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Effective targeting of DC-SIGN by α -fucosylamide functionalized gold nanoparticles.

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Abstract:

Dendritic Cells (DCs), the most potent antigen-presenting cells, play a critical role in the detection of invading pathogens, which are recognized also by multiple carbohydrate-specific receptors. Among them, DC-SIGN is one of the best characterized, with high-mannose and Lewis-type glycan specificity. In this study, we present a potent DC-SIGN targeting device developed using gold nanoparticles functionalized with α -fucosyl- β -alanyl amide. The nanoparticles bound to cellular DC-SIGN and induced internalization as effectively as similar particles coated with comparable amounts of Lewis^X oligosaccharide. They were found to be neutral towards dendritic cell maturation and IL-10 production, thus envisaging a possible use as targeted imaging tools and antigen delivery devices.

Introduction

Dendritic cells (DCs) are the most potent antigen-presenting cells and are responsible for the detection of pathogens.¹ DCs are equipped with multiple carbohydrate-specific receptors, called lectins, that recognize a vast array of glycan determinants on both pathogens and host glycoproteins.² Ligand recognition often results in internalization and routing to the endo-lysosomal pathway for processing and antigen presentation. Some lectins, in addition, are able to trigger specific signaling that modulates the maturation and migration of DCs to secondary lymphoid organs for proper stimulation of T cells.³ Natural carbohydrate ligands for DC lectins include high-mannose *N*-glycans, such as those found on the HIV gp120 envelope-protein, and Lewis-type glycans that also can trigger robust immune responses in humans.⁴⁻⁶ Targeting antigens to DCs via lectins is a well-known strategy explored in the last 20 years in immunotherapy.⁷ Carbohydrate ligands have been used as vehicle to improve DC uptake avoiding adverse immune reactions that may be elicited by monoclonal antibodies.⁸ DC-SIGN is one of the best characterized DC lectins with high-mannose and Lewis-type glycan specificity.⁹ Targeting DC-SIGN on DCs using multivalent carbohydrate-systems is a recent approach that has demonstrated improved uptake, processing, and presentation to antigen-specific T-cells.¹⁰⁻¹³ Additionally, DC-SIGN targeting of appropriate imaging reporters may represent a powerful tool to investigate the cellular dynamics of the immune response in lymphoid organs and in peripheral tissues.¹⁴⁻¹⁵

Our group has recently described the synthesis of a small library of glycomimetic DC-SIGN ligands, characterized by the presence of a β -amino acid tether and a fucosylamide anchor.¹⁶⁻¹⁷ Most of these molecules bind DC-SIGN with an affinity similar to or better than that of the Le^X trisaccharide (Gal β 1-4[Fuc α 1-3]GlcNAc β 1), one of its natural fucose-containing ligands.⁴⁻⁵ In particular, *N*- α -fucosyl- β -alanylamine **1a** (Figure 1), which can be prepared in a single step from fucosyl azide, binds to DC-SIGN with almost the same affinity as Le^X, that has often been used as a DC-SIGN targeting unit in the context of polyvalent constructs.^{11-13,18}

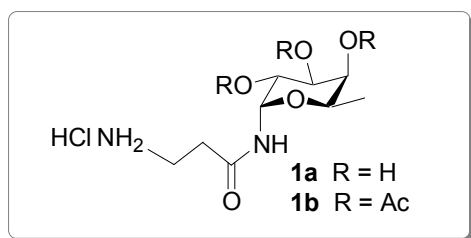


Figure 1. *N*- α -fucosyl- β -alanylamine **1a**, the monovalent targeting device.

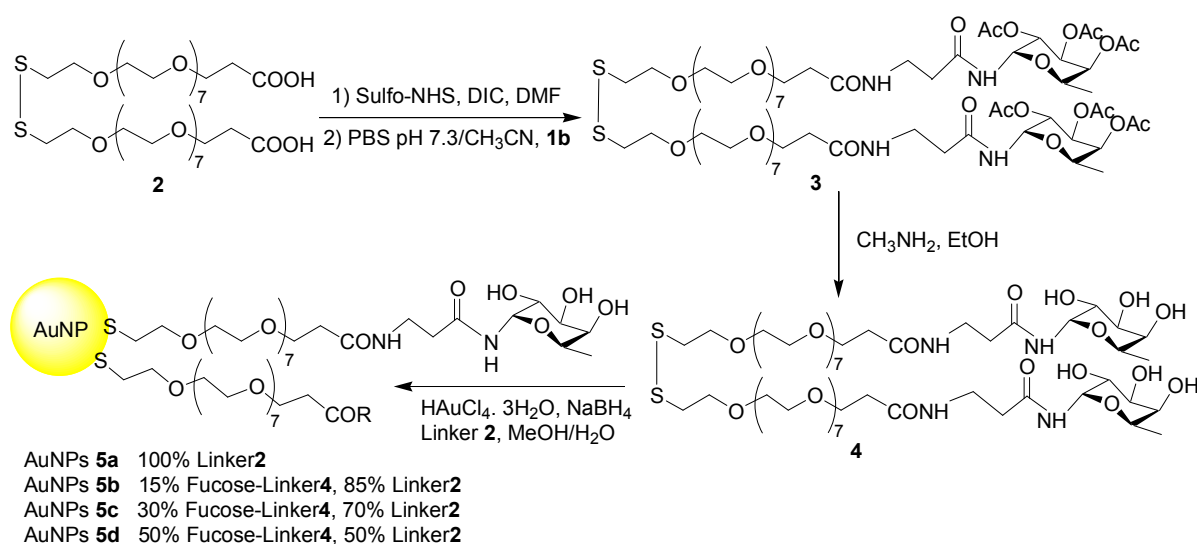
Hence, fucosylamide **1a** could clearly represent an inexpensive and effective alternative to the Le^X trisaccharide to build DC-SIGN targeting-devices. Indeed, some recent studies indicate that even simple monosaccharides can lead to selective nanoparticle recognition and uptake by immune system cells.¹⁹⁻²⁰ We therefore synthesized gold nanoparticles (AuNPs) functionalized with multiple copies of **1a** and measured their ability to interact with DC-SIGN on human DCs and to trigger cellular responses following interaction. AuNPs represent an accessible platform for multivalent presentation. These nanomaterials are relatively easy to synthesize and purify, and their size, shape and coating composition can be finely controlled by choosing the appropriate methodology of preparation.²¹⁻²⁴ Moreover the globular shape of spherical gold nanoparticles allows a defined and stable 3D presentation of the binding ligand. Finally, gold nanoparticles are considered biocompatible and viable for *in vivo* applications.²⁵

Here we show that AuNPs bearing 50% of fucosylamide ligand **1a** compete with gp120 on DC-SIGN expressing cells with a potency comparable to analogous particles functionalized with much more complex DC-SIGN binding oligosaccharides, such as Le^X. We also show that these particles promote DC-SIGN internalization, do not induce DC maturation and do not trigger cytokine responses, as demonstrated for some other DC-SIGN ligands.²⁶ Thus, the AuNPs described in this

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3 manuscript can be considered as excellent DC targeting agents and carriers able to deliver antigenic
4 cargo to DCs through DC-SIGN. They appear to lack any immune modulatory capacity, which
5 makes them suitable for use as a basis for antigen-specific vaccination in autoimmunity or cancer,
6 when used with appropriate adjuvants.
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10 11 12 13 14 15 **Results and discussion**

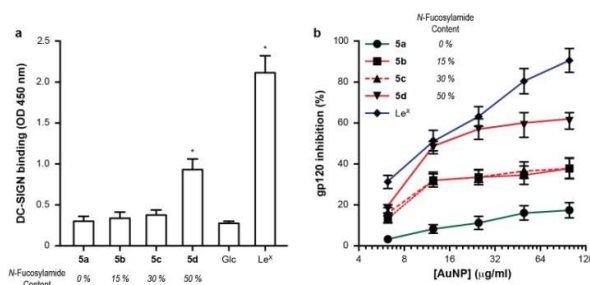
16 The AuNPs were prepared by reduction of tetrachloroauric acid by NaBH₄ in the presence of a thiol
17 (or a mixture of thiols), using Brust's method²⁷ as modified by Penadés (Scheme 1).²⁸ The required
18 thiols were generated starting from tri-*O*-acetyl- α -L- fucosyl- β -alanine **1b**²⁹ that was conjugated to
19 commercially available polyethylene glycolic linker **2**, where the disulfide bridge masks the
20 terminal thiol groups. The divalent linker was activated as a bis *N*-hydroxysulfosuccinimide ester
21 and then treated with **1b** in 4:1 phosphate buffer saline (pH 7.3) : CH₃CN. The desired bis amide **3**
22 was recovered after purification in 30% yield. Acetyl groups removal with 4M CH₃NH₂ in EtOH
23 afforded the free glycosyl amido-PEG **4**, which was used without further purifications. Direct
24 conjugation of the unprotected fucosylamide **1a** to the activated linker was also attempted; however
25 it was complicated by formation of an undesired side-product, presumably the furanose form of the
26 fucose ring. The free thiol function of **4** and/or **2** was unmasked in the presence of AuCl₄H by
27 NaBH₄ reduction of the disulfide bond. The resulting dark suspension of gold nanoparticles was
28 shaken for ca. 2 h at room temperatures and the particles were purified by centrifugal filtration
29 (Vivaspin filters, 10000 MWCO). Using this protocol, the coating composition of the AuNPs is
30 determined by the mixture of thiols used in the reaction. In particular, 15, 30 and 50% mixtures of
31 fucosylamide-derivatized linker **4** and unfunctionalized linker **2** were used, to afford AuNPs **5b-d**,
32 respectively. They were used to investigate the effect of ligand density on AuNP activity. Higher
33 concentrations were not considered, assuming that further increasing the density of the sugar would
34 not improve receptor binding.³⁰ Control nanoparticles AuNP **5a** were also prepared, bearing no
35 fucose ligands. All the particles synthesized were water-soluble and could be re-dissolved after
36 lyophilization. They were characterized by high-resolution transmission electron microscopy (HR-
37 TEM) and by ¹H-NMR (Figure SI-1-4). The HR-TEM analysis showed uniformly dispersed AuNPs
38 characterized by a very small core (ca. 2 nm) and without signs of aggregation. ¹H-NMR spectrum
39 of fucose functionalized AuNPs, confirmed the presence of the sugar on the surface of the
40 nanoparticles and integrations of the proton signals were in agreement with the expected molar ratio
41 of coating molecules.
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24 Scheme 1 Synthesis of the functionalized AuNPs **5a-d**

27 To test whether AuNPs were able to target DC-SIGN, we evaluated the binding of soluble DC-SIGN through a solid-phase DC-SIGN binding assay.³¹ Flat-bottomed 96-well plates were coated with AuNPs and incubated with a DC-SIGN/Fc chimera for 1 h at 37 °C. After washing, DC-SIGN binding was determined by an ELISA using an antibody against the Fc part of DC-SIGN/Fc. (Le^x-AuNP)³² carrying 10% of Le^x were used for comparison. The results (Figure 2a) clearly show binding of DC-SIGN to **5d** (carrying 50% of fucosylamide) and to the Le^x-AuNPs in the presence of Ca⁺⁺ and Mg⁺⁺. As a control, AuNP **5a**, carrying only linker **2**, were not recognized by DC-SIGN. In this experiment, no binding was detected for AuNPs **5b-c**, carrying 15% or 30% of fucosyl-amide **1a**.

42 Because DC-SIGN exists as a tetramer on the membrane of DCs and the soluble DC-SIGN/Fc chimera is a dimer, AuNPs binding was also tested on cellular DC-SIGN, by measuring binding inhibition of fluorescently labelled gp120 beads to a cell line that expresses a mutant form of DC-SIGN unable to internalize (Figure 2b).³³



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5 Figure. 2 AuNPs **5d** are recognized by DC-SIGN and compete with fluorescently-labelled gp120
6 beads. a) ELISA plates were coated with AuNPs (15 μ g/mL) and their binding to soluble DC-SIGN-
7 Fc was measured in a solid-phase assay. b) AuNPs (from 12.5 to 100ng/mL) were co-incubated
8 with K-SIGN_{LLY} cells in presence of fluorescently labelled gp120 beads. The AuNPs ability to bind
9 DC-SIGN was measured by FACS as % on inhibition of the gp120 beads-DC-SIGN interaction.
10 Data represent the mean + S. D. of a triplicate experiment. These experiments are representative of
11 at least three independent experiments.
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20 The results (Figure 2b) clearly show that both **5d** and Le^X-AuNPs (10% Le^X loading) inhibit gp120
21 binding to DC-SIGN in a dose-dependent manner with an approximate IC₅₀ value around 12 ng/mL.
22 Particles **5c** and **5b** carrying 30 and 15% of fucosyl-amide, respectively, showed a lower but
23 significant ability to interact with cellular DC-SIGN. Control AuNPs carrying 100% of linker **2** (**5a**)
24 showed no binding to cellular DC-SIGN. These results establish AuNPs **5d** as a potent and effective
25 DC-SIGN targeting platform.
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30 It has previously been demonstrated that several pathogens can trigger different cytokine responses
31 through DC-SIGN in a carbohydrate-dependent manner.²⁶ This mechanism depends on a fine
32 regulatory balance between C-type lectin receptors (CLRs) and Toll-Like Receptors (TLRs) and has
33 sparked the hypothesis that CLRs, and specially DC-SIGN, are immune homeostatic receptors that
34 can be hijacked by pathogens to escape immune surveillance. Differences in the DC-SIGN-
35 dependent signaling responses were described also using multivalent carbohydrate functionalized
36 polymers (PAA-LeX and PAA-mannose),²⁶ Le^b glycodendrimers,¹¹ dendrimers carrying mannoside
37 mimics³⁴ or gp120 high-mannoses,³⁵ indicating that the characterization of the molecular
38 determinants involved in DC-SIGN activation still needs further research. To study the downstream
39 effects of AuNPs **5** on DC, we analyzed the expression of the DC maturation marker CD86 and of
40 the anti-inflammatory cytokine IL-10. CD86 expression was measured by flow cytometry after
41 incubating DCs with AuNPs (1 μ g/mL) for 16 h. DCs treated with the TLR4 ligand
42 lipopolysaccharide (LPS 10ng/mL), a known inducer of DC maturation, were used as a positive
43 control. The results clearly show that neither the AuNPs **5**, nor the control Le^X-AuNPs induced
44 CD86 up-regulation at the tested concentration (1 μ g/mL) (Figure 3a). DC-SIGN signaling has been
45 reported to synergistically enhance IL-10 expression in the context of simultaneous TLR4
46 triggering.²⁶ This was investigated by assaying IL-10 production by DCs after simultaneous
47 incubation with AuNPs **5** (1 μ g/mL) and LPS (10 ng/mL). After 16 h, cells were centrifuged and the
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presence of IL-10 in the supernatant was determined by ELISA (Figure 3b). AuNPs **5** were not able to enhance LPS mediated IL-10 secretion. On the contrary, a glycopeptide dendrimer carrying Le^b (Le^bD3, Figure 3b) used as positive control, induced LPS mediated IL-10 up-regulation as previously described.¹¹ Thus, AuNPs **5** seem to behave as pure targeting devices and be unable to induce DC maturation or to trigger some of the well-known DC-SIGN mediated signaling-cascades. In this context, it may be worth noting that AuNPs displaying ManNAc mono- and di-saccharides¹⁹ or galactofuranose²⁰ have been reported to induce significant activation of antigen presenting cells.

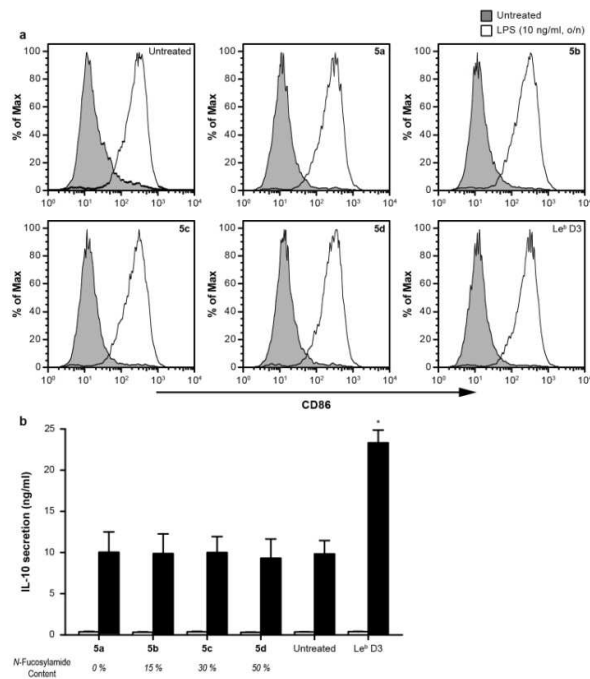


Figure. 3 AuNPs **5** do not induce maturation or IL-10 secretion on DCs. a) Maturation was assessed by CD86 expression human DCs after overnight incubation with AuNPs (grey curves) or LPS (white curve). None of the AuNPs induced DC maturation or altered the typical maturation profile induced by LPS treatment. b) IL-10 secretion (ng/mL) in human DCs after overnight incubation with AuNPs (white bars) and AuNPs + LPS (dark bars), as measured by ELISA. Le^bD3, a Le^b glycopeptide dendrimer,¹¹ was used as positive control. AuNPs do not show any enhancement in LPS-mediated IL-10 secretion.

Finally, *N*-fucosylamide glyco-nanoparticles **5b-5d** were able to induce DC-SIGN internalization, as measured by imaging flow cytometry (Figure 4). The DC-SIGN binding antibody AZN-D1 was used as positive control (Figure 4a); visualization of the internalized protein was achieved using an anti-DC-SIGN, CRD-independent, antibody (CSRD).³⁶ Internalization was achieved at 37 °C only

and it was directly proportional to the amount of *N*-fucosylamide present on the AuNPs (Figure 4b). DC-SIGN internalization is one of the first events occurring after carbohydrate-recognition and it is related to the ability of this lectin to present antigens to the histocompatibility complexes MHC-I and II.³³ The ability of AuNPs **5b-d** to internalize DC-SIGN on DCs suggests that these nanomaterials can be explored for antigen-targeting to DCs.

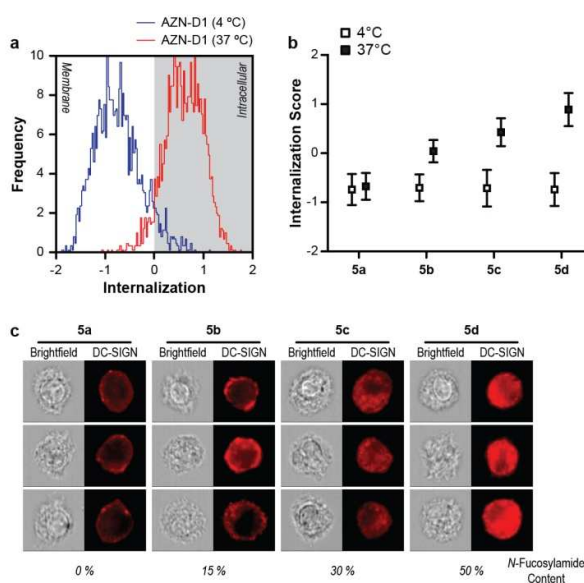


Figure. 4 *N*-fucosylamide glyconanoparticles **5b-d** induce DC-SIGN internalization. a) DC-SIGN internalization is induced using the CRD-binding antibody AZN-D1. The localization of DC-SIGN is investigated using the CRD-independent antibody CSRD. DC-SIGN internalization is only achieved after DCs have been incubated with AZN-D1 at 37 °C. b) Only *N*-fucosylamide nanoparticles **5b-d** were able to induce DC-SIGN internalization. Internalization was directly proportional to the amount of *N*-fucosylamide present on the glyconanoparticles. c) Representative examples of glycan nanoparticle internalization. No internalization observed with control **5a**

Conclusions

In this work we developed potent and effective DC-SIGN targeting gold nanoparticles based on *N*- α -fucosyl- β -alanylamine **1a**. The AuNPs **5b-d**, characterized by different sugar densities (15%, 30% and 50% of fucosylamide) and a diameter of ca. 2 nm, were water soluble and well dispersed. They were found to bind to DC-SIGN and inhibit gp120 binding to DC-SIGN expressing cells with an efficiency comparable to that of similar polyvalent constructs bearing a comparable load (10%) of the much more complex oligosaccharide Le^x. AuNPs **5b-d** induced DC-SIGN internalization,

without triggering DC maturation or the induction of IL-10. Thus they can be regarded as neutral carriers and DC-SIGN targeting devices. Their use as targeted imaging tools and antigen delivery devices will be explored in due course.

Experimental procedures

Chemistry. *General:* All chemicals and solvents were purchased from Sigma-Aldrich, including linker **2**. They all were of reagent grade and were used without further purification. Solvents were dried by standard procedures and reactions requiring anhydrous conditions were performed under Nitrogen atmosphere. *N*-(β -Alanyl)-2,3,4-tri-*O*-acetyl- α -L-fucopyranosyl amine **1b** was prepared according to a reported procedure.²⁹ Mass spectra were obtained with an ESI apparatus Bruker Esquire 3000 plus. Thin layer chromatography (TLC) was carried out with pre-coated Merck F₂₅₄ silica gel plates. Flash chromatography was carried out using the SP1 Flash Purification System by Biotage. ¹H and ¹³C NMR spectra were recorded at 300 K on Bruker AVANCE 400 MHz. Chemical shifts δ are expressed in ppm relative to internal Me₄Si as standard. For transmission electron microscopy (TEM) characterization, a single drop (10 μ L) of solution of gold NPs (ca. 0.1 mg/mL in milliQ water/ethanol) was placed on a holey carbon copper grid (Electron Microscopy Science) and left to dry in air overnight. The specimens were analyzed by a LIBRA200 EFTEM (ZEISS) with a FEG source and accelerating voltage of 200kV. The average diameter of the AuNPs was obtained from evaluation of several TEM micrograph, collected spanning large areas of the sample grid, by means of an in-house algorithm for automatic image analysis.

Synthesis of bis α -fucosyl amide conjugated linker **3.** The disulfide linker **2** (from Sigma-Aldrich, 43 mg, 0.047 mmol) and *N*-hydroxysulfosuccinimide sodium salt (25.5 mg, 0.1175 mmol) were dissolved in dry DMF (1.7 mL) and, under nitrogen and at room temperature, dicyclohexyl carbodiimide (22 μ L, 0.141 mmol) was added. The reaction mixture was stirred for 24 h, and then the solvent was removed under reduced pressure. The crude was dissolved in PBS (2.5 mL) and the tetra-*O*-acetyl- α -fucosyl- β -alanine **1b** (46 mg, 0.1175 mmol) in CH₃CN (0.5 mL) was added. The pH was adjusted to 7.3 by adding NaOH 0.2 N and the reaction mixture was stirred for ca. 24 h monitoring by TLC (CH₃Cl/MeOH 9:1). After reaction completion, the solvent was evaporated under reduced pressure and the crude was purified by automated flash chromatography, eluting with CHCl₃/MeOH (gradient from 95:5 to 80:20). The desired product was collected in 30% yield. (white foam). ¹H NMR (400 MHz, CDCl₃): δ 1.15 (d, 6H, J = 6.1 Hz, CH₃ Fuc), 2.0 (s, 6H, OCOCH₃), 2.03 (s, 6H, OCOCH₃), 2.18 (s, 6H, OCOCH₃), 2.50 (m, 4H, -NHCOCH₂CH₂O-), 2.56 (m, 4H, Fuc-NHCOCH₂CH₂NH-), 2.88 (t, 4H, -OCH₂CH₂SH-), 3.55 (m, 4H, Fuc-NHCOCH₂CH₂NH-), 3.60-3.70 (m, 56H, -OCH₂CH₂O-), 3.71-3.78 (m, 8H, -NHCOCH₂CH₂O-, -

OCH₂CH₂SH-), 4.12 (m, 2H, *H*-5 Fuc), 5.28 (m, 2H, *H*-4 Fuc), 5.36 (m, 2H, *H*-2 Fuc), 5.43 (m, 2H, *H*-3 Fuc), 5.93 (m, 2H, *H*-1 Fuc), 7.32 (m, 2H, Fuc-NHCOCH₂CH₂NH-), 8.16 (m, 2H, Fuc-NHCOCH₂CH₂NH-). ¹³C NMR (100.6 MHz, CDCl₃): δ 173.7, 172.9, 171.4, 170.8, 170.4, 75.1, 71.6, 71.24, 71.22, 71.16, 71.1, 71.0, 70.9, 70.85, 68.6, 67.8, 67.0, 66.5, 38.9, 37.1, 36.3, 21.4, 21.4, 17.0. MS (ESI⁺) m/z: 1599.15 (M+H)⁺, 800.12 (M+2H)²⁺.

Synthesis of 4. The bis- α -fucosyl amide conjugated disulfide **3** (22 mg, 0.014 mmol) was dissolved in a 4M MeNH₂ solution in EtOH (420 μ L, 1.68 mmol) and stirred at room temperature for 2h monitoring by TLC (CH₃Cl/MeOH 9:1). After reaction completion, the solvent was evaporated yielding the final product without further purification. 99% yield. (white foam). ¹H NMR (400 MHz, D₂O): δ 1.03 (m, 6H, CH₃ Fuc), 2.38 (m, 4H, -NHCOCH₂CH₂O-), 2.44 (m, 4H, Fuc-NHCOCH₂CH₂NH-), 2.81 (m, 4H, -OCH₂CH₂SH-), 3.34 (m, 4H, Fuc-NHCOCH₂CH₂NH-), 3.45-3.58 (m, 56H, -OCH₂CH₂O-), 3.59-3.65 (m, 4H, -NHCOH₂CH₂O-), 3.66-3.76(m, 10H, *H*-5 Fuc, *H*-4 Fuc, *H*-3 Fuc -OCH₂CH₂SH-), 3.86 (m, 2H, *H*-2 Fuc), 4.69 (m, 2H, *H*-1 Fuc). ¹³C NMR (100.6 MHz, CDCl₃): δ 175.9, 174.5, 77.2, 72.0, 70.2, 70.1, 70.0, 69.9, 68.9, 68.1, 67.2, 66.5, 37.8, 36.4, 36.2, 35.6, 16.2. MS (ESI⁺) m/z: 1347.7 (M+H)⁺, 674.6 (M+2H)²⁺.

General procedure for nanoparticles preparation

The AuNPs were prepared following a described protocol.²⁸ A 0.012M (3 equiv) solution of the commercially available linker **2** and of the α -fucosyl amide disulfide **4** (in different proportions) in 1:1 MeOH/H₂O was added to a solution of tetrachloroauric acid (0.025M, 1 equiv) in water. An aqueous solution of NaBH₄ (1M, 22 equiv) was then added in four portions, with rapid shaking. The black suspension formed was shaken for an additional 2 h at room temperature. After this time the nanoparticles were purified by centrifugal filtration (Vivaspin filters, 10000 MWCO). The solution was finally lyophilized to afford the desired AuNPs. ¹H-NMR spectra of the obtained AuNPs were recorded. The particle size distribution of the gold nanoparticles was evaluated from HRTEM micrographs by means of an automatic image analyser. The average molecular formula of the nanoparticles was calculated on the basis of the average diameter obtained by TEM.³⁷

AuNPs 5a: (100% linker **2**): Reaction of **2** (50.0 mg, 0.055 mmol) in MeOH/H₂O 1:1 (9 mL) with HAuCl₄·3H₂O (14.6 mg, 0.037 mmol) in 1.5 mL H₂O and NaBH₄ (0.8 mL, 1 M) gave AuNPs **5a** (11.6 mg) as a dark-brown powder. TEM average diameter: 2.2 \pm 1.5 nm, calculated molecular formula and molecular weight: C₁₆₅₆H₃₄₉₆Au₃₀₉O₉₂₀S₉₂, M.W.: 101946. ¹H NMR (400 MHz, D₂O): δ 2.52-2.62 (m, 2H, HOOCCH₂CH₂O-), 3.53-3.80 (m, 32H, CH₂O).

AuNPs 5b: (15% fucose, 85% linker **2**): Reaction of bis- α -fucosyl-amide derivative **4** (5.4 mg, 0.004 mmol) and linker **2** (17.0 mg, 0.0185 mmol) in MeOH/H₂O 1:1 (4.4 mL) with HAuCl₄·3H₂O (7.0 mg, 0.018 mmol) in 720 μ L H₂O and NaBH₄ (400 μ L, 1 M) gave AuNPs **5b** (5.5 mg) as a

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3 dark-brown powder. TEM average diameter: 2.81 ± 0.71 nm, calculated molecular formula and
4 molecular weight: $C_{3174}H_{6554}Au_{807}N_{48}O_{1726}S_{163}$, M.W.: 237194. 1H NMR (400 MHz, D_2O): δ 1.16
5 (m, 0.45H, CH_3 Fuc), 2.42-2.70 (m, 2.3H, -NHCOC H_2 CH $_2$ O- Fuc-NHCOC H_2 CH $_2$ NH-, -
6 HOOCCH $_2$ CH $_2$ O), 3.43 (m, 0.3H, Fuc-NHCOC H_2 CH $_2$ NH-), 3.49-3.92 (m, 32H, CH_2 O), 3.93-4.09
7 (m, 0.45H, *H*-5 Fuc, *H*-4 Fuc, *H*-3 Fuc), 4.12 (m, 0.15H, *H*-2 Fuc), 5.53 (m, 0.15H, *H*-1 Fuc).

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10 **AuNPs 5c:** (30% fucose, 70% linker **2**): Reaction of bis- α -Fucosyl-amide derivative **4** (11.0 mg,
11 0.0082 mmol) and linker **2** (17.0 mg, 0.0185) in MeOH/ H_2O 1:1 (4.6 mL) with $HAuCl_4 \cdot 3H_2O$ (7.8
12 mg, 0.0198 mmol) in 730 μL H_2O and $NaBH_4$ (400 μL , 1 M) gave AuNPs **5c** (5.3 mg) as a dark-
13 brown powder. TEM average diameter: 1.92 ± 0.69 nm, calculated molecular formula and
14 molecular weight: $C_{1488}H_{3013}Au_{225}N_{42}O_{794}S_{71}$, M.W.: 80795. 1H NMR (400 MHz, D_2O): δ 1.15 (m,
15 0.9H, CH_3 Fuc), 2.40-2.68 (m, 2.6 H, -NHCOC H_2 CH $_2$ O- Fuc-NHCOC H_2 CH $_2$ NH-, -
16 HOOCCH $_2$ CH $_2$ O), 3.42 (m, 0.6H, Fuc-NHCOC H_2 CH $_2$ NH-), 3.48-3.92 (m, 32H, CH_2 O), 3.93-4.09
17 (m, 0.9H, *H*-5 Fuc, *H*-4 Fuc, *H*-3 Fuc), 4.13 (m, 0.3H, *H*-2 Fuc), 5.53 (m, 0.3H, *H*-1 Fuc).

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20 **AuNPs 5d:** (50% fucose, 50% linker **2**): Reaction of bis- α -fucosyl-amide derivative **4** (23.5 mg,
21 0.017 mmol) and linker **2** (15.6 mg, 0.017 mmol) in MeOH/ H_2O 1:1 (5.6 mL) with $HAuCl_4 \cdot 3H_2O$
22 (9.0 mg, 0.023 mmol) in 920 μL H_2O and $NaBH_4$ (500 μL , 1 M) gave AuNPs **5d** (8.8 mg) as a
23 dark-brown powder. TEM average diameter: 2.14 ± 0.7 nm, calculated molecular formula and
24 molecular weight: $C_{2098}H_{4148}Au_{314}N_{92}O_{1094}S_{91}$, M.W.: 112937. 1H NMR (400 MHz, D_2O): δ 1.17
25 (m, 1.5H, CH_3 Fuc), 2.42-2.70 (m, 3H, -NHCOC H_2 CH $_2$ O- Fuc-NHCOC H_2 CH $_2$ NH-, -
26 HOOCCH $_2$ CH $_2$ O), 3.46 (m, 1H, Fuc-NHCOC H_2 CH $_2$ NH-), 3.50-3.92 (m, 32H, CH_2 O), 3.93-4.10
27 (m, 1.5H, *H*-5 Fuc, *H*-4 Fuc, *H*-3 Fuc), 4.12 (m, 0.5H, *H*-2 Fuc), 5.53 (m, 0.5H, *H*-1 Fuc).

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30 **Biological assays.** *Cells:* Human monocyte-derived DC were generated from monocytes as
31 previously described.³⁸ Briefly, monocytes were isolated from the blood of healthy donors
32 (Sanquin, The Netherlands) through a sequential Ficoll/Percoll gradient centrifugation. Isolated
33 monocytes (purity > 85 %) were cultured in RPMI 1640 (Invitrogen, USA) supplemented with 10%
34 FCS (BioWhittaker, USA), 1000 IU/ml penicillin (BioWhittaker, USA), 1000 IU/ml streptomycin
35 (BioWhittaker, USA), and 2 mM glutamine (BioWhittaker, USA) in the presence of interleukin-4
36 (IL-4) (500 IU/ml; BioSource, Belgium) and granulocyte-macrophage colony-stimulating factor
37 (GM-CSF) (800 IU/ml; BioSource, Belgium) for 7 days.³⁹ Monocyte differentiation into DC was
38 confirmed by flow cytometric analysis (FACScan, BD biosciences, USA) of the expression of DC-
39 SIGN using the monoclonal antibody AZN-D1⁴⁰ followed by staining with a secondary FITC-
40 labeled anti-mouse antibody (Zymed, San Francisco, CA).

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3 *Stable K562/DC-SIGN_{LL/Y} transfectants*³³ were maintained in RPMI 1640 medium containing 10%
4 Fetal Calf Serum 1000 IU/ml penicillin (BioWhittaker, USA), 1000 IU/ml streptomycin
5 (BioWhittaker, USA), and 2 mM glutamine (BioWhittaker, USA). Transfectants were regularly
6 selected using 1 mg/ml Geneticin (Invitrogen, USA). To check for DC-SIGN expression, cells were
7 incubated with primary antibody (AZN-D1) followed by staining with a secondary FITC-labeled
8 anti-mouse antibody (Zymed, USA) and analyzed by flow cytometry on a FACScan flow cytometer
9 (BD Biosciences, USA). Cell viability was measured using 7-amino actinomycin D (Invitrogen,
10 USA).

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16 **DC-SIGN-Fc ELISA.**³¹ Nunc MaxiSorp plates were coated with 50 μ L AuNPs (15 μ g/mL in
17 coating buffer, 50mM Na₂CO₃, pH=9.7) for 2 h at room temperature. The wells were washed twice
18 with TMS (2x200 μ L) and blocked with 100 μ L TMS with 1% of BSA for 30 min at room
19 temperature. After 1x200 μ L wash with PBS, the wells were incubated at 37°C with 50 μ L DC-
20 SIGN-Fc (3 μ g/mL) in TMS with 1% of BSA for 1 h. The wells were washed four times with TMS
21 (4x200 μ L) and incubated at room temperature with 50 μ L of Goat-anti human HRP (0.8 μ g/mL) in
22 TMS with 1% of BSA for 30 min. After four washes with TMS (4x200 μ L), 100 μ L of substrate
23 solution (3,3',5,5'-Tetramethylbenzidine, TMB, in citric/acetate buffer, pH=4, and H₂O₂) were
24 added and after 4 min at room temperature the reaction was stopped with 50 μ L of H₂SO₄ (0.8M)
25 and the plate was read at 450 nm ELISA reader. All the experiments were performed in triplicate.
26 DC-SIGN/Fc consists of the extracellular portion of DC-SIGN (residues 64 to 404) fused at the C-
27 terminus to a human IgG₁/Fc fragment into the Sig-pIgG₁-Fc vector.⁴¹ DC-SIGN/Fc was produced
28 in CHO-K1 cells by co-transfection of DC-SIGN-Sig-pIgG₁ Fc (20 μ g) and the pEE14 (5 μ g)
29 vector.

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40 **Preparation of gp120-coated beads.** Fluorescently labelled gp120-microbeads were generated in a
41 similar way as was described for ICAM-1 beads.⁴² In brief, carboxylate-modified TransFluoSpheres
42 (488/645 nm, 1.0 μ m, Invitrogen) were coupled to streptavidin (Sigma) followed by incubation with
43 biotinylated F(ab')₂ fragment goat-anti-human IgG Fc fragment (0.5 mg/ml, Jackson Immuno
44 Research) for 2 hours at 37°C. Next, the beads were washed and coupled to purified HIV-1 gp120-
45 Fc protein (1 μ g/ml) by an overnight incubation at 4°C. After extensive washing in ice-cold PBS,
46 beads were reconstituted in 50 μ L of PBS.

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52 **Gp120 binding assay.** Stable K562/DC-SIGN_{LL/Y} transfectants (5×10^4 per well) were incubated
53 with gold nanoparticles for 45 min at 37°C. Next, gp120 fluorescent beads were added to the cells
54 and incubated for another 45 min at 4°C. The percentage of gp120 beads-positive cells was
55 analyzed by flow cytometry (BD Biosciences, USA).
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3 **Flow cytometry analysis of DC surface molecules and cytokine production.** Suspensions of 100
4 μL immature DCs ($1 \cdot 10^6$ cells/mL) were stimulated by addition of AuNPs (1 $\mu\text{g/mL}$) for 16-20
5 hours at 37°C in the presence of 5% CO_2 . 10 ng/mL LPS (*E. coli*, Sigma-Aldrich) served as a
6 control. Cells were then collected by centrifugation, washed with 1% BSA in phosphate-buffered
7 saline and incubated with mouse anti-human CD86 conjugated with phycoerythrin (PE, BD
8 Pharmingen, San Diego, CA). After 30 min incubation at 4°C , cells were analyzed using a flow
9 cytometer (FACS Calibur, BD Biosciences, USA).

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11 **IL-10 ELISA.** For the detection of IL-10, culture supernatants were harvested 24 h after DC
12 stimulation and frozen at -80°C until analysis. Cytokines were measured by ELISA using antibody
13 pairs from eBioscience (The Netherlands) and according to manufacturer's protocols.

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15 **Internalization assay.** DCs were incubated in the presence of gold nanoparticles at either 4°C or
16 37°C for 60 min. Cells were then washed, fixated, stained against DC-SIGN using the polyclonal
17 antibody CSRD³⁶ and an AF488-labeled goat-anti-rabbit antibody, and then prepared for acquisition
18 on the ImageStreamX (Amnis corp., Seattle) imaging flow cytometer. The following laser powers
19 were used: 488 nm at 20 mW and 785 nm at 4.5 mW. Brightfield illumination was set at 800 mW
20 before the acquisition of each sample. Brightfield images were collected in channels 1 and 9.
21 Channels 2 (Atto488) and 6 (granularity) were habilitated for the internalization assay. Cells were
22 acquired at 60x magnification and on the basis of their area (area = the number of pixels in an
23 image reported in square microns). Minimum area for acquisition was set to 50 pixels. A minimum
24 of 15×10^3 cells was acquired per sample at a flow rate ranging between 50 and 100 cells/s. At least
25 2×10^3 cells were acquired from single stained samples to allow for compensation. Compensation
26 samples were acquired with all channels habilitated and with the brightfield illumination and the
27 785 nm laser switched off. A minimum of 5×10^3 cells from the single stained samples were
28 acquired with the same settings as experimental samples to control for over/under compensation.
29 Analysis was performed using the IDEAS v6.0 software (Amnis corp., Seattle) as previously
30 described.⁴³ The internalization score is a log-scaled ratio of the intensity inside the cell
31 (intracellular mask) respect the intensity of the entire cell. Cells that have internalized antigen
32 typically have positive scores while cells that show the antigen still on the membrane have negative
33 scores. Cells with scores around 0 have similar amounts of antigen on the membrane and in
34 intracellular compartments.

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52 **Associated content**

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54 **Supporting Information**
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3 Table with tentative molecular formula. TEM micrographs, size-distribution histograms and ¹H-
4 NMR spectra of gold nanoparticles. This material is available free of charge via the Internet at
5 <http://pubs.acs.org>.
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21 22 **Notes**

23 The authors declare no competing financial interest.
24

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Table of content

A potent DC-SIGN targeting device was developed using gold nanoparticles functionalized with α -fucosyl- β -alanyl amide.

