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Epigallocatechin-3-gallate (EGCG), a green tea polyphenol suppresses bacilli-induced augmented expression of Mycobacterium tuberculosis 85B and proinflammatory TNF-α in human monocytes

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Abstract- Reactive oxygen species (ROS) and tumor necrosis factor (TNF-α), the hallmarks of tuberculosis, are directly induced in human monocytes by *Mycobacterium tuberculosis* (*MTB*). We have previously shown that the expression of 85B in *MTB* infected monocytes correlates positively with both the amount of secreted TNF-α and subsequent mycobacterial growth. In this study, we show the augmented expression of 85B in *MTB* and TNF-α at both the gene and protein levels in *MTB*-infected monocytes were suppressed by EGCG, a green tea polyphenol, in dose-dependent manner. Of notable importance, the anti-inflammatory mechanism of EGCG was mediated by inhibition of NF-κB pathway. Moreover, EGCG enhances the antioxidant potential of monocytes by ameliorating the intramonocyte sIFN-γ levels, glutathione levels and glutathione peroxidase activity, which correlates with the down regulation of TNF-α in *MTB*-infected monocytes. Taken together, this study provides the first novel insight into the possible anti-inflammatory and antioxidant role of EGCG that can be exploited in tuberculosis management.

Index Terms- Epigallocatechin-3-gallate (EGCG), glutathione peroxidase, IFN- γ, ROS, TNF- α

I. INTRODUCTION

Tuberculosis (TB), which claims between 2-3 million lives per year [1], is spiraling out of control at an alarming rate due to prevalence of multidrug-resistant (MDR) strains, emergence of AIDS-related TB [2, 3] and variation in the efficacy of Bacillus Calmette-Guerin (BCG) vaccine with respect to geographic latitude [4]. Moreover, despite of antibiotics helping in complete recovery from TB in non-MDR patients, reports are available where development of antibiotics-induced side effects is evident in host cells. Therefore, research interest has now been shifted in probing compounds from natural sources that have antioxidant, anti-inflammatory and antimycobacterial activity. Thus, after boosting host immunologic responsiveness, these compounds may be particularly useful in the treatment of both TB and MDR-TB.

We have already reported that the bacilli infection in host mononuclear phagocytes results in the induction of reactive oxygen species (ROS) leading to activation and upregulation of host TNF-α [5, 6]. TNF-α in turn, activates and upregulate *M. tuberculosis* 85B which further elevates the host TNF-α levels forming a positive feedback loop [5, 7]. Therefore, the interaction of *MTB* 85B and host TNF-α creates a vicious circle in which each maintains the production of the other. The expression of 85B in *MTB*-infected monocytes correlates positively with both the amount of secreted TNF-α and subsequent intracellular mycobacterial growth [4]. Earlier reports by our group [5, 6] have already established the role of TNF-α as a significant mediator of macrophage activation which enables intracellular killing of *MTB*. However, both beneficial and deleterious effects of TNF-α in host contributing to the pathophysiology of tuberculosis have been reported [8].

EGCG (epigallocatechin-3-gallate), a green tea polyphenol, plays an important role in the prevention of cancer and cardiovascular diseases [9], acts as reversal agent of multidrug resistance (MDR) [10], and show anti-inflammatory property [11]. In this study, we for the first time experimentally demonstrated the incorporation of EGCG as an effective antioxidant and chemo-preventive natural adjunct for the TB treatment. We show that EGCG exerts its potent antioxidant and anti-inflammatory effects on host mononuclear cells infected with *MTB* by suppressing the augmented expression of *MTB* 85B and proinflammatory TNF-α in human monocytes.

II. MATERIALS AND METHOD

2.1 Materials

Epigallocatechin-3-gallate (EGCG), SN50, its analogue SN50/M and Trypan blue were obtained from Sigma–Aldrich (St. Louis, MO, USA). Ficoll-Paque was obtained from Pharmacia (LKB Biotechnology Piscataway, NJ). All other chemicals used were of the highest purity grade available. Blood was obtained from healthy, non-smoking volunteers between 20–40 years of age.

2.2 Strain and media

M. tuberculosis ($H_{37}Rv$) was grown in Middlebrook 7H9 broth supplemented with Middlebrook ADC and characterized as described earlier [4].

2.3 PBMC preparation, infection and cell culture

PBMC (peripheral blood mononuclear cells) (>95% purity) were isolated from buffy coats of blood obtained from healthy donors.

Cells were adhered onto 12-well tissue culture plates (Costar Corp., Cambridge, MA) at 1–2 x 10^6cells/well and resuspended in complete RPMI 1640 medium followed by incubation at 37^0C, 5% CO_2 for 1–2 h adherence. Adhered monocytes were infected with *MTB* at 1:1(bacteria/cell) as reported by us previously [5]. Control cultures received RPMI-1640 medium with 2% autologous serum while experimental cultures received varying doses of EGCG (0-25 µg/ml) immediately after infection. To assess the role of NF-κB, some cultures were pre-treated for 3 min with SN50 and its control analogue SN50/M (100 µg/ml). Then, cultures were harvested after 24 h and cells were lysed in 0.5 ml of TRIZOL Reagent (Invitrogen Inc., Carlsbad, CA). Culture supernatants were stored at -70^0C until use.

2.4 Cell Viability Assay

Cell viability was determined using the MTT Cell Viability Assay kit (R & D Systems) according to the instructions provided in the kit to assess the effect of EGCG (0-25 µg/ml). Control cells were treated exactly the same except that no EGCG was added to the wells. The percentage of viable cells in these experiments was calculated by the formula [(Absorbance value of control/Absorbance value of treated) x100] and the results were expressed as "Viable cells (% of control)." Cell viability was also probed by Trypan blue exclusion assay as described earlier [13].

2.5 Isolation of total RNA and reverse transcription

Total RNA from uninfected as well as infected monocytes was isolated according to the instructions provided in the Qiagen kit (Valencia, CA). Reverse transcription reaction was carried out as described by us earlier [5]. The products obtained were analyzed by electrophoresis on ethidium bromide-stained 2% agarose gels.

2.6 Quantitative real-time RT-PCR

Quantitative real-time RT-PCR was employed to quantify host TNF-α gene transcription as described previously [4-7]. This technique affords a sensitive and specific quantification of individual RNA transcripts [14]. Human R18 housekeeping gene was employed to normalize gene expression. TaqMan PCR primers and probes as well as target-specific RT primer for each assay were designed as described elsewhere [5, 6]. All other conditions were exactly same as reported by us earlier [5]. In each sample, host 18S ribosomal RNA was used as internal control. Expression of TNF-α mRNA was normalized with internal control (host 18S rRNA) in the same sample and was expressed as copies of TNF-α in 10^{10} copies of R18 (equivalent to 1 x10^6 monocytes).

2.7 Assay for glutathione peroxidase (GPx) activity and intramonocyte glutathione (GSH) levels in EGCG-treated infected monocytes.

Glutathione peroxidase (GPx) activity was measured as described elsewhere [15]. Briefly, *MTB*-infected monocytes were co-cultured for 24 h with or without 5 µg/ml EGCG, 100 µg/ml SN50 and 100 µg/ml SN50/M. Cells were then scrapped, sonicated and centrifuged as described earlier [15]. The supernatants were subjected to GPx activity quantification in 100 µl/sample, with continuous photometric monitoring of oxidized glutathione (GSSG) at 37°C. The conversion of NADPH to NADP was evaluated using UV absorbance at 340 nm [16]. GPx activity was calculated after subtraction of the blank value and expressed as µmol of NADPH oxidized/min/mg protein (U/mg protein).

Glutathione (GSH) levels were assayed spectrophotometrically by employing GSH kit (Calbiochem) in treated or control monocytes. 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, according to the instructions provided in the kit and described previously [7].

2.8 Measurement of secreted TNF-α, MTB 85B antigen and IFN-γ by ELISA.

The amount of soluble, secreted TNF-α, *MTB* 85B and sIFN-γ in various culture supernatants was determined using commercial ELISA Kits (R&D systems), according to the instructions provided with the kits.

2.9 Statistical analysis

All experiments were repeated twice to ensure for reproducibility. All assays and quantitative measurements were performed in triplicate. The data was analyzed for statistical significance by paired t test and expressed as mean ± S.E. of three independent experiments. $P < 0.05$ was considered statistically significant.

III. Results

3.1 Effect of EGCG on monocyte viability and housekeeping gene expression

EGCG (0-20 µg/ml; $P<0.001$) failed to show any adverse effect on the viability of monocytes as revealed by MTT assay (Fig. 1A) and Trypan blue exclusion assay (data not shown). Cell viability is expressed as percent mean (±S.E.) viable cells compared to untreated cells (taken as 100% viable).

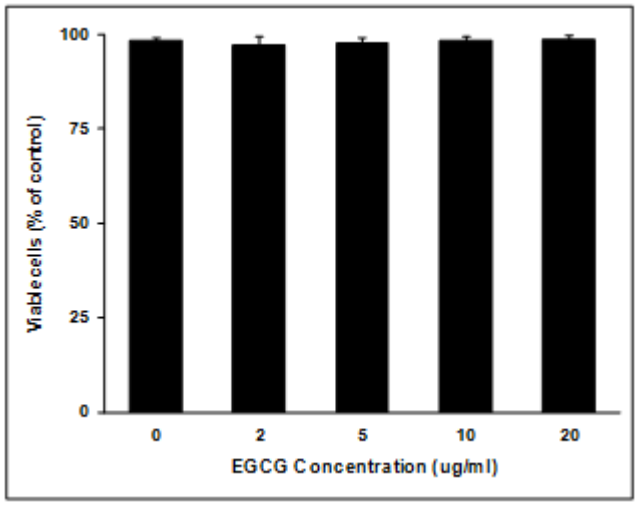

Fig. 1 (A) MTT cell viability assay for dose-effect of EGCG (0–20 µg/ml) on MTB-infected monocytes. Data represents the analysis of three independent experiments in duplicates, which are expressed as mean viable cells (±S. E.) percentage of controls.

Similarly, no effect of EGCG (0-25 µg/ml) was observed on housekeeping genes like R18 gene (18S rRNA) as depicted by quantitative real time RT-PCR (Fig. 1B).This indicates that EGCG does not affect human housekeeping gene transcription non-specifically.

3.2 EGCG downregulates expression of TNF-α and MTB 85B

It has been reported [4, 6] that the expression of *MTB* 85B mRNA increases early during infection of monocytes and

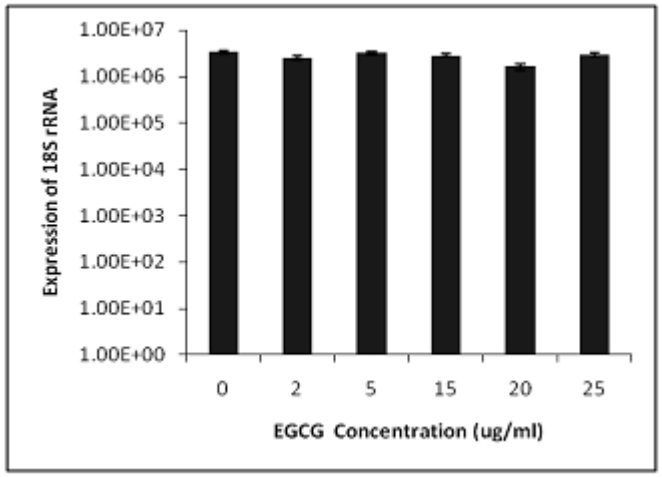

Fig. 1 (B) 'Real-time' quantitative RT-PCR for human housekeeping R18 gene (18S rRNA) in presence of EGCG (0-25 µg/ml), where data are mean (±S.E.) of three experiments

correlates positively with both the amount of secreted TNF-α and subsequent intracellular mycobacterial growth in *MTB*-infected monocytes. An attempt was made to probe the dose-response effect of EGCG on TNF-α and *MTB* 85B gene expression in *MTB*-infected monocytes. Infected/uninfected cultures devoid of EGCG served as controls. The effect of EGCG (0-20µg/ml) on amplification of TNF-α and *MTB* 85B was found to be dose-dependent. The expression of 85B mRNA was normalized with mycobacterial 16S rRNA and expressed as 85B:16S. As revealed by 'real time' RT-PCR, in comparison to the control, a linear downregulation of TNF-α and *MTB* 85B mRNA copy numbers were recorded with 2, 5, 10 and 20µg/ml of EGCG, respectively (Fig. 2A and B). Computation of IC_{50} of EGCG (Figs.2A and B) was found to be ~5 µg/ml.

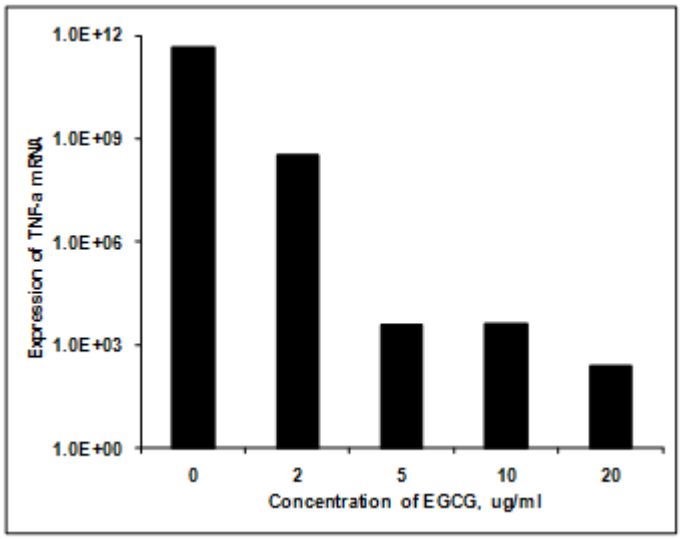

Fig. 2 (A) Dose response effects of EGCG (0-20 µg/ml) on expression of TNF-α by 'real time' RT-PCR in MTB-infected monocytes. Expression of TNF-α mRNA was normalized with host 18S rRNA and expressed as TNF-α in 10^{10} copies of R18 (equivalent to 1 x 10^6 monocytes.)

Similar dose dependent suppressive effects were recorded for the secreted TNF-α and *MTB* 85B proteins upon supplementation with EGCG (0-25µg/ml) (Fig. 2C and D; $P<0.001$). Thus, EGCG potently inhibits the pro-inflammatory cytokine TNF-α and *MTB* 85B antigen.

3.3 EGCG modulates TNF-α level through inhibition of NF-κB

Induction of TNF-α by cellular activation is known to be mediated via NF-κB [17-19]. It has been well documented that TNF-α induced nuclear translocation of NF-κB is inhibited in SN50 peptide-treated human monocytic cell lines [20].

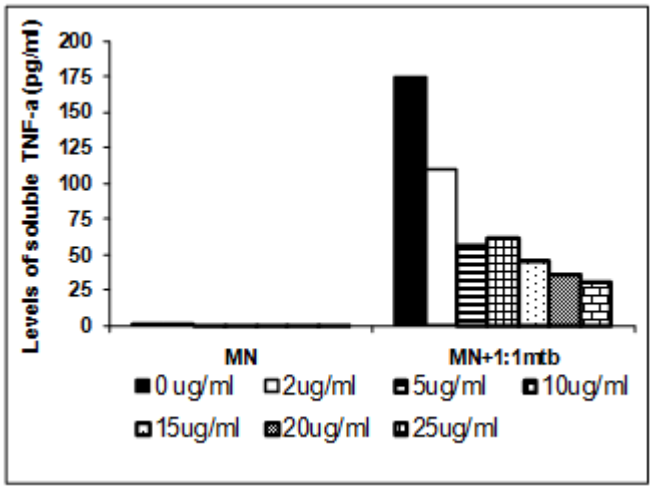

Fig. 2 (B) Dose response effect of EGCG (0-25 µg/ml) on expression of secreted TNF-α protein (pg/ml) in 24 h culture supernatant in MTB-infected monocytes. Data are mean (±S.E.) of three experiments.

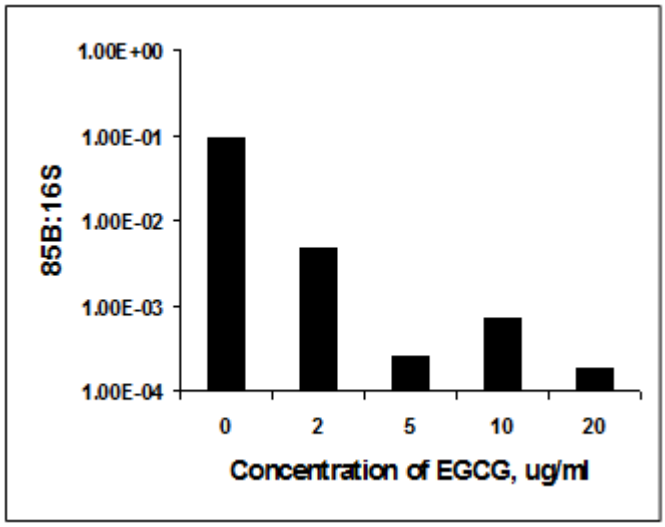

Fig. 2 (C) Dose response effect of EGCG (0-20 µg/ml) on 85B expression in 24 h culture of monocytes infected with MTB assessed by 'real time' RT-PCR. Expression of 85B was normalized with mycobacterial 16S rRNA and represented as 85B:16S ratio. Data are means (± SE) of three experiments.

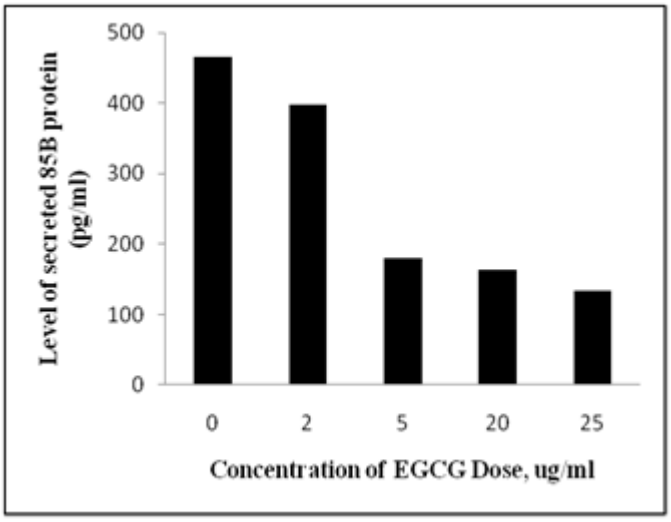

Fig. 2(D) Dose response effect of EGCG (0-25 µg/ml) on the secreted Antigen 85B protein in pg/ml. Antigen 85complex were determined in culture supernatants at 24 h. Data are means (±SE) of three experiments.

Thus, we employed SN50, an inhibitor of NF-κB, to assess the role of NF-κB in EGCG mediated downregulation of TNF-α and MTB-85B expression in MTB-infected monocytes. SN50 (100 µg/ml) was added to monocytes 3 minutes prior to M. tuberculosis infection. At 24 hours, SN50 suppressed endogenous TNF-α mRNA expression in MTB-infected monocytes by around 6.2 logs (P<0.001) in comparison to control cultures devoid of SN50 pre-treatment (Fig. 3A).

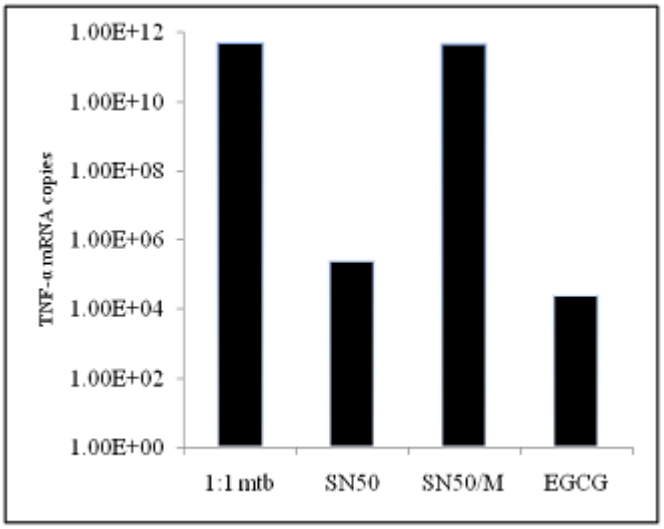

Fig. 3 (A) Effect of inhibition of NF-κB on expression of TNF-α mRNA. Human monocytes were infected with MTB H37Rv (1:1 bacteria/cell) in the presence of 100 ug/ml of SN50 and SN50/M. Total RNA was extracted at 24 h and was assessed for expression of TNF-α mRNA. Data are mean (+S.E.) of three experiments.

Comparable results were observed when EGCG was employed. To ensure that cellular inhibition was specific, we compared the effect of SN50 with its inactive analogue, SN50/M. As expected, SN50/M failed to show any effect on TNF-α mRNA expression (P>0.05) (Fig. 3A). Similarly, SN50 treatment led to decrease in the ratio of 85B:16S in MTB-infected monocytes (Fig. 3B). Therefore, it can be concluded that the increased expression of TNF-α and 85B mRNA in MTB-infected monocytes is mediated mainly via NF-κB inhibition.

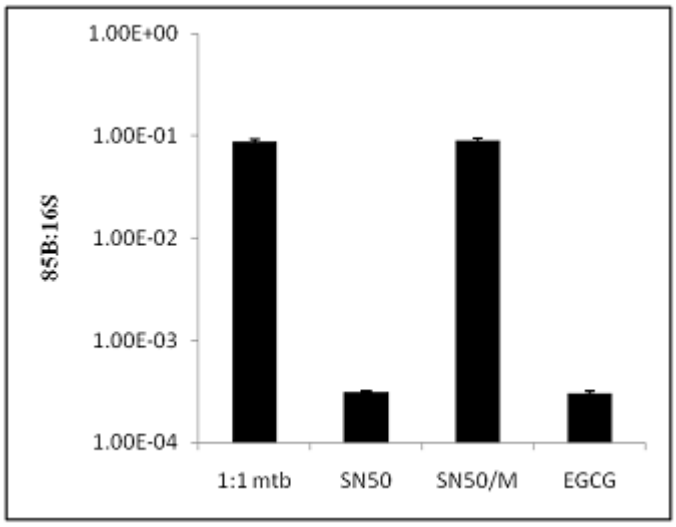

Fig. 3(B) Effect of SN50 (100 ug/ml) and SN50/M (100 ug/ml) on the expression of intramonocyte MTB 85B:16S ratio in M. tuberculosis-infected monocytes after 24 h of infection by 'real time' RT-PCR. MTB-infected monocytes (-) that were devoid of any supplement served as control. The results are mean (± SE) of three experiments.

3.4 EGCG enhances the anti-inflammatory and antioxidant potential of MTB infected monocytes

To probe the anti-inflammatory and antioxidant role of EGCG, we first measured the sIFN-γ levels in MTB-infected monocytes. Uninfected and untreated control monocytes in/of 24 hr cultures exhibited sIFN-γ levels of 262 pg/ml (P>0.001) (Fig. 4A). On the contrary, infected monocytes that were devoid of any EGCG, the sIFN-γ levels were found to be 81 pg/ml (P>0.001) (Fig. 4A). However, addition of EGCG in MTB-infected monocytes exhibited an augmentation in sIFN-γ levels to 233 pg/ml (P>0.001(Fig. 4A). Thus, it appears that down-regulation of sIFN-γ levels in culture supernatant of MTB-infected monocytes is reversed by EGCG.

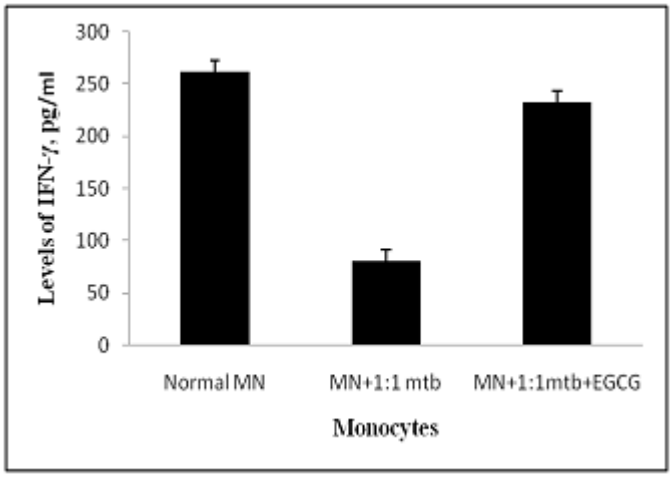

Fig. 4 (A) Assay of sIFN-γ by ELISA in monocytes that were uninfected and infected with MTB H37Rv (1:1 bacteria/cell) and supplemented with EGCG (5 µg/ml). The concentration of sIFN-γ was determined in culture supernatant andexpressed in pg/ml. Data are mean (±S.E.) of three experiments.

Next, the glutathione (GSH) level was investigated in MTB-infected monocytes co-cultured for 24h with and without EGCG. Uninfected monocytes showed 269 pg/ml (P>0.001) GSH level, which was suppressed to 159 pg/ml (P>0.001) in MTB-infected monocytes. An elevation in GSH levels to 210pg/ml (P>0.001) was exhibited upon EGCG treatment (Fig. 4B).

IV. Discussion

Widespread and prolonged usage of antibiotics in treating infection caused by *MTB* has led to the emergence of Multidrug resistance (MDR). Although, mechanisms of MDR and major factors which contribute to it are fairly established, there are evidences to suggest that MDR is a multi-factorial phenomenon which may be contributed by yet unknown mechanisms. EGCG being a potent reversal agent of MDR [21], was selected in the present study. That enhancement of TNF-α and ROS being a significant parameter for *MTB* proliferation in host cells [5-7], this study probed the EGCG-induced regulation of ROS and TNF-α activation in *MTB*-infected human monocytes. TNF-α is implicated in both the pathophysiology and protective host response to *MTB* [22]. Since elevated level of TNF-α are present at the site of *MTB* infection, monocytes isolated from TB patients produce more TNF-α in vitro than those from uninfected donors [23]. Moreover, ROS are also involved in promotion of TB as free radicals generated in *MTB*–infected cells stimulate TNF-α causing *MTB* proliferation. Our findings show that EGCG induce dose dependent suppression of augmented TNF-α and *MTB* 85B at both gene and protein levels in *MTB*-infected monocytes. Also, EGCG ameliorated the sIFN-γ and intramonocyte glutathione levels, which correlate inversely with the down regulation of ROS and TNF-α in *MTB*-infected monocytes. Thus, EGCG exerts potent anti-inflammatory effects on host mononuclear cells infected with *MTB*. EGCG does not affect the human housekeeping gene R18, thereby demonstrating that EGCG did not mediate cellular death, but induce specific inhibition of expression and secretion of pro-inflammatory molecules. The results indicate an appreciable EGCG-induced suppression in endogenous TNF-α mRNA expression in *MTB*-infected monocytes. Elevated expression of TNF-α mRNA and activation of a relevant transcription factor, NF-κB has been reported in monocytes cells treated with *MTB*. Our results are in accordance to our previous findings [5] where induction of TNF-α expression due to *MTB* infection was mediated through activation of NF-κB, as evident by the suppression of TNF-α mRNA in presence of SN50, an inhibitor of NF-κB. It has been previously shown that in electrophoretic mobility shift assay (EMSA), TNF-α induced nuclear translocation of NF-κB was inhibited by SN50 peptide [20]. The NF-κB heterodimer is retained in the cytoplasm in an inactive form through association with one of the IκBa inhibitory proteins. The degradation of Iκb releases active NF-κB, which translocates to the nucleus and regulates gene expression by binding to κB binding sites or by interacting with other transcriptional factors [24]. Our data suggests that EGCG-induced effect involves inhibition of the NF-κB pathway probably by inhibiting the degradation of IκBa, however this remains to be experimentally validated. Since a number of genes involved in inflammatory responses are regulated by NF-κB pathway, thus high magnitude down regulation of the NF-κB pathway by EGCG would predictably reduce the elaboration of NF-κB mediated TNF-α mRNA expression. Based on IC_{50} values, EGCG exerted an appreciable degree of neutralization effects on TNF-α induced action in *MTB* infected human monocytes.

Alteration of redox status upon inflammation is associated with activation of phagocytes and increased formation of reactive oxygen and nitrogen intermediates [25]. Up regulation of sIFN-γ in culture supernatant coupled with simultaneous down regulation of TNF-α at both the gene and protein levels is indicative for anti-inflammatory and protective immune response properties of EGCG. GSH, a known antioxidant, helps in neutralizing ROS generated inside the cell. Similarly GPx, which catalyzes the removal of peroxide ions, as well contribute towards the antioxidant mechanisms. We observed a decline in GSH levels in *MTB*-infected monocytes, thereby correlating with earlier reports that substantial amount of ROS are generated in cells infected with *MTB* due to cellular activation [8, 16]. Intriguingly, augmentation in both GSH levels and GPx activity were observed on treatment with EGCG, thus indicating EGCG to be an effective natural antioxidant combating ROS that was generated as a consequence of cellular activation in *MTB*-infected mononuclear phagocytes.

V. CONCLUSION

In conclusion, our study demonstrates that ameliorated GSH and sINF-γ levels correlate inversely with the downregulation of TNF-α mRNA expression and ROS in monocytes infected with *MTB*. Therefore EGCG, could help in arresting the vicious circle created as a consequence of interaction between *MTB* 85B and host TNF- α, where each maintains the production of the other. The data generated in the present study involving infected human monocytes, strongly indicate EGCG to be a potential adjunct in the treatment of TB. Hence, EGCG may prove to be safe, economic and natural anti-oxidant whose therapeutic potential could be exploited in future for TB management.

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