Metabolic labeling of HIV-1 envelope glycoprotein gp120 to elucidate the effect of gp120 glycosylation on antigen uptake

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The glycan shield on the envelope glycoprotein gp120 of HIV-1 has drawn immense attention as a vulnerable site for broadly neutralizing antibodies and for its significant impact on host adaptive immune response to HIV-1. Glycosylation sites and glycan composition/structure at each site on gp120 along with the interactions of gp120 glycan shield with broadly neutralizing antibodies have been extensively studied. However, a method for directly and selectively tracking gp120 glycans has been lacking. Here, we integrate metabolic labeling and click chemistry technology with recombinant gp120 expression to demonstrate that gp120 glycans could be specifically labeled and directly detected. Selective labeling of gp120 by N-azidoacetyllactosamine (ManNAz) and N-azidoacetylglactosamine (GalNAz) incorporation into the gp120 glycan shield was characterized by MS of tryptic glycopeptides. By using metabolically labeled gp120, we investigated the impact of gp120 glycosylation on its interaction with host cells and demonstrated that oligomannose enrichment and sialic acid deficiency drastically enhanced gp120 uptake by bone marrow–derived dendritic cells. Collectively, our data reveal an effective labeling and detection method for gp120, serving as a tool for functional characterization of the gp120 glycans and potentially other glycosylated proteins.

Glycosylation is among the most ubiquitous post-translational modifications in microbial and mammalian cells, participating in most physiological and pathological processes (1). In particular, glycan interactions with the immune system have important implications in human health and disease (2). One prominent example is the interaction of the HIV-1 envelope spike (i.e. three heterodimers of gp120–gp41 envelope glycoproteins) with the host protein CD4, a key step for viral entry, and infectivity of CD4+ T cells (3–5). gp120 has remarkably high levels of surface glycosylation with glycans contributing to nearly half of its molecular weight and gp120 recognition by broadly neutralizing antibodies (bNAb) (6). In the past decade, a wealth of information has illustrated that instead of being a shield avoiding immune responses, HIV-1 gp120 glycans contribute to the major “sites of vulnerability” targeted by bNAb (3, 4, 8). Moreover, differential glycosylation of gp120 is associated with its antigenicity (recognition by specific antibodies) (9), immunogenicity (antibody elicitation) (11, 12), and T cell recognition and response (13, 14). Therefore, it is critical to further understand immune interactions of gp120 glycans to gain insight into the impact of gp120 glycans in viral infectivity and immune responses to HIV-1.

gp120 contains between 23 and 26 N-linked glycosylation sites throughout its sequence in different virus clades (15–17). gp120 glycans are composed of a mixture of high-mannose, hybrid, and complex glycan structures (18, 19). Depending on the cell type in which the glycoprotein is expressed, the gp120 glycosylation profile varies (10, 16). During the past few years, MS, combined with digestion of gp120 by various proteases and glycosidases, has been widely used for comprehensive glycosylation site analyses of gp120 (15, 17, 19–23). Structure-based studies of the HIV-1 envelope also provided new insights into gp120 glycan structures (24–28). However, functional characterization of gp120 glycosylation by specific targeting of gp120 glycans has been mostly overlooked. In this study, we label gp120 glycans specifically by metabolic labeling and click chemistry and characterize the labeled glycans for their structure and function.

Metabolic labeling of glycoconjugates using bioorthogonal reagents and click chemistry has become a powerful tool in glycobiology, mainly employed for glycoprotein discovery (29–31). Bioorthogonal labeling of glycans on glycoproteins consists of two steps. First, bioorthogonal, azido-functionalized monosaccharide building blocks are incorporated during the cellular glycoprotein biosynthesis (32). Second, glycans bearing azido...
sugars can be selectively reacted with an alkyne reagent linked to a fluorescent dye or biotin through what is called the click reaction (33). Three fully acetylated azido-monosaccharide analogs have been commonly used for metabolic labeling of vertebrate glycans: N-azidoacetylglucosamine (GlcNAz), N-azidoacylmannosamine (ManNAz), and N-azidoacetylgalactosamine (GalNAz). After entry into the cells, GlcNAz is specifically incorporated into intracellular O-GlcNAc–modified glycoproteins (33, 34). ManNAz is converted metabolically to SiaNAz and incorporated into sialic acid on N- and O-linked glycans (33, 34). GalNAz is first converted to UDP-GalNAz through endogenous biosynthesis and then efficiently converted to UDP-GlcNAz, labeling O-GlcNAcylated proteins (35). Meanwhile, MS analysis of GalNAz-labeled N-glycans released from glycoproteins as their permethylated derivatives suggested that GalNAz is incorporated into N-glycans, with no information on the sites at which the azido sugar was incorporated (36). Whereas metabolic labeling of glycoproteins is widely used to probe cell surface and intracellular glycoproteins in living cells, its utilization in targeting specific monosaccharides in recombinantly expressed glycoproteins has been limited (37). Particularly, the direct detection of azido-sugar incorporation into glycoproteins by MS remains poorly delineated.

Here, we demonstrate that gp120 glycans could be metabolically labeled by including azido-modified monosaccharide building blocks in the gp120 expression culture medium. After protein purification and reaction to either fluorophore-labeled or biotinylated dibenzocyclooctynol (DIBO) reagents, gp120 glycans can be specifically detected in fluorescence scanning or Western blotting–based assays, providing a direct tracing method for gp120 glycans. The specific azido-monosaccharide incorporation into certain glycans was verified by glycosidase treatments and characterized by direct MS analysis. Furthermore, we utilize this labeling and detection tool to investigate the effects of gp120 glycosylation on the uptake of gp120 by antigen-presenting cells (APCs). Our findings serve as a versatile tool for tracing and studying glycans on gp120 and potentially other soluble glycoproteins.

**Results**

**gp120 glycans can be labeled with azido analogs and detected by click chemistry**

To metabolically label gp120 glycans, the azido-modified metabolic glycoprotein-labeling reagents GlcNAz, ManNAz, and GalNAz were added together into the FreeStyle™ 293-F cells transfected with gp120 expression plasmid. The azido sugars were metabolically incorporated into N-linked, O-linked, sialic acid–modified, and O-GlcNAz–modified gp120, respectively, through the oligosaccharide biosynthesis pathway (34). Purified gp120 proteins with or without metabolically labeled azido sugars were reacted with Alexa Fluor® 488 DIBO alkyne in vitro and detected by fluorescence scanning after separation by SDS-PAGE. Although the glycoproteins were equally loaded, as shown in Coomassie gel staining (Fig. 1B), only the gp120 metabolically labeled with azido sugars reacted with DIBO reagent and was detected by fluorescence scanning (Fig. 1A). The azido sugar incorporated into gp120 glycans can also be reacted with biotinylated DIBO alkyne reagent and detected by Western blotting using a secondary horseradish peroxidase (HRP)-conjugated avidin antibody (Fig. 1C, top). To validate the band as intact gp120, two gp120-specific antibodies, A32 and 2G12, were used, recognizing a highly conserved conformation-dependent epitope of gp120 (38) and N-linked glycan shield of gp120 (39), respectively (Fig. 1C, middle and bottom).

To optimize the in vitro labeling, gp120 with azido-sugar incorporation was reacted with different concentrations of Alexa Fluor® 488 DIBO alkyne or biotinylated DIBO alkyne and detected by fluorescence scanning (Fig. 1D) or Western blotting (Fig. 1E). Based on this result, 40 μM Alexa Fluor® 488 DIBO alkyne and 500 μM biotinylated DIBO alkyne were used in the subsequent study. These data demonstrate that gp120 glycans are metabolically labeled with azido sugar and specifically detected by either fluorophore-labeled or biotinylated DIBO reagents, providing a direct detection method for gp120 glycans.

**ManNAz and GalNAz are specifically incorporated into gp120 glycans**

To further determine which azido sugar was incorporated into gp120 glycans, individual azido sugars were used to label gp120. Identical concentrations of GlcNAz, ManNAz, and GalNAz were incubated separately with gp120-expressing 293-F cells. Purified gp120 proteins were then reacted in vitro with either fluorophore-labeled DIBO alkyne or biotinylated DIBO alkyne and detected by fluorescence scanning or Western blotting, respectively. Analysis of GlcNAz incubation yielded that GlcNAz was not incorporated into gp120 glycan structure (Fig. 2A). The very faint fluorescent signals from the gp120 sample reacted with Alexa Fluor® 488 DIBO alkyne in GlcNAz-positive and GlcNAz-negative groups in Fig. 2A (top, lanes 2 and 4) are due to low nonspecific/background binding. This observation was confirmed by the Western blotting of gp120 reacted with biotinylated DIBO alkyne, as no specific signal was detected in any group (Fig. 2A, bottom). However, gp120 proteins were equally loaded in each group, as shown in Coomassie gel staining (Fig. 2A, middle).

In contrast to no detectable labeling of gp120 with GlcNAz, ManNAz, and GalNAz, gp120 proteins incubated with both ManNAz and GalNAz reacted with Alexa Fluor® 488 DIBO alkyne or biotinylated DIBO alkyne (Fig. 2B and C). When compared side by side, the fluorescence signal from GalNAz-incorporated gp120 was stronger than the gp120 labeled with ManNAz (Fig. 2D). Increasing amounts of gp120 proteins were loaded on SDS-polyacrylamide gel and reacted with Alexa Fluor® 488 DIBO alkyne to detect by fluorescence scanning (Fig. 2D, left) or stained with Coomassie Blue as a protein-loading control (Fig. 2D, right). These results demonstrate that azido sugars ManNAz and GalNAz, but not GlcNAz, can be effectively incorporated into gp120 glycans during protein expression. In addition, GalNAz-incubated gp120 displays a stronger signal than ManNAz-incubated gp120, suggesting its higher incorporation into gp120 glycan structures.

The incorporation of these nonnatural sugars, together with fluorophore or biotin moiety, did not affect the antigenicity of
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Figure 1. Metabolic labeling and click chemistry detection of gp120 glycans. The purified azido-labeled gp120 proteins specifically reacted with Alexa Fluor® 488 DIBO alkyne were detected by fluorescence scanning in SDS-polyacrylamide gel (A). B, equal concentrations of glycoproteins with or without azido sugar and DIBO fluorophore were loaded on a SDS-polyacrylamide gel and verified by Coomassie gel staining. C, gp120 with or without azido-sugar incorporation was reacted with biotinylated DIBO alkyne and detected by Western blotting using HRP-conjugated avidin (top). gp120 protein loading was verified by gp120-specific antibodies A32 (middle) and 2G12 (bottom). gp120 with azido-sugar incorporation was reacted with increasing concentrations of Alexa Fluor® 488 DIBO alkyne and detected by fluorescence scanning (D) or biotinylated DIBO alkyne and detected by Western blotting (E).

gp120, evidenced by comparable binding to bNAbs specific for the HIV-1 CD4-binding site (VRC01) and glycans (2G12) in an ELISA (Fig. 3).

Treatment of gp120 with various glycosidases verified ManNAz and GalNAz incorporation

To further determine the specificity of gp120 glycan incorporation with azido sugars, glycosidases, also known as glycoside hydrolases, were used to remove specific carbohydrate structures from labeled gp120. Purified gp120 with or without azido-sugar incorporation was further treated with peptide N-glycosidase F (PNGase F) to remove N-linked glycans (i.e. high mannose, hybrid, and complex oligosaccharides) from gp120 glycoproteins (9, 19). The extent of deglycosylation was assessed by band shift in Coomassie gel staining (Fig. 4 (A and B), middle). gp120 proteins before or after PNGase F treatment were reacted with fluorophore-labeled DIBO alkyne or biotinylated DIBO alkyne and detected by fluorescence scanning or Western blotting. Both the fluorescent and biotin signals were absent after PNGase F treatment in both ManNAz- and GalNAz-incorporated gp120 proteins (Fig. 4 (A and B), top and bottom), demonstrating the specific labeling of gp120 by those azido sugars.

ManNAz is metabolically incorporated into sialic acid residues on N- and O-linked cell surface and secreted glycoproteins, whereas GalNAz is utilized as a substitute for GalNAc and incorporated into the core cell surface and secreted O-linked glycoproteins (30, 33, 40). Thus, the specificity of ManNAz incorporation into sialic acid can be verified by sialidase (neuraminidase) treatment to remove azido-labeled sialic acid (10). Purified gp120 proteins with or without ManNAz or GalNAz incorporation were first treated with sialidase A that cleaved all nonreducing terminal sialic acids. Sialidase treatment resulted in a slight loss of molecular weight revealed by the shift in Coomassie gel staining (Fig. 4 (C and D), middle). gp120 proteins with or without sialidase treatment were reacted with fluorophore-labeled DIBO reagent or biotinylated DIBO reagent and detected by fluorescence scanning or Western blotting by SDS-PAGE. Azido-labeled sialic acids were effectively removed by sialidase, as evidenced by the significant
reduction of both fluorescent and biotin signals of gp120 proteins expressed in the presence of ManNAz (Fig. 4C, top and bottom). In contrast to ManNAz, the azide signal from gp120 with GalNAz incorporation was not affected by sialidase treatment (Fig. 4D (top and bottom) and E). These results demonstrated that azido-sugar ManNAz and GalNAz were distinctly incorporated into gp120 glycans. In addition, ManNAz was metabolically incorporated into terminal sialic acid of gp120 glycans, which can be efficiently removed by sialidase treatment.

Characterization of azido glycans by MS

Before characterizing azido-labeled gp120, comprehensive glycomic and glycopeptide analyses were performed on gp120 without azido-glycan labeling. The whole N-glycan profile was characterized by analyzing N-glycans released by PNGase F digestion and demonstrated that the unlabeled gp120 monomer carries both high mannose- and complex-type N-glycans with the latter type being the major N-glycan components (Fig. 5A). Because the 293-F cellular expression system is known to yield glycoproteins bearing N-linked LacdiNAc (GalNAcβ4 GlcNAcβ-) structures (41), we interrogated mass spectra of glycans released from gp120 expressed by 293-F cells for the presence of LacdiNAc and detected very low abundance of LacdiNAc on this particular recombinant protein (pSyn gp120). The relative abundances of the most prevalent glycans possessing LacdiNAc were as follows: Gal1GalNAc1N2M3N2F, 0.8%; GalNAc2N2M3N2F, 0.2%; Gal1GalNAc1N2FM3N2F, 0.2%. Our search for the presence of sialylated LacdiNAc yielded no results. Low abundance of LacdiNAc structures on 293-F–expressed gp120 has been reported previously (42). MS/MS fragmentation at m/z = 1156 (2+), corresponding to the mass of the most abundant LacdiNAc glycan (Gal1GalNAc1N2 M3N2F), revealed an isomeric mixture in which the LacdiNAc structure is the minor component (Fig. 5B).

This observation is consistent with previous reports that the gp120 monomer has significant levels of complex-type glycans (19). N-Glycosylation site occupancy analysis covered all 23 potential N-linked glycosylation motifs (NX(S/T)) in the gp120 sequence. Among those 23 sites, 20 of them are N-glycosylated, whereas three do not carry any N-glycans (Table 1). Furthermore, peptide regions carrying N-glycan (and one O-glycan) were identified and characterized by direct glycopeptide analysis of trypsin-digested samples (Table S1). The glycoproteomic

Figure 2. Specific labeling of gp120 glycans with azido sugars. gp120 molecules incubated with GlcNAz (A), ManNAz (B), or GalNAz (C) were reacted with fluorophore-labeled or biotinylated DIBO alkyne reagents and detected by fluorescence scanning (top) or Western blotting (bottom). Protein loading was verified by Coomassie gel staining (middle). D, labeling efficiency of gp120 glycans were assessed by reacting increasing amounts of gp120 proteins with Alexa Fluor® 488 DIBO alkyne and detecting by fluorescence scanning (left). The GalNAz-incorporated gp120 showed the strongest fluorescently labeled bands, whereas the protein content was comparable (right).
analysis of unlabeled gp120 served as a framework for the MS analysis of azido-labeled gp120.

Purified gp120 proteins individually labeled with GlcNAz, ManNAz, or GalNAz were digested with trypsin, and the resulting glycopeptides were profiled by LC-MS/MS. The azido moiety was detected on gp120 glycopeptides bearing N-linked or O-linked oligosaccharides (Fig. 6A). ManNAz supplementation resulted, as expected, in the incorporation of SiaNAz residues into complex N-linked and O-linked glycans. GalNAz supplementation resulted in the incorporation of GalNAz into O-linked glycans and GlcNAz into high-mannose and complex N-linked glycans. Azido glycans were not detected in gp120 samples produced by GlcNAz supplementation, an observation consistent with fluorescent scanning and Western blotting results (Fig. 2A). The position of azido-modified monosaccharide residues within oligosaccharide structures of glycopeptides could be discerned by MS/MS fragmentation using collision-induced dissociation (CID). A representative spectrum for a glycopeptide ion derived from ManNAz-labeled gp120 demonstrates the incorporation of SiaNAz as a nonreducing terminal, capping residue (Fig. 6B). The MS/MS-CID spectrum of the triply charged intact glycopeptide at \(m/z = 1026\), AKWN339@DTLK (where @ represents the site of N-glycosylation), displays a SiaNAz oxonium ion (minus water) at \(m/z = 315.0936\) (singly charged). The spectrum also displays the neutral loss of SiaNAz from the precursor ion at \(m/z = 1372.5844\) (doubly charged), further verifying that the azide was incorporated into the terminal sialic acid residue. Representative spectra for the same glycopeptide ion, AKWN339@DTLK, derived from GalNAz-labeled gp120 demonstrate the metabolic interconversion of GalNAz to GlcNAz and subsequent incorporation into reducing terminal or branching positions of complex N-glycans (Fig. 6C and D). Three structural isomers were observed from the GalNAz-treated sample. Two isomers eluted during the LC-MS/MS run and presented as a mixture (Fig. 6C). Both of these structures carried the azido moiety on a branching GlcNAc, in one case as the sialylated N-acetyllactosamine (LacNAc) antenna and in the other as the nonsialylated LacNAc antenna. The other structural isomer carrying azide at the GlcNAc core was separated and eluted at a slightly later retention time during the LC-MS run (Fig. 6D).
An essential question regarding metabolic labeling with biorthogonal reagents is whether they incorporate with efficiencies approaching physiologic relevance. Therefore, we undertook the quantification of labeling efficiency for azido-modified glycans on gp120 monomer. Comprehensive analysis of azide incorporation at all N-glycosylation sites of gp120 is extremely challenging technically due to the instability of the azido group under chemical derivatization conditions in common use for conventional MS profiling, including cysteine reduction and alkylation for peptide analysis as well as permethylation for glycosyl composition (Hex1HexNAc1N2M3N2F) (Fig. 7). As shown in Fig. 8, ManNAz- or GalNAz-labeled gp120 was treated with sialidase and detected by Western blotting.

**Figure 4. Glycan incorporation of azido sugar–labeled gp120 characterized by glycosidase treatments.** gp120 with or without ManNAz (A and C) or GalNAz (B and D) incubation/incorporation was treated with (+) or without (−) PNGase F (A and B) or sialidase (C and D) to remove N-glycans or sialic acids, respectively. The proteins were reacted with fluorophore-labeled DIBO alkyne and detected by fluorescence scanning after SDS-PAGE separation (top) or reacted with biotinylated DIBO alkyne and detected by Western blotting (bottom). The molecular weight shifts of the proteins before and after glycosidase treatments were shown by Coomassie gel staining (middle). E, ManNAz- or GalNAz-labeled gp120 was treated with sialidase and detected by Western blotting.

LC-MS/MS analysis of GalNAz-labeled gp120 glycopeptides identified LacdiNAc structures as well. We detected neutral loss of terminal “HexNAz” and a B2 type oxonium fragment ion corresponding to an azide-labeled LacdiNAc (theoretical m/z 448.1674) from N-glycopeptides having LacdiNAc glycosyl composition (Hex1HexNAc1N2M3N2F) (Fig. 7). The presence of the LacdiNAz fragment indicates azide incorporation into LacdiNAc resulting from GalNAz treatment. However, our fragmentation does not distinguish whether the azido-modified monosaccharide is installed as GalNAz or GlcNAz.

**Sialic acid removal enhances gp120 protein uptake by BMDCs.** Because GalNAz can be incorporated into the core/reducing end of gp120 N-glycans as GlcNAz (Fig. 6, A and C), GalNAz labeling serves as a valuable tool for studying glycan function on gp120 through selective enzymatic glycan removal. Here, we investigated the impact of differential glycosylation of gp120 on its uptake by APCs. GalNAz-labeled gp120 was first reacted with Alexa Fluor® 488 DIBO alkyne. Purified fluorophore-labeled gp120 was then incubated between 10 min and 3 h with BMDCs at 37 °C. At indicated time points, cells were collected and tested for the fluorescence signal by flow cytometry. BMDCs were washed extensively with PBS to determine total protein binding and uptake (Fig. 8, A (dotted line) and B (circles)), whereas the protein uptake was specifically measured after cells were washed with acid buffer to remove antigens bound on the cell surface (43) (Fig. 8, A (solid line) and B (triangles)). As shown in Fig. 8B, gp120 was internalized by BMDCs with an uptake plateau observed at 2 h. To study the
role of N-glycans in gp120 uptake, we cleaved all high-mannose structures from gp120 expressed in GnTI−/−/H11002 cells that have been engineered to produce exclusively oligomannose N-glycans (44) by endoglycosidase F1 (Endo F) treatment, leaving a GlcNAz attached to the asparagine (19). After reacting with Alexa Fluor® 488 DIBO alkyne, gp120 uptake was assessed. As expected, the uptake of gp120 without its N-glycan oligosaccharides by APCs was significantly reduced (Fig. 8C). We further examined the potential role of individual glycan residues on gp120 uptake. BMDCs were preincubated with a variety of saccharide inhibitors followed by uptake of gp120 expressed in 293-F cells with GalNAz incorporation and containing high-mannose, hybrid, and complex N-linked oligosaccharides. Whereas no evident inhibition was observed with lactose, galactose, GalNAc, glucose, or GlcNAc coincubation (Fig. 8D and E), mannose and mannan notably inhibited gp120 uptake (Fig. 8F). Interestingly, augmented gp120 uptake was observed upon N-acetylleucaminic acid (sialic acid) coinubcation in a dose-dependent manner (Fig. 8G), suggesting that sialic acid on gp120 surface inhibits gp120 uptake.

Taking this observation further, two GalNAz-labeled gp120 variants, one of which only contains high-oligomannose N-glycans (i.e. expressed in GnTI−/− cells) and the other one specifically lacking sialic acids (expressed in 293-F cells and treated with sialidase) were generated, and their uptake by BMDCs was compared with gp120 expressed in 293-F in the presence of GalNAz. The protein purity and fluorescent intensity were verified by the Coomassie gel staining and fluorescence scanning (Fig. 9A). Uptake by BMDCs was assessed at 37 °C between 10 min and 3 h. In agreement with previous reports (44, 45),
Discussion

Analysis and study of gp120 glycosylation are challenging because gp120 proteins contain varying numbers of potential N-linked glycosylation sites, and each site can be modified by one of several glycan structures (19, 20, 46, 47). Whereas mass spectrometric and X-ray crystallographic approaches have helped to discern certain aspects of the structure and function of the gp120 glycan shield, efforts to specifically target and characterize particular monosaccharide units on gp120 N-glycans have been very limited.

Metabolic incorporation of azido sugars has been effectively used to probe glycosylation of cell surface and intracellular glycoproteins, but labeling of secreted glycoproteins has been understudied (48, 49). Here, we combined the metabolic labeling technology with gp120 protein expression to specifically label glycans on a soluble target protein, providing a tool for tracing and studying the function of specific gp120 glycans. We demonstrated that gp120 glycans could be successfully labeled with azido sugars during protein expression and specifically detected by either fluorophore-labeled or biotinylated secondary reagents. ManNAz and GalNAz were efficiently and specifically incorporated into gp120 N- and O-glycans. However, GlcNAz labeling of the N-glycans was not effective, as GlcNAz does not significantly label mammalian cells (33). Moreover, MS analyses of GalNAz-labeled gp120 directly demonstrated that GalNAz is incorporated into core GlcNAc as well as GlcNAc antenna of high-mannose and complex N-glycans.

The azido-sugar incorporation into cellular glycoproteins was indirectly assessed previously by using various glycosyltransferases (29), glycosidases (50, 51), and N- and O-linked glycosylation inhibitors (50, 52). The direct detection of azide incorporation into glycoproteins by MS has proven to be challenging, restricting the use of metabolic labeling primarily for protein identification rather than for mapping glycosylation (30, 31). Furthermore, MS analysis of labeled glycans released from glycoproteins as their permethylated derivatives has met with limited success (36). To circumvent problems, many of which are caused by sample handling (protein reduction, carboamidomethylation, permethylation), we digested gp120 into glycopeptides and analyzed the glycopeptides directly by MS without further derivatization. Using this strategy, we directly demonstrated that ManNAz was specifically incorporated into gp120 N- and O-glycans. Howev-

Table 1

| Site number | Pro tease used | Potential N-linked site (NX(S/T)) | Known as* | Analysis result |
|-------------|---------------|----------------------------------|-----------|----------------|
| 1           | Chymo/Glu-C   | Asn-82                           | Asn-88    | Glycosylated   |
| 2           | Trypsin       | Asn-129                          | Asn-135   | Glycosylated   |
| 3           | Trypsin       | Asn-132                          | Asn-138   | Glycosylated   |
| 4           | Trypsin       | Asn-135                          | Asn-141   | Not glycosylated |
| 5           | Trypsin       | Asn-148                          | Asn-156   | Glycosylated   |
| 6           | Trypsin       | Asn-152                          | Asn-160   | Glycosylated   |
| 7           | Trypsin       | Asn-179                          | Asn-186   | Not glycosylated |
| 8           | Trypsin       | Asn-180                          | Asn-187   | Glycosylated   |
| 9           | Trypsin       | Asn-233                          | Asn-241   | Glycosylated   |
| 10          | Trypsin       | Asn-234                          | Asn-248   | Glycosylated   |
| 11          | Trypsin       | Asn-258                          | Asn-261   | Glycosylated   |
| 12          | Trypsin       | Asn-268                          | Asn-276   | Glycosylated   |
| 13          | Trypsin       | Asn-287                          | Asn-295   | Glycosylated   |
| 14          | Trypsin       | Asn-293                          | Asn-301   | Glycosylated   |
| 15          | Trypsin       | Asn-323                          | Asn-332   | Glycosylated   |
| 16          | Trypsin       | Asn-330                          | Asn-339   | Glycosylated   |
| 17          | Trypsin       | Asn-346                          | Asn-356   | Glycosylated   |
| 18          | Chymo/Glu-C   | Asn-352                          | Asn-362   | Glycosylated   |
| 19          | Chymo/Glu-C   | Asn-376                          | Asn-386   | Glycosylated   |
| 20          | Chymo/Glu-C   | Asn-382                          | Asn-392   | Glycosylated   |
| 21          | Trypsin       | Asn-387                          | Asn-397   | Glycosylated   |
| 22          | Trypsin       | Asn-399                          | Asn-403   | Not glycosylated |
| 23          | Trypsin       | Asn-434                          | Asn-448   | Glycosylated   |

* Adjusted to common gp120 N-glycan sites.

enrichment of oligomannose N-glycans by GnTI−/− cell expression significantly enhanced the internalization of gp120 by BMDCs, as the uptake plateau was achieved within 20 min (Fig. 9, B and C). Notably, sialidase-treated gp120 also had a significant increase in its rate of uptake by BMDCs compared with gp120-containing sialic acid (Fig. 9, B and C), in accordance with the sialic acid coinubation assay (Fig. 8G). Additionally, the role of differential glycosylation on the uptake of the three gp120 variants was confirmed by confocal microscopy analysis (Fig. 9D). A monosaccharide competition assay was also performed on GalNAz-labeled gp120 variants to reveal the mechanism underlying the increased uptake. The uptake of gp120 molecules expressed in 293-F cells and treated with sialidase A (Fig. 9E) or expressed in GnTI−/− cells (Fig. 9F) was only inhibited by mannose and mannan but not affected by other saccharide inhibitors, indicating that oligomannose N-glycans on gp120 contribute to the uptake of these gp120 variants. These results demonstrated that metabolically labeled gp120 with azido analogs provides a valuable tool to trace and study gp120 glycan function. gp120 glycosylation profile is important with azido analogs provides a valuable tool to trace and study the function of specific gp120 glycans. We demonstrated that gp120 glycans could be successfully labeled with azido sugars during protein expression and specifically detected by either fluorophore-labeled or biotinylated secondary reagents. ManNAz and GalNAz were efficiently and specifically incorporated into gp120 N- and O-glycans. However, GlcNAz labeling of the N-glycans was not effective, as GlcNAz does not significantly label mammalian cells (33). Moreover, MS analyses of GalNAz-labeled gp120 directly demonstrated that GalNAz is incorporated into core GlcNAc as well as GlcNAc antenna of high-mannose and complex N-glycans.

Specific labeling and tracing of gp120 glycans
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A

|       | GlcNAz | ManNAz | Sialic acid | GalNAz |
|-------|--------|--------|-------------|--------|
|       | N.D.   |        | Or          |        |

|       | GlcNAc | GalNAc |
|-------|--------|--------|
|       |        |        |

B

ManNAz-treated

C

GalNAz-treated

D

GalNAz-treated

E

|       | Incorporation ratio (%) |
|-------|-------------------------|
|       | 0.0 | 5.0 | 10.0 | 15.0 | 20.0 |
| ManNAz| N187 | N276 | N339 | N463 |
|       | 12.5 | 11.9 | 8.3  | 12.6  | 17.6 |
| GalNAz| N187 | N276 | N359 | N356 | N463 |
|       | 11.7 | 13.2 | 9.3  | 13.9  | 14.7 |

|       | Incorporation ratio (%) |
|-------|-------------------------|
|       | 0.0 | 5.0 | 10.0 |
| ManNAz| N187 | N276 | N339 |
|       | N463 | N463 | N463 |
|       | n.d | n.d | n.d |
| GalNAz| N187 | N276 | N359 |
|       | N356 | N463 | n.d |
|       | 2.5  | 3.1  | 1.3  | 0.9 | 0.3 |
Incorporation efficiency of ManNAz and GalNAz varied at different sites and glycan structures.

gp120 internalization by APCs can be mediated by cell surface C-type lectin receptors, such as DC-SIGN (44, 53, 54) and mannose receptor (55, 56); therefore, gp120 glycan moieties play an essential role in this process. Because the GalNAz can be incorporated into the GlcNAc residue at the reducing end of N-glycans, we utilized GalNAz-labeled gp120 to investigate the impact of glycosylation on gp120 protein uptake by APCs. Endo F removal of all but core GlcNAc residue dramatically impaired gp120 uptake by BMDCs. Individual monosaccharide residues on gp120 N-glycans were assessed for their role on gp120 internalization by APCs using a variety of glycan inhibitors combined with glycosylation modification through GnTI−/− expression or sialidase treatment. In agreement with previous reports (44, 45), oligomannose N-glycans on gp120 play an essential role in protein uptake by BMDCs, possibly mediated by C-type lectin receptor DC-SIGN in a mannose-dependent manner. Of note, although mouse DC-SIGN has been shown to bind to mannosylated residues similar to its human homologue, differences in the fine specificity of biochemical properties and recognition of carbohydrate ligands between mDC-SIGN and hDC-SIGN have been reported previously (57–59).

Interestingly, preincubation of APCs with exogenous sialic acid or removing sialic acid on gp120 extensively enhanced BMDCs uptake, suggesting an inhibitory effect on gp120 uptake for terminal sialic acids on N-glycan structures. Increased uptake after sialic acid removal from gp120 may involve galactose receptor due to exposed galactose as the terminal sialylated glycans. Alternatively, removal of sialic acid may promote gp120 binding to manno-binding lectin (61, 62). The crystal structures and biochemical analyses of the HIV-1 gp120–gp41 envelope trimers reveal that the glycan shield is mostly composed of high-mannose structures with limited sialylated glycans (19, 28). In addition, DC-SIGN on BMDCs was shown to enhance trans-infection of T cells by the virus (54). Thus, our observation of the increase in BMDCs’ uptake of highly homologous in sequence to the human DC-SIGN and also binds to mannosylated residues similar to its human homologue, differences in the fine specificity of biochemical properties and recognition of carbohydrate ligands between mDC-SIGN and hDC-SIGN have been reported previously (57–59).

Figure 6. Incorporation of azido-modified monosaccharide residues into N- and O-glycans on gp120 glycopeptides. A examples of gp120 glycopeptides detected by LC-MS/MS are shown, using SNFG nomenclature to represent the glycan structures. The glycosylation site detected for each glycopeptide is highlighted in red (● represents the site of N-glycosylation), and the site of azido incorporation is indicated as N₆ and highlighted with a yellow circle. N.D., not detected. B, MS/MS-CID spectrum of m/z 1026 (triply charged), corresponding to the glycopeptide AKWN339@DTLK carrying a monosialylated, monofucosylated, biantennary glycan. C and D, MS/MS-CID spectra corresponding to the same glycopeptide ion in B but harvested from cultures fed with GalNAz. Under these conditions, the azido group is detected on branching (C) and core (D) GlcNAc residues. E, the azido incorporation efficiency of gp120 treated with ManNAz, GalNAz, and GlcNAz was determined, respectively, at each site of the five selected glycopeptides based on peak intensity in MS. n.d., not detected.
desialylated gp120 supports the hypothesis that HIV-1 evolved to decorate its surface with high-mannose patches lacking sia-
lylated complex glycans to enhance lectin-mediated uptake by BMDCs and therefore its infectivity. Further investigation of the involvement of C-type lectin receptors and oligomannose in gp120 binding and uptake by BMDCs may reveal new mech-
anisms for HIV-1 infectivity.

In conclusion, our current study further proves the value of metabolic labeling/click chemistry for tracing and studying gp120 glycans and potentially other glycoproteins, contributing to our understanding of glycans function in the context of a whole glycoprotein.

**Materials and methods**

**Reagents**

The following reagents were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, National Institutes of Health: pSyn gp120 IgG plasmid from Dr. Eun-Chung Park and Dr. Brian Seed (63, 64), HIV-1 gp120 monoclonal antibody (A32) (catalog no. 11438) from Dr. James E. Robinson (65, 66), and HIV-1 gp120 monoclonal antibody (2G12) from Dr. Hermann Katinger (39, 67–70). The azido-modified metabolic glycoprotein-labeling reagents GlcNAz, ManNAz, and GalNAz and the fluorophore-labeled detecting reagent Alexa Fluor®

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**Figure 8.** gp120 glycans modulate uptake of gp120 by BMDCs. A and B, fluorophore-labeled gp120-GalNAz expressed in WT 293-F cells was incubated with BMDCs. Cells were then collected and washed with PBS (dotted line in A and circles in B) to measure combined antigen uptake and binding or with acid buffer (solid line in A and triangles in B) to specifically measure antigen uptake and analyzed by flow cytometry. C, uptake of Alexa Fluor® 488–labeled gp120-GalNAz expressed in GnTI−/− cells with or without Endo F treatment by BMDCs for 2 h was detected by flow cytometry. Gray line, no antigen; blue-shaded, gp120-GalNAz without Endo F treatment; red-shaded, gp120-GalNAz after Endo F. For saccharide inhibition, BMDCs were preincubated with a graded concentration of glycans (lactose, galactose, and acetylgalactosamine (D); glucose and acetylglucosamine (E); mannose and mannan (F); and N-acetylneuraminic acid (G)) at 4 °C for 30 min followed by a 30-min incubation with Alexa Fluor® 488–labeled gp120-GalNAz expressed in 293-F cells. MFI, mean fluorescence intensity. Data are the mean ± S.D. (error bars) *, p < 0.05; **, p < 0.01; ***, p < 0.001.
488 DIBO alkyne were purchased from Life Technologies, Inc. The biotinylated DIBO alkyne used in this study was a gift from Dr. Geert-Jan Boons (University of Georgia) (71, 72). PNGase F and Endo F were gifts from Dr. Kelley Moremen (University of Georgia) (73, 74). All of the following saccharide inhibitors were purchased from Sigma-Aldrich: D-galactose, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, D-mannose, mannan, and N-acetylneuraminic acid. The exceptions were D-lactose (BD Bioscience) and D-glucose (Fisher).

Expression and purification of metabolically labeled gp120

Codon-optimized pSyn gp120 IgG, encoding JR-FL (clade B) gp120, was used as the plasmid for gp120 expression. gp120 was expressed in a serum-free medium as a soluble secreted protein by transient transfection of WT FreeStyle™ 293-F suspension cells (Life Technologies) or HEK293S N-acetylgalactosaminyltransferase I mutant (GnTI−/−/−) cells (ATCC) as described previously (73). Briefly, suspension cultures were maintained at 0.5–3.0 × 10⁶ cells/ml in a humidified CO2 platform shaker incubator at 37 °C. Immediately before transfection, cells were suspended at a 2.5 × 10⁶/ml concentration in fresh Freestyle 293 expression medium (Life Technologies). Transfections of the suspension cultures were accomplished by the direct addition of a 3 μg/ml concentration of the pSyn gp120 plasmid DNA and 9 μg/ml polyethyleneimine (linear 25-kDa polyethyleneimine, Polysciences, Inc., Warrington, PA) to the suspension culture (75). After 24 h, cells were diluted with fresh Freestyle 293 expression medium supplemented with valproic acid (Sigma) to a final concentration of 2.2 mM, and protein production was continued for 3–6 days at 37 °C. For metabolic labeling, azido analogs GlcNAz, ManNAz, and GalNAz were added together or separately 24 h after transfection to a final concentration of 40 μM for each azido sugar. Supernatants were harvested 4 days after transfection for protein purification.

Azido sugar–derived gp120s were purified from transfection supernatant as described previously (16) using Galanthus nivalis lectin-agarose (Vector Laboratories) column chromatography and further purified on a Superdex S200 size-exclusion column (Bio-Rad) to remove contaminants and dimers. The gp120 fractions were collected, desalted, and lyophilized for long-term storage.

Detection of metabolically labeled proteins

Metabolically labeled gp120s (50–100 μg) in 50 μl of TBS buffer were reacted with final concentrations of 40 μM Alexa Fluor® 488 DIBO alkyne or 500 μM biotinylated DIBO alkyne (or a different concentration if indicated) at room temperature in the dark for 1 h. The proteins were separated by SDS-PAGE.
and detected by fluorescence scanning using a Typhoon 9410 variable-mode imager (Amersham Biosciences) or by Western blotting using a secondary HRP-conjugated avidin antibody (Biolegend). Equal protein loading was verified by Coomassie gel staining. To purify gp120 protein with both azido-sugar and fluorophore labeling, a further desalting procedure using a G-25 desalting column (GE Healthcare) was performed following the manufacturer’s protocol to remove unreacted Alexa Fluor® 488 DIBO alkyl reagents.

Antigenicity of gp120 with azido-sugar and fluorophore or biotin were measured by ELISA essentially as described previously (76). Briefly, a 96-well plate was coated with anti-gp120 mAb D7324 (Aalto Bioreagents, Dublin, Ireland) to capture the indicated monomeric gp120. After washing and blocking steps, serially diluted mAbs VRC01 and 2G12 were reacted and detected by HRP-labeled goat anti-human IgG (Santa Cruz Biotechnology, Inc.). Colorimetric detection was performed using 3,3′,5,5′-tetramethyl benzidine solution (Biolegend), and the reaction was stopped using 2 N H₂SO₄.

Glycosidase treatment

To remove N-linked glycans, gp120 was treated with PNGase F and Endo F under denaturing conditions as described previously (73). Briefly, 50–100 μg of purified gp120 with or without azido-sugar incorporation was boiled for 5 min in 50 μL of 20 mM sodium phosphate buffer (pH 7.5) containing 0.05% SDS and 50 mM β-mercaptoethanol. 30 milliunits of PNGase F or 2.5–5 μg (one-tenth of the protein amount) of Endo F was added into the denatured protein for incubation at 37 °C overnight.

Sialidase A (Prozyme) was used to remove sialic acid from gp120 according to the manufacturer’s protocol. Up to 100 μg of gp120 with or without azido-sugar labeling was reconstituted in 14 μL of deionized water. 4 μL of 5× reaction buffer and 2 μL of sialidase A were added and incubated at 37 °C for 3 h. PNGase F– or sialidase A–treated gp120 proteins were separated by SDS-PAGE and analyzed by Coomassie gel staining as well as fluorescence scanning or Western blotting.

Glycomic and glycoproteomic analysis of nonazido-labeled gp120

For gp120 N-glycan profiling, whole N-glycans were released enzymatically by PNGase F, purified, and permethylated according to the method of Anumla and Tylor (77). Briefly, the proteins were resolubilized and denatured by adding 60 μL of 100 mM sodium phosphate, pH 7.5, and 3 μL of 1% SDS, 1 mM β-mercaptoethanol in water. Samples were heated to 100 °C for 5 min and cooled down to room temperature. SDS was removed by adding 7 μL of 1 M KCl followed by incubation on ice and centrifugation at maximal speed for 15 min at 4 °C. The supernatant was recovered and subjected to digestion with 2 μL of PNGase F (10 units/ml) at 37 °C overnight. The released N-glycans were purified from residual enzyme, deglycosylated protein, and other contaminants by passing through a Sep-pak C18 cartridge (Waters). For MS analysis, one-fifth of the permethylated glycans were supplemented with 10 pmol of an exogenous glycan standard (maltotetraose) previously permethylated with isotopically heavy methyl iodide (¹³CH₃I). The sample glycans added to standard were adjusted to a 50-μl total volume and directly infused into an LTQ/Orbitrap mass spectrometer fitted with a nanospray ionization interface (Orbitrap Discovery, Thermo Fisher Scientific). Glycans were detected in FT full MS mode at 30,000 resolution, and an MS/MS-CID spectrum of each glycan ion was acquired by total ion mapping (78), as well as by data-dependent scans (79). Glycan signal intensities were recovered from extracted full MS spectra (Xtract, Thermo Fisher Scientific), and glycan identities were assigned based on exact mass and CID fragmentation.

N-Glycosylation site occupancy of gp120 was determined by an ¹⁸O-labeling procedure described previously (80). Briefly, the sample was reduced by DTT (in ammonium bicarbonate buffer at 50 °C for 1 h) and alkylated by iodoacetamide (room temperature in the dark for 1 h) followed by digestion with sequence grade trypsin or the combination of chymotrypsin/ Glu-C. After the proteolytic digestion, the sample was further deglycosylated with PNGase F in ¹⁸O water (H₂¹⁸O) to convert the glycan-modified asparagine to an ¹⁸O aspartic acid residue and then profiled by LC-nanospray ionization-MS/MS. The instrument used for the analysis was Orbitrap-Fusion equipped with an EASY nanospray source and Ultima 3000 autosampler LC system. The LC separation was performed on a nano-C18 column using a water/acetonitrile gradient with formic acid. Full MS data were collected at 60,000 resolution in FT mode; MS/MS CID data and MS/MS-HCD activation data (both in FT mode) were obtained for each precursor ion.

For direct glycopeptide examination, gp120 samples were reduced, alkylated, and digested with trypsin. The tryptic peptides/glycopeptide were then separated and subjected to LC-MS analysis.

MS analysis of azido-sugar incorporation

For the analysis of azido-sugar incorporation into gp120 by LC directly in-line with MS (LC-MS/MS), two common steps employed in conventional proteomics, reduction and carboximidylation of cysteine, were skipped to prevent modification of the azide group. 10 μg of the protein material was resuspended in 50 mM ammonium bicarbonate and digested with sequential grade trypsin (Promega) at 37 °C overnight. The sample was dried, and an aliquot was dissolved in mobile phase A (0.1% formic acid in distilled H₂O) and filtered with 0.2-μm filters (Nanosep, PALL) for LC-MS analysis. The LC-MS analysis was performed on Orbitrap-Fusion equipped with an EASY nanospray source and Ultimate3000 autosampler LC system (Thermo Fisher Scientific). The sample separation was performed on a nano-C18 column (Acclaim pepMap RSLC, 75 μm × 150 mm, C18, 2 μm) via an 80-min gradient of increasing mobile phase B (80% acetonitrile, 0.1% formic acid in distilled H₂O) at a flow rate of ~300 nl/min into the mass spectrometer. For online MS detection, full MS data were first collected at a resolution of 60,000 in FT mode, and MS/MS with CID, HCD, or ETD activation data (all in FT mode) were obtained for each precursor ion by data-dependent scan (top-speed scan, 3 s). The resulting data were analyzed manually with the use of Byonic software (Protein Metrics). For the preliminary data
analysis of glycopeptides without azide incorporation by Byonic software. Byonic parameters were set to allow 20 ppm of precursor ion mass tolerance and 20 ppm of fragment ion tolerance with monoisotopic mass. The search was performed against a pSyn gp120 sequence with the human/mammalian N-glycan database (default N-glycan database in the Byonic software). Azide-incorporated glycopeptides were searched by manual data interpretation of the raw LC-MS data based on the preliminary data analysis results. Before profiling of azide-incorporated gp120, LC-MS analysis of reduced and alkylated gp120 and direct infusion analysis of released N-linked glycan were performed to define the full diversity of glycans and glycopeptides. These data provided a reference set for searching the azide-incorporated LC-MS results. Graphical representation of monosaccharide residues is consistent with the symbol nomenclature for glycans (SNFG), which has been broadly adopted by the glycomics community (81).

BMDC induction

Dendritic cells were generated from bone marrow as described previously (7). Briefly, bone marrow was flushed out from the tibiae and femurs of 6–8-week-old female BALB/c mice (Taconic Biosciences) and depleted of red blood cells with ACK (ammonium-chloride-potassium) lysing buffer (Thermo Fisher Scientific). The mouse studies were performed under an approved University of Georgia animal use protocol (AUP, A2016 11-022-Y1-A0). The bone marrow cells were first incubated in RPMI 1640 supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin in a T-75 flask (Thermo Fisher Scientific) at 37 °C for 2 h to remove macrophages. The remaining cells were harvested and plated in T-75 flasks (5 × 10⁶ cells/flask) in BMDC induction medium (15 ml of RPMI 1640 supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 1% (v/v) MEM nonessential amino acids, 1 mM sodium pyruvate, 2 mM l-glutamine (Thermo Fisher Scientific), 50 μM β-mercaptoethanol (Gibco), and 20 μg/ml GM-CSF (PeproTech)) at 37 °C in a humidified atmosphere containing 5% (v/v) CO₂. On days 3 and 6, the cell culture was supplemented with 10 ml of fresh medium. BMDCs were collected on day 8 for binding and uptake experiments.

gp120 uptake by BMDCs

BMDCs were incubated with 30 μg/ml Alexa Fluor® 488–labeled gp120-GalNAz in BMDC induction medium without GM-CSF at 37 °C for 0, 10, 20, 30, 60, 120, and 180 min. At each time point, cells were collected and washed twice with either cold PBS or acid buffer (0.2 M acetic acid and 0.15 M NaCl, pH 3) on ice for 4 min to remove residual surface proteins. Then the cells were stained for surface CD11c expression using anti-mouse CD11c antibody (Biolegend, clone N418) and analyzed by flow cytometry for fluorescent signal. Surface staining of cell suspensions was performed in PBS containing 0.1% BSA solution at 4 °C. Samples were analyzed on CyAn (Beckman Coulter, Hialeah, Florida). Data were analyzed using FlowJo software (Treestar, Inc., Ashland, OR).

For confocal labeling and tracing of gp120 glycans

Confocal microscopy, 7 × 10⁴ BMDCs were seeded on an 8-well chamber slide (Thermo Scientific™ Nunc™ Lab-Tek™ Chamber Slide™) and incubated with 150 μg/ml of each of the three types of Alexa Fluor® 488–labeled gp120-GalNAz, respectively, for 0.5 and 1 h. Cells were fixed in 4% polyoxymethylene, and cell nuclei were stained with 2 μg/ml Hoechst 33342 dye (Life Technologies). Images were acquired with a FV1200 confocal microscope (Olympus Life Science).

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References

1. Varki, A. (2017) Biological roles of glycans. *Glycobiology* 27, 3–49 CrossRef Medline
2. Sun, L., Middleton, D. R., Wantuch, P. L., Ozdik, A., and Avci, F. Y. (2016) Carbohydrates as T-cell antigens with implications in health and disease. *Glycobiology* 26, 1029–1040 CrossRef Medline
3. Klein, F., Mouquet, H., Dosenovic, P., Scheid, J. F., Scharf, L., and Nussenzweig, M. C. (2013) Antibodies in HIV-1 vaccine development and therapy. *Science* 341, 1199–1204 CrossRef Medline
4. Kwong, P. D., Mascola, J. R., and Nabel, G. J. (2013) Broadly neutralizing antibodies and the search for an HIV-1 vaccine: the end of the beginning. *Nat. Rev. Immunol.* 13, 693–701 CrossRef Medline
5. Koff, W. C., Burton, D. R., Johnson, P. R., Walker, B. D., King, C. R., Nabel, G. J., Ahmed, R., Bhan, M. K., and Plotkin, S. A. (2013) Accelerating next-generation vaccine development for global disease prevention. *Science* 340, 1232910 CrossRef Medline
6. Barin, F., McLane, M. F., Allan, J. S., Lee, T. H., Groopman, J. E., and Essex, M. (1985) Virus envelope protein of HTLV-III represents major target antigen for antibodies in AIDS patients. *Science* 228, 1094–1096 CrossRef Medline
7. Wen, Y., Wang, H., Wu, H., Yang, F., Tripp, R. A., Hogan, R. J., and Fu, Z. F. (2011) Rabies virus expressing dendritic cell-activating molecules enhances the innate and adaptive immune response to vaccination. *J. Virol.* 85, 1634–1644 CrossRef Medline
8. Pritchard, L. K., Spencer, D. I., Royle, L., Bonomelli, C., Seabright, G. E., Behrens, A. J., Kulp, D. W., Menis, S., Krumm, S. A., Dunlop, D. C., Crispin, D. J., Bowden, T. A., Scanlan, C. N., Ward, A. B., Schief, W. R. et al. (2015) Glycan clustering stabilizes the mannose patch of HIV-1 and preserves vulnerability to broadly neutralizing antibodies. *Nat. Commun.* 6, 7479 CrossRef Medline
9. Raska, M., Czerniewska, L., Moldoveanu, Z., Zachova, K., Elliott, M. C., Novak, Z., Hall, S., Hoelscher, M., Maboko, L., Brown, R., Smith, P. D., Mestecky, J., and Novak, Z. (2014) Differential glycosylation patterns of HIV-1 gp120 is associated with differential recognition of HIV-1 by virus-specific antibodies and cell infection. *AIDS Res. Ther.* 11, 23 CrossRef Medline
10. Raska, M., Takahashi, K., Czerniewska, L., Zachova, K., Hall, S., Moldoveanu, Z., Elliott, M. C., Wilson, L., Brown, R., Jancova, D., Barnes, S., Vrbkova, J., Tomana, M., Smith, P. D., Mestecky, J., et al. (2010) Glycosylation patterns of HIV-1 gp120 depend on the type of expressing cells and affect antibody recognition. *J. Biol. Chem.* 285, 20860–20869 CrossRef Medline
11. Morales, J. F., Morin, T. J., Yu, B., Tatsuno, G. P., O’Rourke, S. M., Theolis, R., Jr., Mesa, K. A., and Berman, P. W. (2014) HIV-1 envelope proteins and V1/V2 domain scaffolds with mannose-5 to improve the magnitude and...
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quality of protective antibody responses to HIV-1. J. Biol. Chem. 289, 20526–20542 CrossRef Medline

12. Zhou, T., Doria-Rose, N. A., Cheng, C., Stewart-Jones, G. B. E., Chuang, G. Y., Chambers, M., Druz, A., Geng, H., McKee, K., Kwon, Y. D., O’Dell, S., Saxty, M., Schmidt, S. D., Xu, K., Chen, L., et al. (2017) Quantification of the impact of the HIV-1 glycan shield on antibody elicitation. Cell Rep. 19, 719–732 CrossRef Medline

13. Li, H., Chien, P. C., Jr., Tuen, M., Visciano, M. L., Cohen, S., Blais, S., Xu, C. F., Zhang, H. T., and Hoe, C. E. (2008) Identification of an N-linked glycosylation in the C4 region of HIV-1 envelope gp120 that is critical for recognition of neighboring CD4 T cell epitopes. J. Immunol. 180, 4011–4021 CrossRef Medline

14. Pritchard, L. K., Harvery, D. J., Bonomelli, C., Crispin, M., and Doores, K. J. (2015) Cell- and protein-directed glycosylation of native cleaved HIV-1 gp120 expressed in CHO cells. Biochemistry 39, 11194–11204 CrossRef Medline

15. Cutalo, J. M., Deterding, L. J., and Tomer, K. B. (2004) Characterization of glycopeptides from HIV-1(SF2) gp120 by liquid chromatography mass spectrometry. J. Am. Soc. Mass Spectrom. 15, 1545–1555 CrossRef Medline

16. Go, E. P., Liao, H. X., Alam, S. M., Hua, D., Haynes, B. F., and Desaire, H. (2016) Composition and antigenic effects of individual glycan sites of a native, fully glycosylated, cleaved HIV-1 envelope trimer. J. Virol. 89, 8932–8944 CrossRef Medline

17. Mizuochi, T., Matthews, T. J., Kato, M., Hamako, J., Titani, K., Solomon, J., and Feizi, T. (1990) Diversity of oligosaccharide structures on the envelope glycoprotein gp 120 of human immunodeficiency virus 1 from the lymphoblastoid cell line H9. Presence of complex-type oligosaccharides with bisecting N-acetylgalactosamine residues. J. Biol. Chem. 265, 8519–8524 Medline

18. Panico, M., Boucle, L., Binet, D., O’Connor, M. J., Rahman, D., Pang, P. C., Canis, K., North, S. J., Desrosiers, R. C., Chertova, E., Koebe, B. F., Bess, J. W., Jr., Lifson, J. D., Haslam, S. M., Dell, A., and Morris, H. R. (2016) Mapping the complex glycoproteome of virion-derived HIV-1 gp120 provides insights into broadly neutralizing antibody binding. Sci. Rep. 6, 32956 CrossRef Medline

19. Pritchard, L. K., Harvey, D. J., Bonomelli, C., Crispin, M., and Doores, K. J. (2015) Cell- and protein-directed glycosylation of native cleaved HIV-1 envelope. J. Virol. 89, 8932–8944 CrossRef Medline

20. Go, E. P., Hawawasam, G., Liao, H. X., Chen, H., Ping, L. H., Anderson, J. A., Hua, D. C., Haynes, B. F., and Desaire, H. (2011) Characterization of glycosylation profiles of HIV-1 transmitted/founder envelopes by mass spectrometry. J. Virol. 85, 8270–8284 CrossRef Medline

21. Behrens, A. J., Vasiljevic, S., Pritchard, L. K., Harvey, D. J., Andve, R. S., Krumm, S. A., Struwe, W. B., Cupo, A., Kamar, A., Zittmann, N., Seabright, G. E., Kramer, H. B., Spencer, D. L., Royle, L., Lee, J. H., et al. (2016) Composition and antigenic effects of individual glycan sites of a trimeric HIV-1 envelope glycoprotein. Cell Rep. 14, 2695–2706 CrossRef Medline

22. Lee, J. H., Ozorowski, G., and Ward, A. B. (2016) Cryo-EM structure of a native, fully glycosylated, cleaved HIV-1 envelope trimere. Science 351, 1043–1048 CrossRef Medline

23. Pancera, M., Shahzad-Ul-Hussan, S., Doria-Rose, N. A., McLellan, J. S., Baier, R. T., Dai, K., Loesgen, S., Louder, M. K., Staupe, R. P., Yang, Y., Zhang, B., Parks, R., Eudailey, J., Lloyd, K. E., Blihn, J., et al. (2013) Structural basis for diverse N-glycan recognition by HIV-1-neutralizing V1-V2-directed antibody PG16. Nat. Struct. Mol. Biol. 20, 804–813 CrossRef Medline

24. Pritchard, L. K., Vasiljevic, S., Ozorowski, G., Seabright, G. E., Cupo, A., Ringe, B., Kim, H. I., Sanders, R. W., Doores, K. J., Burton, D. R., Wilson, I. A., Ward, A. B., Moore, J. P., and Crispin, M. (2015) Structural constraints determine the glycosylation of HIV-1 envelope trimers. Cell Rep. 11, 1604–1613 CrossRef Medline

25. Murin, C. D., Julien, J. P., Sok, D., Stanfield, R. L., Khayat, R., Cupo, A., Moore, J. P., Burton, D. R., Wilson, I. A., and Ward, A. B. (2014) Structure of 2G12 Fab2 in complex with soluble and fully glycosylated HIV-1 Env by negative-stain single-particle electron microscopy. J. Virol. 88, 10177–10188 CrossRef Medline

26. Stewart-Jones, G. B., Soto, C., Lemmin, T., Chuang, G. Y., Druz, A., Kong, R., Thomas, P. V., Wagh, K., Zhou, T., Behrens, A. J., Bylund, T., Choi, C. W., Davison, J. R., Georgiev, I. S., Joyce, M. G., et al. (2016) Trimeric HIV-1-Env structures define glycan shields from clades A, B and G. Cell 165, 813–826 CrossRef Medline

27. Vocadlo, D. J., Hang, H. C., Kim, E. J., Hanover, J. A., and Bertozzi, C. R. (2003) A chemical approach for identifying O-GlcNAc-modified proteins in cells. Proc. Natl. Acad. Sci. U.S.A. 100, 9116–9121 CrossRef Medline

28. Hart, C., Chase, L. G., Hajivandi, M., and Agnew, B. (2011) Metabolic labeling and click chemistry detection of glycoprotein markers of mesenchymal stem cell differentiation. Methods Mol. Biol. 695, 459–484 CrossRef Medline

29. Burnham-Marusich, A. R., Snodgrass, C. I., Johnson, A. M., Kiyoshi, C. M., Buzby, S. E., Gruner, M. R., and Berninsone, P. M. (2012) Metabolic labeling of Caenorhabditis elegans primary embryonic cells with azido-sugars as a tool for glycoprotein discovery. PLoS One 7, e49920 CrossRef Medline

30. Chiang, L., Prescher, J. A., Sletten, E. M., Baskin, J. M., Miller, I. A., Agard, N. J., Lo, A., and Bertozzi, C. R. (2010) Copper-free click chemistry in living animals. Proc. Natl. Acad. Sci. U.S.A. 107, 1821–1826 CrossRef Medline

31. Laughlin, S. T., and Bertozzi, C. R. (2007) Metabolic labeling of glycans with azido sugars and subsequent glycan-profiling and visualization via Staudinger ligation. Nat. Protoc. 2, 2930–2944 CrossRef Medline

32. Luchansky, S. J., Hang, H. C., Saxon, E., Grunwell, J. R., Yu, C., Dube, D. H., and Bertozzi, C. R. (2003) Constructing azide-labeled cell surfaces using polysaccharide biosynthetic pathways. Methods Enzymol. 362, 249–272 CrossRef Medline

33. Boyce, M., Carrico, I. S., Ganguli, A. S., Yu, S. H., Hangauer, M. J., Hubbard, S. C., Kohler, J. J., and Bertozzi, C. R. (2011) Metabolic cross-talk allows labeling of O-linked β-N-acetylgalactosamine-modified proteins via the N-acetylgalactosamine salvage pathway. Proc. Natl. Acad. Sci. U.S.A. 108, 3141–3146 CrossRef Medline

34. Stockinger, M., Buzby, S. E., Bains, S., Sastry, M., Schmidt, S. D., Xu, K., Kong, Y., Geng, H., McKee, K., Wied, A. J., Doores, K. J., Burton, D. R., Wilson, I. A., and Wang, I. A. (2013) Antibody cross-competition analysis of HIV-1 envelope glycoprotein gp120 in complex with soluble and fully glycosylated HIV-1 Env by negative-stain single-particle electron microscopy. J. Virol. 88, 1604–1613 CrossRef Medline
58. Park, C. G., Takahara, K., Umemoto, E., Yashima, Y., Matsubara, K., Matsuda, Y., Clausen, B., E. C., Inaba, K., and Steinman, R. M. (2001) Five mouse homologues of the human dendritic cell C-type lectin, DC-SIGN. *Int. Immunol.* 13, 1283–1290 CrossRef Medline

59. Powlesland, A. S., Ward, E. M., Sadhu, S. K., Guo, Y., Taylor, M. E., and Drickamer, K. (2006) Widely divergent biochemical properties of the complete set of mouse DC-SIGN-related proteins. *J. Biol. Chem.* 281, 20440–20449 CrossRef Medline

60. Manca, F. (1992) Galactose receptors and presentation of HIV envelope glycoprotein to specific human T cells. *J. Immunol.* 148, 2278–2282 Medline

61. Hart, M. L., Saifuddin, M., Uemura, K., Bremer, E. E., Hooker, B., Kawasaki, T., and Spear, G. T. (2002) High mannose glycan and sialic acid on gp120 regulate binding of mannose-binding lectin (MBL) to HIV type 1. *AIDS Res. Hum. Retroviruses* 18, 1311–1317 CrossRef Medline

62. Hart, M. L., Saifuddin, M., and Spear, G. T. (2003) Glycosylation inhibitors and neuraminidase enhance human immunodeficiency virus type 1 binding and neutralization by mannose-binding lectin. *J. Gen. Virol.* 84, 353–360 CrossRef Medline

63. Andrè, S., Seed, B., Eberle, J., Schratt, W., Bülßmann, A., and Haas, J. (1998) Increased immune response elicited by DNA vaccination with a synthetic gp120 sequence with optimized codon usage. *J. Virol.* 72, 1497–1503 Medline

64. Haas, J., Park, E. C., and Seed, B. (1996) Codon usage limitation in the expression of HIV-1 envelope glycoprotein. *Curr. Biol.* 6, 315–324 CrossRef Medline

65. Wyatt, R., Moore, J., Accola, M., Desjardin, E., Robinson, J., and Sodroski, J. (1995) Involvement of the V1/V2 variable loop structure in the exposure of human immunodeficiency virus type 1 gp120 epitopes induced by receptor binding. *J. Virol.* 69, 5723–5733 Medline

66. Moore, J. P., Thali, M., Jameson, B. A., Vignaux, F., Lewis, G. K., Poon, S. W., Charles, M., Fung, M. S., Sun, B., and Durda, P. J. (1993) Immunological analysis of the gp120 surface glycoprotein of human immunodeficiency virus type 1: probing the structure of the C4 and V4 domains and the interaction of the C4 domain with the V3 loop. *J. Virol.* 67, 4785–4796 Medline

67. Mascola, J. R., Lewis, M. G., Stiegler, G., Harris, D., VanCott, T. C., Hayes, D., Louder, M. K., Brown, C. R., Sapan, C. V., Frankel, S. S., Lu, Y., Robb, M. L., Katinger, H., and Bix, D. L. (1999) Protection of macaques against pathogenic simian/human immunodeficiency virus 89.6PD by passive transfer of neutralizing antibodies. *J. Virol.* 73, 4009–4018 Medline

68. Etherad-Moghadam, B., Sun, Y., Nicholson, E. K., Karlsson, G. B., Schenten, D., and Sodroski, J. (1999) Determinants of neutralization resistance in the envelope glycoproteins of a simian-human immunodeficiency virus passaged in vivo. *J. Virol.* 73, 8873–8879 Medline

69. Crawford, J. M., Earl, P. L., Moss, B., Reimann, K. A., Wyand, M. S., Manahan, K. H., Bilska, M., Zhou, J. T., Pauza, C. D., Parren, P. W., Burton, D. R., Sodroski, J. G., Letvin, N. L., and Montefiori, D. C. (1999) Characterization of primary isolate-like variants of simian-human immunodeficiency virus. *J. Virol.* 73, 10199–10207 Medline

70. Buchacher, A., Predl, R., Strutzenberger, K., Steinhauer, W., P., Trkola, A., Purttscher, M., Gruber, G., Tauer, C., Steinfeld, F., and Junghauer, A. (1994) Generation of human monoclonal antibodies against HIV-1 proteins: electrofusion and Epstein-Barr virus transformation for peripheral blood lymphocyte immortalization. *AIDS Res. Hum. Retroviruses* 10, 359–369 CrossRef Medline

71. Mbua, N. E., Li, X., Flanagan-Steet, H. R., Meng, L., Aoki, K., Moremen, K. W., Drickamer, K. (2013) Selective exo-enzymatic labeling of N-glycans on the surface of living cells by recombinant ST6GalI. *Angew. Chem. Int. Ed. Engl.* 52, 13012–13015 CrossRef Medline

72. Ning, X., Guo, J., Wolfert, M. A., and Boons, G. J. (2008) Visualizing metabolically labeled glycoproducts of living cells by copper-free and fast huisgen cycloadditions. *Angew. Chem. Int. Ed. Engl.* 47, 2253–2255 CrossRef Medline

73. Meng, L., Forouhar, F., Thieker, D., Gao, Z., Ramiah, A., Moniz, H., Xiang, Y., Seetharaman, J., Milanina, S., Su, M., Bridger, R., Veillon, L., Azadi, P., Kornhaber, G., Wells, L., Montelione, G. T., Woods, R. J., Tong, L., and Moremen, K. W. (2013) Enzymatic basis for N-glycan sialylation: structure of rat α2-6
Sialyltransferase (ST6GAL1) reveals conserved and unique features for glycan sialylation. *J. Biol. Chem.* **288**, 34680–34698 CrossRef Medline

74. Xiang, Y., Karaveg, K., and Moremen, K. W. (2016) Substrate recognition and catalysis by GH47 α-mannosidases involved in Asn-linked glycan maturation in the mammalian secretory pathway. *Proc. Natl. Acad. Sci. U.S.A.* **113**, E7890–E7899 CrossRef Medline

75. Backliwal, G., Hildinger, M., Chenuet, S., Wulhfard, S., De Jesus, M., and Wurm, F. M. (2008) Rational vector design and multi-pathway modulation of HEK 293E cells yield recombinant antibody titers exceeding 1 g/l by transient transfection under serum-free conditions. *Nucleic Acids Res.* **36**, e96 CrossRef Medline

76. Sanders, R. W., Derking, R., Cupo, A., Julien, J. P., Yasmeen, A., de Val, N., Kim, H. J., Blattner, C., de la Peña, A. T., Korzun, J., Golabek, M., de Los Reyes, K., Ketas, T. J., van Gils, M. J., King, C. R., *et al.* (2013) A next-generation cleaved, soluble HIV-1 Env trimer, BG505 SOSIP.664 gp140, expresses multiple epitopes for broadly neutralizing but not non-neutralizing antibodies. *PLoS Pathog.* **9**, e1003618 CrossRef Medline

77. Anumula, K. R., and Taylor, P. B. (1992) A comprehensive procedure for preparation of partially methylated alditol acetates from glycoprotein carbohydrates. *Anal. Biochem.* **203**, 101–108 CrossRef Medline

78. Aoki, K., Perlman, M., Lim, J. M., Cantu, R., Wells, L., and Tiemeyer, M. (2007) Dynamic developmental elaboration of N-linked glycan complexity in the *Drosophila melanogaster* embryo. *J. Biol. Chem.* **282**, 9127–9142 CrossRef Medline

79. Porterfield, M., Zhao, P., Han, H., Cunningham, J., Aoki, K., Von Hoff, D. D., Demeure, M. J., Pierce, J. M., Tiemeyer, M., and Wells, L. (2014) Discrimination between adenocarcinoma and normal pancreatic ductal fluid by proteomic and glycomic analysis. *J. Proteome Res.* **13**, 395–407 CrossRef Medline

80. Lim, J. M., Aoki, K., Angel, P., Garrison, D., King, D., Tiemeyer, M., Bergmann, C., and Wells, L. (2009) Mapping glycans onto specific N-linked glycosylation sites of *Pyrus communis* PGIP redefines the interface for EPG-PGIP interactions. *J. Proteome Res.* **8**, 673–680 CrossRef Medline

81. Varki, A., Cummings, R. D., Aebi, M., Packer, N. H., Seeberger, P. H., Esko, J. D., Stanley, P., Hart, G., Darvill, A., Kinoshita, T., Prestegard, J. J., Schnaar, R. L., Freeze, H. H., Marth, J. D., Bertozzi, C. R., *et al.* (2015) Symbol nomenclature for graphical representations of glycans. *Glycobiology* **25**, 1323–1324 CrossRef Medline

Specific labeling and tracing of gp120 glycans

78. Aoki, K., Perlman, M., Lim, J. M., Cantu, R., Wells, L., and Tiemeyer, M. (2007) Dynamic developmental elaboration of N-linked glycan complexity in the *Drosophila melanogaster* embryo. *J. Biol. Chem.* **282**, 9127–9142 CrossRef Medline

79. Porterfield, M., Zhao, P., Han, H., Cunningham, J., Aoki, K., Von Hoff, D. D., Demeure, M. J., Pierce, J. M., Tiemeyer, M., and Wells, L. (2014) Discrimination between adenocarcinoma and normal pancreatic ductal fluid by proteomic and glycomic analysis. *J. Proteome Res.* **13**, 395–407 CrossRef Medline

80. Lim, J. M., Aoki, K., Angel, P., Garrison, D., King, D., Tiemeyer, M., Bergmann, C., and Wells, L. (2009) Mapping glycans onto specific N-linked glycosylation sites of *Pyrus communis* PGIP redefines the interface for EPG-PGIP interactions. *J. Proteome Res.* **8**, 673–680 CrossRef Medline

81. Varki, A., Cummings, R. D., Aebi, M., Packer, N. H., Seeberger, P. H., Esko, J. D., Stanley, P., Hart, G., Darvill, A., Kinoshita, T., Prestegard, J. J., Schnaar, R. L., Freeze, H. H., Marth, J. D., Bertozzi, C. R., *et al.* (2015) Symbol nomenclature for graphical representations of glycans. *Glycobiology* **25**, 1323–1324 CrossRef Medline

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