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Antiviral Effect and Ex Vivo CD4$^+$ T Cell Proliferation in HIV-Positive Patients as a Result of CD28 Costimulation

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Because stimulation of CD4$^+$ lymphocytes leads to activation of human immunodeficiency virus–type 1 (HIV-1) replication, viral spread, and cell death, adoptive CD4$^+$ T cell therapy has not been possible. When antigen and CD28 receptors on cultured T cells were stimulated by monoclonal antibodies (mAbs) to CD3 and CD28 that had been immobilized, there was an increase in the number of polyclonal CD4$^+$ T cells from HIV-infected donors. Activated cells predominantly secreted cytokines associated with T helper cell type 1 function. The HIV-1 viral load declined in the absence of antiretroviral agents. Moreover, CD28 stimulation of CD4$^+$ T cells from uninfected donors rendered these cells highly resistant to HIV-1 infection. Immobilization of CD28 mAb was crucial to the development of HIV resistance, as cells stimulated with soluble CD28 mAb were highly susceptible to HIV infection. The CD28-mediated antiviral effect occurred early in the viral life cycle, before HIV-1 DNA integration. These data may facilitate immune reconstitution and gene therapy approaches in persons with HIV infection.

CD4$^+$ T cells contain the major reservoir of HIV-1 in vivo (*1*, *2*). The difficulties involved in inducing proliferation in vitro of autologous CD4$^+$ T cells from patients with HIV-1 infection limit the therapeutic potential for many approaches that involve gene therapy or immune reconstitution of infected persons (*3*). Two obstacles attributed to impaired CD4$^+$ T cell proliferation are a limited clonogenic potential of the uninfected CD4 and CD8 cells and the activation of HIV-1 expression and viral production (*4*). In addition to T cell receptor (TCR) engagement of an antigenic peptide bound to major histocompatibility complex (MHC) receptors, other costimulatory signals are necessary for T cell activation. The most important of the costimulatory signals identified to date is provided by the interaction of CD28 on T cells with its ligands CD80 and CD86 on antigen-presenting cells (*5*). Because CD28 signal transduction can prevent apoptosis in cultures of HIV-infected cells and can induce expression of the *Bcl-X$_L$* cell survival gene (*6*), we tested the hypothesis that costimulation might be limiting in cultures from HIV-infected patients.

We cultured lymphocytes from 10 patients with HIV-1 infection (CD4 counts of 350 to 600 cells/mm^3) in the presence of beads coated with CD3 mAb OKT3 and CD28 mAb 9.3 (Table 1). Cell culture was performed in the absence of exogenous cytokines or feeder cells, as CD28 stimulation provides the necessary costimulus to replace feeder cells (*7*). Figure 1 shows the growth curve of CD4$^+$ T cells from an HIV-infected patient after stimulation by a conventional method [with phytohemagglutinin (PHA) and interleukin-2 (IL-2)] or with CD3 and CD28 mAbs in medium that did not contain antiretroviral agents. In the PHA-stimulated culture, the growth curve revealed an initial exponential expansion and a subsequent plateau phase, resulting in termination on day 18 of the culture (Fig. 1A). This pattern was coincident with increased p24 antigen production and with increased viral burden as measured by a quantitative polymerase chain reaction (PCR) for cellular HIV-1 *gag* (Fig. 1, B to D). In contrast, when cells were cultured with CD3 and CD28 mAbs, exponential cell proliferation was maintained for 50 days (Fig. 1A). Although there was evidence of modest viral expression early in the culture, as indicated by the concentration of p24 on day 8 (Fig. 1B), viral production and proviral DNA decreased to undetectable amounts in the culture (Fig. 1, C and D). Similar results were obtained whether the starting cell population was peripheral blood mononuclear cells (PBMCs) or purified CD4$^+$ T cells; this finding suggested that the enhanced cell proliferation and antiviral effects in the culture stimulated with CD3 and CD28 mAbs were not dependent on CD8$^+$ T cells or accessory cells (Table 2) (*8*).

A quantitative PCR was used to determine amounts of HIV-1 *gag* DNA and RNA in the cultures of lymphocytes from the 10 HIV-positive patients (Table 1). Culture with CD28 mAb resulted in decreased viral burden in all patient-derived cells, including the cells cultured in the absence of antiretroviral agents. HIV-1 *gag* proviral DNA became undetectable in six of seven cultures from patients that were cultured in the absence of antiretroviral agents, and HIV-1 *gag* RNA became undetectable in five of the seven cultures. Culture supernatants were also sampled for p24 antigen at 7- to 14-day intervals. Antigen was not detected in 9 of the 10 patients; in one patient (patient 9; Table 1 and Fig. 1), decreasing concentrations of p24 antigen with time were detected. Virus-free CD4$^+$ T cell proliferation also occurred even when CD8 cells constituted <1% of the cells (Table 2) (*9*).

The increase in the number of CD4 cells was not significantly different in the presence or absence of a combination of antiretroviral agents in three patients (*8*). In uninfected adult blood donors, the average absolute magnitude of the CD28-mediated proliferation of ex vivo polyclonal CD4$^+$ T cells is ~10^{10} or 33 population doublings (*7*). The limits of CD4$^+$ T cell proliferation in HIV-infected patients have not yet been determined, because 7 of 10 cultures were terminated after 4 to 8 weeks of culture and cell proliferation remained in the exponential phase. However, the observed increase appeared to be substantial, with a geometric mean expansion of 6.7 log units in the two CD4$^+$ cell cultures that were continued to plateau phase (Table 1). The mean percent-

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age of $CD4^+$ T lymphocytes in the expanded cultures was 86% and varied from 39 to 99% (*10*). The antigen receptor diversity of the expanded populations of $CD4^+$ T cells was assessed by flow cytometric measurement of the TCR V_β repertoire in seven patients after 4 to 12 weeks in culture. All patients had diverse V_β family expression similar to the input population of cells (*8*), in a manner consistent with a CD28-mediated proliferation of polyclonal $CD4^+$ lymphocytes.

Consistent with previous results with normal cells (*11*), the cells from the HIV-infected patients secreted large amounts of T helper cell type 1 (T_H1) cytokines after stimulation with immobilized CD3 and CD28 mAbs. Cytokine accumulation was measured by enzyme-linked immunosorbent assay (ELISA) in eight of the cultures (*12*). The range was 18 to 215 ng/ml (mean, 74 ng/ml) for IL-2, 5 to 78 ng/ml (mean, 32 ng/ml) for interferon-γ (IFN-γ), 0.005 to 0.25 ng/ml (mean, 0.09 ng/ml) for IL-4, 0.03 to 0.66 ng/ml (mean, 0.32 ng/ml) for IL-5, and 3 to 10 ng/ml (mean, 6.7 ng/ml) for tumor necrosis factor–α (TNF-α). Secretion of the C-C chemokines by CD8 cells has been shown to mediate a noncytotoxic antiviral effect (*13*). The concentration of the C-C chemokines RANTES, MIP-1α, and MIP-1β in supernatants of cultures of CD4 cells did not vary consistently with CD28 costimulation (*8*), and therefore the presence of these chemokines is unlikely to be sufficient to mediate the CD28 antiviral effect. However, our results do not exclude a necessary role for chemokines in the antiviral effect.

To determine whether limiting dilution by discarding cells during culture might enrich the number of uninfected cells and thus account for the loss of HIV-1–infected cells, we performed large-volume culture by addition of medium without removal of cells. As shown in Table 2, the amount of proviral *gag* DNA and p24 antigen decreased in culture at the same rate, regardless of cell discards. We found that CD28 stimulation routinely permitted large-scale culture, in the absence of cell discards and antiretroviral drugs, to $>10^{10}$ $CD4^+$ T cells from patients with intermediate-stage HIV-1 infection. Thus, these experiments indicated that the frequency of HIV-infected $CD4^+$ T cells decreases during culture with CD28 mAb, and therefore that this antiviral effect cannot be attributed to serial replacement of medium and cells with fresh medium during the cell culture process.

The observed reduction in viral load during proliferation of patient lymphocytes after treatment with immobilized CD28 mAb is in contrast to previous studies that showed that the addition of soluble CD28 mAb to cultures of lymphocytes from HIV-1–infected donors resulted in enhanced HIV-1 expression and that in vitro infection of $CD4^+$ T cells was followed by enhanced HIV-1 production (*14*). To assess whether the mode of CD28 stimulation might be important in determining the extent of HIV-1 replication after in vitro infection, we cultured CD8-depleted PBMCs in the presence of high-titer $HIV\text{-}1_{Ba\text{-}L}$ and activated them with CD3 mAb and either immobilized or soluble CD28 mAb. PHA-stimulated cells and cells stimulated with soluble CD28 mAb developed high concentrations of p24 antigen (Fig. 2A). In marked contrast, the cultures stimulated with immobilized CD28 mAb did not contain detectable concentrations of p24. Cultures stimulated with soluble CD3 and CD28 mAbs developed the highest concentrations of p24 (Fig. 2C), consistent with previous observations (*14*). The differences in HIV-1 p24 concentrations were not the result of differences in the strength of T cell activation, because the increase in the numbers of cells during the experiment was equivalent for all forms of activation (Fig. 2B). Results similar to those shown in Fig. 2 and Table 1 were also observed in experiments in which cell cultures containing highly purified $CD4^+$ T cells were used instead of CD8-depleted PBMCs.

These results indicate that, depending on the mode of CD28 receptor engagement, costimulation can enhance or potently inhibit HIV-1 expression or the susceptibility to HIV-1 infection as assessed by p24 concentration in culture supernatants from $CD4^+$ T cells. To distinguish between these possibilities and to ascertain the stage of this antiviral effect in the life cycle of HIV-1 infection, we stimulated cells with PHA or CD28 mAb for 3 days before infection with high-titer HIV-1 and used quantitative PCR to assess the kinetics of full-length *gag* DNA accumulation (Fig. 3). After 4 hours of exposure to HIV-1, cells previously stimulated with either PHA plus IL-2 or immobilized CD3 mAb plus IL-2 had large amounts of viral *gag* DNA within 12 to 24 hours of culture. In contrast, cells stimulated with immobilized CD3 and CD28 mAbs had background or near-background amounts of

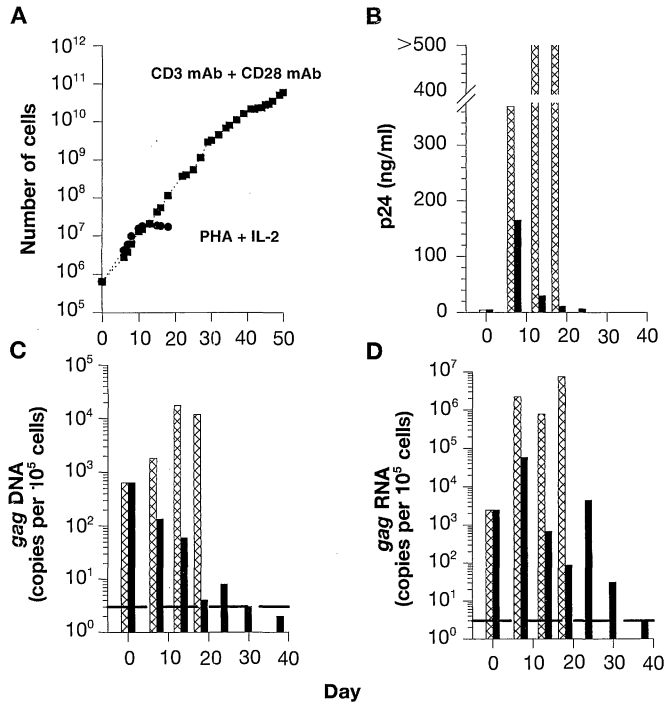

Fig. 1. CD28 mAb–mediated $CD4^+$ T cell proliferation from an HIV-infected patient in the absence of exogenous feeder cells, cytokines, or antiretroviral agents. $CD4^+$ T cells from patient 9 (Table 1) were cultured in either PHA (5 μg/ml, Sigma) plus IL-2 (100 U/ml, Boehringer Mannheim) or beads coated with CD3 and CD28 mAbs, as described below. Cells and supernatants were harvested at 5- to 7-day intervals for analysis of viral burden and cell proliferation. (**A**) Growth curve. The culture stimulated with PHA plus IL-2 was terminated on day 18 because of cytopathic viral effects. (**B**) Concentration of p24 antigen. Cell-free culture supernatants were analyzed for p24 by Coulter ELISA. (**C** and **D**) HIV-1 viral burden. Quantitative measurements of cellular HIV-1 *gag* RNA and proviral DNA were done by PCR with 5×10^6 cells per point, as described below. The horizontal line indicates the lower limit of sensitivity (five copies per 10^5 cells). $CD4^+$ T cells were purified by negative selection [magnetic beads (Dynal) coated with antibodies were used to remove non-CD4 cells (*7*)]. Cells were cultured in plastic tissue culture flasks at 1×10^6 to 2×10^6 cells/ml in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (Hyclone, Logan, Utah), 2 mM L-glutamine, and 20 mM Hepes. Cells were stimulated with immunomagnetic beads (Dynal) that were loaded by tosyl conjugation with equal amounts of CD3 mAb OKT3 and CD28 mAb 9.3 as described (*7*). Beads were added at a ratio of three beads per cell. The cell cultures were monitored by electronic cell sizing (Coulter Counter ZM and Channelyzer 256; Coulter, Hialeah, Florida), and cultures were restimulated with additional beads when the volume of the T cell blasts decreased to <400 fl. No exogenous cytokines or feeder cells were added to the CD28 culture. PCR amplification of β-globin and HIV-1 *gag* with detection by liquid hybridization was used to measure sequential changes in HIV-1 *gag* DNA and RNA during cell culture. Quantitation of HIV-1 *gag* RNA and proviral *gag* DNA in frozen cell pellets was performed as described (*26*).

Table 1. Proliferation, mediated by CD3 and CD28 mAbs, of CD4 cells from HIV-infected donors. PBMCs were obtained from HIV-1–infected persons. The enriched CD4 T cells were cultured with beads coated with CD3 and CD28 mAbs, as described in Fig. 1. Patients had U.S. Centers for Disease Control and Prevention category 2A or 2B infection. The percentage of CD4 cells at culture initiation and at termination and the calculated extent of CD4 cell proliferation are indicated (*25*). Coulter ELISA (Coulter, Hialeah, Florida) was used to detect p24 antigen in culture supernatants at 7- to 14-day intervals; the peak concentration during in vitro culture is indicated. HIV-1 viral burden was determined as described in Fig. 1 at 7- to 14-day intervals (*8*); initial and final values are shown. Culture treatment code: A, azidothymidine (AZT, 1 μM); D, 2′,3′-dideoxyinosine (ddI, 5 μM); N, Nevirapine (0.5 μM). For CD4 cell proliferation, cultures on patients 1 to 8 are submaximal expansions and were terminated while cell proliferation remained in the exponential phase, whereas cultures on patients 9 and 10 were carried to maximum expansion with addition of exogenous IL-2. ND, not done.

Patient	CD4 count (cells/mm^3)	Culture treatment	CD4 cells (%)		CD8 cells (%)		CD4 cell proliferation		Peak p24 concentration (ng/ml)	*gag* RNA (copies per 10^5 cells)		*gag* DNA (copies per 10^5 cells)	
			Initial	Final	Initial	Final	log (number of cells)	Days		Initial	Final	Initial	Final
1	554	A, D	93.2	97.1	1.9	1.2	3.7	35	<0.1	55	<3	<3	<3
2	433	A, D	93.2	95.8	2.2	2.8	2.8	29	<0.1	224	<3	190	20
3	430	A, D, N	93.4	97.2	2.1	3.0	4.0	34	<0.1	7,090	15	1470	51
4	445	None	92.3	98.7	2.3	5.2	3.1	28	<0.1	147	23	128	37
5	355	None	ND	95.8	ND	2.9	4.5	40	<0.1	8	<3	107	<3
6	384	None	91.7	39	1.6	56.3	3.3	36	<0.1	313	<3	267	<3
7	466	None	93.6	95.8	1.6	2.6	3.6	28	<0.1	64	<3	57	<3
8	500	None	82.8	67.7	10.0	35.9	2.2	28	<0.1	267	<3	309	<3
9	401	None	ND	97.8	ND	2.0	6.9	71	0.17	2,448	<10	636	<5
10	413	None	64.1	70.2	2.9	18.7	6.5	61	<0.1	14,037	20	614	<5

Table 2. Outgrowth of uninfected CD4 cells from an HIV-infected donor during large-volume culture. CD4 cells (containing <0.5% CD8 cells) were obtained by two sequential rounds of magnetic bead immunodepletion of non-CD4 cells from an HIV-infected donor (CD4 count, 393 cells/mm^3). The cells were cultured in a 3-liter gas-permeable bag (Baxter) with stimulation by CD3 and CD28 mAbs for 17 days, during which time there were no cell discards (Bag). Alternatively, cells were grown in companion cultures in T25 flasks, with discards as necessary to maintain cells during feeding with fresh medium (Flask). No antiretroviral agents were added to the cultures. Cell samples were collected for quantitative PCR of *gag* DNA, and culture supernatants were tested for p24 content on the indicated days of culture. The extent of cell proliferation was nearly identical in the two culture conditions; bag cells increased from 30×10^6 to 6.7×10^9 cells, and flask cells increased from 5×10^6 to 2.3×10^9 cells.

Day	Culture conditions	*gag* DNA (copies per 10^5 cells)	p24 (pg/ml)
0	Medium only	802	<50
7	Bag	100	565
	Flask	136	667
11	Bag	110	96
	Flask	72	99
14	Bag	19	<50
	Flask	28	<50
17	Bag	21	<50
	Flask	52	<50

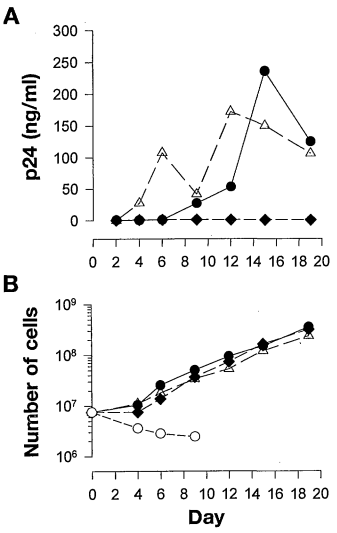

Fig. 2. CD28 can potentiate or inhibit HIV-1 infection of CD4 cells in vitro. (**A** and **B**) CD8-depleted PBMCs were cultured for 2 days in PHA (5 μg/ml) plus IL-2 (100 U/ml) (●), soluble CD3 mAb (100 ng/ml) plus IL-2 (100 U/ml) plus soluble CD28 mAb (1 μg/ml) (△), beads coated with CD3 and CD28 mAbs (◆), or medium only (○) as described in Fig. 1. HIV-1_{Ba-L} (*27*) was also added on day 0 at 2666 times the median tissue culture infectious dose ($TCID_{50}$) per 1×10^6 cells. On day 2, virus was washed out of all cultures, and cells were replated in conditioned media obtained from companion cultures of uninfected cells. Cells were harvested after a further 2 to 17 days of culture, with fresh media added as necessary to maintain the cells at a concentration of 1×10^6 to 2×10^6 cells/ml. Cell-free supernatants were harvested at 2- to 3-day intervals and p24 concentrations were determined by ELISA. Cell counts are averages of two measurements with a Coulter Counter ZM. (**C**) CD8-depleted PBMCs were cultured for 2 days in soluble CD3 mAb (100 ng/ml) plus soluble CD28 mAb (1 μg/ml) (○), soluble CD3 mAb (100 ng/ml) plus IL-2 (100 U/ml) (□), soluble CD3 mAb (100 ng/ml) plus soluble CD28 mAb (1 μg/ml) plus IL-2 (100 U/ml) (△), soluble CD3 mAb (100 ng/ml) plus beads coated with CD28 mAb (▼), or beads coated with CD3 and CD28 mAbs (◆). HIV-1_{Ba-L} was also added on day 0 at 5000 $TCID_{50}$ per 1×10^6 cells. After 2 days, cultures were washed to remove virus and supernatants were harvested as described above.

gag DNA at all time points assessed (Fig. 3A). The antiviral effect could not be attributed to CD3 mAb stimulation alone, as CD4 cells stimulated with immobilized CD3 mAb were infected after exposure to HIV-1, whereas cells exposed to beads coated with CD3 and CD28 mAbs were protected from infection (*8*, *15*). There was specificity for the CD28-mediated antiviral effect because CD4 cells stimulated with beads coated with CD3 and MHC I mAbs were susceptible to infection,whereas beads coated with CD3 and CD28 mAbs rendered the cells resistant to infection (Fig. 3D). Phosphorimager analysis indicated that the amount of *gag* PCR product in cells stimulated with CD3 and CD28 mAbs was less than one-hundredth the amount in cells stimulated with PHA and IL-2. A similar difference was observed when cells infected with HIV-1_{US-1} were stimulated with PHA and IL-2 or with PHA, IL-2, and immobilized CD28 mAb, which indicated that CD28 stimulation could prevent HIV-1 infection in the context of stimulation with PHA and IL-2 (Fig. 3E). Thus, CD28 costimulation conferred marked resistance to HIV-1 infection. The effect did not appear to depend on the strain of virus used

for infection (9), which is consistent with the ability of CD28 costimulation to increase the number of $CD4^+$ cells from multiple patients infected with HIV-1.

The anti-infective effect of CD28 occurred early in the life cycle of HIV-1 infection, before integration. The mechanism was shown to be multifactorial and to involve a prominent protective effect of CD28 stimulation against HIV-1 infection as well as a proliferative advantage of HIV-uninfected $CD4^+$ T cells over HIV-infected cells. Our initial studies indicate that CD4 receptor function remains normal in cells stimulated with CD3 and CD28 mAbs, which suggests that the HIV-1–resistant state is downstream of HIV-1 binding. It is possible that the permissive and inhibitory forms of CD28 costimulation reflect differential signal transduction (*16*) and that distinct forms of signal transduction confer HIV-1–susceptible or HIV-1–resistant states. As was noted above, the CD28-mediated antiviral effect we describe appears to be distinct from that previously described by Levy and others (*17*).

Our results demonstrate that proliferation of polyclonal HIV-1–uninfected $CD4^+$ T cells from HIV-infected donors is possible, and that CD28 stimulation provides a selective proliferative advantage to subsets of cells that do not support infection. Alternatively, CD28 stimulation could inhibit HIV-1 replication or induce death of the HIV-infected cells. With regard to uninfected $CD4^+$ T cells, there are several possible mechanisms whereby CD28 stimulation could provide a selective proliferative advantage. First, CD28 induces the proliferation of a subset of cells that does not include HIV-infected $CD4^+$ T cells. HIV-1 resides primarily in polyclonal $CD4^+$ cells with the memory phenotype (*1, 18*). The selective induction of proliferation of naive $CD4^+$ cells by CD28 mAb is unlikely to explain our results, because stimulation with CD3 and CD28 mAbs has been shown to induce the proliferation of essentially all $CD4^+$ cells from normal individuals, including memory and naive subsets (*7, 19*). Second, in some patients, $CD4^+$ cells of the TCR $V_\beta 12$ family are preferentially infected with HIV-1, and under certain culture conditions HIV-1 replicates 10 to 100 times as efficiently in $CD4^+$ $V_\beta 12$ cells as in control V_β cells (*20*). This possibility was excluded because we did not find a preferential selection against cells that express $V_\beta 12$ in the four cultures that were examined before and after CD28-mediated $CD4^+$ cell proliferation (*21*). Third, HIV-positive individuals have an increasing fraction of $CD28^-$ T cells (*22*), and it is likely that CD28 stimulation confers a proliferative advantage to the $CD28^+$ T cells. It is possible that the viral burden is disproportionately represented in the $CD28^-$ cells. We have also considered that CD28 may selectively stimulate the differentiation of CD4 cells that do not support HIV-1 infection. For example, other investigators have concluded that HIV-1 preferentially replicates in $CD4^+$ T cells producing T_H2-type cytokines (T_H2 and T_H0 cells) (*23*). Further studies will be required to test these notions.

The culture of large numbers of $CD4^+$ T cells from HIV-infected patients has proven difficult. In one study, successful $CD4^+$ T cell proliferation in a subset of HIV-infected patients required the addition of multiple antiretroviral agents to cell culture medium to prevent viral expression (*24*). However, clinical utility was limited by drug-resistant virus breakthrough and a requirement for allogeneic feeder cells to restimulate lymphocytes. Several therapeutic approaches to HIV-1 infection might be facilitated by our results. Ex vivo proliferation of $CD4^+$ T cells may permit immune reconstitution and vaccine therapies involving autologous transfusions of polyclonal or antigen-specific $CD4^+$ T cells into patients. Moreover, autologous transfusions of CD4 lymphocytes might provide the immunologic help necessary to sustain $CD8^+$ T cell function. We have found that although CD28 stimulation can prevent HIV-1 infection and expression, it supports high transduction efficiencies with Moloney leukemia virus–based retroviral vectors (9); hence, culture systems that use CD28 costimulation might be an efficacious way to generate $CD4^+$ T cells for gene therapy as well as immunotherapy. Our results demonstrate a potent CD28-mediated antiviral effect in patients with intermediate-stage HIV infection. Preliminary results from a limited number of patients indicate that the antiviral effect may be less potent in late-stage HIV infection, even though CD28 costimulation still enhances $CD4^+$ T cell proliferation. Finally, our results indicate that in vivo manipulation of CD28 interaction with B7 counterreceptors has the potential to enhance $CD4^+$ T cell proliferation and prevent or limit HIV-1 viral spread in patients.

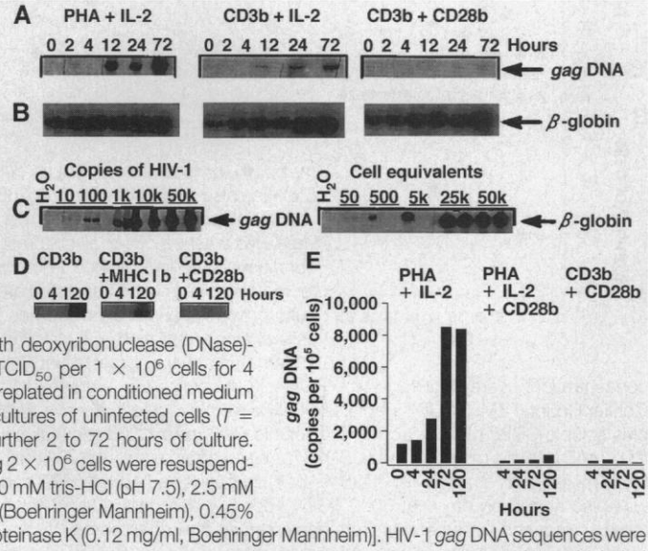

Fig. 3. Immobilized CD28 mAb prevents HIV-1 infection of CD4 cells, as assessed by PCR analysis of HIV-1 *gag*. (**A** and **B**) $CD4^+$ T cells were cultured for 3 days in PHA (5 μg/ml) plus IL-2 (100 U/ml), beads coated with CD3 mAb (CD3b) plus IL-2 (100 U/ml), or beads coated with CD3 and CD28 mAbs (CD3b + CD28b). The cells were collected and incubated with deoxyribonuclease (DNase)-treated HIV-$1_{Ba\text{-}L}$ at 7000 $TCID_{50}$ per 1×10^6 cells for 4 hours, washed three times, replated in conditioned medium obtained from companion cultures of uninfected cells (T = 0), and harvested after a further 2 to 72 hours of culture. Frozen cell pellets containing 2×10^6 cells were resuspended in 200 μl of lysis buffer [10 mM tris-HCl (pH 7.5), 2.5 mM $MgCl_2$, 0.45% Triton X-100 (Boehringer Mannheim), 0.45% Tween 20 (Bio-Rad), and proteinase K (0.12 mg/ml, Boehringer Mannheim)]. HIV-1 *gag* DNA sequences were amplified as described (*26*). The amplified products were detected by liquid hybridization with end-labeled oligonucleotide probes, followed by gel electrophoresis. PCR products were quantitated as described (*26*) with a Molecular Dynamics Phosphorimager. (**C**) Standardization of the PCR products was achieved by parallel amplification of a series of plasmid external control templates. (**D**) CD4 T cells were cultured in IL-2–containing medium as described in (A) and (B) by stimulation with beads coated with CD3 mAb, with CD3 and MHC I mAbs (CD3b + MHC I b), or with CD3 and CD28 mAbs. (MHC I mAb W6/32, which binds to the framework region of HLA class I A, B, and C molecules, served as an isotype and binding control for CD28 mAb 9.3.) Samples were analyzed for *gag* DNA at 0, 4, and 120 hours. (**E**) Immobilized CD28 mAb prevents HIV infection of PHA-stimulated CD4 blasts. $CD4^+$ T cells (95% purity) were cultured for 3 days in medium containing PHA (5 μg/ml) plus IL-2 (100 U/ml), PHA (5 μg/ml) plus IL-2 (100 U/ml) plus beads coated with CD28 mAb, or beads coated with CD3 and CD28 mAbs. The cells were collected and infected with DNase-treated HIV-1_{US1} isolate (*27*) at 1.5×10^3 $TCID_{50}$ per 10^6 cells. Cells were collected for PCR analysis after a further 4 to 120 hours of culture. HIV-1 *gag* and β-globin were quantitated from frozen cell pellets as described above.

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10. Culture with CD3 and CD28 mAbs provides a proliferative advantage for $CD4^+$ over $CD8^+$ lymphocytes with uninfected donors (7) [R. Costello *et al.*, *Eur. J. Immunol.* **23**, 608 (1993)]. However, with HIV-infected donors, the proportion of $CD28^-$ lymphocytes is greater and there are more $CD8^+$ lymphocytes in the input culture, so that further enrichment steps would be required to obtain pure $CD4^+$ lymphocytes at culture termination.
11. T_H1 cytokines predominate in cell cultures when immobilized CD28 mAb is used for stimulation (7). In contrast, when soluble CD28 mAb is used for cell culture, T_H2 cytokines may predominate [C. L. King, R. J. Stupi, N. Craighead, C. H. June, G. Thyphronitis, *Eur. J. Immunol.* **25**, 587 (1995); W. Holter, O. Majdic, F. S. Kalthoff, W. Knapp, *ibid.* **22**, 2765 (1992); T. van der Pouw Kraan, R. de Jong, L. Aarden, *ibid.* **23**, 1 (1993)]. In vivo, CD28-B7 blockade after alloantigenic challenge inhibits T_H1 cytokines but spares T_H2 cytokines [M. H. Sayegh *et al.*, *J. Exp. Med.* **181**, 1869 (1995)]. Thus, the mode of CD28 stimulation may affect T_H1 and T_H2 development [J. A. Bluestone, *Immunity* **2**, 555 (1995); C. B. Thompson, *Cell* **81**, 979 (1995)].
12. Cytokine secretion was tested in cultures from eight patients in Table 1 after 10 to 20 days in culture. Cells were collected and washed, and the extent of cytokine secretion upon restimulation was determined by placing the cells into fresh medium with beads coated with CD3 and CD28 mAbs for 24 hours. Cell-free culture supernatants were analyzed for IL-2, IFN-γ, IL-4, IL-5, and TNF-α by ELISA with the use of commercially available kits [T Cell Diagnostics, Woburn, MA (IL-2); R&D Systems, Minneapolis (TNF-α, IL-4, and IL-5); Endogen, Boston (IFN-γ)].
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15. CD4 cells have a hierarchy of permissiveness to HIV-1 infection that is determined by the mode of stimulation with various mitogens. In most donors, the susceptibility to HIV-1 infection is [soluble CD3 mAb, soluble CD28 mAb] → [concanavalin A, PHA] → [immobilized CD3 mAb, soluble CD28 mAb] → [immobilized CD3 mAb, immobilized CD28 mAb]. The increased susceptibility of CD4 cells to HIV infection after stimulation with soluble antibody has been noted previously (*14*).
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17. We have considered whether the antiviral effect mediated by CD28 costimulation might be a soluble factor or factors previously described by others [C. M. Walker, D. J. Moody, D. P. Stites, J. A. Levy, *Science* **234**, 1563 (1986)]. Given that the factor first described by Levy is secreted by CD8 cells and suppresses viral replication late in the viral life cycle, it is likely that the CD28 effect we have described is distinct. First, as shown in Table 2, the effect mediated by CD28 requires <0.6% CD8 cells, and thus it is either independent of CD8 T cells or dependent on trace amounts of CD8 cells. Second, the CD28 effect acts early in the viral life cycle to prevent infection of CD4, whereas the effect first described by Levy is reported to act late in the viral life cycle to suppress infection. Third, Cocchi *et al.* (*13*) have reported that chemokines are the major mediator of the CD8 antiviral effect, and we have found that the secretion of C-C chemokines is not dependent on CD28 stimulation (*9*).
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Solution Structure of a Two-Base DNA Bulge Complexed with an Enediyne Cleaving Analog

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Nucleic acid bulges have been implicated in a number of biological processes and are specific cleavage targets for the enediyne antitumor antibiotic neocarzinostatin chromophore in a base-catalyzed, radical-mediated reaction. The solution structure of the complex between an analog of the bulge-specific cleaving species and an oligodeoxynucleotide containing a two-base bulge was elucidated by nuclear magnetic resonance. An unusual binding mode involves major groove recognition by the drug carbohydrate unit and tight fitting of the wedge-shaped drug in the triangular prism pocket formed by the two looped-out bulge bases and the neighboring base pairs. The two drug rings mimic helical DNA bases, complementing the bent DNA structure. The putative abstracting drug radical is 2.2 ± 0.1 angstroms from the pro-*S* H5′ of the target bulge nucleotide. This structure clarifies the mechanism of bulge recognition and cleavage by a drug and provides insight into the design of bulge-specific nucleic acid binding molecules.

Bulged structures (regions of unpaired bases) in nucleic acids have been the subject of intense interest (*1*), because they have been implicated as binding motifs for regulatory proteins in viral replication (*2*), as targets for repair enzymes in imperfect homologous recombination (*3*), as products of slipped mispairing in the replication of microsatellite DNA (*4*), as intermediates in frameshift mutations (*5*), and as essential elements in naturally occurring antisense RNAs (*6*).

Neocarzinostatin chromophore (NCS chrom) is unusual among the naturally occurring enediyne antibiotics (*7*) in its ability to attack specifically and exclusively a single residue at a two-base bulge of certain DNA sequences under the influence of general base catalysis (*8*). Under the same conditions NCS chrom cleaves the transactivation response element of human immunodeficiency virus type I viral RNA with high specificity at one of its proposed bulge residues (*9*). Further, studies with long single-stranded DNAs, similar to ones found in some DNA viruses, have revealed related binding-cleavage sites located at bulged sites (*10*). This

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