Differences in the distribution of peripheral blood leukocyte and lymphocyte subsets in pulmonary tuberculosis patients

Diferenças na distribuição de leucócitos e subtipos de linfócitos em sangue periférico de pacientes com tuberculose pulmonar

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Abstract

Introduction: It has been suggested that individuals exposed to Mycobacterium tuberculosis can eliminate the infection through the innate immune response without the participation of the acquired immunity. However, T helper type 1 immune response is critical to control persistent infection. Objective: The purpose of this study was to evaluate the distribution of leukocyte and lymphocyte subsets in peripheral blood of individuals with pulmonary tuberculosis (TB) and positive Tuberculin Skin Test (TST). Methodology: In order to achieve that goal, we have collected blood sample from thirty-four treatment-naïve TB patients, fifty TST-positive and forty-one TST-negative individuals. Results: The evaluation has shown a significant reduction in the number of total lymphocytes, B cells, CD4+ and CD8+ T cells and percentage increase of NK cells, neutrophils and monocytes in TB patients, when compared to TST-positive and TST-negative groups. There was no statistical difference for leucocyte and lymphocyte subsets between TST-positive and TST-negative groups. Peripheral blood white cell counts change significantly at diagnosis. Conclusion: The quantification of these cells may support the diagnosis and monitoring of patients with pulmonary tuberculosis. Keywords: Leucocytes. Lymphocyte subsets. Tuberculosis.

INTRODUCTION

The pathogenesis of tuberculosis (TB) is the result of interactions between the host immune response and mechanisms of bacterial virulence, in which most exposed people do not develop the disease and about half of this individuals remains with latent infection (FLYNN;CHAN, 2001). Although much progress has been made in recent decades, the immunological mechanisms that lead to the TB control are not completely understood. It has been suggested that individuals exposed to Mycobacterium tuberculosis (Mtb) can eliminate the infection through the innate immune response without the participation of the acquired immune response. In this context, the function of neutrophils, monocytes and NK cells are essential to eradicate Mtb infection without inducing measurable T cell sensitization (SCHWANDER; DHEDA, 2010). However, T helper type 1 (Th1) immune response is critical to control...
established infection, and T lymphocytes are more directly involved in the containment of mycobacterial growth by direct lysis of infected cells, cytokine secretion and granuloma formation that may restrain the agent dispersion and prevent infection of new cells (KAUFMANN, 2001). In addition, at the same time that T lymphocytes are important in controlling the disease, its presence also correlates to an increase in mononuclear and caseous lesions required for transmission (COOPER, 2009). Similarly, the presence of neutrophils in tuberculosis is related to both the protection and the clinical severity of the disease (EUM et al., 2010; LEE et al., 2012).

Some authors have reported that tuberculosis infection leads to a decrease of T and B cells with the increase of neutrophils, monocytes and NK cells (BECK et al., 1985; COROMINAS et al., 2004; VEENSTRA et al., 2006). Nevertheless, it is not entirely clear how these cells act to control the infection and the immunopathological consequences of it. The purpose of this study was to evaluate the distribution of leukocyte and lymphocyte subpopulations in peripheral blood of patients with Pulmonary Tuberculosis and positive Tuberculin Skin Test (TST).

**METHODOLOGY**

**Patients and controls**

The study included 125 volunteers, divided into three groups: individuals with pulmonary tuberculosis diagnosed by positive culture and/or acid-fast bacillus (AFB) staining and without antituberculosis treatment; TST-positive individuals with negative AFB staining and culture; TST-negative individuals with no symptoms of tuberculosis and no history of contact with TB patients. All subjects were recruited from a main TB hospital and a TB reference institute in Salvador-Bahia-Brazil. HIV-positive individuals, pregnant women, diabetes patients, drug abusers and autoimmune diseases carriers have been excluded.

**Diagnostic methods**

Bacilloscopy, culture and TST were performed by the institutions of care for patients with tuberculosis. The cutoff used in the TST was less than 5mm of induration, read 72 hours after application of Purified Protein Derivative (PPD) (Statens Serum Institute, Copenhagen, Denmark).

**Counting of white blood cells**

Peripheral blood sample of each volunteer was obtained using vacuum collection tubes with K²EDTA (BD Biosciences). The leukocyte count was carried out in automated blood cell analyzer Model Cell-Dyn 3000 (Abbott, Illinois, USA).

**Lymphocyte Immunophenotyping**

The lymphocyte phenotypes were quantified applying flow cytometry analysis from commercially available assay kits (Lymphogram® and PerfectCount Microspheres™, both from Cytognos – Salamanca – Spain), following the manufacturer’s instructions. Data were acquired by FACS/Calibur® (BD Biosciences) with ten thousand events computed and analyzed using CellQuest ® software (BD Biosciences). Information regarding features such as size and granularity, identified by graphs comprising front light scattering (FSC – Forward Scatter) and side light scattering (SSC – Side Scatter) axes, allowed the selection of lymphocyte population. For the measurement of T cells, B cells and NK cells subpopulations, it has been considered their morphological properties and the fluorescence associated with the presence of monoclonal antibodies specific for each molecule, such as: anti-CD3-PE, anti-CD4-PE/Cy5, anti-CD8-FITC, anti-CD19-FITC and anti-CD56-PE. The labeled cells for each monoclonal display different colors and fluorescence intensity, allowing the discrimination of the lymphocyte subpopulations.

The use of Perfect Count Microspheres™ reagent (Cytognos, Salamanca, Spain) allows calculation of absolute values of subpopulations per uL of blood. The manufacturer provides the number of beads per uL to serve as an internal control to cell quantification.

**Statistical analysis**

Results are expressed as arithmetic median, minimum and maximum. Statistical analysis has been performed using Kruskal-Wallis test for multiple-group comparisons, followed by the Dunn post-test. While Fisher’s exact test was used for the analysis of gender and ethnicity. All statistical analyses have been performed using GRAPHPAD, version 5.0. Statistical significance has been considered for values less than 0.05.

**ETHICS**

All subjects signed an informed consent and the study was approved by the Ethics Committee of Bahia School of Medicine and Public Health (protocol 86/2009).

**RESULTS**

The clinical and demographic characteristics of all patients are listed in Table I. There was no difference in median age among the groups (p=0.82), and with respect to gender, there was a predominance of males among TB patients (p=0.007). The TST-negative group had a higher proportion of whites individuals (25.0%) when compared to TST-positive (12.0%) and TB groups (11.8%, p=0.03). The median induration TST were 14.5mm and 18.0mm for TST-positive and TB groups, respectively.
Differences in the distribution of peripheral blood leukocyte and lymphocyte subsets in pulmonary tuberculosis patients

The total lymphocyte frequency was lower in TB patients (median 17.2%, range: 7 to 48.2%) when compared with the TST-positive (median 30.2%, range: 11.5-61.9%, p < 0.0001) and TST-negative individuals (median 34.8%, range: 15-60%, p < 0.0001). The same profile was observed for the analysis of the absolute value of total lymphocytes (Table 2).

When assessing what lymphocyte phenotypes could be contributing to the decrease in the total number of lymphocytes, it was observed a significant reduction in absolute number of CD4+ and CD8+ T cells and B cells in TB patients (p =0.0009, p = 0.0023 and p=0.0006, respectively, Kruskal-Wallis test), when compared to other groups evaluated in this work. Similar statistical differences for these lymphocyte subtypes were found when compared separately, individuals with TB and negative TST. However, when TB patients were compared to TST-positive individuals a significant reduction in the levels of lymphocytes only occurred for CD4+ T cells (p=0.005) and B cells (p=0.005). No statistical difference was found for absolute count of NK cells among the groups in the present study (Table 2).

On the other hand, regarding the percentage of different phenotypes, only NK cells exhibited a significant increase in the TB group (median 17.4%, range from 4.5 to 29.3%) when compared to the TST-negative individuals (median 13.5 %, range: 2.4 to 38.0%, p <0.038) (Table 2).

### Table 1 – Clinical and demographic features of individuals involved in the study

<table>
<thead>
<tr>
<th>Group</th>
<th>TST-negative</th>
<th>TST-positive</th>
<th>TB</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>35</td>
<td>36</td>
<td>33</td>
<td>0.82</td>
</tr>
<tr>
<td>Min – Max</td>
<td>21 – 58</td>
<td>18 – 58</td>
<td>18 – 57</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (%)</td>
<td>17 (41.5)</td>
<td>22 (44)</td>
<td>21 (61.8)</td>
<td>0.007</td>
</tr>
<tr>
<td>Female (%)</td>
<td>24 (58.5)</td>
<td>28 (56)</td>
<td>13 (38.2)</td>
<td>0.89</td>
</tr>
<tr>
<td>TST induration/mm</td>
<td>0</td>
<td>14.5</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Min – Max</td>
<td>0 – 0</td>
<td>5 – 18</td>
<td>0 – 27</td>
<td></td>
</tr>
<tr>
<td>Etnic Group</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White (%)</td>
<td>10 (25)</td>
<td>12 (6)</td>
<td>4 (11.8)</td>
<td>0.03</td>
</tr>
<tr>
<td>Non-White (%)</td>
<td>31 (75)</td>
<td>44 (88)</td>
<td>30 (88.2)</td>
<td>0.07</td>
</tr>
</tbody>
</table>

(a) Only 19 pulmonary TB patients underwent PPD;
(b) Comparison between TST-negative and TB group (Fisher’s exact test);
(c) Comparison between TST-negative and TST-positive groups (Fisher’s exact test).

### Table 2 – Percentage and absolute numbers of peripheral blood lymphocytes and lymphocyte subtypes in individuals included in the study

<table>
<thead>
<tr>
<th>Group</th>
<th>TST-negative (n= 41)</th>
<th>TST-positive (n= 50)</th>
<th>TB (n=34)</th>
<th>p value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>p value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell count</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absolute lymphocytes/μL</td>
<td>2149 (95-5554)</td>
<td>1944 (977-5251)</td>
<td>1531 (540-3855)</td>
<td>0.0004</td>
<td>&lt;0.0001&lt;sup&gt;c&lt;/sup&gt;, &lt;0.005&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>T cells/μL</td>
<td>1578 (719-4630)</td>
<td>1465 (672-3879)</td>
<td>1092 (389-3020)</td>
<td>0.0004</td>
<td>&lt;0.0001&lt;sup&gt;c&lt;/sup&gt;, &lt;0.005&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD4+ T cells/μL</td>
<td>919 (478-2025)</td>
<td>870 (358-2821)</td>
<td>603 (231-1421)</td>
<td>0.0009</td>
<td>&lt;0.005&lt;sup&gt;c&lt;/sup&gt;, &lt;0.005&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD8+ T cells/μL</td>
<td>535 (197-2064)</td>
<td>450 (148-1571)</td>
<td>313 (98-1365)</td>
<td>0.002</td>
<td>&lt;0.005&lt;sup&gt;c&lt;/sup&gt;, ns&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>B cells/μL</td>
<td>215 (84-695)</td>
<td>223 (53-850)</td>
<td>140 (36-558)</td>
<td>0.0006</td>
<td>&lt;0.005&lt;sup&gt;c&lt;/sup&gt;, &lt;0.005&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>NK cells/μL</td>
<td>286 (80-966)</td>
<td>302 (80-838)</td>
<td>247 (51-1050)</td>
<td>ns</td>
<td>ns, ns&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total lymphocytes %</td>
<td>34.8 (15.0-60.0)</td>
<td>30.2 (11.5-61.9)</td>
<td>17.2 (7.0-48.2)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001&lt;sup&gt;c&lt;/sup&gt;, &lt;0.0001&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>T cells %</td>
<td>75.2 (54.9-89.3)</td>
<td>73.0 (51.8-88.0)</td>
<td>72.3 (52.6-89.0)</td>
<td>ns</td>
<td>ns, ns&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD4+ T cells %</td>
<td>41.9 (26.8-56.0)</td>
<td>42.6 (29.4-57.4)</td>
<td>43.1 (31.8-51.1)</td>
<td>ns</td>
<td>ns, ns&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD8+ T cells %</td>
<td>26.5 (13.0-43.8)</td>
<td>23.4 (7.7-38.5)</td>
<td>25.0 (10.8-43.1)</td>
<td>ns</td>
<td>ns, ns&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>B cells %</td>
<td>11.9 (4.4-25.2)</td>
<td>12.0 (3.3-35.4)</td>
<td>9.9 (4.5-21.7)</td>
<td>ns</td>
<td>ns, ns&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>NK cells %</td>
<td>13.5 (2.4-38.2)</td>
<td>16.8 (4-34.9)</td>
<td>17.4 (4.5-29.3)</td>
<td>0.038</td>
<td>&lt;0.05&lt;sup&gt;c&lt;/sup&gt;, ns&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD4+/CD8+ ratio</td>
<td>1.7 (0.8-4.2)</td>
<td>1.8 (0.9-5.3)</td>
<td>1.7 (0.8-4.5)</td>
<td>ns</td>
<td>ns, ns&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

(a) Kruskal-Wallis test;
(b) Dunn’s post test;
(c) TB group compared to TST-negative group;
(d) TB group compared to TST-positive group.
Non-significant is shown as ns.
It was also observed an increase in the percentage of neutrophils among TB patients (median 67.6%, range: 51.0 to 85.4%) when compared to TST-positive (median 58%; range: 25.6-82%, p <0.0001) and TST-negative individuals (median 60.1%, range: 28.0 to 75.4%, p<0.0001). Regarding the absolute number of these cells, a similar profile with significant increase of level was observed in TB patients when compared to other groups (Table III).

Table 3 – Percentages and absolute numbers of peripheral blood leucocytes in individuals included in the study

<table>
<thead>
<tr>
<th>Group</th>
<th>TST-negative (n= 41)</th>
<th>TST-positive (n= 50)</th>
<th>TB (n=34)</th>
<th>p valuea</th>
<th>p valueb</th>
</tr>
</thead>
</table>
| Cell count             | Median (Min-Max)     | Median (Min-Max)     | Median (Min-Max) | 0.07 | ns, ns  
| Total Leucocytes/μL    | 6100 (3600-10500)    | 6415 (3360-20200)    | 7980 (4040-18800) | 0.07 | ns, ns  
| Neutrophils/μL         | 3849 (1480-1795)     | 3472 (1152-16584)    | 5117 (2222-15886) | 0.001 | <0.05, <0.0001  
| Monocytes/μL           | 404 (129-970)        | 411 (102-1192)       | 609 (228-1242)    | 0.0002 | <0.0001, <0.0001  
| Neutrophils (%)        | 60.1 (28-75.4)       | 58.0 (25.6-82.0)     | 67.5 (51.0-85.4)  | <0.0001 | <0.0001, <0.0001  
| Monocytes (%)          | 6.4 (2.0-13.3)       | 6.0 (2.0-13.3)       | 8.2 (4.5-15.0)    | 0.001 | <0.01, <0.0001  

(a) Kruskal-Wallis test;  
(b) Dunn’s post test.  
(c) TB group compared to TST-negative group.  
(d) TB group compared to TST-positive group. Non-significant is shown as ns.

DISCUSSION

Many studies have reported immunosuppression in tuberculosis with decreased numbers of B and T cells which are reversed after suitable therapy, but few authors have proposed the use of these figures for diagnosis and monitoring of patients with TB (HERNANDEZ et al., 2010; PILHEU et al., 1997; RODRIGUES et al., 2002; VEENSTRA et al., 2006). The present study has shown significant changes in the absolute numbers of neutrophils, monocytes, lymphocytes and their subtypes in patients with pulmonary tuberculosis.

We have detected a higher proportion of men and non-white individuals in the TB group compared to the other groups. These findings are consistent with epidemiological data from our region that show a high incidence of tuberculosis among males and non-whites individuals (BAHIA, 2012). It can be argued that differences in gender and ethnicity could influence the results of our study. However, according to Torres et al. (2013) in research conducted in our city with an admixed population; there is no significant difference in the values of leukocytes and lymphocyte subtypes (except B lymphocytes) with respect to gender in healthy blood donors.

In our research, the TB group presented a significant decrease in percentage and absolute count of total lymphocytes and absolute number of lymphocyte subtypes, with the exception of NK cells, when compared to other study groups. One possible explanation for the low number of lymphocytes and their subtypes in peripheral blood of patients with TB is the migration of these cells to the infection site and granuloma formation. Previous studies have shown that the composition of granuloma involves a variety of T lymphocytes, some B lymphocytes, neutrophils and monocytes, but not NK cells (COSMA et al., 2003; GONZALEZ-JUARRERO et al., 2001). The pathophysiologic mechanism for this lymphopenia is not entirely clear and cell recruitment to form the granuloma is one of the most accepted hypotheses. Another possibility could be an inactivation of lymphocytes caused by Mtb, but this is unlikely to happen because the bacillus seems to benefit from the accumulation of lymphocytes at the inflammation site. The resulting granuloma formation protects Mtb against the host immune response (BARRY et al., 2009).

It is clear that CD4+ T cells are extremely important to control the growth of the tubercle bacillus, since HIV-positive patients and mice deficient in CD4+ T cells have shown to evolve more rapidly to severe tuberculosis (SAUNDERS et al., 2002). Meanwhile, the CD4+ T cells are also related to the increase of immunopathogenesis in the lungs. In our study, there was a significant reduction in CD4+ T cells among TB patients when compared to other...
groups, but no significant difference has been observed among individuals with positive TST, when compared to those with negative TST. The result obtained by Corominas et al. (2004) was similar, with significant reduction of CD4+ T cells in patients with pulmonary tuberculosis, when compared to TST-negative and TST-positive individuals. These data suggest that the active disease, but not latent infection, causes depletion of CD4+ T cells in peripheral blood. Furthermore, CD4+ T cells seem to be the most prevalent in granulomas (TSAI et al., 2006), leading us to suppose that the smaller the amount of these cells in peripheral blood the larger is the quantity of them at the infection site, worsening patient’s prognosis.

A percentage increase in the number of NK cells in peripheral blood of TB and TST-positive patients have been observed when compared to TST-negative individuals. NK cells do not participate in the formation of granulomas and the increased percentage of them in peripheral blood of individuals with TB and positive TST seems to indicate the inability of these cells to migrate to the inflammation site (COSMA et al., 2003; VEENSTRA et al., 2006). Nevertheless, animal studies have confirmed an increase of NK cells in the lung, especially in the first 14 days of infection, returning to normal 21 days after the onset of infection (JUNQUEIRA-KIPNIS et al., 2003), suggesting that it plays an important role in the early stages of tuberculosis by direct lysis of infected cells and cytokine secretion that regulate the activation of macrophages (YONEDA; ELLNER, 1998). Another important NK cell function may be associated with the protection and modulation of the immune response against disseminated tuberculosis, along with monocytes and neutrophils. That may explain why the later ones are present in large numbers in peripheral blood of patients with tuberculosis, when compared to other groups. However, the participation of neutrophils in the tuberculosis pathogenesis needs to be better understood, some authors have reported that these cells offer immune protection and have an important role in the early stages of granuloma formation (MARTINEAU et al., 2007; LEE et al., 2012), while others associate them with the increase transport and clinical severity of the disease (LOWE et al., 2012; EUM et al., 2010).

It has been observed a decrease in the number of B cells in TB patients when compared to other groups. The B cells participate actively in the immune response against tuberculosis by antigen presentation, granuloma formation and cytokine production. Some experiments describe an increase of bacterial load, which is correlated with the increase of neutrophils and immunopathologic effects in B cell-deficient knocked-out mice (VORDERMEIER et al., 1996; MAGLIONE et al., 2007). In our study, the predominance of males among TB patients could explain the low number of B lymphocytes in this group, since Torres et al. (unpublished observations) have reported that men presented decreased values of these lymphocytes when compared to women. Whether or not a small amount of B lymphocyte in peripheral blood may compromise the therapeutic and protective response to tuberculosis, it is still an open question and should be addressed in further studies involving populations susceptible to TB infection.

In the present study, the reduction of CD8+ T cells in peripheral blood of patients with pulmonary tuberculosis was associated with the migration of these cells to the site of infection in order to induce lysis of Mtb infected cells and production of cytokines, especially IFN-gamma. CD8+ T cells deficiency can result in susceptibility to tuberculosis and reactivation of latent infection (VAN PINXTEREN et al., 2000). Some studies have reported a low number of CD8+ T cells in untreated TB patients, but this number return to normal after appropriate therapy (RODRIGUES et al., 2002; VEENSTRA et al., 2006). Probably because it is no longer necessary to recruit these cells from peripheral blood to the inflammation site.

The results obtained here allow us to infer that the determination of leukocyte and lymphocyte subtypes levels do not discriminate between TST-negative and TST-positive subjects. On the other hand, individuals with tuberculosis had decreased levels of B cells, CD4+ and CD8+ T cells with percentage increase of NK cells, neutrophils and monocytes. The quantification of these cells in peripheral blood could aid in the diagnosis and monitoring of patients with TB. The use of flow cytometry has been growing in developing countries, primarily for assistance to HIV patients and could also help in monitoring of TB patients in countries with middle and high incidence of this disease.

REFERENCES
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