IP-HCMC Immunology course 2005

Practical course

Cytokine production determination by intracellular flow cytometry

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The purpose of this practical study is to determine the cytokine levels by intracellular flow cytometry method. This method can be used in both basic and clinical research for human and animal models.

The principle of this technique is to quantify cytokine-producing cells by detecting cytokines by antibody staining and flow cytometry analysis.

The term "flow cytometry" derives from the measurement (meter) of single cells (cyto) as they flow past a series of detectors. The fundamental concept is that cells flow one at a time through a region of interogation where multiple biophysical properties of each cell can be measured at rates of over 1000 cells per second. These biophysical properties are then correlated with biological and biochemical properties of interest. The high through-put of cells allows for rare cells, which may have inherent or inducible differences, to be easily detected and identified from the remainder of the cell population.

Flow Cytometry involves the use of a beam of laser light projected through a liquid stream that contains cells, which when struck by the focussed light give out signals which are picked up by detectors. These signals are then converted for computer storage and data analysis, and can provide information about various cellular properties.

Methodology

I-Isolation of mouse splenocytes

- Mice are killed by exposition to CO_2 during at least 5 minutes
- Dissect the animal to take the spleen
- Dilacerate the spleen in sterile RPMI complemented with PS and transfer the cells in a 15 ml tube.
- After 5 minutes the cell suspension is decanted to a new 15 ml tube.
- Centrifuge 5 min at 300g and wash twice with RPMI
- Resustend the pellet in 10 ml of complete RPMI cuture medium (500 ml RPMI with glutamate supplemented with 50 ml fetal calf serum, 5 ml streptomycin/penicillin antibiotic solution, 5 ml Hepes 1M and 1 ml β2 mercaptoethanol).
- Count cells on a hemocytometer.

II-Cells stimulation

- **Note:** *The cuture times indicated in this protocol are optimised for IL-10 and IFNγ. Culture times for optimal staining of other cytokines will need to be determined empirically by the investigator depending on the type of cell used and the stimulation process.*

- Dilute cells to 2.5 x 10^6cells/ml into two separates culture flasks.
- Add 50 ng/ml PMA + 500 µg/ml Ionomycin + 10 µg/ml Brefeldin A to one flask.
- Incubate flasks at 37°C, 5%CO2 for 3-4 hours.
- Recover cells after stimulation and wash 1X with PBS╣ **WORK ON ICE!!!!**

- **Note:** *Brefeldin A is a fungal metabolite that disrupts the structure and function of the Golgi apparatus. Therefore, protein secretion is inhibited and newly synthetised proteins accumulate inside the cells. Ince it is toxic, Brefeldin A should not be added too long.*

III-<u>Surface markers labelling</u>

- Distribute 1 x 10^6 cells/well in a 96 wells plate and pellet them by centrifugation 5 minutes at 1200 rpm.
- Incubate 30 min with 50 µl of 2.4G2 (anti-FcRγII/III diluted at 1/100)
- Wash cells by adding 200 µl 1 X PBS containing 3%FCS (PBS-3%FCS).
- Pellet by centrifugation 5 minutes at 1200 rpm.
- Incubate for 30 min at 4°C with anti-surface marker phycoerythrin (PE)-or FITC labelled antibody:

  anti-CD4-PE: 1/400

  anti-CD3-FITC: 1/800 diluted in PBS-3%FCS.

- Wash 2 times with PBS-3%FCS.

III-<u>Fixation</u>

- Fix surface labelled cells with 60µl of paraformaldehyde (PFA) 2% final

(dilute PFA%) for 1 hour at room temperature by protecting cells from light exposure.

- Centrifuge plate and wash once with 200 µl PBS-3%FCS-0, 5% saponin or Permwash 1X solution from Becton Dickinson
- Centrifuge 5 min at 1200rpm.

IV-<u>Intracytoplasmic cytokines staining</u>

- Incubate the cells 30 min at 4°C with
- 50 µl anti-IFNγ-APC: 1/50
- or anti- IL-4 APC: 1/50

Allophycocyanin (APC) labelled antibodies diluted in Permwash 1X or 1X PBS-3%FCS-0.5%Saponin. Protect cells from light exposure.

- Wash twice in Permwash 1X or 1X PBS-3%FCS-0.5%Saponin.
- Wash once in PBS 1X-3%FCS.
- Resuspend the cells in 300 µl PBS 1X-3%FCS To do flow cytometry analysis.

Annexe: Reagents, solution disposable

Animals: 8-10 weeks-old mice

Antibodies:

anti-CD4-PE (cloneH129.19)
anti-CD3-FITC (145-2C11)
anti-IFNγ-APC (clone XMG1.2)
anti-IL-4-APC (clone 11B11)
anti-FcRγII/III (clone 24G2)

All antibodies are from BD-Pharmingen.

Reagents:

Fetal calf serum
Formaldehyde 4%
PBS 1 X
Ionomycin calcium salt (sigma ref. I-0634, 1mg)
Brefeldin A (Sigma ref B7651, 5mg)
Phorbol 12 Myristate 13 acetate (PMA, Sigma ref P-8139; 1mg)
Saponin (Sigma ref S-7900)
Paraphormaldehyde (PFA) 4%
Washing and incubation solution: PBS 1X-3% FCS-0,1%N_aN_3 (Azide).
Fixation solution: PFA 2% final
Permeabilisation buffer: Permwash 1X (BD-Pharmingen)
Or 1X PBS-3%FCS-0.5%Saponin

Disposables:

Absorbent paper
Beakers
Centrifuge tubes “falcon” 15 ml
Facs Tubes
96 microwells plates
Tissu culture flasks 75 cm^3
Gloves size L, M, S
Micropipette tips 200-1000µl
Micropipette tips 20-200µl
Sterile pipettes 10 ml
Sterile pipettes 5 ml
Ice

Equipment:

Dissection tools
Cell culture CO_2 incubator
Cell culture Hood
Flow cytometer

Malassez cytometer
Micropipettes 1000µl
Micropipettes 200 µl
Micropipette 20 µl
Microscope
Multichannel pipette 200 µl
Vortex
Refrigerated centrifuge for 15 ml tubes