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**Soluble CD4 and Low Molecular Weight CD4-Mimetic Compounds Sensitize Cells
to be Killed by Anti-HIV Cytotoxic Immunoconjugates**

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Running Title: CD4-Mediated Enhancement of Immunoconjugate Killing

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ABSTRACT

The reservoir of HIV-infected cells that persist in the face of effective anti-retroviral therapy (ART) is the barrier to curing HIV infection. These long-lived CD4⁺ cells carry a functional provirus that can become activated upon immune stimulation. When ART is stopped, this leads to a rapid rebound in viremia. A variety of approaches are proposed to eliminate these cells, many dependent upon expression of virus proteins. We are examining the use of cytotoxic immunoconjugates targeting the HIV envelope protein (Env) as a method to eradicate cells producing virus, and have demonstrated that soluble CD4 enhances the cytotoxic effect of gp41-targeted immunoconjugates. Mechanisms include increased antigen exposure and greater internalization of the immunoconjugate. Here we have tested different protein forms of CD4 and the small molecule CD4-mimetic BNM-III-170 for their effects upon cells expressing cell-surface Env. Effects studied include sensitization to immunoconjugate killing, cell surface antigen expression, viability, and virus secretion. The CD4 proteins and BNM-III-170 produced comparable effects in these Env-expressing cell lines, each sensitizing cells to cytotoxicity by anti-gp41 immunoconjugates. The results provide further evidence that low molecular weight CD4-mimetics produce biologic effects similar to those caused by soluble CD4 itself and suggest additional therapeutic uses for these molecules.

IMPORTANCE

HIV infection can be effectively treated to prevent the development of AIDS, but it cannot be cured. We have attached poisons to anti-HIV antibodies to kill the infected cells that persist even after years of effective antiviral therapy. Here we show that the killing of infected cells can be markedly enhanced by the addition of soluble forms of the HIV receptor CD4 or by mimics of CD4.

INTRODUCTION

HIV infection cannot be cured, except under rare circumstances (1, 2). Patients must remain on life-long anti-retroviral therapy (ART) to suppress viremia and remain free of AIDS-associated morbidity and mortality (3). This is due to a reservoir of long-lived CD4⁺ cells that carry a functional provirus and persist despite years of fully suppressive ART (4-6). Many approaches to eliminating the reservoir depend upon expression of HIV proteins in the reservoir cells, and thus a major effort has been devoted to developing methods to induce HIV expression, so called latency reversing agents (7-13). In this scenario, patients would be protected from this induced virus production by continued or even intensified ART. Several virus activation approaches have been tested clinically and others in animal models, many showing the ability to enhance HIV expression. Initial studies depended upon viral cytopathic effect or host immune responses to eliminate productively infected cells, to little effect. More recently the use of both passive and active immunization has been combined with latency reversal with more promising results (9, 14-17). Our ongoing efforts have been to enhance the ability of anti-HIV monoclonal antibodies (mAbs) to eliminate cells of the persistent reservoir by conjugating them to toxic molecules, making cytotoxic immunoconjugates (CICs).

CICs are bifunctional molecules, one portion of which targets the CIC to the appropriate cell, the other is the toxic moiety, whether a protein toxin (immunotoxins), small drug (antibody-drug conjugate), or high intensity radioisotope (radioimmunoconjugate). We and others have used either anti-Env mAbs or CD4 to target these toxic agents to HIV-infected cells and have shown efficacy in vitro and in animal models (18-36). One CIC, designated CD4-PE40, was tested clinically, with toxicity limiting its utility (37). In both mice and macaques, newer anti-HIV CICs have demonstrated greater efficacy without the dose-limiting toxicity of CD4-PE40 (30, 32, 34, 38). We have tested several large panels of anti-Env mAbs and have found that mAbs to the transmembrane domain of Env, gp41, and in particular to the immunodominant helix-loop-helix

domain (ID-loop) of gp41, make the most effective CICs (33, 39). In the prefusion state of Env this region is both highly mobile and lies close to the cell membrane (40, 41), which may be why it serves as an effective target. We have also shown that the cytotoxic efficacy of these anti-gp41 CICs can be enhanced 30-100X by the addition of soluble CD4 (sCD4), which we attribute to both increased antigen exposure and enhanced internalization (25, 33, 39).

In the absence of CD4, the Env trimer exists in a stable closed conformation (42). Gp120, the external domain of Env, undergoes a conformational shift upon binding its ligand, CD4. Gp120 assumes an open conformation and dissociates from gp41, allowing the latter to form an extended infectious form. Binding of virion and cell-surface Env by sCD4 can induce these changes (42). Multiple different forms of sCD4 have been produced, including a short two domain structure and an Ig-fusion molecule in various conformations (42-46). Some CD4 constructs have been tested in clinical trials with limited success (42, 47). More recently, a series of low molecular weight CD4-mimetic compounds (CD4mc) have been developed that reproduce the biological effects of CD4 itself (13, 48-59). BNM-III-170 is the prototype of many of these compounds. In this publication we have compared the effects of two different protein forms of sCD4 and BNM-III-170 for their effects on Env-antigen exposure and on the ability to sensitize cells to killing by different CICs. We used two Env-expressing cell lines: one infected, one transfected. We find BNM-III-170 accurately reproduces the effects of sCD4. If CICs were to be used therapeutically, combining their use with CD4mc's may serve several purposes: enhancing CIC efficacy, neutralizing virus infection, and eliciting Fc-mediated effector functions including antibody-dependent cellular cytotoxicity (ADCC).

RESULTS

Soluble CD4 and CD4-mimetics sensitize cells to killing by anti-gp41 immunotoxins.

We have tested the ability of the CD4mc BNM-III-170 and two different forms of sCD4, CD4₁₈₃ and CD4-hIgG2, to sensitize cells for killing by anti-HIV CICs. CD4₁₈₃ consists of the two N-terminal domains of CD4, whereas CD4-hIgG2 displays the N-terminal domains as a tetramer. We primarily used an immunotoxin consisting of deglycosylated ricin A chain (dgA) conjugated to the anti-gp41, ID-loop specific mAb 7B2. The target cell lines were persistently infected H9/NL4-3, which expresses the prototype IIIB ENV, and HEK-293T/92UG (henceforth 92UG) transfected to express Env of the Clade C clinical isolate 92UG037.8. These cell lines are unique in that they stably express gp160 on the surface of virtually all cells and demonstrate no cytopathic effects, both essential characteristics for performing cytotoxicity assays. To test CIC-mediated killing, cells were incubated with the CIC and sensitizing agent for 3 days. In Figure 1A, we demonstrate the enhancement of 7B2-dgA-mediated killing in the presence or absence of 20 nM CD4₁₈₃. As we have previously observed (34, 39), the 92UG cells were more sensitive to CIC-mediated killing than H9/NL4-3, but the degree of CD4-mediated enhancement was greater in the H9/NL4-3. Figure 1B confirms the specificity of the cytotoxicity on CD4-sensitized cells, with a thousand-fold difference between the IC₅₀ on 92UG and the untransfected parental line HEK-293T. A comparison of figures 1A and 1B shows a similar difference in the killing of H9/NL4-3 and uninfected H9 by 7B2-dgA.

In Figure 2A, we titrated the concentration of sCD4 or CD4mc necessary to enhance the cytotoxicity of a limiting concentration of 7B2-dgA. Both CD4₁₈₃ and BNM-III-170 enhanced killing of both H9/NL4-3 and 92UG cells. Temsavir had no effect. Figure 2A also demonstrates that CD4₁₈₃ sensitized both cell types to CIC-mediated killing equally, whereas BNM-III-170 had a greater effect on the H9/NL4-3 cells than 92UG. In Figure 2B we titrated 7B2-dgA against constant concentrations of BNM-III-170 (10 μ M with H9/NL4-3 cells, 30 μ M with 92UG), CD4₁₈₃

(20nM), and CD4-hlgG2 (11nM). BNM-III-170 provided equal or greater enhancement of the activity of 7B2-dgA than the two protein forms of sCD4. While both CD4₁₈₃ and CD4-hlgG2 had identical cytotoxicity curves on H9/NL4-3, CD4-hlgG2 was more effective on 92UG. In comparing results in Figure 1A to those in Figure 2B, some variability is observed in the response of H9/NL4-3 to cytotoxicity by 7B2-dgA. We attribute this to differences in the cell populations at the time treatment was initiated, variability that may include growth kinetics, metabolic state, and infection status. For this reason, any panel within the figures in this manuscript use the same cell population as part of a single experiment,

In Figure 3 we compare the dose-response of enhancement of CIC killing by BNM-III-170 and CD4-hlgG2 on the two cell lines. Whereas the efficacy of CD4-hlgG2 was the same on both cells, H9/NL4-3 were significantly more sensitive to BNM-III-170. In sum, the data presented in Figures 2 and 3 demonstrate that BNM-III-170, as well as both forms of sCD4 enhance the efficacy of 7B2-dgA-mediated killing of Env-expressing cells. BNM-III-170 demonstrated a greater effect on the infected H9/NL4-3 than on the 92UG. The concentrations of BNM-III-170 that produce 50% enhancement of CIC cytotoxicity, ~3μM for H9/NL4-3 and ~15μM for 92UG, are similar to those reported for inhibition of virus infectivity and enhancing ADCC (52, 54, 55, 57).

In our search for the most effective mAbs to target CICs to HIV-infected cells, we have produced multiple different dgA-based immunotoxins. In Figure 4, we compare the effects of CD4-hlgG2 and BNM-III-170 on immunotoxins targeted by three different mAbs: 7B2, A32 which binds a CD4-inducible epitope on gp120 and serves as a target for ADCC (60, 61), and PGT126, a clonal sibling of the better-studied PGT128, a broadly neutralizing mAb that binds a complex peptide/glycan epitope at the base of the gp120 V3 loop (62). As we have previously reported, the anti-gp41 CIC, 7B2-dgA is the most effective of the immunotoxins. It is further enhanced by both CD4-hlgG2 and BNM-III-170, as we have shown in Figures 2 and 3. A32-dgA shows marginal efficacy in 92UG when used alone, but measurable killing in the presence of

CD4-hlgG2, and to a lesser degree with BNM-III-170. Neither CD4-hlgG2 nor BNM-III-170 affect the cytotoxicity of PGT126-dgA, which kills 92UG cells, but not H9/NL4-3.

Effects of sCD4 and BNM-III-170 on cellular functions.

We have studied the effects of BNM-III-170, and the two sCD4 forms on viability, virus production, and cell-surface Env expression. To rule out the possibility that the enhanced cytotoxicity we observed was due to a direct effect of binding cell-surface gp120 on cell viability, we measured MTS-dye reduction, a measure of oxidative metabolism, in cells exposed to BNM-III-170 or CD4-hlgG2 for three days (Figure 5A). We tested BNM-III-170 on H9/NL4-3 cells at 22 μ M, which is more than twice the concentration used to sensitize H9/NL4-3 cells in prior figures (10 μ M). There was no effect on viability. BNM-III-170 also has no effect on viability of 92UG cells, tested at 30 μ M, the concentration used in previous experiments (not shown). In the same experiment as Figure 5A, we asked whether engaging the CD4-binding site of gp120 affected virus secretion by H9/NL4-3 cells (Figure 5B). On days 1 and 2, there was no difference between untreated or either of the treated cultures. While the production of virus may have diminished on day 3 in the BNM-III-170 treated cells, the difference was not statistically significant (student t test).

Cell-surface Env expression was studied by indirect immunofluorescence and flow cytometry. Figure 6 shows the median fluorescent intensity (SEM) of binding by anti-gp41 (7B2) and anti-gp120 (VRC01, PGT126, A32, and CD4-hlgG2) mAbs in the presence or absence of BNM-III-170 and CD4₁₈₃. We chose CD4₁₈₃ rather than CD4-hlgG2 to avoid binding by the FITC-conjugated anti-human IgG secondary Ab. Cells were incubated for 24 hours in the presence of the BNM-III-170 (30 μ M), CD4₁₈₃ (20nM), or neither, and then washed. MAb (66nM) and fresh BNM-III-170 or CD4₁₈₃ were added for 1 hr. Following washing, mAb binding was detected with FITC-conjugated anti-human IgG and analysis by flow cytometry. BNM-III-170 and CD4₁₈₃ showed differential effects on the two cell types and toward different epitopes. Binding of

174 anti-gp41 mAb 7B2 was increased by both agents, but to a greater degree by BNM-III-170 in
175 92UG cells and by CD4₁₈₃ in H9/NL4-3 cells. Overall, the binding of 7B2 was enhanced to a
176 greater degree by both agents in H9/NL4-3 cells than in 92UG. These results are consistent
177 with our previously published data regarding sCD4-mediated effects on 7B2 expression (33, 34,
178 39). The anti-gp120 mAb A32 behaved similarly in that both agents enhanced expression of the
179 target epitope, but the increase was smaller and the overall binding of A32 was less than
180 observed with 7B2. Two other anti-gp120 mAbs, VRC01 and PGT126, behave distinctly
181 differently. Both epitopes essentially disappeared from the H9/NL4-3 cell surface on incubation
182 with either CD4₁₈₃ or BNM-III-170. It is clear that gp120 was still present, as shown by the
183 binding of A32 and CD4-hlgG2. The effects on the binding of those same two mAbs on 92UG
184 cells were less pronounced, but showed a difference between the effects of CD4₁₈₃ and BNM-
185 III-170, with the former increasing binding of both PGT126 and VRC01, and the latter
186 decreasing binding of both. We also tested the effect of BNM-III-170 and CD4₁₈₃ on the binding
187 of CD4-hlgG2 to H9/NL4-3 cells. Each diminished the binding, but neither completely. In
188 addition to being tetrameric, CD4-hlgG2 is present in 3.3-fold molar excess to CD4₁₈₃, hence the
189 partial degree of inhibition. In comparing results regarding cell surface antigen expression with
190 those observed for CIC-mediated cytotoxicity (Figure 4), important caveats that will be
191 addressed in the Discussion should be considered. Both killing and binding by 7B2 were
192 enhanced by CD4₁₈₃ and by BNM-III-170. Both A32-dgA and PGT126-dgA were marginally
193 cytotoxic compared to 7B2-dgA. Cytotoxicity of A32-dgA was enhanced by CD4₁₈₃ more so than
194 by BNM-III-170, whereas enhancement of cell surface antigen expression by BNM-III-170 was $\geq$
195 that of CD4₁₈₃. Killing of 92UG cells by PGT126-dgA was unaffected by the presence of either
196 CD4₁₈₃ or BNM-III-170, even though these agents had effects on antigen expression on 92UG.

DISCUSSION

We have examined the ability of CD4-like molecules to sensitize cells for killing by anti-HIV CICs. We also evaluated the effect of these agents on the antigenic structure of cell-surface Env. We studied two different protein forms of sCD4: CD4₁₈₃ and CD4-hIgG2, the former monomeric, the latter tetrameric; and two small CD4mc molecules: BNM-III-170 and temsavir. Both forms of sCD4 and BNM-III-170 enhanced killing by the gp41-targeted immunotoxin 7B2-dgA, and did so on two distinctly different cell lines (Figures 1-4). As we have previously observed, cell surface antigen exposure did not directly correlate with killing by CICs, with target epitope and cell type playing a greater role than degree of mAb binding in defining CIC efficacy (compare Figures 4 and 6).

In the studies reported here, we have focused on measuring cytotoxicity. Because both sCD4 and CD4mc molecules neutralize HIV infectivity, measuring effects of CICs in tissue culture infections is confounded by viral neutralization. On the other hand, none of the CD4-like agents cause any degree of cytotoxicity on the target cells at the doses we are testing, making specific cytotoxicity more straightforward to measure and clearer to interpret. Cytotoxicity assays require target cells that stably express Env and exhibit little or no cytopathic effect, requirements that are difficult to meet. The majority of cell lines that are “persistently” infected with HIV require replenishment with uninfected susceptible cells, as cells resistant to virus infection arise in the tissue culture. When fresh cells are added to an infected culture, cytopathic effect occurs. The adaptation of H9/NL4-3 to maintaining a stable, high level of productive infection results from a 24bp deletion in the HIV accessory gene vpr, and perhaps also cellular alterations. Similarly, it has been difficult to express native Env on the surface of cells by transfection, a feat achieved by the laboratory of Bing Chen at Boston Children’s Hospital with the 92UG cell line. H9/NL4-3 and 92UG represent respectively: infected vs transfected cells, a laboratory adapted strain vs a clinical isolate, and a clade B vs C virus. This provides us with some degree of biologic diversity, but our limited choice of target cells for these assays restricts

the virologic diversity of HIV strains available to us. Each cell type was affected differently by the CICs and by form of CD4 (sCD4 or CD4mc). H9/NL4-3 were more resistant to CIC killing than 92UG (Figures 1, 2B, and 4). We have previously made this observation (34, 39), and attribute this to H9/NL4-3 cells secreting virus, which can be a decoy and bind the CIC before it reaches the cell. However, H9/NL4-3 were also more responsive to CD4-mediated effects (Figures 1 and 2), consistent with NL4-3 being a laboratory-adapted virus (Env derives from the prototype HIV strain IIIB/LAV) cultivated in a cell line, and 92UG a clone of a clinical isolate. It has long been known that an important alteration as HIV becomes “lab adapted” is increased sensitivity to CD4-mediated effects (63). As noted above, H9/NL4-3 cells display a greater variability in sensitivity to 7B2-dgA than do 92UG (compare Figures 1A and 2B). Despite our best efforts to limit this variation, we have yet to identify the factor(s) that affect either the display or internalization of Env in a way to alter sensitivity to 7B2-dgA killing.

We have compared three mAbs for binding to target cells by indirect immunofluorescence and for their ability to deliver an immunotoxin (IT) to the same cells in the presence of no sensitizer, BNM-III-170, CD4₁₈₃, or CD4-hIgG2 (Figures 4 and 6). 7B2-dgA was the most effective IT, even though 7B2 bound to both target cells less well than did PGT126. The efficacy of PGT126-dgA was unaffected by BNM-III-170 or CD4₁₈₃ whereas the binding of PGT126 was. The enhancement of the cytotoxicity of A32-dgA by CD4-hIgG2 was markedly greater than by BNM-III-170, whereas the increase in A32 epitope exposure by BNM-III-170 was greater than that by CD4₁₈₃. We have previously observed this disconnect between degree of epitope exposure and cytotoxicity under differing circumstances (33, 34, 39). Our conclusion is that once binding is above a certain threshold, it is the target epitope that is the major determinant of cytotoxicity, and that the immunodominant helix-loop-helix region of gp41 serves as the best target, rather than any of the major neutralizing sites on Env (33, 39). We posit that the intrinsic mobility of this region, particularly during the CD4-mediated transition of Env from

the closed to open state, combined with its proximity to the cell membrane allows for greater penetration of the CIC's lethal moiety into the cell.

We have used ITs in the current analyses. This has been primarily a matter of convenience rather than a belief that ricin-based ITs are likely to be used clinically. We have considerable experience in making and characterizing dgA conjugates, and these agents are highly effective *in vitro* and in animal models of HIV infection (20, 25, 30, 33, 34, 39). However, the immunogenicity of ITs limits their utility. We found in macaques that efficacy of anti-HIV ITs in reducing viral load waned as anti-IT immune responses arose, 10-14 days post initiation of therapy (34). Attempts to limit immunogenicity by conjugation of the ITs to polyethylene glycol have been only modestly effective. We have previously reported the development of anti-HIV antibody drug conjugates (ADCs) in which small cytotoxic drugs were conjugated to anti-Env mAbs (34). ADCs have proven quite successful against certain cancers (64). However, in treating cancer, the target cell is replicating. This may not be true in using ADCs to target the persistent reservoir of HIV infection, where the provirus may reside in resting cells. We have therefore screened small cytotoxic agents to identify those that will kill resting lymphocytes, as toxins do. We have identified an anthracycline derivative that meets these criteria, have produced effective and specific anti-HIV ADCs by conjugating the drug to mAb 7B2, and have demonstrated differences in mechanisms and kinetics of cell killing mediated by the IT and ADC (S.H. Pincus, manuscript in preparation).

The use of sCD4 was amongst the earliest forms of anti-HIV therapy tested (65, 66). Clinical trials were performed with various forms of sCD4, including CD4-hIgG2 (66) and the IT CD4-PE40 (47). There was little evidence of *in vivo* antiviral efficacy. Additional forms of sCD4 continue to be developed, including those with mutations that enhance binding, novel multimers, and as part of bispecific immunoadhesins with tyrosine sulfated peptides or antibody V-regions (42, 43, 45, 46). In parallel with protein engineering approaches, small molecule inhibitors of the gp120-CD4 interaction that neutralize HIV infection have been developed (13, 48-59). Among

these are temsavir and its FDA-approved prodrug fostemsavir, as well as BNM-III-170, the progenitor of a series of CD4mc compounds of increasing potency. In comparing the ability of temsavir and BNM-III-170 to sensitize cells to CIC-mediated killing, we found that only BNM-III-170 showed activity in these assays (Figure 2A). The mechanism of action of BNM-III-170 has been well studied. Cryo-EM structures demonstrated that Env trimers adopted an open (post-fusion) conformation upon binding by BNM-III-170 (42, 52, 53). Additional congeners of BNM-III-170 with enhanced anti-HIV activity have been synthesized (42, 52, 54, 57, 58). Importantly, HIV-infected cells are sensitized to ADCC-mediated killing by exposure to BNM-III-170 and congeners (13, 48, 49, 52, 54, 55). The *in vivo* ability of BNM-III-170 in combination with ADCC mediating mAbs to suppress the reservoir of HIV-infected cells has been demonstrated in humanized mice (13).

The current study extends the observations regarding ADCC, showing sensitization to cytotoxicity by a distinct mechanism of killing. ADCC occurs at the cell surface with killing mediated by an extracellular interaction (Fc region of anti-HIV mAb binding by the FcR on a killer cell). CIC-mediated killing requires internalization of the conjugate. Moreover, the mAbs that result in effective CIC killing are a minor subset of the mAbs that mediate ADCC, with the most effective CICs targeting the gp41 helix-loop-helix domain (27, 33, 39). This portion of gp41 lies in close proximity to the cell membrane and is highly mobile (40, 41). We believe that the conformational changes induced by sCD4 or CD4mc result in the insertion of the CIC into the cell membrane, which enhances internalization. Thus we have evaluated the effect of BNM-III-170 on a very different functional activity reflecting alterations in different domains of Env.

HIV infection can be treated and AIDS prevented by effective ART, but the infection cannot be cured and patients must remain on lifelong ART (3). The persistence of a reservoir of long-lived T cells that carry a functional HIV provirus remains the barrier to a cure (4-6). It is our

goal to use anti-Env CICs to eliminate these cells, an approach that requires Env expression for the CIC to target the cell. We and others have shown that CICs are highly effective in vitro and in animal models (18-36). One CIC, a fusion protein of CD4 and a fragment of *pseudomonas* exotoxin A, CD4-PE40, underwent a clinical trial and was found to be toxic at doses far lower than seen with similar molecules that have been FDA approved (37). We have reproduced the toxicity of CD4-PE40 in mouse and macaque models of infection, while also demonstrating the safety and efficacy other anti-HIV CICs in the same models (30, 34). If the cells of the reservoir do not produce sufficient amounts of Env while on effective ART, then it may be necessary to induce expression using latency disrupting agents in shock-and-kill protocols (7-12), and then use CICs as the killing agents. The existence of agents, such as CD4-hlgG2 and BNM-III-170 that may be co-administered with CICs and result in sensitization of HIV-infected cells to killing could further enhance the utility of this approach.

In our previous work we have shown that 7B2-dgA could suppress HIV infection in mice carrying HIV-infected tumors and in SHIV-infected macaques (30, 34). In the more physiologic SHIV model the IT was effective in decreasing viral load, but only briefly due to immunogenicity. We are currently testing the efficacy of 7B2-dgA plus CD4-hlgG2 in a more relevant model, using HIV-infected humanized SCID mice whose viremia is fully suppressed by ART. BNM-III-170 has been shown to diminish viral reservoirs in HIV-infected ART-treated humanized mice, when used in combination with ADCC-mediating mAbs (13). Therefore a logical next step in our experiments would be to compare the efficacy of CICs with unarmed ADCC-mediating mAbs in diminishing the reservoir and delaying viral rebound in humanized mice. Optimally these studies would be performed in the presence or absence of latency disrupting agents, so that we can evaluate the full “kick and kill” protocol.

MATERIALS AND METHODS

Cells and reagents. H9/NL4-3 cells are H9 CD4⁺ lymphoma cells persistently infected with the molecular clone of HIV designated NL4-3, itself a hybrid virus with the 3' end (including ENV) derived from HTLV-III_B (67). Through multiple passages H9/NL4-3 maintains a productive infection in virtually all cells (24, 33, 39). The cells were produced at the NIAID Rocky Mountain Laboratories and were the gift of Bruce Chesebro and Kathy Wehrley. Provirus mutations are documented in the following GenBank entries: AF070521.1;L32865.1;L42371.1, and L32837.1. HEK-293T/92UG were transfected to express Env of the Clade C clinical isolate 92UG037.8 in the laboratory of Dr. Bing Chen, Boston Children's Hospital, who kindly shared the cells with us (68). Expression is maintained by periodic selection in 1 µg/mL of puromycin. Cells are maintained in RPMI1640 medium (GIBCO, Thermo Fisher, Waltham MA USA) with 10% fetal bovine serum (Hyclone, Logan UT USA) and grown at 37° in a humidified, 5% CO₂ atmosphere. CD4₁₈₃ consists of the 183 amino terminal peptides of the mature CD4 molecule produced in *E. coli* and was the gift of Upjohn Laboratories (Kalamazoo, MI USA, now owned by Pfizer) and CD4-hIgG2 (44) from Progenics Pharmaceuticals (New York, NY USA). BNM-III-170 was synthesized as described previously (69). Temsavir was purchased from Selleckchem (Houston, TX USA). Both BNM-III-170 and temsavir were dissolved in DMSO at 10 mM prior to adding to tissue culture medium.

Immunotoxins were produced by conjugating deglycosylated ricin A chain (dgA (70), the kind gift of Ellen Vitetta, University of Texas Southwestern) to mAb at a 1:1 mass ratio using the heterobifunctional cross-linking agent succinimidyl 6-[3(2-pyridyldithio) propionamido] hexanoate (SPDP, Pierce Biotechnology, Rockford, IL USA) as described previously (34). MAb 7B2 was originally produced by Dr. James Robinson (Tulane University) and was the gift of Dr. Barton Haynes (Duke University). MAbs PGT126 and A32 were expressed as human IgG1 from synthetic sequences (based on 4YBL_L and 4YBL_H for A32; JN201915.1 and JN201898.1 for PGT126) inserted into pcDNA3.1, expressed in CHO cells and grown in fetal bovine serum that

was precleared on protein G-agarose (GenScript, Piscataway, NJ USA) to remove residual IgG. The IgG in the clarified supernatant was purified on protein G-agarose. PGT126 is clonally related to the better-studied PGT128 and binds a complex epitope consisting of glycan and peptide at the base of the V3 loop of gp120. A32 binds a conformational epitope in the C1 region of gp120. Following conjugation, the ITs were purified on protein G-agarose and characterized by SDS-PAGE showing a mixture of 1:1, 2:1, and 3:1 dgA:mAb ratios.

Cytotoxicity. Cell viability was measured using the MTS dye reduction assay which is a measure of the cell's oxidative phosphorylation. Target cells (1×10^4 H9/NL4-3, 2×10^4 92UG) were suspended in 0.2 mL RPMI 1640 + 10% fetal bovine serum in the presence of CIC with or without enhancing agent. Cells were incubated for 72 hr at 37° humidified 5% CO₂ atmosphere. MTS/PMS substrate (CellTiter AQueous, Promega, Madison WI USA) was added to the wells for the final 3 hr of incubation. At the completion, absorbance (A) at 490 nm was read on a microplate reader (BioTek, Winooski, VT USA). Percent cytotoxicity was calculated according to the formula:

$$\{1 - [(A_{IT} - A_{no\ cells}) / (A_{no\ IT} - A_{no\ cells})]\} \times 100.$$

Flow cytometry. We have used indirect immunofluorescence and flow cytometry to quantify the binding of mAbs to the surface of H9/NL4-3 or 92UG cells. Prior to staining, the cells (4×10^5 /mL in 10 mL RPMI1640 10% fetal bovine serum) were incubated for 24 hr at 37° humidified 5% CO₂ atmosphere in the presence of 30 µM BNM-III-170, 20 nM CD4₁₈₃, or neither. Cells were washed in PBS 1% bovine serum albumin, 0.1% sodium azide (PBA) and resuspended in 100 µL PBA containing mAb (66 nM) and 30 µM BNM-III-170, 20 nM CD4₁₈₃, or neither. Cells were incubated at room temperature for 1 hr in 96 well round-bottom plates, washed twice with PBA and resuspended in 6 nM FITC-conjugated goat anti-human IgG goat anti-human IgG (H + L chain specific, Invitrogen, Waltham MA, USA). Following another 1 hr incubation, the cells were washed and resuspended in PBS 2% paraformaldehyde, and

refrigerated until analyzed. Using an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA), we analyzed $1.5-3 \times 10^4$ events gated by forward and side scatter to exclude debris using FlowJo software (Treestar/BD Biosciences). Data are presented as the median fluorescence intensity $\pm$ standard error of the mean.

P24 antigen detection. We have used an antigen-capture ELISA to measure p24 antigen (71). Triplicate cultures of H9/NL4-3 cells (1×10^5 /mL) were cultured in 24 well plates in 2 mL medium for 72 hr. Samples for testing were removed at 24, 48, and 72 hr. Imulon-2 HB plates (Thermo Fisher) were coated with 200 nM anti-p24 mAb 183-H12-5C (available from the NIH AIDS Reagent Program, ARP) and blocked with blotto (PBS containing 10% nonfat dry milk, 0.2% Tween-20). H9/NL4-3 supernatant samples lysed in 0.1% in PBS or recombinant p24 standards (available from ARP) were diluted into blotto and incubated with the capture mAb for 24 hr at 4°. Plates were washed and 6 nM biotin-conjugated HIV Immune Globulin (ARP) in blotto added and incubated overnight. Plates were again washed and a 1:10000 dilution of Amdex horseradish peroxidase-conjugated streptavidin (Cytiva Life Sciences, Chicago, IL, USA) added for 45 min, then washed off with PBS, and tetramethyl benzidine substrate (Sigma, St. Louis, MO USA) added. The reaction was stopped after 11 min with 2M H₂SO₄. Absorbance at 450 nm was read on a microplate reader. A standard curve (7.5-120 pg/mL) was used to convert absorbance to p24.

Data analysis and display. All cytotoxicity and ELISA results represent the mean and SEM of triplicate samples. Data were plotted using GraphPad Prism (GraphPad Software, Boston, MA USA). Where no error bars are visible, the symbol is larger than the error. Flow cytometry is shown as the median fluorescence and SEM of $1-2 \times 10^4$ events gated by FSC and SSC to contain singlet cells (~80% of collected events). We used the built in statistical packages provided by FlowJo software. In all figures untreated samples are shown as turquoise blue squares or bars, BNM-III-170 as dark blue triangles or bars, CD4₁₈₃ as solid red circles or bars, and CD4-hIgG2 as open red circles or bars.

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FIGURE LEGENDS

Figure 1. Enhancement and specificity of CIC-mediated killing of Cells Expressing HIV

Env. Cells were incubated for 3 days in the presence of the indicated concentration of the CIC 7B2-dgA with or without the addition of 20 nM CD4₁₈₃. For the final three hours MTS/PMS dye was added, and A₄₉₀ determined at the termination. Percent cytotoxicity was determined using the formula described in the text. Results are mean and SEM of triplicate samples. **A.** Demonstration of CD4-mediated enhancement of 7B2-dgA's toxicity on H9/NL4-3 and 92UG cells. **B.** Specificity of CIC-mediated killing is shown on 92UG, untransfected HEK/293T, and uninfected H9 cells. Cells were incubated in the presence of sCD4₁₈₃.

Figure 2. Sensitization to CIC-mediated killing by different forms of sCD4 and CD4mcs.

A. Titration of sensitizing agents in the presence of limiting concentrations of 7B2-dgA: 50 pM on H9/NL4-3 cells, 5 pM on 92UG. **B.** Comparison of different agents' ability to enhance cytotoxicity. Optimal concentrations for each agent and each cell were chosen for comparison: on H9/NL4-3 cells: BNM-III-170 10 μ M, CD4₁₈₃ 20 nM, CD4-hIgG2 11nM; on 92UG cells: BNM 30 μ M, CD4₁₈₃ 20 nM, CD4-hIgG2 11nM. Cytotoxicity was measured by MTS-dye reduction assay three days later. Results are mean and SEM of triplicate samples.

Figure 3. Titration of the sensitization of cells to CIC killing. H9/NL4-3 or 92UG cells were incubated with 7B2-dgA (5 pM on 92UG, 75 pM on H9/NL4-3) in the presence of serial dilutions of BNM-III-170 or CD4-hIgG2. Cytotoxicity was measured by MTS-dye reduction assay three days later. Results are mean and SEM of triplicate samples.

Figure 4. Effects of CD4-hIgG2 and BNM-III-170 on cytotoxicity of CICs targeted to different Env epitopes. H9/NL4-3 and 92UG cells were exposed to the indicated immunotoxins

along with either no sensitizing agent, CD4-hlgG2 (11 nM), or BNM-III-170 (10 μ M on H9/NL4-3, 30 μ M on 92UG). Cytotoxicity was measured three days later. Results are mean and SEM of triplicate samples.

Figure 5. Effect of CD4-IgG2 and BNM-III-170 on cell viability and virus production. A. The viability of H9/NL4-3 or 92UG cells in the presence of CD4-hlgG2 (44nM), BNM-III-170 (22 μ M), or neither for three days was measured by MTS-dye reduction assay. The results are shown comparing the dye reduction in the presence of the sensitizing agents to untreated controls. **B.** Effects of CD4-hlgG2 (44nM), BNM-III-170 (22 μ M), or neither on virus production by H9/NL4-3. Cells were cultured for three days and samples removed at the indicated time points and assayed for production of HIV p24 antigen.

Figure 6. Effect of CD4₁₈₃ and BNM-III-170 on cell surface antigen expression. Prior to staining, the cells were incubated under standard culture conditions for 24 hr in the presence of CD4₁₈₃ (20 nM) or BNM-III-170 (30 μ M), or neither. The cells were washed, resuspended in PBA, and stained by the indicated mAb (66nM) in the presence or absence of the same sensitizing agent at 4° for 60 min. The cells were washed and mAb binding detected with FITC anti-human IgG for 1 hr, washed and fixed in 2% paraformaldehyde. Cells were gated on FSC and SSC, and 1-2 X 10⁴ events were collected. Results show the median and SEM of the fluorescent intensity.