Enzyme-Linked Immunosorbent Spot (ELISPOT) Assay for Detection of Alloreactive Cytokine-Reacting Cells–Detailed Methods

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The ELISPOT assay is a powerful immunologic technique for detecting and characterizing antigen-specific, cytokine-secreting T cells at high resolution. While this technique was originally described in the literature more than a decade ago, recent improvements in membrane design, antibody availability, and the development of computer-assisted analysis devices have now made ELISPOT assays of cellular immune responses both reliable and reproducible. Our laboratory, in conjunction with Paul Lehmann's and Magdelena Tary-Lehmann's laboratories in the Department of Pathology at Case Western Reserve University, has used this assay to characterize cytokine profiles and frequencies of T cells reactive to directly and indirectly presented alloantigens, to minor antigenic determinants and to individual peptide and protein antigens in both mice and humans.[1-14] Here we provide a detailed description of the methods that we have found to be most effective.

ELISPOT ASSAY:

A variation of an ELISA assay in which cytokines are detected as precipitated color spots on a white membrane at the site of secretion by responding cells.

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The assay is generally performed over a 3-4-day period, although it can be accelerated to yield reproducible results for some cytokines in <24h. The steps that can be performed in an accelerated manner are all noted in the detailed description below. As an overview, the ELISPOT procedure involves 8 steps:

- Coating individual wells of sterile ELISPOT plates with a capture antibody for the cytokine or secreted molecule of interest.
- Blocking the plate to reduce background signal.
- Preparing and adding the responder cells, stimulator cells, and/or antigen/mitogen to the wells at appropriate concentrations.
- Incubation period.
- Washing the plate followed by incubating the wells with a second, biotinylated, detection antibody.
- Washing the plate, followed by incubating the wells with a third enzyme-linked reagent.
- Developing the plates with a color substrate.
- Analyzing the results.

Detailed Protocol

Day 1

1. Mark the ELISPOT plate for purposes of orientation and organization. ELISPOT plates made with synthetic white membranes are available from a variety of companies including Fisher Scientific and Millipore. We have obtained the most reproducible and clear results using plates obtained from Cellular Technologies Limited, Cleveland, OH (catalog number M200-50). If only a portion of the plate is required for an individual assay, the remaining wells, if kept dry throughout the entire procedure, can be used at a later time. We have used separate wells on one plate for up to six different experiments without loss of signal and without evidence of contamination.
2. Add coating antibody. In a sterile laminar flow hood (or equivalent environment) primary capture antibodies (see Table 1) are diluted in sterile PBS at the appropriate concentrations

Table 1 | **ANTIBODIES FOR MOUSE ELISPOT ASSAYS**

Coating Antibody			
Antibody	Clone	Source	Final Concentration
IFN-γ	R46A2	Pharmingen[1]	2 µg/mL
IL-2	JES6-1A12	Pharmingen	3 µg/mL
IL-4	11B11	Pharmingen[1]	2 µg/mL
IL-5	TRFK5	Pharmingen[1]	5 µg/mL
2nd Detection Antibody			
Antibody	Clone	Source	Final Concentration
IFN-γ	XMG1.2 -biotin	Pharmingen[1]	1 µg/mL
IL-2	JES6-5H4-biotin	Pharmingen	2 µg/mL
IL-4	BVD6-24G2-biotin	Pharmingen	2 µg/mL
IL-5	TRFK4-biotin	Pharmingen	2 µg/mL

[1]We produce all of these antibodies from hybridomas in our laboratory, but they are also available from Pharmingen.

and 100 µl are placed into each well. As noted, antibodies can be prepared in the laboratory from hybridomas or can be purchased commercially. It is important to note that available antibodies, even those supplied by commercial sources, can vary from batch to batch. We therefore suggest titrating each new batch of antibody over a 10-fold concentration range in preliminary studies prior to using it for any large scale assay.

3. Incubate in humid environment. The plate should be wrapped in plastic wrap, placed in a covered container lined with a moist paper towel to prevent evaporation, and incubated at 4°C overnight; but we have tested plates coated with antibody and maintained at 4°C for up to 90 hours without any deterioration in the quality of the assay. NOTE: Alternatively, the antibody can be incubated in the plate at room temperature for 3-4 hours if one wishes to complete the assay in a shorter time period.

Day 2

1. Block the wells with PBS-1% BSA. Working in a sterile laminar flow hood, empty the coating antibody from the wells by firmly shaking the inverted plate over a catch basin. Block the plate with a sterile solution of PBS-1% BSA, 150 ml/well and incubate for a minimum of 60 minutes at room temperature.
2. Wash with PBS. Dump the liquid from the plate as above and wash the plate three times with sterile PBS, 200 µl/well/wash. Leave the last PBS wash in the wells and keep the plate at room temperature (in the sterile hood) until the antigens and the responder cells are added to the wells.
3. Prepare the responder cells.

A. Assays performed in mice. For antigen-specific responses, we generally study lymph node immune cells (or purified T cells) on days 9-11 after immunization or splenic immune cells on day 21 after immunization. Single cell suspensions of spleen cells are prepared in Hanks Balanced Salt Solution (HBSS) at room temperature and treated with a standard lysis buffer to eliminate RBCs (which can increase the background signal). After a wash in HBSS the cells are counted and resuspended in serum-free HL-1 medium (BioWittaker #77201) to which we add L-glutamine and penicillin-streptomycin. All mouse studies are performed in this HL-1 medium without fetal bovine serum. L-glutamine needs to be re-added to prepared HL-1 medium after 2 weeks because it degrades over that time period. Centrifugation is performed in standard swinging bucket rotors at room temperature for 7 min at ~1200 rpm. All cell preparation procedures (including centrifuge spins) are performed at room temperature and not on ice. Cells are resuspended at 10x concentrations such that addition of 100 µl

RESPONDER CELLS:

These may be lymph nodes or spleen cells from mice or peripheral blood cells from humans. Alternatively purified T cells or CD4 or CD8 cell subsets can be used.

TECHNICAL POINT OF IMPORTANCE:

Optimal antibody concentrations may vary from batch to batch and should be tested over a 5- to 10-fold concentration range prior to use in large assays.

to each well will result in an appropriate final cell concentration in that well. Five hundred thousand to 1 million cells are placed in duplicate or triplicate wells and challenged with either medium alone, specific or control antigens, or a mitogen as a positive control. For many assays it is useful to perform a cell titration (50,000 to 1,000,000 cells per well) to assure that the number of cells in the wells are optimal for detection of the responses. For detection of alloresponses in allograft primed mice (in which cell frequencies are much higher than in naïve or immunized mice) we test draining lymph node or spleen cells or purified T cells at cell concentrations ranging from 1000 to 200,000 cells per well. Cells can be obtained at any time point following placement of the grafts. We have found maximal responses for mice engrafted with fully allogeneic skin to occur on days 8-14 after graft placement. We prefer to use negative selection for T-cell isolation in order to prevent inadvertent activation of the cells during the isolation procedure.

B. Assays performed with human peripheral blood cells. We collect all human samples in green top vacutainer tubes containing heparin. We isolate peripheral blood lymphocytes (PBLs) using Isoprep (Robbins Scientific, Sunnyvale, CA) although any similar cell separation reagent can theoretically be substituted. The whole blood is placed into a centrifuge tube and diluted with an equal amount of sterile PBS. An original volume of Isoprep (half of the total diluted volume of blood) is underlayered and the suspension is centrifuged for 20 minutes at 2000 RPM with the brake off. The lymphocytes at the interface are collected and washed once with HBSS + 5% autologous serum or ABO serum.

The isolated cells can be used immediately in assays or frozen in human cell freezing medium (see below), stored in liquid nitrogen and tested in ELISPOT assays at a later time point. Frozen cells are rapidly thawed and washed in 15-30 ml of HBSS + 5% serum to remove residual DMSO and then counted. We generally count human cells with acrydine orange/ethidium bromide using a UV microscope to absolutely define the number of live cells. All human assays are performed in complete RPMI containing antibiotics, with additional glutamine and 10% high-quality fetal bovine serum (not in HL-1 medium). For detection of alloresponses we resuspend the cells at 1-6 million per ml and use 100 µl per well (100,000-600,000 cells per well). We have generally found ~300,000 PBLs per well to be optimal.

4. Preparation of stimulator cells or antigen presenting cells (APC).

   A. Mouse studies. For most assays we use spleen cells as allostimulator cells or as syngeneic APCs. Single cell suspensions of spleen cells in PBS (50-70 x 10^6/ml) are treated with mitomycin C at a final concentration of 50 µg/ml for 20 minutes at 37°C, followed by three washes in HBSS. The cells are resuspended in HL-1 medium as noted above. Our previous studies revealed that 4-6 x 10^5 stimulator cells per well provided optimal responses. In our experience mitomycin C treatment is superior to irradiation of the cells, as the latter tends to induce cytokine secretion by the stimulator cells. This problem can be fully eliminated if one uses stimulator cells or APCs derived from congenic mice that are knockouts for the cytokine being studied. For some assays investigators may wish to isolate and prepare specific cell types, i.e., dendritic cells, to use as stimulator cells.

   B. Preparation of stimulator cells from humans. For detection of alloreactivity we use frozen aliquots of spleen cells from cadaver donors or frozen aliquots of PBLs from living donors. The cells are isolated using Isoprep as outlined above and frozen in 15-20 million cell aliquots. Once thawed, the stimulator cells are treated with mitomycin C at 50 mg/µl for 30 minutes (NOTE: longer time compared to 20 min for mouse studies) at 37°C, followed by three washes in HBSS + 5% serum. The cells are then counted and resuspended at 3 x 10^6 per ml in complete RPMI and 100 µl (300,000 cells) are used in each

TECHNICAL POINT OF IMPORTANCE:

All handling of cells is performed at room temperature and all washes of human cells are performed in serum containing medium.

well. Human spleen cells are capable of releasing cytokine even if treated with mitomycin C. In our experience, the spontaneous production of IFN-γ, IL-2, and IL-5 by mitomycin C-treated stimulators cells is extremely low, but occasional samples produce a higher background. Spontaneous production of IL-4 is variable and is generally higher than that of the other three cytokines. Studies using serial dilutions of T-cell lines with known specificity and a constant number of stimulator cells suggest that essentially all of the detected spots derive from the responder cells. Still, some investigators may wish to use T-cell depleted APCs for some assays.

5. Preparation of antigens and mitogens. Peptide or protein antigens must be tested at a variety of concentrations to determine optimal responses. We found that for most peptide or protein antigens a final concentration of 0.1-50 μM is a reasonable range with which to begin. We use concanavalin A as a positive control for most mouse studies (final concentration of 2 μg/ml) and phytohemagglutinin (PHA, final concentration 2 μg/ml) as a positive control for human studies.
6. Preparations of donor cell sonicates for evaluation of indirect alloresponses. To evaluate T cells that have been primed through the indirect pathway we test whether purified responder T cells can produce cytokine when stimulated in ELISPOT wells with syngeneic APCs plus donor sonicate, but not with either alone.[10] We have only used this approach to detect indirectly primed mouse T cells and have not yet used this approach for evaluating human peripheral blood responses.
   To prepare the sonicates, single cell suspensions of donor spleen cells (in which the RBCs have been lysed) are prepared in HL-1 medium at concentrations between 20-40 x 10^6/ml. One ml aliquots are sonicated on ice with 10 1-sec pulses at the capacity of 5 Watts using a Sonic Dismembrator (Fisher Scientific) or an equivalent apparatus. The sonicated solution is then frozen in a dry ice/ethanol bath, and thawed in a 37°C water bath to further disrupt the cells. Any residual intact cells or cell membranes are removed by centrifugation at 1200 rpm for 10 minutes at room temperature. NOTE: KEEP THE SUPERNATANT and discard the pellet. Based on our previous studies we use 50 μl of the resultant supernatant per 200 μl final volume in a single ELISPOT well. Individual preparations may vary and we suggest titrating both starting cell number and amount of sonicate in preliminary experiments. We have found that the best responses were detectable using purified T cells isolated from draining lymph nodes 8-10 days following fully allogeneic skin graft placement in the presence of syngeneic APCs and specific sonicates. We therefore suggest using these cells as a positive control to check for adequacy of the sonicate preparations. Sonicates prepared from third party spleen cells can be used as specificity controls.
7. Set up the assay. Dump out the PBS from the washed plates. Add antigens/stimulators/APCs/sonicates and responder cells to the ELISPOT plate to equal a final volume of 200 μl/well. Holding the plate in one hand, gently tap the side of the plate to re-distribute the cells. This is important because otherwise the cells tend to cluster around the edges of the wells and the resultant spots harder to interpret.
8. Incubation. We incubate the IFN-γ and IL-2 plates (both mouse and human) for 24 h at 37°C with 5% CO_2. IL-4 and IL-5 require 48 hours of incubation to obtain optimal results. NOTE: Human IFN-γ plates can be incubated for 16 h (overnight) without loss of signal.

INDIRECT ALLORECOGNITION:

T cells responding through the indirect pathway recognize donor-derived peptides processed presented by recipient antigen presenting cells.

Day 3

1. Dilute the secondary (detection) antibody in PBS-Tween-BSA (see Table 2).
2. The rest of the experiment can be done on the open bench (nonsterile environment). Dump out the plate and wash the wells three times with PBS (200 μl/well/wash). After each wash firmly bang the plate onto a pile of clean paper towels to remove as much liquid as possible.
3. Wash the plate four times with PBS-Tween (200 μl/well/wash). Leave the last PBS-Tween wash on the plate and incubate for 5 minutes at room temperature. The PBS-Tween lyses any residual cells left in the wells.

Table 2 | **ANTIBODIES FOR HUMAN ELISPOT ASSAYS**

COATING ANTIBODY			
Antibody	Clone	Source	Final Concentration
IFN-γ	2G1	Endogen	4 µg/mL
IL-2	5334.21	R&D Systems[1]	6 µg/mL
IL-4	8D4-8	Pharmingen	2 µg/mL
IL-5	TRFK5	Pharmingen	5 µg/mL
2ND DETECTION ANTIBODY			
Antibody	Clone	Source	Final Concentration
IFN-γ	B133.5-biotin	Endogen	1 µg/mL
IL-2	BG5-biotin	Endogen[1]	0.5 µg/mL
IL-4	MP4-25D2-biotin	Pharmingen	2 µg/mL
IL-5	JES1-5A10-biotin	Pharmingen	2 µg/mL

[1]These antibodies have short half-lives at 4°C and should be divided into aliquots and stored at –20°C

4. Dump out the last wash, bang the plate on paper towels as above to dry and add second detecting antibodies, 100 µl/well. Wrap the plate in plastic wrap and incubate overnight at 4°C in a moist-atmosphere container. The assay can be accelerated at this step by performing the incubation at room temperature for 3-4 hours.

Day 4

Development of the spots.

1. Prepare the 3rd reagents as follows:
   For blue spots: alkaline phosphatase-conjugated goat anti-biotin antibody (Vector, Burlingame, CA) is diluted 1:1000 in PBS-Tween-BSA.
   For red spots: Streptavidin-horseradish peroxidase (Dako, Carpenteria, CA) is diluted 1:2000 in PBS-Tween-BSA.
   The color used depends on the type of experiment, the analytic abilities of the image analysis (some computers are better at reading blue versus red spots) and the qualities of the antibodies themselves. Blue spots tend to be a bit larger and somewhat more diffuse than the red spots. Blue spots also develop more rapidly. We have gotten the best results for mouse IFN-γ, IL-2 and IL-5 using blue reagents and mouse IL-4 using red reagents. All human assays are done with red reagents. Two color assays can also be performed to evaluate the production of two different cytokines at once.[15] This requires, however, that one of the antibodies be directly alkaline phosphatase conjugated (blue spots), a procedure that must be done in the laboratory itself (directly enzyme-conjugated antibodies are generally not available commercially). The second color can then be developed using the standard Streptavidin-HRP (red).
2. Dump out the second antibody from the plate and wash three times with PBS-Tween. Add the appropriate 3rd reagent, 100 µl/well and incubate for 90 minutes at room temperature.
3. Near the end of the incubation, prepare the substrates for spot development.
   For blue substrate: add 132 µl of NBT stock and 66 µl of BCIP stock to 20 ml of NBT buffer in a glass container (see below for recipes). Filter through a 0.45µ filter. This quantity is enough to develop one full plate or approximately 100 individual wells.
   For red substrate: add 800 µl AEC to 24 ml AEC buffer. Filter through a 0.45µ filter. Right before using, add 12 µl of 30% H_2O_2 (Sigma, St. Louis, MO). This quantity is enough to develop one full plate or approximately individual 100 wells.
4. Wash plate four times with PBS (200 µl/well). Add the substrate, 200 µl/well. Watch for color development. Expect spots to become visible between 15 and 40 minutes (red) and between 5 and 15 minutes (blue). It is important to stop the reaction when the spots are clearly visible but before the background color becomes too prominent.

Otherwise the wells become too difficult to read. To stop the reaction, wash the plate four times with distilled water.

5. Dry plate at room temperature under a loose foil tent to protect from light. The spots are light sensitive and will fade over time if exposed to light. Once dry, the plates can be stored essentially indefinitely at room temperature if maintained covered in aluminum foil.
6. When the plates are fully dry they can be analyzed by eye using a standard dissecting microscope or can be analyzed using one of several computer assisted image analyzers. We use the Immunospot 2 analyzer available from Cellular Technologies Ltd. (Cleveland OH) that has been specifically designed for this purpose.

TECHNICAL POINT OF IMPORTANCE:

Addition of DMSO must be performed slowly to prevent cell lysis prior to freezing. Use of a double styrofoam box or a controlled slow-freezing apparatus is essential to ensure viability upon thawing.

Reagent Recipes

1X PBS

To make 5 L, dissolve:

1.15 g. NaH2PO4

5.75 g. Na2HPO4

45 g. NaCl

in distilled water and dilute to 5 liters.

PBS + 1% BSA (Blocking Solution)

Sprinkle 3 g BSA (Sigma—St. Louis, MO: A8022) evenly over 300 ml PBS and allow to sit undisturbed for 15 minutes. Filter through a 0.22 mm filter. Store at 4° C.

PBS + 0.025% Tween ± 1% BSA

To 4 L of PBS, add 1 ml of Polyoxyethylenesorbitan Monolaurate (Tween 20) and mix. This makes PBS + 0.025% Tween, which is used as is in the noted wash steps. To make PBS Tween BSA, take 300 ml of the PBS-Tween and evenly sprinkle 3 g BSA over it and allow it to sit 15 minutes without being shaken or stirred in any way. Filter with a 0.22 µm filter. Store at 4°C.

AEC Buffer (0.1 M Sodium Acetate Buffer, pH 5.0)

1. Make 0.2 M Acetic acid: 11.55 ml glacial acetic acid per liter of distilled H_2O.
2. Make 0.2M sodium acetate.
3. Add 148 ml of 0.2 M Acetic acid to 352 ml of 0.2 M sodium acetate.
4. Bring up to 1 L with distilled H_2O.
5. Adjust pH to 5.0; store at 4°C.

AEC

Wear gloves, mask, lab coat, and work in fume hood. Add 1 g AEC (ImmunoPure AEC, Pierce #34004) to 100 mL DMF (N,N-Dimethylformamide, Fisher Scientific #BP1160-500). Wrap bottle in foil and store at room temperature. When bottle is empty, clean bottle with acetone.

NBT Buffer

6.06 g Tris base

2.95 g NaCl

0.5 g MgCl2

Make up to 500 ml with distilled water. Adjust pH to 9.5 with HCl if necessary. Store at 4°C.

NBT Stock

0.25 g NBT (BioRad #170-6532) in 5 ml 70% N,N-Dimethylformamide (DMF). Dissolve the NBT in 1.5 ml water, then add 3.5 ml DMF. Aliquot and store at –20°C.

BCIP Stock

0.25 g BCIP (Sigma #B-0274) in 5 ml 100% DMF. Using a glass stir-rod, smash tablets in a glass beaker. Add DMF. Store at –20°C.

Human Freezing Medium

Solution I: 30 ml good-quality fetal calf serum (FCS)
20 ml RPMI with antibiotics

Solution II: 10 ml dimethyl sulfoxide (DMSO)
40 ml FCS or human ABO serum

Store at 4°C.

The quality of recovery is largely dependent on how rapidly the cells are frozen (and subsequently thawed). We do not freeze more than 20 x 10^6 cells/vial and do not work with more than 5 vials at one time. Work at room temperature.

For each vial: resuspend cells in 0.5 ml Solution I, slowly over 3 min, add 0.5 ml Solution II by drops while agitating the vial. Place in a styrofoam box at room temperature and put this box in a second, larger styrofoam box. Place at 80°C. Move to liquid nitrogen after 12 hours.

Mouse Freezing Medium
30 ml good-quality fetal calf serum (FCS)
4 ml 30% glucose (dissolve 1.2 g D-glucose (Dextrose) in 4 ml deionized water, vortex)
4 ml dimethyl sulfoxide (DMSO)
2 ml RPMI medium
Filter through 0.45 µm filter.
Freezing is performed essentially as outlined above.

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References

1. Lehmann PV, Matesic D, Benichou G et al. Induction of T helper 2 immunity to an immunodominant allopeptide. **Transplantation 1997; 64(2):292-6**.
2. Matesic D, Valujskikh A, Pearlman E et al. Type 2 immune deviation has differential effects on alloreactive $CD4^+$ and $CD8^+$ T cells. **J Immunol 1998; 161(10):5236-44**.
3. Matesic D, Lehmann PV, Heeger PS. High-resolution characterization of cytokine-producing alloreactivity in naive and allograft-primed mice. **Transplantation 1998; 65(7):906-14**.
4. Tary-Lehmann M, Hricik DE, Justice AC et al. Enzyme-linked immunosorbent assay spot detection of interferon-gamma and interleukin 5-producing cells as a predictive marker for renal allograft failure. **Transplantation 1998; 66(2):219-24**.
5. Valujskikh A, Matesic D, Gilliam A et al. T cells reactive to a single immunodominant self-restricted allopeptide induce skin graft rejection in mice. **J Clin Invest 1998; 101(6):1398-407**.
6. Benichou G, Malloy KM, Tam RC et al. The presentation of self and allogeneic MHC peptides to T lymphocytes. **Hum Immunol 1998; 59(9):540-8**.
7. Valujskikh A, Matesic D, Heeger PS. Characterization and manipulation of T cell immunity to skin grafts expressing a transgenic minor antigen. **Transplantation 1999; 68(7):1029-36**.
8. Yip HC, Karulin AY, Tary-Lehmann M et al. Adjuvant-guided type-1 and type-2 immunity: Infectious/noninfectious dichotomy defines the class of response. **J Immunol 1999; 162(7):3942-9**.
9. Heeger PS, Greenspan NS, Kuhlenschmidt S et al. Pretransplant frequency of donor-specific, IFN-gamma-producing lymphocytes is a manifestation of immunologic memory and correlates with the risk of posttransplant rejection episodes. **J Immunol 1999; 163(4):2267-75**.
10. Benichou G, Valujskikh A, Heeger PS. Contributions of direct and indirect T cell alloreactivity during allograft rejection in mice. **J Immunol 1999; 162(1):352-8**.
11. Hartig CV, Haller GW, Sachs DH et al. Naturally developing memory T cell xenoreactivity to swine antigens in human peripheral blood lymphocytes. **J Immunol 2000; 164(5):2790-6**.
12. Valujskikh A, Heeger PS. $CD4^+$ T cells responsive through the indirect pathway can mediate skin graft rejection in the absence of interferon-gamma. **Transplantation 2000; 69(5):1016-9**.
13. Heeger PS, Forsthuber T, Shive C et al. Revisiting tolerance induced by autoantigen in incomplete Freund's adjuvant. **J Immunol 2000; 164(11):5771-81**.
14. Heeger PS, Valujskikh A, Lehmann PV. Comprehensive assessment of determinant specificity, frequency, and cytokine signature of the primed CD8 cell repertoire induced by a minor transplantation antigen. **J Immunol 2000; 165(3):1278-84**.
15. Karulin AY, Hesse MD, Tary-Lehmann M et al. Single-cytokine-producing CD4 memory cells predominate in type 1 and type 2 immunity. **J Immunol 2000; 164(4):1862-72**.