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Original Paper

Diverging biological roles among human monocyte subsets in the context of tuberculosis infection

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Abstract

Circulating monocytes (Mo) play an essential role in the host immune response to chronic infections. We previously demonstrated that CD16^{pos} Mo were expanded in TB (tuberculosis) patients, correlated with disease severity and were refractory to dendritic cell differentiation. In the present study, we investigated whether human Mo subsets (CD16^{neg} and CD16^{pos}) differed in their ability to influence the early inflammatory response against *Mycobacterium tuberculosis*. We first evaluated the capacity of the Mo subsets to migrate and engage a microbicidal response *in vitro*. Accordingly, CD16^{neg} Mo were more prone to migrate in response to different mycobacteria-derived gradients, were more resistant to *M. tuberculosis* intracellular growth and produced higher reactive oxygen species than their CD16^{pos} counterpart. To assess further the functional dichotomy among the human Mo subsets, we carried out an *in vivo* analysis by adapting a hybrid mouse model (SCID/Beige, where SCID is severe combined immunodeficient) to transfer each Mo subset, track their migratory fate during *M. tuberculosis* infection, and determine their impact on the host immune response. In *M. tuberculosis*-infected mice, the adoptively transferred CD16^{neg} Mo displayed a higher lung migration index, induced a stronger pulmonary infiltration of murine leucocytes expressing pro- and anti-inflammatory cytokines, and significantly decreased the bacterial burden, in comparison with CD16^{pos} Mo. Collectively, our results indicate that human Mo subsets display divergent biological roles in the context of *M. tuberculosis* infection, a scenario in which CD16^{neg} Mo may contribute to the anti-mycobacterial immune response, whereas CD16^{pos} Mo might promote microbial resilience, shedding light on a key aspect of the physiopathology of TB disease.

Key words: bacterial infection, cell movement, monocytes, *Mycobacterium* infection, pulmonary infection, tuberculosis

INTRODUCTION

Mycobacterium tuberculosis is the aetiological agent of TB (tuberculosis) that remains one of the most devastating human diseases being responsible for ~1.5 million deaths worldwide each year. Although TB mortality is slowing declining each year, it is still unacceptably high. In fact new, faster and better drugs are urgently needed to better control and deal with TB

Abbreviations: 7-AAD, 7-aminoactinomycin D; APC, allophycocyanin; BAL, bronchial alveolar lavage; BMDM, bone-marrow-derived-macrophage; CCR, CC chemokine receptor; CFU, colony-forming unit; DHR, dihydrorhodamine 123; F-actin, filamentous actin; IL, interleukin; Mo, monocyte(s); OADC, oleic acid/albumin/dextrose/catalase; PE, phycoerythrin; PMN, polymorphonuclear cell; SCID, severe combined immunodeficient; TB, tuberculosis; TGFβ, transforming growth factor β; TNFα, tumour necrosis factor α.

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disease. The success of *M. tuberculosis* infection depends mainly on its ability to elude host immune responses, such as hampering the development of efficient antigen-presenting cells [1]. In this regard, as monocytes (Mo) can replenish tissue antigen-presenting cells (i.e. macrophages and dendritic cells), on demand [2], they represent an ideal target for immune evasion by *M. tuberculosis*.

Human blood Mo are conventionally subdivided into two major subsets based on CD16 expression: $CD14^{+}CD16^{-}$ ($CD16^{neg}$) and $CD14^{+}CD16^{+}$ ($CD16^{pos}$) [3]. We have demonstrated previously that human $CD16^{pos}$ Mo preferentially expand in TB patients, correlate with disease severity [4] and are refractory to dendritic cells differentiation [5]. Furthermore, although CCR5 (CC chemokine receptor 5) and CCR2 are reported to be highly expressed on $CD16^{pos}$ and $CD16^{neg}$ Mo respectively in healthy donors [6,7], we have found that these receptors are similarly expressed in both Mo subsets from patients with severe TB [4], suggesting a major alteration in the chemokine receptor repertoire as a consequence of disease. Together, these results suggest that different biological roles, including the migration capacity, among human Mo subsets might be modulated by the infection with *M. tuberculosis*.

Much of our knowledge on Mo migration and function during *M. tuberculosis* infection derives from the mouse model. Murine Mo subsets are distinguished as the 'inflammatory' $Ly6C^{high}$ and the 'patrolling' $Ly6C^{neg}$ cells. Through gene expression profiling, the $CD16^{neg}$ and $CD16^{pos}$ human Mo subsets have been correlated and compared with mouse $Ly6C^{high}$ and $Ly6C^{neg}$ Mo respectively [8,9]. Interestingly, $Ly6C^{high}$ Mo were found to be the major inducible nitric oxide synthase-producing cell population during pulmonary *M. tuberculosis* infection [10], and thus they may directly defend against mycobacterial infection. Also, increasing evidence implicates $Ly6C^{high}$ Mo in the activation and differentiation of CD4 T-cell responses to mycobacterial infections [11,12]. In spite of this, it was also shown that the CCL2 (CC chemokine ligand 2)–CCR2-dependent recruitment of Mo can be deleterious in part because they harbour live *M. tuberculosis* impairing microbial clearance [13]. Collectively, these reports not only have improved our understanding of murine Mo biology during mycobacterial infections, but also argue for the need to translate this knowledge into the human context. For this purpose, multiple strains of immunodeficient mice have been developed [14–16], such as SCID/Beige (where SCID is severe combined immunodeficient) mice, which lack T- and B-lymphocytes, and have a defect in lysosomal biogenesis. Indeed, the SCID/Beige strain has been successfully used to investigate the role of human Mo subsets in the peritoneal cavity during inflammation [17].

In the present study, we investigated whether human Mo subsets could contribute differently to the early immune response against *M. tuberculosis*. For this purpose, we employed two approaches: (i) an *in vitro* characterization of the capacity of the Mo subsets to migrate and engage a microbicidal response in the TB context; and (ii) an *in vivo* analysis through the adaptation of a hybrid mouse model (SCID/Beige) to transfer each human Mo subset, track their migratory fate during *M. tuberculosis* infection, and determine their impact on the host immune response.

MATERIALS AND METHODS

Ethics statements

The research with human samples was carried out in accordance with the Declaration of Helsinki (2013) of the World Medical Association (further information can be found in the Supplementary Online Data). Experiments involving animals were conducted according to institutional and national guidelines (see the Supplementary Online Data for further details).

Mice

C.B-17 SCID/Beige mice (Taconic Farms, Germantown, NY, U.S.A.) are homozygous for two genetic mutations which render them immunologically incompetent. They have a combined effect of *scid* (no T- and B-cells) and *beige* (defect in lysosomal trafficking regulator gene) mutations. Also, the *beige* mutation results in an impairment of NK (natural killer) cell activity [18]. Consequently, these hybrid mice readily accept foreign cells.

M. tuberculosis growth

All procedures were performed in a laminar flow hood in a biosafety level III laboratory. *M. tuberculosis* H37Rv was grown in Middlebrook 7H9 broth (Difco) supplemented with 10% ADC (albumin/dextrose/catalase) (Difco), 0.05% Tween 80 and 0.5% glycerol to mid-exponential phase before freezing at $-80\,^{\circ}C$. Multiple vials were stored at $-80\,^{\circ}C$ until further use. Frozen *M. tuberculosis* stocks were freshly thawed and reconstituted at room temperature at the time of infection. A vial of stock bacilli was diluted in saline to a final concentration of $(2–4)\times10^{5}$ CFU (colony-forming units) in 0.1 ml (for *in vivo* infections) or $(1–10)\times10^{4}$ CFU in 0.02 ml (for *in vitro* infections). Before infection, each stock vial was plated for CFU enumeration to reconfirm the standard concentration of the inoculum. The CFU concentration of stock vials was calculated by serial dilution and plating in multiple replicates on Middlebrook 7H11 agar (Difco) containing 0.5% glycerol and 10% OADC (oleic acid/albumin/dextrose/catalase) growth enrichment (Becton Dickinson). Plates were incubated for 21 days at $37\,^{\circ}C$ before determination of CFU.

Human Mo subsets isolation

PBMCs (peripheral blood mononuclear cells) were obtained by Ficoll density gradient (Nycomed Pharma) of buffy coats from healthy donors after signed informed consent. Human $CD16^{neg}$ and $CD16^{pos}$ Mo were purified by magnetic-activated cell sorting technology (Miltenyi Biotec), as reported previously [17]. Staining with anti-CD14 and anti-CD16 monoclonal antibodies showed >95% $CD16^{neg}$ Mo in the $CD16^{neg}$ fraction and >85% $CD16^{pos}$ Mo in the $CD16^{pos}$ fraction.

Transmembrane migration assay

Human Mo of each subset (4×10^{5} cells in 75 μl) were placed in the upper chamber of a Transwell insert (5 μm pore size, 96-well plate; Corning Costar), and 230 μl of medium (RPMI 1640 with 0.5% FBS), supernatants from *M. tuberculosis*-stimulated pulmonary epithelial cells (A549) and PMNs (polymorphonuclear cells) or cell-free pleural effusions from TB patients were

placed in the lower chamber. After 3 h, cells that had migrated to the lower chamber were removed and analysed. The relative number of cells migrating was determined on a flow cytometer using Calibrite beads (BD Biosciences), where a fixed number of beads was included in each sample and the number of cells per 1000 beads was evaluated. Data were normalized to the number of initial cells.

3D migration assays

For 3D migration assays, 100 μl of Matrigel^{TM} (BD Bioscience) were polymerized in Transwell inserts as described previously [19]. The top of each matrix insert was seeded with 5×10^4 CD16^{neg} or CD16^{pos} Mo. The lower Transwell chamber was filled with both 250 μl of medium (RPMI 1640 with 0.5 % FBS), and with 250 μl of either pleural fluid effusion from TB patients or supernatants from *M. tuberculosis*-infected pulmonary epithelial cells (A549), PMNs or BMDMs (bone-marrow-derived-macrophages). As control, we used 500 μl of medium from non-infected cells. Cell migration was counted after 72 h, as described previously [19]. The percentage of cell migration was obtained as the ratio of cells within the matrix to the total number of counted cells.

Infection of SCID/Beige mice by *M. tuberculosis*

Briefly, 8–14-week-old SCID/Beige mice were anaesthetized in a gas chamber using sevofluorane at 0.1 ml/mouse, and each mouse was infected by orotracheal instillation with $(2–4)\times10^5$ live bacilli. In parallel, some mice were simultaneously transferred with human CD16^{neg} and CD16^{pos} Mo ($1–5\times10^6$) by orotracheal instillation. Mice were maintained on the holder in a vertical position until normal breathing. Infected mice were maintained in groups of three to five in cages fitted with micro-isolators.

Determination of CFU from tissues

Cell suspensions from lungs and BAL (bronchial alveolar lavage) were thawed rapidly and four serial dilutions of each were made. Dilutions were spread on to duplicate plates containing Bacto Middlebrook 7H10 agar enriched with OADC. Plates were incubated for 21 days before determination of CFU.

Histopathological studies

For histology and automated morphometry analysis, left lungs were fixed by intratracheal perfusion with 4 % formaldehyde for 48 h, sectioned through the hilum and embedded in paraffin. Sections of 4 μm thickness were stained with haematoxylin and eosin. The percentages of infiltrating leucocytes were estimated using an automated image analyser (Q Win Leica).

Immunohistochemistry

Lung paraffin-embedded sections from *M. tuberculosis*-infected SCID/Beige mice were deparaffinized and rehydrated. Heat-induced antigen retrieval was achieved by incubating sections with 10 mM sodium citrate, pH 6.0, in a pressure cooker. The endogenous peroxidase activity was blocked twice by incubating with 6 % H_2O_2 in methanol for 10 min. After blocking with Universal Blocking Buffer (Bio SB), tissue sections were incubated with primary antibodies for 1 h at optimal dilutions determined previously. Primary antibodies against TNFα (tumour necrosis factor α) (rabbit polyclonal IgG), IL-1β (interleukin 1β) (goat polyclonal IgG), TGFβ (transforming growth factor β) (rabbit polyclonal IgG) and IL-10 (goat polyclonal IgG) were used, all purchased from Santa Cruz Biotechnology. Secondary biotinylated antibodies (biotin-conjugated anti-rabbit IgG or biotin-conjugated anti-goat IgG) were used to detect the binding of the primary antibodies and HRP (horseradish peroxidase)-conjugated avidin and DAB (3,3-diaminobenzidine)/H_2O_2 were employed to develop the reaction. Tissue sections were counter-stained with haematoxylin. For morphometry, 15 random fields from each section were studied. The percentage of positive cells for each cytokine was determined using an automated image analyser (Q Win Leica).

Flow cytometry

Cells were incubated with 20 μl of Fc-receptor-blocking agent (Octagam, Octapharma) for 20 min. For the identification of human cells in mice tissue, cells were incubated with anti-human HLA-DR PE (phycoerythrin)-conjugated antibodies (clone G46-6; BD Biosciences) for 30 min at 4 °C. This monoclonal antibody does not cross-react with murine cells [17]. Data were acquired in a Guava Easy flow cytometer (Millipore) and analysed using FCS Express Software (De Novo Software). For the phenotypification of murine cells, cells were incubated with the following antibodies: anti-Ly6C [clone AL-21, APC-Cy7 (allophycocyanin/indotricarbocyanine-conjugated); BD Biosciences], anti-Ly6G (clone 1A8, PE-conjugated; BD Biosciences), anti-CD11b (clone M1/70, FITC-conjugated; eBioscience), and anti-CD11c (clone CHL3, APC-conjugated; BD Pharmingen) for 30 min at 4 °C. Data were acquired in a Cyan ADP flow cytometer.

Kinetics of intracellular replication of *M. tuberculosis* in human Mo

Human Mo of each subset were allow to adhere in 96-well flat-bottomed plates and were cultured in RPMI 1640 with 10 % (v/v) FBS for 24 h (10^5 in 0.2 ml). Adherent cells were co-cultured in triplicate with bacilli at 1:1 ratio. After 2 h of incubation, extracellular bacteria were removed gently by washing four times with pre-warmed PBS. After 2, 24, 48 or 96 h, infected BMDMs were subjected to complete lysis by mixing with 0.1 ml of 0.1 % SDS and incubated at room temperature for 10 min. Lysates were mixed thoroughly ten times and neutralized by the addition of 0.1 ml of 20 % BSA in Middlebrook 7H9 broth. Thereafter lysates were serially diluted and plated, in triplicate, on 7H11 agar plates for CFU determination.

Oxidative burst generation by *M. tuberculosis*-stimulated human Mo subsets

Intracellular reactive oxygen species levels were measured by DHR (dihydrorhodamine 123) assays (Sigma). DHR was dissolved in DMSO (20 μg/ml) and stored in aliquots at −70 °C until use. Briefly, 5×10^5 CD16^{neg} and CD16^{pos} were incubated with 0.1 ml of DHR (5 μg/ml) for 15 min at 37 °C. Afterwards, the γ-irradiated *M. tuberculosis* H37Rv strain, kindly provided by Dr J. Belisle (Colorado State University, Fort Collins, CO,

U.S.A.), was added to the culture at a *M. tuberculosis*/Mo ratio of 5:1 for an additional 20 min. Then, the fluorescent derivative rhodamine 123, was determined by FACS.

F-actin (filamentous actin) polymerization assay

CD16^{neg} or CD16^{pos} Mo isolated from healthy donors were adjusted to a concentration of 2×10^5 cell in 50 μl of PBS and they were left to rest for 30 min at 37 °C. Thereafter cells were treated with 50 μl of cell-free pleural effusion or PBS for different times (0, 5, 15, 60 and 120 s). At the indicated time points, 50 μl of 12 % (w/v) paraformaldehyde was added for 10 min at room temperature. Cells were washed and incubated for an additional 20 min with a solution containing 0.4 μM FITC-labelled phalloidin and 0.1 % Triton X-100 in PBS. The fixed cells were analysed by flow cytometry within 1 h of labelling. Alternatively, F-actin-stained Mo were attached to slides via Cytospin for their microscopic observation.

Statistical analysis

One-tailed paired or unpaired Student's *t* test was applied on datasets with a normal distribution, whereas one-tailed Mann–Whitney (unpaired test) or Wilcoxon (matched-paired test) tests were used otherwise (Prism software). $P < 0.05$ was considered as the level of statistical significance.

RESULTS

In vitro trafficking properties of human CD16^{neg} and CD16^{pos} Mo in response to mycobacteria-induced chemokine gradients

During pathogenic challenge, Mo exit the circulation and migrate towards the site of infection through different environments (e.g. intra-tissular) in response to chemoattractant gradients. Taking into account the differential chemokine receptors repertoire displayed by Mo subsets [4,20], we assessed the capacity of CD16^{neg} and CD16^{pos} Mo to migrate in 2D and 3D environments in response to *M. tuberculosis*-induced chemoattractant gradients. For these *in vitro* migration assays, isolated human Mo subsets were seeded on to the upper chamber of a Transwell system either naked to assess 2D migration (Figure 1A) or filled with a Matrigel matrix (Figure 1B) extracted from tumours, with the lower compartment containing either cell-free pleural fluid effusion from TB patients or conditioned medium derived from *M. tuberculosis*-infected pulmonary epithelial cells (A549) or PMNs. Thereafter migrating cells were quantified. As illustrated in Figure 1(A), CD16^{neg} Mo migrated more efficiently than CD16^{pos} Mo in response to all chemoattractant factors. Across Matrigel, the movement of cells requires proteolytic degradation of the extracellular matrix to create paths and is qualified as 'mesenchymal' [19]. As demonstrated in Figure 1(B), CD16^{neg} Mo were more prone to migrate through Matrigel in response to mycobacteria-derived factors than CD16^{pos} Mo. Since it is recognized that a rapid conversion of G-actin (globular actin) to F-actin is triggered in the initial phase of the chemotactic response [21], we then measured the effect of cell-free pleural fluid effusion from TB patients on actin polymerization in both Mo subsets. In line with their higher motility in different environments, CD16^{neg} Mo increased their F-actin content after exposure to tuberculous pleural fluid effusion in contrast with that observed for CD16^{pos} Mo (Figures 1C and 1D). Another important feature in the Mo recruitment into tissues is the interaction with endothelial and epithelial cells. Then we assessed their capacity to interact with alveolar epithelial cells, and we observed no differences among the Mo subsets in their ability to adhere to A549 cells even when epithelial cells were pretreated with irradiated *Mtb*, cell-free pleural effusions from TB patients or recombinant human TNF-α (Supplementary Figure S1). Therefore, despite showing a comparable ability to interact with lung epithelial cells, both Mo subsets differ in their migratory behaviour with the CD16^{neg} subset being capable of readily responding to mycobacteria-induced chemokine gradients unlike the CD16^{pos} subset.

Microbicidal activities displayed by Mo subsets

Since the antimicrobial activity of human Mo is crucial for the eradication of intracellular pathogens, we investigated whether Mo subsets differ in their ability to control *M. tuberculosis* intracellular growth. We found fewer bacteria growing in CD16^{neg} Mo in comparison with their CD16^{pos} counterpart at different times post-infection when considering the CFU recovered from the whole Mo lysates (Figure 1E). To take into account that *M. tuberculosis*-infected CD16^{pos} Mo display a higher mortality rate than CD16^{neg} Mo [22], we also measured bacterial growth per cell. Accordingly, we confirmed that the CD16^{pos} were more permissive to *M. tuberculosis* replication than CD16^{neg} (Figure 1F). On the basis of these results, we then examined the oxidative microbicidal activity of Mo subsets in response to irradiated *M. tuberculosis*; the killed version was used in order to avoid the well-described suppressive effects mediated by live *M. tuberculosis* [23]. To this end, the conversion of DHR into its fluorescent form triggered by reactive oxygen species such as peroxide and peroxynitrite was analysed in CD16^{neg} and CD16^{pos} Mo after a 20-min challenge with irradiated *M. tuberculosis*. In line with the results from CFU assays, CD16^{neg} Mo induced higher production of oxygen reactive species after *M. tuberculosis* exposure (Figure 1G). Altogether, these results indicate that human Mo subsets differ drastically in the irability to eliminate *M. tuberculosis*.

Potential of CD16^{neg} and CD16^{pos} Mo to traffic from the alveolar spaces to lung parenchyma in an *in vivo* environment

In order to assess the migration patterns of human Mo subsets *in vivo*, we transferred human CD16^{neg} or CD16^{pos} Mo orotracheally into *M. tuberculosis*-infected SCID/Beige mice, and compared their abilities to traffic from the alveolar spaces to the lung parenchyma (see the Supplementary Online Data, Figures S2-4, for further details on the human Mo engraftment). After 24 h, human cells from BAL and lungs were quantified as the ratio between the numbers of recovered HLA-DR^{pos} cells and total inoculated cells. Under infectious conditions, the percentages of HLA-DR^{pos} cells recovered from CD16^{neg} Mo-transferred mice were higher in lungs (Figures 2A and 2B) and lower in BAL (Figures 2C and 2D), which is consistent with the induction of cell recruitment from the alveolar space to the lung parenchyma

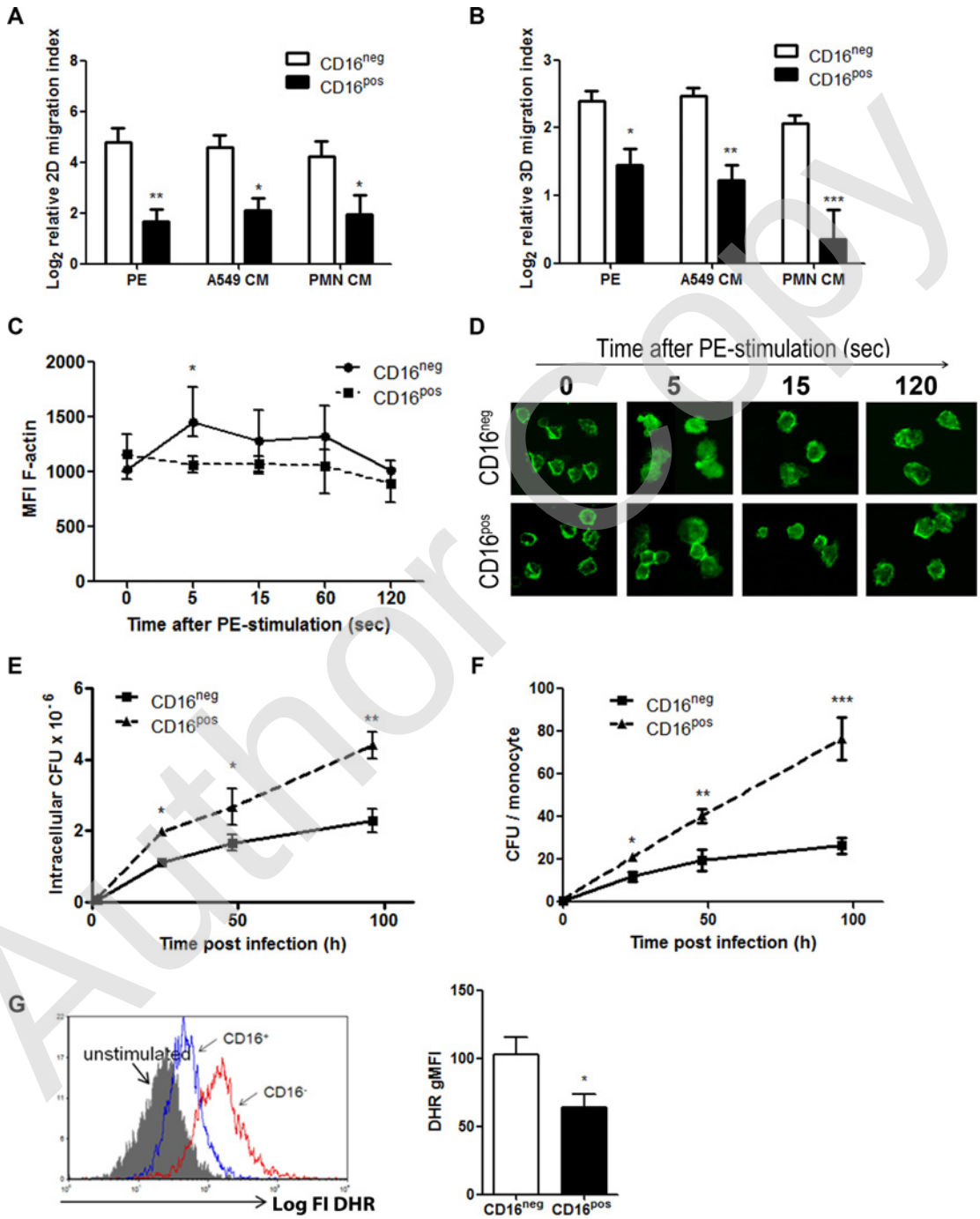

Figure 1 **Migration and microbicidal properties of CD16^{neg} and CD16^{pos} Mo in response to *M. tuberculosis***
Isolated human Mo subsets were seeded on to the upper chamber of a Transwell system (**A**) or on the Matrigel surface (**B**) in response to cell-free pleural effusions from TB patients (PE), conditioned medium (CM) from *M. tuberculosis*-stimulated pulmonary epithelial cells (A549 CM) or PMN (PMN CM). The relative migration index, defined as the number of migrating cells under an inflammatory condition relative to the control medium, is shown for each experimental condition. Results are presented as $\log_2$ ratios, and represent the average for five independent donors of monocytes. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (**C**) Time-course analysis of actin polymerization in human Mo subsets after PE exposure expressed as specific mean fluorescence intensity (MFI). Results are the average for four independent experiments. $^{*}P < 0.05$, CD16^{neg} Mo compared with CD16^{pos} Mo. (**D**) Representative images of F-actin stained in pleural effusion (PE)-treated Mo subsets for the indicated periods. (**E** and **F**) Kinetics of the intracellular CFU obtained from CD16^{neg} or CD16^{pos} Mo infected with *M. tuberculosis*, expressed as absolute CFU numbers (**E**) and CFU numbers relative to viable Mo numbers (**F**). Results are means ± S.E.M. for three independent experiments, each carried out in triplicate. $^{*}P < 0.05$. (**G**) Levels of respiratory burst estimated by DHR fluorescence of unstimulated and *M. tuberculosis*-stimulated CD16^{neg} and CD16^{pos} Mo. A representative histogram is shown. Results are the geometric mean ± S.E.M. fluorescence intensity values for eight independent experiments. $^{*}P < 0.05$.

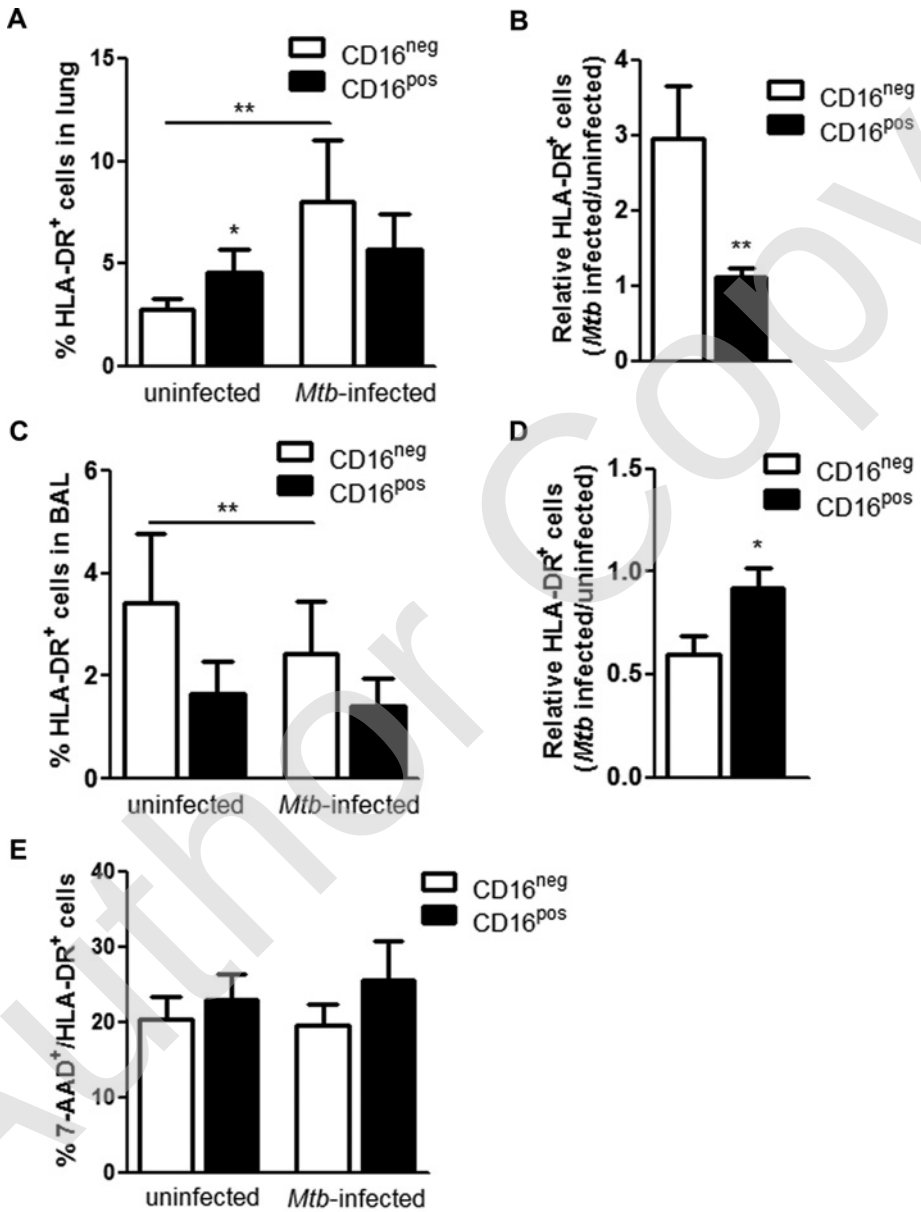

Figure 2 Migration patterns of human Mo subsets in an *in vivo* environment
Percentages of HLA-DR^{pos} human cells derived from CD16^{neg} or CD16^{pos} Mo recovered from lungs (**A**) and BAL (**C**) in uninfected or *M. tuberculosis* (*Mtb*)-infected SCID/Beige mice determined by flow cytometry. Percentages were calculated as numbers of recovered HLA-DR^{pos} cells and total inoculated human cells. Relative recovery of human cells derived from CD16^{neg} or CD16^{pos} Mo of lungs (**B**) and BAL (**D**) were estimated as the numbers of recovered HLA-DR^{pos} cells from *M. tuberculosis* (*Mtb*)-infected mice relative to uninfected mice. Results are means ± S.E.M. for ten independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, CD16^{neg} Mo compared with CD16^{pos} Mo, or comparisons are indicated by lines. (**E**) Percentages of death cells (7-AAD^{pos}) among HLA-DR^{pos} human cells derived from CD16^{neg} or CD16^{pos} Mo recovered from lungs in uninfected or *M. tuberculosis* (*Mtb*)-infected SCID/Beige mice determined by flow cytometry. Results are means ± S.E.M. for five independent experiments.

in response to the infection. Unlike CD16^{neg} Mo, the recovery of HLA-DR^{pos} cells in lungs and BAL from CD16^{pos} Mo-transferred mice did not differ between steady-state and infectious conditions (Figures 2B and 2D). In fact, the percentages of CD16^{pos} Mo recovered under steady-state conditions were higher in lungs and lower in BAL compared with CD16^{neg} Mo (Figures 2A and 2C). Considering that CD16^{pos} circulating Mo are prone to undergo cell death [22], we tested whether this process could be involved during the engraftment of human Mo under infectious conditions. However, no differences between the percentages of 7-AAD^{pos}/HLA-DR^{pos} (7-AAD is 7-aminoactinomycin D) cells recovered from lungs of CD16^{neg} and CD16^{pos} Mo-transferred SCID/Beige mice were found (Figure 2E). Altogether, these results suggest that, unlike human CD16^{pos} Mo, the CD16^{neg} subset is specifically predisposed to respond to *M. tuberculosis* infection by increasing their mobility towards the infected site.

As we transferred human cells into a xenogeneic host, we wondered whether the putative murine orthologue Mo subsets follow the same migration pattern as their human counterparts upon *M. tuberculosis* infection. To this end, we evaluated the dynamics of the murine Ly6C^{high} Mo subset in different tissues from *M. tuberculosis*-infected BALB/c mice. As depicted in Supplementary Figure S5, the percentage of Ly6C^{high} Mo increased in lungs and BAL at early stages of the infection correlating with its decrease in blood, suggesting that this subset migrates very early from blood to the infected site. On the basis of the proposed homology of Mo subsets between species, this result may be in line with our *in vitro* assays described above.

Effect of human CD16^{neg} or CD16^{pos} Mo on the outcome for *M. tuberculosis*-infected SCID/Beige mice

Having found a differential migration pattern to the site of infection between human Mo subsets, we wondered whether the presence of CD16^{neg} or CD16^{pos} Mo during the early stage of the inflammatory response would affect the outcome of the infection against *M. tuberculosis*. We first determined the production of cytokines by immunohistochemistry in lung tissue sections from CD16^{neg} or CD16^{pos} Mo-transferred mice infected (or not) compared with mice that were also infected (or not), but lack human Mo. *M. tuberculosis*-infected mice showed numerous large macrophages with abundant cytoplasm and peripheral nucleus corresponding to activated cells (Figure 3, right-hand panels). These cells were located in the alveolar capillary interstitium and alveolar spaces, being more abundant in CD16^{neg} Mo-transferred mice. We found higher levels of the anti-inflammatory cytokines IL-10 and TGFβ in the lung of CD16^{neg} Mo-transferred mice compared with CD16^{pos} Mo-transferred (Figures 3A and 3B), whereas both Mo subsets induce high levels of TNFα and IL-1β (Figures 3C and 3D). Interestingly, uninfected CD16^{pos} Mo-transferred mice showed high basal IL-1β production.

Another feature of the early inflammatory response is the recruitment of leucocytes to the site of infection. This was evaluated by measuring the infiltration area of murine leucocytes after haematoxylin and eosin staining of lung tissue sections. Under steady-state conditions, CD16^{pos} Mo induced higher recruitment of leucocytes; under infectious conditions, however, the CD16^{neg} Mo induced higher recruitment of leucocytes (Figures 4A and 4B). Therefore, although the transfer of CD16^{pos} Mo was enough to induce the recruitment of IL-1β^{pos} leucocytes in lungs, CD16^{neg} Mo transfer promotes leucocyte attraction and cytokine production upon infection with *M. tuberculosis*.

Finally, the bacterial load associated with airway tissue (lungs and BAL) in CD16^{neg} and CD16^{pos} Mo-transferred infected mice was compared. As shown in Figure 4(C), a significant decrease in the bacterial load was observed in the lungs from mice with adoptive transfer of CD16^{neg} Mo compared with non-transferred or CD16^{pos} Mo-transferred mice. Reciprocally, higher bacillary loads were detected in BAL from mice with adoptive transfer of CD16^{pos} Mo (Figure 4D).

DISCUSSION

Inflammatory cell recruitment is essential for anti-tuberculous host immune defence. In particular, Mo are highly sensitive and reactive to pathogen-derived molecules, and can respond quickly to microbial stimuli to inhibit pathogens at early stages of infection. In the present study, we have shown using different experimental approaches that human Mo subsets have different migration pattern to lungs in response to *M. tuberculosis* infection. In particular, we found that CD16^{neg} human Mo were more prone to migrate in response to mycobacteria-derived gradients. These results are supported further by the restricted actin fibre polymerization induction found in CD16^{neg} Mo upon stimulation with tuberculous pleural effusions, which is central for regulating cellular motility [24]. Despite this, human Mo subsets show a comparable ability to cross the endothelium [25] and to attach to the alveolar epithelium as we have shown. Therefore CD16^{neg} Mo may have enhanced responsiveness to *M. tuberculosis*-induced chemoattractants in comparison with CD16^{pos} Mo. Differences in the concentration of molecular components such as adhesion receptors, cytoskeletal-linking proteins and extracellular matrix ligands may explain the qualitative differences in migratory behaviour between Mo subsets [26]. Hence we propose that, at early events after *M. tuberculosis* infection, CD16^{pos} Mo may display less expression of chemokine receptors that promote the infiltration to lungs compared with CD16^{neg} as stated by others [27]. Yet, it is possible that this unresponsiveness to *M. tuberculosis*-induced chemoattractants displayed by CD16^{pos} Mo may be overcome as the infection progresses, which would be in agreement with our previous finding that CD16^{pos} Mo acquired CCR2 expression in TB patients with severe disease [4].

The immune system in both human and murine contexts is highly conserved and functions in a similar fashion. Nevertheless, when a particular immune cell subtype is compared between the two species, they may display distinct characteristics. Considering that the individual biological functions of human Mo subsets *in vivo* are not completely understood [9], we decided to use the SCID/Beige mice hybrids for modelling the effect of human Mo transference on the outcome of *M. tuberculosis* infection. In fact, we could study the migration of human Mo in response to *M. tuberculosis* infection. This is in agreement with other experimental approaches on humanized mice models in which human bone marrow precursors could differentiate into tissue macrophages, including pulmonary macrophages, under physiological and inflammatory conditions [28,29]. In our hands, this SCID/Beige mice model allows for rapid analysis of human immune cell behaviour because the transferred cells are short-lived and functionally mature. Considering the poor recovery of human-derived cells obtained when transferring the Mo by intravenous route into SCID/Beige mice, we inoculated them orotracheally and examined the migration route from the alveolar space to the lung interstitium. In fact, it has been reported that macrophages transmigrate back to the basal side of the epithelium after phagocytosis in order to participate in the presentation of antigens [30], thus transmigration back to the basal side of the epithelium is an important behaviour of macrophages [31]. In our SCID/Beige model, CD16^{neg} Mo were able to migrate to

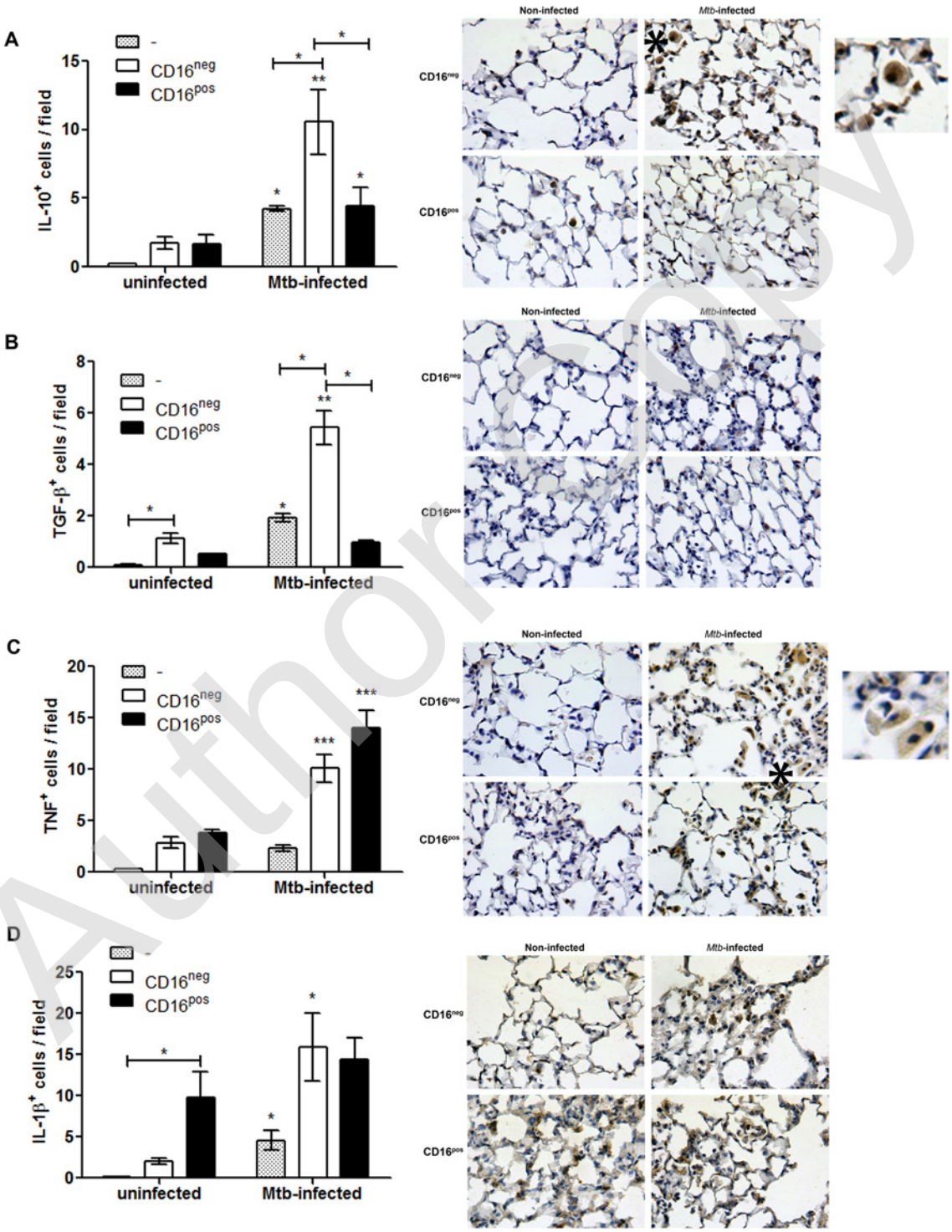

Figure 3 Modulation of local cytokine production by CD16^{neg} and CD16^{pos} Mo

The production of IL-10 (**A**), TGFβ (**B**), TNFα (**C**) and IL-1β (**D**) in lungs from non-transferred (–), or CD16^{neg}- or CD16^{pos}-transferred *M. tuberculosis* (Mtb)-infected or uninfected SCID/Beige mice determined by immunohistochemistry. The percentages of positive cells per field are means ± S.E.M. for eight independent experiments, considering 15 fields. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, *M. tuberculosis*-infected compared with uninfected, or comparisons are indicated by lines. Representative micrographs of cytokines detection by immunohistochemistry comparing infected and non-infected mice, transferred with CD16^{neg} or CD16^{pos} Mo are shown (×400 magnification, haematoxylin counterstain). Macrophages showing an activated phenotype are indicated (asterisks) in *M. tuberculosis* infected mice transferred with CD16^{neg} Mo.

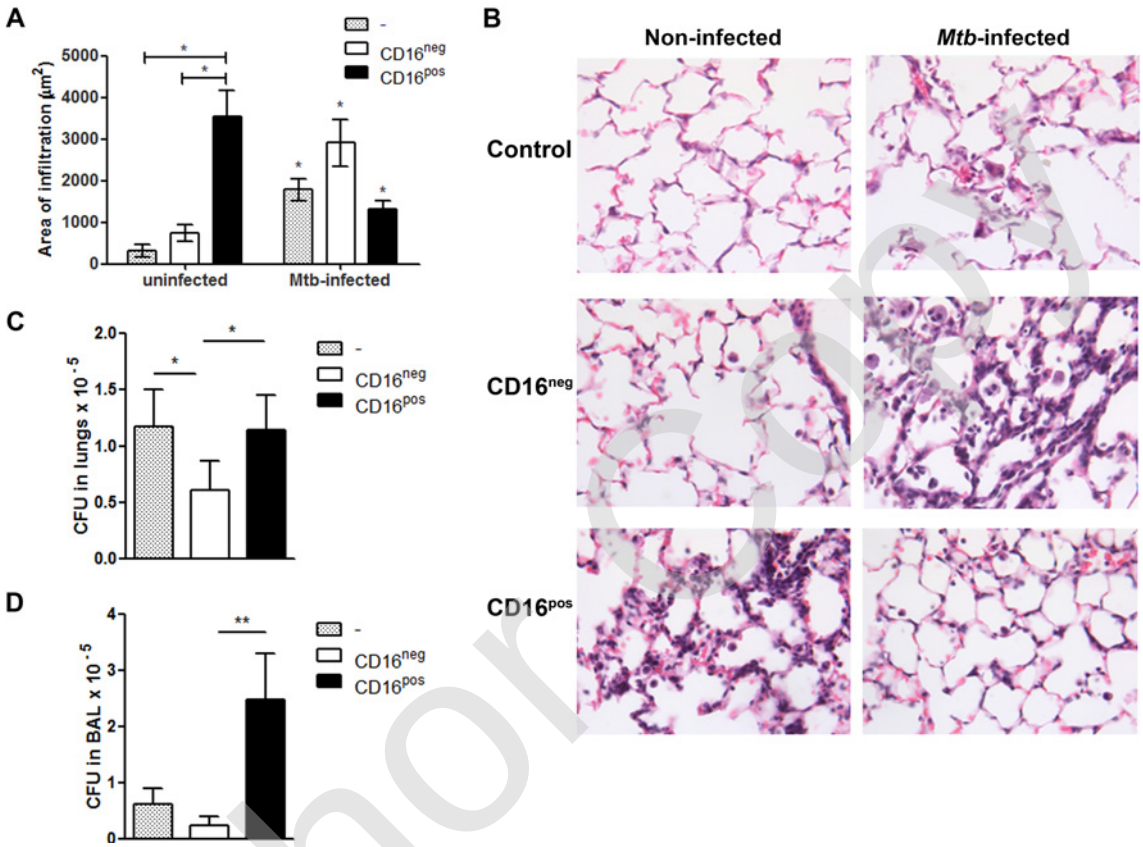

Figure 4 **Impact on pulmonary cell recruitment and mycobacterial loads after CD16^{neg} and CD16^{pos} Mo transference**
(**A**) Morphometric analysis of the inflammatory infiltrate into lungs from non-transferred (−), or CD16^{neg}- or CD16^{pos}-transferred *M. tuberculosis* (Mtb)-infected or uninfected SCID/Beige mice. Results are means ± S.E.M. for five independent experiments, considering 15 random fields. (**B**) Representative micrographs showing the histopathological findings in the lungs from non-transferred (control), or CD16^{neg}- or CD16^{pos}-transferred *M. tuberculosis* (*Mtb*)-infected or uninfected SCID/Beige mice (×400 magnification, haematoxylin and eosin staining). (**C** and **D**) Bacterial burdens were determined in the lungs (**C**) and BAL (**D**) in non-transferred (−), or CD16^{neg}- or CD16^{pos}-transferred *M. tuberculosis*-infected SCID/Beige mice at 24 h post-infection. Results are means ± S.E.M. for eight independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control.

the infected site more efficiently than CD16^{pos} Mo, supporting the differential migration patterns observed *in vitro*.

A limitation of our model is the absence of adaptive immune response from these immunocompromised mice. In this regard, our results require careful consideration given that the effector function of Mo in a normal inflammatory milieu may be significantly different from what we have observed in the present study. Also, this limitation impedes an examination of the effect of each human Mo subset on the initiation of the acquired immune response. Interestingly, it has been demonstrated that infected inflammatory Mo-derived dendritic cells are essential for the transport of *M. tuberculosis* to the local lymph node that, although being relatively inefficient at activating CD4^{+} T-cells, they can release *M. tuberculosis* antigens for uptake and presentation by uninfected resident lymph node dendritic cells [12,32]. These results support the hypothesis that different Mo subsets are committed to specific functions not only at early stages of the infection, as we have demonstrated, but also when the priming of antigen-specific CD4^{+} T-cells may take place. In the present study, we observed a high influx of Ly6C^{high} Mo into lungs and BAL in parallel with a marked decrease in blood at the 14th day post-infection (Supplementary Figures S4C and S4D), when the adaptive immune response is supposed to be initiated [33]. This result suggests that the arrival of this subset at the infected site could be important to modulate the acquired immune response, supporting the already demonstrated essential role of CCR2^{+} inflammatory Mo during *M. tuberculosis*-specific T-cell priming [32]. Moreover, it has been shown recently that Ly6C^{low} Mo are precursors of pulmonary resident macrophages [34], which could be in agreement with our results assuming the equivalence between the murine Ly6C^{low} and the human CD16^{pos} Mo subsets. Although we do not provide direct evidence for this homology, our results support previous reports that postulate Ly6C^{high} as the homologue for CD16^{neg} Mo [9].

In general, tissue macrophages of differing ontological origins coexist, but whether resident and newly recruited macrophages possess similar functions during inflammation is unclear. The lung macrophage populations have been described as

alveolar macrophages, which originate from embryonic haemopoietic stem cells, and CD11b^{pos} macrophages of unknown origin [35]. It is well established that inflammation triggers blood Mo recruitment and differentiation into macrophages [2,35]. Therefore circulating Mo may replenish lung macrophages during TB. Intriguingly, our results suggest that the nature of the Mo subset together with environmental cues can dictate the macrophage functions.

We have observed an increased migration ability of CD16^{pos} human Mo under steady-state conditions in our *in vivo* model, which may be in agreement with their higher expression of genes connected to migration and transendothelial motility (*LSP1*, *LYN*, *CFL1* and *MYL6*), according to the transcriptome analysis in purified Mo subsets from human blood [36]. Despite this, it is important to take into consideration that the inoculation itself might alter steady state dynamics (i.e. injured tissue recruits Mo), making it difficult to draw conclusions on the behaviour of Mo subsets under steady-state conditions.

Additionally, we have shown that human Mo subsets can differently affect the host immune response against *M. tuberculosis*. The recruitment of leucocytes into the alveolar spaces and, even more so, the establishment of balanced pro- and anti-inflammatory cytokine production, are crucial for clearing infections and resolving the inflammatory response. In this regard, we demonstrated that Mo subsets display different abilities to orchestrate cytokine production by pulmonary cells and leucocyte recruitment. Whereas CD16^{neg} Mo induce a balanced production of cytokines together with leucocyte recruitment under infectious conditions, CD16^{pos} Mo induce IL-1β production and leucocyte recruitment under non-infectious conditions. The virulence of pathogens depends on their ability to fine-tune the inflammatory responses to a pitch that is optimal for *M. tuberculosis* growth [37]. Increased disease severity in humans can also occur for fundamentally opposite reasons: an inadequate host immune response to infection or an excessive one [38]. Therefore the balance of inflammation is critical for determining the outcome of infection at a systemic level. Virulent mycobacteria themselves have evolved to disrupt the fine balance of pro- and anti-inflammatory cues required for host protection [39]. In this sense, we speculate that the regulated inflammatory response triggered by CD16^{neg} Mo may prolong host survival by preventing immunopathology. Besides, both human Mo subsets display different abilities to control mycobacterial replication given that human CD16^{pos} Mo are more permissive for *M. tuberculosis* growth and induce lower levels of respiratory burst after *M. tuberculosis* exposure. According to our previous results, the severity of TB in humans is associated with the progressive accumulation of CD16^{pos} Mo in the blood [4], leading to the predominance of a more permissive host cell and possibly contributing to the dissemination of *M. tuberculosis*. As we used Mo subsets isolated from healthy individuals, we consider that our results may explain the migration behaviours that take place during the early events of the infection, given that at advanced stages of the infection the chemokine receptors profile is altered in Mo from TB patients [4]. Therefore it is possible that these more permissive CD16^{pos} Mo may acquire an improved migration ability towards the infected site once the mycobacteria can govern the host–pathogen interaction and promote the disease. This hypothesis needs to be tested. While the present study may be of clinical importance and still requires further validation to compensate for the limitations imposed by our hybrid *in vivo* model, we propose a model for the dynamics of the influx of Mo subsets according to the stage of *M. tuberculosis* infection and how it may affect the ongoing immune response (Supplementary Figure S6).

Our results highlight two main aspects that should be taken into account when evaluating the different roles of Mo subsets in infectious diseases: (i) the existence of intrinsic functional plasticity between CD16^{pos} and CD16^{neg} Mo, including their inherent commitments; and (ii) the extrinsic functional plasticity driven by the specific microenvironment, which explains why a given subset can display different biological functions under steady-state or infectious conditions including immune evasion mechanisms triggered by the pathogen.

Collectively, these results provide clear evidence for a differential contribution of human Mo subsets throughout the course of the infection, shedding light on a key aspect of the physiopathology of TB. Whereas CD16^{neg} Mo may contribute to the antimycobacterial immune response, CD16^{pos} Mo might promote microbial resilience.

CLINICAL PERSPECTIVES

- In 2013, TB was considered the second leading cause of death from a single infectious agent (World Health Organization Global TB Report 2014). Although TB mortality is slowly declining each year, it is still unacceptably high, and, without new and improved TB treatment regimens, including treatment for those suffering from drug-resistant TB and co-infected with HIV/AIDS, the reduction and eventual eradication of the disease cannot be achieved.
- We demonstrate that human monocyte subsets play different roles in promoting or hampering the host immune responses to infection with *M. tuberculosis*.
- The further understanding of how monocyte subsets arrive to the infected site and what they do once there, can shed light on a key aspect of the physiopathology of TB and may help to inform future vaccine and drug development strategies.

AUTHOR CONTRIBUTION

Luciana Balboa, Carmen Sánchez-Torres, María del Carmen Sasiain and Rogelio Hernández-Pando conceived of and designed the experiments. Luciana Balboa, Jorge Barrios-Payan, Erika González-Domínguez, Claire Lastrucci, Geanncarlo Lugo-Villarino, Dulce Mata-Espinoza, Pablo Schierloh and Denise Kviatcovsky performed the experiments. Luciana Balboa, Claire Lastrucci, Geanncarlo Lugo-Villarino, Pablo Schierloh and Denise Kviatcovsky analysed the data. Olivier Neyrolles, Isabelle Maridonneau-Parini, Carmen Sánchez-Torres, María del Carmen Sasiain and Rogelio Hernández-Pando contributed reagents/materials/analysis tools. Luciana Balboa, Claire Lastrucci, Geanncarlo Lugo-Villarino, Olivier Neyrolles, Isabelle Maridonneau-Parini, Carmen Sánchez-Torres, María del

Carmen Sasiain and Rogelio Hernández-Pando contributed to the writing of the paper. All authors read and approved the final paper.

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