

# Specific Killing of HIV-Infected Lymphocytes by a Recombinant Immunotoxin Directed against the HIV-1 Envelope Glycoprotein

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## Abstract

**Background:** 3B3 is a high-affinity anti-gp120 antibody that neutralizes a wide range of primary and laboratory isolates of HIV-1. The parental antibody was isolated from a combinatorial phage display library constructed from bone marrow RNA of an HIV-infected individual. We have generated a highly active immunotoxin using the 3B3 single-chain Fv (scFv) which can specifically kill lymphocytes infected by HIV-1.

**Materials and Methods:** We used recombinant DNA technology to clone the Fv fragment of 3B3 and produce a single-chain Fv (scFv). 3B3 scFv was then fused to a truncated version of *Pseudomonas* exotoxin A (PE38), giving rise to a recombinant immunotoxin 3B3(Fv)-

PE38 that was expressed in *E. coli* and purified to near homogeneity.

**Results:** 3B3(Fv)-PE38 binds with the same affinity as the parental Fab antibody to the MN strain of gp120. The immunotoxin specifically kills a gp120-expressing transfected cell line and a chronically HIV-infected lymphocytic cell line. The immunotoxin is very stable at 37°C, retaining 80% of its original activity after 24 hr.

**Conclusions:** Potent immunotoxins such as 3B3(Fv)-PE38 could be utilized in combination with multidrug cocktails that limit viral replication to help reduce viral reservoirs in patients with AIDS.

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## Introduction

Since the initial isolation of HIV in 1983 and its identification as the causative agent of AIDS (1,2), tremendous efforts have been made to understand the cause and pathogenesis of AIDS, but an effective therapy leading to the cure of

this disease is still in the future. To date, there are several therapeutic drugs available to treat HIV-infected patients, and these lead to prolongation of life and control of symptoms. The major approaches for the treatment of individuals with AIDS or HIV infections are the administration of drugs such as a reverse transcriptase inhibitor, AZT (3'-azido-3'-deoxythymidine), or ddI (2', 3-dideoxyinosine), which act by inhibiting synthesis of the proviral genome after the virion has entered the host cell, and protease inhibitors,

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which block production of infectious virions. Although these agents can effectively inhibit HIV spread *in vitro* and *in vivo*, they do not kill those cells that are already infected. Recently, a highly active antiretroviral therapy (HAART) has shown encouraging results on reduction of viral loads in lymphoid tissues of HIV-infected patients (3,4). In this approach, a cocktail consisting of an HIV-1 protease inhibitor and two reverse transcriptase inhibitors (RTIs) is administered for the treatment of HIV-infected patients. Although significant progress has been made recently in the treatment of HIV-1 infection, we are not yet close to a cure for AIDS.

Immunotoxins are potent cell-killing agents composed of either antibodies or antibody fragments attached to protein toxins made by bacteria or plants (5,6). Ricin, diphtheria toxin, and *Pseudomonas* exotoxin A have been widely used for this purpose. The development of immunotoxins for the therapy of cancer, autoimmune diseases, and other immunological disorders has been ongoing for the past two decades. Our laboratory has focused on immunotoxins made with *Pseudomonas* exotoxin A (PE). Using recombinant DNA technology we have deleted the cell-binding domain and another nonessential portion of PE to generate a molecule (PE38) that retains its cell-killing activity when targeted to cells by antibodies or antibody fragments. Using this approach, several recombinant immunotoxins directed at antigens on cancer cells have been constructed that are capable of curing tumors growing in nude mice. Several of these are currently undergoing clinical trials in leukemias and in colon and breast cancers and have produced significant tumor regression (5,7, and our unpublished data).

In this study, we have made a recombinant toxin containing the Fv portion of an antibody 3B3 that has a high affinity and a broad cross-reactivity with many laboratory and clinical isolates of HIV. The parental antibody was isolated from a combinatorial phage display library constructed from bone marrow RNA of an HIV-infected individual (8) and that neutralizes many different laboratory strains of HIV-1 as well as many primary isolates (9–11). The antibody reacts with the conserved CD4-binding site of gp120, the external subunit of the envelope glycoprotein (12). The resulting immunotoxin 3B3(Fv)-PE38 specifically kills HIV-infected lymphocytes without affecting cells that do not express gp120.

## Materials and Methods

### *Plasmid Constructions and Production of Recombinant Protein*

The plasmid pAra-3B3 encodes the Fab of antibody 3B3 directed against the gp120 glycoprotein of HIV-1 (12). DNA fragments encoding the Fv portion of the heavy ( $V_H$ ) and light ( $V_L$ ) chains of 3B3 antibody were obtained by PCR amplification using pAra-3B3 plasmid DNA as template. High-fidelity *Taq* polymerase (Boehringer Mannheim) was used to avoid PCR errors. The primer pair used to amplify the heavy-chain Fv region was T128 (5'-AAA CAT ATG CAG GTT CAG CTC GAG CAG TCT GGG GCT GAG GTG AAG AAG CCT GGG GCC TCA GTG AAG GTT TCT TGT CAG GCT-3') and T129 (5'-TCC AGA TCC GCC ACC ACC TGA TCC GCC TCC GCC TGA GGA GAC GAT GAC CGT GGT CCC TTT GCC CCA GAC GTC-3'). The primer pair T144 (5'-TCA GGT GGT GGC GGA TCT GGA GGT GGC GGA AGC GAC ATC GAG CTC ACG CAG TCT CCA GGC ACC CTG TCT CTG TCT CCA-3') and T131 (5'-GGA AGC TTT CCT CTC CAG TTT GGT CCC CTG GCC AAA AGT GTA CGA GGA GGC ACC ATA-3') was used to amplify the light-chain Fv region. The plasmid pTKB21.18 that encodes  $V_H$  and  $V_L$  domains of 3B3 antibody connected by a 15 amino acid linker and fused to PE38 was generated by spliced PCR using the purified individual  $V_H$  and  $V_L$  PCR fragments through the primers T128 and T131 and was cloned into the *NdeI-HindIII* site of pUL17. The *NdeI-HindIII* sites fused the inserted fragment in frame to the PE38, the truncated form of *Pseudomonas* exotoxin (13). The vector contains the T7 promoter for expression in Studier's *E. coli* BL21( $\lambda$ DE3) expression system (14). The expression plasmid was confirmed to be correct by DNA sequencing on an ABI 373A sequencer using the Dydeoxy chain terminator sequencing kit.

The immunotoxin 3b3(Fv)-PE38 was expressed in *E. coli* BL21( $\lambda$ DE3). It was then accumulated in inclusion bodies (IBs) and purified as an active immunotoxin molecule, following the method as previously described for other recombinant immunotoxins (15,16).

### *Binding Assays*

The affinities of 3B3(Fab) and 3B3(Fv) were assayed and compared by surface plasmon resonance (BIAcore, Pharmacia Biosensor) assay. Recombinant gp120 from the HIV-1 MN strain was coupled to BIAcore sensor chips (CM5, research

grade, Pharmacia Biosensor) according to the manufacturer's specifications. The 3B3(Fab) and 3B3(Fv)-PE38 were applied to the chips; binding and dissociation ( $k_{\text{ass}}$  and  $k_{\text{diss}}$ ) were determined from association and dissociation curves of the sensor grams with the BIAevaluation software package (Pharmacia Biosensor).  $K_D$  at equilibrium was calculated as  $K_D = k_{\text{diss}}/k_{\text{ass}}$ .

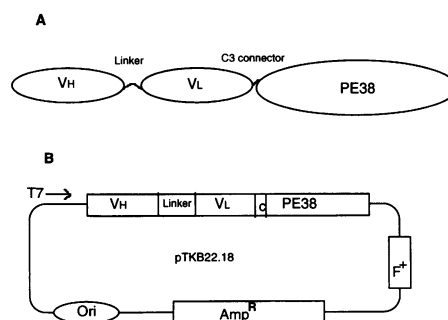
#### Cytotoxicity Assays

The specific cytotoxicity of the CD4-PE40, a fusion protein containing the HIV-binding portion of the human CD4 molecule linked to active regions of *Pseudomonas* exotoxin A and 3B3(Fv)-PE38, was assessed by protein synthesis inhibition assays on ENV15 cells (inhibition of incorporation of tritium-labeled leucine into cellular protein) in 96-well plates as previously described (17). ENV15 cells were generated by transfecting the CHO cell line with a plasmid-encoding HIV-1 envelope glycoprotein. The CHO cell line transfected with control plasmid was used as negative control. The activity of the molecule is defined by the  $IC_{50}$ , the toxin concentration that reduces incorporation of radioactivity by 50%.

To determine cell viability, cell Proliferation Reagent WST-1 (Boehringer Mannheim, Cat. No. 1644 807) assay was performed on HIV-1 chronically infected 8E5 cells (8E5/LAV AIDS Repository #95) and its uninfected parent line A3.01 (A3.01 AIDS Repository #166) cells. Experiments were carried out in 24-well plates. Wells were seeded with either 50,000 cells/well for A3.01 or 100,000 cells/well for 8E5 in 0.5 ml medium. A different amount of immunotoxin was taken in 0.5 ml RPMI-10% fetal bovine serum (FBS) and added in each well and incubated for 5 days. The assays were performed by following the instructions provided with the kit.

#### Stability Assays

The stability of the 3B3(Fv)-PE38 immunotoxins was determined by incubating them at 10  $\mu\text{g}/\text{ml}$  at 37°C in phosphate-buffered saline (PBS) containing 0.2% human serum albumin. Active immunotoxin remaining after incubation was determined by protein synthesis inhibition assays on ENV15 cells.



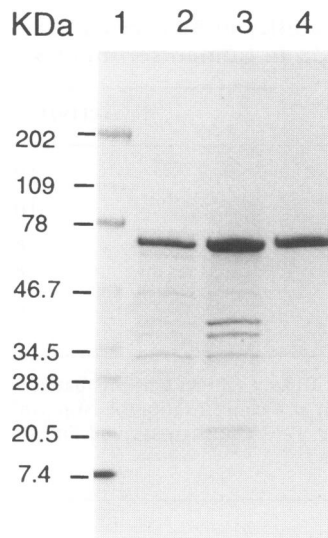
**Fig. 1. Plasmid for expression of 3B3(Fv)-PE38 immunotoxin.** (A) Schematic of 3B3(Fv)-PE38. The V<sub>H</sub> and V<sub>L</sub> portion of antibody 3B3 linked by a 15 amino acid linker and fused to the translocation and ADP ribosylation domains of PE. (B) The expression plasmid pTKB22.18 encodes the V<sub>H</sub> and V<sub>L</sub> domains of antibody 3B3 fused in frame to PE38. The carboxy terminus of V<sub>L</sub> is linked to the amino terminus of the toxin molecule (PE38).

## Results

#### Production and Purification of 3B3(Fv)-PE38 Immunotoxin

To generate plasmids for the expression of 3B3(Fv)-PE38, a gene that codes for the V<sub>H</sub> and V<sub>L</sub> chain variable region of the antibody 3B3 separated by PCR using pAra-3B3 plasmid DNA as template. Schematics showing the immunotoxin fusion protein and the linear composition of the plasmid encoding 3B3(Fv)-PE38 immunotoxin are shown in Figure 1.

*E. coli* BL21 ( $\Delta$ DE3) cells containing the plasmid pTKB21.18 for expression of the 3B3(Fv)-PE38 were grown and induced with isopropylthiogalatoside (IPTG). The fusion protein accumulated in insoluble intracellular inclusion bodies (IBs). These IBs contain almost pure recombinant protein, but in an insoluble and aggregated conformation. To generate protein with a native conformation, we solubilized and reduced the IBs in GuCl and dithiothreitol (DTE), and refolded the protein by dilution in a buffer containing arginine as described in Materials and Methods (15,16). Refolded, soluble monomeric protein was then purified to near homogeneity from other bacterial and improperly folded proteins by ion exchange (Q-sepharose, Mono Q) and size exclusion chromatography. Figure 2 shows the highly purified recombinant immunotoxin recovered after the three column purification steps. Using a standard refolding protocol for Fabs and immunotoxins, about 8% of the input



**Fig. 2. SDS-PAGE analysis of purified recombinant 3B3scFv-PE38 immunotoxin.** Coomassie blue-stained SDS PAGE of 3B3scFv-PE38 immunotoxin samples in reducing loading buffer. Lane 1: prestained protein molecular weight standard; lane 2: total cell extract; lane 3: inclusion bodies; lane 4: purified immunotoxin from sizing column.

protein was obtained as the monomeric single-chain Fv (scFv)-immunotoxin.

#### *Binding Affinity of 3b3(Fv)-PE38 Immunotoxin Towards gp120*

The binding affinity of the parental antibody 3B3 Fab, from which the 3B3(Fv) fragment is made, was improved by complementarity determining region (CDR) walking mutagenesis using phage display technology (12). Frequently, scFvs possess lower binding affinity than the parental whole antibody or Fab (18). To investigate whether 3B3(Fv) retains the same binding affinity as the parental 3B3 Fab, the binding affinities of 3B3 Fab and 3B3 scFv immunotoxin were determined by surface plasmon resonance (BiaCore) assay. For the BiaCore assay, the recombinant 3B3 Fab protein or 3B3(Fv)-PE38 was passed over gp120-coated sensor chips. The gp120 glycoprotein used was from the HIV-1 MN strain. The association and dissociation rates are shown in Table 2. The  $K_D$  at binding equilibrium, calculated as  $K_D = k_a/k_d$ , is 36 nM for the 3B3 and 37 nM for the 3B3(Fv) (Table 1). These data indicate that the 3B3(Fv) molecule has a binding affinity that approximates the parental 3B3 Fab antibody.

**Table 1. Affinity of Ara 3B3 Fab and 3B3scFv-PE38-immunotoxins**

Sample	$k_{\text{ass}}^{\text{ass}}$ ( $\text{M}^{-1} \text{s}^{-1}$ )	$k_{\text{diss}}$ ( $\text{s}^{-1}$ )	$K_D$ (M)
Ara 3B3 Fab	$0.76 \times 10^5$	$2.72 \times 10^{-3}$	$3.6 \times 10^{-8}$
3B3 scFv-PE38	$1.92 \times 10^5$	$7.03 \times 10^{-3}$	$3.7 \times 10^{-8}$

The affinity of Ara 3B3 Fab and 3B3scFv-PE38 was determined by surface plasmon resonance (BiaCore).  $K_{\text{ass}}$ ,  $k_{\text{diss}}$ , and  $K_D$  ( $K_D = k_{\text{diss}}/k_{\text{ass}}$ , binding at equilibrium) were calculated from the sensorgrams using the BIAevaluation software package.

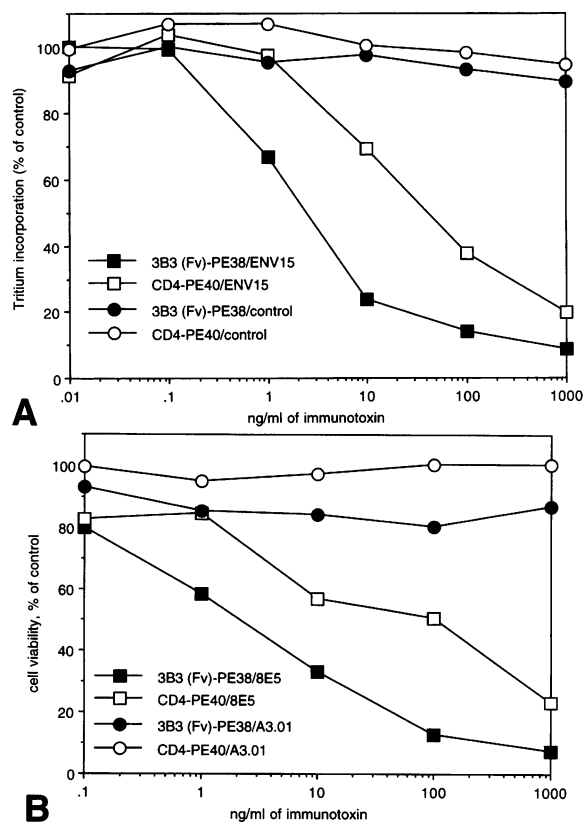
#### *Specific Cytotoxicity of 3B3(Fv)-PE38 Immunotoxin*

Fusion proteins of antibody fragments with PE38 are cytotoxic to antigen-positive cells that bind and internalize the fusion protein but are not cytotoxic to antigen-negative cells. Two assay systems were employed to determine whether the 3B3(Fv)-immunotoxin is selectively internalized and translocated by cells expressing the HIV Env, leading to cytotoxicity. In the first assay, we used ENV-15 cells, a transfected CHO cell line that expresses Env on its surface. CHO cell line transfected with control plasmid was used to determine the nonspecific killing of the immunotoxin. We have also tested the CD4-PE40 immunotoxin, a chimeric protein containing CD4

**Table 2. Specific cytotoxicity of CD4-PE40 and 3B3scFv-PE38 immunotoxins**

Cell Line	gp120	IC <sub>50</sub> , ng/ml	
		CD4-PE40	3B3scFv-PE38
ENV15	+	40	2.5
CHO	-	>1000	>1000
8E5	+	90	2.1
A3.01	-	>1000	>1000

For ENV15 and CHO cell lines, cytotoxicity assays were performed by measuring incorporation of  $^3\text{H}$ -Leu into cellular proteins as described in Materials and Methods. IC<sub>50</sub> is the concentration that causes 50% inhibition of protein synthesis after 20 hr incubation with immunotoxin. For 8E5 and A3.01 cells, WST-1 cell viability assays were performed as described in Materials and Methods. IC<sub>50</sub> is deduced from the concentration that causes 50% reduction of OD ( $A_{450 \text{ nm}}$ ) value after 5 days incubation with immunotoxin.



**Fig. 3. Specific cytotoxicity of CD4-PE40 and 3B3scFv-PE38 immunotoxin.** (A) Cytotoxicity towards gp120-expressing CHO cell line. Squares: ENV15, a gp120-expressing CHO cell line; circles: control CHO cell line. (B) Cytotoxicity towards HIV-1 chronically infected lymphocyte cell line. Squares: 8E5, a lymphocyte cell line that is chronically infected with HIV-1 virus; circles: control parental cell line A3.01. Cell viability assay was performed as described in Materials and Methods.

attached to a truncated form of *Pseudomonas* exotoxin A (19), and compared its activity with 3B3(Fv)-PE38 immunotoxin. As shown in Figure 3A and Table 2, 3B3(Fv)-PE38 is about 16-fold more active on ENV15 cells than CD4-PE40. The  $IC_{50}$  values for 3B3(Fv)-PE38 and CD4-PE40 on ENV15 cells are 2.5 and 40 ng/ml, respectively. Both immunotoxins showed no cytotoxic activity on a CHO control cell line, which does not express Env. In the second assay, we used a human lymphocyte cell line 8E5, which is chronically infected by HIV, and the parental uninfected lymphocyte cell line A3.01 as a negative control. The 8E5 cells constitutively express surface Env and release infectious HIV-1 particles. Figure 3B and Table 2 show that 3B3(Fv)-PE38 can kill 8E5 cells very effectively with an

**Table 3. Stability of 3B3 scFv-PE38 immunotoxin in human serum at 37°C**

Time (hr)	% Activity Remaining
0	100
2	100
4	86
8	86
24	80

Immunotoxin 3B3 scFv-PE38 was incubated with human serum albumin at a concentration of 10  $\mu$ g/ml for the times shown at 37°C and then assayed for cytotoxic activity on ENV15 cells.

$IC_{50}$  of 2.1 ng/ml. In this assay, the immunotoxin is about 40-fold more active than CD4-PE40. Furthermore, the killing is specific because neither molecule has a cytotoxic effect on the HIV-uninfected A3.01 cell line at a concentration of 1000 ng/ml.

#### Stability of 3B3(Fv)-PE38 Immunotoxin

Although Fvs are the smallest functional modules that confer specific antigen binding, often Fv fragments by themselves are unstable. The hydrophobic residues on  $V_H$  and  $V_L$  domains, which are located at the heterodimer interface, are insufficient to prevent dissociation of  $V_L$  and  $V_H$ . This results in aggregation and a reduction in binding affinity (20). In most of the applications for which Fvs are used and in therapy in particular, it is important that the Fvs are stable at 37°C in human serum so they will retain activity for a long time after injection into patients. To analyze if the 3B3(Fv) immunotoxin is stable, we assayed the stability of the 3B3(Fv)-PE38 in human serum albumin at 37°C. The immunotoxin was incubated at 37°C for different periods of time in PBS containing 0.2% human serum albumin at a concentration of 10  $\mu$ g/ml. The remaining activity of the immunotoxin was determined by a protein-synthesis inhibition assay. The results of the stability analyses are shown in Table 3. The 3B3(Fv)-PE38 retains 80% of its activity even after 24 hr incubation at 37°C in human serum albumin.

## Discussion

The HIV antigen envelope glycoproteins (gp120 and gp41) are the only viral proteins displayed on the HIV-infected cell surface that can be recognized by specific antibodies. Since gp120 is exposed on the cell surface of the infected cell, major efforts have been made to target the HIV-infected cells by generating antibodies against the gp120 glycoprotein. Although there is a high degree of inter-isolate sequence variability of gp120, there are a few conserved regions to which antibodies can be generated. Antibody IgG1b12 was isolated from a combinatorial phage display library constructed from bone marrow RNA of an HIV-infected individual (8). This antibody is directed to the CD4 binding site of gp120, and unlike other monoclonal antibodies that are also directed to CD4 binding epitope, it can neutralize many different laboratory strains of HIV-1 as well as many primary isolates (9–11). Both the potency and breadth of neutralization activity of 3B3 were improved over the parental antibody IgG1b12 (12). The resulting antibody, 3B3, thus has several characteristics that make it an attractive reagent for the targeted therapy of AIDS. We have used the Fv portion of antibody 3B3 to produce a recombinant immunotoxin, 3B3(Fv)-PE38, and have shown that this recombinant immunotoxin is very active and specific in killing gp120-expressing cells.

### *Comparison of 3B3(Fv)-PE38 Immunotoxin with Other Immunotoxins Directed against HIV*

A number of different recombinant toxins and recombinant immunotoxins have been made that are directed against either HIV-1 antigens gp120 and gp41 (19,21–23) or against cell-surface markers for T cells (23). The CD4-based toxins have been generated by fusing (19) or conjugating (21) a region of the CD4 molecule containing the gp120 binding site to either the bacterial toxin PE40 or to the plant toxin ricin. The CD4-ricin conjugate can specifically and effectively kill HIV-infected lymphocytes in vitro (21). The immunotoxin anti-gp41 and ricin immunconjugate, which is directed against gp41 of HIV, has also been reported to be effective in vitro on HIV-infected T cells and monocytes (24). But neither of these molecules have been examined in HIV-infected patients.

The most extensively studied chimeric toxin, CD4-PE40, was generated in our laboratory (16).

It is a recombinant chimeric protein containing CD4 linked to a 40,000 molecular weight form of *Pseudomonas* exotoxin A. CD4-PE40 was effective in killing an Env-expressing cell line and chronically HIV-infected cells (19). Also, when CD4-PE40 was used in combination with reverse transcriptase inhibitors that block the viral replication cycles, a synergistic effect was observed, leading to elimination of infectious HIV from human T cell cultures (25). On the basis of these data, preclinical development of CD4-PE40 was carried out and it was found to be very well tolerated by monkeys, so that 250  $\mu\text{g}/\text{kg}$  could be administered daily for 10 days without serious toxicity. Subsequently, phase I clinical trials were performed (26,27). Surprisingly, CD4-PE40 demonstrated very high toxicity in HIV-infected patients with a maximum tolerated dose of only 10  $\mu\text{g}/\text{kg}$ . The major side effect was liver toxicity. No evidence of anti-HIV effect of this protein in this trial was obtained, probably because of the low amount of the drug that could be given to patients. The toxicity of CD4-PE40 is probably due to the CD40-PE40 is probably due to the CD4 portion directing the immunotoxin to the liver, since we have subsequently given several other recombinant immunotoxins to patients at doses of up to 50  $\mu\text{g}/\text{kg}$  without observing dose-limiting liver toxicity (our unpublished data). In addition, a chemical conjugate of PE38 with a whole monoclonal antibody has been given in doses of up to 100  $\mu\text{g}/\text{kg}$  without liver toxicity (7). 3B3(Fv)-PE38 is about 20- to 30-fold more effective in killing gp120-expressing and HIV-infected cells in vitro than CD4-PE40 and should be devoid of the nonspecific toxicity observed with CD4-PE40. We believe that immunotoxins such as 3B3(Fv)-PE38 can have significant utility when used with existing multiple drug strategies. "Triple-drug" therapy could be utilized to remove the soluble antigen, Env, and competing antibodies from the infected patient, since viral titers and competing antibody titers crash following their application. The immunotoxin of selected antibodies could then efficiently target HIV-infected cells for destruction, as they should still express Env on their surface because Env processing is performed by endogenous proteases. Temporary withdrawal of reverse transcriptase inhibitors while maintaining protease inhibitor dosing during 3B3(Fv)-PE38 dosing may facilitate targeting and elimination of infected cells. This could be a curative strategy if viral reservoirs could be efficiently targeted and eliminated. Thus, we believe 3B3(Fv)-PE38 de-

serves further evaluation for the treatment of HIV disease.

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