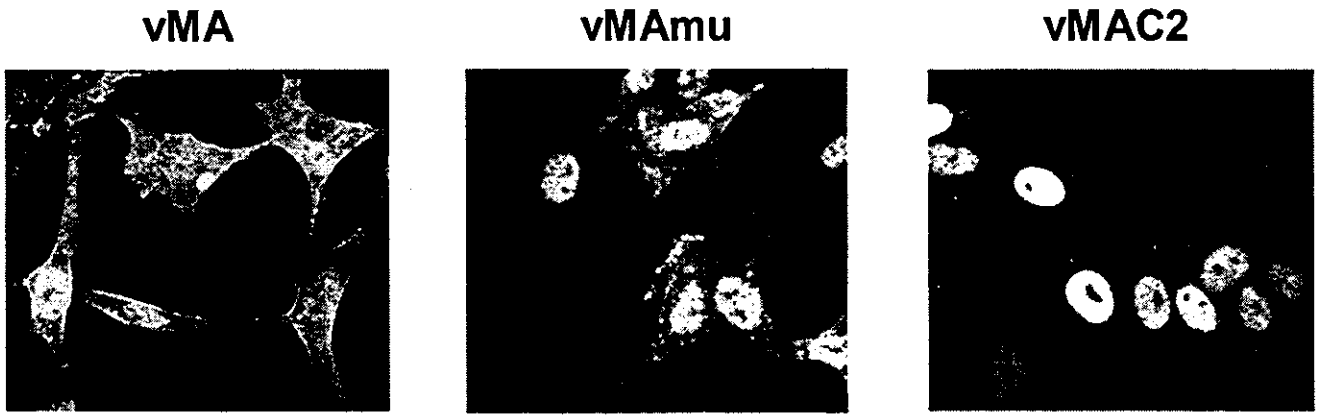

FIG. 9. Intracellular localization of mutant MA proteins. CV-1 cells were infected with vMA, vMAmu, or vMAC2; 24 h later, they were fixed and permeabilized. His tag-conjugated MA proteins were visualized with an anti-His tag Ab. The experiments were performed three times with essentially identical results.

individuals, enhanced $CD4^+$ T-cell responses have been associated with a higher level of virus-specific CTL responses and lower viral loads (22, 46). In this regard, we and others (7, 25, 54) previously found the presence of overlapping CTL epitopes in the leader sequence upstream of the ATG start codon for $Pr65^{gag}$, but not in the MA region, although it has not been determined whether these epitopes are protective or not. However, our previous work clearly demonstrated that the rVV expressing $Pr65^{gag}$ without the leader sequence was as effective as the rVV that expressed $gPr80^{gag}$, indicating that the CTL epitope in the leader sequence is not a requisite for protection against FV infection. Thus, the $CD4^+$ T cells primed with MA might have induced rapid responses of Gag-specific CTL, as well as Env-specific B cells, but the possible importance of Gag-specific CTL responses, if any, has yet to be identified.

Gag-specific $CD4^+$ T cells may also have direct roles in the control of FV infection through their possible cytotoxic activities and production of antiviral cytokines. Direct cytotoxic activities of $CD4^+$ T cells have been described in a number of viral infections (2, 19, 36, 59). A previous study with the FV-infected mouse model, in which direct cytotoxic activities of Env-specific $CD4^+$ T cells were detected, also supported those observations (16). Among $CD4^+$ T-cell clones established from HIV-1-infected individuals with vigorous Gag-specific responses, some displayed virus-specific cytotoxic activities (35, 55). Furthermore, $CD4^+$ T cells have been shown to directly control virus replication by production of gamma interferon in FV infection (10, 18). Thus, $CD4^+$ T cells primed with the MA protein might have contributed to the observed protection against FV infection through multiple effector functions.

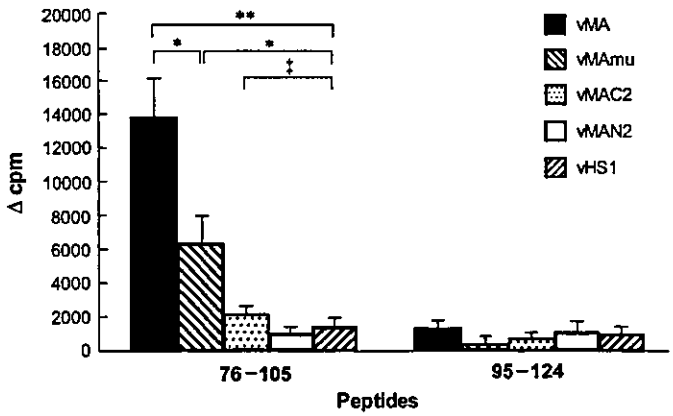

FIG. 10. Different abilities of mutant and truncated MA proteins at priming $CD4^+$ T cells. Proliferative responses of T cells obtained from mice immunized with each rVV were tested by stimulation with peptide 76-105. Each result is expressed as the mean Δcpm for data obtained from four to five mice. Error bars, standard errors of the means. The experiments were performed twice with essentially identical results. Statistically significant differences were observed by Student's t test (*, $P < 0.05$; **, $P < 0.001$). ‡, no significant difference ($P > 0.05$).

The present study has also provided useful information on the structural requirements for effective priming of virus-specific $CD4^+$ T-cell responses by the MA protein. T cells primed in vivo with native MA (vMA) proliferated when stimulated with the Th epitope-harboring peptide 76-105. In contrast, full-length MA lacking the N-terminal myristylation site (vMAmu) and the MA from which the N-terminal 24 residues had been deleted (vMAC2), despite carrying the whole Th epitope, induced only moderate or marginal T-cell responses, respectively, when used to prime T cells in vivo. Of note, their different abilities to elicit the $CD4^+$ T-cell response were well correlated with their efficacies in inducing protection against FV infection in vivo. There was also a correlation between the observed degree of localization of the MA protein at the plasma membrane and its ability to elicit T-cell proliferation and immune protection: By the destruction of the myristylation site, the degree of localization of the MA protein at the plasma membrane was diminished, and the MA lacking the N-terminal 24 residues localized predominantly in the nucleus. These results indicate that the N-terminal region of F-MuLV MA, not just the myristylation site, is responsible for its subcellular localization. A highly basic domain between MA residues 17 and 31 in HIV-1, besides the myristylation signal, has been implicated in membrane binding of the Gag polyprotein (61, 63), and there is a corresponding basic region present between MA residues 17 and 34 in F-MuLV.

Efficient priming of $CD4^+$ T cells by virally encoded proteins is dependent on sufficient levels of antigen expression and delivery of the protein-derived peptides to the MHC class II (MHC II) compartment. Although there is evidence for the activation of $CD4^+$ T cells by viral-DNA-encoded proteins (20, 32, 36), the epitopes displayed on cytoplasmic proteins are usually presented by MHC class I (MHC I) molecules to $CD8^+$ T cells. Epitopes presented by MHC II molecules to $CD4^+$ T cells are mainly derived from extracellular "foreign" proteins taken into cells by endocytotic activities and then degraded in endosomal vesicles. Although retroviral MA is a cytoplasmic protein, there must be some mechanisms for MA to gain access to the cellular site of protein processing involved in peptide presentation on MHC II. It is possible that accumulation of MA at the plasma membrane may result in the formation of aggregates that might be engulfed into phagosomes by mechanisms similar to autophagocytosis. As another possibility, some portions of the MA molecule might be exposed on the outside of the viral envelope, since neutralizing Abs reactive to MA have been detected in some retrovirus infections (3, 5, 38, 50). In addition, there may be an alternative mechanism for the presentation of foreign antigens to $CD4^+$ T cells, in which APC such as macrophages take up whole rVV-infected cells or their fragments by phagocytosis, and the degraded MA is presented on MHC II molecules through the conventional class II pathway. However, the last possibility is unlikely to be the main pathway for MHC II presentation of rVV-generated MA antigens, since the mutant MA proteins expressed by infection of vMAmu and vMAC2, which carried the Th epitope and were detected at a level comparable to that of the native MA expressed by vMA infection, nevertheless failed to induce strong enough $CD4^+$ T-cell proliferation and full protection against FV infection. Therefore, the targeting of MA to the plasma membrane may provide this protein with efficient access to the cellular site for processing and presentation through MHC II pathways, which facilitates induction of the observed immune responses through more-efficient antigen-specific activation of $CD4^+$ T cells. In support of this hypothesis, a recent study demonstrated that a chimeric HIV-1 $p55^{gag}$ protein forced to traffic to the MHC II compartment elicited strong cellular and humoral immune responses in immunized mice (27).

In summary, the results presented here provide compelling evidence that a retrovirus MA peptide is capable of inducing a strong $CD4^+$ T-cell-mediated immune response, which results in effective protection against virus challenge. It will be interesting to design future studies to explore whether there are functional differences between Env-primed and Gag-primed $CD4^+$ T cells, which may be of importance for the development of an effective antiretrovirus vaccine strategy. In addition, the finding that the binding of the MA protein to the plasma membrane is associated with its stronger immunogenicity may lead us to considerations of practical importance about the appropriate immunogenic forms of cytoplasmic proteins when they are considered as candidates for virus-based vaccines.

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Both T and non-T cells with proliferating potentials are effective in inducing suppression of allograft responses by alloantigen-specific intravenous presensitization combined with suboptimal doses of 15-deoxyspergualin

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Abstract

In an MHC class I-disparate combination of mouse strains, a single intravenous injection of donor spleen cells combined with 10 suboptimal doses of 15-deoxyspergualin (DSG) administration was effective in inducing donor-specific suppression of cytotoxic T-lymphocyte (CTL) responses and prolonged survival of the relevant skin allograft. Proliferative potentials of the donor spleen cells were requirement for the induction of suppressed allospecific responses, but both highly purified T cells and non-T cells were equally effective to induce the suppression of CTL responses by intravenous injection. These results have shown that, although working on different mechanisms, DSG is as effective as FK506 or rapamycin in inducing allograft tolerance when used at suboptimal doses along with the donor-specific intravenous presensitization, and an immune mechanism other than well-characterized veto T cells is working in this model in suppressing alloreactive CTL precursors.

Keywords: Donor-specific intravenous presensitization; 15-deoxysupergualin; Skin graft; Tolerance; Veto cell

1. Introduction

The favorable effects of pretransplantation transfusion of donor blood, often referred to as donor-specific transfusion (DST), on allograft survival has been well recognized (for recent reviews see [1,2]). Mechanisms of the effects of DST and influences of different transfusion protocols on the prolongation of graft survival and on recipient immune functions have been studied using various rodent models [3–11]. However, contrasting outcomes can still be observed depending on the degree of disparity at different loci in major histocompatibility complex (MHC) [1,2,11]. In mice, highly reproducible suppression of allograft responses can be induced by a single or repeated intravenous injection(s) of donor spleen cells (donor-specific intravenous presensitization: DSP) in combination with or without suboptimal doses of an immunosuppressive reagent [12–18]. Compelling evidence has indicated that DSP alone suppresses donor antigen-specific proliferative and cytokine-producing responses of recipient T-cells, but cytotoxic T-lymphocyte (CTL) induction is suppressed only by a combination of DSP with antibody-mediated $CD8^+$ T-cell depletion or administration of suboptimal doses of FK506 or rapamycin [12–17]. Cells required

Abbreviations: B6, C57BL/6; Con A, concanavalin A; CTL, cytotoxic T-lymphocyte; DSG, 15-deonxysupergualin; DSP, donor-specific intravenous presensitization; DST, donor-specific transfusion; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; LPS, lipopolysaccharide; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; MMC, mytomycin C; PBBS, phosphate-buffered balanced salt solution; R-PE, R-phycoerythrin.

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for the induction of the suppressed allograft responses by DSP are shown to be radio-sensitive [12] and belong to T-cell populations [12,15], indicating possible roles of veto cells. In the present study, we investigated the efficacy of DSP combined with suboptimal doses of 15-deoxysupergualin (DSG), an immunosuppressive reagent that has mechanisms of action quite different from those of FK506 or rapamycin, in suppressing allograft responses. In addition, we further analyzed cell populations effective in inducing the suppression of allospecific cellular immune responses by highly purifying T and non-T cells using magnetically labeled antibodies.

2. Objectives

The objectives of the present study were (1) to examine if DSG that has mechanisms of action quite different from those of previously tested FK506 and rapamycin is effective in inducing the suppression of allograft responses when used at suboptimal doses in combination with DSP; and (2) to identify a donor cell type(s) and its antigenic and proliferative characteristics required for the effective suppression of allospecific cellular immune responses when used for DSP.

3. Materials and methods

3.1. Mice

C57BL/6 (B6, $H2^b$), B10.QBR ($H2^{bq4}$), and BALB/c ($H2^d$) mice were purchased from Japan SLC, Hamamatsu, Japan, and kept and maintained in animal facilities at Kinki University School of Medicine under specific pathogen-free conditions. B6 and B10.QBR mice differ at the class I D locus, B6 possessing the D^b and B10.QBR the D^q allele, but share the same alleles at other MHC loci. The following experiments were performed under relevant guidelines of the Japanese government and the University, and were approved by the Animal Experiment Committee of the School of Medicine.

3.2. Recipient manipulation and skin grafting

(−)-15-deoxyspegualin was kindly provided by Nippon Kayaku Co., Ltd., Tokyo, Japan as Gusperimus trihydrochloride. A suboptimal dose of 1 mg/kg was given intraperitoneally to recipient B6 mice 10 times at 2-day intervals starting from 8 days before transplantation (Fig. la). For donor-specific presensitization, spleen cells were prepared aseptically from B10.QBR mice and red cells were removed as described in Refs. [14–17,19]. Single-cell suspension was prepared by passing it through sterile nylon mesh, and 1×10^7 cells were injected intravenously into each B6 recipient mouse at 7 days before skin grafting. Skin grafts were prepared by removing 7×7-mm sheets from the tail of B10.QBR mice under ether anesthesia, and transplanted onto the back of each recipient B6 mouse according to the techniques described in Ref. [20]. Bandages were removed on day 7 after transplantation, and graft tissue was observed daily. Skin grafts were determined to be rejected when >80% of the tissue became necrotic as described previously [14–17]. Some recipient mice were killed at 14 days after grafting by cervical dislocation and the spleen was removed for CTL and mixed lymphocyte reaction (MLR) assays as described previously [14–17,19].

3.3. Tissue culture media and reagents

For preparation of spleen cells phosphate-buffered balanced salt solution (PBBS) supplemented with 2% fetal bovine serum (FBS) was used as described in Ref. [19]. To remove erythrocytes, spleen cells were treated with the Tris-buffered ammonium chloride solution, and rinsed three times with PBBS [19]. For tissue culture RPMI 1640 medium supplemented with 5×10^{-5} M 2-mercaptoethanol and 10% heat-inactivated FBS selected for low background stimulation was used. Concanavalin A(Con A), lipopolysaccharide from *Escherichia coli* 0111:B4(LPS), and mytomycin C (MMC) were purchased from Sigma Chemical Co., St. Louis, Missouri.

3.4. CTL and MLR assays

To generate CTL effector cells, 5×10^6 B6 spleen cells were mixed with 1.5×10^6 X-ray (4000 rad)-irradiated B10.QBR or BALB/c stimulator cells, and cultured for 5 days in each well of 24-well tissue culture plates with 2 ml culture medium as described above. Target cells were prepared by stimulating B10.QBR or BALB/c spleen cells with 5 μg/ml Con A for 48 h, and labeling them with 3.7 MBq ^{51}Cr (NEN Life Science Products, Inc., Boston, Massachusetts) per 10^6 cells as described previously [19]. The resultant effector and target cells were mixed at indicated ratios, and the release of ^{51}Cr into the supernate from the 1×10^4 target cells per well of 96-well culture plates in 4 h was measured by using a gamma counter. Levels of antigen-specific target cell lysis were calculated by using a standard formula [19], with maximum release determined by adding 1% Triton X-100 into wells of labeled target cells. For MLR assays, 5×10^5 responder B6 spleen cells were mixed with 4×10^5 4000-rad-irradiated stimulator spleen cells in each well of 96-well tissue culture plates. After culturing for the indicated period, [^{3}H]thymidine (Amersham Life Science, Buckinghamshire, England) was added at 18.5 kBq/well, and the culture was continued for an additional 8 h. Incorporation of [^{3}H]thymidine was measured by using a microplate scintillation counter (TopCount, Packard

Instruments, Meriden, Connecticut) as described previously [19]. The magnitude of antigen-specific proliferation was shown in Δcpm calculated by using a standard formula [19].

3.5. Modification of donor spleen cells and analyses of their antigenicity

B10.QBR spleen cells were prepared as described above, and either irradiated at 1500 or 4500 rad in an X-irradiator, or incubated with 500 μg/ml MMC for 60 min at 37 °C. The treated cells were washed three times with PBBS containing 2% FBS and used as donor cells for DSP. To analyze possible changes in their antigenicity, the above-treated B10.QBR spleen cells, along with untreated ones, were used as stimulator cells and mixed with naive B6 spleen cells in MLR assays as described in Section 3.4. Mixed cultures were incubated for 3, 5 and 7 days, and [^{3}H]thymidine incorporation was measured as described above. Proliferating potentials of the above-treated spleen cells were tested by stimulating them with 5 μg/ml Con A or 10 μg/ml LPS, and [^{3}H]thymidine incorporation was measured at 1, 2, 3, 5 and 7 days after the beginning of culture as described above. Cell surface expression of class I D^q molecules on treated and untreated B10.QBR spleen cells was also analyzed by flow cytometry using an anti-D^q monoclonal antibody (clone KH117, PharMingen, San Diego, California) as described previously [19].

3.6. Purification of T and non-T cells from the spleen

Donor spleen cells were separated into CD90$^+$ T and CD90$^-$, CD45R$^+$ non-T cell populations by using a magnetic cell sorting system. Magnetic microbeads-conjugated anti-mouse CD90 (Thy 1.2) and anti-mouse CD45R (B220) antibodies and CS depletion and VS+ separation columns were purchased from Miltenyi Biotec, Berigische Gladbach, Germany and used according to the manufacturer's instructions. To purify CD90$^+$ T cells, spleen cells were first depleted of B220$^+$ cells and CD90$^+$ cells were positively selected from the B220-depleted population. To purify B220$^+$ non-T cells, spleen cells were first depleted of CD90$^+$ T cells, and B220$^+$ cells were selected from the CD90$^-$ population. Purities of each cell population were confirmed by flow cytometric analyses by using a FACSCaliblur (Becton-Dickinson Immunocytometry Systems, San Jose, California). Fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD3 and R-phycoerythrin (R-PE)-conjugated rat anti-mouse CD19 antibodies and their isotype-matched controls were purchased from PharMingen.

3.7. Statistical analyses

Averages of graft survival periods and magnitudes of proliferative responses between groups of mice were compared by Student's t test. Average percentages of specific lysis at five different effector-to-target ratios were compared between groups as curves by using Hotelling's T^2 test.

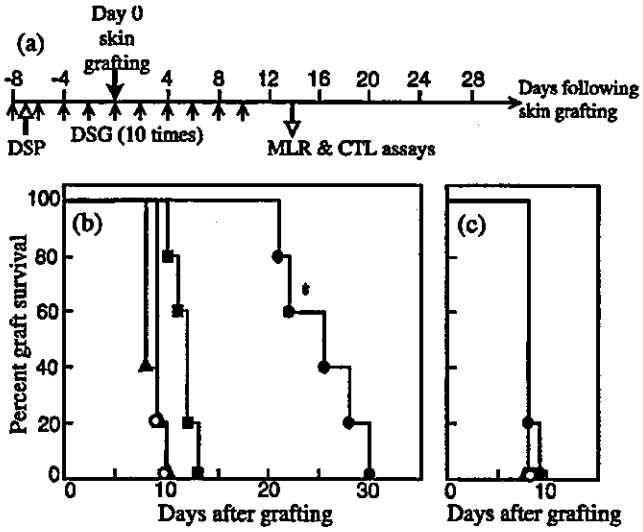

Fig. 1. Experimental protocols and time-courses of graft rejection. (a) Mice were injected with DSG and donor spleen cells as indicated, and received a skin graft. (b) Time-courses of the rejection of BI0.QBR skin graft in untreated B6 mice (○), B6 mice treated with DSP alone (▲), those treated with DSG administration alone (■), or those treated with the combination of DSP and DSG administration (●). *, $P<0.001$. (c) Time-courses of the rejection of BALB/c skin graft in untreated and treated B6 mice. Symbols used are the same as those in b.

4. Results

4.1. Prolongation of skin graft survival by DSP combined with suboptimal doses of DGS in the MHC class I-disparate combination

When B6 mice were treated either by a single DSP or 10 suboptimal doses of DSG injection alone, no significant prolongation of graft survival was observed (Fig. 1b). However, in B6 mice treated with the combination of an intravenous injection of B10.QBR spleen cells and 10 suboptimal dose of DSG injection, skin grafts from the MHC class I-incompatible B10.QBR mice survived significantly longer than those transplanted onto untreated B6 mice. Skin grafts transplanted from the third party BALB/c mice were rejected within 9 days regardless of the treatment of recipient B6 mice (Fig. 1c). These results showed that DSG is as effective as FK506 and rapamycin in inducing the elongation of allograft survival when used at suboptimal doses in combination with DSP.

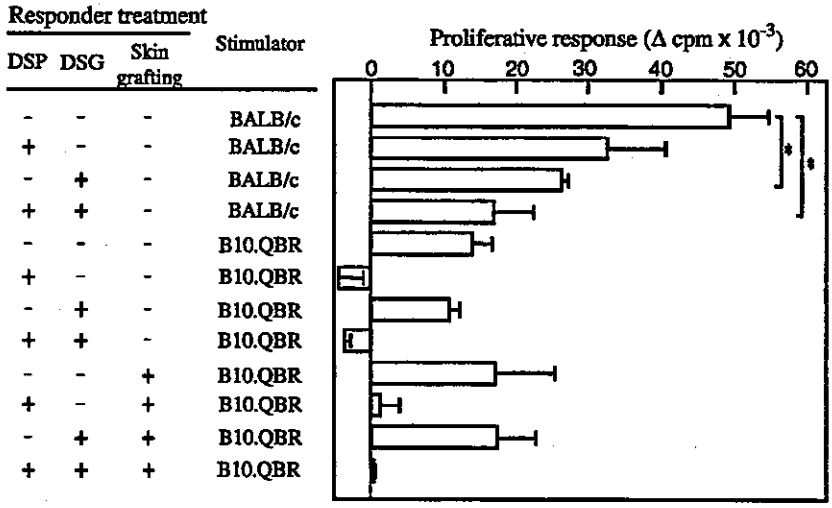

Fig. 2. Proliferative responses of untreated and treated B6 spleen cells to irradiated BALB/c or B10.QBR spleen cells. [^{3}H]thymidine incorporations were measured on days 3, 5 and 7 after the beginning of the MLR responses, and peak responses were always observed on day 5. Thus, average Δcpm values ($n=3$) at day 5 are shown with bars representing S.E.M. *, $P<0.04$.

4.2. Suppression of MLR and CTL responses in B6 mice treated with DSP and/or DSG

When spleen cells from treated and untreated B6 mice with or without skin grafting were tested for allospecific proliferative responses, administration of 10 doses of DSG alone significantly weakened MLR responses to the third party BALB/c stimulator cells, but vigorous proliferative responses were still observed even after DSP with B10.QBR spleen cells plus DSG administration (Fig. 2). In accordance with the previous observations, DSP alone, but not DSG administration alone, completely abolished the ability of B6 spleen cells to respond in MLR assays to MHC class I-disparate B10.QBR simulator cells, and this unresponsiveness was retained in the DSP-treated mice even after being grafted

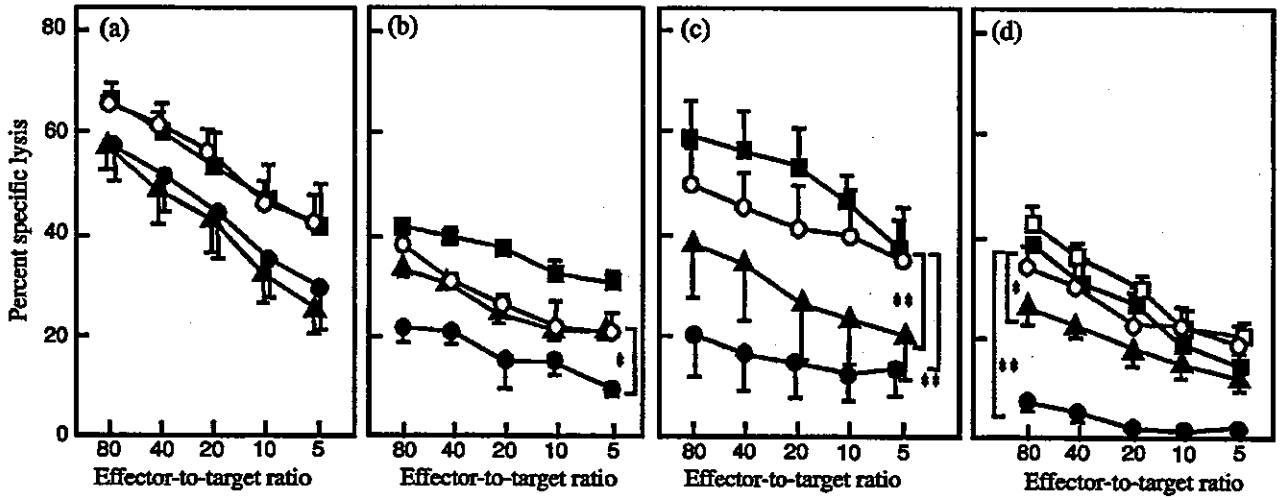

Fig. 3. CTL responses exerted by in vitro-stimulated effector cells derived from untreated and treated B6 mice. (a) Effector cells were induced by in vitro stimulation with irradiated BALB/c spleen cells, and target cells used were labeled BALB/c blast cells. (b–d) Effector cells were induced by in vitro stimulation with irradiated B10.QBR spleen cells, and target cells used were labeled B10.QBR blast cells. In a and b effector cells were prepared from untreated B6 mice (○), B6 mice treated with DSP alone (▲), those treated with DSG administration alone (■), or those treated with the combination of DSP and DSG administration (●). In c B6 mice were treated as above (shown with the same symbols), and received a B10.QBR skin graft. In d effector cells were prepared from untreated B6 mice (○), B6 mice injected with MMC-treated B10.QBR spleen cells plus DSG (▲), those injected with 1500 rad-irradiated B10.QBR spleen cells plus DSG (■), those injected with 4500 rad-irradiated BIO.QBR spleen cells plus DSG (□), or those injected with the combination of untreated BIO.QBR spleen cells and DSG administration (●). Each data point represents the mean from 3 to 6 repeated experiments, and bars represent S.E.M. *, $P<0.03$ by Hotelling's T2 test; **, $P<0.001$ by the same test.

with B10.QBR skin. However, CTL responses against B10.QBR target cells were not significantly affected by DSP alone (Fig. 3b). Only in the group of mice treated with the combination of a single DSP and suboptimal doses of DSG administration, CTL responses to B10.QBR target cells were significantly reduced. Unchanged killing activity of the in vitro-stimulated spleen cells prepared from the treated mice against BALB/c target cells (Fig. 3a) confirmed the alloantigen-specificity of the suppression of CTL activities. Furthermore, significant suppression of CTL activities against B10.QBR target cells was also observed in the B6 mice treated with the combination of DSP and DSG administration even after they received a B10.QBR skin graft (Fig. 3c). CTL activities detected in the untreated control mice and those of the mice treated only with DSG administration were apparently enhanced after skin grafting (Fig. 3b,c), reflecting in vivo priming of CTL precursor cells through alloantigen presentation. However, this priming effect was not observed in mice treated with the combination of DSP and DSG administration. Interestingly, CTL activities detected from the mice treated with a single DSP alone were also significantly lower than those in the control mice when tested after skin grafting (Fig. 3c), probably reflecting the contribution of $CD4^+$ T helper cell functions in the induction of CTL effector cells, which is reduced in the DSP-treated mice (Fig. 2). These data indicated that a significant proportion of CTL precursors were unable to be primed in vivo with the relevant alloantigen when treated with DSP, especially in combination with suboptimal doses of DSG administration.

4.3. Characterization of cellular requirements for the suppression of allograft responses by DSP

To characterize cell properties required for the induction of suppressed allograft responses by DSP, donor spleen cells were treated with several different procedures that modulated their proliferating potentials and antigenicities before intravenous injection. Irradiation (either 1500 or 4500 rad) or MMC treatment of donor spleen cells almost completely abolished their proliferative potentials after Con A or LPS stimulation: no significant incorporation of [^{3}H]thymidine into the treated cells was observed at any one of the four time-points at which the measurements were performed between 1 and 7 days after the beginning of cell cultures. When used as stimulator cells to induce proliferative responses of naive B6 spleen cells in vitro, B10.QBR spleen cells irradiated with 1500 or 4500 rad of X-ray showed significantly reduced antigenicity. They induced only 25% and 15% of peak Δcpm values at day 5 after the begin-ning of the MLR assays, respectively, in comparison with untreated B10.QBR cells, while the stimulatory potential of MMC-treated spleen cells was not significantly reduced. No significant changes in the levels of cell surface expression of D^q molecules between untreated, irradiated, and MMC-treated B10.QBR spleen cells were observed by flow cytometric analyses (data not shown). Interestingly, when used for DSP along with suboptimal doses of DSG, 1500 rad-irradiated spleen cells induced statistically significant but only marginal suppression of MLR responses, and 4500 rad-irradiated spleen cells were not effective at all in inducing the suppression (Table 1). However, MMC-treated B10.QBR spleen cells induced significant suppression of MLR responses when injected intravenously along with DSG administration. When CTL responses of the in vitro-stimulated spleen cells prepared from the untreated and treated B6 mice were compared, irradiated and MMC-treated B10.QBR spleen cells did not induce the suppressive effect that was observed when untreated B10.QBR spleen cells were injected (Fig. 3d), although the injection of MMC-treated B10.QBR spleen cells resulted in slightly reduced CTL activities in comparison with those exerted by the stimulated spleen cells of control untreated mice. These results, especially those with MMC-treated donor cells, indicated that proliferating potentials of donor cells, not just the presence of the relevant alloantigen on their surfaces, are required for the suppression of CTL responses by DSP plus DSG administration.

4.4. Both T and non-T cells were effective in inducing the suppressed allospecific cellular immune responses

To further characterize the donor cells that are involved in the induction of the suppression of allograft responses by DSP and DSG administration, spleen cells used for DSP were separately purified into $B220^-$, $CD90^+$ T and $CD90^+$, $B220^-$ non-T cell populations using a magnetic cell sorter (Fig. 4a). The obtained T-cell population was >98% $CD3^+$ and almost completely devoid of $CD19^+$ B cells, while the non-T cell population was almost completely free from contaminating T cells and comprised of 94% B cells. A single injection of both T and non-T cell populations in combination with suboptimal doses of DSG administration completely abolished the donor antigen-specific proliferative responses in the treated B6 mice (Fig. 4b). Further, CTL responses against B10.QBR target cells were significantly reduced in mice injected with the donor T or non-T cell population (Fig. 4c). As measured by the induction of suppressed alloantigen-specific CTL responses, the purified T cell population was as effective as the whole spleen cells, and purified non-T cells were even more effective and induced almost total suppression of the CTL responses.

5. Discussion

Several different mechanisms have been proposed for the induction of allograft tolerance by DST or DSP in

Table 1
Effects of donor cell irradiation or MMC treatment on the suppression of MLR responses after DSP plus DSG administration

Donor cell treatment	Proliferative responses of recipient spleen cells (Δcpm)	Significance in difference from the control
1500 rad irradiated	7820±528	$P=0.007$
4500 rad irradiated	18 714±2520	NS
MMC treated	2384±468	$P=0.004$
None	−1031±528	$P=0.003$
Control (No DSP)	13 947±2190	

Δcpm values are shown as mean±S.E.M.
NS, not significant.

rodent models. In rats tolerized by DST for heterotopic heart transplantation, recipient immune cells still infiltrated into graft tissues, but cytokine production from the immune cell infiltrated was deeply impaired [1]. Generation of an inhibitory signal from DST-primed host immune cells upon secondary presentation of donor antigens has been reported. In a model using class I L^d-specific T-cell receptor transgenic mice, a single dose of DSP with L^d-expressing spleen cells induced a significant deletion of the donor antigen-reactive T cells, and activated a regulatory subset of T cells in the recipient, which resulted in permanent survival of skin allografts [10]. The above regulatory T cells have been recently characterized as a novel CD4- and CD8- double negative population that exerts Fas-mediated killing of $CD8^+$ effector cells [18]. DST combined with anti-CD154 (CD40 ligand) antibody administration is shown to be effective in tolerance induction for islet grafts, and this model also is apparently mediated by the induction of $CD4^+$ regulatory T cells [2]. For the induction of the above tolerance by DST plus anti-CD154 antibody administration, small resting B cells are sufficient, and T cells are not required in the DST preparation.

In the MHC class I-incompatible model similar to the present study, however, radio-sensitive T cells in the donor cell inoculum is shown to be required for the induction of prolonged skin graft survival associated with reduced CTL precursor frequencies [12,15], suggesting that donor T cells in the DSP preparation may function as veto cells. In the H2 class I-disparate model

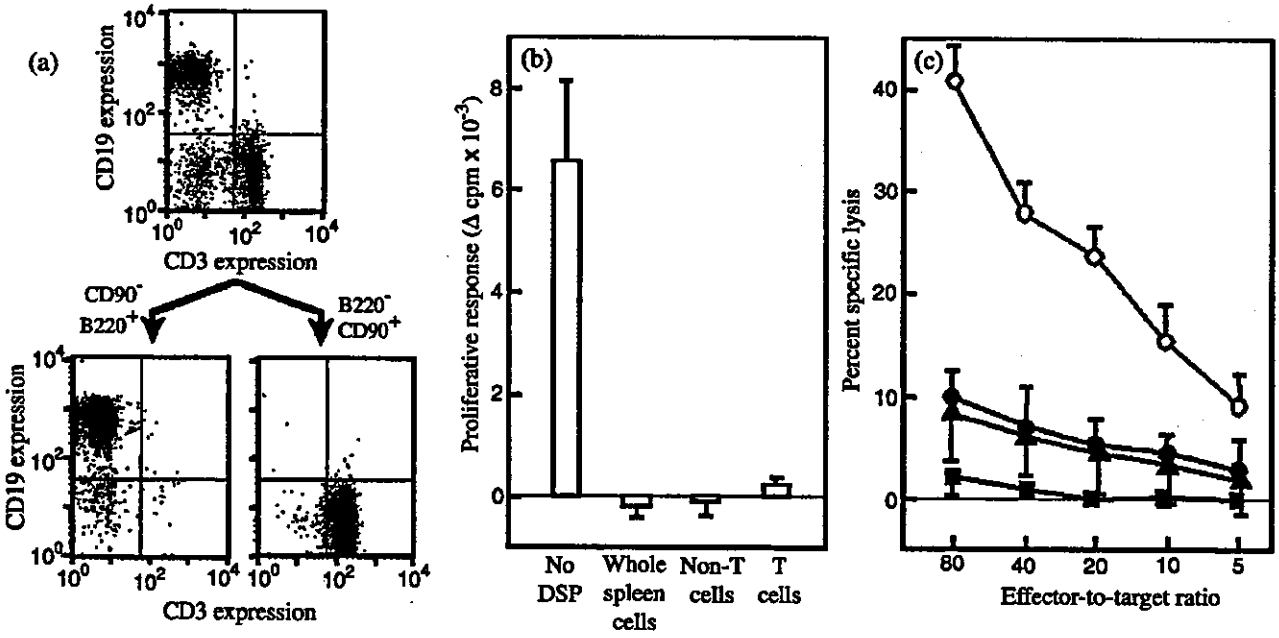

Fig. 4. Identification of cell types in DSP preparation effective in suppressing allospecific cellular immune responses. (a) Flow cytometric analyses confirming purity of injected T and non-T cell populations. (b) Proliferative responses of untreated and treated B6 spleen cells upon stimulation with irradiated B10.QBR spleen cells. Each data show mean±S.E.M. calculated with data obtained from 3 to 6 individuals per group. (c) CTL responses exerted by in vitro-stimulated effector cells prepared from untreated B6 mice (○), B6 mice injected with the purified T cells plus DSG (▲), those injected with the purified non-T cells plus DSG (■), or those injected with the whole B10.QBR spleen cells plus DSG (●). Each data point represents the mean from 3 to 6 repeated experiments, and bars represent S.E.M.

identical to the present combination of B6 recipient and B10.QBR donor mice, it has been shown that (1) a single dose of DSP alone induces a significant reduction in MLR and donor antigen-specific IL-2 production responses, but fails to suppress CTL responses [14]; and (2) DSP combined with either antibody-mediated depletion of $CD8^+$ T cells [14], suboptimal doses or FK506 [16], or suboptimal doses of rapamysin administration [17] results in significant suppression of CTL induction and prolonged skin graft survival. Similar suppression of MLR but not CTL responses was also is observed in rat models of kidney transplantation after DSP [9].

In addition to the previously demonstrated efficacy of FK506 and rapamysin, DSG is now shown to be similarly effective in inducing CTL suppression and prolonged allograft survival when used at suboptimal doses along with a single DSP. FK506 and rapamycin share intracellular receptor molecules, FK-binding proteins, and block the transcriptional activation of interleukin-2 gene in response to T-cell receptor cross-linking or abolish the cell-cycle progression of cytokine-stimulated T cells from G_1 to the S phase, respectively [21]. Mechanisms of action of DSG are quite different from those of FK506 and rapamycin: it suppresses macrophage proliferation [22], blocks pre-T and pre-B cell differentiation [23], and inhibits dendritic cell maturation and antigen presentation [24]. In the present study, suboptimal doses of DSG alone did not affect MLR responsiveness of B6 spleen cells to class I-disparate B10.QBR cells, and CTL induction was significantly suppressed only when DSG was given in combination with DSP. Since DSG is not directly involved in the uncoupling of T-cell receptor signaling and clonal activation, unlike FK506 and rapamycin, effectiveness of DSG in suppressing CTL induction, when combined with DSP, might suggest possible roles of inappropriate antigen presentation and resultant T cell ignorance or anergy [25].

If the induction of T-cell ignorance or anergy is the main mechanism, the expression of relevant alloantigens on the injected cell surfaces, but not functional activities of the injected donor cells, should be sufficient in inducing the suppression of CTL responses and prolonged graft survival. In fact, previous literatures have indicated that cells used for DST can be non-proliferative erythrocytes [7] or even heated blood [8]. However, in the present study, both irradiation and MMC treatment of the donor spleen cells abolished the effect of DSP in suppressing CTL induction. Since T-cell stimulating antigenicity was preserved, albeit reduced, on MMC-treated spleen cells, and MLR responses were indeed largely suppressed in B6 recipient mice injected with MMC-treated B10.QBR spleen cells (Table 1), possible induction of alloantigen-specific regulatory T cells, if any, is also unlikely to be affected by this treatment of donor cells. Rather, a proliferating potential seems to be directly required for the injected donor cells to suppress CTL induction. One possible mechanism that can be affected by irradiation or MMC treatment of the DSP preparation is active involvement of injected donor cells, perhaps as veto cells. Veto phenomenon was originally proposed as a form of antigen-specific suppression of T cells by other lymphoid cells that results in the functional elimination of self-reactive peripheral effector cells [26]. The concept of veto function was later expanded to the inactivation of alloreactive CTL precursor cells upon introduction of allogeneic lymphoid cells [26,27]. Although several different donor cell types are known to exert the veto cell activity when injected intravenously, T cells, especially $CD8^+$ cells, are commonly shown to be the most potent veto cells [26,28,29]. In fact, in the class I-disparate model similar to the present study, the suppression of donor-specific CTL activity by DSP was dependent on the presence of radiosensitive T cells in the injected donor cell preparation [12,15]. Interestingly, however, not only purified T cells but also T cell-depleted $CD90^-$, $B220^+$ cells were effective in inducing almost complete suppression of the donor-specific CTL responses in the present study (Fig. 4). Thus, the results may indicate that the previously described veto T cells are unlikely to be involved in the suppression of CTL induction in the present model. They are rather consistent with the previous finding that as long as relevant MHC molecules are expressed, even transfected fibroblasts can induce immunological unresponsiveness and prolonged graft survival upon intravenous injection [30]. Further studies are required to identify the precise mechanisms by which allospecific CTL responses are suppressed by DSP combined with DSG administration.

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Functionally Impaired HIV-Specific CD8 T Cells Show High Affinity TCR-Ligand Interactions[1]

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We eventually isolated two different clonotypic CD8 T cell subsets recognizing an HIV Pol-derived epitope peptide (IPLTEEAEL) in association with HLA-B35 from a chronic HIV-infected patient. By kinetic analysis experiments, the subsets showed a >3-fold difference in half-lives for the HLA tetramer in complex with the Pol peptide. In functional assays in vitro and ex vivo, both subsets showed substantial functional avidity toward peptide-loaded cells. However, the high affinity subset did not show cytolytic activity, cytokine production, or proliferation activity toward HIV-infected cells, whereas the moderate affinity one showed potent activities. Furthermore, using ectopic expression of each of the TCR genes into primary human CD8 T cells, the CD8 T cells transduced with the high affinity TCR showed greater binding activity toward the tetramer and impaired cytotoxic activity toward HIV-infected cells, corroborating the results obtained with parental CD8 T cells. Taken together, these data indicate that impaired responsiveness of T cells toward HIV-infected cells can occur at the level of TCR-ligand interactions, providing us further insight into the immune evasion mechanisms by HIV. *The Journal of Immunology,* **2004, 173: 5451–5457.**

Our understanding of how HIV avoids control by the human immune system remains incomplete. Although $CD8^+$ CTL are believed to have an important role in the immunopathogenesis of HIV-1 infection, it is not completely clear why viral replication persists and progressive immunodeficiency generally ensues (see recent reviews, 1–5). The findings of several studies show that HIV-specific CTL taken ex vivo can have functional defects that could undermine their control of the virus. For example, whereas most HIV-specific CD8 T cells in patients with chronic HIV disease produced antiviral cytokines on contact with cognate Ag, these cells showed diminished perforin expression and capacity for proliferation compared with CMV-specific T cells (6) and T cells in long term nonprogressors (7), respectively. Such different functional outcomes in T cells can be caused by the quality of T cell activation, such as the strength of TCR engagement and costimulatory or inhibitory interactions (8–10). The kinetics and affinity of interaction between TCR and peptide-MHC complex (pMHC)[3] are the basis of T cell activation. For the most part, longer half-lives of TCR-pMHC interaction correspond to higher T cell activation (11–14). However, in the case of some peptide variants as well as mutations in MHC and/or TCR, a longer half-life was reported to weaken T cell reactivity (15–18). Moreover, it remains unclear what are the functional roles of peripheral T cell subsets that bear TCR with high affinity for a MHC ligand in association with a foreign peptide, because T cells with high affinity for a foreign pMHC appear to be negatively selected in the thymus and not exported to the periphery (19).

In contrast, we and others have generated many CTL lines and clones from HIV-infected patients that were cytotoxic toward HIV-infected cells in vitro in the course of experiments to identify HIV-derived CTL epitopes in previous studies (20). Given that only T cells that were positive for epitope-specific cytolytic activity were reported in these studies, we hypothesized that T cells with negative cytolytic activity toward HIV-infected cells, which may reflect the loss of antiviral effector functions of HIV-specific CTLs in vivo, were concurrently generated, but not further examined, due to their negative activity. Therefore, to examine cell-based mechanisms involved with impaired functions of HIV-specific CD8 T cells, we have again been testing CD8 T cell clones isolated from HIV-infected patients for their lack of killing activity toward HIV-infected cells even though they retained their specificity toward HIV Ags.

In the present study we focused on remarkable functional differences in two different CD8 $\alpha\beta$ T cell subsets (TCR $V\alpha12^+$ and $V\delta1^+$) specific for an HIV Pol-derived epitope peptide (IPLTEEAEL) from a chronic HIV-infected patient. Interestingly, the subsets showed a >3-fold difference in binding activity toward the HLA tetramer in complex with the Pol peptide. The high affinity subset ($V\delta1^+$) showed impaired reactivity toward HIV-infected cells in vitro and ex vivo, whereas the moderate affinity subset ($V\alpha12^+$) had potent reactivity. Additional genetic transfer of each of these TCR genes into human primary CD8 T cells clearly indicated that impaired responsiveness of T cells toward HIV-infected cells can occur at the level of TCR-ligand interactions.

Materials and Methods

Tetramer binding assay

The CTL lines (5×10^4 cells; >60% $tetramer^+$ $CD8^+$ cells) generated by repeated stimulation of the patient's lymphocytes (*HLA-A**2402/*A**2601, *HLA-B**3501/*B**5101) with the Pol peptide (IPLTEEAEL) were first stained with various concentrations of the tetramer at 4 or 37°C for 15 min. The cells were subsequently stained at 4°C for 15 min with anti-CD8-PerCP (BD Pharmingen, San Diego, CA), FITC-conjugated anti-$V\alpha12$

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[3] Abbreviations used in this paper: pMHC, peptide-MHC complex; IRES, internal ribosome entry site; MFI, mean fluorescence intensity.

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TCR (Serotec, Oxford, U.K.), and anti-Vδ1 TCR mAbs. The anti-Vδ1 TCR mAb (A13; provided by L. Moretta, Istituto di Istologia ed Embriologia Generale, Genova, Italy) (21) had been labeled with PE-conjugated Fab specific for the Fc portion of mouse IgG1 (Molecular Probes, Eugene, OR). For the kinetic analysis of tetramer binding, the CTL line was first incubated with 5 μM tetramer at 4°C. A portion of the reaction was removed periodically (2, 5, 10, 15, 30, and 60 min), and the cells were subsequently stained with anti-CD8 and anti-TCR mAbs as described above. For kinetic analysis of the tetramer dissociation, a CTL line was stained with 5 μM tetramer for 60 min at 4°C. Then cells were rapidly washed twice and suspended in 1.5 ml of a buffer (2% BSA in PBS) supplemented with a blocking Ab. A portion of the reaction was then removed periodically (2, 5, 10, 15, 30, and 60 min), and the cells were subsequently stained with the anti-CD8 and anti-TCR Abs. For the flow cytometric analysis, $V\alpha12^+$ or $V\delta1^+$ $CD8^+$ cells were gated and then assessed for their tetramer binding level.

Cytotoxic assay

The cytotoxic activity of CTL clones generated previously (22, 23) was determined by a standard ^{51}Cr release assay as previously described (22). For Pol peptide-pulsed target cells, ^{51}Cr-labeled C1R-B*3501 cells were pulsed with the peptide for 1 h, then incubated with the effector T cells for an additional 4 h at 37°C. For virus-infected target cells, C1R-B*3501 cells or .221-B*3501 cells expressing human CD4 Ag were infected with HIV-1 GagPol-expressing vaccinia virus, HIV-1 LAI, or vesicular stomatitis virus envelope glycoprotein-pseudotyped HIV-1 HXB2D. Note that all these viruses have the same epitope sequence as that used for synthetic Pol peptide (IPLTEEAEL). The cells were subsequently labeled with ^{51}Cr and incubated with the effector T cells for 6 h at 37°C. It should be noted that >70% cells expressed the p24 Gag Ag, as revealed by intracellular flow cytometric analysis of target cells.

Cytokine secretion assay

CTL clones were cocultured with .221-CD4-B*3501 cells, either pulsed with various concentrations of the Pol peptide or infected with HIV-1 LAI for 2 h at 37°C. Brefeldin A (10 μg/ml) was then added, and the culture was continued for an additional 4 h. Then the cells were permeabilized, stained with anti-IFN-γ and TNF-α mAbs (BD Pharmingen), and analyzed by flow cytometry as previously described (23).

Ex vivo activation assay

Cryopreserved PBMC of HIV-positive (1×10^6) or negative donors (5×10^6) were stained with the tetramer at 37°C for 15 min, followed by anti-CD8 and anti-TCR Abs at 4°C for 15 min. The cryopreserved PBMC of the HIV-positive patient were stimulated, or not, with irradiated .221-CD4-B*3501 cells, either pulsed with 100 nM Pol peptide or infected with HIV-1 LAI (>70% p24 Gag^+). The cells were cultured at 37°C for 12 days in RPMI 1640 supplemented with 10% FCS and 200 U of IL-2. A portion of the stimulated cells (2×10^5) was stained as described above.

Construction of retroviral vectors and gene transfer

The genes encoding full-length α and β TCR of CTL 55 (23) and 589 (22) were subcloned into the pGC-based retroviral vector (pGCDNsap[MSCV]; provided by M. Onodera, Tsukuba University, Ibaragi, Japan) (24). The sequence data of the TCR genes are available from DDBJ under accession numbers AB164056, AB164057, AB164620, and AB164621. The genes encoding a murine heat-stable Ag (CD24) or a GFP were also incorporated into the constructs with an internal ribosome entry site (IRES) following the α or β TCR gene to facilitate monitoring of the expression of the α or β TCR gene, respectively, in the transduced cells.

Human primary CD8 T cells were isolated from PBMC of an HIV-negative healthy donor with *HLA-B*3501* using anti-CD8 magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The resultant CD8 T cells were activated over a 3-day period by anti-CD3 mAb (OKT3) coated on the culture dish, then transferred to recombinant fibronectin-coated plates (Takara Shuzo, Otsu, Japan) and incubated for 72 h with the retrovirus supernatant containing the TCR α-IRES-CD24 gene. Transduced T cells expressing CD24 Ags were isolated using PE-labeled anti-CD24 mAb (BD Pharmingen) and anti-PE magnetic beads (Miltenyi Biotec). The isolated cells (>80% of the cells were $CD8^+$ $CD24^+$) were subsequently transduced with another construct containing TCR β-IRES-GFP as described above.

Results

Functional difference in CTL clones in response to HIV-infected cells in vitro

An HIV Pol peptide (IPLTEEAEL) is a CTL epitope endogenously presented by HLA-B*3501 (20). In Pol peptide-stimulated lymphocytes from a patient with chronic HIV infection, we generated two $CD8^+$ αβ T cell clones, designated CTL 55 and 589, that were shown to express TCR Vδ1.1/Vβ13.3 and Vα12.1/Vβ5.6, respectively, on their cell surface (22, 23). It is of note that the genes encoding a Vδ1 variable segment are expressed in ~0.5% of peripheral $CD8^+$ αβ T cells in human healthy individuals and that the Vδ1-bearing TCR αβ recognizes a peptide presented by HLA class I molecules (21, 23). The functional properties of both T cell clones were first tested for their cytotoxic and cytokine production activities in response to cells either pulsed with the Pol peptide or infected with viruses expressing HIV Pol proteins. Peptide titration experiments showed that both clones had substantial cytotoxic activities (Fig. 1*A*). It should be noted that in repeated experiments, CTL 589 appeared to

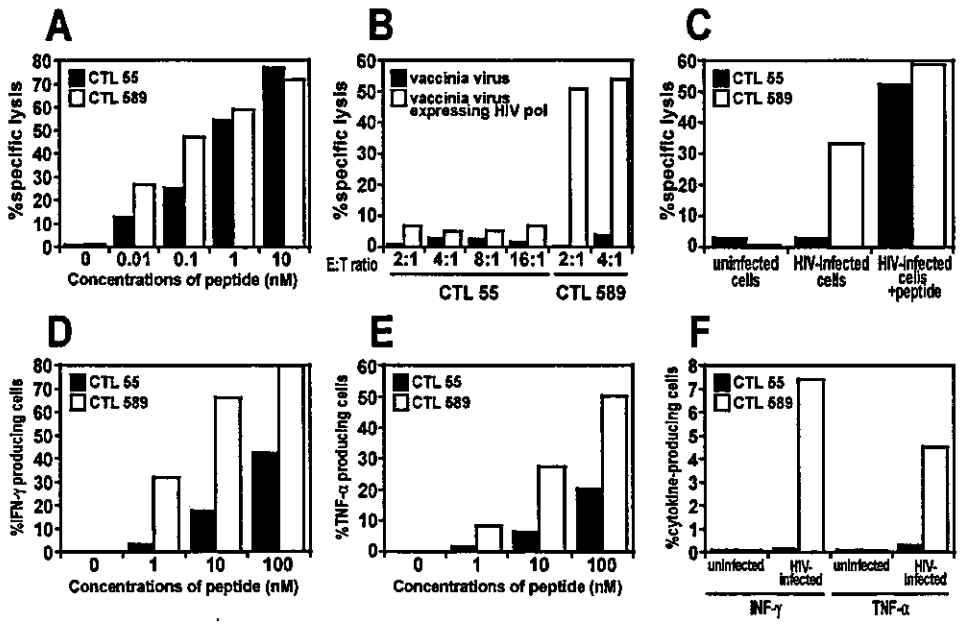


FIGURE 1. Analysis of effector functions of the CTL clones. *A* and *B*, Cytotoxic activity of CTL 55 and 589 toward C1R-B*3501 cells either pulsed with the indicated concentrations of the Pol peptide (*A*) or infected with vaccinia virus expressing HIV-1 GagPol polyproteins at the indicated E:T cell ratios (*B*). *C*, Cytotoxic activity of CTL 55 and 589 toward .221-B*3501 cells uninfected or infected with HIV-1 in the absence or the presence of 100 nM Pol peptide at an E:T cell ratio of 2:1. *D–F*, Intracellular staining for IFN-γ and TNF-α of CTL 55 and 589 in response to .221-B3501 cells either pulsed with the indicated concentrations of the Pol peptide (*D* and *E*, respectively) or infected with HIV-1 at an E:T cell ratio of 1:1 (*F*). Data are shown as the means of duplicate assays for at least three independent experiments.

have more profound cytotoxic activity at low concentrations of the Pol peptide than CTL 55, although the difference was modest under the assay conditions tested (Fig. 1*A*). CTL 589 also showed substantial cytotoxic activities toward cells infected with vaccinia virus expressing HIV-1 Gag-Pol polyproteins (Fig. 1*B*) as well as toward those infected with HIV-1 (Fig. 1*C*). In sharp contrast, CTL 55 was not cytotoxic toward cells infected with either viruses, even at increased E:T cell ratios (Fig. 1, *B* and *C*). Addition of the Pol peptide to the culture medium restored the cytotoxic activity of CTL 55 toward target cells (Fig. 1*C*), indicating the cytotoxic potential of CTL 55 during the assay.

As observed in cytotoxic assays, intracellular cytokine staining experiments showed that both clones had substantial ability to produce IFN-γ (Fig. 1*D*) and TNF-α (Fig. 1*E*) in response to the Pol peptide-loaded cells, although CTL 55 responded to a lesser extent than CTL 589. In response to HIV-infected cells, CTL 55 did not show production of IFN-γ or TNF-α (Fig. 1*F*), whereas CTL 589 produced both cytokines, confirming the impaired responsiveness of CTL 55 toward HIV-infected cells. Moreover, the other Pol peptide-specific CTL clones, 349 and 562, that had TCRs identical with CTL 55 and 589, respectively, showed a similar pattern of functional differences as that observed for CTL 55 and 589 (data not shown), suggesting that the impaired responsiveness toward HIV-infected cells was an inherent property of certain T cell subsets specific to the Pol peptide, rather than of a particular CTL clone.

Different proliferation capacities between the tetramer$^+$ CD8 T cell subsets in response to HIV-infected cells ex vivo

To further investigate the functional difference between these CD8$^+$ T cells toward HIV-infected cells, we examined the ex vivo proliferation capacity of these CD8 T cells in response to HIV-infected cells, because the Pol peptide-specific T cell clonotypes corresponding to CTL 55 and 589 can be exclusively stained by anti-TCR Vδ1 (21) and Vα12 Abs in association with the tetramer. In addition, TCR analysis of CD8$^+$tetramer$^+$ cells of the patient, followed by cell sorting, revealed that all TCR α transcripts that carried either Vδ1- or Vα12-encoded region had the CDR 3α sequence identical with that of TCR 55α or 589α, respectively (data not shown), confirming the T cell clonality and Ag specificity of tetramer$^+$ cells in the flow cytometric analysis.

Firstly, direct analysis of unstimulated peripheral lymphocytes of the HIV-infected patient ex vivo revealed that the frequency of tetramer$^+$ CD8 T cells was ~0.1% of the total CD8 T cell population and that the Vδ1$^+$ and Vα12$^+$ subsets were 75 and 8%, respectively, within this tetramer$^+$ fraction (Fig. 2*B* and Table I), whereas analysis of peripheral lymphocytes of the HIV-negative donors ex vivo revealed that the frequency of tetramer$^+$ CD8 T cells was ~0.02% of the total CD8 T cell population, and that the Vδ1$^+$ and Vα12$^+$ subsets were <5% within this tetramer$^+$ fraction (Fig. 2*A*).

Peripheral lymphocytes of the HIV-infected patient were then stimulated with cells that had been either pulsed with the Pol peptide or infected with HIV-1 and cultured for 12 days. When stimulated with the Pol peptide, frequencies of the Vδ1$^+$ and Vα12$^+$tetramer$^+$ CD8 T cell subsets in the total CD8 T cells were increased 7- and 30-fold (Table I and Fig. 2*D*), respectively, indicating that both subsets had substantial proliferation capacity in response to the Pol peptide-loaded cells. This observation was consistent with our previous finding that CTL 55 and 589 had been generated by repeated stimulation of the patient's lymphocytes by the Pol peptide. In contrast, when stimulated with HIV-infected cells, the frequency of the Vα12$^+$tetramer$^+$ CD8 T cell subset was increased >70-fold, whereas the frequency of the Vδ1$^+$tetramer$^+$ CD8 T cell subset was decreased (Fig. 2*E* and Table I), indicating that the Vδ1$^+$tetramer$^+$ CD8 T cell subset could not respond to HIV-infected cells. Noticeably, the frequency of the tetramer$^+$ CD8 T cell subset, without any Ag stimulation, was virtually constant after a 12-day culture period (Fig. 2*C* and Table I), confirming the Ag-specific proliferation response of the Vα12$^+$tetramer$^+$ subset. It is also of interest that the Vα12$^+$ cells showed more profound proliferation when stimulated by cells infected with HIV than when pulsed with the Pol peptide (Fig. 2 and Table I), suggesting a qualitative difference in Ag presentation to T cells between infected cells and peptide-loaded cells.

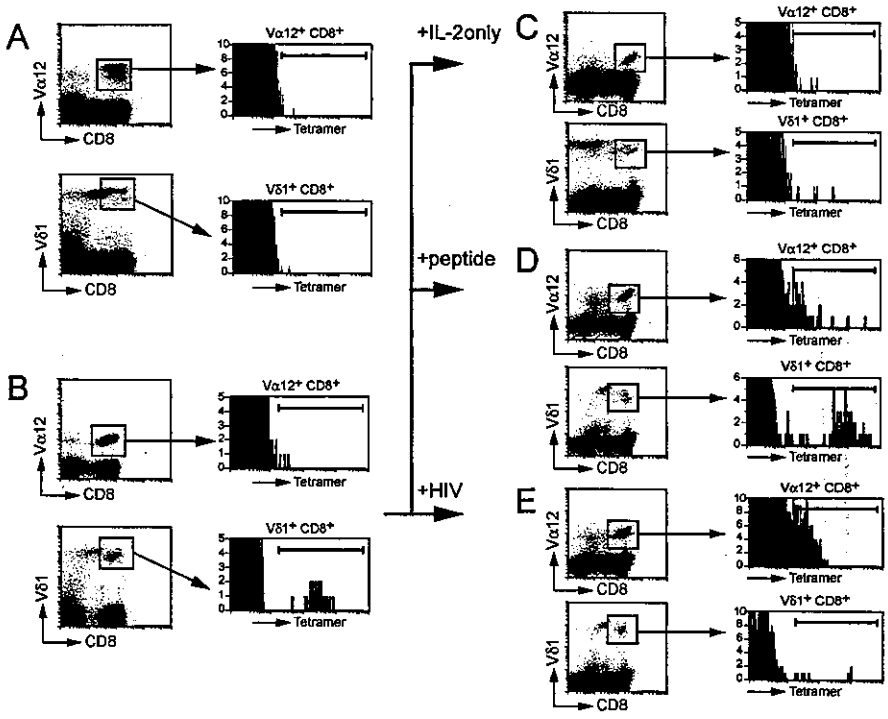


FIGURE 2. Ex vivo analysis of the tetramer$^+$ CD8 T cells in the peripheral lymphocytes in response to Ag stimulation. Cryopreserved PBMC of a representative HIV-negative donor (5×10^6; *A*) and the HIV-infected patient (1×10^6; *B*) were stained with the tetramer and anti-CD8 and anti-TCR Abs. The PBMC of the HIV-infected patient after stimulation with IL-2 alone (*C*) or with .221-B*3501 cells either pulsed with the Pol peptide (*D*) or infected with HIV-1 (*E*) were stained as described above. The Vδ1$^+$ and Vα12$^+$ CD8$^+$ subsets were gated, then analyzed for their levels of tetramer binding. Similar data were obtained in a separate independent experiment. The frequencies and MFI values of the tetramer$^+$ cells in each subset of the HIV-infected patient are summarized in Table I.

Table I. *Summary of ex vivo flow cytometric analysis of tetramer$^+$ CD8$^+$ cells*[a]

Subset	Before Stimulation		+IL-2 Only		+Peptide-Pulsed Cells		+HIV-Infected Cells	
	Cell number	MFI	Cell number	MFI	Cell number	MFI	Cell number	MFI
CD8$^+$Tet$^+$	113	173	46	83.9	1025	525	863	128
CD8$^+$Tet$^+$Vα12$^+$	10	50.9	13	51.9	310	123	755	97.2
CD8$^+$Tet$^+$Vδ1$^+$	86	291	20	105	502	710	12	705

[a] Cell number is the number of cells in the indicated subset per 1×10^5 total CD8 T cells. Mean fluorescence intensity (MFI) for the tetramer in each tetramer$^+$ (Tet$^+$) subset is also shown.

Distinct binding kinetics in HIV-specific CD8 T cell subsets to tetramer

During the flow cytometric analysis, we noticed that the Vα12$^+$ and Vδ1$^+$ subsets had different binding activities toward the tetramer (Fig. 2*D*, for example). Staining CTL clones with the tetramer showed that Vδ1$^+$ CTL 55 had >2-fold greater binding activity than Vα12$^+$ CTL 589 (Fig. 3), whereas both clones showed comparable surface expression of the CD3 Ags (Fig. 3), indicating that the apparent difference in tetramer binding was not due to the surface density of the TCR/CD3 complex. Consistent with this, when the tetramer$^+$ cells were divided into two subsets in the ex vivo flow cytometric analysis, the Vδ1$^+$ cells were mostly found in the tetramer^{high} subset, whereas the Vα12$^+$ cells were in the tetramer^{low} subset (Fig. 4*A*). These observations are interesting, because the extent of T cell activation is known to be sensitive to subtle differences in the duration of TCR-ligand interactions (12, 13, 17).

Because large temperature effects on TCR-pMHC interactions have been noted (12, 25, 26), we first examined the effect of reaction temperature on binding activity toward the tetramer. Pol peptide-stimulated lymphocytes were stained with various concentrations of the tetramer at 4 or 37°C, and the mean fluorescence intensity (MFI) of both subsets was determined by flow cytometry. At either temperature, the Vδ1$^+$CD8$^+$tetramer$^+$ cells showed ~3-fold higher MFI for the tetramer than the corresponding Vα12$^+$ cells (Fig. 4*B*), indicating that both T cell subsets could bind the tetramer with sufficient sensitivity and specificity.

We next examined the kinetic interactions of the tetramer with the Vδ1$^+$ or Vα12$^+$ subset at 4°C, because the level of tetramer binding and its half-life were previously shown to correlate with monomer affinity and off-rate, respectively (13, 27). Although the association of the tetramer with the two subsets was not significantly different (Fig. 4*C*), the dissociation of the tetramer from Vδ1$^+$ cells substantially delayed compared with that from Vα12$^+$ cells (Fig. 4*D*). The half-lives of the tetramer dissociation from the Vδ1$^+$ and Vα12$^+$ subsets were calculated to be 30.0 ± 1.6 and 8.1 ± 0.4 min, respectively. These results suggest that the >3-fold longer half-life of the Vδ1$^+$ subset for interaction with the tetramer than that of the Vα12$^+$ subset was a cause of the impaired antiviral effector functions of the Vδ1$^+$ subset.

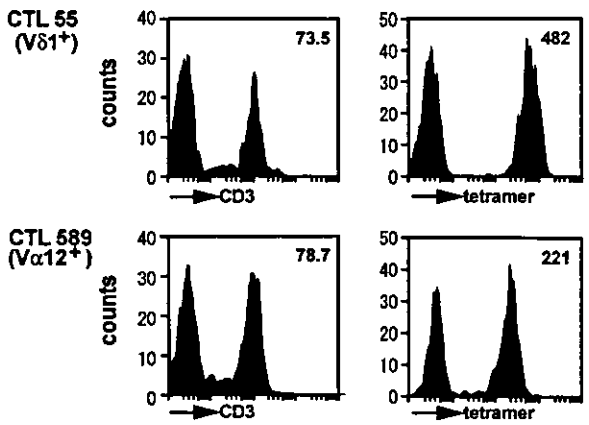


FIGURE 3. Flow cytometric analysis of CTL clones. Two CTL clones, CTL 55 and 589, selected from the Vδ1$^+$ and Vα12$^+$ CD8 T cell subsets, respectively, were stained (■) or not stained (□) with anti-CD3 mAb or the tetramer at 4°C, and analyzed by flow cytometry. MFI values are shown in the *upper right* corners.

Impaired responsiveness of CD8 T cells to HIV-infected cells solely caused by TCR

To clarify whether the high affinity interaction with the tetramer and the impaired responsiveness to HIV-infected cells observed in the Vδ1$^+$tetramer$^+$CD8 T cell subset were caused by the ligand recognition property of the TCR, we transduced human primary CD8 T cells with both TCR genes separately.

Both α and β TCR genes for CTL 55 (Vδ1.1/Vβ13.3) and 589 (Vα12.1/Vβ5.6) were cloned into a pGC-based retrovirus vector with the gene encoding murine CD24 or GFP downstream of IRES for bicistronic expression of α or β TCR genes, respectively (Fig. 5*A*). Human primary CD8 T cells prepared from an HIV-negative donor carrying *HLA-B*3501* were sequentially transduced with α and β TCR genes and analyzed by flow cytometry. As shown in Fig. 5*B*, tetramer$^+$CD8$^+$ subsets reached 15 and 34% of the total lymphocytes for 55 TCR and 589 TCR-transduced cells, respectively, whereas the tetramer$^+$CD8$^+$ subset remained 0.05% of the total lymphocyte population in the case of mock-transduced cells. As measured by the MFI values for the tetramer, the tetramer$^+$CD8$^+$ fraction (gated cells in Fig. 5*B*) of 55 TCR-transduced cells appeared to have 2.8-fold greater binding activity toward the tetramer than that of the 589 TCR-transduced ones. In contrast, both tetramer$^+$CD8$^+$ fractions showed comparable MFI values for CD24 and GFP (within 1.5-fold; Fig. 5, *B* and *C*), which should reflect the expression levels of α and β TCR genes, respectively. These data indicate that the difference in tetramer binding activity observed between the Vδ1$^+$ and Vα12$^+$tetramer$^+$CD8 T cells was solely due to their TCR.

The tetramer$^+$CD8$^+$ fractions of the TCR-transduced cells were then sorted and tested for their cytotoxic activity toward cells either pulsed with the Pol peptide or infected with HIV-1. Both 55 and 589 TCR-transduced cells showed substantial cytotoxic activity toward peptide-loaded cells (Fig. 6*A*), and the activities were as potent as those of their parental CTL clones (cf., Figs. 1*A* and 6*A*). Also, both transduced cells showed modest differences in cytotoxic activities at low concentrations of the Pol peptide (Fig. 6*A*), consistent with the observations made on parental CTL clones (Fig. 1*A*). In contrast, the 55 TCR-transduced cells did not show cytotoxic activity toward HIV-infected cells, whereas 589 TCR-transduced ones killed cells infected with HIV-1 (Fig. 6*B*), again in agreement with the observations made on the parental CTL clones (Fig. 1*C*). These data strongly support our conclusion that the different cytotoxic activities toward HIV-infected cells observed in

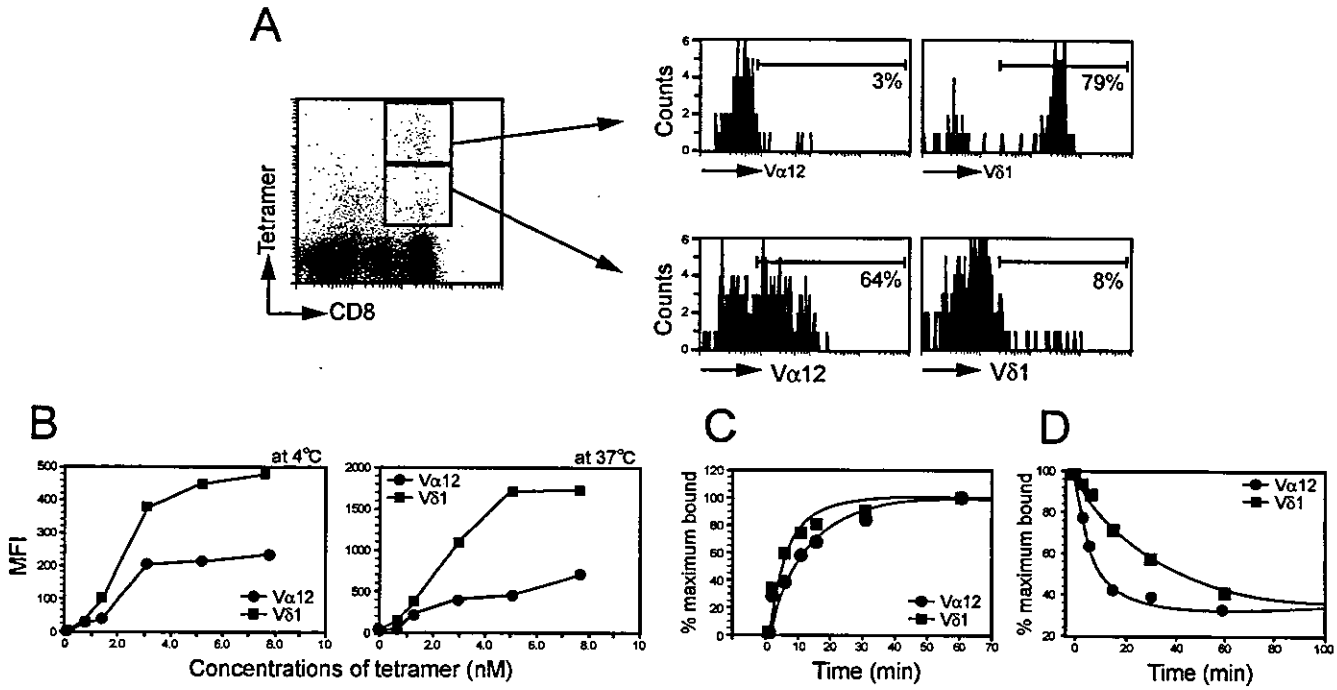


FIGURE 4. Flow cytometric analysis of kinetic interaction between the tetramer and CD8 T cell subsets. *A*, TCR analysis of tetramer^{high} and tetramer^{low} CD8 T cell subsets in Pol peptide-stimulated peripheral lymphocytes of the HIV-infected patient. *B*, Tetramer binding activities of V$\delta1^+$ and V$\alpha12^+$ CD8 T cell subsets determined at 4 or 37°C. *C* and *D*, Association (*C*) and dissociation (*D*) kinetic analysis of interaction between the tetramer and the V$\delta1^+$ and V$\alpha12^+$ CD8 T cell subsets at 4°C. Data are shown as the means of duplicate assays. An independent experiment gave similar results.

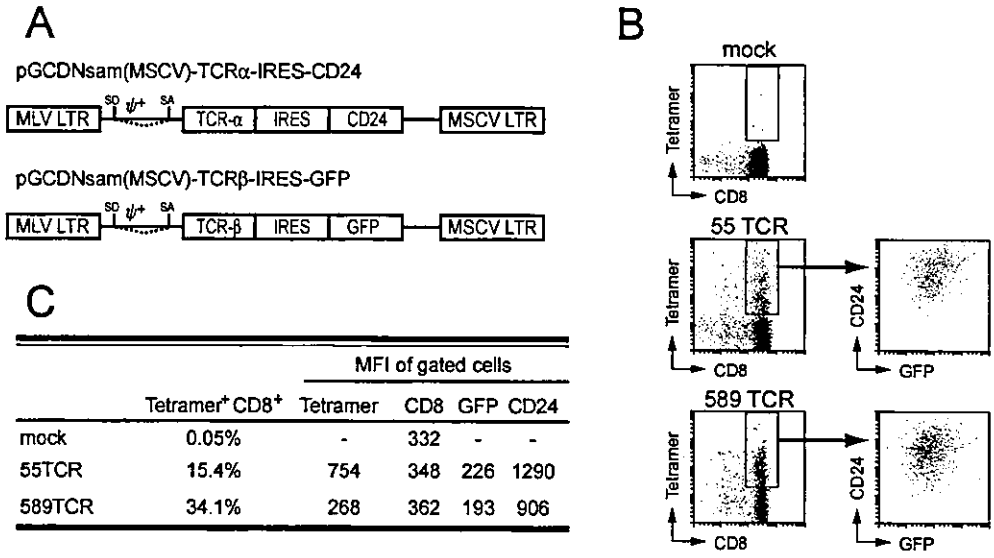
the V$\delta1^+$ and V$\alpha12^+$tetramer$^+$CD8 T cells were solely due to the difference in the TCR.

Discussion

We showed in this study that CD8 T cells with relatively high affinity TCR for an HIV-derived peptide were present in vivo and that these T cells had substantially diminished functional outcomes in response to cells infected with HIV. The difference in functional outcome of the two CD8$^+$tetramer$^+$ T cell subsets (V$\alpha12^+$ and V$\delta1^+$) was evaluated in terms of both the cytotoxic and cytokine production activities of T cell clones in vitro and their proliferation capacity, as assessed by direct ex vivo assays. The results revealed the impaired responsiveness of the higher affinity T cell subset (V$\delta1^+$) to virus-infected cells. In contrast, the V$\delta1^+$ T cells showed functional avidity to cells pulsed with the HIV-derived epitope peptide comparable to that of the other HIV-specific CD8 T cell subset (V$\alpha12^+$), which was competent for effector functions toward HIV-infected cells, indicating that the functional impairment observed in this study did not result from T cell anergy, skewed maturation of CD8 T cells in the periphery, or any defect in signal transduction machinery reported previously (1, 2, 6). There are a myriad of studies that focused on functional differences among Ag-specific (tetramer$^+$) CD8 T cells for HIV (6, 7, 28–30) as well as for other viruses and tumors (31–33), and such studies indicated functional heterogeneity of tetramer$^+$ CD8 T cells. In our study we demonstrated that the duration of TCR-pMHC interactions could have considerable effects on the antiviral effector functions and proliferation capacity of HIV-specific CD8 T cells.

It is known that the T cell is sensitive to subtle differences in the duration of TCR-ligand interactions and that, in general, a longer duration of TCR-pMHC interactions corresponds to higher T cell activation (14, 34, 35). However, Kalergis et al. (18) showed that T cell hybridoma cells transfected with a mutant TCR displayed impaired T cell activation and had an ~2-fold longer half-life for interaction with the tetramer than cells transfected with the wild-type TCR. The present study focused on the human primary CD8 T cells specific for an HIV Ag and showed that a >3-fold increased duration of the TCR-pMHC interaction resulted in impaired, rather than increased, T cell reactivity toward HIV-infected cells, supporting the findings by Kalergis et al. (18). It is likely that HIV-specific T cells have a means to improve their functional avidity to virus-infected cells because only limited numbers of the cognate epitope peptide could be presented on the surface of HIV-infected cells. A serial triggering model (36), based on the T cell integrating the number of TCRs that have interacted with pMHC, predicts that too long an interaction of TCR-pMHC slows the dissociation of TCR from pMHC, so that fewer TCRs are triggered; this may explain our observation. Indeed, we reproducibly observed the different cytotoxic activities of CTL 55 and 589 at low concentrations of the Pol peptide. If the amount of the Pol peptide presented on the surface of virus-infected cells was close to this range, their functional difference in contact with virus-infected cells could be simply explained by their different avidity to low density Ags. However, considering that CTL 55 and 589 showed significant difference in specific killing activity toward HIV-infected cells (<5 and >30%, respectively), the HIV-derived Ag would be preferentially presented at certain sites on the surface of HIV-infected cells with a local density sufficient to activate CTL 589, but not CTL 55. In this regard, it may be helpful to analyze the density and localization of the presenting epitopes on the surface of virus-infected and peptide-pulsed cells using a reagent such as a recombinant Fab specific to the peptide-MHC class I complex.

One of the CTL clones showing the higher affinity TCR-ligand interaction, CTL 55 demonstrated significant differences between their response to peptide-pulsed and virus-infected target cells in this study. It is conceivable that T cells bearing the high affinity TCR can be unduly susceptible to an inhibitory mechanism of HIV-infected cells. Alternatively, a longer duration of TCR-ligand



C

		MFI of gated cells			
	Tetramer$^+$CD8$^+$	Tetramer	CD8	GFP	CD24
mock	0.05%	-	332	-	-
55TCR	15.4%	754	348	226	1290
589TCR	34.1%	268	362	193	906

FIGURE 5. Retrovirus-mediated transduction of human primary CD8 T cells with TCR genes. *A*, Linear representations of the retroviral vector constructs used for transduction with α and β TCR genes. SD, splicing donor; SA, splicing acceptor; ψ^+, packaging signal; MLV, murine leukemia virus; MSCV, murine stem cell virus; LTR, long terminal repeat. *B*, Human primary CD8 T cells isolated from an HIV-negative donor, mock-transduced or transduced with 55 TCR or 589 TCR, and analyzed by flow cytometry. The tetramer$^+$CD8$^+$ subsets were gated, and their expression levels of GFP and CD24 were analyzed. *C*, Summary of frequencies and MFI values of the gated cells. Data shown are similar to those obtained in separate independent experiments.

interaction can lead to recruiting inhibitory receptors or molecules to the sites where the ligand-engaged TCR/CD3 signaling complexes were in action. However, similar different outcomes were observed in CTL 55 when the target cells were infected with vaccinia virus expressing HIV Pol protein, implying the existence of a general inhibitory mechanism of virus-infected cells, rather than an HIV-specific one. It is therefore interesting to see the differences in gene expression profiles of CTL 55 in contact with cells pulsed with the peptide and infected with viruses that had the epitope sequence.

We used an HLA class I tetramer for HIV-specific CD8 T cell subsets to analyze the relationship between affinity and cellular responses. The avidity of tetramer binding gives relative values that can be used as a surrogate for true affinity measurements, such as by surface plasmon resonance (19, 27). Tetramer binding more closely reflects the avidity of the T cell, rather than the intrinsic affinity of the TCR, because the tetramer binding to TCR is measured on the surface of the T cell in the presence of CD3 elements, coreceptors, and other molecules. In our study retrovirus-mediated transfer of TCR genes into human primary CD8 T cells showed that differences in the two CD8 T cells in terms of tetramer binding activity and effector functions toward HIV-infected cells solely depended on the TCR used for transduction. This experimental result clearly indicates that the affinity difference between the two TCRs interacting with the same pMHC ligand is the cause of their distinct responsiveness to HIV-infected cells.

It is reported that the Pol peptide was presented by both HLA-B*3501 and HLA-B*5101, and that CTL 589 cross-recognized the Pol peptide in complex with both HLA molecules (22). Although HLA-B*5101 bound the Pol peptide to a >50-fold lesser extent than HLA-B*3501, it is still possible that CTL 55 and 589 were differently restricted by either HLA-B*3501 or HLA-B*5101, yet specific to the same Pol peptide, because the patient has both *HLA-B*3501 and *HLA-B*5101. However, this is unlikely because CTL 55 did not respond to HLA-B*5101-expressing cells pulsed with the Pol peptide or to those infected with viruses expressing the epitope (H. Tomiyama and T. Ueno, unpublished observations), indicating that the different functional outcomes between CTL 55 and 589 were not due to the difference in their restriction elements.

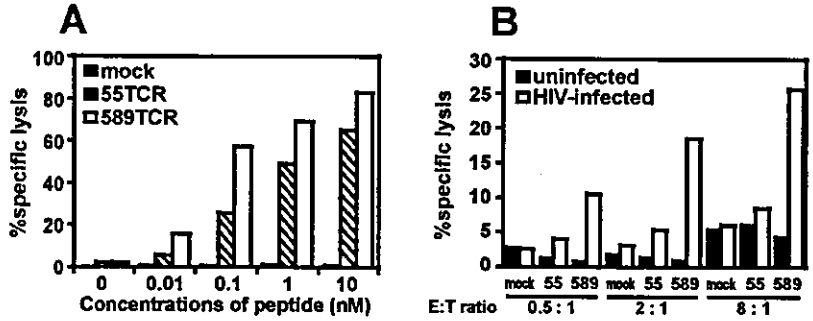


FIGURE 6. Cytotoxic activity of the 55 and 589 TCR-transduced CD8 T cells. The CD8$^+$tetramer$^+$ subsets of the 55 and 589 TCR-transduced cells were sorted (see Fig. 5) and analyzed for their cytotoxic activity toward C1R-B*3501 cells pulsed with the indicated concentrations of the Pol peptide at an E:T cell ratio of 1:1 (*A*). The same set of TCR-transduced cells was assessed for their cytotoxic activity toward C1R-B*3501 cells, infected or not with vesicular stomatitis virus envelope glycoprotein-pseudotyped HIV-1 HXB2D at the indicated E:T cell ratio (*B*). Data are shown as the means of duplicate assays for three independent experiments.

It remains unclear how such a high affinity TCR was generated in the periphery of the patient with chronic HIV infection. It was reported that T cells with high affinity TCR for foreign pMHC appear to be present in the thymus, but that these T cells are eliminated and not exported to the periphery (19). This scenario is most likely because TCRs with high affinity for foreign pMHC show considerable self-reactivity and therefore are negatively selected in the thymus (37). Considering the HIV-infected thymus, a significant increase in the expression of MHC class I molecules was reported, with this MHC class I up-regulation resulting in decreased surface expression of CD8 Ags on thymocytes (38, 39). This could lead to a decrease in the overall avidity of the TCR-CD8 signaling complex for self-pMHC ligands, allowing T cells with high affinity TCR to escape negative selection in the thymus and be exported to the periphery. Although we have only tested limited numbers of T cells, we provide evidence that the high affinity TCR-pMHC interactions can cause an additional level of functional defect in HIV-specific CD8 T cells. Also, it is strongly suggested that such defective CD8 T cells could undermine their control of HIV in vivo.

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Reconstitution of anti-HIV effector functions of primary human CD8 T lymphocytes by transfer of HIV-specific αβ TCR genes

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We redirected the antigen specificity of primary human CD8 T cells by retrovirus-mediated transduction of genes encoding αβ TCR specific to HIV-1 Pol protein. A large polyclonal population of TCR-transduced CD8 T cells showed substantial cytotoxic and cytokine production activities toward target cells either pulsed with the peptide or infected with HIV-1, and their functional activities were comparable to those of the parental CTL clone. Peptide fine-specificity and promiscuous recognition of HLA class I supertypes of the parental CTL clone were also preserved in the TCR-transduced cells. There were no signs of allogeneic responses in these cells, although hybrid TCR dimers consisting of transduced TCR and endogenous TCR were suspected to have been formed in these cells, as the effect of transgene expression on the surface expression of the desired TCR was limited. Moreover, the TCR-transduced cells showed potent inhibitory activity against HIV-1 replication *in vitro*, although the differential surface expression of the desired TCR resulted in differential functional avidity of individual TCR-transduced cells toward the peptide-pulsed target cells. These data suggest that the reconstitution of HIV-specific immunoreactive T cells engineered by genetic transfer of HIV-specific TCR is a potential alternative to immunotherapeutic applications against HIV infections.

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1 Introduction

The transfer of immunoreactive cells into patients, termed adoptive immunotherapy, for the control of viral infections as well as for the treatment of some tumors is an area of considerable interest both in basic research and clinical practice. Adoptive immunotherapy with $CD8^+$ cytotoxic T lymphocytes (CTL) had direct clinical impact on the management of patients at risk for cytomegalovirus and Epstein-Barr virus diseases [1–5]. However, the transfer of HIV-specific CTL lines and clones into HIV-infected patients, although safe, has so far produced only modest results [6–9], despite a line of evidence demonstrating that HIV-specific CTL have an important role in the immunopathogenesis of HIV infection [10].

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Abbreviations: GFP: Green fluorescent protein **IRES:** Internal ribosome entry site **MSCV:** Murine stem cell virus **MFI:** Mean fluorescence intensity

Such poor responses of autologous HIV-specific CTL in patients may be explained by recent findings showing that HIV-specific CTL taken *ex vivo* can have functional defects due to their impaired maturation in the periphery [10–13]. For example, whereas most HIV-specific CD8 T cells in patients with chronic HIV disease produced antiviral cytokines on contact with cognate antigen, these cells showed diminished perforin expression and capacity for proliferation compared with cytomegalovirus-specific T cells [14] and T cells in long-term nonprogressors [15], respectively. Moreover, the evidence that structured treatment interruption-mediated control of viral replication was associated with expansion of virus-specific CD8 T cells with a fully differentiated effector phenotype in patients with treated acute HIV-1 infection [16] strongly supports the importance of functionally mature CD8 T cells in the control of HIV-1 replication.

Redirecting the antigen specificity of T lymphocytes by the transfer of antigen-specific T cell receptor (TCR) genes to T lymphocytes has recently been described as

a potential method of generating large numbers of tumor antigen-reactive T lymphocytes with appropriate effector phenotypes [17, 18]. Using this approach, various investigators demonstrated that retroviral transfer of high-avidity TCR into T lymphocytes is accompanied by the maintenance of the parental T cell avidity and that TCR-transferred T cells preserved peptide fine-specificity in comparison to the parental CTL clone [19, 20]. Furthermore, in a murine model, T cells retrovirally transduced with gene-encoding TCR could expand *in vivo* upon antigen challenge, efficiently home into effector sites, and mediate the rejection of antigen-expressing tumors [21–24].

Although all of these studies were done using TCR specific for tumor antigens, adoptive transfer of TCR-transduced T cells into patients for the control of HIV infections could also have considerable potential. In this regard, it was reported that T cells transduced with the gene encoding a TCR specific for the p17 Gag peptide presented by HLA-A3 into T cells showed cytolytic activity toward both target cells pulsed with the epitope peptide and those infected with HIV-1 [25]. However, such cytolytic activity was observed only after cloning the transduced T cells having the highest surface expression of the transduced TCR [25]. In addition, analysis of the anti-HIV effector function of TCR-transduced T cells, including their cytokine secretion activity and antiviral replication capacity, has not yet been performed.

In the present study, we redirected the antigen specificity of primary human CD8 T cells by sequential transduction of genes encoding αβ TCR specific for an HIV-1 Pol peptide (IPLTEEAEL) using a retrovirus vector system that allows highly sustained transgene expression in the transduced T cells. A large polyclonal population of TCR-transduced CD8 T cells showed peptide fine-specificity and promiscuous recognition of HLA class I supertypes as well as cytotoxic and cytokine production activities that were all comparable to those of the parental CTL clone. Moreover, although the single-cell-sorted TCR-transduced CD8 T cells had a distributed pattern of functional avidity toward the peptide-pulsed target cells, all of the TCR-transduced T cells tested showed comparably potent inhibitory activity toward HIV-1 replication *in vitro*.

2 Results and discussion

2.1 Creation of functional TCR retroviral vectors

CTL clone 589, specific for an HIV Pol peptide ($Pol_{448-456}$; IPLTEEAEL) presented by HLA-B*3501, was previously found to express one β (Vβ5.6) and two in-frame α (Vα10 and Vα12.1) transcripts of TCR [26]. In addition, the transfer of individual α and β chains of TCR to TCR-negative mouse T cell hybridoma cells by use of a Moloney murine leukemia virus (MLV)-based retroviral vector construct pMX [27] revealed that the complex of Vα12.1 TCR and Vβ5.6 was exclusively expressed on the cell surface and solely responsible for the peptide-specific responses [26]. In the present study, both α and β TCR genes of TCR 589 (Vα12.1/Vβ5.6) were cloned into a pGC-based retrovirus vector with the gene encoding mouse CD24 or green fluorescent protein (GFP) downstream of internal ribosome entry site (IRES) for bicistronic expression of α or β TCR genes, respectively (Fig. 1A), in order to facilitate monitoring the expression levels of transduced TCR genes without the need for anti-TCR antibodies. We used a retroviral vector, designated pGCsap(MSCV) [28], in which the 3′-long-terminal repeat (LTR) of pGCsap had been replaced with that of murine stem cell virus (MSCV), allowing sustained expression of the transgene in the transduced T cells [28].

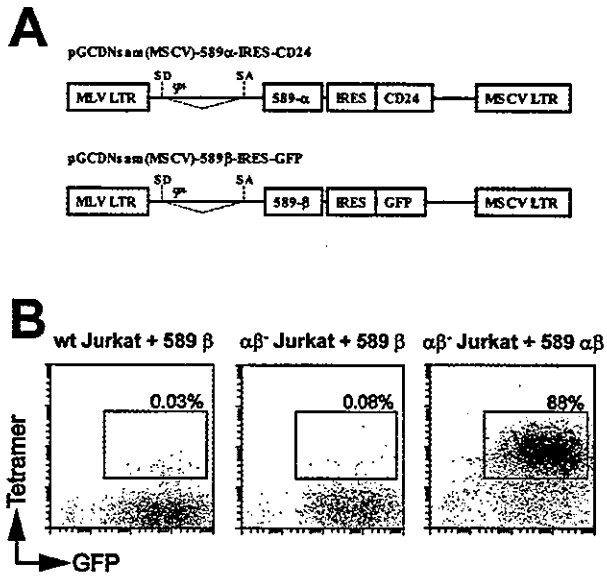

Fig. 1. Creation of functional retroviral vectors for TCR transduction. (A) Linear representation of retroviral vector constructs for transduction of αβ TCR 589 genes. SD and SA, splicing donor and acceptor, respectively; Ψ^+, packaging signal. (B) Wild-type (wt) and TCR-negative variant ($\alpha\beta^-$) of Jurkat cells were transduced with TCR 589 β-IRES-GFP alone, stained with HLA tetramer, and analyzed by flow cytometry. Other $\alpha\beta^-$ Jurkat cells that had been transduced with TCR 589 α-IRES-CD24 were further transduced with TCR 589 β-IRES-GFP and analyzed as above. GFP^+ $tetramer^+$ fractions were gated, and their frequencies within the total cell population are shown. The transduction efficiency for Jurkat cells was >90% as measured by the frequency of the GFP^+ fraction.