above. EMSA was performed using a light shift chemiluminescent EMSA kit (Pierce Biotechnology, USA) according to the manufacturer’s protocol. Briefly, 8 µg of the nuclear proteins and 1 µl of biotin-labeled
NF-κB probe were incubated. For competition assays, 1 µl of unlabeled NF-κB or AP-1 oligonucleotides (200-fold excess of biotin-labeled probe) were added and incubated for 15 minutes before the addition of the biotin-labeled probe. Samples were separated on a 6% non-denaturing acrylamide gel, pre-run for 45 minutes at 100 V, in 0.5×Tris boric acid-EDTA buffer at room temperature for 2 hours at 100 V. Gels were transferred to positively charged nylon membranes where the gel shifts were visualized by a chemiluminescent detection system (Pierce Biotechnology, USA).

**Western blotting**

Denatured cytoplasmic proteins (50 µg per sample) were separated by electrophoresis on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) along with molecular weight markers. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories), which were blocked with 5% nonfat dried milk in Tris-buffered saline that contained 0.1% Tween-20. The membranes were probed with the specific primary antibody (anti-IκBα or anti-β-actin antibody) and an appropriate secondary horseradish peroxidase-conjugated antibody (Boster Biological Technology, China) and visualized using the ECL detection system.

**ELISA**

On the 5th day of DCs culture, half the cells were harvested and pretreated with DEX (10⁻⁷ mol/L) for 48 hours. All DCs were then harvested and washed twice with RPMI 1640, counted and assigned to 12 aliquots and planted in 24-well plates (1×10⁶ cells/ml, 1 ml per well). Treatment protocols were as follows: (1) control group: normal DCs were cultured under normal condition; (2) LPS treated groups: normal DCs were treated with 1 µg/ml of LPS for 2, 4, 6, 12, 24, or 48 hours; (3) DEX treated groups: DEX-pretreated-DCs were cultured under normal condition; (4) DEX+LPS groups: DEX-pretreated-DCs were treated with 1 µg/ml of LPS for 2, 4, 6, 12, 24, or 48 hours. Cell supernatants were collected after LPS stimulation. IL-12p70 and IL-10 were measured by ELISA using the Quantikine Mouse IL-12p70 and IL-10 immunoassays kits from R&D systems according to the manufacturer’s protocol.

**Statistical analysis**

Each experiment was independently repeated at least three times. Data were presented as mean ± standard deviation (SD). SPSS 11.5 was used for data analysis. Statistical differences were evaluated using the unpaired t test and considered significant at the P < 0.05 level.

**RESULTS**

**DEX impaired differentiation of DCs derived from mouse bone marrow**

To determine if DEX stimulation influences differentiation of DCs, mouse bone marrow cells were treated with various concentrations of DEX. Treatment with 10⁻¹¹ mol/L DEX had no significant effect on the expression of CD11c of DCs (68.8%) as compared to normal control (67.7%), 10⁻⁹ mol/L DEX had little effect on the expression of CD11c (60.1%), however DEX at 10⁻⁷ and 10⁻⁵ mol/L significantly inhibited expression of CD11c by DCs, 23.1% and 19.6% (Figure 1). These results demonstrated that DEX impaired DCs differentiation from bone marrow derived progenitors. When the concentration of DEX was more than or equal to 10⁻⁷ mol/L, the effect was more significant. Therefore, we will apply treatment with 10⁻⁷ mol/L DEX for the subsequent study.

**DEX inhibited LPS-induced maturation of DCs derived from mouse bone marrow**

To investigate the DEX influence of LPS-induced maturation of DCs, DCs were treated with DEX (10⁻⁷ mol/L) alone, LPS (1 µg/ml) alone, or DEX+LPS. When LPS was added to the culture it significantly induced high expression of double positive CD86 & CD11c (42.6%) and I-A<sup>+</sup> & CD11c (50.7%) as compared with the control conditions (5.1% and 17.3%). However, DEX treatment...
Figure 2. DEX inhibited LPS-induced maturation of DCs derived from mouse bone marrow. Bone marrow cells from C57BL/6 (H2b) mice were cultured in conditioned medium and treated with LPS alone, DEX alone, or LPS+DEX. Cells cultured under normal condition were used as control. A: Cells were double-stained with FITC anti-mouse CD11c and PE anti-mouse CD86 for 45 minutes at 4°C; B: Cells were double-stained with FITC anti-mouse CD11c and PE-conjugated anti-mouse I-Aβ for 45 minutes at 4°C. Isotypic-matched antibodies were used as negative controls. Cells were examined by flow cytometry for CD11c, CD86 and I-Aβ expression.

significantly inhibited LPS-induced CD86 & CD11c double positive expression (12.9%) and also significantly inhibited LPS-induced I-Aβ & CD11c double positive expression (7.0%) (Figure 2). This result showed that DEX-treated DCs still expressed the low levels of CD86, I-Aβ and CD11c after LPS stimulation, DEX maintained DCs in immature state.

**DEX inhibited NF-κB expression of normal DCs, but had little effect on LPS-induced NF-κB activation**

In order to further study the effect of DEX on DCs differentiation and maturation, we monitored the LPS activation of the NF-κB signal transduction pathway. DCs were pretreated with various concentrations of DEX (10⁻⁵, 10⁻⁷, 10⁻⁹, or 10⁻¹¹ mol/L) for one hour, and then LPS was added for an additional hour. We determined NF-κB activation by EMSA. LPS (0.1 µg/ml) alone induced NF-κB activation (Figure 3A, Lane 3). DEX alone inhibited NF-κB activation in a dose dependent manner (Figure 3A, Lanes 4–7). However, various concentrations of DEX had little effect on LPS-induced NF-κB activation when DCs were pretreated with DEX for 1 hour (Figure 3A, Lanes 8–11). Prolonged DEX-treatment time for 6, 24, or 48 hours partially inhibited NF-κB expression of DCs, and longer DEX exposure produced increased inhibition (Figure 3B, Lanes 4, 6 and 8). However, even the longest exposure of DEX could not completely inhibit LPS induced NF-κB activation (Figure 3B, Lanes 5, 7 and 9).

**NF-κB DNA binding specificity was confirmed by competitive experiment.** NF-κB DNA binding was completely blocked by unlabeled NF-κB, but not unlabeled AP-1 (Figure 3C).

**DEX slightly increased I-κBα expression of normal DCs, and partially inhibited LPS-induced I-κBα degradation**

To determine LPS activation of the TLR4-IκB-NF-κB pathway, DCs were pretreated with various concentrations of DEX (10⁻⁵, 10⁻⁷, 10⁻⁹, or 10⁻¹¹ mol/L) for 1 hour, and then 0.1 µg/ml of LPS was added for an additional hour. The levels of IκBα were determined by Western blotting. LPS alone significantly induced IκBα degradation (Figure 4, Lane 2); various concentrations of DEX slightly increased IκBα expression (Figure 4, Lanes 3–6), but DEX still could not completely block LPS-induced IκBα degradation (Figure 4, Lanes 7–10).

**DEX inhibited LPS-induced IL-12p70 production, but increased LPS-induced IL-10 production by DCs**

To examine whether DEX influences LPS-induced cytokine productions by DCs, DCs were pretreated with DEX (10⁻⁷ mol/L) for 48 hours, and then treated with LPS (1 µg/ml) for 2, 4, 6, 12, 24, or 48 hours. LPS alone induced significant IL-12p70 production by DCs over the time course and DEX almost completely inhibited LPS-induced IL-12p70 production by DCs (Figure 5A). LPS alone also induced significant IL-10 production by DCs. DEX significantly increased LPS-induced IL-10 production by DCs, especially with shorter LPS exposure (4, 6 hours, P <0.01, Figure 5B), suggesting there is a synergistic effect between DEX and LPS on IL-10 production by DCs.

**DISCUSSION**

DCs are professional antigen-presenting cells that are not only critical for the induction of primary immune...
Figure 3. Effect of DEX on NF-κB activation in DCs. A: DCs were treated with LPS (0.1 µg/ml) alone for 1 hour, or treated with various concentrations of DEX (10⁻⁵, 10⁻⁷, 10⁻⁹, 10⁻¹¹ mol/L) alone for 2 hours, or pretreated with various concentration of DEX for 1 hour, then LPS (0.1 µg/ml) was added for an additional hour. Nuclear proteins isolated from DCs without any stimulation were used as control. Blank, no nuclear proteins were added (the same as in following studies). B: DCs were treated with LPS (0.1 µg/ml) alone, or treated with DEX (10⁻⁷ mol/L) alone for 6, 24, 48 hours respectively, or pre-treated with DEX for 5, 23, 47 hours, and then LPS (0.1 µg/ml) was added to treat for an additional hour. C: Nuclear proteins from LPS (0.1 µg/ml, 1 hour) treated DCs were subjected to DNA probe competition experiments using unlabeled NF-κB or AP-1 probes to demonstrate specific NF-κB binding. 200× concentrated unlabeled NF-κB oligonucleotides were used to compete with biotin-labeled NF-κB oligonucleotides, whereas 200× concentrated unlabeled AP-1 oligonucleotide were used as nonspecific control probe.

Figure 4. Effect of DEX on IκBα activation in DCs. DCs were treated with LPS (0.1 µg/ml) alone, or treated with various concentrations of DEX (10⁻⁵, 10⁻⁷, 10⁻⁹, 10⁻¹¹ mol/L) for 2 hours, or pre-treated with various concentrations of DEX for 1 hour, and then added LPS to treat for an additional hour; DCs without any stimulation were used as control. The cytoplasmic proteins were analyzed by Western blotting with antibodies to IκBα or β-actin. β-actin was used as an endogenous reference.

Figure 5. Effects of DEX on LPS-induced IL-12p70 (A) and IL-10 (B) production by DCs. On day five of DCs culture, half of the cells were harvested and pretreated with DEX (10⁻⁷ mol/L) for 48 hours. Normal DCs and DEX pre-treated DCs were collected and washed and treated as follows: (1) control groups: normal DCs were cultured under normal condition; (2) LPS treated groups: normal DCs were only treated with LPS (1 µg/ml) for 2, 4, 6, 12, 24, or 48 hours; (3) DEX treated groups: DEX-pretreated DCs were cultured under normal condition; (4) DEX+LPS groups: DEX-pretreated DCs were treated with LPS (1 µg/ml) for 2, 4, 6, 12, 24, or 48 hours. Cell-free supernatants were collected for detection of IL-12p70 and IL-10 by ELISA. Data are expressed as mean ± SD (n=3). *P <0.01 compared with DEX+LPS group.

responses, but may also be important for the induction of immunological tolerance.1-3 DCs undergo maturation after sensing environmental triggers such as proinflammatory cytokines and/or stimuli that engage TLRs and other pattern-recognition receptors, or CD40.13 Both immature and mature DCs are expected to exert important functions, imDC poorly stimulate T cells and thereby induce T cell tolerance, mDC have a potent capacity to stimulate T cells. DEX is one of the widely used drugs in treatment of many autoimmune and allergic diseases.8 It is very important both for basic research and clinical therapy whether DEX plays an important role in
DC differentiation, maturation and function.

CD11c is a differential marker for DC. When mouse bone marrow cells are cultured in DC conditioned medium for seven days about 70% of the cells are CD11c positive. We found that low doses DEX, 10^{-11} or 10^{-9} mol/L, had little effect on CD11c expression of DCs, but that higher doses, 10^{-7} and 10^{-5} mol/L, significantly impaired CD11c expression. These indicated that DEX inhibited mouse DCs differentiation and, when the concentration of DEX was equal to or greater than 10^{-7} mol/L, the effect was more significant. To further evaluate DEX effects on DC maturation, we examined the CD86 and I-A^b expression on CD11c positive cells in different treatment groups. LPS from Escherichia coli, is a known DC maturation agent, can bind TLR4 on DC to trigger the signal cascade for DC maturation. LPS treatment alone significantly induced CD86 and I-A^b expression of DCs as compared to normal cultured DCs. However, DEX (10^{-7} mol/L) treatment almost completely blocked CD86 and I-A^b expression and LPS stimulation only induced low level expression of CD86 and I-A^b. VanClee et al also found that DEX treated DC expressed low levels of costimulatory molecules, such as CD80 and CD86, as well as major histocompatibility complex (MHC) class II molecules, and this immature phenotype did not retained after the stimulation of maturation. Interestingly, Abe et al demonstrated that despite DEX impaired differentiation of both myeloid DC and plasmacytoid DC, and the suppression of plasmacytoid DC differentiation from bone marrow precursors in vitro, the expression of cell surface MHC class II (I-A^b) and B7 family costimulatory molecules (CD80 and CD86) on plasmacytoid DC was not different between control and DEX-treated cells. These results indicate DEX can impair differentiation and maturation of DCs, but may not inhibit all cell surface molecule expression involved in DC differentiation and maturation, such as I-A^b or B7, in different microenvironments.

An early study showed that despite DEX increased mRNA expression of TLR2, 3 and 4 on DCs derived from human peripheral blood mononuclear cells (PBMCs), their stimulation via TLR-derived signals did not induce NF-κB activation because DEX presence was restricted to the time of DC maturation. Our previous study also demonstrated that DEX increased expression of TLR4 mRNA level in DCs derived from mouse bone marrow. However, it has not been completely clarified whether DEX impairs the TLR4 triggered NF-κB signal transduction pathway. We analyzed signal transduction pathway involved in DC activation by EMSA. DCs were pretreated with various concentrations of DEX for 1 hour, and then treated with LPS. It was seen that DEX could inhibit NF-κB expression by normal DCs in a dose dependent manner. However, DEX had almost no effect on LPS-induced NF-κB activation following DEX pretreatment for one hour. We found that DEX could inhibit NF-κB expression of normal DCs when the DEX treatment time was longer, and the effect was time dependent. However, DEX still could not completely inhibit LPS induced NF-κB activation, even with the longest DEX exposure. I-kB is an upstream regulatory molecule for the NF-κB transcriptional factor that can inhibit NF-κB activation. DEX treatment can slightly increase I-kB expression but it could not completely block LPS induced I-kB degradation.

According to our results, DEX does impair LPS-TLR4 triggered NF-κB activation, however there are other mechanisms involved in the DEX effect on NF-κB activation and DC maturation. Previous studies have demonstrated two mechanisms of glucocorticoid effects on NF-κB activation. First, activated glucocorticoid receptors directly interact with and inhibit activated NF-κB subunits. Second mechanism is involved in the transcriptional activation of the I-kB gene in response to treatment with glucocorticoids. Glucocorticoids can up-regulate the I-kB protein level and function to block nuclear translocation of NF-κB and DNA-binding. On the other hand, LPS can also stimulate DC maturation through other signal transduction pathways, such as PI3-kinase, mitogen-activated protein kinases (MAPKs), and c-Jun pathway. Recently, Zanoni et al reported that mouse DCs stimulated with LPS induced Src-family kinase and phospholipase Cε2 activation, have an influx of extracellular Ca^{2+} and calcineurin-dependent nuclear factor of activated T cell (NFAT) translocation. The initiation of this pathway is independent of TLR4 engagement, and dependent exclusively on CD14. Maybe these results elicit a de novo pathway for LPS-induced DC maturation.

IL-12 and IL-10 are important cytokines secreted by DCs. Splenic DCs constitutively produce low amounts of the inducible IL-12p40 subunit. Ligation of either CD40 or MHC class II molecules on DCs triggers production of high levels of the p70 heterodimer. IL-12 is dominant in directing the development of Th1 cells that produce high amounts of IFN-γ, and influence the Th1-Th2 balance. Our results showed that LPS stimulated DCs to produce IL-12p70 in a time-dependent manner, but DEX pretreatment completely blocked LPS-induced IL-12p70 expression. This result imples that DEX may not only directly impair DC differentiation and maturation, but also indirectly inhibit Th1 development and interfere with Th1-Th2 balance through suppression of IL-12 production by DCs. IL-10 is a pleiotropic cytokine capable of suppressing proinflammatory cytokine production by antigen-presenting cells (APCs) and other cells and is capable of limiting Th1 differentiation. While DEX inhibited IL-12 production by DCs, LPS and DEX showed a synergistic induction of IL-10. This further indicates that DEX may impair Th1 cell development and regulate Th1-Th2 balance by the promotion of IL-10 secretion by DCs.

In summary, we demonstrated that DEX impaired DC
differentiation and LPS-induced maturation through the TLR4-IκB-NF-κB pathway. It also indirectly impaired Th1 development and interfered with Th1-Th2 balance through IL-12 and/or IL-10 secretion by DCs. However, DEX may interact with other signal transduction pathways, such as NFAT, AP-1 or the MAPK pathway, to affect DC differentiation and maturation and clarification of these mechanisms requires further study.

REFERENCES

14. Vanclee A, Schouten HC, Bos GMJ. Murine dendritic cells that are resistant to maturation are unable to induce tolerance to allogeneic stem cells. Transpl Immunol 2006; 16: 8-13.

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