Nerve Growth Factor Promotes TLR4 Signaling-Induced Maturation of Human Dendritic Cells In Vitro through Inducible p75 NTR 1

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Nerve growth factor (NGF), the best-characterized neurotrophic polypeptide of the neurotrophin family, plays important roles in the survival and development of peripheral and central nervous neurons (1). Biological activities of NGF are mediated by two types of its receptors: the low-affinity p75 neurotrophin receptor (p75NTR) and the tyrosine protein kinase receptor p140TrkA (2). In addition to the important roles in the nervous system, NGF and its receptors are also expressed in the immune system (3), and NGF has been shown to be able to affect survival, activation, maturation, and differentiation of a variety of immunocompetent cells, including mast cells, neutrophils, eosinophils, macrophages and monocytes, and B and T lymphocytes. For example, NGF sustains proliferation and differentiation of peripheral blood mononuclear cells (25). Moreover, the increase of NGF production and its receptor expression was observed in the inflammatory and autoimmune diseases. However, up to now, there is no report about the expression of NGF and its receptor on human DCs, and the role of NGF in the regulation of DCs maturation and function in response to TLR ligands. So, the primary aim of this study is to investigate the roles of NGF and its receptors in the regulation of DCs maturation and function. We analyzed the expression of NGF and its receptors on human monocyte-derived DCs (MoDCs) with or without stimulation by ligands of TLRs (22). LPS-initiated TLR4 signaling can activate MAPK and NF-κB pathways, leading to the production of cytokines and resulting in pathogen elimination (23, 24). It has been shown that LPS can induce NGF production and its receptor expression by monocytes/macrophages (25). Moreover, the increase of NGF production and its receptor expression was observed in the inflammatory and autoimmune diseases. However, up to now, there is no report about the expression of NGF and its receptor on human DCs, and the role of NGF in the regulation of DCs maturation and function in response to TLR ligands. So, the primary aim of this study is to investigate the roles of NGF and its receptors in the regulation of DCs maturation and function. We analyzed the expression of NGF and its receptors on human monocyte-derived DCs (MoDCs) with or without stimulation by ligands of TLRs, and survival of memory B cells (4), macrophages (5), and monocytes (6). Also, NGF promotes chemotaxis of mouse macrophages (7) and mast cells (8), activates macrophages (9) and spleen mononuclear cells (10), promotes mast cells maturation (11) and basophils release of inflammatory mediators (12), and enhances the proliferation and differentiation of granulocytes (13). NGF has been shown to synergize with granulocyte-macrophage CSF (GM-CSF) to promote human basophilic cells differentiation (14) and synergize with stem cell factor to support hemopoietic stem cell development (15, 16). NGF was found to be increased during inflammatory responses (17), autoimmune disorders (18, 19), allergic diseases (20) and stress (21). Thus, NGF is also regarded as an important factor involved in the immune responses and pathogenesis of autoimmune diseases.

Dendritic cells (DCs) are an important link of innate immunity and adaptive immunity, and a potent initiator for inflammatory and immune responses upon recognition of the structurally conserved bacterial and viral components termed pathogen-associated molecular patterns through Toll-like receptors (TLRs) (22). LPS-initiated TLR4 signaling can activate MAPK and NF-κB pathways, leading to the production of cytokines and resulting in pathogen elimination (23, 24). It has been shown that LPS can induce NGF production and its receptor expression by monocytes/macrophages (25). Moreover, the increase of NGF production and its receptor expression was observed in the inflammatory and autoimmune diseases. However, up to now, there is no report about the expression of NGF and its receptor on human DCs, and the role of NGF in the regulation of DCs maturation and function in response to TLR ligands. So, the primary aim of this study is to investigate the roles of NGF and its receptors in the regulation of DCs maturation and function. We analyzed the expression of NGF and its receptors on human monocyte-derived DCs (MoDCs) with or without stimulation by ligands of TLRs, and survival of memory B cells (4), macrophages (5), and monocytes (6). Also, NGF promotes chemotaxis of mouse macrophages (7) and mast cells (8), activates macrophages (9) and spleen mononuclear cells (10), promotes mast cells maturation (11) and basophils release of inflammatory mediators (12), and enhances the proliferation and differentiation of granulocytes (13). NGF has been shown to synergize with granulocyte-macrophage CSF (GM-CSF) to promote human basophilic cells differentiation (14) and synergize with stem cell factor to support hemopoietic stem cell development (15, 16). NGF was found to be increased during inflammatory responses (17), autoimmune disorders (18, 19), allergic diseases (20) and stress (21). Thus, NGF is also regarded as an important factor involved in the immune responses and pathogenesis of autoimmune diseases.

**Abbreviations used in this paper: NGF, nerve growth factor; CpG ODN, CpG oligodeoxynucleotide; DC, dendritic cell; LTA, lipoteichoic acid; p75NTR, p75 neurotrophin receptor; p140TrkA, p140 tyrosine protein kinase receptor; TLR, Toll-like receptor; MoDC, monocyte-derived DC; PDTC, Pyrrolidine carbodithioic acid; RTPCR, reverse transcription PCR; Allo-MLR, allogenic mixed-lymphocyte reaction.**

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NGF promotes TLR4 signaling in human DCs

Reagents
Recombinant human β-NGF was obtained from PeproTech. Anti-human p75<sup>NTTR</sup> mAb and human p75<sup>NTTR</sup>/Fc chimera, recombinant human GM-CSF and IL-4, ELISA kits for human NGF, IL-12 p40, IL-1β, IL-6, and TNF-α, were from R & D Systems. LPS (Escherichia coli O26:B6) and FITC-conjugated OVA were from Sigma-Aldrich. FITC-anti-hCD40, PE-anti-hCD80, PE-anti-hCD83, PE-anti-hCD86, PerCP-anti-hHLA-DR, anti-CD14, anti-CXCR4, anti-CCR7, anti-p75 NTTR, and anti-goat IgG labeled with HRP were purchased from Santa Cruz Biotechnology. Nuclear and cytoplasmic extraction reagents (NE-PER) were from Pierce Biotechnology. ERK inhibitor PD98059, p38 MAPK inhibitor SB203580, and NF-κB inhibitor Pyrrolidine carbodithioic acid (PDTC) were from Calbiochem.

Generation and treatment of human monocyte-derived DCs
MoDCs were generated from human peripheral blood CD14<sup>+</sup> monocytes by culturing in 10% FCS-RPMI 1640 medium containing GM-CSF and IL-4 as described previously (26). On day 6, MoDCs (5 × 10<sup>5</sup>/ml) were treated with or without LPS (10 ng/ml) for 12 h and then washed three times with culture medium to exclude the presence of LPS. Then, the MoDCs were stimulated with recombinant NGF at the indicated dosage for different times. Considering that there is a high level of NGF in peripheral blood of some diseases such as in systemic sclerosis (61.3 ng/ml) (27) and also a high concentration of NGF (10–500 ng/ml) is used to investigate its effects on the nervous system and immune cells such as sustaining memory B cell survival (4) and recruitment of human polymorphonuclear leukocyte (29), so we selected 50 ng/ml NGF to stimulate LPS-pretreated MoDCs in our experiments. These LPS-pretreated and NGF-stimulated MoDCs were then collected for the phenotypic and functional analysis.

Reverse transcription-PCR (RT-PCR)
Total RNA was isolated from MoDCs by using TRIzol reagent (Life Technologies) according to the manufacturer’s instructions. The respective primer sequences used for human NGF were: 5′-CAGGACTCACAG GAGCAAGC-3′ and 5′-GGCTTCTCTGTCGACAC-3′; for human p75<sup>NTTR</sup> were: 5′-CTGTTCCTCCTGGCAAGACA-3′ and 5′-GAGATGC...
CACTGTGCTG TG-3' for human p140TrkA were: 5'-H11032 CCATCGTGAA
GAGTGGTCTC-3' and 5'-H11032 GGTGACATTGGCCAGGGTCA-3'. The cDNA was amplified by PCR un-
der the following conditions: 30 to 40 cycles of 30 s at 94°C, 30 – 60 s at
55C-57°C, and 30 s at 72°C, followed by 10 min of elongation at 72°C.

**Flow cytometry**

For phenotypic analysis, MoDCs were stained in PBS using fluorochrome-
conjugated or isotype-matched control Abs for 30 min at 4°C and analyzed by
a FACSCalibur flow cytometer and CellQuest software (BD Biosciences). The values displayed in figures represent the mean fluorescence intensity (MFI) subtracted from the value of isotype-matched control mouse mAbs was analyzed and summary data of MFI on
MoDCs obtained from four donors were shown. Comparisons were per-
formed between LPS/NGF group and LPS alone. Asterisk indicates statistically significant associations ($p < 0.05$).

**Assays for cytokines**

The culture supernatants of MoDCs were collected, centrifuged at 500 ×
g for 5 min, and frozen at −20°C until use for detection of cytokines including human NGF, IL-12 p40, IL-1β, IL-6, and TNF-α by ELISA.

**Allogenic mixed-leukocyte reaction (Allo-MLR)**

The capacity of MoDCs to stimulate proliferation of naive allogeneic T
cells in Allo-MLR was performed as described previously (26). In brief,
T lymphocytes were isolated by positive immunomagnetic depletion of adherent PBMC from healthy volunteers using anti-CD3 magnetic
beads (Miltenyi) Biotech) and used as responders. The irradiated MoDCs (30Gy Co (60)) were used as stimulator cells. T cells were incubated
with the irradiated MoDCs for 4 days at different stimulator:responder ratios. To assess T cell proliferation, cells in each well were pulsed with
1 $\mu$Ci of [3H]-thymidine (Amersham Pharmacia Biotech) during the last
16 h of culture and the incorporated [3H]-thymidine was measured by
liquid scintillation spectroscopy (Wallac1409; Wallac). Results were
expressed as the mean of triplicate assays.

**Western blot analysis**

MoDCs were stimulated with LPS and/or NGF as indicated, and then
lysed with M-PER Protein Extraction Reagent (Pierce) supplemented
with protease inhibitor mixture (Sigma-Aldrich), and protein concent-
trations of the extracts measured by BCA assay (Pierce). Forty micro-
grams of the protein was loaded per lane, subjected to SDS-PAGE,
transferred onto nitrocellulose membranes, and then blotted to detect
the activation of MAPK pathways as described previously (30). For
detection of the nuclear translocation of NF-κB p65, cytoplasmic and
nuclear extracts were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Pierce) and protein concentration was determined with a BCA assay. Nuclear NF-κB p65 subunit was detected by Western blot using specific Ab (31). For analysis of NGF-mediated signal transduction, MoDCs pretreated with 10 ng/ml LPS for 12 h were stimulated by NGF for 15–30 min, and then Western blot was performed to detect the activation of signal pathways. For observation of the roles of signal transduction pathway in the NGF-promoting effect on MoDCs maturation, MoDCs were pretreated with ERK inhibitor PD98059 (25 μM), p38MAPK inhibitor SB203580 (25 μM), or NF-κB inhibitor PDTC (25 μM), respectively, 30 min before stimulation with NGF.

Statistical analysis

Results were given as means ± SD. Experimental results were analyzed for statistical significance (Student’s t test or a paired t test) using the SigmaStat Software package. Statistical significance was determined as p values <0.05 and indicated in the figures (*, p < 0.05; **, p < 0.01).

Results

Effects of TLR ligands on the expression of NGF and its receptors on human DCs

NGF and its receptors have been shown to be expressed on many kinds of immune cells (3). However, we found immature MoDCs expressed low level of NGF but didn’t express any NGF receptors p140TrkA and p75NTR. After MoDCs were stimulated with TLR ligands, p140TrkA expression on MoDCs was also negative. Interestingly, p75NTR expression was induced to be markedly increased by TLR4 ligand LPS, but TLR2 ligand (LTA), TLR3 ligand (Poly I:C), and TLR9 ligand (CpG ODN) could not induce the expression of p75NTR (Fig. 1, A and B). In addition, certain levels of NGF was significantly induced by LPS in MoDCs (Fig. 1C). The results demonstrate that LPS can significantly induce MoDCs to express NGF and p75NTR indicating possible interaction of TLR4 signaling and NGF in the regulation of maturation and function of DCs.

LPS induces human DCs to express NGF and p75NTR in a dose- and time-dependent manner

To further analyze the interaction of TLR4 signaling and NGF in the regulation of DC maturation and function, MoDCs were stimulated with various concentrations of LPS for the indicated time, and the secreted NGF in supernatants was evaluated by ELISA and p75NTR on the surface of MoDCs analyzed by FACS. As shown in Fig. 2, LPS could up-regulate the expression of NGF and p75NTR dose-dependently. At a concentration of 1 ng/ml, LPS was already sufficient to elicit a significant increase in NGF and p75NTR expression.
increase in NGF and p75NTR expression by MoDCs, and the maximal effect of LPS was found at a concentration of 100 ng/ml. At a concentration of 100 ng/ml LPS, there was 5-fold increase of NGF production compared with the basic level of NGF. However, a higher dose of LPS (1/100 g/ml) was not able to induce further expression of NGF and p75NTR. Furthermore, LPS (10 ng/ml) could induce MoDCs to express NGF and p75NTR in a time-dependent manner. With incubation with LPS for 6 h, expression of p75NTR on MoDCs began to increase and reached to approximately maximal induction at 12 h. However, expression of NGF by MoDCs began to increase at 12 h and reached maximal induction at 24 h after incubation with LPS. The inducible expression of p75NTR on MoDCs in response to LPS stimulation indicates that the LPS-pretreated MoDCs may be more sensitive to NGF stimulation, implying that NGF is involved in the regulation of LPS-induced DC maturation.

NGF promotes LPS-induced phenotypic maturation of human DCs

On the basis of the above data, we selected 10 ng/ml LPS to pretreat MoDCs for 12 h to induce p75NTR expression and then stimulate MoDCs with NGF to analyze the effects of NGF on LPS-induced DC maturation. As shown in Fig. 3A, NGF alone, even at a high dosage of 500 ng/ml, could not induce any phenotypic alteration of MoDCs without LPS pretreatment, indicating endogenous and exogenous NGF alone has no effect on the maturation of human MoDCs. However, even low dosage of NGF (10 ng/ml) was found to be able to up-regulate HLA-DR and CD86 expression on LPS-pretreated MoDCs. A higher dosage of NGF (20–500 ng/ml) could further increase the expression of CD40, CD80, CD83, CD86, and HLA-DR on MoDCs pretreated with LPS. The promoting effect of NGF on the phenotypic maturation of MoDCs was dose-dependent and required LPS pretreatment. The LPS-pretreated MoDCs were more sensitive to NGF stimulation, showing that NGF is involved in the regulation of LPS-induced DC maturation.
NGF promotes LPS-induced functional maturation of MoDCs through inducible p75NTR

Consistent with the above result that NGF alone has no effect on maturation of human MoDCs, NGF alone could not induce cytokine production by MoDCs. However, 50 ng/ml NGF could significantly increase the IL-12, IL-1β, IL-6, and TNF-α production by MoDCs pretreated with LPS (10 ng/ml) (Fig. 4A). Interestingly, NGF could not enhance LPS-induced secretion of chemokines including MCP-1, MIP-1β, TARC, and RANTES from MoDCs (data not shown). Further experiments showed that pretreatment of MoDCs with p75NTR receptor-specific antagonist p75NTR/Fc chimera could completely abolish the enhancing effect of NGF on the LPS-induced IL-12, IL-1β, IL-6, and TNF-α production by MoDCs, suggesting that NGF promoted LPS-induced cytokine secretion via inducible p75NTR. IL-12 is regarded as important cytokine secreted by matured and activated DCs to initiate Th1 response, and IL-1β, IL-6, and TNF-α are all important proinflammatory cytokines. So, NGF may promote T cell response and/or inflammatory response initiated by DCs. The observations that NGF-induced further reduction of phagocytosis of FITC-conjugated OVA by LPS-pretreated MoDCs, and such effect could be completely abolished by pretreatment of MoDCs with the p75NTR/Fc chimera (data not shown), further supported the promoting effect of NGF on the LPS-induced DC functional maturation (Fig. 4B). Also accepted, LPS-matured DCs stimulate allogeneic T cell proliferation more significantly than immature DCs. As predicted, NGF could markedly enhance the capacity of LPS-pretreated MoDCs to stimulate proliferation of allogeneic T cells in allo-MLR, and preincubation of MoDCs with p75NTR/Fc chimera could completely abolish the enhancing effect of NGF (Fig. 4C). Therefore, these data convincingly demonstrate that NGF can promote LPS-induced functional maturation of MoDCs via inducible p75NTR.

Activation of p38MAPK and NF-κB pathways is required for the promotion of LPS-induced DCs maturation by NGF

MAPK and NF-κB pathways have been demonstrated to be important in maturation and survival of MoDCs. As shown in Fig. 5A, without pretreatment of MoDCs with LPS, 50 ng/ml NGF alone could not activate MAPK and NF-κB pathways in MoDCs. 10 ng/ml LPS alone could activate p38, ERK, and NF-κB to certain degree, but not JNK, in MoDCs. Interestingly, NGF could further increase the activation of p38, ERK, and NF-κB in MoDCs pretreated with LPS. Thus, the ERK, p38 MAPK, and NF-κB pathways were further activated in LPS-pretreated MoDCs by NGF stimulation. Blockade of p38MAPK and NF-κB pathways with SB203580 and PDTC, respectively, could dramatically inhibit the increased secretion of IL-12p40, TNF-α (Fig. 5B) and the expression of CD86 and HLA-DR (Fig. 5C) from LPS-pretreated MoDCs by NGF. Interestingly, we found that p38MAPK and NF-κB inhibitors could inhibit IL-12p40 secretion from LPS-pretreated, NGF-stimulated MoDCs in a dose-dependent manner (Fig. 5D). However, the blockade of ERK had no such effects. These data suggest that activation of p38 MAPK and NF-κB pathways is required for the promotion of LPS-induced DC maturation by NGF.

Discussion

NGF and functional NGF receptors are expressed by many kinds of hemopoietic and immune cells such as CD34+ cells (32) and monocytes or macrophages. In this study, we find that immature MoDCs express low levels of NGF but do not express any NGF receptors p140TrkA and p75NTR, which are consistent with previous report, that NGF receptors are lost during in vitro differentiation of hemopoietic cells into macrophages and DCs (6). Many immune and inflammatory stimuli, such as LPS and IL-1, can induce expression of NGF and its receptors in immune cells. For examples, LPS can up-regulate NGF and its receptors expression in monocytes or macrophages and B cells (33); TNF-α and IL-1 can promote the release of NGF in rat astrocytes (34), mesangial cells (35), and human microglial cells (36). Our present study showed that LPS, but not other ligands of TLRs including LTA, Poly I:C, and CpG ODN, could stimulate MoDCs to produce NGF and express NGF functional receptor p75NTR in the dose- and time-dependent manner. The fact that many kinds of cells including DCs can express NGF and NGF receptors during immune responses and inflammation indicates that there is a possibility for NGF involvement in the immune response and pathogenesis of autoimmune diseases.

In addition to neurotrophic activity, NGF has been shown to elicit other broad biologic functions of immune and inflammatory cells. In this study, we demonstrate that NGF can promote LPS-induced maturation of DCs both phenotypically and functionally by up-regulating expression of MHC class II and costimulatory molecules CD40, CD83, CD80, and CD86, proportionally by up-regulating expression of MHC class II and pro-inflammatory cytokines including MCP-1, MIP-1α, IL-6, and TNF-α. As we know, DCs are involved in the pathogenesis of some kinds of neurological autoimmune and inflammatory diseases (37). Together with other reports such as the constitutive expression of neurotrophins receptor by Th1 but not Th0/Th2 clones (38), our study may provide another new mechanism for the involvement of NGF in the pathogenesis of neurological autoimmunity and inflammatory diseases through promotion of DCs maturation, thus resulting in more production of proinflammatory cytokines, and activation of T cells causing damages to neurological cells or tissues.

The p75NTR shares a great homology with the receptors belonging to the TNF receptor family such as CD40, CD30 and CD95 (39), which are involved in the regulation of cellular activation and apoptosis. In fact, many reports indicate that NGF has the ability to promote cell death in specific cell types such as developing retina neurons (40) and oligodendrocytes through the p75NTR (41). There are other reports that NGF can protect breast cancer cells (42), monocytes, oligodendrocytes (43), and schwannoma cells (44) from death induction through activating NF-κB via p75NTR. Activation of CD40 on DCs also leads to increased expression of surface MHC, adhesion, and costimulatory molecules, facilitating Ag processing and presentation of DCs. In addition, CD40 is involved in the survival (45) and apoptosis (46) of DCs. Thus, LPS-induced p75NTR on DCs, just like CD40 on DCs, may have other important functions...


