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Impact of antiretroviral and tuberculosis therapies on CD4$^+$ and CD8$^+$ HIV/M. tuberculosis-specific T-cell in co-infected subjects

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ABSTRACT

Background: Human Immunodeficiency Virus (HIV) infection is a risk factor for tuberculosis (TB). Antiretroviral therapy (ART) changed HIV clinical management but it is still unclear how pre-existing HIV/Mycobacterium tuberculosis (Mtb)-specific CD4$^+$ and CD8$^+$ T-cells are restored.

Aim: to evaluate the impact of ART and TB therapies on the functional and phenotypic profile of Mtb-specific antigen-response of CD4$^+$ and CD8$^+$ T-cells in prospectively enrolled HIV-TB co-infected patients.

Methods: ART-naïve HIV-infected patients, with or without active TB or latent TB infection (LTBI), were enrolled before and after starting ART and TB therapies. Peripheral blood mononuclear cells (PBMC) were stimulated overnight with Mtb and HIV antigens (GAG). Cytokine expression and phenotype profile were evaluated by flow cytometry. Cytomegalovirus (CMV) and staphylococcal enterotoxin B (SEB) were also used.

Results: The median of absolute number of CD4$^+$ T-cells increased after ART and TB therapies in all groups analyzed, while the median of absolute number of CD8$^+$ T-cells decreases in HIV and HIV-LTBI groups. Treatments significantly increased the frequency of Mtb-specific CD4$^+$ T-cells in the HIV-LTBI ($p = 0.015$) with a rise of the central memory compartment. The magnitude of the CD4$^+$ T-cell response to HIV-GAG significantly increased in active TB ($p = 0.03$), whereas the magnitude of CMV-specific CD4$^+$ T-cell response decreased in all the groups. Similarly, the treatments increased the number of Mtb-specific CD8$^+$ responders in both HIV-LTBI and HIV-TB groups, whereas the phenotype distribution was dependent on the antigens used and on the stage of infection/disease.

Conclusions: After therapies the median of absolute number and the proportion of CD4$^+$ T-cells increased in all groups whereas the median of absolute count and proportion of CD8$^+$ T-cells decreased in the HIV and HIV-LTBI subjects. Interestingly, an increased frequency of CD4$^+$ T-cell response to RD1 proteins in HIV-LTBI subjects was found. These results contribute to a better understanding of the effect of ART and TB therapies on the modulation of Mtb-specific CD4$^+$ and CD8$^+$ T-cells subsets.

1. Introduction

It is estimated that about one fourth of the world's population is infected with Mtb [1]. In 2016, an estimated 1.0 million (10%) of the 10.4 million people who developed TB worldwide were HIV-infected. In these subjects, HIV infection leads a risk of progression to active TB

Abbreviations: HIV, human immunodeficiency virus; TB, tuberculosis; ART, antiretroviral therapy; Mtb, Mycobacterium tuberculosis; CD, cluster of differentiation; LTBI, latent TB infection; PBMC, peripheral blood mononuclear cells; GAG, group antigens; CMV, cytomegalovirus; SEB, staphylococcal enterotoxin B; N, naïve; CM, central memory; EM, effector memory; E, terminally differentiated effector memory; QFT-GIT, QuantiFERON-TB Gold In-Tube test; BCG, Bacillus Calmette–Guérin; ESAT-6, 6 kDa early secretory antigenic target; CFP-10, 10 kDa culture filtrate protein; RD1, region of difference 1; RPMI-1640, Roswell Park Memorial Institute; ICS, intracellular staining; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FMO, fluorescence minus one; UNS, unstimulated; IFN, interferon; TNF, tumor necrosis factor; IL, interleukin; IQR, interquartile range; FACS, fluorescence-activated cell sorting; PPD, purified protein derivative

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disease that is 21 times greater than individuals who do not have HIV infection [2].

In fact, HIV infection is one of the main and most well known cause for the establishment of active TB disease even at the stage where a profound deficit of $CD4^+$ T-cells count is not yet present [3]. Data suggest that the condition of co-infection HIV-Mtb accelerates the progression of both diseases [4–6]. It has been reported that macrophages co-infected *in vitro* presented higher number of viral copies due to an increase of the HIV replication [7,8]. Probably, the state of immune activation established during TB infection creates an optimal cytokine micro-environment for HIV replication [7,9,10] while the state of immune deficiency could cause an increase of the morbidity and mortality in the co-infection.

ART has significantly changed the clinical outcome of the HIV-infected patients, leading to a decrease of the morbidity and mortality through a reduction of plasma viral copies and an increase of absolute count of $CD4^+$ T-cells [11,12]. Although the clinical improvement due to the ART is evident, many aspects remain to be understood, in particular how far the immune function level is completely restored [13]. HIV-infected patients treated with ART show a variable capacity to re-establish the different co-pathogen specific $CD4^+$ T-cell responses, depending on the nature of pathogen that they identify [14–16]. Regarding the Mtb-specific $CD4^+$ T-cell response, controversial data exist on its restoration upon ART.

$CD4^+$ and $CD8^+$ T-cells show distinct characteristics depending on their differentiation stage [17,18]. Based on the expression of two different and conventional markers, CD45RA and CCR7, is possible to identify different T-cell subpopulations namely: naïve (N), referred to $CD45RA^+$ $CCR7^+$ cells; central memory (CM), as $CD45RA^-$ $CCR7^+$; effector memory (EM), as $CD45RA^-$ $CCR7^-$; and terminally differentiated effector memory (E) T-cells, as $CD45RA^+$ $CCR7^-$ [19].

Studies evaluating the Mtb-response in terms of cytokine profile in HIV co-infected patients naïve or not to ART report contrasting findings [20–24]. Indeed, both polyfunctional and monofunctional cytokine T-cell subsets have been observed in HIV-TB patients and HIV-LTBI [20,22–26]. Sutherland et al. observed that ART therapy induces an increase of the polyfunctional Mtb-specific $CD4^+$ T-cell responses compared to the baseline condition [23], whereas the results of a cross-sectional study of ART-naïve and ART-treated individuals show no significant difference in the frequency and profile of polyfunctional Mtb-specific $CD4^+$ T-cell responses [27].

At the moment it is unclear the impact of ART and TB treatments on the $CD4^+$ and $CD8^+$ T-cells simultaneously evaluated in terms of cytokine expression and maturation phenotype of patients with both co-infections. Therefore, aim of the present study was to assess the role of these therapies in HIV-infected patients naïve to ART with or without Mtb infection (active TB or LTBI) on the absolute number of $CD4^+$ and $CD8^+$ T-cells, on the total cytokine production and memory profile of Mtb-specific antigen-response in comparison with other recall antigen responses, as that to HIV-GAG or CMV.

2. Materials and methods

2.1. Study population and sample collection

This study was conducted at the National Institute of Infectious diseases (INMI) L. Spallanzani and approved by the INMI Ethical Committee (approval number 34/2011). Informed written consent was required to participate in the study. A total of 86 HIV-infected and naïve to ART patients were prospectively enrolled (from 2012 to 2015). They were recently diagnosed for HIV infection, but were not recent infections. Twelve active-TB (HIV-TB) patients were enrolled within 7 days of starting the TB-specific treatment. Active TB microbiologically diagnosed was defined based on the Mtb isolation from sputum culture. Active TB clinically diagnosed was defined based on the clinical and radiological lung lesions associated with TB in the absence of Mtb

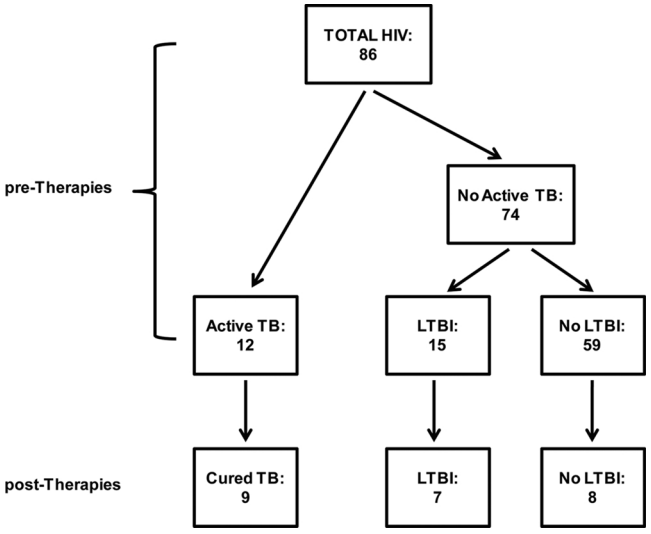

Fig. 1. Flow chart of the patients enrolled for the study. Eighty-six HIV-infected patients naïve to ART were enrolled. Twelve of these patients were HIV-TB. Among the 74 participants without active TB, 15 resulted HIV-LTBI. Among the total participants prospectively enrolled, 24 were followed overtime, 8 without TB co-infection (HIV), 9 HIV-TB and 7 HIV-LTBI. Footnotes: HIV: human immunodeficiency virus; TB: active tuberculosis; LTBI: latent TB infection.

isolated in the sputum that completely recovered after TB-specific treatment for 6 months. Microbiological TB was characterized by first line Mtb drug-sensitive isolates. In the absence of clinical, microbiological and radiological signs of active TB, LTBI was defined based on a positive score to QFT-GIT (Qiagen, Hilden, Germany) [28]. TB therapy was performed for 6 months, as indicated by the standard procedures [29]. We initially prospectively enrolled 86 ART naïve HIV-infected patients. They were compliant to both therapies (ART and/or TB-specific treatment). However most of them were not willing to participate to the study the second time, therefore only 24 (28%) were followed overtime: 8 without TB co-infection (HIV), 7 HIV-LTBI and 9 HIV-TB (6 microbiologically diagnosed and 3 clinically diagnosed) (Fig. 1). Around 70% of the patients followed overtime were BCG-vaccinated; regarding the origin, almost 33% were from South America, 29% from Italy and 21% from Eastern Europe. No significant difference in age or origin was observed comparing all groups while the gender distribution was significantly different among the groups, 50% of female subjects in HIV group, 14.3% and 0% of females subjects in HIV-LTBI and HIV-TB groups respectively (Table 1).

2.2. Stimuli

PBMC were stimulated for 16 h with Mtb-specific antigens: a) a mix of recombinant proteins ESAT-6 and CFP-10 (Lionex, Braunschweig, Germany) (hereafter referred to as RD1 proteins) at 4 μg/ml with a contamination of lipopolysaccharide reported by the manufacturer of less than 0.05 IU/mg for ESAT-6 and equal to 66.7 IU/mg for CFP-10; b) a pool of synthetic overlapping peptides (15 AA in length, with 11 AA of overlapping sequential peptides) corresponding to ESAT-6 and CFP-10 sequences (INBIOS, Naples, Italy) used at 2 μg/ml (hereafter referred to as RD1 peptides).

Regarding HIV-specific stimuli, synthetic peptides (15 AA in length, with 11 AA of overlapping sequential peptides) corresponding to HIV-1 consensus B of HIV-GAG protein were obtained through the Centre for AIDS Reagents, NIBSC and donated by the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (Bethesda, MD). The peptides were mixed to form three different pools: a) pool 1 of HIV-GAG constituted to peptides from 1 to 41 and used at $(2\,\mu g/ml)_{pep}$; b) pool 2 of HIV-GAG constituted to peptides from 42 to 82 and used at (2 μg/

Table 1
Demographic and epidemiological characteristics of the subjects enrolled in the study pre-ART and TB therapy.

	Naïve HIV-infected				
	HIV	HIV-LTBI	HIV-TB	Total	*p* value
N (%)	8 (33.3)	7 (29.2)	9 (37.5)	24 (100)	
Median Age (IQR)	38.0 (29.0–43.5)	37.0 (35.0–41.0)	33.0 (26.5–44.0)	36.5 (30.2–42.7)	0.9[a]
Female gender (%)	4 (50.0)	1 (14.3)	0	5 (20.8)	0.04[b]
Origin (%)					0.2[b]
Italy	5 (62.5)	1 (14.3)	1 (11.1)	7 (29.2)	
Western Europe	1 (12.5)	–	–	1 (4.2)	
Eastern Europe	–	2 (28.6)	3 (33.3)	5 (20.8)	
Africa	–	1 (14.3)	2 (22.2)	3 (12.5)	
South America	2 (25.0)	3 (42.8)	3 (33.3)	8 (33.3)	
BCG status (%)					0.04[b]
Vaccinated	3 (37.5)	6 (85.7)	8 (88.9)	17 (70.8)	
Unvaccinated	5 (62.5)	1 (14.3)	1 (11.1)	7 (29.2)	

HIV: human immunodeficiency virus; LTBI: latent tuberculosis infection; TB: tuberculosis; IQR: interquartile range; BCG: Bacillus Calmette et Guérin.
[a] Kruskal-Wallis test.
[b] Chi-square test.

$ml)_{pep}$; c) pool 3 of HIV-GAG constituted to peptides from 83 to 123 and used at $(2\,\mu g/ml)_{pep}$. We selected the HIV-GAG pool 2 based on our preliminary experiments. CMV lysate (strain AD169) (Experteam, Venice, Italy) at 5 μg/ml and SEB (Sigma, St Louis, MO, USA) at 200 ng/ml were used as an unrelated antigen and positive control, respectively.

PBMC were co-stimulated with anti-CD28 and anti-CD49d monoclonal antibodies (mAb) at 2 μg/ml each (BD Bioscience, San Jose, USA). The following fluorescently conjugated mAb were used: anti-CD3 allophycocyanin (APC)-Vio770, anti-CD8 VioBlue, anti-CD4 peridinin chlorophyll protein (PerCP)-Vio700, anti-CD45RA phycoerythrin (PE)-Vio770, anti-CCR7 VioGreen, anti-IFN-γ APC, anti-TNF-α fluorescein isothiocyanate (FITC) and anti-IL-2 PE (all mAb from Miltenyi Biotec).

2.3. Intracellular staining assay (ICS)

Fresh PBMC were isolated using Ficoll density gradient centrifugation, and 1×10^6 cells/ml were cultured overnight with stimuli (37 °C and 5% CO_2) in 10% fetal bovine serum (PAA Laboratories GmbH, Pasching, Austria) in RPMI-1640 (Gibco, CA, USA). BD Golgi Plug was added after 1 h of stimulation to prevent cytokine secretion. Intracellular staining (ICS) was performed after 16 h of incubation. Unstimulated PBMC served as a negative control. PBMC were stained with mAb for surface markers, fixed in 4% paraformaldehyde and permeabilized with PBS-1% BSA −0.5% saponin-0.1% NaN_3 and then stained with mAb for intracellular cytokines. Cells were fixed again in 2% paraformaldehyde, and at least 200,000 lymphocytes were acquired using a FACSCanto II flow cytometer (BD Biosciences).

2.4. Flow cytometry data analysis

Multiple-parameter flow cytometry data were analyzed using FlowJo (Tree Star Inc., San Carlos, CA), Pestle and SPICE software (provided by Dr. Roederer, Vaccine Research Center, NIAID, NIH, USA, 28). Cells were gated according to forward and side scatter plots and the frequency of total cytokines producing $CD4^+$ and $CD8^+$ T-cells was evaluated, using Boolean combination gates. Background cytokine production in the negative control of the ICS assay was subtracted from each stimulated condition. A positive cytokine response was defined as at least twice the background. A frequency of any cytokine-producing T-cells (IFN-γ and/or TNF-α and/or IL-2) of at least 0.03% was considered as a positive $CD4^+$ and $CD8^+$ T-cell response.

In preliminary experiments, we tested the quality of our fluorescent antibodies using isotype control. Moreover, to properly interpret flow cytometry data, we performed the Fluorescence Minus One (FMO) Control. FMO control contains all the fluorochromes in a panel, except for the one that is being measured. Using FMO it is possible to identify any potential spread of the fluorochromes into the channel of interest.

All donors were responsive to the SEB positive control in the ICS assay. Fig. 2 shows representative flow cytometry analyses of the $CD4^+$ (Fig. 2A) and $CD8^+$ (Fig. 2B) T-cell functional profile in response to Mtb, HIV-GAG, CMV, and SEB stimulation in one HIV-LTBI patient before starting the therapies.

The responses were evaluated in terms of number of responders to the stimulus used and based on the frequency of total cytokine response observed.

Phenotypical analysis of different antigen-memory response of $CD4^+$ and $CD8^+$ T-cells was evaluated by flow cytometry according to the expression of the memory/effector surface markers CD45RA and CCR7. The memory status of antigen-specific $CD4^+$ or $CD8^+$ T-cells was evaluated on differently gated $CD4^+$ and $CD8^+$ T-cells, respectively. In particular, the phenotypical analysis was performed within the gates defined as total $CD4^+$ T-cell response and total $CD8^+$ T-cell response, identifying $CD4^+$ and $CD8^+$ T-cells only in the subjects with a positive cytokine response to the antigens.

The analysis was performed before (pre-therapy) and after a median of 1 year of ART therapy initiation (post-therapy). For patients with active TB or LTBI "post-therapies" indicated during ART and after the end of TB treatment or TB preventive therapy, respectively. The frequency of total cytokine expression (IFN-γ, TNF-α and IL-2) and the phenotype was evaluated by flow cytometry in $CD4^+$ and $CD8^+$ T-cells Ag-specific response.

The analysis of the total enrolled participants was performed blinded to the initial LTBI status of the patients (active TB status was known based on the initial Mtb isolation from sputum culture). Independent blinded analysis was then performed using the same gating strategy by two authors (TC and after, by EP). Concordance of the analyses was 90% and agreement was achieved by discussion.

2.5. Statistical analysis

Data were analyzed using SPSS software (Version 19 for Windows, Italy SRL, Bologna, Italy). For continuous measures, medians and interquartile range (IQR) were calculated, the Kruskal-Wallis and Mann-Whitney tests were used to analyze unpaired data to compare the groups, whereas Wilcoxon test was used for paired data. For non-continuous measures the Chi-square or Fischer's test was used. P values as ≤ 0.05 were considered significant.

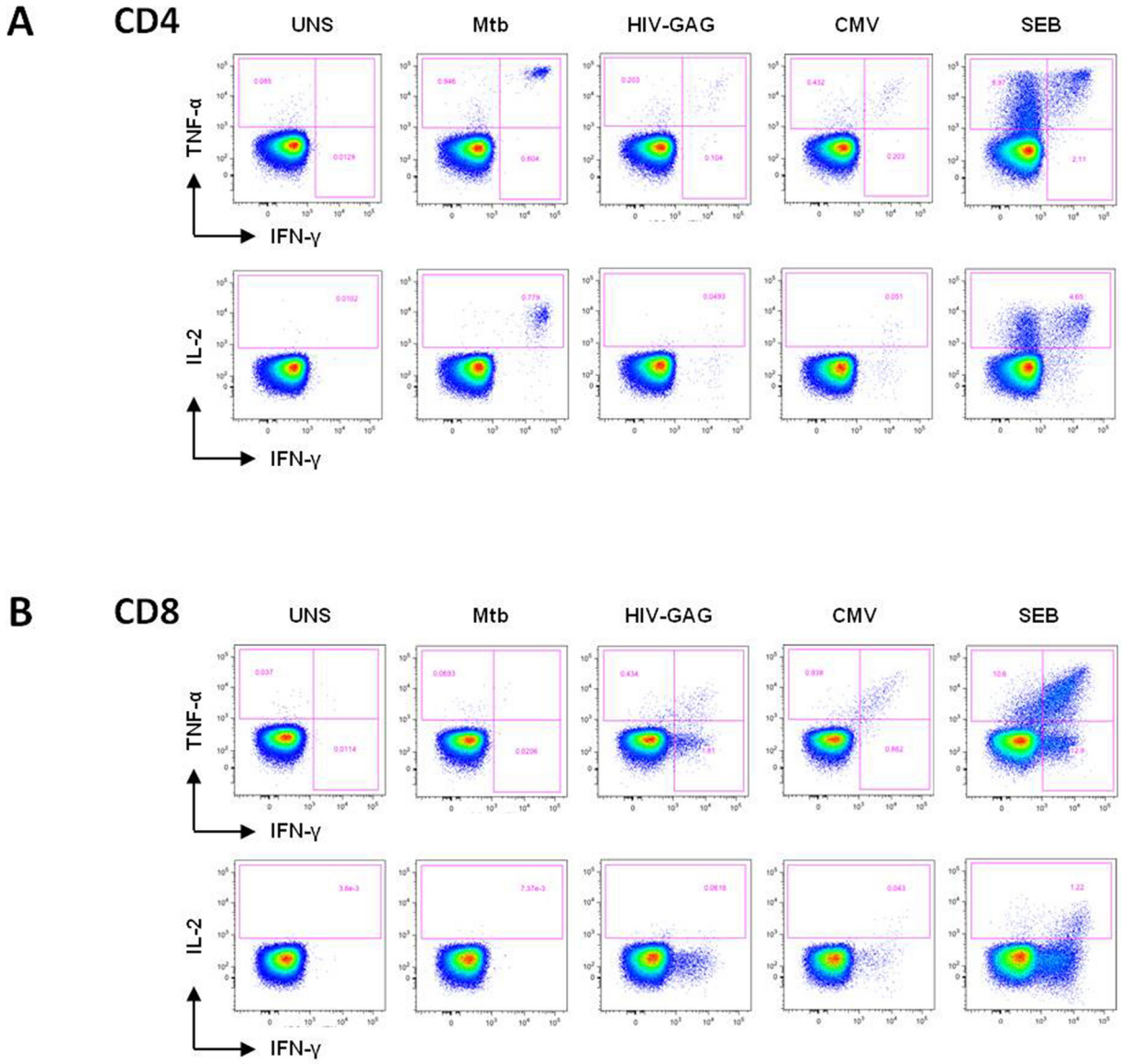

Fig. 2. Cytokine evaluation by flow cytometry plots. Representative flow cytometry plots of the expression of IFN-γ, TNF-α and IL-2 from $CD4^+$ (A) and $CD8^+$ (B) T-cells after stimulation with Mtb (RD1 proteins or peptides), HIV-GAG, CMV and SEB, in one pre-therapies enrolled HIV-LTBI patient. "UNS" indicates unstimulated PBMC. Cells were gated according to forward and side scatter plots and the frequency of total cytokine producing $CD4^+$ and $CD8^+$ T-cells was evaluated, using Boolean combination gates. The frequency of cytokine-producing cells expressed as a percentage of the total $CD4^+$ or $CD8^+$ T-cell population are indicated. Footnotes: Mtb: Mycobacterium tuberculosis; CMV: cytomegalovirus; SEB: staphylococcal enterotoxin B; IFN-γ: interferon-γ; TNF-α: tumor necrosis factor-α; IL-2: interleukin-2.

3. Results

3.1. Comparison of the absolute number of $CD4^+$ and $CD8^+$ T-cells before and after therapies

First, we studied the consistence of $CD4^+$ and $CD8^+$ T-cell populations in the three experimental groups before and after therapies. The $CD4^+$ T-cell absolute counts significantly differed among the analyzed groups at baseline in fact, the median of $CD4^+$ in HIV and HIV-LTBI was 482 cell/mm^3 and 483 cell/mm^3 while in HIV-TB was 253 cell/mm^3. After therapies, the median of $CD4^+$ T-cells absolute number increased in all the groups (521 cell/mm^3, 683 cell/mm^3 and 278 cell/mm^3 for HIV, HIV-LTBI and HIV-TB, respectively) when compared to the pre-therapy values (Table 2). When the relative proportions of $CD4^+$ T-cells were countered the proportions at baseline were higher in HIV and HIV-LTBI (23.7% and 23.4%, respectively) compared to that observed in the HIV-TB group (15.8%). After therapies, percentages of $CD4^+$ T-cells increased in the three groups reaching the value of 30.6% in the HIV group.

$CD8^+$ counts before therapies were 1116 cell/mm^3, 1287 cell/mm^3 and 653 cell/mm^3 for HIV, HIV-LTBI and HIV-TB group respectively; the values decreased after therapies in HIV (782 cell/mm^3) and HIV-LTBI (852 cell/mm^3), while for HIV-TB we observed a weak increase (771 cell/mm^3) when compared to pre-therapy values (Table 2). The proportion of $CD8^+$ T-cells significantly decreased in the following groups after therapies: HIV-LTBI, 56.3% vs 48.2%, $p = 0.018$; HIV-TB 57.7% vs 45.2%, $p = 0.021$ (Table 2).

As expected, the mean number of HIV-RNA (cp/μl) in the three groups, dramatically decreased after ART: 32727 cp/μl pre-ART vs 20 cp/μl post-ART in HIV; 16689 cp/μl pre-ART vs 39 cp/μl post-ART in HIV-LTBI, 63697 cp/μl pre-ART vs 61 cp/μl post-ART in HIV-TB (Table 2). Based on these results, we conclude that the median of $CD4^+$ T-cells absolute number and the median of relative proportion increased after therapy in the three groups of patients, only reaching a

Table 2
Comparison of the absolute number of $CD4^+$ and $CD8^+$ T-cells before and after therapies between HIV-LTBI and HIV-TB patients.

	HIV			HIV-LTBI			HIV-TB		
	Pre-ART	Post-ART	*p* value[a]	Pre-therapies	Post-therapies	*p* value[a]	Pre-therapies	Post-therapies	*p* value[a]
$CD4^+$ T-cell7/mm^3									
Median (IQR)	482 (447–534)	521 (417–635)	0.4	483 (341–1019)	683 (336–878)	0.4512	253 (71–299)	278 (234–456)	**0.05**
$CD4^+$ %									
Median (IQR)	23.7 (21.1–31.7)	30.6 (19.1–35.3)	0.16	23.4 (20.6–44.1)	33.5 (23.4–38.3)	0.6	15.8 (9.7–21.4)	21.4 (14.7–25.6)	0.17
$CD8^+$ T-cell/mm^3									
Median (IQR)	1116 (527–1516)	782 (526–1183)	0.2	1287 (884–1353)	852 (686–1180)	0.31	653 (533–893)	771 (372–1145)	0.5
$CD8^+$ %									
Median (IQR)	59.1 (38.5–64.2)	45.2 (36.5–51.2)	0.07	56.3 (39.3–63.3)	48.2 (25.6–57.5)	**0.018**	57.7 (46.1–61.0)	45.2 (37.7–53.2)	**0.021**
Lymphocytes $x10^3/mm^3$									
Median (IQR)	1.95 (1.43 – 2.38)	1.95 (1.53–2.4)	0.64	2.2 (1.6–2.5)	2.1 (1.8–2.6)	1	1.2 (0.9–1.65)	1.8 (1.35–2.15)	**0.027**
HIV-RNA (cp/μl)									
Median (IQR)	32727 (15344–95370)	20 (20–41)	**0.017**	16689 (2108–40319)	39 (20–2504)[b]	0.16	63697 (6902–537158)	61 (20–47657)[c]	**0.038**

HIV: human immunodeficiency virus; LTBI: latent tuberculosis infection; TB: tuberculosis; IQR: interquartile range; ART: antiretroviral therapy.
The bold values are the p values ≤0.05 to be considered as significant.
[a] Wilcoxon test.
[b] Number of patients that despite ART did not reach the undetectable HIV viral load: 3/7 (43%) HIV-LTBI.
[c] Number of patients that despite ART did not reach the undetectable HIV viral load: 5/9 (55%) HIV-TB.

significant difference in the HIV-TB group as median of absolute number. Differently, the median of $CD8^+$ T-cells absolute count decreased in the group of HIV and HIV-LTBI and this was associated to a decreased of the relative proportion in both groups. Interestingly, in the HIV-TB we observed an increase of the median of $CD8^+$ T-cells absolute number accompanied by a decreased of the relative proportion; this was due to a significant increased number of the total lymphocytes after therapies ($p = 0.027$) (Table 2).

3.2. *Effect of ART and TB therapies on HIV/Mtb-specific $CD4^+$ and $CD8^+$ T-cell responses*

To evaluate whether the dynamics of HIV/Mtb-specific T-cells mirror the reconstitution of the total T-cell populations, we evaluated the magnitude of Mtb-, HIV-GAG-, CMV- and SEB responses, before and after starting the therapies in both $CD4^+$ and $CD8^+$ T-cell populations, measuring the total response of antigen-specific T-cells in terms of total cytokine production (IFN-γ, TNF-α and IL2) comparing HIV-LTBI and HIV-TB groups. We have considered the capability of $CD4^+$ and $CD8^+$ T-cells to produce the different cytokines in relation to the memory differentiation profiles, therefore total intracellular cytokines producing T-cells were observed following stimulation with the above cited antigens by FACS analysis. This value is referred from now on, as the total frequency of any cytokine-producing T-cells (IFN-γ and/or TNF-α and/or IL-2).

3.3. *$CD4^+$ specific T-cell response*

In the HIV-infected group, ART increased the proportion of responders to HIV-GAG (pre-therapy: 38% vs post-therapy: 75%). CMV and SEB responses were detectable in all patients independently of ART (Table 3) (Fig. 3A).

In the HIV-LTBI group, the therapies increased the proportion of responders to RD1 proteins (pre-therapies: 43% vs post-therapies: 100%) and RD1 peptides (pre-therapies: 71% vs post-therapies: 100%) (Table 3). Similarly, the treatments increased the proportions of responders to the recall antigens HIV-GAG (pre-therapies: 43% vs post-therapies: 71%) and CMV (pre-therapies: 86% vs post-therapies: 100%). Conversely, the response to SEB was detectable in all patients independently of the treatments (Table 3).

The frequency of the total $CD4^+$ response to RD1 proteins was significantly higher after ART and TB therapy ($p = 0.015$) (Fig. 3A) and a similar trend, although not significant, was observed in response to RD1 peptides.

Differently, the frequency of CMV-specific $CD4^+$ T-cells was lower after the treatments compared to baseline, whereas the frequency of HIV-GAG-specific and SEB-responding $CD4^+$ T-cells were comparable regardless the treatments (Fig. 3A).

In the HIV-TB group, ART and TB therapies increased the proportion of responders to RD1 proteins and peptides (both pre-therapies: 56% vs post-therapies: 67%), and to CMV (pre-therapies: 33% vs post-therapies: 56%) (Table 3). Otherwise, the number of HIV-TB responders to GAG significantly increased after the therapies (pre-therapies: 0% vs post-therapies: 67%, $p = 0.009$). Conversely, the responses to SEB were detectable in all patients independently of the treatments (Table 3).

The frequency of the total $CD4^+$ response to RD1 proteins and peptides decrease after therapies, from 0.93% to 0.19% for proteins and from 0.9% to 0.09% for peptides, as previously shown in the HIV-uninfected patients [30]. The frequency of SEB $CD4^+$ T-cells response was comparable regardless the treatments (Fig. 3A). Differently, ART and TB therapies significantly increased the frequency of the HIV-GAG-specific T-cell response ($p = 0.03$), whereas the frequency of CMV-specific T-cells was decreased, although not significantly, compared to pre-treatments (Fig. 3A).

To evaluate the impact of the $CD4^+$ T-cell counts across the groups, the absolute number of Ag-specific $CD4^+$ T-cell was calculated, as

Table 3
Impact of ART and TB therapies on the CD4$^+$ T-cell response to specific stimuli in HIV-infected subjects, HIV-LTBI and HIV-TB patients.

	HIV			HIV-LTBI			HIV-TB		
	Pre-ART	Post-ART	*p* value	Pre-therapies	Post-therapies	*p* value	Pre-therapies	Post-therapies	*p* value
RD1 proteins Number of Responder over total (%)	–	–	–	3/7 (43)	7/7 (100)	0.07	5/9 (56)	6/9 (67)	1
RD1 peptides Number of Responder over total (%)	–	–	–	5/7 (71)	7/7 (100)	0.46	5/9 (56)	6/9 (67)	1
HIV-GAG Number of Responder over total (%)	3/8 (38)	6/8 (75)	0.31	3/7 (43)	5/7 (71)	0.59	0/9 (0)	6/9 (67)	**0.009**
CMV Number of Responder over total (%)	6/8 (75)	6/8 (75)	1	6/7 (86)	7/7 (100)	1	3/9 (33)	5/9 (56)	0.64
SEB Number of Responder over total (%)	8/8 (100)	8/8 (100)	1	7/7 (100)	7/7 (100)	1	9/9 (100)	9/9 (100)	1

HIV: human immunodeficiency virus; LTBI: latent tuberculosis infection; TB: tuberculosis; ART: antiretroviral therapy; RD: region of difference; CMV: cytomegalovirus; SEB: staphylococcal enterotoxin B.
Fisher's exact test was used for the comparisons of the number of responders between pre- and post-therapies.
The bold values are the p values ≤ 0.05 to be considered as significant.

described [31].

In the HIV-LTBI group we found a significant increased number of Mtb-specific cells after ART and TB therapies (RD1 proteins $p = 0.015$, RD1 peptides $p = 0.03$) (Fig. 3B).

In the HIV-TB patients the absolute number of Mtb-, CMV- and SEB-responding CD4$^+$ T-cells remained unchanged upon treatments, whereas the number of HIV-GAG-specific CD4$^+$ T-cells significantly increased ($p = 0.03$) (Fig. 3B).

Finally, in the HIV-infected patients, ART did not significantly modify the absolute number of SEB-, HIV-GAG- or CMV-responding CD4$^+$ T-cells (Fig. 3B).

3.4. CD8$^+$ specific T-cell response

In the HIV-infected participants, the number and frequency of responders of CD8$^+$ T-cells to all antigens was evaluated after ART and TB therapies (Table 4 and Fig. 4).

In the HIV-LTBI group, ART and TB therapies increased the proportion of responders to RD1 peptides (pre-therapies: 14% *vs* post-therapies: 43%), and decreased the number of responders to CMV (pre-therapies: 43% *vs* post-therapies: 29%) (Table 4). Differently, the proportion of responders to RD1 proteins, HIV-GAG and SEB remained unchanged upon treatments (Table 4). The frequency of the total response to both Mtb proteins and peptides decreased although not significantly. Frequency of responses to HIV-GAG, CMV and SEB were not significantly modified upon the treatments (Fig. 4A).

In the HIV-TB group, ART and TB therapies increased the proportion of responders to RD1 peptides (pre-therapies: 33% *vs* post-therapies: 77%), HIV-GAG (pre-therapies: 22% vs post-therapies: 44%) and CMV (pre-therapies: 11% *vs* post-therapies: 33%) although not significantly (Table 4). Differently, the proportion of responders to RD1 proteins slightly decreased after the therapies (pre-therapies: 56% vs post-therapies: 44%). Moreover, the number of responders to SEB remained unchanged after the therapies (Table 4). The frequency of the total response to Mtb, HIV-GAG, CMV and SEB did not show any variation after treatments (Fig. 4A).

Finally we did not found any variations in the absolute number of Mtb-, HIV-GAG-, CMV-specific and SEB-CD8$^+$ responding cells comparing pre- and post-therapies in all the groups analyzed (Fig. 4B).

3.5. Effect of ART on the phenotype of pathogen-specific CD4$^+$ and CD8$^+$ T-cells

To further investigate whether the cell maturation phenotype could impact the replenishment capacity of HIV/Mtb-specific CD4$^+$ and CD8$^+$ T-cells, we compared the differentiation profiles of Mtb-, HIV-GAG-, CMV-, and SEB-responding CD4$^+$ and CD8$^+$ T-cells in terms of total cytokine production at baseline (pre-therapies) and post-therapies. Cumulative analyses show that after therapies the majority of the CD4$^+$ T-cell response to Mtb and to the other unrelated antigens was characterized by an increase of the CM and by a decrease of EM subpopulations in all the groups analyzed (Fig. 5A–B). Conversely, the phenotype distribution of CD8$^+$ T-cells showed different patterns. In HIV-LTBI subjects, after therapies an increase of N subsets and a decrease of EM was found in response to RD1 proteins, peptides and to HIV-GAG (Fig. 5A–B). In HIV-TB patients, the EM phenotype was the major subset of CD8$^+$ T-cells in response to all stimuli and did not change overtime, while the E subset was slightly increased after ART and TB therapies in response to RD1 antigens (Fig. 5A–B).

4. Discussion

Although ART causes a progressive reconstitution of the number of CD4$^+$ T-cells in most subjects, the different degree of the recovery of copathogen-specific CD4$^+$ and CD8$^+$ T-cells is poorly defined. In this prospective study, conducted in HIV-infected participants with or without TB co-infection, we evaluated how ART and TB therapies concomitantly contributed to the modulation of the absolute count of CD4$^+$ and CD8$^+$ T-cell, the capacity of these cells to produce several cytokines in response to appropriate antigen stimulation and their different phenotype distribution.

The median of absolute number and the proportion of CD4$^+$ T-cells increased in all groups after therapies, although only in HIV-TB group this increase was significant. Conversely, the median of absolute count and proportion of CD8$^+$ T-cells decreased in the HIV and HIV-LTBI subjects. Interestingly, in the HIV-TB we observed a little increase of the median of absolute number of CD8$^+$ T-cells accompanied by a decreased of the relative proportion due to a significant increased number of the total lymphocyte after therapies.

Regarding the Mtb-specific responses, ART and TB therapies increased the frequency of CD4$^+$ T-cell response to RD1 proteins in HIV-LTBI subjects. This is in line with other reports showing an increased IFN-γ production in response to Mtb-specific antigens after ART [32,33]. Moreover, this data are in agreement with a recent paper [34] showing a decreased frequency of the total cytokine response to PPD in HIV-LTBI compared to the LTBI subjects (without HIV infection). It is known, in fact, that HIV infection causes a depletion of the RD1-specific T-cell clones [34] that ART may, at least partially, recover. In the present study we show that the frequency of RD1-specific CD4$^+$ T-cells found in the cured TB patients (HIV-TB post therapies: median 0.09) is similar to that one found in the HIV-LTBI after therapies (HIV-LTBI: median 0.075) suggesting that this level could be associated to mycobacterial containment.

Interestingly, in the HIV-TB group the therapies significantly increased the CD4$^+$ T-cells counts compared to baseline while the frequency of CD4$^+$ T-cells response to TB-Ags decreased. These results are in agreement with those reported by Day C. et al. [30] showing that in TB patients (without HIV infection), the CD4-specific T-cells response decrease after TB therapy and correlating it with the decrease of the mycobacterial load. In the present manuscript, in those with active TB

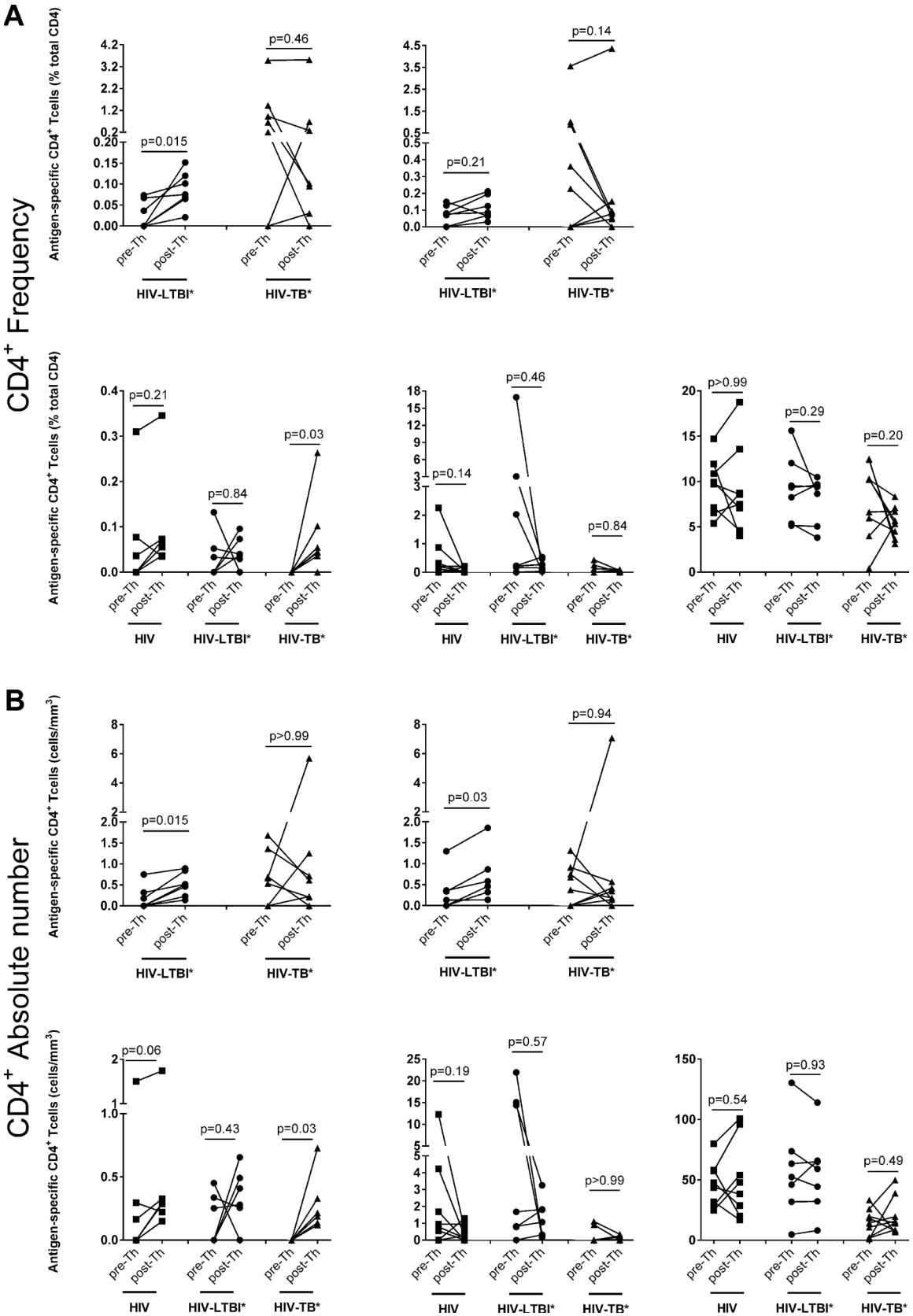

Fig. 3. Effect of ART and TB therapies on RD1 proteins- and RD1 peptides- (Mtb-specific antigens), HIV-GAG-, CMV-, and SEB-specific $CD4^+$ T-cell responses in the different groups of patients analyzed. Frequency (A) and absolute number (B) of RD1 proteins-, RD1 peptides-, HIV-GAG-, CMV- and SEB-specific $CD4^+$ T-cell response before and after ART and TB therapies. Statistical comparisons were performed using a Wilcoxon matched-pairs test and Mann-Whitney un-pairs test. Footnotes: HIV: human immunodeficiency virus; TB: active tuberculosis; LTBI: latent TB infection; RD: region of difference; CMV: cytomegalovirus; SEB: staphylococcal enterotoxin B.

Table 4
Impact of ART and TB therapies on the CD8$^+$ T-cell response to specific stimuli in HIV-infected subjects, HIV-LTBI and HIV-TB patients.

	HIV			HIV-LTBI			HIV-TB		
	Pre-ART	Post-ART	*p* value	Pre-therapies	Post-therapies	*p* value	Pre-therapies	Post-therapies	*p* value
RD1 proteins Number of Responder over total (%)	–	–	–	2/7 (29)	2/7 (29)	1	5/9 (56)	4/9 (44)	1
RD1 peptides Number of Responder over total (%)	–	–	–	1/7 (14)	3/7 (43)	0.56	3/9 (33)	7/9 (77)	0.15
HIV-GAG Number of Responder over total (%)	5/8 (63)	5/8 (63)	1	3/7 (43)	3/7 (43)	1	2/9 (22)	4/9 (44)	0.62
CMV Number of Responder over total (%)	1/8 (13)	1/8 (13)	1	3/7 (43)	2/7 (29)	1	1/9 (11)	3/9 (33)	0.58
SEB Number of Responder over total (%)	8/8 (100)	8/8 (100)	1	7/7 (100)	7/7 (100)	1	9/9 (100)	9/9 (100)	1

HIV: human immunodeficiency virus; LTBI: latent tuberculosis infection; TB: tuberculosis; ART: antiretroviral therapy; RD: region of difference; CMV: cytomegalovirus; SEB: staphylococcal enterotoxin B.
Fisher's exact test was used for the comparisons of the number of responders between pre- and post-therapies.

that are also HIV-coinfected, there is a dissociation between an increased total CD4$^+$ T-cells count, due to the HIV-load reduction by ART, and a decreased CD4-specific response that associate with a reduction of Mtb load due to TB therapy. It is important to mention that the overall functional restoration of CD4$^+$ and CD8$^+$ T-cells is a slowly process, that is evident after more than 2 years of complete viral suppression [35]. Therefore, for a more accurate immune assessment we are planning a subsequent evaluation after a longer observation time.

In this study we show that the number of CD8$^+$ T-cells responders to RD1 peptides increased after therapies in HIV-LTBI and HIV-TB groups, although no significant variation of the CD8-specific response frequency was found. Conversely, using different stimuli, it has been shown a decline of the Mtb peptide-specific CD8$^+$ T-cells after anti-TB therapy in patients with active TB either HIV-TB co-infected [36] or without HIV infection [37]. Therefore, it would be important to study these responses after longer-term ART and using immune panels involving several stimuli. This approach would lead to a more definitive conclusion on the restoration of pathogen-specific immunity.

Finally, comparing active TB versus LTBI, we found that the Mtb-specific CD8$^+$ T-cell frequency (in response to peptides) is higher in the active TB (HIV-TB) compared to those with LTBI (HIV-LTBI) at baseline. This is similar to the findings reported in studies conducted in HIV-uninfected subjects [30,38]. Interestingly, it has also been shown that HIV reduces the Mtb-specific CD8$^+$ T-cell response to ESAT-6/CFP10 proteins in those latently infected [39].

We also assessed the phenotypic and functional characterizations of Mtb-specific CD4$^+$ and CD8$^+$ T-cells to evaluate whether distinct T-cell functional signatures are correlated with a particular skewed effector phenotypes. We found that the therapies increased the Mtb-specific CD4$^+$ CM T-cell response. These data are in line with a previous study demonstrating that TB treatment reduces the frequency of EM PPD-specific CD4$^+$ T-cells while simultaneously increasing the proportion of specific T-cells with CM phenotype [40]. Moreover, it has been demonstrated that the restoration of Mtb-specific CD4$^+$ T-cells depends on the memory status as proportion of the central memory and transitional memory cells present prior to therapy [31]. Similarly, Wilkinson et al. found that ART expanded the CM cell subset [41].

Together these data confirm that CD4$^+$ T-cells are required for an optimal protective immune response against Mtb, especially in the HIV-LTBI subjects that are those at higher risk to progress to disease. This is due to the fact that Mtb-specific CD4$^+$ T-cell are preferentially infected and depleted during early HIV infection [23,42]. ART preferentially reconstitutes the CD4$^+$ T-cells responding to frequently encountered antigens, such as CMV or Mtb but not to tetanus [43–45] indicating the presence of different programs of cell reconstitution.

The analysis of the phenotype distribution of CD8$^+$ T-cell in response to Mtb, HIV-GAG and to CMV antigens highlighted a different phenotype distribution before and after ART and TB therapies. In fact, we found in HIV-LTBI subjects, an increase of CD8$^+$ T-cells with a N phenotype and a decrease of EM in response to RD1 antigens, while in HIV-TB patients, the EM phenotype was the major subset of CD8$^+$ T-cells in response to all stimuli, while the E subset was slightly increased after ART and TB therapies in response to RD1 antigens. Cells with N phenotype may resemble the recently described memory stem cell subset characterized by a greater ability to proliferate and persist long, if compared with CM cells [46,47]. Then, the memory status before the ART could affect the recovery of Ag-specific CD8$^+$ T-cells. Therefore, the response of antigen specific CD8$^+$ T-cells could be related to the stage of Mtb infection/disease, reflecting the ability of the different phenotypes in controlling the pathogen.

The analysis of the cell phenotype distribution in response to viruses correlate with the type of disease induced, acute (Influenza, Yellow Fever, Poliovirus, Hepatitis A, etc) versus chronic disease (CMV, Epstein Barr Virus (EBV), HIV-1, JC Virus (JCV) etc). In this model it was found an association of E phenotype with a controlled chronic infection whereas the EM phenotype was associated with an uncontrolled chronic infection [48]. Our results suggest that this may be also the case for Mtb-specific CD8$^+$ T-cells in HIV-TB patients. From this analysis, it is evident that the type of antigen and the different stage of infection are involved in the phenotype distribution of CD8$^+$ T-cells, but further studies are needed to better attribute any functional role.

A potential limitation of the present study is the relatively small number of participants included. This is the consequence of the fact that the study was conducted in a low TB endemic country in which also HIV prevalence is low, and this implies a fewer number of HIV co-infected subjects. Furthermore, we carried out our study only on the short-term restoration of pathogen-specific immunity (after 1 year of ART and TB therapies). Although the patients recovered the immunological parameters in terms of reduction of HIV replication and improved CD4$^+$ T-cell counts, the study would benefit of monitoring responses after longer-term therapy to provide more definitive conclusions on the restoration of pathogen-specific immunity. On the other hand, the present study was very systematic in terms of groups analyzed (HIV, HIV-LTBI and HIV-TB), experimental tools used (Mtb-specific and unrelated recall antigens employed), integrity and reproducibility of the results obtained, evaluation of the total cytokine responses and memory status in both CD4$^+$ and CD8$^+$ T-cell subsets, which make our findings robust.

In summary, we show here that the dynamics of Mtb- and HIV-GAG- and CMV-specific CD4$^+$ and CD8$^+$ T-cell recovery in HIV co-infected or not co-infected participants are distinct, demonstrating that non all T-cell subsets have the same recovery capacity. Partial reestablishment of functional immunity could affect the choice of the treatment in HIV-infected population. Overall, our findings underscore the complexity of immune reconstitution on ART and TB therapy and the importance of preserving functional immunity with these treatments in HIV-infected participants.

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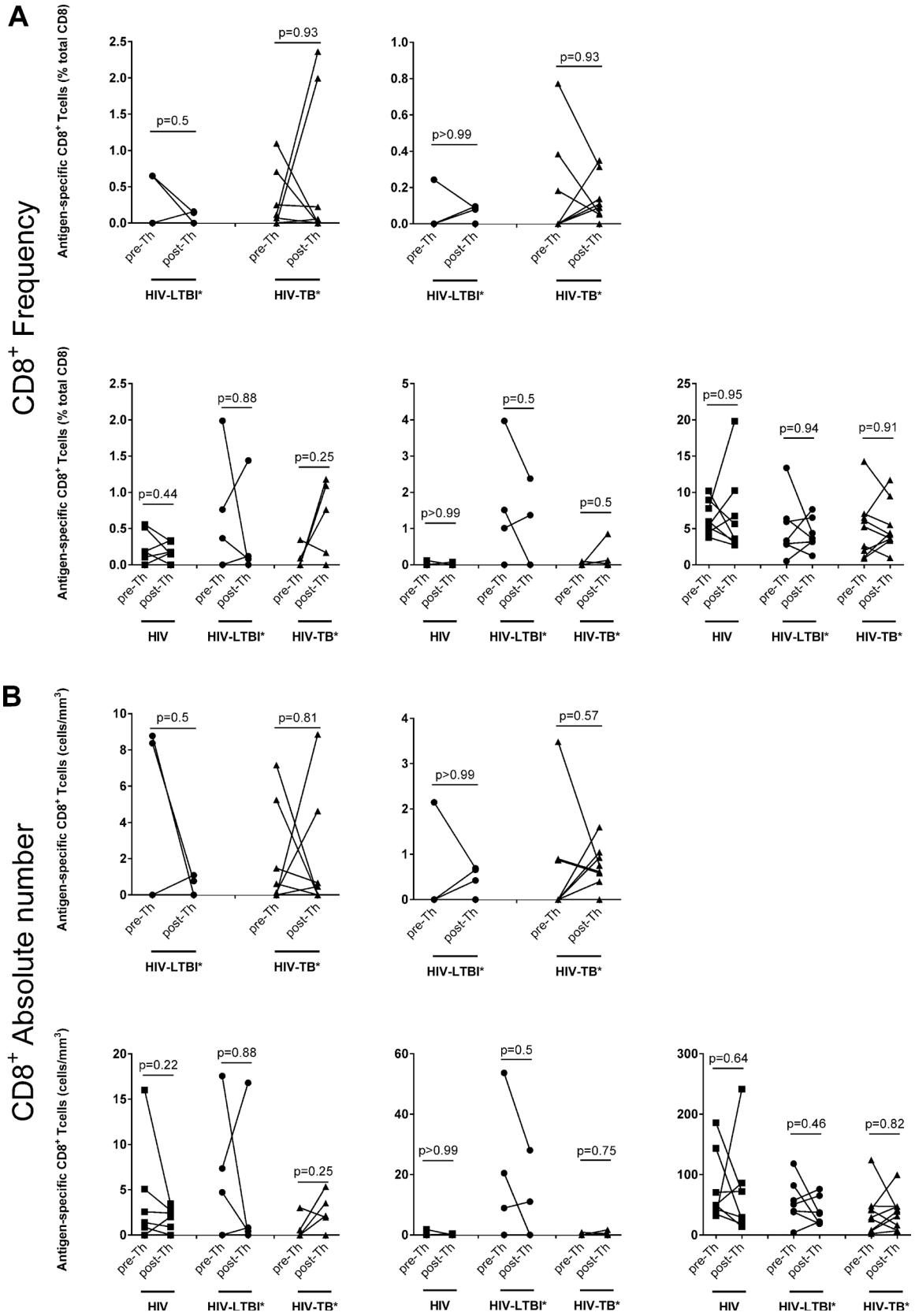

Fig. 4. Effect of ART and TB therapies on RD1 proteins- and RD1 peptides- (Mtb-specific antigens), HIV-GAG-, CMV-, and SEB-specific $CD8^+$ T-cell responses in the different groups of patients analyzed. Frequency (A) and absolute number (B) of RD1 proteins-, RD1 peptides-, HIV-GAG-, CMV- and SEB-specific $CD8^+$ T-cell response before and after ART and TB therapies. Statistical comparisons were performed using a Wilcoxon matched-pairs test and Mann-Whitney un-pairs test. Footnotes: HIV: human immunodeficiency virus; TB: active tuberculosis; LTBI: latent TB infection; RD: region of difference; CMV: cytomegalovirus; SEB: staphylococcal enterotoxin B.

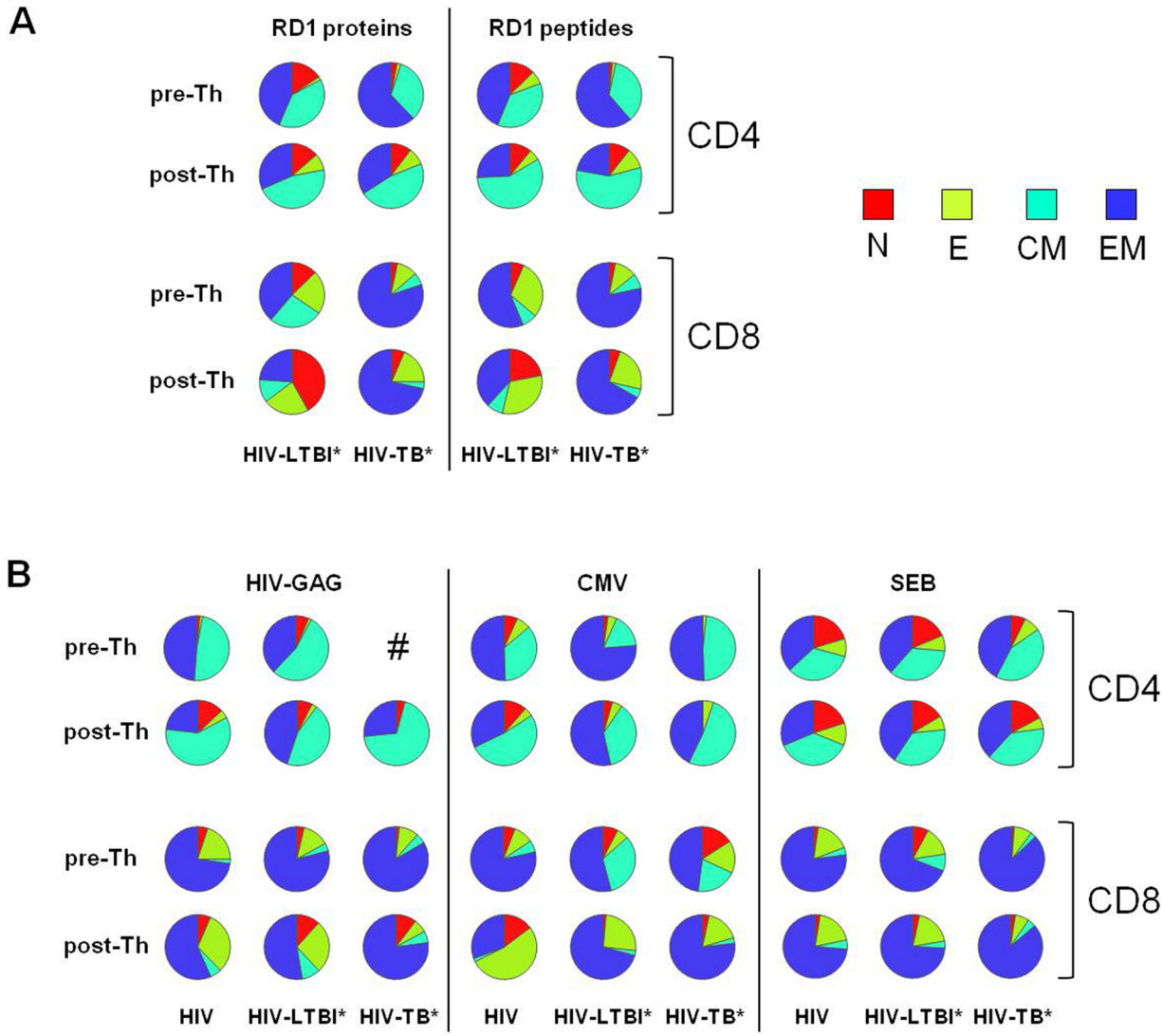

Fig. 5. Memory status of CD4$^+$ and CD8$^+$ T-cells in response to Mtb-, HIV-GAG-, CMV- and SEB-stimuli. Memory status of CD4$^+$ and CD8$^+$ T-cell response was evaluated by flow cytometry according to the surface expression of CD45RA and CCR7 in the gate of total cytokine production CD4$^+$ and CD8$^+$ T-cell. We defined naïve (N) as CD45RA$^+$ CCR7$^+$, central memory (CM) as CD45RA$^-$ CCR7$^+$, effector memory (EM) as CD45RA$^-$ CCR7$^-$, terminally differentiated effector memory T-cells (E) as CD45RA$^+$ CCR7$^-$. Statistical comparisons were performed using a Wilcoxon matched-pairs test. The pie charts represent the proportion of N, CM, EM and E CD4$^+$ and CD8$^+$ T-cells evaluated in the different groups pre- and post-therapies in response to overnight stimulation with Mtb- (A), HIV-GAG-, CMV- and SEB-stimuli (B). Footnotes: HIV: human immunodeficiency virus; TB: active tuberculosis; LTBI: latent TB infection; Mtb: Mycobacterium tuberculosis; RD: region of difference; CMV: cytomegalovirus; SEB: staphylococcal enterotoxin B; N: naïve; CM: central memory; EM effector memory; E: terminally-differentiated effector memory; Th: antiretroviral therapy; *also TB therapy; # no responders.

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