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Exclusive Decoration of Simian Immunodeficiency Virus Env with High-Mannose Type N-Glycans Is Not Compatible with Mucosal Transmission in Rhesus Macaques

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Human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) envelope (Env) proteins are extensively decorated with N-glycans, predominantly of the high-mannose type. However, it is unclear how high-mannose N-glycans on Env impact viral spread. We show that exclusive modification of SIV Env with these N-glycans reduces viral infectivity and abrogates mucosal transmission, despite increasing viral capture by immune cell lectins. Thus, high-mannose N-glycans have opposed effects on SIV infectivity and lectin reactivity, and a balance might be required for efficient mucosal transmission.

Infection with human immunodeficiency virus (HIV) and the ensuing disease, AIDS, continue to claim almost 2 million lives every year (1). HIV employs its envelope (Env) protein to enter host cells, and Env constitutes the sole target for the neutralizing antibody response (2). Moreover, Env and its cellular interaction partners, CD4 and CCR5/CXCR4, are real or potential targets for antiviral therapy (3). Therefore, understanding Env function is a prerequisite to the development of vaccines and novel entry inhibitors.

Env is synthesized as a precursor protein, gp160, in the secretory pathway of infected cells. In the Golgi apparatus, gp160 is processed by furin into the surface unit, gp120, and the transmembrane unit, gp41, which remain noncovalently associated (4). A hallmark of Env is its heavy glycosylation: Env is extensively modified by N-glycans, and its glycan coat protects underlying epitopes from neutralizing antibodies (5, 6). Moreover, the Env glycans are bound by immune cell lectins (7), and these interactions are believed to modulate the efficiency of mucosal transmission by shaping immune responses (8) and by targeting the virus for either transmission or degradation (9–11).

N-glycosylation commences in the endoplasmic reticulum (ER) with the en bloc transfer of a mannose-rich precursor oligosaccharide onto a protein. These oligosaccharides are trimmed by ER and *cis*-Golgi resident enzymes into high-mannose type N-glycans, which are usually further processed into hybrid and complex types upon transport through the Golgi apparatus (11). In contrast, the majority of Env N-glycans are incompletely processed, due to their high density and recessed location, and thus remain in the high-mannose form (12–18). Several studies on HIV and/or simian immunodeficiency virus (SIV) demonstrated that the amounts of high-mannose type N-glycans attached to Env differ markedly between viruses produced in the major viral target cells, T cells and macrophages (19–22). Moreover, some (19, 21, 23) but not all (24, 25) studies suggested that the content of high-mannose type N-glycans negatively correlates with viral infectivity. On the other hand, increased decoration of Env with high-mannose type N-glycans can augment lectin-mediated transfer of HIV from dendritic cells to T cells (20, 23), which might promote viral transmission via the mucosal route. Here, we investigated whether the reduced infectivity or the increased viral transfer by lectins on mucosal cells mirrors the impact of high-mannose type N-glycans on mucosal transmission, the major route of viral spread worldwide.

Production of HIV and SIV in GnTI$^-$ cells reduces viral infectivity in a strain-dependent manner. In order to produce virions with Env proteins exclusively modified with high-mannose type N-glycans, we employed *N*-acetylglucosamine transferase I-deficient (GnTI$^-$) HEK293S cells, since glycan processing in these cells is trapped at the high-mannose state (26). We first investigated whether HIV and SIV produced in GnTI$^-$ and parental cells exhibit differential infectivity. For this, we employed the well-characterized molecular clones HIV-1 NL4-3, HIV-1 JR-CSF, SIVmac239, and SIVmac239/316 Env. The latter SIV clone is identical to SIVmac239 but harbors 9 amino acid exchanges in Env that are associated with macrophage tropism in cell culture. They were identified in viral isolates from a SIVmac239-infected animal that died from simian AIDS and exhibited marked neuropathology and pneumonia (27, 28).

Infection of TZMbl reporter cells with virus stocks normalized

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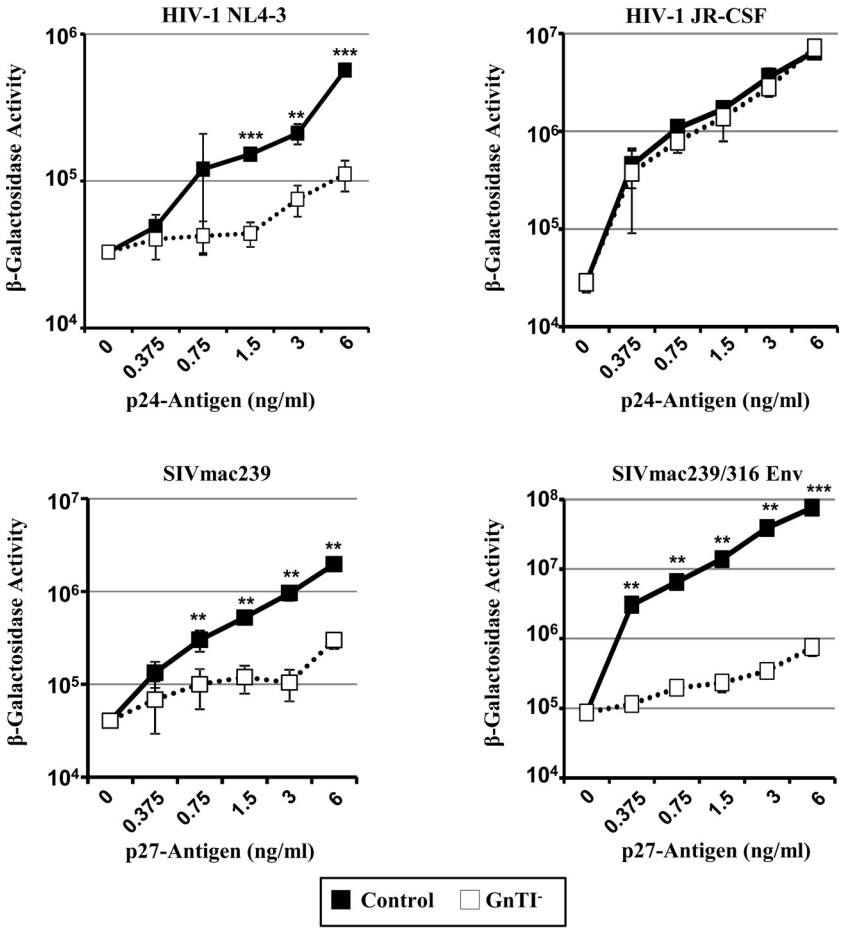

FIG 1 Exclusive modification of Env with high-mannose type N-glycans reduces HIV/SIV infectivity in a strain-dependent manner. TZM-bl cells were incubated with capsid protein-normalized stocks of the indicated viruses produced in GnTI$^-$ or control cells. Input virus was removed at 5 h, and β-galactosidase activity in cell lysates was measured at 72 h postinfection. The results of single representative experiments carried out with quadruplicate samples are shown; error bars indicate standard deviation (SD). Similar results were obtained in two to three separate experiments. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

for capsid protein content revealed that production of HIV-1 NL4-3, SIVmac239, and SIVmac239/316 Env in GnTI$^-$ cells diminished viral infectivity (Fig. 1). In contrast, HIV-1 JR-CSF from GnTI$^-$ and control cells displayed similar infectivity (Fig. 1). Thus, generation of virions in cells that exclusively produce high-mannose type N-glycans can reduce HIV and SIV infectivity, but this effect depends on the molecular clone/strain studied.

SIVmac239/316 Env produced in GnTI$^-$ cells harbors exclusively high-mannose type N-glycans. We focused our subsequent analyses on SIVmac239/316 Env because this virus showed the most profound reduction in infectivity when produced in GnTI$^-$ cells. First, we confirmed that virions generated in GnTI$^-$ cells (termed GnTI-SIV) and control cells (termed wt SIV) are differentially glycosylated. For this, viral particles were concentrated by centrifugation through a sucrose cushion and analyzed by Western blotting (Fig. 2A). Both virus preparations contained more gp160 than proteolytically processed gp120. This effect was not unexpected since earlier data demonstrated reduced cleavage efficiency when large amounts of Env are produced (29). Moreover, gp160/gp120 proteins of GnTI-SIV migrated faster than their counterparts of wt SIV (Fig. 2A), in keeping with incorporation of high-mannose type N-glycans, which exhibit a lower molecular weight than hybrid- or complex-type N-glycans. Indeed, digest with endo-β-*N*-acetylglucosaminidase H (endo H), which selectively removes high-mannose type N-glycans, and peptide-*N*-glycosidase F (PNGase F), which removes all N-linked glycans, confirmed that gp160/gp120 from GnTI-SIV but not wt SIV exclusively harbored high-mannose type N-glycans (Fig. 2A). The slightly faster migration of GnTI-SIV Env bands upon endo H relative to PNGase F treatment most likely reflects a more complete digest. Finally, multiplex capillary gel electrophoresis with laser-induced fluorescence detection (xCGE-LIF) confirmed that gp120 from GnTI-SIV was exclusively modified with high-mannose type N-glycans (not shown). Collectively, our results show that Env of GnTI-SIV is exclusively decorated with high-mannose type N-glycans.

Reduced incorporation of mature Env into GnTI-SIV virions does not account for reduced infectivity. A potential explanation for the low infectivity of GnTI-SIV could be altered incorporation of Env into the virion. The Western blot analysis described above was therefore repeated with virus preparations normalized for capsid protein content. The results showed that roughly 10-fold less gp120 was present in GnTI-SIV relative to wt SIV particles (Fig. 2B), raising the question whether the reduced virion incor-

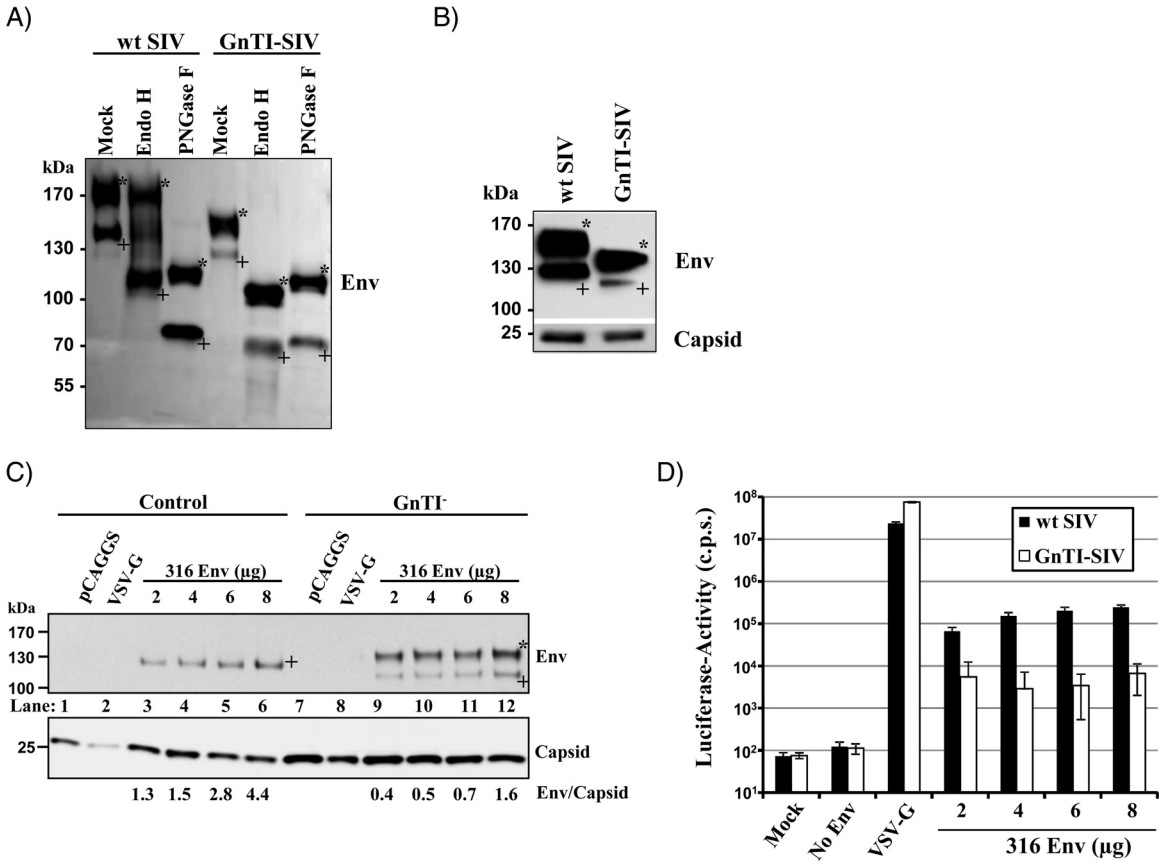

FIG 2 Incorporation of gp120 into GnTI-SIV is reduced, but this defect does not account for the diminished viral infectivity. (A) Concentrated preparations of wt SIV and GnTI-SIV, normalized for equal contents of gp160, were mock treated or digested with endo H or PNGase F, and the migration pattern of Env was analyzed by Western blotting using an antibody against gp120. Similar results were obtained in a separate experiment. (B) Stocks of wt SIV and GnTI-SIV containing equal amounts of capsid antigen were concentrated and analyzed for Env and p27 capsid protein content by Western blotting. Similar results were obtained in three separate experiments. (C) Env-defective HIV-1 particles pseudotyped with SIVmac239/316 Env were produced in GnTI$^-$ and control cells and concentrated, and the Env and p24 capsid protein content was analyzed by Western blotting. Comparable results were obtained in a separate experiment. (D) Particles were produced as described for panel C, and particle infectivity was determined using GHOST CD4$^+$ CCR5$^+$ cells as targets. The results of a single representative experiment performed with quadruplicate samples are shown and were confirmed in two separate experiments. Error bars indicate SD. In panels A to C, bands corresponding to gp160 are marked with an asterisk, and bands corresponding to gp120 are marked with a plus sign.

poration of mature Env may account for the diminished viral infectivity of GnTI-SIV. To clarify this issue, we produced lentiviral pseudotypes in cells transfected to express rising amounts of Env and analyzed particle incorporation of Env (Fig. 2C) as well as infection of target cells (Fig. 2D). In general, pseudotypes generated in control cells were more infectious than their counterparts released from GnTI$^-$ cells. In particular, pseudotypes from control cells, which harbored roughly comparable amounts of gp120 relative to pseudotypes generated in GnTI$^-$ cells (Fig. 2C, compare lanes 4 and 12), were 23-fold more infectious (Fig. 2D), indicating that the reduced infectivity of GnTI-SIV relative to wt SIV was not due to reduced particle incorporation of Env.

Increased binding of GnTI-SIV to high-mannose-specific lectins but absence of mucosal transmission. Binding of Env to soluble lectins can block viral entry into target cells, while engagement of membrane-associated, mucosal lectins might augment mucosal transmission (11, 30). Therefore, we examined whether GnTI-SIV exhibits altered lectin reactivity compared to wt SIV. We found that GnTI-SIV was more sensitive toward inhibition by the soluble mannose-specific lectins cyanovirin-N (CV-N) and *Galanthus nivalis* agglutinin (GNA) than wt SIV (Fig. 3A). In contrast, infectivity of GnTI-SIV and wt SIV was slightly and comparably augmented by *Ulex europaeus* agglutinin (UEA), which recognizes fucose (Fig. 3A). Thus, GnTI-SIV is more readily inhibited by mannose-specific lectins than wt SIV, in line with the exclusive modification of GnTI-SIV with high-mannose type N-glycans.

The mannose-binding lectin DC-SIGN (dendritic cell-specific ICAM3-grabbing nonintegrin, CD209) binds to HIV and SIV Env, and a number of studies (31, 32) have provided evidence that DC-SIGN on dendritic cells promotes mucosal transmission by facilitating viral transfer to adjacent T cells. We employed Raji B cells stably expressing DC-SIGN and the DC-SIGN-related lectin DC-SIGNR (CD209L) to analyze SIV interactions with these lectins. Virus-exposed DC-SIGN$^+$ or DC-SIGNR$^+$ Raji cells transmitted GnTI-SIV with significantly higher efficiency than wt SIV (Fig. 3B), demonstrating that the exclusive decoration of Env with high-mannose type N-glycans can augment viral transfer by mucosal lectins.

The results obtained so far raised the question of whether the decreased viral infectivity for indicator cells or the increased capture by mucosal lectins would be predictive for the efficiency of mucosal transmission of GnTI-SIV. Therefore, we examined

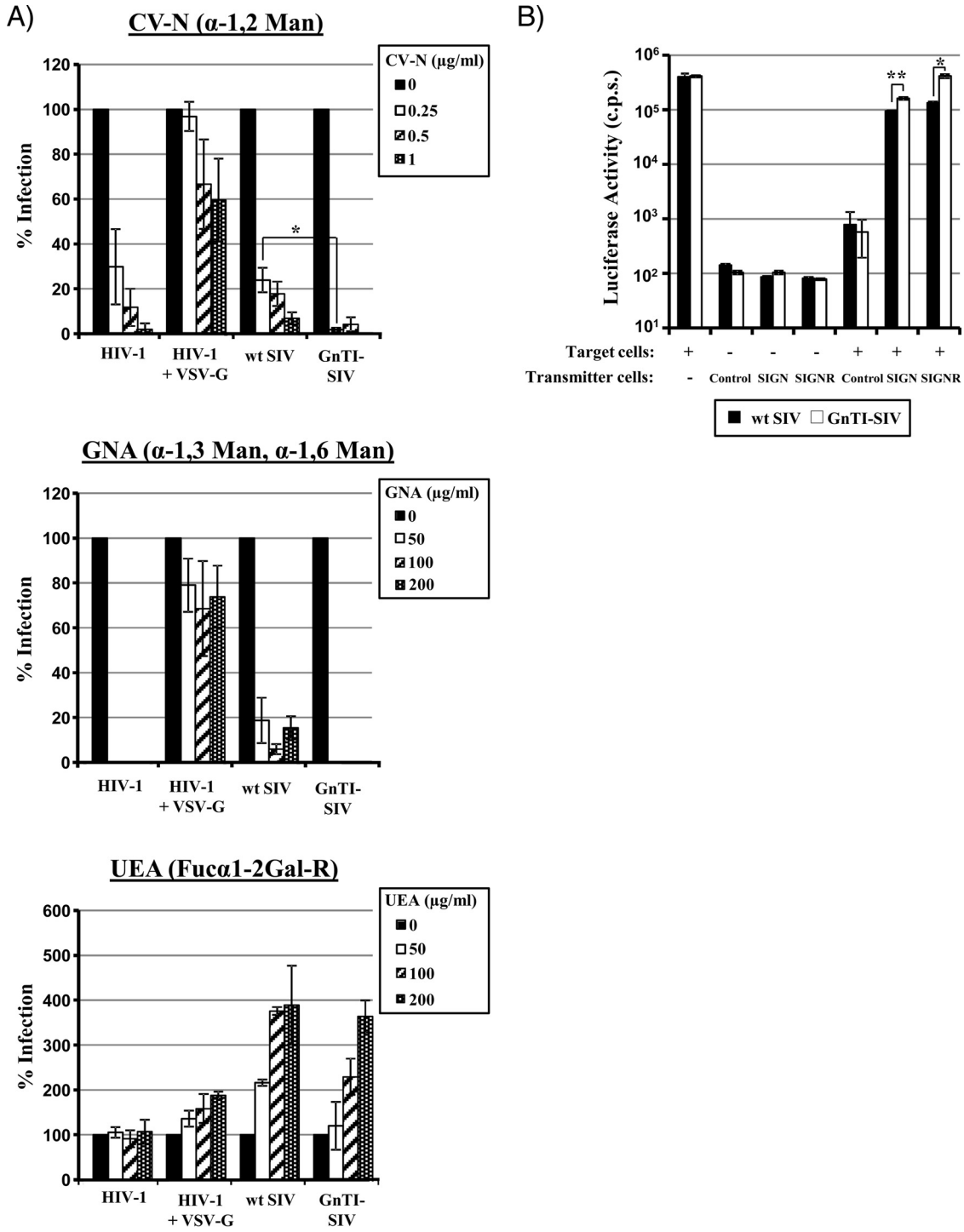

FIG 3 GnTI-SIV is more sensitive to neutralization by soluble, mannose-specific lectins and is better transmitted by mucosal lectins than wt SIV. (A) Infectivity-normalized stocks of wt SIV, GnTI-SIV, HIV-1 NL4-3 (positive control), and HIV-1 NL4-3 pseudotyped with the G protein of vesicular stomatitis virus (VSV-G [negative control]) were incubated with phosphate-buffered saline (PBS) or lectins prior to infection of TZM-bl indicator cells. Input virus was removed at 5 h, and β-galactosidase activity in cell lysates was analyzed at 72 h postinfection. The results of a representative experiment performed with triplicate samples are shown. Infectivity in the absence of lectin was set as 100%, and error bars indicate SD. Similar results were obtained in at least one independent experiment. (B) Parental Raji B cells (control) or Raji cells engineered to express DC-SIGN (SIGN) or DC-SIGNR (SIGNR) were incubated with equal volumes of wt SIV or GnTI-SIV stocks normalized for infectivity. Unbound virus was removed by washing, the transmitter cell lines were cocultured with the CEMx174 R5 target cells, and the luciferase activity in the cell lysates was measured at 72 h post-cocultivation. The results of a single experiment performed with triplicate samples are shown and are representative of two separate experiments. Error bars indicate SD. *, $P \leq 0.05$; **, $P \leq 0.01$. c.p.s., counts per second; UEA, *Ulex europaeus* agglutinin; CV-N, cyanovirin-N; GNA, *Galanthus nivalis* agglutinin.

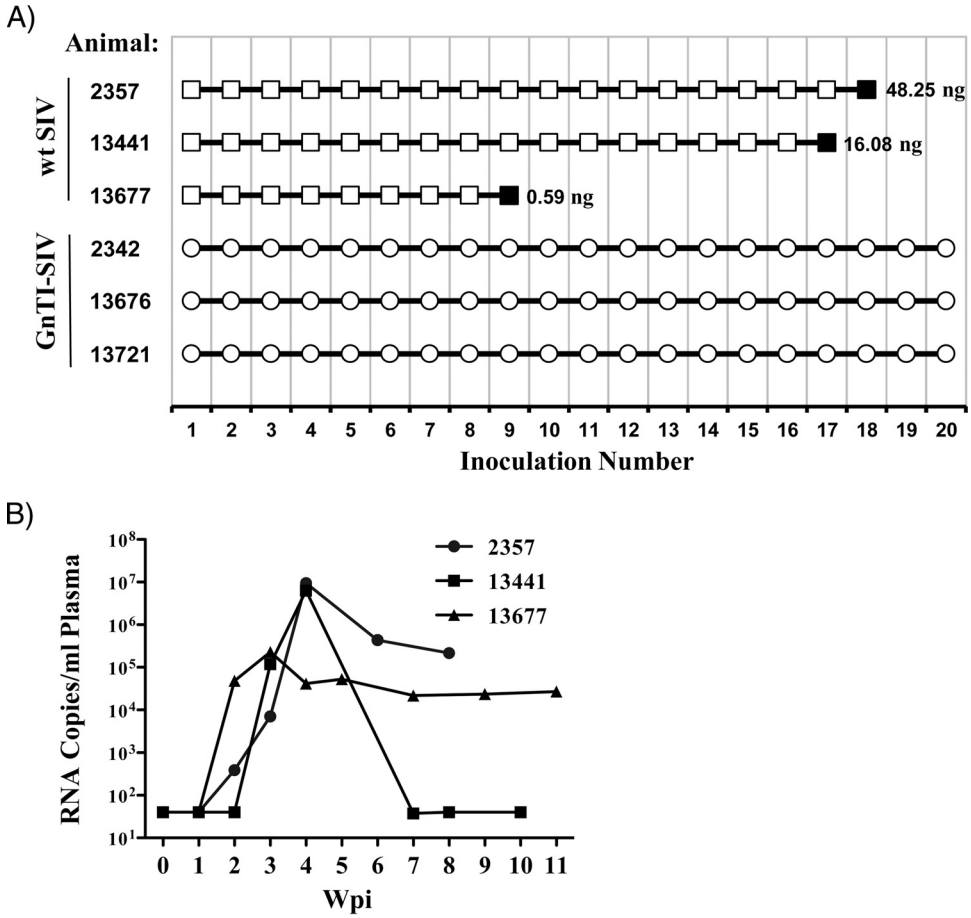

FIG 4 GnTI-SIV is not transmittable through the rectal mucosa of rhesus macaques. (A) Rhesus macaques ($n = 3$) were rectally inoculated with escalating doses of wt SIV and GnTI-SIV, respectively. For this, animals were exposed every 3 weeks to equal volumes of virus stocks normalized for capsid protein content. At inoculation 1, animals were exposed to 0.00009 ng of virus, and doses were escalated by 3-fold increases until inoculation 9, remained constant during exposures 9 to 11 (0.59 ng) and 12 to 15 (1.77 ng), and were then escalated by 3-fold increases until inoculation 20 (434.2 ng). Infection of animals was monitored weekly via SIV-specific reverse transcription-PCR (RT-PCR). RNA positivity is indicated by solid symbols, while negative results are represented by open symbols. (B) The plasma viral load in infected animals was determined in 1- to 2-week intervals by RT-PCR for at least 8 weeks postinfection (Wpi).

GnTI-SIV and wt SIV transmission to rhesus macaques upon repeated rectal inoculation. For this, three rhesus macaques per group were exposed to escalating doses of GnTI-SIV and wt SIV stocks, respectively. The stocks were normalized for equal capsid protein content, and animals were exposed to virus until they became SIV RNA positive or until the maximal virus dose applicable under the chosen conditions was reached. All animals exposed to wt SIV acquired infection (Fig. 4A) and showed peak viral loads of between 10^5 and 10^8 copies per ml (Fig. 4B), which matches previous findings obtained with intravenously inoculated SIVmac239/316 Env (33). In contrast, none of the animals inoculated with GnTI-SIV became SIV RNA positive (Fig. 4A) or seroconverted (not shown), despite exposure to inocula containing up to 433 ng SIV capsid antigen. These findings suggest that GnTI-SIV is unable to overcome the mucosal barrier despite augmented interactions with mannose-specific lectins.

The impact of high-mannose type N-glycans on HIV/SIV infectivity was examined previously, and partially inconsistent results were obtained. A negative correlation between the amount of high-mannose glycans on Env and viral infectivity has been reported based on the analysis of host-cell-specific glycosylation differences (19–21). Moreover, removal of high-mannose type N-glycans from SIV Env was shown to increase viral infectivity (19), and blockade of glycan processing by a mannosidase I inhibitor was demonstrated to reduce HIV-1 YU-2 infectivity without diminishing virion incorporation of Env (23). In contrast to these findings, a separate study documented that HIV-1 LAI and JR-CSF from GnTI$^-$ and control cells were comparably infectious (25). The same authors showed that vectors bearing HIV and SIV Env proteins displayed reduced infectivity upon generation in GnTI$^-$ cells (24). However, it was concluded that the diminished infectivity was due to diminished particle incorporation of Env and not altered Env glycosylation (24).

We found that HIV-1 NL4-3, SIVmac239, and SIVmac239/316 Env, but not HIV-1 JR-CSF, from GnTI$^-$ cells were less infectious than control viruses. The reduced infectivity of SIVmac239/316 Env (GnTI-SIV) relative to wt SIV was paralleled by a reduction of particle incorporation of gp120, but analysis of pseudotypes containing comparable amounts of gp120 revealed that modification with high-mannose type N-glycans and not reduced Env incorporation accounted for the diminished infectivity of GnTI-SIV. We cannot exclude that Env incorporation into HIV-1 NL4-3 and

SIVmac239 from GnTI$^-$ cells was altered relative to control viruses and contributed to the reduced infectivity. However, our findings and previous studies are most compatible with the concept that extensive decoration of HIV and SIV Env proteins with high-mannose type N-glycans can diminish viral infectivity in a viral strain/molecular clone-dependent fashion.

The lectin DC-SIGN binds to glycans on HIV Env and contributes to the ability of dendritic cells to capture and transmit infectious HIV to target T cells *in vitro* (31, 34), although the extent of the contribution is controversial (35–37), and it should be noted that DC-SIGN can also target HIV for degradation (9, 10). Moreover, DC-SIGN can facilitate the establishment of infectious synapses between dendritic cells and T cells (38) and can shape immune responses in a glycan-dependent fashion (8). Thus, DC-SIGN$^+$ dendritic cells might play an important role in mucosal HIV/SIV transmission. DC-SIGN binds to mannose- and fucose-containing glycans (39, 40), and the increased DC-SIGN- and DC-SIGNR-dependent transfer of GnTI-SIV relative to wt SIV was therefore not unexpected. In fact, similar results were obtained by three separate studies (23, 25, 41), although, importantly, two noted that production of virus in GnTI$^-$ cells increased HIV-1 capture by DC-SIGN but not transmission (25, 41). In fact, HIV-1 NL4-3 from GnTI$^-$ cells was slightly less efficiently transmitted than control virus (not shown), suggesting that the impacts of high-mannose type N-glycans on viral transmission might differ between SIV and HIV and/or between viral isolates. In sum, the quantity and quality of the glycan coat are believed to impact mucosal transmission of HIV and SIV, and the forced incorporation of high-mannose type N-glycans into Env increased SIV reactivity with DC-SIGN and potentially other mucosal lectins.

The repetitive rectal exposure of rhesus macaques to escalating doses of wt SIV established infection in all inoculated animals, although an unexpectedly high number of inoculations was required. In stark contrast, none of the GnTI-SIV-exposed animals became infected even after the viral dose was escalated to the maximum, which was applied two times (inoculations 20 and 21) and which contained 7.5-fold more infectious units than were minimally required to establish infection with wt SIV. These findings can be most easily reconciled with a scenario in which reduced SIV infectivity due to extensive decoration of Env with high-mannose type N-glycans might be incompatible with mucosal transmission, a process that encompasses penetration of the mucosa and establishment of a founder virus population (42), and cannot be rescued by augmented lectin reactivity. Our results await confirmation with other SIVs, and it needs to be considered that the effects of high-mannose type N-glycans on infectivity and transmission might differ between SIV and HIV and between viral isolates. Moreover, it cannot be excluded that the reduced Env incorporation into GnTI-SIV relative to wt SIV impacted the efficiency of mucosal transmission. Collectively, however, the results from our study suggest that incorporation of high-mannose type N-glycans into Env has opposed effects on SIV infectivity and lectin-mediated transfer, which may need to be in balance for efficient mucosal transmission. Tipping this balance toward optimal binding to mannose-recognizing lectins may not be compatible with penetrating the mucosal barrier and/or establishing a founder virus population.

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The authors declare they have no competing financial interests.

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