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Patterns of Cytokine Production in Human Immunodeficiency Virus Type 1 (HIV-1)-Specific Human CD8$^+$ T Cells after Stimulation with HIV-1-Infected CD4$^+$ T Cells

Mamoru Fujiwara,[1] Hiroshi Takata,[1] Shinichi Oka,[2] Hiroko Tomiyama,[1] and Masafumi Takiguchi[1]*

Division of Viral Immunology, Center for AIDS Research, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811,[1] and AIDS Clinical Center, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655,[2] Japan

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Although human immunodeficiency virus type 1 (HIV-1)-specific CD8$^+$ T cells can produce various cytokines that suppress HIV-1 replication or modulate anti-HIV-1 immunity, the extent to which HIV-1-specific CD8$^+$ T cells produce cytokines when they recognize HIV-1-infected CD4$^+$ T cells in vivo still remains unclear. We first analyzed the abilities of 10 cytotoxic T-lymphocyte (CTL) clones specific for three HIV-1 epitopes to produce gamma interferon, macrophage inflammatory protein 1β, and tumor necrosis factor alpha after stimulation with epitope peptide-pulsed cells. These CTL clones produced these cytokines in various combinations within the same specificity and among the different specificities, suggesting a functional heterogeneity of HIV-1-specific effector CD8$^+$ T cells in cytokine production. In contrast, the HIV-1-specific CTL clones for the most part produced a single cytokine, without heterogeneity of cytokine production among the clones, after stimulation with HIV-1-infected CD4$^+$ T cells. The loss of heterogeneity in cytokine production may be explained by low surface expression of HLA class I–epitope peptide complexes. Freshly isolated HIV-1-specific CD8$^+$ T cells with an effector/memory or memory phenotype produced much more of the cytokines than the same epitope-specific CTL clones when stimulated with HIV-1-infected CD4$^+$ T cells. Cytokine production from HIV-1-specific memory/effector and memory CD8$^+$ T cells might be a critical event in the eradication of HIV-1 in HIV-1-infected individuals.

Memory and effector CD8$^+$ T cells play an important role in viral eradication through their ability to produce cytokines involved in the suppression of viral replication (6, 10, 15, 26) as well as perforin and granzymes A and B, which are involved in the cytolysis of virus-infected cells (16, 24). The cytokines produced by these cells include gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), and chemokines such as RANTES and macrophage inflammatory protein 1β (MIP-1β). IFN-γ increases the surface expression of HLA molecules and can activate macrophages that predominantly synthesize MIP-1β (8, 25). TNF-α induces apoptosis of human immunodeficiency virus type 1 (HIV-1)-infected cells (17). It has been shown that MIP-1β and RANTES can suppress HIV-1 replication in vitro by inhibiting the entry of HIV-1 via CCR5, while IFN-γ induces cellular proteins which suppress viral replication (2, 6).

A previous study showed that HLA-A2-restricted, HCMVpp65$_{495\text{-}503}$-specific CD8$^+$ T cells expressed various combinations of three cytokines, IFN-γ, TNF-α, and interleukin 2 (IL-2), after peripheral blood mononuclear cells (PBMC) containing these cells from three individuals had been stimulated with HCMVpp65$_{495\text{-}503}$ peptide, suggesting that HCMVpp65$_{495\text{-}503}$-specific CD8$^+$ T cells possess functionally heterogeneous cytokine production (18). This functional heterogeneity may be due to the heterogeneous populations in HCMVpp65$_{495\text{-}503}$-specific CD8$^+$ T cells. Indeed, these cells for the most part are CD8$^+$ T-cell populations with a memory/effector or effector phenotype (9, 23). Additional studies have revealed the heterogeneity of the production of cytokines and cytolytic effector molecules in human CD8$^+$ T cells (21, 23).

It is well known that virus-specific CD8$^+$ T cells can produce cytokines when they recognize virus-infected cells (4, 13, 14). However, virus-specific CD8$^+$ T cells stimulated with virus-infected cells may not produce cytokines more effectively than those stimulated with cells pulsed with an epitope peptide, because the expression of HLA class I–viral epitope peptide complexes is much lower on virus-infected cells than on peptide-pulsed cells. In particular, HIV-1-specific CD8$^+$ T cells may not produce cytokines effectively when they recognize HIV-1-infected cells, because HLA class I molecules have been actively down-regulated, mostly, although not exclusively, by the Nef protein produced by HIV-1-infected cells (4, 7, 20). An analysis of the cytokine production from HIV-1-specific CD8$^+$ T cells stimulated with HIV-1-infected CD4$^+$ T cells is needed to elucidate whether HIV-1-specific CD8$^+$ T cells can recognize HIV-1-infected CD4$^+$ T cells in vivo and to allow the formulation of testable hypotheses on the role of the cytokines from HIV-1-specific CD8$^+$ T cells in the suppression of HIV-1 replication in vivo.

In the present study, we investigated the production patterns of three cytokines, IFN-γ, TNF-α, and MIP-1β, in HIV-1-specific effector CD8$^+$ T-cell clones of the same or different specificities. In addition, we investigated the production of the three cytokines in these cytotoxic T-lymphocyte (CTL) clones and freshly isolated HIV-1-specific CD8$^+$ T cells exposed to

* Corresponding author. Mailing address: Division of Viral Immunology, Center for AIDS Research, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811, Japan. Phone: 81-96-373-6529. Fax: 81-96-373-6532. E-mail: masafumi@kaiju.medic.kumamoto-u.ac.jp.

HIV-1-infected $CD4^+$ T cells whose HLA class I molecules are down-regulated by HIV-1 Nef. The present study elucidates the cytokine production profile of HIV-1-specific $CD8^+$ T cells in response to HIV-1-infected $CD4^+$ T cells.

MATERIALS AND METHODS

CTL clones. Peptide-specific CTL clones were generated from an established peptide-specific bulk CTL culture by seeding 0.8 cell/well into U-bottom 96-well microtiter plates (Nunc, Roskilde, Denmark) together with 200 μl cloning mixture (RPMI 1640 medium supplemented with 10% fetal calf serum [FCS] and 200 U/ml recombinant human IL-2, 5×10^5 irradiated allogeneic PBMC from a healthy donor, and 1×10^5 irradiated C1R-A*2402, C1R-A*3303, or C1R-B*3501 cells prepulsed with 10^{-6} M of the corresponding peptide, Env77-85 [DPNPQEVVL] [19], Gag28-36 [KYKLKHIVW] [12], or Env830-837 [EVAQ RAYR] [11]). Wells positive for growth after about 2 weeks were transferred to 48-well plates together with 1 ml of the cloning mixture. The clones were examined for CTL activity by a standard ^{51}Cr release assay. One Env830-837-specific, HLA-A*3303-restricted CTL clone (clone 1) had been generated previously (11). All CTL clones were cultured in RPMI 1640–10% FCS supplemented with 200 U of recombinant human IL-2/ml and were stimulated weekly with irradiated target cells prepulsed with the appropriate HIV-1 derived peptide.

Antibodies. The peridinin chlorophy protein-conjugated anti-human CD8 monoclonal antibody (MAb) and the ECD-conjugated anti-human CD45RA MAb were purchased from BD Biosciences (San Jose, CA) and the Immunotech Coulter Company (Marseille, France), respectively. Fluorescein isothiocyanate (FITC)-conjugated anti-human CD27, phycoerythrin (PE)-conjugated anti-human IFN-γ, ECD-conjugated anti-human CD28, allophycocyanin (APC)-conjugated anti-human TNF-α, APC-conjugated anti-human IFN-γ, PE Cy7-conjugated anti-human TNF-α, and APC Cy7-conjugated anti-human CD27 MAbs were purchased from PharMingen (San Diego, CA). PE-conjugated anti-human CD28, APC-conjugated anti-human CD8, and FITC-conjugated anti-human MIP-1β MAbs were purchased from DAKO (Glostrup, Denmark). FITC-conjugated anti-HIV-1 p24 MAb KC-57 was purchased from Beckman Coulter (Miami, Fla.). A Cascade Blue-conjugated anti-human CD8 MAb was made by conjugating Cascade Blue (Molecular Probes, Eugene, OR) with the anti-CD8 MAb OKT8.

HLA-peptide tetrameric complexes. HLA class I–peptide tetrameric complexes were synthesized as previously described (3). Briefly, recombinant HLA class I proteins (HLA-A*2402, HLA-A*3303, and HLA-B*3501) and human β_2 microglobulin (β_2m) were produced in *Escherichia coli* cells transformed with the relevant expression plasmids. The heavy chain was modified by deletion of the transmembrane cytosolic tail and COOH-terminal addition of a sequence containing the BirA biotinylation site. Gag28-36, Env830-837, and Env77-85 peptides were used for refolding of the HLA-A*2402, HLA-A*3303, and HLA-B*3501 molecules, respectively. The HLA class I–peptide complexes were refolded in vitro. The 45-kDa complexes were isolated using gel filtration on a Superdex G75 column (Amersham Pharmacia Biotech UK, Ltd., Buckinghamshire, England). Purified complexes were biotinylated with the BirA enzyme (Avidity, Denver, CO). The biotinylated complexes were purified by using gel filtration first on a Superdex G75 column and then on a MonoQ column (Amersham Pharmacia Biotech UK, Ltd, Buckinghamshire, England). HLA class I–peptide tetrameric complexes (tetramers) were mixed with PE-labeled streptavidin (extravidin-PE; Sigma-Aldrich, St. Louis, MO) at a molar ratio of 4:1.

Identification of HIV-1-specific CTLs by flow cytometry. A total of 0.2×10^6 to 1×10^6 cultured cells were mixed with the tetramers at concentrations of 0.02 to 0.04 mg/ml. After incubation at 37°C for 30 min, the cells were washed twice with RPMI 1640–10% FCS, and then an anti-CD8 MAb was added to the cell suspension. The cells were incubated at 4°C for 30 min, and then the cells were washed twice with phosphate-buffered saline (PBS)–10% FCS. The cells were analyzed using a FACSCalibur with CellQuest software (Becton Dickinson, San Jose, CA). The percentage of tetramer-positive cells was measured in CD8-positive cells.

Phenotype analysis of the CTL clones. Cells were stained for 30 min at 4°C using an FITC-conjugated anti-human CD27 MAb, a PE-conjugated anti-human CD28 MAb, an ECD-conjugated anti-human CD45RA MAb, and an APC-conjugated anti-human CD8 MAb; then they were washed twice in PBS supplemented with 10% NCS. The CD27 CD28 CD45RA phenotype of $CD8^+$ cells was analyzed using FACSCalibur.

Cell surface and intracellular cytokine staining. Specific CTL clones were stimulated with stimulator cells prepulsed with the appropriate HIV-1-derived peptide at each concentration at an effector-to-stimulator ratio of 1:1. Stimulator cells were washed in RPMI 1640–10% FCS before use. Cells were incubated for 6 h at 37°C in 5% CO_2. Brefeldin A (Sigma-Aldrich) at a concentration of 10 μg/ml was added 2 h after stimulation. After a 6-h incubation, the cells were washed in PBS supplemented with 20% NCS. Cell surface staining was performed for 30 min at 4°C using a PerCP-conjugated anti-human CD8 MAb; then cells were washed twice in PBS supplemented with 10% NCS. Freshly isolated $CD8^+$ cells from HIV-1-infected individuals were stained with tetramers after a 6-h incubation, followed by staining with ECD-conjugated anti-human CD28, APC Cy7-conjugated anti-human CD27, and Cascade Blue-conjugated anti-human CD8 MAbs.

After a wash, the cells were fixed with 4% paraformaldehyde for 20 min at 4°C and permeabilized with PBS supplemented with 0.1% saponin containing 20% NCS for 10 min at 4°C. The cells were resuspended in permeabilizing buffer and then were stained with a PE-conjugated anti-human IFN-γ MAb or an APC-conjugated anti-human TNF-α MAb at room temperature for 20 min or with an FITC-conjugated anti-human MIP-1β MAb for 30 min at 4°C. When the cells were stained with all three MAbs, they were first stained with the PE-conjugated anti-human IFN-γ MAb and the APC-conjugated anti-human TNF-α MAb at room temperature for 20 min and then with the FITC-conjugated anti-human MIP-1β MAb for 30 min at 4°C. Freshly isolated $CD8^+$ cells were stained with an APC-conjugated anti-human IFN-γ MAb or a PE Cy7-conjugated anti-human TNF-α MAb at room temperature for 20 min or with an FITC-conjugated anti-human MIP-1β MAb for 30 min at 4°C.

Finally, the cells were washed three times with permeabilizing buffer and were resuspended in 2% paraformaldehyde. The percentages of intracellular IFN-γ-, MIP-1β-, and TNF-α-positive cells among tetramer-positive $CD8^+$ cells were analyzed using FACS Aria (Becton Dickinson, San Jose, CA).

Infection of $CD4^+$ T cells with HIV-1. Cultured $CD4^+$ T cells (purity, >98%) were incubated with an HIV-1 clone, NL-432 (1), or with the chimeric virus NL-432gag^{HXB2} for 4 h at 37°C with intermittent agitation. The cells were then washed once and cultured in RPMI 1640–10% FCS medium supplemented with recombinant human IL-2 (200 U/ml). On the following 2 to 7 days, the cells were harvested to determine the percentage of HIV-1-infected cells by measuring p24 antigen-positive cells using FACSCalibur. When HIV-1 p24-positive cells reached more than 40% of the cultured cells, they were used as stimulator cells.

CTL assay. Cytotoxicity was measured by a standard ^{51}Cr release assay as previously described (19). Target cells (2×10^5) were incubated for 60 min with 100 μCi of $Na_2{}^{51}CrO_4$ in saline and washed three times with RPMI 1640 medium containing 10% NCS. Labeled target cells (2×10^3/well) were added into a 96-well round-bottom microtiter plate (Nunc) with the indicated amount of the corresponding peptide. After a 1-h incubation, effector cells were added and the mixtures were incubated for 4 h at 37°C. The supernatants were collected and analyzed with a gamma counter. The spontaneous ^{51}Cr release was determined by measuring the cpm in the supernatant in the wells containing only target cells (cpm spn). Maximum release (cpm max) was determined by measuring the release of ^{51}Cr from the target cells in the presence of 2.5% Triton X-100. Specific lysis was defined as (cpm exp − cpm spn)/(cpm max − cpm spn) × 100, where "cpm exp" is the counts per minute in the supernatant in the wells containing both the target and effector cells. The activities of the CTL clones on target cells pulsed with peptide were tested at an effector-to-target (E:T) ratio of 2:1.

CTL assay for target cells infected with recombinant vaccinia virus. Recombinant vaccinia virus containing the *env* or the *gag/pol* gene of HIV-1 SF2 was generated as described previously (19). Target cells (C1R-A*2402, C1R-A*3303, and C1R-B*3501 cells) were cultured with 10 PFU of recombinant or wild-type vaccinia virus per target cell overnight. These infected cells (2×10^5) were incubated for 60 min with 100 μCi of $Na_2{}^{51}CrO_4$ in saline and then washed three times with RPMI 1640 medium containing 10% NCS. Effector cells were added to the labeled target cells (5×10^3/well), and the mixtures were incubated for 4 h at 37°C. The activities of the CTL clones on target cells infected with recombinant vaccinia virus expressing *env* proteins were tested at an E:T ratio of 2:1.

RESULTS

Production of three cytokines in HIV-1-specific CTL clones. Three SF2-Env77-85-specific, HLA-B*3501-restricted CTL clones, three SF2-Gag28-36-specific, HLA-A*2402-restricted CTL clones, and a further four SF2-Env830-837-specific, HLA-A*3303-restricted CTL clones were established from HIV-1-infected individuals. These CTL clones exhibited specific cytolytic activity in both target cells (C1R-A*2402, C1R-A*3303, or

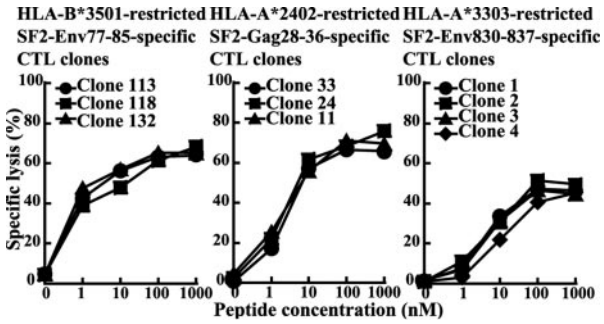

FIG. 1. Cytolytic activities of SF2-Env77-85-specific, SF2-Gag28-36-specific, and SF2-Env830-837-specific CTL clones. Cytolytic activities of three SF2-Env77-85-specific, HLA-B*3501-restricted CTL clones (clones 113, 118, and 132), three SF2-Gag28-36-specific, HLA-A*2402-restricted CTL clones (clones 33, 24, and 11), and four SF2-Env830-837-specific, HLA-A*3303-restricted CTL clones (clones 1, 2, 3, and 4) were tested for C1R-B*3501, C1R-A*2402, and C1R-A*3303 cells pulsed with the corresponding peptides (1 to 1,000 nM), respectively. They were tested at an E:T ratio of 2:1.

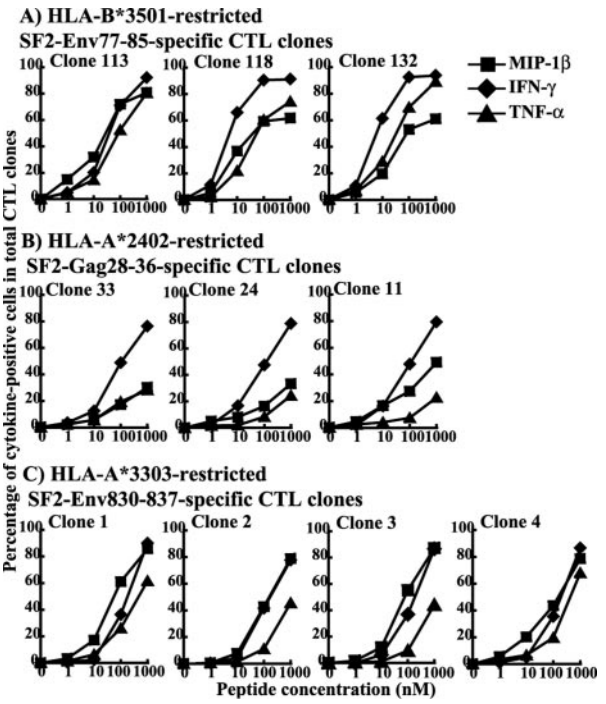

FIG. 2. Intracellular expression of cytokines in SF2-Env77-85-specific, SF2-Gag28-36-specific, and SF2-Env830-837-specific CTL clones responding to epitope peptide-pulsed cells. The 10 CTL clones were incubated for 6 h in the presence of C1R-B*3501, C1R-A*2402, or C1R-A*3303 cells pulsed with specific peptide. Brefeldin A was added 2 h after stimulation, and intracellular staining for IFN-γ, MIP-1β, and TNF-α was carried out using MAbs specific for each cytokine. Cytokine-producing cells were analyzed by flow cytometry. The frequency of each set of cytokine-producing $CD8^+$ cells was measured as the number of cytokine-producing $CD8^+$ cells per total $CD8^+$ cells.

C1R-B*3501 cells) prepulsed with a peptide epitope (Fig. 1) and target cells (C1R-A*2402, C1R-A*3303, or C1R-B*3501 cells) infected with HIV-1 recombinant vaccinia virus (data not shown). The results show no significant difference in cytotoxic activity between CTL clones specific for the same epitopes. We investigated the production of three cytokines, MIP-1β, IFN-γ, and TNF-α, from these HIV-1-specific CTL clones. Intracellular cytokine production by the clones was measured at 6 h after stimulation with the C1R transfectants prepulsed with the epitope peptide. Almost 100% of the cells produced at least one cytokine in all three of the SF2-Env77-85-specific CTL clones, all four of the SF2-Env830-837-specific CTL clones, and all three of the SF2-Gag28-36-specific CTL clones (Fig. 2). Different cytokine production patterns were found among three SF2-Env77-85-specific CTL clones though all of the clones produced IFN-γ more extensively than MIP-1β or TNF-α (Fig. 2A). Different cytokine production patterns were also found for the three SF2-Gag28-36-specific CTL clones (Fig. 2B) and the four SF2-Env830-837-specific CTL clones (Fig. 2C). Clone 11 exhibited much higher production of MIP-1β than of IFN-γ and TNF-α compared with clones 33 and 24. In addition, clones 2 and 3 exhibited much lower production of TNF-α than of IFN-γ and MIP-1β, while the difference in production between these cytokines was at a minimum for clones 1 and 4. These results were confirmed by performing the experiments twice. Thus, the results reveal heterogeneity in the production of these cytokines between CTL clones of the same as well as different specificities. To exclude contamination by T cells that are not epitope-specific CTLs, we stained the CTL clones with an epitope-specific HLA class I tetramer. More than 97% of the cells in nine CTL clones bound the epitope-specific tetramer, while only 92% of the cells bound to the specific tetramer in one (clone 33) of the SF2-Gag28-36-specific CTL clones (Table 1). However, this difference does not seem to be significant for the functions, since the three SF2-Gag28-36-specific CTL clones exhibited the same cytotoxic activity and IFN-γ production.

Since the T-cell clones used in the present study possess cytolytic activity (Fig. 1), they are thought to be mature effector $CD8^+$ T cells. To analyze the relation between the maturation stage of the CTL clone and cytokine production, we stained these CTL clones with anti-CD27, anti-CD28, and anti-CD45RA MAbs. Nine CTL clones were mostly of the $CD27^-$ $CD28^-$ $CD45RA^-$ type, while one (clone 2) of the SF2-Env830-837-specific CTL clones exhibited both $CD27^-$ $CD28^-$ $CD45RA^-$ and $CD27^+$ $CD28^-$ $CD45RA^-$ phenotypes (Table 1). Clone 2, stimulated with SF2-Env830-837 peptide, exhibited a much smaller number of TNF-α-producing cells than of IFN-γ-producing or MIP-1β-producing cells, but this is not a characteristic restricted to clone 2.

Coproduction of three cytokines by HIV-specific CTL clones stimulated with peptide-pulsed cells. The heterogeneity of the CTL clones shown in Fig. 2 suggests that the CTL clones are actually composed of cell populations which produce different combinations of the cytokines. To clarify this, we investigated the coproduction of these three cytokines in the CTL clones by simultaneously staining multiple intracellular cytokines with anti-IFN-γ, anti-MIP-1β, and anti-TNF-α MAbs. The results of the multiple staining of the three SF2-Env77-85-specific CTL clones are shown in Fig. 3A. The SF2-Env77-85-specific CTL clones stimulated with 1,000 nM of specific peptide revealed different patterns of cytokine production. Clone 113 included a high number of cells producing all three of the cytokines (IFN-γ^+ TNF-α^+ MIP-$1\beta^+$) (72.0%). In contrast,

TABLE 1. CD27 and CD28 expression in HIV-1-specific CTL clones

CTL clone	% Tetramer-positive cells	% of total $CD8^+$ $CD45RA^-$ cells with the following phenotype:			
		$CD27^+$ $CD28^+$	$CD27^+$ $CD28^-$	$CD27^-$ $CD28^-$	$CD27^-$ $CD28^+$
Env77-85					
Clone 113	98.2	0.8	1.2	84.8	13.2
Clone 118	99.7	0.2	13.6	86.0	0.2
Clone 132	95.1	0.3	6.3	91.5	1.9
Gag28-36					
Clone 33	91.7	0.0	1.5	97.8	0.7
Clone 24	97.0	0.0	17.2	81.6	1.2
Clone 11	96.0	0.0	19.2	80.7	0.1
Env830-837					
Clone 1	99.5	0.0	13.0	86.0	1.0
Clone 2	99.7	0.0	46.1	53.9	0.0
Clone 3	99.7	0.1	8.9	89.8	1.2
Clone 4	99.2	0.0	0.2	99.8	0.0

clone 118 included IFN-γ^+ TNF-α^+ MIP-1β^+ cells (58.1%) and IFN-γ^+ TNF-α^+ cells (30.6%), while clone 132 included IFN-γ^+ TNF-α^+ MIP-1β^+ cells (54.7%), IFN-γ^+ TNF-α^+ cells (17.8%), and IFN-γ^+ cells (13.1%). This difference between these CTL clones became even more apparent when the CTL clones were stimulated with 10 nM of specific peptide. Clone 113 included MIP-1β^+ cells (16.1%) and IFN-γ^+ MIP-1β^+ cells (7.3%). In contrast, clone 118 included IFN-γ^+ cells (29.1%) and IFN-γ^+ TNF-α^+ cells (17.0%), while clone 132 included IFN-γ^+ cells (27.5%) and IFN-γ^+ MIP-1β^+ cells (20.6%). This difference was consistent over two different experiments performed on different days.

A similar analysis was performed on the four SF2-Env830-837-specific and the three SF2-Gag28-36-specific CTL clones. The seven CTL clones were stimulated with 1,000 nM of specific peptide (Fig. 3B and C). Among SF2-Gag28-36-specific CTL clones, clones 33 and 24 showed similar patterns of cytokine production. They included IFN-γ^+ TNF-α^+ MIP-1β^+ cells (12 to 14%), IFN-γ^+ TNF-α^+ cells (11 to 13%), and IFN-γ^+ MIP-1β^+ cells (14 to 18%). In contrast, clone 11 included IFN-γ^+ TNF-α^+ cells (5%) and IFN-γ^+ MIP-1β^+ cells (34%). The difference became more complex when the clones were stimulated with a lower concentration (100 nM) of the peptide. The major populations of cytokine-producing cells were as follows: for clone 11, IFN-γ^+ cells (24.5%), IFN-γ^+ MIP-1β^+cells (15.4%), and MIP-1β^+cells (7.7%); for clone 33, IFN-γ^+ cells (28.0%), IFN-γ^+ MIP-1β^+cells (9.9%), and IFN-γ^+ TNF-α^+ cells (8.0%); and for clone 24, IFN-γ^+ cells (27.2%), IFN-γ^+ MIP-1β^+cells (9.1%), and IFN-γ^+ TNF-α^+ cells (7.6%). Among SF2-Env830-837-specific CTL clones, clones 1 and 4 showed similar patterns of cytokine production. These included IFN-γ^+ TNF-α^+ MIP-1β^+ cells (50 to 60%), IFN-γ^+ TNF-α^+ cells (13 to 17%), and IFN-γ^+ MIP-1β^+ cells (12 to 14%). In contrast, clones 2 and 3 included IFN-γ^+ TNF-α^+ MIP-1β^+ cells (40 to 42%) and IFN-γ^+ MIP-1β^+ cells (35 to 37%). The difference became more complex when the clones were stimulated with a lower concentration (100 nM) of the peptide. At this concentration the major populations of cytokine-producing cells were as follows: for clone 1, MIP-1β^+ cells (17.6%) and IFN-γ^+ MIP-1β^+cells (10.9%); for clone 2, IFN-γ^+ MIP-1β^+ cells (19.1%), IFN-γ^+ cells (15.7%), and MIP-1β^+ cells (12.4%); for clone 3, MIP-1β^+ cells (20.8%), IFN-γ^+ MIP-1β^+ cells (20.6%), and IFN-γ^+ cells (10.9%); and for clone 4, IFN-γ^+ cells (15.1%), IFN-γ^+ MIP-1β^+ cells (12.0%), and IFN-γ^+ TNF-α^+ MIP-1β^+ cells (10.1%). These differences were also confirmed by two different experiments performed on different days.

Cells expressing all three cytokines were most frequently found in the CTL clones stimulated with the stimulator cells prepulsed with 1,000 nM peptide, while cells expressing either two or all three of the cytokines were not found in the CTL clones stimulated with the stimulator cells prepulsed with 1 nM peptide (Fig. 4). The CTL clones stimulated with stimulator cells prepulsed with 1 nM peptide predominantly produced a single cytokine. These results indicate that CTL clones produce multiple cytokines when they are stimulated with cells prepulsed with a high concentration of an HLA–epitope peptide complex but produce a single cytokine when they are stimulated with cells prepulsed with a low concentration. This implies that HIV-1-specific CTL clones produce a single cytokine when they are stimulated with HIV-1-infected cells because a small number of HIV-1 CTL epitope peptides is presented in HIV-1-infected cells.

Coproduction of three cytokines by HIV-1-specific CTL clones stimulated with HIV-1-infected $CD4^+$ T cells. Nef-mediated down-regulation of HLA class I critically affects the ability of HIV-1-specific CTLs to kill HIV-1-infected cells; this could be the result of a lowered frequency of cytokine-producing cells after stimulation with HIV-1-infected $CD4^+$ T cells. Indeed, our previous study showed that the lower frequency of HIV-1-specific CTL clones produced fewer cytokines by stimulation with Nef^+ HIV-1-infected $CD4^+$ T cells than by stimulation with Nef^- HIV-1-infected $CD4^+$ T cells (22). We investigated the coproduction of the three cytokines by HIV-1-specific CTL clones after stimulation with Nef^+ HIV-1-infected $CD4^+$T cells. Approximately 7 to 9% of the cells in five clones produced cytokines after stimulation with Nef^+ HIV-1-infected $CD4^+$ T cells (Fig. 5). Most of these cytokine-secreting cells produced only a single cytokine but were able to produce any one of the three. Thus, the results indicate that after stimulation with HIV-1-infected $CD4^+$T cells, HIV-1-specific CTL clones can produce various kinds of

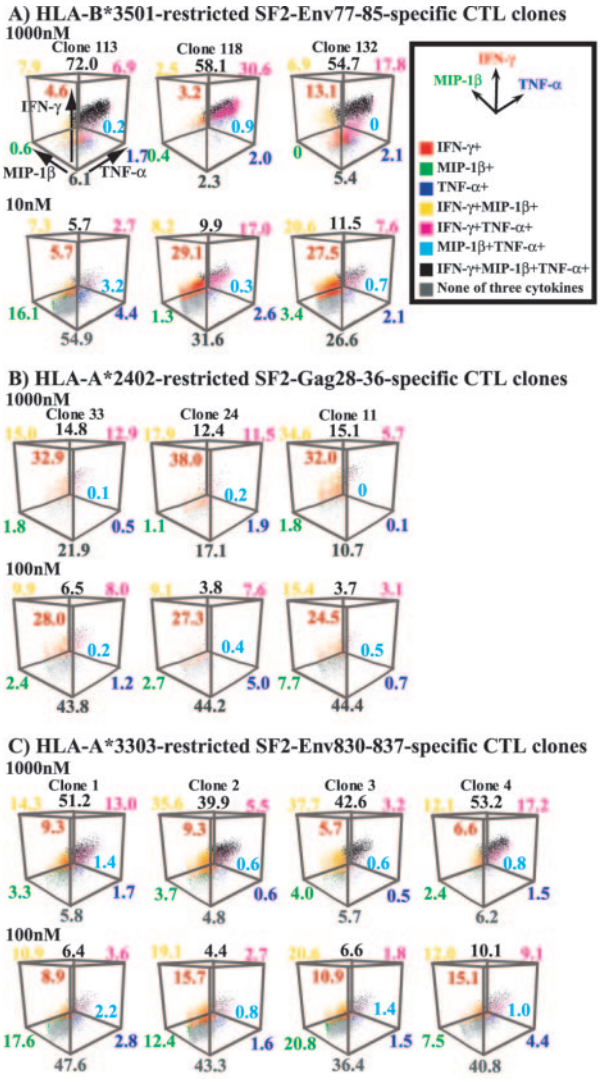

FIG. 3. Coexpression of three cytokines in HIV-1-specific CTL clones responding to epitope peptide-pulsed cells. (A) Env77-85-specific CTL clones. Intracellular cytokine production of three SF2-Env77-85-specific CTL clones was measured 6 h after stimulation with C1R-B*3501 cells prepulsed with the corresponding peptides (1,000 nM and 10 nM). Intracellular staining for the three cytokines IFN-γ, MIP-1β, and TNF-α was simultaneously carried out using three MAbs specific for these cytokines, and then the expression of these cytokines was analyzed by flow cytometry. (B) Gag28-36-specific CTL clones. Intracellular cytokine production of three SF2-Gag28-36-specific, HLA-A*2402-restricted CTL clones (clones 33, 24, and 11) was tested 6 h after stimulation with C1R-A*2402 cells prepulsed with the corresponding peptides (1,000 nM and 100 nM). (C) Env830-837-specific CTL clones. Intracellular cytokine production of four SF2-Env830-837-specific, HLA-A*3303-restricted CTL clones (clones 1, 2, 3, and 4) was tested 6 h after stimulation with C1R-A*3303 cells prepulsed with the corresponding peptides (1,000 nM and 100 nM). Intracellular staining for the three cytokines IFN-γ, MIP-1β, and TNF-α was simultaneously carried out using the three MAbs specific for these cytokines, and then the expression of these cytokines was analyzed by flow cytometry. Fluorescence-activated cell sorter data were analyzed with Paint-A-Gate^{PRO} (BD Biosciences). The frequencies of cells expressing these cytokines are shown as percentages of the total number of cells. Cells expressing these cytokines are shown in a 3-dimensional presentation as follows: none of the three cytokines (gray), IFN-γ only (red), MIP-1β only (green), TNF-α only (blue), IFN-γ and MIP-1β (yellow), IFN-γ and TNF-α (violet), MIP-1β and TNF-α (cyan), and all three cytokines (black).

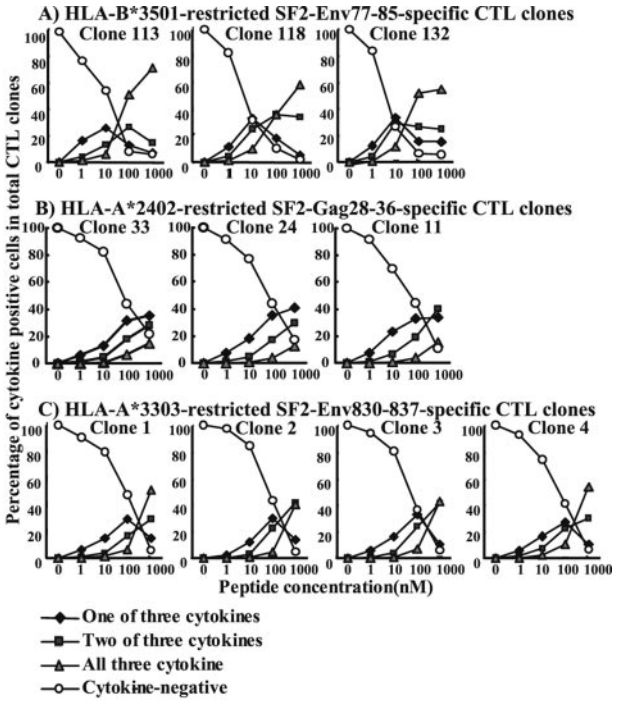

FIG. 4. Kinetics of multiple cytokine expression in the HIV-1-specific CTL clones corresponding to epitope peptide stimulation. The frequency of CTL clones expressing a single cytokine, two cytokines, or all three cytokines was measured 6 h after stimulation with cells prepulsed with the corresponding peptide at different concentrations as follows: the frequency of cells expressing a single cytokine was the sum of the frequency of cells expressing IFN-γ only, MIP-1β only, and TNF-α only in the total CD8$^+$ cells, while the frequency of cells expressing two cytokines was the sum of cells expressing IFN-γ and MIP-1β, IFN-γ and TNF-α, and MIP-1β and TNF-α in total CD8$^+$ cells.

cytokines but that each CTL clone is able to produce only a single cytokine. Heterogeneity in the production of these cytokines among CTL clones of the same specificity is barely noticeable.

Cytokine production of freshly isolated HIV-1-specific CD8$^+$ T cells stimulated with HIV-1-infected CD4$^+$ T cells. The cytokine production results for HIV-1-specific CTL clones stimulated with HIV-1-infected CD4$^+$ T cells suggest the possibility that when HIV-1-specific CD8$^+$ T cells recognize HIV-1-infected CD4$^+$ T cells in HIV-1-infected individuals, they produce various combinations of cytokines but each of them produces only a single cytokine. To clarify the accuracy of this hypothetical picture, we performed ex vivo analysis of the cytokine production of HIV-1 Gag28-36-specific CD8$^+$ T cells after stimulation with Nef$^+$ HIV-1-infected CD4$^+$ T cells (Fig. 6). We isolated CD8$^+$ T cells from fresh PBMC of two HIV-1-infected individuals (KI-144 and KI-158). Analysis using a Gag28-36-specific HLA-A*2402 tetramer as well as anti-CD27 and anti-CD28 MAbs showed that approximately 0.2 to 0.3% of CD8$^+$ T cells were Gag28-36-specific CD8$^+$ T cells and that the Gag28-36-specific CD8$^+$ T cells predominantly have either a CD27$^+$ CD28$^-$ memory/effector phenotype or a CD27$^+$ CD28$^+$ memory phenotype (Fig. 6). We then stimulated the CD8$^+$ T cells with NL-432-infected CD4$^+$ T cells. Approximately

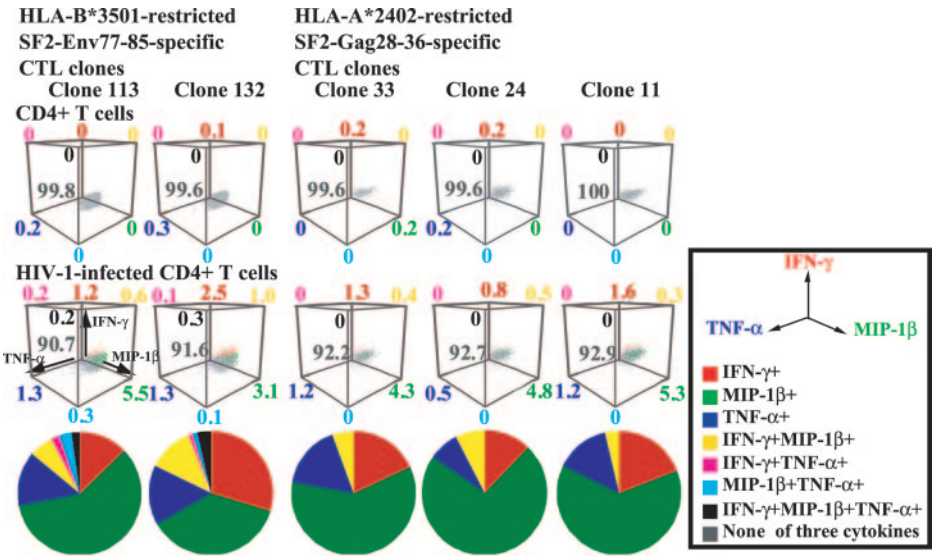

FIG. 5. Coexpression of all three cytokines in HIV-1-specific CTL clones responding to HIV-1-infected $CD4^+$ T cells. Intracellular cytokine production of two SF2-Env77-85-specific CTL clones and three Gag28-36-specific CTL clones was measured 6 h after stimulation with $CD4^+$ T cells infected with the Nef^+ HIV-1 clone NL-432 or NL-432gag^{HXB2}. Intracellular staining for the three cytokines IFN-γ, MIP-1β, and TNF-α was simultaneously carried out using the three MAbs specific for these cytokines, and then the expression of these cytokines was analyzed by flow cytometry. The frequency of cells expressing these cytokines is shown as a percentage of the total number of cells in a 3-dimensional presentation. The frequency of cells expressing these cytokines among total cytokine-producing cells is also shown as a pie chart. Cells expressing these cytokines are shown as follows: none of the three cytokines (gray), IFN-γ only (red), MIP-1β only (green), TNF-α only (blue), IFN-γ and MIP-1β (yellow), IFN-γ and TNF-α (violet), MIP-1β and TNF-α (cyan), and all three cytokines (black).

30 and 10% of Gag28-36-specific $CD8^+$ T cells produced at least one cytokine in PBMC from two different HIV-1-infected individuals (Fig. 6). The frequency of cytokine-producing cells was much higher in freshly isolated Gag28-36-specific $CD8^+$ T cells than in Gag28-36-specific CTL clones. KI-144 cells predominantly produced only MIP-1β or both MIP-1β and IFN-γ, while KI-158 cells predominantly produced only MIP-1β or both MIP-1β and TNF-α. This is in contrast to the finding that Gag28-36-specific CTL clones produced a single cytokine: either MIP-1β, IFN-γ, or TNF-α.

DISCUSSION

In the present study, we employed three kinds of HIV-1-specific CTL clones of the same specificity from the same individual. These CTL clones mostly exhibited an effector phenotype ($CD27^-$ $CD28^-$ $CD45RA^-$) and strong cytolytic activity. The HIV-1-specific CTL clones exhibited functional heterogeneity in the production of three cytokines in clones of the same specificity as well as of different specificities, indicating that HIV-1-specific effector $CD8^+$ T cells, even when of the same specificity, display functional heterogeneity in cytokine production. The mechanism for this heterogeneity of function, however, is still unclear. One possibility is that these CTL clones carry different T-cell receptors (TCR), with the difference in receptor signaling triggering the functional heterogeneity, although it is not understood how different activation of TCR influences cytokine production. The fact that cells expressing different cytokine production patterns do in any event exist in each clone tends to exclude this hypothesis but does support the idea that the cytokine production pattern is determined by various combinations of interacting factors, such as certain specific characteristics of the T cells, including the usage and expression level of TCR, the expression level of accessory molecules, and the relative activation status of the T cells.

Cytokine production by HIV-1-specific CTL clones stimulated with HIV-1-infected $CD4^+$ T cells reflects the response of HIV-1-specific CTLs in vivo much better than cytokine production by such clones stimulated with peptide-pulsed cells. The analysis of HIV-1-specific CTL clones stimulated with HIV-1-infected $CD4^+$ T cells revealed that only 7 to 9% of cells in each CTL clone produce one of the cytokines IFN-γ, TNF-α, and MIP-1β, suggesting limited cytokine production by HIV-1-specific effector $CD8^+$ T cells carrying the $CD27^-$ $CD28^-$ phenotype in vivo. This limited cytokine production may be explained by either or both of two factors: (i) the amount of HLA class I–virus peptide complexes on virus-infected cells is much smaller than that on peptide-pulsed cells; (ii) HLA class I–HIV-1 peptide complexes are down-regulated on the surfaces of HIV-1-infected cells. Previous studies have demonstrated that HIV-1-specific CTL clones may partially suppress HIV-1 replication, although these cells hardly kill HIV-1-infected $CD4^+$ T cells because of the Nef-mediated down-regulation of the HLA-A and -B molecules (20). The partial suppression may be explained by the limited cytokine production of HIV-1-specific CTL clones stimulated with HIV-1-infected $CD4^+$ T cells. Thus, it is hypothesized that cytokines produced by HIV-1-specific $CD8^+$ T cells play an important role in the suppression of HIV-1-replication.

Heterogeneity in cytokine production between CTL clones of the same specificity as well as of different specificities was not found when the CTL clones were stimulated with HIV-1-infected $CD4^+$ T cells. This may be explained by the fact that

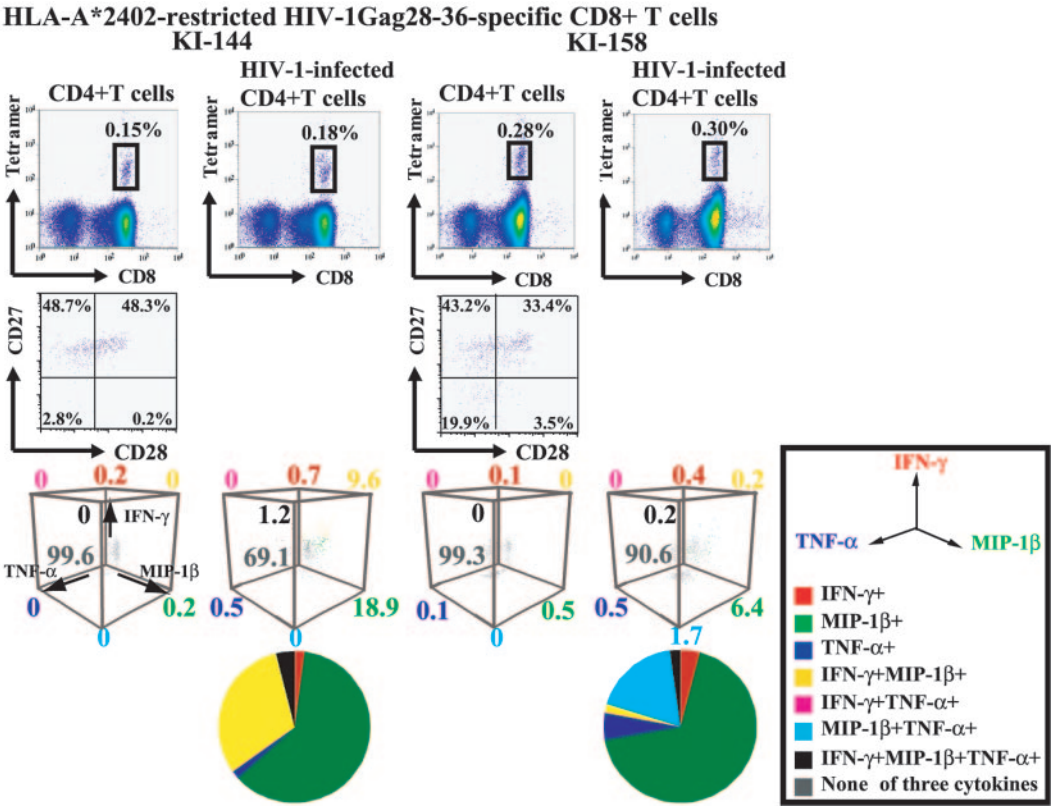

FIG. 6. Coexpression of three cytokines in freshly isolated $HIV_{Gag28-36}$-specific $CD8^+$ T cells responding to HIV-1-infected $CD4^+$ T cells. Intracellular cytokine production of Gag28-36-specific $CD8^+$ T cells was measured 6 h after stimulation with $CD4^+$ T cells infected with the Nef^+ HIV-1 clone NL-432gag^{HXB2}. Intracellular staining for the three cytokines IFN-γ, MIP-1β, and TNF-α was simultaneously carried out using the three MAbs specific for these cytokines, and then the expression of these cytokines was analyzed by flow cytometry. The $CD8^+$ tetramer-positive cells are gated to determine both the frequency of cells expressing these cytokines and the CD27 and CD28 expression. The frequency is shown as a percentage of the total number of cells in a 3-dimensional presentation. The frequency of cells expressing these cytokines among total cytokine-producing cells is also shown in a pie chart. Cells expressing these cytokines are shown as follows: none of the three cytokines (gray), IFN-γ only (red), MIP-1β only (green), TNF-α only (blue), IFN-γ and MIP-1β (yellow), IFN-γ and TNF-α (violet), MIP-1β and TNF-α (cyan), and all three cytokines (black).

heterogeneity in cytokine production between the CTL clones was not found when they were stimulated with cells pulsed with lower concentrations of HIV-1 peptides. Therefore, it is still unclear whether the heterogeneity in cytokine production between CTL clones of the same specificity as well as of different specificities influences disease progression.

A previous study revealed that HLA-A2-restricted, $HCMVpp65_{495-503}$-specific $CD8^+$ T cells are able to produce various combinations of IFN-γ, TNF-α, and IL-2 after PBMC from healthy individuals are stimulated with $HCMVpp65_{495-503}$ peptide, suggesting that the virus-specific $CD8^+$ T cells possess a functional heterogeneity of cytokine production in vivo (18). Since the HCMV-specific $CD8^+$ T cells are heterogeneous with regard to the surface markers CD45RO, CD45RA, CD27, CD28, CD57, and CD62L (18, 21), it is thought that they include various population types, ranging from memory to effector T cells. Therefore, the heterogeneity at maturation of $CD8^+$ T cells may reflect the capacity for functional heterogeneity in cytokine production. Our recent study revealed that $CD8^+$ T cells with effector phenotypes ($CD27^-$ $CD28^-$ $CD45RA^{+/-}$) or memory/effector phenotypes ($CD27^{low}$ $CD28^-$ $CD45RA^{+/-}$) can produce IFN-γ after stimulation of $CD8^+$ T cells with an anti-CD3 MAb (23), indicating that effector and memory/effector $CD8^+$ T cells have a more pronounced ability to produce IFN-γ than memory $CD8^+$ T cells. Thus, the heterogeneity in maturation or differentiation of $CD8^+$ T cells reflects the functional heterogeneity in cytokine production capacity seen in vivo.

Freshly isolated Gag28-36-specific $CD8^+$ T cells produced more cytokines than CTL clones with the same specificity. Since these Gag28-36-specific $CD8^+$ T cells carried either a $CD27^+$ $CD28^-$ memory/effector or a $CD27^+$ $CD28^+$ memory phenotype, it is likely that they are less mature than the CTL clones. These results suggest that HIV-1-specific $CD8^+$ T cells with a $CD27^+$ $CD28^-$ or $CD27^+$ $CD28^+$ phenotype can produce more cytokines than those with an effector phenotype. Approximately 20 to 30% of cytokine-producing $CD8^+$ T cells produced two or three cytokines, whereas most CTL clones of the same specificity produced a single cytokine. This indicates the ability of HIV-1-specific memory and memory/effector $CD8^+$ T cells to produce multiple cytokines when they recognize HIV-1-infected cells. Various differentiation ranges of HIV-1-specific $CD8^+$ T cells, from memory to effector, are found in PBMC from HIV-1-infected individuals (5, 22). HIV-

1-specific $CD8^+$ T cells with a $CD27^+$ $CD28^-$ or $CD27^+$ $CD28^+$ phenotype may play both a direct and an indirect role in the suppression of HIV-1 replication in vivo via cytokines secreted from these $CD8^+$ T cells. Approximately 30 and 10% of Gag28-36-specific $CD8^+$ T cells produced cytokines in KI-144 and KI-158, respectively. This difference might be explained by the fact that approximately 20% of Gag28-36-specific $CD8^+$ T cells from KI-158 carried effector phenotype $CD27^-$ $CD28^-$, whereas no Gag28-36-specific $CD8^+$ T cells from KI-144 carried it.

In summary, HIV-1-specific CTL clones for the most part produced a single cytokine and did not exhibit heterogeneity of cytokine production among clones after stimulation with HIV-1-infected $CD4^+$ T cells, although they exhibited multiple cytokine production and functional heterogeneity of cytokine production between clones after stimulation with HIV-1 peptide-pulsed cells. Freshly isolated HIV-1-specific $CD8^+$ T cells with an effector/memory or memory phenotype produced much greater amounts of the cytokines than CTL clones with the same epitope specificity after stimulation with HIV-1-infected $CD4^+$ T cells. HIV-1-specific $CD8^+$ T cells with an effector/memory or memory phenotype might directly or indirectly play a crucial role in the eradication of HIV-1 via the cytokines secreted from these T cells in HIV-1-infected individuals.

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