Publications:


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Tumour necrosis factor-alpha and nitric oxide response in different categories of tuberculosis patients

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OBJECTIVE: To compare the magnitude of tumour necrosis factor alpha (TNF-α) and nitric oxide (NO) response in different categories of active tuberculosis (TB) patients by ex vivo experiment.

DESIGN: New, relapsed (recurrent), miliary and pleural effusion TB cases were recruited with matched healthy controls. TNF-α and NO were measured from the culture supernatant of peripheral blood monocytes derived from cases and controls with and without challenge with live Mycobacterium tuberculosis H37Rv.

RESULTS: TNF-α and NO production varied significantly among the different categories of TB patients. The magnitude was highest among patients with pleural effusion and lowest in miliary TB cases. In between, progressive decreases in response were noted in new and relapse cases. Overall, positive correlations between TNF-α and NO were noted among the diseased and healthy groups.

CONCLUSION: Distinct TNF-α and NO levels appear to be associated with different clinical forms of TB and might help to assess prognosis and contribute to a better understanding of underlying immunopathological mechanisms.

KEY WORDS: TB; monocytes; relapse; miliary; pleural effusion

AFTER INFECTION, tuberculosis (TB) develops in only ~10% of cases, while ~90% of infected persons do not develop the disease in their lifetime.† Deficiencies in the host immune responses are one of the important factors for determining the pathogenesis of the disease. Mononuclear cells, including macrophages and dendritic cells, play a pivotal role during their initial encounter with Mycobacterium tuberculosis by their intrinsic or innate defence mechanisms.

Reactive nitrogen intermediates (RNIs) are the first line of defence against surviving intracellular pathogens,‡ and in mammalian systems macrophages are the main source of nitric oxide (NO). Production of cytokines, such as tumour necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ) and interleukin 1β (IL-1β), by macrophages are essential in the immune response against mycobacteria. TNF-α is indispensable in the inflammatory response necessary for host defence against M. tuberculosis.‡ Animals lacking the gene for TNF-α or treated with anti-TNF-α antibody show increased susceptibility to M. tuberculosis and other pathogens.‡ TNF-α induces NO generation in macrophages by upregulation of inducible nitric oxide synthase (iNOS). This cytokine is also involved in the enhanced induction of IL-1β and IL-6 in macrophages.‡§ NO induces the production of pro-inflammatory cytokines, including TNF-α.¶,†† NO and related RNIs can kill/inhibit intracellular pathogens such as mycobacteria.¶,†† IFN-γ knockout mice that are not capable of producing NO and RNI in response to tubercle bacilli experience a fulminant course of TB, suggesting a role of NO and RNI in the defence mechanism against M. tuberculosis.¶ There is evidence to suggest that the production of NO and related RNIs correlate with the antimycobacterial effect of murine macrophages. Moreover, iNOS knockout infected mice exhibit a preponderance of pulmonary and splenic mycobacterial load compared to wild-type infected mice.¶ These facts highlight the importance of NO in the restriction of intracellular multiplication of pathogens. The role of TNF-α and NO in different diseases clearly indicates how pro-inflammatory responses are important in disease processes.

Human TB shows a variety of presentations. TB pleuritis is a paucibacillary infection where innate immune cells and associated pro-inflammatory cytokines play an important role in the immunological processes of granulomatous tissue reaction;¶ in new and relapsed pulmonary TB cases, the immune system cannot restrict the pathogen completely, but could confine the pathology to a limited area, while

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Miliary TB is a common manifestation of lymphohematogenous dissemination of tubercle bacilli throughout the lung and other viscera.

Given the above, we hypothesised that innate immune responses might vary with different clinical status of TB. The present study was therefore intended to investigate whether TNF-α and NO response by monocytes were associated with different clinical forms of TB, i.e., new, relapse, miliary and TB with pleural effusion.

MATERIALS AND METHODS

Study population

Twenty-four TB patients (10 new, 10 relapse and 4 miliary) and 10 with tuberculous pleural effusion attending the Chest Clinic and admitted to the Chest Ward of Calcutta National Medical College, Kolkata, India, were recruited from August 2011 to January 2012. The inclusion criteria for pulmonary TB cases were positive sputum smear on Ziehl-Neelsen (ZN) stain and chest X-ray (CXR) consistent with TB. For miliary TB cases, the inclusion criteria were presence of constitutional symptoms suggestive of TB, miliary mottling on CXR and favourable response to anti-tuberculosis treatment during 6-month follow-up. In patients with pleural effusion, the inclusion criteria were CXR suggestive of effusion, positive tuberculin skin test (TST) results, adenosine deaminase criteria were CXR suggestive of effusion, positive tuberculin skin test (TST) results, adenosine deaminase criteria were CXR suggestive of effusion, positive tuberculin skin test (TST) results, adenosine deaminase criteria were CXR suggestive of effusion, positive tuberculin skin test (TST) results, adenosine deaminase criteria were CXR suggestive of effusion, positive tuberculin skin test (TST) results, adenosine deaminase criteria were CXR suggestive of effusion, positive tuberculin skin test (TST) results, adenosine deaminase criteria were CXR suggestive of effusion, positive tuberculin skin test (TST) results, adenosine deaminase criteria were CXR suggestive of effusion, positive tuberculin skin test (TST) results, adenosine deaminase.

Preparation of single-cell suspension and quantification of M. tuberculosis

M. tuberculosis H37Rv was inoculated in Löwenstein-Jensen medium and incubated at 37°C for 3 weeks. The colonies were transferred in normal saline and centrifuged; the supernatant was then removed and the pellet resuspended in normal saline. The suspension was transferred to a new vial containing 4–5 glass beads and subjected to repeated vortexing. The cell suspension was then kept undisturbed overnight to settle down the clumps. Next, the upper part of the cell suspension was carefully transferred to another vial followed by centrifugation and removal of the supernatant. Finally, the pellet was homogeneously resuspended in 1 ml RPMI 1640, and 100 μl of the final suspension was subjected to ZN staining. The stained bacterial sample was diluted and counted in a haemocytometer. After quantification, the final suspension was used for infection of the monocytes.

Infection of monocytes

The volume of mycobacterial suspension was calculated according to the number of monocytes, to achieve a multiplicity of infection of 5. After infection, the monocytes were incubated for 3 h, washed with fresh RPMI to remove any extracellular bacteria and incubated further.

Nitric oxide production assay from cell supernatant

NO generation was estimated by sampling culture supernatants for nitrite, which is a stable product of the NO reaction. Lipopolysaccharide (LPS, 100 ng/ml) and IFN-γ (100 IU/ml) were used as positive controls for NO production. After 48 h, 100 μl volumes of cell-free supernatant were mixed with an equal volume of Griess reagent (0.5% sulfanilamide and 0.05% N-1-naphthyl ethylenediamine dihydrochloride in 2.5% phosphoric acid) and incubated for 10 min at 25°C. Optical densities of the samples were subsequently measured at 550 nm. The nitrite concentration was determined by reference to a standard curve using sodium nitrite (10–100 μM/l) diluted in culture medium.

Tumour necrosis factor-alpha release assay

The level of TNF-α was estimated after 48 h of infection from the cell-free culture supernatant by sandwich enzyme-linked immunosorbent assay kits, per
the manufacturer’s instructions (Immuno Tools GmbH, Friesoythe, Germany).

Statistical analysis
The experiments were performed at least three times, and the data are presented as mean ± standard deviation (SD). One way analysis of variance (ANOVA) was used to compare the TNF-α and NO estimations when five groups were used for comparison, and unpaired two-tailed t-test when two groups were used for comparison using Graph Pad Prism software, version 5.00 (GraphPad Software Inc, La Jolla, CA, USA). Pearson’s correlation coefficient was calculated between TNF-α and NO generation. P < 0.05 was considered significant.

RESULTS

The demographic and clinical characteristics of the patients and healthy controls are summarised in Table 1. The TNF-α and NO released by the monocytes of the four different patient groups and the healthy subjects were measured with and without the stimulation of live M. tuberculosis (H37Rv) and LPS with IFN-γ (Figures 1 and 2). The results showed that, except for the miliary TB cases, unstimulated (control) monocytes from the TB patients produced more TNF-α than the healthy controls. The patterns were similar for NO release by unstimulated monocytes. After stimulation with live virulent M. tuberculosis, the monocytes of the patients with pleural effusion and newly diagnosed patients produced very high levels of TNF-α (respectively 1173 ± 89.63 pg/ml, P < 0.0001 and 912.6 ± 101.5 pg/ml, P < 0.0001) compared to the healthy controls. NO generation of these two categories of patients showed a similar pattern (respectively 33.41 ± 7.77 pg/ml, P < 0.0001 and 30.2 ± 3.96 pg/ml, P < 0.0001). Among the relapsed TB patients, the TNF-α level was found to be significantly low (118.4 ± 31.89 pg/ml, P = 0.0006), whereas no significant change was observed for NO release with the same stimulation (9.1 ± 2.106 pg/ml). These responses were lowest among the miliary TB cases (respectively 40 ± 14.35 pg/ml, P = 0.0016 and 4.675 ± 2.304 pg/ml, P = 0.0006) compared to all other study cases.

As iNOS is transcriptionally under the control of TNF-α and IFN-γ,21 we stimulated the monocytes by LPS with IFN-γ. With these stimulations, monocytes of all the categories of TB patients were found to produce significantly high levels of TNF-α and NO. These responses were highest among the pleural effusion cases (1209 ± 70.95 pg/ml, P < 0.0001 and 51.84 ± 7.54 μM/l, P < 0.0001), followed by new TB cases (1009 ± 154 pg/ml, P < 0.0001 and 38.95 ± 3.7 μM/l, P < 0.0001). Significantly lowered responses of TNF-α and NO were observed in relapsed TB cases (142.3 ± 23.63 pg/ml, P < 0.0001 and 11.67 ± 1.31 μM/l, P < 0.0001). In miliary TB cases, these responses were found to be further lowered (respectively 51.25 ± 18.48 pg/ml, P < 0.0001 and 5.925 ± 1.64 μM/l, P < 0.0001) compared to

Table 1  Demographic and clinical characteristics of the study participants

<table>
<thead>
<tr>
<th></th>
<th>New TB (n = 10)</th>
<th>Relapsed TB (n = 10)</th>
<th>TB with pleural effusion (n = 10)</th>
<th>Miliary TB (n = 4)</th>
<th>Healthy controls (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>42.9 ± 14</td>
<td>31.8 ± 7</td>
<td>30.5 ± 4.5</td>
<td>28.5 ± 9.5</td>
<td>32.3 ± 3.199</td>
</tr>
<tr>
<td>Male:female ratio</td>
<td>3:2</td>
<td>7:3</td>
<td>9:1</td>
<td>4:0</td>
<td>3:2</td>
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<tr>
<td>Chest X-ray</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimal</td>
<td>4 (40)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Moderately advanced</td>
<td>4 (40)</td>
<td>4 (40)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Far advanced</td>
<td>2 (20)</td>
<td>4 (40)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cavity</td>
<td>0</td>
<td>2 (20)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Miliary mottling</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4 (100)</td>
<td>0</td>
</tr>
<tr>
<td>Pleural effusion</td>
<td>0</td>
<td>0</td>
<td>10 (100)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Clinical findings</td>
<td></td>
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<td>Sputum</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Smear positivity</td>
<td>10 (100)</td>
<td>10 (100)</td>
<td>0</td>
<td>2 (50)</td>
<td>0</td>
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<td>Culture positivity</td>
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<td>Pleural fluid</td>
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<td>Smear positivity</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Culture positivity</td>
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<td>0</td>
<td>2 (20)</td>
<td>0</td>
<td>0</td>
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<td>ADA, IU/l</td>
<td>0</td>
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<td>85.2 ± 15.54</td>
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<td>Lymphocyte*</td>
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<td>0</td>
<td>86.15 ± 5.13</td>
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<td>0</td>
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<tr>
<td>Weight, kg</td>
<td>47.2 ± 6</td>
<td>43.8 ± 3</td>
<td>56.2 ± 5</td>
<td>38.6 ± 3</td>
<td>59.8 ± 5.35</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>20.45 ± 1.2</td>
<td>18.34 ± 1.2</td>
<td>26.45 ± 1.3</td>
<td>16.88 ± 1.5</td>
<td>23.54 ± 0.99</td>
</tr>
<tr>
<td>Haemoglobin, g%</td>
<td>11.7 ± 0.7</td>
<td>11.3 ± 0.9</td>
<td>12.5 ± 0.8</td>
<td>10.3 ± 0.7</td>
<td>12.41 ± 0.92</td>
</tr>
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<td>ESR</td>
<td>45.4 ± 1.5</td>
<td>46.3 ± 1.6</td>
<td>35.6 ± 1.5</td>
<td>73.3 ± 1.5</td>
<td>13 ± 2.981</td>
</tr>
</tbody>
</table>

*Percentage of total WBC.

TB = tuberculosis; SD = standard deviation; ADA = adenosine deaminase; BMI = body mass index; ESR = erythrocyte sedimentation rate.
all the study groups after LPS with IFN-γ stimulation. Overall, significant positive correlations between TNF-α and NO production were observed in all of the study cases (Table 2).

**DISCUSSION**

In the present study, we report distinct TNF-α and NO profiles of the innate immune cells among four different groups of TB patients. To our knowledge, this is the first study to evaluate NO and TNF-α responses within a wide variety of active TB patients. Production of NO by innate immune cells is an important phenomenon to restrict the growth or killing of the pathogen in TB. Several studies have demonstrated NO and TNF-α generation by PBMCs, whole blood and from the serum of TB patients with different clinical status. However, studies demonstrating the role of innate immune cells in generating effective immune responses in different clinical types of TB are scarce. For this purpose, we treated the monocytes of four different TB patients with live mycobacterial challenges to evaluate the effective immune responses.

According to our study, TNF-α and NO responses were highest among the TB patients with pleural effusion compared to the other study groups. A previous study also documented very high levels of TNF-α in pleural fluid as well as in the plasma of this category of patients compared to pulmonary TB patients. The present study also showed higher TNF-α and NO release by the monocytes of newly diagnosed TB cases than among healthy controls. These results

<table>
<thead>
<tr>
<th>Study group</th>
<th>Control (unstimulated)</th>
<th>M. tuberculosis-infected</th>
<th>LPS + IFN-γ stimulated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( r_p ) ( P ) value</td>
<td>( r_p ) ( P ) value</td>
<td>( r_p ) ( P ) value</td>
</tr>
<tr>
<td>New TB (n = 10)</td>
<td>0.8724 0.001</td>
<td>0.8437 0.0022</td>
<td>0.9198 0.0002</td>
</tr>
<tr>
<td>Relapsed TB (n = 10)</td>
<td>0.9398 &lt;0.0001</td>
<td>0.7580 0.0111</td>
<td>0.8822 0.0007</td>
</tr>
<tr>
<td>TB with pleural effusion (n = 10)</td>
<td>0.9633 &lt;0.0001</td>
<td>0.9182 0.0002</td>
<td>0.9144 0.0002</td>
</tr>
<tr>
<td>Miliary TB (n = 4)</td>
<td>0.9601 0.0399</td>
<td>0.9643 0.0357</td>
<td>0.7614* 0.0169</td>
</tr>
<tr>
<td>Healthy control (n = 10)</td>
<td>0.6444 0.04</td>
<td>0.810 0.0045</td>
<td>0.9215 0.0002</td>
</tr>
</tbody>
</table>

*Non-significant.

TNF-α = tumour necrosis factor-alpha; NO = nitric oxide; \( r_p \) = Pearson’s correlation coefficient; LPS = lipopolysaccharide; IFN-γ = interferon-gamma; TB = tuberculosis.
are consistent with other studies where monocytes of TB patients showed heightened TNF-α and NO production compared to their healthy counterparts. Previous studies based on alveolar macrophages also demonstrated higher TNF-α and NO release by TB patients than healthy controls. We suggest that the monocytes of new and pleural effusion TB patients were already primed with mycobacterial antigens in vivo, thus resulting in heightened response after ex-vivo stimulation. On the other hand, the healthy controls in our study had no previous exposure to mycobacterial antigens, as evident from their TST negativity.

However, very poor NO generation was observed in relapsed patients, followed by miliary TB cases, where the disease is disseminated. The monocytes of these TB patients could have been anergic due to repeated mycobacterial antigenic exposure in vivo, resulting in poor immune responses after ex-vivo antigenic challenges. Relapsed and miliary TB cases thus showed progressively higher deficiencies of protective innate immune responses than pleural effusion and new TB cases.

The immune responsiveness of immunocompetent individuals varied between the healthy controls and the different categories of TB patients. Differential responses by the monocytes of different groups of subjects toward the same antigenic stimulus indicated the importance of host-related factors in the pathogenesis of TB. Although there have been contradictory reports, we and others found a positive correlation between TNF-α and NO generation by monocytes among almost all of the study groups. In our study, the significant variations in TNF-α and NO responses by monocytes indicated a sharp distinction between every category of TB patients and corroborated with their clinical status.

CONCLUSIONS

The pattern of our data showed clear distinctions between the different clinical types of TB patients by their innate immune responses. Identification of specific immune deficiency could help develop specific strategies for immunotherapy, regardless of the drug resistance patterns of the pathogen. The present study may contribute to a better understanding of individual cell function and immunopathological mechanisms associated with the different clinical forms of TB.

Acknowledgement

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References

OBJECTIF: Comparer l’importance de la réponse du facteur alpha de nécrose tumorale (TNF-α) et de l’oxyde nitrique (NO) dans différentes catégories de patients atteints de tuberculose (TB) active au moyen d’une expérimentation ex vivo.

SCHEMA: On a recruté des cas TB nouveaux, des rechutes (ou reprises), des cas miliaire et d’épanchements pleuraux en même temps que des sujets-contrôle sains. On a mesuré la TNF-α et le NO à partir du surnageant des cultures des monocytes du sang périphérique provenant des cas et des contrôles avec ou sans provocation par Mycobacterium tuberculosis H37 Rv.

RÉSULTATS: La production de TNF-α et de NO a varié de manière significative dans les différentes catégories de patients TB. L’importance de la production est la plus élevée chez les patients atteints d’épanchement pleural et la plus faible dans les cas de TB miliaire. Entre les deux, on a noté une décroissance progressive des réponses entre les cas neufs et les cas de rechute. Au total, on a noté des corrélations positives entre le TNF-α et le NO tant dans les groupes de malades que dans les groupes de bien portants.

CONCLUSION: Les niveaux distincts de TNF-α et de NO semblent en association avec différentes formes cliniques de TB ; ils pourraient aider à estimer le pronostic et contribuer à une meilleure compréhension des mécanismes immunopathologiques sous-jacents.

RESUMEN

OBJETIVO: Comparar la magnitud de la producción de factor de necrosis tumoral α (TNF-α) y de monóxido de nitrógeno (NO) en muestras sanguíneas de diferentes categorías de pacientes con tuberculosis (TB) activa en experimentos ex vivo.

MÉTODO: Se incluyeron en el estudio casos nuevos, recaídas (recurrencias) de TB, TB miliaire y derrames pleurales de origen tuberculoso y los correspondientes testigos sanos emparejados. Se midió la producción de TNF-α y NO en el sobrenadante de cultivos de monócitos de sangre periférica provenientes de los casos y los testigos, antes y después del estímulo con micobacterias viables del tipo Mycobacterium tuberculosis H37Rv.

RESULTADOS: La producción de TNF-α y NO difirió significativamente en las diferentes categorías de pacientes. La producción más alta se observó en los pacientes con derrame pleural y la más baja en los casos de TB miliaire. Se encontraron respuestas decrecientes progresivas en los casos nuevos y en las recaídas. En general, se observó una correlación positiva entre la producción de TNF-α y NO en los grupos de pacientes y de testigos sanos.

CONCLUSIÓN: Las diferencias en la producción de TNF-α y NO parecen presentar una correlación con las diferentes formas clínicas de la enfermedad tuberculosa; estos datos podrían ayudar a evaluar el pronóstico y contribuir a un mejor conocimiento de los mecanismos inmunopatogénicos subyacentes.
INTRODUCTION
CHAPTER 1

TO STUDY THE FREE RADICALS GENERATION AND THEIR ASSOCIATION WITH TB PATIENTS DURING THE DISEASE PROCESS
CHAPTER 2

To measure the expression and release of different pro and anti-inflammatory cytokines secretion by the macrophage cells isolated from tuberculosis patients and healthy individuals.
CHAPTER 3

TO STUDY THE EXPRESSION OF PROTEIN KINASE CS AND TOLL LIKE RECEPTORS IN TUBERCULOSIS.
PUBLICATIONS