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CD3 expression distinguishes two γδT cell receptor subsets with different phenotype and effector function in tuberculous pleurisy

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Summary

Tuberculous pleurisy is a naturally occurring site of *Mycobacterium tuberculosis* (*Mtb*) infection. Herein, we describe the expression of activation, natural killer (NK) and cell migration markers, as well as effector functions from γδT cells in peripheral blood (PB) and pleural effusion (PE) from tuberculosis patients (TB). We observed a decreased percentage of circulating γδT from TB patients and differential expression of NK as well as of chemokine receptors on PB and PE. Two subsets of γδT cells were differentiated by the CD3/γδT cell receptor (γδTCR) complex. The γδTCR^{low} subset had a higher CD3 to TCR ratio and was enriched in Vδ2$^+$ cells, whereas most Vδ1$^+$ cells belonged to the γδTCR^{high} subset. In PB from TB, most γδTCR^{high} were CD45RA$^+$CCR7$^-$ and γδTCR^{low} were CD45RA$^{+/-}$CCR7$^+$CXCR3$^+$. In the pleural space the proportion of CD45RA$^-$CCR7$^+$CXCR3$^+$ cells was higher. Neither spontaneous nor *Mtb*-induced interferon (IFN)-γ production was observed in PB-γδT cells from TB; however, PE-γδT cells showed a strong response. Both PB- and PE-γδ T cells expressed surface CD107a upon stimulation with *Mtb*. Notably, PE-γδTCR^{low} cells were the most potent effector cells. Thus, γδT cells from PB would acquire a further activated phenotype within the site of *Mtb* infection and exert full effector functions. As γδT cells produce IFN-γ within the pleural space, they would be expected to play a beneficial role in tuberculous pleurisy by helping to maintain a T helper type 1 profile.

Keywords: γδ T cell, CD107a, IFN-γ, memory cell, tuberculous pleurisy

Introduction

Although the vast majority of mature T lymphocytes express a heterodimeric αβ T cell receptor (TCR), a small subset (1–5%) of circulating human T cells carries the alternative γδTCR [1,2]. The main differences between αβ and γδT cells concern the diversity of the TCR germline repertoire and antigens recognized by the respective TCR molecules [3]. It has been shown recently that, similar to CD4 and CD8 αβ T cells, Vγ9Vδ2 T cells, the major subset in human peripheral blood, are heterogeneous and comprise distinct populations that can be distinguished on the basis of surface marker expression and effector functions [4–6]. Vδ1 chain expressing γδT cells are a minor subset in blood (10–30% of peripheral γδT cells) [7] that can be activated and expanded in response to lipid extracts of Gram-negative bacteria and polyprenylphosphates [8]. γδT cells display an important number of effector functions leading to proliferation, release of T helper type 1 (Th1) cytokines and cytotoxic activity against pathogen-infected macrophages [9–11]. γδT cells recognize non-peptide phosphorylated metabolites of isoprenoid biosynthesis, (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) being the most potent antigen described. HMBPP is produced by most eubacteria, including *Mycobacterium tuberculosis* (*Mtb*) [12]. Prenyl pyrophosphate antigens do not require antigen uptake, processing or intracellular loading for presentation, but are dependent upon cell-to-cell contact. In this context, recent findings suggest strongly the existence of an antigen-presenting molecule different from the currently known major histocompatibility complex (MHC)/CD1 molecules [13,14]. γδT cells are activated in response to *Mtb* [15,16], and an expansion during mycobacterial infection has been observed in experimental models as well as in secondary challenges with either bacilli Calmette–Guérin (BCG) or virulent *Mtb* [10,17]. Furthermore, γδT cells from BCG-vaccinated individuals expand upon restimulation with mycobacterial antigens and display a memory-like phenotype [18]. However, studies investigating γδT cell function in peripheral blood (PB)

and lungs of patients with TB have provided contradictory results [7,19–21].

Tuberculous pleuritis is a common manifestation of extrapulmonary tuberculosis (TB) and results in an increased pleural vascular permeability that leads to the accumulation of protein-enriched fluid and the recruitment of specific inflammatory leucocytes into the pleural space leading to the clearance of mycobacteria from this cavity [22–24]. *Ex-vivo* and *in vitro* studies have demonstrated that $CD4^+$ cells, together with a marked Th1 environment, are predominant in TB pleural effusion (TB-PE) [25–27]. Other cells present in TB-PE [23,26] may also contribute to mount a protective immune response against *Mtb*. An abundance of immunocompetent cells in the PE enables the study of locally accumulated effector cells, of their cytokine production and of their preferential homing. Considering that tuberculous pleurisy is a naturally occurring site of *Mtb* infection, in this study we have investigated the expression of activation, natural killer (NK) and cell migration-associated markers, and the effector functions from γδT cells in PB and PE from TB patients. Herein, we have demonstrated that γδT cells comprise two distinct subsets that present marked differences in activation state and effector functions in both PB and PE.

Materials and methods

Patients

Thirty-four patients (28 men, six women, aged between 17 and 63 years) with newly diagnosed tuberculous pleuritis were identified at the Servicio de Tisioneumonología, Hospital F. J Muñiz (Buenos Aires, Argentina). Informed consent was obtained from patients according to the Ethics Committee. Patients were evaluated by history and physical examination, complete blood cell count, electrolyte, chest X-ray, human immunodeficiency virus (HIV) and tuberculin skin test status. PE and PB were obtained during diagnostic thoracentesis before initiation of chemotherapy. Exclusion criteria included a positive test for HIV or the presence of concurrent infectious diseases. Effusions were classified as exudates if they fulfilled at least one of the Light *et al.* criteria [28]. TB-PE were defined as exudates with a positive Ziehl–Nielsen stain or Lowenstein–Jensen culture of PE or pleural biopsy specimens.

Thoracentesis and mononuclear cells

PE was obtained as described previously [29]. Biochemical analysis, bacterial cultures and cytological examinations were performed on all PE samples at the Central Laboratory of Muñiz Hospital. PE and PB samples were dispensed into tubes containing heparin and were collected from patients on the same day as thoracentesis. PB from healthy subjects (HS) ($n = 10$; age range 20–55 years) were also evaluated. All HS had received BCG vaccination in childhood and their tuberculin-test status was unknown. Peripheral blood mononuclear cells (PBMC) and PE mononuclear cells (PEMC) were isolated by Ficoll-Hypaque and suspended in RPMI-1640 tissue culture medium (Gibco Laboratories, New York, NY, USA) containing gentamicin (85 μg/ml) and 10% heat-inactivated fetal calf serum (Gibco Laboratories; complete medium). Purity and viability were tested using trypan blue exclusion. PBMC and PEMC (1×10^6 cells/ml) were cultured in Falcon 2063 tubes (Becton Dickinson, Lincoln, NJ, USA) for 24 h at 37°C in a humidified 5% CO_2 atmosphere, in complete medium with or without *Mtb*.

Antigen

The gamma-irradiated *Mtb* H37Rv strain used in this study was provided by J. Belisle (Colorado State University, Denver, CO, USA). Mycobacteria were suspended in phosphate-buffered saline (PBS) free of pyrogen, sonicated and adjusted at a concentration of $\approx 1 \times 10^8$ bacteria/ml [optical density $(OD)_{600} = 1$].

Immunofluorescence analysis

Expression of surface markers on γδT lymphocytes. The following anti-human monoclonal antibodies (mAb) were used: Cy5PE-CD3, fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-CD56, FITC-CD16, FITC-CD94, PE-human leucocyte antigen D-related (HLA-DR), FITC-CD62L, FITC-CD45RO, FITC-CD45RA (e-Bioscience, San Diego, CA, USA), PE-NKG2A and PE-NKG2D (R&D Systems, Minneapolis, MN, USA), FITC- and PE-CD69 (Ancell, Bayport, MN, USA), FITC (e-Bioscience) or PE-pan-γδTCR (BD-Pharmingen, San Diego, CA, USA), PE-CCR7, PE-CXCR3, PE-CD27, PE-Vδ2TCR (BD-Pharmingen) and FITC-Vδ1TCR (Pierce-Endogen, Thermo Scientific, Rockford, IL, USA). Labelled isotype-matched antibodies were also tested. PBMC and PEMC were incubated with the corresponding mAbs for 30 min at 4°C, cells were washed, fixed with 0·5% paraformaldehyde (PFA), suspended in Isoflow™ (BD-Pharmingen) and analysed in a fluorescence activated cell sorter (FACScan) cytometer using Cellquest (BD-Pharmingen) and FSC Express (De Novo Software, Los Angeles, CA, USA) software; 30 000–50 000 events in the lymphocyte gate were acquired. Analysis gates were set on lymphocytes according to forward- and side-scatter properties. Results are expressed as the percentage of positive cells.

Intracytoplasmatic detection of interferon (IFN)-γ

Briefly, PBMC and PEMC (1×10^6 cells/ml) were stimulated with or without *Mtb* for 24 h (ratio of cells to bacteria: 1:2). Brefeldin A (5 μg/ml, Sigma, St Louis, MO, USA) was added

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

Parameter		Peripheral blood	Pleural effusion	*P*-value
Age (years)	29 (17–63)			
Gender	29M/5F			
AFB	21+/13–			
PPD	19+/15–			
ADA		–	100 ± 21	
Cell count, cells/mm^3		7012 ± 1639	2029 ± 1291	$P < 0{\cdot}0001$
Ly, % (range)		21 ± 8 (5–36)	70 ± 12 (44–87)	$P < 0{\cdot}0001$
Ly count, cells/mm^3		1512 ± 564	1746 ± 1021	n.s.
γδT Ly, % (range)		4·5 ± 3·6 (0·6–14·6)	3·0 ± 1·8 (0·4–6·8)	$P < 0{\cdot}05$
γδT Ly, cells/mm^3		64 ± 59	46 ± 44	n.s.

AFB, acid-fast bacillus in sputum or culture; PPD, purified protein derivative skin test; ADA, adenosine deaminase; Ly, lymphocyte; n.s., not significant.

for the final 4 h to block cytokine secretion prior to surface staining of CD3 and γδTCR. Thereafter, cells were fixed and permeabilized according to the manufacturer's instructions (Perm2; BD-Pharmingen). FITC- or PE-anti-IFN-γ (Caltag, Burlingame, CA, USA) was added and incubated for 30 min at room temperature. Cells were washed and analysed by flow cytometry; 30 000–50 000 events were acquired. Results are expressed as a percentage of positive cells on CD3$^+$γδTCR$^+$ cells and as median fluorescence intensity (MFI).

Detection of perforin in γδT cells

PBMC and PEMC were stained with Cy5PE-CD3 and PE-γδTCR mAb. Thereafter, cells were fixed and permeabilized as described above, and stained with FITC-anti-perforin (Ancell, Bayport, MN, USA). Positive cells were analysed by acquiring 30 000–50 000 events; results are expressed as percentage of positive cells and as MFI.

Detection of CD107a on γδT cells

Because degranulation of antigen-responding T cells is associated with acquisition of cell surface CD107a, PBMC and PEMC (1×10^6 cells/ml) were incubated with or without *Mtb* for 18 h, and FITC-CD107a mAB (BD-Pharmingen) was added to the culture during the last 4 h; thereafter, cells were washed once and surface-stained with PE-Cy5-anti-CD3 and PE-anti- γδTCR; 20 000 events were acquired on CD3$^+$γδTCR$^+$ lymphocytes and results are expressed as a percentage of CD107a$^+$ cells.

Statistics

Comparisons of paired PB and PE samples and of different treatments were carried out using the paired Wilcoxon test or the unpaired Mann–Whitney test (non-parametric). A value of $P < 0{\cdot}05$ was assumed as significant. For evaluation of correlations between surface markers, the non-parametric two-tailed Spearman's rank correlation test was used.

Results

Patient characteristics

A total of 34 subjects with tuberculous pleurisy were enrolled in this study. All study participants had newly diagnosed moderate to large pleural effusions. Among them, 11 also had pulmonary disease with positive sputum smears. In addition, PB from 10 healthy donors (HS) was evaluated. Table 1 summarizes selected clinical and laboratory data profiles of TB patients. We found a lower absolute number of lymphocytes (HS = 2175 ± 520 cells/mm^3, TB = 1512 ± 564 cells/mm^3; $P < 0{\cdot}05$) and γδT cells (HS = 102·2 ± 7·6 cells/mm^3, TB = 64 ± 59 cells/mm^3; $P = 0{\cdot}0017$) in PB of TB than in HS. As shown in Table 1, high adenosine deaminase (ADA) was found in PE, which is in accordance with its aetiology [30]. Compared to PB, PE presented higher CD3$^+$ and lower γδT lymphocyte percentages; none the less, no significant differences were observed in absolute numbers.

Characterization of γδT cells in peripheral blood and pleural effusion mononuclear cells from TB patients

It is well known that γδT cells express several NK receptors [31] and that their activity is regulated by both positive and inhibitory signals transduced by cell surface receptors including the C-type lectin family, immunoglobulin (Ig) superfamily and natural cytotoxicity receptors [32–34]. To characterize the activation state as well as NK and chemokine receptor expression, PE-γδT from TB and PB-γδT from TB and HS were analysed by flow cytometry. The percentage of CD69$^+$ γδT cells was higher in PB from TB compared to that of HS and was even greater in PE-γδT (Fig. 1a). Although no differences were detected in the proportion of HLA-DR$^+$ cells between PB from HS and TB, it was significantly higher in PE-γδT cells. PB-γδT from HS showed lower percentages of CD56 and CD16 and higher percentages of NKG2D and NKG2A than TB-PB. Also, a lower proportion of γδT cells expressing CD56, CD16, NKG2D and CD94 molecules was observed in PE than PB from TB. However,

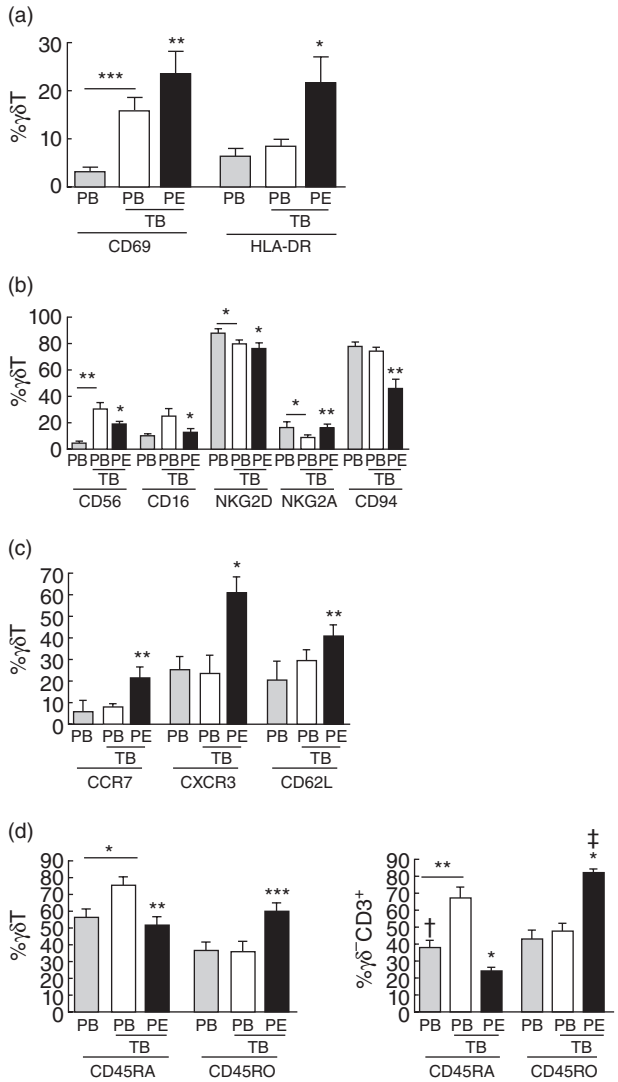

Fig. 1. Characterization of γδT cell in pleural effusion and peripheral blood from tuberculosis (TB) patients and healthy controls. Peripheral blood mononuclear cells (PB) from healthy individuals (grey bars) and TB patients (unfilled bars) and pleural effusion mononuclear cells (PE) from TB patients (black bars) were tested for surface expression of: CD69 and human leucocyte antigen D-related (HLA-DR) activation molecules (a), CD56, CD16, NKG2D, NKG2A and CD94 natural killer (NK) receptors (b) and CCR7, CXCR3 and CD62L migration-associated receptors (c) by flow cytometry in the $CD3^+\gamma\delta T$ cell receptor $(TCR)^+$ gate. CD45RA and CD45RO markers were evaluated in $CD3^+\gamma\delta TCR^+$ (γδT) and $\gamma\delta TCR^-$ ($\gamma\delta^-CD3^+$) T cells (d). Results are expressed as percentage (%) (mean ± standard error of the mean). Statistical differences: PB-TB *versus* PE or PB-TB *versus* PB-healthy subjects (HS): $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}005$; γδT *versus* $\gamma\delta^-CD3^+$: $\dagger P < 0{\cdot}05$, $\ddagger P < 0{\cdot}01$.

the percentage of $NKG2A^+$ γδT cells was higher in PE (Fig. 1b).

Because lymphocyte migration, including γδT cells, depends upon the combined action of adhesion molecules [35] and chemokines and their receptors [36], we evaluated the expression of molecules involved in extravasation (CD62L), migration into lymph nodes (CCR7) and association with a type-1 response (CXCR3). No differences were observed in the frequency of CCR7-, CXCR3- and CD62L-expressing cells between PB-γδT cells from HS and TB. Despite this, a higher percentage of these markers was detected in PE-γδT cells, and this was particularly pronounced for CXCR3 (Fig. 1c).

Naive T cells can be differentiated from those that are activated or have previously encountered antigens (effector/memory cells) by several surface markers, CD45RA and CD45RO being employed widely [5]. γδT cells from PB-TB showed a higher CD45RA percentage than PB-HS with the same proportion in $CD45RO^+$ cells. In addition, lower $CD45RA^+$ and higher $CD45RO^+$ percentages were obtained in PE-γδT cells than in its PB counterpart (Fig. 1d). Having observed the high CD45RA percentage in PB-γδT cells, we wondered whether $CD3^+\gamma\delta^-$ cells would show the same CD45RA/RO pattern of expression; a higher proportion of $CD45RA^+$ cells was detected in PB-TB than in PB-HS, whereas this percentage was lower in PE (Fig. 1d). The latter was due to the enrichment in $CD4^+$ cells (72 ± 2·5% of PEMC) that express mainly PE CD45RO (81·3 ± 2·0%). Interestingly, regarding the CD45RA/RO expression pattern, γδT cells in PB from TB and HS as well as in PE were similar to conventional $CD8^+$ T cells (data not shown).

Because CD45RA alone is not sufficient to identify naive cells, CD27 and CD11a were employed as additional markers and these cells were identified by $CD45RA^+/CD27^{bright}/CD11a^{dull}$ expression [4,5]. To rule out that $CD45RA^+$ cells were naive, CD27 was employed as a second marker. Although in TB patients 40 ± 2% of PB and 68 ± 3% of PE-γδT cells were $CD27^+$, its expression was very low (MFI: PB = 66 ± 5; PE = 95 ± 30) compared to that of $CD3^+\gamma\delta^-$ cells (MFI: PB = 250 ± 100; PE = 150 ± 60; $n = 5$), suggesting that $CD45RA^+$ γδT cells did not fit the naive phenotype.

Two populations of γδT cells are differentiated by CD3/γδTCR complex expression

According to TCR and CD3 expression we were able to differentiate $\gamma\delta TCR^{high}$ and $\gamma\delta TCR^{low}$ subpopulations in PB from 25 of 38 (66%) TB patients and 14 of 24 (58%) HS (Fig. 2a). $\gamma\delta TCR^{low}$ was the prevailing subset in PB and PE, and no differences in the $\gamma\delta TCR^{high}/\gamma\delta TCR^{low}$ ratio were observed in either HS or TB samples (Fig. 2b). Furthermore, a significant correlation between CD3 and TCR was achieved only when high and low subpopulations were considered as two independent groups both in PB and in PE (Fig. 2c), and no correlation was found when high and low γδT were taken as a single population (PB: $r = 0{\cdot}1611$, $P = 0{\cdot}4627$; PE: $r = -0{\cdot}0270$, $P = 0{\cdot}9005$). When V gene usage was analysed in $\gamma\delta TCR^{high}$ and $\gamma\delta TCR^{low}$ subsets, we found that most $V\delta1^+$ corresponded to $\gamma\delta TCR^{high}$ and $V\delta2^+$ cells were largely represented in the $\gamma\delta TCR^{low}$ subset in PB from both HS and TB as

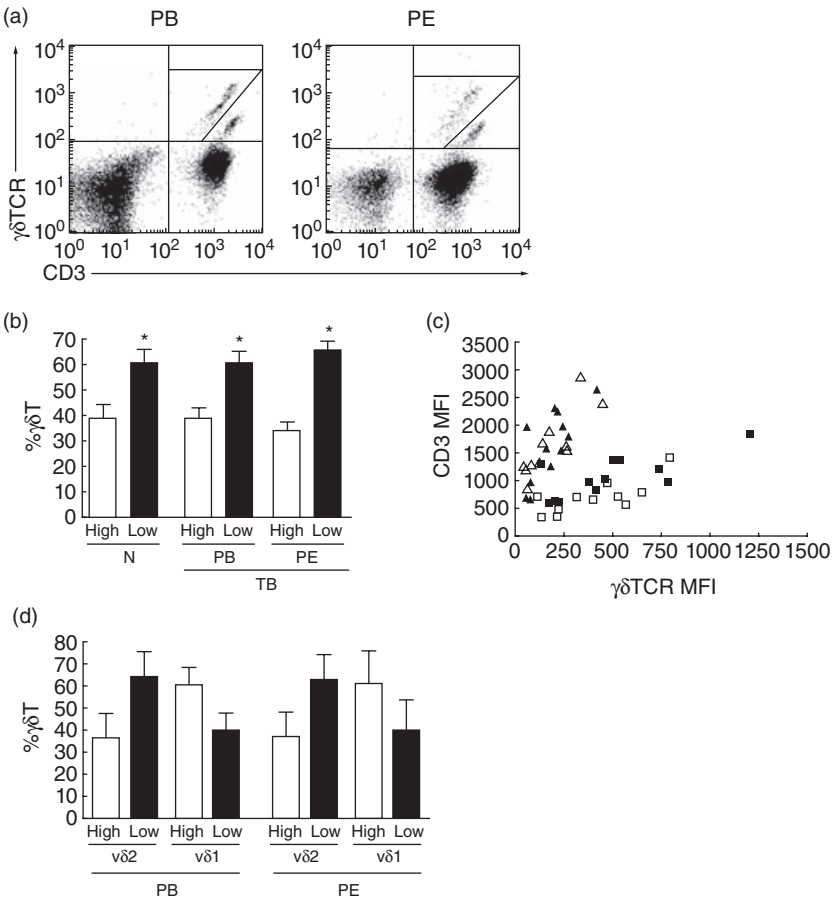

Fig. 2. CD3/γδT cell receptor (TCR) complex expression differentiates two populations of γδT cells. According to their CD3 and γδTCR expression, two γδT cell populations from peripheral blood (PB) and pleural effusion (PE) mononuclear cells were defined by flow cytometry: γδTCR^{high} (HIGH) and γδTCR^{low} (LOW). (a) A representative dot-plot from a tuberculosis (TB) patient is shown. (b) Results are expressed as percentage of γδTCR^{high} and γδTCR^{low} subsets among total γδT cells (mean ± standard error of the mean). Statistical differences: high *versus* low, $^*P < 0{\cdot}05$. (c) Correlation between CD3 and γδTCR median fluorescence intensity (MFI) in γδTCR^{high} and γδTCR^{low} from PB (■ PB-high and (▲ PB-low) and PE (□ PE-high and (△PE-low). Two-tailed Spearman's rank correlation test. PB-high: $r = 0{\cdot}8091$, $P < 0{\cdot}005$; PB-low: $r = 0{\cdot}6165$, $P < 0{\cdot}05$; PE-high: $r = 0{\cdot}6154$, $P < 0{\cdot}05$; PE-low: $r = 0{\cdot}8671$, $P < 0{\cdot}001$. (d) Vδ2$^+$ and Vδ1$^+$ were distributed asymmetrically in γδTCR^{high} and γδTCR^{low} subsets ($n = 6$).

well as in PE (Fig. 2d). Despite this asymmetrical distribution, almost half the γδTCR^{high} cells expressed the Vδ2 chain (data not shown). Therefore, our results suggest that γδT cells can be divided into two subsets according to the TCR complex, with γδTCR^{low} having a higher CD3/TCR ratio.

Phenotypical differences of γδTCR^{high} and γδTCR^{low} subsets from PB and PE cells in TB patients

Considering the γδTCR^{high} and γδTCR^{low} subsets identified in PB and PE, we wanted to determine whether there were differences in the CD45RA and CD45RO markers. As shown in Fig. 3a and b, in PB almost all γδTCR^{high} expressed CD45RA, whereas in γδTCR^{low} both CD45RA/RO isoforms were expressed similarly. γδTCR^{low} from PE showed the highest proportion of CD45RO and, although a high percentage of CD45RA was detected in γδTCR^{high}, CD45RO was also expressed (Fig. 3c). Furthermore, most circulating Vδ1 were CD45RO-negative, while in contrast Vδ2 were CD45RO-positive, in accordance with previous reports (data not shown) [5]. According to these markers, in those TB and HS in whom two subpopulations of γδ T cells were not observed, these belonged to the γδTCR^{low} subset.

Additional markers were assessed to define further the phenotype of these subsets. As shown in Fig. 4a, the percentages of CCR7$^+$ and CXCR3$^+$ were higher in PB-γδTCR^{low} compared to those in PB-γδTCR^{high}, whereas no differences were detected in CD62L, CD69, HLA-DR, CD16 and CD94 (data not shown). Although the percentages of CCR7, CXCR3, CD62L and CD69 were slightly higher in PE-γδTCR^{low}, they did not reach statistical significance (Fig. 4b). Additionally, the proportion of HLA-DR$^+$ cells did not differ between both subsets, and CD16, CD56 and CD94 were significantly lower in PE-γδTCR^{low} (data not shown).

Effector functions of γδTCR^{high} and γδTCR^{low} subsets from PB and PE cells

In order to determine whether the memory phenotype of γδT cells correlates with their effector functions, markers of cytotoxic activity, such as perforin and lysosomal-associated membrane protein 1 (LAMP-1/CD107a), as well as IFN-γ production, were evaluated. As shown in Fig. 5a, the percentage of perforin-positive cells found in PB-γδT was reduced in PE-γδT, as it also was in MFI (PB = 485 ± 200; PE = 390 ± 210, $n = 9$, $P < 0{\cdot}01$). Among perforin-positive cells, PB-γδTCR^{high} expressed a higher percentage than PB-γδTCR^{low} and these frequencies were decreased in both subsets from PE (Fig. 5b). Spontaneous degranulation assessed by CD107a was observed in 6 ± 2% of *ex vivo*

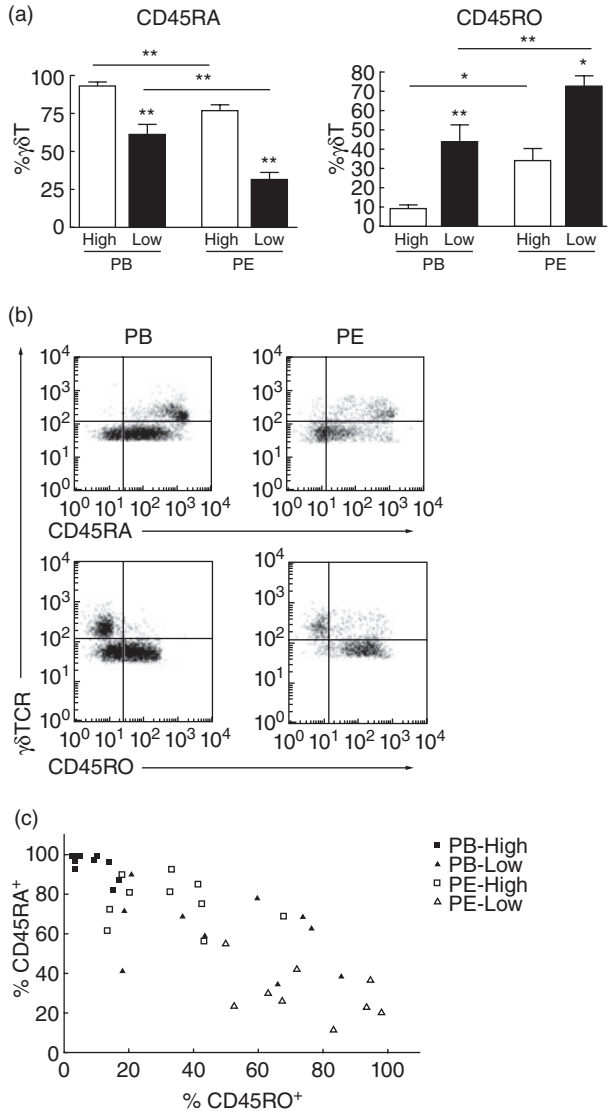

Fig. 3. CD45RA and CD45RO expression in γδT cell receptor (TCR)^{high} and γδTCR^{low} subsets from peripheral blood (PB) and pleural effusion (PE) mononuclear cells cells. CD45RA/RO markers were evaluated in high and low γδT subpopulations. (a) Percentage of CD45RA$^+$ or CD45RO$^+$ cells among high and low population is shown (mean ± standard error of the mean). Statistical differences: high *versus* low or PB *versus* PE: $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$. (b) Representative dot-plots. (c) Correlation between CD45RA$^+$ and CD45RO$^+$ cells.

PB-γδT and 12 ± 5% of PE-γδT cells, and these percentages did not increase in untreated cells during *in vitro* culture, the percentage of CD107a$^+$ from PE being significantly higher than that from PB-γδT cells ($P < 0{\cdot}05$; Fig. 5c). Upon stimulation with *Mtb*, the percentage of CD107a$^+$-γδT cells was increased in PB and PE, with a stronger response in the latter being slightly higher, although not statistically significant, in γδTCR^{low} in both PB and PE. *Mtb* induced degranulation in all subsets but differences between high and low subsets were found only in PE (Fig. 5d).

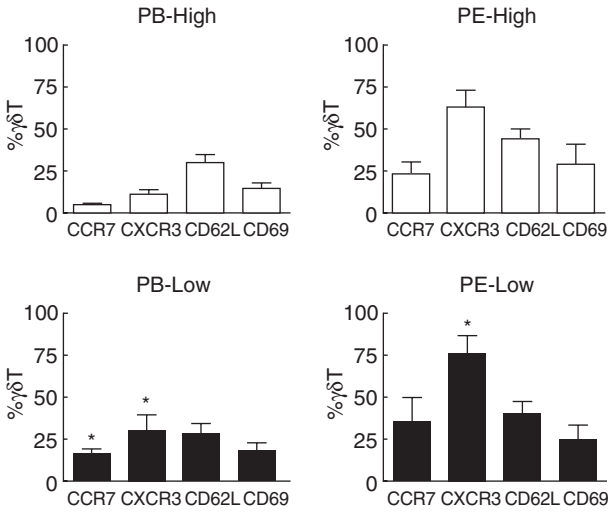

Fig. 4. CCR7, CXCR3, CD62L and CD69 expression in γδT cell receptor (TCR)^{high} and γδTCR^{low} subsets from peripheral blood (PB) and pleural effusion (PE) mononuclear cells. The expression of CCR7, CXCR3 and CD62L migration markers as well as CD69 activation molecules were evaluated in γδTCR^{high} and γδTCR^{low} cells from PB and PE. Statistical differences: high *versus* low: $^*P < 0{\cdot}05$.

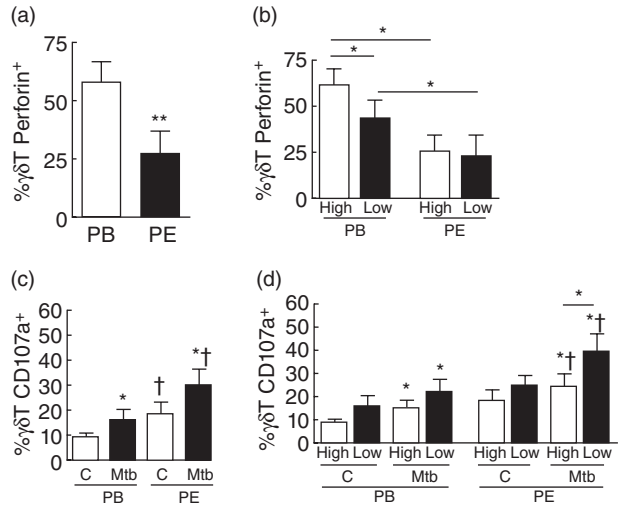

Fig. 5. Perforin and CD107a expression in γδT cells from peripheral blood (PB) and pleural effusion (PE) mononuclear cells cells. Perforin expression was determined *ex vivo* in total γδ T cell (a) or γδT cell receptor (TCR)^{high} and γδTCR^{low} cells (b) from PB and PE. Results are expressed as percentage of positive cells among γδT cell subpopulations. Statistical differences: high *versus* low: $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$. PB and PE were cultured for 18 h with or without *Mycobacterium tuberculosis* (*Mtb*). CD107a surface expression was determined on total γδT cells (c) or γδTCR^{high} and γδTCR^{low} (d) subpopulations. Results are expressed as percentage of positive cells. Statistical differences: PB *versus* PE, $\dagger P < 0{\cdot}05$; C *versus Mtb* or high *versus* low $^*P < 0{\cdot}05$.

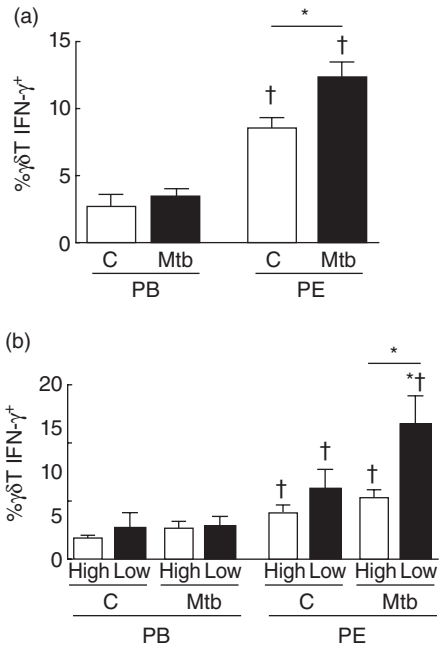

Fig. 6. Spontaneous interferon (IFN)-γ production by PE-γδT cells is increased upon *Mtb* stimulation. Peripheral blood (PB) and pleural effusion (PE) mononuclear cells were cultured for 18 h with or without *Mtb* and IFN-γ expression was evaluated in total γδT cells (a) or γδT cell receptor (TCR)^{high} and γδTCR^{low} (b) populations by flow cytometry. Results are expressed as percentage of positive cells. Statistical differences: PB *versus* PE, †$P < 0{\cdot}05$; control *versus Mtb* or high *versus* low *$P < 0{\cdot}05$.

IFN-γ is a key cytokine in the immune response against *Mtb*, and γδ T cells have been proposed as an early source for this cytokine; therefore, we evaluated IFN-γ production. While PB-γδT cells from healthy donors produced IFN-γ upon stimulation with *Mtb* [control = 2·9 ± 0·5%, *Mtb* = 8·3 ± 1·5%, mean ± standard error of the mean (s.e.m.), $n = 10$, $P < 0{\cdot}05$], PB-γδT cells from TB patients showed impaired IFN-γ production (Fig. 6a), in accordance with previous reports [37]. PE-γδT cells showed high percentages of both spontaneous and *Mtb*-induced IFN-γ production (Fig. 6a). Remarkably, these IFN-γ-producing cells belonged mainly to the γδTCR^{low} subset (Fig. 6b). In addition, while 28 ± 5% of Vδ2$^+$ cells were IFN-γ$^+$ with a ≈2·0-fold increase in expression (FITC-IFN-γ$^+$ MFI ± s.e.m.; control = 47 ± 17, *Mtb* = 98 ± 14), 9 ± 4% of vδ1$^+$ cells were IFN-γ$^+$ without a significant increment in MFI (PE-IFN-γ$^+$ MFI ± s.e.m.; control = 129 ± 39, *Mtb* = 158 ± 48, $n = 3$).

Discussion

In this study we have shown phenotypical and functional differences in circulating and recruited pleural fluid γδT cells from TB patients. Contradictory results have been reported in circulating γδT cell numbers, being increased or remaining constant during active TB disease [20,21,38,39], and their loss has been associated with the involvement of the CD95/CD95ligand apoptotic pathway [19,38,40]. In this study, we also found a lower percentage of γδT cells in PE than in its PB counterpart, but the decrease was not reflected in absolute numbers; this could be due to T cell enrichment in the pleural space. We have also shown that circulating γδT cells from TB patients have an activated phenotype, as reported previously [39]. The different proportions of NK and chemokine receptor-expressing cells between PB and PE, as well as the high percentage of CD69 and MHC class II markers in PE that we observed, may be ascribed to the microenvironment of the pleural effusion where cytokines/chemokines and antigens can modulate their expression [24,25].

Differences in the expression of CD16 [41], perforin [42] and MHC class II [43] have been employed to identify subpopulations of γδT cells and in this study we have demonstrated that CD3/TCR complex expression can also discriminate two subpopulations. CD3 expression was higher in γδTCR^{low} than in γδTCR^{high}, the former subset being the most representative in PB and PE. It has been proposed that after antigen encounter γδTCR is down-regulated to turn cells hyporesponsive [41] but, in our hands, what distinguishes high and low subsets appears to be a different CD3/γδTCR ratio. Although γδTCR^{low} and γδTCR^{high} were enriched in Vδ2 and Vδ1 cells, respectively, we were not able to assign any gene usage to each subset because only a partial overlap was found. In line with this differential distribution most Vδ1 cells were CD45RO-negative, resembling γδTCR^{high} cells, whereas most Vδ2 cells were CD45RO-positive, resembling the γδTCR^{low} subset.

Inflammatory and chemotactic mediators released by leucocytes or stromal cells are likely to represent a predominant mechanism, whereby the recruitment of cells is regulated tightly, to reach the site of *Mtb* infection. In particular, pleural mesothelial cells are responsible in part for initiating the inflammatory response by recruiting mononuclear cells from the vascular compartment into the pleural space through chemokine receptors and their ligands [44]. A weak CCR7 expression in *ex-vivo* γδT cells from healthy individuals has been found, the majority being CD45RO$^+$ cells [45], and stimulation with heat-killed extracts of *Mtb* down-modulates CCR5 expression on γδT cells [45]. γδTCR triggering induces early and transient CCR7 up-regulation, regarded as a marker of early activation in these cells, together with high CXCR3 expression [46]. We have also observed consistently a weak CCR7 and CXCR3 expression in *ex vivo* PB-γδT cells from TB and HS, although the majority were CD45RA$^+$ cells. In this context, it has been proposed that the CD45RA$^+$ marker can be lost upon antigen stimulation and reacquired afterwards in the absence of TCR triggering or homeostatic cytokines [47,48].

γδTCR^{high} from PB-TB displayed a CD45RA$^+$CCR7$^-$ CXCR3$^-$CD27^{low} phenotype but carried the largest amount of perforin; therefore they are not naive cells. In contrast,

γδTCR^{low} from PB-TB showed a higher percentage of CCR7$^+$ and CXCR3$^+$ as well as decreased CD45RA$^+$ cells, suggesting that they are activated and ready for extravasation. Also, PB-γδTCR^{low} carried lesser amounts of perforin and higher spontaneous CD107a expression, but upon *Mtb* stimulation neither γδTCR^{high} nor γδTCR^{low} from PB produced IFN-γ. Although PB-γδT cell anergy has been reported in TB and HIV patients [37], the absence of co-stimulatory signals along with the presence of systemic inhibitors in the periphery could also be the cause of their hyporesponsiveness.

Within the pleural space, *ex vivo* γδTCR^{high} and γδTCR^{low} cells showed the highest proportion of CXCR3 and CCR7 receptors, suggesting that they would be able to migrate to other sites of microbial infection as well as draining reactive lymph nodes. In line with this, a high proportion of CXCR3$^+$ cells has been reported in γδT and CD4$^+$ cells [24], which coincides with the high levels found in the tuberculous pleural fluid of IFN-γ-inducible ligands IP-10 and MIG [49,50]. In addition, both subsets showed decreased perforin content, suggesting that they have already exerted their cytotoxic effector function. Additionally, spontaneous IFN-γ production by high and low subsets was detected with a trend towards increased CD107a surface expression. Remarkably, upon *Mtb* stimulation, effector functions of both IFN-γ production and degranulation were exerted mainly by PE-γδTCR^{low}. This may be due in part to Vδ2 enrichment within γδTCR^{low}, which are known to respond to *Mtb* antigens [8,40]. Activation of Vγ9Vδ2 T cells is regulated by NK receptors such as the heterodimer CD94/NKG2A, that strongly inhibits the killing of MHC class I targets, and the NKG2D receptor that enhances γδT cell response by engagement to MHC class-I-related chains (MIC)A ligand [33,51]. It has been shown that in the absence of antigen, NKG2D$^+$ γδT cells do not lyse MICA$^+$ targets; however, in the presence of non-peptide antigens, MICA$^+$ targets are susceptible to lysis by γδT cells [33]. Bacterial infections up-regulate MICA expression on antigen-presenting cells (APC) enhancing TCR-dependent activation of γδT cells [33]. Accordingly, we have observed recently enhanced MICA but not HLA-class I expression on APC from PE [29], and in this study we have shown that NKG2D was expressed in the majority of γδT cells. Hence, MICA/NKG2D interaction may be involved in enhancing effector functions within PE-γδT cells [33]; however, the involvement of other innate receptors cannot be ruled out [52]. Consistent with the high effector functions of PE-γδTCR^{low} cells, this subset is mainly CD45RO$^+$. Thus, CD45RA might be lost in those cells infiltrating the site of infection where *Mtb* antigens are present, resulting in the phenotype observed in PE.

Our results suggest strongly that at the site of infection, recruited γδT cells from PB acquire a further activated phenotype displaying effector functions. As γδT cells within the pleural space produce IFN-γ, they would be expected to play a beneficial role in tuberculous pleurisy by helping to maintain a Th1 profile necessary for the resolution of infection. Whether high and low γδTCR belong to divergent lineages or correspond to the same subset with different activation status remains to be established.

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Disclosure

The authors have no conflicts of interest.

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