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Original Paper

Human pleural B-cells regulate IFN-γ production by local T-cells and NK cells in a *Mycobacterium tuberculosis*-induced delayed hypersensitivity reaction

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Abstract

DTH (delayed type hypersensitivity) reactions are secondary cellular immune responses that appear 24–72 h after antigen exposure. Tuberculous pleurisy is a common manifestation of extrapulmonary TB (tuberculosis) and is considered a human model of Th1-mediated DTH. In order to identify functional cross-talk among cellular populations sited in this inflammatory microenvironment, we analysed phenotypic and functional features of human B-cells isolated from the PF (pleural fluid) of TB patients. Freshly isolated PF-B-cells displayed a lower expression of CD20, CD1d and HLA-DR, and a higher expression of CD95, CD38, CD25, CXCR3 (CXC chemokine receptor 3) and CXCR4 (CXC chemokine receptor 4) than their PB (peripheral blood) counterparts, suggesting a non-classical *in situ* activation. Although memory PF-T-cell frequencies were increased, the frequencies of memory PF-B-cells were not. We demonstrated that, upon stimulation with γ-irradiated *M. tuberculosis*, mycobacterially secreted proteins or a lectin mitogen, PF-B-cells had a strong activation and produced IL-10 by a mechanism that was dependent on bystander activation of $CD19^-$ PF cells. Besides, within PF cells, B-cells diminished *in vitro M. tuberculosis*-induced IFN (interferon)-γ production by T-cells and NK (natural killer) cells in an IL-10-dependent manner. Finally, we found that the lower the frequency of B-cells, the higher the ratio of IFN-γ/IL-10 within PF. Thus our results suggest that B-cells can regulate a human DTH reaction induced by *M. tuberculosis*.

Key words: B-cell, delayed type hypersensitivity (DTH), interleukin 10 (IL-10), Th1 profile, tuberculous pleurisy

INTRODUCTION

TB (tuberculosis) is the leading cause of death from a single infectious pathogen [1]. Although the role of T-cell-mediated immunity in the containment of *Mycobacterium tuberculosis* is well known, the role of B-cells in the host defence against *M. tuberculosis* has not been clearly defined yet. In this regard, there is growing clinical and experimental evidence for an active role of B-cells in intracellular infections [2,3]. In fact, different studies have shown contradictory results regarding the frequency of B-cells in PB (peripheral blood) of TB patients [4,5]. Nevertheless, studies on human tuberculous lung tissue have identified dominant structures of follicle-like B-cells, suggesting that the cross-talk between B- and T-cells may be critical for the containment of *M. tuberculosis* [6,7].

Among the clinical manifestations of extrapulmonary TB, pleurisy is the most common form of primary TB associated with young adults, but may also develop as a complication of primary pulmonary TB, which is considered a relatively benign form of the disease since it may resolve without chemotherapy [8]. The presence of mycobacterial antigens in the pleural space elicits an intense cellular immune response initially characterized

Abbreviations: ADA, adenosine deaminase; BCR, B-cell receptor; CCR, CC chemokine receptor; CD40L, CD40 ligand; CD62L, CD62 ligand; CFP, culture filtrate protein; CXCR, CXC chemokine receptor; Cy5, indodicarbocyanine; DC-SIGN, dendritic cell-specific intracellular adhesion molecule-3 grabbing non-integrin; DTH, delayed type hypersensitivity; IFN, interferon; IL, interleukin; IL-10R, IL-10 receptor; mAb, monoclonal antibody; MFI, mean fluorescence intensity; NK, natural killer; PB, peripheral blood; PF, pleural fluid; PMN, polymorphonuclear cell; PWM, poke weed mitogen; rh, recombinant human; TB, tuberculosis; TLR, Toll-like receptor.

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Table 1 Clinical and demographic heterogeneity of PF samples

PF samples from TB ($n = 55$) and non-TB ($n = 6$) patients were characterized. The number of samples (n) or years of age [median (minimum–maximum)] are indicated. PF samples were classified as exudates or transudates following Light's criteria [17]. Clinical presentation of TB was based on X-ray findings. PF cytology was performed by microscopic examination of MGG (May–Grünwald)-stained samples. Classification of PF as 'lymphocytic' or 'atypical' was determined according to Cheng et al. [18]. TB patients were stratified as function of days after initiation of antibiotic treatment. For the cancer patients, one had an adenoma, one had a mesothelioma and three were of undetermined origin.

Parameter	PF samples from TB patients	PF samples from non-TB patients
Sex (n)		
Male	45	5
Female	10	1
Age (years)	31 (19–60)	41 (29–63)
Light's criteria (n)		
Exudate	55	6
Transudate	0	0
Clinical presentation (n)		
Pleural	43	–
Pleuropulmonary	12	–
PF cytology (n)		
Lymphocytic	49	2
Atypical	6	4
Antibiotic treatment (n)		
0 day	29	–
<10 days	18	–
>10 days	8	–
Aetiology (n)		
Cancer¶	–	5
Hydatid cyst	–	1

by abundant PMNs (polymorphonuclear cells) and MΦs (macrophages), followed by NK (natural killer) cells, γ/δ T-cell and Th cells, together with accumulation of their soluble mediators [9–16]. This local inflammatory response is considered as a DTH (delayed type hypersensitivity) reaction in which $CD4^+$ T-cells are the major effector population, making tuberculous pleurisy a relevant model for studying human DTH reactions [12,17].

In the present study, we determined the phenotype and function of B-cells from the PF (pleural fluid) and PB of TB patients in order to better understand their role at the site of the *M. tuberculosis*-induced human DTH reaction.

MATERIALS AND METHODS

Patients and healthy blood donors

Patients with newly diagnosed moderate and large pleural effusions were identified at the Servicio de Tisioneumonología, Hospital F.J Muñiz, and the Instituto Vaccarezza Buenos Aires, Argentina from 2007 to 2012. The research has been carried out in accordance with the Declaration of Helsinki (2013) of the World Medical Association, and has been approved by the Ethics Committees of both of the institutions mentioned above. Written informed consent was obtained before sample collection. Physical examination, complete blood cell count, electrolytes, chest X-ray, HIV test, ADA (adenosine deaminase) and LDH (lactate dehydrogenase) in PF were performed by the institutions. The diagnosis of tuberculous pleurisy was based on a positive Ziehl–Nielsen stain or Lowestein–Jensen culture from PF and/or histopathology of pleural biopsy, and was confirmed further by an *M. tuberculosis*-induced IFN (interferon)-γ response [15] and an ADA-positive test [8]. None of the patients had multidrug-resistant TB. Exclusion criteria included a positive HIV test or the presence of concurrent infectious diseases. PF and PB samples were obtained as described previously [16].

Table 1 provides details about the demographics, Light's criteria [17], PF cytology [18], antibiotic treatment and clinical presentation of the patients and controls. Buffy coats from healthy donors were obtained from the Blood Transfusion Service, Hospital Fernandez, Buenos Aires, Argentina.

Mononuclear cells

PF and PB were collected into 50 ml polystyrene tubes (Corning) containing heparin. PBMCs (PB mononuclear cells) and PFMCs (PF mononuclear cells) were isolated by Ficoll–Hypaque gradient centrifugation (Pharmacia) and were suspended in complete medium [RPMI 1640 (Gibco) containing gentamycin (85 μg/ml; Gibco), penicillin (100 units/ml; Sigma) streptomycin (100 μg/ml; Sigma) and 10% heat-inactivated FBS (Gibco)]. Cells numbers, purity and viability were determined using the exclusion dye Trypan Blue.

Antigens

The γ-irradiated *M. tuberculosis* H37Rv strain and CFPs (culture filtrate proteins) were kindly provided by J. Belisle (Colorado State University, Fort Collins, CO, U.S.A.). Mycobacteria were suspended in pyrogen-free PBS, sonicated and adjusted to a concentration of $\approx 10^8$ bacteria/ml ($D_{600\,nm} = 1$).

Reagents

Human T memory-Activator Dynabeads® coated with anti-human CD3, CD28 and CD137 were from Invitrogen. PMA, ionomycin and PWM (poke weed mitogen) were from Sigma. rh (recombinant human) IL (interleukin)-10, rhIL-21 and rhIL-4, neutralizing azide-free purified rabbit polyclonal anti-IL-10, anti-IL-21 and anti-IL-4 were from Peprotech. As a control in the neutralization experiments, Protein A-purified rabbit IgG from pre-immune serum was used (kindly provided by M. Izturiz, Academia Nacional de Medicina, Buenos Aires, Argentina). The purified anti-CD19 mAb (monoclonal antibody) was from Becton Dickinson. FITC-, PE- (phycoerythrin), PE–Cy5- (indodicarbocyanine), PerCP–Cy5.5 or APC (allophycocyanin)-conjugated mAbs against CD62L (CD62 ligand), CD80, CD86, TLR (Toll-like receptor) 2, TLR4, HLA-DR and CD1d were from e-Bioscience. CD32, PD-L1 and PD-L2 were from Becton Dickinson. Mannose receptor, DC-SIGN (dendritic cell-specific intracellular adhesion molecule-3 grabbing non-integrin), CCR (CC chemokine receptor) 1, CCR2, CCR5, CXCR (CXC chemokine receptor) 3, CXCR4 and CX_3CR1 (CX_3C receptor 1) were from R&D Systems. CD3, CD69, CD19 and CD5 were from Biolegend. CD20, CD25 and MHC-II were from Ancell. IFN-γ and IL-10 were from Caltag, and were used along with an isotype-matched mAb.

PF-B-cell isolation and depletion

PF-$CD19^+$ cells were positively selected by pre-incubation of $\sim 2\times10^7$ PFMCs at 4 °C for 30 min with anti-CD19 mAb. Then, magnetic selection of PFMCs was performed using goat anti-(mouse IgG)-coated MACS (magnetic-activated cell sorting) (Miltenyi Biotec), according to the manufacturer's instructions. This procedure yielded less than 5% of $CD3^+/CD56^+/CD14^+$ cells in PF-$CD19^+$ and less than 0.5% of B-cells in PF-$CD19^-$, as determined by FACS analysis.

In-vitro stimulation of PBMCs, PFMCs, PF-$CD19^+$ and PF-$CD19^-$

PBMCs, PFMCs and PF-$CD19^+$-depleted mononuclear cells (PF-$CD19^-$) from TB patients were cultured at 10^6 cells/ml in complete medium. Given their low numbers per sample, purified PF-$CD19^+$ cells were cultured at 10^5 cells/ml. Mitogen, *M. tuberculosis* antigens and bead concentrations, as well as the stimulation time (48 h), were chosen according to our previous studies [10,16,19]. Where indicated, PF-$CD19^-$ cells were mixed with PF-$CD19^+$ cells setting their relative ratio to 10:1 ($10^6/10^5$ cells/ml) to reach their relative *in vivo* frequencies. For intracellular cytokine determinations, cells were cultured in polystyrene round-bottomed tubes (Falcon™; Becton Dickinson) at a final volume of 1 ml and Brefeldin A (5 μg/ml; Sigma) was added 4–6 h before staining. For cytokine secretion assays, cells were cultured in 96-well round-bottomed microplates (Falcon™) at final volume of 0.2 ml for 48 h. As positive control experiments, PMA/ionomicyn were added overnight. Finally, plates were centrifuged 10 min at 300 ***g*** before harvesting the supernatants for ELISA.

Flow cytometry analysis for cell-surface and intracellular protein expression

Surface marker expression was evaluated on freshly isolated or cultured PBMCs and PFMCs by staining $(0.1–1)\times10^6$ cells for 20 min at 4 °C with adequate amount and combinations of labelled mAb in ~50 μl volume of PBS/1% FCS. For intracellular cytokine determinations, after surface staining, as indicated above, cells were fixed, permeabilized and stained with FITC-conjugate anti-IFN-γ or PE-conjugated anti-IL-10 (IntraPrep™; Immunotech), according to the manufacturer's instructions. Data acquisition [at least $(0.5–5)\times10^5$ events] was carried out using FACScan or FACScalibur flow cytometers both equipped with CellQuest software (Becton Dickinson). For data analysis, we defined electronically a region that included PF and PB lymphocyte/lymphoblast (R1, see Figure 3A), according to forward and side-scatter parameters using the FCS Express V3 program (De Novo Software™). B-cells were defined as R1-gated events with an $CD19^+CD3^-$ phenotype, and NK cells and T-cells as $CD56^+/CD3^-$ and $CD3^+$ events respectively.

Cytokine ELISA

IL-10 and IFN-γ were determined in cell-free PF and culture supernatants, following the manufacturer's recommendations. Samples were stored at -70 °C until the assays were performed. PF was diluted 1:10 and culture supernatants 1:20 for IFN-γ determinations. For IL-10 determinations, no sample dilutions were performed. When different cell numbers were used, the cytokine production was normalized to cell numbers for each condition.

Statistical analysis

Comparisons of paired PBMCs and PFMCs and cell-free PF samples were performed using the Wilcoxon (non-parametric) pairwise test. Comparisons of non-paired data (i.e. normal compared with patients' data or between different groups of patients) were performed using an unpaired Mann–Whitney *U* test (non-parametric). For multiple comparisons among the *in vitro* treatments, one-way ANOVA for repeated measures with a Bonferroni's post-hoc test was applied. A Spearman two-tailed (non-parametric) test was used for correlation analysis. A $P < 0.05$ value was taken as significant.

RESULTS

Frequency and memory status of B-cells remain invariant between PB and PF

It has been demonstrated that the frequencies of $CD4^+$ T-cells are increased in tuberculous PF, whereas PMNs, NK cells and monocytes are decreased compared with PB [9–11,13–16]. Thus, to determine the B-cell frequency in both compartments, freshly

isolated PBMCs and PFMCs derived from patients with pleurisy (TB and no-TB), as well as PBMCs from healthy subjects, were stained with anti-CD19 and anti-CD3 mAbs and analysed by FACS. $CD19^+$ lymphocyte/lymphoblast cells were considered as B-cells. We found that B-cell frequencies between PF- and PB-TB and no-TB did not differ, as the proportions of PB-B-cells and PF-B-cells were similar to PB-B-cells from healthy subjects (Figure 1A).

As T-cell effector memory recruitment is a hallmark of tuberculous pleurisy [9], we investigated whether memory B-cells were also accumulated in PF-TB. Therefore the co-expression of surface IgD and CD27 was determined in $CD19^+$-gated PBMCs and PFMCs from TB patients, as well as in PB-MCs from healthy subjects, allowing the characterization of naïve B-cells ($IgD^+/CD27^-$), non-switched memory B-cells ($IgD^+/CD27^+$), switched memory B-cells, human B1 cells and plasma cells ($IgD^-/CD27^+$), and a double-negative cell population ($IgD^-/CD27^-$), which includes a recently described $CD27^-$ memory subset and a subset of plasma cells [20–23]. As shown in Figure 1(B), although the frequencies of memory or naïve B-cells did not differ between the PB and PF from TB patients, a reduction in non-switched memory PB-B-cells and an increase in double-negative PF-B-cells were observed in TB patients compared with PB from healthy subjects. In addition, the frequencies of switched memory B-cells ($CD27^+IgD^-$ or $CD27^+IgM^-$) were similar in PB- and PF-TB (Figure 1B), which was in contrast with the increased memory-like phenotype of PF-T-cells ($CD3^+/CD45R0^+/CD45RA^{low/-}$) from the same PF-TB samples (Figure 1C).

PF-B-cells enhance homing molecules and display an activated phenotype

Next, we tested whether freshly isolated PB-B-cells and PF-B-cells express a differential pattern of homing receptors. Among the chemokine receptors assessed, the percentages of $CXCR3^+$ cells and CXCR4 MFI (mean fluorescence intensity) were increased in PF-B-cells (Figure 2A), whereas no significant differences in CCR1, CCR2, CCR5 and CX_3CR1 expression were detected (results not shown). Then, we determined whether B-cells were also activated in PF, as in other pleural cell populations [9–16]. Compared with PB-B-cells, PF-B-cells had higher CD95/Fas, CD38, CD25 and CD45R0 expression and lower CD32 and CD1d expression (Figure 2B), indicating B-cell activation [24–26]. On the other hand, several molecules known to be augmented in BCR (B-cell receptor)-activated B-cells were not modified on PF-B-cells (i.e., CD62L, CD69, CD80, CD86, PD-L1, PD-L2 and CD5; Figure 2B). Similarly, the expression levels of the pattern-recognition receptors TLR2, TLR4, mannose receptor and DC-SIGN were undetectable in PB-B-cells or PF-B-cells (results not shown). Interestingly, the expression of the B-cell marker CD20 was lower in PF-B-cells than in PB-B-cells (Figure 2C), which is in accordance with the down-regulation of this protein upon B-cell activation and/or differentiation [24]. In addition, HLA-DR levels were reduced in PF-B-cells without a concomitant reduction in whole MHC-II molecules (Figure 2D). Taken together, these results indicate that the environment of tuberculous pleurisy induces a non-classical activation phenotype on B-cells.

Mycobacterial antigen-induced PF-B-cell activation is dependent on PF bystander-activated $CD19^-$ cells

Considering that PF-B-cells from TB are pre-activated, we investigated whether they respond differentially to *ex vivo* stimulation compared with PB-B-cells. Thus paired samples of PBMCs and PFMCs were stimulated with PWM or *M. tuberculosis* antigens, such as irradiated bacteria (H37Rv strain) or CFPs, and the CD69 activation marker was determined within the $CD19^+$ or $CD19^-$ gates. We found that PWM and *M. tuberculosis* antigens induced CD69 in $CD19^+$ cells from PBMCs and PFMCs, with this activation being higher in PFMCs and more pronounced following stimulation with *M. tuberculosis* antigens (Figure 3A). Furthermore, as shown in the representative dot plots (Figure 3A), PWM induced CD69 expression in $CD19^-$ cells from PB and PF, whereas *M. tuberculosis* antigens induced CD69 expression only in PF-$CD19^-$ cells. Therefore we determined whether the strong activation of PF-B-cells upon stimulation with *M. tuberculosis* antigens might be a bystander cell effect instead of BCR-mediated antigen recognition. To address this, PBMCs and PFMCs were incubated with beads coated with anti-human CD3, CD28 and CD137 that activate effector memory T-cells (1:10 bead/cell ratio) and the frequencies of $CD69^+$ cells were analysed within the B-cell gate. T-cell-activator beads induced CD69 expression in both PB-B-cells and PF-B-cells, which was greater in PFMCs (Figure 3A), indicating that T-cell activation is sufficient to induce PF-B-cell activation to similar levels as the stimuli did. Moreover, $CD19^-$ cells from PBMCs and PFMCs were activated by T-cell-activator beads, but PF-$CD19^-$ cells had a more efficient activation (Figure 3A). Interestingly, when *ex vivo* stimulation was performed using $CD19^+$-purified PFMCs, neither PWM nor *M. tuberculosis* antigens induced significant levels of CD69 compared with PMA/ionomycin (Figure 3B). Therefore the fact that PF-B-cells are only activated by *M. tuberculosis* antigens in the presence of PF-$CD19^-$ cells and that PF-$CD19^-$ cells are activated by those stimuli indicate that $CD19^-$ cells may contribute to the activation of B-cells by a bystander-cell-dependent mechanism.

IL-10 production in PF-B-cells induced by mycobacterial antigens is dependent on PF-$CD19^-$ cells

Given that activated B-cells can exert immunoregulatory functions through the secretion of certain cytokines [2,26–32], the intracellular expression of IL-10 was evaluated in PFMCs stimulated with PMW or *M. tuberculosis* antigens within the $CD19^+$ and $CD19^-$ cell gates. We found that, upon stimulation for 48 h, the frequencies of $IL\text{-}10^+$ cells increased in PF-B-cells and in PF-$CD19^-$ cells, which were higher in the former cells (Figure 4A). As B-cells are at a low proportion in PFMCs, we assessed their contribution to the overall IL-10 production. Therefore total PFMCs, $CD19^+$-depleted PFMCs and $CD19^+$-purified PFMCs were stimulated as indicated above, and IL-10 secretion was measured by ELISA. PFMCs secreted high amounts of IL-10

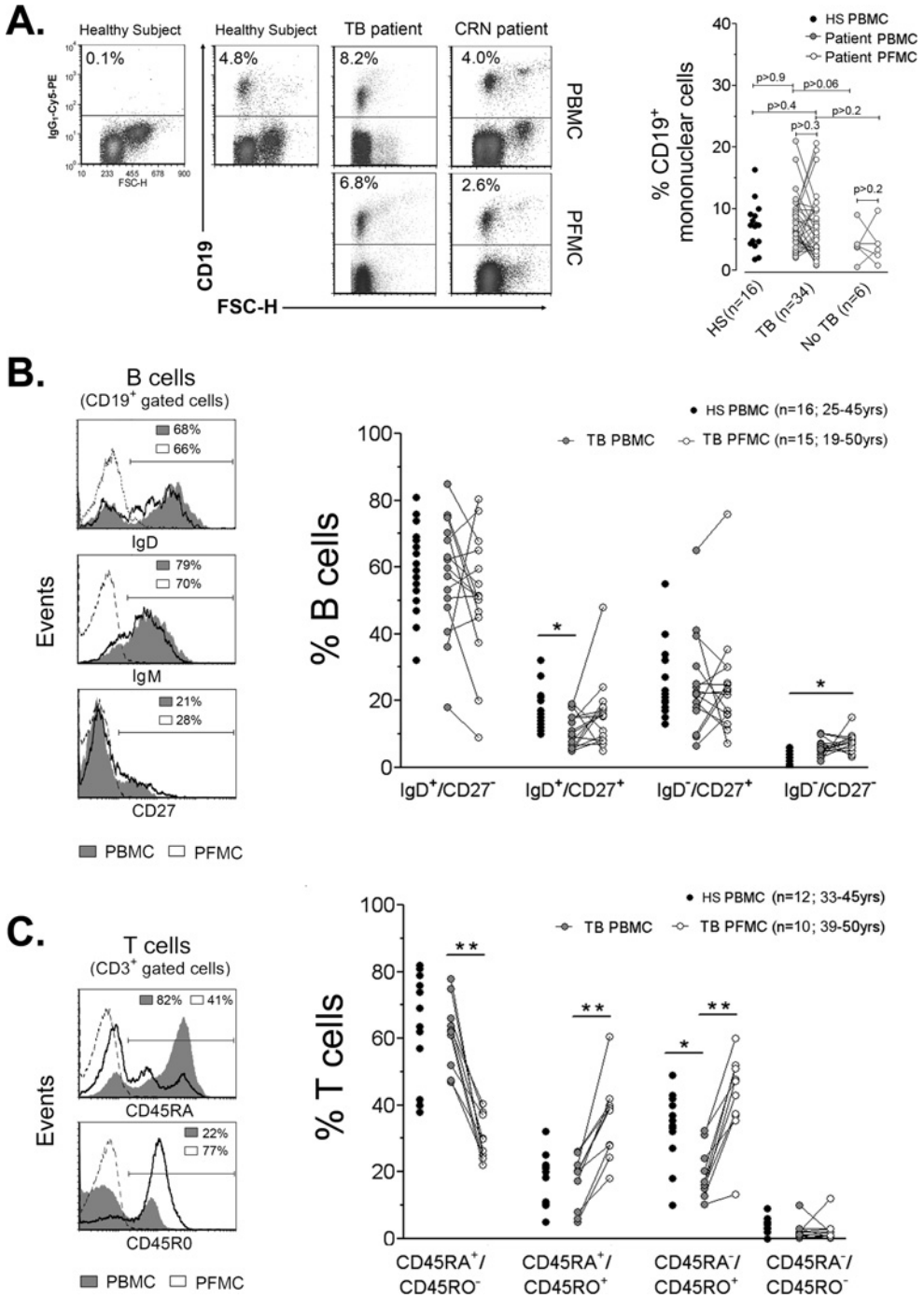

Figure 1 **B-cell frequency and differentiation stage in PBMCs and PFMCs**

Percentages of $CD19^+$, B-cells and T-cells expressing naïve/memory markers were determined in PBMCs and PFMCs from patients with TB and with non-tuberculous pleural exudates (No TB), as well as PBMCs from healthy subjects (HSs). (**A**) Percentage of $CD19^+$ cells within PBMCs and PFMCs from TB ($n = 34$) and No-TB ($n = 6$), as well as of PBMC from healthy subjects ($n = 16$). None of these groups were statistically different. (**B**) Percentage of B-cells ($CD19^+$ within the lymphocyte/lymphoblast gate) expressing the B-cell naïve/memory markers (IgD, IgM and CD27) in PBMCs and PFMCs from TB patients ($n = 15$), as well as in PBMCs from healthy subjects ($n = 16$). $^*P < 0.05$ compared with TB-PBMC $IgD^+/CD27^+$ and TB-PFMC $IgD^-/CD27^-$ (Mann–Whitney U test). (**C**) Percentage of T-cells ($CD3^+$ lymphocyte/lymphoblast gate) expressing CD45RA and CD45RO in PBMCs and PFMCs from TB patients ($n = 19$), as well as in PBMC from healthy subjects ($n = 12$). $^*P < 0.05$ compared with TB-PBMCs for $CD45RA^-/CD45R0^+$ (Mann–Whitney U test); $^{**}P < 0.005$ compared with TB-PFMCs (Wilcoxon matched pair test). In (**B** and **C**), dark grey histograms correspond to PBMCs, empty histograms to PFMCs and dashed histograms to control isotype. Ages range are indicated.

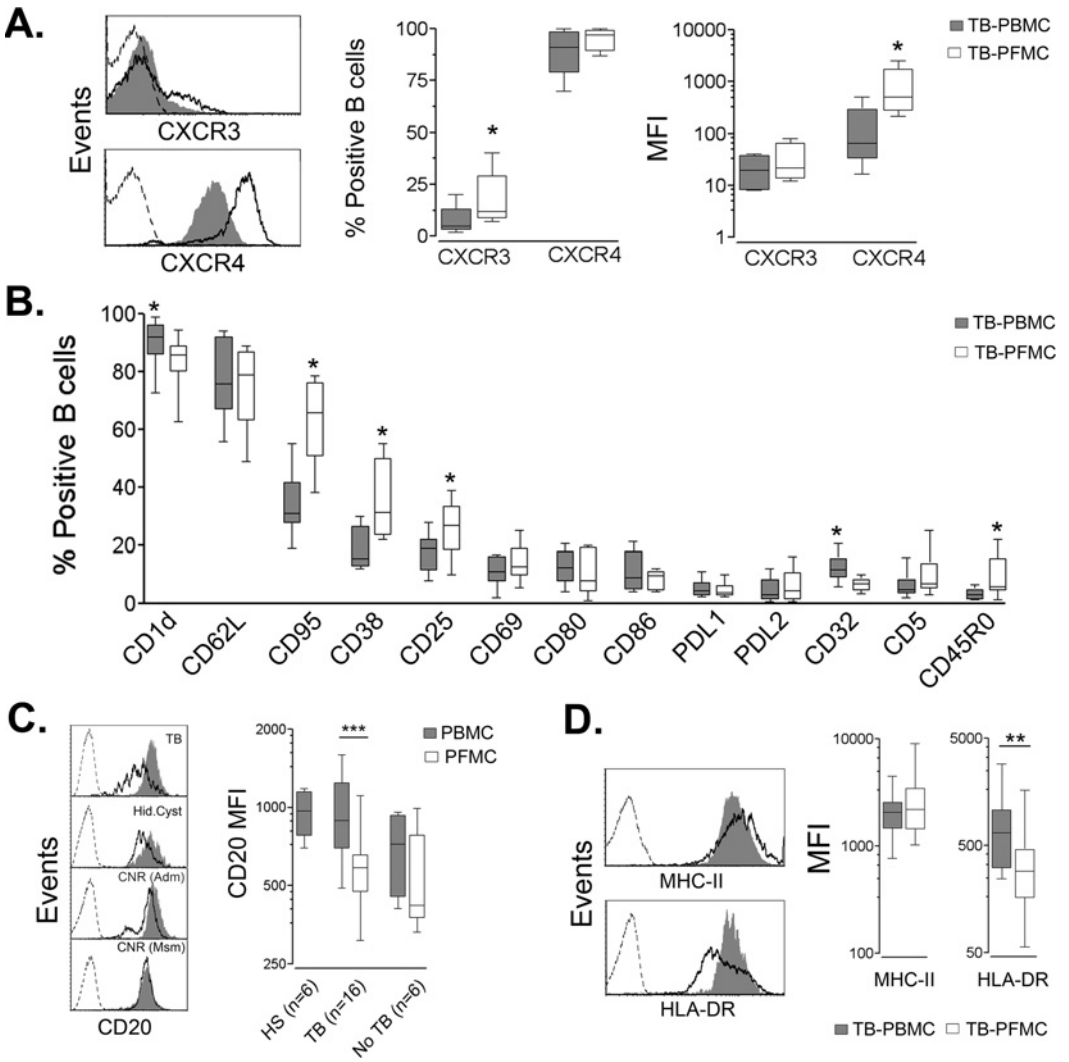

Figure 2 **B-cell expression of homing receptors and activation markers in PBMCs and PFMCs**

Expression and percentage of B-cells positive for the indicated molecules were determined in PBMCs or PFMCs. FACS analysis was performed in gated $CD3^-/CD19^+$ lymphocytes/lymphoblast (B-cells). (**A**) Percentage of positive B-cells and MFI for CXCR3 and CXCR4 in paired TB samples (box and whiskers plots indicate the median, 25–75% percentile and range, $n = 8$). Broken line indicates the isotype control. (**B**) Percentage of B-cells expressing the indicated cell markers in TB patients ($n = 10$). (**C**) CD20 MIF in B-cells from TB ($n = 16$), No-TB ($n = 6$) patients and healthy subjects ($n = 6$). Representative traces in a TB patient and three No-TB patients [Hyd.Cyst, hidatidosis; CNR (Adm), adenoma cancer; CNR (Msm), mesothelial cancer]. (**D**) MHC-II and HLA-DR MIF in B-cells of paired samples from TB patients ($n = 10$). $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.0005$ compared with TB-PBMCs (Wilcoxon paired test).

upon stimulation with PWM or *M. tuberculosis* antigens, which were reduced by depletion of $CD19^+$ cells (Figure 4B), indicating a significant contribution of PF-B-cells to the overall IL-10 production. Besides, $CD19^+$-purified PFMCs did not produce IL-10 upon stimulation with PWM or *M. tuberculosis* antigens, but they did produce high levels of IL-10 upon PMA/ionomycin stimulation, showing that $CD19^+$-purified PFMCs are able to secrete IL-10, which rules out a purification process artefact. Therefore IL-10 production induced by *M. tuberculosis* antigens in PF-B-cells involves the presence of activated $CD19^-$-PF cells, as we have shown for B-cell activation. Next, we investigated the involvement of T-cell-co-stimulation ligands [CD40L (CD40 ligand)] or Th-derived soluble factors (IL-21, IL-4 and IL-13) in IL-10 secretion by PF-B-cells [25–27,33]. Our results showed that the inhibition of these molecules did not modify the *M. tuberculosis*-induced IL-10 production by PFMCs and, furthermore, addition of IL-21 and/or IL-4 did not induce IL-10 secretion in PFMCs (Figure 4C, and results not shown).

Frequency of PF-B-cells inversely correlates with the IFN-γ/IL-10 ratio in cell-free PF

Thus far, our results have indicated that *ex vivo*-activated PF-B-cells may be a significant source of IL-10, a homoeostatic cytokine that regulates Th1 inflammatory responses. Given that tuberculous pleurisy is a well-characterized example of DTH, we investigated the correlation of the frequency of B-cells in the PF

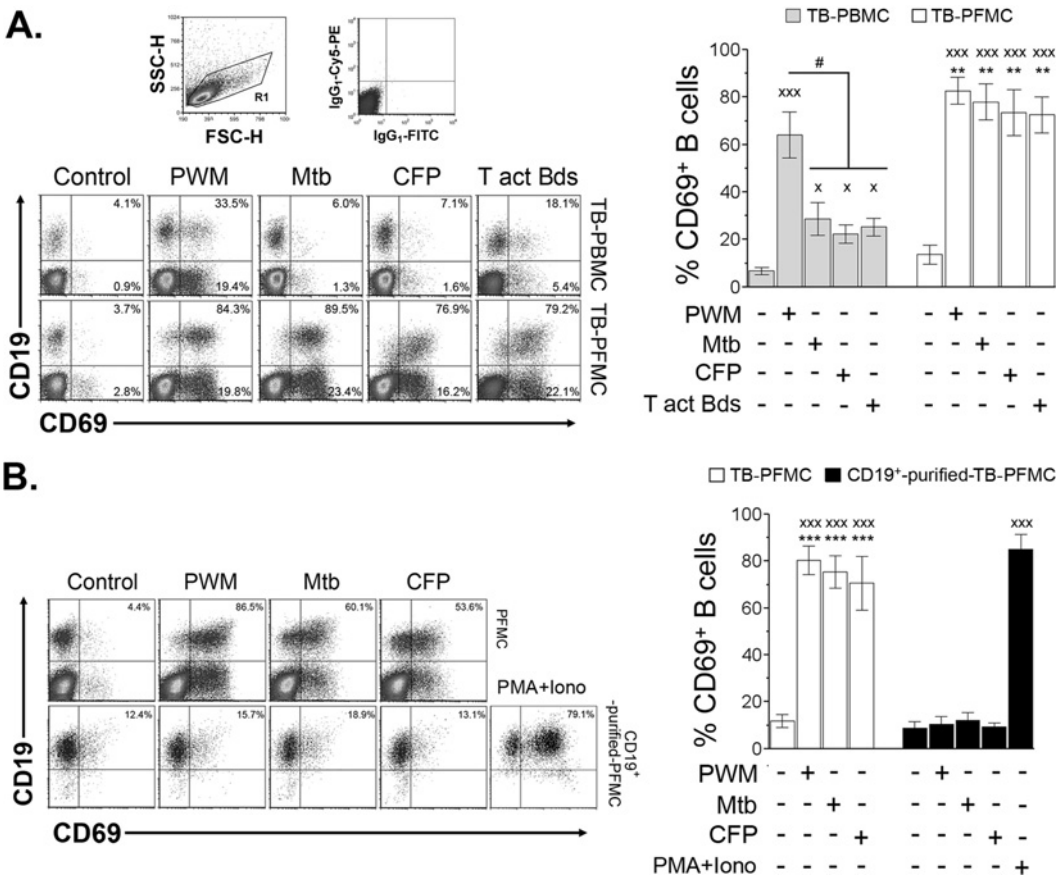

Figure 3 ***In vitro*** **B-cell activation in TB-PBMCs, TB-PFMCs and CD19$^+$-purified TB-PFMCs**

PBMCs, PFMCs or CD19$^+$-purified PFMCs from TB patients were cultured alone (Control) or stimulated with PWM (1 μg/ml), *M. tuberculosis* (Mtb; 10:1 bacteria/cell ratio), CFP (1 μg/ml), T-memory activator beads (1:10 T act Bds/cell ratio) or PMA (10 ng/ml) plus ionomycin (1 μg/ml) (PMA + Iono) for 48 h and CD69 expression was determined. (**A**) left-hand panel, representative dot plots showing cellular activation in lymphocyte/lymphoblast-gated cells (R1). Numbers in the upper-right quadrants indicate the percentage of CD69$^+$ B- (CD19$^+$) cells and the numbers in the lower-right quadrants indicate the percentage of CD69$^+$ non-B- (CD19$^-$) cells. Right-hand panel, quantification of the percentage of CD69$^+$ cells in gated CD19$^+$ lymphocyte/lymphoblast cells. Values are means $\pm$ S.E.M., $n = 10$. $^{x}P < 0.05$ and $^{xxx}P < 0.005$ compared with the control; $^{\#}P < 0.05$ compared with the other treatments; $^{**}P < 0.01$ compared with the same treatment in TB-PBMCs. (**B**) TB-PFMCs or CD19$^+$-purified TB-PFMCs were stimulated as indicated above. Left-hand panel, representative dot plots of CD69 and CD19 expression in TB-PFMCs and CD19$^+$-purified TB-PFMCs. Numbers in upper right quadrants indicate the percentage of CD69$^+$ B-cells. Right-hand panel, quantification of the percentage of CD69$^+$ B-cells in TB-PFMCs and CD19$^+$-purified TB-PFMCs. Values are means $\pm$ S.E.M., $n = 10$. $^{xxx}P < 0.005$ compared with the control; $^{***}P < 0.005$ compared with the same treatment in CD19$^+$-purified PFMCs (repeated-measures one-way ANOVA, Bonferroni post-hoc test).

from TB patients with the cytokine profile present in the same sample. Despite not finding any significant associations with clinical data or cytological findings (see Supplementary Figure S1 at http://www.clinsci.org/cs/127/cs127ppppadd.htm), we observed that the levels of IL-10 and IFN-γ were directly correlated within PF from TB (Figure 5A). Interestingly, the percentage of CD19$^+$ cells on PFMCs was inversely correlated with IFN-γ, but not with IL-10 (Figure 5A). More importantly, the IFN-γ/IL-10 ratio, which is indicative of the pro-/anti-inflammatory cytokine balance, was inversely correlated with PF-B-cell frequencies (Figure 5B).

Previous studies have shown an association between the regulatory functions of human B-cells with CD19$^+$/CD5$^+$/CD1d$^{+/hi}$ and/or with CD19$^+$/CD24^{hi}/CD38^{hi} phenotypes [30–32]. Consistent with this, we observed that the percentage of CD38^{hi} ($n = 8$) or CD1d^{hi} ($n = 10$) in PF-B-cells was not increased compared with PB-B-cells in TB patients (results not shown). In addition, no significant differences were found in the percentage of CD19$^+$/CD5$^+$/CD1d$^+$ between PF-B-cells and PB-B-cells ($P > 0.09$; $n = 10$). Furthermore, when the percentage of CD19$^+$/CD5$^+$/CD1d$^+$ PFMCs was correlated with the level of IL-10 present in PF, no statistical relationship was observed (Figure 5B), suggesting that PF-B-cells with the conventional 'B reg' phenotype are not quantitatively related to the PF cytokine profile. In addition, it has been shown recently that plasmablast/plasma cells, besides their conventional function as antibody-secreting cells, are able to produce a variety of cytokines regulating cellular responses [34]. In this regard, we correlated the

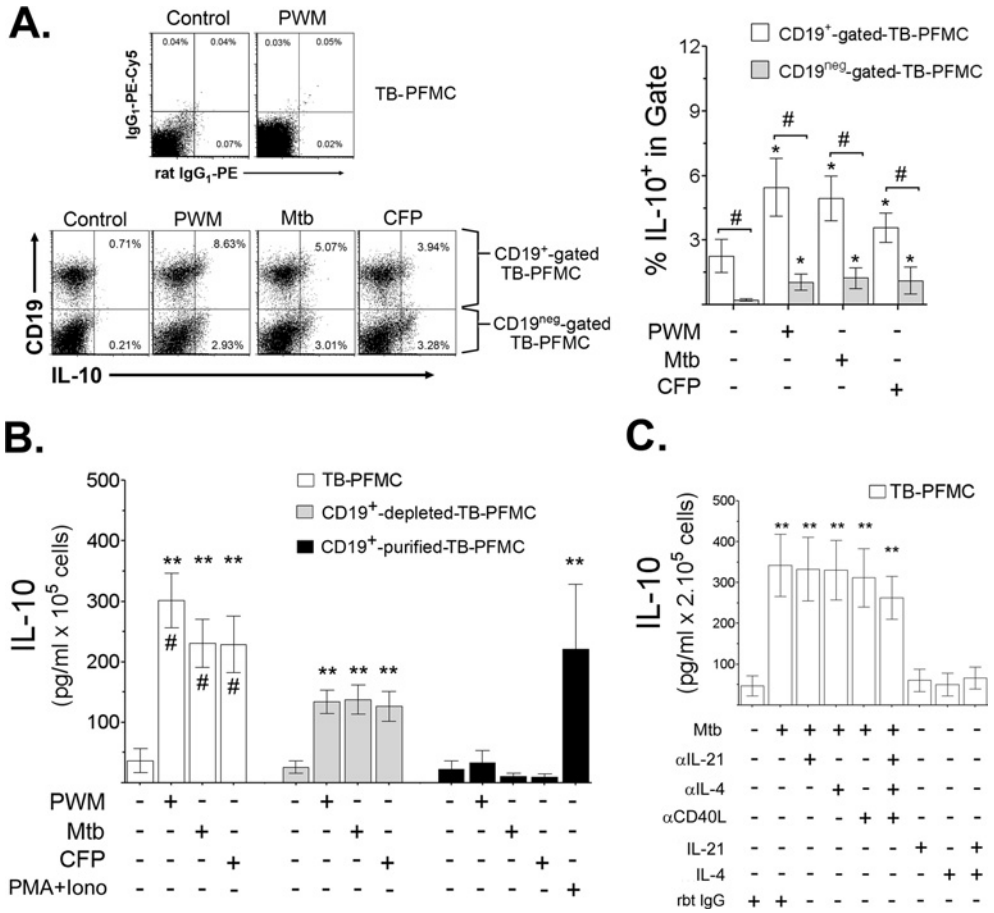

Figure 4 **IL-10 production by PF-B-cells from TB patients**

PFMCs (10^6/ml), $CD19^+$-depleted-PFMCs (10^6/ml) or $CD19^+$-purified PFMCs (10^5/ml) from TB patients were cultured in complete medium alone (control) or with the treatments as indicated in Figure 3. (**A**) left-hand panel), representative dot plots showing surface CD19 and intracellular IL-10 expression in lymphocytes/lymphoblast gates from TB-PFMCs. Numbers in upper-right quadrants indicate the percentage of $IL\text{-}10^+$ on $CD19^+$ and the numbers in the lower-right quadrants indicate the percentage of $IL\text{-}10^+$ on $CD19^-$ cells. Isotype controls are also depicted. Right-hand panels, quantification of the percentage of IL-10 positive cells in the $CD19^+$ cells gate ($CD19^+$-gated-TB-PFMC) and in $CD19^-$ cells from TB-PFMC ($CD19^-$-gated-TB-PFMC). Values are means $\pm$ S.E.M. ($n = 10$). $^*P < 0.05$ compared with control; $^\#P < 0.05$ (repeated-measures one-way ANOVA, Bonferroni post-hoc test). (**B**) IL-10 production was determined by ELISA. Data were normalized to equivalent cell numbers and are shown as the means $\pm$ S.E.M. ($n = 12$ $^{**}P < 0.01$ compared with control; $^\#P < 0.05$ compared with the same treatment in $CD19^+$-depleted TB-PFMCs. (**C**) TB-PFMCs ($n = 11$) were stimulated with *M. tuberculosis* in the presence of neutralizing antibodies (α) against hIL-21 (5 μg/ml), IL-4 (5 μg/ml) and CD40L (5 μg/ml), or rhIL-4 and/or rhIL-21 (10 ng/ml). Statistical differences: treatment vs control: $^{**}P < 0.01$ compared with control (repeated-measures one-way ANOVA, Bonferroni post-hoc test). Purified rabbit IgG (rbt IgG; 2 μg/ml) used as an antibody control did not modify *M. tuberculosis*-induced IL-10 production.

amount of PF cytokines with the presence of PF antibodies that recognize a variety of *M. tuberculosis* antigens. In our hands, antibodies directed to protein antigens [i.e., anti-PPD (purified protein derivate), -HSP10KD (10 kDa heat-shock protein) or -Rv3763] or non-protein antigens [i.e., anti-mannan, -ManLAM (mannose capped lipoarabinomannan) or -total lipid extract] were not correlated with the amounts of IL-10 or IFN-γ in PF from TB patients ($n = 27$, $P > 0.05$; see Supplementary Figure S2A at http://www.clinsci.org/cs/127/cs127ppppadd.htm). Moreover, when we analysed the percentage of plasmablast/plasma cells in TB-PFMCs, we did not observe any difference with respect to PBMCs from TB patients or health subjects ($n = 16$, $P > 0.05$; see Supplementary Figure S2B).

Down-modulation of *M. tuberculosis*-induced-IFN-γ production by PF-B-cells depends on IL-10

Next, we investigated whether PF-B-cells modulated a previously characterized *M. tuberculosis*-evoked *ex vivo* response [15,16]. To assess this, IFN-γ production was measured in PFMCs and $CD19^+$-depleted PFMCs stimulated with *M. tuberculosis* antigens. $CD19^+$-depleted PFMCs produced higher amounts of IFN-γ than PFMCs (Figure 6A), suggesting that PF-B-cells regulate the Th1 response. It is worth noting that, as the ELISA data were normalized to identical numbers of $CD19^-$ cells in each condition, the increase in IFN-γ production by $CD19^+$-depleted PFMCs cannot be explained by the enrichment of IFN-γ-producing populations. Next, to assess the role of IL-10 in

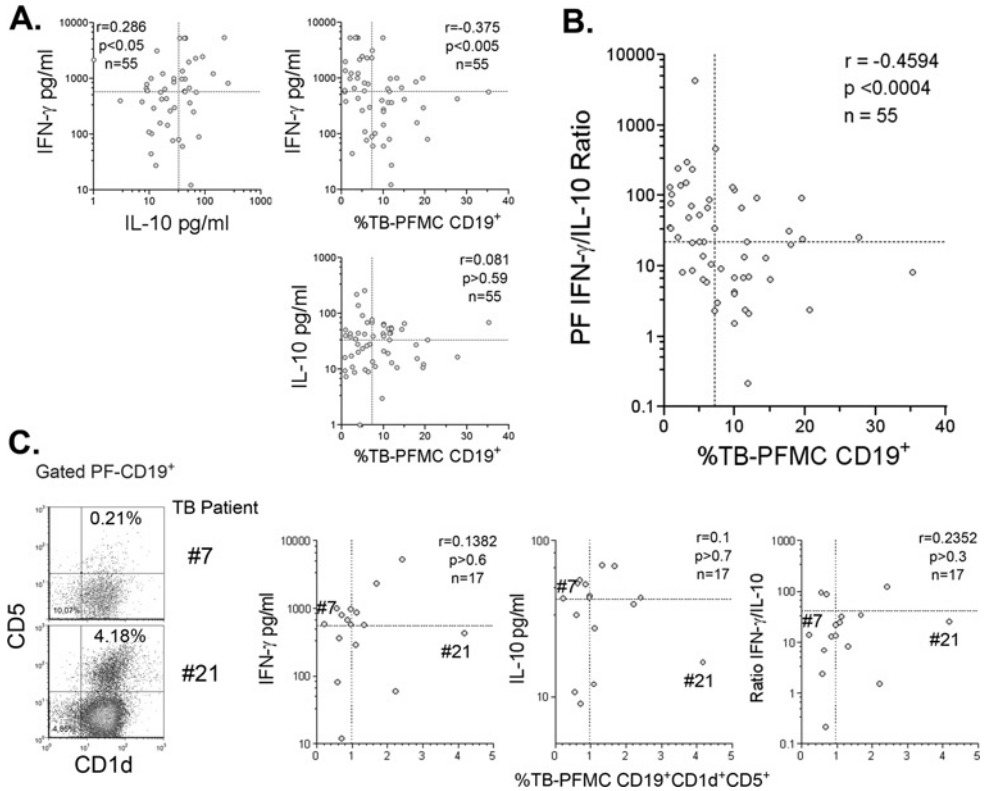

Figure 5 **Correlation of PF-B-cell frequency with the pleural IFN-γ/IL-10 ratio in TB patients**
(**A**) IFN-γ and IL-10 levels were correlated with each other or with the percentage of $CD19^+$ PFMCs ($n = 55$). (**B**) Correlation between the IFN-γ/IL-10 ratio and the percentage of $CD19^+$ PFMCs ($n = 55$). (**C**) Correlation of IFN-γ, IL-10 and the IFN-γ/IL-10 ratio with the percentage of $CD19^+/CD5^+/CD1d^+$ PFMCs ($n = 17$). Dot plots are two TB patients with very disparate frequencies of $CD19^+/CD5^+/CD1d^+$ PFMCs are shown. Spearman's test correlation factor (r), P values and number of patients (n) are indicated. Median values are indicated as broken lines.

the B-cell-mediated regulatory effect, fixed numbers of $CD19^+$-depleted PFMCs (2×10^5 cells/well) were stimulated with *M. tuberculosis* and co-cultured with autologous $CD19^+$-purified PFMCs (10:1 ratio). We found that *M. tuberculosis*-induced IFN-γ production by $CD19^+$-depleted PFMCs was indeed reduced by the addition of $CD19^+$-purified PFMCs, which was recovered by IL-10 neutralization (Figure 6B). Furthermore, this reduction was also achieved by the addition of IL-10 to $CD19^+$-depleted PFMCs (Figure 6B), suggesting that B-cells exert a regulatory function on $CD19^-$ from PF by an IL-10-dependent mechanism.

As T-cells and NK cells are the main sources of IFN-γ within PFMCs [9,10], we determined whether PF-B-cells modulated IFN-γ production by these populations. Thus $CD19^+$-depleted-PFMCs were stimulated with *M. tuberculosis* in the absence or presence or $CD19^+$-purified PFMCs and intracellular IFN-γ expression was analysed within the $CD56^+/CD3^-$ (NK cell) and $CD3^+$ (T-cell) gates. The presence of $CD19^+$-purified PFMCs reduced *M. tuberculosis*-induced IFN-γ expression in T-cells and NK cells. Moreover, addition of anti-IL-10 abrogated IFN-γ inhibition, and IL-10 mimicked the effect of $CD19^+$-purified PFMCs (Figure 6C). Taken together, these results show that IL-10 secretion by PF-B-cells regulates IFN-γ production by T-cells and NK cells.

DISCUSSION

In the present study, we investigated the role of B-cells in a human DTH reaction using tuberculous pleurisy as a model [8,12]. We found that, in the pleural and blood compartments from patients with tuberculous pleurisy, the proportions of B-cells were conserved within the peripheral ranges found in healthy subjects and was not dependent on the aetiology of the pleurisy (TB or no-TB). In addition, we did not detect changes in the frequencies of memory and naïve B-cells between PB and PF from patients with tuberculous pleurisy, but we observed a reduction in non-switched memory B-cells ($CD27^+/IgD^+$) in PB and an increase in $CD27^-/IgD^-$ PF-B-cells in patients with tuberculous pleurisy when compared with age-matched PB from healthy subjects, which might implicate an infection-driven outcome [20,21].

M. tuberculosis triggers an inflammatory response in the pleural space, resulting in the recruitment of specific inflammatory cells to the site of infection [8–17]. In particular, increased amounts of chemoattractants such as IP-10 (IFN-inducible protein-10), MIG (monokine induced by IFN-γ) and SDF-1 (stromal cell-derived factor-1) were found in PF compared with plasma from TB patients [35,36]. In line with this, we found that the expression of CXCR4 and the frequency of $CXCR3^+$ cells were augmented in PF-B-cell from TB patients, as has been

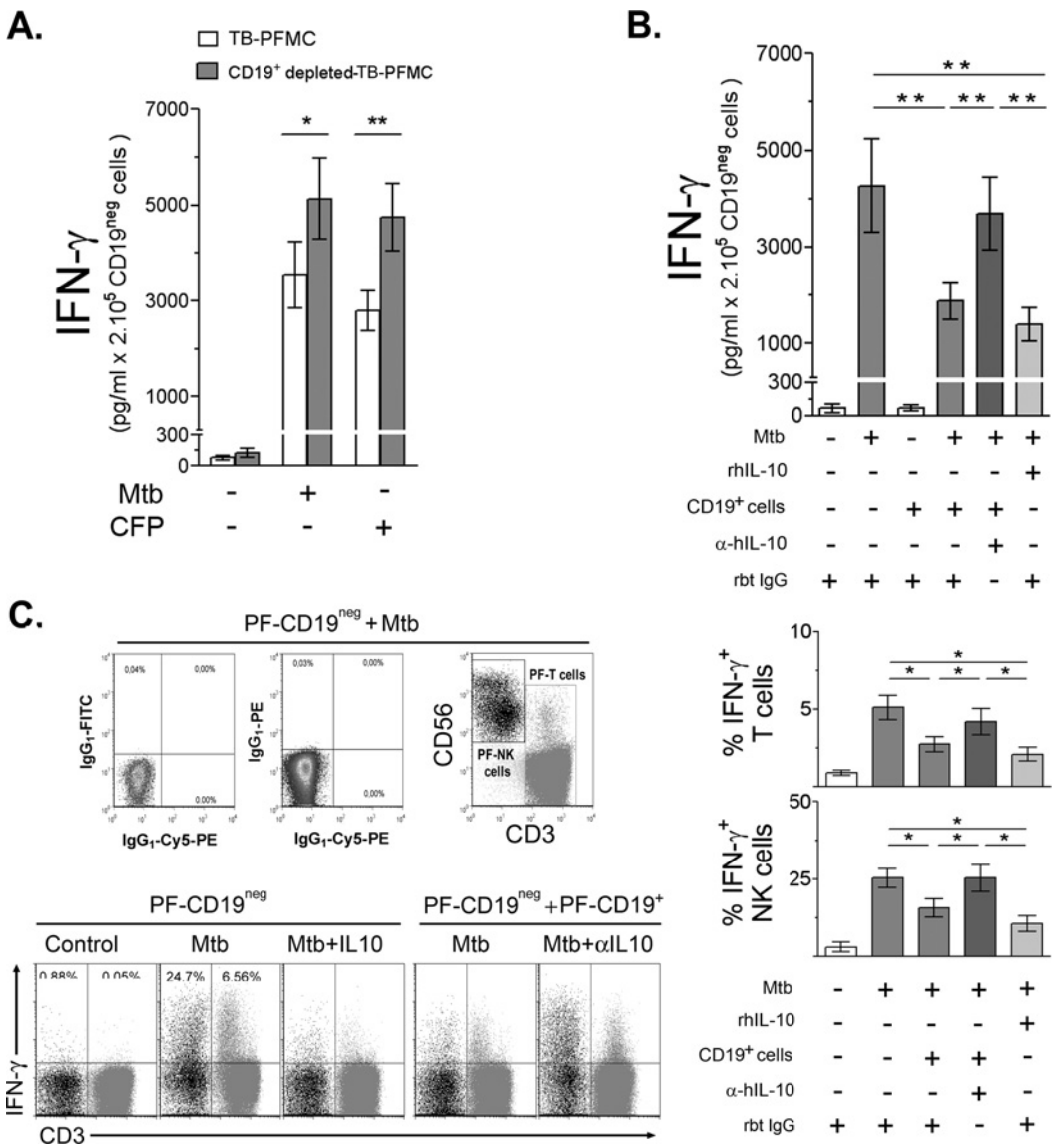

Figure 6 **B-cell-mediated regulation of IFN-γ production by T-cells and NK cells in TB-PFMCs**

(**A**) TB-PFMCs or CD19$^+$-depleted TB-PFMCs were cultured in complete medium (control) or stimulated with CFP or *M. tuberculosis* as indicated above for 48 h, and IFN-γ was determined by ELISA. Results are means $\pm$ S.E.M. ($n = 10$). All treatments were significantly higher compared with control supernatants. $^*P < 0.05$ and $^{**}P < 0.005$. (**B**) CD19$^+$-depleted TB-PFMCs were cultured without or with CD19$^+$-purified TB-PFMCs (CD19$^+$) in complete medium (control) or stimulated with *M. tuberculosis* in the absence or presence of rhIL-10 (1 ng/ml) or anti-IL-10 (α-hIL-10; 1 μg/ml) for 48 h, and IFN-γ production was determined by ELISA. Values are means $\pm$ S.E.M. ($n = 12$). $^{**}P < 0.005$ (repeated-measures one-way ANOVA, Bonferroni post-hoc test). (**C**) CD19$^+$-depleted TB-PFMCs were cultured alone or with CD19$^+$-purified TB-PFMCs as indicated in (**B**), and intracellular IFN-γ expression was determined within the T-cell and NK cell gates. Left-hand panel, isotypes, gating strategy and analysis of a representative TB patient. Numbers in upper quadrants indicate the percentage of IFN-γ^+ cells within NK-cell or T-cell gates. Purified rabbit IgG (2 μg/ml) was used as an antibody control. Right-hand panel, quantification of the percentage of IFN-γ^+ in T-cells (CD3$^+$) and in NK cells (CD56$^+$/CD3$^-$). Values are means $\pm$ S.E.M., $n = 10$. $^*P < 0.05$ (repeated-measures one-way ANOVA, Bonferroni post-hoc test).

observed in a similar study [37]. Consistent with that study, we also observed signs of B-cell activation in PF from TB patients, including down-regulation of CD20 and CD1d molecules [23,24] and up-modulation of several activation markers (Figure 2B). However, the classical feature of antigen-experienced B-cells, such as enrichment of molecules for antigen presentation and TLRs, as well as CD62L shedding, was not verified in PF-B-cells, suggesting that the environment of tuberculous pleurisy induces an atypical differentiation pathway for B-cell activation [24,26].

A remarkable observation in our present study was that *in vitro* PF-B-cells, but not PB-B-cells, were strongly activated by *M. tuberculosis* antigens. This response was dependent on the presence of bystander non-B-cells, since the single activation of

effector memory PF-T-cells achieved similar levels of CD69$^+$ B-cells as did stimulation with PWM and *M. tuberculosis* antigens. Indeed, neither PWM nor *M. tuberculosis* antigens were able to activate CD19$^+$-purified PF-B-cells, indicating that activation signals triggered by BCR-specific and/or TLRs are not sufficient. Regarding IL-10 production by PF-B-cells, our results showed that neither the whole bacterium nor its secreted proteins induced IL-10 production in purified PF-B-cells, indicating that BCR stimulation is not involved or not sufficient. This result is in clear contrast with those found in mice [38], although this difference may be attributed to the anatomical source of B-cells or to the antigen employed, i.e. heat-inactivated *M. tuberculosis* antigen H37Ra. We have shown that IL-10 production by PF-B-cells required CD19$^-$ co-operation, but was not dependent on CD40/CD40L, IL-4 or IL-21, although other co-stimulatory molecules on T-cell–B-cell interactions could be involved [39]. We have also shown that IL-10-producing B-cells modulate IFN-γ production by pleural effector lymphocytes in a Th1 context, which is consistent with the reported ability of a B-cell subset, mostly CD5$^+$/CD1d^{high} cells, to suppress IFN-γ production of naïve and memory CD4$^+$ T-cells from healthy subjects under Th1-polarizing conditions or upon polyclonal stimulation [29–31]. However, in our hands, the majority of PF-CD19$^+$ cells were CD5$^-$ and the percentage of CD19$^+$/CD5$^+$/CD1d$^+$ PFMCs were not correlated with the amounts of IL-10 in PF. In addition, we did not find any evidence supporting a regulatory role of antibody-secreting cells (CD19$^+$/CD27^{hi}/CD38^{hi}) in PFMCs from TB patients. As far we are aware the present study is the first to report that IFN-γ production by human NK cells can be modulated by IL-10-producing B-cells. Supporting this, PF-NK cells from TB patients express IL-10R (IL-10 receptor) (?????, Q2 unpublished work), as reported previously for PB-NK cells from healthy subjects [40]. Although additional experiments are necessary, considering results from the present study and our previous study [10], we speculate that IL-10 produced by PF-B-cells could be acting at two levels to inhibit IFN-γ production by PF-NK cells: (i) indirectly by diminishing IL-12 production by antigen-presenting cells in PF [41], and (ii) directly through IL-10R signalling. Finally, our *ex vivo* assays showing that PF-B-cell-derived IL-10 modulates IFN-γ were reinforced further by the negative correlation between the frequency of B-cells and the ratio of IFN-γ/IL-10 at the site of infection.

CLINICAL PERSPECTIVES

- TB is the leading cause of death from a single infectious pathogen. Although the role of T-cell-mediated immunity in the containment of *M. tuberculosis* is well known, the role of B-cells in the host defence against *M. tuberculosis* has not been clearly defined yet.
- In the present study, for first time in a human model of a DTH reaction (tuberculous pleurisy), we have described a feedback loop involving locally activated B-cells, which modulate the inflammatory response of NK and T-cells upon *M. tuberculosis* stimulation.
- Thus, as it has been proposed for autoimmune diseases, human B-cells with regulatory potential also exert homoeostatic functions on clinically relevant DTH reactions.

AUTHOR CONTRIBUTION

Pablo Schierloh and María Sasiain conceived and designed the experiments. Pablo Schierloh, Verónica Landoni and Luciana Balboa performed the experiments. Rosa Musella, Jorge Castagnino, Graciela de Casado, Domingo Palmero and Eduardo Moraña recruited, diagnosed and treated the patients, and obtained the relevant clinical data. Eduardo Moraña performed the toracocentesis for PF collection. Pablo Schierloh, Verónica Landoni, María Sasiain, Graciela de Casado and Domingo Palmero contributed reagents/materials/analysis tools. Pablo Schierloh, María Sasiain and Verónica Landoni analysed the data and wrote the paper.

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SUPPLEMENTARY ONLINE DATA

Human pleural B-cells regulate IFN-γ production by local T-cells and NK cells in a *Mycobacterium tuberculosis*-induced delayed hypersensitivity reaction

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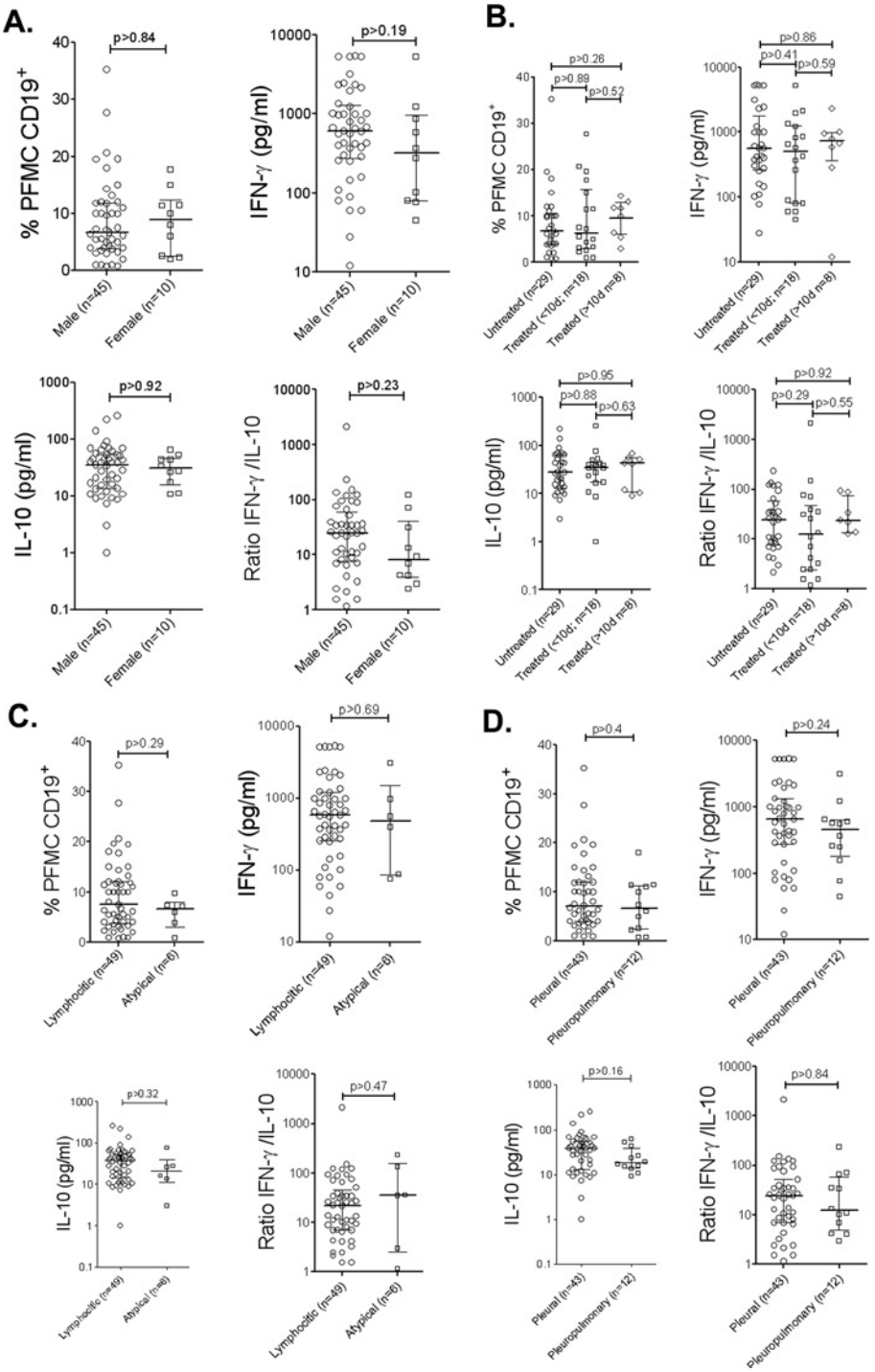

Figure S1 Absolute and relative amounts of PF cytokines and B-cell frequencies with respect to demographic and clinical features of the TB patients

PF B-cell frequency (% PFMC CD19$^+$), PF IFN-γ amounts (IFN-γ pg/ml), PF IL-10 amounts (IL-10 pg/ml) and the PF IFN-γ/IL-10 ratio present in TB patients discriminated by groups are shown. (**A**) Male or female TB patients. (**B**) Patients without antibiotic treatment (untreated), with $<$10 days of antibiotic treatment or with $>$10 days of antibiotic treatment (treated). (**C**) Typical pleural effusion cytology characterized by lymphocytic cell preponderance (Lymphocitic) or atypical pleural effusion cytology showing abnormal numbers of reactive mesothelial and/or phagocytic cells [Atypical (reactive cells)] [1]. (**D**) Patients with TB pleurisy or with pleuropulmonary TB. Median (horizontal line) and interquartile range (error bars) are indicated. None of the parameters were statistically different between the groups.

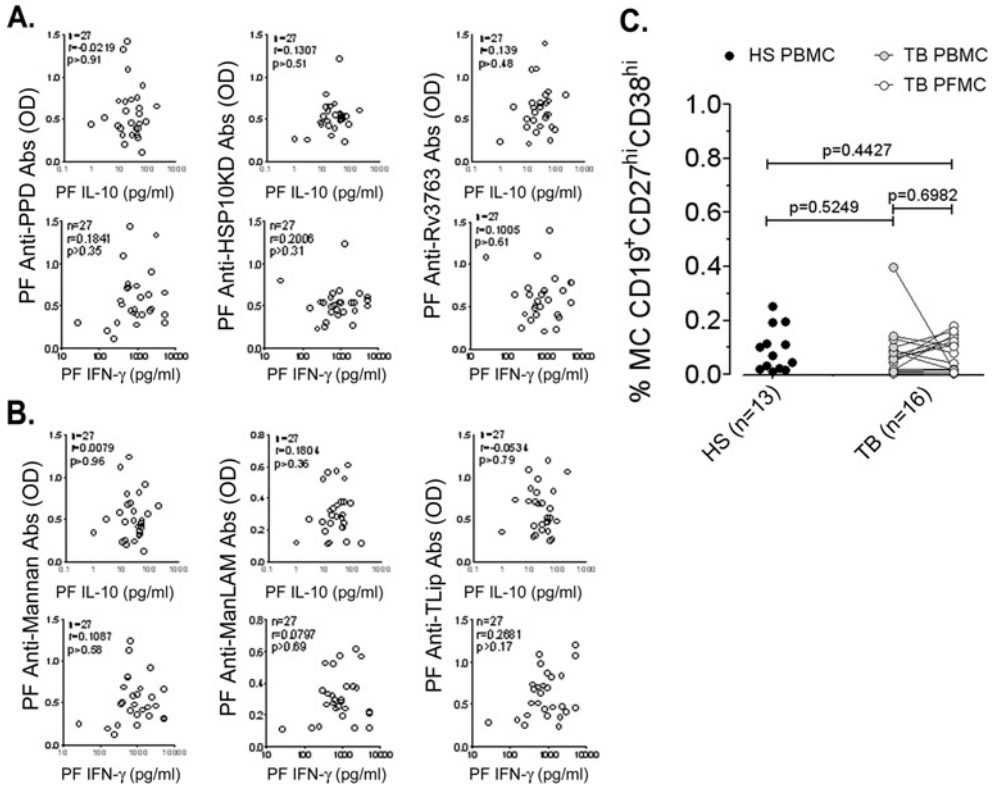

Figure S2 **PF cytokine levels with respect to PF antibodies and the frequency of PB and PF plasmablast/plasma cells**
An indirect ELISA assay for the detection of PF antibodies (Abs) that binds to plate-adsorbed *Mycobacterium tuberculosis* H37Rv-derived antigens was performed as described previously [2]. Optimal PF dilutions were established experimentally and were 1:1000 for purified protein derivate (PPD), mannan and total lipid extract (TLip), and 1:800 for mannose capped lipoarabinomannan (ManLAM), 10 kDa heat-shock protein (HSP10KD) and 19 kDa lipoprotein (Rv3763). (**A**) Correlation analysis for PF IL-10 and PF IFN-γ with respect to the amount of PF anti-PPD, -HSP10KD and -Rv3763 protein antigens ($n = 27$; Spearman test). None of the correlations were statistically significant. (**B**) Correlation analysis for PF IL-10 and PF IFN-γ with respect to the amount of PF anti-Mannan, -ManLAM and -TLip non-protein antigens ($n = 27$; Spearman test). (**C**) Frequency of plasmablast/plasma cells were determined among mononuclear cells from healthy donors (HS, $n = 13$) and TB patients (TB, $n = 16$). The immunophenotype of plasmablast/plasma cells was defined as $CD19^+/CD27^{hi}/CD38^{hi}$ according to Caraux et al. [3]. For detection of this small cell subpopulation, at least 2×10^5 events gated on FSC (forward side scatter)/SSC (side scatter) lymphocyte/lymphoblast region were acquired. Unpaired (HS compared with TB) data were compared with a Mann Whitney U-test. Paired (TB-PBMC compared with TB-PFMC) were compared with Wilcoxon signed rank test. None statistically significant differences were found. OD, absorbance.

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