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Clinical & Experimental Immunology
The Journal of Translational Immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi: 10.1111/cei.13544

Th22 response induced by *Mycobacterium tuberculosis* strains is closely related to severity of pulmonary lesions and bacillary load in patients with multi-drug-resistant tuberculosis

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Accepted for publication 21 October 2020
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Summary

The role of interleukin-22 (IL-22) in the pathogenesis or tissue repair in human tuberculosis (TB) remains to be established. Here, we aimed to explore the *ex-vivo* and *in-vitro* T helper 22 (Th22) response in TB patients and healthy donors (HD) induced by different local multi-drug-resistant (MDR) *Mvcobacterium tuberculosis* (*Mtb*) strains. For this purpose, peripheral blood mononuclear cells from drug-susceptible (S-TB) MDR-TB patients and HD were stimulated with local MDR strains and the laboratory strain H37Rv. IL-22 and IL-17 expression and senescent status were assessed in CD4$^+$ and CD8$^+$ cells by flow cytometry, while IL-22 amount was measured in plasma and culture supernatants by enzyme-linked immunosorbent assay (ELISA). We found lower IL-22 amounts in plasma from TB patients than HD, together with a decrease in the number of circulating T cells expressing IL-22. In a similar manner, all *Mtb* strains enhanced IL-22 secretion and expanded IL-22$^+$ cells within CD4$^+$ and CD8$^+$ subsets, being the highest levels detected in S-TB patients. In MDR-TB, low systemic and *Mtb*-induced Th22 responses associated with high sputum bacillary load and bilateralism of lung lesions, suggesting that Th22 response could be influencing the ability of MDR-TB patients to control bacillary growth and tissue damage. In addition, in MDR-TB patients we observed that the higher the percentage of IL-22$^+$ cells, the lower the proportion of programmed cell death 1 (PD-1)$^+$ or CD57$^+$ T cells. Furthermore, the highest proportion of senescent T cells was associated with severe lung lesions and bacillary load. Thus, T cell senescence would markedly influence Th22 response mounted by MDR-TB patients.

Keywords: IL-22, multi-drug-resistant tuberculosis, *Mycobacterium tuberculosis* strains, Th22 response

Introduction

Interleukin-22 (IL-22), a member of the IL-10 cytokine family, is produced by cells from the innate and adaptive immune system, including tissue-resident innate lymphoid cells (ILCs), natural killer (NK) cells, macrophages, NK T cells and activated CD4$^+$, CD8$^+$ and γδ T cells [1]. In mice, antigen-specific IL-22 production is driven by T helper type 1 (Th1) and Th17 cells, while in humans IL-22 can be released by Th17 cells as well as a different subset of T helper cells, called Th22 cells, which produce IL-22 and tumor necrosis factor (TNF)-α and not IL-17 or interferon (IFN)-γ [2–4]. IL-22 binds to its cognate heterodimeric receptor comprised of the subunits IL-22R1 and IL-10R2. IL-22R is expressed in epithelial cells and fibroblasts of diverse tissues, but mainly in skin and mucosal epithelial cells promoting tissue proliferation, regeneration and healing, suggesting that IL-22 is involved in protection from tissue damage at mucosal surfaces [3,5]. Conversely, IL-22 stimulates the production of anti-microbial peptides by epithelial cells, mediating early mucosal defense against bacteria that cause intestinal disease and pneumonia in mouse models [3]. However, its association with a number of human diseases, including inflammatory bowel disease,

psoriasis and rheumatoid arthritis, suggests that IL-22 has an important role in inflammatory processes [6].

Recent studies show that *Mycobacterium tuberculosis* (*Mtb*) induces expression of IL-22R1 on infected macrophages from mice, macaques and humans [7,8]. Also, IL-22 signaling inhibits intracellular growth of H37Rv and Erdman strains by enhancing phagolysosomal fusion and calgranulin production in infected human macrophages, suggesting a protective role of IL-22 in mycobacterial infection [9,10]. However, results obtained in either experimental or human tuberculosis (TB) are not clear enough. While in the mouse the absence of IL-22 does not affect acute infection with H37Rv or Erdman strains, IL22 deficiency increases the bacillary load in chronic stages of infection with the hypervirulent strain HN878 [7]. In human TB, IL-22 was found in bronchoalveolar lavage and pleural effusions from TB patients as well as in macaques infected with *Mtb*, suggesting that IL-22 could be involved in the pathogenesis of the disease or tissue repair [9]. Also, a subset of $CD4^+$ T cells bearing membrane-bound IL-22 (m-IL-22) that inhibit *Mtb* intracellular replication in macrophages by cell–cell interaction has been demonstrated in H37Rv-infected peripheral blood mononuclear cells (PBMCs) from latently infected individuals and *Mtb*-infected macaques [9], which suggests that these cells could evolve into effectors cells after *de-novo* IL-22 production and to exert anti-microbial functions. Even though IL-22 is one of the few cytokines with secretory and membrane-bound forms, the mechanism by which it is retained in the membrane is not yet known [8]. Although a negative association has been demonstrated between IL-22 and IFN-γ in purified derivative protein (PPD)-stimulated PBMCs from drug-susceptible TB patients [11], drug-susceptible strains of the Beijing lineage with recognized virulence and high replication capacity induce low levels of IL-22, IFN-γ and IL-1β in PBMC of healthy individuals, suggesting that the low induction of an inflammatory response could be an evasion mechanism in epidemiologically successful emerging strains [12].

In Argentina, a total of 11 670 TB cases were reported throughout the country in 2018 resulting in an incidence of 26·23 per 100 000 inhabitants, being 2·25% of the cases caused by *Mtb* strains resistant at least to one anti-TB drug, and almost 50·0% of these strains showed resistance to isoniazid and rifampicin [multi-drug-resistant (MDR)-TB], the main anti-TB drugs [13]. In Argentina, MDR-TB was first reported in the 1990s in patients hospitalized with AIDS [14], and epidemiological and genotyping studies identified strains of outbreak M (Haarlem 2, H2) in Buenos Aires and Ra (Latin American–Mediterranean 3, LAM3) and Rb (Tuscany) in Rosario [15]. Currently, M causes approximately 29% of cases of MDR-TB and Ra 11·3%, demonstrating that they are adapted to their geographical niche and have found mechanisms to evade the immune response of the host [16]. In previous work we demonstrated that, in opposition to the laboratory strain H37Rv, M and Ra strains induce an altered T helper type 1/T helper type 2 (Th1/Th2) profile in T cells from PPD^+ healthy donors (HD) and from patients with TB [17]. Also, both strains induce a strong Th17 response in MDR-TB patients through differential expansion of $IL\text{-}17^+IFN\text{-}\gamma^-$ T cells that was modulated by the genetic background of the infecting *Mtb* strain and its bacterial burden, suggesting that *Mtb* strains manipulated the host immune response [18,19]. Therefore, the aim of the present work was to explore *ex-vivo* and *in-vitro* Th22 responses induced by different local MDR *Mtb* strains in patients with active pulmonary MDR-TB and HD.

Materials and methods

Study period

This study was carried out from August 2016 to May 2019.

Patients

Peripheral blood samples (n = 86) were obtained from patients with MDR-TB (n = 59) hospitalized at F. J. Muñiz Hospital (MH), Buenos Aires City, and from drug-susceptible pulmonary TB patients (S-TB, n = 27), provided by Dr A. A. Cetrángolo Hospital (CH), Vicente López, Buenos Aires Province, Argentina.

Informed consent was obtained from patients according to the guidelines of the ethics committee of each hospital. All TB cases were diagnosed at MH and CH by the presence of clinical respiratory symptoms and chest radiography and the use of microbiology methods, such as sputum smear examination for acid-fast bacilli (AFB) detection and cultures on Löwenstein-Jensen (LJ) medium and/or MGIT960 (Becton Dickinson, Buenos Aires, Argentina), to isolate *Mtb* [20,21]. At the time of the blood collection, all TB patients had received fewer than 15 days of the specific anti-TB treatment.

Drug-susceptibility testing to first- (isoniazid, rifampicin, streptomycin, ethambutol and pyrazinamide) and second-line anti-TB drugs (ethionamide, cycloserine, amikacin, linezolid, kanamycin, p-aminosalicylic acid, levofloxacin, moxifloxacin) was also performed at MH and CH by the proportion method on LJ and/or MGIT SIRE kit (Becton Dickinson) [22,23].

Exclusion criteria included HIV co-infection, the presence of concurrent infectious diseases or non-infectious conditions (cancer, diabetes or steroid therapy) and children aged under 15 years. Clinical and epidemiological data

from patients were recovered. The information included location of the disease and severity of the lesion, smear examination, cultures and drug-susceptibility testing results and age, gender, residence, being a health-care worker, previous treatment history, household contact with a TB case, co-morbidities and laboratory data, such as white blood cell count, percentage of lymphocytes, monocytes, neutrophils, eosinophils, basophils.

All MDR-TB and S-TB patients showed radiological advanced pulmonary disease (MDR-TB: 76% showed bilateral and 24% unilateral cavities; S-TB: 53% with bilateral cavities, 47% unilateral cavities). Five of 59 MDR-TB patients had a negative sputum smear examination and 92% (54 of 59) of MDR-TB were smear-positive (AFB^+, $n = 41$; AFB^{++}, $n = 8$; AFB^{+++}, $n = 5$), while all S-TB were AFB smear-positive (AFB^+, $n = 13$; AFB^{++}, $n = 4$; AFB^{+++}, $n = 9$) at the time of the study. All TB patients had a positive culture for *Mtb*. Twenty-five HD were included as controls. All the HD included in the study had PPD-negative and IFN-γ release assay (IGRA)-negative results, indicating that they did not have latent TB infection.

The demographic data, including age, gender and ethnicity, are listed in Table 1. Supporting information, Fig. S1 shows the drug-resistance profile of the *Mtb* strains isolated from the MDR-TB patients studied.

Mononuclear cells

PBMCs were isolated from heparinized blood by Ficoll-Triyosom centrifugation and suspended in RPMI-1640 medium (HyClone, Thermo Scientific, Fremont, CA, USA) containing 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated fetal calf serum (Gibco/Invitrogen, Carlsbad, CA, USA) (complete RPMI). PBMC were counted in a Neubauer chamber using Türk stain.

Table 1. Demographic data of TB patients and healthy donors involved in the study

	MDR-TB	S-TB	HD
Number of participants	59	27	25
Age (years)			
Median (25th–75th percentiles)	29 (23–43)	34 (22–42)	30 (27–46)
Gender			
Men/women	33 M/29W	13M/14W	12M/13W
Ethnicity (*n*)	30 Argentines	24 Argentines	20 Argentines, 3 Peruvians
	20 Peruvians	3 Bolivians	
	3 Paraguayans		
	6 Bolivians		

MDR-TB = multi-drug-resistant tuberculosis patients; S-TB = sensitive drug tuberculosis patients; HD = healthy donors.

Antigens

MDR strains M (H2) and Ra (LAM3) and the reference laboratory strain H37Rv were grown in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI, USA) at 37°C in 5% CO_2. Mycobacteria were harvested in the logarithmic phase, washed three times, and the bacterial pellets were inactivated by gamma irradiation. Thereafter, pellets were suspended in pyrogen-free phosphate-buffered saline (PBS), sonicated and suspended at an optical density of 1 at 600 nm ($\approx 10^8$ bacteria/ml), and stored at −20°C until use.

PBMC cultures

In order to detect *ex-vivo* $IL\text{-}22^+$ cells, PBMCs (1×10^6 cells/ml) were cultured in complete RPMI medium for 6 h in the presence of brefeldin A to block cytokine secretion. Then, these cells were tested for their expression of IL-22 by flow cytometry [fluorescence-activated cell sorter (FACS)] analysis.

To evaluate *Mtb*-mediated $IL\text{-}22^+$ cell expansion, 1×10^6 PBMC/ml were cultured in complete RPMI medium in 1·5 ml polypropylene tubes at 37°C in a humidified 5% CO_2 atmosphere with strains M, Ra or H37Rv at a 2 : 1 *Mtb* to PBMC ratio. Phorbol myristate acetate (PMA, 10 ng/ml) plus ionomycin (0·25 μmol/ml) was also used as positive stimulation control. PBMC cultures were performed first at two different times (2 and 6 days), in order to establish the appropriate day to perform the experiments. As the highest IL-22 expression was observed in 6-day stimulated PBMC (Supporting information, Fig. S2), further experiments were performed only in 6-day cultures.

Then, cultured cells were tested for their senescent status through CD57 and programmed cell death 1 (PD-1) surface expression and IL-22 and IL-17 surface and intracellular expression by FACS analysis. PBMC supernatants were also collected and tested for secreted IL-22 by enzyme-linked immunosorbent assay (ELISA).

Flow cytometry analysis

(A) For surface membrane expression of IL-22 (m-IL-22) and senescent markers, 6-day-cultured PBMC were incubated with the following monoclonal antibodies (mAb): phycoerythrin-cyanin 5.5 (PE-Cy5.5)/PE-anti-human CD4 or anti-human-CD8 (Biolegend, San Diego, CA, USA), fluorescein isothiocyanate (FITC)-anti-human CD57 (eBioscience, San Diego, CA, USA), PE-anti-human CD279 (PD-1) (eBioscience), FITC-anti-human IL-22 (R&D Systems, Minneapolis, MN, USA) or the corresponding isotypes for 30 min at 4°C.

(B1) To detect *ex-vivo* expression, PBMC cultured for 6 h with brefeldin A (5 μg/ml) were surface-stained for CD4 or CD8 expression with PE-Cy5.5/PE-anti-CD4 or anti-CD8 mAb (BioLegend). Then, cells were fixed with paraformaldehyde (PFA) 0·5%, permeabilized with Perm2 (BD Bioscience), according to the manufacturer's

instructions, and finally stained with FITC-anti-IL-22, PE-anti-IL-17 or the corresponding isotypes (R&D Systems) for a further 30 min.

(B2) To evaluate *in-vitro* expression, PBMC were cultured for 6 days alone or with the mentioned *Mtb* strains and brefeldin A (5 μg/ml) was added for the last 4-6 h of the culture to block cytokine secretion. Then, cells were surface-stained for CD4 or CD8 expression with PE-Cy5.5/PE-anti-CD4 or anti-CD8 mAb (BioLegend), fixed with PFA 0·5%, permeabilized with Perm2 (BD Bioscience), according to the manufacturer's instructions, and finally stained with FITC-anti-IL-22, PE-anti-IL-17 or the corresponding isotypes (R&D Systems) for a further 30 min, following the previously described protocol [18,19].

In both cases (A and B), stained cells were washed and fixed, and then suspended in Isoflow (BD Bioscience). Then, 60 000 events were acquired for each cell preparation, using a flow cytometer BD FACScalibur-3 colors (BD Bioscience) with CellQuest software acquisition. The lymphocyte gate was set according to forward- and side-scatter parameters, excluding cell debris and apoptotic cells. FCS Express 7 Plus software (De Novo Software, Los Angeles, CA, USA) was used for analysis. Results were expressed as a percentage of positive cells within the $CD4^+$ and $CD8^+$ cells subsets or absolute number of $CD4^+$ and $CD8^+$ IL-22^+ cells/mm^3.

The levels of IL-22 in plasma and 6-day PBMC culture supernatants were assessed using a commercial ELISA kit (BioLegend), according to the manufacturer's instructions. IL-22 sensitivity was 16 pg/ml, range = 16–2000 pg/ml.

Statistical analysis

Data were analyzed using GraphPad Prism version 7.00 (GraphPad Software Inc., San Diego, CA, USA). Results were expressed as medians and 25th–75th percentiles. The non-parametric Kruskal–Wallis test was used to compare data from TB patients and healthy individuals, followed by the Mann–Whitney *U*-test to compare two groups. Friedman's test was performed to compare data within each group, followed by Wilcoxon's rank sum test. Correlations were performed by the non-parametric Spearman's rank correlation test. All statistical analyses were two-sided, and the significance level adopted was $P < 0{\cdot}05$.

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Results

Ex-vivo Th22 response is diminished in patients with active tuberculosis

We first determined the levels of IL-22 in plasma from MDR-TB and S-TB patients and HD and, as shown in Fig. 1a, lower IL-22 amounts were detected in plasma from MDR-TB and S-TB patients compared to HD. Although IL-22 values from MDR-TB were slightly lower than those from S-TB, no significant differences were observed ($P = 0{\cdot}1250$). However, in line with this, the absolute number of circulating $CD4^+$ expressing intracytoplasmatic IL-22 (ic-IL-22) was lower in both groups of patients than in HD (Fig. 1b), according to the slight

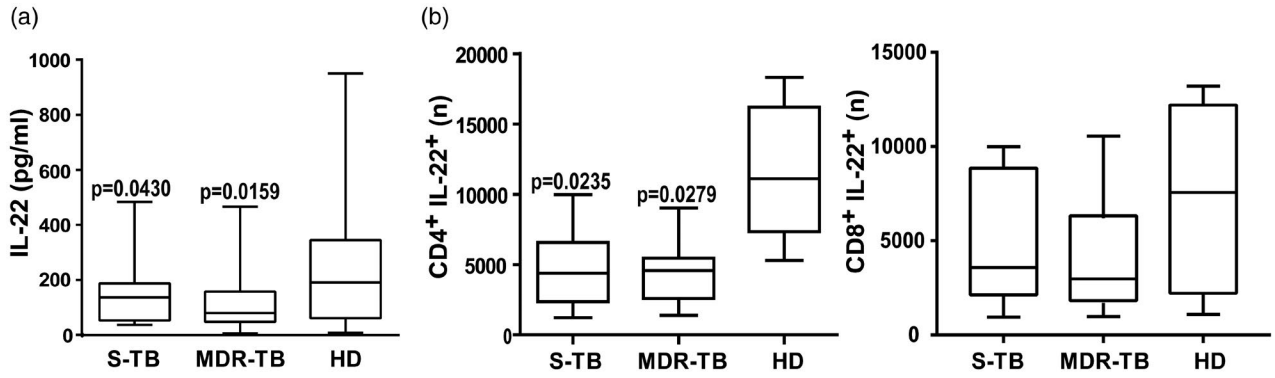

Fig. 1. *Ex-vivo* T helper type 22 (Th22) response in patients with active tuberculosis (TB) and healthy donors. (a) Interleukin (IL)-22 secretion was detected in plasma from patients with drug-susceptible (S-TB, $n = 27$) and multi-drug-resistant [(MDR)-TB, $n = 59$] tuberculosis and healthy donors (HD, $n = 25$). Results are expressed as pg/ml. Medians and 25th–75th percentiles are shown. Statistical differences: *P*-values are shown for TB patients *versus* HD values. (b) Intracytoplasmatic IL-22 expression was determined in recently isolated peripheral blood mononuclear cells (PBMCs) from S-TB ($n = 27$) and MDR-TB patients ($n = 59$) and HD ($n = 25$), as described in Materials and methods. The percentages of $CD4^+$IL-22^+ and $CD8^+$IL-22^+ cells were determined by flow cytometry and individual absolute number was then calculated. Results are expressed as median and 25th–75th percentiles. Statistical differences: *P*-values are shown for TB patients *versus* HD values.

decrease in the absolute number of $CD4^+$ and $CD8^+$ cells detected in peripheral blood (Table 2).

In-vitro Th22 response induced by *Mtb* strains is increased in TB patients

We further determined if $CD4^+IL\text{-}22^+$ and $CD8^+IL\text{-}22^+$ cells from TB patients differed in their ability to expand in response to M and Ra MDR strains. For this purpose, PBMC were cultured for 6 days alone (non-stimulated) or with M and Ra strains and the laboratory H37Rv strain or PMA, and then the proportion of cells expressing ic-IL-22 or bearing membrane-bound IL-22 (m-IL-22) was determined within $CD4^+$ and $CD8^+$ subsets by FACS.

As shown in Figs. 2a,b and in Supporting information Fig. S3, the percentage of ic-IL-22^+ cells within $CD4^+$ and $CD8^+$ cells (ic-IL-22^+/CD4 and ic-IL-22^+/CD8, respectively) was slightly higher in unstimulated PBMC cultures from S-TB patients compared to MDR-TB and HD. All *Mtb* strains enhanced the percentage of ic-IL-22^+ cells in both groups of TB patients and HD, with no differences detected among strains. Interestingly, while a marked increase in the percentage of ic-IL-22^+/$CD4^+$ and ic-IL-22^+/$CD8^+$ was observed in S-TB patients compared to HD, data from MDR-TB patients did not significantly differ from that of healthy controls. We did not observe a significant difference in IL-22 expression levels between PMA and *Mtb*-stimulated cells when stimulation was made through the *Mtb* strains (Supporting information, Fig. S4).

When membrane-bound IL-22 was determined in cultured PBMC, the proportion of m-IL-22^+/CD4 and m-IL-22^+/CD8 was similar in unstimulated PBMC from TB patients and HD, and for ic-IL-22, an increase in the percentage of m-IL-22^+ cells within both cell populations was observed upon stimulation with all *Mtb* strains, being the highest values detected in $CD4^+$ cells from S-TB (Fig. 2c,d).

Conversely, while similar IL-22 amounts in supernatants from non-stimulated PBMC were observed in all groups, higher values were detected in supernatants from S-TB and MDR-TB patients than in HD (Fig. 2e). Therefore, PBMC from S-TB and, to a lesser extent, those from MDR-TB patients showed a higher *in-vitro* Th22 response to *Mtb* antigens compared to HD, suggesting that circulating memory Th22 $CD4^+$ and $CD8^+$ cells are able to expand in response to *Mtb* antigens.

Mtb-induced IL-22^+IL-17^+ cells are increased in S-TB patients

Considering that IL-22 can be expressed and released not only by Th17 cells, but also by T cells which do not produce IL-17 [2–4], we determined the intracellular expression of both cytokines in $CD4^+$ and $CD8^+$ cells from TB patients and HD. As shown in Fig. 3a, the percentage of IL-22^+IL-17^+ cells in $CD4^+$ and $CD8^+$ subsets were increased in non-stimulated PBMC from S-TB patients compared to MDR-TB and HD. An expansion of this subset was observed in both groups of TB patients, observing the highest values in S-TB patients (Fig. 3a,c). Upon *Mtb* stimulation, the proportion of IL-22^+IL-17^+ cells expanded in $CD4^+$ from MDR-TB and S-TB patients by all strains and in $CD8^+$ subsets from S-TB patients by Ra and M strains. In all cases, S-TB patients showed the highest values. With regard to the percentage of IL-22^+IL-17^- cells within $CD4^+$ and $CD8^+$ cells, no significant differences were observed among groups (Fig. 3b,d). Also, upon *Mtb* stimulation, a slight enhancement of the percentage of IL-22^+IL-17^- cells was only observed within the $CD4^+$ cells from S-TB patients. Therefore, our results suggest that the differences in *Mtb*-induced IL-22 expression in T cells observed among S-TB patients, MDR-TB patients and HD could be the consequence of differential expansion of IL-22^+IL-17^+ and IL-22^+IL-17^- subsets.

Correlation of *ex-vivo* and *in-vitro* Th22 response from TB patients with clinical data

We then wondered in *ex-vivo* and *in-vitro* IL-22 secretion and the proportion of $CD4^+$ and $CD8^+IL\text{-}22^+$ cells correlated with clinical data, such as the bacillary load, or disease severity according to the presence of the unilateral or bilateral pulmonary lesions with or without cavities.

Table 2. $CD4^+$ and $CD8^+$ levels in TB patients and healthy donors

	MDR-TB	S-TB	HD
Leukocytes (n/mm^3)	8484 (4400–15 600)	10 200 (3900–16 700)	6500 (4000–9800)
Lymphocytes % (n/mm^3)	20 (8·2–49·4)	19·3 (7–40)	35 (20–45)
	1620 (122·2–3458)	1837 (702–4114)	2090 (1600–3800)
$CD4^+$ cells % (n/mm^3)	40·56 (24·07–63·7)	46·87 (35·02–72·91)	51 (43–58)
	653 (374–1236)	728 (511–1441)	1071 (903–1218)
	*P = 0·0235	*P = 0·0279	
$CD8^+$ cells% (n/mm^3)	18·53 (5·26–32·64)	18·21 (13·45–25·01)	29 (22–35)
	354 (232–476)	315·4 (221·4–644)	609 (462–735)

MDR-TB = multi-drug-resistant tuberculosis; S-TB = susceptible tuberculosis; HD = healthy donors.
*P-values for S-TB and MDR-TB *versus* HD.

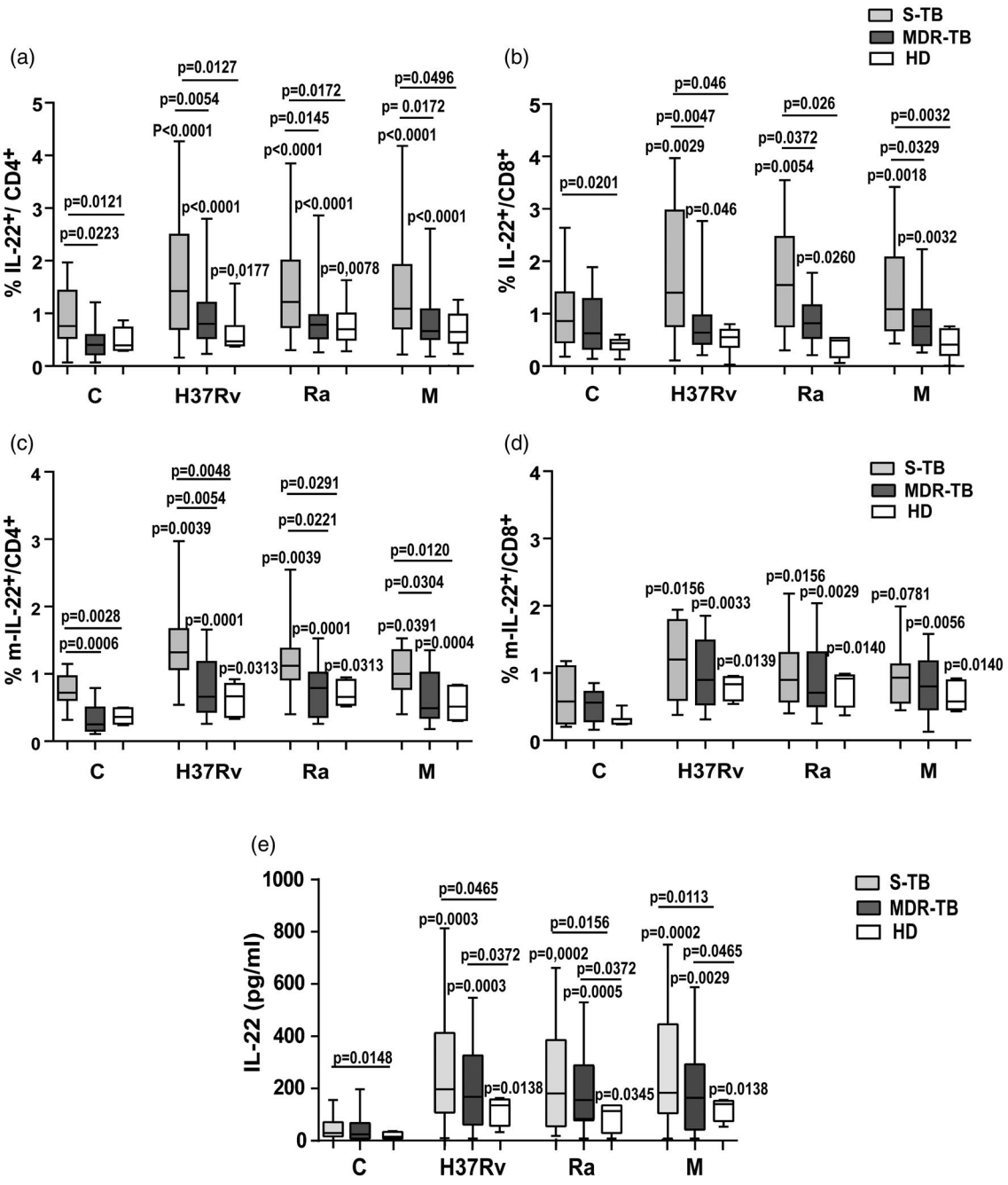

Fig. 2. *In-vitroMycobacterium tuberculosis* (*Mtb*)-induced T helper type 22 (Th22) response in tuberculosis (TB) patients and healthy donors (HD). (a–d) Peripheral blood mononuclear cells (PBMCs) from 27 S-TB, 59 multi-drug-resistant (MDR)-TB and 25 HD were cultured for 6 days alone (control) or with M, Ra and H37Rv strains. Then, intracellular interleukin (IL)-2 expression [intracytoplasmatic (ic)-IL-22] (a,b) and membrane-bound IL-22 expression (m-IL-22) (c,d) was determined in $CD4^+$ and CD8+ T cells by fluorescence activated cell sorter (FACS). Results are expressed as percentages of ic-IL-22^+ within $CD4^+$ and $CD8^+$ cell subsets (percentages of ic-IL-22^+/$CD4^+$ and ic-IL-22^+/$CD8^+$) and percentages of m-IL-22^+ cells in $CD4^+$ and $CD8^+$ cell subsets (percentages of m-IL-22^+/$CD4^+$ and m-IL-22^+/$CD8^+$). Median and 25th–25th percentiles are shown. Statistical differences: *P*-values are shown for strains *versus* control (above each bar) and for S-TB *versus* MDR-TB or HD (above each line connecting bars). (e) IL-22 amounts (pg/ml) were determined in PBMC supernatants by enzyme-linked immunosorbent assay (ELISA). Results are expressed as median and 25th–75th percentiles; statistical differences: *P*-values for strains *versus* control (above each bar) and for S-TB *versus* HD (above each line connecting bars) are shown.

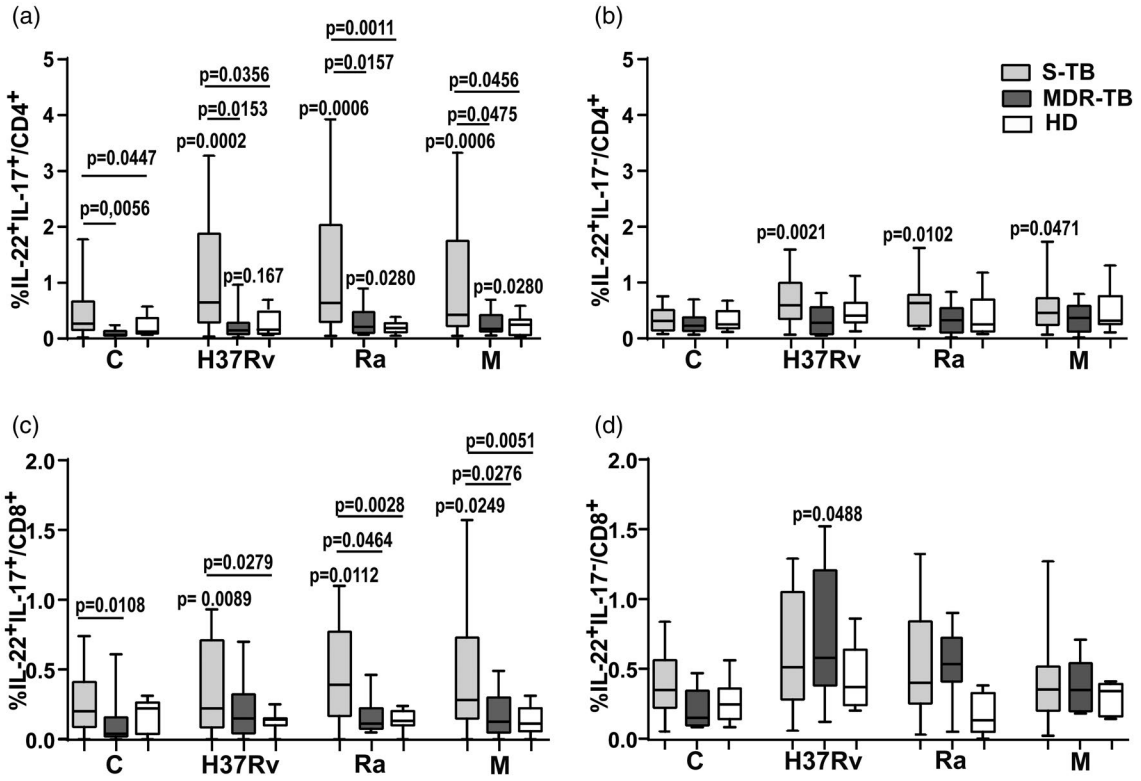

Fig. 3. Differential *Mycobacterium tuberculosis* (*Mtb*)-induced expansion of interleukin (IL)-22$^+$IL-17$^+$ and IL-22$^+$IL-17$^-$ cells in tuberculosis (TB) patients. Peripheral blood mononuclear cells (PBMCs) from 27 susceptible (S)-TB and 59 multi-drug-resistant (MDR)-TB patients and 25 healthy donors (HD) were cultured for 6 days alone or with *Mtb* strains. Then, intracellular expression of IL-22 and IL-17 was determined by fluorescence-activated cell sorter (FACS) analysis. Results are expressed as percentages of IL-22$^+$IL-17$^+$ and IL-22$^+$IL-17$^-$ cells within CD4$^+$ (a,b, respectively) and percentages of IL-22$^+$IL-17$^+$ and IL-22$^+$IL-17$^-$ cells within CD8$^+$ cells (c,d, respectively). Medians and 25th–75th percentiles are shown; statistical differences: *P*-values are shown for strains *versus* control (above each bar) and for S-TB *versus* MDR-TB and HD (above each line connecting bars).

No associations were seen between Th22 response and the disease progression time, the time that symptoms appeared or a history of previous TB in MDR-TB and S-TB patients. Furthermore, all patients included in the study were untreated or with fewer than 15 days of anti-TB treatment at the time of blood sampling, so no association between Th22 response and time of treatment was observed.

Interestingly, the higher sputum bacillary load and the greater severity of the lesion observed in MDR-TB patients, the lower percentage of ic-IL-22 expression in CD4$^+$ and CD8$^+$ T cells ($P < 0{\cdot}05$) (Fig. 4a–d). In this context, while 30% of patients with low bacillary load in sputum had unilateral cavities, only 8% of those with high bacillary load had unilateral lesions. Although the same tendency was observed in S-TB patients, differences were not significant (data not shown). In addition, we observed low proportion of IL-22$^+$IL-17$^+$ cells within CD4$^+$ and CD8$^+$ subsets in those MDR-TB patients with high bacillary load (Fig. 4e,f). Also, MDR-TB patients with high bacillary load in sputum showed lower IL-22 secretion by *Mtb*-stimulated PBMC than those MDR-TB patients with low or no bacillary load (Fig. 4g); the same cytokine pattern was observed in patients with bilateral cavities (Fig. 4h). Altogether, these results suggest that a Th22 response could be influencing the ability of MDR-TB patients to control bacillary growth and tissue damage.

MDR-TB patients show high CD57 and PD-1 expression in cultured CD4$^+$ and CD8$^+$ T cells

Antigen-specific T cell dysfunction and up-regulation of inhibitory receptors such as PD-1 have been associated with persistent antigen stimulation in chronic infections such as TB [24]. Therefore, we wondered whether the differences in Th22 response observed among MDR-TB patients could be associated with the proportion of senescent CD4$^+$ and CD8$^+$ T cells. As shown in Fig. 5a,b and Supporting information, Fig. S5, the percentages of PD-1$^+$ cells within CD4$^+$ and CD8$^+$ T cell subsets (% PD-1$^+$/CD4 and % PD-1$^+$/CD8) was markedly increased in non-stimulated PBMC from MDR-TB patients compared to S-TB and HD, and *Mtb* strains did not enhance the PD-1

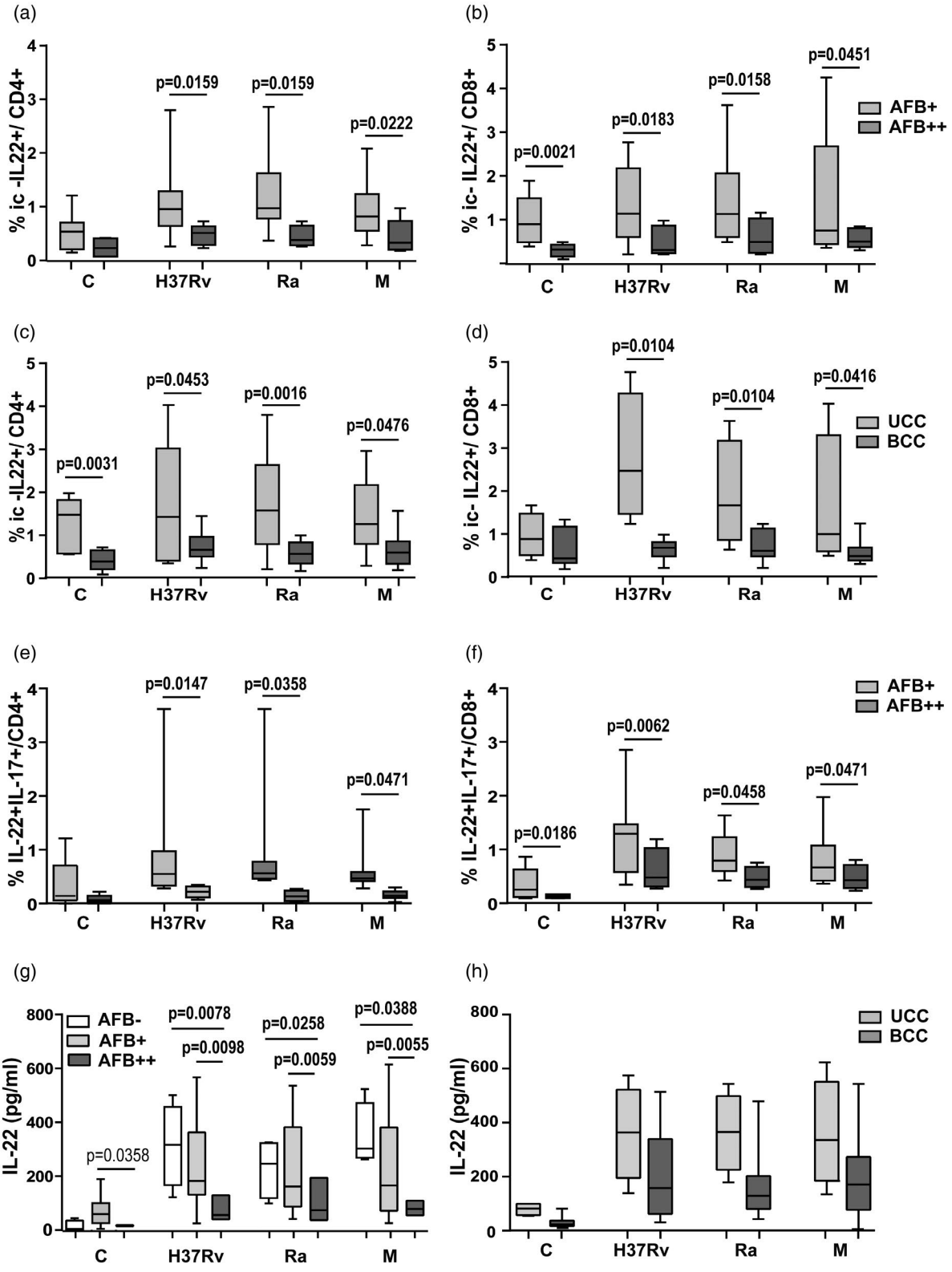

Fig. 4. *Ex-vivo* and *in-vitro* T helper type 22 (Th22) response from tuberculosis (TB) patients associated with clinical data. Multi-drug-resistant (MDR)-TB patients were grouped according to the number of acid-fast bacilli (AFB) detected in sputum smear (AFB $^{-}$: 0 bacilli in 100 fields; AFB^{+}: 10–99 bacilli in 100 fields; AFB^{++}: 1–10 bacilli/field in 50 observed fields) or the magnitude of pulmonary lesions (UCC = unilateral with cavity; BCC = bilateral with cavity), and then the percentages of IL-22^{+} and IL-22^{+}IL-17^{+} cells within CD4^{+} and CD8^{+} subsets were calculated for each group. (a,b) Medians and 25th–75th percentiles of percentages of IL-22^{+} cells/CD4^{+} (a) and IL-22^{+} cells/CD8^{+} cells (b) in AFB^{+} and AFB^{++} patients; statistical differences: *P*-values for AFB^{+}*versus* AFB^{++} are shown. (c,d) Medians and 25th–75th percentiles of percentages of IL-22^{+}/CD4^{+} cells (c) and IL-22^{+}/CD8^{+} cells (d) in UCC and BCC patients; statistical differences: *P*-values for UCC *versus* BCC are shown. (e–f) Medians and 25th–75th percentiles of percentages of IL-22^{+}IL-17^{+} cells/CD4^{+} (e) and IL-22^{+}IL-17^{+}/CD8^{+} cells (h) in AFB^{+} and AFB^{++} patients; statistical differences: *P*-values for AFB^{+}*versus* AFB^{++} are shown. (h) IL-22 amounts (pg/ml) in peripheral blood mononuclear cell (PBMC) supernatants from AFB^{-}, AFB^{+} and AFB^{++} patients (mean and 25th–75th percentiles). (g) IL-22 amounts (pg/ml) in PBMC supernatants from UCC and BCC patients (mean and 25th–75th percentiles). Statistical differences: *P*-values for AFB^{++}*versus* AFB^{-} or AFB^{+} patients are shown.

expression in any T cell populations from TB patients and HD. Furthermore, we observed an inverse correlation between the percentages of ic-IL-22^{+} and PD1^{+} within CD4^{+} cells and CD8^{+} in TB patients (Fig. 5c,d). Similarly, MDR-TB patients with bilateral cavities showed the highest percentage of PD1^{+} cells in CD4^{+} and CD8^{+} subsets (Supporting information, Fig. S6).

Conversely, we evaluated the surface expression of CD57, an antigen reported to be present in low proliferative CD4^{+} and CD8^{+} T cells undergoing late stages of differentiation that are increased in chronic immune activation [25,26]. As observed in Fig. 5e,f and Supporting information, Fig. S7, similarly to PD1 expression, MDR-TB patients showed the highest values of percentages of CD57^{+}/CD4 and CD57^{+}/CD8. Also, an inverse correlation was observed between the percentages of ic-IL-22^{+} cells and CD57^{+} cells within CD4^{+} and CD8^{+} subsets in TB patients (Fig. 5g,h).

Altogether, these results suggest that increased senescent markers such as PD-1 and CD57 expression could be partially involved in the low *Mtb*-induced Th22 response observed in those MDR-TB patients with severe lung damage and/or bacillary load.

Discussion

Increasing evidence indicates that IL-22 would have a role in anti-mycobacterial response, but it is still matter of debate whether increased susceptibility to disease and the severity of TB is a consequence of low Th22 responses. In our study, we evaluated *ex-vivo* and *in-vitro Mtb*-induced Th22 response in active pulmonary TB patients and HD. We observed that: (1) MDR-TB and S-TB patients show low IL-22 amounts in plasma with a decreased number of circulating CD4^{+} and CD8^{+} cells expressing IL-22; (2) independently of the strain, low *in-vitro Mtb*-induced IL-22 secretion and decreased expansion of total IL-22^{+} and IL-22^{+}IL-17^{+} cells in CD4^{+} and CD8^{+} subsets was detected in MDR-TB when compared to S-TB patients; (3) remarkably, in MDR-TB patients, low IL-22 secretion and reduced percentage of total IL-22^{+} and IL-22^{+}IL-17^{+} cells associates with high bacillary load and severity of the disease and finally, (4) in MDR-TB patients, the proportion of senescent PD-1^{+} and CD57^{+} cells in CD4^{+}/CD8^{+} subsets was markedly increased and inversely correlated with the *Mtb*-induced Th22 response, and associates with the severity of the lung lesions.

The literature concerning IL-22 amounts in serum from TB patients is contradictory. While some authors did not observe significant differences in circulating IL-22 concentrations [27], others reported lower serum IL-22 amounts in pulmonary TB patients compared with latent *Mtb* infection [28,29]. Herein, and in agreement with the latter works, we observed lower levels of IL-22 in plasma from S-TB and MDR-TB patients than in HD, in accordance with the low absolute number of circulating T cells expressing IL-22 observed in both groups of patients. Given that increased IL-22 levels were found in bronchoalveolar fluid from pulmonary TB patients compared with those obtained from healthy controls [30–32], and in pleural and pericardial effusions than in peripheral blood from patients with extrapulmonary TB [31,33], the low number of circulating Th22 cells could be due to the recruitment of antigen-specific IL-22-producing T cells to the site of the lesion to fulfill its regenerative function of the epithelium [6], as also observed in a non-human primate model [34].

Conversely, similar low circulating Th22 T cells in macaques infected with MDR V791 strain and drug-sensitive Erdman strains upon 6 weeks' infection suggested that infection with MDR strains resembles infection with drug-sensitive *Mtb* [35]. In our work, similar numbers of circulating Th22 cells were detected in MDR-TB and S-TB patients in the absence of antigen stimulation, which could explain the lack of differences in systemic IL-22 levels, and reflects the attempt of TB patients to control *Mtb* infection and tissue damage through the influx of potentially Th22 cell infection to the site of infection, independently of the nature of the strain.

It has been reported that S-TB patients show a higher percentage of memory CD4^{+} and CD8^{+} T cells that express intracellular IL-22 in response to *M. bovis* culture filtrate or PPD [27,36], as well as an increased expansion of PPD-specific CD4^{+} and CD8^{+} cells with membrane-bound IL-22 than in healthy individuals [36]. In accordance, in S-TB patients we observed a marked expansion of CD4^{+} and CD8^{+} cells with ic-IL-22 or m-IL-22 and increased

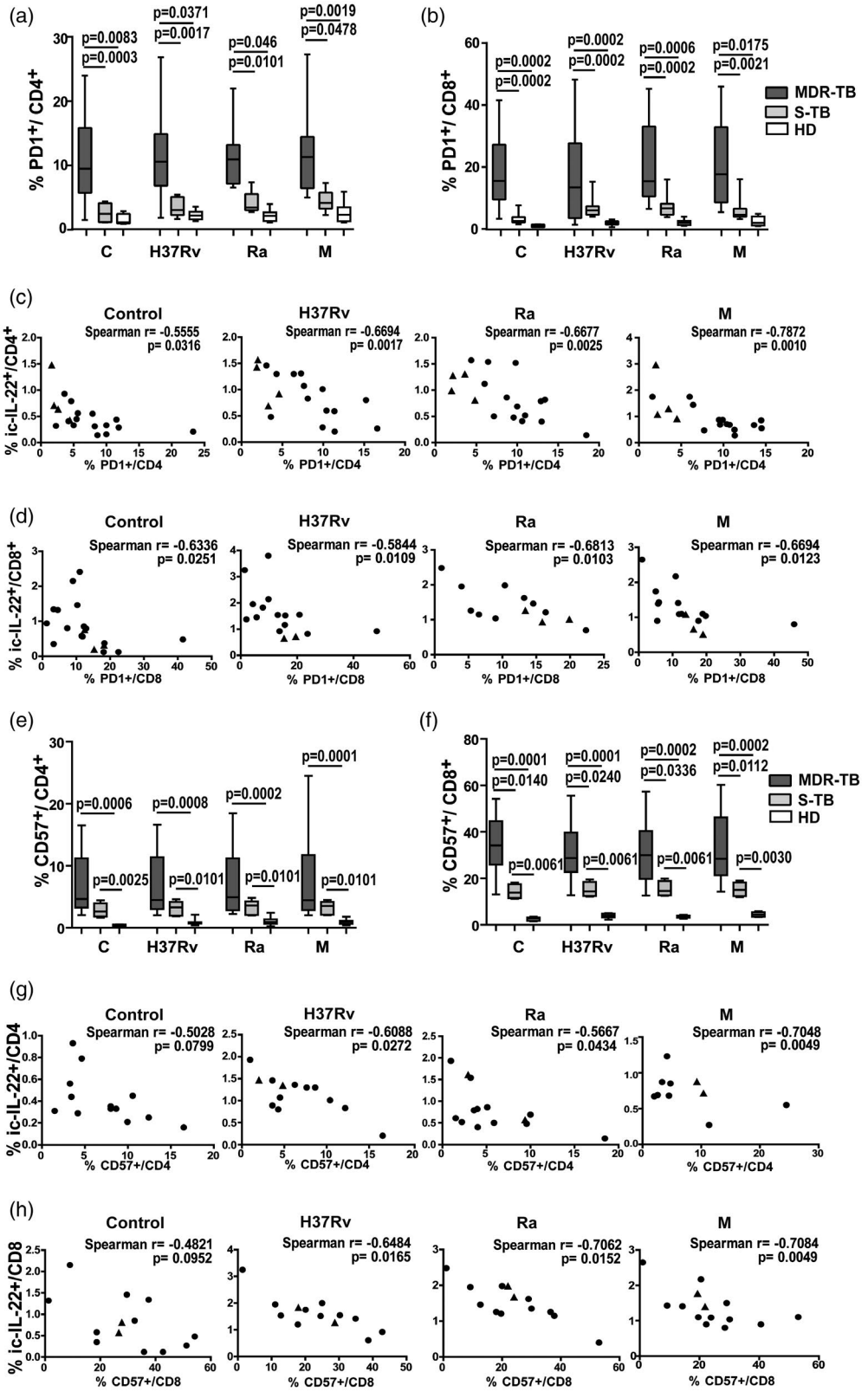

Fig. 5. Programmed cell death 1 (PD-1)$^+$ and CD57$^+$ cells are markedly increased in peripheral blood mononuclear cells (PBMCs) from multi-drug-resistant tuberculosis (MDR)-TB patients; 6 day-cultured PBMC from 23 drug-susceptible (S)-TB, 50 MDR-TB and 20 healthy donors (HD) were tested for their PD-1 and CD57 surface expression in CD4$^+$ and CD8$^+$ cells by fluorescence activated cell sorter (FACS). (a,b) Figures show mean and 25th–75th percentiles of percentages of PD-1$^+$ cells/CD4$^+$ (a) and PD-1$^+$ cells/CD8$^+$ (b); statistical differences: *P*-values for TB *versus* HD and for MDR-TB *versus* S-TB are shown above lines connecting bars. (c,d) Correlation between percentages of IL-22$^+$ cells/CD4$^+$ and PD-1$^+$ cells/CD4$^+$ (c) and of IL-22$^+$ cells/CD8$^+$ and PD-1$^+$ cells/CD8$^+$ (d); individual data and Spearman's rho coefficients are shown. (e,f) Figures show mean and 25th–75th percentiles of percentages of CD57$^+$ cells/CD4$^+$ (e) and CD57$^+$ cells/CD8$^+$ (f); statistical differences: *P*-values for TB *versus* HD and for MDR-TB *versus* S-TB are shown. (g,h) Correlation between percentages of IL-22$^+$ cells/CD4$^+$ and CD57$^+$ cells/CD4$^+$ (c) and percentages of IL-22$^+$ cells/CD8$^+$ and CD57$^+$ cells/CD8$^+$ (d); individual data and Spearman's rho coefficients.

IL-22 secretion upon stimulation with H37Rv or MDR strains. Considering that a negative association has been shown between IL-22 and IFN-γ in PPD-stimulated PBMCs from S-TB patients [11], and both MDR-TB and S-TB patients show decreased IFN-γ expression in PBMC stimulated with M, Ra and H37Rv strains than HD [17], we expected an increased IL-22 expression in both groups of TB patients. However, *Mtb*-induced expansion of IL-22$^+$ cells and IL-22 secretion were lower in MDR-TB than in S-TB patients, suggesting that circulating T cells from the former patients would be slightly less prone to expand and secrete IL-22 independently of their deficient IFN-γ response.

Conversely, while unspecific activation of human naive CD4$^+$ T cells in Th17 polarization conditions results in IL-22 secretion by mainly Th17 cells [37], BCG or PPD mainly enhance the expansion of memory CD4$^+$IL-22$^+$ cells that do not express IL-17 in S-TB patients and HD [30]. In this study, we observed that IL-22$^+$IL-17$^+$ and IL-22$^+$IL-17$^-$CD4$^+$/CD8$^+$ cells are expanded in differently in MDR-TB and S-TB patients, this difference being possibly due to our use of whole *Mtb* bacilli or intrinsic factors of patients' cells. Interestingly, MDR-TB patients showed a lower proportion of IL-22$^+$IL-17$^+$ cells than S-TB patients, which could account for the differential *in-vitro* Th22 response detected in both groups of TB patients.

In our study, we observed that despite their subtle differences in the *in-vitro* induction of Th1, Th2 and Th17 responses [17,19,38], H37Rv, M and Ra strains expanded in a similar manner in IL-22$^+$ T cells in TB patients. As M, Ra and H37Rv strains belong to the European–American lineage, which is the most prevalent in our country both in MDR and S-TB strains, our results are in accordance with van Laarhovene *et al.* [12], demonstrating that this lineage induces similar expansion of Th22 cells.

Regarding MDR-TB patients and their Th22 response, our study showed that the bacillary load is related to Th22 response: the higher sputum bacillary load, the lower the Th22, and these differences could not be ascribed to the efficacy of the treatment because all the patients had fewer than 15 days of treatment. Also, all MDR-TB patients included in this study showed severe lung compromise; however, those with bilateral cavities showed lower IL-22 secretion and a reduced percentage of IL-22$^+$ and IL-22$^+$IL-17$^+$ T cells. Taken together, our results suggest that the marked inability to mount a specific Th22 response could be predisposed to greater lung damage and bacillary load, although further studies are necessary to determine the role of both IL-22 subsets in tissue repair mechanisms in the context of TB.

Sustained high expression of PD-1 has been associated with T cell dysfunction in chronic viral infections and tumors [39]. In human TB, high PD-1 expression has been observed in T cells from peripheral blood and pleural effusion of patients with TB pleurisy [40] and in circulating CD4$^+$ T cells from S-TB patients with a positive AFB sputum smear [24]. In our study, we observed a marked increase in the percentage of CD4$^+$ and CD8$^+$ PD-1$^+$ cells in MDR-TB compared to S-TB patients, despite 92% of MDR-TB and all S-TB patients having a positive sputum smear at the time of the study. Given that MDR-TB patients expend more time to access to an effective anti-TB treatment until the diagnosis of drug resistance, the persistent high bacillary load could promote an excessive exhaustion of memory T cells in these patients. In this context, we found that the higher PD-1 expression, the lower IL-22$^+$ number of cells in TB patients; however, MDR-TB with extensive lung damage showed the high proportion of PD-1$^+$ cells. In conclusion, our results suggest that the regulation of *in-vitro* Th22 response is mediated by PD-1, as has been previously reported [41], and that this senescent signaling pathway could impact upon the control of tissue damage. In the same context, we observed that terminally differentiated CD57$^+$ T cells with low proliferative ability to recall antigens [25] were notably increased in MDR-TB patients compared to S-TB and, as observed for PD-1, CD57 expression inversely correlates with the IL-22 response in TB patients, indicating that circulating senescent T/terminally differentiated T cells could affect the *Mtb*-induced expansion of memory Th22 cells.

In summary, we have observed that in MDR-TB patients, the magnitude of the *Mtb*-induced Th22 response closely associates with the senescent status of CD4$^+$ and CD8$^+$ T cells. In addition, in these patients low *ex-vivo* and *in-vitro Mtb*-induced Th22 responses were associated with high bacillary load and extensive lung involvement, reinforcing the role of IL-22 in the control of mycobacteria and tissue repair mechanisms.

Acknowledgements

This work was funded by grants from Florencio Fiorini Foundation (research grants in biomedical sciences, year 2018), the National Agency for Scientific and Technological Promotion (PICT 2015-0491) and CONICET (PIP 112-2013-0100202).

Disclosures

The authors declare no conflicts of interest.

Author contributions

I. B. R and d. l. B. S. designed the study and the experiments, analyzed the data and wrote the manuscript. I. B. R. and M.A. performed the experiments. S. M. C. revised and corrected the manuscript. G. A., G. M. P., M. L. P. D. J. and M. N. S. contributed with blood samples, performed TB diagnosis and drug-susceptible testing, collected epidemiological and clinical data of patients and corrected the manuscript.

Data Availability Statement

Data available on request from the authors. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Fig S1. The number of MDR-TB patients grouped according to the resistance profile of *Mtb* clinical isolates and country of origin is depicted in the graph.

Fig S2. PBMC from 6 healthy donors were cultured alone or with H37Rv, Ra and M strains for 2 and 6 days and then, cells were tested for the intracytoplasmic expression of IL-22 by FACS. Results were expressed as percentage of $IL\text{-}22^+$ cells in $CD4^+$ and $CD8^+$ subsets (% $IL\text{-}22^+/CD4^+$ and % $IL\text{-}22^+/CD8^+$, respectively). % $IL\text{-}22^+/CD4^+$ and % $IL\text{-}22^+/CD8^+$ (median and 25^{th}-75^{th} percentiles) detected in 2- and 6-days cultures were depicted. Statistical differences: p values for 2 days *versus* 6-days -stimulated PBMC are shown.

Fig S3. Intracellular IL-22 (ic-IL-22) staining in PBMC cultured for 6 days alone (Control) and with M, Ra and H27Rv strains of *M. tuberculosis*; dot plots from 1 MDR-TB patient representative of 59 patients and 1 healthy donor (HD) representative of 25 individuals studied are shown. Numbers in upper-right quadrant represent the percentage of IL-22+ cells within live $CD4^+$ and $CD8^+$ cell gates.

Fig S4. PBMC from 5 S-TB patients and 5 HD were cultured for 6 days alone (Control, C), H37Rv strain (*Mtb* to PBMC ratio 2:1) and PMA (10 ng/mL) plus Ionomycin (0.25 μmol/mL) and then, the percentage of $ic\text{-}IL\text{-}22^+$ cells within live $CD4^+$ and $CD8^+$ cells was determined by FACS. **A and B:** dot plots from one representative S-TB patient were shown. Numbers in upper-right quadrant represent the % $ic\text{-}IL\text{-}22^+/CD4^+$ and % $ic\text{-}IL\text{-}22^+/CD8^+$ cells. **C:** % $IL\text{-}22^+/CD4^+$ and

% IL-22$^+$/CD8$^+$ cells (median and 25th-75th percentiles) are depicted.

Fig S5. Surface PD-1 staining in PBMC cultured for 6 days alone (Control) and with M, Ra and H27Rv strains of *M. tuberculosis*; dot plots from 1 MDR-TB patient representative of 50 patients and 1 healthy donor (HD) representative of 20 individuals studied are shown. Numbers in upper-right quadrant represent the percentage of PD-1+ cells within live CD4$^+$ and CD8$^+$ cell gates.

Fig S6. MDR-TB patients were grouped according to the severity of lung lesions (UCC: unilateral with cavity; BCC: bilateral with cavity) and then, mean and 25th to 75th percentiles of % PD-1$^+$ cells/ CD4$^+$ and of % PD-1$^+$ cells/ CD8$^+$ was calculated for each group. Statistical differences: p values for BCC *versus* UCC patients are shown.

Fig S7. Surface CD57 staining in PBMC cultured for 6 days alone (Control) and with M, Ra and H27Rv strains of *M. tuberculosis*; dot plots from 1 MDR-TB patient representative of 50 patients and 1 healthy donor (HD) representative of 20 individuals studied are shown. Numbers in upper-right quadrant represent the percentage of CD57 cells within live CD4$^+$ and CD8$^+$ cell gates.