Author’s response to reviews

Title: Lymphocyte apoptosis in children with central nervous system tuberculosis

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Author’s response to reviews: see over
Reviewer: Malgorzata Mrugacz

Reviewer's report

An interesting study assess spontaneous peripheral T-lymphocyte apoptosis in children with central nervous system tuberculosis before and after starting chemotherapy. The aim of this study is well defined, however should be placed in Introduction section (not in Methods), both in the abstract and in the text. The study excluded the problematic group with positive for HIV. The data regarding subjects (e.g. age) are presented too chaotic. The methods are appropriate and well described but the authors should shortly describe these methods in the abstract. The results are sound, however the description of the statistical analysis is not clear (p 0.000?). The manuscript adheres to the relevant standards for reporting and data presentation. The discussion is adequate and conclusions are connected with the results. The title and abstract accurately convey what has been found. The writing is acceptable.

- <<The aim of this study is well defined, however should be placed in Introduction section (not in Methods), both in the abstract and in the text.>>

In order to take into account this important suggestion of the reviewer, the authors have inserted the following sentences in the introduction section, in the abstract and in the text.

Abstract section
<<In the present study, we analyze spontaneous T-lymphocyte (PBT) apoptosis in the peripheral blood of children with Central Nervous System tuberculosis, before and after chemotherapy, and compare the results with healthy controls.>>

Introduction section
<<In this study, we analyze spontaneous T-lymphocyte (PBT) apoptosis and subsets CD3+, CD4+ and CD8+, including the analysis of apoptotic CD8+/CD28+ T cells, in the peripheral blood of children with Central Nervous System tuberculosis, before and after starting anti-tubercular treatment, and of healthy control children.>>

- <<The data regarding subjects (e.g. age) are presented too chaotic>>

The following sentence has been modified to answer the reviewer’s comment:

Material and methods Section, page 5

[10 boys, 8 girls; mean age (±SD) 3.8(3.4); range 0.5–12 years]
[10 boys, 7 girls; mean age (±SD) 5.6(3.2); range 2–12 years]

- <<The methods are appropriate and well described but the authors should shortly describe these methods in the abstract>>

In order to take into account this important suggestion of the reviewer, the authors have modified the following sentences in the methods section in the abstract

<<A case-control study was conducted from January 2002 to June 2009. It included 18 children with CNS TB and 17 healthy controls. Spontaneous apoptosis of PBTs, including CD4+, CD8+ and CD8+/CD28+ T cells, was evaluated after 24 h and 72 h of culture in complete medium, using the Annexin V detection test. Analysis was conducted before and after chemotherapy, and apoptotic markers CD95 (Fas) and Fas ligand (FasL) expression were evaluated. >>

- <<The results are sound, however the description of the statistical analysis is not clear (p 0.000?).>>

The following statistical results have been modified to answer the reviewer’s comment in all the sentences in the Results Section (see also …...):

Reviewer: Anna Dubaniewicz

Reviewer’s report:

The manuscript is very interesting but needs some comments:
1. Regarding the Abstract section:
   • The Author should avoid words like interestingly

   This word has been removed from the abstract section

   • The Authors should shortly introduce in background why important is apoptosis of lymphocytes T in the etiopathogenesis of TB
In order to take into account this important suggestion of the reviewer, the authors have inserted the following sentences in the background section of the abstract:

Progress in our understanding of the apoptosis mechanisms involved in the pathogenesis of tuberculosis suggests that *Mycobacterium tuberculosis* can actively interfere with the apoptosis of infected cells. *In vivo* studies have been performed in adult populations but have not focused on the process in children.

• The Authors should supplement this section with:
  - number of included patients and healthy controls
  - used methods
  - a specification of tested lymphocytes: it was percentage or number?
  - values of obtained results, in current section we see only discussion, not results

In order to take into account this important suggestion of the reviewer, the authors have inserted the following sentences in the results section of the abstract:

<<Higher percentages of apoptotic T-cells and CD4 lymphocytes were isolated from children with acute phase CNS TB than from the children in the control group. The difference between the two groups decreased significantly after 60 days of specific treatment. As regards the illness group, high levels of Fas ligand expression were detected on lymphocyte populations, associated with a high percentage of Fas positive cells, before and after treatment. Contrary to the CD4+ apoptosis profile, we did not find any significant difference in total CD8⁺ cell apoptosis between the illness and control groups, whereas percentage of apoptotic CD8⁺/CD28⁺ T cells was significantly higher in the children with acute phase disease than in the healthy controls.>>

2. Regarding the Introduction section:
• The Authors should supplement this section with some epidemiological information about occurrence of TB in Italy

In order to take into account this important suggestion of the reviewer, the authors have inserted the following sentences in the introduction section in the text:

<<In Italy, like in many other industrialized nations, tuberculosis is a relatively rare disease. In the last few years, the incidence of tuberculosis has been less than 10 cases/100.000 population (in 2009 the rate was 7/100.000 population, corresponding to just over 4.200 cases) while according to WHO estimates the prevalence of latent infections is 12% (corresponding to just over 7.200.000 latent infections) [1].>>
The Authors should shortly introduce in background why important is apoptosis of lymphocytes T in the etiopathogenesis of TB and why FAS and CD28 markers were tested.

In my opinion, part of general information of Fas and CD28 should be removed to this section.

In order to take into account this important suggestion of the reviewer, the authors have rewritten this part in the introduction section of the text; moreover the related references have been modified and CD28 was removed from this section:

<<The relevant role of apoptosis (programmed cell death) in the development of the immune system in children has recently been highlighted in studies on the efficacy of the tuberculosis vaccine [7].

Current studies have demonstrated that \textit{M. tuberculosis} actively interferes with the apoptosis of infected cells \textit{in vitro} as a means of virulence, and that dysregulation of the host’s lipid metabolism is a major pathway for generating pathology and promoting necrosis over apoptosis [8,9]. However, the importance of apoptosis as a virulence mechanism \textit{in vivo} and interaction of apoptotic mechanisms with the host cytokine response have been largely unexplored until recently, and only now is this area coming into focus [9,10].

A number of alternative mechanisms of peripheral T-cell apoptosis have been defined. Distinction has been made between active apoptosis, which follows stimulation and activation of the T cells, and passive apoptosis which occurs as a result of withdrawal of the sustaining cytokines following activation [11-14]. Activation of death receptors by their ligands can initiate apoptosis. Fas and TNF receptors are more clearly understood than others, and their important role in peripheral T cell apoptosis has been demonstrated. Fas/FasL interaction is particularly important in initiating activation induced cell death (AICD) of CD4$^+$ T cells [15-17].

Understanding the specific mechanisms by which children’s T cells fail to contain Mycobacterium tuberculosis infection could offer potential new targets for appropriate vaccines. In addition, studying the defects in infant immune responses that explain poor bacterial control may contribute
more broadly to our understanding of severe forms of tuberculosis such as Central Nervous System tuberculosis. >>

3. Regarding the Material and method section:
• We don’t know if tested children with CNS-TB had pulmonary TB or not?
• How many tested patients had M. tuberculosis positive CSF culture?
• What about of familiar TB?

In order to take into account this important suggestion of the reviewer, the authors have inserted the following sentences in the Material and method section:

<<CSF culture and PCR were positive in 60% and 90% of patients respectively. Resistant strains to 1 or more anti-tuberculosis drugs were found in 4 children and their adult sources. Seven patients (38%) had pulmonary TB. The source of infection was documented for 9 patients (50%): grandparents for 4 of them, parents for 3, a teacher for 1, and a neighbour for 1. >>

4. Regarding the Results section:
• This section should be divided into paragraphs with particular subheadlines

The Results section has been divided into two paragraphs to answer the reviewer’s comment:

• The Authors should be supplement this section with the results of analysis of blood count and information about number or percentage of tested T-cells

In order to take into account this important suggestion of the reviewer, the authors have inserted the following sentences and table 1 in the Results section and modified the figure legends:

<<T lymphocytes, Fas and Fas ligand during Central Nervous System tuberculosis

Mean (±SD) total lymphocyte and CD3+ lymphocyte counts in peripheral blood of patients in the acute phase of disease and of the controls are shown in table 1. There was no significant difference between the two groups (p > 0.05)

T-Lymphocyte subset apoptosis during Central Nervous System tuberculosis
Mean (±SD) CD4⁺, CD8⁺ and CD8⁺/CD28⁺ lymphocyte counts in patients during the acute phase of the disease and in the controls are shown in table 1. There was no significant difference between the two groups (p > 0.05)

Table 1: Peripheral blood lymphocyte populations in acute CNS Tuberculosis patients and controls

<table>
<thead>
<tr>
<th></th>
<th>Acute phase</th>
<th>Control</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes (x10⁹/l)</td>
<td>6.77(3.6)</td>
<td>6.58(2.8)</td>
<td>0.863</td>
</tr>
<tr>
<td>CD3⁺ T-lymphocytes (x10⁹/l)</td>
<td>6.20(3.2)</td>
<td>5.11(2.1)</td>
<td>0.245</td>
</tr>
<tr>
<td>CD4⁺ T-lymphocytes (x10⁹/l)</td>
<td>2.17(1.2)</td>
<td>2.28(0.8)</td>
<td>0.753</td>
</tr>
<tr>
<td>CD8⁺ T-lymphocytes (x10⁹/l)</td>
<td>1.33(0.6)</td>
<td>1.09(0.4)</td>
<td>0.176</td>
</tr>
<tr>
<td>CD28⁺ T-lymphocytes (x10⁹/l)</td>
<td>0.36(0.2)</td>
<td>0.27(0.1)</td>
<td>0.105</td>
</tr>
</tbody>
</table>

*mean values (±SD)

- The Authors should clearly state that T-cells were stimulated or not

*Apoptosis detection and peripheral blood T cells (PBTs) phenotype analysis* T-cells were not stimulated

Moreover, in order to take into account this important suggestion of the reviewer, the authors have modified the following sentences in the Results Section:

<< The analysis of apoptotic CD8⁺/CD28⁺ T cells showed impaired cell apoptosis in the acute phase of disease (acute vs control after 24h and 72 h of incubation p<0.0005 and p=0.002 respectively). In this case, the difference between CNS tuberculosis patients and healthy control children decreased after 90 days of specific treatment (p>0.05) (Figure 4). >>

- Why did the Authors write “p 0.019” without “=”?

I have inserted “=”

5. Regarding the Discussion section:
- This section should be entirely rewritten:
- at the beginning of this section, the Authors should be introduce their results with following discussion of obtained results with others studies and in the end, explain obtained results in the context immunological background; in current study, the Discussion section, the explanation of obtained results needs entirely more immunological knowledge;
- the sentences were the same in both the Discussion in the Abstract/Conclusions section.

In order to take into account this important suggestion of the reviewer, the authors have rewritten the Discussion section. This was not easy because there is a lack of relevant data available in paediatric literature. Consequently it is difficult to put together a discussion based on specific data as paediatric studies, especially studies on children under two years old, are difficult to find. Moreover, available data concern viral pathologies and etiologies but not bacterial and particular pathogens like *M. tuberculosis* infection in paediatric age. We hope, therefore, that the reviewer understands our difficulty in elaborating a discussion in this kind of scientific context.