

# Prospective clinical utility and evolutionary implication of broadly neutralizing antibody fragments to HIV gp120 superantigenic epitope

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## ABSTRACT

**Background:** Neutralizing antibodies (Abs) that neutralize diverse strains found across the world are needed to develop effective HIV immunotherapies and topical immunomicrobicides. Residues 421–433 constitute a conserved epitope overlapping the CD4 receptor binding and B cell superantigenic sites of gp120. Abs to this epitope from uninfected humans bind and catalyze the hydrolysis of gp120, resulting in modest HIV neutralization. We describe here the anti-HIV properties of Ab fragments from patients with lupus, a disease rarely coincident with HIV infection.

**Methods:** Single chain Fv (scFv) clones were selected from phage displayed lupus repertoires using gp120 and its electrophoretic analogs (E-gp120). Neutralization of clinical HIV isolates by purified Ab fragments was measured by p24 assays using peripheral blood mononuclear cells. gp120, E-gp120 and E-416-433 binding and hydrolysis were determined by ELISA and electrophoresis. Framework (FR) replacement was by PCR mutagenesis.

**Results:** Selected scFv clones displayed gp120 binding and hydrolyzing activities attributable to 421–433 peptide recognition. Sub- $\mu$ g to  $\mu$ g/ml scFvs neutralized all CR5-dependent clade A, B, C, D and AE viruses tested (18–20 strains). scFv potencies and neutralization breadths were superior to IgG b12. Pseudoviruses derived from neutralizable clinical isolates were not neutralized. CXCR4 strains were neutralized poorly. Replacements of VH FR1 and FR3 in a VH4-family scFv with the FRs from a VH3-family scFv improved the binding of E-gp120 and E-peptide, consistent with the belief that superantigens are recognized at FRs, and contrasting with adaptive recognition of conventional antigens at the hypervariable CDRs.

**Conclusion:** The unprecedented potency and breadth of HIV neutralization revive hopes of producing effective immunotherapeutic and microbicial Abs at affordable cost. Recognition at the FRs of Abs suggests that an ancient antigenic homolog of the gp120 superantigenic region guided the evolution of germline genes encoding the HIV neutralizing Abs. Supported by NIH.

## INTRODUCTION

The mutability of the immunodominant epitopes expressed by the HIV envelope protein gp120 confounds the ability of the immune system to produce antibodies that can neutralize diverse strains found across the world. The gp120 site responsible for binding to host CD4 receptors (CD4bs), however, is relatively unchangeable, as the virus must maintain its infectivity. One such conserved component of the CD4bs is composed of residues 421–433 (1,2). Abs to this epitope may be hypothesized, therefore, to recognize and impede infection by diverse HIV strains. This epitope is also a component of the B cell superantigen (SAG) site expressed by gp120 (hereafter designated CD4bs-SAG epitope). SAGs sites are recognized by antibodies (Abs) found in the preimmune repertoire without requirement of adaptive sequence diversification of Ab variable (V) domains. Framework regions (FRs) in Ab V domains are important in SAG recognition, whereas interactions with conventional antigens are dominated by the complementarity determining regions (CDRs). The ability of IgG class Abs to bind the gp120 SAG site in non-infected individuals is associated with reduced risk of contracting HIV infection (3). Some Abs possess a serine protease like enzymatic activity, permitting them to hydrolyze peptide bonds following noncovalent binding of polypeptide epitopes. We reported the catalytic hydrolysis of gp120 and weak HIV neutralization by preimmune IgM and IgA class Abs from humans without HIV infection that is attributable to recognition of the CD4bs-SAG epitope (4,5).

B cell SAG epitopes do not generally induce the adaptive improvement of Abs. Other than our report of improved Abs to the CD4bs-SAG epitope produced by long-term survivors of HIV infection (Abstract THA0305), there are no examples of neutralizing Abs to this epitope induced by the infection. Several clinical reports have noted the rare coexistence of HIV/AIDS and the autoimmune disease systemic erythematous (6). Sera from lupus patients contain increased levels of Abs that bind a synthetic peptide spanning the CD4bs-SAG (7). We have previously reported an HIV neutralizing single chain Fv (VL-Li-VH; VL and VH denote the light and heavy chain V domains, and L, a linker peptide) from a phage displayed scFv repertoire of lupus patients (8).

We report here the identification and characterization of scFv clones from lupus patients with binding and catalytic activities directed to the CD4bs-SAG epitope. The scFvs displayed exceptionally broad neutralization of CR5-dependent HIV strains expressing diverse envelope sequences at picogram/ml-nanogram/ml concentrations, reviving the hope of an effective immunotherapeutic reagent against R5-dependent HIV strains at affordable costs. The scFv V domains were extensively mutated. Chimeragenesis studies indicated the importance of FRs in recognition of the CD4bs-SAG epitope, indirectly suggesting improvement of epitope contacts with the FRs as a novel pathway for obtaining improved Abs.

## METHODS

**Electrostatic probes.** The preparation and characterization of electrostatic probes (designated E-gp120 or E-peptides) of full length gp120 (clade B MN strain), synthetic analogs of the CD4bs-SAG epitope and the irrelevant peptide VIP containing phosphonate diesters and biotin at lys side chains or the C terminus is described (9–11). Two CD4bs-SAG epitopes were studied, gp120 residues 421–433 and LPSRI-421–433 (consensus clade B sequence). The LPSRI peptide region corresponds to gp120 residues 416–420 thought to regulate the 421–433 epitope conformation.

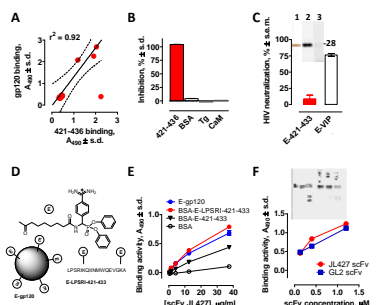
**Phage scFvs.** The phage scFv library from lupus patients is described (12). Phage selections with gp120 was as in (8). Phages incubated in E-gp120 solution were precipitated with PEG, fractionated on an anti-biotin antibody column, and bound phages were eluted at low pH. scFvs were purified from bacterial periplasmic extracts to electrophoretic homogeneity following expression in soluble form (metal affinity chromatography by means of the his6 tag) (12).

**Binding and hydrolytic activity.** Immobilized antigens in ELISA plates were allowed to bind the scFvs. Bound complexes were detected using anti-c-myc IgG (the scFvs contain a c-myc peptide tag). Hydrolysis of biotinylated gp120 was determined by a decrease of low mass fragments by electrophoresis and densitometry (9).

**scFv chimeras.** FR and CDR replacements of scFv GL2 and scFv JL427 were swapped by PCR-based substitution using mutagenic primers encoding the individual regions and pHEN2 plasmid.

**HIV neutralization:** This was determined by p24 measurement following infection of peripheral blood mononuclear cells by clinical HIV isolates or reporter pseudovirus assays as described (8, 13).

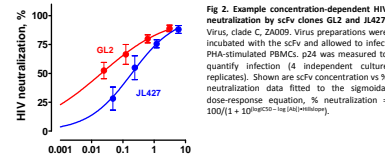
## RESULTS



**Fig. 1. scFv recognition of the CD4bs-SAG epitope.** A, Correlated binding of lupus scFv to full-length gp120 and CD4bs-SAG epitope (residues 421–436). Bacterial periplasmic extracts conjugated with human scFvs from lupus patients were tested for binding to gp120 or albumin conjugated synthetic 421–436 peptide (MN strain sequence) by ELISA. The 3 scFv clones with greatest gp120 activity in the figure were identified by phage selection of the scFv library using immobilized gp120. The remaining clones were identified by screening randomly picked clones for gp120 binding (A500–0.3). Pearson correlation,  $r=0.92$ ,  $P<0.0007$ . B, Competitive inhibition of gp120 binding by CD4bs-SAG epitope. scFv JL427 purified by affinity chromatography (46  $\mu$ g/ml) was allowed to bind immobilized gp120 in ELISA plates in the presence of soluble peptide 421–436, BSA, thyroglobulin (Tg) and calmodulin (CaM) (1  $\mu$ M). scFv binding was measured using anti-c-myc antibody. C, Inhibition of scFv JL427 neutralizing activity by E-421–433. JL427 scFv (1.5  $\mu$ g/ml) was preincubated (0.5 h) with E-421–433 (100  $\mu$ M), control E-VIP HIV-1 virus (strain ZAO09, clade C) were added and infection of PMNSs was measured. Inset, Anti-c-myc stained blot of scFv JL427 (lane 1); streptavidin stained blot of E-421–433 and E-VIP tested scFv JL427 (lane 2 and 3, respectively). D, E-probe structures. E, The electrostatic phosphonate linked to CD4bs-SAG epitope or full-length gp120 at lys side chains. F, Identification of an improved CD4bs-SAG probe, E-LPSRI-421–433. ELISA data showing dose-dependent scFv JL427 binding to immobilized E-LPSRI-421–433, BSA conjugated E-LPSRI-421–433, BSA conjugated E-421–433 and control BSA. F, Recognition of CD4bs-SAG epitope by scFv GL2. Dose-dependent scFv GL2 binding to immobilized E-LPSRI-421–433. Inset, Hydrolysis of gp120 by scFv GL2. Streptavidin-peroxidase stained SDS-electrophoresis gel of Bt-gp120 (0.1  $\mu$ M) incubated with purified scFv GL2 (11.2  $\mu$ g/ml, 64 h incubation). Control scFv 18B analyzed in parallel is devoid of cleavage activity. Diluent lane, gp120 incubated with buffer instead of scFv.

Selecting Antigen	# of clones, tested	# of clones, neutralizing	Potency (EC50, $\mu$ g/ml)
gp120	52	25	17
E-gp120	14	24	0

**Table 1. HIV neutralization (clade C, RS, ZA009) by lupus scFv fragments selected using gp120 or E-gp120.** The scFvs were purified by Ni-NTA chromatography and tested for neutralization using ZAO09 (clade C, RS).



**Fig. 2. Example concentration-dependent HIV neutralization by scFvs clones GL2 and JL427.** Virus, clade C, ZA009. Virus preparations were incubated with the scFv and allowed to infect PHA-stimulated PMNSs. p24 was measured to quantify infection (4 independent culture replicates). Shown are scFv concentration vs. % neutralization.  $IC_{50}$  values were determined by sigmoidal dose-response equation, % neutralization =  $100 / (1 + 10^{(IC_{50} - [Ab]) / (IC_{50} - [Ab])})$ .

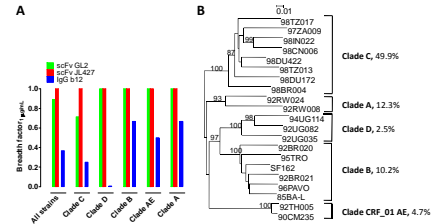
Clade	Strain	scFv JL427	scFv GL2	IC <sub>50</sub> (µg/ml)	Potency factor
C	98T2013	<0.002	2.2	>10	>3125
	98DU172	0.002 (-4.5)	NT (-4.5)	9.9 (1.3)	1226
	98DU422	0.015 (-4.5)	0.015 (-4.5)	10.0 (0.7)	667
	98BR004	<0.030	1.69	>10	333
	97ZA009	0.064	0.008	8.43	132
	98T2017	<0.040	<0.040	1.63	>41
	98M022	<0.032	<0.024	0.866	>21
B	97ZA012	0.092	NT	0.15	76
			Geometric mean	187	52
			Geometric mean	1372	61
D	92UG035	<0.0036	0.079	4.83	>1342
	92UC082	<0.0036	0.26	4.83	>1342
	94UG114	<0.0036	0.1	2.36	>656
			Geometric mean	1657	30
B	92BR021	<0.0036	0.13	1.29	>356
			Geometric mean	19	>19
			Geometric mean	>6	0.04
AE	CM235	<0.0036	0.63	>10	>2778
	92TH005	0.022	0.84	0.8	36
			Geometric mean	316	4
A	97USSN64	0.033	0.55	0.27	>9
	92RW008	0.54	0.58	1.64	3
	92RW024	<0.090	0.2	0.13	>1.4
		Geometric mean	3	3	0.5
		Geometric mean of all strains	10532	3433	9

**Table 2. Neutralization of CR5-dependent HIV strains by scFv JL427, scFv GL2 and monoclonal IgG b12.** Assays as in Fig. 2. IC<sub>50</sub> values are from least-square fit sigmoidal dose-response curves. For potency comparison, data for IgG b12 are included for each strain. “Potency factor” represents scFv potency relative to IgG b12 computed as  $IC_{50}(b12)/scFv$ . Assays were conducted in the labs of Carl Hanson and David Montefiori. All strains are CR5-dependent. IC50 values of assay observed in reporter pseudovirus assays are in parentheses.

Clade	Strain	V16	V17	V18	V19	V20	V21	V22	V23	V24	V25	V26	V27	V28	V29	V30	V31	V32	V33	Mutations
C	98DU172	L	D	C	R	I	K	D	I	I	R	M	W	D	D	D	D	D	D	A
	97ZA009	L	P	C	R	I	K	D	I	I	R	M	W	D	D	D	D	D	D	A
	98T2017	L	P	C	R	I	K	D	I	I	R	M	W	D	D	D	D	D	D	A
	98BR004	L	P	C	R	I	K	D	I	I	R	M	W	D	D	D	D	D	D	A
B	98M022	L	P	C	R	I	K	D	I	I	R	M	W	D	D	D	D	D	D	A
	98BR004	L	P	C	R	I	K	D	I	I	R	M	W	D	D	D	D	D	D	A
	98BR004	L	P	C	R	I	K	D	I	I	R	M	W	D	D	D	D	D	D	A
	98BR004	L	P	C	R	I	K	D	I	I	R	M	W	D	D	D	D	D	D	A
D	92UG035	L	P	C	R	I	K	D	I	I	R	M	W	D	D	D	D	D	D	A
	92UC082	L	P	C	R	I	K	D	I	I	R	M	W	D	D	D	D	D	D	A
	94UG114	L	P	C	R	I	K	D	I	I	R	M	W	D	D	D	D	D	D	A
	92BR021	L	P	C	R	I	K	D	I	I	R	M	W	D	D	D	D	D	D	A
AE	CM235	L	P	C	R	I	K	D	I	I	R	M	W	D	D	D	D	D	D	A
	92TH005	L	P	C	R	I	K	D	I	I	R	M	W	D	D	D	D	D	D	A
	92TH005	L	P	C	R	I	K	D	I	I	R	M	W	D	D	D	D	D	D	A
	92TH005	L	P	C	R	I	K	D	I	I	R	M	W	D	D	D	D	D	D	A

**Table 3. Sequence of CD4bs-SAG epitope of RS HIV strains neutralized by scFv JL427.** Red, non-conservative replacement; green, conservative replacement compared to clade C consensus sequence.

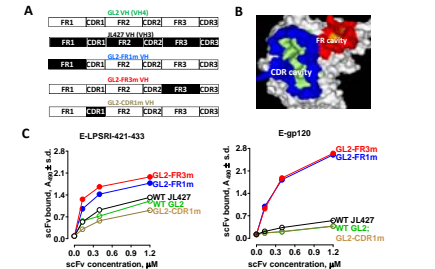
Despite the indicated mutations, all strains tested were neutralized at  $\mu$ g/ml – ng/ml concentrations. Viral escape by mutations at the indicated positions, therefore, should be minimal.



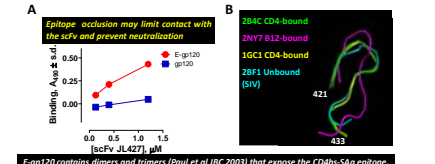
**Fig. 3. Breadth of HIV neutralization by scFvs GL2 and JL427.** “Breadth factor<sub>100%</sub>” represents: (Number of strains neutralized by scFv with  $IC_{50} < 1 \mu$ g/ml) / (Total number of strains tested in Table 2). B, Phylogenetic relationship of HIV RS-dependent strains neutralized by scFv JL427. The tree was derived by nucleotide sequence alignment of the gp120 sequences of the neutralizable strains (CLUSTALW multiple sequence alignment program), using the neighbour joining method (PHYMLIP 3.2 package) after exclusion of gaps. Horizontal branch lengths are drawn to scale. Bar indicates 0.01 nucleotide substitution per site. Right, estimated percent global prevalence as % of total infections.

Clade	Corceptor	Strain	IC50 (µg/ml)	IgG b12
B	X4	92H1599	<0.03	0.11
	X4	B2167	>10	0.26
	X4	BR014	1.8	NT
	X4	TYBE	>10	NT
D	X4	92UG001	>10	0.15
	X4	92UC086	>10	1.63
	X4	92UC086	>10	NT
	X4	90UC266	>10	NT

**Table 4. Poor scFv JL427 neutralization of X4-dependent strains.** Assays as in Fig. 2 and Table 2.



**Fig. 4. Evidence for recognition of CD4bs-SAG epitope by scFv framework regions.** A, Schematic representations of the VH domains contained in wild type (WT) scFv JL427, wildtype scFv GL2, FR1-swapped scFv GL2 (GL2-FR1m) chimera, FR3-swapped scFv GL2 (GL2-FR3m) chimera and FR1-swapped scFv GL2 (GL2-FR1m) chimera. Black and white boxes, respectively, are regions derived from scFv JL427 and scFv GL2. The mutants contained the VL domain of scFv GL2. B, scFv GL2 molecular model. Surface of scFv model showing FR and CDR cavities. The rims and bottom surface of the cavities are shown in different colors. Aggregate number of sequence divergences at scFv GL2 and scFv JL427 V domains compared to their germline counterparts were respectively, 7 and 55. Of these mutations, 3 and 1 sequence divergences attributable to somatic hypermutation were located, respectively, in the FRs and CDRs of scFv GL2. The corresponding number of divergences for scFv JL427 were, respectively, 11 and 12. C, Improved E-LPSRI-421-433 and E-gp120 recognition by FR1 and FR3 chimeras of scFv GL2. ELISA data showing scFv binding to immobilized E-LPSRI-421-433 (x4) conjugate; 70 ng peptide equivalent/well or E-gp120 (100 ng/well).



**Fig. 5. Epitope occlusion hypothesis explaining poor scFv neutralization of CXCR4-dependent strains.** A, ELISA using immobilized gp120 and E-gp120. B, Superimposed CD4bs-SAG epitope structures (residues 421–433) from published crystal structures of various monomer gp120 isoforms. (Piang et al. PMID 16281895; Zhou et al. PMID 17332175; Kwoing et al. PMID 9541577; Chen et al. PMID 15729334)

## CONCLUSIONS

- Selection of the lupus scFv phage library with gp120 and E-gp120 identified scFvs recognizing the CD4bs-SAG epitope (Fig. 1). There was no evidence of scFv polyreactivity as reported for certain neutralizing anti-HIV antibodies (Fig. 18).
- scFvs that catalyzed the hydrolysis of monomer gp120 neutralized the clade C strain ZA009 more potently than reversibly binding scFvs (Fig. 15, Fig. 2 and Table 1). However, monomer gp120 hydrolysis was an insufficient guide for scFv reactivity with various strains. Reversibly binding scFv JL427 neutralized many strains better than the catalytic scFv GL2, presumably because of the differences in the viral epitope structure that diminish reactivity with the catalyst (Table 2).
- scFv JL427 neutralizing 20 diverse CR5-dependent HIV strains. Breadth of neutralization and potencies were superior to the anti-CD4bs IgG b12 (Table 2, Fig. 3).
- scFv JL427 was “universally” neutralizing despite sequence divergences at certain CD4bs-SAG epitope positions (Table 3). This suggests a low probability of viral escape via mutation in the CD4bs-SAG epitope of RS strains. However, conversion from RS- to X4-corceptor dependence represents a potential route for viral escape (Table 4, see below).
- CXCR4-dependent strains were neutralized poorly compared to RS strains with similar CD4bs-SAG epitopes (Table 2). Remote mutations and post-translational structural modifications can influence HIV neutralization by antibodies. We hypothesize that X4 strains contain a sterically occluded CD4bs-SAG that is comparatively inaccessible to the scFv. Structurally perturbed monomer gp120 expressed a more exposed CD4bs-SAG epitope, suggesting epitope occlusion as an explanation for poor X4 strain neutralization (Fig. 5). Pseudoviruses with envelopes identical in sequence to the neutralizable RS-strain were also superior to the scFvs, presumably because of insufficient CD4bs-SAG exposure (Table 2).
- Unlike conventional antigens, B cell SAG recognition entails recognition by antibody V domains, particularly VH3 family FRs. Replacement of the FR1/FR3 of the VH4 family scFv GL2 by corresponding FRs of the VH3 family scFv JL427 generated chimeric scFvs with improved CD4bs-SAG recognition (Fig. 4). Adaptive improvements of CD4bs-SAG recognition by V domain FRs driven by an unidentified antigen, therefore, is a feasible mechanism to produce neutralizing antibodies.
- The observed FR involvement is consistent with the theory that SAG recognition is a germline V gene function that arose over millions of years of evolution, presumably to counter a primordial virus expressing a CD4bs-SAG epitope analog of modern HIV. The identification of an endogenous retroviral sequence with nucleotide similarity to the CD4bs-SAG epitope supports the theory (14).
- In view of their breadth and potency of HIV neutralization, the scFvs are candidates for systemic therapy of HIV infection and topical application for prevention of mucosal HIV transmission.

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