In Vivo gp41 Antibodies Targeting the 2F5 Monoclonal Antibody Epitope Mediate Human Immunodeficiency Virus Type 1 Neutralization Breadth[▽]

Xiaoying Shen,^{1,2} Robert J. Parks,^{1,4} David C. Montefiori,^{1,2} Jennifer L. Kirchherr,^{1,4} Brandon F. Keele,⁶ Julie M. Decker,⁶ William A. Blattner,⁵ Feng Gao,^{1,4} Kent J. Weinhold,^{1,2,3} Charles B. Hicks,⁴ Michael L. Greenberg,^{2†} Beatrice H. Hahn,⁶ George M. Shaw,⁶ Barton F. Haynes,^{1,3,4} and Georgia D. Tomaras^{1,2,3*}

Duke Human Vaccine Institute¹ and Departments of Surgery,² Immunology,³ and Medicine,⁴ Duke University Medicine Center, Durham, North Carolina 27710; Division of Epidemiology and Prevention, Institute of Human Virology, University of Maryland, Baltimore, Maryland 21201⁵; and Department of Medicine, University of Alabama at Birmingham, Birmingham, Alabama 35294-0024⁶

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The broadly neutralizing human monoclonal antibodies (MAbs) 2F5 and 4E10, both targeting the highly conserved human immunodeficiency virus type 1 (HIV-1) envelope membrane proximal external region (MPER), are among the MAbs with the broadest heterologous neutralizing activity and are of considerable interest for HIV-1 vaccine development. We have identified serum antibodies from an HIV-infected subject that both were broadly neutralizing and specifically targeted MPER epitopes that overlap the 2F5 epitope. These MPER-specific antibodies were made 15 to 20 months following transmission and concomitantly with the development of autoantibodies. Our findings suggest that multiple events (i.e., genetic predisposition and HIV-1 immune dysregulation) may be required for induction of broadly reactive gp41 MPER antibodies in natural infection.

The induction of broadly neutralizing antibodies, like the membrane-proximal external region (MPER) monoclonal antibodies (MAbs) 2F5 and 4E10, is a major goal for antibody-based human immunodeficiency virus type 1 (HIV-1) vaccine strategies. These antibodies neutralize 80% and 100% of transmitted viruses (19), respectively. MPER-specific broadly neutralizing antibodies are very rarely made in HIV-1 infection (7, 29), and in the rare cases where such antibodies were identified, the target region of the antibodies was either undefined (2) or was attributed to the 4E10 epitope region (18, 27). Broadly neutralizing antibodies specifically targeting the 2F5 epitope regions have never been identified in HIV-1-positive (HIV-1⁺) patients. Vaccine strategies aimed at eliciting 2F5- or 4E10-like antibodies have been of great interest to researchers, but so far, the efforts have been largely futile (6, 8, 20, 25), partly due to the lack of understanding of the mechanism for the production/inhibition to the production of such antibodies. Both 2F5 and 4E10 MAbs were originally obtained from Epstein-Barr virus-immortalized B-cell clones generated from pooled peripheral blood mononuclear cells of six HIV-1-infected patients (4). Information is not available about the specific patient(s) from whom these B-cell clones were derived, and therefore, neither the presence nor the levels of circulating antibodies in the subject(s) are known.

A fundamental question is whether subjects fail to routinely

make 2F5- and 4E10-like antibodies because of host immune regulatory constraints or because the Env epitopes presented to host B cells are not in the correct envelope conformation. The lack of production of these types of antibodies has been suggested to be caused by either a lack of correct conformation of the neutralizing MPER epitopes, immune diversion by a nonneutralizing MPER epitope (21), or downmodulation of neutralizing MPER antibody responses by nonneutralizing MPER antibodies (1). In addition, recent studies have shown that MAbs 2F5 and 4E10 have lipid polyreactivity (12, 14) and long hydrophobic CDR3 loops that do not interact with gp41 but rather are available to reside near the virion lipid bilayer (1, 22, 23). Thus, 2F5 and 4E10 MAbs are polyreactive and have CDR3 motifs suggestive of autoantibodies, giving rise to the alternative hypothesis that immune tolerance mechanisms might play a role in limiting induction of 2F5- and 4E10-like antibodies (12, 14).

Approximately 1 to 10% of HIV-1-infected subjects eventually develop potent and broadly reactive neutralizing antibodies (13), but few of these broadly neutralizing antibody specificities have been mapped. When HIV-2/HIV-1 chimeras are used, less than 1% of HIV-1⁺ subjects have either 2F5- or 4E10-like neutralizing antibodies (7, 11, 29). Most broadly neutralizing antibodies in chronic HIV-1⁺ sera may be against the CD4 binding site (7). Li et al. (17) elegantly demonstrated the CD4 binding site specificity of broadly neutralizing antibodies in sera from two subjects. Other strategies, involving envelope panning of phage display libraries from pooled bone marrow of HIV-1⁺ subjects, identified cross-reactive anti-gp41 MAbs, but these MAbs were not found to be similar to 2F5 or 4E10 MAbs (30).

* Corresponding author. Mailing address: Duke Human Vaccine Institute, Rm. 4079, MSRBII, 106 Research Drive, Durham, NC 27710. Phone: (919) 681-5598. Fax: (919) 684-5230. E-mail: gdt@duke.edu.

† Present address: b3 Bio., Inc., Research Triangle Park, NC 27709.

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FIG. 1. 2F5 MAb competitive ELISA screening of 2F5-like activity in HIV-1⁺ patients. Serum samples were tested for their potency in competing with biotinylated 2F5 MAb in binding to HIV-1 JRFL gp140. The 2F5-equivalent concentrations (2F5 Eq) were calculated from a 2F5 MAb standard curve.

Given the rare occurrence of broadly neutralizing antibodies (2, 7, 13, 18, 27), genetic factors, such as those that predispose to abnormal tolerance mechanisms in autoimmune disease, are likely important. If tolerance mechanisms are solely responsible for limiting induction of MPER neutralizing antibodies, then when they are made (rarely), they should appear immediately after HIV-1 transmission. However, if immune tolerance is not the sole mechanism, then in addition to genetic factors, broadly reactive neutralizing antibodies may appear later in the course of HIV-1 infection, after HIV-1-induced immune dysregulation and after prolonged antigen stimulation (5). Indeed, a recent study (27) concluded that the amount of time following infection was a significant factor affecting neutralization breadth, in addition to antibody avidity and plasma viral load.

In this study, we have identified gp41 MPER 2F5-like antibodies in sera from an HIV-1⁺ subject with broad neutralizing activity and demonstrated this specificity to be responsible for neutralization breadth. We found that it took 15 to 20 months following infection for the MPER-neutralizing antibodies to develop, and these antibodies appeared coincident with the development of Jo-1 and double-stranded DNA (dsDNA) autoantibodies.

MATERIALS AND METHODS

Subjects studied. The Trinidad Seroconverter Cohort was described previously (3). Patient SC44 was an antiviral-naïve female patient. All experiments were performed in compliance with the relevant laws and institutional guidelines. Ethics approval for this study was obtained from Duke University Medical Center. Written informed consent was obtained from all participants.

Viruses, antibodies, and peptides. The TND_669S envelope clone was obtained from a Trinidad Cohort patient plasma sample by bulk PCR and subse-

FIG. 2. Neutralization of TND_669S and TND_669L Env pseudoviruses by HIV-1⁺ sera or antibodies against HIV-1. TND_669S and TND_669L are two HIV-1 isolates that vary by one amino acid in their envelope sequences. TND_669S is 279-fold more sensitive to 2F5 MAb neutralization (black and gray bars, plotted on the secondary y axis) and 275-fold more sensitive to 4E10 MAb neutralization, whereas its sensitivities to various other neutralizing antibodies targeting gp120 and to virus entry inhibitor T20 are not significantly higher than those of TND_669L (data not shown). SC44 neutralized TND_669S 11-fold more potently than it did TND_669L, while another broadly neutralizing serum sample, IBB JT, neutralized TND_669S only 2-fold more potently than it did TND_669L. Values shown above each bar are either the serum IC₅₀s (in cases of serum samples) or the MAb IC₅₀s (in cases of 2F5 MAb).

quent molecular cloning. The TND_669S/K665N envelope was produced by introduction of a single K665N mutation into TND_669S *env* by use of a QuikChange XL site-directed-mutagenesis kit. Production and titration of Env pseudoviruses were done as previously described (16). Production and characterization of HIV-2 chimeric viruses containing the HIV-1 MPER epitopes were done as previously described (29).

HIV-1 gp41 MAbs 2F5 and 4E10 were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (Hermann Katinger) (4, 26, 31).

Two peptides containing the 2F5 epitope sequence (2F5 peptides) were used: 2F5₆₅₂₋₆₇₁-QKEKNEQELLELDKWLWN and 2F5₆₅₇₋₆₇₁-EQELLELDKWLWN. These peptides were custom-made by Primm Biotech and CPC.

Neutralization assay. Production of pseudoviruses and neutralization assays were performed as previously reported (16). Briefly, full-length *env* clones in pcDNA3.1D/V5-His-TOPO vector were cotransfected into 293T cells with an *env*-deficient HIV-1 backbone (pSG3Δ*env*) by using FuGENE 6 transfection reagent (Roche Applied Science, Basel, Switzerland). Tissue culture fluid was harvested after 24 to 36 h of incubation and filtrated through 0.44 μm filters, and fresh fetal bovine serum was added to the virus stock to make a final concentration of 20%. The 50% tissue culture infectious dose of each virus preparation was determined with TZM-bl cells. Briefly, serial diluted virus stocks were used to infect TZM-bl cells on 96-well flat-bottom plates for 48 h. The cells were then lysed and the number of relative luminescence units (RLU) determined using the BriteLite assay system (PerkinElmer, Inc., Waltham, MA). Wells with luciferase luminescence levels 2.5-fold higher than that of the cell-only control were considered positive for virus infection. The 50% tissue culture infectious doses were calculated using the Reed-Muench formula.

Peptide absorption neutralization assay. Peptide absorption neutralization assays were performed by preincubating antibodies/sera with the 2F5 peptide for 1 h, followed by the neutralization assay as described above.

2F5 binding ELISA and 2F5 competitive inhibition ELISA. The binding enzyme-linked immunosorbent assay (ELISA) and the 2F5 competitive inhibition ELISA were performed as previously described (1). A 2F5 peptide containing the consensus epitope sequence (2F5₆₅₂₋₆₇₁-QKEKNEQELLELDKWLWN) was used in the direct binding ELISA, and the JRFL gp140 CF oligomer Env

FIG. 3. Specificity of neutralizing activity for the 2F5 epitope. (A) 2F5 MAb positive-control data for the peptide absorption neutralization assay. The 2F5 peptide alone did not affect virus entry (infectivity of 2.58×10^5 RLU with peptide and 2.53×10^5 RLU with virus only). On the basis of this dose response, 3 μM of 2F5 peptide was used in subsequent screening assays of patient sera. (B) 2F5 and 4E10 MAb controls for the K665N reduction assay. The K665N mutant was resistant to 2F5 MAb neutralization but remained sensitive to 4E10 MAb neutralization. (C) Absorption of the neutralization activity of SC44 27-month-postenrollment serum by the 2F5 peptide. The percentages above the bars are the relative levels of 2F5-like neutralizing activity, calculated as follows: $(IC_{50} \text{ without peptide} - IC_{50} \text{ with peptide}) / IC_{50} \text{ without peptide}$. SC03 represents a control HIV⁺ serum sample that was identified to contain nonneutralizing antibodies which bind to the 2F5 peptide. (D) Differential neutralization of the 2F5-sensitive and 2F5-resistant viruses by SC44 27-month-postenrollment serum, in contrast to that of the control serum (from SC03). Bars show the neutralization titers (IC_{50} s) of the serum against the 2F5-sensitive virus (TND_669S, in black) and the 2F5-resistant virus (TND_669S/K665N, in gray). The percentages indicate the relative levels of the 2F5-like neutralizing activity, calculated as follows: $(IC_{50} \text{ for TND_669S} - IC_{50} \text{ for TND_669S/K665N}) / IC_{50} \text{ for TND_669S}$.

protein was used in the 2F5 competitive inhibition ELISA. For the 2F5 competitive inhibition ELISA, plates coated with gp140 protein were incubated with a 1:50-diluted test sample or a serially diluted unlabeled 2F5 MAb (for generation of a standard curve) before biotinylated 2F5 MAb was added and allowed to bind. Binding of biotinylated 2F5 MAb was then detected with the use of streptavidin-alkaline phosphatase followed by alkaline phosphatase substrate.

Autoantibody detection. Autoantibodies were measured by the FDA-approved AtheNA Multi-Lyte ANA II test kit from Zeus Scientific, Inc., per the manufacturer's instructions and as described previously (14). The cutoff value for all antibodies in this assay is 120 AtheNA U/ml. HIV-2/HIV-1 MPER neutralization assays were performed as previously described (11).

Affinity column purification. Purification of 2F5 epitope-binding antibodies from SC44 serum/plasma samples was performed using an AminoLink Plus immobilization kit (Pierce), following the manufacturer's instructions. Briefly, 1.5 mg of a 2F5 peptide, QQEKNEQELLELDKWASLWN (CPC Scientific, San Jose, CA, and Primm Biotech, Cambridge, MA), was coupled to the beads following the pH 10 coupling procedure. Serum/plasma samples were diluted 1:2

with the washing/binding buffer and then pulled through the peptide-conjugated column in 1.5- to 2-ml batches. The eluted fractions were combined and concentrated using Centricon 30 (Millipore) columns.

RESULTS

Screening sera for 2F5-like antibodies. We tested sera from subjects chronically infected with HIV-1 for antibodies that block the binding of 2F5 MAb to the HIV-1 gp140 oligomer in a 2F5 competitive ELISA. In this initial screen, we found 3 subjects out of 311 total U.S. and Trinidad clade B subjects chronically infected with HIV-1 that blocked greater than 50% binding of biotinylated 2F5 MAb. Two of these subjects with 2F5 blocking activity did not have 2F5 epitope-specific neu-

FIG. 4. Characterization of SC44 serum/plasma antibodies purified by affinity columns coupled with the 2F5 peptide. (A) Tests for neutralizing activities. The 27-month-postenrollment plasma sample (7.5 ml) was purified with an affinity column conjugated with the MPER peptide (QOEKNEQELLELDKWASLWN, containing the 2F5 binding site), and the purified antibody (SC44 puri) was concentrated to ~500 μ l. The total protein concentration in the purified fraction was measured to be 1.36 mg/ml. The SC44 purified fraction (in red) was then tested for neutralization of TND_669S (solid lines), absorption of neutralization activity by the MPER peptide (dotted lines), and abrogation of neutralization of the TND_669S/K665N pseudotyped virus (dashed lines), side by side with 2F5 MAb neutralization controls (in blue). The IC_{50} s of the SC44 puri antibody and the 2F5 MAb were 1.09 and 0.027 μ g/ml, respectively. (B) 2F5 competitive ELISA. Approximately 6 ml of SC44 serum (around 27 months postenrollment) was pooled and purified through a 2F5 peptide affinity column, and the purified antibody was concentrated to 350 μ l. The purified antibody fraction was then tested in a 2F5 competitive ELISA. The black line shows the standard curve generated with unlabeled 2F5 MAb. The gray drop line indicates the optical density (OD) of the purified SC44 antibody at a 1:50 dilution.

tralizing activity in peptide absorption neutralization assays (not shown), and the 2F5 MAb blocking activity was shown to reflect serum nonneutralizing antibodies against MPER that sterically blocked 2F5 MAb binding as previously published for one of these two subjects (1). The remaining subject, SC44, with broadly neutralizing and 2F5 epitope-specific neutralizing antibody activity, was followed for 4 to 5 years, along with 11 other subjects from the same cohort (Fig. 1).

To determine whether these antibodies competing with 2F5 MAb were neutralizing antibodies, SC44 sera were tested for neutralization of an HIV-1 envelope clone, TND_669S, which is ~275-fold more sensitive to 2F5 and 4E10 MAb neutralization than the wild-type envelope. SC44 sera neutralized TND_669S Env-pseudotyped virus 11-fold more potently than the wild-type virus, indicating the possible existence of neutralizing antibodies against MPER (Fig. 2).

Antibody epitope specificity. To characterize the neutralizing specificities of MPER antibodies in SC44 sera, we utilized a 2F5 peptide absorption neutralization assay, wherein neu-

tralization of the TND_669S Env-pseudovirus was performed after preincubation of the SC44 serum with a 2F5 MPER epitope peptide (QOEKNEQELLELDKWASLWN). As a positive control, neutralization of the TND_669S pseudovirus by the 2F5 MAb was inhibited by absorption by the 2F5 epitope peptide in a dose-dependent manner (Fig. 3A). Screening of HIV-1-infected sera in this assay showed absorption of neutralization activity by the 2F5 epitope peptide in SC44 serum (Fig. 3C). The 2F5 epitope peptide was able to absorb 58% of the neutralizing activity of SC44 serum from 27 months postenrollment after the acute infection syndrome, while the neutralizing activities of other serum samples tested were absorbed $\leq$ 20%.

Next, we further determined whether the Env gp41 MPER amino acids required for 2F5 neutralization were also required for SC44 serum neutralization. We used an Env clone, TND_669S/K665N, generated by introduction of the K665N mutation into the TND_669S envelope. The K665N mutation is located in the DKW core region of the ELDKWAS 2F5 epitope and has been shown to abrogate 2F5 binding (26).

TABLE 1. SC44 serum from 33 months postenrollment neutralizes 92% of viruses in the standard reference panel, in addition to SF162.LS virus^a

Pseudovirus	SC44 neutralization potency (IC ₅₀) ^b		
	33-mo plasma	2F5 MAb	HIVIG
SF162.LS	2,025	0.7	5
6535.3	42	4.6	148
QH0692.42	29	3.6	943
SC422661.8	99	1.0	312
TRO.11	174	>25	243
AC10.0.29	45	0.7	570
RHPA4259.7	120	12.6	343
THRO4156.18	274	>25	419
REJO4541.67	82	0.6	193
TRJO4551.58	565	>25	542
WITO4160.33	33	0.7	530
PVO.4	<20	>25	900
CAAN5342.A2	102	4.0	600

^a Anti-MPER antibodies in serum are responsible for neutralization breadth. Heterologous neutralization of SC44 serum and MPER purified antibody were measured against the Standard Reference Panel of Subtype B HIV-1 Env Clones (16).

^b IC₅₀s for SC44 33-month-postenrollment plasma are expressed as reciprocals of dilution factors. IC₅₀s for 2F5 and HIVIG (purified IgG from pooled sera of HIV-1-infected patients) are expressed in µg/ml.

The TND_669S/K665N Env pseudovirus was resistant to 2F5 MAb neutralization but remained highly sensitive to 4E10 MAb neutralization (Fig. 3B). Thus, a reduction in serum neutralization titer for TND_669S/K665N Env pseudovirus compared to that for TND_669S Env pseudovirus indicated the requirement of the 2F5 epitope of the envelope for the neutralizing potency of the serum tested. This is precisely what was seen with SC44 serum; the neutralizing titer (50% inhibitory concentration [IC₅₀]) of the 27-month-postenrollment serum against the TND_669S/K665N pseudovirus was 68% lower than that against the TND_669S pseudovirus (Fig. 3D). Taken together, these data indicated that ~70% of the neutralizing activity of the SC44 serum against TND_669S Env pseudovirus required the presence of a 665K core amino acid of the 2F5 ELDKWA epitope and that ~60% of the neutralizing activity was absorbed by a gp41 MPER 2F5 epitope-containing peptide.

In addition, sera from SC44 also bound a recombinant gp41 construct in which the HR-2 MPER was stabilized in a gp41 intermediate conformation (9), with a binding antibody mid-point titer of 1.82×10^4 (four-parameter logistic curve fit), which was ninefold greater than that for other sera with MPER binding activity (not shown).

Purification of 2F5-reactive antibodies from human sera. To directly characterize the specificities of the antibodies mediating the neutralizing activity in SC44 serum, antibodies specific for the 2F5 epitope were purified by an affinity column conjugated with a peptide containing the 2F5 nominal epitope (QQEKNEQ ELLELDKWASLWN) (1). The affinity-purified and concentrated fraction of SC44 plasma neutralized TND_669S with an IC₅₀ of 1.09 µg/ml (2F5 MAb neutralized the same virus with an IC₅₀ of 0.027 µg/ml) (Fig. 4A). The lower IC₅₀ of the purified fraction may be partly due to the fact that the antibodies are purified by 2F5 peptide binding ELISA, where any antibody that binds to the peptide will be retained in the purified fraction. Some

TABLE 2. MPER affinity-purified antibody from 45 months postenrollment neutralizes 100% (6/6) of tested viruses from the standard reference panel, in addition to SF162.LS virus^a

Pseudovirus	SC44 neutralization potency (IC ₅₀) ^b		
	Purified fraction ^c	Flowthrough fraction ^c	42/45-mo plasma
SF162.LS	219	959	1,301
PVO.4	276	23	63
TRO.11	188	37	164
RHPA4259.7	261	30	91
THRO4156.18	313	<20	NT
TRJO4551.58	229	515	930
CAAN5342.A2	169	<20	23

^a Anti-MPER antibodies in serum are responsible for neutralization breadth. Heterologous neutralization of SC44 serum and MPER purified antibody were measured against the Standard Reference Panel of Subtype B HIV-1 Env Clones (16).

^b IC₅₀s are expressed as reciprocals of dilution factors. NT, not tested.

^c The SC44 "purified" and "flowthrough" fractions were the bound and unbound fractions of plasma from patient SC44 at 42 and 45 months postenrollment (combined), obtained from affinity column purification by use of beads conjugated to the 2F5 peptide. Only seven pseudoviruses were tested, due to the limited amount of affinity-purified, SC44 MPER-reactive IgG.

of the antibodies purified could be binding but nonneutralizing antibodies. The 2F5-equivalent antibody concentration in the SC44 serum, calculated from the neutralization curves of the MPER peptide-purified antibody and from a 2F5 MAb standard curve, was 2.2 µg/ml. The specificity of the affinity-purified antibody to the 2F5 MPER epitope was tested using the HIV-1 Env-pseudotyped virus with the K665N mutation. The affinity-purified SC44 antibody did not neutralize the HIV Env-pseudotyped virus with the K665N mutation (Fig. 4A). Importantly, neutralization of the SC44 affinity-purified antibody fraction was blocked by addition of the 2F5 peptide (QQEKNEQELLELDKWASLWN) (Fig. 4A). The concentration of the affinity-purified SC44 antibody was calculated to be 51.6 µg/ml 2F5 MAb equivalents by the 2F5 competitive ELISA (Fig. 4B), which translated into ~3.0 µg/ml 2F5 equivalents in the original serum. The subtype of immunoglobulin (Ig) that was responsible for this 2F5-like activity in the MPER peptide-purified fraction of SC44 serum was tested in an IgG subtyping ELISA and determined to be IgG1 (data not shown). This affinity-purified fraction also neutralized the HIV-2/HIV-1 MPER chimeric virus (11) containing the 2F5 epitope (HIV-2_{7312A}C7) at an IC₅₀ of 100 µg/ml but did not neutralize the HIV-2 control virus lacking HIV-1 MPER sequences (HIV-2_{7312A}) (data not shown) (7).

Broadly neutralizing activity of SC44 serum. To investigate the contribution of the MPER-sensitive antibody in SC44 serum to heterologous neutralizing activity, SC44 serum/plasma samples were evaluated for neutralization breadth in the TZM-bl HIV-1 Env-pseudotyped virus assay against a standard reference panel of subtype B HIV-1 Env clones (Tables 1 and 2) (16). The 33-month-postenrollment serum from SC44 neutralized 11 out of 12 virus isolates (92%) in the standard clade B reference panel, in addition to the easy-to-neutralize SF162.LS pseudovirus (Table 1). Furthermore, antibodies purified from SC44 42/45-month-postenrollment plasma samples (plasma samples from 42 months and 45 months postenrollment were combined 1:1 for this purification) were tested against six selected viruses from the reference panel,

FIG. 5. Ontogeny of the 2F5-like-antibody activity in patient SC44. The kinetics of the 2F5-like neutralizing activity in SC44 over the course of HIV-1 infection was assessed by a K665N reduction assay (A) and a 2F5 binding ELISA (B) of longitudinal serum samples of SC44. (A) Neutralization titers for 2F5-sensitive virus TND_669S (dark blue) and 2F5-resistant virus TND_669S/K665N (light blue) are shown with the bars and plotted on the primary y axis. The corresponding viral loads over the infection course are shown with the gray line and plotted on the secondary y axis. The first viral load measurement shown is below the limit of detection (<100 copies/ml). The relative levels of 2F5-like activity, as indicated by K665N reduction levels in this assay (calculated as described for Fig. 4D), are shown above each group of bars. Neutralizing anti-MPER antibodies developed after 12 months from the time of enrolment into the acute infection cohort, as indicated by a 41% difference in the results for the reduction neutralization assay, which then peaked at a 65 to 70% difference by 27 months. (B) Direct 2F5 peptide binding ELISA was performed as previously described (1). Multiple serum samples (from both acute and chronic stages of infection) from each patient were diluted 1:50 and tested in the 2F5 peptide binding ELISA.

and six out of six (100%) of the isolates were neutralized. The MPER antibody-depleted fraction (flowthrough) had significantly less neutralizing activity against five out of six virus isolates from the standard panel, whereas the majority of the neutralizing activity against the easy-to-neutralize SF162.LS pseudovirus and also against one of the standard panel viruses, TRJO4551.58, remained in the flowthrough (Table 2). These results indicate that the MPER affinity-purified antibodies contributed significantly to the neutralization breadth of the serum. Of note, the purified antibodies also neutralized 2F5-resistant isolates, suggesting the contribution of additional neutralizing antibody species that are different from MAb 2F5 in SC44 serum, or suggesting that the 2F5-like activity in SC44 serum is not identical to the 2F5 MAb activity.

Ontogeny of 2F5-like-antibody development after HIV-1 transmission. SC44 serum samples over a 4-year period were studied to determine the time of onset of anti-MPER neutralizing activity, using a neutralization assay for paired pseudoviruses TND_669S (2F5 sensitive) and TND_669S/K665N (2F5 resistant). Neutralizing anti-MPER antibodies were not present at 1 and 7 months after acute infection but developed after 12 months following the time of enrolment into the acute-infection cohort, as indicated by a 41% difference in the results for the reduction neutralization assay, which then peaked at a 65 to 70% difference by 27 months (Fig. 5A). These neutralization data were in accordance with the kinetics of serum 2F5 MAb blocking activity (Fig. 1). The existence of 2F5-like antibodies in the sera of SC44 and the kinetics of this activity were also confirmed by a binding ELISA using a 2F5 peptide as a target (Fig. 5B).

FIG. 6. Detection of autoantibodies in patient SC44. Results are shown for detection of anti-dsDNA (A) and anti-Jo-1 (B) antibody in patient SC44 sera. The units are AtheNA U/ml, with 120 U/ml as the positivity cutoff.

Autoimmune antibodies in SC44. The 2F5-like neutralizing activity in SC44 rose late in infection, similar to the timing required for overall neutralization breadth to occur both in these subjects and as reported by others (11). This is consistent with, but does not prove, the assumption that one factor for induction of broadly neutralizing antibodies is delayed maturation of the antibody response in the context of persistent antigen (5). However, this is not the only factor, since these antibodies were rarely found in our study (in approximately 0.3% of subjects). Other possible factors that might explain the delayed development of the 2F5-like MPER-neutralizing antibody include the gradual onset of immune dysregulation occurring over time following HIV-1 transmission, a genetic predisposition to make autoantibodies, or both. To examine these possibilities, we determined autoimmune antibody levels in longitudinal SC44 serum samples. Interestingly, patient SC44 developed autoantibodies against dsDNA (Fig. 6A) and Jo-1 (Fig. 6B) over the time that directly coincided with the development of the MPER neutralizing antibody (Fig. 5A). SC44 was the only patient among a total of 96 screened that was positive for anti-dsDNA and one of two patients among the 96 screened for autoantibodies that were positive for anti-Jo-1. Additionally, SC44 7-month (IgM) and 33-month (IgG and IgM) sera were also positive for anti-cardiolipin autoantibodies (Fig. 7). Of note, SC44 was diagnosed with syphilis at the time of study enrollment, and the presence of anti-CL antibodies was likely related to syphilis coinfection. However, the level of anti-dsDNA antibody in SC44 was remarkable. It was 207 U/ml at 33 months (Fig. 6A), while the other 96 patients tested were all negative and most of them had levels well below 50 U/ml (data not shown). The development of these autoantibodies, coincident with development of the MPER broadly neutralizing antibody, strongly suggests that the developments of both types of antibodies were dependent on dysregulation of B-cell tolerance control mechanisms.

Virus envelope sequence analysis. The viral envelope was sequenced at two time points to investigate whether the presence of the anti-MPER neutralizing antibodies exerted immune pressure in vivo. HIV-1 gp41 sequences were amplified from plasma samples at both enrollment and 45 months postenrollment by single-genome amplification (SGA) as pre-

viously described (15). Figure 8 shows the alignment of the 2F5 epitope region of four SC44 *env* SGA sequences from enrollment and 29 sequences from chronic infection (45 months). There are no mutations in the 2F5 epitope region that were

FIG. 7. Detection of anti-cardiolipin antibodies in SC44 serum. Anti-cardiolipin IgG, IgA, and IgM concentrations were measured for SC44 sera from the 7-month-, 15-month-, and 33-month-postenrollment time points. The cutoffs for different antibody species are listed in the table in panel B. ** indicates positive values.

FIG. 8. Alignment of the 2F5 MAb epitope region of SC44 SGA sequences. SGA of envelope sequences from HIV-1 viruses was performed as previously described (15, 24). Early sequences were derived from plasma samples at enrollment, and late sequences were derived from 45-month-postenrollment plasma samples.

previously identified as capable of mediating enhanced sensitivity or resistance to neutralization by 2F5 MAb (31). However, the MPER antibodies in SC44 may have epitope specificities different from those of the 2F5 MAb. In addition, we also examined the MPER sequences for possible contribution of a unique MPER structure to the development of the anti-MPER antibodies; however, there are no mutations in the MPER region that are specific for the SC44 virus (compared to virus sequences in LANL) (data not shown).

DISCUSSION

In this study, we have characterized for the first time neutralizing antibodies reacting with an epitope in the gp41 membrane-proximal region that overlaps with that of the rare 2F5 MAb antibody in an HIV-1-infected patient, and these antibodies are responsible for the neutralization breadth of the serum neutralizing activity. Importantly, these antibodies did not arise in acute infection but rather developed from 12 to 27 months after infection, coincident with the development of multiple autoantibodies.

We have found that SC44, the only patient out of 311 selected that presented a 2F5-like neutralizing activity, developed autoimmune antibodies over the course of HIV infection, suggesting possible involvement of immune dysregulation in the development of these MPER antibodies. Findings of autoantibodies in SC44, however, do not completely rule out contributions of viral factors and other components of the host immune system that may have contributed to the delayed development of the 2F5-like neutralizing activity in SC44. Sequence analysis of Env clones obtained from SC44 did not

show evidence of a unique MPER structure that would have contributed to the elicitation of the neutralizing MPER antibodies. However, the optimal structure of the MPER for eliciting broadly neutralizing antibodies has not yet been determined. Furthermore, characterization of the virus populations over time in SC44 and in other regions outside the immediate proximity of MPER may yield additional insights.

A previous report identified the presence of 4E10-resistant viral variants in a clade C subject perinatally infected with HIV-1, suggesting that broadly neutralizing MPER antibodies may provide selective pressure on virus replication (10), although in vitro selection studies (18a) have shown that 2F5- and 4E10-resistant mutants are difficult to develop and may confer lower infectivity. In our study, characterization of chronic SC44 SGA sequences at the 2F5 epitope region did not support the development of 2F5 escape mutants. However, further investigation of the potential immune pressure imposed by the specific anti-MPER antibodies in SC44 is the subject of additional studies.

Finally, identification of 2F5-like antibodies and, if possible, 4E10-like antibodies from other patients, and characterization of their immune statuses, will improve our understanding of the correlation between autoimmunity and the development of anti-MPER antibodies. Understanding of the mechanism of development/suppression of anti-MPER antibodies will provide critical guidance for relevant HIV-1 vaccine strategies.

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