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_J Immunol_ published online 17 February 2010
http://www.jimmunol.org/content/early/2010/02/17/jimmunol.0900439

Supplementary Material  http://www.jimmunol.org/content/suppl/2010/02/15/jimmunol.0900439.DC1

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Glutathione-Redox Balance Regulates c-rel–Driven IL-12 Production in Macrophages: Possible Implications in Antituberculosis Immunotherapy

Kaiser Alam,* Sheikh Ghousunnissa,* Shiny Nair,* Vijaya Lakshmi Valluri, † and Sangita Mukhopadhyay*

The glutathione-redox balance, expressed as the ratio of intracellular reduced glutathione (GSH) and oxidized glutathione, plays an important role in regulating cellular immune responses. In the current study, we demonstrate that alteration of glutathione-redox balance in macrophages by GSH donors like cell-permeable glutathione ethyl ester reduced or N-acetyl-L-cysteine (NAC) can differentially regulate production of IL-12 cytokine in macrophages. A low concentration of NAC increased IL-12 p40/p70 production, whereas at high concentration, IL-12 production was inhibited due to increased calmodulin expression that binds and sequesters c-rel in the cytoplasm. Although NAC treatment increased the IκBα phosphorylation, it failed to increase TNF-α levels due to enhanced expression of suppressor of cytokine signaling 1, which specifically prevented nuclear translocation of p65 NF-κB. We demonstrate that NAC at 3 mM concentration could increase bacillus Calmette-Guérin–induced IFN-γ production by PBMCs from patients with active tuberculosis and shifts the anti–bacillus Calmette-Guérin immune response toward the protective Th1 type. Our results indicate that redox balance of glutathione plays a critical role in regulating IL-12 induction in native macrophages, and NAC can be used in tailoring macrophages to induce enhanced Th1 response that may be helpful to control tuberculosis and other pathophysiological disorders. The Journal of Immunology, 2010, 184: 000–000.
Materials and Methods

Isolation of monocyte-derived macrophages

Monocyte-derived macrophages (MDMs) were purified following the method as described earlier (19). Briefly, PBMCs were isolated from healthy volunteers by Ficoll-Hypaque (Sigma-Aldrich, St Louis, MO) density gradient centrifugation. Cells were washed and cultured in RPMI 1640 medium (Invitrogen, Grand Island, NY) supplemented with 10% FBS and antibiotics (Invitrogen). Macrophages were generated by culturing adherent monocytes in RPMI 10 supplemented with 0.1 ng/ml GM-CSF (Sigma-Aldrich) at 37˚C 5% CO2 for 1 wk. Medium was replaced with fresh RPMI-10 containing 0.1 ng/ml GM-CSF on alternate days.

Macrophage stimulation assay

The RAW 264.7 macrophages were obtained from the National Centre for Cell Science, Pune, India, and maintained in DMEM (Invitrogen) containing 10% FBS and antibiotics (DMEM-10). MDM or RAW 264.7 macrophages were plated at a density of 3 × 105 cells/ml and treated with various concentrations of N-acetyl-L-cysteine (NAC, Sigma-Aldrich) or glutathione ethyl ester reduced (GSH-OEt) (Sigma-Aldrich). Wherever electrophoretic transfer, the nitrocellulose membranes were incubated with 37˚C overnight. Nuclear extracts were prepared from nonidet P-40–lysed cells as described earlier (20). Briefly, at 2 h posttreatment with NAC (20 mM), cells were washed and mounted in Vectashield medium and imaged using an Nikon fluorescence microscope (Nikon DX1, Nikon, Tokyo, Japan). For checking suppressor of cytokine signaling 1 (SOCS1) expression, RAW 264.7 macrophages were treated with various concentrations of NAC. After 1 h, cells were fixed, permeabilized, and incubated with goat anti-SOCS1 Ab (Santa Cruz Biotechnology, Santa Cruz, CA) followed by incubation with anti-goat FITC (Sigma-Aldrich). Cells were washed and mounted in Vectashield medium and imaged using a Nikon fluorescence microscope (Nikon DX1, Nikon).

MTT assay

RAW 264.7 macrophages were either left untreated or treated with various concentrations of NAC and cultured as 5 × 104 cells/well in 96-well tissue culture plates in 200 µl volume of medium. After 48 h, MTT (Sigma-Aldrich) was added as 1 mg/ml and incubated further for 4 h (21). Cells were lysed overnight using 100 µl lysis buffer. The absorbance was determined at 570 nm.

IB analysis

For detecting total and phosphorylated IκBα and CaM levels, cells were lysed using lysis buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl2, 100 mM NaF, 100 mM sodium orthovanadate, and 1 mM EGTA) followed by centrifugation for 20 min at 14000 × g at 4˚C. Nuclear extracts were prepared from nonidet P-40–lysed cells as described earlier (20, 21) to detect the levels of p30, p65 and c-rel. Equal amounts of the extracts were separated by 10% SDS-PAGE. Following electrophoretic transfer, the nitrocellulose membranes were incubated with mouse Ab to CaM (Millipore, Bedford, MA) or rabbit Ab to either phosphorylated or total IκBα (Cell Signalling Technology, Beverly, MA) or rabbit Ab to p50 or p65 or c-rel transcription factor (Santa Cruz Biotechnology). Membranes were washed and incubated with either anti-mouse or anti-rabbit Ig coupled to HRP (Sigma-Aldrich). Bound enzyme was detected by ECL following the manufacturer’s protocol (GE Healthcare, Little Chalfont, U.K.) as described earlier (21). Equal loading of protein was confirmed by measuring the β-actin level in the same extracts by IB.

Flow cytometric evaluation of pp38 MAPK

The RAW 264.7 macrophages (1 × 106) were treated with various concentrations of NAC for 45 min and then fixed with 1.5% formaldehyde (Sigma-Aldrich). After washing, permeabilization was carried out with freshly prepared ice-cold methanol (90% for 30 min) on ice. Cells were washed twice in suspension buffer (0.5% BSA in PBS) and incubated with mouse Ab (100 times diluted in staining buffer) to pp38 MAPK (Cell Signalling Technology) for 30 min at 37˚C. Cells were washed in staining buffer and probed with anti–mouse–FITC conjugate (Sigma-Aldrich). Flow cytometry was carried out on BD FACSVantage SE (BD Biosciences). The post flow cytometric data were analyzed using CellQuest data analysis software (BD Biosciences).

EIA for measuring CaM level

An EIA method involving competitive binding of anti-CaM mAb was used to measure cytoplasmic CaM levels as described earlier (21, 22). Briefly, RAW 264.7 macrophages were either left untreated or treated with various concentrations of NAC for 1 h. In some experiments, RAW 264.7 macrophages were pretreated with 10 μM SB203580 for 30 min followed by treatment with 20 mM NAC. After 1 h, whole-cell extracts were prepared and incubated with mouse anti-CaM mAb (Milloi) at a ratio of 10:1 and transferred to EIA plates that were previously coated with recombinant CaM (Sigma). Postincubation for 2 h at 37˚C, plates were washed with PBS containing 0.05% (v/v) Tween-20 (Sigma-Aldrich) and incubated with goat anti-mouse Ig-HRP (Sigma-Aldrich) for 1 h. HRP activity was detected by using o-phenylenediamine tetrahydrochloride (Sigma-Aldrich) at 0.5 mg/ml in citrate-phosphate buffer (pH 5.4) containing 1 μl hydrogen peroxide (Qualigens, Mumbai, India). The reaction was terminated using 1 N H2SO4, and the absorbance values were measured at 490 nm. CaM levels in the test samples were expressed as the fold change over the untreated control (21).

Measurement of endogenous reactive oxygen species level

The intracellular reactive oxygen species (ROS) level was measured using a fluorescent dye, 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Sigma-Aldrich) as described earlier (23). Briefly, cells (3–5 × 104) were treated with either various concentration of NAC for 10–15 min or a fixed concentration of 3 mM NAC for various time periods. Cells were then incubated with 5 μM DCFH-DA for 15 min in dark. The stained cells were analyzed on a BD Biosciences flow cytometer (BD FACSVantage SE, BD Biosciences).

Immunoprecipitation assay

The immunoprecipitation (IP) assay was carried out as described earlier (21). Whole-cell extracts (200 µg/ml) prepared from various groups were incubated with 4 µg/ml target-specific Ab for 3 h at 4˚C. Fifteen microliters of protein A/G-Sepharose (Santa Cruz Biotechnology) was added to each preparation and incubated further for 2 h at 4˚C. Beads were washed extensively with IP buffer containing 25 mM HEPES (pH 7.3), 0.1 M NaCl, 10% glycerol, 0.05% Tween X-100, and 2 mM EDTA. The coimmunoprecipitated proteins were detected by Western blotting using appropriate combinations of primary and secondary Abs.

Transient transfection assay

The phosphorylation-defective IκBα (ΔNκBα) plasmid construct and dominant-negative mutant of p38 (pNp38) construct were kind gifts from Jurgen Heesemann (Max von Pettenkofer-Institut für Hygiene und Medizinische Mikrobiologie, Munchen, Germany) and Jiahuai Han (Scripps Research Institute, La Jolla, CA), respectively. Cells were transfected with 10 μg of the construct using Lipofectamine 2000 (Invitrogen) as described earlier (20). The control group was transfected with same amount of backbone vector alone. At 24 h posttransfection, cells were either left untreated or treated with 3 or 20 mM NAC, and IL-12 p40 was estimated after 48 h in the culture supernatants by EIA. In some experiments, RAW 264.7 macrophages were transfected with control small interfering RNA (siRNA) or SOCS1-specific siRNA (both from Santa Cruz Biotechnology) following the method as described by the manufacturer. After 24 h, cells were treated with medium or 3 mM NAC and harvested either after 1 h for measuring SOCS1 mRNA levels by semiquantitative RT-PCR or for detecting the nuclear p65 levels by Western blotting or cultured for 48 h to estimate TNF-α production by EIA and NO production by Griess reaction.

Nitrite estimation

The accumulated nitrite resulting from NO production by the stimulated macrophages in culture was measured using the Griess reaction (24, 25). The assay was performed in 96-well plates using equal volumes of Griess...
reagent (1% sulfanilamide and 0.1% naphthylethylenediamine [1:1] in 2.5% orthophosphoric acid) and sample. The plates were read at 550-nm absorbance. Nitrite concentrations were calculated based on a standard curve read from a prepared standard solution of sodium nitrite.

**Intracellular GSH and GSSG assays**

GSH and GSSG levels were measured by enzymatic recycling assay as described earlier (26). Briefly, RAW 264.7 macrophages (3 × 10⁶ cells/ml) were treated with varying concentrations of NAC for 1 h and suspended in ice-cold extraction buffer (1% Triton X-100 and 0.6% sulfosalicylic acid) and lysed by freezing and thawing. The extracts were centrifuged (10,000 × g) for 5 min at 4°C, and the supernatants were used to estimate GSH and GSSG levels. To measure total glutathione content, 50 μl of the samples as well as GSH (Sigma-Aldrich) or GSSG (Sigma-Aldrich) standards were added in the microtitre plate. A total of 100 μl glutathione assay mix solution containing 1.66 mg/ml NADPH (Sigma-Aldrich), 1.66 mg/ml 5,5′-dithio-bis-(2-nitrobenzoic acid) (Sigma-Aldrich) and 2 U/ml glutathione reductase (Sigma-Aldrich) were added, and then the rate of change in absorbance was measured at 405 nm. To assay GSSG, the GSH present in the sample was derivatized by adding 2 μl 2-vinylpiridine and 6 μl triethanolamine to a 100-μl aliquot of supernatant. After 1 h of incubation at 25°C, GSSG was measured in the same way as GSH was quantified. The amount of GSH and GSSG for each sample was determined from the standard curve.

**Lymphocyte proliferation assay**

Peripheral blood was obtained from patients with TB reported to the Directly Observed Treatment—Short-course Clinic of Mahavir Hospital and Research Centre, Hyderabad, India. The diagnosis of the patients with TB was confirmed by tuberculin skin test, radiographic examination, observation of acid-fast bacilli in sputum, and clinical symptoms. These patients were negative for HIV virus. The PBMCs from heparinized blood samples from patients with TB (n = 33) were isolated using gradient centrifugation in Ficoll-Hypaque (Sigma-Aldrich) solution as described elsewhere (27) and prepared at 2.5 × 10⁶ cells/ml in RPMI 1640 (Invitrogen) medium containing 10% FBS (Invitrogen) and antibiotics (RPMI-10). Cell suspensions (3 × 10⁶/200 μl/well) were dispensed into 96-well, flat-bottom microtitre plates (Nunc, Roskilde, Denmark) and maintained at 37°C in 5% CO₂ incubator. PBMCs from various groups were treated with a fixed concentration of NAC (3 mM) and cultured in the presence of BCG as recall Ag. After 4 d, culture supernatants were harvested for estimating TNF-α and IL-5 cytokines secreted in the culture supernatants by ELISA. T cell proliferation was measured by MTT assay after 4 d. The bioethics committee of Mahavir Hospital and Research Centre, Hyderabad, India. The diagnosis of the patients with TB was confirmed by tuberculin skin test, radiographic examination, observation of acid-fast bacilli in sputum, and clinical symptoms. These patients were negative for HIV virus. The PBMCs from heparinized blood samples from patients with TB (n = 33) were isolated using gradient centrifugation in Ficoll-Hypaque (Sigma-Aldrich) solution as described elsewhere (27) and prepared at 2.5 × 10⁶ cells/ml in RPMI 1640 (Invitrogen) medium containing 10% FBS (Invitrogen) and antibiotics (RPMI-10). Cell suspensions (3 × 10⁶/200 μl/well) were dispensed into 96-well, flat-bottom microtitre plates (Nunc, Roskilde, Denmark) and maintained at 37°C in 5% CO₂ incubator. PBMCs from various groups were treated with a fixed concentration of NAC (3 mM) and cultured in the presence of BCG (1 × 10⁷). In some experiments, macrophage population from PBMC was purified as described in Materials and Methods. The adherent cells were ~90% macrophages by staining. The macrophages were pretreated for 1 h with 3 mM NAC. The cells were washed and used as APCs. Proliferation assays were performed using purified T cells added at a concentration of 3 × 10⁵ cells/well with autologous macrophages added at 1 × 10⁵ cells/well. Cell suspensions (200 μl/well) were dispensed into 96-well, flat-bottom microtitre plates (Nunc) and cultured in the presence of BCG as recall Ag. After 4 d, culture supernatants were harvested for estimating TNF-α and IL-5 cytokines secreted in the culture supernatants by ELISA. T cell proliferation was measured by MTT assay after 4 d. The bioethics committee of Mahavir Hospital and Research Centre and Centre for DNA Fingerprinting and Diagnostics approved the current study, and informed consent was obtained from all the subjects.

**Statistical analysis**

Data were expressed as mean ± SD. Statistical comparisons were made using either Student t test or by one-way ANOVA followed by Tukey’s honestly significant difference (HSD) tests. The significance level was set at p < 0.05.

**Results**

**Intracellular glutathione-redox balance affects IL-12 p40/p70 induction in macrophages**

To examine the effect of intracellular glutathione-redox balance on cytokine production in macrophages, we treated RAW 264.7 macrophages with various concentrations of NAC, a known precursor of glutathione (21, 28) to alter the GSH and the GSSG concentrations and the GSH/GSSG ratio. The cells were treated with increasing concentrations (0.3–20 mM) of NAC for 1 h, and intracellular glutathione-redox status in these macrophages was quantified following enzymatic recycling assay. It was observed that treatment of NAC resulted in increased intracellular GSH in a dose-dependent manner up to a concentration of 3 mM (Fig. 1A), but the intracellular GSSG levels were not significantly changed within this range of NAC (Fig. 1B). However, higher concentrations of NAC (10 or 20 mM) increased GSSG levels by ~3- or 4-fold when compared with the medium-treated control macrophages (Fig. 1B). Intracellular glutathione-redox balance was indicated as GSH/GSSG (29). The GSH/GSSG balance showed predominantly a reducing state when macrophages were treated with NAC up to 3 mM due to increased levels of intracellular GSH (Fig. 1C, compare bar 4 with bar 1). However, the redox balance shifts toward more oxidizing state at higher concentrations of NAC due to an increase in intracellular GSSG level (Fig. 1C, compare bar 6 with bar 1).

Next, we investigated whether changes in the intracellular glutathione-redox balance in macrophages can modulate the levels of various innate cytokines like IL-12, IL-10, and TNF-α. IL-12 is known to be a heterodimeric protein of 70 kDa, composed of two subunits, IL-12 p35 and IL-12 p40, and regulation of biologically active IL-12 p70 is found to be dependent upon transcriptional regulation of the gene encoding the IL-12 p40 subunit (21, 30). We therefore treated RAW 264.7 macrophages with various concentrations of NAC, and at 48 h posttreatment, the levels of IL-12 p40, IL-12 p70, IL-10, and TNF-α were measured in the culture supernatants by ELISA. It was observed that NAC at low concentrations activated induction of IL-12 p40 (Fig. 2A) and IL-12 p70 (Fig. 2B). Maximum induction of IL-12 p40 (Fig. 2A) and IL-12 p70 (Fig. 2B) was observed at 3 mM NAC (Fig. 2A, 2B, compare bar 4 with bar 1). However, these levels were decreased with subsequent higher concentrations of NAC (i.e., 10–20 mM) (Fig. 2A, 2B, compare bars 5 and 6 with bar 4). The decrease was not due to cell cytotoxicity by higher concentrations of NAC used, because cell viability remained unchanged as revealed by MTT assay (Fig. 2C). The dose-dependent increase of IL-12 p40 was also reflected at the mRNA level, where maximum expression was observed in an environment rich in reduced glutathione (3 mM NAC) (Supplemental Fig. 1). Exogenous treatment of RAW 264.7 macrophages with GSH-OEt, a permeable form of glutathione, alters the intracellular GSH and GSSG levels in macrophages in dose-dependent manners (data not shown) (28) and similarly influences IL-12 p40 induction (Fig. 2D). These data indicate that IL-12 p40 induction is directly regulated by the glutathione-redox status. Pretreatment of RAW 264.7 macrophages with BSO, a pharmacological inhibitor of GSH synthesis (26), decreased GSH levels in macrophages treated with 3 mM NAC as expected (28) (Supplemental Fig. 2) and inhibited IL-12 p40 induction (Fig. 2E), confirming that GSH has a positive effect on IL-12 p40 induction. A dose-dependent effect of NAC on IL-12 p40 induction was also observed in peritoneal macrophages from BALB/c mice (data not shown) and monocyte-derived macrophages (Fig. 2F). These results indicate that the effect of NAC on IL-12 p40 transcription was not limited to transformed cell lines only. The same culture supernatants that were used to measure IL-12 p40 and IL-12 p70 levels (Fig. 2A, 2B) were checked for IL-10 and TNF-α production. There was no induction of TNF-α, although a slight increase in IL-10 was noticed only in the group treated with 20 mM NAC (Fig. 2G).

**Nuclear c-rel level is regulated by the macrophage’s glutathione-redox state**

Because the intracellular GSH/GSSG balance was found to influence IL-12 p40 induction, we next investigated the possible molecular mechanisms involved in such regulation. There are reports that indicated that the redox balance can affect rel family transcription factors to a large extent (31). Among the rel factors,
c-rel is known to play a central role in IL-12 p40 transcription (21, 32). Therefore, nuclear c-rel level was measured in cells treated with varying concentrations of NAC, and it was found to be markedly increased in the cells treated with 3 mM NAC (Fig. 3A, compare lane 4 with lane 1). However, nuclear c-rel levels were found to be decreased with higher NAC concentrations (Fig. 3A, compare lanes 5 and 6 with lane 4). The total c-rel level was not affected by the changes in the GSH/GSSG balance (data not shown). These data indicate that intracellular glutathione balance probably affects nuclear translocation of the c-rel transcription factor. The c-rel is known to be controlled upstream by the cytoplasmic IκBα, which sequesters c-rel in the cytoplasm (20, 33). During macrophage activation, IκBα is phosphorylated, releasing the cytoplasmic c-rel to translocate to the nucleus. Therefore, we examined whether intracellular glutathione-redox has a direct effect on the levels of phosphorylation and degradation of IκBα. Because we observed that NAC at 3 mM concentration increased IL-12 p40 expression (Fig. 2A), we speculated that NAC at this concentration would also increase the phosphorylation of IκBα and its subsequent degradation. In a pilot experiment, it was found that levels of IκBα phosphorylation were highest at 45–60 min post-NAC treatment (data not shown). Therefore, in the subsequent experiments, levels of IκBα phosphorylation were examined at 45 min after treating cells with various concentrations of NAC by IB. We found that IκBα

FIGURE 1. NAC modulates the intracellular glutathione-redox status in dose-dependent manner. The RAW 264.7 macrophages were treated with various concentrations of NAC for 1 h. Cells were harvested and lysed, and the intracellular concentrations (mean ± SD) of GSH (A), GSSG (B), and the GSH/GSSG (C) in all groups were quantified following enzymatic recycling assay. Data are representative of three independent experiments. p value was calculated by Tukey’s HSD test.

FIGURE 2. IL-12 induction in macrophages is regulated by the intracellular glutathione-redox status. The RAW 264.7 macrophages were treated with various concentrations of NAC. After 48 h, culture supernatants were collected to measure IL-12 p40 (mean ± SD) (A) and IL-12 p70 (mean ± SD) (B) levels. C, The cell cytotoxicity in these groups was measured by MTT assay. D, IL-12 p40 (mean ± SD) induction was examined in RAW 264.7 macrophages treated with cell-permeable GSH-OEt. E, IL-12 p40 (mean ± SD) induction in response to various concentrations of NAC was measured in RAW 264.7 macrophages pretreated with BSO, a pharmacological inhibitor of GSH synthesis. F, IL-12 p40 (mean ± SD) induction was quantified in MDMs treated with various concentrations of NAC. G, Induction of IL-10 (mean ± SD) and TNF-α (mean ± SD) in NAC-treated RAW 264.7 macrophages was measured by EIA. Data are representative of three independent experiments. p value was calculated by Tukey’s HSD test.
phosphorylation was increased by both 1 and 3 mM NAC (Fig. 3B, compare lanes 3 and 4 with lane 1), suggesting that intracellular GSH/GSSG balance at NAC concentration of 1–3 mM probably targets the IkBα phosphorylation and degradation to increase nuclear c-rel level (Fig. 3A) and IL-12 p40 induction (Fig. 2A). To confirm this hypothesis, we next transfected RAW 264.7 macrophages with phosphorylation-defective IkBα (ΔIkBα) plasmid construct or with backbone vector (pRC/CMV), and IL-12 p40 induction was measured in these macrophages posttreatment with 3 mM NAC. It was observed that NAC at 3 mM concentration failed to increase IL-12 p40 in ΔIkBα-transfected cells (Fig. 3C, compare bar 4 with bar 2), indicating that IkBα plays a role in upregulation of IL-12 p40 in the presence of 3 mM NAC.

It is interesting to note that although nuclear c-rel and IL-12 p40 were upregulated in a macrophage environment rich in GSH (treated with 3 mM NAC), their levels were decreased in a macrophage environment rich in GSSG (i.e., when treated with 10 and 20 mM NAC). Therefore, we speculated that the resultant decrease in the level of IL-12 p40 was due to inhibition of IkBα phosphorylation by NAC at these concentrations. However, levels of IkBα phosphorylation were actually increased in these cells (Fig. 3D, compare lanes 3 and 4 with lane 1). These results hint that the oxidized state of glutathione probably regulates nuclear c-rel levels by targeting different signaling cascades in macrophages.

CaM is involved in the regulation of nuclear c-rel and IL-12 p40 by GSSG

CaM protein, a highly conserved, ubiquitously expressed, intracellular sensor for calcium (34), is known to interact with IkBα-released c-rel and inhibit its nuclear transport (21, 35). Therefore, we speculated that macrophages rich in GSSG may also have higher levels of CaM. To validate this hypothesis, RAW 264.7 macrophages were treated with various concentrations of NAC for 1 h, and levels of CaM were measured by EIA (21) as well as IB. It was found that CaM expression was increased in macrophages treated with 10 and 20 mM NAC as compared with the group treated with medium alone (Fig. 4A, bars 4 and 5, 4B, lanes 5 and 6). CaM expression was not affected by 3 mM NAC (Fig. 4A, bar 3, Fig. 4B, lane 4), indicating that GSSG plays an important role in increasing the CaM level in macrophages. Next, we examined whether CaM was directly involved in the suppression of nuclear c-rel and IL-12 p40 by the intracellular GSSG. RAW 264.7 macrophages were treated with 20 mM NAC in the absence or presence of 5 μM TFP, a known pharmacological inhibitor of CaM activity (36). It was found that both c-rel (Fig. 4C, compare lane 4 with lane 2) and IL-12 p40 (Fig. 4D) levels were increased in cells treated with 20 mM NAC along with TFP as compared with the macrophages treated with 20 mM NAC alone. These data clearly indicate that GSSG targets the CaM signaling to inhibit c-rel translocation to the nucleus, and, consequently, IL-12 p40 gene transcription levels are reduced. To further confirm involvement of CaM in such regulation, levels of c-rel bound to CaM were directly measured by IP. Whole-cell extracts were prepared from RAW 264.7 macrophages treated with medium or 3 mM or 20 mM NAC and were immunoprecipitated with anti-CaM Ab and then IB with anti-c-rel Ab. We found that an increased amount of c-rel was coimmunoprecipitated in the group treated with 20 mM NAC as compared with groups treated with either medium or 3 mM NAC (Fig. 4E). These observations indicate a direct role of CaM in the GSSG-mediated regulation of IL-12 p40. Recently, we demonstrated that ROS play important roles in the redox-mediated regulation of CaM expression (21). Therefore, we speculated that NAC at 20 mM concentration probably increased ROS levels, which in turn downregulated nuclear c-rel and IL-12 p40. To test this, RAW 264.7 macrophages were treated with 20 mM NAC, and ROS production was measured at various time points by flow cytometry. We found that NAC at 20 mM concentration decreased intracellular ROS levels in a time-dependent manner (Fig. 5A),

**FIGURE 3.** Nuclear c-rel level is regulated by the glutathione-redox balance of macrophages. RAW 264.7 macrophages were treated with varying concentrations of NAC as indicated. A, Nuclear extracts were prepared, and c-rel levels in the nuclear extracts were measured by IB using anti-c-rel Ab. B, The total and phosphorylated IkBα in the groups treated with 0.3, 1, and 3 mM NAC for 45 min were measured by IB using total and phosphorylated anti-IkBα Ab. C, IL-12 p40 (mean ± SD) induction was measured in RAW 264.7 macrophages transfected with either ΔIkBα or pRC/CMV. D, IB analysis of total and phosphorylated IkBα in macrophages treated with 10 and 20 mM NAC for 45 min. p value was calculated by Student t test.
which could be due to its direct quenching effect. Results shown in Fig. 5 indicated that NAC inhibited intracellular ROS in a dose-dependent manner. These data indicate a ROS-independent mechanism of IL-12 p40 regulation by GSSG via CaM–c-rel signaling.

In the previous section, we have observed that NAC at higher concentrations (10 and 20 mM) increased CaM levels independent of ROS, suggesting possible involvement of some other stress-related signaling pathways in such regulation. Recently, we reported that one of the signaling pathways in regulating IL-12 p40 upstream of CaM could be the p38 MAPK (37). Therefore, we assumed that upregulation of CaM expression by GSSG could be due to activation of the p38 MAPK pathway. To test our assumption, first we determined the time point at which 20 mM NAC induced the highest levels of phosphorylation of p38 MAPK, and a treatment of 45 min was found to be optimum (data not shown). Next, we compared the levels of phosphorylated p38 MAPK in macrophages treated with various concentration of NAC for 45 min, and it was found that p38 MAPK phosphorylation was significantly increased only when NAC was used at 10 mM and 20

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**FIGURE 4.** CaM plays an important role in the GSSG-mediated regulation of c-rel and IL-12 p40. RAW 264.7 macrophages were treated with various concentrations of NAC. A, After 1 h, cells were harvested, and whole-cell extracts were prepared. CaM levels (mean ± SD) in the cell extracts were determined by EIA as described in Materials and Methods and expressed as the fold changes over unstimulated control. B, CaM levels in these groups were measured also by Western blotting using anti-CaM Ab. In another experiment, macrophages were treated with 20 mM NAC in the absence or presence of 5 μM TFP (a known pharmacological inhibitor of CaM activity). The nuclear c-rel was measured by Western blotting using anti-c-rel Ab (C), and the intracellular IL-12 p40 levels were measured by immunofluorescence microscopy (D). Again, the whole-cell extracts prepared from 3 mM and 20 mM NAC were incubated with anti-CaM Ab for 3 h at 4°C, and then protein A/G-Sepharose was added to the mixture and was further incubated for 2 h at 4°C. Coimmunoprecipitated c-rel was detected by Western blotting using anti-c-rel Ab (E, top lane). The same preparation was also used to detect CaM level using anti-CaM Ab (E, middle lane). Data are representative of at least three experiments.

**FIGURE 5.** NAC inhibits intracellular ROS in concentration-dependent manner. RAW 264.7 macrophages were treated with either 3 mM NAC for various time points (A) or with various concentrations of NAC for a fixed time point of 10–15 min (B). Cells were then incubated with 5 μM DCFH-DA for 15 min in dark, and the stained cells were analyzed on a BD Biosciences flow cytometer. Data are representative of four independent experiments.
mM concentrations (Fig. 6A). These data indicate that p38 MAPK phosphorylation increases in macrophages when the intracellular GSSG levels are higher. To corroborate direct involvement of p38 MAPK in the regulation of CaM as well as IL-12 p40 in macrophages rich in GSSG, we used SB203580, a pharmacological inhibitor of p38 MAPK (37). Accordingly, RAW 264.7 macrophages were left untreated or pretreated with 10 μM SB203580 for 30 min and then treated with 20 mM NAC. Nuclear c-rel levels were measured after 1 h by IB, and intracellular IL-12 p40 expressions were checked after 6 h by immunofluorescence microscopy. Levels of CaM in whole-cell extracts were measured after 1 h by EIA. It was found that the presence of SB203580 increased nuclear c-rel (Fig. 6B, compare lane 4 with lane 2) as well as intracellular IL-12 p40 (Fig. 6C) expression in macrophages even treated with 20 mM NAC. SB203580 decreased the CaM level in macrophages even in the presence of 20 mM NAC (Fig. 6D), indicating that p38 MAPK plays an important role in the GSSG-mediated regulation of CaM expression in macrophages, which subsequently controls IL-12 induction. To further confirm the regulatory role of p38 MAPK on CaM signaling in macrophages rich in GSSG, DNp38 was used. RAW 264.7 macrophages were transfected either with backbone vector (pCDNA3) or with DNp38 plasmid, and at 24 h posttransfection, both the groups were treated with 20 mM NAC. It was observed that there was an upregulation of nuclear c-rel (Fig. 6E, compare lane 4 with lane 2) as well as IL-12 p40 (Fig. 6F, compare bar 4 with bar 2) in the DNp38-transfected group as compared with the group transfected with the pCDNA3 backbone vector alone. These results support the view that an increase in GSSG levels in macrophages resulted in activation of p38 MAPK, in turn upregulating CaM expression, which subsequently downregulated IL-12 p40 induction by directly binding and sequestering c-rel in the cytoplasm.

**FIGURE 6.** p38 MAPK is involved upstream of CaM in the regulation of c-rel and IL-12 p40 in RAW 264.7 macrophages rich in GSSG. A. RAW 264.7 macrophages were treated with 3, 10, and 20 mM NAC for 45 min, and p38 MAPK phosphorylation in each group was measured by flow cytometry using phosphorylated anti-p38 MAPK Ab. Nuclear c-rel (B), IL-12 p40 (C), and CaM (mean ± SD) (D) levels were compared in groups treated with 20 mM NAC in the absence or presence of 10 μM SB203580. In another experiment, RAW 264.7 macrophages were transfected with either the DNp38 or the backbone vector pCDNA3. Cells were treated with 20 mM NAC and either harvested after 1 h to examine the nuclear c-rel by Western blotting (E) or cultured for 48 h to measure IL-12 p40 (mean ± SD) by EIA (F). Data are representative of three independent experiments. p value was calculated by Student t test.

It is known that the c-rel as well as other NF-κB factors like p50 and p65 are all bound to the IκBα in the cytoplasm and are released to translocate to the nucleus after IκBα gets phosphorylated and degraded (20, 38–40). Because increased phosphorylation and degradation of IκBα was noticed at 3 mM NAC concentration (a situation with a higher intracellular GSH/GSSG ratio), it was expected that similar to c-rel (Fig. 3A), more p50 and p65 NF-κB would be available in the nucleus to start transcription of the NF-κB-dependent genes like TNF-α and inducible NO synthase (iNOS) (23–25, 41). However, we found that NAC at 3 mM concentration did not activate TNF-α (Fig. 2G). We therefore next examined the mechanism responsible for specific suppression of TNF-α and NO but not IL-12 in these macrophages. It is known that SOCS1 negatively regulates NF-κB activation by specifically targeting p65 NF-κB through proteasome-mediated degradation (42). SOCS1 was found to directly interact with p65 and enhanced its ubiquitination presumably by its ubiquitin ligase-like activities. However, the p50 levels remain largely unaffected. Because NAC treatment augmented IL-12 synthesis, but not TNF-α (largely regulated by p65), we speculated that NAC probably induces SOCS1, which in turn negatively affects nuclear translocation of p65 NF-κB by virtue of its preferential targeting of p65 to the proteasome (42). Therefore, we next examined the levels of SOCS1 upon treatment with 3 mM NAC (Fig. 7A) or 5 mM GSH-OEt (Supplemental Fig. 3), and we found that these treatments increased SOCS1 expression levels. Coimmunoprecipitation assay using anti-SOCS1 Ab clearly revealed that SOCS1 strongly interacted with the p65 subunit of NF-κB (Fig. 7B), and, as a result, nuclear p65 levels were barely detectable (Fig. 7C). However, SOCS1 did not show significant interactions with p50 NF-κB and
activates SOCS1 that prevents p65 NF-κB (Figs. 3A, 12 (Fig. 2B) c-rel (Fig. 7B), making them available for nuclear translocation (Figs. 3A, 7C) and to activate c-rel/p50-dependent genes like IL-12 (Fig. 2A). Therefore, it appears that NAC/GSH-OEt specifically activates SOCS1 that prevents p65 NF-κB from translocation to the nucleus without affecting c-rel and p50 NF-κB. As a result, p65-dependent TNF-α and iNOS genes (23–25, 41) were not induced in these macrophages, whereas c-rel/c-rel or c-rel/p50-dependent genes like IL-12 (20) were induced.

This was further confirmed by gene-silencing experiments using SOCS1-specific siRNA. The RAW 264.7 macrophages were transfected with control siRNA (RAW-control) or SOCS1-specific siRNA (RAW-SOCS1si), and after 24 h, the cells were treated with 3 mM NAC and either harvested after 1 h to check the SOCS1 mRNA level by RT-PCR and the nuclear p65 level by Western blotting or cultured for 48 h to measure TNF-α and NO production in the culture supernatants. It could be observed that depletion of SOCS1 by SOCS1-specific siRNA (Supplemental Fig. 4) resulted in an increased nuclear p65 level in the RAW-SOCS1si group as compared with RAW-control group when treated with 3 mM NAC (Fig. 8A, compare lane 4 with lane 2). Concomitantly, an increase in the production of TNF-α as well as NO was also observed in RAW-SOCS1si group as compared with the RAW-control group (Fig. 8B, 8C, compare bar 4 with bar 2). Collectively, these results indicate that SOCS1 induction is increased in RAW 264.7 macrophages treated with 3 mM NAC, which prevents nuclear translocation of IkBα-released p65 NF-κB but not p50 or c-rel. This explains the possible mechanisms by which 3 mM NAC upregulates IL-12 but not TNF-α or NO production in these macrophages.

Anti-BCG Th1 response in PBMCs from TB patients is increased by 3 mM NAC

It has been shown that the T cell responses in patients with TB with active infection are mostly skewed toward the Th2 type, which favors intracellular survival of the M. tuberculosis bacilli (17, 43). We found in this study that 3 mM NAC increased IL-12 induction in macrophages. IL-12 is known to favor the Th1 response. Therefore, we assumed that NAC at 3 mM concentration might act as a Th1 adjuvant and activate the Th1 response in patients with TB. We therefore checked whether the anti-BCG Th1 response was increased by NAC in PBMC cultures obtained from patients with active TB infection. PBMCs from 43 patients with TB were incubated with Mycobacterium bovis BCG in the absence or presence of 3 mM NAC. After 96 h, culture supernatants were harvested to measure the levels of IFN-γ and IL-5 by EIA and T cell proliferation by MTT assay. We found that NAC increased T cell proliferation to BCG (Fig. 9Ai) with a tendency of increased IFN-γ (Fig. 9Aii) and decreased IL-5 (Fig. 9Aiii) induction. In our initial experiments, we found that 3 mM NAC increased IL-12 p40 in MDM and PMA-differentiated THP-1 macrophages stimulated with BCG (data not shown). To critically assess the possible role of macrophages in the activation of Th1 response by 3 mM NAC, we isolated macrophages from individual patients with TB and incubated for 2 h with medium or 3 mM NAC. The cells were washed to remove any external NAC and used as APCs. The purified T cells were cultured with autologous macrophages (as APCs) pretreated with medium or NAC (3 mM) along with BCG. After 96 h, culture supernatants were tested for the levels of IL-5 and IFN-γ cytokines. The data revealed that the anti-BCG T cell response was downregulated (Fig. 9Bi) and was skewed toward the Th1 type with higher production of IFN-γ (Fig. 9Bii) and decreased production of IL-5 (Fig. 9Biii) in the groups that received NAC-treated macrophages as APCs compared with the groups receiving medium-treated macrophages as APCs. These results demonstrate that NAC (3 mM) can be used as an immunomodulator to activate Th1 response in patients with TB.

Discussion

Intracellular glutathione homeostasis plays a major role in the maintenance of intracellular redox environment and regulates a number of critical cellular functions (44). One of the initial events in many inflammatory responses is the depletion of intracellular GSH (44). Growing evidence suggests that an altered cellular redox has a profound role in inflammation through the activation of various kinases and redox-sensitive transcription factors such as NF-κB and p50 proteins, which differentially regulate the genes encoding various proinflammatory cytokines (44, 45). The mechanisms by which GSH and GSSG balance regulate the induction of various proinflammatory cytokines remains unclear. In the current study, we demonstrate that the GSH/GSSG redox balance could modulate IkBα signaling and the levels of CaM expression in macrophages, which subsequently influences the nuclear c-rel translocation and thereby regulates the levels of IL-12. Although some previous studies reported that in LPS-activated macrophages IL-12 p40 expression can be regulated by GSH ethyl esters by involving the JNK pathway (46), we found that glutathione-redox–mediated regulation of IL-12 in native unstimulated macrophages involves p38 MAPK and CaM protein.

FIGURE 7. NAC targets the SOCS1 to regulate nuclear translocation of p65 NF-κB in RAW 264.7 macrophages. A, RAW 264.7 macrophages were either left untreated or treated with 0.3, 1, and 3 mM NAC for 1 h. The cells were washed and checked for SOCS1 expression levels by immunofluorescence microscopy. B, In another experiment, macrophages were either left untreated or treated with 3 mM NAC for 1 h. The whole-cell extracts were prepared. The cell lysates were immunoprecipitated (IP) with goat anti-SOCS1 Ab, and the precipitates were immunoblotted (IB) with rabbit anti-p50 or anti-p65 or anti–c-rel Ab. C, The p50 and p65 levels were measured in the nuclear extracts prepared from macrophages either left untreated or treated with 0.3, 1, and 3 mM NAC for 1 h by Western blotting using anti-p65 or anti-p50 Ab. Data are representative of five independent experiments.
To study the effect of glutathione-redox balance in resting macrophages, we used NAC, a very widely used cell-permeable antioxidant that is converted into GSH by γ-glutamylcysteine synthetase (47). We found that NAC, at lower concentrations (up to 3 mM), steadily increased the GSH levels without significantly affecting its conversion to its oxidized form, GSSG. However, when NAC was used at higher concentrations (10–20 mM), there was a significant conversion of GSH to GSSG, resulting in a low GSH/GSSG ratio. Therefore, it appears that in unstimulated macrophages, NAC at low concentrations acts as an antioxidant and at higher concentrations, it turns out to be a pro-oxidant. Interestingly, such a concentration-dependent role reversal is not

**FIGURE 8.** Specific knockdown of endogenous SOCS1 by siRNA increases nuclear translocation of p65 and production of TNF-α and NO in RAW 264.7 macrophages treated with 3 mM NAC. RAW 264.7 macrophages were transiently transfected with control siRNA or SOCS1-specific siRNA and after 24 h, cells were treated with medium alone or with 3 mM NAC. Cells were harvested either after 1 h to measure nuclear levels of p65 by Western blotting using anti-p65 Ab (A) or cultured for 48 h to measure production of TNF-α (mean ± SD) by EIA (B) and NO (mean ± SD) by Griess reaction (C). Data are representative of three independent experiments. $p$ value was calculated by Student $t$ test.

**FIGURE 9.** Cell proliferation and IFN-γ response of PBMCs from patients with TB to BCG is increased by NAC. PBMCs (3 × 10^5/well) harvested from patients with TB ($n=33$) were incubated with BCG (1 × 10^5/well) in the absence or presence of 3 mM NAC. After 4 d, cell proliferation was measured by MTT assay (Ai). The induction of IFN-γ (Aii) and IL-5 (Aiii) in the culture supernatants of all the patients was measured by EIA. In another experiment, macrophages were purified from individual patients with TB ($n=10$) and pretreated with 3 mM NAC. Cells were washed and cultured (1 × 10^5/well) with autologous nylon wool-purified T cells (3 × 10^5/well) in the presence of BCG (1 × 10^5/well). After 4 d, culture supernatants were harvested, and cell proliferation was measured by MTT assay (Bi). The levels of IFN-γ (Bii) and IL-5 (Biii) in various culture supernatants were measured by EIA. Each circle represents an individual patient. $p$ value was calculated by Student $t$ test.
GLUTATHIONE-REDOX REGULATES c-rel AND IL-12 PRODUCTION

rare for NAC. In TNF-α–stimulated endothelial cells, NAC could attenuate TNF-α–induced ICAM-1 expression at low concentrations, but at higher concentrations, ICAM-1 expression was enhanced (47). Interestingly, in several in vivo studies, a high dose of NAC was found to act as a pro-oxidant rather than an antioxidant. High doses of NAC were found to be pro-oxidative in function in healthy rat striatum (48). Similarly, a low dose of NAC protected rats against endotoxin-mediated oxidative stress, whereas a high dose increased their mortality presumably because of its pro-oxidant action (49). NAC at high doses was also found to act as a pro-oxidant, as it could decrease the GSH levels and increase the GSSG levels in healthy human subjects (50). It will be pertinent to mention in this paper that NAC can also act as pro-oxidant in the presence of certain compounds like vitamin B12b (51). Nevertheless, NAC can act as pro-oxidant or antioxidant depending on the pre-existing redox state. Pretreatment of NAC was found to counteract the ethanol-induced oxidative stress in liver; however, on the contrary, posttreatment was found to aggravate the liver injury, as NAC behaved as a pro-oxidant in such circumstances (52). Similarly, pretreatment of NAC was able to protect LPS-induced intrauterine fetal death and growth retardation, whereas posttreatment aggravated these situations as a pro-oxidant (53). Therefore, it appears that whether NAC may act as a pro-oxidant or antioxidant depends on some factors like concentration and the existing redox status. As we have used unstimulated cells in our experiments, which already have an existing pool of GSH, addition of NAC at high concentration is likely to shoot the cellular GSH levels abruptly high, leading to production of some kind of cellular stress that probably rapidly converted part of the large GSH pool into GSSG and thus skewed the GSH/GSSG ratio toward a pro-oxidant state. Although NAC is not known to change the expression levels of genes involved in the glutathione recycling, but is known to alter the activities of these enzymes in TNF-α–stimulated endothelial cells (47), it may be possible that the activities of these enzymes are differently altered by different concentrations of NAC in unstimulated cells, leading to such biphasic activities of NAC.

When we examined the effect of NAC-regulated glutathione-redox on the production of macrophage innate cytokines, it was found that a high GSH/GSSG ratio, induced by lower concentrations of NAC, significantly increased IL-12 production. However, at higher concentrations, IL-12 production is significantly inhibited, and NAC did not have any apparent cytotoxic effect at these higher concentrations. Interestingly, the levels of TNF-α did not significantly change in all of the concentrations of NAC used. The concentration-dependent regulation of IL-12 by NAC was found to be due to differential nuclear translocation of c-rel, which is known to be critical for IL-12 transcription (20) despite increased IκB phosphorylation in all of the concentrations of NAC used. The differential nuclear c-rel levels could be attributed to an increased level of CaM protein only at higher concentrations of NAC. Recently, it has been shown that CaM can interact and regulate the activities of IκBα-released NF-κB family members by differentially regulating nuclear localization of c-rel and p65, as CaM-bound p65 could localize into the nucleus, whereas CaM-bound c-rel is sequestered and retained in the cytoplasm (35). We found that an GSSG-rich environment imparted by treating the macrophages with high concentrations of NAC could result in an increased level of cytoplasmic CaM protein, which in turn sequesters the c-rel to prevent its nuclear translocation. Earlier, we demonstrated a link between the CaM expression and IL-12 p40 regulation (21). To the best of our knowledge, in this study, we report for the first time that a changing GSH/GSSG balance can alter the levels of CaM protein and thereby affect the levels of IL-12 p40 in native macrophages. The CaM protein was found to be regulated by the redox-balance through the p38 MAPK, as we demonstrated earlier (37).

Interestingly, although there was increased IκBα phosphorylation in all of the concentrations of NAC used, the levels of TNF-α remained largely unchanged. The c-rel as well as the other NF-κB factors like p50 and p65 are known to be sequestered by the IκBα in the cytoplasm and are released to translocate to the nucleus upon phosphorylation of IκBα (38–40). Therefore, we expected an increase in the nuclear levels of p50 and p65 NF-κB factors along with c-rel as observed. However, we found that NAC could increase the nuclear levels of p50, but the p65 levels were barely detectable under identical conditions. This observation probably explains our inability to detect TNF-α, whose transcription is largely controlled by p65 (23, 41). Because SOCS1 is known to negatively regulate NF-κB activation by specifically targeting p65 through proteasome-mediated degradation (42), we speculated a direct role of SOCS1 in NAC-mediated inhibition of nuclear translocation of p65. SOCS1 was found to directly interact with p65 and enhanced its ubiquitination, presumably by its ubiquitin ligase-like activities. Because NAC treatment (3 mM) augmented IL-12 synthesis but not TNF-α, we assumed that NAC probably induces SOCS1, which in turn negatively affects nuclear translocation of p65 by virtue of its preferential targeting of p65 to the proteasome (42). Therefore, when we examined the levels of SOCS1 in the presence of NAC, we found that SOCS1 expression levels were increased, which was found to strongly interact with the p65 subunit of NF-κB, preventing its nuclear translocation. The role of SOCS1 was further confirmed by gene-silencing experiments using SOCS1-specific siRNA. We found that silencing of SOCS1 in RAW 264.7 macrophages enhanced both the nuclear p65 level and production of TNF-α as well as NO in the presence of 3 mM NAC. Therefore, also, glutathione-redox specifically attenuates p65-dependent activation of TNF-α and iNOS/NO in macrophages through specific activation of SOCS1. Therefore, glutathione-redox appears to play a pivotal role in regulating macrophage signaling involving the CaM and SOCS1 to differentially regulate various NF-κB family-dependent cytokines.

Interestingly, there has been intense debate regarding the exact effect of NAC on NF-κB activity. It is reported that 20 mM NAC downregulates basal NF-κB DNA binding in RAW 264.7 macrophages and also inhibits LPS-stimulated NF-κB DNA binding in these cells (54). In contrast, few studies indicate that NAC fails to inhibit LPS-induced activation of NF-κB in RAW 264.7 macrophages (55); yet in other studies, NAC has been shown to inhibit activation of NF-κB in alveolar macrophages induced by TNF-α (56). A role of IκB kinase α is also proposed in the NAC-mediated regulation of NF-κB signaling in TNF-α–stimulated endothelial cells (47). These apparent discrepancies in the role of NAC in regulating NF-κB signaling could be explained by the fact that NAC behaves differently when used at different concentrations as well as differences in redox status. Also, NAC may specifically induce different sets of genes depending on the type and redox status of the cell. The complex interactions of these gene products probably dictate the course of NF-κB–mediated signaling, whether it be activated or inhibited. In fact, our data at least partly explain how NAC can differentially regulate the nuclear translocation of different members of the NF-κB family and therefore promote transcription of IL-12 and TNF-α differently. A detailed study in the upstream signaling cascades may be helpful for better understanding the differential effect of NAC on NF-κB–dependent signaling in macrophages activated with various stimulators.

In summary, our data underscore the critical importance of glutathione-redox status in regulating the induction of innate
cytokines in macrophages. This can interestingly constitute a point of hijacking the host immune response by the intracellular macrophage pathogens like *M. tuberculosis*. IL-12 is known to activate the Th1 cell immune response (30) crucial for inducing protection against the intracellular pathogens like *M. tuberculosis* (57). Therefore, it is possible that these pathogens modulate the GSH/GSSG balance in macrophages, which could be initiated by receptor triggering during host-pathogen interaction and thus polarize the immune environment to its favor by modulating the IκBα-Ca2+ signaling pathway. Th1 immune response is known to be suppressed in patients with active TB infection (17). Interestingly, studies also indicate that patients with TB have an altered glutathione balance (18). In this case, our results demonstrate that NAC, when used at 3 mM concentration, could improve anti-BCG Th1 response in PBMC cultures obtained from patients with active TB. Therefore, NAC-mediated manipulation of intracellular glutathione balance can be used to devise immune therapeutics to improve the anti-BCG Th1 response in patients with TB as well as the BCG-based immunotherapy in cancer treatment (58). This study also provides possible insights into devising strategies to pharmacologically regulate IL-12 induction by manipulating GSH and GSSG levels in macrophages. However, it should be used with caution for immunotherapeutic applications, as higher concentrations may lead to development of undesired or opposite effects.

**Acknowledgments**

We thank Niteen Pathak for technical assistance in flow cytometry and Dr. Sudip Ghosh (National Institute of Nutrition, Hyderabad, India) for valuable suggestions.

**Disclosures**

The authors have no financial conflicts of interest.

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