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CD4 mimetics sensitize HIV-1-infected cells to ADCC

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HIV-1-infected cells presenting envelope glycoproteins (Env) in the CD4-bound conformation on their surface are preferentially targeted by antibody-dependent cell-mediated cytotoxicity (ADCC). HIV-1 has evolved a sophisticated mechanism to avoid exposure of ADCC-mediating Env epitopes by down-regulating CD4 and by limiting the overall amount of Env at the cell surface. Here we report that small-molecule CD4-mimetic compounds induce the CD4-bound conformation of Env, and thereby sensitize cells infected with primary HIV-1 isolates to ADCC mediated by antibodies present in sera, cervicovaginal lavages, and breast milk from HIV-1-infected individuals. Importantly, we identified one CD4 mimetic with the capacity to sensitize endogenously infected ex vivo-amplified primary CD4 T cells to ADCC killing mediated by autologous sera and effector cells. Thus, CD4 mimetics hold the promise of therapeutic utility in preventing and controlling HIV-1 infection.

HIV-1 | envelope glycoproteins | gp120 | CD4 mimetics | ADCC

Worldwide, it is estimated that more than 35 million people are living with HIV. In 2013 alone, around 2.1 million people became newly infected with HIV, and 1.5 million people died from AIDS (1). Measures to prevent HIV-1 transmission are desperately needed. Prevention of HIV-1 transmission and progression likely requires approaches that can specifically target and eliminate HIV-1-infected cells. Interestingly, there is increasing evidence supporting a role of antibody (Ab)-dependent cell-mediated cytotoxicity (ADCC) in controlling HIV-1 transmission and disease progression (2–8). Analysis of the correlates of protection in the RV144 vaccine trial suggested that increased ADCC activity was linked with decreased HIV-1 acquisition (9), and Abs with potent ADCC activity were isolated from some RV144 vaccinees (10). Recent studies reported that the viral accessory proteins Nef and Vpu protect HIV-1-infected cells from anti-HIV-1 envelope (Env)-mediated ADCC responses (11–14). Importantly, we and others reported that Env in the CD4-bound conformation was preferentially targeted by ADCC-mediating Abs and sera from HIV-1-infected individuals (11, 12, 15, 16), which represent a significant proportion of anti-Env Abs elicited during natural HIV infection (11, 17). However, the vast majority of circulating HIV-1 strains worldwide express functional Nef and Vpu proteins, which limit the exposure of CD4-induced (CD4i) Env epitopes at the surface of infected cells, likely preventing ADCC responses.

Theoretically, agents promoting the CD4-bound Env conformation should expose CD4i epitopes that are readily recognized by ADCC-mediating Abs and sera from infected individuals (11, 12, 15, 16, 18), resulting in the sensitization of HIV-1-infected cells to ADCC. Importantly, modulating Env conformation at the surface of HIV-1-infected cells has become feasible as a result of the availability of small CD4-mimetic compounds (CD4mc). The prototypes of such compounds, NBD-556 and NBD-557, were discovered in a screen for inhibitors of gp120-CD4 interaction (19). These small-molecule ∼337-Da compounds and recent derivatives (DMJ-I-228, JP-III-48) bind in the Phe-43 cavity (20–22), a highly conserved ∼150-Å^3 pocket in the gp120 glycoprotein located at the interface of the inner domain, outer domain, bridging sheet, and CD4 receptor (23). CD4mc block gp120-CD4 interaction and induce thermodynamic changes in gp120 similar to those observed during CD4 or soluble CD4 (sCD4) binding (24). Accordingly, these small molecules, as well as sCD4, can promote the transition of Env to the CD4-bound conformation, thus sensitizing HIV-1 particles to neutralization by otherwise nonneutralizing CD4i Abs (17, 25). Additional strategies using scaffolded miniproteins targeting critical gp120 elements required for CD4 interaction allowed the identification of CD4 mimetics

Significance

The prevention of HIV-1 transmission and progression likely requires approaches that can specifically eliminate HIV-1-infected cells. Rationally designed CD4-mimetic compounds (CD4mc) have been shown to efficiently inhibit viral entry and sensitize HIV-1 particles to neutralization by otherwise nonneutralizing CD4-induced antibodies. Here we found that CD4mc can also sensitize HIV-1-infected cells to antibody-dependent cell-mediated cytotoxicity (ADCC). Indeed, CD4mc induced the CD4-bound conformation of HIV-1 envelope glycoproteins, exposing CD4-induced epitopes recognized by easy-to-elicit antibodies present in sera, cervicovaginal lavages, and breast milk from HIV-1-infected individuals. Importantly, we provide evidence that CD4mc can efficiently sensitize primary CD4 T cells from HIV-1-infected individuals to ADCC mediated by autologous sera and effector cells. Therefore, CD4mc might represent an attractive approach to prevent and control HIV-1 infection.

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with nanomolar affinity for gp120 (26). One of these variants, M48U1, displayed remarkably potent neutralization of three HIV-1 isolates (27). Its crystal structure in complex with HIV-1 gp120 was recently solved, showing that M48U1 engages the Phe-43 cavity in a manner similar to that of CD4 (28); thus, M48U1 might induce gp120 to adopt the CD4-bound conformation and expose CD4i epitopes. Previous studies exploring the antiviral properties of CD4mc were performed on viral particles (17, 25, 27). However, whether these compounds are able to engage the large amounts of Env present at the surface of infected cells and modulate Env conformation in a way that allows exposure of ADCC-mediating epitopes is currently not known. In this study, we show that CD4mc strongly sensitize HIV-1-infected primary CD4 T cells to ADCC mediated by sera, cervicovaginal fluids, and breast milk from HIV-1-infected individuals, as well as help eliminate infected, ex vivo-expanded primary CD4 T cells from HIV-1-infected individuals. Therefore, CD4mc possess three valuable complementary antiviral properties: direct inactivation of viral particles, sensitization of viral particles to neutralization by otherwise nonneutralizing Abs, and sensitization of HIV-1-infected cells to ADCC-mediated killing.

Results

Env–CD4 Interaction Enhances Recognition of HIV-1-Infected Cells by Sera from HIV-1-Infected Individuals. We recently reported that Env interaction with the CD4 receptor at the surface of infected cells is critical for efficient ADCC activity mediated by monoclonal Abs targeting CD4i Env epitopes (12) or by sera from HIV-1-infected individuals (11). Env-CD4 interaction is modulated by the HIV-1 accessory proteins Nef and Vpu, which are known to decrease cell surface levels of CD4 (29, 30). In addition to its role in CD4 degradation, Vpu also antagonizes a restriction factor, Tetherin/bone marrow stromal antigen 2 (BST-2), which normally inhibits retroviral release (31, 32). Viruses lacking Vpu remain trapped at the cell surface, resulting in an accumulation of exposed Env (11–14). Therefore, Nef and Vpu can indirectly modulate Env–CD4 interaction at the surface of infected cells through CD4 and BST-2 down-regulation (11, 12). Cells infected with viruses defective for both Nef and Vpu present enhanced levels of CD4 and Env at the cell surface, resulting in the exposure of CD4i Env epitopes recognized by ADCC-mediating Abs such as A32 and HIV-1$^+$ sera (11, 12) (Fig. 1 *A* and *B*). However, if the ability of Env to interact with CD4 is decreased by a change near the CD4-binding site (D368R) (23, 33), Env CD4i epitopes are poorly exposed, resulting in decreased interaction with CD4i Abs and HIV-1$^+$ sera (11, 12) (Fig. 1 *A* and *B*).

CD4 Mimetics Sensitize HIV-1-Infected Cells to ADCC Mediated by Sera from HIV-1-Infected Individuals. We explored the capacity of different CD4mc to promote the CD4-bound conformation of Env, and thereby enhance Env recognition at the surface of HIV-1-infected cells by sera from HIV-1-infected individuals. sCD4 is the recombinant human CD4 protein lacking the transmembrane domain and cytoplasmic tail, and it is known to induce conformational changes in Env to some extent, similar to those induced by CD4 expressed on target cells. sCD4 induces formation of the bridging sheet and the coreceptor binding site, but certain gp120 epitopes, including potent ADCC targets in the C1–C2 region (A32-like epitopes), remain occluded in sCD4-triggered Env trimers (34, 35). These epitopes become exposed on virions only on the interaction of Env trimers with host CD4, indicating that binding membrane-anchored CD4 provides an additional energy component that is not provided by sCD4 (35). Rationally designed CD4mc (JP-III-48, DMJ-I-228) engage gp120 within the Phe-43 cavity (22) and can act as CD4 agonists, inducing thermodynamic changes in the Env trimer more similar to those observed during membrane CD4 binding (20, 24). Importantly, compounds of this class have been shown to sensitize HIV-1 particles to neutralization by CD4i and V3 nonneutralizing vaccine-elicited Abs (25). Fig. 1 demonstrates that Env present at the surface of cells infected with a wild-type (wt) virus is barely recognized by HIV-1$^+$ sera. This is a result of efficient CD4 down-regulation by the virus: Env cannot engage with CD4, and therefore remains in the unbound conformation, preventing CD4i epitope exposure (11, 12, 16). CD4mc (JP-III-48, DMJ-I-228) and sCD4 promote the exposure of Env CD4i epitopes, resulting in enhanced recognition of Env at the surface of HIV-1-infected cells by HIV-1$^+$ sera. As expected, when the ability of the virus to down-regulate CD4 is impaired by deleting *nef* (nef$^-$ or nef$^-$vpu$^-$), CD4mc do not enhance Env recognition by HIV-1$^+$ sera. In the absence of Nef, CD4 accumulates at the cell surface and interacts with Env; thus, in this case, CD4 blocks access to the Phe-43 cavity (11, 12), effectively competing for Env interaction. Cells infected with a wt virus express little Env at the cell surface because of the BST-2-counteracting effect of Vpu (11, 12), explaining why the enhancement by CD4mc is small. Deletion of *vpu* results in enhanced Env expression at the cell surface, likely resulting from prevention of viral release by BST-2 (11–14) (Fig. S1); in this context, CD4mc can engage more Env at the cell surface, resulting in a more pronounced effect on Env recognition by HIV-1$^+$ sera. Under these conditions, infected cells treated with CD4mc reach the same level of recognition as cells infected with a nef$^-$vpu$^-$ virus (Fig. 1 *C* and *D*). Similar results were observed with M48U1, a miniprotein CD4 mimic that also engages the gp120 Phe-43 cavity with nanomolar affinity (28) (Fig. S2). Interestingly, CD4mc JP-III-48 enhanced recognition of an Env variant (D368R) unable to efficiently engage CD4. Thus, Env adoption of the CD4-bound conformation, but not CD4 interaction per se, appears to be required to expose CD4i epitopes at the cell surface (Fig. S3).

Importantly, CD4mc enhancement of Env recognition by sera from several HIV-1-infected individuals (Fig. 1 *D*–*F*) is translated into higher ADCC killing of infected cells by effector peripheral blood mononuclear cells (PBMCs) (Fig. S4). It is worth noting that the effect of CD4mc on Env detection and sensitization to ADCC is also observed when primary CD4 T cells from healthy individuals, rather than CEM-NKr cells, are used as target cells (Fig. 2). Interestingly, we observed that the effect of CD4mc was more pronounced when primary CD4 T cells were used. This could be a result of enhanced levels of Env presented at the surface of these cells compared with CEM-NKr (Fig. S5).

CD4 Mimetics Enhance Recognition and Killing of Cells Infected with Primary HIV-1 Strains. To ensure that sensitization of HIV-1-infected cells by CD4 mimetics was also observed when using full-length clinically relevant primary HIV-1 isolates, we infected primary CD4 T cells with extensively characterized infectious molecular clones (IMCs) constructed from two transmitted/founder (T/F) and their corresponding 6-mo consensus sequences (36–39). Primary viruses are known to exhibit low Env reactivity and, as such, have little or no intrinsic exposure of CD4i epitopes (40). JP-III-48 and DMJ-I-228 CD4 mimetics were able to significantly enhance recognition of cells infected with the four primary viruses by HIV-1$^+$ sera (Fig. 3*A*); cells infected with T/F and 6-mo IMCs from CH58 exhibited a greater enhancement of recognition compared with the T/F and 6-mo IMCs from CH77 (Fig. 3). This is likely related to the levels of Env present at the surface of infected cells; cells infected with CH58 T/F or 6-mo viruses expressed higher surface Env levels than cells infected with CH77 T/F or 6-mo strains (Fig. S6). Altogether, these results indicate that Env conformational changes induced by CD4mc are not just a function of TF viruses but are also maintained during chronic infection.

We found that JP-III-48 binds monomeric gp120 from the YU2 strain of HIV-1 with higher affinity than DMJ-I-228 (Table S1). As a result, JP-III-48 exhibits much more potent inhibitory activity against two HIV-1 strains (Table S1); this suggests that the ability of JP-III-48 to bind and/or induce conformational changes in the functional HIV-1 Env trimer is superior to that of DMJ-I-228. Accordingly, JP-III-48 was more effective at stimulating ADCC than

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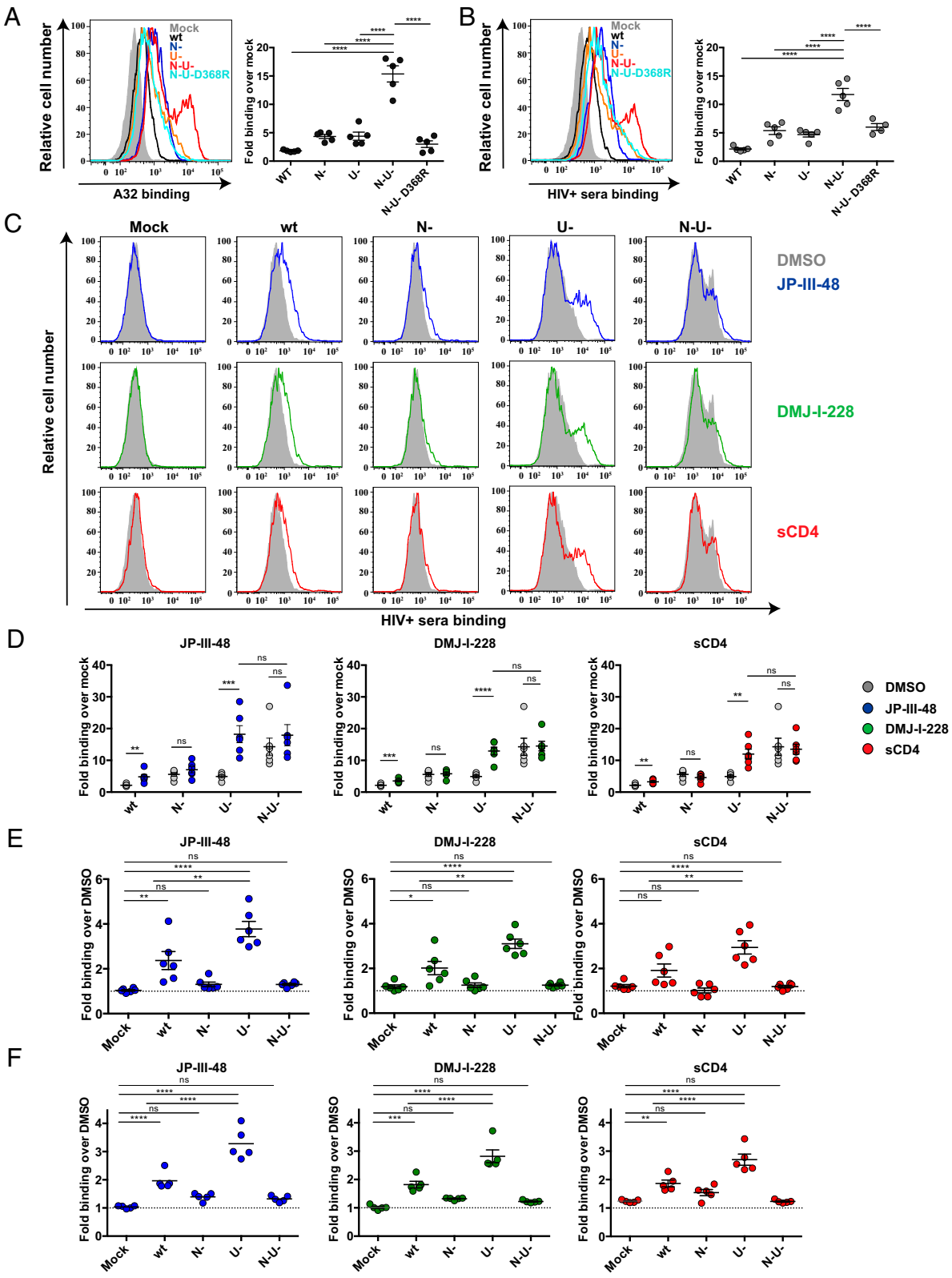

Fig. 1. Env interaction with CD4 or CD4mc enhances recognition of HIV-1-infected cells by sera from HIV-1-infected individuals. Cell-surface staining of CEM. NKr cells infected with NL4.3 GFP ADA-based viruses [wt, lacking Nef (*N*–), Vpu (U–), or both Nef and Vpu (*N*–U–)] and expressing wt Env or D368R Env (*N*–U–D368R) with (*A*) A32 mAb or (*B*) sera from an HIV-1-infected donor. Shown in *A* and *B* are histograms depicting representative staining of infected cells (GFP^+). (*Right*) Fold increase of staining relative to mock-infected cells. (*C*) Staining of mock-infected or HIV-1-infected cells with sera from an HIV-1-infected donor in the presence of CD4 mimetics or vehicle (DMSO). (*D*) Quantification of data presented in *C* as fold binding over mock in 6 independent experiments. In *E*, the data were calculated as a fold increase between CD4 mimetic-treated cells over cells treated with the vehicle (DMSO) only. (*F*) Effect of CD4 mimetics on the recognition of infected cells (as described in *C*) by sera from 5 additional HIV-1-infected individuals. Error bars indicate mean ± SEM. Statistical significance was tested using (*A, B, E*) ordinary one-way ANOVAs, (*D*) an unpaired *t* test, or (*F*) paired one-way ANOVAs (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).

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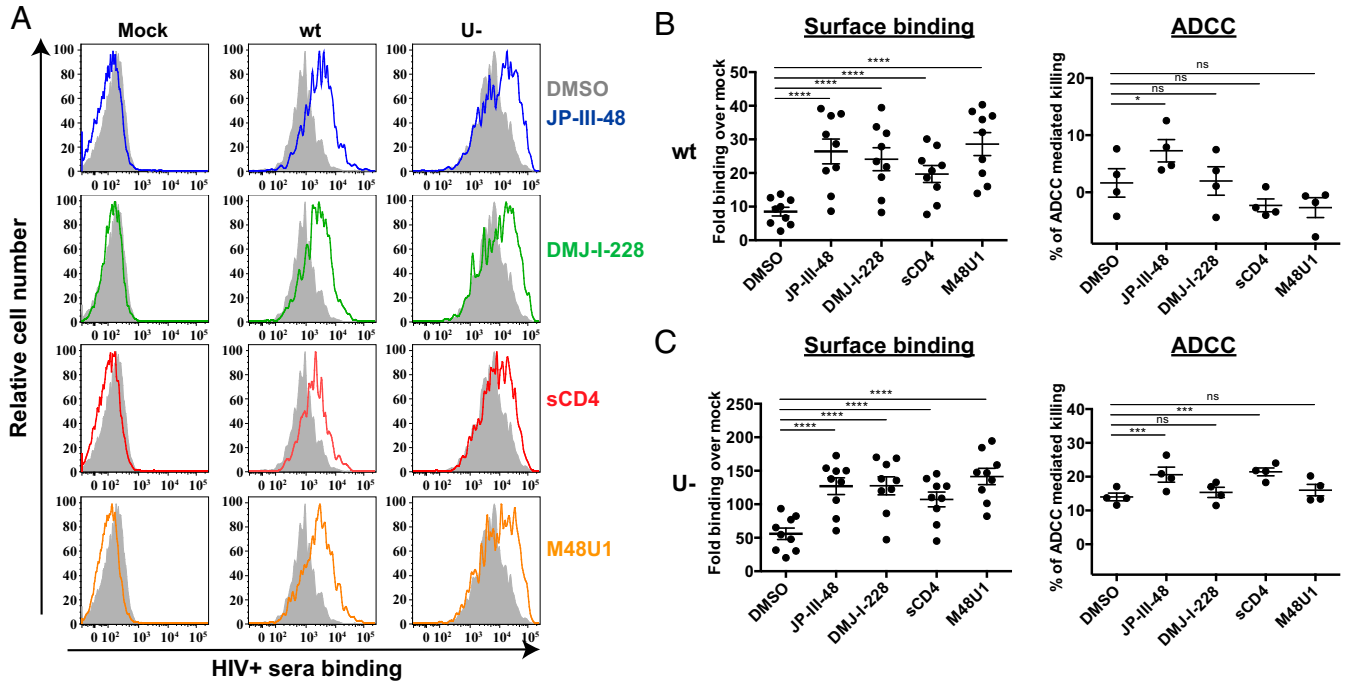

Fig. 2. CD4 mimetics enhance recognition and ADCC-mediated killing of HIV-1-infected primary CD4 T cells. Staining of primary CD4 T cells infected or not with NL4.3 GFP ADA-based viruses either wt or lacking Vpu (U−) with sera from nine HIV-1-infected individuals in the presence of CD4 mimetics or the vehicle (DMSO). (*A*) Histograms depicting representative staining of infected (GFP^+) or mock infected cells with HIV-1^+ sera. (*Left*) Quantification of cell-surface staining of wt (*B*) or U− (*C*) infected cells, as fold binding over mock. (*Right*) Ability of the sera to mediate ADCC-mediated killing of infected primary CD4-T cells in the presence of CD4 mimetics or the vehicle (DMSO), using autologous PBMCs. Error bars indicate mean ± SEM. Statistical significance was tested using a paired one-way ANOVAs test (*$P < 0.05$; ***$P < 0.001$; ****$P < 0.0001$).

DMJ-I-228 (Fig. 3 and Table S1). sCD4 did not enhance recognition or killing of infected cells. The effect of M48U1 was less than that observed with CD4mc for CH77 T/F or 6-mo strains. The quaternary architecture of some primary Envs might pose constraints against proteins engaging the Phe-43 cavity; because of their smaller size (300–400 Da), CD4mc could bypass such constraints.

The CD4mc JP-III-48 Sensitizes HIV-1-Infected Cells to ADCC Mediated by Abs Present in Cervicovaginal Fluids, Breast Milk, and Early-Infection Sera. Figs. 1–3 indicate that CD4mc can sensitize HIV-1-infected cells to ADCC mediated by sera from chronically HIV-1-infected individuals. Previous studies (17) have suggested that some Abs against CD4i Env epitopes are elicited early during the course of HIV-1 infection. Therefore, we evaluated whether anti-Env Abs elicited during the first few weeks of infection could eliminate HIV-1-infected cells in the presence of CD4mc. Because we consistently observed enhanced sensitization of HIV-1-infected cells to ADCC by JP-III-48 compared with the other CD4 mimetics tested in this study (Figs. 2 and 3), we selected JP-III-48 for the next set of experiments. We obtained sera from six early HIV-1 converters (within 4 wk of the estimated date of infection) from the Fonds de Recherche Québec Santé-AIDS Montreal Primary HIV-1-infection cohort and assessed their ability to mediate ADCC of primary CD4 T cells infected with T/F CH58. Sera from early converters did not recognize Env at the surface of infected cells, nor did they mediate ADCC in the absence of CD4 mimetics. However, JP-III-48 addition allowed Env recognition and significantly enhanced killing of infected cells by these sera (Fig. 4*A*). These observations suggest that administration of CD4mc during the early phase of HIV-1 infection might benefit infected individuals by contributing to the elimination of infected cells. Abs able to mediate ADCC have also been reported in cervicovaginal lavages (CVLs) (18, 41, 42) as well as in breast milk (7, 43) from HIV-1-infected women. CVLs from HIV-1-infected Beninese commercial sex workers and breast milk from HIV-1-infected Malawian women (43) were evaluated for their ability to mediate ADCC of primary infected CD4 T cells. As observed for sera from early seroconverters, in the absence of CD4-mimetics, CVLs and BM samples were unable to detect Env or mediate ADCC of HIV-1-infected primary CD4 T cells. However, addition of the CD4mc JP-III-48 significantly enhanced Env detection and killing of these cells (Fig. 4 *B* and *C*). This is in agreement with a previous report that found that HIV-1-Env-specific IgG in genital mucosal compartments preferentially targets Env in the CD4-bound conformation and can mediate ADCC (18). Sera, CVL, and breast milk samples from uninfected individuals failed to recognize Env in the presence or absence of the CD4mc JP-III-48 (Fig. 4, *Left*).

A CD4mc Sensitizes ex Vivo-Amplified Primary CD4 T Cells from Viremic Untreated HIV-1-Infected Individuals to ADCC-Mediated Killing. To evaluate the potential of a CD4mc to sensitize endogenously infected cells, we purified primary CD4 T cells from four viremic untreated HIV-1-infected individuals. Cells were activated with phytohemagglutinin (PHA) for 36 h and then cultured in the presence of recombinant (r)IL-2. Six days after activation, viral replication was measured by intracellular p24 staining, and Env was detected at the surface of $p24^+$ cells with autologous and heterologous HIV-1^+ sera (Fig. 5 *A*–*D*). Significantly, in the four tested individuals, the CD4mc JP-III-48 enhanced Env detection by both autologous and heterologous sera, whereas activated primary CD4 T cells from uninfected subjects were not recognized in the absence or presence of the CD4mc (Fig. S7). Importantly, enhanced recognition of infected cells by HIV^+ sera resulted in a significant increase in ADCC killing mediated by autologous effector PBMCs (Fig. 5 *E* and *F*). These results highlight the potential of CD4mc to sensitize HIV-1-infected cells to ADCC in viremic HIV-1-infected individuals.

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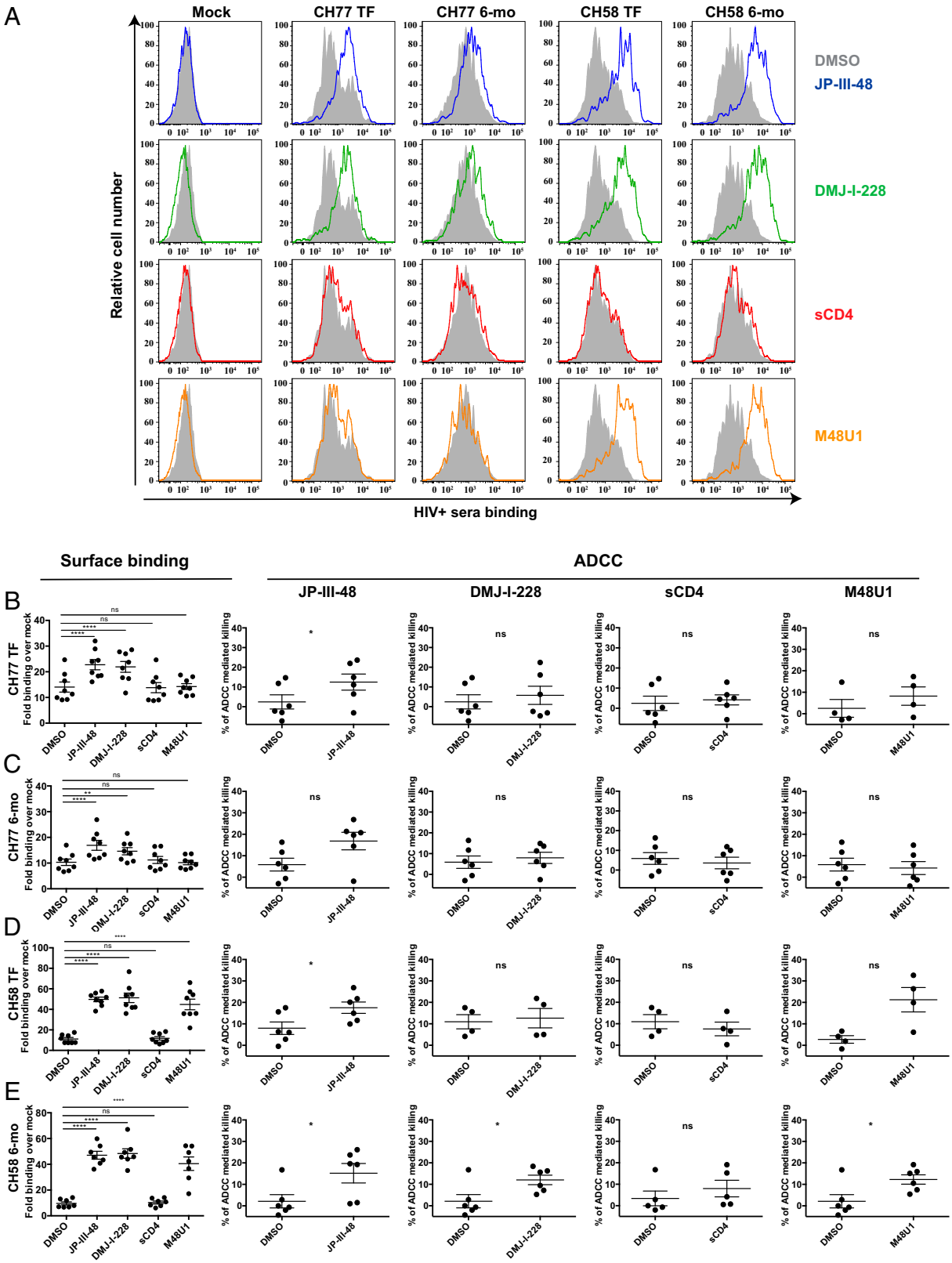

Fig. 3. CD4 mimetics sensitize primary CD4 T cells infected with HIV-1 primary isolates to ADCC. Staining of primary CD4 T cells infected or not with infectious molecular clones (IMCs) representing T/F and corresponding 6-mo consensus viruses from two longitudinally followed patients CH77 and CH58 with sera from eight HIV-1-infected individual in the presence of CD4 mimetics or vehicle (DMSO). (*A*) Histograms depicting representative staining of infected ($p24^+$) or mock-infected cells. (*Left*) Quantification of cell-surface staining of CH77 T/F (*B*), CH77 6-mo (*C*), CH58 T/F (*D*), and CH58 6-mo (*E*) infected cells, as fold binding over mock. (*Right*) Ability of the sera to mediate ADCC-mediated killing of infected primary CD4-T cells in presence of CD4 mimetics or the vehicle (DMSO), using autologous PBMCs. Error bars indicate mean ± SEM. Statistical significance was tested using a paired one-way ANOVAs test for the left panels and a paired *t* test for the right panels (*$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$).

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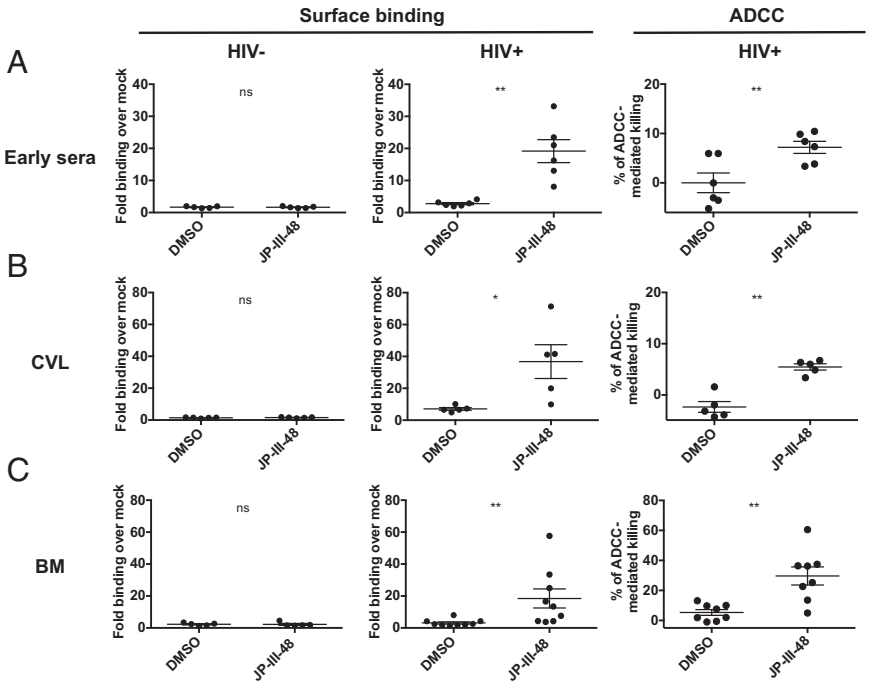

Fig. 4. The CD4mc JP-III-48 sensitizes primary CD4 T cells infected with primary HIV-1 isolates to ADCC mediated by sera from early converters, CVLs, and breast milk from HIV-1-infected individuals. Staining of primary CD4 T cells infected or not with T/F CH58 virus (CH58 T/F) with samples obtained from uninfected (HIV-negative) donors (first column) or from HIV-1-infected individuals (second and third columns). Sera from early converters (*A*), CVLs (*B*), or breast milk (*C*) were used at 1/1,000 (sera) and 1/100 (CVL and breast milk) dilutions to stain infected cells (first and second columns) or to mediate ADCC by autologous PBMCs in the presence of vehicle alone or JP-III-48 CD4 mimetic (third column). Error bars indicate mean ± SEM. Statistical significance was tested using a paired *t* test or a Wilcoxon matched-pairs signed rank test for breast milk samples (*$P < 0.05$; **$P < 0.01$).

Discussion

The HIV-1 accessory proteins Nef and Vpu help protect HIV-1-infected cells from ADCC by decreasing the overall amount of Env at the cell surface (11–14), and by down-regulating CD4 (11, 12), which otherwise engages Env, induces the CD4-bound conformation and exposes CD4i epitopes. These epitopes are readily recognized by several well-established ADCC-mediating Abs isolated from chronically infected individuals or RV144 vaccinees (12) or by sera from HIV-1-infected individuals, which are known to have a large fraction of nonneutralizing CD4i Abs (11, 17). However, the vast majority of new HIV-1 infections worldwide are mediated by viruses coding for functional Nef and Vpu proteins and expressing Envs with low intrinsic reactivity (40); these factors limit the exposure of CD4i epitopes that would otherwise be recognized by CD4i Abs with the potential to mediate ADCC (11, 12, 16). It is becoming increasingly clear that HIV-1 has evolved multiple means to avoid exposure of Env CD4i epitopes at the surface of infected cells (11, 12). Therefore, strategies aimed at exposing these epitopes by either targeting Vpu and/or Nef or by engaging Env to induce the exposure of these epitopes might sensitize HIV-1-infected cells to ADCC. Recent advances in the development of a series of CD4 mimetics that engage Env in a CD4-like manner and that induce similar conformational changes, resulting in the exposure of CD4i epitopes, permitted us to test this strategy. We observed that CD4mc can indeed expose CD4i epitopes at the surface of infected primary CD4 T cells and effectively sensitize them to ADCC killing mediated by CVLs, breast milk, and sera from early and chronic HIV-1-infected individuals. This was observed with a variety of HIV-1 variants, notably including primary T/F viruses and their 6-mo counterparts. CD4mc were more efficient than protein-based CD4 mimetics (sCD4, M48U1) at sensitizing cells infected with primary HIV-1-isolates to ADCC. The quaternary architecture of primary Envs may resist engagement with proteins such as sCD4 and M48U1; the smaller size of the CD4mc might allow them to bypass such structural constraints. The resistance of most primary HIV-1 Envs to sCD4 engagement may explain why sCD4 exhibited only minimal in vivo potential as an antiviral agent (44). CD4mc have been shown to sensitize HIV-1 virions to neutralization by otherwise nonneutralizing CD4i Abs that can easily be generated through vaccination with CD4-bound stabilized gp120s (25, 45, 46). Importantly, these types of Abs were isolated from RV144 vaccinees and possessed ADCC potential (10, 12). Therefore, one practical use of the observed ADCC and neutralization sensitization might be the enhancement of vaccine efficacy by CD4mc. A frustrating aspect of HIV-1 vaccine development is the difficulty of eliciting Abs that potently neutralize diverse strains of virus (47–49). Therefore, the dual effect of these CD4mc (i.e., sensitization of HIV-1 viral particles to neutralization and infected cells to ADCC by CD4i Abs) might help limit viral transmission. Such CD4i Abs are elicited early and in a high proportion of infected individuals (11, 17) and can also be elicited by vaccination with "stabilized gp120 cores" that have been engineered to assume the CD4-bound state (45). One can envision a multicomponent vaccine regimen in which one of the immunogens is a stabilized gp120 core that elicits CD4i Abs and CD4mc administered orally or in a microbicide formulation that could sensitize a range of T/F viruses and infected cells to inhibition by the vaccine-elicited Abs. Finally, our observation that endogenously infected, ex vivo-amplified CD4 T cells can be sensitized to ADCC mediated by autologous sera suggest that CD4mcs could be useful for the "kill" part of the "shock and kill" strategy being pursued to purge the HIV reservoir (50). Moreover, by targeting both viral particles and infected cells, CD4 mimetics might represent an alternative and/or complementary approach to currently available drugs for preventing viral transmission and treating HIV-1-infected individuals.

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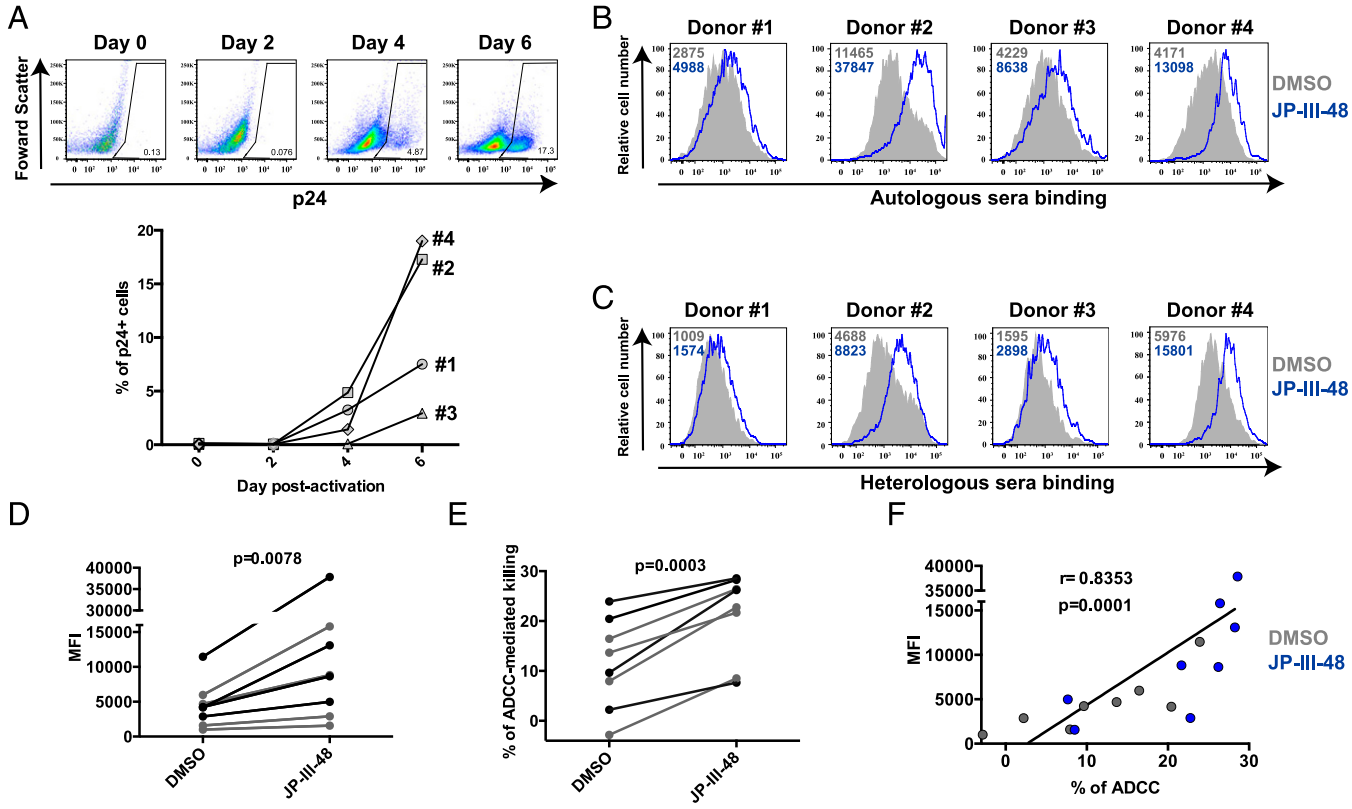

Fig. 5. The CD4mc JP-III-48 sensitizes ex vivo-expanded endogenously infected primary CD4 T cells from viremic antiretroviral therapy (ART)-naive individuals to ADCC-mediated killing by autologous sera. (*A*) The top panel show percentages of p24$^+$ CD4 T cells expanded after activation from a representative HIV-1-infected untreated viremic patient, while the bottom panel represents the percentages of p24$^+$ CD4 T cells expanded after activation for four HIV-1-infected viremic patients (Table S2). Six days postactivation, cells were stained with (*B*) autologous sera or (*C*) heterologous HIV$^+$ sera in presence of the CD4mc JP-III-48 or the vehicle (DMSO). The data shown in *B* and *C* are histograms depicting staining of p24$^+$ cells and are presented as mean fluorescence intensity (MFI). (*D*) Data shown represent paired values MFI obtained with autologous sera (depicted in black) and heterologous HIV$^+$ sera (depicted in gray) in the presence of vehicle alone (DMSO) or the CD4mc JP-III-48. (*E*) Six days postactivation, cells were used as target cells to evaluate their susceptibility to ADCC by autologous PBMCs in the presence of the CD4mc JP-III-48 or the vehicle (DMSO) by autologous sera (depicted in black) or heterologous HIV$^+$ sera (depicted in gray). (*F*) A positive correlation was observed between the staining intensity of autologous and heterologous HIV$^+$ sera on CD4 T cells and their ability to mediate ADCC. Statistical significance was tested using (*D*) the Wilcoxon matched-pairs signed rank test, (*E*) a paired *t* test, and (*F*) a Spearman correlation.

In summary, we report that CD4mc induce the CD4-bound conformation of Env, exposing CD4i epitopes recognized by easy-to-elicit Abs present in sera, CVLs, and breast milk from HIV-1-infected individuals. CD4mc effectively sensitize HIV-1-infected cells to ADCC and thus might have therapeutic utility for preventing transmission and decreasing the number of infected cells in HIV-1-infected individuals.

Materials and Methods

Cell Lines and Isolation of Primary Cells. 293T human embryonic kidney (obtained from ATCC), CEM.NKr cells (obtained from David Evans, Harvard Medical School), and primary cells were grown as previously described (12, 51). CD4 T lymphocytes were purified from rested PBMCs by negative selection and activated as previously described (11).

Plasmids and Site-Directed Mutagenesis. pNL43-ADA(Env)-GFP.IRES.Nef proviral vectors (deleted for *vpu*, *nef*, and *nef and vpu* or expressing the D368R Env variant) and the VSVG-encoding plasmid (pSVCMV-IN-VSV-G) were previously described (12, 51). T/F and corresponding 6-mo consensus IMCs of patients CH58 and CH77 were inferred, constructed, and biologically characterized as described (36–39).

Viral Production, Infections, and Ex Vivo Amplification. Vesicular stomatitis virus G-pseudotyped NL4.3 GFP-encoding ADA-based viruses were produced and titrated as previously described (12). Viruses were then used to infect ~20–30% of CEM.NKr cells or primary CD4 T cells from healthy donors by spin infection at 800 × *g* for 1 h in 96-well plates at 25 °C. To expand endogenously infected CD4 T cells, primary CD4 T cells were isolated from PBMCs obtained from viremic untreated HIV-1-infected individuals. Purified CD4$^+$ T cells were activated with PHA-L at 10 μg /mL for 36 h and then cultured for 6 d in RPMI-1640 complete medium supplemented with rIL-2 (100 U/mL).

CD4 Mimetics. sCD4 and the miniprotein M48U1 were produced and purified as previously described (26, 52). The CD4-mimetic small molecules JP-III-48 and DMJ-I-228 were synthesized as described previously (20, 21). The compounds were analyzed, dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 10 mM, aliquoted, and stored at −20 °C. Each compound was then diluted to 50 μM in PBS for cell surface staining or in RPMI-1640 complete medium for ADCC assays.

Flow Cytometry Analysis of Cell Surface Staining and ADCC Responses. Cell surface staining was performed as previously described (11, 12). Binding of HIV-1-infected cells by sera (1:1,000 dilution), breast milk (1:100 dilution), or concentrated CVLs (1:100 dilution) was performed 48–72 h after in vitro infection or 6 d postactivation for endogenously infected, ex vivo-amplified cells at 37 °C in the presence of the CD4 mimetics JP-III-48 (50 μM), sCD4 (10 μg/mL), or M48U1 (100 nM) or with equivalent volume of vehicle (DMSO). Cells infected with HIV-1 primary isolates or for ex vivo amplification were stained intracellularly for HIV-1 p24, using the Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences) and then fluorescent anti-p24 mAb (PE-anti-p24, clone KC57; Beckman Coulter/Immunotech; 1:100 final concentration). The percentage of infected cells (p24$^+$ or GFP$^+$ cells) was determined by gating the living cell population on the basis of viability dye staining. Samples were analyzed on an LSRII cytometer (BD Biosciences), and data analysis was performed using FlowJo vX.0.7 (Tree Star).

Measurement of serum-mediated ADCC was performed with a previously described assay (12, 15) after 48–72 h for in vitro infection or 6 d postactivation

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for endogenously infected ex vivo-amplified cells using a 1:1,000 final concentration of serum, 1:100 dilution of concentrated CVLs or breast milk samples. The percentage of cytotoxicity was calculated with the following formula: (relative count of GFP^+ cells in Targets plus Effectors) − (relative count of GFP^+ cells in Targets plus Effectors plus A32 or serum)/(relative count of GFP^+ cells in Targets), as described (12, 15). For cells infected with HIV-1 primary isolates or for ex vivo amplification, infected cells were identified by intracellular staining for HIV-1 p24. In that context, the percentage of cytotoxicity was calculated with the following formula: (% of $p24^+$ cells in Targets plus Effectors) − (% of $p24^+$ cells in Targets plus Effectors plus serum)/(% of $p24^+$ cells in Targets) by gating infected lived target cells.

Statistical Analyses. Statistics were analyzed using GraphPad Prism version 6.01 (GraphPad). P values <0.05 were considered significant; significance values are indicated as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$.

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