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MECHANISMS

Nerve growth factor promotes expression of costimulatory molecules and release of cytokines in dendritic cells involved in Th2 response through LPS-induced p75NTR

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Abstract

Introduction: Nerve growth factor (NGF) plays an important role in asthmatic inflammatory responses. However, the effects of NGF on dendritic cells (DCs) in asthmatic inflammation remain unknown. Therefore, we examined the effects of NGF on co-stimulatory molecules and the release of cytokines after ovalbumin (OVA) and a low dose of LPS (low LPS) stimulation of dendritic cells. **Methods:** Bone-marrow-derived dendritic cells (BMDCs) were collected from 6- to 8-week-old wild or TLR4^{-/-} mice. BMDCs were treated with OVA and/or low LPS for 12h, and then stimulated with NGF for 24h. ELISA and flow cytometry were performed to measure TSLP, IL-6, IL-10, and IL-12 production and MHCII and CD86 expression on BMDCs. BMDCs were exposed to p75 neurotrophin receptor (p75NTR) inhibitor (TAT-Pep5) or NF-κB inhibitor (QNZ) 30 min prior to NGF 1 h after NGF intervention, the levels of RelA and RelB in cytoplasmic and nuclear were detected by west blot. Co-cultured BMDCs with naïve CD4⁺ T cells, and ELISA was used to detect IL-4 and INF-γ levels. **Results:** NGF was found to markedly promote OVA and low LPS-induced expression of MHCII, CD86, secretion of TSLP and IL-6, and Th2-response-stimulating capacity of BMDCs. NGF affected BMDCs through LPS-induced p75NTR expression. TAT-Pep5 or QNZ could attenuate the promotive effect of NGF. **Conclusions:** NGF facilitates OVA with lowLPS-induced maturation of mouse BMDCs through LPS-up-regulated p75 NTR via activation of NF-κB pathways, providing another mechanism for the involvement of NGF in the Th2 response.

Keywords

Nerve growth factor, dendritic cells, ovalbumin, Th2 response

History

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Introduction

The number of patients with asthma has increased rapidly over the past 20 years. It has been estimated conservatively that there are currently 300 million asthma patients worldwide, with 1 in every 250 deaths attributable to asthma (Global Burden of Asthma 2014). Asthma is a common chronic inflammatory disease of the airway that involves multiple inflammatory cells. Mounting evidence shows that during an asthmatic episode, dendritic cells (DCs) play a vital role in T cell activation and Th2/Th1 ratio offsetting, which are initial allergen-associated asthmatic responses [1–5]. During asthmatic inflammatory responses, DCs present antigens to T cells, thereby activating CD4⁺ naïve T cells and furthering differentiation towards Th2 cells. This process offsets the Th2/Th1 ratio and increases the secretion of inflammatory cytokines, including interleukin 4 (IL-4), IL-5, and IL-13, which participate in chronic allergic airway inflammation [5–7].

Some epidemiological investigation showed that LPS exposure increases individual susceptibility and the severity of asthma [8–10]. Toll-like receptor 4 (TLR4), expressed on the surface of antigen-presenting cells (APCs), recognizes LPS and plays a key role in the development of asthmatic symptoms [11]. Airway inflammation was significantly decreased in TLR4^{-/-} mice compared with wild-type mice [12]. Stimulation of an ovalbumin (OVA)-sensitized mouse model with a low dose of LPS (lowLPS) significantly increased the bronchoalveolar lavage fluid (BALF), cell propagation, eosinophil infiltration, pulmonary inflammation, and airway mucus secretion, and draining lymph nodes caused type-2 T-helper cell (Th2) inflammatory responses [13]. It has been demonstrated that Th2 inflammatory responses in OVA-sensitized mice activated the DCs via the TLR4 signaling pathway [14].

Nerve growth factor (NGF) is a member of the NGF family. It plays a central role in the regulation of neurogenesis, neural differentiation, neural survival, neural remodeling, and neural repair [15]. The 15 NGF has also been found to serve other functions, especially in allergic disease [16]. Increased levels of circulating NGF have been reported in patients with allergic diseases such as bronchial asthma [17, 18]. Similar result was found in animal models of asthma and the increased NGF levels have been shown to positively correlated with airway

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hyperresponsiveness levels, [19–21] Th2 airway inflammation, and airway remodeling [21–23]. After injection of an anti-NGF antibody or small interfering RNA targeting NGF injection, asthmatic symptoms in mice were significantly alleviated [19, 23]. NGF promotes the occurrence of allergic immune responses via corresponding receptors that are bound to the surfaces of the cell, such as tropomyosin receptor kinase A (p140trkA) and p75 neurotrophin receptor (p75NTR) [24, 25].

Currently, the effects of NGF in DCs in asthmatic inflammation remain largely unknown. In the present study, we investigated whether NGF promoted expression of the costimulatory molecule and cytokine release in DCs induced by OVA with lowLPS and further explored the potential mechanisms.

Materials and methods

Animals and main reagents

C57BL/6 mice were purchased from the SLAC Animal Laboratory (Hunan, China). C57BL/10ScNJNju mice (TLR4^{-/-} mice) and C.Cg-Tg (DO11.10) 10Dlo/JNju mice were purchased from the Nanjing Biomedical Research Institute of Nanjing University (Jiangsu, China). All animals were raised in a facility that was free of specific pathogens. The experiments were conducted in 6–8-week-old mice, 30 wild-type C57BL/6 mice, 6 TLR4^{-/-} mice, and 6 DO11.10 mice were used in this study, which was performed in 2015. All the experimental processes were approved by the Animal Protection and Utilization Committee of the Xiangya School of Medicine of Central South University and conducted according to guidelines published by the U.S. National Institutes of Health.

The PC12 cell line (KG111) was purchased from Nanjing KeyGen Biotech. Co. Ltd. (Nanjing, China). Recombinant mouse granulocyte/macrophage colony-stimulating factor (GM-CSF) (415-ML-010), IL-4 (404-ML-010), IL-2 (402-ML-020), and NGF (1156-NG-100) were purchased from R&D. CD11c immunomagnetic beads (130-092-465) and the Naive CD4⁺ T Cell Isolation Kit (130-093-227) were purchased from Miltenyi Biotec. In addition, 2-mercaptoethanol (ES-007-E) was purchased from Merck Millipore. Fetal bovine serum (FBS, 12007C-500ML), LPS (L4391-1MG), and OVA (A5503-1G) were purchased from Sigma. Detoxi-Gel Endotoxin Removing Gel (20344) was purchased from Invitrogen.

A ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit (L00350) was purchased from GenScript. Anti-mouse CD11c fluorescein isothiocyanate (FITC) (11-0114), anti-mouse MHCII phycoerythrin (PE) (12-5321), anti-mouse CD86 APC (17-0862), and the corresponding isotype control antibodies were purchased from eBioscience. Anti-mouse p75NTR (D4B3) and anti-mouse Relb antibodies (4922) were purchased from Cell Signaling Technology. Anti-NF-κB p65 (ab32536) and anti-mouse Trk A (ab76291) antibodies were purchased from Abcam. TAT-Pep5 (506181) was purchased from Calbiochem. QNZ (EVP4593 10 nM) was purchased from Selleck. IL-6 (ELM-IL6), IL-10 (ELM-IL10), IL-4 (ELM-IL4), INF-γ (ELM-INFγ), and IL-12 (ELM-IL12 P40P70) enzyme-linked immunosorbent assay (ELISA) kits were purchased from RayBiotech, Inc. The thymic stromal lymphopoietin (TSLP) (434107) ELISA kit was purchased from BioLegend.

LPS removal and OVA production

Following the requisite instructions, Detoxi-Gel Endotoxin Removing Gel was used to remove 99% of all LPS content from the purchased OVA. The purchased OVA was purified, and the ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit was then used to measure the LPS content according to the manufacturer's instructions. LPS content was measured at less than 1 endotoxin unit/mL for all 3 detection stages. Purified OVA was used in the experiments.

Bone-marrow-derived dendritic cells (BMDCs) culture

The method proposed by Weimershaus and van Endert was modified and then used to develop the BMDCs culture [26]. The BMDCs and TLR4^{-/-} BMDCs were collected and inoculated on a culture plate with a cell density of 10⁶/mL using a 1,640 medium of GM-CSF (20 ng/mL), IL-4 (10 ng/mL), 2-mercaptoethanol (50 μM), L-glutamine (4.5 μM), fetal bovine serum (10%), penicillin (100 U/ml) and streptomycin (100 μg/ml). The BMDCs were inoculated on culture plates on day 0. The medium was changed by half every other day. On the sixth day, following the requisite instructions, immunomagnetic beads were used to screen out CD11c positive cells (CD11c⁺BMDCs) (CD11c⁺BMDCs purity levels determined via flow cytometry were generally higher than 95%). BMDCs were then cultured in a GM-CSF and IL-4-free medium that was otherwise similar to the aforementioned medium for the subsequent experiment.

Treatment of BMDCs

First, on day 6, BMDCs were treated with pOVA (100 μg/mL) with/without LPS (10 ng/ml) for 12 h and then washed 3 with culture medium to exclude the presence of OVA and LPS. Then, the BMDCs were stimulated with NGF at a concentration of 100 ng/mL 24 h later [27, 28]. Cell morphology was observed with an inverted microscope. BMDC surface molecule expression (CD86 and MHCII) was determined using flow cytometry. Second, for the experiment to determine the concentration gradient of NGF, BMDCs were stimulated using pOVA (100 μg/mL) + lowLPS (10 ng/mL) for 12 h after washed 3 times the BMDCs were stimulated with recombinant NGF at the different dosage (0 ng/mL, 20 ng/mL, 50 ng/mL, 100 ng/mL, and 200 ng/mL) for 24 h then cells were collected, the expression of BMDCs surface molecules (CD86 and MHCII) was determined by flow cytometry.

Based on these results, an NGF concentration of 100 ng/mL was selected for the subsequent experiment. Third, 6, 12, 18, and 24 h after 100 ng/mL NGF intervention, TSLP, IL-6, IL-10, and IL-12 levels of cytokines released by BMDCs were examined using the ELISA method. Fourthly, for the NGF receptor expression determination experiment, Cultured BMDCs of the wild and TLR4^{-/-} mice were divided into a control group, pOVA+LPS, and pOVA+LPS+NGF group. In accordance with the aforementioned method, respective PBS, pOVA+lowLPS, and pOVA+lowLPS were individually added to one these 3 groups to stimulate BMDCs for 12 h. then washed in the 1,640 medium three times, after which isometric PBS, PBS, and NGF were individually added to one these three groups to stimulate the BMDCs for 24 h. All BMDCs were

collected, and surface molecule expression was determined by flow cytometry.

A Western blot was used to examine BMDCs NGF receptor expressions (TrkA and p75 (NTR)). Fifthly the corresponding NGF receptor inhibitor (TAT-Pep5 20 $\mu\text{g}/\text{mL}$) or NF- κB inhibitor (QNZ 10 nM) was added 30 min before the NGF intervention; 1 h after addition of NGF, the BMDCs' cytoplasmic and nuclear proteins were extracted, and the RelA and RelB expression levels were determined; 24 h after the NGF intervention, the BMDCs surface molecule (CD86 and MHCII) expression was determined by flow cytometry. The culture supernatant was also collected, and the release of culture supernatant cytokines was determined using an enzyme-linked immunosorbent assay (ELISA).

PC12 cells culture

PC12 cells were cultured in DMEM supplemented with 10%FCS. Half the medium was changed every other day. To induce differentiation, NGF at a concentration of 25 ng/mL was added to medium 5 days before Western blot.

Western blot analysis

BMDCs or PC12 cells were extracted, collected, and washed twice in PBS. Following the requisite instructions, radio immunoprecipitation assay lysis buffer (Beyotime) with phenylmethanesulfonyl fluoride (PMSF) (Beyotime) was then used to extract the protein. The lysis product was centrifuged for 20 min at 12,000 rpm, and the supernatant (the total protein) was collected. When necessary, the cytoplasmic and nuclear proteins were separately extracted using the cytoplasmic and nuclear protein extraction kits (Beyotime), respectively.

Following the requisite instructions, 200 μL of the PMSF-containing cytoplasmic protein extraction reagent was added to the samples, which were vortexed for 5 s and then placed in an ice bath for 15 min. Cytoplasmic protein extraction reagent B was then added to the samples, which were vortexed for 5 s and then placed in an ice bath for 1 min. Each sample was then centrifuged for 5 min at 12,000 rpm, and the supernatant (cytoplasmic protein) was collected. After the residual supernatant in each sample was fully removed, PMSF-containing nuclear protein extraction reagent was added. Each sample was then

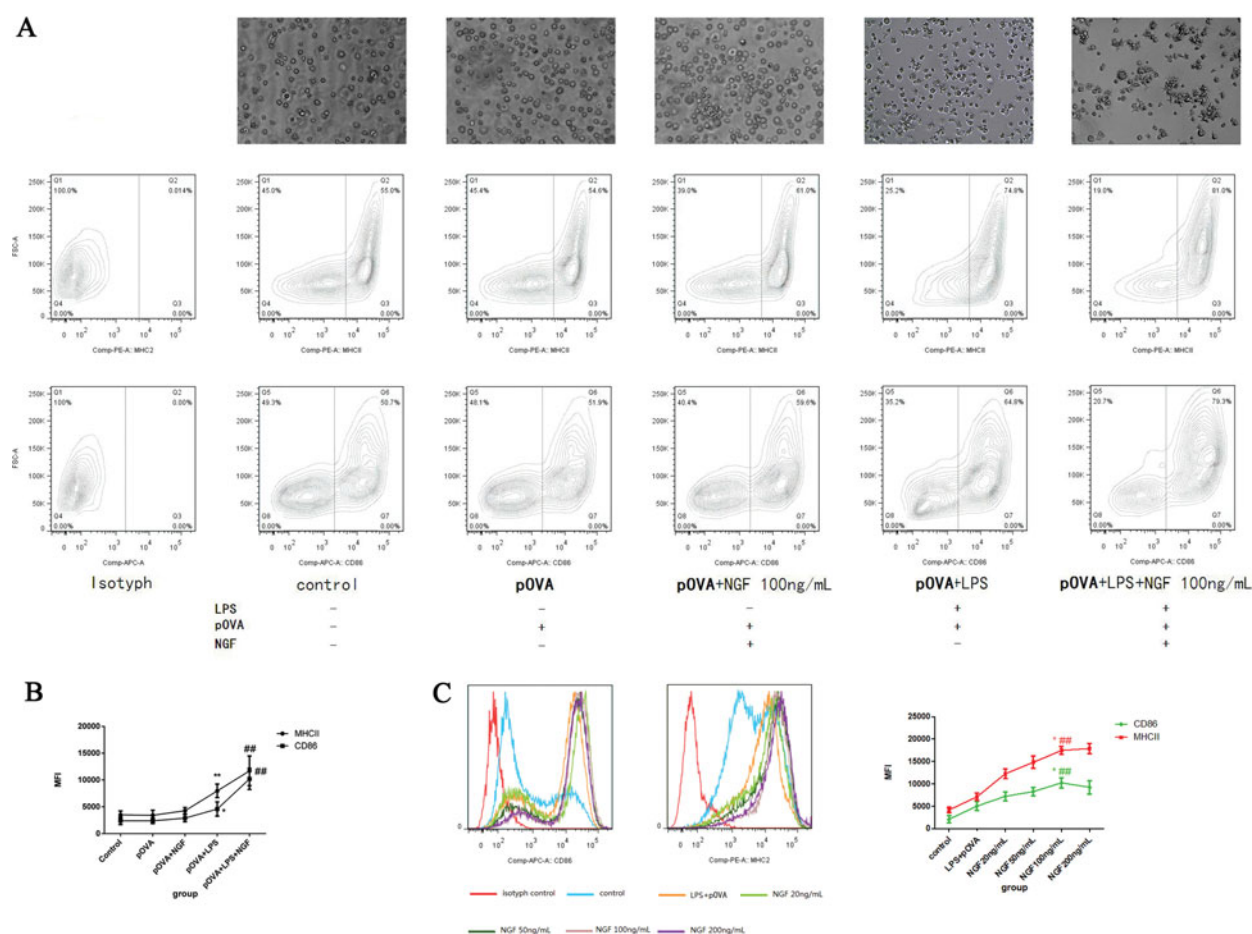


Figure 1. NGF promotes BMDCs Costimulatory molecule expression due to OVA+lowLPS. OVA and NGF interventions were conducted in BMDCs with or without LPS. The percentage of MHCII⁺CD86⁺BMDCs not increased after stimulation with pOVA alone, after addition of pOVA alongside lowLPS increased significantly. The relative number of MHCII⁺CD86⁺BMDCs increased more dramatically after addition of NGF than in the pOVA+LPS group. Determining the MFI (mean fluorescence intensity) of MHCII and CD86 in BMDCs produced the same results (* $P < 0.05$, ** $P < 0.01$ compared with the control group, ### $P < 0.01$ compared with the OVA+LPS group). Different NGF concentrations were then used to stimulate the BMDCs. The results demonstrated that NGF promoted the expression of MHCII and CD86 in BMDCs OVA+lowLPS in a dose-dependent manner. The expression of MHCII and CD86 in BMDCs increased with increasing NGF concentrations (0, 20, 50, and 100 ng/mL). Peak expression of MHCII and CD86 on BMDCs surfaces was determined at NGF concentrations of 100 ng/mL. However, increasing the NGF concentration from 100 ng/mL to 200 ng/mL did not increase BMDCs surface molecule expression further. (* $P < 0.05$ when comparing CD86 and MHCII levels between 100 ng/mL and 50 ng/mL NGF concentrations; ### $P < 0.01$ when comparing CD86 and MHCII levels of the LPS+OVA+NGF (100 ng/mL) group with those of the LPS+OVA group.) This experiment was performed in triplicate.

placed in an ice bath for 30 min, during which the sample was vortexed once every 2 min. Each sample was then centrifuged for 10 min at 12,000 rpm, and the supernatant (nuclear protein) was collected. A bicinchoninic acid protein assay kit was used to measure the protein concentration (Beyotime).

The protein samples (50 μ g) along with the molecular weight marker (Bio-Rad) were loaded onto a 10% polyacrylamide gel, after electrophoresis the protein was transferred to a polyvinylidene fluoride membrane (Millipore Corporation). The membranes were blocked in western blocking buffer (Beyotime) for 2 h at 20°C. The protein-bearing membrane was then incubated with the corresponding primary antibody overnight at 4°C. The diluted primary antibody concentrations used in the experiment were as follows: anti-p140trkA (1:1,000), anti-p75NTR (1:1,000), anti-Relb (1:1,000), and anti-RelA (1:100,000). The membrane was then incubated in horseradish peroxidase-coupled secondary antibody for 2 h following the requisite instructions; BeyoECL Plus (Beyotime) was used to develop the protein bands, which were imaged using the Bio-Rad ChemiDoc MP imaging system. Grey protein band values were analyzed using ImageJ.

Flow cytometry

BMDCs were centrifuged at 1,000 rpm for 10 min, collected, and washed twice with PBS. PBS was then added to the BMDCs to adjust the cell concentrations to 1×10^6 cells/mL. FITC-, APC-, and PE-labeled anti-mouse CD11c, CD86, and

MHCII antibodies and the corresponding isotype control antibodies were each added to an Eppendorf (EP) tube with BMDCs (volume: 100 μ L). Each EP tube was incubated at 4°C for 30 min in the dark. BMDCs in each tube were then washed 3 times with PBS, re-suspended in 500 μ L of PBS, and examined by flow cytometry. FlowJo software was used to analyze and calculate geometric fluorescence intensities.

Co-culture of BMDCs with CD4⁺ naive T cells

BMDCs (1×10^5) were treated with lowLPS+OVA, with or without NGF and p75 inhibitor. Untreated DCs were used as control. CD4⁺ naive T cells were purified from spleens of DO11.10 transgenic mice using CD4⁺ Miltenyi MACs beads according to the manufacturer's instructions. CD4⁺ naive T cells (5×10^5) were incubated with 1×10^5 BMDCs to obtain a final T cell/BMDCs ratio of 5:1. Cultured cells were incubated for 48 h at 37°C. The medium was then removed, and fresh complete medium containing 10 ng/mL mouse rIL-2 was added. After incubation for 72 h at 37°C, the cell suspension was collected for cytokine analysis by ELISA.

ELISA

The suspended cells were collected and centrifuged for 10 min at 1,000 rpm, and the supernatant was then collected and stored at -80°C for subsequent examinations. Following the requisite instructions, ELISAs were used to measure cytokine concentrations (IL-6, IL-10, IL-12, TSLP, IL-4, and INF- γ) in the cell supernatants.

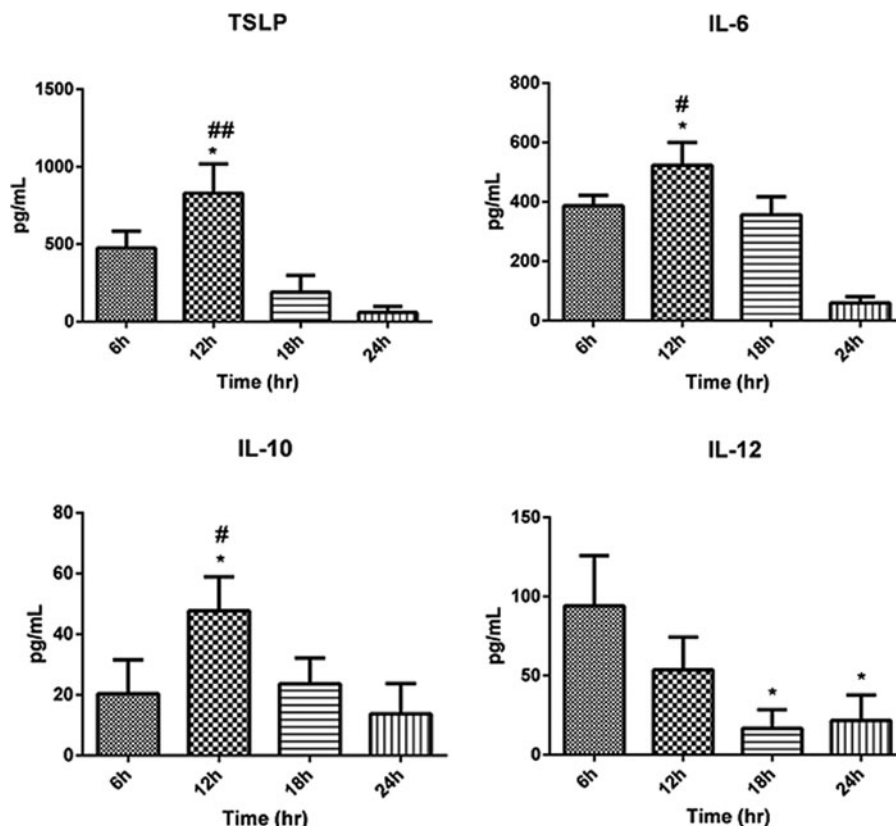


Figure 2. NGF promotes the release of cytokines from BMDCs through OVA+lowLPS. BMDCs production of TSLP, IL-6, IL-10, and IL-12 was determined by ELISA at 4 different time points (6 h, 12 h, 18 h, 24 h). Data are expressed as mean $\pm$ SE from 3 independent experiments. (* $P < 0.05$ compared with cytokine production at 6 h, # $P < 0.05$ and ## $P < 0.01$ compared with cytokine production at 18 h).

Statistical methods

The experimental data were statistically analyzed using Graph-Pad Prism5 software. All of the data are expressed as means $\pm$ SD. Student *t*-tests were used to examine the groups for differences. *P*-values of < 0.05 were considered statistically significant.

Results

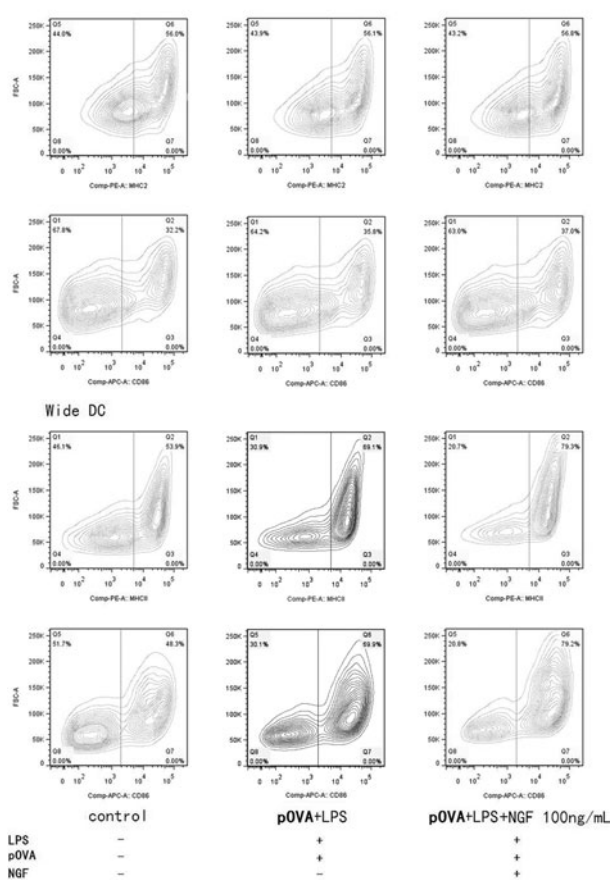
NGF promotes the expression of BMDCs costimulatory molecule due to OVA+lowLPS

First, whether NGF could regulate the costimulatory molecule expression of BMDCs, OVA, and NGF interventions was determined in BMDCs with or without LPS. The results indicated that the relative number of MHCII⁺CD86⁺BMDCs did not increase after stimulation with pOVA alone. After an addition of lowLPS alongside pOVA, the relative number of MHCII⁺CD86⁺BMDCs increased significantly. The relative number of MHCII⁺CD86⁺BMDCs increased more obviously in the pOVA+LPS+NGF group than in the pOVA+LPS group. Determining the MFI (mean fluorescence intensity) of MHCII and CD86 in BMDCs produced the same results (compare the pOVA+LPS group with the control group: MHCII 7973.7 ± 732.2 versus 3515.3 ± 419.8 $P < 0.01$ CD86 4599.7 ± 778.1 versus 2394.3 ± 402.9 $P < 0.05$, compare the pOVA+LPS+NGF group with the pOVA+LPS group: MHCII 11703.3 ± 1597.2 versus 7973.7 ± 732.2 $P < 0.01$ CD86 11209.7 ± 1139.7 versus 4599.7 ± 778.1 $P < 0.01$) (Figures 1A, B). To further analyze NGF in the regulation of MHCII and CD86 in BMDCs, BMDCs with different NGF concentrations were then used to stimulate the BMDCs. The results demonstrated that NGF promoted the expression of MHCII and CD86 in BMDCs OVA+lowLPS in a dose-dependent manner. The expression of MHCII and CD86 in BMDCs increased with increased NGF concentrations (0, 20, 50, and 100 ng/mL) (Figure 1C). Peak expressions of MHCII and CD86 on BMDCs surfaces were obtained at NGF concentrations of 100 ng/mL. However, increasing the NGF concentration from 100 ng/mL to 200 ng/mL did not further increase BMDCs surface molecule expression. Therefore, 100 ng/mL of NGF was used in the following studies (compare NGF100 ng/mL group with NGF50 ng/mL group: MHCII 17466.7 ± 1484.4 versus 14833.3 ± 2400.7 $P < 0.05$ CD86 10236.3 ± 1956.2 versus 8282.7 ± 1597.4 $P < 0.05$, compare NGF100 ng/mL group with LPS+OVA group MHCII 17466.7 ± 1484.4 versus 4178.7 ± 992.2 $P < 0.01$ CD86 10236.3 ± 1956.2 versus 2096.0 ± 1358.5 $P < 0.01$) (Figure 1C).

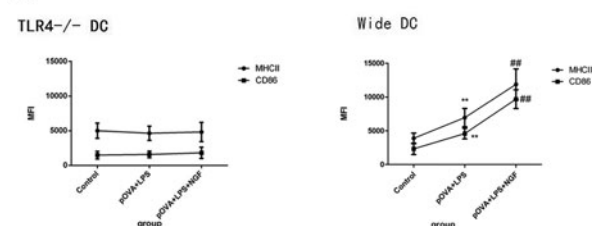
NGF promotes the release of cytokines from BMDCs due to OVA+lowLPS

OVA and lowLPS were used to treat cells as described above. NGF (100 ng/mL) was added to the BMDCs, and the culture supernatant was collected 6 h, 12 h, 18 h, and 24 h after stimulation. As shown, 100 ng/ml NGF could significantly increase the TSLP, IL-6, and IL-10 production by BMDCs pretreated with OVA+lowLPS BMDCs. In addition, 12 h after NGF stimulation, TSLP and IL-6 IL-10 reached maximal induction, then started a decline that lasted for 24 h (compare 12h

A TLR4^{-/-} DC



B



C

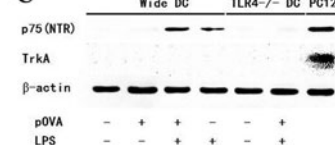


Figure 3. NGF promotes BMDCs maturation due to OVA+lowLPS after LPS-induced p75NTR expression in BMDCs. NGF did not have a promotion effect on TLR4^{-/-} BMDCs surface molecule expression ($*P < 0.05$ relative to the control group); (C) Western blotting was used to detect expression of NGF receptors, p140trkA and p75NTR, in wild-type DCs or TLR4^{-/-} DCs under stimulation of OVA, LPS, or OVA+lowLPS. PC12 served as the positive control. TLR4^{-/-} BMDCs did not express NGF receptors. p75NTR was expressed on the surfaces of BMDCs in the presence of TLR4 signaling, but p140trkA was not. (C) BMDCs did not express p140trkA after LPS stimulation. This experiment was performed in triplicate.

with 6h(pg/mL): IL-6 523.3 ± 44.1 versus 386.7 ± 20.1 $P < 0.05$, IL-10 47.7 ± 6.5 versus 20.3 ± 6.5 $P < 0.05$, TSLP 829.3 ± 108.8 versus 476.0 ± 62.8 $P < 0.05$, compare 12 h with 18 h(pg/mL): IL-6 523.3 ± 44.1 versus 356.0 ± 35.4 $P < 0.05$, IL-10 47.7 ± 6.5 versus 23.7 ± 4.9 $P < 0.05$, TSLP

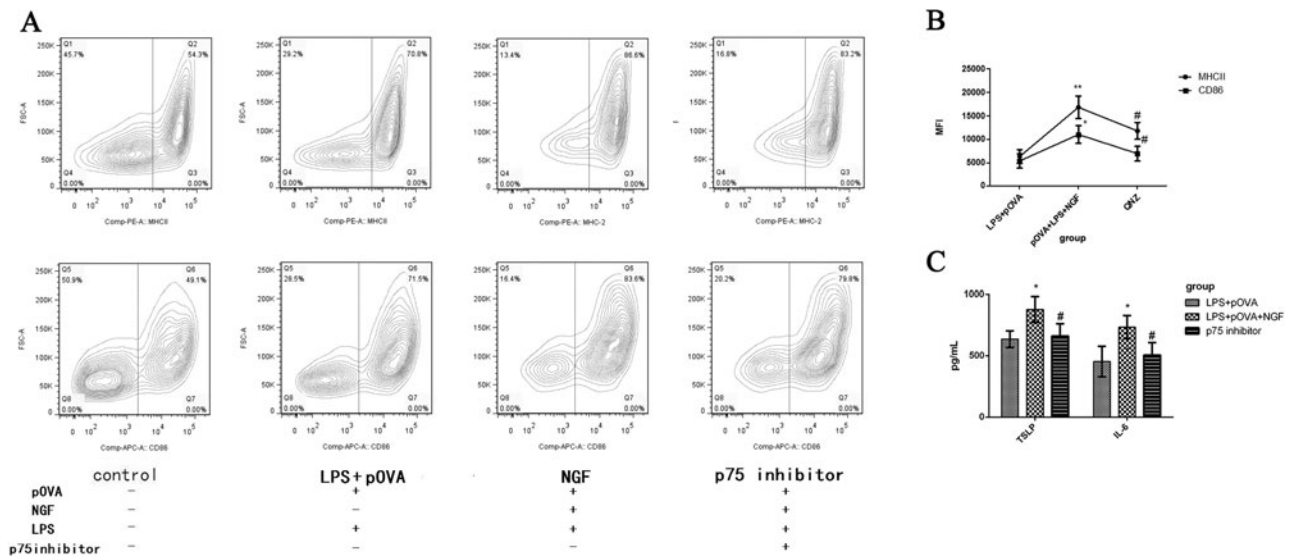


Figure 4. NGF promotes BMDCs costimulatory molecule expression and cytokine release via inducible p75NTR. The p75NTR inhibitor TAT-Pep5 (20 μ g/mL) was added 30 min before NGF intervention. (A) p75NTR inhibitor significantly reduced expression costimulatory molecules (MHCII and CD86) of BMDCs. Corresponding MFI value variations (* $P < 0.05$ and ** $P < 0.01$ NGF when comparing the NGF group with the LPS+OVA group; # $P < 0.05$ when comparing the p75 inhibitor group with the NGF group). (C) TSLP and IL-6 levels in the BMDCs culture supernatant were measured. Levels of TSLP and IL-6 in the BMDCs culture supernatant were highest in the LPS+pOVA+NGF group and they decreased after p75NTR inhibitor pretreatment. (# $P < 0.05$, ## $P < 0.01$). This experiment was performed in triplicate.

829.3 $\pm$ 108.8 versus 191.0 $\pm$ 62.0 $P < 0.01$). After NGF intervention, the level of IL-12 decreased to its lowest level at 18 h (compare 18 h with 6 h (pg/mL): IL-12 16.7 $\pm$ 6.8 versus 94.0 $\pm$ 18.4 $P < 0.05$, compare 24 h with 6 h (pg/mL): IL-12 21.7 $\pm$ 9.3 versus 94.0 $\pm$ 18.4 $P < 0.05$). These findings indicate that NGF may promote OVA+lowLPS induced the BMDCs to release TSLP, IL-6, and IL-10 (Figure 2).

NGF promotes BMDCs costimulatory molecule expression and the release of cytokines due to OVA+lowLPS after LPS-induced p75NTR expression in BMDCs

In the present study, OVA+lowLPS were used to stimulate TLR4^{-/-} BMDCs for 12 h; however, no changes in the expression of TLR4^{-/-} BMDCs costimulatory molecules (MHCII and CD86) were observed (Figures 3A, 3B). NGF did not have a stimulatory effect on pretreated TLR4^{-/-} BMDCs surface molecule expression (Figures 3A, 3B). Thus, western blotting was conducted to measure NGF receptor expression (p140trkA and p75NTR) in wild-type and TLR4^{-/-} BMDCs under different pretreatment conditions. TLR4^{-/-} BMDCs did not express NGF receptors. Interestingly, p75NTR but not p140trkA was expressed on surfaces of BMDCs in the presence of TLR4 signaling. BMDCs only expressed p75NTR when both LPS and TLR4 were present and did not express p140trkA under any conditions (Figure 3C). These results indicate that LPS-induced p75NTR expression in BMDCs and helped NGF perform its function.

NGF promotes OVA+LowLPS-induced BMDCs expression of costimulatory molecule and cytokine release via inducible p75NTR

Biological activities of NGF are mediated by two types of receptors: p75NTR and p140trkA [19]. Based on the p75NTR was expressed in BMDCs, the p75NTR inhibitor (TAT-Pep5) was added 30 min before NGF for intervention. Adding the

p75NTR inhibitor not only significantly reduced expression MHCII and CD86 (Figures 4A and B) but also decreased secretion of cytokines (TSLP and IL-6) (Figure 4C) of BMDCs. The expression of MHCII and CD86 on the surfaces of BMDCs decreased after p75NTR inhibitor treatment (compare p75 inhibitor group with NGF group: MHCII 11803.3 $\pm$ 1803.1 versus 16833.3 $\pm$ 2413.2 $P < 0.05$, CD86 6962.7 $\pm$ 1570.3 versus 11036.0 $\pm$ 1874.6 $P < 0.05$). Also, the levels of TSLP and IL-6 in the BMDCs culture supernatant were highest in the LPS+pOVA+NGF group and decreased after p75NTR inhibitor pretreatment (compare p75 inhibitor group with NGF group (pg/mL): TSLP 660.0 $\pm$ 100.0 versus 876.7 $\pm$ 106.0 $P < 0.05$, IL-6 506.7 $\pm$ 100.7 versus 733.3 $\pm$ 94.5 $P < 0.05$).

NGF promotes BMDCs maturation and cytokine release due to lowLPS and OVA via the canonical and less-canonical NF- κ B signaling pathways

The NF- κ B signaling pathway is closely related to the maturation and antigen-presenting functions of DCs and particularly to RelB, which is a transcription factor that belongs to the non-canonical NF- κ B signaling pathway [20]. BMDCs were stimulated as before. Changes in BMDCs cytoplasmic and nuclear RelA and RelB levels were examined 1 h after NGF treatment. As shown, total levels of RelA and RelB in BMDCs increased following NGF treatment. RelA and RelB translocated from the cytoplasm to the nucleus indicate that NGF could further increase the activation of RelA and RelB in BMDCs pretreated with OVA+lowLPS, TAT-Pep5 could suppress this phenomenon (Figure 5A).

The NF- κ B pathway inhibitor QNZ pretreatment significantly decreased the levels of BMDCs costimulatory molecules (CD86 and MHCII) as well as supernatant TSLP and IL-6 concentrations (Figures 5B, C, and D). Expression of CD86 and MHCII on BMDCs surfaces decreased significantly in

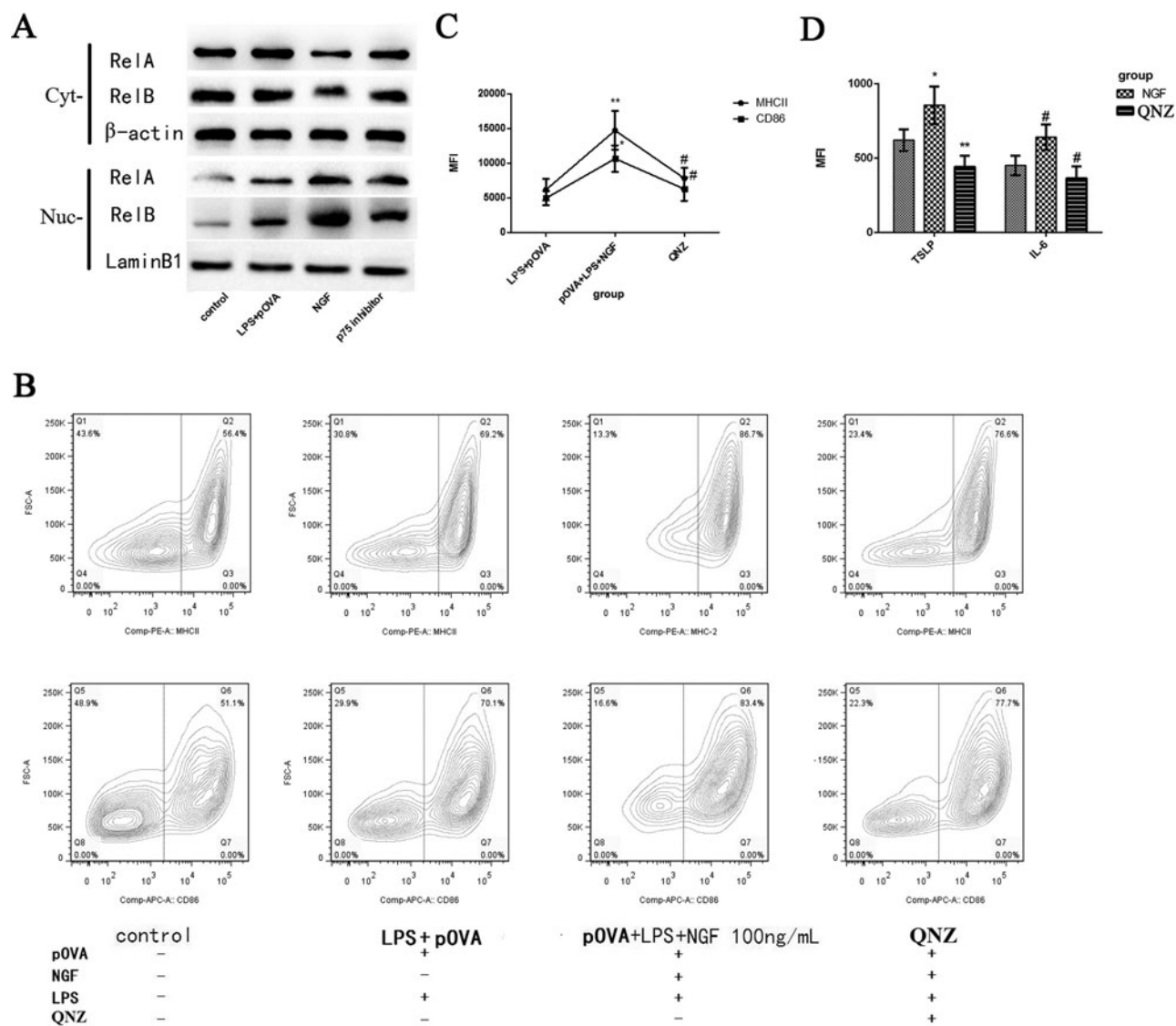


Figure 5. NGF promotes BMDCs maturation and cytokine release due to OVA+lowLPS via the NF-κB signaling pathway. One hour after NGF treatment. (A) Total levels of RelA and RelB in BMDCs increased after NGF treatment. RelA and RelB translocated from the cytoplasm to the nucleus, TAT-Pep5 could suppress this phenomenon to an extent ($P < 0.05$ compared with the LPS+OVA group). (B, C) Pretreatment with the NF-κB pathway inhibitor QNZ pretreatment significantly decreased the levels of BMDCs CD86, and MHCII * $P < 0.05$ ** $P < 0.01$ relative to the NGF group with the LPS+pOVA group; # $P < 0.05$ relative to the QNZ group with the NGF group). (D) Supernatant TSLP and IL-6 concentrations, # $P < 0.05$, ## $P < 0.01$). This experiment was repeated 3 independent times.

the QNZ pretreatment group (compare QNZ group with NGF group: MHCII 7793.0 ± 1550.4 versus 14700 ± 2787.5 $P < 0.05$, CD86 6259.3 ± 1684.6 versus 10638.3 ± 1923.6 $P < 0.05$). Also, TSLP and IL-6 secretion in the BMDCs culture supernatant significantly decreased in the QNZ treatment group (compare QNZ group with NGF group(pg/mL): TSLP 440.0 ± 75.5 versus 855.3 ± 125.6 $P < 0.01$, IL-6 363.3 ± 80.8 versus 640.0 ± 85.4 $P < 0.05$). The data suggest that NGF promotes BMDCs maturation and cytokine release through both canonical and less-canonical NF-κB signaling pathways.

NGF could stimulate the BMDCs to adaptive Th2 immunity

The effects of NGF on the ability of BMDCs to stimulate CD4⁺ T cell cytokine production were examined using naïve CD4⁺ T cells. BMDCs were co-cultured with DO11.10 naïve CD4⁺ T cells as described previously [21]. Compared with

the control group, CD4⁺ T cells co-cultured with OVA+lowLPS-stimulated BMDCs modestly induced the expression of IL-4, whereas highest levels of IL-4 were measured in the LPS+OVA+NGF group (compared LPS+pOVA+NGF group with LPS+pOVA group (pg/mL) 106.0 ± 8.1 versus 62.7 ± 8.2 $P < 0.05$). Moreover, the p75NTR inhibitor decreased the levels of IL-4 to a certain degree (compared p75 inhibitor group with LPS+pOVA+NGF group (pg/mL) 78.0 ± 4.2 versus 106.0 ± 8.1 $P < 0.05$). The levels of INF-γ were not significantly different in the 4 groups. The ratio of IL-4/INF-γreached the highest in the LPS+OVA+NGF group compared with the LPS+OVA group (0.243 ± 0.036 versus 0.118 ± 0.008 $P < 0.05$). Furthermore, the p75NTR inhibitor decreased the ratio of IL-4/INF-γ(compared p 75 inhibitor group with LPS+pOVA+NGF group 0.149 ± 0.010 versus 0.243 ± 0.036 $P < 0.05$) (Figure 6). These results indicate that NGF could stimulate the BMDCs to adaptive Th2 immune responses.

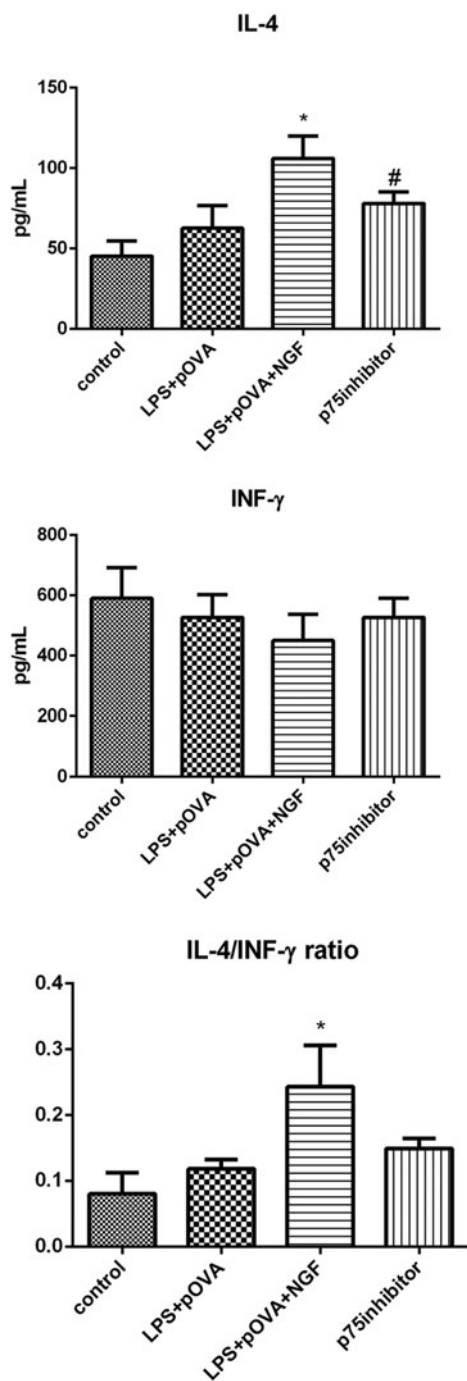


Figure 6. NGF stimulates the BMDCs to Th2 inflammation. The effects of NGF on the ability of BMDCs to stimulate CD4⁺ T cell cytokine production were examined using naïve CD4⁺ T cells. IL-4 and INF-γ levels in cultured medium were measured. CD4⁺ T cells co-cultured with OVA+lowLPS-stimulated BMDCs induced the expression of IL-4 to a moderate extent, whereas the highest levels of IL-4 were measured in the LPS+OVA+NGF group. The p75NTR inhibitor decreased the levels of IL-4 to an extent. The levels of INF-γ were not significantly different in the 4 groups. The ratio of IL-4/INF-γ was higher in the LPS+OVA+NGF group than in the LPS+OVA group ($P < 0.05$). This experiment was performed in triplicate.

Discussion

NGF is a critical factor in the development of asthma [16, 20, 23]. This study demonstrated that NGF might promote OVA+lowLPS-induced MHCII and CD86 expression and that TSLP and IL-6 are produced in BMDCs. The occurrence of

a Th2 asthmatic inflammatory response depends on the presence of DCs, which are important immune cells that connect the innate and acquired immune systems, play an essential role in asthmatic inflammatory responses, and directly determine T lymphocyte differentiation [1, 29, 30]. APCs require CD86, which belongs to the B7 family, to activate the T cells [31]. Soluble CD86 levels in sera of asthma patients are higher than those in sera of healthy individuals.

Patients with high CD86 levels have lower values of forced expiratory volume in 1 second and higher numbers of peripheral blood eosinophils [32]. Asai-Tajiri Y et al. found that downregulation of CD86 in mouse asthma models by short interfering RNAs could ameliorated OVA-induced airway eosinophilia, airway hyperresponsiveness, and the elevations of OVA-specific IgE in the sera and IL-5, IL-13, and CCL17 in the bronchoalveolar lavage fluid [33]. Meanwhile, Zhang et al. reported that TSLP secreted by DCs due to lowLPS stimulation promotes Th2 inflammation [34]. In addition, IL-6 promotes the development of asthma, whereas IL-12 and IL-10 inhibit Th2 inflammation [35–37].

Kim and Jones reported that epinephrine pre-treatment enhanced surface expression of MHCII, CD80 and CD86 and induced a significant decrease of IL-12p70 and a significant increase of IL-23 and IL-10 cytokine production in BMDCs. These changes corresponded with increased IL-4 and IL-17A, but not IFN-γ cytokine production by CD4(+) T cells [38]. Similar effect had been found in the present study, NGF can promote Th2 inflammation by inducing TSLP and IL-6 secretion via up-regulation of CD86 and MHCII expression on DC surfaces. The current study may provide another new mechanism for the involvement of NGF in asthma inflammation. Thus may increase production of cytokines, and activation of Th2 cells can damage the airway.

We further explored the mechanism of NGF. Interactions between NGF and immune cells occur via NGF receptors [39, 40]. It has been shown that immature DCs do not express NGF receptors, p140trkA and p75NTR; however, stimulation with a lowLPS (10 ng/mL) induces p75NTR expression in DCs in both humans and mice, though neither TLR3 nor TLR7/8 caused NGF receptor expression in DCs [27, 28]. In another study, the asthmatic airway inflammatory reaction of an animal model stimulated by OVA +lowLPS was significantly more intense than that caused by OVA with agonists such as TLR3 and TLR7/8 [41]. In the present study, BMDCs did not express p75NTR without LPS stimulation, indicating that the promotive effect of NGF on BMDCs also requires activation of TLR4 signaling.

DC maturation depends on RelB, which is the final product of the non-canonical NF-κB signaling pathway [42]. RelB is highly expressed in APCs and regulates the maturation and immune function of DCs [43]. *In vivo* and *in vitro* experiments have demonstrated that silencing RelB expression in DCs using small interfering ribonucleic acids significantly inhibits DC maturation and decreases the ability of DCs to stimulate specific T-cell responses [44]. RelB expression and such genes determine DC immune response capacities. This newly discovered NF-κB signaling pathway is also called the “less-canonical NF-κB pathway” [45]. In the present study, not only RelB but also RelA levels in BMDCs increased and accumulated in the nucleus after stimulation with NGF. The expression

of BMDCs costimulatory molecules and release of BMDCs cytokines decreased after NF- κ B inhibitor treatment, suggesting that NGF may have exerted its biological effects by activating NF- κ B signaling after combining with p75NTR on the surfaces of BMDCs.

In summary, the results of the present study show that NGF likely promotes BMDCs expression of costimulatory molecules and cytokine release due to OVA+lowLPS stimulation and that the effects of NGF involved in Th2 response through LPS-induced p75NTR. The current study suggests another new mechanism for the involvement of NGF in the Th2 response. This study nevertheless, has its own limitations. First, it is only an *in vitro* experiment and lacks animal models. A lot of airway cells could secrete NGF and DCs in airway also affected by other cytokines such as GM-CSF, so it is difficult to study the function of NGF on DCs in animal models. Second, although BMDCs are widely used for *in vitro* analysis, the existence of an *in vivo* equivalent is unclear.

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