Mouse ELISPOT-Assay Protocol

To check frequency of cytokine (IFN-γ, IL-5, IL-2, IL-10, IL-4) positive cells following in vitro Ag-specific activation.

Schedule

DAY-0

Calculate # of animals x # of organs x # of cytokines x # of antigens (dilutions, controls) and # of replicates to get # of plates to be coated with primary cytokine antibody.

Set up 96-well plate map

Pre-coating of ELISPOT 96-well plates with primary antibodies:

- new „white" plates from Whatman/Polyfiltronics, or white plastic nitrocellulose plates from Millipore
- 100µl of sterile PBS with
  - anti-mIFN-γ (R46A2 hybridoma or Pharmingen), final 4µg/ml
  - anti-mIL-2 (Pharmingen), final 2µg/ml
  - anti-mIL-4 (11B11-hybridoma or Pharmingen), final 4µg/ml
  - anti-mIL-5 (TRFK-5-hybridoma or Pharmingen), final 5µg/ml
  - anti-mIL-10 (not tested yet – Pharmingen), final ? µg/ml
- incubate o/n, 4°C, humidified chamber (Tupper, with soaked paper towels)

DAY-1

Before getting the mice preparation of:

Collection of blood/serum samples
Bleeding of mice by **cardiac puncture**: prepare Halothane glass cylinder, cotton, syringes, needles, and Eppendorf vials labeled.

cHL-1 medium;
calculation of volume:
a) proliferation assay = # organs x # antigens x # replicates x 0.1ml
b) cytokine supernat. = # organs x # antigens x # replicates x 0.5ml
c) ELISPOT = # cytokines x # organs x # antigens x # replicates x 0.1ml

x # of cell suspensions
d) **spleen; 10^7/ml (final cell concentration = 10^6/well)**
e) **LN; 5x10^6/ml**

Add L-glutamin, and Pen/Strep, sterile filter.

(when not testing ex vivo peptide response, one can also successfully use RPMI or DMEM, 10% FCS; with other antigens!)

1) continue preparing ELISPOT plates
   - wash plates 3x with sterile PBS (200µl)
   - block 1hr with sterile PBS with „highgrade“ (cell culture, BSA fraction V, Sigma) 1% BSA (200µl)
   - wash 3x with sterile PBS (200µl)

2) Plate out the antigens in fresh cHL-1 in ½ of the final volume (at **2x concentration**):

   a) medium (cHL-1)
   b) Ovalbumin: OVA final 13.5µg/ml; „optimal“ for OVA T cell clones = 2.7µg/ml
   c) Concanavalin A (final 2µg/ml) = from stock (100µg/ml stock)

in this case with Balb/c for relevant (I-A^d) and irrelevant (I-A^k) spleen APC or DC

in respective replicates of ELISPOT plates

And, into 96-well (triplicates) for **proliferation assay** (same cell number as ELISPOT)
and, into 2x24-well plates (pipette 500µl of 2x Ag solution + 500µl of 10^7/ml cell solution) or 2x96-well plates for generation of **cytokine supernatants** (48hrs). Store at 4°C until 1hr before the cells will be added, then pre-warm in incubator at 37°C.

Warm up cHL-1 medium to 37°C.

Prepare for organ preparation: keep instruments in 75% EtOH (or flame); prepare petri dishes (# of organs x 10ml DMEM); and 50ml tube top cell filter, syringes to mash organs.

Get mice; do blood collection first, then by neck dislocation kill groups of 3-8 (depending on organ preparation time), mash organs with stamp of syringe, pour through cell filter into 50ml tube.

Count cells (or estimate $80x10^6$ per spleen with „normal“ size): set up fluorescence microscope (sign on/off etc. needs at least 20min to warm up); get acridine orange / ethidium bromide dye solution, mix 1:2 (to 1:10) of cells with dye (put dye on parafilm or microtiter plate), count green cells (living=acridin orange cells) and red (ethidium bromide = dead cells),
Put antigen loaded 96- and 24-well plates into 37°C incubator.

Spin cells 10min, RT, 1200rpm; resuspend with proper volume of cHL-1 to get final cell concentration of spleen cells = 10^7/ml and LN = $5x10^6$/ml.
Get antigen loaded plates and pipette (use tips with wide opening!!) the second ½ of volume to microtiter or 24-well plates; do the magic shake! (= tap the plates slightly at both sides in order to distribute cells evenly on the bottom of the wells) and
incubate at 37°C, 7% CO_2.: **- IFN-γ , IL-2, for 24hrs**
- IL-4 and -5 for 48hrs (mostly from afternoon to the second day morning = 40hrs; be careful not to disturb cells while incubating)

DAY-2

1) Continue after 24hrs with IFN-γ plates, later or next morning IL-5 plates (do not **bang** the ELISPOT plates, but shake out carefully):
   - wash 3x PBS

- wash 4xPBS/Tween (0.05% = 500µl in 1L), let sit in the last wash for at least 5min
- remove washing solution

- add secondary antibody in sterile 100µl PBS/Tween/1% BSA

for mIL-2:	- Biotin-labeled	= Biotin-rat anti-mouse IL-2, Pharmingen, 0.5mg/ml, final 2µg/ml
for mIL-4:	- Biotin-labeled	= Biotin-rat anti-mouse IL-4, Pharmingen, 0.5mg/ml, final 4µg/ml
for mIL-5:	- „indirect“	= TRFK-4 (hybridoma, or Pharmingen), final 4µg/ml
for mIFN-γ:	- HRP-labeled	= XMG1.2-HRP (Pharmingen) final (= 1µg/ml)

- incubate in humidified box (Tupper) o/n at 4°C

DAY-3

anti mIFN-γ „direct“ (= XMG1.2 conjugated with HRP)
- wash IFN-γ (XMG1.2-HRP) plates/wells 3x PBS, leave PBS
- AEC solution: take 24ml AEC buffer, pH 5.0 (storage)
  add 0.8ml AEC (= ImmunoPure AEC, 3-amino-9-ethylcarbazole, Pierce #34004, 100mg in 10ml dimethylformamide (DMF), chemical cabin, RT, wear gloves!)
  sterile filter with 150ml filter units (.45µm)
  before adding to plate: add 12µl of H_2O_2 (stock 30%, Fisher # H325-100, stored at 4°C)
- shake out plate
- add 200µl/well of AEC solution
- after 15 (IFN-γ) or 45-60min shake out plate
- wash with tap water 3x, let dry (hood)

anti mIL-5 (unlabeled)
- wash IL-5 (unlabelled TRFK-4) plates gently 3x PBS/TWEEN (with squirt bottle)
- add mouse anti-rat IgG_{2a}-HRP (ZYMED, South San Francisco, FAX (415) 871-4499; # 03-9620, 1ml, HRP-mouse mAb anti-rat IgG_{2a}), coupled mAb (frige 4°C at sink) diluted 1:300 in PBS/TWEEN-BSA 1% (40µl in 12ml)
- incubate at least 2hrs, RT
- wash 3x PBS, leave PBS
- then add substrate.... see above

anti mIL-2, -4, -10-Biotin
- wash plates gently 3x PBS/TWEEN, let sit in wash #4
- add Streptavidin-HRP (Dako; frige 4°C at sink), 1:2000, in PBS/Tween/1%BSA, 200µl
- incubate at least 2hrs, RT
- wash 3x PBS, leave PBS
- then add substrate.... see above

Development

a) XMG-HRP: is turning dark, but lightens with drying
b) XMG-Biotin: does **not light up** with drying!!

(in general: leave substrate at least 15 but at max. 60min)