MATERIALS
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MATERIALS AND METHODS:

Materials
Monoclonal anti-TNF-α antibody, N-acetyl-cysteine (NAC), SN50 (an inhibitor of NF-κB) and its analogue SN50/M, Epigallo-catachin-3-gallaterum, Bovine Serum Albumin, Coomassie Brilliant Blue G-250 and R-250, p-nitrophenyl phosphate, anti-human IgG alkaline phosphate conjugate, Tween-20, dithiothreiotol, phenylmethylsulphonyl fluoride, standard protein markers, protein A sepharose CL-4B, sodium azide, ethidium bromide, chloroform, isoamyl alcohol, reduced glutathione, glutathione reductase, cumene hydroperoxide and reduced β-nicotinamide adenine dinucleotide phosphate were from Sigma Chemical Company, U.S.A. SN50 is a hybrid peptide containing nuclear localization sequence of p50 subunit of NF-κB heterodimer and has been shown to completely inhibit the translocation of NF-κB in human cell lines at 100 μg/ml (Lin et al., 1995).

Ficoll-Paque was from Pharmacia, Piscataway, NJ, U.S.A. Middlebrook 7H9 broth, Middlebrook ADC enrichment fluid, RPMI-1640 medium were from HiMedia, India. MTT cell viability assay kit and immunoassay kits for TNF-α were of R & D Systems, U.S.A. 12-wells tissue culture plates were obtained from Techno Plastic Products (TPP), Switzerland. Polystyrene microtitre flat bottom ELISA plates having 96 wells (7 mm diameter) were from NUNC, Denmark. A colorigenic substrate p-nitrophenyl phosphate was obtained from C.S.I.R. Centre for Biochemicals, New Delhi. Asparagine, citric acid, magnesium sulphate, dipotassium hydrogen phosphate, ferric ammonium citrate and glycerol were obtained from Qualigens, India. Sodium pyruvate was obtained from SRL, India. All other chemicals were of highest grade available.

Study subjects:
For isolation of peripheral blood mononuclear cells (PBMC's) or serum, venous blood was obtained from patients with systemic lupus erythematosus (SLE),
tuberculosis (TB) and SLE-TB admitted or attending O.P.D. at J.N. Medical College Hospital of A.M.U, Aligarh. Also, for some experiments involving in vitro infection with pathogenic strain of MTB for using the infected cells as valuable antigens / inhibitors in immunoassays, we obtained healthy nonsmoking adult volunteers with no history of tuberculosis or positive tuberculin skin test. The SLE sera showed high titer anti-DNA antibodies and fulfilled the American College of Rheumatology (formerly American Rheumatism Association) revised criteria for the classification of SLE (Arnett et al., 1988). Serum samples were decomplemented by heating at 56°C for thirty minutes and stored in small aliquots at -20°C until use with 0.1 percent sodium azide as preservative.

Exclusion criteria
Patients who have already received steroids/cyclophosphamide and other immunosuppressant as well as patients having hepatitis, septicemia, multi organ failure, were excluded from the study.

Methods

Determination of protein concentration:
Protein was estimated by the methods of Lowry et al. (1951) and Bradford (1976) (data not shown).

(A) Protein estimation by the Lowry (Folin-Ciocalteau) method:
Protein estimation by this method involves complexing of the protein's peptide bonds with Cu^{2+} under alkaline conditions (Lowry et al., 1951). The resultant Cu^{+} appears to catalyze the oxidation of tyrosine and tryptophan residues by reducing phosphomolybdotungstate anions in the Folin reagent (a mixture of sodium tungstate, molybdate and phosphate), added subsequently. This reaction develops a blue colour due to the formation of heteropolymoiybdenum blue, which can be quantified by its absorbance at 660 nm.

Reagents:

(i) Folin-Ciocalteau reagent
The reagent was diluted 1:4 with distilled water before use.

(ii) Alkaline copper reagent
The components of alkaline copper reagent were prepared as follows:

(a) 2% sodium carbonate in 100 mM NaOH
(b) 0.5% copper sulphate in 1% sodium potassium tartarate

The working reagent was prepared fresh before use by mixing the two components in the ratio 50:1, respectively.

Procedure:
To 1.0 ml of protein sample was added 5.0 ml of freshly prepared alkaline copper reagent. After thorough mixing, the reaction mixture was allowed to stand at room temperature for 10 minutes, followed by the addition of 1.0 ml of 1:4 times diluted Folin-Ciocalteau reagent. The contents were mixed immediately. The reaction was allowed to proceed for 30 minutes at room temperature and each tube was subsequently monitored at 660 nm. The protein content of the unknown sample was determined by using bovine serum albumin to construct a standard calibration curve.

(B) Protein estimation by the Bradford method:
This method is based on strong binding of the dye Coomassie Brilliant Blue G-250, in acidic medium, to protein hydrophobically and at positively charged groups (Bradford, 1976). In the environment of these positively charged groups, protonation is suppressed and a blue color develops ($\lambda_{max}$=595 nm).

Preparation of dye:
100 mg of Coomassie Brilliant Blue G-250 was dissolved in 50 ml of 95% ethanol and 100 ml of 85% (v/v) orthophosphoric acid was added. The resulting solution was diluted to a final volume of 1.0 litre and filtered through Whatman No. 1 filter paper to remove undissolved particles.
Procedure:
To 1.0 ml of solution containing 10–100 μg protein was added 5.0 ml of dye solution. The contents were mixed thoroughly by vortexing. The absorbance was read at 595 nm after 5 minutes against a reagent blank.

Polyacrylamide gel electrophoresis of proteins:
Polyacrylamide gel electrophoresis was performed under denaturing conditions as described by Laemmli (1970) (data not shown as serum samples from patients were analyzed in comparison to normal controls for preliminary experiments).

Reagents:
(i) Acrylamide-bisacrylamide (30:0.8)
A stock solution of 30% acrylamide containing 0.8% bisacrylamide was prepared by dissolving 30 gm of acrylamide and 0.8 gm of bisacrylamide in a total volume of 100 ml. The solution was stored at 4°C in an amber coloured bottle.

(ii) Resolving gel buffer
A stock solution was prepared by dissolving 36.3 gm Tris base in 48 ml of 1 N HCl. The contents were mixed, pH adjusted to 8.8 and the final volume brought to 100 ml with distilled water.

(iii) Stacking gel buffer
6.05 gm Tris was dissolved in 40 ml distilled water, pH adjusted to 6.8 with 1 N HCl and the final volume adjusted to 100 ml with distilled water.

(iv) Electrode buffer
3.03 gm Tris, 14.4 gm glycine and 1.0 gm SDS were dissolved in distilled water, pH adjusted to 8.3 and the final volume made up to 1.0 litre with distilled water.

(v) Sample buffer
(a) 6.0 gm of Tris was dissolved in 80 ml distilled water and pH adjusted to 6.8 with phosphoric acid. The final volume was brought to 100 ml with distilled water.

(b) 1.0 mg of bromophenol blue and 12.5 ml of glycerol were added to 12.5 ml of the above solution. β-mercaptoethanol was added just before use.

Recipe for 10–20% Gradient Gel

<table>
<thead>
<tr>
<th>Reagents</th>
<th>10%</th>
<th>20%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide-bisacrylamide (30:0.8)</td>
<td>5.0 ml</td>
<td>10 ml</td>
</tr>
<tr>
<td>Resolving gel buffer</td>
<td>3.8 ml</td>
<td>3.8 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>150 μl</td>
<td>150 μl</td>
</tr>
<tr>
<td>10% Ammonium persulphate</td>
<td>50 μl</td>
<td>50 μl</td>
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<tr>
<td>TEMED</td>
<td>10 μl</td>
<td>10 μl</td>
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Resolving Gel (total volume: 30 ml)

<table>
<thead>
<tr>
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<th>20%</th>
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</thead>
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<tr>
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<td>50 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 μl</td>
<td>10 μl</td>
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</table>

The final volume was raised to 15 ml each with distilled water.
**2.5% Stacking Gel (total volume: 10 ml)**

<table>
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<th>Acrylamide-bisacrylamide (30:0.8)</th>
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<tbody>
<tr>
<td>Stacking gel buffer</td>
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<tr>
<td>10% SDS</td>
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</tr>
<tr>
<td>10% Ammonium persulphate</td>
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<td>25 μl</td>
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</table>

The final volume was raised to 10 ml with distilled water.

**Recipe for 7.5% SDS-PAGE (total volume: 10 ml)**

<table>
<thead>
<tr>
<th>Acrylamide-bisacrylamide (30:0.8)</th>
<th>2.5 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolving gel buffer</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>100 μl</td>
</tr>
<tr>
<td>10% Ammonium persulphate</td>
<td>50 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 μl</td>
</tr>
</tbody>
</table>

The final volume was raised to 10 ml with distilled water.

**Procedure:**

The glass plates (18 cm x 16 cm) were soaked in chromic acid and thoroughly washed with tap water followed by a final rinse with distilled water and ethanol. The plates were dried and sealed with 1% agarose and 1.5 mm thick spacers. The reagents were mixed and poured between the glass plates. The resolving gel was allowed to polymerize at room temperature, following which, the stacking gel was layered on top. A well-forming comb was inserted immediately and the gel was left to polymerize at room temperature. In case of gradient gels, a gradient of resolving gel was formed with the help of a gradient former (Bio-Rad, model 385). After ensuring complete polymerization, the protein samples (25–100 μg) in one-fourth volume of sample buffer were electrophoresed at 80 volts.
at room temperature. The gels were stained using 0.25% Coomassie Brilliant Blue R-250 or with silver stain reagent.

**Silver staining:**

Silver staining was done by the method of Merril et al. (1983). Briefly, the gel was incubated in 40% methanol and 12% acetic acid for 45 minutes followed by incubation in 50% ethanol for 30 minutes. Next the gel was treated with 0.02% hypo (sodium thiosulphate) for 1 minute. After washing with distilled water, the gel was placed in 0.2% silver nitrate (with 0.05% v/v formaldehyde), washed again with distilled water, and transferred to a 6% solution of sodium carbonate (with 0.05% v/v formaldehyde). After colour development, the gel was washed with distilled water and treating the gel with 3% v/v acetic acid and 5% v/v methanol arrested the reaction. All the reagents used in this procedure were freshly prepared.

**Agarose gel electrophoresis:**

Agarose gel was prepared by bringing 2% agarose to molten state in electrophoresis buffer (0.04 M Tris acetate, pH 8.0 containing 0.002 M EDTA). Molten agarose was poured on the gel tray and allowed to solidify for 1 hour at room temperature. The nucleic acid samples in one-tenth volume of stop-mix (30% ficoll, 0.025% xylene cyanole FF and 500 mM EDTA in 10X TAE buffer) were electrophoresed for 2–4 hours at 30 mA in the same buffer. The gel was stained with ethidium bromide (0.5 µg/ml).

**Isolation of IgG by affinity chromatography:**

Protein A sepharose CL-4B was employed as the affinity matrix. It was swelled in 10 mM PBS, pH 7.4 at room temperature for 12 hours. The swelled matrix was washed with PBS and packed in a column having a dimension of 0.9 cm x 5 cm. The packed column was washed with 0.1 M sodium citrate, pH 3.0 in order
to elute any bound material. This was followed by equilibration of the packed matrix with 5 volumes of PBS, pH 7.4. Serum diluted with equal volume of PBS, pH 7.4 was loaded onto the column and allowed to bind at a flow rate of 20 ml/hour. Unbound protein was eliminated with PBS and absorbance of the effluent monitored till a negative absorbance was obtained at 280 nm. The bound IgG was eluted with 0.58% acetic acid in 0.85% sodium chloride. To prevent the effect of acidic elution buffer on IgG, fractions were collected in 1 M Tris-HCl, pH 8.5. The fractions containing IgG were monitored at 280 nm. The IgG concentration was determined considering 1.4 OD_{280} = 1.0 mg IgG/ml. The isolated IgG was dialyzed against 10 mM PBS, pH 7.4 and stored at -20°C with 0.1% sodium azide. To check the purity of IgG, the samples were subjected to 7.5% SDS-PAGE.

**Gel retardation assay:**
The binding of TB-IgG with *M. tuberculosis* sonic extract proteins as well as with MTCF proteins was analyzed by altered electrophoretic mobility on SDS-PAGE under non-reducing conditions. *M. tuberculosis* proteins were allowed to interact with TB-IgG for 2 hours at 37°C and overnight at 4°C. This resulted in the formation of immune complex (IC). Thereafter, the complex was electrophoresed on 7% SDS-PAGE under non-reducing conditions for 2 hours at 80 V. The electrophoresed gels were visualized by staining with 0.25% Coomassie Brilliant Blue R-250.

**Enzyme-linked immunosorbent assay (ELISA):**
Antibodies were detected and quantified by ELISA using polystyrene flat-bottom microtitre plates as solid phase. The method described by Alam et al (1992) and Islam and Ali (1998) was followed for the assay.

**Buffers and reagents:**
(i) Tris-buffered saline (TBS)
10 mM Tris, 150 mM NaCl, pH 7.4
(ii) Tris-buffered saline Tween-20 (TBS-T)
20 mM Tris, 144 mM NaCl, 2.68 mM KCl and 1.0 ml/litre Tween-20, pH 7.4

(iii) Bicarbonate buffer
15 mM sodium carbonate, 35 mM sodium bicarbonate, pH 9.6

(iv) Substrate buffer (for anti-human IgG alkaline phosphatase conjugate)
15 mM sodium carbonate, 35 mM sodium bicarbonate and 2 mM magnesium chloride, pH 9.6
Substrate: 0.5 mg/ml of p-nitrophenyl phosphate

(A) Direct binding ELISA:
Polystyrene microtitre plates were incubated with 100 µl of protein antigen (30 µg/ml in carbonate/bicarbonate buffer, pH 9.6) for two hours at room temperature followed by overnight incubation at 4°C. The plates were washed thrice with TBS-T and unoccupied sites blocked by 150 µl of BSA (1.5% in TBS, pH 7.4) for 4–6 hours at room temperature. Serially diluted sera in TBS were added to antigen-coated as well as control (antigen uncoated) wells. The antigen-antibody interaction was allowed to proceed for two hours at room temperature followed by overnight incubation at 4°C and subsequently the plates were washed four times with TBS-T in order to remove the unbound antibodies. Bound antibodies were assayed by means of appropriate anti-immunoglobulin alkaline phosphatase conjugate using p-NPP as substrate. The reaction was stopped with 3 N NaOH and the absorbance of each well was monitored at 405 nm on an ELISA microplate reader. Each sample was coated in duplicate and the results were expressed as a mean of A_{test} – A_{control}.

(B) Inhibition ELISA:
The antigen binding specificity of antibody was determined by inhibition experiments (Hasan et al., 1991). Varying concentration of inhibitors (0–20
μg/ml) were mixed with a constant amount of antiserum or IgG. The mixture was incubated for two hours at 37°C followed by overnight incubation at 4°C. The resulting immune complex was employed in the immunoassay instead of serum. The rest of the steps were as in direct binding ELISA. The results were expressed as percent inhibition.

\[
\text{Percent inhibition} = (1 - \frac{A_{\text{inhibited}}}{A_{\text{uninhibited}}}) \times 100
\]

Preparation of mycobacteria:

Virulent laboratory-adapted \( M. \text{tuberculosis} \) (H37Rv) were grown in Middlebrook 7H9 broth supplemented with Middlebrook ADC enrichment fluid at 37°C in 5% CO₂. Mycobacterial cultures were harvested at mid-logarithmic (14 days) phase. Aliquots of the stock were kept at −70°C until use. The viability of the stock remained >99% at 1 year. Before use, aliquots were defrosted, then vortexed with glass beads for 15 minutes, followed by equilibration at 37°C for 45 minutes. This treatment results in single cell suspension of mycobacteria (Toossi et al., 1996).

Isolation of mycobacterial antigens:

Mid-logarithmic mycobacterial cultures (14 days) were heat-killed at 100°C for one hour in a boiling water bath, followed by centrifugation at 10,000 rpm for 30 minutes at 4°C. The culture filtrate containing secreted proteins was separated and stored at −20°C until further use.

(A) Desalting of \( M. \text{tuberculosis} \) culture filtrate (MTCF) proteins:

The supernatant was sterilized by filtration through a 0.22 micron pore size membrane (Millipore Corp., USA). MTCF was concentrated 50 fold by
ammonium sulphate and dialyzed against 10 mM PBS, pH 7.4 for desalting. The protein content was determined by the method of Lowry et al. (1951) against a BSA standard. The culture filtrate preparations were stored in small aliquots at −20°C.

(B) *M. tuberculosis* sonic extract (MTSE) proteins:
Heat-killed bacilli were washed with sterile TBS, pH 7.4 and suspended in sonicating buffer containing 1 mM DTT, 1 mM PMSF, 1.0 μl/ml β-mercaptoethanol and proteinase inhibitors cocktail in TBS, pH 7.4. Sonicating at 4°C for 15 minutes in a sonicator disrupted the cell suspension and the cell debris was removed by ultra centrifugation at 20,000 rpm for 15 minutes. The protein content was determined by the method of Lowry et al. (1951). The sonic extract preparations were stored at −20°C in small aliquots until required.

**Preparation of RPMI-1640 medium:**

Dehydrated RPMI-1640 medium of one unit vial (16.3 gm) was suspended in 950 ml of tissue culture-grade water at room temperature with constant, gentle stirring until the medium was completely dissolved. The container was rinsed with tissue culture grade water to remove all traces of powder and added to the above solution. 3.7 gm sodium bicarbonate was added to the medium and stirred until dissolved. The final volume was brought to 1000 ml with tissue culture grade water. The medium was sterilized immediately by filtering through a sterile membrane filter with a porosity of 0.22 micron using positive pressure rather than vacuum to minimize the loss of carbon dioxide, and stored at 4°C till use.

**Preparation of PBMC:**

**Step 1:** In order to isolate peripheral blood mononuclear cells (PBMCs), 60 ml of blood was drawn from a healthy volunteer into 60 cm³ syringes containing
3.8 units heparin/ml. The heparinized blood, in 15 ml aliquots, was transferred to sterile 50 ml polypropylene centrifuge tubes and diluted 1:1 with sterile 10 mM, PBS, pH 7.4 at room temperature, followed by gentle mixing by inverting the tube a few times.

**Step 2:** Diluted blood was under layered with 15 ml of Ficoll-Paque at room temperature using an 18 gauge spinal needle. Care was taken to prevent mixing of the layers. The gradient was centrifuged at 1800 rpm for 30 minutes at room temperature with the centrifuge brake turned off.

**Step 3:** Using a sterile pipette, the upper clear layer containing plasma was removed. The PBMCs appeared as a dense white band (buffy layer) above the red blood cells and granulocytes layer. This was removed with another sterile pipette. The banded cells were combined in 10 ml aliquots.

**Step 4:** Ten milliliters of banded PBMCs were diluted with 25 ml of PBS in sterile 50 ml polypropylene centrifuge tubes and centrifuged at 1100 rpm for 12 minutes at room temperature to remove platelets, which remain in the supernatant. The PBMC pellets were combined to four tubes, diluted in 30 ml PBS and centrifuged at 1100 rpm for 10 minutes at room temperature. This wash was repeated.

**Step 5:** The pellets were then combined and resuspended in 30 ml complete medium (CM) (RPMI-1640 medium containing 2 M L-glutamine, 25 mM HEPES, and no antibiotics). An aliquot was diluted 20-fold and counted using a hemocytometer under a light microscope using 10x ocular and 40x objective.

**Preparation of autologous serum for monocyte culture:**

From the same donor, 30 ml of blood was drawn without anticoagulant and transferred to serum separator tubes. The blood was allowed to clot for at least 30 minutes, then centrifuged at 3000 rpm for 15 minutes at room temperature.
and the serum filtered through a sterile 0.22 µm filter unit. Autologous serum can be stored for a year or longer at −20°C.

Cell culture:

PBMCs (5x10^6 cells/well) were added in 12-wells tissue culture plates in complete RPMI-1640 medium, and were subsequently incubated at 37°C, 5% CO₂ for 1–2 hours for adherence. Thereafter, non-adherent cells were removed by washing the plates extensively 4 times with RPMI-1640 medium. The adherent monocytes were cultured in RPMI-1640 supplemented with 2% autologous serum, followed by overnight resting at 37°C, 5% CO₂. This population of adherent cells is up to 95% monocytes, as observed by cytostaining and is 39% viable (Toossi et al., 1996). Prior to infection, the plates were washed twice with RPMI-1640 medium.

Infection and co-culture of monocytes with supplements:
Healthy monocytes were infected with *M. tuberculosis* (H₃₇Rv) at 1:1 (bacteria/cell) in 30% autologous unheated serum for 90 minutes at 37°C, 5% CO₂. Subsequent to this, the infected monocytes were washed four times with complete medium. Cells harvested at this time point were considered as time zero after infection (t₀). Other cultures received RPMI-1640 medium with 2% autologous serum. As per experimental design, some cultures, immediately after infection received 10 ng/ml anti-TNF-α antibodies, varying doses of EGCG (0-25 µg/ml), 10 nM reduced glutathione, 10 mM NAC, 100 µg/ml SN50 and SN50/M. Cultures were then harvested after 24 hours and cells were lysed in 0.5 ml of TRIZOL Reagent (Invitrogen Inc. Carlsbad, CA, USA) for RNA extraction or in protein lysate buffer for other studies. The cell-free culture supernatants were kept at −70°C.

Treatment with EGCG monocytes viability assay:
The effect of EGCG (0-25 µg/ml) on the viability of monocytes from patients with SLE, TB and SLE-TB was assessed by using MTT Cell Viability Assay Kit (R & D Systems) according to the manufacture’s instructions provided.
Reagents supplied in the kit:

<table>
<thead>
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<th>Component</th>
<th>Quantity</th>
<th>Storage conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTT reagent</td>
<td>25 ml</td>
<td>2 – 8°C</td>
</tr>
<tr>
<td>Detergent reagent</td>
<td>250 ml</td>
<td>18 – 24°C</td>
</tr>
</tbody>
</table>

Assay procedure:
Adherent monocytes were gently scraped with RPMI-1640 medium. After this, monocytes (3 x 10^4 cells/well in 100 µl) were added in 96-well tissue culture plates. Cells were incubated in RPMI-1640 with 2% autologous serum containing varying doses of EGCG for 24 hours at 37°C, 5% CO₂. After 24 hours, 10 µl of MTT reagent (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) was added to each well and incubation was continued for an additional two hours. When a purple precipitate was clearly visible under the microscope, 100 µl of detergent reagent was added to all wells, including control wells and incubated for two hours in the dark at 20°C. After incubation, the precipitate was solubilized and the absorbance of the resulting solution was measured at 570 nm using a microplate reader. Control cells were treated exactly the same except that no EGCG added to the wells. The percentage of viable cells was calculated by the formula as described by Islam et al. (2000) and the results are expressed as "Viable cells (% of control cells)".

\[
\text{Viable monocytes (% of control cells)} = \frac{\text{Absorbance value of control cells}}{\text{Absorbance value of treated cells}} \times 100
\]

Trypan blue exclusion assay for monocytes viability:
Adherent monocytes were gently scraped with RPMI-1640 medium. Trypan blue suspension (1.6 mg/ml in saline solution) was added to the monocytes at a final concentration of 0.8 mg/ml. The cells were kept at 37°C for 7 minutes in a CO₂ chamber (5%), mounted on a hemocytometer and then observed under light
microscope. The cells taking up Trypan blue (dead cells), and cells excluding the dye (viable cells) were counted. Percentage of viable cells was calculated by the following formula:

\[
\text{% Cell viability} = \frac{\text{Total viable cells (unstained)}}{\text{Total cells (stained plus unstained)}} \times 100
\]

**TNF-α Immunoassay**

The concentration of TNF-α in various culture supernatants as well as in serum of patients was determined by use of a commercial ELISA Kit (R & D Systems). This assay employed the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for TNF-α was available pre-coated onto a microplate. Standards and samples were pipetted into the wells and the immobilized antibody bound any soluble TNF-α present. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TNF-α was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and colour developed in proportion to the amount of TNF-α bound in the initial step. The colour development was stopped and the intensity of the colour was measured.

**Reagents supplied in the kit:**

**TNF-α microplate** - 96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against TNF-α.

**TNF-α conjugate** - 21 ml of polyclonal antibody against TNF-α conjugated to horseradish peroxidase, with preservatives.

**TNF-α standard** - 10 ng of recombinant human TNF-α in a buffered protein base with preservatives, lyophilized.

**Assay diluent RD1F** - 6 ml of a buffered protein base with preservatives. It contained a precipitate and was mixed well before and during use.
Calibrator diluent RD6-35 - 21 ml of animal serum with preservatives.

Wash buffer concentrate - 21 ml of a 25-fold concentrated solution of buffered surfactant with preservatives.

Colour reagent A - 12.5 ml of stabilized hydrogen peroxide.

Colour reagent B - 12.5 ml of stabilized chromogen (tetramethylbenzidine).

Stop solution - 6 ml of 2 N sulphuric acid.

Plate covers - 4 adhesive strips.

Working reagents:

Wash buffer - 20 ml of wash buffer concentrate was diluted into deionized or distilled water to prepare 500 ml of wash buffer.

Diluted calibrator diluent RD6-35 - 20 ml of calibrator diluent RD6-35 was mixed with 80 ml of deionized or distilled water to yield 100 ml of diluted calibrator diluent RD6-35.

Substrate solution - Colour reagents A and B were mixed together in equal volumes within 15 minutes of use to form substrate solution. It was protected from light.

TNF-α standard - TNF-α standard was reconstituted with 1.0 ml of distilled water. This reconstitution produced a stock solution of 10,000 pg/ml. The standard was allowed to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Assay procedure:

50 µl of assay diluent RD1F was added to each well of 96 well polystyrene microplate coated with a mouse monoclonal antibody against TNF-α. Thereafter, 200 µl of standards, samples, or control per well was added, covered with the adhesive strip provided and incubated for 2 hours at room temperature. The plate was washed four times by filling each well with wash buffer using a squirt bottle.
After washing, 200 µl of TNF-α conjugate was added to each well, covered with a new adhesive strip and incubated for 1 hour for cell culture supernatants and 2 hours for serum samples at room temperature. After four washings with wash buffer, 200 µl of substrate solution was added to each well and incubated for 20 minutes at room temperature in the dark, a blue colour appeared. Thereafter, 50 µl of stop solution was added to each well to stop the reaction. Then the absorbance of each well was determined within 30 minutes, using a microplate reader set to 450 nm. The cut off or lower limit of sensitivity was 4.4 pg/ml.

Glutathione peroxidase assay

The activity of glutathione peroxidase (GPx) was measured as described elsewhere (Mohandas et al., 1984; Mates et al., 1999). The oxidized glutathione (GSSG) produced during GPx reaction was immediately reduced by NADPH and glutathione reductase. Therefore, the rate of NADPH consumption was regarded as the rate of GSSG formation during the GPx reaction. Patients as well as healthy control monocytes were co-cultured for 24 hours with or without 10 mM NAC, 100 µg/ml SN50, 100 µg/ml SN50/M and 0-25 µg/ml EGCG. Thereafter, cells were gently scraped with lysis buffer containing protease inhibitors (50 mM Tris/HCl, pH 7.4; 1 mM EDTA; 500 mM PMSF). The cell suspension was homogenized with a sonicator on ice and centrifuged at 10,000 rpm for 10 minutes. Protein concentrations of supernatants were determined by the method of Bradford with BSA as the standard, and were subjected to GPx activity determination. The reaction mixture (1.0 ml) containing 50 mM potassium phosphate (pH 7.0), 1 mM sodium azide, 2 mM GSH, 0.2 mM NADPH, 1 unit/ml glutathione reductase, 1.5 mM cumene hydroperoxide, and 20–100 µl of samples were incubated at 25°C for 5 minutes. The reaction was initiated by the addition of cumene hydroperoxide. The kinetic change was spectrophotometrically recorded at 340 nm (37°C) for 3 minutes. GPx activity was calculated after
subtraction of the blank value, as μmol of NADPH oxidized/minute/mg protein (U/mg protein).

**Intracellular Glutathione Assay.**

Glutathione (GSH) levels in treated or control monocytes were assayed by spectrophotometry, using a GSH assay kit (Calbiochem) as previously described by us (Hasan et al., 2007). Monocytes were mixed with equal volume of ice cold 5% metaphosphoric acid and centrifuged at 3000 rpm for 15 min. Supernatants were used for GSH assay, as per the manufacturer's instruction.

**Assay for Malondialdehyde (MDA) levels.**

As a matter of fact, free radicals, because of their unstable and transient nature are difficult to measure directly. Their tendency to cause lipid peroxidation has been used as an indirect measure. Hence estimation of lipid peroxides (markers of lipid peroxidation), was carried out by measuring MDA (Malondialdehyde) as described earlier (Philpot, 1963).

**Principle:** One Molecule of MDA reacts stoichiometrically with two molecules of thiobarbituric acid (TBA) at pH 3.5. The pink color chromogen can be measured spectrophotometrically at 532 nm.

**Procedure:** For assay 1 ml of serum was mixed with 2.5 ml of 20% trichloroacetic acid (TCA) and 1 ml of 0.67% of aqueous solution of TBA. The mixture was heated for 30 minutes in boiling water bath, the pink pigment was extracted with 2 ml of n-Butanol and its absorbance was read at 532 nm against n-butanol as blank:

\[
\text{OD of sample} \times \text{Conc. of standard MDA} = \frac{\text{MDA}}{\text{OD of standard}}
\]
RNA extraction

After lysis of monocytes in 0.5 ml TRIZOL Reagent, the cell lysates were agitated with glass beads to complete cell wall disruption. After cooling on ice the tubes were again subjected to repeated disruption as above. The tubes were cooled and 200 µl of chloroform was added to each sample, followed by vortexing for 2 minutes, and centrifugation at 3000 rpm for 5 minutes. Samples were then transferred to fresh eppendorf tubes and centrifuged at 14,000 rpm for 15 minutes at 4°C. The aqueous layer was harvested and transferred to a fresh tube. After addition of 100 µl Cleanascite (CPG Inc., Lincoln Park, NJ, USA), samples were gently rocked for 10 minutes and then centrifuged at 14,000 rpm for one minute. The aqueous layer obtained was mixed with 500 µl of chloroform-isoamyl alcohol (24:1) and vortexed. RNA was precipitated using 50 µl of 1 M sodium acetate, and 475 µl of isopropanol at -20°C for 3 hours in the presence of glycogen. This was followed by centrifugation at 14,000 rpm and the pellet obtained was washed two times with 75% ethanol, and resuspended in 87 µl DEPC-water. DNAase 1 digestion (10 µl of 10X DNAase 1 buffer in 0.5 M Tris pH 7.5, 0.1 M MgCl₂, 1 mM DTT; and 50 µg/ml BSA, 2.0 µl RNAase inhibitor; 10U RNAase free DNAase 1) was employed to remove DNA. The reaction was stopped by the addition of 700 µl of 0.5 M NH₄OAc and the RNA was re-extracted using 500 µl of acid phenol-chloroform (1:1). The aqueous layer was harvested, extracted again with chloroform-isoamyl alcohol and precipitated.

Reverse transcriptase polymerase chain reaction (RT-PCR)

The DNAase-treated RNA was subjected to reverse transcription using oligo(dT) primers with SuperScript II reverse transcriptase (Invitrogen, Life Technologies, USA) according to the manufacturer's instructions. RNA (2 µg) was transcribed into cDNA in a 20 µl reaction volume containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 0.5 mM of each deoxynucleotide triphosphate, 25
µg/ml oligo(dT)_{12-18} primers and 10 U/µl of SuperScript II reverse transcriptase, at 42°C for 50 minutes. The reaction was then stopped by heating at 70°C for 15 minutes followed by rapid chilling on ice.

PCR: The primers used as described by our laboratory previously (Hasan et al., 2006). cDNA for β-actin was amplified with various primer sets supplied by (Stratagene, La Jolla, CA, USA). For PCR, 2.0 µl of each cDNA sample was used as template in the PCR amplification. The reactions were carried out in a 50 µl reaction volume containing 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM of each of the four dNTPs, 2U of Taq DNA polymerase (Invitrogen) and 0.2 µM of each forward and reverse primers. After initial denaturation for 2 minutes at 95°C, 35 cycles at 95°C for 15 seconds, 60°C for 45 seconds were performed, followed by 72°C for 1 minute. The reaction products were visualized by electrophoresis in 2% agarose after staining with 0.5 µg/ml ethidium bromide.

Quantitative real-time RT-PCR:
Real-time quantitative reverse transcriptase PCR (RT-PCR), which is the latest innovation in the field of PCR technology, provides a sensitive, reproducible, and accurate method for determining mRNA levels in tissues or cells. The method is based on the detection of a fluorescent signal produced and monitored during the amplification process, without the need for post-PCR processing (Heid et al., 1996).

Two important findings led to the discovery of real-time PCR. First, the Taq polymerase has a 5'-3' exonuclease activity (Holland et al., 1991), apart from its polymerase activity. Second, dual-labelled fluorogenic oligonucleotide probes have been created which emit a fluorescent signal only upon cleavage, based on the principle of fluorescence resonance energy transfer (Cardullo et al., 1988). In the TaqMan assay (Applied Biosystems, Foster City, CA, USA), these two principles are combined. In this system a probe, the so-called TaqMan probe, is designed to anneal to the target sequence between the classical forward and
reverse primers. The probe is dually labelled, with a reporter fluorochrome (eg., 5-carbofluorescein, or FAM) at one end and a quencher dye (eg., N,N,N',N'-tetramethyl-6-carborhodamine, or TAMRA) at the other end. In the intact probe, the fluorescence emission of the reporter dye will be absorbed by the quencher dye. The probe will be degraded during the extension phase by the 5'–3' exonuclease activity of the Taq polymerase, separating the reporter and quencher, thus resulting in an increase in reporter fluorescence emission. The amount of fluorescence released is directly proportional to the amount of product generated in each PCR cycle and thus can be applied as a quantitative measure of PCR product formation.

Procedure:

Internal fluorescent hybridization probes were used in ABI Prism 7700 Detection System (ABI/PerkinElmer (PE) Biosystems, Foster City, CA, USA), which allows the sensitive and specific quantification of individual host (Hartel et al., 1999), as well as M. tuberculosis RNA, transcripts (Wilkinson et al., 2001) by quantitative real-time RT-PCR. TaqMan™ PCR primers and probes as well as target-specific RT primer for each assay were designed as described elsewhere (Wilkinson et al., 2001; Islam et al., 2004). We have reported the primer and probe sequences used for R18, TNF-alpha and iNOS earlier (Islam et al., 2004, Hasan et al., 2006; Singh et al., 2002), and are also given below. All probes were dually labeled with FAM at the 5' end and TAMRA at the 3' end. The proximity of the dye (FAM) and the quencher (TAMRA) on the intact probe prevents detection of any fluorescence. However, degradation of the probe during the course of PCR allows the release and detection of FAM (Holland et al., 1991). The primer and probe sequences used for R18, TNF-alpha and iNOS were as given below:

R18:

**RT primer:** GACGGTATCTGATC;
**reverse primer:** 5'-CAT TCT TGG CAA ATG CTT TC-3';
**forward primer:** 5'-CGC CGC TAG AGG TGA AAT TC-3';
**TaqMan probe:** 5'-6FAM-ACC GGC GCA AGA CGG ACC AGA-TAMRA-3'.

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TNF-alpha:

RT primer: GGTTTCTACAACA;
reverse primer: 5'-GTTCGAGAAGATGATCTGACTGCC-3';
forward primer: 5'-AGGCCGGTGCTTGTTCCCTCA-3';
TaqMan probe: 5'-6FAM-CCAGAGGGAAGAGTTCCAGGGAC-TAMRA-3'.

iNOS:

RT primer: 5'-CTCTggTCAAAC-3';
forward primer: 5'-AgCggg-ATgACTTTCCAAGA-3';
reverse primer: 5'-ATAATggACCCCAgg-CAAgATT-3';
TaqMan Probe: 5'-AMCCATAAggCCAAagggATTTTAACCTTgCAg-3'.

The PCRs for all amplifications were similar: 5 μl of each cDNA, 20 μl of Taqman Universal PCR Master Mix (PE Biosystems), which contains optimal amount of AmpliTaq Gold DNA polymerase (which protects against amplicon carryover) and of dNTPs, and optimal amounts of probe and primers calibrated to allow measurement of the targets. First, cDNA was synthesized in the presence of 0.5 μl of murine leukemia virus enzyme (Invitrogen, USA)/reaction and 10 μM each RT primer, dNTPs, and other substrate. Conditions for PCR were similar for all products (1 cycle of 2 minutes at 50°C and 1 cycle of 10 minutes at 95°C and then 40 cycles of 15 seconds at 95°C and 1 minute at 60°C). The cycle threshold for each sample was compared with the cycle threshold values of known amounts of a standard DNA constructed for each target and amplified simultaneously. To assure lack of DNA contamination in the RNA samples, in some experiments, a duplicate tube of sample with no RT enzyme was included as control. DNA contamination remained negligible. In each sample, host 18S ribosomal RNA was used as the internal control. Expression of TNF-α mRNA and iNOS were corrected to internal control (host 18S rRNA) in the same sample and was expressed as copies of TNF-α or iNOS in 10^{10} copies of R18 (equivalent to 1 x 10^6 monocytes).
**Statistical analysis:**

Results were analyzed by paired t-test and the data expressed as mean ± SEM of six to eight experiments unless otherwise specified. \( P<0.05 \) was considered statistically significant.