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**Interleukin-10 protects Lipopolysaccharide-induced Neurotoxicity in Primary
Midbrain Cultures by Inhibiting the Function of NADPH Oxidase**

Li Qian, Michelle Block, Sung-Jen Wei, Chiou-feng Lin, Jeffrey Reece, Hao Pang,
Belinda Wilson, Jau-Shyong Hong, and Patrick M Flood

Comprehensive Center for Inflammatory Disorders, University of North Carolina, Chapel
Hill, NC 27599,USA (L.Q., P.M.F.)

Neuropharmacology Section, Laboratory of Pharmacology and Chemistry, National
Institute of Environmental Health Sciences, National Institutes of Health, Research
Triangle Park, North Carolina 27709, USA (L.Q., M.B., C.-F.L., H.P., B.W., J.-S.H.)

The National Center for Toxicogenomics, National Institute of Environmental Health
Sciences, Research Triangle Park, North Carolina, USA (S.-J.W.)

Confocal Microscopy Center, Laboratory of Signal Transduction, National Institute of
Environmental Health Sciences, Research Triangle Park, North Carolina, USA (J.R.)

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Address correspondence and reprint requests to Dr. Patrick M. Flood, The Comprehensive Center for Inflammatory Disorders, University of North Carolina, Chapel Hill, NC 27599-7455. E-mail address: pat_flood@dentistry.unc.edu telephone: 919-966-1538; fax: 919-966-3683

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Abbreviations used in this paper: IL, interleukin; MPP⁺, 1-methyl-4-phenylpyridinium; DA, Dopaminergic; PD, Parkinson's disease; ROS, reactive oxygen species; iNOS, inducible nitric oxide synthase; TH-IR, tyrosine hydroxylase-immunoreactive; NO, Nitric Oxide; PGE₂, Prostaglandin E₂; COX-2, cyclooxygenases-2; PHOX, NADPH oxidase; DCFH-DA, Dichlorodihydrofluorescein Diacetate; OX-42, CR3 complement receptor; SOD, superoxide dismutase. TNF, tumor necrosis factor; LPS, lipopolysaccharide;

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ABSTRACT

The role of anti-inflammatory cytokines in Parkinson's disease is not completely understood. In this study, using mesencephalic neuron-glia cultures, we report that both pre-treatment and post-treatment of rat mesencephalic neuron-glia cultures with interleukin (IL)-10, a natural immune modulator, reduced lipopolysaccharide (LPS)-induced DA neurotoxicity. The main purpose of this study was to elucidate the molecular mechanism underlying IL-10-elicited neuroprotection. IL-10 significantly inhibited LPS-induced production of tumor necrosis factor (TNF)- α , nitric oxide, and extracellular superoxide in microglia cells. In addition, using reconstituted neuron and glia cell cultures, IL-10 was shown to be neuroprotective only in the presence of microglia. More importantly, IL-10 failed to protect DA neurons in cultures from mice lacking NADPH oxidase (PHOX), a key enzyme for extracellular superoxide production in immune cells, suggesting the critical role of PHOX in IL-10 neuroprotection. This conclusion was further supported by the finding that IL-10 inhibited LPS-induced translocation of the cytosolic subunit of NADPH oxidase p47^{phox} to the membrane. When JAK1 signaling pathway was blocked, IL-10 failed to attenuate LPS-induced superoxide production, indicating that the JAK1 signaling cascade mediates the inhibitory effect of IL-10. Together, our results suggest that IL-10 inhibits LPS-induced DA neurotoxicity through the inhibition of PHOX activity in a JAK1 dependent mechanism.

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INTRODUCTION

The pathogenesis of several neurological disorders, including Parkinson's disease (PD), Alzheimer's disease, multiple sclerosis and the AIDS dementia complex, has been closely associated with localized inflammatory responses in the brain (PL McGeer, 1988; Liu and Hong, 2003; Rosi et al., 2005). It has been suggested that this inflammation may play a role in a delayed and progressive degeneration of dopaminergic (DA) neurons in the substantia nigra, which is a hallmark of PD (McGeer PL, 1988). Recent studies have implicated a critical role of inflammation induced by microglia in neurodegenerative diseases, including PD. While activation of microglia serves an important protective function in immune surveillance by removing foreign microorganisms (Aloisi, 1999), overactivation of microglia followed by overproduction of pro-inflammatory factors have been shown to result in neuronal death in the brain (McGuire et al., 2001; Sriram et al., 2002). The midbrain region that encompasses the substantia nigra is particularly rich in microglia (Kim et al., 2000), therefore, activation of nigral microglia and release of these pro-inflammatory neurotoxic factors may be a crucial component of the degenerative process of DA neurons in PD.

Since these pro-inflammatory neurotoxic factors also exhibit immunoregulatory functions necessary for normal immune responses, the microglial response to inflammatory stimuli must be tightly regulated to avoid over-activation and disastrous neurotoxic consequences (Liu and Hong, 2003). It has been shown that DA neurons are particularly sensitive to the lethal effects of oxidative stress, including nitric oxide (NO) and superoxide (Jenner and Olanow, 1996; Greenamyre et al., 1999). Previous reports

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from our laboratory have shown that inhibition of microglial production of extracellular superoxide was most effective in protecting DA neurons, suggesting that superoxide was the dominant neurotoxic factor for these DA neurons (Liu et al., 2000a). NADPH oxidase (PHOX), an enzyme that is a major source of superoxide in immune cells, is highly expressed in microglia. Both *in vivo* and *in vitro* studies using PHOX-deficient mice have clearly demonstrated reduced DA neurotoxicity induced by lipopolysaccharide (LPS) in PHOX^{-/-} compared to PHOX^{+/+} wild-type mice (Qin et al., 2004). Moreover, PHOX activity may also regulate the levels of tumor necrosis factor (TNF)- α production by microglia following LPS stimulation (Qin et al., 2004), indicating that PHOX not only mediates superoxide production, but also controls the levels of other pro-inflammatory neurotoxic factors produced by activated microglia. Therefore, the neurotoxicity mediated by microglia is a complex puzzle that potentially involves a number of distinct pro-inflammatory responses by microglia. The exact mechanism and kinetics by which microglia mediate neurotoxicity and the signals that control their pro-inflammatory response have yet to be determined.

Interleukin (IL)-10 is a pleiotropic cytokine that plays a critical role in the regulation of inflammatory responses and immune reactions, acting on both hematopoietic and non-hematopoietic cells (Kremlev and Palmer, 2005). Increasing evidence indicates that IL-10 has the ability to improve neurological outcome after CNS injury, and this ability relies on its anti-inflammatory effects (Grilli et al., 2000; Kremlev and Palmer, 2005). For example, IL-10 has neuroprotective properties against glutamate-induced (Bachis et al., 2001) or hypoxic–ischemic (Dietrich et al., 1999) neuronal death, and against LPS- or interferon-induced oligodendrocyte cell death (Molina-Holgado et

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al., 2001). It was also reported that IL-10 counteracts acute effects of endotoxin on cerebral metabolism, microcirculation, and oxygen tension during hypoxia–ischemia in the perinatal brain (Froen et al., 2002). While it is well known that IL-10 inhibits known inflammatory responses of microglia (Hu et al., 1999; Kremlev and Palmer, 2005), the interplay between glial cells, neurons and induced pro-inflammatory mediators leading to CNS pathology is complex and incompletely understood. Therefore, further assessment of the functional contribution of IL-10 critically depends on the elucidation of downstream secondary signaling mechanisms.

The main purpose of this study was to elucidate the molecular mechanism underlying IL-10-elicited neuroprotection. We show that IL-10 has significant protective effects on LPS-induced DA neurotoxicity through its inhibition of microglia activation. The finding that IL-10 is acting to inhibit PHOX activity, which then results in the inhibition of a wide array of pro-inflammatory mediators produced by activated microglia, offers new insights into our understanding the etiology and eventual treatment of neurodegenerative diseases such as PD.

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MATERIALS AND METHODS

Animals

NADPH oxidase-deficient (gp91phox^{-/-}) and wild-type C57BL/6J (gp91phox^{+/+}) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Breeding of the mice was performed to achieve timed pregnancy with the accuracy of ± 0.5 days. Timed-pregnant Fisher F344 rats were obtained from Charles River Laboratories (Raleigh, NC). Housing and breeding of the animals were performed in strict accordance with the National Institutes of Health guidelines.

Reagents

The recombinant rat IL-10 was obtained from R&D system (Minneapolis, MN). JAK inhibitor I and LPS (strain O111:B4) were purchased from Calbiochem (San Diego, CA). Cell culture reagents were obtained from Invitrogen (Carlsbad, CA). [³H] DA (30 Ci/mmol) was obtained from Perkin-Elmer Life Sciences (Boston, MA), and the monoclonal antibody against the CR3 complement receptor (OX-42) was purchased from Chemicon International (Temecula, CA). The polyclonal anti-tyrosine hydroxylase antibody was a generous gift from Dr. John Reinhard (GlaxoSmithKline, Research Triangle Park, NC). The Vectastain ABC kit and biotinylated secondary antibodies were purchased from Vector Laboratories (Burlingame, CA). The fluorescence probe Dichlorodihydro- fluorescein Diacetate (DCFH-DA) was obtained from Calbiochem (La Jolla, CA). Rabbit anti-p47^{phox} was obtained from Upstate (Lake Placid, NY). FITC-conjugated goat anti-rabbit IgG was obtained from Jackson ImmunoResearch

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Laboratories. Rabbit anti-GAPDH was obtained from Abcam (Cambridge, MA). Mouse anti-gp91^{phox} was purchased from BD Transduction Laboratories (San Jose, CA).

Cell lines

The rat microglia HAPI cells were a generous gift from Dr James R. Connor (Cheepsunthorn et al., 2001) and were maintained at 37°C in DMEM supplemented with 10% FBS, 50 U/ml penicillin and 50 µg/ml streptomycin in a humidified incubator with 5% CO₂/95% air.

Primary mesencephalic neuron-glia culture

Neuron-glia cultures were prepared from the ventral mesencephalic tissues of embryonic day 14-15 rats or day 13-14 mice, as described previously (Liu et al., 2000a; Gao et al., 2002). Briefly, dissociated cells were seeded at 1×10^5 /well and 5×10^5 /well to poly-D-lysine-coated 96-well and 24-well plates respectively. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air, in MEM containing 10% fetal bovine serum, 10% horse serum, 1 g/L glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 µM nonessential amino acids, 50 U/ml penicillin, and 50 µg/ml streptomycin. Seven-day-old cultures were used for treatment. At the time of treatment, immunocytochemical analysis indicated that the rat neuron-glia cultures were made up of 11% microglia, 48% astrocytes, 41% neurons, and 1% tyrosine hydroxylase immunoreactive (TH-IR) neurons. The composition of the neuron-glia cultures of NADPH oxidase-deficient mice was very similar to that of the wild-type mice consisting of 12% microglia, 48% astrocytes, 40% neurons, and 1% TH-IR neurons.

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Primary Mesencephalic Neuron-Enriched Cultures

Midbrain neuron-enriched cultures were established as described previously (Gao et al., 2002). Briefly, 24 h after seeding the cells, cytosine β -D-arabinozide was added to a final concentration of 10 μ M to suppress glial proliferation. Three days later, cultures were changed back to maintenance medium and were used for treatment 7 days after initial seeding.

Primary Microglia-Enriched Cultures

Rat microglia-enriched cultures with a purity of >98%, were prepared from whole brains of 1-day-old Fischer 344 rat pups as described previously (Liu et al., 2000b). For superoxide assays, 10^5 cells were grown overnight in 96-well culture plates before use.

Mesencephalic Microglia-depleted Cultures

Mesencephalic neuron-glia were seeded at 5×10^5 /well in 24-well plates. Microglia were depleted by 1 mM L-leucine methyl ester for 72 h (Liu et al., 2000b). The cultures stained with OX-42 antibody showed less than 0.1% microglia.

DA Uptake Assay

[3 H]-DA uptake assays were performed as described (Liu et al., 2000b). Briefly, cells were incubated for 20 min at 37°C with 1 μ M [3 H]-DA in Krebs-Ringer buffer (16 mM sodium phosphate, 119 mM NaCl, 4.7 mM KCl, 1.8 mM CaCl₂, 1.2 mM MgSO₄, 1.3 mM EDTA, and 5.6 mM glucose; pH 7.4). After washing three times with ice-cold Krebs-Ringer buffer, the cells were collected in 1 N NaOH. Radioactivity was determined by

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liquid scintillation counting. Nonspecific DA uptake observed in the presence of mazindol (10 μ M) was subtracted.

Immunostaining

DA neurons were recognized with the anti-TH antibody and microglia were detected with the OX-42 antibody, which recognizes the CR3 receptor as described previously (Gao et al., 2002). Briefly, formaldehyde (3.7%)-fixed cultures were treated with 1% hydrogen peroxide (10 min) followed by sequential incubation with blocking solution for 30 min, primary antibody overnight at 4°C, biotinylated secondary antibody for 2 h, and ABC reagents for 40 min. Color was developed with 3,3'-diaminobenzidine. For morphological analysis, the images were recorded with an inverted microscope (Nikon, Tokyo, Japan) connected to a charge-coupled device camera (DAGE-MTI, Michigan City, IN) operated with the MetaMorph software (Universal Imaging Corporation, Downingtown, PA). For visual counting of TH-IR neurons, nine representative areas per well of the 24-well plate were counted under the microscope at 100 \times magnification by three individuals. The average of these scores was reported.

Nitrite and TNF- α Assays

The production of NO was determined by measuring the accumulated levels of nitrite in the supernatant with Griess reagent and the release of TNF- α was measured with a rat TNF- α enzyme-linked immunosorbent assay kit from R and D System (Minneapolis, MN, USA), as described (Liu et al., 2002).

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Prostaglandin E₂ (PGE₂) production

PGE₂ in supernatant was measured with a PGE₂ EIA kit from Cayman (Ann Arbor, MI, USA) according to the manufacturer's instructions.

Superoxide Assay

The production of superoxide was determined by measuring the superoxide dismutase (SOD)-inhibitable reduction of the tetrazolium salt WST-1 (Peskin and Winterbourn, 2000; Tan and Berridge, 2000). Neuron-glia or microglia-enriched cultures in 96-well culture plates were washed twice with HBSS without phenol red. Cultures were then incubated at 37°C for 30 min with vehicle control (water) or IL-10 in HBSS (50 µl/well). Then, 50 µl of HBSS with and without SOD (50 U/ml, final concentration) was added to each well along with 50 µl of WST-1 (1 mM) in HBSS, and 50 µl of vehicle or LPS (10 ng/ml). Thirty minutes later, absorbance at 450 nm was read with a SpectraMax Plus microplate spectrophotometer (Molecular Devices Corp, Sunnyvale, CA). The difference in absorbance observed in the absence and presence of SOD was considered to be the amount of superoxide produced, and results were expressed as percentage of vehicle-treated control cultures.

Assay of Intracellular Reactive Oxygen Species (ROS)

Intracellular oxidative stress was measured by Dichlorodihydrofluorescein-Diacetate (DCFH-DA). DCFH-DA enters cells passively and is deacetylated by esterase to nonfluorescent DCFH. DCFH reacts with ROS to form DCF, the fluorescent product (Liu et al., 2001). DCFH-DA was dissolved in methanol at 10 mM and was diluted 500-fold in

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HBSS to give DCFH-DA at 20 μ M. The cells were exposed to DCFH-DA for 1 h and then treated with HBSS containing the corresponding concentrations of LPS for 2 h. The fluorescence was read immediately at wavelengths of 485 nm for excitation and 530 nm for emission using a SpectraMax Gemini XS fluorescence microplate reader (Molecular Devices). The value subtracted by control group was viewed as the increase of intracellular ROS.

Real Time RT-PCR Analysis

For real time quantitative PCR, RNA was obtained using the RNeasy Mini Kit from QIAGEN. DNase (Ambion, Austin, TX) was used to digest DNA at 37°C for 30 min, the integrity of the RNA was assessed by visualization of high 18S and 28S ribosomal RNA peaks as well as a small amount of 5S RNA. Quantity and quality assessment using a UV/VIS spectrophotometer was performed at 260 nm and 280 nm. OD 260/280 ratio greater than 1.8 was an acceptable indicator of good RNA quality. 1 μ g of total RNA was reverse-transcribed using random hexamers and MMLV reverse transcriptase. The SYBR green DNA PCR kit (Applied Biosystems, Foster City, CA) was used for real time PCR analysis. The relative differences in expression between groups were expressed using cycle time values normalized to GADPH. The relative differences between control and treatment groups were calculated and expressed as relative increases setting control as 100%. The sequence of the oligonucleotide primers used were from rat: TNF- α : 5'-TCGTAGCAAACCACCAAGCA-3' and 5'-CCCTTGAAGAGAACCTGG GAGTA-3'; Inducible nitric oxide synthase (iNOS): 5'-ACATCAGGTCGGCCATCAC T-3',5'-CGTACCGGATGAGCTGTGAATT-3'; cyclo-oxygenases-2 (COX-2): 5'-CCAGCAG

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GCTCATACTGATAGGA-3' and 5'-GCAGGTCTGGGTCGAACTTG-3'; GAPDH: 5'-CCTGGAGAAACCTGCCAAGTAT-3' and 5'-AGCCCAGGATGCCC TTTAGT-3'.

Subcellular Fractionation and Western Blot Analysis

Subcellular fractionation were performed as described (Yuan et al., 1996). HAPI cells were lysed in hypotonic lysis buffer (1 mM EGTA, 1 mM EDTA, 10 mM β -glycerophosphate, 10 mM NaF, 1 mM sodium orthovanadate, 2mM MgCl₂, 10 mM DTT, 1 mM PMSF, and 10 ug/ml each leupeptin, aprotinin, and pepstatin A), incubated on ice for 30 min, and then subjected to Dounce homogenization (20~25 stokes, tight pestle A). The lysates were loaded onto sucrose in lysis buffer (final 0.5 M) and centrifuged at 1600 \times g for 15 min, the supernatant above the sucrose gradient was used as cytosolic fractions after centrifugation at 150,000 \times g for 30 min. The pellets solubilized in 1% NP-40 hypotonic lysis buffer were used as membranous fraction. Equal amounts of protein (20 μ g per lane) were separated by 4~12% Bis-Tris Nu-PAGE gel and transferred to polyvinylidene difluoride membranes (Novex, San Diego, CA). Membranes were blocked with 5% nonfat milk and incubated with rabbit anti-p47^{phox} antibody (1: 2000) or rabbit anti-GAPDH (1:2000), or mouse anti-gp91^{phox} (1:2000) for 1 h at 25°C. Horse radish peroxidase-linked anti-rabbit or mouse IgG (1:3000) for 1h at 25°C, ECL+Plus reagents (Amersham Biosciences Inc., Piscataway, NJ) were used as a detection system.

Confocal microscopy

HAPI cells seeded in dish at 5×10^4 cells/well were treated with LPS for 10 min in the absence or presence of IL-10 pretreatment for 1 h. Cells were fixed with 3.7%

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paraformaldehyde in PBS for 10 min. After washed with PBS, cells were incubated with rabbit polyclonal antibody against p47^{phox}. Cells were then washed and incubated with FITC-conjugated goat anti-rabbit antibody. Focal planes spaced at 0.4- μ m intervals were imaged with a Zeiss 510 laser scanning confocal microscope (63 \times PlanApo 1.4 numerical aperture objective) equipped with LSM510 digital imaging software. The signal of p47^{phox} (FITC- p47^{phox}; green) and the merge view of cell morphology and p47^{phox} (Phase plus FITC- p47^{phox}) are shown.

Statistical Analysis

The data were presented as the means \pm SE. For multiple comparisons of groups, ANOVA was used. Statistical significance between groups was assessed by paired Student's *t* test, with Bonferroni's correction. A value of $P < 0.05$ was considered statistically significant.

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RESULTS

Effect of IL-10 on LPS-induced degeneration of DA neurons

We first sought to determine if LPS-induced inflammation by glial cells leading to destruction of DA neurons could be inhibited by the anti-inflammatory cytokine IL-10. Mesencephalic neuron–glia cultures were pretreated with IL-10 for 1 h and then stimulated with LPS for 7 days. The degeneration of DA neurons was then determined by [³H] DA uptake assay and numeration of the TH-IR neurons. The [³H]-DA uptake assay showed that LPS treatment reduced the capacity of the cultures to take up DA to approximately 60% of the vehicle control and this LPS-induced reduction was prevented by a dose-dependent pretreatment with IL-10 (Fig. 1A). At 10 and 30 ng/ml IL-10, LPS-induced decrease in DA uptake was completely restored while IL-10 alone at this concentration range did not affect DA uptake levels in the cultures. A similar protective effect was obtained with IL-10 when counting the number of TH-IR neurons after immunostaining (Fig. 1B). Thus, LPS-induced loss of TH-IR neurons was prevented by IL-10 pretreatment in a concentration-dependent manner with a significant effect at 10 and 30 ng/ml. Morphological inspection revealed that LPS treatment not only decreased the number of TH-IR neurons, but also caused a loss of neuronal process, and these characteristics were also reversed by 10 ng/ml of IL-10 pretreatment (Fig. 1C).

Microglia, but not astroglia, mediated IL-10's neuroprotective effect against LPS-induced DA neurotoxicity

Our mesencephalic neuron–glia cultures contain ~10% microglia and ~50% astrocytes in addition to DA and other neurons, we sought to evaluate what cell type of glia mediated

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the neuroprotective effect of IL-10. Previous report shows the expression of IL-10 receptors both in microglia and astroglia (Ledeboer et al., 2002). Using immunostaining we found that neurons from both mouse and rat cultures do not express detectable levels of IL-10 receptors (data not shown). Therefore, we investigated whether microglia or astroglia cells were the targets mediating the inhibitory effects of IL-10 on LPS-induced neurotoxicity by performing reconstitution experiments where we added either microglia or astroglia back to neuron- enriched cultures.

As shown in Fig. 2A, LPS alone, IL-10 alone or the combination of LPS and IL-10 did not affect the DA uptake capacity in either neuron-enriched cultures or neuron-astroglia cultures. In contrast, addition of 10% of microglia back to the neuron-enriched cultures reduced the capacity of DA uptake to 52% by LPS, similar to the original neuron-glia cultures, and this reduction can be reversed by IL-10. In addition, when 1-methyl-4-phenylpyridinium (MPP⁺), which is known to damage DA neurons directly, was added to neuron-enriched or microglia-depleted cultures, IL-10 failed to show any protection (Fig. 2B), suggesting that the neuroprotective effect of IL-10 is not a direct effect on neurons. These results demonstrate that it is the microglia, not astroglia, which serve as the target of IL-10-mediated neuroprotection against LPS induced neurodegeneration.

IL-10 pretreatment suppresses LPS-induced release of pro-inflammatory mediators and their gene expression

Mesencephalic neuron–glia cultures treated with LPS displayed a significant enlargement of cell size from predominantly resting round and small cells to activated rod- and/or amoeboid-shaped cells (Kreutzberg, 1996), and intensified OX-42 immunoreactivity, a

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marker for the activation of microglia (Fig. 3A). The LPS-stimulated activation of microglia was suppressed in neuron–glia cultures pretreated with IL-10, while IL-10 alone did not show a significant effect on microglia activation. We also found that pretreatment with 3~30 ng/ml IL-10 significantly blocked LPS-stimulated NO production and iNOS mRNA expression (Fig. 3B), TNF- α production and mRNA expression (Fig. 3C), and PGE₂ production and COX-2 mRNA expression (Fig. 3D). In addition, IL-10 at 10 and 30 ng/ml, but not 3 ng/ml, significantly attenuated the LPS-induced ROS production, which including extracellular superoxide and intracellular ROS production (Fig. 3E and Fig. 3F). Interestingly, the two concentrations of IL-10 found to significantly inhibit both ROS and TNF- α production were identical to the concentrations that correspond with the DA neuroprotective effect of IL-10 in the LPS induced mesencephalic neuron-glia cultures (Fig. 1), while the 3 ng/ml dose (which has little if any protective effect) shows no significant inhibition of the generation of ROS but significantly inhibits NO and PGE₂ production, and shows significant but not complete inhibition of TNF- α production. Therefore, our data suggest that the neuroprotective effect of IL-10 is due, at least in part, to a reduction in LPS-induced oxidative stress and perhaps to the partial inhibition of TNF- α production in glial cells.

IL-10 inhibits LPS-induced translocation of cytosolic subunit of PHOX p47^{phox} to the membrane.

It has previously been shown that activation of PHOX activity requires cytosolic component p47^{phox} becomes phosphorylated, and translocates to the plasma membrane to associate with cytochrome b558 to assemble into an active enzyme complex (Liva et al.,

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1999). Since IL-10 showed potent effects on the ROS production, we sought to determine if IL-10 inhibits PHOX activation by preventing the translocation of the PHOX cytoplasmic subunit p47^{phox} from cytosol to membrane following LPS stimulation. Using confocal light scanning microscopy, we observed that LPS initiated the translocation of cytosolic p47^{phox} to the membrane as early as 3 min following LPS treatment (data not shown). Ten min after LPS stimulation, almost all the FITC fluorescence signal clustered in the membrane (Fig. 4A, panel III and IV), and the addition of IL-10 significantly prevented the translocation (Fig. 4A, panel V and VI). While vehicle (Fig. 4A, panel I and II) or IL-10 alone (Fig. 4A, panel VII and VIII) treated cells, p47^{phox} mainly localized in the cytosol. Consistent to the results of confocal study, western blot assay clearly showed an increase in the immunoreactivity of p47^{phox} in the membrane of HAPI cells 3 and 10 min after LPS treatment and this increase in the immunoreactivity was significantly blocked in the presence of IL-10 (Fig. 4B). Therefore, it is likely that IL-10-mediated inhibition of superoxide production by LPS is through the inhibition of p47^{phox} translocation.

PHOX plays an important role in IL-10-mediated protection against LPS-induced neuron degeneration

The above-mentioned results indicate that IL-10 reduced the LPS-induced production of superoxide through the inhibition of PHOX activity by blocking p47^{phox} translocation, thus, we further studied the role of ROS in IL-10 mediated neuroprotection by determining if IL-10 could protect LPS-induced neurodegeneration in PHOX-deficient cells. Neuron-glia cultures were prepared from PHOX^{-/-} mutant and PHOX^{+/+} wide-type

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mice. As shown in Fig. 5A, LPS treatment of neuron-glia cultures prepared from PHOX^{+/+} mice reduced [³H]-DA uptake by 42%; while IL-10 at 10 ng/ml significantly attenuated the decrease (Fig. 5A). In contrast, LPS treatment significantly reduced the uptake capacity by only 21% in PHOX^{-/-} mice, but IL-10 failed to show any protective effect. Consistent to the results of DA uptake, while LPS-induced TNF- α production in PHOX^{-/-} mice is significantly less than that seen in PHOX^{+/+} mice, IL-10 at 10 ng/ml was able to significantly reduce TNF- α production in PHOX^{+/+} mice but not in PHOX^{-/-} mice (Fig. 5B).

IL-10 inhibits superoxide production through the JAK-STAT pathway

We further studied the mechanism underlying the inhibitory effect of IL-10 on LPS-induced increase in superoxide production. Since IL-10 receptor signaling functions by activating the JAK1/STAT3 pathway, an inhibitor of JAK1 was used for this purpose. As shown in Fig. 6, the addition of JAK1 inhibitor eliminated the inhibitory effect of IL-10 on superoxide production by LPS-stimulated microglia. The inhibitory effect of IL-10 on LPS-induced superoxide production was also significantly inhibited when anti-IL-10 receptor antibody was used to block the action of IL-10 in these cultures (data not shown). These results suggest that IL-10 binds to its receptor to activate the JAK1/STAT3 pathway, leading to the inhibition of superoxide production by microglia.

Post-treatment with IL-10 was still effective in protecting LPS-induced DA neuronal damage.

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Since the production of most inflammatory mediators occurred within a few hours after stimulation of glial cells with LPS, we sought to determine if post-treatment with IL-10 was still effective in protecting DA neurons from LPS-induced toxicity. Neuron-glia cocultures were first treated with LPS for 3, 6, or 12 h, then IL-10 was added to the cultures and incubation was continued for another 7 days. We found that post-treatment with IL-10 up to 6 h after LPS exposure still showed a protective effect (Fig. 7A). However, when IL-10 was added at 12 h post LPS treatment, no significant protective effect was seen. When we analyzed nitric oxide and TNF- α production, we found that they were consistently inhibited only when IL-10 was added 3 h after LPS treatment, but not when added at 6 or 12 h after exposure to LPS (Fig. 7B and C). In contrast, superoxide production was significantly inhibited by the addition of IL-10 even up to 6 h after LPS treatment (Fig. 8). Thus, among the pro-inflammatory factors released from microglia, the inhibition of superoxide by IL-10 was best-correlated with its protection against LPS-induced DA neuron damage.

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DISCUSSION

LPS-induced degeneration of DA neurons in mesencephalic neuron-glia cultures is a useful *in vitro* model for studying the mechanism and identifying the potential therapeutic implications on inflammation-mediated neurodegeneration (Liu and Hong, 2003). Using this well-characterized model, we sought to determine if and how the major anti-inflammatory cytokine, IL-10, which regulates the production of pro-inflammatory mediators by macrophage and glial cells, could be effective in diminishing inflammation-induced neurodegeneration *in vitro*. Our results showed that IL-10 exerted potent effects in inhibiting LPS-induced inflammation and neuronal destruction. Three salient features of this protective role of IL-10 were observed in this study: 1) both pre-treatment and post-treatment with IL-10 showed strong neuroprotection in LPS-induced DA neuron degeneration; 2) the neuroprotective effect of IL-10 was mediated through the inhibition of microglia activation, and 3) PHOX is a major target of IL-10 mediated neuroprotection.

It has been reported that IL-10 has a major regulatory effect by inhibiting the production of a number of pro-inflammatory mediators by activated glial cells (Heyen et al., 2000). Our study demonstrates a neuroprotective effect of IL-10 in LPS-induced DA neurotoxicity. Further studies indicate that IL-10 has no direct effect on either neurons or astroglia, instead, the neuroprotective effect of IL-10 was mediated through the inhibition of microglial over-activation by LPS. This observation is novel and critical to further characterize the mechanisms by which microglia mediate neurotoxicity after LPS stimulation. These results confirm those previously obtained from our laboratory (Gao et al., 2002) and others (Tzeng et al., 2005) that microglia are the key players responsible

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for inflammation-related neuronal damage. In addition to the neuroprotective effect seen when IL-10 was added before LPS, we also observed that IL-10 can inhibit inflammation-induced neuronal degeneration when added up to 6 h after LPS exposure. The effectiveness in neuroprotection with post-treatment of IL-10 suggests that the release of this immune modulator after brain inflammation may play a critical role in suppressing the over-activation of microglia and preventing the excessive damage of neurons.

Increasing evidence has shown that oxidative stress plays a very important role in PD (Smith and Zigmond, 2003; Jackson-Lewis and Smeyne, 2005). DA neurons in the substantia nigra are uniquely vulnerable to oxidative stress (Jenner, 1998; Greenamyre et al., 1999). The higher sensitivity of DA neurons to oxidative damage so far has been attributed in large part to their known reduced antioxidant capacity, increased accumulation of iron, and the concentration of neurochemicals such as dopamine that are prone to oxidative modification. The fact that IL-10 significantly inhibits the production of superoxide induced by LPS within a few minutes after stimulation, led us to examine this factor in greater details by using PHOX-deficient mutant mice. The findings that IL-10 could significantly lessen the LPS-induced DA uptake reduction in cells from wild-type mice, but has no significant protective effect on cells from PHOX^{-/-} mice (Fig. 5A) strongly support the contention that the protective effect of IL-10 is most likely mediated through the inhibition of PHOX activity. Since we are measuring ROS production from primary midbrain cultures, we can not rule out the possibility that cells other than microglia are producing superoxide, and that PHOX is not the only enzyme which may play a role in ROS production. However, only microglia express LPS receptors and are

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activated by LPS, and therefore no other cell is likely to produce ROS upon stimulation with LPS. In addition, no extracellular superoxide production has been detected in cultures from PHOX^{-/-} mice (Qin et al., 2004), suggesting that PHOX is the only enzyme involved in superoxide production in these cultures. Activation of PHOX in microglia not only increases the production of superoxide, but indirectly increases the intracellular ROS concentration, possibly through the conversion of superoxide to H₂O₂, which is membrane permeable. Increase of intracellular ROS can intensify the activation of NF-κB, which leads to higher TNF-α and PGE₂ production (Liu and Hong, 2003; Qin et al., 2004). In addition, it was reported that PHOX inhibitors prevented LPS/IFNγ-induced degradation of IκBα, and thus, inhibited the activation of NFκB (Pawate et al., 2004). However, the ability to activate NF-κB-dependent genes such as TNF-α in PHOX^{-/-} cells suggests that PHOX plays an important but not exclusive role in regulating inflammation in microglial cells. These data are consistent with the notion that PHOX is a major effector of neurotoxicity, as well as the target of IL-10.

It has been found by a number of laboratories that IL-10 is a potent inhibitor of NF-κB activity (Ehrlich et al., 1998), primarily by inhibiting the transcriptional binding of NF-κB to κB sites in inflammatory mediators (Baldwin, 1996). However, others found that the direct anti-inflammatory effect of IL-10 is independent of the activity of NF-κB (Clarke et al., 1998), and rather targets the JAK/STAT pathway to selectively inhibit transcription of inflammatory genes (Murray, 2005). So far, the target of the IL-10-activated inhibitory effect still remains controversial. By inhibiting JAK1, a primary signaling kinase for the IL-10 receptor signaling, we demonstrate that the inhibition of NADPH oxidase by IL-10 is mediated through the inhibition of the JAK1 signaling

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pathway, which in turn prevents the translocation of cytosolic subunit p47^{phox} to the membrane, rather than a direct inhibition of PHOX activity. The inhibition of both LPS-induced and PMA-induced (data not shown) superoxide production by IL-10, and reversal of this inhibition by a JAK1 inhibitor, lend further support of this possibility.

Microglia play a critical role in the pathogenesis of various neurological disorders, not only because of their role as pro-inflammatory cytokine-producing cells, but also by playing a major regulatory role in limiting inflammation (Gehrmann et al., 1995). For example, it is known that resident microglial cells in the CNS produce IL-10 and express the IL-10 receptor, and therefore IL-10 may play an important autocrine role in the regulation of acute brain inflammation (Ledeboer et al., 2002). The potent anti-inflammatory and immune regulatory property of IL-10 suggests that it has the potential to be of major therapeutic value in the treatment of PD. In addition, IL-6 has also been described as a neuroprotectant and is released mainly from astrocytes after LPS stimulation (Bolin et al., 2005). It is possible that IL-6 and IL-10 have a similar role in neuroprotection, and they might even act in concert to regulate chronic inflammation in the brain. However, both IL-6 and IL-10 have been shown to be most effective anti-inflammatory cytokines only in acute inflammatory responses. It is yet to be determined what role IL-10 plays in regulating chronic CNS inflammation in PD. In addition, the level of the immune response in the brain necessary to effectively control infections without resulting in neuropathology is yet to be understood. Consequently, much work remains to determine if IL-10, either therapeutically delivered or produced in the CNS, plays an important anti-inflammatory role by limiting the activation of microglia and

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promoting a well regulated immune response that is lacking during the pathophysiology
of CNS disorders.

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FOOTNOTES

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Address correspondence and reprint requests to Dr. Patrick M. Flood,
The Comprehensive Center for Inflammatory Disorders, University of North Carolina,
Chapel Hill, NC 27599-7455. E-mail address: pat_flood@dentistry.unc.edu telephone:
919-966-1538.

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FIGURE LEGENDS

FIGURE 1. IL-10 is neuroprotective against LPS-induced neurotoxicity. Rat primary mesencephalic neuron-glia cultures seeded in a 24-well culture plate with 5×10^5 rat midbrain cells, then pretreated with various concentrations of IL-10 for 1 h before the addition of 10 ng/ml LPS. Seven days later, the LPS-induced dopaminergic neurotoxicity was quantified by the [^3H] DA uptake assay (A); the immunocytochemical analysis, including TH-IR neuron counts (B); and the representative pictures of immunostaining (C). Results were expressed as a percentage of the vehicle-treated control cultures and were the means \pm SE. from three independent experiments in triplicate. * $P < 0.05$, ** $P < 0.01$ compared with the LPS-treated cultures.

FIGURE 2. Effect of IL-10 on neurodegeneration is mediated through microglia. (A): 10 ng/ml of IL-10 or LPS were added to the following types of cell cultures: (NG): original neuron-glia cultures; (N+10%MG): reconstituted cultures by adding 10% of microglia to the neuron-enriched cultures; (N+50%AS): reconstituted cultures by adding 50% of astroglia to the neuron-enriched cultures, and (N): neuron-enriched cultures. (B): Midbrain microglia-depleted cultures (*open bars*) and neuron-enriched cultures (*closed bars*) were prepared as described in materials and methods. Cells were pretreated for 1 h with 10 ng/ml IL-10 followed by treatment with 0.5 μM MPP $^+$. The [^3H] DA uptake measurements were performed 8 days after the treatment. Results were expressed as a percentage of the vehicle-treated control cultures and were the means \pm SE from four independent experiments in triplicate. * $P < 0.05$ compared with the LPS-treated cultures. ** $P < 0.01$ compared with the vehicle-treated control cultures.

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FIGURE 3. Effect of IL-10 on LPS-induced production of pro-inflammatory factors and their gene expression in microglial. Mesencephalic neuron–glia cultures treated with LPS displayed the characteristics of activated microglia and intensified OX-42 immunoreactivity when analyzed on day 7 following LPS stimulation (A). IL-10's effect on LPS-induced production of NO, TNF- α and PGE2 (B-D respectively, closed bars) or iNOS, TNF- α , and COX-2 mRNA expression (B-D respectively, open bars) were shown. Protein levels of these factors are expressed as absolute values. Effects of IL-10 on LPS-induced production of superoxide (E) as % of control; and intracellular ROS (F) as absolute absorbance. Enriched microglia cells were treated with the vehicle control or IL-10 for 1 h before the addition of LPS (10 ng/ml). For analysis of mRNA, total RNA was harvested 3 h after LPS treatment, followed by real time RT-PCR analysis of iNOS, TNF- α , and COX-2 using specific primers. IL-10 alone has no effect on the protein or mRNA production of these inflammatory mediators. The results are the means \pm SE. of three experiments performed in triplicate. *P<0.05, **P<0.01, and ***P<0.001 compared with the LPS-treated cultures.

FIGURE 4. Effect of IL-10 on cytosolic p47^{phox} protein translocation. (A): Immunofluorescence and confocal microscopy analysis of p47^{phox} localization in LPS-stimulated HAPI cells. HAPI cells were treated with LPS for 10 min in the absence or presence of IL-10 pretreatment for 1 h. Cells were stained with rabbit polyclonal antibody against p47^{phox}, then washed and incubated with FITC-conjugated goat anti-rabbit antibody. The signal of p47^{phox} (FITC-p47^{phox}; on left) and the merge view of cell morphology and p47^{phox} (Phase plus FITC-p47^{phox}, on right) are shown. The inset shown

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in the left hand side of each treatment condition shows the location of FITC-p47^{phox} in a single, randomly selected cell. (B): HAPI cells were pretreated with vehicle or IL-10 (10 ng/ml) for 1h, followed by LPS treatment for 3~10 min. Subcellular fractions were isolated to perform western blot analysis. c: cytosolic extract; m: membrane extract. GAPDH is as an internal cytosolic control, gp91^{phox} as an internal membrane control. Each experiment has been performed three times.

FIGURE 5. Microglial NADPH oxidase is the target of IL-10 inhibition in LPS-induced neurotoxicity. PHOX^{+/+} and PHOX^{-/-} mice neuron-glia cultures were pretreated with vehicle or IL-10 (10 ng/ml) for 1 h, followed by LPS treatment. Neurotoxicity was assessed by DA uptake (A). TNF- α production (B) was measured by ELISA. Results are expressed as % of the control culture (A), and pg/ml (B) respectively, and are the means \pm SE of 3 individual experiments in triplicate in each experiment. * $P < 0.05$ compared with LPS treated culture. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ compared with the vehicle-treated control cultures.

FIGURE 6. IL-10 inhibits superoxide production through JAK1 signal pathway.

Enriched microglia cells were treated with the vehicle control or JAK1 inhibitor (50 nM) for 30 min before the addition of IL-10 (10 ng/ml), 1 h later, LPS (10 ng/ml) was added in the cultures. Extracellular superoxide was measured as SOD-inhibitable reduction of WST-1. Results were expressed as % of the control culture, and are the means \pm SE from 3 independent experiments in triplicate. * $P < 0.05$ compared with the LPS-treated cultures. # $P < 0.05$ compared with the vehicle-treated control cultures.

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FIGURE 7. Effect of IL-10 post-treatment in rat neuron-glia cocultures. The cultures were first treated with LPS, then 3, 6, or 12 h later IL-10 was added to the cultures. Neurotoxicity was assessed by DA uptake, TNF- α , and nitric oxide production. Results are expressed as % of the control cultures (*A*) and absolute value (*B* and *C*) and are the means \pm SE of 3 individual experiments in triplicate in each experiment. * P <0.05, ** P <0.01, *** P <0.001 compared with the LPS-treated cultures.

FIGURE 8. Effect of IL-10 post-treatment on superoxide production in rat neuron-glia cocultures. The cultures were first treated with LPS, then 6 h later IL-10 was added to the cultures. 1 h later, superoxide was assayed. Results are expressed as % of the control cultures and are the means \pm SE of 3 individual experiments in triplicate in each experiment. * P <0.05 compared with the LPS-treated cultures.

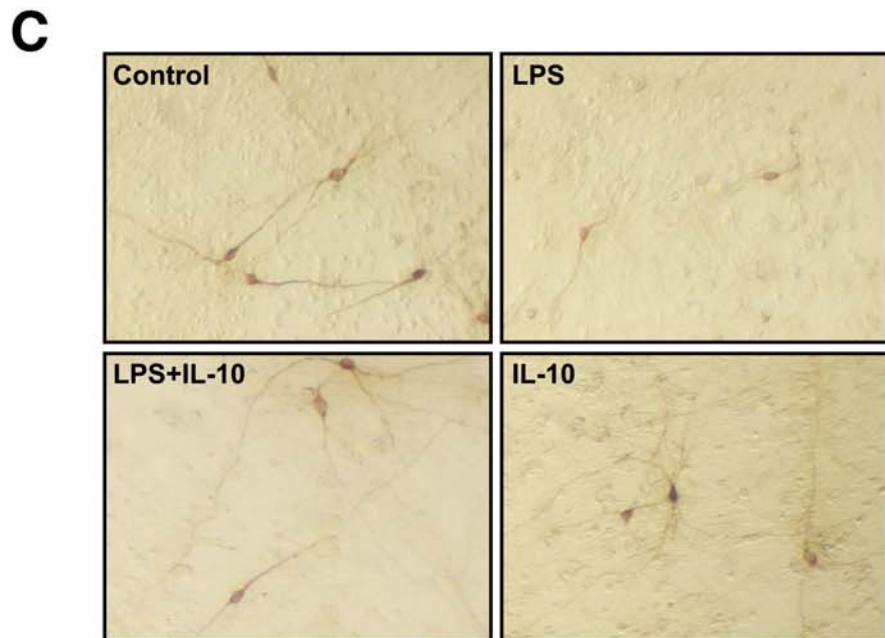
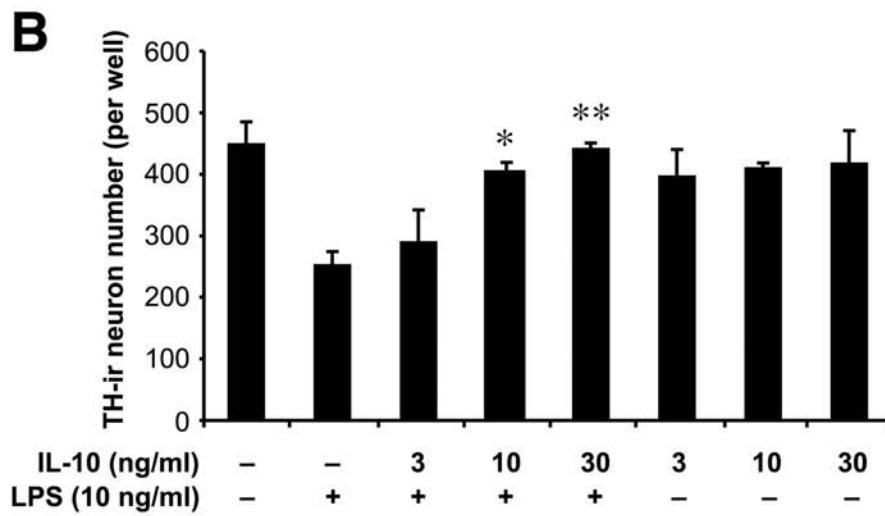
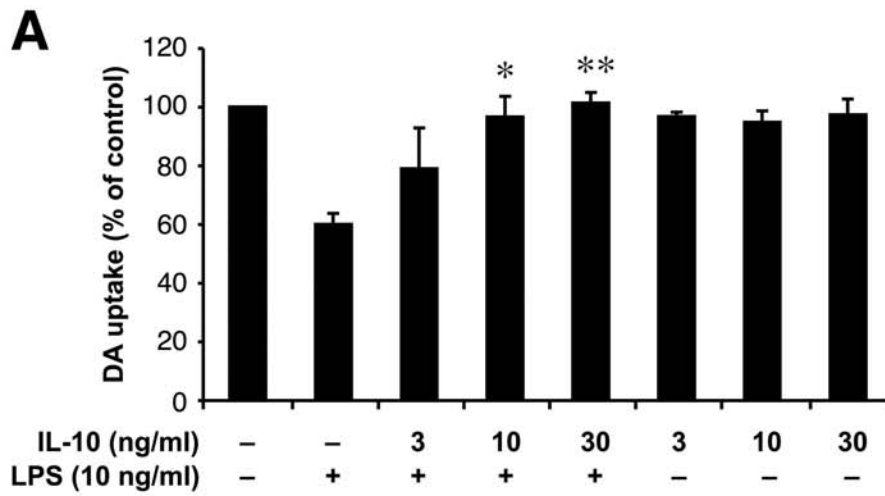
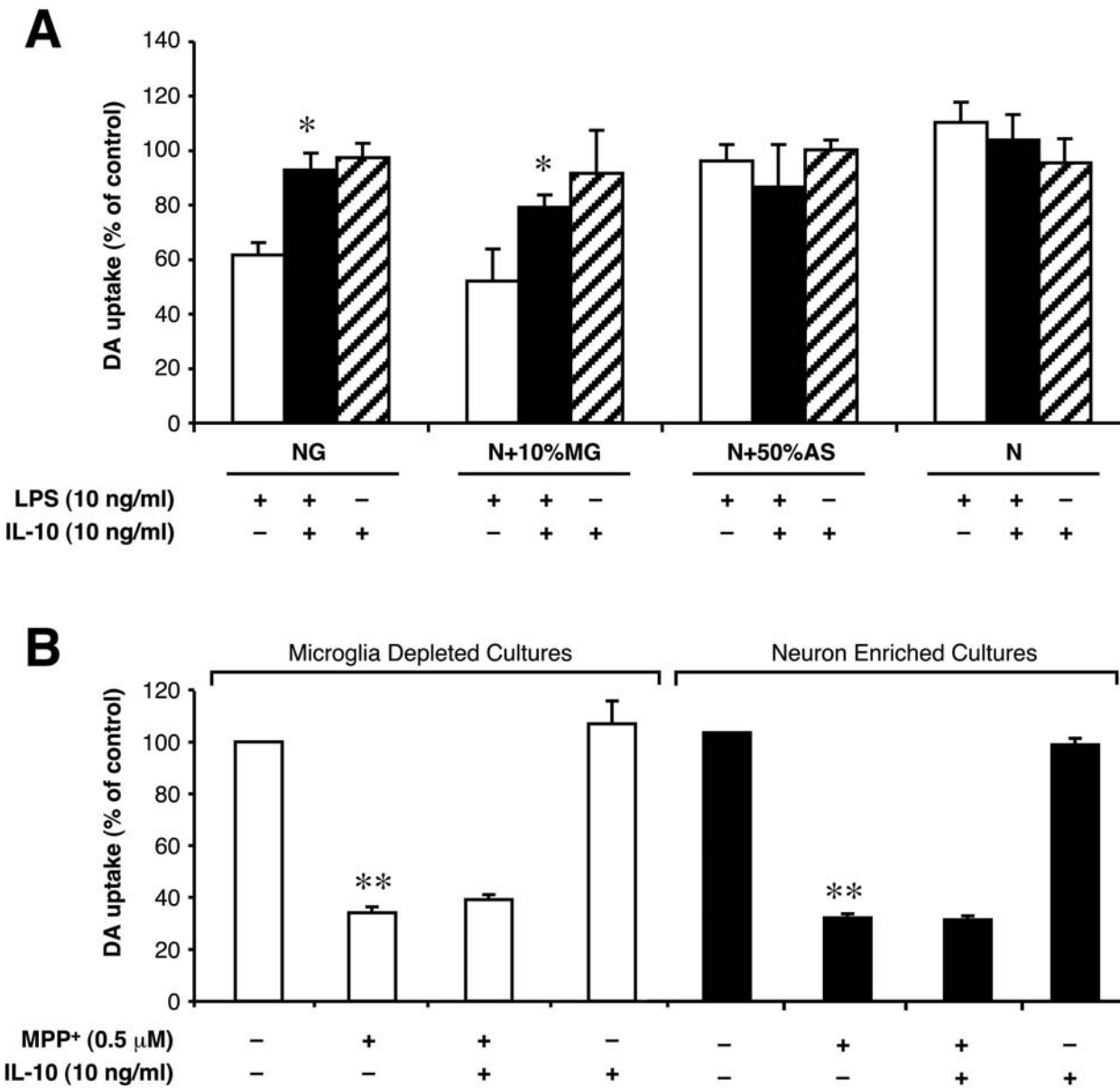
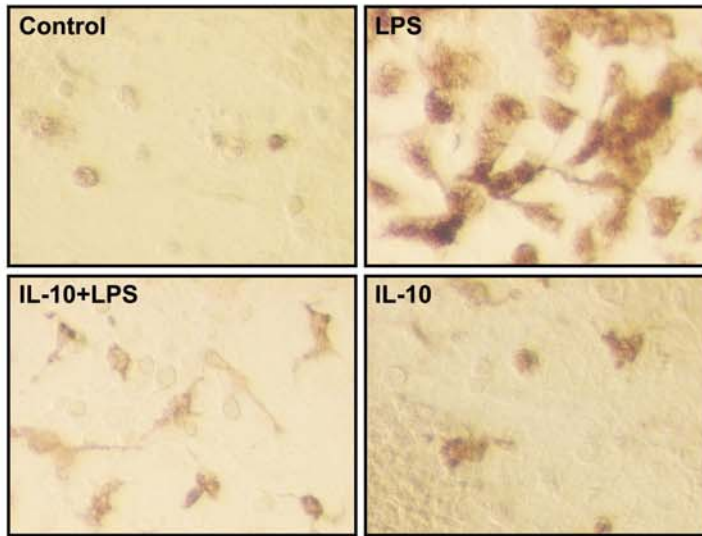


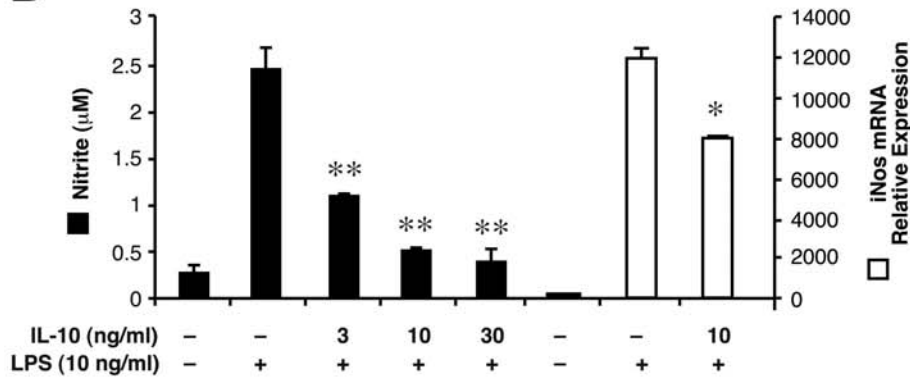
Figure 2



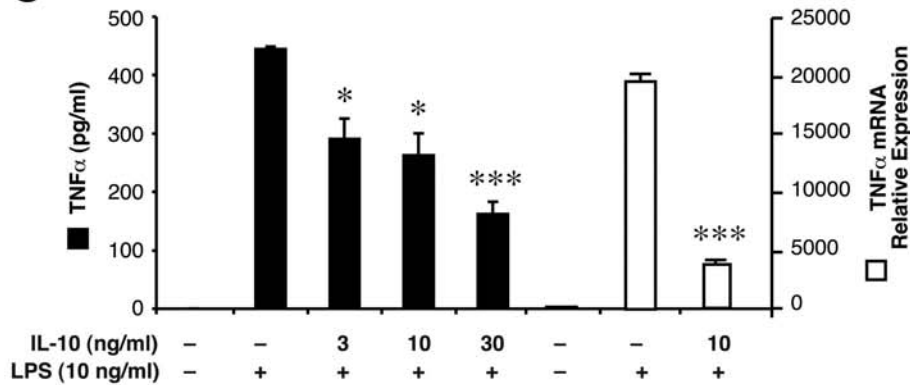
A



B



C



D

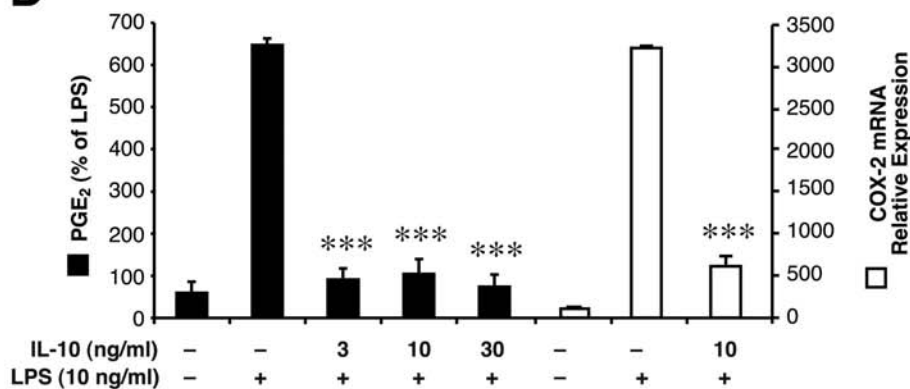
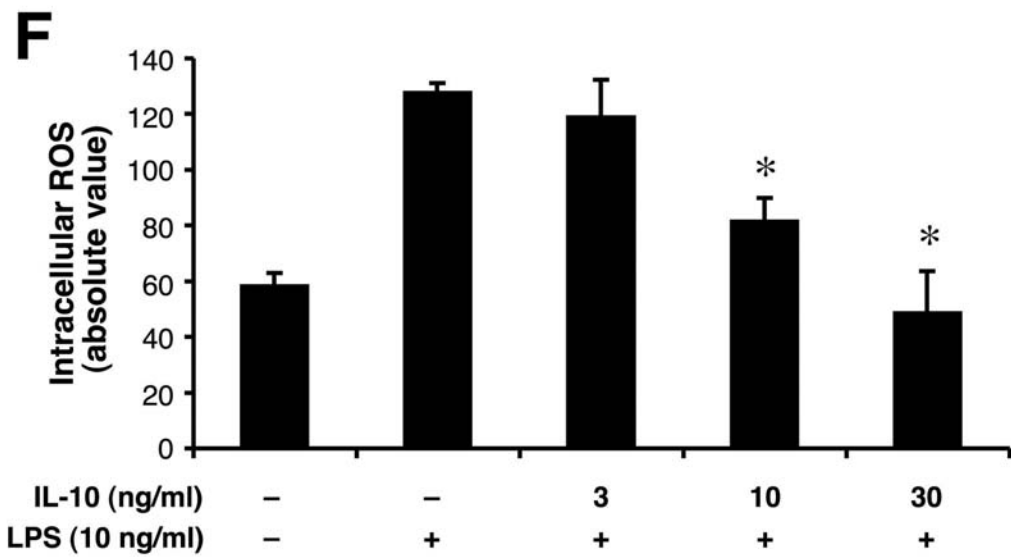
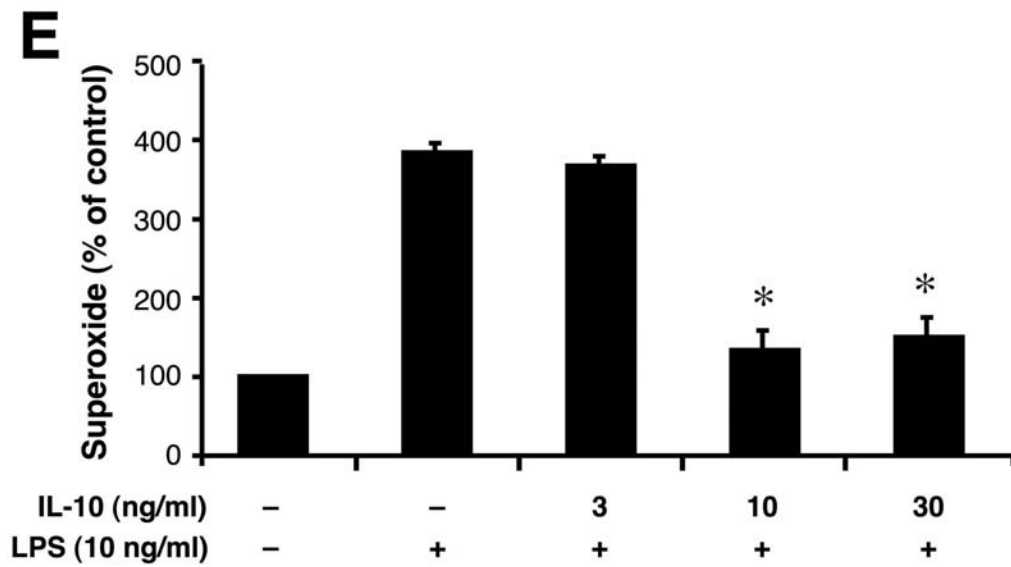
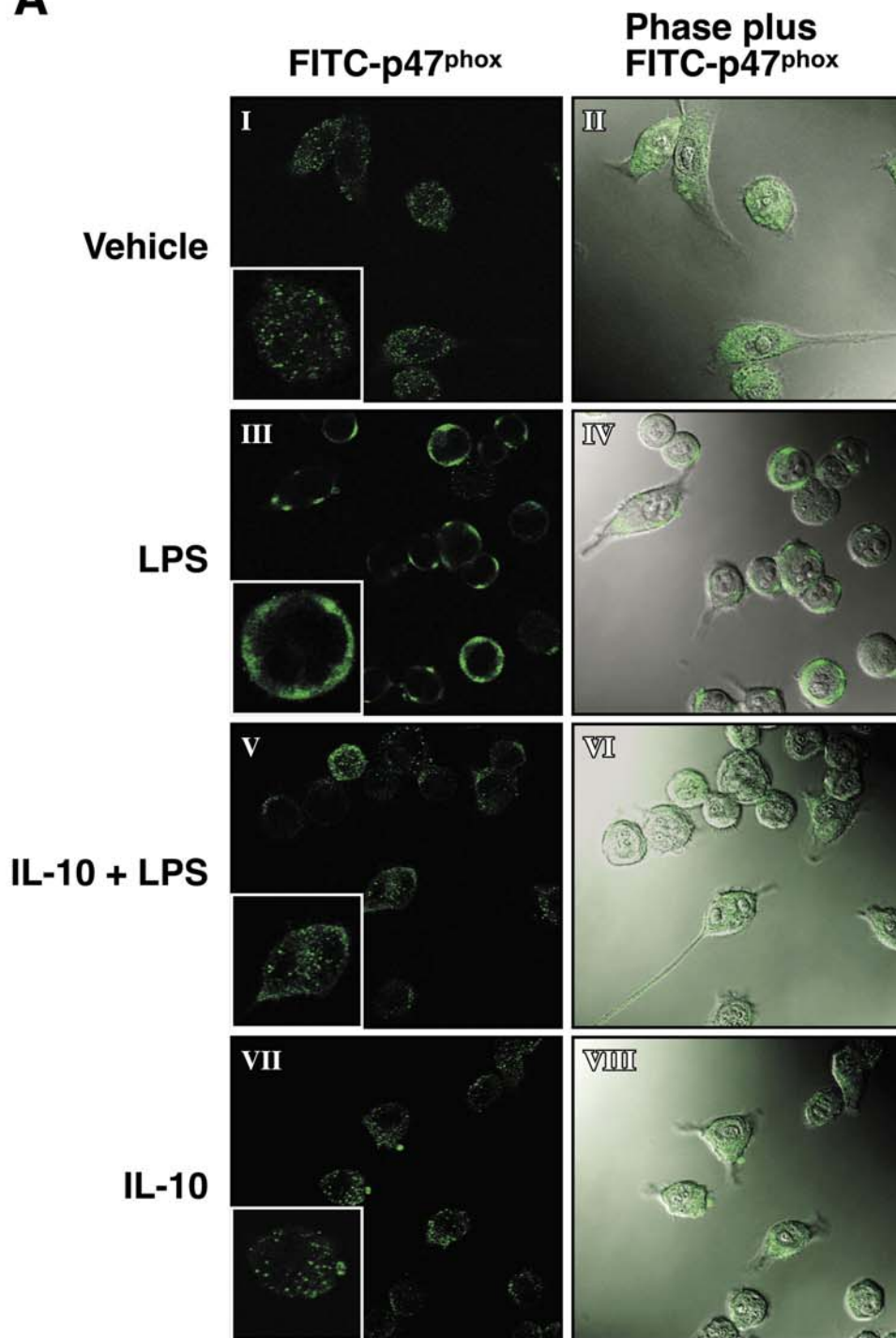


Figure 3



A



B

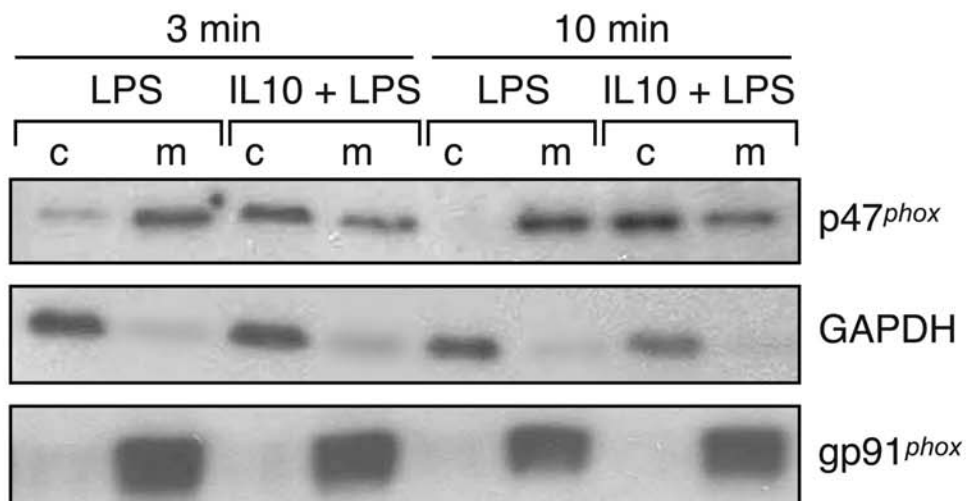


Figure 5

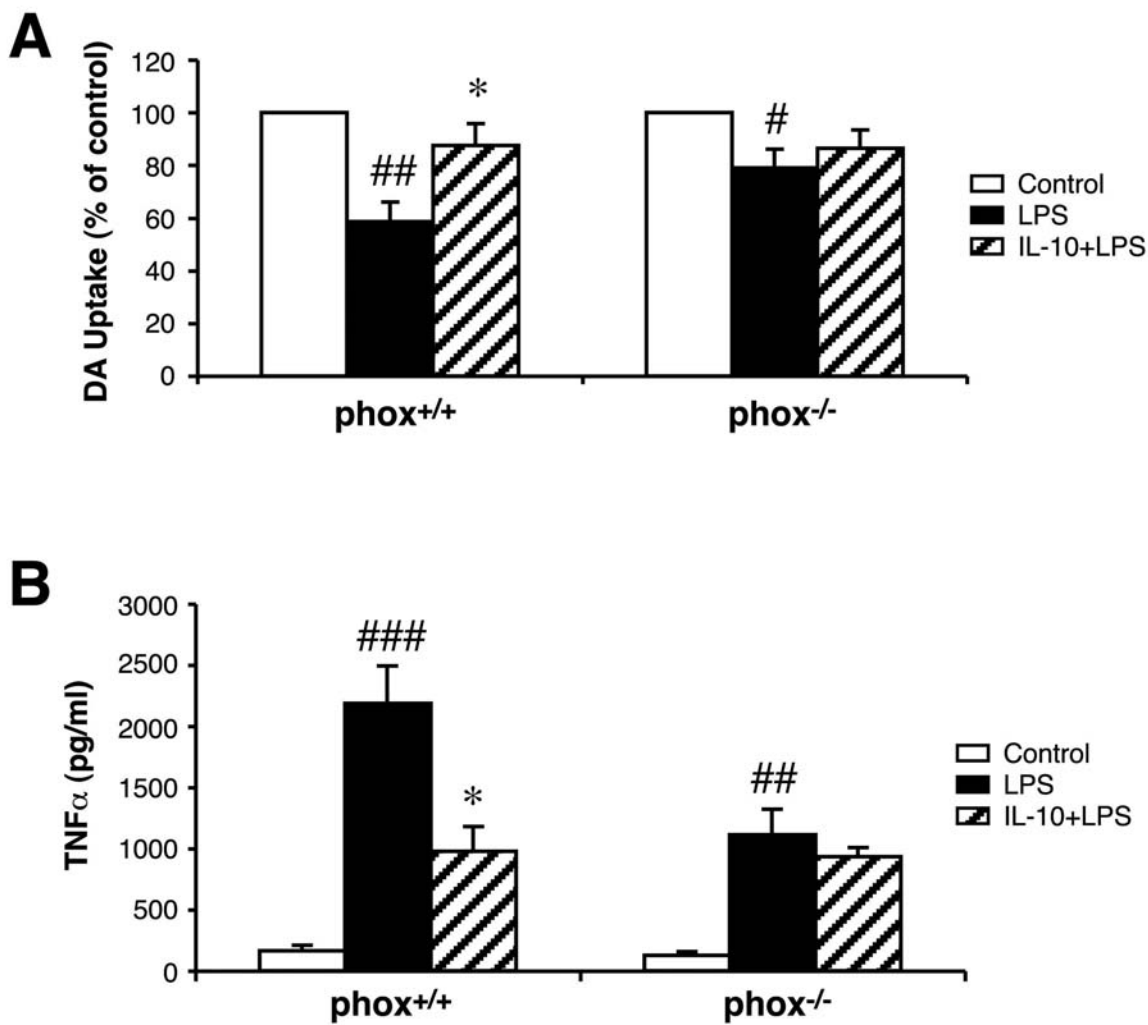
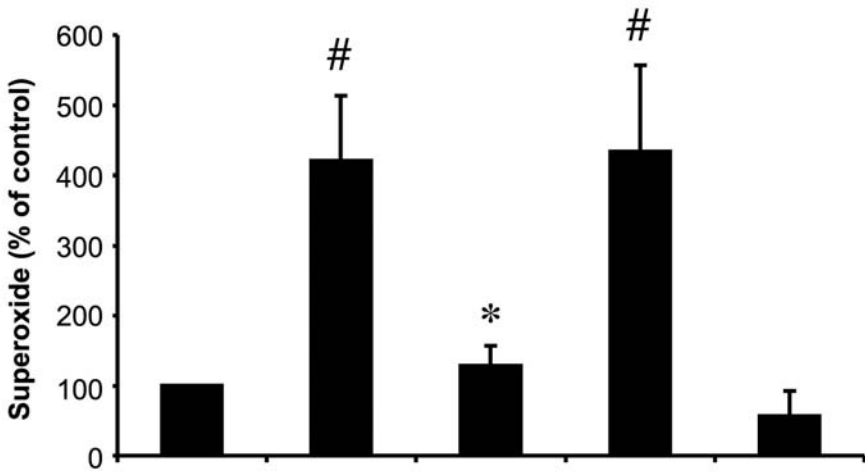


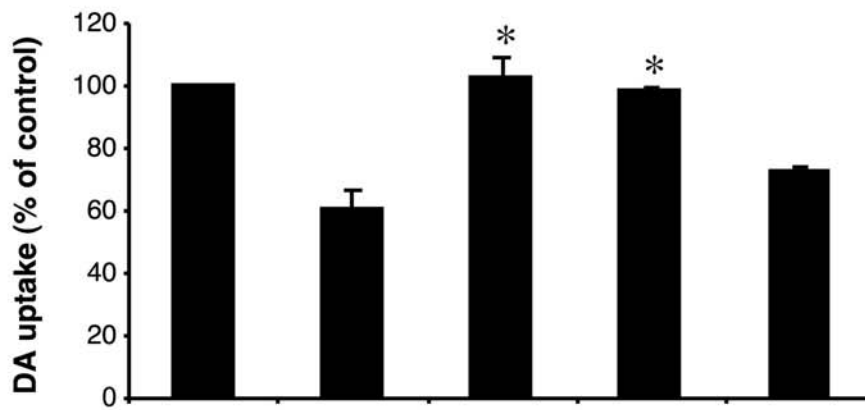
Figure 6

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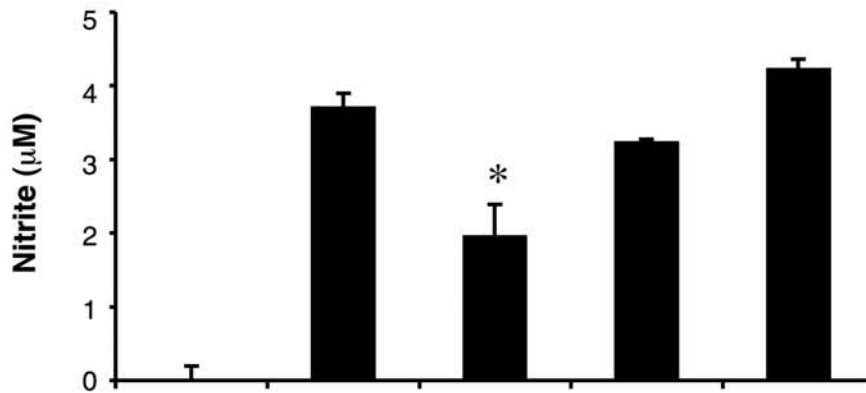
JAK1 inhibitor (50 nM)	-	-	-	+	+
IL-10 (10 ng/ml)	-	-	+	+	-
LPS (10 ng/ml)	-	+	+	+	-

A



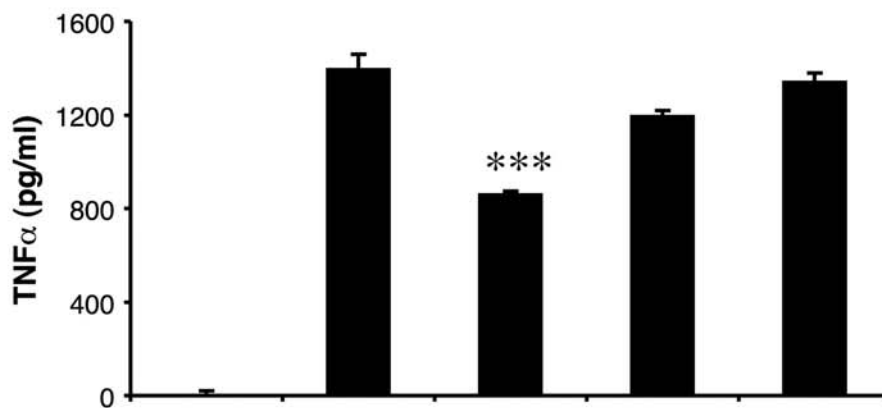
LPS (10 ng/ml)	-	+	+	+	+
IL-10 (10 ng/ml)	-	-	+	+	+
Time point of adding IL-10 (h)			3	6	12

B



LPS (10 ng/ml)	-	+	+	+	+
IL-10 (10 ng/ml)	-	-	+	+	+
Time point of adding IL-10 (h)			3	6	12

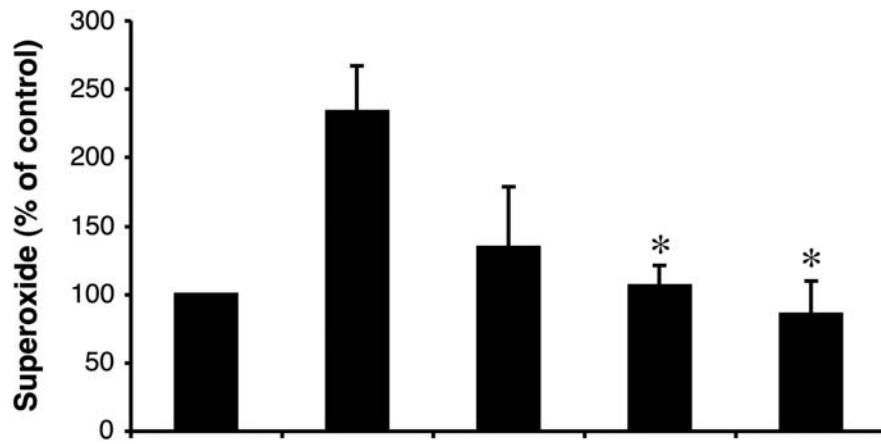
C



LPS (10 ng/ml)	-	+	+	+	+
IL-10 (10 ng/ml)	-	-	+	+	+
Time point of adding IL-10 (h)			3	6	12

Figure 8

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LPS (10 ng/ml)	-	+	+	+	+
IL-10 (ng/ml)	-	-	3	10	30
Time point of adding IL-10 (h)			6	6	6

Time point of adding IL-10 (h)