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Oxidative Modulation of the Glutathione-redox Couple Enhances Lipopolysaccharide-induced Interleukin 12 P40 Production by a Mouse Macrophage Cell Line, J774A.1

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Interleukin (IL)-12 plays a key role in determining the immune response pattern that results in maturation of Th0 to Th1 and Th2. To investigate the correlation between intracellular redox state and IL-12 production in macrophages, cells from the mouse cell line J774A.1 were treated with reagents modulating the glutathione-redox couple before stimulation with lipopolysaccharide (LPS). It was found that the glutathione reductase inhibitor, 1,3-bis (2-chloroethyl)-1-nitrosourea, markedly augmented LPS-induced IL-12p40 production particularly when it was added for 24 h before LPS stimulation, whereas the glutathione-synthesis inhibitor, L-buthionine-(S,R)-sulfoximine, suppressed IL-12p40 production. The profile of IL-12p40 augmentation correlated well with the profile of intracellular glutathione oxidation (GSSG) and the activation profile of nuclear transcription factor κB (NF-κB), suggesting that GSSG is important in NF-κB activation which leads to IL-12p40 production. Our results indicate that the glutathione-redox couple plays an important role in the augmented production of IL-12p40 and thus in influencing immune response patterns.

Keywords: LPS; IL-12; Glutathione; BCNU; BSO; NF-κB

INTRODUCTION

Interleukin (IL)-12 is secreted by antigen-presenting cells (APCs) such as dendritic cells and macrophages, and plays a key role in directing helper T cells (Th0) to mature into two different cell subsets, Th1 and Th2.[1–4] In the presence of IL-12, Th0 maturation is polarized towards Th1 that secrete IL-2 and interferon (IFN)-γ and preferentially promotes cell-mediated immunity as well as IgG2a production by B cells. In the absence of IL-12 on the other hand, Th0 maturation is polarized towards Th2 that secrete IL-4 and IL-5, and preferentially promote humoral immunity, including IgE and IgG1 production. The bioactive IL-12 molecule (p70) is a heterodimer that consists of two disulfide-bonded subunits, p35 and p40.[5–8] The p40 subunit also exists as a monomer or a homodimer, the latter serving as an antagonist of p70 by competitively binding to the IL-12 receptor of helper T cells.[9,10]

Glutathione is the most abundant thiol in cells; it detoxifies reactive oxygen intermediates (ROI) such as hydrogen peroxide (H_2O_2), and maintains the intracellular redox state via conversion from its reduced form (GSH) to the oxidative form (GSSG). It has been reported that the nuclear transcription factor κB (NF-κB) that regulates expression of various inflammatory genes, is activated by oxidative stresses induced by inhibitors of glutathione synthesis or reduction as well as by H_2O_2 added exogenously.[11–14] Conversely, activation of NF-κB is suppressed by an antioxidant thiol precursor of GSH, *N*-acetyl cysteine (NAC).[15–17] On the other hand, NF-κB was recently shown to mediate lipopolysaccharide (LPS) or superantigen-induced IL-12p40 production by macrophages.[18–21]

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Although these findings suggest that oxidative stress may enhance, whereas reductive stress may suppress, IL-12p40 production, no study to date has proved this directly. In one study ultraviolet irradiation, which is known to induce ROI, was shown to augment IL-12p40 production.[22] Evidence that seems to contradict the theory that IL-12 production is augmented by oxidative stress comes from a report describing glutathione depletion *in vivo* and *in vitro*. The report showed suppression of IL-12 (p40 + p70) production by APCs and a shift in the immune response from Th2 to Th1 in response to *in vivo* administration of NAC.[23–28] Thus, it remains unclear how oxidative stress affects IL-12 production by APCs.

To clarify the relationship between oxidative stress, the glutathione-redox couple in particular, and IL-12 production, we treated a mouse macrophage cell line, J774A.1, with an inhibitor of glutathione reductase, and an inhibitor of glutathione synthesis, and measured LPS-induced IL-12p40 production. We measured intracellular GSH and GSSG concentrations to determine the redox state of the J774A.1 cells. Finally, we analyzed the activation status of NF-κB in J774A.1 cells treated with these regents to assess the correlation between the three parameters, redox, NF-κB, and IL-12 production.

MATERIALS AND METHODS

Cell Culture and Glutathione Inhibitors

Macrophages from the mouse cell line J774A.1 were cultured in 24-well microplates (Corning 25820) in RPMI 1640 medium containing 10% (v/v) fetal bovine serum and antibiotics (RPMI medium). When the cell culture reached 70–80% confluency, various concentrations of 1,3 bis (2-chloroethyl)-1-nitrosourea (BCNU) (Sigma, C-0400), L-buthionine-(S,R)-sulfoximine (BSO) (Sigma, B2515), were added to the medium. The cells were incubated for 24 or 0 h, and LPS (Sigma, L-5024) (final concentration of 300 ng/ml in most cases) was added to the cell culture for 12 h to stimulate IL-12p40 production.

Enzyme-linked Immunosorbent Assay (ELISA) for IL-12p40

IL-12p40 was measured in the culture supernatant of LPS-stimulated J774A.1 cells using a sandwich ELISA system. A monoclonal antibody specific for p40 (Genzyme, clone C15.6) was used as the capture antibody, and a biotinylated monoclonal antibody specific for p40 (Genzyme C17.8) was used as the detection antibody. The binding signal was detected spectrophotometrically at 492 nm as the color developed with horseradish peroxidase-conjugated streptavidin and *o*-phenylene diamine.

Measurement of Intracellular Glutathione Content

Intracellular glutathione content was measured by high-performance liquid chromatography (HPLC) according to reported methods[29,30] with several modifications. In brief, J774A.1 cells were suspended in 40 mM boric acid and 10 mM tetraborate buffer containing 10.5 mg/ml L-serine, 0.5 mg/ml bathophenanthroline disulfonate sodium salt, and 1 mg/ml iodoacetic acid then subjected to freeze-thawing and sonication to destroy the cell membranes. After centrifugation, the supernatant was diluted with an equal volume of a solution containing 0.4 M boric acid, 10 mg/l cresol red and 100 μM glutamylglutamine (as an internal standard) in 10% (v/v) perchloric acid (for deproteinization) and centrifuged again. The supernatant was adjusted to pH 8.6–8.8 using 2 M lithium chloride, 5 mg/ml of dansyl chloride in acetonitrile was added and the solution was incubated for 2 h at 25°C in the dark. After incubation, the solution was applied to an aminoprophyl silica gel column and eluted with methanol–acetic acid buffer and the dansylated material was estimated using HPLC (Shimazu, LC-9A) equipped with a fluorescence detector (Nippon Bunko, FP-1520).

Electrophoretic Mobility Shift Assay (EMSA)

J774A.1 cells ($0.2–1 \times 10^7$ total cells) were washed with PBS and suspended for 5 min in a hypotonic lysis buffer containing 10 mM HEPES–KOH (pH 7.8), 0.05% (v/v) Nonidet P-40, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and a protease inhibitor cocktail (0.25 mM 4-(2-aminoethyl) benzenesulfonyl fluoride, 0.2 μM aprotinin, 12.5 μM bestatin, 3.8 μM *trans*-epoxysuccinyl-L-leucylamido (4-guanidino) butane, 5 μM leupeptin and 2.5 μM pepstatin A (Wako, Tokyo, Japan). After a 1-min spin at 2,000*g*, the pellet was resuspended in a high-salt extraction buffer containing 50 mM HEPES–KOH (pH 7.8), 420 mM KCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, 0.5 mM PMSF, 2% (v/v) glycerol and the protease inhibitor cocktail then allowed to incubate for 30 min with vigorous mixing every 10 min. Nuclei were then centrifuged for 15 min at 12,000*g* to pellet the cell debris and the remaining clear layer (nuclear extract) was collected. The protein concentration was measured using a commercial kit (Bio-Rad, Hercules, CA) and the solution was diluted to 2 μg/μl and kept frozen at −80°C until EMSA was performed.

The binding activity of NF-κB in nuclear extracts of J774A.1 cells to its consensus oligonucleotide

(5′-GTACGGAGTATCCAGCTCCGTAGCATGCAA-ATCCTCTGG-3′, Promega, Madison, WI) was evaluated by EMSA, using a non-radioactive EMSA kit, (DIG Gel Shift Kit: Roche, Mannheim, Germany). The NF-κB consensus oligonucleotide was end-labeled with digoxigenein (DIG) using terminal transferase. Binding reactions were carried out at room temperature for 15 min. The reaction mixtures contained 10 μg of total protein, 20 mM HEPES (pH 7.6), 30 mM KCl, 1 mM EDTA, 1 mM DTT, 0.2% (v/v) Tween 20, 50 μg/ml poly (dI–dC), 5 μg/ml poly L-lysine and 15.54 fmol of the DIG-labeled probe (total volume of 10 μl). Protein–DNA complexes were separated from protein-free DNA by non-denaturing electrophoresis in 5% (w/v) polyacrylamide gels. The gels were run at 4°C in 22.25 mM Tris–HCl (pH 8.0) buffer containing 22.25 mM boric acid and 0.5 mM EDTA at a constant voltage, and electroblotted onto a positively charged nylon membrane (Hybond-N^+: Amersham Pharmacia Biotech, Tokyo, Japan) at a constant current. The non-labeled NF-κB-specific oligonucleotide (1,925 fmol) was used as a specific competitor to confirm specific binding. A non-labeled Oct2A consensus oligonucleotide (5′-GTACGGAGTATCC-AGCTCCGTAGCATGCAAATCCTCTGG-3′) (1,925 fmol) was used as an irrelevant competitor. The DNA was cross-linked to the membrane using UV light (254 nm for 3 min) then the membrane was treated with alkaline phosphatase-conjugated anti-DIG-antibody. The chemiluminescent substrate CSPD® was added and chemiluminescent signals were detected by exposing the membrane on X-ray film. The film was photographed using a digital camera (Kodak, DC-120 zoom) and then quantified using ImageQuant software (Molecular Dynamics).

RESULTS

Oxidative Modulation of the Glutathione-redox Couple Augments LPS-induced IL-12 P40 Production

To examine whether modulation of the glutathione-redox couple influences IL-12 production, a mouse macrophage cell line, J774A.1, was incubated for 36 h with two different concentrations of either BCNU (an inhibitor of glutathione reductase) or BSO (an inhibitor of glutamate–cysteine ligase). During the final 12 h of incubation, the cells were stimulated with 300 ng/ml LPS to induce IL-12 production. The IL-12p40 molecules secreted in the supernatant were measured using ELISA. Because J774A.1 cells do not produce detectable amounts of the p35 subunit,[27] the IL-12p40 detected in this study was thought to be a p40-monomer plus a p40-homodimer. LPS-induced IL-12p40 production appears to be augmented in a dose-dependent manner in J774A.1 cells treated with BCNU (1.4 and 1.9-fold stimulation at 20 and 100 μM BCNU, respectively) (Fig. 1). In contrast BSO slightly inhibited LPS-induced IL-12p40 production (0.9 and 0.7-fold at 20 and 100 μM BSO, respectively) although this effect was not statistically significant.

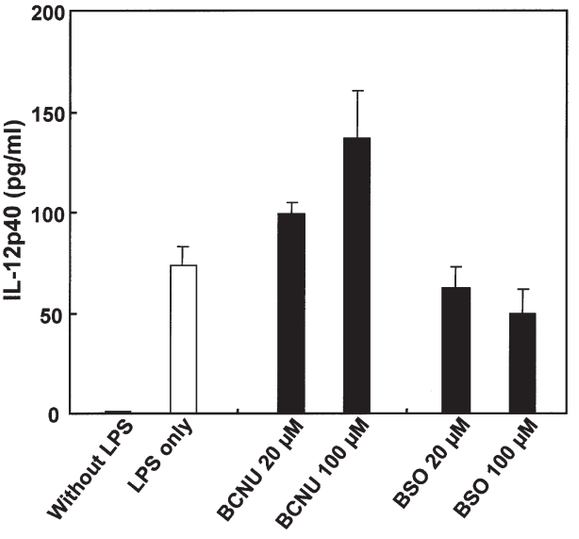

FIGURE 1 Effect of glutathione inhibitors on LPS-induced IL-12p40 production by J774A.1 cells. The cells were incubated with two different concentrations (absciassa) of BCNU and BSO for 36 h. During the final 12 h, the cells were stimulated with 300 ng/ml LPS. The results for IL-12p40 production are means ± SD from duplicate experiments. By way of comparison with LPS only, a *t*-test gave the following *P* values: 0.02 (BCNU 20 μM), 0.01 (BCNU 100 μM), 0.2 (BSO 20 μM), and 0.2 (BSO 100 μM).

Augmentation by BCNU is Dependent on LPS Concentration and Treatment Period

Because BCNU stimulated LPS-induced IL-12p40 production, its effect was analyzed further with respect to LPS concentration and incubation period. In an experiment using various concentrations of LPS (from 3 to 3000 ng/ml), augmentation of IL-12p40 production by BCNU was greater at higher concentrations of LPS (Fig. 2). An LPS concentration of 300 ng/ml was chosen for subsequent experiments. In the time-course study, J774A.1 cells were incubated with 100 μM BCNU for 36 and 12 h (24 and 0 h before LPS stimulation). Augmentation of IL-12p40 production was observed only when BCNU was present for the full 36 h prior to assay (Fig. 3). Indeed when present for only 12 h IL-12p40 production appeared to be inhibited.

Intracellular Glutathione Change

To assess the redox state of J774A.1 cells after BCNU treatment, intracellular glutathione content was measured using HPLC. J774A.1 cells were incubated

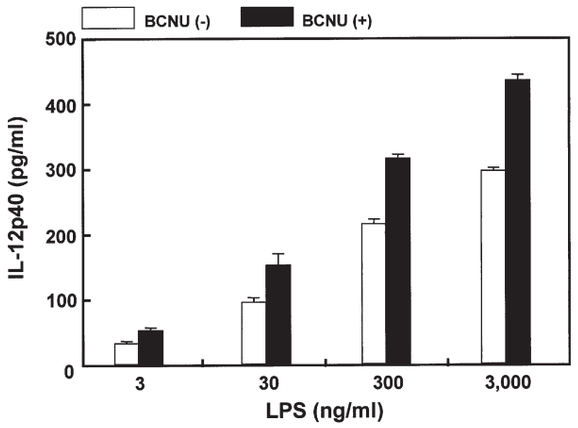

FIGURE 2 Effect of BCNU on IL-12p40 production by J774A.1 cells stimulated with various doses of LPS. Cells were incubated with 100 μM BCNU for 36 h. During the final 12 h, cells were stimulated with the indicated concentration of LPS. The results for IL-12p40 production are means ± SD from triplicate experiments. By way of comparison with the group of BCNU (−), a *t*-test gave the following *P* values; 0.002 (LPS 3 ng/ml), 0.004 (LPS 30 ng/ml), 0.00003 (LPS 300 ng/ml), and 0.000008 (LPS 3,000 ng/ml).

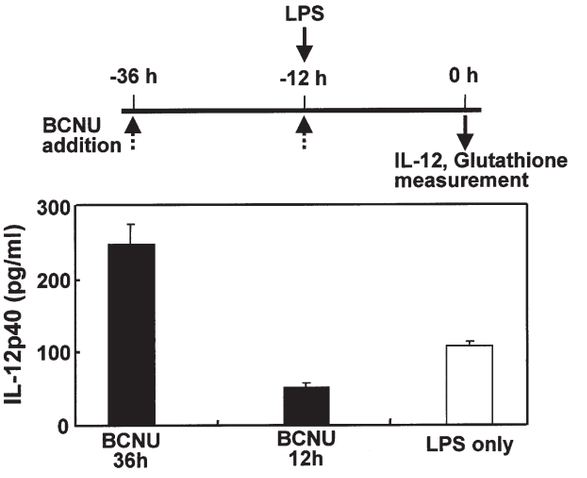

FIGURE 3 Time-course study of the effect of BCNU treatment on IL-12p40 production by J774A.1 cells. BCNU (100 μM) was added into the culture at the time indicated at the top of the figure, then the cells were stimulated with 300 ng/ml LPS for 12 h. The results for IL-12p40 production are means ± SD from duplicate experiments. In a comparison with LPS only *P* values obtained from a *t*-test were as follows: 0.0001 (12 h), and 0.001 (36 h).

with 100 μM BCNU for 36 h, and stimulated with 300 ng/ml LPS during the final 12 h. Although BCNU was expected to lower GSH and raise GSSG due to its inhibitory effect on glutathione reductase, both GSH and GSSG levels were elevated in the treated J774A.1 cells relative to control (LPS only) (Table I). The time-course study revealed that the increase in GSSG caused by BCNU treatment occurred later than the change in GSH. This resulted in a lower GSSG ratio (GSSG/total glutathione) after 12 h than at 36 h. The increase in the GSSG ratio at 36 and 12 h of BCNU treatment was 2.1 and 1.0-fold compared with the control (LPS only), respectively.

NF-κB Activation

The effect of glutathione inhibitors on NF-κB activation was analyzed. J774A.1 cells were incubated with BCNU or BSO for 36 h, during the final 12 h of which cells were stimulated with LPS. The nuclear extract was prepared and EMSA was performed. The specificity of the assay was confirmed by the use of non-labeled specific and irrelevant nucleotide competitors (Fig. 4A). BCNU treatment for 36 h augmented LPS-induced NF-κB activation; the augmentation was 1.6-fold higher than in controls (LPS only) (Fig. 4B). In contrast, BCNU treatment for 12 h and BSO treatment for 36 h did not change or rather inhibited NF-κB activation slightly (0.9-fold in both cases). In conclusion, the activation profile of NF-κB corresponded closely to the profile of both IL-12 production and the GSSG ratio in these studies.

DISCUSSION

Although it is known that activation of NF-κB is augmented by oxidative stress, and several recent studies have demonstrated that expression of the IL-12p40 gene is mediated by NF-κB,[11–14,18–21] there has not been a study in which the three key parameters involved in oxidative stress-induced IL-12 production have been simultaneously examined, namely cellular redox status, NF-κB activation and IL-12 production itself. The present study demonstrated that LPS-induced IL-12p40 production by a macrophage cell line, J774A.1, was augmented markedly by BCNU. This augmentation was associated with an increase in the ratio of oxidative to

TABLE I Effect of BCNU on intracellular glutathione content of J774A.1 cells

	BCNU 36 h	BCNU 12 h	LPS only
GSH (pmol/μg)	31.0 ± 1.3	25.0 ± 0.1	16.8 ± 0.7
GSSG (pmol/μg)	0.67 ± 0.06	0.26 ± 0.01	0.18 ± 0.02
GSSG ratio	0.021 ± 0.0010	0.010 ± 0.0003	0.010 ± 0.0006

Means ± SD for three independent experiments. *P* values obtained from a *t*-test for comparison with LPS only, were as follows: 0.002 (12 h) and 0.003 (36 h) for GSH; 0.01 (12 h) and 0.03 (36 h) for GSSG; 0.5 (12 h) and 0.002 (36 h) for GSSG ratio.

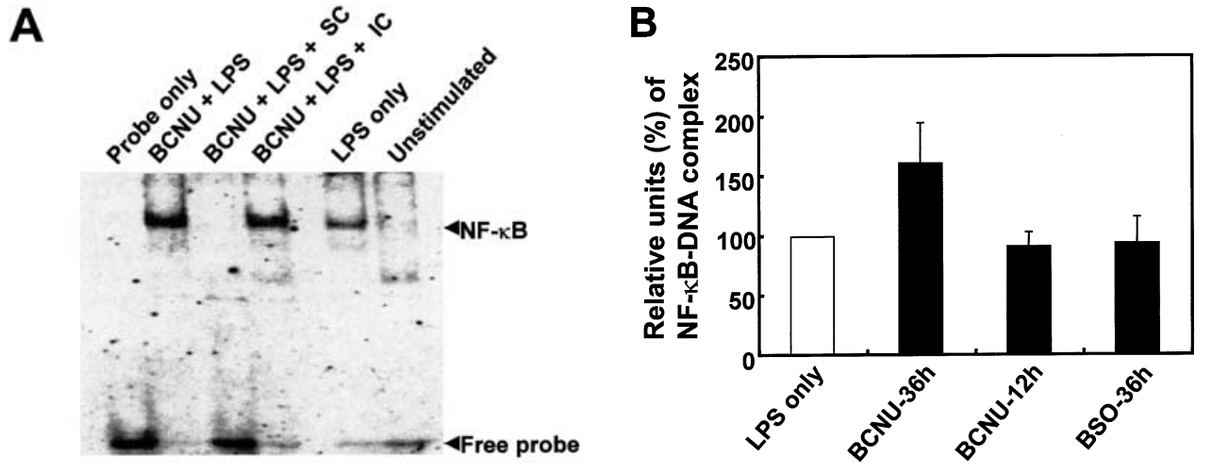

FIGURE 4 NF-κB activation of J774A.1 cells treated with glutathione inhibitors. (A) Cells were incubated with BCNU (100 μM) for 36 h, and in the presence or absence of 300 ng/ml LPS for the final 12 h. A nuclear extract was prepared from the cells and used for EMSA with a DIG-labeled NF-κB-specific probe, non-labeled NF-κB-specific oligonucleotide (specific competitor: SC), or non-labeled Oct2A-specific oligonucleotide (irrelevant competitor: IC). (B) Cells were incubated with BCNU (100 μM) and BSO (100 μM) for 36 h and then stimulated with 300 ng/ml LPS for the final 12 h. In the case of BCNU, 12 h incubations were also performed. The nuclear extract from the cells of each experiment was used for EMSA with a DIG-labeled NF-κB-specific oligonucleoitde. The chemiluminescent signals were detected by exposing the membrane to X-ray film. The film was quantified and the results were represented in relative units (%) of NF-κB-DNA complex. The results for NF-κB activation are means ± SD from four independent experiments. *P* values obtained from a *t*-test comparison with LPS only were as follows: 0.02 (36 h) and 0.1 (12 h) for BCNU and 0.3 (36 h) for BSO.

reduced forms of glutathione, a major redox-controlling thiol in the cell. The activation profile of NF-κB corresponded well to that of glutathione content and that of IL-12 production. Taken together these results indicate that oxidative modulation of the glutathione-redox couple augments NF-κB activation and results in stimulation of IL-12p40 production.

Notwithstanding the fact that BCNU is an inhibitor of glutathione reductase and was therefore expected to increase GSSG and decrease GSH in BCNU-treated J774A.1 cells, both GSH and GSSG were increased. Because glutamate-cysteine ligase, which is a key enzyme in GSH synthesis, has been shown to be activated by oxidative stress, it is possible that activation of this enzyme led to an increase in the GSH content.[31,32] In the time-course study of BCNU treatment, the increase in GSSG occurred later than that of GSH, and resulted in a lower GSSG ratio (GSSG/total glutathione) at 12 h than at 36 h of BCNU treatment. This suggests that oxidative stress induced by BCNU initially activated GSH synthesis and, subsequently, to maintain the intracellular redox state, glutathione peroxidase oxidized GSH to GSSG. GSSG probably accumulated in the cell due to the inhibition of glutathione reductase, resulting in the time-dependent increase of the GSSG ratio. In accordance with this increase, LPS-induced NF-κB activation and IL-12 production were augmented markedly after 36 h of BCNU treatment but not after 12 h of treatment. Thus, the results from the time-course study support the hypothesis that the higher GSSG ratio causes the observed NF-κB activation and IL-12p40 production.

Although BSO, an inhibitor of glutathione synthesis, could be considered a type of oxidative reagent, it suppressed both NF-κB activation and IL-12p40 production slightly. This result is consistent with reports demonstrating that a glutathione-depleting reagent, diethyl maleate, suppressed IL-12 (p40 + p70) production and led to a Th2-polarized immune response.[23,25,26] Because BSO almost completely depleted both GSH and GSSG in J774A.1 cells (data not shown), one possible explanation for the suppressive effect of BSO could be, suggested by Dröge *et al.*[33] that NF-κB requires GSSG for its translocation to nuclei. BSO recently has been shown to inhibit NF-κB activation through cytoplasmic accumulation of IκB.[34]

Because IL-12p40 is one of two subunits in the bioactive p70 (p35 + p40) heterodimer molecule, the augmentation of p40 production by modulation of the glutathione-redox couple would be expected to lead to augmented secretion of the p70 molecule that induces Th1-polarized immune responses. However, we were unable to confirm this in J774A.1 cells, because this cell line does not produce enough p35 mRNA or p70 heterodimer to be detectable by RT-PCR or ELISA, respectively, even after LPS stimulation.[29] Recently, p35 gene expression has been reported to be regulated by NF-κB,[35] which suggests that the expression of both p35 and p40, and therefore p70, is augmented by oxidative modulation of the glutathione redox couple. In a preliminary study using mouse peritoneal macrophages, we have

obtained results that support this idea. If this is the case, it would be important to examine whether p35 or p40 is more strongly affected by the redox state because the p35/p40 ratio will determine the ratio of p70-heterodimer/p40-homodimer and because the p40-homodimer is known to serve as an antagonist to the Th1-inducing activity of p70 by competitively binding to the IL-12 receptors of helper T cells.[9,10] Further studies to clarify the relationship between the glutathione-redox system, IL-12p40/p70 ratios, and the Th1/Th2 balance are currently in progress in our laboratory.

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