

Evaluation of cell-mediated immunocompetence using ELISPOT assay

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Abstract

The systemic suppression of immunity is commonly observed in cancer patients. Since immunotherapeutic treatments are targeted to induce effective immune responses against tumor, the capability of patient cell-mediated immunity (CMI) may make a major difference in the immunotherapy outcome. The skin test for hypersensitivity to recall antigens, widely used for patient selection in immunotherapy clinical trials, does not provide a quantitative measure of reactivity and often generates false positive as well as false negative results. We evaluated PBMC from a group of 21 normal donors for the reactivity to 6 common recall antigens (tetanus toxoid, candida albicans, cytomegalovirus (CMV), tuberculosis purified protein derivative (PPD), mumps, and varicella zoster virus (VZV)), mixture of CEF (CMV, Epstein-Barr virus (EBV), and influenza) peptides, and phytohemagglutinin (PHA) using IFN γ ELISPOT assay. The donors demonstrated various patterns and levels of antigen recognition. Individual patterns were highly reproducible from day to day. Most of the donors demonstrated reactivity to mumps (100% of donors responded, average 139 IFN γ secreting cells/300,000 PBMC (Spot Forming Cells, SFC)) and VZV (86%, average 80 SFC). CMV induced the highest level of reactivity (71% responded, average 462 SFC). Statistical analysis of the results enabled calculations of the positivity thresholds for the T-cell immune responses to these antigens. Such thresholds may be useful for the real time evaluation of cell-mediated immunocompetence in prospective clinical trials participants. We suggest that incorporation of this extended and more quantitative methodology may enable selection of an appropriate patient population likely to benefit from the immunotherapy and increase a frequency of positive responses to the treatment.

Materials and Methods

PBMC Isolation

Blood samples were obtained from 21 normal volunteers. 20-30 mL of blood was collected in sodium heparin vacutainers, and PBMC were isolated by density separation on Ficoll gradient. Cells were counted and cell viability was evaluated using the Guava PCA Flow cytometer (Guava Technologies, Hayward, CA).

Antigens

The following antigens were used for stimulations: Tetanus Toxoid (5 IU/mL, Cylex, Columbia, MD, or Calbiochem, San Diego, CA, at 20 ng/mL), Candida Albicans (20 μ g/mL, Cylex or Greer, Lenoir, NC), CMV (10 μ g/mL, Cylex), PPD (0.5 U/mL, Cylex), Mumps (36 μ g/mL, Cylex), VZV (17.6 μ g/mL, Cylex), CEF peptide pool (2 μ g/mL of each peptide, CTL). Stimulation with PHA (2.5 μ g/mL, Sigma, St. Louis, MO) was used as a positive control.

ELISPOT Assay

The PVDF-bottomed 96-well plates (MSIPN41W50, Millipore, Billerica, MA) were coated overnight at 4°C with anti-IFN γ capture antibodies (4 mg/mL, M700A, Pierce, Rockford, IL) in sterile PBS. Plates were washed once with PBS and blocked for at least 2 hours with PBS+4% BSA, then washed three times with PBS. 200,000-300,000 PBMC/well were plated in 100 μ L of CTL serum-free media (Cellular Technology Ltd., Cleveland, OH). Antigens were added in 100 μ L volume and tested in triplicate. Plates were incubated at 37°C and 5% CO $_2$ for 24-2 hours, then washed twice with PBS and three times with 0.05% Tween-PBS. Biotinylated anti-IFN γ antibodies (1 mg/mL, M701B, Pierce, Rockford, IL) in 1% BSA-PBS buffer were added to the plates and plates were incubated overnight at 4°C in a humidity chamber. Plates were washed three times with PBS-Tween and once with PBS, and then Streptavidin HRP-conjugate (BD Biosciences, San Diego, CA) was added at a 1:1000 dilution in 1% BSA-PBS and left for 2 hours at room temperature. Plates were washed twice with Tween-PBS and 4 times with PBS. The AEC substrate (BD Biosciences) was prepared and color reaction was developed for 5-15 minutes after addition of substrate to the plate. The numbers of spots in wells were evaluated using the ImmunoSPOT ® Analyzer Series 3B (Cellular Technology Ltd., Cleveland, OH).

Results

I. Tested 21 normal donors by IFN γ ELISPOT; five of them were tested at several time points. The donors demonstrated distinct patterns and levels of antigen recognition (Fig. 1), which were highly reproducible from day to day (Fig. 2).

II. Evaluated responses to the antigens using the following criteria for positivity:

- Greater than 2 x background
- At least 2 StDev above background
- Background + StDev $_{\text{background}} <$ reading - StDev $_{\text{reading}}$

III. Positivity thresholds for each pathogen were calculated using 2 StDev cutoff above the average value for negative donors

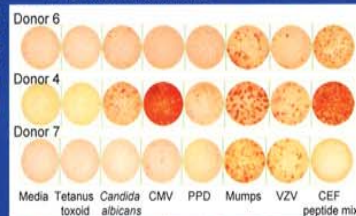


Fig. 1 IFN γ ELISPOT profiles of donors 4, 6, and 7. The donors demonstrate distinct patterns and levels of antigen recognition

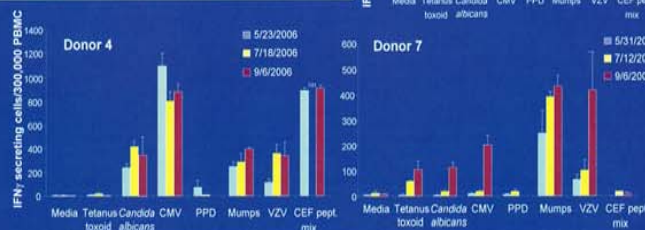


Fig. 2 Changes in the IFN γ ELISPOT profiles of Donor 1, Donor 4, and Donor 7 in a period over 3 months. Donor 7 was immunized with tetanus vaccine on 6/16/2006

Table 1. Positivity thresholds for seven antigens

Antigen	Media	Tetanus Toxoid	Candida Albicans	CMV	PPD	Mumps	VZV	CEF pept. mix
Average 21 donors	9	9	57	301	5	139	68	293
StDev	12	16	98	515	12	114	90	300
Donors positive		6	12	15	1	21	18	19
% positive donors		29	57	71	5	100	86	90
Average positive		25	83	462	55	139	80	363
StDev positive		22	120	560	NA	114	93	336
Average negative		3	4	1	3	NA	1	4
StDev negative		4	6	2	5	NA	2	0
Positivity cut off (2 StDev above average negative) after subtraction of background								
Cut off		11	16	5	12	NA	5	5

Conclusions

- The donors demonstrated distinct patterns and levels of antigen recognition, which were highly reproducible from day to day
- Most donors reacted to mumps (100% of donors responded, average 139 SFC) and VZV (86%, average 80 SFC). CMV induced the highest level of reactivity (71% responded, average 462 SFC).
- ELISPOT assay is an excellent tool for evaluation of cell-mediated immunocompetence
- We suggest that the incorporation of ELISPOT as an extended and more quantitative methodology may enable the selection of an appropriate patient population likely to benefit from immunotherapy and increase frequency of positive responses to the treatment.



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