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 through-put 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.

n=8	Male / female	Age at sample time	Nadir CD4 T cell	Sample CD4 T cells*	Sample CD8 T cells*	Sample HIV*	Place of Infection
Median	6/2	43	595	1,026	840	<50	Perth -5, Burma -1 Zambia-1, Not stated -1
Range		30 - 62	414 - 832	660 - 1,537	506 -1254	40-38,019	

Table 1: The patient cohort demographics . * = 1/8 samples had no data on CD4/CD8 T cell counts or HIV load at sample time

Antigens: HLA class 1 restricted HIV peptides were synthesized by Invitrogen (Melbourne, Australia). Peptides were reconstituted in dimethyl sulphoxide to 10mg/mL and stored at stored at -20ºC prior to dilution for use in assay. 1mg/mL stock solutions were prepared prior to screening by FluoroSpot assay and the same 1mg/mL stocks were used to evaluate positive FluoroSpot responses by flow cytometry. Peptide final concentration was 5mg/mL in the FluoroSpot assay and flow cytometry.

IFNγ/IL-2 dual colour FluoroSpot assay Cryopreserved PBMC were thawed and left to settle overnight at 37ºC under $C0_2$. Cells were counted, re-suspended at 1 million cells/mL and anti-CD28 antibody (0.1µg/mL) was added to enhance detection of IL-2 producing cells. IFNγ/IL-2 precoated plates (FluoroSpot kit-FSP-0102-10 Mabtech, Sweden) were washed and blocked for ≥ 30minutes (10% FCS/RPMI-1640). 100µls of cells were dispensed in duplicate wells with either: HIV peptide (final concentration 5µg/mL), positive (anti-CD3 antibody [0.1µg/mL]) or negative (media alone) controls and incubated overnight (37ºC under $C0_2$). Next day, plates were washed and IFNγ and IL-2 cytokine spots were developed using the Mabtech kit reagents as illustrated in Figure 1a. Plates were read immediately on the AID *i*Spot reader with software 5.0 B7337. IFNγ positive spots fluoresce green and IL-2 spots are red. Dual IFNγ/IL-2 spots are 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 (SFU) after background removal.

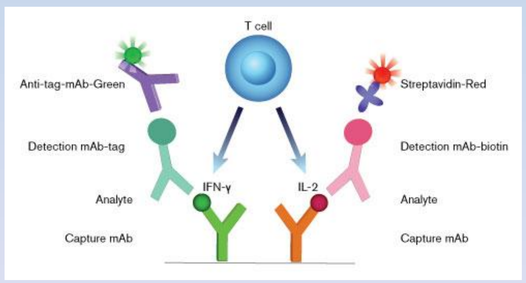


Figure 1a

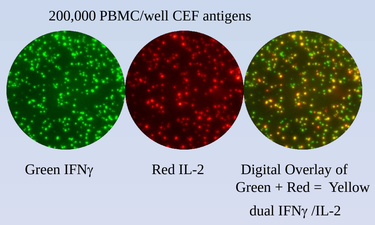


Figure 1b

Figure 1 shows a cartoon image of the FluoroSpot assay (1a) obtained from the web (1). Figure 1 b shows an example of positive green IFNγ spots, IL-2 red spots and dual IFNγ/IL-2 yellow spots generated after overnight CEF-stimulation of PBMC.

IFNγ/IL-2 flow cytometry assay

Peptide positive responses obtained by FluoroSpot screening were further evaluated by flow cytometry. 0.5 to 1.0ml of thawed PBMC (1million cells/mL) were stimulated for 6 hours with either: HIV peptides, SEB (1µg/mL) or media alone. Anti-CD49 (1µg/mL), anti-CD28 (1µg/mL) and Brefeldin A (5µg/mL) were added immediately. The reaction was stopped after 6 hours with cold PBS/1%FCS. Cells were washed, stained for membrane receptor antibodies (CD3 V450, CD8 PE CF594, CD45RA APC-H7, CCR7 PeCy7, CD57 APC, then fixed and permeabilised prior to performing intracellular staining with IFNγ AF488, IL-2 PerCp-Cy5.5, TNFα AF700 and Perforin PE. Samples were analysed on the Gallios flow cytometer (Beckman Coulter). Results are limited to intracellular production of IFNγ and IL-2 by CD8 T cells for the current presentation.

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

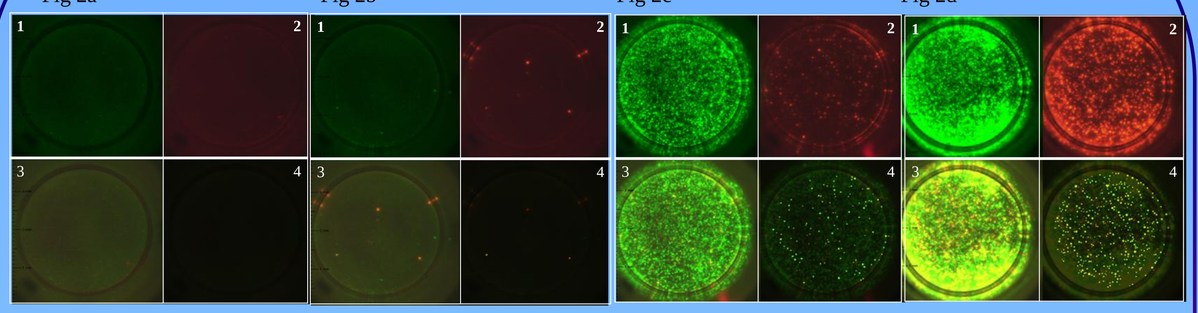


Figure 2. The addition of anti-CD28 enhances the detection of IL-2 producing cells without increasing the background of IFNγ or IL-2 spots: 2a (cells alone - no anti-CD28), 2b (cells alone + anti-CD28), 2c (cells + anti-CD3 antibody with no anti-CD28), 2d (cells + anti-CD3 antibody + anti-CD28). Each figure is divided into 4 quadrants showing 4 images from the same well captured by the AID iSpot 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 30-7,170 SFU), n = 67. 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).

Fig 3a

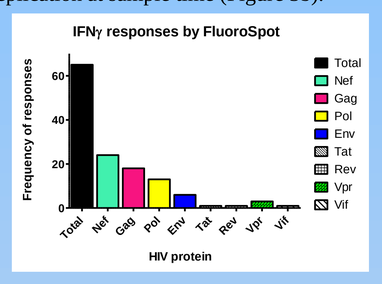


Fig 3b

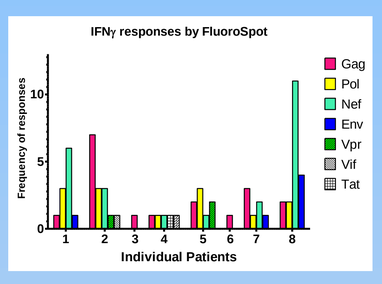
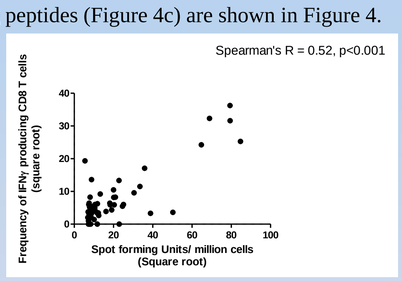


Figure 3 The frequency of IFNγ responses detected by FluoroSpot assay across the 8 patients (3a) and IFNγ responses observed in individual patients (3b)

Comparison of IFNγ responses detected by FluoroSpot and flow cytometry: Sufficient cells were available to evaluate 63/67 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 (a response less than the median 105 (range 30-7170) SFU, n= 61) was highly likely to result in a negative response when the same sample was assessed by flow cytometry (Table 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.

Fig 4a



Median IFNγ SFU	IFNγ+ flow	IFNγ- flow
<105	16	14
>105	28	3

Table 2

p = 0.0016
Fishers exact test

Fig 4b

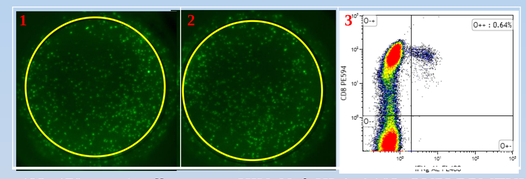


Fig 4c

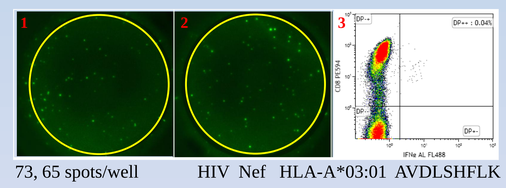


Figure 4 Comparison of IFNγ responses detected by FluoroSpot and flow cytometry assays (4a), a table showing fishers exact test (Table 2), duplicate FluoroSpot images (Figure 4b I & 2 of a high IFNγ inducing peptide with flow plot (4b 3) and a low IFNγ inducing peptide with flow plot (Figure 4c 1, 2 & 3)

Table 3 Results from patient cohort. Patients 6, 7 and 8 (red box) are now receiving therapy. Few peptides induced IL-2 cytokine by FluoroSpot thus negating comparative analysis of dual IFNγ/IL2 producing cells between FluoroSpot assay and flow cytometry. * samples with <1500 IFNγ SFU after anti-CD3 antibody stimulation, ** samples with <7500 IFNγ SFU after anti-CD3 antibody stimulation.

Patient	IFNγ Screen Fl-Spot	IL-2	HIV proteins recognised by FluoroSpot	IFNγ CD8 T cell by flow cytometry
1	11/15	Not detected	4	6/9
2	13/55	High background	6	13/13
3	1/32*	Not detected	1	1/1
4	5/19	1/19	5	0/5
5	8/28**	2/28	4	5/8
6	1/68*	Not detected	1	1/1
7	7/25	Not detected	5	4/7
8	19/34	5/34	5	16/19

Table 3

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 through put screening compared with flow cytometric methods. However, further evaluation of antigen-specific cytokine production by fluorometric detection is required.

References: (1) FluroSpot images in figure 1 were obtained from the website: http://en.wikipedia.org/wiki/File:Fluorospot_staining.png

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