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A nonself sugar mimic of the HIV glycan shield shows enhanced antigenicity

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Antibody 2G12 uniquely neutralizes a broad range of HIV-1 isolates by binding the high-mannose glycans on the HIV-1 surface glycoprotein, gp120. Antigens that resemble these natural epitopes of 2G12 would be highly desirable components for an HIV-1 vaccine. However, host-produced (self)-carbohydrate motifs have been unsuccessful so far at eliciting 2G12-like antibodies that cross-react with gp120. Based on the surprising observation that 2G12 binds nonproteinaceous monosaccharide D-fructose with higher affinity than D-mannose, we show here that a designed set of nonself, synthetic monosaccharides are potent antigens. When introduced to the terminus of the D1 arm of protein glycans recognized by 2G12, their antigenicity is significantly enhanced. Logical variation of these unnatural sugars pinpointed key modifications, and the molecular basis of this increased antigenicity was elucidated using high-resolution crystallographic analyses. Virus-like particle protein conjugates containing such nonself glycans are bound more tightly by 2G12. As immunogens they elicit higher titers of antibodies than those immunogenic conjugates containing the self D1 glycan motif. These antibodies generated from nonself immunogens also cross-react with this self motif, which is found in the glycan shield, when it is presented in a range of different conjugates and glycans. However, these antibodies did not bind this glycan motif when present on gp120.

2G12 | gp120 | oligosaccharide | glycoconjugate | glycomimetic

HIV-1 is thought to have infected up to 60 million people since its discovery over 20 years ago (1). Of those infected, more than 20 million have died, with the vast majority of individuals affected being from developing countries (1). An effective vaccine is, therefore, paramount to combat the epidemic. The HIV-1 envelope spike, critical for viral infectivity, consists of a compact, unstable trimer of the glycoproteins gp120 and gp41 (Fig. 1*A*) and is a key target for design of an antibody-based HIV-1 vaccine. The envelope spike undergoes rapid evolution in each individual patient, resulting in enormous sequence heterogeneity among individual isolates of HIV-1 (2). Moreover, neutralization-sensitive epitopes on gp120 and gp41 are either difficult to access or shielded from recognition by the immune system by an extensive display of host-derived *N*-glycans (3, 4). Nevertheless, a small group of rare, broadly neutralizing antibodies (b12, 2G12, 2F5, 4E10, Z13) against gp120 and gp41 have been previously isolated from HIV-1-infected patients that provide protection against viral challenge in animal models (5–9) as well as the more recent discovery of new highly potent human antibodies (PG9, PG16) (10). Structural analyses have revealed how they broadly neutralize HIV-1 and the mechanism by which the virus normally evades detection by the immune system (8, 11–16). Identification of antigens that could generate similar types of broadly neutralizing antibodies is, therefore, an important step in the development of an HIV-1 vaccine.

From this small group, broadly neutralizing antibody 2G12 is uniquely capable of recognizing sugars on the immunologically "silent" carbohydrate face of gp120 (part of HIV's glycan shield) and escaping immune tolerance (11, 17, 18). Carbohydrates are typically considered poor immunogens. One major limitation to the recognition and, hence, immunogenicity of carbohydrate structures is their exhibition of microheterogeneity. A single protein may display many variations of carbohydrate structure (glycoforms) (19) that can result in a polyclonal and reduced antigenic response (20). Furthermore, viruses typically rely on host glycosylation machinery; their glycosylation patterns are, therefore, inevitably similar to that of the host. As a result, host immune mechanisms should normally recognize such sugars as self and display tolerance, thus not eliciting antibodies to host-derived sugars. Carbohydrate–protein interactions tend also to be weaker than protein–protein interactions (μM rather that nM) (21, 22) further reducing affinities of antibodies to glycans. Large glycan structures can also shield underlying peptide epitopes, thereby further reducing the effectiveness of the immunogenic response (4).

It is therefore remarkable that, in spite of the expected poor immunogenicity of carbohydrate epitopes, 2G12 recognizes a conserved cluster of oligomannose residues on the silent face of gp120 with high affinity (Fig. 1*A* and *B*) (11). The crystal structure of 2G12 (11) revealed a unique domain-exchanged dimeric structure; the variable domains of the heavy chains (V_H) swap over to form an extended binding surface that includes a novel $V_H/V_{H'}$ interface in addition to two conventional V_H/V_L combining sites (11), and that greatly enhances the carbohydrate-antibody affinity to the nanomolar range (11). Thus, it is the cluster of high-mannose sugars on gp120 (Fig. 1*A*) that creates novel epitopes that are recognized as foreign by the immune system and are the ultimate target of the domain-exchanged 2G12. Structural studies (11, 23), binding assays (11, 17, 18, 23, 24), enzymatic hydrolysis (17), and mutational analysis (17) have highlighted terminal mannosides and, in particular, the D1 arm (Fig. 1*B*) as key binding motifs for 2G12. This unique binding solution reveals a potential vaccine strategy that may be accessible by

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Data deposition: The coordinates and structure factors for 2G12/D-fructose, 2G12/C-6 methyl monosaccharide **10**, and 2G12/C-6′′′ methyl tetrasaccharide **5** crystal structures have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 3OAY, 3OAZ, and 3OB0, respectively).

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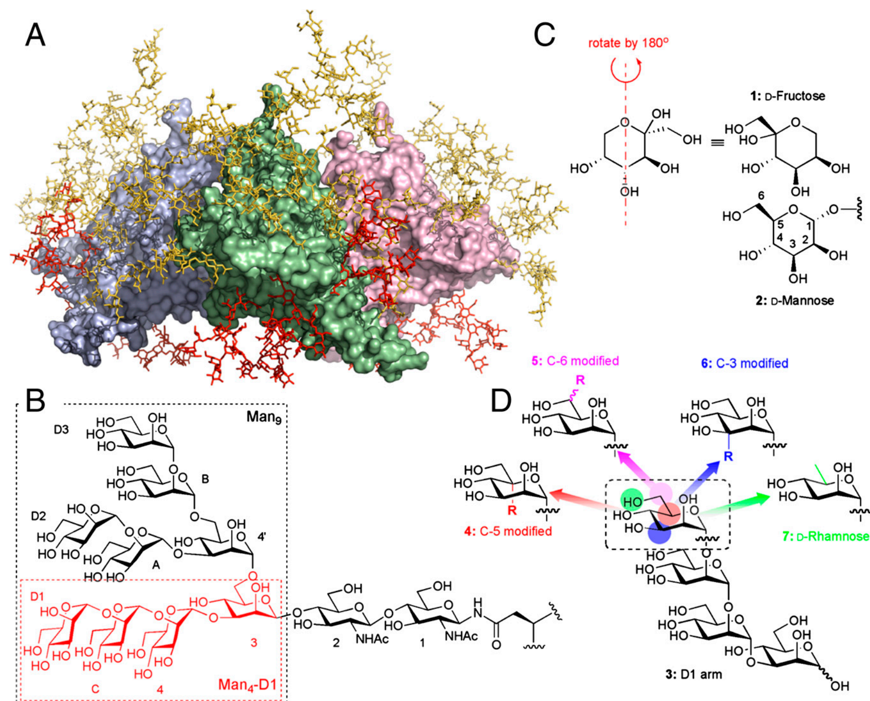


Fig. 1. Carbohydrate epitopes on gp120 and mannose mimics. (*A*) Model of the gp120 component of the trimeric HIV-1 envelope spike based on the structure of core gp120, (51) with three gp120 monomers shown in purple, green, and pink. Carbohydrate chains are shown in yellow. The high-mannose cluster, corresponding to glycans attached to Asn295, Asn332, Asn339, Asn386, and Asn392, which is recognized by 2G12, is represented in red. Figure was made using PyMOL (52). (*B*) 2G12 high-mannose carbohydrate epitope. (*C*) D-fructose as a 1-deoxy-5-hydroxy analogue of D-mannose. (*D*) Proposed nonself, D1-arm ligands (R = alkyl).

targeting these conserved oligomannose clusters on gp120 as antigens, and that may be probed by using 2G12 as a recognition template. The potential therapeutic value of the 2G12 epitope as an attractive HIV-1 vaccine target has recently been highlighted by the finding that 2G12 can protect macaques against HIV-1 challenge at remarkably lower serum neutralizing titers than most other broadly neutralizing antibodies (25).

To date, the use of glycans containing the natural sugar D-mannose or variants in a number of elegantly designed oligosaccharide constructs have failed to elicit 2G12-like antibodies (26–33). The antibodies generated fail to cross-react with gp120 (26, 30) and may reflect an inability to break tolerance for these host-generated self sugars. Moreover, syntheses of fragments (or modified fragments) of the high-mannose glycans found on gp120 have failed to reveal a monovalent ligand that binds more tightly to 2G12 than the D1 arm (Man_4) or $Man_{8–9}GlcNAc_2$ (Fig. 1*B*); indeed, modifications or truncations have created weaker binders (26–33). Elegant studies using a three-component conjugate have shown that reactivity toward cancer antigens containing self-carbohydrates can be enhanced by attaching them to scaffolds containing Toll-like receptor and related ligands that increase antigen-presenting cell uptake and modulate cytokine responses (34). However, the generation of antigens in which the carbohydrate epitope itself is successfully engineered to be both nonself and more antigenic has not heretofore been described.

We sought to address these challenges by adopting a design and synthesis strategy inspired by the prior unexpected observation that, from a panel of monosaccharides, D-fructose (**1**) binds most strongly, some 9 times greater than D-mannose (**2**) itself, to 2G12 (11). The keto-sugar fructose is absent from protein glycans, yet when displayed in its pyranose form and rotated by 180° it strongly resembles D-mannose, differing only in anomeric and C-5 substituents (Fig. 1*C*). This enhanced binding affinity for an unusual sugar not found in protein epitopes suggested that a hybrid nonself sugar could be designed, based on the structure of D-fructose, that might engage additional interactions (binding to additional residues perhaps by virtue of the additional substitution found in D-fructose in proximity to C-5 compared to D-mannose) in the 2G12 binding site.

We report here the synthesis and insertion of unnatural sugars into oligomannose constructs to break tolerance and create nonself glycans with high affinity to 2G12. We identify a unique nonself D1 arm mimic that displays better inhibition of 2G12/gp120 binding than the natural D1 arm, both as an isolated sugar and in protein–sugar conjugates, and reveal the mechanism of inhibition of this most potent monovalent 2G12 ligand known. We also uncover the molecular mechanism by which D-fructose achieves better inhibition of 2G12/gp120 binding than D-mannose. We further show that this nonself glycan can elicit higher titers of antibodies that can react with the self glycan D1 arm motif, a motif that is found in the glycan shield of HIV. However, these antibodies do not neutralize HIV.

Results

Crystal Structure of Fab 2G12 in Complex with D-Fructose. To understand the molecular basis for the higher affinity of 2G12 for D-fructose over D-mannose (and Manα1-2Man) (11) and aid our design strategy, we determined the crystal structure of Fab 2G12 in complex with the nonself monosaccharide to 1.95 Å resolution (see *SI Appendix*, *SI Materials and Methods* and Table S1). The electron density for D-fructose at the two primary combining sites of the domain-exchanged Fab dimer is excellent and readily interpretable and reveals D-fructose adopts a pyranose form that does, indeed, resemble D-mannopyranose in the 2G12 binding sites (Fig. 2*A*). The structure validates our previous postulate that the contacts formed by D-fructose with 2G12 would be similar to those made by the terminal mannoside in Manα1-2Man (11) (Fig. 2*A*). The C-5 hydroxyl in D-fructose forms an additional direct H bond with Ser^{H100A} O and four additional water-mediated H bonds with Ala^{H31} O, Leu^{H100} O, Ser^{H100A} O, and Tyr^{L94} Oη. These additional water-mediated interactions engaging the C-5 substituent appear to explain why D-fructose is a better inhibitor of 2G12/gp120 binding than D-mannose. Moreover, this enhanced network of H bonds is also likely to be responsible for the higher affinity of Fab 2G12 for D-fructose over Manα1-2Man because it also mimics the direct H bond between O3 in the next mannose of the disaccharide and Ala^{H31} O. A total of 173 Å^2 of molecular surface on Fab 2G12 and 155 Å^2 of molecular surface on D-fructose are buried in the complex, with 10 direct and 10 water-mediated H bonds and 42 van der Waals interactions in each antigen binding site. This crystal structure confirms that additional substituents around a term-

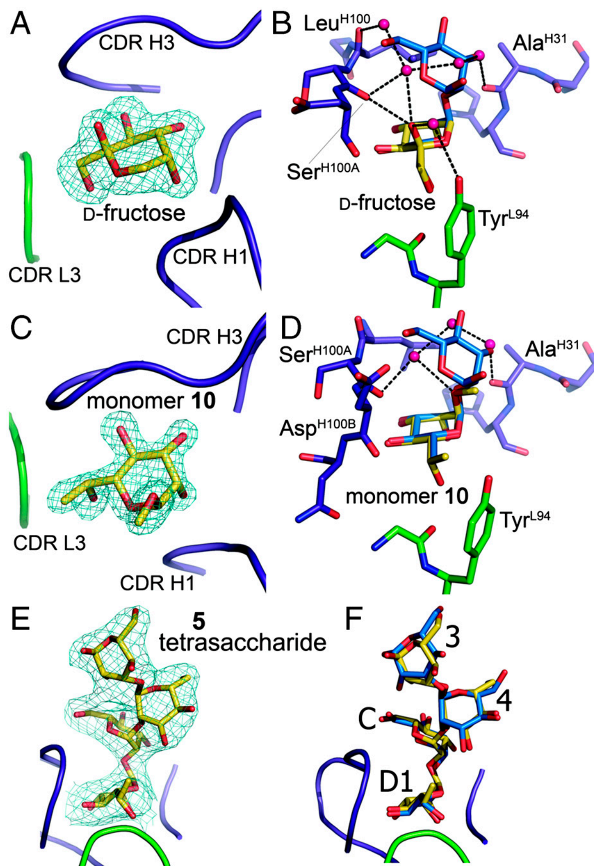


Fig. 2. Crystal structures of Fab 2G12 in complex with D-fructose, C-6 methyl monosaccharide **10** and C-6′′′ methyl tetrasaccharide **5**. (*A*) F_o–F_c simulated annealing omit map of D-fructose bound to Fab 2G12 contoured at 3σ. The light and heavy chains of Fab 2G12 are shown in green and purple, respectively, and the CDR loops are labeled. (*B*) Overlay of D-fructose (shown in yellow) with Manα1-2Man (shown in blue) from a previous Fab 2G12 structure (11). Additional direct and water-mediated H bonds (H bonds are shown as black dashed lines; waters are shown as pink spheres) in the Fab 2G12/D-fructose structure are shown. (*C*) F_o–F_c simulated annealing omit map of C-6 methyl monosaccharide **10** (shown in stick format) bound to Fab 2G12 contoured at 3σ. (*D*) Overlay of the modified monosaccharide (shown in yellow) with Manα1-2Man (shown in blue) (11). Additional water-mediated H bonds in the Fab 2G12/C-6 methyl monosaccharide **10** structure are shown. (*E*) F_o–F_c simulated annealing omit map of C-6′′′ methyl tetrasaccharide **5** bound to Fab 2G12 contoured at 2.5σ. (*F*) Overlay of the modified tetrasaccharide (shown in yellow) with the D1 arm of Man_7 (shown in blue) (23). The mannose residues are labeled. Figure was made using PyMOL (52).

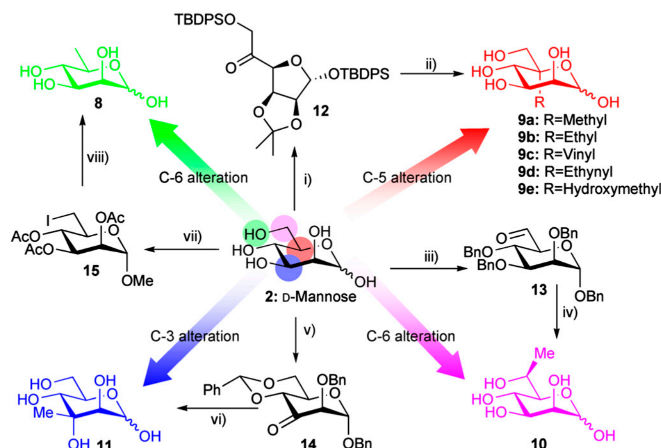
inal mannoside mimic (here D-fructose) could mediate beneficial interactions.

Modeling, Synthesis, and Affinity for 2G12 of Nonself Monosaccharides. Based on this understanding of the mechanism of enhanced D-fructose binding by 2G12, we sought to design 2G12 antigens that incorporated D-fructose-like, nonself monosaccharides. The D1-arm tetrasaccharide (**3**) was chosen as a scaffold on which to mount these nonself prototypes because, in its natural form, it shows near maximal interaction with 2G12 compared to other oligomannose fragments (23, 24).

To investigate to what extent such and other nonself substitutions on the D-mannose structure could be tolerated in the 2G12 binding site, we carried out docking studies of substitutions to D-mannose in complex with Fab 2G12. This modeling revealed that nonself substitutions not only at C-5 (as in D-fructopyranose), but also at C-3 and C-6 (see *SI Appendix*, Fig. S1), could be potentially tolerated by 2G12 and might enhance binding (11). Based on these results, a representative target panel of nonself oligosaccharides **3-7** containing substitutions and modifications at the C-3, C-5, and C-6 positions of the terminal mannoside was designed for synthesis (Fig. 1*D*).

As a first step, a larger panel of eight nonself, monosaccharide sugars was synthesized and evaluated prior to selection of the most potent for introduction onto the terminus of the D1 arm. Monosaccharides **8**, **9a-e**, **10**, and **11** (Scheme 1) were prepared from parent sugar D-mannose via an oxidation and addition procedure that allowed late-stage diversification for rapid substituent variation. Thus, carbonyl compounds **12**, **13**, and **14** were each regioselectively protected to allow selective access to OH-3, OH-6, and OH-5, respectively; this, in turn, allowed alteration and substituent introduction at C-3, C-6, and C-5. Oxidation under Swern conditions allowed mild preparation of these target carbonyl intermediates. Reaction of **12**, **13**, and **14** with appropriate organometallic nucleophiles followed by global deprotection gave **9a-e**, **10**, and **11**. These synthetic routes were high yielding and allowed ready introduction of varied substituents with high stereoselectivity (Scheme 1). Absolute configuration of stereochemistry in **10** was confirmed using nOe NMR analysis of a 4,6 benzylidene derivative; nOe was also used to confirm the configuration of C-3 derivative **11** (see *SI Appendix*).

A competitive ELISA assay was used to assess the ability of these nonself monosaccharides to inhibit binding of 2G12 to HIV-1 glycoprotein gp120 (Fig. 3). Of the fructose-like monomers with substitution at the C-5 position (**9a-e**), those with large substituent groups (vinyl **9c** and ethynyl **9d**) that are rotationally restricted by hybridization were found to have much higher IC_{50} values than D-mannose. These results suggested that larger *R* groups are not tolerated in the 2G12 binding site, an observation consistent with the modeling experiments (see *SI Appendix*). However, smaller and less-restricted substitutions at C-5 (compounds methyl **9a**, ethyl **9b**, and hydroxylmethyl **9e**) as well as C-3 methyl-modified **11** and 6-deoxy **8** structures were all found to have IC_{50} values similar to the natural sugar D-mannose. These results indicated that such designed substitutions were indeed tolerated in the 2G12 binding site. Excitingly, from this panel, C-6 methyl-substituted compound **10** emerged with an IC_{50} value not only more potent than D-mannose but even more



Scheme 1. Synthesis of nonself monosaccharides. Reagents and conditions: (*i*) 2,2-dimethoxypropane, *p*TsOH, DMF, 73%, then TBDPSCl, imidazole, DCM, 95%, then c.HCl, MeOH, 82%, then TBDPSCl, imidazole, DMF, 79%, then DMSO, $C_2O_2Cl_2$, DCM, −78 °C → RT, 97%; (*ii*) RMgBr, THF or Bu_3SnCH_2OPMB, BuLi, THF, 76%, then TBAF, THF, then TFA, H_2O; (*iii*) BnOH, AcOH, 60%, then TrtCl, DMAP, Pyr, 80 °C, 77%, then BnBr, NaH, DMF, 99%, then AcOH, ethanol, 80 °C, 90%, then DMSO, $C_2O_2Cl_2$, DCM, −78 °C → RT, 97%; (*iv*) MeMgBr, THF, then H_2, Pd/C, MeOH, 95%; (*v*) BnOH, AcOH, 60%, then dimethoxybenzaldehyde, *p*TsOH, DMF, 75 °C, 61%, then DIBAL-H, toluene, −40 °C, 76%, DMSO, Ac_2O; (*vi*) MeMgBr, THF, then H_2, Pd/C, MeOH; (*vii*) MeOH, AcCl, then I_2, PPh_3, imidazole, THF 65 °C, then Ac_2O, Pyr; (*viii*) H_2, Pd/C, NEt_3, MeOH, then AcOH, H_2SO_4, then NaOMe, MeOH.

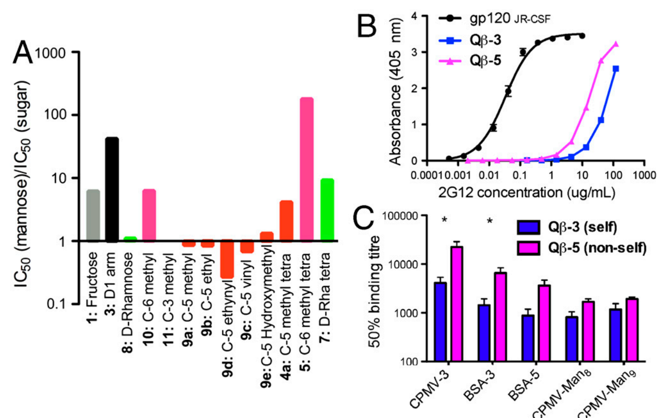
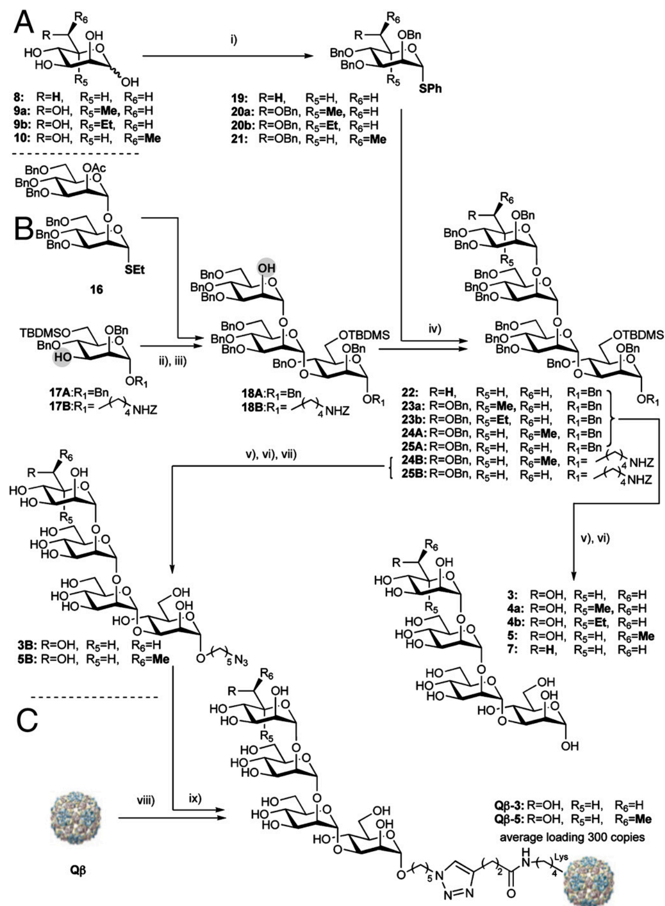


Fig. 3. (*A*) IC_{50} values of synthetic monosaccharides and tetrasaccharides compared to the IC_{50} of D-mannose monosaccharide in the binding of 2G12 to gp120. See *SI Appendix*. (*B*) ELISA assay of binding of 2G12 to Qβ glycoconjugates **Qβ-3** and **Qβ-5**. (*C*) Average 50% maximum binding titer of serum against oligomannose glycoconjugates elicited by immunization with **Qβ-3** and **Qβ-5** (measured by ELISA). For each group, $n = 4$. $* = p < 0.05$ in *t*-test.

potent than D-fructose. This compound is the most potent monosaccharide 2G12/gp120 inhibitor reported to date.

Synthesis and Affinity for 2G12 of Tetrasaccharide D1-Arm Mimics. Based on these encouraging results, key representative, nonself, monosaccharides were next incorporated at the terminus of the oligosaccharide D1 arm. A $1 + 2 + 1$ oligosaccharide assembly strategy was adopted that was intended not only to be high yielding and allow easy deprotection but to also allow late-stage introduction of diversity at both reducing and nonreducing termini (Scheme 2). The central Manα1-2Man disaccharide block **16** was synthesised prior to formation of the α1-3 linkage to give trisaccharide **18**. The terminal residues were added last (Scheme 2 *A* and *B*) using perbenzylated thiophenyl glycosyl donors **19**, **20a**, **20b**, and **21** and DMTST as an activating agent (35). These donors (**19**, **20a**, **20b**, and **21**) were prepared in four high yielding steps from the corresponding unprotected monomers **8**, **9a**, **9b**, and **10** (Scheme 2*A*). Following assembly, tetrasaccharides **22**, **23a**, **23b**, **24A**, and **25A** were readily deprotected in high yields under standard conditions to yield target D1-arm sugars **3**, **4a**, **4b**, **5**, and **7**.

All but one D1-arm sugar variant inhibited binding of gp120 to 2G12 (Fig. 3*A*). The least potent tetrasaccharide **4a** (modified at C-5′″ with a methyl substituent, where each substituent is denoted according to sugar residue from reducing to nonreducing terminus X, X′, X″, X′″) displayed an IC_{50} 10 times higher than the D1 arm **3** (4 times lower than that of monomeric D-mannose). Modeling data suggested that substitution at the C-5′″ position causes changes in the conformation of mannose ring C (*SI Appendix*, Fig. S2) thereby reducing interaction of this mannose ring with the 2G12 binding site (see *SI Appendix*). This was further supported by the result that tetrasaccharide **4b** modified at C-5′″ with a bulkier ethyl substituent showed no inhibition at the concentrations tested. Encouragingly, C-6′″ deoxygenated tetrasaccharide **7** had only a 4 times higher IC_{50} than the D1 arm (6 times lower than that of D-mannose) and suggested alterations at C-6′″ of the terminal mannose residue, such as removal of a hydroxyl group, were well tolerated by 2G12. This result was strikingly confirmed by C-6′″-methyl tetrasaccharide **5**, which not only binds better than the other nonself variants but has a more potent binding IC_{50} value than the D1 -arm **3** itself to 2G12 (over 4 times lower than the D1 arm). This is the only nonself D1-arm derivative to show better inhibition of 2G12/gp120 binding than the natural D1-arm and the most potent monovalent 2G12 ligand known.



Scheme 2. (*A*) Synthesis of nonself glycosyl donor building blocks. Reagents and conditions: (*i*) Ac_2O, Pyr then PhSH, $BF_3 \cdot OEt_2$, DCM then NaOMe, MeOH then NaH, BnBr, DMF. (*B*) Synthesis of nonself tetrasaccharides with (*B*) and without (*A*) linker group glycoconjugate synthesis. Reagents and conditions: (*ii*) DMTST, TTBP, DCM, 4 Å mol sieves, −78 °C → RT; (*iii*) NaOMe, MeOH: (*iv*) DMTST, TTBP, DCM, 4 Å mol sieves, −78 °C → RT; (*v*) AcOH, H_2O, 50 °C; (*vi*) Pd/C, H_2, MeOH,; (*vii*) TfN_3, NH_4HCO_3, $CuCl_2$, DCM, H_2O. (*C*) Glycoconjugate synthesis. Reagents and conditions: (*viii*) alkynyl *N*-hydroxysuccinimide ester ($HCC(CH_2)_2CO_2$-NHS), 15% DMSO, TRIS buffer pH 7.0; (*ix*) aminoguanidine, $CuSO_4$, tris(3-hydroxypropyltrazolylmethyl)amine, sodium ascorbate, PBS (0.1 M, pH 7.0).

Crystal Structures of Fab 2G12 in Complex with C-6 Methyl-Substituted Monosaccharide 10 and C-6′″-Methyl Tetrasaccharide 5. To elucidate the structural basis of the higher affinity of 2G12 for C-6′″-methyl-substituted tetrasaccharide **5** over the natural D1-arm **3**, we determined crystal structures of Fab 2G12 in complex with both C-6 methyl-substituted compound **10** and C-6′″-methyl tetrasaccharide **5** to 1.75 Å and 2.85 Å resolution, respectively (see *SI Appendix*, *SI Materials and Methods* and Table S1). In the former high-resolution structure, compound **10** is bound at the two primary combining sites of the Fab dimer with extremely well-defined electron density (Fig. 2*C*). As in the Fab 2G12/D-fructose structure, the contacts formed by the modified monosaccharide with 2G12 are similar to those made by the terminal mannose in Manα1-2Man (11) (Fig. 2*D*). However, the C-6 methyl group forms additional van der Waals interactions with the aromatic side chain of Tyr^{L94} and Asp^{H100B} O, which results in partial burial of the hydrophobic methyl group and appears to account for the enhanced affinity of 2G12 for compound **10** over D-mannose. Moreover, similar to the Fab 2G12/D-fructose structure, a water-mediated H-bond relay bridges the anomeric oxygen in compound **10** with Ala^{H31} O and Ser^{H100A} O and mimics the direct H bond between O3 in the reducing terminal mannose in Manα1-2Man disaccharide (11) and Ala^{H31} O and further

explains the stronger affinity of 2G12 for the modified monosaccharide over Manα1-2Man. A total of 197 $Å^2$ of molecular surface on Fab 2G12 and 182 $Å^2$ of molecular surface on C-6 methyl monosaccharide **10** are buried in the complex, with 9 direct and 9 water-mediated H-bonds and 61 van der Waals interactions in each antigen binding site.

Although the Fab 2G12/C-6‴ methyl tetrasaccharide **5** cocrystals were highly anisotropic and diffracted to modest resolution, the electron density for the entire modified tetrasaccharide is also well-defined at both primary combining sites (Fig. 2*E*). The tetrasaccharide is bound with an overall conformation similar to that of the D1 arm in Man_7, Man_8, and $Man_9GlcNAc_2$ in complexes with Fab 2G12 (11, 23) (Fig. 2*F*). The buried surface area is approximately 300 $Å^2$ for Fab 2G12 and 295 $Å^2$ for the nonself, D1-arm mimic **5**. Together, the Fab 2G12/C-6 methyl monosaccharide **10** and Fab 2G12/C-6‴ methyl tetrasaccharide **5** structures uncover the molecular basis for the higher affinity of 2G12 for C-6‴-methyl tetrasaccharide **5** over Man_4. That C-6‴-methyl tetrasaccharide **5** adopts the same overall conformation at the antigen binding sites of 2G12 as the D1 arm of $Man_9GlcNAc_2$ suggests that the interactions with the C-6‴ methyl group are the only difference between the mechanism of 2G12 binding to the modified tetrasaccharide and the D1 arm.

Synthesis of Glycoconjugates for Immunogenicity Studies. Having identified a nonself modification that showed enhanced 2G12 antigenicity, we next investigated whether this correlated with enhanced immunogenicity. Both C-6‴-methyl-tetrasaccharide **5** and D1-arm tetrasaccharide **3** were equipped with a five-carbon linker terminating with a reactive azide group (Scheme 2*B*). Reaction with alkyne-modified lysine residues on the surface of the virus-like particle Qβ using copper-catalyzed azide-alkyne chemistry (Scheme 2*C*) (36, 37) created glycan shield mimetics **Qβ-3** and **Qβ-5** that bound 2G12 with nanomolar affinity (Fig. 3*B*). Comparable glycan loadings (average ∼300 per particle) was confirmed by MALDI mass spectrometry (*SI Appendix*, Fig. S7*A*), which also showed slight differences in distribution of the sugars across the capsid subunits. The enhanced IC_{50} observed for isolated C-6‴-methyl-tetrasaccharide **5** over D1 arm also correlated with an enhancement in affinity of 2G12 for the glycoconjugate **Qβ-5** that contained **5** as compared to that bearing only D1 arm (**Qβ-3**).

Immunogenicity of Nonself Glycan 5. New Zealand white rabbits were immunized with a prime and three boost immunizations of either self conjugate **Qβ-3** or nonself **Qβ-5**, and the 50% maximum binding titers were measured in an ELISA format. In all cases, higher titers of mannose-reactive antibodies were generated from immunization with the nonself glycoconjugate **Qβ-5** compared to the self conjugate **Qβ-3** (Fig. 3*C*). The increased titers were, importantly, most significant for reactivity toward D1 arm (**3**) on two unrelated protein scaffolds, cowpea mosaic virus (**CPMV-3**) and bovine serum albumin (**BSA-3**), but were also observed for Man_8 and Man_9 glycans as well as **5** (Fig. 3*C*). Both immunogens showed a similar time course trend in D1-arm-specific antibody production (see *SI Appendix*). Only moderate reactivity was observed against CPMV particles displaying the linker and one mannose sugar (see *SI Appendix*) confirming specificity for the sugar moiety. Indeed, depletion of this linker reactivity did not result in significant decreases in D1-arm-specific antibodies. The ability of the nonself glycoconjugate **Qβ-5** to elicit HIV-reactive antibodies was assessed by measuring both binding to recombinant gp120 and the ability to neutralize HIV pseudovirus. This assay revealed that there was no binding by the antibodies to gp120 and no neutralization (Figs. S8 and S9).

Discussion

Our design and synthetic strategy has led to identification of nonself modifications to the D1 arm that show enhanced antigenicity to 2G12. Although nonself modifications to the terminus of the D1 arm are tolerated in the 2G12 binding site, it is clear that several mechanisms influence the antigenicity of these glycans for the 2G12 antibody. Small alterations at the C-3 and C-5 positions of D-mannose, as well as removal of the hydroxyl group at the C-6 position (Scheme 1, **8**, **9a**, **9b**, and **11**), can be tolerated in the 2G12 binding site to create binding affinity similar to that of D-mannose. Specific tolerance was also observed for the tetrasaccharides: **4a** (C-5 methyl) and **7** (C-6 deoxy) both show binding levels similar to that of D1-arm **3**. However, when the size of the alteration is increased and/or its conformations restricted by carbon hybridization (**9c** and **9d**), these modifications become much less tolerated in the 2G12 binding site leading to IC_{50} values much higher than D-mannose. For the tetrasaccharide **4b**, with the bulkier ethyl group at the C-5‴ position, there is no inhibition of 2G12/gp120 binding. Our modeling studies (see SI Appendix) suggest that large substituents at the C-5 position lead to alterations in the conformation of the mannose ring C (Fig. 1*B*) resulting in reduced interaction of this sugar with the 2G12 binding site.

Modifications that lead to enhancement of the inhibition of 2G12/gp120 binding arose from additional interactions of the nonself modification with the 2G12 binding site. This hypothesis is supported by the crystal structures of D-fructose and C-6 methyl monosaccharide **10**. In the case of D-fructose, the additional direct and water-mediated interactions between the C-5 hydroxyl and Ala^{H31} O, Leu^{H100} O and Ser^{H100A} O appear to explain why this sugar in its pyranose form is an inhibitor of 2G12/D-mannose binding. The additional van der Waals contacts between the designed C-6-methyl modification and the aromatic side chain of Tyr^{L94} appear to correlate with the higher affinity of 2G12 for the modified monosaccharide **10** over D-mannose and even D-fructose. The finding that 2G12 uses the same mode of recognition for the nonself C-6‴-modified tetrasaccharide **5** as for the natural D1 arm, apart from the beneficial additional interactions formed by the designed C-6‴-methyl group modification, led us to evaluate the immunogenicity of this antigen in vivo.

Conjugates that contain this nonself glycan elicited antibodies that react with the self glycan, suggesting that the additional methyl group, although enhancing antigenicity, does not dominate the specificity of the resulting antibody binding sites. It should be noted that previous examples of glycoconjugates designed as vaccines [containing, e.g., *S*-linked gangliosides (38) or *N*-propionylated polysialic acids (39)] have led to antibodies being generated that were highly complementary to the modification in preference to the unmodified sugar. Although here HIV-reactive antibodies were not elicited, higher titers of self-D1 arm (**3**) reactive antibodies were elicited by immunization with the nonself glycoconjugate compared to the self glycoconjugate; it also cannot be discounted that other differences such as loading distribution may play a role. One possible central factor is the need to also elicit domain-exchanged antibodies. Immunization is, therefore, potentially limited by the presentation mode of the glycan on conjugates (such as in Qβ particles), and alternative clustered presentations of this nonself glycan to better mimic the glycan shield of HIV are now under investigation. It is also important to note that given the uniqueness of 2G12 and its domain-exchanged structure, the as-yet unknown conditions required for such exchange might be difficult to achieve. Such a structure or similar might therefore only be formed very rarely in response to vaccine challenge.

In conclusion, based on the previous observation that D-fructose is a more potent inhibitor of 2G12 binding to gp120 than D-mannose (11), a series of nonself modifications have been introduced into the terminus of the D1 arm of high-mannose sugars that represents an unusual self-carbohydrate epitope in HIV-1 gp120. These modified sugars are not only well tolerated in

the 2G12 binding site but also enhance antigenicity. In particular, introduction of a methyl group at the C-6′″ position (in tetrasaccharide **5**) of the terminal mannose residue of the D1 arm enhances binding to 2G12 compared to the natural D1 arm found on gp120. Crystal structures of this modified sugar with 2G12 in comparison to structures of 2G12 with unmodified sugars (11, 23) and D-fructose enabled us to elucidate the mechanism of this enhanced affinity as well as the increased affinity of D-fructose for 2G12 compared to D-mannose. HIV-reactive antibodies were not elicited. The generation of HIV neutralizing activity or even gp120 binding activity from antibodies using synthetic conjugates as immunogens has, thus far, been elusive for the field in general. However, higher titers of self-D1 arm (**3**) reactive antibodies were elicited from the nonself glycan than the self. The exact mechanism of this enhanced immunogenicity is uncertain, yet the observed cross-reactivity between natural and unnatural glycans may have broader significance and potential use in other vaccine programs directed toward other (e.g., microbial or fungal) pathogens. We are currently investigating alternative presentations of these nonself D1-arm mimics to more effectively mimic the clustered oligomannose glycans of HIV in an attempt to elicit domain-exchanged 2G12-like antibodies or other neutralizing antibodies. When displayed in the "correct" format this nonself glycan could be an important component of a carbohydrate anti-HIV vaccine.

Materials and Methods

Chemical Synthesis. Details of the synthesis of all compounds can be found in the *SI Appendix*. Briefly, glycosylations typically used acceptor (1 equiv), thioglycoside donor (1.2 equiv), and dimethylthiosulfonium triflate (4 equiv) at −78 °C in dichloromethane.

Biological Assays. Details of all binding assays, immunization protocols, and titer determinations can be found in the *SI Appendix*.

Crystal Structure Determinations and Analyses. Full details can be found in the *SI Appendix*. Briefly, Fab 2G12 fragments were prepared as previously described (11) and concentrated to 20 mg/mL. For each complex, the solid sugar ligand was added to the Fab solution to saturation. Cocrystals were cryoprotected with glycerol or sodium malonate. Data collection and refinement statistics are summarized in *SI Appendix*, Table S1.

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