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Brief Conclusive Report

Paradoxical role of CD16$^+$CCR2$^+$CCR5$^+$ monocytes in tuberculosis: efficient APC in pleural effusion but also mark disease severity in blood

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ABSTRACT

The role of CD16$^-$ and CD16$^+$ Mo subsets in human TB remains unknown. Our aim was to characterize Mo subsets from TB patients and to assess whether the inflammatory milieu from TB pleurisy modulate their phenotype and recruitment. We found an expansion of peripheral CD16$^+$ Mo that correlated with disease severity and with TNF-α plasma levels. Circulating Mo from TB patients are activated, showing a higher CD14, CD16, and CD11b expression and *Mtb* binding than HS. Both subsets coexpressed CCR2/CCR5, showing a potential ability to migrate to the inflammatory site. In tuberculous PF, the CD16$^+$ subset was the main Mo/MΦ population, accumulation that can be favored by the induction of CD16 expression in CD16$^-$ Mo triggered by soluble factors found in this inflammatory milieu. CD16$^+$ Mo in PF were characterized by a high density of receptors for *Mtb* recognition (DC-SIGN, MR, CD11b) and for lipid-antigens presentation (CD1b), allowing them to induce a successful, specific T cell proliferation response. Hence, in tuberculous PF, CD16$^+$ Mo constitute the main APC population; whereas in PB, their predominance is associated with the severity of pulmonary TB, suggesting a paradoxical role of the CD16$^+$ Mo subset that depends on the cellular localization. *J. Leukoc. Biol.* 90: 69–75; 2011.

Introduction

Mo/MΦ are the primary target of *Mtb*, and their innate capacity to deal with *Mtb* defines the early progression of the infection [1]. Human circulating Mo are heterogeneous in morphology, phenotype, and function [2, 3]. According to the differential expression of CD14 and CD16, Mo are classified in CD14^{high}/CD16$^-$ and CD14^{low}/CD16$^+$ subsets. In HS, the former subset (CD16$^-$) comprises 80–90% of circulating Mo, and the latter subset (CD16$^+$) constitutes the remaining 5–10% [4]. It was subsequently shown that Mo subsets also differ in the pattern of chemokine receptors [5–7], cytokines, adhesion molecules, and scavenger receptors [8]. Several studies have reported an increase in the number of peripheral CD16$^+$ Mo in acute inflammation [9] and infectious diseases [10–14] including TB [15]. The recruitment of Mo into inflamed tissues is largely determined by their response to chemokines and their expression patterns of chemokine receptors. Upon infection with *Mtb*, alveolar MΦ secrete chemokines such as MIP-1α, MIP-1β, and RANTES, mediating the trafficking of Mo, which express CCR2 and CCR5 [16]. In this context, CCR5 is expressed on CD16$^+$ Mo, whereas CCR2 is on CD16$^-$ Mo [5].

Among clinical manifestations of TB, pleurisy is of particular interest, as it may be resolved without therapy, and patients are known to undergo a relatively effective immune response against *Mtb* [17]. The initial recognition of the bacteria is considered to be critical to mount a protective, antimycobacterial immune response. Accordingly, several receptors have been involved in *Mtb* uptake and recognition, such as CD14, MR [18], complement receptor 3 (formed by CD11b and CD18 subunits) [19], and DC-SIGN [20]. The recognition of *Mtb* triggers the inflammatory response in the pleural space, where mesothelial cells are known to produce multiple chemokines that provide the chemotactic gradient for the influx of inflammatory cells [21]. Particularly, higher amounts of TNF-α, TGF-β, IL-10 [22], IFN-inducible protein-10, monokine induced by IFN-γ, IL-8, MCP-1, and MIP-1α were found in PF

Abbreviations: AFB=acid fast bacilli, CD62L=CD62 ligand, DC-SIGN=DC-specific intercellular adhesion molecule 3-grabbing nonintegrin, FSC=forward-scatter, HS=healthy subjects, MΦ=macrophage(s), MFI=mean fluorescence intensity, Mo=monocyte(s), MR=mannose receptor, *Mtb*=*Mycobacterium tuberculosis*, *Mtb*-FITC=FITC-labeled *Mycobacterium tuberculosis*, PB=peripheral blood, PF=pleural effusions, SSC=side-scatter, TB=tuberculosis, TST=tuberculin skin test

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than in PB from patients with TB [23]. In agreement with this, increased amounts of RANTES and MCP-1 were found in BAL fluid from TB patients [24].

The aim of the present study was to characterize Mo subsets in PB from TB and to assess whether the inflammatory milieu found in tuberculous pleurisy could modulate their phenotype and recruitment. For this purpose, we evaluated Mo phenotype together with their activation state and function.

MATERIALS AND METHODS

Patients and healthy blood donors

A total of 80 TB patients (70 pulmonary and 10 tuberculous pleurisy) and 30 healthy blood donors were studied. Their age and gender are detailed in **Table 1**. TB and cancer patients (median age=52 years; range=28–76 years; 80% male) were diagnosed at the Servicio de Tisioneumonología, Hospital F. J. Muñiz (Buenos Aires, Argentina). Written, informed consent was obtained according to Ethics Committee from the Hospital Institutional Ethics Review Committee. Exclusion criteria included a positive HIV test and the presence of concurrent infectious diseases or noninfectious conditions (cancer, diabetes, or steroid therapy). TB was diagnosed by sputum smear staining for AFB and/or sputum culture [25]. According to chest X-ray findings, pulmonary disease was classified into three groups: mild for patients without cavities, moderate for patients with unilateral cavities, and advanced for patients with bilateral disease. Effusions were classified as exudates according to Light et al. [17] criteria. PB samples from HS were provided by the Blood Transfusion Service, Hospital Fernandez. J. Muñiz. All HS had received bacillus Calmette-Guerin vaccination in childhood, and their TST status was unknown.

Thoracentesis and mononuclear cells

PF were obtained as described previously [26]. Paired samples of PB and PF mononuclear cells were isolated by Ficoll-Hypaque and suspended in RPMI-1640 tissue-culture medium (Hyclone, Logan, UT, USA) containing gentamicin (85 μg/ml) and 10% heat-inactivated FCS (Gibco Laboratories, Invitrogen, Carlsbad, CA, USA).

Reagents

The following FITC, PE, and/or PerCP/Cy5.5 mAb against: CD14, CCR5, CD86, and HLA-DR (BD Biosciences PharMingen, San Diego, CA, USA); CD16, CD54, CD62L, TLR-2, and TLR-4 (eBioscience, San Diego, CA, USA); CCR1, CCR2, DC-SIGN, and MR (R&D Systems, Minneapolis, MN, USA); CD11b and CD1b (Ancell, Bayport, MN, USA); CX3CR1 (MBL, Nagoya, Japan); and isotype-matched mAb were used. Anti-CD11b (eBioscience), anti-CD14 (Immunotech, Marseille, France), and anti-MR and anti-DC-SIGN (R&D Systems) neutralizing mAb were used in blocking experiments. Cytochalasin B was purchased from Sigma-Aldrich (St. Louis, MO, USA). The γ-irradiated *Mtb* H37Rv strain was kindly provided by Dr. John Belisle (Colorado State University, Denver, CO, USA).

Flow cytometric analysis of cell surface phenotype

Briefly, 2×10^5 cells were labeled with FITC, PE, or PerCP/Cy5.5 mAb as described previously [27] and acquired in a FACScan cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). The Mo population was gated according to its FSC and SSC proprieties. The percentage of positive cells and the MFI were analyzed using FCS Express V3 software (De Novo Software, Los Angeles, CA, USA).

Cytokine measurement

Plasma IL-10, TNF-α, and TGF-β amounts were measured by ELISA using commercial kits (eBioscience) according to the manufacturer's instructions.

Binding assays

Irradiated *Mtb* was labeled by incubation with FITC as described previously [27]. Mo (2×10^5) from HS or TB patients (10% mild, 30% moderate, and 60% advanced) were incubated with *Mtb*-FITC (five *Mtb*:one Mo) for 90 min at 37°C or 4°C. Then, cells were washed extensively to remove unbound bacteria and labeled with anti-CD16 and anti-CD14, and the percentage and MFI of *Mtb*-FITC$^+$ Mo were measured by flow cytometry. To verify the involvement of phagocytosis, control experiments were performed with cells pretreated for 30 min with the inhibitor of actin polymerization, cytochalasin B (10 μg/ml, Sigma-Aldrich). When indicated, cells were incubated for 30 min at 4°C with anti-CD11b (2 μg/ml) or anti-CD14 (4 μg/ml) neutralizing mAb before *Mtb*-FITC addition.

Proliferation assay

Mononuclear cells from PB or PF purified by Ficoll-Hypaque (1×10^5) were cultured in the presence or not of *Mtb* (ratio, one *Mtb*:one cell) in round-bottom 96-well plates (Corning, Corning, NY, USA) for 5 days. Thereafter, 0.5 μCi/well [methyl-^{3}H]thymidine (PerkinElmer, Boston, MA, USA) was added for the last 18 h, and radioactivity was measured in a liquid scintillation counter. The

TABLE 1. Clinical Characteristics of the Study Populations

	Diagnosis		
	HS	Pulmonary TB	TB pleurisy
n	30	70	10
Age (range)	30 (20–45)	36 (23–50)	30 (20–55)
Gender	15 F/15 M	25 F/45 M	2 F/8 M
TST+, %	ND	25	ND
Days of evolution (range)	–	41 (7–90)	ND
AFB[a], %	–	1+, 33 2+, 30 3+, 33	ND
Leukocyte count, mean ± SEM, cell/μL	8200 ± 420	9093 ± 318	7400 ± 900
Lymphocyte mean ± SEM, %	32 ± 15	28 ± 8	21 ± 12
Mo mean ± SEM, %	6 ± 3	8 ± 3	6 ± 3
Cell count (range), cells/μL	–	–	2300 (500–3000)
Mononuclear cells mean ± SEM, %	–	–	71 ± 15

Clinical characteristics of HS; Pulmonary TB, patients with pulmonary TB; and TB pleurisy, patients with TB pleurisy. [a]AFB in sputum or culture: 1+, 2+, 3+ AFB are defined according to the International Union Against Tuberculosis and Lung Disease (IUATLD)/World Health Organization (WHO) quantification scale.

results were expressed as counts of [^{3}H]thymidine incorporation/min (cpm). Blockage of CD14, CD11b, DC-SIGN, MR, or isotype-dependent T cell proliferation was carried out by adding the blocking mAb at the start of the culture.

Influence of soluble factors on CD16 expression

PB mononuclear cells (1×10^6) from HS were incubated with a pool of sera from 30 pulmonary TB patients (6% mild, 40% moderate, and 54% advanced) or cell-free supernatants from 10 tuberculous PF or a pool of sera from 30 HS at 10% for 18 h at 37°C in 5% CO_2. Afterward, cells were washed twice and stained with the specific mAb.

Statistical analysis

Wilcoxon paired test was used for comparison of paired PB and PF samples and Kruskal-Wallis test followed by Dunn's multiple comparison test when comparing more than two groups. Correlation analysis was performed with Spearman two-tailed (nonparametric) test. A $P < 0.05$ value was assumed as significant. All analyses were done using Prism 4 software (GraphPad, San Diego, CA, USA).

RESULTS AND DISCUSSION

In chronic infections, circulating Mo arrive to the infected site and constitute a central feature of the host response, as they represent a large pool of precursors of MΦ and DCs. Although MΦ and DCs are important constituents of the normal lung, during *Mtb* infection, Mo recruitment could modulate immunity and affect the outcome of the disease [28]. Hence, we compared the phenotype of blood Mo from TB patients and HS with respect to a range of markers thought to be relevant for the immune response in TB. Clinical characteristics from pleurisy or pulmonary TB and HS are summarized in Table 1. No differences were found in terms of age and sex distribution or in the number and percentages of leukocytes among groups (Table 1). As it is shown in **Table 2**, Mo from pulmonary TB displayed higher CD14, CD16, CD11b, TLR-2, TLR-4, CCR2, and CCR5 expression than Mo from HS, whereas the expression of CD54, CD62L, CCR1, and CX3CR1 was similar between the groups (Table 2). As Mo from TB patients expressed higher CD11b levels, a specific receptor for *Mtb* entry, we wondered if they also showed an increased *Mtb* binding and phagocytosis. We found that Mo from TB patients showed higher *Mtb*-FITC cell association levels than Mo from HS (**Fig. 1A**). Moreover, *Mtb*-FITC binding was significantly inhibited by CD11b but not by CD14 blockage, confirming the involvement of the former receptor in the uptake of *Mtb*. Thus, circulating Mo from TB patients show a high degree of activation and a more mature phenotype compared with HS, highlighting the systemic impact of the disease. Additionally, the more activated phenotype observed in Mo from TB patients might indicate their advanced commitment for a specific function and could explain the altered differentiation toward DCs already described in these Mo [27, 29].

Thereafter, we evaluated the Mo population based on CD16 expression, and we found a higher percentage of circulating CD16$^+$ Mo in TB patients than in HS (**Fig. 2A** and **B**), which is in accordance with previous reports [15]. The high frequency of CD16$^+$ Mo in TB patients could not be ascribed to a selective increase of this subset in PB, as the proportion of Mo and leukocyte counts did not differ between HS and TB patients. Furthermore, the percentage of CD16$^+$ Mo correlated with the extent and type of chest X-ray findings; e.g., the higher the percentage of CD16$^+$ Mo, the more severe disease, although the number of bacilli in sputum smears and symptoms evolution did not correlate (Fig. 2C). As CD16 can be induced by TGF-β, TNF-α, and IL-10 in Mo and MΦ [30], their amounts were measured in plasma from HS and TB patients. Plasma amounts of TNF-α were associated with the percentage of CD16$^+$ Mo in TB patients (Spearman r=0.52; P=0.04), and 86% of the samples from HS had no detectable levels of TNF-α (Fig. 2D). In contrast, TGF-β and IL-10 did not correlate with the percentage of CD16$^+$ Mo in TB patients (data not shown), as has been observed in other chronic, infectious diseases [31, 32]. It is well known that TNF-α has a wide range of activities in inflammatory and immune responses, and it plays an essential role in host resistance against *Mtb* infection. In addition, a high-level expression of TNF-α is associated with failure to resolve ongoing inflammation and with triggering of pathological manifestations [33]. Hence, as the ex-

TABLE 2. Phenotypic Characterization of Mo and Mo Subsets from HS and Patients with TB

	HS			TB		
	Mo	CD16$^-$	CD16$^+$	Mo	CD16$^-$	CD16$^+$
CD14	619 ± 38	575 ± 41	549 ± 53	828 ± 50[a]	673 ± 45[a]	756 ± 48[a,b]
CD16	25 ± 3	–	261 ± 28	45 ± 5[a]	–	269 ± 18
CD11b	683 ± 43	726 ± 42	907 ± 47[b]	1149 ± 140[a]	1000 ± 136[a]	1248 ± 147[a,b]
CD54	369 ± 34	385 ± 46	525 ± 37[b]	321 ± 30	291 ± 85	528 ± 94[b]
CD62L	76 ± 24	83 ± 27	7,5 ± 3[b]	64 ± 23	74 ± 24	13 ± 6[b]
TLR-2	53 ± 5	48 ± 8	65 ± 7[b]	68 ± 3[a]	73 ± 6[a]	87 ± 5[a,b]
TLR-4	14 ± 1	11 ± 3	17 ± 1[b]	22 ± 2[a]	20 ± 1[a]	27 ± 1[a,b]
CCR2	43 ± 6	67 ± 4	47 ± 3[b]	80 ± 9[a]	85 ± 1[a]	83,5 ± 3[a]
CCR5	34 ± 3	27 ± 4	41 ± 3[b]	569 ± 55[a]	320 ± 67[a]	491 ± 105[a,b]
CCR1	11 ± 2	12 ± 2	8 ± 1[b]	13 ± 2	13 ± 2	16 ± 2[a,b]
CX3CR1	73 ± 6	71 ± 4	165 ± 12[b]	77 ± 9	70 ± 8	130 ± 11[a,b]

Cell surface markers from circulating Mo from HS or patients with pulmonary TB were measured by flow cytometry. Results are expressed as MFI. When indicated, Mo subsets were gated according to CD16 expression. Standard errors from 20 to 40 independent experiments are shown. [a]Statistical differences for HS versus TB, $P < 0.05$; [b]statistical differences for CD16$^+$ versus CD16$^-$, $P < 0.05$.

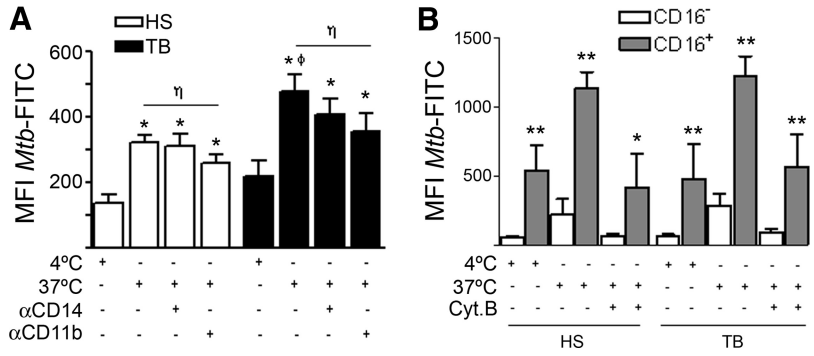

Figure 1. *Mtb* uptake in blood Mo populations. Uptake of *Mtb*-FITC was measured in PB mononuclear cells by flow cytometry. PB mononuclear cells were incubated at 4°C or at 37°C, with or without cytochalasin B. Mo were first gated according to their FSC and SSC profiles, and anti-CD14-PE/Cy5.5 staining after CD16-PE/Cy5 channel fluorescence was measured within the Mo gate. *Mtb* association was determined by measuring *Mtb*-FITC MFI. (A) MFI of *Mtb*-FITC in the whole Mo population from HS (open bars) and TB patients (solid bars) at 4°C and 37°C, with or without neutralizing anti-CD11b and anti-CD14 mAb. Statistical differences for treatment versus 4°C: $*P < 0.05$; for blockage versus 37°C: $^{\eta}P < 0.05$; and for HS versus TB: $^{\Phi}P < 0.05$ (n=10). (B) MFI of *Mtb*-FITC in each Mo subset according to CD16 expression: CD16$^-$ (open bars) and CD16$^+$ (shaded bars) from HS and TB patients at 4°C and 37°C, with or without cytochalasin B. Statistical differences for CD16$^-$ versus CD16$^+$: $**P < 0.01$; $*P < 0.05$ (n=10).

pansion of CD16$^+$ Mo in TB patients correlated with the disease severity and with the amounts of circulating TNF-α, the peripheral inflammatory Mo profile seems to be associated with an active inflammation background and to be clinically relevant. As Mo from TB patients showed a higher *Mtb*-FITC association and also an increased proportion of CD16$^+$ Mo than HS, we evaluated the *Mtb*-binding capacity of each Mo subset in both groups. As it is shown in Fig. 1B, although CD16$^+$ Mo displayed a more elevated *Mtb*-FITC association than CD16$^-$ Mo, no differences were found between the same subsets from HS and TB patients when the assays were carried out at 37°C, at 4°C, or even in the presence of cytochalasin B, indicating that same Mo subsets from HS and TB patients do not differ in their binding or phagocytic capacity. Therefore, we conclude that the elevated *Mtb*-binding levels observed in TB patients (Fig. 1A) can be ascribed to the increased proportion of CD16$^+$ cells, which show higher *Mtb* association than CD16$^-$ Mo and not to an enhanced, intrinsic binding capacity displayed by the Mo subsets in TB patients. Next, we wondered whether the shift toward CD16$^+$ from CD16$^-$ Mo in PB from TB patients could explain the differences observed in Mo surface markers between HS and TB patients. Thus, we determined surface molecules that are differentially expressed on Mo subsets from HS. Both Mo subsets from TB patients showed higher CD14 expression than Mo subsets from HS, and the highest level was found in the CD16$^+$ subset (Table 2). Besides, CD16$^+$ Mo from HS showed elevated CD54, TLR-2, TLR-4, CCR5, and CX3CR1 and lower CCR1, CCR2, and CD62L expression than CD16$^-$ Mo, and these findings are in accordance with previous reports [6, 34], but in contrast with those studies, we observed high CD11b expression in this subset. With regard to chemokine receptor pattern expression, it has been described that CCR5 and CX3CR1 are highly expressed on CD16$^+$ Mo and

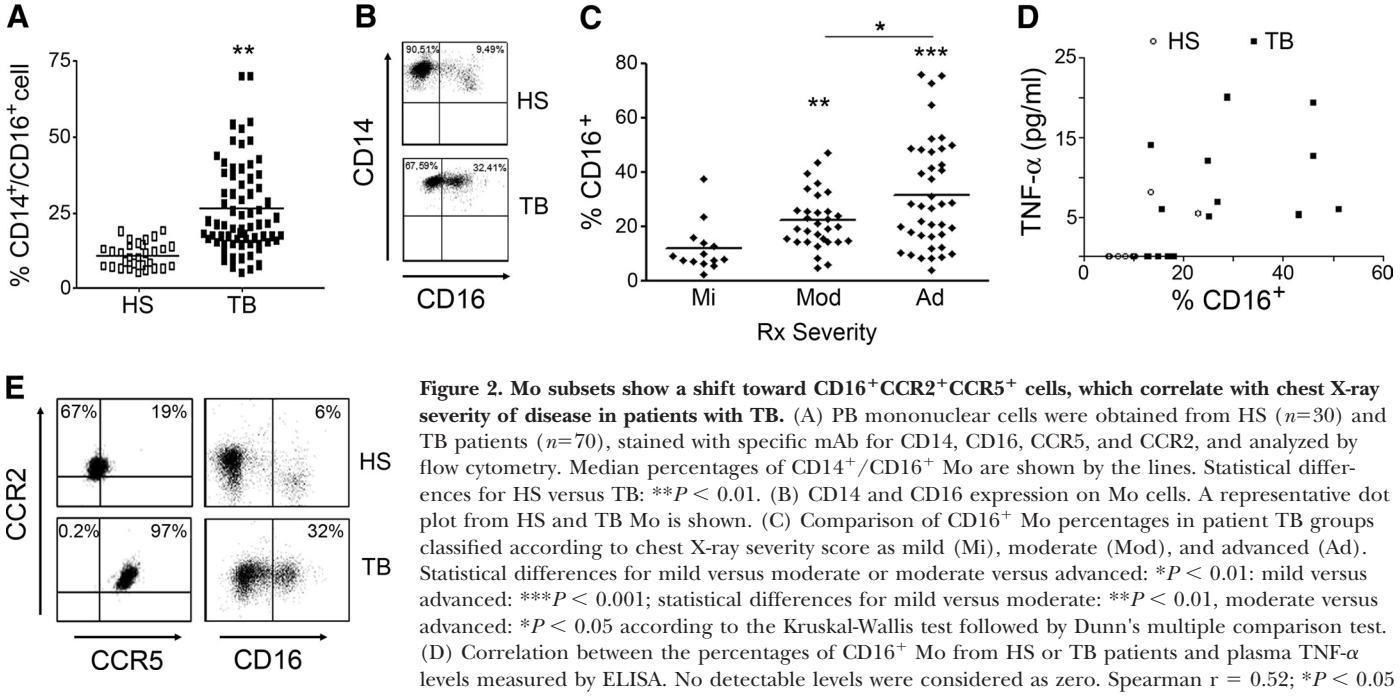

Figure 2. Mo subsets show a shift toward CD16$^+$CCR2$^+$CCR5$^+$ cells, which correlate with chest X-ray severity of disease in patients with TB. (A) PB mononuclear cells were obtained from HS (n=30) and TB patients (n=70), stained with specific mAb for CD14, CD16, CCR5, and CCR2, and analyzed by flow cytometry. Median percentages of CD14$^+$/CD16$^+$ Mo are shown by the lines. Statistical differences for HS versus TB: $**P < 0.01$. (B) CD14 and CD16 expression on Mo cells. A representative dot plot from HS and TB Mo is shown. (C) Comparison of CD16$^+$ Mo percentages in patient TB groups classified according to chest X-ray severity score as mild (Mi), moderate (Mod), and advanced (Ad). Statistical differences for mild versus moderate or moderate versus advanced: $*P < 0.01$: mild versus advanced: $***P < 0.001$; statistical differences for mild versus moderate: $**P < 0.01$, moderate versus advanced: $*P < 0.05$ according to the Kruskal-Wallis test followed by Dunn's multiple comparison test. (D) Correlation between the percentages of CD16$^+$ Mo from HS or TB patients and plasma TNF-α levels measured by ELISA. No detectable levels were considered as zero. Spearman r = 0.52; $*P < 0.05$ (n=15). (E) CD16, CCR5, and CCR2 expression gated on the Mo population according to CD14 expression; a representative dot plot from HS and TB Mo is shown.

CCR2 on CD16− Mo [6, 35]. Accordingly, this pattern expression was observed in Mo subsets from HS; however, this profile was indeed altered in Mo from TB patients so that both subsets coexpressed CCR2 and CCR5 (Fig. 2E). In addition, CD16+ Mo from TB patients expressed higher levels of CCR1 than CD16− Mo, which is in contrast with that observed in HS. Remarkably, CX3CR1 levels on CD16+ Mo from TB patients were lower than from HS, as has also been described in other infectious diseases [36, 37]. In line with this, the down-regulation of CX3CR1 together with the up-regulation of CCR2 expression on CD16+ Mo in TB patients contribute to diminish the difference in the chemokine receptor expression between the subsets. Hence, we consider that the conventional Mo subset classification, according to the differential chemokine receptor expression, can be altered under a chronic infection; consequently, it could not be used as a loyal marker to distinguish both subsets in pathological conditions. It is known that in many infections and immune-mediated inflammatory responses, the recruitment of Mo and MΦ into inflamed tissues is mediated by CCR2 and CCR5 in response to the MCPs, MIP-1α, MIP-1β, and/or RANTES [38]. In this context, a lesser in vivo migratory capacity of CD16+ Mo to the site of infection, because of their lack of CCR2 expression, has been suggested [34]. Interestingly, both Mo subsets from TB expressed high CCR2 and CCR5 levels; therefore, they would be able to respond to RANTES and MIP-1α, chemokines found in high amounts at the site of *Mtb* infection [23, 24]. Hence, it is likely that both subsets have a high ability to migrate to the site of infection.

Tuberculous pleurisy provides a good model to study immune response in vivo, as the immunological reactivity against *Mtb* is compartmentalized in the pleural space [39]. So, we determined the Mo population in PF from 10 patients with tuberculous pleurisy and five patients with cancer and compared the phenotype of CD14+ cells from paired PB and PF samples. Clinical data from patients with TB pleurisy were depicted above in Table 1. Mo from tuberculous PF showed a remarkably higher expression in CD16, TLR-2, TLR-4, CCR5, CCR2, CD11b, and CD14 receptors than their PB counterpart (**Table 3** and **Fig. 3A** and **B**). Furthermore, both Mo subsets contribute to the phenotype in PF, displaying higher levels of CD14, CD11b, CCR2, and CCR5 than their PB counterpart. In addition, the percentage of CD16+ Mo as well as CCR5+ and double-positive CCR5/CCR2 Mo observed in PB was increased further in PF (Fig. 3A), and the CD16+ population was up to 80% of total CD14+ Mo from PF. In contrast, Mo from cancer PF did not show the increase in the CD16+ Mo subset or CCR5/CCR2 expression (Fig. 3A and B), suggesting that the infection, instead of the inflammation per se, may induce the accumulation of CD16+ cells in PF. Importantly, CCR5 can mediate Mo activation in *Mtb* infection by binding heat shock protein 70 from *Mtb* [40], so its increase in PF could be modulated by interaction with *Mtb* antigens present in PF. As we found that the CD16+ population was up to 80% of total CD14+ in PF, we wondered whether this subset expressed receptors involved in *Mtb* recognition and antigen presentation. CD16+ from PF showed a higher expression of C-type lectins (DC-SIGN and MR), the integrin subunit CD11b, TLRs (TLR-2 and TLR-4), the costimulatory molecule CD86, and antigen-presenting molecules of peptides (HLA-DR) and lipids (CD1b) than in PB (Table 3). Moreover, among APCs from tuberculous PF, 32 ± 8% were DC-SIGN+, and 40 ± 7% were MR+, which is in accordance with previous results [26]. Then, the contribution of these receptors in the induction of the lymphoprolifetative response was evaluated, using mononuclear cells from PB or PF stimulated with irradiated *Mtb*. In accordance with that described when purified protein derivative or mycobacterial antigens were used [41, 42], an increased proliferative response in PF cells compared with PB was observed, and this response was strongly inhibited by the blockage of CD11b and CD14 and to a lesser extent, by DC-SIGN and MR (Fig. 3C). In line with this, the majority of CD16+ Mo from PF showed a high expression of DC-SIGN, MR, and CD11b—key receptors involved in the uptake and subsequent *Mtb* antigen presentation (Fig. 3D)—and this would predict a superior APC capability.

It is known that TB pleurisy is generally a self-limited disease, indicating that these patients mount a relatively effective immune response against *Mtb* [39]; therefore, CD16+ Mo are likely to play

TABLE 3. Phenotypic Characterization of Mo from PB and PF from Patients with TB

	PB			PF		
	Mo	CD16−	CD16+	Mo	CD16−	CD16+
CD14	876 ± 165	778 ± 130	910 ± 142[a]	2,142 ± 420[b]	1400 ± 320[b]	2,250 ± 428[a,b]
CD16	17 ± 4	–	261 ± 28	240 ± 22[b]	–	406 ± 95[b]
CD11b	950 ± 200	902 ± 165	1354 ± 178[a]	1,500 ± 250[b]	1,405 ± 224[b]	1786 ± 286[a,b]
TLR-2	92 ± 11	53 ± 8	96 ± 13[a]	147 ± 12[b]	103 ± 32[b]	173 ± 24[a,b]
TLR-4	22 ± 5	22 ± 3	31 ± 5[a]	60 ± 6[b]	52 ± 8[b]	65 ± 7[a,b]
CCR2	72 ± 15	77 ± 14	68 ± 8	253 ± 88[b]	224 ± 83[b]	198 ± 74[b]
CCR5	89 ± 24	52 ± 17	92 ± 25[a]	450 ± 61[b]	252 ± 71[b]	472 ± 65[a,b]
HLA-DR	54 ± 15	53 ± 18	105 ± 31[a]	256 ± 32[b]	198 ± 34[b]	286 ± 22[a,b]
CD86	79 ± 11	79 ± 8	101 ± 14[a]	224 ± 12[b]	222 ± 15[b]	233 ± 27[b]
DC-SIGN	–	–	–	12 ± 3	9 ± 2	41 ± 4[b]
MR	–	–	–	17 ± 4	13 ± 5	28 ± 3[b]
CD1b	–	–	–	56 ± 7	36 ± 8	89 ± 11[b]

Cell surface markers were measured by flow cytometry on Mo from PB and PF of patients with tuberculous pleurisy. Results are expressed as MFI. When indicated, Mo subsets were gated according to CD16 expression. Standard errors from 10 independent experiments are shown. [a]Statistical differences for CD16+ versus CD16−, $P < 0.05$; [b]statistical differences for PB versus PF, $P < 0.05$; –, nondetectable expression.

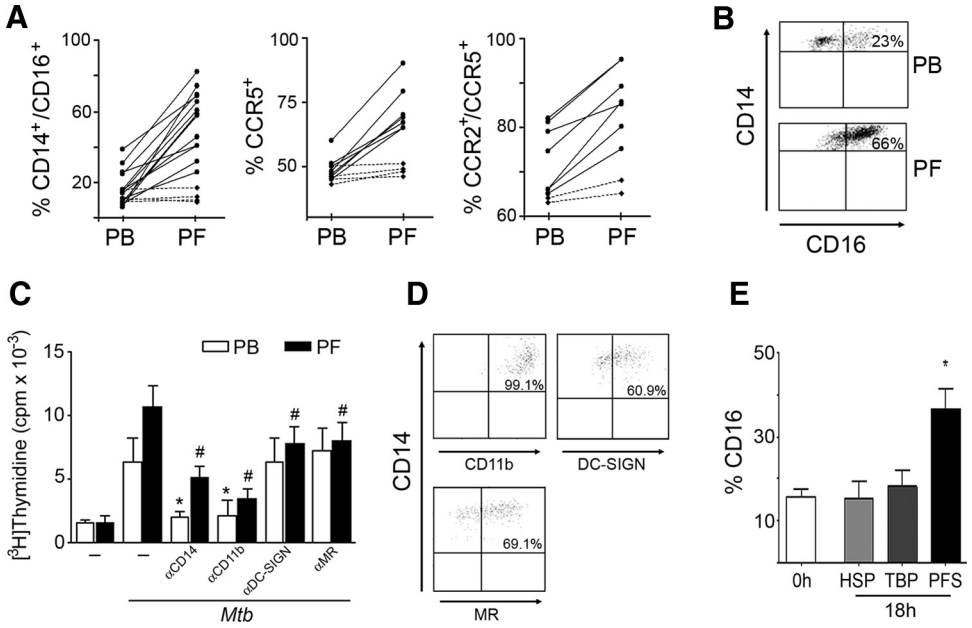

Figure 3. CD16$^+$ is the major Mo subset in tuberculous PF. PB and PF mononuclear cells were obtained from TB or cancer pleurisy, stained with specific mAb for CD14/CD16, and analyzed by flow cytometry. (A) Percentages of CD14$^+$/CD16$^+$, CCR5$^+$, or CCR2$^+$/CCR5$^+$ cells in paired PB and PF from tuberculous (solid lines) and cancer (dotted lines) pleurisy. (B) CD14 and CD16 expression on PB and PF and a representative dot plot. (C) Specific proliferation against *Mtb* in paired PB (open bars) and PF (solid bars) mononuclear cells. When indicated, neutralizing anti-CD14, CD11b, DC-SIGN, or MR mAb were used. Statistical differences for *Mtb* versus *Mtb* plus blockage: $^*P < 0.05$ in PB; *Mtb* versus *Mtb* plus blockage: $^\#P < 0.05$ in PF (n=10). (D) Representative dot plots of CD14 and CD11b, DC-SIGN, and MR expression on CD16$^+$ cells from PF. (E) PB mononuclear cells from HS were incubated with a pool of sera from HS (HSP) or from TB patients (TBP) or with cell-free supernatants from PF TB (PFS) for 18 h. Percentages of CD14$^+$/CD16$^+$ on PB mononuclear cells at 0 h or at 18 h with 10% HS, TB patients, or PF TB were determined by flow cytometry. Statistical differences for 18 h versus 0 h; $^*P < 0.05$ (n = 10).

a positive role against *Mtb* at the site of infection. Host defense against tuberculous pleurisy involves infiltration of the PB mononuclear cells into the pleural space; however, the mechanisms regulating Mo migration remain unclear. Although CD16$^+$ Mo is the main subset among CD14$^+$ cells in PF, we hypothesized that both peripheral Mo subsets have a high ability to migrate to the site of infection because of their peculiar chemokine receptor profile. So, we speculated that the origin of CD16$^+$ cells in PF might be the result of two processes, such as direct CD16$^+$ Mo transmigration from blood and in situ acquisition of CD16 expression induced by soluble mediators in those transmigrated CD16$^-$ Mo. To assess if soluble factors are able to induce CD16 expression on CD16$^-$ Mo, PB mononuclear cells from HS were incubated with a pool of sera from HS or from TB patients or of cell-free supernatants from tuberculous PF for 18 h, and then CD14 and CD16 expression was determined. As it is shown in Fig. 3E, sera from neither HS nor TB patients modified the proportion of the CD16$^+$ subset in HS Mo, but cell-free supernatants from tuberculous PF did increase it. As serum from TB patients did not affect CD16 levels, we consider that the association found between TNF-α plasma levels and the frequency of peripheral CD16$^+$ Mo may be the result of TNF-α production by CD16$^+$ Mo [43] rather than by induction of CD16 expression by TNF-α. Hence, at the site of infection, CD16$^+$ Mo/MΦ might be the result of their transmigration from blood and CD16 acquisition induced in situ by soluble mediators and/or *Mtb* antigens. Besides, induction of CD16 and MR expression occurs during Mo differentiation into MΦ; therefore, it is likely that transmigrated CD16$^-$ may contribute to the pool of CD16$^+$ cells found in PF by their differentiation into MΦ-like cells. However, the involvement of differential transmigration or selective apoptosis of Mo subsets induced by *Mtb* cannot be excluded, and this is a matter of current investigation.

In summary, we have described a paradoxical role of the CD16$^+$ Mo subset that depends on the cellular localization. Circulating Mo from TB patients are activated and show a potential ability to migrate to the inflammatory site mediated by CCR2/CCR5. In tuberculous, PF CD16$^+$ Mo express receptors for *Mtb* recognition and antigen presentation and constitute the main APC population. In PB, their predominance is associated with extensive tissue destruction, fibrosis, cavitary lesions, as well as with TNF-α plasma levels, highlighting the role of CD16$^+$ Mo in human TB. Our results contribute to the better understanding of the role of Mo in the balance between protective immune responses and immunopathology in TB.

AUTHORSHIP

L.B. performed experiments, designed research, analyzed data, and wrote the manuscript. M.M.R. performed experiments. J.I.B., C.A.S.G., P.S., N.Y., and L.G. contributed to laboratory work. R.M.M., J.C., and E.A. selected patients and collected blood and PF samples. S.B. and M.C.S. contributed to the study's conception. M.A. contributed to the study's conception and design.

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