First Membrane Proximal External Region–Specific Anti-HIV1 Broadly Neutralizing Monoclonal IgA1 Presenting Short CDRH3 and Low Somatic Mutations

Fahd Benjelloun, Zeliha Oruc, Nicole Thielens, Bernard Verrier, Gael Champier, Nadine Vincent, Nicolas Rochereau, Alexandre Girard, Fabienne Jospin, Blandine Chanut, Christian Genin, Michel Cogné and Stephane Paul

*J Immunol* published online 1 August 2016
http://www.jimmunol.org/content/early/2016/07/30/jimmunol.1600309

---

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2016/07/30/jimmunol.1600309.DCSupplemental.html

**Subscriptions**
Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscriptions

**Permissions**
Submit copyright permission requests at: http://www.aai.org/ji/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/cgi/alerts/etoc
First Membrane Proximal External Region–Specific Anti-HIV1 Broadly Neutralizing Monoclonal IgA1 Presenting Short CDRH3 and Low Somatic Mutations

Fahd Benjelloun,* Zeliha Oruc, † Nicole Thielens,‡§‖ Bernad Verrier,‖ Gaël Champier,§ Nadine Vincent,* Nicolas Rochereau,* Alexandre Girard,* Fabienne Jospin,* Blandine Chaunt,* Christian Genin,* Michel Cogné,† and Stephane Paul*

Mucosal HIV-1–specific IgA have been described as being able to neutralize HIV-1 and to block viral transcytosis. In serum and saliva, the anti-HIV IgA response is predominantly raised against the envelope of HIV-1. In this work, we describe the in vivo generation of gp41-specific IgA1 in humanized o1KI mice to produce chimeric IgA1. Mice were immunized with a conformational immunogenic gp41-transfected cell line. Among 2300 clones screened by immunofluorescence microscopy, six different gp41-specific IgA with strong recognition of gp41 were identified. Two of them have strong neutralizing activity against primary HIV-1 tier 1, 2, and 3 strains and present a low rate of somatic mutations and autoreactivity, unlike what was described for classical gp41-specific IgG. Epitopes were identified and located in the hepted repeat 2/membrane proximal external region. These Abs could be of interest in prophylactic treatment to block HIV-1 penetration in mucosa or in chronically infected patients in combination with antiretroviral therapy to reduce viral load and reservoir. The Journal of Immunology, 2016, 197: 000–000.

I mmunoglobulin A are the major mediators of immunological defense at mucosal surfaces. Studies focus on the role of mucosal env-specific secretory IgA (sIgA) present in genital fluids from infected women highlight their ability to neutralize HIV primary isolates in vitro and to inhibit HIV transcytosis across epithelial cells (1, 2). This neutralizing property has also been demonstrated for systemic and mucosal IgA in HIV-1+ and exposed seronegative (ESN) individuals (3, 4), but the magnitude and frequency of HIV-specific sIgA seem to be very low during chronic infection (5). The initial Ab response to HIV-1 can be detected as circulating anti-gp41 IgG followed by production of anti-gp120 Abs a few weeks later, but none of these responses are able to efficiently neutralize the infecting strain (6). The maturation of broadly neutralizing Abs (bNAbs) takes several months (7) and goes through different steps of somatic mutations and modifications (8, 9) before becoming more specific to known sites of vulnerability of the HIV envelope such as the CD4 binding site, glycan V3 region, V1/V2 loop, and gp41, especially the membrane proximal external region (MPER) domain (9, 10). Neutralizing IgAs have been described at the mucosal level with specificity against gp41-specific epitopes such as QARILA VERY, Kennedy, or MPER (1, 11, 12). MPER is a highly conserved region in gp41 involved in the early stages of HIV attachment and infection (13). This region comprises the epitopes of well-described human monoclonal IgG broadly neutralizing Abs such as ELDKWA for 2F5, WF (N/D) IT for 4E10, and Z13 (14) for 10E8 (15). Recent studies have described the potency in targeting MPER for the generation of Abs with increased breadth, potency, and high-affinity binding. Indeed, structural observations from the crystal structure of the interaction of modified bNAbs and epitopes have provided clues to address gp41 and its different fusion intermediate states. New broadly neutralizing Abs like PGT122 and 35O22 have been also described, recognizing an unknown epitope overlapping the gp41–gp120 interface, and these Abs present potent neutralizing activity (9, 16). Moreover, the role of cholesterol and the lipid context in the mechanisms of broad neutralization by enhancing the presentation of MPER to B cells has been highlighted (17–19). Nevertheless, most of the studied bNAbs are IgGs (14). To date, very few studies have described the role of broadly neutralizing anti-MPER IgA and their involvement in the protection from and/or neutralization of HIV (20, 21) during experimental anti-HIV vaccine trials. The role of mucosal protective neutralizing IgA against mucosal transmission of HIV is unclear and remains controversial (5).
The aim of our study was to elicit and to characterize recombinant human monoclonal IgA neutralizing Abs that target the C-terminal region of gp41. To improve the immunogenicity of our Ag, the HEK 293-gp41 MSD cell line that allows expression of a folded, transmembrane gp41 has been used for immunization (22). Transfected cells were injected into humanized chimeric mice αIK1 to produce gp41-specific humanized chimeric IgA1 (23). In this article, we describe the use of this mouse model to generate potent neutralizing IgA Abs. High-affinityAbs were produced as mAbs after immortalizing and selecting specific Ab-producing cells through hybridoma derivation (24). Immunization in mice allowed the generation of six highly potent gp41-specific IgA-producing clones. From these clones, two Abs presented high recognition of gp41 and potent neutralization activity in vitro against both HIV-1 laboratory–adapted virus and primary isolates from different clades. The IgA1 clones F4.30 and C6.11 display high and low avidity to gp41, respectively, but high neutralizing potency against different HIV-1 strains. These two Abs recognize a large conformational epitope that overlaps the highly conserved epitope of 4E10 and present low rates of autoreactivity, unlike some previously described MPER-specific IgG and present a very low rate of somatic mutation (25).

Materials and Methods

Reagents

The following materials were obtained from the NIH AIDS Reference and Research Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health: primary HIV-1 isolates BAL, 92UG029, 92UG001, 92US660, HIV-1 G3, CAM1970, 92BR025, SF162, LAI, the HIV-1 subtype B (MN), and Env Peptides (15-mer) Complete medium for HeLa cells (97/2154, clade C8) and 3D6, 4E10, and 2F5 mAbs were obtained from Polymun Scientific (Vienna, Austria). The gp41 ectodomain from HXB2 strain was produced in Escherichia coli in our laboratory. Human HEK 293 cell lines were obtained from the American Type Culture Collection, and SP2/0 cells were obtained from UMR Centre National de la Recherche Scientifique 6101 (Limoges, France). HEK293-gp41 MSD were developed and characterized in our research group (26).

Immunization of mice and mAb production

αIK1 mice (6–8 wk old) were immunized with 50 μg of naked DNA (plasmid display heated repeat (HR)-1-PID-HHR2 used to develop the HEK293-gp41 MSD) in 2.5 ml of Ringer solution; this hydrodynamics-based immunization were realized by i.v. injection via the tail vein. Next, two groups of mice were immunized with 106 cells in IFA (Sigma-Aldrich), one i.p. injection with 106 cells in PBS at day 60.

Binding to HEK293-gp41 MSD

Membrane binding to gp41 on HEK293-gp41 MSD cells was also studied by flow cytometry. A total of 5 μg/ml IgA or positive control as 4E10 or 2F5 were incubated 45 min at 37°C. The cells were washed three times and then incubated with R-PE goat anti-human IgA (Abliance, Besançon, France). The anti-MPER IgA were incubated 45 min at 37°C and then washed three times and incubated with R-PE goat anti-human IgA (Abliance, Besançon, France). During acquisition of data, at least 10,000 events were analyzed on FACScan (BD Biosciences, Pont de Claix, France).

Production and purification of anti-MPER IgA

Hybridomas were expanded in DMEM high glucose–L-glutamate medium (PAA, Velizy-Villacoublay, France) supplemented with 10% FCS (Life Technologies), 0.1 mmol/l hypoxanthine, 100 μmol penicillin, and 100 mg/ml streptomycin in 96-well plates. The hybridoma cells were cultured for 2 d in 96-well plates and fixed in cold acetone. IgA were incubated for 30 min on fixed cells at a concentration of 5 μg/ml/well. After three washes with PBS containing 0.2% Tween 20, cells were incubated for 30 min with FITC-conjugated goat anti-human IgG or anti-human IgA as appropriate (Abliance, Besançon, France). Different anti-gp41 human mAbs 4E10, 2F5, and 3D6 at 10 μg/ml were used as positive controls and an anti--c-enzyme conjugated with FITC was used as positive control of gp41 expression. The labeling was visualized by fluorescence microscopy (Nikon TE 2000 Microscope, Burlingame, CA) using NIS-Elements software.

Measure of neutralizing activity

Measure of the neutralizing activity of purified gp41-specific Abs was performed using T cell line adapted strain LAI (clade B) or primary isolates of clade A (92UG029), B (BAL, SF162, 92US660, and QHO), C (92BR025), G (HIV-1 G3), CRF02 AG (CAM1970), and D (92UG001). Neutralizing activities were measured in duplicate and repeated three times. For LAI assay, CD4+/CXCR4+ SupT1 cells were incubated at 37°C under 5% CO2 in DMEM supplemented with 10% FCS. Infection was performed in 96-well, round-bottom plates. Purified Abs (50 μl) were incubated with an equal volume of virus containing a 100% tissue culture infective dose (TCID50) for 2 h at 37°C. Then, 3 × 105 SupT1 cells/well were added. At day 1, infected cells were washed twice with tissue culture medium. Supernatants were collected 7 d postinfection, and p24 was quantified by ELISA (ABL). Neutralizing activity of purified gp41-specific Abs was determined as described previously (27).

Briefly, PBMCs were isolated from healthy donors (from EFS Auvergne Loire, France) and stimulated with PHA (5 μg/ml) and 200 U/ml recombinant human IL-2 (Abcys, Paris, France). The cells were infected with a 100 TCID50 dose for 3 h at 37°C in the presence of purified Abs, and after 48 h, the cells were washed twice. Infected supernatants were collected 7 d postinfection, and p24 was quantified by ELISA (ABL). Neutralizing activity of purified Abs was also tested on stimulated PBMC infected with CAM1970, 92UG029, 92US660, 92BR025, 93BR025, HIV-1 G3, and 92UG001 primary HIV-1 isolates as described previously. The
cells were infected with a 100 TCID50 dose in the presence of serially diluted Ab. The supernatants were collected 10 d postinfection, and p24 measurement was performed. The percentage of neutralization was calculated as the reduction of p24 production. IC50 and IC80 were determined as the lowest Ab concentration able to confer 50 and 80% of neutralization, respectively.

Production of pseudoviruses COT6.15 and mutants

Pseudoviruses were generated by transfecting the COT6.15env plasmid or derived mutant (28, 29) with pSG3Denv plasmid, using the Lyovec transfection reagent (InvivoGen) in HEK293 cells in a petri dish. The production of pseudoviruses was confirmed by quantification of p24 produced, and their infectivity and measurement of TCID50s were quantified by infecting TZM-Bl cells (JC53-bl) with serial dilutions of the supernatant in triplicate in the presence of DEAE-dextran (30 mg/ml; Sigma-Aldrich, St. Louis). The infection was monitored 24 h later by evaluating luciferase activity, using the Bright Glo reagent (Promega, Madison, WI) following the manufacturer’s instructions. Luminescence was measured in a Berthold Tristar multilabel counter during 5 s/well (Berthold THOIRY, France). The TCID50 was calculated as described previously (30). Wells with relative light unit readings of 10 times that of the negative control were considered as positive.

Measurement of the avidity of anti-MPER IgA1

Biacore analyses were conducted on a BIAcore 3000 Instrument (GE Healthcare, Velizy-Villacoublay, France). Recombinant gp41 (HXB2), gp140 (97/2CN54), and gp120c41 (HXB2; Abcys Eurobio, Courtaboeuf, France) were coated on a 96-well plate (Maxisorp; Nunc) at 5 

μg/ml in 0.1 mol/l sodium carbonate buffer (pH 9.6) overnight at 4˚C. The plates were washed with PBS containing 0.05% Tween 20 and blocked with the same buffer and 4% BSA for 1 h at 37˚C. The TCID50 was calculated as described previously (30). Wells with relative light unit readings of 10 times that of the negative control were considered as positive.

Study of the autoreactivity of anti-MPER IgA

Autoreactivity to human epithelial (HEp-2) cells was determined by indirect immunofluorescence on slides using FITC-conjugated goat anti-human IgA polyclonal Ab (Abliance, Besançon, France). IgA were all tested at 25 

μg/ml. Autoreactivity of Abs was also tested by specific ELISA with purified cardiolipin (CLP) (Thermofisher, Illkirch, France) as described previously (31).

Competition assay

To study the ability of anti-MPER IgA to recognize the same epitope as well-known anti-MPER IGg previously described, a competition assay was performed by ELISA.

The protein gp120c41 (HXB2; Abcys Eurobio, Courtaboeuf, France) were coated on a 96-well plate (Maxisorp; Nunc) at 5 

μg/ml in 0.1 mol/l sodium carbonate buffer (pH 9.6) overnight at 4˚C. The plates were washed with PBS containing 0.05% Tween 20 and blocked with the same buffer and 4% BSA for 1 h at 37˚C. After washing, 100 μl of 2F5 and 4E10 (0–20 

μg/ml) diluted in PBS were added to the wells and incubated 2 h at 37˚C. After washing, 100 μl of the competition Abs were added to the wells at the concentration of 5 

μg/ml, diluted in PBS+BSA 1%, and incubated 1 h at 37˚C. After washing, detection was performed using

FIGURE 1. Characterization of MPER-specific IgA1. (A) Coomassie blue staining and Western blot analysis of MPER-specific IgA. 10 μg of purified IgA were loaded on a 9% acryl/bis-acryl gel under nondenaturing conditions. Detection of IgA was revealed with a polyclonal goat-anti-human IgA (Sigma-Aldrich). Size of monomers, dimers, and polymers are indicated. (B) Indirect immunofluorescence of HEK 293 cells transfected with MPER-specific IgA. Immunoreactivity of purified IgA (500 ng) was revealed by a rabbit polyclonal antibody labeled with FITC. No binding of MPER-specific IgA was observed on untransfected cells (ctrl-). 3D6 was used as positive control. (C) Neutralizing activity of MPER-specific IgA1 on primary and laboratory HIV-1 strains. 4E10 and 2F5 were used as positive controls. Dose-dependent neutralization was performed within a concentration range of 0.156–10 μg/ml. The experiments were performed three times with similar results.
Table I. Neutralizing activity of MPER-specific IgA

<table>
<thead>
<tr>
<th>HIV-1 Strains</th>
<th>IC50 (µg/ml)</th>
<th>IC50 (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab</td>
<td>92US660 (CCR5; B)</td>
<td>BAL (CCR5; B)</td>
</tr>
<tr>
<td>2F5 IgA</td>
<td>8.62</td>
<td>10</td>
</tr>
<tr>
<td>4E10 IgG</td>
<td>2–3</td>
<td>0.15</td>
</tr>
<tr>
<td>4E10 (410 m)</td>
<td>0.15</td>
<td>0.09</td>
</tr>
<tr>
<td>C6.11</td>
<td>2–3</td>
<td>0.15</td>
</tr>
<tr>
<td>SC16C13</td>
<td>2–3</td>
<td>0.15</td>
</tr>
<tr>
<td>SF162 (CCR5; B)</td>
<td>8.62</td>
<td>10</td>
</tr>
<tr>
<td>92BR025 (CCR5; C)</td>
<td>1</td>
<td>0.15</td>
</tr>
<tr>
<td>SF162 (CCR5; B)</td>
<td>8.62</td>
<td>10</td>
</tr>
<tr>
<td>92US660 (CCR5; B)</td>
<td>8.62</td>
<td>10</td>
</tr>
<tr>
<td>92UG001 (CCR5/CXCR4; D)</td>
<td>8.62</td>
<td>10</td>
</tr>
<tr>
<td>CAM1970 (CCR5; CRF02AG)</td>
<td>2–3</td>
<td>0.16</td>
</tr>
<tr>
<td>92UG029 (CXCR4; A)</td>
<td>2–3</td>
<td>0.15</td>
</tr>
<tr>
<td>SF162 (CCR5; B)</td>
<td>8.62</td>
<td>10</td>
</tr>
<tr>
<td>LAI (CXCR4; B)</td>
<td>8.62</td>
<td>10</td>
</tr>
<tr>
<td>COT6.15</td>
<td>2</td>
<td>0.15</td>
</tr>
</tbody>
</table>

2F5A IgA and 4E10 IgG are used as controls. IC50 or IC80 < 0.1 µg/ml. IC50 or IC80 < 10 µg/ml.

HRP-conjugated goat anti-human IgA (MP Biomedical, Strasbourg, France) and TMB substrate (Tebu bio, Le Perray en Yvelines, France). The absorbance was read at 450 nm. All assays were performed in duplicate (Multiskan Microplate Photometer; Thermo Scientific).

**Sequencing and analysis**

Sequencing was performed from purified DNA from pooled hybridomas to analyze the H chain nucleotide sequence and the characteristics of CDRH3 (B Cell Design, Limoges, France). The characterization of Abs sequence was performed using Integrates Sequence Tools and ExPASy.org/Protparam

**Statistical analysis**

The statistical difference in vitro experiments between controls and Abs was assessed using Student’s t test (GraphPad software; GraphPad, San Diego, CA). A p value < 0.05 has been considered statistically significant. The whole results were obtained from the mean of at least a triplicate or two triplicates. The significances were verified according to an ANOVA test with Bonferroni posttests to compare the row means. The correlations were obtained after a Spearman correlation for nonparametric data.

**Results**

To induce gp41-specific neutralizing Abs (NAbs), immunization of humanized transgenic mice (23) were performed using HEK293-gp41MSD human cell line that allowed native shape expression of a gp41 (22). After immunizations, >2300 hybridoma B cell clones were isolated and immortalized. Supernatants of all individual B cell clones were tested by immunoﬂuorescence on HEK293-gp41MSD and untransfected cells (Fig. 1B, Supplemental Fig. 1). Among the hybridomas, only six clones presented highly specific recognition of the HEK293-gp41MSD cells. From those clones, IgA1 were produced and purified (Fig. 1A). Recombinant chimeric IgA are produced both as monomers, dimers, and polymers (Fig. 1A). The average concentration of IgA was with a range between 86 and 295 ng/µl before purification.

**Elicited IgA1 are specific to gp41 ectodomain**

To confirm the specificity of purified IgA1, immunofluorescence on HEK 293-gp41MSD was performed. Only clones with high speciﬁc recognition HEK 293-gp41MSD were selected (Fig. 1B). This screening allowed the selection of six IgA1 clones (F4.5, F4.6, F4.30, C6.11, C6.13, and SC16C13) (Fig. 1B).

**F4.30 and C6.11 are highly potent and broadly neutralizing Abs of tier 1, 2, and 3 primary HIV-1 strains**

The neutralizing activities of purified MPER-specific IgA1 were tested on different strains. Among them, F4.30 exhibited a strong neutralization activity with a high proportion of neutralized primary isolates (>80% of tested strains) with very low IC80 and IC50 in the range of few nanograms per milliliter (Fig. 1C, Table I). F4.30 neutralized 92UG029, LAI, and COT6.15 strains with a high proportion of neutralized primary isolates (>80% of tested strains) with very low IC80 and IC50 in the range of few nanograms per milliliter (Fig. 1C, Table I). F4.30 neutralized 92UG029, LAI, and COT6.15 strains with a strong and higher potency (IC80 < 1 µg/ml) than those obtained for 4E10 IgG or 2F5 IgA with LAI and those reported for 10E8. F4.30 neutralized BAL and 92UG001D strains more efficiently than 4E10 with IC80 five times lower for BAL but higher than for 2F5 IgA (3.3, 17.19, and 0.81 µg/ml, respectively). F4.30 showed stronger neutralization potency for 92UG001D strain with an IC80 of 8.62 µg/ml that is 47 and 4 times lower than those obtained for 4E10 (410 µg/ml) and 2F5 (36 µg/ml), respectively. SF162 and 92BR025 strains were weakly neutralized by F4.30, and the IC80 was lower than for 4E10. C6.11 neutralized efficiently the infection of 70% of the tested strains with an IC50 < 1 µg/ml. C6.11 neutralized 92US660 and CAM1970 with the same potency as 4E10 and 2F5 IgA, whereas LAI, COT6.15, and HIVG3 were neutralized with lower IC80. BAL, 92UG029, and 92UG001 were moderately neutralized by C6.11, but its IC80 is lower than 4E10. C6.11 neutralized SF162 and 92BR025 with an IC80 of 13.27 and...
13.37 μg/ml, respectively, 20 times lower than 4E10 or 10E8 but close to 2F5 IgA. SC16C13 neutralized 30% of the tested strains with an IC80 of 137 μg/ml. 92UG029, 92US660, and CAM1970 were neutralized with IC80 of 1 μg/ml (equivalent to 4E10 and 2F5). F4.6 and C6.13 strongly neutralized only three of the tested strains and F4.5 presented weak neutralization except for 92US660 and HIV-1 G3 that were neutralized with IC80 of 1 μg/ml (Fig. 1C, Table I).

gp41-specific IgA1 recognize different epitopes in the MPER region

To characterize the neutralizing epitopes recognized by the more potent IgA1 F4.30 and C6.11, the binding of purified IgA1 to peptide epitopes of the Env glycoprotein (clade B) and to recombinant gp41 or gp140 were measured (Supplemental Table I). The specificity of IgA1 was directed to the MPER and HR2 regions. F4.30 and C6.11 recognized the 2F5 neutralizing epitope on MPER. C6.11 recognized the two neutralizing epitopes ELDKWA and WFD/NIT on MPER, whereas F4.30 recognized the ELDKW motif and the HR2 region. All recombinant IgA1 were able to strongly recognize both the gp41 ectodomain and the gp140 glycoprotein (Supplemental Table I).

Anti-MPER IgA1s have a strong avidity for gp41

IgA1 abilities to recognize the gp41 glycoprotein were measured using surface plasmon resonance. Kinetic experiments were performed using different targets as the gp41 ectodomain (clade B), the gp140 (clade C) and the gp120cgp41 (clade B). After immobilization of the target proteins, MPER-specific IgA1 (F4.30 and C6.11) as well as 4E10 or 3D6 IgGs at various concentrations (0.5–160 nM) were tested (Fig. 2B). F4.30 recognized gp140 with a lower avidity (KD of 18.8 nM). C6.11 recognized weakly the gp120c41 precluding determination of the KD value.

F4.30 and C6.11 are able to recognize both viral particles and infected cells

To demonstrate the ability of IgA1 to efficiently recognize HIV-1 particles, an ELISA was performed with coated viral particles of both laboratory and primary strains. As a negative control, an anti-OVA IgA1 was used. Free virions from BAL, CAM1970, LAI, and SF162 strains were strongly recognized by C6.11 and F4.30 (Supplemental Table III). The anti-OVA IgA1 did not bind to CAM1970, BAL, and SF162 viral particles and showed a very weak binding to the LAI strain (Supplemental Table III). The binding to free viral particles is probably the mechanism involved in the neutralizing activity of IgA1. An infection assay with TCLA LAI and SupT1 cell line was performed to demonstrate the ability of IgA1 to specifically recognize HIV-1–infected cells (Supplemental Table III). The binding to free viral particles is probably the mechanism involved in the neutralizing activity of IgA1. An infection assay with TCLA LAI and SupT1 cell line was performed to demonstrate the ability of IgA1 to specifically recognize HIV-1–infected cells (Fig. 2C). The percentage of p24+/IgA1+ cells was 12% with a mean fluorescence intensity ratio (infected/uninfected) of 5.1 for C6.11. F4.30 was not able to recognize >10% of infected cells with a mean fluorescence intensity of 4.3. No binding was observed with anti-OVA IgA1.
Neutralizing epitopes of IgA1 are larger than 2F5 or 4E10 epitope. Mapping of neutralizing epitopes was performed by measuring their neutralizing activities with HIV-1 COT6.15 pseudoviruses and compared with mutated pseudoviruses with alanine substitutions in MPER residues (662–682) as described previously (32). As described for well-characterized MPER-specific IgG as 2F5 or 4E10, IgA1 neutralizing activity was highly sensitive to substitution of the W666 tryptophan and the K667 lysine residues that are central in the 2F5 recognition (14). For F4.30, a downstream-extended 2F5 epitope merged with the upstream 4E10 epitope 667KNLWSWF D/N/S I675 seems to be crucial for neutralization. However, mutation of the T676 residue also reduced the neutralizing potency of F4.30. C6.11 was also sensitive to the substitution of W666, W670, and L669 residues also crucial in 2F5 neutralization activity. C6.11 was also sensitive to the mutation of the C-Term residues of MPER (674D/N/SITKWLWYI682). The F4.30 and C6.11 epitopes are probably larger than 4E10 and 2F5 epitopes. It was confirmed by the determination of an IC80 COT mutant versus IC80 COT wild-type ratio of for each Ala substitution.

F4.30 and C6.11 are not autoreactive/polyreactive
A property common to the previously characterized MPER-specific mAbs 2F5 and 4E10 is their ability to cross-react with self-antigens (33). In addition, binding to both the cell membrane and the Env trimer is thought to be important for optimal neutralization. Autoreactivity of IgA1 was first tested on HEp-2 cells by immunofluorescence (Fig. 3B). F4.30, C6.11, anti-OVA IgA negative control, and anti-DNA IgG positive control were used at 25 μg/ml. Assay were performed in triplicate. Original magnification ×20.

FIGURE 3. (A) Screening of the neutralizing activity of MPER-specific IgA on COT6.15 pseudoviruses and COT6.15 mutants. MPER sequence is shown in each panel. For each mAb and mutant, the value of SD is calculated for the neutralization from two independent experiments realized in triplicate. The critical residues for neutralization are indicated in red. (B) Analysis of MPER-specific IgA autoreactivity on HEp-2 cells by immunofluorescence. F4.30, C6.11, anti-OVA IgA negative control, and anti-DNA IgG positive control were used at 25 μg/ml. Assay were performed in triplicate. Original magnification ×20.

Neutralizing epitopes of IgA1 are larger than 2F5 or 4E10 epitope. Mapping of neutralizing epitopes was performed by measuring their neutralizing activities with HIV-1 COT6.15 pseudoviruses and compared with mutated pseudoviruses with alanine substitutions in MPER residues (662–682) as described previously (32). As described for well-characterized MPER-specific IgG as 2F5 or 4E10, IgA1 neutralizing activity was highly sensitive to substitution of the W666 tryptophan and the K667 lysine residues that are central in the 2F5 recognition (14). For F4.30, a downstream-extended 2F5 epitope merged with the upstream 4E10 epitope 667KNLWSWF D/N/S I675 seems to be crucial for neutralization. However, mutation of the T676 residue also reduced the neutralizing potency of F4.30. C6.11 was also sensitive to the substitution of W666, W670, and L669 residues also crucial in 2F5 neutralization activity. C6.11 was also sensitive to the mutation of the C-Term residues of MPER (674D/N/SITKWLWYI682). The F4.30 and C6.11 epitopes are probably larger than 4E10 and 2F5 epitopes. It was confirmed by the determination of an IC80 COT mutant versus IC80 COT wild-type ratio of for each Ala substitution.

F4.30 and C6.11 are not autoreactive/polyreactive
A property common to the previously characterized MPER-specific mAbs 2F5 and 4E10 is their ability to cross-react with self-antigens (33). In addition, binding to both the cell membrane and the Env trimer is thought to be important for optimal neutralization. Autoreactivity of IgA1 was first tested on HEp-2 cells by immunofluorescence (Fig. 3B). F4.30, C6.11, anti-OVA IgA negative control, and anti-DNA IgG positive control were used at 25 μg/ml. Assay were performed in triplicate. Original magnification ×20.

Neutralizing epitopes of IgA1 are larger than 2F5 or 4E10 epitope. Mapping of neutralizing epitopes was performed by measuring their neutralizing activities with HIV-1 COT6.15 pseudoviruses and compared with mutated pseudoviruses with alanine substitutions in MPER residues (662–682) as described previously (32). As described for well-characterized MPER-specific IgG as 2F5 or 4E10, IgA1 neutralizing activity was highly sensitive to substitution of the W666 tryptophan and the K667 lysine residues that are central in the 2F5 recognition (14). For F4.30, a downstream-extended 2F5 epitope merged with the upstream 4E10 epitope 667KNLWSWF D/N/S I675 seems to be crucial for neutralization. However, mutation of the T676 residue also reduced the neutralizing potency of F4.30. C6.11 was also sensitive to the substitution of W666, W670, and L669 residues also crucial in 2F5 neutralization activity. C6.11 was also sensitive to the mutation of the C-Term residues of MPER (674D/N/SITKWLWYI682). The F4.30 and C6.11 epitopes are probably larger than 4E10 and 2F5 epitopes. It was confirmed by the determination of an IC80 COT mutant versus IC80 COT wild-type ratio of for each Ala substitution.

F4.30 and C6.11 are not autoreactive/polyreactive
A property common to the previously characterized MPER-specific mAbs 2F5 and 4E10 is their ability to cross-react with self-antigens (33). In addition, binding to both the cell membrane and the Env trimer is thought to be important for optimal neutralization. Autoreactivity of IgA1 was first tested on HEp-2 cells by immunofluorescence (Fig. 3B). F4.30, C6.11, anti-OVA IgA negative control, and anti-DNA IgG positive control were used at 25 μg/ml. Assay were performed in triplicate. Original magnification ×20.

F4.30 and C6.11 are not autoreactive/polyreactive
A property common to the previously characterized MPER-specific mAbs 2F5 and 4E10 is their ability to cross-react with self-antigens (33). In addition, binding to both the cell membrane and the Env trimer is thought to be important for optimal neutralization. Autoreactivity of IgA1 was first tested on HEp-2 cells by immunofluorescence (Fig. 3B). F4.30, C6.11, anti-OVA IgA negative control, and anti-DNA IgG positive control were used at 25 μg/ml. Assay were performed in triplicate. Original magnification ×20.

F4.30 and C6.11 are not autoreactive/polyreactive
A property common to the previously characterized MPER-specific mAbs 2F5 and 4E10 is their ability to cross-react with self-antigens (33). In addition, binding to both the cell membrane and the Env trimer is thought to be important for optimal neutralization. Autoreactivity of IgA1 was first tested on HEp-2 cells by immunofluorescence (Fig. 3B). F4.30, C6.11, anti-OVA IgA negative control, and anti-DNA IgG positive control were used at 25 μg/ml. Assay were performed in triplicate. Original magnification ×20.

F4.30 and C6.11 show low levels of mutation and have a short and low hydrophobic H chain third CDR
We next aimed to understand whether F4.30 and C6.11 share the same characteristics as 2F5, 4E10, or 10E8, which have a long and hydrophobic H chain third CDR (CDRH3) and a high rate of somatic mutation. The H chains of F4.30 and C6.11 were sequenced and the CDRH3 of each clone was analyzed and compared with 2F5 and 10E8 (34). The sequences of the H chain of C6.11 and F4.30 are identical and could come from the same original clone (Fig. 4A). However, the sequence of the L chains differs from two residues. F4.30 and C6.11 present a shorter CDRH3 with 9-aa residues, whereas 2F5 and 10E8 (accession numbers JX645769/JX645770) contains 24 and 22 residues respectively (Fig. 4B). The CDRH3 of F4.30 and C6.11 is less hydrophobic than 2F5 but more hydrophobic than 10E8 with a hydropathicity score of −0.833, −0.225, and −1.386, respectively. By comparing the sequence of the constant H chain of each Ab and the VDJ allele precursor, we observed that both F4.30 and C6.11 H chains come from the IGHV3-602 F with which they share 98.96% of identity, the IGHJ2*1 with 95.74% identity, and IGHD2-1*01F. The alignment of the variable domain of the H chains of F4.30 and C6.11 with 2F5, 10E8, and PG9 showed matches of 51.67, 40.83, and 49.17% respectively (data not shown).

Discussion
Recently, many studies have demonstrated the role of Nabs during the course of HIV-1 infection. New advances allowed to elicit Abs directed against different key epitopes, such as the quaternary neutralizing epitopes of the viral envelope (35, 36). Passive ad-
**ministration of rare human envelope-specific monoclonal bNAbs**

**Moreover, gp120-specific Abs seem to be one of the correlates of protection in the recent RV144 vaccine trial (40, 41) even if the mucosal immune response was not investigated. The presence of systemic IgA (mainly monomeric) directed against certain gp120 specificities were associated with higher rates of infection. However, other specific IgA were not associated with infection risk (40, 42, 43). In contrast, the presence of IgA in saliva and serum, directed against different key regions of gp160 (CD4 binding site, V2 loop or the MPER) and able to neutralize different HIV-1 strains in vitro, has been associated to protection in ESN (1, 12). Moreover, several studies have described the potential ability of mucosal sIgA to locally block infection (39, 44).**

**FIGURE 4. Sequencing of the C region of the H and L chain of F4.30 and C6.11. (A) Alignment of the sequences of the H chain of F4.30 and C6.11 and the consensus sequence. Nucleotides that are different from the consensus sequence are highlighted in yellow, and the corresponding residues are in blue and highlighted in yellow. (B) “Pearl necklace” representation of the amino acid sequence of the H chains of F4.30 and C6.11 and characterization of their CDRH3. Amino acid sequence of the H chain of F4.30 and C6.11 as well as 2F5 and 10E8. CDRH1 are represented in red, CDR2 in orange, and CDRH3 in purple. The length of the CDRH3 (number of amino acids) and the score of hydropathicity are mentioned under each sequence.**

**The α1KI mice model allows the production of chimeric monomeric, dimeric, and polymeric IgA1 with equivalent rate of glycosylation similar to those encountered in human (48). Cross-linking of mIgA+ B cells residing in MALTs mediates local IgA secretion responses in this model (49, 50). As for IgG, a B cell stage with mIgA expression is necessary to mount IgA Ab responses (51). That both early and late B cell development can be ensured by expression of membrane α chain (HC) was shown in the α1KI model where α-mouse was replaced with an Ig HC α1 gene (45). In such mice, a partial pre-B defect was reminiscent of data in mice with premature expression of membrane HC. BCR density was lower in α1KI than in wt mice, and was associated with increased abundance of both long-lived and short-lived plasma cells.**

**In this model, a prime boost strategy using naked DNA as a prime by hydrodynamic i.v. route was used to increase the immunogenicity of the gp41 (23). Using HEK293-gp41MSD cells as a boost gave rise to six clones able to specifically recognize gp41 in a conformational shape. Among these clones, two Abs (F4.30 and C6.11) showed a high neutralizing potency for different strains of HIV. The neutralizing activity for F4.30 and C6.11 are very similar to the one reported for the 10E8 another bNAbs gp-41–specific IgG but higher**
than for 4E10 or 2F5 (both in IgG and IgA backbone). It could be very important to assess the breadth of the neutralizing activity against a wide range of pseudoviruses from National Institutes of Health panels. Moreover, the solubility of these two clones seems to be higher than for 10E8 (52). F4.30 recognizes both MPER neutralizing epitopes ELDKW and WFD/NIT and the HR2 regions with different efficiency. C6.11 bound the KWA motif in the ELDKWA 2F5 neutralizing epitope. These data correlated with the results obtained by surface plasmon resonance using immobilized gp41, gp140, and gp120c41 proteins. The high neutralization activity of F4.30 is also correlated to a high level of recognition of free viral particles and the absence of autoreactivity. The high neutralization capacities in comparison with the well-known IgG 4E10 could be explained by a better recognition of the gp41 conformational shape by F4.30.

Screening of the F4.30 binding site and competition assay suggest that the epitope could be conformational or discontinuous and involve HR2 and other regions. In fact, the polymeric nature of IgA suggests that they could bind large functional epitopes possibly located on adjacent proteins such as gp41 and gp120 during the infection process or to HR1/HR2 domains in a fusion complex state.

Other studies have described purified neutralizing IgA Abs from patients directed against different key regions on the gp160 surface including the super antigenic site on gp120 and the CD4 binding site (53), which are also discontinuous epitopes. Furthermore, the recently described bNAb 35022 recognizes and efficiently binds a large epitope corresponding to the interface of gp120 and gp41 (9, 16). Structural studies have described the ability to enhance the neutralization potency of well-known Abs like 10E8 by presenting MPER in a different context using a hydrocarbon stapled peptide that reinforces unique architectures, such as the helix-kink-helix MPER motif that presents a new fashioning of peptide structure (19). It will be interesting to make such studies with F4.30 and C6.11.

Numerous studies have also shown that the advent of heterologous neutralizing Abs in a subset of HIV patient appears many years postinfection, what is explained by multiple rounds of modifications and maturation that are exerted by a continually evolving pathogen (7, 54). These mutations lead to different features common to some neutralizing Abs including an exceptional high rate of somatic mutations and a long hydrophobic CDRH3 (8, 55). In fact, a long CDRH3 is observed for Abs that target and penetrate the envelope glycan shield like PG9, PG16, and PGT145 or the 10E8 that targets MPER (15, 56). However, 35022 that recognizes a different site of vulnerability on the glycoprotein envelope uses a novel mechanism of glycan-protein recognition, combining a protruding FW3 with CDRH1, H2, and H3 to form a “bowl” that holds glycan. FW3 and CDRH3 provide the top edges of the bowl and interact with the protein surface of gp120, whereas CDRH1 and H2 are recessed and hold/reconize glycan. This structural mechanism of recognition contrasts with the extended CDRH3-draping glycan observed with other Abs that penetrate the glycan shield such as PG9 and PGT128. Whereas 35022 Ab possesses a CDRH3 composed of 14 aa that is shorter than 4E10 or the 2F5 Ab but presents an insertion of 8 aa in framework 3. This high rate of somatic mutation and framework 3 insertions is a feature of other HIV-specific bNAbs (9, 16, 57, 58). F4.30 and C6.11 presented a low rate of mutation with a high score of similarity to the consensus VDJ alle. This suggests a weak maturation of the F4.30 and C6.11 clones and can be explained by an exposition to a small number of immunogen variants because of the immunization protocol. Furthermore, F4.30 and C6.11 presented a short CDRH3 with a low hydrophobicity in comparison with 2F5, which does not exclude reactivity with a complex glycosylated epitope but discards the idea of a deeply hidden region buried within the glycan shield.

An important inconvenience of well-known MPER-specific Abs as 2F5 and 4E10 is that they cross-react with self-antigens. F4.30 and C6.11 do not present any autoreactivity or recognition of self-antigens as phospholipids. Studies of potent Abs such as 2G12 (59) described the capacity of monomeric or dimeric 2G12 to mediate Ab-dependent cellular cytotoxicity or to activate the complement system in vitro (60, 61). The capacity of F4.30 to mediate Ab-dependent cellular cytotoxicity or Ab-dependent cell-mediated virus inhibition against infected cells remains to be investigated. This work strengthens our previous studies of the role of anti-MPER IgA present in parotid saliva of ESN and HIV+ individuals (1, 12, 28). As for M66.6 and M66 2F5-like Abs (62), MPER-specific IgA1 are similar but present different activities of recognition and binding for gp41. It may suggest that both Ab lineages evolved under similar constraints for recognition and neutralization of HIV-1, whereas functional elements within CDR-like glycosylation may differ during this evolution and impact their properties.

F4.30 and C6.11 IgA have the capacity to strongly neutralize >80% of the HIV-1 strains tested. The avidity of IgA for its Ag was not correlated to neutralizing activity. C6.11 has a weak Ag avidity but presents a high cross-clade neutralizing activity. The profile of the higher neutralizing IgA Abs is of intermediate avidity for the recognition of MPER, high recognition of free viral particles, and an absence of auto reactivity.

These two mAbs (F4.30 and C6.11) are to our knowledge the first recombinant neutralizing cross-clade IgA1. There are, however, remaining questions with the αK1 chimeric model such as the relevance with regard to a normal physiological development of Abs, the proportion in those mice of LB1 that do not develop into memory B cells, LB2, or B cells of the marginal zone, and the impact of a prior IgA switch in the B cell compartment. αK1 is a promising chimeric model for the eliciting of new humanized Abs. Further understanding of the interaction of F4.30 with gp41 at the atomic level would require crystallographic studies. MPER-specific IgA1 could be now tested in vivo to block the entry of HIV-1 after recent viral exposure or to reduce, after systemic administration, viral burden, and the massive CD4 cell depletion in HIV-1 reservoirs such as the intestine. Potent mAbs acquire their neutralization potency from their ability to block a functionally important site that is critical for viral entry as seen for the CD4 binding site Abs. Nonetheless, the breadth and potency of F4.30 demonstrates a conserved site of gp41 is an important target Ag for HIV neutralization. The highly conserved MPER is a target of potent, non-self-reactive neutralizing Abs, confirming the high interest in MPER-based HIV vaccine design.

Acknowledgments
We thank Philip Lawrence for critical reading of the manuscript. We thank Isabelle Bally for assistance and access to the surface plasmon resonance platform of the Partnership for Structural Biology in Grenoble. We also thank Dr. Elin Gray and Prof. Lynn Morris for providing the COT6.15 platform of the Partnership for Structural Biology in Grenoble. We also thank Dr. Elin Gray and Prof. Lynn Morris for providing the COT6.15 plasmids. This work used the platforms of the Grenoble Instruct centre (ISBG, UMS 3518 CNRS-CEA-UJF-EMBL).

Disclosures
The authors have no financial conflicts of interest.

References


The Journal of Immunology


