HIV-1 gp41-specific monoclonal mucosal IgAs derived from highly exposed but IgG-seronegative individuals block HIV-1 epithelial transcytosis and neutralize CD4$^+$ cell infection: an IgA gene and functional analysis

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AIDS is mainly a sexually transmitted disease, and accordingly, mucosal tissues are the primary sites of natural human immunodeficiency virus type-1 (HIV-1) transmission. Mucosal immunoglobulin A (IgA) antibody specific for HIV-1 envelope gp41 subunit is one correlate of protection in individuals who are highly sexually exposed to HIV-1 but remain persistently IgG seronegative (HEPS). Understanding these peculiar IgAs at the gene and functional level is possible only with monoclonal IgAs. We have constructed a mucosal Fab IgA library from HEPS and have characterized a series of HIV-1 IgAs specific for gp41 that, *in vitro*, are transcytosis-blocking and infection-neutralizing. Characterization of their IgA genes shows that Fab specific for the gp41 membrane-proximal region harbors a long heavy-chain CDR3 loop (CDRH3) similar to the two broadly neutralizing IgG monoclonal antibodies, 2F5 and 4E10. Furthermore, the selected Fab IgA shows extensive somatic mutations that cluster in the CDR regions, indicating that affinity maturation due to an antigen-driven process had occurred in HEPS individuals, presumably upon multiple exposures to HIV. This analysis of HEPS monoclonal IgA gives a unique opportunity to correlate an antibody function (resistance to a pathogen *in vivo*) with an antibody gene. Such neutralizing monoclonal IgAs could be used in microbicide formulation.

INTRODUCTION

Human immunodeficiency virus type-1 (HIV-1) is mainly a sexually transmitted virus. Accordingly, mucosae covering the genital and the gastrointestinal tract are the principal sites of entry for HIV-1 worldwide. Establishing selective protection against HIV-1 infection at these sites, either by inducing a mucosal HIV-specific response by a vaccine or by characterizing specific antiviral agents for inclusion in microbicide formulation, has remained a challenge for two decades.

Over the years, one group of individuals has emerged that remains IgG-seronegative, despite repeated unprotected sexual intercourse with seropositive partners as first described by Mazzoli.[1] In these individuals, either women or men, referred to as Highly Exposed to HIV-1 but Persistently IgG Seronegative (HEPS), protection against HIV-1 correlates with various immune determinants (reviewed in refs. 2–4). In addition to cellular and innate immune responses, and more recently the enzyme apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G (APOBEC3G),[5] a HIV-specific humoral response at mucosal sites, namely an IgA response, has been associated with protection from infection in several cohorts of HEPS patients with different genetic backgrounds.[6–8] One characteristic of these IgAs, detected mainly in vaginal washes and urine, but in one instance also

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in the serum, is selective recognition of the transmembrane subunit, gp41, of HIV-1 envelope but not of the gp120 subunit. Multiple studies have shown that such HEPS-derived IgA could block, *in vitro*, numerous mucosal HIV-1 entry pathways, including HIV-1 transcytosis across human epithelial cells, and $CD4^+$ T-cell infection by primary HIV-1 isolates.[9–11] The main molecular determinants on gp41 recognized by these IgAs are the conserved regions.[12,13] Accordingly, such IgAs are capable of cross-clade neutralization.[10] Nonetheless, overall, the rationale behind the apparent protective mechanism in HEPS remains both elusive[4] and controversial[14,15] due essentially to variations in the status of HEPS individuals (couple/sex workers, duration and frequency of unprotected intercourse, opportunistic mucosal infections, etc.), the technique and antigens to quantify immunogenicity, etc. In-depth studies of such IgAs would require the production of monoclonal antibodies.

In contrast, in HIV-1-infected individuals, the antibody response to infection develops against various epitopes of both gp120 and gp41, with response rarely specific to conserved regions being the most suitable for viral neutralization (i.e., protection). Despite the frequent occurrence of HIV-1 envelope-specific IgA with *in vitro* neutralizing activities in secretions of HIV-1-infected individuals,[11] studies have mainly focused on the IgG response. To date, only five broadly neutralizing HIV-1 reactive monoclonal IgGs have been isolated. IgGs 2G12 and b12 are specific for gp120 and the other three, IgGs 2F5, 4E10, and Z13, the latter being a recombinant IgG, are directed against the membrane-proximal region (MPR) of the gp41 subunit, the most conserved region (reviewed by Ofek *et al.*[16]). These rare, broadly neutralizing IgGs were all isolated from blood or bone marrow B cells derived from unfortunately ill-characterized HIV-infected individuals (reviewed by Ferrantelli and Ruprecht[17]). To date, no neutralizing anti-HIV-1 monoclonal antibody of the IgA class has been isolated.

The rationale for focusing on IgA for fighting mucosal infection is that secretory IgA (S-IgA) and secretory IgM (S-IgM) are major components of the mucosal immune response. S-IgA has a well-documented role in prevention of viral infection by immune exclusion and under dimeric form intracellular neutralization, although in contrast to antibodies at other mucosal surfaces, IgG is quantitatively the predominant isotype in the female genital tract. Other research groups and ours have shown that *in vitro*, gp41-specific IgAs block infection of mucosal target cells. This occurs either by blocking adherence of virus to epithelial cells preventing HIV-1 transcytosis[11,18] or by way of neutralizing the virus within epithelial cells, thus redirecting the virus in the apical compartment,[19] or by binding the virus in the lamina propria, in turn blocking infection of mononuclear and dendritic cells (reviewed by Huang *et al.*[20]). Several such neutralizing IgAs are specific for P1, a peptide we have described earlier.[21] Peptide P1 (P1; amino acid (aa) 648–683) covers the entire gp41-MPR, which is recognized to control HIV fusion neutralization/infection of $CD4^+$ cells,[22] and consequently the ELDKWA and NWFDIT epitopes, which are recognized by IgGs 2F5, 4E10, and Z13. The amino acids adjacent to the MPR allows P1 to bind the mucosal receptor for HIV, that is the glycosphingolipid galactosyl ceramide (GalCer).[21,23] P1 is determinant in mucosal entry of HIV-1, as it mediates, through mucosal GalCer, HIV-1 internalization in epithelial cells as well as in mucosal dendritic cells before viral transfer to $CD4^+$ cells.[21,24] P1 shows at physiological pH a three-dimensional structure that resembles the one it adopts within the viral spike,[25] and can form oligomers,[11] as gp41 does within the spike at the viral surface. Mucosal immunization with P1 fused to cholera toxin B subunit, which serves as a potent adjuvant, results in the induction of P1-specific mucosal IgA antibodies with potent neutralizing activity against transcytosis across a tight epithelium and against $CD4^+$ cell infection.[26,27] However, gp41, especially under the trimeric conformation it adopts within the spike at the virus surface, may contain additional conformational epitopes targeted by antibodies interfering with HIV-1 infection.[28,29]

To characterize in detail the mucosal IgA response in HEPS individuals, we generated a mucosal Fab IgA library from cervical B cells derived from HEPS Cambodian women, either commercial sex workers or women living with a seropositive partner. The library was screened on P1 and a trimeric gp41 extracellular domain construct. Selected clones were characterized immunochemically and functionally for their *in vitro* potential to block HIV-1 transcytosis across epithelial cells and to neutralize HIV-1 infection of $CD4^+$ cells. Moreover, the Fab genes were analyzed at the molecular level. Such a characterization offers a unique opportunity to establish definitively, at the molecular level, the existence of a mucosal humoral immune response with *in vitro* protective characteristics in HEPS individuals.

RESULTS

Construction of an IgA Fab-κ/λ library from B cells of Cambodian HEPS individuals

We first analyzed the anti-HIV-1 envelope humoral response in the genital secretions of HEPS women. As detected in preliminary studies by antigen gp41- and gp120-specific ELISA (**Supplementary Figure S1** online) and confirmed by western blot (data not shown), 50% of Cambodian HEPS individuals had gp41-specific, but not gp120-specific, IgAs in their cervico-vaginal secretions. In contrast, broad anti-HIV-1 response was detected in secretions of HIV-1 seropositive individuals, and HIV-1-specific IgAs was absent in secretion from an HIV low-risk control individual. Furthermore, no HIV-1 envelope-specific IgG could be detected, confirming the IgG sero-negative status of the HEPS individuals analyzed (data not shown).

Subsequently, cervical B-lymphocytes of those HEPS individuals having gp41-specific IgAs in their secretions were used to construct a phage displayed IgA-κ/λ library by standard techniques, but using a primer set able to recognize all functional human V genes[30] (**Supplementary Table SI**). It resulted in an IgA-κ/λ Fab library containing ~1×10^7 independent clones, the expected size for an immune antibody library.[31]

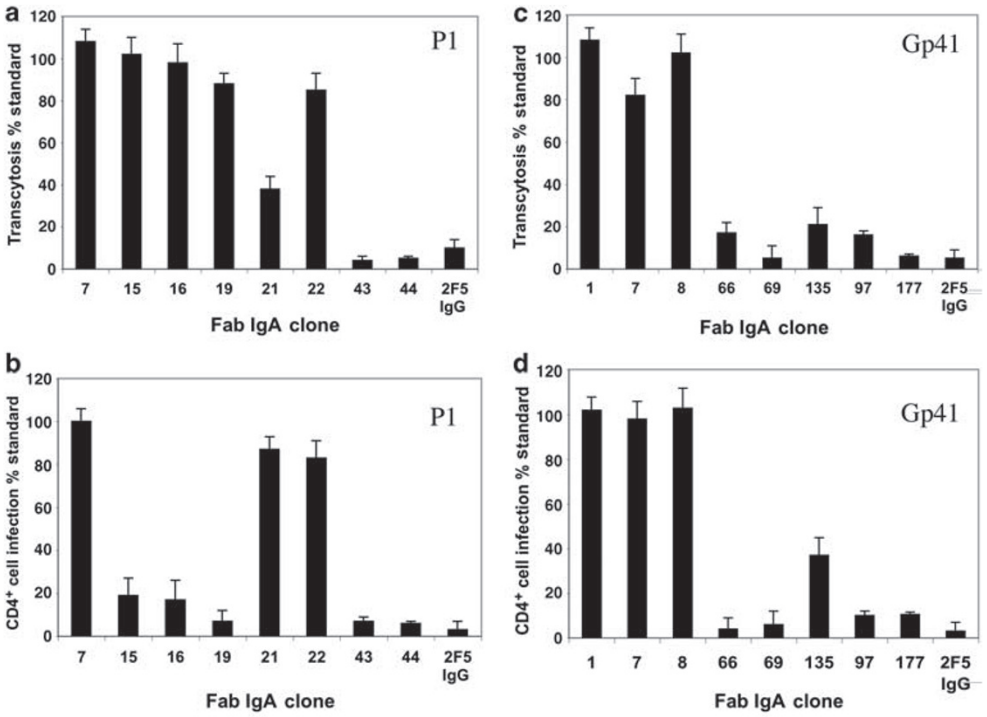

Figure 1 Human immunodeficiency virus type-1 (HIV-1) blocking activities of Fabs from the Highly Exposed to HIV-1 but Persistently IgG Seronegative (HEPS) IgA library specific for P1 or trimeric gp41-ΔMPR (membrane-proximal region). Fabs from the IgA Fab library were screened on peptide P1 (P1) or trimeric gp41-ΔMPR (gp41). Soluble Fabs (100–300 ng ml^{-1}) specific for (**a** and **b**) P1 or (**c** and **d**) gp41 were evaluated for their functional activity to block HIV-1 transcytosis (**a** and **c**) or to neutralize HIV-1 entry into the reporter CD4$^+$ CXCR4$^+$ HeLa—LTR lacZ cells (**b** and **d**). Blocking of transcytosis and neutralization of infection activities are expressed as percentage (%) of transcytosis or infection in the presence of control IgG. Data for representative sets of the clones analyzed, either specific for P1 (8 of 17 analyzed) or for gp41 (8 of 20 analyzed) are shown. IgG 2F5 (1 μg ml^{-1}) served as a positive control. Error bars represent the mean of at least two independent experiments performed using X4 tropic NDK subtype D HIV-1-infected cells for transcytosis or cell-free X4 tropic Lai HIV-1 for infection experiments.

IgA Fabs specific for the conserved MPR P1 of gp41

To identify new neutralizing antibodies specific for gp41, we first focused on the gp41-MPR, namely the peptide P1 (aa 649–684).[11]

The library was screened by three successive rounds of panning on P1, coated on ELISA plates with decreasing coating concentration at each round, ranging from 250 to 30 μM. At all these concentrations, P1 remains oligomeric.[11] Of 100 clones analyzed after completion of the panning, 20 bound specifically to P1 in ELISA, with an optical density (OD) >0.6 (data not shown). These specific binder clones were directly evaluated for their functional neutralizing activity against HIV-1 transcytosis and CD4$^+$ T-cell infection. As shown in **Figure 1a** and **b**, some clones could efficiently block transcytosis or CD4$^+$ T-cell infection. We focused on clones 43 and 44 that could block both cell entry mechanisms by >90%. Sequencing heavy and light chains from these two clones followed by an analysis using the IMGT/V-QUEST software showed that these two clones were identical. Hereafter they will be referred to as Fab 43. The deduced amino-acid sequences of the heavy and light chains of clone 43 are shown in **Figure 2a**.

IgA Fabs specific for MPR-deleted trimeric gp41

We next designed a panning strategy to select Fab IgA clones specific for regions of gp41 other than P1. Hence, the library was first depleted of P1-specific binder phages by a round of panning on P1. Non-binder phages were then subjected to three sequential rounds of panning on decreasing concentrations (from 50 to 12 μM) of the trimeric gp41-ΔMPR, a trimeric recombinant gp41 protein[32] devoid of 2F5 and 4E10 epitopes. Of 100 clones analyzed after panning completion, 17 bound specifically to gp41 by ELISA, with an OD >0.6 (data not shown). **Figure 1** shows that at the functional level, the selected gp41-specific binders that blocked HIV-1 transcytosis (**Figure 1a** and **b**) also efficiently blocked CD4$^+$ T-cell infection (**Figure 1c** and **d**), with the broadly neutralizing IgG 2F5 serving as a positive control. Sequencing of nine gp41-specific clones (designated 59, 66, 69, 82, 97, 105, 125, 154, and 177) showed that two of those (59 and 69) were identical and the seven others (69, 82, 97, 105, 125, 154, 177) had identical heavy chains but different light chains.

Three gp41-specific clones with efficient HIV-1 blocking activities (**Figure 1c** and **d**) were chosen for further analysis, two of them having identical heavy chains but different light chains (**Figure 2b**), lambda (λ) for Fab 69 and kappa (κ) for Fab 177. The third, Fab 66, had heavy and light λ-chain sequences different from those of Fabs 69 and 177 (**Figure 2b**).

P1- and gp41-specific IgA Fab characterization

DNA from selected phages was subcloned into the pASK88 vector,[33] a vector designed for high levels of Fab production. Using

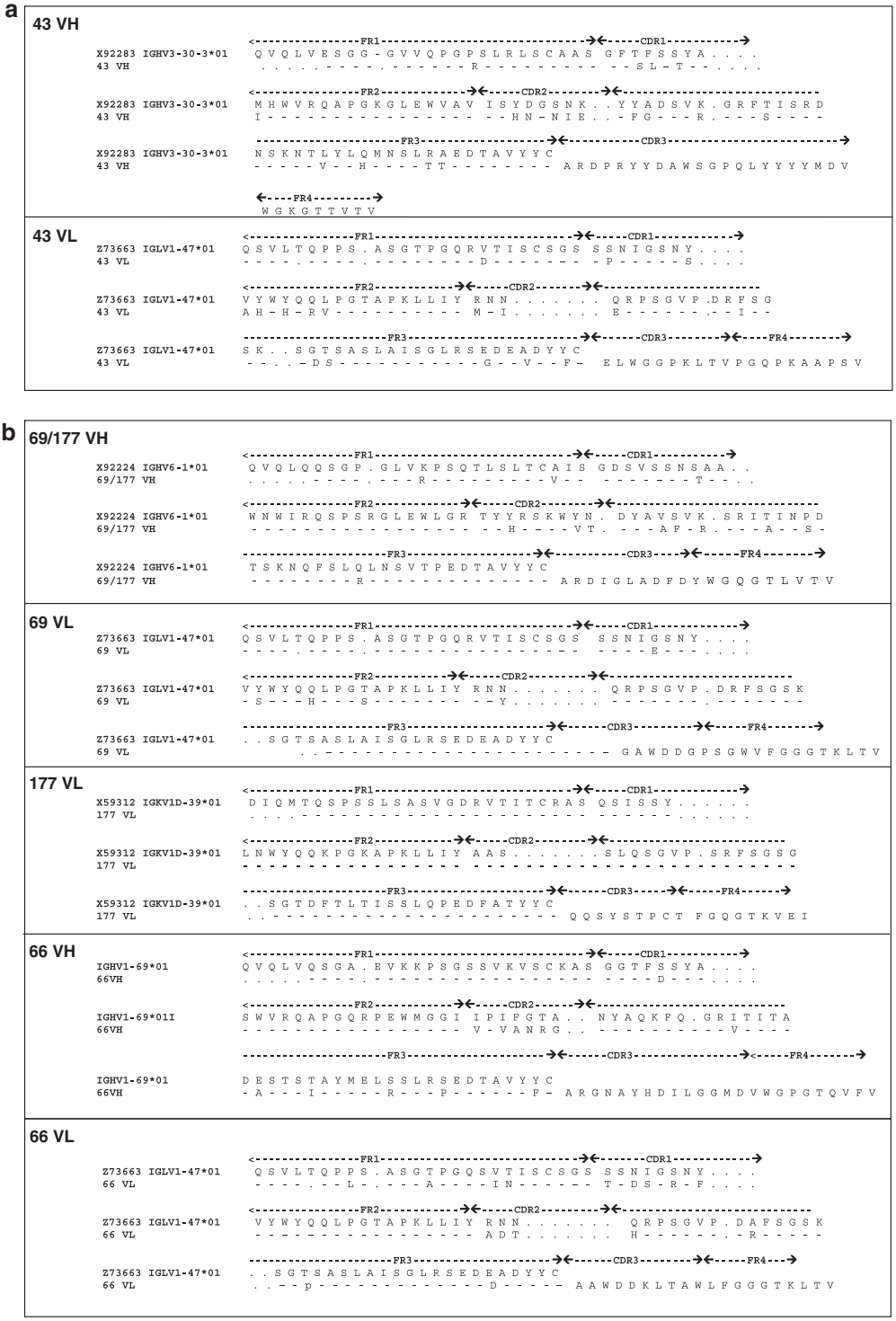

Figure 2 Sequences of the variable regions of the gp41-specific Fabs obtained from Highly Exposed to HIV-1 but Persistently IgG Seronegative (HEPS). Alignment of (**a**) Fab clone 43 or (**b**) Fab clones 69, 177, and 66 variable regions of the heavy chain (VH) or the light chain (VL) with the sequences encoded by the closest germline gene, using the IMGT/V-QUEST library. FR, framework region; CDR, complementary-determining region. Dot (.), gaps; hyphen (**–**), amino-acid identity.

this expression system, the resulting Fab 43 bound peptide P1 in a concentration-dependent manner as well as a trimeric gp41 recombinant protein containing 2F5 and 4E10 epitopes (gp41-MPR) (**Figure 3a**).

P1 covers a highly conserved region of gp41,[34] which can vary among clade C viruses, the latter causing the increasing AIDS pandemic in Asia, especially in China. Importantly, Fab 43 bound specifically to a peptide P1 from a clade C virus mutated

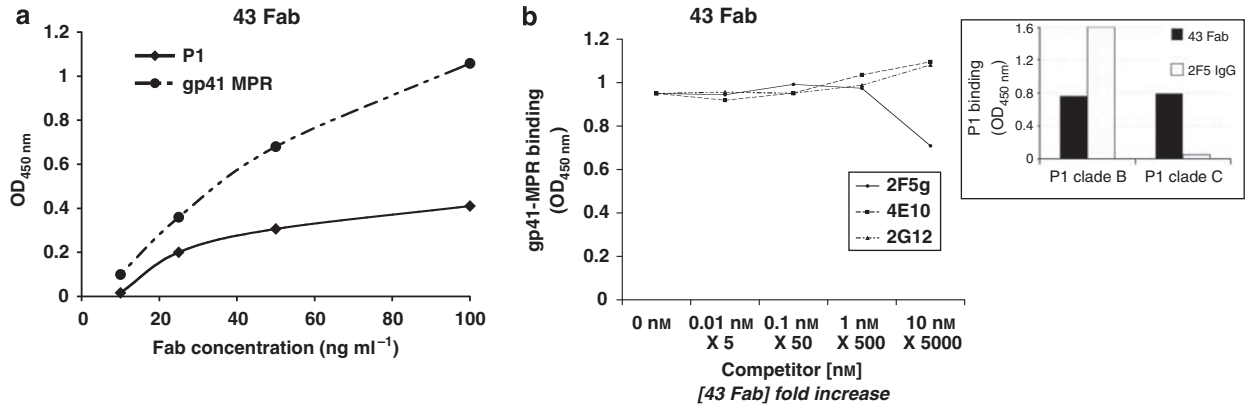

Figure 3 (**a**) Fab clone 43 binding to P1 and to gp41-MPR (membrane-proximal region) occurs in a dose-dependent manner. The specificity of Fab 43, produced using the pASK88 vector, for peptide P1 (coated at 250 ng ml^{-1}) (diamonds, solid line) or trimeric gp41-MPR (coated at 500 ng ml^{-1}) (filled circles, dashed line) was evaluated by ELISA. Specific binding ($OD_{450\,nm}$) is plotted as a function of Fab 43 concentration (ng ml^{-1}). The experiment shown is representative of at least three independent experiments. (**b**) Fab 43 binds to P1 at epitopes other than the linear hexapeptides, ELDKWA or NWFDIT, recognized by IgGs 2F5 and 4E10. Competitive binding of Fab 43 for P1 (coated at 250 ng ml^{-1}) in the presence of an excess of IgG 4E10 (specific for NWFDIT sequence on gp41) squares, dashed line) or 2F5 (specific for ELDKWA sequence on gp41) (circles, solid line) or 2G12 (specific for gp120) (triangles, dotted and dashed line) used as a negative control was evaluated in a competitive ELISA, as described in Methods. Specific binding ($OD_{450\,nm}$) is plotted as a function of competitor concentration (nM) and fold increase as compared with Fab 43 concentration (italic characters). The experiment shown is representative of at least three independent experiments. (Inset) Fab 43 bound specifically a peptide P1 from a clade B and C virus mutated in the 2F5 and 4E10 epitopes in contrast to 2F5 IgG that bound only to P1 clade B. P1 clade B or C was coated on ELISA plates before the addition of Fab 43 (filled bars) or 2F5 IgG (open bars), and peptide-binding was detected by ELISA. Peptides were coated at 250 ng ml^{-1}. Specific binding is directly proportional to OD at 450 nm. The experiment shown is representative of at least three independent experiments.

in the 2F5 and 4E10 epitopes, whereas, as expected, IgG 2F5 could not do so (**Figure 3b**, inset). Accordingly, IgGs 2F5 and 4E10 could not compete with Fab 43 for P1 (clade B) and gp41-MPR binding in competitive ELISA (**Figure 3b**). These results indicated that Fab 43 binds to P1 at epitopes other than the linear hexapeptides, ELDKWA or NWFDIT, possibly still involving the 2F5- or 4E10-like epitopes, but as a non-linear structure. The P1-specific epitope is most likely conformational, because Fab 43 did not bind any linear 15 amino-acid overlapping peptides covering the P1 region (data not shown).

Fabs 66, 69 and 177 produced using the pASK88 vector system bound to gp41-ΔMPR in a concentration-dependent manner (**Figure 4a**). The epitope specificity of Fabs 66, 69 and 177 was evaluated by ELISA using successive 15 amino-acid HIV-$1_{H\times B2}$ overlapping peptides from residues 535 to 688, each differing by a sequential block of 5 amino acids. None of these overlapping gp41 peptides was recognized by the Fab clones, in contrast to IgG 2F5, which bound to ELDKWA-containing peptides with an $OD_{450\,nm}$ >1 (data not shown). These data suggest that epitopes recognized by these Fabs are conformational. Accordingly, binding of Fabs 177 and 69 to gp41-MPR was strongly decreased by guanidine-HCl denaturation of the antigen. Binding of Fab 66 was less affected and binding of IgG 2F5, used as a positive control, was not at all affected by guanidine-HCl denaturation (**Figure 4b**).

Next, competitive binding of Fabs 66, 69 and 177 to gp41 was evaluated by competitive ELISA. Interaction of Fab 177 with gp41-MPR was completely inhibited by a 20-fold excess of Fab 69 (**Figure 4c**) and vice versa (data not shown). As these two Fabs have identical heavy chains but different light chains, these results suggest that the heavy chain of these Fabs participates in the binding to gp41, the light chain modulating the affinity. Accordingly, the isolated heavy chain, obtained by deleting the light chain gene from the vector, could not bind gp41 (data not shown). The interaction of Fab 66 with gp41 was almost completely inhibited by an excess of Fab 177, but conversely, interaction of gp41 with Fab 177 was only partially inhibited by an excess of Fab 66 (**Figure 4c**). This suggests that these two Fabs recognize different but overlapping epitopes with different affinities. The competition between Fabs 69 and 66, each containing a λ-light chain, could not be evaluated in such an assay (see Methods).

Finally, none of the Fab clones that were specific either for P1- or gp41-bound gp120 or for various irrelevant antigens, such as bovine serum albumin, DNA, cardiolipin, or hemagglutinin (HA) peptide (**Figure 4d**), as has been suggested by Haynes *et al.*[35] for 4E10 and 2F5, but not confirmed by others.[36,37]

Gp41-specific Fab IgAs from HEPS block transcytosis of HIV-1 across a tight epithelial barrier

We previously reported that mucosal gp41-specific IgA from HIV-1-seropositive patients blocked HIV-1 transcytosis either intracellularly within epithelial cells or before HIV-1 penetration into the epithelium[11,19] Similarly, and in agreement with the result shown in **Figure 1** for the HIV-1 clade D, X4 tropic laboratory isolate NDK, the gp41-specific Fab clones isolated from our HEPS individuals blocked transcytosis of primary R5 tropic isolates from both B and C clades, with IC90 < 50 ng ml^{-1} (**Figure 5**). IgA Fabs' blocking activity for R5 tropic viruses (IC90 = 150 ng ml^{-1} for the various clones) is much higher than

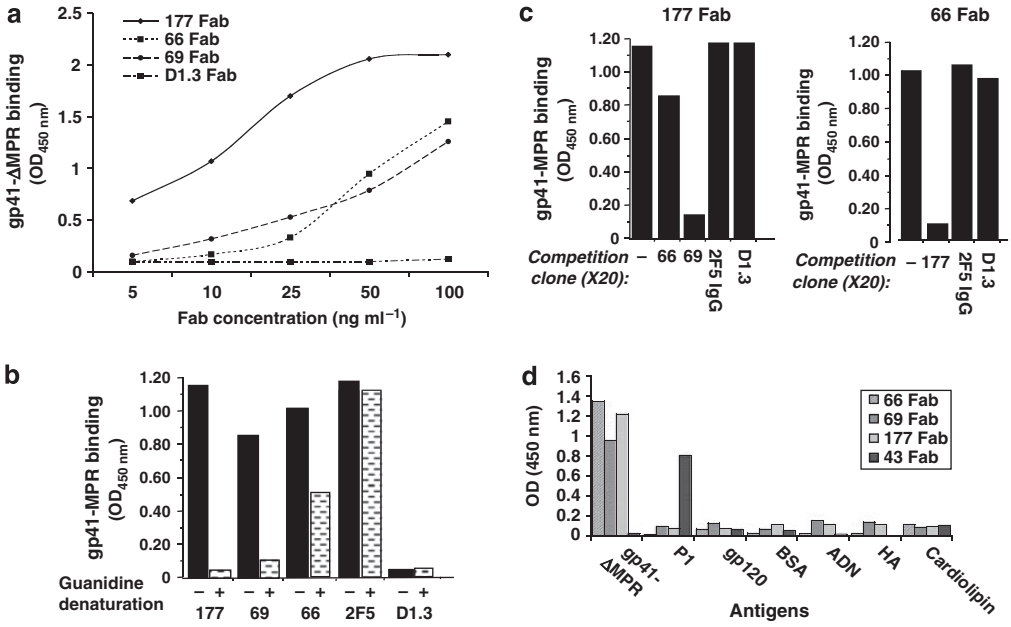

Figure 4 (**a**) Fab clones bind specifically to gp41-ΔMPR (membrane-proximal region) in a dose-dependent manner. Fab produced using the pASK88 vector was evaluated for their specificity of binding to gp41-ΔMPR (coated at 250 ng ml^{-1}) by ELISA. Fab 177 (diamonds, solid line), Fab 66 (squares, dotted line) and Fab 69 (circles, dashed line) are specific for gp41; D1.3 (large squares, dotted and dashed line) was used as a negative control. The experiment shown is representative of at least three independent experiments. (**b**) Fab 177-, 69-, and 66-specific epitopes on gp41 are conformational. The effect of guanidine-mediated denaturation of the gp41-MPR on the binding of Fabs 177, 69, and 66 was evaluated by comparing Fab binding with gp41-MPR or to guanidine-denatured gp41-MPR by ELISA as in **Figure 3**. 2F5 IgG specific for binding to the linear ELDKWA epitope and nonspecific D1.3 IgGs were used as controls. Recombinant proteins were coated at 250 ng ml^{-1}. Concentration of Fabs 66 and 69 and IgGs 2F5 and D1.3 was 100 ng ml^{-1}, and that of Fab 177 was 25 ng ml^{-1}. The experiment shown is representative of at least three independent experiments. (**c**) Competitive binding of Fabs 66, 69, and 177 to gp41-MPR. Competition ELISAs were performed as in **Figure 3b**. In the left panel, binding of Fab 177 to gp41-MPR was competed partially by Fab 66 and markedly by Fab 69; in the right panel, Fab 66 binding to gp41-MPR was competed by Fab 177. In both panels, 2F5 specific for the ELDKWA epitope on Gp41 and nonspecific IgG D1.3, used as controls, showed no competition. Concentration of Fabs 66 was 100 ng ml^{-1}, and that of Fab 177 was 25 ng ml^{-1}. The experiment shown is representative of at least three independent experiments. (**d**) Gp41 Fabs from Highly Exposed to HIV-1 but Persistently IgG Seronegative (HEPS) are not polyspecific. Polyspecificity was evaluated by ELISA comparing antibody binding to gp41 and peptide P1 with binding to irrelevant antigens, such as gp120, bovine serum albumin (BSA), DNA, hemagglutinin peptide (HA), and cardiolipin as described in the Methods section. Concentration of Fabs 66 and 69 was 100 ng ml^{-1}, and that of Fabs 177 and 43 was 25 ng ml^{-1}. The experiment shown is representative of at least three independent experiments.

that of IgG 2F5 (IC90 = 1 μg ml^{-1}). Interestingly, Fab IgAs were slightly more potent at blocking R5 tropic than at blocking the laboratory adapted, otherwise very infectious, clade D NDK, X4 virus. Cross-neutralization between X4 and clade B and C R5 tropic isolates confirms that the epitopes recognized by HEPS-derived Fab IgAs are conserved at either the sequence and/or the conformational level.

Antibodies isolated from the HEPS IgA library neutralize *in vitro* infection of mononuclear cells

The next important event in HIV-1 transmission at mucosal sites is the infection of mucosal dendritic cells, CD4$^+$ T lymphocytes, or macrophages that initiates the spread of the infection. We therefore evaluated with different assays performed independently in three different laboratories the ability of the Fabs to neutralize HIV-1 infection of various target cells. Fabs 66 and 69 specific for trimeric gp41-ΔMPR and Fab 43 specific for P1 neutralized macrophage infection by R5 tropic, clade B HIV-1 (BaL) with an IC90 ≅ 30–45 ng ml^{-1}, similar to IgG 2F5 (**Table 1**). Fab 177 exhibited a much more potent neutralizing activity, with an IC90 100 lower (0.3 ng ml^{-1}) (**Table 1**). In contrast, transforming Fab 177 from an IgA to IgG isotype (by switching the CH1 alpha for a CH1 gamma) drastically diminished Fab 177 neutralizing activity. Thus, in the IgG form, Fab 177 showed an IC90 = 25 ng ml^{-1} similar to that of the other library Fabs and to the IgG 2F5. IgA Fab 177 was also highly neutralizing in the very sensitive assay based on pseudovirus infection of TZM-bl cells (**Table 1**).[38] In addition, IgA Fab 177 neutralized dendritic cell infection by the R5 tropic HIV-1 JR-CSF isolates with an IC50 similar to that of 2F5IgG (data not shown).

We further concentrated on Fab 177 because of its higher neutralizing activity compared with the other gp41-ΔMPR-specific clones. Using both human CD8$^+$ T-cell-depleted peripheral blood mononucleared cells (PBMCs) and CD4$^+$ T cells as targets, Fab 177 neutralized HIV-1 infection by two HIV-1 R5 tropic clade B clones—either JR-CSF or the field isolate 93BR029, R5 tropic clade B virus—in a concentration-dependent manner (**Table 2**). As a control, pre-incubation of virus with Zidovudine (AZT) completely inhibited infection to a level comparable with mock-infected cells (**Supplementary Figure S2**). In

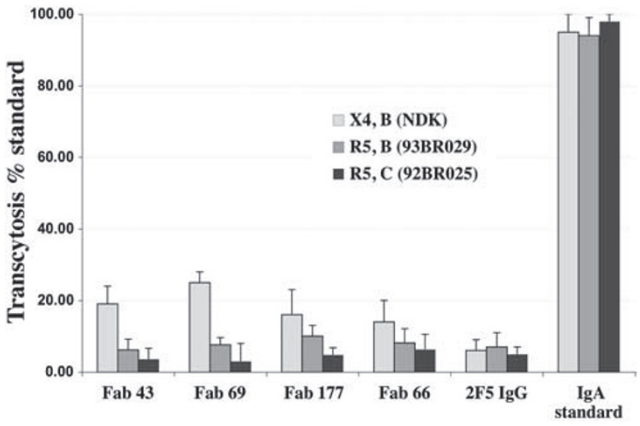

Figure 5 gp41-specific Fabs from Highly Exposed to HIV-1 but Persistently IgG Seronegative (HEPS) block human immunodeficiency virus type-1 (HIV-1) transcytosis. Transcytosis of HIV-1 induced by HIV-1-infected cells across a tight monolayer of epithelial cells was monitored as described in Methods section. CEM chronically infected with X4 tropic clade D HIV-1 (light gray bars), PBMCs infected by R5 tropic clade B (dark gray bars), or PBMCs infected by clade C (black bars) isolates were used to initiate transcytosis. Fabs (50–150 ng ml^{-1}), or control 2F5 IgG (1 μg ml^{-1}) or nonspecific IgA (150 ng ml^{-1}) were pre-incubated with HIV-1-infected cells before inoculation of the HIV-1-infected cells at the apical pole of the epithelial monolayer. Transcytosis was evaluated 2 h later by measuring the p24 content in the basal medium. Results are presented as percentage of transcytosis in the presence of nonspecific IgA (far right). Error bars represent the mean of at least three independent experiments.

this neutralization assays, Fab 177 was again much more potent in neutralizing HIV-1 than was IgG 2F5 (**Table 2**). Using CD8$^+$ T-cell-depleted PBMCs, the IC50 was 6 nM for Fab 177 vs. 60 nM for IgG 2F5, and the IC70 was 60 nM for Fab 177 vs. 200 nM for IgG 2F5. The difference between Fab 177 and IgG 2F5 was even more dramatic against infection of CD4$^+$ T cells with Fab 177 displaying a more than 10-fold higher efficiency than IgG 2F5 (IC90 = 60 nM).

Molecular characterization of the P1-specific Fab 43

Although the vast majority of anti-HIV-1 antibody gene analyses has been performed on IgG, little is known for IgA. Differences between mucosal IgA and IgG can be expected, as the mucosal IgA repertoire is highly compartmentalized.[39]

Diversity in the primary B-cell repertoire is created by the combinatorial rearrangement of different variable (V), diversity (D), joining (J), gene segments, by junctional diversity and by the association of different heavy and light chains.[40,41] An additional diversity can arise during development of the immune response by somatic hypermutation.

Antibody binding and specificity are mediated by the variable regions of the heavy (H) and light (L) chains.[42] Each variable region comprises three hypervariable subregions referred to as complementary-determining regions CDR1, CDR2, and CDR3 separated by four framework (FR1, FR2, FR3, and FR4) regions. Each H-region is encoded by three germline segments: variable (V_H), diversity (D_H), and joining (J_H). Although CDR1 and CDR2 are encoded by V_H, the CDR3 is encoded by the D_H segment and the beginning of the J_H segment. Therefore,

Table 1 gp41-specific Fabs from HEPS neutralize macrophage and TZM-bl cell infection by R5 tropic HIV-1

			Macrophages infection with HIV-1 R5, B (BaL)	CD4$^+$ cell TZM-bl infection assay with HIV-1 R5 (QH0692.42)	
Clone	**Specificity**	**Isotype**	**IC90% (ng ml^{-1})**	**IC50% (ng ml^{-1})**	**IC90% (ng ml^{-1})**
43 Fab	Gp41: P1	Fab IgA	31	ND	ND
66 Fab	Gp41	Fab IgA	45	>4,500	>4,500
69 Fab	Gp41	Fab IgA	32	ND	ND
177 Fab	Gp41	Fab IgA	0.5	670	960
D1.3 Fab	Neg. CTRL	Fab IgA	ND	>4,470	>4,470
177 Fab	Gp41	Fab IgG	25	ND	ND
2F5	GP41 ELDKWA positive CTRL	IgG	30	2,560	>6,670
D1.3 Fab	Negative CTRL	Fab IgG	>5,500	ND	ND

HEPS, Highly Exposed to HIV-1 but Persistently IgG Seronegative; HIV-1, human immunodeficiency virus type-1; IC, inhibition concentration; ND, not determined.

Table 2 gp41-specific Fabs from HEPS neutralize CD4$^+$ T cells infection by R5 tropic HIV-1

		CD8$^+$-depleted PBMC		CD4$^+$ T cells	
HIV-1 strain	**Clone**	**IC50% (nM)**	**IC70% (nM)**	**IC50% (nM)**	**IC90% (nM)**
R5, B 93BR029	177 Fab IgA	ND	2	ND	60
R5, B JR-CSF	177 Fab IgA	6	60	ND	60
	2F5 IgG	60	200	200	> 600

HEPS, Highly Exposed to HIV-1 but Persistently IgG Seronegative; HIV-1, human immunodeficiency virus type-1; IC, inhibition concentration; ND, not determined.

Table 3 Gene characteristics of gp41-specific Fabs from HEPS compared with the closest germline gene

			Mutations		R:S				
Clone	Germline gene	Homology (%)	FR	CDR	FR	CDR	DH	JH	CDR3 length (aa)
(a)									
43	IGHV3-30-3*01	91	9	8	0.81	2.75	IGHD3-3*01	IGHJ6*03	22
69	IGHV6-1*01	94	8	4	9	∞	IGHD2-8*02	IGHJ4*02	11
177	IGHV6-1*01	94	8	4	9	∞	IGHD2-8*02	IGHJ4*02	11
66	IGHV6-69*01	89	7	7	2.25	5	IGHD3-9*01	IGHJ5*02	15

			Mutations		R:S		
Clone	Germline gene	Homology (%)	FR	CDR	FR	CDR	JL
(b)							
43	IGLV1-47*01	92	13	4	14	4	Non-defined
69	IGLV1-47*01	96	4	2	5	∞	IJLJ3*02
177	IGKVID-39*01	100					IJLJ1*01
66	IGLV1-47*01	87	8	9	0.6	11	IJLJ3*02

aa, amino acids; CDR, complementary determining region; FR, framework region; HEPS, Highly Exposed to HIV-1 but Persistently IgG Seronegative; R:S, replacement (R) to silent (S) nucleotide change ratio.
Rearranged VH genes of clones (**a**) 43, 66, 69, and 177 and (**b**) VL genes of clones 43, 66, 69, and 177, their most homologous germline gene, percentage of nucleotide homology to their respective gene, absolute number of mutations, the R:S ratio in CDR and FR regions, and amino-acid length of the CDR, DH, JH, and JL segments used.

CDRH3 is the loop most diverse in composition and length (estimated potential diversity of 10^{23} sequences).[43] V_H genes are divided in seven families (V_H1–V_H7), each of which is distinguished by shared nucleotide homologies of >80% in conserved regions.[42] The largest gene family, V_H3, comprises about half of the expressed V_H repertoire in adult peripheral B cells.

The light chain is formed by rearrangement of a germline variable gene (V_L) with a joining gene (J_L). The CDRs 1 and 2 are encoded by the V_L segment and the CDR3 by the 3′-end of the V_L and the 5′-end of the J_L segments. In human, there are two types of light chains: κ (60%) and λ (40%). The diversity of the repertoire is generated not only by rearrangement of V_H, D_H, and J_H segments, and V_L and J_L segments but also by various possible combinations between heavy and light chains and by somatic mutations that clusters in CDR regions.

Analysis of the VH segment: Fab 43 showed a long CDRH3 of 22 amino acids, characteristics of HIV-1 envelope neutralizing antibodies

Characteristics of the CH1 region indicated that Fab 43 was of the IgA1 isotype. The Ig H variable region of Fab 43 (**Figure 2a**) consists of a VH gene from the largest gene family, VH3, in conjunction with the D3-3*01 allele of DH family, and these are followed by the JH6 gene segment. A 91% nucleotide homology was found with the germline gene VH3-30*01, differing by 34 nucleotides and 17 amino-acid residues (9 in CDR and 8 in FR regions) (**Table 3a**). The CDR regions had the highest level of nucleotide replacement mutations, with a replacement (R) to silent (S) nucleotide change ratio (R:S) of 2.75. Mutations in CDRH1 and CDRH2 lead to amino-acid changes of 25 and 50% compared with the germline gene VH3-30-3*01 (**Table 4**).

Strikingly, the heavy chain CDR3 loop (CDRH3) is very long, containing 22 amino acids. Such an extended CDRH3 has been described as one molecular characteristic of the rare HIV-1 broadly neutralizing IgGs, either specific for gp120, namely IgGs 2G12 and b12, or specific for gp41-MPR, namely IgGs 2F5 and 4E10.[44–46]

D(H) region usage

The D(H) regions of Fab 43 was compared with the immunoglobulin D(H) locus and showed homology with the D3-3 segment, with 21 nucleotides homologous out of 31 (68% homology) and with 8 additional nucleotides at the 5′-end and 10 additional ones at the 3′-end of the germline sequence. These changes translate to a 22 amino-acid residue-long extended CDR3 (**Supplementary Table SII**).

Analysis of the VL segment

The light chain of Fab 43 belongs to the λ-type. Its nucleotide sequence showed 92% homology with the germline gene VL1-47*01, differing by 20 nucleotides and 17 amino-acid residues (4 in CDR and 13 in FR regions) (**Table 3b**). Surprisingly, the percentage of amino-acid changes was high in both FR (especially in FR2) and CDR regions (**Table 4**). Somatic mutations in FR and CDR regions in Fab 43 (evaluated by R:S ratios) were greater in FR than for CDR regions (**Table 3b**). No alignment for the J segment was found.

Molecular characterization of trimeric-gp41-specific Fab clones

As for Fab 43, these gp-41-ΔMPR-specific Fabs were all of the IgA1 isotypes. In addition to the identity of sequences of heavy

Table 4 Molecular characteristics of gp41-specific Fab IgA clones 43, 66, 69, and 177 genes

	Clone 43		Clone 66		Clones 69 and 177	
	% Mutations related to					
Region	IGHV3-30-3*01 aa changes	IGLV1-47*01 aa changes	IGHV1-69*01 aa changes	IGLV1-47*01 aa changes	IGHV6-1*01 aa changes	IGLV1-47*01 clone 177 has 100% homology with IGKV1D-30*01 aa changes
FR1	0	3.84	0	19.23	7.69	0
FR2	5.88	29.41	11.76	5.88	0	23
FR3	20.51	17.94	12.83	7.69	15.38	0
CDR1	25	16.66	16.66	41.66	8.33	8.33
CDR2	50	20	60	30	30	10

aa, amino acid; CDR, complementary-determining region; FR, framework region.

and light chain variable regions between two clones (59 and 69), identical heavy chains but different light chains found in six other clones (69, 97, 105, 125, 154, and 177) indicate considerable heavy chain promiscuity, as observed *in vivo*.[47] Such promiscuity is also not uncommon for Fabs isolated from phage display libraries.[46]

Analysis of the expressed V_H genes

The IgH variable region of Fabs 69 and 177 comprised a VH gene from the VH6 family, in conjunction with the D2-8 allele of the DH family, followed by the JH4 gene segment. In contrast, the IgH variable region of Fab 66 consists of a VH gene from the VH1 family in association with the D3-9 allele, followed by the JH5 gene (**Table 3a**).

Analysis of the VH region showed that Fabs 69 and 177 were closely related to the germline gene VH6-1*01 (94% homology). Fab 66 showed a greater diversity from germline, as it was only 89% homologous to the closest germline gene VH1-69*01 (**Table 3a**).

Analysis of somatic mutations in FR and CDR regions of the 69, 177, and 66 gp41-reactive Fab clones showed that the R:S ratios were highest in the CDR compared with the FR region, as expected for affinity-matured antibodies, only for clone 66 (**Table 3a**).

The Fab 66 VH-rearranged gene differed by 25 nucleotides and 14 amino-acid residues (7 in FRs and 7 in CDRs) from the closest germ line VH1-69*01 (**Table 3a**). Mutations in CDR1 and CDR2 of Fab 66 leads to an exchange of 16.66 and 60%, respectively, compared with the germline gene VH1-69*01, whereas mutations in FR2 and FR3 lead to changes in amino acids of 11.76 and 12.83%, respectively (**Table 4**). Fab 69 VH- and Fab 177 VH-rearranged genes differed by 14 nucleotides and 12 amino-acid residues (8 in FRs and 4 in CDRs) from the closest germ line VH6-1*01 (**Table 3a**). The R:S value was higher for FR than for CDR regions (**Table 3a**) because of a high level of replacement mutations in FR3 (15.38%). These data suggest that this CDR loop participates in the antigen binding as has been shown for other anti-HIV-1 antibodies (reviewed in Viau and Zouali[42]). Mutations in CDR1 and CDR2 lead to changes of 8.33 and 30% amino acids, respectively, compared with the germline gene VH6-1*01 (**Table 4**). Surprisingly, all nucleotide mutations identified in the CDR2 of these clones lead to amino-acid alterations (that explains the ∞ value of the R:S ratio) (**Table 3a**).

D(H) region usage

The D(H) regions of trimeric gp41-specific Fabs were compared with the immunoglobulin D(H) locus. **Supplementary Table SII** shows the assignment of the single D(H) segments of Fabs 66, 69 (and 177) with the D(H) locus. Fab 66 showed homology with the D3-9 segment, with 15 homologous nucleotides out of 32 (47% homology). It also had 6 additional nucleotides and 1 additional P insertion at the 5′-end and 10 additional nucleotides at the 3′-end of the germline, resulting in a prolonged CDRH3 of 15 amino-acid residues. The Fabs 69 and 177 showed homology with the D2-8 segment, with 14 out of 31 homolog nucleotides (45% homology) resulting in a CDRH3 of 11 amino-acid residues long.

Analysis of the expressed V_L gene

Fabs 69 and 66 were of λ-type and were derived from the germline gene family VL1. In contrast Fab 177 had a κ-light chain of the VK1 gene family. The J segment belonged to the J3 family for Fabs 69 and 66 and to J1 for Fab 177 (**Table 3b**). Comparison of the nucleotide sequences of these Fab light chains with the sequence of the germline gene VL1-47*01, as already shown for the heavy chain, indicated a high nucleotide homology for Fab 69 (96%) but lower nucleotide homology (87%) for Fab 66 (**Table 3b**). An analysis of somatic mutations indicated that the Fab 69 VL gene differed by eight nucleotides and six amino-acid residues (four in FR and two in CDR) from the germ line VL1-47*01, whereas the Fab 66 VL gene differed by 36 nucleotides but only 17 amino acids (8 in FR and 9 in CDR) from the germ line VL1-47*01 (**Table 3b**). There was only 1 silent mutation but 11 replacement mutations in the CDR loops that explain the R:S ratio of 11 for clone 66 (**Table 3b**). Surprisingly, mutations in CDR1 and CDR2 of Fab 66 produced greater amino acid changes in CDR1 (41.66%) compared with CDR2 (30%) (**Table 4**), suggesting

the participation of the CDR1 in antigen binding. For Fab 69, CDRs contain only two replacement mutations and no silent mutation (R:S ratio value is ∞) (**Table 3b**).

Clone 177 has 100% nucleotide homology with the germline gene VK1D39*01.

DISCUSSION

AIDS is mainly a sexually transmitted disease. It is therefore essential to design and establish anti-HIV-1 strategies for protection against mucosal transmission of HIV, which is exemplified in HEPS individuals.

Here we report on the construction, by genetic engineering from mucosal cervical B lymphocytes of HEPS women, and characterization of new human monoclonal Fab IgAs, specifically directed against gp41. Resulting monoclonal Fab IgAs show potent HIV-1 neutralizing activities against both transcytosis of HIV-1 across epithelial cells and infection of $CD4^+$ T cells by HIV. Access to the specific individual genes of HEPS IgA provides unambiguous evidence of their occurrence *in vivo*, in contrast to earlier studies reporting only on polyclonal IgA fractions from biological fluids, which lacked direct evidence for the presence and functional roles of particular anti-HIV-directed antibodies, as pointed out recently.[14,48,49] As the result of their functional activities *in vitro* reported here, the monoclonal Fab IgAs we describe are the first antibodies that are unquestionably correlated with protection from HIV-1 infection in HEPS individuals.

Upon sexual transmission, HIV-1 virus encounters two main target cells, mucosal epithelial and $CD4^+$ T cells. These cells are protected from HIV-1 infection *in vitro* by the gp41-specifc IgAs described in this study. Mucosal epithelial cells, the initial target of the virus at mucosal surfaces, usually remain uninfected *in vivo*. But in contrast to its customary function as a physical barrier, the mucosal epithelium is used opportunistically by the virus as a tunnel for HIV-1 transcytosis to provide access to subluminal mononucleated $CD4^+$ T cells and dendritic cells, the first target cells actually infected by the virus in the submucosa.[50]

One therefore could suggest that in HEPS women, neutralizing gp41-specific IgA present in the submucosa, after synthesis from mucosal IgA lymphoblasts, or in the cervico-vaginal secretions after crossing the mucosal epithelium,[51] block HIV-1 either before viral entry into the epithelial cell monolayer or within the submucosa after HIV-1 transcytosis of the epithelium, if the initial blockade had been partial or if HIV-1 had translocated into the submucosa through the pluristratified epithelia.

In vitro both IgG and secretory IgA from mucosal secretion have been shown to interfere with the earliest events in HIV-1 entry by transcytosis across epithelial barriers.[19,52] The IgA Fabs from HEPS women that we have characterized, efficiently block transcytosis of HIV-1 with IC90 $< 50–150\,ng\,ml^{-1}$. Blocking activity does not result from interference with the local budding process of infected cells at the virological synapse (data not shown), as shown earlier for other gp41-specific IgAs;[11] this is not surprising for small Fab monovalent antibodies. Fab 43, specific for the gp41 region that binds the glycosphingolipid GalCer, the epithelial receptor of the virus, most likely interferes with HIV-1 binding to GalCer. The anti-infective activity of the set of Fabs specific for other regions in gp41 suggests that other epitopes are important for HIV-1 interaction with epithelial cells. Such epitopes could be the caveolin binding site 1 (ref. 53) regions recently described to be targets of IgG neutralizing infection, or QARILAV epitope within the HR1 region of gp41, the target of HIV-1 neutralizing IgA.[13] However, the Fabs described here did not bind these epitopes when linear in ELISA assays (data not shown). Alternatively, new conformational epitopes could be unmasked on gp41 following interaction of HIV-1 envelope with the epithelial surface involving HIV-1 envelope subunits, gp120 and gp41.[50] Interestingly, the Fabs from HEPS women were more potent at blocking transcytosis than were two IgGs that had been transformed into IgAs (IgA b12: IC90 $>800\,ng\,ml^{-1}$);[54] (2F5 IgA: IC90 $>3\,\mu g\,ml^{-1}$).[55] The role of the IgA constant region in the transcytosis blocking activity of the Fabs, either by contributing to the conformation of the IgA paratope, or for other undefined reason, is under investigation.

Neutralization of infectivity of HIV-1 in various $CD4^+$ mononuclear target cells has been evaluated in independent tests performed in three separate laboratories and conducted in a completely blinded manner. All results show a good neutralization potential of the HEPS Fabs. Furthermore, the IC90 of the Fab IgA *in vitro* HIV-1 neutralization in $CD4^+$ cells is low as compared with the IC90 of antibodies of the IgG isotype, such as 2F5. Altogether, the HIV-blocking efficiency of the Fabs for epithelial and $CD4^+$ mononuclear cells suggests that the low concentration of IgA that is present in genital secretions could be active *in vivo*.

Our functional studies indicate that at least three interdependent factors determine the neutralizing function of antibodies present in HEPS: (i) first, the epitope specificity, for conformational epitopes conserved among clades at the three-dimensional level; (ii) second, the paratope of the antibody, essentially comprising the antibody variable domains that show some common features with HIV-specific neutralizing IgGs; and (iii) third, as described here for the first time, the antibody isotype based on the constant antibody regions, namely CH1 alpha.

Relative to the epitope specificity, HEPS IgAs appears to recognize conformationally conserved regions of gp41, present at least in both clade B, X4 as well as R5 tropic, and clade C R5 tropic viruses. These epitopes differ from those targeted by the 2F5 and 4E10 IgG, which are linear epitopes that are poorly conserved between clade B and clade C viruses.

With regard to the paratope, some characteristics of the genes of the Fabs selected from the HEPS mucosal IgA library are strikingly similar to those of HIV-specific IgGs isolated from the blood or bone marrow of HIV-1-infected individuals. Similarities include: (i) extensive somatic mutations that cluster in the CDR regions, indicating that an antigen-driven process is operative, (ii) an unexpectedly high ratio of replacement to silent (R:S) mutations present in the FR3 region, suggesting the possible participation of this loop in HIV-1 binding by stabilizing the antibody paratope (reviewed by Viau and Zouali[42]). These similarities are an addi-

tional argument in favor of HIV-antigen-driven induction of IgAs in HEPS individuals. In addition, several clones retrieved upon gp41 screening conserve the same heavy chain but have different light chains, as exemplified by clones 69 and 177. These findings suggest that the heavy chain is determinant in epitope binding but that the light chain contributes synergistically in the affinity for the epitope. This finding is in agreement with the recently described contribution of heavy and light chain CDR regions of IgG 2F5 for its target.[56] Accordingly, the expression of only the heavy chain from our Fab library of IgAs results in a total loss of specificity for gp41.

Finally, regarding the IgA isotype, transformation of Fab 177 IgA into its IgG counterpart by replacing the CH1 alpha by a CH1 gamma dramatically reduces the neutralization potential, despite an equivalent affinity for its cognate epitope on gp41 as measured by ELISA (data not shown). It suggests that the conserved CH1 region of the antibody contributes significantly to its paratope structure and function. The gain of neutralizing activity, brought by the CH1 alpha to the antibody variable region, probably due to specific spatially arranged sets of charged and hydrophobic regions will be further studied by crystallography.

The development of the humoral immune response is an evolving process characterized by affinity maturation (reviewed in Tonegawa[57]). HEPS individuals are subjected to a repeated antigenic stimulation at mucosal sites due to repeated unprotected intercourse (2–6 events of unprotected intercourse per week for serodiscordant couples and 1–15 unprotected intercourses per day for sex workers) and thereby frequent, repeated exposure to HIV. Therefore, one could expect to find affinity-matured antibodies at mucosal sites in these individuals because of antigen-driven selection over a period of months or even years. Indeed, when each expressed sequence of Fab clones derived from the mucosal IgA library was compared with the germline gene sequence with the closest homology, the sequence showed extensive somatic mutations, especially in the CDR2 (**Table 4**) consistent with affinity maturation. Furthermore, there is a high level of mutation in the FR regions (**Table 4**), but overall the ratio of R:S mutations, indicating selection for diversity during antibody maturation, was higher in the CDR loops. In particular, all nucleotide mutations identified in the CDR1 and CDR2 of both chains of Fab 69 lead to amino-acid mutations.

An important role in antigen recognition and binding has been assigned to the heavy chain CDRH3,[58] which can harbor binding sites of a wide range of length and shape.[58,59] In this regard, P1-specific Fab 43 shows the typical molecular characteristics of broadly and potently neutralizing antibodies against HIV-1, such as IgGs 2F5, 4E10, and b12 (**Supplementary Table SIII**). Hence, this set of antibodies harbors a long CDRH3. Comparison with IgGs specific for the same MPR region is particularly relevant, with IgG 2F5 having a CDRH3 of 24 aa in length, and the recombinant IgG, Z13, having 19 aa in length. A long CDRH3 loop, similar to the one present in Fab 43, most likely adopts a finger-like conformation. This conformation would allow the antibody either to penetrate closer to its specific epitope,[44] especially those with hydrophobic regions such as the MPR,[56,60] or to penetrate within the lipid membrane from which the epitope emerges—here, the viral membrane from which the MPR emerges—or to interact with the target cell membrane, thereby increasing the antibody neutralizing potential.

Antibodies are often generated too late in the natural history of acute infection to contribute to host defenses, but they are likely to be much more effective if they are present before infection and before virus diversification, that is, if supplied locally in sufficient concentration to block entry and spread of the virus. Accordingly, it has been shown[61] that vaginal administration of the broadly neutralizing human monoclonal antibody, b12, can protect macaques from vaginal infection by simian–human immunodeficiency virus. Alternatively, a vaccine, most likely a mucosal one, able to induce mucosal IgG or IgA, could interrupt initial events associated with mucosal transmission of HIV-1.[62,63]

The first generation of microbicides against HIV-1 transmission based on agents nonspecific for virus has failed. The use of antiretroviral agents as an alternative is now widely explored. However, such strategy may also become harmful or inefficient, especially in women in the long term, because of changes in virological parameters induced by antiretroviral drugs.[64] Therefore, development of virus-specific agents is still worth exploring. The neutralizing gp41-specific IgA derived from HEPS individuals and therefore "naturally" induced may be one of such agents. These antibodies have evolved slowly during multiple exposures to HIV, they have accumulated high-affinity, antigen-binding somatic mutations, and therefore they might be the best "selected" to neutralize different HIV-1 strains. Such IgA antibodies, when topically applied, would target not only cell-free virus but also any infectious virus released locally from infected cells, thereby blocking HIV-1 entry into mucosal tissues, the primary site of HIV-1 transmission worldwide.

This study also suggests that using mucosal vaccines containing a few key peptides corresponding to epitopes that are recognized by IgA antibodies from HEPS individuals could be an additional way to induce locally a sufficient concentration of neutralizing antibodies to abort infection at the point of entry and to prevent the expansion and broadcasting of the virus to distal regions.[26,27,65] Along with cellular immunity ($CD8^+$ cytotoxic T lymphocytes), vaccine-induced neutralizing antibodies against conserved HIV-1 envelope domains at the mucosal level could offer protection against HIV-1 infection or disease.

METHODS

Peptides and recombinant proteins

Peptides P1 (aa 630–685), clade B HXB2 HIV-1,[21] and clade C (Bw96Bw0502 clone): SQNQQEKNEKDLLALDSWQNLWNWFSIT NWIWYIK were chemically synthesized (purity >95%) by Eurogentec (Beerse, Belgium). Gp41 constructs[32] with sequence from the HXB2 HIV-1 clone were kindly provided by Mymetics (Nyon, Switzerland and Protein X'pert, Grenoble, France) and comprise the following: (1) trimeric gp41-ΔMPR: aa 472-530-SGGRGGS-612-664 (patented by Mymetics, reference no. PCT/IB2004/002433) and (2) trimeric gp41-MPR 524-SGGRGGS-618-682. Residue numbering is given according to position in gp160 of CXCR4 tropic HIV-1_{HXB2}, clade B.

Study population and sampling

In this study, an HIV-1-infected control and 52 HEPS women, either living with a stable HIV-1 seropositive partner (41) or sex workers (11), were recruited at the Anonymous and Free Voluntary Counseling and Testing Center of the Pasteur Institute (Phnom Penh, Cambodia). The inclusion criteria for HEPS were unprotected sexual intercourse for more than 1 month at the time of enrollment. Serodiscordant couples lived together for more than 4 months (mean = 9 years, range: 4 months– 22 years), and seropositivity of the partner was known for a mean of 1.2 years (range: 4 months–7 years), with 2–6 unprotected episodes of intercourse per week over a mean of 11 months (range: 3 months–2 years). Sex workers experienced 1–15 unprotected episodes of intercourse per day for more than 3 months (mean = 11 months, range: 3 months–2 years). In all cases, counseling and information on HIV-1 and safe sex were provided. All individuals were asked to return 6 months later for follow-up. Medical teams conducted regular and free medical follow-up for the seropositive individuals, including prophylactic treatment of opportunistic infections when appropriate. Enrolled participants gave informed consent and completed a questionnaire covering the frequency of sexual relations and other sexual behaviors. All HEPS women were confirmed as seronegative, as tested by PCR of blood as well as of serology. All individuals studied were free of CCR5-Δ32 gene mutations, even in the heterozygote state.

Cervico-vaginal samples were collected at ~4 days before ovulation. Samples containing blood were eliminated. Cervico-vaginal secretions were collected using a sterile pipette by inserting in the vaginal vault 3 ml of sterile PBS (phosphate-buffered saline) containing protease inhibitors and antibiotics (gentamicin, fungizone, penicillin, streptomycin 50 $\mu g\,ml^{-1}$). Fluid was re-aspirated with the same pipette, and centrifuged at 600×g for 10 min. Supernatants were aliquoted into five tubes, snap-frozen, and stored at −80 °C. Cervical B cells were collected by inserting a cytobrush (Acellon multi Medscan, Fumouze Lab., Levallois-Perret, France, ref no. A00121) into the cervix according to Musey *et al.*[66] Collected cells were released from the brush by immediate immersion in 3 ml of RPMI 1640 medium containing antibiotics. After centrifugation at 600×g for 10 min, the cell pellets were immediately re-suspended in 1 ml of Trizol, snap-frozen, and stored at −80°C.

Samples containing blood or traces of seminal fluids, as determined by the presence of prostate antigen using the SEMA kit Humagen Fertility Diagnostics, Charlottesville, VA), were eliminated together with their respective cytobrush samples.

Analysis of cervico-vaginal secretions

ELISA. Concentrations of total IgG and IgA in cervico-vaginal secretions were measured by sandwich ELISA,[11,18] using polyclonal goat anti-human IgG or IgA (Biosys, Burlingame, CA) for coating and polyclonal goat anti-human IgG (Nordic, Tilburg, The Netherlands) or polyclonal goat anti-human IgA (Nordic) for detection; standards were purified human serum IgG (Sigma, St Louis, MI, I2511) or purified human colostral IgA (Sigma, I2636). To quantify HIV-1 envelope-specific IgA, ELISA plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with gp160 (300 ng per well), gp120 (150 ng per well), or gp41 (150 ng per well).[11,18] After incubation of the coated plates with individual samples (1/2 dilution in PBS + Tween 0.5%), specific IgAs were detected with biotinylated goat anti-human IgA (1:5,000; Biosys,) plus streptavidin-horseradish peroxidase (Sigma, 0.5 $\mu g\,ml^{-1}$).

Construction and panning of the combinatorial library

A combinatorial Fab α/κλ library was constructed according to Barbas.[67] RNAs from cervical B cells of HEPS with gp41-specific IgA in their secretions ($n = 22$) were extracted with Trizol (Invitrogen Life Technology, Paisley, UK) as recommended by the manufacturer.

To amplify all human heavy and light chain V-region gene segments, we used a set of oligonucleotide primers as described by Sblattero and Bradbury[30] (**Supplementary Table SI**). PCR products were cloned into the phagemid vector, pComb3X, under the control of the LacZ promoter (provided by Dr C Barbas III, The Scripps Institute, La Jolla, CA), to yield the Fab IgA library. The helper phage was VCSM13 carrying resistance to kanamycin. Briefly, the library was constructed by sequential cloning into the phagemid vector, pComb3X, of the light chain using *Sac*I and *Xba*I followed by the Fd fragment using *Xho*I and *Spe*I. The phage-displayed Fab library was generated by transforming the resulting DNA into electrocompetent *E. Coli XL1 Blue (Tet^r, F+)*. The resulting IgA κ/λ library expressed on the surface of filamentous M13 phage contained ~1×10^7 independent clones.

Panning of the library was carried out on two different antigens, P1 and gp41-ΔMPR, using standard protocols.[68] Peptide P1 was immobilized on ELISA plates (Peptide Immobilizer, Exiqon, Vaedbeck, Denmark) with a range of concentrations (decreasing), all being that at which P1 forms oligomer (250–30 μM).[21] Specific bound phages were processed for three additional panning rounds on decreasing concentrations of P1. Unbound phages were collected and used for the second panning on trimeric gp41-ΔMPR immobilized in immunotubes (range from 50 to 12 μM) (Maxisorp, NUNC) followed by three successive panning rounds with further decreasing antigen concentration.

Expression and purification of soluble Fabs

Constructs for the expression of soluble Fabs were prepared by subcloning the DNA into the pASK88 vector[33] originally designed for IgG1. Therefore, the entire IgA Fd (V_H–C_H1) and light chain (V_L–C_L) coding genes were inserted sequentially into pASK88 between *Pst*I and *Nco*I (V_H–C_H1) and *Sac*I and *Hin*dIII (V_L–C_L) sites, respectively. For indicated Fab IgA clones that were transformed into Fab IgG, the CH1 alpha was replaced by the CH1 gamma domain of human IgG1. To serve as control, lysozyme-specific D1.3 human IgG1 was produced using the same pASK88 vector provided by Skerra.[33]

Soluble Fabs were prepared by transfecting the vectors into the *E. coli* strain JM83. Production of functional Fab was performed as previously described.[69] Briefly, cultures were grown in 1 l of Luria Bertani medium containing 100 $\mu g\,ml^{-1}$ of ampicillin, and the expression was induced for 14 h at 22 °C by the addition of 0.2 $mg\,l^{-1}$ of anhydrotetracycline (ACROS Chimica, Thermo-Fisher Sci. Inc, Roskilde, Denmark) at an $OD_{550\,nm}$ of 0.5. Fabs were purified from the periplasmic fraction of the *E. coli* pellet by immobilized metal affinity chromatography using a GE Healthcare Kit (GE Healthcare, Europe Gmbh, Munich, Germany, 17-5286-01, HisTrap FF crude).

ELISA

The concentration of Fab was measured by sandwich ELISA using goat anti-human IgA (Caltag, Burlingame, CA) and biotinylated mouse anti-human Ig light chains, either κ or λ (BD Pharmingen, Franklin Lakes, NJ), with a standard curve generated from a standard human IgA (Jackson ImmunoResearch Laboratories, West Grave, PA).

Specificity of the Fab clones was detected by coating microtiter plates (NUNC-Immuno Plate MaxiSorp Surface, or Peptide Immobilizer (Exiqon) plate for P1) with either recombinant gp41s (trimeric gp41-ΔMPR or trimeric gp41-MPR), recombinant gp120, peptide P1, or unrelated antigens, including bovine serum albumin, DNA, and HA peptide from Influenza (Roche, Basel, Switzerland) (250 ng per well). Incubation was performed overnight at 4 °C. Fab binding was detected with biotinylated mouse anti-human κ- or anti-humanλ-light chains. All experiments were performed with Fabs from at least three independent purifications, each carried out in duplicate. For competitive binding ELISA to compare different Fabs, varying concentrations of each Fab were used to compete for the binding to gp41-MPR constructs. When indicated, competition for binding to gp41-MPR was performed using 2F5 IgG antibody. For comparisons between Fabs with different light chain type, the competitor Fab was added at a 20× higher concentration than the competed Fab. Binding of competed Fab was detected with a biotinylated mouse anti-human light-chain-specific antibody (either κ or λ according to the Fab tested). Cardiolipin ELISAs were performed as described.[35]

CD4$^+$ target cells

Peripheral blood samples from 20 healthy donors were depleted of CD8$^+$ T cells with RosetteSep Cocktail (StemCell Technologies, Grenoble, France) and mononuclear cells were isolated using the Ficoll-Hypaque technique (Europe Gmbh, Munich, Germany). After stimulation for 2 days with 2 μg ml^{-1} phytohemagglutinin (Sigma-Aldrich, St Louis, MO), the CD8$^+$ depleted PBMCs (T cells and monocytes) were used for infection and neutralization experiments. Alternatively, we used CD4$^+$ T cells purified from PBMCs using human CD4$^+$ T-cell enrichment kits (StemCell Technologies).

Virus stock preparation

A stock of HIV-1$_{JR-CSF}$ (clade B, R5 tropic) was prepared on a large scale by transfecting 293T cells with a plasmid containing the DNA sequence of JR-CSF (NIH, Germantown, MD).[24] The cell culture supernatant was concentrated, separated into single-use aliquots, and stored at −80 °C. Virus concentration was quantified by measuring p24 antigen by ELISA (Innotest HIV-1 Antigen mAb, Innogentics). HIV-1 primary isolate, 93BR029 (clade B, R5), and BaL isolate have been obtained through the NIH AIDS Reagent Program, and amplified on PBMCs, as described.[24,70]

HIV-1 neutralization assays

Single-cycle neutralization assay. The neutralization activity of Fabs was evaluated on CD8$^+$ T-cell-depleted PBMCs, or on CD4$^+$ T cells infected with the R5 HIV-1$_{JR-CSF}$ using intracellular p24 antigen (p24-Ag) staining, as described[70] (M. Bomsel Laboratory, unpublished data). Briefly, HIV-1 (3.3 μg of p24 per ml, which produces infection of roughly 8% target cells) was incubated with serial dilutions of the Fab for 1 h at 37 °C before addition to PHA-stimulated cells (1.5×10^5 cells) for 36 h in the presence of IL-2. The rest of the assay was performed as previously described.[70] In some experiments, 10 μM Zidovudine (AZT) (NIH AIDS Reagent Program) was included, before virus infection. Flow cytometry acquisitions, utilizing at least 20,000 events per condition, and analysis were performed using a Becton Dickinson (Franklin Lakes, NJ) FACSCalibur instrument and Cytomix RXP software. Live cells initially gated by forward and side scatter were analyzed for intracellular expression of p24-Ag as described previously.[71]

At least three independent experiments, each performed in triplicate, were performed. Neutralization was defined in the percentage of cells infected in the absence of antibody. The neutralizing titer was defined as the inhibitory concentration of the Fab (interpolated between successive dilutions performed in triplicate) that neutralized 50, 70, and 90% (IC50, IC70, and IC90, respectively) of the virus. IgG 2F5 was used as a positive control of neutralization.

Neutralization tests in macrophages using an R5 HIV BaL isolate (C. Moog Laboratory) were performed as described previously.[70]

Neutralization assays with envelope-pseudotyped viruses

Neutralization was also evaluated using a very sensitive test based on viruses pseudo-typed with an HIV-1 envelope (L. Lopalco Laboratory)—here the HIV-1 R5 tropic QH0692.42 envelope. Neutralization correlates directly with the reduction in luciferase reporter gene expression after a single round of virus infection in JC53-BL cells (also termed TZM-bl cells) as described previously.[72] The antibody concentrations causing 50% (IC50), 70% (IC70), and 90% (IC90) reductions in luciferase reporter gene production were determined by regression analysis.

HIV-1 transcytosis

Inhibition of HIV-1 transcytosis across monolayers of the HT29 epithelial cell line induced upon contact with HIV-1-infected PBMC with appropriate controls was performed as previously described.[11,73] PBMCs were infected with a R5 tropic clade B or clade C HIV-1 isolate as described.[74] Fabs were pre-incubated with HIV-1-infected PBMCs (0.8 million cells per point) for 30 min at 4 °C before the addition of the PBMCs at the apical side of epithelial cell monolayers (1 million cells) grown in a two-chamber system. Alternatively, the lymphocytic cell line CEM, chronically infected by the NDK (clade D, X4 tropic) clone was used.[73] Transcytosis of HIV-1 was evaluated by measuring p24 in the basolateral medium by ELISA (Beckman Coulter, Villepinte, France) after 2 h of apical contact of the epithelial monolayer with HIV-1-infected cells and was expressed as percentage of transcytosis in the absence of antibody. For each condition, at least three independent experiments were performed. IgG 2F5 was used as a positive control at 1 μg ml^{-1}.

Nucleic acid sequencing and sequence analysis

Sequencing of each Fab was performed on purified plasmid DNA with an automated DNA sequencer using appropriate primers and a Taq fluorescent dideoxynucleotide terminator cycle sequencing kit (Applied Biosystems, Foster City, CA).

The homology with known germ line VH and VL (Vκ, Vλ genes) was calculated by alignment of Fab sequences (from the 5′-end of framework 1 to the 3′-end of framework 3) to the GeneBank/EMBL and IMGT (Human Ig set) databases using NCBI BLAST and IMGT softwares. For analysis of light and heavy chain framework (FR) and complementary-determining region (CDR) sequences, we used the IMGT/V-QUEST software (http://imgt.cines.fr).[75] In this analysis, the most 5′-nucleotide for the CDR3 loop was considered to be the first nucleotide after codon 104 (Cys) of the variable region and the most 3′-nucleotide of CDR3 loop was assigned to the last nucleotide before codon 118 (W for H-chain and F for L-chain) downstream of the conserved motifs G-X-G, according to IMGT/V-QUEST software.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

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DISCLOSURE

The authors declared no conflict of interest.

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