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J Immunol 2012; 189:5367-5381; Prepublished online 22 October 2012;
doi: 10.4049/jimmunol.1200981
http://www.jimmunol.org/content/189/11/5367

Supplementary Material
http://www.jimmunol.org/content/suppl/2012/10/22/jimmunol.1200981.DC1

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Antibodies to a Superantigenic Glycoprotein 120 Epitope as the Basis for Developing an HIV Vaccine

Stephanie A. Planque,* Yukie Mitsuda,* Yasuhiro Nishiyama,* Sangeeta Karle,* Stephane Boivin,* Maria Salas,† Mary-Kate Morris,† Mariko Hara,* Guangling Liao,*+1 Richard J. Massey,‡ Carl V. Hanson,† and Sudhir Paul*,‡

Failure to induce synthesis of neutralizing Abs to the CD4 binding determinant (CD4BD) of gp120, a central objective in HIV vaccine research, has been alternately ascribed to insufficient immunogen binding to Abs in their germline V region configuration expressed as BCRs, insufficient adaptive mutations in Ab V regions, and conformational instability of gp120. We employed peptide analogs of gp120 residues 421–433 within the CD4BD (CD4BDcore) to identify Abs produced without prior exposure to HIV (constitutive Abs). The CD4BDcore peptide was recognized by single-chain Fv fragments from noninfected humans with lupus that neutralized genetically diverse strains belonging to various HIV subtypes. Replacing the framework region (FR) of a V_{H}3-family single-chain Fv with the corresponding V_{H}3-family FRs from single-chain Fv JL427 improved the CD4BDcore peptide-binding activity, suggesting a CD4BDcore binding site outside the pocket formed by the CDRs. Replacement mutations in the FR site vicinity suggested the potential for adaptive improvement. A very small subset of serum CD4BDcore-specific serum IgAs from noninfected humans without autoimmune disease isolated by epitope-specific chromatography neutralized the virus potently. A somatic mutations was induced by immunization with a CD4BDcore peptide analog containing an electrophilic group that binds vicinity suggested the potential for adaptive improvement. A very small subset of serum CD4BDcore-specific serum IgAs from noninfected humans without autoimmune disease isolated by epitope-specific chromatography neutralized the virus potently. A very small subset of serum CD4BDcore-specific serum IgAs from noninfected humans without autoimmune disease isolated by epitope-specific chromatography neutralized the virus potently. A very small subset of serum CD4BDcore-specific serum IgAs from noninfected humans without autoimmune disease isolated by epitope-specific chromatography neutralized the virus potently. A very small subset of serum CD4BDcore-specific serum IgAs from noninfected humans without autoimmune disease isolated by epitope-specific chromatography neutralized the virus potently. A very small subset of serum CD4BDcore-specific serum IgAs from noninfected humans without autoimmune disease isolated by epitope-specific chromatography neutralized the virus potently. A very small subset of serum CD4BDcore-specific serum IgAs from noninfected humans without autoimmune disease isolated by epitope-specific chromatography neutralized the virus potently. A very small subset of serum CD4BDcore-specific serum IgAs from noninfected humans without autoimmune disease isolated by epitope-specific chromatography neutralized the virus potently. A very small subset of serum CD4BDcore-specific serum IgA by immunization with a CD4BDcore epitope that is available for amplification for vaccination against HIV. The Journal of Immunology, 2012, 189: 5367–5381.

The HIV-1 surface expresses noncovalently associated oligomeric gp120–gp41 complexes that have been the targets of numerous experimental vaccines. Consensus has developed that inducing Abs to one or more structurally conserved epitopes is required for protection against genetically diverse HIV strains responsible for the pandemic (broadly neutralizing Abs). Most Abs to gp120 bind its mutable epitopes. Conserved gp120 epitopes essential for the viral life cycle are poorly immunogenic (1, 2). Binding of the mostly conserved gp120 conformational determinant to the host CD4 receptors (CD4 binding determine [CD4BD]) initiates infection. Broadly neutralizing Abs to the CD4BD have been identified in HIV-infected patients (2–4). Although the CD4BD is vulnerable to host immunity, Abs with the correct specificity are not produced in sufficient amounts for effective protection against the infection. Various CD4BD epitopes are not equally susceptible to Ab neutralization. Some Abs bind the CD4BD of monomer gp120, but fail to neutralize HIV (5). Deviations from the native CD4BD conformation were suggested to underlie the failure of monomer gp120 to induce the synthesis of broadly neutralizing Abs (1). Genetically engineered oligomeric gp120 also failed to induce broadly neutralizing Abs (6). The mAb b12 to a conformational epitope that overlaps the CD4BD outer domain region expresses comparatively broad neutralizing activity (2, 7). An immunogen reverse engineered to be conformationally complementary to the b12 binding site did not induce neutralizing Abs (8).

These difficulties have inspired divergent hypotheses suggesting that the failure of the CD4BD to induce neutralizing Abs may reflect an intrinsic weakness of the humoral immune system. Inducing Ab synthesis involves initial weak immunogen binding to BCRs with V regions encoded by germline genes (Abs associated with signal-transducing proteins), followed by immunogen-driven clonal expansion of B cells and selection of BCRs containing mutated CDRs with improved Ag-binding affinity. The BCR repertoire expressed constitutively prior to contact with immunogen is diverse and large, composed of paired V_{H} and V_{L} domains generated from ~500 V, D, and J germline genes by V-(D)-J gene recombination, a step that introduces sequence diversity in CDR3 (9). The diversity of constitutive BCRs is innate in the sense that it is generated randomly with no requirement for contact with immunogen. Dimitrov and coworkers (10) suggested that an unusually low binding affinity of conserved HIV epitopes for constitutive BCRs...
precludes B cell recruitment into the adaptive differentiation pathway (Fig. 1A). Conversely, neutralizing mAbs from HIV-infected patients that bind discontinuous gp120 outer domain epitopes overlapping the CD4BD contain extremely dense V region somatic mutations (11, 12), prompting the hypothesis of an intrinsic incompetence of B cells in generating sufficiently mutated Abs.

Our approach to HIV vaccine design focuses on the exposed, mostly conserved 421–433 residues located in the β20/β21 turn of the gp120 bridging sheet that provide essential contributions in high-affinity CD4 recognition (CD4BDcore) as determined by mutagenesis and crystallography (13–16). Importantly, the CD4BDcore appears to contribute only minimally to the CD4BD-overlapping outer domain epitopes recognized by neutralizing Abs from infected patients (3, 12). Several groups have reported that Abs produced spontaneously by humans without HIV infection or vaccination (constitutive Abs) recognize the CD4BDcore-spanning superantigenic determinant of gp120 and synthetic peptides containing the CD4BDcore (17–20). Superantigenic epitopes are recognized by Abs containing V regions in the germline configuration with no requirement for an immunogen-driven Ab response (21, 22). The binding occurs mostly at germline gene-encoded structural elements in the framework regions (FRs) and certain CDR residues outside the traditional Ag-binding pocket formed by the CDRs (22–24). Upon completion of the noncovalent CD4BDcore-binding step, a subset of the constitutive Abs proceeds to catalyze the hydrolysis of gp120 by a nucleophilic mechanism (19, 20). However, superantigenic recognition by constitutive Abs comes at a cost. Like other microbial superantigenic epitopes (21), the CD4BDcore is poorly immunogenic. Infected humans and animals immunized with gp120 rarely generate neutralizing Abs to the CD4BDcore by adaptive immune mechanisms (25, 26). Unlike the stimulatory effect of Ag binding at the CDRs, noncovalent superantigen-FR binding downregulates further B cell differentiation (Fig. 1A) (21). We and others have suggested that epitopes essential for microbial survival have evolved superantigenic character as an immune evasion mechanism that precludes an efficient adaptive Ab response (27, 28).

Capitalizing on the CD4BDcore superantigenicity for HIV vaccination may be feasible if the constitutive Abs neutralize the virus. Braun and colleagues (29) reported highly variable binding of the gp120 superantigen determinant by serum IgGs in a large group of noninfected humans, with low binding activity serving as a predictor for increased incidence of subsequent HIV infection. However, the gp120-binding Abs from the sera and a phage library from noninfected humans did not neutralize HIV detectably under commonly employed tissue culture conditions (20, 22, 30), raising doubt about the functional relevance of the constitutive Abs. We hypothesize the existence of a broad range of constitutive Abs with varying CD4BDcore reactivity of which a small subset is capable of potent and broad HIV neutralization (Fig. 1B, top panel).

The physiological restriction on adaptive Ab induction must be overcome to develop a CD4BDcore-based vaccine. There is no precedent for a clinically useful Ab response that targets a B cell superantigen epitope. We reported electrophilic analogs of CD4BDcore-spanning peptides that bind the constitutive Abs noncovalently, coordinated with covalent electrophilic binding to Ab nucleophilic sites (19, 20). Like the noncovalent superantigen binding sites, nucleophilic sites are expressed constitutively by secreted Abs and membrane-bound BCRs (31). We also reported mAbs to the CD4BDcore with HIV-neutralizing activity raised by immunization with an electrophilic analog of full-length gp120. This suggested covalent stimulation of B cells as a strategy to induce the synthesis of Abs to the CD4BDcore. Classical vaccines depend on the somatic hypermutation process to develop neutralizing Abs. If a constitutive Abs subset with neutralizing activity directed against genetically divergent HIV strains exists, amplifying this subset could protect against HIV infection even without an improvement of the Abs via somatic hypermutation (Fig. 1B, middle panel). The extent to which the immunogen targets the minority of B cells producing strongly neutralizing constitutive Abs is an important factor. An immunogen that amplifies only moderately neutralizing constitutive Abs will be a less effective vaccine (Fig. 1B, bottom panel).

We describe evidence for epitope-specific constitutive Abs from noninfected humans with potent ability to neutralize diverse HIV strains identified using the electrophilic CD4BDcore-spanning peptide probes. Structural analysis and mutagenesis studies of the constitutive human Abs supported a contribution of the FRs in CD4BDcore recognition and the potential of adaptive Ab improvement. A monoclonal murine IgM isolated following immunization with an electrophilic CD4BDcore peptide recognized the CD4BDcore specifically and displayed broad HIV neutralization despite being free of V gene somatic mutations. The findings validate constitutive CD4BDcore recognition by Abs as a novel principle supporting HIV vaccine development.

Materials and Methods

Polyepitope probes

Proteins were obtained from these sources: OVA, BSA, thyroglobulin, and calmodulin from Sigma-Aldrich (St. Louis, MO); soluble CD4 (sCD4; 4 domain) from Problin Science (Meriden, CT); recombinant gp120 (MN strain) from Immunodiagnostics (Woburn, MA); and biotinylated BSA from Pierce (Rockford, IL). Biotinylated gp120 (1.8 mol biotin/mol), E-gp120 (24.8 mol phosphonate/mol), and biotinylated E-gp120 (1.2 mol biotin/mol; 33 mol phosphonate/mol) were prepared by linking phosphonate diesters and biotinamidothiolyx to Lys side chains, as described (31, 32). Nonelectrophilic peptide 421–436 conjugated to BSA (NE-421–436; 6 mol peptide/mol) and electrophilic E-421–433 with biotin at the N terminus (E-416–433 with biotin at the N terminus (E-416–433a); and E-416–433 conjugated to keyhole limpet hemocyanin (KLH) (E-416–433b; 2036 mol peptide/mol) or to BSA (E-416–433c; 6.1 mol peptide/mol) contained the consensus subtype B gp120 421–433 residues and were prepared as described previously (4, 31, 33). Cys141 in the 416–433 peptides was replaced with Ser to preclude intermolecular S-S bonding. Phosphonate diester groups were located at the C terminus in E-421–433 and at Lys521 and Lys532 side chains in E-416–433a, E-416–433b, and E-416–433c. Control biotinylated E-vasoactive intestinal peptide (VIP) was prepared, as described previously (34). The chemical identity of all peptides was verified by electrospray ionization mass spectrometry. Ala-containing mutant 416–433 peptides, nonelectrophilic NE-416–433 peptide, and sequence-shuffled 416–433 peptide (50416–433, GQSKWEPAKRNLVMIQ) were synthesized in the PEPscreen platform, and their structural identity was verified by matrix-assisted laser desorption time-of-flight mass spectrometry by Sigma-Aldrich (4).

Abs

The use of human subjects and vertebrate animals was approved by our Institutional Committee for Protection of Human Subjects and the Animal Welfare Committee. The animal protocol adheres to American Association for the Accreditation of Laboratory Animal Care International guidelines. Written informed consent was provided by humans who were studied. The healthy humans had no evidence of infectious or other disease (age range 28–48 y; 5 males, 5 females). Abs were purified from sera or tissue culture fluids to electrophoretic homogeneity using immobilized protein G (IgG), anti-IgA Ab, or anti-IgM Ab (35, 36). All Ab subunit bands stainable with Coomassie Blue were stainable with Abs to H (γ, α, μ) or L chain (κ, λ) stained by SDS-electrophoresis. The IgA fraction from serum (2 mg) was purified further by affinity chromatography on E-416–433 conjugated to agarose (4).

Single-chain Fv (scFv) clones were selected from a phagelid library from three systemic lupus erythematosus patients free of HIV infection by binding to immobilized gp120 (36), followed by two further selection rounds in which 1012 CFUs of the phages were treated for 2 h with biotinamidothiolyx-E-gp120 (6.1 μg) in PBS (0.13 mM NaCl, 137 mM NaH2PO4, 2.7 mM KCl [pH 7.4]), immune-complexed phages were captured on agarose-conjugated anti-biotin Ab, and bound phages were eluted with acid pH buffer (37). gp120 (36) and E-gp120 (38)
express the CD4BDcore as a component of the larger CD4BD in a conformation reaction with neutralizing Abs. A subset of nucleophilic Abs identified based on covalent binding to electrophilic E-gp120 expresses catalytic activity (32). The scFv clones contained a His6 tag and a c-myc tag. Secreted scFv expressed by Escherichia coli HB2151 cells was purified by metal-affinity chromatography and quantified by c-myc dot blotting (37). Mutated scFv GL2-GL2Rm and GL2-GL2Rm were prepared by synthesizing the V4 GL2 domain genes in which the nucleotide-sequence corresponding to aa 1–30, 66–94, or 50–65 (Kabat numbering) were replaced by the corresponding V4 GL2 sequence, followed by cloning of the mutant V4 domain gene in place of the wild-type V4 domain of scFv GL2 in pHEN2 vector via the ApaLI/NorI sites (Muragenex, Hillsborough, NJ). scFv GL2-GL2Rm was prepared by replicating the wild-type scFv GL2 vector with the mutated primers encoding JL427 VH V4 GL2 and JL427 VH GL2 GCATGCACTGGATCCGCCAGCACCC-3’ (38). Monoclonal IgG1 b12 used as a reference reagent was from the Scripps Research Institute (La Jolla, CA, USA). D. Burton) and monoclonal IgG 4B6 directed to phosphatidylinerine was from Abcam (Cambridge, MA).

Full-length IgG1 was prepared by inserting clone JL427 V1 and V4 domains adjacent to the human κ domain (BioGllNot sites; pML-JL427-HL vectors) or γ1C domains (EcorHindIII sites; pML-JL427-HG1) respectively (39). The L and H chain vectors were coexpressed in NS0 cells, and IgG in the culture supernatant was purified as before. mAbs were prepared from female BALB/c mice (The Jackson Laboratory; 4–5 wk) immunized by nasal instillation of E-416–433b (100 μg peptide equivalents/20 μl in 5 μl heat-labile E. coli enterotoxin Arg1–27 Glyy mutant adjuvant; provided by J. Clements, Tulane University, New Orleans, LA) at weekly intervals for 6 wk. Hybridomas were prepared by fusing spleenocytes and peritoneal cells 3 d after the final injection with NS-1 myeloma cells (32). Monoclonal cell lines were obtained by limiting dilution from wells secreting E-416–433c–binding IgMs. IgM was purified from concentrated tissue culture fluid (Cen trium, YM10 filter; Millipore, Billerica, MA) by chromatography on immobilized anti-IgM Ab and a Superose 6 column (Pharmacia, Piscataway, NJ). scFv GL2 and V1 domains directed to the CD4BD core (19, 20, 23, 24). Use of E-gp120 (20A, 2B), a probe that forms covalently assembled oligomers, enabled improved detection of the Abs (38). The peptide probes containing the 421–433 region are flexible and can assume alternate conformations with varying affinity for sCD4 (42–44). Electrophilic E-416–433 and E-416–433 were also validated previously as specific probes for constitutive Abs with CD4BD core specificity (4, 19, 20). The E-416–433 peptide containing the N-terminal 416–420 extension binds the Abs at somewhat superior levels compared with E-421–433 (38), and both peptide bind sCD4 with affinity lower than gp120 (4). The probes contain a peptide epitope that binds Abs noncovalently coordinated with covalent binding of the electrophilic phosphonate to naturally occurring nucleophilic Ab sites (Fig. 2C). A subset of nucleophilic Abs can catalyze the cleavage of peptide bonds. However, the nucleophilic sites are also expressed by noncatalytic Abs deficient in active site structures needed to complete the catalytic reaction cycle (31). Consequently, the electrophilic peptides are combined probes for the noncatalytic and catalytic Ab subsets. The CD4BDcore sequence is largely conserved in diverse HIV strains (see Supplementary Table I for example sequence divergences). The 421–433 CD4BDcore sequence in E-416–433 and E-421–433 corresponds to the consensus HIV-1 subtype B sequence. The 421–433 sequence in E-gp120 also corresponds to the subtype B consensus sequence except for a divergence at residue 429. Recombinant CD4BDcore-specific Abs

The paired V1–V4 domains of scFv constructs are surrogates of Ab binding sites. We initially became interested in the potential protective role of Abs to the CD4BDcore from evidence suggesting that these Abs are increased in patients with the autoimmune disease lupus (45) and comments in the literature about the rare coexistence of lupus and HIV infection (46). A CD4BDcore-reactive scFv clone obtained by fractionating a phage library from noninfected humans (lupus patients) was described previously (36). In this study, we describe the structure–function properties of two scFv clones with broad and potent HIV neutralizing activity from the same library (scFv JL427 and scFv GL2).
We fractionated the library by binding to immobilized gp120. Of 12 scFv clones obtained by gp120 fractionation, 5 displayed detectable binding to immobilized gp120 by ELISA. Four of the 5 gp120-binding clones neutralized HIV subtype C strain ZA009 in an initial screening assay consisting of three-point concentration–dependence curves (example data in Fig. 3A; Supplemental Fig. 2).

**FIGURE 1.** Alternative reasons underlying failed induction of Abs to the CD4BD and constitutive Abs as the basis for HIV vaccination. (A) X, detrimental B cell event; ✓, our proposed basis for HIV vaccination. Middle panel, B cell developmental events necessary for neutralizing Ab synthesis. Right panel, The CD4BDcore is a superantigenic epitope that binds the FRs of constitutive BCRs noncovalently, but this causes B cell downregulation. Left panel, Alternatively, B cells are unable to accumulate sufficient V gene mutations needed for CD4BD binding (3), or the initial affinity of germline BCRs for the CD4BD is too low (10). NAbs, neutralizing Abs. (B) Constitutive CD4BDcore-reactive Abs pertinent to HIV vaccination. Top panel, The innate germline Ab repertoire contains a wide range of Abs with varying CD4BDcore reactivity and HIV neutralizing potency. A small Ab subset with the greatest CD4BDcore reactivity is hypothesized to possess potent and broad neutralizing activity (black). Middle panel, An electrophilic vaccine expressing a CD4BScore that mimics the viral CD4BDcore conformation accurately will selectively amplify the Ab subset with greatest CD4BDcore reactivity and neutralizing activity effective against genetically divergent HIV strains. Bottom panel, A vaccine that mimics the viral CD4BDcore imperfectly will amplify an Ab subset with lesser CD4BDcore reactivity and neutralizing activity (gray).

**FIGURE 2.** Probes for CD4BDcore-binding Abs. (A) Electrophilic peptide and gp120 analogs. The probes contain residues 421–433 constituting the CD4BDcore. The N-terminal LPSRI residues in E-416–433 stabilize the 421–433 epitope conformation (42). The electrophilic phosphonate is located at Lys421 and Lys432 side chains in E-416–433 and at the C terminus in E-421–433. Biotin placed at the N terminus of some probes enabled detection of immune complexes. BSA and KLH conjugates of the electrophilic peptides were used, respectively, for ELISA and immunization. NE-416–433 and NE-421–436 are the nonelectrophilic peptide probes, and Sh416–433 is the shuffled-sequence, control peptide. E-VIP is an irrelevant control peptide. E-gp120 contains electrophilic phosphonate groups at surface-accessible Lys side chains. (B) Substituent structure. R1 and R2 denote, respectively, the electrophilic phosphonate and positively charged amidino-linker substituents. (C) Coordinated noncovalent and covalent probe binding by Abs. Initial noncovalent binding of amino acids constituting the CD4BDcore epitope (black ovals) to the Ab paratope imparts specificity to the subsequent covalent binding of the electrophilic phosphonate (E) by a nucleophilic (Nu) Ab site (31). Bt, Biotinamidohexanoyl.
reports binding and neutralization data for individual clones). An identically purified extract from bacteria harboring the empty pHEN2 vector was devoid of neutralizing activity. scFv JL427 and scFv JL606, the two clones with greatest strain 97ZA009-neutralizing potency, also neutralized additional HIV strains (subtype C strain BR004 and subtype B SF162; Supplementary Fig. 2). As scFv JL427 displayed superior neutralizing potency across the three strains tested in the initial studies, this clone was picked for further analysis (mean IC₅₀ for scFv JL427 and scFv JL606, respectively, 0.053 and 0.649 μg/ml). There was no correlation between the magnitude of gp120 binding and neutralizing potency of the four clones (e.g., scFv JL427 and scFv JL606 displayed nearly equivalent gp120 binding, but differing average neutralizing potency). However, none of scFv clones devoid of gp120-binding activity neutralized HIV detectably (n = 7; 2.5 μg scFv/ml; p < 0.01 versus scFv group with binding activity, n = 5, Fisher’s exact test).

We reported the ability of E-gp120 to induce the synthesis of Abs that hydrolyze gp120 (32). To identify scFv clones with catalytic activity, the gp120-fractionated library was subjected to further fractionation using E-gp120. Of 21 scFv clones screened, 1 scFv displayed slow gp120 hydrolytic activity evident after prolonged incubation (Fig. 3B; scFv GL2; 2.3% biotinylated gp120 hydrolyzed/h at 0.36 μM scFv, gp120 substrate concentration 100 nM; measured as in Ref. 32). This clone was also positive for gp120 binding by ELISA (A490 0.25). scFv GL2 neutralized HIV strain 97ZA009 potently, and it was picked for further analysis (Supplemental Fig. 2).

Example concentration–dependence data verifying the scFv neutralizing activity in independent scFv preparations are shown in Fig. 3C. To verify HIV neutralization within the physiological Ab scaffold, we also prepared full-length IgG JL427 containing the scFv VH and VL domains. The purified IgG displayed concentration-dependent neutralizing activity (Fig. 3C). A few neutralization assays included the reference monoclonal IgG b12 directed to the CD4BD epitope located in the gp120 outer domain (2). scFv JL427 displayed superior potency compared with IgG b12 (IC₅₀ geometric mean and range, respectively: strain 98TZ013, 0.017 and 29.2; 92UG082, 0.004 and 4.8; strain SF162, 0.011 and 0.721). The viability of PBMCs treated for 4 d with scFv JL427 (60 μg/ml) or PBS was comparable, ruling out nonspecific scFv cytotoxicity (respectively, 84 and 74%). scFv JL427 did not neutralize irrelevant viruses (20 μg scFv/ml; Dengue II virus, St. Louis encephalitis virus, and West Nile virus), suggesting specific HIV neutralizing activity.

Both scFv JL427 and scFv GL2 displayed E-416–433b-binding activity, with scFv JL427 displaying 12.0-fold superior binding (Fig. 4A). E-gp120 binding by scFv JL427 was also superior to the binding by scFv GL2 (by 2.5-fold; Fig. 4B). The differing E-416–433b/E-gp120–binding ratios for the two scFv clones suggest a nonidentical fine specificity of CD4BDcore recognition (scFv JL427, 7.3; scFv GL2, 1.5; determined from scFv concentrations yielding A490 values of 0.2). scFv JL427 was bound by E-421–433, but not the irrelevant probe E-VIP (Fig. 4C, inset). Its HIV neutralizing activity was inhibited by E-421–433, but not E-VIP (Fig. 4C). As further evidence of specificity, the binding of scFv JL427 to immobilized peptide NE-421–436 devoid of the phosphonate groups was inhibited competitively by soluble peptide NE-421–436, but not irrelevant proteins (Fig. 4D). The scFv specificity behavior differs from the previously described polyreactive binding of structurally unrelated Abs by nonneutralizing gp120-binding recombinant Fabs isolated from a noninfected human (30). Also, unlike the scFv, the previously described Fabs did not display any clear specificity for the CD4BDcore within the gp120 sequence.

The breadth of neutralization was tested using a panel of 17 HIV-1 strains. Both scFv clones neutralized all subtype A, B, C, D, and AE strains tested. scFv JL427 displayed superior mean neutralization potency across the panel of strains (Table I; IC₅₀ geometric mean and range, respectively: scFv JL427, 0.014 μg/ml and 0.001–0.169 μg/ml; scFv GL2, 0.337 μg/ml and 0.002–2.218 μg/ml; strains neutralized at >50% levels at the lowest tested Ab concentration tested in Table I were not included in geometric mean computation). Taken together, the data indicate potent and broad HIV neutralization due to specific CD4BDcore binding by the scFv clones.

scFv GL2 hydrolyzed recombinant gp120 slowly (see above). Its lesser average neutralizing potency compared with the non-hydrolytic scFv JL427 suggests that hydrolysis of gp120 does not contribute appreciably in virus neutralization. From the rate of recombinant gp120 hydrolysis, only 0.08 and 9.6% of the viral gp120 will be hydrolyzed, respectively, at the mean 50% neu-

![FIGURE 3.](https://www.jimmunol.org/ Downloaded from http://www.jimmunol.org/ by guest on June 1, 2017)
FIGURE 4. CD4BD\textsuperscript{core} peptide analog binding by scFv JL427 and scFv GL2. (A) E-416–433b binding. Binding of the purified scFv to immobilized KHL-conjugated E-416–433b (70 ng/well). Values are corrected for nonspecific binding to KLH. An identically purified extract of E. coli harboring empty pHEN2 vector without the scFv gene did not display detectable binding. Values are mean ± SD. (B) E-gp120 binding. Binding of purified scFv to immobilized E-gp120 (100 ng/well). Values are corrected for nonspecific binding. An identically purified extract of E. coli harboring empty pHEN2 vector without the scFv gene did not display binding. Values are mean ± SD. (C) Inhibition of scFv JL427 neutralizing activity by E-421–433. Neutralization of HIV subtype C strain 97ZA009 was determined in the presence of E-421–433 or control E-VIP (final concentration, 100 μM). Values are means ± SEM of four culture replicates. Inset. Streptavidin-stained, reducing SDS-gel blots showing adducts of the SEM of four culture replicates. Anti–c-myc staining of c-myc tag in the scFv. (D) scFv JL427-binding specificity. Binding of the scFv (46 μg/ml) to immobilized peptide NE-421–436 was measured in the absence or presence of soluble NE-421–436 or the irrelevant peptide NE-421–436 was measured in the absence or presence of soluble NE-421–436 or the irrelevant proteins BSA, thyroglobulin, and calmodulin (1 μM). Binding (A490) in absence of soluble competitor was 1.8 ± 0.1. Values are corrected for nonspecific binding observed for an extract of E. coli with empty pHEN2 vector devoid of the scFv gene.

V domain structure

From sequence conservation studies, Karray et al. (23) suggested 16 V\textsubscript{H} region amino acids likely to underlie preferential binding of neutralizing concentration of scFv GL2 (0.337 μg/ml) during its preincubation with HIV (1 h) and the subsequent culture with PBMCs (4 d).

Table I. Cross-subtype HIV neutralizing activity of scFv clones

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\textsuperscript{a}IC\textsubscript{50} and IC\textsubscript{50} values were interpolated from concentration–dependence curves using primary, coreceptor CCR5-dependent HIV isolates and PBMCs as hosts.

Neutralizing activity at the indicated lowest scFv concentration tested was greater than 80%. scFv concentrations tested ranged from a lower limit of 0.0004–15 μg scFv/ml. Assay accuracy is greatest at 50% neutralization. For all strains, neutralization exceeded 80% at the highest scFv concentration tested. r\textsuperscript{2} values for fitted curves with an IC\textsubscript{50} that could be determined by interpolation were 0.43–0.99.

NT. Not tested.
activity observed by introducing the mutations into the scFv were quantitatively nonidentical, presumably because of fine differences in the CD4BD<sub>core</sub> conformation expressed by the two test Ags. It may be concluded that FR1, FR3, and CDR2 of scFv JL427 provide important contributions in CD4BD core recognition.

Molecular modeling of scFv JL427 suggested that the 30-residue FR1, the 32-residue FR3, and the rest of CDR2 are spatially separated from the Ag-binding pocket composed of the CDRs (Fig. 6A). Four of the 17 CDR2 residues approach the rim of the CDR-binding pocket, but the rest of CDR2 is well separated from the pocket. In contrast, the CDR1 segment (which was without effect on the binding activity) lines the CDR-binding pocket. Local changes in backbone FR1, FR3, and CDR2 topography of the wild-type and mutated scFv GL2 were evident, but the overall structure of these segments was mostly maintained (Fig. 6B–D; r.m.s. deviation for FR1, FR3, and CDR2 mutants, respectively, 1.47, 0.86, and 1.10 Å). The overall wild-type and mutant scFv protein backbones were largely superimposable, suggesting the absence of global conformational changes (r.m.s. deviation for scFv GL2<sub>FR1mut</sub>, scFv GL2<sub>FR3mut</sub>, and scFv GL2<sub>CDR2mut</sub> backbones, respectively, 1.84, 0.14, and 0.32 Å). The levels of amino acid identities and conservations, respectively, between the wild-type and mutant segments were as follows (ClustalW2 grouping; Supplemental Table II): 19 and 6 residues in FR1 (length 30 residues), 20 and 8 residues in FR3 (length 32 residues), and 6 and 8 CDR2 residues (length 17 residues; the wild-type scFv GL2 CDR2 is 1 residue shorter compared with the mutant). Ligand binding sites consist of noncontiguous or contiguous flat surfaces or concavities and protrusions (49). No marked con-

### Table II. Ab sequence properties

<table>
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<th>Property</th>
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<th>scFv GL2</th>
<th>IgM 2G9</th>
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Family assignments according to Kabat/Wu database (69). Germline assignments according to IgBLAST (blast.ncbi.nlm.nih.gov). V<sub>-(D)-J</sub> junctional deletions and insertions deduced using IMGT/V-QUEST (70). R/S ratio computation excludes mutations attributable to V<sub>-(D)-J</sub> junctional diversification and PCR primer-annealing region. The Kabat/Wu and IMGT/V-QUEST databases differ in demarcation of CDRs and FRs. R/S ratios computed from both databases are reported. R/S ratios predicted for a random mutational process are as follows: V<sub>H</sub>-CDRs, 3.56/1; V<sub>L</sub>-CDRs, 4.16/1; V<sub>H</sub>-FRs, 2.88/1; V<sub>L</sub>-FRs, 2.84/1 (71). GenBank accession numbers (http://www.ncbi.nlm.nih.gov/genbank/) for scFv JL427, scFv GL2, IgM 2G9 V<sub>H</sub>, and IgM 2G9 V<sub>L</sub> are, respectively, AF329462, JQ343847, JQ412130, and JQ338486. Replacement mutations in scFv V regions were (germline residues listed first, Kabat numbering): scFv JL427 V<sub>H</sub>, G16:R, S33:G, N35:H, S52:G, S52a:R, S54:G, T56:H, I57:T, Y58:N, A74:S, S77:T, M82:I; scFv JL427 V<sub>L</sub>, A11:V, T14:A, T28:S, Q38:H, L39:F, S50:R, N52:D, D60:A; scFv GL2 V<sub>H</sub>, G10:R, E16:G, G32:S, S35b:G; scFv GL2 V<sub>L</sub>, none. IgM V<sub>H</sub> and V<sub>L</sub> regions contain no somatic mutations. scFv JL427 V<sub>H</sub> contains all 16 Karray residues, 15 encoded by the germline gene, and 1 acquired by somatic mutation (G10, Q13, R19, A23, T28, Y12, G54, K64, K65, Y79, Q81, N82a, K75, N82a). scFv GL2 contains 3 identities (Y59, K64, K75), 4 conservative deviations (T28:S, Y32:S, Y79:S, N82a:S), and 9 nonconservative deviations at the Karray positions. Six of the 7 identities/conservations are germline gene encoded. Conservation of Karray residues was computed as in (23). IgM 2G9 contains 7 identities (G10, Q13, Y32, G54, Y59, K75, N82a), 1 conservative deviation (T28:S), and 8 nonconservative deviations at the Karray positions.

![FIGURE 5. Contribution of scFv JL427 FRs to CD4BD<sup>core</sup> peptide binding. (A) scFv JL427 FR-containing mutants. The FR1, FR3, CDR1, or CDR2 segments of the V<sub>H</sub>-family scFv JL427 were inserted in place of the corresponding V<sub>H</sub>-family scFv GL2 segments, designated, respectively, GL2<sub>FR1mut</sub>, GL2<sub>FR3mut</sub>, GL2<sub>CDR1mut</sub>, and GL2<sub>CDR2mut</sub>. (B) Increased E-416–433b binding by scFv JL427 FR-containing mutants. Values (means ± SD) represent fold increase of mutant binding relative to wild-type scFv GL2, computed as the ratio of scFv concentrations displaying A<sub>490</sub> 0.25 (A<sub>490</sub> 0.25 observed at 23 ± 1 μg/ml wild-type scFv). scFv constructs were purified by metal-affinity chromatography. E-416–433b (70 ng/well). (C) Increased E-gp120 binding by scFv JL427 FR-containing mutants. Values (means ± SD) represent fold increase of mutant binding relative to wild-type scFv GL2 computed as the ratio of scFv concentrations displaying A<sub>490</sub> 0.25 (A<sub>490</sub> 0.25 observed at 36 ± 2 μg/ml wild-type scFv). scFv constructs were purified by metal-affinity chromatography. E-gp120 (100 ng/well). (D) Example of E-gp120–binding data for scFv GL2<sub>FR3mut</sub>. Values are means ± SD corrected for nonspecific binding.](http://www.jimmunol.org/doi/abs/10.5382)
cavity or protrusion was noted in the scFv FR1/FR3/CDR2 regions outside the CDR-binding pocket, arguing against a lock-and-key fit CD4BDcore-binding model. This leaves the interpretation of surface chemical forces at a comparatively flat surface as the explanation for FR-CD4BDcore-binding interactions.

The V<sub>H</sub>/V<sub>L</sub> domain CDR3 sequences of both scFv clones have been diversified by V-(D)-J gene recombination, a process completed prior to the immunogen-driven phase of B cell differentiation. The role of immunogen-driven selection in Ab maturation can be reliably assessed from the R/S ratios outside the V-(D)-J junctions (50). R/S ratio for scFv JL 427 V<sub>H</sub> FRs, V<sub>H</sub> CDRs, and V<sub>L</sub> CDRs, but not the V<sub>L</sub> FRs exceeded the predicted ratio for the random mutational process (Table II), suggesting selection of the replacement mutations. A further indication of immunogen-driven selection of replacement mutations was the finding of a contiguous surface composed of eight Karray residues belonging to FR1, FR3, and CDR2 extended by two FR3 and four CDR2 residues by somatic mutation (Fig. 6E; individual residues constituting the contiguous surface are identified in Supplemental Table II). The probability of finding six somatic replacements in surface contiguity with the eight Karray residues by random chance alone is minuscule [P = (13/82)<sup>6</sup> = 1.6 × 10<sup>-5</sup>, where 13 is the total number of V<sub>H</sub> gene replacement mutations and 82 is the V<sub>H</sub> gene length less the 8 Karray residues]. All 14 residues forming the contiguous surface are located well outside the CDR-binding pocket.

Constitutive CD4BDcore-specific polyclonal Abs

We previously used electrophoresis assays to demonstrate specific covalent binding of E-421–433 and E-416–436 by a subset of constitutive IgAs purified from the pooled serum of noninfected humans without autoimmune disease (example in Fig. 7A, inset) (19, 20). In the current study, we observed detectable E-416–436a binding by the individual polyclonal IgA preparations from all five healthy, noninfected human subjects tested (determined as in Fig. 7A; combined intensity of the IgA H and L chain adduct bands with E-416–436a bands in arbitrary volume units 222,370 ± 96,185, mean ± SD; range 106,648–354,872). To evaluate the functional significance of the Abs, we tested HIV neutralization

![Figure 6](https://example.com/figure6.png)

**FIGURE 6.** scFv JL427 models. (A) Two scFv JL427 surface views. The CDR-binding pocket (green) is well separated from the V<sub>H</sub> domain FR1 (white) and FR3 (pink). The V<sub>H</sub>-CDR2 residues (brown) are located mostly outside the CDR-binding pocket. V<sub>L</sub>-CDR1 residues are shown in magenta. Remaining V<sub>H</sub> and V<sub>L</sub> surfaces are shown in dark blue and light blue, respectively. (B) Superimposition of scFv GL2 wild-type (red) and mutant (white) V<sub>H</sub>-FR1. The r.m.s. deviation is 1.47 Å. (C) Superimposition of scFv GL2 wild-type (red) and mutant (white) V<sub>H</sub>-FR3. The r.m.s. deviation is 0.86 Å. (D) Superimposition of scFv GL2 wild-type (red) and mutant (white) V<sub>H</sub>-CDR2. The r.m.s. deviation is 1.10 Å. (E) Contiguous scFv JL427 V<sub>H</sub> domain 14-residue surface composed of Karray residues and somatically replaced residues. The 8 Karray residues in this stretch belong to V<sub>H</sub> FR1, FR3, or CDR2 (yellow, Arg<sup>59</sup>, Lys<sup>64</sup>, Tyr<sup>70</sup>, Gln<sup>81</sup>, Asn<sup>82</sup>, Tyr<sup>85</sup>, Lys<sup>86</sup>, Gly<sup>96</sup>); note that CDR2 residues Tyr<sup>85</sup>, Lys<sup>86</sup>, and Gly<sup>96</sup> have been reclassified as FR3 residues in the IMGT database). The contiguity is extended on the left by four somatic replacements (Gly<sup>54</sup>, His<sup>56</sup>, Thr<sup>57</sup>, Asn<sup>58</sup>), and on the right by two somatic replacements (Ser<sup>74</sup>, Thr<sup>77</sup>) shown in red.

![Figure 7](https://example.com/figure7.png)

**FIGURE 7.** Neutralizing activity of constitutive CD4BDcore-specific Abs. (A) Neutralizing CD4BDcore-specific IgA subset from sera of noninfected humans. Pooled serum IgA from 10 healthy humans without HIV infection was fractionated on E-416–433 conjugated to agarose, unbound IgA was collected, and noncovalently bound IgA was recovered by acid elution. Covalently bound IgA was recovered by treating the gel with pyridine 2-aldoxime methiodide and elution with the acid buffer. HIV neutralization was measured using human PBMC hosts and subtype C HIV strain 97ZA009 (coreceptor CCR5 dependent). Values are means ± SEM of four replicates. Inset, Streptavidin-peroxidase–stained reducing SDS-electrophoresis gels showing binding of E-421–433 by total serum IgA loaded on the E-416–433 column. Lanes 1–3, IgA incubated with E-421–433 for 1, 4, and 20 h, respectively. Lanes 4–6, IgA incubated with control E-VIP for 1, 4, and 20 h, respectively. IgA, 80 µg/ml; E-421–433 or E-VIP, 10 µg/ml. Complexes of E-421–433 with the H chain subunit (65 kDa) and L chain subunit (25 kDa) are evident. (B) Weak neutralization by unfractionated serum IgM from noninfected humans. Polyclonal IgM purified by chromatography on immobilized anti-µ-chain Abs from pooled human serum was tested for neutralization of strain 97ZA009 by the low-sensitivity method (preincubation of IgM–HIV mixture in 10% FBS for 1 h) or high-sensitivity method (preincubation of IgM–HIV mixture in 0.66% FBS for 24 h). Means ± SEM of four culture replicates. (C) IgM neutralization specificity. Inhibition of human serum IgM (10 µg/ml) neutralizing activity monitored by the high-sensitivity assay, as in (B), in the presence of E-421–433 (100 µM) or irrelevant E-VIP (100 µM). The control incubation received diluent containing 1% DMSO instead of the peptides.
by the IgA. The purified serum IgA fractions did not neutralize HIV detectably when tested by the low-sensitivity assay commonly employed to measure Ab effects on PBMC infection (Fig. 7A; 1-h HIV preincubation with IgA in diluent containing 10% FBS). Two CD4BDcore-specific Ab subsets were isolated from a pool of 10 subjects’ serum IgA by chromatography on immobilized E-416–433, as follows: 1) noncovalently bound Abs recovered by acid elution; and 2) covalently bound Abs recovered by treatment with 2-pyridine aldoxime, a reagent that cleaves the nucleophile–electrophile covalent bond. The same affinity chromatography procedure was applied previously to isolate epitope-specific Abs from HIV-infected patients (4). Only 0.04 and 0.005% of the total serum IgA from noninfected humans was recovered, respectively, in the noncovalently and covalently bound fractions. Both CD4BDcore-specific fractions, but not the nonbinding IgA in the column flow-through, neutralized HIV (Fig. 7A; IC50 for noncovalently bound and covalently bound IgA, respectively, 4.2 and 0.5 ng/ml). The affinity chromatography procedure entails extensive washing of the column that can be expected to remove Abs with weak CD4BDcore reactivity. The studies suggest that a small minority of highly CD4BDcore-reactive constitutive IgAs expresses sufficiently robust neutralizing activity for consideration as effective anti-HIV Abs.

Serum IgG (29) and IgM (17, 19) from noninfected humans are reported to bind the CD4BDcore-containing superantigen determinant. Similarly, mononclonal human IgMs from patients with Waldenström’s macroglobulinemia displayed variable but specific recognition of E-421–433 (19). Like the serum IgA prior to affinity chromatography on immobilized E-416–433, pooled serum IgM from noninfected humans (n = 10) did not neutralize HIV in the commonly used low-sensitivity assay, but the high-sensitivity assay detected IgM neutralizing activity (24-h HIV–IgM preincubation in 0.66% FBS; Fig. 7B). The weak neutralizing activity was attributable to CD4BDcore recognition, evident from competitive inhibition of the activity by E-421–433, but not the irrelevant peptide E-VIP (Fig. 7C).

**Induction of constitutive CD4BDcore-specific IgMs**

To obtain unambiguous evidence for HIV neutralization by CD4BDcore-reactive Abs with no requirement for V region somatic mutations, we studied murine IgM class Abs induced by immunization with E-416–433b. E-416–433b is an electrophilic immunogen that can bind covalently to nucleophilic BCRs. IgMs are the first Abs secreted by B lymphocytes and often contain sparse V region somatic mutations (50). We proposed covalent BCR binding by electrophilic immunogens as a cellular stimulation strategy for inducing the synthesis of Abs to superantigen epitopes (38). In principle, immunogen-driven clonal expansion of the pre-existing CD4BDcore-specific B cell subset alone may permit increased Ab synthesis with no requirement for further Ab improvement by the somatic mutation pathway (Fig. 1A). Immunization of mice with E-416–433b induced the production of serum IgM (Fig. 8A) and IgG with E-416–433c–binding activity (serum IgM titer 1:2,500; serum IgG titer 1:35,000; no E-416–433c binding of either Ab class detected at 1:100 preimmune serum). We focused on the IgM Abs. Of 1,199 hybridoma wells screened, 77 wells were positive for E-416–433c–binding IgMs (A490 0.2–3.0). The V domains of six IgMs with the highest E-416–433c–binding activity were sequenced. Four IgMs contained V regions exactly identical in sequence to their germline VH and

**FIGURE 8.** CD4BDcore-specific IgM induced by E-416–433 immunization. (A) Amplified E-416–433c binding by serum IgM from E-416–433b-immunized mice. Serum was obtained on day 42 after initiation of immunization. Data are means ± SD of pooled serum dilutions (n = 8 mice). (B) Gel filtration of IgM 2G9. Superpose 6 FPLC column (0.4 ml/min; 50 mM Tris-HCl, 0.1 M glycine, 0.15 M NaCl, 0.1 mM CHAPS [pH 7.8]). Arrows indicate marker proteins: thyroglobulin (660 kDa), ferritin (440 kDa), catalase (232 kDa), and IgG (150 kDa). Inset, Reducing SDS-electrophoresis and immuno blot of IgM 2G9. Stained with Coomassie Blue (lane 2) and anti-μ/κ Ab (lane 3). Lane 1, Marker proteins with mass shown in kDa. The 50-kDa band in lanes 2 and 3 was identified previously as a μ-chain fragment (36). (C) Neutralization of CCR5-dependent HIV subtype A, B, C, and D strains by IgM 2G9. For strain 97ZA009, the activity of a second IgM preparation is included (IgM 2G9 preparation 2). Values are means ± SEM of four independent culture replicates. (D) Competitive inhibition of IgM 2G9 binding to immobilized E-416–433c by soluble proteins and peptides. IgM (30 μg/ml) binding to E-416–433c (70 ng/well) was measured in the absence or presence of increasing concentrations of E-416–433a, sCD4, gp120, control Sh416–433, and control OVA. Residual binding was computed as percentage of the binding in the absence of inhibitors (A490 0.67 ± 0.10).
V<sub>1</sub> gene counterparts (clones 2G9, C11, 1F4, and 2G2; E-416–433c–binding activity at 30 μg IgM/ml in A490 U, respectively, 0.49 ± 0.01, 0.45 ± 0.01, 0.51 ± 0.01, and 0.94 ± 0.04).

IgM 2G9 was picked as a representative E-416–433c–binding germline Ab for further studies. Its V<sub>H</sub> and V<sub>L</sub> regions are encoded, respectively, by germline genes V2-2*02 and KV10-96*01 (Table II). Twenty-five of the 30 V<sub>H</sub>-FR1 positions, 28 of the 32 V<sub>L</sub>-FR3 positions, and 10 of the 16 V<sub>H</sub>-CDR2 positions in IgM 2G9 are occupied by identical residues or conservative substitutions compared with the corresponding FRs of human scFv JL427 (Supplemental Table II). The IgM was composed of H and L chain subunits with the anticipated mass, and a predominantly pentameric species was evident under non-denaturing conditions (Fig. 8B). IgM 2G9 neutralized all four strains drawn from subtypes A, B, C, and D (Fig. 8C; IC<sub>50</sub> range 0.002–0.51 μg/ml). A second independent IgM 2G9 preparation also neutralized subtype C strain ZA009 (IC<sub>50</sub> 2.9 and 8.5 μg/ml in two assays). It may be concluded that IgM 2G9 neutralizes genetically diverse HIV strains despite the use of germline V gene sequences free of somatic mutations.

Specificity of the IgM for the CD4BD<sup>core</sup> was evident from competitive inhibition of E-416–433c binding by E-416–433a, gp120, and scCD4, but not OVA (Fig. 8D). As scCD4 binds E-416–433c specifically (4), it is predicted to inhibit IgM binding to E-416–433c by steric hindrance. We also evaluated the role of individual 416–433 residues by measuring competitive inhibition of IgM–E-416–433c binding in the presence of wild-type peptide NE-416–433 and mutant 416–433 peptides containing Ala replacements. Attenuated competitive inhibition by 2.1- >166-fold was evident for 9 of the 17 aa positions tested (Table III). The control shuffled-sequence peptide (sh416–433) did not inhibit the binding (<8.4 ± 6.2% inhibition; mean ± SD). The amino acids at 5 of the 9 positions are also important for scCD4 binding (13, 15, 16).

**Host cell effects**

Autoantibodies reactive with Ags expressed by PBMCs can inhibit HIV infection by an anti-host cell mechanism (51). A cross-reaction of anti-CD4BD<sup>core</sup> Abs with a PBMC Ag may explain their observed neutralizing activity. Abs to phosphatidylserine inhibit HIV infection in PBMC cultures by inducing β-chemokine release from monocytes, resulting in saturation of the chemokine coreceptors needed for HIV entry into the cells (40). Contaminant LPS (endotoxin) in Ab preparations can also inhibit HIV infection by inducing β-chemokine release from the cells (52–54).

Without the C region (Fc), an anti-phospholipid Ab did not inhibit HIV infection of PBMCs (40). The scFv clones reactive with the CD4BD<sup>core</sup> in the current study do not contain the Fc region, arguing against an anti-phospholipid Ab-like neutralizing mechanism. Inhibition of HIV infection by anti-phospholipid Abs was completely blocked by Abs to MIP-1α or MIP-1β (40). In contrast, the HIV neutralizing activity of IgG JL427 was maintained in the presence of Abs to MIP-1α, MIP-1β, and RANTES (Fig. 9). In the presence of excess anti-MIP-1α Ab, an increase of basal infection (no IgG JL427) was evident compared with the control nonimmune IgG. In previous studies, MIP-1α inhibited the infection of PBMCs potently, and neutralizing Abs to constitutively produced β-chemokines in PBMCs and T cell cultures enhanced the infection, consistent with CCR5 availability as a factor regulating the infection (54, 55). The differences in IgG JL427 neutralizing activity in the control nonimmune IgG versus experimental cultures containing Abs to MIP-1α, MIP-1β, and RANTES were within the range of experimental error (<25%). β-chemokine–mediated inhibition of infection, therefore, is not a factor in HIV neutralization by the anti-CD4BD<sup>core</sup> Ab.

To further rule out the β-chemokine mechanism and other potential neutralization mechanisms due to Ab recognition of a PBMC Ag, we tested whether the neutralizing activity is dependent on the duration of HIV preincubation with test Abs to the CD4BD<sup>core</sup>. The PBMCs were exposed to the Abs for equivalent durations at all data points. Time-dependent neutralization is observed in this experimental protocol only if the Ab targets a viral Ag, whereas the level of neutralization remains constant if the target Ag is located on the PBMCs. Progressively increasing neutralization by IgG JL427 and IgM 2G9 as a function of increasing time of HIV–Ab preincubation was observed (Fig. 10A, 10B). As expected, the time dependency was lost at a saturating IgG JL427 concentration, and the curve shifted to the left at a subsaturating IgM 2G9 concentration. Consistent with findings that the β-chemokine–mediated inhibition of infection by Abs to phosphatidylserine does not require Ab–HIV contact (40), the inhibitory effect of a control Ab to phosphatidylserine remained constant regardless of the length of Ab–HIV preincubation (Fig. 10C). Another infection-inhibiting mechanism was reported for an Ab to phosphatidylinositol, internalization of Ab-coated HIV by macrophages, followed by release of β-chemokines (56). However, the infection was not impeded if macrophages were pretreated with this Ab prior to contact with the virus, suggesting functionally futile Ab consumption by excess cellular lipid. In contrast, IgG JL427 to the CD4BD<sup>core</sup> pretreated with PBMCs maintained the HIV neutralizing activity (percentage of neutralization observed in PBMCs cocultured with IgG JL427 3 h prior to addition of virus versus simultaneous coculture with Ab and virus, respectively: 92 ± 2 and 93 ± 3; 0.5 μg IgG JL427/ml, strain 97ZA009). Taken together with finding that the neutralizing activity is maintained in the presence of anti-chemokine

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Binding of IgM 2G9 (30 μg/ml) to immobilized E-416–433c was measured in the absence or presence of increasing concentrations of wild-type and mutant 416–433c peptides containing Ala substitutions at individual positions (0.4–50 μg/ml). A490 in absence of competitor peptide was 0.67 ± 0.10. IC<sub>50</sub> values were determined from plots of percentage of residual binding (computed relative to binding without competitor peptide) versus competitor peptide concentration fitted to the equation: residual binding (%) = 100(1 + 10<sup>(IC<sub>50</sub>-log(Competitor peptide)) / log(Competitor peptide)). Loss of IgM binding activity is evident from an increased (IC<sub>50mutation peptide/IC<sub>50wild-type peptide) ratio. “None” indicates changes in binding ratios <1.5-fold. IC<sub>50</sub> for the wild-type peptide was 27 μg/ml. Residues reported to contribute in binding of gp120 to CD4 are marked “X,” as determined by crystallography (PDB 2B4C) (13) or site-directed mutagenesis (15, 16).
Abs, the data indicate that HIV neutralization by the anti-CD4BD<sup>core</sup> Abs occurs by recognition of HIV free of host cell artifacts.

Inhibition of HIV infection by LPS is highly variable depending on the LPS source, the test virus strain, host PBMC source, and monocyte content. In the report of Geonnotti et al. (54), most test LPS preparations did not inhibit infection in about half of the assays, but, when observed, the inhibitory effect was potent (median IC<sub>50</sub> for LPS in assays showing inhibition: 30–210 pg/ml). Another paper from the same group reported the requirement for very large amounts of LPS for inhibition of infection (IC<sub>50</sub> 4 μg LPS/ml) (40). In our hands, an authentic LPS preparation inhibited infection of three PBMC preparations by HIV strain 97ZA009 variably (IC<sub>50</sub> 1,800 pg/ml for one PBMC preparation and >100,000 pg/ml for the other two preparations). The LPS concentration at the 50% neutralizing concentrations of purified scFv JL427 (0.2 pg/ml), IgG JL 427 (0.2 pg/ml), and IgM 2G9 (undetectable) was below the threshold needed to interfere with HIV infection. Unlike the inconsistent inhibitory effect of authentic LPS, the scFv clones consistently neutralized the infection of all test PBMC preparations (n = 20) by all test HIV strains (n = 17) with high potency.

The explanation of interfering host cell effects is also inconsistent with the following: 1) specific inhibition of scFv JL427 and IgM 2G9 neutralizing activity by the CD4BD<sup>core</sup> peptide analog, and 2) potent neutralization by the CD4BD<sup>core</sup>-specific IgA fraction from noninfected humans, but not the flow-through from the affinity column. Similar studies ruled out host cell artifacts as the explanation for HIV neutralization by anti-CD4BD<sup>core</sup> Abs from HIV-infected subjects (4) and mice immunized with E-gp120 (38).

**Discussion**

Using the commonly used low-sensitivity neutralizing assay, we isolated a very small subset of CD4BD<sup>core</sup>-reactive IgAs produced spontaneously with no prior exposure to HIV that neutralized HIV potently. The IgAs were found in sera from humans without HIV infection or autoimmune disease. Like IgGs, class-switched IgAs usually contain somatically mutated V regions. Constitutive production of Abs to the CD4BD<sup>core</sup> is not restricted to the IgA classes. We and others reported constitutive Abs of the IgG (29) and IgM (17, 19) class with this specificity. Rare scFv clones from humans without HIV infection also recognized the CD4BD<sup>core</sup> specifically and neutralized genetically diverse HIV strains, verifying constitutive production of the neutralizing Abs. Additional observations supporting the pre-existing CD4BS<sup>core</sup> specificity include binding of the Abs to CD4BD<sup>core</sup> spanning peptides E-416–433 and E-421–433, but not irrelevant peptides; inhibition of the binding to E-416–433 by sCD4 and gp120, but not an irrelevant protein; and the previously documented catalytic cleavage of gp120 dependent on initial noncovalent binding to the CD4BD<sup>core</sup> (19, 20).

Lupus patients produce CD4BD<sup>core</sup> peptide-binding Abs at increased levels (45), and clones with this specificity may be overrepresented in our scFv library. We noted the possibility of an unusual immune response to endogenous retroviral and exogenous bacterial Ags resembling the CD4BD<sup>core</sup> as the cause (28). However, sera from noninfected humans without autoimmune disease also contain Abs to the gp120 superantigen site (19, 20, 29), and finding of potent CD4BD<sup>core</sup>-directed IgAs in the current study indicates that the autoimmune disease is not a prerequisite for

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**FIGURE 9.** Maintenance of IgG JL427 neutralizing activity in the presence of Abs to β-chemokines. Shown are p24 levels of PBMC lysates infected for 4 d by HIV strain 97ZA009. PBMCs were mixed with the 2.5 μg/ml (A) or 15 μg/ml (B) goat anti-β-chemokine IgG or control goat nonimmune IgG and immediately cocultured with HIV pretreated with diluent or IgG JL427 (100 ng/ml, 1 h). The same anti-MIP-1α and MIP-1β IgG tested in this experiment completely blocked the β-chemokine–mediated inhibition of HIV infection by anti-phospholipid Abs (40).

**FIGURE 10.** Dependence of Ab neutralizing activity on duration of pretreatment with HIV while holding constant the exposure of PBMCs to Ab at all data points (4 d). (A) IgG JL427 to the CD4BD<sup>core</sup>. Shown are neutralizing activities (mean ± SEM) following preincubation of HIV strain 97ZA009 with the IgG (0.1 or 0.5 μg/ml) for 0, 1, 3, and 24 h. PBMCs were maintained in the presence of Ab throughout until harvest of extracellular and intracellular p24 from the culture wells. (B) IgM 2G9 to the CD4BD<sup>core</sup>. Methods were as in (A). HIV was treated with IgM 2G9 (0.3 or 1.3 μg/ml) in 0.66% FBS for the indicated durations. (C) Control IgG 4B6 to phosphatidylserine. Methods were as in (A). As predicted for inhibition of infection via an anti-host cell mechanism, the effect of this Ab was independent of the length of HIV–Ab pretreatment.
constitutive production of the Abs. Other groups have proposed insufficient gp120-binding affinity of Abs containing V regions in their germline configuration and insufficient V region somatic mutations following exposure to HIV as explanations for infrequent production of adaptively matured neutralizing Abs to conserved HIV epitopes like the CD4BD (10). Neither explanation applies to constitutive Abs directed to the CD4BDcore epitope. We observed broad HIV neutralization by the CD4BDcore-specific scFv clones. One of the clones contained minimal V region mutations (scFv GL2), and the other was more extensively mutated. Although neutralizing Abs to the CD4BDcore are difficult to induce adaptively, a small subset of these Abs is present constitutively.

We conceive the constitutive BCR repertoire as a source of diverse Abs with varying CD4BDcore reactivity, of which only a small subset displays sufficient fine specificity relevant to HIV vaccination. The specificity most likely depends both on the initial events that commit B cells to use of a particular pair of rearranged germline VH–VL genes and the subsequent mutational changes. The gp120 superantigen site binds most strongly to human VH3-family Abs (22), but interactions with Abs from other V gene families are also evident (19, 48). In the current study, the VH3-family scFv (clone JL427) expressed superior CD4BDcore binding and HIV neutralizing activity compared with the VH4-family scFv GL2. Swap mutation studies suggested that the FR1, FR3, and CDR2 segments of scFv JL427 lying outside the traditional CDR-based Ag-binding pocket provide important contributions in CD4BDcore-binding activity. scFv JL427 contains all 15 germline VHgene-encoded amino acids and 1 somatically-acquired replacement previously suggested to be important in binding the gp120 superantigen site (23). Mechanisms that can diversify the constitutive FR-based repertoire and explain the production of rare CD4BDcore-specific neutralizing Abs are as follows: 1) varying binding activity of the 97 VH3 germline genes and the remaining 166 VH1–VH7 gene families; 2) a direct contribution from the VH domain, suggested by the CD4BDcore recognition activity of certain free L chain subunits (48); 3) binding site structural alterations caused by VH-L domain pairing; and 4) remote structural changes in the CDRs. In particular, the VH CDR3 is subject to substantial length and sequence changes during V-D-J gene rearrangement prior to contact with immunogen. Sub-A˚ngstrom-to-A˚ngstrom level movements of the backbone and amino acid side chains attendant to remote V domain structure alterations can impact their reactivity with Ags (37). The VH CDR3 of both CD4BDcore-reactive scFv clones in the present report contains extensive structural changes due to the gene rearrangement mechanism.

Exploiting the constitutive neutralizing activity for HIV vaccination requires identification of an immunogen that stimulates clonal expansion and differentiation of B cells producing the CD4BDcore-reactive Abs. Improving the CD4BDcore reactivity by V gene somatic mutations is also desirable. Unlike stimulatory CDR binding of traditional immunogens, FR binding to superantigens downregulates B cells (21). Only limited escape from the downregulatory effect seems feasible under physiological circumstances. The protein A superantigen site induced transient clonal proliferation prior to deletion of B cells (57), low-level CD4BDcore-binding Abs were detected in HIV-infected patients (26), and CD4BDcore-specific class-switched IgAs with broad and potent HIV neutralizing activity were identified in patients with prolonged HIV infection over two decades (4). An immunogen-driven regulation of CD4BDcore-reactive Ab production by noninfected humans is also conceivable, as the CD4BDcore contains partial sequence identity with a protein from the commensal bacterium Streptococcus gordonii and certain endogenous retroviral sequences (28). Our structural studies suggested that scFv JL427 contains a FR-based CD4BDcore binding site with somatic replacement mutations, a sign of immunogen-driven selection. The spatially separated CD4BD-core pocket also contained somatic replacements. A structural precedent for two binding sites expressed within a single Fv is provided by mAbs raised by immunization with E-gp120, the CD4BDcore binding site dominated by the FRs, and a second CDR pocket that binds a physically distinct gp120 epitope (38). We hypothesized that simultaneous CDR engagement of the second epitope furnishes a stimulatory signal compensating for downregulatory CD4BDcore binding to the FRs, thereby amplifying neutralizing Ab synthesis by B cells. In principle, binary engagement of the FR-based site and CDR-binding pocket by two epitopes expressed by a yet-to-be identified physiological immunogen may explain amplified production of Abs without the occurrence of HIV infection.

Electrophilic immunogens are first-generation vaccine candidates holding the potential of inducing a broadly neutralizing Ab response. Immunization with E-gp120 induced a more robust class-switched Ab response compared with gp120 devoid of the electrophilic groups (58). In addition to the binary FR-CDR-binding mechanism, our studies on the immunogenicity of E-gp120 suggested covalent electrophile binding at nucleophilic BCR sites as an independent stimulatory factor that may bypass the downregulatory effect of noncovalent CD4BDcore–BCR binding (38). E-416–433 lacks a second CDR-binding epitope, but it contains the electrophilic phosphate and peptide epitope available for covalent BCR binding coordinated with specific noncovalent binding at the FR-based site. IgMs often contain few or no V region somatic mutations, as they are produced from plasma cells developed early in immunogen-driven phase of the response, prior to extensive somatic V region mutations and IgG/IgA class-switching accompanying the germinal center reaction (50). To evaluate constitutive Ab amplification free of somatic mutation requirements, we analyzed the IgM Abs in E-416–433–immunized mice. The mice mounted a polyclonal IgM response with E-416–433–binding activity. A monoclonal IgM induced by E-416–433 expressed specific E-416–433–binding activity that was inhibited competitively by gp120 and CD4 despite a complete absence of somatic mutations in its V regions. This IgM also neutralized diverse HIV strains. Clonal expansion of B cells with constitutive CD4BDcore recognition activity alone with no reliance on immunogen-driven V region mutations, therefore, is a valid basis to induce the synthesis of broadly neutralizing Abs. With respect to effective vaccination, maintaining and improving the neutralizing activity as the immune responses mature to class-switched IgG/IgA Abs is an important objective. Others have reported both increased and decreased HIV neutralization by the IgM version of Abs to certain HIV epitopes containing the same V domains compared with their smaller IgG/IgA counterparts (59, 60). The IgM scaffold supports comparatively rapid catalytic cleavage of gp120 compared with IgG (19), and multivalent epitope recognition by IgM may allow more avid noncovalent binding. However, the large IgM size may cause sterically hindered epitope binding, and IgMs are found in blood at concentrations lower than the IgGs.

To selectively amplify the small subset of constitutive Abs with the most potent CD4BDcore reactivity, the electrophilic immunogen must mimic the native HIV CD4BDcore conformation with sufficient accuracy and rigidity (Fig. 1B, middle panel). Imperfect conformational mimicry will amplify constitutive Abs with lesser neutralizing potency. Similarly, an immunogen that expresses an insufficiently rigid CD4BDcore conformation can be anticipated to amplify a constitutive Ab subset with lesser neutralizing potency due to the induced-fit binding mechanism (Supplemental Fig. 1B). Moreover, the process of immunogen-driven selection of muta-
tions over the course of Ab maturation will improve immunogen-binding activity, not native CD4BDcore-binding activity, and a conformationally deviant immunogen may select FR mutations that cause loss of neutralizing activity. Inducing a CDR-based neutralizing Ab response is even more onerous. Synthetic peptides such as the CD4BDcore peptides can assume alternate conformations depending on their microenvironment. The induced-fit binding mechanism will force a flexible immunogen that binds the CDRs into an inappropriate conformation, resulting in induction of nonneutralizing Abs (Supplemental Fig. 1B; see Ref. 61 for review). The importance of conformational considerations is evident from reports of vigorous IgG production induced by non-electrolytic CD4BDcore peptides that displayed inconsistent HIV neutralizing activity and varying binding specificity (33, 62, 63). Importantly, the immune response to the nonelectrolytic peptide immunogens is most likely dominated by traditional Abs with CDR-based binding sites, as the noncovalent superantigen epitope–FR binding downregulates B cells.

The utility of constitutive Abs for HIV vaccination depends on the veracity with which the primary virus–PBMC neutralization assay reports a genuine anti-virus Ab effect. The assay is often cited as the “gold standard” for determining HIV neutralization in tissue culture because it approximates the natural process of infection in vivo. However, discrepant neutralization by Abs to various HIV epitopes in the primary virus–PBMC assay versus the pseudovirus-engineered reporter cell assay has been noted (64–68), including the CD4BDcore epitope. The CD4BDcore-specific Abs in long-term survivors of HIV infection (4) or induced in mice by E-gp120 immunization (38) displaying neutralizing activity in the primary virus/PBMC assay did not impede pseudovirus entry into genetically engineered host cells expressing CD4 and chemokine coreceptors (TZM/Bl cells) (68). scFv JL427 at concentrations showing readily detectable neutralization in the PBMC-based assay was also ineffective in the TZM/Bl-based assay (12 µg/ml). The underlying reason remains to be elucidated, but overexpression of the HIV coreceptors and an altered CD4BDcore conformation on the pseudovirus surface are potential factors (68). Inhibition of HIV infection due to β-chemokine–mediated Ab host cell effects and contaminant endotoxin was noted in the PBMC assay (40, 54). We ruled out these explanations from findings that neutralization by anti-CD4BDcore Abs was preserved in the presence of anti-β-chemokine Abs and occurred by a mechanism involving recognition of HIV, not host cells. The neutralization increased progressively with increasing time of Ab contact with HIV while holding PBMC exposure to Abs constant; enriched neutralizing activity was evident in the epitope-specific subset of polyclonal Abs; CD4BDcore-reactive recombinant Abs displayed neutralizing activity; the CD4BDcore peptide inhibited Ab neutralization competitively; a neutralizing mAb was induced by immunization with a CD4BDcore-containing immunogen; and this Ab recognized amino acids within the CD4BDcore important for CD4 binding. It may be concluded that the PBMC-based assay is a valid measure of specific CD4BDcore recognition and HIV neutralization by constitutive Abs.

In summary, our studies reveal the constitutive Ab repertoire as a novel basis for inducing broadly neutralizing Abs expressing FR-based specificity for the CD4BDcore. The structural properties of a broadly neutralizing Ab fragment from noninfected humans were consistent with adaptive improvement of the constitutive Abs under the influence of an unidentified immunogen. Electroplatic immunogen-driven amplification of constitutive IgM Abs with unmutated V genes appears feasible. Previous findings of slow appearance of broadly neutralizing anti-CD4BDcore Abs in patients with prolonged HIV infection (4) and the more rapid induction of such Abs observed in mice immunized with E-gp120 (38) also support a vaccination strategy that amplifies and improves the neutralizing function of constitutive Abs.

Acknowledgments

We thank colleagues at the University of Texas Medical School at Houston for technical assistance, contributions in preparing recombiant Abs, HIV neutralization assays, and sequence analysis; Dr. David Montefiori for performing the TZM-BI neutralization assays; Dr. Robert Chiles for performing flavivirus neutralization assays; and Drs. Victoria Polonis, Christina Ochsnerbauer, and Seth Pincus for critiquing the vaccine approach and the neutralization assay methods.

Disclosures

S.A.P., Y.N., R.J.M., and S.P. have a financial interest in Covalent Bioscience and in patents concerning HIV vaccination. The other authors have no financial conflicts of interest.

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