The Journal of Immunology

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J Immunol 2011; 187:5299-5309; Prepublished online 17 October 2011;
doi: 10.4049/jimmunol.1101889
http://www.jimmunol.org/content/187/10/5299

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Semen Clusterin Is a Novel DC-SIGN Ligand

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The C-type lectin receptor dendritic cell-specific ICAM-3–grabbing nonintegrin (DC-SIGN) is an important player in the recognition of pathogens by dendritic cells. A plethora of pathogens including viruses, bacteria, parasites, and fungi are recognized by DC-SIGN through both mannose and fucose-containing glycans expressed on the pathogen surface. In this study, we identified semen clusterin as a novel DC-SIGN ligand. Semen clusterin, but not serum clusterin, expresses an extreme abundance of fucose-containing blood-type Ags such as Le^x and Le^y, which are both excellent DC-SIGN ligands. These motifs enable semen clusterin to bind DC-SIGN with very high affinity (K_d 76 nM) and abrogate the binding of HIV-1 to DC-SIGN. Depletion of clusterin from semen samples, however, did not completely prevent the ability of semen to inhibit the capture of HIV-1 by DC-SIGN, supporting that besides clusterin, semen contains other DC-SIGN ligands. Further studies are needed to characterize these ligands and define their contribution to the DC-SIGN–blocking activity mediated by semen. Clusterin is an enigmatic protein involved in a variety of physiologic and pathologic processes including inflammation, atherosclerosis, and cancer. Our results uncover an unexpected heterogeneity in the glycosylation pattern of clusterin and suggest that the expression of high concentrations of fucose-containing glycans enables semen clusterin to display a unique set of biological functions that might affect the early course of sexually transmitted infectious diseases. *The Journal of Immunology*, **2011, 187: 5299–5309.**

Dendritic cells (DCs) are highly specialized professional APCs with a unique ability to activate resting T cells and regulate the adaptive immune response (1). DCs express a diverse array of pattern-recognition receptors (PRRs) responsible for the recognition of invading pathogens. These PRRs include TLRs, C-type lectin receptors (CLRs), nucleotide oligomerization domain-like receptors, and RIG-1–like receptors. Each of these families of PRRs recognizes different sets of pathogen-associated molecular patterns (2, 3).

CLRs recognize specific carbohydrates on the pathogen surface. This leads to the internalization of pathogens by DCs favoring Ag processing and presentation of pathogen-derived peptides to naive T cells. This family of PRRs includes DC-SIGN, Dectin-1, DC immunoreceptor, DC-associated C-type lectin, blood DC Ag-2, macrophage galactose-type lectin-1, Langerin, DEC-205, C-type lectin-like receptor-1, and mannose receptor (2, 4). Recent reports have shown that the function of CLR is not merely restricted to the uptake of pathogens. These receptors express signaling motifs in their cytoplasmic tails or associate with signaling complexes and activate different signaling pathways after the recognition of pathogen glycans (4, 5). Hence, similar to TLRs, CLRs are also able to shape the course of the adaptive immune response.

The lectin DC-SIGN binds different carbohydrate structures expressed on the pathogen surface, such as mannose-containing glycoconjugates and fucose-containing Lewis Ags (Le^x, Le^y, Le^a, and Le^b) (6, 7). A huge number of pathogens are recognized by DC-SIGN including viruses (HIV-1, hepatitis C virus, CMV, dengue, Ebola, severe acute respiratory syndrome-associated coronavirus, HSV, H5N1, West Nile virus, and measles virus), bacteria (*Helicobacter pylori*, *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Streptpcoccus pneumoniae*, and *Leptospira interrogans*), fungi (*Candida albicans* and *Aspergillus fumigatus*), and parasites (*Leishmania* and *Schistosoma mansoni*) (6, 8). Although DC-SIGN might play an important role in the capture and internalization of these pathogens for processing and Ag presentation, some pathogens appear to be able to misuse DC-SIGN to infect target cells, circumvent Ag processing, alter TLR-mediated signaling, skew T cell responses, and spread the infection (6, 8).

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Received for publication June 28, 2011. Accepted for publication September 15, 2011.

This work was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas, the Buenos Aires University School of Medicine, the Agencia Nacional de Promoción Científica y Tecnológica (Argentina), the Curie Institute, and INSERM (France). The Mass Spectrometry facility (MALDI-TOF and MALDI-TOF/time of flight) used in this study was funded by the European Community, the Région Nord-Pas de Calais (France), and the Université des Sciences et Technologies de Lille I.

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The online version of this article contains supplemental material.

Abbreviations used in this article: CLR, C-type lectin receptor; 2D-PAGE, two-dimensional PAGE; DC, dendritic cell; DC-SIGN, dendritic cell-specific ICAM-3–grabbing nonintegrin; ECD, extracellular domain; MS, mass spectrometry; m/z, mass-to-charge ratio; NHS, *N*-hydroxysuccinimide; PRR, pattern-recognition receptor; TOF, time of flight.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1101889

We have previously reported that human semen, even when used at very high dilutions ($1:10^4$ to $1:10^5$), inhibits the recognition of HIV by DC-SIGN (9). This observation was recently confirmed in two independent reports (10, 11). During the course of studies directed to analyze the ability of semen to enhance HIV infection mediated by fragments of prostatic acid phosphatase termed semen-derived enhancer of viral infection, Kim and colleagues (10) found that semen potently inhibits DC-SIGN–mediated transmission of HIV. A similar finding was also reported by Stax and colleagues (11). In the present report, we identified semen clusterin as a novel high-affinity DC-SIGN ligand. Clusterin is an enigmatic glycoprotein found in a variety of tissues, in a secreted or a nuclear form (12–16). Secretory clusterin delays apoptosis of cancer cells, whereas nuclear clusterin triggers cell death (15, 16). The secreted form of clusterin consists of two 40-kDa chains derived from a single precursor polypeptide: α (corresponding to residues 206–427) and β (corresponding to residues 1–205), linked by five disulfide bonds (13, 14, 17). Clusterin is implicated in an array of physiological functions, including control of complement assembly and activation, inhibition of the NF-κB pathway, inhibition of stress-induced protein damage, facilitation of clearance of unfolded proteins, and modulation of cell survival (18–21). Clusterin expression is disregulated in many types of cancer, and it seems to play a role in tumor progression and metastasis (14, 15). Clusterin is also involved in the development of Alzheimer's disease (12). The presence of high concentrations of clusterin in seminal plasma (0.4–15.0 mg/ml) was reported previously (22–24), and a role in sperm development has also been proposed (24).

We show in this study that semen clusterin, but not serum clusterin, bears a set of complex glycans with high affinity for DC-SIGN. These glycans mediate the effective binding of semen clusterin to DC-SIGN, thus efficiently interfering with the binding of HIV to DCs.

Materials and Methods

Reagents

Recombinant human DC-SIGN–Fc chimera, monoclonal IgG anti-human DC-SIGN, monoclonal IgG anti-human clusterin, goat polyclonal IgG anti-human clusterin, sheep polyclonal IgG anti-human fibronectin (R&D Systems), secondary labeled Abs (Jackson ImmunoResearch Laboratories), recombinant clusterin (Biovendor), PNGase-F (Roche), and α 1-3,4 fucosidase (Calbiochem) were used. Human clusterin ELISA kit (R&D Systems) was also used.

Seminal plasma samples

Semen samples were collected from healthy donors (aged 25–45 y) as previously described (9). Informed consent was obtained from each patient before sperm collection.

Two-dimensional gels

Seminal plasma samples (usually ~50 μg proteins) were precipitated with a two-dimensional clean-up kit (Amersham Biosciences). Pellets were dissolved in two-dimensional sample buffer (7 M Urea, 2 M thio-urea, 4% CHAPS, 20 mM DTT, and 0.5% ampholytes). IPG strips (Invitrogen and GE Bioscience) were rehydrated at room temperature. Isoelectric focusing was carried out on IEF-cells (Bio-Rad) for 15 min at 175 V, 45 min slope up to 2000 V, and until 5000 V h are reached. Strips were re-equilibrated (6 M Urea, 75 mM Tris HCl [pH 6.8], 2% SDS, and 21% glycerol v/v) and placed on Zoom Gels (Invitrogen; or self-made gradient gels) 4–12%. Gels were run in MOPS (or Tris-glycine) running buffer. The run gels were stained with Coomassie (LabSafe Gel blue G-Bioscience) or silver nitrate or were transferred onto polyvinylidene difluoride membrane (Millipore).

Mass spectrometry

Gel slices were cut out from gels and washed in 25 mM NH_4HCO_3 (pH 8) and acetonitrile. Samples were reduced, alkylated, and digested overnight at 30°C with Trypsin (Roche). Peptides were extracted from gel with H_2O/acetonitrile and formic acid (30, 65, and 5%) in a sonicator. Peptides were separated using an Ultimate nanocapillary HPLC system (Dionex) with a PepMap C18 nanocolumn. Peptide mixtures were loaded on a precolumn and washed with loading solvent (95% H_2O/acetonitrile 5% and 0.1% formic acid). They were transferred to an analytical C18 nanocapillary column and eluted at a flow rate of 135 nl/min using the following gradient: solvent A containing 95% H_2O/acetonitrile 5% and 0.1% formic acid, and solvent B containing 80% acetonitrile 20% H_2O and 0.085% formic acid. The eluted peptides were passed via a nanospray ion source directly into a quadrupole time of flight (TOF) spectrometer QSAR pulsar (Applied Biosystems/MDS Sciex). Tandem mass spectrometry (MS) spectra were submitted to the search engine Mascot (Matrixscience, London, U.K.) and Phenyx (GeneBio, Geneva, Switzerland).

Purification of clusterin

Purification was performed by affinity chromatography using 3 mg polyclonal goat IgG anti-clusterin Abs (R&D Systems) coupled to an *N*-hydroxysuccinimide (NHS)-activated HiTrap column and a pool of serum or seminal plasma samples obtained from five different donors prepared in the presence of a complete protease inhibitor mixture (Santa Cruz Biotechnology). The retained material was eluted using an elution buffer containing 1 M glycine-HCl at pH 2.8 and analyzed by SDS-PAGE (under reducing or nonreducing conditions) and Coomassie staining (LabSafe Gel blue; G-Bioscience).

Cell lines and virus

Viruses and cell lines were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health. The CCR5-using HIV-1 BaL isolate, as well as the primary HIV-1 isolates 93BR020.1 (subtype F, using either CXCR4 or CCR5) and 96USHIPS4 (subtype B, using either CXCR4 or CCR5), were grown and concentrated as previously described (25). Virus input into assays was a function of p24 Ag concentration (ELISA; Abbot Murex).

Immunoprecipitation and Western blotting

Seminal plasma (10% v/v in PBS) was depleted of IgG Abs by passage over a protein G-Sepharose column (GE Bioscience). Western blot using DC-SIGN–Fc chimera was carried out as follows. Samples were analyzed by SDS-PAGE (4–12% polyacrylamide gel under reducing conditions), transferred onto a nitrocellulose blot, treated with DC-SIGN–Fc chimera (1 μg/ml in a buffer containing 20 mM TRIS, 150 mM NaCl, 1 mM $CaCl_2$, and 2 mM $MgCl_2$ for 4 h at 4°C), and stained with a secondary peroxidase-conjugated goat anti-human IgG (Jackson ImmunoResearch Laboratories). Alternatively, membranes were treated with polyclonal goat IgG anti-clusterin or sheep IgG anti-fibronectin and stained with secondary peroxidase-conjugated donkey anti-goat IgG or anti-sheep IgG, respectively (Jackson ImmunoResearch Laboratories).

Binding of clusterin to DC-SIGN–expressing Raji cells and DCs

Binding assays were carried out by incubating Raji DC-SIGN cells or DCs (2×10^5/0.1 ml) with different concentrations of seminal plasma, purified clusterin, or recombinant clusterin for 60 min at 4°C in 96-well U-bottom plates. The cells were washed twice with cold PBS, stained with polyclonal goat IgG anti-clusterin and FITC-labeled donkey IgG anti-goat IgG, and analyzed by using an FACS flow cytometer and CellQuest software (BD Biosciences).

Enzymatic treatments of semen clusterin

Clusterin purified from seminal plasma (3–10 μg) was treated with 2 U PNGase F for 15 h at 37°C following the manufacturer's instructions or with 0.5 mU α 1-3,4 fucosidase for 3 h at 37°C in 50 mM sodium phosphate buffer (pH 5). Enzymes were inactivated by heating at 95°C for 5 min.

Quantitation of cellular apoptosis by Annexin V binding and flow cytometry

Annexin V binding to DCs was performed using an apoptosis detection kit (Immunotech, Marseille, France).

HIV-1 binding assays

Binding assays were performed as previously described (9). In all cases, 1×10^6 DCs, Raji–DC-SIGN cells, activated PBMC, or macrophages were incubated with different concentrations of clusterin (either recombinant or purified from seminal plasma) for 30 min at 37°C in a final volume of 200

μl, and then HIV-1 stocks, containing the indicated amounts of p24, were added. Cells were incubated for 90 min at 37°C, washed thoroughly, pelleted, lysed, and assayed for HIV p24 Ag by ELISA.

HIV-1 infection assays

Infection assays were carried out by incubating DCs (1×10^5/200 μl complete medium) or activated PBMCs (2×10^5/200 μl complete medium and 10 μg/ml PHA for 48 h) with or without different concentrations of clusterin, for 30 min at 37°C. Cells were then exposed to HIV (5 ng p24) for 90 min at 37°C and then washed thoroughly. DCs were next cultured for 15 d in a final volume of 200 μl in 96-well flat-bottom plates in complete culture medium supplemented with 10 ng/ml IL-4 and GM-CSF. PBMCs were cultured for 15 d in a final volume of 200 μl in 96-well flat-bottom plates in complete culture medium supplemented with IL-2 (10 U/ml; R&D Systems). Supernatants harvested at different times were assayed for HIV p24 Ag by ELISA. Alternatively, PBMC were intracellular stained with FITC-conjugated anti-p24 Ag Abs (KC57 clone; Beckman Coulter) and analyzed by FACS.

HIV-1 transmission assays

HIV-1 transmission assays were carried out by incubating DCs or Raji–DC-SIGN cells (10^6 cells in 200 μl complete medium) with or without different concentrations of clusterin for 30 min at 37°C. Cells were then exposed to HIV (5 ng p24) for 90 min at 37°C. The cells were then washed thoroughly and suspended in culture complete medium. DC cultures were supplemented with 10 ng/ml IL-4 and GM-CSF. HIV transmission to PBMCs, previously activated by IL-2 (10 U/ml; R&D Systems) and PHA (10 μg/ml; Sigma-Aldrich) for 2 d, was performed by incubating 5×10^4 DCs with 2×10^5 PMBC in a final volume of 200 μl in 96-well U-bottom plates. Supernatants harvested at different times were assayed for p24 Ag by ELISA. HIV-1 transmission to SupT-1 cells was carried out by incubating 5×10^4 HIV-pulsed Raji–DC-SIGN cells with 1×10^5 SupT-1 cells in a final volume of 200 μl in 96-well U-bottom plates. Supernatants harvested at different times were assayed for p24 Ag by ELISA.

Analysis of DC-SIGN interaction with clusterin by surface plasmon resonance

Flow cells of a Biacore CM4 sensor chip (Biacore) were activated for 10 min with a mixture of 0.2 M N-ethyl-N'-(diethylaminopropyl)-carbodiimide and 0.05 M NHS. Clusterin purified from seminal plasma (1.25 μg/ml in 10 mM sodium acetate buffer [pH 4.2]) was then injected for 1 min, after which remaining activated groups were blocked with 1 M ethanolamine (pH 8.5). Typically, this procedure allowed the coupling of ~200 resonance units of clusterin. For binding assays, DC-SIGN, produced as described (26), was injected at 25 μl/min for 10 min over the clusterin functionalized surface. The formed complexes were rinsed with running buffer (50 mM Tris, 0.15 M NaCl, 4 mM $CaCl_2$, and surfactant P20 0.005% [pH 7.4]) for 5 min, after which the surface was regenerated with a 4-min pulse of running buffer containing 50 mM EDTA instead of $CaCl_2$.

Release, preparation, and MS analysis of N-glycans

Semen clusterin and serum clusterin (BioVendor) were reduced and carboxyamidomethylated followed by sequential tryptic and peptide *N*-glycosidase F digestion and Sep-Pak purification. Exoglycosidase digestions, permethylation of the freeze-dried glycans, MALDI-TOF–MS of permethylated glycans, and linkage analysis were performed as described elsewhere (27). Briefly, hydrogen fluoride treatment of *N*-glycans was performed by treatment with 48% HF at 0°C for 20 h. After treatment, *N*-glycans were permethylated. Tandem MS data were acquired using a 4800 MALDI-TOF/TOF (Applied Biosystems) mass spectrometer. The collision energy was set to 1 kV, and argon was used as collision gas. Samples were dissolved in 20 μl methanol, and 1 μl was mixed at a 1:1 ratio (v/v) with 1 μl 2,5-dihydrobenzoic acid (20 mg/ml in 70% methanol in water) as matrix.

Preparation of human DCs and macrophages

PBMC were isolated from blood of healthy donors by density gradient centrifugation using Ficoll-Hypaque (GE Healthcare). $CD14^+$ cells were obtained using CD14 microbeads (Miltenyi Biotec). To obtain DCs, monocytes were cultured for 6 d with 10 ng/ml IL-4 and GM-CSF, as described (28). To obtain macrophages, monocytes were cultured in RPMI 1640 medium for 2 h on 24-well plates; after this period, cells were resuspended on complete culture medium containing 10 ng/ml GM-CSF and cultured for 6 d.

Depletion of clusterin from seminal plasma

Depletion was performed by affinity chromatography using 3 mg polyclonal goat IgG anti-clusterin Abs (R&D Systems) coupled to an NHS-activated HiTrap column.

Gel filtration chromatography

Gel filtration analysis of seminal plasma was performed using an FPLC-Superdex 200 10/300 column (Amersham Bioscience). The calibration of the column was performed using thyroglobulin (669 kDa), ferritin (440 kDa), adolase (158 kDa), albumin (67 kDa), OVA (43 kDa), and chymotrypsinogen A (25 kDa) as protein standards. The running buffer was PBS, and 0.5 ml seminal plasma (50% v/v in PBS) was applied to the column at a flow rate of 0.4 ml/min at room temperature. Fractions of 1 ml were collected. The elution of proteins was followed by measuring the OD at 280 nm. The amount of protein (Bradford), the concentration of clusterin (ELISA), and the ability to inhibit the binding of HIV-1 to DC-SIGN–expressing Raji cells were assessed in all of the collected fractions.

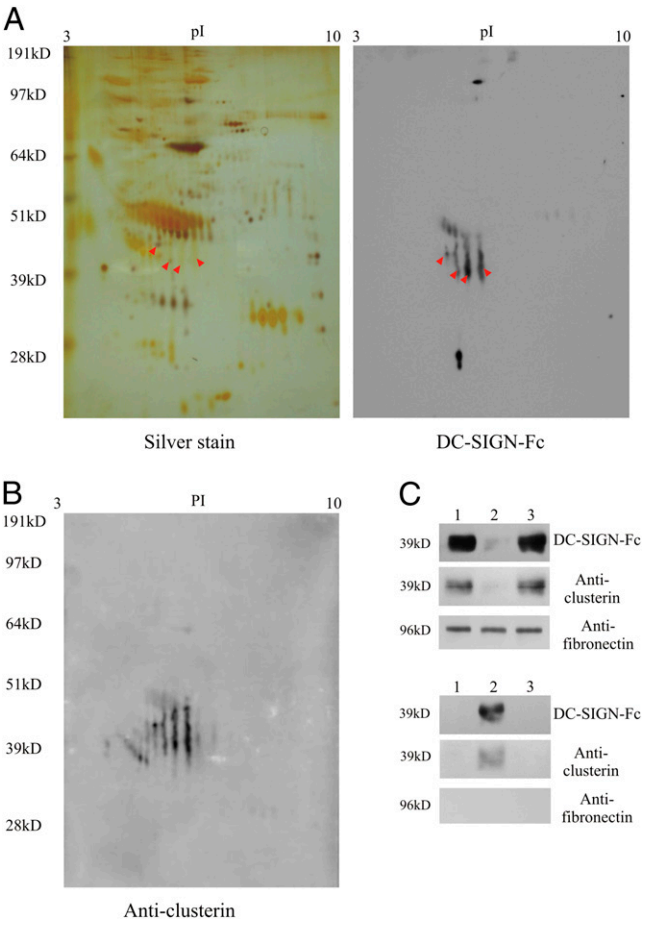

FIGURE 1. Identification of clusterin as a novel DC-SIGN ligand present in human semen. *A*, Human seminal plasma was run in two-dimensional SDS-PAGE and stained with silver nitrate (*left panel*) or blotted onto a nitrocellulose membrane. DC-SIGN–binding proteins were revealed using DC-SIGN–Fc and HRP-conjugated goat anti-human IgG (*right panel*). The arrowheads show the spots selected for MS analysis. *B*, The membrane shown in *A* (*right panel*) was stripped and probed again with goat anti-clusterin Abs and HRP-conjugated anti-goat IgG. *C*, Seminal plasma was incubated overnight with protein G-Sepharose alone (*lane 1*), protein G-Sepharose coupled with goat anti-clusterin Abs (*lane 2*), or protein G-Sepharose coupled with control goat IgG (*lane 3*). The samples were then centrifuged, and the supernatants were collected. Retained material was eluted at acidic pH. Both the supernatants (*upper panel*) and the retained fractions (*lower panel*) were analyzed by Western blot using DC-SIGN–Fc, goat polyclonal anti-clusterin Abs, or goat polyclonal anti-fibronectin Abs as a control.

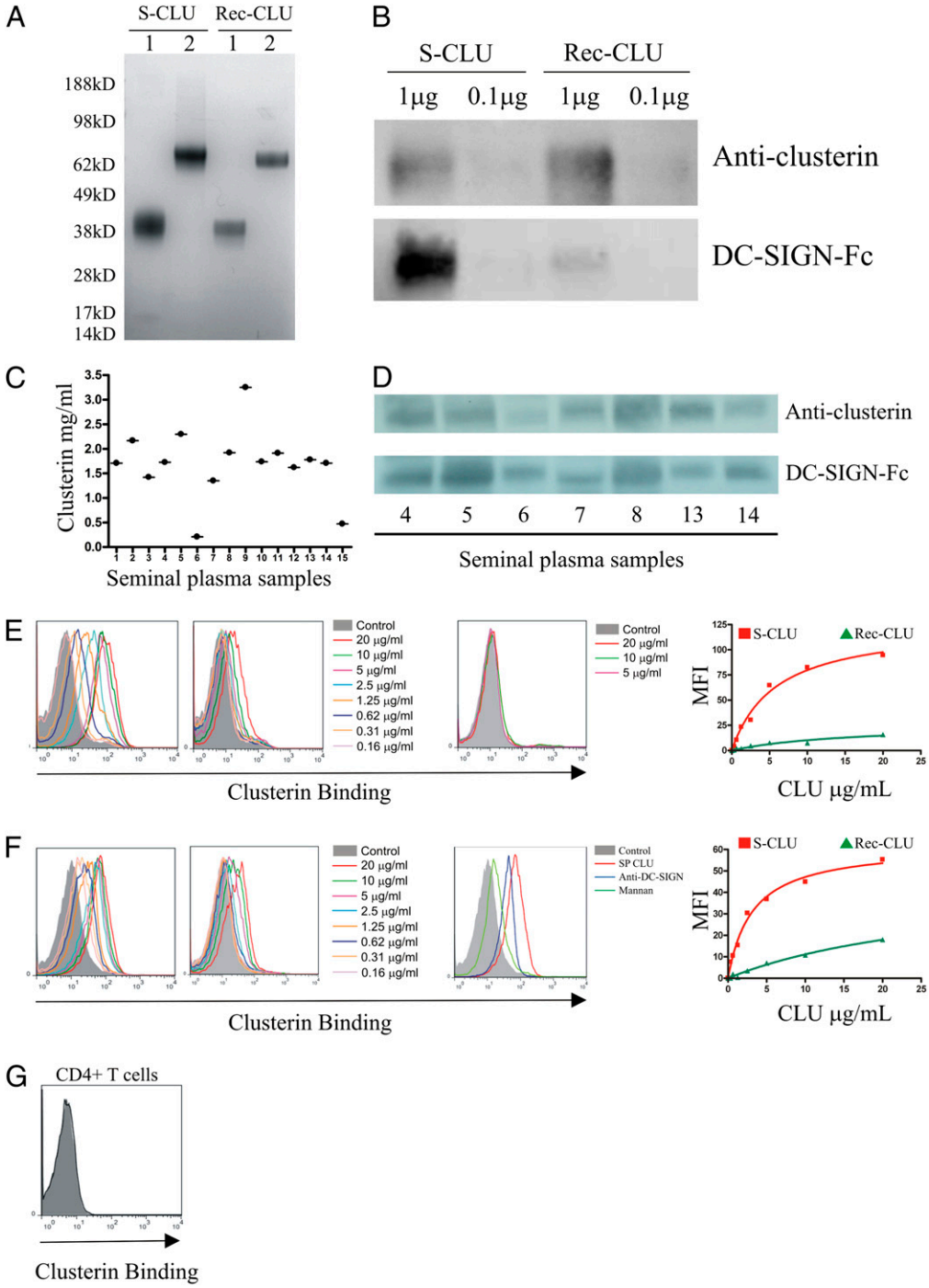

FIGURE 2. Analysis of semen clusterin binding to soluble and cell-associated DC-SIGN. *A*, Purified semen clusterin (S-CLU) and recombinant clusterin (Rec-CLU) were run on SDS-PAGE under reducing (*lane 1*) and nonreducing (lane 2) conditions and stained using Coomassie blue. *B*, Western blot of purified semen clusterin and recombinant clusterin (1.0 and 0.1 μg) was revealed using goat anti-clusterin polyclonal IgG or DC-SIGN–Fc chimera. *C*, The concentration of clusterin was determined in seminal plasma samples from 15 different donors by ELISA. *D*, The ability of clusterin to bind DC-SIGN was tested on seminal plasma samples from seven different donors by Western blot. *E*, Raji DC-SIGN$^+$ cells were cultured with different concentrations of purified semen clusterin (*left histograms*) or recombinant clusterin (*center histograms*) for 60 min at 4˚C. Clusterin binding was then analyzed by FACS. No binding of clusterin was observed using DC-SIGN (−) Raji cells (*right histograms*). In the *right panel*, binding of the different concentrations of semen clusterin (red) and recombinant clusterin (green) is shown as the mean fluorescence intensity. In all cases, a representative experiment (n = 6–8) is shown. *F*, DCs were cultured with different concentrations of purified semen clusterin (*left histograms*) or recombinant clusterin (*center histograms*) for 60 min at 4˚C. Clusterin binding was then analyzed by FACS. *Right histograms* depict the inhibition of clusterin binding by mannan (5 mM) or Abs directed to DC-SIGN. In the *right panel*, binding of the different concentrations of semen clusterin (red) and recombinant clusterin (green) is shown as the mean fluorescence intensity. *G*, Activated PBMC were cultured with 20 μg/ml of semen clusterin for 60 min at 4˚C. Clusterin binding was then analyzed by FACS. Binding of clusterin to PBMC was analyzed in the gate of CD3$^+$CD4$^+$ cells. Representative experiments are shown (n = 3 to 4).

Statistics

All statistical comparisons were performed by using ANOVA. The p values <0.01 and <0.05 were considered statistically significant.

Results

Identification of a DC-SIGN binding protein in human semen

To study whether semen contains proteins that interact directly with DC-SIGN, we analyzed seminal plasma using two-dimensional PAGE (2D-PAGE) and Western blot with DC-SIGN–Fc. Fig. 1*A* (*left panel*) shows a typical 2D-PAGE of seminal plasma total proteins revealed by silver staining. Western blot with DC-SIGN-Fc (Fig. 1*A*, *right panel*) revealed a group of proteins of relatively acidic pH (~5.5–6) and a molecular mass of 40–45 kDa. Four spots corresponding to these DC-SIGN–Fc-interacting proteins were cut off the 2D-PAGE and analyzed by MS. The peptides sequenced from these spots identified clusterin as the main DC-SIGN–interacting protein (Supplemental Table I). Abs to clusterin revealed the same spots on the two-dimensional membrane (Fig. 1*B*). In addition, the DC-SIGN–binding protein was retained on protein G-Sepharose coupled with goat polyclonal anti-clusterin Abs and could be recovered by acid elution (Fig. 1*C*). We conclude that semen clusterin is a novel ligand for DC-SIGN.

Semen clusterin binds soluble and cell-associated DC-SIGN

To analyze clusterin–DC-SIGN interaction in further detail, we purified semen clusterin using affinity chromatography (see *Materials and Methods*). The identity of the purified protein was confirmed using MS (data not shown) and Western blot. We also used recombinant commercial clusterin produced in human HEK 293 cells. Similar to recombinant clusterin, semen clusterin showed a molecular mass ~70–75 kDa in nonreducing conditions and a single band of molecular mass ~40 kDa after reduction (Fig. 2*A*). Consistent with the results shown in Fig. 1, purified semen clusterin effectively bound DC-SIGN, as detected by Western blot with DC-SIGN–Fc (Fig. 2*B*). Surprisingly, recombinant clusterin bound weakly to DC-SIGN–Fc in Western blots, although it was well revealed by anti-clusterin Abs (Fig. 2*B*). We reasoned that the recombinant clusterin could fail to bind DC-SIGN because a difference in the posttranslational modifications as compared with semen clusterin. Moreover, in agreement with previous reports, we observed that seminal plasma contains high concentrations of clusterin (ranging from 0.2–3.5 mg/ml) (Fig. 2*C*). In all of the samples tested, we found that clusterin effectively bound to DC-SIGN–Fc in Western blots (Fig. 2*D*). We conclude that recombinant clusterin and semen clusterin display distinct DC-SIGN–binding capacities.

To study the ability of semen clusterin to bind DC-SIGN on intact cells, we used DC-SIGN–expressing Raji cells and monocyte-derived DCs. The phenotype of the DCs used in this study is shown in Supplemental Fig. 1. The cells were incubated with increasing concentrations of clusterin, and the binding was revealed using FACS. Purified semen clusterin, but not recombinant clusterin, effectively binds to Raji cells expressing DC-SIGN, but not to untransfected Raji cells (Fig. 2*E*). Similarly, semen clusterin binds much more efficiently to DCs than recombinant clusterin. As expected, clusterin binding was inhibited by the C-type lectin inhibitor mannan or by Abs directed to DC-SIGN (Fig. 2*F*). We conclude that semen clusterin, but not recombinant clusterin, efficiently binds to both soluble and cell-associated DC-SIGN.

We next analyzed whether semen clusterin might be able to bind to $CD4^+$ T cells. Using PBMC and analyzing both the gate of $CD3^+CD4^+$ ($CD4^+$ T cells) (Fig. 2*G*) and $CD4^-$ cells (data not shown), we did not detect any binding of semen clusterin.

Semen clusterin binds DC-SIGN with high affinity

Surface plasmon resonance technology was used to further analyze DC-SIGN binding to clusterin. The isolated and monomeric carbohydrate recognition domain of DC-SIGN, prepared as described, did not interact with semen clusterin, whereas the tetrameric and full-length extracellular domain (ECD) of the lectin displayed effective binding (Fig. 3A). Sensorgrams were then obtained by injection of a concentration range of recombinant ECD (21–160 nM) over surfaces functionalized with 200 resonance units of clusterin (Fig. 3*B*). Analysis of the equilibrium data using Scatchard plots (Fig. 3*C*) provides an affinity value, K_d, of 76 ± 3.8 nM (mean ± SEM, $n = 3$). Therefore, semen clusterin binds DC-SIGN with high affinity.

Semen clusterin binds DC-SIGN through fucose-rich glycans

To investigate if other natural forms of clusterin also bind DC-SIGN, we next purified clusterin from human serum. As shown in Fig. 4*A*, serum clusterin displays a different electrophoretical mobility as compared with semen and to recombinant clusterin (suggesting again differences in their glycosylation patterns). Like recombinant clusterin, serum clusterin does not bind with DC-SIGN (Fig. 4*A*). The glycosylation pattern of serum clusterin was analyzed previously (13, 29). Serum clusterin contains six N-glycosylation sites modified with usual N-glycans, the most abundant being mono- and bisialo antennary structures with low levels of fucose and some sialyl-Lewis structures. These types of glycans would not be expected to bind to DC-SIGN (6, 7). We performed the analysis of the N-glycans isolated from serum clusterin and found similar results (Fig. 4*B*).

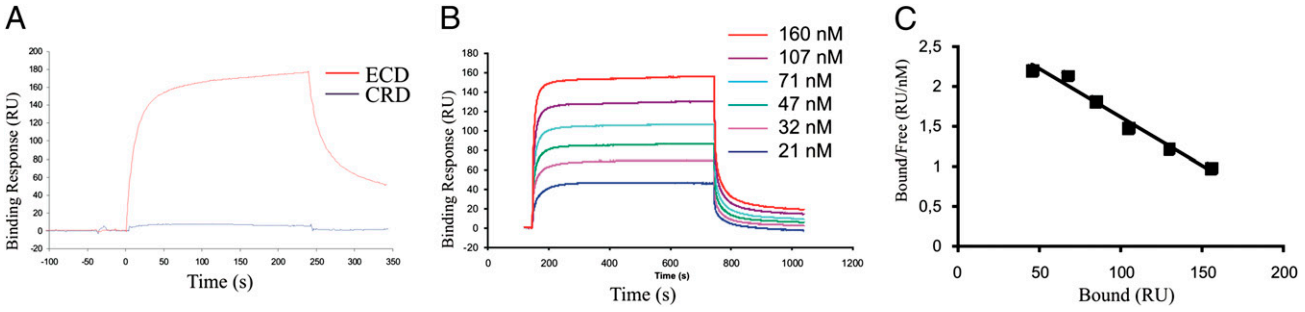

FIGURE 3. Analysis of semen clusterin binding to DC-SIGN by surface plasmon resonance. *A*, The binding of the full-length and tetrameric ECD (red) or the monomeric carbohydrate recognition domain (CRD, blue) (160 nM) to immobilized clusterin was recorded as a function of time. *B*, Overlay of sensorgrams showing the binding of different concentrations of DC-SIGN ECD to immobilized clusterin. *C*, Scatchard plot of the equilibrium binding data recorded on the sensorgrams at the end of the association phase.

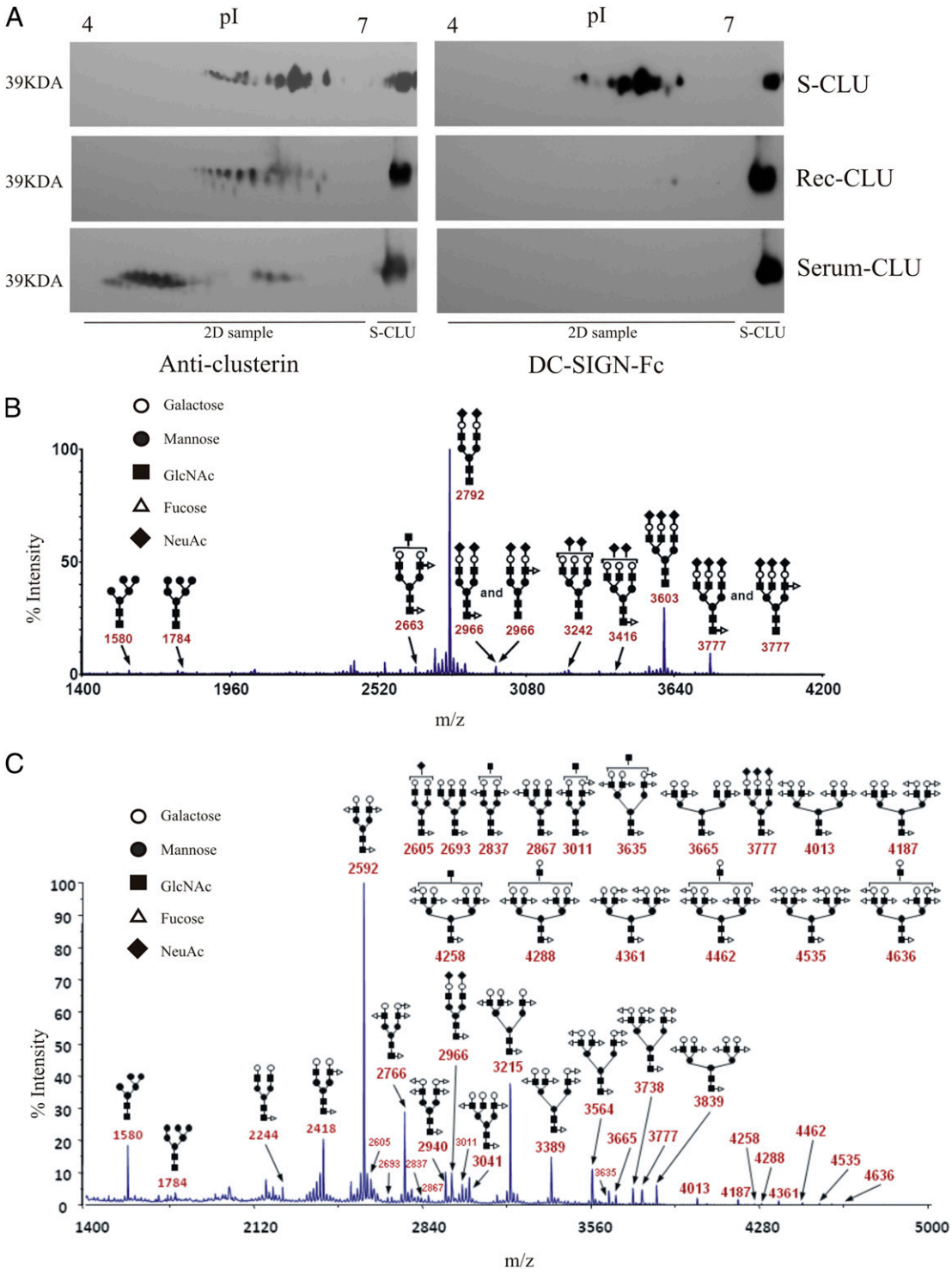

FIGURE 4. Analysis of clusterin glycans. *A*, Semen clusterin, recombinant clusterin, and serum clusterin were run in two-dimensional SDS-PAGE, blotted onto a nitrocellulose membrane, and revealed with goat anti-clusterin IgG (*left panel*) or DC-SIGN–Fc chimera (*right panel*). As a control, in all cases, semen clusterin (5 μg) was run only in the second dimension, in the lane usually reserved for a marker or ladder (in the right of each gel). *B*, MALDI-MS analysis of glycans from serum clusterin was performed as described in *Materials and Methods*. Annotations are based on compositional information provided by MALDI molecular weights complemented by MALDI-TOF/TOF sequencing, exoglycosidase digestions, hydrofluoric acid treatment, and linkage analysis experiments (data not shown). *C*, MALDI-MS analysis of semen clusterin glycans was performed as described in *Materials and Methods*. Only one branching pattern for triantennary structures is shown. Galactose (open circles); mannose (closed circles); GlcNAc (closed squares); fucose (open triangles); and NeuAc (closed diamonds).

These results suggest that semen clusterin bears glycans different from the ones present in serum or recombinant clusterin and that these glycans may mediate binding to DC-SIGN. We therefore analyzed the nature of the glycans present in semen clusterin by MALDI-MS analysis (30). As shown in Fig. 4*C*, the analysis of semen clusterin glycans revealed a characteristic and unusual glycosylation pattern. The N-glycans isolated from semen clusterin showed an extreme abundance of highly fucosylated glycans

ranging from biantennary (mass-to-charge ratio [m/z] 2940) to triantennary (m/z 3041, 3215, 3564, and 3738) and tetra-antennary glycans (m/z 4535, 4013, and 3839). All of these glycans bear the Lewis X or Lewis Y motifs, which are both excellent DC-SIGN ligands. Therefore, semen clusterin displays a unique glycosylation pattern, very different from the one found in serum clusterin.

Semen clusterin inhibits the binding of HIV and the infection of DCs

To analyze whether semen clusterin inhibited the interaction between DC-SIGN and HIV-1, experiments were performed using DCs and DC-SIGN–expressing Raji cells. Binding of HIV was evaluated after a short (90-min) incubation with the cells by

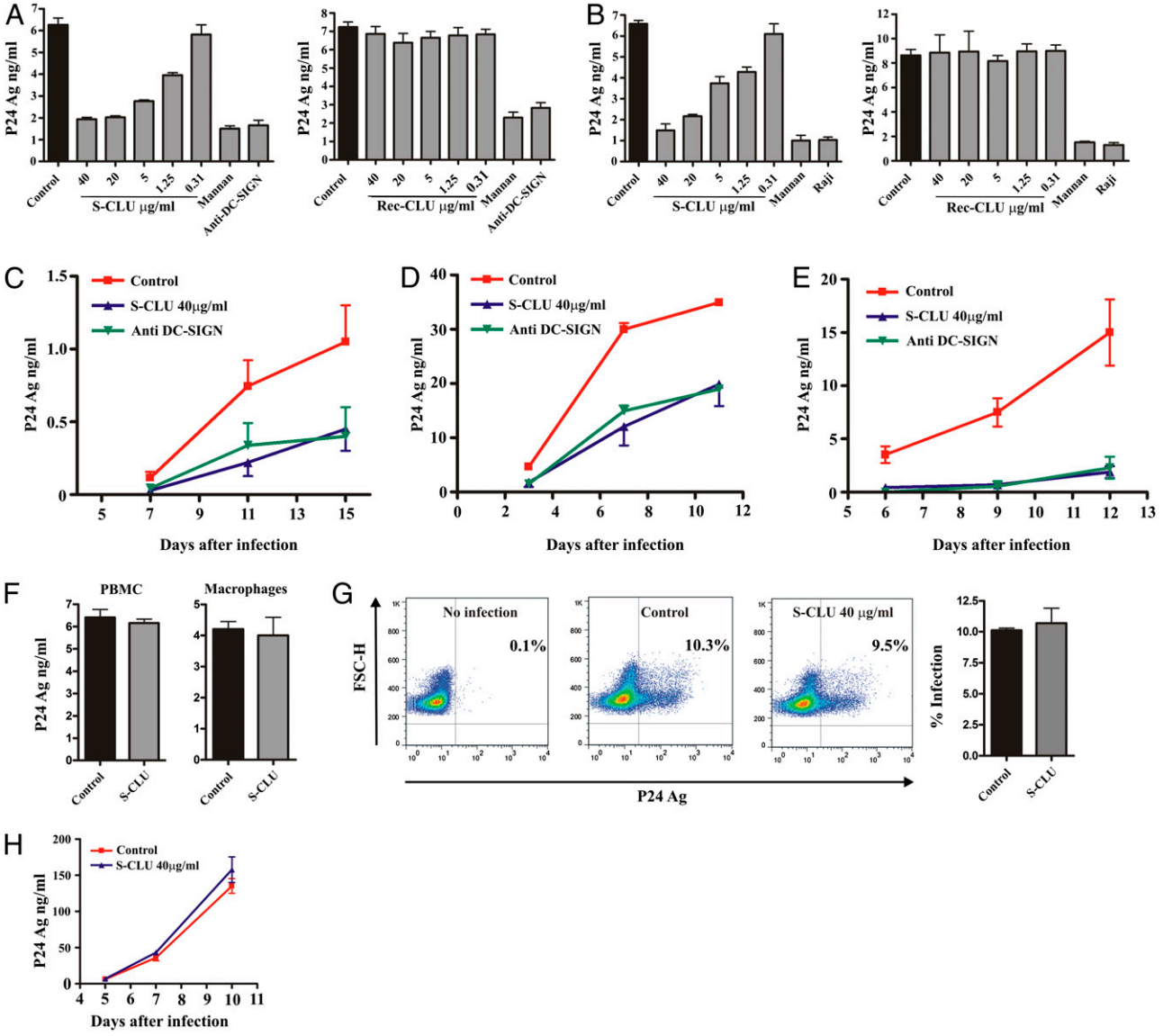

FIGURE 5. Inhibition of DC-SIGN–mediated binding and infection of DCs by HIV-1. DCs (*A*) or Raji DC-SIGN$^+$ cells (*B*) were incubated for 30 min at 37˚C with different concentrations of semen clusterin or recombinant clusterin, mannan (1 mg/ml), or a blocking MAb directed to DC-SIGN. Cells were then cultured with HIV-1 BaL (5 ng of p24, 90 min at 37˚C), washed thoroughly, lysed, and assayed for p24 Ag by ELISA. A representative experiment (n = 5–8) is shown. HIV-1 binding to Raji cells, which do not express DC-SIGN, is also shown. *C*, To analyze DC infection, cells were incubated for 30 min at 37˚C with different concentrations of semen clusterin or blocking mAb directed to DC-SIGN, exposed to HIV-1 BaL (5 ng of p24, 90 min at 37˚C), washed, and cultured for 15 d. Supernatants harvested at different time points were assayed for p24 Ag by ELISA. A representative experiment (n = 3) is shown. *D*, DCs were incubated with semen clusterin (40 μg/ml) (blue), PBS (control, red), or blocking anti–DC-SIGN Abs (green) for 30 min at 37˚C. Cells were then exposed to HIV-1 BaL (5 ng p24, 90 min at 37˚C). After this period, cells were thoroughly washed, and HIV-1 transmission to activated PBMCs was assessed as described under *Materials and Methods*. A representative experiment (n = 4) is shown. *E*, Raji DC-SIGN cells were incubated with semen clusterin (40 μg/ml) (blue) or PBS (control, red) for 30 min at 37˚C. Cells were then exposed to HIV-1 IIIB (5 ng p24, 90 min at 37˚C). After this period, cells were thoroughly washed and *trans*-infection of the T cell line SupT-1 was assessed. HIV-1 transmission mediated by Raji cells, which do not express DC-SIGN (green), is also shown. A representative experiment (n = 4) is shown. *F*, Activated PBMCs (*left panel*) or macrophages (*right panel*) were incubated with 40 μg/ml of semen clusterin for 30 min at 37˚C. Cells were then cultured with HIV-1 BaL (5 ng of p24, 90 min at 37˚C), washed thoroughly, lysed, and assayed for p24 Ag by ELISA. A representative experiment (n = 3) is shown. *G* and *H*, To analyze CD4$^+$ T cell infection, activated PBMCs were incubated in the absence (control) or presence of semen clusterin (40 μg/ml) for 30 min at 37˚C. Cells were then exposed to HIV-1 IIIB (5 ng p24, 90 min at 37˚C), washed, and cultured for 10 d. *G*, At day 7, cells were harvested and assayed for intracellular p24 Ag staining by FACS. The infection was analyzed in the gate of CD4$^+$ T cells, and representative dot plots are shown. The bars show the mean of a representative experiment performed by triplicate (n = 3). *H*, Supernatants harvested at different time points were assayed for p24 Ag by ELISA. A representative experiment (n = 3) is shown.

measuring p24 Ag by ELISA. As shown in Fig. 5, semen clusterin, but not recombinant clusterin, markedly inhibited the binding of the CCR5-using HIV-1 BaL by both DCs (Fig. 5*A*) and DC-SIGN–expressing Raji cells (Fig. 5*B*). Similar results were observed using the CXCR4-dependent HIV-1 IIIB or the primary isolates 93BR020.1 and 96USHIPS4 (Supplemental Fig. 2). Likewise, both the infection of DCs (Fig. 5*C*) and the transmission of HIV-1 to T lymphocytes mediated by DCs (Fig. 5*D*) were markedly inhibited by semen clusterin. A similar inhibitory effect was mediated by blocking Abs directed to DC-SIGN (Fig. 5*C*, 5*D*). In contrast, using DC-SIGN–expressing Raji cells, we observed that semen clusterin almost completely abrogates the transmission of HIV-1 to T lymphocytes (Fig. 5*E*).

Because the production of virus by DCs is strongly dependent on cell viability, Supplemental Fig. 3*A* and 3*B* show that incubation of DCs with semen clusterin (40 μg/ml) for 6 d did not affect DC viability. We conclude that semen clusterin inhibits DC-SIGN–mediated HIV binding to DCs, the infection of DCs, and the transmission of HIV to T lymphocytes.

Further experiments were then performed to analyze whether semen clusterin might be able to inhibit the binding of HIV to activated PBMC and macrophages. Contrasting with the observations made in DCs, we found that semen clusterin did not inhibit the binding of HIV-1 by these cells (Fig. 5*F*). Moreover, Fig. 5*G* and 5*H* show that semen clusterin did not inhibit the infection of activated PBMC by HIV-1. We conclude that the anti-HIV activity of semen clusterin is selectively exerted on DCs.

To directly address the role of glycans in the binding of semen clusterin to DC-SIGN, we next treated semen clusterin with PNGase-F, which cleaves all N-linked glycans. Coomassie blue analysis of SDS-PAGE gels showed effective deglycosylation of semen clusterin (Fig. 6*A*, *left panel*). Western blot with DC-SIGN–Fc showed that semen clusterin completely lost any detectable DC-SIGN–binding activity after treatment with PNGase-F (Fig. 6*A*, *right panel*). Similar results were observed after treatment of semen clusterin with fucosidase (Fig. 6B), which selectively cleaves fucose residues. Both deglycosylated and defucosylated clusterin failed to inhibit HIV binding by DC-SIGN–expressing Raji cells, indicating that fucose residues are indispensable for inhibiting HIV binding (Fig. 6*C*, 6*D*). We conclude that semen clusterin glycan moeties are required for the binding to DC-SIGN and for the inhibition of HIV capture by DC-SIGN.

To analyze whether clusterin accounted for the inhibitory effect exerted by semen on HIV capture mediated by DC-SIGN, we performed additional experiments using clusterin-depleted semen samples. Depletion of clusterin was performed by affinity chromatography, as described under *Materials and Methods*. More than 90% of the clusterin content was eliminated from semen samples, as revealed by ELISA. As shown in Fig. 7*A*, depletion of clusterin markedly reduced the ability of semen to inhibit the capture of HIV by DC-SIGN–expressing Raji cells. However, depletion of clusterin did not completely prevent the ability of semen to inhibit the capture of HIV-1, supporting that besides clusterin, semen might contain other DC-SIGN ligands.

Recently, Stax and colleagues (11) proposed that mucin-6 in seminal plasma is a novel DC-SIGN ligand. The authors fractioned seminal plasma by size-exclusion chromatography and reported that the high m.w. fractions of seminal plasma containing mucin-6 (among other proteins) bind DC-SIGN and block DC-SIGN–mediated transmission of HIV-1 to $CD4^+$ T lymphocytes. We re-examined in this study these findings by analyzing whether these high m.w. fractions of seminal plasma also contain clusterin. Seminal plasma was fractionated using an FPLC-Superdex 200 10/300 column (Amersham Bioscience). The elution of proteins was followed by measuring the OD at 280 nm, and the amount of protein in each fraction was quantified by the Bradford assay (Fig.

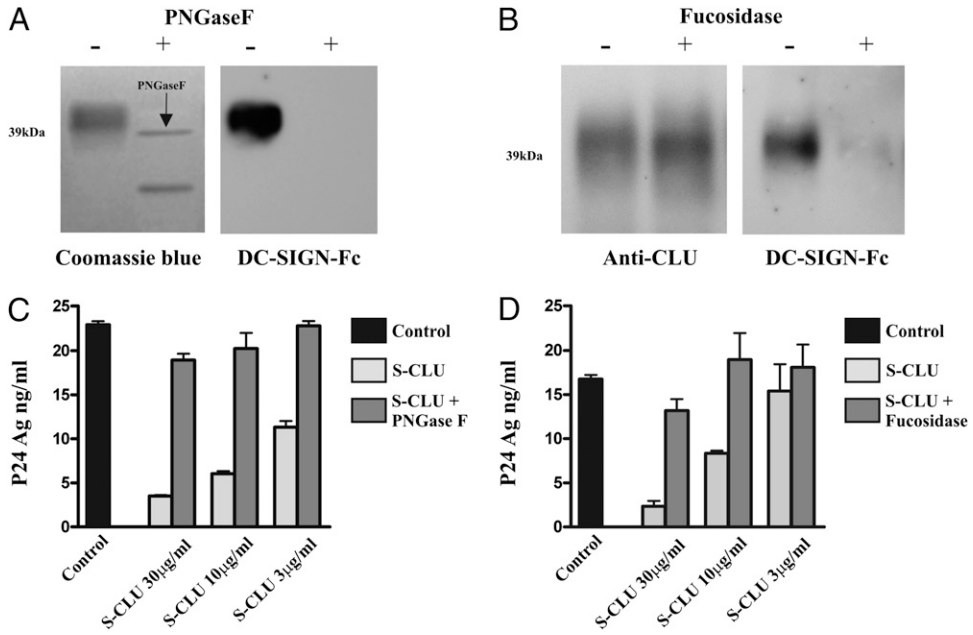

FIGURE 6. Analysis of the role of semen clusterin glycans in the binding to DC-SIGN and the inhibition of HIV capture. *A*, Semen clusterin was treated with PNGase F (2 units, 15 h at 37˚C). Then it was run on SDS-PAGE and stained with Coomassie blue (*left panel*), or blotted onto a nitrocellulose membrane, treated with DC-SIGN–Fc chimera (*right panel*), and revealed with HRP-conjugated goat anti-human IgG. *B*, Semen clusterin was treated with α 1-3,4 fucosidase (0.5 units, 3 h at 37˚C). Then it was run on SDS-PAGE, blotted onto a nitrocellulose membrane, and revealed with goat anti-clusterin IgG (*left panel*) or DC-SIGN–Fc chimera (*right panel*). A representative experiment is shown. *C* and *D*, Semen clusterin was treated with PNGase F (2 units, 15 h at 37˚C) or α 1-3,4 fucosidase (0.5 units, 3 h at 37˚C) as described under *Materials and Methods*. Raji DC-SIGN cells were incubated for 30 min at 37˚C with different concentrations of untreated, PNGase F-treated, or α 1-3,4 fucosidase-treated clusterin. Cells were then cultured with HIV-1 BaL (5 ng of p24, 90 min at 37˚C), washed thoroughly, lysed, and assayed for p24 Ag by ELISA. A representative experiment is shown (n = 2 to 3).

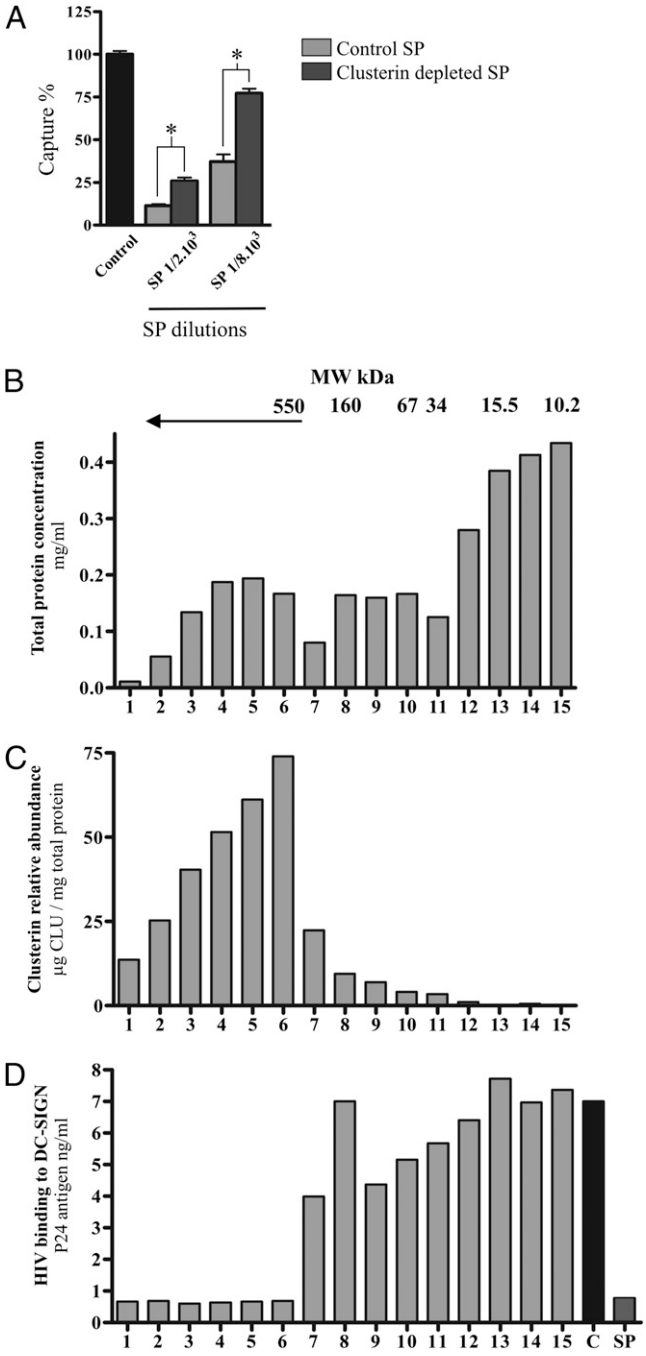

FIGURE 7. Analysis of the role of clusterin in the inhibition of HIV-1 recognition by DC-SIGN mediated by semen. *A*, Seminal plasma was depleted of clusterin by affinity chromatography. More than 90% of the clusterin content was eliminated from semen samples, as revealed by ELISA. Raji DC-SIGN cells were incubated for 30 min at 37°C with different dilutions (1:2 × 10^3 or 1:8 × 10^3) of control seminal plasma or clusterin-depleted seminal plasma. Cells were then cultured with HIV-1 BaL (5 ng of p24, 90 min at 37°C), washed thoroughly, lysed, and assayed for p24 Ag by ELISA. Data represent the mean ± SEM of three experiments carried out in triplicate. Asterisk represents statistical significance ($p < 0.01$) versus controls. *B–D*, Seminal plasma was fractionated using an FPLC-Superdex 200 10/300 column (Amersham Bioscience). The elution of proteins was followed by measuring the OD at 280 nm. The amount of protein in each fraction was quantified by the Bradford assay (*B*), and the concentration of clusterin was determined by ELISA (*C*). All of the collected fractions were adjusted to a final protein concentration of 100 µg/ml, and the ability of each fraction to inhibit the binding of HIV-1 BaL to DC-SIGN–expressing Raji cells was assessed (*D*). The capture of HIV-1 mediated by DC-SIGN–expressing Raji cells incubated with HIV-1 in the absence of seminal plasma (controls) or in the presence of whole seminal plasma (dilution 1:10^3) is shown in the C and SP bars, respectively. *B–D*, A representative experiment ($n = 3$) is shown.

7*B*). We first analyzed the ability of each fraction to inhibit the binding of HIV-1 by DC-SIGN–expressing Raji cells. Consistent with the results reported by Stax and colleagues (11), we found that the inhibitory activity of seminal plasma was restricted to the high molecular mass fractions (>550 kDa) (Fig. 7*C*, 7*D*). Of note, these fractions were shown to contain high concentrations of clusterin. A close correlation was found between the ability of each fraction to inhibit the binding of HIV-1 to DC-SIGN–expressing Raji cells and their clusterin content (Fig. 7*C*, 7*D*). In fact, all of the fractions with high concentrations of clusterin almost completely block the binding of HIV-1 to DC-SIGN, whereas no inhibition was observed for clusterin-poor fractions. As previously described for serum clusterin, the presence of semen clusterin in the high molecular fractions of seminal plasma could be explained considering the ability of clusterin to form aggregates or interact with a variety of proteins due to its intrinsic chaperone-like activity (21, 31–33).

Discussion

In this study, we identified clusterin as a DC-SIGN–binding protein on two-dimensional Western blot assays of total seminal plasma. The identity of semen clusterin was unequivocally confirmed using specific Abs as well as MS. The purified protein binds not only to soluble DC-SIGN in Western blot assays but also to DC-SIGN expressed on DCs and Raji DC-SIGN$^+$ cells. Moreover, the analysis of clusterin glycans by MALDI-MS revealed an extreme abundance of highly fucosylated glycans that bear the Lewis^x or Lewis^y motifs, which are both excellent DC-SIGN ligands. These motifs enable semen clusterin to bind DC-SIGN with very high affinity (K_d 76 nM) and to effectively inhibit HIV-1 binding to DC-SIGN. Of note, a complete inhibition of HIV-1 recognition by DC-SIGN was observed using 40 µg/ml of semen-purified clusterin, a concentration almost 25 fold-lower than the concentrations of clusterin found in normal semen.

In a recent paper, Stax and colleagues (11) confirmed our previous results showing that semen effectively inhibits the binding of HIV to DC-SIGN (9). Using gel filtration chromatography, they also showed that high molecular fractions of seminal plasma containing mucin-6 (among other proteins) binds DC-SIGN and blocks DC-SIGN–mediated transmission of HIV-1 to CD4$^+$ T cells. Consistent with the ability of clusterin to form high m.w. complexes with different proteins (31–35), we found in this study that the high molecular fraction of seminal plasma also contains very high concentrations of clusterin. Moreover, we found a strong correlation between the ability of each fraction to inhibit the binding of HIV-1 to DC-SIGN and their clusterin content. Might clusterin and mucin-6 act together to inhibit the recognition of HIV-1 by DC-SIGN? Experiments using either purified mucin-6 or mucin-6–depleted semen samples are required to define this point.

Taken together, our observations and the results published by Stax and coworkers (11) suggest that semen might contains a set of different proteins able to block the recognition of carbohydrate structures expressed on the pathogen surface by DCs. Because DC-SIGN–expressing DCs are mainly located in the subepithelial mucosa of the vagina and the rectum (36, 37), an open question is how semen clusterin might gain access to the subepithelium to interfere the recognition of pathogens by DC-SIGN. Epithelial microabrasions in the mucosa, which are detected in 60% of healthy women after consensual intercourse (38, 39), would allow

semen compounds to access subepithelial DCs. Moreover, a variety of sexually transmitted diseases trigger both local inflammatory responses and disruption of epithelium integrity (40–42). Finally, semen components might gain access to DCs through an alternative route (e.g., by binding to DC projections that extend to, or near, the luminal surface of the challenged epithelium) (43, 44).

Recent studies indicate that DC-SIGN not only mediates the uptake of pathogens but also exerts profound regulatory effects on the function of DCs (4, 5). Hence, the high concentrations of semen clusterin (~1 mg/ml) and its high affinity for DC-SIGN suggest that semen clusterin might modulate the immune response induced in the receptive partner against sexually transmitted pathogens. In fact, blockade of DC-SIGN might impair the ability of pathogens to misuse DC-SIGN as a receptor to infect target cells, circumvent Ag processing, subvert immune effector mechanisms, and spread the infection.

The role of semen clusterin might not be restricted to infectious processes. It is well known that semen suppress a number of immune responses mediated by both the innate and adaptive immune system (45, 46). These immunosuppressive actions appear to play an important role in human reproduction. They enable spermatozoa to survive in the female reproductive tract, induce a state of nonresponsiveness against sperm Ags, promote a tolerogenic response to paternal alloantigens favoring maternal acceptance of the conceptus at implantation, and avoid allogeneic fetal rejection (47–51). Gringhuis and coworkers (5) have recently demonstrated that mannose- and fucose-expressing ligands induced different effects on the function of DCs. Mannose-expressing ligands triggers an inflammatory response mediated by DCs, whereas fucose-expressing ligands suppress the ability of DCs to produce the inflammatory cytokines IL-6 and IL-12, enhancing the production of IL-10. The abundance of highly fucosylated glycans in semen clusterin suggest that it might contribute to switch the function of DCs into a regulatory profile promoting a tolerogenic response to paternal alloantigens in the female reproductive tract. Semen clusterin might promote a tolerogenic profile acting, not only on DCs, but also on DC precursors. It has been recently shown that DC-SIGN ligation greatly affects DC differentiation from DC precursors compromising their normal function (52).

Clusterin is an enigmatic protein involved in a variety of physiologic and pathologic processes including inflammation, atherosclerosis, and cancer (12–16). The diverse set of functions mediated by clusterin is usually attributed to the existence of two alternatively spliced forms of the clusterin gene that encode secretory or nuclear clusterin (15, 16). Our results uncover an unexpected heterogeneity in the glycosylation pattern of clusterin and, more interestingly, indicate that clusterin may display different biological functions according to its glycosylation pattern.

Clusterin expression is disregulated in many types of cancer, and it appears to play a role in tumor progression (15, 53). Moreover, changes in glycosylation are strongly associated with the development of cancer and metastasis (54–56). Recent findings indicated that tumor-related glycoforms of carcinoembryonic Ag, MUC-1, and Mac-2–binding protein are specific ligands for C-type lectins expressed on DCs such as DC-SIGN and instruct DC to drive Th2-mediated responses that, unlike Th1 responses, do not contribute to tumor eradication (56–61). It is tempting to speculate that tumor-related glycoforms of clusterin, similar to semen clusterin, might also bind to DC-SIGN, favoring tumor evasion of the immune response.

Acknowledgments

We thank the Laboratory of Proteomic Mass Spectrometry at Institut Curie, Paris, France.

Disclosures

The authors have no financial conflicts of interest.

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