Structural Requirements for Multimerization of the Pathogen Receptor Dendritic Cell-specific ICAM3-grabbing Non-integrin (CD209) on the Cell Surface*

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The myeloid C-type lectin dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN, CD209) recognizes oligosaccharide ligands on clinically relevant pathogens (HIV, Mycobacterium, and Aspergillus). Alternative splicing and genomic polymorphism generate DC-SIGN mRNA variants, which have been detected at sites of pathogen entrance and transmission. We present evidence that DC-SIGN neck variants are expressed on dendritic and myeloid cells at the RNA and protein levels. Structural analysis revealed that multimerization of DC-SIGN within a cellular context depends on the lectin domain and the number and arrangement of the repeats within the neck region, whose glycosylation negatively affects oligomer formation. Naturally occurring DC-SIGN neck variants differ in multimerization competence in the cell membrane, exhibit altered sugar binding ability, and retain pathogen-interacting capacity, implying that pathogen-induced cluster formation predominates over the basal multimerization capability. Analysis of DC-SIGN neck polymorphisms indicated that the number of allelic variants is higher than previously thought and that multimerization of the prototypic molecule is modulated in the presence of allelic variants with a different neck structure. Our results demonstrate that the presence of allelic variants or a high level of expression of neck domain splicing isoforms might influence the presence and stability of DC-SIGN multimers on the cell surface, thus providing a molecular explanation for the correlation between DC-SIGN polymorphisms and altered susceptibility to HIV-1 and other pathogens.

Dendritic cells (DCs)4 link the innate and adaptive branches of the immune response by virtue of their capacity to recognize pathogen-specific structures (1) via pathogen-associated molecular pattern receptors (2). Immature DCs express a number of lectins and lectin-like molecules, which endow them with a broad capacity for pathogen recognition, as they mediate the specific recognition of parasitic, bacterial, yeast, and viral pathogens (3, 4). Dendritic cell-specific ICAM-3-grabbing non-integrin (DC-SIGN, CD209) is a type II membrane C-type lectin (5, 6) abundantly expressed in vivo on myeloid DC and macrophage subpopulations (5–12), as well as in vitro generated monocyte-derived dendritic cells (MDDCs) and alternatively activated macrophages (12–14). DC-SIGN binds a large array of pathogens, including HIV (15), Ebola (16), hepatitis C (17–19), and Dengue virus (20) and Leishmania amastigotes and promastigotes (21, 22), Mycobacterium tuberculosis (23, 24), Aspergillus fumigatus (25) and Candida albicans (26) via mann- and Lewis oligosaccharides-dependent interactions (27, 28). In addition, DC-SIGN appears to mediate DC contacts with naïve T lymphocytes through its recognition of ICAM-3 (6), DC trafficking through interactions with endothelial ICAM-2 (8), and DC-neutrophil interactions by interacting with the CD11b/CD18 integrin (29).

Structurally, DC-SIGN contains a carbohydrate-recognition domain, a neck region composed of eight 23-residue repeats, and a transmembrane region followed by a cytoplasmic tail containing recycling and internalization motifs (5, 30–32). Analysis of recombinant molecules has revealed that the monomeric lectin domain has low affinity for carbohydrates, whereas full-length DC-SIGN molecules form tetramers through their neck domain, thus allowing high affinity recognition of specific ligands (33–35). In addition to this prototypical structure, alternative splicing events generate DC-SIGN isofrom transcripts whose presence exhibits inter-individual variations (36). The numerous DC-SIGN isofrom transcripts reported to date include an alternative cytoplasmic tail, an absent transmembrane region, truncated lectin domains, and a variable number
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of repeats within the neck domain (36). Moreover, the 23-residue repeat region of DC-SIGN is polymorphic at the genomic level (37, 38). Five different alleles for the DC-SIGN neck domain have been identified to date, whose presence correlates with altered susceptibility to HIV-1 transmission (38). The functional relevance of the DC-SIGN neck variants has been further suggested by their detection at mucosal HIV transmission sites (39). Given the involvement of the neck domain in recombinant DC-SIGN multimerization, we hypothesized that the existence of this large array of polymorphic variants might have an impact on the repertoire of pathogen recognition by dendritic cells, as well as on the establishment of interactions between dendritic cells and other cell types. We have characterized naturally occurring alternative splicing isoforms, allelic variants and mutant isoforms of DC-SIGN in terms of surface receptor multimerization and adhesive and pathogen-recognition capabilities, and found that the lectin domain contributes to DC-SIGN multimerization on the cell surface, that glycosylation of the neck domain negatively regulates formation of multimers, and that a neck domain with a single 23-residue repeat is sufficient to mediate DC-SIGN multimerization on the cell surface. Functional comparison of the distinct constructs revealed that the basal multimerization of DC-SIGN does not correlate with enhanced binding to endogenous or pathogenic ligands, indicating that pathogen-induced cluster formation predominates over the basal multimerization capability of the DC-SIGN molecule and is the driving force for the DC-SIGN-dependent pathogen capture and internalization.

Isolation and Structural Characterization of Alternatively Spliced DC-SIGN Isoforms

DC-SIGN isoforms were isolated by reverse transcription-PCR on RNA from MDDCs of a healthy donor. Reverse transcription-PCR was performed essentially as described previously (41). DC-SIGN mRNA was optimally amplified after 35 cycles of denaturation (95 °C, for 45 s), annealing (62 °C, for 45 s), and extension (72 °C, for 1 min), followed by a 10-min extension step at 72 °C. Oligonucleotides used for amplification of the coding region of the prototypical DC-SIGN isoform 1A (DC-SIGN 1A) mRNA were CD209s (5′-GGGGATTAGGAGTGGAGATGAGCCTGAC-3′) and CD209as (5′-CCCCAGAGCTATTGTCTGAGCAG-3′) (6, 36). Amplification of DC-SIGN isoforms was accomplished using the primer pairs CD209s/CD209as, CD209as/CD209as, and CD209lb/CD209as. The oligonucleotide CD209soluble (5′-GATACAGGCTTACGTGGTGCA-3′) spans through the exon Ic/exon III junction previously described for potentially soluble transmembrane-lacking DC-SIGN isoforms. The oligonucleotide CD209lb (5′-GGGAATTTCATGCTGGCTCTTCA-C3′) includes the alternative translation initiation site found in exon lb, which originates the DC-SIGN 1B isoforms (Fig. 1A). PCR-generated fragments were resolved in agarose gels, purified, sequenced, and cloned into pCDNA3.1(−) vector.

Identification of DC-SIGN Polymorphic Isoforms and Generation of His- and FLAG-containing DC-SIGN Expression Vectors

Three DC-SIGN allelic variants (-D3, -D5, and -D7) were identified by PCR on genomic DNA from 300 independent donors. Amplification of the DC-SIGN neck domain-encoding exon was carried out on 300 ng of genomic DNA using oligonucleotides CD209-4F, (5′-GGGAATTCTGCTGACCTGAGCCTGAC-3′) and CD209-4R, (5′-CCCCACTTCTTCAGGTGGATG-3′). After 35 cycles of denaturation (95 °C, for 45 s), annealing (61 °C, for 30 s), and extension (72 °C, for 90 s), followed by a 10-min extension step at 72 °C, PCR-generated fragments were resolved in agarose gels, purified, cloned into pcDNA4-TOPO vector (Invitrogen), and sequenced.

Swapping of the neck domains between the allelic variants and the prototypic form of DC-SIGN was done after introduction of silent mutations creating restriction sites at Val63 (KpnI) and Ala247/Ala248 (Sacl) in pCDNA3.1-DC-SIGN 1A and the allelic variants in pCR4-TOPO. Oligonucleotides used for mutagenesis included: DC-SIGN-Val63s (5′-TGTCCAAAGTG-TCCAGTTTCACCCAGCTCCA-TGACTACG-3′), DC-SIGN-Val63as (5′-CTGACTTATGGAGCTGGGTACCTTGGGACA-TTTGCAAC-3′), DC-SIGN-247/248s (5′-GCTGAGGCCTGGGGAAGCCGGGTGAACCTTGGCACC-3′), and DC-SIGN-247/248as (5′-GATCCAGGCTTACGTGGTGCA-3′). The resulting plasmids (pCDNA3.1-DC-SIGN-D3, pcDNA3.1-DC-SIGN-D5, and pcDNA3.1-DC-SIGN-D7) were verified by sequencing.

An expression vector for N-terminal-His epitope-containing DC-SIGN 1A (pCDNA3.1-DC-SIGN 1A-His) was created by PCR on pCDNA3.1-DC-SIGN 1A using oligonucleotides

EXPERIMENTAL PROCEDURES

Generation of MDDCs

Human peripheral blood mononuclear cells were isolated from buffy coats from healthy donors over a Lymphoprep (Nycomed, Norway) gradient according to standard procedures. Monocytes were purified from peripheral blood mononuclear cells by magnetic cell sorting using CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and immediately subjected to the dendritic cell differentiation protocol (40). Monocytes were cultured for 5–7 days in complete medium with 1000 units/ml granulocyte macrophage-colony stimulating factor (Schering-Plough, Kenilworth, NJ) and 1000 units/ml recombinant interleukin-4 (1000 units/ml), as described before (14).

Cells

The acute monocytic leukemia cell line THP-1, and the erythroleukemic K562 were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (complete medium). COS-7 and HEK293T cells were grown in Dulbecco’s modified Eagle’s medium 10% fetal calf serum. THP-1 differentiation was induced by treatment with phorbol 12-myristate 13-acetate (10 ng/ml), Bryostatin (10 nM), either alone or in combination with interleukin-4 (1000 units/ml), as described before (14).
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Flow Cytometry and Antibodies

Cellular phenotypic analysis was carried out by indirect immunofluorescence, using FITC-labeled goat anti-mouse antibody (Serotec, Oxford, UK). Monoclonal antibodies used for cell surface staining included MR1 (directed against the lectin domain of DC-SIGN), and the supernatant from the mouse myeloma P3-X63Ag8 (X63) was used as the control. All incubations were done in the presence of 50 μg/ml human IgG to prevent binding through the Fc portion of the antibodies. Flow cytometry analysis was performed with an EPICS-CS (Coulter Científica, Madrid, Spain) using log amplifiers.

Immunofluorescence

Cells were resuspended in PBS and allowed to adhere onto poly-L-lysine-coated coverslips for 60 min at 37 °C. After a brief washing step with PBS, cells were fixed and permeabilized in a 1:1 solution of acetone:methanol for 10 min at −20 °C, washed, and stained with the MR1 monoclonal antibody (13) followed by an incubation with an FITC-labeled goat anti-mouse antibody. Coverslips were mounted in fluorescent mounting medium (DakoCytomation, Carpinteria, CA), and representative fields were photographed through an oil immersion lens on a Nikon Eclipse E800 microscope equipped for epifluorescence or by confocal microscopy.

Cell Surface Protein Labeling and Precipitation

For labeling, immature MDDCs were washed with PBS 1 mM EDTA, resuspended in PBS, pH 8.0, and incubated in 0.5 mg/ml bixinamidohexanoyl acid 3-sulfo-N-hydroxysuccinimide ester sodium salt (Pierce) for 30 min at 4 °C. Cells were extensively washed in PBS and lysed using 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.025% sodium azide, 1% Brij 58 (Sigma-Aldrich), 1 mM iodoacetamide, 2 mM Pefabloc (Alexis Biochemicals, Lausen, Switzerland), and 2 μg/ml aprotinin, antipain, leupeptin, and pepstatin. For precipitation of biotin-labeled proteins, Streptavidin-agarose (Sigma-Aldrich) was added to the lysates, and the mixture incubated for 1 h at 4 °C. After centrifugation, beads were extensively washed in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.025% sodium azide, 0.1% Brij 58, resuspended in 3× Laemmli sample buffer (2% SDS, 6.25 mM Tris base, 10% glycerol), and boiled. Eluted material was resolved by SDS-PAGE under reducing or non-reducing conditions and subsequent Western blot with polyclonal antibodies specific for DC-SIGN. Coprecipitation of DC-SIGN 1A/DC-SIGN 8dΔL or DC-SIGN 1A/His/DC-SIGN-D3/-D5-FLAG hetero-oligomers was performed on lysates from transiently transfected COS-7 with MR1 antibody as previously described (42), and precipitated material was detected with specific polyclonal antibodies, or using anti-His antibody for precipitation and anti-FLAG-HRP antibody for detection of precipitated material, respectively.

Cross-linking Experiments

Cross-linking experiments were performed using the water-soluble cross-linking agent dithiobis(succinimidylpropionate) (DTSSP) according to the manufacturer’s instructions (Pierce). Briefly, immature MDDC was washed with PBS 1 mM EDTA, resuspended in 1 ml of PBS, and incubated in the presence of 100 μl of 10 mM DTSSP in sodium citrate 5 mM, pH 5.0, for 30 min at room temperature. Stop solution (20 mM Tris-HCl, pH 7.5) was added (15 min at room temperature), and cells were washed twice with PBS. Total cell lysates were obtained in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.025% sodium azide, 0.5% Nonidet P-40, 1 mM iodoacetamide, 2 mM Pefabloc (Alexis Biochemicals), and 2 μg/ml aprotinin, antipain, leupeptin, and
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peptatin (Nonidet P-40 lysis buffer), 10 μg of each lysate was subjected to SDS-PAGE as described for Western blot experiments. For cleaving the cross-linker agent, lysates were incubated with 5% β-mercaptoethanol in Laemmli sample buffer.

**Generation of Polyclonal Antisera against DC-SIGN Structural Domains**

Peptides based on the sequence of the sixth repeated domain of the DC-SIGN neck region (GELPEKSQOEYQELRTLKAAV), and the region between residues 6 and 33 of the cytoplasmic tail (EPRLQLGLLIEEQLRLGFRQTRGYSKS), were synthesized by the multiple antigen peptide system (13). New Zealand White rabbits were immunized by subcutaneous injection of each peptide (for DSG-1 and DSG-2 antisera) or the recombinant DC-SIGN lectin domain (for DSG-4 antisera) expressed in bacteria (0.5 ml of a 1 mg/ml solution in PBS) in complete Freund’s adjuvant (1:1) on day 0 and in incomplete Freund’s adjuvant (1:1) on days 21 and 42. Rabbits were bled on day 49, and serum was assayed for DC-SIGN recognition in Western blot experiments.

**Functional Characterization of DC-SIGN Isoforms and Mutants**

*C. albicans* and *A. fumigatus* Binding Assays—Conidia were labeled with 0.1 mg/ml FITC for 1 h at room temperature and extensively washed. For conidia-binding assays (25), cells were washed, resuspended in complete medium, and pretreated for 20 min at room temperature with anti-DC-SIGN (MR1) or an isotype-matched irrelevant antibody (X63). Then cells were incubated with FITC-labeled *A. fumigatus* or *C. albicans* conidia at the indicated ratios for 30 min at room temperature. After extensive washing, cells were fixed with 2% paraformaldehyde in PBS for 1 h at 4 °C, washed, and analyzed by flow cytometry.

**DC-SIGN-dependent Adhesion Assays—**DC-SIGN-dependent adhesion was evaluated using *Saccharomyces cerevisiae* mannan as specific ligand. 96-well microtiter EIA II-Linbro plates were coated overnight with mannan at 50 μg/ml in PBS at 4 °C, and the remaining sites were blocked with 0.5% bovine serum albumin for 2 h at 37 °C. Cells were labeled in RPMI 0.5% bovine serum albumin with the fluorescent dye 2′,7′-bis-(2-carboxyethyl)-5-((and)-6)-carboxyfluorescein acetoxyethyl ester (Molecular Probes, The Netherlands) at 37 °C and then preincubated for 20 min with either the isotype-matched control antibody or an irrelevant antibody (X63) for 20 min at 4 °C. Then, cells were incubated with a phycoerythrin-labeled polyclonal antiserum against human IgG (Beckman Coulter), and analyzed by flow cytometry.

**Sugar-coated Fluorescent Bead Binding to DC-SIGN—**Synthetic fluorescein-labeled fucose- or Lewis^a^-containing polycyclic amide beads (FITC-PAA-Nac-Gal, FITC-PAA-Fuc, and FITC-PAA-Le^a^) were obtained from Lectinex (Moscow, Russia). After washing with PBS and 1 mM EDTA, transiently transfected HEK293T cells were resuspended in complete medium, and sugar-PAA-FLU beads were added to a final concentration of 20 μg/ml and incubated at 37 °C for 30 min. After extensive washing, cells were fixed for 1 h at room temperature, and analyzed by flow cytometry. For inhibition assays, cells were preincubated for 10 min at room temperature with either MR1 antibody or an irrelevant antibody (X63) in complete medium before beads addition. Results from binding assays were expressed as “Binding Index,” which represents the DC-SIGN-dependent binding relative to DC-SIGN expression levels according to the formula: Binding index = (mean fluorescent intensity (MFI) of cells plus beads − MFI of cells plus beads in the presence of MR1)/MFI after MR1 staining/MFI after staining with X63.

**NMR Experiments**

Binding of soluble glucocmannan from *Candida utilis* (IF) to DC-SIGN transfectants was done by basic Saturation Transfer Difference, as previously described (43).

**Western Blot**

Total cell lysates were obtained in Nonidet P-40 lysis buffer, and 10 μg of each lysate was subjected to SDS-PAGE under reducing or non-reducing conditions and transferred onto an Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, MA). After blocking of the unoccupied sites with 5% nonfat dry milk in 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Tween 20, protein detection was performed using the SuperSignal West Pico chemiluminescent system (Pierce). Detection of DC-SIGN was carried out using polyclonal antiserum against the C-terminal 20-residue peptide of DC-SIGN (C-20, sc-11038, Santa Cruz Biotechnology, Santa Cruz, CA), amino
acids 61–200 (H-200, sc-20081, Santa Cruz Biotechnology), or polyclonal antisera raised against peptides based on the sixth 23-residue repeats within the DC-SIGN neck region (DSG-1), against a 28-residue peptide from the DC-SIGN cytoplasmic tail (DSG-2), or against the whole lectin domain (DSG-4).

**Carbohydrate Affinity Precipitations**

For precipitation of mannan- and N-acetylgalactosamine-binding proteins, transiently transfected HEK293T or COS-7 cells (3 × 10⁶) were lysed in Nonidet P-40 lysis buffer. Then, 200 µl of each lysate was taken to 1 ml with Nonidet P-40 lysis buffer and incubated with 50 µl of mannan- or N-acetylgalactosamine-agarose (Sigma-Aldrich) for 12 h at 4 °C. After extensive washing in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.025% sodium azide, 0.05% Nonidet P-40, bound proteins were eluted by boiling the agarose beads in 3× Laemmli sample buffer.

**RESULTS**

**Range of DC-SIGN Alternatively Spliced Isoforms—MDDCs** express a high number of alternatively spliced DC-SIGN mRNA species (36), which are also found at mucosal HIV transmission sites (39). To determine the range of DC-SIGN mRNA species found in MDDC from a single donor, three different sets of primers were designed to specifically amplify the prototypic DC-SIGN mRNA (DC-SIGN 1A), or species encoding either an alternative cytoplasmic domain (DC-SIGN 1B) or lacking the transmembrane domain (DC-SIGN ΔTM) (Fig. 1A). Sequencing of the amplified fragments resulted in the identification of DC-SIGN mRNA species encoding for variants with SDS eluted and non-bound materials were resolved by SDS-PAGE and DC-SIGN detection accomplished with specific polyclonal antibodies.

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**FIGURE 1. Detection of DC-SIGN isoforms on monocyte-derived dendritic cells.** A, schematic representation of the DC-SIGN mRNA and the position of exons I–VI, genomic organization; ATG, translational start sites; CYT, cytoplasmic domain; TM, transmembrane region. B, schematic structure of the major PCR fragments obtained from RNA of immature MDDCs. C–F, lysates from COS-7 cells transiently transfected with expression vectors for DC-SIGN 1A, a chimeric construct lacking the lectin domain (8dΔL) or an empty vector (Mock) (C), precipitated material from surface (biotin-labeled) immature MDDCs (D), lysates from THP-1 cells differentiated with Bryostatin (Bryo) in the presence or absence of interleukin-4 (E), or lysates from immature MDDCs either untreated or incubated with the cross-linking agent DTSSP (F) were resolved by SDS-PAGE under non-reducing or reducing conditions (in the presence of β-mercaptoethanol, β-MSH). The gels were then subjected to Western blot using polyclonal antisera against the neck domain (DSG-1 in C and F; H-200 in E), against the cytoplasmic tail of DC-SIGN (DSG-2) (C, D, and F) or against the C-terminal 20-amino acid peptide of DC-SIGN (C-20) (C). The specificity of the distinct antisera is indicated in each panel. Thin lines indicate the position of bands with higher mobility than the full-length DC-SIGN isoform. In D, the biotin-labeled proteins precipitated with streptavidin-agarose (SP) were analyzed in parallel with whole cell extracts (WE) and proteins in the supernatant or non-precipitated (SN).
alternative cytoplasmic tails and potentially soluble isoforms, with each group including transcripts differing in the neck domain or the carbohydrate-binding region (Fig. 1B). Therefore, and in agreement with previous reports (14, 36, 39), the DC-SIGN gene gives rise to a large number of alternatively spliced mRNA species, most of which differ in the number of 23-residue repeats within the neck domain, previously demonstrated to mediate multimerization of recombinant DC-SIGN (34).

Next we generated polyclonal antisera specific for either the neck domain (DSG-1) or the prototypic cytoplasmic tail of DC-SIGN 1A (DSG-2). Both DSG-1 and DSG-2 specifically detected the 44-kDa band of the prototypic full-length isoform DC-SIGN 1A, as well as a deletion mutant lacking the lectin domain (8dΔL), whereas a polyclonal antisera against the 20 C-terminal residues of the lectin domain (C-20) only detected the full-length molecule (Fig. 1C).

To determine the degree of DC-SIGN multimerization on MDDCs, cell surface proteins were biotin-labeled, and streptavidin pulled-down material was analyzed for the presence of DC-SIGN. Under non-reducing conditions, the DSG-2 antisera detected distinct several bands corresponding to DC-SIGN monomers, dimers, trimers, tetramers, and high order multimers either in the whole extracts and the pull-down (Fig. 1D, left panel, lanes WE and SP, respectively), which suggests that DC-SIGN multimers are found on the cell surface of MDDCs. Analysis of cell surface DC-SIGN molecules from MDDC under reducing conditions also revealed the presence of additional higher mobility bands that were also recognized by the DSG-2 antisera (Fig. 1D, right panel, lane SP). The same pattern was detected in total lysates of dendritic-like THP-1 cells (14) using a polyclonal antisera against the whole neck region of the molecule (Fig. 1E), and similar bands could be detected in MDDC lysates with both DSG-1 and DSG-2 antisera (Fig. 1F). Therefore, DC-SIGN isoforms can be detected on the cell surface of monocyte-derived dendritic cells, although to a lower extent than the full-length DC-SIGN 1A.

**Contribution of the Lectin Domain to DC-SIGN Multimerization on the Cell Membrane**—DC-SIGN multimer formation in MDDC could be readily identified by SDS-PAGE (Fig. 1D) (33, 44). In fact, although treatment with the membrane-impermeable cross-linker DTSSP enhanced the formation of high order multimers, DC-SIGN monomers, dimers, and multimers were readily detected by C-20, DSG-1, and DSG-2 antisera under non-reducing conditions (Fig. 1F). The detection of DC-SIGN multimers was almost completely prevented in the presence of reducing agents (Fig. 1, D and F), indicating that disulfide bridges contribute to multimerization. Because all Cys residues within the lectin domain are engaged in intramolecular disulfide bridges (27), we determined the effect of mutating Cys37, the only DC-SIGN cysteine residue outside of the lectin domain and located within the cytoplasmic tail. Mutation of Cys37 had no effect on the degree of formation of DC-SIGN multimers (Fig. 2B, left panel), suggesting that multimerization could be dependent on cysteine residues within the lectin domain. The lectin domain was then removed from either the DC-SIGN prototypic isoform (8dΔL) or from an isoform with only six repeats (6dΔL) (Fig. 2A). Both 8dΔL and 6dΔL constructs displayed greatly reduced multimerization ability (Fig. 2B, right panel), which indicates that, although DC-SIGN multimerization might be mediated by the neck region (34, 35, 45), it requires or is stabilized by the lectin domain of the molecule.

Along this line, the presence of lectin domain-lacking constructs (8dΔL, 7dΔL, and 6dΔL) had a negative impact on the degree of multimerization of DC-SIGN 1A, as we observed a lower level of DC-SIGN 1A multimers in the presence of these deletion constructs (Fig. 2C). This could be explained by an increased formation of heteromultimers (formed by DC-SIGN 1A and constructs lacking the lectin domain), which might exhibit lower stability in the presence of denaturing detergent, thus precluding its detection. If so, the existence of heteromultimers could be demonstrated by coprecipitation experiments on lysates from cells cotransfected with DC-SIGN 1A and 8dΔL. The fact that the 8dΔL isoform was pulled down after immunoprecipitation of lectin domain-containing molecules with the MIR monoclonal antibody (Fig. 2D) confirms that lectin domain-lacking constructs associate with the prototypic DC-SIGN 1A isoform, suggests that heteromultimers of DC-SIGN 1A and 8dΔL are more sensitive to the presence of denaturing agents than DC-SIGN 1A homomultimers and confirms a role for the lectin domain of DC-SIGN in the formation of stable oligomers.

**Structural Requirements of the Neck Domain for DC-SIGN Multimerization**—Although the neck domain is absolutely required for the formation of multimers of recombinant non-glycosylated DC-SIGN and DC-SIGNR (34, 35, 45), its role in DC-SIGN multimerization on the cell membrane remains unclear. To address this issue, we analyzed the pattern of multimerization of the prototypic full-length molecule (1A), naturally occurring (4d, 4d’, 2d, and 1d) or in vitro generated (3d) isoforms differing in the number and order of the neck region repeats, and constructs mutated at the N-linked glycosylation site (1AN/Q and 1dN/Q) (Fig. 3A). Transient transfection revealed that the distinct DC-SIGN isoforms differed in their ability to form oligomers. A high proportion of full-length DC-SIGN 1A appeared as multimers, whereas deletion of half the neck region (4d) resulted in a considerable reduction of high order multimers (Fig. 3A). By contrast, isoforms 3d and 2d, whose neck regions are composed of three and two repeats, exhibited an oligomerization ability roughly similar to that of the full-length molecule, whereas isoform 1d showed the weakest oligomerization (Fig. 3A). These results indicate that the presence of at least two repeats within the neck region is sufficient for DC-SIGN multimerization. On the other hand, the lower multimerization of 4d suggests that there is no direct correlation between the length of the neck region and oligomerization, and that the distinct repeats within the neck region might not be functionally equivalent. This hypothesis was confirmed when comparing the low multimerization capability of 4d (composed of neck repeats 1, 6, 7, and 8) with the normal (similar to 1A) oligomerization pattern of 4d’ isoform, whose neck region is composed of repeats 1, 2, 3, and 8 (Fig. 3A), thus confirming that multimerization capability of DC-SIGN on the cell membrane is dependent not only on the number of neck repeats but also on their arrangement, and that the repeats
within the neck region of DC-SIGN are not functionally interchangeable.

In agreement with the results obtained after transient transfection, the 4d isoform also exhibited a greatly reduced proportion of DC-SIGN multimers when stably expressed in K562 cells, whereas multimerization of 2d isoform was similar to that of DC-SIGN 1A (Fig. 3B), a finding also observed after transfection in T lymphoblastoid Jurkat cell (data not shown). Functional analysis of the three isoforms in K562 transfectants revealed that 1A, 4d, and 2d bound soluble C. utilis glucomannan (IF), as determined by one-dimensional saturation transfer difference (43, 46) (Fig. 3C), and were internalized after MR1-mediated engagement (Fig. 3D). Therefore, it can be concluded that the degree of multimerization of functional DC-SIGN isoforms on the cell surface is cell-type independent and does not influence the ligand-induced internalization of the molecule.

The inability of the first repeat to mediate multimerization (1d in Fig. 3A), and the fact that it contains the only potential N-glycosylation site of DC-SIGN, prompted us to determine the contribution of glycosylation to DC-SIGN oligomerization. Replacement of Asn^{80} for Gln in the context of the full-length molecule (1AN/Q) greatly increased the proportion of DC-SIGN multimers (compare 1A and 1AN/Q in Fig. 3A) and suggests that glycosylation of the first neck repeat negatively affects DC-SIGN multimerization. The negative influence of glycosylation on multimerization was even more evident upon analysis of the 1dN/Q mutant, whose neck domain is formed only by the first repeat with the Asn^{80}/Gln replacement. Unlike the 1d isoform, oligomers (and even high order multimers) of the 1dN/Q mutant could be easily detected (Fig. 3A). In fact, and like in the case of 1AN/Q, no 1dN/Q monomers were observed under non-reducing conditions (Fig. 3A). Therefore,
glycosylation of the first repeat in the neck region impairs multimerization of DC-SIGN molecules.

Influence of Multimerization on DC-SIGN Pathogen Recognition—Despite the differences in their ability to multimerize, transient transfection of the whole range of constructs previously assayed revealed that all of them are capable of binding *Candida* yeasts and *Leishmania* amastigotes to a similar extent (supplemental Fig. S1A). To rule out the subtle differences in pathogen binding among the distinct constructs we evaluate *Candida* and *L. pifanoi* amastigotes binding by cells expressing decreasing levels of three naturally occurring isoforms (1A, 4d, or 2d) isoforms. DC-SIGN expression at different time points is shown relative to the initial cell surface expression (100%, upper panel), and was determined by flow cytometry. For the three stable transfectants, the MFI (lower number) and the percentage of positive cells (upper number) at time zero are shown.

FIGURE 3. Multimerization capacity of DC-SIGN isoforms and constructs. A and B, lysates from transiently transfected HEK293T cells (A) or K562 cells stably transfected (B) with the indicated DC-SIGN constructs (see upper drawing) or a mock construct were analyzed by SDS-PAGE and subjected to Western blot with the DSG-2 polyclonal antiserum. In B, two different clones of K562-DC-SIGN 1A were analyzed, whose relative level of DC-SIGN expression is indicated by a dark triangle. C, binding of *C. utilis* glucomannan (IF) to K562 cells stably transfected with the indicated DC-SIGN isoforms by means of one-dimensional saturation transfer difference NMR. The lower profile represents the \(^1\)H NMR spectrum of IF in PBS at 298 K. For comparative purposes, two subpopulations of K562-DC-SIGN 1A, which differ in their DC-SIGN cell surface expression level, were assayed. The graph illustrates the signal intensity yielded by each transfectant (y-axis) and the chemical shift (δ) in parts per million (ppm). D, monoclonal antibody-induced internalization of DC-SIGN isoforms in K562 cells stably transfected with the 1A, 4d, or 2d isoforms. DC-SIGN expression at different time points is shown relative to the initial cell surface expression (100%, upper panel), and was determined by flow cytometry. For the three stable transfectants, the MFI (lower number) and the percentage of positive cells (upper number) at time zero are shown.
avoid pathogen-induced clustering effects on the membrane, we assess the ability of the distinct DC-SIGN constructs to be retained by sugars after membrane solubilization. As shown in Fig. 4A (upper panels), except 1d, all DC-SIGN constructs were specifically retained by mannan (a polysaccharide that blocks most DC-SIGN interaction). However, analysis of molecules not retained by mannan (supernatant) revealed that constructs 1A, 1AN/Q, 4d, and 1dN/Q are retained with higher efficiency than the 4d/H11032, 3d, and 2d constructs (Fig. 4A, lower panels). Monomers were preferentially retained by mannan within the strong mannan-binding and N-glycosylation-containing constructs (1A and 4d) (lanes 1A and 4d in the left panels of Fig. 4A). By contrast, those exhibiting lower binding to mannan (4d’, 3d, and 2d) were preferentially retained as multimers, as monomers were almost exclusively detected in the supernatant (lanes 4d’, 3d, and 2d in left panels of Fig. 4A). Furthermore, and in agreement with the negative effect of N-glycosylation on DC-SIGN multimerization, the 1AN/Q and 1dN/Q constructs were preferentially retained as multimers. Therefore, functional analysis of detergent-solubilized cellular DC-SIGN demonstrates that multimer formation compensates for the lower mannan-binding affinity of certain DC-SIGN constructs after membrane solubilization, an effect that becomes even more evident when less-than-optimal sugar ligands (NAc-Gal) were used, which only retained lectin multimers (Fig. 4B). Therefore, this set of data indicates that the number and arrangement of the repeats within the neck domain directly influences the specificity and the sugar-binding ability of the DC-SIGN lectin domain.

To further evaluate the relevance of DC-SIGN cell surface multimerization on ligand binding, cell surface expressed 1d and 1dN/Q constructs were compared in their ability to bind FITC-PAA-Fucose and Lewis* beads. 1dN/Q, which appears almost exclusively as multimers, displayed a stronger bead-binding activity than 1d, whose multimers can barely be detected, and the same finding was observed at three distinct cell surface expression levels (high, left panel; middle, middle panel; and low, right panel). These results further support the involvement of cell surface DC-SIGN multimerization in ligand binding, and establish N-linked-glycosylation as a critical parameter for the DC-SIGN ligand-binding activity on the cell surface.
Structural and Functional Characterization of Polymorphic Variants of DC-SIGN—The above results demonstrate that the neck region is an important determinant in the ligand-binding activity of DC-SIGN on the cell surface. It has been reported that, among the polymorphisms in the DC-SIGN gene (38, 49–52), those affecting the length of the neck domain correlate with altered susceptibility to HIV-1 infection (38). In fact, similar findings have been reported in the case of the related DC-SIGNR and susceptibility to HIV-1 and severe acute respiratory syndrome infection (53–55). To evaluate the functional significance of polymorphic DC-SIGN neck domains, three distinct allelic variants, whose neck domains contain only seven repeats, were identified at the genomic DNA and RNA level (Fig. 5, A and B) and functionally characterized. These polymorphic variants lack repeats 3, 5, or 7, but their multimerization ability (Fig. 5B), cell surface expression (Fig. 5C), and ligand-induced internalization capability (Fig. 5D) were found to be indistinguishable from that of the prototypic molecule. Moreover, the three variants displayed unaltered capacity for recognition of Leishmania and Aspergillus (supplemental Fig. S2A) and mediated cellular binding to Ebola GP1-Fc and Mannan (supplemental Fig. S2B) and were retained by agarose-bound mannan after membrane solubilization (supplemental Fig. S2C), and mediated binding of C. utilis glucomannan to cells as determined by one-dimensional saturation transfer difference NMR (supplemental Fig. S2D). Therefore, DC-SIGN polymorphic variants lacking a single neck domain repeat (3, 5, or 7) exhibit functional activities that are similar to those exhibited by the prototypic DC-SIGN molecule.

Because altered susceptibility to infections has been mostly observed in individuals with heterozygosity at the DC-SIGN (or DC-SIGNR) gene (38, 53–55), we next evaluated the influence of DC-SIGN polymorphic variants (-D3, -D5, and -D7, Fig. 5B) on the expression, multimerization, and functional capability of the prototypic molecule when expressed on the same cell. Transient transfection experiments demonstrated that the expression of any of the polymorphic variants had no influence on the DC-SIGN 1A total or cell surface expression (Fig. 6, A).
Like in the case of the 1A/8dΔ1/cotransfectants, heterooligomers might have an increased sensitivity to denaturing agents. However, coimmunoprecipitation experiments with epitope-tagged molecules demonstrated that the DC-SIGN 1A molecules preferentially formed homo-oligomers, and associate weakly to seven repeat-containing polymorphic variants (1% of the prototypic DC-SIGN molecules are engaged in hetero-oligomer formation) (Fig. 6C). Therefore, the shorter polymorphic variants can be expressed on the cell surface but tend to form homo-oligomers and associate very weakly with the prototypic DC-SIGN 1A full-length isoform. This result would imply that cells heterozygous at the DC-SIGN gene might almost exclusively express homo-oligomers on the cell surface. We tested this hypothesis by analyzing the DC-SIGN isoform expression and multimerization state in MDDCs from a donor previously identified as a CD209 heterozygote (see Fig. 5A). The prototypic and shorter variant of DC-SIGN were expressed to a similar extent in heterozygous dendritic cells, but no evidence was found of hetero-oligomer formation (Fig. 6D), confirming that DC-SIGN multimerization takes place preferentially among variants whose neck region has identical structure.

The low percentage (or impaired stability) of lectin heterooligomers might explain the reduced pathogen-binding capacity exhibited by cells coexpressing allelic variants of DC-SIGNR (53) and the correlation between DC-SIGN/DC-SIGNR neck region heterozygosity and susceptibility to viral infection (38, 53–55). Consequently, DC-SIGN-dependent activities of cells coexpressing different DC-SIGN allelic variants were evaluated on transiently transfected cells. As shown in Fig. 6B, coexpression of the DC-SIGN-D7 isoform (which lacks the seventh neck domain repeat) did not significantly affect the ability of the prototypic DC-SIGN 1A isoform to bind immobilized mannan. Along the same line, capture of fucose- or Lewisx-coated polyacrylamide beads by DC-SIGN 1A was not affected by the coexpression of the DC-SIGN-D3 isoform (which lacks the third neck domain repeat) (Fig. 7A and not shown). These results indicated that expression of polymorphic variants with shorter
neck domains does not significantly alter the pathogen recognition ability of cells expressing the prototypic DC-SIGN 1A isoform.

Finally, because sugar precipitation had previously allowed the identification of functional differences among alternatively spliced isoforms (Fig. 4), lysates from MDDCs coexpressing DC-SIGN 1A and DC-SIGN-D7 were subjected to precipitation with mannan-agarose. Whereas a single band (corresponding to DC-SIGN 1A) was specifically retained from the 1A/1A dendritic cells, both DC-SIGN 1A and DC-SIGN-D7 isoforms were equally retained by mannan-agarose when the dendritic cell lysate from the 1A/-D7 donor was used (Fig. 7B). Therefore, DC-SIGN 1A and DC-SIGN-D7 are retained by mannan to a similar extent, confirming that shorter neck polymorphic variants of DC-SIGN retain their sugar-recognition ability, showing no differences from that of the prototypic full-length DC-SIGN 1A molecule.

DISCUSSION

DC-SIGN-dependent binding and uptake of clinically relevant pathogens by dendritic cells relies on the lectin ability to bind mannos- and fucose-containing glycans (56). Studies on recombinant molecules have demonstrated that the avidity of such interactions is mediated through multimerization of the lectin, which is accomplished through intermolecular associations mediated by the neck domain of the molecule (27, 34). The neck region of DC-SIGN is composed of eight 23-amino acid repeats, which are encoded in a single exon whose polymorphism has been already demonstrated (38, 51). In fact, DC-SIGN alleles with 4–9 repeats within the neck region-coding exon have been described (51), and heterozygosity at this specific exon correlates with altered susceptibility to HIV-1 infection (38). Besides, numerous DC-SIGN alternatively spliced isoforms have been described at the mRNA level (14, 36). The combination of alternative splicing and genomic polymorphism predicts that a large repertoire of DC-SIGN protein isoforms might exist, most of which would differ in the size of the neck domain (14, 36, 38, 51). However, to date, the functional characterization of DC-SIGN isoforms and allelic variants on the cell membrane had not been addressed. In the present manuscript we present evidences that 1) DC-SIGN alternatively spliced mRNA species give rise to proteins that are expressed at the cell membrane on monocyte-derived dendritic cells, cell lines, and transfectants; 2) DC-SIGN alternatively spliced isoforms differ in their multimerization capability and sugar-binding ability; 3) the presence of two repeats within the neck domain is sufficient for DC-SIGN multimerization; 4) the neck domain repeats are not functionally interchangeable, because the number and arrangement of repeats within the neck domain critically determines the multimerization and ligand-binding ability; 5) the lectin domain of DC-SIGN stabilizes or contributes to the neck region-dependent multimerization of DC-SIGN, which is negatively influenced by the N-linked glycosylation of the first neck domain repeat; 6) basal multimerization of the molecule does not predict the pathogen-binding ability and does not correlate with ligand-induced internalization; and 7) polymorphic variants differing in neck domain composition can self-associate, but multimerize very poorly with the prototypic full-length molecule, suggesting that the DC-SIGN molecules on the cell surface predominantly appear as homo-multimers. The data here presented constitutes the first demonstration that alternative splicing and polymorphic variants of DC-SIGN are expressed on monocyte-derived dendritic cells, where they exhibit altered multimerization and carbohydrate-binding abilities (splicing variants) and tend to segregate...
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from the prototypic molecule forming homo-multimers (polymorphic variants with a shorter neck domain).

Whether DC-SIGN and related lectins are bona fide pathogen-recognition receptors or antigen-binding receptors whose function is subverted by pathogens is still unclear (4, 57). Our results indicate that the various alternatively spliced isoforms differ in their ability to be retained by immobilized mannan, whereas all of them are equally efficient in terms of pathogen binding. We hypothesize that the large amount of DC-SIGN ligands on the surface of interacting pathogens compensate for the distinct affinity and multimerization ability of the isoforms. If this is the case, pathogen-induced formation of DC-SIGN-containing clusters on the cell surface would counterbalance for the diminished multimerization ability of certain isoforms and would justify the large range of pathogens bound and internalized via DC-SIGN. Therefore, according to this hypothesis, isoforms would have a physiological role (increasing the range of soluble antigens bound and internalized by DC-SIGN), but would not have a major impact on the range of pathogens bound by DC-SIGN. Further studies are needed to clarify these issues, because it is currently unknown whether the basal multimerization of DC-SIGN on the cell surface (33, 44) is exclusively mediated by intermolecular interactions or is a soluble ligand-induced event. In this regard, all the experiments performed in the present study were done after extensive washing of the cells with EDTA, to prevent any carbohydrate-DC-SIGN interaction that might affect multimerization of the molecule on the cell surface.

Sequence analysis has allowed the definition of 23-residue repeats within the neck region of DC-SIGN, which is sometimes divided into 7.5 repeats to account for the presence of an unrelated and unique sequence at the N-terminal half of the first repeat (34). Ultracentrifugation and cross-linking of recombinant truncated DC-SIGN molecules have established that removal of the two N-terminal repeats only partially affected the tetramerization ability, whereas recombinant proteins containing only repeats 7–8 formed partially dissociating dimers. This has led to the proposal that repeats close to the lectin domain mediate dimer formation while the membrane proximal repeats are required for tetramer formation (34). Our results with transient and stable transfectants of the naturally occurring DC-SIGN 4d and 2d isoforms, which include repeats 1, 6, 7, and 8 and 1 and 2 (Fig. 3A), indicate that the two more N-terminal domains are sufficient for multimerization in a cellular context, a fact further confirmed by the very different multimerization capability of the 4d (1, 6, 7, and 8) and 4d′ (1, 2, 3, and 8) isoforms. The importance of repeats 1 and 2 for the ability of DC-SIGN to multimerize in the cell membrane is even more evident when considering that the DC-SIGN 1d isoform (containing only repeat 1) does not multimerize, and that removal of the N-glycosylation site (1dN/Q mutant) allows multimerization within a cellular context. In addition, the 3d construct, which includes the first repeat followed by the N-terminal half of repeat 2, the C-terminal half of repeat 7 and the entire repeat 8, also exhibits an efficient multimerization capability within a cellular context. Therefore, essential residues for multimerization can be mapped to the sequence GELSE at the beginning of the second repeat, which includes a serine residue unique among the repeats and contributes to the multimerization ability of recombinant DC-SIGNR (58). These results demonstrate the critical role of repeats 1 and 2 for DC-SIGN multimerization, because repeat 1 is capable of mediating multimer formation, and the mere presence of repeat 2 appears sufficient to overcome the inhibitory effect of the N-glycosylation at repeat 1. These results are compatible and extend previous data on the multimerization capability of recombinant DC-SIGN/DC-SIGNR molecules, and establish neck glycosylation as an important parameter to limit the degree of DC-SIGN multimerization in the cell.

The combination of genomic polymorphism and alternative splicing at the DC-SIGN gene results in the generation of a large number of isoforms/allelic variants of the molecule. Considering their variable multimerization capability, and the higher avidity displayed by multimers, it is tempting to speculate that the existence of all these variants might endow macrophages and dendritic cells with a broader repertoire of ligand-binding affinity and/or specificity. In fact, the ability of mannan-agarose to differentially retain the various DC-SIGN splicing isoforms (Fig. 4) would support this hypothesis. On the other hand, an alternative function for the numerous DC-SIGN isoforms could be the modulation of full-length DC-SIGN-dependent functions. In this regard, and like the lectin domain-lacking chimeric constructs (Fig. 2), isoforms with truncated lectin domains might reduce the effective concentration of full-length DC-SIGN molecules on the cell membrane, thus impairing its multimerization on the cell surface and, consequently, the binding and uptake of pathogens/ligands containing limiting amounts of sugar ligands.

Regarding polymorphic variants, our results indicate that the number of DC-SIGN allelic variants is greater than previously thought. The study of Barreiro and Liu (38, 51) has defined polymorphisms within the neck region of DC-SIGN and classified them according to the number of repeats. However, and at least within the Spanish population, the allelic variants containing only 7 neck repeats are not structurally identical, and three distinct alleles have been identified which differ in the missing repeat within the neck domain (D3, D5, and D7). Therefore, it is likely that most of the previously defined CD209 alleles are really heterogeneous in terms of the arrangement of the neck domain repeats they contain. On the other hand, and despite the association found between neck domain heterozygosity at the CD209 and CD209L genes and altered susceptibility to HIV-1 (38, 55), hepatitis C (59, 60), or severe acute respiratory syndrome infection (53), the polymorphic variants that we have characterized exhibit similar homo-multimerization capability and pathogen- and carbohydrate-binding specificity as the full-length molecule. However, the polymorphic variants containing seven repeats (−D3, −D5, and −D7) exhibit a very weak ability to assemble into hetero-multimers with the full-length DC-SIGN 1A prototypic molecule, as hetero-multimers cannot be observed by Western blot and an extremely low percentage of the −D3 variant can be coprecipitated with DC-SIGN 1A (Fig. 6). This result is in contrast to the reported ability of recombinant polymorphic forms of DC-SIGNR to engage in stable homo- and hetero-tetramers (58). However, we feel that this is only an apparent discrepancy, because the N-linked glycosyla-
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tion of the N-terminal neck repeat limits the extent of multimerization of the molecule within a cellular context (Fig. 2) and, therefore, recombinant molecules (which are devoid of glyco-sylation) might display an enhanced tendency to multimerize. Whether the reduced ability of DC-SIGN polymorphic variants to associate with the full-length molecule contributes to the altered susceptibility of heterozygous individuals to various infections remains to be determined. However, the preferential formation of homo-multimers in heterozygous individuals must lead to a reduction (50%) in the number of multimers containing the full-length DC-SIGN 1A molecule, what might affect the recognition of pathogens with a limiting amount of carbohydrate ligands. The fact that CD209 gene promoter polymorphisms, thought to affect DC-SIGN cell surface levels, also associate with altered susceptibility to HIV-1 (52), Dengue (37), and tuberculosis (49) is compatible with the above explanation. Consequently, although further studies are required, our results demonstrate that expression of neck domain splicing and allelic variants influence the presence and stability of DC-SIGN multimers on the cell surface, and provide relevant clues about the underlying molecular mechanisms for the association between DC-SIGN polymorphisms and altered susceptibility to clinically relevant pathogens.

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3903
A

Supplementary Figure 1

| Pathogen                | Mock | 1A | 1AN/Q | 4d  | 4d' | 3d  | 2d  | 1d  | 1d-N/Q |
|-------------------------|------|----|-------|-----|-----|-----|-----|-----|--------|
| C. albicans yeasts      | 7.1% |79.9%|      |6.8% |75.5%| 4.4%| 9.0%|11.4%|        |
| L. infantum amastigotes | 84.1%| 84.3%|11.4% |88.0%| 88.8%|17.9%|14.5%|11.4%|4.4%    |

% cells with bound pathogens

C. albicans yeasts

L. infantum amastigotes

Pathogen

Mock
1A
1AN/Q
4d
4d'
3d
2d
1d
1d-N/Q

B

% cells with bound spores

% cells with bound spores

3 2 1 0.5
1A
4d
2d

1A
4d
2d

Mock
1A
1AN/Q
4d
4d'
3d
2d
1d
1d-N/Q

1.5 0.75

81.8 (9.9) 79.2 (8.0) 74.4 (5.7) 39.4 (1.2)
77.7 (5.8) 66.8 (3.0) 32.0 (0.9)
79.3 (7.0) 52.7 (2.2) 29.2 (0.8)

% cells with bound spores

3 2 1 0.5

3 1.5 0.75

3 1.5 0.75

µg DNA transfected

Supplementary Figure 1
Supplementary Figure 1.- Pathogen-binding capacity of DC-SIGN isoforms and mutants.- A, B. HEK293T cells transiently expressing the indicated DC-SIGN isoforms/mutants (A) or decreasing levels of DC-SIGN 1A, 4d or 2d (B), were incubated with fluorescent *C. albicans* yeasts and *L. infantum* amastigotes and the percentage of cells with bound pathogens was determined by flow cytometry. Cell surface expression of each DC-SIGN construct is indicated (thick lines, DC-SIGN; thin lines, control antibody for (A) and one profile for each distinct amounts of transfected plasmids in (B) (3 µg, thick line; 2 or 1.5 µg, thin line; 1 µg, dashed line; 0.75 and 0.5 µg, dotted line). In each case, the Mean Fluorescence Intensity and the percentage of positive cells are shown. The experiment was done three times with similar results, and a representative experiment is shown.

Supplementary Figure 2.- Pathogen and sugar-binding capacity of DC-SIGN polymorphic variants.- A. K562 cells stably transfected with the indicated DC-SIGN variants were incubated with fluorescent *C. albicans* yeasts and *L. infantum* amastigotes, in the presence of either X63 or MR1 antibodies as indicated, and the percentage of cells with bound pathogens was determined by flow cytometry. In each case, the first number indicates the percentage of cells with bound amastigotes or conidia, and the Mean Fluorescence Intensity of the whole cell population is indicated in parenthesis. B. Adhesion to immobilized mannann (upper panel) or binding of Ebola GP1-Fc (lower panel) of K562 cells stably expressing the indicated DC-SIGN variants in the presence of a blocking (MR1) or an irrelevant antibody (X63) antibody. For comparative purposes, two subpopulations of K562-DC-SIGN 1A with different DC-SIGN cell surface expression levels were assayed. C. Lysates from COS-7 cells transiently transfected with the indicated DC-SIGN polymorphic variants were incubated with mannann-agarose. After extensive washing, bound (Eluted, left panel) and non-bound proteins (Supernatant, right panel) were resolved by SDS-PAGE under non-reducing conditions and subjected to Western blot with DSG-2. D. Binding of *Candida utilis* glucomannan (IF) to K562 cells stably transfected with the indicated DC-SIGN polymorphic variants by means of 1D Saturation Transfer Difference NMR. The lower profile represents the 1H NMR spectrum of IF-S in PBS at 298 K. Graph illustrates the signal intensity yielded by each transfectant (y-axis) and the chemical shift (δ) in parts per million (ppm).
Structural Requirements for Multimerization of the Pathogen Receptor Dendritic Cell-specific ICAM3-grabbing Non-integrin (CD209) on the Cell Surface
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