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## Incomplete immunological recovery following anti-tuberculosis treatment in HIV-infected individuals with active tuberculosis

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**Background & objectives:** *Mycobacterium tuberculosis* infection has been shown to result in increased HIV replication and disease progression in HIV-infected individuals through increased immune activation. The objective of this study was to correlate plasma levels of immune activation markers with the presence of tuberculosis (TB) in HIV-infected and uninfected individuals, and to study the changes following anti-tuberculosis treatment.

**Methods:** Plasma markers of immune activation - neopterin, beta-2-microglobulin ( $\beta$ 2M) and soluble tumour necrosis factor alpha receptor type I (sTNF $\alpha$ -RI) were measured by ELISA in 42 HIV positive TB patients (HIV+TB+) undergoing a six-month course of TB chemotherapy. Thirty seven HIV+ persons without active TB, 38 TB patients without HIV infection, and 62 healthy volunteers served as controls.

**Results:** Plasma levels of all three markers were elevated in HIV+ individuals, more so in those with active TB. When HIV+ individuals were further categorized based on CD4+ T cell counts, HIV+TB+ patients with CD4+ T cells counts  $\leq 200$  cells/ $\mu$ l were found to have the highest levels at baseline with a steep fall in neopterin and sTNF $\alpha$ -RI during treatment, but in most instances the levels did not drop to normal.  $\beta$ 2M levels remained persistently high despite completing TB treatment.

**Interpretation & conclusions:** The findings of the study suggest that both HIV and TB act synergistically to activate the host immune system. Although ATT was effective in clearing *M. tuberculosis* infection, a high proportion of HIV+ TB patients continued to have levels well above the normal range, indicating that underlying immune activation persists despite TB treatment. None of the markers were specific enough to be used to assess cure of TB.

**Key words** Anti-tuberculosis treatment - HIV-1 - immune activation - *Mycobacterium tuberculosis* -neopterin - sTNF $\alpha$ -RI -  $\beta$ -2-microglobulin

HIV-infected individuals are shown to be more susceptible to tuberculosis (TB)<sup>1</sup>. Levels of HIV in the bloodstream increase 5- to 160-fold in HIV-infected people who develop active TB<sup>2</sup>. There is evidence to suggest that *Mycobacterium tuberculosis*-mediated

viral production perhaps occurs through antigen-specific activation and infection of responding T cells<sup>3</sup>. Although cellular activation is essential to mount an effective host immune response to invading pathogens, paradoxically the marked systemic immune activation

that accompanies HIV-1 infection *in vivo* may play an important role in sustaining phenomenal rates of HIV-1 replication in infected persons<sup>3</sup>. Moreover, by inducing CD4+ T cell loss by apoptosis, immune activation may further be central to the increased rate of CD4+ T cell turnover and eventual development of CD4+ lymphocytopenia<sup>4</sup>. In addition to HIV-1-induced immune activation, opportunistic infections such as TB provoke activation of immune cells and further impact the rate of HIV-1 replication and CD4+ T lymphocyte turnover<sup>5</sup>. Such stimuli may also lead to genotypic and phenotypic changes in the virus pool. Together, these various immunological effects on the biology of HIV-1 may potentially enhance disease progression in HIV-infected persons and may ultimately outweigh the beneficial aspects of antiviral immune responses.

Infection with *M. tuberculosis* results in a state of immune activation, more so, when there is concomitant HIV infection<sup>6</sup>. Three diagnostic markers are best documented to have significance in relation to prognosis of HIV infection; these include HIV viral load, CD4+ T cell levels and plasma levels of soluble markers of immune activation<sup>7</sup>. There has been a suggestion that some markers could be used to assess response to TB treatment in HIV infected patients<sup>8</sup>. In this study an attempt was made to correlate plasma levels of immune activation markers neopterin, beta-2-microglobulin ( $\beta_2M$ ), and soluble tumour necrosis factor alpha-receptor type I (sTNF $\alpha$ -RI) with the presence of active TB in HIV-infected and uninfected individuals, and to study the changes following anti-tuberculosis treatment (ATT).

### Material & Methods

**Study population:** Forty five HIV+ individuals with newly diagnosed active pulmonary TB (HIV+TB+) were recruited from a hospital in Chennai, south

India, treating co-infected patients. Thirty seven HIV+ individuals without active TB (HIV+TB-) and 39 TB patients without HIV infection (HIV-TB+) along with 62 healthy laboratory volunteers served as controls. All the HIV positive and TB patients included in this study were selected from a cohort of individuals recruited from the HIV/TB clinic at the Tuberculosis Research Centre (TRC), Chennai, India, during the period 2002-2004 to participate in an ongoing controlled clinical trial for HIV/TB at TRC. The study was approved by the Institutional Ethics Committee.

The diagnosis of TB was based on clinical features, chest X-ray as well as sputum smear examination for presence of acid-fast bacilli, and confirmed by culture for *M. tuberculosis*. After pretest counselling and obtaining informed consent from the patients, their HIV status was determined using a rapid HIV test which could detect HIV-1 as well as HIV-2 infections (HIV TRI-DOT, J. Mitra & Co., India). Positive results were confirmed using a second rapid HIV test (Comb AIDS, Span Diagnostics, India) or Western blot (J. Mitra, India). HIV-2 was not detected in any of the subjects tested. None of the patients were receiving ATT or anti-retroviral therapy (ART) at the time of intake to the study.

**Samples:** Venous blood (10 ml) was collected from all individuals in EDTA-coated vacutainer tubes (Becton Dickinson, USA). After the initial (baseline) blood draw, all TB patients were started on a six-month short course chemotherapy regimen containing rifampicin (450/600 mg based on body weight), isoniazid (600 mg), pyrazinamide (1500 mg) and ethambutol (1200 mg) thrice weekly for two months, followed by rifampicin (450 mg) and isoniazid (600 mg) thrice weekly for four months. In addition, all HIV-positive patients with CD4+ T cell counts  $\leq 200$  cells/ $\mu$ l of blood were given co-trimoxazole double strength one tablet daily. All

**Table.** Changes in levels of plasma immune activation markers from baseline to the end of six months of anti-TB treatment among HIV-infected and uninfected TB patients

Immune activation markers	HIV+TB+				HIV-TB+ (n=37)	
	CD4+ T cell counts $\leq 200$ cells/ $\mu$ l (n=24)		CD4+ T cell counts $> 200$ cells/ $\mu$ l (n=18)		0 month	6 month
	0 month	6 month	0 month	6 month		
Neopterin (ng/ml)	24.4 $\pm$ 5.9	15.0 $\pm$ 5.9	12.5 $\pm$ 2.7	10.7 $\pm$ 4.4	13.8 $\pm$ 3.5	7.3 $\pm$ 2.1*
$\beta_2$ -M (ng/ml)	5241 $\pm$ 1101	3700 $\pm$ 292	4643 $\pm$ 809	4776 $\pm$ 761	4979 $\pm$ 983	4462 $\pm$ 836
sTNF $\alpha$ -RI (ng/ml)	4.2 $\pm$ 0.6	3.0 $\pm$ 0.3*	3.4 $\pm$ 0.5	2.7 $\pm$ 0.4	3.6 $\pm$ 0.5	2.0 $\pm$ 0.3*

Values are expressed as mean  $\pm$  standard error of mean; \* $P < 0.05$  (0 vs. 6 month within each group); HIV+TB+: HIV positive patients with active TB; HIV-TB+: HIV negative TB patients;  $\beta_2M$ : beta 2 microglobulin; sTNF $\alpha$ -RI: soluble tumour necrosis factor  $\alpha$ -receptor type I

TB drugs were acquired through the Revised National Tuberculosis Control Program (RNTCP), India. At the end of six months of chemotherapy, blood was again collected from all TB patients (both HIV positive and HIV negative) who were on ATT. Blood was obtained only at one time point from all other study subjects. Total and differential white blood cell counts were determined using an automated hematology analyzer (ABX, France). Plasma was separated and stored frozen at  $-80^{\circ}\text{C}$  until use.

*Determination of CD4+ T cell count:* Percentage of CD4+ T cells in peripheral blood was determined by staining whole blood samples with fluorescence tagged monoclonal antibodies and analyzing them by flow cytometry using a FACSsort flow cytometer (Becton Dickinson, USA), employing the Cell Quest software (Becton Dickinson, USA).

*Estimation of plasma levels of immune activation markers:* Commercially available enzyme-linked immunosorbent assays were used to measure plasma concentrations of neopterin (IBL, Hamburg, Germany),  $\beta 2\text{M}$  (Immunotech, Beckman Coulter) and sTNF $\alpha$ -RI (Biosource International). All assays were performed according to the manufacturers' protocol. Every plate included identical set of standards and ten per cent of the samples were tested in duplicate to rule out inter- and intra-test variability.

*Statistical analysis:* Only TB patients (HIV-infected or uninfected) who became sputum smear and culture negative after six months of ATT and showed clinical and radiological improvement, were included for the analysis.

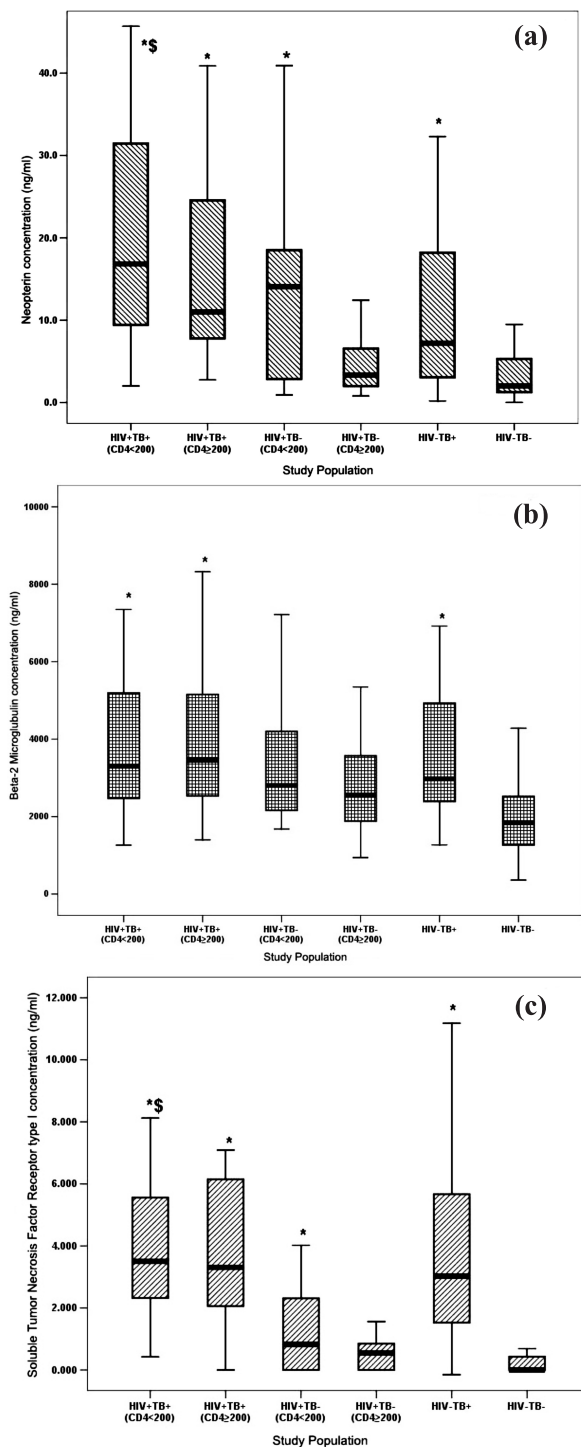
All statistical analyses were performed using the Statistical Package for Social Sciences, version 8.0 (SPSS Inc, Chicago). The results in each group were expressed as mean  $\pm$  SE (standard error of mean). Wilcoxon rank test and Mann-Whitney U test were used to compare paired and unpaired data respectively. Data from the different groups of study participants were also compared by ANOVA. Statistical significance was defined as  $P < 0.05$ . Correlations between various parameters within each patient group were calculated using the Spearman's rank correlation coefficient.

## Results

Three of the HIV+TB+ patients and one HIV-TB+ patient continued to be culture positive for *M. tuberculosis* and were therefore excluded from the analysis. The HIV negative and HIV positive TB

patients did not differ in body mass index (BMI), degree of smear positivity or chest X-ray grading at baseline. However, as expected, CD4+ T cell numbers were significantly lower in the HIV positive subjects than in HIV negative cases. Based on CD4+ T cell counts, the HIV+TB+ group ( $n=42$ ) was further divided into two subgroups, one with CD4+ T cell counts  $< 200$  ( $n=24$ ) and another with CD4+T  $> 200$  cells/ $\mu\text{l}$  of blood ( $n=18$ ); the former subgroup had a mean CD4+ T cell count of  $97 \pm 27$  and the latter had a mean CD4+ T cell count of  $488 \pm 55$ . The HIV+TB- group ( $n=37$ ) was again divided into two categories, those with CD4+T cell counts  $< 200$  ( $n=17$ ) and those with CD4+T cell counts  $> 200$  cells/ $\mu\text{l}$  of blood ( $n=20$ ); while the former subgroup had a mean CD4+ T cell count of  $108 \pm 12$ , the latter had a mean CD4+ T cell count of  $362 \pm 35$  cells. The HIV-TB+ group had mean CD4+ T cell counts of  $678 \pm 59$  cells/ $\mu\text{l}$  of blood. CD4+ T cell counts were not measured in healthy individuals.

At the time of diagnosis of active TB, plasma levels of neopterin were found to be significantly elevated above normal in both HIV and TB patients ( $P < 0.05$ ), with the highest levels seen in HIV+TB+ patients with CD4+ T cell counts  $\leq 200$  cells/ $\mu\text{l}$  ( $24.4 \pm 5.9$  ng/ml) (Fig. a). HIV-positive patients without active TB had significantly lower levels of plasma neopterin than that of CD4+ T cell count matched HIV+TB+ patients ( $11.9 \pm 2.8$  ng/ml vs.  $24.4 \pm 5.9$  ng/ml in those with CD4+ T cell counts  $\leq 200$  cells/ $\mu\text{l}$ , and  $4.8 \pm 2.1$  vs.  $12.5 \pm 2.7$  in those with  $> 200$  cells/ $\mu\text{l}$   $P < 0.001$ ), but higher than healthy controls ( $3.9 \pm 0.6$  ng/ml). Baseline levels of  $\beta 2\text{M}$  were also significantly higher in HIV+ as well as TB patients when compared to healthy controls ( $P < 0.05$ ) (Fig. b). Among the HIV+TB+ patients, those with CD4+ T cell count  $> 200$  cell/ $\mu\text{l}$  of blood had plasma levels of  $\beta 2\text{M}$  similar to that seen in HIV-TB+ patients ( $4643 \pm 809$  ng/ml in the former group and  $4979 \pm 983$  ng/ml in the latter group), but lower than in HIV+TB+ patients with low CD4+ T cell counts ( $\leq 200$  cells/ $\mu\text{l}$ ) and advanced HIV disease ( $5241 \pm 1101$  ng/ml). HIV+TB- individuals had lower levels of these markers than those dually infected with HIV and TB. Plasma levels of sTNF $\alpha$ -RI were found to be significantly elevated above normal in both HIV and TB patients ( $P < 0.05$ ), with the highest levels seen in HIV+TB+ patients with CD4+ T cell  $\leq 200$  cells/ $\mu\text{l}$  ( $4.2 \pm 0.6$  ng/ml) (Table, Fig. c). HIV+TB- patients had significantly lower levels of sTNF $\alpha$ -RI than that of CD4+ T cell count matched HIV+TB+ patients ( $1.6 \pm 0.4$  ng/ml vs.



**Fig.** Plasma levels of immune activation markers in the study groups. **(a)** Neopterin, **(b)** beta-2-microglobulin, **(c)** soluble TNF $\alpha$  receptor type I. In the figure the bars represent median values, boxes represent quartile 1 and quartile 3 and whiskers represent the range of the data set. HIV+TB+: HIV positive patients with active TB (n=42); HIV-TB+ : HIV negative TB patients (n=39); HIV+TB-: HIV positive individuals without active TB (n=37); HIV-TB-: Healthy controls (n=62). \* $P$ <0.05 in comparison with healthy controls;  $^{\$}$  $P$ <0.05 in comparison with both the groups of HIV+TB- individuals.

$4.2 \pm 0.6$  ng/ml in those with CD4+ T cell counts  $\leq 200$  cells/ $\mu$ l, and  $0.6 \pm 0.1$  vs.  $3.4 \pm 0.5$  in those with CD4 counts  $>200$  cells/ $\mu$ l  $P$ <0.001), but higher than healthy controls ( $0.4 \pm 0.1$  ng/ml).

At the end of ATT, CD4+ T cell counts increased from  $97 \pm 27$  to  $271 \pm 127$  in the HIV+TB+ patients with CD4+ T cell counts  $\leq 200$  cells/ $\mu$ l of blood ( $P$ <0.05). The CD4/CD8 ratio also improved from  $0.14 \pm 0.08$  to  $0.21 \pm 0.04$  in this group. On the other hand, there was no significant improvement either in the CD4+ T cell count or CD4/CD8 ratio in HIV+TB+ patients with CD4+ T cell counts  $>200$  cells/ $\mu$ l at baseline.

Following ATT, mean sTNF $\alpha$ -RI levels declined significantly in the HIV+TB+ group with CD4+ T cell counts  $>200$  cells/ $\mu$ l as well as in the HIV-TB+ group (Table). A significant decrease in plasma neopterin levels was also observed in the HIV-TB+ group, but the decline was not significant in the HIV+TB+ group.  $\beta$ 2M levels remained persistently elevated at the end of six months of ATT in all TB patients.

Further, at the end of ATT, while 90 per cent of HIV+TB+ patients had neopterin levels above the mean + 2 SD (standard deviation) of the normal value, only 10 per cent of individuals in the HIV-TB+ group continued to have levels above this range. This proportion was 69 and 30 per cent for sTNF $\alpha$ -RI and 50 and 41 per cent for  $\beta$ 2M, respectively.

A significant negative correlation was observed between baseline CD4+ T cell count and circulating levels of immune activation markers (CD4+ T cell counts vs. neopterin  $r^2 = -0.297$ ;  $P$ <0.05;  $\beta$ 2M =  $-0.339$ ,  $P$ <0.05 and sTNF $\alpha$ -RI =  $0.299$ ,  $P$ <0.05). However, there was no significant correlation between levels of immune activation and CD4+ T cell counts at the end of ATT.

### Discussion

Immune stimulation occurs very early in HIV infection, and evidence of it can be found in the first serum samples that demonstrate seroconversion<sup>9,10</sup>. Not only does a specific immune response occur, but widespread activation of CD4+ and CD8+ T cells, B cells, natural killer (NK) cells and macrophages also occurs<sup>11,12</sup>. Immune cell activation leads to increased production of many cytokines and increased expression of cytokine receptors and soluble products of activation that can be identified and quantitated by measurements in plasma or serum. There is evidence to show that the presence of clinical TB in HIV infection

results in continuous cellular activation, which in turn leads to conditions that favour viral replication and disease progression<sup>7,13-16</sup>. However, the association of these changes with treatment of TB and clinical outcome in the co-pathogenesis of HIV and TB needs to be evaluated.

Earlier studies have demonstrated that markers of immune activation can predict the future rate of decrease in CD4+ T cells<sup>17</sup>, and that progression to AIDS can be predicted more accurately by combining the CD4+ T cell counts with levels of soluble activation markers<sup>18</sup>. Further, changes in soluble activation markers have been found to be more closely related to viral load than to CD4+ T cells changes<sup>19</sup>. Increased neopterin levels in blood and urine may be viewed as an indication of immune activity<sup>17,20-22</sup>. Levels of  $\beta$ 2M in plasma strongly reflect the degree of immune system activation, and increased concentration of these molecules have been reported to predict progression of HIV infection to AIDS<sup>23,24</sup>. Level of sTNF- $\alpha$  receptors correlate with their induction by TNF- $\alpha$ , and promote active expression of latent HIV in monocytes and lymphocytes<sup>25</sup>. All three markers have been studied extensively and shown to increase early in HIV infection and correlate with HIV disease progression<sup>18,26,27</sup>. However, their prognostic value in HIV+ individuals who have concurrent TB is not clear. We therefore made an attempt to measure circulating levels of immune activation markers in HIV+ individuals with and without active TB, and monitor changes, accompanying successful ATT.

At the time of diagnosis of active TB, plasma levels of neopterin,  $\beta$ 2M and sTNF $\alpha$ -RI were significantly elevated in both HIV+ and HIV negative TB patients when compared to healthy controls, with the highest levels seen in the most immunosuppressed HIV+TB+ patients. This also corroborates with an earlier study wherein we demonstrated that highest levels of the proinflammatory cytokine IFN- $\gamma$ , whose surrogate marker is neopterin, was present in HIV+TB+ patients with CD4+ T cell counts <200 cells/ $\mu$ l<sup>28</sup>. Aziz *et al*<sup>29</sup> also reported progressive increase in levels of IFN- $\gamma$  and neopterin when HIV-seropositive individuals were stratified on the basis of CD4+ T cell count, with the highest levels being present in the category with CD4+ T cell counts  $\leq$ 200 cells/ $\mu$ l.

After six months of ATT, plasma levels of neopterin and sTNF $\alpha$ -RI were found to decline significantly in both HIV positive and HIV negative TB patients.

In spite of the significant decline following ATT, post-treatment neopterin levels were significantly higher than normal values in a larger proportion of dually infected individuals than those with TB alone. This observation suggests that though ATT results in successful elimination of the pathogen in six months, the effect of TB on the host immune system in HIV positive individuals may take much longer to resolve, or that the underlying HIV disease is the cause for ongoing immune activation. In a previous study from our Centre Immanuel *et al*<sup>30</sup> reported that though serum neopterin levels declined with ATT, their levels continued to be higher in HIV+TB+ patients than in HIV-TB+ patients<sup>30</sup>.

Lawn *et al*<sup>8</sup> suggested that sTNF $\alpha$ -RI was predominantly associated with TB and  $\beta$ 2M with HIV and these could therefore serve as relatively independent markers of TB and HIV disease activity in HIV co-infected patients. In our study, comparison of pre- and post-treatment levels of sTNF $\alpha$ -RI showed that both the HIV+TB+ group with CD4+ T cell count >200 cells/ $\mu$ l and TB+HIV- group showed a significant decline in circulating levels of sTNF $\alpha$ -RI following ATT. However, 69 per cent of individuals in the HIV+TB+ group and 30 per cent of individuals in the HIV-TB+ group had levels above the normal range after six months of ATT. Further, among HIV+TB- patients sTNF $\alpha$ -RI levels were significantly increased above normal in those with low CD4+ T cell counts, suggesting that this marker is not specific for TB. Elevated levels of  $\beta$ 2M were found to persist at the end of six months of ATT in all groups.  $\beta$ 2M levels represent the activity of several cytokines throughout the body and is therefore a relatively nonspecific marker of immune activation.  $\beta$ 2M has been reported to be elevated in infectious diseases that affect lymphocytes, including cytomegalovirus, hepatitis, mononucleosis, and HIV infection<sup>31</sup>. Our data suggested that none of these markers were specific enough to be used to assess response to ATT especially in individuals with HIV. The conventional techniques of sputum smear and culture, radiography and clinical outcome will continue to be used till further sensitive and specific biomarkers are discovered. Our study had certain limitations as the viral load was not estimated for the HIV patients and follow-up time was limited.

In conclusion, our findings showed that plasma concentration of immune activation markers were elevated in patients infected with HIV and that the increase was related to the stage of HIV disease. HIV

and TB have additive effects on the immune system which may contribute to the mutually unfavourable effects of co-infection. Further incomplete immunological recovery after six months of ATT indicating that rate of immune restoration does not match that of microbiological clearance and resolution of TB<sup>32</sup>.

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