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Mycobacterium tuberculosis promotes T$_h$17 expansion *via* regulation of human dendritic cells toward a high CD14 and low IL-12p70 phenotype that reprograms upon exogenous IFN-γ

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Abstract

The capacity to develop protective immunity against mycobacteria is heterogeneously distributed among human beings, and it is currently unknown why the initial immune response induced against *Mycobacterium tuberculosis* (Mtb) does not provide proper clearance of this pathogen. Dendritic cells (DCs) are some of the first cells to interact with Mtb and they play an essential role in development of protective immunity against Mtb. Given that Mtb-infected macrophages have difficulties in degrading Mtb, they need help from IFN-γ-producing CD4+ T cells propagated *via* IL-12p70-producing DCs. Here we report that Mtb modifies human DC plasticity by expanding a CD14+ DC subset with weak IL-12p70-producing capacity. The CD14+ Mtb-promoted subset was furthermore poor inducers of IFN-γ by naive CD4+ T cells, but instead prompted IL-17A-producing RORγT+ CD4+ T cells. Mtb-derived peptidoglycan and mannosylated lipoarabinomannan partly recapitulated the subset partition induced by Mtb. Addition of IFN-γ, but neither IL-17A nor IL-22, which are potentially produced by Mtb-exposed γ/δ-T cells in mucosal linings, inhibited the differentiation toward CD14+ DCs and promoted high-level IL-12p70 in Mtb-challenged DCs. We conclude that Mtb exploits DC plasticity to reduce production of IL-12p70, and that this process is entirely divertible by exogenous IFN-γ. These data suggest that strategies to increase local IFN-γ production in the lungs of tuberculosis patients may boost host immunity toward Mtb.

Keywords: co-stimulatory molecules, IL-17, mannosylated lipoarabinomannan, monocytes, naive CD4 T cells

Introduction

Mycobacterium tuberculosis (Mtb) is spread through aerosols and enters the body through the lungs (1). Characteristic for the immune response against Mtb is the development of a granuloma in the lungs, consisting of an aggregate of various lymphocytes (2). This limits bacterial spread, but also limits interaction of immune cells with infected cells after the granuloma has formed. Therefore, the early immune response against Mtb is of vital importance to whether the infection will be cleared, or a latent infection will manifest. Obviously, the number of people able to naturally resist Mtb infection is not known, but with one-third of the world's population currently infected (3), clearly many people do not develop a proper early immune response against Mtb being able to clear the infection.

The first cells Mtb interacts with are considered to be alveolar macrophages and myeloid dendritic cells (DCs) (4–6). Normally when micro-organisms are taken up by macrophages they are degraded in the lysosomal compartment (7, 8). Mtb, however, has developed a technique to divert this by inhibiting fusion of the phagosomes with the lysosomes, and by inhibiting autophagy (9). Consequently, Mtb resides as an intracellular microbe surviving within lung-resident macrophages. Clearance of this type of pathogen most essentially requires a CD4+ T$_h$1 immune response, which is needed to activate the phagolysosomal process in infected macrophages in an antigen-specific manner, and further to provide help for CD8+ cytotoxic

T lymphocytes capable of killing infected cells (10–13). Because DCs are the only cells capable of activating naive CD4+ T cells, the DC response to Mtb is considered a critical factor for how the immune response to Mtb evolves. Unlike inside macrophages, Mtb is not able to grow inside DCs (14). Upon uptake of Mtb, DCs migrate to the draining lymph nodes (4, 6). Here they interact with naive T cells, presenting Mtb-derived antigen and providing co-stimulatory and differentiation signals for the T cells (15). After activation, T cells migrate back to the infection site, where they help control the infection (1). However, currently little is known about the phenotype of the T cells present at the site of infection at early stages.

DC-produced IL-12p70 is necessary for induction of a T_h1 response (16, 17), which is enhanced by IL-27 (18, 19). IL-1β, IL-6 and IL-23 are important for expansion of human T_h17 cells (20–23) that function to recruit neutrophils *via* IL-17 secretion. However, the role of T_h17 cells during an Mtb infection in humans has not yet been elucidated (24). IL-10 acts as a general immunosuppressive cytokine, and the cytokine plays a specific role during Mtb infection by inhibiting autophagy (25, 26). The remarkable functional plasticity of DCs to induce these different types of responses depends on which signals the DCs receive from the environment (27). For example, activation of toll-like receptor (TLR)2 or nucleotide oligomerization domain (NOD) 2 in monocyte-derived DCs (moDCs) gives rise to very low levels of IL-12p70, while a combined activation of the two pattern recognition receptors (PRRs) results in intermediate amounts of IL-12p70 (28). Stimulation of TLR4 gives even higher amounts of IL-12p70 (29), while simultaneous triggering of C-type lectin receptors (CLRs) DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN, CD209) and the mannose receptor (MR, CD206) inhibits TLR4-mediated IL-12p70 production, perhaps *via* increased IL-10 production (30, 31). It is well established that Mtb contains ligands for both TLR2 and NOD2 through its display of lipoproteins and muramyl dipeptide, but it has also been suggested that Mtb contains non-canonical ligands for TLR4 (32–34). Furthermore, Mtb contains genomic DNA that can signal through intracellular DNA sensors, TLR9 and stimulator of IFN genes (STING), to induce type I IFNs (35, 36) which have been shown to boost IL-12p70 production in an autocrine manner (37). Based on this insight it is therefore curious that Mtb only induces very small, almost undetectable amounts of IL-12p70 in moDCs (28, 38–40).

Since the discovery of moDCs, they have been described as being CD14-low/negative (41), and CD14 is mainly considered a monocytic marker. However, CD14+ DCs do exist *in vivo* in the skin (42, 43). These CD14+ dermal DCs have been described as having a tolerogenic character, e.g. by promoting generation of Tregs (44). The tolerance-inducing compound vitamin D3 has furthermore been shown to induce up-regulation of CD14 on otherwise CD14-neg DCs (44, 45).

Here we assayed how the phenotype of Mtb-stimulated moDCs diverges from a classical T_h1-promoting DC subset and identify up-regulation of CD14 as one of the major differences between the two DC phenotypes. The up-regulation of CD14 on moDCs was partly recapitulated by a combined stimulation with Mtb-derived peptidoglycan (PGN) and mannosylated lipoarabinomannan (ManLAM), and in addition to a diminished induction of a T_h1 response, the Mtb-expanded CD14+ moDCs also increased the propagation of T_h17 A cells. Finally, we provide data showing how IFN-γ, but not IL-17A or IL-22, can abrogate Mtb-propagated CD14+ development.

Methods

Reagents

The following reagents were obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH: γ-irradiated whole cells from Mtb strain H37Rv (NR-14819), purified PGN from Mtb H37Rv (NR-14853) and purified ManLAM from Mtb H37Rv (NR-14848). *Escherichia coli* LPS (O26:B6) was purchased from Sigma-Aldrich. Recombinant human IFN-γ (cat. # 285-IF), IL-17A (317-ILB) and IL-22 (782-IL) were purchased from R & D Systems. The following anti-human antibodies were used in the studies: CD1a/PE (clone# HI149), HLA-DR/v500 (G46-6), CD86/v450 (2331(FUN-1)), PD-L1/PE-Cy7 (M1H1), DC-SIGN/FITC (DCN46), MR/APC (19.2) (all from BD Biosciences), CD14/PE-Cy7 (61D3), CD45RA/APC (HI100) (all from eBioscience), CD16/FITC (3G8), CD40/PerCP-Cy5.5 (5C3) (both from Biolegend) and CD4/ECD (SFCI12T4D11) (Beckman Coulter).

Human blood samples

Human buffy coats were acquired from the blood bank at the National Hospital of Denmark (Rigshospitalet) collected from anonymous healthy donors. Blood samples were handled in accordance with guidelines put forward in the 'Transfusion Medicine Standards' by the Danish Society for Clinical Immunology (www.dski.dk). The buffy coats were obtained from 450 ml whole blood donations in citrate phosphate dextrose anticoagulant. Whole blood was fractionated to separate the majority of erythrocytes and plasma. The buffy coat contains ~90% of the leukocytes from the 450 ml of blood and was used within 5.5 h upon blood draw.

Cell purification and culture

PBMCs were isolated from human buffy coats by Ficoll-Paque (GE Healthcare) density centrifugation. The PBMCs were labeled with magnetic particle-linked CD14 antibodies and isolated using MACS (Miltenyi). Cells were cultured in RPMI 1640 (Lonza) containing 2% (volume to volume) L-glutamine (Lonza), 10% fetal bovine serum (FBS; Lonza), 1% penicillin, streptomycin (Lonza), 50 μM β-mercaptoethanol (Sigma-Aldrich) and 10 mM HEPES (Lonza). Cells were kept in a humidified 37°C, 5% CO_2 incubator. For differentiation of monocytes into immature DCs, the medium was supplied with 20 ng ml^{-1} (≥160 U ml^{-1}) recombinant human GM-CSF and 30 ng ml^{-1} (≥150 U ml^{-1}) recombinant human IL-4 (both CellGenix). The cells were grown in polystyrene 6-well plates (Nunc) at a concentration of 2×10^6 ml^{-1}. Monocyte cultures were supplied with fresh medium containing fresh GM-CSF and IL-4 on day 3, and non-adherent immature DCs were harvested on day 6. In some experiments CD1a-hi moDCs were enriched by MACS as described (46). The purity of CD1a-hi

moDCs were >92% for all donors. Immature DCs were plated at a cell concentration of 10^6 ml^{-1} and stimulated with 500 ng ml^{-1} LPS, 10 μg ml^{-1} PGN, 50 μg ml^{-1} ManLAM and 500 μg ml^{-1} Mtb (final concentrations). The concentration of Mtb was determined in dose–response experiments (Supplementary Figure E1, available at *International Immunology* Online). Right before adding Mtb, clumps of bacteria were disrupted through syringe shearing by passing the solution 10 times through continuously smaller needles in the order 18-, 21-, 23-, 25- and 27-gauge. Recombinant human IFN-γ, IL-17A and IL-22 was added at 100 ng ml^{-1}.

ELISA

ELISA was performed according to the manufacturer's instructions. Supernatants of stimulated and unstimulated DCs were harvested after 24 h and kept at −40°C until analysis. The following ELISA kits were used in this study: human IL-1β, IL-6, IL-10, IL-12p70, IL-27, IFN-γ (all R&D Systems) and IL-23p19p40 (eBioscience).

Flow cytometry

Flow cytometry was performed according to standard procedures. The wash and staining buffer consisted of PBS with 1% FBS and 0.1% Na-azide (Sigma-Aldrich). Before staining, the cells were incubated for 10 min in staining buffer with 2% allogeneic human AB plasma (Rigshospitalet) to limit non-specific binding of antibodies. Monocytes were analyzed right after isolation, immature moDCs were analyzed right after harvesting, and stimulated/unstimulated moDCs were analyzed after 24 h. FMO (fluorescence minus one) controls were tested for all antibody combinations and found to be lower or identical to negative control cells (monocytes for HLA-DR, CD86, CD40, PD-L1, DC-SIGN and MR; and LPS-stimulated moDCs for CD14). Dead cells were stained with a near infrared necrotic cell marker (Invitrogen) according to the manufacturer's instructions. The dye reacts with amines exposed in cells with a compromised cell membrane.

CD4+ naive T-cell co-culture

PBMCs were enriched for CD3+ cells by Dynabeads FlowComp human CD3-kit (Invitrogen), which enables removal of magnetically labeled antibodies before cell sorting. CD3-enriched PBMCs were labeled with antibodies against CD4/ECD and CD45RA/APC and sorted on MoFlo XDP (Beckman Coulter) according to CD4+CD45RA++. Purity of T cells were assayed on FACSCanto II and were >97% for all donors. Prior to the co-culture moDCs were stimulated with the respective stimuli for 6 h. moDCs were washed, counted and added in a ratio of 1:20 to naive T cells. T cells and moDCs were co-cultured for 6 days. Supernatants were harvested, and T cells were re-stimulated with 10 ng ml^{-1} phorbol myristate acetate (PMA; Sigma-Aldrich) and 1 μg ml^{-1} ionomycin (Sigma-Aldrich) for 6 h. After 1 h 10 μg ml^{-1} brefeldin A (BD Biosciences) was added.

Statistics

All data were tested for a Gaussian distribution using a D'Agostino and Pearson omnibus test. Data that did not follow a Gaussian distribution were either log-transformed or analyzed by a non-parametric statistical method. When comparing two samples, the Student's paired *t*-test or the Wilcoxon matched-pairs signed-rank test was used. When comparing more than two samples an analysis of variance (ANOVA) or the Kruskal–Wallis test was used. All correlation analyses are based on Pearson or Spearman correlations. Prism 5 (Graphpad Software) was used to conduct the analyses.

Results

Mtb induces a IL-12p70-low, CD14+ moDC phenotype

To examine how the phenotype of Mtb-activated moDCs diverges from a classical T_h1-promoting phenotype, we compared the Mtb-induced response pattern to moDCs stimulated with a prototypic IL-12p70-inducing ligand; LPS derived from *E. coli* (Fig. 1). LPS induced significantly higher levels of especially IL-12p70 but also IL-27 compared with Mtb, while Mtb induced higher levels of IL-1β and IL-23 than seen for LPS-treated moDCs (Fig. 1A). No difference in IL-6 and IL-10 production was observed for the two stimuli. To study if these results were associated with differences in surface marker expression, we compared the expression levels of a range of surface markers between Mtb- and LPS-stimulated moDCs (Fig. 1B and C). No difference was observed for the antigen-presenting molecule HLA-DR nor for the co-inhibitory receptor PD-L1, while minor differences were found in the expression levels for the co-stimulatory molecules CD40 and CD86 as well as the CLRs DC-SIGN and MR. However, a large difference in CD14 expression was evident for the LPS- and Mtb-activated moDCs. On average, a 32-fold induction of CD14 by Mtb relative to LPS was seen, which was due to a subpopulation of moDCs that dramatically increased CD14 expression after Mtb stimulation (Fig. 1B). As IL-4 has previously been reported to be responsible for down-regulation of CD14 (47) and was left out of the medium during culture with LPS and Mtb to avoid a non-physiological bias of our results, we also investigated the phenotype of moDCs left unstimulated for 24 h to determine the baseline level of CD14 shifting. While the unstimulated moDCs up-regulated CD14 compared with immature moDCs, their average CD14 expression was still 8-fold less than that observed for Mtb-activated moDCs. Notably, the expression level of CD14 on the CD14+ moDC subset induced by Mtb was even higher than the CD14 expression on undifferentiated monocytes (Fig. 1B). Importantly, the stimulation of the moDCs with Mtb did not reduce the viability of these cells compared with unstimulated or LPS-stimulated moDCs (Fig. 1D).

Mtb-specific CD14+ moDCs are mainly CD16-neg

CD14 is classically used as a marker of monocytes along with the Fcγ receptor III, also known as CD16. To further examine the division of Mtb-challenged moDCs into a CD14-neg and a CD14+ subset, we assayed CD14 and CD16 expression profiles (Fig. 2A and B). Monocytes phenotyped immediately after isolation clearly displayed two populations with a predominant CD14+CD16-neg population and a minor CD14+CD16+ subset, as is routinely seen
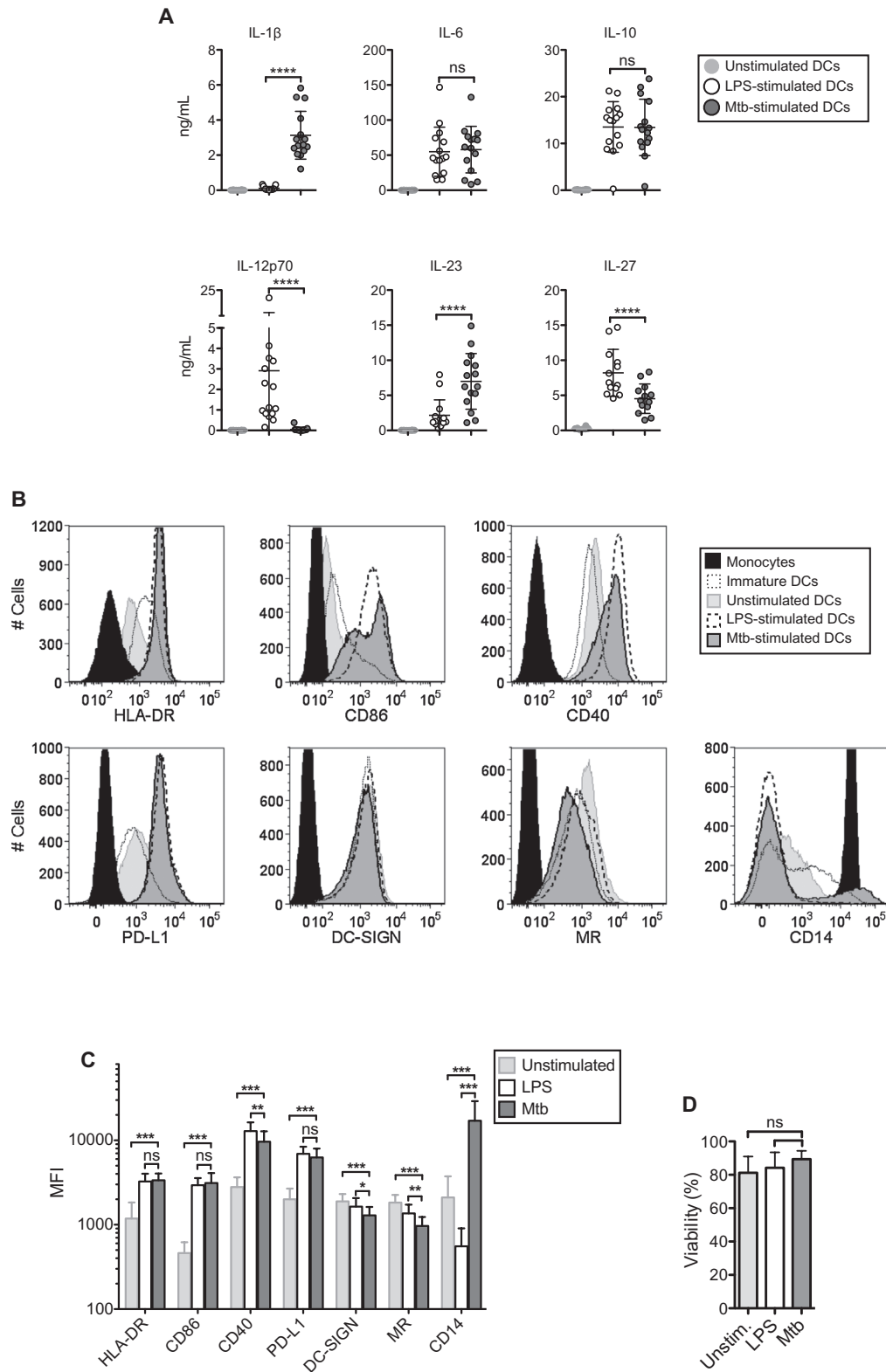
Fig. 1. Comparison of response pattern between LPS- and Mtb-activated DCs. MoDCs were stimulated with Mtb, LPS or left unstimulated for 24 h. Secreted cytokines were assayed by ELISA (A) and surface marker expression and viability was determined by flow cytometry (B–D). Data on monocytes harvested before DC differentiation, and immature DCs harvested at day 6 of differentiation are also displayed in the histograms (C). Each dot in the plots in A represents one donor. (B) Displays a representative donor from the data summarized in C. Error bars represent standard deviation, and LPS versus Mtb or versus unstimulated cells were tested for statistically significant differences using either the Student's two-tailed paired *t*-test or a Wilcoxon matched-pairs signed-rank test (comparing LPS versus Mtb), or an ANOVA with Tukey's post-hoc test (comparing all three conditions). *n* = 6–21, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
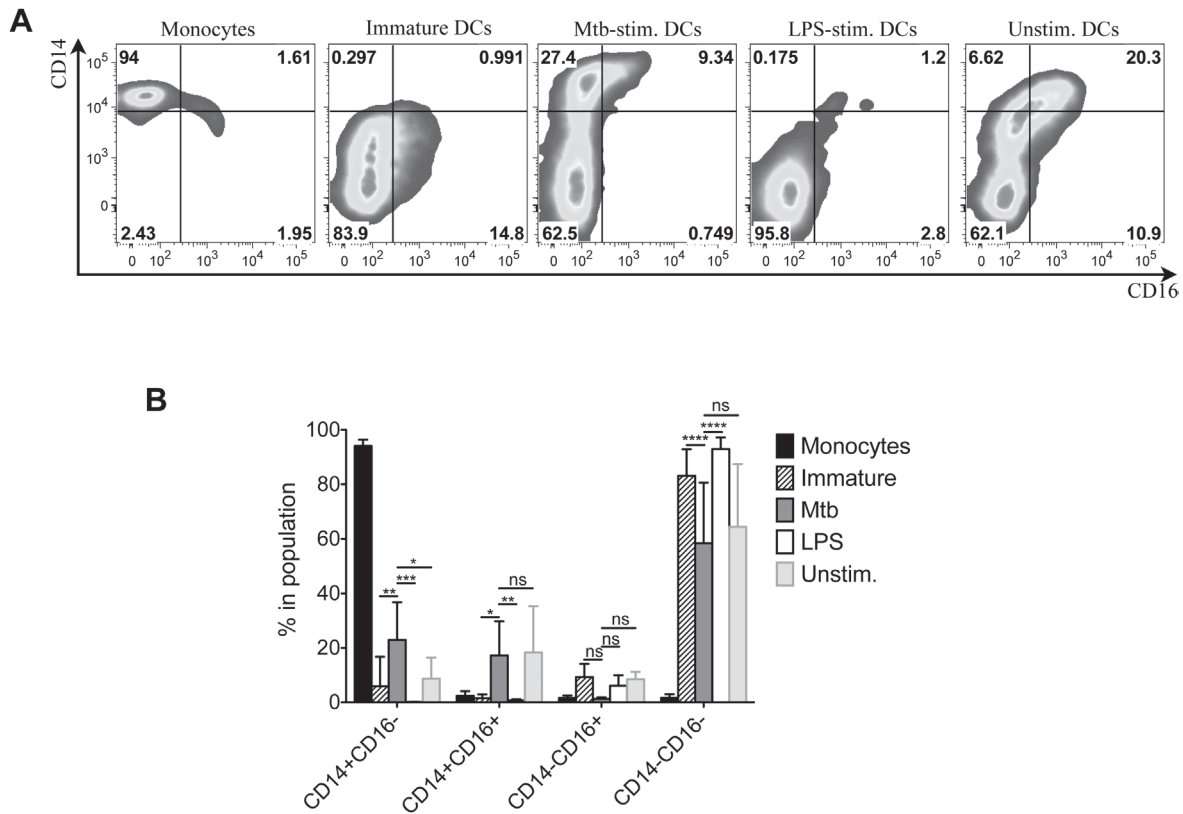
Fig. 2. Extended phenotypic analysis of Mtb-induced moDC subsets. Monocytes were harvested at day 0, immature moDCs were harvested at day 6, and Mtb/LPS-activated or unstimulated moDCs were harvested after 24 h of additional incubation. Subsequently, flow cytometry was used to assay phenotypic differences in CD14+ and CD14-neg subsets. (A and B) Division of subsets based on expression of CD14 and CD16. The numbers in each gate represents the frequency inside the specified gate. Error bars represent standard deviation, and data were tested for statistical significance using an ANOVA with Bonferroni post-hoc test ($n = 8$), $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

for human monocytes. Immature moDCs had lost most CD14 expression while maintaining some degree of CD16 expression. Upon Mtb stimulation, one-third of the moDCs up-regulated CD14; with the CD16+ fraction being CD14+, while LPS stimulation lead to almost a complete removal of CD16. As shown in Fig. 1, unstimulated moDCs also up-regulated CD14, but using CD16 as an additional marker showed that the CD14+CD16-neg subset made up a significantly larger fraction among Mtb-stimulated moDCs than for unstimulated moDCs (Fig. 2B). The other three CD14/CD16 populations did not show a significant difference between Mtb-stimulated and unstimulated moDCs, but even though the CD14+CD16+ population size was similar on an average basis, the mean fluorescence intensity of CD14 was consistently higher for the Mtb-induced cells than for unstimulated moDCs (Figs 1C and 2A).

Of notice, moDCs from 2 out of 10 donors assayed did not exhibit this 'plastic' Mtb-imposed shift in CD14-CD16 display but instead showed a CD14-CD16 expression pattern similar to that observed for LPS-treated cells (data shown are only for 'plastic' donors). MoDCs from these donors were not able to up-regulate CD14 in response to Mtb nor when left unstimulated and may reflect the heterogeneity of the human population, as previously described by our group (46).

Mtb-propagated CD14+ moDCs promote induction of a T_h17 response rather than T_h1 induction

After showing that Mtb exposure propagated a CD14+ moDC subset, we speculated whether this subset was associated with a differential cytokine production. Therefore, the percentage of Mtb-induced CD14+ moDC subset were correlated with the cytokine secretion from Mtb-challenged moDCs per individual blood donor (Fig. 3A). The data showed a clear inverse correlation between %CD14+ moDCs induced by Mtb and the level of IL-12 family cytokines (IL-12p70, IL-23 and IL-27) and IL-1β. Production of IL-6 and IL-10, however, showed no correlation with the Mtb-induced regulation of moDC subsets (Fig. 3A). In line with the pattern of moDC cytokine production, the %CD14+ moDCs was significantly inversely correlated with the level of IFN-γ produced by CD4+ naive allogenic T cells co-cultured with Mtb-challenged moDCs (Fig. 3B–D). In contrast, IL-17A and IL-10 produced by co-cultured naive T cells showed a general trend to positively correlate with %CD14+ moDCs (Fig. 3B and D). Furthermore, induction of the T_h17-specific transcription factor RORγT in co-cultured CD4+ naive allogenic T cells was strongly correlated with the proportion of CD14+ moDCs (Fig. 3D). Mtb did not induce the Treg-specific transcription factor FoxP3 (data not shown). Collectively, the data suggest that the induction of CD14+ moDCs leads to reduced production of IL-12 family
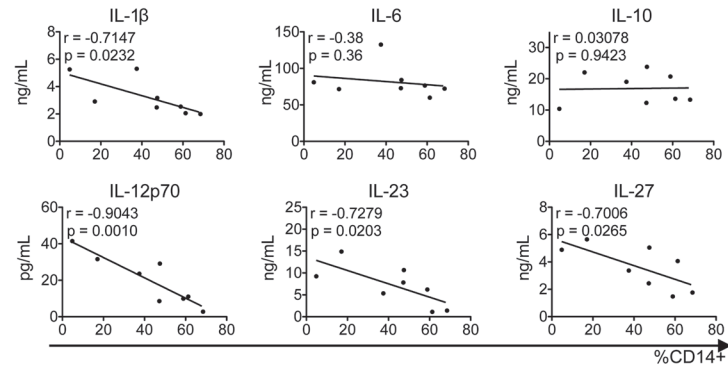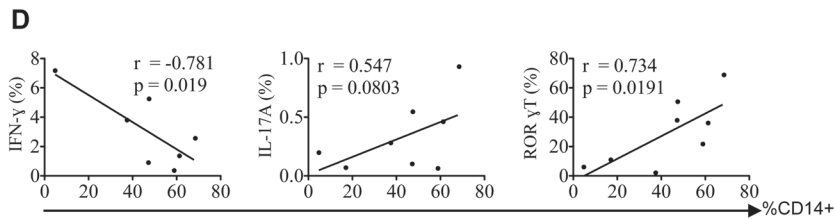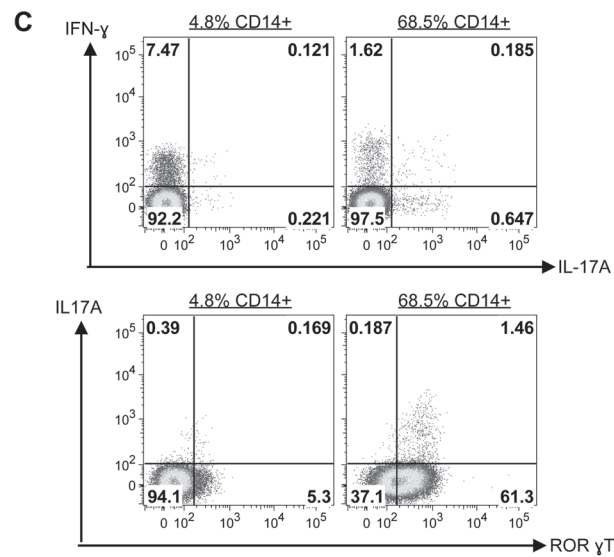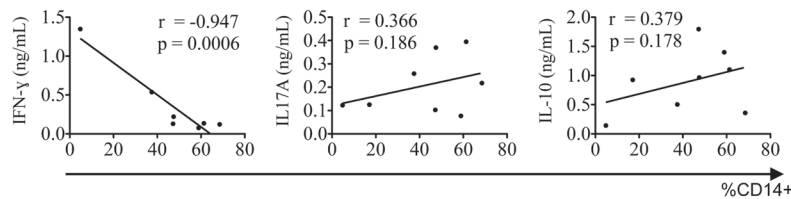
A Mtb-stimulated DC-derived cytokines

B T cell cytokines

C

D

Fig. 3. The effect of Mtb-induced CD14 expression on DC and naive CD4+ T-cell cytokine production. MoDCs were cultured for 24 h (A) or 6 h followed by a 6-day co-culture with allogenic CD4+CD45RA++ sorted T cells (B–D). Supernatants from DCs and T cells were assayed by ELISA (A and B), and CD14 expression on moDCs as well as intracellular cytokine production by PMA/ionomycin re-stimulated T cells was assayed by flow cytometry. Expression of the T_h17-specific transcription factor RORγT was determined by intracellular flow cytometry. (A and B) The percentage of Mtb-induced CD14+ moDCs were correlated to DC (A) or T-cell (B) cytokine production. Correlation analyses were performed using a Pearson or a Spearman correlation test ($n = 7$–8). Each dot in A, B and D represents one donor. (C) Display of the intracellular cytokine and transcription factor staining for a representative donor from each end of the range of observed levels of %CD14+ induction. The actual %CD14+ moDCs is displayed above each FACS plot. The numbers in each gate represent the frequency inside the specified gate. (D) Shows all the donors assayed as in C.

cytokines, decreased IFN-γ levels in CD4+ T cells and induction of IL-17A-producing T$_h$17 cells.

PGN and ManLAM partly promotes the CD14+ moDC subset

Mtb expresses a range of pathogen-associated molecular patterns (PAMPs), and two of the most prominent Mtb-associated PAMPs are PGN and ManLAM (48). Therefore, we wondered if one or both of these PAMPs were responsible for the up-regulation of CD14 in moDCs (Fig. 4). Challenge of moDCs with Mtb-derived PGN resulted in distribution of the moDCs into a CD14+ and CD14-neg population in a manner similar to Mtb, whereas ManLAM alone skewed the populations similarly to unstimulated moDCs (Fig. 4A). However,
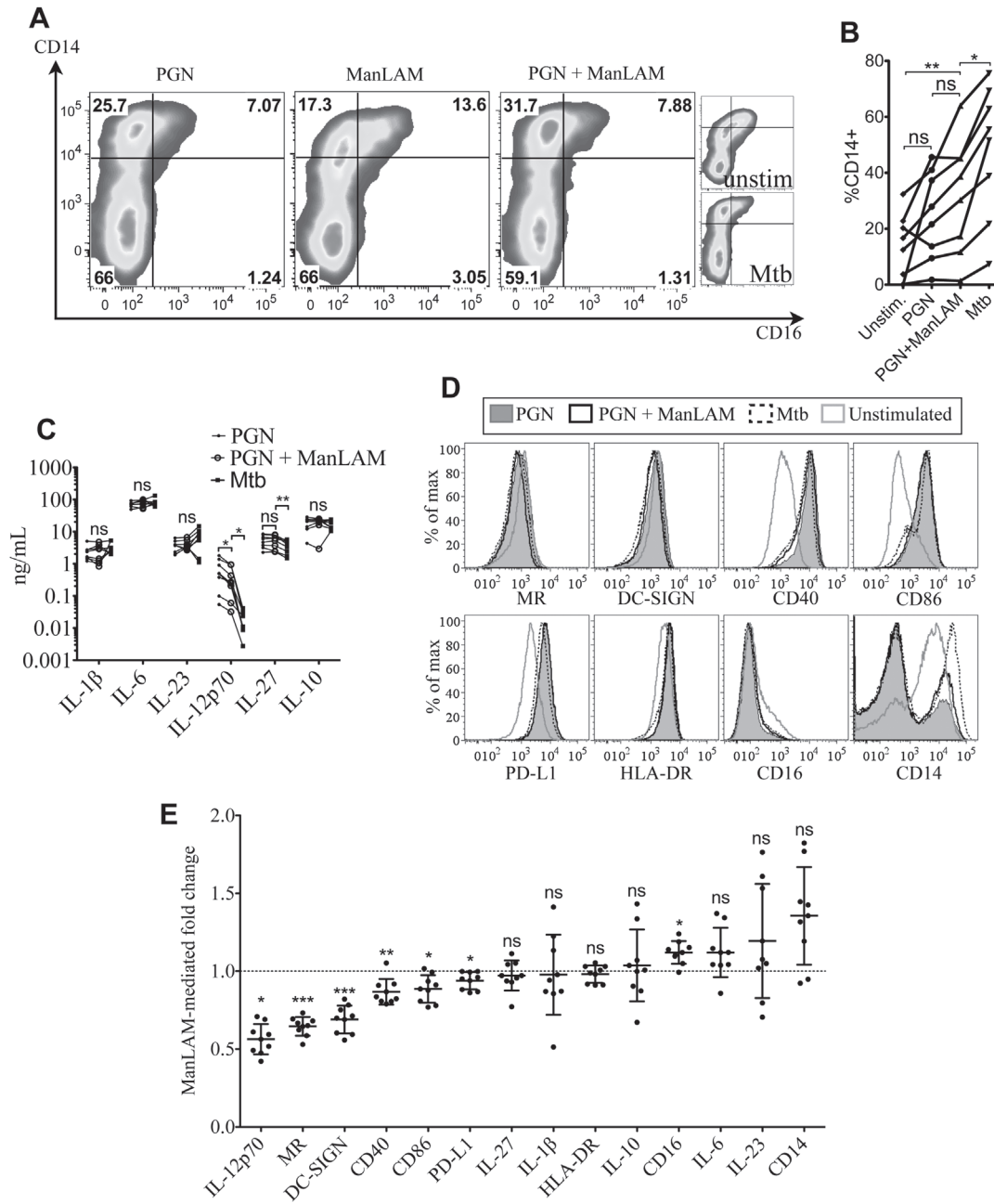

Fig. 4. Role of PGN and ManLAM in CD14+ moDC propagation. MoDCs were stimulated with Mtb-derived PGN and ManLAM alone or in combination for 24 h and analyzed by flow cytometry (A, B, D and E) and ELISA (C and E). (A) Representative flow cytometry plot for the division of moDCs according to CD14 and CD16 expression patterns. The numbers in each gate represent the frequency inside the specified gate. (B) Percentage of CD14+ moDCs for all donors (*n* = 8). (C) Level of cytokines produced by PGN stimulation alone (left) or combined with ManLAM (right). (D) Representative histograms for ManLAM-mediated changes of surface marker expression in PGN-activated moDCs. (E) Collected ManLAM-mediated fold changes in PGN-induced phenotype for all assayed surface markers and cytokines ordered by increasing mean fold change. Each dot represents one donor, and the error bars represent standard deviation. Data were tested for statistical significance by ANOVA by Tukey's post-hoc test. (in B), and the Student's two-tailed paired *t*-test or the Wilcoxon matched-pairs signed-rank test (in C and E), *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

simultaneous stimulation with PGN and ManLAM induced a more profound increase in the overall CD14+ population in moDCs from most donors (Fig. 4A and B). ManLAM has previously been shown to increase IL-10 and decrease IL-12p70 production in LPS-triggered moDCs (30, 31). Therefore, we speculated whether regulatory effects of ManLAM on PGN-activated moDCs could explain the observed increase in CD14+ moDCs. To test this, we measured the cytokines produced by moDCs stimulated with PGN or the combination of PGN and ManLAM (Fig. 4C). Stimulation with ManLAM alone did not induce production of any cytokines above the detection level of the assays (data not shown). Using flow cytometry, we concurrently investigated whether ManLAM induced any noticeable differences in the display of surface markers on PGN-activated moDCs (Fig. 4D). When ordering the differences in cytokines and surface markers according to the largest effects, it was apparent that only production of the cytokine IL-12p70 and expression of the surface markers MR and DC-SIGN were consistently down-regulated by ManLAM (Fig. 4E). IL-1β, IL-6, IL-10, IL-23 and IL-27 were diversely regulated in the donor pool and no clear effect of ManLAM addition on production of these cytokines was observed. Surface display of CD40, CD86, PD-L1 and HLA-DR (Fig. 4D and E) was down-regulated by ManLAM addition for most donors. CD16 and CD14 were up-regulated for most of the donors, with CD14 displayed the highest average up-regulation of all assayed cytokines and markers. Collectively, these data demonstrated that Mtb-derived PGN and ManLAM synergistically modify moDCs to program the Mtb-promoted CD14+, IL-12p70-low phenotype.

IFN-γ but neither IL-17A nor IL-22 inhibits Mtb induction of CD14+ Mtb-challenged moDCs

γδT cells, naturally present as intra-epithelial lymphocytes, have been shown to produce the cytokines IFN-γ, IL-17A and IL-22 in response to Mtb challenge (49). To investigate their individual effect on Mtb-mediated induction of CD14+ moDCs, we stimulated moDCs with Mtb alone or in combination with each of these cytokines (Fig. 5). Addition of IL-17A or IL-22 did not affect the induction of the CD14+ moDCs by Mtb (Fig. 5A). Conversely, IFN-γ administration completely inhibited the expansion of CD14+ moDCs by Mtb, resulting in a DC phenotype similar to that induced by LPS (Fig. 5A and B). IFN-γ added as a single stimulus also inhibited the incubation-induced generation of intermediate CD14 expression seen for unstimulated moDCs. IL-17A and IL-22 did not modify the cytokine production from Mtb-activated moDCs, while IFN-γ modulated the production of most of the cytokines assayed (although only significantly for IL-10 and IL-12p70) (Fig. 5C and E). Most remarkably was the substantial increase in IL-12p70 production by IFN-γ addition, and also the production of IL-23 and IL-27 was consistently increased in all donors. IL-6 showed a slight, though insignificant, up-regulation in most donors, while IL-1β showed no persistent regulation. IL-10 production was significantly inhibited upon addition of IFN-γ. Regarding surface marker expression, addition of IFN-γ resulted in an extensive down-regulation of CD14 and a more modest down-regulation of CD16, indicating an IFN-γ-mediated reversal of the Mtb-induced propagation of

monocyte markers on moDCs (Fig. 5D and E). MR was not modulated by IFN-γ, while HLA-DR, PD-L1 DC-SIGN, CD40 and CD86 were all increased upon IFN-γ addition. Of note, IFN-γ alone did not have any effect on CD16, MR, HLA-DR or DC-SIGN, while PD-L1, CD40 and CD86 were up-regulated intermediately compared with the LPS- or Mtb-stimulated moDCs (Fig. 5D). None of the assayed markers were modulated by IL-17A or IL-22 (data not shown), suggesting that Mtb-activated moDCs respond to exogenous IFN-γ, and not to IL-17A and IL-22.

Discussion

Various subpopulations of DCs are known to co-exist in human lungs and may arise based on different progenitors, and the plastic regulation of phenotypes upon exposure to tissue-derived factors and infectious agents (50–53). Recent reports suggest that some of these DCs are derived from monocytes (54, 55), suggesting an *in vivo* relevance for moDCs in response to lung infections such as Mtb. However, CD14+ DCs have not been reported in the lungs, which may be due to technical issues such as CD14 not being assayed or gated away in a lineage cocktail (50–53). Mtb stimulation of moDCs *in vitro* has been reported by others to induce close to no IL-12p70 (28, 38–40), and it has been suggested that exogenous IFN-γ is needed for moDCs to produce IL-12p70 in response to Mtb (28). The present data elucidate the basis for the low level of IL-12p70 in Mtb-activated moDCs by demonstrating that mainly CD14-neg moDCs induce IL-12p70 secretion, and moreover that the presence of a CD14+ subset keeps IL-12p70 low. Furthermore, we found that PAMPs from Mtb regulate the plasticity of moDCs toward expansion of a CD14+ subset and low level of IL-12p70, also repressing propagation of IFN-γ-producing CD4+ T-cells while promoting IL-17A-producing RORγT+ CD4+ T cells (T$_h$17). Intriguingly, this phenotypic shift in Mtb-challenged moDCs was observed to be fully reversed by IFN-γ treatment, therefore suggesting that the role for IFN-γ in boosting IL-12p70 is partly *via* inhibition of DC differentiation toward a CD14+ phenotype.

We observed that Mtb-derived PGN and ManLAM in combination propagated immature moDCs to expand a CD14+ subset analogous to that observed after Mtb treatment. It is therefore possible that other micro-organisms expressing identical or similar PAMPs can induce immune modulations similarly to Mtb. In contrast to stimulation by PGN + ManLAM or Mtb, our data show that the prototypic IL-12p70-inducing stimulus LPS leads to further down-regulation of CD14 compared with immature moDCs. An explanation for the down-regulation of CD14 by LPS could be due to internalization of the TLR4/MD-2/CD14 receptor complex upon ligand binding. However, as we observed that IFN-γ also mediated a down-regulation of surface CD14, a more likely mechanism is that CD14 is shedded upon endogenous enzymatic activity, as has been demonstrated for monocytes stimulated with LPS or IFN-γ (56). It has been speculated that the physiological role of CD14 shedding is to generate soluble CD14 needed for CD14-neg leukocytes to respond to LPS (57). The role of CD14 shedding or down-regulation of surface CD14 in the IL-12p70-producing moDC subset could also be to limit a propagating immune response, as DCs induced to produce
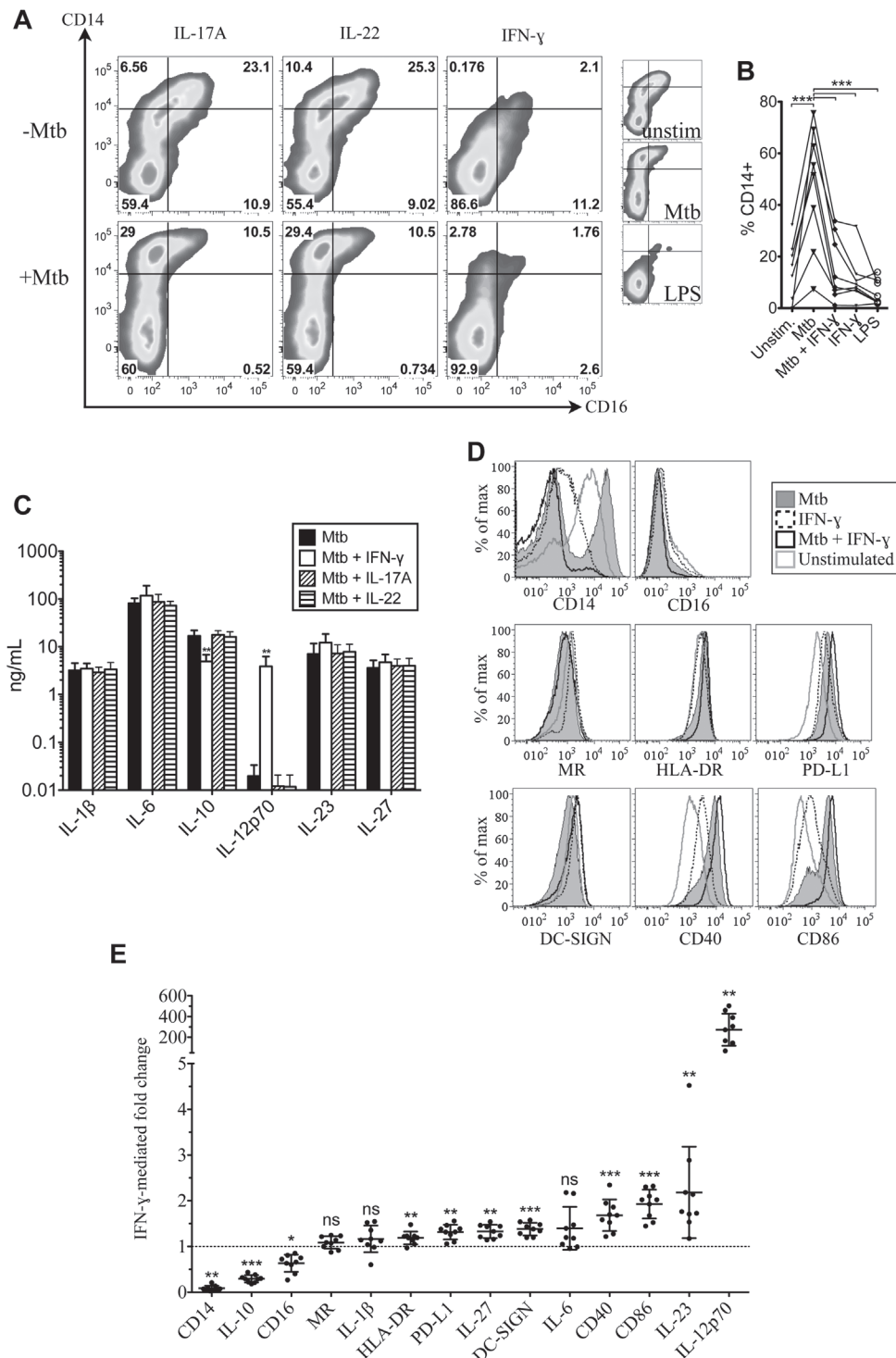
Fig. 5. Role of IL-17A, IL-22 and IFN-γ in the induction of CD14+ moDCs. MoDCs were stimulated with Mtb and/or recombinant IL-17A, IL-22 or IFN-γ at 100 ng ml^{-1} for 24 h and analyzed by flow cytometry (A, B, D and E) and ELISA (C and E). (A) Representative flow cytometry plot of CD14 and CD16 expression patterns of moDCs. The numbers in each gate represent the frequency inside the specified gate. (B) Percentage of CD14+ moDCs for all donors (n = 8). (C) Level of cytokines produced by CD1a+ moDCs after stimulation with Mtb alone or in combination with recombinant IL-17A, IL-22 or IFN-γ. Significance is based on comparison with stimulation with Mtb alone, and non-significant differences are not displayed. (D) Representative histograms for IFN-γ-mediated changes in Mtb-induced moDC phenotype. (E) Collected IFN-γ-mediated fold changes in Mtb-induced moDC phenotype for all assayed surface markers and cytokines ordered by increasing mean fold change. Each dot represents one donor, and the error bars display the standard deviation. Data were tested for statistical significance by Tukey's post-hoc test (in B), a Kruskal–Wallis test with a Dunn's *post-hoc* test (in C), and the Student's two-tailed paired *t*-test or the Wilcoxon matched-pairs signed-rank test (in E), *P < 0.05, **P < 0.01, ***P < 0.001.

IL-12p70 would not need to sense additional LPS. In this sense it is important to recognize that MD-2, CD14 and TLR4 all cooperate in activation of TLR4. Furthermore, Mtb-stimulated moDCs may also up-regulate CD14 to be sensitized for TLR4-triggered apoptosis, as has been described for murine terminally differentiated DCs (58).

In addition to the connection between surface CD14 and IL-12p70 production, the present data suggest that there is an association between CD40 expression and IL-12p70 production. Three points of evidence supports this notion: (i) LPS-activated moDCs release copious amounts of IL-12p70 and highly express CD40 compared with Mtb-activated moDCs, (ii) ManLAM inhibits both CD40 and IL-12p70 production in PGN-activated moDCs and (iii) IFN-γ boosts both CD40 expression levels and IL-12p70 production by moDCs. Since CD40 is a key co-stimulatory molecule during antigen presentation, this could indicate that moDCs that produce high levels of IL-12 family cytokine are more prone to have strong interactions with naive T cells, as has been demonstrated in a murine model system (59). The combined lack of IL-12p70 and reduced CD40 expression in moDCs stimulated with Mtb could indicate that Mtb possesses an ability to prime the immune system away from an expansion of T_h1 and CD8+ T cells, which represent the adequate immune phenotype for clearance of Mtb from infected macrophages. This phenomenon may be causative for the inadequate removal of Mtb within lungs of infected individuals, but this hypothesis will require adequate *in situ* justification in future studies.

Collectively, the observed Mtb-induced phenotype in moDCs could indicate a minimal T_h1 induction during early infection phases, if in the absence of IFN-γ. However, the pulmonary environment may influence how DCs respond to Mtb, as γδT cells potentially present at the site of infection could produce IFN-γ in response to Mtb (49). To our knowledge, the presence of γδT cells in the lungs at steady state has not been demonstrated in humans, while in macaques, small numbers of γδT cells have been found in the bronchoalveolar lavage fluid 2 weeks after BCG vaccination (60). However, if cells that mediate IFN-γ priming are not present in the lung during the initiation of the Mtb infection, or if they do not interact with the relevant DC subsets, this important priming signal for expansion of IL-12p70-producing DCs, and hence early T_h1 development, could be absent. Depending on other environmental cues, the γδT cells may also produce IL-17A or IL-22 instead of IFN-γ and thereby not induce IL-12p70 production in DCs. However, even though IL-17A and IL-22 do not affect the differentiation of Mtb-stimulated moDCs alone, the combination, or if present together with IFN-γ, could potentially modulate the DC phenotype. It will be interesting in future studies to assay this hypothesis. Apart from γδT cells, T_h1 and T_h17 cells can also produce these cytokines; however, due to an activation and expansion-based delay in the CD4+ T-cell immune response after Mtb infection (1), these cells will not affect DC differentiation upon initial encounter with Mtb.

Even though one-third of the world's population is infected with Mtb, certain individuals may be able to develop an early T_h1 response, and therefore clear the infection. Obviously, there are no data available about whether this is actually happening in humans. However, it could be speculated that if this was possible, it would be in the type of donor that is not able to up-regulate CD14 on DCs in response to Mtb (2 out of 10 donors assayed in this study). Furthermore, it could be speculated that donors developing active disease are the type of donors with the highest CD14 up-regulation on DCs in response to Mtb. It would be very interesting, in future studies, to compare CD14 expression on moDCs from Mtb-infected individuals that do and do not develop active TB.

In summary, Mtb holds the capacity to promote CD14+ subset generation in human moDCs and reduce overall IL-12p70 production needed for T_h1 priming. This Mtb promoted a DC phenotype was fully reversed by exogenously administered IFN-γ. Due to the observed ability of IFN-γ to regulate the phenotype of Mtb-exposed moDC, strategies for improving local IFN-γ production in tuberculosis granulomas could represent a way to improve Mtb clearance in latently infected patients.

Supplementary data

Supplementary data are available at *International Immunology* Online.

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