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Constrained Intracellular Survival of *Mycobacterium tuberculosis* in Human Dendritic Cells[1]

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Dendritic cells (DCs) are likely to play a key role in immunity against *Mycobacterium tuberculosis*, but the fate of the bacterium in these cells is still unknown. Here we report that, unlike macrophages (Mϕs), human monocyte-derived DCs are not permissive for the growth of virulent *M. tuberculosis* H37Rv. Mycobacterial vacuoles are neither acidic nor fused with host cell lysosomes in DCs, in a mode similar to that seen in mycobacterial infection of Mϕs. However, uptake of the fluid phase marker dextran, and of transferrin, as well as accumulation of the recycling endosome-specific small GTPase Rab11 onto the mycobacterial phagosome, are almost abolished in infected DCs, but not in Mϕs. Moreover, communication between mycobacterial phagosomes and the host-cell biosynthetic pathway is impaired, given that <10% of *M. tuberculosis* vacuoles in DCs stained for the endoplasmic reticulum-specific proteins Grp78/BiP and calnexin. This correlates with the absence of the fusion factor *N*-ethylmaleimide-sensitive factor onto the vacuolar membrane in this cell type. Trafficking between the vacuoles and the host cell recycling and biosynthetic pathways is strikingly reduced in DCs, which is likely to impair access of intracellular mycobacteria to essential nutrients and may thus explain the absence of mycobacterial growth in this cell type. This unique location of *M. tuberculosis* in DCs is compatible with their T lymphocyte-stimulating functions, because *M. tuberculosis*-infected DCs have the ability to specifically induce cytokine production by autologous T lymphocytes from presensitized individuals. DCs have evolved unique subcellular trafficking mechanisms to achieve their Ag-presenting functions when infected by intracellular mycobacteria. *The Journal of Immunology*, 2003, 170: 1939–1948.

Mycobacterium tuberculosis, the causative agent of tuberculosis, affects up to one-third of the world population. The ability of the bacterium to create active or latent disease relies, at least in part, on the powerful mechanisms it has evolved to parasitize host macrophages (Mϕs)[5] (1). Although Mϕs appear to be central for mycobacterial replication, interactions of the bacilli with other leukocytes, such as dendritic cells (DCs), are likely to play a key role in immunity to *M. tuberculosis.* DCs exhibit the unique ability to ingest pathogens at the site of infection and to migrate to secondary lymphoid organs, such as lymph nodes, where they present pathogen-derived Ags to naive T lymphocytes (2). Priming and activation of mycobacterial Ag-specific T lymphocytes are essential for protection against tuberculosis (3, 4). In vitro studies have shown that human and murine DCs undergo maturation on infection with various mycobacterial species, such as *M. tuberculosis*, the vaccine strain *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) and *Mycobacterium avium* (5–13). Mycobacterium-infected DCs up-regulate MHC and costimulatory molecules and acquire the ability to prime naive T lymphocytes in vitro and in vivo when reinjected into animals. In vivo, it is likely that DCs contribute to the generation of protective cellular immunity against mycobacteria, in essence by polarizing T lymphocyte reactivity toward a type 1 profile. Indeed, in response to mycobacterial infection, DCs produce IL-12 (8, 10, 12), the function of which is to activate NK cells and naive T lymphocytes to produce type 1 cytokines (14) such as IFN-γ, which are essential for protection against mycobacterial infections (3, 4). A recent ex vivo study in mice has demonstrated that DCs are the major leukocyte population involved in early activation of naive T lymphocytes after infection with *M. bovis* BCG (15). Interactions between DCs present in the airway and mycobacteria are thus likely to be critical for mounting a protective immune response and for determining the outcome of infection. However, interactions of *M. tuberculosis* with DCs remain poorly documented, at least on the subcellular level. In particular, the ability of mycobacteria to replicate in DCs relative to Mϕs remains controversial (10, 15, 16), and the trafficking pathway of mycobacteria in DCs is still unknown, whereas it has been well delineated in Mϕs.

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[5] Abbreviations used in this paper: Mϕ, macrophage; DCs, dendritic cells; BCG, bacillus Calmette-Guérin; ciM6PR, cation-independent mannose 6-phosphate receptor; TACO, tryptophan-alanine-rich coat protein; NSF, *N*-ethylmaleimide-sensitive factor; GFP, green-fluorescent protein; PFA, paraformaldehyde; PBS-PFA, PBS-4% PFA; TR, Texas Red; PPD, purified protein derivative; TGN, *trans*-Golgi network; ER, endoplasmic reticulum; Grp78/BiP, 78-kDa glucose-regulated protein.

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M. tuberculosis entry into Mϕs involves various surface receptors (17) and ends in the bacteria residing in vacuoles or phagosomes. Since the discovery by Armstrong and d'Arcy Hart (18) that phagosomes containing viable *M. tuberculosis* do not fuse with ferritin-loaded host cell lysosomes, numerous studies have shed light on the nature and the mechanisms of biogenesis of the mycobacterial vacuole (19). In particular, compared with phagolysosomes containing inert particles, the mycobacterial phagosome is only mildly acidic (pH $\sim$ 6.2), a feature thought to rely on the exclusion of the vesicular H^+ATPase from the vacuole (20). In addition, the vacuole does not accumulate late endosomal and/or lysosomal proteins, such as the cation-independent mannose 6-phosphate receptor (ciM6PR) (21) or the small GTPases Rab7 and Rab9 (22–24). By contrast, the mycobacterial phagosome displays reduced clearance of early endosomal proteins, such as the small GTPase Rab5a (22) or the actin-binding coronin 1-like for tryptophan-alanine-rich coat protein (TACO) (24, 25), although the exact role of TACO in phagosome biogenesis remains to be fully elucidated (26). Furthermore, the phagosome is accessible within minutes to exogenously administered molecules, such as the fluid phase marker HRP or recycled transferrin, and it is enriched in transferrin receptor/CD71 (27, 28), indicating that intracellular mycobacteria maintain access to extracellular components and possibly essential nutrients, such as iron, via the host cell recycling pathway. The mycobacterial phagosome is subjected not only to fusion but also to fission events, because vesicular materials, containing mycobacterial Ags, can traffic from the phagosome to other subcellular compartments where Ags can be processed and loaded onto presentation molecules (29–31). Thus, despite its limited fusogenicity towards organelles of the deep endocytic pathway, the mycobacterial phagosome appears to be very dynamic indeed. This feature correlates with the continuous presence of various fusion and docking factors, such as the ATPase *N*-ethylmaleimide-sensitive factor (NSF), onto the phagosomal membrane, whereas these factors are rapidly lost by phagolysosomes containing inert particles (24, 32).

Comparing concomitantly infected Mϕs and DCs, we report that unlike human Mϕs, monocyte-derived DCs do not allow *M. tuberculosis* intracellular growth, even though bacteria are not killed. Mycobacterial persistence in DCs was found to be associated with location of bacilli within vacuoles that exhibited immature characteristics but reduced communication with the host cell recycling and biosynthetic pathways. This is probably a consequence of infection-induced maturation of DCs, and this process most likely impairs access of intracellular mycobacteria to extracellular components, including possible essential nutrients such as iron and cholesterol. Reduced connection of the mycobacterial vacuole with the DC recycling and biosynthetic pathways did not impair the stimulating capacity of DCs for autologous T lymphocytes from presensitized individuals.

Materials and Methods

Mycobacteria

M. tuberculosis strain H37Rv was grown in 7H9 broth or 7H11 agar supplemented with 10% oleic acid-albumin-dextrose catalase (Difco, BD Biosciences, Mountain View, CA) and glycerol and lacking Tween 80 or any other detergent because this could result in alteration of mycobacterial cell wall and virulence (33). Liquid cultures were grown for up to 12 days and stored at -80°C in 1- to 2-ml aliquots with 10% glycerol; these were then used for cell infection. Aliquots were enumerated after eliminating clumps by 25 to 30 passages through a needle (26-gauge 3/8; 0.45 $\times$ 10 for intradermal injection; BD Biosciences), and serial 10-fold dilutions in sterile distilled water with 1% BSA were made. Green-fluorescent protein (GFP)-expressing *M. tuberculosis* strain H37Rv (GFP-*M. tuberculosis*) carried the pEGFP plasmid (gift from G. Stewart, Imperial College, London, U.K.), which encodes resistance to hygromycin and harbors the *gfp* gene under the control of the mycobacterial P*hsp60* constitutive promoter. In line with other reports (34), the presence of GFP in *M. tuberculosis* did not alter the growth ability of the bacilli under axenic conditions relative to wild-type *M. tuberculosis* (data not shown). Recombinant bacteria were cultured in the presence of 50 μg/ml hygromycin B (Boehringer Ingelheim, Ingelheim, Germany). Before infection, viability of mycobacteria, evaluated by the propidium iodide exclusion method, was always >90%. In some experiments, bacteria were killed either by heating for 20 min at 95°C or by a 30-min incubation at 37°C in PBS-4% paraformaldehyde (PBS-PFA). After fixation, PFA was extensively washed off with PBS.

DCs, Mϕs, and infection

Blood mononuclear cells from healthy volunteers (Etablissement Français du Sang, Paris, France) were isolated by Ficoll-Paque (Pharmacia, Peapack, NJ) centrifugation. T, B, and NK cells were depleted using M-450 Pan T/CD2 and M-450 Pan B/CD19 Dynabeads (Dynal, Oslo, Norway). Recovered cells, hereafter referred to as monocytes, were seeded at 2×10^6 cells/well in six-well plates (Falcon; BD Biosciences, Pont de Claix, France) in 3 ml RPMI 1640 (BD Biosciences, Invitrogen, Paris, France) supplemented with 10% heat-inactivated FCS (Dutscher, Brumath, France), L-glutamine, GM-CSF (10 ng/ml; Schering-Plough, Kenilworth, NJ), and IL-4 (20 ng/ml) for DC cultures, or with Mϕ-CSF (M-CSF; 50 ng/ml; R&D Systems, Minneapolis, MN) for Mϕ cultures. Monocytes were allowed to differentiate into DCs or Mϕs for 6 days at 37°C in 5% CO_2 humidified atmosphere. In some cases, Mϕs were prepared as above, but with GM-CSF (10 ng/ml) instead of M-CSF. Cultures were fed every 2 days with complete medium containing full doses of cytokines. GM-CSF- and IL-13-monocyte-derived DCs were prepared as reported (35). Unless specified, DCs refers to GM-CSF/IL-4-induced monocytes and Mϕs to M-CSF-induced monocytes. In some cases (indicated in the figure legends), FCS was replaced by human AB pooled sera (Établissement Français du Sang). Before infection, *M. tuberculosis* H37Rv (expressing or not the GFP) was rapidly thawed and incubated overnight in 30 ml of 7H9 with glycerol and hygromycin. Bacteria were then washed three times with and resuspended in 1 ml RPMI 1640. Clumps were disassociated by 25–30 passages through a needle, followed by 5 min of sedimentation. The density of bacteria in the supernatant was checked at OD_{600} and correlated to the numeration of the aliquot to allow 1-to-1 bacterium-per-cell infection. This was performed in six-well plates with 2×10^6 cells in 3 ml medium containing the respective cytokines for Mϕs and DCs. After 6 h of incubation at 37°C, infected cells were washed three times in RPMI 1640 to remove extracellular bacteria and were incubated in fresh medium for a further 1–7 days. In experiments on the antimycobacterial effect of GM-CSF, M-CSF was replaced by GM-CSF 18 h before infection of Mϕs. Unless otherwise indicated, cytokines were maintained in culture throughout the course of infection. After 7 days of infection, the percentages of dead cells (dextran blue exclusion assay) were 25–40% for DCs and 50–75% for Mϕs. Enumeration of intracellular bacteria was performed at day 0 (6 h postinfection), day 1, day 3, day 5, and day 7. Cell supernatant was collected and centrifuged at 12,000 rpm to recover potential extracellular bacteria. Mϕs and DCs were lysed by cold distilled water with 0.1% Triton X-100. Bacteria were enumerated as previously mentioned and plated on 7H11. CFUs were scored after 3 wk at 37°C.

Flow cytometry analysis

DCs and Mϕs were harvested at indicated times and resuspended in 200 μl of PBS-2% heat-inactivated FCS. The cell suspension was then incubated for 30 min at 4°C with the appropriate Abs (listed below) at optimal dilutions. Cells were washed and fixed with PBS-PFA. Fluorescence was analyzed on a total of 10^4 cells per sample using FACSCalibur and CellQuest software (BD Biosciences). PE-conjugated anti-CD14 and FITC-conjugated anti-CD1a Abs were from BD Biosciences and BD PharMingen, respectively.

Confocal microscopy analysis

Mϕs were cultured and infected onto glass coverslips and fixed for 30 min in PBS-PFA. DCs were allowed to adhere for 15 min on polylysine-coated coverslips before fixation. Cells were then permeabilized with 0.05% saponin and immunostained as described (31). Coverslips were observed under a TCS4D confocal microscope (Leica, Deerfield, IL), and sections <0.8 μm were scored and treated using AdobePhotoshop software (Adobe Systems, Mountain View, CA). Primary Abs were as follows: anti-Rab11, -Rab6, -calnexin, anti-M6PR (Affinity Bioreagent, Golden, CO); anti-NSF (Synaptic Systems, Göttingen, Germany), anti-TACO (gift from J. Pieters, Basel Institute for Immunology, Basel, Switzerland); anti-vacuolar H^+ATPase (Synaptic Systems); anti-Grp78/Bip (StressGen Biotechnologies, Victoria, Canada), anti-Golgin97 (Molecular Probes, Eugene, OR).

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Cy3-conjugated anti-mouse and -rabbit secondary Abs were from Amersham Biosciences UK (Little Chalfont, U.K.). Cy5-conjugated anti-mouse Abs were from Jackson ImmunoResearch Laboratories (West Grove, PA). Alexa594-conjugated anti-goat Abs were from Molecular Probes. In experiments using Lysotracker DND-99 (Molecular Probes), infected cells were incubated with the dye at 200 nM for 5 min at 37°C before fixation.

Colocalization of GFP-*M. tuberculosis* with the markers was quantified by counting at least 200 phagosomes in preparations of two different donors.

Transferrin and dextran uptake

Cells were deprived of transferrin by incubation in serum-free RPMI 1640 for 2 h at 37°C, washed in RPMI 1640, and incubated for 1 h at 4°C or 37°C with Texas Red (TR)-conjugated transferrin (50 μg/ml; Molecular Probes) in an excess of BSA (2 mg/ml) to saturate nonspecific endocytosis. Cells were washed twice in cold RPMI 1640 and incubated for 5 min at room temperature with (25 mM citric acid, 24.5 mM sodium citrate, 280 mM sucrose, pH 4.6) to remove extracellular transferrin. After 0–2 h chase with excess unlabeled transferrin (5 mg/ml + 2 mg/ml BSA), cells were fixed PBS-PFA and observed by confocal microscopy or flow cytometry. FITC-conjugated dextran (Sigma-Aldrich, St. Louis, MO) was pulsed at 1 mg/ml for 1 h at 4°C or 37°C. Cells were washed with cold PBS and fixed with PBS-PFA before flow cytometry analysis.

Cytokine detection in supernatants

DCs were left uninfected, incubated with heat-killed *M. tuberculosis*, or infected with live mycobacteria as described above and cultured for 48 h. Then, 10^5 DCs were cultured for 48 h with 10^6 autologous T lymphocytes enriched as reported (36). IL-2, IL-4, IL-5, IL-6, and IFN-γ levels in culture supernatants were then assessed by ELISA according to the manufacturer's recommendations (R&D Systems). As controls, supernatants were collected from cultures of noninfected or *M. tuberculosis*-infected DCs alone, DCs alone incubated with heat-killed *M. tuberculosis*, DCs mixed with T lymphocytes without any stimulus, and T lymphocytes that were incubated or not with 20 μg/ml purified protein derivative (PPD; Sigma-Aldrich) with or without added DCs.

Results

Human DCs do not support intracellular replication of M. tuberculosis *H37Rv*

DCs derived from monocytes cultured with GM-CSF and IL-4 did not support intracellular replication of *M. tuberculosis* H37Rv (Fig. 1*A*). The bacterial load was slightly decreased (0.3- to 0.4-log reduction in CFUs) between days 0 and 7, a value unlikely to reflect active killing of bacteria by infected cells. In contrast, Mϕs derived from the same monocytes cultured with M-CSF, permitted intracellular replication of bacteria with a 26- to 28-h doubling time. This observation was confirmed using DCs prepared from monocytes treated with GM-CSF and IL-13 (35). Again, mycobacteria persisted at almost constant levels in these cells, without any obvious replication (Fig. 1*B*). Because GM-CSF has previously been reported to restrict mycobacterial growth in monocyte-derived Mϕs (37), we ascertained that our findings in DCs were not due to the presence of the cytokine per se. To this end, we compared *M. tuberculosis* growth in GM-CSF-differentiated and in M-CSF-differentiated Mϕs in the presence of either M-CSF or GM-CSF during the infection period. The three culture conditions allowed mycobacterial growth at almost identical rates (Fig. 1*C*). Thus, it is very unlikely that the constrained survival of *M. tuberculosis* observed in DCs was due to the presence of GM-CSF per se in the culture medium.

Our data are apparently at variance with a previous report (16), which could be due to the fact that GM-CSF and IL-4 were there

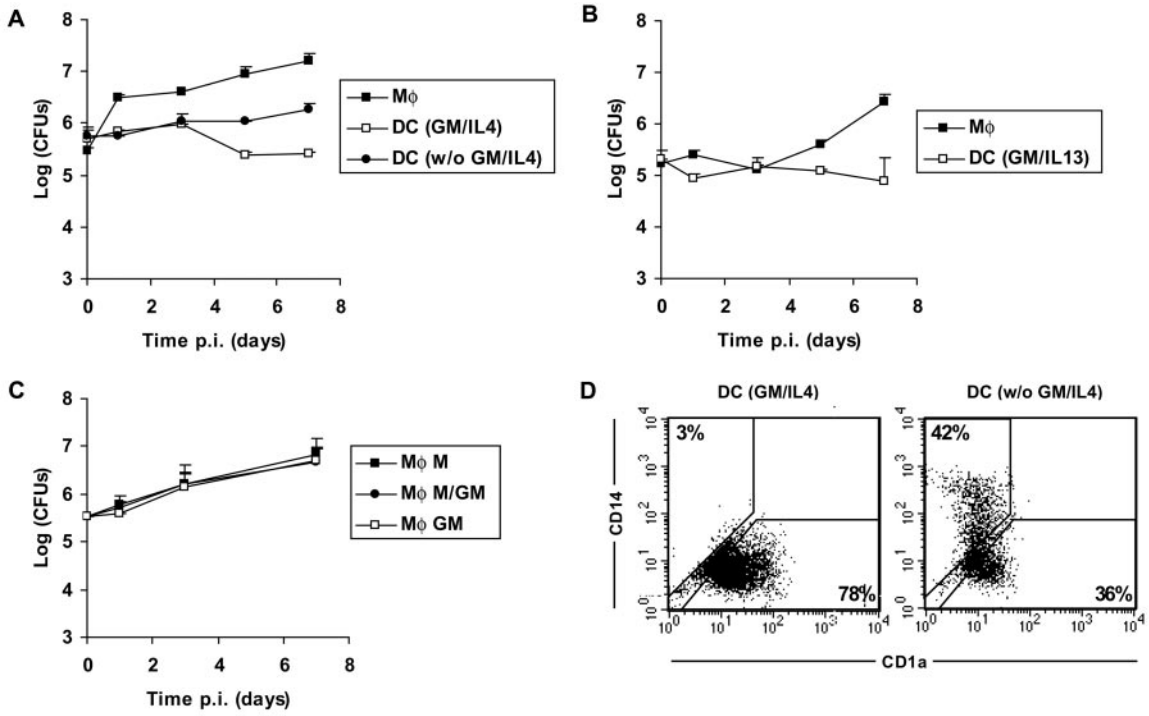

FIGURE 1. DCs restrain *M. tuberculosis* H37Rv replication in vitro. *A*, Mϕs (■) were obtained from M-CSF-induced monocytes; DCs were obtained from monocytes cultured with GM-CSF (GM)/IL-4; cytokines were left in the DC culture medium supplemented with 10% human AB serum throughout the course of infection (□) or discontinued (●) before infection; CFU numbers were determined at the indicated time points. *B*, Mϕs (■) were obtained as in *A*; DCs were obtained from monocytes cultured with GM-CSF/IL-13 (□); cytokines were left in the culture medium throughout the course of infection. *C*, Mϕs were obtained as in *A* (■ and ●) or from GM-CSF-treated monocytes (□); cytokines were maintained throughout the course of infection (● and □) or M-CSF was replaced by GM-CSF (●). *D*, CD1a and CD14 expression by 48-h-infected DCs when GM-CSF/IL-4 was either kept in the culture (*left*) or discontinued (*right*). Data in *A–C* are mean values (±SD) from triplicate experiments using monocyte-derived Mϕs and DCs from three different donors. Data presented in *D* are from one representative experiment of three with as many different donors. Comparable results were obtained whether cells were cultured and infected in the presence of human AB serum or FCS (data not shown). w/o, Without.

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removed from the culture once the cells were infected. By doing the same, we found indeed that, in line with previous findings (38), >40% of the cells reverted from a $CD1a^+CD14^-$ DC phenotype to a $CD1a^-CD14^+$ Mϕ-like phenotype over a 48-h infection period (Fig. 1*D*) and that mycobacterial growth then resumed, albeit to a lesser extent than in Mϕs (Fig. 1*A*).

In our experiments, as reported (39), infection often resulted in a preliminary stationary-like 48- to 72-h phase during which the net bacterial load remained relatively constant, but after which growth either started (in Mϕs) or not (in DCs). This initial stage might reflect the time required for the microbe to arrange its intracellular niche. Thus, we investigated comparatively in Mϕs and DCs whether the contrasting behavior of *M. tuberculosis* could be explained, at least in part, by the different intracellular compartments available to the bacillus in the two cell types.

The maturation of the M. tuberculosis *vacuole is arrested in DCs*

In Mϕs, based on studies showing that mycobacterial phagosomes fail to acidify and to fuse with host cell late endosomes and lysosomes, pathogenic mycobacteria are thought to reside in a mildly acidic compartment that is arrested at an early stage of the endocytic pathway (19). Confocal microscopy examination of *M. tuberculosis*-infected cells showed that most mycobacterial vacuoles failed to acidify in DCs (Fig. 2*A*) as in Mϕs (data not shown), as attested by lack of accumulation of the acid tropic dye LysoTracker (Molecular Probes) in vacuoles for up to 5 days after initial infection. PFA-killed *M. tuberculosis*, used as a control, were readily transported within 24 h to acidic (LysoTracker-positive) phagolysosomes in both DCs (Fig. 2*A*) and Mϕs (data not shown). As reported in Mϕs (20), the lack of vacuole acidification in DCs was associated with the absence of H^+ATPase onto the vacuolar membrane, whereas H^+ATPase was abundant on phagolysosomes containing PFA-killed *M. tuberculosis* (data not shown). Mycobacterial vacuoles also lacked the late endosome-specific marker ciM6PR in both Mϕs and DCs (data not shown).

Several mechanisms have been proposed to account for the ability of pathogenic mycobacteria to arrest phagosome maturation. Among these, is the role of coronin 1-like TACO, which appears to be actively retained on the surface of *M. bovis* BCG phagosomes in mouse Mϕs as a specific mean to avoid phagolysosome fusion (25). In our experiments, TACO was detected on 55–65% of *M. tuberculosis* vacuoles in both Mϕs and DCs during the 5-day infection period (Fig. 2*B*). Taken together, these data indicate that the *M. tuberculosis* vacuole has characteristics of an immature endosome in both Mϕs and DCs and are consistent with previous findings. Indeed, had the bacilli been killed in DCs, then most of them would have been detected in acidic phagolysosomes, because arrest of phagosome maturation is a feature of live mycobacteria only. On the other hand, had DCs routed the bacilli to an acidic compartment, then the bacilli would have been killed over time,

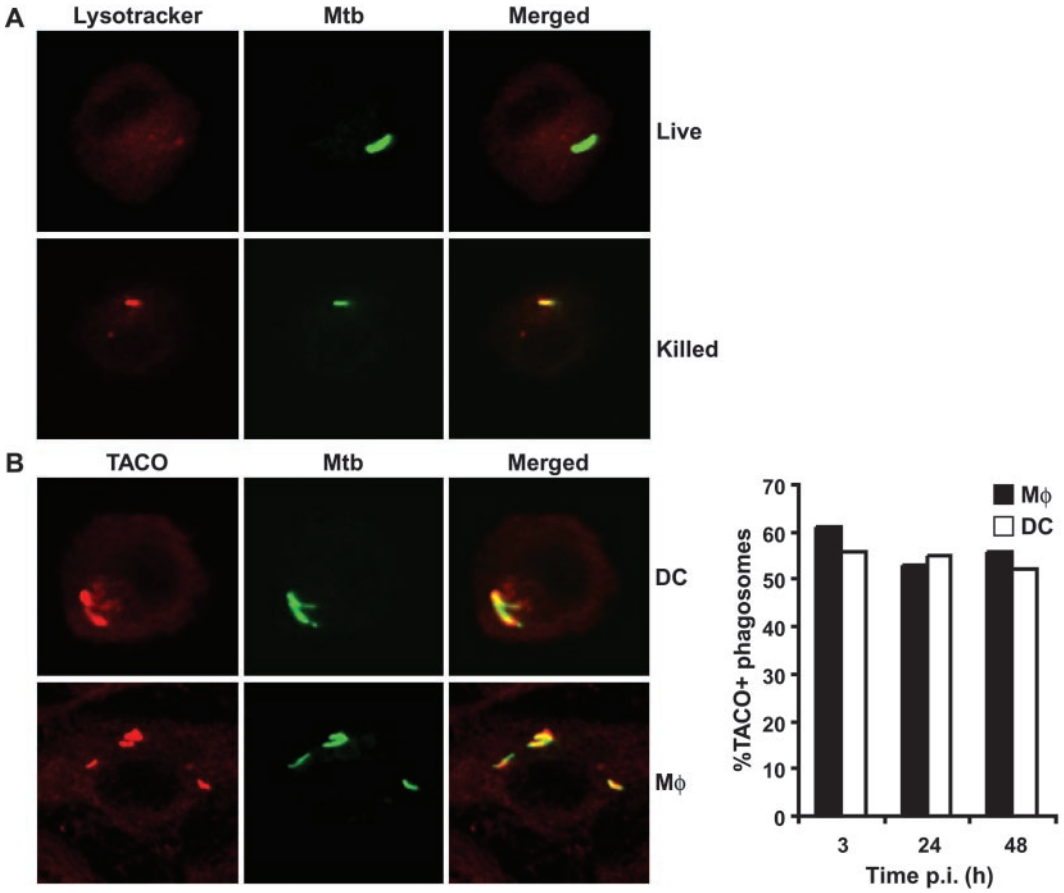

FIGURE 2. The maturation of the *M. tuberculosis* vacuole is arrested in DCs. *A*, DCs infected for 48 h with live (*top*) or PFA-killed GFP-*M. tuberculosis* (Mtb) (*bottom*) were stained with the acid-tropic dye LysoTracker. *B*, DCs (*top*) or Mϕs (*bottom*) infected for 48 h with live GFP-*M. tuberculosis* were stained for the coronin 1-like TACO. Data are representative of three (*A*) and six (*B*) experiments performed on monocyte-derived DCs and Mϕs from as many different donors. The graphs indicate the percentages of TACO-positive phagosomes assessed by counting >200 phagosomes in preparations of one representative donor of two examined. No significant difference was observed between infection days 2 and 5. In Mϕs, TACO was also detected at the plasma membrane, but due to the strong staining on phagosomes the signal had to be reduced. p.i., Postinfection.

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because *M. tuberculosis* does not survive at acidic pH (40). This was not the case.

M. tuberculosis *vacuoles have no access to exogenous material in DCs*

Survival of mycobacteria within Mϕs is thought to rely, at least in part, on continuous access of the microbes to extracellular nutrients through permanent fusion events of the bacterial phagosome with host cell endosomes (19). We thus reasoned that constrained survival of *M. tuberculosis* inside DCs could be due to reduced access of the mycobacterial vacuole to endosomes. Uptake of the fluid phase marker dextran was almost abolished in infected DCs compared with uninfected cells, whereas it remained constant in infected Mϕs (Fig. 3*A*). This results most likely from DC maturation upon *M. tuberculosis* infection. To assess whether receptor-mediated endocytosis was also diminished in infected DCs, we pulsed the cells with TR-labeled transferrin. The conditions of transferrin uptake used here allowed staining of the recycling compartment only, because pulsed TR-transferrin was chasable within 1 h by an excess unlabeled transferrin (data not shown). As for dextran, transferrin uptake was strikingly diminished in infected DCs but not in Mϕs (Fig. 3*B*). Consequently, transferrin could not reach *M. tuberculosis* vacuoles in DCs, even after up to a 2-h pulse (Fig. 3*B*). These results suggest that *M. tuberculosis* vacuoles have no access to recycling endosomes in DCs only. To strengthen this finding, we stained infected cells with the recycling endosome-specific small GTPase Rab11. As shown in Fig. 3*C*, 45–65% of phagosomes retained GTPase Rab11 in Mϕs over the 5-day infection period, whereas Rab11 was detected on only 10–15% of vacuoles in DCs already at 3 h postinfection. Rab11 is present on recycling vesicles and the *trans*-Golgi network (TGN) in various cell types (41), including Mϕs (42), and it has once been reported to be present on *M. bovis* BCG phagosomes in murine Mϕs (24). In our experiments, it is unlikely that Rab11 enrichment on mycobacterial

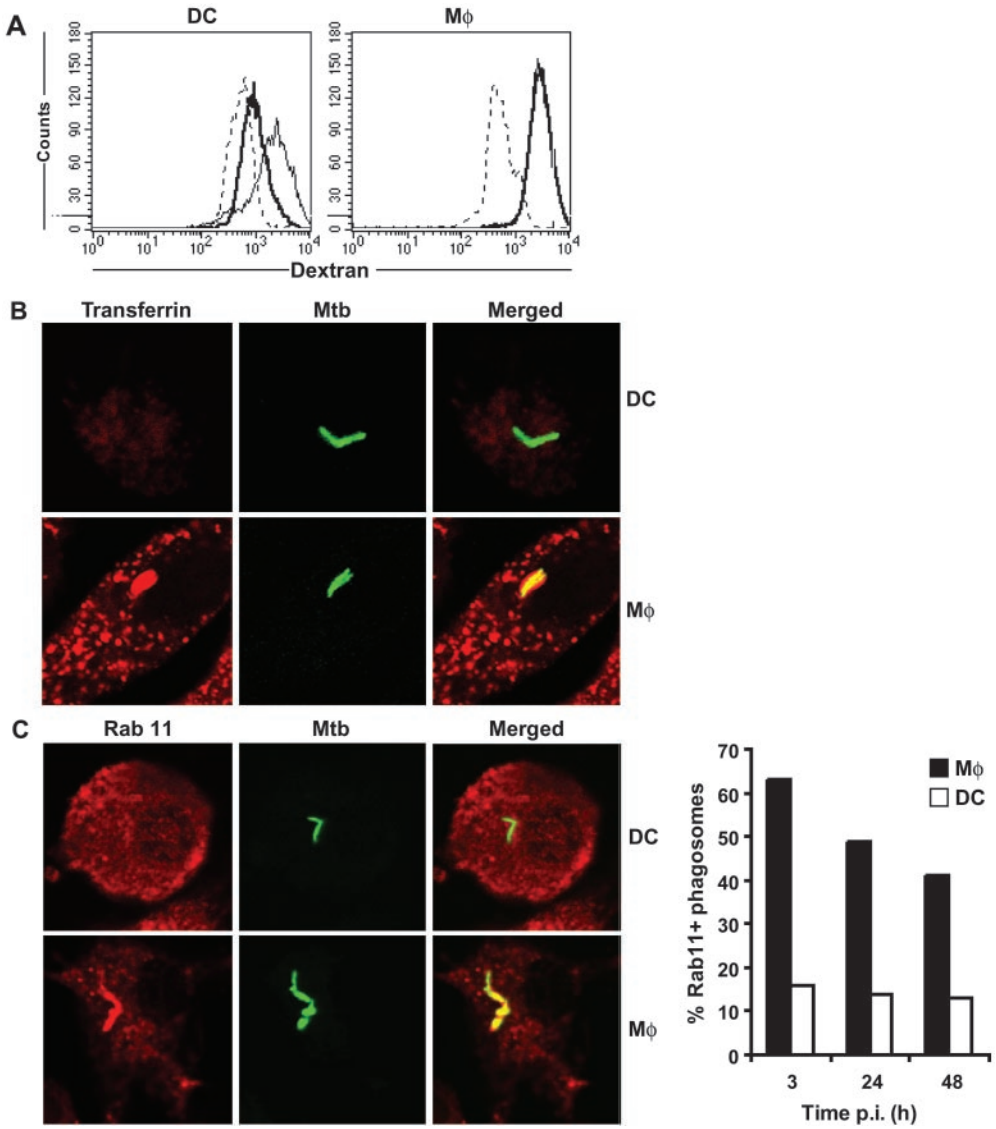

FIGURE 3. Mycobacterial vacuoles in DCs are inaccessible to endosomes. Mϕs or DCs infected for 48 h with *M. tuberculosis* (*A*) or GFP-*M. tuberculosis* (*B* and *C*) were analyzed by flow cytometry (*A*) or confocal microscopy (*B* and *C*) for uptake of FITC-conjugated dextran (*A*) or TR-transferrin (*B*), or Rab11 staining (*C*). *A*, Dashed lines, controls at 4°C; bold lines, infected cells; thin lines, uninfected cells. *C*, Percentages of Rab11-positive phagosomes assessed by counting >200 phagosomes in preparations of one donor of two examined. Confocal microscopy images are representative of two (*A* and *B*) and four (*C*) independent experiments performed on monocyte-derived DCs from as many different donors. No significant difference was observed between infection days 2 and 5. p.i., Postinfection.

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phagosomes in Mϕs reflected communication of the vacuoles with the TGN, since confocal microscopy analysis clearly indicated that *M. tuberculosis* vacuoles were devoid of TGN-specific markers Rab6 and Golgin97 (data not shown). Thus, it is rather likely that the presence of Rab11 on phagosomes in Mϕs but not in DCs reflects differential access of the vacuoles to recycling endosomes in the two cell types.

M. tuberculosis *vacuoles have no access to the biosynthetic pathway in DCs*

We next examined the possible communication between mycobacterial phagosomes and the host cell biosynthetic pathway, which could allow intracellular mycobacteria to access additional nutrients including host cell lipids. We found that in infected Mϕs, 30–40% and ~55% of *M. tuberculosis* phagosomes stained for the endoplasmic reticulum (ER)-specific proteins calnexin and 78-kDa glucose-regulated protein (Grp78/BiP), respectively, during a 5-day infection period. In contrast, only ~10% of *M. tuberculosis* vacuoles in DCs were positive for these markers 48 h after phagocytosis (Fig. 4, *A* and *B*). As controls, phagolysosomes containing PFA-killed *M. tuberculosis* did not stain for calnexin in either Mϕs or DCs (data not shown). These data suggest that the mycobacterial vacuole remains in continuous communication with the ER in Mϕs but not in DCs.

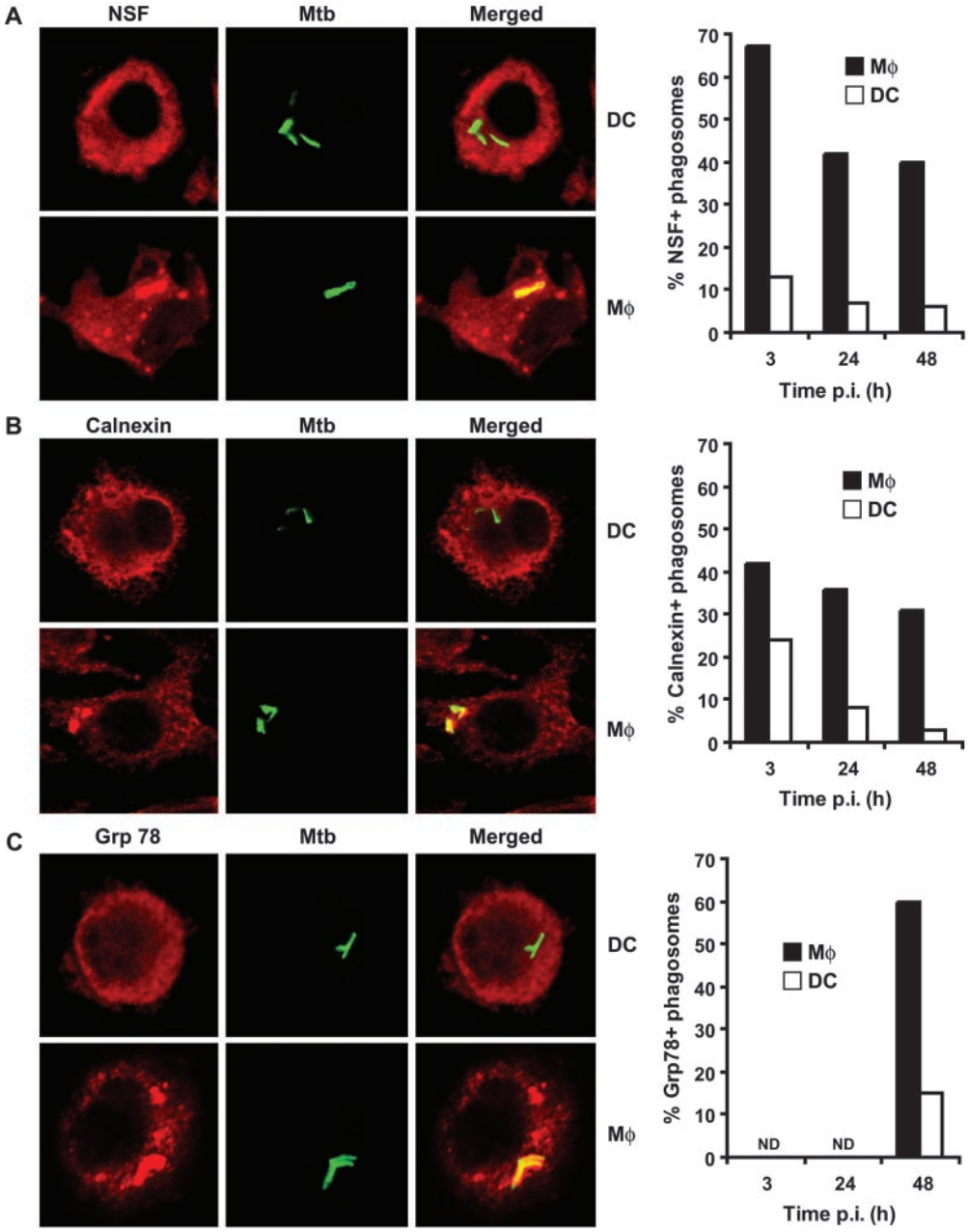

FIGURE 4. Intracellular *M. tuberculosis* communicates with the cell biosynthetic pathway in Mϕs but not in DCs. Mϕs and DCs were infected for 48 h with GFP-*M. tuberculosis* (Mtb) and stained for the endoplasmic reticulum-specific markers calnexin and Grp78/BiP (*A* and *B*), and the fusion factor NSF (*C*). The graphs indicate percentages of calnexin-, Grp78/BiP-, or NSF-positive phagosomes, respectively, assessed by counting >200 phagosomes in preparations of one donor of two examined. Confocal microscopy images are representative of four (*A* and *B*) and three (*C*) independent experiments performed on monocyte-derived DCs and Mϕs from as many different donors. No significant difference was observed between infection days 2 and 5. *C*, NSF was detected in Mϕ cytosol, but due to the strong staining on the phagosomes, the signal had to be reduced. p.i., Postinfection.

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Because fusogenicity of the mycobacterial vacuole was globally reduced in DCs, we examined whether this correlated with the lack of fusion factors onto the vascular membrane in this cell type. The ATPase NSF, together with soluble NSF attachment protein, is required in a broad range of vesicular docking and fusion events (43), and NSF was recently shown on *M. bovis* BCG phagosomes in J774 Mϕ-like cells (24, 32). Consistent with these results, the ATPase NSF was continuously retained on the phagosome membrane in Mϕs, but the mycobacterial vacuoles in DCs were deficient of this marker (Fig. 4*B*).

M. tuberculosis-*infected DCs present mycobacterial Ags to T lymphocytes*

Finally, we examined whether the particular location of mycobacteria in DCs could perturb their ability to present mycobacterial Ags to T lymphocytes (5). Thus, we incubated *M. tuberculosis*-infected DCs with autologous T lymphocytes from donors known to have been previously sensitized against *M. tuberculosis* by *M. bovis* BCG vaccination, which is mandatory in France to assess their capacity to produce IL-2, IL-6, IFN-γ, and IL-5. Under these conditions, the T lymphocytes were stimulated to produce cytokines in culture supernatants, especially IL-2 and IFN-γ and to a lesser degree IL-5 and IL-6, to levels comparable with or greater than those of noninfected DCs pulsed with PPD from *M. tuberculosis* H37Rv or with heat-killed bacteria (Fig. 5). Infected DCs alone as well as T cells alone were unable to produce IL-2 (Fig. 5), and IL-4 was undetectable under any of the conditions used here (data not shown).

Discussion

In mammals, mycobacteria face several lines of defense, the first of which are professional phagocytic cells, including DCs and Mϕs. These two cell types are dedicated to different functions; whereas Mϕs ingest and degrade microbes at the site of infection, DCs take up pathogens and migrate to secondary lymphoid organs where they present pathogen-derived Ags to T lymphocytes. In concomitantly cultured Mϕs and DCs, our data indicate that these cells behave very differently when infected with *M. tuberculosis* in that, contrary to Mϕs, DCs are not permissive to mycobacterial growth. These findings conflict with a report in which *M. tuberculosis* was found to replicate in human DCs (16). This discrepancy is likely due to the culture conditions used during the infection period, namely whether or not differentiating cytokines GM-CSF and IL-4 are maintained throughout the course of infection. Indeed, removal of the differentiating cytokines during the infection period resulted in reversion of a great proportion of DCs into Mϕ-like cells and in resuming mycobacterial growth. Such reversion could also account for the 10% increase in $CD14^+$ cells after 4 h of infection of murine DCs, recently reported by Bodnar et al. (7). Again these authors did not maintain the differentiating cytokine GM-CSF after infection, and murine DCs were found to be permissive to *M. tuberculosis* growth. We also demonstrated that GM-CSF per se could not account for the constrained *M. tuberculosis* survival in DCs, even though this cytokine was reported once to restrict mycobacterial growth in Mϕs (37).

Our data are in line with the report by Jiao et al. (15), who found that *M. bovis* BCG persists but does not replicate in splenic DCs of experimentally infected mice. A similar discrepancy between DCs and Mϕs has also been reported for other intracellular bacteria. For instance, *Listeria monocytogenes* does not replicate in murine DCs, in vitro in human DCs (44) or in vivo in rats (45). In parallel with our observation, *Salmonella typhimurium* does not replicate but persists over time in murine DCs (46). Furthermore, it has been shown that several *S. typhimurium* genes, such as *phoP*, essential for survival and replication of the bacterium in Mϕs, are dispensable in DCs, suggesting that the intracellular compartment occupied by the microbe differs in the two cell types (46).

The different behavior of *M. tuberculosis* in Mϕs and DCs prompted us to characterize the vacuole occupied by the bacillus in the two cell types. To survive inside Mϕs, pathogenic mycobacteria have developed mechanisms to resist intracellular degradation and to keep access to essential nutrients by intercepting the

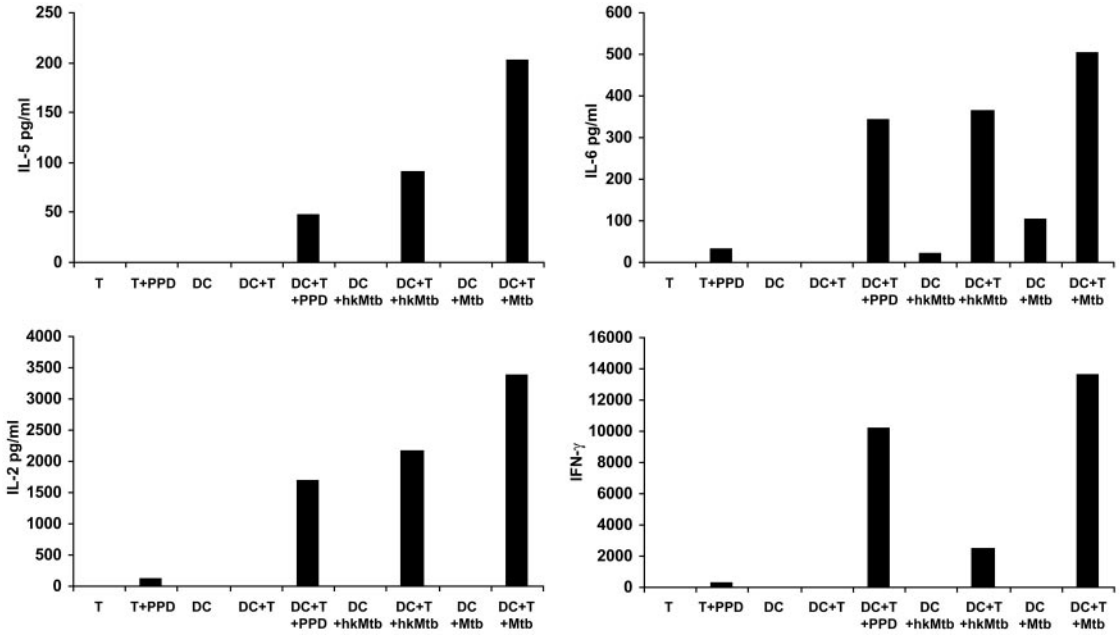

FIGURE 5. *M. tuberculosis* (Mtb)-infected DCs stimulate autologous T lymphocytes (T) to produce cytokines. DCs infected 2 days previously were cultured for 48 h with autologous T lymphocytes, and IL-2, IL-5, IL-6, and IFN-γ were then assessed in culture supernatants. As controls, T lymphocytes were cultured alone or in the presence of PPD, noninfected DCs and T lymphocytes were incubated or not with PPD, or DCs were mock-infected with heat-killed (hk) mycobacteria. Data are representative of three experiments corresponding to as many different donors.

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host cell recycling pathway (19, 27, 28). Our data that mycobacterial phagosomes stain for Rab11 in Mϕs is in line with such assumption. Indeed, Rab11 GTPase localizes to transferrin receptor-containing pericentriolar recycling compartments in a variety of polarized cells (47). Rab11 also localizes to a recycling compartment in mouse Mϕs (42), even though the cells lack a morphologically distinct pericentriolar recycling compartment. It is also present on the TGN, but we have shown that *M. tuberculosis* phagosomes do not communicate with the TGN since they failed to accumulate TGN-specific markers Rab6 and Golgin97. On the contrary, Rab11 was detected in very few mycobacterial vacuoles in DCs, suggesting that the vacuoles have limited access to recycling vesicles in these cells. Indeed, use of labeled transferrin allowed us to demonstrate that transferrin cannot reach the mycobacterial compartment in infected DCs. Such reduced endocytosis, which is mediated by the transferrin receptor (48), most likely results from DC maturation (49). In our experiments, *M. tuberculosis* infection led to maturation of DCs, as illustrated by up-regulation of surface CD83 and other markers, including MHC-II and CD86 (data not shown). Dextran uptake, which is mediated both via the mannose receptor and macropinocytosis (50), was also strongly diminished in infected DCs. It is likely that the global maturation-induced reduction in endocytic processes impairs *M. tuberculosis* to access exogenous nutrients, and thus to impair replication in DCs.

Although communication between mycobacterial phagosomes and Mϕ recycling endosomes has been extensively studied, accessibility of the vacuoles to the cell biosynthetic pathway has been suggested only once in *M. avium*-infected murine Mϕs (51). We found that a significant proportion of mycobacterial phagosomes stained strongly for the ER-specific markers calnexin and Grp78/BiP in Mϕs only. Whether this results from a specific mycobacterial ER-targeting strategy still remains to be elucidated. A more probable explanation relies on an attractive model of membrane homeostasis recently proposed by Gagnon et al. (52), which suggests that ER-derived material could interact directly with phagosomes to keep particles in closed compartments while the plasma membrane engaged in phagosome formation recycles back to the cell surface to maintain a stable cell size. Interestingly, according to this model, infected mature DCs that no longer endocytose and phagocytose would not require ER-derived material to accumulate on bacterial vacuoles, which is what we have observed.

The overall reduced fusogenicity of *M. tuberculosis* vacuoles in DCs consistently correlated with the paucity of this compartment in the ATPase NSF, a factor involved in a broad range of intracellular membrane fusion events (43). Several intracellular pathogens reside in NSF-rich vacuoles, indicating that they represent dynamic compartments involved in permanent fusion and fission events with other intracellular organelles. Two such examples are *S. typhimurium*, which actively recruits NSF on the phagosomal membrane in murine Mϕ-like J774 cells as a specific means to promote phagosomal fusion with early endosomes (53) and *M. bovis* BCG phagosomes in J774 cells (32).

Finally, our data suggest that the unique location occupied by *M. tuberculosis* within DCs does not disturb their T lymphocyte-stimulating function, inasmuch as infected DCs had the ability to activate T lymphocytes to produce cytokines, especially IFN-γ. However, one cannot rule out that lymphocytes were then activated by DCs that had ingested material containing mycobacterial Ags, including apoptotic bodies and exosomes (54), in addition to, or instead of, directly infected DCs.

The different behavior of *M. tuberculosis* in Mϕs and in DCs may reflect differences in the receptors involved in bacterial uptake by the two cell types. For example, DCs have surface receptors such as the recently identified DC-specific ICAM3 grabbing nonintegrin (DC-SIGN/CD209), that are not expressed on most Mϕ types, and that are dedicated to Ag uptake (55). We have recently demonstrated that *M. tuberculosis* enters human DCs after binding to DC-SIGN (56). By contrast, the complement receptor type 3 and the mannose receptor, which are involved in the phagocytosis of mycobacteria by Mϕs, appeared to play a minor role, if any, in mycobacterial binding to DCs. Intracellular route of ingested particles mostly differs depending on the receptors engaged in particle ligation and phagocytosis, and the trafficking of DC-SIGN-bound mycobacteria is now being investigated. Recent reports have also shown profound differences in the phagosome biology of Mϕs relative to DCs. For example, DCs but not Mϕs can present exogenous Ags to $CD8^+$ T lymphocytes via a cytosol-dependent pathway involving an as yet unidentified phagosome-to-cytosol transporter (57).

Taken together, our findings might be interpreted in two different but not exclusive ways. One might hypothesize that DCs have evolved specific mechanisms for priming T lymphocytes after homing from the lungs to lymph nodes, while maintaining mycobacteria alive in vacuoles, reminiscent of the retention compartment reported in a mouse DC line (58, 59). Indeed, we and others have shown that mycobacterial Ag presentation by MHC molecules is far more efficient when bacteria are viable (31). This might allow T lymphocytes to respond to Ags produced in replicating mycobacterium-infected cells, such as alveolar Mϕs. On the other hand, this might offer the possibility to the microbe to use DCs as a vehicle to spread inside the host and reach the lymphoid tissues. In our experiments, *M. tuberculosis*-infected DCs exhibited a migratory phenotype ($CCR5^-$, $CXCR4^+$) (L. Tailleux, O. Neyrolles, J. L. Herrman, P. H. Lagrange, and J. C. Gluckman, unpublished data) while maintaining a relatively constant bacterial load for up to 7 days, which is more than enough for DCs to migrate from the periphery to secondary lymphoid organs (60). Similar observations have been made in *M. tuberculosis*-infected murine DCs (7). Recent reports suggest that various pathogens can exploit the migratory properties of mature DCs to disseminate within the host. For example, *Leishmania* spp. have been shown to persist within DCs in lymph nodes, suggesting that DCs are impaired in their ability to kill this pathogen, which can then manipulate DCs for its own invasion strategy (61). In the case of mycobacteria, this might explain such clinical phenomena as mediastinal lymphadenitis, usually associated with primary *M. tuberculosis* infections. A spread to local lymph nodes might also account for persistence of the pathogen and chronic stimulation of the immune system. Whether *M. tuberculosis* is in a dormant-like state in DCs still remains to be firmly defined. These possibilities are currently being investigated using in vitro-infected DCs and lymph node biopsies from patients with tuberculosis.

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