Reduced Phagocytic Capacity of Blood Monocyte/Macrophages in Tuberculosis Patients Is Further Reduced by Smoking

Mahshid Aryanpur1, Esmaeil Mortaz2,3,4, Mohammad Reza Masjedi5, Payam Tabarsi6, Johan Garssen7, Ian M. Adcock4, Alireza Mozafarian3, Hooman Sharifi1

1 Tobacco Prevention and Control Research Center, National Research Institute of Tuberculosis and Lung Diseases (NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran
2 Department of Immunology, National Research Institute of Tuberculosis and Lung Diseases (NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran
3 Chronic Respiratory Diseases Research Center, National Research Institute of Tuberculosis and Lung Diseases (NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran
4 Airways Disease Section, National Heart and Lung Institute, Imperial College London, London, UK
5 Chronic Respiratory Diseases Research Center, National Research Institute of Tuberculosis and Lung Diseases (NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran
6 Clinical Tuberculosis and Epidemiology Research Center, National Research Institute of Tuberculosis and Lung Diseases (NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran
7 Division of Pharmacology and Pathophysiology Utrecht Institute for Pharmaceutical Sciences, Faculty of Sciences, Utrecht University, Utrecht, Netherlands

Received: 11 October 2015; Received in revised form: 20 January 2016; Accepted: 31 January 2016

ABSTRACT

Tuberculosis (TB) and tobacco use are two major alarming global health issues posing immense threats to human populations. Mycobacterium tuberculosis (MTB) by activation of macrophages could induce the sequences of cells activation and releases of inflammatory cytokines such as CXCL-8, IL-12 and TNF-α which in turn induces the immune system network. However no information is available on other activity of cells by MTB and smoking. In the current study we aimed to investigate the serum levels TNF-α, CXCL-8 and phagocytosis capacity in tuberculosis patients with and without smoking.

103 subjects entered the study including 61 new diagnosed pulmonary TB patients (23 smokers and 38 nonsmokers) and 42 control healthy subjects. The phagocytosis of fluorescein isothiocyanate dextran (FITC-dextran) in blood monocytes/macrophages through flowcytometry was assessed. Serum levels of TNF-α and CXCL-8 were analyzed by ELISA methods.

A lower percentage of cells from TB patients who smoked [50.29% (43.4-57.2), p<0.01] took up FITC-dextran after 2h compared to non-smoking TB subjects [71.62% (69.2-74.1)] and healthy cases [97.45% (95.9-99.1)]. Phagocytic capacity was inversely correlated with cigarette smoking as measured by pack years (r=-0.73, p<0.001). The serum levels of TNF-α and CXCL-8 were significantly higher in the TB patients who smoked compared to the TB non-smoker group (p<0.001, p<0.01 respectively).

Blood monocytes/macrophages from TB patients have reduced phagocytic capacity which is further reduced in TB patients who smoke. Smoking enhanced serum levels of TNF-α and CXCL-8 suggesting a greater imbalance between the proinflammatory and anti-inflammatory factors in these patients.

Keywords: Chemokine CXCL8; Phagocytosis; Smoker; TNF-alpha; Tuberculosis
INTRODUCTION

The association between tobacco smoke and tuberculosis (TB) has been considered recently. Tuberculosis (TB) and tobacco use are two major alarming global health issues posing immense threats to human populations. Most of the morbidity and mortality from tuberculosis occurs in developing countries, which incidentally, smoking is still the most common problem. Mycobacterium Tuberculosis (Mt) is an intracellular pathogen of human mononuclear phagocytes. As with many other bacteria, the interaction of Mt with innate and adaptive immune mechanisms specifies the infection prognosis. Mt has developed mechanisms to evade from immune surveillance at many steps in the host response. Mt may impair the recognition of phagocytic activity of macrophages and neutrophils in different ways.

It has been proposed that the correlation of TB and smoking could be due to the inhibitory effect of nicotine on production of TNF-α by the lung macrophages which make the patients susceptible to the development of progressive latent infection to the active form.

Smoking impairs the phagocytic function of alveolar macrophages. Both smoking and tuberculosis induce apoptosis of macrophages. Cigarette smoke activates macrophages to produce a local inflammatory response, but nicotine as important component of smoke suppresses the antigen presentation function to develop a specific immune response. Chronic exposure to cigarette smoke reduces expression of surface proteins related to antigen presentation by macrophages.

Macrophage/monocyte is important in the function of the immune system and macrophage-derived cytokines play role in the immune system. Mt infection recruit the Inflammatory cells in the lungs and these infiltrating cells constitute into a construction named granuloma that controls and contains infection. The formation process of granulomas and its influencing factors are unknown but it seems that the TNF-α plays an important role in this process. TNF-α produced by the macrophages which activated by endotoxins. Treatments that modulate TNF-α production are reported to be associated with higher susceptibility of patients to TB disease and may reactivate the latent form of diseases.

Interleukin-8 (CXCL8) is found to be chemotaxis attraction and regulation of white blood cell production which can affect the pathogenesis of intensive infectious diseases such as TB by suppressing the normal immune response to Mt that can lead to granuloma formation. Although monocytes and macrophages infected with Mt are the main sources of CXCL8 production, it can also be produced by neutrophils and respiratory epithelial cells as well. It is probable that CXCL8 is responsible for aggregation of neutrophils at infection sites in TB patients, however, it has not been proven yet.

Cigarette smoking is a major risk factor for acute and chronic respiratory tract infections and can be expected to affect human health. Modulation of macrophage function by tobacco and smoking has been extensively reviewed. Aqueous phases of cigarette smoke with more than 5000 compounds exist in blood stream and those particles could affect on inflammatory cells. These compound enter to the cells and activate / suppress the activity of macrophage.

In fact MTB by activation of macrophages via dectin receptor activate the sequences of cells activation and release of inflammatory cytokines such as CXCL-8, IL-12 and TNF-α release which in turn induces the immune system network. However we don’t know how phagocytosis suppression happened in TB and cigarette smoke patients. Only we could speculate that smoking suppress the capacity of cells which induces phagocytosis.

Our study goal was to define smoking effects on the phagocyte capacity of blood monocytes/macrophages and levels of inflammatory mediators such as TNF-α and CXCL-8 in TB patients.

MATERIALS AND METHODS

103 subjects including 61 newly diagnosed pulmonary TB patients (23 smokers and 38 nonsmokers) and 42 controls healthy cases were studied. Smoker patients had history of 26.4±16.3 pack-year smoking. Control and non-smoker TB patients were selected from people with no history of passive smoking.

Samples were selected from both genders, aged 18 years and older. Inclusion criteria were as follows: being diagnosed with active pulmonary TB based on the classification extracted from the WHO treatment
M. Aryanpour, et al.

guidelines\textsuperscript{34} and being classified as a category I patients (new TB cases). Subjects were excluded if they had only extra pulmonary TB (involving CNS, pericardium, adrenal gland etc.), had multi-drug resistant TB at diagnosis, had HIV/AIDS, were an active IV drug abuser, were classified as category II (relapse, treatment failure, and treatment after default) or category III (chronic TB), fulfilled the eligibility criteria but were not willing to participate in the study or were unable to comprehend the contents of the informed consent form.

Chemicals and Reagents
Fluorescein isothiocyanate (FITC)-dextran (average molecular mass, 40 kDa) was purchased from Sigma-Aldrich (St. Louis, Mo., USA). CXCL-8 (R&D system,USA),TNF-\(\alpha\) (Invitrogen ,USA) ELISA kits, Ficoll- hypeaque (Pharmacia, Uppsala, Sweden), FCS (fetal Calf serum, Greiner Bio-One, Germany).

Isolation of Monocytes/Macrophages from Peripheral Blood
Venous blood was drawn from cases in all groups; healthy volunteers and TB patients and harvested in coagulated tubes containing the 1/10 vol of 3.8\% sodium citrate. Peripheral blood mononuclear cells (PBMC) were prepared by Ficoll-hypaque density gradient isolation by centrifugation of freshly drawn blood. PBMC were seeded for 1h in a cell culture flask before non-adherent cells were removed. Adherent cells were considered as a monocyte/macrophage subgroup of PBMC. Thereafter, the cells were washed once with PBS (1X) and suspended in buffer containing 1ml PBS-1\% Fetal calf serum (FCS). We stained the cells for CD14 marker and then analyzed by flow cytometry (FACS Calibour), which was always in the range of 90\%-95\%. The isolated monocytes were used freshly or frozen at -130°C for later use.

Phagocytosis Assays
Phagocytosis was assayed by flow cytometry using FITC-dextran.\textsuperscript{35} The removed cells (5×10\(^5\)) were suspended in 100\μl PBS including 1\% bovine serum albumin and incubated together with FITC-dextran (0.1mg/ml) at 37°C or 4°C for 2hrs as described previously.\textsuperscript{36} Incubation was stopped by mixing with 2ml ice-cold PBS that included 1\% human serum and 0.02\% sodium azide. The cells were washed three times, with cold PBS and FACS Calibour flow cytometer (FACS Calibour, BD bioscience, USA) was used for analysis. Specific FITC-dextran uptake of each sample was expressed as the total FL1 fluorescence intensity at 37°C minus total FL-1 fluorescence intensity at 4°C. Relative endocytosis activity was calculated using the formula specific FITC-dextran uptake of sample/specific FITC-dextran uptake of control × 100%.

Trypan Blue Quenching
In order to eliminate the effects of membrane adherent FITC-Dextran, phagocytosis was evaluated by comparing the intensity of green fluorescence (FITC) before and after trypan blue quenching of membrane-bound, labeled dextran. For stopping extracellular fluorescence, after exposure to FITC-Dextran, cells were pelleted by centrifugation (10,000 xg at 4°C for 5 min) and then resuspended in ice-cold 0.2\% Trypan blue in 1x PBS (100\μl) as described earlier.\textsuperscript{37} Then 10000 cells were analyzed by flow cytometry.

TNF-\(\alpha\) and CXCL-8 Measurements
Drawing venous blood samples was performed between 7:30 and 8:00 AM in the morning before having breakfast and for the smoker subjects before the first cigarette of the day. Samples were immediately centrifuged 10 minutes at a speed of 1500 rpm. Prior to analysis the serum was stored at -20°C. The serum levels of TNF-\(\alpha\) and CXCL-8 were analyzed by using the mentioned ELISA kits according to manufacture Instruction.\textsuperscript{38} The detection threshold level in both kits was 31.2 pg/ml.

Statistical Analysis
Results are presented as means ±standard error of the mean (S.E.M.). SPSS v.18.0 software was used to analyze the data. Since some of the data sets were not normally distributed, group comparisons were performed using non parametric tests and Kruskal Wallis H test.Spearman’s rank correlation coefficients were calculated \(p<0.05\) was considered significant.

RESULTS
The important Demographic character of study populations was shown below (Table 1).
Table 1. Demographic characteristics of smoker and nonsmoker patients with TB and without TB

<table>
<thead>
<tr>
<th>Variables</th>
<th>Healthy Controls</th>
<th>TB-Non-smokers</th>
<th>TB-Smoker</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>42</td>
<td>38</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>53.9±4.3</td>
<td>54.2±6.5</td>
<td>53.7±5.2</td>
<td>NS</td>
</tr>
<tr>
<td>Sex(Male %)</td>
<td>30(71%)</td>
<td>17(74%)</td>
<td>11(73%)</td>
<td>NS</td>
</tr>
<tr>
<td>Pack/Years</td>
<td>0</td>
<td>0</td>
<td>26.4±16.3</td>
<td></td>
</tr>
<tr>
<td>Marriage status (% single)</td>
<td>15(35.7%)</td>
<td>14(36.8%)</td>
<td>8(34.7%)</td>
<td>NS</td>
</tr>
<tr>
<td>Job(manual)</td>
<td>18(42.9%)</td>
<td>18(47.4%)</td>
<td>12(52.2%)</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Results presented as Mean ±SD where appropriate, NS, not significant

Smoker TB Patients Showed Lower Phagocytosis Capacity

A lower percentage of peripheral blood monocyte/macrophage cells from smoking TB subjects [50.29% (43.4-57.2)] took up FITC-dextran after 2h compared with non-smoking TB patients [71.62% (69.2-74.1)] and healthy controls [97.45% (95.9-99.1)] (Figure 1). Phagocytosis was significantly reduced in non-smoking TB subjects compared to healthy controls (Figure 1). Inhibition of phagocytosis by cytochalasine D (10mg/ml) prevented FITC-dextran uptake (data not shown) and cells cultured at 4°C showed significant reduced phagocytosis (data not shown).

We further evaluated in smoker's phagocytosis capacity suppression according smoking pack-years, there was an inverse correlation between the suppression of phagocytosis and pack-years (r=-0.73, p<0.001). There was no significant correlation between suppression of phagocytosis and any demographic factors including age, sex, job, education and marriage status.

TB Smokers Have Higher Serum Levels of Inflammatory Cytokines

The serum levels of TNF-α and CXCL-8 were significantly higher in the smoking TB group than in the healthy non-smoking subjects (p<0.01; Figures 2 and 3). Serum TNF-α was also higher in smoking TB patients compared to non-smoking TB subjects (p<0.05, Figure 2).

Figure 1. Suppression of phagocytosis in TB and TB+Smoker patients.

Uptake of FITC-dextran by peripheral blood monocytes/macrophages was measured by relative fluorescence intensity after 2hrs in healthy control subjects and in smoking and non-smoking TB patients. Data are presented as mean±S.E.M. of experiments. *p<0.05, **p < 0.01 versus the study's healthy subjects.
Figure 2. Serum levels of TNF-α in healthy controls (n=42) and smoking (n=23) and non-smoking (n=38) TB patients. Data are presented as mean±S.E.M. *p<0.05, **p<0.01 versus healthy subjects.

There was no correlation between TNF-α levels and pack years or demographic factors between smokers TB and smokers. In contrast, there was a positive correlation between CXCL-8 increase and pack years in the smoking TB group (r=0.58, p<0.05).

Figure 3. Serum levels of CXCL8 in healthy controls (n=42) and smoking (n=23) and non-smoking (n=38) TB patients. Data are presented as mean±S.E.M. **p<0.01 versus healthy subjects.
DISCUSSION

In the current study blood monocytes/macrophages from TB patients showed reduced phagocytic capacity which is further reduced in TB patients who smoke. Besides, smoking enhanced serum levels of TNF-α and CXCL8 in TB patients above that seen in healthy controls. TNF-α, but not CXCL8, serum levels were also elevated in TB patients who smoked compared to those who were non-smokers.

According to the WHO, more than 30% of world's population may be infected with Mtb, and in most high-burden regions, it ranks after cardiovascular disease, as the second problem. Each year 8.6 million people develop TB disease and 1.3 million die of the disease. The association between tobacco smoking and tuberculosis has been described recently. The biological mechanisms that affect smoking risk on TB infection include decrease in ciliary function, alterations in macrophage number and response. Due to suppression effects of tobacco smoking on the immune response, studies of this effect on the macrophages recently much appreciated. We have shown that TB patients have a reduced phagocytic capacity compared to healthy subjects and this is further attenuated in TB patients who smoke. Previous studies have revealed a lower phagocytosis of smokers in comparison with non-smokers.

One of the most important roles of macrophages in the lungs of smokers is clearing the particles entered the lungs through tobacco smoking. In this regard, it is of interest to assess the effects of tobacco smoke on phagocytic capacity of macrophages. Accordingly, macrophages isolated from the lungs of smoke-exposed mice were found to have decreased capacity to bind and internalize yeasts. The larger phagolysosomes found in lung macrophages of smoker subjects compared to non-smokers indicate that regardless of its influence on phagocytosis, smoking suppresses the breakdown of ingested particles in lysosomes. These observations suggest that a central feature of TB pathogenesis involves mycobacterial interference with, or evasion of, phagocyte activation pathways which normally lead to the killing of ingested organisms.

This finding is congruent with results of previous studies reporting a significant increase in activation of TNF-α pathway in cigarette smokers. Production of CXCL8 in epithelial cells is also shown to increase through exposure to bacterial endotoxin and TNF-α. No consensus has been reached on the role of CXCL-8 in pathogenesis of TB. Several studies have shown that multiple growth factors, cytokines and other mediators might be able to interact with bacterial pathogens influencing the release of inflammatory mediators. It has been proposed that cytokine releases could be modulated by smoking. Our data indicate that CXCL8 could be associated to TB infection in patients who smoke as there was a further increase of serum CXCL-8 levels and a direct correlation with cigarette smoking as defined by pack years in these patients. It can be concluded from this observation that a significant exposure of cigarette smoke is necessary for activating inflammatory processes. Other evidences were indicated the serum and BAL fluid’s CXCL-8 concentration increase in active TB patients, even in some cases it reached to 8.9 times the normal limits. The increased levels of CXCL-8 in the host response against TB infection may be considered as an immune response. However, the precise role of CXCL-8 in TB infection remains unclear and merits further study specially with considering more control group as non TB smoker.

In conclusion, the results of this study revealed that smoking could play role in imbalance of immune system in TB patients which may indicate by differences in phagocytosis and expression of pro-inflammatory mediators. Importantly, both phagocytosis impairment and CXCL-8 levels elevation were modified in TB smokers and it was correlated with smoking exposure. This highlights the important interactions of cigarette smoking and evasion of immune surveillance in TB patients. We speculate that serum TNF-α, CXCL-8 and phagocytosis may considered as useful biomarkers for anticipating smoker's immunologic response in TB patients.

ACKNOWLEDGEMENTS

The authors would like to appreciate the National Research Institute of Tuberculosis and Lung Diseases (NRITLD) and Shahid Beheshti University of Medical Sciences who granted this project.

REFERENCES

M. Aryanpour, et al.


Attenuation Release of TNF and CXCL-8 in TB and Smoking


51. Khair OA, Devalia JL, Abdelaziz MM, Sapsford RJ,


