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Nerve Growth Factor Downregulates Inflammatory Response in Human Monocytes through TrkA

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Nerve growth factor (NGF) levels are highly increased in inflamed tissues, but their role is unclear. We show that NGF is part of a regulatory loop in monocytes: inflammatory stimuli, while activating a proinflammatory response through TLRs, upregulate the expression of the NGF receptor TrkA. In turn, NGF, by binding to TrkA, interferes with TLR responses. In TLR-activated monocytes, NGF reduces inflammatory cytokine production (IL-1β, TNF-α, IL-6, and IL-8) while inducing the release of anti-inflammatory mediators (IL-10 and IL-1 receptor antagonist). NGF binding to TrkA affects TLR signaling, favoring pathways that mediate inhibition of inflammatory responses: it increases Akt phosphorylation, inhibits glycogen synthase kinase 3 activity, reduces IκB phosphorylation and p65 NF-κB translocation, and increases nuclear p50 NF-κB binding activity. Use of TrkA inhibitors in TLR-activated monocytes abolishes the effects of NGF on the activation of anti-inflammatory signaling pathways, thus increasing NF-κB pathway activation and inflammatory cytokine production while reducing IL-10 production. PBMC and mononuclear cells obtained from the synovial fluid of patients with juvenile idiopathic arthritis show marked downregulation of TrkA expression. In ex vivo experiments, the addition of NGF to LPS-activated juvenile idiopathic arthritis to both mononuclear and damage. In conclusion, this study identifies a novel regulatory mechanism of inflammatory responses through NGF and its receptor TrkA, for which abnormality may have pathogenic implications for chronic inflammatory diseases. The Journal of Immunology, 2014, 192: 3345–3354.

The neurotrophin nerve growth factor (NGF) is a well-known regulator of differentiation, plasticity, and phenotype of sensory and sympathetic neurons during the entire lifespan (1). Because of its dynamic control of innervation, trophism, and synthesis of neurotrophins in neurons, the production of NGF in the peripheral tissues is strictly regulated, because alterations in NGF tissue concentrations lead to neuropathy, hyperalgesia, and pain (2). Nevertheless, as demonstrated by an increasing number of studies on inflammatory and autoimmune diseases, inflammation can greatly enhance the synthesis of NGF in the tissues (3). In vitro, inflammatory cytokines induce NGF production by a variety of cells, such as fibroblasts, epithelial, endothelial, glial, neuronal, and immune cells [for a recent review, see Bracci-Laudiero (4)]. Because NGF concentrations in inflamed tissues appear to be correlated with the extent of inflammation and/or clinical severity, it has been inferred that NGF has a proinflammatory action. This hypothesis is in apparent contradiction with in vivo data in animals. Indeed, experiments in animal models of inflammation, such as models of multiple sclerosis, colitis, and UV irradiation, have shown that the administration of NGF in vivo reduces disease severity, decreasing tissue inflammation and immune cell infiltration and enhancing IL-10 production (5–8). At present, it is not known why NGF production is upregulated in inflamed tissues or how this can affect the cellular and molecular mechanisms triggered during the inflammatory response.

Immune cells are responsive to NGF and indeed express the NGF receptors TrkA and p75-NTR. TrkA is specific for NGF, whereas p75-NTR is a pan-neurotrophin receptor and binds all neurotrophins with equal affinity. TrkA expression is considered necessary to elicit the biological response of NGF. As demonstrated in studies performed on neuronal cells, binding of NGF to TrkA activates Ras, PI3K, phospholipase Cγ1, and downstream signaling pathways, including MAPKs (9). Very little information is available on the signaling pathways elicited by TrkA activation in immune cells (10–12).

To provide insight into the levels of NGF in inflamed tissues, we focused our attention on monocytes, which are key players in the initiation and perpetuation of inflammation and a critical link between innate and adaptive immunity. Monocytes recognize pathogen-associated molecular patterns and endogenous danger signals via germline-encoded pattern recognition receptors,
such as TLRs (13). Signaling downstream of these receptors engages common effector mechanisms, including NF-κB/AP-1–dependent transcription of proinflammatory cytokines and chemokines (14–16). The amount and type of cytokines released after the TLR activation regulate the differentiation and functional state of dendritic cells and influence T cell activation and differentiation (17). Aberrant stimulation of TLRs or dysregulation of TLR signaling pathways results in increased expression of cytokines and chemokines, creating a vicious inflammatory cycle that contributes to the initiation and progression of chronic inflammatory diseases and autoimmune diseases such as rheumatoid arthritis (18, 19).

Because previous studies have shown that LPS stimulation through TLR4 upregulates TrkA expression in monocytes (20, 21), in the current study, we investigated in these cells whether NGF addition affects the expression and release of cytokine production after TLR activation and the intracellular pathways involved. Our results show that, through the activation of TrkA signaling, NGF influences TLR ligand–activated pathways, resulting in a downregulation of inflammatory cytokine production and the induction of anti-inflammatory mediators. We demonstrate that TrkA activation enhances AKT phosphorylation, inhibits glycogen synthase kinase 3 (GSK3) activity, and decreases IkB phosphorylation and p65 NF-κB nuclear translocation, while increasing p50 NF-κB nuclear binding, leading to a potentiation of endogenous anti-inflammatory mechanisms. We also report that mononuclear cells of patients with juvenile idiopathic arthritis (JIA) have reduced expression of TrkA. This results in the absence of NGF-mediated inhibition of inflammatory cytokine release after ex vivo TLR stimulation, suggesting that a physiological anti-inflammatory pathway is defective in JIA patients.

**Materials and Methods**

**Chemicals and Abs**

Endotoxin-free LPS was purchased from Sigma-Aldrich (St. Louis, MO), and lipopolysaccharide from *Staphylococcus aureus* (LPS) and Pam3CSK4 (PAM) were supplied by InvivoGen (San Diego, CA). NGF was purified according to Bocchini and Angeletti (22). Polyclonal Ab against TrkA was from Santa Cruz Biotechnology (Santa Cruz, CA), and phospho-TrkA was from R&D Systems (Minneapolis, MN). Polyclonal Abs against Akt, phospho-Akt (Ser473), phospho-IκB (Ser536), and phospho-GSK3β (Ser21) were from Cell Signaling Technology (Danvers, MA); mAbs and polyclonal Abs against p65 NF-κB were from Santa Cruz Biotechnology; mAb against tubulin was from Sigma-Aldrich. HRP-conjugated secondary Abs were from Jackson ImmunoResearch Laboratories. For cellular cultures, neutralizing NGF Ab, goat Ab against TrkA, and recombinant human TrkA Fc chimera were from R&D Systems, and purified rabbit and goat IgG were from Zymed Laboratories (San Francisco, CA).

The specific PI3K inhibitors LY294002 [2-(4-Morpholinyl)-phenethyl]-4H-1-benzopyran-4-one] and wortmannin were purchased from Calbiochem. From the two GSTK3 pharmacological inhibitors used, LiCl was from Sigma-Aldrich, whereas GSTK3 inhibitor AR-18 was a kind gift from Dr. Hernandez (Madrid, Spain).

**Human monocyte cultures**

PBMC were obtained from buffy coats of healthy donors after centrifugation over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradients. Monocytes were separated from lymphocytes by Percoll (Pharmacia) gradients, as described previously (23). Monocyte preparations were >95% pure, as assessed by flow cytometry.

Monocytes were cultured at a concentration of 2 × 10^6/ml in RPMI 1640 (BioWhittaker, Walkersville, MD) and 5% heat-inactivated and endotoxin-free FCS (HyClone Labs, Logan, UT). For experiments with TLR stimulation, monocytes were either treated or not treated with 10 ng/ml LPS, 2 μg/ml LTA, 2 μg/ml PAM, and 200 ng/ml NGF for 18 h. In a set of experiments to assess TNF-α release, the conditioned media were collected after 3 h. In experiments with NGF inhibition, cells were incubated with 5 μg anti-NGF for 3 h and then with 10 ng/ml LPS or 2 μg LTA with or without the addition of 100 ng/ml NGF. For experiments with TrkA inhibition, cells were incubated with 3 μg/ml affinity-purified anti-TrkA Ab or 3 μg/ml TrkA-Fc chimera for 3 h and then stimulated with LPS and LTA with or without the addition of 50 ng/ml NGF. For experiments with Akt and GSK3 inhibition, cells were pretreated with 1 μM wortmannin, 25 μM LY 294002, 40 μM LiCl, or 25 μM AR-18 and then stimulated with 10 ng/ml LPS with or without the addition of 100 ng/ml NGF. In all of these conditions, the conditioned media were collected after 18 h of incubation.

**RNA extraction and real-time PCR analysis**

Total RNA was isolated from monocytes using the RNeasy mini kit (Qiagen), according to the manufacturer’s instructions. A total of 1 μg total RNA from each sample was used for first-strand cDNA synthesis using the Superscript VILO cDNA synthesis Kit (Invitrogen, Carlsbad, CA). TrkA, p75-NTR, IL-6, IL-1β, and IL-10 gene expression levels were measured by real-time quantitative PCR. PCR reactions were performed on the ABI PRISM 7900 HT Sequence Detector (Applied Biosystems, Foster City, CA) platform using TaqMan Universal Master Mix (Applied Biosystems). TrkA, p75-NTR, IL-6, IL-1β, and IL-10 expression were tested using Assays on Demand reagents (p75-NTR, Hs00182120_m1; IL-6, Hs00985639_m1; IL-1b, Hs00174097_m1; and IL-10, Hs00961622_m1; Applied Biosystems). Each measurement was performed in duplicate. TaqMan Endogenous Control human β-actin (4326351E; Applied Biosystems) was used as the housekeeping gene. Relative quantification was performed using the comparative threshold cycle (Ct) method, and results were expressed in arbitrary units (AU). Expression levels were calculated as 2^(-ΔΔCt) and then compared with each other, whereas fold changes were calculated using the 2^-ΔΔCt equation (24).

**Cytokine assays**

Conditioned media were collected from monocytes obtained from different donors and incubated for 18 h with TLR ligands with or without the addition of NGF. The media were stocked at −70°C, and different cytokine concentrations were analyzed simultaneously. Human IL-6, IL-1 receptor antagonist (IL-1ra), IL-1β, IL-8, TNF-α, and IL-10 were measured in cell–culture supernatants using ELISA kits purchased from R&D Systems. Human NGF Elisa Kit was from Promega (Madison, WI). All of the assays were performed according to the manufacturer’s instructions. The detection limit of the assay was 9.3 pg/ml for IL-6, 39.06 pg/ml for IL-1ra, 7.8 pg/ml for IL-1β, 31.25 pg/ml for IL-8, 15.62 pg/ml for IL-10, 15.6 pg/ml for TNF-α, and 7.8 pg/ml for NGF.

**Western blot analysis**

To study the effects of TrkA activation on TLR signaling, monocytes in RPMI 1640 0.1% FCS were either treated or not treated with 10 ng/ml LPS, 2 μg/ml LTA, 2 μg/ml PAM, and 100 ng/ml NGF for 30 min. For experiments with TrkA inhibition, cells were instead incubated with 3 μg/ml affinity-purified anti-TrkA Ab or 3 μg/ml TrkA-Fc chimera for 3 h in RPMI 0.1% FCS and then stimulated with LPS and LTA with or without the addition of 50 ng/ml NGF for 30 min. Monocytes were then lysed in ice-cold RIPA buffer containing: 50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.1% SDS, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 μM each aprotinin, leupeptin, and pepstatin, and 25 mM NaF (all from Sigma-Aldrich). Equal amounts of protein were released by SDS-PAGE. Proteins were transferred to nitrocellulose membranes (Amersham Life Sciences, Little Chalfont, U.K.) and probed with Abs using standard procedures. Ninety-five percent-bound Abs were detected by chemiluminescence with ECL (Amersham Life Sciences).

**p65 NF-κB nuclear translocation**

Freshly isolated monocytes were treated with 10 ng/ml LPS in the presence or absence of 100 ng/ml NGF for 30 min. After treatment, cells were fixed for 20 min in 4% paraformaldehyde in PBS, permeabilized in cold methanol for 5 min, blocked with 2% BSA for 20 min, and immunostained for p65 NF-κB. Confocal images were acquired using the Olympus Fluoview FV1000 confocal microscope equipped with FV10-ASW version 2.0 software (Olympus), and fluorochrome unmixing was performed by the acquisition of automated sequential collection of multichannel images to reduce spectral crosstalk between channels. Quantification of p65 NF-κB nuclear fluorescence intensity was calculated using FV10-ASW Olympus software (Olympus). Briefly, nuclei were delimited using Hoechst labeling, and mean fluorescence intensity (MFI) of p65 NF-κB labeling was quantified in nuclear, cytoplasmic, and total cell areas. A minimum of three different fields and at least 40–60 nuclei were analyzed per condition. A total of 220 nuclear and cytoplasmic MFIs were acquired for each condition in four independent experiments.

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NGF decreases inflammatory cytokine production induced by TLR activation. Monocytes stimulated with ligands of TLR4 (10 ng/ml LPS) and TLR2 (2 μg/ml LTA and 2 μg/ml PAM) release inflammatory cytokines. The addition of 100 ng/ml NGF significantly decreases IL-6 (A), IL-1β (B), TNF-α (C), and IL-8 (D) production induced by TLR activation. The reduction of inflammatory cytokines is blocked by neutralizing anti-NGF Ab (aNGF; 5 μg/ml). The effect of NGF on cytokine production is dose dependent, with a maximal effect at 100 ng/ml (E). The graphs in (A), (B), and (D) represent the mean of nine independent experiments ± SEM. (E) is representative of three independent experiments,

Two × 10^6 monocytes were stimulated with LPS with or without the addition of NGF. After 1 h incubation, the cells were pelleted and washed with cold PBS. Nuclear extracts were prepared using the NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific, Rockford, IL), and protein concentrations were determined (Bio-Rad protein assay; Bio-Rad, Munich, Germany). Nuclear extracts were used to determine p50 DNA binding activity using the NF-κB p50 transcription factor kit (TransAM, Carlsbad, CA), which permits a more sensitive and quantitative readout of the activation of p50NF-κB than EMSA (25). Ten micrograms protein per well nuclear extracts was added to 96-well plates with prebound specific consensus sequences following the manufacturer’s instructions. The chemiluminescent signal was measured with a Bio-Rad Benchmark Plus microplate spectrophotometer (Bio-Rad).

**Patients with JIA**

JIA is a term that collectively refers to a group of chronic childhood arthropathies that represent the most common rheumatic condition in children (26). Twenty-five children with JIA were included in the study. Diagnosis of JIA and of JIA subtypes was based on the International League of Associations for Rheumatology classification criteria. Fifteen patients had persistent oligoarticular JIA, 7 had extended oligoarticular JIA, and 3 had polyarticular JIA. The mean age was 8.9 y (range 1.77–18.88), and the mean disease duration was 4.5 y (range 0.19–13.73). All patients had active disease at the time of sampling. Synovial fluid samples were obtained at the time of intrarticular steroid injection. PBMC and mononuclear cells from synovial fluid (SFMC) were isolated by standard Ficoll-Hypaque density centrifugation and immediately frozen for mRNA extraction and analysis or for protein extraction and Western blot. In one set of experiments, freshly purified PBMC and SFMC from JIA patients were used to assess the effect of NGF on cytokine production. Cells were cultured at a concentration of 1 × 10^6/ml in RPMI 1640, 10% FCS with or without the addition of 10 ng/ml LPS, and 100 ng/ml NGF, and the supernatants were collected after 18 h. The study was approved by the Ethical Committee of the Bambino Gesù Children’s Hospital, and written informed consent was obtained from the parents of each child.

**Statistical analysis**

Data are presented as mean ± SEM or SD, as appropriate. Statistical analysis of the results was performed using nonparametric tests. All statistical analyses were performed using the Prism software (GraphPad, La Jolla, CA). Data were considered statistically significant when p < 0.05.

**Results**

**Effects of NGF on TLR-induced inflammatory cytokine production**

To gain more information on the effects of high levels of NGF in inflamed tissues (3), monocytes were stimulated with TLR2 and TLR4 ligands in the presence or absence of NGF. As shown in Fig. 1A, the addition of NGF at the time of stimulation with TLR ligands (LPS, PAM, and LTA) induced in human monocytes a reduction in IL-6 release. This reduction was neutralized using anti-NGF Ab. When we tested the effect of NGF on the expression and release of other inflammatory cytokines such as IL-1β (Fig. 1B), TNF-α (Fig. 1C), and IL-8 (Fig. 1D), we observed a similar inhibition in TLR-activated monocytes. Also, in these experiments, anti-NGF Abs abolished the effect of NGF. The effect of NGF on the production of inflammatory cytokines in TLR-activated monocytes was dose dependent, with a maximal effect at a concentration of 100 ng/ml (Fig. 1E). In the absence of TLR stimulation, addition of 100 ng/ml of NGF alone did not induce cytokine release. IL-6, IL-1β, and TNF-α concentrations were undetectable in conditioned media of both unstimulated (Us) and NGF-treated monocytes and data are represented as percentages of variations in cytokine release in LPS-tr. samples with and without NGF. *p < 0.05, **p < 0.01 comparing TLR ligands with and without NGF.
cytes (Us + NGF). In addition, no change in IL-8 basal production was observed (Us, 2360 ± 551 pg/ml; NGF-treated, 2406 ± 592 pg/ml).

Effects of NGF on TLR-induced anti-inflammatory cytokine production

To evaluate whether NGF affects the production of anti-inflammatory mediators, released as a negative-feedback loop, we analyzed, in the same experimental conditions, the production of IL-10 and IL-1ra. In the absence of TLR ligands, IL-10 mRNA expression is expressed in negligible amounts (Us, 0.021 ± 0.003 AU), and no effect of NGF is observed (+NGF, 0.023 ± 0.004 AU). In the presence of LPS or LTA stimulation, addition of NGF increased mRNA expression (Fig. 2A) and release of IL-10 (Fig. 2B).

Although IL-10 itself inhibits the expression of proinflammatory cytokines and thereby limits the inflammatory response, IL-1ra binds to the IL-1Rs. IL-1ra is known to be induced by inhibitory cytokines and thereby limits the inflammatory response, produced by TLR activation. After TLR stimulation, monocytes release IL-10 and IL-1ra. In the presence of NGF, IL-1ra secretion induced by LPS was increased (Fig. 2C). Taking into account the previously shown decrease in IL-1β release (Fig. 1C), NGF addition resulted in a significant decrease in the IL-1β/IL-1ra molar ratio from 1.58 ± 0.3 in LPS-treated monocytes to 0.75 ± 0.2 in LPS + NGF–treated cells (p < 0.01).

Effects of TLR ligands on TrkA expression

As the expression of only one of the two NGF receptors, TrkA, is markedly upregulated following TLR activation, we hypothesized that NGF affects TLR signaling through TrkA-activated pathways. TLR stimulation results in activation of specific inflammatory, as well as anti-inflammatory, intracellular pathways. The latter include the PI3K pathway, which has been shown to inhibit inflammatory cytokine production and activate anti-inflammatory mechanisms in monocytes (16, 28). Because it is well known that in neuronal cells (9) NGF binding to TrkA activates the PI3K pathway, we focused on this pathway involved in both TrkA- and TLR-driven signaling.

We found that NGF addition at the time of TLR activation enhanced Ser473 phosphorylation of Akt (Fig. 4A), a downstream effector of PI3K activated in a phosphatidylinositol 3,4,5-triphosphate–dependent manner. In Us monocytes and in the absence of TLR ligands, no significant changes in Akt phosphorylation were observed after NGF addition. In monocytes activated with LPS or LTA, the effect of NGF on Akt phosphorylation was confirmed by densitometric analyses of immunoblots from four independent experiments, which indicated a significant increase in the level of phospho-Akt (p < 0.05; Fig. 4A1). One essential function of Akt is the phosphorylation and subsequent inactivation of GSK3 (29). We found that NGF significantly increased inhibitory phosphorylation in Serβ of GSK3 (Fig. 4B, 4B1) as assessed with densitometric analysis of four independent experiments (p < 0.05). Inhibition of Akt phosphorylation using two pharmacological inhibitors of PI3K, Wortmannin and LY294002, abolished the NGF-induced reduction in IL-6 release (Fig. 4C). Indeed, as expected (30, 31), both inhibitors enhanced IL-6 production in monocytes treated with either LPS or LTA + NGF. In keeping with the involvement of this pathway, pretreatment of monocytes with LiCl and AR-18 (32), two GSK3 inhibitors, led to a strong decrease in IL-6 production, amplifying the inhibitory effect of NGF (Fig. 4C).

Because the production of inflammatory cytokines after TLR activation in monocytes is primarily under the control of the NF-κB pathway, we also investigated the effects of NGF treatment on this pathway. The addition of NGF resulted in decreased IκB phosphorylation (Fig. 4D, with densitometric quantification of four independent experiments in Fig. 4D1). Consistent with a reduced degradation of IκB, we observed a reduced p65 NF-κB nuclear translocation in LPS-activated monocytes treated with NGF (Fig. 4E). The videodensitometric analysis of the fluorescence intensity (Fig. 4F) showed that NGF addition induced a significant decrease in the p65 NF-κB immunofluorescence in the nuclear compartment of LPS-treated cells (p < 0.001). Although translocation of p65 NF-κB in the nucleus is reduced, p50NF-κB DNA-
In this group of experiments, the MFI in the nuclear compartment with increased nuclear translocation of p65 NF-κB in LPS + NGF + anti-TrkA Ab–treated cells (Fig. 5E) or an increase in IL-10 production (Fig. 5F), demonstrating that these effects are mediated by NGF binding to TrkA. Moreover, in the presence of TrkA antagonists, NGF addition to LPS-activated monocytes did not cause a decrease in IL-6 production (Fig. 5D). The two TrkA antagonists also abolished the NGF effect on GSK3 in LPS-activated monocytes, as indicated by Western blot (E). One of three independent experiments is shown in (E).

Effects of TrkA inhibition on TLR signaling in monocytes

To demonstrate that the effects of NGF on cytokine production and intracellular signaling are mediated through TrkA, we used two TrkA-specific antagonists, the soluble TrkA receptor and a neutralizing TrkA Ab. The soluble TrkA receptor is a recombinant chimeric molecule with the extracellular portion of TrkA fused to the Fc region of human IgG1 (TrkA-Fc chimera). These antagonists inhibit the binding of NGF to TrkA through two different mechanisms, as demonstrated in neuronal cells (33, 34). As our results indicate, this inhibition also occurs in monocytes: the incubation of LPS-activated monocytes with the neutralizing TrkA Ab abolished TrkA phosphorylation induced by NGF (Fig. 5A). In LPS-activated monocytes, NGF induces a strong phosphorylation of Akt at Ser9 (Fig. 5B). The two TrkA antagonists also abolished the NGF effect on GSK3 in LPS-activated monocytes, as demonstrated by the reduction in the inhibitory phosphorylation of GSK3β at Ser9 (Fig. 5B). The two TrkA antagonists also abolished the inhibitory effect on IkB phosphorylation induced by NGF in LPS-activated monocytes (Fig. 5C), and this was associated with increased nuclear translocation of p65 NF-κB (Fig. 5D).

FIGURE 3. TrkA and p75NTR expression in monocytes activated by TLR ligands. Quantification by real-time PCR of TrkA and p75 mRNA expression showed that freshly isolated monocytes (t0) express high levels of TrkA that decrease after 24 h in culture (Us). Addition of LPS (10 ng/ml) strongly induces TrkA expression, whereas p75 is not affected (A, B). The study of the kinetics of TrkA and p75NTR mRNA expression in human monocytes stimulated with LPS (C) or LTA (D) confirmed that whereas TrkA expression is modulated after TLR activation, p75NTR expression levels remain stable. The results in (C) and (D) are expressed as AU obtained after normalization with the housekeeping gene actin and represent the mean of four independent experiments ± SEM.

Freshly isolated monocytes were incubated with LPS, LTA, or PAM for 24 and 48 h. Lysates of cells were tested for TrkA and tubulin control expression by Western blot (E). One of three independent experiments is shown in (E).
FIGURE 4. Effects of NGF on TLR signaling and cytokine production. Freshly isolated monocytes were starved for 3 h in RPMI 1640 0.1% FBS, stimulated with LPS or LTA in the presence or absence of NGF for 30 min, and then lysed. In TLR-activated monocytes, the addition of NGF enhances Akt phosphorylation in Ser473 (A) and GSK3β inhibitory phosphorylation in Ser9 (B). Figures in (A) and (B) are representative of one of four experiments, and (A1) and (B1) show the mean values obtained from the densitometric analysis. Monocytes were pretreated with inhibitors of Akt, wortmannin (WT; 1 μM), and LY294002 (LY; 25 μM) or with inhibitors of GSK3 (iGSK3), LiCl (40 mM), and AR-18 (25 μM) for 30 min and then incubated with LPS in the presence or absence of NGF for 18 h (C). IL-6 secretion was enhanced in the presence of Akt inhibitors, and the effect of NGF (Figure legend continues)
production (median value 3591 pg/ml; IQR 11224–2157; and median value 618 pg/ml; IQR 1478–270, respectively) was not reduced in the presence of NGF. Overall, these data suggest that reduced expression of TrkA in JIA patients may contribute to the downregulation of a physiological anti-inflammatory mechanism mediated by NGF.

was abolished. In contrast, the inhibition of GSK3 strongly reduced IL-6 synthesis. The results represent the mean of five independent experiments ± SEM. **p < 0.01 comparing LPS + NGF and LPS without NGF, according to Wilcoxon statistical analysis. Freshly isolated monocytes were treated with LPS in the presence or absence of NGF for 30 min and then lysed to evaluate IκB phosphorylation (DO, D1). Starved cells activated for 30 min as described in (A) and (B) were also immunostained for confocal microscopy analysis of p65 NF-κB nuclear translocation (D). The images are representative of three independent experiments. Green, p65 NF-κB; dark blue, nucleus; white/yellow, p65 NF-κB in the nucleus. Scale bar, 20 μm. IL-6 (E) and IL-10 (F) secretion in supernatants after 18 h of treatment was quantified by ELISA. The results represent the mean of four independent experiments ± SEM. *p < 0.05, **p < 0.01.
Discussion

In this study, we show that, by binding to TrkA, NGF dampens the inflammatory response in monocytes activated with TLR ligands, and we identify TrkA-activated pathways that interact with TLR signaling.

The results obtained in the current study show that although monocytes express both TrkA and p75NTR, only the expression of TrkA is selectively upregulated in the presence of TLR stimulation, whereas the expression of p75NTR remains stable. These results confirm and extend previous studies on TrkA expression in monocytes (20, 21, 35), demonstrating that the increase in TrkA expression is a characteristic response not only to TLR4 ligand activation but also to TLR2 ligand activation, suggesting that this could be a generalized phenomenon induced by TLRs. We found that this upregulation of TrkA expression, induced by TLR activation, allows NGF to limit the production of inflammatory cytokines, dampening the inflammatory response.

Stimulation of monocytes by TLRs leads to a cascade of intracellular signaling events that ultimately result in the production of inflammatory mediators that are crucial for effective clearance of pathogens. Dysregulation of TLR signaling pathways leads to increased expression of cytokines and chemokines, causing tissue damage and chronic inflammation (18). As a number of studies have shown, when TLRs are stimulated, there is a concomitant activation of inflammatory and anti-inflammatory pathways (28), the latter being needed to limit the inflammatory response and avoid tissue damage. Hence, a balance needs to be maintained between activation and downregulation of this response. Our findings show that, after TLR activation, NGF binding to TrkA activates intracellular pathways that interfere with TLR signaling, potentiating endogenous negative-feedback mechanisms that regulate excessive inflammation.

We show that the addition of NGF to TLR-activated monocytes inhibits the production of proinflammatory cytokines, including IL-1β, IL-6, and TNF-α and increases the production of the anti-inflammatory cytokines IL-10 and IL-1ra. These results are in keeping with a previous observation showing increased release of IL-10 by LPS-activated monocytes in the presence of NGF (23).

The anti-inflammatory action of NGF is mediated by the TrkA receptor. Our data show for the first time, to our knowledge, that in TLR-stimulated monocytes, similarly to what happens in neuronal cells, NGF binding to TrkA specifically autophosphorylates tyrosine residues of this receptor and activates signaling through the PI3K/Akt pathway, which influences the downstream signaling of TLRs. Several studies have shown that in monocytes, the PI3K/Akt pathway, activated in response to TLR stimulation, is one of the main pathways responsible for the downregulation of TLR ligand-induced inflammatory responses (16, 29). Indeed, phosphorylation of Akt, a downstream effector of PI3K, inhibits GSK3 activity (36, 37) and results in the suppression of NF-κB–dependent transcription of proinflammatory cytokine genes, while concomitantly inducing the expression of IL-10 and IL-1ra (27, 34). The

FIGURE 6. TrkA mRNA expression in JIA patients. TrkA mRNA expression was analyzed in PBMC and SFMC of 25 children affected with JIA. As shown in (A), the expression of TrkA is extremely reduced in JIA PBMC compared with PBMC of healthy children of similar age (control [Ctrl]). A similar decreased expression of TrkA characterizes SFMC of the inflamed synovia. The results are expressed as AU and obtained after normalization with the housekeeping gene actin. The box plot indicates median, 25th, and 75th percentile values, and the bars represent the minimum and maximum values obtained from sample analysis. In the matched samples, the expression of TrkA mRNA in the cells obtained from the inflamed synovia (SFMC) is only slightly increased (B) compared with JIA PBMC and does not even reach the constitutive expression levels of PBMC from healthy donors (A). Western blot confirmed that TrkA is almost absent in PBMC and SFMC from JIA patients, whereas is constitutively expressed in PBMC from controls (Ctrl) (C). In addition to low TrkA, SFMC from JIA patients have a barely detectable constitutive secretion of NGF (9.4 ± 1.3 pg/ml). On the contrary, the NGF concentration in the matched synovial fluids (syn fluid) is ∼2 ng/ml (D). JIA PBMC and SFMC were stimulated ex vivo with LPS with or without the addition of NGF. The production of IL-6 induced by LPS activation was decreased by NGF in Ctrl PBMC, whereas in PBMC and SFMC of JIA patients, the addition of NGF was not able to downregulate IL-6 production (E). **p < 0.01. norm. exp., normalized expression.
key role of the PI3K/Akt pathway in mediating the observed effects of NGF on cytokine production is supported by the use of pharmacological inhibitors of Akt, such as wortmannin and LY294002 (30), which restored the production of IL-6 while inhibiting the production of IL-10 (38).

We found consistently that, in TLR-activated monocytes, NGF significantly enhances phosphorylation of Akt and inhibition of GSK3, which, as one might expect (39), affect the NF-κB pathway. Indeed, we found that IκB phosphorylation, necessary for IκB degradation, resulting in NF-κB activation and nuclear translocation (40), is reduced by NGF addition to LPS-activated monocytes. In these cells, as expected, we observed an associated decrease in p65 NF-κB nuclear localization. While reducing nuclear translocation of p65 NF-κB, NGF treatment enhances p50 NF-κB levels in the nucleus of LPS-activated monocytes as indicated by the increase in p50 homodimer DNA binding activity. The increase in p50 homodimer activity is known to represent an important anti-inflammatory mechanism used to repress inflammatory cytokine production in monocytes and macrophages (40, 41). All of these effects of NGF on NF-κB pathway are consistent with our data on the NGF-induced decrease in inflammatory cytokines for which production is under NF-κB control.

The central role of the TrkA receptor in mediating these anti-inflammatory effects of NGF was demonstrated by using two TrkA antagonists, an anti-TrkA–neutralizing Ab and the TrkA-soluble receptor (TrkA chimera), which block the binding of NGF to TrkA (33, 34) through two different mechanisms. Addition of these two inhibitors to LPS-activated monocytes led to blocking of NGF-induced TrkA phosphorylation and, consequently, reduced Akt phosphorylation, decreased inhibitory phosphorylation of GSK3 in Ser9, and enhanced phosphorylation of IκB and NF-κB nuclear translocation. As a consequence, blocking TrkA resulted in an increase in the production of IL-6 and IL-1β and a decrease in the production of IL-10 by LPS-activated monocytes.

We have previously shown that NGF decreases HLA-DR and CD86 expression in LPS-activated monocytes, reducing the Ag-presenting capacity and costimulatory function of monocytes (23). This observation, together with the results of the current study, leads to the hypothesis that the monocyte lineage is the target of the anti-inflammatory immunosuppressive effects of NGF through the TrkA receptor, for which expression is induced by TLR activation to maintain homeostasis and avoid prolonged inflammation and subsequent tissue damage.

Our in vitro findings regarding the anti-inflammatory effect of NGF provide a possible mechanistic explanation for the effects of in vivo manipulation of NGF in animal models of inflammation. Indeed, administration of NGF in experimental autoimmune encephalomyelitis (EAE) delays the onset of clinical symptoms and prevents the full development of EAE lesions (5, 42, 43). Conversely, the neutralization of NGF in EAE leads to an increase in tissue inflammation and disease severity (44). Very similar results were reported in a colitis model, in which NGF neutralization resulted in exacerbated mucosal damage and increased disease severity (6, 7). Interestingly, in both models, the results suggested that increased IL-10 levels might be involved in mediating NGF anti-inflammatory effects (5, 6). Altered Th cell balance and reduced IFN-γ production were also reported in these models after NGF manipulation (5, 42). It is tempting to speculate that the effect of NGF on monocytes might affect downstream adaptive immunity with changes in effector T cell differentiation.

Whatever the complexity of the downstream effects of NGF, it appears reasonable to hypothesize a physiological loop in which inflammatory stimuli, while activating TLR and an inflammatory response, also upregulate TrkA and NGF expression, which, in turn, modulates TLR signaling and activates regulatory anti-inflammatory pathways to limit excessive tissue damage. As previously mentioned, it is well known that chronic inflammatory diseases, such as JIA or rheumatoid arthritis, are characterized by high levels of NGF in the inflamed tissues (45–47). To evaluate whether these high levels of NGF exert their full inhibitory potential, we investigated the levels of TrkA expression in PBMC or SFMC of patients with JIA and compared them with age-matched controls. Our results demonstrate markedly reduced expression of TrkA in mononuclear cells of JIA patients. This reduced expression characterizes not only PBMC but also from the site of inflammation (i.e., synovial fluid). Even in SFMC, the expression of TrkA is lower than the constitutive expression levels of quiescent PBMC obtained from healthy controls. Analysis of the matched synovial fluids has shown that a high concentration of NGF is present locally but does not seem to modulate inflammatory cytokine production. However, based on the decreased expression of TrkA, we hypothesized that the high levels of NGF present at inflammatory sites may not exert their full regulatory potential. Confirming the hypothesis, we found in ex vivo experiments that the addition of NGF to LPS-activated PBMC and SFMC from JIA patients fails to reduce the production of IL-6, in contrast with our observations in LPS-activated PBMC of healthy donors following addition of NGF. These data suggest that, although NGF is present in high concentrations in the inflamed joints, its anti-inflammatory action is impaired by TrkA downregulation. The decreased expression of TrkA therefore promotes an imbalance between proinflammatory and anti-inflammatory intracellular pathways. This imbalance might represent a novel mechanism contributing to the development and maintenance of chronic inflammation. Further studies aimed at evaluating TrkA expression in other inflammatory diseases that target tissues other than the synovium may help to clarify whether this is a general mechanism in chronic inflammatory diseases. Studies aimed at understanding the mechanisms and factors that affect TrkA expression during the course of inflammatory diseases might provide useful additional information before these findings are translated into possible approaches for the treatment of chronic inflammatory diseases. In this respect and bearing in mind the anti-inflammatory effect mediated through TrkA, due caution should be exercised during the implementation of approaches based on anti-TrkA Abs, such as those aimed at reducing neuropathic pain (48, 49), and in vivo and ex vivo inflammatory responses should be closely monitored.

In summary, NGF, through the TrkA receptor, is one of the signals that is involved in endogenous regulatory feedback mechanisms during the inflammatory response; by showing reduced expression of TrkA in children with chronic arthritis, we provide evidence that this regulatory feedback mechanism may be altered in chronic inflammatory disease.

Disclosures

The authors have no financial conflicts of interest.

References
