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PIVOTAL ADVANCE

Pivotal Advance: The promotion of soluble DC-SIGN release by inflammatory signals and its enhancement of cytomegalovirus-mediated *cis*-infection of myeloid dendritic cells

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RECEIVED JULY 2, 2010; REVISED SEPTEMBER 13, 2010; ACCEPTED SEPTEMBER 13, 2010. DOI: 10.1189/jlb.0710386

ABSTRACT

DC-SIGN is a member of the C-type lectin family. Mainly expressed by myeloid DCs, it is involved in the capture and internalization of pathogens, including human CMV. Several transcripts have been identified, some of which code for putative soluble proteins. However, little is known about the regulation and the functional properties of such putative sDC-SIGN variants. To better understand how sDC-SIGN could be involved in CMV infection, we set out to characterize biochemical and functional properties of rDC-SIGN as well as naturally occurring sDC-SIGN. We first developed a specific, quantitative ELISA and then used it to detect the presence sDC-SIGN in in vitro-generated DC culture supernatants as cell-free secreted tetramers. Next, in correlation with their inflammatory status, we demonstrated the presence of sDC-SIGN in several human body fluids, including serum, joint fluids, and BALs. CMV infection of human tissues was also shown to promote sDC-SIGN release. Based on the analysis of the cytokine/chemokine content of sDC-SIGN culture supernatants, we identified IFN-γ and CXCL8/IL-8 as inducers of sDC-SIGN production by MoDC. Finally, we demonstrated that sDC-SIGN was able to interact with CMV gB under native conditions, leading to a significant increase in MoDC CMV infection. Overall, our results confirm that sDC-SIGN, like its well-known, counterpart mDC-SIGN, may play a pivotal role in CMV-mediated pathogenesis. *J. Leukoc. Biol.* **89: 329–342; 2011.**

Introduction

DC-SIGN is a TM type II protein, which belongs to a family of calcium-dependent lectins diversely used by human APCs, such as tissue-residing myeloid DCs, alveolar and LN macrophages, and endothelial cells from liver sinusoids [1–4]. DC-SIGN contains a CRD that is highly conserved in lectins and a neck region consisting of Ig-like domain repetitions, which mediate a pH-dependent oligomerization of DC-SIGN monomers and thus, increase affinity with its ligands [5–8]. During monocyte differentiation toward MoDCs, DC-SIGN expression has also been reported to be induced by IL-4 and to be negatively regulated by IFNs, TGF-β, and dexamethasone [9].

DC-SIGN is a receptor for self-glycoproteins, such as ICAM-3/2 [10, 11], and is also able to recognize high mannose-containing structures and fucosylated Lewis blood Ags ($Le^{x/y/b/a}$) [7, 12], expressed by several pathogenic microorganisms including viruses, bacteria, yeasts, and parasites (for review, see ref. [13]). Interactions between DC-SIGN and pathogenic-derived sugar moieties have been shown to play a prominent role in vivo in favoring the capture and internalization of microbes [14], with or without the help of other Ag-capture receptors, such as the macrophage mannose receptor (CD206), DEC-205 (CD205) [15], or the mac-

Abbreviations: CBA=cytokine bead array, CBP=cord blood precursor, CRD=carbohydrate recognition domain, CRP=C-reactive protein, DC-SIGN=dendritic cell-specific ICAM3-grabbing-nonintegrin, FSF=foreskin fibroblast, gB=glycoprotein B, HEK=human epithelial kidney, IE/E=immediate early/early, IP=immunoprecipitation, IP-10=IFN-inducible protein 10, Marimastat=N-[2, 2-dimethyl-1-(methylcarbamoyl)propyl]-2-[hydroxyl(hydroxycarbamoyl)methyl]-4-methyl-pentanamide, MBL=mannan-binding lectin, mDC-SIGN=membrane-associated DC-specific ICAM-3-grabbing-nonintegrin, MMP=matrix metalloproteinase, MoDC=monocyte-derived DC, MW_{meas}=measured MW, MW_{theor}=theoretical MW, RA=rheumatoid arthritis, sDC-SIGN=soluble DC-specific ICAM-3-grabbing-nonintegrin, TBGFP, a genetically modified TB40/E-based strain encoding the GFP under the control of an immediate early gene promoter, Tm=melting temperature, TM=transmembrane

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

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rophage galactose-type C-type lectin [16]. In contrast to other C-type lectins, several transcripts have been described for DC-SIGN, most likely originating from alternative splicing and potentially leading to the expression of sDC-SIGN proteins [17]. As described already for the DC-SIGN homologue, DC-SIGNR (or CD209 ligand) [18], such cDNAs have been reported by others to encode nonsecreted sDC-SIGN molecules [19]. In addition, the same study showed that sDC-SIGN displayed no functional activity in terms of ICAM-3-dependent cosignaling compared with mDC-SIGN.

Some years ago, we demonstrated that CMV, like HIV, is able to bind to DCs via DC-SIGN and enter them more easily than the free viral particles [20]. Consequently, the DC-SIGN-mediated increase in virus entry resulted in a strong CMV infection of MoDC. The study in question shed new light on the molecular interactions that favor DC infection by CMV and especially the pivotal role played by DC-SIGN as a docking and internalizing receptor. CMV is a widespread herpesvirus that infects 40–100% of the populations worldwide. It induces lifelong viral persistence and may cause severe disease in immunocompromised individuals, such as those with HIV or transplant patients. The major CMV host entry sites are myeloid DC-containing peripheral tissues, especially oropharyngeal and genital mucosa. In the present study, we investigated expression regulation and functional properties of naturally occurring as well as rsDC-SIGN variants. We demonstrated that such variants can promote CMV infection of MoDCs, as already shown for their counterparts, mDC-SIGN. These data suggest that sDC-SIGN variants may play a crucial role in the CMV-mediated pathogenesis.

MATERIALS AND METHODS

Sample collection and patients

After signing an informed consent, peripheral or cord blood and BAL samples, as well as vaginal mucosa explants, resulting from a partial or complete hysterectomy (vaginal tumor resection or prolapsus), were collected and used in our experiments, in accordance with protocols approved by the local ethical committee. BAL samples were isolated from hospitalized-infected (CMV primary infection, influenza, coronavirus, pneumococcus, or hemofilus influenza) or polytrauma patients. BAL were classified as "inflammatory" or "noninflammatory" according to the CRP serum concentration (respectively, $[CRP]_{serum}>5$ mg/ml, and $[CRP]_{serum}<5$ mg/ml).

Viruses, reagents, and antibodies

Two endotheliotropic human CMV strains, TB40/E and VHL/E, were kindly provided by Dr. Christian Sinzger (Institute for Medical Virology and Epidemiology, University of Tubingen, Tubingen, Germany) [21]. TBGFP was a kind gift of Dr. Martin Messerle (Department of Virology, Hannover Medical School, Hannover, Germany). All viruses were propagated, purified, and titered as described [22]. For gel filtration analyses, the rCRD of DC-SIGN was kindly provided by Dr. Franck Fieschi (Institute for Structural Biology, Grenoble, France). This molecule was produced in *Escherichia coli*. Anti-DC-SIGN antibodies were obtained from Dr. Bernard Verrier (FRE2736 CNRS-bioMérieux, Lyon, France; mAb clone 3E1A8, purified and biotinylated) from the French Agency for AIDS Research program (mAb clone 1B10; AC14.1 [20]) or purchased from BD Biosciences (San Jose, CA, USA; mAb clone DCN46) or Santa Cruz Biotechnology (Santa Cruz, CA, USA; pAb H-200). Mouse mAb against specific human surface Ags were used in a direct immunostaining assay: PE-cyanin5-conjugated anti-CD1a (clone BL6), PE-conjugated anti-CD80/83/86 (clones MAB104, HB15a, and HA5.2B7), and conjugated isotypic control mAb (Beckman Coulter, Fullerton, CA, USA); and PE-conjugated anti-HLA DR (clone L243) and FITC-conjugated anti-DC-SIGN/CD209 (#DCN46; BD Biosciences). Neutralizing polyclonal antibodies directed against human CXCL-8/IL-8, CXCL-10/IP-10, IFN-γ, or IL-6 were purchased from R&D Systems (Minneapolis, MN, USA).

Cells and in vitro DC differentiation

HEK 293T clones were cultured in 8% FCS DMEM supplemented with 2 mM glutamine. HEK cells were used for transient recombinant protein expression assays. DCs were generated in vitro from adult blood monocytes according to a modified version of the protocol described previously by Sallusto and Lanzavecchia [23]. Briefly, $CD14^+$ monocytes were highly enriched from PBMCs of healthy donors by a negative magnetic depletion using hapten-conjugated CD2, CD3, CD19, CD56, CD66b, and Glycophorin A antibodies (StemSep™ human monocyte enrichment kit, Stem Cell Technologies, Vancouver, Canada). Routinely, two enrichment steps resulted in >98% pure $CD14^+$ cells. Purified $CD14^+$ monocytes were cultured in six-well plates (NUNC, Thermo Scientific, Rochester, NY, USA) for 6 days with RPMI-1640 medium, supplemented with 8% FCS (BioWhittaker, Cambrex, Charles City, IA, USA) and 20 ng/ml human rIL-4 and 100 ng/ml human rGM-CSF (Peprotech EC Ltd., London, UK). Every 2 days, one-half of the medium was replaced by fresh IL-4/GM-CSF-supplemented medium. At Day 5, virtually all cells displayed the typical phenotype $CD1a^+$, $CD14^-$, HLA-DR^-, $CD80^{low}$, $CD86^{low}$, $CD83^-$, DC-$SIGN^+$ of immature MoDCs, as assessed by flow cytometry. Alternatively, DCs were generated from $CD34^+$ CBPs, as described already elsewhere [17]. Immunomagnetically purified CBPs were cultured in medium supplemented with 20 ng/ml stem cell factor and 50 ng/ml GM-CSF (R&D Systems). TNF-α (10 ng/ml) was added on Day 7, and on Day 11 of culture, IL-4 (10 ng/ml) was added to induce DC-SIGN synthesis for 3 additional days.

cDNA cloning

Total RNA was isolated from MoDC using the TRIzol™ reagent isolation method according to the manufacturer's instructions (Invitrogen Corp., Carlsbad, CA, USA). Total RNA was then reverse-transcribed with the M-MLV RT and polydT oligonucleotide (Promega, Madison, WI, USA). DC-SIGN-encoding cDNAs were amplified by PCR using a high-fidelity Taq polymerase (Roche Diagnostics, Meylan, France) with the following oligonucleotides: forward 5′-AAGAATTCGACTACAAGGATGACGATGACAAGGGAATGAGTGACTCCAAGGAA-3′, allowing to insert a FLAG tag-encoding sequence, and reverse 5′-TATTATGCATCTACGCAGGAGGGGAATTCTT -3′. PCR products were directly cloned at the *EcoRI* sites of the pET21a prokaryotic expression vector (Novagen, VWR International S.A.S, Fontenay sous bois, France) or alternatively, of the pCDNA3.1 plasmid (Invitrogen Corp.). TM-missing cDNA were screened using two internal primer pairs to amplify, respectively, all and TM-containing cDNA: forward/reverse DC-SIGN_SM/DC-SIGN_AS (Tm=59.8°C, 5′-CTCCAAGGAACCAAGACTGC-3′/Tm=59.8°C, 5′-TTGTTGGGCTCTCCTCTGTT-3′) and forward/reverse DC-SIGN_SS/DC-SIGN_AS (Tm=55.3°C, 5′-AACTCCTCTCCTTCACGC-3′). Retained, TM-missing cDNAs were then sequenced by the dideoxynucleotide termination method (Genomexpress, Meylan, France) and submitted to a Basic Local Alignment Search Tool request (http://blast.ncbi.nlm.nih.gov/Blast.cgi). For clarity reasons, our constructions consisting of the sDC-SIGN1A type I isoform encoding cDNA cloned into pET21a and pCDNA3.1 vectors and were renamed pET21-FLAG-sDCSIGN1AT1 and pCDNA3.1-FLAG-sDCSIGN1AT1, respectively. The sDC-SIGN1A type III-encoding sequence was also cloned into the pET21a plasmid and produced similarly as described below for sDCSIGN1AT1.

Recombinant protein expression

For protein production, pET21a-FLAG-sDCSIGN1AT1 and pET21a-FLAG-sDCSIGN1AT3 recombinant plasmids were transferred to Rosetta™ (DE3) pLysS-competent cells (Novagen, a Merck company, Germany). This modified bacterial strain is usually used to synthesize native recombinant eukaryotic protein, even in the absence of a signal peptide. The recombinant protein expression was induced for 6–12 h by the addition of 1 mM IPTG to a growing culture at 30°C, according to the manufacturer's instructions, and in the presence of 34 μg/ml chloramphenicol (C0378, Sigma-Aldrich, St. Louis, MO, USA). IPTG-induced cells were harvested and lysed directly with the bacterial-protein extraction reagents (Thermo Scientific). Both sDC-SIGN isoforms were then purified by a two-step affinity chromatography consisting of one passage on mannan-conjugated agarose (elution was done with 50 μg/ml mannan in TBS), followed by a second passage on a M2 affinity resin-loaded column at low pressure (Sigma-Aldrich). Then, rsDC-SIGN elution was obtained with a 100-μg/ml FLAG peptide solution (in TBS), according to Sigma-Aldrich protocols. After the second round of purification, each eluted fraction was concentrated onto Centricon-3 filtration devices (Millipore, Bedford, MA, USA) to allow the contaminating FLAG peptide to be removed. Each treated fraction was then analyzed by Western blot and silver nitrate staining to estimate purity (90–95% estimated purity was usually achieved). Positive fractions were then pooled and stored in glycerol (50% v/v) at −80°C until used. sDC-SIGN concentration was measured by ELISA for every FLAG-sDC-SIGN batch production.

Gel filtration

When required, samples were submitted to gel filtration chromatography to allow MW determination of native multimer or monomer proteins. Briefly, 0.5–2 ml DC-SIGN-containing samples were loaded onto a HiPrep 26/60 Sephacryl S-200 HR column (GE Healthcare, Waukesha, WI, USA) and separated according to their MW. Fractions (1 ml) were collected and analyzed by Western blot or ELISA to document the presence or absence of DC-SIGN. Mean elution volumes were then determined for each tested sample, thus enabling the calculation of DC-SIGN MW using MW standards (Bio-Rad France, Marnes-la-Coquette, France).

sDC-SIGN ELISA titration

A specific sDC-SIGN ELISA was developed in our laboratory. A polyclonal anti-DC-SIGN antibody (clone H-200, Santa Cruz Biotechnology) was coated onto presaturated Reactibind 96-well plates, according to the manufacturer's instructions (Pierce Biotechnology, Rockford, IL, USA). After washing plates, samples (sera, BAL, synovial fluid, cell culture supernatants, etc.) were diluted in TBS or left undiluted, mixed with an equal volume of TBST containing the biotin-conjugated 3E1 anti-DC-SIGN mAb (0.2 μg/ml), and incubated further for 1 h at room temperature. A final incubation step with a HRP-conjugated Neutravidin™ solution (Pierce Biotechnology) allowed for the detection of sDC-SIGN with tetramethylbenzidine, a specific chromogenic HRP substrate. Absorbance was read at 450 nm and 570 nm (background substraction) on a Labsystems Multiskan microplate reader (Labsystem Multiskan MS, Finland) and analyzed with the Biolise™ software (Version 2.0, Labtech International, UK). Quantitative sDC-SIGN concentration determination was achieved when ODs were compared with a standard curve, obtained with FLAG-sDC-SIGN1AT1 protein, produced in *E. coli*. Validation tests of our homemade ELISA sDC-SIGN are presented in **Table 1**.

DC-derived exosome and supernatant separation

Exosomes were prepared from the supernatant of 6-day-old IL-4/GM-CSF-differentiated MoDCs cultured in complete medium using a simplified version of a protocol reported previously by Raposo et al. [24]. Three successive centrifugations at 300 g (5 min), 1200 g (20 min), and 10,000 g (30 min) were performed to eliminate cells and debris. Supernatants were ultracentrifuged at 100,000 *g* for 2 h onto a D20/30% sucrose gradient-density cushion (d=1.217). The exosome-enriched pellet was resuspended in

TABLE 1. sDC-SIGN ELISA Validation Test

sDC-SIGN concentrations (ng/ml)	Sandwich ELISA (H200/3E1*)[a]	No capture antibody (H200)	sDC-SIGN standard solution pretreatments					
			No secondary antibody (3E1*)[a]	Homotypic ELISA (3E1/3E1*)[a]	IP DC-SIGN (1B10)[b]	IP DC-SIGN (H200)[b]	IP control (1F10)[b]	Mannan
50 ($OD_{450/570nm}$)	1.10 ± 0.12	0.03 ± 0.01	0.032 ± 0.01	0.05 ± 0.04	0.04 ± 0.04	0.03 ± 0.01	1.057 ± 0.2	0.03 ± 0.01
5 ($OD_{450/570nm}$)	0.19 ± 0.05	0.03 ± 0.02	0.033 ± 0.01	0.04 ± 0.03	0.03 ± 0.01	0.03 ± 0.01	0.19 ± 0.08	0.03 ± 0.01

ODs at 450 nm (570 nm as a reference filter) were measured for two FLAG-sDC-SIGN1AT1 concentrations (50 and 5 ng/ml) in different settings. Values are indicated as medians of four distinct sets of experiments ± the SD. [a]3E1*, Biotinylated form of the anti-DC-SIGN 3E1 clone; [b]IP, DC-SIGN IP (control mAb is directed against LIF; clone 1F10).

PBS and kept frozen for further experiments, and clarified supernatant was concentrated with an Amicon centrifugal filter device (10 kDa cut-off, Millipore). Total protein contents of supernatants and exosome pellets were quantified using the BCA assay (Pierce Biotechnology).

Functional assays

Cytokines and chemokines were quantified into cell/tissue culture supernatants or biological fluids by the CBA flex set (BD Biosciences). When needed, cervical or vaginal epithelium biopsies were infected with TB40/E CMV strain (6×10^5 pfu/cm^2) or activated with 100 ng/ml LPS (*E. coli* 0111:B4; Sigma-Aldrich) for varying times (from 10 min to 36 h). sDC-SIGN concentrations were simultaneously determined in samples by ELISA. sDC-SIGN-induced expression assays were performed with fully differenciated immature MoDCs (6 days). Cells were starved in IL-4 for 16 h; i.e., only GM-CSF was added to the culture medium before being subcultured under various conditions, i.e., supplementation with recombinant cytokines or chemokines (CXCL8/IL-8, CXCL10, IFN-γ, and IL-6; Peprotech EC Ltd.). When required, immature MoDCs (Day 6) or FSF were subcultured with a single MOI with the TBGFP CMV strain previously incubated with various amounts of FLAG-sDC-SIGN1AT1 for 2 h at 37°C. After 48 h, cells were harvested and analyzed by flow cytometry. Percentages of GFP$^+$ cells directly reflected the infectious rate, as assessed already in our former work [20].

Western and dot-blot analyses

Total proteins were obtained from concentrated cell culture supernatants or cell lysates. When required, HEK 293T cells were transiently transfected with the pCDNA3.1-FLAG-sDCSIGN1AT1 eukaryotic expression vector using the Exgen 500 transfection reagent according to the manufacturer's instructions (Euromedex, France). After 3 days, cells were harvested, washed three times with PBS, and resuspended into a lysis buffer (50 mM Tris, pH=7.5, 150 mM NaCl, 0.5% Triton X-100), supplemented with a protease inhibitor cocktail (P8340; Sigma-Aldrich) and cleared from nuclei by centrifugation at 10,000 g. For each kind of experiment, indicated total protein amounts were loaded and separated onto 10% acrylamide SDS-PAGE gels before being transferred to nitrocellulose membranes. Saturated membranes (2 h, room temperature in 0.1% TBST, 5% nonfat milk) were then hybridized with specific primary antibodies against DC-SIGN (3E1A8 or 1B10 clones) or actin (rabbit polyclonal antibody; Sigma-Aldrich) and secondary HRP-conjugated goat anti-mouse or rabbit mAb (Amersham Pharmacia Biotech, Sweden). Proteins of interest were finally detected using an ECL detection kit (Pierce Biotechnology).

Alternatively, protein lysates were diluted in TBS and spotted onto appropriate nitrocellulose membranes using a Minifold-I Dot-Blot system (Schleicher & Schuell, Germany). Then, membranes were saturated and hybridized as described in the previous paragraph.

Immunohistochemistry

Human cervical or vaginal mucosa explants were collected by Prorfessor C. Hocke (Department of Gynecological Surgery, General Hospital of Bordeaux, France), in accordance with international ethical rules. Explants were extracted as circular pieces of ~1 cm^2 and submitted or not to CMV infection (VHL/E; 6×10^5 pfu/cm^2). OCT (Sakura Finetek USA, Inc., Torrance, CA, USA)-embedded frozen tissue sections were air-dried for 30 min, washed in PBS (pH 7.4), and fixed/permeabilized in 1:1 vol:vol cold acetone/methanol at 4°C for 10 min. The fixed sections were saturated using PBS containing 0.5% BSA and 10% normal goat serum (Sigma-Aldrich) at room temperature for 1 h. Subsequently, the sections were incubated with the anti-DC-SIGN DCN46 mAb or the anti-IE/E CMV Ag antibody (Argene SA, Varilhes, France), followed by incubation with an Alexa® 488-conjugated goat anti-mouse mAb (Molecular Probes, Invitrogen Corp.). Concomitantly, nuclei were stained with DAPI. Slides were then washed twice in PBS for 5 min and rinsed in distilled water before being air-dried. Stained sections were mounted with Dako fluorescent mounting medium (Dako, Carpinteria, CA, USA) and analyzed on a SP5 confocal microscope (Leica Microsystems, Germany).

Statistical analyses

Statistics were generated with the GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA). Unpaired sample comparisons were performed using a Mann-Whitney nonparametric rank test.

RESULTS

Cloning, expression, and ELISA titration of sDC-SIGN

A previous study by Mummidi and colleagues [17] reported the cloning of cDNA, potentially coding for sDC-SIGN. To determine whether corresponding proteins exist, we first isolated DC-SIGN-encoding cDNAs from monocyte- or CD34$^+$ cord blood cell-derived DCs by PCR. The primer used in this PCR allowed for the insertion of a FLAG tag-encoding sequence in the 5′ position (**Fig. 1A**). Only 10 selected cDNA clones were submitted to a second round of PCR to retain the sole TM region lacking cDNAs, thought to probably code for sDC-SIGN (Fig. 1B). Four of the 10 clones were not amplified by the TM-specific internal PCR (Clones 2, 3, 6, and 7; Fig. 1A and B). These cDNA clones lacking the TM exon were sequenced and blasted against known cDNA libraries. Then, we confirmed that three out of these four isolated cDNAs were completely identical to a sequence reported previously by Mummidi et al. [17] (i.e., sDC-SIGN1A type I isoform-encoding sequence; GenBank Accession Number NM_001144896) and that the last one encoded a spliced variant, the sDC-SIGN1A type III (GenBank Accession Number AY042227). We then subcloned sDC-SIGN1A type I- and III-encoding cDNAs (called thereafter FLAG-sDC-SIGN1AT1 and T3) into prokaryotic and eukaryotic expression plasmids as required. An ELISA was produced simultaneously to measure sDC-SIGN isoform concentrations in cell culture supernatants or in any other biological fluids. The FLAG-sDC-SIGN1AT1 protein of prokaryotic origin was used to obtain a standard curve and set up our homemade ELISA (Fig. 1C). Although our sDC-SIGN ELISA was moderately sensitive with a typical concentration range of 100–0.1 ng/ml, we were able to validate its specificity. In fact, by means of selective IP experiments on sDC-SIGN standard solutions, we could demonstrate that 1B10 mAb or H-200 pAb (the coating pAb), directed against DC-SIGN, abrogated sDC-SIGN detection, whereas an irrelevant mAb had no effect, i.e., FLAG-sDC-SIGN1AT1 was still detected by ELISA (Table 1). Interestingly, a homotypic ELISA, i.e., the same antibody (clone 3E1) used as the primary (capture) and secondary antibody, led to low signal detection. This observation evoked the possibility that the FLAG-sDC-SIGN1AT1 protein was expressed as multimers. This issue is addressed at a later stage in this paper.

sDC-SIGN released by DCs generated in vitro

Previous studies have reported that TM-lacking encoding DC-SIGN sequences are transcribed as soluble cytoplasmic pro-

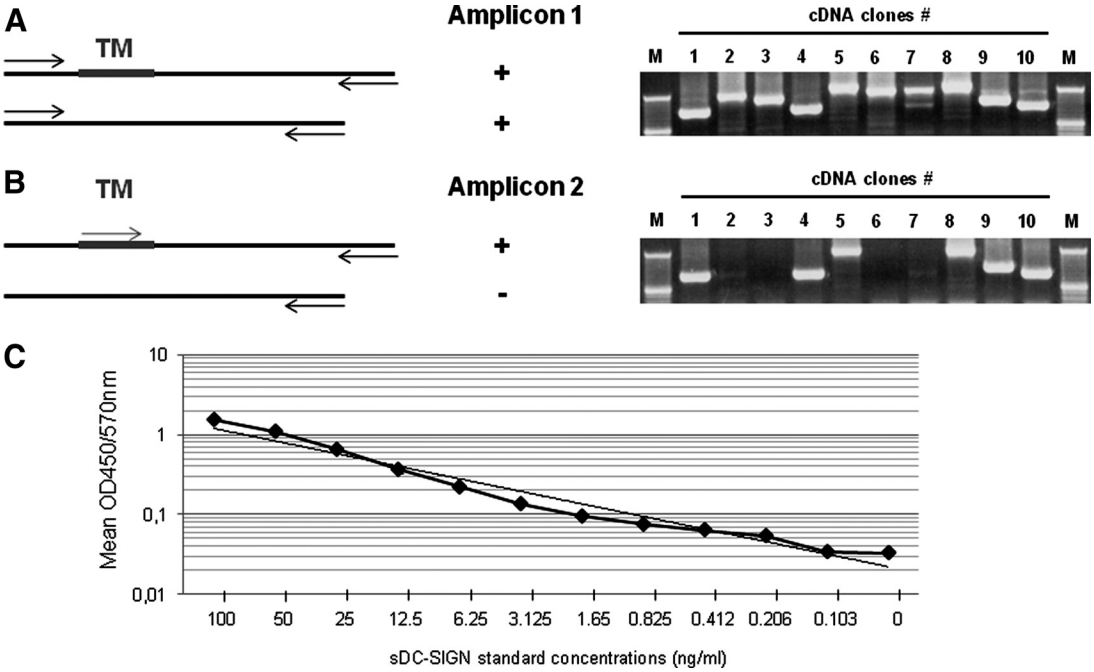

Figure 1. Cloning expression and sDC-SIGN ELISA development. (A) Schematic representation of the full-length DC-SIGN-encoding cDNA amplification by RT-PCR. The DC-SIGN_SM/DC-SIGN_AS primer pair was used here to amplify all DC-SIGN sequences denoted by the "+" sign under "Amplicon 1" (product size range from 1041 to 633 bp). Amplicons were separated on an agarose gel (1%), and DNA was labeled with ethidium bromide. (B) The legend here is identical to the previous one except for the primer pair used to amplify only TM-containing cDNA DC-SIGN-encoding sequences (DC-SIGN_SS/DC-SIGN_AS). A "–" sign indicated under "Amplicon 2" means that no amplicon could be detected on the gel. M, molecular weight marker. (C) Standard curve of FLAG-sDC-SIGN1AT1 titration. Data was plotted as $OD_{450\ nm}$ (with a 570-nm reference filter) on the *y*-axis against sDC-SIGN concentrations (ng/ml) on the *x*-axis. OD values are representative of four distinct experiments.

teins but not secreted by the sDC-SIGN-expressing transfectant cells or immature MoDCs [19]. It was our hypothesis that the lack of secreted proteins in culture supernatants may be a result of a secretion defect or the lack of a signal peptide [25]. Of note, certain proteins, such as IL-1α, FGF2, or galectin-1/3, are secreted, despite being devoid of a signal peptide [26–29]. As a result, we surmised that the same could be true for sDC-SIGN. Thus, using our quantitative and specific ELISA, we observed the presence of potential sDC-SIGN isoforms in 10× concentrated cell culture supernatants of mDC-SIGN-expressing DCs. In vitro DCs were generated from adult monocytes or $CD34^+$ CBPs, according to already well-known protocols [17, 23, 30]. Somewhat surprisingly, increasing quantities of sDC-SIGN were found in both DC-derived culture supernatants in relation to postdifferentiation time (**Fig. 2A** and **B**). sDC-SIGN appearance in culture supernatants was correlated with the addition of rIL-4. This observation was consistent with previously published observations showing that upon differentiation, IL-4 was required to induce mDC-SIGN expression [9]. To know whether sDC-SIGN and mDC-SIGN expressions could be differentially regulated during differentiation, MoDCs were generated with several cytokine cocktails (IL-4/GM-CSF, IFN-α/GM-CSF, and IL-13/GM-CSF) or IL-4/IL-13 alone. All combinations, except the one using IFN-α, were able to induce sDC-SIGN and mDC-SIGN to the same extent, as assessed by Western blot (Fig. 2C and D). Taken together, these results provided strong evidence that sDC-SIGN may be secreted by in vitro-generated DCs and that sDC-SIGN and mDC-SIGN were regulated in a similar manner in the course of DC differentiation.

sDC-SIGN isoforms are secreted as exosome-free multimers and do not result from proteolytic cleavage by MMPs

We next tried to purify sDC-SIGN from MoDC culture supernatants to definitely prove that this molecule is derived from

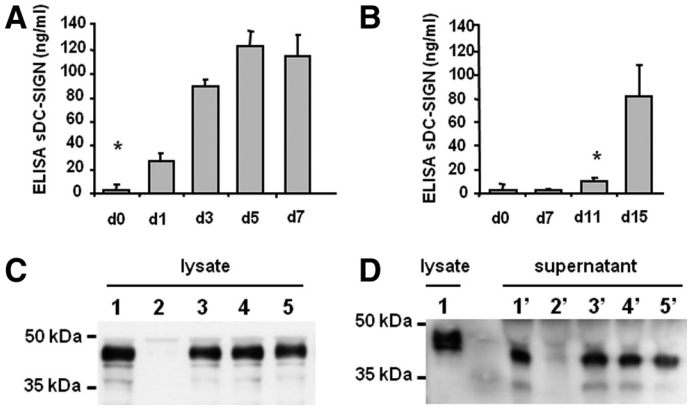

Figure 2. sDC-SIGN is released by DCs in the course of their differentiation. Culture supernatants from (A) MoDC or (B) $CD34^+$ hematopoietic stem cells were tested for the presence of sDC-SIGN by ELISA. (A and B) Asterisks indicate addition of exogenous IL-4 to culture media. (C) Western blot analysis of cell lysates (Lanes 1–5) and (D) 10× concentrated culture supernatants (Lanes 1′–5′) obtained from MoDC generated with IL-4/GM-CSF (Lanes 1 and 1′), IFN-α/GM-CSF (Lanes 2 and 2′), IL-13/GM-CSF (Lanes 3 and 3′), IL-4 (Lanes 4 and 4′), or IL-13 (Lanes 5 and 5′). The biotin-conjugated 3E1 mAb clone (anti-DC-SIGN) was used as the detection antibody.

an alternative splicing event, as expected in previous studies [17]. However, we failed to purify sufficient amounts of high-purity sDC-SIGN for N-terminal sequencing. To circumvent these difficulties, we alternatively treated MoDCs with a broad-spectrum inhibitor of MMPs (i.e., a disintegrin and metalloprotease/TACE family), called Marimastat (British Biotech, UK), which was supposed to have no effect on sDC-SIGN release. Differentiating DCs were incubated with increasing doses of Marimastat. At Day 6, mDC-SIGN expression was analyzed by flow cytometry, and sDC-SIGN was quantified in culture supernatants by ELISA (**Fig. 3A**, respectively, black and open bars). Despite the use of high concentrations of Marimastat (10 μM), no modification of sDC-SIGN versus mDC-SIGN expression patterns could be observed, thus weighing in favor of a sliced, sequence-derived product and not a shedding of mDC-SIGN by MMPs.

Like many cell types, DCs are able to secrete 60–80 nm membrane vesicles, called exosomes. To know whether sDC-SIGN isoforms could be secreted as exosome-borne proteins, we separated the exosome-enriched fraction from cleared IL-4/GM-CSF-derived MoDC culture supernatant, according to the modified protocol published by Raposo et al. [24], and titered sDC-SIGN in all samples by ELISA, i.e., medium alone, nonultracentrifuged supernatant, ultracentrifuged supernatant, and exosome-enriched pellet. The results are described in Fig. 3B. sDC-SIGN was retained almost completely in the supernatant cleared from exosomes by ultracentrifugation, as it was shown to be absent from the exosome-enriched fraction (p). We concluded that sDC-SIGN was not expressed as exosome-associated proteins.

Several groups have provided strong evidence that mDC-SIGN or its homologue DC-SIGNR is expressed as homotetramers to increase affinity for their ligands [5, 7, 31]. Tetramerization is dependent on the neck length consisting of various Ig-like domain repeats [5, 32] and also on the extracellular pH [8]. The question we posed ourselves was whether this was the case for sDC-SIGN using gel filtration. Here again, we were unable to purify sufficient sDC-SIGN from MoDC cultures to perform our analysis. As a result, we produced rFLAG-sDC-SIGN1AT1 and T3 in *E. coli* and separated them according to their apparent MW by gel filtration. For each sample, fractions were collected and analyzed by ELISA. The results are shown in Fig. 3C. sDC-SIGN quantities were plotted against elution volumes, and elution volume of the maximum sDC-SIGN quantities for each sample was determined on the plot. Column precalibration allowed us to calculate each apparent MW_{meas} of the most abundant sDC-SIGN isoform found in each sample. Thus, the FLAG-sDC-SIGN1AT1-associated peak was eluted with 52 ml, corresponding to an apparent MW of 175 kD. An approximate, MW_{theor} of 46 kD was calculated on specialized websites (MW_{theor}; http://www.expasy.org/) for FLAG-sDC-SIGN1AT1. As MW_{meas} was about four times larger than MW_{theor} (ratio=3.8), we concluded that FLAG-sDC-SIGN1AT1 could be expressed as tetramers in nondenaturing and nonreducing conditions. Applying the same procedure for the FLAG-sDC-SIGN1AT3, it was estimated that unlike sDC-SIGN1AT1, sDC-SIGN1AT3 could probably be

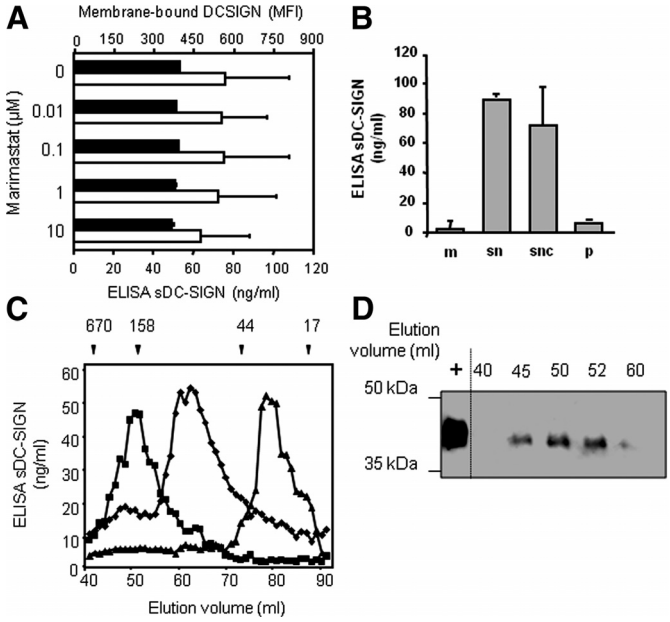

Figure 3. Characterization of the releasing mode and oligomerization status of MoDC-derived sDC-SIGN. (A) Dose-dependent effect of Marimastat™ on mDC-SIGN (MFI, mean fluorescence intensity: black bars, cytometric analysis) and sDC-SIGN (ELISA) expression by 6-day immature MoDC (open bars). (B) ELISA titration of sDC-SIGN in membrane ghosts (m), 10× concentrated culture supernatant (sn), 10× concentrated ultracentrifuged culture supernatant (snc; 100,000 g, 2 h), and post-ultracentrifugation pellet of the culture supernatant (p). Total protein (50 μg) was used for sDC-SIGN titrations for all tested samples (BCA total protein quantification). (C) The oligomerization status analysis of FLAG-sDC-SIGN1AT1/T3 was studied by gel filtration. Fractions (500 μl-sized), each containing 100-μg purified FLAG-sDC-SIGN1AT1 (■), FLAG-sDC-SIGN1AT3 (◆), and CRD alone (approximately 19 kDa; ▲; monomeric control), were separated onto a gel filtration column (GE Healthcare). Elution fractions were collected (1 ml) and analyzed further by ELISA to quantify sDC-SIGN, except for the CRD. Indeed, CRD was devoid of the neck region recognized by the H-200-coating anti-DC-SIGN pAb. Thus, CRD was quantified in eluted fractions by BCA. The results are plotted as the sDC-SIGN concentrations, according to the elution volume. Standard MW are indicated by black arrowheads on the top of the graph and positioned at their corresponding elution volume (thyroglobulin=670,000 kD; bovine γ-globulin=158,000 kD; chicken OVA=44,000 kD; equine myoglobin=17,000 kD). (D) Five concentrated, FLAG-sDC-SIGN1AT1-associated, peak-surrounding fractions were analyzed by Western blot. Each fraction is characterized by its elution volume, indicated for each lane on the top of the gel. FLAG-sDC-SIGN1AT1 was used as a positive control (200 ng loaded in the "+" lane).

found in culture medium as a dimer (MW_{meas}=81 kD; MW_{theor}=32 kD; ratio=2.5). Purified CRD (~19 kD) was used as a control here, as it was unable to multimerize. Peak-associated fractions of the FLAG-sDC-SIGN1AT1 gel filtration run were pooled and concentrated ten- to 15-fold onto centrifugal filter units (10 kDa cutoff) and finally analyzed by Western blotting in denaturing and reducing conditions. The results are shown in Fig. 3D. In this setting, we only observed FLAG-sDC-SIGN1AT1 as a 40- to 45-kDa pro-

tein, suggesting that FLAG-sDC-SIGN1AT1 is expressed as a homotetramer in native conditions. All of our data indicate that sDC-SIGN proteins are secreted in a MMP-independent manner as exosome-free homotetramers, at least for full-length sDC-SIGN variants.

sDC-SIGN is overexpressed in body fluids upon inflammation

We demonstrated above that sDC-SIGN is secreted by human blood-borne, precursor-derived DC-SIGN$^+$ DCs in vitro. Thus, we assumed that it could be secreted by DCs in vivo. Our study first analyzed human sera from healthy donors by Western blot (n=21; **Fig. 4A**). Much to our surprise, sDC-SIGN was detected in 19 of the 21 sera with a heterogeneity of expression between individuals. Following this, we tested a broader panel of human serum from healthy donors (n=62) by ELISA. Consistently with the Western blot analysis, almost all sera contained detectable amounts of sDC-SIGN ranging from 0 to >150 ng/ml with a mean value of 65.36 ng/ml (min=0 ng/ml; max=154 ng/ml; median=72 ng/ml; 25th percentile=32.41 ng/ml; 75th percentile=95.75 ng/ml; Fig. 4B). The serum sDC-SIGN concentrations were comparable with the lower amounts of MBL variants, which are found in human serum in a broad range of concentrations, depending on their genotype [33]. Further gel-filtration experiments demonstrated that serum sDC-SIGN was more probably expressed as tetramers, which was consistent with our previous results obtained with rFLAG-sDC-SIGN1AT1 (Supplemental Fig. 1). mDC-SIGN has previously been associated with Th2 responses, at least in vitro [34, 35]. However, certain experimental findings came to our attention, showing that DC-SIGN may be up-regulated in inflammatory diseases such as Crohn's disease [36, 37]. We hypothesized that sDC-SIGN may be found in inflammatory body fluids. For this purpose, BALs were harvested from patients suffering from diverse lung diseases, including primary CMV infections, and tested for the presence of sDC-SIGN. When segregated according to the inflammatory status of samples (based on diagnoses, i.e., viral infections, etc.), the quantity of sDC-SIGN was clearly higher within inflammatory (mean=24.4 ng/ml) compared with noninflammatory BAL (mean=1.37 ng/ml; P=0.0005; Fig. 4C). To confirm these observations, we compared sera and joint fluids from RA versus osteoarthritis-suffering patients. Joint fluids from RA patients were indeed prototypical inflammatory fluids, whereas joint fluids from os-

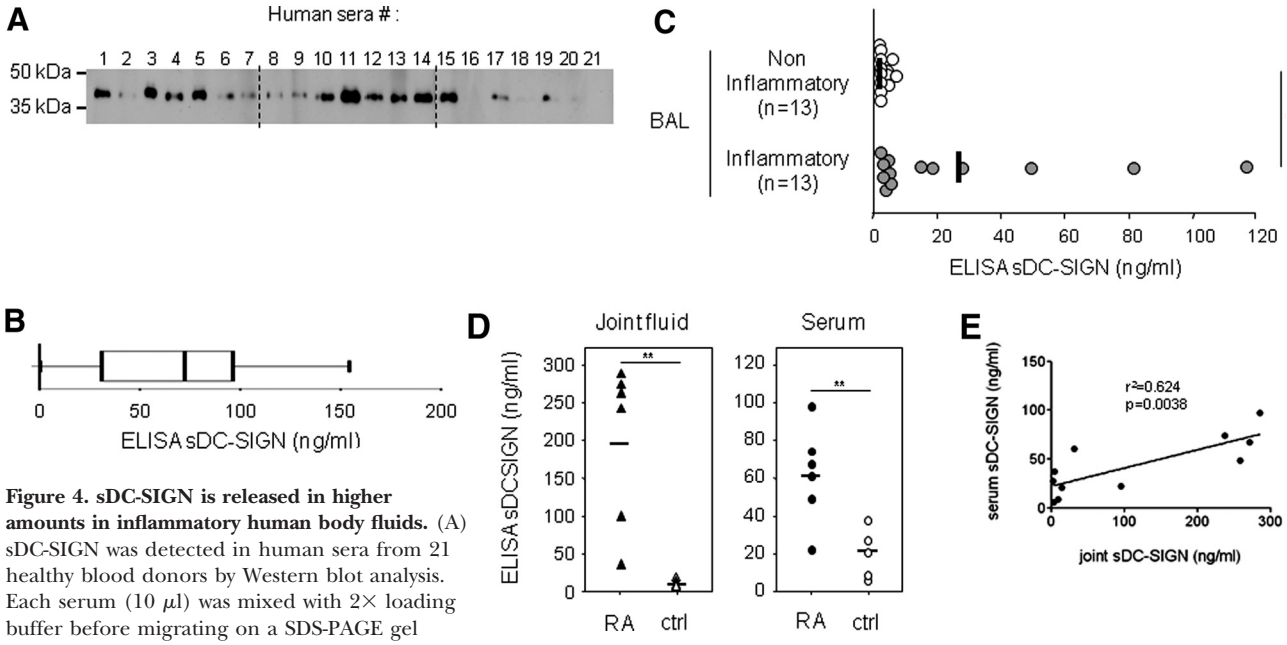

Figure 4. sDC-SIGN is released in higher amounts in inflammatory human body fluids. (A) sDC-SIGN was detected in human sera from 21 healthy blood donors by Western blot analysis. Each serum (10 μl) was mixed with 2× loading buffer before migrating on a SDS-PAGE gel (10% acrylamide) under reducing conditions. After transfer onto a nitrocellulose membrane, sDC-SIGN was detected by immunoblotting with the mAb 3E1 (anti-DC-SIGN). Dashed lines indicate separation among three distinct digitalized gel pictures. (B) sDC-SIGN was quantified in the serum of 62 healthy volunteers by ELISA. Values are indicated in ng/ml and have been displayed as a box-and-whisker plot, done with the GraphPad Prism software (mean serum sDC-SIGN concentration=65.36 ng/ml; min=0 ng/ml; max=154 ng/ml; median=72 ng/ml; 25th percentile=32.41 ng/ml; 75th percentile=95.75 ng/ml). (C) BAL from patients were collected and analyzed by sDC-SIGN ELISA. Samples were separated according to their inflammatory status [i.e., $(CRP)_{serum}$<5 mg/ml=noninflammatory]. Mean sDC-SIGN values (black bars) were calculated for both groups (n=13): inflammatory = 24.4 ng/ml versus noninflammatory = 1.37 ng/ml. Comparison of mean values was done using a Mann-Whitney test. The significant difference is indicated by **P = 0.0005. (D) sDC-SIGN was quantified by ELISA in paired samples of joint fluids (left panel, n=5) and sera (right panel, n=6) and from RA versus osteoarthritis-suffering patients. For each type of biological fluid, comparison between the two groups was performed with a Mann-Whitney test. The significant differences are indicated by ** in each panel (joint fluid, P=0.006; serum, P=0.02). (E) Correlation graph displaying sDC-SIGN concentrations in serum (y-axis) compared with those measured in joint fluids from the same patients (x-axis). The correlation coefficient (r^2=0.624) and the corresponding P value (P=0.0038) are indicated near the linear regression curve.

teoarthritis were considered as mechanical fluids marked by the absence of inflammatory cytokines and chemokines (Supplemental Fig. 2). As shown in Fig. 4D (left panel), there was a highly significant difference between sDC-SIGN amounts in RA versus control joint fluids (P=0.006). When analyzing the related sDC-SIGN concentrations in the sera of the same patients (Fig. 4D, right panel; P=0.02), a similar difference could be observed, thus promoting the conclusion that sDC-SIGN expression was closely correlated to the inflammatory status of these human biological fluids (Fig. 4E). Taken together, these results largely confirmed the fact that sDC-SIGN is produced in distinct biological fluids, and its expression appears to be up-regulated by inflammatory disorders, as exemplified here in the case of RA.

CMV infection-induced inflammation of genital mucosa causes an up-regulation of sDC-SIGN secretion

On the basis of previous results of this study showing that higher amounts of sDC-SIGN are generally found in inflammatory, virally infected BAL (Fig. 4C), we next endeavored to ascertain whether such inflammation was dependent on viral infection. We therefore infected freshly isolated mucosal explants of human origin (vagina or cervix biopsies) with a high

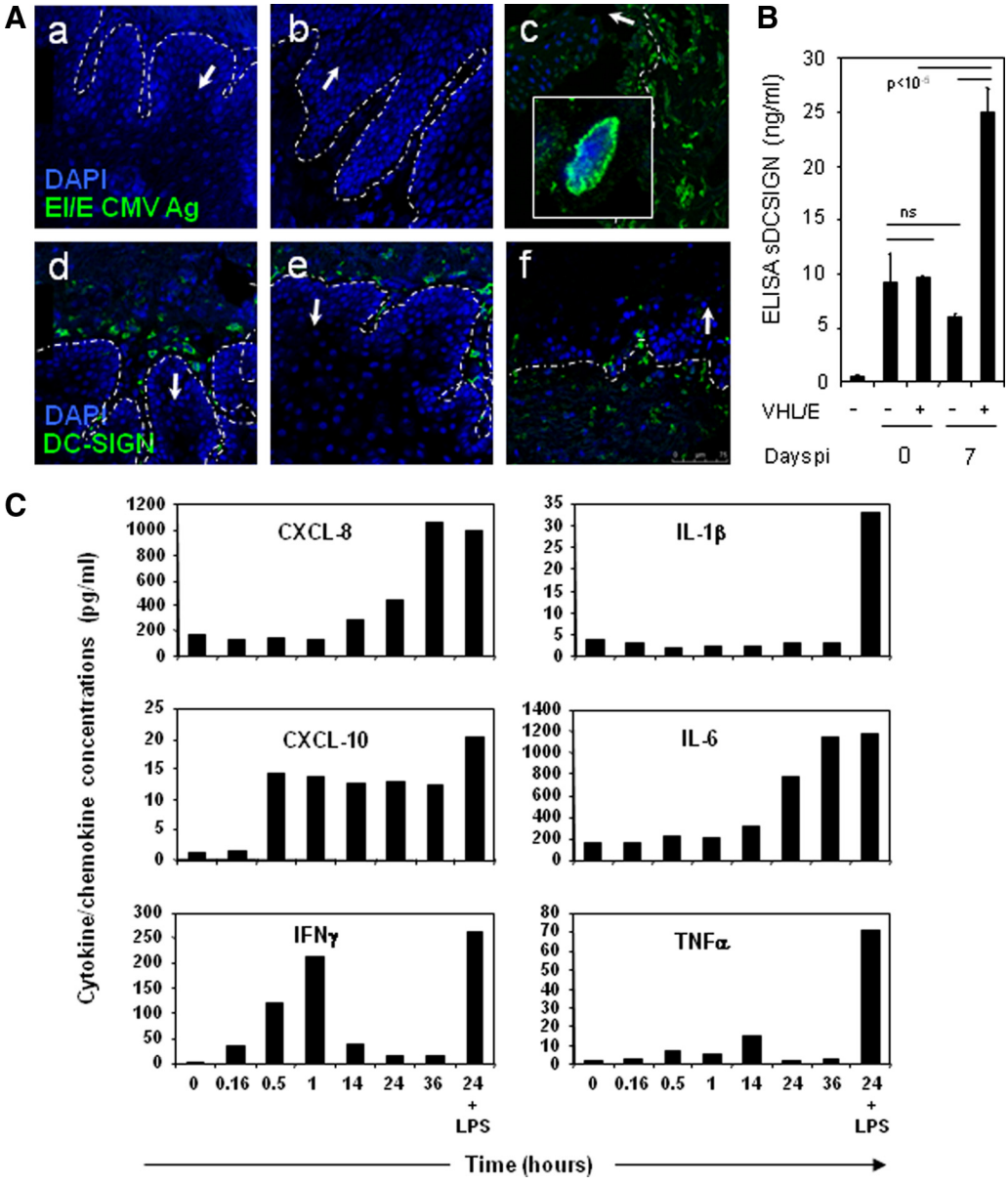

Figure 5. CMV infection-induced inflammation of mucosal explants promotes sDC-SIGN release. (A) Frozen sections were analyzed by confocal imaging 2 h after sampling (a and d), after a 7-day subculture in DMEM (b and e), or after infection with CMV (VHL/E strain) and a 7-day subculture in DMEM (c and f). Nuclei were counterstained with DAPI (blue), and IE/E CMV Ag (a–c) or DC-SIGN (d–f) was stained with primary, specific antibodies, followed by incubation with Alexa 488-conjugated goat anti-mouse mAb. (c) The inset represents a high magnification picture of an IE/E Ag-positive nucleus. The interface between the mucosa and the *lamina propria* is highlighted by white dashed lines. White arrows indicate the lumen location. (B) CMV-infected (VHL/E strain) or noninfected mucosal explant culture supernatants were harvested at Day 0 and 7 days post-infection (pi) and submitted to sDC-SIGN quantification by ELISA. The results are representative of at least three independent experiments. Statistical significances are represented as P values on the graph and are identical between "Day 0-no VHL/E" versus "Day7-no VHL/E" and "Day 0-no VHL/E" versus "Day 0-with VHL/E" (ns) on the one hand and between "Day 0-with VHL/E" versus "Day 7-with VHL/E" and "Day 7-no VHL/E" versus "Day 7-with VHL/E" on the other hand. (C) Inflammation-associated cytokines and chemokines were quantified by the CBA (BD Biosciences) in CMV-infected (VHL/E; 600,000 pfu/cm^2) or LPS-treated (100 ng/ml; 24 h) explant culture supernatants, which were harvested at different time-points post-treatment: 0.16 (10 min), 0.5 (30 min), 1, 14, 24, or 36 h postinfection. Human IFN-γ, TNF-α, CXCL-8 (IL-8), CXCL-10 (IP-10), IL-1β, and IL-6 were quantified simultaneously in each sample after being diluted (1/10 dilution). All of these results were representative of two independent experiments.

viral load and further incubated them for 7 days before freezing. Frozen tissue sections were stained to reveal nuclear IE/E CMV Ag in infected cells or DC-SIGN. As shown in **Fig. 5A**, DC-SIGN$^+$ cells were located exclusively in the *lamina propria* (Fig. 5A, d and e) of freshly isolated explants (Fig. 5A, a) or of 7-day noninfected explants (Fig. 5A, b), whereas after a 7-day infection period, DC-SIGN$^+$ cells were distributed equally among the mucosal epithelium and the *lamina propria* (Fig. 5A, f), as shown by a sharp nuclear staining of IE/E CMV Ag-positive cells (Fig. 5A, c). In the course of these experiments, explant culture supernatants were collected and analyzed by ELISA to quantify secreted DC-SIGN. The results are described in Fig. 5B. Upon infection, sDC-SIGN release was ~2.5 times higher in explants when compared with the basal sDC-SIGN release after 7 days in noninfected explant supernatants, as well as in freshly isolated explant supernatants. We then confirmed that CMV infection contributed to the establishment of an inflammatory environment by showing that typical cytokines (IL-6 and IFN-γ) or chemokines (CXCL-8/IL-8 and CXCL-10/IP-10) were overproduced in explant supernatants upon infection (Fig. 5C). LPS stimulation for 24 h was used as a positive control of cytokine/chemokine release. Together, these results clearly demonstrate that sDC-SIGN expression could be up-regulated by a viral infection of freshly isolated peripheral tissues.

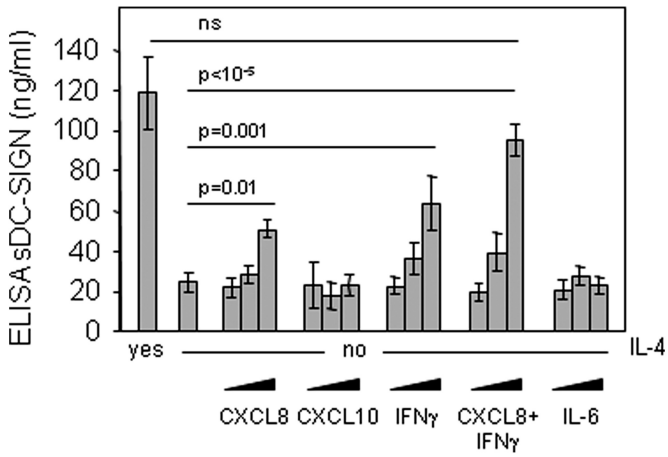

Figure 6. sDC-SIGN secretion by immature, fully differenciated MoDC is positively regulated by CXCL-8/IL-8 and IFN-γ, alone or in combination. IL-4-starved MoDC (6 days) were cultured with increasing doses of human rCXCL-8/IL-8, rCXCL-10/IP-10, rIL-6 (0.1, 1, 10 ng/ml), or rIFN-γ (10, 100, 1000 UI/ml). One condition consists of a mix of CXCL-8/IL-8 and IFN-γ at their respective low, intermediate, and high concentrations mentioned above. MoDC supernatants were analyzed after 48 h by ELISA to measure corresponding sDC-SIGN quantity. All of these results were representative of at least three independent experiments. Statistical significances are represented as *P* values.

sDC-SIGN expression is up-regulated by CXCL-8/IL-8 and IFN-γ in MoDC culture supernatants

We next sought to identify proinflammatory cytokines or chemokines that might be responsible for the induction of sDC-SIGN up-regulation. Focusing on molecules overexpressed in explant culture supernatants upon CMV infection or LPS activation, experiments were performed using fully differentiated immature MoDC, subcultured with various doses of human rCXCL-8/IL-8, rCXCL-10/IP-10, rIFN-γ, and rIL-6, alone or in combination. To minimize the effect of exogenous IL-4, which is necessary to differentiate MoDC but also able to induce sDC-SIGN, potentially leading to misleading conclusions, cells were first starved of IL-4. In that setting, sDC-SIGN release was up-regulated significantly by IFN-γ and to a lesser extent, by CXCL-8/IL-8 in a dose-dependent manner when compared with the spontaneous level of sDC-SIGN secretion by IL-4-starved cells (i.e., "no IL-4" experimental conditions; respectively, $P=0.01$ and $P=0.001$), whereas no significant effect could be shown after adding exogenous CXCL-1/IP-10 or IL-6 (**Fig. 6**). It should be noted that CXCL-8/IL-8 and IFN-γ act in an additive but not synergistic manner to induce sDC-SIGN production by IL-4-starved immature MoDCs. The stimulation level in that case is equivalent to the "IL-4 alone" experimental condition. IL-6 unresponsiveness is most likely a result of the absence of a functional receptor on the cell surface, as no significant staining for gp130, i.e., the signal-transducing chain of IL-6R, could be observed by flow cytometry (data not shown). In accordance with our previous data, we hereby demonstrated that sDC-SIGN is re-induced by exogenous IFN-γ and/or CXCL-8/IL-8 in fully differentiated immature MoDC cultures.

sDC-SIGN is functional and promotes the CMV infection of MoDC

We demonstrated above that sDC-SIGN secretion is promoted by an inflammatory setting and even upon CMV infection of human tissue explants. Thus, it remained to be determined whether sDC-SIGN has a protective or facilitating effect on CMV infection. First, we tested the functionality of FLAG-sDC-SIGN1AT1 through its ability to interact directly with the CMV envelope gB, which we identified previously as a ligand for mDC-SIGN [20]. Lysates of transiently transfected HEK cells were spotted on a nitrocellulose membrane and incubated further with FLAG-sDC-SIGN1AT1 or specific mAb as a detection control to document interactions between gB and sDC-SIGN under native conditions (**Fig. 7A**). When incubated with FLAG-sDC-SIGN1AT1, gB$^+$ cell lysates could be revealed by a HRP-conjugated anti-FLAG antibody, whereas the anti-FLAG alone did not provide any signal. FLAG-sDC-SIGN1AT1$^+$ HEK cell lysates were used as positive controls for DC-SIGN and FLAG detection. An additional actin detection was used as a loading sample control. These results suggested that the FLAG-sDC-SIGN1AT1 was functional. However, the functional role of sDC-SIGN during the CMV infection remained unclear. On the basis of the MBL-mediated inhibition of HIV susceptibility reported in the literature, we first hypothesized that sDC-SIGN might neutralize the CMV infection of MoDC. In this setting, we were unable to block

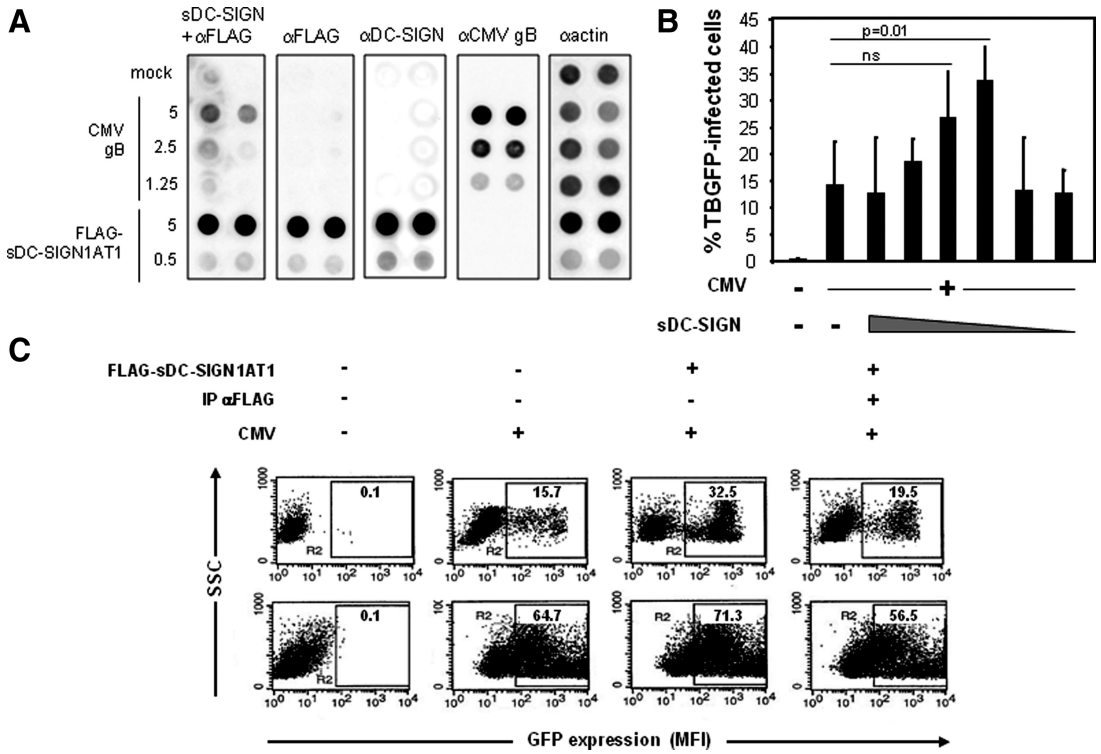

Figure 7. sDC-SIGN is functional and promotes CMV infection of MoDC. (A) Interaction between CMV gB and FLAG-sDC-SIGN1AT1 was assessed by dot blot under native conditions. Lysates of transiently transfected HEK cells were spotted in duplicate onto a nitrocellulose membrane according to the following order (from the top to the bottom of the membrane): mock (empty pRC/CMV vector)-, CMV gB (respectively, with 5, 2.5, and 1.25 μg gB-encoding plasmid, pRC/CMV-CMVgB)-, and FLAG-sDC-SIGN1AT1-expressing cells (respectively, transfected with 5 and 0.5 μg pCDNA3.1-FLAG-sDCSIGN1AT1 vector). CMV gB was selectively detected with a first incubation with a FLAG-sDC-SIGN1AT1 solution (5 μg/ml in TBS, 0.05% Tween, 5% creamed milk), followed by a HRP-conjugated anti-FLAG mAb (clone M2; 1/10,000) at room temperature. Control detections were made using only the HRP-conjugated anti-FLAG mAb or alternatively, mAb directed against DC-SIGN (clone 3E1, biotinylated), CMV gB (clone HCMV37), or F-actin, respectively, revealed by a HRP-conjugated streptavidin or goat anti-mouse IgG. (B) A single dose of TBGFP (MOI=20) was preincubated with or without increasing amounts of FLAG-sDC-SIGN1AT1 (final concentrations: 400, 200, 100, 50, 25, and 12.5 ng/ml) for 30 min (4°C), prior to being added to MoDC for a further 2-h incubation at 37°C. Cells were analyzed after 24 h by flow cytometry to determine the percentage of GFP-positive cells (i.e., early infected cells). These experiments were performed with MoDC obtained from four distinct, healthy blood donors. (C) The same experiments were performed with MoDC (a single donor; upper panels) or FSF (lower panels) in the presence or absence of a preadsoption step on a mannan-conjugated agarose matrix. GFP$^+$ cells (i.e., CMV-infected cells) are indicated for each panel inside the gate containing positive cells. These results are representative of two independent experiments performed with MoDC from distinct donors. SSC, Side-scatter.

MoDC infection, even using higher concentrations of FLAG-sDC-SIGN1AT1 (data not shown). In addition, as mDC-SIGN expression was reported to be responsible for MoDC CMV cis-infection, we assumed that FLAG-sDC-SIGN1AT1 may function as a promoter of the infection. For this purpose, MoDC were infected with TBGFP CMV (MOI=1) for 24–48 h in the presence of decreasing amounts of FLAG-sDC-SIGN1AT1 (from 400 to 12.5 ng/ml). The GFP$^+$ MoDC, indicating the percentage of early infected cells, was estimated by flow cytometry. Infected MoDC frequencies were approximately twice as high (33.1±6%) as the control infection (14.8±7.2%) when incubated with 50 ng/ml FLAG-sDC-SIGN1AT1-supplemented culture medium (P=0.01; Fig. 7B). A 100-ng/ml concentration was not sufficient to mediate a significant infection enhancement when compared with control infection. Surprisingly, it was observed that FLAG-sDC-SIGN1AT1 concentrations higher than 100 ng/ml and lower than 50 ng/ml did not modify the infectious rate of MoDC. To confirm that the deleterious effect that we could observe was specific to sDC-SIGN, we conducted similar experiments but only using an optimal amount of FLAG-sDC-SIGN1AT1 (50 ng/ml), which was first captured or not onto a mannan-conjugated resin (Fig. 7C, upper panels). Here again, the addition of FLAG-sDC-SIGN1AT1 doubled the GFP$^+$ MoDC percentage (32.5% vs. 15.7%). This enhanced infection was readily a result of FLAG-sDC-SIGN, as it was abrogated by a preincubation step of the recombinant lectin on mannan-conjugated agarose. As a negative control, we also performed the same assay with FSF, which we reported previously as being prone to CMV infection in a DC-SIGN-independent manner ([20]; Fig. 7C lower panels). All of these results led us to conclude that FLAG-sDC-SIGN1AT1 is functional and is able to facilitate CMV infection of MoDCs.

DISCUSSION

We previously reported the crucial role of mDC-SIGN as a docking and internalizing receptor for CMV on MoDC [20]. Simultaneously, other groups reported the existence of potentially sDC-SIGN variants at the cDNA level, generated by alternative splicing of the TM-encoding exon [17]. Soluble but nonsecreted and nonfunctional DC-SIGN proteins were described by others [19]. To better delineate the role of an extended DC-SIGN repertoire on CMV infection pathogenesis, the present study appraised the biochemical properties, the regulation, and the role of recombinant as well as naturally occurring sDC-SIGN variants in the CMV cis-infection of MoDC.

Martinez et al. [19] had previously shown that sDC-SIGN was produced by transfected cells. They reported that sDC-SIGN failed to be secreted in the cell culture supernatant but instead, was retained in the cytoplasm. Strangely, they sought to test its ability to promote the transmission of cosignals to T cells in comparison with mDC-SIGN but to no avail. In the present study, we developed and used a specific and quantitative ELISA to demonstrate the secretion of sDC-SIGN in concentrated MoDC culture supernatants. The fact that Martinez et al. [19] failed to detect sDC-SIGN in culture supernatant may be a result of the lack of sensitivity of their own ELISA and the absence of a culture supernatant concentration step before measuring sDC-SIGN.

What is more, in keeping with the work of Mummidi et al. [17], we cloned cDNAs lacking the TM-encoding exon and coding for putative sDC-SIGN from distinct, in vitro-generated DC populations. In our study, a broad-spectrum inhibitor of MMPs was used to avoid a possible shedding of mDC-SIGN at the plasma membrane. Nevertheless, MoDC and tissue-residing DCs expressed two or three other membrane-associated MMPs that might not be targeted by MMP inhibitors [38]. However, MMP inhibition did not result in a difference of sDC-SIGN and mDC-SIGN expression by MoDCs. These results all confirmed previous observations reporting sDC-SIGN expression as a soluble, full-length molecule. Another crucial issue was to check whether sDC-SIGN could be expressed as an exosome-associated protein, as suggested in a recent report [39]. Briefly, exosomes derived from DC-SIGN$^+$ murine bone marrow-derived DCs were shown to express DC-SIGN on the basis of cytometric analyses. On the other hand, as a result of very slight DC-SIGN staining of exosomes, these results were not convincing and had to be interpreted with caution. In this case, based on the measurement of sDC-SIGN concentrations with our ELISA, we could undoubtedly argue in favor of the expression of sDC-SIGN as exosome-free molecules. The final proof of the secretion of a full-length sDC-SIGN would be to sequence the naturally occurring sDC-SIGN protein, but we failed to purify sufficient amounts of it to obtain any kind of irrefutable proof.

Feinberg et al. [5] previously demonstrated that mDC-SIGN was expressed as tetramers at the plasma membrane of transfected cell lines. The mDC-SIGN tetramerization was dependent on the presence of at least five Ig-like domain repeats in the neck region. Another study also provided sound evidence that immature MoDCs express mDC-SIGN tetramers, which are supposed to physically interact with the coreceptor CD4 [40]. Here, by gel filtration analyses under native conditions (i.e., neutral pH and isotonic buffer), we proved that not only recombinant but also blood-borne sDC-SIGN consisted of tetramers, thus confirming results published previously by others [5, 8].

We next demonstrated that IL-4 and IL-13 were potent inducers of sDC-SIGN and mDC-SIGN. These results were in line with those published by Relloso and colleagues [9] in a previous report. In addition, we did not test IFN-γ during monocyte differentiation in DCs, but we tested IFN-α, which was sufficient to prevent DC-SIGN expression when added at the very beginning of MoDC differentiation. In contrast, it had no effect when added after 2 days postdifferentiation (data not shown), strongly suggesting that DC-SIGN expression was irreversible. This idea was borne out by a former study reporting the IL-4-dependent and irreversible up-regulation of PU.1, a specific transcription factor of the myeloid lineage, mainly responsible for the induction of DC-SIGN expression in MoDCs and monocyte-derived macrophages during their in vitro differentiation [41, 42]. In this study, besides IL-4 or IL-13, we used fully differentiated immature MoDCs as a cellular model to document what inflammatory soluble factors could be involved in the sDC-SIGN secretion that we detected in several human body fluids. Surprisingly, IFN-α and most probably, type I IFNs, in general (although this remains to be experimentally ascertained), were shown to impair DC-SIGN during cell differentiation, whereas IFN-γ was identified as a good inducer of sDC-SIGN by fully differentiated immature MoDC. CXCL-8/IL-8 was also demonstrated to promote sDC-SIGN expression but to a lesser extent. When added together as exogenous cytokines on IL-4-starved MoDCs, we observed additive effects on sDC-SIGN secretion, suggesting that they may be both required to induce optimal sDC-SIGN secretion, albeit weakly. Even at higher doses of IFN-γ and/or CXCL-8/IL-8, we were unable to attain the sDC-SIGN secretion level obtained with IL-4 or IL-13 in vitro. To explain why, in such a case, IFN-γ induced and did not repress sDC-SIGN secretion by MoDCs, one could argue once again that fully differentiated immature MoDCs have a distinct transcription program coordinated by distinct transcription factors (i.e., PU.1, etc.) compared with monocytes during their differentiation.

In genital human mucosa, after 7 days of ex vivo CMV infection, we noted a significant increase of sDC-SIGN secretion by residing DCs. The analysis of the cytokine content of infected biopsy supernatants confirmed the presence of IFN-γ and CXCL-8/IL-8 in sufficient amounts to imagine their direct involvement in the induction of sDC-SIGN. In these experiments, no difference of mDC-SIGN expression by dermal DCs could be observed by immunostaining. Based on our own results, we expected that sDC-SIGN induction could be correlated with the same up-regulation of membrane isoforms. However, it is our belief that the explanation for this discrepancy can be found in a recent study reported by Liu et al. [18], indicating that the vast majority of DC-SIGNR, a closely related DC-SIGN gene product found in HIV mucosal inoculation sites, i.e., anal and genital mucosa, was encoded by TM

lacking spliced cDNA sequences [17]. These data pointed to a differential expression of sDC-SIGN versus mDC-SIGNR isoforms in virally infected peripheral tissues. Despite being unable to document any differential expression of DC-SIGN in vitro, we cannot rule out this possibility in vivo. To better understand if this were the case, discriminating antibodies are currently being generated in our laboratory. Interestingly, former studies provided strong evidence that RA synovia contained high levels of CXCL-8/IL-8, which may be a result of viral infection caused by CMV [43]. Here, these molecules were detected in high amounts in infected BAL or RA joint fluids, suggesting that they could also be sDC-SIGN inducers in vivo. Moreover, CXCL-8/IL-8 was up-regulated in the female genital tract in the course of coinfection with CMV and HIV-1 [44]. This observation substantially supported our sDC-SIGN induction data in genital mucosa explants. CXCL-8/IL-8 was already known as an efficient neutrophil and macrophage chemoattractant, and a link had been established between high serum concentrations and the CMV disease occurrence in patients after liver allotransplantation [45].

The analysis of the sDC-SIGN content in sera from healthy volunteers showed a high heterogeneity of concentrations. On the basis of previous reports in the literature, we endeavored to forge a link between sDC-SIGN concentrations and a Th2 cytokine environment, marked by high amounts of IL-4/13 in sera and BAL from atopic patients. Unfortunately, we were unable to show significant correlation (data not shown). In contrast, we demonstrated that high amounts of sDC-SIGN could be correlated to high amounts of cytokines and chemokines, usually overexpressed upon inflammation in diverse body fluids, such as serum, BAL, or joint fluids, from patients suffering from autoimmune, inflammatory, or infectious diseases. These results match the data reporting an increased number of mDC-SIGN-expressing macrophages in BAL from *Mycobacterium tuberculosis*-infected patients [3], as well as in RA synovia [4]. Several studies have already reported a link between high frequencies of mDC-SIGN$^+$-infiltrating cells and the proinflammatory environment found in Crohn's disease lesions [37]. One can easily imagine that these mDC-SIGN$^+$ cells may also be able to secrete sDC-SIGN upon inflammation and/or infection. Thus, on the basis of these unexpected results, we suggest that DC-SIGN, whether sDC-SIGN or mDC-SIGN, may be considered as a new marker of tissue inflammation.

Our final investigation focused on sDC-SIGN functions. Several studies have already documented the fact that rsDC-SIGN was able to neutralize HIV and dengue virus infection of transfectants or MoDC at similar mean concentrations (i.e., from 0.5 to 50 μg/ml [46, 47]). Although Navarro-Sanchez et al. [47] reported convincing data, they used truncated rsDC-SIGN, only consisting of the DC-SIGN CRD domain, which was shown to be expressed as monomers in solution by others [5]. CRD monomers had a limited affinity for their ligands in comparison with tetramers [40]. Here, we provided strong evidence that FLAG-sDC-SIGN1AT1 was capable of interacting with the CMV gB, already known to be a ligand of mDC-SIGN [20]. Using our tetrameric rsDC-SIGN, we were unable to inhibit CMV infection of FSF, even when sizeable sDC-SIGN amounts were used (up to 17 μM; data not shown). We then hypothesized that sDC-SIGN could function as an enhancer of CMV infection. As expected, low FLAG-sDC-SIGN1AT1 concentrations (varying from 10 to 100 ng/ml) allowed a significant increase in the percentage of IE/E CMV Ag$^+$ immature MoDC from three distinct blood donors, whereas sDC-SIGN concentrations below and above this range had no potentializing effect. To explain this bell-curve effect, we proposed a hypothetical model at low but constant viral input. According to this model, at high sDC-SIGN concentrations, free virions are rapidly complexed, leading to an impaired binding and internalization of CMV into MoDC. Between 100 and 10 ng/ml, sDC-SIGN may serve as an opsonin with CMV virions with an optimal stoichiometry leading to the capture and internalization of a maximum number of sDC-SIGN-immobilized virions. When using very low sDC-SIGN concentrations (below 10 ng/ml), the majority of virus is free, and the infection efficacy decreases to levels of infection without sDC-SIGN, as we postulated that sDC-SIGN-opsonized CMV was captured more efficiently by MoDC than free virions. This suggests the existence of one or several yet-unknown opsonic receptors for sDC-SIGN, which may not be able to interact with previously reported, truncated rsDC-SIGN [5, 46]. A novel study is under progress in our lab to fulfil the identification of such a receptor. However, based on the literature, we discuss putative candidates. First, ICAM-2 and -3, but not ICAM-1, are ligands for mDC-SIGN [10, 11]. However, only ICAM-1 is expressed by MoDC [48]. Although we cannot rule out, so far, the possibility for sDC-SIGN to interact with ICAM-1, the involvement of ICAM-2/3 in the sDC-SIGN-mediated infection enhancement in MoDC is likely to be inconceivable. Second, mDC-SIGN complexes have the propensity to form multimers (di-, tri-, and tetramers, depending on the neck length [7]). As a consequence, one can imagine that sDC-SIGN, whether bound to CMV virions or not, may be able to dock on mDC-SIGN to form aggregates with pre-existing lectin homodimers or trimers. New experiments are needed to confirm this hypothesis. We finally considered a last potential candidate receptor enabling the sDC-SIGN-mediated CMV infection enhancement, the CD11b molecule, also known as the αM integrin, a macrophage antigen-1 or complement receptor 3 component when associated with the α2 integrin. CD11b is highly expressed by immature MoDC [49]. Interestingly, it has been involved recently in the uptake of mannosylated liposomes by macrophages in cooperation with a murine homologue of mDC-SIGN [50]. Furthermore, CD11b has also been described to function as a facilitating agent for HIV opsonization by immature MoDC in a mDC-SIGN-dependent manner [49]. It is thus highly tempting to speculate on the fact that sDC-SIGN-opsonized CMV particles may interact directly with CD11b, leading to their internalization and thus, promoting MoDC infection. Further experiments are obviously needed to confirm this hypothesis.

In summary, our work has strengthened the notion that DC-SIGN, whether sDC-SIGN or mDC-SIGN, should be considered as an inflammatory rather than a regulatory marker. Our results also raised the question of the existence of a cell surface receptor for sDC-SIGN-opsonized CMV virions on MoDC. As

such, our findings shed new light on the diversity of the DC-SIGN repertoire and extend our knowledge of the use of mDC-SIGN as well as sDC-SIGN by CMV to divert the human innate immune response to its own benefit.

AUTHORSHIP

N.P. and J-M.H. performed experiments, analyzed results, and generated figures and table; V.L. and B.V. generated the anti-DC-SIGN antibody 3E1A8 and performed experiments; C.H. provided patient care and performed tissue explant biopsies; F.H. designed the research and was responsible for the project and manuscript preparation; and P.N., L.V., and B.V. participated in the data analyses. All authors reviewed the paper and had access to raw data.

ACKNOWLEDGMENTS

This work was supported by an institutional grant from INSERM Avenir (National French Health Institute), the Aquitaine, and the Pays de Loire regions (J-M.H.'s salary). We thank Olivier Neyrolles for his critical reading of the manuscript, the Etablissement Français du Sang d'Aquitaine for blood samples, and the Bordeaux Imaging Center (BIC; confocal imaging).

DISCLOSURE

All authors certify that they have no conflicts of interest or competing financial interests.

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KEY WORDS:
opsonin · C-type lectin · inflammation