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# Cross-Reactive Cytotoxic T Lymphocytes against Human Immunodeficiency Virus Type 1 Protease and Gamma Interferon-Inducible Protein 30

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**The gamma interferon (IFN-γ)-inducible protein 30 (IP-30) signal peptide −11 to −3 (LLDVPTAAV) is a prominent self peptide expressed with the class I human histocompatibility leukocyte antigen A2 (HLA-A2). Stimulation of peripheral blood mononuclear cells (PBMC) from HLA-A2 human immunodeficiency virus type 1 (HIV-1)-infected individuals with an HLA-A2-restricted HIV protease (PR) peptide 76-84 (LVGPTPVNI) activated cytotoxic T lymphocytes (CTL) against the IP-30 signal peptide. Since HIV-1 PR 76-84 stimulated CD8$^+$ T cells from these individuals to secrete IFN-γ, we tested whether the activation of IP-30-specific CTL in vitro resulted from T-cell cross-reactivity or from up-regulation of IP-30 by IFN-γ. Neither high levels of exogenous IFN-γ nor incubation of PBMC with other HIV peptides triggering substantial IFN-γ release activated IP-30-specific CTL. Although the IP-30 signal peptide did not stimulate IFN-γ release from freshly isolated PBMC, it activated CTL in vitro against itself and HIV PR 76-84. Peptide-stimulated IFN-γ release, cold target inhibition, and HLA-A2/immunoglobulin dimer-mediated binding and depletion of effector cells all indicated that in vitro stimulation with HIV PR 76-84 or the IP-30 signal peptide activated a comparable population of cross-reactive effector cells. Neither IP-30 nor HIV PR 76-84 activated CTL against themselves following in vitro stimulation of PBMC from non-HIV-infected HLA-A2 individuals. Peptide titrations indicated higher-avidity T-cell interactions with HIV PR 76-84 than with the IP-30 signal peptide. These data indicate that HIV PR 76-84 is a heteroclitic variant of the IP-30 signal peptide −11 to −3, which has implications for immune memory and autoimmunity.**

Viral infections can trigger immunopathology or autoimmunity through a variety of mechanisms. Immunopathology results when the antiviral immune response targeting infected or otherwise sensitized host cells damages self tissues, such as in viral hepatitis. Autoimmunity results from the selective activation and expansion of normally quiescent self-reactive B or T lymphocytes. Molecular mimicry between viral and self proteins, selective up-regulation of certain host proteins, virus-induced changes in the processing of host proteins, enhanced antigen-presenting cell (APC) function, and proinflammatory environmental conditioning can all facilitate induction of autoimmunity or immunopathology by viral infections (2, 9, 14, 20, 21, 22, 23, 26). While even acute infections can trigger immunopathology and autoimmunity, the persistent influence of chronic viral infections increases the probability of these immunological side effects.

Human immunodeficiency virus type 1 (HIV-1) establishes chronic infection in essentially 100% of cases and has been associated with both immunopathology and autoimmunity. Immunopathology related to the pronounced CD8$^+$ T-cell activation characteristic of HIV infection includes skin rashes, alveolitis, sicca syndrome, lymphadenopathy, neuritis, and vasculitis (4, 5, 6, 12, 13, 29). Autoreactive CD8$^+$ T cells specific for self peptides selectively overexpressed in HIV-infected cells have also been identified in HIV-infected individuals (7, 10). In this context, both the proimmune cytokine gamma interferon (IFN-γ) itself and a number of IFN-γ-responsive genes are expressed at elevated levels in lymphatic tissue when HIV replication is not fully suppressed (16). This alters the protein expression pattern and enhances the antigen-presenting capacity of IFN-γ-responsive cells, which could subsequently favor activation of cross-reactive T cells against IFN-γ-inducible proteins.

The signal peptide −11 to −3 (LLDVPTAAV) from an IFN-γ-inducible protein (IP) termed IP-30 is a dominant self peptide expressed in the context of human histocompatibility-linked leukocyte antigen A2 (HLA-A2) (28). IP-30 was originally identified as an IFN-γ-inducible protein ubiquitously expressed in IFN-γ-stimulated peripheral blood mononuclear cells (PBMC) and distributed in the lysosomes (17). More recently, IP-30 was shown to localize within major histocompatibility complex (MHC) class II compartments (3) and therein catalyze reduction of disulfide bonds in proteins endocytosed by APCs (18). Therefore, IP-30 is also referred to as γ-IFN-inducible lysosomal thiol reductase. Due to the central role of IP-30 in antigen processing and presentation and the prominent expression of the IP-30 signal peptide in the context of HLA-A2, we reasoned that T-cell tolerance would be strictly enforced against the IP-30 signal peptide and chose it as a control HLA-A2 binding peptide for studying HIV-specific HLA-A2-restricted CD8$^+$ T-cell responses. Although we never

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observed ex vivo IFN-γ release by $CD8^+$ T cells in response to the IP-30 signal peptide, CTL against HLA-A2 target cells pulsed with IP-30 signal peptide arose following in vitro stimulation of PBMC with the HIV protease (PR) peptide 76-84 (LVGPTPVNI). Since activation of IP-30-specific CTL was associated with strong ex vivo IFN-γ release in response to the PR 76-84 peptide and IFN-γ increases IP-30 expression levels, we tested whether in vitro induction of IP-30 signal peptide-specific CTL by HIV PR 76-84 reflected the impact of elevated IFN-γ levels or cross-reactivity between the two peptides. We found that, in HIV-infected HLA-A2 individuals, the $CD8^+$ T-cell response against HIV PR 76-84 included self-reactive $CD8^+$ T cells specific for the IP-30 signal peptide. These cross-reactive CTL were readily activated in vitro by either HIV PR 76-84 or the IP-30 signal peptide itself. The IP-30 signal peptide also activated CTL in vitro against HIV PR 76-84. Such cross-reactivity may have both pathological potential, in the context of autoimmunity against APCs, and adaptive potential in the context of an IFN-γ-responsive self peptide acting as a surrogate peptide supporting the maintenance or propagation of a select subset of antiviral CTL.

## MATERIALS AND METHODS

**Study population.** Subjects for this study were a subset of HLA-A2 individuals nested within a study cohort of HIV-infected individuals attending the St. John's General Hospital HIV Clinic, St. John's, Newfoundland, Canada. Laboratory personnel expressing HLA-A2 served as non-HIV-infected controls. Informed consent was obtained for blood collection, and this study was given ethical approval by the Memorial University Faculty of Medicine Human Investigation Committee. Blood was drawn concurrently for these studies and for standard clinical follow-up, which included plasma virus load measurement, blood chemistry, and lymphocyte subset analysis.

**Lymphocyte isolation and cell culture.** Whole blood was collected by venipuncture into acid-citrate-dextrose-treated Vacutainer receptacles, and PBMC were isolated with Ficoll-Paque PLUS (Amersham Biosciences AB, Uppsala, Sweden). Cells were washed twice with phosphate-buffered saline (PBS) plus 1% fetal calf serum (FCS) and resuspended in lymphocyte medium consisting of RPMI 1640 with 10% FCS, 100 IU/ml of penicillin, 100 μg/ml of streptomycin, 2 mM L-glutamine, 10 mM HEPES buffer solution, and $2 \times 10^{-5}$ M 2-mercaptoethanol (all from Gibco Invitrogen Corporation, Grand Island, NY). HLA class I-A and B types of all donors were determined using commercial kits (One Lambda, Canoga, CA) as previously described (25). Autologous B-lymphoblastoid cell lines (BLCL) were generated by Epstein-Barr virus transformation of peripheral blood B cells. Briefly, 2.5 ml of supernatant from marmoset B95-8 leukocytes (ATCC CRL 1612; Rockland, ME) was passed through an 0.45-μm-pore-size sterile filter (Millipore, Bedford, MA) and added to $5 \times 10^6$ freshly isolated PBMC. These cells were then cultured for 24 h, washed, and maintained in medium with 20% FCS and 1 μg/ml of cyclosporine until sufficient growth occurred to cryopreserve several aliquots of the cell line.

**IFN-γ ELISPOT assay.** Microtiter assay plates (Multiscreen; Millipore, Bedford, MA) were coated with 100 μl of 15 μg/ml of anti-IFN-γ monoclonal antibody (MAb) 1-D1K (Mabtech, Stockholm, Sweden) overnight at 4°C. The plates were then washed six times with PBS, and $2 \times 10^5$ PBMC added in medium to duplicate wells were incubated overnight with 0.4 μM peptide of interest. Negative control wells containing PBMC alone and positive control wells containing PBMC and 5 μg/ml of purified phytohemagglutinin (ICN Biomedicals Inc., Aurora, OH) were included in each assay. After overnight incubation, plates were washed as above and 100 μl/well of 1-μg/ml biotinylated anti-IFN-γ MAb 7-B6-1 (Mabtech, Stockholm, Sweden) was added for 2 h. Wells were washed six more times, and 100 μl of streptavidin alkaline phosphatase conjugate (Mabtech, Stockholm, Sweden) diluted 1:1,000 was added in for 1 h. Plates were again washed six times, and 100 μl of chromogenic alkaline phosphatase substrate (Bio-Rad Laboratories, Hercules, CA) diluted 1:10 in Tris buffer, pH 9.5, was added. After 30 min, plates were washed with tap water to terminate color reactions and then air dried. Spots were counted with an automated ELISPOT counter (Zellnet Consulting Inc., New York, NY). Responses were considered positive if the number of spots was more than twice the negative control and >50/$10^6$ PBMC. Synthetic peptides used (Genemed Synthesis, San Francisco, CA) were all >95% pure.

**Peptide-specific CTL stimulation.** Peptide-specific CTL were generated in vitro as previously described (15). Briefly, $5 \times 10^6$ PBMC were pulsed with 100 μM peptide in 100 μl of medium for 1 h at 37°C. The cells were then resuspended at $2.5 \times 10^6$ cells/ml in medium supplemented with 25 ng/ml of recombinant human interleukin 7 (IL-7) (R&D Systems, Minneapolis, MN). On day 3 after initiation of culture, 10 U/ml of IL-2 (Hoffmann-La Roche Ltd., Nutley, NJ) was added, and cells were tested 7 to 11 days after IL-2 addition for cytotoxicity against peptide-pulsed BLCL.

**Cytotoxicity assays.** Autologous or partially HLA-matched BLCL target cells were incubated in 1 ml of medium with 100 μCi of $Na_2{}^{51}CrO_4$ (Amersham Biosciences, Piscataway, NJ) for 1 h at 37°C. Labeled cells were then washed three times and transferred to U-bottomed 96-well plates (Corning Inc., Corning, NY) at $5 \times 10^3$ cells/well in 50 μl of medium. To sensitize target cells, individual peptides were added to the appropriate wells for 1 h at 37°C at a final concentration of 20 μM in a total of 100 μl of medium. For cold target inhibition experiments, unlabeled target cells were incubated with peptides for 1 h, washed, and added to duplicate wells at the desired cold/labeled target ratios. Sufficient effector cells were added to attain desired effector-to-target cell (E:T) ratios, and the volume of each well was adjusted to 300 μl. Assays ran for 5 h, after which 125 μl of supernatant was removed from each well and counted in a Wallac 1280 gamma counter. Specific lysis was calculated as (experimental $^{51}$Cr release − spontaneous $^{51}$Cr release)/(maximal $^{51}$Cr release − spontaneous $^{51}$Cr release) × 100. Specific lysis of >10% above background controls was considered positive. Spontaneous $^{51}$Cr release was <15% in all assays reported.

**Intracellular flow cytometry.** Stimulator cells were generated by incubating $1.0 \times 10^5$ BLCL for 1 h with 20 μM peptide at 37°C and 5% $CO_2$ for 1 h. BLCL incubated as above without peptide served as negative controls. Peptide-stimulated CTL were added to the stimulator cells at a 5:1 ratio and incubated for 5 h at 37°C and 5% $CO_2$. Brefeldin A (Sigma-Aldrich, Oakville, Ontario, Canada) was added to cells for the final 4 h at 10 μg/ml. Cells were then washed with fluorescence-activated cell sorting (FACS) buffer containing 5 mM EDTA, 0.5% bovine serum albumin, and 0.02% sodium azide (all from Sigma) in PBS; incubated with phycoerythrin-labeled anti-human CD8 (Caltag, Hornby, Ontario, Canada) at 4°C for 20 min; and then washed again with FACS buffer. Cells were then fixed with fixation buffer A (DAKO Cytomation Inc., Mississauga, Ontario, Canada), permeabilized with Perm buffer (DAKO), and incubated with fluorescein isothiocyanate-conjugated mouse anti-IFN-γ MAb (Caltag) at 4°C for 20 min. The cells were then washed, resuspended in 1% paraformaldehyde, and analyzed on a FACScalibur flow cytometer.

**Flow cytometry and T-cell depletion with HLA-A2:Ig dimer.** Empty HLA-A2:immunoglobulin (Ig) dimer protein (BD Biosciences Pharmingen, Mississauga, Ontario, Canada) was loaded with peptide per manufacturer's instructions. Briefly, HLA-A2:Ig dimer was mixed with 160 M excess peptide in PBS, pH 7.2, and incubated overnight at 37°C. Lyophilized peptides were dissolved at 50 mg/ml in dimethyl sulfoxide and diluted 1:25 in sterile PBS, pH 7.2. Empty HLA-A2:Ig dimer served as a negative control. Peptide-stimulated PBMC (CTL lines) were incubated at $10^6$ cells per 50 μl of FACS buffer with 8 μl of FcR blocking reagent (Miltenyi Biotec, Auburn, CA) at 24°C for 10 min. After this, 2 μg of peptide-loaded HLA-A2:Ig dimer was added and the cells were incubated at 4°C for 1 h. Cells were then washed twice with FACS buffer followed by a second incubation with 8 μl of FcR blocking reagent (Miltenyi) at 24°C for 10 min. Phycoerythrin-conjugated A85-1 MAb (rat anti-mouse IgG1) (BD Biosciences Pharmingen) was then added, and cells were incubated at 4°C for 30 min. The cells were then washed twice in FACS buffer and resuspended in 1% paraformaldehyde for analysis on a FACScalibur flow cytometer (BD Biosciences).

For depletion experiments, cells were incubated with peptide-loaded HLA-A2:Ig dimer as above, washed in PBS supplemented with 0.5% bovine serum albumin and 2 mM EDTA, pH 7.2, and incubated with a 10:1 ratio of goat anti-mouse IgG-coated magnetic beads (Dynal Inc., Brown Deer, WI) at 4°C for 45 min. Bead-bound cells were removed by magnetic attraction, and the unbound cells were tested for cytotoxicity against selected target cells.

## RESULTS

**HIV PR 76-84 activates CTL against the IP-30 signal sequence peptide in HIV-infected individuals expressing HLA-A2.** We originally chose the HIV PR 76-84 peptide LVGPT PVNI for in vitro stimulation of CTL in order to study cross-

reactivity between this peptide and a variant incorporating the I84V mutation associated with HIV protease inhibitor resistance (19). Following in vitro stimulation with the HIV PR 76-84 peptide LVGPTPVNI, we observed specific lysis of HLA-A2 target cells pulsed with the IP-30 signal sequence peptide LLDVPTAAV in an individual with a high frequency of PBMC producing IFN-γ in response to HIV PR 76-84. Therefore, we tested three additional individuals with strong ex vivo IFN-γ responses to HIV PR 76-84 for CTL against IP-30 following in vitro stimulation with HIV PR 76-84 (Fig. 1a). Although there was no demonstrable ex vivo IFN-γ release in response to the IP-30 signal sequence peptide in any case, even with fourfold-higher levels of peptide than normally used (data not shown), in vitro stimulation of PBMC from all four individuals with HIV PR 76-84 activated CTL against target cells pulsed with the IP-30 signal sequence peptide (Fig. 1b to e). None of six non-HIV-infected HLA-A2 individuals generated CTL against the IP-30 signal sequence peptide following in vitro stimulation of their PBMC with HIV PR 76-84 as shown for four of those six controls (Fig. 1f to i). The HIV-infected individuals tested had HLA-A2 as their only common HLA class I-A or B molecule (039-A2,24;B18,73; 065-A2,3;B51,62; 078-A1,2;B18,41; 081-A2;B7,61), but to confirm that recognition of IP-30 signal sequence peptide was restricted by HLA-A2, we did cytotoxicity assays using partially matched BLCL target cells and C1R transfectants expressing either HLA-A2 or HLA-A3 as their only HLA class I molecule. Killing of target cells pulsed with the IP-30 signal sequence peptide was restricted to those target cells expressing HLA-A2 (Fig. 2). These data show that cross-reactive HLA-A2-restricted CTL against the IP-30 signal sequence peptide can be expanded in vitro by stimulating PBMC from HIV-infected individuals, but not uninfected controls, with HIV PR 76-84.

**IFN-γ does not indirectly activate IP-30-specific CTL.** To determine whether exposure to the HIV PR 76-84 peptide could activate IP-30-specific CTL indirectly through IFN-γ production and increased IP-30 expression, we incubated PBMC with high levels of IFN-γ or with other peptides known to induce IFN-γ release ex vivo from the PBMC of HIV-infected individuals expressing HLA-A2. Exposure of PBMC to the same in vitro conditions used to activate peptide-specific CTL with addition of 500 U/ml of exogenous IFN-γ did not induce IP-30-specific CTL (data not shown). To test whether localized endogenous IFN-γ production activated IP-30-specific CTL, we incubated PBMC from other HLA-A2 individuals with additional HIV peptides known to trigger IFN-γ production in the context of HLA-A2 or other coexpressed HLA molecules. Despite triggering ex vivo IFN-γ production and activating strong in vitro CTL responses against themselves, none of the peptides HLA-A2-restricted reverse transcriptase (RT) 33-41 (ALVEICTEM), HLA-B44-restricted HIV RT 203–212 (EELRQHLLRW), and HLA-B57-restricted HIV Gag 147-155 (LSPRTLNAW) activated CTL against IP-30 (Table 1). These data indicate that up-regulation of IP-30 expression through IFN-γ release does not account for in vitro activation of CTL against the IP-30 signal peptide with PBMC from HIV-infected individuals expressing HLA-A2.

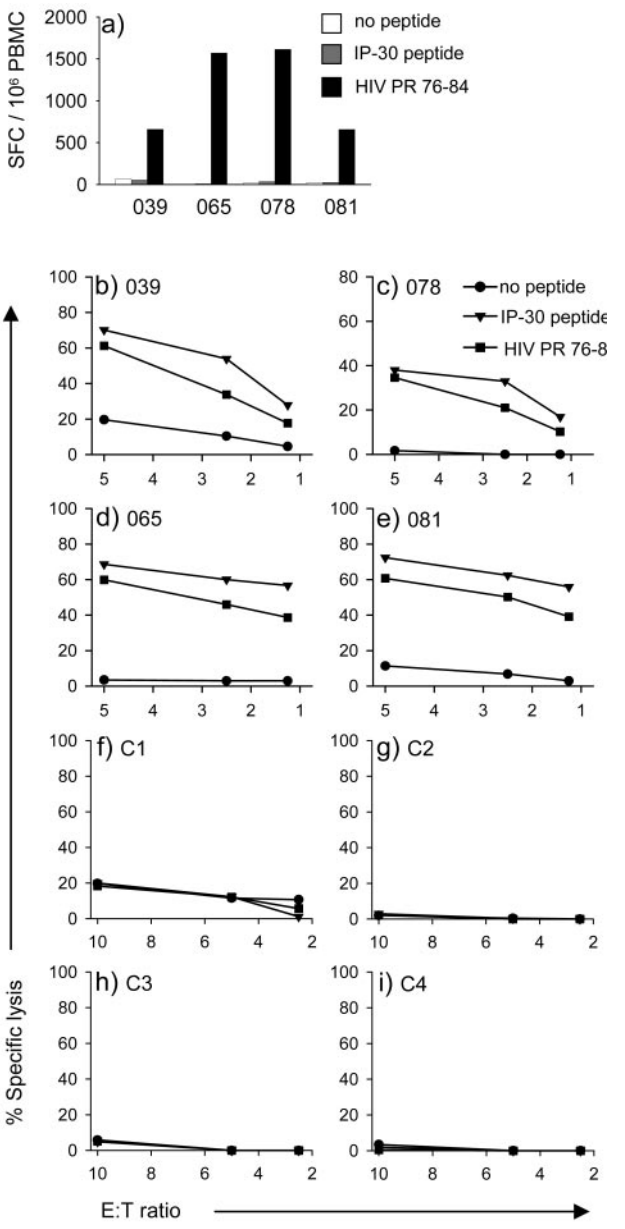


FIG. 1. Frequency of $CD8^+$ T cells in PBMC from four HIV-infected HLA-A2 individuals who produced IFN-γ in response to stimulation with HIV PR 76-84, IP-30 −11 to −3, or no peptide (a) and specific cytotoxicity of T cells from those HIV-infected HLA-A2 individuals (b to e) and four non-HIV-infected HLA-A2 controls (f to i) stimulated in vitro with HIV PR 76-84 against autologous BLCL target cells pulsed with HIV PR 76-84 or IP-30 −11 to −3 peptides.

**In vitro stimulation with the IP-30 self peptide activates self-reactive and anti-HIV CTL.** Although it didn't trigger ex vivo IFN-γ release, we tested whether in vitro stimulation with the IP-30 signal peptide activated CTL against itself and/or the HIV PR 76-84 peptide previously shown to activate IP-30-specific CTL in vitro. We stimulated PBMC from the same donors who generated anti-IP-30 CTL in response to HIV PR 76-84 in vitro with the IP-30 signal peptide. In all four cases, this activated both CTL against target cells pulsed with the IP-30 signal peptide and HIV-specific CTL against HIV PR

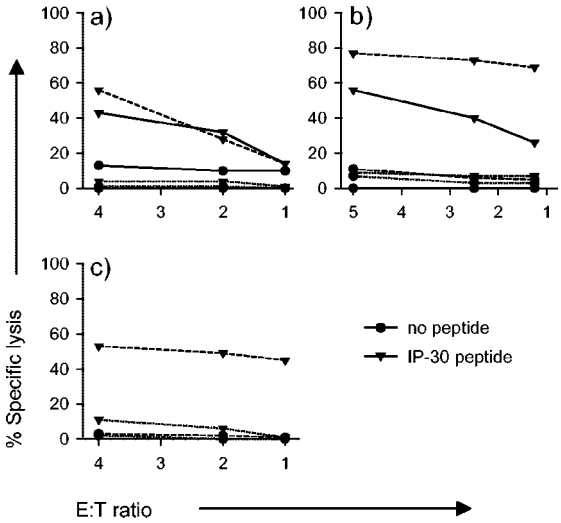


FIG. 2. Specific cytotoxicity of T cells from subjects 039 (a and c) and 081 (b) stimulated in vitro with HIV PR 76-84 against autologous (solid lines), HLA-A2-matched (dashed lines), and mismatched (dotted lines) BLCL pulsed with IP-30 −11 to −3 peptide. Panel c shows specific cytotoxicity against peptide-pulsed C1R transfectants expressing HLA-A2 (dashed lines) or A3 (dotted lines) only.

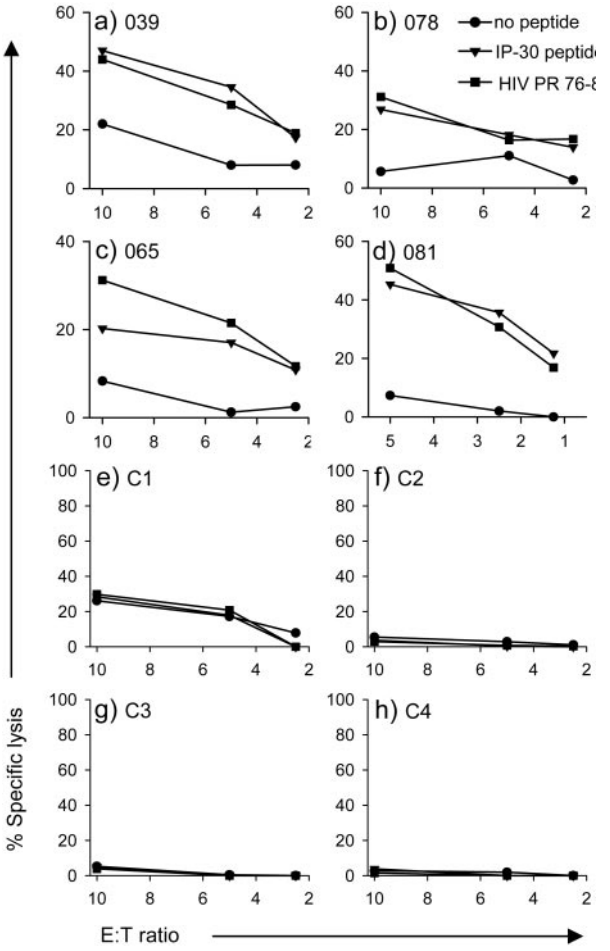


FIG. 3. Specific cytotoxicity of T cells from four HIV-infected HLA-A2 individuals (a to d) and four non-HIV-infected HLA-A2 controls (e to h) stimulated in vitro with IP-30 −11 to −3 against autologous BLCL target cells pulsed with HIV PR 76-84 or IP-30 −11 to −3 peptides.

76-84 (Fig. 3a to d). At the level of in vitro CTL activation, there was clear cross-reactivity between HIV PR 76-84 and the IP-30 signal peptide, and despite not inducing IFN-γ release ex vivo, the IP-30 signal peptide was effective at in vitro stimulation of CTL against both itself and the PR 76-84 peptide. Stimulation of PBMC from non-HIV-infected HLA-A2 controls with the IP-30 signal peptide did not activate CTL against IP-30 in any case (Fig. 3e to h), indicating that, in vivo, HIV infection primes $CD8^+$ T cells for in vitro activation with the IP-30 signal peptide.

**Cross-reactivity between HIV PR 76-84 and IP-30 signal peptide-specific CTL.** Although the IP-30 signal peptide did not trigger ex vivo IFN-γ production, once PBMC from subject 081 were stimulated in vitro with either the IP-30 signal peptide (Fig. 4a to d) or HIV PR 76-84 (Fig. 4e to h), the cells did release IFN-γ when exposed to BLCL pulsed with the IP-30 signal peptide (Fig. 4b and f). A similar fraction within each of the individual in vitro-expanded $CD8^+$ T-cell populations produced IFN-γ in response to either the IP-30 signal peptide or PR 76-84 peptide (Fig. 4b versus c and f versus g), and there was no additive effect from combining sets of stimulators pulsed separately with one or the other peptide (Fig. 4d and h). The same pattern was observed with $CD8^+$ T cells from subject 039 (data not shown), suggesting that a largely overlapping set of $CD8^+$ T cells responds to the different peptides.

To confirm that CTL against HIV PR 76-84 and the IP-30

TABLE 1. Activation of CTL by IFN-γ-inducing HIV peptides other than PR 76–84

| Subject | HLA | HIV peptide | HLA restriction | SFC/$10^6$ PBMC[a] | % Specific lysis[b] | |
|---|---|---|---|---|---|---|
| | | | | | HIV peptide | IP-30 −11 to −3 |
| 034 | A2,3;B35,60 | RT 33–41 | A2 | 330 | 28/15/8 | 7/3/0 |
| 043 | A2,32;B14,62 | RT 33–41 | A2 | 65 | 60/44/43 | 5/0/3 |
| 126 | A2;B44,62 | RT 203–212 | B44 | 2,710 | 50/39/17 | 0/0/0 |
| 131 | A2;B7,44 | RT 203–212 | B44 | 940 | 58/24/18 | 6/3/0 |
| 071 | A1,2;B18,57 | Gag 147–155 | B57 | 1,175 | 40/23/6 | 1/1/0 |
| 149 | A1,2;B57,61 | Gag 147–155 | B57 | 325 | 36/20/14 | 0/0/1 |

[a] Spot-forming cells (SFC) were enumerated by IFN-γ ELISPOT following incubation of PBMC with the indicated peptides.
[b] Cytotoxicity following in vitro stimulation of PBMC with the indicated HIV peptide was measured against autologous BLCL pulsed with either the stimulating peptide or IP-30 −3 to −11 signal peptide. Lysis values reported are at E:T ratios of 5:1, 2.5:1, and 1.25:1, respectively, with background lysis against unpulsed targets subtracted.

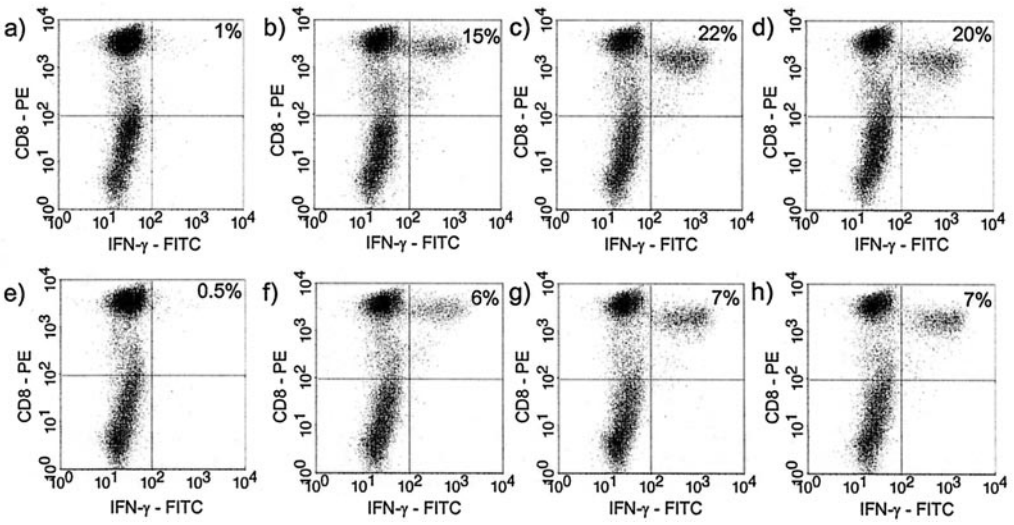


FIG. 4. Intracellular IFN-γ production by $CD8^+$ T cells stimulated in vitro for 14 days with IP-30 −11 to −3 signal peptide (a to d) or HIV PR 76-84 (e to h) and exposed for 5 h to autologous BLCL pulsed with either no peptide (a and e), IP-30 −11 to −3 signal peptide (b and f), or PR 76-84 (c and g) or pulsed with each peptide individually and combined as stimulators (d and h). The percentage of $CD8^+$ T cells producing IFN-γ is denoted in the upper right quadrant of each plot. PE, phycoerythrin; FITC, fluorescein isothiocyanate.

signal peptide were actually cross-reactive and not reciprocally activated through some form of bystander mechanism, we did flow cytometry and depletion experiments with peptide-loaded HLA-A2:Ig dimer and carried out cold target inhibition assays. Flow cytometry with the HLA-A2:Ig dimer loaded with HIV PR 76-84 revealed a similar-size population of dimer-positive cells (~25%) following stimulation of PBMC from subject 065 with either HIV PR 76-84 (Fig. 5a) or the IP-30 signal peptide (Fig. 5b). The HLA-A2:Ig dimer loaded with IP-30 signal peptide failed to stain T cells within either the HIV PR 76-84 or IP-30 signal peptide in vitro-stimulated CTL populations. Similar results were obtained with subjects 039 and 081 (data not shown). When PBMC from non-HIV-infected HLA-A2 individual C1 were stimulated in vitro with either HIV PR 76-84 (Fig. 5c) or the IP-30 signal peptide (Fig. 5d), flow cytometry with the HLA-A2:Ig dimer loaded with HIV PR 76-84 revealed no dimer-positive cells relative to the empty dimer control. Identical results were obtained with stimulated PBMC from non-HIV-infected HLA-A2 individual C2 (data not shown).

Since the HLA-A2:Ig dimer loaded with HIV PR 76-84 stained T cells within both HIV PR 76-84 and IP-30 signal peptide-stimulated CTL populations, we used this dimer for depletion experiments. Depletion of effector cells from subject 081 with the HIV PR 76-84-loaded HLA-A2:Ig dimer and goat-anti-mouse IgG-coupled magnetic beads reduced killing of HLA-A2 target cells pulsed with the IP-30 signal peptide to background levels and substantially reduced killing of HLA-A2 target cells pulsed with HIV PR 76-84, whether the effector cells were generated by stimulation with HIV PR 76-84 (Fig. 6a) or the IP-30 signal peptide −11 to −3 (Fig. 6b). Depletion of effector cells from subjects 039, 065, and 078 produced equivalent results (data not shown).

When CTL were raised in vitro by stimulation with either the IP-30 signal peptide or HIV PR 76-84, killing of HLA-A2 target cells pulsed with IP-30 signal peptide or HIV PR 76-84 was effectively inhibited by cold target cells pulsed with HIV PR 76-84 (Fig. 6c). Cold target cells pulsed with the IP-30 signal peptide inhibited killing of HLA-A2 target cells pulsed with the IP-30 signal peptide but had little effect on killing of HLA-A2 target cells pulsed with HIV PR 76-84 (Fig. 6d).

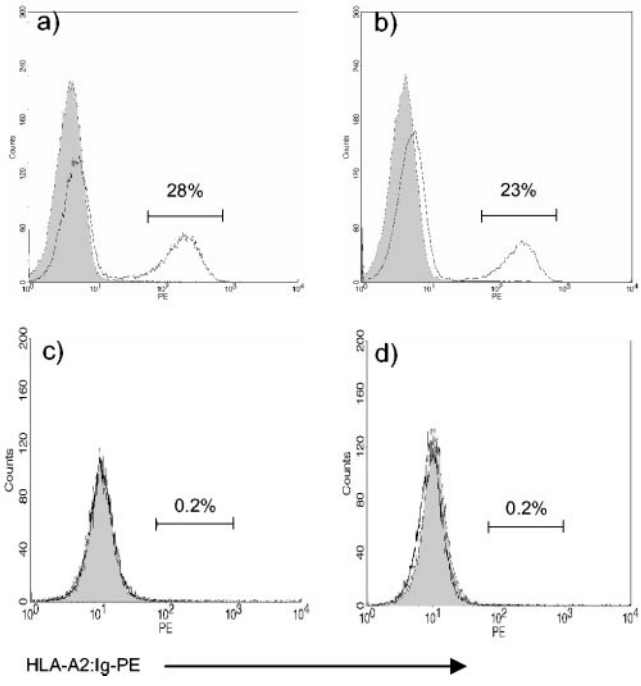


FIG. 5. Immunofluorescent labeling of T cells from HIV-infected subject 065 (a and b) and non-HIV-infected HLA-A2 control C1 (c and d) following in vitro stimulation with HIV PR 76-84 (a and c) or IP-30 −11 to −3 (b and d) with HLA-A2:Ig dimer loaded with either HIV PR 76-84 (unshaded histogram) or no peptide (shaded histogram). PE, phycoerythrin.

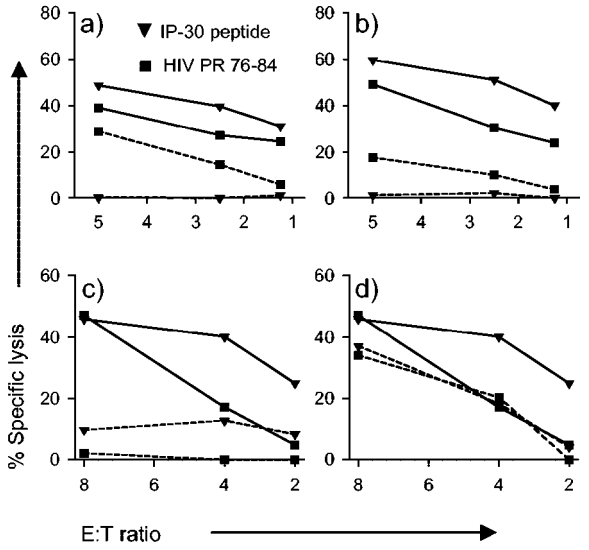


FIG. 6. Effect of depleting cells binding the HLA-A2:Ig dimer loaded with HIV PR 76-84 (a and b, dashed lines) on specific cytotoxicity of effector cells from subject 081 stimulated in vitro with HIV PR 76-84 (a) or IP-30 −3 to −11 (b) against autologous BLCL target cells pulsed with HIV PR 76-84 or IP-30 −3 to −11 and cold target inhibition of cell-mediated cytotoxicity against BLCL pulsed with either the IP-30 −11 to −3 signal peptide or HIV PR 76-84. HLA-A2-expressing BLCL cold targets were pulsed with no peptide (c and d, solid lines), HIV-PR 76-84 (c, dashed lines), or IP-30 −11 to −3 (d, dashed lines). Cold targets were used at a 5:1 cold-to-hot target ratio to inhibit killing by HIV PR 76-84-stimulated CTL at the E:T ratios shown.

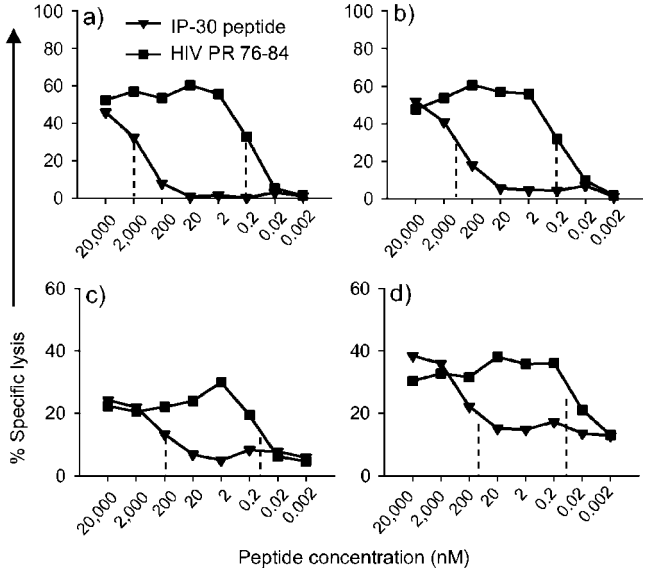


FIG. 7. Peptide titration curves for cytotoxicity of effector cells from subjects 078 (a and b) and 065 (c and d) activated by in vitro stimulation with HIV PR 76-84 (a and c) or IP-30 −11 to −3 (b and d) at an E:T ratio of 5:1 against autologous BLCL targets pulsed with decreasing concentrations of either HIV PR 76-84 or IP-30 −11 to −3 as indicated by the symbols. Dashed vertical lines to the *x* axis indicate the peptide concentrations at which killing of peptide targets fell to 50% of maximum values.

Similar results were observed with CTL from subjects 078 and 081 (data not shown). Thus, both functional and phenotypic assays demonstrate cross-reactivity between the IP-30 signal peptide and HIV PR 76-84 and indicate that, with PBMC from HLA-A2 HIV-infected individuals, the IP-30 signal peptide can effectively activate CTL against HIV PR 76-84 in vitro.

**The CTL interaction with HLA-A2/IP-30 self peptide is of lower avidity than that with HLA-A2/HIV PR 76-84.** Escape of IP-30-specific CTL from thymic deletion, inability to stimulate ex vivo IFN-γ release, inefficient inhibition of killing with IP-30 signal peptide-pulsed cold targets, and failure of the HLA-A2:Ig/IP-30 peptide dimer to stain IP-30-specific CTL suggest that the T-cell receptor interaction with the HLA-A2/IP-30 signal peptide complex is of relatively low avidity (25, 26). We compared the avidity of CTL from subjects 065 and 078 generated by in vitro stimulation with either HIV PR 76-84 or the IP-30 signal peptide for HLA-A2 target cells pulsed with the corresponding peptides by titrating peptide concentrations in cytotoxicity assays. Regardless of whether the CTL were generated by in vitro stimulation with HIV PR 76-84 (Fig. 7a and c) or the IP-30 signal peptide (Fig. 7b and d), killing of target cells pulsed with HIV-PR 76-84 fell to 50% of baseline killing at <0.2 nM HIV-PR 76-84, an approximately 1,000-fold-lower peptide concentration than that with killing of target cells pulsed with the IP-30 signal peptide. While a lower affinity of HLA-A2 binding for the IP-30 peptide than HIV PR 76-84 could also explain these results, this is unlikely for several reasons. Firstly, IP-30 was identified as a very prominent self peptide in the context of HLA-A2 molecules purified from lymphoid cells (28). Secondly, the IP-30 signal peptide sequence fits the motif for HLA-A2 binding and ranks higher than HIV PR 76-84 using the two most common algorithms for predicting HLA binding affinity. With the http://bimas.thr.cit.nih.gov/molbio/hla_bind, IP-30 −11 to −3 scores 47.3 and HIV PR 76-84 scores 1.6. Using the Rammensee lab-developed site http://www.syfpeithi.de/, the IP-30 −11 to −3 and HIV PR 76-84 peptides score 28 and 18, respectively.

## DISCUSSION

The potential for molecular mimicry to activate cross-reactive T cells during viral infections relates to the extent of peptide mimicry, from the perspective of host HLA molecules and T-cell receptors, and to the degree of self-tolerance imposed upon T cells reactive with the analogous self peptides. Self-reactive T cells that avoid thymic and peripheral deletion may persist in the periphery in an effectively tolerant state invoked by their inactivation, suppression, or ignorance. While this is normally a stable situation, perturbations accompanying viral infections, including the introduction of more immunogenic variants of self peptides, may challenge the tolerance imposed on self-reactive T cells. In this study, we found that, following HIV infection, $CD8^+$ T cells reactive against a self peptide derived from IP-30 could be readily activated and expanded in vitro by exposure to either the foreign HIV PR 76-84 peptide or the self IP-30 −11 to −3 signal peptide. Despite acquiring the capacity to expand and differentiate into CTL in vitro in response to self peptide, the $CD8^+$ T cells cross-reactive with HIV PR 76-84 didn't release IFN-γ ex vivo when exposed to the self peptide. This presumably reflects the

relatively low avidity of the T-cell receptor/HLA-A2/IP-30 signal peptide interaction that allowed the IP-30-specific T cells to originally escape thymic deletion and persist in the periphery without effecting symptomatic autoimmunity (24, 27). Low avidity relative to the HIV PR 76-84 peptide recognition was reflected in an approximately 1,000-fold-lower concentration of HIV PR 76-84 than IP-30 −11 to −3 required to trigger 50% maximal lysis of peptide-pulsed targets, inefficient inhibition of cytotoxicity against targets pulsed with HIV PR 76-84 by cold targets pulsed with IP-30 −11 to −3, and failure of the HLA-A2:Ig dimer loaded with IP-30 −11 to −3 to stably bind the cross-reactive $CD8^+$ T cells.

The higher-avidity interaction with HIV PR 76-84 expands cross-reactive $CD8^+$ T cells specific for the IP-30 signal peptide in vivo and in so doing increases their sensitivity to activation and expansion by the IP-30 signal peptide itself, at least in vitro. This situation, effected by viral infection, parallels a proposed approach to therapeutic vaccination for cancer where synthetic peptide variants are used to activate T cells against self peptides expressed on tumor cells, when the self peptides themselves do not activate the T cells (8). In these cases, self-reactive T cells react with higher avidity against synthetic heteroclitic variant peptides and, once activated and expanded by exposure to the variants, selectively attack tumor cells, which express higher levels of the analogous self peptide than do normal host cells (8). In this study, we identified a viral peptide that acts as the synthetic heteroclitic variant activating self-reactive T cells against a self peptide expressed at higher levels in lymphoid cells exposed to IFN-γ. Activation, by HIV PR 76-84, of cross-reactive CTL against an IFN-γ-inducible protein widely expressed in lymphoid cells could have dangerous implications for autoimmunity, especially when elevated IFN-γ levels during periods of viral replication up-regulate IP-30 expression (16). As in the selective targeting of tumor cells by autoreactive CTL, activation and proliferation of IP-30-specific CTL against a backdrop of elevated IFN-γ levels could selectively target IFN-γ exposed APCs expressing elevated levels of IP-30 and thereby contribute to the APC dysfunction and lymphoid architectural destruction characteristic of progressive HIV infection. However, there is as yet no evidence of any relationship between HIV disease progression and CTL against HIV PR 76-84. Of 46 HLA-A2 HIV-infected individuals whom we tested by IFN-γ ELISPOT, 17 had T cells reactive with HIV PR 76-84 and 7 of these 17 generated HIV PR 76-84 and IP-30 −11 to −3 cross-reactive CTL following in vitro peptide-specific stimulation. While it is clearly a subset of HLA-A2 HIV-infected individuals who possess these CTL, there was no clear indication that this subset had a clinically distinct pattern of disease progression, and longitudinal studies will be needed to address this issue.

Current models of T-cell selection hold that $CD8^+$ thymocytes are positively selected through interaction with self peptides ubiquitously expressed with the MHC class I molecules of cortical epithelial cells. Subsequent negative selection eliminates only those $CD8^+$ T cells that react with high avidity against endogenous peptide/MHC class I molecule complexes expressed on bone marrow-derived APC at the corticomedullary junction or in the medulla (24). Therefore, selection of $CD8^+$ T cells with the capacity for high-avidity interactions with foreign peptide/MHC class I complexes is absolutely dependent upon those $CD8^+$ T cells engendering low-avidity self-reactivity. Within the normal environmental milieu, such low-avidity self-reactive T cells are nonpathogenic; however, increased expression of the self peptide; alterations in the environment; or activation, proliferation, and differentiation of self-reactive CTL in response to foreign peptides could shift the situation towards symptomatic autoimmunity. The ability of the IP-30 signal peptide itself to activate and expand CTL in vitro, once they have been activated in vivo by HIV PR 76-84, suggests that the in vivo activation and/or expansion of cross-reactive CTL lowers the threshold for breaking the effective tolerance of these T cells for self tissue. While the potential negative consequence of CTL against uninfected lymphoid cells is obvious, as long as the relatively low avidity of CTL against IP-30 protects against symptomatic autoimmunity, there could be substantial advantages to cross-reactivity between antiviral CTL and a prominent self peptide up-regulated by proimmune cytokines such as IFN-γ. One previously identified positive consequence of activation of low-avidity self-reactive T cells can be selective targeting of tumor cells. In the case of the self peptide that we identified as functionally analogous to an HIV CTL epitope, its broad distribution in lymphoid cells, prominent basal representation in HLA-A2 molecules, and up-regulation in response to IFN-γ could all potentially benefit the anti-HIV CTL response. Based on the premise that cross-reactive self peptides are required for homeostatic maintenance of anti-HIV memory T cells when HIV replication is undetectable, the broad distribution and prominent representation of IP-30 −11 to −3 may convey an advantage on anti-HIV CTL manifesting lower-avidity recognition of that particular self peptide. In this context, we observed a persistent high frequency of $CD8^+$ T cells against HIV PR 76-84 in one individual (subject 081), despite antiretroviral suppression of HIV replication to undetectable levels for over 6 years. When HIV replication or any another event that elevates IFN-γ levels occurs, the selective up-regulation of IP-30 might favor propagation of those antiviral T cells with the capacity for functional recognition of IP-30 −11 to −3. The differential avidity would ensure an impact only on those cross-reactive memory T cells previously activated by higher-avidity interactions with foreign peptides and possibly allow a self peptide to selectively activate antiviral CTL. Thus, both in terms of their homeostatic maintenance and the potency of their response to secondary challenge, CTL reactive with self peptides such as IP-30 −11 to −3 may have a significant advantage.

Although we identified CTL against IP-30 −11 to −3 specifically through their cross-reactivity with HIV PR 76-84, it is quite feasible that a variety of CTL restricted to HLA-A2 would have lower-avidity interactions with IP-30 −11 to −3. Minimal sequence homology between IP-30 −11 to −3 and HIV PR 76-84 as well as between other reported cross-reactive CTL epitopes such as the influenza A virus matrix protein peptide MI 58-66 (GILGFVFTL) and HIV p17 77-85 (SLYN TVATL) illustrates the surprising level of promiscuity of $CD8^+$ T-cell recognition of peptides with a common restricting element (1). Previous studies showed that a broad T-cell receptor repertoire is selected even when thymic presentation is restricted to a single MHC/peptide combination (11). The surrogate function of self peptides in positive selection of T cells

with high avidity for foreign peptides may be recapitulated in the periphery following initial activation of these T cells during infection if the expression pattern of the surrogate self peptide is appropriate. Depending upon the relative stringency of requirements for expression levels and avidity threshold to serve as a positively selecting surrogate peptide in the periphery versus during thymic selection, the autoreactivity that drives positive selection of the T-cell repertoire may also play a major role in evolution of the T-cell response against foreign agents.

In summary, this study demonstrates cross-reactivity between HIV PR 76-84 and IP-30 −11 to −3 at the level of $CD8^+$ T-cell recognition. The interaction with IP-30 is of lower avidity, and the $CD8^+$ T cells require in vivo priming with HIV PR 76-84 in order to respond to IP-30 −11 to −3 in vitro. Although such cross-reactivity raises the possibility of autoreactivity against APCs, in the context of our current understanding of T-cell selection, the IP-30 −11 to −3 signal peptide appears to be an ideal surrogate peptide for selection, maintenance, and expansion of $CD8^+$ T cells against foreign agents.

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