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Preparation, characterization and immunogenicity of HIV-1 related high-mannose oligosaccharides-CRM_{197} glycoconjugates

Anna Kabanova · Roberto Adamo · Daniela Proietti · Francesco Berti · Marta Tontini · Rino Rappuoli · Paolo Costantino

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Abstract The dense glycan shield on the surface of human immunodeficiency virus type 1 (HIV-1) gp120 masks conserved protein epitopes and facilitates virus entry via interaction to glycan binding proteins on susceptible host cells. The broadly neutralizing monoclonal antibody 2G12 binds a cluster of high-mannose oligosaccharides on the gp120 subunit of HIV-1 Env protein. This oligomannose epitope is currently being considered for the design of a synthetic vaccine. The cluster nature of the 2G12 epitope suggests that a multivalent antigen presentation is important to develop a carbohydrate-based vaccine candidate. In this work we describe the development of neoglycoconjugates displaying clustered HIV-1 related oligomannose carbohydrates. We exploited flexible polyamidoamine (PAMAM) scaffold to generate four- and eight-valent sugar clusters of HIV-1-related oligomannose antigens Man_4, Man_6 and Man_9. The multivalent presentation of oligomannoses increased the avidity of Man_4 and Man_9 to 2G12. The synthetic glycodendrons were then covalently coupled to the protein carrier CRM_{197}, formulated with the adjuvant MF59, and used to immunize two animal species. Oligomannose-specific IgG antibodies were generated; however, the antisera failed to recognize recombinant HIV-1 gp120 proteins. We conclude that further structural vaccinology work is needed to identify an antigen presentation that closely matches *in vivo* the structure of the epitope mapped by 2G12.

Keywords HIV · High-mannose · Glycoconjugate · PAMAM · Vaccine

Abbreviations

CHO	Carbohydrate
Man	Mannose
RT	Room temperature
vol	Volume
GNL	Galantus nivalis lectin
i.m.	Intramuscular
sc.	Subcutaneous
HSA	Human serum albumin
IC_{50}	Inhibitor concentration causing fifty % signal reduction

Introduction

Worldwide human immunodeficiency virus (HIV) pandemic involves approximately 33 million people with 2.7 million new infections and 2 million deaths each year [1]. It is generally believed that an effective prophylactic weapon against HIV-1 could be a vaccine capable of eliciting both neutralizing antibodies and T-cell responses. However, numerous defense mechanisms help HIV-1 to

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A. Kabanova · R. Adamo · D. Proietti · F. Berti · M. Tontini · R. Rappuoli · P. Costantino (✉)
Novartis Vaccines and Diagnostics, Research Center,
Via Fiorentina 1,
53100 Siena, Italy
e-mail: paolo.costantino@novartis.com

A. Kabanova
Department of Experimental Evolutionary Biology,
University of Bologna,
via Zamboni 33,
40126 Bologna, Italy

evade host immune attacks directed against HIV envelope (Env) neutralization epitopes by means of frequent mutations, structural occlusions achieved by protein complex formation and heavy glycosylation [2–4]. The latter leads to formation of so-called "glycan shield" that masks conserved protein epitopes [5, 6]. This shield provides to the virus an additional source of antigen heterogeneity due to the numerous glycoforms in which proteins can exist and, being produced by the host glycosylation machinery, is expected to induce immune tolerance. Nevertheless, a unique carbohydrate epitope mapped by the human broadly neutralizing monoclonal antibody 2G12 was discovered on the surface of Env gp120. This makes HIV glycans potential candidates for an anti HIV-1 vaccine [7].

The neutralizing carbohydrate epitope of gp120 consists of a cluster of terminal α-D-Man-(1,2)-α-D-Man residues (Manα1-2-Man) on the D1 and D3 arms of $Man_9GlcNAc_2$ residues [8, 9]. An extended antibody binding surface is formed by a unique heavy chain variable domain-swapped configuration, which favors the possibility of multiple interactions with mannose surface [6]. Man_4, Man_6 and Man_9 derivatives of natural $Man_9GlcNAc_2$ oligosaccharide (Fig. **1**a) were proposed as possible "building blocks" of a future glycoconjugate vaccine. This is because they possess Manα1-2-Man units that are essential for 2G12 recognition and have been proved to interact with 2G12 in binding and inhibition assays [8, 10–12].

The cluster nature of the 2G12 epitope suggests the importance of multivalent presentation of oligomannoses in developing glycoconjugate molecules as possible candidate vaccines. Synthetic high-mannose clusters of 2-, 4- and higher valence, compared to monovalent sugars, showed enhanced binding to 2G12 and up to 110 times lower IC_{50} when used as inhibitors of the interaction between antibody and gp120 [13–15]. Two types of clustering scaffold have been investigated so far for HIV related antigens. In one case high mannoses were randomly oriented by a flexible linker around a galactose core [12, 16]. In a different study a semirigid cyclic peptide scaffold served to position the carbohydrate moieties at the correct distance as defined by the crystal structure of gp120 [13, 14]. The latter strategy seemed to provide a better mimic of the native epitope, but the sterical constrains of the model led to incorporation of a lower number of carbohydrate chains [17]. In summary, up to now three HIV related glycoconjugates have been used for *in vivo* studies: the monovalent Man_4 conjugated to BSA [18], the bivalent $Man_9GlcNAc_2$ on the cyclic peptide scaffold conjugated to *Neisseria meningitidis* outer membrane protein complex (OMPC) [17], and the galactose-based tetravalent $Man_9GlcNAc_2$-cluster conjugated to tetanus toxoid T-helper peptide [16]. None of them elicited "2G12-like" response that cross-reacted with HIV Env proteins.

In our laboratory we have investigated four- and eight-valent polyamidoamine (PAMAM) dendrons as scaffolds to make clusters with HIV-1 related high-mannose oligosaccharides (Fig. **1**b). PAMAMs appeared attractive, due to their potential low immunogenicity [19, 20] and built-in surface functionalities, which provide multiple sites for sugar incorporation. The high-mannose oligosaccharide clusters have been coupled to CRM_{197}, a non-toxic mutant of diphtheria toxin already extensively used as carrier for glycoconjugate vaccines in humans [21–23]. Formulated with the MF59 adjuvant, an oil-in-water buffered emulsion of 5% squalene, 0.5% Tween 80 and 0.5% Span 85 that has been shown to be effective for intramuscular immunization [24–26], the glycoconjugates were tested in rabbits and mice. Here we report their preparation, structural characterization, antigenic and immunogenic properties.

Results

Oligomannose cluster synthesis and characterization

High mannose oligosaccharides equipped with a six-carbon amino linker at the reducing end were converted into the corresponding succinimidyl adipate esters by reaction with ten-fold molar excess of disuccinimidyl adipate linker, so that formation of dimers could be avoided. After purification from the excess of bifunctional linker by precipitation and subsequent washings with ethylacetate, the activated oligosaccharides were dried and reacted with $PAMAM_4$t-Boc or t-$PAMAM_8$t-Boc (Fig. 1b). The glyco-PAMAMs were purified on a C4 hydrophobic interaction cartridge where the excess of unreacted oligosaccharides eluted in the flow-through. Fractions containing the fully derivatized PAMAM were identified by ESI Q-TOF MS.

Glyco-$PAMAM_4$ dendrons were analyzed by direct infusion of the sample into Q-TOF system, glyco-$PAMAM_8$ dendrons were analyzed by UPLC coupled to Q-TOF. The general pattern of the ESI-MS spectra contained molecular ion peaks related to the fully derivatized PAMAMs as major species and a slight fragmentation of molecules due to the loss of Man units starting from the molecular ion peak, as better evidenced in the deconvoluted spectra (Fig. 2, Online resource 1).

The ESI-MS analysis of the Man_4PAMAM_8t-Boc UPLC peak (Fig. 2a) showed multiple molecular ions corresponding to the Man_4PAMAM_8t-Boc molecule (8,760.14 Da) bearing different charges, the most abundant of which being the 5+ molecular ion, although 3+, 4+ and 6+ ions were also present.

Prominent ions in the spectrum of Man_9PAMAM_4t-Boc (7,587.11 Da) were the 4+ charged (Fig. 2b). The presence

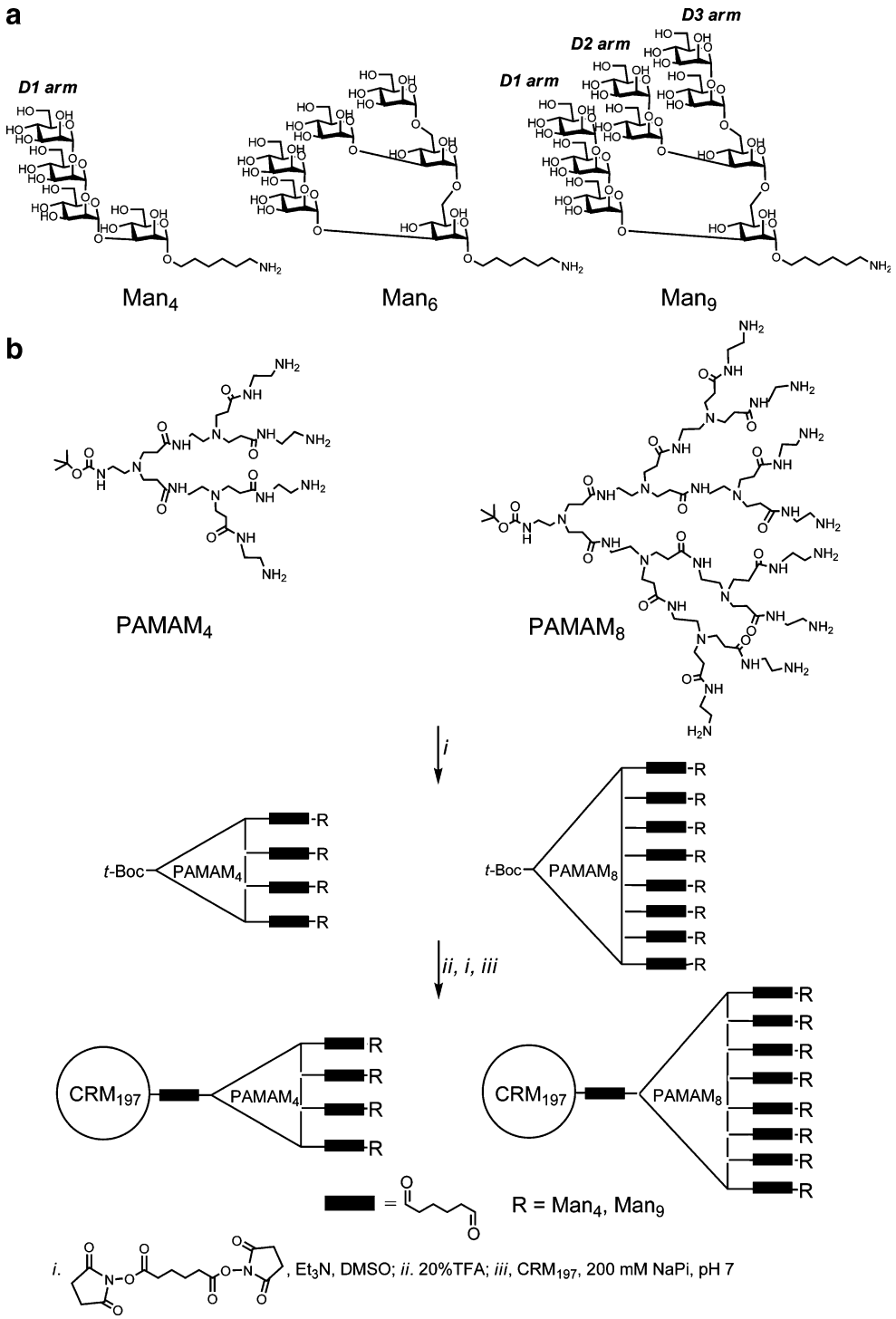

Fig. 1 Structures of oligomannoses (**a**). Synthesis of PAMAM-oligosaccharide clusters (**b**)

of a molecular ion with a mass of 4,157.13 Da was attributed to the product of two Man_9 molecules with a $PAMAM_4$ minor contamination in which a cyclization between two branches subsequent to the loss of an ethylenediamine unit (60 Da) had occurred. This contamination is most likely a side product from incomplete reaction during the synthesis of PAMAM dendrimer [27].

Man_4PAMAM_4t-Boc (4,346.05 Da) was characterized by the presence of the 3+ and the 4+ molecular ions as most intense peaks (Online resource 1). The ESI spectrum of Man_9PAMAM_8 t-Boc (15,242.25 Da) (Online resource 1) presented the 6+ ion as prominent peaks and two minor contaminations: the first corresponded to the $PAMAM_8$ derivatized with two Man_9 molecules; and, the second to the $PAMAM_8$ derivatized with six Man_9 moieties and a bridging between two ethylendiamines through an adipate molecule. However, we considered the Man_9PAMAM_8 cluster as the most abundant product.

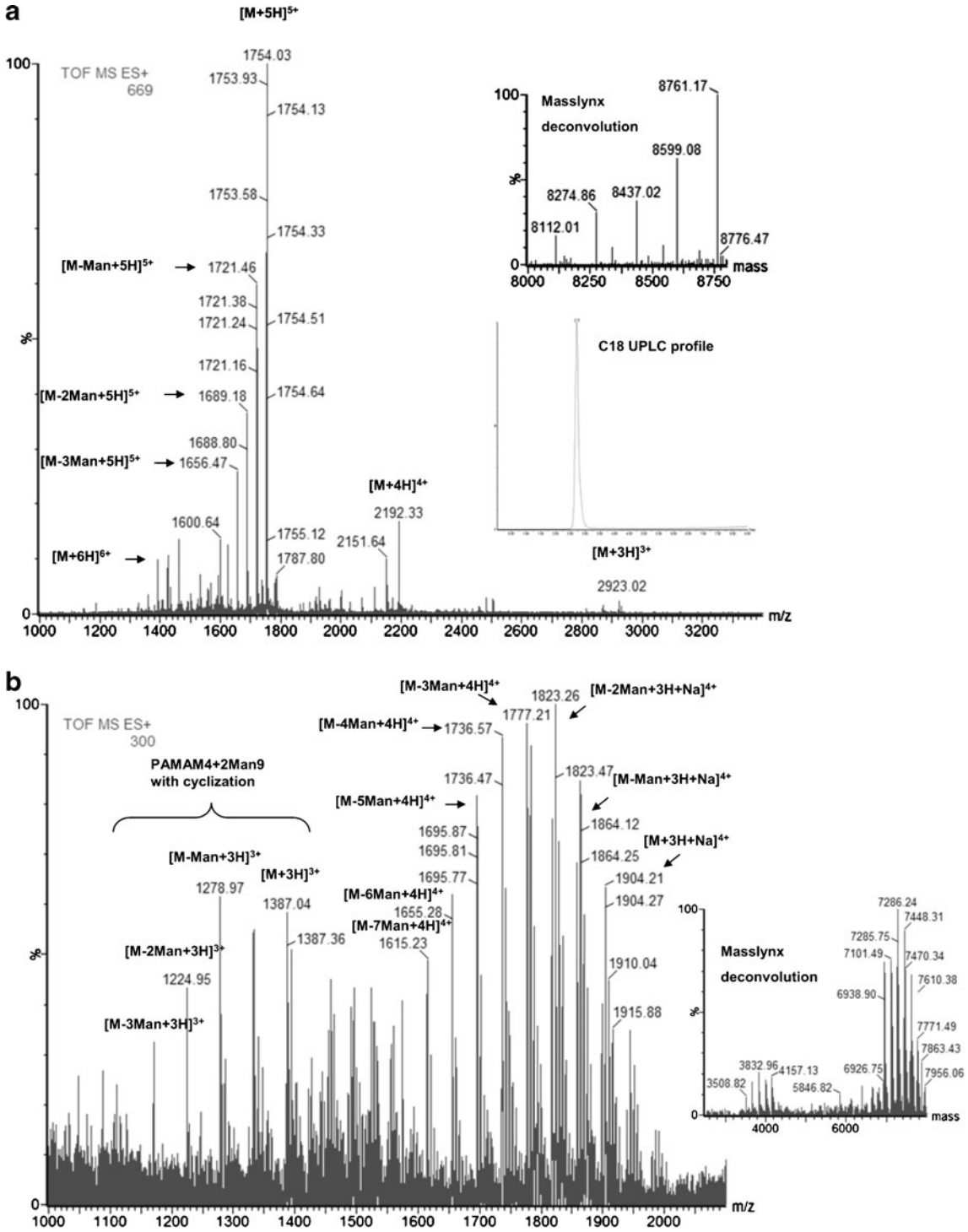

Fig. 2 Mass Spectrometry profiles of Man_4PAMAM_8 (**a**) and Man_9PAMAM_4 (**b**)

The glyco-PAMAMs were further characterized by ^1H NMR spectroscopy. The proton spectra of Man_9PAMAM_4t-Boc, Man_9PAMAM_8t-Boc, Man_4PAMAM_4 and Man_4PAMAM_8 (the last two after t-Boc removal) showed signals that are characteristic of the synthetic oligomannose with the six-carbon amino linker, the disuccinimidyl adipate linker, PAMAM and, when not removed, t-Boc. The results obtained from the quantitative analysis of the NMR spectra were in good agreement with the expected values and with the ESI Q-TOF MS data, confirming that the major species in the glyco-PAMAMs preparations were represented by the fully derivatized dendrons (Online resource 2).

The yields for cluster formation and purification varied from 46–77% for $PAMAM_4$-based dendrons and 28–30%

for $PAMAM_8$-based dendrons. The variability of yields could be explained by different efficiency of the dendron elution from C4 cartridge during the purification process. However, since our primary goal was to examine the immunogenic properties of the glyco-PAMAMs, we did not investigate this point further.

Immunochemical characterization of oligomannose antigens

In order to determine the relative ability of the different oligomannose systems to bind 2G12 we performed competitive experiments using surface plasmon resonance (SPR). HIV protein gp140 UG37 was immobilized on a Biacore CM5 chip, and 2G12 with and without inhibitors was injected over it. Initial screening of monovalent oligomannoses showed inferior inhibitory capacity of Man_6 as compared to Man_4 and Man_9. In fact, 1.3 mM Man_4 and 0.6 mM Man_9 inhibited gp140-2G12 interaction by 89% and 64%, respectively, while 1.3 mM Man_6 showed only 17%. We therefore concentrated our attention on Man_4 and Man_9 antigens and explored if clustering influences the binding ability to 2G12.

SPR inhibition assay evidenced lower IC_{50} values for $PAMAM_4$ and $PAMAM_8$ clusters, as compared to their respective monovalent oligosaccharides (Fig. 3; see Online resource 3 for SPR sensorgrams). In particular IC_{50} of Man_4PAMAM_4 and Man_9PAMAM_4 clusters were 9.1 and 10.2 times lower than IC_{50} of Man_4 and Man_9, respectively; moreover IC_{50} of Man_4PAMAM_8 and Man_9PAMAM_8 clusters were 2.3 and 3.9 times lower than IC_{50} of Man_4PAMAM_4 and Man_9PAMAM_4, respectively. The absolute IC_{50} values were comparable for both Man_4 and Man_9 clusters. Thus multivalent presentation of oligomannose increased their avidity to 2G12, and the smaller D1-armed Man_4 competed for 2G12 at the same level as D1D3-armed Man_9.

Synthesis of CRM_{197} glycoconjugates

A well-established way to improve poor immunogenicity of carbohydrate antigens is the conjugation to a protein carrier that provides T cell epitopes. We therefore coupled our high-mannose oligosaccharides, plain or PAMAM-clustered, to the lysine residues of CRM_{197}. Using disuccinimidyl adipate linker chemistry (Fig. 1b) we synthesized a panel of glycoconjugates that have been characterized by carbohydrate/protein ratio and SDS-PAGE (Fig. 4, Table 1). The average molar loading of glyco-PAMAMs onto the protein, as determined by chemical analyses, was 10 and 6 for Man_4PAMAM_4 and Man_9PAMAM_4, respectively, while decreased to 4 and 2 for $PAMAM_8$ clusters of Man_4 and Man_9. The reason for these differences could be found in the increased steric hindrance due to $PAMAM_8$. In SDS-PAGE the glycoconjugates migrated with diffuse bands covering a region consistent with the expected increase of Mw and suggesting a certain heterogeneity of the glycoconjugate molecules likely due to the multiple conjugation sites on CRM_{197} represented by 39 lysine residues in its structure (Fig. 4) [28].

Glycoconjugates immunogenicity in rabbits and mice

Initially we tested in rabbits the immunogenicity of Man_4- and Man_9-PAMAM glycoconjugates compared to Man_9-CRM_{197}. Man_4/Man_9-$PAMAM_4$ and Man_4/Man_9-$PAMAM_8$ glycoconjugates were tested at 20 and 5 μg carbohydrate dose respectively, in groups of 2–4 rabbits. In all cases, the antigens were formulated with MF59 adjuvant.

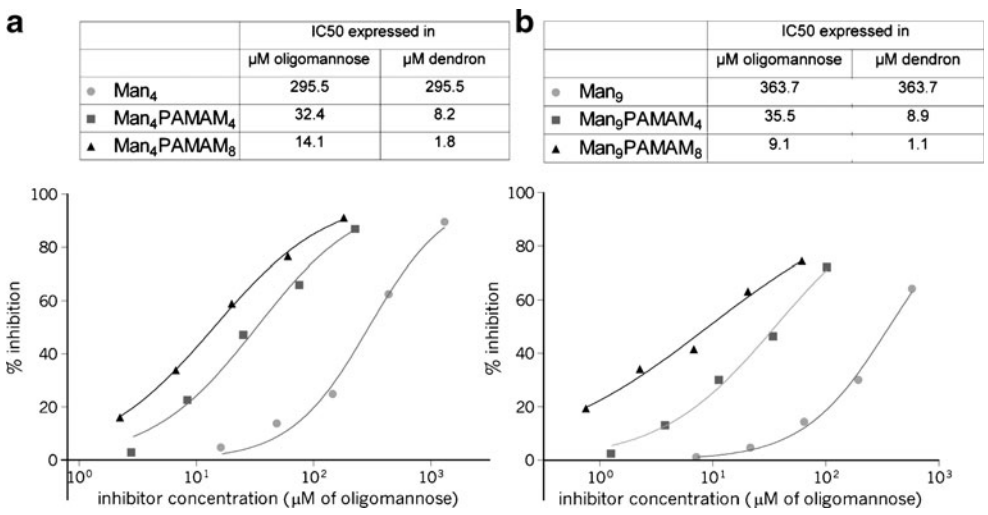

Fig. 3 Biacore inhibition of 2G12-gp140 interaction by monovalent and clustered Man_4 (**a**), and monovalent and clustered Man_9 (**b**). Inhibition was calculated as difference in maximal binding

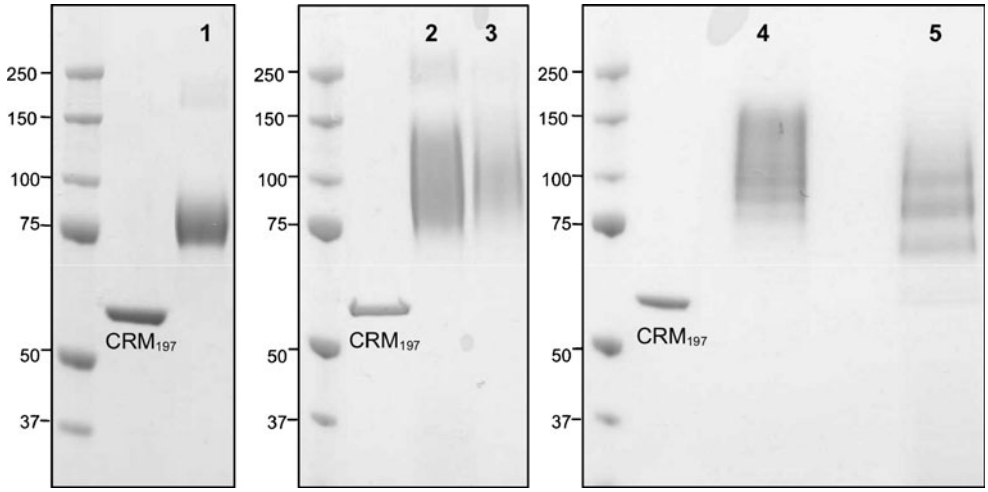

Fig. 4 SDS-PAGE of Man_9-CRM_{197} (1), Man_4PAMAM_4-CRM_{197} (2), Man_9PAMAM_4-CRM_{197} (3), Man_4PAMAM_8-CRM_{197} (4) and Man_9PAMAM_8-CRM_{197} (5)

We first assessed the antibody response against Man_9, by ELISA using Man_9 conjugated to HSA via squarate linker as coating reagent. The different protein and coupling chemistry were used in order to reveal only oligomannose-specific antibodies. As reported in Fig. 5a, b, c all glycoconjugates induced Man_9-specific IgG titer. In particular, Man_9 glycoconjugates, clustered or plain, elicited a stronger antibody response in comparison to Man_4 antigens. We did not detect significant carbohydrate-specific IgM titers (data not shown). The specificity of detected antibodies was further investigated in a competitive ELISA assay in which Man_9 inhibits the binding of antisera to Man_9-squarate-HSA. As shown in Fig. 6, complete inhibition was reached for all rabbit sera, however differences in IC_{50} values were observed in the order $Man_9PAMAM_4/PAMAM_8$-CRM_{197} < Man_9-CRM_{197} < $Man_4PAMAM_4/PAMAM_8$-CRM_{197}, suggesting that $Man_9PAMAM_4/PAMAM_8$-CRM_{197} conjugates elicited anti-Man_9 antibodies of higher affinity.

Following the main goal of this study, we then examined the cross-reactivity of the rabbit sera against HIV-1 gp120 proteins. Several clade B gp120 proteins were coated onto ELISA microplates and tested against pools of rabbit post immunization sera. None of the gp120 proteins showed cross-reactivity with antiserum panel. Meanwhile, as expected, 2G12 and *Galantus nivalis* lectin (GNL) did recognize everyone (Fig. 7).

In order to collect data with a different animal model we additionally tested Man_4PAMAM_4-CRM_{197}, and Man_9PAMAM_4-CRM_{197} in mice at 1 μg carbohydrate dose. Anti-Man_9 antibodies were clearly elicited by the Man_9 conjugate, while the response of Man_4PAMAM_4-CRM_{197} was weak (Fig. 5d). Also in this case, when we tested the antisera against HIV-1 gp120, no cross-reaction was observed (Fig. 7).

Moreover, we examined the presence of anti-CRM_{197} antibodies in pools of sera from rabbit and mice immunized with the different conjugates. In all cases anti-carrier antibodies have been induced. Interestingly, in rabbit, PAMAM-based conjugates seemed to induce a lower anti-carrier response as compared to Man_9-CRM_{197}. This could be explained with a shielding of relevant T- or B-cell epitopes of CRM_{197} by the glyco-PAMAM haptens (Fig. 8).

Table 1 Chemical characteristics of high-mannose glycoconjugates

Gel line (Fig.4)	Antigen	Mw[a] of the hapten portion[b] (Da)	Average molar ratio of hapten[b] to protein[a]	CHO/Protein % wt/wt	Expected glycoconjugate average Mw[a] (Da)
1	Man_9-CRM_{197}	1,688	17	43.8	87,165
2	Man_4PAMAM_4-CRM_{197}	4,360	10	43.6	100,130
3	Man_9PAMAM_4-CRM_{197}	7,602	6	58.4	102,350
4	Man_4PAMAM_8-CRM_{197}	8,776	4	39.0	95,971
5	Man_9PAMAM_8-CRM_{197}	15,262	2	44.1	91,679

[a] Values rounded to the nearest integer

[b] Hapten is defined as oligosaccharide or glycodendron with adipate linker

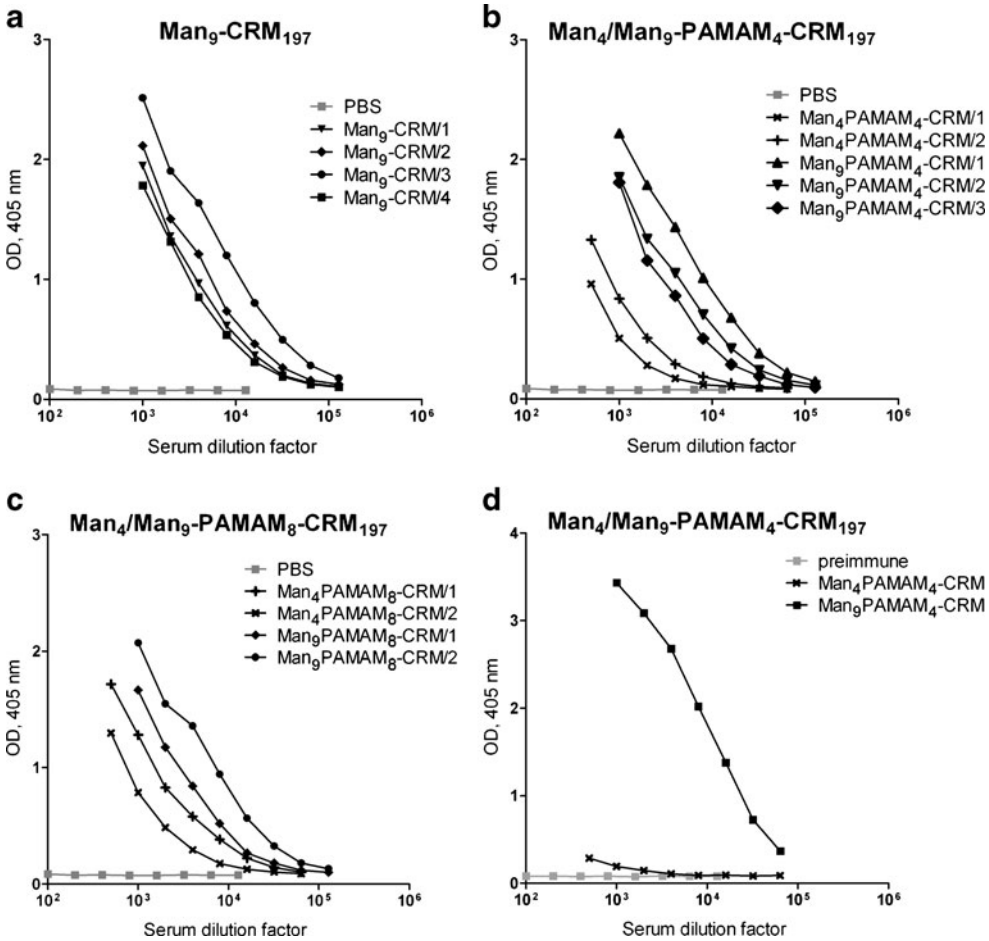

Fig. 5 Anti Man_9 antibodies induced by high-mannose oligosaccharides-CRM_{197} conjugates as detected by ELISA in single rabbit (**a**, **b**, **c**) and pooled mice (**d**) antisera

Discussion

HIV is characterized by a densely glycosylated surface, which enhances the effectiveness of immune escape and is implicated in viral dissemination [29, 30]. Human broadly neutralizing antibody 2G12 and mannose-binding lectin cyanovirin-N were found to recognize high-mannose oligosaccharides on the surface of HIV-1 gp120. Both demonstrated anti-HIV activity at nanomolar level [31, 32]. Moreover, both 2G12 or cyanovirin-N so far have shown no autoimmune property, probably due to their strict specificity to dense oligomannose surfaces that have not been observed among human glycoproteins [9, 33]. This suggested that high-mannose oligosaccharides are feasible targets for a vaccine aiming at eliciting "2G12"-like antibodies. Up to now various synthetic approaches have been applied to prepare clusters with the aim of mimicking the 2G12 epitope. However, none of the obtained molecules reported gp120 cross-reactive immune response virtually due to initial low antigen affinity to 2G12 [16–18]. The affinity increase that can be achieved by multivalent presentation of carbohydrate ligands prompted us to explore PAMAM dendrons that offer high coupling valence and are poor immunogens *per se* [19, 20]. This convinced us that the utilization of such scaffolds could provide more control in spatial presentation of sugars than conjugation to Lys residues of carrier protein, allowing a better emulation of the dense arrangement of oligomannoses on the gp120

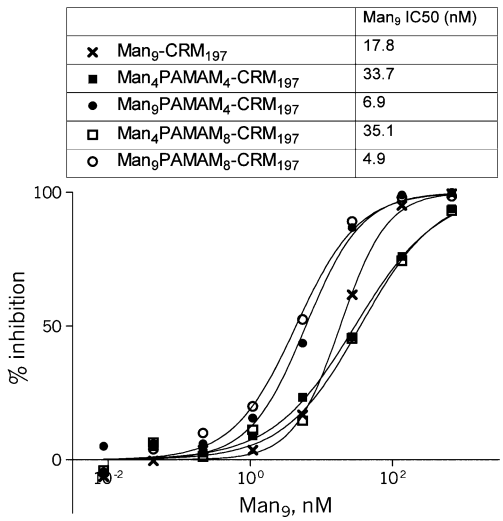

Fig. 6 Specifity of rabbit antibodies induced by high-mannose oligosaccharides-CRM_{197} conjugates as evidenced by the ability of Man_9 to inhibit the binding of pooled antisera to Man_9-squarate-HSA

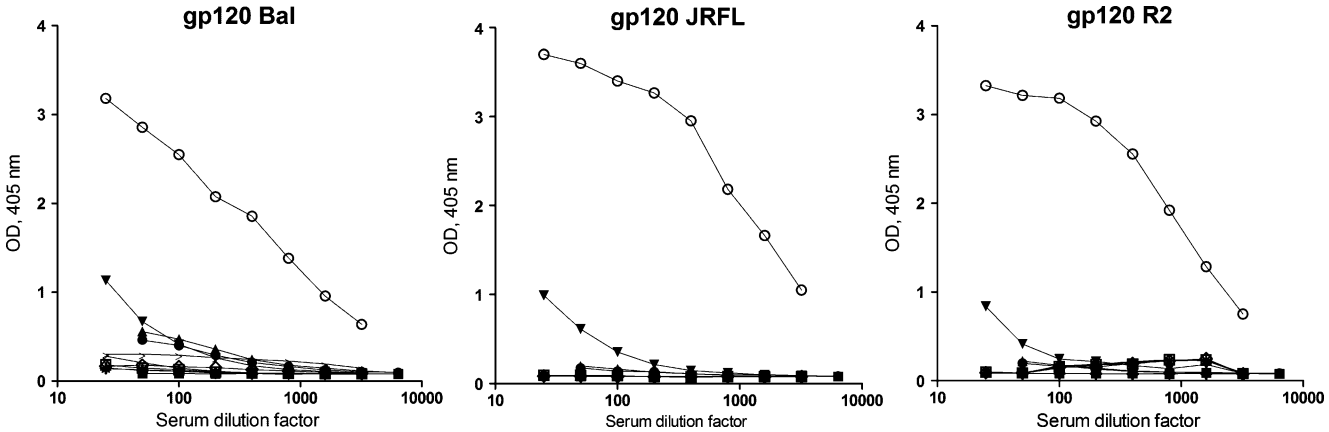

Fig. 7 Binding of pooled animal antisera to HIV-1 gp120 glycoproteins. Rabbit antisera: PBS-MF59 (+), Man_9-CRM_{197} (□), Man_4-$PAMAM_4$-CRM_{197} (<), Man_9PAMAM_4-CRM_{197} (>), Man_4PAMAM_8-CRM_{197} (◊) and Man_9PAMAM_8-CRM_{197} (*); mice antisera: preimmune (■), Man_4PAMAM_4-CRM_{197} (▲), Man_9PAMAM_4-CRM_{197} (●); GNL (○) and 2G12 (▼). GNL and 2G12 have 2 and 10 μg/mL at first graph point, respectively; two-fold dilution scheme was applied

glycan. In a recent study nine- and 27-valent oligomannose dendrimers showed similar affinity and inhibition capacity in binding 2G12 to gp120 [15], suggesting that rising cluster valence above nine moieties would not necessarily lead to further increase in the 2G12 affinity.

Here we report the first *in vivo* study with glycoconjugates containing four- and eight-valent high-mannose oligosaccharide-PAMAM dendrons. Our glycoconjugates consisted of the HIV-1 related carbohydrate antigens clustered onto the PAMAM dendrons and subsequently conjugated to CRM_{197}, which is well known for its excellent properties as carrier for bacterial oligo- and polysaccharides and is widely used in licensed glycoconjugate vaccines [22, 28, 34–36]. The antigens were formulated with the potent MF59 adjuvant, which was shown to be effective in boosting both cellular and humoral immune response and is commonly used for seasonal flu vaccination

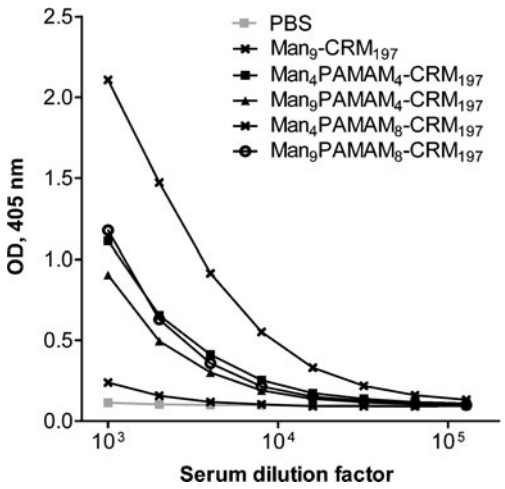

Fig. 8 Binding of pooled rabbit antisera to CRM_{197}

[24–26]. The combination of the above-mentioned factors was designed to confer to the glycoantigens improved immunogenic features.

The oligomannoses for the development of our glycoantigens were chosen on the basis of biochemical, biophysical and crystallographic evidences available in the literature [6, 8, 10, 15, 37, 38]. Man_4, Man_6 and Man_9 candidates possessed terminal Manα1-2-Man units that were shown to be essential for 2G12 recognition [8, 9]. Although we did expect that all three oligosaccharide candidates would demonstrate 2G12 reactivity, Man_6 antigen showed low potency as inhibitor compared to Man_4 and Man_9 oligosaccharides in SPR studies. This may indicate that trisaccharide α-D-Man-(1-2)-α-D-Man-(1-2)-α-D-Man, present in both Man_4 and Man_9, is required for the affinity interaction. This observation is in line with the structural requirements of ligand binding for the high-mannose-specific lectin cyanovirin-N [31]. 2G12 recognition of Man_6 observed in the glycoarray studies indicates that this oligosaccharide benefits from dense multivalent display on the surface of microarray slide [10]. Nevertheless, its structural features might not be sufficient to provide enough 2G12 affinity in case of a lower density carbohydrate presentation.

Having this information in our hands, we focused only on Man_4 and Man_9 antigens. The preparation of high-mannose glycodendrons was based on the activation of the amino groups present in the oligosaccharide linkers with an excess of disuccinimidyl adipate, followed by the reaction of activated oligosaccharides with t-Boc-protected PAMAM and subsequent purification with C4 hydrophobic interaction cartridge. The effect of oligosaccharide multivalent presentation was evidenced by the enhancement of glycodendrons capacity to inhibit 2G12-gp140 interaction (Fig. 3). After hydrolysis of the t-Boc

group and activation of the amino function again with disuccinimidyl adipate, the clusters with four and eight oligomannose antennae were conjugated to CRM_{197}. As a result we were able to synthesize glycoconjugates with 39–58% carbohydrate content, which is significantly higher than previous studies reporting a 15–19% range (elaborated from ref. [16–18]).

Immunization of rabbits and mice with MF59-formulated CRM_{197} glycoconjugates of Man_4 and Man_9 antigens induced specific anti-Man_9 antibodies (Fig. 5). Man_9-conjugates induced stronger response and higher affinity antibodies (Fig. 6) as compared to the Man_4-conjugates, which can be easily explained considering the structural differences between Man_4 and Man_9. However, neither the four- nor the eight-valent flexible PAMAM dendrons antigens induced gp120 cross-reactive antibodies (Fig. 7), indicating that the presentation of oligomannose sugars was not sufficient to mimic the native carbohydrate epitopes. Previous studies conducted with glycoconjugates prepared from high-mannose oligosaccharides clustered onto scaffolds and then linked to different carriers, such as BSA, OMPC or tetanus toxoid T-helper peptide, have also failed to induce antibodies cross reactive with HIV-1 gp120 [16–18]. The cluster approach was used also for synthetic *Candida albicans* β-mannan epitopes, but did not result in a superior immunogenicity as compared to the non-clustered control [39].

In the present work the different model based on the use of PAMAM dendrons as a way to display the HIV-1 related oligomannoses in a clustered form on the surface of CRM_{197} confirms the difficulties in the identification of a suitable carbohydrate-based anti-HIV vaccine candidate. The failure of high-mannose-PAMAM-CRM_{197} conjugates to provide antibodies with affinity for gp120 could be explained with inappropriate spacing of oligomannose antennae in the synthesized clusters, too much flexibility introduced by the presence of two subsequent six-carbon spacer chains between the oligomannoses and the PAMAM core, or too wide separation among the cluster molecules on the carrier protein surface. These issues should be definitively addressed in the future work for a synthetic anti-HIV vaccine.

Recently reported data on the rabbit "2G12"-like serum response to immunization with Man_8-reach mutant yeast cells suggests that the oligomannose density exposure is likely to be one of the dominating factors for designing HIV glycoantigens [40–42]. This outcome, which might be due to the abundant high-mannose glycosylation of yeast proteins comprising approximately 100% of protein weight (elaborated from ref. [40] and [42]), suggests that finding the right density and exhibition of oligomannose surface could be key for the search of best mimics of the native 2G12 epitope.

Experimental section

Materials

Synthetic high-mannose oligosaccharides, equipped with an amino linker, were purchased from Ancora Farmaceuticals (MA, USA); their characterization data can be found in online resource 4. $PAMAM_4$ and $PAMAM_8$ were kindly provided by Professor G. Catelani (University of Pisa, Italy). CRM_{197} were internally produced in Novartis V&D, Siena, Italy. The 2G12 antibody, HIV protein gp140 UG37 (clade A strain 92/UG/037, a.a. 32–662, NCBI protein database No. AAC97548, catalog no. ENV001) was purchased from Polymun Scientific (Vienna, Austria). HIV gp120 Bal (a.a.32–518, GenBank No. M68893, catalog no. IT-001-002p), gp120 R2 (a.a.41–520, GenBank No. AF128126, catalog no. IT-001-0029p), and gp120 JRFL (a.a.34–518, GenBank No. U63632, catalog no. IT-001-0024p) were purchased from Immune Technology Corp. (New York, US). Polymun and Immune Technology recombinant proteins are expressed in CHO and 293T cells, respectively. Biotinylated Galantus Nivalis Lectin (GNL) was purchased from Vector Laboratories, CA, US.

Analytical methods

Total saccharide concentration was determined by HPAEC-PAD analysis (ICS-3000 Dionex system). Briefly, oligomannose carbohydrate preparation was hydrolyzed in 2 M trifluoracetic acid for 2 h at 100°C, dried and then dissolved in water. 20 μL of sample were injected into CarboPac PA1 analytical column (250 mm×4 mm i.d., Dionex) with CarboPac PA1 guard column (50 mm×4 mm i.d., Dionex). Isocratic separations were performed using a 30-min 16 mM NaOH followed by a 5-min 500 mM NaOH regeneration step and 15-min re-equilibration, set to a flow rate of 1.0 $mLmin^{-1}$. Monosaccharide peaks were detected directly by using quadruple-potential waveform pulsed amperometry on a gold working electrode and an Ag/AgCl reference electrode. Raw data were elaborated on a Chromeleon 6.8 chromatography software (Dionex) with application of 0.5–10 μg/mL mannose calibration curve. Rapid hexose quantification was performed by Phenol-H_2SO_4 method [43]. Protein concentration was determined by Micro BCA kit (Thermo Fisher Scientific). Sodium-dodecylsulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed using NuPAGE® 4–12% Bis Tris pre-cast gels (Invitrogen).

ESI Q-TOF MS analyses

Analyses by direct sample injection were performed in a Micromass Q-Tof Micro system (Waters MS Technologies,

UK) diluting the samples 1:200 (v/v) or less in 0.1% formic acid, 1:1 (v/v) acetonitrile:water. For LC-Mass analyses the Q-tof Micro system was coupled to an UPLC system (ACQUITY UPLC System, Waters, UK). Chromatographic separations of samples diluted in water were performed on 2.1 mm i.d.×50 mm ACQUITY BEH C18 1.7 μm column (Waters Corp., USA). Elution was performed with a linear gradient of 2–50% B for 8 min, then 50–100% B for 1.5 min, reconditioned 2% B for 2 min each cycle, where A = water with 0.1% formic acid and B = acetonitrile with 0.1% formic acid. Each cycle duration was 13 min at a flow rate 0.4 mL/min. 10 μL aliquots of sample were loaded. HPLC peak detection was performed by total ion current and best peak intensity measurement.

TOF MS analysis was performed operating in positive ion mode (ESI). The nebulization gas was set to 800 L/h at a temperature of 250°C, the cone gas set to 50 L/h, and the source temperature set to 100°C. The capillary and cone voltages were 3,500 V and 30 V, respectively. The Q-Tof Micro was operated with collision energy of 5 V. The data acquisition rate was set to 0.1 s with a 0.1 s inter-scan delay. The raw data were analyzed by the Micromass MassLynx applications manager Version 1.0, using Max-ent3 for deconvolution (Waters, UK). The general strategy for assigning peaks to glycodendrimers involved: 1) identification of a pair or series of ions in the spectra separated by the mass of a mannose saccharide (162 Da); and, 2) assigning individual peaks of these distributions.

NMR analyses

All the lyophilized samples of Man_4PAMAM_4, Man_9PAMAM_4, Man_4PAMAM_8 and Man_9PAMAM_8 were dissolved in deuterium oxide (Aldrich) and the solutions were inserted in 5-mm NMR tubes (Wilmad). Proton NMR experiments were recorded at 25°C on a Bruker Avance™ III 400 MHz spectrometer, using a 5 mm broadband probe (Bruker). The TOPSPIN™ 2.1 software package (Bruker) was used for data acquisition and processing. All the ^{1}H-NMR spectra were collected and standard one-pulse experiments, with 32 k data points, were collected over a 10 ppm spectral width. The transmitter was set at the HDO frequency, which was also used as reference signal (4.79 ppm).

PAMAM cluster synthesis and purification

In a typical experiment Man_4, Man_6 or Man_9 synthetic oligosaccharide with a six-carbon amino linker at the reducing end (20 μmol) were treated with disuccinimidyl adipate (200 μmol) in 0.3 mL DMSO containing 43 μmol of triethylamine. After 2 h of vigorous stirring the activation of sugar was checked by TLC performed on aluminium plates coated with silica gel 60 Å F_{254} (Merck) with detection by charring with 10% ethanolic H_2SO_4. The activated oligosaccharide was purified by precipitation in nine volumes of ethylacetate; the pellet obtained by centrifugation was washed twice with 1 mL of ethylacetate and vacuum dried.

The succinimidyl-activated oligosaccharides were then coupled to $PAMAM_4$ and $PAMAM_8$ with stoichiometry of 8:1 and 20:1 mol/mol, respectively. The reaction was carried out in 0.1 mL DMSO containing 20 μL/mL triethylamine at RT. Cluster formation was monitored by HPLC-ESI MS analysis and in some cases a further addition of activated oligosaccharide was performed in order to maximize the formation of the desired cluster. The reaction mixture was then lyophilized and dissolved in water. The excess of unreacted oligosaccharide was removed by hydrophobic interaction on a C4 column (0.5 mL resin, Bioselect, Grace Vydac) activated with methanol and preconditioned with water and eluted with a stepwise gradient of methanol (0–80% in water). Fractions of 2 mL were analyzed by TLC and ESI Q-TOF MS; and those containing fully substituted PAMAM were dried to remove methanol.

$PAMAM_4$-Boc ESI MS *m/z* ($C_{37}H_{76}N_{14}O_8$): found 845.56 ($(M+H)^+$, calc. 845.60), 423.27 ($(M+2H)^{2+}$, calc. 423.31). *$PAMAM_8$-Boc* ESI MS *m/z* ($C_{37}H_{76}N_{14}O_8$): found 879.70 ($(M+2H)^{2+}$, calc. 879.62), deconv. 1,758.37 ($(M+H)^+$, calc. 1,758.24). *Man_4PAMAM_4-Boc* ESI MS *m/z* ($C_{181}H_{320}N_{18}O_{100}$): found 1,450.48 ($(M+3H)^{3+}$, calc. 1,450.54), 1,458.80 ($(M+3H+Na)^{3+}$, calc. 1,457.87), 1,396.45 ($(M+3H\text{-}Man)^{3+}$,calc. 1,396.49), deconv. 4,347.36 ($(M+H)^+$, calc. 4,347.06), 4,369.24 ($(M+Na)^+$, calc. 4,369.04), 4,185.32 ($(M+H\text{-}Man)^+$, calc. 4,185.01). *Man_9PAMAM_4-Boc* ESI MS *m/z* ($C_{301}H_{520}N_{18}O_{200}$): found 1,898.62 ($(M+4H)^{4+}$, calc. 1,898.87), 1,904.21 ($(M+3H+Na)^{4+}$, calc. 1,904.36), 1,858.09 ($(M+4H\text{-}Man)^{4+}$,calc. 1,858.33), deconv. 7,587.53 ($(M+H)^+$, calc. 7,588.12), 7,610.38 ($(M+Na)^+$, calc. 7,610.10), 7,425.80 ($(M+H\text{-}Man)^+$, calc. 7,426.06). *Man_4PAMAM_8-Boc* HPLC t_R= 2.728 min; ESI MS *m/z* ($C_{365}H_{644}N_{38}O_{200}$): found 1,754.03 ($(M+5H)^{5+}$, calc. 1,754.06), 1,721.46 ($(M+5H\text{-}Man)^{5+}$, calc. 1,721.63), 1,689.18 ($(M+5H\text{-}2\ Man)^{5+}$, calc. 1,689.20), 2,192.33 ($(M+4H)^{4+}$, calc. 2,192.32), 2,152.64 ($(M+4H\text{-}Man)^{4+}$, calc. 2,151.79), 2,111.22 ($(M+4H\text{-}2Man)^{4+}$, calc. 2,111.25), deconv. 8,761.17 ($(M+H)^+$, calc. 8,761.15), 8,599.08 ($(M+H\text{-}Man)^+$, calc. 8,599.09), 8,437.02 ($(M+H\text{-}2\ Man)^+$, calc. 8,437.04). *Man_9PAMAM_8-Boc* HPLC t_R=2.172 min; ESI MS *m/z* ($C_{605}H_{1044}N_{38}O_{400}$): found 2,179.85 ($(M+7H)^{7+}$, calc. 2,179.72), 2,156.65 ($(M+7H\text{-}Man)^{7+}$, calc. 2,156.55), 2,133.57 ($(M+7H\text{-}2\ Man)^{7+}$, calc. 2,133.39), 2,542.94 ($(M+6H)^{6+}$, calc. 2,542.83), 2,515.60 ($(M+6H\text{-}Man)^{6+}$, calc. 2,515.81), 2,488.67 ($(M+6H\text{-}2Man)^{6+}$, calc. 2,488.79), deconv. 15,240.77 ($(M+H)^+$,

calc. 15,243.26), 15,079.57 ($(M+H\text{-}Man)^+$, calc. 15,081.21), 14,918.34 ($(M+H\text{-}2\ Man)^+$, calc. 14,919.15).

Competitive surface plasmon resonance

The experiments were carried out with a BiaCore X100 system in a HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 0.005% surfactant Tween 20, pH 7.4). For coupling two flow cells of a CM5 chip (GE Healthcare) were activated by injection of EDC/NHS mixture for 7 min at 10 μL/min, followed by injection of 1 μg/mL gp140 UG37 in sodium acetate pH 4.5 over the channel two until the target level was reached; both were then blocked with 1.0 M ethanolamine pH 8.5 for 7 min at 10 μL/min. Final immobilization level of gp140 was 550 RU. 2G12 solution with and without carbohydrate inhibitors was injected over both channels, and the binding profile was obtained by subtraction of the blank signal in channel one from the gp140 UG37 signal in channel two. 2 μg/mL 2G12 was incubated with 0–1,300 μM carbohydrate inhibitor for 15 min at 37°C before the analysis. Analyte was injected at 5 μL/min for 800 sec, followed by 500 sec dissociation and 50 sec of regeneration with 10 mM glycine, 3 M NaCl pH 2.0. Sensorgrams were elaborated on the Biacore X100 software package. Inhibition percentage was calculated as $(B_{no\ inhibitor}-B_{inhibitor})*100/B_{no\ inhibitor}$, where B is the binding level with reference subtraction (buffer sample). The binding level corresponds to the RU signal at 780 sec, 20 s before the end of sample injection. Fitting of inhibition curves and calculation of IC50 values was performed on the Graphpad Prism software using variable slope model (Graphpad Prism Inc.).

Conjugation of oligomannose and oligomannose glycodendrimers to CRM_{197}

t-Boc protecting groups in glycodendrimers were cleaved by reaction in 20% trifluoracetic acid (TFA) for 2 h at RT. The removal of t-Boc was verified by MS analysis, and the samples were extensively dried under vacuum to remove TFA. Monovalent oligosaccharides or deprotected glycodendrimers were then activated with disuccinimidyl adipate and purified by ethylacetate precipitation as reported above. The activated oligosaccharides were then conjugated in 200 mM sodium phosphate pH 7.2 to CRM_{197} (10–20 mg/mL) with a stoichiometry of 30:1 or 40:1 glycodendrimer:protein (mol/mol). After overnight incubation at 37°C, conjugates were then purified from the excess of unconjugated carbohydrate using ultrafiltration spin columns with 30 kDa or 50 kDa cut-off (Vivaspin, Sartorius). The purified glycoconjugates were analyzed for their protein and carbohydrate content and by SDS-PAGE.

Conjugation of Man_9 to HSA via diethyl squarate chemistry The synthetic oligosaccharide (20 mmol) was treated with 3,4-diethoxy-3-cyclobuten-1,2-dione (150 mmol) in 0.1 mL 1:1 vol ethanol:100 mM sodium phosphate pH 7.0. After overnight incubation with vigorous stirring activation of sugar was checked by TLC. The excess of linker was removed by hydrophobic interaction C18 column (C18-E, Strata, Phenomenex) after 3 CV water and 3 CV ethylacetate washing steps with final methanol elution. Target fractions were dried to remove methanol. The activated oligosaccharides were conjugated in 200 mM sodium borate pH 9.2 to CRM_{197} (10–20 mg/mL) with a stoichiometry of 30:1 (mol/mol). Purification and characterization was performed as described above. This glycoconjugate has been used in ELISA as coating reagent for anti Man_9 antibodies determination.

Animal immunizations

Animal experimental guidelines set forth by the Novartis Animal Care Department were followed in all animal studies performed. Groups of 2–4 female white Zealand rabbits (2 kg weight) were immunized on days 1, 21 and 35 with 5 or 20 μg carbohydrate antigens or with PBS both formulated 1:1 (v/v) with MF59 and delivered in a final volume of 250 μL, intramuscularly into both quadriceps. Sera were collected on days 20, 34 and 42.

Groups of 8 female Balb/c mice were immunized on days 1, 14 and 28 with 1 μg carbohydrate antigens or PBS both formulated with MF59 and delivered in a volume of 150 μL by subcutaneous injection. Sera were collected on day 0, 27 and 42.

ELISA

a) Determination of anti Man_9-specific antibodies. 96-well Maxisorp plates (Nunc, Thermo Fisher Scientific) were coated with 100 μL/well of a 1 μg/mL solution of Man_9-squarate-HSA in PBS. Plates were incubated overnight at +4°C, then washed three times with TPBS (PBS with 0,05% Tween 20, pH 7.4) and blocked with 100 μL/well of 2% BSA (Sigma-Aldrich) for 1 h at 37°C. Subsequently, each incubation step was followed by triple TPBS wash. Sera, prediluted 1:25–1:1,000 in 2% BSA-TPBS, were transferred into coated- plates (200 μL) and then serially two-fold diluted followed by 2 h incubation at 37°C. Then 100 μL/well of 1:10,000–1:20,000 diluted appropriate alkaline phosphatase-conjugated secondary antibody (Sigma Aldrich) were added and plates incubated for 1 h at 37°C. Then, 100 μL/well of 1 mg/mL pNPP disodium hexahydrate (Sigma Aldrich) in 1 M diethanolamine (pH 9.8) was distributed onto plates. After 30 min of development at

RT plates were read at 405 nm with a microplate spectrophotometer. Antibody titers were defined as the reciprocal of those dilutions that gave an optical density (OD) three times higher than the average OD of preimmune or mock-immunized sera. *b) Competitive ELISA.* Based on the previous ELISA assay an appropriate dilution of each rabbit antiserum pool was chosen in order to give an approximate absorbance of 1.0. Prediluted antisera were incubated with varying concentrations of Man_9 in a final volume of 100 μL. For the rest, the protocol was followed as described above. Inhibition percentage was calculated as difference in maximal binding according to the equation

$(OD_{no\ inhibitor}-OD_{inhibitor})*100/OD_{no\ inhibitor}$, where $OD_{no\ inhibitor}$ is a mean of four replicates. Each OD value was subtracted by the value of background OD (sample without antisera). Inhibition curve fitting and IC_{50} calculation was performed as described above for the competitive SPR assay. *c) Evaluation of anti HIV-1 gp120 and anti-CRM specific antibodies.* In order to evaluate the ability of rabbit or mouse immune sera to recognize HIV-1 gp120 glycoproteins or CRM_{197}, the ELISA protocol described above has been repeated but using 100 ng/well coating of three different HIV-1 clades Bal, JRLF and R2, and CRM_{197}.

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