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Enhancement of HIV-Specific CD8 T Cell Responses by Dual Costimulation with CD80 and CD137L[1]

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HIV-specific CD8 T cell responses are defective in chronic HIV infection. In this study, we report that costimulation with either CD137L (4-1BBL) or CD80 (B7.1) enhanced the Ag-specific expansion and acquisition of effector function by HIV-specific memory CD8 T cells. Ag-specific T cells from recently infected donors showed maximal expansion with single costimulatory molecules. Dual costimulation of T cells from recently infected donors or from healthy donors responding to influenza epitopes led to enhanced responses when the accumulation of cytokines was measured. However, accumulation of regulatory cytokines, particularly IFN-γ, led to inhibition of further Ag-specific CD8 T cell expansion in the cultures. This inhibition was relieved by neutralization of IFN-γ or of IFN-γ, TNF, and IL-10. Thus, strong costimulation of T cells in vitro can lead to induction of regulatory cytokines at levels that limit further T cell expansion. In marked contrast, T cells from long-term (>4 years) infected HIV$^+$ donors exhibited reduced Ag-specific CD8 T cell expansion, reduced CD4 T cell responses, and minimal cytokine accumulation. Dual costimulation with both 4-1BBL and B7.1 enhanced responses of T cells from long-term infected subjects to a level similar to that obtained with T cells from early in HIV infection. Experiments with purified CD8 T cells showed that B7.1 and 4-1BBL could act directly and synergistically on CD8 T cells. Taken together, these data suggest that 4-1BBL and B7.1 have additive or synergistic effects on HIV-specific CD8 T cell responses and represent a promising combination for therapeutic vaccination for HIV. ***The Journal of Immunology,*** **2005, 175: 6378–6389.**

The appearance of cytotoxic lymphocytes correlates with a dramatic decrease in viral load in primary HIV infection (1–4). However, CTL control eventually fails in most individuals, perhaps due to a decrease in CD4 T cell help, viral escape mutants, or exhaustion of the renewal mechanism for CD4 T cells. Later in infection, HIV-specific cytotoxic CD8 T cells express less perforin than CD8 T cells specific for other viruses (5, 6) and exhibit defects in proliferation (6). A small percentage of patients, termed long-term nonprogressors, control the infection well for extended periods of time (7), with strong CTL responses, unimpaired proliferation, perforin expression (6), and most importantly strong CD4 helper responses (8).

CD4 T cell responses are critical for the generation and/or maintenance of effective CD8 T cell memory responses (9–12). Thus, it is likely that defective CD4 T cell responses contribute to the functional deficiencies of HIV-specific CTLs: HIV preferentially infects HIV-specific CD4 T cells (13), the $t_{1/2}$ of an infected CD4 T cell in vivo is ~1 day (14), and viremic subjects lack functional IL-2-producing CD4 cells (15, 16). Indeed, recent evidence shows that deficient HIV-specific CD8 T cell responses are due to declining CD4 T cell help and can be restored in vitro by providing CD4 T cells from the acute stages of infection (17).

To overcome immune deficiencies of HIV-infected individuals due to compromised CD4 T cell help, direct costimulation of CD8 T cells might be beneficial. Costimulation plays an important role in the activation and survival of T cells. The best characterized costimulatory receptor/ligand interaction is CD28/B7 (18, 19). CD28 costimulatory signals regulate IL-2 production and T cell survival, and lower the threshold for T cell activation (18, 19). CD28 also promotes the increase in T cell metabolism needed for cell proliferation (20).

The importance of additional costimulatory molecules, most notably members of the TNFR family, has recently been elucidated. These include OX40, CD27, and 4-1BB (21, 22). 4-1BB is expressed on activated T cells (23), NK cells (24), and a subset of dendritic cells (25, 26). Its ligand, 4-1BBL, is expressed on activated APC (22). In mouse models, 4-1BB is important in recall CD8 T cell responses to viruses and in maintenance of effector-memory CD8 T cells late in the primary response (27–30). Administration of agonistic anti-4-1BB Abs can rescue the nearly absent CD8 T cell response to influenza virus in CD28$^{-/-}$ mice,

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suggesting that 4-1BB is a potent costimulatory molecule for priming CD8 T cells independently of CD28 (30, 31). 4-1BB also plays a role in prolonging T cell survival (32, 33) via the NF-κB-dependent induction of Bcl-x_L and Bfl-1 (34). Although 4-1BB has the capacity to activate both CD4 and CD8 T cells (22), it shows a preferential role on CD8 T cells in antiviral and antitumor immunity in mice (29, 35–37).

4-1BB costimulation also enhances expansion, cytokine production, and cytolytic effector functions of human T cells, with effects on both $CD4^+$ and $CD8^+$, $CD28^-$, and $CD28^+$ T cells (33, 38–42). However, 4-1BB is expressed more rapidly and to higher levels on human CD8 compared with CD4 T cells (39). Recent work has shown that 4-1BBL delivered to syngeneic APC via recombinant adenoviral vectors is an efficient adjuvant for human antiviral CD8 T cell responses, resulting in enhanced expansion and differentiation of mature effector cells with high levels of perforin, granzyme A, and cytolytic activity (43).

The finding that 4-1BBL costimulation is effective in driving antiviral CD8 T cells toward a fully activated effector state (43) prompted us to explore whether costimulation via 4-1BBL might improve the activation of CD8 T cells from HIV-infected donors. To this end, we compared 4-1BBL and B7.1 costimulation, either separately or in combination for enhancement of HIV-specific CD8 responses. Although there were quantitative differences in the response to costimulation using T cells from recently vs chronically HIV-infected individuals, there was clear enhancement of CD8 T cell responses in all cases, and additive or synergistic effects on T cell responses from chronically infected donors when the two costimulatory molecules were combined.

Materials and Methods

Tetramers, Abs, peptides, and cytokine detection and neutralization

Class I HLA-peptide tetramers were generated, as described (43, 44). Abs for CD3, CD4, and CD28 were purified on protein G-Sepharose and labeled with FITC or biotin. Abs for CD4, CD8, CD14, CD27, CD28, CD62L, CD69, and CD45RA were purchased from eBioscience, and Abs for 4-1BBL, B7.1, CCR7, IFN-γ, perforin, and HLA-A,B,C were purchased from BD Pharmingen. Bcl-x_L-specific Ab was purchased from Southern Biotechnology Associates. The Influenza HLA-A2-restricted GILGFVFTL ($M1_{58-66}$) and EBV HLA-A2-restricted GLCTLVAML ($BMLF1_{280-288}$) peptides and the HIV HLA-B7-resticted IPTRIRQGL ($env_{843-851}$) peptide (purified to >90%) were obtained from the Alberta Peptide Institute, with others obtained from Sheldon Biotechnology Centre: HIV HLA-B8-restricted FLKEKGGL (nef_{90-97}) and HLA-A2-restricted SLYNTVATL (gag_{77-85}). TGF-β was detected by Opti-ELISA kits from BD Biosciences, while IL-10, IFN-γ, TNF-α, IL-6, IL-4, and IL-2 were detected with the Th1/Th2 cytometric bead array from BD Biosciences. Purified culture-grade neutralizing Abs for IFN-γ (10 μg/ml) and TNF-α (5 μg/ml) were obtained from BD Biosciences. Purified culture-grade neutralizing Abs for IL-10 (40 ng/ml) and all isotype control Abs for the neutralizing assay were obtained from eBiosciences. In pilot experiments, the neutralizing Abs were titrated to determine the dose that had maximal effect on T cell expansion.

Flow cytometry

Samples were stained with tetramers at 37°C for 15 min, followed by a wash with cold buffer. All subsequent Ab stains were done on ice. For intracellular cytokine staining, samples were restimulated with 5 μM peptide for 5 h in the presence of GolgiPlug (BD Biosciences). Cells were stained for surface markers, followed by intracellular staining with anti-cytokine Abs. The CytoFix/CytoPerm kit (BD Biosciences) was used to fix and permeabilize the cells for intracellular staining. FlowJo software was used for data analysis.

Generation of recombinant adenoviruses

Replication-defective adenovirus 5 recombinants expressing human 4-1BBL or B7.1 were generated by two-plasmid rescue method, as previously described (43).

Determination of peptide reactivity via ELISPOT and design of peptide matrices

The HIV peptide sets (National Institutes of Health AIDS Research and Reference Reagent Program) used for stimulation were 15 aa with 11-aa overlaps (Gag, Env, Nef, Tat, Rev, Vpr, Vpu, Vif) or 20 aa with 10-aa overlaps (Pol). Pools containing 2–15 peptides were prepared and organized into matrice peptide pools such that each peptide was present in two pools within each peptide matrix. IFN-γ secretion by HIV-specific cells was quantified using the ELISPOT assay, with 10^5 PBMCs/well and final concentration of each peptide at 2–4 μg/ml. Medium alone was used as a negative control, and anti-CD3 or a pool of 21 peptides derived from CMV, EBV, and Influenza was a positive Ag control stimulus (CEF pool; National Institutes of Health Reagent Bank). A positive response was defined as being 3 SDs above that seen for uninfected subjects and at least 3-fold greater than the autologous negative control wells. The identity of candidate stimulatory peptides identified in the peptide-pool matrix ELISPOT assay was confirmed by IFN-γ ELISPOT using optimal peptide at 4 μg/ml as stimuli.

Determination of presumed infection date

When available, the presumed date of HIV infection was estimated for each individual using clinical and laboratory data as well as patient history information. The following guidelines proposed by the Acute HIV Infection and Early Disease Research Program sponsored by the National Institutes of Allergy and Infectious Disease Division of AIDS were used to estimate the date of infection: the date of the first indeterminate Western blot minus 35 days; the date of a positive HIV RNA test or p24 Ag assay available on the same day as a negative HIV EIA test minus 14 days. The date of onset of symptoms of an acute retroviral syndrome minus 14 days was also used to estimate the date of infection. Information obtained from questionnaires addressing the timing of high-risk behavior for HIV transmission was used when available to confirm the presumed date of exposure.

Patients, T cell purification, and APC preparation

All subjects gave informed consent as approved by the University of Toronto, the University of Montreal, and the McGill University Health Care Centre ethics committees. Leukaphoresis samples were obtained from HIV-infected patients, and PBMC were isolated by Ficoll-Paque^{Plus} gradient centrifugation. PBMC were frozen in 10% DMSO in 50/50 FCS/medium mixture and stored at −150°C. Patients were HLA typed using the amplification-resistant mutation system (ARMS-PCR; Pel-Freez Clinical Systems).

For costimulation cultures, fresh or freshly thawed PBMC were plated at 4–5 million/well of a 48-well plate for 1 h to allow the monocytes to attach to the plastic wells. The nonadherent fraction was removed and kept overnight at 37°C. After washing the adherent cells, control Adv, 4-1BBL-Adv, or B7.1-Adv was added at a multiplicity of infection of 100, and the plate was centrifuged at 37°C at 3000 rpm for 1 h. After overnight incubation with adenovirus and peptides, APC were washed twice with prewarmed medium. T cells were purified with a Pan T cell negative selection kit from Miltenyi Biotec (MACS), with purity routinely better than 99.5%. For some experiments, CD8 T cells were purified using the CD8 T cell negative selection kit from Miltenyi Biotec, and were used only if a purity of >90% was achieved. Purified T cells were added to adherent cell cultures at a concentration of 1 million/well of a 48-well dish and stimulated for 8 days. No exogenous IL-2 was added to any experiments with total T cells. For purified CD8 T cells, we compared effects of costimulation in the absence as well as in the presence of low dose IL-2 (0.04 U/ml). Note that the overnight incubation of nonadherent PBMC resulted in the loss of some $tetramer^+$ T cells, but their CCR7 phenotype changed little after this incubation (data not shown). The starting populations of $tetramer^+$ T cells were determined immediately before adding to the APC.

At the end of the culture, HIV p24 was analyzed by ELISA (AIDS Reagent and Reference Program). However, no p24 was detected in the cultures, suggesting that costimulation had not led to excessive virus production in the cultures (data not shown).

CTL assays

For HLA-A2 donors, we used the T2 cell line as targets. For HLA-$B7^+$ or HLA-$B8^+$ donors, autologous EBV-transformed B lymphoblastoid cell line (B-LCL)[4] were used. For some repeat experiments (data not shown in the figures), the HLA-B7-transfected C1R cell line was used as a target, with identical results. When T2 cells were used as targets, they were pulsed

[4] Abbreviation used in this paper: B-LCL, B lymphoblastoid cell line.

with 10 μM HLA-A2-restricted peptides overnight. The next day, targets were labeled with 200 μCi of $Na_2{}^{51}CrO_4$ and incubated with effector T cells for 4 h. Supernatant was analyzed for the release of radioactive chromium. In the case of B-LCL or C1R-B7, the target cells were incubated with 10 μM respective peptide during the 1-h labeling with $Na_2{}^{51}CrO_4$.

Statistical analyses and normalization calculations

Unpaired two-tailed Student's *t* test with 95% confidence intervals was used for all statistical comparisons. The resulting *p* values are indicated in figures and text. In Figs. 3 and 6, one to four experiments were conducted for each donor, and results are reported as average ± SD for each donor. For experiments with replicates per donor, the mean for each donor was compared with the overall mean, and the statistics are then performed on those means. Weighted median was calculated as the median of the individual donor means.

Results

Delivery of costimulatory molecules to APC from HIV-infected individuals using recombinant adenovirus vectors

To analyze the role of costimulation in HIV-specific cytotoxic T cell memory responses, we used recombinant replication-defective adenoviruses expressing 4-1BBL or B7.1 to deliver costimulatory molecules to monocytes, as previously described (43). Dose titration of recombinant adenovirus showed that optimal expression of both molecules occurred at a multiplicity of infection of between 50 and 100 (data not shown). Adherent monocytes from different stages of infection express 4-1BBL and B7.1 similarly at this multiplicity of infection (Fig. 1). No 4-1BBL or B7.1 was detected following control adenovirus infection. Furthermore, up-regulation of MHC molecules due to recombinant adenovirus was similar between donors at different stages of infection (43).

Phenotypic characterization of T cells from early vs chronic HIV infection

HLA type and the HIV epitopes recognized by each donor were determined, as described in *Materials and Methods* (Table I). As will be discussed below, during the course of these experiments, we noted quantitative differences in the response to costimulation in recently vs long-term infected donors. Therefore, based on these findings, we have retrospectively divided the data set into two groups, early (infected ≤14 mo) and chronic (infected >4 years), for the purposes of comparison. Starting frequencies of HIV-specific tetramer$^+$ T cells in the unstimulated PBMC ranged from 0.2 to 2.6% of CD8 T cells.

To analyze the starting populations of T cells in cultures, we looked at the frequency of MHC I/peptide tetramer-binding T cells and their surface expression of CD28, CD27, CD45RA, and CCR7. It has been suggested that the CD27$^+$CD28$^+$ antiviral CD8 T cells represent effector T cells at the early stages of differentiation (45). Progressive maturation results in the loss of CD28, followed by the loss of CD27, to yield terminally differentiated CD27$^-$CD28$^-$ memory T cells. Phenotypic characterization based on CD27 and CD28 expression revealed a decreased proportion of CD27$^+$CD28$^+$CD8 T cells and a compensatory increase in the proportion of CD27$^-$CD28$^-$ T cells in HIV subjects that had been infected for longer than 4 years (Fig. 2*a*). Thus, the division of donors into two groups based on duration of infection is supported by the CD28/CD27 phenotype observed in the two groups, and is consistent with previous reports that chronic exposure to HIV results in depletion of the CD28$^+$CD27$^+$CD8 T cell subset (45). Analysis of HIV-specific CD8 T cells based on CD45RA and CCR7 expression, markers that are used to distinguish T cell memory phenotype, showed that, as expected, the tetramer$^+$ T cells were largely found in the CD45RA$^-$ subset (Fig. 2*b*). The CCR7 phenotype was quite variable between donors and did not show statistically significant differences between the two groups.

Enhancement of HIV-specific CD8 T cell expansion by 4-1BBL and B7.1 costimulation

Leukapheresis samples from HIV$^+$ individuals were used as a source of both adherent monocytes (used as APC) and purified T

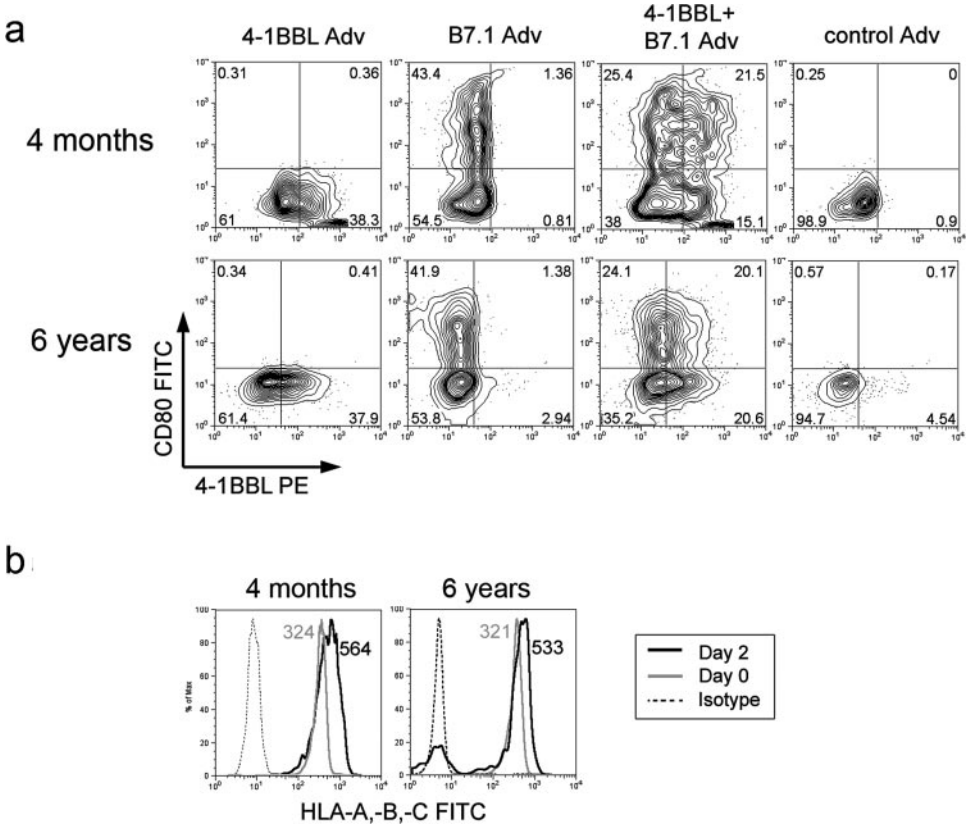

FIGURE 1. Expression of 4-1BBL and B7.1 on adherent cells from HIV-infected donors following adenovirus transfer. *a*, Adherent monocytes from recently and chronically infected HIV$^+$ donors were infected with recombinant adenovirus(es), as indicated. Expression of 4-1BBL vs B7.1 is shown. *b*, Expression of HLA class I (A,B,C) before (gray lines) and after (black lines) infection with adenovirus, with mean fluorescence intensity indicated. Dotted lines indicate staining with isotype control Ab. Representative of four donors.

Table I. *HIV donor characteristics at time of leukapheresis*[a]

Donor	Sex	Age	Duration of Infection	HIV RNA (copies/ml)	CD4 Count	On HAART	Treatment[b]	Responder Epitope(s)
Chronic								
HTM 303	M	43	6.4 years	78,196	325[c]	6.3 years	AZT, 3TC, various PI	A2-SL9
HTM 305	M	39	6.3 years	<50	222	4 years	AZT, 3TC, IND	B8-FLKE
HTM 327	M	38	4.3 years	<50	529	3.5 years	3TC, AZT, Efavirenz	B8-FLKE
OM8	M	43	12 years	76	620	NA	None	B8-FLKE
OM4(LTNP)	M	57	12 years	<50	834	NA	None	A2-SL9
OM9(LTNP)	M	41	10 years	108	978	NA	None	A2-SL9
Early								
PI023	M	33	4 mo	2,830	470	2 wk	Viracept, Combivir	B8-FLKE
PI025–02	M	46	6.5 mo	244,029	580	NA	None	B8-FLKE
PI025–03	M	46	14 mo	91,762	322	NA	None	B8-FLKE
PI026	M	40	7 mo	880	870	2 wk	Combivir, Sustiva	B7-IPTR
OM18	M	32	1.5 mo	103,493	630	2 wk	RTV, ABC	A2-SL9
OM5	M	57	5 mo	495,852	820	NA	None	A2-SL9

[a] After analysis of responses to dual costimulation yielded different results depending on the length of infection, HIV$^+$ subjects were divided into two groups: individuals at early stages of infection (<18 mo) and chronically infected (>4 years). The chronically infected group includes two long-term nonprogressors. OM8 was classified as a chronic progressor at the time of leukapheresis. Subject PI025 has two samples, from 2002 (02) at 6.5 mo and 2003 (03) at 14 mo postinfection. OM donors are from the Toronto Cohort and the remainder are from the Quebec cohort. Peptides used are: SL9, gag_{77-85}; FLKE, nef_{90-97}; IPTR, $env_{843-851}$.

[b] AZT, Zidovudine; 3TC, Lamivudine; IND, Indinavir; RTV, Ritonavir; ABC, Abacavir.

[c] HTM 303 CD4 count is an average of observations taken 4 mo before leukapheresis (386) and 6 mo after (264).

cells. Pilot experiments determined the optimal peptide concentration for each epitope, and this same peptide dose was used for each donor to allow comparison. Following overnight incubation of the adherent cell layer with control or recombinant adenoviruses and control or HIV epitope peptide, the monolayers were washed, and purified T cells from the same donor were added to the cultures. T cell expansion was analyzed 8–9 days later using MHC I/peptide tetramers (Fig. 2). The response to 4-1BBL and B7.1 costimulation for four HIV-infected subjects at different stages of infection is shown in Fig. 3*a*. The results are reported as the percentage as well as the total numbers of tetramer-positive CD8 T cells recovered, with the same trend for both measurements (Fig. 3*a*). We consistently observed enhancement of HIV-specific CD8 T cell expansion using Ag and either 4-1BBL or B7.1 costimulation alone compared with responses to Ag plus control adenovirus. No expansion of CD8-HIV-specific T cells was observed in the presence of control Ag (Fig. 3*a*).

This enhancement of Ag-specific CD8 T cell expansion by costimulation was observed for several different HLA alleles and epitopes (Fig. 3*a* and data not shown). In 24 independent experiments, with 11 different HIV$^+$ subjects, costimulation with 4-1BBL resulted in 1.8- to 87.5-fold greater response (mean 18.8) of HIV-specific T cells, and costimulation with B7.1 resulted in 2.1- to 88.2-fold greater response (mean 23.4) of HIV-specific T cells compared with the response observed with control adenovirus or control Ag alone. In T cells from recently infected HIV$^+$ donors, maximal proliferation occurred with individual costimulatory molecules, with no additional CD8 T cell expansion in response to dual costimulation. In contrast, T cells from chronically infected donors showed more limited expansion in response to individual costimulatory molecules and additive or synergistic effects on proliferation when 4-1BBL and B7.1 were combined.

Fig. 3*b* summarizes the fold enhancement of HIV-specific CD8$^+$ T cells by costimulation (as measured using MHC tetramers) defined as the response to Ag and costimulation over the response to Ag plus control adenovirus. Fig. 3*c* shows the same data plotted as weighted median. In both plots, the mean responses of T cells from recently infected donors were greater than those from chronically infected donors. This decreased response in chronic vs recently infected donors was statistically significant for 4-1BBL ($p < 0.001$; Fig. 3, *b* and *c*) and showed a similar trend for B7.1 ($p = 0.04$; Fig. 3, *b* and *c*). In contrast, with dual costimulation, the response of the chronically infected donors was no different from those early in infection ($p = 0.69$; Fig. 3, *b* and *c*). It should be noted that two donors in the long-term infected group are classified as long-term nonprogressors. If one removes these from the calculation, the difference between early and chronic for response to 4-1BBL still shows a similar trend ($p = 0.04$), the difference to B7.1 costimulation becomes nonsignificant, and again the response to dual costimulation is similar between the two groups.

Similar results on the role of costimulation on T cell expansion were obtained when we examined the frequency of IFN-γ-producing cells in four different donors following peptide restimulation, after the 8-day stimulation (Fig. 3*d*). Conversion of the frequencies of IFN-γ or tetramer$^+$ cells to total cell numbers again gave similar results (data not shown).

Costimulation enhances the number of cells with effector function, and for long-term infected subjects increases the level of perforin per cell

Costimulation also enhanced the level of cytotoxic activity exhibited by the CD8 T cells (Fig. 4*a*). In general, we found that the level of killing was proportional to the frequency of Ag-specific CD8 T cells in the cultures. For long-term infected donors, dual costimulation during the 8-day culture resulted in increased killing by the CTL effectors when compared with individual costimulatory molecules (Fig. 4*a*). With cultures from early in HIV infection, as was the case for Ag-specific T cell expansion, dual costimulation resulted in no additional cytotoxicity (Fig. 4*a*).

When perforin levels were analyzed in the tetramer$^+$ population, T cells from chronically infected subjects showed enhancement with costimulation over peptide alone (Fig. 4*b*). In contrast, T cells from early in HIV infection showed increased perforin levels in response to Ag-pulsed APC, without additional costimulatory molecules (Fig. 4*b*). These results suggest that HIV-specific memory T cells from early HIV infection, despite a CD69$^-$ resting phenotype (data not shown), are more readily activated. With long-term infected donors, costimulation not only enhances numbers of effector cells, but effector function per cell, as measured by perforin levels.

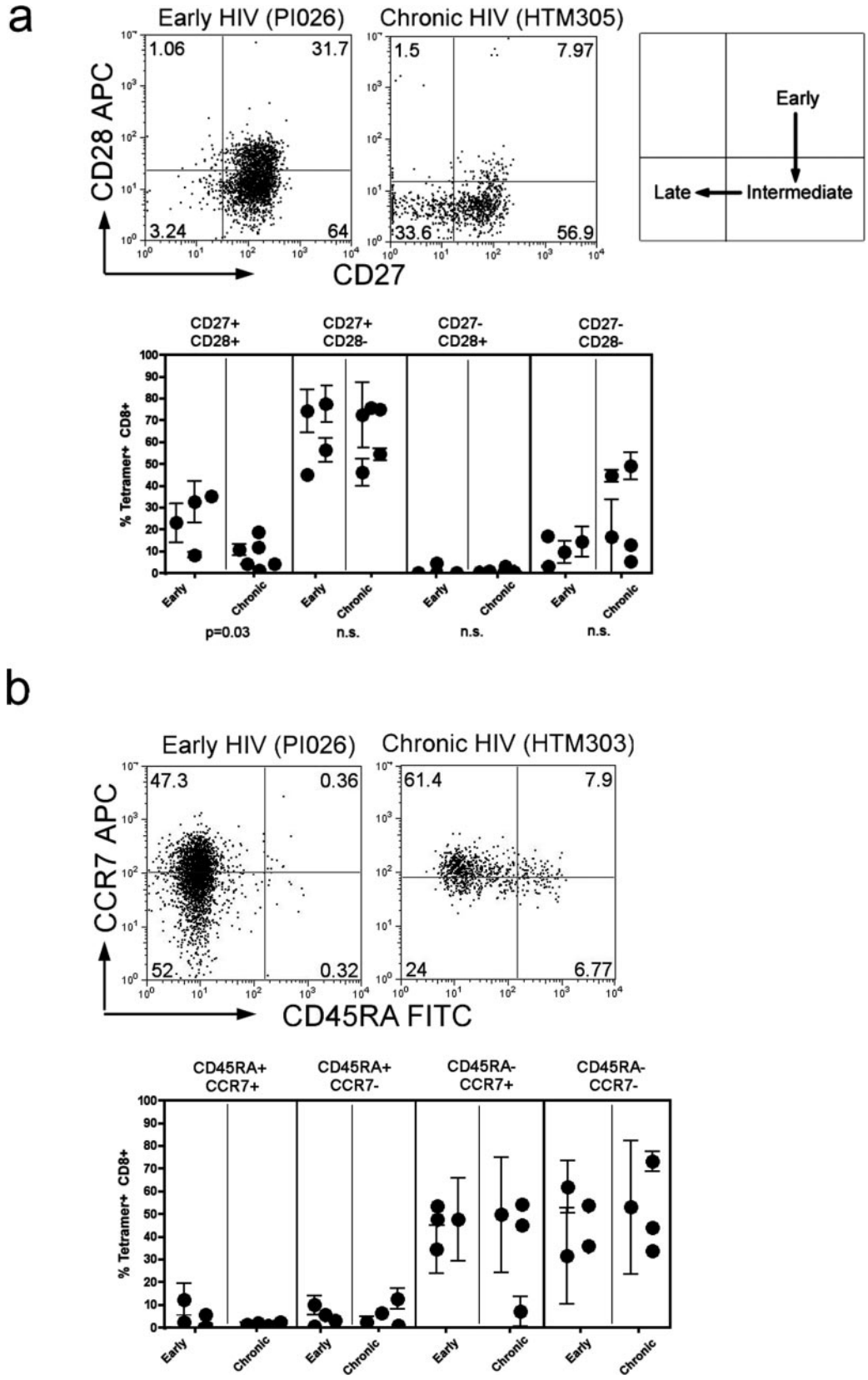

FIGURE 2. Phenotypic characterization of unstimulated HIV-specific T cells in early and chronic HIV subjects. *a*, Expression of CD28 and CD27 on HIV-specific $CD8^+$ T cells (FLKE specific) in a representative early (*left*) and chronic (*right*) HIV subject. Percentage of cells found in each quadrant is shown for four early and six chronically infected donors in the *bottom plot*. Each data point represents one donor, and SDs are indicated for donors in which multiple determinations were made. Student's *t* test was used to determine the differences between the two groups, with the *p* value indicated below relevant panels (n.s. = not significant). *b*, Expression of CD45RA and CCR7 on HLA-B8-FLKE $tetramer^+$ and HLA-A2-SL9 $CD8^+$ T cells in representative early (*left*) and chronic (*right*) HIV subjects. Percentage of cells found in each quadrant is shown for four early and four chronic HIV subjects in the *bottom plot*. SD is indicated for donors for which repeat experiments were done. No statistical significance was found (n.s.).

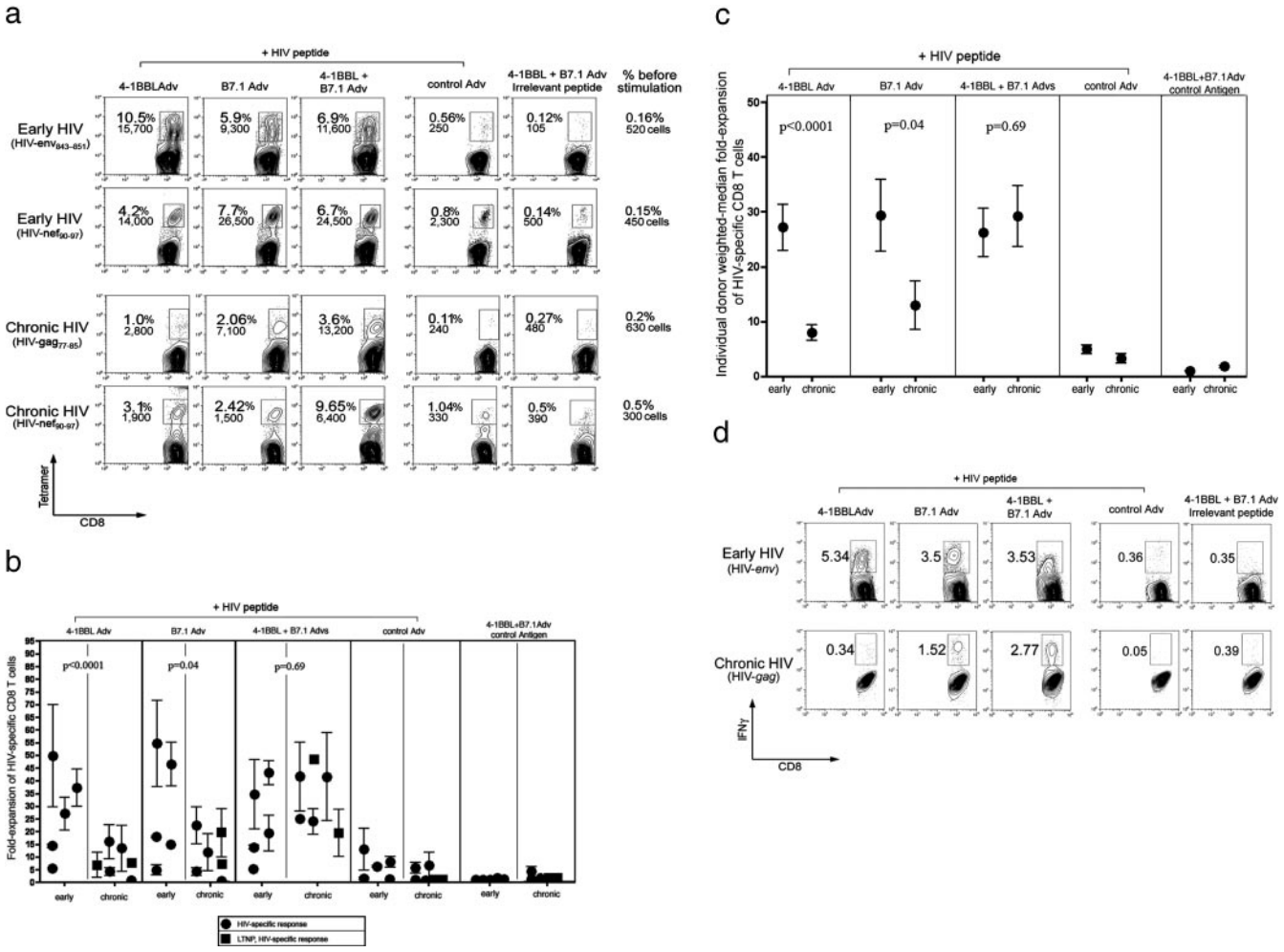

FIGURE 3. Costimulation enhances expansion of HIV-specific T cells. *a*, Ag-specific expansion of tetramer$^+$ T cells from recently (early) and long-term (chronic) infected HIV subjects in response to costimulation with B7.1, 4-1BBL, or both. Adenovirus vectors used to modify the donor APC are indicated at the *top* of each column. Specific CD8 epitope peptides are included in each culture except for the *right-most panel*, which shows the response to dual costimulation with an irrelevant (melanoma) peptide. Plots are gated on CD8$^+$ T cells with the percentage of CD8 T cells that are tetramer positive indicated (*top number*) and the actual number of tetramer$^+$ T cells recovered (*bottom number*). *b*, Fold expansion of HIV-specific CD8 T cells in response to 4-1BBL, B7.1, or dual costimulation in cultures from early vs chronic HIV infection. Student's *t* test was used to determine statistical significance, with the *p* values indicated. Each data point represents the mean for a single donor, repeated one to four times, depending on sample availability, with SD indicated for those donors in which repeat experiments were done. *c*, Weighted median of fold expansion determined from means from each individual donor in response to 4-1BBL, B7.1, or dual costimulation in cultures from early vs chronic HIV infection. Statistics were calculated by comparing mean for each donor with the overall mean. Student's *t* test was used to determine statistical significance, with the *p* values indicated. Error bars indicate the SE in the experiment. *d*, IFN-γ production by HIV-specific CD8 T cells after restimulation with HIV peptides. Representative data from early and chronic HIV subjects are shown.

The prosurvival molecule bcl-x_L was found to be increased with costimulation in two of three experiments, but expression level did not relate to the duration of infection. The combination of both costimulatory molecules resulted in no additional enhancement of bcl-x_L expression, as single costimulatory molecules were sufficient to up-regulate bcl-x_L (Fig. 4*c*).

Cytokine accumulation in cultures from early vs chronic HIV subjects

To determine the overall level of cytokines produced in the cultures, we used a quantitative cytometric bead array assay. Culture supernatant from four recently infected HIV subjects, four chronic donors, as well as four healthy individuals responding to influenza peptide was assayed. Cultures of T cells from healthy donors or early HIV accumulated large amounts of IFN-γ (>16,000 pg/ml) and moderate amounts of IL-10 and TNF-α (Fig. 5*a*) in the dual costimulation cultures. The accumulation of cytokines in dual costimulation cultures showed additive or synergistic effects in both groups of donors when compared with individual costimulatory molecules (Fig. 5*a*). Thus, in cultures from early HIV or healthy controls, additive effects of dual costimulation are observed in the accumulation of cytokines, but not in Ag-specific CD8 T cell expansion (Fig. 2*a*). In contrast, cultures from most chronic HIV donors accumulated minimal amounts of cytokines, even in the presence of dual costimulation (Fig. 5*a*). IL-2 levels were quite low in all the cultures, most likely due to consumption. We also tested for the presence of TGF-β by ELISA, but none was detected (data not shown). Although expansion of IFN-γ-producing CD8 T cells and tetramer$^+$ T cells was clearly Ag specific (Fig. 3), costimulation-dependent cytokine production was also observed independently of added influenza or HIV peptide

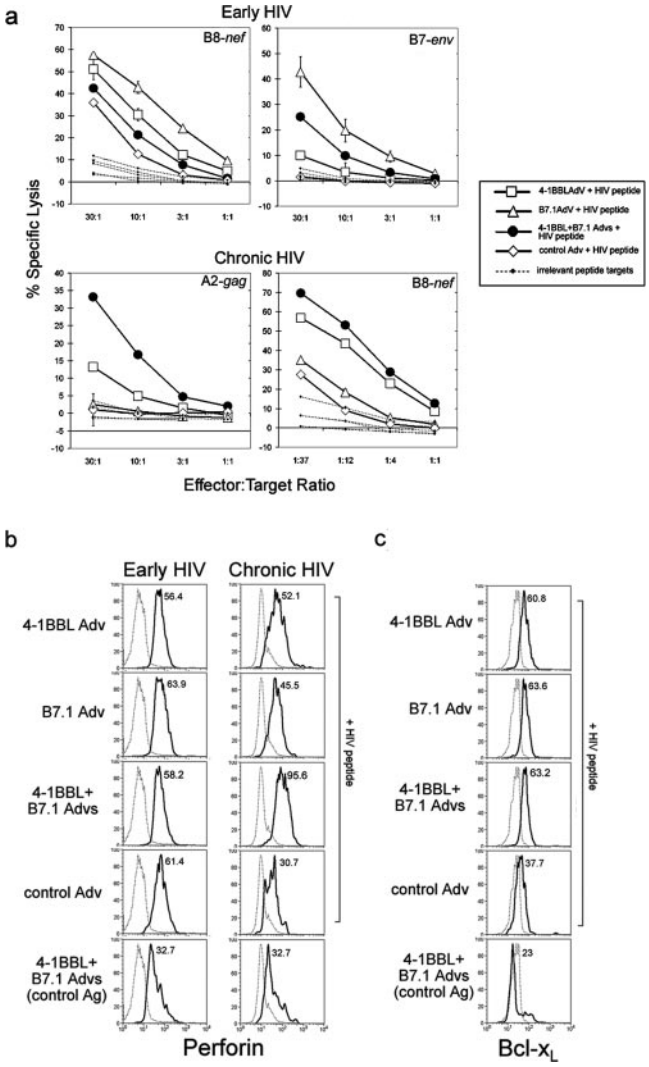

FIGURE 4. Effector function and bcl-x_L expression of expanded HIV-specific CD8 T cells. *a*, Cytolytic activity of HIV-specific CD8 T cells, measured in a chromium release assay. For HLA-$A2^+$ individuals, peptide-pulsed T2 cells were used as targets. Autologous EBV-transformed B-LCL were used for epitopes resticted by other HLA alleles. Representative of seven donors. *b*, Perforin expression in $CD8^+$ $tetramer^+$ T cells, as measured after 8 days of stimulation with the costimulatory condition indicated on the *left*. Solid lines represent perforin staining; dotted lines control Ab staining. The mean fluorescence intensity for each stimulatory condition is indicated. Representative of nine donors. *c*, Up-regulation of bcl-x_L in $CD8^+$ $tetramer^+$ cells. No difference was observed between early and chronic HIV-infected individuals (data not shown). Solid lines represent bcl-x_L staining; dotted lines control Ab staining. Mean fluorescence intensity of each stimulatory condition is indicated. Representative of three donors.

epitopes (Fig. 5*a*). We attribute this to T cell responses to the recombinant adenovirus used to deliver costimulatory molecules, as most individuals have been exposed to adenoviruses (46). Experiments with healthy donors confirmed that three of three healthy subjects demonstrated recall T cell responses to adenovirus V (data not shown).

Cytokine accumulation in dual costimulation cultures from early HIV or healthy donors limits further T cell expansion

Recently, it has become apparent that strong costimulation, either through constitutive expression of costimulatory ligands or in the presence of strong inflammatory signals, can lead to production of cytokines at levels sufficient to limit immune responses (47–50). Thus, we hypothesized that the strong costimulatory signal obtained with dual costimulation of cultures from early HIV and healthy donors might cause cytokine levels to reach inhibitory concentrations.

To further test this hypothesis, we neutralized cytokines using mAbs, starting at day 4 of culture. Day 4 was chosen to allow cytokines to stimulate CD8 T cell expansion early in the response (51), but to neutralize the cytokine once it started to accumulate. As shown in Fig. 5*b*, neutralization of IFN-γ alone was sufficient to restore additive or synergistic effects of dual costimulation in two of four healthy donors (Fig. 5*b*, donor 1). In the remaining two, neutralization of IFN-γ, TNF-α, and IL-10 in combination restored additive or synergistic effects of dual costimulation (Fig. 5*b*, donor 2). Neutralization of IL-10 alone had a modest effect on enhancing CD8 T cell expansion in three separate experiments (data not shown). Cytokine neutralization had the most pronounced effect on dual costimulation cultures, with minor and often inhibitory effects in individual costimulation cultures, consistent with a positive role for IFN-γ in CD8 T cell expansion at low doses (51). Therefore, strong responses to dual costimulation in healthy donor cultures result in accumulation of cytokines, particularly IFN-γ, at levels limiting the expansion of antiviral CD8 T cells. In contrast, T cells from chronic HIV respond poorly to the extent that cytokine production is greatly reduced, and allows enhanced T cell expansion in response to dual costimulation.

Effect of costimulation on CD4 T cell responses in cultures from early and late in HIV infection

In the experiments reported in Figs. 3–5, we used total T cell populations to analyze responses of Ag-specific CD8 T cells to costimulation. Thus, although we only add CD8 T cell epitopes to the cultures, the presence of CD4 T cells contributes to the response. Examination of the cultures for numbers of blasting CD4 T cells, based on forward vs side scatter, showed that some CD4 T cells were activated in response to costimulation (Fig. 6). However, the number of blasting CD4 T cells at the end of the 8-day culture was consistently lower in cultures from chronic HIV donors, compared with subjects early in infection. This was most apparent in the 4-1BBL-stimulated cultures (Fig. 6, $p = 0.01$), but also affected the B7.1 (Fig. 6, $p = 0.04$) and dual costimulation cultures (Fig. 6, $p = 0.02$). These data are consistent with the published observations that CD4 T cell help is decreased in chronic HIV infection. Moreover, the data suggest that dual costimulation, although augmenting CD4 T cell responses in both chronic and early donors, does not lead to correction of the CD4 T cell response difference in early vs chronic donors.

Effect of costimulation on isolated CD8 T cells

As noted in Fig. 6, there was significant expansion of CD4 T cells in the cultures of total T cells. Thus, effects of costimulation on CD8 T cell expansion could be attributed to direct or indirect effects. To determine whether 4-1BBL and B7.1 could have direct effects on CD8 T cell expansion, we isolated CD8 T cells using negative selection. CD8 preparations of >90% purity were used. Fig. 7 shows the results of costimulation of these highly enriched CD8 T cell cultures with 4-1BBL, B7. 1, or both. Because levels of IL-2 in the pure CD8 T cell cultures can be limiting, we conducted the analysis in both the presence and absence of added IL-2 at low dose (0.04 U/ml). As shown in Fig. 7, in highly enriched CD8 T cell cultures, both recently and

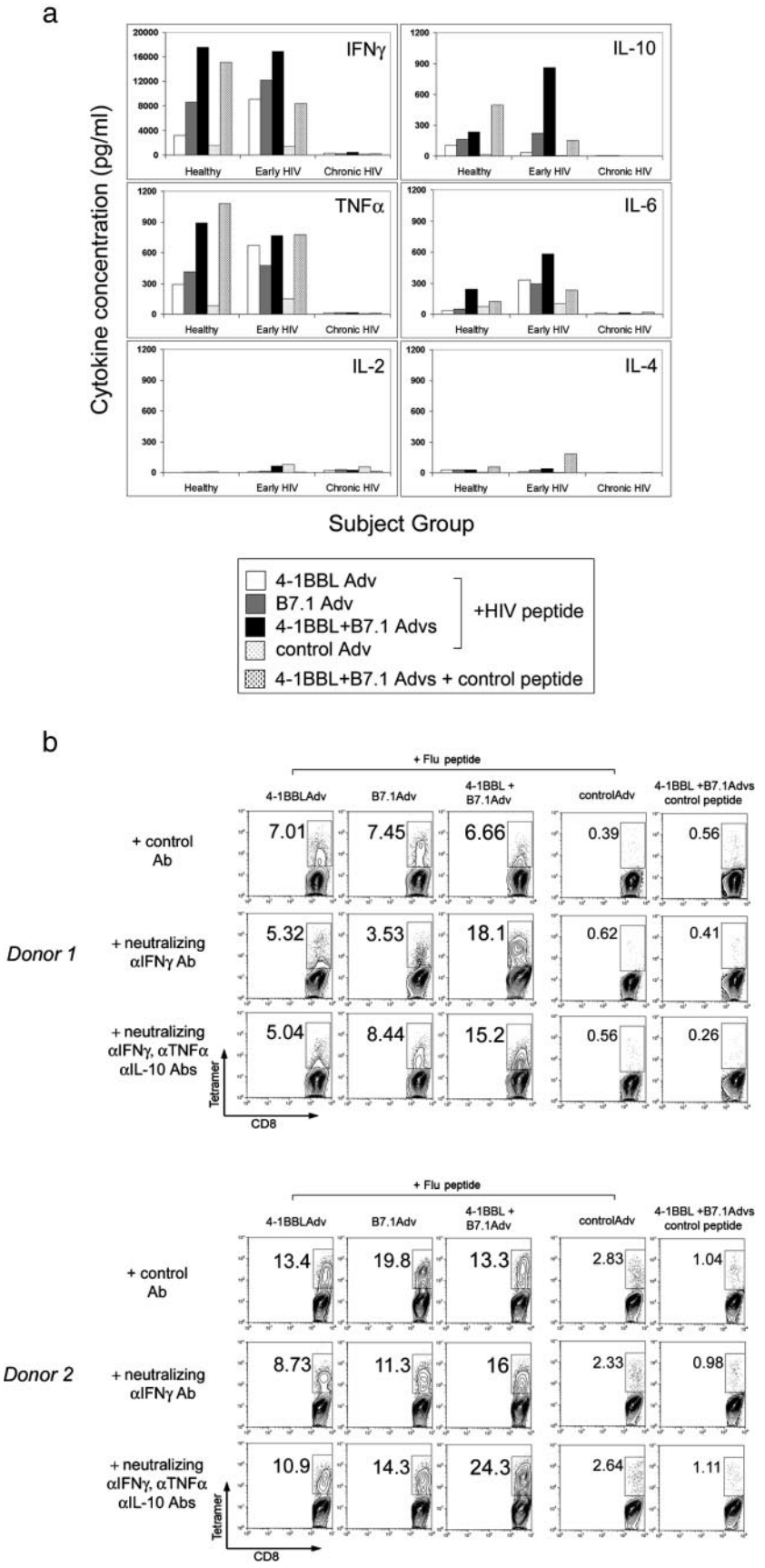

FIGURE 5. Cytokine accumulation in response to costimulation and effect of cytokine neutralization. *a*, Supernatant cytokine accumulation after 8 days of stimulation, as measured by cytometric bead array in the three groups of subjects, with stimulation conditions indicated in the legend. HIV subjects' T cells were stimulated with HIV peptide or control peptide, whereas healthy donor T cells were stimulated with influenza peptide or control peptide. Note the higher scale on the IFN-γ plot. Results are shown for one of each type of donor and are representative of four healthy, four early HIV, and four chronic HIV subjects. *b*, Neutralization of IFN-γ alone or in combination with TNF-α and IL-10. Neutralizing Abs to cytokines were added at day 4, and the tetramer-positive cells were enumerated after 8 days of stimulation. Representative of four healthy donors.

chronically infected donors showed enhanced CD8 T cell responses with dual costimulation. Although exogenous IL-2 further enhanced responses, some donors showed strong CD8 T cell expansion in the absence of IL-2. There was no difference between early and chronic donors in terms of response to costimulation in these assays, suggesting that a major contribution

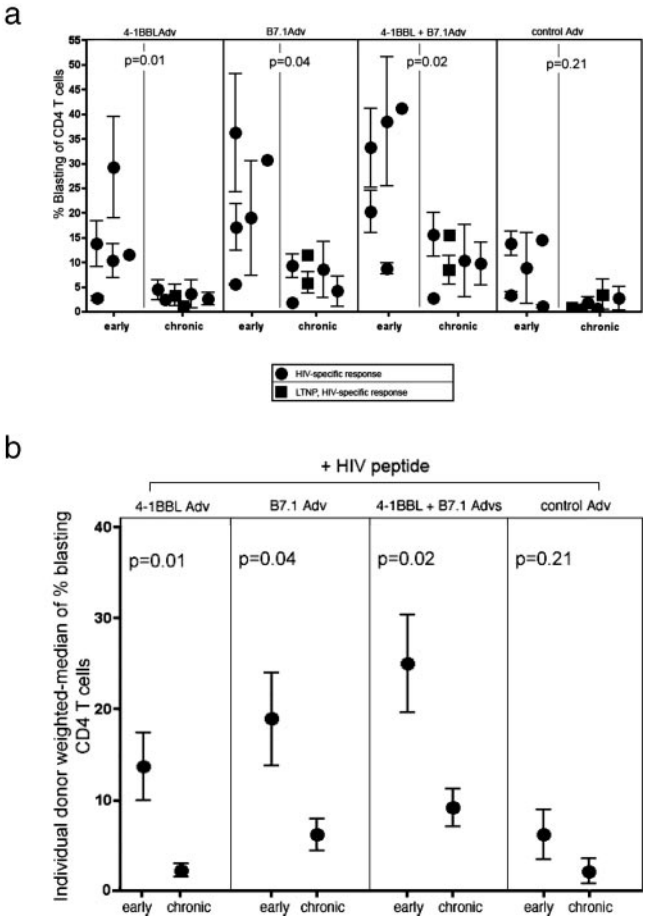

FIGURE 6. Analysis of CD4 T cell activation in cultures stimulated with CD8 epitopes and costimulatory ligands. *a*, Percentage of blasting CD4 T cells in each culture was assessed based on forward vs side scatter profiles at the end of the 8-day cultures. The costimulatory conditions used for each culture are indicated above each plot. Subjects are divided into early HIV or chronic HIV, as in Table I. Long-term nonprogressors are identified with a separate symbol in the chronic category. Where repeat measurements were done, results for each donor are reported as mean ± SD. All samples contained only CD8 epitope HIV peptides. No specific CD4 T cell epitopes were added. This CD4 T cell response is costimulation dependent and at least partially represents the response to adenovirus components (data not shown). *b*, The same data are plotted as weighted median (median of the mean for each donor). Statistics were calculated by comparing the mean for each donor with the overall mean.

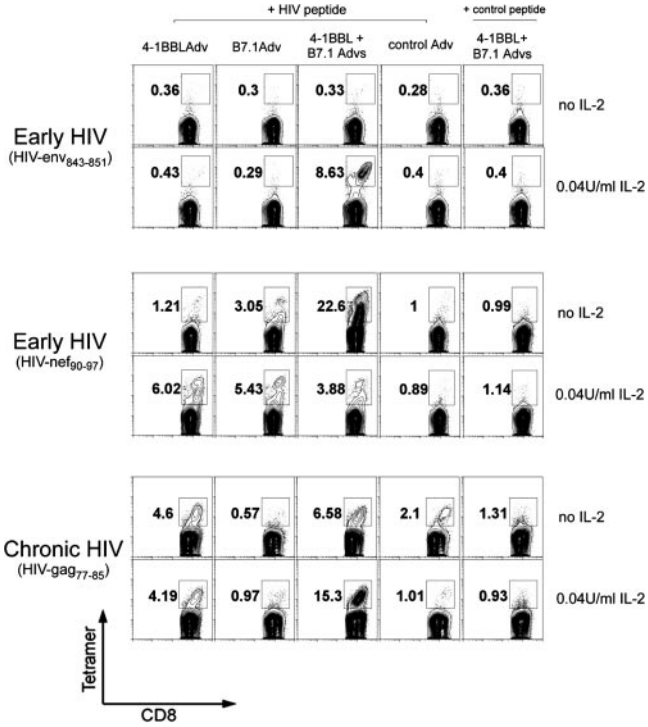

FIGURE 7. Effect of costimulation on highly enriched CD8 T cells in the absence or presence of low dose IL-2. CD8 T cells, purified using negative selection, were added to adenovirus-modified, peptide-pulsed monocytes, as described in *Materials and Methods*. Enriched CD8 T cells from each donor were tested in the absence or in the presence of low dose IL-2 (0.04 U/ml). In total, we tested six donors for responses of isolated CD8 T cells to 4-1BBL and four donors for responses to dual costimulaion. In all donors, at least under some conditions, 4-1BBL induced CD8 T cell expansion and there was enhancement with dual costimulation.

to the inhibitory cytokine effect seen in total T cell cultures was the presence of CD4 T cells.

Discussion

In this study, we show that costimulation with either B7.1 or 4-1BBL, delivered to donor APC by recombinant adenoviruses, can augment the expansion, cytokine production, and cytolytic effector function of HIV-specific CD8 T cells. With total T cell cultures, the responses of chronically infected HIV$^+$ donor T cells were reduced compared with those of recently infected donors, consistent with the known decrease in CD8 T cell responses late in HIV infection (6). However, dual costimulation with B7.1 plus 4-1BBL appeared to compensate for the reduced responses in chronic HIV cultures, raising the level of Ag-specific T cell expansion to the level observed in early HIV cultures.

In this study, we focused primarily on total T cell cultures, as we wished to more closely mimic a therapeutic context, in which CD8 T cell responses to Ag plus costimulation would be induced in the presence of a full complement of T cells, all with a potential to contribute to the response. Analysis of cultures for the number of blasting CD4 T cells revealed that cultures from recently infected donors showed greater CD4 T cell responses than cultures from chronic donors, particularly with costimulation. At least a part of this CD4 Th cell response was due to recall responses to adenovirus components (data not shown), consistent with most adults being seropositive for adenovirus exposure (46). Because we add only a minimal HIV CD8 T cell epitope to the cultures, it is likely that recall responses to adenovirus components provide CD4 help in this culture system. The finding that the chronic HIV subjects had reduced CD4 T cell responses is consistent with evidence suggesting that a loss of CD4 help is responsible for declining CD8 T cell responses in HIV (17).

With total T cell cultures from six of six chronically infected subjects, dual costimulation with 4-1BBL and B7.1 had synergistic or additive effects on both CD8 T cell expansion and development of effector function and showed responses that were equivalent to those of recently infected subjects responding to single costimulatory molecules. With healthy donors or recently infected HIV$^+$ donors, the combination of the two costimulatory ligands also resulted in increased T cell activation when monitored at the level of cytokine accumulation in the cultures (Fig. 5). However, accumulation of cytokines, particularly IFN-γ, resulted in inhibition of further CD8 T cell expansion and obscured the effect of dual costimulation on T cell responses. Neutralization of IFN-γ alone restored the additive effects of dual costimulation with two of four healthy donors (Fig. 5*b*). When IFN-γ, TNF-α, and IL-10 were

neutralized in combination, enhanced CTL expansion to dual costimulation was seen in all four healthy donors examined (Fig. 5*b*). Thus, dual costimulation with B7.1 and 4-1BBL leads to enhanced responses in both early and chronic HIV, but the increased accumulation of regulatory cytokines in this in vitro model limits the expansion of Ag-specific CD8 T cells from healthy donors and most likely has the same effect in the T cell cultures from early HIV.

There are several examples in the literature whereby strong costimulation, particularly in the presence of an inflammatory signal, can result in cytokine production at levels that limit T cell responses (47–50). Constitutive expression of the costimulatory TNF ligand CD70 leads to depletion of B cells by an IFN-γ-dependent mechanism (47), and can lead to profound immunosuppression in mice (52). Similarly, stimulation by agonistic anti-4-1BB Abs in a mouse model of rheumatoid arthritis leads to induction of an IFN-γ-producing CD8 T cell subset that results in immunosuppression (50). In the present studies, the frequency of IFN-γ-producing CD8 T cells was generally proportional to the frequency of tetramer$^+$ T cells detected: in early HIV, we observed maximal numbers of IFN-γ-producing CD8 T cells in the presence of a single costimulatory molecule, whereas in cultures from chronic HIV there were additive/synergistic effects on the number of IFN-γ-producing HIV-specific CD8 T cells. Thus, an increase in the frequency of IFN-γ-producing Ag-specific CD8 T cells in response to dual costimulation does not appear to explain the accumulation of IFN-γ in the dual costimulation cultures. Difference in levels of cytokines accumulating in these cultures may be in part due to the greater number of responding CD4 T cells in the early HIV cultures compared with chronic HIV cultures (Fig. 6). These findings most likely explain our previous inability to observe synergy between B7.1 and 4-1BBL in both human and mouse costimulation assays, in which it is likely that a similar accumulation of cytokines occurred with dual costimulation (43, 53).

The lower overall response of T cells from chronically infected donors was not due to differences in expression of MHC I or adenovirus-delivered costimulatory molecules on the APC (Fig. 1), nor was it due to a lower starting population of Ag-specific CD8 T cells, or increased activation-induced cell death of the Ag-specific T cells (data not shown). Examination of the surface phenotype of the Ag-specific memory T cells at the onset of culture showed a reduced population of CD28$^+$CD27$^+$ tetramer$^+$ memory CD8 T cells in chronic HIV infection, as previously noted (45). A decrease in CD28$^+$CD27$^+$ T cells correlated with chronic as compared with early infection and correlated with the weaker Ag-specific CD8 T cell response as well as decreased CD4 T cell response observed. In this regard, it is of interest that two donors classified as long-term nonprogressors segregate with the chronically infected group in terms of response to dual costimulation (Fig. 3), and also show a chronically infected phenotype, as evidenced by the loss of CD28$^+$CD27$^+$ T cells.

In the present study, there was a wide variation in the CCR7 expression by the starting population of HIV-specific CD8 T cells. In contrast, Champagne et al. (54) had shown that the majority of HIV-specific CD8 T cells are CCR7$^-$. However, subjects used in their study were all viremic and therapy naive. In our study, only half the patients in each group are therapy naive, and some exhibit lower viremia than the cutoff used in the Champagne study. These factors most likely explain the differences we see in CCR7 expression.

Although chronically infected donors showed fewer CD28$^+$CD8$^+$ tetramer-positive cells at the start of the culture, CD28 expression was clearly detectable on a substantial proportion of the expanded CD8 tetramer-positive population at the end of the 8-day cultures, even though the mean fluorescence intensity for CD28 staining was lower than that on unstimulated T cells (data not shown). Whether the expression of CD28 on these CD8 T cells reflects preferential outgrowth of CD28$^+$ Ag-specific T cells or re-expression of CD28 on T cells during culture could not be distinguished in these experiments. However, the expression of CD28 on the expanded CD8 T cells is consistent with the finding that B7.1 can directly costimulate the HIV-specific CD8 T cells (Fig. 7). We also examined the ability of Ag-specific CD8 T cells to up-regulate 4-1BB following stimulation and found that both groups of donors showed strong Ag-dependent up-regulation of 4-1BB on the CD8 T cells independently of added B7.1 (data not shown).

It has been suggested that costimulation with 4-1BB might be detrimental to anti-HIV therapy because of the possibility of enhancing viral replication due to enhanced proliferation of activated T cells (55). However, we detected no virus at the end of the 8-day culture period as monitored using p24 ELISA (data not shown); thus, any effects on enhanced viral replication may be compensated for by enhanced cytolytic effector function.

Treatment of HIV patients with antiretroviral therapy can restore CD4 T cell counts and improve immune function. However, the differences between the chronically infected and recently infected subjects did not correlate with treatment, as there were treated and untreated subjects in both groups, and it was not possible to dissect the response based on prior treatment with the small cohort studied.

It has been suggested that chronic stimulation during HIV infection might lead to accelerated aging of the immune system, as there are many similarities between HIV patients and the elderly, including an inversion of the CD4/CD8 ratio, susceptibility to common infections, preferential IFN-γ production, and accumulation of late-differentiated CD8$^+$CD28$^-$ T cells (45, 56). In this regard, it is of interest that 4-1BB costimulation has a similar synergistic benefit when combined with CD28 costimulation in old mice, but not young mice (57), consistent with the results shown in this study for early vs chronic HIV-specific responses in total T cell cultures.

Experiments with isolated CD8 T cells suggest that 4-1BBL and B7.1, particularly in combination, can directly stimulate CD8 T cells even in the absence of added IL-2. The CD8 T cell purity was checked at the beginning and end of the cultures. It was found that in the dual costimulation cultures, there was a small population (6–9%) of CD4 T cells at the end of the culture period. Nevertheless, based on the strong responses to costimulation observed in these highly enriched (>90%) CD8 T cell cultures (Fig. 7), it appears likely that there is a significant direct effect of costimulation on the CD8 T cells.

The distinction between recently and chronically infected donors with respect to strength of response and effect of dual costimulation was not observed with the enriched CD8 T cell cultures. This suggests that CD4 T cells most likely contributed to inhibitory cytokine production under conditions of strong costimulation in the total T cell cultures. However, with added IL-2, we also sometimes observed inhibition of costimulation with dual costimulation in the isolated CD8 T cell cultures, suggesting that CD8 T cells may also contribute to the accumulation of inhibitory cytokines under some circumstances.

In conclusion, in this study, we show that 4-1BBL or B7.1 delivered to syngeneic monocytes using recombinant replication-defective adenovirus augments peptide-specific HIV responses of CD8 T cells from donors at early and chronic stages of HIV

infection. Increased responses were observed at the level of Ag-specific CD8 T cell expansion, cytolytic activity, cytokine production, and up-regulation of survival signals. Experiments with highly enriched CD8 T cells suggested that at least part of these effects can be direct. In total T cell cultures, CD8 T cells from chronically infected donors exhibited reduced responses to 4-1BBL or B7.1 provided individually, when compared with responses of T cells from early in HIV infection. This defect is most likely due to reduced CD4 T cell help. However, dual costimulation with B7.1 and 4-1BBL resulted in increased CD8 T cell expansion and effector function in six of six chronically infected donors, suggesting that dual costimulation with B7.1 and 4-1BBL represents a promising strategy for therapeutic immunization of HIV-infected subjects.

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Disclosures

The authors have no financial conflict of interest.

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