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We have observed that CD28 costimulation of CD4+ cells can have differential effects on HIV replication. Triggering the CD28 molecule on peripheral blood CD4+ cells during stimulation with anti-CD3 Abs enhances virus production following acute infection with HIV. Endogenous virus production in CD4+ cells from HIV-infected individuals is also increased by this procedure. The enhanced virus production occurs equally when anti-CD28 Abs and soluble forms of the natural ligands for CD28, CD80 Ig, and CD86 Ig are used to trigger CD28 on CD4+ cells during stimulation. This increased virus replication is observed only when the source of CD28 costimulation is removed immediately after stimulation and before infection. Continued exposure of CD4+ cells to anti-CD3 and CD28 Ab beads following acute infection prevents virus production. These findings may have relevance to therapeutic approaches aimed at inhibiting HIV replication by CD28 costimulation. The Journal of Immunology, 1998, 161: 6223–6227.

Cellular activation is necessary for efficient HIV replication in infected cells (1). For example, CD4+ T cells, the major reservoir of HIV-1 in vivo, require the engagement of the TCR/CD3 complex in the presence of IL-2 to become productively infected by HIV (2). Although resting cells can be infected, virus production does not occur unless these cells are stimulated (3–5). Moreover, the extent of virus released from the infected cell is directly related to the level of cell activation (5, 6).

Optimal HIV production in CD4+ T cells requires not only the engagement of the TCR/CD3 complex but also a costimulatory signal provided by the CD28 molecule on the cell surface (7). Blocking the CD28 ligands, CD80 and CD86, during stimulation of PBMC reduces the ability of these cells to support HIV replication (8). Moreover, CD28 costimulation using anti-CD28 Abs or following coculture with cell lines expressing CD80 and CD86 enhances the level of HIV production when compared with cells stimulated with anti-CD3 Abs alone (9).

An apparent contradiction to the above observations, recent studies have demonstrated that exposure to anti-CD3 and CD28 Abs immobilized on Sepharose beads appears to induce CD4+ cells to resist productive infection by strains of HIV that are able to infect macrophages (M-tropic) but not T cell lines (TCL-tropic) (10). This arrest in virus production is not only apparent by a decrease in virus particles present in the culture fluids but also by a reduction in proviral DNA in acutely and naturally infected cells (10, 11). The differences in the outcomes of these recent studies vs the earlier ones (9) showing enhanced HIV replication following CD28 costimulation have been attributed to the use of soluble instead of immobilized anti-CD28 Ab during stimulation (10). Following CD28 costimulation, an increased production of β-chemokines (11) and/or decreased expression of CCR5 chemokine receptor (12) have been suggested to be involved in blocking HIV infection of CD4+ cells. Both mechanisms can prevent entry of M-tropic viruses that use CCR5 as a coreceptor (reviewed in Ref. 13). Because CD28 costimulation appears to render CD4+ cells resistant to infection with HIV, its potential therapeutic use for ex vivo expansion of CD4+ cells from HIV-infected individuals followed by reconstitution has been proposed (10, 14).

In previous studies, we demonstrated that CD28 costimulation of CD8+ cells increased their capacity to suppress HIV replication (15). To determine whether triggering the CD28 molecule during stimulation could be used in the treatment of HIV-infected individuals, we further evaluated what effect CD28 costimulation would have on HIV infection of CD4+ cells. Our present findings demonstrate that CD28 costimulation, using anti-CD3 and CD28 Ab coupled to beads, can lead to enhanced or reduced HIV production in CD4+ cells depending on whether the cells are continuously exposed to the Ab beads during culture.

Materials and Methods

Subjects

Peripheral blood was collected by venipuncture in Vacutainer tubes containing sodium heparin (Becton Dickinson, Rutherford, NJ). Blood from HIV-seronegative donors was provided by The Blood Centers of the Pacific (San Francisco, CA) or came from randomly selected healthy individuals employed at the University of California, San Francisco (UCSF). The two HIV-infected individuals used in our study were volunteers with CD4+ cell counts of 682 and 164 cells/μl, respectively, and their percentages of CD4+ cells were 25 and 12, respectively. Both subjects were asymptomatic, had viral loads <500 viral RNA copies/ml at the time of the study, and were taking AZT and 3TC (Glaxo Wellcome Pharmaceuticals, Research Triangle Park, NC. The subject with 164 CD4+ cells/μl was also taking Viracept (Agouron Pharmaceuticals La Jolla, CA). The biologic phenotype of the viruses present in the blood of the infected individuals was determined by the MT2 assay (16). The studies received the approval of the Committee on Human Research, UCSF.

Isolation of PBMC and their cell subsets

PBMC were obtained by Ficoll-Hypaque (Sigma, St. Louis, MO) gradient centrifugation (17). The CD4+ cells were isolated from the PBMC by...
positive selection using magnetic beads bearing anti-CD4 mAb (Dynal, Lake Success, NY) (18). Beads were removed from the cells by Detach-a-bead (Dynal) according to the manufacturer’s instructions. Purity of the cells obtained by the immunomagnetic bead isolation procedure was >95% CD4⁺, <1% CD8⁺, <1% CD19⁺, <1% CD56⁺, and <1% CD14⁺ as determined by flow cytometry (FACSort, Becton Dickinson) (19).

**Culture medium, cytokines, and reagents**

RPMI 1640 medium (BioWhittaker, Walkersville, MD) supplemented with 10% heat-inactivated (56°C, 30 min) FBS (Gemini Bio-Products, Calabasas, CA), 2 mM glutamine (BioWhittaker), and 100 U/ml penicillin, and 100 μg/ml streptomycin (BioWhittaker) was used as the culture medium (complete medium). Stimulation of purified CD4⁺ cells (3 × 10⁶) for use in the acute infection was done in the presence of 1 ml of the complete medium containing 100 U/ml of recombinant human (rh) IL-2 (gift from Glaxo-Wellcome) over 3 days. The mouse anti-human CD3 mAb (clone X53; Immunotech, Bar Harbor, ME) was used to stimulate the CD4⁺ cells was coupled to tosyl-activated magnetic Sepharose beads according to the manufacturer’s instructions (Dynal). The optimal anti-CD3 Ab bead:CD4⁺ cell ratio used in our study was 2:1. This ratio was determined by the maximal incorporation of tritiated thymidine by CD4⁺ cells incubated for 3 days in the presence of various numbers of beads compared with cells cultured in the absence of the beads in a final volume of 0.2 ml of complete medium (Fig. 1A). In experiments evaluating costimulation of CD4⁺ cells, anti-CD28 Ab (clone CD28.2; Immunotech) was added together with equal amounts of anti-CD3 Ab to tosyl-activated beads and used at a optimal bead-to-cell ratio of 2:1 (Fig. 1B). The CD80lg and CD90lg fusion proteins were used to costimulate CD4⁺ cells (provided as a gift by Philip Morton, Monsanto, St. Louis, MO) were mixed with an equal amount of anti-CD3 Ab before coupling them to magnetic rat anti-mouse IgG2a (Fc) beads according to the manufacturer’s instructions (Dynal). These beads were used at an optimal bead-to-cell ratio of 2:1 (data not shown).

**Acute infection of CD4⁺ cells**

Following stimulation of the CD4⁺ cells, the magnetic beads were removed and the cells were washed three times in calcium- and magnesium-free HBSS (CMF-HBSS, BioWhittaker). The stimulated CD4⁺ cells were then treated with polybrene (2 μg/ml for 30 min; Sigma) and infected with 100 × 50% tissue culture infectious dose (TCID₅₀) of HIV-1SF162, HIV-1SF128, HIV-1SF33, HIV-1SF162/HIV-1SF28A, or HIV-1Bal for 1 h. The HIV-1SF33 isolate is a highly cytopathic, TCL-tropic, and syncytium-inducing (SI) virus (20). The other isolates are nonsyncytium-inducing (NSI) M-tropic viruses (21, 22). All viruses were cultured only in PBMC and not T cell lines. The β-chemokines, RANTES, MIP-1α, and MIP-1β, have been shown to block infection of the M-tropic but not the TCL-tropic viruses (23). The infected cells were washed three times with CMF-HBSS, resuspended in complete medium containing 100 U/ml of rhIL-2, and plated in 96-well flat-bottom plates (Falcon Labware, Lincoln Park, NJ) at 10⁵ cells/0.2 ml using six replicates/group. Cultures were passed every 3–4 days by removing one-half the volume of medium and replacing it with fresh complete medium containing 100 U/ml of rhIL-2. The culture fluids that were removed were then monitored for particle-associated reverse transcriptase activity (24). In some experiments, the anti-CD3 and CD28 Ab beads were added to the cells every 7–10 days at a bead-to-cell ratio of 2:1.

**Results**

**HIV replication following CD28 costimulation of CD4⁺ cells**

Initially, we determined the effect that triggering the CD28 molecule on CD4⁺ cells during CD3 stimulation would have on the sensitivity of these cells to infection with HIV. Purified CD4⁺ cells from HIV-seronegative donors were treated with magnetic beads coated with both anti-CD3 and CD28 Ab. As controls, CD4⁺ cells from the same donors were exposed to beads to which only anti-CD3 Ab or anti-CD28 Ab was coupled. Following stimulation, the CD4⁺ cells were infected with 100 TCID₅₀ of either the M-tropic HIV-1SF162 or the TCL-tropic HIV-1SF33 isolate. The results indicated both types of HIV strains could replicate in the CD28-costimulated CD4⁺ cells (Fig. 2). Peak HIV replication of both strains (645 × 10³ and 840 × 10³ cpm/ml, respectively) was observed 10 days following the initiation of infection. Exposure to anti-CD3 Ab coupled to beads alone led to moderate (146 × 10³ cpm/ml) levels of HIV-1SF162 replication 10 days after infection (Fig. 2A). Moreover, at the time of infection β-chemokine production was elevated in CD28-costimulated cells (112 ng/ml MIP-1α, 63 ng/ml MIP-1β, and 7 ng/ml RANTES) compared with the same cells stimulated with anti-CD3 Ab beads alone (67.9 ng/ml MIP-1α, 37.8 ng/ml MIP-1β, and 3.2 ng/ml RANTES) Ab.

CD28 costimulation using anti-CD3 and CD28 Ab beads significantly enhanced the mean peak level of HIV-1SF162 replication in CD4⁺ cells (416.2 × 10³ cpm/ml) from seven different donors relative to a different aliquot of the same CD4⁺ cells stimulated with anti-CD3 Ab beads alone (233.3 × 10³ cpm/ml) (p = 0.018; Wilcoxon signed rank test). The ability of the M-tropic viruses to infect CD28-costimulated CD4⁺ cells was not limited to the HIV-1SF162 strain because these cells were also susceptible to infection by the HIV-1SF28A and HIV-1Bal M-tropic isolates (data not shown). Replication of HIV-1SF33 appeared to be similar when CD4⁺ cells were exposed to anti-CD3 Ab beads or anti-CD3 and anti-CD28 Ab beads (Fig. 2B). Both strains of HIV-1 did not replicate to detectable levels in CD4⁺ cells exposed to anti-CD28 Ab beads alone (Fig. 2).
HIV replication in CD4<sup>+</sup> cells costimulated with the CD80 and CD86 molecules

Because engagement of the CD28 molecule with anti-CD28 Ab during stimulation enhanced the replication of HIV in CD4<sup>+</sup> cells, we next determined if the natural ligands (i.e., the CD80 and CD86 molecules) for the CD28 receptor could replace these Abs in the costimulation process. CD4<sup>+</sup> cells from HIV-seronegative individuals were cocultured with beads coated with both anti-CD3 Ab plus CD80Ig and anti-CD3 Ab plus CD86Ig before infection with HIV-1SF162. Every 3–4 days culture fluids were removed and monitored for reverse transcriptase activity (24). The data are the mean of results from six replicate cultures. The results are representative of two separate experiments using different donors.

Production of HIV from CD28-costimulated CD4<sup>+</sup> cells of HIV-infected individuals

Treatment of CD4<sup>+</sup> cells from HIV-infected individuals with immobilized anti-CD3 and CD28 Abs ex vivo has been reported to decrease endogenous virus production and proviral DNA to undetectable levels (10). Therefore, we determined if triggering the CD28 molecule during stimulation would affect the production of virus from naturally infected CD4<sup>+</sup> cells from HIV-seropositive individuals. One of the subjects studied was clinically healthy and infected with a dominant NSI virus (Fig. 4A); the other individual had an AIDS diagnosis and was infected with an SI virus strain (Fig. 4B) (see Materials and Methods). CD4<sup>+</sup> cells from both HIV-infected individuals were exposed to beads to which either anti-CD3 Ab alone or anti-CD3 Ab together with CD80Ig and CD86Ig coupled to magnetic beads were bound. The cells were then infected with 100 TCID<sub>50</sub> of HIV-1SF162 and cultured for 13 days. Every 3–4 days, culture fluids were removed and monitored for reverse transcriptase activity (24). The data are the mean of results from six replicate cultures. Results are representative of three separate experiments using different donors.
The production of HIV is prevented by constant exposure of CD4+ cells to anti-CD3 and CD28 Abs.

Our current findings that CD28-costimulation induces higher levels of HIV replication differs from those of a recent study by other investigators that reported CD28-costimulation blocks HIV replication (10, 11). In examining the possible reason(s) for the difference, we evaluated whether constant exposure of CD4+ cells to the Ab beads would change the dynamics of virus infection. This procedure was used because other investigators, when studying the effects of CD28-costimulation on HIV production by infected CD4+ cells, added the anti-CD3 and CD28 Ab beads to their culture whenever the CD4+ cell size decreased (10, 14). CD4+ cells were isolated from HIV-seronegative individuals and stimulated for 3 days with anti-CD3 and CD28 Ab coupled to beads. The cell-bead mixture was then split into two groups; the beads were either removed or left in culture during acute infection. HIV could be detected within 7–10 days in culture when the beads were removed from the cells before virus inoculation (Fig. 5). HIV replication was delayed for up to 7 days when the beads were present with the cells during virus infection (data not shown). Furthermore, the addition of fresh anti-CD3 and CD28 Ab beads every 7–10 days following infection led to prolonged suppression of HIV replication (Fig. 5), even as long as 5 wk following infection. Only after removal of the beads (e.g., 25 days postinfection) did virus production take place (Fig. 5B). The kinetics of virus production from these cells was similar to that observed with the infected cells in which the beads were initially removed. These findings indicate that continual CD28-costimulation can suppress the production of HIV by infected CD4+ cells.

**Discussion**

These studies were undertaken to determine whether or not CD28-costimulation would lead to productive infection of CD4+ cells. They were conducted because we considered the possible clinical
application of using CD28 costimulation in HIV-infected people to increase the ability of CD8+ cells to suppress HIV replication (15). We found that engagement of the CD28 molecule on CD4+ T cells from HIV-seronegative individuals during stimulation through the TCR/CD3 complex enhanced HIV production following acute infection of the cells in culture (Fig. 2). This result was observed whether anti-CD28 Ab or the natural ligands for CD28 (CD80 or CD86) (Fig. 3) were used to trigger the CD28 molecule. Moreover, the enhanced HIV replication (both NSI and SI phenotype) was noted following similar CD28 costimulation of naturally infected CD4+ cells from HIV-seropositive individuals (4). This finding is in agreement with the studies by Smithgall et al. (9), who demonstrated that optimal production of HIV from CD8+ cell-depleted PBMC of asymptomatic HIV-infected individuals occurred when accessory cells bearing CD80 and CD86 were present during stimulation.

Our findings differ from those of Levine et al. (10), which showed that activation of CD4+ T lymphocytes with immobilized anti-CD3 and CD28 Ab induced resistance to productive infection by M-tropic viruses. As stated above, using anti-CD3 and CD28 Ab coupled to beads (including those obtained from these authors; data not shown), we found that the CD28-costimulated CD4+ cells were susceptible to acute infection by M-tropic viruses. The effect of CD28-costimulation of CD4+ cells on acute infection by the TCL-tropic strain appeared to be minimal, which probably reflects the rapid growth kinetics of this strain of HIV (21). Importantly, we noted that the differences observed between the two studies were based on whether or not the anti-CD3 and CD28 Ab beads were removed from the CD4+ cells after stimulation. In the experiments conducted by Levine et al., the anti-CD3 and CD28 Ab beads were left on and replenished (Fig. 5). The beads had to be added to the cells every 7–10 days or more, because the beads were removed from the CD4+ cells when their cell size decreased to those of resting cells (10, 14). In our studies, removing the beads immediately after stimulation led to virus production within 7–10 days postinfection, whereas no HIV replication was observed when the beads were left on and replenished (Fig. 5). The beads had to be added to the cells every 7–10 days or more, because the virus was released in culture (data not shown). Even when the beads were removed from the cells 25 days after prolonged CD28-costimulation, virus was still recoverable (Fig. 5B). The virus replication, two passages after removal of the beads, was similar to that observed after the initial infection of cells in which beads were removed after stimulation, before acute infection (Fig. 5B). These findings suggest that HIV can infect CD4+ cells constantly exposed to anti-CD3 and CD28 Ab beads but virus production occurs only after the source of CD28 costimulation is removed.

Other investigators have demonstrated that CD28 costimulation of CD4+ cells enhances β-chemokine production and loss of CCR5 expression (11, 12). Both processes can prevent entry of M-tropic viruses into the cell and virus production (13). Our studies suggest that CD28 costimulation does not effectively block the initial infection of CD4 cells by the M-tropic virus; however, further spread of the virus in the cultures appears to be prevented. We have observed, in preliminary studies, that the CD28 costimulation leads to an increase in β-chemokine levels in the culture fluid. Only by maintaining the CD28 costimulation of CD4+ cells through the addition of Ab beads every 7–10 days are high levels of β-chemokines maintained (our unpublished observations). Thus, our results support those of others (11) suggesting a relationship between high β-chemokine production and reduced virus replication in the cultured CD4+ cells. Further studies are under way to evaluate the effect of constant CD28 costimulation on infection of CD4+ cells by both NSI and SI strains. Whether therapeutically approaches can be developed to mimic these conditions of costimulation in vivo remains to be determined.

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