The Interleukin-2 Fusion Protein, DAB_{389}IL-2, Inhibits the Development of Infectious Virus in Human Immunodeficiency Virus Type 1–Infected Human Peripheral Blood Mononuclear Cells

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DAB_{389}IL-2 is a genetically engineered fusion protein that reduces human immunodeficiency virus type 1 (HIV-1) replication in activated, interleukin (IL)-2 receptor (IL-2R)–expressing human peripheral blood mononuclear cells (PBMC). The level of infectious virus released by cultured PBMC after treatment with DAB_{389}IL-2 was measured by a quantitative microculture assay. The inhibition of p24 antigen production was also evaluated in cultures that differed in duration of infection and activation state. Although the activation state of the cell and the time of DAB_{389}IL-2 addition to the cultures influenced its anti-HIV activity, DAB_{389}IL-2 treatment decreased levels of infectious HIV-1 to 0.1%–6.5% of untreated cell levels. DAB_{389}IL-2 also decreased p24 antigen expression to 10%–48% of controls, even when added as late as 8 days after acute infection. Mutational variants of DAB_{389}IL-2 that lack catalytic activity or IL-2R binding are without anti-HIV activity.

DAB_{389}IL-2 is a genetically engineered fusion protein that decreases human immunodeficiency virus type 1 (HIV-1) production in acutely infected peripheral blood mononuclear cell (PBMC) cultures [1, 2]. DAB_{389}IL-2 binds to the high-affinity interleukin (IL)-2 receptor (IL-2R) on the target cell surface and is internalized by receptor-mediated endocytosis [3, 4]. Following receptor-mediated uptake and endosomal processing, the N-terminal fragment A of the diphtheria toxin portion of the molecule is released into the cytosol, resulting in the inhibition of both cellular and viral protein syntheses. Because DAB_{389}IL-2 treatment may result in lysis of cells with productive HIV-1 infections, questions exist about the infectivity of the target cell cytosol. In addition, some reports indicate that the IL-2R on the host cell may be down-regulated during the course of HIV infection [5–8]. Should this be the case, down-regulation of the IL-2R on an infected cell may interfere with the ability of DAB_{389}IL-2 to display anti-HIV activity.

In this study, we examined the ability of DAB_{389}IL-2 and two mutational variants, $DA_{glu53}B_{389}$IL-2 and $DAB_{389}IL\text{-}2_{\Delta 8\text{-}19}$, to mediate inhibition of protein synthesis and infectious virus production in activated PBMC. We also examined the ability of these fusion proteins to inhibit p24 antigen production in unstimulated and activated HIV-1–infected cells that were exposed to the fusion protein at different times after acute infection.

Materials and Methods

IL-2 fusion proteins. DAB_{389}IL-2 (lots 4A19HA2 and 3H22HA2), $DAB_{389}IL\text{-}2_{\Delta 8\text{-}19}$ (lot 5B06CP2), and $DA_{glu53}B_{389}$IL-2 (lot 5B15CP2) were provided by Seragen Inc. Protein concentrations of the two DAB_{389}IL-2 lots, as determined by amino acid composition and the bicinchoninic acid method (Pierce, Rockford, IL), were 0.196 and 0.322 mg/mL, respectively. Evaluated by a protein synthesis inhibition bioassay, specific activities of these lots were comparable.

[^{14}C]leucine incorporation assay. Human PBMC from normal, healthy volunteers were isolated from 60 mL of whole blood collected by venipuncture into a heparinized syringe. Lymphocyte-rich mononuclear cells were separated by centrifugation on a ficoll–sodium diatrizoate density gradient and resuspended (10^6/mL) in RPMI 1640 (JRH Biosciences, Lenexa, KS) containing 2 m*M* L-glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin, and 15% heat-inactivated fetal bovine serum. OKT3 (Ortho Diagnostics, Raritan, NJ) was added at concentrations found to be optimal. After a 72-h incubation in a 5% CO_2–humidified atmosphere at 37°C, cells were washed and dispensed (10^5 cells, 100 μL) into microtiter wells containing DAB_{389}IL-2, recombinant (r) IL-2, $DA_{glu53}B_{389}$IL-2, $DAB_{389}IL\text{-}2_{\Delta 8\text{-}19}$, or medium alone. After an 18-h incubation, the culture medium was removed and replaced with leucine-free medium (Dulbecco's MEM; Life Technologies GIBCO BRL, Gaithersburg, MD) buffered with 25 m*M* HEPES (JRH Biosciences) and containing 1.5 μCi/mL [^{14}C]leucine (DuPont-NEN Research Products, Boston). Cultures were harvested 2 h later on glass fiber filters (Skatron, Sterling, VA), and uptake of label was determined by liquid scintillation counting (model LS6000IC; Beckman, Fullerton, CA). The mean counts per minute of triplicate wells of fusion protein-treated cultures was expressed as a percentage of the mean counts per minute in triplicate wells of the control cultures. Estimates of the concentration of fusion protein resulting in 50% inhibition (IC_{50}) of [^{14}C]leucine incorporation were made from a graph of the log fusion protein concentration versus percentage of control [^{14}C]leucine incorporation.

HIV-1 infection. HIV-1 LAV stock was grown from the chronically infected H9 cell line (NIH AIDS Research and Reference Reagent Program, Bethesda, MD). The filtered and titrated virus was used to infect activated or unstimulated PBMC. PBMC were prepared by ficoll-hypaque density gradient fractionation of donor

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blood samples. PBMC were activated with 3 μg/mL phytohemagglutinin (PHA; Sigma, St. Louis) for 72 h at 37°C, in RPMI 1640 (Sigma) containing 20% fetal bovine serum (Sigma), 30 U/mL IL-2 (Hemagen Diagnostic, Columbia, MD), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma). PBMC that were not deliberately mitogenically activated were prepared in the same manner but without the addition of PHA to the medium and without the incubation period. The PHA-stimulated and -unstimulated PBMC were washed with PBS before HIV-1 infection. One reverse-transcriptase unit of virus per 10 cells was added to the PBMC, which were then incubated for 2 h at 37°C. Unbound HIV-1 was removed by washing the infected cells with PBS.

p24 antigen assay. The supernatants of DAB_{389}IL-2– and non–DAB_{389}IL-2–treated HIV-infected PBMC cultures were collected at scheduled times. p24 antigen levels were determined with a p24 antigen assay kit (Coulter, Miami, FL) according to the manufacturer's instructions.

Quantitative PBMC microculture assay (MIC). The procedure for the quantitative PBMC microculture assay is described in the AIDS Clinical Trial Group virology manual for HIV laboratories [9]. In brief, 3 mL of cell suspension (10^6 PBMC/mL) or culture supernatant was collected from each DAB_{389}IL-2–treated or –untreated culture, respectively. The cell suspension or supernatant was serially diluted (1:5) and mixed with PHA-activated normal donor PBMC. These cultures were incubated for 14 days; the supernatant was then assessed for p24 antigen levels. The number of infectious units per million (IUPM) PBMC was calculated with the formula of Spearman-Karber using RMLP software (DataWorks Development, Seattle).

Statistics. Variance analysis was used to examine the association between the time that DAB_{389}IL-2 was added to cells and p24 antigen production, and between the concentration of DAB_{389}IL-2 added to cells and p24 antigen production. The effect of DAB_{389}IL-2 added at different days after infection was examined by adjusting for the DAB_{389}IL-2 concentration; the effect of DAB_{389}IL-2 added at different concentrations was examined by adjusting for the day after infection that DAB_{389}IL-2 was added to the cells. The significance of the association between the variances was presented as the *P* value ($< .05$ was considered significant).

Results

DAB_{389}IL-2 mediates IL-2R–specific inhibition of protein synthesis in activated normal human PBMC. Activated human PBMC were admixed with the DAB_{389}IL-2 fusion protein in the presence and absence of 10^{-7} *M* rIL-2 or rIL-4. In cultures to which no competitor was added, the IC_{50} of control levels of [^{14}C]leucine incorporation was 4.2×10^{-12} *M* (figure 1). The addition of rIL-4 to the cultures resulted in little, if any, inhibition of the action of DAB_{389}IL-2 (IC_{50}, 6.6×10^{-12} *M*). Complete blocking of the ability of DAB_{389}IL-2 to mediate protein synthesis inhibition, however, was observed when a comparable molar dose of rIL-2 was added to the cultures.

Requirement for a catalytically intact DAB_{389}IL-2 molecule that can bind to the IL-2R. The genetically modified fusion protein molecules, $DAB_{389}IL\text{-}2_{\Delta 8\text{-}19}$ and $DA_{glu53}B_{389}$IL-2, were used to investigate the requirement for intact IL-2R binding and catalytic activity, respectively. $DAB_{389}IL\text{-}2_{\Delta 8\text{-}19}$ retains the catalytic activity of DAB_{389}IL-2 but is impaired in its ability to bind to the IL-2R because of a deletion in the native molecule's IL-2 sequence. While retaining the IL-2R–binding capability of native DAB_{389}IL-2, $DA_{glu53}B_{389}$IL-2, on the other hand, is impaired in catalytic activity because of an amino acid substitution at position 53 in the catalytic portion of the molecule. As shown in figure 2, $DAB_{389}IL\text{-}2_{\Delta 8\text{-}19}$ was markedly diminished in its ability to inhibit protein synthesis in these cultures. In contrast to an IC_{50} of 1.8 p*M* for native DAB_{389}IL-2, the median IC_{50} for cultures receiving $DAB_{389}IL\text{-}2_{\Delta 8\text{-}19}$ was 6.6 n*M*, representing a 3700-fold loss in potency. At physiologic concentrations, the $DA_{glu53}B_{389}$IL-2 fusion protein stimulates [^{14}C]leucine incorporation similar to that observed when rIL-2 is added to the cultures. These studies indicate that DAB_{389}IL-2 must be catalytically active and capable of binding to the IL-2R to effectively mediate protein synthesis inhibition.

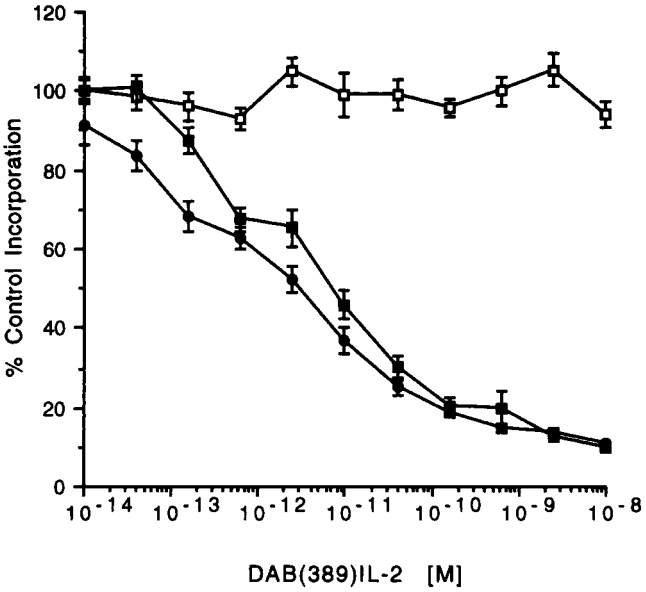

Figure 1. Inhibition of [^{14}C]leucine incorporation by DAB_{389}IL-2 is IL-2 receptor–specific. Activated human PBMC were treated for 18 h with various doses of DAB_{389}IL-2 in absence (●) or presence (□) of 10^{-7} *M* recombinant (r) IL-2 or 10^{-7} *M* rIL-4 (■). Mean [^{14}C]leucine incorporation in triplicate cultures containing fusion protein is expressed as % of that measured in cultures to which no fusion protein was added. Data from 5 experiments are shown ± SE.

Levels of HIV-1 released from DAB_{389}IL-2–treated human PBMC. PHA-activated HIV-1–infected PBMC were allowed to replicate virus for different periods of time before being treated with the IL-2 fusion protein. DAB_{389}IL-2 was added to the cultures either immediately (day 0) or 5 days after infection (day 5). The cultures were harvested 72 h later, and their contents (representing total virus in the system) were assessed for levels of infectious virus with the MIC assay. Dose-dependent inhibition of infectious virus production was observed in

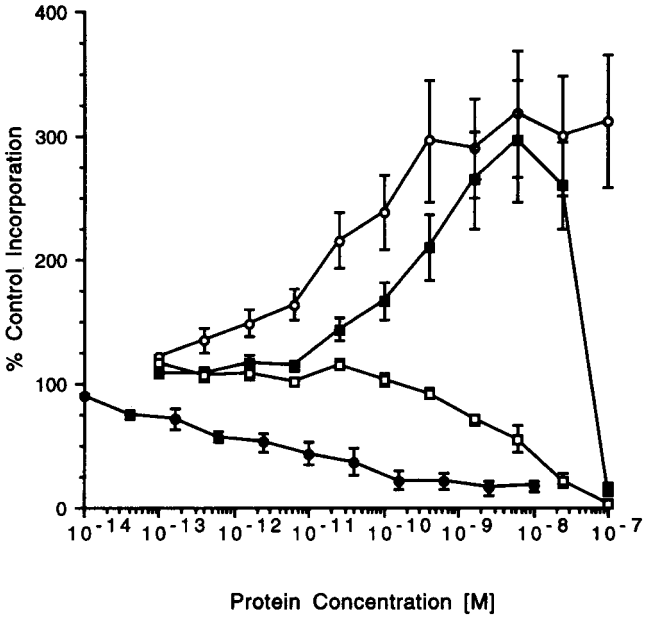

Figure 2. Uptake of [^{14}C]leucine by cultures treated with $DAB_{389}IL\text{-}2$, $DAB_{389}IL\text{-}2_{D8\text{-}19}$, $DA_{glu53}B_{389}IL\text{-}2$, and recombinant (r) IL-2. Activated human PBMC were treated for 18 h with various concentrations of $DAB_{389}IL\text{-}2$ (●), $DAB_{389}IL\text{-}2_{D8\text{-}19}$ (□), $DA_{glu53}B_{389}IL\text{-}2$ (■), or rIL-2 (○). Mean [^{14}C]leucine incorporation in triplicate cultures containing rIL-2 and various fusion proteins is expressed as % of that measured in cultures to which no rIL-2 or fusion protein was added. Data from 4 experiments are shown ± SE.

PBMC cultures that had been treated with $DAB_{389}IL\text{-}2$ immediately after infection (table 1). Untreated control cell suspensions contained >5608 IUPM, a value that represents the upper limit of quantifiable virus in the assay.

When the same concentrations of $DAB_{389}IL\text{-}2$ were added to cultures after the virus had been allowed to replicate for 5 days, $DAB_{389}IL\text{-}2$ consistently inhibited infectious virus production in a dose-dependent manner regardless of whether total virus in the system or the amount of virus specifically released into culture supernatants was measured. Relative to cultures in which $DAB_{389}IL\text{-}2$ was added immediately after infection, however, increased concentrations of fusion protein were required to comparably suppress infectious virus production.

Neither mutational variant of $DAB_{389}IL\text{-}2$ could inhibit the replication of infectious virus, even when added at doses as high as 10^{-9} *M*. These data indicate that the anti-HIV activity of $DAB_{389}IL\text{-}2$ is specifically directed at the IL-2R on HIV-1–infected cells and requires an active catalytic moiety on the fusion protein for its expression.

p24 antigen production and its inhibition by $DAB_{389}IL\text{-}2$ in extended acute infections. On various days after an acute infection, $DAB_{389}IL\text{-}2$ was added to the cultures of HIV-1–infected PBMC, and levels of p24 antigen were assessed 72 h later. Dose-dependent inhibition of p24 antigen production was observed on each test day (table 2). Although the ability of $DAB_{389}IL\text{-}2$ to inhibit p24 antigen production diminished with time after infection, the addition of $DAB_{389}IL\text{-}2$ (10^{-11} *M*) as late as 8 days after infection nevertheless resulted in significant inhibition of p24 antigen production. The earlier $DAB_{389}IL\text{-}2$ was added after infection, however, the more pronounced was the inhibition of p24 antigen production.

The p24 antigen production in HIV-1–infected PBMC that had not been deliberately activated by mitogen prior to infection was exceedingly low at all time points tested. However, $DAB_{389}IL\text{-}2$ added at doses $\geq 10^{-11}$ *M* significantly inhibited p24 antigen production in day 8 postinfection cultures. The dose-dependent inhibition of p24 antigen expression by

Table 1. Levels of infectious HIV-1 released from $DAB_{389}IL\text{-}2$–treated PBMC.

Assay, fusion protein	*M*	Total HIV-1 (IUPM)		HIV-1 released into supernatant (IUPM)	
		Day 0	Day 5	Day 0	Day 5
Dose-response					
$DAB_{389}IL\text{-}2$	10^{-9}	8	363	<1	6
$DAB_{389}IL\text{-}2$	10^{-11}	1122	5608	63	2503
$DAB_{389}IL\text{-}2$	10^{-13}	5608	>5608	2503	5608
None		>5608	>5608	>5608	>5608
Control molecule					
$DAB_{389}IL\text{-}2$	10^{-9}	10	ND	1.2	ND
$DA_{glu53}B_{389}IL\text{-}2$	10^{-9}	>5608	ND	>5608	ND
$DAB_{389}IL\text{-}2_{\Delta 8\text{-}19}$	10^{-9}	>5608	ND	5608	ND
None		>5608	ND	>5608	ND

NOTE. MIC, quantitative PBMC microculture (assay); ND, not done; IUPM, infectious units per million. Results shown are representative experiment of 7 dose-response and 2 control molecule assays. Infectious virus was cultured from total cell suspension or from supernatant alone. $DAB_{389}IL\text{-}2$ was added on day 0 or day 5 after infection; infectious virus was assessed 72 h later with MIC assay. Goodness-of-fit of MIC assays = 1 (by AIDS Clinical Trials Group standards).

Table 2. p24 antigen production after DAB_{389} IL-2 treatment of HIV-1–infected PBMC.

		Day*					
Mitogen, fusion protein	*M*	3	5	6	7	8	$P^{\dagger}$
PHA							
DAB_{389}IL-2	10^{-9}	1.4 ± 0.2	24.1 ± 0.1	31.8 ± 0.1	ND	51.8 ± 0.4	.0007
DAB_{389}IL-2	10^{-11}	1.7 ± 0.1	51.5 ± 0.2	62.3 ± 0.2	ND	72.4 ± 0.5	.0027
DAB_{389}IL-2	10^{-13}	35.1 ± 0.3	82.3 ± 0.0	131.0 ± 0.1	ND	110.7 ± 0.2	.0837
None		56.7 ± 0.0	198.0 ± 0.3	152.0 ± 0.3	ND	108.0 ± 0.7	
						$P^{\ddagger} = .0202$	
None							
DAB_{389}IL-2	10^{-9}	0.9 ± 0.0	0.4 ± 0.1	0.3 ± 0.1	0.2 ± 0.3	0.5 ± 0.3	.0287
DAB_{389}IL-2	10^{-11}	0.8 ± 0.7	0.3 ± 0.6	0.3 ± 0.1	0.4 ± 0.1	0.9 ± 0.2	.0351
DAB_{389}IL-2	10^{-13}	1.0 ± 0.0	0.6 ± 0.1	0.9 ± 0.0	0.4 ± 0.5	3.1 ± 0.9	.3719
None		0.8 ± 0.9	0.4 ± 0.6	1.4 ± 0.1	3.9 ± 0.2	5.0 ± 0.2	
						$P^{\ddagger} = .0481$	
PHA							
$DA_{glu53}B_{389}$IL-2	10^{-9}	92.2 ± 0.3	161.3 ± 0.4	150.7 ± 0.2	144.2 ± 0.5	141.2 ± 0.2	
$DAB_{389}IL\text{-}2_{\Delta 8\text{-}19}$	10^{-9}	55.9 ± 0.1	114.6 ± 0.2	131.8 ± 0.7	140.0 ± 0.4	112.7 ± 0.2	
None		59.1 ± 0.2	171.3 ± 0.5	141.0 ± 0.3	132.2 ± 0.3	112.7 ± 0.9	
None							
$DA_{glu53}B_{389}$IL-2	10^{-9}	0.6 ± 0.0	0.6 ± 0.0	1.6 ± 0.5	1.4 ± 0.0	4.5 ± 0.5	
$DAB_{389}IL\text{-}2_{\Delta 8\text{-}19}$	10^{-9}	0.6 ± 0.0	0.6 ± 0.0	1.2 ± 0.4	2.8 ± 0.1	3.2 ± 0.2	
None		0.6 ± 0.0	1.0 ± 0.3	1.0 ± 0.4	1.4 ± 0.5	5.6 ± 0.7	

NOTE. PHA, phytohemagglutinin; ND, not done. p24 antigen levels (ng/mL) were measured in culture supernatants 72 h after addition of DAB_{389}IL-2.
* Day DAB_{389}IL-2 added after infection.
† *P* for effect of DAB_{389}IL-2 concentration on inhibition of p24 antigen production, adjusting for days.
‡ *P* for overall effect of day on p24 antigen production, adjusting for DAB_{389}IL-2 concentration.

DAB_{389}IL-2 showed a significant association of increasing efficiency by days. The two mutational variants of DAB_{389}IL-2 had no inhibitory effect on p24 antigen production in cultures of either mitogenically stimulated or unstimulated HIV-1–infected PBMC.

Discussion

In this study, we evaluated the ability of DAB_{389}IL-2 to reduce infectious virus production in HIV-1–infected normal human PBMC. The results described herein confirm and extend earlier observations that the IL-2 fusion proteins, DAB_{486}IL-2 and DAB_{389}IL-2, decrease HIV-1 replication in virally infected PBMC in a dose-dependent and IL-2R–specific manner [1, 2]. In those studies, HIV-1 mRNA and p24 antigen production were inhibited in PBMC infected with an HIV-1 laboratory strain, fresh clinical isolates, and a zidovudine-resistant strain. The present study was designed to evaluate two aspects of DAB_{389}IL-2 antiviral activity, including the ability to reduce the levels of infectious virus released from cells at an early and late time point after infection and the ability to inhibit viral replication at various points during an extended acute infection. The two assays used in this study have been commonly used to identify surrogate markers for evaluating drug efficacy in numerous clinical studies.

The addition of DAB_{389}IL-2 to activated HIV-1–infected human PBMC resulted in greatly diminished viral replication and release of infectious virus. When added on the day of HIV-1 infection, DAB_{389}IL-2 (10^{-9} *M*) inhibited the production and release of >99.9% of the infectious virus produced by control cultures. Although delaying the addition of the IL-2 fusion protein by 5 days permitted the replication of more virus by the infected cells, DAB_{389}IL-2 (10^{-9} *M*) was nevertheless able to inhibit 94% of the total infectious virus production and 99.9% of the infectious virus release. Similar results were observed in extended acute infections in which viral replication was assessed by production of p24 antigen. In these studies, DAB_{389}IL-2 treatment (10^{-9} *M*) of activated PBMC 3 days after HIV-1 infection reduced p24 antigen production to 2.5% of that of control cultures. The addition of 10^{-9} *M* DAB_{389}IL-2 as late as 8 days after HIV-1 infection still resulted in inhibition of 52% of p24 antigen production.

The antiviral effects associated with DAB_{389}IL-2 treatment of HIV-infected PBMC were dose-related in both assay systems and required a catalytically active fusion protein that could bind to the IL-2R. Mutational variants of DAB_{389}IL-2 that either had impaired catalytic activity ($DA_{glu53}B_{389}$IL-2) or impaired IL-2R binding ($DAB_{389}IL\text{-}2_{\Delta 8\text{-}19}$) were unable to inhibit the production of either infectious virus or p24 antigen. These observations are consistent with those from our studies of the mutational variants of DAB_{389}IL-2 in activated human PBMC, in which we showed

that inhibition of cellular protein synthesis by DAB_{389}IL-2 requires both IL-2R binding and a catalytically active fusion protein.

Several studies have shown that HIV-1 infection can also occur in nondividing cells [10, 11]. Although p24 antigen levels in our studies were exceedingly low compared with those produced by activated HIV-1–infected PBMC, inhibition of p24 antigen production in these cells was also observed when DAB_{389}IL-2 was added to the cultures, notably late in the extended acute infection period (day 8). These results suggest that under the conditions of extended culture, the initially unstimulated cells acquired IL-2R. Acquisition or expression of IL-2R by mitogenically unstimulated cultured HIV-1–infected PBMC may either be the result of mitogenic conditions that develop with extended culture or may be related to the presence of the HIV-1 virion itself [12, 13].

In view of these and earlier observations, further clinical studies are suggested to evaluate DAB_{389}IL-2 either as a monotherapy or in combination with other agents. By targeting the HIV-1–infected lymphocyte, rather than the virus itself, DAB_{389}IL-2 treatment may be able to circumvent the classical viral resistance mechanisms that have limited the utility of monotherapies that are strictly virus-directed [14, 15].

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