

## ELISPOT

- Recognition of MHC-peptide complex by TCR will lead to activation of T cells and production of cytokines detected by Elispot.

- One of the most sensitive technique to measure cytokine

- Secreted cytokines are directly captured by antibodies coated on the Elispot plates which will avoid diffusion and dilution of the cytokine in the supernatant, degradation by proteases or binding to soluble cytokine receptors possibly present in the supernatant.

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Each spots represents a footprint of the original cytokine-producing cells (Czerkinsky et al 1988)	Plates for the coating of capture antibodies   I Nitrocellulose (e.g. HA-Multiscreen Plates, Multiscreen Immobilon, Millipore)   - High capacity to bind antibodies and proteins -   - High sensitivity -   - Spots : small size without modification with time. -   - Risk of Background (high sensitivity, nitrocellulose may sometimes activate the cells). -   II PVDF (Polyvinylidene diffluoride) -   - Low binding capacity of proteins (need to moisten the plates with ethanol) -   - Less background - -   III Plastic plates (Ex : Maxisorb plates) -   - Intermediate capacity to bind proteins - -   - Less expensive than nitrocellulose or PVDF - Rapid appearance of spots which increase in size and may fusion leading to difficulties for the counting.
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## Antibodies for Elispot

- The binding capacity of plates for Elispot is generally lower than for Elisa plates.

Use higher concentrations of antibodies for the coating

- Capture antibodies will be in close contact with cells : avoid antibodies contaminated with LPS or containing azide or other potential activators of cells.

- First list of antibodies available for Elispot (McCutcheon M . J Immunol Methods 1997)

- Different companies sell antibodies for Elispot (Diaclone, BD Pharmingen, Mabtech, R&D...) 2<sup>rd</sup> PSU Workshop on Tumour Immunology and Immunotherapy, December 15-20, 2003. Prince of Songkla University, Université Pierre et Marie Curie and Institut Paster



1-2 hours at 37°C with : - PBS 5% Bovine Serum Albumin (BSA)

SATURATION STEP

### - PBS 2% Milk

Avoid saturation with Fetal Calf Serum or human serum because it may contain proteins which will non specifically activate the cells leading to background.

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#### Antigen presenting cells or not during the Elispot assay

For CD4 T cells Absolute requirement of antigen presenting cells ( PBMC, dendritic cells, monocytes or cells expressing MHC class II molecules plus antigen) For CD8 T cells (human) Different APC have been tested: - T2 + β2 microglobuline TAP deficient cells which can be loaded with high concentrations of peptides No HLA class II molecules (risk of NK cytotoxicity or alloreactivity)

Since CD8 T cells express MHC class I molecules, direct loading with peptides without APC have been tested.









### **Direct Elispot:**

- Cells stimulated or not are directly put in the Elispot plates
- To sensitize the test, cells (CD8 or CD4-T cells) may be purified

### Indirect Elispot:

- Cells are first *in vitro* amplified with antigen for 1 week and then tested in an Elispot assay
- Low dose of IL-2 (10U/ml) are added during this amplification step

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Elispot may reveal the production of many cytokines Each of them present some peculiar properties and characteristics which have to be taken into account for the Elispot assay - Optimal results are obtained with time of incubation: . less of 24 hours: IFNy, IL-2, IL-5, IL-6 . more than 24 hours: IL-10, IL-4 - Size of spots Small (IL-4, TNFa) Large (IFNy) - Background often observed with IL-10 because adhesion of monocytes in the plates results in IL-10 production

# ADVANTAGES OF THE ELISPOT TECHNIQUE

- Detect functional T lymphocytes

- May be used even without the knowledge of MHCpeptide complex recognized by T cells (APC pulsed with pool of peptides or vectorized antigens, tumour cells as target).

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- High sensitivity

- Possible automation

## LIMITS OF THE ELISPOT TECHNIQUE

- DETECTS ONLY NON ANERGIC T CELLS WITH THE ABILITY TO PRODUCE THE CYTOKINE TESTED.

- DOES NOT ALLOW THE PHENOTYPING OF T CELLS PRODUCING THE CYTOKINE
- DOES NOT ALLOW THE SORT OR PURIFICATION OF SPECIFIC T LYMPHOCYTES PRODUCING THE CYTOKINES.

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## INDICATIONS OF THE ELISPOT TECHNIQUE

- Analysis of the frequency of T cell precursors producing cytokines (TH1, TH2, TH3...). Analysis of the polarisation of T lymphocytes.

- Identification of specific T lymphocytes with characterization of their polarisation.

NEW DEVELOPMENT USING THE ELISPOT PRINCIPLE

Rationale for the development of a multi-cytokines Elispot

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- In most cases, the Elispot procedure detects only one secreted cytokine, which constitutes a major drawback for the characterization of the various T cell subpopulations.

- For example, IFNy can be secreted by type 0, type 1 and Tr1 T cells, and IL-10 is produced by both type 2 and Tr1 cells.

- The detection of T lymphocytes simultaneously producing multiple cytokines will therefore help to better characterize subpopulations of T cells.

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Other groups have encountered difficulties in interpreting these mixed coloured spots when immuno-enzymatic dual colour Elispot assays were used. - Karulin AY et al J Immunol 2000 - Okamoto Y. et al Immunopharmacology 1998

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# Development of a fluorospot assay to detect single cells that simultaneously produce multiple cytokines

- Fluorescence will provide better discrimination and characterization of double cytokine producing cells than does an enzymatic reaction

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- Availability of a large range of fluorophores.















The number of cells producing IFNy and IL-2 in the single colour fluorospot matched the sum of the frequencies of the single producers and double producers of IFNy/IL-2 detected in the two colour assay

	Number of spots per well	
Fluorospot IL-2	<b>48</b> [47,	7-48,3]
Fluorospot IFN <del>y</del>	<b>59</b> [58,	7-59,3]
Dual color Fluorospot IL-2 / IFNγ	Single IL-2 spots: Single IFNy spots: Dual spots:	<b>46</b> [44,6-47,4] <b>58</b> [56,3-59,8] <b>4</b> [3-5]

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After activation of PBMC, the same frequency of double IFN $\gamma$ /IL-2 producing cells was observed by the fluorospot assay and cytometry



Dual color IL-2/IFNy fluorospot

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Intra-cytoplasmic IL-2 and IFNγ detection by cytometry

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## Detection of Tr1 regulatory T cells

- The hallmark of Tr1 cells is their production of high levels of IL-10, moderate amounts of IFN $\gamma$  and IL-5, but little or no IL-2 or IL-4 (Roncarolo et al., 2001).

- Tr1 cells are involved in down-regulation of immune responses *in vitro* and *in vivo*, partly via production of the immunosuppressive cytokines IL-10 and TGF $\beta$  (Roncarolo et al., 2001).

- Immunization of patients with antigen-pulsed immature dendritic cells has been reported to elicit Tr1 specific T cells with a suppressive activity on effector type 1 T cells (Dhodapkar et al., 2002).



The fluorospot assay will be useful for detailed analysis of T lymphocytes in various disease states (allergy, cancer, infectious diseases), in which an imbalance of T cell subpopulations is suspected but also for a better characterization of a polarized specific immune response.

It will also help to better identify subpopulations of T cells.

Gazagne A et al. J Immunol Methods 2003 2<sup>rd</sup> PSU Workshop on Tumour Immunology and Immunotherspry, December 15-20, 2003. Prince of Songlisk University, University Enter et Marie Curie and Institut Pasteur



