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Context-dependent IL-6 potentiation of interferon-gamma-induced IL-12 secretion and CD40 expression in murine microglia

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Abstract

Interleukin-6 (IL-6) is produced by neurons, astrocytes, and microglia, and elevated levels of IL-6 within the CNS have been documented in multiple neurological disorders including Alzheimer's disease, stroke, epilepsy, attention deficit disorder, cerebral palsy, and multiple sclerosis. Here, we sought to understand how IL-6 regulates microglial signal transduction and their immune properties. Using highly enriched cultures of neonatal murine microglia we show that IL-6 alone has direct effects on microglia as it activates STAT3 and extracellular signal-regulated kinase pathways in a time- and dose-dependent fashion and it enhances interferon-gamma (IFNγ)-stimulated IL-12 secretion. However, other immune properties were only weakly modulated by IL-6 when administered without the soluble IL-6 receptor (sIL-6R). For instance, IFNγ-induced expression of the co-stimulatory molecule, CD40 was dependent on sIL-6R administration. IL-6 with or without sIL-6R did not affect major histocompatability complex class II expression. In granulocyte–macrophage colony-stimulating factor (GMCSF)-induced dendritic cell-like microglia, IL-6/sIL-6R and IFNγ stimulated an even greater increase in CD40 expression compared with primary microglia. Altogether, our results demonstrate that microglial responses to IL-6 are not simple in that the effects of IL-6 are context-dependent. In particular, the presence or absence of sIL-6R, IFNγ or GMCSF will alter the type and amplitude of their response.

Keywords: CNS, co-stimulatory molecules, granulocyte–macrophage colony-stimulating factor, inflammation, soluble interleukin-6 receptors.

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Interleukin-6 (IL-6), a 26 kDa, alpha-helical protein, is produced locally in the nervous system by astrocytes, microglia, and neurons, and is important in activating infiltrating macrophages, regulating B cell growth and differentiation as well as inducing T cell responses (Frei *et al.* 1989). IL-6 is a member of a family of cytokines that include ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), cardiotrophin-1, oncostatin M, and IL-11, which are structurally similar and share glycoprotein 130 (gp130) as a signal transducer. They bind to specific receptors and induce either homodimerization of the 'beta' signal transducing component gp130 (in the case of IL-6 and IL-11) or heterodimerization of gp130 and LIF receptor beta (in the case of CNTF, LIF, cardiotrophin-1, and oncostatin M). Dimers of beta components activate JAK and subsequently activate STAT, mitogen-activated protein kinase, and additional signaling pathways (Stahl *et al.* 1994).

Whereas gp130 is ubiquitously expressed, IL-6 receptor (IL-6R) is more selectively expressed. However, soluble IL-6 receptor (sIL-6R) can be generated via proteolysis of

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Abbreviations used: AD, Alzheimer's disease; BSA, bovine serum albumin; CNTF, ciliary neurotrophic factor; ERK, extracellular signal-regulated kinase; GMCSF, granulocyte–macrophage colony-stimulating factor; HRP, horseradish peroxidase; IFNγ, interferon-gamma; IL, interleukin; JAK, Janus kinase; LIF, leukemia inhibitory factor; MHC, major histocompatability complex; MS, multiple sclerosis; PBS, phosphate-buffered saline; PBS-T, 0.05% Tween-20 in PBS; PTP1B, protein tyrosine phosphatase 1B; sIL-6R, soluble IL-6 receptor; STAT, Signal Transducers and Activators of Transcription; TNF, tumor necrosis factor.

membrane-bound IL-6R or by differential mRNA splicing (Mullberg *et al.* 1994; Holub *et al.* 1999). The presence of sIL-6R allows cells that do not express membrane-bound IL-6R or that express low levels of the IL-6R to respond to IL-6 (Igaz *et al.* 2000). Alone, sIL-6R does not appear to transduce a signal (Peters *et al.* 1996).

Elevated IL-6 levels have been observed in the CSF and plasma of multiple sclerosis (MS) patients (Frei *et al.* 1991; Maimone *et al.* 1991; Navikas *et al.* 1996), and IL-6 immunoreactivity has been observed in acute and chronically active MS plaques (Maimone *et al.* 1997). The concentration of sIL-6R is also increased in the CSF of MS patients compared with patients with other inflammatory neurological disorders (Michalopoulou *et al.* 2004). In animal models for MS, IL-6 deficient mice are completely resistant to experimental autoimmune encephalomyelitis induced by myelin oligodendrocyte glycoprotein (Eugster *et al.* 1998; Samoilova *et al.* 1998). Similarly, in Alzheimer's disease (AD) patients' IL-6 levels are consistently elevated in the CSF and IL-6 immunoreactivity is increased in cortical senile plaques, which indicate the acute-phase state of AD (Bauer *et al.* 1991; Blum-Degen *et al.* 1995). In animal studies, exogenous IL-6 induces several inflammatory responses as seen in AD brains, including expression of β-amyloid precursor protein, production of acute-phase proteins, and gliosis [reviewed by Vandenabeele and Fiers (1991)]. In addition, IL-6 levels are elevated in amniotic fluid, cord blood, and brain sections of infants with periventricular white matter damage, which is associated with a number of pediatric neurological disorders that include, cerebral palsy, epilepsy and attention deficit, and hyperactivity disorder (Yoon *et al.* 1996, 1997, 2003). Overproducing IL-6 in transgenic mice causes a neurologic syndrome that includes ataxia, seizure, and tremor, and a marked and sustained astrogliosis as well as microgliosis (Campbell *et al.* 1993; Chiang *et al.* 1994). In these animals, increased production of pro-inflammatory cytokines, including IL-1 and tumor necrosis factor (TNFα), and intercellular adhesion molecule-1 (ICAM-1), are found in the brain. It suggests that IL-6 mediates several inflammatory responses in the CNS and that it is involved in the pathogenesis of MS, cerebral palsy, epilepsy, attention deficit, hyperactivity disorder, and AD.

In a recent study we showed that IL-6 increased the expression of TNFα and IL-1β and that IL-6 stimulates microglia to secrete molecules that are toxic for spinal cord motor neurons (Krady *et al.* 2008). These studies were conducted using enriched cultures of primary rat microglia. As several groups have used IL-6 knockout and IL-6 overexpressing transgenic mice to gain insights into the functions of IL-6 within the nervous system (Campbell *et al.* 1993; Penkowa *et al.* 1999), it is surprising that there have not been systematic studies to evaluate whether IL-6 directly affects microglia, as such knowledge is fundamental to interpret the results from past and future studies on genetically modified mice. Therefore, here we used highly enriched microglial cultures from neonatal mouse brains to systematically evaluate the signal transduction pathways activated by IL-6, how IL-6 affects the production of pro-inflammatory cytokines, and whether IL-6 modulates microglial antigen presenting and co-stimulatory molecule expression. As IL-6 null mice have been used to model T-cell-mediated diseases (Samoilova *et al.* 1998), we also asked how IL-6 would affect microglia in the context of interferon-gamma (IFNγ). Altogether, our results demonstrated that murine microglia do respond to IL-6 directly, but the resultant effects of IL-6 are context-dependent. In particular, we show that the presence or absence of sIL-6R, IFNγ, or granulocyte–macrophage colony-stimulating factor (GMCSF) will alter the type and amplitude of microglial responses.

Materials and methods

Materials

Recombinant rat CNTF was purchased from Alomone (C-245, Jerusalem, Israel), and recombinant mouse IL-6 (406-ML-005), recombinant mouse IL-6 receptors (1830-SR-025), recombinant mouse IFNγ (485-MI-100), and recombinant mouse granulocyte–macrophage colony-stimulating factor (415-ML-010) were purchased from R&D Systems (Minneapolis, MN, USA). Recombinant mouse LIF2010 was purchased from Millipore (Billerica, MA, USA). Anti-STAT3 (9132), phospho-STAT3-tyr705 (9131), phospho-ERK1/2 (9101), and ERK1/2 (9102) antibodies were purchased from Cell Signaling Technologies (Beverly, MA, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Real-time PCR primer pairs for Ptpn1 (PPM05101A) and 18S (PPM57735A) were purchased from SuperArray (Frederick, MD, USA). Standard laboratory chemicals and reagents were purchased from Sigma (St Louis, MO, USA), and VWR (West Chester, PA, USA) or Fisher (Pittsburgh, PA, USA). Tissue culture reagents were purchased from Invitrogen (Carlsbad, CA, USA).

Enriched microglial cultures

Primary mixed glial cultures were prepared from P0-2 mouse brains. Briefly, C57BL/6 mouse pups were killed by decapitation and whole brains excluding cerebelli and olfactory bulbs were isolated. The meninges were removed, tissues were enzymatically digested using Accutase (AT104, Innovative Cell Technologies, San Diego, CA, USA), mechanically dissociated, and the cell suspension was passed through 100 μm cell strainers before centrifuging at 400 *g* for 7.5 min. Cells were counted using a hemocytometer in the presence of 0.1% trypan blue and plated into 75-cm^2 tissue culture flasks at a density of 2×10^5 viable cells/cm^2 in minimum essential medium (11090-99; Invitrogen) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/100 μg/mL penicillin/streptomycin, and 0.6% glucose (MEM-10C). Media was changed every 3 days after plating. On day 9, the mixed glial cultures were shaken on a Labline (Melrose Park, IL, USA) (#3525) orbital shaker at 250 rpm for 60–75 min to dislodge microglial cells. The non-adherent cells after shaking were plated onto 6-well or 12-well plates at 8×10^4 viable

cells/cm^2, and incubated at 37°C for 30 min to allow microglial cells to adhere. The wells were rinsed extensively with minimum essential medium to eliminate non-adherent cells and debris. The enriched microglial cultures were fed with 2 mL/well (for 6-well plates) or 1 mL/well (for 12-well plates) of hormone-supplemented media that contained 1% fetal bovine serum, 0.66 mg/mL bovine serum albumin (BSA), 100 μg/mL D-biotin, 5 ng/mL insulin, 1 ng/mL selenium, 40 μg/mL iron poor transferrin, 2 mM glutamine, 15 mM HEPES buffer, and 100 U/100 μg/mL penicillin/streptomycin [microglial cell defined medium (MCDM)]. Purity of the enriched murine microglial cultures was confirmed to be > 99% by CD11b and A2B5 staining.

Protein isolation

After cytokine treatments, microglial cells were washed three times with phosphate-buffered saline (PBS), then lysed in buffer containing a final concentration of 1% Triton-X-100, 10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.5% nonidet P-40, 1 mM EDTA, 0.2% EGTA, 0.2% sodium orthovanadate, and 1 μL/mL protease inhibitor cocktail (P8340-1ML, Sigma). Samples were rocked at 4°C for 15 min. DNA was sheared using a 21-gauge needle prior to centrifugation at 9300 *g* for 10 min at 4°C. Protein concentrations from the supernatants were determined using the bicinchoninic acid colorimetric assay (23225, Pierce, Rockford, IL, USA). Protein lysates were aliquoted and stored at −20°C until needed.

Western blotting

Ten to fifteen micrograms of protein isolated from the microglial cells was separated on 7% Tris acetate polyacrylamide gels (EA03552BOX; Invitrogen), electrophoresed at 150 V for 80 min, and transferred at 300 mA for 80 min to nitrocellulose membranes (LC2000; Invitrogen). Membranes were stained with 0.1% Ponceau S in 5% acetic acid to confirm proper transfer of proteins. The membranes were blocked for 1 h at 22°C in 10% (w/v) non-fat dry milk diluted in 0.05% Tween-20 in PBS (PBS-T). Membranes were incubated overnight at 4°C on a rocking platform in primary antibody diluted in 1% BSA/PBS-T. Following incubation with the primary antibody, the blot was extensively washed with PBS-T for 30 min and then incubated for 1.5 h at room temperature with secondary antibody conjugated to HRP diluted in 1% BSA/PBS-T. The membrane was then washed extensively in PBS-T for 30 min prior to visualization using Renaissance™ Chemiluminescence (NEL104; NEN Life Science, Boston, MA, USA). Antibodies from Cell Signaling were diluted in 1 : 1000, HRP-conjugated donkey anti-rabbit secondary antibody was used at 1 : 5000. Images were obtained and quantified using a UVP imaging system with LabWorks software (UVP, Upland, CA, USA); densitometric values were normalized to total STAT3 or ERK1/2 or Ponceau S, as appropriate, and then each sample was normalized to the untreated controls within each experiment.

Luminex assay

Enriched murine microglial cultures were treated with cytokines for 20 h and supernatants were collected and stored at −20°C until assayed. The Beadlyte® Mouse Multi-Cytokine Detection System 2 (48-004) was purchased from Millipore (Billerica, MA, USA). The samples were assayed following the manufacturer's protocols with additional washing to remove excess biotin from the culture medium and analyzed using a Luminex 100 (Austin, TX, USA).

Flow cytometry

Enriched murine microglia were treated with cytokines for 24 h, and detached from the cultureware using Accutase for 10 min. The Accutase was inactivated by adding an equal volume of MEM-10C and cells were collected using a cell scraper. Cells were washed twice with fluorescence-activated cell sorting (FACS) buffer containing Ca^{2+}, Mg^{2+}-free PBS, 0.5% BSA, and 0.02% sodium azide. The cell suspension was incubated for 10 min on ice in anti-Fc antibody (553142; BD Pharmingen, San Jose, CA, USA) diluted 1 : 50. The cells were then incubated in anti-major histocompatability complex (MHC) class II antibody conjugated with Fluorescein isothiocyanate (FITC) (553551; BD Pharmingen, San Jose, CA, USA) diluted 1 : 25 and anti-CD40 antibody conjugated with Allophycocyanin (APC) (558695; BD Pharmingen) diluted 1 : 100 for 1 h on ice in the dark. Cells were washed twice and fixed in 1% paraformaldehye. Ten thousand cells were measured on a BD FACSCalibur at the UMDNJ Flow Cytometry Core Facility (Newark, NJ, USA) using the Cell Quest program. All samples were prepared in triplicate and the geometric mean and percent positive cells were determined after subtracting the background determined with isotype control antibodies.

Reverse transcription and real time RT-PCR

One microgram of RNA was reverse-transcribed to cDNA using the Omniscript RT Kit (205111; Qiagen, Valencia, CA, USA) plus RNase Inhibitor (10777-019; Invitrogen), random nonamer (R7647, Sigma), and Oligo (dT)12-18 Primer (18418-012; Invitrogen). One hundred nanogram of cDNA were mixed with Sybr Green Master Mix (204143; Qiagen) with primer pairs at a final concentration of 400 nM. Samples were analyzed with relative quantification method using an Applied Biosystems ABI Prism 7700 SDS PCR instrument (Foster City, CA, USA). The cycling conditions were 95°C for 10 min for hot start, PCR cycling was set for 95°C for 15 s (denaturation) and 59°C for 1 min (elongation).

Statistical analysis

Data were analyzed using one-way ANOVA followed by Tukey's *post hoc* test or Student's *t*-test to detect significant difference between the means with $p < 0.05$.

Results

IL-6 and LIF activate STAT3 and ERK pathways in cultured murine microglia

Interleukin-6 binds itself to its specific IL-6R and recruits two gp130 components to activate JAK/Tyrosine kinase (Tyk), which leads to activation of STAT and mitogen-activated protein kinase proteins. Prior to initiating studies to evaluate how IL-6 affected microglial immune functions, we performed dose and time effect experiments and analyzed the phosphorylation of STAT3 and ERK1/2 proteins as read-outs. Enriched murine microglial cultures were stimulated with IL-6 at 10 ng/mL for 5, 20, and 40 min and phosphorylation of STAT3 at Tyr705 residues (one band around 90 kDa) was analyzed. Phosphorylation of STAT3 increased 21-fold at 5 min, 43-fold at 20 min, and remained elevated 22-fold at

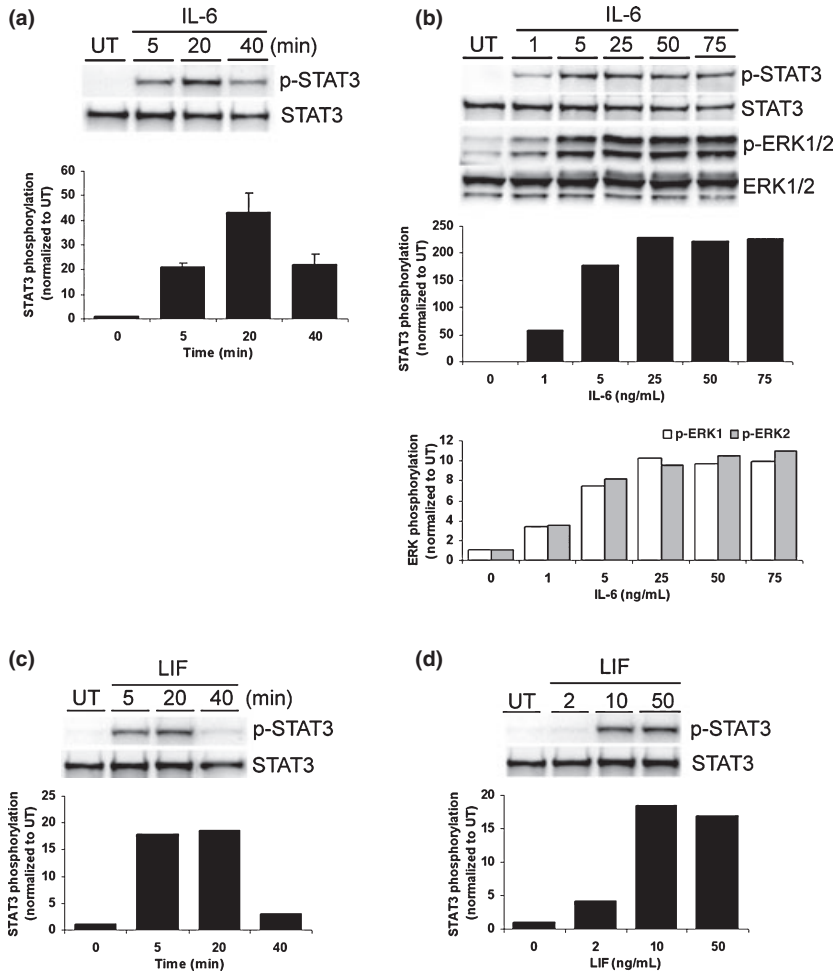


Fig. 1 IL-6 and LIF activate STAT and ERK pathways in microglia. Enriched murine microglial cultures were treated with IL-6 or LIF or left untreated (UT). Following treatment 15 μg of protein lysates from each sample were analyzed by western blotting. Panel a shows representative western blots and densitometry quantification performed using a UVP chemi-imaging system for phosphorylated STAT3 tyr705 and total STAT3 when microglia were treated with IL-6 (10 ng/mL) for 5, 20, and 40 min or left untreated. Values represent the means ± SEM from three independent experiments. Panel b shows representative western blots and densitometry quantification for phosphorylated STAT3 Tyr705, total STAT3, phosphorylated ERK1/2, and total ERK1/2 at 20 min after IL-6 stimulation at 1, 5, 25, 50, and 75 ng/mL. Panel c shows representative western blots and densitometry quantification for phosphorylated STAT3 Tyr705 and total STAT3 when microglia were treated with LIF (10 ng/mL) for 5, 20, and 40 min or left untreated. Values are averaged from two independent experiments. Panel d shows representative western blots and densitometry quantification for phosphorylated STAT3 Tyr705 and total STAT3 at 20 min after LIF stimulation at 2, 10, and 50 ng/mL. Values are averaged from two independent experiments.

40 min compared with untreated cells (Fig. 1a). We also examined phosphorylation of STAT3 and ERK after stimulating with IL-6 at 1, 5, 25, 50, and 75 ng/mL. For both STAT3 Tyr705 (one band around 90 kDa) and ERK (two bands between 40 and 45 kDa), the maximum effect of IL-6 was achieved at 25 ng/mL of IL-6 (Fig. 1b). Thus, the calculated ED_{50} for IL-6 was 2–3 ng/mL. These results were similar to our previous work on rat microglia stimulated with recombinant rat IL-6 (Krady *et al.* 2008). We compared the murine microglial responses to LIF and found that the phosphorylation of STAT3 was similar to that for IL-6; however, the time–course was shifted. At 10 ng/mL of LIF, STAT3 phosphorylation on Tyr705 increased to 18-fold by 5 min and this level was sustained for at least 20 min, but declined to threefold by 40 min (whereas IL-6 stimulated STAT3 phosphorylation remained elevated 22-fold at this time point; Fig. 1c). To establish a dose-effect curve for LIF, microglia were treated with LIF at 2, 10, and 50 ng/mL for 20 min and phosphorylation of STAT3 tyr705 was found to be increased by 4-, 18-, and 17-fold, respectively (Fig. 1d). The maximum effect of LIF was reached by 10 ng/mL. Thus the calculated ED_{50} for LIF on murine microglia was 3–4 ng/mL.

IL-6 or the combination of IL-6 and sIL-6R induces phosphorylation of STAT3, ERK, and STAT1 in a similar magnitude in murine microglia

To delineate whether signaling by the combination of IL-6 and sIL-6R was mediated by gp130, we examined phosphorylation of STAT3 and ERK in microglia after cytokine stimulation. The concentration of IL-6 was used at 5 ng/mL, which was two times the ED_{50} determined by STAT3 and ERK1/2 phosphorylation, whereas the concentration of sIL-6R was used at 200 ng/mL, which was in excess of the molar concentration of IL-6. We found that both IL-6 and the combination of IL-6 and sIL-6R activated STAT3, ERK, and STAT1 (Fig. 2a–c). Densitometry showed that IL-6 and IL-6/sIL-6R induced similar levels of STAT3, ERK, and STAT1 phosphorylation.

IL-6 collaborates with IFNγ to increase secretion of IL-12p70

During an immune response microglia secrete cytokines to activate resident neural cells as well as infiltrated immune cells. Therefore, we questioned how IL-6 family cytokines would affect the production of pro-inflammatory cytokines.

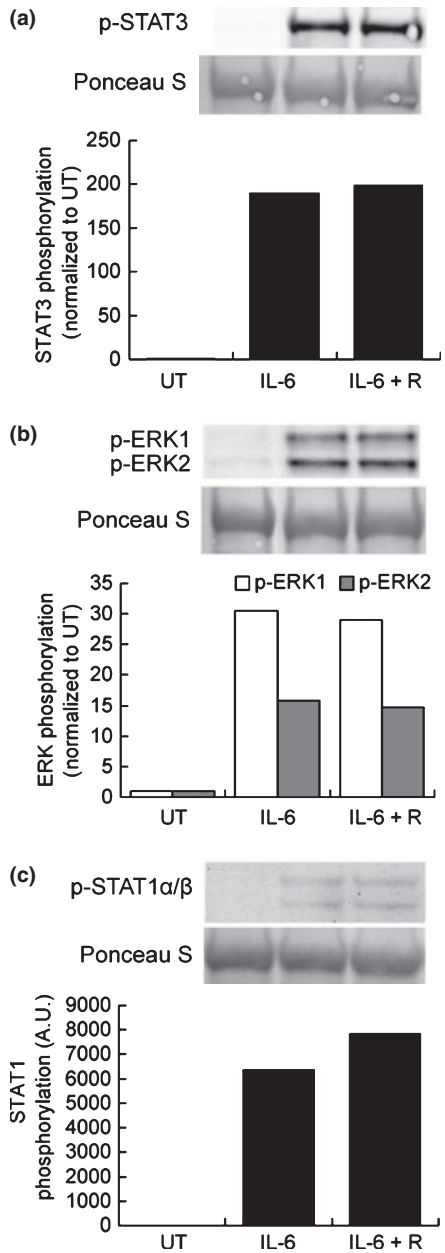


Fig. 2 IL-6 or the combination of IL-6 and sIL-6R activates signaling transduction of a similar magnitude in murine microglia. Panel a, b, and c show enriched murine microglial cultures which were treated with IL-6 (5 ng/mL), the combination of IL-6 (5 ng/mL) and sIL-6R (200 ng/mL) or left untreated (UT) for 20 min. Fifteen micrograms of protein lysates from each sample were analyzed by western blotting. Representative western blots and densitometry quantification using a UVP chemi-imaging system for phosphorylated STAT3 Tyr705, phosphorylated ERK1/2, and phosphorylated STAT1α/β were shown. Data are representative of three separate experiments.

We used a beadlyte kit to measure the levels of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, TNFα, GMCSF, and IFNγ in microglial culture supernatants. The phosphorylation of

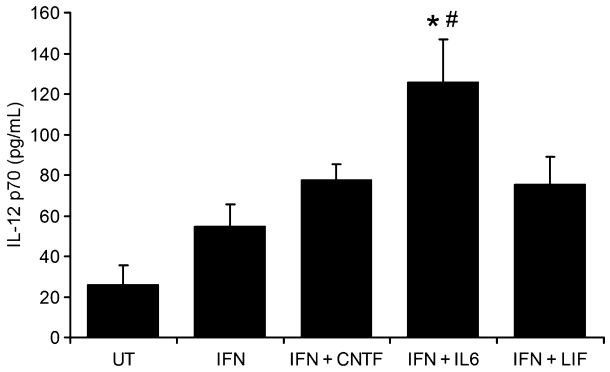


Fig. 3 IL-6 collaborates with IFNγ to increase IL-12p70 secretion from microglia. Microglia were treated with IFNγ (10 ng/mL) alone (IFN), IFNγ (10 ng/mL) plus CNTF (10 ng/mL) (IFN + CNTF), IL-6 (5 ng/mL) (IFN + IL-6) or LIF (10 ng/mL) (IFN + LIF), or left untreated (UT) for 20 h. Supernatants were collected and stored at –20°C until assayed. Values represent the means ± SEM from five independent experiments. *$p < 0.001$ versus UT, #$p < 0.01$ versus IFN by one-way ANOVA followed by Tukey's *post hoc* test.

signal transducers can occur within minutes as we showed, whereas the expression and secretion of soluble proteins often requires new protein synthesis, post-translational modifications, and secretion mechanisms which require hours. Therefore, cultured microglia were treated for 20 h with IFNγ alone, IFNγ plus IL-6, CNTF or LIF, or left untreated, whereupon collected supernatants were analyzed. We found that IL-6 collaborated with IFNγ to increase microglial secretion of IL-12p70. None of the other cytokines analyzed were either increased or decreased by IL-6, LIF, or CNTF (Table S1). Basal levels of IL-12p70 were 41.2 ± 8.9 pg/mL in untreated cells, and upon stimulation with IFNγ, IL-12p70 levels were increased to 64.8 ± 5.6 pg/mL. Treating with IFNγ plus IL-6 further increased the concentration to 126.1 ± 18.3 pg/mL ($p < 0.01$ versus IFNγ; Fig. 3). At the concentrations used, neither CNTF nor LIF altered the secretion of IL-12p70.

IL-6 in the presence of sIL-6R potentiates the effect of IFNγ on CD40, but not MHC class II, expression in murine microglia

Expression of antigen presenting and co-stimulatory molecules on microglia is required for antigen presentation. IFNγ is secreted from Th1 cells and known to strongly induce the expression of antigen presenting and co-stimulatory molecules in microglia. To understand how IL-6 modulated microglial antigen presentation we examined whether IL-6 alone or in combination with sIL-6R would affect the expression of the antigen presenting molecule, MHC class II, and the co-stimulatory protein, CD40, on IFNγ-stimulated microglia. Microglial cultures were treated with IFNγ alone, IFNγ plus IL-6 or IFNγ plus IL-6, and sIL-6R, or left

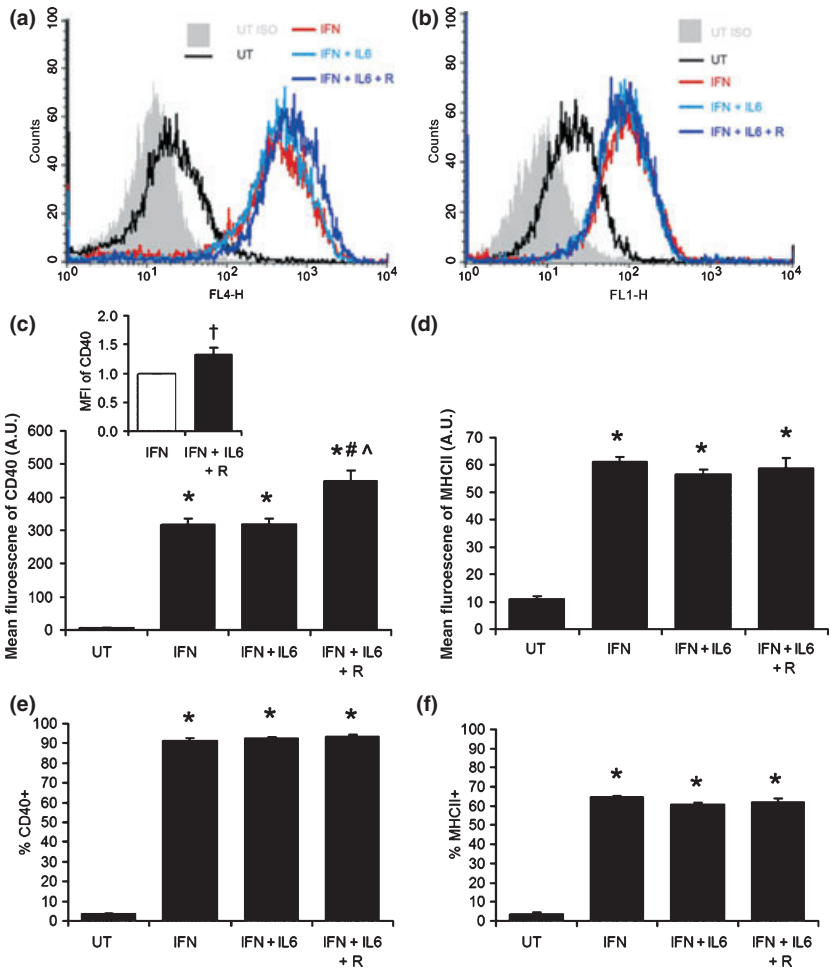


Fig. 4 IL-6 in the presence of sIL-6R potentiates the effect of IFNγ on CD40, but not MHC class II, expression in murine microglia. Microglia were treated with IFNγ (10 ng/mL) (IFN), IFNγ plus IL-6 (25 ng/mL) (IFN + IL6) or IFNγ plus a combination of IL-6 (25 ng/mL) and sIL-6R (200 ng/mL) (IFN + IL6 + R), or left untreated (UT) for 24 h. Ten thousand cells were collected and analyzed by flow cytometry for CD40 (APC) and MHC class II (FITC) expression. Panel a shows a representative histogram for CD40 while Panel b shows that for MHC class II. (c) Mean fluorescence of CD40 and inset shows averaged values from three independent experiments as normalized to IFNγ alone (†$p < 0.05$ by Student's *t*-test). (d) Mean fluorescence of MHC class II; e, percentage of CD40 positive cells; f, percentage of MHC class II positive cells. Values represent the means ± SEM from triplicates in one experiment. *$p < 0.001$ versus UT, #$p < 0.01$ versus IFN, ^$p < 0.01$ versus IFN + IL6 by one-way ANOVA followed by Tukey's *post hoc* test. Data are representative of three independent experiments.

untreated for 24 h. The concentration of IFNγ was used at 10 ng/mL, which was 10-fold of the ED_{50} determined by MHC class II expression in a mouse microglial cell line, EOC20 (data not shown). Cells were collected and analyzed by flow cytometry for surface expression of CD40 and MHC class II. Both fluorescence intensity and percentage positive cells were analyzed. In unstimulated microglia, the expression of CD40 was low (3.7%). Upon stimulation with IFNγ, the signal intensity for CD40 was induced 49-fold when compared with untreated microglia ($p < 0.001$). IL-6 plus sIL-6R further increased the expression of CD40 (approx. 40%) compared with IFNγ alone ($p < 0.01$; Fig. 4). IL-6 in the absence of the sIL-6R had no effect on the levels of CD40 expression induced by IFNγ ($p > 0.05$). The percentage of cells that expressed CD40 was also increased by IFNγ ($p < 0.01$), but the percentage of CD40+ cells was not further increased by IL-6 with or without sIL-6R ($p > 0.05$), which was likely because of high percentage of CD40+ after IFNγ stimulation (> 90%). Whereas IL-6/sIL-6R enhanced the IFNγ-induced expression of CD40, it did not increase that of MHC class II. IFNγ increased MHC class II expression 5.5-fold and percentage of MHC class II positive cells 18-fold ($p < 0.001$), and adding IL-6 alone or IL-6 plus sIL-6R was without effect ($p > 0.05$). CD80 expression was also evaluated. The expression of CD80 was low in untreated microglia and remained unaltered after stimulation of IFNγ alone or with IL-6/sIL-6R (data not shown).

IL-6 in the presence of sIL-6R potentiates the effect of IFNγ on CD40, but not MHC class II, expression in dendritic cell-like microglia

As microglia can differentiate into dendritic cell-like cells when stimulated with GMCSF, we were interested in whether IL-6 family cytokines would exert different effects upon microglia in this state versus in their less differentiated state. Enriched microglial cultures were treated with GMCSF at 10 ng/mL and the dose was chosen according to previously published studies (Re *et al.* 2002). The expression of CD11c, a dendritic cell marker, was analyzed by flow cytometry. In the unstimulated primary microglia, fewer than 5% of cells were positive for CD11c. After culturing in GMCSF for 2 or 8 days, approximately 16% or 80%, respectively, of the cells

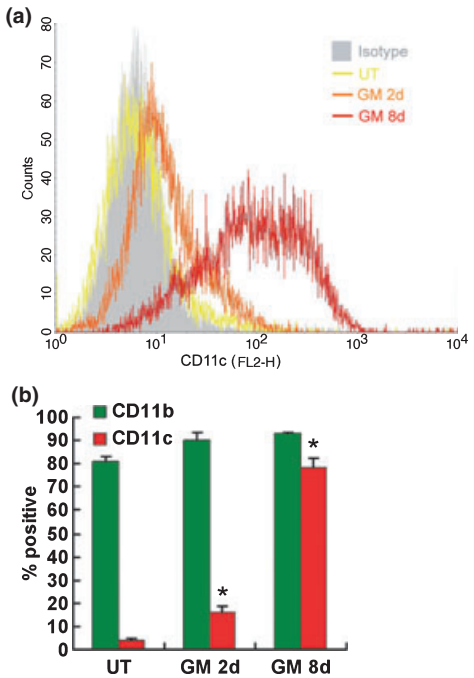


Fig. 5 GMCSF induces microglia to differentiate into dendritic cell-like microglia. Panel a shows a representative histogram for CD11c (PE). Panel b shows the percentage of CD11b and CD11c positive cells after GMCSF treatment (10 ng/mL) for 2 or 8 days. Values represent the means ± SEM from at least four independent experiments. *$p < 0.005$ versus untreated (UT) by one-way ANOVA followed by Tukey's *post hoc* test.

were positive for CD11c ($p < 0.005$; Fig. 5a and b). The expression of CD11b, which is a macrophage lineage marker also expressed on microglia, remained high.

On the seventh day in GMCSF, the dendritic cell-like microglia (80% CD11c-expressing cells) were stimulated with IL-6, IL-6 plus sIL-6R, IFNγ, IFNγ plus IL-6, IFNγ plus IL-6, and sIL-6R or left untreated for 24 h. Cells were collected and analyzed by flow cytometry for CD40 and MHC class II expression. The expression of CD40 was increased 2.3-fold following IFNγ treatment ($p < 0.005$; Fig. 6e). Stimulating with IL-6 showed a trend towards inducing CD40 but averaged values from three separate experiments showed the increase to be around 30% compared to IFNγ alone, which was not statistically significant ($p > 0.05$; Fig. 6e inset). However, the combination of IFNγ with IL-6 and sIL-6R strongly induced CD40 expression 4.5-fold compared to IFNγ alone ($p < 0.05$). The percentage of CD40+ was also increased from 25.5% to 72.5% (2.8-fold) after stimulation with IL-6 plus sIL-6R compared with IFNγ alone ($p < 0.05$; Fig. 6g). By contrast, IL-6 or IL-6 plus sIL-6R in absence of IFNγ failed to induce MHC class II or CD40 ($p > 0.05$), and they failed to potentiate IFNγ-induced MHC class II expression ($p > 0.05$) while the signal intensity and percentage of positive cells of MHC class II was increased by IFNγ stimulation 2.7- and 3.9-fold, respectively ($p < 0.005$; Fig. 6f and h). As neither IL-6 nor IL-6/sIL-6R had any effect on MHC class II or CD40 expression, these data indicate that there was a synergistic effect of IL-6 and IFNγ on CD40 expression.

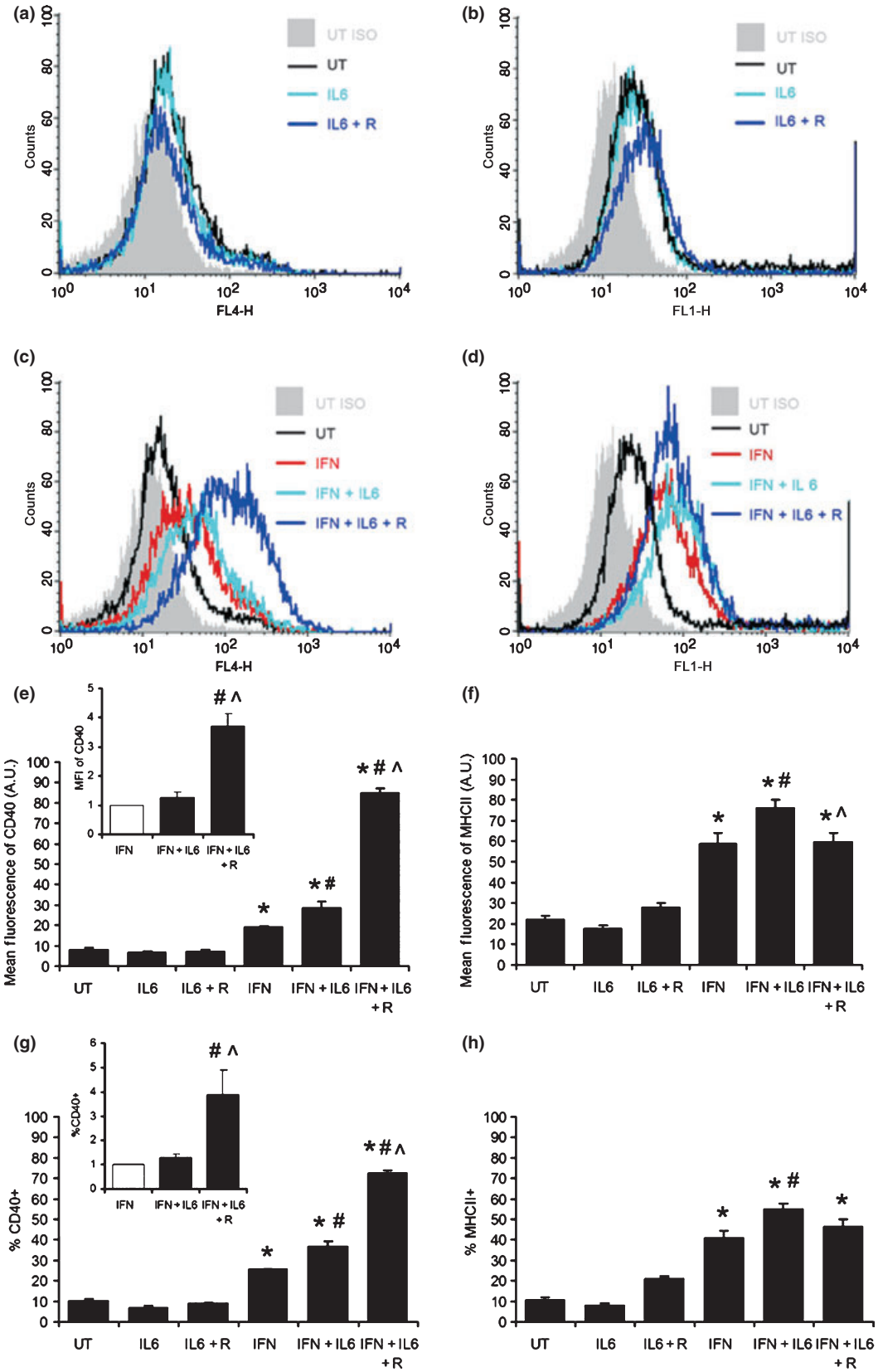
IL-6 and LIF, but not CNTF, increase mRNA of protein tyrosine phosphatase 1B

To evaluate the counter-regulatory effects of IL-6 on microglial stimulated responses, cultured microglia were treated with CNTF, IL-6, or LIF or left untouched for 6 h. Total RNA was isolated, reverse transcribed to cDNA, and used for real-time PCR analysis. IL-6 and LIF increased the transcripts for protein tyrosine phosphatase 1B (PTP1B) ($p < 0.05$), but CNTF was without effect ($p > 0.05$; Fig. 7). We also examined mRNA levels for protein inhibitor of activated STAT2 (PIAS2), PIAS3, and PTP non-receptor type substrate 1. However, none of them were regulated by IL-6, CNTF, or LIF.

Discussion

Interleukin-6 is a systemically and locally produced cytokine that possesses both anti- and pro-inflammatory activities, and it is involved in inflammation-associated neurological diseases such as MS, cerebral palsy, and AD. Here, we sought to understand which signal transduction pathways were activated by IL-6 in microglia and how IL-6 regulated their production of cytokines and expression of antigen presenting and co-stimulatory molecules. We showed that (i) IL-6 induced an increase in STAT3 and ERK phosphorylation in newborn microglial cultures in a dose and time-dependent manner; (ii) IL-6 alone or co-administering IL-6 and a soluble form of the IL-6 alpha receptor induced STAT3, ERK, and STAT1 phosphorylation in a similar magnitude; (iii) IL-6, but not CNTF or LIF, potentiated IFNγ-induced IL-12p70 secretion; (iv) the combination of IL-6 and sIL-6R potentiated the effect of IFNγ on CD40 expression whereas IL-6 alone was without effects; (v) neither IL-6 nor the combination of IL-6 and sIL-6R enhanced IFNγ-induced MHC class II expression; (vi) IL-6 and sIL-6R strongly potentiated IFNγ-induced CD40 in the GMCSF-induced dendritic cell-like microglia; and (vii) IL-6 induced PTP1B. Cumulatively, our results demonstrated that the effects of IL-6 on microglia depended on the presence of sIL-6R, IFNγ, or GMCSF, which altered the type and amplitude of their response.

Soluble IL-6 receptors are generated via proteolysis of membrane-bound IL-6 receptors or differentially spliced mRNA (Mullberg *et al.* 1994; Holub *et al.* 1999) and sIL-6R acts with IL-6 as an agonist on cells that are not responsive to IL-6. In several inflammatory diseases, trans-signaling of IL-6 through sIL-6R has been shown to promote persistent inflammation (Rose-John *et al.* 2006). It is conceivable that IL-6/sIL-6R activates novel pathways that are not stimulated



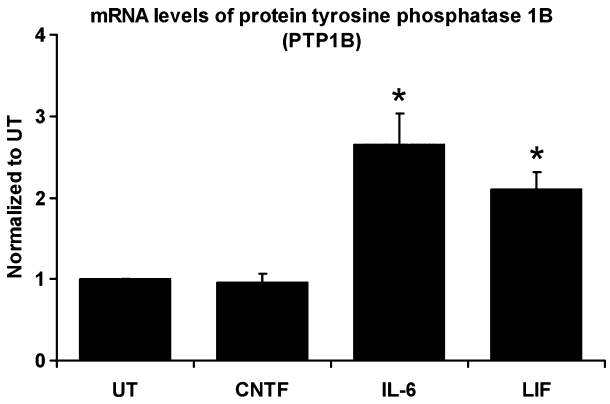


Fig. 7 IL-6 and LIF, but not CNTF, increases mRNA of protein tyrosine phosphatase 1B (PTP1B). Microglia were treated with CNTF (10 ng/mL), IL-6 (5 ng/mL), and LIF (10 ng/mL) or left untreated (UT) for 6 h. Total RNA was isolated, reverse-transcribed to cDNA, and used for real-time PCR analyses for protein tyrosine phosphatase 1B (PTP1B) using a primer to 18S RNA for normalization. Data are normalized to untreated and then averaged from four independent experiments. Values represent the means ± SEM. *$p < 0.05$ versus UT by one-way ANOVA followed by Tukey's *post hoc* test.

by IL-6 alone, which act independently or cooperatively with known IL-6 signaling pathways, such as STAT and ERK, in microglia. Our results showed that sIL-6R potentiated the effect of IL-6 on CD40 expression. Similar results were obtained for cyclooxygenase 2 expression (Lin 2008). This led us to hypothesize that the addition of sIL-6R activated either STAT3 or ERK pathway more vigorously and, therefore, we examined the phosphorylation of STAT3 and ERK by IL-6 in the presence or absence of sIL-6R. However, we found that IL-6 and IL-6/sIL-6R induced similar levels of STAT3 and ERK phosphorylation. Previous studies also showed that STAT1, PU 1, and Spi-B mediated the induction of CD40 expression by IFNγ in macrophages (Nguyen and Benveniste 2000; Wesemann *et al.* 2002). We found that both IL-6 and IL-6/sIL-6R elicited STAT1α/β phosphorylation in microglia but IL-6 alone did not increase CD40 expression. Overall, these data suggest that activation of these pathways is not sufficient to induce CD40.

As reviewed above, IL-6 was found in the CSF of MS and AD patients, and the time-course of IL-6 expression was consistent with a role for IL-6 as a pro-inflammatory cytokine that participated in disease progression (Maimone *et al.* 1991; Blum-Degen *et al.* 1995). LIF was also produced locally in the CNS by microglia and astrocytes, and it has been shown to activate microglia and astrocytes and to exacerbate CNS inflammation (Banner *et al.* 1997; Kerr and Patterson 2004). Yet, a third member of this family, CNTF, was produced in astrocytes in the CNS and in Schwann cells in the PNS (Stockli *et al.* 1991; Dobrea *et al.* 1992), and the production of CNTF coincided with the remyelination phase of virus-induced demyelination in the spinal cord (Albrecht *et al.* 2003). Therefore, we compared the effects of IL-6, LIF, and CNTF in their capacity to induce IL-12p70 secretion from microglia. IL-12p70 is a 70 kDa glycoprotein consisting of a 40 kDa subunit (IL-12p40) and a 35 kDa subunit (IL-12p35), and it is secreted from macrophages and APCs to induce differentiation of naïve T cells to Th1 cells as well as to expand T cells (Mullen *et al.* 2001). IL-12p70 has been implicated in the T-cell-mediated CNS demyelinating diseases like MS and experimental autoimmune encephalomyelitis (Gran *et al.* 2004). Cultured microglia were capable of secreting IL-12p70 in response to IFNγ and lipopolysaccharide (LPS) stimulation (Becher *et al.* 1996; Xiao *et al.* 1996; Aloisi *et al.* 1997). IL-6 null animals were deficient in myelin oligodendrocyte glycoprotein-reactive T cells that were differentiated into Th1 and Th2 effector cells, suggesting a (presumably indirect) role for IL-6 in Th1 and Th2 differentiation (Samoilova *et al.* 1998). Therefore, we tested the hypothesis that IL-6 family cytokines collaborate with IFNγ to increase IL-12p70 secretion. Our analyses showed that IFNγ alone did not significantly increase the secretion of IL-12p70 in microglia, but that co-administering IL-6 with IFNγ increased IL-12p70 secretion. These data confirm the validity of our hypothesis. Interestingly, neither CNTF nor LIF when administered with IFNγ increased IL-12p70 release. Although these cytokines shared gp130 as a signal transducer, they appeared to regulate microglial properties differently.

Fig. 6 IL-6 in the presence of sIL-6R potentiates the effect of IFNγ on CD40, but not MHC class II, expression in dendritic-cell like microglia. After continuous growth in GMCSF (10 ng/mL) for 7 days, the dendritic cell-like microglia were treated with IL-6 (25 ng/mL) (IL6), a combination of IL-6 (25 ng/mL) and sIL-6R (200 ng/mL) (IL6 + R), IFNγ (10 ng/mL) (IFN), IFNγ (10 ng/mL) plus IL-6 (25 ng/mL) (IFN + IL6) or IFNγ (10 ng/mL) plus a combination of IL-6 (25 ng/mL) and sIL-6R (200 ng/mL) (IFN + IL6 + R), or left untreated (UT) for 24 h. Ten thousand cells were collected and analyzed by flow cytometry for CD40 and MHC class II expression. Panel a and c show representative histograms for CD40 (APC) while Panel (b) and (d) show representative histograms for MHC class II (FITC). (e) Mean fluorescence of CD40 and inset shows averaged values from three independent experiments as normalized to IFNγ alone. (f) Mean fluorescence of MHC class II. (g) Percentage of CD40 positive cells and inset shows averaged values from three independent experiments as normalized to IFNγ alone. (h) Percentage of MHC class II positive cells. Values represent the means ± SEM from triplicates in one experiment. *$p < 0.005$ versus UT, #$p < 0.05$ versus IFN, ^$p < 0.05$ versus IFN + IL6 by one-way ANOVA followed by Tukey's *post hoc* test. Data are representative of three independent experiments.

Microglia can become competent APCs and here we show that after stimulating with IFNγ microglia readily express MHC class II and CD40. The combination of IL-6 and sIL-6R further enhanced IFNγ-induced CD40 expression by 40%. More interestingly, in GMCSF-induced dendritic cell-like microglia, the combination of IL-6 and sIL-6R strongly increased IFNγ-induced CD40 expression as measured by mean fluorescence intensity and the percentage of positive cells, 3.76- and 3.82-fold, respectively (averaged values from three independent experiments). Therefore, we concluded that IL-6/sIL-6R synergized with IFNγ to increase CD40 expression. By contrast, IL-6/sIL-6R had no effect on the IFNγ-induced MHC class II expression in primary or dendritic cell-like microglia. In addition, the flow cytometry analyses showed that CD40 and MHC class II were expressed in an overlapping fashion after IFNγ stimulation in microglia. Unstimulated microglia expressed low levels of both markers. After stimulating with IFNγ, almost all of the cells that expressed MHC class II also expressed CD40 and some cells only expressed CD40 (59.4 ± 8.7% were MHCII+/CD40+; 28.5 ± 6.5% were CD40+/MHCII−; 0.8 ± 1.0% were CD40−/MHCII+). IL-6 or IL-6 plus sIL-6R did not change the percentage of CD40+/MHCII+, CD40+/MHCII− or CD40−/MHCII+ cells induced by IFNγ ($p > 0.05$).

The negative regulatory mechanisms for JAK/STAT signaling have been widely studied. Protein tyrosine phosphatases (PTP) including SHP-2, PTP1B and PTPεC, and CD45 dephosphorylate JAKs (Kim *et al.* 1998; Schaper *et al.* 1998; Tanuma *et al.* 2000; Irie-Sasaki *et al.* 2001; Myers *et al.* 2001). Among them, the current study revealed that PTP1B was induced in microglia by IL-6 and LIF, but not CNTF. PTP1B can dephosphorylate JAK2 and Tyrosine kinase 2 (Tyk2) to suppress downstream signaling cascades (Myers *et al.* 2001). Our result suggests that PTP1B induced by IL-6 and LIF will, in turn, suppress IL-6 signaling and prevent microglia from continuously responding to cytokine stimulation, which may also include interferons because they also signal through JAKs. We also observed that stimulating microglia with IFNγ suppressed IL-6 receptor mRNA levels by 50% (unpublished data), suggesting that IFNγ reduced microglial responsiveness to IL-6. These results indicated that both endogenous negative regulators and external stimuli could reduce microglial responses to IL-6.

Altogether, our studies demonstrated that microglial responses to IL-6 were not simple in that the effects of IL-6 were context-dependent. In particular, the presence or absence of sIL-6R, IFNγ, or GMCSF will alter the type and amplitude of their response. We showed that IL-6 alone had direct effects on microglia as it stimulated STAT3, ERK, and STAT1 phosphorylation and the stimulated secretion of IL-12 did not require sIL-6R co-administration. However, other indices of immune function which we analyzed were only weakly modulated by IL-6 when administered without sIL-6R. For instance, the IFNγ-induced expression of the co-stimulatory molecule, CD40 was dependant on sIL-6R administration. In addition, our analyses revealed that although IL-6, CNTF, and LIF were structurally related, they did not have identical effects on microglia, which warranted additional studies.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Cytokines detected in the supernatants of murine microglial cultures.

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