Rapid development of glycan-specific, broad, and potent anti–HIV-1 gp120 neutralizing antibodies in an R5 SIV/HIV chimeric virus infected macaque

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It is widely believed that the induction of a broadly neutralizing antibody (bNAb) response will be a critical component of a successful vaccine against HIV. A significant fraction of HIV-infected individuals mount bNAb responses, providing support for the notion that such responses could be elicited through vaccination. Infection of macaques with simian immunodeficiency virus (SIV) or SIV/HIV chimeric virus (SHIV) has been widely used to model aspects of HIV infection, but to date, only limited bNAb responses have been described. Here, we screened plasma from 14 R5-tropic SHIV-infected macaques for broadly neutralizing activity and identified a macaque with highly potent cross-clade plasma NAb response. Longitudinal studies showed that the development of broad and autologous NAb responses occurred coincidentally in this animal. Serum-mapping studies, using pseudovirus point mutants and antigen adsorption assays, indicated that the plasma bNAbs are specific for epitopes that include carbohydrates and are critically dependent on the glycan at position 332 of Env gp120. The results described herein provide insight into the development and evolution of a broad response, suggest that certain bNAb specificities may be more rapidly induced by immunization than others, and provide a potential model for the facile study of the development of bNAb responses.

Understanding the complex interplay of HIV-1 and the immune system in infected individuals may inform HIV-1 vaccine design. A series of studies have demonstrated that roughly 5–30% of HIV-1–infected individuals develop broadly neutralizing antibodies (bNAbs) over time (1–3), dependent upon the criteria used to define breadth and potency of neutralization. When passively administered, bNAbs have been shown to protect against infection by chimeric simian HIV (SHIV) challenge in macaque models (4–10). However, no immunogens developed to date have succeeded in eliciting significant bNAb responses. Understanding the development of such responses during natural infection may provide important clues for designing more appropriate immunogens.

A number of studies have characterized the antibody specificities mediating plasma neutralization breadth and potency in HIV-1–infected individuals (1, 11–13). Furthermore, a few longitudinal studies have examined the factors associated with the development of breadth, and although there are some inconsistencies, it has been suggested that broad neutralization correlates with time postinfection, plasma viremia levels, $CD4^+$ T-cell counts at set-point, and binding avidity to the envelope protein (1, 14, 15). Though these studies have provided some insight into the factors associated with the development of bNAbs, detailed longitudinal studies involving bNAbs of different specificities would greatly improve our understanding of the evolution and maturation of broad responses.

SHIVs express the HIV envelope glycoprotein and can therefore be used to evaluate HIV-1 Env-specific neutralizing antibody (NAb) responses. However, although the SHIV/macaque model has been extensively used to evaluate vaccine efficacy, the ability of SHIV-infected macaques to mount highly potent bNAb responses has not yet been demonstrated. Developing a SHIV that is capable of eliciting broad and potent NAb responses, and tracking the evolution of this immune response, might provide unprecedented insight into the factors associated with the development of bNAbs.

Recently, a pathogenic R5-tropic SHIV with the env from a molecularly cloned derivative of HIV-1_{Ada} (pHIV-1_{AD8}) was developed by serial passage of viral "swarms" in macaques (16). To determine whether any of the $SHIV_{AD8}$-infected macaques developed bNAbs, we screened plasma from 14 infected animals for neutralizing activity. Of these, one macaque displayed extraordinarily potent cross-clade plasma NAb responses. Neutralization assays were carried out using samples taken at serial time points, and indicated that the development of broad plasma neutralization was unusually rapid and coincided with the development of autologous NAbs. Furthermore, serum-mapping studies suggested that the bNAbs interact with carbohydrates and are critically dependent on the N332 glycan. The results described herein suggest considerable promise for the SHIV/macaque model in the dissection of bNAb responses to HIV.

Results

Analysis of Plasma Neutralization Breadth. We previously reported that some $SHIV_{AD8}$-infected rhesus monkeys generated autologous NAbs, which generally correlated with levels of set-point viremia (16). Here, plasma samples from 14 $SHIV_{AD8}$-infected macaques were tested for the development of autologous NAbs using either replication-competent (RC) or pseudovirus targets in the TZM-bl cell system (Table S1). Consistent with the previous study, we were only able to demonstrate evidence of virus neutralization in five of 14 infected animals. In two of the five macaques, partial neutralization was detected at a plasma dilution of 1:20. In two of the three remaining monkeys (CJ58 and CJ8B), high NAb titers declined over time to levels <1:100 by year 3 postinfection (Table S1). A single macaque (CE8J) generated high, sustained autologous NAb titers against both RC and pseudovirus targets in the TZM-bl assay.

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Plasma from the five SHIV_{AD8}-infected macaques, which had generated autologous NAbs, were next tested for neutralizing activity against a cross-clade HIV-1 pseudovirus panel containing tier 2 isolates, that had previously been shown to be predictive of neutralization breadth and potency against a larger number of isolates (Fig. 1*A*) (2). Only macaque CE8J developed broad and potent plasma-neutralizing responses. A plasma sample, collected at 77 wk postinfection from macaque CE8J, neutralized five of six HIV-1 pseudoviruses from the cross-clade panel, and notably, four of these viruses were neutralized with exceptional potency ($\text{IC}_{50s} > 1/1500$ plasma dilution). Based on these results, we then tested the macaque CE8J plasma for neutralization against a larger cross-clade pseudovirus panel containing 21 tier 2 isolates; 71% of the viruses were neutralized with extraordinary potency ($\text{IC}_{50s} > 1/1500$ plasma dilution), confirming the remarkably broad and potent activity (Fig. 1*B*).

To determine the component of the CE8J plasma responsible for the broad neutralizing activity, we adsorbed the plasma IgG with either Protein A-Sepharose beads or blank control beads and tested the depleted fractions for neutralizing activity. We observed that no neutralizing activity was present in the Protein A-depleted fraction (Fig. S1), demonstrating that the IgG component of the plasma mediates the broad neutralizing activity.

Evolution of Plasma Neutralization Breadth and Potency in Macaque CE8J. To investigate the development of plasma neutralization breadth and potency in macaque CE8J, we tested 17 plasma samples taken at serial time points (between 20 and 115 wk postinfection) for neutralizing activity. Potent cross-clade plasma neutralization developed between 32 and 36 wk postinfection (Fig. 2). Interestingly, potent neutralizing responses against the autologous virus also developed between these time points. The plasma neutralization potency against most isolates generally either plateaued or increased slightly until 80 wk postinfection, at which point there was a substantial drop in potency against most isolates (Fig. 2 and Fig. S2). Notably, the diminished neutralization potency may have resulted from the sharp decline in memory CD4^{+} T cells and/or spike in viral load, which also occurred at this time point (Fig. S3) (16).

Dissecting the Antibody Specificities Mediating CE8J Plasma Neutralization Breadth. We next sought to define the antibody specificities mediating broad plasma neutralization in macaque CE8J. As a first approach, we investigated whether the bNAb specificities reacted with recombinant gp120 by adsorbing the plasma with JR-FL gp120-coated beads and then testing the flow-through fraction for neutralizing activity against a cross-clade pseudovirus panel (Fig. S4). Indeed, the broad and potent plasma-neutralizing activity could be depleted on the gp120-coated beads, suggesting that gp120-specific antibodies were mediating the breadth of neutralization. Next, we examined the role of the variable loops in forming the epitopes recognized by the plasma bNAbs by performing plasma adsorptions with $\text{gp120}_{\text{JR-FL}}$ ΔV1/V2 and ΔV3-coated beads. Interestingly, the broad plasma-neutralizing activity could be partially adsorbed by $\text{gp120}_{\text{JR-FL}}$ ΔV1/V2 but not by $\text{gp120}_{\text{JR-FL}}$ ΔV3 (Fig. S4).

Because the results described herein suggested that the epitopes recognized by the bNAb specificities may be in proximity to or contiguous with the V3 loop, we next tested the plasma for neutralizing activity against a panel of JR-CSF mutants containing single point mutations in or adjacent to the V3 loop (Fig. 3*A*). Interestingly, we found that the N332A mutation, which results in the removal of a glycosylation site at the base of the V3 loop, completely abolished plasma-neutralizing activity against $\text{HIV-1}_{\text{JR-CSF}}$. To determine whether this mutation also affected broad plasma-neutralizing activity, we tested the plasma for neutralization against a cross-clade panel of pseudoviruses incorporating the N332A mutation (Fig. 3*B*). Indeed, the plasma-neutralizing activity was significantly diminished against all of the mutant pseudoviruses on the panel except for 92RW020. The lack of plasma-neutralization sensitivity to 92RW020 N332A may suggest that the N332 glycan dependency is somewhat isolate-specific, as has been observed for other glycan-dependent and glycan-specific bNAbs (17). Notably, the N332 glycan is important for formation of the epitopes recognized by the glycan-specific bNAb 2G12 and the recently described PGT bNAbs (18, 19). Furthermore, serum-mapping studies have demonstrated that this glycan is critical for broad and potent serum-neutralizing activity in a significant proportion of HIV-1–infected humans (13, 14).

A

			Clade A		Clade B		Clade C	Clade CRF01_AE	Autologous virus	negative control
macaque	weeks post infection	score	92RW020	94UG103	JR-CSF	YU2	MGRM-C26	92TH021	SHIV_{AD8}	SIVmac239
DA24	41	<0.37	<100	<100	<100	<100	<100	<100	<100	<100
DBJE	42	<0.37	<100	<100	<100	<100	<100	<100	<100	<100
CJ58	156	<0.37	<100	<100	<100	<100	<100	<100	<100	<100
CJ8B	180	<0.37	<100	<100	<100	<100	<100	<100	<100	<100
CE8J	77	3.04	>2700	400	>2700	1800	>2700	<100	>2700	<100

B

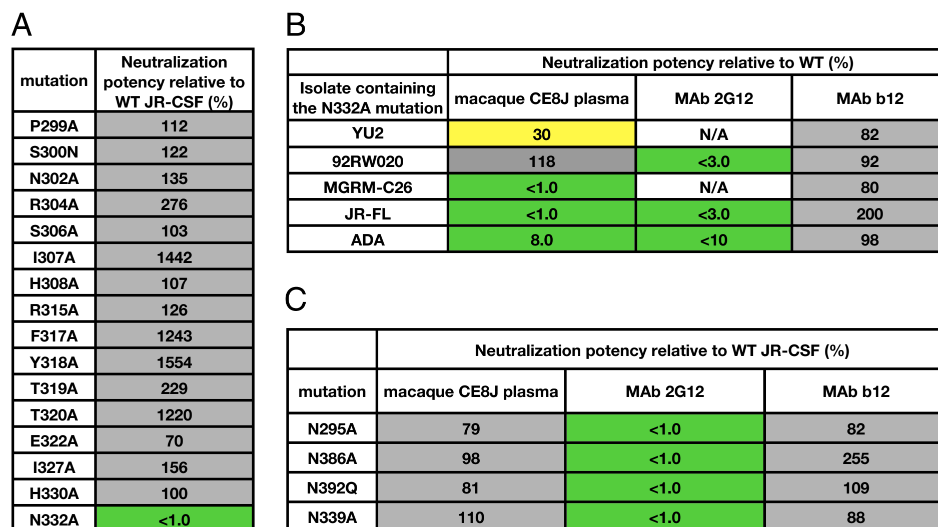
Clade	Isolate	IC_{50} (1/serum dilution)
A	92RW020	20299
	KNH1144	285
	94UG103	400
	92UG037.8	3368
B	JR-CSF	9378
	YU2	1800
	JR-FL	7232
	92BR020	11417
	89.6	8753
	QH0692.42	631
	6535.3	39032
	ADA	2679
C	MGRM-C26	15726
	DU422	4123
	DU156	12398
	93IN905	19600
	DU172	1600
	ZM249M	100
	ZM214	6400
	CAP210.2	200
CRF01_AE	92TH021	<100

Fig. 1. Cross-clade neutralizing activity of macaque plasma. (*A*) Plasma from 5 SHIV_{AD8}-infected macaques were tested for neutralizing activity against a cross-clade tier 2 pseudovirus panel, the autologous virus (SHIV_{AD8}), and a negative control virus (SIVmac239). Values represent the plasma dilution at which 50% neutralization was detected. Scores were designated based on an algorithm incorporating breadth and potency (2). (*B*) Plasma from macaque CE8J was tested against a larger cross-clade pseudovirus panel containing 21 isolates. Boxes are color coded as follows: gray, $\text{IC}_{50} < 1{:}100$; green, $1{:}100 < \text{IC}_{50} < 1{:}300$; yellow, $1{:}300 < \text{IC}_{50} < 1{:}500$; orange, $1{:}500 < \text{IC}_{50} < 1{:}1{,}000$; red, $\text{IC}_{50} > 1{:}1{,}000$.

weeks post infection	score	JR-CSF	MGRM-C26	92RW020	YU2	92BR020	JR-FL	94UG103	92TH021	$SHIV_{AD8}$	SIVmac239 (neg. control)
20	0.37	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100
24	0.37	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100
29	0.37	<100	<100	<100	100	<100	<100	<100	<100	<100	<100
32	0.91	100	<100	<100	350	400	<100	<100	<100	450	<100
36	1.76	<100	805	250	1684	1880	100	<100	<100	811	<100
40	2.28	<100	1600	1337	1014	>2700	1066	<100	<100	3282	<100
46	2.70	250	>2700	>2700	1500	>2700	1657	<100	<100	2873	<100
52	2.75	735	>2700	>2700	1261	>2700	1063	<100	<100	1899	<100
57	2.60	400	>2700	>2700	900	>2700	735	<100	<100	1709	<100
60	2.57	300	>2700	2134	1300	>2700	640	<100	<100	1324	<100
67	2.69	960	>2700	>2700	800	>2700	760	<100	<100	1181	<100
72	2.75	1456	>2700	>2700	870	>2700	740	<100	<100	1907	<100
77	3.28	>2700	>2700	>2700	1800	>2700	>2700	400	<100	5193	<100
80	2.54	1336	1586	>2700	400	1901	720	<100	<100	1181	<100
86	2.31	1002	735	2495	200	2007	550	<100	<100	510	<100
101	2.22	1173	640	2343	250	>2700	450	<100	<100	397	<100
115	2.57	1095	1050	>2700	350	>2700	1295	<100	<100	1106	<100

Fig. 2. Development of broad neutralization in macaque CE8J. Plasma samples collected from macaque CE8J at serial time points were tested for neutralizing activity against a cross-clade pseudovirus panel containing tier 2 viruses, the autologous $SHIV_{AD8}$ virus, and a negative control virus (SIVmac239). Scores were designated based on an algorithm incorporating breadth and potency (2). Boxes are color coded as follows: gray, $IC_{50} < 1{:}100$; green, $1{:}100 < IC_{50} < 1{:}300$; yellow, $1{:}300 < IC_{50} < 1{:}500$; orange, $1{:}500 < IC_{50} < 1{:}1{,}000$; red, $IC_{50} > 1{:}1{,}000$.

Plasma bNAbs in Macaque CE8J Bind to the Glycan Shield of gp120. Based on our results, we next investigated whether the plasma bNAbs bound to epitopes similar to that of 2G12, which recognizes a cluster of high-mannose glycans composed of N332, N339, and N392 (18). Mutation of individual glycans at positions 339 and 392 as well as 295 and 386 in JR-CSF pseudovirus had no effect on plasma-neutralizing activity (Fig. 3*C*), demonstrating that the plasma bNAb specificities bind to epitopes distinct from that recognized by 2G12. As a second approach, we adsorbed the plasma with TM-Pst1, a yeast glycoprotein displaying homogenous $Man_8GlcNAc_2$ glycans that has high affinity for 2G12 and can inhibit 2G12 neutralization of pseudoviruses (20). Interestingly, a large fraction of the broad and potent plasma-neutralizing activity could be adsorbed with TM-Pst1, suggesting that the broadly neutralizing plasma antibodies bind directly to $Man_8GlcNAc_2$ glycans (Fig. S5). We further investigated the role of glycans in recognition by the plasma bNAbs using the glucosidase analog *N*-butyldeoxynojirimycin (NB-DNJ), which blocks the removal of glucose residues attached to the D1 arm of the precursor $Glc_3Man_9GlcNAc_2$ (21) and has been shown to inhibit 2G12 binding (18). Similar to results obtained for 2G12, treatment of pseudoviruses with NB-DNJ abolished CE8J plasma-neutralizing activity, suggesting that the terminal mannose of the D1 arm of $Man_9GlcNAc_2$ is important for formation of the epitopes recognized by the CE8J plasma bNAb specificities (Fig. S6). Together, these results indicate that the plasma bNAbs bind to glycan-specific epitopes, which may overlap with that of 2G12 and the recently described PGT MAb epitopes.



A

mutation	Neutralization potency relative to WT JR-CSF (%)
P299A	112
S300N	122
N302A	135
R304A	276
S306A	103
I307A	1442
H308A	107
R315A	126
F317A	1243
Y318A	1554
T319A	229
T320A	1220
E322A	70
I327A	156
H330A	100
N332A	<1.0

B

Isolate containing the N332A mutation	Neutralization potency relative to WT (%) macaque CE8J plasma	MAb 2G12	MAb b12
YU2	30	N/A	82
92RW020	118	<3.0	92
MGRM-C26	<1.0	N/A	80
JR-FL	<1.0	<3.0	200
ADA	8.0	<10	98

C

mutation	Neutralization potency relative to WT JR-CSF (%) macaque CE8J plasma	MAb 2G12	MAb b12
N295A	79	<1.0	82
N386A	98	<1.0	255
N392Q	81	<1.0	109
N339A	110	<1.0	88

Fig. 3. Broad neutralization of macaque CE8J plasma is critically dependent on the N332 glycan. (*A*) Neutralizing activity of macaque CE8J plasma against selected JR-CSF alanine mutants. (*B*) Neutralizing activity of macaque CE8J plasma against a cross-clade pseudovirus panel containing the N332A mutation. mAbs b12 and 2G12 are included for comparison. N/A, data are not available because 2G12 does not neutralize these isolates. (*C*) Neutralizing activity of macaque CE8J plasma against selected JR-CSF glycan mutants. mAbs b12 and 2G12 are included for comparison. Percent neutralization potency relative to the WT pseudovirus was calculated using the equation (IC_{50} variant/IC_{50} WT) × 100. Boxes are color coded as follows: gray, 50–2,000%, yellow, 10–50%; green, <10%.

PNAS PNAS PNAS

Evolution of the N332A-Sensitive, Broadly Neutralizing Specificity in the Plasma of Macaque CE8J. The broad plasma-neutralizing activity in macaque CE8J developed at 36 wk postinfection and was maintained after this time point, suggesting either rapid development of a single bNAb specificity (i.e., the N332A-sensitive specificity) or sequential development of broad and potent neutralizing antibodies with distinct specificities. To investigate this question, we tested the plasma samples collected at serial time points for sensitivity to the N332A mutation (Fig. 4*A*). Indeed, all of the serial plasma samples exhibited a loss of neutralization activity against the N332A-containing pseudovirus variants, indicating that the N332A-sensitive bNAb specificity was rapidly induced and subsequently maintained over time. A similar result was observed with the autologous $SHIV_{AD8}$ virus containing an N332A mutation, suggesting that both the autologous and heterologous neutralizing antibody responses in this macaque are mediated by the N332A-sensitive specificity (Fig. 4*B*).

Neutralization Escape SHIV Variant Emerges in Macaque CE8J. The neutralization experiments described herein were all performed using pseudotyped virus prepared from a recently described pathogenic $SHIV_{AD8}$ molecular clone carrying an env gene present in the swarm virus stock generated from animal CK15 at week 42 postinfection (22). To ascertain whether the neutralization sensitivity of the input swarm had changed during the 2-y infection of animal CE8J, the replication-competent $SHIV_{AD8}$ inoculum (Lymph Node Virus) (16) or virus collected at the time of euthanasia at week 117 postinfection (W.117) were incubated with plasma collected at weeks 0, 50, and 87 and assayed for 28 h in TZM-bl cells. The input $SHIV_{AD8}$ was neutralized by the week 50 and 87 plasma samples, whereas the week 117 virus swarm was resistant to neutralization at both of these time points. A comparison of env gene sequences in the $SHIV_{AD8}$ inoculum with those present in the swarm virus at week 117 revealed consistent changes affecting all of the variable and the C3 regions of gp120 (Fig. S7) and is concordant with the neutralization escape properties of the virus circulating at weeks 50 and 87.

Discussion

Numerous studies have shown that ~5–30% of HIV-1–infected donors develop broad and potent neutralizing serum, depending on the criteria used to define neutralization breadth and potency. Here, we observed that one of the 14 $SHIV_{AD8}$-infected macaques tested (~7%) developed broad and potent plasma-neutralizing activity, which is within the range that has been observed in humans. However, a larger number of infected macaques will need to be screened to make more robust comparisons of neutralization patterns in human and macaque sera. Importantly, macaque CE8J developed plasma-neutralizing activity with comparable breadth and potency as HIV-1 "elite neutralizers" (2), which, to our knowledge, represents the most broad and potent NAb response observed to date in the SHIV/macaque model. Therefore, the results demonstrate that the rhesus monkey immune system is capable of generating exceptionally potent bNAb responses during natural infection, further validating the use of the SHIV/macaque model to evaluate vaccine candidates.

Interestingly, macaque CE8J developed broad and potent responses by 9 mo postinfection, which is much faster than observed for most HIV-1 elite neutralizers, who generally only develop such responses 2–3 y or more postinfection. Also, in stark contrast to observations in HIV-1–infected humans, the autologous and broadly neutralizing responses in macaque CE8J developed simultaneously, and both responses appeared to be mediated by the N332A-sensitive bNAb specificity. The mechanisms underlying the development of bNAbs in a single $SHIV_{AD8}$-infected monkey are currently under investigation and may relate to the quality and/or quantity of B-cell or $CD4^+$ T-cell responses, or host genetics. Of note, many of the 14 $SHIV_{AD8}$-infected animals in this study exhibited similar or higher levels of plasma viremia than macaque CE8J (Fig. S8), suggesting that the failure to develop broadly reactive neutralizing activity is likely not due to low levels of antigenic stimulation.

A common feature of $SHIV_{AD8}$-infected macaques seen here is the relatively modest fraction that develop autologous NAbs. In our entire cohort of moneys infected with $SHIV_{AD8}$ derivatives (swarm virus stocks prepared during infection or at the time of euthanasia or a pathogenic molecular clone [$SHIV_{AD8MV}$]), only nine of 40 animals developed autologous NAbs (Table S2). When detected, neutralization was observed whether the target virus used in the assay was an exact match for the virus inoculated into an animal, and in some cases was not sustained over time. At present we have no explanation for the resistance of $SHIV_{AD8}$-infected macaques to generate autologous NAbs,

A

weeks post infection	JR-FL	JR-FL N332A	MGRM-C26	MGRM-C26 N332A
20	<100	<100	<100	<100
24	<100	<100	<100	<100
29	<100	<100	<100	<100
32	<100	<100	<100	<100
36	100	<100	805	<100
40	1066	<100	1600	<100
46	1657	<100	>2700	<100
52	1063	<100	>2700	<100
57	735	<100	>2700	<100
60	640	<100	>2700	<100
67	760	<100	>2700	<100
72	740	<100	>2700	<100
77	>2700	<100	>2700	<100
80	720	<100	1586	<100
86	550	<100	735	<100
101	450	<100	640	<100
115	1295	<100	1050	<100

B

weeks post infection	$SHIV_{AD8}$	$SHIV_{AD8}$ N332A
20	<100	<100
24	<100	<100
29	<100	<100
32	450	<100
36	811	<100
40	3282	300
46	2873	100
52	1899	300
57	1709	100
60	1324	200
67	1181	<100
72	1907	<100
77	5193	<100
80	1181	<100
86	510	<100
101	397	100
115	1106	<100

Fig. 4. Evolution of the N332A-sensitive bNAb specificity in macaque CE8J plasma. (*A*) Plasma samples collected at serial time points were tested for neutralizing activity against JR-CSF and MGRM-C26 pseudovirus variants containing an N332A mutation. (*B*) Plasma samples taken at serial time points were tested for neutralizing activity against $SHIV_{AD8}$ containing an N332A mutation. Boxes are color coded as follows: gray, IC_{50} < 1:100; green, 1:100 < IC_{50} < 1:300; yellow, 1:300 < IC_{50} < 1:600; orange, 1:600 < IC_{50} < 1:1,000; red, IC_{50} > 1:1,000.

although the tier 3 status of the parental HIV-1_{Ada} may be a contributing factor.

Our epitope-mapping studies indicate that the plasma bNAbs bind to an epitope critically dependent on the gp120 N332 glycan, perhaps overlapping that of 2G12 and/or the recently described PGT antibodies. Importantly, broad and potent serum-neutralizing activity in a significant proportion of HIV-1–infected donors has been mapped to N332A-sensitive epitopes (13, 14), and broad and highly potent monoclonal antibodies have recently been isolated from such donors (19). Given the rapid generation of bNAbs in this macaque, and the prevalence of bNAbs against N332A-sensitive epitopes in humans, it will be of interest to determine whether bNAbs against glycan epitopes are more rapidly elicited during natural infection and whether they can be more easily induced by vaccination. Future longitudinal studies on infected or vaccinated donors will reveal whether this is the case.

Materials and Methods

Animal Experiments. Virus inoculations, phlebotomies, lymphocyte subset analysis, and plasma viral load determination were performed as described previously (16). All animal experiments were done at the National Institutes of Health in compliance with the guidelines of the National Institute of Allergy and Infectious Diseases Animal Care and Use Committee.

Pseudovirus Production and Neutralization Assays. Pseudoviruses incorporating single-alanine substitutions were generated by transfection of 293T cells with an Env-expressing plasmid and an Env-deficient genomic backbone plasmid (pSG3ΔEnv), as described previously (23). For generation of NB-DNJ–treated pseudoviruses, 2 mM of the glycosidase inhibitor was added at the time of transfection (17). Pseudoviruses were harvested 72 h posttransfection for use in neutralization assays. Neutralizing activity was assessed using a single round of replication pseudovirus assay and TZM-bl target cells, as described previously (24). Virus neutralization assays using replication competent virus stocks [the starting $SHIV_{AD8}$ lymph node virus inoculum (16) and the virus recovered from macaque CE8J at the time of euthanasia at week 117 (W.117 virus)] were performed as described previously (16).

Serum Adsorptions. Serum adsorptions with Protein A-Sepharose beads (GE Healthcare) were performed by incubating 20 μL plasma with ∼250 μL of a 50% slurry of Protein A-Sepharose beads for 2 h at room temperature. The beads were then pelleted by centrifugation, and the Protein A-depleted fraction was collected. Serum adsorptions with antigen-coupled beads were performed using tosyl-activated magnetic beads, as described previously (25). A total of 1 mg of gp120 or 1 mg of TM-Pst1 was used for bead coupling. Two rounds of adsorption were performed to ensure complete removal of antigen-specific antibodies. Functional antibodies were eluted from beads by exposing the beads to series of increasingly acidic conditions, as described (25).

ELISAs. Ninety-six-well ELISA plates were coated overnight at 4 °C with 50 μL PBS containing 50 ng of goat anti-human IgG Fc (Pierce), 100 ng of gp120, or 100 ng TM-Pst1 per well. The wells were washed 4× with PBS containing 0.05% Tween-20 and blocked with 3% BSA at room temperature for 1 h. Serial dilutions of plasma or mAb were then added to the wells, and the plates were incubated at room temperature for 1 h. After washing 4×, goat anti-human IgG F(ab′)2 conjugated to alkaline phosphatase (Pierce), diluted 1:1,000 in PBS containing 1% BSA and 0.025% Tween-20, was added to the wells. The plate was incubated at room temperature for 1 h, washed 4×, and the plate was developed by adding 50 μL of alkaline phosphatase substrate (Sigma) to 5 mL alkaline phosphatase staining buffer (pH 9.8), according to the manufacturer's instructions. The optical density at 405 nm was read on a microplate reader (Molecular Devices).

Virus Isolation and Sequence Analysis. The W.117 virus stock was prepared from peripheral blood mononuclear cell (PBMC) and lymph node samples collected from macaque CE8J at the time of euthanasia at week 117 postinfection. Lymph node and PBMC suspensions were cocultivated with ConA-stimulated PBMC from an uninfected animal as described previously (16). Entire env genes were amplified from the starting $SHIV_{AD8}$ lymph node virus inoculum and W.117 virus by RT-PCR, using primer pairs described previously (16), and then cloned for sequence analysis.

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