Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/cei.12227

$CD4^+CD25^{high}$forkhead box protein 3^+ regulatory T lymphocytes suppress interferon-γ and CD107 expression in $CD4^+$ and $CD8^+$ T cells from tuberculous pleural effusions

L. Geffner,*[1] J. I. Basile,*[1]
N. Yokobori,* C. Sabio y García,*
R. Musella,† J. Castagnino,†
M. C. Sasiain* and S. de la Barrera*
*IMEX-CONICET, Academia Nacional de Medicina, and †Departamento de Neumonología, Hospital Francisco Muñiz, Buenos Aires, Argentina

Accepted for publication 15 October 2013
Correspondence: S. de la Barrera, IMEX-CONICET, Academia Nacional de Medicina, Pacheco de Melo 3081, 1425 Buenos Aires, Argentina.
E-mail: sdelab@hematologia.anm.edu.ar

[1]Both authors contribute equally to this study.

Summary

Tuberculous pleural effusion is characterized by a T helper type 1 (Th1) profile, but an excessive Th1 response may also cause tissue damage that might be controlled by regulatory mechanisms. In the current study we investigated the role of regulatory T cells (T_{reg}) in the modulation of Th1 responses in patients with tuberculous (TB) pleurisy. Using flow cytometry we evaluated the proportion of T_{reg} ($CD4^+CD25^{high}$forkhead box protein 3^+), interferon (IFN)-γ and interleukin (IL)-10 expression and CD107 degranulation in peripheral blood (PB) and pleural fluid (PF) from patients with TB pleurisy. We demonstrated that the proportion of $CD4^+CD25^+$, $CD4^+CD25^{high}FoxP3^+$ and $CD8^+CD25^+$ cells were increased in PF compared to PB samples. *Mycobacterium tuberculosis* stimulation increased the proportion of $CD4^+CD25^{low/neg}IL\text{-}10^+$ in PB and $CD4^+CD25^{low/neg}IFN\text{-}\gamma^+$ in PF; meanwhile, $CD25^{high}$ mainly expressed IL-10 in both compartments. A high proportion of $CD4^+CD107^+$ and $CD8^+CD107^+$ cells was observed in PF. T_{reg} depletion enhanced the *in-vitro M. tuberculosis*-induced IFN-γ and $CD4^+$ and $CD8^+$ degranulation responses and decreased $CD4^+IL\text{-}10^+$ cells in PF. Our results demonstrated that in TB pleurisy T_{reg} cells effectively inhibit not only IFN-γ expression but also the ability of $CD4^+$ and $CD8^+$ cells to degranulate in response to *M. tuberculosis*.

Keywords: CD107, pleural effusions, T regulatory, tuberculosis

Introduction

Among the many clinical manifestations of tuberculosis (TB), pleuritis is of particular interest as it may be resolved without therapy, and patients are known to undergo a relatively effective immune response against *Mycobacterium tuberculosis*. However, half of patients recidivate during the next 5 years in the absence of chemotherapy [1]. TB pleural effusion is caused by a severe delayed-type hypersensitivity reaction in response to the rupture of a subpleural focus of *M. tuberculosis* infection, and although it often occurs in conjunction with pulmonary infiltrates typical of post-primary tuberculosis, it may also manifest as a primary feature of the disease [2]. TB pleurisy results in an increased pleural vascular permeability leading to the accumulation of protein-enriched fluid and recruitment of specific inflammatory leucocytes into the pleural space leading to the clearance of mycobacteria from this cavity. Lymphocytes are the predominant cell type, and *in-vivo* and *in-vitro* studies have demonstrated that *M. tuberculosis*-specific T cells are recruited and expand clonally at the site of infection [3,4]. An increase of $CD4^+$ T cells belonging to the T helper type 1 (Th1) subset with selective concentrations of interferon (IFN)-γ, tumour necrosis factor (TNF)-α and interleukin (IL-12) [5–9], as well as IFN-γ-inducible chemokines [10], have been detected in pleural fluid (PF).

Although Th1 responses are necessary for the effective control of TB infection they may also cause tissue damage. In line with this, IFN-γ may mediate the clinical manifestation of TB pleuritis, such as fever, exudative pleural effusion and tissue necrosis [11]. For this reason, it has been proposed that regulatory T cells (T_{reg}) and Th2 responses might play important regulatory functions in protecting the host from collateral tissue damage by regulating excessive Th1 responses.

High levels of $CD4^+CD25^{high}$forkhead box protein 3 ($FoxP3^+$) T_{reg} cells have been detected in peripheral blood mononuclear cells (PBMC) from TB patients [12–15], and an *in-vivo* enrichment of T_{reg} cells has been observed in tuberculous PF [12,16]. *In-vitro* depletion of circulating and

Table 1. Clinical, cytological and laboratory profile from patients with tuberculous pleurisy.

Parameter		PBMC	PFMC
Age	25 (22·5–30·75)		
Gender	10 male/6 female		
AFB	6+/10–		
PPD	10+/5–		
Pulmonary disease	6/16		
ADA			96·6 (71·0–106·0)
Cell counts, cells/mm^3		7400 (6100–8500)	1600 (800–2500)
Lymphocyte, %		19 (16·5–21·5)	77 (67·5–85)
Lymphocyte counts, cells/mm^3		1445 (1075–1699)	1190 (668–1875)

AFB: acid-fast bacillus in sputum; PPD: purified protein derivative skin test; ADA: adenosine deaminase; PBMC: peripheral blood mononuclear cells; PFMC: pleural fluid mononuclear cells.

local site-derived CD4$^+$CD25^{high} T lymphocytes suggest a role of T_{reg} in the suppression of systemic and local immune responses to *M. tuberculosis* antigens [12,16]. Also, it has been demonstrated recently that the alveolar lung compartment is enriched in CD4$^+$CD25$^+$FoxP3$^+$ T_{reg} cells that suppress the ability of alveolar and monocyte-derived macrophages to restrict the growth of *M. tuberculosis*, suggesting that T_{reg} cells also subvert anti-mycobacterial immunity in human TB [17].

It is widely accepted that control of human TB depends on fully activated CD8$^+$ T cells that not only release Th1 cytokines such as IFN-γ and TNF-α, but also lyse *M. tuberculosis*-infected macrophages [18,19] and reduce *M. tuberculosis* viability [20]. In a previous work, we demonstrated an *in-vitro M. tuberculosis*-induced expansion of T_{reg} cells in PBMC from active TB patients that efficiently suppress antigen-induced IFN-γ expression and the ability of CD8$^+$ cytotoxic T lymphocytes (CTL) to lyse *M. tuberculosis*-pulsed macrophages. Moreover, T_{reg} depletion enhanced surface CD107a expression on CTL [21]. CD107a and CD107b are intracellular proteins normally found in lysosomes that are expressed transiently on CTL surfaces upon exocytosis of cytotoxic granules during target cell lysis [22,23]. Furthermore, CD107a-expressing CD8$^+$ T cells are shown to mediate cytolytic activity in an antigen-specific manner [23], demonstrating that CD107a expression on the cell surface is a marker of cytotoxic CD8$^+$ T cell degranulation/activation. In the current study we investigated the role of T_{reg} cells in the modulation of IFN-γ and CD107 expression in human tuberculous pleurisy. Our results demonstrated that upon encountering *M. tuberculosis*, T_{reg} are functionally active in pleural effusions by modulating CD4$^+$ and CD8$^+$ T cell-mediated IFN-γ and CTL responses.

Materials and methods

Ethical approval

Ethical approval for the study was obtained from Ethic Committee of Hospital F. J. Muñiz, according to the principles laid down in the Declaration of Helsinki recommendations guiding physicians in biomedical research involving human subjects. The study was conducted in accordance with the standards for clinical research of the Universidad Nacional de Buenos Aires, Argentina.

Patients

Patients with newly diagnosed moderate and large pleural effusions were identified at the Hospital F. J. Muñiz, Buenos Aires, Argentina. Informed, written consent was obtained from all patients and healthy individuals and the study was approved by the Hospital F. J. Muñiz Ethic Committee. Patients were evaluated by a history and physical examination, with routine investigations including testing for HIV infection, chest radiography, microbiological sputum examination, whenever possible, and aspiration of pleural fluid for biochemical, cytological and detailed microbiological (Ziehl–Nielsen stain and culture for bacterial pathogens) evaluation. Exclusion criteria included HIV positivity or the presence of concurrent infectious diseases. Pleural effusions and blood samples were obtained from patients during thoracentesis before the initiation of chemotherapy. Diagnosis of pleural effusion from TB aetiology was based on demonstration of *M. tuberculosis* on pleural fluid smear (by the Ziehl–Neelsen stain) and growing *M. tuberculosis* in pleural fluid specimens (by Lowenstein–Jensen-positive culture) and/or histopathology corroboration. A total of 16 patients were included; among them, six also had pulmonary disease. Table 1 summarizes selected clinical and laboratory data of TB patients. Blood samples were also obtained from 10 purified protein derivative (PPD$^+$) healthy volunteers (four males and six females, aged between 26 and 60 years).

Thoracocentesis and pleural biopsy

PF was collected by therapeutic thoracocentesis, as described previously [24]. Specimens were subjected to routine biochemical analysis, including tests for total

protein, glucose, lactate dehydrogenase and differential cell counts. Bacterial cultures and cytological examinations were performed on all PF in the central laboratory at Hospital F. J. Muñiz. A second sample was dispensed into 50-ml polystyrene tubes containing heparin to obtain mononuclear cells. Peripheral blood samples were also collected on the same day of thoracocentesis.

Mononuclear cells

Peripheral blood and pleural fluid mononuclear cells were isolated from heparinized blood and PF (PBMC and PFMC, respectively) by Ficoll-Hypaque gradient centrifugation and suspended in RPMI-1640 tissue culture medium (HyClone®; Thermo Scientific, Rockford, IL, USA) containing 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated fetal calf serum (Invitrogen Gibco®, Carlsbad, CA, USA) (complete medium).

CD25-depletion

In order to deplete lymphocytes with high CD25 expression, 1×10^7 PBMC or PFMC were incubated with a limiting concentration of anti-CD25 antibody (0·1 μg/10^7 cells; eBioscience, San Diego, CA, USA) for 30 min at 4°C, washed with phosphate-buffered saline (PBS) and mixed with goat anti-mouse immunoglobulin (Ig)G-coated magnetic beads (Invitrogen Dynal, Oslo, Norway) by gentle rolling at 4°C for 30 min. Non-rosetted cells (CD25-depleted PBMC) were separated using a magnet. Generally, one cycle of treatment was sufficient for an effective depletion as assessed by flow cytometry. Approximately 90 ± 3% of $CD4^+CD25^{high}$ T cells were eliminated from PB and PF samples after depletion, while $CD4^+CD25^{low}$ and $CD8^+CD25^+$ T cells were slightly reduced (11 ± 4% and 10 ± 5%, respectively). To compare cytokine production and CD107 expression, CD25-depleted PBMC or PFMC were suspended in complete medium ensuring that the number of cells/ml of each subset was the same as in total cultured PBMC.

Antigen

The gamma-irradiated *M. tuberculosis* H37Rv strain used in this study was provided by J. Belisle (Colorado University, Co, USA). Mycobacteria were suspended in PBS free of pyrogen, sonicated and adjusted at a concentration of 1×10^8 bacteria/ml [optical density $(OD)_{600\ nm} = 1$].

PBMC and PFMC cultures

In-vitro antigen-specific effector T cells were evaluated by culturing total or CD25-depleted PBMC or PFMC (2×10^6 cells/ml in complete medium) for 18 h in polystyrene tubes (BD Falcon™, San Jose, CA, USA) at 37°C in 5% CO_2 atmosphere with or without *M. tuberculosis* in a 2:1 *M. tuberculosis* to PFMC/PBMC ratio. Cells were then tested for cytokine, CD25, FoxP3 and CD107 expression.

Immunofluorescence analysis

Surface membrane expression. The following monoclonal antibodies (mAbs) and the corresponding isotypes were used to evaluate surface marker expression in fresh and 18-h cultured PBMC or PFMC: phycoerythrin-cyanin 5 (PE-Cy5) or fluorescence-activated cell sorter (FITC)-conjugated anti-CD4 and anti-CD8, FITC-anti-CD127 (eBioscience) and PE-anti-CD25 (BD Bioscience).

Intracellular expression of FoxP3 transcription factor. FoxP3 expression was detected using the FITC anti-human FoxP3 staining set (eBioscience), according to the manufacturer's instructions. An isotype-matched antibody was used as control (eBioscience).

CD107 surface expression. The frequency of $CD4^+$ and $CD8^+CD107^+$ cells was evaluated in total or CD25-depleted PBMC and PFMC cultured for 18 h with or without *M. tuberculosis*, with the addition of FITC-anti-CD107 mAb (BD Bioscience) for the last 4 h. Cells were then stained with PE-Cy5 or PE-anti-CD4 or anti-CD8 mAb (BD Bioscience).

Intracellular cytokine expression. Intracellular IL-10 and IFN-γ expression was determined in 18-h cultures from PBMC and PFMC with the addition of brefeldin A (5 μg/ml; Sigma Chemical Co., St Louis, MO, USA) for the last 4 h; thereafter cells were stained with anti-CD4, anti-CD8 and anti-CD25, and fixed with 0·5% paraformaldehyde, permeabilized with FACS™ permeabilizing solution 2 (BD Bioscience) before FITC- or PE-labelled anti-IL-10 (BD Bioscience), and anti-IFN-γ (Invitrogen, Camarillo, CA, USA) or the corresponding isotypes were added.

Surface or intracellular stained cells were analysed by flow cytometry; 20 000 events were acquired on a FACSCan cytometer (BD Bioscience) using CellQuest. FCS express software (De Novo Software, Los Angeles, CA, USA) was used for the analysis. Lymphocyte gates were set according to forward- and side-scatter parameters, excluding cell debris and apoptotic cells. Results were expressed as percentages of positive cells in a lymphocyte population or within $CD4^+$ or $CD8^+$ T cells.

Statistics

Data were analysed using Graphpad Prism version 5·0 (Graphpad Software Inc., San Diego, CA, USA). Results were expressed as medians and 25–75th percentiles. The non-parametric Kruskal–Wallis test was used to compare data from TB patients and healthy individuals, followed by

Table 2. Increased levels of $CD4^+CD25^+$ cells in pleural fluid (PF) and peripheral blood (PB) from patients with tuberculous pleurisy.

	TB-PF		TB-PB		N-PB	
	%	Cells/mm^3	%	Cells/mm^3	%	Cells/mm^3
Total $CD4^+$ T cells	60·0 (55·1–65·2)#	477 (366–771)	34·2 (22·3–42·8)*	500 (280–853)*	53·1 (45·8–56·0)	1113 (962–1176)
$CD4^+CD25^+$	13·0 (7·6–18·2)#	157 (54–234)	5·6 (3·8–8·1)*	168 (74–238)*	4·7 (4·1–6·7)	135 (48–188)
$CD4^+$ $CD25^{high}$	2·5 (2·0–3·0)#	30 (26–77)	1·7 (1·1–2·1)*	56 (33–72)*	1·1 (0·7–1·6)	43 (16–54)
$CD4^+CD25^{low}$	12·1 (8·4 ± 15·6)#	123 (77–156)	5·6 (3·8–8·1)*	152 (89–281)*	3·4 (2·7–5·2)	115 (57–150)
$CD25^{high}FoxP3^+$ in CD4	2·3 (1·5–2·8)#	31 (15–61)	1·5 (1·0–2·0)*	59 (36–83)*	1·0 (0·6–1·4)	23 (11–27)
Total $CD8^+$ T cells	23·4 (19·6–25·0)#	147 (101–192)	39·3 (25·7–43·1)	441 (333–742)	31·5 (29·1–35·0)	662 (611–735)
$CD8^+CD25^+$	4·0 (3·5–5·9)#	34 (4–169)	1·8 (1·5–2·3)*	13 (6–109)	0·6 (0·4–1·3)	13 (8–17)
$CD25^+$ $FoxP3^+$ in CD8	0·6 (0·5–1·6)#	5 (2–13)	0·3 (0·1–0·8)	5 (3–14)	0·4 (0·2–0·5)	3 (2–4)

Mononuclear cells isolated from paired samples of PF and PB obtained from 16 patients with tuberculous pleurisy (TB-PF and TB-PB, respectively) as well as PB from 10 healthy individuals (N-PB) were tested for the expression of surface CD4, CD8 and CD25 and intracellular forkhead box P3 (FoxP3) by flow cytometry. Results are expressed as percentage (%) of (a) total $CD4^+$ and $CD8^+$ cells, $CD4^+CD25^+$ and $CD8^+CD25^+$, $CD4^+$ expressing high or low CD25 expression ($CD4^+$ $CD25^{high}$ and $CD4^+CD25^{low}$) within the lymphocytes gates, (b) percentage of $CD25^+FoxP3^+$ cells within the $CD4^+$ and $CD8^+$ subsets ($CD25^{high}FoxP3^+$ in CD4 or CD8). Absolute numbers (cells/mm^3) were also calculated. Median and 25–75% percentiles are shown. Significant differences between TB-PB *versus* N-PB: $*P < 0·05$; TB-PB *versus* TB-PF: $\#P < 0·05$.

Mann–Whitney *U*-test. Friedman's test was performed to compare data from pleural and peripheral blood samples, followed by Wilcoxon's test. Correlations were performed by the non-parametric Spearman's rank test. All statistical analyses were two-sided, and the significance level adopted was for *P*-values of <0·05.

Results

The proportion of $CD4^+CD25^+$ T cells is increased in TB-PFMC

As shown in Table 2, the percentage and absolute number of $CD4^+$ T cells were decreased in PBMC from TB (TB-PB) when compared to PBMC from N (N-PB), while no differences were observed in CD8 T cells. The percentages of $CD4^+CD25^+$ and $CD8^+CD25^+$ cells were higher in TB-PB that in N-PB. Conventional and regulatory $CD4^+$ T cells were analysed discriminating low or high CD25 expression on the basis of CD25 mean fluorescence intensity (MFI) within the CD4 population. We observed that TB-PB showed a higher percentage of $CD4^+CD25^{low}$ and $CD4^+CD25^{high}$ cells when compared to N-PB. When we evaluated the expression of FoxP3, considered a marker of natural T_{reg} cells, a higher proportion of $CD25^{high}FoxP3^+$ was detected within the $CD4^+$T subset from TB-PB than in N-PB, while no differences were found in the proportion of $CD25^+FoxP3^+$ within the $CD8^+$ subset (Table 2).

PFMC from TB (TB-PF) showed a higher percentage but a similar absolute count of $CD4^+$T cells than TB-PB (Table 2). The proportion of total $CD4^+CD25^+$, $CD4^+$ $CD25^{high}$ and $CD4^+CD25^{low}$ cells were higher in TB-PF than in TB-PB. Also, an increased percentage of $CD25^+FoxP3^+$ within $CD4^+$ cells was observed in TB-PF compared to TB-PB (Table 2); 65–97% were $CD127^{NEG}$ in PF and in PB (data not shown), confirming their condition of T_{regs}. However, no differences in the absolute number of $CD4^+CD25^+$ and $CD4^+CD25^{high}FoxP3^+$ cells were observed between PB and PF samples. The percentage and absolute number of $CD8^+$ T cells were diminished in TB-PF when compared to TB-PB; however, the percentage of $CD25^+$ cells was enhanced in TB-PF. Although the percentage of $CD25^+FoxP3^+$ cells was also enhanced in the $CD8^+$ subset, it represents only 17·3% (2·9–25·2) of $CD8^+CD25^+$ cells from TB-PF, suggesting that $CD8^+CD25^+$ cells were mainly classically activated T cells.

IL-10 and IFN-γ are expressed differentially in PBMC and PFMC from TB patients

In order to delineate the cytokine profile of $CD4^+$ and $CD8^+$ T cells, the intracellular expression of IL-10 and IFN-γ was determined in PB and PF from TB patients upon stimulation with *M. tuberculosis* for 18 h. As shown in Fig. 1a,c, *M. tuberculosis* enhanced the proportions of $CD4^+IL\text{-}10^+$ and $CD8^+IL\text{-}10^+$ cells in PF and PB samples, the highest values being observed in PB from TB patients. Conversely, the percentage of $CD4^+$ and $CD8^+$ T cells expressing IFN-γ was increased upon *M. tuberculosis* stimulation, but the highest values were observed in TB-PF (Fig. 1b,c). Although 85–97% of $CD4^+CD25^{high}$ cells expressed IL-10 in PF and PB samples, the main IL-10 expression was found in $CD4^+CD25^{low/neg}$ cells from PB. Furthermore, IFN-γ was expressed mainly by $CD4^+CD25^{low/neg}$ cells in PF (Fig. 1d).

CD107 expression is enhanced in $CD4^+$ and $CD8^+$ T cells from PFMC

Considering that CD107a cell surface expression has been described as a marker of cytotoxic T cell degranulation/activation [22,23] and is closely associated with the ability of T cells to produce IFN-γ [23], CD107 expression was

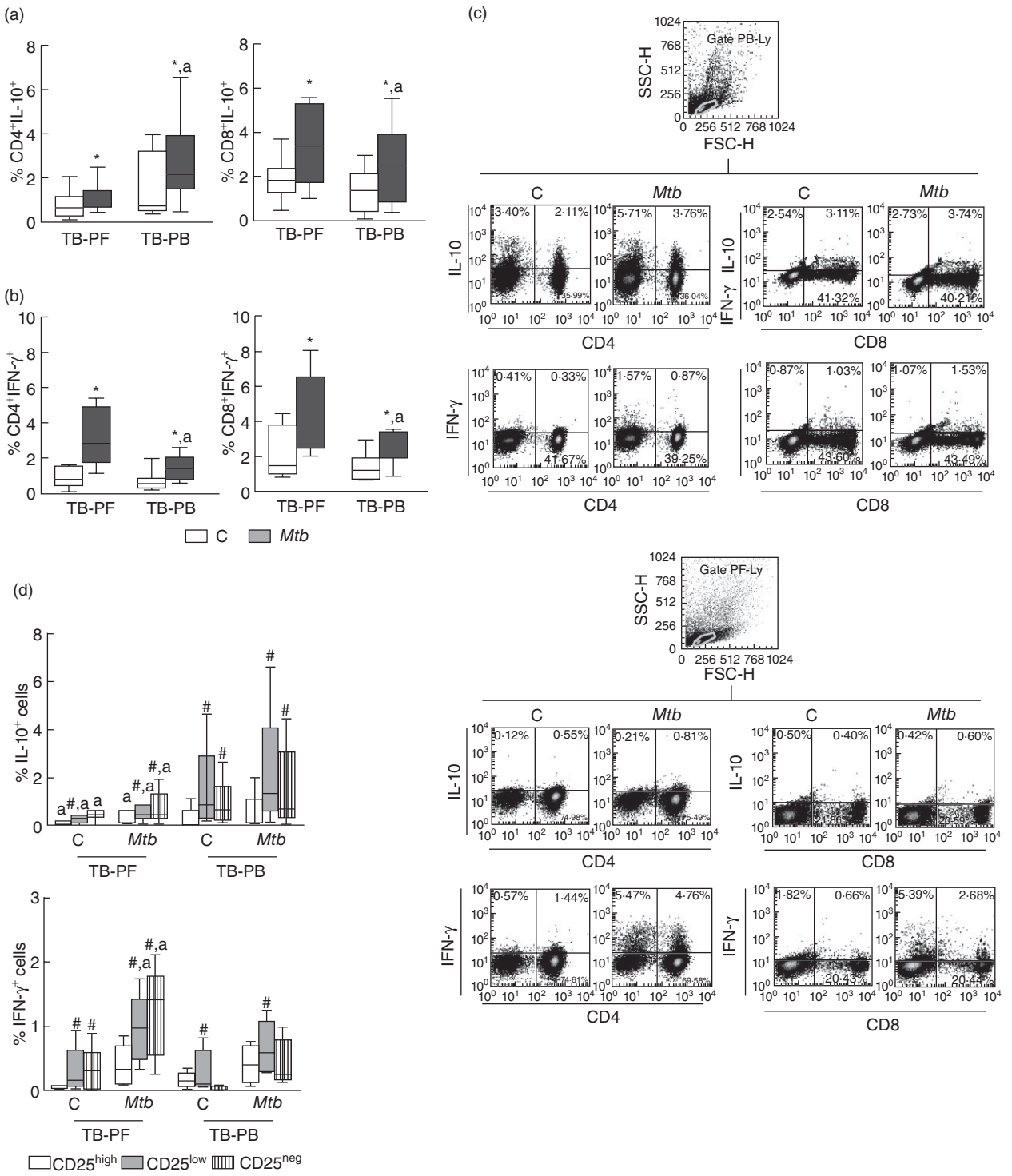


Fig. 1. Interleukin (IL)-10 and interferon (IFN)-γ are expressed differentially in peripheral blood mononuclear cells (PBMC) and pleural fluid mononuclear cells (PFMC) from tuberculosis (TB) patients. TB-PF and TB-PB cells from 14 TB patients were cultured for 18 h alone (control, C) or with *Mycobacterium tuberculosis*. Then cells were tested for the intracellular expression of IL-10 and IFN-γ by flow cytometry. (a) Percentage of $CD4^+$ IL-10^+ and $CD8^+$ IL-10^+ cells within the lymphocyte gates. Results are expressed as median and 25–75 percentiles. (b) Percentage of $CD4^+$ IFN-γ^+ and $CD8^+$ IFN-γ^+ cells within the lymphocyte gates (median and 25–75 percentiles). (c) Representative dot-plots from one TB patient are shown. Numbers in the upper-right quadrant represent the percentage of $CD4^+$IL-10^+, $CD8^+$IL-10^+, $CD4^+$IFN-γ^+ and $CD8^+$IFN-γ^+ cells within live lymphocyte gate in PB (gate PB-Ly) and PF samples (gate-PF-Ly). (d) Percentage of IL-10^+ and IFN-γ^+ cells within the $CD4^+CD25^{high}$, $CD4^+CD25^{low}$ and $CD4^+CD25^{neg}$ subsets from non-stimulated (control, C) and *M. tuberculosis*-stimulated TB-PB and TB-PF cells. (a–c) Statistical differences: $^*P < 0{\cdot}05$ for *M. tuberculosis*-stimulated *versus* control cells, $^aP < 0{\cdot}05$ for TB-PF *versus* TB-PB values, $\#P < 0{\cdot}05$ for $CD4^+CD25^{low/neg}$ *versus* $CD4^+CD25^{high}$.

Fig. 2. CD107 expression is enhanced in $CD4^+$ and $CD8^+$ T cells from tuberculosis-pleural fluid (TB-PF). (a) TB-PF and TB-periheral blood (PB) cells from 14 TB patients were cultured for 18 h alone (c) or with *Mycobacterium tuberculosis* and then tested for their CD107 surface expression by flow cytometry. Results are expressed as percentage of $CD4^+CD107^+$ and $CD8^+CD107^+$ cells within the lymphocyte gates (median and 25–75 percentiles). Statistical differences: $^*P < 0{\cdot}05$ for *M. tuberculosis*-stimulated *versus* control cells; $^aP < 0{\cdot}05$ for TB-PF *versus* TB-PB. (b) Representative dot-plots from one TB patient are shown. Numbers in the upper-right quadrant represent the percentage of $CD4^+CD107^+$ and $CD8^+CD107^+$ in live lymphocyte gate in PB (gate PB-Ly) and PF samples (gate-PF-Ly). (c) Correlation between %$CD4^+/CD8^+$ $CD107^+$ and %$CD4^+/CD8^+$ IFN-γ^+ in control and *M. tuberculosis*-stimulated TB-PB (C-TB-PB, *M. tuberculosis*-TB-PB), TB-PF (C-TB-PF and *M. tuberculosis*-TB-PF). Individual data and Spearman's rho coefficients are shown.

evaluated on $CD4^+$ and $CD8^+$ T cells upon stimulation with *M. tuberculosis* for 18 h. As shown in Fig. 2a,b, *M. tuberculosis* induced CD107 expression on $CD8^+$ and $CD4^+$ T cells from TB-PB and TB-PF. The percentages of $CD4^+CD107^+$ and $CD8^+CD107^+$ cells in PF were higher than in their PB counterpart and confined to $CD4^+/CD8^+CD25^{low/neg}$ cells (data not shown). The percentage of $CD107^+$ cells also correlated with the percentage of IFN-γ^+ cells in *M. tuberculosis*-stimulated PB and PF from TB (Fig. 2c).

T_{reg} cells suppress M. *tuberculosis*-induced degranulation and IFN-γ expression in $CD4^+$ and $CD8^+$ T cells from TB-PF

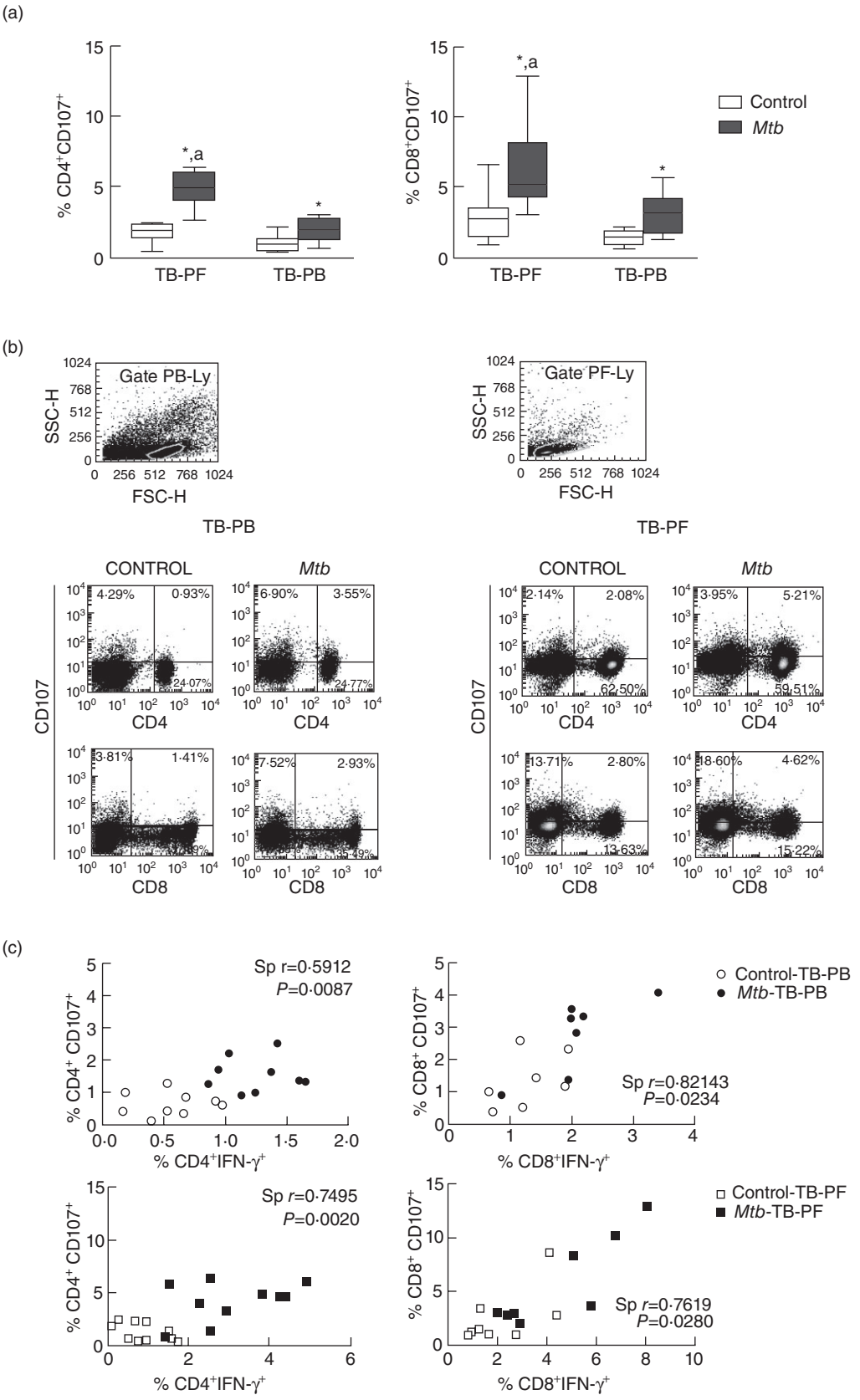
Finally, we wondered whether $CD4^+CD25^{high}$ T_{reg} were able to modulate *in-vitro M. tuberculosis*-induced $CD4^+$ and $CD8^+$ T cell responses in PF. For this purpose, PBMC and PFMC were depleted of $CD25^{high}$ cells by magnetic methods and cultured for 18 h alone or with *M. tuberculosis*; cells were then tested for their expression of IL-10, IFN-γ and CD107. As shown in Fig. 3a, $CD25^{high}$ depletion decreased the percentage of *M. tuberculosis*-stimulated $CD4^+$IL-10^+ and $CD8^+$IL-10^+ cells from PF and PB. In contrast, *M. tuberculosis*-stimulated PB and PF cells depleted of $CD25^{high}$ cells showed increased percentages of $CD4^+$ and $CD8^+$ cells expressing IFN-γ (Fig. 3b) and degranulating $CD4^+$ and $CD8^+$ (Fig. 3c). Again, a direct correlation was observed between $CD107^+$ and IFN-γ^+ cells in PB and PF samples (Spearman's *r*, PB samples: CD4 = 0·7727, $P = 0{\cdot}0053$ and CD8 = 0·6894, $P = 0{\cdot}0064$; PF samples: CD4 = 0·6713, $P = 0{\cdot}0168$ and CD8 = 0·8912, $P < 0{\cdot}0001$). These results suggested that T_{reg} efficiently suppressed *M. tuberculosis*-induced IFN-γ in $CD4^+$ and $CD8^+$ T cells, a fact that impacted directly on their ability to degranulate in response to *M. tuberculosis* even in a Th1 microenvironment such as observed in PF.

Association between T_{reg} and effector T cells and clinical features

We wondered whether the proportion of T_{reg} and $CD4^+$ and $CD8^+$ T cells that express intracellular IL-10, IFN-γ or surface CD107 correlated with clinical data, such as the presence of concomitant pulmonary tuberculosis and acid-fast bacillus (AFB) in sputum using Ziehl–Neelsen-stained smears, antigen load (number of bacilli/field), PPD-positive skin test and days of evolution. As shown in Fig. 4a, the proportion of $CD4^+CD25^{high}$ cells in PB and PF from patients with combined pleural and pulmonary disease tuberculosis was higher than in patients with pleural TB, while no differences were observed in the proportion of $CD4^+CD25^{low}$ cells. Furthermore, higher percentages of IL-10^+ cells (Fig. 4b), together with lower IFN-γ^+ and $CD107^+$ cell levels (Fig. 4c,d, respectively), were observed in those patients with combined pleural and pulmonary TB. All patients with combined pleural and pulmonary TB had positive sputum smears and moderate to advanced disease at the time of the study, while most of the patients with pleural TB had unilateral PF and negative sputum smears; therefore, it is tempting to speculate that bacterial load and/or severity of the disease could be influencing T_{reg} expansion and, in turn, the Th1 response in the pleural space.

Discussion

Immune response to infection is a complex balance between successful induction of proinflammatory responses against the pathogen and anti-inflammatory responses required to limit damage to host tissues. In this context, T_{reg} lymphocytes, which were initially found to control inflammation mediated by autoimmune diseases, play an important role in the control of the inflammatory process induced by infectious pathogens [25,26]. Increased levels of $CD4^+CD25^{high}FoxP3^+$ T_{reg} cells have been detected in PBMC from pulmonary TB patients compared to uninfected [12,15] or healthy infected [13] subjects. *Ex-vivo* depletion of circulating $CD4^+CD25^{high}$ T cells from TB patients also resulted in enhanced IFN-γ production and CTL response upon stimulation with mycobacterial antigens, suggesting a role of T_{reg} in the pathogenesis of TB [12,14,21]. Also, decreased or increased T_{reg} proportions have been demonstrated in samples from different sites of *M. tuberculosis* infection such as pleural, ascitic or pericardial fluids and in the lung alveolar compartment [12,16,17,27,28]. In this study, we showed that the proportion of $CD4^+CD25^{high}$ $FoxP3^+$ T_{reg} cells was increased in PBMC from patients with pleural TB compared to healthy infected controls, as was also observed in other studies [12,16,28]. In agreement with the results reported by other authors [16], $CD4^+CD25^{high}$ $FoxP3^+$ T_{reg} cells were detected in TB-PF, indicating that these cells are recruited efficiently to the site of infection. However, as we did not include a control group, such as


(a)
% CD4+CD107+
% CD8+CD107+
*,a
*
Control
Mtb
TB-PF
TB-PB
(b)
Gate PB-Ly
Gate PF-Ly
SSC-H
FSC-H
TB-PB
TB-PF
CONTROL
Mtb
CD107
CD4
CD8
4·29%
0·93%
24·07%
6·90%
3·55%
24·77%
2·14%
2·08%
62·50%
3·95%
5·21%
59·51%
3·81%
1·41%
7·52%
2·93%
13·71%
2·80%
13·63%
18·60%
4·62%
15·22%
(c)
Sp r=0·5912
P=0·0087
Sp r=0·82143
P=0·0234
Sp r=0·7495
P=0·0020
Sp r=0·7619
P=0·0280
% CD4+ CD107+
% CD8+ CD107+
% CD4+IFN-γ+
% CD8+IFN-γ+
Control-TB-PB
Mtb-TB-PB
Control-TB-PF
Mtb-TB-PF

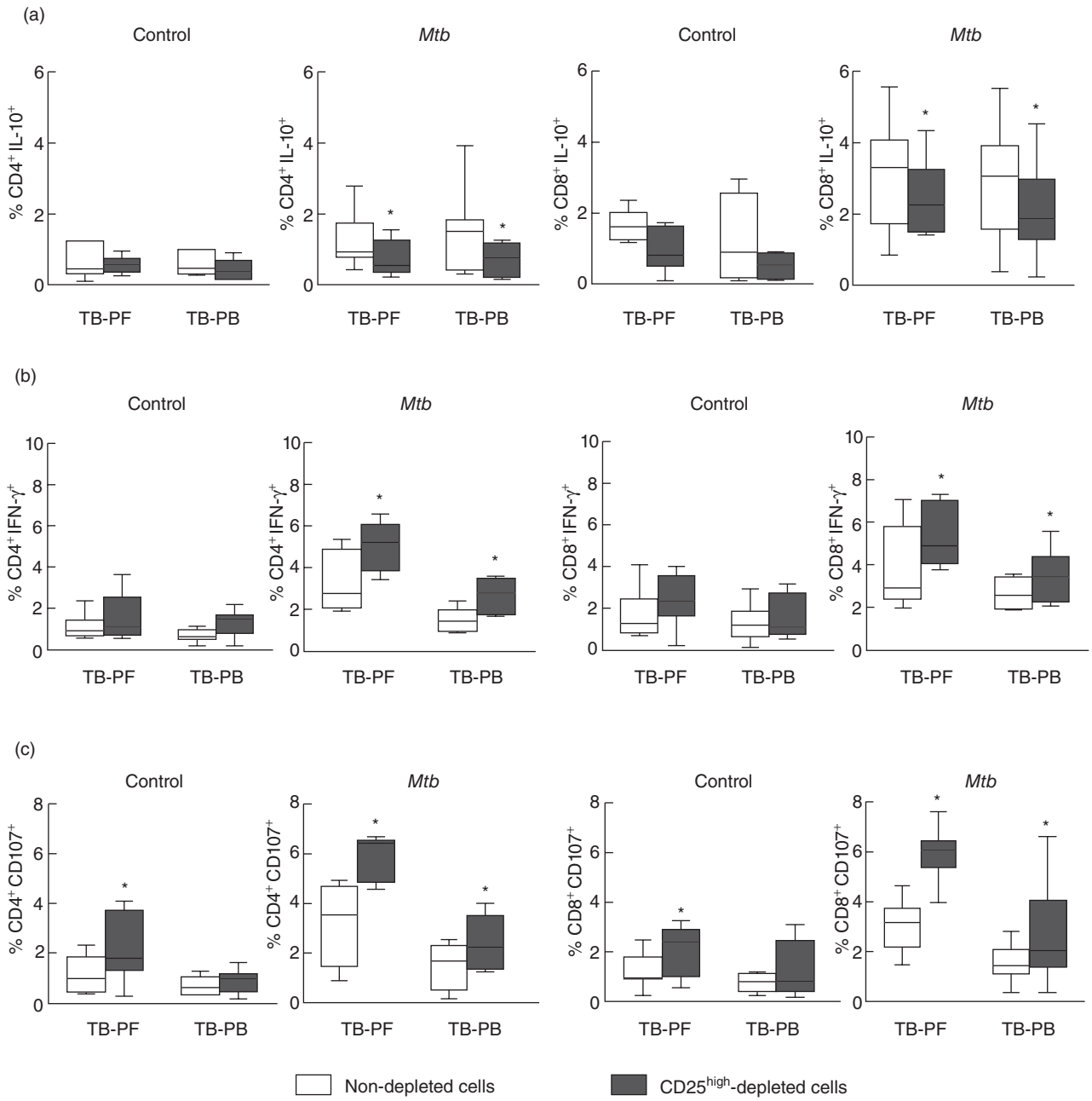


Fig. 3. Regulatory T cells (T_{reg}) suppress *Mycobacterium tuberculosis*-induced degranulation and interferon (IFN)-γ expression in $CD4^+$ and $CD8^+$ T cells from tuberculosis-pleural fluid (TB-PF). TB-peripheral blood (PB) and TB-PF from 10 TB patients were depleted of $CD25^+$ cells by magnetic methods. $CD25^{high}$-depleted and non-depleted cells were cultured for 18 h alone or with *M. tuberculosis* and tested for intracellular interleukin (IL)-10 (a) and IFN-γ (b) or and surface CD107 expression (c). Results are expressed as a percentage of $CD4^+$ and $CD8^+$ cells expressing IFN-γ, IL-10 and CD107 within the lymphocyte gates (median and 25–75 percentiles). For all graphs, statistical differences between non-depleted and $CD25^{high}$-depleted PBMC: $^*P < 0{\cdot}05$ (Wilcoxon's rank sum test).

patients with non-tuberculous pleurisy, we cannot rule out that T_{reg} recruitment could be a response to *M. tuberculosis* infection or a host mechanism to reduce inflammation in the pleural compartment.

As demonstrated in this study, *M. tuberculosis* induced a high proportion of IL-10^+ T cells in PB, whereas high percentages of IFN-γ^+ T cells were found in PF samples, which is in concordance with other studies [7,27,29,30]. Therefore, our results confirm that in spite of the systemic Th2 profile observed in TB patients a switch to a local Th1 profile is observed within the tuberculous PF. It is well known that a Th1 microenvironment favours CD4 and CD8 CTL differentiation and survival [31,32]. In line with this, we found that the increased proportions of *M. tuberculosis*-

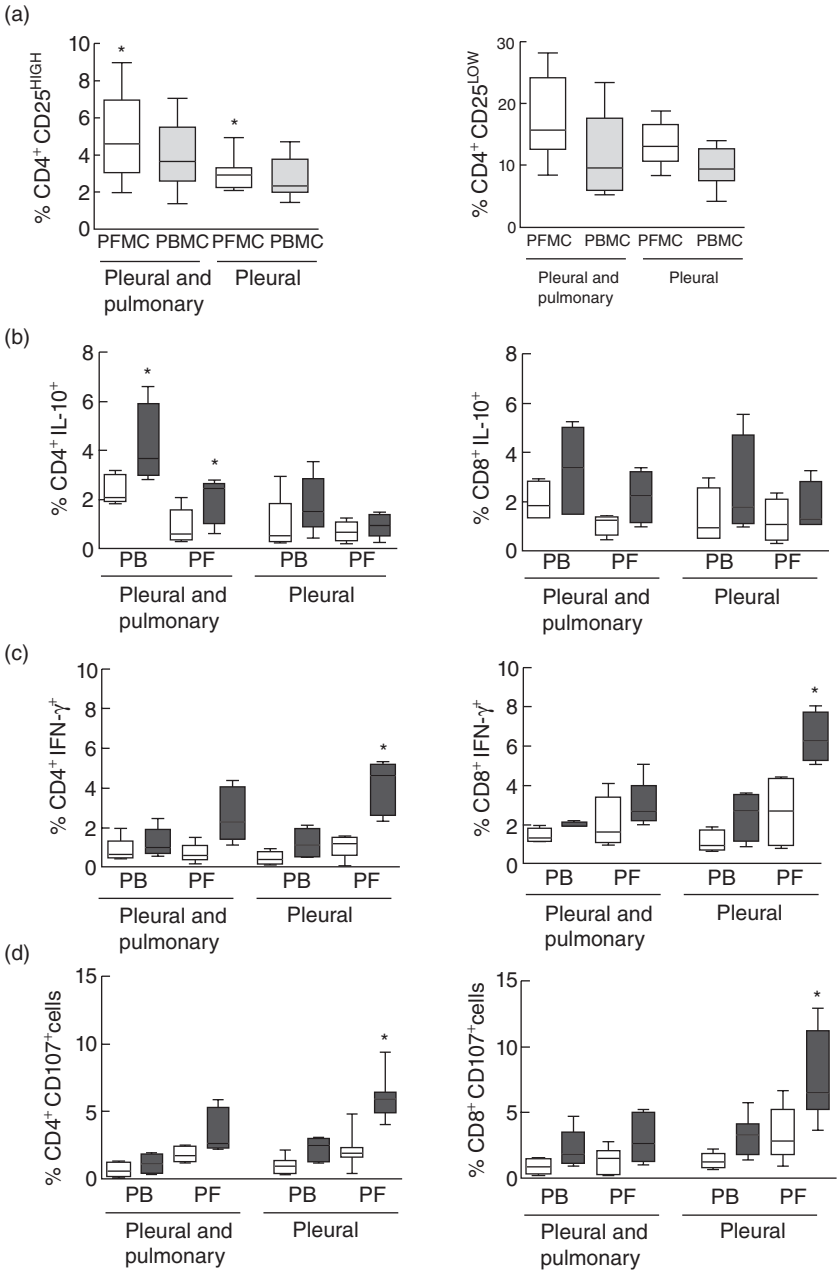


Fig. 4. Patients with combined pleural and pulmonary tuberculosis (TB) showed differential regulatory T cells (T_{reg}) and effector T cell frequencies in peripheral blood and pleural effusions. TB patients were grouped according to the presence (pleural and pulmonary) or absence (pleural) of combined pulmonary tuberculosis. Then, the medians and 25th to 75th percentiles of (a) %$CD4^+CD25^{high}$ and $CD4^+CD25^{low}$ cells, (b) %$CD4^+$ IL-10^+ and $CD8^+$ IL-10^+ cells, (c) %$CD4^+$ IFN-γ^+ and $CD8^+$ IFN-γ^+ cells and (d) %$CD4^+$ $CD107^+$ and $CD8^+$ $CD017^+$ cells were calculated for each group (mean and 25–75th percentiles). Statistical differences, patients with pleural and pulmonary tuberculosis *versus* patients with pleural tuberculosis: $^*P < 0{\cdot}05$.

induced $CD4^+CD107^+$ and $CD8^+CD107^+$ cells correlated closely with the proportions of *M. tuberculosis*-induced IFN-γ^+ cells in PF, as we observed previously in circulating *M. tuberculosis*-induced CD8 CTL [21]. *M. tuberculosis*-induced CTL has been ascribed to the CD8 subset, although cytolytic $CD4^+$ T cells specific for *M. tuberculosis* have also been reported [33–35]. Similarly, CD1-restricted $CD4^+$CTL differentiation has been demonstrated in response to mycobacterial lipids [36]. Hence, the high proportion of $CD4^+$CTL suggests that $CD4^+$ cells expressing IFN-γ and/or with lytic capacity are also attracted at the site of infection and may contribute, in concert with $CD8^+$CTLs, to the lysis of infected antigen-presenting cells. In this context, the negligible bacterial load observed in patients with tuberculous pleurisy could be related, among other factors, to the high CTL function mediated by $CD4^+$ and $CD8^+$ T cells.

Little is known about the role of T_{reg} in a clear Th1 environment such as TB pleurisy. It has been demonstrated that early secreted antigenic target protein 6 (ESAT-6)- and bacilli Calmette–Guérin (BCG)-induced IFN-γ production by PB and PFMC from TB patients is correlated inversely with the percentage of $CD4^+CD25^+FoxP3^+$ T_{reg} cells [28] and their depletion with increased $CD4^+CD25^{neg}$ proliferation [16,37]. In this study we showed that T_{reg} depletion

decreased the percentage of $CD4^+IL\text{-}10^+$ and $CD8^+IL\text{-}10^+$ cells in PB as well as in PF samples. Although it is tempting to speculate that a 37–80% decrease in *M. tuberculosis*-induced IL-10 expression could be due to partial depletion of activated $CD25^{low}$ cells, it is likely that IL-10 was induced by T_{reg} from $CD4^+CD25^{neg}$ cells, as observed by other authors [38].

It has been proposed that T_{reg}-mediated suppression could account for the lack of *M. tuberculosis*-induced IFN-γ and CTL effector functions in TB [12,15,21], the latter function being down-regulated by T_{reg} through direct cell-to-cell contact [39]. In this way, suppression of the influenza vaccine-mediated $CD8^+$CTL response by human circulating T_{reg} cells involves down-regulation of IFN-γ and perforin expression as well as conjugate formation by CTL with target cells [40]. Herein, we showed that depletion of $CD25^{high}$ cells increased IFN-γ and CD107 expression in $CD4^+$ and $CD8^+$ cells from PB and from PF cells, although co-cultures of isolated T_{reg} and non-T_{reg} cells were not performed to confirm the suppressor functionality of PB and PF T_{reg} cells. Both CD107 and IFN-γ expression in $CD25^{high}$ depleted cells were related closely to each other. Thus, in accordance with the suppressor ability described for peripheral T_{reg}, these cells indeed inhibit IFN-γ expression, and in turn the ability of $CD4^+$ and $CD8^+$ cells to degranulate, in response to *M. tuberculosis* in TB pleurisy. Considering that CTL activity has been associated with control of mycobacterial growth through the killing of infected macrophages and also to tissue damage, T_{reg} might contribute to down-regulating excessive CTL activity and protect the host from extensive tissue damage in the pleural space. In line with this, our study demonstrated that in PB and PF samples from patients with combined pleural and pulmonary TB there were increased T_{reg} frequencies compared to those patients with pleural TB. It has been demonstrated that IL-4 induces $CD4^+CD25^+$ T_{reg} cell from $CD25^{neg}$ PB-T cells [41], this cytokine being associated with the severity of the disease [42,43] and detected in bronchoalveolar lavage (BAL) samples [44]. As all patients with combined pleural and active TB showed moderate to severe lung disease and positive sputum smear, an increased frequency of T_{reg} in PB and PF might be associated with bacterial burden or inflammation related to active disease, as observed by other authors [21,45–47]. In these patients, high IL-10 expression and suppression of IFN-γ and CTL response in PB and PF samples could be the consequence of a protective mechanism to limit pulmonary and extra-pulmonary inflammation.

Acknowledgements

This work was supported by grants from the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, PAE-PICT 07-2328, PAE-PICT 07-2329 and PICT 2010-0599).

Author contributions

L. G., J. I. B., N. Y. and C. S. B. were responsible for sample preparation and data collection. R. M. and J. C. were responsible for patient recruitment and selection and for sample collection. M. C. S. and S. de la B. were responsible for study design, data analysis and manuscript preparation.

Disclosure

None of the authors have either a commercial or other association that might pose a conflict of interest. No one involved in the publication process has a financial or other beneficial interest in the products or concepts mentioned in the submitted manuscript or in competing products that might bias his or her judgement.

References

1 Ellner JJ, Barnes PF, Wallis RS, Modlin RL. The immunology of tuberculous pleurisy. Semin Respir Infect 1988; **3**:335–42.
2 Berger HW, Mejia E. Tuberculous pleurisy. Chest 1973; **63**:88–92.
3 Pettersson T, Klockars M, Hellstrom PE, Riska H, Wangel A. T and B lymphocytes in pleural effusions. Chest 1978; **73**:49–51.
4 Lucivero G, Pierucci G, Bonomo L. Lymphocyte subsets in peripheral blood and pleural fluid. Eur Respir J 1988; **1**:337–40.
5 Barnes PF, Mistry SD, Cooper CL, Pirmez C, Rea TH, Modlin RL. Compartmentalization of a CD4+ T lymphocyte subpopulation in tuberculous pleuritis. J Immunol 1989; **142**:1114–9.
6 Mitra DK, Sharma SK, Dinda AK, Bindra MS, Madan B, Ghosh B. Polarized helper T cells in tubercular pleural effusion: phenotypic identity and selective recruitment. Eur J Immunol 2005; **35**:2367–75.
7 Sharma SK, Mitra DK, Balamurugan A, Pandey RM, Mehra NK. Cytokine polarization in miliary and pleural tuberculosis. J Clin Immunol 2002; **22**:345–52.
8 Jalapathy KV, Prabha C, Das SD. Correlates of protective immune response in tuberculous pleuritis. FEMS Immunol Med Microbiol 2004; **40**:139–45.
9 Zhang M, Gately MK, Wang E *et al.* Interleukin 12 at the site of disease in tuberculosis. J Clin Invest 1994; **93**:1733–9.
10 Okamoto M, Kawabe T, Iwasaki Y *et al.* Evaluation of interferon-gamma, interferon-gamma-inducing cytokines, and interferon-gamma-inducible chemokines in tuberculous pleural effusions. J Lab Clin Med 2005; **145**:88–93.
11 Barbosa T, Arruda S, Chalhoub M *et al.* Correlation between interleukin-10 and *in situ* necrosis and fibrosis suggests a role for interleukin-10 in the resolution of the granulomatous response of tuberculous pleurisy patients. Microbes Infect 2006; **8**:889–97.
12 Guyot-Revol V, Innes JA, Hackforth S, Hinks T, Lalvani A. Regulatory T cells are expanded in blood and disease sites in patients with tuberculosis. Am J Respir Crit Care Med 2006; **173**:803–10.
13 Hougardy JM, Place S, Hildebrand M *et al.* Regulatory T cells depress immune responses to protective antigens in active tuberculosis. Am J Respir Crit Care Med 2007; **176**:409–16.
14 Hougardy JM, Verscheure V, Locht C, Mascart F. *In vitro* expansion of CD4+CD25highFOXP3+CD127low/– regulatory T cells from peripheral blood lymphocytes of healthy *Mycobacterium tuberculosis*-infected humans. Microbes Infect 2007; **9**:1325–32.

15 Roberts T, Beyers N, Aguirre A, Walzl G. Immunosuppression during active tuberculosis is characterized by decreased interferon-gamma production and CD25 expression with elevated forkhead box P3, transforming growth factor-beta, and interleukin-4 mRNA levels. J Infect Dis 2007; **195**:870–8.

16 Qin XJ, Shi HZ, Liang QL, Huang LY, Yang HB. CD4+CD25+ regulatory T lymphocytes in tuberculous pleural effusion. Chin Med J (Engl) 2008; **121**:581–6.

17 Semple PL, Binder AB, Davids M, Maredza A, van Zyl-Smit RN, Dheda K. Regulatory T cells attenuate mycobacterial stasis in alveolar and blood-derived macrophages from patients with tuberculosis. Am J Respir Crit Care Med 2013; **187**:1249–58.

18 Canaday DH, Wilkinson RJ, Li Q, Harding CV, Silver RF, Boom WH. CD4(+) and CD8(+) T cells kill intracellular *Mycobacterium tuberculosis* by a perforin and Fas/Fas ligand-independent mechanism. J Immunol 2001; **167**:2734–42.

19 Stenger S, Mazzaccaro RJ, Uyemura K *et al*. Differential effects of cytolytic T cell subsets on intracellular infection. Science 1997; **276**:1684–7.

20 Stenger S, Hanson DA, Teitelbaum R *et al*. An antimicrobial activity of cytolytic T cells mediated by granulysin. Science 1998; **282**:121–5.

21 Geffner L, Yokobori N, Basile J *et al*. Patients with multidrug resistant tuberculosis display impaired Th1 response and enhanced regulatory T cells levels in response to M and Ra outbreak multidrug resistant *Mycobacterium tuberculosis* strains. Infect Immun 2009; **77**:5025–34.

22 Wolint P, Betts MR, Koup RA, Oxenius A. Immediate cytotoxicity but not degranulation distinguishes effector and memory subsets of CD8+ T cells. J Exp Med 2004; **199**:925–36.

23 Betts MR, Brenchley JM, Price DA *et al*. Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. J Immunol Methods 2003; **281**:65–78.

24 Schierloh P, Yokobori N, Aleman M *et al*. Increased susceptibility to apoptosis of CD56dimCD16+ NK cells induces the enrichment of IFN-gamma-producing CD56bright cells in tuberculous pleurisy. J Immunol 2005; **175**:6852–60.

25 Sakaguchi S, Sakaguchi N. Regulatory T cells in immunologic self-tolerance and autoimmune disease. Int Rev Immunol 2005; **24**:211–26.

26 Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. Cell 2008; **133**:775–87.

27 Sharma PK, Saha PK, Singh A, Sharma SK, Ghosh B, Mitra DK. FoxP3+ regulatory T cells suppress effector T-cell function at pathologic site in miliary tuberculosis. Am J Respir Crit Care Med 2009; **179**:1061–70.

28 Chen X, Zhou B, Li M *et al*. CD4(+)CD25(+)FoxP3(+) regulatory T cells suppress *Mycobacterium tuberculosis* immunity in patients with active disease. Clin Immunol 2007; **123**:50–9.

29 Saha PK, Sharma PK, Sharma SK, Singh A, Mitra DK. Recruitment of Th1 effector cells in human tuberculosis: hierarchy of chemokine receptor(s) and their ligands. Cytokine 2013; **63**:43–51.

30 El Fenniri L, Toossi Z, Aung H *et al*. Polyfunctional *Mycobacterium tuberculosis*-specific effector memory CD4+ T cells at sites of pleural TB. Tuberculosis (Edinb) 2011; **91**:224–30.

31 Williams MA, Bevan MJ. Effector and memory CTL differentiation. Annu Rev Immunol 2007; **25**:171–92.

32 Mescher MF, Curtsinger JM, Agarwal P *et al*. Signals required for programming effector and memory development by CD8+ T cells. Immunol Rev 2006; **211**:81–92.

33 Mutis T, Cornelisse YE, Ottenhoff TH. Mycobacteria induce CD4+ T cells that are cytotoxic and display Th1-like cytokine secretion profile: heterogeneity in cytotoxic activity and cytokine secretion levels. Eur J Immunol 1993; **23**:2189–95.

34 Ottenhoff TH, Mutis T. Specific killing of cytotoxic T cells and antigen-presenting cells by CD4+ cytotoxic T cell clones. A novel potentially immunoregulatory T–T cell interaction in man. J Exp Med 1990; **171**:2011–24.

35 De La Barrera SS, Finiasz M, Frias A *et al*. Specific lytic activity against mycobacterial antigens is inversely correlated with the severity of tuberculosis. Clin Exp Immunol 2003; **132**:450–61.

36 Bastian M, Braun T, Bruns H, Rollinghoff M, Stenger S. Mycobacterial lipopeptides elicit CD4+ CTLs in *Mycobacterium tuberculosis*-infected humans. J Immunol 2008; **180**:3436–46.

37 Wu C, Zhou Q, Qin XJ, Qin SM, Shi HZ. CCL22 is involved in the recruitment of CD4+CD25 high T cells into tuberculous pleural effusions. Respirology 2010; **15**:522–9.

38 Zheng SG, Wang JH, Gray JD, Soucier H, Horwitz DA. Natural and induced CD4+CD25+ cells educate CD4+CD25– cells to develop suppressive activity: the role of IL-2, TGF-beta, and IL-10. J Immunol 2004; **172**:5213–21.

39 Piccirillo CA, Shevach EM. Cutting edge: control of CD8+ T cell activation by CD4+CD25+ immunoregulatory cells. J Immunol 2001; **167**:1137–40.

40 Trzonkowski P, Szmit E, Mysliwska J, Dobyszuk A, Mysliwski A. CD4+CD25+ T regulatory cells inhibit cytotoxic activity of T CD8+ and NK lymphocytes in the direct cell-to-cell interaction. Clin Immunol 2004; **112**:258–67.

41 Skapenko A, Kalden JR, Lipsky PE, Schulze-Koops H. The IL-4 receptor alpha-chain-binding cytokines, IL-4 and IL-13, induce forkhead box P3-expressing CD25+CD4+ regulatory T cells from CD25–CD4+ precursors. J Immunol 2005; **175**:6107–16.

42 Dlugovitzky D, Bay ML, Rateni L *et al*. *In vitro* synthesis of interferon-gamma, interleukin-4, transforming growth factor-beta and interleukin-1 beta by peripheral blood mononuclear cells from tuberculosis patients: relationship with the severity of pulmonary involvement. Scand J Immunol 1999; **49**:210–7.

43 Seah GT, Scott GM, Rook GA. Type 2 cytokine gene activation and its relationship to extent of disease in patients with tuberculosis. J Infect Dis 2000; **181**:385–9.

44 Nolan A, Fajardo E, Huie ML *et al*. Increased production of IL-4 and IL-12p40 from bronchoalveolar lavage cells are biomarkers of *Mycobacterium tuberculosis* in the sputum. PLoS ONE 2013; **8**:e59461.

45 Green AM, Mattila JT, Bigbee CL, Bongers KS, Lin PL, Flynn JL. CD4(+) regulatory T cells in a cynomolgus macaque model of *Mycobacterium tuberculosis* infection. J Infect Dis 2010; **202**:533–41.

46 Singh A, Dey AB, Mohan A, Sharma PK, Mitra DK. Foxp3+ regulatory T cells among tuberculosis patients: impact on prognosis and restoration of antigen specific IFN-gamma producing T cells. PLOS ONE 2012; **7**:e44728.

47 Masood KI, Rottenberg ME, Salahuddin N *et al*. Expression of *M. tuberculosis*-induced suppressor of cytokine signaling (SOCS) 1, SOCS3, FoxP3 and secretion of IL-6 associates with differing clinical severity of tuberculosis. BMC Infect Dis 2013; **13**:13.