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Induction of Th1/Th17 immune response by *Mycobacterium tuberculosis*: role of dectin-1, mannose receptor, and DC-SIGN

Elena Zenaro, Marta Donini, and Stefano Dusi[1]

Department of Pathology, Section of General Pathology, University of Verona, Verona, Italy

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ABSTRACT

Mtb influences DC activity and T cell-mediated immune responses. We show that the treatment of immature monocyte-derived DC with Mtb elicited the formation of mature DC, producing TNF-α, IL-1β, IL-6, and IL-23 and instructing $CD4^+$ cells to secrete IFN-γ and IL-17. Mtb-induced cytokine release by DC depended on dectin-1 receptor engagement, whereas MR or DC-SIGN stimulation inhibited this process. A selective dectin-1 binding by the receptor agonist glucan was sufficient to enable DC to generate Th1/Th17 lymphocytes, showing features comparable with those induced by Mtb-treated DC. Interestingly, DC-SIGN or MR engagement inhibited Th17 and increased Th1 generation by glucan- or Mtb-treated DC. Our results indicate that Mtb modulates the lymphocyte response by affecting DC maturation and cytokine release. Dectin-1 engagement by Mtb enables DC to promote a Th1/Th17 response, whereas DC-SIGN and MR costimulation limits dectin-1-dependent Th17 generation and favors a Th1 response, probably by interfering with release of cytokines. *J. Leukoc. Biol.* 86: 1393–1401; 2009.

Introduction

In peripheral tissues, immature DC capture antigens by specialized pathogen recognition receptors and then undergo maturation and migrate to lymphoid organs, where they process antigens and present them to T lymphocytes [1–5]. These receptors, whose engagement modulates the functional activity of DC, include C-type lectin receptors and TLRs [6, 7]. DC regulate the immune response by stimulating the differentiation of $CD4^+$ lymphocytes into Th effectors, such as Th1, Th2, and Th17 cells [8–11], characterized by the production of different patterns of cytokines, which released by DC-stimulated T lymphocytes and by DC themselves, orchestrate the course of infective diseases, leading to eradication of the pathogens, chronicization of the infection, or autoaggressive events.

Mtb has evolved several strategies to survive in infected individuals, including the ability to interfere with DC functions. For instance, engagement of DC-SIGN receptor by mycobacterial cell-wall component ManLAM inhibits the LPS-generated signaling leading to DC maturation [12, 13]. Moreover, infection of monocytes with Mtb interferes with IFN-α-induced monocyte differentiation into fully competent DC, leading to formation of macrophage-like cells with impaired capacity to prime IFN-γ-producing T lymphocytes [14]. Interestingly, cell wall-associated α-glucan induces monocytes to differentiate into DC with the same altered phenotype and functionality observed in DC infected with Mtb [15], suggesting that some isolated mycobacterial components can reproduce the effects of the whole Mtb.

Mice infected with Mtb develop a Th1 and Th17 immune response [16–18]. It has been demonstrated that Mtb interacts with dectin-1 of DC [19, 20] and that dectin-1 engagement by curdlan induces Th17 generation in in vitro [21] and in vivo [22] mouse models.

Although the role of Th17 cells in host protection has not been clarified completely, it has been suggested that IL-17 produced by these lymphocytes could trigger the induction of chemokines, which attract the leukocytes to infected tissues [23–25]. It has also been reported that the ability of IL-17-producing $CD4^+$ T cells to control Mtb infection in mouse models is limited [16, 26], although Umemura et al. [27] found an impaired granuloma formation in bacillus Calmette-Guerin-infected lungs of IL-17-deficient mice. An efficient generation of Th17 cells depends on IL-23, IL-1β, IL-6, and TNF-α release [28–34], and an altered secretion of these cytokines has important consequences on the inflammatory outcome of mycobacterial infections [16, 24, 35].

On the basis of the above-mentioned reports, it is possible that subversion of DC maturation or changes in the pattern of cytokines secreted by DC upon Mtb interaction with selected receptors could represent mechanisms by which Mtb drives the human Th lymphocytes response. The aim of our investiga-

Abbreviations: DC=dendritic cell(s), DC-SIGN=DC-specific intercellular adhesion molecule-grabbing nonintegrin, h=human, IDEAS=ImageStream Data Exploration and Analysis Software, ManLAM=mannosylated lipoarabinomannan, MNE=mean normalized expression, MR=mannose receptor, Mtb=*Mycobacterium tuberculosis*, ROR=orphan retinoid nuclear receptor, T-bet=T box expressed in T cells

1. Correspondence: Dipartimento di Patologia, Sezione Patologia Generale, Università di Verona, Strada Le Grazie 8, 37134 Verona, Italy. E-mail: stefano.dusi@univr.it

tions was to individuate these mechanisms as well as the DC pathogen recognition receptors involved in these processes.

MATERIALS AND METHODS

Reagents and antibodies

Reagents used include: RPMI 1640, ultraglutamine 1, and 10% low endotoxin FBS (Lonza, Walkersville, MD, USA); rhGM-CSF and rhIL-4 (PeproTech, Rocky Hill, NJ, USA); biglycan from bovine cartilage, glucan from baker's yeast *Saccharomyces cerevisiae*, laminarin from *Laminaria digitata*, and PMA (Sigma Chemical Co., St. Louis, MO, USA); Ultra Pure *Escherichia coli* LPS (0111: B4 strain, InvivoGen, San Diego, CA, USA). Whole cell γ-irradiated Mtb strain H37Rv and ManLAM from Mtb H37Rv were provided by Colorado State University (Fort Collings, CO, USA; Contract HHSN266200400091c). All of the reagents and solutions were prepared using clinical pyrogen-free water and were tested by a *Limulus* amebocyte lysate assay kit (Microbiological Associates, Walkersville, MD, USA) to determine the endotoxin content, which was found to be always <0.125 unit/ml. Antibodies used include: CD83 (HB15e) and CD1a (HI149; Becton Dickinson, San Jose, CA, USA); CD80 (2D10), CD86 (IT2.2), HLA-DR (L243), HLA-I (W6/32), CD40 (HB14), and CD14 (M5E2; Biolegend, San Diego, CA, USA); CD1c (AD5-8E7, Miltenyi Biotec GmbH, Auburn, CA, USA); MR (HM2056, Hbt, Uden, The Netherlands); dectin-1 (MAB1859, R&D Systems, Minneapolis, MN, USA); DC-SIGN (ab13487, Abcam, Cambridge UK); mouse isotype control (Sigma Chemical Co.); IL-17A (eBio64DEC17), IFN-γ (4S.B3), and IL-4 (MP-4-25D2; eBioscience, San Diego, CA, USA).

DC and lymphocyte preparation and culture

DC were generated from monocytes obtained from the blood bank of the University of Verona (Italy) as discarded material after preparation of therapeutic blood products. Monocytes isolated from buffy coats of healthy donors by Ficoll-Hypaque and Percoll (Amersham Biosciences, UK) density gradients, as described previously [36], were purified using the Human Monocyte Isolation Kit II (Miltenyi Biotec GmbH). The final monocyte population was 99% pure, checked by FACS analysis. $CD4^+$ lymphocytes were isolated from the lymphocyte fraction of the Percoll gradient with the EasySep Negative Selection Human $CD4^+$ T Cell Enrichment Kit (StemCell Technologies, Vancouver, BC, Canada). The final $CD4^+$ lymphocyte population was 99% pure, checked by FACS analysis. To preserve T cells during the differentiation of monocytes into DC, $CD4^+$ T cells were spun down, resuspended in freezing medium (low endotoxin FBS+10% DMSO), and kept in a liquid nitrogen freezer. Control experiments demonstrated that the responsiveness to DC from frozen T cells was comparable with that of fresh T lymphocytes. To generate DC, monocytes were incubated at 37°C, 5% CO_2, for 5–6 days at 1×10^6/ml in six-well tissue-culture plates (Lonza, Walkersville, MD, USA) in RPMI 1640 supplemented with heat-inactivated 10% FBS, 2 mM L-glutamine, 50 ng/ml GM-CSF, and 20 ng/ml IL-4.

ELISA and intracellular cytokine staining

Cytokine production in culture supernatants was determined by ELISA, according to the manufacturer's instructions: IL-10 (range 1.2–300 pg/ml), TNF-α (range 1.4–1000 pg/ml), IL-6 (range 0.6–500 pg/ml), IFN-γ (range 2–600 pg/ml), and IL-4 (range 0.6–500 pg/ml; ImmunoTools GmbH, Friesoythe, Germany); IL-12 (range 4–500 pg/ml), IL-23 (range15 pg/ml–2.0 ng/ml), IL-1β (range 4–500 pg/ml), and IL-17 (range 4–500 pg/ml; eBioscience). T cell intracellular cytokine staining was done upon 20 ng/ml PMA, 1 μM ionomycin, and brefeldin A (Biolegend) stimulation for the final 6 h of culture. Cells were incubated with a fluorescent-conjugated antibody anti-CD4 (IgG1, RPA-T4, Biolegend) and then with fixation/permeabilization buffer (420801 and 421002, Biolegend). Cells were stained with anti-IL-17A, IFN-γ, and IL-4 fluorescent-conjugated antibodies, acquired on a FACScan flow cytometer (BD Biosciences, Mountain View CA, USA), and analyzed using FCS Express 3 (De Novo Software, Thornhill Ontario, Canada).

T lymphocyte proliferation assays

Autologous $CD4^+$ T lymphocytes were cultured for 5 days in 96-well plates (2×10^5 cells/well/200 μl), alone or with 50 μg/ml Mtb and/or receptor ligand-activated DC (2×10^4 cells) and then incubated 12 h with 10 μM BrdU (B5002, Sigma Chemcal Co.). Incorporated chemical was detected by anti-BrdU antibody (FastImmune anti-BrdU FITC with DNase, Becton Dickinson). Cells were stained with 7-amino-actinomycin D (Becton Dickinson) for the exclusion of nonviable cells, acquired on a FACScan flow cytometer (Becton Dickinson), and analyzed using FCS Express 3 (De Novo Software).

Flow cytometric analysis

Live cells were washed twice with PBS and incubated 30 min with 10% human male AB serum to prevent nonspecific binding. For immunofluorescence staining, mouse anti-human CD1a, CD1c, CD14, CD80, CD83, CD86, CD40, HLA-I, and HLA-DR were used (see Reagents and antibodies). Annexin-V-Alexa 568 (Roche, Indianapolis, IN, USA) was used to detect apoptotic cells. FACScan flow cytometer (Becton Dickinson) and FCS Express 3 (De Novo Software) were used to analyze cells.

Real-time PCR

RT-PCR has been performed as described [37]. Briefly, total RNA extracted from 10^6 DC or T cells using the RNeasy mini kit (Qiagen, Crawley, UK) was used as a template for the RT reaction, using random hexamers and SuperScript II RT (Invitrogen, Carlsbad, CA, USA). Oligonucleotide primers (Invitrogen) are available under the following entry code in the public database RTPrimerDB (medgen.ugent.be/rtprimerdb/): GAPDH (3539), T-bet (7752), RORγC (7756), GATA3 (7757), IL-23R (7755), IL-17A (7754), IL-6 (3545), IFN-γ (7753), TNF-α (3551), IL-23 p19 (7758), IL-1β (7760), IL-22 (7759). Triplicate RT-PCR reactions for each sample were performed in 20 μl containing 20 ng cDNA, SYBR Premix Ex Taq (Takara, Tokio, Japan), and primers (200 nM). The PCR reactions were performed in 96-well plates using the DNA Engine Opticon 2 system (MJ Research, Waltham, MA, USA). Amplification plots were analyzed using Opticon Monitor Software, Version 2.02 (MJ Research). Data were calculated with Q-Gene software (www.BioTechniques.com) and expressed as MNE units after GAPDH normalization [38]. In DC-T cell coculture experiments, RNA was extracted from whole mixed cell populations.

Image stream

DC prestimulated 24 h with Mtb were cocultured 5 days with autologous $CD4^+$ T cells. PMA 20 ng/ml, ionomycin 1 μM, and brefeldin A were added for the final 6 h of culture. Cells were fixed, permeabilized, then stained with anti-IL-17A and anti-IFN-γ fluorescent-conjugated antibodies, and acquired by the Amnis ImageStream cytometer (Amnis, Seattle, WA, USA): data analysis was performed using IDEAS (Amnis). In our experiment, we have tested 1×10^6 cells/100 μl. IDEAS application builds a matrix of spectral compensation values by using control files, which contain fluorescence values of cells stained with one single fluorochrome as well as unlabeled cells. To set the instrument, $CD4^+$ cells cocultured 5 days with Mtb-matured DC were fixed, permeabilized, and left unstained as well as stained with anti-IL-17A, anti-IFN-γ, or anti-CD4 fluorescent-conjugated antibodies and acquired by the Amnis ImageStream cytometer. Moreover, immature DC, cocultured 5 days with $CD4^+$ T cells, were used as a negative fluorescent control to exclude false fluorescent-positive cells. Debris and cell aggregates were excluded, and individual cells were identified by gating on size and aspect ratio. Individual cell populations were identified by gating on $CD4^+$ cells and confirmed by visual inspection of the fluorescence pattern.

Statistical analysis

Statistical analysis was performed with SigmaStat for Windows, Version 2.0, using a one-way ANOVA. Statistical significance was established at $P < 0.001$.

RESULTS

Mtb induces TNF-α, IL-6, IL-1β, and IL-23 production by DC and enables DC to trigger a Th1/Th17 response

We first analyzed the effect of γ-irradiated Mtb on the DC maturation process and cytokine release: Monocytes were cultured for 5 days with GM-CSF and IL-4 to obtain immature DC expressing the differentiation markers CD1a and CD1c but not the monocytes marker CD14 (**Fig. 1A**). These cells were then challenged for 24 h with Mtb (Mtb-matured DC) or induced with LPS (LPS-matured DC) as control. Mtb and LPS increased the expression of the DC maturation markers CD80, CD86, CD83, HLA-DR, HLA-I, and CD40 (Fig. 1A). Moreover, Mtb caused a remarkable TNF-α, IL-6, IL-1β, and IL-23 production and a weak IL-12 secretion (Fig. 1B). Mtb-induced TNF-α, IL-6, IL-23, and IL-12 secretion was lower than that obtained upon DC treatment with LPS (Fig. 1B). In contrast, IL-1β production by Mtb-matured DC was comparable with that observed in LPS-matured DC (Fig. 1B).

We then examined the capacity of Mtb-matured DC to instruct the functional activity of T lymphocytes. A MLR demonstrated that these DC induced the proliferation of autologous $CD4^+$ lymphocytes as efficiently as control, LPS-matured DC (Fig. 1C). Moreover, Mtb-matured DC triggered a remarkable IL-17 and IFN-γ production (Fig. 1D) but no IL-4 or IL-10 secretion (results not shown) by $CD4^+$ cells. LPS-matured DC used as control triggered a remarkable IFN-γ but a weak IL-17 production (Fig. 1D). Stimulation of $CD4^+$ lymphocytes with Mtb in the absence of DC or in the presence of immature DC did not result in substantial $CD4^+$ lymphocyte proliferation (Fig. 1C), IL-17 or IFN-γ (Fig. 1D), IL-4, or IL-10 (results not shown) production.

To further characterize the lymphocytes obtained upon incubation of $CD4^+$ cells with Mtb-matured DC, we performed a RT-PCR analysis to investigate the expression of IL-23R, IL-17A, RORC transcription factor, and IL-22, selective for Th17 cells [39], as well as of T-bet transcription factor and IFN-γ, peculiar to Th1 cells [39]. Lymphocytes challenged with Mtb-matured DC expressed mRNA levels of IL-23R, IL-17A, RORC, IL-22, T-bet, and IFN-γ higher than lymphocytes cocultured with immature DC (Fig. 1E). Lymphocytes cultured with LPS-matured DC showed increased levels of T-bet, IFN-γ, and to a lesser extent, IL-22 but not of IL-23R, IL-17A, and RORC transcripts (Fig. 1E). Moreover, lymphocyte incubation with immature DC or Mtb-matured DC did not result in relevant changes of the GATA3 transcript, peculiar to Th2 lymphocytes (Fig. 1E). GATA3 mRNA was increased slightly only upon T cell incubation with LPS-matured DC (Fig. 1E). These results show that Mtb-matured DC induce the Th1 and Th17 generation, whereas LPS-matured DC trigger a prevalent Th1 response, confirming the data depicted in Figure 1D.

Furthermore, we analyzed $CD4^+$ lymphocytes by intracellular staining to assess the relative amounts of IL-17- and IFN-γ-producing T cell populations generated upon coculture with Mtb-matured DC. An analysis by the ImageStream cytometer showed that Mtb-matured DC induced the formation of a heterogeneous lymphocyte population, including T cell subsets selectively producing IL-17 or IFN-γ, as well as lymphocytes secreting IL-17 and IFN-γ (Fig. 1F). Th17, Th1, and Th1/Th17 subsets represent, respectively, 38%, 17%, and 12% of $CD4^+$ cells, as assessed by IDEAS (see Materials and Methods). These results demonstrate that Mtb-matured DC stimulate $CD4^+$ lymphocytes with formation of cells showing features of Th17, Th1, or Th1 and Th17 cells.

Mtb triggers cytokine production through engagement of DC receptor dectin-1

We then sought to individuate the DC receptors responsible for Mtb-induced cytokine release. First of all, we performed a preliminary screening of the most important DC receptors by analyzing the cytokine secretion by immature DC stimulated with purified glucan, a dectin-1 agonist [40], biglycan that binds to MR [41], or ManLAM, agonists of DC-SIGN and MR [7, 42, 43]. Notably, ManLAM, used in our experiments, was purified from Mtb H37Rv (see Materials and Methods). We found that DC stimulation with glucan caused TNF-α, IL-6, IL-1β, and IL-23 (**Fig. 2A**) but no IL-12 secretion (not shown). Biglycan and ManLAM were unable to elicit a substantial TNF-α, IL-1β, IL-23 (Fig. 2A), and IL-12 (not shown) release. However, ManLAM, but not biglycan, triggered a weak IL-6 production (Fig. 2A). Interestingly, simultaneous addition of glucan and biglycan or ManLAM resulted in inhibition of glucan-dependent IL-1β, IL-23, and to a lesser extent, TNF-α release. Contemporaneous addition of glucan and biglycan resulted in IL-6 secretion comparable with that observed upon cell stimulation with glucan alone, whereas this secretion was increased upon addition of glucan plus ManLAM (Fig. 2A). To investigate the role of dectin-1 further, we also performed experiments by using the dectin-1 agonist curdlan [21, 22], alone or in combination with biglycan or ManLAM, and we observed a cytokine secretion similar to that obtained with glucan (results not shown).

Taken together, these results suggest that dectin-1 is involved in the mechanisms of TNF-α, IL-1β, IL-23, and IL-6 production by DC and that DC-SIGN or MR engagement inhibits the dectin-1-dependent signals responsible for the release of some cytokines.

We then investigated whether the results obtained with soluble receptor agonists could reflect the effect of the interactions between Mtb and DC receptors. A RT-PCR analysis demonstrated that incubation of immature DC with the dectin-1 receptor antagonist laminarin or a dectin-1-blocking antibody decreased Mtb- and glucan-induced TNF-α, IL-1β, IL-23, and IL-6 expression (Fig. 2B). Moreover, DC incubation with MR- or DC-SIGN-blocking antibodies increased the Mtb-induced mRNA levels of these cytokines (Fig. 2C). The effect of dectin-1-, MR-, and DC-SIGN-blocking antibodies on cytokine mRNA expression was detectable at 4 h (Fig. 2, B and C) and to a lesser extent, at 8 h and 12 h (results not shown) after DC treatment with Mtb or glucan. These results have been confirmed by ELISA experiments showing that incubation of immature DC with a dectin-1-blocking antibody decreased, whereas DC incubation with MR- or DC-SIGN-blocking antibodies increased the Mtb-induced TNF-α, IL-6, IL-1β, and IL-23 secretion (Fig. 2D). Moreover, we found that DC treatment with biglycan or ManLAM decreased the Mtb-triggered

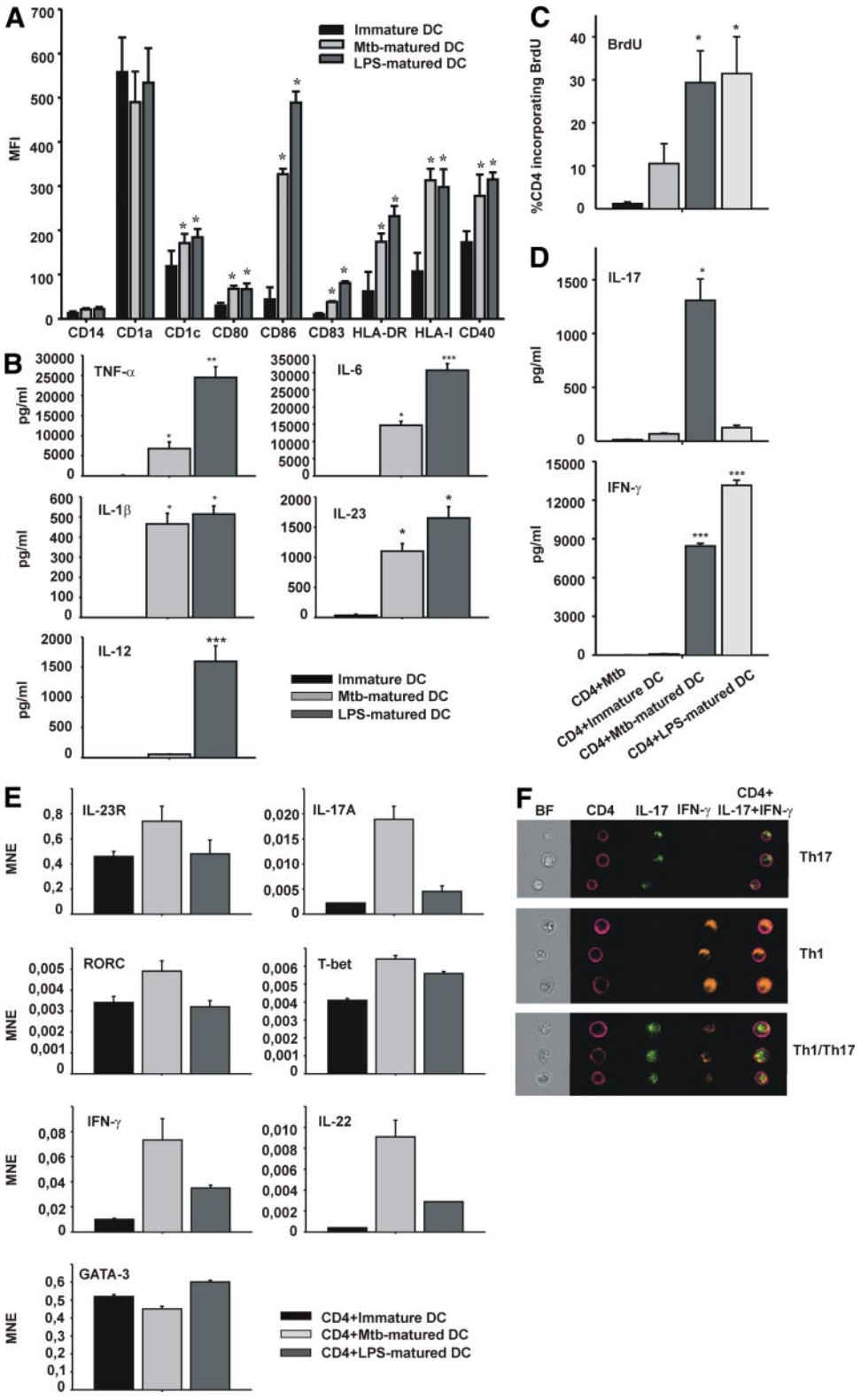

Figure 1. Mtb enables DC to induce a Th1/Th17 response. Monocytes were incubated for 5 days with 50 ng/ml GM-CSF and 20 ng/ml IL-4 to obtain immature DC and then treated (24 h) with 50 μg/ml Mtb (Mtb-matured DC) or 10 ng/ml LPS (LPS-matured DC) to induce DC maturation. FACS analysis was performed to evaluate the DC surface expression of differentiation and maturation markers. MFI, Mean fluorescence intensity (A). Cytokine release was determined by ELISA in DC culture supernatants (B). Results are mean + SD of three (A) and five (B) experiments. Mtb-matured DC and LPS-matured DC versus immature DC, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (A and B). Immature DC and Mtb- or LPS-matured DC were incubated 5 days with purified $CD4^+$ lymphocytes (CD4): BrdU incorporation was analyzed by FACS (C), and IL-17 and IFN-γ release was analyzed by ELISA (D). As control, $CD4^+$ lymphocytes were incubated with Mtb without DC (CD4+Mtb; C and D). Results are mean + SD of five (C) and three (D) experiments. $CD4^+$ lymphocytes cultured with Mtb- or LPS-matured DC versus $CD4^+$ lymphocytes cultured with immature DC, *, $P < 0.05$; ***, $P < 0.001$ (C and D). mRNA expression of lymphocyte markers was tested by RT-PCR. RNA was extracted from a whole mixed cell population: One experiment representative of four is shown (E). Lymphocytes cocultured 5 days with Mtb-matured DC, immunofluorescent-stained and analyzed by the ImageStream cytometer: The images are representative of different lymphocyte subsets (F).

TNF-α, IL-6, IL-1β, and IL-23 release into cell culture supernatant (Fig. 2E).

These results indicate that Mtb induces TNF-α, IL-6, IL-1β, and IL-23 production, mainly by activating dectin-1, whereas MR or DC-SIGN engagement limits the secretion of these cytokines. Differently from the data obtained by stimulating DC with soluble receptor agonists (Fig. 2A), the results illustrated in Figure 2E show that DC-SIGN or MR engagement inhibited the Mtb-induced production of all the cytokines, including IL-6. This discrepancy could depend on a different

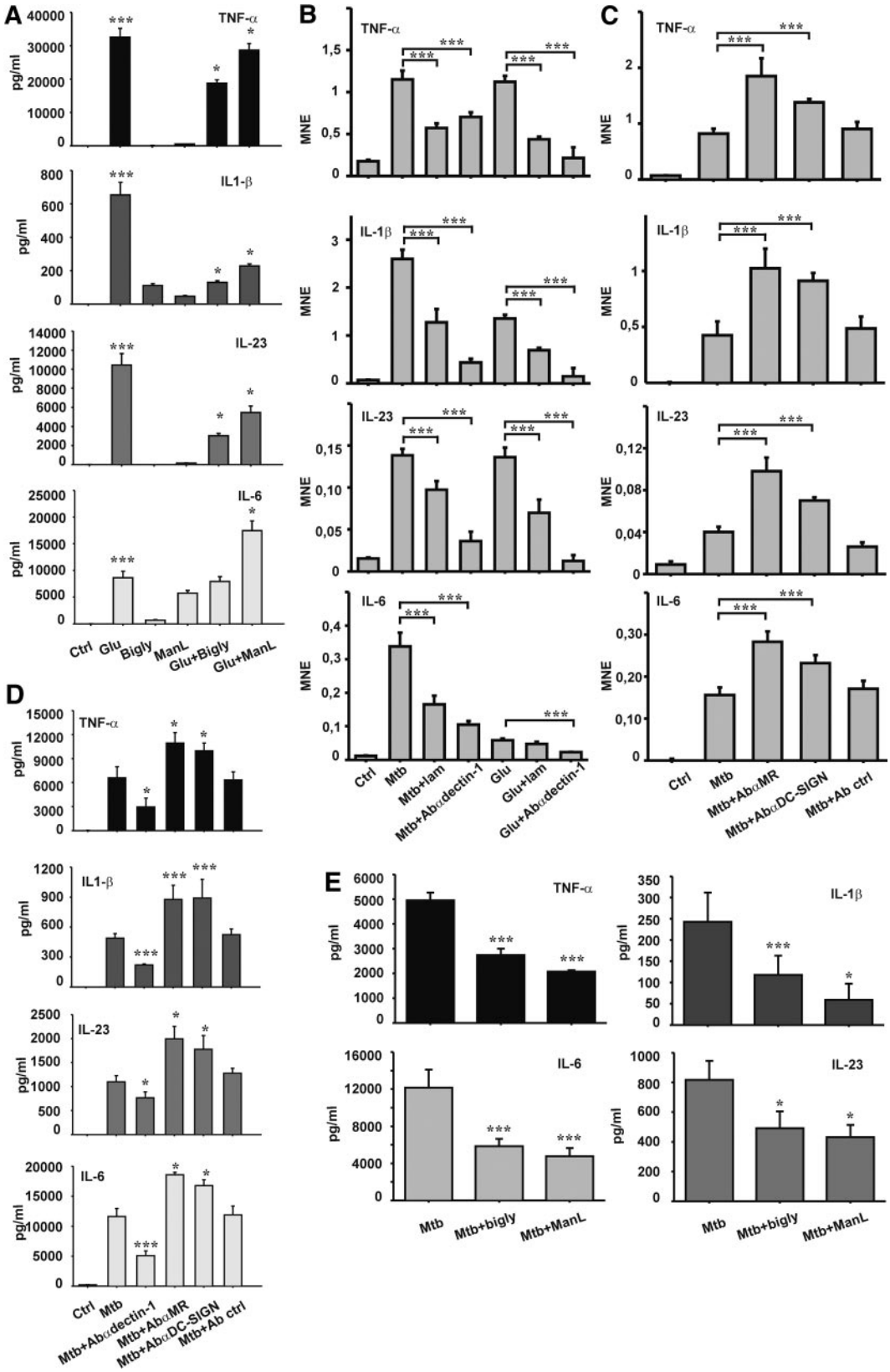

Figure 2. Effect of dectin-1, MR, and DC-SIGN engagement on cytokine expression by DC. Immature DC were cultured for 24 h in the absence (Ctrl) or presence of 15 μg/ml glucan (Glu), 10 μg/ml biglycan (Bigly), 10 μg/ml ManLAM (ManL), or a combination of glucan with biglycan (Glu+Bigly) or ManLAM (Glu+ManL). Cytokine release was evaluated by ELISA in culture supernatants. Results are expressed as the mean value + SD of three independent experiments. Glucan-treated DC versus control DC, ***, $P < 0.001$; biglycan- or ManLAM-treated DC in the presence of glucan versus glucan-treated DC, *, $P < 0.05$ (A). Immature DC were preincubated (30 min) with 500 μg/ml laminarin (lam) or 10 μg/ml dectin-1 (Abαdectin-1; B)-, MR (AbαMR; C)-, or DC-SIGN (AbαDC-SIGN; C)-blocking antibodies; an irrelevant antibody (Mtb+Ab ctrl) was used as control (C). DC were then challenged for 4 h with 50 μg/ml Mtb (B and C) or 15 μg/ml glucan (B). Ctrl, Untreated DC (B and C). TNF-α, IL-1β, IL-23, and IL-6 expression was analyzed by RT-PCR. (B and C) One experiment representative of three: laminarin- or blocking antibody-treated DC versus Mtb- or glucan-stimulated DC, ***, $P < 0.001$. Immature DC were cultured (24 h) with 50 μg/ml Mtb in the presence of 10 μg/ml dectin-1-, MR-, or DC-SIGN-blocking antibodies (D) or 10 μg/ml biglycan (Mtb+Bigly) or ManLAM (Mtb+ManL; E). An irrelevant antibody was used as control (D). Cytokine secretion in culture supernatant was investigated by ELISA (D and E). Results are the mean value + SD of four experiments: Mtb-treated cells cultured in the presence of blocking antibodies (D), biglycan (E), or ManLAM (E) versus DC treated with Mtb alone, *, $P < 0.05$; ***, $P < 0.001$.

pattern of receptors engaged in Mtb-treated DC as compared with DC treated with mixed soluble receptor agonists.

Dectin-1 stimulation enables DC to induce Th1/Th17 generation, whereas DC-SIGN and MR engagement limits dectin-1-dependent Th17 generation and favors Th1 response

As dectin-1, DC-SIGN, and MR regulate the release of TNF-α, IL-1β, IL-23, and IL-6 (Fig. 2), and it is known that these cytokines are involved in Th1/Th17 generation [28–34], we investigated the role of these receptors on the mechanisms of T cell response. We incubated glucan-stimulated DC with $CD4^+$ T cells to assess whether a selective dectin-1 stimulation is sufficient to enable human DC to induce a Th1/Th17 response: We found that under these experimental conditions, $CD4^+$ lymphocytes produced IL-17 (**Fig. 3A**) and IFN-γ (Fig. 3B). A characterization of these lymphocytes by RT-PCR showed that they express RORC, IL-23R, IL-17A, IL-22, IFN-γ, and T-bet mRNA (Fig. 3C). These features are similar to those of $CD4^+$ lymphocytes cultured with Mtb-treated DC (Fig. 1E).

DC incubated with ManLAM or biglycan in the absence of glucan were unable to trigger a substantial cytokine production by $CD4^+$ lymphocytes (Fig. 3, A–D). Notably, addition of biglycan or ManLAM resulted in an inhibition of IL-17 (Fig. 3A) and an increase of IFN-γ (Fig. 3B) release by $CD4^+$ lymphocytes incubated with glucan-treated DC. Accordingly, addition of biglycan or ManLAM decreased Th17 and enhanced Th1 marker expression by $CD4^+$ lymphocytes cocultured with glucan-stimulated DC (Fig. 3C). We then explored whether ManLAM and biglycan caused similar effects on lymphocytes incubated with Mtb-treated DC. Figure 3D shows an ELISA, demonstrating that biglycan or ManLAM inhibited IL-17 and increased IFN-γ release by $CD4^+$ lymphocytes incubated with Mtb-challenged DC. Similar results have been obtained by intracellular staining experiments (**Fig. 4**). Therefore, it is conceivable that glucan and Mtb induce Th1/Th17 generation through similar mechanisms of dectin-1 stimulation and that DC-SIGN and MR engagement interferes with these mechanisms, favoring Th1 and inhibiting Th17 generation. This effect is likely a result of DC-SIGN- and MR-induced changes of cytokine patterns generated by glucan- or Mtb-stimulated DC (Fig. 2).

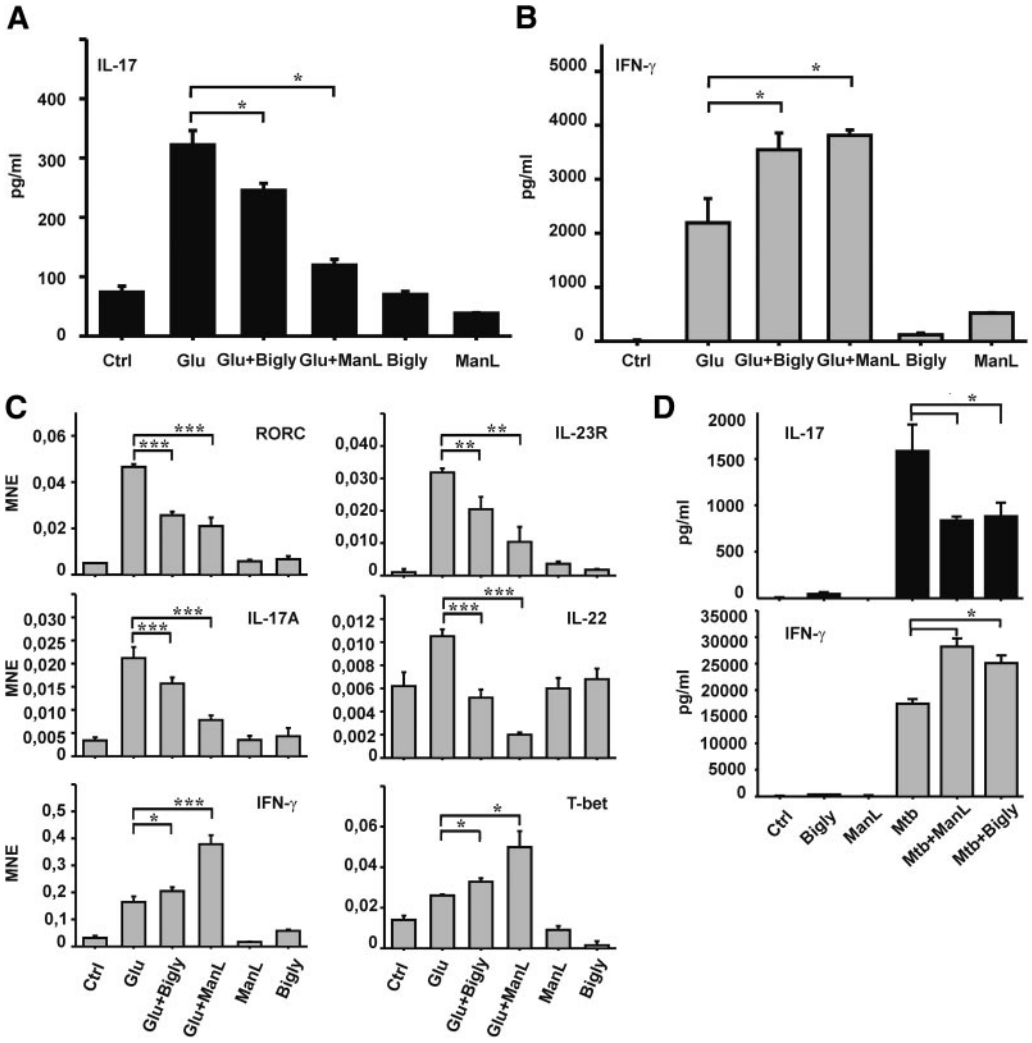

Figure 3. A selective dectin-1 stimulation is sufficient to enable DC to instruct Th1/Th17 generation, whereas MR or DC-SIGN engagement affects a dectin-1-dependent lymphocyte response. DC cultured for 24 h in the absence or presence of 15 μg/ml glucan, 10 μg/ml biglycan, 10 μg/ml ManLAM, or a combination of glucan with biglycan or ManLAM were incubated (5 days) with $CD4^+$ lymphocytes. IL-17 (A) and IFN-γ (B) release in the culture supernatant was analyzed by ELISA. Results are the mean + SD of five independent experiments (A and B). The mRNA expression of RORC, IL-23R, IL-17A, IL-22, IFN-γ, and T-bet was analyzed by RT-PCR: One experiment representative of four independent ones is shown (C). Biglycan- or ManLAM-treated DC in the presence of glucan versus glucan-treated DC, *, $P < 0.05$ (A–C); **, $P < 0.01$ (C); ***, $P < 0.001$ (C). Immature DC were cultured for 24 h with 50 μg/ml Mtb in the absence (Mtb) or presence of 10 μg/ml ManLAM or biglycan (D). Cytokine secretion in culture supernatant was investigated by ELISA: Results are the mean + SD of four independent experiments. Mtb-treated cells cultured in the presence of biglycan or ManLAM versus DC treated with Mtb alone, * $P < 0.05$ (D).

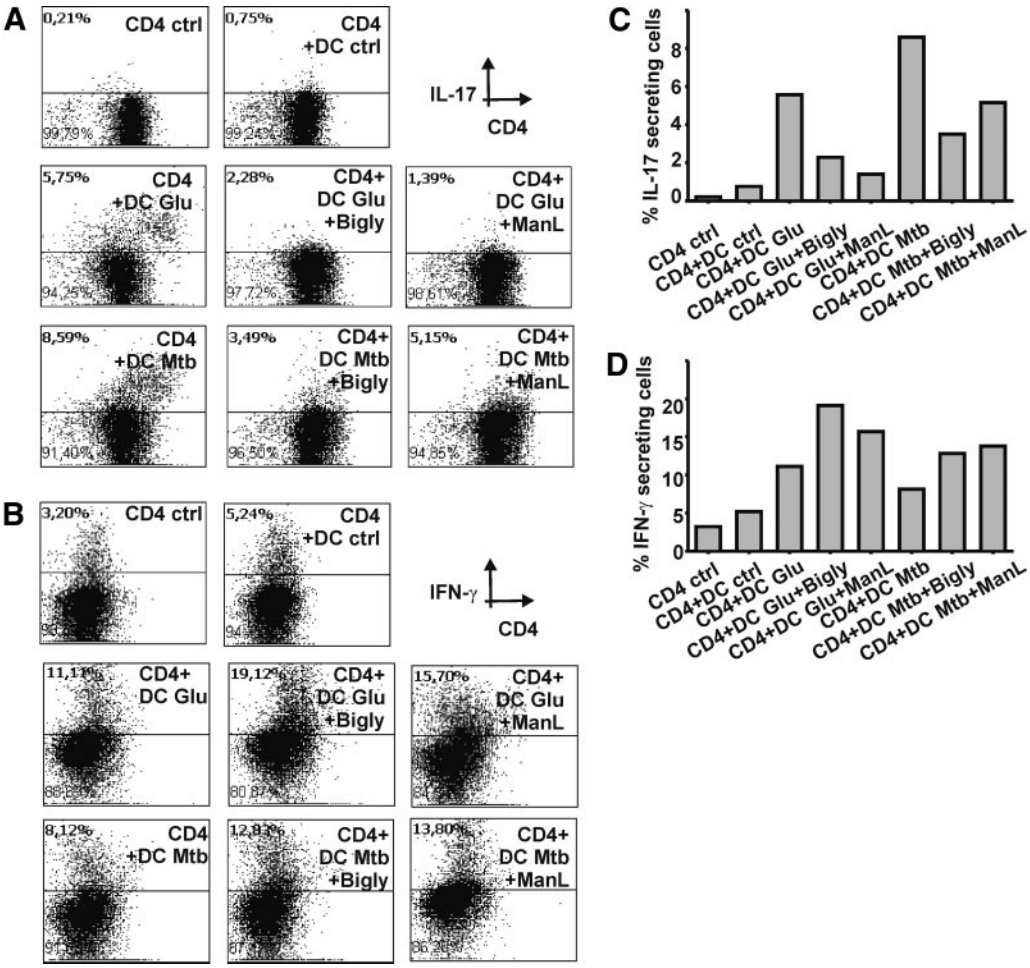

Figure 4. Effects of MR or DC-SIGN engagement on IL-17 and IFN-γ production by lymphocytes cocultured with glucan- or Mtb-treated DC. $CD4^+$ lymphocytes were cultured for 5 days with DC stimulated for 24 h with glucan in the absence (CD4+DC Glu) or presence of biglycan (CD4+DC Glu+Bigly) or ManLAM (CD4+DC Glu+ManL), as well as with DC stimulated for 24 h with Mtb in the absence (CD4+DC Mtb) or presence of biglycan (CD4+DC Mtb+Bigly) or ManLAM (CD4+DC Mtb+ManL). IL-17 (A) and IFN-γ (B) production was detected by intracellular staining using fluorescence-conjugated, specific antibodies and analyzed by FACS. The results illustrated in A and B are displayed as histogram profiles in C and D, respectively. One experiment representative of three independent ones is shown.

DISCUSSION

Here, we show that interaction of γ-irradiated Mtb with human DC leads to DC maturation and IL-1β, IL-6, IL-23, and TNF-α release. Moreover, Mtb enables DC to induce the secretion of IFN-γ and IL-17 by $CD4^+$ lymphocytes. Our results agree with the reports that during mycobacterial infection in mice, IFN-γ- and IL-17-producing T cells are generated [16–18]. It is known that IL-1β, IL-6, IL-23, and TNF-α play a role in induction of the Th17 response in human and mouse models [28–34]. Therefore, our data indicate that Mtb triggers the Th17 response by mechanisms involving the production of these cytokines by human DC.

It has been demonstrated that Mtb interacts with dectin-1 of DC [19, 20]. Here, we show that IL-1β, IL-6, IL-23, and TNF-α secretion is mainly a result of dectin-1 receptor engagement by Mtb and that a selective dectin-1 engagement is sufficient to enable DC to trigger the Th1/Th17 response, independently of stimulation of other DC receptors.

These findings indicate that dectin-1 could be an important receptor involved in induction of Th1/Th17 generation and agree with previous results showing that mouse DC stimulated with the dectin-1 agonist curdlan release IL-6, IL-23, and TNF-α and are able to prime Th1/Th17 cells [21, 22]. Obviously, we cannot exclude that other DC receptors could cooperate with dectin-1 in induction of a Th1/Th17 response. For instance, it has been reported that TLR2 engagement programs DC to promote IL-17 generation [44] and that TLR2 collaborates with dectin-1 to induce IL-23 production in mouse DC [45]. However, Manicassamy et al. [22] published that TLR2 signaling suppresses IL-23 production and Th17 and Th1 responses, whereas dectin-1 engagement induces IL-23 and increases Th17- and Th1-mediated autoimmune responses in an in vivo mouse model. Therefore, the role of TLR2 in lymphocyte activation by DC remains to be fully elucidated. Notably, some authors demonstrated that TLR2 is not involved in interactions between mycobacteria and DC [19, 46, 47], thus ruling out an effect of this receptor on a Mtb-induced T cell response.

Some authors [48–50] showed that LPS-stimulated DC induce T lymphocytes to produce IL-17. Here, we report that LPS-treated DC induce a remarkable Th1 response but a weak IL-17 generation. Our results agree with those of Acosta-Rodriguez et al. [28], who show that human monocyte-derived DC activated by LPS do not induce IL-17 secretion efficiently.

DC stimulation with Mtb or glucan leads to a low or absent IL-12 secretion, in agreement with previous reports [51]. However, we found that in association with IL-17 release, DC stimulated with Mtb or dectin-1 agonists always trigger IFN-γ pro-

duction by $CD4^+$ lymphocytes, as observed previously [21, 24]. These results are not surprising, as it is known that a simultaneous Th1 and Th17 response can take place in the absence of significant amounts of IL-12 [16, 28], although this cytokine plays an important role in induction of IFN-γ secretion by lymphocytes [52].

$CD4^+$ lymphocytes activated by Mtb- or glucan-treated DC display Th17-associated RORC, IL-23R, IL-22, and IL-17A transcripts, as well as Th1-associated T-bet and IFN-γ mRNA, suggesting that Mtb and glucan enable DC to induce the response of lymphocytes through similar mechanisms, likely involving dectin-1-dependent cytokine release. A further characterization of lymphocytes stimulated by Mtb-treated DC evidenced the presence of T cells selectively producing IL-17 or IFN-γ as well as lymphocytes secreting both of these cytokines. Therefore Mtb causes a complex activating condition leading to formation of Th1, Th17, and Th1/Th17 lymphocyte subsets.

In our experiments, we used human $CD4^+$ T cells, containing naïve and memory T cells. Therefore, it remains to be determined whether Th1/Th17 populations obtained by us derive from naïve or memory cell subsets. To clarify this point, coculture experiments performed with DC and isolated naïve or memory T cells should be performed. However, as DC promote IL-17 production exclusively in memory T cells [44, 53], it is likely that the Th17 and Th1/Th17 populations obtained by us result from the expansion of memory cells present in the blood of the donors.

Here, we also report that DC-SIGN or MR engagement leads to a decreased, Mtb-dependent IL-23, IL-1β, IL-6, and TNF-α production by DC, whereas MR- or DC-SIGN-blocking antibodies enhanced the Mtb-induced expression of these cytokines. These effects are probably a result of an interference of MR and DC-SIGN on dectin-1-associated signals. Experiments performed with soluble receptor agonists, in which simultaneous addition of glucan and biglycan or ManLAM results in depressed cytokine release, support this hypothesis. Interestingly, DC incubation with DC-SIGN or MR agonists inhibits the Th17 generation and increases the Th1 response induced by glucan- or Mtb-treated DC.

To our knowledge, this is the first demonstration that DC-SIGN and MR engagement interferes with the dectin-1-dependent mechanisms leading to Th17 generation by human DC. During our investigations, we found that DC-SIGN engagement also inhibits the LPS-dependent cytokine production (results not shown), suggesting that these receptors could represent more general inhibitors of proinflammatory cytokine release [41]. The exact mechanisms by which dectin-1 signaling pushes the Th17 response, and the simultaneous MR/DC-SIGN stimulation preferentially drives Th1 generation remain to be investigated. These changes probably reside in different patterns/amounts of cytokines and/or different surface molecules expressed by DC upon engagement of these receptors.

The role of Th17 in the immune response to tuberculosis remains unclear [16]. It is likely that IL-17 is involved in the control of mycobacteria-induced inflammation, acting on neutrophil and mononuclear cell accumulation [23–25, 54–56] as well as on neutrophil survival [57]. However, it has been shown that the Th17 pathway could be associated with defective pathogen clearance, as well as failure to resolve inflammation and to initiate protective immune responses [58]. Moreover, it has been found that IL-17-producing cells play a modest role in protecting against intracellular mycobacteria [26]. Therefore, Mtb could induce monocyte-derived DC to increase the IL-17 production by lymphocytes to promote its own survival and the chronicization of the disease. These effects are a part of a more complex strategy by which Mtb subverts the functions of the cells involved in the immune response and escapes the defensive mechanisms of the host organisms.

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