A Dual Color ELISPOT Assay

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The ELISPOT assay is considered by many to be a gold standard for monitoring specific cellular immune responses. It can detect single cells secreting molecules of interest (1-3). This assay is highly sensitive, quantitative, easy to use and amenable to high throughput (4). Until recently assays using chromophores to detect the secretion of cytokines from single cells was limited by the difficulty of detecting mixed color spots (5;6).

Chronic viral infections such as HIV and hepatitis C infection, where the virus persists at high levels, are characterized by defective virus specific immunity. In virally infected individuals who spontaneously control their infection and maintain low viral loads such as infection with cytomegalovirus, Epstein-Barr virus or in slow progressor HIV-infected subjects, CD4+ T cells are more likely to maintain the ability to secrete IL-2 (7) and CD8+ T cells dual IFN-gamma and IL-2 secretion (8) than chronically infected HIV infected subjects with uncontrolled viremia. The typical response in HIV disease progressors by both CD4+ and CD8+ T cells is HIV-specific IFN-gamma secretion only. Betts et al. extended these studies by measuring additional functions such as secretion of MIP-1b, CD107a as a marker for lytic activity and TNF-alpha. They demonstrated that T cell responses to HIV can be polyfunctional and that HIV-infected slow progressors are more likely to have polyfunctional responses than subjects exhibiting HIV disease progression (9). The high throughput qualities of the ELISPOT assay make it a good choice for screening PBMC for repertoire specificity, response magnitude and breadth and how these parameters change with time or following therapeutic interventions. Therefore, a dual cytokine ELISPOT assay able to capture information on IFN-gamma and IL-2 secretion has potential relevance for studies on immune responsiveness in the context of viral infections such as HIV where disease outcome is associated with the antigen specific IL-2 and IFN-gamma/IL-2 but not IFN-gamma secretion.

In order to capture information on both IL-2 and IFN-gamma secretion simultaneously we designed a dual color ELISPOT assay (10). An advantage of using a dual color assay for detecting polyfunctional cellular responses is the requirement for half the number of cells to measure both cytokines together than would be needed to detect either cytokine alone, a factor that is an asset in human studies where blood volume restriction often limits cell availability. Another potential advantage the dual color

ELISPOT assay as an immune-monitoring strategy is that it is both practical and affordable in resource-poor settings where state of the art techniques such as polychromatic intracellular cytokine staining cannot be easily implemented. This assay detects three immunologically distinct T cell populations: IL-2 and IFN-gamma single secretors and dual cytokine secretors.

The dual color ELISPOT assay procedure begins with coating the wells of an ELISPOT plate with optimized concentrations of capture antibodies for both IFN-gamma and IL-2. Using HIV peptides sets available from the NIH AIDS Research Reference and Reagent Program as stimuli for cells from slow progressor HIV infected subjects we showed that functional responses for all three cytokine secretion patterns can be detected at an optimal input of 200 000 cells/well. Because IL-2 secreting cells are less frequent than IFN-gamma secreting cells, they seem to be the limiting factor for the lower end of the cell input number. Cytokine secretion is detected using the following sequential steps with washes in between: 1) a mixture of detection antibodies including a biotin conjugated anti-IL-2 and an FITC-conjugated anti-IFN-gamma antibody (Ab), 2) a mixture of enzymatic conjugates including horseradish peroxidase conjugated anti-FITC monoclonal Ab (Jackson ImmunoResearch Laboratories, Ltd, West Grove PA) and streptavidin alkaline phosphatase (Jackson ImmunoResearch Laboratories, Ltd.), 3) Vector Blue substrate solution (Vector Laboratories, Burlingame, CA) and 4) amino ethyl carbazole (AEC, Sigma-Aldrich). These steps result is the development of red spot from cells secreting IFN-gamma only, blue spots from cells secreting IL-2 only and purple spots from cells secreting both cytokines (Fig 1). The use of Vector Blue, which produces a medium blue color is essential to the discrimination of the purple mixed color spots.

Figure 1A

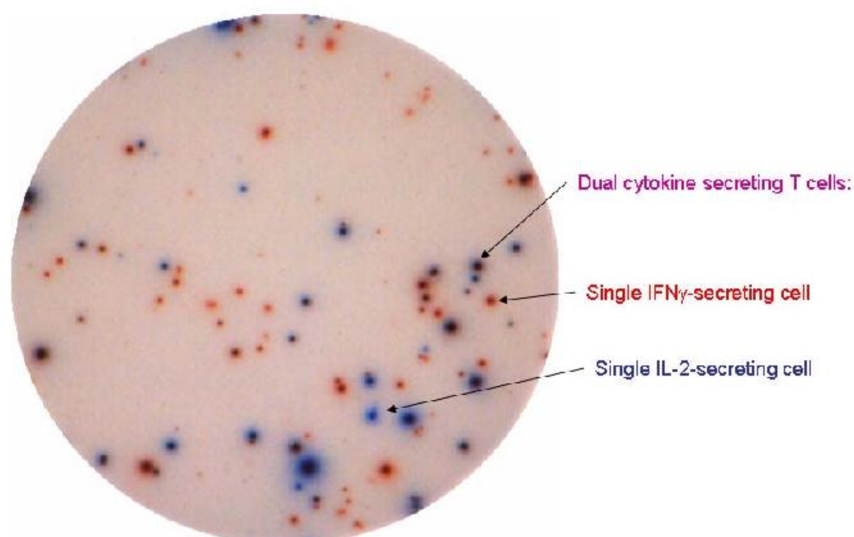

Figure 1B

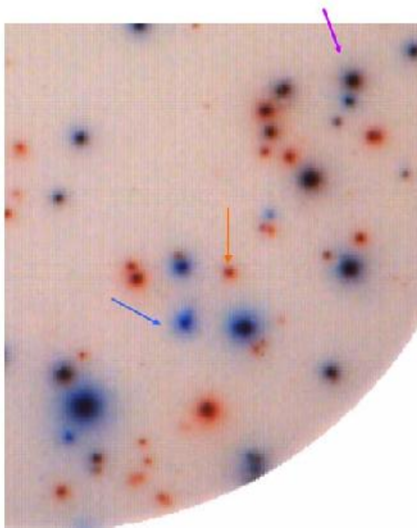

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Figure 1. Well from a dual color ELISPOT assay detecting cells secreting IFN-gamma only, IL-2 only and both IFN-gamma and IL-2. PBMC (2 x 105 cells) from an HIV-infected slow progressor were incubated with a stimulatory peptide on Multiscreen IP plates for 28 hours. Red spots correspond to cell secreting IFN-gamma, blue spots to cells secreting IL-2 and purple spots to cells secreting both IFN-^{3} and IL-2. Panel A shows spot corresponding to the 3 cytokine secretion patterns, Panel B shows a 3-fold magnification of the well in Panel A with color coded arrows pointing to 3 individual spots.

Sensitivity and compensation thresholds are established using at least two positive control wells (PBMC stimulated with a positive control peptide or peptide pool and anti-CD3), one coated with anti-IFN-gamma capture Ab only (red color spots) and the other with anti-IL-2 capture Ab only (blue color spots). Sensitivity is set for each color separately. Detection of blue and red colored-spots in anti-IFN-^{3} and anti-IL-2 only coated wells, respectively was then compensated out using the automated spot reader softwares imbedded algorithms. A dual spot was determined as the intersection of two single color thresholds. Counts obtained from the software were then audited well by well. Reading can be done using a CTL Immunospot reader or a Zeiss ELISPOT reader.

We determined that by 28 hours of stimulation PBMC cytokine secretion for both IFN-gamma and IL-2 was optimal in this assay format. In addition, the within- and between-assay variations were both within the range reported by others for single color ELISPOT assay (11). Cell subset depletion experiments demonstrated that the responses detected could be either CD8+ or CD4+ T cell dependent (10).

Other groups have designed multiple-color ELISPOT (5;6) or FLUOROSPOT assays (12;13). The assay developed by Gazagne et al. is dependent on fluorescent antibodies and results are viewed on an instrument equipped with fluorescent reading capability. Although having advantages, this system requires access to instrumentation able to detect different fluorescent signals simultaneously and fluorescent antibodies. A colorimetric system may be more flexible and adaptable to different laboratory settings. Although, Okamoto et al. and Karulin et al. also reported the development of a dual cytokine ELISPOT assay they focused on its application for evaluation of Th1/Th2 cytokine balance. The assay developed by Okamoto et al. was for dual IL-2 and IL-4 detection, but few dual color spots were reported. This could have resulted from the cytokine pair selected or from difficulties in interpretation of mixed color spots. Karulin et al used NBT/BCIP (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) as a substrate for alkaline phosphatase conjugated Abs. In our hands this substrate produces dark blue/purple spots that when combined in a dual color ELISPOT format with red spots developed by AEC, make it difficult to distinguish spots from cell secreting both cytokines, i.e. the red color is hidden by the dark blue/purple color.

Although the detection of IL-2 was not affected by capture of both cytokines in a single well, we have shown that when capturing both IL-2 and IFN-gamma the magnitude of IFN-gamma was decreased by an average of around 16%. This phenomenon has been reported by others and may result from the sequestration of IL-2 (which is known to induce IFN-gamma production) by capture Abs rather than biochemical interference in the test system such as competition of capture Ab binding to the membrane (14;15).

In conclusion, we have developed and optimized a dual color ELISPOT for the simultaneous detection of antigen-specific IFN-gamma and IL-2 secreting cells. This method can be adapted to detect other cytokine combinations and is amenable to immune monitoring in large-scale clinical trials.

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