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Inverse Correlation of Maturity and Antibacterial Activity in Human Dendritic Cells[1]

Maike Buettner,* Christoph Meinken,* Max Bastian,* Rauf Bhat,* Elmar Stössel,* Gerhard Faller,† George Cianciolo,§ Joachim Ficker,‡ Manfred Wagner,‡ Martin Röllinghoff,* and Steffen Stenger[2]*

Dendritic cells (DCs) are a key part of host defense against microbial pathogens, being part of the innate immune system, but also instructing the adaptive T cell response. This study was designed to evaluate whether human DCs directly contribute to innate immunity by killing intracellular bacteria, using tuberculosis as a model. DCs were detected in bronchoalveolar lavage samples indicating that DCs are available for immediate interaction with *Mycobacterium tuberculosis* (*M. Tb*) after inhalation of the pathogen. The phenotype of DC in bronchoalveolar lavage closely resembles monocyte-derived immature DC (iDC) according to the expression of CD1a, CD83, and CCR7. The antimicrobial activity of iDC against intracellular *M. Tb* inversely correlated with TNF-α-release and was enhanced by treatment with anti-TNF-α Abs. Differentiation of iDC into mature DC by addition of TNF-α or activation via Toll-like receptors further reduced killing of *M. Tb*. The antibacterial activity against intracellular *M. Tb* of all DCs was significantly lower than alveolar macrophages. Therefore, the maintenance of a pool of DCs at the site of disease activity in tuberculosis, and the maturation of these DC by TNF-α provides a mechanism by which *M. Tb* escapes the innate immune system. *The Journal of Immunology,* **2005, 174: 4203–4209.**

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Dendritic cells (DCs)[3] are unique in their ability to ingest pathogens at the site of infection and to migrate to secondary lymphoid organs, where they present pathogen-derived Ags to naive T lymphocytes (1). DCs are present in epithelial layers, including alveolar spaces in the lung, where they create a tight surveillance network (2). This localization suggests that they present a first line of defense against inhaled foreign particles, including microbial pathogens (3). A major human pathogen predominantly infecting its host by the aerosolic route is *Mycobacterium tuberculosis* (*M. Tb*), the causative agent of tuberculosis. Recent work demonstrated that mycobacteria-infected DC accumulate in the spleens of *M. Tb*-infected mice (4) and are detectable in granulomas of infected rats (5) and humans (3, 6). In vitro experiments revealed that *M. Tb* is phagocytosed by human DC (7), mainly by the interaction between the mycobacterial lipoarabinomannan and DC-specific ICAM-1-grabbing nonintegrin (DC-SIGN) (3, 8). This indicates that the interaction of mycobacteria and DCs plays a special role because macrophages, the major host cells of *M. Tb*, do not express significant levels of DC-SIGN (9). Although these studies shed light on the molecular mechanisms of the uptake of mycobacteria, not much information is available about the intracellular fate of phagocytosed *M. Tb* in DCs.

We recently demonstrated that human monocyte-derived DC are inferior to macrophages in limiting *M. Tb* growth (10), but this observation is dependent on the culture conditions which may influence the maturity of the DC (11). Insight into the growth of mycobacteria in DC is particularly important in view of the increasing excitement for new vaccination strategies against tuberculosis based on the injection of mycobacteria-infected DC (12–14). Therefore, we sought to identify host and pathogen-derived factors modulating the intracellular growth of virulent *M. Tb* in human DC. We demonstrate that mediators supporting the maturation of DCs interfere with antibacterial activity, thereby providing a novel functional feature distinguishing immature and mature DC.

Materials and Methods

Cell culture reagents

Cells were cultured in RPMI 1640 (Biochrom) supplemented with 10% heat-inactivated FCS (Sigma-Aldrich), glutamine (2 mM; Sigma-Aldrich), 10 mM HEPES, 13 mM $NaHCO_3$, 100 μg/ml streptomycin, and 6 μg/ml penicillin (all from Biochrom). In experiments involving the infection of cells with *M. Tb*, FCS was replaced by pooled human serum (generated from the blood of healthy volunteers) to optimize the phagocytosis of the bacteria and no antibiotics were added. Amphotericin B (5.6 μg/ml, Sigma-Aldrich) was added to cultures of bronchoalveolar lavage (BAL) to suppress the growth of fungi from the physiological flora of the donors.

Cytokines, Abs, and reagents

The following cytokines and Abs were used in this study: recombinant human (rh) TNF-α (Endogen), rhIL-1, rhIL-6, rhIL-10 (all from R&D Systems), PGE_2 (Sigma-Aldrich), GM-CSF (Novartis), IL-4 (Strathmann Biotec), M-CSF, anti-TNF-α, goat IgG, anti-IL-10, anti-CCR7 (all from R&D Systems), anti-CD1a-FITC (Serotec), anti-MHC class II-FITC, anti-CD14-PE, anti-CD86-PE (all from BD Biosciences), anti-CD83-PE (Beckman-Coulter). FITC-conjugated secondary Abs and streptavidin-PE were purchased from Dianova. Isotype controls were all purchased from BD

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[3] Abbreviations used in this paper: DC, dendritic cell; AM, alveolar macrophage; BAL, bronchoalveolar lavage; *M. Tb*, *Mycobacterium tuberculosis*; MOI, multiplicity of infection; iDC, immature DC; DC-SIGN, DC-specific ICAM-1-grabbing nonintegrin.

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Biosciences. LPS from *Salmonella minnesota* was purchased from Sigma-Aldrich. Nineteen-kilodalton lipoprotein from virulent *M. Tb* (strain H37Rv) was synthesized by EMC Microcollections. LMP-420 (2-NH_2-6-Cl-9-[(5-dihydroxyboryl)-pentyl] purine), an inhibitor of TNF-α transcription, was a gift from LeukoMed. LMP-420 was stored at $-20°C$ as aliquots of a 10-mM stock solution in DMSO (Sigma-Aldrich).

Bronchoalveolar lavage

Alveolar macrophages (AM) were obtained from the BAL fluid of patients who underwent bronchoscopy for diagnostic purposes. Patients given a diagnosis of an infectious lung disease or a disease afflicting the alveolar space were not considered for further analysis except for quantification of DCs. The diagnosis of tuberculosis or squamous cell carcinoma was given retrospectively according to the final microbiological (growth of *M. Tb*) or pathological diagnosis. BAL (50–70 ml) was filtered through a cell strainer (70 μm; BD Biosciences) and centrifuged (1200 rpm, 10 min) at 4°C. Aliquots of the cell-free BAL were stored at $-70°C$ until measurement of the concentration of TNF-α. AM were isolated by plating total cells in six-well plates at a density of 1×10^6/ml overnight for adherence. Nonadherent cells were removed by vigorous washing with PBS (Biochrom). Purity of the cells was confirmed by α-naphthyl-acetate esterase staining (Sigma-Aldrich) and was found to be above 95% in all experiments. Flow cytometry confirmed the purity of the population (CD3 $<$ 1%, CD19, CD56, CD66, CD1a negative). Viability was above 96% in all experiments as determined by trypan blue dye exclusion.

Generation of DCs and macrophages

PBMC from healthy donors were isolated from buffy coats obtained by Ficoll-Hypaque (BD Biosciences) density centrifugation. Cells were allowed to adhere to culture flasks (Nunc) for 1 h at 37°C, after which the nonadherent cells were removed by vigorous washing with PBS. Cells were detached by incubation with Mg^{2+}- and Ca^{2+}-free PBS containing 1 mM EDTA for 10 min and analyzed by flow cytometry. Cell surface staining (MHC class II, CD14, CD11b) showed that the adherent population contained $>$95% monocytes. Monocyte-derived macrophages were generated by culturing monocytes in the presence of M-CSF (500 U/ml) for 7 days. To generate immature DC (iDC) the adherent fraction was cultured in the presence of GM-CSF (1000 U/ml) and IL-4 (10 ng/ml). Cytokines and half of the medium were replaced after 3 days of culture. After a total of 7 days, nonadherent cells ($>$85% of the total population) were harvested and used for the subsequent experiments (15–17). To induce maturation, DCs were cultured for an additional 24–48 h with different stimuli: 1) LPS (20 ng/ml), 2) 19 kDa lipoprotein (2 μg/ml) (18), and 3) TNF-α alone (10 ng/ml) or in combination (maturation mix (19)) with PGE_2 (1 μg/ml), IL-6 (1000 U/ml), and IL-1β (2 ng/ml).

Growth of M. Tb

M. Tb (virulent strain H37Rv) was grown in suspension with constant, gentle rotation in roller bottles (Corning) containing Middlebrook 7H9 broth (BD Biosciences) supplemented with 1% glycerol (Roth), 0.05% Tween 80 (Sigma-Aldrich), and 10% Middlebrook oleic acid, albumin, dextrose, and catalase enrichment (BD Biosciences). Aliquots from logarithmically growing cultures were frozen in PBS containing 10% glycerol and representative vials were thawed and enumerated for viable CFU on Middlebrook 7H11 plates. Bacterial viability was above 90% (BacLight; Molecular Probes). Because clumping of mycobacteria is a common problem that can influence the validity and reproducibility of the experiments we undertook several precautions to minimize clumps. First, culture conditions (rotation, Tween 80) were chosen to support the growth of single cell suspensions. Second, before in vitro infection, *M. Tb* bacilli were sonicated to disrupt small aggregates of bacteria. Third, the multiplicity of infection (MOI) was selected such that there were only two to three bacilli per cell.

Infection of DCs and macrophages

DCs and macrophages were infected with single cell suspensions of *M. Tb*. DC were harvested after 4 h (slightly adherent cells were detached by vigorous pipetting) and centrifuged at 800 rpm for 8 min. This low-speed centrifugation selectively spins down DCs while extracellular bacteria remain in the supernatant. After three cycles of centrifugation, the majority of extracellular bacteria ($>$99%) were removed as determined by auramine-rhodamine stain (Merck) and plating of the supernatants after the final centrifugation. As infected macrophages are adherent to the plastic flask, extracellular bacteria were removed by vigorous washing. Adherent cells were then detached by treatment with EDTA, counted, and plated at a concentration of 5×10^5 cells/ml in a 24-well plate. The efficiency of infection, as quantitated by acid fast staining of control cultures on Permanox chamber slides (Nunc), was routinely quantitated for every experiment and varied with the MOI (MOI 0.5–5) in a range of 5–35%. For the direct comparison of growth in AM, iDC, and mature DC, the MOIs were adjusted such that the efficiency of infection was similar for all cell populations ($\pm$5%). The microscopic evaluation of infected cells by auramine-rhodamine stain confirmed the absence of any mycobacterial aggregates. Cell viability of infected cells was determined by trypan blue exclusion and was $>$90% in all experiments.

Quantification of mycobacterial growth

To ensure the reliable quantification of intracellular *M. Tb* we used two independent methods for measuring mycobacterial growth: 1)CFU: infected cells were lysed with 0.3% saponin (Sigma-Aldrich) to release intracellular bacteria. At all time points, an aliquot of unlysed infected cells was harvested and counted. This allowed an exact quantification of cells as well as the determination of cellular viability by trypan blue exclusion. Recovery of cells was $>$70% in all experiments, with cell viability regularly exceeding 90% of total cells. Lysates of infected cells were resuspended vigorously, transferred into screw caps, and sonicated in a preheated (37°C) water bath sonicator for 10 min. Aliquots of the sonicate were diluted 10-fold in 7H9-medium. Four dilutions of each sample were plated in duplicates on 7H11 agar plates and incubated at 37°C and 5% CO_2 for 21 days. 2) Incorporation of tritium-labeled uracil ([^{3}H]uracil) (Pharmacia): uptake of [^{3}H]uracil into the mycobacterial RNA was determined as described (10). Briefly, 1×10^6 infected cells were cultured in duplicates. At the end of the incubation period, cells were lysed (0.3% saponin), resuspended vigorously, and transferred into screwcaps. Lysates were centrifuged in an aerosol-tight microfuge (3000 rpm, 20 min) and resuspended in 100 μl of 7H9 medium. Lysates were then transferred into 96-well round-bottom plates and incubated in the presence of 3 μCi of [^{3}H]uracil. After 24 h, mycobacteria were killed by treatment with 4% paraformaldehyde for 30 min. The mycobacteria were harvested onto glass fiber filters (Inotech) and [^{3}H]uracil-incorporation was measured in a beta-counter (Berthold Technologies). Background radioactivity in uninfected cells was below 200 cpm in all experiments.

To estimate the metabolic activity of extracellular *M. Tb*, 2×10^6 bacteria were plated in 100 μl of CM plus 10% FCS without antibiotics in a 96-well plate. All wells were set up in triplicates. Cultures were incubated for 96 h whereby 1 μCi of [^{3}H]uracil was present during the last 24 h. After inactivation of the bacteria (100 μl of paraformaldehyde, 30 min), incorporation of [^{3}H]uracil was determined as described above.

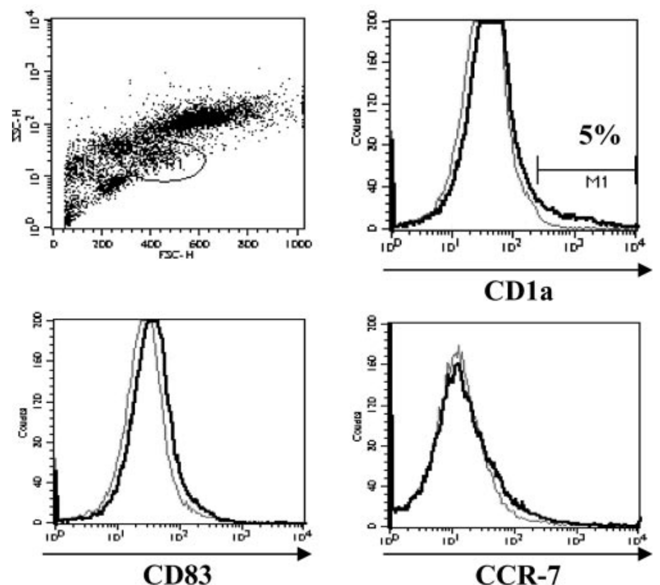

FIGURE 1. DCs in the BAL express an immature phenotype. Leukocytes from BAL were isolated and stained for CD1a, CD83, and CCR7 (black lines) or the respective isotype controls (gray lines). The histograms present the positive cells within the gate of intermediate size and granularity (R1). Because of the low frequency of DCs, 5×10^5 cells per sample were acquired. The experiment shows a representative example of 93 independent donors.

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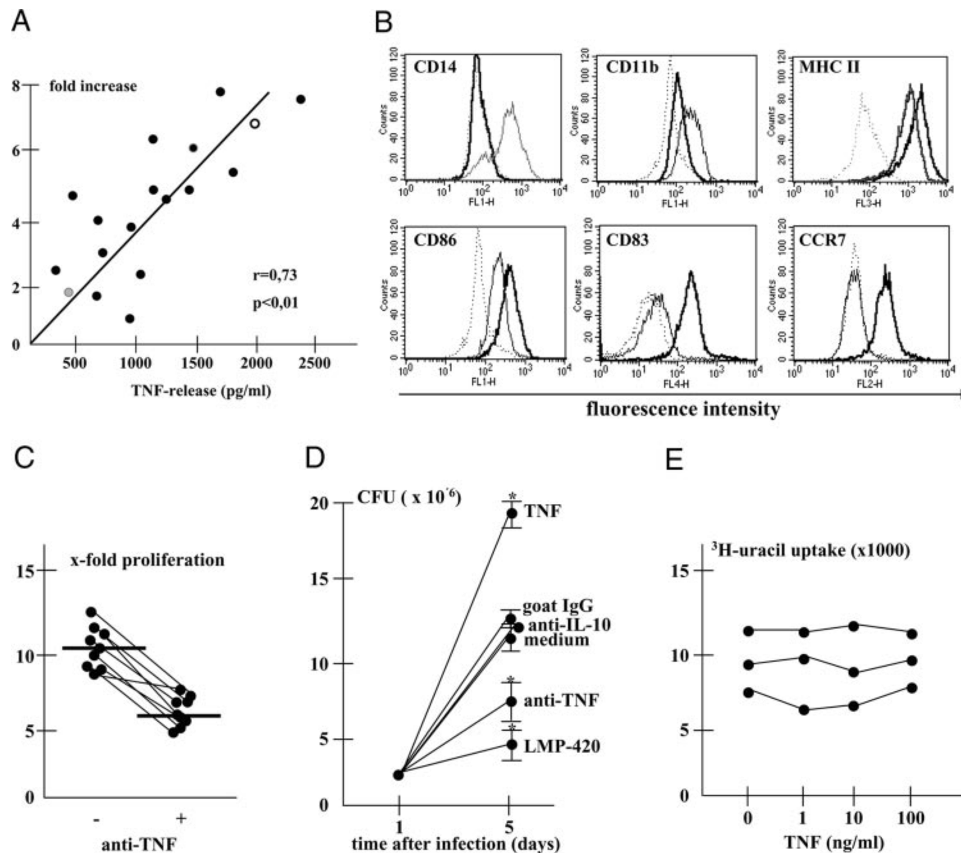

FIGURE 2. Antimicrobial activity of DC conversely correlates with TNF-α release. *A*, iDCs were generated from the blood of 18 different donors and infected with *M. Tb* (MOI 5). Supernatants were removed and measured for TNF-α content after 18 h. Mycobacterial growth in the same cultures was determined after 5 days of incubation. X-fold proliferation was calculated from the difference in CFU between day 1 and 5. The values of the coefficient *r* and the significance are given and were determined by the Spearman rank correlation. The solid line represents a regression line. *B*, After 48 h, DCs from two donors analyzed for growth and TNF-α release (gray and open circles in *A*) were detached using EDTA, fixed with paraformaldehyde (2%) and stained for cell surface markers as indicated. The histogram depicts the isotype (dotted line), DC infected with a strain inducing high (1512 pg/ml, black line) or low amounts of TNF-α (498 pg/ml, gray lines). *C*, DC were generated from nine different donors and infected with *M. Tb* (MOI 0.5) for 4 h. Infected cells were cultured in the presence or absence of anti TNF-α or control goat IgG (20 μg/ml). Cytokines or Abs were present throughout the culture period. The x-fold proliferation between day 1 and 5 was calculated from CFU determined by plating cell lysates. The black horizontal bars indicate the median increase. *D*, DC were infected and stimulated as described above. In addition, LMP-420 (10 mM), an inhibitor of TNF-α transcription and synthesis or anti IL-10 (20 μg/ml), was added. Cell lysates were plated immediately after infection and after 5 days of incubation. Shown is one representative experiment of five with similar results. The error bars depict SD calculated from the CFU of four dilutions, each performed in duplicates. Asterisk indicates significant differences as compared with the medium or goat IgG control ($p < 0.01$). *E*, A total of 2×10^6 mycobacteria were seeded in a 96-well plate in 200 μl of CM plus 10% FCS and incubated in the presence of TNF-α (0–100 ng/ml) for 72 h. Cultures were continued for an additional 24 h in the presence of 1 μCi of [^{3}H]uracil. Uptake of radioactivity was measured after deactivating the bacteria with paraformaldehyde. Presented are the results of three independent experiments.

Measurement of TNF-α (ELISA)

For measurement of TNF-α, sandwich ELISA was used and performed exactly as suggested by the supplier (Endogen). The sensitivity was 15 pg/ml in all experiments.

Flow cytometry

A total of 2×10^6 (BAL) or 2×10^5 cells (DCs) were resuspended in 200 μl of staining buffer (2% FCS, 1% NaN_3, PBS without Mg^{2+}/Ca^{2+}) and incubated with unconjugated or conjugated Abs for 30 min at 8°C. Samples were washed twice in staining buffer and, if necessary, incubated for an additional 30 min at 8°C with goat anti-mouse-FITC Abs (1:500). Cells were then fixed in 2% paraformaldehyde and stored at 8°C until analysis in a FACScan flow cytometer. Data were analyzed using CellQuest Prosoftware (BD Biosciences).

Immunohistochemistry

Tissue sections from the lungs of 12 tuberculosis patients that had undergone surgery due to hemoptysis were provided from the Institut für Pathologie, Klinikum Nürnberg. A universal decloaking solution (Biocarta) was applied for dewaxing the sections. Ags were demasked by heating the slides (121°C for 30 s and 85°C for 10 s) in a Decloaker chamber (Biocare Medical) using the decloaking solution as a buffer. Nonspecific binding was blocked with PBS/10% goat serum (Promocell)/Tween 20 (Sigma-Aldrich) before anti-DC-SIGN (Loxo) was added at a 1/100 dilution for 30 min. A biotinylated secondary Ab (Biocarta) and streptavidin-peroxidase (DakoCytomation) were used to label cells that had bound Abs. The staining was developed with aminoethylcarbazole (Zytomed) for 20 min and the nuclei were counterstained for 30 s in hematoxylin (Biocarta).

Statistical analysis

Data are presented as mean value ± SEM except where stated otherwise. Student's *t* test was used to determine statistical significance between two differentially treated cultures. Differences were considered significant if $p < 0.05$.

Results

DCs in the BAL have an immature phenotype

To determine the phenotype of DCs that are immediately available for the uptake of inhaled bacilli, we performed flow cytometry on

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Table I. *Modulation of DC maturation by infection with* M. Tb [a]

	Immature DC			Mature DC	
	Before infection	After infection	After infection + anti-TNF	Before infection	After infection
CD14	298 ± 37	202 ± 28*	276 ± 32**	Negative	Negative
MHC class II	2185 ± 231	2297 ± 145	2314 ± 316	3876 ± 376	3767 ± 432
CD86	1823 ± 57	1905 ± 107	1755 ± 138	2694 ± 323	2548 ± 340
CD83	Negative	112 ± 87	42 ± 32**	295 ± 65	312 ± 43
CCR7	Negative	87 ± 39	19 ± 6**	253 ± 32	279 ± 32

[a] iDCs and mature DC were labeled with Abs and analyzed by flow cytometry before or 5 days after infection with *M. Tb* (MOI iDCs: 0.5; MOI mature DC: 2). The numbers give the average MFI ± SD of five independent experiments. The MFI of the isotype controls was subtracted from the MFI of the specific staining.

*, $p < 0.01$ as compared to DC prior to infection.

**, $p < 0.01$ as compared to iDCs after infection without anti TNF.

leukocytes isolated from human BAL. To account for the low frequency of DCs at least 5×10^5 cells were acquired per sample. DCs were detected by staining with CD1a (20) and were located in a distinct area of intermediate size and granularity (Fig. 1, *upper left panel*). Although 0.4–0.6% (mean range in 93 donors) of total BAL cells expressed CD1a, only a minority of the cells (<0.05%) stained positively for CD83 or CCR7, indicating that DCs lining the alveoli have an immature phenotype (Fig. 1). These results indicate that besides AM, iDC are potential host cells for inhaled *M. Tb*.

Antimicrobial activity of DCs conversely correlates with TNF-α release

The frequency of DC in the BAL is too low (Fig. 1) to allow for isolation, infection, and measurement of mycobacterial proliferation. Because the phenotype of DC derived from BAL resembles that of monocyte derived iDC, we performed the following studies with iDC. We infected iDC derived from 18 individuals with identical amounts (MOI 2) of the identical stock solution of *M. Tb*. TNF-α release in the supernatants was determined after 18 h and culture was continued for 4 days to measure mycobacterial multiplication. We found a striking correlation between the amount of TNF-α released in the supernatant and the mycobacterial growth (Fig. 2*A*), indicating that TNF-α-release was a good predictor for the antibacterial activity of iDC. As shown for one representative donor (Fig. 2*A*, open circle), release of high amounts of TNF-α (1976 pg/ml) yielded DC with a mature phenotype (CD14^{neg}, CD11b^{low}, MHC class II^{high}, CD86^{high}, CD83^{high}, CCR7^{pos}) (Fig. 2*B*, dark lines). In contrast, DCs of another donor secreted only 498 pg/ml TNF-α after infection (gray circle in Fig. 2*A*) and failed to develop a mature phenotype (Fig. 2*B*, gray line). These findings suggested that TNF-α release decreases the antibacterial activity of iDC by promoting the development of mature DC.

Neutralization of TNF-α reduces mycobacterial proliferation in iDC

Because iDC release TNF-α after taking up virulent *M. Tb* (Fig. 2*A*), we reasoned that neutralization of TNF-α will interfere with the maturation of DC and hence increase antimycobacterial activity. To test this hypothesis we infected iDC of nine different donors and added anti-TNF-α Abs or goat IgG. In nine of nine donors anti-TNF-α Abs reduced mycobacterial growth from a median multiplication rate of 12 to 7 (Fig. 2*C*). The effect was even more pronounced when TNF-α synthesis was inhibited pharmacologically by LMP-420, an inhibitor of TNF-α-transcription (Fig. 2*D*). Inhibition of TNF-α-activity concomitantly prevented the maturation of DC (CD83^{low}, CCR7^{low}, CD14^{high}) (Table I). The inhibition of maturation by anti-TNF Abs was not complete, even though we confirmed the absence of immunoreactive TNF-α by ELISA (data not shown). Most likely, other cytokines such as IL-1β, which is secreted by infected iDCs (range, 290–476 pg/ml; data not shown), are responsible for this effect. Addition of exogenous TNF-α to the cultures further accelerated growth of *M. Tb* (Fig. 2*D*) and maturation of iDC (data not shown). In contrast, IL-10 Abs had no impact on the proliferation of mycobacteria (Fig. 2*D*) which is in agreement with the lack of IL-10 in the supernatants of iDC infected with a low mycobacterial inoculum (data not shown) (10). To rule out a direct effect of TNF-α on the proliferation of *M. Tb* we measured the influence of TNF on the metabolic activity of extracellular bacteria. In three experiments even high concentrations of TNF-α (up to 100 ng/ml) had no impact on the incorporation of [^{3}H]uracil (Fig. 2*E*). Taken together, these findings highlight the inverse correlation between TNF-α-induced maturation of iDC and growth inhibition of intracellular mycobacteria.

Mature DCs have poor antibacterial activity

To investigate whether poor antimycobacterial activity is a general feature of mature DC, we differentiated iDC by culture with TNF-α alone, the activation of TLR 1/2 (19 kDa lipoprotein), LPS, or a maturation mixture before infection. Because mature DC are less phagocytic than iDC the MOI was adjusted (iDC 0.5; mature DC 2) to achieve an equal efficiency of infection (19 ± 5%, numbers on top of the bars). Survival of intracellular *M. Tb* was then compared in mature DC and iDC derived from the same donor. TNF-α, 19 kDa lipoprotein, LPS, and the maturation mixture resulted in the development of mature DC (data not shown) with reduced ability to contain mycobacterial growth as compared with

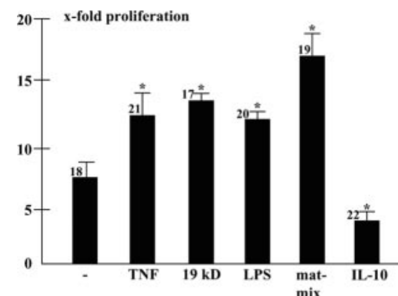

FIGURE 3. Mature DCs have poor antibacterial activity. iDCs were incubated in the presence of TNF-α (10 ng/ml), 19 kDa lipoprotein (5 μg/ml), LPS (20 ng/ml), a maturation mixture (10 ng/ml TNF-α, 1 μg/ml PGE_2, 2 ng/ml IL-1β, and 10 ng/ml IL-6) or IL-10 (10 ng/ml) for 48 h. Mature DC were infected with *M. Tb* (iDC: 0.5; mature DC: MOI 2) and the CFU were determined immediately after the end of the pulse infection and after 5 days of culture. The numbers on top of the bars indicate the average efficiency of infection in percent. The average of seven independent experiments ± SEM is shown (* indicates statistical significance of treated iDC as compared with control iDC; $p < 0.01$).

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untreated iDC (Fig. 3). In contrast, IL-10-treatment converted iDC to macrophage-like cells with increased antibacterial activity (10). During the 5 days of infection iDCs up-regulated the maturation markers CD83 and CCR7 (Table I). Once differentiated, mature DCs do not revert into macrophage-like cells despite the absence of GM-CSF and IL-4 during the 5-day period of infection (Table I). These findings illustrate that mature DCs are less efficient in killing *M. Tb* than iDC, regardless of whether maturation is induced via TNF-α or alternative mediators.

iDCs are less efficient in killing M. Tb *than macrophage*

To compare antibacterial activity of DC with alternative host cells of *M. Tb*, AM, blood-derived macrophages, and iDCs were derived from 12 different donors and infected with *M. Tb*. The MOI was adjusted for the different host cells to obtain a similar initial bacterial load (AM: 0.5; macrophages: 1; iDCs: 3). Numbers of intracellular bacteria were determined immediately after the 4-h pulse-infection and after 5 days of culture by plating cell lysates. Mycobacteria multiplied 4-fold in AM, 4-fold in macrophages, and 14-fold in iDCs (Fig. 4), indicating that DCs are limited in their ability to constrain mycobacterial proliferation as compared with macrophages.

DCs accumulate in the lung of patients with tuberculosis

To determine whether the interaction of DCs and *M. Tb* may be important at the site of disease, we compared the number of DC in the BAL of tuberculosis patients and patients suffering from noninfectious lung disease by quantifying the percentage of CD1a-expressing cells (as described in Fig. 1). In 29 tuberculosis patients, the number of DCs was significantly increased as compared with 39 patients suffering from neoplasms of the lung (median 2.2 vs 0.5%, $p < 0.01$) (Fig. 5*A*). Bronchiolar DC in tuberculosis patients predominantly showed an immature phenotype (MHC class II^{int}, $CD80^{low}$, $CD86^{low}$, $CD14^{low}$, $CD83^{neg}$, $CCR7^{neg}$ (data not shown). Because our patients all had paucibacillary disease (no acid fast rods in the BAL) and the concentrations of TNF-α in the BALs were below the level of detection (data not shown) it is not surprising that we failed to detect mature DC. DCs are not only present at the site of entry of tubercle bacilli, but are also detectable in the inflammatory infiltrate in the lung tissue of tuberculosis patients (Fig. 5*B*, *left panel*). Remarkably, DC-SIGN-positive cells were completely absent in 127 well-organized lung granulomas from 12 different patients (Fig. 5*B*, *right panel*). This indicates that iDC are involved in the development, but not in the maintenance of tuberculous granulomas in which bacterial growth is efficiently contained in the infected host.

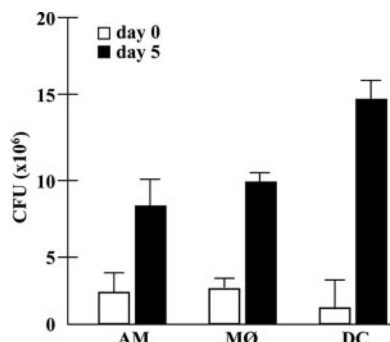

FIGURE 4. iDC are less efficient in killing *M. Tb* than macrophages. Mϕ and DC were generated from the peripheral blood of healthy donors and AM were isolated from patients undergoing bronchoscopy for diagnostic purposes. Cells were then infected with *M. Tb* (AM: MOI 0.5; Mϕ: MOI 1; iDC: MOI 3) and after 4 h extracellular bacteria were removed by vigorous washing (AM, Mϕ) or differential centrifugation (iDC). Selected samples were lysed immediately and plated to determine the initial bacterial load. After 5 days of culture the number of CFU in the cell lysates was determined. The average $\pm$ SD of 12 donors is shown (Mϕ and iDC were derived from the same donor for each individual experiment). Differences between AM and DC as well as Mϕ and DC were statistically significant ($p < 0.01$). The difference between AM and Mϕ was not statistically significant.

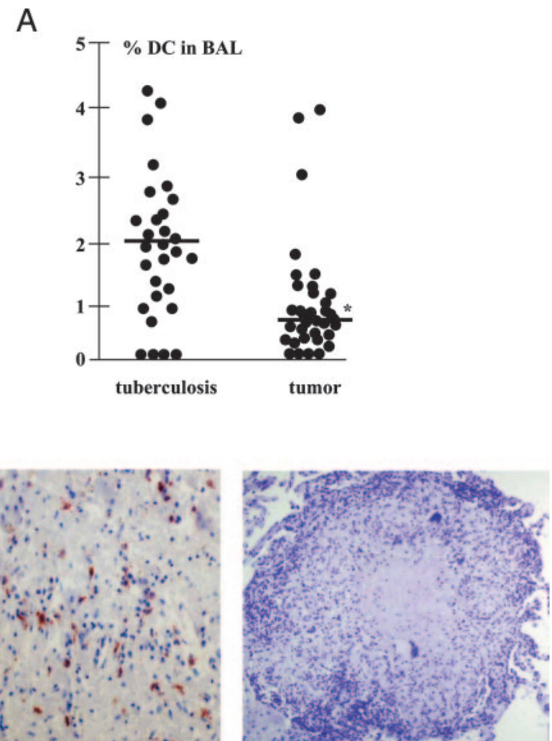

FIGURE 5. DCs at the site of disease in human tuberculosis. *A*, Cells were isolated from BAL of 29 tuberculosis patients and 39 patients that were later diagnosed with carcinoma of the lung. The percentage of CD1a-positive cells was determined as outlined in Fig. 1. At least 5×10^5 cells were acquired per sample. The horizontal line shows the median of all donors investigated. According to Student's *t* test for unpaired samples, the difference between the two groups is statistically significant (*, $p < 0.01$). *B*, Tissue sections from the lung were dewaxed, and the Ags were demasked by heat-treatment and stained for DC-SIGN. Aminoethylcarbazole was used as a substrate, and positive cells appear red-brown. Shown are representative areas from two different patients that underwent lung surgery due to hemoptysis. One donor was receiving tuberculosis treatment since 6 wk (*left panel*), the other donor (*right panel*) had finished 6 mo of therapy and was smear-negative before developing hemoptysis (magnification, $\times 400$).

Discussion

The major aim of our study was to investigate the direct antibacterial activity of human DC against intracellular *M. Tb*. Our experiments reveal that 1) TNF-α supports the growth of *M. Tb* in iDCs by inducing DC maturation; 2) there is a decline in the ability to kill *M. Tb* during the development of monocytes to mature DC, 3) these findings may be relevant in vivo because iDC accumulate in the BAL of tuberculosis patients and are present in the cellular infiltrate in the lung. We conclude that DC maturation provides a mechanism by which *M. Tb* can evade the host defense. In this context, it is essential to consider both beneficial and harmful effects of DC maturation in combating microbial pathogens.

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Uptake of viable mycobacteria by DCs is likely to support protective immunity by several mechanisms: First, a panel of cytokines that orchestrate adaptive immune responses is selectively secreted by *M. Tb*-infected DC but not macrophages. Most notably, in vitro experiments showed that IL-12 release that is of paramount importance for the development of a protective Th1 response is restricted to *M. Tb*-infected DC, whereas macrophages preferentially produce TNF-α, IL-1, and IL-6 (21, 22). Similarly, the selective secretion of type I IFN by *M. Tb* infected human DC will stimulate and recruit NK cells (23, 24), which are capable of killing intracellular *M. Tb* (25–27). However, these in vitro observations may not reflect the net effect of type I IFNs in vivo, because—at least in mice—type I IFNs aggravate the course of tuberculosis (28) and NK cells are not substantially involved in protection (29).

A second important function of DCs is the transport of Ags from the epithelium to the draining lymph nodes where adaptive immunity is initiated (1). In tuberculosis, the pathogen itself induces the up-regulation of CCR7, the major chemokine receptor for trafficking leukocytes to secondary lymphoid organs (30). Remarkably, the up-regulation of CCR7 by *M. Tb* is restricted to DC, highlighting the relevance of this interplay for the induction of protective immunity. Theoretical and experimental approaches suggest that a delay in DC trafficking to the lymph nodes will alter the outcome of tuberculosis (31).

Third, DCs have a special role in initiating T cell responses in tuberculosis. They are essential for activating conventional T cells in the lung, because the local AM are poor APCs (32–35). In addition, DCs are unique in their ability to activate unconventional T cell subsets via the Ag presenting molecules HLA-E and CD1 that are absent on macrophages. A subset of reactive cells are $CD8^+$ cytolytic T cells (36–42) that may be particularly important because they release granulysin and directly kill intracellular bacteria (43, 44).

In contrast DCs, could be exploited by mycobacteria as a safe haven preventing uptake and destruction by activated macrophages. Our results favor this hypothesis because DCs are less efficient in constraining mycobacterial growth than macrophages (Fig. 1). Furthermore, by their release of TNF-α, DCs can promote mycobacterial proliferation in human DCs (Fig. 3), AM (45), and monocytes (46). As already pointed out in the context of type I IFNs, the biological effects of TNF-α have to be carefully related to the in vivo evidence that demonstrates the crucial contribution of TNF-α to protection against tuberculosis in mice (47–50) and humans (51, 52). The protective effect of TNF-α in vivo is most likely mediated by its impact on cellular trafficking and the formation of granulomas rather than by activating antibacterial activity in mycobacterial host cells.

Our finding that virulent *M. Tb* converts iDC into mature DC with reduced antibacterial activity extends the repertoire of evasion mechanisms that mycobacteria have acquired to circumvent immune recognition. First, lipoarabinomannan, one of the major components of the mycobacterial cell wall, suppresses IL-12 release, thereby interfering with the development of protective adaptive immunity (8, 53). Second, infection of iDC results in down-regulation of the nonclassical Ag presenting molecule CD1, thereby preventing the establishment of CD1-restricted immune responses (54) which contribute to protective immunity (55). Finally, *M. Tb* inhibits the development of mature DC by infecting myeloid precursors (56–58). The majority of these evasion mechanisms are restricted to viable bacteria and virulent strains (54, 56, 57) suggesting an important role for these effects in natural infection.

Clearly, future studies are required to understand why mature DC, as compared with iDCs, are less able to control intracellular *M. Tb* growth. DNA analyses comparing gene expression profiles in immature and mature DCs revealed several candidate molecules (59). Most notably, maturation of human iDC by CD40 ligation resulted in an increased gene expression of chemokines (e.g., RANTES), receptors for cytokines (e.g., GM-CSF receptor), and the retinoic acid receptor, all of which have been implicated in modulating the growth of intracellular *M. Tb* in human macrophages (60–62). Ongoing studies are investigating the possibility that a single or a combination of these molecules influence the antibacterial activity of DCs.

The cross-talk between DC and the human pathogen *M. Tb* initiates a plethora of biological events that bias the immune response. Whether the balance is tipped in favor of the host or the pathogen is most likely dependent on the composition of the cellular influx and the local cytokine environment in the lung. These interactions should be carefully considered before designing vaccination strategies that use DC as vehicles for live mycobacteria.

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Disclosures

G. Cianciolo has a financial interest in LeukoMed.

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