ABSTRACT
Abstract

One of the hallmarks of immune system is the tremendous potentiality to discriminate between self and non-self. Systemic Lupus Erythematosus (SLE) - a prototype autoimmune disorder, is a chronic, life-long and potentially fatal disease, which is characterized by unpredictable exacerbations and remissions with protean clinical manifestations. SLE is a complex disorder affecting predominately young populations and shares similarities with HIV infection which is well known associated with tuberculosis (HIV-Tuberculosis) as regards the propensity for multiple organ involvement, potentially life-threatening episodes, and need for sophisticated monitoring. The clinical features of SLE are protean and may mimic infectious mononucleosis, lymphoma, or other systemic disease. The origin of autoantibody production in SLE is still poorly understood till date. However, a role has been suggested for an antigen driven process, spontaneous B-cell hyper-responsiveness, or impaired immune regulation. Thus, the etiology of SLE still remains poorly understood and warrants in-depth investigation for the management of the disease.

Evidences exist for the involvement of mycobacterial protein antigens in the pathogenesis of autoimmune disease. Furthermore, established evidences exist for autoimmune mediated destruction of islet cells of pancreas in the pathogenesis of Type-1 Diabetes mellitus for which various markers have been identified.

The direct and or indirect role of proinflammatory cytokine namely TNF-α in SLE has been indicated. TNF-α exerting its effects through its receptors namely TNFR-I and TNFR-II plays a crucial role in host defense. Inhibition of TNF-α clearly predisposes to certain infections, such as granulomatous infections like TB. Inhibition of TNF-α may also play a role in autoimmunity although the pathophysiologic mechanisms are uncertain. Furthermore, cases of TB have been reported in patients treated with TNF-α-antagonists. The risk for TB in RA/SLE patients is associated with multiple other factors including age, country of origin or current residence, exposure history to persons with TB, concomitant
therapy with other immunomodulators including corticosteroids, and disease activity. Focus has now shifted to development of compounds from natural sources that have anti-inflammatory and antimycobacterial activity. By boosting host immunologic responsiveness, these compounds may be particularly useful in the treatment of autoimmune disorders as well as drug-resistant tuberculosis. Our study involves the incorporation of such a compound, namely EGCG – a green tea polyphenol, as the natural herbal component for SLE and SLE-TB management.

Thus, the present study was carried out to probe the possible involvement of mycobacterial protein / nucleic acid antigen(s) acting as an alternate auto antigen for autoimmune disorders by employing various techniques such as direct binding and competition ELISA, cell culture with and without modulators, ‘real time’ RT-PCR, etc. Thus, it is hoped that the present study would help in the better understanding about the contributory role of \textit{M. tuberculosis} infection in the morbidity and mortality of patients with SLE.

Cellular signalling by TNF-\( \alpha \) is mediated mainly through activation of NF-\( \kappa \)B. During characterization studies in order to assess the role of NF-\( \kappa \)B in the expression of TNF-\( \alpha \) in SLE, TB and SLE-TB monocytes, we employed SN50, an inhibitor of NF-\( \kappa \)B. From our results, it was apparent that the induction of TNF-\( \alpha \) expression was mediated through activation of NF-\( \kappa \)B, because TNF- \( \alpha \) was suppressed when SN50 was present in cultures. The inactive analogue of SN50 (i. e. SN50M) did not have any effect. Thus, as previously reported in macrophages, this study also shows that cellular activation is associated with augmentation of expression of both TNF-\( \alpha \) in monocytes of patients with SLE, TB and SLE-TB.

Cell viability and potential cytotoxicity of EGCG, if any, on monocytes obtained from patients with SLE, TB and SLE-TB were determined for the concentrations (0-25 \( \mu \)g/ml) employed in this study using trypan blue and MTT assays, where viability of \(~98-99\%\) was observed. Interestingly, no effect of EGCG was observed on human housekeeping genes like R18 rRNA, thereby demonstrating
that the effect of EGCG was not mediated by cellular death, but rather by specific inhibition of expression as well as secretion of TNF-α.

To the best of our understanding, in our characterization studies, we show for the first time that EGCG exerts potent anti-inflammatory effects on host mononuclear cells obtained from patients of SLE, TB and SLE-TB, as evidenced by a strong inhibition of the pro-inflammatory cytokine TNF-α. The results indicate an appreciable suppression in soluble TNF-α secretion with EGCG.

TNF-α production in monocytes is regulated at multiple intracellular levels, beginning with transcription. Augmented expression of TNF-α mRNA and activation of a relevant transcription factor, NF-κB have been reported in monocytic cells infected with *M. tuberculosis*. Induction of TNF-α expression was mediated through activation of NF-κB, as evidenced by the suppression of secreted TNF-α protein in the presence of SN50, an inhibitor of NF-κB. On the contrary, SN50/M, an inactive analogue of SN50, failed to show any such effect. It has been well established that TNF-α induced nuclear translocation of NF-κB is inhibited by SN50 peptide as demonstrated in EMSA. In view of it, our data demonstrated that this effect involved inhibition of the NF-κB pathway induced by EGCG, probably by inhibiting the degradation of IκBα. The NF-κB heterodimer is retained in the cytoplasm in an inactive form through association with one of the IκBs inhibitory proteins.

In continuation to the above, a role for the antioxidant enzyme glutathione peroxidase (GPx) in MTB-infected and autoimmune mononuclear cells was investigated with respect to various modulators employed in this study. In the present study, reduced / suppressed GPx activity in patient monocytes that were untreated or treated with SN50/M was recorded, thereby concurring with earlier reports that substantial amounts of ROS are being generated in patient cells due to cellular activation. Enhancement of GPx activity in monocyte cultures from patients with SLE, TB and SLE-TB after addition of NAC, a precursor of the *in vivo* antioxidant glutathione, indicates reversal of impaired neutralizing mechanisms. Surprisingly, GPx activity was observed to be further augmented
when EGCG was co-cultured instead of NAC, indicating EGCG to be an effective natural antioxidant combating ROS, generated as a consequence of cellular activation in mononuclear phagocytes. Moreover, in continuation to the above, our data further shows the lipid peroxidation-induced augmented MDA levels in culture supernatants of monocytes from patients with SLE, TB and SLE-TB were appreciably suppressed or down-regulated with EGCG.

Our results are strongly indicative for the appreciable correlation between autoimmune diseases like SLE and *Mycobacterium tuberculosis* infection. Inhibition ELISA results strongly suggests that all the TB positive sera selected in this study were having a high degree of specificity towards MTB Ag85B (30kDa). Similarly, our data show high degree of recognition of anti-DNA antibodies found in SLE sera by native dsDNA. The achievement of 50% inhibition in antibody activity in both the above diseases i. e. TB and SLE at a very low inhibitor concentrations are indicative for the presence of highly specific respective antibodies in all the sera selected for this study.

One of the interesting findings of this study was that the SLE patients were found to be more susceptible to TB development than the vice-versa case. This is evident from the data indicating that autoantibodies found in SLE sera strongly recognized both native dsDNA as well as mycobacterial Ag85B 30kDa, whereas anti-TB antibodies found in TB sera strongly recognized only Ag85B 30kDa, whereas it exhibited low or negligible recognition with native dsDNA. However, antibodies found in sera of patients suffering with both TB along with SLE (SLE-TB) revealed high specificity for both the antigens i. e. native dsDNA and MTB 30kDa.

An attempt was also made to evaluate the immuno-binding by generating in-vivo conditions i. e. by using cultured monocytes that were infected with bacilli (TB patients) as well as monocytes from SLE patients. The high percent maximum inhibitions as well 50% inhibitions in anti-TB and anti-DNA activities at extremely low inhibitor concentrations are suggestive for the appreciably high affinity
immuno-interaction occurring between anti-TB antibodies and anti-DNA autoantibodies with antigens in monocyte protein lysates as well as respective dsDNA of TB patients and SLE patients respectively.

A striking finding in the present study is the EGCG as well as reduced glutathione-induced down-regulation in binding of anti-TB antibodies and anti-DNA antibodies with antigens present in protein lysates prepared from monocyte of TB patients and SLE patients respectively. Similar actions of reduced glutathione in monocytes obtained from blood of patients having both TB along with SLE further substantiates the antioxidant-induced suppression in immuno-binding. Furthermore, these results are correlating appreciably with the amount of TNF-α secreted in different monocyte cultures undertaken in this study.

To have further in-sight, the present study involves utilization of dsDNA isolated from monocyte cultures of SLE, TB and SLE-TB patients that were either untreated or treated with EGCG, NAC, SN50 and SN50M, an in turn, employed as antigens / inhibitors in immunoassays against anti-DNA SLE antibodies and anti-TB antibodies respectively. Thus, interesting important observations were made. We observed that purified anti-DNA antibodies from SLE patients exhibited high degree of recognition / specificity against dsDNA isolated from monocytes of patients with SLE, TB and SLE-TB respectively. However, this high magnitude specificity / binding of purified anti-DNA antibodies from SLE patients was reduced or suppressed appreciably towards respective dsDNA isolated from monocytes of patients with SLE, TB and SLE-TB respectively that were treated with EGCG, NAC or SN50. On the contrary, anti-TB antibodies exhibited high binding only with dsDNA isolated from monocytes of patients with TB, but failed to show any significant recognition / binding with dsDNA isolated from monocytes of patients with SLE. Furthermore, when monocyte cultures of SLE, TB and SLE-TB patients that were treated with EGCG, NAC and SN50, and in turn, respective dsDNA isolated and employed in ELISA, reduced or insignificant binding was observed against anti-dsDNA antibodies from SLE patients or anti-TB antibodies from TB patients. Thus, in view of the fact that native DNA is a
poor immunogen, and that, the exact trigger of anti-DNA production in SLE still remains poorly understood, where DNA has been thought to act only as a cross-reacting antigen, the results indicate possible involvement of Mycobacterium tuberculosis protein(s) / nucleic acid antigens(s) in providing an alternate trigger / origin for autoantibody production in systemic lupus erythematosus (SLE). Moreover, the data generated in the present study is suggestive for the fact that reactive oxygen species (ROS) generated as a consequence of stress of any kind in autoimmune SLE results in the activation of proinflammatory cytokine TNF-α, which in turn results in the production of anti-DNA auto antibodies. These SLE patients having high ROS levels become highly susceptible to MTB infection. Upon MTB infection, the ROS and TNF-α potentiates or activates the MTB 85B replication in SLE-TB patients. Such an activation of ROS or TNF-α and in turn the SLE and TB proliferation could be arrested or limited by the usage of EGCG- a green tea polyphenol and reduced glutathione as revealed by our data. In conclusion, based on characterization studies, followed by immunological data, it can be inferred from the present study that:

1. There exist high basal levels of TNF-α in sera as well as in monocyte cultures of patients with SLE, TB and SLE-TB.
2. Activation of monocytes by *M. tuberculosis* infection in SLE patients induces the expression of both TNF-α at both the gene and protein levels.
3. Both RNI and ROI, induced early after infection of SLE monocytes, increases expression of TNF-α.
4. Activation of monocytes by *M. tuberculosis* initiates a cascade of events whereby a vicious circle may exist in which expression of host inflammation and mycobacterial products amplify one another.
5. EGCG (0-25 µg/ml) exhibited no toxic effect on the viability of human monocytes.
6. EGCG inhibits the expression of TNF-α protein production in a dose-dependent manner in 24 hr monocyte cultures from patients with SLE, TB and SLE-TB, and that, it is mediated mainly via NF-κB.
7. EGCG ameliorates the glutathione peroxidase activity in monocytes from patients with SLE, TB and SLE-TB.
8. EGCG suppresses the augmented MDA levels in monocytes from patients with SLE, TB and SLE-TB.
9. All the *M. tuberculosis* and SLE sera involved in this study showed a high degree of specificity for Ag85B (30 kDa) and native dsDNA respectively.
10. Mycobacterial 30 kDa protein antigen (Ag85B) as well as protein lysates prepared from monocytes of *M. tuberculosis* patients were recognized appreciably by anti-tuberculosis antibodies present in *M. tuberculosis* sera, whereas non-mycobacterial native dsDNA showed poor recognition with the same anti-tuberculosis antibodies.
11. On the contrary, both non-mycobacterial native dsDNA and protein lysates prepared from monocytes of SLE patients as well as mycobacterial 30 kDa protein antigen (Ag85B) were found to be recognized appreciably by anti-DNA autoantibodies present in SLE sera.
12. Furthermore, co-culturing of monocytes obtained from *M. tuberculosis*, SLE or *M. tuberculosis*-SLE with 10 nM of reduced glutathione showed amelioration of ROS and TNF-α induced actions, which in turn, subsequently suppressed the immuno-bindings observed in monocytes of *M. tuberculosis* and SLE patients cultured without glutathione.
13. Our data shows that SLE patients are more susceptible to *M. tuberculosis* development, as ROS and TNF-α in SLE patients could activate the replication of Ag85B (30 kDa) after bacilli infection.
14. Finally, immunoaffinity purified anti-DNA antibodies from SLE patients recognized dsDNA isolated from monocytes of both SLE and TB patients, but on the contrary, anti-TB antibodies recognized dsDNA only from monocytes from TB patients but not SLE patients.