

Fine Mapping the Interaction Between Dendritic Cell-Specific Intercellular Adhesion Molecule (ICAM)-3-Grabbing Nonintegrin and the Cytomegalovirus Envelope Glycoprotein B

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Background. Human cytomegalovirus (HCMV) is a leading cause of virally induced congenital disorders and morbidities in immunocompromised individuals, ie, transplant, cancer, or acquired immune deficiency syndrome patients. Human cytomegalovirus infects virtually all cell types through the envelope glycoprotein complex gH/gL/gO with or without a contribution of the pentameric gH/gL/pUL128L. Together with gH/gL, the HCMV envelope glycoprotein B (gB) contributes to the viral fusion machinery.

Methods. We previously showed that gB is a ligand for the C-type lectin dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) contributing to HCMV attachment to and infection of DC-SIGN-expressing cells. However, the features of the DC-SIGN/gB interaction remain unclear. To address this point, the role of glycans on gB and the consequences of mutagenesis and antibody-mediated blockades on both partners were examined in this study.

Results. We identified DC-SIGN amino acid residues involved in this interaction through an extensive mutagenesis study. We also showed the importance of high-mannose *N*-glycans decorating the asparagine residue at position 208, demonstrating that the antigenic domain 5 on gB is involved in the interaction with DC-SIGN. Finally, antibody-mediated blockades allowed us to identify DC-SIGN as a major HCMV attachment receptor on monocyte-derived dendritic cells.

Conclusions. Taken together, these results have permitted us to fine-map the interaction between DC-SIGN and HCMV gB.

Keywords. antibody-mediated blockade; attachment; cytomegalovirus; DC-SIGN; glycoprotein B.

Human cytomegalovirus (HCMV) is a highly prevalent beta-herpesvirus, ie, 40–90 worldwide. The virus is usually asymptomatic, yet it causes diseases in newborns and immunocompromised hosts, eg, transplant, cancer, and acquired immune deficiency syndrome patients [1]. Although neutralizing antibodies (NAb) were shown to target the pentameric complex [2, 3], the HCMV envelope glycoprotein B (gB) remains a target for the humoral response [4, 5]. Human cytomegalovirus gB was recently shown to assemble with gH/gL to form the

fusion machinery that enables virus to penetrate cells [6, 7]. Five gB antigenic domains ([AD] 1 to 5) are recognized by NAb [8]. Human cytomegalovirus gB belongs to the class III fusion proteins with the herpes simplex virus (HSV)-1 gB [9], the Epstein-Barr virus gB [10], the baculovirus gp64 [11], the Thogotovirus glycoprotein [12], as well as the prototypical vesicular stomatitis virus (VSV)-G envelope glycoprotein [13].

Human cytomegalovirus gB interacts with the dendritic cell-specific intercellular adhesion molecule (ICAM)-3-grabbing nonintegrin (DC-SIGN) [14]. Dendritic cell-SIGN is expressed by immature monocyte-derived dendritic cells (MDDC) and macrophage subsets [15, 16]. It is a type II C-type lectin composed of a short N-terminal intracytoplasmic tail, seven and a half conserved 23-amino acid repeats forming the “neck” region involved in tetramerization [17–19], and a C-terminal calcium-dependent carbohydrate-recognition domain (CRD) containing 2 calcium sites enabling ligand binding [15]. Dendritic cell-SIGN tetramers display a high avidity for fucose or high-mannose sugars [20]. It recognizes endogenous ligands such as ICAM-2 and -3 known to stabilize interactions between DC and endothelial cells or naive T-cells, respectively

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[15, 21]. It also functions as a receptor for many viral glycoproteins (for review, see [22]). Nevertheless, the interaction between DC-SIGN and gB remains poorly characterized. To address this point, we examine the role of glycans, mutagenesis, and antibody-mediated blockades.

METHODS

Ethic Statements

Blood samples from anonymous healthy volunteers were obtained from the Etablissement Français du Sang, the French blood bank (EFS, Nantes, France) in accordance with authorizations by the “Ministère français de l’enseignement supérieur et de la recherche” and the “Comité de protection des personnes” (Agreement numbers: DC-2013-1832/DC-2014-2206).

Cells

Monocytes were isolated by immunomagnetic separation (Miltenyi Biotec, Bergisch Gladbach, Germany) or by elutriation (DTC, Nantes University Hospital/Biogen Ouest, Nantes, France) from healthy donors (>95% purity). Monocytes were differentiated into MDDC as previously described [23] with 20 ng/mL recombinant human interleukin-4 (CellGenix, Freiburg, Germany), 100 ng/mL granulocyte-macrophage colony-stimulating factor (Gentaur, Paris, France), and 2% human serum albumin (Vialebex, LFB, Coutaboeuf, France). U937, HEK293T cells (American Type Culture Collection number CRL-3216; LGC Standards, Teddington, UK), and MRC-5 (RD Biotech, Besançon, France) were propagated as described elsewhere [14].

Viruses and Reagents

TRI, an in-house clinical isolate (no sequence data available yet), 3 low passage HCMV laboratory strains (Toledo, TB40/E-GFP and VHL/E; a gift from Dr. Christian Sinzger) [24, 25], and BAC4-based HCMV gB-mutated viruses were propagated on MRC-5 cells and used as clarified viral supernatants for *trans*-infection experiments at a multiplicity of infection (MOI) ranging from 2 to 10. Cytomegalovirus (CMV) supernatants were also titered by quantitative polymerase chain reaction (qPCR) as described elsewhere [26]. Chinese hamster ovary (CHO)-derived recombinant HCMV gB and human immunodeficiency virus (HIV)-1 IIIB gp120 were purchased from Sanofi Pasteur (Marcy l’Etoile, France) and ImmunoDx (Woburn, MA), respectively. Glycoproteins were used either as purified or fluorescence-conjugated reagents (Molecular Probes, Thermo Fisher Scientific, Waltham, MA). Antibodies against AD-4/5 (clones SM5-1 and 1G2) and AD-1/2 (clones ITC33, ITC39, ITC48, ITC52, ITC63, and ITC88) [27] were kindly provided by Professor Michael Mach and Dr. Mats Ohlin, respectively. A polyclonal antibody (pAb) against gB was purchased from Sino Biological ([10202-RP01-100], Beijing, China). Fifteen antihuman DC-SIGN antibodies from Dendritics SA (Lyon, France) were used in this study. Four clones, 103G2.07, 111E3.04, 102E11.06, and 111H2.02, are directed against the neck region (amino acids [AA] 96–257),

whereas 9 (clones 104B4.01, 103F12.01, 109H12.03, 113B11.02, 106A4.01, 120E12.03, 108H8.05, 105E9.01, and 114F1.08) were targeting the CRD (AA, 258–404). We considered that clone 108C7.01 recognized both the extracellular proximal region ([ECPR] AA 59–95) and the CRD, whereas clone 102F10.04 bound to the ECPR alone (see [Supplementary Table 2](#)). Four other commercially available anti-DC-SIGN monoclonal antibodies (mAb), clones MR1 (Bio-Rad AbDSerotec, Oxford, UK), AZN-D1 (Beckman Coulter France, Villepinte, France), 1B10, and an anti-neck (H200, Santa Cruz Biotechnology Inc., Heidelberg, Germany), were also used here. Antibodies targeting platelet-derived growth factor receptor (PDGFR) α (aR1), the β_1 chain integrin (MAR4), and the $\alpha_v\beta_3$ integrin (23C6) were purchased from BD Biosciences (Franklin Lakes, NJ). Antibodies were used at 20 μ g/mL for blockade experiments.

Viral Envelope Glycoprotein Binding Assay

Cells were resuspended in a binding buffer (Tris-buffered saline, 1 mM CaCl₂, 2 mM MgCl₂; 0.1% bovine serum albumin) and seeded in 96-well plates at 1×10^5 cells/wells. Dendritic cell-SIGN/gB interaction blockade was performed by incubating cells with antibodies for 30 minutes at 4°C. Conjugated gB or HIV-1 IIIB gp120 (2 μ g/mL) were added to cells for 20 minutes at 4°C without washing. After 3 washes with ice-cold binding buffer, cells were analyzed on a LSR II flow cytometer (BD Biosciences, Franklin Lakes, NJ) with FlowJo (Tree Star, Ashland, OR). Surface plasmon resonance was carried out with soluble gB and recombinant DC-SIGN extracellular domain [28].

Transinfection Experiments

Cells were incubated with blockers for 30 minutes at 4°C if necessary before adding virus (MOI = 2) without washing. After a subsequent 2-hour incubation at 37°C, cells were washed and cultured with 70%–80% confluent MRC-5 cells for 48–72 hours. Cells were fixed in acetone/water 9:1 (volume/volume) and further stained with an Alexa Fluor 488-conjugated anti-IE/E antigen antibody (8B1.2; Millipore, Burlington, MA). Percentages of IE/E+ cells were determined on 4 fields per condition with Fiji (SciJava consortium; <http://scijava.org/>).

Statistical Analysis

Statistical tests were performed using the Graph Pad Prism 5.0 software (Graph Pad Software Inc., La Jolla, CA). On-way analysis of variance was applied to all data of this manuscript. *P* values below or equal to .05 were considered significant. Additional materials and methods are available in [Supplementary Materials](#).

RESULTS

Dendritic Cell-Specific Intercellular Adhesion Molecule-3-Grabbing Nonintegrin Binds to Glycoprotein B Through Its Carbohydrate Recognition Domain

Although HCMV gB is known as a DC-SIGN ligand, it is not clear whether this interaction is restricted to the DC-SIGN CRD

[14]. To that purpose, HEK293T cells were modified to express wild-type (WT) DC-SIGN (AA 1–404; UnitProtKB, Q9NNX6) or 2 deletion mutants, respectively, lacking neck repeats (AA 1–80 in frame with AA 253–404, called Δ neck) or the CRD (AA 1–252, called Δ CRD) in fusion with the enhanced green fluorescent protein (eGFP) [29]. All cells expressed comparable eGFP levels and DC-SIGN cell surface expression as well (Figure 1A). We showed that gB interacts with CRD-containing DC-SIGN molecules and does not require the neck repeats (Figure 1A and B).

Then, we sought to identify CRD AA involved in this interaction. We hypothesized that AA taking part to the calcium ion coordination or sugar binding could be detrimental [20, 30]. Single-point mutants were generated and further expressed in HEK293T cells. Antineck staining showed similar DC-SIGN expression across all cell lines (Supplementary Figure 1). Their ability to bind gB was then assessed by flow cytometry (Figure 1C). E347, N349, E354, N365, and D366 form the calcium binding “site 2” and enable contact with high-mannose sugars as well [30, 31]. Expectedly, mutations at these positions precluded interaction with gB (Figure 1D). Similarly, mutants D320A, E324A, N350A, and D355A lost their ability to optimally bind gB, assuming that it was likely due to substantial fold changes in the calcium binding “site 1” as proposed for HIV-1 gp120 [32]. Here, F313Y, Q323E, and K368A DC-SIGN mutations were ineffective (Figure 1D). Moreover, we confirmed that the E354Q within site 2 broke the interaction [33]. The V351 residue was shown to discriminate between endogenous and pathogen-derived ligands such as ICAM-3 and HIV-1 gp120 or hepatitis C virus E1/E2, respectively [32, 34, 35]. In this study, we analyzed 2 mutations, ie, V351G and V351T. The V351G mutant lost its binding capacity to gB, suggesting that this AA is as important as its human herpesvirus (HHV)-8 counterpart and ICAM-3 [36]. It is interesting to note that a methyl group substitution of the V351 by a hydroxyl group of a threonine conserved a nominal binding activity, suggesting that subtle variations in the DC-SIGN fold could determine its ability to recognize unrelated ligands. Taken together, these results demonstrate that gB utilizes the same CRD AA as the HHV-8 gB or even ICAM-3.

Recombinant Glycoprotein B Contains High-Mannose Sugars

Glycoprotein B and HIV-1 gp120 are both able to bind to DC-SIGN, although their respective folds are distinct. We postulated that their glycan covers might be similar. Prototypical *N*- or *O*-glycans are depicted in the Figure 2A. Culture wells were coated with various lectins from plant or mammalian origins to characterize glycans on CHO-derived gB. Human immunodeficiency virus-1 gp120 was used as a control of a high mannose-containing glycoprotein. In line with other pioneering studies on gp120 [20], we observed dose-dependent binding of both glycoproteins to DC-SIGN, concanavalin A, and, to a lesser

extent, to wheat germ agglutinin (WGA), suggesting that high mannose-containing *N*-glycans were decorating gB and gp120 (Figures 2B and C). Binding to *Datura stramonium* Agglutinin, and also to a lesser extent to WGA (due to its double specificity), confirmed the presence of *N*-acetylglucosamine. A weak binding to *Maclura pomifera* agglutinin (MPA) at the highest gB concentration suggested that *O*-glycans were almost absent. No significant signal was observed with *Sambucus nigra* agglutinin.

Next, we used whole HCMV virions to perform the same kind of experiments. Here, virus attachment to lectins was quantified by qPCR. Again, DC-SIGN and WGA retained HCMV particles, suggesting the presence of high-mannose sugars on viral particles. It is interesting to note that *O*-glycans were readily recognized by MPA on virions most likely on glycoproteins other than gB. Apparently, no sialic acid (SA) residues could be detected on virions. These results demonstrated that CHO-derived gB and HIV-1 gp120 are decorated with high-mannose sugars on *N*-glycans. We also showed that gB and whole HCMV particles share similar glycans contents except for *O*-glycans.

High-Mannose Sugars Decorating Glycoprotein B Enable Its Binding to Dendritic Cell-Specific Intercellular Adhesion Molecule-3-Grabbing Nonintegrin

Next, we examined the contribution of gB glycans to the interaction with DC-SIGN. Hence, various glycosidases were used to selectively remove sugars from gB. Although a moderate decrease of the gB molecular weight (MW) was observed after a mannosidase treatment, the PNGase F digestion induced a clear shift in the apparent gB MW, confirming that *N*-glycans account for a substantial part of the molecule (Figure 3A). Neuraminidase (NA)-treated gB also exhibited an altered electrophoretic mobility compared with nontreated gB, likely indicating an unexpected presence of SA based on results shown in Figure 3.

We then measured the affinity of DC-SIGN for untreated or deglycosylated gB by surface plasmon resonance spectroscopy. Apparent dissociation constants ($K_{d\ app}$) were calculated from the sensorgrams presented in Figure 3B. The $K_{d\ app}$ values were extrapolated using a kinetic model (Figure 3B). Untreated gB had a $K_{d\ app}$ of 14 pM (Figure 3C). Sialic acid removal did not seem to alter the binding to immobilized DC-SIGN extracellular domain ($K_{d\ app} = 25$ pM), indicating that SA are not important, in line with our results in Figure 2D. In contrast, the mannosidase activity led to a marked $K_{d\ app}$ increase (149 pM), suggesting a function for terminal mannose residues. The PNGase F-treated gB had an even higher $K_{d\ app}$ (230 pM) in that setting, confirming that *N*-glycans are crucial (Figure 3C). The relatively limited $K_{d\ app}$ increase upon removal of *N*-glycans was associated to an incomplete PNGase F digestion in regular conditions [37]. By adding sodium dodecyl sulfate to the PNGase F or using the trifluoromethanesulfonic acid to chemically deglycosylate gB, we confirmed our hypothesis (Supplementary Figure 2).

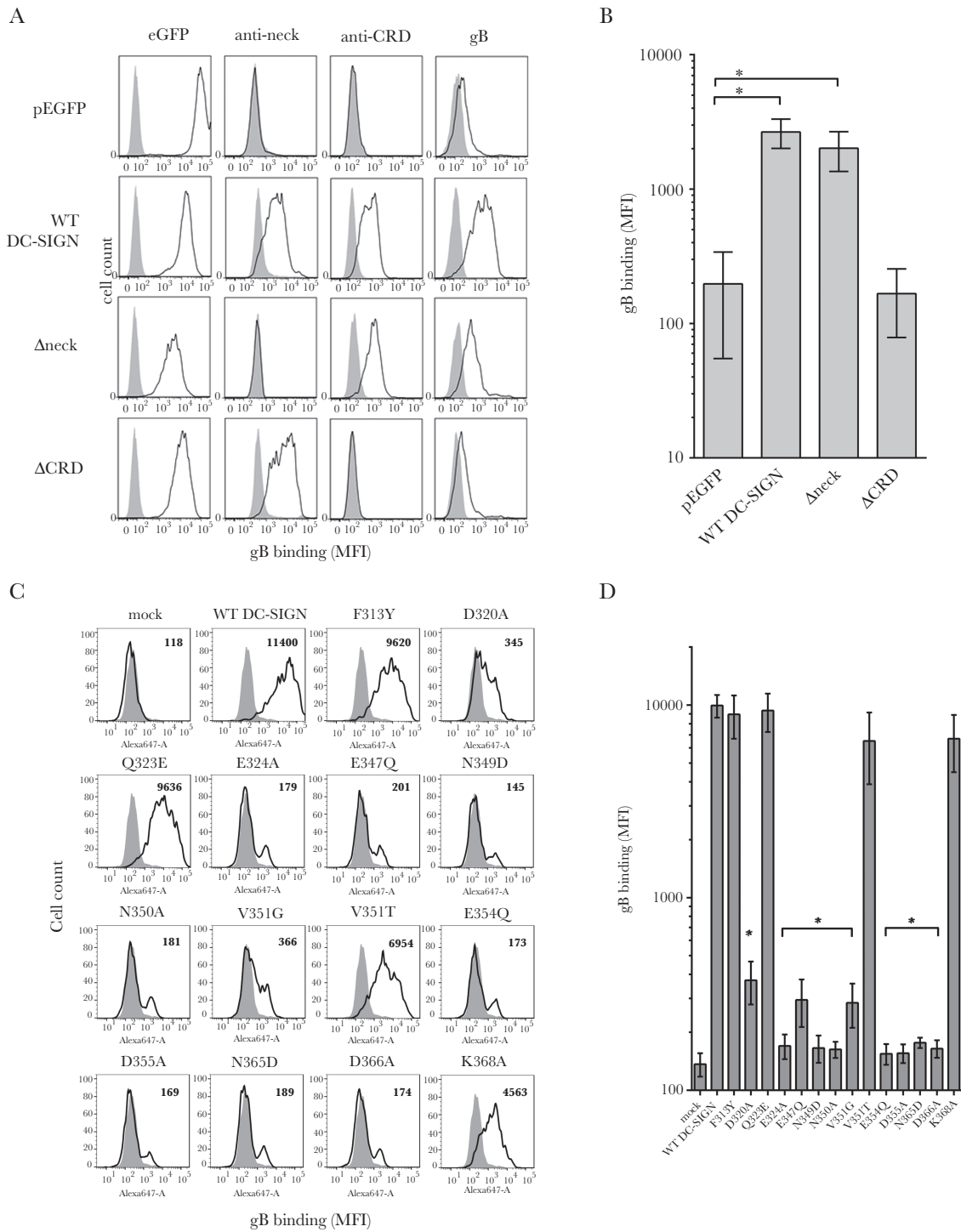


Figure 1. Dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) binds the glycoprotein B (gB) through its carbohydrate recognition domain. (A) Histograms showing DC-SIGN expression of wild-type (WT) DC-SIGN or deletion mutants lacking the DC-SIGN neck repeat (Δ neck) or the carbohydrate-recognition domain (Δ CRD) regions fused to enhanced green fluorescent protein (eGFP). The eGFP allowed a rapid quantitation of the DC-SIGN expression level on stably transfected HEK293T (left panels), except for the pEGFP-transfected cells (first line). The 2 centered columns represent extracellular staining of DC-SIGN with an antineck (clone H-200) and an anti-CRD (clone 1B10) antibody, respectively. The ability of DC-SIGN variants to bind recombinant biotinylated human cytomegalovirus (HCMV) gB is represented in right panels. Gray histograms display nontransfected HEK293T cell fluorescence background. (B) Quantitative measurements of the binding of recombinant biotinylated HCMV gB (2 μ g/mL) onto WT DC-SIGN or Δ neck- and Δ CRD-expressing cells compared with a control cell line (pEGFP). Biotinylated HCMV gB was revealed with 1 μ g/mL antigen-presenting cell-conjugated streptavidin. Values are expressed as mean fluorescence intensities ($n = 4$; *, $P < .05$; one-way analysis of variance [ANOVA] with multiple comparison tests). (C) Histograms showing the binding of recombinant Alexa Fluor 647-conjugated HCMV gB (4 μ g/mL, mean fluorescence intensity [MFI]) on HEK293T cell lines expressing WT or mutated DC-SIGN on their surface. Values indicated for each histogram represent MFI. These results are representative of 3 independent experiments. (D) Quantitative results showing the behavior of mutated DC-SIGN compared with the WT form towards the binding of recombinant Alexa Fluor 647-conjugated HCMV gB ($n = 3$). Statistically significant results were marked by an asterisk (*, $P < .05$; one-way ANOVA with multiple comparison tests).

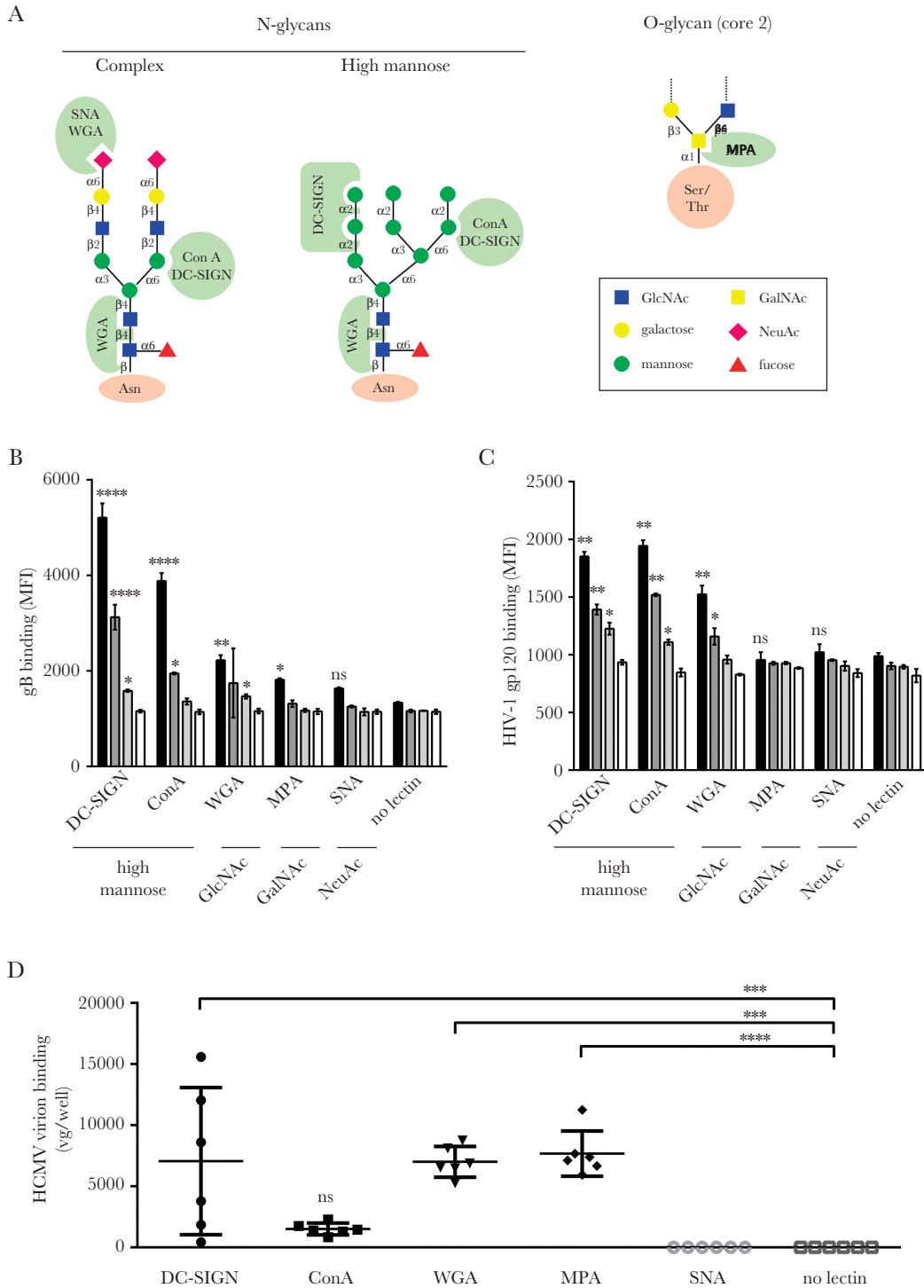


Figure 2. Recombinant soluble or particle-associated human cytomegalovirus (HCMV) glycoprotein B (gB) preferentially interacts with high mannose-specific and *N*-glycan-specific lectins. (A) Schematic representation of typical *N*- or *O*-glycans potentially harbored by glycoproteins. Sugar linkage is indicated between sugar residues. Green zones represent lectin specificities. Lectin binding assay revealing interactions between Alexa 488-conjugated gB (B) or human immunodeficiency virus (HIV)-1 IIIB gp120 (C) and various recombinant plant and animal lectins of known specificities immobilized on plastic 96-well plates. Serial dilutions of each viral glycoprotein were used in this assay: 10 µg/mL (black), 2.5 µg/mL (dark gray), 0.4 µg/mL (light gray), and no gB (open bars). Lectin specificities are indicated beneath each lectin. Results are shown as mean fluorescence intensity (MFI) (n = 3). The “no lectin” condition was used for both recombinant glycoproteins as a negative control providing a basal level of unspecific binding for fluorescence-labeled glycoproteins. (D) Binding of HCMV infectious particles (TB40/E strain, BAC4) to plastic-immobilized plant and animal lectins. A single input of virus was equivalent to 8×10^6 viral genomes/well (vg/well) (n = 6). Mean values are represented by horizontal bars for each type of lectin (****, $P < .0001$; ***, $P < .001$; **, $P < .01$; *, $P < .05$; one-way analysis of variance with multiple comparison tests). Abbreviations: DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin; ConA, concanavalin A; MPA, *Maclura pomifera* agglutinin; ns, nonsignificant; SNA, *Sambucus nigra* agglutinin; WGA, wheat germ agglutinin.

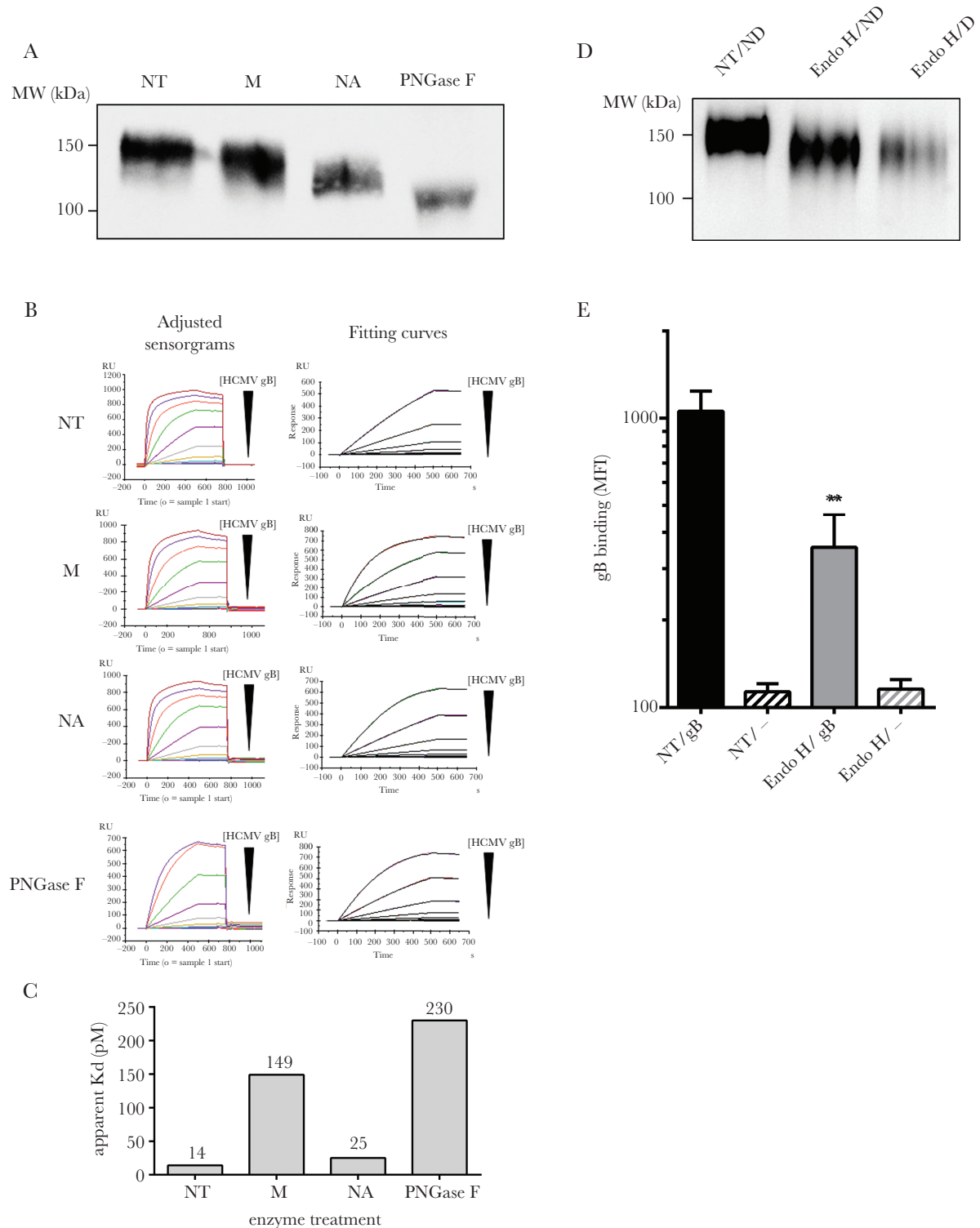


Figure 3. High mannose-containing *N*-glycans on glycoprotein B (gB) are crucial for its interaction with dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN). (A) Western blot analysis showing the detection of recombinant soluble gB with or without enzymatic treatments to remove specifically particular glycan residues. M, NA, and PNGase F stand for the α (1-2,3,6)-mannosidase, the α (2 to 3,6)-neuraminidase, and the peptide-*N*-glycosidase F, respectively. (B) Adjusted sensorgrams and fitting curves (kinetic model 1:1 binding) for $K_{d,app}$ (apparent K_d) measurements. Black triangles indicate decreasing human cytomegalovirus (HCMV) gB concentration ([HCMV gB]) with 10-fold dilution steps. (C) The affinity of HCMV gB for the extracellular domain of DC-SIGN was determined by surface plasmon resonance ($K_{d,app}$) before and after deglycosylation with Man, Neu, or PNGase F. (D) Western blot analysis showing from left to right untreated gB, Endo Hf-digested gB in nondenaturing conditions (ND; O/N, 37°C, no denaturation buffer), and Endo Hf-digested gB in denaturing conditions, ie, 1 hour at 37°C with denaturation buffer. (E) Biotinylated HCMV gB was treated with Endo Hf in nondenaturing conditions or left untreated (NT) and further incubated with wild-type DC-SIGN-expressing HEK293T cells. The HCMV gB fixation was revealed as described in the [Supplementary Materials](#). Abbreviation: MW, molecular weight.

Dendritic cell-SIGN is a high-mannose sugar specific lectin. We looked for the presence of high-mannose sugars on gB. Electrophoretic mobilities of Endo Hf-treated or untreated gB were compared (Figure 3D). Endo Hf removed high mannoses on gB in denaturing (D) and in non-denaturing (ND) conditions but with an extended incubation time (O/N) compared with untreated gB. We showed that Endo Hf-treated gB significantly lost its ability to interact with WT DC-SIGN-expressing HEK293T cells compared with untreated gB (Figure 3E). Altogether, these data show that high-mannose on *N*-glycans but not SA are crucial for the gB/DC-SIGN interaction.

Antibodies Against Antigenic Domains 4 and 5 Impair the Glycoprotein B/Dendritic Cell-Specific Intercellular Adhesion Molecule-3-Grabbing Interaction

To better define the DC-SIGN/gB interaction, we first used mAbs against AD-1 (clones ITC33, ITC39, ITC48, ITC52, and ITC63B) and AD-2 (clone ITC88) for blockade experiments [27]. None of these mAbs blocked the binding except the polyclonal, which demonstrated that AD-1 or -2 were not involved (Figure 4A). Two mAbs, clones SM5-1 and 1G2 recognizing AD-4 and AD-5, respectively [8], were further tested. Results showed a partial but significant blocking activity of both antibodies up to 48% loss of binding on DC-SIGN⁺ U937 or MDDC, suggesting that AD-4 and AD-5 could be responsible for the interaction with DC-SIGN (Figure 4B). It is noticeable that DC-SIGN appeared as the only cellular receptor for gB because the pAb-mediated blockade totally abrogated binding on DC-SIGN⁺ U937 compared with parental cells (Figure 4B, left graph). Those antibodies were also used to block HCMV *trans*-infection by DC-SIGN⁺ cells, which relies on the transfer of DC-SIGN⁺ cells-immobilized HCMV virions, ie, either stuck on the plasma membrane or endocytosed, to HCMV permissive cells. We were surprised to find that although 1G2 consistently blocked half of the *trans*-infection by DC-SIGN⁺ U937 cells, the SM5-1 clone totally abrogated it (Figure 4C). We hypothesized that this was due to the SM5-1 ability to block the pre-fusion to intermediate or postfusion transition, thus impairing fusion and eventually the infection of MCR-5 cells [38]. Similar results were obtained with 4 low-passage HCMV strains, ie, TB40/E-GFP, VHL/E, Toledo, and a clinical isolate from our center (TRI) on MDDC (Figure 4D). Therefore, we concluded that AD-5 and AD-4 were both responsible for the interaction with DC-SIGN.

Mutating the Asparagine At Position 208 on Glycoprotein B Impairs Its Interaction With Dendritic Cell-Specific Intercellular Adhesion Molecule-3 and Subsequent Transinfection

Antigenic domain-4 and AD-5 are heavily *N*-glycosylated on HCMV gB [39]. To identify the high-mannose sugar-containing *N*-glycans recognized by DC-SIGN on gB, all putative *N*-glycosylation sites were mutated and expressed in HEK293T

cells (see Methods, Supplementary Figure 3, and Supplementary Table 1). Wild-type or mutated gB in non-denatured cell lysates were immobilized on a nitrocellulose membrane. The capacity of DC-SIGN-Fc to bind to gB in all conditions was calculated on a normalized gB expression. Values ranged from 0.24 for the negative control (ie, empty pRC-CMV vector) to 1.15 for WT gB. The N208A mutation located in AD-5 led to a significant DC-SIGN-Fc binding decrease, suggesting that *N*-glycans on this residue are important, although other mutants found in AD-4 and AD-5 (from N281A to N409A) behaved similarly (Figure 5A).

We sought to determine whether such a mutation could alter the binding of a whole HCMV particle. To that end, we took advantage of the “en-passant” mutagenesis strategy developed by Tischer et al [40] to modify the *UL55* sequence in the genome of the TB40 HCMV strain (BAC4) to generate gB-mutated virions. Virions bearing N208A, N417A, N447A, N452A, and N585A mutations were reconstituted with similar yields (Figure 5B) reaching a plateau at day 16 postinfection (approximately 1×10^7 genome copies/mL), indicating that mutations did not notably alter growth kinetics during reconstitution (Figure 5C). Then, we demonstrated that the N208A mutation exhibited a significantly altered *trans*-infection ability (Figure 5D). We showed that all mutated and WT virions were infecting MRC-5 cells similarly at the same time point, ie, day 2 postinfection (Figure 5E). We concluded from these results that gB interacts with DC-SIGN mainly through high-mannose containing *N*-glycans on the asparagine at position 208.

Antibody-Mediated Targeting of Dendritic Cell-Specific Intercellular Adhesion Molecule-3 Blocks Glycoprotein B Binding and Neutralizes *Trans*-Infection

Next, we looked at the blockade of the DC-SIGN/gB interaction with specific Abs to DC-SIGN. First, we characterized the specificity of 15 anti-DC-SIGN mAbs by flow cytometry on WT DC-SIGN, Δ neck- or Δ CRD-expressing cell lines. We identified 9 anti-CRD mAbs (7 to 15). Monoclonal Ab 1 was listed as an anti-CRD or -ECPR antibody (Supplementary Table 2). All mAbs were tested in parallel with commercial Abs, 1 anti-neck (H200), and 3 additional anti-CRD antibodies (1B10, AZN-D1, and MR-1), to block gB binding to DC-SIGN. Almost all anti-CRD mAbs including commercial ones were moderate to high inhibitors of this interaction. For mAbs 12 to 15, a total abrogation of gB binding was even observed (Figure 6A). Similar results were obtained with MDDC, confirming that targeting the DC-SIGN CRD almost totally abrogates gB binding (Figure 6B). To clarify whether other known HCMV receptors could participate to this interaction, we analyzed expression of PDGFR α [41], β_1 chain integrin [42], and $\alpha_v\beta_3$ integrin [42] on U937 cells and MDDC. Whereas MDDC displayed low to moderate expression levels for all tested receptors, U937 expressed the β_1 chain integrin and PDGFR α . However, none

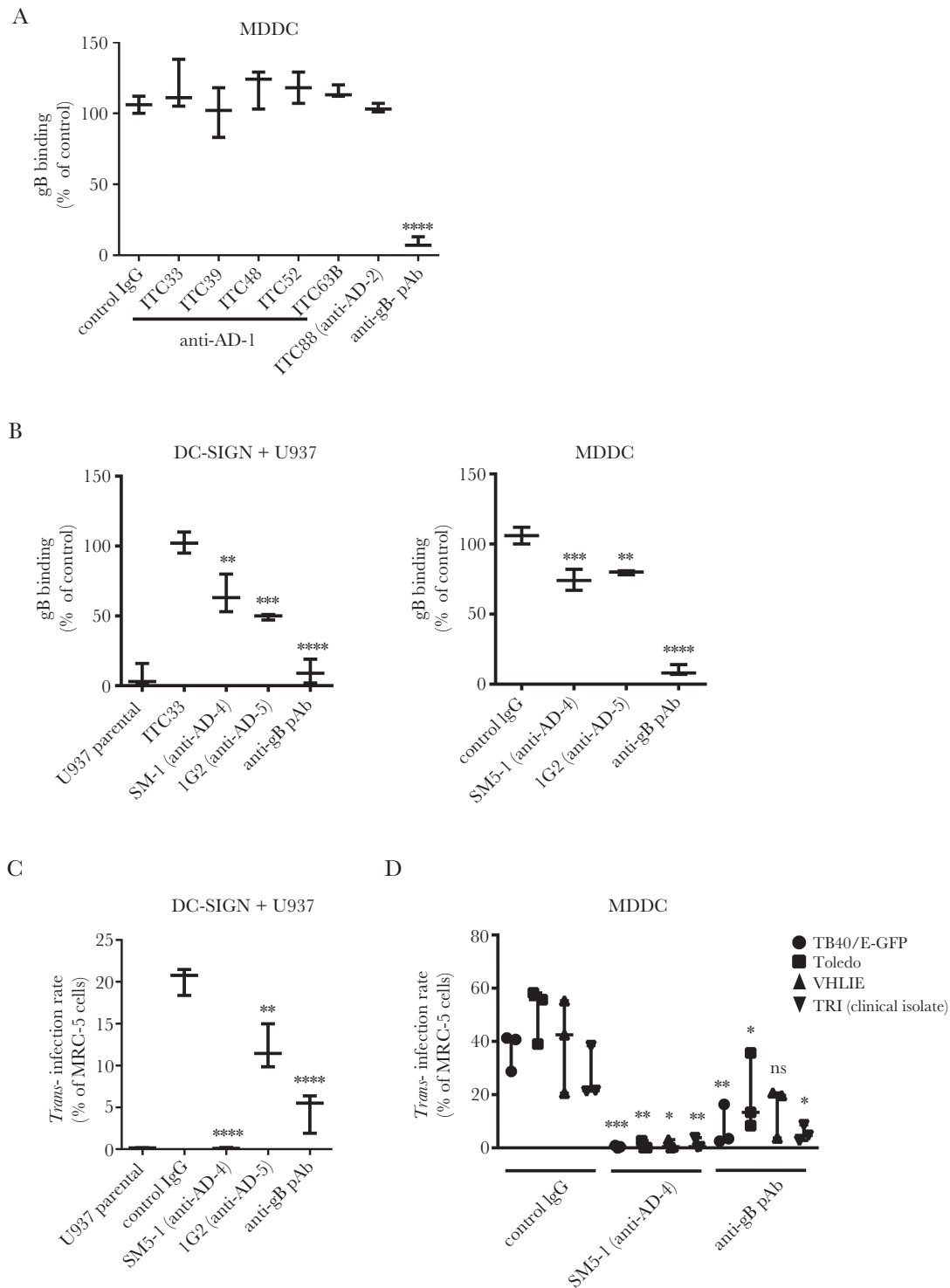


Figure 4. Antigenic domains (AD)-4- and AD-5- but not AD-1/2-specific antibodies inhibit the dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN)/human cytomegalovirus (HCMV) glycoprotein B (gB) interaction and the DC-SIGN-dependent *trans*-infection of various HCMV strains. (A) Anti-AD-1 (ITC33, ITC39, ITC48, ITC52, and ITC63B) monoclonal antibodies (mAbs), anti-AD-2 (ITC88) mAbs, or a polyclonal anti-HCMV gB serum were used as potential competitors of the HCMV gB binding to immature DC-SIGN⁺ monocyte-derived dendritic cells (MDDCs). Binding of HCMV gB was assessed by flow cytometry. Binding intensities are represented as mean percentages of the maximum binding ($n = 3$), ie, without any antibody, and compared with a control immunoglobulin (IgG). (B) Inhibition of gB binding on DC-SIGN⁺ U937 cells compared with the parental counterpart (left panel) or on day 6 immature MDDCs (right panel) by anti-AD-4 (clone SM5-1), anti-AD-5 (clone 1G2), and an anti-gB polyclonal antibody (pAb). Binding intensities are represented as mean percentages of the maximum binding, ie, without any antibody ($n = 5$). (C) Graph showing *trans*-infection results obtained with parental vs DC-SIGN⁺ U937 cells and the TB40/E-GFP strain (multiplicity of infection [MOI] = 2). (D) Similar *trans*-infection experiments with MDDCs loaded with various low-passaged HCMV strains (VHL/E, Toledo, and TB40/E) and a clinical isolate (TRI) (MOI = 2). Asterisks represent significant results compared with the control IgG condition (****, $P < .0001$; ***, $P < .001$; **, $P < .01$; *, $P < .05$; one-way analysis of variance with multiple comparison tests). Abbreviation: ns, nonsignificant.

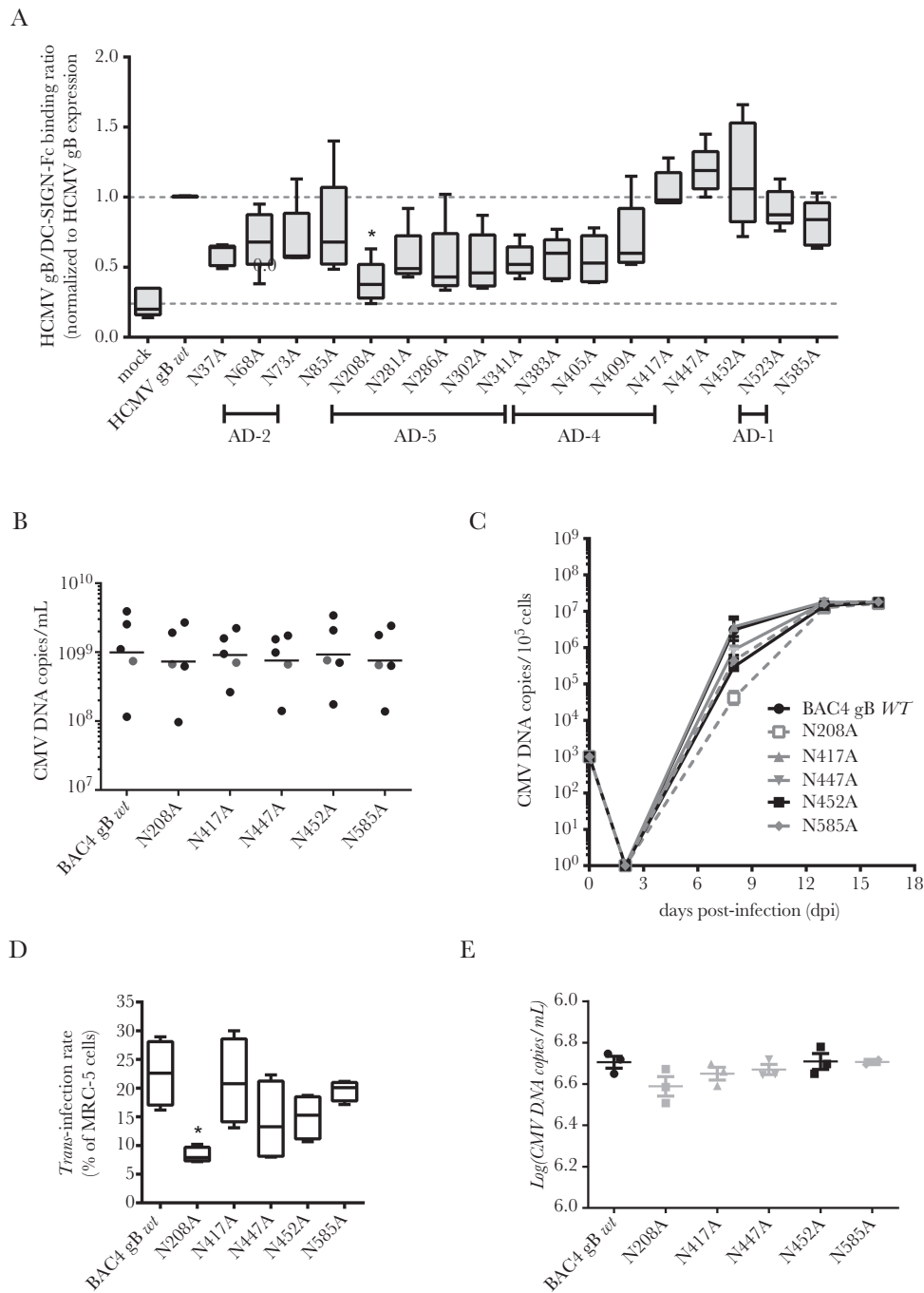


Figure 5. Mutating the asparagine into an alanine at the position 208 of the human cytomegalovirus (HCMV) gBAA sequence selectively impairs both the HCMV glycoprotein B (gB)/dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) interaction and the DC-SIGN-dependent HCMV *trans*-infection. (A) Quantitative assessment of the interaction of DC-SIGN-Fc (2 μ g/mL) on membrane-immobilized cell lysates of HCMV gB either mutated or wild-type (WT)-expressing HEK293T cells ($n = 5$). Mean ratios measuring the interaction between WT HCMV gB and DC-SIGN-Fc were normalized to 1 (top gray dashed line), whereas the “mock” condition determines unspecific DC-SIGN-Fc binding on cells transfected with an empty pRC-CMV plasmid (bottom gray dashed line). (B) Five independent viral supernatants were reconstituted for BAC4 gB *wt* and its mutated versions, ie, N208A, N417A, N447A, N452A, and N585A from BAC-transfected MRC-5 (see [Supplementary Materials](#)). The CMV deoxyribonucleic acid (DNA) copy quantification per volume unit by quantitative polymerase chain reaction (qPCR) for all stocks produced in this work is shown here. Grey dots represent stocks from 1 reconstitution. (C) The growth kinetics of the parental HCMV strain (gB WT) or the virus variants with the indicated mutations in the gB protein on MRC5 cells was assessed at day 2, 8, 13, and 16 postinfection. MRC-5 (10^5) cells were initially infected with a viral dose equivalent to 10^3 genome copies. Virus amounts are given as genome copies per 10^5 cells. (D) Wild-type (BAC4 gB *wt*) and mutated virions (N208A, N417A, N447A, N452A, and N585A) were used to load monocyte-derived dendritic cells (multiplicity of infection [MOI] = 2) for *trans*-infection experiments. *Trans*-infection rates represent the percentages of HCMV-infected MRC-5 cells, ie, IE/E antigen-positive among total MRC-5 cells stained at day 2 postinfection (*, $P < .05$; one-way analysis of variance with multiple comparison tests; $n = 4$). Results are displayed as a minimum to maximum representation; the box plots show the 10th percentile, the mean (intermediate bars in boxes), and the 90th percentile. (E) In parallel, we collected supernatants of MCR-5 infected with the same amount of virus compared with the one used in *trans*-infection experiments (MOI = 2). Quantifications by qPCR (CMV deoxyribonucleic acid [DNA] copies/mL) of these supernatants are shown here ($n = 3$ except for the N585A supernatant where $n = 2$).

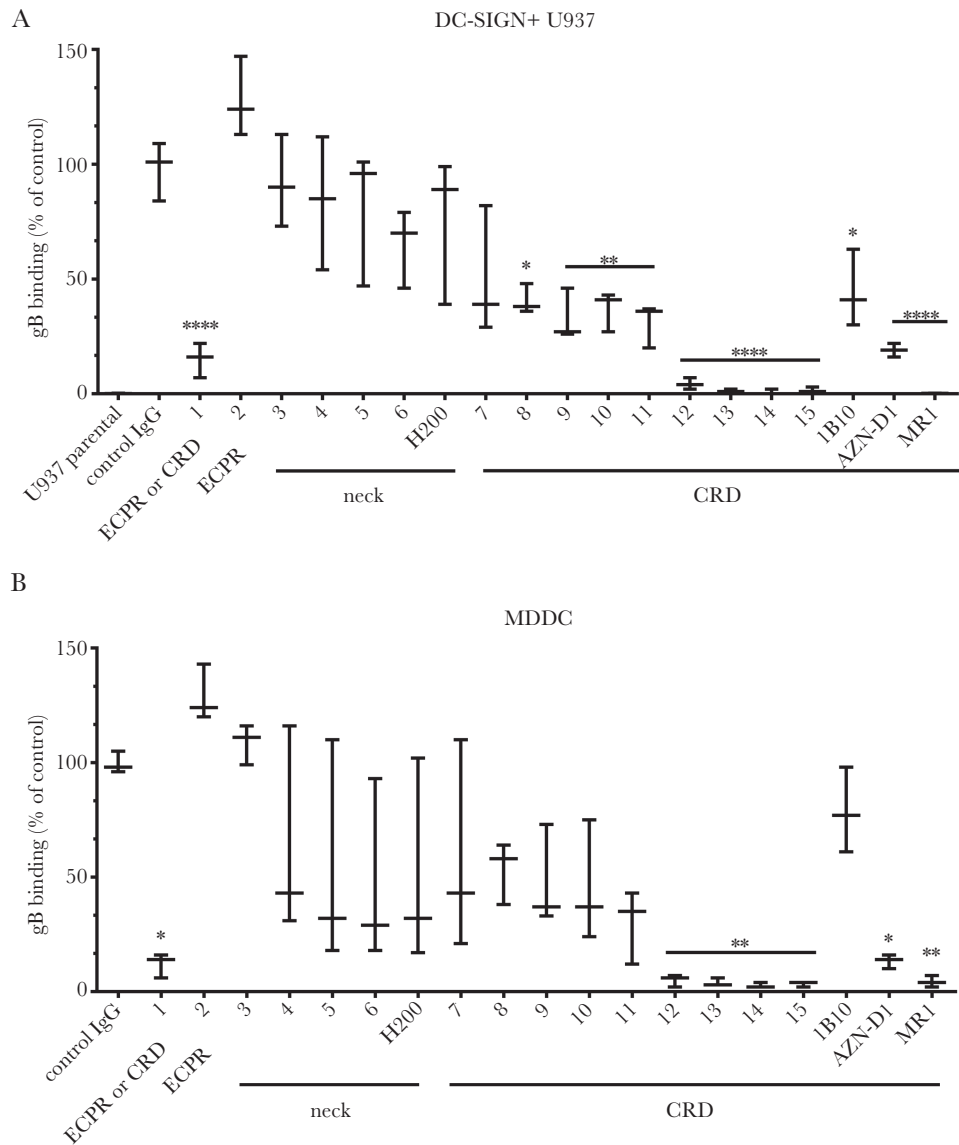


Figure 6. Anti-dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) carbohydrate-recognition domain (CRD) antibodies abrogate the DC-SIGN/human cytomegalovirus (HCMV) glycoprotein B (gB) interaction. The binding of conjugated gB (2 µg/mL) to (A) parental or DC-SIGN⁺ U937 cells or (B) immature monocyte-derived dendritic cells (MDDC) was assessed by flow cytometry. Fifteen available anti-DC-SIGN antibodies directed against the neck, extracellular proximal region (ECPR), or CRD regions were compared with the H200 (anti-neck), 1B10, AZN-D1, and MR-1 (anti-CRD) commercially available monoclonal antibodies for their ability to block the DC-SIGN/HCMV gB interaction. Binding intensities are represented as mean percentages of the maximum binding (n = 5), ie, in the absence of any antibody, and compared with treatment with an irrelevant control immunoglobulin (IgG). Asterisks represent significant results (****, $P < .0001$; **, $P < .01$; *, $P < .05$; one-way analysis of variance with multiple comparison tests).

of these HCMV receptors could be linked to our observations. Therefore, we concluded that DC-SIGN is the major, if not the only, attachment receptor for gB on MDDC and DC-SIGN⁺ U937 cells.

Finally, we tested some anti-DC-SIGN CRD mAbs in *trans*-infection blockade experiments. All were shown to drastically neutralize the TB40/E-GFP *trans*-infection by MDDC or DC-SIGN⁺ U937 cells (62% to 75% reduction; [Figure 7A](#) and [Supplementary Figure 5](#)). Similar results were obtained with VHL/E and Toledo and with an in-house-generated clinical

isolate (TRI) (inhibitions ranging from 67% to 83%, 70% to 86%, and 79% to 87.5%, respectively; [Figures 7B–D](#)). Taken together, these results establish that blocking the DC-SIGN CRD prevents gB binding and almost completely neutralizes HCMV *trans*-infection.

DISCUSSION

In this study, we characterized the DC-SIGN/HCMV gB interaction. We identified DC-SIGN AA residues involved through an extensive mutagenesis study. We also demonstrated the

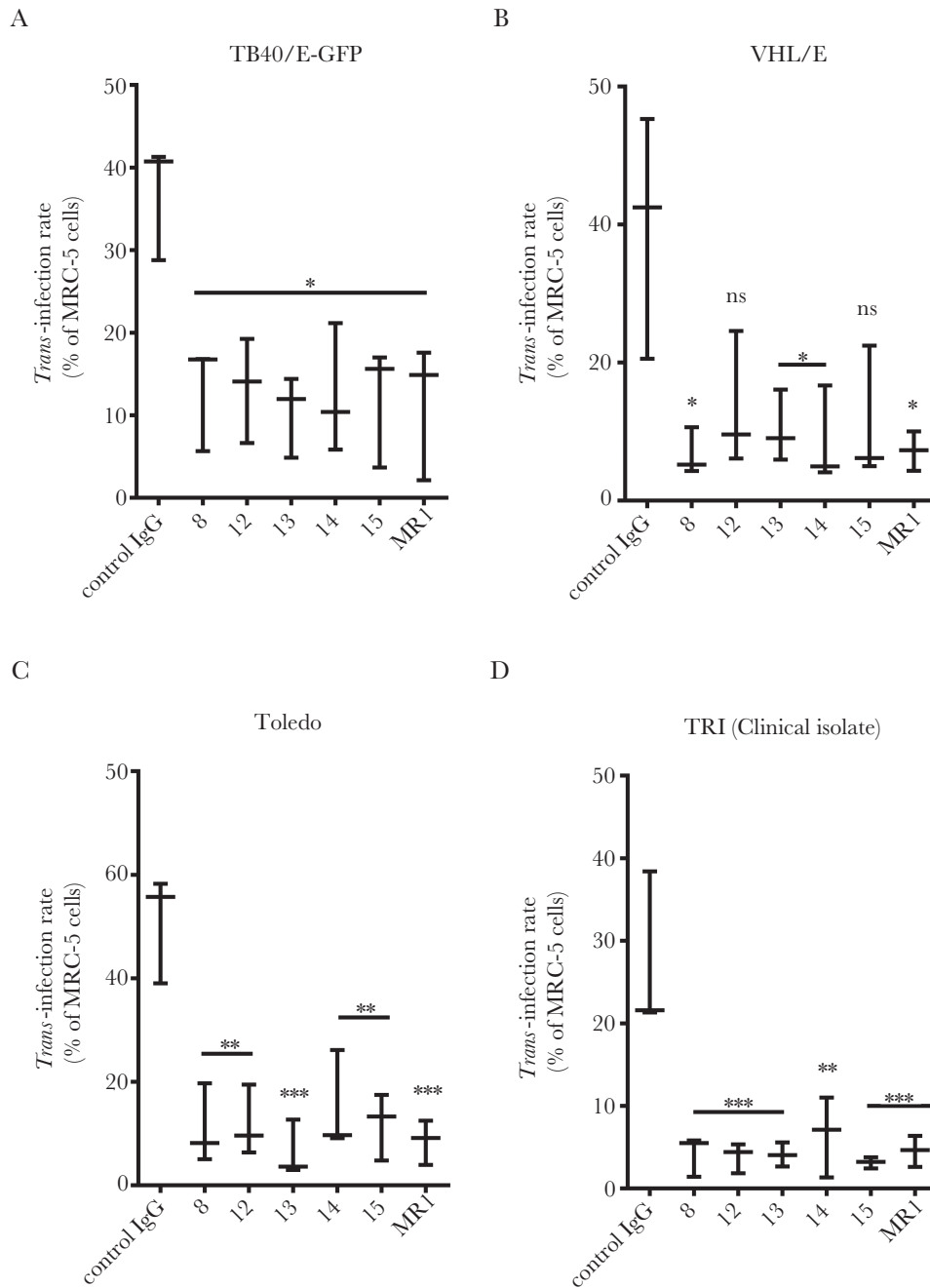


Figure 7. Blocking dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) carbohydrate-recognition domain (CRD) strongly impairs human cytomegalovirus (HCMV) *trans*-infection by monocyte-derived dendritic cells (MDDC). (A) Antibody-mediated blockade of HCMV *trans*-infection by MDDC. Six anti-DC-SIGN CRD antibodies were used to interfere with the capture and subsequent transmission to highly permissive MRC-5 cells. Monoclonal antibodies were used to block the capture and further transmission of HCMV (TB40/E-GFP strain, multiplicity of infection = 2). Similar experiments showing the *trans*-infection of the VHL/E (B) and Toledo (C) strains as well as a clinical isolate from our research center, TRI (D). Asterisks represent significant results compared with the control immunoglobulin (Ig)G condition (***, $P < .001$; **, $P < .01$; *, $P < .05$; one-way analysis of variance with multiple comparison tests). Abbreviation: ns, nonsignificant.

importance of high mannose *N*-glycans decorating the asparagine residue in position 208 on gB, although others might be involved too. Finally, antibody-mediated blockades allowed us to identify DC-SIGN as a major attachment molecule for HCMV on MDDC.

Mutating DC-SIGN has confirmed a crucial role for the 2 Ca^{2+} binding sites and the glycan binding pocket in the

recognition of gB, arguing in favor of a common interaction mode for all DC-SIGN viral ligands described to date, ie, HIV-1 gp120 or HSV-1/HHV-8 gB [36, 43]. Su et al [33] showed that turning the V351 into an alanine abrogated the HIV-1 gp120 binding. Our observation is consistent with this and indicates that a glycine residue instead of a valine at position 351 is deleterious. In contrast, replacement of this valine by a threonine,

both having similar electron densities, has no effect, indicating that the fold matters at this position. In this study, we showed that the DC-SIGN neck is dispensable for gB binding, although 2 anti-neck mAbs, ie, 4 and 6 (Supplementary Figure 5), were shown to block HCMV *trans*-infection. We hypothesized that the neck-dependent DC-SIGN microclustering at the plasma membrane was dispensable for soluble gB fixation to DC-SIGN but mandatory for the HCMV attachment to its target cells by permitting multiple ligand/receptor interactions.

We identified gB AD-5 and, more precisely, high mannose-containing *N*-glycans harbored by the asparagine, at least in position 208, as responsible for the recognition of DC-SIGN. Based on structural homologies and functional studies, Sharma et al [44] proposed that HCMV gB could be classified as a class III fusion protein. Class III fusion proteins exist as pre- and post-fusion states and possibly as conformational intermediates [45]. By analogy to a VSV-G pre-fusion crystal structure, a first model of a putative pre-fusion HCMV gB conformation was proposed by Spindler et al [38]. More recently, a study based on electron cryotomography showed for the first time that membrane-associated HSV-1 gB should exist because at least 2 distinct conformations reported as pre- and postfusion conformations [46]. Due to a high degree of similarity between HSV-1 and HCMV gB sequences, it is tempting to speculate that the latter exists at least as pre- and postfusion states. It is interesting to note that in the pre-fusion model reported by Zeev-Ben-Mordehai et al [46], AD-5 and AD-4 protrudes out of HCMV gB, potentially providing an accessible platform for DC-SIGN. In contrast, in line with a former work [47], Fontana et al [48] propose that the pre-fusion model described by Zeev-Ben-Mordehai et al [46] could be an intermediate between the pre- and postfusion states. They also suggested that the fusion loop points toward the viral membrane instead of pointing toward the cellular target membrane in the pre-fusion model of HSV-1 gB. In that setting, we can assume that DC-SIGN might be long enough to contact AD-4/AD-5 regardless of their respective locations into the pre-fusion model. Further investigations are needed to establish whether DC-SIGN can interact with HCMV gB pre-fusion conformation only or if it can adapt itself to distinct conformational states of HCMV gB.

CONCLUSIONS

In conclusion, we characterized the DC-SIGN/gB interaction in the present study. This work provides the molecular bases to understand how some anti-CRD mAbs can efficiently block the gB/DC-SIGN interaction. It should also help to rationally design other blockers of this interaction with antiviral properties. We propose that the DC-SIGN/gB interaction represents the most important HCMV attachment receptor on MDDC. Whether this holds true with other human tissue-resident DC or macrophages expressing DC-SIGN remains to be established. The relevance of our results now has to be challenged

in appropriate models. To date, few single-nucleotide polymorphisms described in the 5' untranslated region of the *CD209* gene are associated with variable expression of DC-SIGN on MDDC [49]. Unexpectedly, the GG genotype (rs735240), which leads to a decreased DC-SIGN expression on MDDC, was shown to correlate (1) with increased CMV reactivation or disease in allogeneic stem cell transplantation patients [49] and (2) with decreased CMV infection-free survival in kidney transplant patients [50]. Further investigations are now required to reconcile our *in vitro* results with clinical observations.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

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