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An assay for functional dendritic cell-specific ICAM-3-grabbing nonintegrin (DC–SIGN) inhibitors of human dendritic cell adhesion

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ABSTRACT

We report a new dendritic cell adhesion assay, using either immature or mature dendritic cells, for identifying functional dendritic cell-specific ICAM-3-grabbing nonintegrin (DC–SIGN) inhibitors. Because immature dendritic cells are responsible for pathogen binding and invasion, this in vitro assay provides an important link between in vitro results and pathogen-based in vivo assays. Furthermore, this assay does not require laborious expression, refolding, and purification of DC–SIGN carbohydrate recognition domain or extracellular domain as receptor-based assays. The assay power evaluated with Z and Z' parameters enables screening of compound libraries and determination of IC_{50} values in the first stage of DC–SIGN inhibitor development.

Dendritic cells (DCs)[1] are specific antigen recognition cells that drive the immune response in its earliest stages because they are likely to be the first cells to encounter invading pathogens [1]. Their specific receptors are involved in antigen recognition and reuptake that triggers DC maturation. On DC maturation, captured antigen is processed and the resulting antigen fragments are presented on the major histocompatibility complex (MHC). The latter presents the antigen fragments to T helper cells, harnessing their immune repertoire [2].

Many of the recognition and adhesion functions of DCs are attributed to DC-specific ICAM-3-grabbing nonintegrin (DC–SIGN), a C-type lectin expressed by DCs whose function was first reported by Geijtenbeek and coworkers [3]. As an adhesion molecule, DC–SIGN interacts with ICAM-2 on endothelial cells to induce transendothelial migration and mediates clustering of DCs with naive T cells through binding of ICAM-3. Furthermore, DC–SIGN acts as an antigen recognition receptor that binds numerous glycoproteins on the pathogen surface and modulates the immunological response to several pathogens [2]. However, the very same mechanism is used by several pathogens to overcome the response of the immune system [3–5]. For example, HIV-1 targets DC–SIGN but escapes lytic processing by DCs. Bound to DC–SIGN, HIV-1 is protected from destruction in blood, thereby using DCs as a "Trojan horse" to invade the host organism [3a,4]. This makes DC–SIGN a target of interest in the design of new anti-infectives. Several groups have recently demonstrated that inhibition of DC–SIGN, either by designed glycoconjugates or by antibodies, prevents pathogen attachment to DCs or, thus, prevents the infection of other immune cells [5].

We have reported the design and synthesis of glycomimetic compounds that bind to DC–SIGN [6,7]. Reina and coworkers reported a pseudo-1,2-mannobioside (**1a** in Fig. 1) that had an approximately 2-fold greater antiviral activity in the Ebola infection model (IC_{50} = 0.62 mM) than the corresponding 1,2-mannobiose (IC_{50} = 1.19 mM) (**2** in Fig. 1) [6]. **1b**, a precursor of **1a**, inhibited DC–SIGN adhesion with IC_{50} = 1.1 mM, as measured by surface plasmon resonance (SPR) [6,8]. Recently, we reported a trisaccharide mimetic, pseudo-man3 (**3**), that inhibits DC–SIGN potently with IC_{50} = 130 μM [8]. In another study, we designed and synthesized α-fucosylamides (**4** and **5** in Fig. 1) that mimic the Lewis^x structure (**6**). These compounds bound to DC–SIGN with IC_{50} = 0.35 to 0.50 mM, which is a moderate improvement over the corresponding Lewis^x (IC_{50} = 0.80 mM), as measured by SPR assay [7].

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[1] *Abbreviations used:* DC, dendritic cell; MHC, major histocompatibility complex; DC–SIGN, DC-specific ICAM-3-grabbing nonintegrin; SPR, surface plasmon resonance; DPBS, Dulbecco's phosphate-buffered saline; FBS, fetal bovine serum; rhGM–CSF, recombinant human granulocyte macrophage colony-stimulating factor; rhIL-4, recombinant human interleukin 4; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; MTS, 3-(4,5-dimethylthiazol-2-*yl*)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; CFSE, carboxyfluorescein succinimidyl ester; SDS, sodium dodecyl sulfate.

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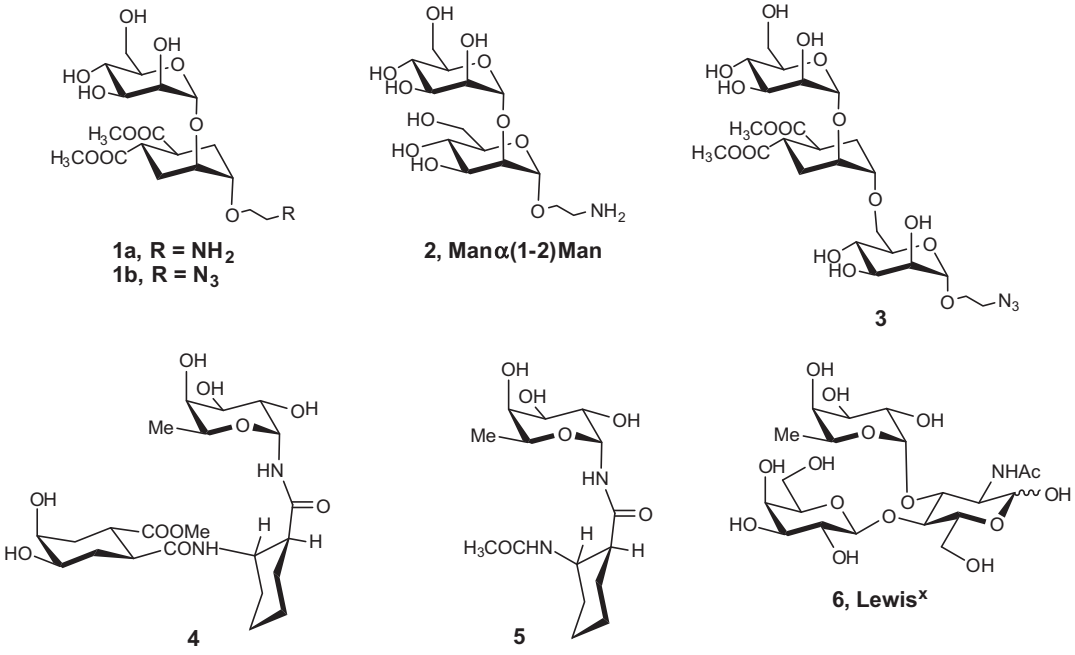

Fig.1. Glycomimetic ligands of DC–SIGN.

In the aforementioned studies, we mostly used an SPR-based assay that detects inhibition of ligand binding to DC–SIGN [7,9]. This assay requires laborious expression, refolding, and purification of DC–SIGN carbohydrate recognition domain or extracellular domain, chip preparation, and SPR measurements that are fairly time-consuming [9–11]. For the reasons stated, we tried to obtain a less laborious assay to identify new DC–SIGN inhibitors and determine their IC_{50} values. Furthermore, to ascertain whether our compounds could act as functional inhibitors of DC–SIGN, we sought an effective and robust DC–SIGN-specific assay that was cell based, that is, a DC–SIGN-mediated DC binding/adhesion assay. Cell-based assays have numerous advantages over those based on isolated receptors. They are based on a relevant function in a self-contained living entity, they detect allosteric modulators that might not be detected by assays using isolated receptors, and they give an early indication of cytotoxicity. In addition, if the target is an intracellular one, they indicate the degree of cell penetration of the compound. Thus, cell-based assays provide important links in "screening cascades" aimed at relating in vitro results to in vivo effects [12]. An assay reported by Borrok and Kiessling uses transfected 293FT cells with expressed DC–SIGN instead of primary cells (DCs) [13]. However, the results cannot be simply extrapolated to deduce an inhibitory effect on the adhesion properties of DCs due to the innate DC differentiation-dependent characteristics that affect DC–SIGN adhesion [14].

We have developed a DC–SIGN-mediated DC adhesion assay to identify functional DC–SIGN inhibitors that act on immature and mature DCs. The suitability of the assay for screening purposes has been evaluated on the basis of its power, robustness, and reproducibility.

Materials and methods

Preparation and culture of DCs

Buffy coats from the venous blood of normal healthy volunteers were obtained by the Blood Transfusion Center of Slovenia according to institutional guidelines. The study was approved by the national medical ethics committee of the Ministry of Health, Republic of Slovenia, and written consent was obtained before collection of specimens.

Peripheral blood mononuclear cells were isolated using Lympholyte-H (Cedarlane Laboratories, Ontario, Canada). The cells were washed twice with Dulbecco's phosphate-buffered saline (DPBS), counted, and used as the source for immunomagnetic isolation of CD14-positive cells (Miltenyi Biotec, Bergisch Gladbach, Germany). These were cultured in RPMI 1640 medium (Cambrex) supplemented with 10% fetal bovine serum (FBS), gentamicin (50 μg/ml, Gibco, Paisley, UK), 500 U/ml recombinant human granulocyte macrophage colony-stimulating factor (rhGM–CSF, Gentaur, Paris, France), and 400 U/ml recombinant human interleukin 4 (rhIL-4, Gentaur). On day 2, half of the medium was exchanged with starting quantities of rhGM–CSF (500 U/ml) and rhIL-4 (400 U/ml). After 5 days, nonadherent immature DCs were harvested and characterized by flow cytometry as $CD1a^{hi}$, $CD83^{-}$, $CD86^{low}$, and $HLA^{-}DR^{low}$ (data not shown). Cells were counted and resuspended in the medium containing 500 U/ml rhGM–CSF and 20 ng/ml lipopolysaccharide (LPS) and were cultured for a further 2 days [15].

Flow cytometry analysis of DC–SIGN level

The levels of membrane markers were determined by flow cytometry using fluorescently labeled antibodies. Human peripheral blood monocytes were differentiated into immature DCs for 5 days using rhGM–CSF (800 U/ml) and rhIL-4 (1000 U/ml) (both from PeproTech EC, London, UK). To obtain mature DCs, nonadherent cells were collected on day 5 and matured for a further 2 days using LPS (20 ng/ml). On days 5 and 7, the cells were collected by centrifugation. Antibody was added, and the cells were incubated at room temperature for 15 min in the dark. They were then washed twice with DPBS and resuspended in 2% paraformaldehyde. To determine levels of DC–SIGN expression on immature or mature DCs, we used monoclonal phycoerythrin-labeled anti-DC–SIGN antibody (BioLegend, San Diego, CA, USA) and determined the mean fluorescence index relative to the isotype-matched control antibody.

Measurement of DC adhesion with MTS colorimetric assay

Wells of a 96-well culture plate (Nunc Nunclon Δ surface) were precoated, overnight at 4 °C with 50 μl of mannan (1.0 mg/ml, Sigma, St. Louis, MO, USA) or Lewis^x trisaccharide (5 μg/ml, Carbosynth, Berkshire, UK) in carbonate buffer (pH 9.6). The wells were then washed once with phosphate-buffered saline (PBS) and incubated with 1% bovine serum albumin (BSA) in PBS for 30 min at room temperature. Mature DCs were harvested, washed with PBS, and resuspended at 5×10^5 cells/ml in the medium (RPMI 1640) containing 500 U/ml rhGM–CSF and 20 ng/ml LPS at a concentration of 5×10^5 cells/ml. Then 1.0 μl of 50 mM stock solution of DC–SIGN inhibitors in dimethyl sulfoxide (DMSO) or 2.5 μl of Lewis^x trisaccharide (1 mg/ml) in DMSO was added to the wells. The same volume of DMSO was used in the control experiment. Then 50 μl of the mature DC suspension was added to the wells of the 96-well culture plate. Cells were allowed to attach for 30 min, 90 min, and 3 h. Wells were then washed twice with PBS, and 100 μl of medium was added. Each experiment was done in quadruplicate for treated and control cells. To determine total mature DCs, one quadruplicate of wells was not washed with PBS, but 50 μl of medium was added. After the last incubation, CellTiter 96 One Solution Cell Proliferation Assay (MTS [3-(4,5-dimethylthiazol-2-*yl*)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium] colorimetric assay, Promega, Madison, WI, USA) was used to assess the adhesion of cells according to the manufacturer's protocol [16]. The quantity of formazan product, measured by absorbance at 490 nm, is directly proportional to the number of living cells in culture. Cell adherence was determined by the following equation:

$$\text{cell adherence } (\%) = (A_{tx} - A_{blank}/A_{total\ DC} - A_{blank}) \times 100, \qquad (1)$$

where A_{tx} and $A_{total\ DC}$ are the absorbance of formazan determined for cells washed after different time points and for all DCs, respectively, and A_{blank} is the absorbance of the formazan added to the medium alone.

Measurement of DC adhesion with CFSE fluorescence assay

Wells of a 96-well culture plate (Nunc Nunclon Δ surface or Nunc MaxiSorp) were precoated with 50 μl of mannan (1.0 mg/ml, Sigma) in carbonate buffer (pH 9.6) overnight at 4 °C. Wells were then washed once with PBS and incubated with 1% BSA in PBS for 30 min at room temperature. Immature or mature DCs were harvested, washed with PBS, and labeled with 2.5 μM carboxyfluorescein succinimidyl ester (CFSE) according to the manufacturer's protocol (Molecular Probes/Invitrogen, Eugene, OR, USA) [17] and resuspended in the medium containing 500 U/ml rhGM–CSF and either 1000 U/ml rhIL-4 or 20 ng/ml LPS at a concentration of 5×10^5 cells/ml. Anti-DC–SIGN antibodies H200 and 1B10 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or 1.0 μl of 50 mM stock solution of DC–SIGN inhibitors or 2.5 μl of Lewis^x trisaccharide (1 mg/ml) were added to the wells. PBS or DMSO was used in controls. Then 50 μl of mature or immature DC suspension was added to wells of a 96-well culture plate. Cells were allowed to attach for 90 min. Wells were then washed twice with PBS, and 50 μl of lysis buffer (25 mM Tris and 0.1% sodium dodecyl sulfate [SDS]) was added. Each experiment was done in quadruplicate for each concentration of inhibitor used and for control cells. To estimate the total mature DCs added to wells, one quadruplicate of wells was not washed with PBS, but only lysis buffer was added directly. CFSE is colorless and nonfluorescent until the acetate groups are cleaved by intracellular esterases to yield highly fluorescent CFSE that reacts with intracellular amines, forming fluorescent conjugates. The fluorescence was measured at 520 ± 10 nm with a Tecan Saphire microplate reader (Tecan Group, Männedorf, Switzerland), excitation wavelength 490 nm, and is directly proportional to the number of adhered cells. Cell adherence was determined by Eq. (1), where A_{tx} and $A_{total\ DC}$ are the fluorescence of CFSE determined for cells washed after different times and for all DCs, respectively, and A_{blank} is the background fluorescence of buffer.

Statistical analysis

Z' and Z factors were calculated using the reported method as follows: $Z' = 1 - (3 \times SD_{100\%\ adhesion} + 3 \times SD_{blank})/(mean_{100\%\ adhesion} - mean_{blank})$ and $Z = 1 - (3 \times SD_{sample} + 3 \times SD_{blank})/(mean_{sample} - mean_{blank})$ [18], where SD represents standard deviation. A parametric paired and unpaired two-tailed *t* test calculator was used to assess statistical difference between mean adhesion data and IC_{50} (GraphPad Software [http://www.graphpad.com/quickcalcs/ttest1.cfm?Format=SD] and Table 1) [19]. The IC_{50} values were calculated by fitting mean values of cell adherence to the logistic (sigmoid) equation by nonlinear least squares curve fitting using OriginPro 8 software (OriginLab, Northampton, MA, USA).

Results

Flow cytometry analysis of DC–SIGN level

To develop a DC–SIGN-specific cell-based assay, we first analyzed the level of DC–SIGN expression with flow cytometry. DC–SIGN expression on both mature and immature DCs was measured with fluorescently labeled, DC–SIGN-specific antibodies. In agreement with previous reports [20], we observed higher expression of DC–SIGN on immature DCs (Fig. 2). The level of DC–SIGN expression was reasonably high enough even for mature DCs, so both differentiation types of DCs could be used for assay development.

Time-dependent inhibition of DC adhesion

To determine optimal incubation time, DC adhesion was measured with the MTS colorimetric assay methodology using Nunc Nunclon Δ surface 96-well culture plates coated with mannan and Lewis^x because both bind to DC–SIGN [21]. Mature DCs were allowed to adhere in the presence of two known DC–SIGN adhesion inhibitors (**1b** and **4**) at concentrations of 1 mM. After three time intervals (30, 90, and 210 min), DC binding was quantified to establish the optimal DC adhesion interval, as shown in Fig. 3. In the case of positive control (total observed adhesion) and compound **1b**, the adhesion was independent of time, whereas for compound **4**, a moderate change in DC adhesion occurred after 90 min for both mannan- and Lewis^x-coated plates. Thus, a cell attachment interval of 90 min was chosen for further assay development.

Inhibition of DC–SIGN-mediated binding using the MTS colorimetric assay

Nunc Nunclon Δ surface 96-well culture plates were coated with either mannan or Lewis^x, and mature DCs were allowed to adhere in the presence of two concentrations of a known DC–SIGN inhibitor (**1b**, **3**, or **4**), and of Lewis^x as control (95 μM). DC adhesion was quantified with the MTS colorimetric assay. The two-point test was used to evaluate assay quality and identify candidates for IC_{50} measurement (Fig. 4). The assay data clearly indicated that the tested compounds inhibited DC adhesion in given concentrations, but the ratios between 100% adhesion and blank absorbance (signal-to-background ratios) were too small and indicated that the colorimetric MTS assay failed to quantify the adhered DCs properly. Accordingly, Z' values were unsuitable for screening and for IC_{50} determination without significant errors.

Table 1
Assay reproducibility.

Experiment	Mean ± standard error (% adhesion)	Standard deviation (% adhesion)	S/B	Z	Z'	P_{xy} (paired t test)[a]
Reference	44.82 ± 3.40	6.80	86.1	0.540	0.793	/
Plate to plate	49.60 ± 3.47	6.93	37.0	0.558	0.778	$P_{12} = 0.806$
Day to day	48.44 ± 4.02	8.03	31.3	0.538	0.748	$P_{13} = 0.632$
Different blood donors	47.48 ± 4.36	8.72	48.8	0.432	0.727	$P_{14} = 0.926$

[a] *xy* Values refer to the experiment number of which mean adhesion data were compared with *t* test.

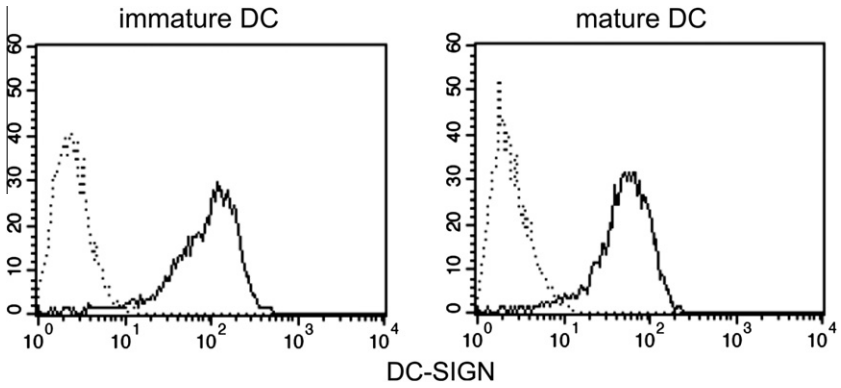

Fig.2. Relative levels of DC–SIGN expressed on immature and mature DC surfaces obtained with flow cytometry. Traces show the fluorescence obtained with an isotype-matched control (dotted line) and the specific DC–SIGN count (solid line) obtained for each cell line with a DC–SIGN-specific monoclonal antibody.

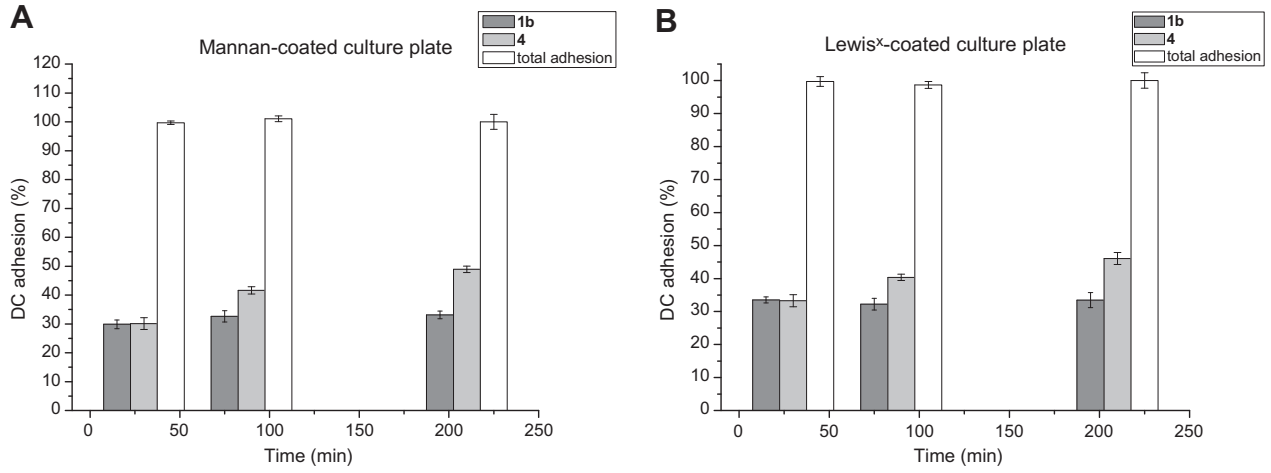

Fig.3. DC adhesion was measured with the MTS colorimetric assay using Nunc Nunclon Δ surface 96-well culture plates coated with either mannan (A) or Lewis^x (B). In both cases, 1-mM concentrations of compounds **1b** and **4** were used. The adhesion was calibrated to 100% at the time interval of 210 min.

Relative quantification of DC adhesion and time-dependent inhibition of DC adhesion with CFSE methodology

Based on the above-noted observation, we sought an effective and sensitive method to quantify the relative percentage of DC adhesion. The new quantification method required a well-retained cell-tracing reagent that withstands all of the washing procedures and provides at least two orders of magnitude signal-to-background ratio. To avoid Beer–Lambert law deviations, a fluorescence- or chemiluminescence-based cell-tracing reagent is needed. After careful examination of available reagents, a CellTrace CFSE Cell Proliferation Kit (Molecular Probes/Invitrogen) was chosen for further assay development [17]. Before assay validation and IC_{50} determination, relative quantification of DC adhesion was performed with CFSE methodology to assess the optimal number of cells for a suitable response, as measured in relative fluorescence units (Fig. 5). Based on these results, a number of approximately 2.5×10^5 cells/well was chosen for further assay. Namely, measurement of DC adhesion using 2.5×10^5 cells/well gave a suitable fluorescence output with more than two orders of magnitude signal-to-background fluorescence ratio and high dynamic range (S/B = 135, 3493 ± 246.8 RFU vs. 23.3 ± 5.06 RFU for blank). Furthermore, a high Z' value (0.782) indicated acceptable quality for screening purposes.

Optimal incubation time was also determined with CFSE methodology. Mature DCs were allowed to adhere to mannan-coated plates in the presence of two known DC–SIGN adhesion inhibitors (**1b** and **4**) at concentrations of 1 mM. After three time intervals (30, 90, and 210 min), DC binding was quantified. In the case of positive control (total observed adhesion), we observed a slight increase in adhesion after 90 min compared with 30 min and practically the same adhesion as after 210 min (Fig. 6). At 210 min,

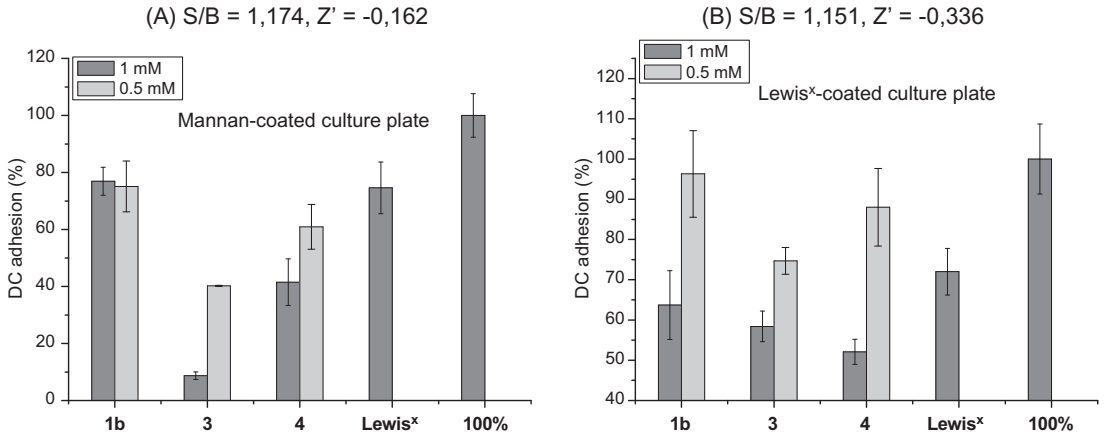

Fig.4. Two-point test of known DC–SIGN inhibitors (**1b**, **3**, **4**, and Lewis^x at a concentration of 95 μM and with no inhibitor–100% adhesion) with MTS colorimetric assay methodology using Nunc Nunclon Δ surface 96-well culture plates coated with mannan (A) and Lewis^x (B). Signal-to-background ratios (S/B) and Z' values were calculated to establish assay quality.

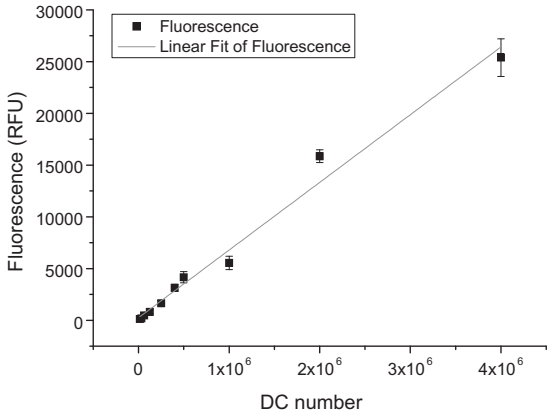

Fig.5. Fluorescence of DCs labeled with CFSE and lysed according to the manufacturer's protocol. Data are mean values ± standard errors of four replicates. Data were fitted to a linear curve ($y = 245.1 + 0.00654x$, y = fluorescence, x = DC number) with $R^2_{linear} = 0.983$ as a measure of goodness of fit.

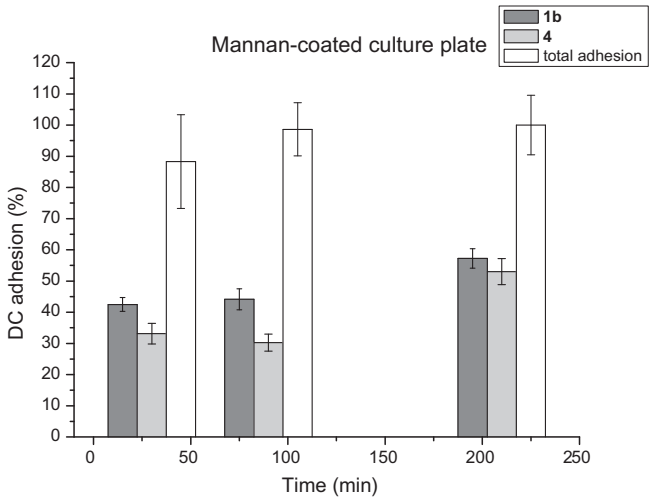

Fig.6. DC adhesion was measured with the CFSE fluorescence assay methodology using Nunc Nunclon Δ surface 96-well culture plates coated with mannan and 1-mM concentrations of compounds **1b** and **4**. The adhesion was calibrated to 100% at the time interval of 210 min.

however, a significant decrease in inhibition of adhesion for both **1b** and **4** occurred. Thus, a cell attachment interval of 90 min was chosen for further assay development.

Reproducibility of assay

Reproducibility of the assay was assessed with a control experiment. DC–SIGN-mediated binding inhibition was measured with CFSE fluorescence assay methodology using Nunc Nunclon Δ surface 96-well culture plates coated with mannan. Mature DCs were allowed to adhere in the presence of 0.5 mM compound **3** on the separate plates at different time intervals (intra- and interday) and using mature DCs obtained from different donors. The experiment was performed in quadruplicate, and the adhesion data were evaluated with standard deviation, signal-to-background ratio, and Z and Z' factors calculated (Table 1). These indicate that assay in the majority of cases fulfills the criteria for high-throughput screening robustness (Z and $Z' > 0.5$) [18]. We have also tested the difference between adhesion means with a paired two-tailed t test [19]. By conventional criteria for P values, the differences between mean adhesion data are considered to be not statistically significant and demonstrate good reproducibility of the assay.

Inhibition of DC–SIGN-mediated DC adhesion with DC–SIGN-specific antibodies

DCs express various mannose-binding C-type lectins, including DC–SIGN, and all of them could cause DC adhesion to mannan-coated plates [22]. However, DC–SIGN has a higher affinity for more complex mannose residues in specific arrangements (as found in mannan), and it does not bind single terminal mannose residues [11]. To assess whether DC adhesion to mannan-coated plates could be inhibited by DC–SIGN-specific antibodies in a concentration-dependent manner, DC adhesion was measured against different concentrations of DC–SIGN-specific antibodies 1B10 and H200 [23]. The results demonstrate that DC adhesion can be abolished by DC–SIGN-specific antibodies and is concentration dependent (Fig. 7). It should be noted that 100% inhibition of DC adhesion was not observed with any of the tested antibodies and that the maximum inhibition achieved was 30% (observed for 1B10). This indicates that DC adhesion to mannan-coated plates is DC–SIGN mediated but does not rely solely on DC–SIGN, in accordance with the fact that DCs express various mannose-binding receptors.

Cell-based assays should be validated not only for statistical reproducibility (e.g., Z factors) but also for the pharmacological

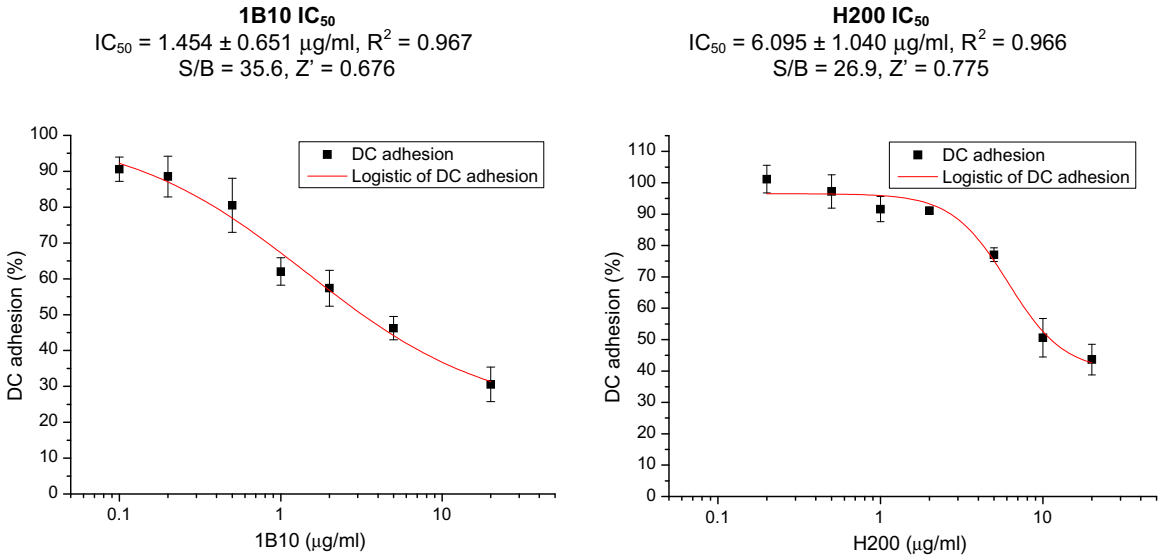

Fig.7. Inhibition of DC–SIGN-mediated adhesion by two high-potency DC–SIGN-specific antibodies: 1B10 and H200. DC–SIGN-specific mature DC adhesion was measured with CFSE fluorescence assay methodology using Nunc Nunclon Δ surface 96-well culture plates coated with mannan. The dose–response curves were generated against DC adhesion expressed in percentage of adhesion. Data are mean values ± standard errors of four replicates and were fitted to the logistic (sigmoid) equation by nonlinear least squares curve fitting using OriginPro 8 software.

parameters (e.g., IC_{50}) of reference compounds [11]. As a consequence, we have calculated IC_{50} for 1B10 and H200 to establish whether the assay is sensitive enough for complete evaluation of reference antibodies.

Sensitivity of DC–SIGN-mediated adhesion using a CFSE fluorescence assay

To establish that the assay is sensitive enough even for low-potency reference compounds, IC_{50} for either mannose- or fucose-based DC–SIGN inhibitors (**3** or **4**) was determined. Mature DCs were allowed to adhere in the presence of various concentrations (0.005, 0.01, 0.05, 0.2, 0.5, 0.75, 1.0, and 2.5 mM) of DC–SIGN inhibitors (**3** and **4**), and IC_{50} values were calculated from fitted logistic curves (Fig. 8). Although some authors question whether the R^2 value is the most appropriate weighting factor for goodness of fit [24], it should be stressed that the R^2 values of both fitted curves were exceptionally high (>0.99). The calculated IC_{50} values for both high-potency DC–SIGN-specific antibodies and low-potency DC–SIGN inhibitors **3** and **4** clearly demonstrate that the assay distinguishes between high- and low-potency inhibitors.

The observed inhibition of adhesion for both **3** and **4** was not complete even in high concentrations of both inhibitors, again suggesting participation of cell adhesion molecules other than DC–SIGN in binding to mannan-coated plates.

Borrok and Kiessling observed significant differences (~10-fold) in IC_{50} values of compounds when comparing fluorescence binding and cell adhesion assays. In contrast, the IC_{50} values for compounds

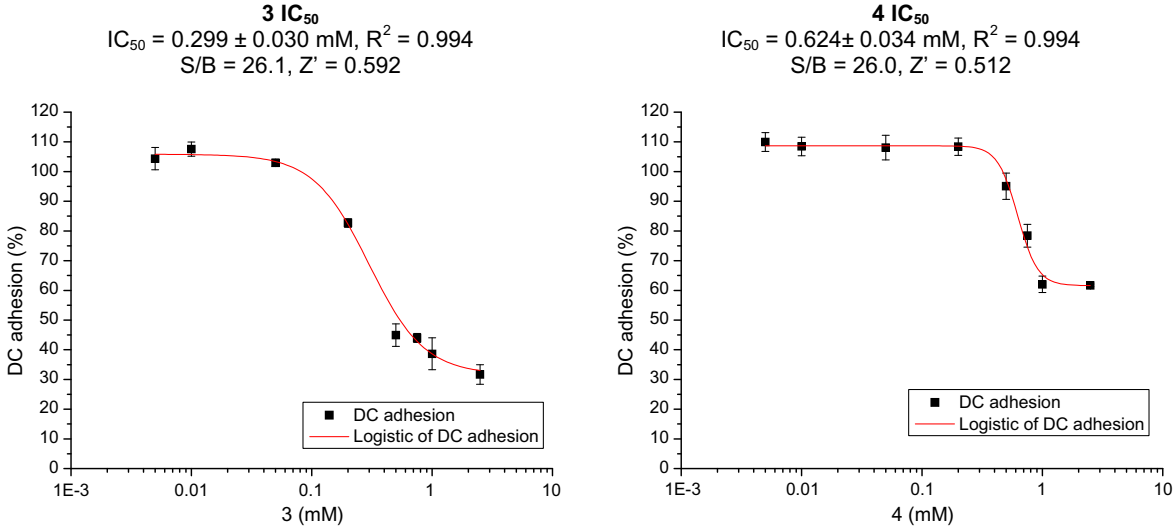

Fig.8. IC_{50} values of low-potency DC–SIGN inhibitors **3** and **4** determined by CFSE fluorescence assay methodology using Nunc Nunclon Δ surface 96-well culture plates coated with mannan. The dose–response curves were generated against DC adhesion expressed in percentage of adhesion. Data are mean values ± standard errors of four replicates and were fitted to the logistic (sigmoid) equation by nonlinear least squares curve fitting using OriginPro 8 software.

Table 2
Comparison of IC_{50} values for mature and immature DCs.

Compound/ antibody	IC_{50} of mDCs (μg/ml or mM)[a]	95% CI of mDCs (μg/ml or mM)	IC_{50} of iDCs (μg/ml or mM)	95% CI of iDCs (μg/ml or mM)	*P* (unpaired *t* test)
H200	6.095 ± 1.040	4.440–7.750	0.630 ± 0.053	0.546–0.714	>0.0001
1B10	1.454 ± 0.651	0.418–2.490	0.413 ± 0.129	0.208–0.618	0.0201
3	0.299 ± 0.030	0.251–0.347	0.472 ± 0.054	0.386–0.558	0.0014
4	0.624 ± 0.034	0.570–0.678	0.318 ± 0.082	0.188–0.448	0.0005

Note. mDC, mature dendritic cell; iDC, immature dendritic cell; CI, confidence interval.
[a] g/ml refers to concentrations of antibodies H200 and 1B10; mM refers to inhibitory concentrations of compounds **3** and **4**.

3 and **4** (299 and 624 μM, respectively) are on the same order of magnitude and in the same rank order as those obtained with SPR (130 and 350 μM for **3** and **4**, respectively) [8].

Influence of DC differentiation type on DC adhesion

Immature DCs express an even higher number of DC–SIGN molecules on their surface, but their use in the first step of assay development was abandoned because of their unfavorable properties; that is, in contrast to mature DCs, immature DCs tend to readhere after being moved to another well. To compare the adhesion properties of immature and mature DCs, we measured IC_{50} values for two DC–SIGN-specific antibodies (1B10 and H200) and for two inhibitors (**3** and **4**) using CFSE fluorescence assay methodology. The values were compared according to *P* values (unpaired *t* test) and using confidence intervals (95% assuming that IC_{50} values are normally distributed) (Table 2).

IC_{50} values obtained for H200, 1B10, and **4** with immature DCs decreased significantly when compared with those obtained with mature DCs.

Discussion

A human DC adhesion assay for assessing potential functional DC–SIGN inhibitors has been established using mature and immature DCs. The power of the assay, based on observed *Z* and *Z′* factor values and its reproducibility, enables robust screening of compound libraries. Because immature DCs are known to be responsible for pathogen invasion via DC–SIGN with a Trojan horse mechanism, this in vitro assay may replace pathogen-based assays for screening purposes [6,25].

A significant advantage of the new assay is the use of a fluorescence-based reagent. An MTS-based colorimetric assay resulted in low signal-to-background ratios and unacceptably low *Z′* values due to background turbidity from living cells. MTS reagent is added after the last incubation and could be absorbed nonspecifically to the well surface even if the surface is blocked with BSA. Moreover, colorimetric methods are limited by Beer–Lambert's law deviation that, in the best possible case, leaves a maximum of three orders of magnitude dynamic range. In this respect, fluorescence- and chemiluminescence-based methods have a great advantage over colorimetric methods. On the contrary, the DCs are labeled with CFSE before adhesion to cell culture plates, enabling complete removal of residual unbound CFSE reagent before addition to plate wells. Thus, low background signal of CFSE provides acceptable signal-to-background ratios and high *Z′* values.

The CFSE-based assay results reported in the current work are consistent with in vitro SPR results on isolated receptor given that comparable inhibitory activity was observed for compounds **3** and **4** (IC_{50} values = 299 and 624 μM, respectively) compared with SPR (IC_{50} values = 130 and 350 μM for **3** and **4**, respectively) [7,8].

In agreement with previous reports [18], we observed that immature DCs express a higher level of DC–SIGN than mature DCs, and notable DC–SIGN expression was also observed in mature DCs. On this basis, we expected that the differentiation type of DCs could affect DC–SIGN-mediated adhesion properties. To test this hypothesis, IC_{50} values were determined for two DC–SIGN-specific antibodies (1B10 and H200) and for two known DC–SIGN inhibitors (mannose-based **3** and fucose-based **4**) with both mature and immature DCs. Indeed, we observed that IC_{50} values differ according to DC differentiation type; the IC_{50} values obtained with immature DCs for 1B10, H200, and **4** were significantly lower than those obtained with mature DCs. Thus, we conclude that DC–SIGN-mediated adhesion properties of immature DCs are more affected by the majority of tested DC–SIGN inhibitors.

In conclusion, these results demonstrate that the established cell-based assay provides a novel cellular platform for discovering new DC–SIGN inhibitors that may act as anti-infectives. To the best of our knowledge, this assay is the first to establish a clear link with results observed on isolated receptors (SPR). Moreover, mature and immature DCs can be used in the assay, enabling further study of functional differences between the distinct differentiation stages of DCs.

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