

The Lysispot assay reveals HIV-specific T cells can lyse targets without secreting IFN γ directly *ex vivo*, and the frequencies of IFN γ -secreting to cytotoxic cells vary both amongst HIV-specific and in comparison to CMV, EBV responses within individual patients

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Abstract:

CD8⁺ T cells are believed to contribute to the containment of the virus and the delay of disease progression in HIV-infected patients. However, the frequencies of HIV-specific CD8⁺ T cells, as measured by IFN γ secretion and tetramer binding, often do not correlate with a delay in disease progression during chronic infection. Using the Lysispot and Elispot assays, we measured the frequencies of cytotoxic and IFN γ -secreting T cells responding to overlapping peptides specific for Gag, Nef, Env, and Pol consensus B sequences. PBMC taken directly *ex vivo* from the majority of HIV-infected subjects contained significant frequencies of HIV-specific cells that killed targets within five hours directly *ex vivo*. The relative frequencies of IFN γ -secreting and cytotoxic cells varied markedly between different HIV peptide pools within the same patient; and some responses included T cells able to lyse targets without secreting IFN γ . This finding indicates the use of IFN γ production alone to measure changes in the breadth of the HIV-specific T cells response may be misleading. Also, neither the CTL:IFN γ ratios nor the total *ex vivo* CTL frequencies specific for different HIV proteins were consistently decreased in comparison to responses specific for two other chronic viral infections, human cytomegalovirus and Epstein Barr virus, within the same subjects. These results do not support the model of 'pre-terminal differentiation' of HIV-specific CD8⁺ T cells. Further analysis of the frequency of directly cytotoxic HIV-specific T cells may be of considerable value in the assessment of disease progression and the potential efficacy of HIV vaccines.

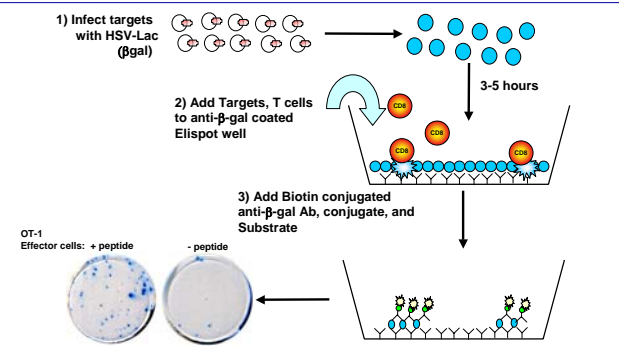


Figure 1. The Lysispot Assay¹ Target cells are infected with the HSV-Lac amplicon² and cultured for 5 hours to allow an abundant amount of β -galactosidase to accumulate within each cell. After this incubation, the target cells are washed and added to an Elispot well coated with anti- β -gal antibodies. Effector cells are added, and if cytotoxic cells are present and lyse target cells, β -gal will be released in a localized area and bound by the coating antibody. At the end of the assay, biotinylated anti- β -gal antibodies, conjugate, and substrate are added to visualize the spots.

Materials and Methods:

Peptides: Overlapping 15mer peptide sets spanning the entire Gag, Pol, Nef, and Env consensus sequences of HIV-1 subtype B (NIH AIDS Research and Reference Program, Rockville, MD). Peptides were pooled into 11 Env, 5 Gag, 8 Pol, and 2 Nef pools and used at a concentration of 1 μ g/ml as antigens in the IFN γ Elispot and Lysispot assays. Three immunodominant CMV peptides were pooled: the NLPVPMVATV peptide, specific for the pp65 peptide (HLA-A2 specific), the TPRTVGGGAM peptide, (HLA-B*57 specific), and the EFWFDANDH peptide, (HLA-B*57 specific). Three immunodominant EBV peptides were pooled: the RAKFKQLI peptide (HLA-B*57 specific), the RPEPEIRL peptide (HLA-B*7 specific), and the GLCTLVAML (HLA-A2 specific) peptide. These peptides were used at a concentration of 1 μ g in the Elispot and Lysispot assays.

Selection of HIV peptide pools for the IFN γ Elispot and Lysispot assays: To conserve PBMC and autologous B cell targets during the analysis of cytotoxicity, a limited set of peptide pools were analyzed in simultaneous IFN γ Elispot and Lysispot assays using autologous B cells from each patient pulsed with the selected peptide pools. For HIV+ patients, the majority of selected pools induced significant IFN γ responses in the preliminary screen in each subject. Subjects with no significant IFN γ responses to any pool (i.e. HIV-negative subjects) were stimulated with peptide pools randomly selected from the pools that stimulated significant CTL frequencies from HIV+ subjects.

IFN γ Elispot and Lysispot Assays: Elispot plates (Millipore, #82ER097M8 or MAIPN4550) were coated with either anti-IFN γ antibody (5 μ g/ml, 50 μ l/well in PBS, Mabtech #mAb 1-D1K) or anti- β -gal antibody (diluted 1:1000, 50 μ l/well in PBS, Sigma #G-8021). Plates were coated for a minimum of 1 hour at room temperature, and washed two times with PBS before cells were added. Target cells were added to non-toxic culture treated dishes and transfected with the HSV-Lac amplicon² at a MOI of 5. After one hour, cells were removed and added to a 15ml conical tube and washed twice with complete media. B cells were resuspended at a concentration of 1x10⁶/ml and pulsed with peptides for 90 minutes. After pulsing, cells were washed twice with warm complete media. Typically 15-30,000 antigen-presenting cells, and 7x10⁶ - 5x10⁶ PBMCs were added per well. The total volume in each well was 100 μ l, including effector cells. The plates were incubated for 3-5 hours. Overall, the frequencies of IFN γ producing cells for each peptide pool in a 40-hour assay, using endogenous APCs, were similar to the frequencies obtained in the shorter assay using the peptide-pulsed autologous B cells as antigen presenting cells (data not shown). Thirty minutes before the end of the assay, biotinylated anti- β -gal (diluted 1:1000, Sigma #B-0271) or biotinylated anti-IFN γ (1 μ g/ml, Mabtech #mAb7-B6-1) antibodies were added in complete medium as an additional 50 μ l/well. The final concentrations of the biotinylated antibodies in the wells were 1:2000 (anti- β -gal) or 0.83 μ g/ml (anti-IFN γ). At the end of the assay, plates were washed 2-3 times with PBS1, and streptavidin-Alkaline Phosphatase diluted 1:1000 in PBS1TB was added overnight at 4°C. Plates were again washed with PBS1, and substrate mix (Vector Laboratories, Cat. #SK5300) was added. The plates were rinsed with tap water when spots were clearly visible. An aliquot of the transfected antigen-presenting cells were cultured separately for the duration of the Lysispot assay. These cells were then fixed and stained with a X-gal substrate to determine the percent transfected.

Calculation of results: Three potential backgrounds were subtracted from the Lysispot values, (1) the spontaneous lysis of the HSV-Lac transfected B cell targets, (2) the increased spontaneous lysis of the B cells due to pulsing with particular peptide pools; and (3) the non-peptide-specific lysis of the B cells by the PBMCs. The third background could potentially be due to the ability of PBMCs to lyse target cells due to EBV reactivity, HSV reactivity, a cytotoxic response to serum proteins, etc. In order to accurately deduct these non-peptide specific Lysispots, the frequency of peptide-specific cytotoxic cells was calculated using the equation D₂ - (B₁ - C₁ - A₁). Each letter in the equation represents the average spot number of triplicate wells containing the following cell combinations: A - unpulsed B cells, B - B cells pulsed with a particular peptide pool, C - PBMC + unpulsed B cells, D - PBMC + B cells pulsed with a particular peptide pool. For some samples the PBMC + unpulsed B cell control wells were increased to six replicates and located on different portions of the plate to ensure changes in spot number were not due to the location of the wells. The percent of targets transfected was consistently greater than 95 percent.

Statistical Guidelines used in the HIV assay: The 1-tailed P value T test was used to compare net Lysispot frequencies between HIV uninfected and infected individuals. The Mann-Whitney test was used to discern significant IFN γ and cytotoxic frequencies in comparison to wells containing PBMC + B cells without the Peason product moment correlation coefficient was used to compare the net IFN γ and CTL frequencies per HIV+ subject with CD4 counts, viral load.

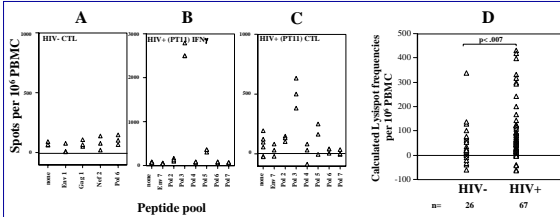


Figure 2. The frequencies of HIV-specific cytotoxic cells can be measured directly *ex vivo* from chronically infected subjects using the Lysispot assay.

An example of the cytotoxic cell frequencies detected from an HIV- subject (A). An example of PBMC from an HIV-infected patient which produced IFN γ in response to two HIV peptide pools (B); one of these pools also induced significant cytotoxicity (C). Graph D summarizes the results obtained from all subjects. The net Lysispot frequencies (calculated as described in the Materials/Methods section) were compared between 26 HIV peptide pools measured in 6 different HIV-subjects, and a total of 67 HIV peptide pool responses measured in 9 HIV+ patients. Comparing all results by 1-tailed P value T test, there was a highly significant increase in HIV-specific cytotoxicity in the HIV-infected group versus the uninfected group (p<0.007).

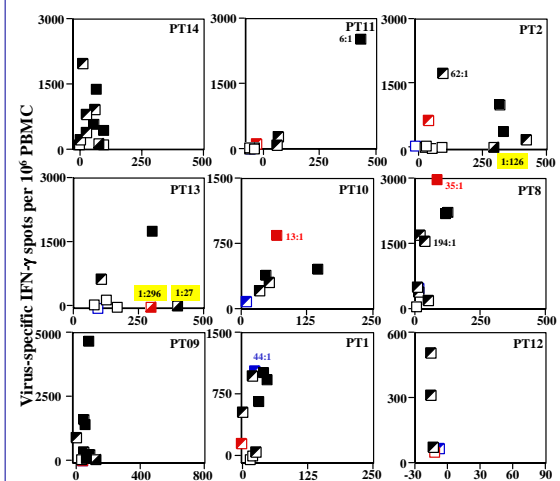


Figure 3. The frequencies of IFN γ -secreting to cytotoxic cells vary both amongst HIV-specific and in comparison to CMV, EBV responses within individual patients.

The net frequencies of cytotoxic and IFN γ -secreting cells in response to HIV, CMV, and EBV peptide pools from nine HIV+ patients were calculated. These data demonstrate: 1) the frequencies of HIV-specific T cell responses with distinct qualitative properties (cytokine secretion and cytotoxicity) can differ dramatically both within and between individual patients, 2) HIV-specific IFN γ :CTL ratios could be either higher (e.g. subject PT10) or lower (e.g. subject PT13) than responses against one or both of the other two chronic viral infections, 3) Surprisingly, 2 out of 3 peptide pools, initially chosen as IFN γ -negative pools, showed HIV peptide-specific cytotoxicity, and also, one CMV response was detected with significant frequencies of cytotoxic but not IFN γ secreting cells (PT13) (highlighted ratios). These results demonstrate that the cytotoxic function of HIV-specific T cells does not always correlate with IFN γ , and is not necessarily impaired compared to other chronic infections within the same subject.

There was no correlation between either the magnitude of the cytotoxic response or the IFN γ :CTL ratio and the responses to the different HIV proteins (Env, Gag, Nef, or Pol) (data not shown).

Patient ID (Group)	Sex	Age	Ethnicity	Time since HIV-1 diagnosis (years)	CD4+ T cell count (cells/ μ l)	HIV RNA load (copies/ml plasma)	HAART
1	M	58	Afr Am	3	462	3,107	NEVER
12	M	36	Cauc	10	838	322	NEVER
9	F	43	Hisp	7	648	2,232	OFF
10	F	53	Cauc	7	150	5,718	ON
2	F	39	Hisp	9	207	316,887	OFF
11	F	42	Afr Am	15	281	232,727	OFF
8	F	48	Cauc	13	262	131,060	OFF
13	M	31	Cauc	7	123	31,663	ON
3	M	39	Afr Am	9	17	206,011	OFF
14	M	35	Afr Am	10	157	75,000	ON

Table 1. Clinical and demographic characteristics of the HIV+ study individuals. Abbreviations: Afr Am = African-American; Cau= Caucasian; Hisp= Hispanic

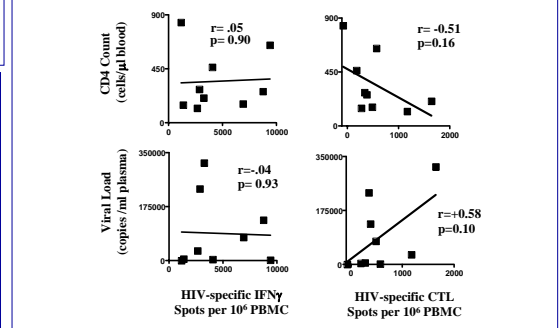


Figure 4. Close association between the frequencies of cytotoxic cells with parameters of HIV disease progression. The clinical status of an HIV infected patient is typically defined by two parameters, the viral load and the number of CD4 cells within the patient's blood. We were interested in comparing the frequencies of *ex vivo* HIV-specific CTL with these parameters to determine if the frequencies of circulating cytotoxic cells correlate with disease progression. There is a suggestive trend indicating that the frequencies of CTLs (and not IFN γ secreting cells) may correlate positively with viral load, and inversely with CD4 counts.

Conclusions:

- 1) Peptide-specific cytotoxic cells are detectable *ex vivo* from HIV- infected patients.
- 2) The ratio of IFN γ to cytotoxic cells can vary amongst different HIV-specific peptide pools within individual patients.
- 3) Significant frequencies of HIV-specific T cells in two patients (PT2 and PT13) and CMV-specific cells in one patient (PT13) lyse targets without secreting IFN γ .
- 4) After comparisons of HIV, CMV, and EBV responses from several patients, no HIV-specific impairment of cytotoxicity is evident.
- 5) There is a substantial relationship between the net frequencies of HIV-specific Lysispots (and not IFN γ Elispots) in chronically infected patients.

References:

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