Deciphering the molecular bases of *Mycobacterium tuberculosis* binding to the lectin DC-SIGN reveals an underestimated complexity

Sylvain PITARQUE*[1], Jean-Louis HERRMANN‡[1], Jean-Luc DUTEYRAT†, Mary JACKSON§, Graham R. STEWART††, François LECOINTE‖, Bruno PAYRE†, Olivier SCHWARTZ¶, Douglas B. YOUNG††, Gilles MARCHAL‖, Philippe H. LAGRANGE‡, Germain PUZO*, Brigitte GICQUEL§, Jérôme NIGOU*[2] and Olivier NEYROLLES§**[2,3]

*Institut de Pharmacologie et de Biologie Structurale, Département Mécanismes Moléculaires des Infections Mycobactériennes, CNRS UMR 5089, 205 route de Narbonne, 31077 Toulouse Cedex 4, France, †Université Paul Sabatier, Centre de Microscopie Electronique Appliquée à la Biologie, route de Narbonne, 31077 Toulouse Cedex 4, France, ‡Université Lariboisière-Saint Louis, Equipe d'Accueil EA3510, Hôpital Saint-Louis, Service de Microbiologie, 1 avenue C. Vellefaux, 75010 Paris, France, §Unité de Génétique Mycobactérienne, 28 rue du Dr Roux, 75015 Paris, France, ‖Institut Pasteur, Unité de Génétique Mycobactérienne, 28 rue du Dr Roux, 75015 Paris, France, ¶Institut Pasteur, Groupe Virus et Immunité, 28 rue du Dr Roux, 75015 Paris, France, **CNRS URA 2172, 28 rue du Dr Roux, 75015 Paris, France, and ††Centre for Molecular Microbiology and Infection, Department of Biological Sciences, South Kensington Campus, Imperial College London, London SW7 2AZ, U.K.

Interactions between dendritic cells and *Mycobacterium tuberculosis*, the aetiological agent of tuberculosis in humans, are thought to be central to anti-mycobacterial immunity. We have previously shown that *M. tuberculosis* binds to human monocyte-derived dendritic cells mostly through the C-type lectin DC-SIGN (dendritic-cell-specific intercellular molecule-3-grabbing non-integrin)/CD209, and we have suggested that DC-SIGN may discriminate between mycobacterial species through recognition of the mannose-capping residues on the lipoglycan lipoarabinomannan of the bacterial envelope. Here, using a variety of fast- and slow-growing *Mycobacterium* species, we provide further evidence that mycobacteria recognition by DC-SIGN may be restricted to species of the *M. tuberculosis* complex. Fine analyses of the lipoarabinomannan molecules purified from these species show that the structure and amount of these molecules alone cannot account for such a preferential recognition. We propose that *M. tuberculosis* recognition by DC-SIGN relies on both a potential difference of accessibility of lipoarabinomannan in its envelope and, more probably, on the binding of additional ligands, possibly including lipomannan, mannose-capped arabinomannan, as well as the mannosylated 19 kDa and 45 kDa [Apa (alanine/proline-rich antigen)] glycoproteins. Altogether, our results reveal that the molecular basis of *M. tuberculosis* binding to DC-SIGN is more complicated than previously thought and provides further insight into the mechanisms of *M. tuberculosis* recognition by the immune system.

Key words: dendritic-cell-specific intercellular molecule-3-grabbing non-integrin (DC-SIGN), dendritic cell, lipoarabinomannan, mycobacteria, tuberculosis.

INTRODUCTION

Among APCs (antigen-presenting cells), DCs (dendritic cells) are thought to play a central role in immunity against *Mycobacterium tuberculosis*, the aetiological agent of TB (tuberculosis) in humans [1,2]. It has recently been shown that *M. tuberculosis* binds to human monocyte-derived DCs through the C-type lectin DC-SIGN (dendritic-cell-specific intercellular molecule-3-grabbing non-integrin)/CD209 almost exclusively [3–5]. By contrast, the receptors involved in *M. tuberculosis* binding to macrophages, namely the complement receptors and the mannose receptor, played only minor roles, if any, in binding of the tubercle bacillus to DCs, although they were expressed by the cells. Furthermore, we have shown that DC-SIGN was expressed in pulmonary DCs from human lung parenchyma, and that mycobacterial antigens could be detected in DC-SIGN-expressing DCs in lymph-node biopsies from patients with TB, strongly suggesting that *M. tuberculosis* interactions with DC-SIGN occur *in vivo* during the course of TB [5].

The ability of DC-SIGN to form tetramers appears to be a key feature of its lectin activity [6,7]. Among carbohydrates, DC-SIGN has high affinity for mannose-rich oligosaccharides [8,9]. The envelope of mycobacteria is exceptionally rich in manno-conjugates, including glycolipids, lipoglycans and glycoproteins. Binding of DC-SIGN to *M. tuberculosis* was fully inhibited by ManLAM [mannose-capped LAM (lipoarabinomannan)], an abundant lipoglycan of the mycobacterial envelope, suggesting that ManLAM may constitute a DC-SIGN ligand of the bacillus [5]. Moreover, terminal mannose caps are critical for ManLAM binding to DC-SIGN [10,11], which may explain why DC-SIGN recognizes the slow-growing ManLAM-containing species *M. tuberculosis* [5] and *M. bovis* bacillus Calmette-Guérin ('BCG') [5,12], but not the fast-growing mycobacterial species *M. smegmatis*, *M. fortuitum* and *M. chelonae* [10], in which the LAM is either capped with phosphoinositide motifs (PILAM) or not capped (AraLAM) [13–15]. Altogether, these results led us to suggest that DC-SIGN may discriminate between mycobacteria species through recognition of the mannose caps on the LAM molecules [10].

Unexpectedly, we report here that other slow-growing mycobacteria not belonging to the TB complex, namely *M. marinum*, *M. avium*, *M. kansasii*, *M. xenopi* and *M. gordonae*, bind to

Abbreviations used: ADC, albumin/dextrose/catalase; Apa, alanine/proline-rich antigen (*apa* is its gene); APTS, 1-aminopyrene-3,6,8-trisulphonate; APC, antigen-presenting cell; *aph*, gene coding for aminoglycoside phosphotransferase; CE-LIF, capillary electrophoresis–laser-induced fluorescence; ConA, concanavalin A; DC-SIGN, dendritic-cell-specific intercellular molecule-3-grabbing non-integrin; FCS, fetal-calf serum; LAM, lipoarabinomannan; ManLAM, mannose-capped LAM; AraLAM, non-mannose-capped LAM; PILAM, phosphoinositide-capped LAM; LM, lipomannan; ManAM, mannose-capped arabinomannan; PIM, phosphatidylinositol mannoside; MPI, mannosylphosphatidyl-*myo*-inositol; TB, tuberculosis.

[1] These authors contributed equally to the work.

[2] These authors share the senior authorship and contributed equally to the work.

[3] To whom correspondence should be addressed (email neyrolle@pasteur.fr).

DC-SIGN as poorly as fast-growing species do. The exact structure of the mannose-capping moieties in LAMs from *M. marinum*, *M. kansasii*, *M. xenopi* and *M. avium* as well as the ability of these LAMs to inhibit the binding of *M. tuberculosis* to DC-SIGN were determined. Altogether our results strongly suggest that ManLAM is most likely not the (only) DC-SIGN ligand within the *M. tuberculosis* cell envelope, although ManLAM may be differentially accessible in the cell wall of *M. tuberculosis* complex species as compared with in that of other slow-growing species. We also suggest that additional ligands, possibly including LM (lipomannan) and the mannose-containing 19 kDa and 45 kDa glycoproteins, might participate in *M. tuberculosis* binding to DC-SIGN, which may account for the restricted ability of the lectin to recognize *M. tuberculosis* and close relatives of the TB complex. Altogether, our results uncover an as yet unsuspected complexity in mycobacteria recognition by the innate immune system, and stresses the great care that has to be taken when using purified molecules to identify the ligands responsible for microbial binding to immune receptors.

MATERIALS AND METHODS

Cells, mycobacteria and binding assays

HeLa and HeLa::DC-SIGN cells [16] were cultured in RPMI 1640 (Invitrogen) supplemented with 10 % (v/v) foetal-calf serum (Dutscher, Brumath, France). Mycobacterial strains were cultivated as pellicles in 7H9 medium supplemented with 10 % ADC (albumin/dextrose/catalase; Difco) at the appropriate temperature. Binding and inhibition of binding experiments were performed using previously published procedures [5,10]. In particular, bacteria were passaged 20 times through a needle [26-gauge; 3/8 in (0.95 cm); 0.45 mm × 10 mm; for intradermal injection; BD Biosciences] to minimize clumping. After such treatment, clumping was comparable between species. Species with the highest tendency to form clumps included species of the TB complex, as well as *M. marinum*, *M. xenopi* and *M. kansasii*. Therefore differences in binding to DC-SIGN cannot be attributed to differential clumping between species. A 19-kDa-glycoprotein-null mutant of *M. tuberculosis* H37Rv [17] was cultivated in the presence of 50 μg/ml hygromycin. Two 45 kDa [Apa (alanine/proline-rich antigen)]-null mutants of *M. tuberculosis* H37Rv were generated by classical allelic exchange procedures [18] and were cultivated in the presence of 20 μg/ml kanamycin. Briefly, a pUC18-derived plasmid (pLA34.2) containing a 2 kb DNA fragment encompassing the *apa* gene [19] was digested by KpnI, blunt-ended using T4 DNA polymerase, and dephosphorylated using calf intestinal phosphatase (all enzymes from Invitrogen). The pUC4K plasmid, containing the *aph* (aminoglycoside phosphotransferase) gene encoding resistance to kanamycin, was digested with HincII in order to extract the *aph* gene. The kanamycin cassette was cloned into digested pLA34.2 using T4 DNA ligase, creating an insertion of *aph* into the *apa* gene. Ligated vectors were subsequently digested with EcoRI and BamHI in order to extract the *aph*::*apa* fusion fragment, which was subsequently blunt-ended and dephosphorylated as described above for cloning into a SmaI-digested pXyl4 plasmid. Ligation allows the fusion between *aph*::*apa* and the *xylE* gene for selection of transformants [18]. The resulting plasmid was then digested by BamHI in order to extract the *aph*::*apa*::xylE fragment that was subsequently cloned into BamHI-digested and dephosphorylated pPR27 vector [18]. Using previously described procedures [18], this vector was used to generate *apa*-deficient strains of *M. tuberculosis* H37Rv. Inactivation of the *apa* gene in the selected strains was verified by PCR using an oligonucleotide (Kmout) that is present as an inverted repeat at the 3′ and 5′ ends of the *aph* insertion gene, together with flanking primers [45Fd (45 forward) and 45Rv (45 reverse)] in the *apa* sequence. Kmout: 5′-CCCCCCCCCCCCTGCAG-3′; 45Fd: 5′-ATGCATCAGGTGGACCCCAAC-3′; 45Rv: 5′-GAATCCTCCAACCGGGTTGT-3′. The size and sequence of the amplification products were as expected (results not shown). *M. smegmatis* expressing 19 and 45 kDa glycoproteins were obtained by transforming *M. smegmatis* mc^2155 with pSMT3-19 [20] and pLA34.2 [19] plasmids respectively. Bacteria were grown in medium containing the appropriate antibiotics.

LAM purification and mannose caps and fatty acid analyses

LAM molecules were purified as previously described [21,22]. Briefly, the cells were de-lipidated by several extractions with chloroform/methanol (1:1, v/v). De-lipidated cells were disrupted by sonication. The cellular glycans and lipoglycans were further extracted by refluxing the broken cells in 50 % ethanol at 65 °C. Contaminating proteins and glucans were removed by enzymatic degradation using proteases and α-amylase treatments followed by dialysis. The resulting extract was submitted to hydrophobic-interaction chromatography on an octyl-Sepharose CL-4B column (Amersham Biosciences), allowing the separation of glycans and lipoglycans. The resulting lipoglycans, LAM, LM and PIMs (phosphatidylinositol mannosides), were separated according to their size by gel-permeation chromatography using a Bio-Gel P-100 column (Bio-Rad). Mannose caps were analysed and quantified by CE-LIF (capillary electrophoresis–laser-induced fluorescence) after mild acid hydrolysis of LAM and APTS (1-aminopyrene-3,6,8-trisulphonate) derivatization as previously described [23]. Fatty acids were analysed by GC and GC/MS, as methyl esters derivatives, after alkaline hydrolysis of LAM and methylation using 10 % (w/w) BF_3 in methanol [22].

Biotin hydrazide labelling of purified LAM and LM

A 10 μg portion of LAM or LAM plus LM mixture were dissolved in 30 μl of 0.1 M ammonium acetate buffer, pH 5.5 (buffer A), containing 1 mM sodium metaperiodate (Merck) and incubated at 4 °C in the dark. The reaction was stopped by the addition of 10 μl of 80 mM sodium bisulphite (Sigma) in buffer A. After 5 min incubation at room temperature, 20 μl of buffer A containing 5 mM biotin hydrazide (Sigma) was added. The reaction was stopped by dialysis against water, and biotinylated lipoglycans were dried under vacuum.

Biotin hydrazide labelling of mycobacteria

Surface-exposed oxidizable carbohydrates were labelled with biotin hydrazide after periodate oxidation [24]. Around 3 g (wet weight) of fresh cell cultures was washed twice with PBS and resuspended in 5 ml of 0.1 M sodium acetate buffer, pH 5.5 (buffer B), containing 15 mM sodium metaperiodate (Merck). After a 20 min incubation at 4 °C in the dark with gentle rotation, 5 ml of buffer B containing 30 mM sodium bisulphite (Sigma) was added to stop the reaction. After 5 min incubation, oxidized cells were centrifuged and resuspended in 10 ml of buffer B containing 5 mM of biotin hydrazide (Sigma). After a 2 h incubation at room temperature with gentle rotation, cells were washed three times with PBS. Bacterial viability after treatment was determined by counting colony-forming units on Middlebrook 7H11 (with oleic acid/ADC supplement; Difco) agar plates using mycobacteria grown with shaking in 7H9 medium/ADC/Tween 80, and was shown to be about 98 %.

SDS/PAGE and Western blot

Lipoglycans were purified (see above) from biotin hydrazide-labelled bacteria and untreated bacteria. The fractions containing pure lipoglycans were analysed by SDS/PAGE (8 μg of each fraction), followed by periodic acid/$AgNO_3$ staining [25] or Western blotting on to nitrocellulose. After transfer of lipoglycans for 1 h at 15 V, Western blots were probed with alkaline phosphatase-conjugated avidin (Sigma). Nitrocellulose membrane was blocked for 2 h at room temperature with 4 % BSA, 0.2 % Tween 20 in TBS (Tris-buffered saline). After extensive washing with 1 % BSA and 0.05 % Tween 20 in TBS, the membrane was incubated with alkaline phosphatase-conjugated avidin (1:17000 in TBS) for 2 h at room temperature. After extensive washing, alkaline phosphatase activity was detected by addition of 165 μg/ml 5-bromo-4-chloroindol-3-yl phosphate ('BCIP'; Promega) and 330 μg/ml NitroBlue Tetrazolium ('NBT'; Promega) in 0.1 M Tris/HCl buffer containing 5 mM $MgCl_2$ and 0.1 M NaCl, pH 9.6. Biotinylated BSA (0.1 μg; Sigma) and *in vitro* biotinylated *M. xenopi* LAM (5 μg) and *M. bovis* BCG LAM/LM (5 μg) were used as positive controls.

ELISA

The different lipoglycans (300 ng/50 μl) obtained from biotin hydrazide-labelled bacteria and untreated bacteria in two independent experiments were adsorbed in the wells of microtitre plates (Nunc) by air drying at room temperature. The wells were blocked for 2 h at 37 °C with 5 % (w/v) BSA and 0.2 % Tween 20 in TBS, then extensively rinsed with washing buffer (0.5 % BSA/0.02 % Tween 20 in TBS). A 100 μl portion of monoclonal CS-35 anti-LAM antibody (1:1000 in washing buffer) or rabbit anti-BCG serum (1:500 in washing buffer) or alkaline-phosphatase-conjugated avidin (1:1000 in washing buffer) was added for 2 h at 37 °C. After extensive washing, 100 μl of alkaline-phosphatase-conjugated polyvalent anti-mouse IgGs (Sigma; 1:3000 in washing buffer) was added in wells containing anti-LAM antibody, and 100 μl of alkaline-phosphatase-conjugated mouse monoclonal anti-rabbit IgGs (clone RG16; Sigma; 1:5000 in washing buffer) in those wells containing anti-BCG serum. After incubation at 37 °C for 2 h and extensive washing, alkaline phosphatase activity was detected by addition of 100 μl of 1 mg/ml *p*-nitrophenyl phosphate in 0.1 M Tris/HCl buffer, containing 5 mM $MgCl_2$/0.1 M NaCl, pH 9.6.

Flow cytometry

Bacteria were washed twice in PBS containing 2 % (v/v) FCS (fetal-calf serum), resuspended in the same buffer containing CS-35 anti-LAM mouse monoclonal antibody (1:10) and incubated for 1 h at 4 °C. Bacteria were then washed three times in PBS/FCS, resuspended in the same buffer containing fluorescent-dye-Cy3-conjugated goat anti-mouse antibodies (Amersham; 1:100), and incubated for 1 h at 4 °C. After three washes in PBS/FCS, bacteria were analysed for red fluorescence by flow cytometry. A total of 10000 bacteria were acquired per sample.

RESULTS

Mycobacteria binding to DC-SIGN

Previous studies have demonstrated that DC-SIGN recognizes slow-growing *M. tuberculosis* as well as its close relative, the vaccine strain *M. bovis* BCG, but not the non-pathogenic fast growing species *M. chelonae*, *M. fortuitum* and *M. smegmatis* [5,10,12]. These results arose from binding experiments performed at 4 °C using recombinant HeLa cells expressing or not expressing the lectin. In the present study we used the same experimental assay to evaluate whether other non-TB slow-growing mycobacterial species (Figure 1A) could bind to DC-SIGN. In accordance with our previous results, fast-growing mycobacterial species (*M. chelonae*, *M. smegmatis* and *M. fortuitum*) bound poorly to DC-SIGN-expressing HeLa cells as compared with non-transfected cells (Figure 1B). By contrast, species of the TB complex (*M. bovis* BCG and clinical isolates of *M. tuberculosis*, *M. bovis*, and *M. africanum*) bound to DC-SIGN-expressing HeLa cells up to 11-fold more than to non-recombinant HeLa cells (Figure 1B). Surprisingly all the other slow-growing non-TB complex species we tested (clinical isolates of *M. marinum*, *M. kansasii*, *M. avium*, *M. xenopi* and *M. gordonae*) bound to DC-SIGN as poorly as the fast-growers did (Figure 1B). Similar results were obtained using laboratory strains of mycobacteria (results not shown). These results suggested that: (i) the structure of the ManLAM molecules, especially the presence and number of terminal mannose caps, could differ among slow-growers, resulting in a lower affinity for DC-SIGN, and/or that (ii) ManLAMs could be differentially exposed on the surface of the various species, and/or that (iii) additional TB complex-specific ligands could be the natural DC-SIGN ligands within the *M. tuberculosis* envelope. The rest of the present study aims at assessing these non-mutually exclusive hypotheses.

LAM structure and ability to bind to DC-SIGN

It has previously been demonstrated that both the acyl chains and the mannose caps played a crucial role in DC-SIGN/ManLAM interaction *in vitro* [10]. The results above may suggest that LAM molecules are differentially capped among slow-growing species. LAM molecules were purified from *M. tuberculosis* H37Rv, *M. bovis* BCG, and the clinical isolates of *M. avium*, *M. marinum*, *M. xenopi* and *M. kansasii* that were used in the binding assay above. All the four last-named species contained LAM with a similar SDS/PAGE migration pattern to that of *M. tuberculosis* H37Rv or *M. bovis* BCG ManLAMs (results not shown). They were first analysed for their mannose-cap content. All the LAMs were ManLAMs, since they contained the typical manno-oligosaccharide units found in *M. tuberculosis* or *M. bovis* BCG ManLAMs, i.e. mono-, ($\alpha 1 \rightarrow 2$)-di- and ($\alpha 1 \rightarrow 2$)-tri-mannosyl units [25–27] (Figure 2). All the ManLAMs possessed a total number of caps that was at least as high as that found for *M. tuberculosis* ManLAM (Figure 2). So, even if the number of caps may vary from one batch of LAM to another, it was clear that none of the investigated mycobacteria had a LAM significantly less capped than that of *M. tuberculosis*. Dimannoside units were the most abundant motifs in *M. tuberculosis* and *M. bovis* BCG ManLAMs, as previously reported [23], but also in *M. avium* and *M. xenopi* ManLAMs. Trimannoside units were always the less abundant motifs, but, surprisingly, ManLAMs from *M. kansasii* and *M. marinum* had the monomannoside motif as abundant as, or more abundant in the latter case, than the dimannoside one (Figure 2). We then analysed the fatty acid contents of the various ManLAMs after alkaline hydrolysis. They all showed a similar acylation pattern, i.e. containing palmitic and tuberculostearic acids as the main fatty acids, with stearic and octadecenoic acids being present in smaller amounts, except in *M. marinum* ManLAM, which contained these latter fatty acids in higher amounts than tuberculostearic acid (results not shown). Altogether, the structural analysis of the caps and MPI (mannosylphosphatidyl-*myo*-inositol) domains of ManLAMs from *M. avium*, *M. marinum*, *M. xenopi* and *M. kansasii* did not reveal any dramatic structural difference that would predict inability of these molecules to bind to DC-SIGN.

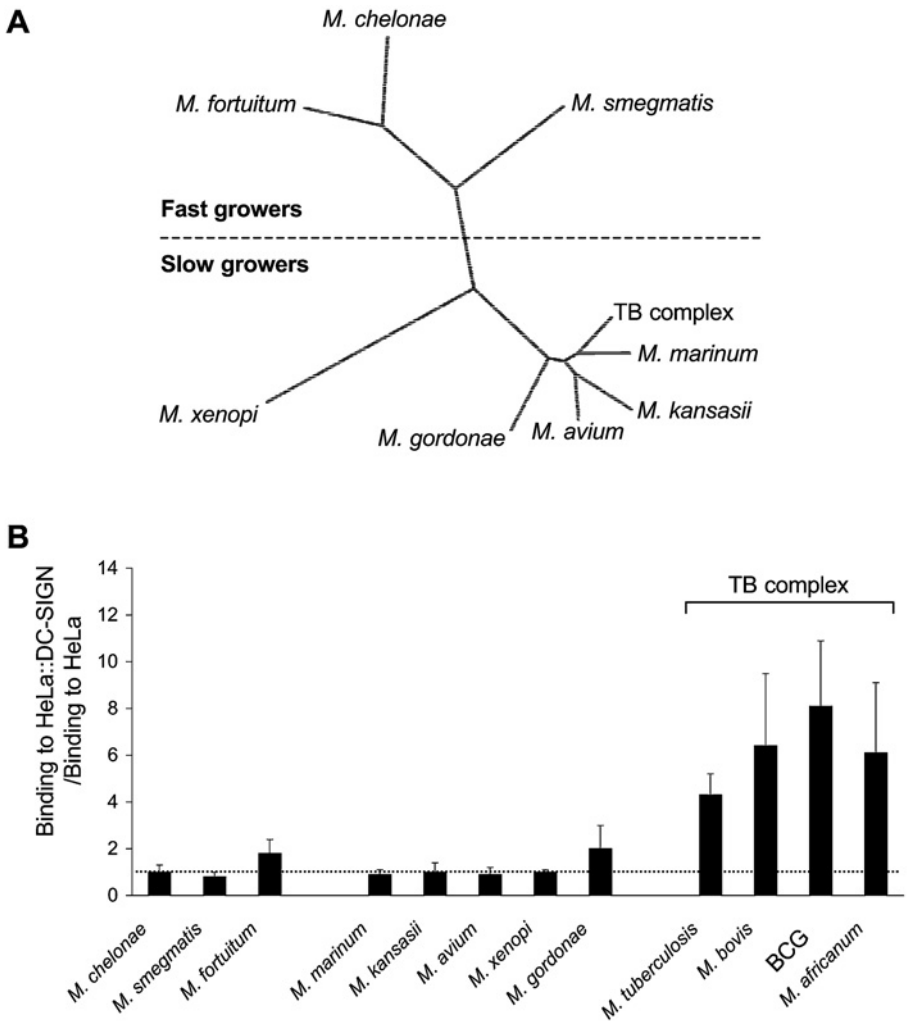

Figure 1 DC-SIGN has high affinity for species of the *M. tuberculosis* complex

(**A**) Phylogenetic tree of the mycobacteria species used in the present study, based on partial 16 S RNA sequences. (**B**) Epithelial HeLa-derived cells expressing or not DC-SIGN (HeLa::DC-SIGN and HeLa respectively) were infected with fast-growing (*M. chelonae*, *M. smegmatis*, *M. fortuitum*), slow-growing non-TB (*M. marinum*, *M. kansasii*, *M. avium*, *M. xenopi*, *M. gordonae*) or TB complex (*M. tuberculosis* H37Rv, *M. bovis*, *M. bovis* BCG, and *M. africanum*) species at a multiplicity of infection of 1 bacterium/cell. Bacterial binding was evaluated after 4 h at 4 °C by counting colony-forming units. Results are means (±S.D.) of the ratio between binding to HeLa::DC-SIGN and binding to HeLa for three to five independent experiments. A ratio of 1 (dotted line) indicates no binding to the lectin.

We nevertheless investigated the potency of the different purified ManLAMs, as compared with *M. tuberculosis* ManLAM, to inhibit *M. tuberculosis* binding to DC-SIGN. All the ManLAMs, when used in a concentration range of 1–10 μg/ml, inhibited *M. tuberculosis* binding to DC-SIGN to comparable extents (Figure 3). Similar results were obtained with ManLAM purified from laboratory strains of mycobacteria (results not shown). In conclusion, the inability of the non-TB slow-growers to bind to DC-SIGN was not a result of the presence of LAM molecules with lower affinity for the receptor within their envelope.

LAM accessibility to the mycobacterial cell surface

Since the structure of LAMs and their affinity for the receptor do not account for the differential ability of the various non-TB slow-growers to bind to DC-SIGN, we next sought to determine whether differential exposition of the lipoglycans on the mycobacterial surface could explain this phenomenon. To investigate the presence of ManLAM on the mycobacterial cell surface, surface-exposed carbohydrates were labelled with biotin hydrazide after periodate oxidation of the cells [24,28], and subsequently purified ManLAMs were assessed for biotin tagging. The experiments were first carried out and tested on *M. bovis* BCG, a species that binds to DC-SIGN. Biotin labelling has been widely used to identify cell-surface proteins or glycoconjugates [29]. However, in order to ensure that biotin labelling was indeed restricted to surface components of *M. bovis* BCG, labelled cells were examined for their cell-wall integrity. As previously reported [24], the bacteria retained around 98 % viability after labelling, indicating that this treatment does not significantly affect cell integrity (results not shown). Scanning-electron-microscopic examination showed that the morphology of labelled bacteria was not dramatically altered when compared with that of unlabelled control bacteria (results not shown). Moreover, arabinogalactan, a polysaccharide known to be imbedded in the mycobacterial cell wall, was not tagged with biotin, in contrast with AM (arabinomannan), a polysaccharide known to be exposed on the cell surface ([30]; results not shown). Altogether these data suggested

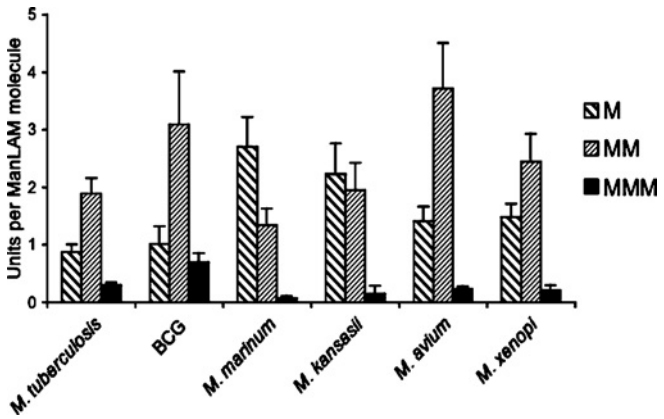

Figure 2 ManLAMs of *M. tuberculosis* H37Rv, *M. bovis* BCG, *M. avium*, *M. xenopi*, *M. marinum* and *M. kansasii* have similar mannose caps contents

Mannose caps were analysed and quantified by CE–LIF after mild acid hydrolysis of LAM and APTS derivatization as previously described [23]. M, α-D-Man*p*; MM, α-D-Man*p*-(1 → 2)-α-D-Man*p*; MMM, α-D-Man*p*-(1 → 2)-α-D-Man*p*-(1 → 2)-α-D-Man*p*.

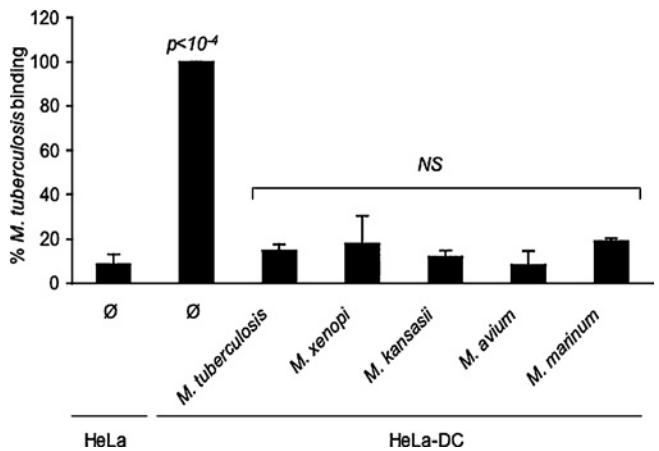

Figure 3 ManLAMs of non-TB slow-growing mycobacteria can inhibit *M. tuberculosis* binding to DC-SIGN

HeLa or HeLa::DC-SIGN cells were preincubated with 10 μg/ml ManLAM from *M. tuberculosis*, *M. xenopi*, *M. kansasii*, *M. avium* or *M. marinum*, or saline (Ø) for 1 h at 4 °C and infected with *M. tuberculosis* H37Rv as described in Figure 1. Bacteria binding was measured as described in Figure 1. Data are expressed as percentages of binding relative to control values (100 %, preincubation of HeLa::DC-SIGN cells with saline), and the means (± S.D.) for three independent experiments are shown. *P* values are given as assessed by Student's *t* test comparison with HeLa cells. *NS*, not significant.

that biotin labelling was restricted to the *M. bovis* BCG cell surface. This approach was thus applied for the comparative analysis of ManLAM accessibility to cell-surface labelling between bacteria with a different ability to bind to DC-SIGN. *M. bovis* BCG, a bacterium able to bind to DC-SIGN, and *M. xenopi*, a bacterium unable to bind to DC-SIGN, were labelled with biotin. Labelled and control bacteria were subsequently submitted to ManLAM purification as previously described. The lipoglycan fractions were analysed by SDS/PAGE, followed by $AgNO_3$ staining (Figure 4A) and contained only ManLAM and LM. Western blotting using alkaline-phosphatase-conjugated streptavidin revealed two bands in both lipoglycan fractions from biotin-labelled *M. bovis* BCG and *M. xenopi* (Figure 4B, lanes 3 and 6 respectively). These bands unambiguously corresponded to ManLAM and LM, since they had the same migration pattern

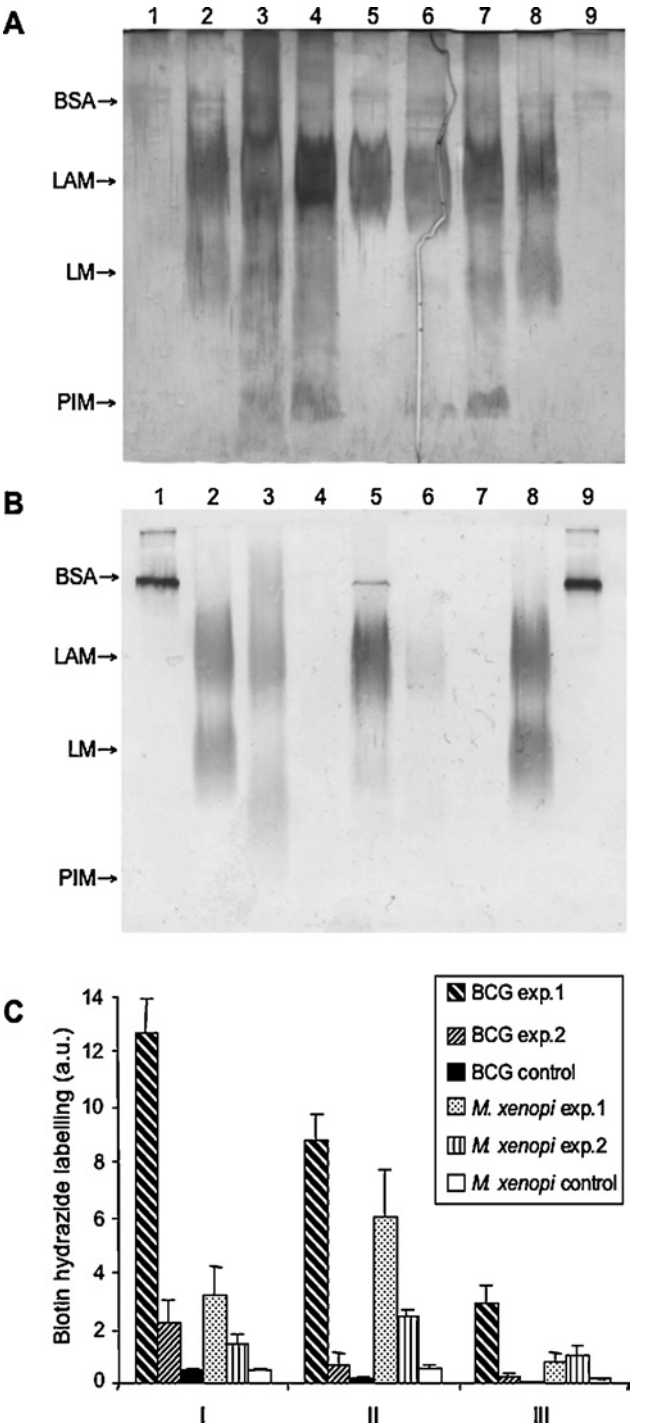

Figure 4 Analyses of lipoglycans from control and biotin hydrazide-labelled *M. bovis* BCG and *M. xenopi*

(**A**) Periodic acid/$AgNO_3$ staining of SDS/PAGE gel. (**B**) Western blot probed with alkaline phosphatase-conjugated avidin. Lanes 1 and 9, biotinylated BSA standard (0.1 μg); lanes 2 and 8, *in vitro* biotinylated *M. bovis* BCG LAM and LM standards (5 μg); lanes 3 and 4, lipoglycans from biotin hydrazide-labelled and control *M. bovis* BCG respectively (8 μg); lane 5, *in vitro* biotinylated *M. xenopi* LAM standard (5 μg); lanes 6 and 7, lipoglycans from biotin hydrazide-labelled and control *M. xenopi* respectively (8 μg). (**C**) Quantification of biotin hydrazide labelling by ELISA. Two independent experiments of labelling were performed on both *M. bovis* BCG and *M. xenopi*. Portions (300 ng) of lipoglycans from labelled and control bacteria were adsorbed on the microtitre wells and probed with alkaline phosphatase-conjugated avidin. The absorbance values are given without correction (I) or corrected by the response to the monoclonal CS-35 anti-LAM antibody (II) or by the response to a rabbit anti-BCG serum (III). Abbreviation: a.u., absorbance units.

as that of ManLAM and LM, as revealed by silver staining (Figure 4A), and also as that of ManLAM and LM standards labelled with biotin hydrazide (Figure 4B, lanes 2, 5 and 8). As expected, ManLAM and LM from control cells were not revealed (Figure 4B, lanes 4 and 7). These results showed that ManLAM and LM from both *M. bovis* BCG and *M. xenopi* are accessible to biotin-hydrazide labelling and are thus likely to be exposed on the cell surface of both mycobacteria species. In order to check whether one species could express more lipoglycans on the surface than the other, both mycobacteria were labelled in two independent experiments and labelling was quantified by ELISA. Indeed the comparison of relative intensities of labelling was hardly able to be evaluated on the glycoblots. The results are summarized in Figure 4(C). Each lipoglycan fraction arising from the different experiments for both bacteria was adsorbed on the ELISA plate at the same concentration. However, in order to take into account differences of binding between biotinylated and native ManLAM or between ManLAM from *M. xenopi* and *M. bovis* BCG, the intensity of labelling determined by the activity of alkaline-phosphatase-conjugated avidin was normalized according to three different parameters: (i) the concentration of lipoglycans loaded in the microtitre wells (uncorrected value), (ii) the response to the monoclonal CS-35 anti-LAM antibody and (iii) the response to a rabbit anti-BCG serum. Whatever the type of normalization used, the hierarchy between the signals obtained with the different lipoglycan fractions remained the same (Figure 4C). However, the intensity of labelling from one experiment to another for the same species was dramatically different. The data obtained did not allow one to conclude that one species, most particularly *M. xenopi*, had lipoglycans less exposed than the other. Accordingly we next compared the exposure of LAM epitopes at the cell surface of *M. bovis* BCG and *M. xenopi* by flow-cytometric analysis using the monoclonal CS-35 anti-LAM antibody. The fluorescence intensity of *M. bovis* BCG cells (Figure 5A) was found to be, on average, about 2–3-fold higher than that of *M. xenopi* cells (Figure 5B). These data suggest that the epitopes recognized by the antibody, i.e. the hexa-arabinofuranosyl motifs of the arabinan domain [31], are significantly less exposed at the surface of *M. xenopi* cells. Since this epitope is shared by ManLAM and its related glycan ManAM, one can conclude that these two mannoconjugates are globally less expressed at the surface of *M. xenopi* than they are on the surface of *M. bovis* BCG. However, it seems unlikely that this 2–3-fold decrease in ManLAM density at the surface of *M. xenopi* can alone explain the inability of this species to bind to DC-SIGN.

Other possible non-ManLAM DC-SIGN ligands

Altogether, these data would suggest that ManLAMs are not the crucial or the only mycobacterial ligands responsible for the different abilities of slow-growing mycobacteria to bind to DC-SIGN. We thus wished to evaluate whether other mycobacterial compounds, different from ManLAM, could bind to DC-SIGN in solution and might constitute additional ligand candidates for the lectin. The mycobacterial envelope is exceptionnally rich in mannosylated molecules, including (lipo)glycans and (lipo)glycoproteins [30]. Among (lipo)glycans, when tested at 10 μg/ml, we found that LM was able to inhibit *M. tuberculosis* binding to DC-SIGN-expressing HeLa cells as efficiently as ManLAM did, whereas ManAM was also inhibitory, but to a lesser extent (Figure 6A). Among glycoproteins, similar results were obtained using purified 19 and 45 kDa antigens, the latter being less efficient (Figure 6A). Altogether these results indicate that these components bind to the lectin in solution and thus are possible

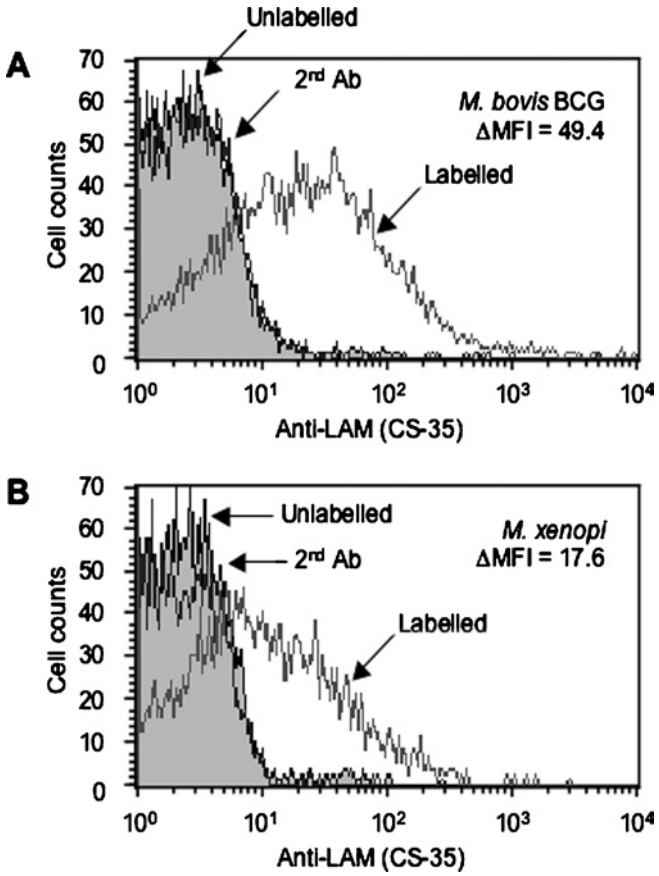

Figure 5 Flow-cytometric analysis of *M. bovis* BCG and *M. xenopi* labelling with the monoclonal CS-35 anti-LAM antibody

M. bovis BCG (**A**) or *M. xenopi* (**B**) were incubated with anti-LAM monoclonal antibody CS-35, which was revealed by fluorophore-Cy3-conjugated anti-mouse IgG antibody and analysed by flow cytometry ('Labelled'). Analysis was carried on 10000 bacteria. Controls consisted of unlabelled bacteria ('Unlabelled') and bacteria incubated with secondary antibody only ('2nd Ab'). ΔMFI, mean fluorescence intensity variation between CS-35-labelled bacteria and bacteria incubated with secondary antibody only. The Figure is representative of two independent experiments.

ligands of *M. tuberculosis*. Since 45- and 19-kDa-deficient mutants of *M. tuberculosis* are available [17], we evaluated the ability of these strains, as compared with their wild-type counterparts, to bind to DC-SIGN. Binding of both mutants did not differ significantly from that of the wild-type strains (Figure 6B). In an attempt to check whether 19 and 45 kDa antigens may be recognized by DC-SIGN in the context of a mycobacterial cell envelope, we evaluated the ability of recombinant 19- or 45-kDa-antigen-expressing *M. smegmatis* (a species that does not naturally express these antigens) to bind to DC-SIGN-expressing HeLa cells. Recombinant strains did not bind to the cells more efficiently that the wild-type strain (Figure 6C). Altogether, these results indicate that purified ManAM, LM, and 19 and 45 kDa molecules constitute additional DC-SIGN ligand candidates within the *M. tuberculosis* cell envelope, and that 19 or 45 kDa proteins alone cannot account for mycobacterial binding to the lectin.

DISCUSSION

The results we present here reveal that the molecular bases of DC-SIGN binding to *M. tuberculosis* and close relatives of the TB complex are more complicated than previously thought.

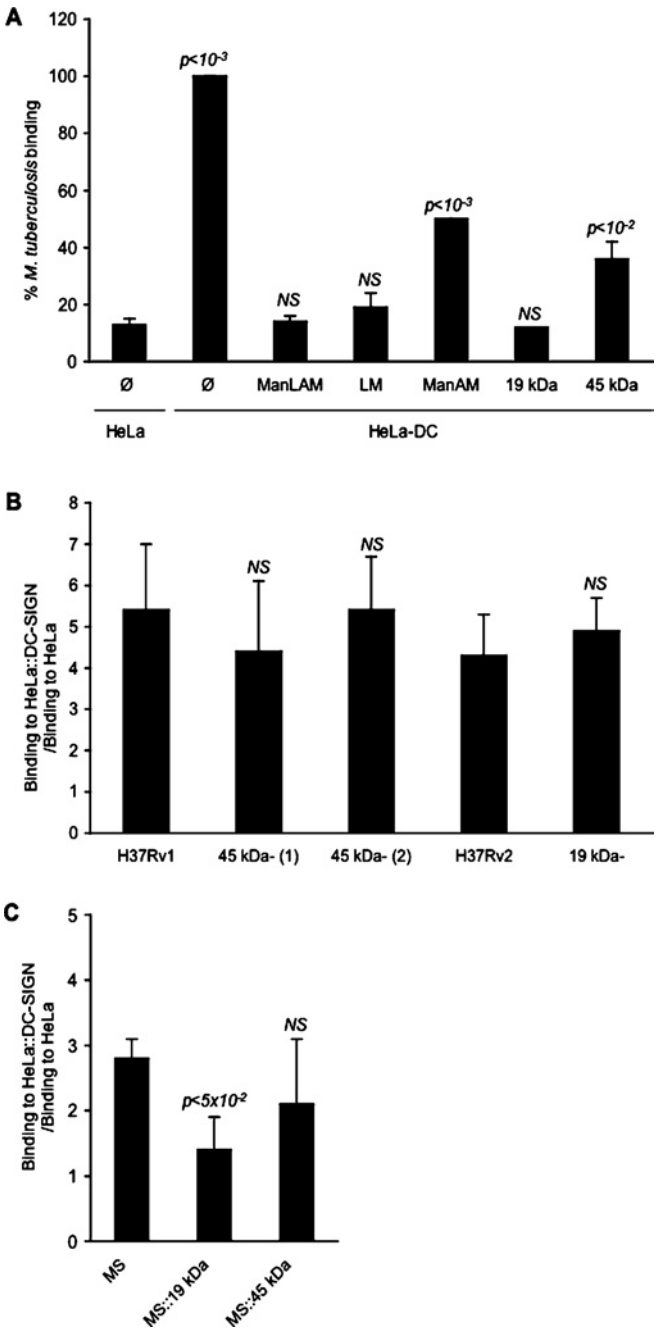

Figure 6 Other non-ManLAM *M. tuberculosis*-derived ligands can inhibit *M. tuberculosis* binding to DC-SIGN

(**A**) HeLa or HeLa::DC-SIGN cells were preincubated with 10 μg/ml *M. tuberculosis*-derived ManLAM, LM, ManAM, 19 kDa antigen, 45 kDa antigen or saline (Ø) for 1 h at 4 °C and infected with *M. tuberculosis* H37Rv as described in Figure 1. Bacteria binding was measured as described in Figure 1. Data are expressed as described in Figure 3 and represent the means (+S.D.) for three independent experiments. *P* values are given as assessed by a Student's *t* test comparison with HeLa cells. (**B**) HeLa::DC-SIGN and HeLa cells were infected with 45 kDa-protein-null (two strains) or 19 kDa-protein-null (one strain) mutants of *M. tuberculosis*, or with their wild-type counterparts (H37Rv1 and H37Rv2). Bacterial binding was evaluated as described in Figure 1. Data are expressed as described in Figure 1 and represent the means (+S.D.) for three independent experiments. *P* values are given as assessed by a Student's *t* test comparison with wild-type strains. *NS*, not significant. (**C**) HeLa::DC-SIGN and HeLa cells were infected with 45 kDa- or 19 kDa-protein-expressing recombinant *M. smegmatis* (MS) or the wild-type strain. Bacterial binding was evaluated as described in Figure 1. Data are expressed as described in Figure 1 and represent the means (+S.D.) for three independent experiments. *P* values are given as assessed by a Student's *t* test comparison with the wild-type strain. *NS*, not significant.

ManLAM was initially proposed as a possible DC-SIGN ligand within the *M. tuberculosis* cell envelope because: (i) it could bind to DC-SIGN-Fc chimeras in solution [12], (ii) it could inhibit *M. tuberculosis* and *M. bovis* BCG binding to DC-SIGN-expressing recombinant cells [5], (iii) removal of the mannose caps on ManLAM abrogated the inhibitory effect of the molecule [10], (iv) synthetic mannose caps can bind to DC-SIGN–Fc chimaeras in solution [11], and (v) fast growers, such as *M. smegmatis*, hardly bound to the lectin, and PILAM isolated from this species could not inhibit *M. tuberculosis* binding to DC-SIGN [5,10,12]. Altogether, these results led us to propose that DC-SIGN may discriminate between saprophytic fast-growing and pathogenic or potentially pathogenic slow-growing *M.* species through the selective recognition of mannose caps on LAMs [10,32,33]. Nevertheless, to formally demonstrate that ManLAM indeed constitutes a DC-SIGN ligand within the *M. tuberculosis* envelope, LAM-deficient strains would be required. Unfortunately, such strains are not yet available, since mycobacterial lipoglycan biosynthesis pathways remain largely unknown and since the arabinan domain of LAM seems to be essential in *M. tuberculosis* (G. Stadthagen and M. Jackson, unpublished work).

The results reported here strongly challenge our first hypothesis, as other slow-growing species out of the TB complex, namely *M. marinum*, *M. kansasii*, *M. avium*, *M. xenopi* and *M. gordonae* could not bind to DC-SIGN-expressing recombinant HeLa cells, although fine analyses revealed that they contained ManLAM in similar amounts and with chemical structures similar to those of ManLAM from *M. tuberculosis*. We particularly focused our structural analyses on the molecule moieties previously shown to be required for ManLAM binding to the receptor, i.e. the MPI anchor and the mannose caps [10,32,33]. All the LAMs investigated showed an acylation pattern similar to that observed in *M. bovis* BCG or *M. tuberculosis* ManLAMs, and possessed a total number of caps, [mono-, ($\alpha 1 \rightarrow 2$)-di- and ($\alpha 1 \rightarrow 2$)-tri-mannosyl units] that was at least as high as the one found in *M. tuberculosis* ManLAM (Figure 2). Dimannoside units were the most abundant motifs in *M. tuberculosis* and *M. bovis* BCG ManLAMs, as previously reported [23,26], but this was the case for *M. avium* and *M. xenopi* ManLAMs. However, surprisingly, ManLAMs from the clinical isolates of *M. kansasii* and *M. marinum* had the monomannoside motif as abundant as, or more abundant than, in the latter case, the dimannoside one, a structural feature only observed so far in ManLAM from an ethambutol-resistant *M. avium* isolate [34]. The case of *M. marinum* is of particular interest in this aspect, because this species is thought to be the one phylogenetically closest to the TB complex (Figure 1A). The crystal structure of the carbohydrate-recognition domain of DC-SIGN bound to an oligosaccharide showed that the interactions with the DC-SIGN Ca^{2+} site are unusual because they involve an internal, rather than a terminal, sugar [8]. Thus the length of the mannose cap chains might be a key parameter determining ManLAM affinity for DC-SIGN. The presence of the monomannoside units as the most abundant motifs on *M. marinum* and *M. kansasii* ManLAMs could thus possibly result in a weaker affinity of these ManLAMs for DC-SIGN as compared with that of *M. tuberculosis*, which have dimannoside units as the main capping motifs. However, such an explanation would not be valid for *M. avium* and *M. xenopi* ManLAMs. Finally we found that these slight structural differences among the different ManLAMs had no effect on their ability to bind to DC-SIGN. Indeed, as an additional proof of the high similarity among ManLAMs from the various slow growers studied here, we have shown that all molecules could inhibit *M. tuberculosis* binding to DC-SIGN-expressing cells as efficiently as *M. tuberculosis*-derived ManLAM did.

In order to tentatively explain the selective ability of DC-SIGN to recognize species of the TB complex, we have raised several non-mutually exclusive hypotheses. We first proposed that ManLAMs from non-TB slow growing mycobacteria might have an altered structure precluding a high affinity for the receptor. This explanation can now be excluded. We also hypothesized that ManLAMs may be differentially exposed and thus differentially accessible within the cell envelopes of the different species. Using *M. bovis* BCG and *M. xenopi* as archetypes of slow-growing mycobacteria able and unable respectively to bind to DC-SIGN, we investigated this possibility by two complementary approaches: (i) by comparison of ManLAM tagging after labelling of the mycobacterial cell surface with biotin hydrazide following periodate oxidation of the cells, and (ii) by comparison of cell-surface labelling by the monoclonal CS-35 anti-LAM antibody. The first approach indicated that both mycobacterial species expressed ManLAM and LM at their cell surface. However, the data obtained did not allow one to conclude that *M. xenopi* had less ManLAM exposed than *M. bovis* BCG. The second approach demonstrated that the hexa-arabinofuranosyl motifs of the arabinan domain shared by both ManLAM and ManAM are significantly less exposed at the surface of *M. xenopi* cells than at that of *M. bovis* BCG. However, it seems unlikely that the 2–3-fold decrease in ManLAM plus ManAM density at the surface of *M. xenopi* we observed can alone explain the complete inability of the bacterium to bind to DC-SIGN.

We finally investigated whether additional ligands may account for the ability of DC-SIGN to recognize species of the TB complex. We identified such possible ligands, namely LM, ManAM, and the 19 and 45 kDa glycoproteins. However, LM and AM are ubiquitously found, and in comparable amounts, in mycobacteria and, to our present knowledge, these molecules share, for a given species, the structure of the corresponding parts of LAM, i.e. the lipomannan core and the polysaccharidic moiety respectively [35–37]. Thus variations in the structure of these molecules or in their amount are not likely to explain the inability of the corresponding species to bind to DC-SIGN. In contrast, 19 and 45 kDa glycoproteins are of particular interest. The 19 kDa antigen from *M. tuberculosis* has been proposed to be mannosylated on the basis of ConA (concanavalin A) binding experiments [38]. Binding of the protein to ConA has been shown to be mediated by oligosaccharides that are O-linked to a cluster of threonine residues (S^{6}TTGSGETTTAAGTTASP^{23} in the mature lipoprotein) in the N-terminal part of the polypeptide, near the acylated cysteine residue. *In silico* examination of the complete genomes from other strains of the TB complex, namely *M. bovis* and *M. microti*, revealed that this threonine cluster is fully conserved across species. However, the cluster is not conserved, but replaced by a serine cluster in 19 kDa homologues in *M. avium* (SAPSSSASSSSTSASA) and *M. marinum* (STTSGGESSSTGSTSAST). Interestingly the serine cluster from the *M. avium* 19 kDa homologue cannot bind to ConA *in vitro*, suggesting that the species lacks serine glycosyltransferases and thus that 19 kDa homologue is not O-glycosylated in *M. avium* [39]. The situation is still unknown in *M. marinum*. If confirmed, such a differential glycosylation pattern of the 19 kDa antigen among species may account, at least in part, for the selective recognition of the TB complex by DC-SIGN. However, the 19 kDa is likely not the only DC-SIGN ligand within the *M. tuberculosis* cell wall, as a 19 kDa-null mutant of *M. tuberculosis* could still bind to the lectin. Similarly the Thr^{10}, Thr^{18}, Thr^{27} and Thr^{277} residues that are O-glycosylated in the 45 kDa (Apa) antigen [40,41] are not conserved in the *M. avium* and *M. marinum* Apa homologues, which again may account for the selective recognition of the TB complex by DC-SIGN. However, as for the 19 kDa antigen, two 45 kDa-null mutants of *M. tuberculosis* could bind to DC-SIGN as well as their wild-type counterpart did, indicating that Apa expression alone is not sufficient to explain *M. tuberculosis* binding to the lectin. This hypothesis is strengthened by the fact that 19- or 45-kDa-protein-expressing recombinant *M. smegmatis* did not bind to DC-SIGN-expressing HeLa cells more efficiently than their wild-type counterparts. However, it is noteworthy that the cell envelope context in *M. smegmatis* is dramatically different from that in *M. tuberculosis*. The construction of multiple knock-outs might be useful in the future in investigating the co-operation of several ligands in the binding of *M. tuberculosis* to DC-SIGN.

Altogether, our results seriously challenge current views on mycobacteria recognition by DC-SIGN, as they strongly suggest that DC-SIGN binding to *M. tuberculosis* and relatives of the TB complex does not rely on the binding of ManLAM only, but rather depends on the recognition of other ligands, possibly including lipoglycans, glycans and glycoproteins, and eventually on their differential accessibility within the mycobacterial cell envelope. Although our present results raise more queries than they provide answers, they likely uncover the genuine complexity of DC-SIGN–mycobacteria interactions. Moreover, they also stress the great care that has to be taken when making conclusions about ligand specificity from experiments using purified molecules. This is particularly true with *M. tuberculosis*, which has an envelope exceptionally rich in glycoconjugates and, most particularly, mannoconjugates. However, it can be extended to other microbes, in the context of either DC-SIGN or other receptors. Indeed, the demonstration that a purified molecule is able to (i) inhibit the binding of a bacterium to a receptor or (ii) bind to the receptor itself, is not necessarily predictive of the role of this compound in the binding of the whole bacterium. Moreover, one cannot exclude the possibility that purified molecules added in competition assays may mask DC-SIGN epitopes on the surface of the bacterial envelope. In addition, HeLa cells have been recently reported to express low amounts of mannose-receptor mRNA, which, if leading to expression of the mannose-receptor polypeptide and binding of mannosylated molecules, may interfere with DC-SIGN expression and/or conformation in the transfected HeLa cells we used in our experiments [42]. To overcome such an eventual bias, soluble molecules and genetically modified mycobacteria will be assessed using purified recombinant DC-SIGN, knowing that all the results obtained will have to be finally confirmed with monocyte-derived dendritic cells.

Finally, the apparent restriction of mycobacteria recognition by DC-SIGN to the *M. tuberculosis* complex is of interest on immunological and evolutionary perspectives. Indeed, it is tempting to suggest that pathogenic mycobacteria have adapted to exploit DC-SIGN to suppress host immunity [12,32,43,44], since it has been shown that binding to DC-SIGN results in interleukin-10 production by activated human dendritic cells *in vitro* [12]. However, this may well be in favour of the host, for instance to limit inflammation-induced immunopathology [32,33]. Besides the question of the nature of the DC-SIGN ligand(s) on the whole bacterium, the ability of soluble molecules to bind DC-SIGN and other receptors is still of interest, as it has been reported that mycobacterial compounds, including ManLAM, are delivered from infected macrophages to non-infected bystander dendritic cells [24,28,45,46]. As such, these molecules may stimulate bystander cells through DC-SIGN, even though they are not ligands of the receptor within the whole bacterium, and this may induce a variety of signalling events that have yet to be understood. Future *in vitro* and *in vivo* studies should help to provide a better understanding of the functional consequences of the interaction of mycobacteria, as well as soluble secreted mycobacterial compounds, with DC-SIGN and other innate immune receptors.

We thank Colorado State University (NIH contract NIAID No1-AI-40091) for generously providing *M. tuberculosis*-purified LAM, 19 and 45 kDa antigens and CS-35 monoclonal anti-LAM antibody. This research project was co-financed by the European Commission, within the Sixth Framework Programme (contract no. LSHP-CT-2003-503367). The text represents the authors' views and does not necessarily represent a position of the Commission, who will not be liable for the use made of such information. S.P. and J.N. gratefully acknowledge Dr Patricia Constant (Institut de Pharmacologie et de Biologie Structurale) for skilful help with cell culturing.

REFERENCES

1 Kaufmann, S. H. (2001) How can immunology contribute to the control of tuberculosis? Nat. Rev. Immunol. **1**, 20–30
2 Flynn, J. L. and Chan, J. (2001) Immunology of tuberculosis. Annu. Rev. Immunol. **9**, 93–129
3 Curtis, B. M., Scharnowske, S. and Watson, A. J. (1992) Sequence and expression of a membrane-associated C-type lectin that exhibits CD4-independent binding of human immunodeficiency virus envelope glycoprotein gp120. Proc. Natl. Acad. Sci. U.S.A. **89**, 8356–8360
4 Geijtenbeek, T. B., Torensma, R., van Vliet, S. J., van Duijnhoven, G. C., Adema, G. J., van Kooyk, Y. and 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
5 Tailleux, L., Schwartz, O., Herrmann, J., Pivert, E., Jackson, M., Amara, A., Legres, L., Dreher, D., Nicod, L., Gluckman, J. et al. (2003) DC-SIGN is the major *Mycobacterium tuberculosis* receptor on human dendritic cells. J. Exp. Med. **197**, 121–127
6 Mitchell, D. A., Fadden, A. J. and Drickamer, K. (2001) A novel mechanism of carbohydrate recognition by the C-type lectins DC-SIGN and DC-SIGNR. Subunit organization and binding to multivalent ligands. J. Biol. Chem. **276**, 28939–28945
7 Feinberg, H., Guo, Y., Mitchell, D. A., Drickamer, K. and Weis, W. I. (2005) Extended neck regions stabilize tetramers of the receptors DC-SIGN and DC-SIGNR. J. Biol. Chem. **280**, 1327–1335
8 Feinberg, H., Mitchell, D. A., Drickamer, K. and Weis, W. I. (2001) Structural basis for selective recognition of oligosaccharides by DC-SIGN and DC-SIGNR. Science **294**, 2163–2166
9 Guo, Y., Feinberg, H., Conroy, E., Mitchell, D. A., Alvarez, R., Blixt, O., Taylor, M. E., Weis, W. I. and Drickamer, K. (2004) Structural basis for distinct ligand-binding and targeting properties of the receptors DC-SIGN and DC-SIGNR. Nat. Struct. Mol. Biol. **11**, 591–598
10 Maeda, N., Nigou, J., Herrmann, J. L., Jackson, M., Amara, A., Lagrange, P. H., Puzo, G., Gicquel, B. and Neyrolles, O. (2002) The cell surface receptor DC-SIGN discriminates between *Mycobacterium* species through selective recognition of the mannose caps on lipoarabinomannan. J. Biol. Chem. **278**, 5513–5516
11 Koppel, E. A., Ludwig, I. S., Hernandez, M. S., Lowary, T. L., Gadikota, R. R., Tuzikov, A. B., Vandenbroucke-Grauls, C. M., van Kooyk, Y., Appelmelk, B. J. and Geijtenbeek, T. B. (2004) Identification of the mycobacterial carbohydrate structure that binds the C-type lectins DC-SIGN, L-SIGN and SIGNR1. Immunobiology **209**, 117–127
12 Geijtenbeek, T., van Vliet, S., Koppel, E., Sanchez-Hernandez, M., Vandenbroucke-Grauls, C., Appelmelk, B. and van Kooyk, Y. (2003) Mycobacteria target DC-SIGN to suppress dendritic cell function. J. Exp. Med. **197**, 7–17
13 Chatterjee, D. and Khoo, K. H. (1998) Mycobacterial lipoarabinomannan: an extraordinary lipoheteroglycan with profound physiological effects. Glycobiology **8**, 113–120
14 Nigou, J., Gilleron, M. and Puzo, G. (2003) Lipoarabinomannans: from structure to biosynthesis. Biochimie **85**, 153–166
15 Guerardel, Y., Maes, E., Elass, E., Leroy, Y., Timmerman, P., Besra, G. S., Locht, C., Strecker, G. and Kremer, L. (2002) Structural study of lipomannan and lipoarabinomannan from *Mycobacterium chelonae*. Presence of unusual components with α1,3-mannopyranose side chains. J. Biol. Chem. **277**, 30635–30648
16 Sol-Foulon, N., Moris, A., Nobile, C., Boccaccio, C., Engering, A., Abastado, J. P., Heard, J. M., van Kooyk, Y. and Schwartz, O. (2002) HIV-1 Nef-induced upregulation of DC-SIGN in dendritic cells promotes lymphocyte clustering and viral spread. Immunity **16**, 145–155
17 Yeremeev, V. V., Stewart, G. R., Neyrolles, O., Skrabal, K., Avdienko, V. G., Apt, A. S. and Young, D. B. (2000) Deletion of the 19 kDa antigen does not alter the protective efficacy of BCG. Tuber. Lung Dis. **80**, 243–247
18 Pelicic, V., Jackson, M., Reyrat, J. M., Jacobs, Jr, W. R., Gicquel, B. and Guilhot, C. (1997) Efficient allelic exchange and transposon mutagenesis in *Mycobacterium tuberculosis*. Proc. Natl. Acad. Sci. U.S.A. **94**, 10955–10960
19 Laqueyrerie, A., Militzer, P., Romain, F., Eiglmeier, K., Cole, S. and Marchal, G. (1995) Cloning, sequencing, and expression of the *apa* gene coding for the *Mycobacterium tuberculosis* 45/47-kilodalton secreted antigen complex. Infect. Immun. **63**, 4003–4010
20 Abou-Zeid, C., Gares, M. P., Inwald, J., Janssen, R., Zhang, Y., Young, D. B., Hetzel, C., Lamb, J. R., Baldwin, S. L., Orme, I. M. et al. (1997) Induction of a type 1 immune response to a recombinant antigen from *Mycobacterium tuberculosis* expressed in *Mycobacterium vaccae*. Infect. Immun. **65**, 1856–1862
21 Ludwiczak, P., Gilleron, M., Bordat, Y., Martin, C., Gicquel, B. and Puzo, G. (2002) *Mycobacterium tuberculosis* phoP mutant: lipoarabinomannan molecular structure. Microbiology **148**, 3029–3037
22 Nigou, J., Gilleron, M., Cahuzac, B., Bounery, J. D., Herold, M., Thurnher, M. and Puzo, G. (1997) The phosphatidyl-*myo*-inositol anchor of the lipoarabinomannans from *Mycobacterium bovis* bacillus Calmette–Guérin. Heterogeneity, structure, and role in the regulation of cytokine secretion. J. Biol. Chem. **272**, 23094–23103
23 Nigou, J., Vercellone, A. and Puzo, G. (2000) New structural insights into the molecular deciphering of mycobacterial lipoglycan binding to C-type lectins: lipoarabinomannan glycoform characterization and quantification by capillary electrophoresis at the subnanomole level. J. Mol. Biol. **299**, 1353–1362
24 Beatty, W. L., Rhoades, E. R., Ullrich, H. J., Chatterjee, D., Heuser, J. E. and Russell, D. G. (2000) Trafficking and release of mycobacterial lipids from infected macrophages. Traffic **1**, 235–247
25 Venisse, A., Berjeaud, J. M., Chaurand, P., Gilleron, M. and Puzo, G. (1993) Structural features of lipoarabinomannan from *Mycobacterium bovis* BCG. Determination of molecular mass by laser desorption mass spectrometry. J. Biol. Chem. **268**, 12401–12411
26 Chatterjee, D., Lowell, K., Rivoire, B., McNeil, M. R. and Brennan, P. J. (1992) Lipoarabinomannan of *Mycobacterium tuberculosis*. Capping with mannosyl residues in some strains. J. Biol. Chem. **267**, 6234–6239
27 Torrelles, J. B., Khoo, K. H., Sieling, P. A., Modlin, R. L., Zhang, N., Marques, A. M., Treumann, A., Rithner, C. D., Brennan, P. J. and Chatterjee, D. (2004) Truncated structural variants of lipoarabinomannan in *Mycobacterium leprae* and an ethambutol-resistant strain of *Mycobacterium tuberculosis*. J. Biol. Chem. **279**, 41227–41239
28 Beatty, W. L., Ullrich, H. J. and Russell, D. G. (2001) Mycobacterial surface moieties are released from infected macrophages by a constitutive exocytic event. Eur. J. Cell Biol. **80**, 31–40
29 Wilchek, M. and Bayer, E. A. (1988) The avidin–biotin complex in bioanalytical applications. Anal. Biochem. **171**, 1–32
30 Daffe, M. and Draper, P. (1998) The envelope layers of mycobacteria with reference to their pathogenicity. Adv. Microb. Physiol. **39**, 131–203
31 Kaur, D., Lowary, T. L., Vissa, V. D., Crick, D. C. and Brennan, P. J. (2002) Characterization of the epitope of anti-lipoarabinomannan antibodies as the terminal hexaarabinofuranosyl motif of mycobacterial arabinans. Microbiology **148**, 3049–3057
32 Tailleux, L., Maeda, N., Nigou, J., Gicquel, B. and Neyrolles, O. (2003) How is the phagocyte lectin keyboard played? Master class lesson by *Mycobacterium tuberculosis*. Trends Microbiol. **11**, 259–263
33 Tailleux, L., Gicquel, B. and Neyrolles, O. (2005) *Mycobacterium tuberculosis* and dendritic cells: who's manipulating whom? Curr. Immunol. Rev. **1**, 101–105
34 Khoo, K. H., Tang, J. B. and Chatterjee, D. (2001) Variation in mannose-capped terminal arabinan motifs of lipoarabinomannans from clinical isolates of *Mycobacterium tuberculosis* and *Mycobacterium avium* complex. J. Biol. Chem. **276**, 3863–3871
35 Besra, G. S., Morehouse, C. B., Rittner, C. M., Waechter, C. J. and Brennan, P. J. (1997) Biosynthesis of mycobacterial lipoarabinomannan. J. Biol. Chem. **272**, 18460–18466
36 Gilleron, M., Nigou, J., Cahuzac, B. and Puzo, G. (1999) Structural study of the lipomannans from *Mycobacterium bovis* BCG: characterisation of multiacylated forms of the phosphatidyl-*myo*-inositol anchor. J. Mol. Biol. **285**, 2147–2160
37 Nigou, J., Gilleron, M., Brando, T., Vercellone, A. and Puzo, G. (1999) Structural definition of arabinomannans from *Mycobacterium bovis* BCG. Glycoconj. J. **16**, 257–264
38 Herrmann, J. L., O'Gaora, P., Gallagher, A., Thole, J. E. and Young, D. B. (1996) Bacterial glycoproteins: a link between glycosylation and proteolytic cleavage of a 19 kDa antigen from *Mycobacterium tuberculosis*. EMBO J. **15**, 3547–3554
39 Herrmann, J. L., Delahay, R., Gallagher, A., Robertson, B. and Young, D. (2000) Analysis of post-translational modification of mycobacterial proteins using a cassette expression system. FEBS Lett. **473**, 358–362
40 Dobos, K. M., Swiderek, K., Khoo, K. H., Brennan, P. J. and Belisle, J. T. (1995) Evidence for glycosylation sites on the 45-kilodalton glycoprotein of *Mycobacterium tuberculosis*. Infect. Immun. **63**, 2846–2853
41 Dobos, K. M., Khoo, K. H., Swiderek, K. M., Brennan, P. J. and Belisle, J. T. (1996) Definition of the full extent of glycosylation of the 45-kilodalton glycoprotein of *Mycobacterium tuberculosis*. J. Bacteriol. **178**, 2498–2506
42 Fernandez, N., Alonso, S., Valera, I., Vigo, A. G., Renedo, M., Barbolla, L. and Crespo, M. S. (2005) Mannose-containing molecular patterns are strong inducers of cyclooxygenase-2 expression and prostaglandin E_2 production in human macrophages. J. Immunol. **174**, 8154–8162

43 Geijtenbeek, T. B. and van Kooyk, Y. (2003) Pathogens target DC-SIGN to influence their fate DC-SIGN functions as a pathogen receptor with broad specificity. APMIS **111**, 698–714
44 Kaufmann, S. H. and 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
45 Xu, S., Cooper, A., Sturgill-Koszycki, S., van Heyningen, T., Chatterjee, D., Orme, I., Allen, P. and Russell, D. G. (1994) Intracellular trafficking in *Mycobacterium tuberculosis* and *Mycobacterium avium*-infected macrophages. J. Immunol. **153**, 2568–2578
46 Winau, F., Kaufmann, S. H. and Schaible, U. E. (2004) Apoptosis paves the detour path for CD8 T cell activation against intracellular bacteria. Cell Microbiol. **6**, 599–607

Received 29 April 2005/20 July 2005; accepted 10 August 2005
Published as BJ Immediate Publication 10 August 2005, doi:10.1042/BJ20050709