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ORIGINAL ARTICLE

Role of *Mycobacterium avium* lysate INF-γ, IL-17, and IL-2 ELISPOT assays in diagnosing nontuberculous mycobacteria lymphadenitis in children

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Abstract

Nontuberculous mycobacteria are the most frequent cause of chronic cervical lymphadenitis in childhood. The aim of the study was to evaluate the performance of IL-2, IL-17, and INF-γ in-house enzyme-linked immunospot assays using a *Mycobacterium avium* lysate, in order to identify a noninvasive diagnostic method of nontuberculous mycobacteria infection. Children with subacute and chronic lymphadenopathies or with a previous diagnosis of nontuberculous mycobacteria lymphadenitis were prospectively enrolled in the study. Sixty children with lymphadenitis were included in our study: 16 with confirmed infection (group 1), 30 probable infected (group 2) and 14 uninfected (group 3). Significantly higher median cytokine values were found in group 1 vs group 2, in group 1 vs group 3, and in group 2 vs group 3 considering IL-2-based enzyme-linked immunospot assay ($p = 0.015$, $p < 0.001$, $p = 0.004$, respectively). INF-γ-based enzyme-linked immunospot assay results were significantly higher in group 2 vs group 3 ($p = 0.010$). Differences between infected and uninfected children were not significant considering IL-17 assays ($p = 0.431$). *Mycobacterium avium* lysate IL-2 and INF-γ-based enzyme-linked immunospot assays seem to be promising noninvasive diagnostic techniques for discriminating children with nontuberculous mycobacteria lymphadenitis and noninfected subjects.

Keywords *Mycobacterium avium* · Nontuberculous mycobacteria · Lymphadenitis · Children · ELISPOT

Introduction

Lymphadenopathy is a common medical condition in childhood, reported in 38–45% of healthy children every year [1, 2]. Nontuberculous mycobacteria (NTM) are the most frequent cause of chronic cervical lymphadenitis in children. In particular, *M. avium-intracellulare complex* (MAC) is the causative agents of more than 75% of cases of NTM lymphadenitis [3, 4]. NTM lymphadenitis usually affects children below 4 years of age and it is characterized by unilateral and painless neck mass. The clinical course is unpredictable, varying from spontaneous regression to fistulization [5, 6]. The diagnosis is difficult as noninvasive tests are lacking and the gold standard remains culture of lymph nodes after surgical excision [7, 8]. Moreover, culture requires a long period of time to become positive and has low sensitivity, ranging from 40 to 50% of lymph nodes suspected for NTM infections [9]. Patients with NTM infection can have positive tuberculin skin test (TST), because the purified protein derivative preparation, derived from *M. tuberculosis* (MTB), shares a number of antigens with several NTM species, including MAC. On the contrary, interferon-gamma release assays (IGRAs) are usually negative in children with MAC infection, but cross reaction with other NTM species are possible [10]. Therefore, laboratory tests able to identify accurately NTM infection are needed. Previous studies reported an important role of interferon-γ

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(IFN-γ), interleukin-2 (IL-2), and interleukin-17 (IL-17) in protection against mycobacteria [11–14]. IFN-γ is produced by CD4+ Th1 cells, which are essential in activation of microbicidal effector mechanisms of macrophages. IL-2 is also secreted by Th1 cells and by dendritic cells in response to mycobacterial infections [11–14]. In the last decade, several new T cell subsets, including Th17 cells, have been studied. Th17 cells produce IL-17, which seems to be increased in patients with tuberculosis and with NTM infections, but its role is not clear.

The aim of this study is to evaluate the performance of in-house IFN-γ-, IL-2-, and IL-17-based enzyme-linked immunospot (ELISPOT) assays by using a *M. avium* lysate in children with lymphadenitis in order to identify possible immunological markers for the diagnosis of NTM infection.

Materials and methods

Study design

Children (≤ 18 years) with subacute and chronic lymphadenopathies or with a previous diagnosis of NTM lymphadenitis referred to the Infectious Disease Unit, Anna Meyer Children's University Hospital, Florence, Italy, were prospectively enrolled between 1 July 2016 and 31 December 2017.

Children with congenital or acquired immunodeficiency, lymphoproliferative disorder or with MTB infection were excluded from the study.

Demographic data and past medical history were collected for each child and recorded into the study database. All children underwent clinical examination, TST and venipuncture for IGRAs (Quantiferon-TB-Gold In tube or QuantiFERON-TB Gold Plus since September 2017), lymphocyte subsets and investigations for the most frequent infectious causes of lymphadenopathy (data not included in the study database). For each child enrolled an additional sample of blood (3 mL) was obtained in occasion of venipuncture for the study test.

When samples from surgical drainage or lymph node excisional surgery were available, histology, microscopy, cultural and molecular tests for bacteria and mycobacteria were performed.

Information regarding type and length of anti-mycobacterial treatment was recorded into the study database.

This study was approved by the ethical committee of Anna Meyer Children's University Hospital, Florence, Italy. All the parents/tutors of the children enrolled gave their written consent to the study.

Study groups

Confirmed NTM infections were defined in case of positive culture on surgical samples and included in group 1. Probable NTM infections were included in group 2 on the basis of the following criteria [15–22]:

1) Previous healthy children
2) Unilateral and painless lymphadenitis with hard or floating consistency and purple color of the overlying skin without systemic symptoms or signs
3) Colliquative necrosis, microcalcifications, and/or fistulization at ultrasound imaging
4) Negativity of serologic tests for common infectious causes of lymphadenopathy in children
5) Induration ≥ 5 mm at tst
6) Negative igras
7) Inflammatory reaction with necrotizing granulation tissue at histology of surgical specimens
8) Negative results of polymerase chain reaction for *Bartonella henselae*, *Staphylococcus aureus*, *Streptococcus pyogenes*, and MTB in surgical biopsy

Finally, children with alternative diagnosis were classified as uninfected and included in group 3.

Laboratory tests

ELISPOT assays were performed at the Department of Experimental and Clinical Medicine, University of Florence, Italy. All the other laboratory tests were performed at the Anna Meyer Children's University Hospital, Florence, Italy, using standardized techniques and according to manufacturers' instructions.

Tuberculin skin test

TST was administered by trained nurses and performed according to the Mantoux method. The transverse skin induration was recorded in millimeters (mm) after 48–72 h directly by a pediatrician of the Infectious Disease Unit or by the general physician. Following the American Academy of Pediatrics guidelines, a positive TST was defined as an induration size ≥ 10 mm. Also, induration size between 5 and 10 mm was recorded [10].

Ultrasound imaging

Ultrasound imaging was performed at the Radiology Department of Anna Meyer Children's University Hospital, Florence, Italy, at the first evaluation and repeated after 2 months.

Preparation of *Mycobacterium avium* lysate

Whole cell lysates of *Mycobacteriun avium* strain HMC02 [23] were obtained from bacterial cultures, collected and

treated for mechanical lysation using a beadbeater (Biospec Products, Bartlesville, USA). Samples were then subjected to heat-treatment at 80°C for 2 h for sterilization. Quick Start™ Bradford protein assay (Bio-Rad. California, USA) was performed on *M. avium* lysate samples to quantify the amount of protein, according to manufacturers' instructions. Samples were stored at −80°C until further usage.

PBMCs isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples of each enrolled subject by Ficoll-Hypaque density gradient centrifugation. Briefly, blood was layered on top of Lymphoprep™ (Fresenius Kabi Norge, Oslo, Norway) and centrifuged at 800×*g* for 25 min at room temperature without brake. PBMCs layer was harvested and washed two times in PBS, pH 7.4. Cells were resuspended into RPMI 1640 complete medium (supplemented with L-glutamine 1%, beta-mercaptoethanol 1%, Na-pyruvate 1%, nonessential aminoacids 1%, penicillin 50.000U, and streptomycin 50 mg), with 10% fetal bovine serum in order to have a concentration of 2×10^6 PBMCs/ml.

ELISPOT assays

In-house ELISPOT assays detecting IL-2, IL-17, and INF-γ (Mabtech AB, Nacka Strand, Sweden) were performed on PBMCs isolated. For each patient and for each of the three cytokines, cells were seeded in 96-well plates coated with anti-IL-2 or IL-17 or IFN-γ antibody in duplicate in a concentration of 2×10^5 per well. Only medium as negative control (to subtract the basal cytokine production) or *Mycobacterium avium* lysate (10 μg/ml) or positive control mAb anti-CD3 were added to the cell suspension. Cells were incubated in a 37°C humidified incubator with 5% CO2 for 24 h for IFN-γ and IL-2 ELISPOT and for 48 h for IL-17 ELISPOT. At the end of the culture period, plates were washed and incubated for 2 h with the related detection antibody (IL-2-II-biotin, MT-504-biotin, 7-B6–1-biotin). Streptavidin complex was then added for 2 h, followed by the 3,3′,5,5′-tetramethylbenzidine substrate solution. The ELISPOT plates were incubated for 10 min at room temperature in the dark until spots were visible. The reaction was stopped and the plate was dried. The number of spot-forming cells (SFCs) was counted by an automated ELISPOT reader using the AID ELISPOT Software Version 3.2.3. Results were expressed as number of SFCs per million PBMCs. The average of SFCs counted into negative control wells was subtracted from the average of the SFCs detected into wells with lysate. Laboratory workers were blind to the clinical status of participants.

Statistical analysis

Median and interquartile ranges (IQRs) were calculated for continuous measurements in the study groups (i.e. age, intervals between lymphadenopathies onset and study test, TST, treatment length). The Wilcoxon-Mann-Whitney test was used to compare continuous measurements and to test relationships in unpaired analysis. Qualitative variables were reported as number and percentage and compared by means of χ-square (or Fisher test, when appropriate). Receiver operating characteristic (ROC) curve analysis was conducted to determine the best IL-2, IL-17 and IFN-γ ELISPOT result thresholds in discriminating between children with NTM infection or uninfected, relatively to *M. avium* lysate and correspondent sensitivity and specificity were reported. The area under the ROC curve (AUC) and 95% confidence intervals (CIs) were also calculated. To define the best cut-off for each cytokine, the Youden's index (= Sensitivity −[1-Specificity]) was applied. Statistical analysis was performed using the statistical software SPSS for Windows, version 25.0, $p < 0.05$ was considered statistically significant.

Results

Overall, 60 children (26 males and 34 females) with lymphadenitis were enrolled. The majority of lymphadenitis (96.7%) were cervical and only two (3.3%) children had inguinal lymphadenitis. Sixteen, 30, and 14 children were included in group 1, group 2, and group 3, respectively. Children were included in group 3 in case of a final diagnosis different from NTM infection, proven or presumed. In particular, two children were diagnosed with bacterial lymphadenitis due to *Staphylococcus aureus*, one child with branchial cyst, 6 children had an histology showing hyperplastic inflammation, negativity of microbiological tests and no other signs/symptoms suspected for NTM infection. Moreover, five more children which did not undergo surgery, were included in group 3 because of prompt spontaneous improvement and absence of ultrasound changes related to NTM infection.

Main features of the study population are summarized in Table 1. Median age at onset of lymphadenitis was 44.5 (30.0–69.9) months. When age was compared, no significant differences were found among study groups, probably because of the low number of patients enrolled. As matter of fact, the median age of children in group 1 was lower compared to children in the other groups.

The median interval between lymphadenopathy onset and study enrolment was 8.71 (IQRs 2.6–19.0) months and no significant differences were found in this interval

Table 1 Main features of children included in study groups

	All children ($n = 60$)	Group 1 ($n = 16$)	Group 2 ($n = 30$)	Group 3 ($n = 14$)	p
Age at lymphadenopathy onset (months), median (IQRs)	44.5 (30.0–69.9)	29.4 (22.6–57.0)	49.6 (33.5–78.0)	44.5 (40.5–54.2)	0.114
Time between lymphadenopathy onset and test (months), median (IQRs)	8.7 (2.6–19.0)	4.6 (2.7–18.7)	10.3 (2.6–19.7)	5.5 (1.5–18.6)	0.594
Undergo surgery, *n* (%)	53 (88.3)	16 (100)	28 (93.3)	9 (64.2)	0.005
Complete surgical excision, *n* (%)	32 (60.4)	10 (62.5)	17 (60.7)	5 (55.5)	0.942
Incision and drainage, *n* (%)	21 (39.6)	6 (37.5)	11 (39.3)	4 (44.5)	
Time between lymphadenopathy onset and surgery (weeks), median (IQRs)	13.4 (8.5–28.1)	12.2 (6.8–16.1)	17.9 (9.8–28.2)	37.5 (5.2–85.9)	0.095
Undergo re-intervention, *n* (%)	10 (16.6)	3 (18.7)	7 (23.3)	0 (0.00)	0.044
Treated with antimycobacterial drugs, *n* (%)	37 (61.6)	16 (100.0)	21 (70.0)	0 (0.0)	< 0.001
Treated with antimycobacterial drugs before study test, *n* (%)	31 (51.6)	14 (87.5)	17 (80.95)	n/a	0.680
Length of antimycobacterial treatment before the study test (weeks), median (IQRs)	8.8 (5.0–15.0)	10.9 (5.3–17.3)	8.1 (4.7–13.4)	n/a	0.597
Length of total antimycobacterial treatment (weeks), median (IQRs)	20.15 (8.7–23.1)	16 (13.4–23.7)	10.57 (8.0–24.8)	n/a	0.292

n/a not applicable

comparing the different groups. However, a wide inter-patients variability was observed, with a minimum value of 0.5 months and a maximum value of 86.5 months. No patients had Bacille-Calmette-Guerine vaccination in their past medical history.

TST and IGRAs results

TST was performed in 58/60 (96.7%) patients included in the study. TST resulted positive in 6 (40.0%) patients in group 1 and 6 (20.7%) patients in group 2. Moreover, 6.6% and 20.7% of patient in group 1 and group 2 had induration between 5 and 9 mm, respectively. In one child included in group 2 the diameter of induration was not reported. All patients included in group 3 had negative TST.

IGRAs were performed in 55/60 (91.7%) children and resulted negative in all patients.

Ultrasound features

Ultrasound imaging was performed in 54 (90.0%) of patient at first evaluation and in 41 (68.3%) of children after a period of 2 months of follow-up. Comparing ultrasound imaging between NTM infected (probable plus definite disease) children and those uninfected, colliquative necrosis was significantly more frequent in NTM infected children, both at first and second evaluation ($p = 0.024$ and $p = 0.04$, respectively).

Moreover, in 6 children (3 in group 1 and 3 in group 2) microcalcifications at first ultrasound imaging were reported and in one child included in group 2 also fistulization was present.

Histopathology and culture results

Fifty-three (88.3%) children included in the study underwent surgery (Table 1). Re-intervention was needed in 10 (16.6%) patients, in particular in 3 children included in group 1 and in 7 patients in group 2.

Among patients in group 1, histopathology showed inflammation with necrotizing granuloma in all patients. Presence of acid-fast bacilli (AFB) in the tissue was reported in 6 (37.5%) patients. Culture on surgical biopsy resulted positive for *M. avium* in 12 patients. Remaining 4 children resulted to be infected by *M. marseillense*, *M. malmoense*, *M. intracellulare*, and *M. bolletii.* Culture resulted positive after a median time of 2 (IQRs 2–4) weeks.

Among children in group 2, histopathology showed an inflammation with necrotizing granuloma in all surgical specimens. Presence of AFB in the tissue specimens was reported in 1 child.

Among patients in group 3, histopathology showed hyperplastic inflammation in 5 children, neutrophilic inflammation in 2 patients, a dermoid cyst in 1 patient, and inflammation with nonnecrotizing granuloma in 1 patient.

Antibiotic treatment for NTM infection

A significantly higher number of children included in group 1 received anti-mycobacterial treatment compared to group 2

($p = 0.013$). Medical treatment of patients included in the study groups is summarized in Table 1. All children in group 1 and 21 (70%) children included in group 2 were treated with antimycobaterial drugs, while no children in group 3 received medical treatment. Clarithromycin and rifampicin was the most used combination among both group 1 and 2 (68.7% and 46.6%, respectively).

Considering only children of group 1 and 2, when lengths of treatment before study test were compared, no significant difference was observed. Similarly, no differences were noted in total length of therapy and in length of treatment before and after surgery ($p = 0.51$, $p = 0.156$, respectively).

ELISPOT assay results

M. avium lysate IL-2, IFN-γ, and IL-17 ELISPOT assay results are summarized in Figs. 1, 2, and 3. Significantly higher median cytokine values were found in group 1 vs group 2 ($p = 0.015$), in group 1 vs group 3 ($p < 0.001$), and in group 2 vs group 3 ($p = 0.004$) considering IL- 2 ELISPOT responses to *M. avium* lysate. IFN-γ ELISPOT assay results were significantly higher in group 2 vs group 3 ($p = 0.010$), while no differences were observed between group 1 and group 3. Moreover, differences in IL-17 ELISPOT assay results between study groups were not significant.

Comparing children with probable or confirmed NTM lymphadenopathy (group 1 and group 2) vs NTM uninfected children (group 3), significantly higher IL-2- and IFN-γ-based ELISPOT results were found in NTM infected children ($p < 0.001$, $p = 0.010$, respectively), while no difference in IL-17 ELISPOT results was detected (Table 2).

In the group 1, four patients were infected by NTM other than *M. avium*; their median value and IQR for IL-2, IFN-γ and IL-17 ELISPOT were respectively 113.4 (92.9–149.2), 380.3 (85.5–734.25), and 31.2 (19.5–57.3). To determine if their inclusion into group 1 could affect statistical results of IL-2, IFN-γ, and IL-17 ELISPOT we demonstrated no impact in statistical significance because they are heterogeneously distributed as reported in Table 3.

ROC analyses showed 87.5% sensitivity and 85.7% specificity in discriminating between group 1 and group 3 considering response to *M. avium* lysate by IL-2 ELISPOT assay, for a cut off of 68.9 SCF per million PBMCs. The area under the ROC curve was 0.924 (95% IC 0.828–1.00) (Fig. 4a).

IFN-γ ELISPOT assay performance was poorer. Sensitivity and specificity in discriminating between group 1 and group 3 were respectively 81.3% and 71.4%, for a cut off of 63.4 SCF per million PBMCs. Area under the ROC curve was 0.703 (95% IC 0.490–0.916) (Fig. 4b).

Even poorer was the performance of *M. avium* lysate IL-17 ELISPOT assay. ROC analyses demonstrated 50% sensitivity and 71.4% specificity in discriminating between group 1 and group 3, for a cut off of 42.67 SCF per million PBMCs. Area under the ROC curve was 0.583 (95% IC: 0.374–0.791). Furthermore, ROC analysis between group 1 without the four "non *M.avium*" NTM infected children results and group 3 showed 91.7% sensitivity and 85.7% specificity for a cut off of 68.9 SFC per million PBMCs in IL-2 ELISPOT, 83.3% sensitivity and 71.4% specificity for a cut off of 63.4 SFC per million PBMCs in IFN-γ ELISPOT and 58.3% sensitivity and 71.4% specificity for a cut off of 42.7 SFC per million PBMCs in IL-17 ELISPOT, thus finding the same best cut off for the group1 versus group 3 for IL-2 and IFN-γ ELISPOT and a lower best cut off in IL-17 ELISPOT only.

Comparing results of IL-2, IFN-γ and IL-17 *M. avium* lysate ELISPOT assay of children included in group 1 and group

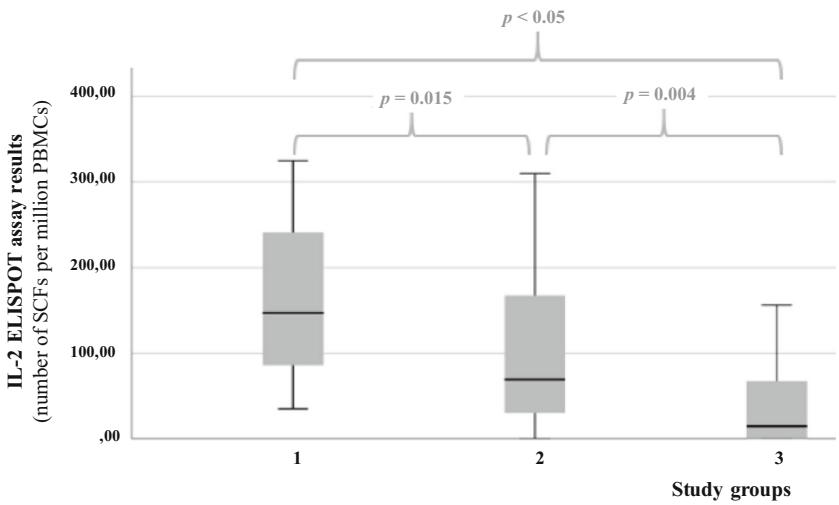

Fig. 1 *M. avium* lysate IL-2 ELISPOT assay results in children included in study groups (results are expressed as spot-forming colonies per million PBMCs)

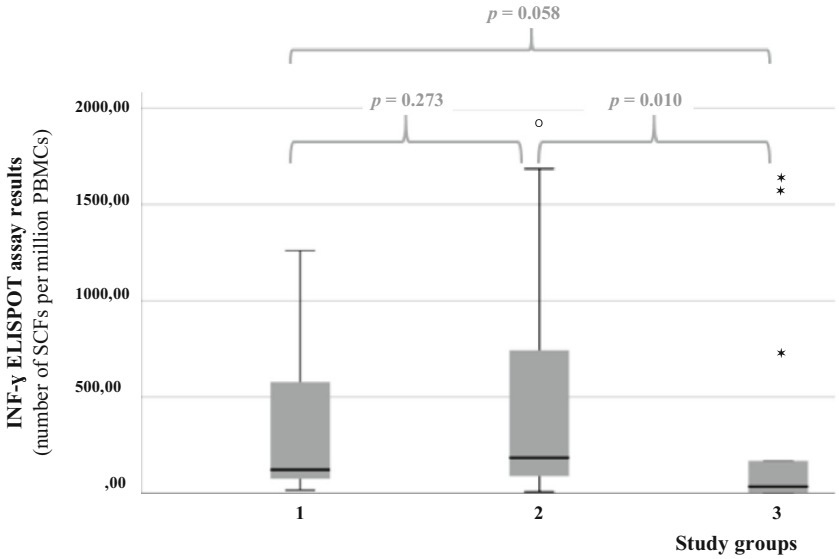

Fig. 2 *M. avium* lysate INF-γ ELISPOT assay results in children included in study groups (results are expressed as spot-forming colonies per million PBMCs)

2, significant differences were found according to time at test with respect to the onset of clinical signs. In particular, IL-2 ELISPOT assay results were significantly higher in patients who performed the test more than 3 months after lymphadenopathy onset vs those who had been tested before ($p = 0.001$). On the contrary, IFN-γ ELISPOT results were significantly higher in those children who performed the test within 3 months from lymphadenopathy onset ($p = 0.025$). No significant differences were noted in IL-17 ELISPOT results in the same subgroups ($p = 0.260$) (Table 4).

Moreover, IL-2-, IFN-γ-, and IL-17-based ELISPOT assay results were not significantly different in children in group 1 and 2 who had taken anti-mycobacterial drugs before study test vs. those who did not ($p = 0.690$, $p = 0.131$, $p = 0.972$, respectively).

Finally, no differences in IL-2-, IFN-γ-, and IL-17-based ELISPOT results were observed in children who needed re-intervention compared to those who underwent surgery only once ($p = 0.239$, $p = 0.802$, $p = 0.294$, respectively).

Discussion

The diagnosis of NTM lymphadenitis remains a challenge for pediatricians. Previous studies indicated that the combination of positive TST and negative IGRAs could suggest NTM infection. However, it is reported that TST results negative in almost one third of NTM infections [24, 25]. In our study, this percentage was higher, reaching 60% of negative TST in confirmed NTM infected children. However, an additional 6.6% of NTM infected children had TST induration between 5 and 9 mm. With the aim of increasing the sensitivity of intradermal skin tests, some authors suggested the use of antigens

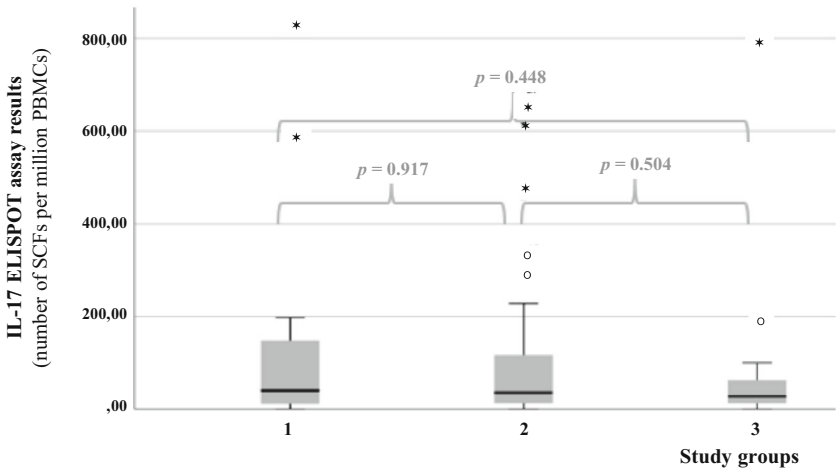

Fig. 3 *M. avium* lysate IL-17 ELISPOT assay results in children included in study groups (results are expressed as spot-forming colonies per million PBMCs)

Table 2 *Mycobacterium avium* lysate IL-2, IL-17, and INF-γ ELISPOT assay results in NTM infected and uninfected children

Mycobacterium avium lysate	NTM infected (probable and confirmed) ($n = 46$)	NTM uninfected ($n = 14$)	p
IL-2 ELISPOT median (IQRs)*	95.8 (36.8–192.2)	14.5 (2.0–58.6)	< 0.001
INF-γ ELISPOT median (IQRs)*	157.5 (78.7–674.6)	33.7 (4.0–140.0)	0.010
IL-17 ELISPOT median (IQRs)*	36.9 (11.46–130.0)	27.5 (12.5–57.2)	0.431

*Results are expressed as median and interquartile range of spot-forming colonies per million PBMCs

derived from NTM, but several studies reported a high percentage (6–32%) of false positive results [24, 25]. Diagnostic difficulties are increased by the fact that IGRAs could result positive in patients infected by few NTM species. In fact, IGRAs are based on response to ESAT-6 and CFP-10, which are proteins synthetized also in *M. gastri, M. kansasii, M. marinum, M. riyadhense*, and *M. szulgai* [26]. In our study, IGRAs resulted negative in all children, probably because no patients were infected by the above mentioned NTM species.

Our results show that *M. avium* lysate IL-2 and IFN-γ ELISPOT assays could be helpful for diagnosing NTM infection in children with lymphadenopathies, while IL-17-based ELISPOT should be avoided for its poorer performance.

Only a study on IFN-γ ELISPOT for the diagnosis of NTM infection is available in literature [26]. In this study, T.SPOT.TB test has been modified using a purified protein derivative (PPD) antigen mixture stimulation of PBMCs. The PPD mixture contained several mycobacterial antigens that cross-react with BCG, MTB and many NTM species. The modified T.SPOT.TB test showed an estimated sensitivity of 1.00 and specificity of 0.81, which are higher than sensitivity and specificity of our *M. avium* lysate IFN-γ ELISPOT assay (81.3% and 71.4%, respectively). However, the modified T.SPOT.TB could not differentiate between MTB or NTM infection and BCG-vaccinated children.

Previous studies evaluated the performance of IL-2 ELISPOT assay in addition to IFN-γ-based assay in diagnosing MTB infection and in discriminating active from latent tuberculosis (TB) [27, 28]. Our study is the first one which analyses the performance of IL-2/IFN-γ-based ELISPOT in NTM infection. The sensitivity of *M. avium* lysate IL-2 ELISPOT in discriminating NTM infected and uninfected children reached 87.5%, with a specificity of 85.7%. Moreover, the specificity could have been influenced by possible asymptomatic exposure to NTM in the control group.

Finally, a poor performance of IL-17 ELISPOT assay in distinguishing between NTM infected and uninfected children was observed. The role of IL-17 in mycobacteria infections remains still unclear. It has been recently reported that IL-17 is involved in the early host immune response to MAC. Persistent high levels of IL-17 are associated with tissue-damaging inflammation and negative outcomes [29, 30]. In our study, we also observed that anti-mycobacterial treatment did not affect the performance of IL-2 and IFN-γ ELISPOT assay and that results are not predictive of re-intervention.

Finally, an interesting finding was that the number of spots detectable by IL-2 ELISPOT assays was higher in children with lymphadenopathies onset more than 3 months before. On the contrary, the number of spots in IFN-γ ELISPOT was higher in those who had more recent lymphadenopathies. This result probably reflects the increased number of IL-2 secreting T cells and the reduced number of IFN-γ secreting T-cells in patients with less recent onset of NTM lymphadenopathies, probably due to low bacterial replication and low antigen load. These data are similar to those observed in children with MTB infection. In fact, it has been reported in several studies that the number of IL-2 secreting T cells in latent TB seems to be higher than those found in patients with active TB [27, 28, 31]. On the

Table 3 *Mycobacterium avium* lysate IL-2, IL-17, and INF-γ ELISPOT assay results in NTM infected (excluding non *M. avium* NTM) and uninfected children

Mycobacterium avium lysate	NTM infected (probable and confirmed, excluding "non *M.avium* NTM") ($n = 42$)	NTM uninfected ($n = 14$)	p
IL-2 ELISPOT median (IQRs)*	91.0 (35.62–186.44)	14.5 (2.0–58.6)	< 0.001
INF-γ ELISPOT median (IQRs)*	157.5 (82.0–603.75)	33.7 (4.0–140.0)	0.009
IL-17 ELISPOT median (IQRs)*	40.62 (13.12–149.05)	27.5 (12.5–57.2)	0.412

*Results are expressed as median and interquartile range of spot-forming colonies per million PBMCs

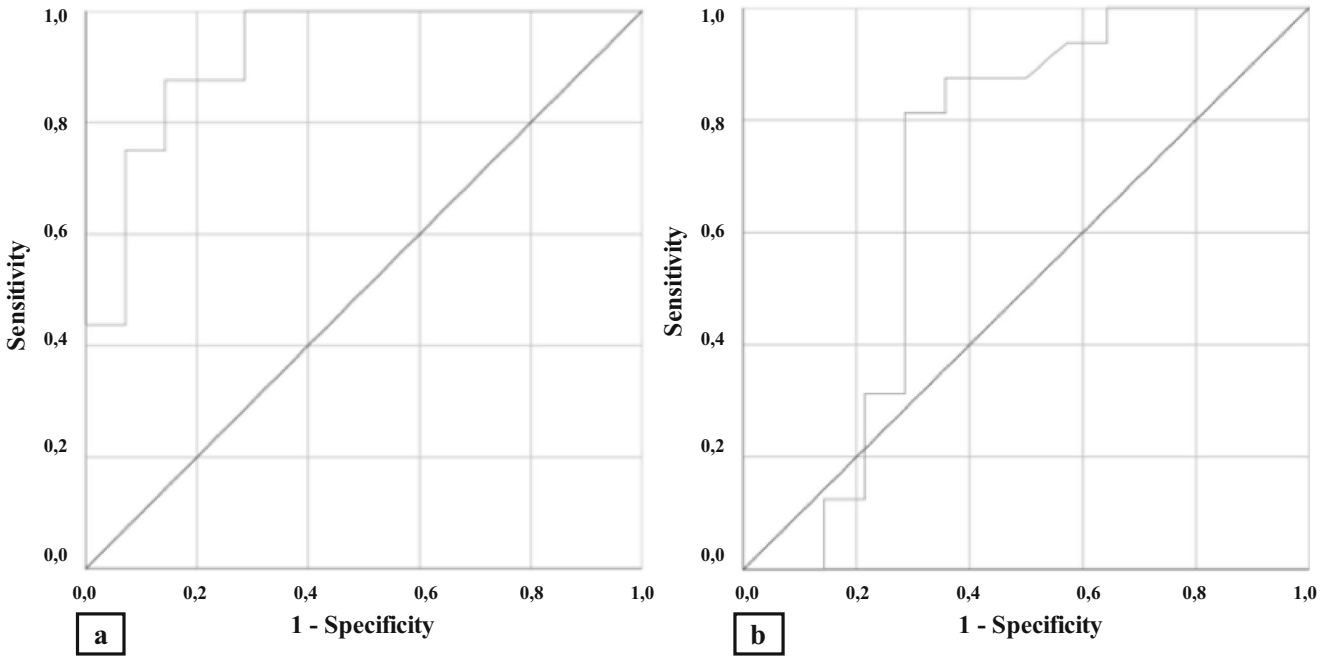

Fig. 4 A receiver operator characteristic (ROC) plot is shown, illustrating sensitivity and specificity of *M. avium* IL-2 (**a**) and IFN-γ (**b**) ELISPOT results in discriminating children NMT infected (n = 16) and uninfected (n = 14)

contrary, more recently, Zhang et al. have demonstrated that, after being stimulated with ESAT-6 and CFP-10, frequencies of IL-2, IFN-γ, and both IFN-γ/IL-2 secreting T-cells were all higher in patients with active TB than in patients with latent TB. Therefore, other studies are needed to clarify these observations [32].

Our study has several limitations. The small number of children enrolled with confirmed NTM infection is the main limitation of our study. Therefore, the role of IL-2- and IFN-γ-based ELISPOT assays in distinguishing between NTM infected and uninfected children should be confirmed in larger prospective pediatric studies. Moreover, the definition of study groups was based not only on surgical specimen cultures, but also on laboratory tests and clinical criteria. We excluded children with MTB infection according to TST/IGRAs and chest-radiograph results so we cannot completely exclude that TB infection could influence study test performance.

In conclusion, although our investigations were performed in a small pilot study and results should be confirmed in larger pediatric population, IL-2- and IFN-γ-based ELISPOT assays seem to be helpful in distinguishing between NTM infected and uninfected children. Considering that at present, a non-invasive test for diagnosing NTM lymphadenopathy in childhood is not available, IL-2- and IFN-γ-based ELISPOT assays could help in early diagnosis to address children to the right therapeutic approach.

Table 4 *Mycobacterium avium* lysate IL-2, IL-17, and INF-γ ELISPOT assay results in probable and defined NMT infected children according to time between lymphadenopathy onset and study test

Mycobacterium avium lysate	Children tested within 3 months from lymphadenopathy onset (n = 13)	Children tested over 3 months from lymphadenopathy onset (n = 33)	p
IL-2 ELISPOT median (IQRs)*	87.5 (36.2–138.8)	97.1 (41.2–211.0)	0.001
INF-γ ELISPOT median (IQRs)*	415.4 (84.5–1042.3)	149.6 (77.5–513.7)	0.025
IL-17 ELISPOT median (IQRs)*	30.0 (2.5–86.2)	43.7 (17.5–202.1)	0.260

*Results are expressed as median and interquartile range of spot-forming colonies per million PBMCs

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

This article does not contain any studies with animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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