Evaluating mouse HBV-specific CD8+ T cell responses by ELISPOT assay

Immunology Assays

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Introduction

- Immunocompetent mouse models that allow for the investigation of virus replication and the immune response to HBV include hydrodynamic injection, HBV genome transduction with adenovirus or adeno-associated virus, and HBV transgenic mice [1].
- A number of different techniques can be used to measure the magnitude, specificity, and functionality of HBV-specific CD8+ T cells in mice, each having its own advantages.
- The enzyme-linked immunospot (ELISPOT) assay is highly sensitive, cost effective, measures the frequency of functional T cells, involves relatively little initial optimization, and requires minimal specialized equipment and training.
- A common application of ELISPOT is to measure IFN-gamma production after stimulation of mouse splenocytes with CD8+ T cell peptide epitopes, which has previously been described in greater detail as a chapter in the book Methods in Molecular Biology: Hepatitis B Virus [2].

Materials and Reagents

1. 70 µm pore nylon cell strainers
2. 1 mL syringe
3. 15 mL conical tubes
4. 50 mL conical tubes
5. Absorbent paper or paper towels
6. Multichannel pipette
7. Sterile reagent/cell reservoirs
8. Hank’s balanced salt solution (HBSS; Gibco)
9. ACK red blood cell lysis buffer (Thermo)
10. Fetal bovine serum (FBS)
11. Complete media: RPMI 1640, 10% FBS, 2 mM L-glutamine, 50 µg/mL penicillin, 50 U/mL streptomycin
12. Mouse IFN-gamma ELISPOT set (BD Biosciences; catalog #551083): anti-mouse IFN-gamma capture antibody, biotinylated anti-mouse IFN-gamma detection antibody, streptavidin-HRP reagent, 96-well ELISPOT plate
13. 3-Amino-9-ethylcarbazole (AEC) substrate set (BD Biosciences)
14. Dulbecco’s phosphate buffered saline (PBS)
15. PBS-Tween solution: PBS with 0.05% Tween-20
16. PBS-FBS solution: PBS with 10% FBS
17. HBV peptide stocks
18. Phorbol 12-myristate 13-acetate (PMA)
19. Ionomycin

20. Dissecting microscope or automated ELISPOT reader

Experimental Procedures

1. Splenocyte preparation a. Collect the mouse spleen in a 15 mL conical tube containing 3-5 mL HBSS. As an alternative to HBSS, either serum free RPMI or RPMI containing 1% FBS can also be used. b. For each spleen, place a 70 µm pore strainer on the top of a 50 mL conical tube. c. Transfer HBSS and spleen into the strainer, allowing the HBSS to pass through into the tube. d. Disrupt the spleen through the strainer using a plunger from a 1 mL syringe. As the connective tissue of the spleen is disrupted, the splenocytes will pass through the strainer into the tube. Continually wash the strainer by addition of 3 - 5 mL HBSS while disrupting the spleen. e. Centrifuge at 300 × g for 5 min at 4° C to pellet the cells. f. Remove liquid from the tube and dissociate the cell pellet from the bottom by gently flicking andshaking the tube. Using too much mechanical force during suspension steps can damage the cells and reduce yields. Suspend the pellet in 2 mL of ACK lysis buffer and incubate at room temperature for 5 min. Do not leave cells in ACK lysis buffer for extended periods of time, as doing so can result in lysis of lymphocytes. g. Add 8 mL of HBSS to the tube and immediately centrifuge at 300 × g for 5 min at 4° C. h. Decant the supernatant and wash cells in 5 mL HBSS, followed by centrifugation at 300 × g for 5 min at 4° C. Suspend pellet in 5 mL of complete medium and keep on ice. i. Count cells using a hemocytometer or automated cell counter.
2. IFN-gamma ELISPOT assay a. This procedure is adapted from the manufacturer's protocol for the BD Biosciences mouse IFN-gamma ELISPOT set (catalog #551083). Procedures for other manufacturers may vary. b. On the day before the assay will be performed, dilute anti-mouse IFN-gamma capture antibody according to the manufacturer's lot-specific recommendation in PBS, and add 100 µL of diluted antibody per well to an ELISPOT plate. c. Incubate the plate overnight at 4° C. Although the ideal incubation period is overnight, a minimum time of 4 hours can be done. d. Remove the capture antibody solution, and wash wells once with complete media. Block wells by adding 200 µL/well complete media and incubating at room temperature for 2 hrs. e. Prepare peptides for stimulation by diluting in complete media at a concentration of 20 µg/mL (final concentration will be 10 µg/mL after 1:1 dilution with cell suspension). Although mouse $CD8^+$ T cell responses to many HBV antigen epitopes have been described, the responses to some epitopes are stronger than others. The HBV S protein 191-202 (IPQSLDSWWTSL; L^d), 353-360 (VWLSVIWM; K^b), and 364-372 (WGPSLYSIL; D^d) epitopes, the HBcAg 87-95 (SYVNTNMGL; K^d)and 93-100 (MGLKFRQL; K^b) epitopes, and the Pol 140-148 (HYFQTRHYL; K^d) epitope are particularly immunogenic [3-10]. Alternatively, pools of overlapping peptides that cover the entire antigen coding sequence can be used. f. Dilute cells in complete media to a concentration of 2×10^6 cells/mL. Cells may need to be diluted more or less depending on the responses expected in a given experiment. It is recommended that a pilot experiment be performed to determine the optimal cell concentration. Keep in mind that the responses obtained using different cell numbers may not be linear, as both the interactions between T cells and antigen presenting cells as well as the actual number of T cells will be affected by dilution. g. Discard blocking solution and add 100 µL each of diluted cells (2×10^5 cells/well) and diluted peptides to the appropriate wells. It is recommended that duplicate wells be used for each experimental condition. h. As an unstimulated negative control, a well for each cell sample should also be included that contains cells but lacks peptide (add 100 µL of complete media without peptide). This control will be important to subtract the nonspecific responses. As a positive control, a well can be included for each sample in which the cells are stimulated with a polyclonal T cell activator such as PMA (20 ng/mL) plus ionomycin (1 µg/mL). i. To exclude that false spots might be generated by the reagents alone, a background control well without the cells can also be included. j. Depending on the parameters of the specific experiment, other controls are also appropriate and recommended. For example: cells from a control group of mice subjected to a mock/sham experimental manipulation that are stimulated with peptide; cells from an experimental group of mice stimulated with an irrelevant peptide (rather than no peptide). k. Incubate the ELISPOT plate overnight at 37° C / 5% CO_2 in a cell culture incubator. To prevent formation of irregular spots, avoid disturbing the plate after addition of the cells. l. Discard cells and media from the plate, and wash wells twice with deionized or Milli-Q water, allowing wells to soak for 3-5 min

during each wash. m. Wash wells 3× with PBS-Tween. Avoid touching the surface of the membrane in the wells during washing of the plate. Generally, to quickly remove cells or wash buffer, the plate can be inverted and flicked over a sink (or if appropriate, removed by vacuum suction in a biological safety cabinet) and tapped against absorbent paper to remove residual wash buffer. n. Dilute biotinylated anti-mouse IFN-gamma detection antibody according to the manufacturer's lot-specific recommendation in PBS-FBS. After removal of the final wash, add 100 µL of diluted antibody to each well. o. Incubate the plate at room temperature for 2 hrs. Dispose of detection antibody solution, and wash wells 3× with PBS-Tween. p. Dilute streptavidin-HRP reagent 1:100 in PBS-FBS, and add 100 µL of the diluted enzyme conjugate to each well. Incubate at room temperature for 1 hr. q. After incubation, discard the streptavidin-HRP solution and wash wells 4× with PBS-Tween, soaking 1-2 min during each wash. Wash wells an additional 2× with PBS. r. Prepare substrate solution by adding 20 µL of AEC chromogen (1 drop) to 1 mL of AEC substrate. Add 100 µL of final solution to each well. s. While monitoring spot development, incubate the plate for 5-60 min at room temperature. When spots have developed sufficiently so that they are readily visible, stop the reaction by washing wells with water. Do not allow the plate to overdevelop. t. Allow the plate to dry at room temperature, removing the rubber backing to facilitate drying. Spots will continue to develop as the plate dries, and contrast between spots and background will improve. **3. Quantification of T cell response** a. Using a dissecting microscope to magnify the wells, count the number of spots in each well. Ideally, the number of spots will fall in the range between a minimum of 10-20 and maximum of ~100-200. Numbers fewer than 10 may be difficult to distinguish from background, and greater than 200 are difficult to accurately quantify. Alternatively, an automated spot counter can be used to enumerate the spots (Cellular Technology Ltd.). b. Spots will vary in size and intensity, and it is important to maintain well-to-well consistency in how the spots are counted. In wells with very few spots, there will be a tendency to count very small or light spots, but to undercount these in wells that have many or larger/darker spots, which should be avoided. c. To quantify the specific response, subtract the corresponding "no peptide" control sample from each animal from the peptide-stimulated sample. The number of non-specific background spots in the unstimulated and background control wells should ideally be relatively low in comparison to the number of specific spots in the peptide-stimulated wells. d. Data are often enumerated as specific IFN-gamma spot forming cells (SFC), and normalized to 10^6 total cells (i.e. "Specific IFN-gamma SFC per 10^6 splenocytes"). e. One limitation of the ELISPOT assay using total splenocytes is the inability to rigorously ascribe a response to a specific cell type, such as $CD8^+$ T cells. However, if using well-defined peptide epitopes for stimulation that are known to not be cross-reactive for $CD4^+$ T cells, there is a reasonable degree of likelihood that the response is $CD8^+$T cell-specific. However, specificity can be further confirmed by using magnetic bead separation (Miltenyi Biotech) to enrich or deplete specific cell populations from the splenocytes prior to use in the assay.

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