Evaluation of the dual IFNγ/IL-2 FluoroSpot assay with flow cytometry for detection of HLA-restricted HIV-specific T-cell responses in HIV controllers

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Introduction

The IFNγ ELISPot assay is widely used for high-throughput screening of HIV-specific responses in studies of HIV infection and vaccine studies. However, dual production of IFNγ/IL-2 and increased proliferative capacity may be associated with better natural control of HIV infection. Here, we evaluated a novel FluoroSpot assay enabling the identification of dual IFNγ/IL-2 producing antigen-specific cells and compared it with intracellular cytokine staining by standard flow cytometry in individuals with natural control of HIV-infection.

Study plan: Cryopreserved PBMCs from HIV-infected Viraemic controllers and Elite controller patients (n = 8) identified in the WA HIV patient database in 2008 were screened for HLA-restricted HIV-specific responses using the dual colour IFNγ/IL-2 FluoroSpot assay. Positive peptide responses by FluoroSpot assay were further evaluated for intracellular IFNγ/IL-2 production by flow cytometry and results of the FluoroSpot assay and flow cytometry were compared.

Methods

Patient samples: No patients were receiving antiretroviral medication at sample time, although one patient experienced AZT and 3TC in 1996 and had discontinued treatment from 1999. 4/8 patients had <50 HIV RNA copies/mL; 7/8 patients expressed one of the protective HLA alleles: A*74, B*14, B*27, B*52 or B*57:01. The patient demographics are shown in Table 1.

Table 1: The patient cohort demographics. * p = 0.18 samples had no data on CD4/CD8 T cell counts or HIV load at sample time

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<tr>
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<th>Male</th>
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<th>Age at sample time</th>
<th>Median</th>
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<tr>
<td>HLA Class restricted HIV</td>
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<td>IFNγ/IL-2 FluoroSpot assay</td>
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Antigens: HLA class I restricted HIV peptides were synthesized by Invitrogen (Melbourne, Australia). Peptides were reconstituted in dimethyl sulfoxide to 10 mg/mL and stored at -20°C prior to dilution for use. 1 mg/mL stock solutions were prepared prior to screening by FluoroSpot assay and the same 1 mg/mL stocks were used to evaluate positive FluoroSpot responses by flow cytometry. Peptide final concentration was 5 mg/mL in the FluoroSpot assay and flow cytometry.

IFNγ/IL-2 dual colour FluoroSpot assay: Cryopreserved PBMCs were thawed and left to settle overnight at 37°C under CO2. Cells were counted, re-suspended in 1 ml of 55% media and anti-CD28 antibody (0.1 µg/mL) was added to enhance detection of IL-2 producing cells. IFNγ/IL-2 precoated plates (FluoroSpot kit-FSP-0010-120 Mahtech, Sweden) were washed and blocked for 10 min (10% FCS/RPMI-1640). 100,000 cells were dispensed in duplicate wells with either: HIV peptide (final concentration 5 µg/mL), positive anti-CD28 antibody (0.1 µg/mL), or negative (media alone) controls and incubated overnight (37°C under CO2). Next day, plates were washed and IFNγ and IL-2 cytokine spots were developed using the Mahtech kit reagents as illustrated in Figure 1a. Plates were read immediately on the AID Spot Reader with software 5.0B7337. IFNγ positive spots fluoresce green and IL-2 spots are red. Dual IFNγ/IL-2 spots were generated from a digital overlay of the green and red images of the same well and appear yellow (Figure 1b). Positive responses were defined as > 50 spots/million cells (SPU) after background removal.

Results: Firstly, the addition of anti-CD28 antibody to PBMC enhances the detection of IL-2 in the FluoroSpot assay (Figure 2).

Figure 2: The addition of anti-CD28 enhances the detection of IL-2 producing cells without increasing the background of IFNγ or IL-2 spots: 24 cells alone - no anti-CD28, 24 cells alone + anti-CD28, 24 cells and anti-CD28 antibody with no anti-CD28, 24 cells and anti-CD28 antibody with anti-CD28. Each figure is divided into 4 quadrants showing # images from the same well stained with the AID-SPOT reader: 1) green fluorescence IFNγ, 2) red fluorescence IL-2; 3) overlay of red and green images, 4) overlay with yellow dual cytokine producing cells highlighted.

IFNγ/IL-2 responses by FluoroSpot assay: IFNγ responses were detected to 67 of 276 HLA restricted epitopes evaluated across 8 patients (Figure 3a). Limited IL-2 responses were detected to 8 of 276 HIV peptides, although 43/55 IL-2 peptide positive responses were observed in one patient. No dual IFNγ/IL-2 antigen specific responses were detected. The majority of IFNγ responses were detected to Nef and Gag epitopes, median 112 (range 30.7-1701 SFU), n = 61. Dual IFNγ/IL-2 producing cells were detected in samples from all patients after anti-CD3 antibody stimulation with samples from 3/8 patients >700 SFU. Patients displayed wide variation in the frequency of detectable HLA-restricted HIV specific responses despite all patients naturally controlling their viral replication at sample time (Figure 3b).

Comparison of IFNγ responses detected by FluoroSpot and flow cytometry: Sufficient cells were available to evaluate 63/87 positive IFNγ FluoroSpot responses by flow cytometry. 46/63 peptides induced IFNγ by flow cytometry indicating a reduced sensitivity by <27%. When IFNγ responses for both assays were compared, the magnitude of the IFNγ response by FluoroSpot correlated with the magnitude of the IFNγ response by flow (Figure 4). However a low IFNγ response by FluoroSpot (response less than the median 105 (range 30-1710 SFU), n = 61) was highly likely to result in a negative response when the same sample was assessed by flow cytometry (Figure 2) p = 0.0016 Fishers exact test. Representative plots of high IFNγ (Figure 4b) and low IFNγ inducing peptides (Figure 4c) are shown in Figure 4.

Summary and Conclusions: Dual IFNγ/IL-2 responses were detected in samples from all patients after anti-CD3 antibody stimulation and antigen-specific IFNγ responses were detected by FluoroSpot assay. Greater breadth of IFNγ responses was detected by FluoroSpot when compared with flow cytometry. In conclusion FluoroSpot assay may be more promising for high throughput screening compared with flow cytometric methods. However, further evaluation of antigen-specific cytokine production by flow cytometric detection is required.

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