Published in final edited form as:

Mol Neurobiol. 2015 August ; 52(1): 587–600. doi:10.1007/s12035-014-8889-0.

PGE₂ inhibits IL-10 production via EP2-mediated β -arrestin signaling in neuroinflammatory condition

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Abstract

Regulatory mechanisms of the expression of interleukin 10 (IL-10) in brain inflammatory conditions remain elusive. To address this issue, we used multiple primary brain cell cultures to

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Competing Interests:

The authors declare no competing financial interests.

study the expression of IL-10 in lipopolysaccharide (LPS)-elicited inflammatory conditions. In neuron-glia cultures, LPS triggered well-orchestrated expression of various immune factors in the following order: tumor necrosis factor α (TNF- α), cyclooxygenase-2 (COX-2), prostaglandin E₂ (PGE₂) and lastly IL-10, and these inflammatory mediators were mainly produced from microglia. While exogenous application of individual earlier-released pro-inflammatory factors (e.g. TNF- α , IL-1 β or PGE₂) failed to induce IL-10 expression, removal of LPS from the cultures showed the requirement of continuing presence of LPS for IL-10 expression. Interestingly, genetic disruption of *tnf- α* , its receptors *tnf-r1/r2*, and *cox-2* and pharmacological inhibition of COX-2 activity enhanced LPS-induced IL-10 production in microglia, which suggests negative regulation of IL-10 induction by the earlier-released TNF- α and PGE₂. Further studies showed that negative regulation of IL-10 production by TNF- α is mediated by PGE₂. Mechanistic studies indicated PGE₂-elicited suppression of IL-10 induction was eliminated by genetic disruption of the PGE₂ receptor EP2 and was mimicked by the specific agonist for the EP2, butaprost, but not agonists for the other three EP receptors. Inhibition of cAMP-dependent signal transduction failed to affect PGE₂-mediated inhibition of IL-10 production, suggesting a G-protein-independent pathway was involved. Indeed, deficiency in β -arrestin-1 or β -arrestin-2 abolished PGE₂-elicited suppression of IL-10 production. In conclusion, we have demonstrated that COX-2-derived PGE₂ inhibits IL-10 expression in brain microglia through a novel EP2- and β -arrestin-dependent signaling pathway.

Keywords

microglia; IL-10; PGE₂; COX-2; EP2; β -arrestin

Introduction

Neuroinflammation is a self-defensive attempt by the central nervous system (CNS) to remove harmful stimuli (e.g. pathogens, damaged cells, autoimmunogens, or toxins) and to initiate the healing process [1,2]. The neuroinflammatory process has been recognized as an orchestrated stereotype, which contains three stages: initiation, propagation, and resolution [3]. Like peripheral inflammation, the neuroinflammatory response must be tightly regulated and actively terminated to prevent unnecessary tissue destruction [4]. At the resolution stage, organisms terminate inflammation by different mechanisms, which include rapid degradation of pro-inflammatory mediators, production and release of anti-inflammatory cytokines interleukin 10 (IL-10) and transforming growth factor β (TGF- β), desensitization of receptors, and apoptosis of inflammatory cells [5]. Failure in the resolution of inflammation leads to chronic inflammation and progressive cellular damage [6]. Indeed, emerging evidence has documented a pivotal role of chronic neuroinflammation in the pathogenesis of neurodegenerative diseases [7,8]. However, the precise mechanism orchestrating the resolution of acute neuroinflammation remains poorly understood.

As a potent anti-inflammatory cytokine, IL-10 plays an important role in resolving inflammation and maintaining immune homeostasis in various tissues including the CNS [9,10]. For example, IL-10-deficient mice spontaneously develop inflammatory bowel disease [11]. During the recovery phase of CNS inflammation, IL-10 regulates microglial phagocytosis, represses expression and release of pro-inflammatory mediators, and

suppresses antigen presentation [12–15]. Given the crucial role of IL-10 in neuroinflammation resolution and neuronal survival, elucidation of the mechanism governing the regulation of IL-10 expression is of paramount importance. In general, IL-10 production quickly rises at the later stage of neuroinflammation following the release of most pro-inflammatory factors [16]. To date, several intracellular signaling pathways (e.g. MAPK) and transcriptional factors (e.g. SP1) known to regulate expression of most pro-inflammatory mediators enhance IL-10 promoter activity and mRNA expression in various immune cells [17,18,10]. However, complex interactions and cross-regulation between different immune factors during neuroinflammation have hampered efforts to determine precise signal pathways responsible for regulation of IL-10 production.

Several studies have demonstrated that early release of immune factors can influence late-onset IL-10 production via an autocrine fashion [17,19–28]. Neutralization of the earlier-released pro-inflammatory factors TNF- α and IL-1 β using antibodies or genetic ablation of type I interferon leads to significant reduction in LPS-induced IL-10 production in macrophages [17,26]. Alternatively, phagocytosis of apoptotic cells or exogenous application of TNF- α , IL-1 β , IL-6, PGE₂, ATP, or adenosine directly elicits IL-10 production in a variety of immune cells [16,19–25]. These findings indicate the influence of autocrine signals by earlier-released immune factors on IL-10 induction during inflammatory process. Additionally, two recent studies have reported that pre-addition of PGE₂ not only blunts production of earlier-released pro-inflammatory factors, but also inhibits later-on IL-10 expression in rat microglia co-stimulated with LPS and interferon- γ [27] and in CD4⁺ T cells activated with IL-23 and IL-1 β [28]. Inhibition of IL-10 production by PGE₂ pre-addition may go through suppressing the earlier-released pro-inflammatory factors. The reason for these seemingly conflicting results and the mechanism underlying IL-10 regulation by earlier-released immune factors is not clear. In the present study, we investigate regulatory mechanism by which early-released pro-inflammatory factors from microglia influence later IL-10 production. Our findings show negative regulation of IL-10 induction by earlier-released TNF- α and PGE₂ from LPS-elicited microglia. Moreover, we found EP2 receptor-mediating G-protein-independent β -arrestin signalings are responsible for PGE₂-induced suppression of IL-10 production.

Materials and Methods

Animals

tnf- α ^{-/-}, *tnf-r1/r2^{-/-}*, *cox-2^{-/-}*, *ep2^{-/-}*, *β -arrestin-1^{-/-}* [29], *β -arrestin-2^{-/-}* [30] mice and their age-matched wildtype controls were obtained from the Jackson Laboratory (Bar Harbor, ME) or from NIEHS. Pregnant Fischer 334 rats were purchased from Charles River Laboratories. Housing and breeding of the animals were performed humanely and with regard for alleviation of suffering following the *National Institutes of Health Guide for Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources 1996). All procedures were approved by the NIEHS Animal Care and Use Committee.

Recombinant proteins, protein kinase inhibitors, and reagents

LPS (*E. coli* O111:B4) was obtained from EMD Chemicals, Inc. (Darmstadt, Germany). Recombinant rat TNF- α and IL-1 β protein were purchased from R&D Systems (Minneapolis, MN). Wortmannin, U0126, and PD98059 were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Actinomycin D, PMA (phorbol myristate acetate) and polymyxin B were purchased from Sigma-Aldrich (Saint Louis, MO). Pyrochrome chromogenic endotoxin testing reagent was purchased from Associates of Cape Cod, Inc. (East Falmouth, MA). Rp-cAMPs and SP600125 were purchased from Enzo Life Sciences, Inc. (Farmingdale, NY) and Abcam Inc. (Cambridge, MA) respectively. The following reagents were purchased from Cayman chemical (Ann Arbor, MI): PGE₂, 17-phenyl trinor prostaglandin E₂ (17-p T PGE₂), Butaprost, Sulprostone, CAY10598, 5-bromo-2-(4-fluorophenyl)-3-(4-methylsulfonyl) thiophene (Dup-697), N-(2-cyclohexyloxy-4-nitrophenyl) methanesulfonamide (NS-398), and SB216763.

Preparation of primary neuron-glia, mixed-glia, microglia-enriched and astrocyte-enriched cultures

Mesencephalic neuron-glia cultures were prepared from the mesencephalon of embryos at gestation day 14 ± 0.5 Fischer 334 rats as previously reported [31]. Briefly, mesencephalic tissues were dissected and dissociated with a mild mechanical trituration. Cells were seeded to 24-well (5×10^5 cells/well) culture plates precoated with poly-D-lysine (20 μ g/ml) and maintained in 0.5 ml/well of MEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 10% heat-inactivated horse serum (HS), 1 g/L glucose, 2mM L-glutamine, 1mM sodium pyruvate, and 0.1mM nonessential amino acids. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂/95% air and were replenished with 0.5 ml/well fresh medium 3 days later. Seven-day after seeding, cultures were treated with vehicle or desired reagents in MEM containing 2% FBS, 2% HS, 2 mM L-glutamine, and 1mM sodium pyruvate. At the time of treatment, the neuron-glia cultures were made up of about 10% microglia, 50% astrocytes, and 40% neurons. The cell composition was not different among different genotypes. For neuron-enriched culture, dividing glia were depleted from neuron-glia cultures 48 hours after seeding with 8–10 μ M of cytosine β -D-arabinofuranoside (Ara-C; Sigma-Aldrich, St. Louis, MO) for three days. These cultures contained 99% neurons and less than 1% glia, and treated two days later.

Primary mixed-glia cultures were prepared from whole brains of postnatal day 1 pups from rats, wildtype (C57BL/6J) mice or gene knockout mice [31]. Disassociated brain cells were seeded onto 6-well (1×10^6 cells/well) culture plates and maintained in 1 ml/well DMEM/F-12 supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids. The medium was changed every 3 days. After reaching confluence at 11–12 days after plating, the cultures contained about 80% astrocytes and 20% microglia and were used for treatment. The cell composition of mixed-glia cultures was not different among different genotypes.

Astroglia-enriched cultures were prepared from mixed-glia cells treated with L-leucine methyl ester (LME, 1.5 mM) 2 day after cell seeding [31]. After incubation with LME for 3 days, these cells were replaced with fresh medium without LME. This procedure removes

~99.5 % of the microglia from the original mixed-glia cultures in 2 days after changing medium, which was the time for treatment.

Microglia-enriched cultures were prepared from the whole brains of 1-day-old rodents as previously reported [31]. Briefly, brain tissues, devoid of meninges and blood vessels, were dissociated by a mild mechanical trituration. The isolated cells (5×10^7 cells) were seeded in 150 cm² culture flasks in DMEM/F12 containing 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 50 U/ml penicillin, and 50 µg/ml streptomycin. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂/95% air, and the medium was changed 4 days later. On reaching confluence (12–14 days), the microglia were separated from astroglia by shaking the flasks for 30 minutes at 180 rpm. The enriched microglia were 98% pure.

Quantitative real time-PCR

Total cellular RNA of cells was isolated using RNeasy Mini kit (QIAGEN, Valencia, CA), and first-strand cDNA was synthesized using MuLV reverse transcriptase (Applied Biosystems, Foster City, CA) according to the manufacturer's instruction. After reverse transcription reaction, quantitative real-time PCR analysis was performed to amplify cDNA by using SYBR-Green Master mix (Applied Biosystems, Foster City, CA). PCR condition was as follows: hold at 95 °C for 10 minutes and start 40 cycles at 95 °C for 15 seconds and 60 °C for 1 minute. Data were normalized to GAPDH expression. The primers were designed with Vector NTI software (Invitrogen). The sequences of the primers were the following: mouse TNF- α forward primer 5' GAC CCT CAC ACT CAG ATC ATC TTC T 3'; mouse TNF- α reverse primer 5' CCT CCA CTT GGT GGT TTG CT 3'; mouse COX-2 forward primer 5' TGA TAT GTC TTC CAG CCC ATT G 3'; mouse COX-2 reverse primer 5' AAC GGA ACT AAG AGG AGC AGC 3'; mouse iNOS forward primer 5' GCC ACC AAC AAT GGC AAC A 3'; mouse iNOS reverse primer 5' CGT ACC GGA TGA GCT GTG AAT T 3'; mouse IL-10 forward primer 5' CCC TTT GCT ATG GTG TCC TTT C 3'; mouse IL-10 reverse primer 5' CAA AGG ATC TCC CTG GTT TCT C 3'; rat IL-10 forward primer 5' ACC TGG TAG AAG TGA TGC CCC 3'; rat IL-10 reverse primer 5' GGT CTT CAG CTT CTC TCC CAG G 3'; rat and mouse GAPDH forward primer 5' TTC AAC GGC ACA GTC AAG GC 3'; rat and mouse GAPDH reverse primer 5' GAC TCC ACG ACA TAC TCA GCA CC 3'; rat and mouse β -actin forward primer 5' GTA TGA CTC CAC TCA CGG CAA A 3'; rat and mouse β -actin reverse primer 5' GGT CTC GCT CCT GGA AGA TG 3'.

Measurement of TNF- α , PGE₂ and IL-10

TNF- α , PGE₂ and IL-10 in the culture medium were measured with the commercial ELISA kits from R&D Systems (Minneapolis, MN).

Protein extraction and western blot—Western blot analysis was performed using cell extract from wildtype, β -arrestin-1KO, and β -arrestin-2KO mixed-glia cultures 14 days after cell seeding. After washed twice with cold PBS, cultured cells were homogenized with protein lysis buffer (Pierce, Rockford, IL) with Protease Inhibitor Mini Tablets (Pierce, Rockford, IL) and 1 mM PMSF for 30 minutes on ice and then centrifuged at 15,000 rpm for

15 minutes. Supernatant was collected as cytosolic protein fraction. Protein concentrations were determined using the bicinchoninic acid (BCA) Protein Assay Reagent (Pierce, Rockford, IL). Protein samples were boiled at 100°C for 10 minutes and resolved on 4–12% SDS-PAGE. Nonspecific protein binding was blocked in blocking buffer at RT for 1 hour (5% milk, 20 mM Tris–HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20). Immune analysis was performed using anti-EP2 antibody (Santa Cruz Biotechnology, Dallas, Texas) in wildtype, β -arrestin-1KO and β -arrestin-2KO cells. An antibody against GAPDH (abcam, Cambridge, MA) was used as loading control. Densitometric analysis of immunoblots was performed using the AlphaImager 2200 digital imaging system (Digital Imaging System, CA, USA).

MTT cell viability assay—Viability of the treated cells with PGE₂ or specific agonists for four EP receptors was measured with Cell Proliferation kit I (MTT) assay from Roche Applied Science (Branford, CT).

Statistical analysis

All of the results were expressed as the mean $\pm$ SEM or the means and the coefficient of variation of at least three independent experiments. Standard curves were plotted, and the data that were obtained within the linear range of the curve. In addition, RT-PCR results were normalized to their respective internal loading controls GAPDH or β -actin. Results in figures 1A–C, 3D, 4B, and 5B–D were analyzed by non-parametric Mann & Whitney by Prism 6.0 (GraphPad Software Inc., CA). Results in figures 1E, 3A–C and E as well as supplemental figures 1B, 2C, and 5 were analyzed by non-parametric Kruskal-Wallis by Prism 6.0 (GraphPad Software Inc., CA). Results in figures 1D, 2, 3F, 3G, 4C–E, and 5B–E as well as supplemental Figures 2A, 2B, 3B, 3C, 4, 6 were analyzed by two-way ANOVA with preplanned contrast comparisons against the wildtype group or against the individual treated group by Prism 6.0 (GraphPad Software Inc., CA). In all cases, P values less than 0.05 were considered statistically significant.

Results

Microglia are the major source of LPS-elicited IL-10 production in neuron-glia cultures

A variety of peripheral immune cells (e.g. monocytes, lymphocytes, and mastocytes) are known to produce IL-10. We observed up-regulation of IL-10 mRNA in the brain of the mice received an intraperitoneal injection of LPS. However, which cell type(s) (neurons, astroglia or microglia) in inflamed brain can secrete IL-10 is still unclear. To determine the cell type(s) producing this cytokine in the brain, we examined levels of IL-10 in 3 different primary cultures treated with LPS: neuron-enriched (more than 99% neurons and less than 1% microglia), astroglia-enriched (more than 99% astroglia and less than 1% microglia) and microglia-enriched cultures (more than 98% microglia) [31]. Only in enriched microglia but not enriched astroglia or neurons, LPS induced extracellular secretion of IL-10 (Table 1). Similarly, TNF- α , which is mainly produced from microglia, was undetectable in the culture medium of neuron-enriched cultures and astroglia-enriched cultures (Table 1). These results indicate that microglia, but not neurons or astroglia, are the major source of pro-

inflammatory factors and anti-inflammatory cytokine IL-10 in the LPS-induced neuroinflammation.

Induction profiles of pro-inflammatory factors and IL-10 from LPS-treated microglia

To investigate regulation of IL-10 induction, instead of using enriched microglial cultures, we used neuron-glia cultures (containing 10% microglia, 40% neurons and 50% astroglia) or mixed-glia cultures (containing 20% microglia and 80% astroglia) for two reasons: 1) microglia coexist and constantly interact with neurons or astroglia in the brain, thus co-cultures better mimic the physiological microenvironment and 2) microglia stay healthier and survive longer in co-culture with neurons and astroglia [32,33]. After treatment of mixed-glia cultures with LPS, well-orchestrated, time-dependent mRNA expression of several pro-inflammatory factors was observed: while TNF- α peaked at 3 hours, COX-2 crested at 6 hours and preceded the appearance of IL-10 mRNA, which peaked at 24 hours (Fig. 1A). It is interesting to note the different patterns of mRNA expression between IL-10 and the earlier-released proinflammatory factors. While mRNA levels of these pro-inflammatory factors quickly declined to 10–20% of maximum levels within 24 hours after LPS treatment, the level of IL-10 mRNA peaked at 24 hours and still sustained at a high level at 48 hours (up to 69% of the maximum levels after LPS treatment; Fig. 1A). Similar to the expression of mRNA, a time-related pattern of increase in supernatant levels of these immune factors was observed (Fig. 1B). The sequential release of pro-inflammatory factors and IL-10 after LPS treatment prompted us to examine possible roles of pro-inflammatory factors in IL-10 induction.

Earlier-released factors fail to induce IL-10 production

Based on the observed pattern of sequential release of immune factors (Fig. 1A, B), we initially hypothesized that LPS-induced earlier-secreted pro-inflammatory factors initiated IL-10 expression via an extracellular autocrine cascade. To test this hypothesis, we compared the production of IL-10 in neuron-glia cultures treated with LPS, pro-inflammatory factors (TNF- α , IL-1 β or PGE₂), or PMA (a potent PKC activator that is often used to activate NADPH oxidase) [34,35]. In contrast to LPS, TNF- α , IL-1 β , and PGE₂, alone or in combination, failed to induce IL-10 production in neuron-glia cultures (Fig. 1C). To investigate whether IL-10 induction can be continued in the absence of LPS, we neutralized LPS by using antibiotic polymyxin B (a cyclic amphipathic peptide) [36] at 24 hours following LPS treatment. While showing no toxicity to microglia and neurons (Supplemental Fig. 1), polymyxin B (10 μ g/ml) terminated IL-10 induction by LPS (Fig. 1D). Moreover, we prepared microglial conditioned medium (MCM) from microglia-enriched cultures 12 hours after LPS treatment, which contained earlier-released pro-inflammatory factors but not IL-10. After incubated with polymyxin B or vehicle, the MCM was added to the neuron-glia cultures. The vehicle-pretreated MCM containing LPS and microglia-derived inflammatory factors induced secretion of TNF- α , PGE₂ and IL-10 into the supernatant of neuron-glia cultures (Fig. 1E). However, the polymyxin B-pre-incubated MCM lost its ability to stimulate neuron-glia cultures to produce IL-10, although TNF- α and PGE₂ from MCM can be detected in the supernatant (Fig. 1E). Taken together, our findings suggest that IL-10 induction is independent of earlier pro-inflammatory factors during LPS-

elicited neuroinflammation and continued presence of LPS-induced intrinsic cellular signaling events is required for the induction of IL-10 expression.

Signaling pathways mediate LPS-induced increase in IL-10 production

Although regulatory intrinsic signaling events of IL-10 expression has been studied in peripheral immune cells, such as macrophages, dendritic cells, and T helper cells (10), little is known in brain microglia. We next investigated potential intrinsic cellular and molecular mechanisms underlying the induction of IL-10 in the CNS. We first found that toll-like receptor 4 (TLR4) was essential for LPS-elicited IL-10 expression in microglia (supplement Fig. 2A), which is consistent to previous reports [37]. In addition to TLR4, LPS has been reported to directly associate with Mac-1 receptor (CD11b/CD18) triggering intracellular signals [38]. Our results showed that IL-10 production in Mac-1 receptor-deficient mixed-glia cells was 50% lower than wildtype cells after LPS challenge (Supplemental Fig. 2B). Thus, both TLR4 and Mac-1 participated in LPS-elicited induction of IL-10. To further search for the downstream signaling molecules, we used inhibitors of a variety of protein kinases and NF- κ B. The results showed that ERK1/2, p38, JNK, and NF- κ B, but not PKA and PKC, are required for LPS-induced expression of IL-10 (Supplemental Fig. 2C). These data indicated that the induction of IL-10 gene expression was programmed once the TLR4 and Mac-1 receptors are stimulated by LPS.

Disruption of TNF- α signal decreases PGE₂ production but increases IL-10 production after LPS treatment

After finding out early-released pro-inflammatory factors did not show stimulatory effects on IL-10 production (Fig. 1), we next investigated whether they play a negative regulatory role in LPS-induced IL-10 production. Previous studies have shown that TNF- α can positively regulate the expression of other late pro-inflammatory mediators (e.g. iNOS/NO, IL-1 β , and COX-2/PGE₂) in monocytes, macrophages and microglia [7,39]. In this study, genetic disruption of TNF- α or its receptors TNF-R1/R2 was used to study roles of endogenous TNF- α in LPS-induced IL-10 production. Immunocytochemistry using antibody against ionized calcium-binding adapter molecule 1 (Iba-1) exhibited similar cell number of microglia among wildtype, TNF- α -deficient, and TNF-R1/R2-deficient mixed-glia cultures (Supplemental Fig. 3A). TNF- α was detectable in both wildtype and TNF-R1/R2-deficient mixed-glia cultures, but not in TNF- α -deficient cells (Supplemental Fig. 3B). In comparison with wildtype cells, TNF- α -deficient and TNF-R1/R2-deficient mixed-glia cells expressed lower levels of COX-2 and iNOS mRNA at 6 hours (Supplemental Fig. 3C) and produced less PGE₂ at 24 hours after LPS treatment (Fig. 2A). Thus, our finding also indicated that earlier-released TNF- α exerts an inductive effort on the pro-inflammatory factors via an autocrine mechanism during LPS-induced inflammatory event. Surprisingly, IL-10 production in both TNF- α -deficient and TNF-R1/R2-deficient mixed-glia cultures was significantly higher than that of wildtype cells in response to LPS (Fig. 2B). These results indicated TNF- α negatively regulated LPS-induced IL-10 production in a receptor-dependent manner.

PGE₂ inhibits LPS-induced IL-10 expression at the transcriptional level

To determine if the inhibitory effect of endogenous TNF- α on LPS-induced IL-10 production was via its downstream modulator PGE₂ (Fig. 2), exogenous PGE₂ was added to neuron-glial cultures at 24 hours after LPS treatment, the time point of maximum production of endogenous PGE₂. Addition of PGE₂ inhibited IL-10 production measured 24 hours later in a concentration-dependent manner (Fig. 3A). Moreover, levels of IL-10 mRNA rapidly decreased by 50% at 6 hours after PGE₂ addition and were further reduced to less than 5% at 24 hours compared with LPS-treated groups (Fig. 3B). The blockage of *de novo* mRNA transcription by actinomycin D abolished the inhibitory effect of PGE₂ on IL-10 mRNA transcription, indicating that PGE₂ down-regulated IL-10 at the transcription level (Fig. 3C). Furthermore, PGE₂ at 1 to 10 nM concentrations did not affect cell viability (Fig. 3D) indicating its inhibitory effect on IL-10 expression did not result from toxicity to the cells.

Inhibition of COX-2 activity or disruption of *cox-2* gene augments LPS-induced IL-10 production

Alternatively, pharmacological inhibition of COX-2 activity and ablation of PGE₂ production using cells deficient in the *cox-2* gene were employed to study the role of PGE₂ in regulating the expression of IL-10. COX-2 specific inhibitors NS-398 and Dup-697 enhanced LPS-elicited IL-10 production in a dose-dependent manner (Fig. 3E) suggesting that PGE₂ plays a negative role in LPS-induced IL-10 production. This conclusion was further supported by additional experiment showing that LPS-induced increases in supernatant IL-10 levels were higher in *cox-2*^{-/-} cultures compared with wildtype control cultures in a dose dependent manner (Fig. 3F). A separate time-course experiment revealed more pronounced increases in LPS-elicited IL-10 release in *cox-2*^{-/-} microglia than wildtype cells mainly at later time points (96 and 120 hours), but not earlier time points (Fig. 3G). It is interesting to note that there was no significant reduction in the release of TNF- α (3 hours; Supplemental Fig. 4A), IL-1 β (24 hours; data not shown), and NO (24 hours; data not shown) after LPS challenge in COX-2-deficient glial cells compared with wildtype cells, indicating certain degree of specificity in PGE₂-elicited inhibition on IL-10 expression. Taken together, our findings indicated that COX-2/PGE₂ axis critically down-regulated LPS-induced IL-10 production during the neuroinflammatory process.

PGE₂ acts specifically on the EP2 receptor to suppress LPS-induced IL-10 production

PGE₂ binds to four different G-protein coupled receptor subtypes (EP1–4), elicits distinct second messages and mediates different signaling pathways [40]. By using both receptor agonists and receptor-deficient microglia, we examined which subtype(s) of the PGE₂ receptor was responsible for its down-regulation of IL-10. Among four specific agonists for individual EP1–4 receptors, the specific EP2 agonist, butaprost, mimicked the inhibitory effect of PGE₂ on LPS-induced IL-10 production in a dose-dependent manner (Fig. 4A). In contrast, specific agonists for EP1 (17-p T PGE₂), EP3 (sulprostone), and EP4 (CAY10598) were unable to reduce IL-10 production (Fig. 4A). None of the EP agonists affected cell viability at concentrations used in this study (Fig. 4B). Thus, EP2, but not other PGE₂ receptors (EP1, EP3, and EP4) mediated PGE₂-elicited inhibition of IL-10 expression. Moreover, in response to LPS treatment, EP2-deficient mixed-glia cultures produced less

PGE₂ at 24 hours (Fig. 4C) and higher IL-10 at 72 to 120 hours than wildtype cultures (Fig. 4D). Deficiency in EP2 eliminated the inhibitory effect of PGE₂ on LPS-induced IL-10 production (Fig. 4E). Together, these results indicate that the EP₂ receptor mediates PGE₂ induction and governs the negative regulation of PGE₂ on IL-10 production.

Deficiency in β -arrestin genes blocks the negative regulation of PGE₂ on IL-10 induction

It is well established that the binding of PGE₂ to the EP2 receptor activates G α s and triggers the cAMP-dependent PKA pathway [20]. The PKA pathway regulates multiple cellular functions including gene transcription via phosphorylation of a myriad of downstream targets. We therefore investigated whether the PKA signal pathway participated in the inhibitory effect of PGE₂ on LPS-induced IL-10 production. The results showed that the PKA inhibition by PKA inhibitor Rp-cAMPs failed to reverse PGE₂-mediated suppression of IL-10 production (Fig. 5A). In addition, inhibitors of MAPK, PI3K, or GSK3 β also exhibited no effect on the negative regulation of IL-10 by PGE₂ (Supplemental Fig. 5). These results suggest that an alternative non-G protein signal may mediate the inhibitory effect of PGE₂ on IL-10 induction.

Members of β -arrestin family have recently been implicated in agonist-mediated desensitization of GPCRs and suppression of specific cellular responses to stimuli such as hormones or neurotransmitters [41]. Both β -arrestin-1 and β -arrestin-2 are expressed at high levels in the CNS [42]. Our recent studies indicate that the activation of GPCR β 2-adrenergic receptor by low doses of salmeterol exhibited potent inhibitory effects on microglial activation through a β -arrestin-dependent pathway [43]. This prompted us to investigate the role of β -arrestin-1 and β -arrestin-2 in PGE₂-mediated down-regulation of IL-10. Genetic disruption of β -arrestin-1 or β -arrestin-2 did not significantly affect LPS-induced production of TNF- α (Supplemental Fig. 6) and PGE₂ in mixed-glia cultures (Fig. 5B). β -arrestin-1^{-/-} and β -arrestin-2^{-/-} mixed-glia cultures produced slightly higher amounts of IL-10 protein after LPS treatment compared with wildtype cultures (Fig. 5C, D). Moreover, PGE₂ addition was unable to inhibit IL-10 induction in mixed-glia cultures deficient in β -arrestin-1 or β -arrestin-2 gene (Fig. 5E). Wildtype, β -arrestin-1-deficient, and β -arrestin-2-deficient mixed-glia cultures express similar amount of EP2 receptor (Fig. 5F). It indicates that genetic deletion of β -arrestin-1 and β -arrestin-2 genes did not alter EP2 degradation. Thus, PGE₂ acted on the EP2 to activate a β -arrestin-dependent but G-protein-cAMP/PKA-independent pathway to inhibit IL-10 induction during neuroinflammation.

Discussion

Chronic neuroinflammation is a crucial contributor to the pathogenesis of progressive neurodegeneration [7,8]. Persistent injurious stimuli (e.g. toxins, pathogens, toxic products from injured neurons) and failed resolution of acute neuroinflammation can flip a protective immune response to chronic destruction to CNS tissues. Anti-inflammatory cytokine IL-10 plays an important counter-regulatory role in resolving inflammation within the periphery and the CNS [9,10]. However, the precise mechanisms governing the regulation of IL-10 induction in brain microglia remain largely undetermined. The present study demonstrated that LPS-induced expression of IL-10 was not induced by earlier released pro-inflammatory

factors via autocrine fashion. We further showed that that induction of IL-10 expression is mainly regulated by LPS-elicited activation of intracellular intrinsic signaling pathways such as MAPK and NF- κ B (Fig. 6) rather than the paracrine regulation by the earlier released pro-inflammatory factors. Previous report indicates that these signaling pathways also play a critical role for LPS-induced pro-inflammatory factors in microglia [44]. Further studies to determine specific and key intrinsic signal pathways/molecules for IL-10 production might provide potential molecular therapeutic targets in neuroinflammation-associated disease in the future. Surprisingly, TNF- α -deficient and TNF-R1/R2-deficient mixed-glia cultures released more IL-10 and less pro-inflammatory factors in response to LPS than wildtype cultures. Among the pro-inflammatory factors examined, we found that COX-2-derived PGE₂ inhibited LPS-induced IL-10 production through EP2-mediated activation of PKA-independent, β -arrestin-dependent signaling cascade in brain microglia (Fig 6). This finding identified a critical role of the coupling between pro-inflammatory cascade (TNF- α -COX-2-PGE₂) and IL-10 in the regulating immune resolution.

Most of our knowledge regarding roles of IL-10 in immune resolution and its regulatory pathways has come from the studies on the peripheral immune system. In contrast, much less is known in the CNS. One of the conspicuous differences is the cell type producing IL-10. Multiple immune cells in the peripheral system are capable of producing IL-10. Our data showed that microglia, not neuron and astroglia, are the sole source of IL-10 in the brain (Table 1). Increasing evidence indicates that although microglia and peritoneal macrophage share the cell lineage of myeloid progenitor, they have distinct properties in modulating innate or adaptive immune responses. For example, our preliminary data showed that microglia do not exhibit LPS tolerance upon the second addition of LPS, which was opposite to what has been reported in peripheral macrophages [45]. In view of the differences mentioned, it is important to elucidate the regulation of IL-10 expression in the CNS, since failure in the resolution of acute neuroinflammation has been proposed as a critical event leading to chronic neuroinflammation and neurodegeneration.

Various inflammatory mediators have been shown to affect IL-10 production. Direct addition of individual cytokines (e.g. TNF- α and IL-6) at relatively high concentrations (20–100 ng/ml) into cultures of peripheral macrophages or neutrophils enhances LPS-elicited IL-10 production [16]. In contrast, addition of different individual pro-inflammatory factors alone or in combination failed to increase the expression of IL-10 in our neuron-glia cultures (Fig. 1C). Moreover, removal of LPS from cellular media, which contain early pro-inflammatory factors, by polymyxin B (Fig. 1D, E) indicated that LPS-induced earlier released pro-inflammatory factors did not induce the late-release of IL-10. Instead, the continued presence of LPS is necessary for inducing and maintaining the production of IL-10. Our findings are similar to a previous report showing that LPS-treated CD25-positive lymphocytes produced IL-10 without secretion of pro-inflammatory cytokines in the human colon [46]. These findings may imply LPS regulates the expression of IL-10 likely via intrinsic cellular signaling events and is independent of the earlier-released pro-inflammatory factors.

One of the important findings from this study was the demonstration that TNF- α negatively regulated IL-10 production through PGE₂. Compared with wildtype microglia, TNF- α or

TNF-R1/R2-deficient microglia showed a decrease in PGE₂ production (Fig. 2A) and an increase in IL-10 production (Fig. 2B) after LPS treatment. Indeed, among factors measured, PGE₂ addition was able to reduce IL-10 production not only in wildtype (Fig. 3A, B), but also in TNF- α -deficient or TNF-R1/R2-deficient mixed-glia cells (data not shown). Thus, the earlier released pro-inflammatory factors TNF- α and PGE₂ play an inhibitory role in regulating the expression of IL-10 in LPS-induced immune cascades. Our studies also suggest that PGE₂ being released between earlier-released pro-inflammatory factors, such as superoxide, TNF- α , IL-1 β and NO and late-released IL-10, may be strategically critical in regulating the resolution of neuroinflammation.

To further elucidate regulatory mechanisms of PGE₂ on LPS-induced IL-10 production, a series of experiments was performed. Initially, we demonstrated the potent inhibitory influence of PGE₂ (Fig. 3A, B) on IL-10 in LPS-elicited neuroinflammation. Direct post-stimulation of EP receptors with specific agonists revealed that butaprost, an EP2 specific agonist, was the only one showing the inhibitory activity (Fig. 4A). Here, our findings on IL-10 inhibition by post-treatment with PGE₂ (24 hours after LPS challenge) were rather different from a recently published report [27] in which pre-treatment of rat microglia with PGE₂ inhibits IL-10 production after LPS and interferon- γ co-stimulation through suppressing pro-inflammatory factor release. The evidence of the negative regulation of IL-10 by PGE₂ was further supported by the findings that interruption of COX-2 function by two COX-2 inhibitors (Fig. 3E) and deletion of the *cox-2* gene (Fig. 3F, G) enhanced LPS-induced IL-10 production. We also provided the evidence indicating that the negative regulation of PGE₂ on IL-10 expression was at the gene transcriptional level (Fig. 3B, C). Taken together, strong evidence indicates that both exogenous and endogenous PGE₂ play a critical role in downregulating the expression of IL-10 in microglia.

EP2 signaling broadly regulates expression of many inflammatory genes and participate in peripheral and central inflammatory responses. In LPS-stimulated peripheral macrophage, EP2 receptor activation further up-regulates COX-2, iNOS, gp91^{phox} (the catalytic subunit of NADPH oxidase), IL-6 and IL1 β , amplifying M1 pro-inflammatory response [47]. Microarray analysis of brain microglia with conditional EP2 deletion and unsupervised hierarchical clustering show down-regulation of 116 inflammatory genes ($\sim$ 1.5-fold) and up-regulation of 20 inflammatory genes ($\sim$ 1.5-fold). Of particular interest, COX-2 is highly down-regulated with EP2 deletion. Moreover, EP2 agonist butaprost potentiates LPS-induced COX-2 mRNA expression [47]. Our results revealed reduced PGE₂ production in EP2-deficient microglia compared with wildtype microglia (Fig. 4C). These findings together suggest a feed-forward cycle in which EP2-mediated COX-2 up-regulation triggers more PGE₂ production leading to enhanced EP2 activation and pro-inflammatory responses. In theory, the increase in IL-10 production in *ep2*^{-/-} cultures (Fig. 4D) could result from the actual loss of the receptor and/or the decrease in PGE₂ production in *ep2*^{-/-} cells (Fig. 4C). The inability of additional PGE₂ to inhibit IL-10 production in *ep2*^{-/-} cells (Fig. 4E) indicates the actual loss of the EP2 receptor, but not reduced production of PGE₂, is responsible for the increased IL-10 production in *ep2*^{-/-} cultures. In addition, the agonist specific for the EP2 receptor, but not agonists of other EP receptors (EP1, EP3 and EP4),

dose-dependently suppressed IL-10 production (Fig. 4A). These results together indicate the EP2 dependency of PGE₂-mediated IL-10 inhibition.

We extended our study to include the downstream signaling of PGE₂-EP2 in the regulation of IL-10 expression. It is well characterized that the binding of PGE₂ to EP2 activates G protein Gas and triggers cAMP-dependent PKA pathway. Failure in altering PGE₂-mediated inhibition of LPS-induced IL-10 production by PKA inhibition (Fig. 5A), suggests an alternative G protein-independent signaling pathway is responsible for the negative regulation of PGE₂ on IL-10 induction. Members of β -arrestin family have recently been implicated in agonist-mediated desensitization of GPCRs and suppression of specific cellular responses to stimuli (e.g. hormones or neurotransmitters). Indeed, β -arrestins have been mainly described as a signaling pathway inhibitor by inducing intracellular segregation and/or degradation of receptors. However, recent reports indicated that besides down-regulating G-protein coupled receptors β -arrestins also serve as multifunctional adaptors and scaffolds to signal different pathways in a G-protein-independent fashion. Our previous reports showed that EP2 receptor activation could activate multiple signaling pathways such as c-Src, ERK1/2, STAT3 and AKT through activation of β -arrestins [48,29,49]. Deficiency in β -arrestin-1 [48,29,49] and β -arrestin-2 (our unpublished data) reduced these signal pathways, but did not affect the level of G-protein-dependent cAMP and PKA activation. Genetic deletion of *β -arrestin-1* and *β -arrestin-2* did not alter EP2 degradation (Fig. 5F), but abolished the inhibitory effect of PGE₂ on IL-10 induction (Fig. 5E). Thus, β -arrestin regulated the suppression of IL-10 production by PGE₂-EP2 through β -arrestin downstream signaling.

In conclusion, this study identified a negative regulatory role of PGE₂ on IL-10 induction in brain microglia and demonstrated that such negative regulation is mediated by EP2- β -arrestin-dependent pathway, thereby elucidating a novel mechanistic basis for neuroinflammation resolution by PGE₂ and IL-10. Our findings suggest that pharmacological regulation of neuroinflammation resolution through targeting EP2- β -arrestin-dependent signaling cascade may mitigate neuroinflammation-mediated neurodegeneration and may become a promising therapeutic strategy for neurodegenerative diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by the Intramural Research Program of the NIH/NIEHS (ES090082; ES025043), the National Natural Science Foundation of China, and the award to high-level innovative and entrepreneurial talents of Jiangsu Province of China. We thank Anthony Lockhart for the assistance with animal colony management and maintenance of the timed pregnant mice.

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Fig. 1. Sequential release of inflammatory factors and their lack of effects on LPS-induced increase in IL-10 production in LPS-treated mixed glia cultures

A. Mixed-glia cultures from C57BL/6J mice were treated with LPS at 15 ng/ml concentration. mRNA levels of pro-inflammatory factors ($TNF-\alpha$ and $COX-2$), anti-inflammatory factor $IL-10$, and $GAPDH$ (as an internal control) were detected by RT-PCR at different time points as indicated. Data were expressed as percentage of maximum gene expression following LPS treatment. Results are from three independent experiments performed in triplicate. **B.** Measurement of $TNF-\alpha$, PGE_2 and $IL-10$ release into the supernatant of mixed-glia cultures from C57/6J mice treated with LPS at different time points as indicated. **C.** Neuron-glia cultures were treated with a variety of immune stimuli as indicated. After 48 hours of LPS treatment, $IL-10$ production in the supernatant of treated cells was detected by ELISA. **D.** After 24 hours of LPS (15 ng/ml) incubation, neuron-glia cultures were treated with polymyxin B (10 μ g/ml) or vehicle. $IL-10$ production in the supernatant of these treated cells was detected by ELISA at indicated time points following polymyxin B addition. **E.** Microglia conditioned medium (MCM) from microglia-enriched cultures with LPS treatment for 12 hours was collected. Polymyxin B at 10 μ g/ml concentration was used to neutralize LPS in this MCM. MCM, MCM plus polymyxin B, or polymyxin B alone was added into neuron-glia cultures. Production of $TNF-\alpha$ (3 hours), PGE_2 (12 hours), and $IL-10$ (48 hours) in the supernatant of these cultures was detected by ELISA. Data were expressed as means $\pm$ SEM from three independent experiments run in triplicate. *, $p < 0.05$, compared with corresponding vehicle-treated control cultures. #, $p < 0.05$, compared with corresponding MCM-treated cultures.

Fig. 2. Deficiency in *tnf-α* or *tnf-r1/r2* gene decreases PGE₂ expression, but increases IL-10 production after LPS treatment

Mixed-glia cultures from wildtype and TNF-α-deficient and TNF-R1/R2-deficient mice were treated with LPS (15 ng/ml). Production of PGE₂ (A) and IL-10 (B) in the supernatant of these cells was measured at indicated time following LPS treatment by ELISA. Results are shown as the mean ± SEM from 3 independent experiments. *, $p < 0.05$, compared with vehicle-treated control cultures. #, $p < 0.05$, compared with corresponding LPS-treated wildtype cultures.

Fig. 3. PGE₂ negatively regulates LPS-induced IL-10 production

Exogenous PGE₂ decreases LPS-induced IL-10 expression. **A.** Neuron-glia cultures were treated with PGE₂ at different concentrations following LPS treatment for 24 hours. IL-10 production in the supernatant was detected 24 hours after PGE₂ addition by ELISA. **B.** After LPS treatment for 24 hours, neuron-glia cultures were stimulated with PGE₂ (10 nM). mRNA transcripts of IL-10 were detected by RT-PCR at 1, 6, and 24 hours after PGE₂ addition. **C.** Pre-incubation of neuron-glia cultures with LPS (15 ng/ml) for 24 hours was followed by the addition of actinomycin D (1 μg/ml) with or without co-treatment with PGE₂ (10 nM). Expression of IL-10 mRNA was measured by RT-PCR at 15, 30, 45, and 60 minutes after the addition of actinomycin D. Results of RT-PCR were represented as percentage of LPS-treated cultures. **D.** MTT assay revealed no change in microglial viability in microglia-enriched cultures treated with PGE₂ for 6 hours. **E.** Inhibition of COX-2 activity augments LPS-induced IL-10 production. Neuron-glia cultures were pre-treated with specific COX-2 inhibitors Dup-697 (1–100 nM) and NS-398 (1–10 nM) for 30 minutes followed by stimulation with LPS (15 ng/ml). IL-10 levels in the culture supernatant were measured at 48 hours by ELISA. Deletion of *cox-2* gene increases LPS-induced IL-10 production. **F.** Wildtype and COX-2-deficient mixed-glia cultures were treated with LPS at different concentrations as indicated. IL-10 production in the supernatant of these cells at 96 hours after LPS treatment was detected by ELISA. **G.** Mixed-glia cultures from wildtype and COX-2-deficient mice were treated with LPS at the concentration of 15 ng/ml. Expression levels of and IL-10 in the supernatant were detected by ELISA at indicated time points after LPS treatment. Data are expressed as means ± SEM from three independent experiments performed in triplicate. *, *p* < 0.05, compared with corresponding vehicle-treated control cultures. #, *p* < 0.05, compared with corresponding LPS-treated cultures. &, *p* < 0.05, compared with corresponding LPS-treated wildtype cultures.

Fig. 4. PGE₂ specifically acts on the EP2 receptor to suppress IL-10 production

A. After treatment of neuron-glia cultures with LPS (15 ng/ml) for 24 hours, four specific agonists for individual EP receptors including 17-phenyl trinor prostaglandin E₂ (17-p T PGE₂) for EP1, butaprost for EP2, sulprostone for EP3, and CAY10598 for EP4 were added. IL-10 production in the supernatant was detected by ELISA 24 hours after the addition of EP agonists (48 hours after LPS treatment). Wildtype and EP2-deficient mixed-glia cultures were treated with 15 ng/ml LPS. **B.** Specific EP receptor agonists have no effect on microglial viability in microglia-enriched cultures 6 hours after the treatment. Levels of PGE₂ (**C**) and IL-10 (**D**) in the supernatant were measured at indicated time points by ELISA. **E.** Mixed-glia cultures from wildtype and EP2-deficient mice were stimulated with PGE₂ at indicated concentrations following 24 hours of LPS treatment. IL-10 production in the supernatant was detected 24 hours after PGE₂ treatment. Results are shown as the mean $\pm$ SEM from three independent experiments performed in triplicate. *, $p < 0.05$, compared with corresponding vehicle-treated control cultures. #, $p < 0.05$, compared with corresponding LPS-treated cultures. &, $p < 0.05$, compared with corresponding LPS-treated wildtype cultures.

Fig. 5. Deficiency in β -arrestin genes abolishes the inhibitory effect of PGE₂ on IL-10 production

A. PGE₂ and Rp-cAMPs (10 μM; PKA inhibitor) were added into rat neuron-glia cultures after these cultures were treated with LPS for 24 hours. IL-10 release into the supernatants was detected at 48 hours following LPS treatment by ELISA. Production of PGE₂ (**B**) and IL-10 (**C**, **D**) in the supernatant of LPS-treated wildtype and β -arrestin 1- or 2-deficient mixed-glia cultures was detected at indicated time points by ELISA. **E.** After LPS challenge for 24 hours, mixed-glia cultures from wildtype and β -arrestin 1- or 2-deficient mice were stimulated with PGE₂. IL-10 level in the supernatant was detected 24 hours after PGE₂ addition (48 hours after LPS treatment). IL-10 production in (**E**) was represented as percentage of LPS-treated group. **F.** Wildtype, β -arrestin-1-deficient, and β -arrestin-2-deficient mixed-glia cultures express similar amount of EP2 receptor. The experiment has been performed three times. Results are shown as the mean $\pm$ SEM. *, $p < 0.05$, compared with corresponding vehicle-treated control cultures. #, $p < 0.05$, compared with corresponding LPS-treated cultures. &, $p < 0.05$, compared with corresponding LPS-treated wildtype cultures. NS: non-significant.

Fig. 6. Regulatory mechanism of early pro-inflammatory cascade on IL-10 expression in the neuroinflammatory condition

Our working model illustrated that sequential release of pro-inflammatory factors suppresses later IL-10 production in LPS-stimulated microglia. In time frame of neuroinflammatory cascade, IL-10 expression was later than most pro-inflammatory factors such as TNF- α , COX-2 and PGE₂. Directly treated cells with early released pro-inflammatory factors or microglia conditioned medium from enriched microglia treated with LPS failed to initiate IL-10 production in the absence of LPS. Thus, it is plausible that IL-10 induction was dependent on intrinsic signal events such as MAPK and NF- κ b pathways, but not in need of early released pro-inflammatory factors. Conversely, deficiency in *tnf- α* and *cox-2* gene significantly decreased PGE₂ production but increased IL-10 production. Exogenous addition of PGE₂ inhibited LPS-induced IL-10 production via EP2-mediating G protein-independent β -arrestin signaling pathway. Together, our results showed that an extracellular autocrine cascade from TNF- α and PGE₂ plays a negative role in the regulation of LPS-induced IL-10 production in microglia.

Table 1

Microglia are the major source of the LPS-induced IL-10 production

	Neuron-enriched cultures	Astroglia-enriched cultures	Microglia-enriched cultures
TNF-α (pg/ml)	Undetectable	Undetectable	1546 $\pm$ 26
IL-10 (pg/ml)	Undetectable	Undetectable	789 $\pm$ 76

Following treatment of three types of rat primary cultures with LPS (15 ng/ml) or vehicle, supernatant levels of TNF- α at 3 hours and IL-10 at 48 hours were measured. The data were presented as means $\pm$ SEM from three independent experiments performed in triplicate. The expression level of TNF- α and IL-10 in vehicle groups is undetectable (<15.6 pg/ml).