

# Generation of HIV-1 potent and broad neutralizing antibodies by immunization with postfusion HR1/HR2 complex

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**Background:** The envelope glycoproteins are major targets for HIV vaccines. The N-terminal and the C-terminal regions of the gp41 interact to form six helix bundles that are responsible for the fusion between the viral and the target cell membranes. Monoclonal antibodies (Abs) able to disrupt the formation of this complex or to interfere with it could inhibit HIV fusion. Most of the well described gp41-specific broadly neutralizing Abs target conformational epitopes within the membrane proximal region of gp41 (MPER) and recognize linear peptides.

**Method and results:** In this study, a stable human transfected cell line, expressing a well folded heptad repeat regions 1 (HR1)/HR2 postfusion complex was developed. Transfected cells were highly immunogenic in mice and allowed the generation of 40 complex specific B-cell clones. Three of them were able to neutralize efficiently both HIV-1 laboratory adapted virus and primary isolates from different clades. Two neutralizing Abs (Nabs) FC-2 and FC-3 bound to a recombinant folded gp140 and blocked with a high potency HR1/HR2 fusion complex formation *in vitro*. The conformational epitopes of the three antibodies are different to 2F5, 4E10, D5 or NC-1 and mainly located in the MPER region. Abs were capable of inhibiting syncytium formation by blocking spatial interactions between HR1 and HR2 regions.

**Conclusion:** These findings suggest that immunogenicity of gp41 is achievable and that the use of a fusion complex expressing human cell line is a highly potent immunogen to generate neutralizing antibodies against gp41 envelope glycoprotein.

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## Introduction

The HIV-1 envelope (Env) glycoprotein is the main target for neutralizing antibodies. A major challenge in HIV-1 vaccine research remains to identify an immunogen that has the capacity to induce high titers of broadly neutralizing antibodies [1–3]. The HIV-1 genome and especially its env gene is highly variable between and

within clades [4], which is partly responsible for the difficulty in developing a suitable vaccine candidate [5]. Env-specific neutralizing antibodies mainly target the spikes on the surface of infectious virions [2,3,6].

Trimeric Env is composed of the receptor binding domain gp120, which is noncovalently associated with the membrane-anchored fusion protein gp41. Infection

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of target cells is initiated by the attachment of Env to the CD4 cell count receptor [7,8], which triggers conformational changes that expose the hypervariable loop 3 (V3) [9], thus, priming it for coreceptor CCR5 or CXCR4 interaction [10,11]. Together CD4 cell count and coreceptor (CCR5 or CXCR4) interactions are thought to induce conformational changes in the fusion protein subunit resulting in exposure and subsequent insertion of the fusion peptide into the target cell membrane which produces the fusion intermediate prehairpin structure that bridges viral and cellular membranes [6,12]. During this process two helical heptad repeat regions 1 (HR1) and 2 (HR2) are transiently exposed [13] allowing interaction with peptide inhibitors of fusion such as T-20 [14,15]. This structure consists of a bundle of six  $\alpha$ -helices, in which three C-terminal Heptad Repeat peptides pack in an antiparallel manner against a central three-stranded coiled-coil formed by the HR1 regions of the same gp41 monomers [16–18]. Subsequent refolding of the prehairpin structure into the postfusion conformation leads to the contact of viral and cellular membranes catalyzing pore formation and membrane fusion [16,17,19–22].

The fusion-intermediate conformation of gp41 is an attractive target for neutralizing antibodies due to its relative high sequence conservation and low degree of glycosylation. These antibodies are considered to be able to prevent HIV-1 infection by interfering with post-attachment steps leading to virus membrane fusion.

Cross-clade neutralizing antibodies 2F5, 4E10 and Z13 target the membrane proximal region most likely during epitope exposure in the fusion-intermediate prehairpin conformation [23–25]. Although conformation and lipid environment may be crucial for their activities, these mAbs bind to linear epitopes of membrane proximal region of gp41 [26–30]. 2F5 is mapped to the conserved sequence ELDKWA [31], whereas 4E10 and Z13 recognize an epitope involving the sequence NWF(D/N)IT located in the C-Term domain of the 2F5 epitope [32]. The mAbs 3D6 and 5F3 exhibiting low neutralizing activity restricted to limited HIV-1 clades recognized the GCSGKLICT-TAVPW 17 AA gp41 region [33] or an epitope involving the sequence (QNQQEKNE) located upstream to the MPER region, respectively. The recombinant mAb CAP206-CH12 recently described used a portion of the distal MPER from HXB2 and neutralized a subset of HIV-1 pseudoviruses [34]. Failure to elicit broadly neutralizing antibodies against the membrane proximal external region of HIV-1 gp41 reflects the difficulty of mimicking its neutralization-competent structure. The pivotal role of the transmembrane region of the gp41 in MPER exposure has been recently demonstrated [35].

Antibodies directed against HR1/HR2 complex exist in the sera of HIV-1-infected individuals. Specific HR1/HR2 antibodies have been purified from patients with an

HR1/HR2 (C34) fusion complex and exhibit potent neutralizing activity against various primary isolates [36]. Several HR1-specific monoclonal antibodies targeting the prehairpin conformation of gp41 have been isolated from phage display libraries with variable neutralization profiles depending on the assays used. NC-1 is a murine monoclonal antibody (mAb) that specifically recognizes the six helix bundle core of the HIV-1gp41 [37]. NC-1 antibody is devoid of neutralizing activity. The D5 mAb was isolated from a naive human library and identified as an antibody directed against the highly conserved hydrophobic pocket in the groove formed by the trimeric coiled-coil of the gp41 N-helices [38]. MAb DN9 from a Fab library has been generated from bone marrow RNA from an HIV-1-infected individual [39], whereas the rabbit single chain mAb 8K8 was derived from a phage library [39] prepared from rabbits immunized with a gp41 HR1 mimetic [40]. More recently, HK20 mAb has been isolated from immortalized memory B cells of an HIV-1-infected individual, which targets the conserved hydrophobic pocket in HR1 [41,42]. HK20 seems to have an important breadth of neutralization. However, the HK20 neutralizing activity remains very low and seems to be target-cell dependent. Although this region is highly immunogenic, immunization strategies employing HR1 peptide mimetics led to the generation of a polyclonal antibody response capable of neutralizing only Tier 1 primary isolates [43–45]. Crystal structures of N-HR mimetic 5-helix with specific Fabs suggest that both neutralization properties and affinity for the target can be attributed to the differences in the interactions of the CDR-H2 loop with the antigen [46].

In this study, we focused on eliciting neutralizing antibodies that target HR1/HR2 complex of gp41. To improve the immunogenicity of the complex, we have developed a human HEK 293 cell line that expressed a transmembrane folded prehairpin fusion complex on its surface. This immunogen is recognized by well known antibodies targeting HR1/HR2 complex such as NC-1 or D5. Transfected cells were highly immunogenic in mice and allowed the generation of 40 complex-specific B-cell clones. Three Nabs are specific for gp41 and two (FC-2 and FC-3 for Fusion Complex) bound efficiently to a recombinant folded gp140 and recognized very specifically HR1/HR2 fusion complex. These antibodies were also able to efficiently block the formation of the complex and to inhibit syncytium formation. Finally, the three Abs demonstrate distinct profiles of neutralization both on HIV-1 laboratory adapted virus and primary isolates from different clades.

## Methods

### Reagents

The following materials were obtained through the NIH AIDS Research and Reference Reagent Program,

Division of AIDS, NIAID, NIH: pSVIII-93BR025.9 plasmid (Gene Bank number U09126), primary HIV-1 isolates (SF162, 92US660, and 93BR025), the SHIV 89.6P Subtype B env (20-mer) Peptides Complete Set, NC1, 2F5, 5F3, NC-1 mAbs and the CHO-WT cell line. Gp140 recombinant protein (strain 97/CN/54, clade C/B) and 3D6, 4E10, Z13 mAbs were obtained from Polymun Scientific (Vienna, Austria). Gp41 ectodomain from HXB2 strain was produced in *Escherichia coli*. D5 mAbs was kindly provided by Dr Miller (Merck & Co, Rahway, New Jersey, USA) [38]. Human HEK 293 and Hela CD4<sup>+</sup> cell lines were obtained from American Type Culture Collection. SP2/0 cells were obtained from Dendritics (Lyon, France).

### Cloning of gp41

The plasmid pSVIII-92BR025.9, containing the full sequence of clade C HIV-1 envelope protein, was used as a template for the amplification of the HR1-PID-HR2-TM by using specific primers. A stop codon has been inserted at the end of the gp41 TM region. The PCR products obtained were purified by Qiagen purification kit (Courtaboeuf, France), then were digested with SmaI/PstI and cloned in the pDisplay expression vector (Invitrogen, Carlsbad, California, USA). Plasmids were then transformed into *E. coli* strain DH5 $\alpha$  using thermal shock method followed by extraction and purification using Qia-prep Miniprep kits (Qiagen) and large-scale Midiprep kits (Qiagen). The quality of the constructs was verified by the enzymatic digestion method and by automated DNA sequencing.

### Establishment of gp41 expressing cell line

HEK 293 cell culture was maintained in Dulbecco's modified Eagle medium with stable L-glutamine and high glucose level (Sigma-Aldrich, St. Louis, Missouri, USA) supplemented with 10% fetal bovine serum and 1X nonessential amino acids at 37°C with 5% CO<sub>2</sub>. Transfection was made using LYOVEC (Invitrogen) as according to the manufacturer's recommendation. The selective agent G418 (Roche Applied Science, Indianapolis, Indiana, USA) was added to a final concentration of 0.5 mg/ml 48 h after transfection. After 10 days of culture, transfected cells were analyzed by immunofluorescence. Then, limiting dilution of transfected cells has been made. Analysis of gp41 expression on HEK 293-gp41<sup>MSD</sup> (MSD = Membrane Spanning Domain of gp41) cells was performed both by flow cytometry and by immunofluorescence.

To study the expression of the gp41 at the cell surface by flow cytometry, HEK 293-gp41<sup>MSD</sup> cells were tested with different antigp41 human monoclonal antibody, Z13, 2F5, 5F3 at 5  $\mu$ g/ml for 30 min at 4°C. The cells were washed three times and then incubated with R-Phycoerythrin R-PE goat antihuman IgG (Sigma) for 30 min at 4°C. After washing, cells were analyzed by flow

cytometry. At least, 10 000 events were analyzed on FACScan (Becton Dickinson, Pont de Claix, France)

The antigp41 mAbs (3D6, 2F5, and 4E10) were used at 15  $\mu$ g/ml for the validation of the cell line by indirect immunofluorescence and using the antimyc conjugated with fluorescein isothiocyanate (FITC) for direct immunofluorescence followed by a second step with FITC conjugated goat antihuman IgG. The visualization of labeling was observed by fluorescent microscopy software (Nikon TE 2000 Microscope, Burlingame, California, USA) using NIS-Elements software.

### Immunization of mice and monoclonal antibody production

BALB/c mice (6–8 weeks old,  $n=3$ ) were intraperitoneally (i.p) immunized with  $2 \times 10^6$  cells of HEK 293-gp41<sup>MSD</sup> in complete Freund's adjuvant (CFA) (Sigma Aldrich) followed by one i.p. injection with  $2 \times 10^6$  cells in incomplete Freund's adjuvant (IFA) (Sigma Aldrich) at day 14, one i.p. injection with  $2 \times 10^6$  cells in PBS at day 21 and finally one intravenous boost with  $4 \times 10^6$  cells in PBS at day 28. Use of CFA/IFA adjuvants allow to reduce the number of human HEK cells for immunization, to increase the number of accessible epitopes in the emulsion and finally to facilitate the migration of APCs to the spleen. All mice were sacrificed 3 days after the last boost. The splenocytes were fused immediately with myeloma cells SP20 by using polyethylene glycol-1000 as fusion reagent. The hybridoma cells were cultured in DMEM-F12-Glutamax medium supplemented with 10% horse serum (Gibco), 0.1 mmol/l hypoxanthine, 0.5% azaserine, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin in 96-well plates, until the colonies of hybridoma cells were grown. Initially, the hybridoma cells were diluted in round-bottom 96-well plates by limiting dilutions to obtain 0.3 cell per well and cultured for 7 days.

### Specificity of monoclonal antibodies by enzyme-linked immunosorbent assay

The specificity and the reactivity of hybridomas were measured by enzyme-linked immunosorbent assay (ELISA) against HEK 293-gp41<sup>MSD</sup> cell line to identify the specific antibody-producing clones. Briefly, the HEK 293-gp41<sup>MSD</sup> cells were cultured for 2 days in 96-well plates and fixed in cold acetone. Then, hybridoma supernatants were harvested after 6 days, and then incubated for 30 min on fixed HEK 293gp41<sup>MSD</sup> cells. Antibody binding was then revealed with HRP-conjugated sheep antimouse IgG (Biosys) at a 1:200 dilution in PBS for 30 min at 37°C. The substrate o-phenylenediamine di-hydrochloride (OPD) substrate (Sigma) was added to the plate. Optical density was measured at 492 nm. Forty clones were selected, exhibiting a strong reactivity against the cell line. Positive clones showing neutralization activity were expanded using a high density culture system (Integra cell line CL1000; Integra Biosciences; Abbott Lab., Abbott Park,

Illinois, USA) [47]. After sodium sulfate precipitation, the mAbs were purified by anion exchange chromatography on a Hyper-D column (Sepracar, France).

### Analysis of the monoclonal antibodies specificity by flow cytometry

To test the binding specificity of the generated antibodies, we used flow cytometry to analyze FC-1, FC-2 and FC-3 specificity against HEK 293-gp41<sup>MSD</sup> cell line. Cells were separated from the culture flasks using versene buffer (GIBCO, Grand Island, New York, USA). The cells were washed twice with RPMI 1640 without phenol red containing 5% horse serum and 0.1% sodium azide (FACs buffer). Gp41 expressed cells ( $1 \times 10^6$ ) were incubated with 100  $\mu$ l of the three generated antibodies at 15  $\mu$ g/ml for 30 min at 4°C. Cells were washed and incubated with 100  $\mu$ l FITC-labeled goat antimouse IgG (diluted 1:10.000 in FACs buffer) for 30 min at 4°C. Subsequent to washing, the cells were analyzed on a FACScan flow cytometer (Becton Dickinson, Pont de Claix, France).

### Virus neutralization assays

Measure of the neutralizing activity of purified gp41-specific antibodies 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 (93BR025) and D (92UG001). Neutralizing activities were tested in duplicate and repeated three times.

For LAI assay, CD4<sup>+</sup>/CXCR4<sup>+</sup> SupT1 cells were incubated at 37°C under 5% CO<sub>2</sub> in DMEM medium supplemented with 10% FCS. Infection was performed in 96-well rounded bottom plates. Purified antibodies or hybridoma supernatants (50  $\mu$ l) in the case of the primary screening were incubated with an equal volume of virus containing a 100 TCID<sub>50</sub> dose for 2 h at 37°C. Then,  $3 \times 10^5$ /well of SupT1 cells were added. The next day, infected cells were washed twice with tissue culture medium. Supernatants were collected 7 days postinfection and p24 ELISA measurement was performed (HIV-1 p24 Antigen Capture Assay; Advanced Bioscience Laboratories, Inc. USA).

For the BaL strain, neutralizing activity of purified antibodies was determined as previously described [36]. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors (generous gift from EFS Auvergne Loire, France) and stimulated with phytohaemagglutinin (5  $\mu$ g/ml) and 200 U/ml of recombinant human interleukin-2 (Abcys, Paris, France). The cells were infected with a 100 TCID<sub>50</sub> dose for 2 h at 37°C in the presence of purified antibodies and after 48 h, the cells were washed twice. Infected supernatants were collected 7 days postinfection and ELISA p24 measurement was performed.

Neutralizing activity of purified antibodies was also tested on stimulated PBMC infected with 92UG029, 92US660,

92BR025, 93BR025, HIV-1 G3 and 92UG001 primary HIV-1 isolates as previously described. The cells were infected with a 100 TCID<sub>50</sub> dose in the presence of serially diluted antibody. The supernatants were collected 14 days postinfection and p24 measurement was performed. In the particular case of the primary HIV-1 isolates SF162 and QHO, neutralizing activity was measured as previously described [48,49].

The percentage of neutralization was calculated as the reduction of p24 production (for BaL 92UG029, 92US660, 92BR025, 93BR025, HIV-1 G3 and 92UG001) or intracellular p24 staining (for SF162 and QHO) compared with the control value of p24 obtained in the absence of antibody. IC<sub>50</sub> and IC<sub>80</sub> were determined as the antibody concentration able to confer 50 and 80% of neutralization, respectively.

### Epitope mapping of monoclonal antibodies

ELISA was carried out using the SHIV 89.6P (subtype B) env 20-mer peptides complete set that cover and overlap the gp41. Briefly, each peptide was coated on a 96-well plate (Maxisorp, Nunc) at 5  $\mu$ g/ml in 0.1 mol/l sodium carbonate buffer (pH 9.6) overnight at 4°C. The plates were washed with tris-buffered saline (TBS; 144 mmol/l NaCl, 25 mmol/l Tris -HCL, pH 7.5) containing 0.5% tween 20 and blocked with the same buffer and 2% BSA for 1 h at 37°C. After washing, 100  $\mu$ l of the generated antibodies (5  $\mu$ g/ml) diluted in TBS were added to the wells and incubated 2 h at 37°C. After washing, the detection was done by using horseradish peroxidase HRP-conjugated goat antimouse IgG and OPD substrate. The measurement of absorbance was read at 492 nm. All assays were performed in duplicate (Multiskan Microplate Photometer, thermo scientific).

### Competition assays for antibody epitope mapping

With  $1 \times 10^6$  cells HEK 293-gp41<sup>MSD</sup> were first incubated with FC-1, FC-2, FC-3, 2F5, 4E10, D5 or NC-1 unlabeled antibodies at different concentrations (500, 250, 125 or 50  $\mu$ g/ml) during 30 min at room temperature. After washing, cells were then incubated with Alexa647 labeled FC-1, FC-2 or FC-3 for 30 min at room temperature. FC-1, FC-2 and FC-3 labeling was done accordingly the manufacturer instructions as described by Molecular Probes (Alexa Fluor R 647 Protein Labeling Kit, InVitroGen). After washing, cells were then analysed on a FACScan flow cytometer (Becton Dickinson, Pont de Claix, France).

### HIV Envelope-mediated cell-cell fusion assay

The  $4 \times 10^5$  chinese hamster ovary (CHO) cells (target cells) stably transfected with a HIV-1 HXB2 Env expressing vector pEE 14 (CHO-WT; Env cells) were added in each well of 24-well culture plate in glutamine-deficient minima essential medium containing 400  $\mu$ mol/l methionine sulfoximine (Gibco). Purified monoclonal

antibodies were then added at different concentrations into the wells and mixed adequately. After 4 h incubation at 37°C under 5% CO<sub>2</sub>, 4 × 10<sup>5</sup> HeLa CD4 cells were added to each well. After 22 h incubation at 37°C under 5% CO<sub>2</sub>, syncytia were stained by Giemsa (Sigma) and counted under a microscope. The number of syncytia was calculated at five different fields in a well.

### Inhibition of fusion complex formation

The proteins HUG037H2 (92UG037 clade A) and HTH22H2 (92TH022 clade EA) representing the HR2 region and the protein HBR025H1 (92BR025 clade C) representing the HR1 region were produced in *E. coli* and purified as previously described [36]. To measure the ability of mAbs to block HR1/HR2 complexes formation, a specific ELISA assay was developed. A Ni-NTA HisSorb plate (Qiagen) was coated with purified HR2 (500 µg/ml) at room temperature for 2 h. Purified HR1 (200 µg/ml) was mixed with the three mAbs (10 µg/ml) or with an irrelevant anti-OVA mAb at 37°C for 45 min. The mixture was then added to wells followed by adding D5 anti-gp41 and horseradish peroxidase HRP-conjugated goat antihuman IgG and OPD substrate sequentially. The optical density was measured at 492 nm.

### Statistical analysis

The statistical difference in in-vitro experiments between control and antibodies was assessed using Student's *t* test (Statistica 5.1 software; Statsoft Inc., Maison-Alfort, France). A *P* value at least 0.05 has been considered statistically significant.

## Results

In order to induce gp41-specific neutralizing antibody, we have decided to generate a potent conformational immunogen based on a human cell line that allowed the expression of a natural conformational membrane-bound gp41 as present in viral particles.

### Construction and characterization of the human HEK 293-gp41<sup>MSD</sup> cell line

The HR1-PID-HR2 was amplified on the plasmid pSVIII-92BR025.9 with specific primers. The HR1-PID-HR2 fragment encodes a fragment of 516 base pairs or 172 amino acid residues that has been cloned in frame into pDisplay vector and verified by DNA sequencing (Fig. 1a). Recombinant plasmid was then used to transfect the human HEK 293 cells as described in the methods section. After selection, a clonal dilution was realized at 0.5 cell/well and more than 50 individual clones were analyzed for transmembrane gp41 expression. One clone has been selected on the basis of its stability and the high level of gp41 expression both on the surface and in the cytoplasmic content of the HEK 293-gp41 transfected

cells by immunofluorescence (Fig. 1b). Gp41 transmembrane expression was confirmed by specific immunolabeling of HEK 293-gp41<sup>MSD</sup> cells with the well known gp41-specific IgG (Z13, 2F5 and 5F3) but not with a well known gp120-specific antibody b12 as shown in Fig. 1c. All the three antibodies recognized very specifically the HEK 293-gp41<sup>MSD</sup> transfected cells as compared to untransfected cells.

To evaluate the formation of the gp41 postfusion complex on the HEK 293-gp41<sup>MSD</sup> cells, NC-1 and D5 mAbs specific of conformational epitopes were used (Fig. 1d). We observed that almost all the HEK 293-gp41<sup>MSD</sup> cells were stained with NC-1 specific for the six-helix bundle postfusion conformation. Only, 19% of cells were stained with D5 mAb, which indicates that the HR1 epitope is weakly accessible and that the expressed fusion complex might be an intermediate conformation.

### Specificity of the three monoclonal antibodies by flow cytometry and ELISA

The HEK 293-gp41<sup>MSD</sup> cells were used to immunize BALB/C mice as described in the method section. Immunizations were made in presence of Freund's adjuvant to optimize antigen presentation. The micelle structure obtained by performing a stable emulsion between adjuvant and cellular proteins from transfected cells allows the immune system to present a higher number of protein epitopes than most other adjuvants. The presence of plasma membranes of *Mycobacterium tuberculosis* in adjuvant allows the recruitment of competent dendritic cells at the injection site so far superior to all the compounds in the proposed unit.

Three successive immunizations were made and more than 200 hybridoma clones were obtained. Sera from immunized mice were shown to exhibit specific binding to HEK 293-gp41<sup>MSD</sup> cells and not to untransfected cells and potent neutralizing activity on the LAI HIV-1 strain (Supplementary Figures 1A and 1B, <http://links.lww.com/QAD/A292>). However, this neutralizing activity remains very low. Hybridomas were tested for their immunoreactivity against HEK 293 or HEK 293-gp41<sup>MSD</sup> cells. Culture supernatants of confluent cells were analyzed by immunofluorescence. Among the 200 clones, 40 exhibited a strong specific reactivity against the HEK 293-gp41<sup>MSD</sup> cells. The other clones were all specific for untransfected cells revealing the strong immunogenicity of HEK 293 in mouse.

Supernatants of these 40 clones were screened for their neutralizing activity in a neutralizing LAI/SupT1 assay. Among the 40 clones, only three (referred as FC-1, FC-2 and FC-3) exhibited a strong neutralizing activity *in vitro* (more than 80% neutralization with supernatant diluted to 1/4), neutralizing activity of the other 37 clones being less than 80%.

The three clones were amplified and produced at high density to obtain high amount of each specific antibody. Hybridoma supernatants were pooled and used to purify specific IgG on a Hyper-D column. Purity of the antibody was estimated higher than 95% (Supplementary File 2, <http://links.lww.com/QAD/A292>). The production rate of each individual antibody has been quantified around 10 µg/ml of hybridoma supernatant.

Specificity of the mAbs was tested by both flow cytometry analysis on HEK 293-gp41<sup>MSD</sup> and specific ELISA assay. A concentration of 5 µg/ml of mAbs allowed the detection of gp41 on the cell surface of HEK 293-gp41<sup>MSD</sup> with a great specificity (Fig. 1e). FC-2 and FC-3 seems to recognize better gp41 conformation at the HEK 293-gp41<sup>MSD</sup> cell surface. None of the three clones recognize untransfected cells (Fig. 1e). The specificity of the three mAbs by cell-coated ELISA with the HEK 293-gp41<sup>MSD</sup> cells was also confirmed. Again, FC-2 and FC-3 seem to have a better affinity (data not shown).

### Mapping of binding sites of the three monoclonal antibodies

Mapping of the specific epitopes of each mAb was determined by ELISA with a SHIV 89.6P (subtype B) env peptides complete set (Table 1). Binding of each mAb was also tested on recombinant gp140 from Clade C/B (CN54) produced in CHO as positive control (Table 1). FC-1 recognized a linear epitope located in the HR2 region (peptide 4767; AA 671–690; FDITNWLWYIRLFIMIVGGL), whereas FC-2 and FC-3 bound specifically to a conformational epitope formed by the HR1 region (peptide 4753; AA 531–560; ASVTLTV-QARLLLSGIVQQQ) and to the same fragment of the HR2 recognized by FC-1 (peptide 4767; AA 623–661; FDITNWLWYIRLFIMIVGGL).

In an attempt to further identify the ability of the three mAbs to detect the spatial structure of the HR1/HR2 fusion complex, we measured the capacity of these antibodies to specifically recognize different fusion complexes generated *in vitro*. We used the N36/C34 and the complex formed by mixing the two well recognized peptides 4753/4767 as a fusion complex model (Table 1). The formation of the complexes has been verified by ELISA with D5 and NC-1 antibodies. FC-2 and FC-3 bound with a great efficiency to the complex formed with 4753/4767 peptides and to a reduced level with other constructed complexes based on HR1 and HR2 domains produced in *E. coli* (data not shown). No binding of the different mAbs was observed with the N36/C34 complex. Absence of binding to N36/C34 complex is probably due to the absence of the 13 residues of the reactive 4753 peptide in N36 sequence. According to the mapping, the C34 sequence was not included in the C-Term region recognized by FC-2 and FC-3. A possible correspondence between the reactivity of the individual peptide 4753 and the absence of N36/

C34 recognition could suggest that the SGIVQQQ sequence is contained in the HR1 epitope part of FC-2 and FC-3. FC-1 seems to recognize a linear epitope located in HR1.

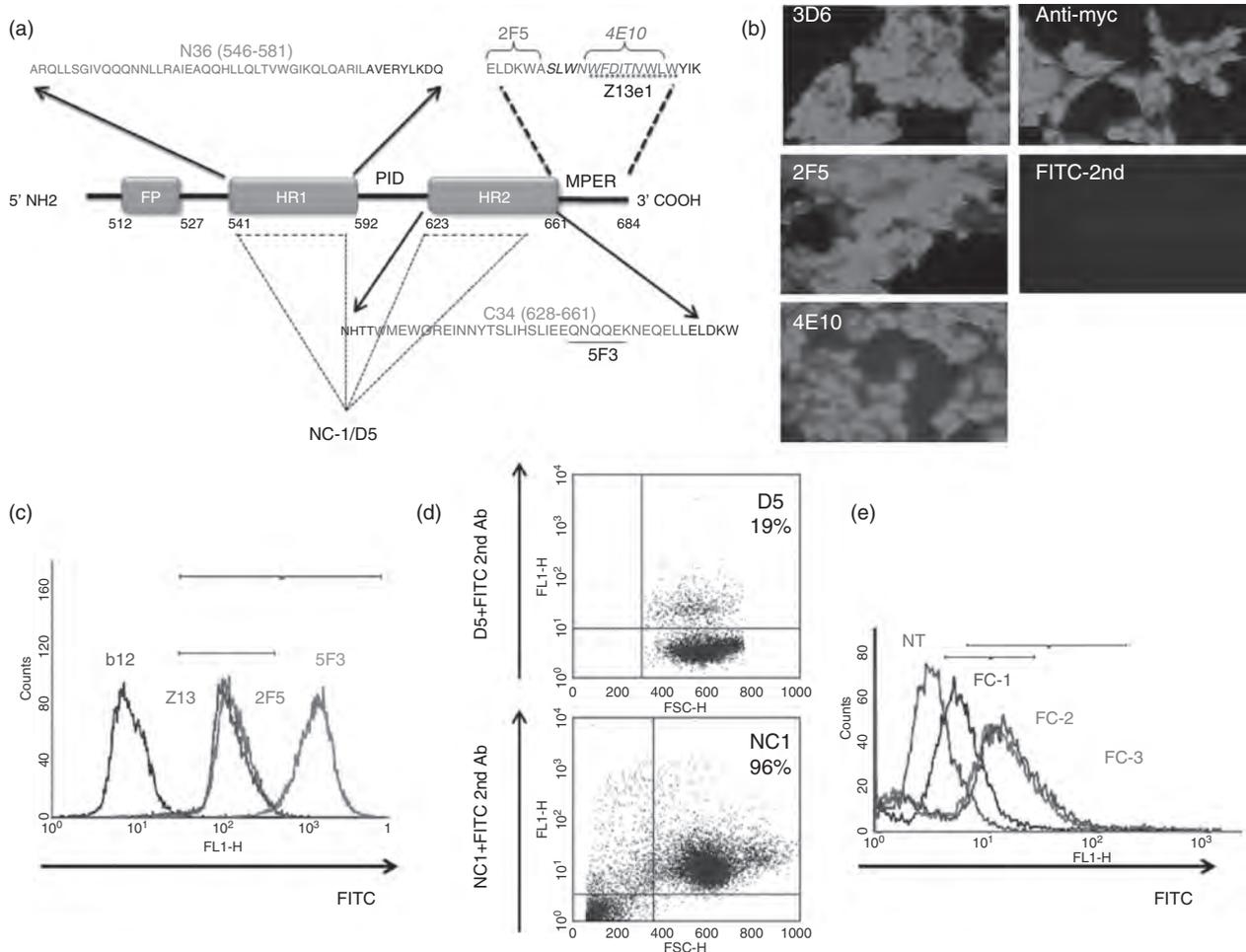
### Competition assays for antibody epitope mapping

Epitopes for FC-1, FC-2 and FC-3 were also studied by competition experiments on HEK 293-gp41<sup>MSD</sup> cell line. Briefly, saturating concentrations of different gp41-specific antibodies were used before labeling of cells with Alexa647 labeled FC-1, FC-2 or FC-3 (Fig. 2). These experiments indicate first that the epitopes of FC-1, FC-2 and FC-3 are different that those described for 2F5, 4E10, D5 or NC-1. The epitopes are certainly conformational in the view of the heterogeneous inhibition observed. Epitope of FC-1 has been mapped in HR2 and none of the antibodies tested is capable of blocking its binding to HEK 293-gp41<sup>MSD</sup> cell line (Fig. 2a). FC-2 epitope is very close from FC-3 and 4E10 epitopes (Fig. 2b). FC-3 epitope seems to be conformational and encompass a region containing FC-2, 2F5 and 4E10 epitopes probably MPER. FC-2 and mainly FC-3 epitopes seems to be masked by NC-1 that is specific for the six-helix bundle postfusion conformation. Thus, indicate the capacity of FC-2 and FC-3 to block the fusion complex formation. Any competition was observed with D5 specific for the HR1 region.

### Virus neutralization of the three monoclonal antibodies against primary HIV-1 isolates from different clades

To investigate the neutralizing effect of the three antibodies, we first confirmed the neutralizing activity against the TCLA strain LAI. The clones FC-1, FC-2 and FC-3 are able to efficiently neutralize at 80% the LAI strain at 0.78, 12.5 and 1.5 µg/ml, respectively. Next, the 3 mAbs were also analyzed for their ability to neutralize primary HIV-1 isolates of clade A (92UG029), clade B (BaL, SF162, QHO692 and 92US660), clade C (92BR025) and clade D (92UG001). The overall results of neutralization (IC<sub>50</sub> and IC<sub>90</sub>) are summarized in Table 2. Neutralization curves obtained for the representative primary isolates 92BR025, 92UG001, 92US660 and 92UG029 are described in Fig. 3 as examples of neutralizing or none-neutralizing strains.

The three antibodies were able to efficiently neutralize BaL, SF162, 92UG029, 92UG001 93BR025, HIV-1 G3 and 92BR025 primary isolates with IC<sub>90</sub> concentrations ranging between 0.6 and 100 µg/ml. FC-2 was the more potent of the three Nabs regarding IC<sub>90</sub> ( $P < 0.05$ ) but also as it is the only able to neutralize efficiently the Tier 2 primary isolate QHO692. None of them were able to decrease replication of 92US660 virus replication by 80%. As expected by the use of clade C gp41 expressing cell line, the three clones were able to neutralize the 92BR025 clade C strain. These data indicate that two of the three



**Fig. 1. Construction and validation of the HEK 293-gp41<sup>MSD</sup> cell line.** (a) Schematic representation of the cloned HIV-1 gp41 domain (HR1-PID-HR2) in pDisplay vector. The amino-acid residues and their position number are indicated. Epitopes for 2F5, 4E10, Z13, 5F3, D5 and NC-1 are described with the N36 entire sequence. (b) Transmembrane HR1-PID-HR2 expression at the HEK 293-gp41<sup>MSD</sup> cell surface was detected by immunofluorescence with human anti-gp41 3D6, 2F5, 4E10 followed by FITC-2nd antibody, with FITC antimyc or with FITC-2nd antibody alone. (c) Histogram generated by flow cytometry analysis shows the detection of the gp41 at the cell membrane surface of HEK 293-gp41<sup>MSD</sup> cell line using anti-gp41 antibodies Z13, 2F5, 5F3 followed by FITC-2nd antibody. Labeling of HEK 293-gp41<sup>MSD</sup> cell line with the gp120-specific antibody b12 antibody was used as negative control. (d) Measure of the binding of NC-1 or D5 mAb followed by FITC-2nd antibody to HEK 293-gp41<sup>MSD</sup> by flow cytometry analysis. The upper right quadrant represents the percentage of NC1<sup>+</sup> or D5<sup>+</sup> HEK 293-gp41<sup>MSD</sup> cells. Labelling of HEK 293-gp41<sup>MSD</sup> cell line with the gp120-specific antibody b12 was used as negative control (data not shown). (e) Specificity of FC-1, FC-2 and FC-3 by flow cytometry analysis on HEK 293-gp41<sup>MSD</sup> cell line. Abs staining was revealed with an antimouse IgG secondary antibody coupled with FITC. Staining of untransfected cells (NT) with a mix of FC-1, FC-2 and FC-3 was used as negative control. FP, fusion peptide; HR1, heptad repeat region 1; PID, principal immunodominant region; HR2, heptad repeat region 2; MPER, membrane proximal external region.

monoclonal antibodies inhibit a broad spectrum of clade neutralization ( $P < 0.05$ ). In comparison with the well known gp41-specific 2F5 or 4E10 also tested in these experiments, neutralizing activity of FC-2 and FC-3 are significantly ( $P < 0.05$ ) higher on primary isolates from clade A, B, C, D and G. Moreover, comparison with the two well known fusion complex specific antibodies D5 and NC-1 indicate that FC-2 and FC-3 have a significantly higher neutralizing activities ( $P < 0.05$ ).

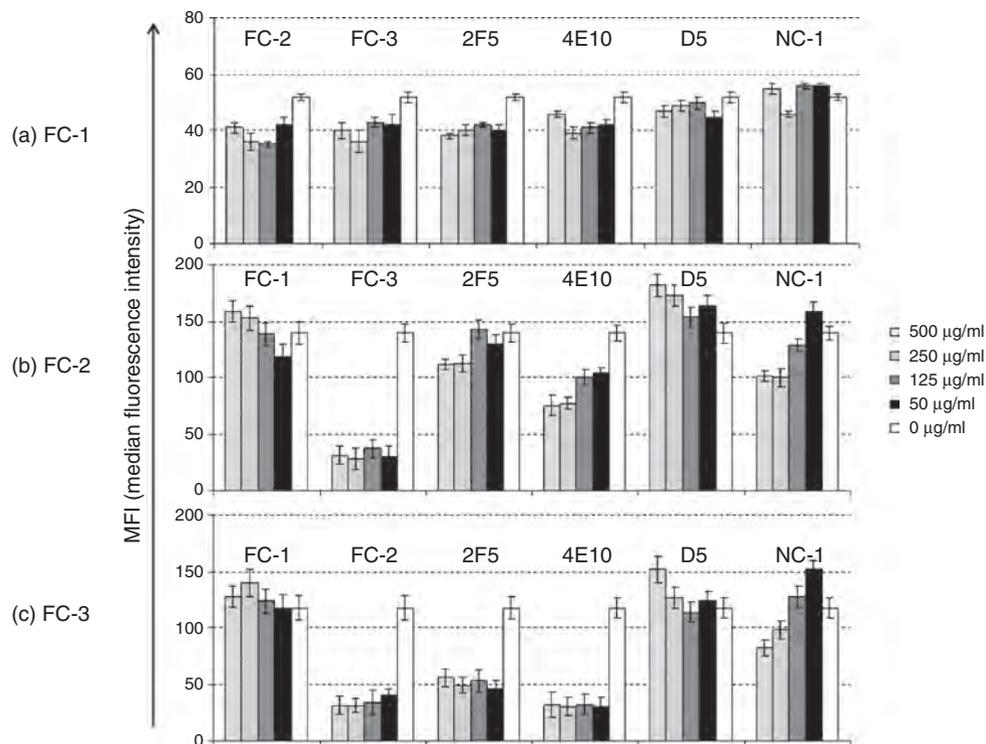
### Cell-cell fusion inhibition

Interestingly, all three mAbs could inhibit HIV-1 Env-mediated membrane fusion of CHO-WT and Hela CD4 at a 10–15  $\mu\text{g/ml}$  level (Fig. 4). FC-1 inhibited significantly syncytia formation by 40%, FC-2 by 70% and FC-3 by 60% when syncytia were counted under microscope (Fig. 4b). T20 that blocks the gp41-dependent virus-cell fusion, was used as a control in the same cell fusion system [50]. T20 showed a complete inhibition of syncytium formation at 2.5  $\mu\text{g/ml}$ .

**Table 1. Epitope mapping by ELISA of the three clones using the SHIV 89.6P envelope library.**

Peptide Name	Amino acid position and sequence	Reactivity (optical density)				
		FC-1	FC-2	FC-3	D5	NC-1
4753 (FPPR; SHIV 89.6P)	531–550 ASVTLTVQARLLLSGIVQQQ	nb	0.76 ± 0.04	0.45 ± 0.01	nt	nt
4754 (HR1; SHIV 89.6P)	541–560 LLLSGIVQQQNLLRAIEAQ	nb	nb	nb	nt	nt
4755 (HR1; SHIV 89.6P)	551–570 NNLLRAIEAQNNMLRLTVWG	nb	nb	nb	nt	nt
4756 (HR1; SHIV 89.6P)	561–580 QNMLRLTVWGKQLQARVLA	nb	nb	nb	nt	nt
4757 (HR1; SHIV 89.6P)	571–590 IKQLQARVLALERYLRDQQL	nb	nb	nb	nt	nt
4758 (HR1; SHIV 89.6P)	581–600 LERYLRDQQLMGIVGCSGKL	nb	nb	nb	nt	nt
4759 (PID; SHIV 89.6P)	591–610 MGIWGCSSGKLICTTSVPWNV	nb	nb	nb	nt	nt
4760 (PID; SHIV 89.6P)	601–620 ICTTSVPWNVSWNSKSVDDI	nb	nb	nb	nt	nt
4761 (PID; SHIV 89.6P)	611–630 SWSNKSVDIWNMTWMEWE	nb	nb	nb	nt	nt
4762 (HR2; SHIV 89.6P)	621–640 WNNMTWMEWEREIDNYTDYI	nb	nb	nb	nt	nt
4763 (HR2; SHIV 89.6P)	631–650 REIDNYTDYIYDLLEKSQTQ	nb	nb	nb	nt	nt
4764 (HR2; SHIV 89.6P)	641–660 YDLLEKSQTQEQEKNEKELLE	nb	nb	nb	nt	nt
4765 (MPER; SHIV 89.6P)	651–670 QEKNEKELLELDKASLWNW	nb	nb	nb	nt	nt
4766 (MPER; SHIV 89.6P)	661–680 LDKWASLWNWFDITNWLWYI	nb	nb	nb	nt	nt
4767 (MPER; SHIV 89.6P)	671–690 FDITNWLWYIRLFIMIVGGL	0.47 ± 0.02	0.91 ± 0.03	0.68 ± 0.01	nt	nt
4768 (MPER; SHIV89.6P)	681–700 RLFIMIVGGLIGLRIVFAVL	nb	nb	nb	nt	nt
4769 (MPER; SHIV89.6P)	691–710 IGLRIVFAVLSIVNRVRQGY	nb	nb	nb	nt	nt
N36/C34 complex	546–581 SGIVQQQNLLRAIEAQQ HLLQLTVWGKQLQARI 628–661 WMEWDREINNYTSLIHSLI EESQNQQEKNEQELL	nb	nb	nb	0.81 ± 0.04	0.64 ± 0.09
4753/4767 complex	531–550 ASVTLTVQARLLLSGIVQQQ 671–690 FDITNWLWYIRLFIMIVGGL	nb	1.1 ± 0.1	0.94 ± 0.08	0.59 ± 0.05	0.64 ± 0.08
Gp140 (CN54; clade B/C)		nb	0.89 ± 0.01	0.95 ± 0.01	0.58 ± 0.1	0.74 ± 0.07
Gp41 (HXB2; clade B)		0.66 ± 0.046	0.885 ± 0,09	1.245 ± 0,11	0.783 ± 0,08	1.01 ± 0,07

Each individual peptide tested is mentioned. Recombinant gp140 and N36/C34 complex have been also tested. Data represent mean values ±SD of three determinations. Optical density values were all corrected for nonspecific binding in blank controls. nb, no detectable binding; nt, not tested.

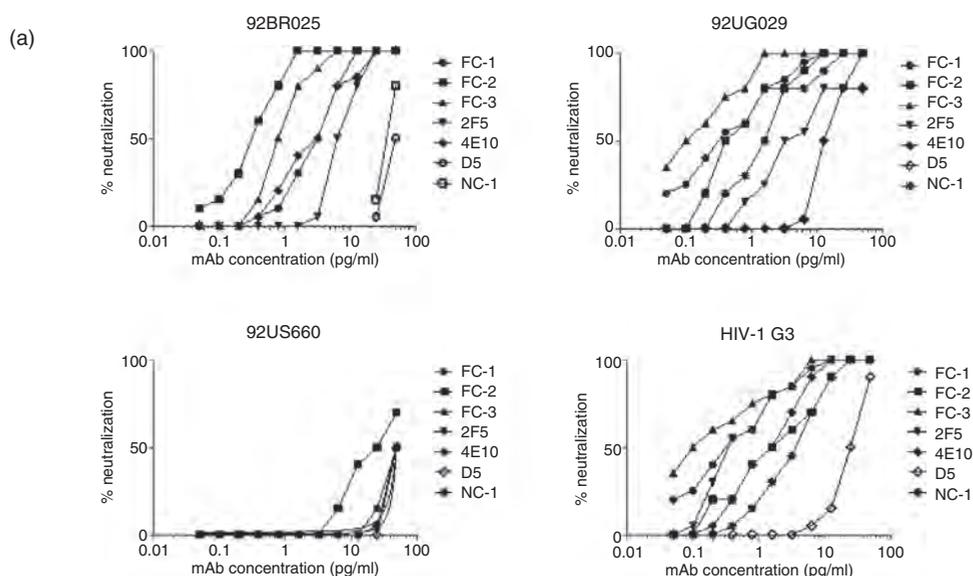


**Fig. 2. Epitope mapping of FC-1, FC-2 and FC-3 by competition assay on HEK 293-gp41<sup>MSD</sup> cell line.** Cells were incubated with saturating concentration of different unlabeled antibodies against different regions of gp41 (FC-1, FC-2, FC-3, 2F5, 4E10, D5 and NC-1) and then revealed with Alexa647 labeled FC-1 (a), FC-2 (b) and FC-3 (c). The values of median fluorescence intensity (MFI) were monitored by flow cytometry and reported on histograms.

**Table 2. Neutralizing activities of FC-1, FC-2 and FC-3 on laboratory strains and primary HIV-1 strains.**

Strain (Tropism, Clade)	[NAbs] for IC <sub>90</sub> /IC <sub>50</sub> (μg/ml)													
	FC-1		FC-2		FC-3		D5	NC-1	4E10		2F5			
LAI (CXCR4; B)	0.78	0.16	12.5	3.1	1.5	0.31	>100	25	>100	25	6.25	3.1	12.5	6.25
BAL (CCR5; B)	12.5	3.1	12.5	6.25	25	12.5	n.d.	n.d.	n.d.	n.d.	25	12.5	50	25
92UG029 (CXCR4; A)	1.5	0.31	1.5	0.31	0.78	0.16	50	12.5	12.5	3.1	50	12.5	25	6.25
SF162 (CCR5; B)	100	25	10	1.5	100	12.5	>100	50	>100	50	>100	50	>100	50
QHO692 (CCR5; B)	>100	10	100	10	>100	100	n.d.	n.d.	n.d.	n.d.	>100	25	>100	50
92US660 (CCR5; B)	>100	50	>100	25	>100	50	>100	50	>100	12.5	>100	50	>100	50
93BR025 (CCR5; B)	3.1	1.5	1.5	0.78	0.78	<0.78	n.d.	n.d.	n.d.	n.d.	3.1	0.78	3.1	1.5
92BR025 (CCR5; C)	6.25	3.1	0.78	<0.78	1.5	0.78	>100	25	>100	12.5	6.25	3.1	12.5	6.25
92UG001 (CCR5/CXCR4; D)	3.1	0.78	3.1	0.31	0.78	0.06	25	12.5	12.5	6.25	25	12.5	12.5	3.1
HIV-1 G3 (CCR5; G)	12.5	3.1	12.5	3.1	6.25	1.5	50	25	12.5	3.1	6.25	3.1	12.5	6.25

The table contains the antibodies concentration in μg/ml of the three purified mAbs used to obtain a neutralization of HIV infection ([NAbs]) at 50% or 90% as described in the method section. D5, NC-1, 4E10 and 2F5 mAbs were used as controls. (n.d. not done). The MAb neutralization titers have been color-coded as follows: numbers with a white background indicate an IC<sub>90</sub> of >12.5 μg/ml, numbers with a medium grey background indicate 3.1 μg/ml > IC<sub>90</sub> >12.5 μg/ml and numbers with a dark grey background indicate an IC<sub>90</sub> of < 3.1 μg/ml.



	Number of syncytia / field	% of inhibition
FC-1	31, 35, 33, 38, 28	40±5 ( <i>P</i> < 0.05)
FC-2	25, 13, 17, 15, 16	70±10 ( <i>P</i> < 0.05)
FC-3	24, 20, 32, 10, 23	60±10 ( <i>P</i> < 0.05)
T20	0, 0, 0, 2, 10	100±10 ( <i>P</i> < 0.05)
untreated	49, 51, 67, 55, 53	0

**Fig. 3. In-vitro biological activities of FC-1, FC-2 and FC-3.** (a) Neutralizing activity of FC-1, FC-2 and FC-3 against four representative primary isolates 92BR025, 92US660, 92UG029 and HIV-1 G3. These mAb were tested for dose-dependent neutralization within a concentration range of 50–0.06 μg/ml, according to the neutralization assay protocol described in Methods. For each mAb and virus combination, the neutralization from one experiment, out of three repeated experiments, is shown. (b) Inhibition of the HIV-1 Env mediated syncytium formation between Hela CD4 and HIV-1 HXB2 Env-transfected CHO cells (CHO-WT) by FC-1, FC-2 and FC-3 at 10 μg/ml. T20 has been used as positive control of inhibition at 2,5 μg/ml. Table containing the number of syncytia counted per field for each condition and the median of the % of cell to cell fusion inhibition. Each experiment has been repeated five times with similar results, *P* values calculated by a Student *t* test between untreated group and inhibitory conditions are indicated.

## Blocking of heptad repeat regions 1 and 2 complex formation

We have developed a convenient method for detecting the formation of the gp41 fusion complex to study the inhibitory activity of mAbs. To evaluate the in-vitro capacity of the three mAbs to interfere with the fusion complex formation, we have used two highly stable complex models composed of recombinant HR1 (clade C) and HR2 (clade A and EA) domains produced in *E. coli* and available in the laboratory. These complexes are associated as dimer or trimer (data not shown) and are well recognized by D5 and also NC1 mAbs specific of the fusion complex (Fig. a and b, respectively) indicating the correct folding of the six helix bundle core of the HIV-1gp41. Moreover, preincubation of the two complexes with D5 or NC-1 mAb does not alter complex recognition by FC-2 and FC-3 but reduce binding of FC-1 (data not shown).

We demonstrated that FC-1, FC-2 and FC-3 blocked efficiently and specifically two different heterologous HR1/HR2 trimeric complexes formation within 55, 74 and 79% for HBR025H1/HUG037H2 and 70, 85 and 95% for HBR025H1/HTH22H2, respectively (Fig. c and d). The inhibitions were highly significant for FC-2 and FC-3 in comparison to FC-1 and 2F5. No inhibition was measured with the irrelevant murine IgG2. These results suggest that the three mAbs bound to HR1 or HR2 protein and interfere with the association between HR1 and HR2 regions.

## Discussion

HIV-1 envelope glycoprotein is one the major target for humoral immune response in viral infection. Neutralizing antibodies were mainly directed against trimeric form of the envelop glycoprotein. Recent results report the presence of neutralizing antibodies directed against quaternary epitopes that are exposed on the YU2 gp140-folden trimer or directed against gp41 [5]. The high degree of variation in gp120 sequence and of glycosylation coupled with the highly conserved sequence of gp41 has long rendered gp41 an attractive target for inhibitors and NAb [32,51].

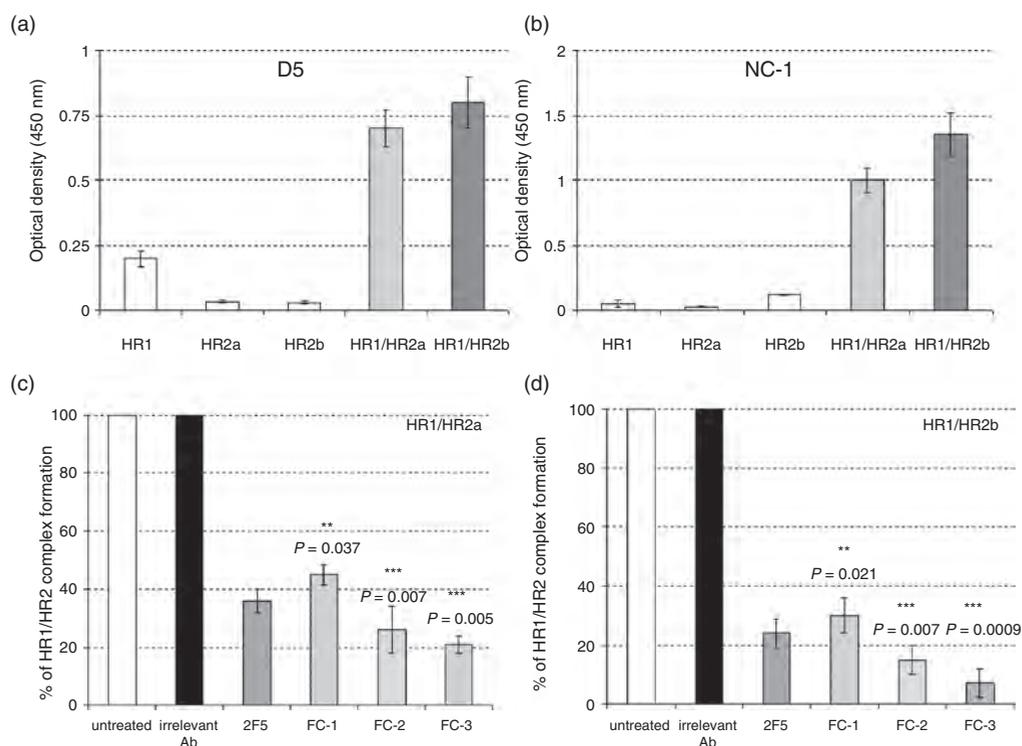
Entry inhibitors are a new class of drugs for the treatment of HIV infection. Enfuvirtide (T20) is the first compound of this family to be approved for clinical use [52]. T20 is a synthetic peptide that mimics an HR2 fragment of gp41, blocking the formation of a six-helix bundle structure that is critical in the fusion process. This compound is a potent therapeutic option as rescue therapy in combination with other active antiretroviral drugs against different HIV-1 variants, including all group M subtypes and group O [53]. The main mechanism of resistance to enfuvirtide depends on the selection of changes in a ten

amino-acid domain between residues 36–45 in the HR1 region of gp41. Single and double mutations in this region have been shown to result in high-level resistance to enfuvirtide [54,55].

Gp41 is highly immunogenic and elicits antibodies in almost all HIV-1-infected individuals. These titers are 25-fold to 625-fold higher than that of anti-gp120 antibody [56]. In addition, gp41 differs from gp120 in that all regions of the protein appear to be targeted by the human antibodies [57]. MAb directed against gp41 region may neutralize viruses by blocking the formation of the fusion complex with target cells during infection [32,58,59]. Several broadly neutralizing HIV-1 monoclonal antibodies that bind to gp41 have been already described as 4E10, 2F5 or more recently 10E8 [1–3]. The majority of gp41-specific antibodies are mainly targeted to the MPER region.

The heptad repeat regions HR1 and HR2 of HIV-1 gp41 can be associated to form hetero-oligomers through helical coiled-coil interactions that are believed to play a key role in virus-induced membrane fusion. The HR1/HR2 complex was proposed to be the core structure of the fusion-active conformation of gp41. The high degree of conservation in the gp41 N-HR and C-HR sequences among HIV-1 strains of different clades makes these structures potential targets for inhibition. Antibodies that are able to interact with the core of the putative fusion-active gp41 may be useful in further unveiling the mechanism of HIV-induced membrane fusion [60]. Peptides representing these repeats are potent inhibitors of HIV infection [44]. They appear to block virus entry by mimicking helices of the six-helix bundle and forming a peptide-gp41 complex, which interferes with the formation of the gp41 six-helix bundles in a dominant negative manner. An earlier study concluded that six-helix bundles can form prior to membrane fusion and that the prehairpin fusion intermediates can be accessible to antibodies [45]. The formation of the HR1-PID-HR2-TM viral structure was required because it is only transiently exposed during fusion of the virus with the host cell. Indeed, for this reason, neutralizing antibodies targeting this region are rarely elicited by natural infection or immunization. Some studies also indicate that such type of antibodies are autoimmune antibodies which normally cannot be induced in healthy individual. However, a very recent study on the neutralizing potential of gp41 specific maternal antibodies and their correlation with HIV transmission in HIV-infected mother-child pairs highlight important epitopes in gp41 (HR1 and MPER) that appear to be associated to protection [61].

To generate such type of antibodies specific to the six-helix bundle structure present in the gp41 postfusion conformation, a stable human cell line expressing HR1-PID-HR2 gp41 domain from a clade C primary strain at



**Fig. 4. Inhibition of HR1/HR2 complex formation by FC-1, FC-2 and FC-3.** Recognition by ELISA of two distinct fusion complexes obtained by mixing of HBR025H1 (HR1; 92BR025) and HUG037H2 (HR2a; 92UG037) or HTH22H2 (HR2b; 92TH022) with D5 (a) and NC1 (b) mAb. Inhibition of the two HR1/HR2 complex formations (c and d) using FC-1, FC-2 FC-3, 2F5 or mouse IgG2 as an irrelevant mAb (10  $\mu$ g/ml). Complex formation was revealed with D5 antibody. Each experiment has been repeated three times with similar results. The *P* values are indicated.

the cell surface has been produced. Clade C strain has been chosen as a representative of most currently circulating HIV-1 viruses. These cells have been used to immunize mice and to generate hybridomas producing gp41-specific antibodies. Antibodies were screened for their ability to inhibit the LAI adapted T-cell virus in the SupT1 model. Although this type of screening could result in a bias toward selection of Abs neutralizing TCLA strains only, the three mAbs selected in this study (FC-1, FC-2 and FC-3) also neutralize at least seven of the eight primary isolates tested using conventional PBMC neutralization assay. With this assay, the IC<sub>90</sub> of two fusion complex specific antibodies D5 and NC1 (non neutralizing antibody) and 2F5 and 4E10 was often observed at concentrations higher than 20  $\mu$ g/ml [37,39,62–66]. FC-2 mAb and to a less extent FC-3 are able to neutralize at low concentrations most of the primary strain tested. In particular, FC-2 seems to be more potent than D5, NC-1, 2F5 or 4E10 in our neutralization assays. The relative higher neutralization efficiency of FC-2 and FC-3 in comparison with FC-1 could also be explained by a better recognition of gp41 conformation of these two mAbs. Envelope variability of the 92US660 primary strain could explain the apparent resistance of this strain for the three mAbs (data not shown).

Two mAbs (FC-2 and FC-3) have the capacity to recognize both the native envelope glycoprotein gp41 region and a new conformational epitope composed by the folding in the fusion complex of a first peptide located in the HR1 region (SGIVQQQ) and a second one located in the C-terminal part of gp41 containing HR2 (FDITNWLWYIRLFIMIVGGL). This epitope is probably a conformational epitope present on the quaternary structure of the HR1/HR2 (pre)fusion complex as it has been previously described for the E1/E2 complex epitope of 2F5 or for the polar segment and MPR [67,68]. Thus, explains the strong effect of FC-2 and FC-3 on the inhibition of HR1/HR2 complex formation. FC-1 is reactive with a linear epitope located in HR2/MPER as previously described with other antibodies (2F5 or 4E10). Competition experiments indicate that FC-1 epitope is certainly a linear epitope contained in HR2 region. The epitopes for FC-2 and FC-3 seems to be different and conformational. FC-2 epitope is very close from FC-3 and 4E10 epitopes. FC-3 epitope seems to be conformational and encompass a region containing FC-2, 2F5 and 4E10 epitopes probably MPR.

The three mAbs interfered with the gp41 conformational changes by blocking the formation of the fusion active gp41 six-helix bundle as demonstrated by two different

assay systems (cell–cell fusion test and ELISA test). The ability of other anti-gp41 specific mAbs as 2F5 or 4E10 to block fusion was observed at a range of concentration about 100 µg/ml by coculturing the MOLT/CCR5 cells with the NL4–3/BaL isolates and with CD4 T cells [69]. Previously, Massanella *et al.* produced four mAbs in mice after immunization with recombinant soluble gp41 produced in *E. coli*. These four mAbs bind to the recombinant soluble gp41 and recognize the native envelope glycoprotein gp160. All four purified mAbs could inhibit HIV-1 Env-mediated membrane fusion (syncytium formation) by 40–60% at 10 µg/ml but with low neutralizing activity against common HIV-1 strains.

In this work, a cell line that expressed a conformational gp41 in a cell-membrane context to induce potent neutralizing antibodies has been used as immunogen. The use of this cell line allow us to generate highly potent antibodies with an high specificity for the prefusion complex. The three antibodies have the capacity to strongly interfere with HR1/HR2 complex formation and to inhibit very efficiently the HIV-1 infectious process. A better activity of FC–2 and FC–3 were observed on HR1/HR2 complexes containing heterologous regions than those contained in the HEK 293-gp41<sup>MSD</sup> cell line used for immunization. The capacity of these antibodies to mediate ADCC or ADCVI against infected cells has not yet been studied. These mAbs will first be humanized in order to prevent Human Anti-Mouse Antibody immune responses both in monkeys and in humans. Such type of antibodies reacting specifically with the fusion complex could be used, alone or associated, as therapy to block the entry of HIV-1 after a recent viral exposition or to reduce, after systemic administration, the viral burden and the massive CD4 cell count depletion in HIV-1 reservoirs as the intestine. The capacity of these antibodies to block the fusion process could be also tested on T20 resistant strains.

It appears that the fusion complex epitope when expressed in a cell-membrane context is strongly immunogenic and is able to elicit neutralizing antibody. The proximity between the fusion complex and the lipid bilayer of the transfected cell line is probably involved in the correct assembly and exposure to the surface of the cells. This study re-explores and demonstrate the great interest of gp41 as immunogen to induce cross-clade neutralizing antibodies. Approaches based on conformational HR1/HR2 complexes should be further explore to evaluate their ability to induce protective immunity.

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## Conflicts of interest

There are no conflicts of interest.

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