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:**

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<tr>
<th>Calculation of volume:</th>
<th>a) proliferation assay = # organs x # antigens x # replicates x 0.1ml</th>
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<td>b) cytokine supernat. = # organs x # antigens x # replicates x 0.5ml</td>
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<td>c) ELISPOT = # cytokines x # organs x # antigens x # replicates x 0.1ml</td>
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x # of cell suspensions

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<th>d) spleen; 10⁷/ml (final cell concentration = 10⁶/well)</th>
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<td>e) LN; 5x10⁶/ml</td>
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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<sup>d</sup>) and irrelevant (I-A<sup>k</sup>) 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<sup>7</sup>/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<sup>6</sup> 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<sup>7</sup>/ml and LN = 5x10<sup>6</sup>/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% CO₂:
   - 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

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**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 H₂O₂ (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-IL IgG₂a-HRP (ZYMED, South San Francisco, FAX (415) 871-4499; # 03-9620, 1ml, HRP-mouse mAb anti-IL IgG₂a), 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)