Protein Cell 2010, 1(9): 859–870
DOI 10.1007/s13238-010-0101-3

Protein & Cell

RESEARCH ARTICLE

Identification of four novel DC-SIGN ligands on *Mycobacterium bovis* BCG

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Received June 29, 2010 Accepted August 3, 2010

ABSTRACT

Dendritic-cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN; CD209) has an important role in mediating adherence of *Mycobacteria* species, including *M. tuberculosis* and *M. bovis* BCG to human dendritic cells and macrophages, in which these bacteria can survive intracellularly. DC-SIGN is a C-type lectin, and interactions with mycobacterial cells are believed to occur via mannosylated structures on the mycobacterial surface. Recent studies suggest more varied modes of binding to multiple mycobacterial ligands. Here we identify, by affinity chromatography and mass-spectrometry, four novel ligands of *M. bovis* BCG that bind to DC-SIGN. The novel ligands are chaperone protein DnaK, 60 kDa chaperonin-1 (Cpn60.1), glyceraldehyde-3 phosphate dehydrogenase (GAPDH) and lipoprotein lprG. Other published work strongly suggests that these are on the cell surface. Of these ligands, lprG appears to bind DC-SIGN via typical protein-glycan interactions, but DnaK and Cpn60.1 binding do not show evidence of carbohydrate-dependent interactions. LprG was also identified as a ligand for DC-SIGNR (L-SIGN; CD299) and the *M. tuberculosis* orthologue of lprG has been found previously to interact with human toll-like receptor 2. Collectively, these findings offer new targets for combating mycobacterial adhesion and within-host survival, and reinforce the role of DC-SIGN as an important host ligand in mycobacterial infection.

KEYWORDS DC-SIGN, *Mycobacteria*, lectins

INTRODUCTION

Tuberculosis is the world's most prevalent infectious disease affecting a third of the global human population. The causative agent of tuberculosis, *Mycobacterium tuberculosis*, avoids the destructive capacity of the host immune system by residing inside the phagosome of host mononuclear phagocytes (Armstrong and Hart, 1975; Clemens and Horwitz, 1995; Sturgill-Koszycki et al., 1996). Many studies have shown that *M. tuberculosis*, *M. paratuberculosis* and *M. bovis* BCG can bind to dendritic-cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN/CD209) to promote entry into human dendritic cells (DCs) and alveolar macrophages (Geijtenbeek et al., 2003; Maeda et al., 2003; Tailleux et al., 2003; Pitarque et al., 2005; Appelmelk et al., 2008). A recent study indicates that a mutation of DC-SIGN causing lower expression is protective against tuberculosis-induced lung cavitation (Vannberg et al., 2008). DC-SIGN is a 44 kDa type II transmembrane protein that consists of a carbohydrate recognition domain, neck domain, transmembrane domain and cytoplasmic tail. It is expressed mainly on DCs and on selected macrophage populations including alveolar macrophages (Geijtenbeek et al., 2000a; Lee et al., 2001; Maeda et al., 2003). DC-SIGN is a calcium-dependent lectin and has a high affinity for mannosylated surfaces, forming tetrameric complexes when binding to high mannose glycoproteins, such as HIV gp120 (Geijtenbeek et al., 2000b; Feinberg et al., 2001; Mitchell et al., 2001; Appelmelk et al., 2003). DC-SIGN has been shown to bind lipolysaccharide Le^x and mannose structures found on bacteria, such as *Helicobacter pylori*, *Klebsiella pneumonia* and *M. tuberculosis* (Appelmelk et al., 2003; Geijtenbeek et al., 2003; Tailleux et

al., 2003; van Kooyk and Geijtenbeek, 2003). Using purified cell wall components from mycobacteria, DC-SIGN was shown to bind lipoarabinomannan (LAM) structures from *M. tuberculosis, M. bovis* and *M. bovis* BCG, all of which express mannose-capped LAM (ManLAM). However, LAM purified from *M. smegmatis* did not bind DC-SIGN, since it expresses uncapped LAM, so-called AraLAM. Similarly, LAM from *M. avium* bound poorly to DC-SIGN since it expresses single mannose residue attachments and thus presents lower mannoside density (Geijtenbeek et al., 2003; Maeda et al., 2003). ManLAM was therefore believed to be the major ligand on *M. tuberculosis* for binding to DC-SIGN (Maeda et al., 2003; Tailleux et al., 2003). However, later studies showed that removal of the mannose-cap in experiments using whole bacteria did not appear to have a dramatic effect on DC-SIGN binding. The faster growing mycobacteria such as *M. smegmatis* or *M. avium* could also bind DC-SIGN despite not having the mannose caps, suggesting that other components in the mycobacterial cell wall were also binding DC-SIGN. Mannosylated lipoproteins found on the cell surface of mycobacteria such as 19 kDa lipoprotein lpqH/Rv3763 and a 45 kDa lipoprotein were shown to contribute to the binding of DC-SIGN to the bacteria (Pitarque et al., 2005; Appelmelk et al., 2008). These studies have revealed that the binding interaction of DC-SIGN to *M. tuberculosis* is more complicated than originally perceived, and suggests that there may be more potential DC-SIGN ligands present on *M. tuberculosis*.

In this study we set out to demonstrate DC-SIGN binding to *M. bovis* BCG as a model organism for *M. tuberculosis*. We explored the binding characteristics of DC-SIGN to whole *M. bovis* BCG and also observed the binding characteristics of a closely related protein, DC-SIGNR (DC-SIGN-*Related*/L-SIGN/CD299) to the mycobacterium. DC-SIGNR shares 77% amino acid sequence identity with DC-SIGN (Soilleux et al., 2000). Using affinity chromatography, we purified and identified four novel DC-SIGN binding ligands of *M. bovis* BCG: chaperone protein DnaK (DnaK), 60 kDa chaperonin-1 (Cpn60.1), glyceraldehyde-3 phosphate dehydrogenase (GAPDH) and lipoprotein lprG.

RESULTS AND DISCUSSION

We set out first to confirm the binding of DC-SIGN to whole *M. bovis* BCG, using lung surfactant protein A (SP-A) and BSA as positive and negative controls respectively. We also compared the binding of DC-SIGN to that of DC-SIGNR. By flow cytometry, we found that the binding of DC-SIGN and DC-SIGNR to whole *M. bovis* BCG is dose-dependent (Fig. 1), reaching a maximum at a protein input of about 10 μg per 5×10^8 cells (Fig. 2A). SP-A also binds dose-dependently, while BSA does not bind. Binding of DC-SIGN and DC-SIGNR are predominantly Ca^{2+}-dependent, as binding is reduced by ~80% in the presence of EDTA (Fig. 2B) compared with binding in 5 mM $CaCl_2$. Binding of SP-A appears less dependent on Ca^{2+} ions, as binding is reduced < 50% in EDTA. Mannose (50 mM) inhibits the binding of DC-SIGN and SP-A by less than 20%, while binding of DC-SIGNR is reduced by about 70% (Fig. 2B). These findings are compatible with the view that DC-SIGN, DC-SIGNR and SP-A are all likely to be binding to several bacterial ligands and the results with mannose and EDTA suggest more than one mode of binding. For DC-SIGNR, the results are consistent with its binding mainly (~80%) via its calcium-dependent carbohydrate binding site. For DC-SIGN and SP-A, a much smaller proportion (10%–20%) of binding may be mediated via these sites, and other binding occurs via Ca^{2+}-independent sites, and also via Ca^{2+} dependent sites that do not constitute the canonical carbohydrate binding site. Similar diversity for modes of binding of SP-A to viable and apoptotic mammalian cells has been observed previously (Jäkel et al., 2010a, b, c).

To identify macromolecules on the mycobacterial cell surface to which DC-SIGN is binding, *M. bovis* BCG lysates were passed through a DC-SIGN affinity chromatography column. Bound proteins were eluted with buffer containing EDTA. The eluted proteins were then concentrated and resolved by SDS-PAGE. From the gel (Fig. 3) four visible bands can be seen at 74, 60, 37 and 27 kDa. As a control *M. bovis* BCG lysates were passed through a control column made of underivatised Sepharose in the same way. No protein was detected in the eluted fractions of the control column, indicating no non-specific binding interactions (not shown). The 74, 60, 37 and 27 kDa bands were cut from the gel and analyzed by MALDI-TOF tryptic peptide fingerprinting mass spectrometry, and database searches carried out against both NCIBr and SwissProt. The bands were identified as chaperone protein DnaK, 60 kDa chaperonin (Cpn60.1), glyceraldehyde-3-phosphate dehydrogenase (GADPH) and lipoprotein lprG, respectively (Table 1). All of these have the same protein sequence in *M. tuberculosis* as in *M. bovis* BCG (Table 1). Two other minor candidates, CTP synthase and ATP synthase beta subunit (Table 1) were not considered further.

DnaK and Cpn60.1 are collectively known as heat shock proteins or chaperone proteins. Cpn60.1 generated the highest protein score, with nine peptide sequences matched. These peptide sequences cover 38.51% of the protein sequence (Table 1). The protein ran at ~60 kDa on a SDS-PAGE gel and was calculated to have a mass of 55,877 Da from the amino acid sequence (Fig. 3 and Table 1). The second highest protein score was for DnaK. This protein band produced four matching peptides sequences which contribute 11.2% sequence coverage. It ran at ~70 kDa on SDS-PAGE and had a calculated mass from the amino acid sequence of 66,830 Da (Fig. 3 and Table 1).

Toward the C-terminal of Cpn60.1, there is one possible N-linked glycosylation site at $N^{506}AS$ (Fig. 4). This potential

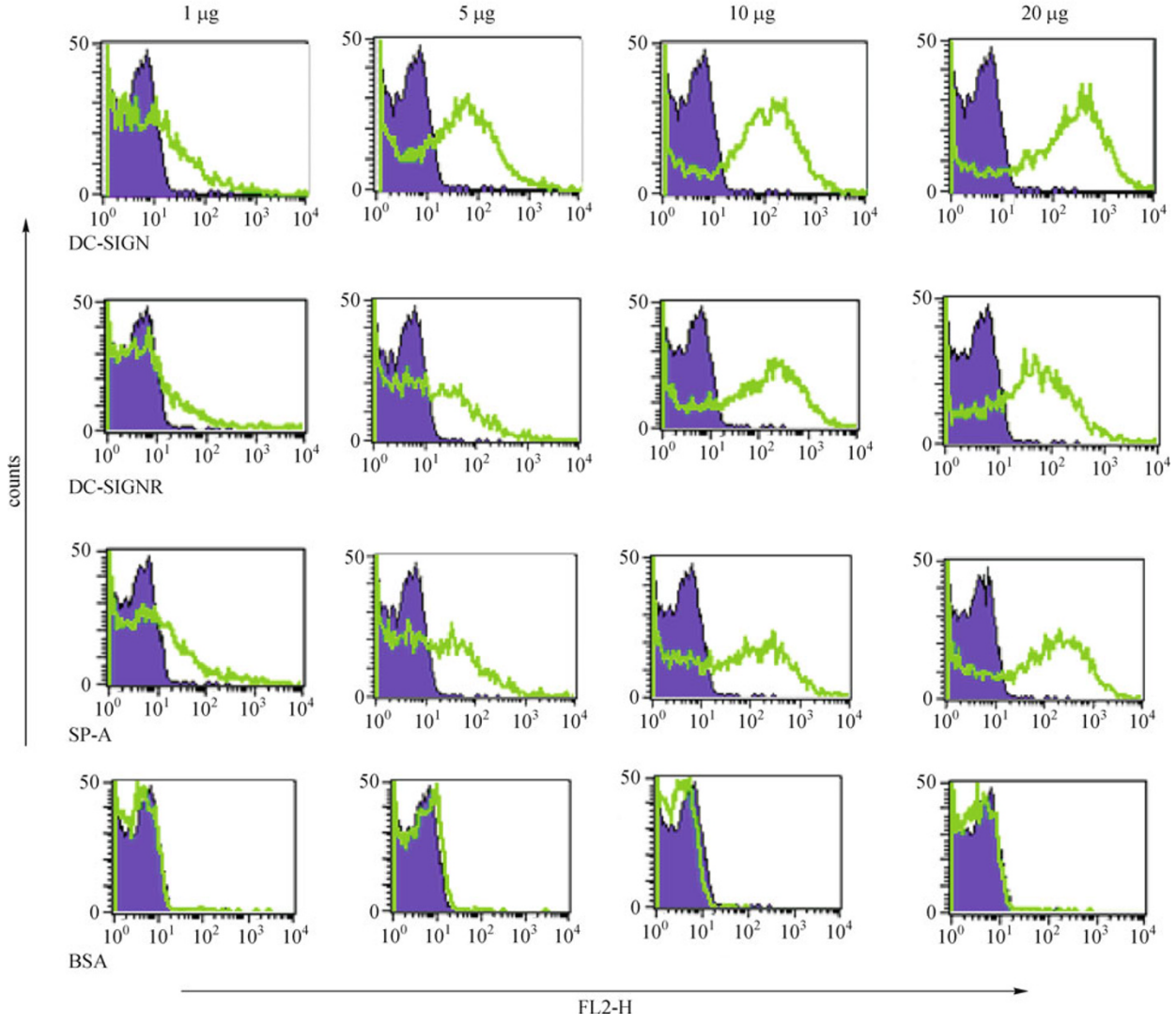

Figure 1. DC-SIGN, DC-SIGNR and SPA bind to whole *M. bovis* BCG. *M. bovis* BCG cells were washed in PBS and fixed in paraformaldehyde. Bacteria alone (solid) or bacteria incubated with protein (open) (5×10^8 cells) were incubated with either biotinylated-DC-SIGN, biotinyalted-DC-SIGNR, SP-A or biotinylated-BSA (1, 5, 10, 20 μg, respectively). SP-A binding was detected by incubating the cells with a biotinylated monoclonal anti-SPA antibody. All cells were then treated with streptavidin-PE and binding was measured using fluorescent cytometry. Biotinylated-BSA was used as a negative control. Results are representative of three independent experiments.

N-linked glycosylation site occurs in one of the Cpn60.1 peptides identified during mass spectrometry. This indicates that the site was not occupied by an oligosaccharide otherwise the peptide molecular mass would have been affected and unidentifiable during analysis. The site may be partially occupied indicating that there may be another population of this protein with an N-linked glycan present at N^{506}. However, the form of this protein identified after capture by the affinity column was not glycosylated at this position, and it is therefore very unlikely that DC-SIGN binds to this ligand via its Ca^{2+}-dependent lectin activity. Similarly, no potential N-linked glycosylation sites for DnaK were found (Fig. 4), suggesting that it also is not bound to DC-SIGN via N-glycans. From the current literature it is unknown whether these proteins undergo any O-linked glycosylation, but use of *in silico* O-glycosylation prediction tools available at the EXPASy (**Ex**pert **P**rotein **A**nalysis **Sy**stem) proteomics server (http://expasy.org/tools/; Gasteiger et al., 2003) indicates no predicted O-glycosylation in either protein.

A recent study (Hickey et al., 2009) showed that DnaK is located at the cell-surface of *M. tuberculosis.* There are no published data on the localization of Cpn60.1, but a related protein, Cpn60.2 was also shown to be on the cell surface of *M. tuberculosis*, and has a role in the adherence of *M.*

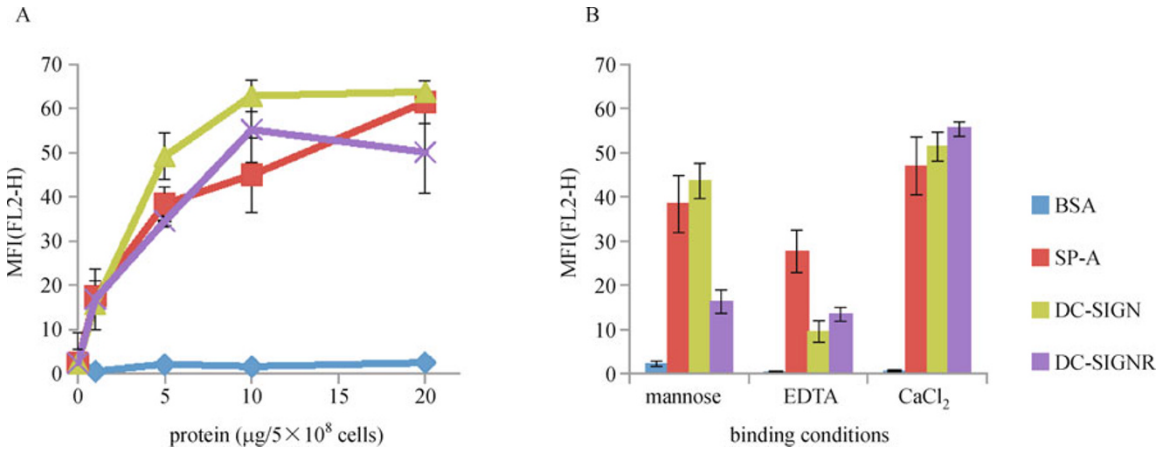

Figure 2. Binding of DC-SIGN, DC-SIGNR and SPA to whole *M. bovis* BCG. *M. bovis* BCG cells were washed in PBS and fixed in paraformaldehyde. (A) 5×10^8 cells were incubated with 1, 5, 10, 20 μg biotinylated-DC-SIGN, biotinylated-DC-SIGNR, SP-A and biotinylated-BSA in the presence of 5 mM $CaCl_2$. After incubations, SP-A was detected using a biotinylated monoclonal anti-SP-A antibody. Cells were then incubated with streptavidin-PE and binding was measured by flow cytometry. MFI (FL2-H) indicates the mean fluorescent intensity of PE-positive cells. Results are expressed as the mean of three independent experiments ± SD. (B) 5×10^8 cells were incubated with 20 μg biotinylated-DC-SIGN, biotinylated-DC-SIGNR, SP-A and biotinylated BSA in the presence of 50 mM mannose plus 5 mM $CaCl_2$, or 5 mM EDTA or 5 mM $CaCl_2$ without mannose. Biotinylated-BSA was used as a negative control. Cells were treated as above. Results are expressed as the mean of two independent experiments ± SD.

tuberculosis to macrophages (Hickey et al., 2009). Cpn60.1 and Cpn60.2 show 61% amino acid sequence identity (Kong et al., 1993). Hickey et al. (2009) showed that macrophages formed specific interactions with *M. tuberculosis*, which could be inhibited by pre-incubation with increasing concentrations of Cpn60.2 or by blocking surface localized Cpn60.2 with $F(ab')_2$ antibody. This was supported by showing that purified Cpn60.2 could bind to the surface of macrophages. Although DnaK was also shown to be located at the mycobacterial cell-surface, Hickey et al. (2009) could not show consistent binding via DnaK to macrophages using antibodies to block the reaction. This may have been due to a lack of appropriate anti-DnaK antibodies. In *Listeria monocytogenes*, DnaK has been shown to facilitate phagocytosis of the pathogen into macrophages (Hanawa et al., 1999). The same authors observed that wild type bacteria were endocytosed more than DnaK knockouts. Once inside the macrophage DnaK was shown not to be essential for multiplication within the cell although it was necessary for cell entry. Studies looking at the pathogenic role of the DnaK and its co-chaperone DnaJ, in *Salmonella enterica* serovar Typhimurium revealed that they are both essential for internalising the bacteria within epithelial cells and survival within macrophages (Takaya et al., 2004).

Cpn60.1 and Cpn60.2 are potent immunomodulatory proteins in the host. Cpn60.1 has been shown to be a more potent activator of stimulatory proinflammatory cytokines (Friedland et al., 1993; Lewthwaite et al., 2001; Hu et al., 2008). Despite chaperones being more commonly known as cytosolic proteins, many pathogenic bacteria express these proteins at the cell-surface possibly to promote attachment to host cells and mediate internalization. Cpn60 proteins have been reported to demonstrate these functions in *Helicobacter pylori, Clostridium difficile, Hemophilus ducreyi* and *Salmonella enterica* serovar Typhimurium (Yamaguchi et al., 1996; Frisk et al., 1998; Hennequin et al., 2001). Here we demonstrate that Cpn60.1 can also interact with DC-SIGN and propose that this could aid the entry of mycobacterial cells into DC or macrophage.

GAPDH was also identified as one of four DC-SIGN binding ligands in this study. Running at ~37 kDa on SDS-PAGE (Fig. 3), GAPDH was identified with three peptide matches, covering 15.04% of the protein sequence. The calculated mass of the protein is 35,955 Da and two potential N-linked glycosylation sites are present in the sequence, $N^{53}ST$ and $N^{154}AS$ (Table 1, Fig. 4). These two potential N-linked glycosylation sites may be occupied by carbohydrate structures required for DC-SIGN binding via its CRD. This protein has significant homology to the GAPDH enzymes indentified in Group A *Streptococcus*, enteropathogenic *E. coli*, and *Candida albicans* (Parker and Bermudez, 2000). GAPDH is an important enzyme in both prokaryotic and eukaryotic metabolism that catalyzes a step of glycolysis, converting glyceraldehyde-3-phosphate to glycerate 1,3-bisphosphate. GAPDH is more commonly recognized as a cytosolic enzyme found on the inner surface of the cell membrane. Even though there is no apparent signal sequence or stretch of hydrophobic residues to indicate a transmembrane region (Fig. 4), studies have reported that a 37 kDa protein homologous to GAPDH is expressed on the

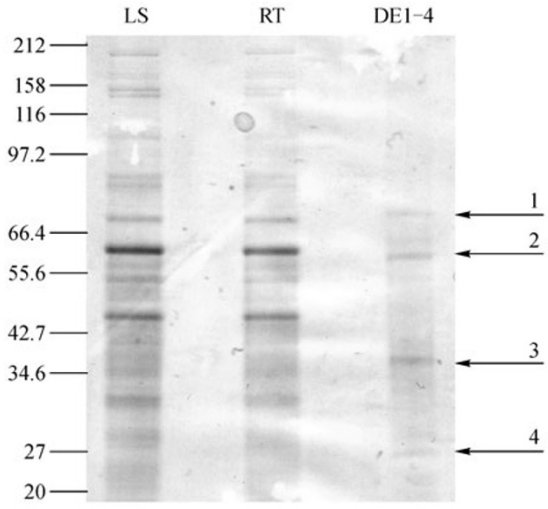

Figure 3. *M. bovis* BCG lysate proteins binding to immobilised DC-SIGN. DC-SIGN Sepharose was incubated with *M. bovis* BCG lysate in 10 mM Hepes, 140 mM NaCl, 5 mM $CaCl_2$ pH 7.4. The Sepharose was placed in a column and washed and bound proteins were eluted with 10 mM Hepes, 140 mM NaCl, 5 mM EDTA pH 7.4. Eluted fractions were concentrated with Strataclean beads and prepared in reducing conditions for analysis by SDS-PAGE. Concentrated eluates were run on 4%–12% gradient gel. As a negative control, underivatised Sepharose was incubated with the lysate in the same way (results not shown). LS, 2 µL of *M. bovis* BCG lysate; RT, 2 µL lysate proteins not bound to the column ("run-through"); DE1–4, concentrated eluted fractions 1–4 from the DC-SIGN column. Bands marked by black arrows were used for mass spectrometry analysis. Results are representative of three independent experiments.

outer cell membrane of hematopoietic cells (Allen et al., 1987) and also on many microorganisms such as Group A *Streptococcus*, enteropathogenic *E. coli*, *Candida albicans*, *Mycobacterium avium* and *Schistosoma mansoni* (Goudot-Crozel et al., 1989; Pancholi and Fischetti, 1992; Kenny and Finlay, 1995; Gil-Navarro et al., 1997; Parker and Bermudez, 2000). *M. avium* expresses GAPDH on its cell surface, whereupon GAPDH can bind to human epidermal growth factor. In the presence of recombinant human epidermal growth factor the rate of growth of *M. tuberculosis* and *M. avium* is rapidly increased (Parker and Bermudez, 2000).

Another DC-SIGN ligand purified by affinity chromatography was identified as lprG, a 24 kDa lipoprotein. LprG actually runs with an apparent molecular weight of 27 kDa on SDS-PAGE (Fig. 3) and was identified with only one peptide hit with a protein score of 70.07, covering 7.62% of the protein sequence. The calculated mass of the protein is 24,547 Da (Fig. 4). The identification of lprG was supported by Western blot analysis. As shown in Fig. 5, in eluted fractions DE1–2, DE3–4 and DE5–6 from DC-SIGN affinity chromatography, a strong band can be seen representing lprG. LprG has two potential N-linked glycosylation sites, one of which ($N^{83}PT$) is unoccupied or only partially occupied since it lies in one of the peptides identified by mass spectrometry. The other site, $N^{185}AT$ may be occupied. Ligand blot analysis (Fig. 6) of whole *M. bovis* BCG lysate incubated with either ^{125}I-DC-SIGN or ^{125}I-DC-SIGNR revealed that DC-SIGN and DC-SIGNR both bind the same protein at around 27 kDa, which corresponds to lprG in our SDS-PAGE system, and is the only ligand detected by this method. DC-SIGN and DC-SIGNR binding to lprG can therefore still occur when the mycobacterial protein has been denatured by SDS-PAGE. This strongly suggests that lprG binds to DCSIGN predominantly or entirely via protein-carbohydrate interactions.

In other studies looking at the importance of lprG in *M. tuberculosis*, knockout of the lprG operon was shown to attenuate *M. tuberculosis*, indicating that it has a prominent role in the pathogenic behavior of the bacterium (Bigi et al., 2004). Furthermore, lprG has been identified as a ligand for TLR-2 on macrophages, and lprG-TLR-2 interactions lead to reduced MHC class II presentation (Gehring et al., 2004). There is also growing evidence indicating that intracellular signaling via DC-SIGN modifies transduction pathways downstream from TLRs, driving immunosuppressive responses (Gringhuis et al., 2007, 2009).

Several other *M. tuberculosis* lipoproteins that are either glycosylated or presumed to be glycosylated also have been identified as key antigens with immunomodulatory functions (Herrmann et al., 2000). LpqH (19 kDa) was confirmed to have seven O-linked glycosylation sites (Herrmann et al., 2000). It has the same protein sequence in *M. tuberculosis* as in *M. bovis* BCG and was previously identified as a ligand for DC-SIGN (Pitarque et al., 2005) possibly binding via glycans. We were unable accurately to detect lipoproteins below

Table 1 Peptide hits of proteins eluted from DC-SIGN affinity chromatography

band No. (Fig. 3)	molecular weight (kDa) (SDS-PAGE)	protein name	peptide sequence	protein score	calculated mass (kDa)	occurrence
1	74	chaperone protein dnaK	4 peptides matched: LLGSFELTGIPPAPR, DVLLLDVTPLSLGIETK, IQEGSGLSKEDIDR, GVNPDEVVAVGAAL-QAGVLKGEVK, 11.2% sequence coverage	120.7	66.83	*Mycobacterium bovis* BCG (Pasteur 1173P2), *Mycobacterium bovis, Mycobacterium tuberculosis*
1	74	CTP synthase	1 peptide matched: GLTASSLGQLLTAR, 2.38% sequence coverage	52.86	63.635	*Mycobacterium bovis* BCG (Pasteur 1173P2), *Mycobacterium bovis, Mycobacterium tuberculosis*
2	60	60 kDa chaperonin 1	9 peptides matched: AADAVSEALLASATPVSGK, AFGGPTVTNDGVTVAR, LVAAGVNPIALGVGIGK, AAVEEGIVPGGGASLIHQAR, SAVLNASSVAR, EVGLEVLGSAR, AMEVGMDKLADTVR,, ESVEDAVAAAK, TGIAQVATVSSRDEQIGDLV-GEAMSK, 26.90% sequence coverage	409.71	55.877	*Mycobacterium bovis* BCG (Pasteur 1173P2), *Mycobacterium bovis, Mycobacterium tuberculosis*
2	60	ATP synthase beta-subunit	1 peptide matched: TISLQPTDGLVR, 2.46% sequence coverage	27.99	53.094	*Mycobacterium bovis* BCG (Pasteur 1173P2), *Mycobacterium bovis, Mycobacterium tuberculosis*
3	37	glyceraldehyde-3-phosphate dehydrogenase	3 peptides matched: LVDLVTLVGK, AAALNIVPTSTGAAK, YYDAPIVSSDIVTDPHS-SIFDSGLTK, 15.04% sequence coverage	65.65	35.955	*Mycobacterium bovis* BCG (Pasteur 1173P2), *Mycobacterium bovis, Mycobacterium tuberculosis*
4	27	lipoprotein lprG precursor	1 peptide matched: TLSGDLTTNPTAATGNVK, 7.62% sequence coverage	70.07	24.547	*Mycobacterium bovis* BCG (Pasteur 1173P2), *Mycobacterium bovis, Mycobacterium tuberculosis*

20 kDa in the affinity chromatography experiment shown in Fig. 3 due to limitations in the SDS-PAGE system used, but in Fig. 6 (ligand blotting) no band in the position of lpqH is seen. This suggests either that lprG is a much better ligand (more abundant or higher affinity) or that lpqH does not bind via glycans.

LprG binds to both DC-SIGN and DC-SIGNR. DC-SIGNR is expressed in the liver, lymph nodes but has also been described in the lung (Pöhlmann et al., 2001; Jeffers et al., 2004). In humans, both DCs and alveolar macrophages express DC-SIGN in the lungs. Although DC-SIGNR has a different expression pattern from DC-SIGN, it has similar binding properties to DC-SIGN (Bashirova et al., 2001; Mitchell et al., 2001; Pöhlmann et al., 2001). While DC-SIGN has been shown to mediate endocytosis and protein trafficking as a recycling receptor and the release of bound ligand at reduced pH, DC-SIGNR does not endocytose nor demonstrate pH-sensitive ligand binding (Guo et al., 2004).

DC-SIGN has been implicated as an important receptor in the establishment of *M. tuberculosis* infection. Although many DC-SIGN ligands have been identified at the cell-surface of the mycobacterium, studies suggested that there were more ligands present that had not yet been identified. Here, we have shown DC-SIGN binds to whole *M. bovis* BCG in both Ca^{2+}-dependent and Ca^{2+}-independent modes. We have identified four novel ligands for DC-SIGN. Of these only one,

Figure 4. Protein sequences of the identified eluted proteins. Protein sequences of the four identified proteins eluted from DC-SIGN affinity chromatography. (A) Chaperone protein DnaK (DnaK), (B) chaperone protein 60 (Cpn60.1), (C) glyceraldehyde 3-phosphate dehydrogenase (GADPH), and (D) lipoprotein lprG (lprG). In *red*, peptides identified by MS-MS, and in *purple*, potential N-linked glycosylation sites. Sequences were obtained from databases as described in the text.

lprG appears to bind predominantly via the glycan binding site. LprG is also a ligand for DC-SIGNR. Dendritic cells present in the lung migrate in order to prime T lymphocytes in the lymph nodes. It is believed that *M. tuberculosis* resides within the phagosome of the DC and exploits the migration thereby circulating within the host undetected (Fenton and Vermeulen, 1996; Henderson et al., 1997; Banchereau and Steinman, 1998). The discovery of new DC-SIGN binding ligands: DnaK, Cpn60.1, GAPDH and lprG, may help further research into designing inhibitors to prevent interactions between DC-SIGN and *M. tuberculosis* with the aim of blocking uptake and intracellular survival of mycobacterial cells.

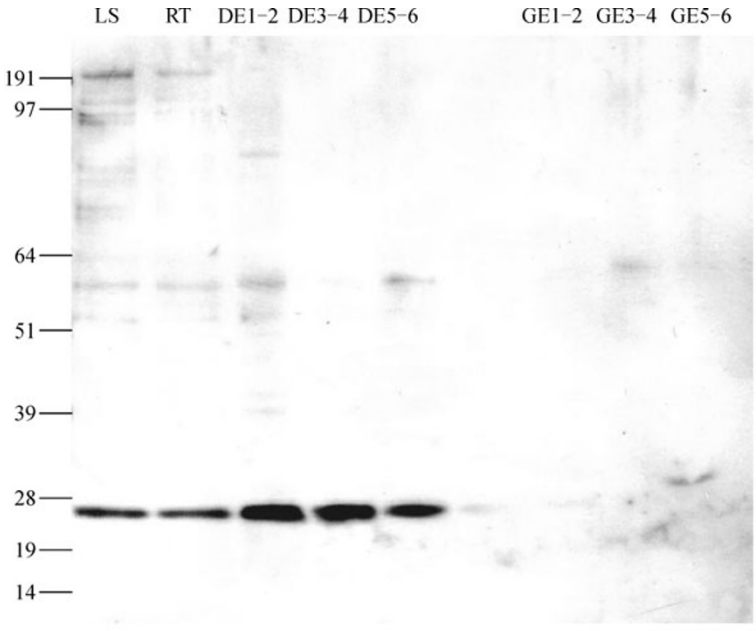

Figure 5. Western blot confirmation of lprG binding to DC-SIGN-Sepharose. SDS-PAGE of concentrated eluted fractions were transferred to a PVDF membrane and blocked. The membrane was incubated with rabbit anti-lprG antiserum, then washed and incubated with goat anti-rabbit-horseradish peroxidase (HRP)-conjugated antibody. The membrane was washed and exposed to Enhanced Chemiluminescence Western Blot Detection Reagents. The bands were visualized by exposing the membrane to X-ray film for a few seconds. Results are representative of 2 independent experiments. LS, lysate; RT, run-through; DE1–6, eluted fractions from the DC-SIGN column; GE1–6, eluted fractions from the guard (underivatised Sepharose) column.

MATERIALS AND METHODS

Mycobacterial cultures

Liquid cultures of *Mycobacterium bovis* BCG (Pasteur strain) were grown as described previously (Carroll et al., 2009) in Middlebrook 7H9 liquid medium containing 0.2% (*v/v*) glycerol, 0.05% (*v/v*) Tween-80, and 10% (*v/v*) albumin-dextrose-catalase (ADC, BD BBL Prepared Culture Medium: Becton Dickinson, Oxford, UK). Fresh cultures were inoculated from 1 mL glycerol stock of *M. bovis* BCG to generate a 100 mL culture. The 'first passage' was grown for four to five days at 37°C in roller bottles at 2 rpm until the bacteria had reached the exponential growth phase (OD_{600nm} = 0.80−1.00). Only the first passages of the strains were used for experimental work.

Preparation of cell lytates

M. bovis BCG cell cultures (200 mL) were harvested at exponential phase and cells were washed three times in 137 mM NaCl, 2.6 mM KCl, 8.2 mM Na_2HPO_4 and 1.5 mM KH_2PO_4, pH 7.4 (PBS). Cells were resuspended in 3 mL 10 mM Tris, 140 mM NaCl, 0.5% Triton X-100, pH 7.5 in the presence of protease inhibitors (Protease Inhibitor Cocktail, Roche Diagnostics, Mannheim Germany) and kept on ice for 5 min. The cells were then ribolysed in ribolysing tubes containing Lysing Matrix B (MPBiomedicals, Illkirch, France) for 45 s at speed setting 6.5 in a ribolyser (FastPrep FP120). Lysate was placed on ice for 5 min before being spun down. To reduce viscosity, mycobacterial lysate was incubated with 10 µg/mL of RNase A (R4642 Sigma Aldrich,Poole UK) for 30 min at 37°C. Lysate buffer was adjusted to 2.5 mM $CaCl_2$, 2.5 mM $MgCl_2$ and incubated with 10 µg/mL DNase II (D4138, Sigma Aldrich) for 30 min at 37°C. The lysate was then stored at −20°C until needed.

Protein Preparations

Recombinant, tetrameric DC-SIGN and DC-SIGNR (complete extra-cellular domains, lacking the transmembrane segment) were made and purified as described previously (Mitchell et al., 2001). These were used in either unmodified, biotinylated or radioiodinated form. Biotinylation was performed using N-hydroxysuccinimide biotin (Sigma-Aldrich, Poole, UK) at a molar ratio of 20:1 reagent : protein at pH 8.4, 4°C for 60 min. Radioiodination was done as a standard iodogen-catalyzed reaction (Krarup et al., 2007) with 50 µg of protein in PBS and 250 uCi of $Na^{125}I$ (GE Healthcare, UK, product IMS-30). SP-A was purified from human alveolar proteinosis broncho-alveolar lavage fluid as described by Jäkel et al. (2010a).

Flow cytometry

M. bovis BCG (5×10^8 cells) were fixed in 1.5% paraformaldehyde in PBS, 2 mM $CaCl_2$. Cells were washed in 100 µL 10 mM Hepes, 140 mM NaCl, 5 mM $CaCl_2$, pH 7.4 (assay buffer) and resuspended in 150 µL of the same buffer. Cells were incubated with 0, 5, 10, 20 µg of biotinylated-DC-SIGN or biotinylated-DC-SIGNR for 1 h at room temperature in assay buffer. Incubations were also carried out in the presence of 50 mM mannose and 5 mM EDTA as potential inhibitors

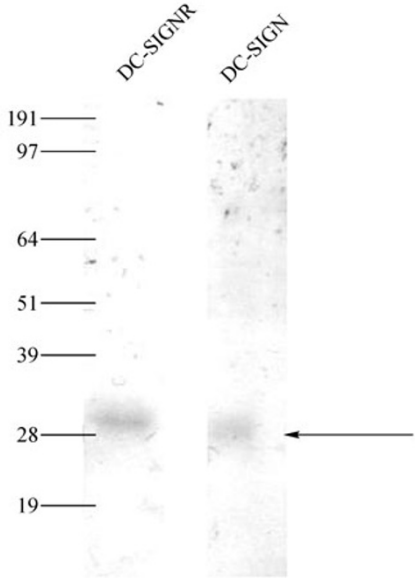

Figure 6. Radiolabelled DC-SIGN and DC-SIGNR binding to *M. bovis* BCG blot. SDS-PAGE of *M. bovis* BCG lysate was run and protein bandswere transferred to a PVDF membrane, blocked and incubated with 15 mL of 350,000 dpm/mL of either ^{125}I-DC-SIGN or ^{125}I-DC-SIGNR. The bands were then visualized by exposing the membrane to X-ray film for 1 week.

of binding to *M. bovis* BCG. Cells were washed and incubated with 1:200 dilution of Streptavidin-PE solution (554061 BD Pharmingen, Oxford, UK) for 40 min in 100 µL assay buffer and fixed in 180 µL of 1.5% paraformaldehyde in PBS, 2 mM $CaCl_2$. Binding to the cells was measured by flow cytometry using a FACScan instrument (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Aquisition and processing of data from 10,000 cells per sample were carried out with the CellQuest software (Becton Dickinson). Surfactant protein-A (SP-A) was used as positive control (Downing et al., 1995; Pasula et al., 1997; Weikert et al., 1997) and was detected using a biotinylated anti-SP-A monoclonal antibody (AntibodyShop, Gentofte, Denmark); biotinylated BSA was used as a negative control for binding to *M. bovis* BCG.

DC-SIGN Sepharose

Soluble recombinant DC-SIGN extracellular domain protein (2 mL, 1 mg/mL) in 10 mM Hepes, 140 mM NaCl, 5 mM $CaCl_2$, pH 7.5 was incubated with 1 mL hydrated CNBr-activated Sepharose (GE Healthcare, Chalfont St. Giles, UK) for 2 h at room temperature with rotation. The resin was washed twice in 1 M NaCl and then incubated in 3 mL 100 mM ethanolamine, pH 8.8 for 2 h at room temperature with rotation. The resin was washed twice in 1 M NaCl and stored in 25 mM Hepes, 150 mM NaCl, 5 mM EDTA, pH 7.5. Fifteen percent of the DC-SIGN supplied remained unbound, as assessed by measuring protein OD_{280} in the supernatant after binding.

DC-SIGN affinity chromatography

Capacity of the DC-SIGN-Sepharose for capturing glycoprotein ligand was confirmed using a test solution containing 100 µg of yeast invertase (20% oligomannose by mass) loaded onto the column in 1 mL of 10 mM Hepes, 140 mM NaCl, 5 mM $CaCl_2$ pH 7.4 (equilibration buffer) and eluted with 10 mM Hepes, 140 mM NaCl, 5 mM EDTA pH 7.4 (eluting buffer). Successful capture and elution of ligand was visualized by SDS-PAGE. The DC-SIGN-Sepharose column was regenerated with 20 mM Hepes, 2 M NaCl, 10 mM EDTA pH 7.4 (regeneration buffer). The column was then equilibrated with equilibration buffer. Lysate treated with RNase and DNase was diluted with one volume of 20 mM Hepes, 140 mM NaCl, 7.5 mM $CaCl_2$, pH 7.5 to obtain 5 mL with a protein concentration of about 5 mg/mL. As a control, a second column (1 mL) was made from underivatised Sepharose (guard column) and prepared in equilibration buffer. Lysate (5 mL) was added to the guard column and the beads were stirred at intervals during an incubation period of 2 h at 4°C. The lysate was then run off and loaded onto the DC-SIGN column. Beads were resuspended and incubated with the lysate as above. Both columns were washed exhaustively with equilibration buffer. Bound ligands were eluted with eluting buffer and 0.5 mL fractions collected. Eluted proteins were detected by reading OD_{280}, and positive fractions were pooled and the protein concentrated by binding to 40 µL Strataclean beads (Stratagene, Cedar Creek, TX, USA) per mL of eluted fraction. Beads were incubated with eluates on a rotary stirrer for 2 h. Beads were spun down and prepared for analysis by SDS-PAGE.

SDS-PAGE

SDS-PAGE was performed using the Invitrogen NuPAGE® system (Invitrogen, Cambridge, UK). Samples were prepared as described by Fairbanks et al. (1971). A total of 20 µL Strataclean beads per concentrated fraction were prepared in reducing conditions for SDS-PAGE and loaded per well.

Western blotting

SDS-PAGE was run with SeeBlue® Plus2 Prestained Standard (Invitrogen) to facilitate band size estimation. Protein bands were transferred to a polyvinylidene fluoride (PVDF) microporous membrane (Millipore, Billerica, Massachusetts, USA) in 48 mM Tris-HCl, 39 mM glycine, 20% (*v/v*) methanol, pH 8.3 (transfer buffer) for 4 h using a semi-dry blotter (Whatman International Ltd. Banbury, UK). The membrane was blocked with PBS, 0.2% Tween-20, 1 mg/mL BSA for 2 h. The membrane was washed with PBS, 0.2% Tween-20, 0.5 mM EDTA (washing buffer) and incubated with 1:300 dilution of rabbit anti-lprG antiserum (Bigi et al., 1997) in PBS, 1 mg/mL BSA for 3 h at room temperature. The membrane was washed in washing buffer and incubated with 1:10,000 dilution goat anti-rabbit-horseradish peroxidase-conjugated antibody (Sigma Aldrich, A0545) in PBS, 1 mg/mL BSA for 1 h. The membrane was washed in washing buffer and exposed to Enhanced Chemiluminescence Western Blot Detection Reagents (GE Healthcare) for detection. Bands were detected by exposing the membrane to X-ray film.

Ligand blotting

SDS-PAGE of reduced *M. bovis* BCG lysate was run and protein bands were transferred to a PVDF microporous membrane and blocked as above. The membrane was washed with 25 mM Hepes,

150 mM NaCl, 5 mM $CaCl_2$, 0.02% Tween-20 pH 7.4 and incubated with 15 mL of 350,000 dpm/mL of either ^{125}I-DC-SIGN or ^{125}I-DC-SIGNR for 2 h at room temperature. The membrane was washed with 25 mM Hepes, 150 mM NaCl, 5 mM $CaCl_2$, 0.02% Tween-20 pH 7.4 and bands were visualized by exposing the membrane to X-ray film in a lightproof cassette for 1 week.

Mass spectrometry

Protein bands from SDS-PAGE gels were stained with either SafeStain (Invitrogen) or Coomassie Blue R-250 stain (Fairbanks et al., 1971) and destained in 10% (*v/v*) acetic acid, 10% (*v/v*) ethanol. Individual bands were excised and subjected to MS-MS analysis. Mass spectrometric analysis was carried out using a Q-TOF 1 (Micromass, Manchester, UK) coupled to a CapLC (Waters, Milford, USA). In-gel trypsin digestion was carried out as described by Shevchenko et al. (2006). Tryptic peptides were concentrated and desalted on a 300 μm id/5 mM C18 pre-column and resolved on a 75 μm id/25 cm C18 PepMap analytical column (LC packings, San Francisco, CA, USA). Peptides were eluted to the mass spectrometer using a 45 min 5%–95% (*v/v*) acetonitrile gradient containing 0.1% (*v/v*) formic acid at a flow rate of 200 nL/min. Spectra were acquired in positive mode with a cone voltage of 40 V and a capillary voltage of 3300 V. The MS to MS/MS switching was controlled in an automatic data-dependent fashion with a 1 s survey scan followed by three 1 s MS/MS scans of the most intense ions. Precursor ions selected for MS/MS were excluded from further fragmentation for 2 min. Spectra were processed using ProteinLynx Global Server 2.1.5 and searched against the SwissProt_55.6 and NCBInr_20080718 databases using the MASCOT search engine (Matrix Science, London, UK). Database searches were performed with the taxonomy restricted to Mycobacteria. Carbamidomethyl cysteine was set as a fixed modification and oxidised methionine as a potential variable modification. Data was searched allowing 0.1 Da error on all spectra and up to one missed tryptic cleavage site.

ABBREVIATIONS

Cpn60.1, 60 kDa chaperonin-1; DC, dendritic cell; DC-SIGN/CD209, dendritic-cell-specific intercellular adhesion molecule-3-grabbing non-integrin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

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