Mycobacterium bovis Bacillus Calmette-Guérin infects DC-SIGN$^-$ dendritic cell and causes the inhibition of IL-12 and the enhancement of IL-10 production

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Abstract: The only available vaccine against tuberculosis is *Mycobacterium bovis* Bacillus Calmette Guérin (BCG), although its efficacy in preventing pulmonary tuberculosis is controversial. Early interactions between dendritic cells (DC) and BCG or *Mycobacterium tuberculosis* (Mtb) are thought to be critical for mounting a protective antimycobacterial immune response. Recent studies have shown that BCG and Mtb target the DC-specific C-type lectin intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) to infect DC and inhibit their immunostimulatory function. This would occur through the interaction of the mycobacterial mannosylated lipoarabinomannan to DC-SIGN, which would prevent DC maturation and induce the immunosuppressive cytokine interleukin (IL)-10 synthesis. Here, we confirm that DC-SIGN is expressed in DC derived from monocytes cultured in granulocyte macrophage-colony stimulating factor (GM-CSF) and IL-4 and show that it is not expressed in DC derived from monocytes cultured in GM-CSF and interferon-α (IFN-α). We also demonstrate that DC-SIGN$^-$ DC cultured in GM-CSF and IFN-α are able to phagocytose BCG and to undergo a maturation program as well as DC-SIGN$^+$ DC cultured in IL-4 and GM-CSF. We also show that BCG causes the impairment of IL-12 and the induction of IL-10 secretion by DC, irrespective of DC-SIGN expression. Finally, we demonstrate that the capacity to stimulate a mixed leukocyte reaction of naïve T lymphocytes is not altered by the treatment of both DC populations with BCG. These data suggest that DC-SIGN cannot be considered as the unique DC receptor for BCG internalization, and it is more interesting that the mycobacteria-induced immunosuppression cannot be attributed to the engagement of a single receptor. *J. Leukoc. Biol.* 78: 106–113; 2005.

Key Words: *tuberculosis · phagocytosis · immunosuppression · DC receptors*

INTRODUCTION

Estimates indicate that one-third of the world's population is infected with *Mycobacterium tuberculosis* (Mtb), but infection does not usually lead to active disease [1, 2]. In fact, in most cases of primary Mtb infection, the individual is asymptomatic and noninfectious, although infected by dormant mycobacteria. This clinical latency often extends for the lifetime of the individual. Acute, active tuberculosis (TB) can result in a small percentage of tuberculin-positive, latently infected individuals, probably as a result of the lack of initiation/maintenance of an appropriate immune response [3]. Reactivation of latent infections can occur in response to perturbations of the immune response, thus ensuing active TB. However, in many cases of active TB, an obvious immunodeficiency is not found [1]. The immune response limiting and switching the infection off during primary TB is presumably initiated when upon exposure to Mtb, the efficient antigen-capturing tissue immature (Im) dendritic cells (DC) [4] are transformed into strongly T cell-stimulatory, mature (m)DC, which migrate with high efficiency into draining lymph nodes. In these compartments, the stimulatory capacity of mDC ultimately leads to effector T cell differentiation and memory T cell expansion, which in turn, confer protection against Mtb in the lung [2, 5–7]. Mtb may persist by suppressing microbicidal activities of host macrophages and by ultimately subverting cell-mediated immune responses capable of eradicating the infection, but these latter mechanisms are still not understood completely [8, 9]. As DC are of fundamental importance in establishing a protective immune response against Mtb, it is conceivable that DC may represent strategic targets of Mtb immune evasion mechanisms. We have shown previously that Mtb can alter the differentiation of monocytes into DC capable of promoting a T helper cell type 1 response [10–12] and hypothesized that this phenomenon could represent an escape mechanism explaining the diversion of the recall immune response during reactivation TB.

In addition, the interaction of structural components of mycobacteria such as Mtb and Bacillus Calmette Guérin (BCG) with DC-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) has been proposed recently as crucial to the infection of DC and to potentially determine immuno-

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Received January 21, 2005; accepted March 16, 2005; doi: 10.1189/jlb.0105037.

suppression [13, 14]. DC-SIGN is a type II transmembrane protein that belongs to the C-type lectin family, and it is expressed by monocyte-derived DC, as well as by tissue DC of virtually all body compartments [15, 16]. DC have been shown to interact with several pathogens including virus, bacteria, fungi, and protozoa through DC-SIGN [17]. In particular, the interaction of Mtb or BCG with DC-SIGN has been reported as one of the major examples of how this receptor can influence DC function [13, 14]. However, most of the studies have been conducted using DC derived from monocytes cultured with granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin (IL)-4 [18, 19]. This is a relevant issue, as it has been demonstrated that DC-SIGN expression during DC differentiation is primarily induced by IL-4, and transforming growth factor-β, interferon-α (IFN-α), or dexamethasone prevent its IL-4-dependent induction on monocytes [20]. The possibility exists that alternative in vitro methods of DC generation, which may reflect different in vivo microenvironments, lead to the differentiation of DC with different expression or function of DC-SIGN. In particular, GM-CSF plus IFN-α is a method that may mimic the in vivo DC differentiation from monocytes recruited in an inflammatory milieu, where type I IFN is released as a danger signal [21]. Given the known capability of IFN-α to prevent DC-SIGN expression, in this study, we investigated the kinetics of DC-SIGN expression and the functional consequences of the interaction of mycobacteria with DC derived from monocytes cultured with GM-CSF and IFN-α.

MATERIALS AND METHODS

Reagents

Recombinant IL-4 was purchased by from R&D Systems (Minneapolis, MN), IFN-α from Alfa Wasserman S.p.A. (Alanno PE, Italy), and GM-CSF (Leucomax) from Sandoz (Basel, Switzerland). Tritiated thymidine was from Amersham (Little Chalfont, UK). Phorbol 12-myristate 13-acetate, ionomycin, and brefeldin-A were from Sigma Chemical Co. (St. Louis, MO). RPMI 1640 (Euroclone Ltd., UK) was used, supplemented with 100 U/ml kanamycin, 1 mM glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, and 10% fetal bovine serum (Hyclone, Logan, UT; complete medium).

Growth of mycobacteria

BCG (ATCC 27291) was grown in Middlebrook 7H10 agar (Difco Laboratories, Detroit, MI) at 37°C under a humidified 5% CO_2 atmosphere for 2 weeks. Bacterial suspensions were prepared by dispersing colonies with glass beads in RPMI 1640. The tubes were vortexed and allowed to stand for 30 min to let larger particles settle. The upper supernatant was stored at −80°C until use. Colony-forming units were counted by the standard viable count technique in Middlebrook 7H10 agar plates [12].

Monocyte isolation, BCG infection, and DC generation

Peripheral blood mononuclear cells were purified from heparinized blood obtained by healthy donors on a density gradient (Lymphoprep, Nycomed Pharma AS, Oslo, Norway) as described previously [22].

Monocytes were sorted using anti-CD14-labeled magnetic beads (magnetic cell sorter, Miltenyi Biotec, Germany) according to the manufacturer's instructions and cultured for 6 days at the concentration of 4×10^5cells/ml in complete medium containing GM-CSF (50 ng/ml) and IFN-α (1000 U/ml) or GM-CSF and IL-4 (1000 U/ml). Infection of DC generated with GM-CSF plus IL-4 (IL-4 DC) or with GM-CSF plus IFN-α (IFN-α DC) was performed at day 5 of culture with single-cell suspensions of BCG at the multiplicity of infection (MOI), DC:mycobacterium, of 1:6. The efficiency of infection was quantitated by counting intracellular mycobacteria in cells stained with the Kinyoun method [23] after overnight (o/n) incubation. When indicated, on day 5, DC were stimulated o/n with 0.1 μg/ml lipopolysaccharide (LPS) from *Escherichia coli* (Sigma Chemical Co.) to induce their maturation.

Reverse transcriptase-polymerase chain reaction (RT-PCR) and real-time PCR

Total RNA was extracted from 10^6 cells using an RNeasy mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Total RNA (0.5–3 μg) was treated with 8 units RQ1 DNase I (Promega, Madison, WI) in 40 mM Tris-HCl (pH 8), 10 mM $MgSO_4$, and 1 mM $CaCl_2$ for 1 h at 37°C. RNA was extracted with phenol-chloroform, ethanol-precipitated, and resuspended in H_2O. An aliquot of DNase-treated RNA was tested for DNA contamination with β-actin primers. Total RNA was then reverse-transcribed 1 h at 42°C with 0.5 μg random primers, 5 μl ImProm-II reaction buffer (Promega), 3 mM $MgCl_2$, and 0.5 mM each nucleotide and 1 μl ImProm-II RT (Promega) in a final volume of 25 μl. PCRs were carried out in 20 μl reaction volume as follows: a 3-min denaturation at 95°C, followed by 40 min at 95°C, 40 min at 62°C, and 90 min at 72°C. cDNA was amplified for 25 cycles using human β-actin sense (5′-TCCTGTGGCATCCACGAAACT-3′) and antisense (5′-GAAGCATTTGCGGTGGACGAT-3′) primers for 36 cycles with DC-SIGN sense (5′-AGAGTGGGGTGACATGAGTG-3′) and antisense (5′-GAAGTTCT-GCTACGCAGGAG-3′) primers [24] and for 30 cycles with mannose receptor (MR) sense (5′-GGTCGGATGGATGGCTCTG-3′) and antisense (5′-GCTGAT-GGACTTCCTGGTAAC-3′) primers. All primers flanked introns for DNA-genomic product discrimination. Serial 1:2 dilutions of cDNA from THP-1 cells were amplified with β-actin primers to establish the exponential range of amplification. The PCR fragments were separated on 1.2% agarose gels. For real-time quantitation, cDNA was amplified with the primers described above, with the exception of DC-SIGN, which was amplified using a different sense primer (5′-CACCCCTGTCCCTGGGAAT-3′) to produce a shorter PCR product (465 bp). PCR was performed in 20 μl with iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) in an iCycler iQ (Bio-Rad) for 40 cycles as follows: 40 min at 95°C, 40 min at 62°C, and 60 min at 72°C. To verify the absence of primer dimers in the reaction, a final melting curve was generated with temperature increases from 55°C to 95°C. All amplifications were tested in triplicate. A nontemplate control was run in every assay. Threshold cycle (C_t) data were collected, ΔC_t was calculated as the C_t difference between the gene of interest and the β-actin, and comparative gene expression was established by the $\Delta\Delta C_t$ method. Finally, data were expressed as fold induction using the $2^{-\Delta\Delta Ct}$ formula [25]. A standard curve for the three primer pairs was determined using known 1:10 serial dilutions of PCR products as starting material to assess amplification efficiency over a wide range of template (data not shown). *P* values were determined by uncoupled two-tailed Student's *t*-test.

Fluorescein-activated cell sorter analysis

We used the following monoclonal antibodies (mAb): fluorescein isothiocyanate (FITC)-conjugated anti- DR, -CD1a, -CD83, and -CD86; phycoerythrin (PE)-conjugated anti-CD14, -CD80, and -DC-SIGN, purified mouse immunoglobulin G1 (IgG1) anti-CD11b, and appropriate isotypic controls, purchased from PharMingen (San Diego, CA). IgG1 mAb (clone 3.29) anti-MR was kindly provided by Antonio Lanzavecchia (Institute of Research in Biomedicine, Bellinzona, Switzerland). Human adsorbed, PE-conjugated goat anti-mouse IgG1 (Southern Biotechnology Assoc., Birmingham, AL) was used in association with anti-CD11b and anti-MR. Cells were harvested and washed in phosphate-buffered saline (PBS) containing 1% fetal calf serum (FCS) and 0.1% NaN_3 (staining buffer) and stained using the above-mentioned antibodies or appropriate isotype controls for background determination.

Stained cells were analyzed by flow cytometry using a FACScan cytometer (Becton Dickinson, Mountain View, CA) equipped with Cellquest software (Becton Dickinson). Fluorescence intensity was evaluated by computerized analysis of dot plots or histograms generated by at least 5000 viable cells.

Phagocytosis assay

IL-4 DC and IFN-α DC were incubated o/n with BCG at the MOI of 1:6 and then washed by low-speed centrifugation (100 *g*). Cells were then transferred

onto polylysine-coated culture slides (Falcon, Seattle, WA) for 1 h. Finally, slides were stained by the Kinyoun or Giemsa method. In addition, capture and internalization of BCG were evaluated using FITC-conjugated BCG. Briefly, 10^9 bacteria were labeled by incubation of 0.5 mg FITC/ml (Sigma Chemical Co.) in PBS at room temperature for 1 h. BCG was then washed four times to remove unbound FITC and resuspended in RPMI plus 10% FCS. IL-4 DC and IFN-α DC were incubated with FITC-conjugated BCG (MOI=1:6) and centrifuged at 100 *g*, and then the percentage of cells that bound FITC-conjugated bacteria was measured by flow cytometry. Phagocytosis was determined by comparing the intensity of green fluorescence (FITC) before and after trypan blue quenching of membrane-bound, labeled bacteria [13].

Priming of naïve T cells

DC were cultured at different cell numbers with 3×10^4 cord blood $CD4^+$ T cells purified by indirect magnetic sorting with the $CD4^+$ T cell isolation kit by Miltenyi Biotec. The proliferative response was measured after 7 days by ^{3}H-thymidine (0.5 μCi/well) incorporation.

Cytokine determination

After 5 days, the supernatant of DC cultures was removed for IL-4 determination, and cells were washed, adjusted to 4×10^5cell/ml, and cultured in the presence or absence of 0.1 μg/ml LPS or BCG (MOI=1:6) for an additional 24 h. Supernatants were examined for cytokines by enzyme-linked immunosorbent assay (ELISA) using commercially available kits (R&D Systems), according to the manufacturer's instructions. Detection limit of the assay was 15 pg/ml.

Statistical analysis

Data were analyzed using the Statview 4.1 program (Abacus Concepts, Berkeley, CA). The statistical significance of the difference between groups of data with a normal distribution was determined by the ANOVA test with Bonferroni-Dunn post-tests.

RESULTS

DC differentiated in IFN-α and GM-CSF do not express DC-SIGN

To investigate DC-SIGN expression along DC differentiation, monocytes were cultured for 5 days in the presence of IL-4 DC or IFN-α DC, and a kinetic analysis of receptor appearance was performed. As shown in **Figure 1**, monocytes do not express DC-SIGN, but the receptor is detectable on their membrane, starting from 24 h of culture with GM-CSF and IL-4 (data not shown) and reaching its highest intensity of expression after 48 h of culture. By contrast, monocytes that are induced to differentiate into DC in the presence of IFN-α plus GM-CSF do not express DC-SIGN at any time of the culture. To determine whether the lack of DC-SIGN surface expression in IFN-α DC was associated with decreased transcription, DC-SIGN mRNA levels were measured in IL-4 DC and IFN-α DC at different times of the culture. **Figure 2** shows that DC-SIGN mRNA levels are detected by RT-PCR in IL-4 DC after 36 cycles. Quantitation by real-time RT-PCR showed barely detectable DC-SIGN mRNA in IFN-α-treated DC: mRNA levels after 12 h, 48 h, and 5 days were, in fact, 39.4, 44.6, and 43.3 times lower than in IL-4 DC, respectively.

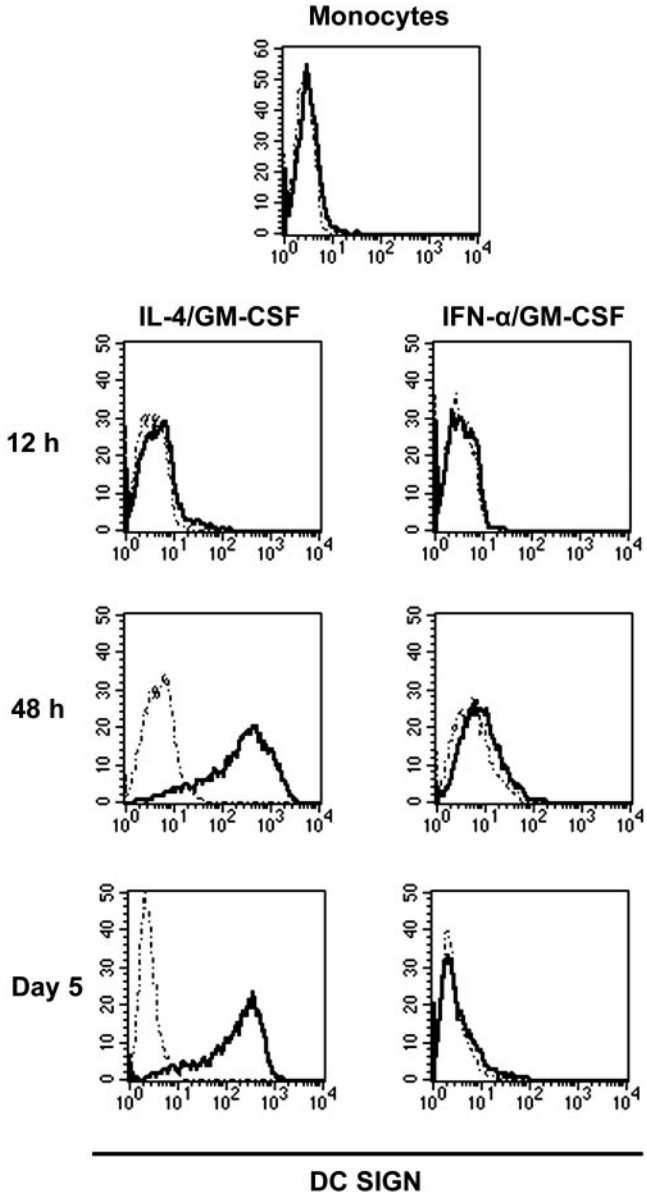

Fig. 1. Kinetics analysis of DC-SIGN expression. Monocytes were cultured in the presence of GM-CSF plus IL-4 or GM-CSF plus IFN-α and analyzed at time 0 and after 12 h, 48 h, and 5 days of culture by flow cytometry. Cytometric analysis was performed by surface staining with PE-conjugated anti-DC-SIGN mAb (solid-line histograms). Dotted-line histograms indicate isotype control mAb. One representative experiment out of three is shown.

IFN-α DC phagocytose BCG

DC-SIGN has been considered the major receptor for Mtb and BCG on immature DC. For this reason, we asked if capture and internalization of mycobacteria would occur in IFN-α DC lacking DC-SIGN expression with the same kinetics and efficiency as IL-4 DC. As shown in **Table 1**, a lower percentage of IFN-α DC (37%) binds FITC-BCG after 2 h of incubation, as compared with IL-4 DC (65%). However, trypan blue quenching of noninternalized FITC-conjugated bacteria revealed that the percentage of cells that internalize bound bacteria is comparable between the two DC populations (IL-4 DC=52.3% and IFN-α DC=48.4%), suggesting that DC-SIGN may increase the BCG binding rate, but other receptor(s) may also be involved in BCG binding and internalization. Then, we tested the ability of the two DC populations to phagocytose BCG after o/n incubation by counting Kinyoun-stained bacteria in DC allowed to adhere on polylysin-precoated slides. We observed

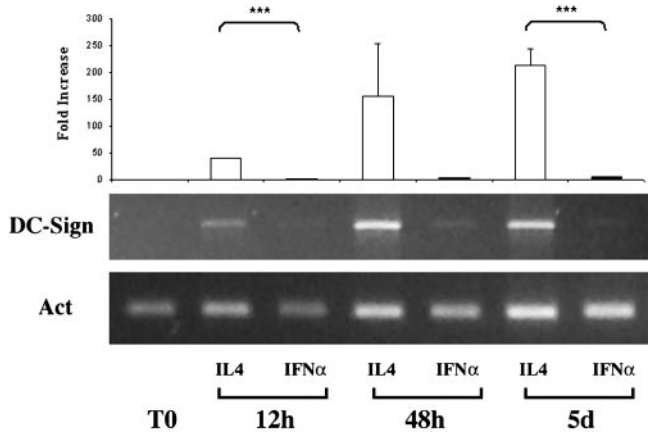

Fig. 2. Analysis of DC-SIGN mRNA by RT-PCR and real-time PCR. RT-PCR and real-time quantitative analysis of DC-SIGN mRNA expression in monocytes incubated with GM-CSF and IL-4 (IL4) or GM-CSF IFN-α (IFNα). Total RNA was extracted at time 0 (T0) and after 12 h, 48 h, and 5 days. RT-PCR quantification was performed for 25 cycles for β-actin (Act) and 36 cycles for DC-SIGN, as described in Materials and Methods. Band sizes were as expected from cDNA amplification: 315 bp for β-actin and 1237 bp for DC-SIGN. SYBR Green real-time RT-PCR was performed for 40 cycles. Each sample was tested in triplicate, and the ΔC_t was calculated, normalizing to β-actin. Differential gene expression was calculated by the $\Delta\Delta C_t$ method, and values were expressed as fold induction compared with baseline levels, which was IFN-α DC at 12 h for DC-SIGN, and DC-SIGN mRNA at T0 was undetectable. ***, $P < 0.01$.

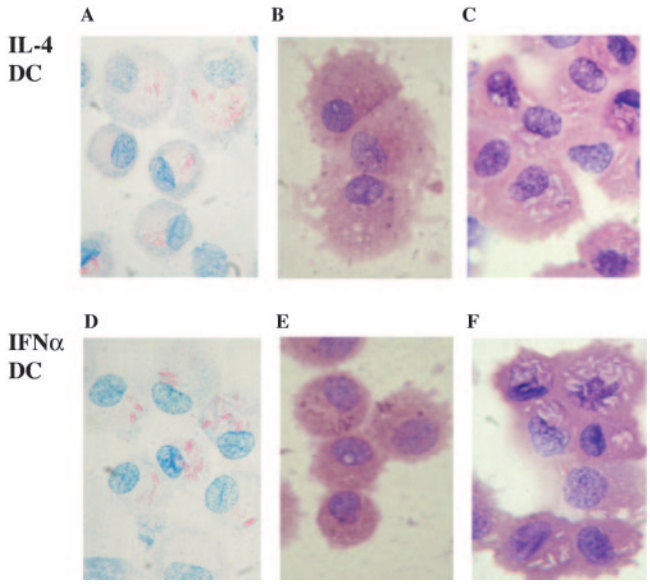

Fig. 3. Phagocytosis of BCG by IFN-α DC and IL-4 DC, which were incubated o/n with BCG at the MOI of 1:6 (A, C, D, and F) or left uninfected (B and E) and then transferred onto polylysine-coated culture slides. After 1 h of incubation to allow DC adherence, slides were stained by the Kinyoun (A and D) or Giemsa (B, C, E, and F) method.

that IL-4 and IFN-α DC are equally associated with high numbers of BCG (**Fig. 3, A** and **D**). In addition, Giemsa staining of DC showed cytoplasmic vacuoli in BCG-infected cells (Fig. 3, C and F) but not in uninfected cells (Fig. 3, B and E), suggesting that bacteria are not only associated but also ingested into phagosomes.

IFN-α DC were also shown by flow cytometry to remain DC-SIGN$^-$ after infection with BCG, ruling out the possibility that an o/n incubation with the mycobacterium would induce DC-SIGN expression on these cells (data not shown).

These data indicate that IFN-α DC phagocytose BCG, irrespective of DC-SIGN expression, but internalization occurs with a reduced efficiency in comparison with IL-4 DC.

IFN-α DC express MR and complement receptor 3 (CR3)

It has previously been demonstrated that Mtb and BCG use different receptors to bind and enter cells of the innate immune system [26, 27]. Among these molecules, the MR and CR3 were shown to be relevant for mycobacteria uptake and internalization. By cytometric analysis, we could demonstrate that IFN-α DC express MR and CR3 with the same fluorescence intensity as IL-4 DC (**Fig. 4A**). In particular, as the MR expression was described previously, as strictly dependent on IL-4 stimulation of monocytes [28, 29], we expanded our investigation on MR expression in IFN-α DC by means of conventional as well as real-time PCR and showed that MR mRNA expression is comparable in IL-4 DC and IFN-α DC, although the MR gene activation occurs early on DC differentiation in the presence of IL-4. In particular, we detected MR mRNA induction in IL-4 DC after 12 h of culture, which is 1.9 times higher than in IFN-α DC (Fig. 4B). However, at later culture times, we observed that MR mRNA induction in IFN-α DC is 2.9 and 1.9 times higher than IL-4 DC (at 48 h and 5 days, respectively). Last, at any time of the differentiation cultures, we could measure IL-4 in GM-CSF and IFN-α-treated monocytes (data not shown). This result ruled out the possibility that IFN-α-cultured monocytes secrete IL-4, which in turn, would induce MR expression.

Taken together, these data indicate that except for DC-SIGN, IL-4 DC and IFN-α DC share the same pattern of phagocytic receptors that could be involved in mycobacteria uptake and internalization.

TABLE 1. Flow Cytometric Evaluation of FITC-BCG Phagocytosis

	IL-4 DC	IFN-α DC
Surface-bound bacteria[a]	65 ± 28	37 ± 36.3
Internalized bacteria[b]	34 ± 14.5	18 ± 14.9
Ratio[c]	52.3	48.6

[a] % Fluorescent DC; [b] % fluorescent DC after trypan blue quenching of external fluorescence; [c] % internalized/surface-bound bacteria.

BCG infection promotes phenotypic and functional maturation of IFN-α DC

Next, we investigated the functional outcome of IFN-α DC infection by BCG as compared with IL-4 DC. As summarized in **Table 2**, both DC populations acquire a mature phenotype after o/n incubation with BCG, as demonstrated by the increased expression of major histocompatibility complex class II, CD86, and to a lesser extent, CD80 molecules. Moreover, both BCG-infected populations, when challenged in mixed leukocyte reaction (MLR) with allogeneic, naïve CD4$^+$ T cells,

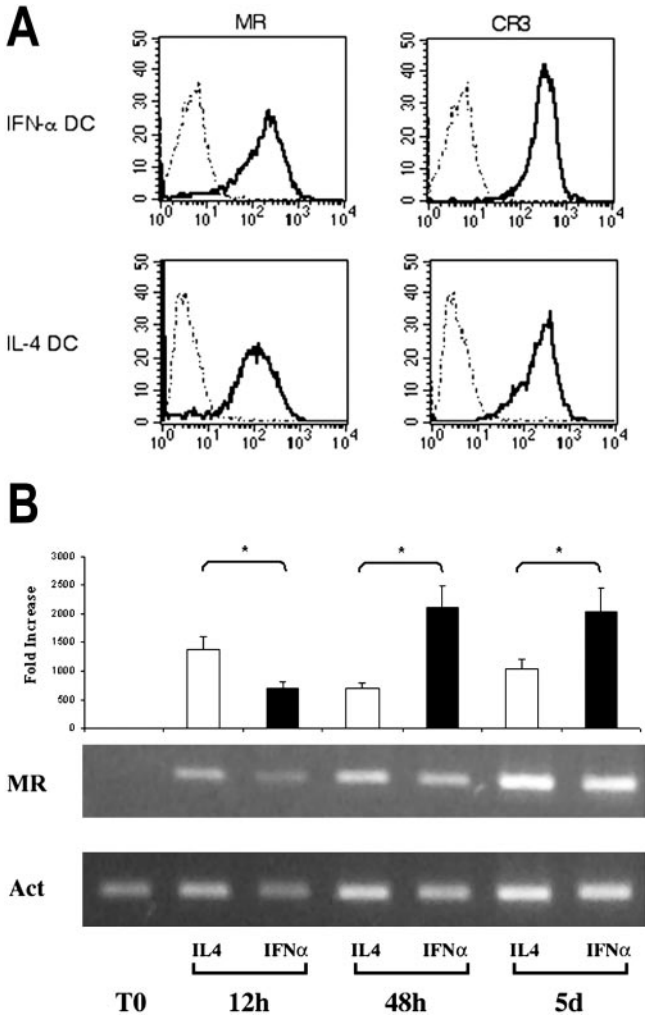

Fig. 4. Expression of MR and CR3 on IFN-α DC and IL-4 DC, which at day 5 of culture, were stained with purified mouse IgG1 anti-CR3 or anti-MR in association with PE-conjugated goat anti-mouse IgG1. Dotted-line histograms indicate isotype control mAb. One representative experiment out of four is shown. RT-PCR and real-time quantitative analysis of MR mRNA expression in monocytes incubated with GM-CSF and IL-4 (IL4) or GM-CSF and IFN-α (IFNα). Total RNA was extracted at time 0 (T0) and after 12 h, 48 h, and 5 days. RT-PCR quantification was performed for 25 cycles for β-actin (Act) and 30 cycles for MR, as described in Materials and Methods. Band sizes were as expected from cDNA amplification: 315 bp for β-actin and 370 bp for MR. SYBR Green real-time RT-PCR was performed for 40 cycles. Each sample was tested in triplicate, and the ΔC_t was calculated, normalizing to β-actin. Differential gene expression was calculated by the $\Delta\Delta C_t$ method, and values were expressed as fold induction compared with baseline levels, which was T0. *, $P < 0.05$.

induce T cell priming and proliferation (**Fig. 5**), indicating a fully competent antigen-presentation function, which is a prerogative of mature DC. These data suggest that BCG can infect DC, irrespective of DC-SIGN expression, inducing the same maturation program in IFN-α DC and IL-4 DC.

BCG-infected IFN-α DC and IL-4 DC exhibit a similar cytokine profile

It has been demonstrated that Mtb and BCG target DC-SIGN to infect DC and to induce IL-10 production. Therefore, we asked whether the lack of DC-SIGN could influence the cytokine production pattern of IFN-α DC after BCG infection. Although the amount of IL-10 and IL-12 produced by LPS-stimulated DC varies greatly among different donors, we could demonstrate that IL-4 DC secrete a higher amount of IL-12 and a lower amount of IL-10 than IFN-α DC when stimulated with LPS (**Fig. 6**). However, if infected with BCG, IFN-α DC fail to synthesize IL-12 as well as IL-4 DC. It is striking that upon infection with BCG, IFN-α DC produce higher amounts of IL-10 than IL-4 DC (Fig. 6). It has been suggested that Mtb and BCG target DC-SIGN to suppress DC function through the production of the anti-inflammatory cytokine IL-10. Our results indicate that even in the absence of DC-SIGN, as in the case of IFN-α DC, BCG infection induces an immunosuppressive cytokine profile. These data further suggest that alternative DC-mycobacteria interactions may be involved. This is also true for IL-12 inhibition, which occurs indifferently in IL-4 DC and IFN-α DC.

DISCUSSION

In this paper, we show that DC generated from monocytic precursors using GM-CSF and IFN-α lack the expression of DC-SIGN. We also demonstrate that DC-SIGN$^-$ DC are able to phagocytose BCG and to undergo a maturation program as well as DC-SIGN$^+$ DC cultured in IL-4 and GM-CSF. Moreover, BCG causes the impairment of IL-12 and the induction of IL-10 secretion by DC, irrespective of DC-SIGN expression.

These data suggest that DC-SIGN cannot be considered as the unique receptor for BCG internalization, and it is more interesting that it cannot be considered as the exclusive receptor responsible for mycobacteria-induced immunosuppression.

DC-SIGN is a type II transmembrane protein that belongs to the C-type lectin family, and it is expressed by DC derived from monocytes cultured with GM-CSF and IL-4, dermal DC,

TABLE 2. Flow Cytometric Analysis of DC Maturation Markers

	IL-4 DC			IFN-α DC		
	ImDC	+LPS	+BCG	ImDC	+LPS	+BCG
DR[a]	59 ± 0.7	150 ± 111*	168 ± 96*	108 ± 39	232 ± 138*	200 ± 101*
CD83	4.2 ± 2.0	14 ± 5.9*	14 ± 6.4*	3.3 ± 1.0	7.3 ± 1.7*	6.3 ± 1.1*
CD80	13 ± 4.6	76 ± 38.7*	51 ± 26*	16 ± 6.8	63 ± 31.8*	25 ± 7.4*
CD86	42 ± 7.6	252 ± 111*	205 ± 69*	40 ± 4.9	170 ± 64*	120 ± 53.2*

[a] Values are reported as mean ± SD of the mean intensity of fluorescence measured in seven independent experiments. * Values are statistically different from the respective values of ImDC.

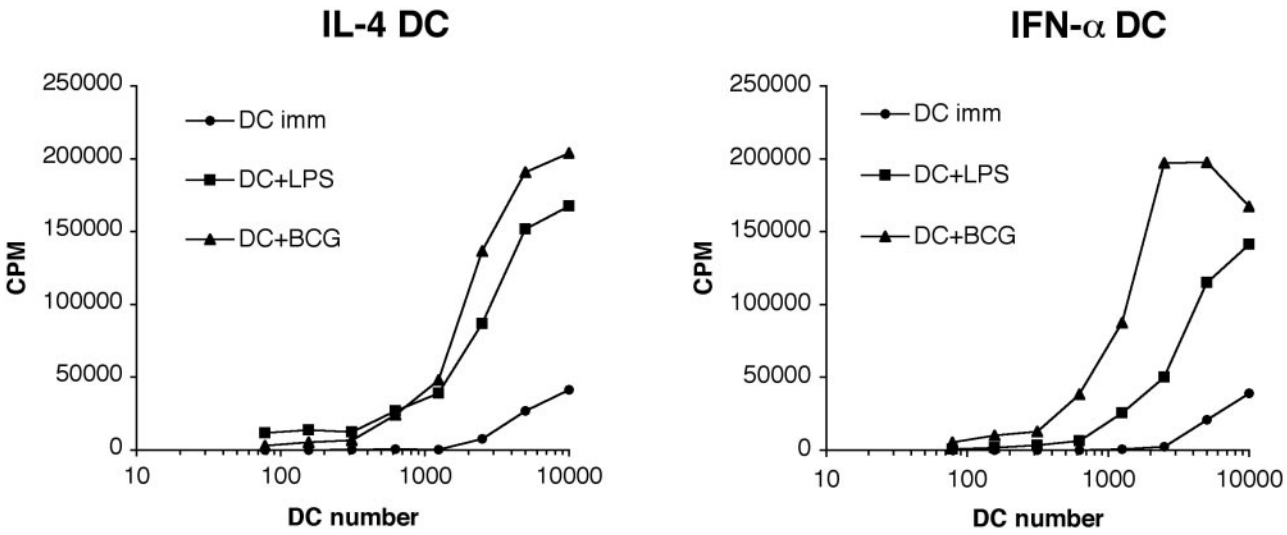

Fig. 5. Priming of naïve T cells by IL-4 DC and IFN-α DC in a MLR. Immature IL-4 DC and IFN-α DC (DC imm), stimulated with LPS (DC+LPS) or infected with BCG at MOI 1:6 (DC+BCG), were cultured at different cell numbers with 3×10^4 cord blood-purified CD4+ T cells. The proliferative response was measured after 7 days by thymidine incorporation. The background proliferation of T cells alone was <1000 counts per minute (cpm). This experiment was performed with five different donors, and one representative is shown.

and interstitial DC in the lungs, intestine, rectum, cervix, placenta, as well as in lymph nodes [15, 16]. DC-SIGN was originally proposed as a receptor for human immunodeficiency virus involved in the DC-dependent lymphocyte infection [30]. DC-SIGN was further characterized as a binder for fucose and mannose-containing carbohydrates, which are present on various other pathogens such as Ebola virus [31, 32], cytomegalovirus [33], hepatitis C virus [34], *Leishmania* [35], *Schistosoma mansoni* [36], *Candida albicans* [37], *Helicobacter pylori* [38], and mycobacteria [14]. In particular, the interaction of Mtb with DC-SIGN has been reported as one of the major examples of how a pathogen can subvert the function of DC through the interaction with a single receptor [13, 14]. In fact, it has been proposed that an indirect sign of immunosuppression caused by Mtb through DC-SIGN binding and signaling is represented by the increased secretion of the immunosuppressive cytokine IL-10 associated with a decrease secretion of IL-12 [13].

It is interesting that in this paper, we show that DC derived from monocytes cultured with GM-CSF and IFN-α do not express DC-SIGN, and the expression of this receptor on monocytes induced to differentiate into DC in the presence of GM-CSF and IL-4 starts as early as 24 h after the beginning of the culture and continues until day 5. These data were paralleled by the analysis of DC-SIGN mRNA by conventional as well as real-time PCR, which confirmed the kinetics of DC-

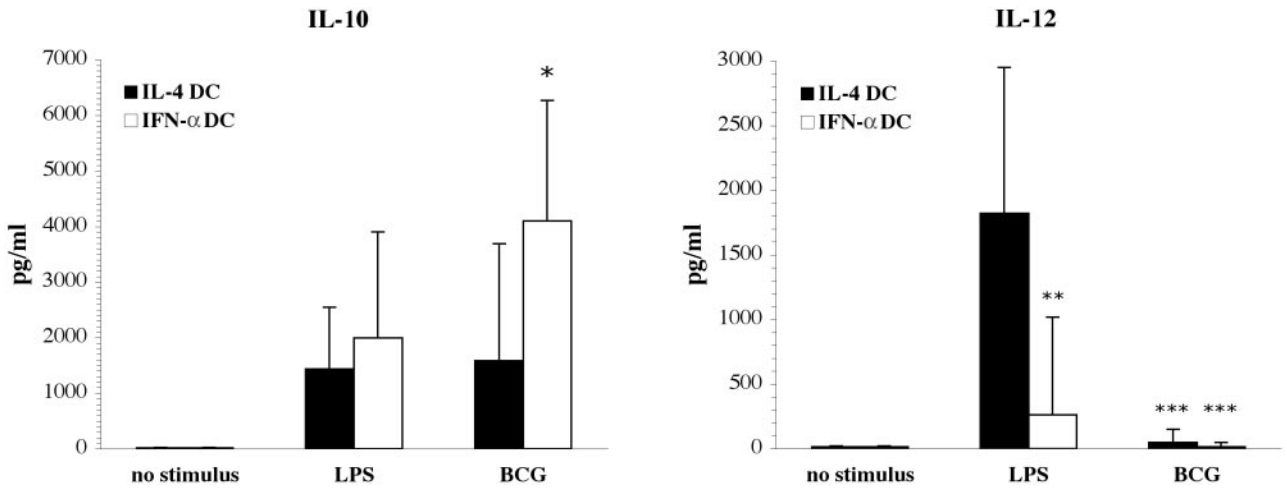

Fig. 6. Cytokine production by IL-4 DC and IFN-α DC. Immature IL-4 DC and IFN-α DC at day 5 of culture were washed, adjusted to 3×10^5cell/ml, and left unstimulated (no stimulus), stimulated with LPS, or infected with BCG at MOI 1:6 for an additional culture of 24 h. Supernatants were examined for cytokines by ELISA. Values indicate the mean ± SD of eight independent experiments. Detection limit of the assay, 15 pg/ml. *, The secretion of IL-10 by IL-4 DC treated with BCG versus IFN-α DC treated with BCG was significantly different ($P<0.05$). **, The secretion of IL-12 by IFN-α DC treated with LPS versus IL-4 DC treated with LPS was significantly different ($P<0.05$). ***, The secretion of IL-12 by IFN-α DC or IL-4 DC treated with BCG versus IFN-α DC or IL-4 DC treated with LPS was significantly different ($P<0.05$).

SIGN expression in IL-4 DC and its absence in IFN-α DC. Conversely, we show that IFN-α DC express other receptors involved in Mtb recognition and internalization, such as MR and CR3, with the same fluorescence intensity as IL-4 DC. It is generally believed that the expression of MR is strictly dependent on IL-4 [28, 29, 39]. However, IL-4 is not necessarily secreted in those in vivo compartments in which monocytes are required to differentiate into DC. Our data show that MR is induced in DC, which derive from monocytes cultured with IFN-α and GM-CSF, and provide the original demonstration that DC have the possibility to express MR in an IL-4-independent manner. These findings are relevant, as IFN-α DC are functionally equivalent to IL-4 DC as antigen-presenting cells (APC) and are candidates to renew the pool of tissue DC during infections caused by viruses or bacteria, including Mtb, which cause the release of IFN-α as a danger signal [40, 41].

Upon infection with BCG, IFN-α DC and IL-4 DC undergo functional maturation, in terms of up-regulation of costimulatory molecules and higher capacity of T cell priming. However, they acquire a peculiar cytokine production profile, characterized by an increase in IL-10 and absence of IL-12 secretion. The capacity of both DC populations to induce T cell priming and proliferation is apparently in contrast with the high IL-10 secretion by IFN-α DC. However, we investigated the capacity of DC to stimulate naïve T lymphocytes in a MLR to measure their antigen-presentation capacity as a feature of mDC. The role of cytokine(s) secreted by DC in this assay is underestimated, as DC are washed extensively after o/n incubation with BCG. However, this washing procedure allows a fine evaluation of the APC function through the reproducible calculation of the DC:T lymphocyte ratio and allows verification of whether DC have ingested the mycobacteria and analysis of their phenotype at the moment of T cell encounter.

In any case, if this subversion of cytokine production by DC may be considered an indirect sign of immunosuppression, our data indicate that this phenomenon is caused by mycobacteria independently on DC-SIGN expression.

In addition, IFN-α and IL-4 DC were capable of binding and internalizing BCG with the same efficacy, although IFN-α DC showed a reduced efficiency. In fact, in short-term cytofluorimetric tests, FITC-BCG was associated with and engulfed by a higher percentage of IL-4 DC than IFN-α DC. However, after o/n incubation, Kinyoun and Giemsa stainings revealed the same degree of BCG phagocytosis by the two DC populations. These data suggest that BCG can infect DC-SIGN$^-$ DC through the engagement of alternative receptors. As a consequence, the indication of DC-SIGN as the unique Mtb receptor on DC cannot be confirmed in our system, which suggests, instead, a revaluation of the role accomplished by other receptors in the uptake of mycobacteria by DC.

As DC are known to be low-permissive to Mtb growth [42], it is to be underlined that an increase in mycobacterial internalization by DC should not necessarily be considered as a negative phenomenon, i.e., a mechanism to enhance mycobacterial intracellular growth and replication. On the contrary, the presence on DC of a high-affinity receptor for mycobacteria, such as DC-SIGN, may be considered as a further tool for antigen capture and presentation of mycobacterial antigens as a result of the known ability of DC-SIGN to internalize its ligands and target them to late endosomes/lysosomes [43]. As a consequence, after mycobacteria capture in the tissues, DC would mature, with the down-regulation of CC chemokine receptor 5 (CCR5) and expression of CCR7 [40], and migrate toward draining lymph nodes, where they are known to play a pivotal role in the priming of mycobacterial-specific T lymphocytes.

Conversely, the capacity of mycobacteria to modify the profile of cytokine secretion by DC seems related to their capacity to interact with DC independently on DC-SIGN expression. Our results are in agreement with previous data showing that the synthesis of IL-12 by DC derived from monocytes treated with Mtb [12], BCG [10], or mannosylated lipoarabinomannan (manLam) [44] is inhibited. As this effect could only be observed if monocytes were incubated with mycobacteria or manLam at the beginning of the culture, i.e., when monocytes do not express DC-SIGN, these data further support the hypothesis that mycobacteria and their manLam can induce the block of IL-12 synthesis through signaling, not exclusively related to DC-SIGN. In addition, it is to underline that it is difficult to extrapolate whether the secretion of a certain cytokine array in vitro may result in an immunosuppressive response in vivo. In fact, we believe that the kinetics and the body compartments in which mycobacteria-DC interactions occur in vivo should also be considered in trying to understand the pathogen mechanisms of virulence. The secretion of certain cytokines by DC interacting with mycobacteria could influence the inflammatory milieu in which the interaction occurs, but it is not clear whether the same cytokines are secreted in the secondary lymphoid organs by migrated DC, which could be functionally exhausted and low-responsive to further stimuli provided by T cells.

In conclusion, DC-SIGN represents an important receptor for the recognition, internalization, and possibly presentation of mycobacterial antigens, but our data indicate that it cannot be considered as the target for the immunotherapy of mycobacterial infections [13]. In fact, the role of DC-SIGN in the suppression of DC function is not so fully defined to encourage therapeutic approaches aimed at blocking its function, which could result, instead, in a decreased presentation of mycobacterial antigen.

ACKNOWLEDGMENTS

This work was supported by Istituto Superiore di Sanità Grants #50F/G and 5303 ISS-NIH. We thank Prof. Antonio Cassone and Prof. Mara Torrisi for their helpful comments and critical reading of the manuscript.

REFERENCES

1. Flynn, J. L., Chan, J. (2001) Immunology of tuberculosis. *Annu. Rev. Immunol.* **19,** 93–129.
2. Kaufmann, S. H. E. (2001) How can immunology contribute to the control of tuberculosis? *Nat. Rev. Immunol.* **1,** 20–30.
3. Lauzardo, M., Ashkin, D. (2000) Phthisiology at the dawn of the new century. *Chest* **117,** 1455–1473.

4. Sertl, K., Takemura, T., Tschachler, E., Ferrans, V. J., Kaliner, M. A., Shevach, E. M. (1986) Dendritic cells with antigen-presenting capability reside in airway epithelium, lung parenchyma, and visceral pleura. *J. Exp. Med.* **163,** 436–451.
5. Lanzavecchia, A., Sallusto, F. (2000) Dynamics of T lymphocyte responses: intermediates, effectors, and memory cells. *Science* **290,** 92–97.
6. Lanzavecchia, A., Sallusto, F. (2001) Regulation of T cell immunity by dendritic cells. *Cell* **106,** 263–266.
7. Kaufmann, S. H., Schaible, U. E. (2003) A dangerous liaison between two major killers: *Mycobacterium tuberculosis* and HIV target dendritic cells through DC-SIGN. *J. Exp. Med.* **197,** 1–5.
8. Pulendran, B., Palucka, K., Banchereau, J. (2001) Sensing pathogens and tuning immune responses. *Science* **293,** 253–256.
9. Schluger, N. W., Rom, W. N. (1998) The host immune response to tuberculosis. *Am. J. Respir. Crit. Care Med.* **157,** 679–691.
10. Gagliardi, M. C., Teloni, R., Mariotti, S., Iona, E., Pardini, M., Fattorini, L., Orefici, G., Nisini, R. (2004) Bacillus Calmette-Guerin shares with virulent *Mycobacterium tuberculosis* the capacity to subvert monocyte differentiation into dendritic cell: implication for its efficacy as a vaccine preventing tuberculosis. *Vaccine* **22,** 3848–3857.
11. Mariotti, S., Teloni, R., Iona, E., Fattorini, L., Romagnoli, G., Gagliardi, M. C., Orefici, G., Nisini, R. (2004) *Mycobacterium tuberculosis* diverts α interferon-induced monocyte differentiation from dendritic cells into immunoprivileged macrophage-like host cells. *Infect. Immun.* **72,** 4385–4392.
12. Mariotti, S., Teloni, R., Iona, E., Fattorini, L., Giannoni, F., Romagnoli, G., Orefici, G., Nisini, R. (2002) *Mycobacterium tuberculosis* subverts the differentiation of human monocyte into dendritic cell. *Eur. J. Immunol.* **32,** 3050–3058.
13. Geijtenbeek, T. B., Van Vliet, S. J., Koppel, E. A., Sanchez-Hernandez, M., Vandenbroucke-Grauls, C. M., Appelmelk, B., Van Kooyk, Y. (2003) Mycobacteria target DC-SIGN to suppress dendritic cell function. *J. Exp. Med.* **197,** 7–17.
14. Tailleux, L., Schwartz, O., Herrmann, J. L., Pivert, E., Jackson, M., Amara, A., Legres, L., Dreher, D., Nicod, L. P., Gluckman, J. C., Lagrange, P. H., Gicquel, B., Neyrolles, O. (2003) DC-SIGN is the major *Mycobacterium tuberculosis* receptor on human dendritic cells. *J. Exp. Med.* **197,** 121–127.
15. Kammerer, U., Eggert, A. O., Kapp, M., McLellan, A. D., Geijtenbeek, T. B., Dietl, J., van Kooyk, Y., Kampgen, E. (2003) Unique appearance of proliferating antigen-presenting cells expressing DC-SIGN (CD209) in the decidua of early human pregnancy. *Am. J. Pathol.* **162,** 887–896.
16. Geijtenbeek, T. B., Torensma, R., van Vliet, S. J., van Duijnhoven, G. C., Adema, G. J., van Kooyk, Y., Figdor, C. G. (2000) Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell* **100,** 575–585.
17. van Kooyk, Y., Geijtenbeek, T. B. (2003) DC-SIGN: escape mechanism for pathogens. *Nat. Rev. Immunol.* **3,** 697–709.
18. Cella, M., Sallusto, F., Lanzavecchia, A. (1997) Origin, maturation and antigen presenting function of dendritic cells. *Curr. Opin. Immunol.* **9,** 10–16.
19. Sallusto, F., Lanzavecchia, A. (1994) Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor α. *J. Exp. Med.* **179,** 1109–1118.
20. Relloso, M., Puig-Kroger, A., Pello, O. M., Rodriguez-Fernandez, J. L., de la Rosa, G., Longo, N., Navarro, J., Munoz-Fernandez, M. A., Sanchez-Mateos, P., Corbi, A. L. (2002) DC-SIGN (CD209) expression is IL-4 dependent and is negatively regulated by IFN, TGF-β, and anti-inflammatory agents. *J. Immunol.* **168,** 2634–2643.
21. Santini, S. M., Lapenta, C., Logozzi, M., Parlato, S., Spada, M., Di Pucchio, T., Belardelli, F. (2000) Type I interferon as a powerful adjuvant for monocyte-derived dendritic cell development and activity in vitro and in Hu-PBL-SCID mice. *J. Exp. Med.* **191,** 1777–1788.
22. Nisini, R., Paroli, M., Accapezzato, D., Bonino, F., Rosina, F., Santantonio, T., Sallusto, F., Amoroso, A., Houghton, M., Barnaba, V. (1997) Human CD4+ T-cell response to hepatitis δ virus: identification of multiple epitopes and characterization of T-helper cytokine profiles. *J. Virol.* **71,** 2241–2251.
23. Giacomini, E., Iona, E., Ferroni, L., Miettinen, M., Fattorini, L., Orefici, G., Julkunen, I., Coccia, E. M. (2001) Infection of human macrophages and dendritic cells with *Mycobacterium tuberculosis* induces a differential cytokine gene expression that modulates T cell response. *J. Immunol.* **166,** 7033–7041.
24. Turville, S. G., Arthos, J., Donald, K. M., Lynch, G., Naif, H., Clark, G., Hart, D., Cunningham, A. L. (2001) HIV gp120 receptors on human dendritic cells. *Blood* **98,** 2482–2488.
25. Giulietti, A., Overbergh, L., Valckx, D., Decallonne, B., Bouillon, R., Mathieu, C. (2001) An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. *Methods* **25,** 386–401.
26. Ernst, J. D. (1998) Macrophage receptors for *Mycobacterium tuberculosis*. *Infect. Immun.* **66,** 1277–1281.
27. Le Cabec, V., Cols, C., Maridonneau-Parini, I. (2000) Nonopsonic phagocytosis of zymosan and *Mycobacterium kansasii* by CR3 (CD11b/CD18) involves distinct molecular determinants and is or is not coupled with NADPH oxidase activation. *Infect. Immun.* **68,** 4736–4745.
28. Martinez-Pomares, L., Reid, D. M., Brown, G. D., Taylor, P. R., Stillion, R. J., Linehan, S. A., Zamze, S., Gordon, S., Wong, S. Y. (2003) Analysis of mannose receptor regulation by IL-4, IL-10, and proteolytic processing using novel monoclonal antibodies. *J. Leukoc. Biol.* **73,** 604–613.
29. Egan, B. S., Abdolrasulnia, R., Shepherd, V. L. (2004) IL-4 modulates transcriptional control of the mannose receptor in mouse FSDC dendritic cells. *Arch. Biochem. Biophys.* **428,** 119–130.
30. Geijtenbeek, T. B., Kwon, D. S., Torensma, R., van Vliet, S. J., van Duijnhoven, G. C., Middel, J., Cornelissen, I. L., Nottet, H. S., KewalRamani, V. N., Littman, D. R., Figdor, C. G., van Kooyk, Y. (2000) DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. *Cell* **100,** 587–597.
31. Alvarez, C. P., Lasala, F., Carrillo, J., Muniz, O., Corbi, A. L., Delgado, R. (2002) C-type lectins DC-SIGN and L-SIGN mediate cellular entry by Ebola virus in cis and in trans. *J. Virol.* **76,** 6841–6844.
32. Baribaud, F., Doms, R. W., Pohlmann, S. (2002) The role of DC-SIGN and DC-SIGNR in HIV and Ebola virus infection: can potential therapeutics block virus transmission and dissemination? *Expert Opin. Ther. Targets* **6,** 423–431.
33. Halary, F., Amara, A., Lortat-Jacob, H., Messerle, M., Delaunay, T., Houles, C., Fieschi, F., Arenzana-Seisdedos, F., Moreau, J. F., Dechanet-Merville, J. (2002) Human cytomegalovirus binding to DC-SIGN is required for dendritic cell infection and target cell trans-infection. *Immunity* **17,** 653–664.
34. Gardner, J. P., Durso, R. J., Arrigale, R. R., Donovan, G. P., Maddon, P. J., Dragic, T., Olson, W. C. (2003) L-SIGN (CD 209L) is a liver-specific capture receptor for hepatitis C virus. *Proc. Natl. Acad. Sci. USA* **100,** 4498–4503.
35. Colmenares, M., Corbi, A. L., Turco, S. J., Rivas, L. (2004) The dendritic cell receptor DC-SIGN discriminates among species and life cycle forms of Leishmania. *J. Immunol.* **172,** 1186–1190.
36. Appelmelk, B. J., van Die, I., van Vliet, S. J., Vandenbroucke-Grauls, C. M., Geijtenbeek, T. B., van Kooyk, Y. (2003) Cutting edge: carbohydrate profiling identifies new pathogens that interact with dendritic cell-specific ICAM-3-grabbing nonintegrin on dendritic cells. *J. Immunol.* **170,** 1635–1639.
37. Cambi, A., Gijzen, K., de Vries, J. M., Torensma, R., Joosten, B., Adema, G. J., Netea, M. G., Kullberg, B. J., Romani, L., Figdor, C. G. (2003) The C-type lectin DC-SIGN (CD209) is an antigen-uptake receptor for *Candida albicans* on dendritic cells. *Eur. J. Immunol.* **33,** 532–538.
38. Bergman, M. P., Engering, A., Smits, H. H., van Vliet, S. J., van Bodegraven, A. A., Wirth, H. P., Kapsenberg, M. L., Vandenbroucke-Grauls, C. M., van Kooyk, Y., Appelmelk, B. J. (2004) *Helicobacter pylori* modulates the T helper cell 1/T helper cell 2 balance through phase-variable interaction between lipopolysaccharide and DC-SIGN. *J. Exp. Med.* **200,** 979–990.
39. Cipriano, I. M., Mariano, M., Freymuller, E., Carneiro, C. R. (2003) Murine macrophages cultured with IL-4 acquire a phenotype similar to that of epithelioid cells from granulomatous inflammation. *Inflammation* **27,** 201–211.
40. Lande, R., Giacomini, E., Grassi, T., Remoli, M. E., Iona, E., Miettinen, M., Julkunen, I., Coccia, E. M. (2003) IFN-αβ released by *Mycobacterium tuberculosis*-infected human dendritic cells induces the expression of CXCL10: selective recruitment of NK and activated T cells. *J. Immunol.* **170,** 1174–1182.
41. Muller, H., Kruger, S. (1994) Immunohistochemical analysis of cell composition and in situ cytokine expression in HIV- and non-HIV-associated tuberculous lymphadenitis. *Immunobiology* **191,** 354–368.
42. Tailleux, L., Neyrolles, O., Honore-Bouakline, S., Perret, E., Sanchez, F., Abastado, J. P., Lagrange, P. H., Gluckman, J. C., Rosenzwajg, M., Herrmann, J. L. (2003) Constrained intracellular survival of *Mycobacterium tuberculosis* in human dendritic cells. *J. Immunol.* **170,** 1939–1948.
43. Engering, A., Geijtenbeek, T. B., van Vliet, S. J., Wijers, M., van Liempt, E., Demaurex, N., Lanzavecchia, A., Fransen, J., Figdor, C. G., Piguet, V., van Kooyk, Y. (2002) The dendritic cell-specific adhesion receptor DC-SIGN internalizes antigen for presentation to T cells. *J. Immunol.* **168,** 2118–2126.
44. Johansson, U., Ivanyi, J., Londei, M. (2001) Inhibition of IL-12 production in human dendritic cells matured in the presence of Bacillus Calmette-Guerin or lipoarabinomannan. *Immunol. Lett.* **77,** 63–66.