The 2nd PSU International Teaching Platform on Tumour Immunology and Immunotherapy

Jointly organized by

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

December 15 – 20, 2003

At The Department of Biomedical Sciences

Faculty of Medicine, Prince of Songkla University,

Hat Yai, Songkhla, Thailand

Lab Course Handout

(December 18-20, 2003)

Sylvie Garcia, Institut Pasteur

Eric Tartour, Université René Descartes

Adrien Six, Université Pierre et Marie Curie et Institut Pasteur

Cytokine production determination by ELISPOT and intracellular flow cytometry

The purpose of this practical study is to determine the cytokine levels by two different methods, the ELISPOT and intracellular flow cytometry methods. The objective of this 3-day lab course session is to demonstrate the ease of use and the power of these two techniques which can be applied in both basic and clinical research.

In this handout, you will find a detailed protocol for the experimental steps that will be carried out during the next days, as well as some reference information on the techniques, protocols, reagents and equipments.

Although these techniques routinely apply to the follow-up of anti-tumour immune response, it was not possible to implement them on actual patient samples during this 3-day lab course. In order to make this course as practical as possible for participants, we selected a mouse model for the experiments that you will perform. The protocols described below are fully applicable to human samples; the references for human reagents are given in Annexe D.

Additional information on these techniques can be read in the following sections:

- Annexe E : Techniques for the detection of specific T-lymphocytes – Application to the immunomonitoring of cancer vaccine protocols, Eric Tartour
- Annexe F : Detection of functional T lymphocytes, Eric Tartour
- Annexe G : Few notions in flow cytometry and intracellular cytokine detection, Sylvie Garcia
- Annexe H : Rentzsch, C. et al. (2003) - Clinical Cancer Research *Vol. 9, 4376–4386.*

Analysis of Results and Discussion

Conclusion

More and more research findings demonstrate the importance of studying cytokine up- and down-regulation in immunocompetent leucocytes. Studies in humans clearly show that Th1 dominated responses are effective in protection against several intracellular microorganisms and usually drive their complete clearance or at least contribute to reduce their load. The important physiologic function of Th2 cells may be the regulation or suppression of some immune responses (*e.g.* regulation of inflammatory processes in non-lymphoid tissues, B-cell coactivation in secondary lymphoid organs).

Although the choice of the methods to implement is still under discussion, cytokines are now routinely measured, during fundamental or clinical research projects, in order to estimate the anti-tumoral response of the immune system. This will be discussed along the lab course through lectures and article discussion with your trainer.

Annexe A : Tentative schedule

Date	***Activity***
Dec. 17, evening	- *Isolation of mouse splenocytes* - *Activation in culture with PMA/Ionomycin/BrefeldinA*
Dec. 18, Morning	- Cell counting and fixation - Introduction and general instructions - Introduction on Elispot and intracellular staining
Dec. 18, Afternoon	- Intracellular staining - Demonstration of Elispot revelation
Dec. 19, Morning	- Group 1: Acquisition on flow cytometer - Group 2: Reading and analysis Elispot/Exercise session
Dec. 19, Afternoon	- Group 2: Acquisition on flow cytometer - Group 1: Reading and analysis Elispot/Exercise session (if time allows)
Dec. 20, Morning	- Analyze flow cytometry results - Analyze ELISPOT results
Dec. 20, Afternoon	- Short presentation of results by each group - Discussion of results 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.

In the following lists "E" indicates reagents of equipment required for ELISPOT when "I" indicates reagents of equipment required for intracellular flow cytometry analysis.

Animals

- 8-week-old albino Swiss mice E/I

Antibodies

- Anti-IFNγ capture antibody (BD-Pharmingen Ref. 551216; 1 mg) E
- Anti-IFNγ biotinylated detection antibody (BD-Pharmingen Ref. 554410; 0.5 mg) E
- Streptavidin Alcaline Phosphatase (BD-Pharmingen Ref. 554065; 1 mL) E
- Coat 96 well Multiscreen plate MAHA S45 10 (Millipore) E
- Anti-mouse CD4 FITC antibody (BD-Pharmingen Ref. 553047; 0.5 mg) I
- Anti-mouse IFNγ PE antibody (BD-Pharmingen Ref. 554412; 0.1 mg) I
- Anti-mouse CD8α Percp antibody (BD-Pharmingen Ref. 553036; 0.1 mg) I
- Anti-mouse CD3ε APC antibody (BD-Pharmingen Ref. 553066; 0.1 mg) I

Culture media, solutions and reagents

- Bovine serum albumin (Sigma Ref. A7030) E/I
- Demineralised water E/I
- Ethanol 70% (Prolabo Réf : UN1170) E
- Fetal calf serum (ATGC Biotechnologies Ref. 04-001-1B) E/I
- Formaldehyde 37% (Sigma Ref. F-1635) I
- Ionomycin calcium salt (Sigma Ref. I-0634; 1 mg) E/I
- Brefeldin A (Sigma Ref. B7651; 5 mg) I
- NaN_3 (Sigma Ref. S-2002) I
- PBS (50 tablets Sigma Ref. P4417-50 or 1X Invitrogen Ref. 14190094) E/I
- Phorbol 12 Myristate 13 acetate (PMA; Sigma Ref. P-8139; 1 mg) E/I

- Saponin (Sigma Ref. S-7900) I
- Tween 20 (Merck Eurolab Ref. 822184) E
- β2-mercaptoethanol I
- RPMI 1640 culture medium with glutamax (Invitrogen Ref. 61870-010) I
- Hepes buffer 1M solution in distilled water I
- Penicillin-Streptomycin 10000U/10 mg/mL (Invitrogen) I
- Washing buffer PBS 1X, 0.1% Tween 20: For one plate, dilute 100 µl Tween 20 in 100 ml PBS 1X. E
- Cell suspension buffer: PBS 1X, 2% fetal calf serum, 0.1% NaN_3 I
- Fixation buffer: 10.8 mL 37% formaldéhyde in 89.2 mL PBS 1X I
- Permeabilization buffer: washing buffer with 0.1% Saponin I
- Washing buffer: PBS 1X, 2% fetal calf serum, 0.1% NaN_3 I

Disposables

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

- Cell culture plate (x6 or x12 wells) I

Equipment

- Dissection tools (scissors, forceps…) E/I
- Cell culture CO_2 incubator E/I
- Cell culture hood E/I
- ELISPOT plate reader (optional) E
- Flow cytometer (FACScalibur, Becton Dickinson) I
- Incubator (37°C) E
- Refrigerated centrifuge (for 15/50 mL tubes) E/I
- Benchtop centrifuge E/I
- Malassez cytometer E/I
- Microfuge tube racks E/I
- Micro-pipettes 1000 µL E/I
- Micro-pipettes 200 µL E/I
- Micro-pipettes 20 µL E/I
- Microscope E/I
- Multi-channel pipettes E
- Vortex E/I

Annexe C : Detailed protocols

Cytokine secretion by T-cell populations

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.

a-8. Dilute cells to $2.5x10^6$ cells/mL into two separate culture flasks.

a-9. For intracellular staining, add 50 ng/mL PMA and 500 µg/mL Ionomycin to one flask.

a-10. Incubate flasks at 37°C, 5% CO_2.

a-11. After 5 hours of culture, add Brefeldin A (10 µg/mL).

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.

a-12. The remaining cells will be incubated directly in the ELISPOT plates with or without PMA/Ionomycin.

b. ELISPOT protocol

The principle of this technique is to detect and measure cytokine-producing cells which are revealed by a coloured spot following the capture of cytokine that they produce by the anti-cytokine antibodies coated on the ELISPOT plates. Each spot reveals the presence of a cytokine-producing cell. It is somehow possible to estimate the amount of cytokine produced by the relative size of the spots. See the following Annexes for additional information:

- Annexe E : Techniques for the detection of specific T-lymphocytes – Application to the immunomonitoring of cancer vaccine protocols, Eric Tartour

- Annexe F : Detection of functional T lymphocytes, Eric Tartour

b-1. Coating:

- Coat 96 well Multiscreen plate MAHA S45 10 (Millipore) with rat anti-mouse IFNγ mAb clone R4-6A2 at 2-4 μg/ml diluted in PBS (100μl/well).
- Cover the plate and incubate for 4-24 hrs at room temperature.

b-2. Saturation:

- Empty the plate and wash five times with PBS 1X.
- Add 200 μl per well of culture medium containing fetal calf serum and incubate for one hour at 37°C, 7% CO_2.

b-3. Cell transfer and incubation:

- Discard the blocking solution.
- Wash once with 100 μL PBS 1X.

Note: Typically add 100 μL of cell suspension per well containing serial dilutions of responder cells (10^6, 5.10^5, $2.5.10^5$… cells/well, 2 wells/dilution), 5.10^5 irradiated splenocytes per well as feeder cells, with or without 1 μg/mL of peptide (specific antigen). The best way is to prepare serial dilutions of responder cells (2.10^7, 10^7, 5.10^6… cells/mL), add 50 μL/well of these cell suspensions and then add 50 μL of complete medium containing 10^7 feeder splenocytes/ml, and +/- 2μg/mL of specific peptide.

Note: Cells to be tested with or without stimulation/inhibition agents are incubated in the ELISPOT plate wells in 100 μL of culture medium. A positive control is usually needed for each experiment: PBMC stimulated with 100 ng/mL PMA and 10 μM Ionomycin.

- Dilute splenocytes prepared above in culture medium to a concentration of 10^6 cells per mL.
- Separate into two halves.
- Add 100 ng/mL PMA and 10 μM Ionomycin to one half.
- Add 100 μL cells to ELISPOT wells (4 wells with PMA/Ionomycin; 4 wells without).
- Incubate overnight (15-20 hours) at *37°C in an incubator with 5% CO_2 without shaking.*

Note: It is critical to make sure the ELISPOT plate is not shaken nor moved during incubation so that cytokines produced by one cell are captured around that cell and do not diffuse in medium.

b-4. Revelation (for one plate):

- Empty the plate by taping over absorbent paper.
- Wash twice with H_2O-Tween 0.05%, then wash five times in PBS-Tween 0.05%, and pat dry.

- Dilute biotinylated rat anti-mouse γIFN mAb clone XMG1.2 at 4 μg/mL in PBS-Tween 0.05%-1% BSA and distribute 100 μL/well.
- Incubate the plate for 2-4 hours at room temperature.
- Empty the plate and wash five times with PBS-Tween 0.05% and pat dry.
- Dilute (1/1000) streptavidin-phosphatase alkaline in PBS-Tween 0.05%-1% SVF. Add 100 μL/well and incubate for 2-4 hours at room temperature.
- Empty the plate and wash plate five times PBS-Tween 0.05% and pat dry. Wash the plate once in PBS without Tween and pat dry.
- Add 100 μL/well of BCIP/NBT substrate and incubate at room temperature until blue spots develop (15 min).
- To stop the revelation reaction, wash three times with demineralised water, carefully removing the excess of water and air dry the plate. Quantitate spots. Reading the plates will be better after one night; keep the plates in the dark (wrapped in aluminium foil).

b-5. Calculation, results and interpretation

- The number of spots is determined for each well representing the number of cytokine-producing cells.
- When available, an automatic ELISPOT plate reader can be used; in this case, it becomes possible to give an estimation of the spot intensity in addition to the number of spots.
- A well is considered positive when one counts more than 10 spots per 10^5 cells above background.

c. 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 G : 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.

<u>Alternatively,</u> 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.

c-1. Experimental procedure for intracellular staining:

- Transfer cells for staining in 15 mL.

Each group will perform intracellular staining of cells stimulated or not with PMA/Ionomycin.

- Cells are washed twice in cold "suspension buffer" and centrifuged 5 minutes at 1500 rpm.
- Resuspend cells to a final concentration of $2.5x10^6$ cells/mL in suspension buffer.
- Transfer 200 µL of the cell suspension in a FACS tube.
- Add 200 µL of "fixation buffer".
- Carefully resuspend the cell pellet by pipetting in order to avoid aggregates.
- Incubate for 20 min at room temperature.
- Wash twice with 2 mL cold PBS 1X and centrifugation 5 min at 1500 rpm.
- Resuspend the cell pellet in 100 µL of a mixture of the 4 antibodies diluted in the "permeabilization buffer" (anti-CD4 FITC 1/200, anti-γIFN 1/50, anti-CD8 percP 1/50, anti-CD3 APC 1/50).
- Incubate for 30 min on ice in the dark.
- Wash twice with 2 mL cold PBS 1X and centrifugation 5 min at 1500 rpm.
- Resuspend the cells in 500 µL PBS and run the sample on the flow cytometer as soon as possible.

c-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.

Annexe D : Reagents and alternative protocol used for humans

d. Samples used for human samples

- IFNγ PVDF ELISPOT kit (DIACLONE Ref. 856.051.010 for 10 plates or 856.051.015 for 15 plates). E

  Note: Each kit contains capture and detection antibodies, saturation buffer (skimmed milk), streptavidine-alcaline phosphatase and BCIP/NBT substrate. ELISPOT kits for many other cytokines can be purchased by the same supplier.

- ELISPOT sterile PVDF plates (Diaclone 856.111.111 for 5 plates) E
- Anti-CD3 FITC antibody (BD-Pharmingen Ref. 555332) I
- Anti-IFNγ antibody PE (Diaclone Ref. 855.002.010) I
- Isotype-matched mouse IgG1 antibody FITC (BD-Pharmingen Ref. 555748) I
- Isotype-matched mouse IgG1 antibody PE (BD-Pharmingen Ref. 555749) I
- Saturation solution (2% skimmed milk): For one plate, dissolve 0.2 g skimmed milk in 10 ml PBS 1X. E
- Dilution buffer PBS 1X, 1% BSA: For one plate, dissolve 0.2 g de BSA (Sigma Ref. A-7030) in 20 ml PBS 1X. E

e. ELISPOT protocol used for human samples

e-1. Coating:

- Transfer 100 µL of 70% ethanol per ELISPOT plate wells and wait for 10 min at room temperature.
- Empty the plate and wash 3 times with 100 µL PBS 1X.
- Dilute 100 µL of capture antibody in 10 mL PBS 1X and distribute 100 µL per well. Incubate *overnight at 4°C.*

e-2. Saturation:

- Empty the plate and wash once with 100 µL PBS 1X.
- Distribute 200 µL per well of 2% skimmed milk and incubate for 2 hours at room temperature.

e-3. Cell transfer and incubation:

- Empty the plate by taping over absorbent paper.

- Wash once with 100 µL PBS 1X.

Note: Cells to be tested with or without stimulation/inhibition agents are incubated in the ELISPOT plate wells in 100 µL of culture medium. A positive control is usually needed for each experiment: PBMC stimulated with 100 ng/mL PMA and 10 µM Ionomycin.

- Dilute splenocytes prepared above in culture medium to a concentration of 10^6 cells per mL.
- Add 100 ng/mL PMA (SIGMA Réf : P8139) and 10 µM Ionomycin (SIGMA Réf : I-0634).
- Add 100 µL cells to ELISPOT wells.
- Incubate overnight (15-20 hours) at *37°C in an incubator with 5% CO_2 without shaking.*

Note: It is critical to make sure the ELISPOT plate is not shaken nor moved during incubation so that cytokines produced by one cell are captured around that cell and do not diffuse in medium.

e-4. Revelation (for one plate):

- Empty the plate and tap dry.
- Add 100 µL PBS 1X with 0.1% Tween. Incubate 10 min at 4°C.
- Wash 3 times with PBS 1X-0.1% Tween.
- Dilute 100 µL detection antibody in 10 mL PBS 1X with 1% BSA. Distribute 100 µL in each well. Incubate 1h30 at 37°C.
- Empty the plate and wash 3 times with PBS 1X-0.1% Tween.
- Dilute 10 µL streptavidine-alcaline phosphatase in 10 mL PBS 1X-1% BSA and distribute 100 µL per well. Incubate 1 h at 37°C.
- Empty the plate and wash 3 times with PBS 1X-0.1% Tween carefully removing the excess of solution.
- Distribute 100 µL BCIP-NBT solution per well and follow for the revelation during 5 to 20 min.
- To stop the revelation reaction, wash 3 times with demineralised water, carefully removing the excess of water and dry the plate. Reading the plates will be better after one night; keep the plates in the dark (wrapped in aluminium foil).

e-5. Calculation, results and interpretation

- The number of spots is determined for each well representing the number of cytokine-producing cells.

- When available, a automatic ELISPOT plate reader can be used; in this case, it becomes possible to give an estimation of the spot intensity in addition to the number of spots.
- A well is considered positive when one counts more than 10 spots per 10^5 cells above background.

f. Protocol used human samples for IFNγ detection by intracellular flow cytometry

f-1. Experimental procedure for intracellular staining:

- Transfer cells for staining in 15 mL.
- Cells are washed twice in cold "suspension buffer" and centrifuged 5 minutes at 1500 rpm.
- Resuspend cells to a final concentration of $2.5.10^6$ cells/mL in suspension buffer.
- Transfer 200 µL of the cell suspension in a FACS tube.
- Add 10 µL of anti-CD3 FITC antibody.
- Incubate on ice for 30 min.
- Wash twice in cold PBS 1X and centrifugation 5 min at 1500 rpm.
- Resuspend the cell pellet in 500 µL of cold PBS 1X.
- Add 500 µL of "fixation buffer".
- Carefully resuspend the cell pellet by vortexing in order to avoid aggregates.
- Incubate for 20 min at room temperature.
- Wash twice with 2 mL cold PBS 1X and centrifugation 5 min at 1500 rpm.
- Resuspend the cell pellet in 300 µL of "permeabilization buffer" and incubate for 10 min at room temperature.
- Wash twice with 2 mL cold PBS 1X and centrifugation 5 min at 1500 rpm.
- Resuspend the cell pellet in 300 µL of "permeabilization buffer" and add 10 µL of anti-cytokine antibody (anti-IFNγ antibody in our case).
- Incubate for 30 min at room temperature in the dark.
- Wash twice with 2 mL cold PBS 1X and centrifugation 5 min at 1500 rpm.

- Resuspend the cells in 500 μL PBS and run the sample on the flow cytometer as soon as possible.

f-2. Flow cytometer: acquisition and analysis

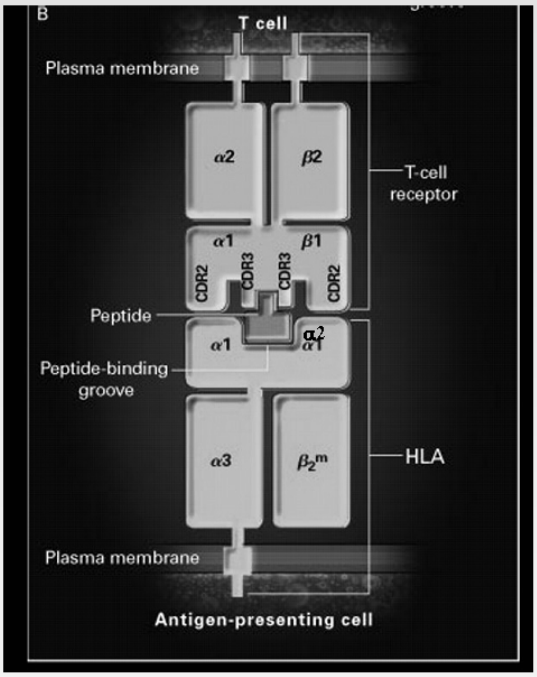
- The use of the flow cytometer will be demonstrated by an instructor for acquisition and analysis of data.
- Results are expressed as a percentage of positive cells after normalization by the isotype-matched controls.

Annexe E : Techniques for the detection of specific T-lymphocytes – Application to the immunomonitoring of cancer vaccine protocols, Eric Tartour

TECHNIQUES FOR THE DETECTION OF SPECIFIC T-LYMPHOCYTES
APPLICATION TO THE IMMUNOMONITORING OF CANCER VACCINE PROTOCOLS

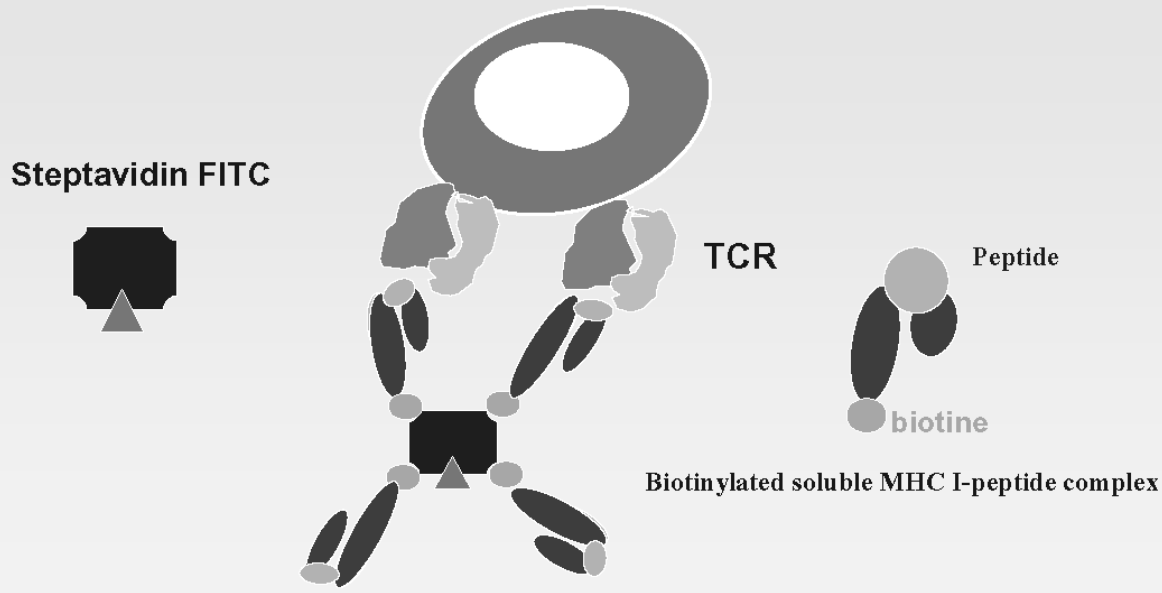
PEPTIDE MHC TETRAMER STAINING

INTERACTION BETWEEN TCR AND MHC-PEPTIDE COMPLEXES

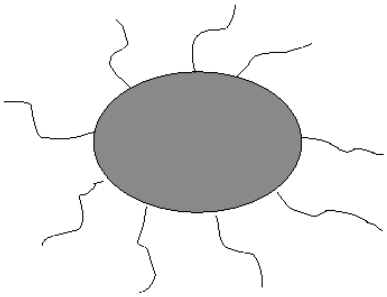


Klein et Sato NEJMed 2000

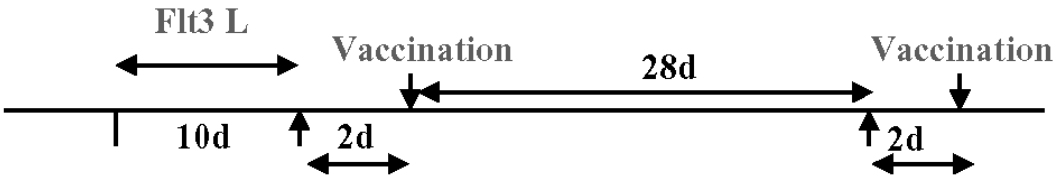
MHC PEPTIDE TETRAMERIC COMPLEX STAINING



Biotinylated soluble MHC I-peptide complex



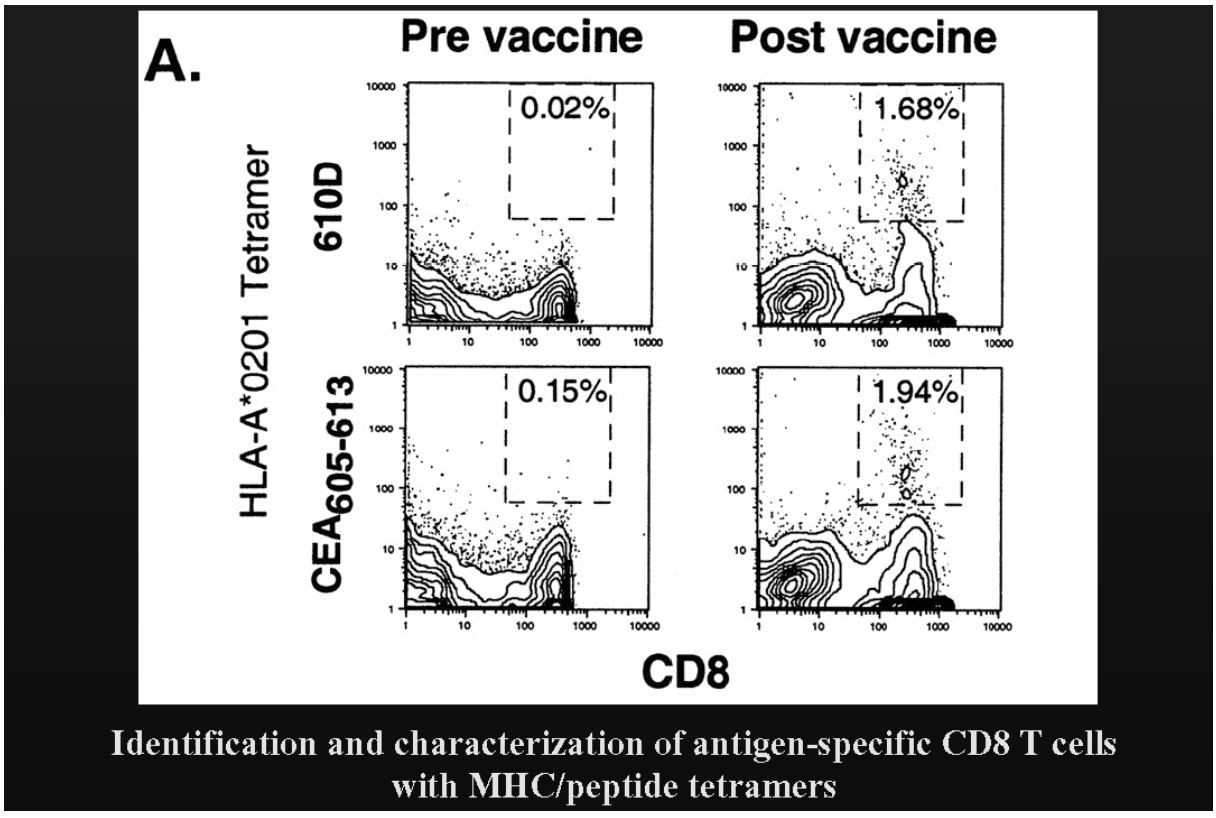
Pulse DC with modified peptide
CEA 605-613$^{Asp\text{-}Asn\ (610)}$



- Cytapheresis
- DC purification
(Density gradient)

- Cytapheresis
- DC purification
(Density gradient)

Phase I : 12 patients
(Colon adenocarcinomas and non small lung carcinomas)



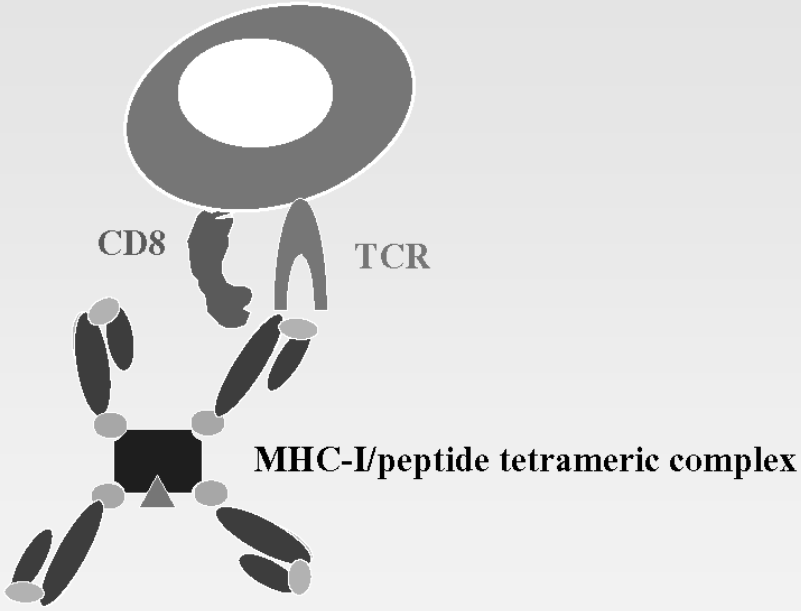
Identification and characterization of antigen-specific CD8 T cells with MHC/peptide tetramers

Correlation between immune and clinical responses* .

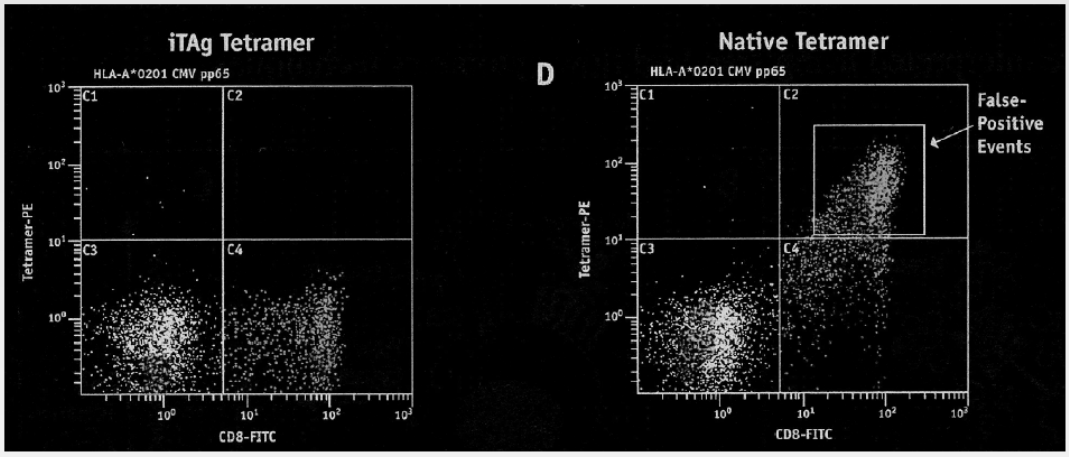
Patient	Clinical Response	Tetramer+ Prevaccine%	Tetramer+ Postvaccine%
1	PD	0.08	0.25
2	PD	0.03	0.08
3	**SD**	0.15	**1.11**
4	PD	0.18	0.04
5	**CR-10Mo**	0.4	**1.03**
6	PD	0.10	0.31
7	PD	0.26	0.49
8	**SD**	0.43	**1.05**
9	PD	0.16	0.07
10	PD	0.24	0.5
11	**CR-10Mo**	0.28	**1.03**
12	**MR**	0.12	**1.68**

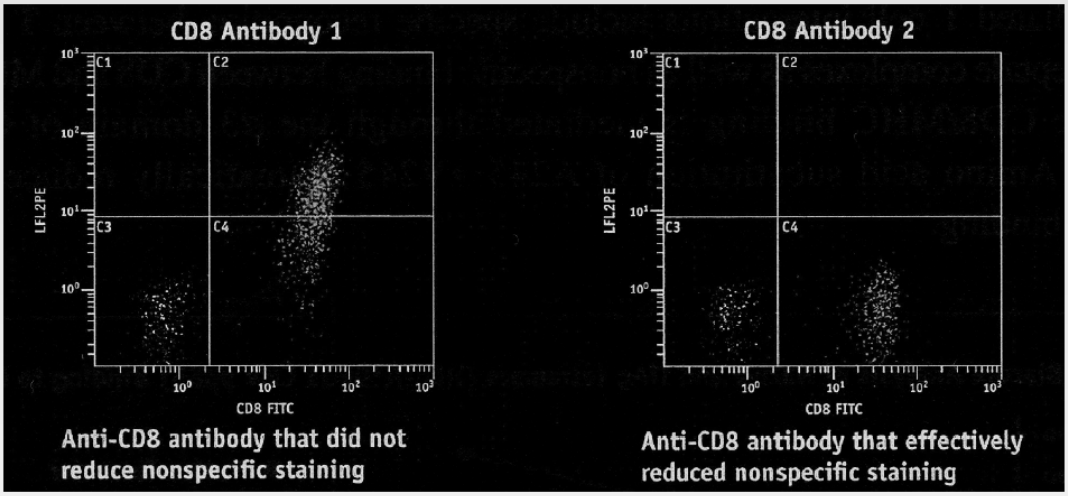
*** P = 0.002**

Non antigen dependent interaction between CD8 and non polymorphic region of MHC-I



Addition of mutations in amino-acids of HLA molecules involved in the interaction between monomorphic region of HLA and CD8 molecules permits the reduction of non specific binding of tetramers.

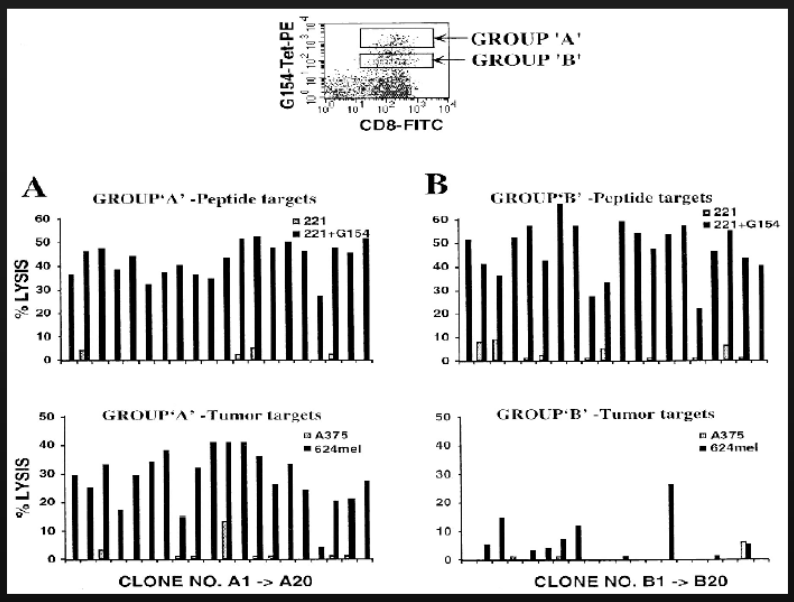




Sensitivity of this technique :

Threshold of detection 0.1-0.01% of specific T cells : $1/10^{-3}$ to $1/10^{-4}$

Although previous studies used the intensity of tetramer staining as a measure for recognition efficiency (or functional avidity) (Yee C et al J Immunol 1999)…

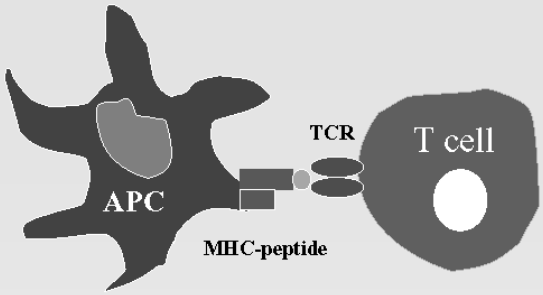


Isolation of high avidity, tumor-reactive T cell clones from a heterogeneous population following sorting of tetramer^{high} CD8^{+} T cells

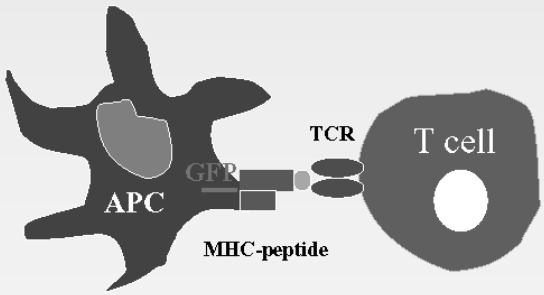
Yee C J Immunol 1999

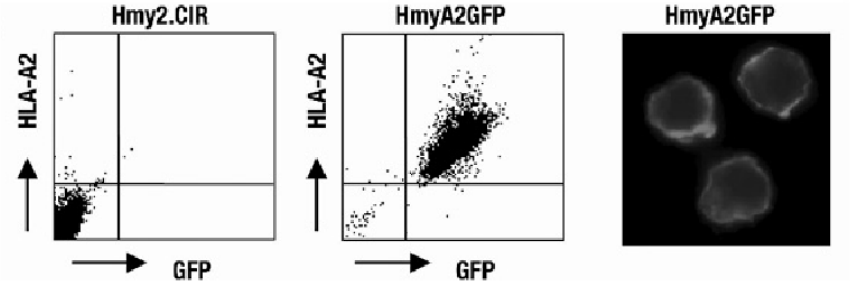
Although previous studies used the intensity of tetramer staining as a measure for recognition efficiency (or functional avidity) (Yee C et al J Immunol 1999)…

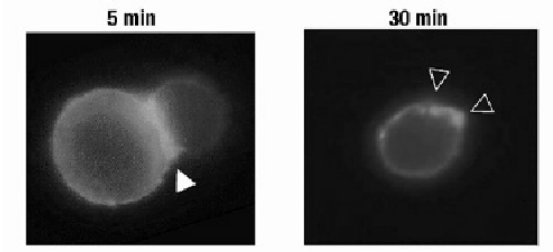
recent evidence suggests that tetramer staining does not directly correlate with recognition efficiency. (Echkakir H et al. PNAS 2002)(Dutoit V. Eur J Immunol 2002)



During peptide MHC-TCR specific recognition, peptide-MHC clusters are acquired by CD8+ T cells and internalized through the TCR (Stinchcombe JC Immunity 2001)(Huang JF Science 1999)

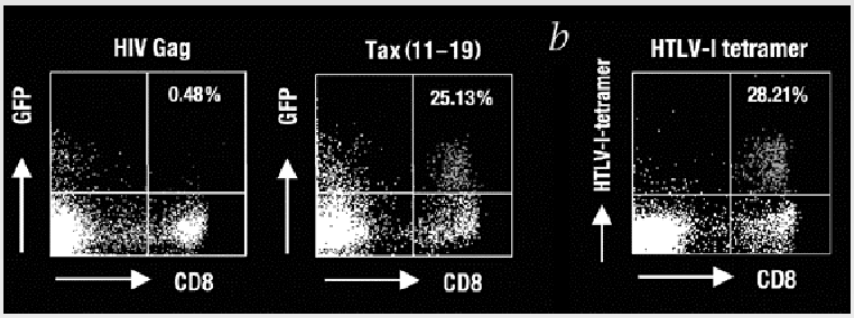






Peptide–HLA-GFP complexes are acquired by HLA-A*201–restricted HTLV-I–specific CTL clone

Tomaru U. Nat Med 2003



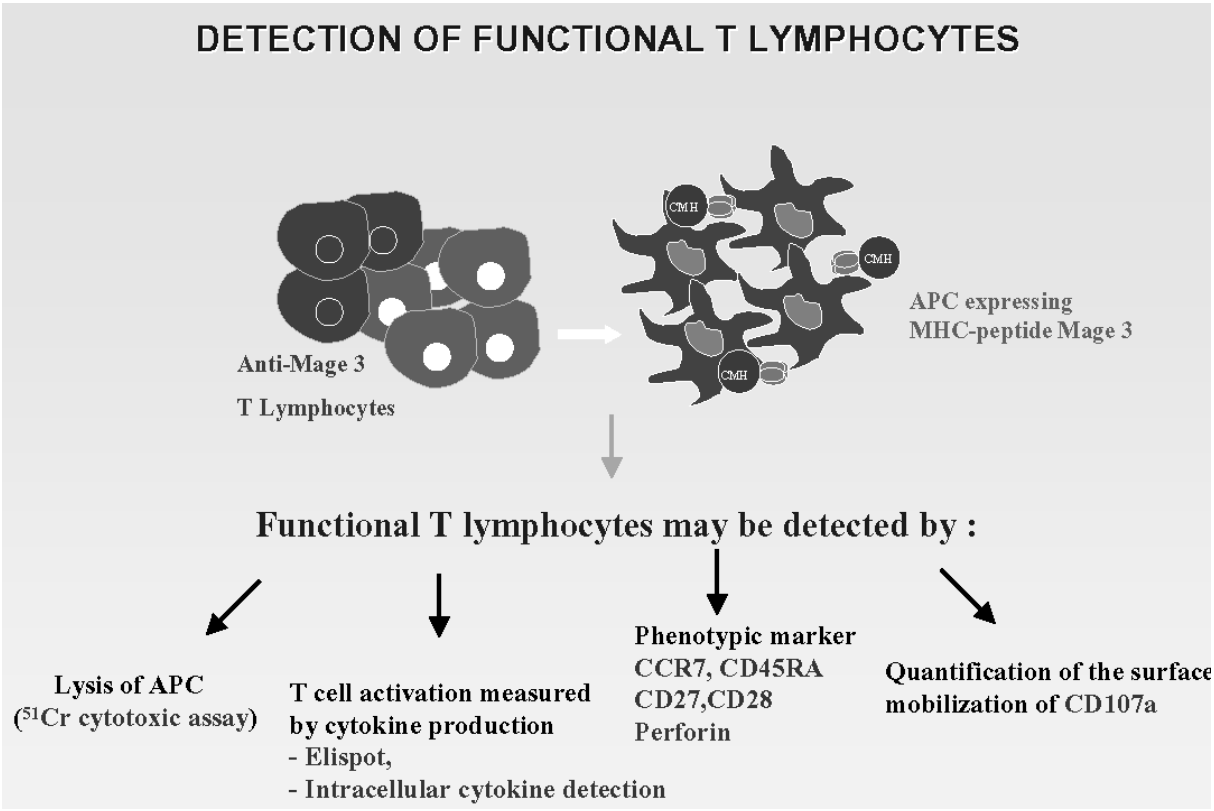
Tax (11-19) specific HLA-GFP acquisitionby CD8+ T cells from bulk PBMCs of HLA-A201 HTLV-1 infected patients.

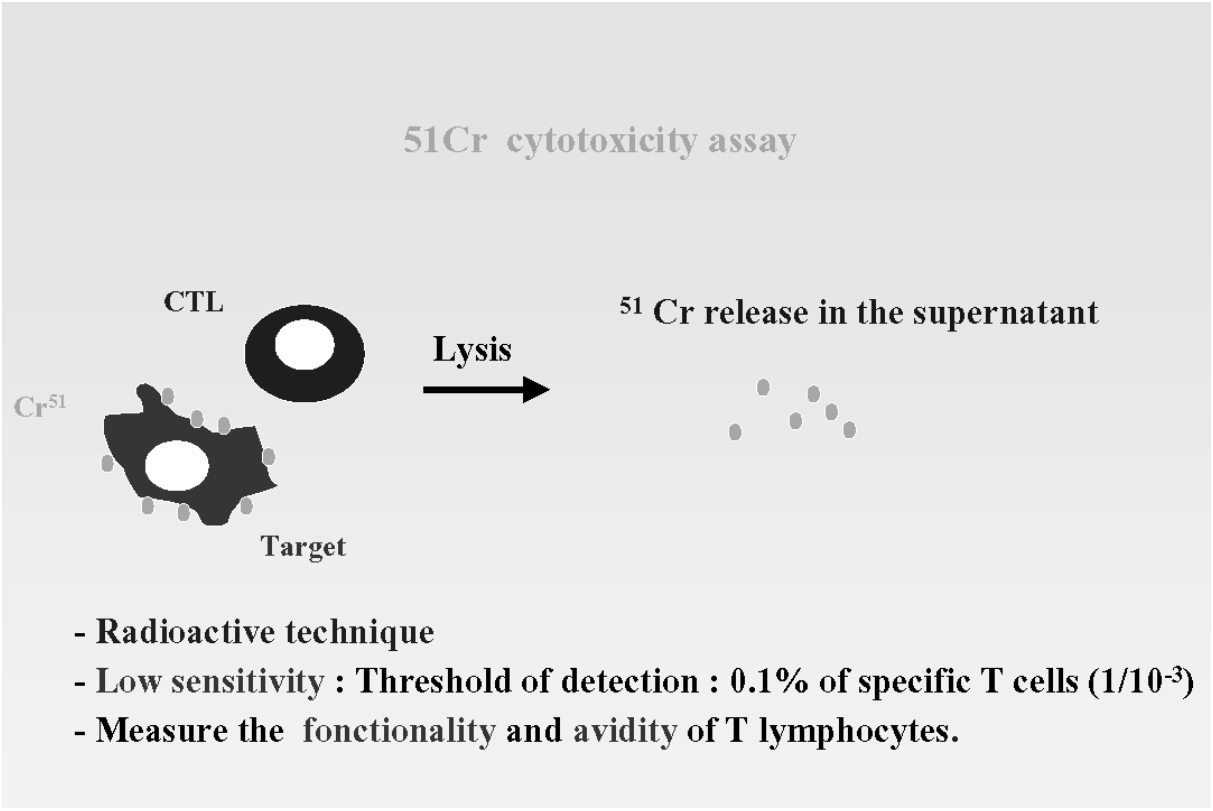
Tomaru U. Nat Med 2003

- MHC-peptide tetramer staining enumerates all specific T cells but does not discriminate functionnal or anergic specific T cells

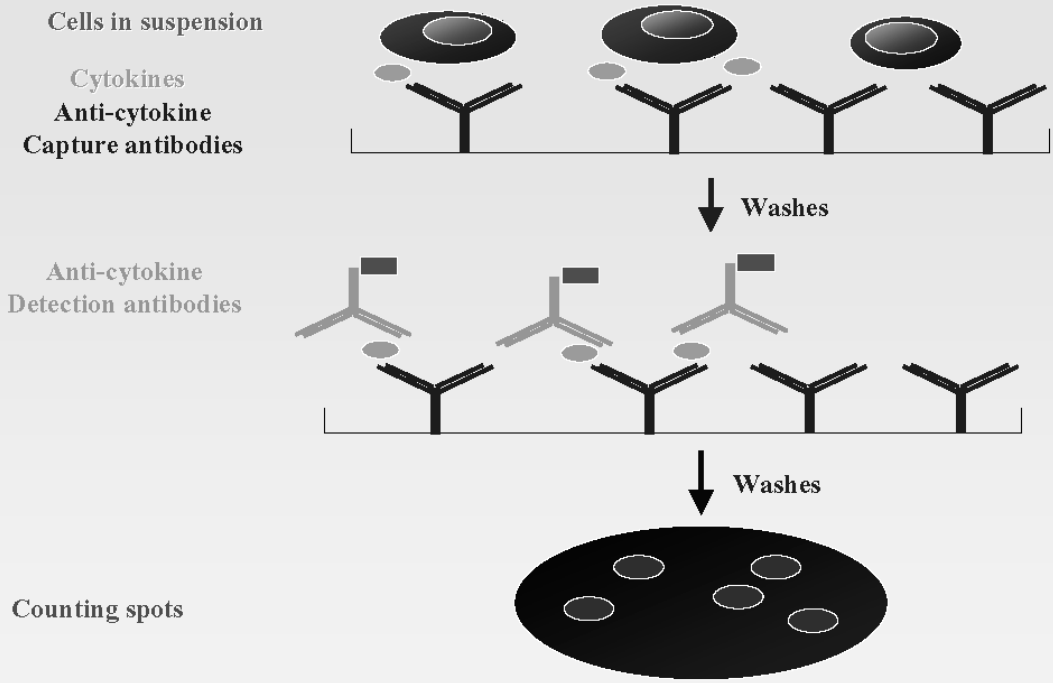
- The avidity and the potential cytotoxic of these T cells are also not analyzed by these technique.

- Functional avidity or recognition efficiency of T cells is now emerging as a key factor in the effectiveness of an antigen specific T cell response.


DETECTION OF FUNCTIONAL T LYMPHOCYTES
CMH
CMH
CMH
APC expressing
MHC-peptide Mage 3
Anti-Mage 3
T Lymphocytes
Functional T lymphocytes may be detected by :
Lysis of APC
(51Cr cytotoxic assay)
T cell activation measured
by cytokine production
- Elispot,
- Intracellular cytokine detection
Phenotypic marker
CCR7, CD45RA
CD27,CD28
Perforin
Quantification of the surface
mobilization of CD107a


51Cr cytotoxicity assay
CTL
Cr51
Lysis
51 Cr release in the supernatant
Target
- Radioactive technique
- Low sensitivity : Threshold of detection : 0.1% of specific T cells (1/10-3)
- Measure the fonctionality and avidity of T lymphocytes.

ELISPOT



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.

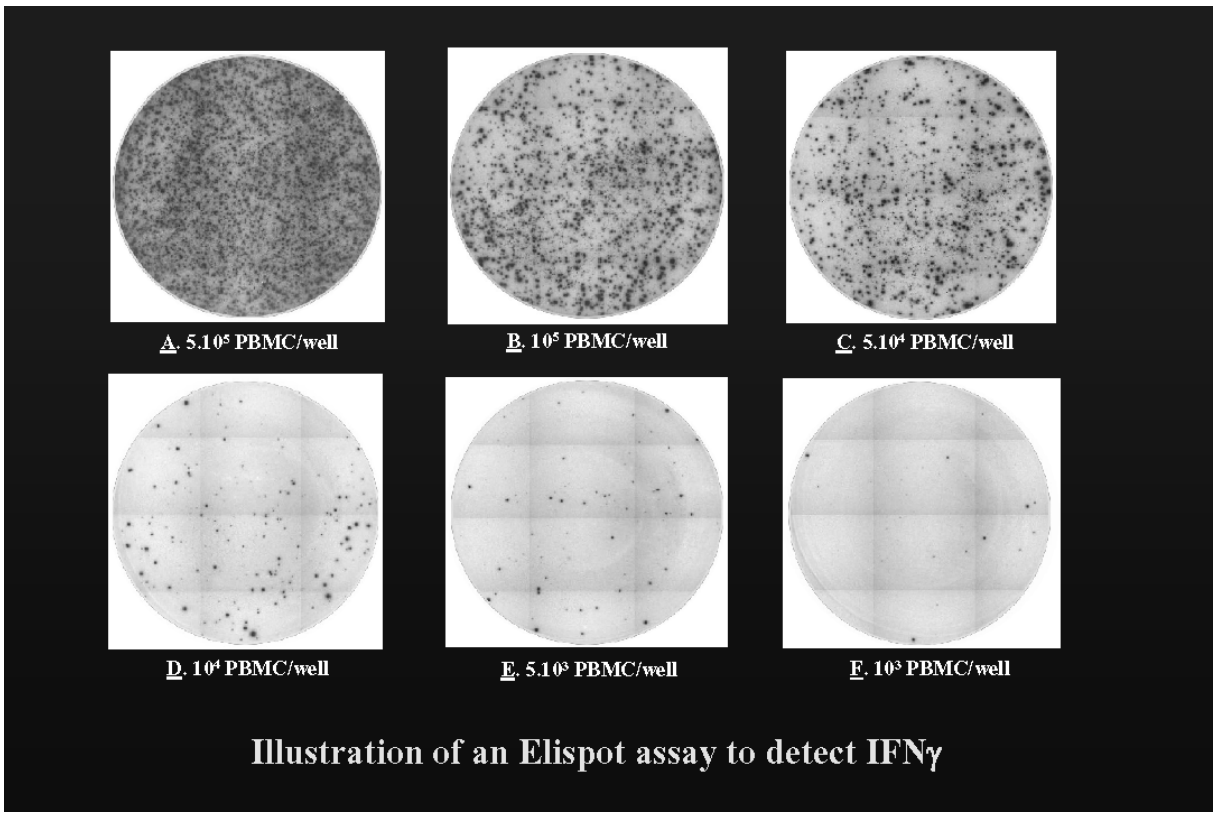


Illustration of an Elispot assay to detect IFNγ

SENSITIVITY OF ELISPOT

- The plate of Elispot is saturated for concentrations of PBMC above : 10^5

→ Theoretically : Threshold of detection : $1/10^5$ (0.001%)

- In routine analysis :
the test is considered positive if > 5-10 spots per 10^5 cells.

Sensitivity : $1/10^4$ et $1/10^5$

ADVANTAGES OF THE ELISPOT TECHNIQUE

- Detect functional T lymphocytes

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

- High sensitivity

- Possible automation

LIMITS OF THE ELISPOT TECHNIQUE

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

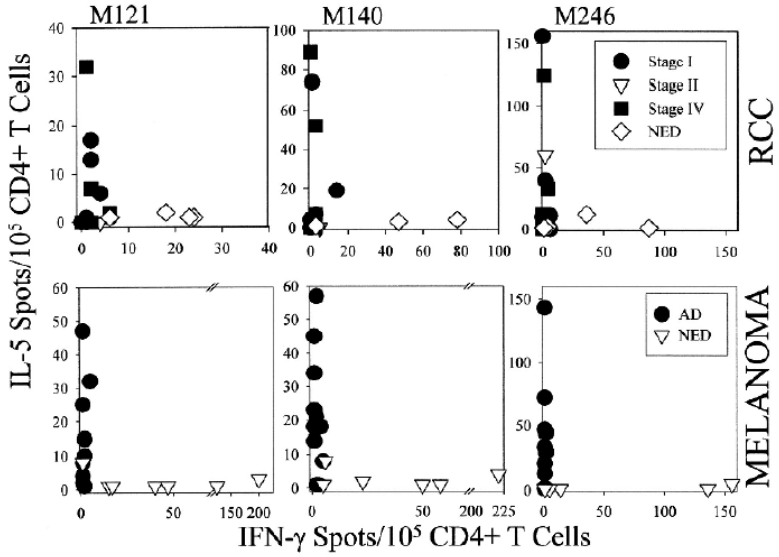
- DOES NOT ALLOW THE PHENOTYPING OF T CELLS PRODUCING THE CYTOKINE

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

INDICATIONS OF THE ELISPOT TECHNIQUE

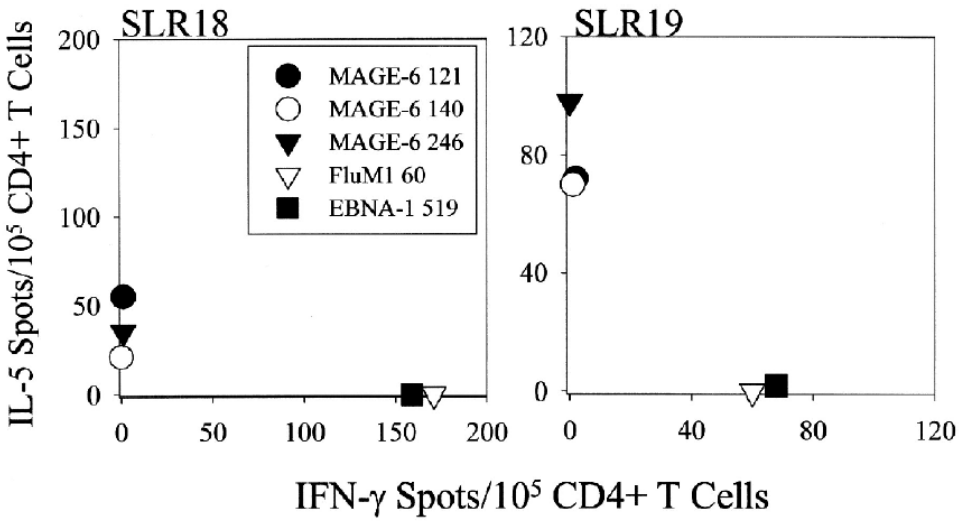
- Analysis of the frequency of T cell precursors producing cytokines (TH1, TH2, TH3…). Analysis of the polarisation of T lymphocytes.

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



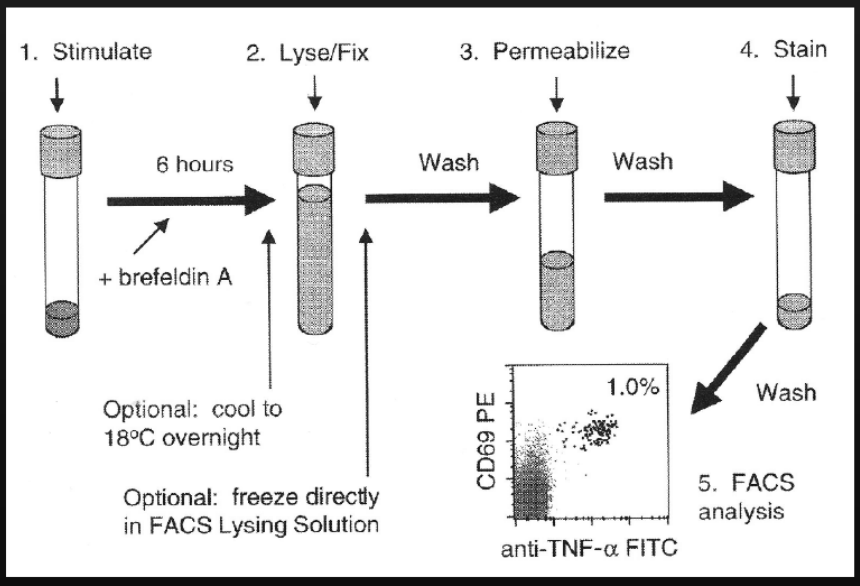
Analysis of the TH1-type vs TH2 type CD4+ T cell response to Mage-6 peptides in HLA-DRβ10401 in RCC or melanoma patients.

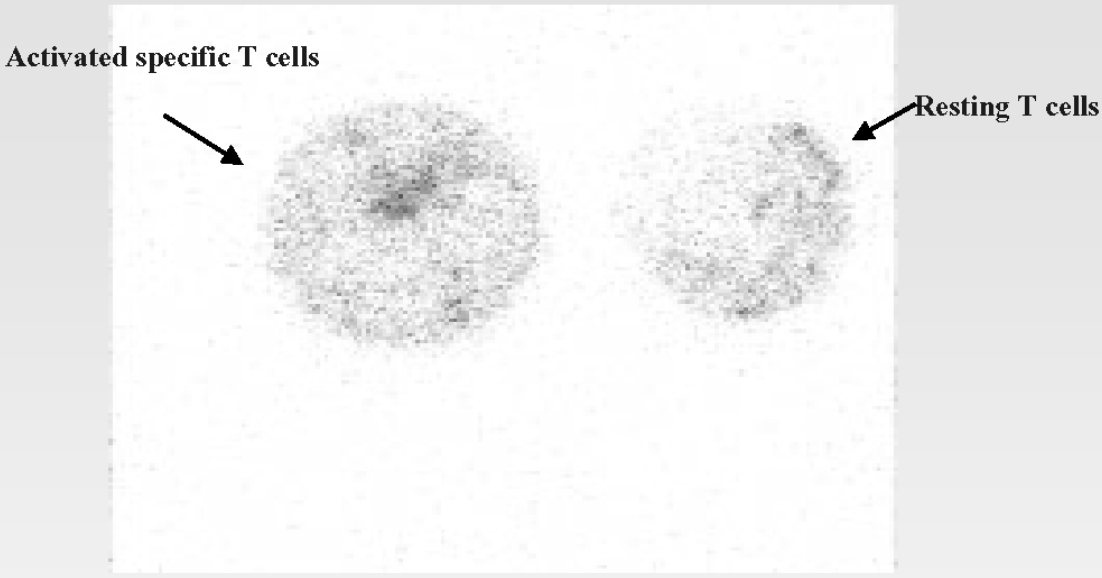
Tasumi et al J. Exp Med 2002



Peripheral blood from cancer patients display TH2-type reactivity to Mage-6 epitope but TH-1 reactivity to viral epitopes.

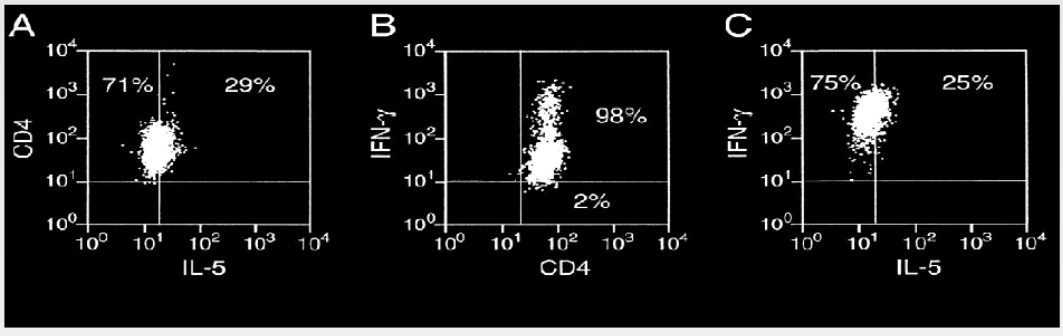
DETECTION OF SPECIFIC T LYMPHOCYTES BY MEASURING INTRACELLULAR CYTOKINE BY FACS ANALYSIS





Activated and resting T lymphocytes identified by the expression of CD3 (green) and producting or not IL-2 (red)

Single-cell analysis of IFN- and IL-5 synthesis by clone -CD4 anti-HA



Graham CM J Immunol 1998

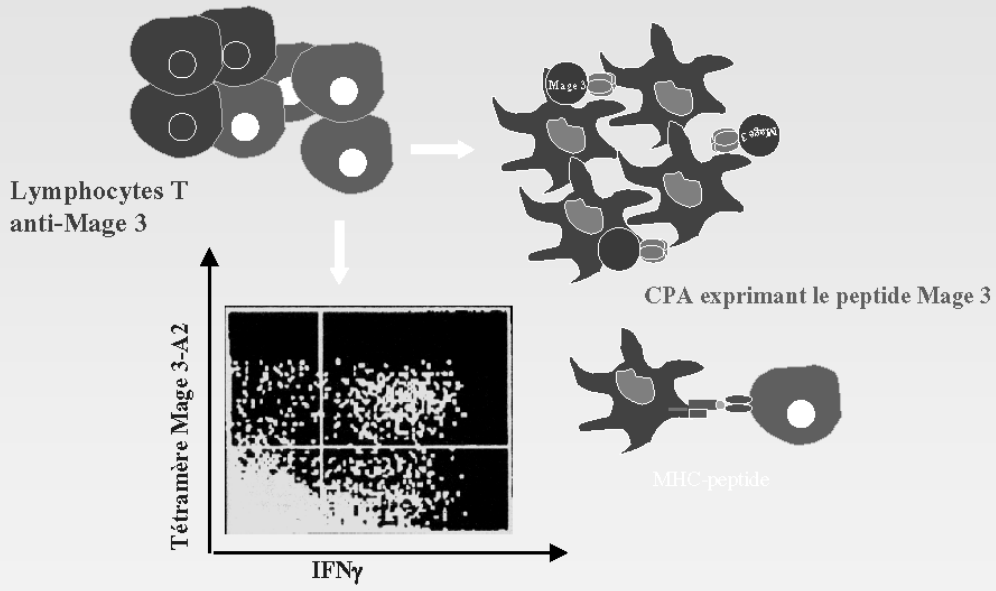
DETECTION OF SPECIFIC T LYMPHOCYTES BY INTRACELLULAR CYTOKINE ANALYSIS USING CYTOMETRY

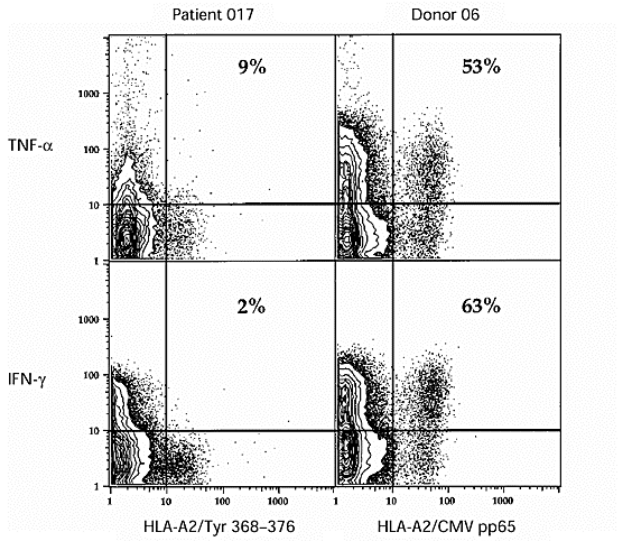
- SENSITIVITY : Ability to detect 0.1 to 1% specific T cells ($1/10^{-2}$-$1/10^{-3}$)

- Identification of functional specific T lymphocytes

- Possibility to phenotype but not to sort specific T lymphocytes producing cytokines.

COUPLING MHC-PEPTIDE TETRAMER ANALYSIS WITH DETECTION OF INTRACELLULAR CYTOKINES





Intracellular cytokine staining of antigen-specific T cells.

Tyrosinase-specific CD8+ T cells from patient 017 and CMV-specific CD8+ T cells from donor 06 were stained for the expression of TNFα and IFNγ after stimulation with PMA and ionomycin for 6 h (last 3 h with Brefeldin A)

Lee et al Nat Med 1999

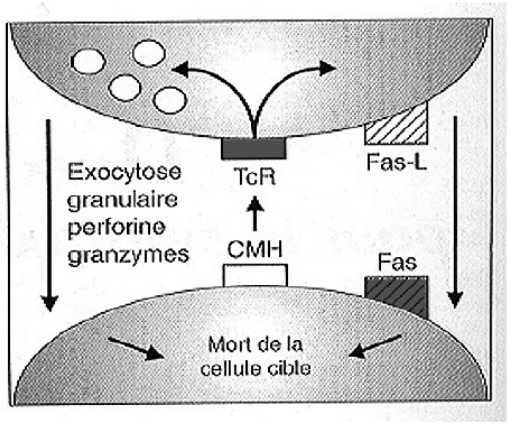
Comparative analysis of techniques to detect specific T lymphocytes

	ELISPOT	INTRACELLULAR CYTOKINES	MHC TETRAMERE	CYTOTOXICITY ^{51}Cr
Sensitivity	10^4-10^5	10^3	10^3-10^4	10^3
T cell detection				
- fonctional	+	+	+	+
- anergic	-	-	+	-
Phénotypic analysis	-	+	+	-
Sort of cells	-	-	+	-
Automation	+	-	-	-

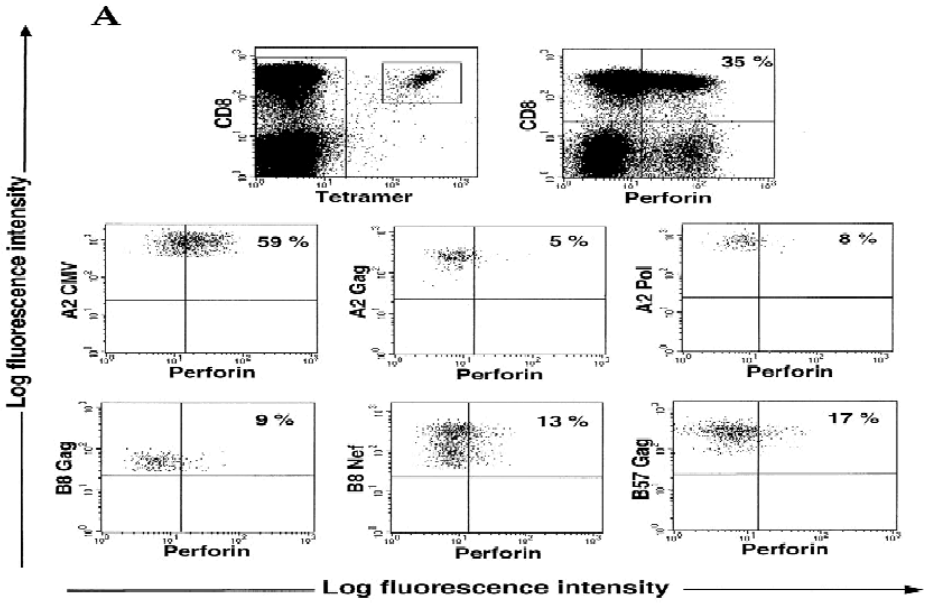
ANALYSIS OF CYTOLYTIC POTENTIAL OF CD8- T CELLS

- Labelling the target with : ^{51}Cr (standard cytotoxic ^{51}Cr assay)
  : **fluorescent dyes** (flow based killing assay)

- Assessment of perforin or granzyme cell content (Intracellular cytometry)
  release by CD8-T cells (Elispot)



Two main mechanisms responsible of the cytotoxicity mediated by CD8-T-cells



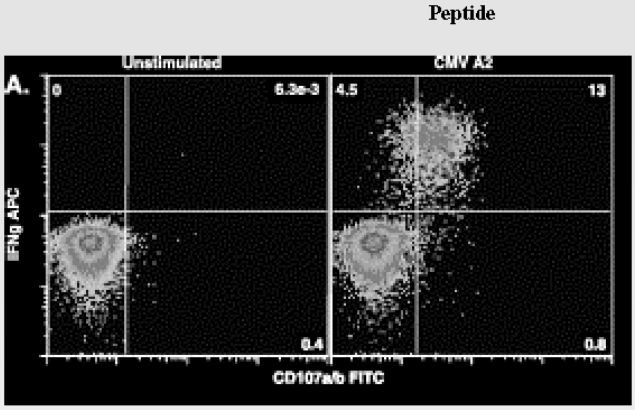
HIV specific CD8+ T cells express low levels of perforin

Appay V J. Exp Med 2000

Lytic granules of CD8-T cells contain a dense core composed of various proteins including perforin and granzymes surrounded by a lipid bilayer containing lysosomal associated membrane glycoproteins (LAMPS) including CD107a (LAMP-1), CD107b (LAMP-2) and CD63 (LAMP-3).

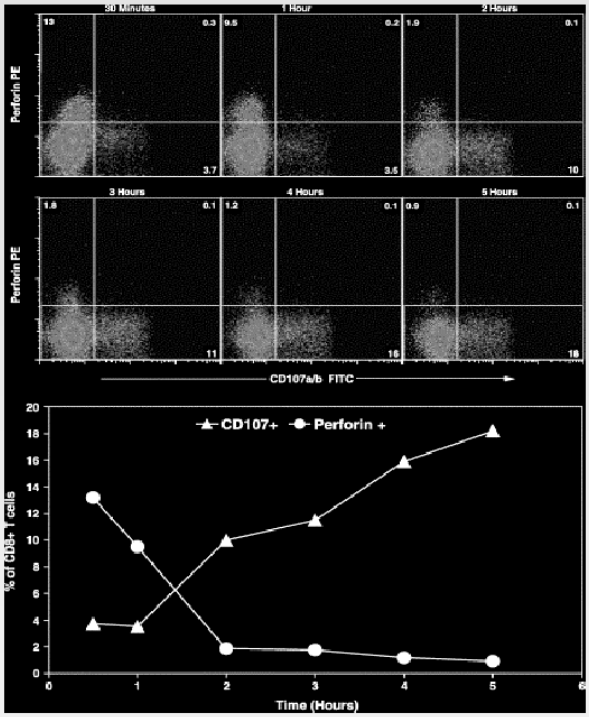
Cumulative exposure of granular membrane proteins (CD107a and b) on the cell surface of responding antigen-specific T cells provides a marker of degranulation.

Significant expression of cell surface CD107a and b can be observed as early as 30 min following stimulation of primary CD8+T cells, and reaches a maximum by 4h.



CD107a is expressed by ex vivo activated antigen-specific CD8+ T cells

Betts MR J Immunol Methods 2003



Betts MR J Immunol Methods 2003

Acquisition of cell surface CD107 is correlated with a loss of intracellular perforin

Cytolytic activity of tetramer-positive CD107a+ and CD107a- clones directed against gp100

Sample 10545

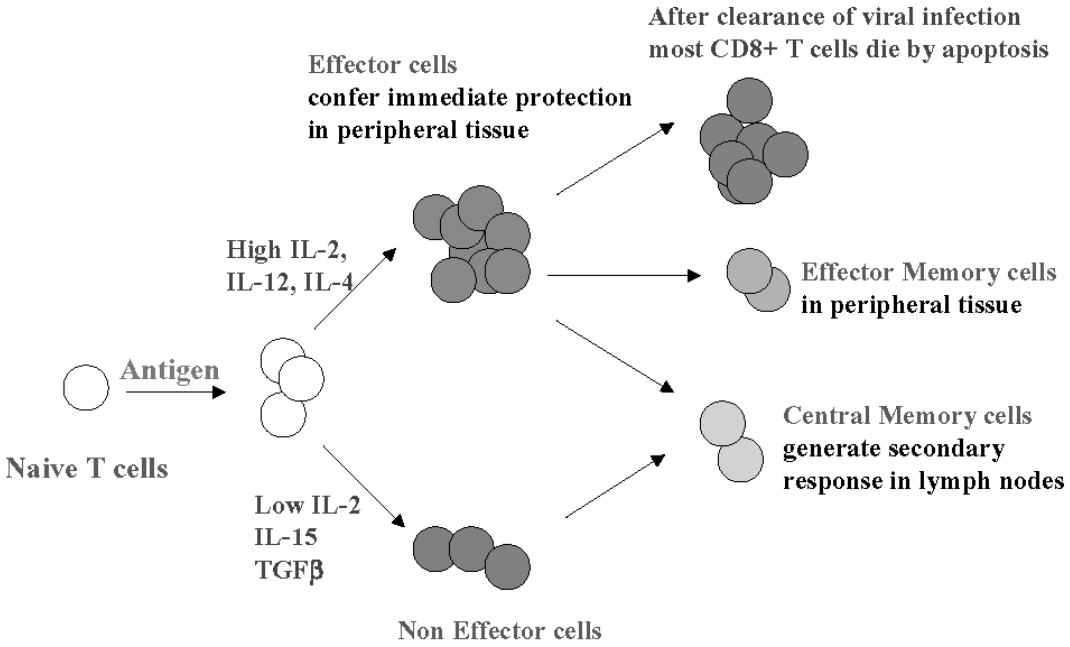
Tetramer-positive CD107a+ clones				Tetramer-positive CD107a− clones			
Malme-3M	mel526	A375	RE	Malme-3M	mel526	A375	RE
31	38	−1	10^{-12} M	0	0	0	10^{-8} M
20	22	−1	10^{-10} M	8	4	−1	10^{-9} M
20	22	−1	10^{-11} M	11	7	0	10^{-9} M
27	29	−1	10^{-11} M	24	20	1	10^{-10} M
20	15	−1	10^{-11} M	2	1	0	10^{-8} M
—	—	—	—	4	8	−1	10^{-9} M
—	—	—	—	1	5	−1	10^{-8} M
23.6	25.2	−1	Averages	7.1	6.4	−0.3	Averages

Sample 10356

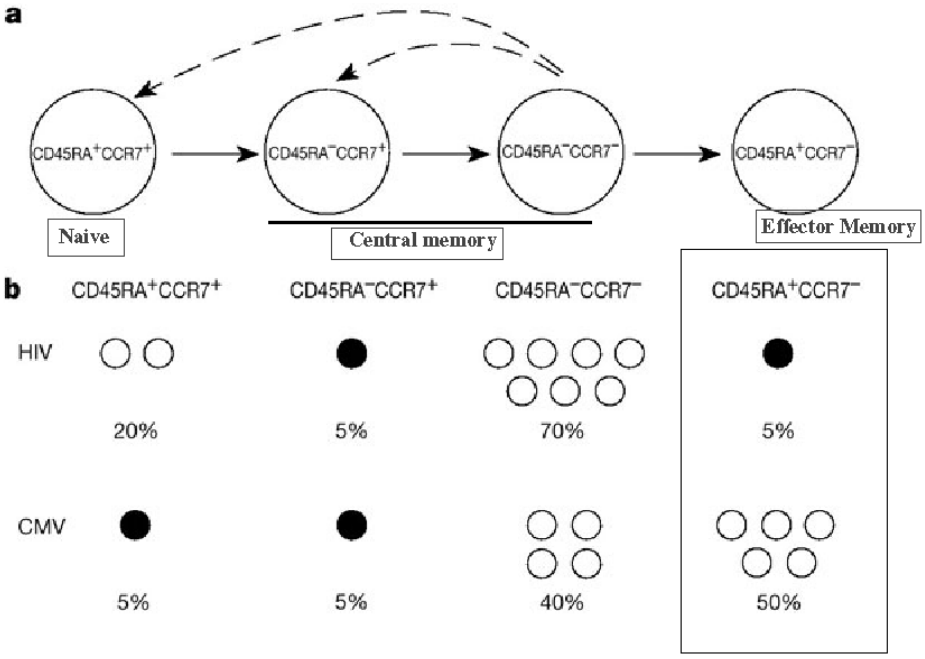
Tetramer-positive CD107a+ clones				Tetramer-positive CD107a− clones			
Malme-3M	mel526	A375	RE	Malme-3M	mel526	A375	RE
40	42	1	10^{-11} M	2	6	0	10^{-8} M
37	32	2	10^{-11} M	2	5	0	10^{-7} M
40	42	3	10^{-11} M	1	3	1	10^{-7} M
33	32	3	10^{-10} M	42	47	0	10^{-11} M
32	34	2	10^{-10} M	2	5	1	10^{-9} M
39	51	1	10^{-12} M	—	—	—	—
36.8	38.8	2	Averages	9.8	13.2	0.4	Averages

Rubio V
Nature Med 2003

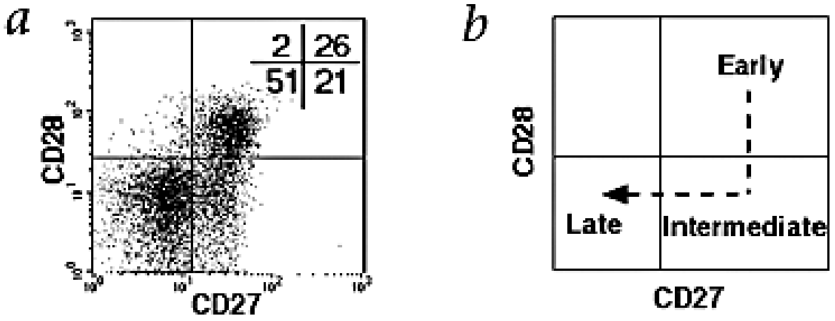
Phenotypic markers to identify functional (effector T cells)



Pathways for memory T cell generation

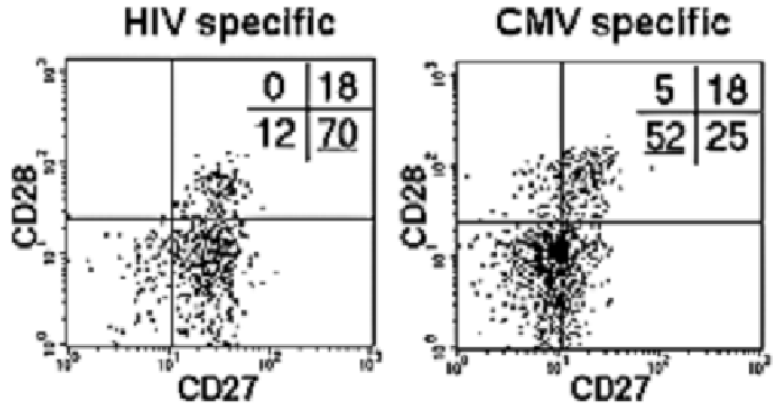


Differences in the composition of the HIV and CMV specific memory CD8+ T cell subsets



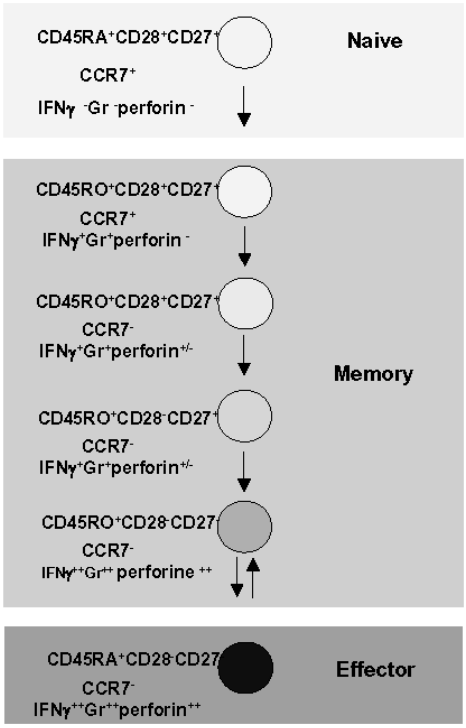
Model of antigen-specific CD8+ T cell differentiation

Appay V Nature Med 2002



CD28/CD27 expression on virus-specific CD8+T cells during chronic viral infection

Appay V Nature Med 2002

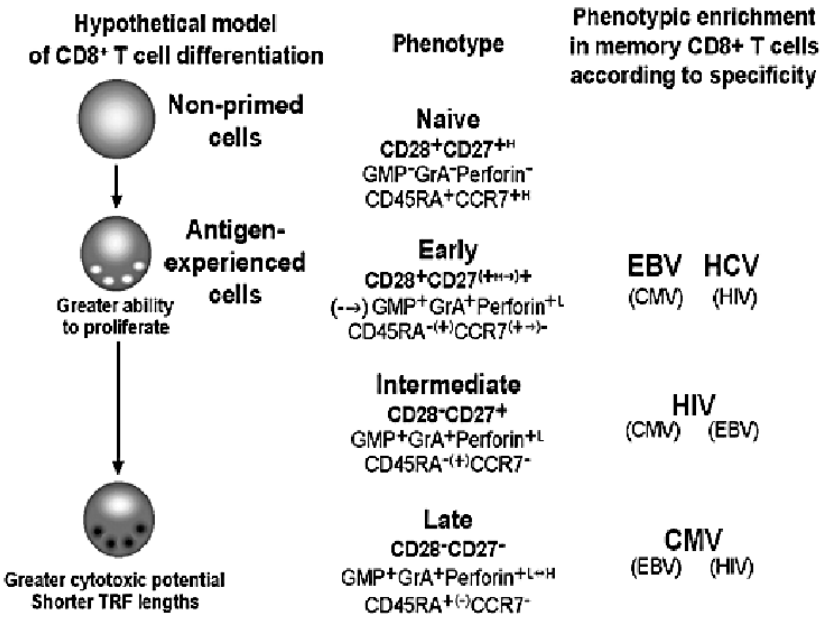


Gr: Granzyme

Différenciation of CD8-T cells

Van Baarle et al.

Trends in Immunology 2002



	CCR7	CD62L	Perforin	CD45RA	CD44	CD122 β chain IL-2R/IL-15R
Central memory CD8-T cells	+	+	-	-	+	-
Effector Memory CD8 T cells	-	-	+	+	+++	+

Phenotypic markers of effector and central memory CD8 - T cells in mice

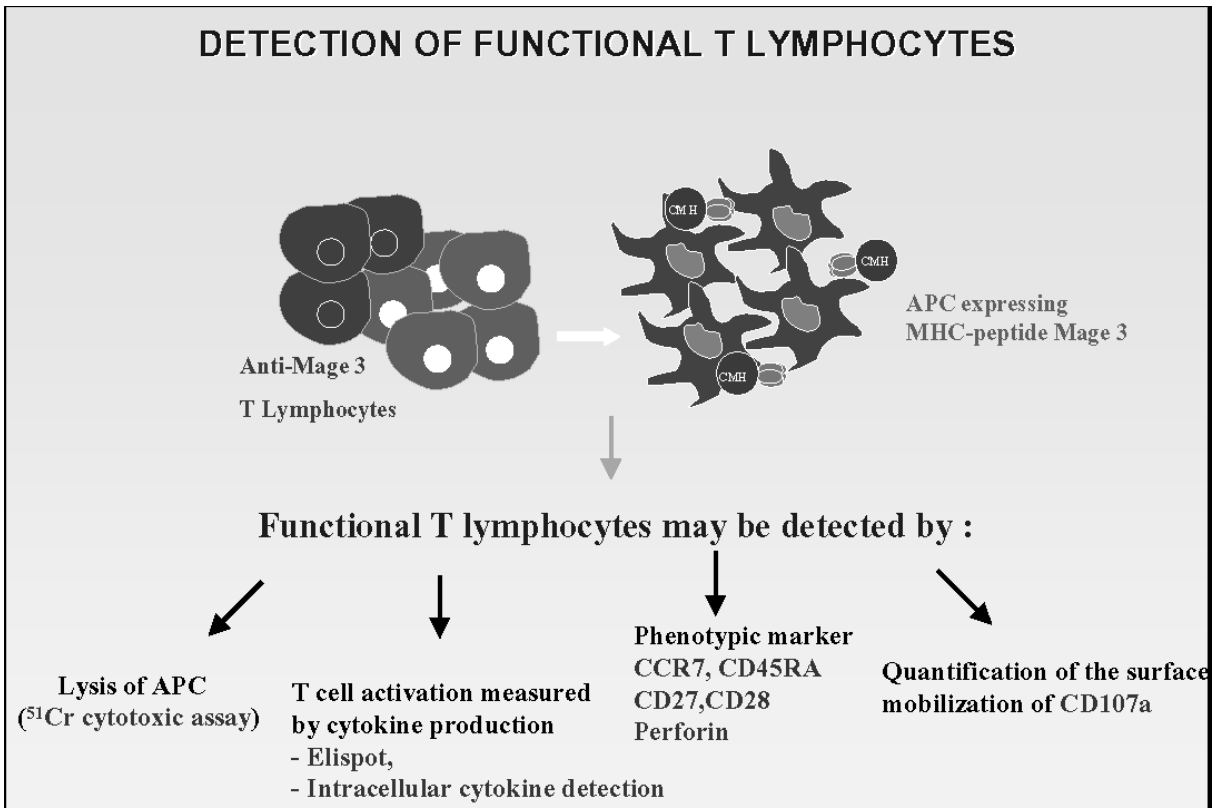
CONCLUSION AND PERSPECTIVES

-Numerous techniques for the detection and characterization T-lymphocytes have been reported during these last years. They will allow to better discriminate functional activity and anergic T-cells.

-Surrogate T lymphocytes markers of the in vivo functional activity of these T cells have to be selected among different parameters.

- This monitoring will guide the improvement of strategies for the development of cancer vaccines.

Annexe F : Detection of functional T lymphocytes, Eric Tartour

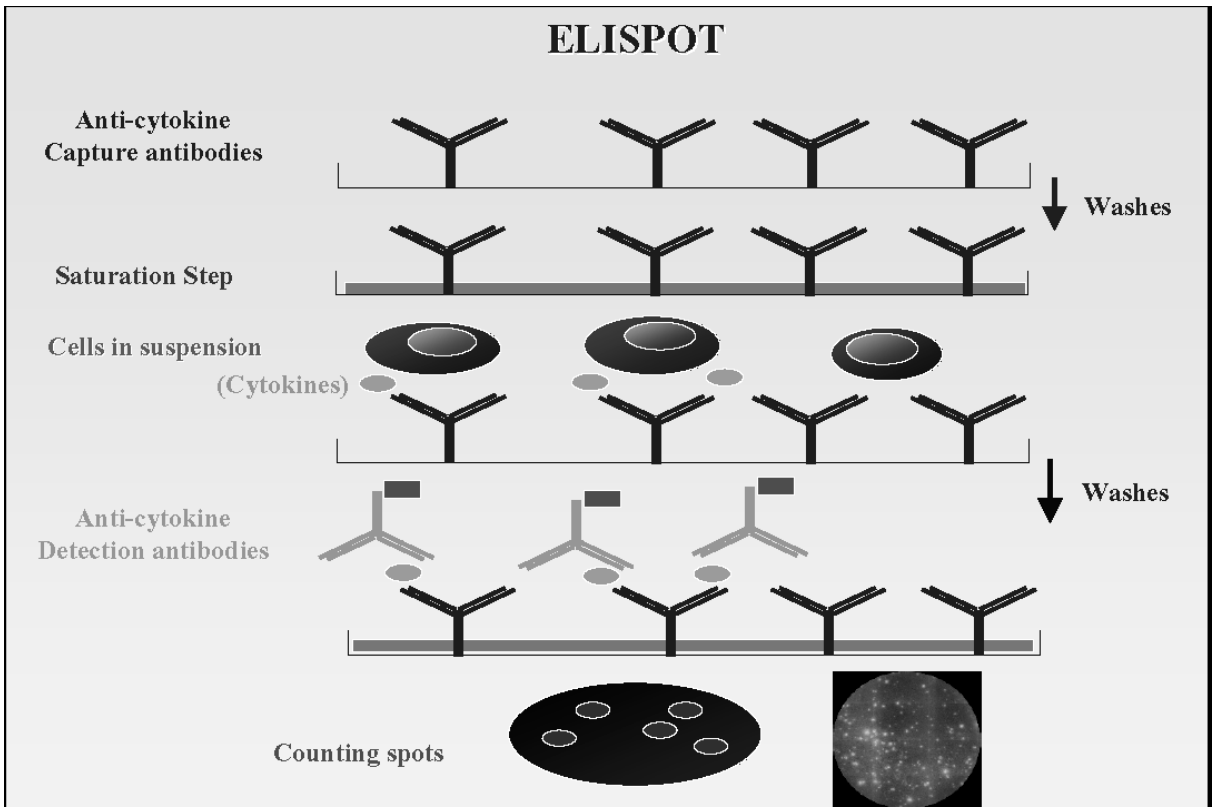


ELISPOT

- 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.

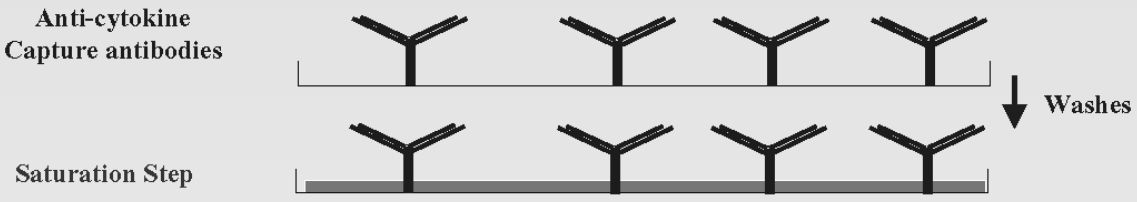
→ Use higher concentrations of antibodies for the coating

- 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



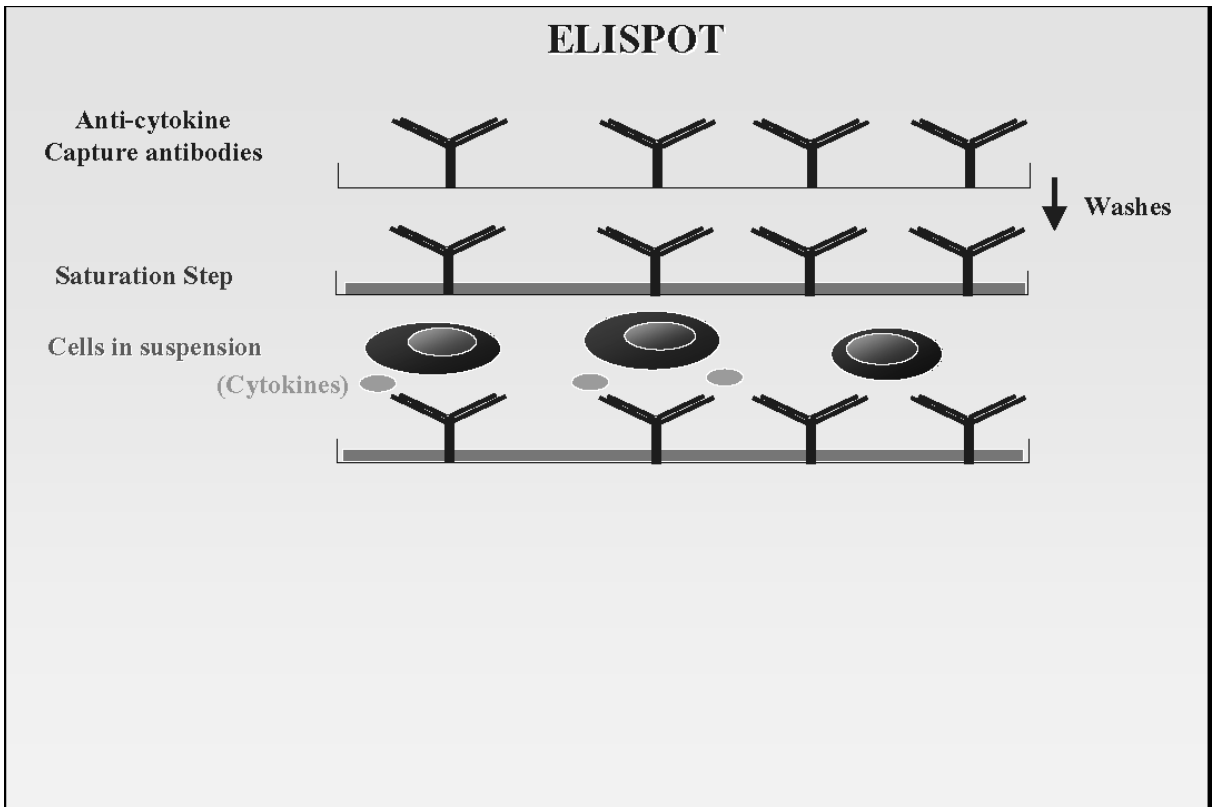
SATURATION STEP

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)

ELSEVIER

Journal of Immunological Methods 283 (2003) 91 98

JIM Journal of Immunological Methods

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A Fluorospot assay to detect single T lymphocytes simultaneously producing multiple cytokines

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Abstract

Various subpopulations of T lymphocytes—i.e. Type 1, Type 2, Tr1 T cells—play a major role in the homeostasis of the immune system and in the pathogenesis of many inflammatory and auto-immune diseases. At present, in the absence of specific surface markers, these T cells can only be reliably distinguished on the basis of their cytokine production profile. The Elispot assay detects cytokine-producing cells, but in most cases can detect only one secreted cytokine, which represents a major limitation of this technique.

We have developed a Fluorospot assay to detect single cells that simultaneously produce multiple cytokines. The Fluorospot assay permits the detection of regulatory T cells with an immunosuppressive activity, identified by their coexpression of IL-10 and IFNγ. Polarized type 1 and type 2 specific tetanus toxoid T cells are also directly detected using a dual color Fluorospot. This technique will therefore be useful for detailed analysis of T lymphocytes in various disease states in which an imbalance of T cell subpopulations is suspected, but will also provide a better characterization of polarized specific immune responses.

Keywords: Elispot; Cytokine; T-lymphocytes

1. Introduction

Various subpopulations of T lymphocytes, i.e. Type 1, Type 2, TH3 and Tr1 T cells, which play a major role in homeostasis of the immune system, have been characterized. Although many attempts have been made over the past decade to identify specific cell surface markers, these various types of lymphocytes

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0022-1759/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.jim.2003.08.013

are mainly distinguished, at present, on the basis of their cytokine production profile.

Type 1 T cells are identified by their expression of IL-2, IFNγ and TNFβ, whereas type 2 T cells secrete IL-4, IL-5, IL-10 and IL-13. TH3 cells produce TGFβ and variable amounts of IL-10 and IL-4. Type 0 cells are considered to be precursor cells with a mixed phenotype (Fridman and Tartour, 1997). The hallmark of Tr1 cells is their production of high levels of IL-10 and TGFβ, moderate amounts of IFNγ and IL-5, but little or no IL-2 or IL-4 (Roncarolo et al., 2001). The detection of these T cell subpopulations is crucial to determine and analyze the type of immune response induced by antigen priming. For instance, type 1 T cells provide a helper activity for T cell-mediated immunity, whereas type 2 T cells are important for B cell development and antibody production (Mosmann and Sad, 1996). 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 TGFβ (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 and Steinman, 2002).

An imbalance in the relative numbers of these various T cell subpopulations may affect the state and progression of several diseases, including infectious, allergic and auto-immune disorders, but also cancers (Yssel and Groux, 2000; Clerici et al., 1998).

The enzyme-linked immunospot (Elispot) assay, based on solid phase immunoenzyme technology, is able to detect single cells producing various immunoreactive substances, such as cytokines. The end result of this test is a set of colored spots, each representing a "footprint" of the original cytokine-producing cells (Czerkinsky et al., 1988). However, 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, IFNγ can be secreted by type 0, type 1 and Tr1 T cells, and IL-10 is produced by both type 2 and Tr1 cells. To overcome this limitation of the Elispot, we have developed a Fluorospot assay, which is a modification of the Elispot based on the use of multiple fluorescent labeled anti-cytokines detection antibodies. This test permits the detection of single cells producing multiple cytokines.

2. Methods

2.1. Cells

The B-EBV cell line V.1 was a gift from Dr U. Blank (Institut Pasteur, Paris) and the Sezary cells, SeAx, were kindly provided by Dr Keld Kaltoft (University of Aarhus, Denmark).

Cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM glutamine (Sigma, Saint Quentin Fallavier, France), 5 mM sodium pyruvate, and 50 μM 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin.

2.2. Cell purification

Mononuclear cells from pleural effusion were enriched by a Ficoll-Hypaque gradient. CD3+CD4+ T cells were then purified by magnetic cell sorting using a MiniMACS device (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of recovered cells ranged between 97% and 99%.

2.3. Elispot

The Elispot assay for the detection of IFNγ was purchased from Diaclone (Besançon, France).

2.4. Fluorospot

Ninety-six-well polyvinylidene difluoride flat-bottom plates (Millipore, Molsheim, France) previously treated with ethanol were coated overnight at 4 °C with 100 μl of mouse monoclonal cytokine-specific antibodies (mAbs) at 10 μg/ml in PBS. All anti-human cytokine mAbs (Anti-IFNγ, Anti-IL-2, Anti-IL-5, Anti-IL-10) used were obtained from Diaclone.

The references for all antibodies and amplification reagents used for this assay have been summarized in Table 1.

The plates were then blocked with 2% milk in PBS for 2 h at room temperature and washed twice with PBS containing 0.05% Tween. Cells in a volume of 100 μl were then added in serial dilutions in duplicate and were incubated for various times ranging from 18 h to 48 h at 37 °C in a humidified atmosphere of 5% $C0_2$ in air. The plates were then

Table 1
List and references for all antibodies and amplification reagents used for the Fluorospot assay

	Fluorospot IFNγ	Fluorospot IL-2	Fluorospot IL-5	Fluorospot IL-10
Capture Ab	Mouse IgG1 anti-IFNγ Clone B-B1	Mouse IgG1 anti-IL-2 Clone B-G5	Rat IgG2a anti-IL-5 Clone BZ 25	Mouse IgG1 anti-IL-10 Clone $B.N_{10}$
Detection Ab	Biotinylated or fluorescein-labeled Mouse anti-IFNγ Clone B-G1	Biotinylated rabbit polyclonal anti-IL-2	Biotinylated goat polyclonal anti-IL-5	Biotinylated mouse IgG2b anti-IL-10 Clone $B.T_{10}$
Amplification step	Anti-fluorescein rabbit IgG labeled with Alexa Fluor 488 or Phycoerythrin-conjugated streptavidin (Dako)	Biotinylated goat anti-rabbit IgG (Southern Biotechnology) and Phycoerythrin-conjugated streptavidin (Dako)	Phycoerythrin-conjugated streptavidin (Dako)	Phycoerythrin-conjugated streptavidin (Dako)

washed and 100 μl of labeled anti-cytokine detection antibodies diluted in PBS containing 1% BSA was added.

For the detection of IFNγ, 100 μl of a fluorescein-labeled mouse monoclonal anti-IFNγ antibody (2 μg/ml) was added for 1.5 h at 37 °C and the signal was amplified with 15 μg/ml of an anti-fluorescein rabbit IgG conjugated with Alexa Fluor 488 (Molecular Probes, Eugene, OR) for 1 h at 37 °C in a volume of 100 μl. This amplification step was essential for detection of a fluorescent signal.

When the phycoerythrin fluorophore was selected to reveal the IFNγ Fluorospot, a biotinylated antibody directed against IFNγ was used as the detection antibody. Phycoerythrin-conjugated streptavidin was then added to the amplification step (Table 1).

For the detection of IL-2, IL-5 or IL-10, 100 μl of biotinylated rabbit polyclonal anti-IL-2 or biotinylated goat polyclonal anti-IL-5 or biotinylated monoclonal anti-IL-10 antibodies (Diaclone) were added for 1.5 h at 37 °C. All these antibodies were used at a concentration of 1.5 μg/ml. An amplification step with 100 μl of biotinylated goat anti-rabbit IgG (500 ng/ml) (Southern Biotechnology, Birmingham AL) for 45 min at 37 °C was added for the detection of IL-2-producing cells. This amplification step for IL-2 increased the signal but satisfactory results could still be obtained when it was omitted. After washing, the reaction was revealed with 15 μg/ml of phycoerythrin-conjugated streptavidin (Dako, Trappes, France) for 1 h at 37 °C.

Spots were read and counted using a stereomicroscope (Carl Zeiss, Le Pecq, France) equipped with a fluorescent reading system.

For dual color Fluorospot assays (IFNγ-IL-2, IFNγ-IL-5, IFNγ-IL-10), two pairs of anti-cytokine antibodies were simultaneously included without any change in the protocol.

To ensure that the reaction was specific and to eliminate cross-reactivity between the different anti-cytokine antibodies, pairs of anti-cytokine antibodies were split and one anti-cytokine antibody was replaced by an irrelevant isotype control antibody or a second anti-cytokine antibody not recognizing the same cytokine as the first reagent used. In all cases, no spot was observed.

During the double IFNγ/IL-2 Fluorospot assay, to avoid a possible cross-reactivity between the biotinylated goat anti-rabbit IgG used for the amplification step of IL-2 and the rabbit IgG anti-fluorescein used to reveal IFNγ, we did not add these reagents simultaneously. We first incubated the cells with the biotinylated goat anti-rabbit IgG for 1.5 h at 37 °C and then performed three washings with PBS containing 0.05% Tween. Finally, the anti-fluorescein rabbit IgG conjugated with Alexa Fluor 488 was added for 1 h at 37 °C.

2.5. Cell activation

Peripheral blood mononuclear cells (PBMC) were activated with either phorbol 12-myristate 13-acetate (PMA; 100 ng/ml) and ionomycin (10 μM) or a combination of 10 μg/ml of anti-CD3 mAb (Beckman Coulter, Villepinte, France) and 1 μg/ml of anti-CD28 mAb (Beckman Coulter) for the indicated incubation times.

For the detection of anti-tetanus toxoid specific T cells, PBMC (10^5 cells/well) were incubated with 20

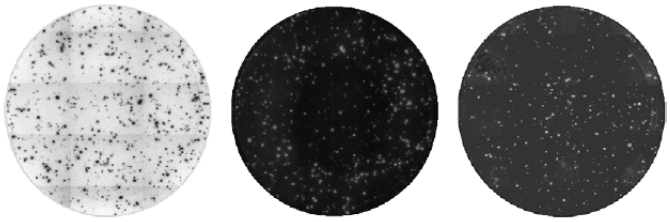

Fig. 1. Illustration of an Elispot and a Fluorospot assay. PBMC (3×10^3) were activated with PMA (100 ng/ml) and ionomycin (10 μM) for 18 h. IFNγ-producing cells were detected with either an alkaline phosphatase Elispot (left) or a Fluorospot assay using Phycoerythrin (middle) or Alexa Fluor 288 (right) conjugated antibodies.

μg/ml of tetanus toxoid (Statens Serum Institute, Copenhagen, Denmark) for 48 h directly in the Fluorospot assay plates.

3. Results

3.1. Development of a Fluorospot assay

During the development of a Fluorospot assay for the detection of cytokine-producing cells, different fluorophores including phycoerythrin (Fig. 1, middle) and Alexa Fluor 488 (Fig. 1, right) were successfully employed. A linear relationship was observed between the number of activated mononuclear cells added to the well and the number of fluorescent spots representing individual cells that secreted IFNγ (Fig. 2). Three different protocols were compared to detect IFNγ-producing cells. The sensitivity of the Fluorospot assay was similar for each fluorophore used (Phycoerythrin or Alexa fluor 488) and comparable to conventional Elispot procedures revealed by alkaline phosphatase (Figs. 1 and 2).

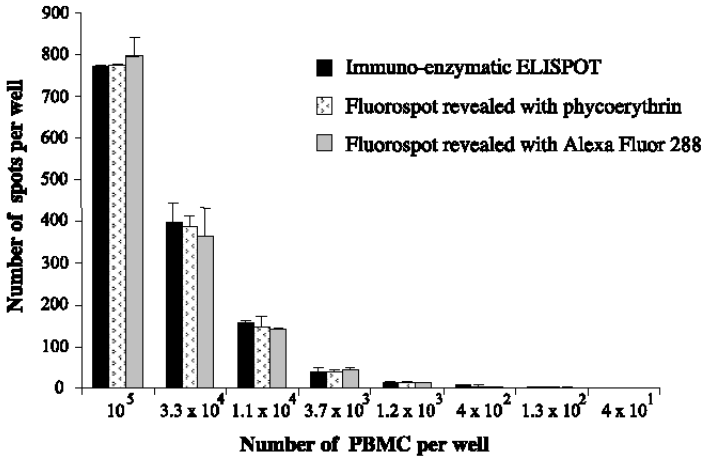

Fig. 2. Analysis of the sensitivity of the Fluorospot assay compared to Elispot. Different concentrations of PBMC were stimulated as in Fig. 1 with PMA and ionomycin. The number of IFNγ-producing cells was compared between Elispot and Fluorospot assays. All experiments were performed in triplicate and non-stimulated cells were introduced as negative controls.

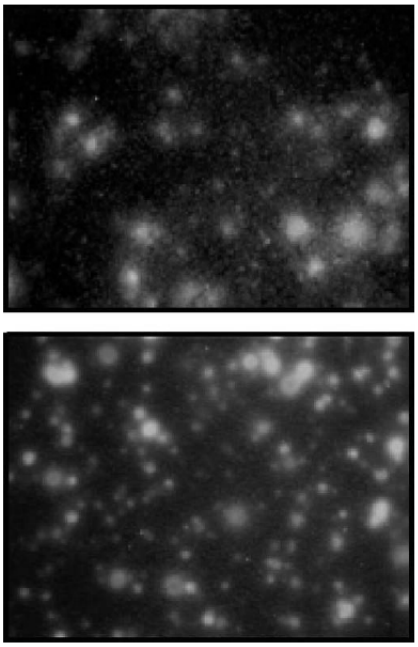

Fig. 3. Dual color Fluorospot for the detection of double cytokine-producing cells. PBMC (5×10^4) were stimulated with PMA and ionomycin on PVDF plates. Double IFNγ and IL-5 (top) or IFNγ and IL-2 (bottom)-producing cells were characterized by a dual color Fluorospot assay. Green spots corresponded to IFNγ secreting cells (top and bottom), whereas red spots were the hallmark of IL-5 (top) or IL-2 (bottom)-producing cells. Yellow spots corresponded to cells coexpressing IFNγ and IL-5 (top) or IFNγ and IL-2 (bottom). No spot was observed when resting cells were used for the dual color Fluorospot.

3.2. Dual color Fluorospot with single cell resolution

A dual color Fluorospot assay was then validated to measure cytokines coexpressed by individual cells. As expected, when cells were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin, green spots corresponding to IFNγ-producing cells (Fig. 3) or red spots representing either IL-5 (Fig. 3, top) or IL-2 (Fig. 3, bottom) secreting cells, were observed. No spot was detected in the absence of cell activation (data not shown). In addition, mixed color (yellow) fluorescent spots representing IFNγ/IL-5 double positive cells (Fig. 3, top) and IFNγ/IL-2 (Fig. 3, bottom) were easily identified. In contrast, we and other groups have encountered difficulties interpreting these mixed colored spots when immunoenzymatic dual color Elispot assays were used (data not shown) (Okamoto et al., 1998; Karulin et al., 2000). Fluorescence, therefore, clearly provides better discrimination and characterization of double cytokine-producing cells than does an enzymatic reaction.

3.3. Accuracy and specificity of the Fluorospot assay

To validate the sensitivity of this dual color Fluorospot, peripheral blood mononuclear cells (PBMC) were stimulated with PMA and ionomycin, and the frequency of IL-2- and IFNγ-producing cells was tested with the single color or double color Fluorospot procedure. The number of cells producing IFNγ and IL-2 in the single color Fluorospot matched the sum of the frequencies of single and double IFNγ/IL-2 secreting cells detected on the dual color assay (with a 5% error) (Table 2). These results indicate that the dual color Fluorospot does not miss single- and double-expressing cells.

Using intracytoplasmic cytokine analysis performed by cytometry in permeabilized cells, the same frequency of double IFNγ/IL-2-producing cells was observed, which reinforces the accuracy of the test (data not shown).

In addition, to confirm that mixed color spots truly correspond to double-expressing cells and not random superimposition of spots derived from different cells, we mixed IFNγ-producing cells that did not secrete IL-2 with IL-2-secreting cells that did not produce IFNγ. No double color Fluorospot was recorded, which supports the correlation between the presence

Table 2
Accuracy of the dual color IL-2 and IFNγ Fluorospot assay

	Number of spots per well
Fluorospot IL-2	48 (47 50)
Fluorospot IFNγ	59 (56 64)
Dual IL-2/IFNγ Fluorospot	IL-2 spots: 46 (44 48)
	IFNγ spots: 58 (56 60)
	Mixed spots: 4 (3 6)

PBMC (10^4) were activated with PMA and ionomycin for 18 h. IFNγ- and/or IL-2-producing cells were detected with single color IL-2 or IFNγ Fluorospot or dual color IL-2 and IFNγ Fluorospot. The results represent the mean (± S.D.) of triplicate wells and are representative of three experiments.

of mixed spots and double cytokine-producing cells (Fig. 4).

3.4. The Fluorospot assay permits the detection of Tr1-like cells and polarized specific T cells

As a potential application of this Fluorospot assay is to detect T cell subpopulations based on their cytokine secretion profile, we purified CD4+CD3+ T cells from pleural effusions derived from patients with breast cancer. When these cells were stimulated with anti-CD3 and anti-CD28 mAbs, single IFNγ- and IL-10-producing cells were clearly identified (Fig. 5, top). We also observed some cells that simultaneously produced IFNγ and IL-10 and which may have been related to Tr1-like cells (Fig. 5, top). No double color spot was detected in the absence of stimulation (data not shown).

When cells were activated with PMA ionomycin, a known inhibitor of IL-10 induction (Schwarz et al., 1995), red spots corresponding to IL-10-secreting cells were no longer observed, which reinforces the specificity of the results (Fig. 5, bottom). Resting cells did not produce IFNγ (data not shown).

Tumor infiltrating T cells producing IL-10 and IFNγ have been previously detected in cancer patients by PCR (Wang et al., 1995; Rabinowich et al., 1996).

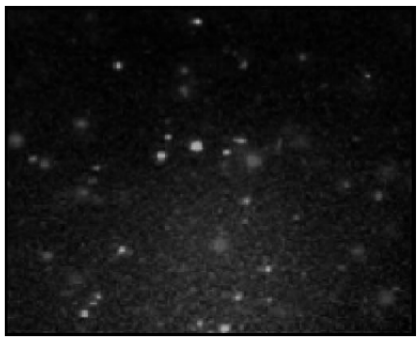

Fig. 4. Specificity of the mixed color spots in the dual color Fluorospot assay. B-EBV.V.1 (5×10^4) and SeAx (10^4) cells were mixed and activated with PMA ionomycin, an inducer of IFNγ without IL-2 in BEBV.V.1 and IL-2 without IFNγ in SeAx cells. IFNγ and IL-2 production was assessed with a dual color Fluorospot. Green and red spots corresponded to IFNγ and IL-2 secreting cells, respectively.

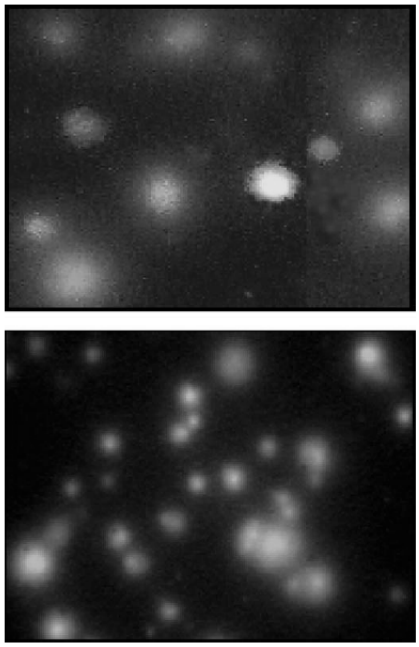

Fig. 5. Detection of Tr1-like cells in pleural effusions. Highly purified CD3+CD4+ T cells from a pleural effusion derived from a breast cancer patient were directly activated in the PVDF plates with anti-CD3 (10 μg/ml) and anti-CD28 (1 μg/ml) (top) or PMA ionomycin (bottom) for 48 h. An IFNγ and IL-10 dual color Fluorospot was used to detect IFNγ- and/or IL-10-producing cells. Green and red spots corresponded to IFNγ and IL-10 secreting cells, respectively. Yellow spots corresponded to cells coexpressing IFNγ and IL-10.

However, in contrast to the Fluorospot assay, coexpression of these cytokines, which is essential for characterization of regulatory T cells with an immunosuppressive activity, could not be analyzed by the previous techniques.

Another advantage of this Fluorospot assay is that it can be used to assess directly polarization of specific immune responses in the same well. Using tetanus toxoid as a model antigen, we directly demonstrated the presence of IFNγ- and IL-5-secreting tetanus toxoid-specific T cells in the blood of healthy patients (Fig. 6). No double IFNγ/IL-5-producing cells were observed in these experiments, which could indicate that these specific T cells were already engaged in a Type 1 or Type 2 differentiation pathway

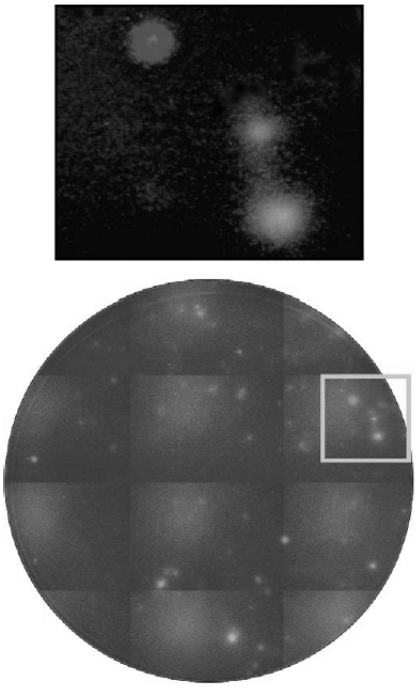

Fig. 6. Detection of polarized specific tetanus toxoid T cells. Bottom: PBMC (10^5 cells/well) were sensitized with tetanus toxoid (20 μg/ml) for 48 h. IFNγ and IL-5 production was assessed with a dual color Fluorospot. Green and red spots corresponded to IFNγ and IL-5 secreting cells, respectively. Top: Greater enlargement of a top right hand quadrant.

(Fig. 6). As a control, no spot was observed when PBMC were not pulsed with antigen (data not shown).

4. Discussion

We have developed a Fluorospot assay for the characterization of single cells secreting multiple cytokines. The sensitivity of the Fluorospot assay is the same as that of the conventional Elispot assay and these two techniques are approximately twice as sensitive as intracytoplasmic staining with FACS analysis for cytokine detection (Helms et al., 2000; Whiteside, 2000).

Unlike intracytoplasmic staining, in which cytokine secretion must be inhibited to increase the signal, the Fluorospot assay directly visualizes the cytokine actually secreted by pharmacologically untreated cells. In addition, detection of a cytokine by cytometry cannot always be equated with secretion of that cytokine, and the assay does not, therefore, measure a cellular function (Whiteside, 2000).

This Fluorospot assay can directly visualize, in the same reaction, the balance between specific type 1 and type 2 T cells. This test will therefore be useful to monitor patients with chronic hepatitis C treated with IFNα-based regimens, in which the initial change in the ratio of type 1 and type 2 HCV-specific CD4 T cells is indicative of treatment outcome (Cramp et al., 2000). A predominant tumor antigen-specific type 2 CD4 T cell response and a frequent decrease of a type 1 specific response has also been reported in cancer patients (Tatsumi et al., 2002).

The use of an immunoenzymatic dual color Elispot assay failed to easily discriminate between double cytokine secreting cells because of difficulties in the interpretation of mixed color spots (Okamoto et al., 1998; Karulin et al., 2000). In this study, we have demonstrated that the Fluorospot assay is better adapted than the Elispot to distinguishing single from double cytokine-producing cells. This property is directly relevant for the analysis of an immune response. For example, during a primary immune response, 80 90% of the population of T cells that express IFNγ fail to coexpress IL-2. Similarly, coexpression of the Type 2 cytokine is rare. However, following the second stimulation, the coexpression pattern more closely resembles a Type 1- and Type 2-like pattern and the incidence of Type 0-like cells is decreased (Bucy et al., 1995).

The ability to detect cells that coexpress cytokines may also have direct applications in the follow-up of HIV and cancer patients.

Various recent studies have shown a correlation between the number of HIV-specific CD4 T cells that secrete both IFNγ and IL-2 and a good clinical outcome in HIV infection, while no difference in the number of specific CD4 T cells secreting only IL-2 or IFNγ was observed between patient groups with different clinical prognosis (Boaz et al., 2002; Sieg et al., 2001).

In melanoma patients immunized with a preparation of heat-killed *Mycobacterium vaccae*, the induction of

IL-2 producing T cells was associated with improved survival (Maraveyas et al., 1999). Surprisingly, the number of T cells secreting IFNγ or coexpressing IL-2 and IFNγ was not associated with a better outcome.

This study has focused on validation of the dual color Fluorospot assay mainly applied to cytokine production by T cells, but the availability of a large range of fluorophores should permit the extension of this technique to multiparameter analysis of molecules secreted by various cell types.

Acknowledgements

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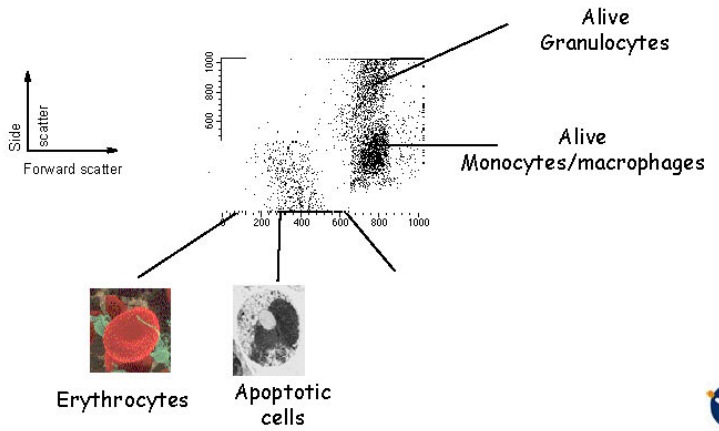
Annexe G : Few notions in flow cytometry and intracellular cytokine detection, Sylvie Garcia

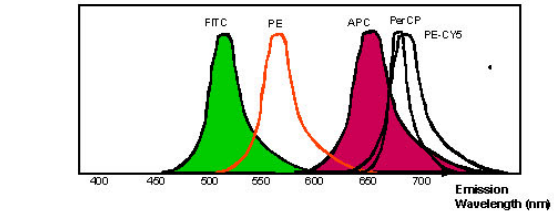
Few notions in flow cytometry and intracellular cytokine detection

Sylvie Garcia, Institut Pasteur, Paris

Detection of the blood subsets using SSC/FCS parameters



Usual dye coupled with antibodies



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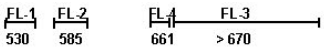
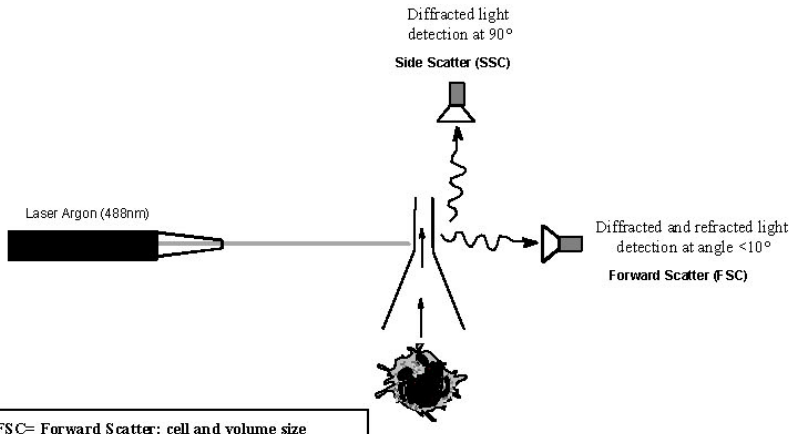


FITC : Fluorescein Isothiocyanate
PE: Phycoerythrin
APC: Allophycocyanin (*)
perCP: Peridinin Chlorophyll Protein
PE-Cy5: PE + Cyanine-5

Size (FCS)-granularity (SSC) detection



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

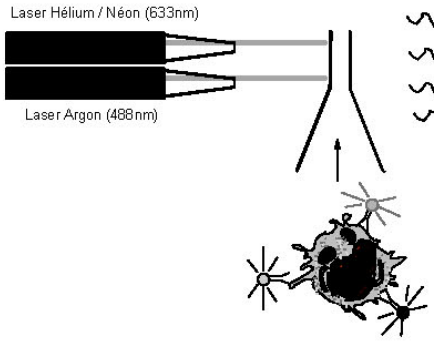
Subsets detectable by flow cytometry

	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

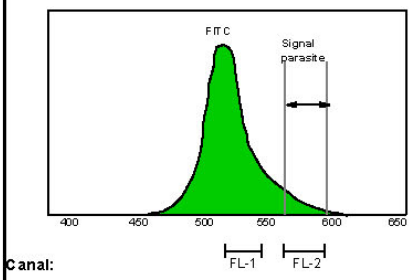
Fluorescence detection



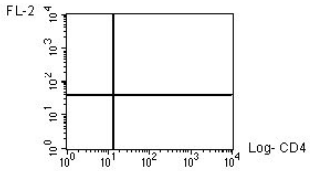
Cytokines/Cytokine receptors detected by flow cytometry

	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

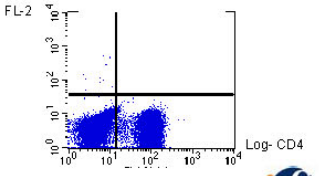
Settings: the compensation problem



CD4 FITC staining not compensated in FL-2

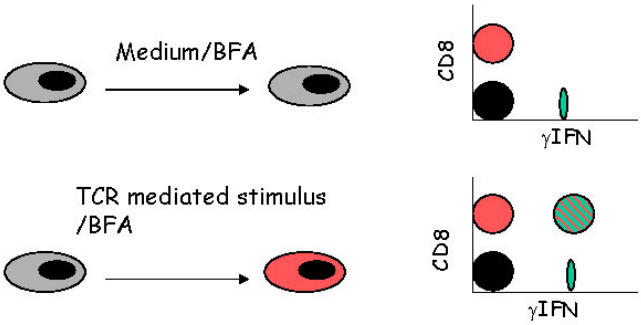


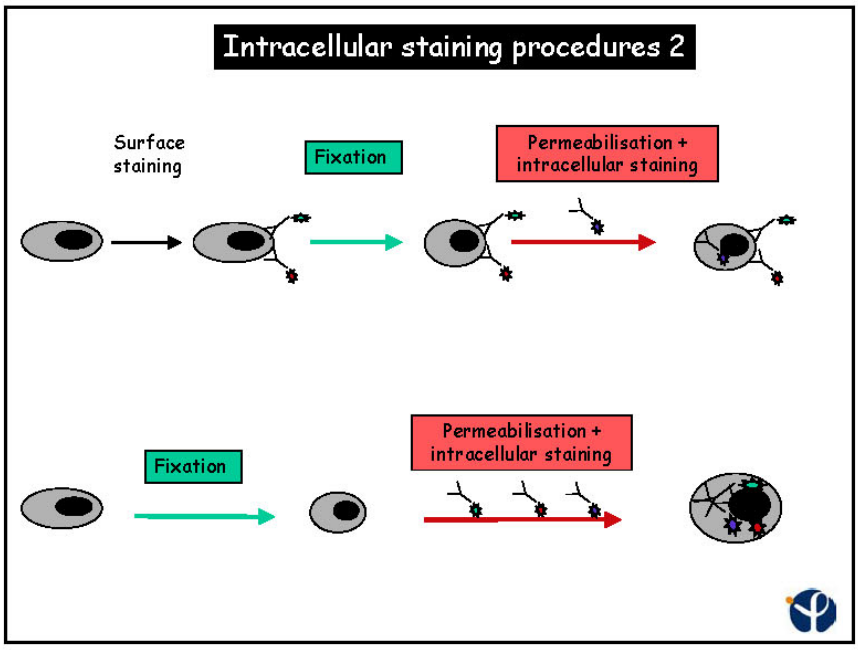
CD4 FITC staining compensated in FL-2



Intracellular staining procedures 1

4-ON culture step:
cytokine accumulation for further detection




Intracellular staining procedures 2
Surface staining
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Fixation
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Annexe H : Rentzsch, C. *et al.* (2003) - Clinical Cancer Research *Vol. 9, 4376–4386.*

Evaluation of Pre-existent Immunity in Patients with Primary Breast Cancer: Molecular and Cellular Assays to Quantify Antigen-Specific T Lymphocytes in Peripheral Blood Mononuclear Cells

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ABSTRACT

Purpose: **Breast cancers are known to frequently (over)express several well-characterized tumor-associated antigens (TAAs) such as carcinoembryonic antigen, MUC-1, Her-2/neu, and cancer/testis antigens such as NY-ESO-1, SSX-2, and members of the MAGE family. Whereas in melanoma patients, the detection of pre-existing T cell responses to tumor-associated differentiation antigens was a rationale to initiate several vaccination strategies, little is known thus far concerning tumor-specific immunity in breast cancer patients. The objectives of our study were (*a*) to modify and compare different immunodiagnostic T cell assays with regard to their suitability for clinical applications and (*b*) to determine endogenous TAA-specific T cell immunity of breast cancer patients at the time point of primary diagnosis.**

Experimental Design: **Using MUC-1- and Her-2/neu-derived HLA-A*0201-restricted peptides as model antigens, we analyzed antigen-dependent IFN-γ release of T cells by enzyme-linked immunospot (ELISpot) assay, intracellular cytokine flow cytometry (CytoSpot), and quantitative real-time PCR. As an assay independent of T cell function, we performed tetramer staining.**

Results: **In our hands, the quantitative real-time PCR method is most sensitive and a feasible screening test to perform an "immunological staging" of cancer patients. By doing this, we detected in 7 of 13 (54%) of HLA-A*0201$^+$ breast cancer patients a pre-existent specific cellular immune response to at least one of the investigated TAAs (MUC-1, Her-2/neu, carcinoembryonic antigen, NY-ESO-1, and SSX-2). Four of 21 patients (19%) were found to have a significant Her-2/neu-specific T cell response as defined by a stimulation index ≥ 2 (range, 10–88).**

Conclusions: **Although the clinical relevance of endogenous TAA-specific immunity remains unclear, our findings suggest that patients with primary breast cancer can mount a T cell immune response to their tumor that might be beneficially enhanced by TAA-dependent vaccination strategies in the adjuvant situation.**

INTRODUCTION

The characterization of TAAs[3] recognized by cellular or humoral effectors of the immune system has opened new perspectives for cancer therapy. Immunomodulating strategies such as peptide-based approaches or cellular cancer vaccines are considered as potential adjuvant therapies in breast cancer patients either to treat minimal residual disease or to prevent relapse (1, 2). These strategies are based on the assumption that the T cell repertoire of individuals contains TAA-specific CTL precursors or tumor-primed memory T cells and that the patient's immune system can be sensitized to TAAs of the patient's own tumor. However, little is known about the incidence and magnitude of a pre-existing tumor-specific cellular immune response in patients with primary breast cancer.

Breast cancer cells frequently overexpress TAAs such as CEA, MUC-1, and Her-2/neu known to elicit HLA-restricted CTLs (3–10). Furthermore, Her-2/neu proves a target for therapeutic antibodies used in clinical studies (11, 12). The family of C/T antigens, first identified in malignant melanoma, includes the most promising candidates for clinical vaccination approaches due to their tumor-restricted expression pattern (13). Multiple C/T antigens, for example, members of the MAGE and BAGE family, SSX-2, and NY-ESO-1, are ubiquitously ex-

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[3] The abbreviations used are: TAA, tumor-associated antigen; CEA, carcinoembryonic antigen; C/T, cancer/testis; qRT-PCR, quantitative real-time PCR; PBMC, peripheral blood mononuclear cell; HS, human AB serum; PMA, phorbol-12-myristate-13-acetate; mAb, monoclonal antibody; Ab, antibody; PE, phycoerythrin; FAM, 6-carboxy-fluorescein; TAMRA, 6-carboxytetramethyl-rhodamine.

pressed in breast cancer as demonstrated by PCR and immunohistochemistry (14).

In several studies investigating PBMCs of melanoma patients, it was demonstrated that the T cell repertoire frequently contains precursors against tumor differentiation antigens such as Melan-A, tyrosinase, or C/T antigens such as NY-ESO-1 (15–19). These findings were the rationale to initiate several TAA-specific vaccination strategies in melanoma and renal cell carcinoma (13, 20). Recently, it was demonstrated that in contrast to healthy donors, melanoma patients show a non-naive phenotype in fractions of Melan-A-specific T cells revealing tumor-dependent immune activation. Furthermore, vaccinations using Melan-A-derived peptides were able to increase the frequency of specific T cells and changed their differentiation stage from naive to memory cells (21).

In breast cancer patients, little information exists about endogenous TAA-specific cellular immune responses. A study analyzing patients with advanced, Her-2/neu-overexpressing breast or ovarian cancer for Her-2/neu-specific immunity showed proliferative PBMC responses to the recombinant antigen in only 10% of the cases. However, in case of HLA-A*02$^+$ patients, no CD8$^+$ T cell response to a defined Her-2/neu HLA-A*02 epitope (E75, Her-2/neu$_{369-377}$) could be detected (22). Another study analyzing breast cancer patient T cells from the bone marrow demonstrated tumor-reactive memory T cells by ELISpot in 10 of 17 patients (23). Interestingly, approximately 70% of CD3$^+$ T cells in the bone marrow of breast cancer patients were CD45R0$^+$ memory T cells (24). Among those, Her-2/neu- and MUC-1-specific HLA-tetramer-binding CD8$^+$ T cells were detectable in a few cases that were able to mount a cytotoxic response, whereas T cells isolated from the blood of the same patients were not able to do so (23).

MUC-1 potentially represents an ideal antigen for immunotherapy because it is overexpressed on a variety of human epithelial tumor cells in an underglycosylated form and proved a target for humoral and cellular immune responses. MUC-1-specific CTL lines stimulated *in vitro* were shown to lyse MUC-1-expressing tumor cells (including breast cancer cells) in a HLA-restricted manner (6, 7, 25). Using MUC-1-derived HLA-A*0201-restricted peptides together with viral peptides (HCMV, EBV, and influenza) as model antigens, we compared different assays to monitor antigen-specific T cells. As functional tests, we analyzed antigen-dependent IFN-γ release of T cells by either ELISpot assay, intracellular cytokine flow cytometry (CytoSpot), or quantification of mRNA encoding IFN-γ using qRT-PCR. Using tetramers, we were able to detect specific T cells independent of their functionality.

Analyzing the pre-existent cellular immune response to frequently expressed breast cancer-associated TAAs, we questioned whether breast cancer patients could have evidence of TAA-specific cellular immunity at the time point of primary diagnosis. In parallel, we investigated blood samples of healthy donors. In approximately 54% of HLA-A*0201$^+$ breast cancer patients, a pre-existing specific cellular immune response to at least one of the investigated TAAs (MUC-1, Her-2/neu, CEA, NY-ESO-1, or SSX-2) could be detected. Nineteen percent of the patients showed pre-existent CD8$^+$ T cell reactions related to Her-2/neu$_{369-377}$ and Her-2/neu$_{654-662}$. However, the clinical significance of this level of tumor-associated immunity is currently unknown.

MATERIALS AND METHODS

Patients and Blood Samples. Healthy donors and patients were selected on the basis of HLA-A*02 antigen expression. Blood samples were obtained after informed consent from patients with primary breast cancer before surgery. Patients enrolled in this study had not received any neoadjuvant or adjuvant chemotherapy, hormone therapy, or radiotherapy before sample collection. PBMCs were isolated from heparinized blood by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density centrifugation, washed twice with PBS (Life Technologies, Inc.), and cryopreserved in FCS and 10% DMSO. Vials contained 1×10^7 cells/ml at maximum and were frozen gradually to −80°C before storage in liquid nitrogen.

Synthetic Peptides. Peptides were synthesized by solid-phase Fmoc chemistry using a peptide synthesizer 432A (Applied Biosystems, Foster City, CA). Identity and purity (>90%) of the peptides were analyzed by reversed-phase high-performance liquid chromatography and matrix-assisted laser desorption/ionization/time-of-flight mass spectrometry. Peptides were dissolved in DMSO at 10 mg/ml, further diluted in H_2O_{bidest} to a final concentration of 1 mg/ml, and stored at –20°C. The following HLA-A*0201-binding peptides were synthesized: CEA$_{571-579}$ (YLSGANLNL); MAGE-1$_{278-296}$ (KVLEYVIKV); NY-ESO-1$_{108-116}$ (SLAQDAPPL); NY-ESO-1$_{86-94}$ (RLLEFYLAM); NY-ESO-1$_{159-167}$ (LMWITQCFL); SSX-2$_{103-111}$ (RLQGISPKI); MUC-1$_{950-958}$ (STAPPVHNV); MUC-1$_{12-20}$ (LLLLTVLTV); Her-2/neu$_{369-377}$ (E75; KIFGSLAFL); Her-2/neu$_{654-662}$ (GP2; IISAVVGIL); HCMV pp56$_{495-503}$ (NLVPMVATV); influenza matrix protein flu$_{58-66}$ (GILGFVFTL); EBV EBNA-6$_{284-293}$ (LLDFVRFMGV); EBV IE63$_{259-267}$ (GLCTLVAML); and RNA-dependent helicase p68$_{146-154}$ (YLLPAIVHI).

Peptide Presensitization of PBMCs. Cryopreserved PBMCs were thawed in RPMI 1640 supplemented with 10% heat-inactivated HS (ccPro, Neustadt, Germany), 1% penicillin/streptomycin, and 4 mM L-glutamine (all from Life Technologies, Inc., Eggenstein, Germany). PBMCs were seeded into round-bottomed 96-well plates (Costar, Bodenheim, Germany) at a concentration of 1×10^5 cells/well and cultured overnight for reconstitution. Thereafter, PBMCs were harvested, washed, and used for functional assays (*ex vivo* testing). For peptide presensitization, PBMCs containing CD8$^+$ effector T cells as well as subsets of antigen-presenting cells were stimulated directly with peptide antigens added in final concentrations of 5 μg/ml. Presensitized PBMCs were harvested after 7 days of peptide stimulation and either stained directly using tetramers or restimulated for an additional 2 h using peptide-pulsed T2 cells as antigen-presenting cells before further functional analysis (peptide presensitization). The same antigens were used for the peptide presensitization as well as the restimulation period.

As positive controls, PBMCs were activated nonspecifically by incubation with a combination of the calcium ionophore A 23187 (ionomycin, 250 nM; Sigma, Taufkirchen, Germany) and PMA (60 nM; Sigma).

ELISpot Assay. ELISpot assay was carried out as described previously (26). In brief, multiscreen 96-well nitrocellulose plates (Millipore, Bedford, the Netherlands) were coated overnight at 4°C with antihuman IFN-γ mAb (1-D1K; 2 μg/ml; Mabtech, Stockholm, Sweden) diluted in 0.1 M carbonate-bicarbonate buffer (pH 9.6). Plates were blocked with RPMI 1640 containing 10% HS for 2 h at 37°C. Different numbers of PBMCs (6.25×10^4 to 10×10^4) presensitized with peptide for 7 days or not (see above) were added to each well and coincubated with 3.5×10^4 peptide-pulsed T2 cells for 18 h in RPMI 1640 and 10% HS. T2 is an antigen processing defective cell line that does express HLA-B/C at rather low levels and expresses higher levels of HLA-A*0201 (27). The antigen pulse was performed in serum-free X-Vivo medium (Biowhittaker, Verviers, Belgium) at a peptide concentration of 5 μg/ml for 2 h. Background controls were performed using p68-pulsed T2 cells as stimulator cells and shown in comparison to TAA and viral antigen stimulations, thereby excluding natural killer and unspecific cellular responses. As a positive control, PBMCs were stimulated with a combination of 250 nM ionomycin and 60 nM PMA. After incubation, plates were washed extensively with PBS and 0.05% Tween 20 (Sigma) and further incubated with 100 μl/well anti-IFN-γ Ab (7-B6-1-biotin; 0.2 μg/ml; Mabtech). After incubation for 3 h at room temperature, plates were washed and developed for additional 2 h with streptavidin-alkaline phosphatase (1 μg/ml; Mabtech). Spots were visualized by adding the substrate (5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium; Sigma) and counted automatically using an automated ELISpot reader (Zeiss Vision, Göttingen, Germany).

Intracellular Fluorescence-activated Cell Sorter Staining for IFN-γ (CytoSpot). PBMCs were presensitized with antigens or not as described above. Cells were harvested and resuspended at 2×10^6 cells/ml in RPMI 1640 and 10% HS. The cultures were restimulated with 2×10^6 cells/ml peptide-pulsed T2 cells (see above). As a positive control, PBMCs were stimulated with a combination of ionomycin and PMA. After 2 h of incubation, 0.7 μl/ml Golgi-Stop-Solution (PharMingen, San Jose, CA) was added, and the incubation period was extended to 12 h at 37°C, 5% CO_2. Cells were then labeled with PE-, FITC- or Tricolor-conjugated Abs by incubation on ice for 30 min. For intracellular labeling, cells were fixed and permeabilized for 30 min on ice using Cytoperm/Cytofix solution followed by washing steps with Washing Buffer (all from PharMingen). Nonspecific binding was measured using PE-conjugated isotype-matched mouse Ig. The following Abs were used: anti-CD8-Tricolor (Caltac, Burlingame, CA); anti-IFN-γ-PE (PharMingen); and anti-CD4-FITC (Becton Dickinson, Heidelberg, Germany). After washing, stained cells were analyzed by flow cytometry (FACSCalibur; Becton Dickinson).

Tetramer Staining. HLA-A*0201 tetrameric complexes were produced as described previously, with minor modifications (28, 29). The HLA-A*0201-binding peptide used for the refolding was KIFGSLAFL from Her-2/neu. Tetramers were assembled by mixing biotinylated monomers with streptavidin-PE (Molecular Probes, Eugene, OR) at a 4:1 ratio. PBMCs were presensitized with peptides as described above, except that they were collected after 6 days of culture. Staining of the PBMCs was performed using 10 μg/ml tetrameric complex in 50 μl of PBS, 0.01% NaN_3, 2 mM EDTA, and 2% FCS for 30 min at 4°C in the dark. Cells were counterstained using mAbs CD8-Tricolor (Caltac) and mAb CD4-FITC (Becton Dickinson) for 30 min at 4°C. After extensive washing, stained cells were analyzed by flow cytometry (FACSCalibur; Becton Dickinson) by appropriate gating on $CD8^+$ cells and excluding $CD4^+$ cells.

RNA Isolation and cDNA Synthesis. PBMCs were presensitized with antigens or not as described above. PBMCs that experienced a presensitization were restimulated with 1×10^4 peptide-loaded T2 cells per well. Unspecific stimulations with ionomycin/PMA were used as positive controls. After 2 h of restimulation, $1–3 \times 10^5$ cells were harvested, lysed in 800 μl of Trizol (Invitrogen, Karlsruhe, Germany), and stored at –80°C for a maximum of 30 days before RNA extraction. Total RNA was isolated using Trizol according to the manufacturer's instructions for small cell numbers (Invitrogen). After extraction, RNA was resuspended in 10 μl of RNase-free water (Promega, Madison, WI). Five μl of RNA were reverse transcribed into cDNA using the Omniscript kit (Qiagen, Hilden, Germany) and 10 units of RNAsin (Promega) according to the manufacturer's instructions and stored at –20°C until use.

Quantification of IFN-γ-specific mRNA Expression by Real-Time PCR. Quantification of IFN-γ gene expression was performed by using the ABI Prism 7700 sequence detection system (Perkin-Elmer, Foster City, CA) with probes and primers as described elsewhere (30–32). Amplification of a CD8 sequence for normalization was performed in separate tubes with primers and probes as described previously (31). Probes were labeled at the 5′ end with the reporter dye molecule FAM (emission $\lambda_{max.}$ = 518 nm) and at the 3′ end with the quencher dye molecule TAMRA (emission $\lambda_{max.}$ = 582 nm). The qRT-PCR was performed with the TaqMan Universal Master Mix (Applied Biosystems) using 5 μl of 1:10 diluted cDNA, fluorescence-labeled probe at a final concentration of 150 nM, and primers at final concentrations of 400 nM in a reaction volume of 20 μl. Cycling conditions were as follows: one cycle (50°C, 2 min; 95°C, 10 min) followed by 40 cycles (95°C, 15 s; 60°C, 1 min).

Cycle threshold values of IFN-γ were normalized to cycle threshold values of CD8. The relative expression was defined as relative value in comparison with the arbitrary expression value 1 achieved with T cells stimulated with the self-antigen p68.

qRT-PCR primers and probes were as follows: IFN-γ, 5′-AGCTCTGCATCGTTTTGGGTT-3′ (forward), 5′-GTTCCATTATCCGCTACATCTGAA-3′ (reverse); and FAM-TCTTGGCTGTTACTGCCAGGACCCA-TAMRA (probe); and CD8, 5′-CCCTGAGCAACTCCATCATGT-3′ (forward), 5′-GTGGGCTTCGCTGGCA-3′ (reverse), and FAM-TCAGCCACTTCGTGCCGGTCTTC-TAMRA (probe).

RESULTS

Assessment of Antigen-specific T Cells by ELISpot, CytoSpot, and IFN-γ-specific mRNA Quantification in the Blood of Healthy Donors. The quantification of mRNA encoding T cell activation markers for immune assessment was described to be rather sensitive but is still investigational. Therefore, we first performed IFN-γ-specific qRT-PCR using PBMCs *ex vivo* or after short-time *in vitro* stimulation to detect antigen-specific T cells in the peripheral blood. In a second step, we try

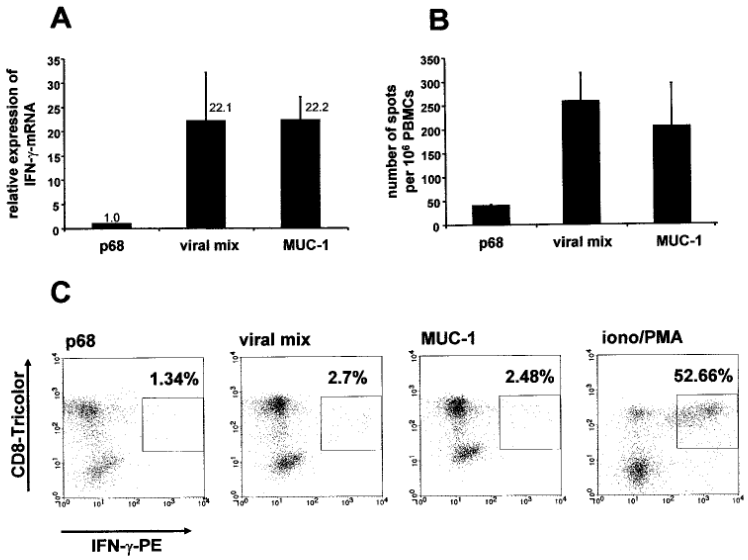

Fig. 1 *Ex vivo* analysis of virus- and MUC-1-specific T lymphocytes using IFN-γ mRNA quantification, ELISpot, and CytoSpot. *A*, 1×10^5 PBMCs of a healthy blood donor (HD5) were cocultured with 5 μg/ml MUC-$1_{950-958}$, p68 (negative control; expression value set to 1), or a mix of four HLA-A*0201-restricted viral peptides (positive control; see "Materials and Methods") and tested after 2 h (*ex vivo*) for increase of IFN-γ-specific mRNA by qRT-PCR. IFN-γ-specific mRNA expression was normalized to CD8 expression and given as an *x*-fold increase (stimulation index) in comparison with p68 stimulations. *B*, PBMCs were tested in parallel in an ELISpot assay. PBMCs were stimulated *ex vivo* for 18 h on ELISpot plates, thereby analyzing their IFN-γ secretion. The diagram shows the number of spots in 10^6 PBMCs. *C*, in parallel, PBMCs of HD5 were stimulated *ex vivo* for 10 h using peptide-loaded T2 cells and stained for intracellular IFN-γ (CytoSpot). A p68 stimulation served as background control; antigen-independent activation using ionomycin and PMA served as positive control. Cells were double-stained using PE-labeled IFN-γ antibodies and anti-CD8-Tricolor.

to confirm qRT-PCR results using additional functional assays such as ELISpot and CytoSpot or function-independent tetramer staining. To do so, a mixture of four HLA-A*0201-restricted viral peptides (HCMV, EBV, and flu; see "Materials and Methods") was used as recall antigens with an expected detectable frequency of responding T cells in the blood of healthy donors. In addition, a MUC-1 epitope (MUC-$1_{950-958}$) was included in the investigation. The assays were performed with cryopreserved PBMCs, which were thawed and cultured overnight to allow reconstitution of the cells. Initial optimization experiments were conducted by simply exposing bulk PBMCs to the peptide antigens for 2 h without the addition of further cytokines (*ex vivo* testing). In parallel, PBMCs from the same donors underwent an *in vitro* stimulation for 7 days in the presence of the indicated peptides, followed by a restimulation period for an additional 2 h with peptide-loaded T2 cells (peptide presensitization). In both settings, stimulations with p68 served as background controls, whereas ionomycin/PMA-induced activation was performed as positive control to estimate assay conditions.

Using this approach, we were able to detect in approximately 60% of healthy blood donors virus-specific T cell responses at various degrees *ex vivo*. An example for detecting antigen-specific T cells *ex vivo* without peptide presensitization is given in Fig. 1. As shown in Fig. 1*A*, $CD8^+$ T cells responded *ex vivo* with a 22-fold increase of IFN-γ-specific mRNA to viral antigens when related to the p68 stimulation. In this case, a similar induction was observed after MUC-$1_{950-958}$ stimulation. This result was confirmed by the ELISpot assay, revealing a frequency of 204 MUC-$1_{950-958}$-specific and 258 virus-specific T cells in 1 million PBMCs (Fig. 1*B*) as well as by the CytoSpot staining, detecting 2.5% MUC-$1_{950-958}$-specific and 2.7% virus-specific $CD8^+$ T cells (Fig. 1*C*). Negative results of the IFN-γ qRT-PCR could also be confirmed using ELISpot as well as tetramer staining (data not shown).

Methods to Detect T Cells Specific for Her-2/neu in the Blood of Breast Cancer Patients. Patient blood samples are usually limited and have to be cryopreserved to allow parallel testing of samples obtained at different time points under immunotherapy. Furthermore, the frequencies of TAA-specific T cells are expected to be rather low. Therefore, we evaluated the functional T cell assays with regard to their feasibility and transferability to the needs of the clinic using Her-2/neu as a model antigen.

Figs. 2 and 3 show the results of two breast cancer patients (BCP8 and BCP9) with a pre-existing cellular immune response specific for Her-2/neu. PBMCs of BCP8 were analyzed either *ex vivo* or after peptide presensitization with Her-2/$neu_{369-377}$ and viral antigens, respectively. The importance of presensitization

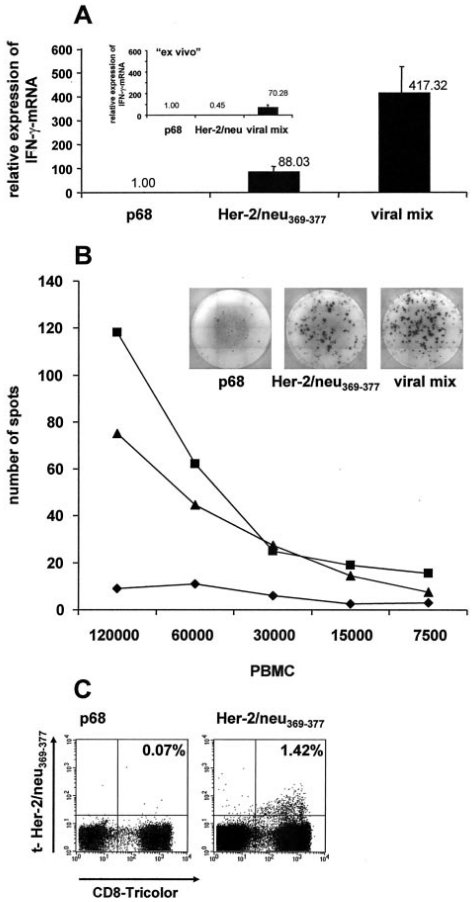

Fig. 2 Assessment of Her-2/neu$_{369-377}$-specific immunity in breast cancer patient BCP8 using IFN-γ mRNA quantification, ELISpot, and tetramer staining. *A*, 1×10^5 PBMCs of a breast cancer patient (BCP8) were cocultured with 5 μg/ml Her-2/neu$_{369-377}$, p68 (negative control; expression value set to 1), or a mix of four HLA-A*0201-restricted viral peptides (positive control; see "Materials and Methods"). T cells were tested for increase of IFN-γ-specific mRNA by qRT-PCR either after 2 h (*ex vivo*) or after peptide presensitization for 7 days followed by an additional 2-h restimulation with 1×10^4 peptide-loaded T2 cells. IFN-γ-specific mRNA expression was normalized to CD8 expression and given as an *x*-fold increase (stimulation index) in comparison with p68 stimulations. *B*, PBMCs of patient BCP8 were tested in parallel in an ELISpot assay. The indicated peptides were used for *in vitro* presensitization. PBMCs were restimulated in varying concentrations for 18 h on ELISpot plates using p68-loaded (♦), Her-2/neu$_{369-377}$-loaded (▲), or viral antigen-loaded (■) T2 cells, thereby analyzing their IFN-γ secretion. The diagram shows the number of spots for different cell concentrations. *Inset*, photographs illustrate ELISpot wells at the highest PBMC concentration (1.2×10^5). *C*, in parallel, PBMCs of patient BCP8 were harvested after 6 days of peptide presensitzation and stained directly using Her-2/neu$_{369-377}$-loaded HLA-A*0201-specific tetramers. A p68 stimulation served as negative control. Cells were triple-stained with PE-labeled tetramers, anti-CD8-Tricolor, and anti-CD4-FITC mAbs.

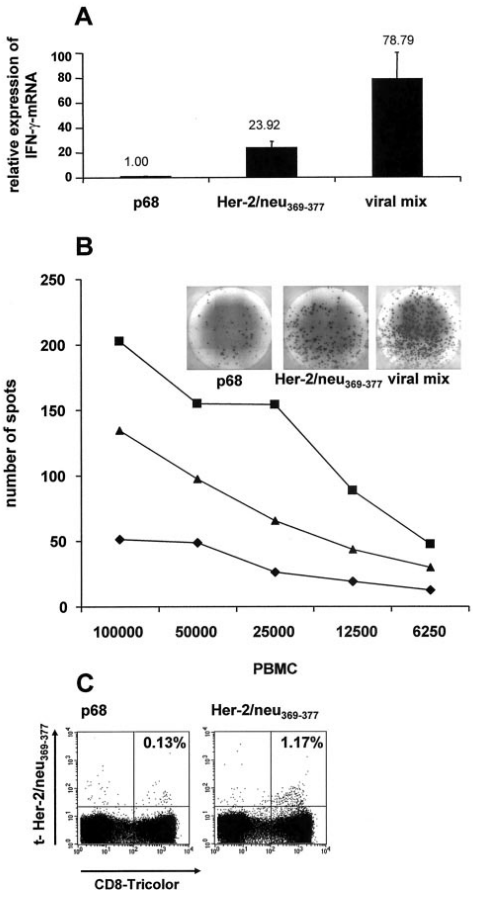

Fig. 3 Analysis of Her-2/neu$_{369-377}$-specific T lymphocytes of breast cancer patients BCP9 after peptide presensitization. Example of functional and phenotypic analyses using PBMCs of another breast cancer patient (BCP9). Assays were performed as described in the Fig. 2 legend.

with peptides for demonstrating TAA-specific reactivity is illustrated in Fig. 2*A*. T lymphocytes obtained directly from the blood of BCP8 did not respond within 2 h of antigen exposure to Her-2/neu$_{369-377}$ but to viral peptides by mRNA synthesis encoding IFN-γ. Testing 20 breast cancer patients, no or minimal TAA-specific T cell reactivity was observed in the absence of peptide presensitization (data not shown). In contrast, examples shown in Figs. 2 and 3 demonstrated strong Her-2/neu reactivity after Her-2/neu$_{369-377}$ presensitization of the PBMCs, in comparison with the background controls stimulated with p68 (BCP8, 88-fold; BCP9, 23-fold). Similarly, the reactions against the viral peptide mix were augmented by specific peptide presensitization (Fig. 2*A*).

In accordance with our data analyzing MUC-1-specific T cell responses, Her-2/neu-specific reactions shown by increase of IFN-γ-specific mRNA could be confirmed by the ELISpot assay. Figs. 2*B* and 3*B* show the number of spots obtained after 1 week of peptide presensitization. In the mRNA quantification as well as in the ELISpot assay, Her-2/$neu_{369-377}$-specific responses were lower (although clearly above the p68 background) than the viral antigen-induced reactions. Tetramer staining as a nonfunctional assay revealed a distinct population of Her-2/$neu_{369-377}$/HLA-A*0201-tetramer$^+$ and CD8$^+$ T cells in both patients (Figs. 2*B* and 3*B*). These populations are likely to be specific because far fewer cells were detected after p68 stimulation. After TAA presensitization, negative results of the IFN-γ qRT-PCT could be confirmed using ELISpot as well as tetramer analyses (data not shown).

In general, analyzing T cell reactions toward one antigen together with the proper control reactions needed approximately 0.9×10^6 PBMCs in case of IFN-γ-specific qRT-PCR (triplicates), 1.2×10^6 PBMCs for the CytoSpot or tetramer staining, and 1.8×10^6 PBMCs for the ELISpot assay (triplicates).

Pre-existent Immunity to Her-2/neu in Patients with Breast Cancer and Healthy Donors. Having confirmed the sensitivity and feasibility of the qRT-PCR-based T cell assay, we screened PBMCs of HLA-A*0201$^+$ healthy donors and breast cancer patients, respectively, to compare Her-2/neu-specific T cell frequencies. Although low levels of reactivity were detected in a few patients, the presensitization step appeared essential to warrant detection of TAA-specific T cells at rather low frequencies. Blood samples of patients were taken before surgery. To exclude a potentially therapy-based immunosuppression, none of the patients included in this study received any neoadjuvant chemotherapy or hormone therapy. In some cases, the tumor's Her-2/neu status could be evaluated using the DAKO classification.

PBMCs of 20 breast cancer patients and 10 healthy donors were analyzed for Her-2/neu-dependent IFN-γ-specific mRNA increase after *in vitro* presensitization (Table 1). Her-2/$neu_{369-377}$ (E75) and Her-2/$neu_{654-662}$ (GP2), both described to be immunodominant epitopes of Her-2/neu, were used for stimulation.

Table 1 summarizes the results of Her-2/neu-dependent IFN-γ mRNA quantification, normalized to the CD8 expression. A reaction was considered positive if it was 2-fold above the background control (p68 stimulation) plus 2-fold SD. All individuals tested showed a clear increase of IFN-γ-mRNA in response to ionomycin/PMA stimulation (data not shown). Four of 20 patients (19%) and none of the healthy donors (0 of 10 donors) showed an up-regulation of IFN-γ mRNA after stimulation with Her-2/neu epitopes. The quantity of the reactions was rather high, with stimulation indices of 10.0, 23.9, 78.2, and 88.0, respectively. Comparable high stimulation indices were only shown for virus antigen-dependent reactions (see Table 2). Due to limited knowledge of antigen expression in the patient tumors, it was not possible to correlate Her-2/neu expression and T cell response. However, it is noteworthy that high responders could either overexpress Her-2/neu at the primary tumor (BCP8, DAKO score 2+) or not (BCP9, DAKO score 0).

Table 1 Her-2/neu-stimulated increase of IFN-γ-specific mRNA in T lymphocytes of (A) breast cancer patients and (B) healthy volunteers

A. Breast cancer patients

Breast cancer patient	Her-2/neu expression of patient's tumor[b]	Peptide presensitization[a] p68	Peptide presensitization[a] Her-2/$neu_{369-377}$ and* Her-2/$neu_{654-662}$
BCP1	0	1 (±0.45)	0.27 (±0.14)[c]
BCP2	n.a.[d]	1 (±0.24)	0.20 (±0.06)
BCP3	0	1 (±0.38)	2.68 (±1.35)
BCP4	n.a.	1 (±0.19)	0.56 (±0.28)
BCP5	0	1 (±0.09)	0.34 (±0.24)
BCP6	n.a.	1 (0.43)	1.76 (±0.63)
BCP7	0	1 (±0.37)	1.21 (±0.38)
BCP8	2+	1 (±0.31)	**88.03 (±19.91)**
BCP9	0	1 (±0.25)	**23.92 (±5.18)**
BCP10	n.a.	1 (±0.17)	0.27 (±0.07)*
BCP11	0	1 (±0.11)	1.19 (±0.20)*
BCP12	n.a.	1 (±0.54)	1.29 (±0.60)*
BCP13	3+	1 (±0.04)	0.29 (±0.15)*
BCP14	n.a.	1 (±0.37)	0.58 (±0.16)*
BCP15	n.a.	1 (±0.28)	0.66 (±0.17)*
BCP16	n.a.	1 (±0.57)	0.72 (±0.34)*
BCP17	n.a.	1 (±0.11)	0.75 (±0.06)*
BCP18	n.a.	1 (±0.43)	0.47 (±0.17)*
BCP19	n.a.	1 (±0.05)	**78.25 (±5.56)***
BCP20	0	1 (±0.11)	3.68 (±2.56)*
BCP22	n.a.	1 (±0.41)	**10.02 (±2.92)***

B. Healthy volunteers

Healthy donor	Peptide presensitization[a] p68	Peptide presensitization[a] Her-2/$neu_{369-377}$ and* Her-2/$neu_{654-662}$
HD1	1 (±0.31)	0.47 (±0.18)[c]
HD2	1 (±0.17)	0.76 (±0.10)
HD3	1 (±0.21)	0.52 (±0.22)
HD4	1 (±0.17)	0.34 (±0.14)
HD6	1 (±0.86)	0.25 (±0.25)*
HD7	1 (±0)	2.4 (±0.26)*
HD8	1 (±0.03)	1.19 (±0.53)*
HD9	1 (±0.42)	1.86 (±1.43)*
HD10	1 (±0.17)	0.83 (±0.25)*
HD11	1 (±0.27)	0.92 (±0.42)*

[a] PBMCs (3×10^5) of breast cancer patients prior therapy or healthy donors were cocultured with 5 μg/ml Her-2/$neu_{348-356}$ or p68. PBMCs were tested for IFN-γ-specific mRNA by qRT-PCR after peptide presensitization for 7 days followed by an additional 2-h restimulation with 3×10^4 peptide-loaded T2 cells.

[b] Her-2/neu expressions in patient's tumors were detected by immunohistochemistry and quantified using the DAKO score.

[c] IFN-γ-specific mRNA expression was normalized to CD8 expression and expressed as a *x*-fold increase (stimulation index) in comparison to the p68 stimulations. SDs are given in parentheses. A reaction was considered as positive (bold letters) lying 2-fold above the background control (1 for p68 stimulation) plus 2-fold SDs.

[d] n.a., not analyzed; *, Presensitization was performed with both peptides in parallel. Other samples were stimulated only with Her-2/$neu_{369-377}$.

TAA- and Virus-specific T Cells in Blood of Breast Cancer Patients and Healthy Volunteers. Antigens other than Her-2/neu are frequently overexpressed in breast cancer such as CEA (40–50%; Ref. 33) and MUC-1 (>90%) (25, 33, 34). In addition, members of the C/T-antigen family such as

MAGE-1, NY-ESO-1, and SSX-2 were detected in breast cancer lesions, although at lower frequencies [5–25% (35–39)]. In this context, it was of interest to look for T cells specific for one of these TAAs after peptide presensitization.

Table 2 summarizes the results of TAA-dependent IFN-γ mRNA quantification, normalized to CD8 expression. A reaction was considered as positive if it was 2-fold above the background control (p68 stimulation) plus 2-fold SD. Again, all individuals tested showed a clear increase of IFN-γ mRNA in response to ionomycin/PMA stimulation (data not shown). In addition, IFN-γ mRNA quantification was performed after peptide presensitization using a mixture of HLA-A*0201-restricted viral antigens (cytomegalovirus, EBV, and flu) to get an impression of the functional immune status of tested individuals.

Thirteen of 21 (62%) breast cancer patients (Table 2A) and 6 of 11 (54%) healthy donors (Table 2B) did show a significant reaction toward viral antigens. Positive reactions ranged from 5.1–417.3, demonstrating a high interindividual heterogenicity in mounting a virus-specific T cell memory response. However, patients with no detectable virus-specific T cell activation did not show TAA-specific responses either.

In contrast to MUC-1_{12-20}, MUC-$1_{950-958}$-specific T cell reactions were commonly detected in patients (46%) as well as in healthy donors (28%). CEA- and NY-ESO-1-specific responses were more limited and restricted to breast cancer patients (each was seen in 1 of 10 responders). SSX-2-related T cell responses were shown for one breast cancer patient (of 10 cases). Surprisingly, we could also see an IFN-γ mRNA increase in response to these C/T antigens in one of the healthy donors (HD8). No T cell reactivity at all could be observed toward a MAGE-1 epitope.

DISCUSSION

There is a considerable interest to validate T cell detection assays as surrogates for immune competence and vaccine potency. Besides sensitivity and robustness, several aspects have to be taken into account with regard to their clinical usefulness, *i.e.*, the need for use of frozen/thawed PBMC samples to analyze batches gained during therapy in parallel and the avoidance of collecting large blood samples. Therefore, antigen-dependent cytokine release assays, on either the protein or mRNA level, and tetramer staining represent powerful tools to assess the specificity and magnitude of T cell responses.

In this study, we first tested the feasibility of the qRT-PCR method to assess antigen-specific T cell reactions in the peripheral blood. PBMCs were directly stimulated *ex vivo* using HLA-A*0201-restricted epitopes derived from influenza, EBV, and cytomegalovirus, allowing antigen presentation among the sample cells. In response to the given peptides, IFN-γ-specific mRNA was quantified after 2 h of stimulation. The signal was normalized using CD8-specific mRNA as reference, which is not likely to be sensitive to the peptide stimulus applied in the time frame of the assay (31, 32). In contrast to ELISpot and CytoSpot assays, IFN-γ mRNA quantification does not take into account the variations in the frequency of the cell subset targeted by the stimulus. Therefore, one of the major problems in the interpretation of qRT-PCR results is discriminating a positive from a negative result (40). We considered a positive result an at least 2-fold IFN-γ increase above the mean expression obtained in control stimulations plus a 2-fold SD. By doing so, we were able to detect *ex vivo* virus-specific T cell responses in the majority of tested individuals. More importantly, we could confirm positive and negative qRT-PCR results by additional independent functional assays such as ELISpot and CytoSpot or by tetramer staining, irrespective of T cell function. Due to the assay conditions using PBMCs, the magnitude of positive T cell responses correlated in a rather relative but not absolute manner: qRT-PCR-, CytoSpot-, and tetramer-reacting T cells could be restricted to the $CD8^+$ subset; whereas ELISpot reacting T cells could not. In addition, T cell frequencies defined by tetramer staining include functional as well as anergic $CD8^+$ T cells.

In comparison with cellular immunity directed against viral recall antigens, tumor-specific T cell reactions represent another quality of immune responses due to the restricted immunogenicity of self-antigens and the tolerizing mechanisms provided by the tumor itself. Therefore, the expected frequency of TAA-reacting T cells is limited and might lie beyond the threshold level of successful detection. Although low levels of reactivity have been seen *ex vivo* with a few patients using the qRT-PCR assay in our study, the presensitization step appeared essential to warrant a certain detection of TAA-specific T cells in unimmunized volunteers.

In our hands, the combination of peptide presensitization with the qRT-PCR technique is practicable, most sensitive, and a suitable screening test for immunological staging that needs only small blood samples. This method was previously shown to represent a useful tool for the monitoring of patients with cancer undergoing immune manipulation. Kammula *et al.* (31) identified T cell reactivity toward epitopes used for active-specific vaccination of melanoma patients by quantifying IFN-γ transcript levels in PBMCs obtained before and after treatment. The evidence of vaccine-induced immune responses obtained with qRT-PCR correlated with results obtained with classic *in vitro* sensitization assays, intracellular cytokine detection by fluorescence-activated cell-sorting analysis, and tetramer staining (32, 41). However, evaluating TAA-specific T cell reactions directly *ex vivo* in individuals not receiving immunomodulating therapies seemed to be rather difficult at times (42, 43). Confirming our data, Jäger and co-workers (19, 43, 44) demonstrated that in the case of patients with advanced tumors overexpressing NY-ESO-1, strong antigen-specific reactivity was best seen when combining *in vitro* peptide presensitization of T cells together with ELISpot or Cytospot techniques. Additionally, a more recent publication (45) described the complete absence of *ex vivo* T cell responses in breast cancer patients toward a small panel of TAAs such as Her-2/neu, CEA, and Ep-CAM using the ELISpot assay without TAA presensitization.

Several studies analyzing immune responses to melanoma-associated differentiation antigens and MUC-1 revealed that cancer patients as well as healthy donors can show high frequencies of Melan-A-specific naive $CD8^+$ T cells or MUC-1-specific memory T cells (17, 46, 47). Thus, we performed an evaluation of volunteers without cancer together with breast cancer patients to assess the prevalence of immunity in an unimmunized breast cancer population. We further decided to analyze blood samples before surgery and adjuvant therapy because of (*a*) the suggestion that pre-existing antitumoral im-

Table 2 TAA- and viral antigen-stimulated increase of IFN-γ-specific mRNA in T lymphocytes of (A) breast cancer patients and (B) healthy volunteers

A. Breast cancer patients

Breast cancer patient	Peptide presensitization[a]								
	p68	Viral peptides[b]	$CEA_{571-579}$	$SSX\text{-}2_{103-111}$	NY-ESO-$1_{108-116}$ NY-ESO-1_{86-94}	NY-ESO-$1_{159-167}$	$MAGE\text{-}1_{278-286}$	$MUC\text{-}1_{950-958}$	$MUC\text{-}1_{12-20}$
BCP1	1 (±0.45)	6.59 (±2.30)[c]	n.a.[d]	n.a.	n.a.	n.a.	0.67 (±0.14)	n.a.	n.a.
BCP2	1 (±0.24)	**7.7 (±1.44)**	n.a.	n.a.	n.a.	n.a.	0.74 (±0.18)	n.a.	n.a.
BCP3	1 (±0.38)	**122.36 (±32.92)**	n.a.	n.a.	n.a.	n.a.	1.77 (±0.97)	n.a.	n.a.
BCP4	1 (±0.19)	**11.96 (±2.22)**	n.a.	n.a.	n.a.	n.a.	0.03 (±0.04)	n.a.	n.a.
BCP5	1 (±0.09)	**9.13 (±0.72)**	n.a.	n.a.	n.a.	n.a.	0.41 (±0.23)	n.a.	n.a.
BCP6	1 (±0.43)	0.66 (±0.35)	n.a.	n.a.	n.a.	n.a.	1.27 (±1.24)	n.a.	n.a.
BCP7	1 (±0.37)	**27.28 (±7.62)**	n.a.	n.a.	n.a.	n.a.	1.34 (±0.45)	n.a.	n.a.
BCP8	1 (±0.31)	**417.32 (±109.40)**	n.a.	n.a.	n.a.	n.a.n.a.	1.95 (±0.50)	n.a.	n.a.
BCP9	1 (±0.23)	**78.79 (±21.15)**	n.a.	n.a.	n.a.	n.a.	2.35 (±0.47)	n.a.	n.a.
BCP10	1 (±0.17)	1.21 (±0.26)	n.a.	0.18 (±0.02)	n.a.	0.20 (±0.02)	n.a.	0.17 (±0.04)	0.22 (±0.03)
BCP11	1 (±0.11)	**21.78 (±4.22)**	0.56 (±0.16)	0.46 (±0.04)	1.32 (±0.11)	1.77 (±0.91)	n.a.	**18.38 (±2.08)**	0.44 (±0.08)
BCP12	1 (±0.54)	n.a.	n.a.	n.a.	0.92 (±0.41)	n.a.	n.a.	**17.09 (±6.85)**	n.a.
BCP13	1 (±0.04)	**66.95 (±20.76)**	0.94 (±0.14)	n.a.	Tc0.72 (±0.44)	1.09 (±1.05)	n.a.	**8.14 (±1.40)**	n.a.
BCP14	1 (±0.37)	0.54 (±0.16)	0.89 (±0.37)	0.82 (±0.29)	0.38 (±0.16)	0.95 (±0.31)	n.a.	0.47 (±0.14)	0.45 (±0.17)
BCP15	1 (±0.28)	0.36 (±0.12)	0.67 (±0.14)	1.06 (±0.47)	0.70 (±0.18)	0.60 (±0.20)	n.a.	0.78 (±0.34)	0.77 (±0.17)
BCP16	1 (±0.57)	0.73 (±0.34)	0.52 (0.33)	2.07 (±2.19)	0.88 (±0.39)	0.59 (±0.28)	n.a.	1.27 (0.53)	n.a.
BCP17	1 (±0.11)	**17.39 (±1.51)**	0.37 (±0.09)	0.34 (±0.04)	0.91 (±0.30)	0.76 (±0.08)	n.a.	**22.01 (±2.52)**	0.34 (±0.06)
BCP18	1 (±0.43)	4.32 (±2.47)	17.51 (±39.2)	1.44 (±0.53)	0.70 (±0.36)	0.70 (±0.27)	n.a.	3.84 (±1.47)	0.87 (±0.30)
BCP19	1 (0.05)	**13.64 (0.76)**	**12.08 (0.73)**	**51.45 (±4.93)**	**15.45 (±1.97)**	n.a.	n.a.	**1063.8 (±37.24)**	n.a.
BCP20	1 (±0.11)	7.09 (±3.69)	n.a.	1.41 (±0.75)	6.15 (±2.08)	n.a.	n.a.	**3.72 (±0.75)**	5.15 (±3.27)
BCP21	1 (±0.46)	**27.10 (±9.21)**	0.40 (±0.51)	1.31 (±0.73)	0.42 (±0.20)	0.50 (±0.37)	n.a.	1.99 (±1.12)	0.28 (±0.12)
BCP22	1 (±0.41)	**18.13 (±5.24)**	2.06 (0.83)	6.19 (±2.56)	5.01 (±2.08)	2.48 (±1.09)	n.a.	3.73 (±1.14)	4.03 (±1.29)

B. Healthy volunteers

Healthy donor	Peptide presensitization[a]								
	$p68_{146-154}$	Viral peptides[b]	$CEA_{571-579}$	$SSX\text{-}2_{103-111}$	NY-ESO-$1_{108-116}$ NY-ESO-1_{86-94}	NY-ESO-$1_{159-167}$	MAGE-1	$MUC\text{-}1_{950-958}$	$MUC\text{-}1_{12-20}$
HD1	1 (±0.31)[e]	4.52 (±1.38)	n.a.	n.a.	n.a.	n.a.	0.34 (±0.22)	n.a.	n.a.
HD2	1 (±0.17)	**5.13 (±0.65)**	n.a.	n.a.	n.a.	n.a.	0.67 (±0.15)	n.a.	n.a.
HD3	1 (±0.21)	**41.36 (±7.14)**	n.a.	n.a.	n.a.	n.a.	1.39 (±0.27)	n.a.	n.a.
HD4	1 (±0.17)	**23.18 (±3.99)**	n.a.	n.a.	n.a.	n.a.	1.15 (±0.23)	n.a.	n.a.
HD5	1 (±0.27)	**22.39 (±4.44)**	4.36 (±1.18)	1.60 (±0.33)	3.15 (±0.64)	1.69 (±0.33)	n.a.	**61.0 (±16.14)**	1.89 (±0.4)
HD6	1 (±0.86)	2.0 (±1.28)	0.10 (±0.06)	0.84 (±0.64)	0.44 (±0.64)	0.71 (±0.48)	n.a.	6.4 (±3.98)	0.8 (±0.47)
HD7	1 (±0)	1.88 (±1.66)	1.36 (±0.51)	1.53 (±0.03)	1.17 (±0.14)	2.64 (±1.58)	n.a.	1.3 (±0.01)	2.3 (±0.08)
HD8	1 (±0.03)	**37.92 (±1.45)**	0.63 (±0.11)	**12.0 (±3.38)**	0.22 (±0.11)	0.58 (270.25)	n.a.	0.98 (±0.30)	1.54 (±0.19)
HD9	1 (0.42)	**1.89.36 (±56.62)**	0.66 (±0.35)	1.26 (±0.90)	7.78 (±3.41)	0.64 (±0.29)	n.a.	**11.7 (±3.53)**	15.2 (±10.0)
HD10	1 (±0.17)	1.04 (±0.38)	1.18 (±0.38)	1.57 (±0.36)	0.91 (±0.56)	1.38 (±0.38)	n.a.	1.26 (±0.56)	n.a.
HD11	1 (±0.27)	0.81 (±0.45)	0.84 (±0.27)	1.29 (±0.46)	1.03 (±0.45)	1.74 (±0.70)	n.a.	0.46 (±0.41)	3.2 (±3.1)

[a] PBMCs (3×10^5) of breast cancer patients before therapy or of healthy donors were cocultured with 5 μg/ml of different TAA-peptides, p68, or a mix of viral peptides. PBMCs were tested for IFN-γ-specific mRNA by qRT-PCR after peptide presensitization for 7 days followed by an additional 2-h restimulation with 3×10^4 peptide-loaded T2 cells.

[b] Mix of four HLA-A*0201-restricted viral peptides: flu_{58-66} EBNA-6-$EBV_{284-293}$, IE63-$EBV_{259-267}$, and pp65 $HCMVA_{495-503}$.

[c] IFN-γ-specific mRNA expression was normalized to CD8 expression and expressed as a x-fold increase (stimulation index) in comparison to the p68 stimulations. SDs are given in parentheses. A reaction was considered as positive (bold letters) lying 2-fold above the background control (p68 stimulation) plus 2-fold SDs.

[d] n.a., not analyzed.

munity depends on antigen persistence, (*b*) the unpredictable influence of therapeutic interventions, and (*c*) our former failure in detecting CEA-, MAGE-1-, or Her-2/neu-specific T cells using tetramers in breast cancer patients receiving chemotherapy.[4]

Her-2/neu appears to be the most prominent and best analyzed tumor antigen associated with breast cancer. However, most of the studies defining Her-2/neu as an immunogenic antigen in patients whose tumors overexpress this oncogene represent individual case reports (9, 48, 49). To our knowledge, our study is the first extensive evaluation of pre-existing immunity directed against HLA A*0201 restricted epitopes of Her 2/neu. We could find Her-2/neu-specific $CD8^+$ T cell responses in 4 of 22 HLA-$A*0201^+$ breast cancer patients and in none of the investigated healthy blood donors. This is in contrast to a study by Disis *et al*. (22), who could not detect (despite peptide presensitization before ELISpot analysis) pre-existent T cells specific for Her-2/$neu_{369-377}$ in eight breast cancer patients overexpressing Her-2/neu. As expected, we saw the highest Her-2/$neu_{654-662}$-specific reactivity in a patient overexpressing Her-2/neu (BCP8; DAKO score, 2+). Surprisingly, strong Her-2/neu-specific T cell reaction was demonstrated in another patient without Her-2/neu overexpression (BCP9; DAKO score, 0), probably representing an example for the selective pressure of Her-2/neu immunity and emergence of antigen loss variants. In addition, strong Her-2/neu overexpression did not necessarily provoke a T cell response (BCP13; DAKO score, 3+), probably due to gaps in the patient's T cell repertoire, tumor-mediated tolerance, or lack of appropriate diagnostic tools. However, due to our limited knowledge of Her-2/neu expression patterns in the patients analyzed here, it is not possible to make a clear correlation between antigen expression and T cell response. A comprehensive study analyzing cellular and humoral immunity pointed out that Ab and T cell response to NY-ESO-1 occurred only in patients with NY-ESO-1-expressing tumors (43). However, a study is ongoing to determine whether there is a correlation between the antigen expression pattern in tumors and the presence of tumor-specific immunity. Furthermore, it would be of interest to analyze the status of activation of circulating tumor-related T cells to explain their paradoxical coexistence with unimpaired tumor growth.

Because of its ubiquitous expression pattern in breast cancer, we were also interested in MUC-1-specific immunity (34). MUC-1-derived epitopes were already included in clinical studies, and several investigators demonstrated evidence for only MUC-1-specific T cell responses after MUC-1-based vaccinations (7, 50, 51). In contrast to these findings, we could frequently detect MUC-$1_{950-958}$-specific T cells in unimmunized healthy volunteers (28%) as well as in breast cancer patients (46%). Because MUC-1-specific T_H1 responses could be detected in multiparous women, it was speculated that there is a natural immunization against MUC-1 epitopes during pregnancy (47). Although our collective of patients includes multiparous women, this was not the case for the healthy volunteers included in our study. However, it is of interest that we could not detect any pre-existing immunity toward MUC-1_{12-20}, which turned out to be immunodominant in vaccinations using peptide-pulsed dendritic cells (7). At the moment, we cannot explain the high magnitude of MUC-$1_{950-958}$-specific T cell responses in healthy donors and have started investigations comparing the functional state of these T cell populations and their capacity to lyse MUC-1-expressing tumor cells.

In our study, positive reactions toward cancer-associated antigens such as CEA, SSX-2, NY-ESO-1, and MAGE-1 seemed to be exceptional. Despite its limited expression pattern to tumors, testis, or placenta, we were surprised to find one healthy donor reacting to SSX-2.

In summary, with exception of Her-2/neu, our study revealed no distinct differences in TAA-related cellular immunity between cancer patients and a control group consisting of healthy volunteers. However, in approximately 54% of HLA-$A*0201^+$ breast cancer patients, a pre-existent specific cellular immune response to at least one of the investigated TAAs MUC-1, Her-2/neu, CEA, NY-ESO-1, or SSX-2 could be detected. Nineteen percent of the patients showed pre-existent $CD8^+$ T cell reactions related to Her-2/$neu_{369-377}$ and Her-2/$neu_{654-662}$. Although the clinical relevance of an endogenous TAA-specific immunity remains unclear, our findings suggest that patients with primary breast cancer can mount a T cell immune response to their tumor that might be beneficially enhanced by TAA-dependent vaccination strategies in the adjuvant situation.

[4] S. Kayser, S. Stumm, S. Stevanovic, and B. Gückel, unpublished results.

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