


RESEARCH NOTE

T-cell responses against tuberculin and sensitin in children with tuberculosis and non-tuberculosis mycobacterial lymphadenopathy

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ABSTRACT

Multi-colour flow cytometry was applied to determine T-cell-specific interferon-γ, interleukin-2 and tumour necrosis factor-α expression in children with tuberculosis and non-tuberculosis mycobacterial lymphadenopathy (NTM-L). In vitro stimulation of peripheral blood mononuclear cells with purified protein derivative from Mycobacterium tuberculosis (tuberculin) and M. avium (sensitin) revealed differential recognition of tuberculin and sensitin in both study groups. Ratios of tuberculin-specific and sensitin-specific T-cell proportions in individual patients discriminated between children with tuberculosis or NTM-L. These findings have the potential to improve the differential diagnosis of mycobacterial infections.

Keywords Childhood tuberculosis, flow cytometry, intracellular cytokine staining, non-tuberculosis mycobacterial, sensitin, tuberculin

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Lymphadenopathy in children can be caused by M. tuberculosis or non-tuberculosis mycobacteria (NTM) [1]. Rates of non-tuberculosis mycobacterial lymphadenopathy (NTM-L) in children have increased during recent years in industrialized countries, whereas tuberculosis rates are decreasing [2,3]. Differential diagnosis of tuberculosis and NTM-L has major implications for the treatment procedure but can only be accomplished by bacterial culture lasting at least 3 weeks. Consequently, presumptive diagnosis that relies on clinical findings, patient anamnesis and tuberculin skin test (TST) guides the treatment choice.

Different approaches have been evaluated to improve the differential diagnosis of tuberculosis and NTM-L. On the one hand, the TST induration sizes induced by tuberculin have been analysed [4,5]. These studies identified differences in the mean induration size between NTM-infected and M. tuberculosis-infected individuals. However, high variances between individuals and the marked overlap between the study groups rendered induration sizes insufficient as biomarkers, especially in paediatric cases [4]. On the other hand, concomitant TST analyses of tuberculin and sensitin (dual skin testing) have been performed [4,6]. Dual skin testing and calculation of the ratio of responses against each antigen discriminated between NTM and M. tuberculosis-infected individuals. However, high variances between individuals and the marked overlap between the study groups rendered induration sizes insufficient as biomarkers, especially in paediatric cases [4]. On the other hand, concomitant TST analyses of tuberculin and sensitin (dual skin testing) have been performed [4,6]. Dual skin testing and calculation of the ratio of responses against each antigen discriminated between NTM and M. tuberculosis infection in adults [6,7], whereas results from studies in children were less convincing [8]. These ambiguous results may be caused by cross-reactivity against the antigens prevalent in both tuberculin and sensitin preparations.
Peripheral blood (3 mL) was obtained from ten children with culture-confirmed tuberculosis and nine children with culture-confirmed NTM-L. Intracellular analyses of cytokines, namely interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α), and interleukin-2 (IL-2), were used as readouts after short-term in vitro restimulation.

Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation, (Biocoll; Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions. PBMCs (2 × 10^5 cells/well) were then stimulated with tuberculin, sensitin (each 10 µg/mL) (Statens Serum Institute, Copenhagen, Denmark), or *Staphylococcus enterotoxin B* (1 µg/mL) (Sigma-Aldrich, St. Louis, MI, USA), or were unstimulated for 20 h in 200 µL of RPMI (Gibco) containing 5% human serum (Sigma) and 1% L-glutamine (Sigma). During the final 15 h, brefeldin A was added to avoid release of cytokines from the Golgi apparatus. Cells were then fixed and permeabilized using BD Biosciences (New Jersey, USA) cytokix/cytoperm, following the manufacturer’s instructions, before monoclonal antibody (mAb) mixtures were added. These mAb mixtures contained anti-IFN-γ mAb (APC-labelled), anti-IL-2 mAb (fluorescein isothiocyanate-labelled), and anti-TNF-α mAb (Alexa 700-labelled) (all BD Biosciences), combined with anti-CD4 mAb (APC-Cy7-labelled), anti-CD3 mAb (Pacific Blue-labelled), and anti-CD45RO mAb (PE-Cy7-labelled). An LSRII flow cytometer (BD Biosciences) was used for measurement, and FCS express software (De Novo, Los Angeles, CA, USA) was used for data analyses (see Fig. S1 for gating procedures). The Mann-Whitney U-test was used to determine significant differences in cytokine-expressing T-cell proportions between study groups, and Student’s t-test was applied to compare ratios of stimulation values. These tests were selected on the basis of Kolmogorov-Smirnov normality testing with Lilliefors’ correction.

CD4+ T-cells were the predominant cytokine-producing T-cell population after antigen-specific in vitro stimulation (data not shown). The proportions of IFN-γ-expressing, IL-2-expressing and TNF-α-expressing T-cells after restimulation with tuberculin in PBMCs from children with tuberculosis were only slightly higher than those in PBMCs from children with NTM-L (Fig. 1a). For sensitin, slightly higher IFN-γ-expressing, IL-2-expressing and TNF-α-expressing T-cell propor-

### Table 1. Clinical characteristics of children with tuberculosis (A–I) and children with non-tuberculosis mycobacterial lymphadenopathy (J–R)

<table>
<thead>
<tr>
<th>Case</th>
<th>Age</th>
<th>Gender</th>
<th>TST* (mm)</th>
<th>Quantiferon® - TB Gold</th>
<th>T-SPOT® TB</th>
<th>Pathogen (from gastric lavage, bronchoscopy, or extirpated lymph nodes)</th>
<th>Clinical features</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5</td>
<td>F</td>
<td>30</td>
<td>Positive</td>
<td>Positive</td>
<td><em>M. tuberculosis</em></td>
<td>Primary pulmonary tuberculosis; culture-confirmed</td>
</tr>
<tr>
<td>B</td>
<td>9</td>
<td>M</td>
<td>30</td>
<td>Positive</td>
<td>Positive</td>
<td><em>M. tuberculosis</em></td>
<td>Primary pulmonary tuberculosis; culture-confirmed</td>
</tr>
<tr>
<td>C</td>
<td>4</td>
<td>M</td>
<td>20</td>
<td>Positive</td>
<td>Positive</td>
<td><em>M. tuberculosis</em></td>
<td>Primary pulmonary tuberculosis; culture-confirmed</td>
</tr>
<tr>
<td>D</td>
<td>8</td>
<td>M</td>
<td>8</td>
<td>Negative</td>
<td>Negative</td>
<td><em>M. tuberculosis</em></td>
<td>Primary pulmonary tuberculosis; culture-confirmed</td>
</tr>
<tr>
<td>E</td>
<td>10</td>
<td>M</td>
<td>30</td>
<td>Positive</td>
<td>Positive</td>
<td><em>M. tuberculosis</em></td>
<td>Primary pulmonary tuberculosis; culture-confirmed</td>
</tr>
<tr>
<td>F</td>
<td>14</td>
<td>M</td>
<td>20</td>
<td>Positive</td>
<td>Positive</td>
<td><em>M. tuberculosis</em></td>
<td>Primary pulmonary tuberculosis; culture-confirmed</td>
</tr>
<tr>
<td>G</td>
<td>4</td>
<td>F</td>
<td>20</td>
<td>Positive</td>
<td>Positive</td>
<td><em>M. tuberculosis</em></td>
<td>Primary pulmonary tuberculosis; culture-confirmed</td>
</tr>
<tr>
<td>H</td>
<td>5</td>
<td>F</td>
<td>5</td>
<td>Positive</td>
<td>Positive</td>
<td><em>M. tuberculosis</em></td>
<td>Multiresistant primary pulmonary tuberculosis; culture-confirmed</td>
</tr>
</tbody>
</table>

Here we used flow cytometry, a highly sensitive and specific method, to determine specific T-cell responses against purified protein derivative from *M. tuberculosis* (tuberculin) and purified protein derivative from *M. avium* (sensitin) in children with tuberculosis or NTM-L. Intracellular analyses of cytokines, namely interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α), and interleukin-2 (IL-2), were used as readouts after short-term in vitro restimulation.

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tions were detected in PBMCs of children with NTM-L than in those of children with tuberculosis (p < 0.05; for all cytokines) (Fig. 1b). No differences were detected in the T-cell responses to the superantigen *Staphylococcus* enterotoxin B between the study groups (data not shown). Owing to the strong variations in cytokine responses of individual donors, the percentages induced by tuberculin and sensitin overlapped markedly between the study groups (Fig. 1a,b). Therefore, comparison of IFN-γ-secreting, IL-2-secreting and TNF-α-secreting T-cell proportions...
specific for tuberculin or sensitin was insufficient to discriminate between children with tuberculosis and those with NTM-L.

Next, the CD4\(^+\) T-cell responses to tuberculin and sensitin were determined for each individual donor. Significantly higher proportions of tuberculin-specific T-cells were detected in children with tuberculosis (Fig. 1c) and significantly higher proportions of sensitin-specific T-cells in children with NTM-L (Fig. 1d) (p <0.05; for all cytokines). As the relative proportions of tuberculin-specific and sensitin-specific T-cells correlated strongly in individual patients (p <0.01 for all three cytokines in both study groups, data not shown), cross-reactivity against the antigens is likely, as has been described by others [5].

Consequently, the ratios of cytokine-expressing T-cell proportions induced by tuberculin and sensitin were determined in individual patients from both study groups. The individual cytokine ratios revealed highly significant differences between the study groups for IFN-\(\gamma\) (p <0.001), IL-2 (p <0.001), and TNF-\(\alpha\) (p <0.001) (Fig. 1e). Notably, ratios of cytokine-expressing T-cell proportions revealed non-overlapping results for all cytokines between children with tuberculosis and those with NTM-L (Fig. 1e). These results were in accordance with studies comparing dual skin testing with tuberculin and sensitin in adults, and demonstrated for the first time that the analysis of T-cell responses against both mycobacterial protein preparations in parallel can contribute to improved differential diagnosis in children [7].

In particular, the high specificity of flow cytometry-based analyses and the enrolment of strictly stratified study groups (Table 1) may account for the contradictory results obtained in previous studies concerning dual skin testing in children [8]. The present study suggests that both sensitin and tuberculin preparations contain exclusive immunogenic proteins. For \textit{M. tuberculosis}, specific immunodominant proteins have been identified and are already being used in diagnostic tests [9,10]. These IFN-\(\gamma\) release assays have shown improved specificity and sensitivity as compared to the TST, but are probably less reliable if used for the diagnosis of immunocompromised patients [11]. Immunity based on serological analyses against components specifically expressed by \textit{M. avium} has been analysed in patients with tuberculosis as well as in those with NTM-L [12,13], with promising results from a very recent study in adults [13].

Our assay provides the possibility of detecting T-cell immunity specific for \textit{M. avium} infection. This is a major difference from the IFN-\(\gamma\) release assays, which detect \textit{M. tuberculosis} infection. Therefore, we consider our finding to be an initial step to characterize the influence of co-infection with NTM on tuberculosis and other infectious diseases (e.g. human immunodeficiency virus (HIV)). Especially in regions with high incidences of NTM infections, these co-infections are relevant [14].

Furthermore, in immunocompromised donors (e.g. HIV-infected), false-negative results of the TST and of IFN-\(\gamma\) release assays are prevalent, and hence the number of truly \textit{M. tuberculosis}-infected individuals among these patients is underestimated [11]. Ongoing studies will determine the efficacy of our assay in detecting mycobacterial infections in this group of patients.

In conclusion, the combined analyses of cytokine-expressing T-cell proportions specific for tuberculin or sensitin in individual patients rather then the response to one of these antigens discriminated between children with tuberculosis and those with NTM-L. All three cytokines, IFN-\(\gamma\), TNF-\(\alpha\) and IL-2, were equally suited for discrimination. We consider our findings an initial step to: (i) identify sensitin-specific components; (ii) analyse the impact of NTM co-infections on other infectious diseases (e.g. in patients with HIV infection); and (iii) develop a fast and reliable method for discrimination between children with tuberculosis and those with NTM-L.

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TRANSPARENCY DECLARATION

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.
Fig. S1. Gating procedures of flow cytometry analyses to determine tuberculin-specific and sensitin-specific T-cell proportions.

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REFERENCES


RESEARCH NOTE

Distribution of genes encoding iron uptake systems among enteroaggregative Escherichia coli strains isolated from adults with irritable bowel syndrome

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ABSTRACT

The distribution of genes encoding different iron acquisition systems in enteroaggregative Escherichia coli (EAEC) from adults with irritable bowel syndrome and from healthy controls was examined using a PCR assay. As many as 95.5% of EAEC carried the chuA gene coding for a haem receptor, and the majority of these strains also had yersiniobactin-encoding genes. Apart from yersiniobactin, enterobactin was the siderophore most frequently associated with EAEC among those strains examined. Genes encoding aerobactin and salmochelin siderophores were less frequent in the group of EAEC.

Keywords Enteroaggregative Escherichia coli, haem receptor, iron uptake systems, irritable bowel syndrome, yersiniobactin

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