The 3rd PSU International Teaching Platform

Immunological Activity Determination of Medicinal Plants

Jointly organized by

Prince of Songkla University, Université Pierre et Marie Curie (Paris 6) and Institut Pasteur

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Faculty of Medicine, Prince of Songkla University,

Hat Yai, Songkhla, Thailand

Lab Course Handout

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Immunological Activity Determination of Medicinal Plants

Introduction

The purpose of this 6-day training is to study the immunomodulatory effect of a natural substance, in particular its action on T lymphocyte activation. We have chosen to study the action of a bacterial toxin suspected to target T cell activation. For this purpose, the handout gives a background on the effect of a related bacterial toxin, the effect of which has already been characterized. After going through this background, as well as the different available biomaterial, reagents and experimental protocols, you will be asked to implement a true scientific process: problem definition, choice of strategy, experimental design and implementation, data collection and analysis, interpretation and discussion.

Due to time constraints, it is suggested that the participants arrange in groups of six or eight people and focus their attention on a limited number of tests as indicated by your instructors. It is recommended that each group consults with the other groups in order to address different and complementary questions. At the end of the week, the methodological approach and results of the different groups will be presented for group discussion. This should allow all participants to familiarize with standard and up-to-date techniques and procedures commonly used in immunology (flow cytometry & ELISA), as well as allow them to make a better and critical use of the theoretical concepts and techniques learnt during training or from the literature.

This handout gives, in a first part, a presentation of the subject which should lead you to hypotheses which you will try to answer through experiments. In the second part, you will find a series of experimental protocols describing the techniques which you can implement as well as the list of the available biologic material and reagents. It will be up to you to choose among these protocols, by adapting them if necessary.

Consequently, you will take care to properly document your strategy, experimental design and implementation. This will allow you to report to other groups at the end of the week so that critical comparison of selected strategies and techniques can be made. The objective of this reporting is to achieve capacity to approach the problem, to set hypotheses, to implement experiments to test these hypotheses, to analyze and to discuss the obtained results.

Introductory background

Upon activation through engagement of the T cell receptor which recognize the MHC/peptide complex together with the appropriate second signal provided by costimulatory molecules, adhesion molecules, cytokines… T cells are activated. Upon this activation, they can proliferate and/or secrete cytokines which in turn will provide help for other cell type activation.

A number of molecules derived from natural products, in particular those found in herbal and traditional medicine, exhibit immunomodulatory properties: they can either activate or inhibit activation of T cells (or other cell type of the immune system). Molecules that can activate T cell subsets are typically classified as antigens, superantigens or mitogens. Antigens are specifically recognized in the context of MHC molecules; therefore the frequency of responding T cells is very low, in the range of 10^{-3} to 10^{-5}. On the contrary, mitogens (such as PHA or ConA) will activate all T cells regardless of their specificity. Such a massive activation will usually turn into unresponsiveness by exhaustion.

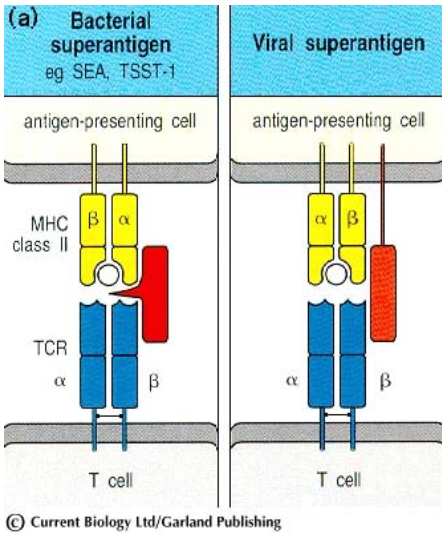


Figure 1: Superantigens bind directly to T cell receptors (TCR) and to MHC molecules.

Superantigens comprise a group of molecules, produced by different microorganisms such as bacteria (Staphylococci, Streptococci, Yersinia, Mycoplasma) and viruses. They have been implicated in the pathogenesis of several diseases including food poisoning, septic shock and some autoimmune syndromes. Superantigens can modulate the activity of T cells but with intermediate properties between those of mitogens and conventional antigens. Superantigens can activate T cells in an MHC-unrestricted, unspecific way by interacting directly with the TCR-Vβ chain and the MHC class II molecule outside the peptide-binding groove area (see

Figure 1). Bacterial superantigens have been shown to bind certain TCRBV regions. Therefore, the frequency of T cell responding to a superantigen is typically around 1-20%. Here again, activation of a TCRV-specific T cell subset will result in exhaustion of the activated T cells. This can lead to hyporesponsiveness to a secondary challenge by the same superantigen or a specific antigen.

The group of the *S. aureus*-derived superantigens mainly comprises the enterotoxins A-D (SEA-D) and the toxic shock syndrome toxin-1 (TSST-1). The influence of bacterial superantigens on the immune system is well investigated particularly in animal models. After intravenous injection of superantigen (1 μg of SEB) in mice, almost all $V\beta8^+$ T cells become activated, leading to a substantial production of cytokines. In the initial phase of T cell activation, 1-4 hours after injection of superantigens, TNF-α, IL-2, IL-4, IL-6 and IFN-γ are produced in the serum. This initial phase in followed by a deletion and anergy of SEB-reactive cells (see Figure 2). T cells activated by superantigens undergo apoptosis after a distinct number of cell divisions[1].

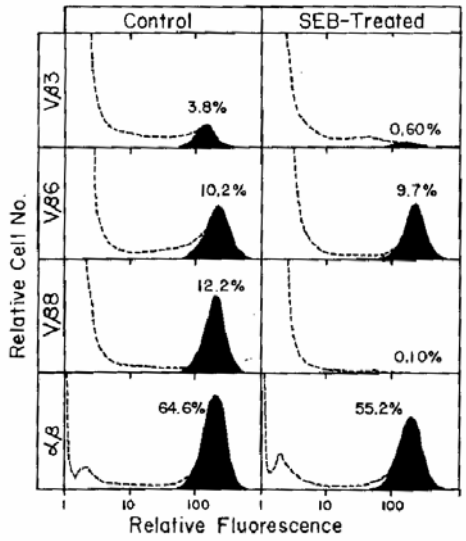


Figure 2: Effect of in vivo SEB treatment in mouse[2].

The interaction between superantigen and T cells does not always lead to activation, proliferation, and expansion of T cells that express the appropriate TCRV elements. The activation of T cells requires two signals: one is delivered by TCR engagement and the

[1] Miethke T. *et al.* (1995) *Int Arch Allergy Immunol* 106:3-7; Renno T. *et al.* (1999) J Immunol 162:6312-5.

[2] White J. *et al.* (1989) *Cell* 56:27-35.

second happens via the interaction of APC-associated costimulatory molecules with their respective ligands on the T cell. In the absence of relevant costimulatory signals, the engagement of the TCR by superantigen induces T cell anergy. Conversely, in the presence of elevated levels of a cytokine, such as TNF-α or IFN-γ, reengagement of the TCR by superantigen in preactivated T cells can lead to a process of programmed cell death (known as apoptosis) which can be followed by selective deletion of superantigen-specific T cells (*Figure 3*).

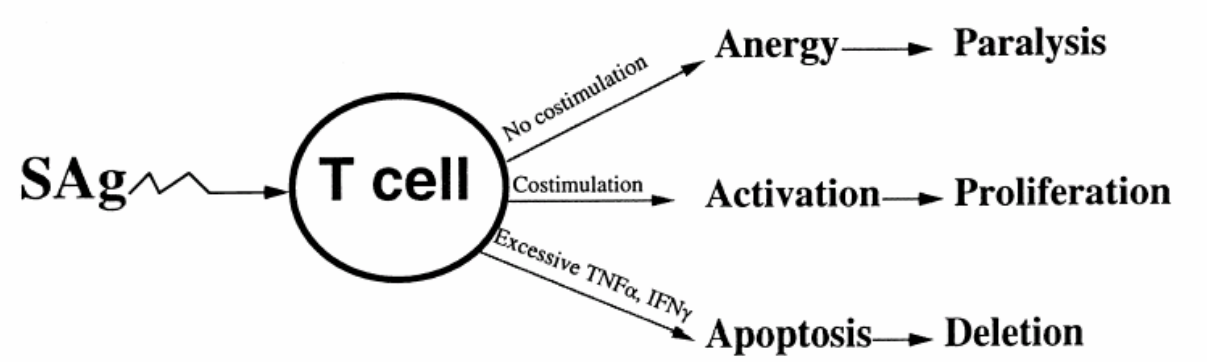


Figure 3: Possible fate of T cells that encounter superantigen (SAg) *in vivo*.

We have isolated a new bacterial toxin which is suspected to show immunomodulatory properties. Based on the available biomaterial and reagents (Annexe B), as well as technique protocols presented in Annexe C, you will design relevant experiments in order to characterize the properties of this bacterial toxin. A tentative schedule for the following days is provided in Annexe A; it will be completed during the first with your instructors.

For additional information on relevant techniques:

- Annexe D : Few notions in flow cytometry and intracellular cytokine detection, Sylvie Garcia
- Annexe E : Detection of functional T lymphocytes by ELISPOT, by Eric Tartour, Professor of Immunology, Université Renée Descartes – Paris 5

Annexe A : Tentative schedule

Date	***Activities***
Dec. 5, evening	- *Isolation of mouse splenocytes* - *Activation in culture with PMA/Ionomycin/BrefeldinA*
Dec. 6, Morning	- Workshop presentation and objectives (1h) - Problem analysis and rationale, choice of strategy (3h)
Dec. 6, Afternoon	- Experimental design and planning (2h) - Start of experiments: *Isolation of mouse lymphocytes and activation in culture*
Dec. 7, Morning	- Introduction to flow cytometry (S. Garcia) (1h30) - Experimental design and planning
Dec. 7, Afternoon	- Experiments
Dec. 8, Morning	- Experiments - Presentation on Quantitative PCR strategies
Dec. 8, Afternoon	- Experiments
Dec. 9, Morning	- Experiments
Dec. 9, Afternoon	- Experiments
Dec. 10, Morning	- End of experiments - Data analysis
Dec. 10, Afternoon	- Data analysis - Preparation of group presentations
Dec. 11, Morning	- Group presentations - Discussion of results
Dec. 11, Afternoon	- General discussion and conclusion

Annexe B : Reagents, solutions, disposable and equipment

Note: Please use care to keep the biological reagents at -20°C or +4°C as indicated.

Animals

- 8-week-old BALB/c mice

Antibodies

- Anti-mouse CD4 FITC antibody (BD-Pharmingen Ref. 553047)
- Anti-mouse CD4 APC antibody (BD-Pharmingen Ref. 553051)
- Anti-mouse IFNγ PE antibody (BD-Pharmingen Ref. 554412)
- Anti-mouse CD8α Percp antibody (BD-Pharmingen Ref. 553036)
- Anti-mouse CD3ε APC antibody (BD-Pharmingen Ref. 553066)
- Anti-mouse Vβ6 FITC antibody (BD-Pharmingen Ref. 553193)
- Anti-mouse Vβ8 FITC antibody (BD-Pharmingen Ref. 553861)
- Mouse IFNγ ELISA Ready-SET-Go! (eBiosciences 88-7314-22)

Culture media, solutions and reagents

- Bovine serum albumin (Sigma Ref. A7030)
- CFSE (Interchim, 25mg, ref: C1157)
- Concanavalin A (Sigma Ref. C5275)
- Demineralised water
- DMSO (Sigma Ref. D4540)
- Ethanol 70% (Prolabo Réf : UN1170)
- Fetal calf serum (ATGC Biotechnologies Ref. 04-001-1B)
- Ionomycin calcium salt (Sigma Ref. I-0634; 1 mg)
- Brefeldin A (Sigma Ref. B7651; 5 mg)
- NaN_3 (Sigma Ref. S-2002)
- PBS (50 tablets Sigma Ref. P4417-50 or 1X Invitrogen Ref. 14190094)
- Phorbol 12 Myristate 13 acetate (PMA; Sigma Ref. P-8139; 1 mg)

- Saponin (Sigma Ref. S-7900)
- Tween 20 (Merck Eurolab Ref. 822184)
- β2-mercaptoethanol
- RPMI 1640 culture medium with glutamax (Invitrogen Ref. 61870-010)
- Hepes buffer 1M solution in distilled water
- Penicillin-Streptomycin 10000U/10 mg/mL (Invitrogen)

Disposables

- 1.5 mL microfuge tubes
- Absorbent paper
- Beakers (for liquid waste)
- Centrifuge tubes "Falcon" 15 mL
- Centrifuge tubes "Falcon" 50 mL
- FACS tubes 5 ml (Falcon Ref. 352052)
- Gloves latex L
- Gloves latex M
- Gloves latex S
- Micropipette tips (200-1000 µL)
- Micropipette tips (20-200 µL)
- Sterile Petri dishes (for organ collection and isolation of lymphocytes)
- Sterile pipettes 10 mL
- Sterile pipettes 5 mL
- Tissue culture flasks 75 cm^3
- Cell culture plate (x6 or x12 wells)

Equipment

- Dissection tools (scissors, forceps…)
- Cell culture CO_2 incubator
- Cell culture hood

- ELISA plate reader
- Flow cytometer (FACScalibur, Becton Dickinson)
- Incubator (37°C)
- Refrigerated centrifuge (for 15/50 mL tubes)
- Benchtop centrifuge
- Malassez cytometer
- Microfuge tube racks
- Micro-pipettes 1000 µL
- Micro-pipettes 200 µL
- Micro-pipettes 20 µL
- Microscope
- Multi-channel pipettes
- Vortex

Annexe C : Detailed protocols

Analysis of T cell activation
Measure of proliferation and cytokine detection

a. Isolation of mouse splenocytes

a-1. One mouse is killed by exposition to CO_2 during at least 5 minutes.

a-2. The animal is dissected and the spleen is taken.

a-3. The spleen is dilacerated in PBS 1X and the cell suspension is transferred to a 15 mL tube.

a-4. After 5 minutes, the cell suspension is decanted to a new 15 mL tube.

a-5. Centrifuge (5 min at 300 x *g*) and wash twice in PBS 1X. Carefully remove the supernatant.

a-6. Resuspend in 10 mL of complete RPMI culture medium (500 mL RPMI w/Glutamate supplemented with 50 mL fetal calf serum, 5 mL streptomycin/penicillin antibiotic solution, 5 mL Hepes 1M and 1 mL β2-mercaptoethanol).

a-7. Count cells on a hemocytometer.

b. Cell culture and activation

This section describes the cell culture step that you will implement in order to test the effect of your molecule with regard to T cell activation and cytokine production. It is therefore dependent upon your choice of experiment, as well as the nature and number of controls.

This cell culture step will be performed on 12- or 24-well cell culture plates in 2 mL culture medium for each well, as follows:

b-1. For analysis of cell proliferation with CFSE, see CFSE labelling protocol section c below "Measure of proliferation by CFSE staining" before proceeding.

b-2. Dilute cells with RPMI culture medium to $2x10^6$ cells/mL.

b-3. Distribute 1 mL of cells per culture well.

b-4. Depending on the conditions to be tested, add 1 mL medium with or without "additives" to the appropriate final concentration:

- PMA (50 ng/mL) & Ionomycin (500 ng/mL)
- ConA (1 µg/mL)

- Toxin X (typical concentration: 1 µg/mL)

b-5. Incubate flasks at 37°C, 5% CO_2.

b-6. For intracellular staining, add Brefeldin A (10 µg/mL) will be added later between 4 to 8 hours before cell collection and staining.

N.B.: Brefeldin A if a fungal metabolite which disrupts the structure and function of the Golgi apparatus. Therefore, protein secretion is inhibited and newly synthesized proteins accumulate inside de cells. Since it is toxic, Brefeldin A should not be added too long (NOT longer than 10 hours; then fix the cells).

N.B.: Brefeldin A should NOT be added when testing for cytokine production by ELISA or ELISPOT.

c. Measure of proliferation by CFSE staining

CFSE (carboxyfluorescein diacetate, succinimidyl ester) is a fluorescent dye that is widely used for the analysis of cell generation and proliferation. This fluorescent SE (succinimidyl ester) covalently couples to both intracellular and cell-surface proteins by reaction with lysine side chains and other available amine groups. When cells divide, the SE labeling is distributed equally between the daughter cells, which are, therefore, half as fluorescent as the parents. As a result, each successive generation in a population of proliferating cells is marked by a halving of cellular fluorescence intensity that is readily followed by flow cytometry. Using flow cytometric analysis of CFSE labeling, one can resolve up to ten successive generations of lymphocytes (see Figure 4).

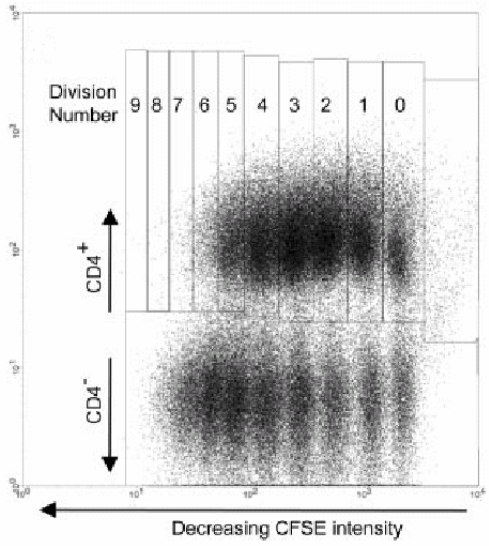


Figure 4: Mitogen-induced proliferative responses measured using CFSE incorporation.

See Annexe D : Few notions in flow cytometry and intracellular cytokine detection, Sylvie Garcia for further information.

c-1. Prepare the stock solution of CFSE in DMSO at 5 mM. Aliquot in small volume and keep it frozen at –20°C.

c-2. Resuspend the cells at 2.10^7/mL in PBS (make sure that the PBS is suitable to the cell survival – good pH).

c-3. Add the same volume of CFSE at 5 µM (2X=1/1000) and mix gently.

c-4. The final concentration of cells will be 10^7/mL and of CFSE 2.5 µM.

c-5. Incubate for 10 min at room temperature, agitating gently from time to time.

c-6. Wash once in a big volume of complete medium (5 % FCS).

c-7. Resuspend the cells at 2.10^6/mL of complete medium.

c-8. After cell culture with the appropriate stimuli (see above), cells can be analyzed for CFSE staining with a flow cytometer.

NB: Check by Facs that the totality of the cells is stained as an homogeneous peak.

d. IFNγ detection by intracellular flow cytometry

The principle of this technique is to detect and measure cytokine-producing cells detecting intracellular cytokines by antibody staining and flow cytometric analysis. In the present experiment, we will detect IFNγ producing cells. See Annexe D : Few notions in flow cytometry and intracellular cytokine detection, Sylvie Garcia for further information.

Note: The phenotypic characterization of cells of interest (T lymphocytes, B lymphocytes…) is achieved by performing a standard membrane staining with a relevant monoclonal antibody before fixating the cells. It is important to include the relevant isotype-matched controls.

Alternatively, the phenotypic characterization of cells can be performed intracellularly and concomitantly with the cytokine detection. This method will be used during this practical course.

For simultaneous analysis of activation markers, the first method (surface staining) will be preferred.

d-1. Experimental procedure for intracellular staining:

- Transfer cells for staining in 15 mL.
- Cells are washed twice in cold "suspension buffer" (PBS 1X, 2% fetal calf serum, 0.1% NaN_3) and centrifuged 5 minutes at 1500 rpm.
- Resuspend cells with 500 µL of fixation buffer (1% paraformaldéhyde in PBS 1X).
- Carefully resuspend the cell pellet by pipetting in order to avoid aggregates.
- Incubate for 10-20 min at room temperature.

Note: shorter time for fixation is recommended if cells to be tested are fragile.

- Wash once with 5 mL PBS 1X and centrifugation 5 min at 1500 rpm.
- Resuspend the cell pellet in n x 100 µL of PBS 1X (n being the number of FACS stainings to be performed).

- Dispatch 100 µL in n microfuge tubes.
- Centrifuge and resuspend cells in 100 µL of a mixture of the appropriate antibodies diluted in the "permeabilization buffer" (PBS 1X, 2% fetal calf serum, 0.1% NaN_3, 0.1% Saponin).
- Incubate for 30-60 min on ice in the dark.

  Note: longer time will cause "unspecific" staining.
- Wash twice with 1 mL washing buffer (PBS 1X, 2% fetal calf serum, 0.1% NaN_3) and centrifugation microfuge tube 5 min at 2000 rpm.
- Resuspend cells in 500 µL PBS or washing buffer and run the sample on the flow cytometer as soon as possible.

d-2. Flow cytometer: acquisition and analysis

- The use of the flow cytometer will be demonstrated by an instructor for acquisition and analysis of data.
- Results are expressed as a percentage of positive cells after normalization by the isotype-matched controls.

e. Cytokine production determined by ELISA

The IFN-γ cytokine level produced in cell culture supernatant is measured with the eBioscience mouse IFNγ ELISA Ready-SET-Go! kit, as described by the manufacturer.

e-1. Add 100 µl of capture antibody (Ab), diluted as indicated on Certificate of Analysis, to each well. Incubate overnight at 4°C.

e-2. Wash x3 with PBS-0.05% Tween.

e-3. Block wells with 200 µl of Assay Diluent (diluted 1 to 5 in DI water). Incubate for 1 hour at room temperature (RT).

N.B.: <u>Do not</u> add NaN_3 since it inhibits HRP activity.

e-4. Wash x3 with PBS-0.05% Tween.

e-5. Add 100 µl of samples or standards in each well (see standard preparation procedure on Certificate of Analysis). Incubate for 2 hours at RT.

e-6. Wash x5 with PBS-0.05% Tween.

e-7. Add 100 µl/well of detection antibody (biotinylated anti-human cytokine mAb), diluted in 1X Assay Diluent as indicated on certificate of Analysis. Incubate for 1 hour at RT.

e-8. Wash x5 with PBS-0.05% Tween.

e-9. Add 100 µl/well of enzyme reagent (avidin-horseradish peroxidase conjugate) , diluted in 1X Assay Diluent as indicated on certificate of Analysis. Incubate for 30 min at RT.

N.B.: It is critical that the Assay Diluent does not contain any NaN_3 since it inhibits HRP activity.

e-10. Wash x7 with PBS-0.05% Tween.

e-11. Add 100 µl/well of TMB (3,3',5,5'-Tetramethylbenzidine) substrate solution. Incubate 15 min at RT.

e-12. Stop reaction with 50 µl/well of H_2SO_4 2N.

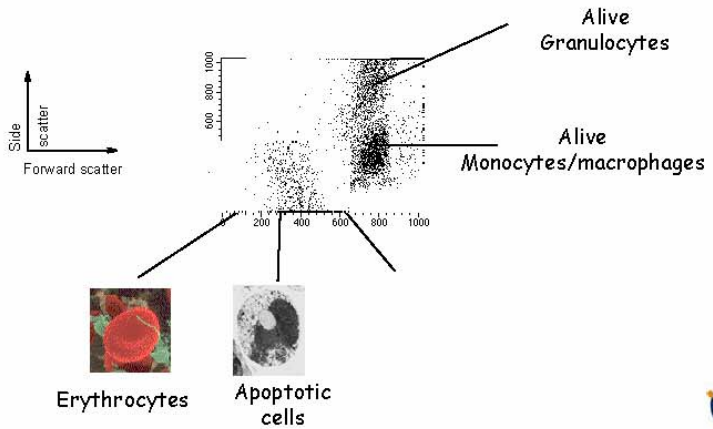
e-13. Estimate O.D. plates at 450 nm with a spectrophotometer for 96-well plates

Annexe D : Few notions in flow cytometry and intracellular cytokine detection, Sylvie Garcia

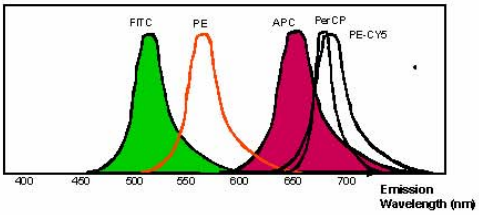
Few notions in flow cytometry and intracellular cytokine detection

Sylvie Garcia, Institut Pasteur, Paris

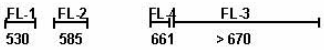
Detection of the blood subsets using SSC/FCS parameters



Usual dye coupled with antibodies

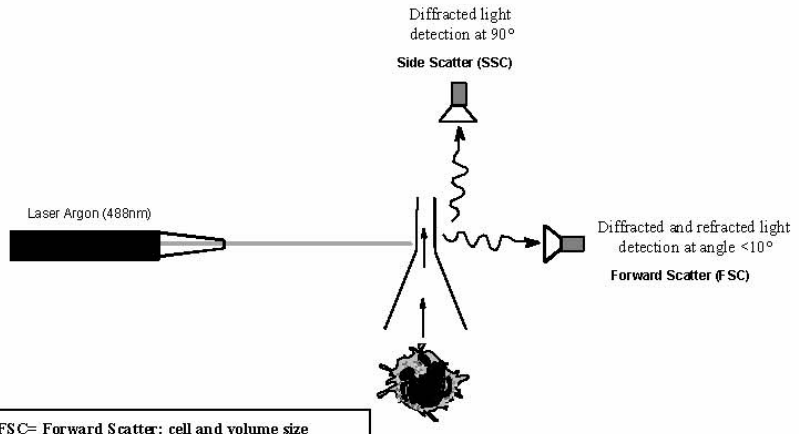


Channel:



FITC : Fluorescein Isothiocyanate
PE: Phycoerythrin
APC: Allophycocyanin (*)
perCP: Peridinin Chlorophyll Protein
PE-Cy5: PE + Cyanine-5

Size (FCS)-granularity (SSC) detection



FSC= Forward Scatter: cell and volume size
SSC= Side scatter: cell complexity and granularity

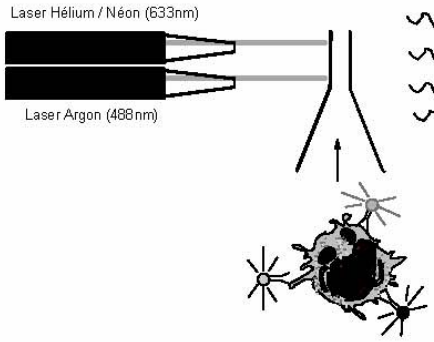
Subsets detectable by flow cytometry

	Human	Mouse
- Leukocytes:	CD45	CD45 (2 alleles: Ly5.1, Ly5.2)
- T Lymphocytes :	CD2, CD3, CD4, CD8, αβ, γδ (different chains)	
- NK:	NK: CD16, CD56, KIR	NK: CD56, NK1.1, KIR
- B Lymphocytes:	CD19, CD20	
- Monocytes / macrophages:	CD4, CD44	CD11b, CD14
- Granulocytes:	CD15	Anti-GR1
- DC:	CD11c	

Detection of activation/differentiation by cytometry

	Human	Mouse
Naïve/Memory	CD45RA,CD45RO, CD62-L, CCR7	CD45RB,CD44, CD62-L
Resting/Effector	CD69, CD25, HLA-DR	CD69, CD25

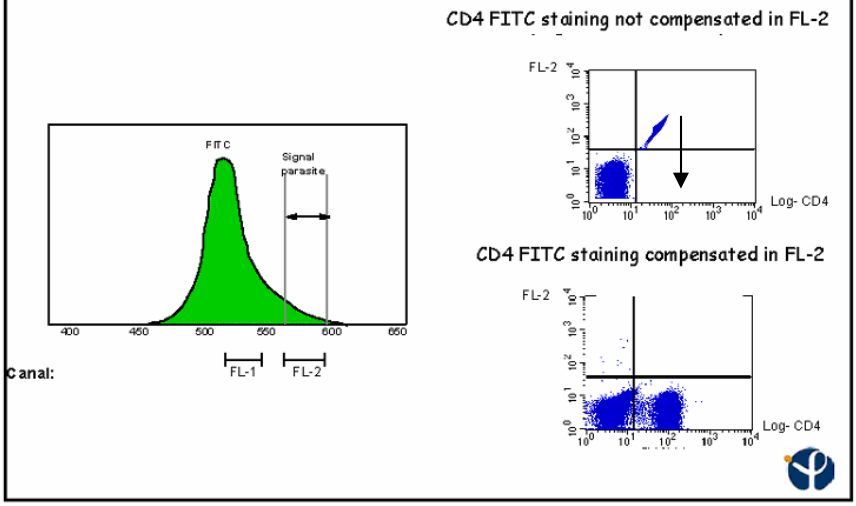
Fluorescence detection



Cytokines/Cytokine receptors detected by flow cytometry

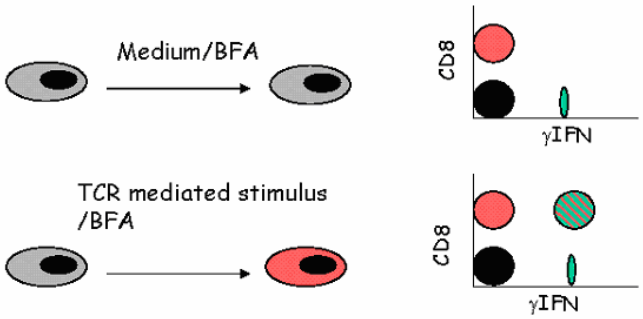
	Human	Mouse
Cytokines	IL-1, IL-2, IL-4, IL-6, IL-8, IL-13, TNFα, γIFN	IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12, GM-CSF, γIFN, TNFα
Cytokine receptors	G-CSF-R, GM-CSF-R, IL-2R, IL-3R, IL-5R, γc, IL-10R, IL-12R,	IL-4Rα, IL-3R, IL-6R IL-7Rα, IL-12Rβ, γ IFN, TNF-R, IL2R, γc, IL-15R, IL-10R

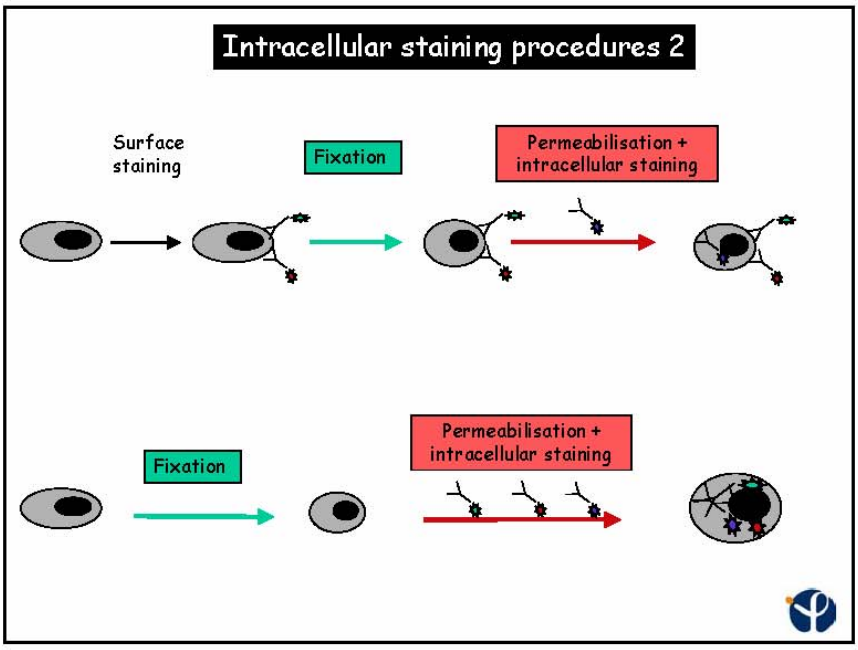
Settings: the compensation problem



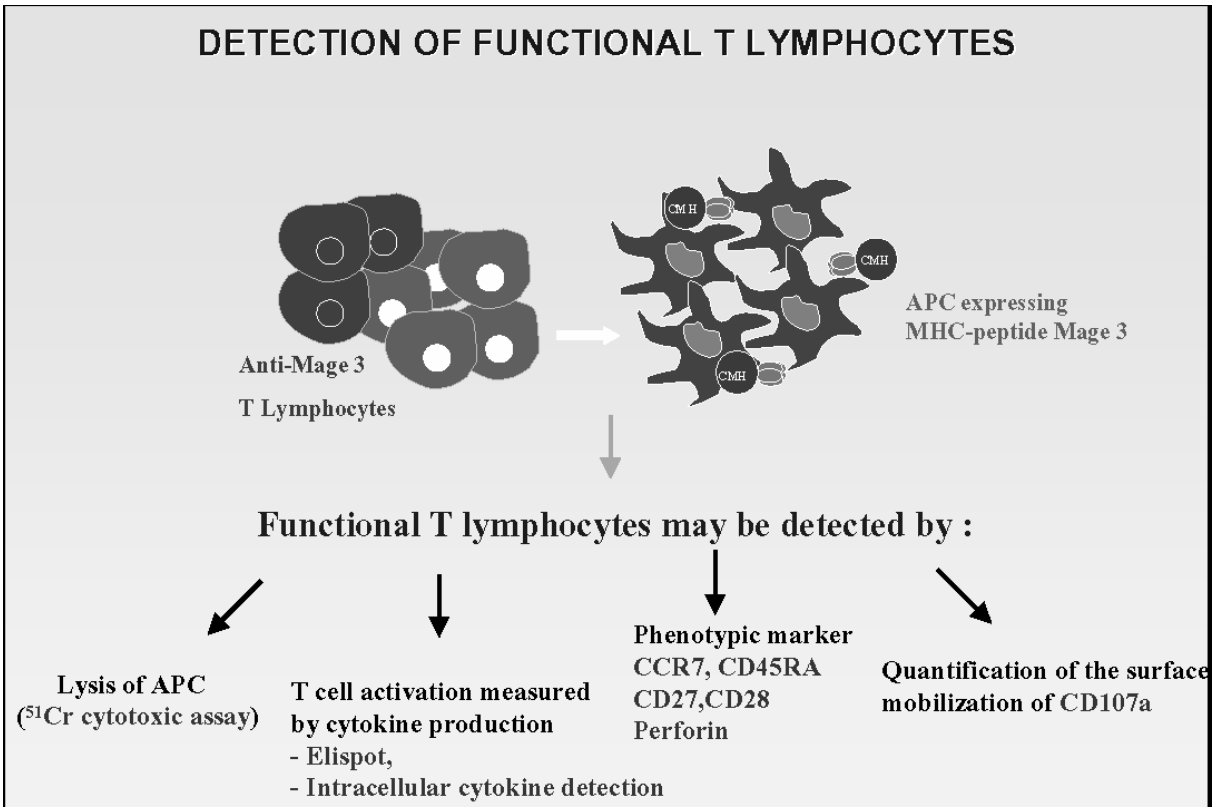
Intracellular staining procedures 1

<u>4-ON culture step:</u>
cytokine accumulation for further detection




Intracellular staining procedures 2
Surface staining
Fixation
Permeabilisation + intracellular staining
Fixation
Permeabilisation + intracellular staining

Annexe E : Detection of functional T lymphocytes by ELISPOT, by Eric Tartour, Professor of Immunology, Université Renée Descartes – Paris 5

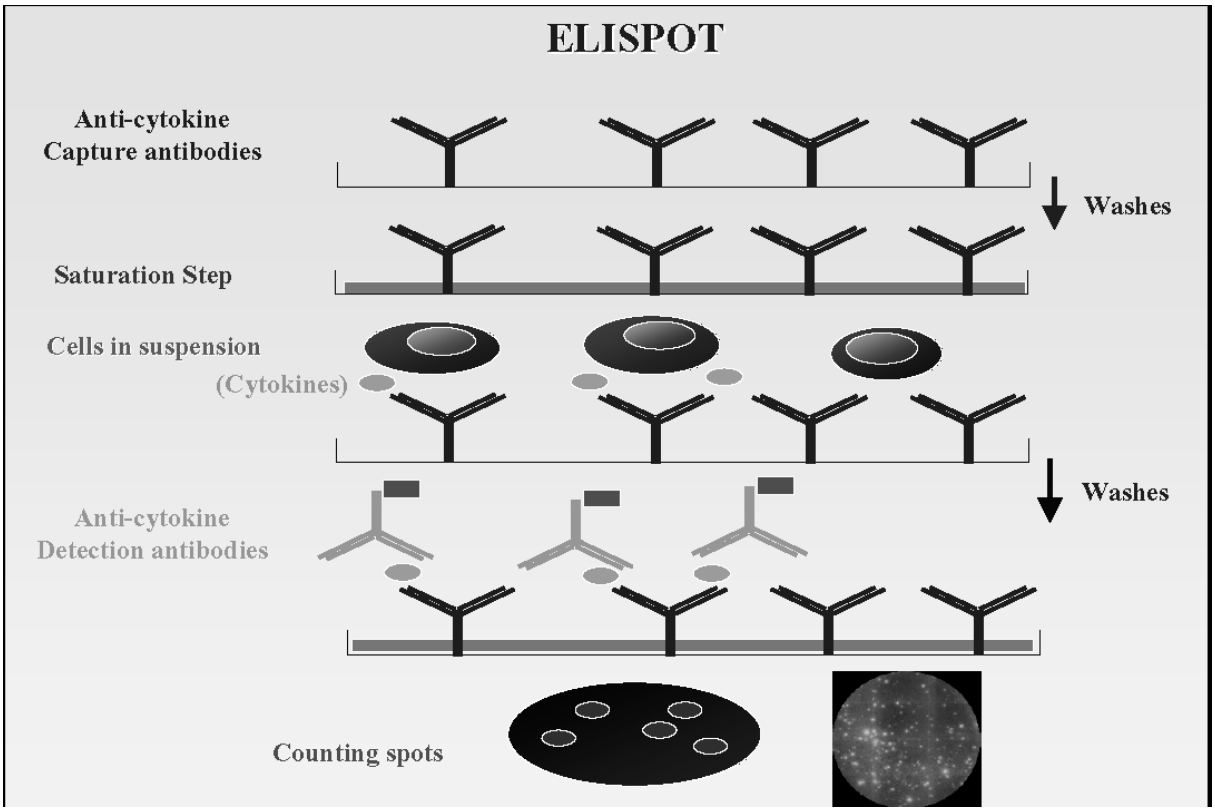


ELISPOT

- 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.



Each spots represents a footprint of the original cytokine-producing cells

(Czerkinsky et al 1988)

Plates for the coating of capture antibodies

I Nitrocellulose : (Ex : 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

Antibodies for Elispot

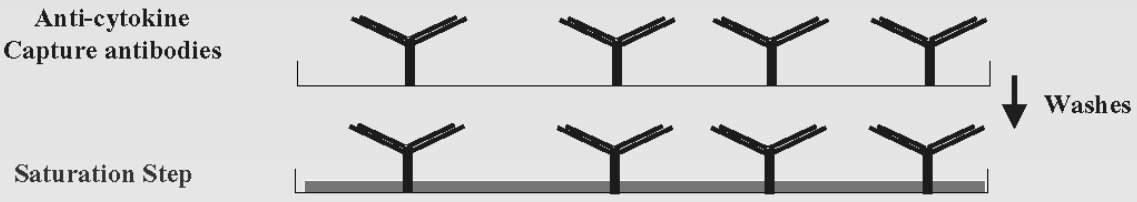
- The binding capacity of plates for Elispot is generally lower than for Elisa plates.
→ Use higher concentrations of antibodies for the coating

- 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...)

ELISPOT



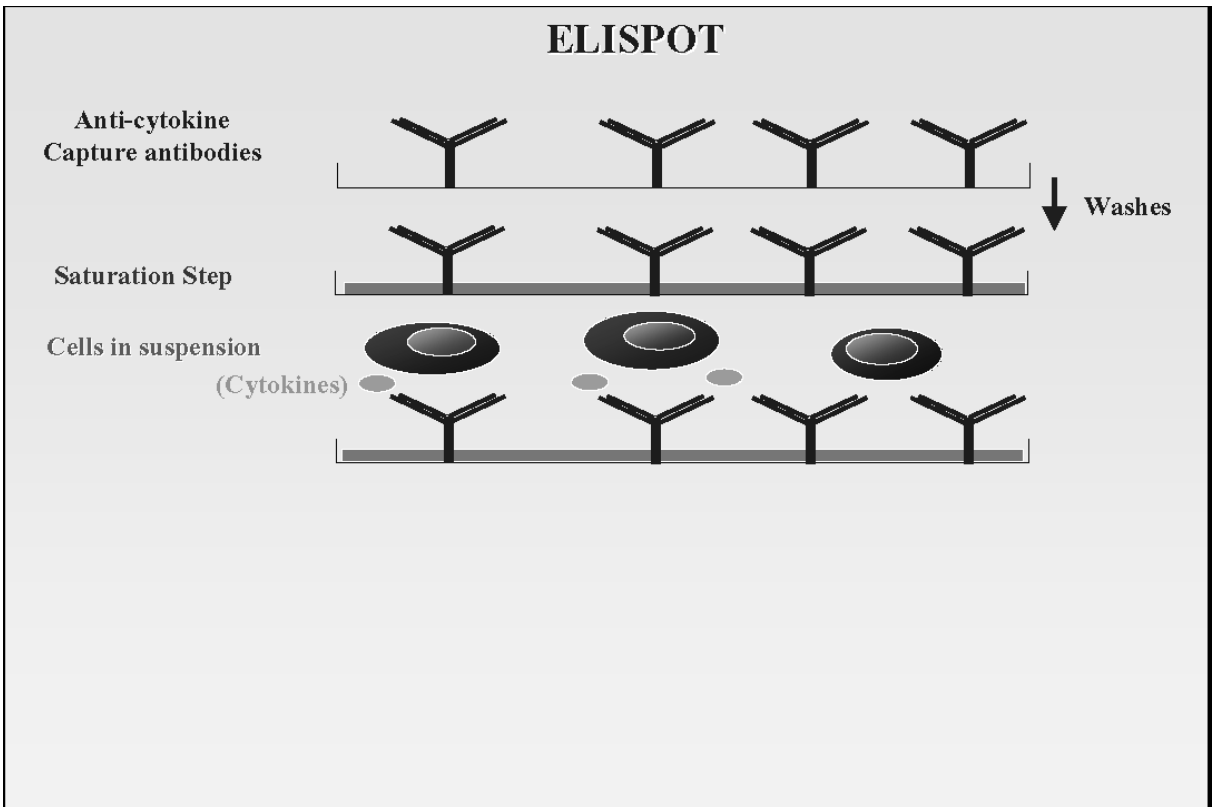
SATURATION STEP

1-2 hours at 37°C with :

- PBS 5% Bovin Serum Albumin (BSA)

- PBS 2% Milk

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


ELISPOT
Anti-cytokine
Capture antibodies
Washes
Saturation Step
Cells in suspension
(Cytokines)

Annexe F : Bibliography

Intracellular flow cytometry:

1. Jung T., *et al.* (1993) Detection of intracellular cytokines by flow cytometry. *J. Immunol. Methods*, 159, 197-207.
2. Assenmacher M., *et al.* (1994) Flow cytometric determination of cytokines in activated murine T helper lymphocytes : expression of interleukin-10 in interferon γ and in interleukin-4 expressing cells. *Eur. J. Immunol.* 24, 1097-101.
3. Openshaw P., *et al.* (1995) Heterogenety of intracellular cytokine synthesis at the single-cell level in polarized T helper 1 and T helper 2 population. *J. Exp. Med.* 182, 1357-1367.
4. Ledru, E., *et al.* (1998) Differential susceptibility to activation-induced apoptosis among peripheral Th1 subsets: correlation with Bcl-2 expression and consequences for AIDS pathogenesis. *J. Immunol.* 160, 3194-3206.
5. Ledru, E., *et al,* (2003) A nonsecreted variant of interleukin-4 is associated with apoptosis: implication for the T helper-2 polarization in HIV infection. *Blood* 101, 3102-3105.