Antigen Processing and Presentation Protocols

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Purification of 20S Proteasomes

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1. Introduction

Proteasomes are large multicatalytic proteinases located in the nuclei and cytoplasm of all eukaryotic cells. Proteasomes are composed of four heptameric rings stacked to form a hollow cylinder (length 16–20 nm, diameter 11–12 nm). The outer two rings are composed of α-subunits, while β-subunits, which contain the active sites, comprise the inner two rings. Proteasomes from archaebacteria contain only one type each of α- and β-subunits. Eukaryotic proteasomes are more divergent; yeast proteasomes have seven different α- and seven different β-subunits, each occupying a unique position in the ring. Only three of the seven yeast β-subunits contain the N-terminal threonine necessary for activity *(1–2)*.

Mammalian 20S proteasomes have seven different α- and ten different β-subunits, and have been classified into two groups. The so-called "constitutive" proteasomes contain three catalytic β-subunits: PSMB5 (X or MB1), PSMB6 (Y or δ), and PSMB7 (Z). These subunits can be replaced in "immunoproteasomes" by the IFN-γ-inducible catalytic β-subunits PSMB8 (LMP7), PSMB9 (LMP2), and PSMB10 (MECL-1), respectively *(1–3)*. Although there are eight possible combinations of catalytic subunits in the β rings, proteasomes with mixtures of constitutive and immune subunits are not favored *(4)*. Replacement of constitutive catalytic subunits with the IFN-γ-inducible subunits has been shown to change the proteasome activities against fluorogenic peptide and protein substrates *(2,5)*. It has been shown that proteasomes are responsible for generation of cytosolic peptides 7–13 amino acids in length, which are presented on cell surfaces in association with major histocompatibility complex class I (MHC-I) molecules *(1,3)*. The IFN-γ-inducible subunits are not essential for MHC-I antigen presentation, but it is thought that the additional

peptide diversity resulting from the presence of immunoproteasomes increases antigen presentation efficiency and/or repertoire, thus enhancing the immune response. Not only do proteasomes produce peptides for MHC-I presentation, but they are the primary nonlysosomal protein degradation machinery in eukaryotic cells, and are important in cell cycle regulation and transcription factor activation as well.

Although both types of proteasomes are present to some degree in almost every tissue *(6)*, mouse livers are highly enriched in constitutive proteasomes, and bovine pituitary proteasomes have almost no inducible subunits *(5)*. In addition, a homogeneous population of constitutive proteasomes can be purified from mouse H6 cells grown in the absence of IFN-γ *(7)*. On the other hand, preparations from spleens are highly enriched in immunoproteasomes *(5)*. Further enrichment can be obtained by hydrophobic interaction column (HIC) chromatography *(8)*.

Proteasomes are easy to purify, because they are relatively stable, they are present in large quantities in most tissues, and, since they are much larger (750 kDa) than most other cellular proteins, they can be separated from the bulk of cellular constituents early in the purification. Many different protocols are available for proteasome purification *(8–19)*; among these, three different protein purification strategies are common: separation based on size (such as gel filtration chromatography or ultracentrifugation), anion exchange chromatography, and hydrophobic interaction chromatography. Based on these three strategies, the method described here has been used to generate proteasomes that are 95–99% pure, from mouse livers, spleens, and muscles. Generally, 3 mg proteasomes can be purified from 20 g mouse spleens, a yield consistent with other reports *(8–19)*. Moreover, because proteasome structure is highly conserved from yeast to human, the following method should be easily adaptable to proteasome purification from other tissues and species.

The first day of purification involves collection of the tissues, homogenization, and centrifugation. The homogenization buffer includes 150 m*M* NaCl to reduce nonspecific interactions, and to help dissociate 20S proteasomes from the PA28 proteasome activator *(20)*. Two successive centrifugations yield cell lysates cleared of cellular debris, mitochondria and other organelles. A final 5-h ultracentrifugation step pellets the 20S proteasomes, while leaving smaller cell matrix proteins in the supernatant. The pellet is then suspended in buffer, and proteasomes are fractionated from contaminants through successive anion-exchange and HIC chromatography steps.

The anion-exchange column chromatography step is accomplished using a diethylaminoethyl (DEAE)-Sepharose matrix. In buffer B at pH 7.7 (*see* **Subheading 2.1., step 5**), proteasomes have a net negative charge, and bind the column matrix. More positively charged proteins pass through. As the salt con-
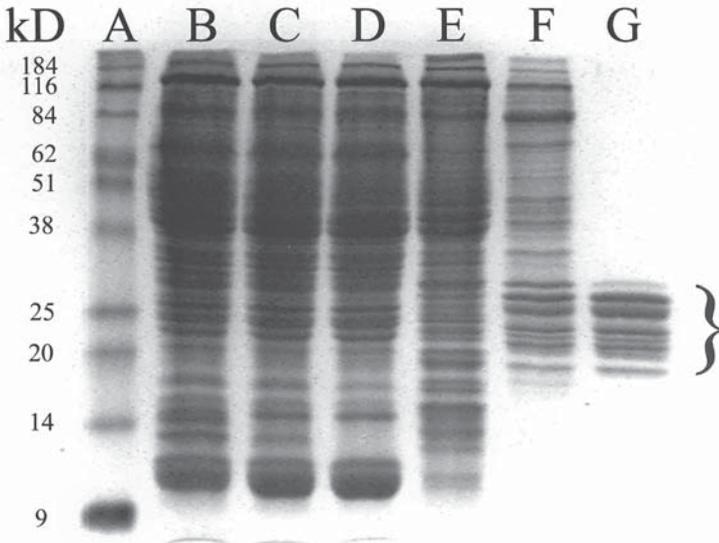
Fig. 1. SDS-PAGE of samples from each step of proteasome purification. The following samples were separated by standard SDS-PAGE on a 12% polyacrylamide gel, and stained with 0.1% Coomassie Brilliant Blue R250. **(A)** Benchmark Prestained Protein Ladder (Gibco-BRL, Rockville, MD, 10 µL); **(B)** homogenate (50 µg); **(C)** 10,000*g* supernatant (50 µg); **(D)** 1-h 100,000*g* supernatant (50 µg); **(E)** 5-h 100,000*g* pellet (35 µg); **(F)** DEAE active fractions (15 µg); **(G)** HIC active fractions (5 µg). The bracket indicates the position of proteasome bands in the gel. Molecular masses of the ladder proteins are indicated at left.

centration of the buffer increases during the gradient elution step, proteins of increasingly negative charge are eluted from the column, providing the basis for purification. It is evident from Coomassie-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the pooled fractions **(Fig. 1**, lane F) that most of the contaminants are removed by DEAE-Sepharose chromatography **(Fig. 2)**. The remaining contaminants are removed from the proteasomes by HIC chromatography **(Fig. 3)**. Hydrophobic proteins bind to the matrix when in contact with a high-salt buffer; less hydrophobic proteins pass through. As the salt concentration (and thus polar quality) of the buffer on the column is decreased with a reverse salt gradient, proteins of an increasingly hydrophobic nature are able to pass into the buffer and elute from the column. Proteasome activity elutes from the HIC column coinciding with a single, isolated peak of protein **(Fig. 3)**, which contains only proteasome proteins **(Fig. 1**, lane G). When the purified 700 kDa enzyme is separated on a denaturing SDS-PAGE gel stained with Coomassie Brilliant Blue, multiple protein bands
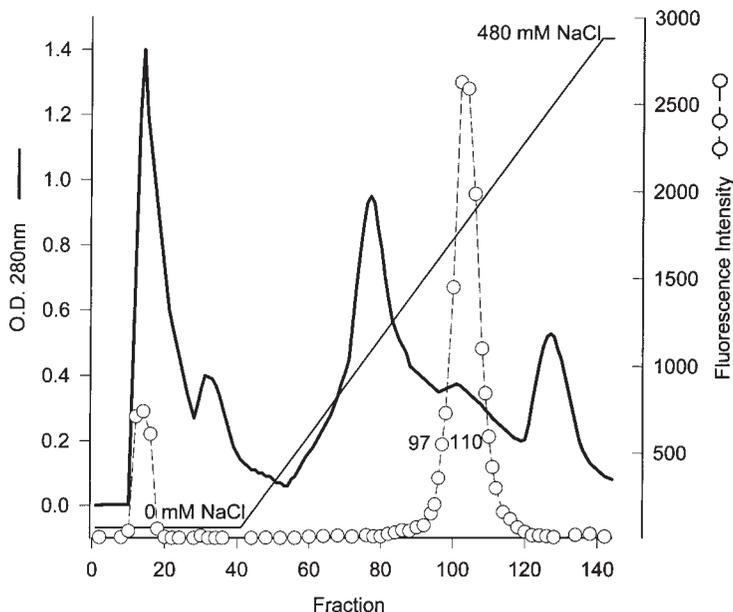
Fig. 2. DEAE column chromatography. The 5-h pellet was dissolved in buffer, centrifuged, and loaded onto the DEAE-Sepharose column. Bound proteins were eluted with a NaCl gradient (—). Samples (35 μL of each 4.5 mL fraction) were tested for LLVY-AMC hydrolysis (o---o). A small amount of activity in the void peak may indicate the presence of proteasomes, possibly because the amount of protein in the starting material exceeded the binding capacity of the column. The proteasome eluted at 250–350 m*M* NaCl. Relative protein content (—) showed that the major protein contaminants were excluded from the pooled active fractions (fractions 97–110). Note that some active fractions on either side of the peak were not pooled, in favor of reducing contaminating proteins. We have shown with SDS-PAGE that, if DEAE-Sepharose proteasomes fractions are pooled too widely, the purified mouse liver and spleen proteasomes contain contaminants between 60 and 80 kD.

are visible in the range of 22–35 kDa. Although preparations of pure eukaryotic proteasomes contain at least 14 different subunits, not all of these may be visible as distinct protein bands on Coomassie-stained SDS-PAGE gels, because of their similarity in molecular weight.

Several things are apparent when examining the proteasome purification table (**Table 1**) and the SDS-PAGE gel (**Fig. 1**). The initial centrifugation steps are required to remove insoluble material, and to concentrate the material for subsequent purification. However, these steps do not result in a great overall enrichment for proteasomes. After the 5-h centrifugation step, there is a great reduction in the amount of protein present, as well as in total proteasome activ-
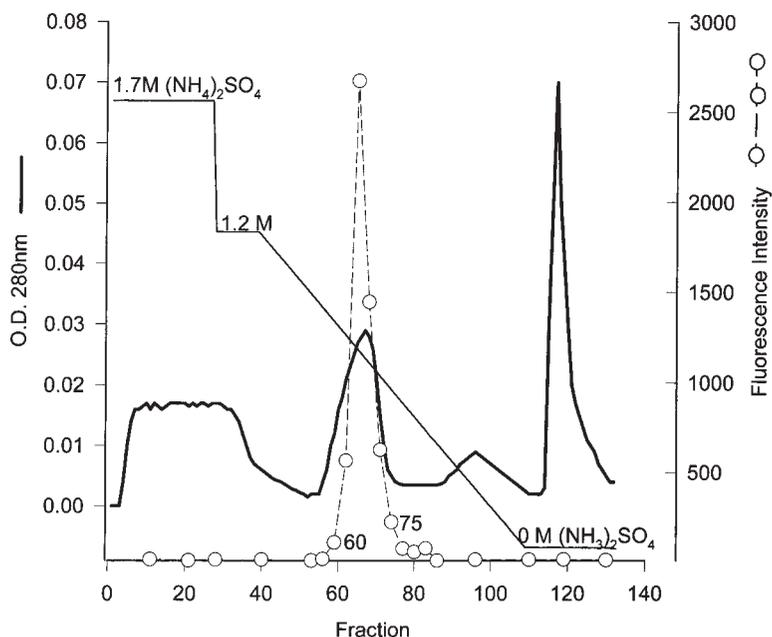
Fig. 3. HIC column chromatography of proteasomes from DEAE-Sepharose. Pooled DEAE-Sepharose fractions were brought to 1.7 M $(NH_4)_2SO_4$ and loaded onto an HIC column equilibrated with 1.2 M $(NH_2)_2SO_4$. Bound proteins were eluted with an $(NH_2)_2SO_4$ gradient (—). Each fraction (4.5 mL) was buffer exchanged, and 35 µL were tested for LLVY-AMC hydrolysis (o---o). The major activity eluted between 0.9 and 0.6 M $(NH_2)_2SO_4$ coincident with a single peak of protein (—). Nearly the entire proteasome activity peak was pooled (fractions 60–75).

ity. The yields in the first three steps are elevated by the presence of activators relative to the yields in the final three steps, because of the separation of proteasomes from PA28 and other lower-mol-wt activators after **step 3**. Although most activity remained in the 5-h supernatant, quantitative Western blotting indicates that approx 60% of the proteasomes from the 1-h supernatant are recovered in the 5-h pellet (data not shown). As evidenced by both the purification table and the gel, the greatest improvements in purification occur during the chromatography steps, resulting in a 54-fold final purification.

At each purification step, the fraction containing proteasomes is determined by an assay for hydrolysis of fluorogenic peptides. Three proteasome activities, corresponding to the three active β-subunits, are commonly assayed. Subunits Z or MECL-1 are responsible for the trypsin-like activity, which cleaves peptides on the carboxyl side of a basic residue (lysine or arginine). Subunits X or LMP 7 are responsible for the chymotrypsin-like activity, which cleaves after a hydro-

Table 1.
Purification of Proteasomes from 220 B6 Mouse Spleens (19.0 g)

Purification step	Protein (mg)	Specific activity[a]	Purification factor (X)	Total activity[b]	Yield (%)
Homogenate	2860	4.0	1.0	11440	100
10,000g supernatant	1739	4.8	1.2	8365	73
1-h 100,000g supernatant	1490	5.3	1.3	7942	69
5-h 100,000g supernatant[c]	1280	3.7	0.9	4672	41
5-h 100,000g pellet	145	12.6	3.2	1828	16[d]
DEAE active fractions	18	36.4	9.1	675	5.9
HIC active fractions	2.1	217	54.2	383	3.3

[a]nmol LLVY-AMC hydrolyzed/mg protein/h.
[b]nmol LLVY-AMC hydrolyzed/h.
[c]The 5-h 100,000g supernatant was not used for proteasome purification. The data for this fraction was included only to enable comparison of the 1-h supernatant and the 5-h supernatant and pellet.
[d]20S proteasome protein recovery from the 1-h 100,000g supernatant (assayed by quantitative Western blotting) during this step is approx 60%. The recovery of activity is artificially depressed by loss of the proteasome activator, PA28, during this step.

phobic residue (e.g., tyrosine, phenylalanine, leucine, or tryptophan). Subunits δ or LMP 2 are responsible for the peptidyl-glutamyl-peptide bond-hydrolyzing (PGPH) activity, which cleaves after acidic residues (glutamate or aspartate). Common substrates used for these activities are, respectively, N-t-BOC-Leu-Arg-Arg-7-amido-4-methylcoumarin (LRR-AMC), N-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (LLVY-AMC), and N-CBZ-Leu-Leu-Glu-β-naphthylamide (LLE-βNA). The fluorogenic groups of these substrates, 7-amino-4-methyl-coumarin or β-naphthylamide, increase in fluorescence when released from the peptide by proteolysis. Bulky groups (N-tert-butoxy-carbonyl [N-t-BOC], N-succinyl, or benzyloxycarbonyl [N-CBZ]) which block the peptide substrates at the amino terminus, render them indigestible by aminopeptidases, and help to identify proteasome activity in impure fractions.

Proteasomes are often referred to as "latent" or "active" *(21)*. Upon activation, one or more of the activities of latent proteasomes, especially a protein-

degrading activity, may dramatically increase. Activation can be caused by a variety of treatments (i.e., incubation with KCl, low concentrations of SDS or lipids, dialysis against water, or heat treatment) and the activities that are affected seem to differ, depending on starting material and purification procedure. The mechanism of proteasome activation is unknown, but evidence suggests that proteasome activation is accompanied by conformational changes or by proteolytic cleavage. Including 15–20% glycerol in all buffers during proteasome purification helps to maintain proteasomes in a latent state *(6)*. Because glycerol also increases proteasome stability and yield, we have included 15–20% glycerol in all purification steps after homogenization. This must be diluted or removed in order to measure proteasome activity.

Using the following method, proteasomes can easily be purified with standard chromatography equipment in 5 working days, or considerably less time if fast protein liquid chromatography (FPLC) or high performance liquid chromatography (HPLC) is used. The method has been organized into 1-d steps, although it is not difficult to find alternate stopping points, if necessary. Equivalent column chromatography methods for FPLC or HPLC are included in **Notes 2** and **6**.

2. Materials
2.1. Homogenization and Centrifugation

1. Stainless steel scissors and other tools necessary for dissection and tissue collection.
2. Buffer A (250 mL): 50 mM Tris-HCl, pH 7.5, 250 mM sucrose, 150 mM NaCl. Make fresh, keep chilled on ice.
3. Tissue homogenizer with a saw-tooth generator appropriate for homogenizing tissue and fibrous materials (e.g., PowerGen Model 125, Fisher Scientific, Pittsburgh, PA).
4. Ultracentrifuge capable of centrifugation at 100,000g, and ultracentrifuge tubes.
5. Buffer B (20 mL): 20 mM triethanolamine (TEA) (Sigma, St. Louis, MO), pH 7.7, 150 mM NaCl, 15% glycerol. Make 2 L; the remainder will be used in **Subheading 3.2, step 1**.

2.2. DEAE-Sepharose Column Preparation and Chromatography

1. Buffer B (2 L, *see* above).
2. Buffer C (1 L): 20 mM TEA, pH 7.7, 500 mM NaCl, 15% glycerol.
3. Buffer D (200 mL): 20 mM TEA pH 7.7, 1 M NaCl.
4. DEAE-Sepharose Fast Flow anion-exchange column chromatography matrix (Pharmacia), approximately 65 mL.
5. Chromatography column: 35 cm length $\times$ 1.6 cm inner diameter, volume = 70 mL.
6. Peristaltic pump.

7. UV monitor, chart recorder, and fraction collector.
8. Gradient maker.

2.3. Assay for Peptidase Activity

1. N-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (Sigma): Make 3 mM solution in dimethylsulfoxide (DMSO), and store at –20°C.
2. N-t-BOC-Leu-Arg-Arg-7-amido-4-methylcoumarin (Sigma): Make 4 mM solution in DMSO, and store at –20°C.
3. N-CBZ-Leu-Leu-Glu-β-naphthylamide (Sigma): Make 5 mM solution in DMSO and store at –20°C.
4. 7-amino-4-methylcoumarin (Sigma).
5. Apyrase (Sigma): Make 50 U/mL solution in 1X assay buffer (*see* **step 6**) and store at –20°C.
6. 5X assay buffer: 250 mM Tris-HCl, pH 8.3 at 25°C, 50 mM MgCl$_2$. Store at 4°C for up to 3 mo.
7. 96-well microtiter plates.
8. Fluorescent plate reader (e.g., CytoFluor 4000, PE Biosystems, Framingham, MA). Excitation and emission filters are required: 370 and 430 nm, respectively, for measurement of AMC-containing substrates, or 333 and 450 nm, respectively, for measurement of βNA-containing substrates.

2.4. HIC Chromatography

1. Buffer E: 20 mM Tris-HCl, pH 7.0, 1.2 M ammonium sulfate (NH$_4$)$_2$SO$_4$.
2. Buffer F: 20 mM Tris-HCl, pH 7.0, 0.2 M (NH$_4$)$_2$SO$_4$.
3. Buffer G: 20 mM Tris-HCl, pH 7.0.
4. HIC matrix: phenyl-650M (Toyopearl, Montgomeryville, PA), approx 20 mL.
5. Column: 12 cm length × 1.6 cm id, volume = 24 mL.
6. (NH$_4$)$_2$SO$_4$.
7. Glycerol.
8. Gradient maker.

2.5. Buffer Exchange of HIC Fractions for Assay

1. Buffer-exchange spin columns (e.g., Bio-Spin Chromatography Columns, Bio-Rad, Hercules, CA).
2. Buffer B.

2.6. Protein Concentration, Determination, and Storage

1. Enzyme dilution buffer: 50 mM Tris-HCl pH 7.5, 20% glycerol, 5 mM MgCl$_2$.
2. Protein determination reagent (e.g., Bio-Rad Protein Assay, Bio-Rad).
3. Bovine serum albumin (0.5 mg/mL), or other suitable protein standard for protein determination.
4. Protein concentration devices (e.g., Centriplus 50 concentration devices, Amicon, Beverly, MA).

3. Method

3.1. Day 1: Homogenization and Centrifugation

For the highest possible yield of proteasomes, all purification steps should be performed as rapidly as possible at 4°C, and chill all buffers and equipment that will contact proteasome preparation. The following method assumes 30 g of mouse livers as a starting material. The process can be easily scaled for much larger or smaller amounts of starting material by adjusting buffer amounts and column sizes proportionally. **Step 1** requires 8–10 h to complete, and can be finished in 1 d.

1. Obtain the starting material from freshly collected and euthanized animals, plants, or cell cultures; rinse in three changes of ice-cold buffer A, and store in ice-cold buffer A for a few minutes, until homogenization. Alternatively, tissues may be frozen until use (*see* **Note 1**).
2. Weigh the tissue to be homogenized. Add ice-cold buffer A in a ratio of 10 mL/g of tissue, and mince tissues with scissors in the buffer.
3. Separate mixture into 100-mL batches in beakers. Homogenize tissues with a tissue homogenizer at medium speed for 30 s, then place beaker on ice for 30 s. Repeat 3X; avoid foaming or warming the mixture. For larger volumes or tougher tissues, perform the same procedure in a Waring blender (four 1-min bursts with 30-s extraction intervals on ice.)
4. To clear the cell lysate of nuclei and other debris, centrifuge the homogenate at 10,000g (20 min).
5. Centrifuge the homogenate supernatant in an ultracentrifuge at 100,000g (1 h) to remove organelles.
6. Centrifuge the 100,000g supernatant in an ultracentrifuge at 100,000g (5 h). During this step, the proteasomes are pelleted. When starting with mouse livers or spleens, the pellet is a clear, reddish gel.
7. Remove the supernatant with a pipet, and gently suspend the pellet into 15 mL buffer B with an ice-cold Dounce homogenizer. Avoid introducing bubbles or foam, which will denature proteins in the suspension. Keep the suspension on ice overnight.

3.2. Day 1: DEAE-Sepharose Column Preparation and Equilibration

Column preparation is most efficiently accomplished during the long centrifugation steps of the first day. The second day can then be devoted to DEAE-Sepharose chromatography and peptidase assays.

1. Assemble the following apparatus at 4°C in a walk-in or chromatography refrigerator.
2. Standardize the peristaltic pump so that flow can be accurately measured between 0.1 and 2 mL/min. Select a tubing setup that can easily be attached and removed from the column without introducing air bubbles into the column matrix.

3. Set up the pump, column, UV monitor, fraction collector, and chart recorder.
4. Pack and prepare the column according to the manufacturer's instructions. Matrix capacity depends on the amount of proteins in the sample that bind at the buffer pH (7.7 in this case), therefore determination of necessary column size is empirical. However, the column capacity for thyroglobulin, which has a comparable molecular mass to proteasomes, is 3.1 mg protein/mL of matrix. We find 65 mL of matrix is sufficient when purifying proteasomes from 30 g of mouse livers.
5. Equilibrate the column with buffer B at approx 1 mL/min. Alternatively, the column can be equilibrated overnight at a very slow flow rate (0.l mL/min). Be sure to have sufficient buffer in the reservoir, so that the column will not run dry, in which case it will need to be repacked. The column is equilibrated when the effluent pH is equal to the pH of buffer B entering the column, and the baseline of the chart recorder becomes flat. Five column vol are usually sufficient.

3.3. Day 2: DEAE-Sepharose column chromatography

Alternative method for FPLC/HPLC users (*see* **Note 2**):

1. We find that, as the proteasome suspension sits on ice overnight, a white precipitate forms that contains very little proteasome activity. Remove the precipitate by centrifugation at 10,000g (20 min).
2. Set the UV monitor sensitivity at full-scale (absorbance unit range = 2.0). Using the chart recorder at 1 mm/min, record the UV absorbance while the column is equilibrating.
3. When the column is equilibrated, load the proteasome suspension from **step 1** onto the column at a relatively slow flow rate (0.5 mL/min). If the column size has been scaled up or down, adjust the flow rate accordingly. In general, larger diameter columns can sustain a greater flow rate.
4. Once the sample is loaded, wash the column in buffer B at a flow of 1.5 mL/min.
5. An increase in the absorbance units at 280 nm, seen as a peak on the chart recorder trace, indicates unbound protein flowing through the column. Collect this void peak in a beaker on ice until the chart recorder trace returns to baseline. Save the void peak to assay for proteasome activity.
6. While collecting the void peak, prepare 0–500 m*M* NaCl gradient (*see* **Note 3**).
7. Once the trace returns to baseline, start the gradient, and collect 7-mL fractions in 13×100 mm test tubes. The proteasomes elute from the column after one-third of the gradient has entered the column. Keep the fractions covered at 4°C.
8. After completing the gradient, wash the column with buffer C until the chart recorder trace returns to baseline.
9. Wash the column with buffer D to elute any tightly bound proteins, and store the column in 20% EtOH at 4°C.

3.4. Day 2: Assay for Peptidase Activity

1. Every second fraction should be tested for LLVY-AMC-hydrolyzing activity. Assign each fraction to be tested to a well of a 96-well microtiter plate. Additionally, include two wells for a negative control.

2. Into each well, add 5X assay buffer (10 µL), 3 mM LLVY-AMC substrate (5 µL), and, last, DEAE fraction (35 µL). The negative control receives 35 µL of buffer B instead of sample.
3. Cover microtiter plate and incubate at 37°C for 30 min.
4. If the plate will not be read immediately on a fluorescence plate reader, then stop hydrolysis with 150 µL cold EtOH (95%).
5. Read the plate with a fluorescence plate reader (370 nm excitation filter, 430 nm emission filter for LLVY-AMC and LRR-AMC; 333 nm excitation filter, 450 nm emission filter for LLE-βNA). At the time the assays are performed, a standard curve for the instrument (AMC concentration [nm] vs fluorescence units) should be prepared with several concentrations of 7-amino-4-methyl-coumarin (AMC) (i.e., in a range of 0–1.0 nmol/well). Because the fluorescence readings are dependent on the UV source intensity, which deteriorates with time, these standard curves should be prepared frequently.
6. Subtract the background fluorescence (negative control values) from each well.
7. To ensure that the substrate is present in saturating quantities, the fluorescence values of active fractions should increase proportionally with the amount of sample.
8. Pool the fractions containing proteasome activity. If there is more than one peak, it may be worth assaying the fractions for LLE-AMC and LRR-AMC hydrolysis to determine which is the proteasome peak (*see* **Note 4**). For details on deciding which fractions to pool, refer to **Note 5** and **Fig. 2**.
9. Store the pooled fractions overnight on ice.

3.5. Day 3: HIC Chromatography

Alternative method for FPLC/HPLC users (*see* **Note 6**):

This is the final step in the proteasome purification. In liver, spleen, and muscle, if column fractions have been pooled conservatively, no contaminants are visible on one-dimensional (*see* **Fig. 1**) and two-dimensional Coomassie-stained SDS-PAGE gels. The HIC column chromatography step takes 6–8 h.

1. Prepare and equilibrate the HIC column (*see* **Note 7**).
2. Slowly add $(NH_4)_2SO_4$ directly to pooled DEAE fractions to a final concentration of 1.7 M (add 0.225 g $[NH_4]_2SO_4$), mixing continuously at 4°C, until $(NH_4)_2SO_4$ is dissolved.
3. When UV trace has flattened into a baseline, the pooled fractions may be loaded onto the HIC column at a flow of 0.5 mL/min. Collect the void peak into a beaker on ice.
4. Wash the column with buffer E at a flow of 1.0 mL/min until trace returns to baseline. Meanwhile, set up a 1.2–0.2 M $(NH_4)_2SO_4$ gradient according to **Note 3**, using 50 mL of buffer E as the starting buffer, and 50 mL of buffer F as the ending buffer.
5. Start the gradient, and collect 5-mL fractions in 13 × 100 mm tubes. Proteasomes elute just after the midpoint of the gradient, and quickly lose activity without glycerol in the buffer. Add 1.25 mL glycerol (final concentration 20%) to each

    tube, and mix thoroughly with collected fractions as soon as possible.

6. After completing the gradient, wash with buffer F until the chart recorder trace returns to baseline.
7. Store tubes covered at 4°C overnight.
8. To elute tightly bound proteins, wash column with buffer G. Store column in buffer E at 4°C.

3.6. Day 4: Buffer Exchange and Assay of HIC Fractions

1. Proteasome activity is inhibited by $(NH_4)_2SO_4$, so fractions need to be buffer-exchanged before the proteasome assay. Choose the middle one-third of the fractions; from this set, buffer exchange 100 μL of every other fraction into buffer B using spin columns according to the manufacturer's instructions (spin columns can be re-used if stored in water at 4°C). Alternatively, overnight dialysis can be used to achieve a buffer exchange (*see* **Note 8**).
2. Test the buffer-exchanged fractions for LLVY-AMC-hydrolyzing activity as described in **Subheading 3.4.**, **steps 1–4, 6**. If the proteasomes are pure, and fractions have been pooled conservatively at each step, the LLVY-AMC-hydrolyzing activity should elute from the HIC column simultaneously with a single peak of protein (*see* **Fig. 3**).
3. In order to determine which fractions to pool, it may be helpful to run a SDS-PAGE gel of each fraction containing proteasome activity, as well as adjacent fractions, to check for contaminants. Use fractions that have been buffer-exchanged; otherwise, the high $(NH_4)_2SO_4$ content in the samples will disrupt the electrophoresis.
4. Pool fractions that contain highest levels of proteasome activity and no contaminants. If proteasomes still contain impurities, consider running a smaller DEAE column or a gel-filtration column (*see* **Note 9**).

3.7. Day 5: Proteasome Concentration, Protein Determination, and Storage

1. Buffer-exchange and concentrate the pooled fractions using Centriplus 50 devices or another comparable device. After at least a 10-fold concentration according to the manufacturer's instructions, dilute the sample to original volume with enzyme dilution buffer and reconcentrate. After three iterations of concentration and dilution, the $(NH_4)_2SO_4$ concentration will be 5 m*M* or less.
2. Measure the protein concentration of the concentrate using Bio-Rad Protein Assay kit according to the manufacturer's instructions. A proteasome concentration of 1–2 mg/mL makes a convenient working stock for running electrophoresis gels or enzyme assays.
3. Store proteasomes in 100-μL aliquots in 1.5-mL screw-cap micro-centrifuge tubes at –80°C. Under these conditions, purified proteasomes will retain activity for many months. It is best not to thaw and refreeze proteasomes more than 2–3 × because of changes (including loss of activity) that may occur to the enzyme.

4. Notes

1. It is possible, and convenient, to collect the starting material (e.g., mouse livers) 1 d or more prior to beginning the homogenization step of the purification. This reduces the time required to complete the first day of proteasome purification. Tissues can be stored at –80°C for many months without loss of proteasome activity. For long-term tissue storage, rinse starting material in three changes of Buffer A, blot away excess buffer with filter paper, and store at –80°C or below in a suitable container (a 50-mL conical screw-cap tube works well).

2. For FPLC users, the Bio-Rad UnoQ6 column is a good alternative to DEAE-Sepharose open-column anion-exchange chromatography. A comparable FPLC anion-exchange column is the MonoQ (Amersham Pharmacia). Two buffers (prepared with HPLC-grade water, filtered through 0.22-μm filters, and degassed before use) are required: buffer 1 (1 L): 20 mM TEA pH7.7, 15% glycerol; and Buffer 2 (1L): 20 mM TEA, pH 7.7, 15% glycerol, 1 M NaCl. The 6 mL UnoQ6 column has a high binding capacity and will accommodate at least 60 mg protein, while retaining maximal resolution. Equilibrate the column in 15% Buffer 2 for 5 column vol before loading sample. Because the buffers contain glycerol, run the pumps at a much slower flow rate than maximum, in order to avoid high back pressures and column compaction (i.e., 3 instead of 8 mL/min for the UnoQ6 column). After loading the protein, wash the column with 5 vol of 15% buffer 2, or until UV trace returns to baseline. Collect the void to test for possible flowthrough proteasome activity. Elute proteins from the column with a gradient of 15–50% Buffer 2 over 10 column volumes, and 50–100% Buffer 2 over 5 column vol, collecting 2-mL fractions. Wash column with 100% Buffer 2 until trace returns to baseline. Test every second fraction for LLVY-AMC-hydrolyzing activity, as described in **Subheading 3.4.** Mouse liver proteasome activity elutes between 250–300 mM NaCl from the UnoQ6 column.

3. Gradient makers can be purchased, but an inexpensive and effective alternative merely requires two identical 100-mL bottles and a piece of tubing. Tape the bottles together, and label one bottle "starting buffer" and the other "ending buffer." Fill the starting buffer bottle with exactly 75 mL Buffer B, and the ending buffer bottle with exactly 75 mL Buffer C. Put a small stir-bar in the starting buffer. Using a syringe, fill the tubing with ending buffer (do not draw the buffer from the 75 mL; use a different source). With the tubing clamped off so that no buffer escapes, put the ends of the tubing in the bottles, making sure that they reach the bottoms of each bottle. Unclamp the tubing. Connect a second piece of tubing from the starting buffer to the column, and set the gradient maker to stir gently on a stir plate. As the starting buffer is depleted, the resulting siphon from the ending buffer creates a salt gradient. The buffer levels in both bottles should be equal throughout the gradient.

4. When testing chromatography fractions for proteasome activity, it is convenient to assay only the chymotrypsin-like activity (LLVY-AMC-hydrolyzing activity), because it is usually high, and therefore easily detectable in dilute solutions. How-

ever, it may be desirable to test fractions for trypsin-like (LRR-AMC-hydrolyz-
ing) and PGPH (LLE-βNA-hydrolyzing) activities during early stages of purifi-
cation, if contaminating proteases capable of LLVY-AMC hydrolysis are present.
Fractions containing proteasomes will show increased fluorescence for all three
substrates. For mouse liver proteasomes, LLVY-AMC should be assayed at a
final concentration of 300 μM, LRR-AMC at 400 μM, and LLE-βNA at 500 μ*M*.
The 26S proteasome, which contains the 20S proteasome as its catalytic core,
also can hydrolyze all three substrates. Although it rapidly loses activity in the
absence of adenosine triphosphate, and is therefore an unlikely contaminant, apy-
rase (5 μL/well of a 50 U/mL stock) can be included in the assay to inhibit the
26S proteasome.

5. It is best to pool fractions very conservatively, taking only the fractions with
   highest activity and excluding any shoulders of the proteasome peak, which usu-
   ally indicate contaminants. It is helpful to determine the precise location of
   contaminants by analyzing proteolytically active and adjacent fractions with
   Coomassie- or silver-stained SDS-PAGE. Generally, it is wiser to cut away as
   many contaminants as possible and lose some activity earlier in the purification.
   This ensures that subsequent purification steps will result in better separation of
   proteins, thus producing higher final yields of pure proteasomes.

6. The Alkyl Superose HR 5/5 (Pharmacia, 1 mL column vol) is a good choice for
   an FPLC hydrophobic interaction column. Prepare two buffers with HPLC-grade
   water, filter through 0.22-μm filters, and degas before use: Buffer 3 (20 m*M* Tris-
   HCl, pH 8.0, 2.0 *M* $(NH_4)_2SO_4$); Buffer 4 (20 m*M* Tris-HCl, pH 8.0). Bring
   sample to 2 *M* $(NH_4)_2SO_4$ by slowly adding $(NH_4)_2SO_4$ directly to pooled anion
   exchange fractions (add 0.225 g $[NH_4]_2SO_4$) per ml, mixing continuously at 4°C
   until $(NH_4)_2SO_4$ is dissolved. With mouse livers, many of the remaining proteins
   precipitate at 2 *M* $(NH_4)_2SO_4$; remove these by centrifugation at 10,000*g* for 20 min.
   In mouse livers, this precipitate contained less than 3% of the remaining
   proteasome activity. Load the sample onto the column, and wash with 5 mL
   Buffer 3, collecting the void peak. Run the gradient from 0–50% Buffer 4 over 15 mL
   (15 column vol), collecting 1 mL fractions. Run a gradient from 50–100% Buffer
   4 over 5 mL. Wash the column with 20 vol of 100% Buffer 4. The proteasome
   activity elutes between 1.6 and 1.4 *M* $(NH_4)_2SO_4$; immediately add 0.2 mL glyc-
   erol to each fraction between 1.2 and 1.8 *M* $(NH_4)_2SO_4$ and mix well, to preserve
   proteasome activity. Proceed with buffer exchange and activity assay as described
   in **Subheading 3.6.**

7. HIC open-column equilibration essentially follows method steps in **Subheading
   3.2.** Pack and prepare a chromatography column (12 × 1.6 cm) with 20 mL
   Toyopearl 650M matrix, according to the manufacturer's instructions. Set the
   UV detector to 280 nm, with an absorbance unit range of 0.2, and the chart recor-
   der speed at 1 mm/min. Equilibrate with Buffer E (at 0.1–0.2 mL/min overnight,
   or up to 1 mL/min) until chart recorder trace has returned to baseline, and buffer
   has stabilized at pH 7.0.

8. Dialysis can be used for buffer exchange instead of the spin columns. Split 4 L enzyme dilution buffer into two 2-L flasks, and chill to 4°C. Clip each fraction into dialysis tubing, and dialyze while stirring at 4°C against 2 L enzyme dilution buffer (4 h); dialyze against the second change of buffer overnight.

9. In our experience, mouse liver, spleen, and muscle proteasomes are very pure after HIC chromatography (*see* **Fig. 1**, lane G), if chromatography fractions have been pooled conservatively. If proteasomes are not pure enough, try running a second anion-exchange column of smaller dimensions. Another possibility is gel filtration chromatography, which has the advantages of buffer-exchanging the proteasomes into enzyme dilution buffer while retaining higher enzyme yields. Some proteasome purification protocols use gel filtration chromatography as a size-based separation step rather than the differential ultracentrifugation steps described in this protocol (e.g., *see* **refs. 12, 15,** and **19**). Pharmacia makes a gel filtration column suitable for purification of large proteins at low pressures (Sephacryl S-300 HR, HiPrep 16/60), as well as FPLC gel filtration columns (Superose 6 HR 10/30). Set the UV monitor to an absorbance unit range of 0.1, equilibrate, and run the column in enzyme dilution buffer + 100 m*M* NaCl (salt may be included to minimize interactions between proteins and column matrix). After proteasome purity has been established through SDS-PAGE analysis and proteasome activity assays, concentrate and store proteasomes according to **Subheading 3.7.**

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2

Use of Proteasome Inhibitors to Examine Processing of Antigens for Major Histocompatibility Complex Class I Presentation

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1. Introduction

Proteasomes are multicatalytic proteases present in the nucleus and cytosol of eukaryotic cells. The central catalytic core, the 20S proteasome, consists of four heptameric rings, the central two of which contain the catalytic β-sub-units, members of a new family of threonine (Thr)-proteases. The outer rings, made of α-subunits, bind the regulators that control the substrate specificity of the proteasome. The binding of a 19S regulator to each end of the 20S core creates the 26S proteasome, which degrades ubiquitinated substrates in an adenosine triphosphate-dependent manner *(1,2)*.

Proteolytic degradation of cytosolic substrates is the chief source of antigenic peptides that are presented by major histocompatibility complex class I (MHC-I) molecules. The involvement of proteasomes in the generation of class I ligands was suggested by their intracellular distribution and multiple proteolytic activities and by the fact that genes encoding two of the β-subunits are located in the MHC, and are controlled by cytokines in parallel with class I molecules and other proteins associated with antigen (Ag) processing and presentation *(3–5)*. The introduction of proteasome inhibitors to cellular studies enabled the demonstration of the dominant role of this protease in cellular protein turnover and its involvement in the generation of class I ligands *(6)*.

Currently, there are four kinds of commonly used proteasome inhibitors, all of which, through different mechanisms, base their activity on the modification of the gamma oxygen (Oγ) on the N-terminal, active residue of Thr in one or more of the catalytic β-subunits (for a review on the mechanisms of the inhibitors, see **ref. *7***). These are:

Table 1
Molecular Weights, and Concentrations and Solvents used for Stock Solutions of the Inhibitors and the Negative Control AcLLM

Inhibitor	Mol wt	Solvent	Concentration
zLLL	475.6	DMSO Ethanol	40 mM
AcLLnL	383.5	DMSO Ethanol	40 mM
zLLnV	461.61	DMSO Ethanol	10 mM
AcLLM	401.6	DMSO Ethanol	25 mM
Lactacystin	376.4	DMSO H$_2$O	40 mM
CLβL	213.2	DMSO Acetonitrile	
NLVS	722.6	DMSO	10 mM

1. Tripeptide aldehydes. Used in initial studies, they are not specific for proteasomes, but affect other proteases, particularly calpains *(8)*. They form a reversible hemiacetal covalent bond with N-terminal Thr *(9)*.
2. Lactacystin *(10)*, and its active form, *clasto*-lactacystin β-lactone CLβL *(11)*, a *Streptomyces* sp. natural product, which covalently and irreversibly blocks proteasome activity; this is, so far, the most specific inhibitor of the proteasome, but some inhibitory effects on other proteases have been reported *(12)*.
3. Peptidyl-vinylsulfones, which form a covalent bond with the active group, and are also irreversible *(13)*.
4. Boronic salts, a group of potent inhibitors that only recently has started to be thoroughly studied *(14)*. Their binding to the active site is reversible.

An important limitation to the use of proteasome inhibitors is that none of the inhibitors exclusively affects the proteasome. To ascertain that proteasome inhibition is the cause (but not necessarily the proximal cause, *see* below) of the effect observed, appropriate controls must be performed. For the peptide aldehydes, there are several related compounds that block a similar spectrum of cellular proteases without affecting the proteasome (one of them, N-acetyl-leucyl-leucyl-methioninal [Ac-LLM], is included in **Table 1**). Also, at least two mechanistically different kinds of proteasome inhibitors should yield comparable results.

The first step in using proteasome inhibitors should be to assess the optimal inhibitor concentration, defined as the minimal concentration that completely blocks the cellular degradation of a proteasome substrate. Chimeric proteins,
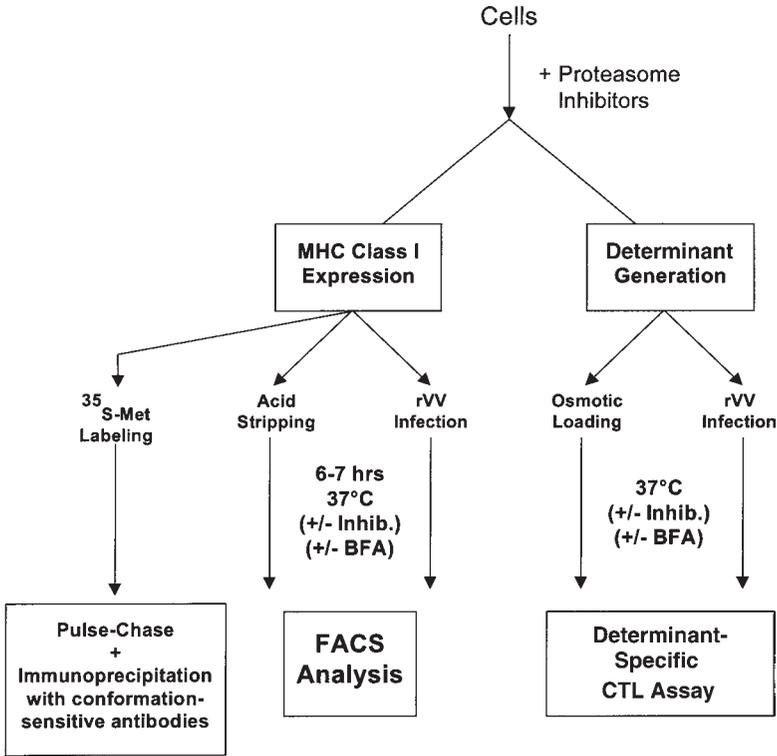
Fig. 1. Scheme of the different methods described to study the effect of proteasome inhibitors on Ag processing for MHC-I presentation.

composed of an N-terminal moiety of ubiquitin, followed by a destabilizing amino acid, according to the N-end rule *(15,16)*, and a target protein, are commonly used substrates. The method detailed here uses one of these proteins, UbRNP, in which the nucleoprotein (NP) from influenza virus A/NT/60/68 is preceded by ubiquitin and a destabilizing residue of arginine Arg *(17)*. The protein is expressed as a recombinant vaccinia virus (rVV), and pulse-labeling of the infected cells, followed by chases at different times, provides a good estimate of proteasome activity. The same principle could be used with other metabolically unstable proteins expressed in different ways (endogenously expressed, transfected, and so on).

Predominantly there are two different methods of investigating the role of proteasomes in MHC-I presentation (*see* **Fig. 1**). One way is to examine the effects of proteasome inhibitors on the maturation and cell surface expression of newly synthesized MHC-I molecules, which are peptide binding-dependent. One approach to accomplish this is to pulse-label the cells, in the absence or

presence of inhibitors, and to lyse them after different times of chase. The lysates
are then incubated at 37ºC, a treatment that renders peptide-receptive (or empty)
molecules unable to bind antibodies (Abs) that recognize only folded class I mol-
ecules. As a control, synthetic peptides, which bind to the class I allele studied,
are added to the lysates before the 37°C incubation. Cells with a compromised
peptide delivery to class I molecules in the endoplasmic reticulum (ER) will
have fewer molecules recognized by the Ab in the absence of exogenous peptide.
Another approach is to estimate by flow cytometry the cell surface expression of
newly synthesized class I molecules. This is done either by destroying cell sur-
face molecules by acid treatment, then allowing for new ones to be expressed in
the presence or absence of the inhibitors, or by infection with rVV-expressing
MHC-I Ags different from those endogenously expressed by the infected cell,
and, as before, following their cell surface expression.

The second method is to examine the effects of proteasome inhibitors on the
generation of particular class I-peptide complexes, using either peptide–MHC-
specific cytotoxic T-lymphocytes (CTL) or monoclonal antibodies (mAbs) spe-
cific for these particular complexes *(18,19)*. This requires that the class
I–peptide complexes studied are not expressed before proteasome inhibition.
There are two chief methods of accomplishing this. The most widely used strat-
egy entails transient expression of the substrate, which is achieved by either
viral infection of the target cells or by loading of the purified protein into the
cytosol; alternatively, cells constitutively expressing a target Ag are
acid-stripped to remove existing complexes, and the effect of proteasome
inhibitors on regeneration of peptide–class I complexes is determined.

As alluded to above, one must exercise caution when interpreting results
from any experiment using proteasome inhibitors. Even brief inhibition of
proteasomes has protean effect on cells, including reduction of ubiquitin pools
(20), induction of a stress response *(21–23)*, interference with cell cycle pro-
gression *(24)*, and either enhancement or prevention of apoptosis, depending
on the cell type *(25,26)*. Cytosolic proteases different from the proteasome
may contribute to Ag presentation *(27–32)*. A candidate protease, as well as an
inhibitor that blocks this protease (but not proteasomes) have been described,
and may prove of great relevance to the field *(33,34)*.

2. Materials
2.1. Inhibitor Stocks

Table 1 shows a list of commercially available proteasome inhibitors com-
monly used in studies of Ag presentation, solvents, and concentrations of stock
solutions. These are carbobenzoxy-leucyl-leucyl-leucinal (zLLL, also known
as MG132); N-acetyl-leucyl-leucyl-norleucinal (AcLLnL, calpain inhibitor I);
carbobenzoxy-leucyl-leucyl-norvalinal (zLLnV, MG115); AcLLM, calpain

inhibitor II, a control inhibitor which does not affect the proteasome; lactacystin; CLβL, the active component of lactacystin; and 4-hydroxy-5-iodo-3-nitrophenylacetyl-leucyl-leucyl-leucyl-vinylsulfone (NLVS). Stocks should be stored at –20°C or below, and are stable for at least a few months. Aqueous solutions are not recommended for storage, because the half life of the inhibitor is reduced, most dramatically in the case of CLβL. Some useful information about the inhibitors can be obtained on-line from some of the manufacturer's Websites, particularly Calbiochem-Novabiochem (La Jolla, CA [www.calbiochem.com]) or Affinity Research (Exeter, UK [www.affinity-res.com]).

2.2. Cell Lines

There is no cell line specifically recommended for any of the methods described. The choice of cell line will depend mostly on the class I molecule, the determinant studied, and susceptibility to virus infection. Cells commonly used in this kind of studies include P815 (mastocytoma, H-2^d), L929 (fibroblast, H-2^k), EL4 (thymoma, H-2^b), LB27.4 (lymphoblastoid, H-2$^{d/b}$), or transfectants of the class I-deficient human cell line, H2MY2.C1R. Some of these cells are also available as transfectants expressing other class I molecules. Alternatively, class I molecules can be expressed using rVVs. Most mouse lymphocyte cell lines are resistant to VV infection.

2.3. Determination of the Effective Inhibitor Concentration

All solutions for tissue culture should be sterile.

1. ^{35}S-L-methionine (Met) (10 mCi/mL).
2. Phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin (BSA) (PBS/BSA).
3. Methionine (Met) starving medium: Met-free, serum-free medium (RPMI or Dulbecco's modified Eagle's medium [DMEM]), containing 20mM HEPES.
4. PBS containing 10 mM L-Met (PBS–Met).
5. Iscove's modified DMEM containing 7.5% fetal calf serum (FCS) and L-Met (I/Met).
6. rVV expressing the chimeric protein UbRNP.
7. Laemmli´s sodium dodecyl sulfate-polyacrylamide gel electrophoresis SDS/PAGE sample buffer, 2X solution, containing 0.5% (v/v) 2-mercaptoethanol.
8. Protease inhibitor cocktail. Boehringer Mannheim's (Indianapolis, IN) Complete inhibitors work well for this purpose. The cocktail is usually prepared as a 25X stock solution in water.
9. PhosphorImager (Molecular Dynamics, Sunnyvale, CA, or Fuji, Medical Systems, Stamford, CT), with software for quantitation of protein bands.

2.4. Conformational Stability of Newly Synthesized Class I Molecules

1. Same reagents needed for metabolic labeling as indicated in **Subheading 2.3.**
2. Lysis buffer: 50 mM Tris-HCl, pH 7.3, 100 mM NaCl, 1 mM EDTA, and 2% Triton X-100.

3. Protease inhibitors (*see* **Subheading 2.3.**).
4. Glass fiber filters (Whatman, Clifton, NJ).
5. Trichloroacetic acid (TCA), 10% (w/v).
6. Synthetic peptides with sequences known to bind class I. Prepare a 10 mg/mL stock solution in DMSO, and store at less than –20°C.
7. Protein A- or protein G-agarose (50% suspension). Use 30–40 μL of the 50% suspension per sample.
8. Abs specific for conformationally sensitive epitopes in MHC-I molecules. These Abs fail to recognize class I molecules unfolded after incubation at 37°C. Some Abs that share this characteristic include MA2.1 (ATCC clone no. HB54, specific for HLA-A2 and -B17), B22 (H2-D^b), Y-3 (HB176, H2-K^b), 30–5–7 (HB31, H2-L^d), and 34-5-8S (HB102, H2-D^d).
9. Wash buffers for the immunoprecipitations *(35)*:
   a. 10 m*M* Tris-HCl, pH 7.4, 1 m*M* EDTA and 0.5% Nonidet P-40 (NTE).
   b. NTE containing 0.5 *M* NaCl.
   c. NTE containing 0.15 *M* NaCl and 0.1% SDS.
   d. 10 m*M* Tris-HCl, pH 7.4, and 0.1% Nonidet P-40.
10. PhosphorImager with analysis software.

2.5. Acid Stripping of Class I Molecules and Cytofluorographic Analysis

1. 300 m*M* Glycine, pH 2.5, containing 1% BSA.
2. Brefeldin A (BFA) stock, 25 mg/mL in methanol (BFA may be purchased least expensively from Sigma, St. Louis, MO).
3. Anticlass I mAbs for use in cytofluorographic analysis. They are available from different manufacturers, unlabeled or conjugated to fluorescein isothiocyanate (FITC), as well as other fluorophores.
4. If the anticlass I Ab is not directly labeled, a FITC-conjugated anti-immunoglobulin Ab, specific for the anticlass I Ab.
5. Ethidium homodimer (Molecular Probes, Eugene, OR). Stock solution in PBS–BSA (100 μg/mL).
6. Flow cytometer.
7. The materials shown would be the same in the case of rVV-expressed class I Ags, but adding those needed for VV infection, and that are included in **Subheading 2.3.**

2.6. Effect of Inhibitors on Presentation of Defined Determinants

2.6.1. Infection of Target Cells with VV

1. All reagents needed for vaccinia virus infection, as described in **Subheading 2.3.**
2. rVV viruses expressing the proteins of interest. Controls, including a wild type VV, or an irrelevant rVV, would be included as well.
3. BFA stock (25 mg/mL) in methanol.
4. Iscove's modified DMEM containing FCS (7.5%).
5. Na^{51}CrO$_4$, 10 mCi/mL.

6. Peptide-specific CTLs.
7. γ-counter.

2.6.2. Osmotic Loading of Substrates

1. Hypertonic medium: RPMI containing 0.5 *M* sucrose, 10% polyethylene glycol 1000 and 10 m*M* HEPES, pH 7.2. Prepare fresh, and warm to 37°C before adding to the cells. The medium should also contain the substrate protein at high concentration (on the order of 20 mg/mL).
2. Hypotonic medium: 60% RPMI in water. As before, warm it to 37°C before adding.
3. Other reagents as in **Subheading 2.6.1.**

3. Methods

3.1. Determination of Effective Inhibitor Concentration

The method given is based on the reduced half life of the chimeric protein, UbRNP, with an Arg residue between ubiquitin and the NP from influenza virus. The protein, however, is completely stable in the presence of proteasome inhibitors. As a control, the full-length NP or the stable UbMNP, with a stabilizing Met, instead of Arg, can be used. All forms are expressed as rVV (*see* **Note 1**).

1. Wash the cells with PBS–BSA (318g in a benchtop centrifuge), and resuspend them at 10^7 cells/mL in the same buffer now containing the rVV-expressing UbRNP, at a multiplicity of infection (MOI) of 10 PFU/cell. The high cell concentration enables efficient virus adsorption.
2. After 1 h at 37°C, mixing every 10–15 min, add medium to reach a final cell density of 10^6 cells/mL. Incubate at 37°C for another 30 min.
3. Wash the cells with warm PBS buffer, and resuspend them in Met-free medium, containing the desired concentration of the inhibitor (*see* **Note 2**), at a density of 5 x 10^6 cells/mL. Incubate 20 min at 37°C (*see* **Note 3**).
4. Wash the cells, and resuspend them again in Met-free medium, containing the appropriate amount of inhibitor (final cell density of 10^7 cells/mL). Add 10–20 μCi of ^{35}S-Met per 2 x 10^6 cells. Incubate for 1 min at 37°C (*see* **Note 4**).
5. Add ice-cold PBS–Met. Wash the cells, and make aliquots of 2×10^6 each.
6. Separate one aliquot for time 0, and lyse, as described in **step 7**, or freeze immediately on dry ice. The remaining aliquots are resuspended in 1 mL of I/Met, containing the corresponding inhibitors, and incubated for different times before being lysed. For UbRNP, chase times of 10, 30, 60, and 120 min are sufficient to estimate the half-life of the protein.
7. Lyse the cells with 100 μL boiling sample buffer of SDS-PAGE, containing protease inhibitors, and boil for 5 min (*see* **Note 5**).
8. Separate the proteins in a 9% SDS-PAGE gel. Fixed and dried gels are exposed to a PhosphorImager screen for an appropriate time, and imaged using the PhosphorImager. For normalization of samples, one of the metabolically stable

VV proteins, which may be seen in the scanned image, can be used as an internal standard in the different chase times.

3.2. Effect of Proteasome Inhibitors on MHC-I Ag Presentation

3.2.1. Conformational Stability of Newly Synthesized Class I Molecules

The method described takes advantage of the conformational instability, detected by mAbs, of empty class I molecules. An alternative method is the analysis of the transport of newly synthesized class I molecules from the ER to the Golgi, which takes place only after peptide binding. It can be detected by resistance of the carbohydrate groups in the class I molecules to digestion by endoglycosydase H, which is acquired in the Golgi.

1. Incubate the cells for 30 min at 37°C in Met-free medium, with the appropriate concentration(s) of inhibitors (*see* **Note 6**). Use 2×10^6 cells/immunoprecipitation.
2. Pellet the cells, and resuspend them at 10^7 cells/mL in the Met-free medium. Add 200 µCi of ^{35}S-Met/2×10^6 cells, and incubate for 15 min at 37°C.
3. Add an excess of ice-cold PBS–Met, wash the cells once, and make the appropriate number of 2×10^6 cell aliquots.
4. Save one aliquot for time = 0, and resuspend the rest in 1 mL I/Met, containing the corresponding inhibitor. Incubate them at 37°C for the desired intervals.
5. Pellet the cells, and lyse them on ice in 100 µL of lysis buffer, for 30 min at 0°C.
6. Pellet the nuclei by spinning the cells at 15,000*g* at 4°C for 15 min. Harvest the supernatant.
7. Estimate the amount of radioactivity incorporated in the different samples by spotting 5 µL of each sample, in triplicate, on glass-fiber filters. Wash filters with 10% TFA (w/v), dry, and place in appropriate vials with biodegradable scintillation fluid, and count the filters in a β-counter.
8. Adjust the volumes of each sample used for immunoprecipitation, so that each sample contains the same amount of incorporated ^{35}S-Met. Prepare two aliquots with each sample.
9. To one of the aliquots, add a class I-binding synthetic peptide (final concentration 5 µg/mL). Incubate all the samples, with or without peptide, at 37°C for 2 h, then incubate the extracts on ice.
10. Load control and class I-specific Abs to protein A/G-agarose by rotating the beads (30–40 µL/sample) with the Ab preparations (~20 µg Ab/sample) for 1 h at 4°C. Wash the beads with PBS. Resuspend in PBS containing 10% lysis buffer (to make approx a 50% slurry).
11. First incubate extracts with beads coupled to the irrelevant Ab, in a shaker, for 2 h at 4°C. This step will clear the lysates from proteins that bind nonspecifically to the Ab-coupled beads. After pelleting, transfer the supernatant to a new tube containing the beads coupled to the conformation-sensitive anti-class I Ab. Incubate for 2 h in a shaker at 4°C.

12. Pellet the beads and harvest (keep the supernatant for use with other anti-class I Abs). Wash the beads with 1 mL of each of the wash buffers, and boil in 100 μL of 2X sample buffer containing β-mercaptoethanol.
13. Separate the proteins by SDS-PAGE and expose dried gels to PhosphorImager screens and analyze with the PhosphorImager.

3.2.2. Cytofluorographic Analysis of Cell Surface Class I Molecules

3.2.2.1. ACID STRIPPING OF CLASS I-ASSOCIATED PEPTIDES

This method can be used for the analysis of the effects of proteasome inhibitors on the cell surface expression of MHC-I molecules that had been destroyed by acid stripping. It can be used, as well, for class I molecules expressed by rVVs. In this case, cells are infected with the recombinant viruses in the presence of the inhibitors, as in **steps 1** and **2** of **Subheading 3.1.** Cells are harvested for analysis at least 7 h after infection. The method described uses fluorescence-activated cell sorting, but a cytotoxicity (CTL) assay may also be performed in some circumstances.

1. Incubate cells with the inhibitor, as in **Subheading 3.2.1.** Include enough cells to have at least 5×10^5 cells per sample per Ab staining.
2. Pellet cells, and resuspend in 300 mM glycine, pH 2.5, containing 1% BSA (use 100 μL glycine buffer/2×10^6 cells).
3. Incubate for 3 min at 37°C, and neutralize immediately with a large excess of medium.
4. Pellet cells, and resuspend in culture medium containing the appropriate inhibitor. A control with BFA (5 μg/mL), a drug that blocks transport from the ER to the Golgi, and thus blocks class I cell surface expression should be included.
5. Incubate for 5–8 h at 37°C, rotating.
6. Pellet cells. The next steps should all be carried out on ice and in buffers containing 0.02% NaN$_3$, to prevent internalization of cell surface molecules (*see* **Note 7**). Wash cells in ice-cold PBS containing 0.2% BSA.
7. Stain for 1 h on ice with an Ab specific for the class I molecule of interest. A volume of 50 μL dilution is sufficient for as many as 1×10^6 cells. The Ab can be labeled with FITC, or unlabeled. Incubations should be performed in round bottom 96-well polystyrene plates. Pellet by allowing centrifuge to reach 650 g, then setting timer to 0. Remove liquid by a single hard flick, and tap plates hard to resuspend cells prior to addition of next reagent. Use 270 μL for washes.
8. If the first Ab is unlabeled, wash cells in PBS–BSA, and incubate with a FITC-labeled Ab, specific for the class I Ab used, and incubate for 1 h.
9. In both cases, i.e., whether using labeled or unlabeled anticlass I Ab, wash the cells with PBS–BSA and finally resuspend in 400 μL PBS–BSA containing 10 μg/mL ethidium homodimer (*see* **Note 8**). Using a multichannel pipetor transfer cells to 1 mL conical tubes arrayed in a 96-well format, and keep on ice.
10. Analyze the cells in a flow cytometer, gating out dead cells as identified by positive staining for ethidium homodimer. Conical tubes are inserted into the stan-

dard tube, and simply flicked out into the biohazard waste when the sample has been analyzed. Ten thousand events, or more, may be counted. The mean channel florescence values can be used to estimate the amount of class I expressed in the cell surface, with the background levels given by the cells incubated in the presence of BFA.

3.2.3. Effect of Inhibitors on Presentation of Defined Determinants

3.2.3.1. Infection of Target Cells with rVV

The method described here uses vaccinia as an expression vector, but it can be used with different viruses (*see* **Note 9**). BFA is added before ^{51}Cr labeling, in order to avoid exposing CTLs to proteasome inhibitors. BFA prevents the presentation of peptides generated after inhibitor removal.

1. Incubate the cells with the appropriate amount of the inhibitors (*see* **Subheading 3.2.1.**). Include 1.5 x 10^6 cells per final target cell group. As a negative control, an irrelevant rVV should be included, (*see* **Note 10**).
2. Wash the cells with PBS–BSA, and proceed with the infections as in **steps 1** and **2** of **Subheading 3.1.** The length of incubation after infection depends on the protein expressed by the rVV and the particular determinants studied. Usually, 3–5 h are required after the initial 1-h infection.
3. Before labeling, add BFA to a final concentration of 10 mg/mL, incubate 5–10 min at 37°C, and pellet the cells. BFA (5 µg/mL) will now be included in all the media used in washes, and during the CTL assay.
4. Pellet cells, leaving 20–50 µL medium, and add 100 µCi ^{51}Cr/1 × 10^6 cells. Incubate for 1 h at 37°C.
5. Add 5 mL culture medium and pellet cells. Aspirate as much media as possible, resuspend cells in warm medium (with BFA) and incubate for 10–15 min at 37°C. Pellet, and again scrupulously remove the supernatant. Finally, resuspend the labeled cells in medium, containing BFA, at a density of 10^5 cells/mL.
6. Add 100 µL/well (10^4 cells) of the cell suspension in round-bottom 96-well plates. These may contain different numbers of the peptide-specific CTL, in 100 µL medium, in order to have different effector-to-target ratios. The final volume in the wells should be 200 µL. Data points are obtained at least in triplicate.
7. Incubate at least 4–6 h at 37°C in a CO$_2$ incubator.
8. Harvest 100 µL supernatant, and count the released ^{51}Cr in a γ-counter. The spontaneous release is obtained from target cells incubated in the absence of CTL, and the total release, by incubation in Triton X-100 (final concentration 1% v/v). The specific release is calculated by the formula:

$$\frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Total release} - \text{Spontaneous release}} \times 100$$

3.2.3.2. Osmotic Loading of Substrates

The method is essentially identical to the one described in the previous Subheading, replacing the infection with the osmotic loading described below *(36)* (*see* **Note 11**). Care must be taken to control for the presence of antigenic peptides in the preparation.

1. Incubate cells with the appropriate amount of the inhibitors (*see* **Subheading 3.2.1.**).
2. Pellet 1.5 x 10^6 cells and resuspend in 500 µL warm hypertonic medium containing approx 20 mg/mL of the protein substrate. Incubate for 10 min at 37°C.
3. Add 14 mL warm hypotonic medium, and incubate for 3 min at 37°C.
4. Pellet cells, and proceed as in the previous subheading, adding BFA before labeling.

4. Notes

1. A method commonly used in the references relies on the accumulation of ubiquitinated proteins in the presence of proteasome inhibitors, because of their compromised degradation. These modified proteins appear as high-mol-wt smears in Western blots of gels from extracts of treated cells, developed with anti-ubiquitin. The caveat of this method is that it is not a quantitative approach, and it is difficult, if not impossible, to distinguish between partial inhibition of proteasomes and total inhibition.
2. The range of concentrations to test varies with the inhibitor and the cell type used, but is always within the µ*M* range. With lactacystin, a range of 5–100 µ*M* would be recommended, although concentrations as high as 500 µ*M* have been reported. In the case of zLLL, concentrations range from 0.5 to 50 µ*M* (concentrations in the higher range affect protein biosynthesis). AcLLnL has been used at concentrations from 2.5 to 250 µ*M*.
3. In vaccinia infected cells after 2–3h of infection at an MOI of 10, most of the endogenous gene expression has been shut off, and biosynthesized proteins are mostly restricted to early VV gene products. Because rVVs generally use the early–late 7.5K promoter, this enables detection of the NP band in the gels of total cell lysates relatively well separated from other proteins. This is relevant for nuclear proteins, such as flu NP, in which recovery after lysis in nonionic detergents is not complete, making quantitative immunoprecipitation impossible.
4. Longer labeling periods, in the absence of inhibitors, result in a considerable degradation of UbRNP during the labeling time (less than 60% left is sometimes observed after a 5-min pulse). If longer times of labeling are required, this should be taken into account. This is also relevant when UbMNP is used as a control, because some co-translational degradation of the protein is observed, presumably before removal of the ubiquitin moiety, whereas the final product is stable. Such an effect is not observed, however, with the wild type NP.
5. Often, the lysate is too viscous to handle easily. This can be solved by shearing the DNA with a probe sonicator, passing through a 23 G needle, or keeping the lysates overnight at 4°C.

6. The incubation time required to inactivate proteasomes varies with the inhibitor and cell type. A safe estimate would be at least 15 min with peptide aldehydes and 30 min with lactacystin (which must to be converted to the CLβL form, which represents both the cell-membrane-permeable and active form *(37)*. For example, with HeLa cells, we have seen that times as short as 5 min with 25 μ*M* zLLL before pulse are enough to protect UbRNP from proteasomal degradation. For 100 μ*M* lactacystin, however, more than 15 min were required.

7. The method described here uses live, unfixed cells for flow cytometry analysis. Staining with ethidium homodimer, which stains the nuclei of dead cells, allows gating of live cells. The method can be used with fixed cells, as well, and gating can be performed by incubating with ethidium homodimer prior to the fixation step (cells must be washed thoroughly, to prevent carryover of the dye and postvital staining).

8. VV infection is associated with an increase in staining of live cells, with up to a 10-fold increase in ethidium homodimer or propidium iodide. The cells are still distinguished from nonviable cells, which are at least 10-fold brighter.

9. Flu infections can be very useful here, because the class I-restricted response is well characterized for many alleles. In the particular case of influenza, the infection (20 hemagglutinating U/cell) should be carried out in AIM (MEM, Gibco-BRL, Rockville, MD, 0.1% BSA, and 20 m*M* HEPES, pH 6.8), instead of PBS–BSA. It is essential to wash cells to remove FCS, which blocks viral adsorption.

10. It is advisable, whenever feasible, to control for the effect of the inhibitors on gene expression (vaccinia or any other gene, viral or not). A seemingly ideal tool are rVVs expressing minigenes that code for the presented peptide. However, the number of peptide–MHC complexes this generates far exceeds the sensitivity threshold of the CTL. Therefore, variations in the amount of complexes in the cell surface may not be detected, a limitation that is often overlooked. The use of Abs specific for MHC–peptide complexes, or extraction and quantitation of the presented peptides, can solve this problem. The effect on gene expression can also be tested by FACS analysis of cell surface expression of virus-encoded proteins. In influenza virus infections, for example, cell surface expression of neuraminidase was mostly reduced, compared to nontreated controls, when cells were treated overnight with 10 μ*M* lactacystin, and could not even be detected when cells were treated with 100 μ*M* lactacystin.

11. An alternative is electroporation of the protein into the cell. Exogenous loading suffers from the difficulty of obtaining abundant and consistently pure substrate protein. Ovalbumin, commercially available, has been successfully used by different groups in this kind of experiment, but one should be cautious as to whether the results can be extrapolated to other substrates, particularly endogenous Ags. In this sense, viral infections offer much more flexibility.

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3

Tracing the Route Taken by Peptides and Major Histocompatibility Complex Class I Molecules in Presentation of Exogenous Antigens

Christopher C. Norbury

1. Introduction

Since the discovery of cross priming by Bevan *(1)* nearly thirty years ago, a large amount of work has focused on defining the mechanisms that account for this in vivo phenomenon. Following the discovery that the majority of major histocompatibility complex (MHC-I)-bound peptides are derived from endogenous (intracellular) sources *(2,3)*, a paradigm was established that exogenous (extracellular) antigens (Ags) are presented on MHC-I molecules and endogenous Ags are presented on MHC-II. In more recent years, accumulating evidence using a number of model systems, including presentation of bacterial *(4,5)*, particulate *(6)* and soluble *(7,8)* Ag, has challenged that paradigm.

With the evidence that this paradigm may not be correct has come the realization that presentation of exogenous Ag on MHC-I may be required for the generation of a productive cytotoxic T-lymphocyte (CTL) response to tumors, bacteria, and viruses. In all of these cases, Ag may not be expressed endogenously by the professional Ag-presenting cells (APC, e.g., dendritic cells or activated macrophages), which are almost certainly required to prime a productive CD8^{+} T-cell response. Thus the study of presentation of exogenous Ag on class I, both in vitro and in vivo *(9)*, has assumed much greater significance.

In vitro investigation of exogenous processing has resulted in the description of at least two major routes of Ag trafficking for exogenous presentation. The first involves a similar pathway to that for the presentation of the majority of MHC-II-restricted determinants. Ag is internalized and degraded in endosomes, before loading MHC-I molecules already present in the endocytic

pathway. Although this pathway may resemble the class II pathway closely, the exact details of Ag and MHC-I trafficking are relatively uninvestigated. Ag may be degraded endosomally, and peptides may bind class I molecules within the endosomes, or may be regurgitated and bind to cell-surface class I molecules. If the peptide does bind MHC-I molecules within endosomes, the class I molecules may be trafficked directly to the endosomal compartment, or may recycle from the cell surface to this site. In contrast, a novel pathway that involves transfer of endosomal Ag into the cytosol of APC appears to be more straightforward. The mechanism of release from an endosomal compartment to the cytosol is unknown, but, once in the cytosol Ag appears to be treated in a manner similar to endogenous Ags. Proteasomal degradation, transport into the endoplasmic reticulum (ER) via transporter of antigen presentation (TAP), and new synthesis of class I molecules are required.

A great deal of further investigation is required in the area of exogenous presentation. The possibility that exogenous Ags can prime a CD8^{+} T-cell response allows the design of a large number of vaccine strategies. Priming with DNA or nonreplicating vaccine vectors may exploit presentation of exogenous Ags on MHC-I, to allow generation of a CD8^{+} T-cell response. To date, the majority of the studies performed have examined presentation of a single determinant (Ova 257-264 SIINFEKL) derived from chicken ovalbumin. Whether the rules defined for presentation of this Ag are applicable to presentation of other Ags remains to be seen.

1.1. Readouts

The approach that can be taken to study Ag trafficking is severely limited by the readout assay being used. This will be determined by the availability of T-cells recognizing peptide derived from the Ag being studied. Although detection of peptide on MHC complexes on the cell surface may be possible with a specific Ab, because a few such antibodies (T-Ags) *(10,11)* do exist, but the availability of an antibody recognizing the peptide of choice is unlikely. In addition, the nature of exogenous presentation is such that Ag loaded into a cell at low concentrations is competing with Ags being synthesized by the cell. Thus, only low concentrations of peptide–MHC complexes, which may be beneath the detection level of the T-Ags, are likely to be present on the cell surface. However, outlined below are three possible readouts that may be used in an experimental system examining presentation of exogenous Ags on MHC-I. In each of the outlines, the term Ag is used to describe intact protein that does not bind to MHC-I, rather than the minimal determinant peptide or a longer derivative that may bind directly to MHC-I.

1.1.1. Restimulation of Previously Primed Cells

APCs are pulsed with intact Ag, then incubated with spleen cells (or blood cells in humans) from previously primed donors *(11,12)*. APC are pulsed with

Ag (*see* **Note 1**), then incubated with the donor cells for 5–6 d, before assaying in a chromium (^{51}Cr) release assay for activity against peptide pulsed or infected targets. Restimulation of previously primed cells will occur with low levels of peptide–MHC complexes on the surface of the APC, and also when only a small proportion of the APC population are presenting Ag. However, the system is difficult to manipulate, especially regarding to the use of inhibitors, because of the long time periods involved.

1.1.2. Direct Pulsing of APCs with Ag and Use as Targets in a ^{51}Cr-Release Assay

APCs are labeled with ^{51}Cr, then pulsed with Ag, before exposure to previously activated cytotoxic killer cells. The major disadvantage with this method is that only a portion of the APCs may present exogenous Ag. Because the readout in a ^{51}Cr release assay is percentage of maximum lysis, a low to negligible readout may result. Use of an established cell line or clone, however, does add additional sensitivity to low numbers of peptide–MHC complexes on the cell surface.

1.1.3. Exposure of T-Cell Hybridoma to Ag-Pulsed APCs

APCs are pulsed with Ag, then exposed to a T-cell hybridoma, which may provide an indirect assay of interleukin 2 production *(13)* or a direct assay of T-cell activation, if the hybridoma carries the *lacZ* gene under the control of the interleukin 2 promoter *(14,15)*. The *lacZ* hybridomas have the added advantage that activation can be assessed on a single-cell basis, allowing recognition of rare events within an APC population *(8)*.

1.2. Strategies

Once a suitable readout system has been established, one may proceed with investigating the trafficking of Ag and MHC-I molecules. Initially, it is imperative to rule out peptide contamination in the Ag preparation. Failure to do so will leave the possibility that peptide contaminants in the Ag preparation are binding to MHC-I directly, either at the cell surface or at another site.

Once peptide contamination has been ruled out, a number of approaches can be used to study uptake of the Ag into the APC. This chapter focuses on one approach in which Ag is conjugated to a fluorophore (fluorescein isothiocyanate, [FITC]), followed by fluorimetric quantitation of Ag uptake. At this point, inhibitors of phagocytosis (cytochalasin B and D) and of fluid phase endocytosis (amiloride and amiloride analogs), as well as blockade of receptor-mediated endocytosis with an excess of unlabeled Ag, can reveal the mechanism of Ag uptake.

Once the mechanism of Ag uptake has been established, the time course of presentation, along with the sensitivity of presentation to various inhibitors

Table 1
Properties of Inhibitors

Inhibitor	Mode of action	Inhibits
Cytochalasin D	Prevents actin polymerization	Phagocytosis (plus a minor component of macropinocytosis in some cells)
Amiloride	Blocks the Na+/H+ proton pump	Blocks fluid phase uptake
DMA	Blocks the Na+/H+ proton pump	Blocks fluid phase uptake
Chloroquine	Prevents endosomal acidification	Blocks endosomal protease action
Leupeptin	Peptide aldehyde that binds to endosomal thiol proteinases	Blocks endosomal proteolysis mediated by some proteinases
Ammonium chloride	Prevents endosomal acidification	Blocks endosomal protease action
zLLL	Tripeptide aldehyde that binds reversibly to the proteasome in the active site	Blocks the degradation of cytosolic proteins by the proteasome
Lactacystin	Binds covalently to proteasome	Blocks the degradation of cytosolic proteins by the proteasome
Brefeldin A	Blocks coat formation on intracellular vesicles, collapsing the Golgi complex back into the ER.	Prevents transport of ER resident MHC-I molecules (and other proteins) to the cells surface

(*see* **Table 1**), can give an indication of the trafficking of both Ag and MHC-I molecules prior to presentation at the cell surface. Ag introduced into the cytosol by electroporation *(16)* or osmotic lysis of pinosomes *(17)* can be degraded and small numbers of peptides presented in complex with MHC-I on the cell surface in less than 1 h. However, maximal presentation may take as long as 5 h (**8**). In contrast, presentation of exogenous Ags via an endosomal route may reach maximal presentation in less than 1 h *(4)*. Sensitivity of presentation to the proteasome inhibitors (carbobenzoxy-leucyl-leucyl-leucinal [zLLL], or lactacystin) indicates a requirement for cytosolic processing, and thus endosome-to-cytosol transport prior to presentation. However, sensitivity to inhibitors of endosomal proteases (chloroquine, leupeptin, and ammonium chloride [NH$_4$Cl]) may indicate a requirement for endosomal processing.

Although the inhibitors represent a powerful tool in the study of processing and presentation of exogenous Ags on MHC-I, their effects can easily be misinterpreted. The methods outlined will provide a strong indication of the pathway taken by Ag and of the site of interaction with MHC-I. When reaching a conclusion, however, the specificity of the inhibitors within the assay used must be questioned. In addition to its published effects, an inhibitor may also affect cell viability, endocytosis of the Ag, Ag degradation, or MHC-I trafficking. As a result, it is imperative to perform adequate controls when using inhibitors, and only to draw conclusions from the consensus of results obtained from inhibitor studies.

2. Materials

2.1. Inhibitor Stocks

Inhibitor stocks should be prepared as outlined in **Table 2**. Dimethyl sulfoxide (DMSO) and EtOH can affect the results (particularly in the internalization assays), if these solvents are included at intolerably high concentrations. Thus, equal amounts of solvent should be included as controls in all experiments.

2.2. Cell Lines

No cell line is specifically recommended for any of the methods described. Indeed, presentation of exogenous Ags may occur only in some of the cell types tested, depending on specific mechanisms of endocytosis, intracellular trafficking, or proteolysis for presentation to occur. Tissue culture cell lines, such as P815, EL4, RMA, or T2 cells, are commonly used (*see* **Note 2**), but, for greater relevance to in vivo studies, some groups have studied presentation in ex vivo APCs such as macrophages *(8)* or dendritic cells *(7)*, or in cell lines directly derived from these ex vivo cells *(6,18)*.

2.3. Ruling Out Peptide Contamination

2.3.1 Pulsing Fixed Cells with Ag

1. Freshly prepared solution of 1% paraformaldehyde, pH 7.2–7.4.
2. Phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) (PBS–BSA).
3. 0.2 *M* glycine in PBS.
4. Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS).
5. Titrated amounts of Ag peptide, representing the minimal Ag determinant of the Ag studied, from 10^{-6} *M* to 10^{-14} *M* in DMEM, 10% FCS (*see* **Note 3**).
6. T-cell readouts as discussed (*see* **Notes 4, 5** and **6**).

Table 2
Use of Inhibitors

Inhibitor	Stock concentration	Working concentration	Preincubation time
Cytochalasin D	1 mM in DMSO	10 µM	45 min on ice
Amiloride	600 mM in DMSO	3 mM	10 min
DMA	300 mM in DMSO	100 µM	10 min
Chloroquine	10 mM in medium	100 µM	Add during Ag pulse
Leupeptin	1 mM in medium	15 µM	Add during Ag pulse
Ammonium chloride	1 M in medium	5–20 mM	Add during Ag pulse
zLLL	40 mM in DMSO	5–10 µM	Add following Ag pulse
Lactacystin	40 mM in DMSO		Add following Ag pulse
Brefeldin A	5 mg/mL in EtOH	5 µg/mL	Add following Ag pulse

2.3.2. Assessing Requirement for Exogenous β$_2$-Microglobulin

1. Equal amounts of DMEM + 10% FCS and DMEM lacking 10% FCS.
2. Titrated amounts of Ag peptide, representing the minimal Ag determinant of the Ag studied, from 10^{-6} M to 10^{-14} M in DMEM, 10% FCS.
3. T-cell readouts, as discussed.

2.4. Internalization Assays

2.4.1. FITC Labeling of Ag

1. Dialysis tubing.
2. 0.1 M Na$_2$CO$_3$/NaHCO$_3$ buffer, pH 9.2.
3. 5 (and 6)-carboxyfluorescein, succinimidyl ester, mixed isomers, dissolved in DMSO.
4. Stock solution of 500 mM NH$_4$Cl.
5. PBS, pH 7.4.
6. Biogel P-10 column.

2.4.2. Fluorimetric Quantitation of Ag Uptake

1. 0.22 µm filter.
2. 2X concentrated Ag solution in PBS/BSA (1 mg/mL), preheated to 37ºC.
3. Large excess volume (minimum 1 L) ice-cold BSS–BSA.

4. 0.1% Triton X-100 solution in BSS–BSA.
5. Microcentrifuge.
6. Spectrofluorometer.

2.5. Localization of Ag-MHC Interaction

2.5.1. Time-course of Presentation

1. Essentially the same as for **Subheading 2.3.1.**

2.5.2. Use of Inhibitors

1. Stock solutions of inhibitors as outlined in **Table 2**.

3. Methods

3.1. Ruling Out Peptide Contamination

Any study of the presentation of exogenous Ags on MHC-I must rule out peptide contamination in the Ag preparation. Despite attempts to remove peptide from Ag preparations by gel filtration, centrifugation, or other methods, it is possible that peptide contamination that is undetectable biochemically may still be present. Such peptide contamination will dramatically affect the results, and may be misleading.

3.1.1. Pulsing Fixed Cells with Ag

Cells lightly fixed with paraformaldehyde still express peptide-receptive MHC-I molecules at the cell surface, and are able to present peptide–MHC-I complexes to T-cells. Therefore, fixed cells represent an ideal way to screen for peptide contamination in the absence of cellular processes. In addition, over prolonged periods, this assay can also reveal the action of serum proteases in the generation of MHC-I binding peptides.

For protein Ags and inactivated infective agents (inactivated viruses, and so on):

1. Wash APC, aspirate medium, and add a small amount of freshly prepared 1% paraformaldehyde. Incubate for exactly 10 min at room temperature (RT) (*see* **Note 7**).
2. Add an excess of 0.2 *M* glycine in PBS to quench the fixation reaction. Incubate at RT for 20 min.
3. Wash extensively (5×) with PBS–BSA (*see* **Notes 8** and **9**).
4. Pulse fixed APC with a high dose of Ag for 6 h (or, minimally, for the length of time that Ag is pulsed onto live cells).
5. In parallel, pulse APC with titrated amounts of Ag peptide.
6. Wash extensively (5×) in BSS–BSA, then resuspend in regular culture medium, and expose to T-cells.

For inactivated infective agents (inactivated viruses, and so on) only:

1. Perform the assay as above, to rule out peptide contamination.
2. Pulse live APC with Ag, and, in parallel, pulse APC with titrated amounts of peptide.
3. Full inactivation of the agent must be established. Although assays specific for the live infective agent, such as viral fusion assays, may indicate that the agent is fully inactivated, activity may still remain that is sufficient to allow presentation of exogenous Ag. To rule this out, presentation of an alternative determinant from a nonstructural protein (structural proteins may be present in the Ag preparation) must be examined in a way similar to the Ag of interest.

3.1.2. Assessing Requirement for Exogenous β_2-Microglobulin

As outlined in **ref. *19*** presentation of exogenous peptide normally requires β_2-microglobulin (β_2m). Therefore, in addition to testing for the presence of exogenous peptide in the Ag preparation, it is also prudent to investigate the requirement for β_2m. For some intact Ags, however, β_2m is also an absolute requirement for exogenous presentation *(20)*. If presentation is independent of exogenous β_2m but, it can be concluded that peptide contamination is probably not a factor, whereas the reverse is not always true.

1. Harvest APC, and wash extensively (5×) in serum free medium (*see* **Note 10**).
2. Incubate APC either in medium containing fetal bovine serum, or in medium lacking serum, for 48 h prior to Ag pulsing (*see* **Note 11**).
3. Aliquot the cells grown in serum and the cells grown in the absence of serum into 10 tubes, with an equal number of cells in each.
4. In parallel, pulse eight tubes of the APC grown in serum or in the absence of serum with titrated (10^{-6}–$10^{-13}M$) amounts of the peptide, representing the minimal determinant of the Ag studied, for 30 min (*see* **Note 12**).
5. Pulse one of the remaining tubes in each group with intact Ag for the same time period. The remaining tube of cells is a negative control.
6. Following pulsing with peptide or Ag, wash extensively (5×) in BSS–BSA.
7. Resuspend in regular culture medium, and incubate with T-cells.

3.2. Internalization Assays

3.2.1. FITC Labeling of Ag

It is essential to demonstrate internalization of Ag before degradation and peptide loading of MHC-I. Studies with inhibitors may contribute evidence that Ag is internalized, but only measurement of uptake can provide clear details of the rate of endocytosis and of the effectiveness of inhibitors. To perform such analysis, it is necessary to label Ag with a marker enabling measurement of endocytosis. A number of possible ways of labeling proteins exist, such as iodination or biotinylation, but labeling with fluorophores, particularly FITC, is probably the easiest. In addition, the labeled protein can then also be examined microscopically, to give additional information about intracellular localization.

1. Prepare Ag solution (*see* **Note 13**) at a minimal concentration of at least 2 mg/mL.
2. Dialyze the Ag preparation overnight at 4°C against 0.1 M Na_2CO_3–$NaHCO_3$ buffer, pH 9.2.
3. Dissolve FITC slowly into DMSO, to give a final concentration of 1 mg/mL.
4. Add a 20-fold molar excess of FITC slowly to the Ag preparation, in 5 µL aliquots, while stirring in an ice bath.
5. Allow the conjugation reaction to proceed in the dark for 8 h at 4°C, while stirring continually.
6. Bring the Ag preparation to RT and add NH_4Cl to a final concentration of 50 mM. Allow the reaction to quench for 2 h at RT.
7. Pre-equilibrate a Biogel P-10 column to pH 7.4, by running PBS through it.
8. Pass the Ag conjugate through the Biogel P-10 column, and collect the conjugated Ag, free of unconjugated FITC.
9. Determine the protein concentration using a conventional Bradford assay.

3.2.2. Fluorimetric Quantitation of Ag Uptake

Although uptake of a fluorescently labeled Ag can be quantitated on a cytofluorograph, it is difficult to rule out cell-surface binding, and quantitation by lysis of the cells is more accurate. The inclusion of inhibitors of phagocytosis (cytochalasin D) or of fluid-phase endocytosis (amiloride or analogs), or an excess (10×) of unlabeled Ag (to block receptor-mediated endocytosis) may give information about the contribution of these various endocytic pathways to Ag uptake.

1. Pass the soluble Ag preparation through a 0.2 µm filter, to remove aggregates that may affect the mechanism of Ag uptake (*see* **Note 14**).
2. Incubate a known number (1×10^6 works well) APCs at 37ºC in a small quantity of BSS–BSA (*see* **Note 15**).
3. Add an equal volume of prewarmed 2X Ag solution, and incubate for various time periods (e.g., 10, 20, and 30 min at 37ºC), to allow measurement of the rate of endocytic uptake.
4. Add an excess (at least 10-fold) of ice-cold BSS–BSA, wash, and repeat.
5. Incubate cells on ice for 5 min, then wash once more with ice-cold BSS–BSA. Repeat this step 5×.
6. Aspirate the remaining BSS–BSA (*see* **Note 16**), and lyse cells in 0.5mL Triton X-100.
7. Remove crude membrane contamination from lysates by centrifugation at 10,000g for 2 min.
8. Fluorescence is read in a spectrofluorometer, exciting at 480–500 nm and measuring at 520–540 nm.

3.3. Localization of Ag–MHC Interaction

3.3.1. Time-Course of Presentation

The amount of time it takes for an Ag to be endocytosed, processed, and presented as a peptide in complex with MHC-I will give a strong indication of

the route of processing. When an endosomal route of processing occurs prior to presentation, appreciable levels of peptide MHC complexes may be found on the cell surface 30–45 min after Ag administration *(4)*. In contrast, in studies in which a cytosolic route of trafficking has been implicated, up to 6 h *(8)* may be required for maximal presentation.

1. Pulse APCs with Ag, then wash extensively (5×) to remove all Ag (*see* **Note 17**).
2. Chase cells for various time periods in regular culture medium at 37ºC.
3. Prepare a fresh solution of 1% paraformaldehyde in PBS (pH 7.2–7.4).
4. Add fixative to cells for 10 min, then wash thoroughly (minimum 3×)
5. Prepare a solution of 0.2 *M* glycine in PBS, and add in excess to the fixed cells. Allow to incubate at RT for 10 min.
6. Wash a minimum of 3× with an excess of PBS.
7. Resuspend in regular culture medium, and incubate with responding T-cells.

3.3.2. Use of Inhibitors

Inhibitors are a useful tool that can give an indication of the trafficking pathways of both MHC-I and Ag during presentation of exogenous Ags. A list of inhibitors (by no means complete) that are commonly used to investigate the mechanism of class I presentation of exogenous Ags, along with short descriptions of their action, can be found in **Table 1** (*see* **Notes 18–26**).

For instance, inhibition by the proteasome inhibitors zLLL or lactacystin implies trafficking of exogenous protein through the cytosol prior to presentation, and sensitivity to NH_4Cl or leupeptin implies trafficking through the cytosol. However, the results from the use of any one inhibitor, or group of inhibitors, cannot, in itself, implicate a particular route of trafficking. Rather, the consensus between the effects of inhibitors affecting widely differing processes is strongly suggestive of cytosolic or endosomal trafficking.

Concentrations of stock and working solutions of inhibitors, and the lengths of time required for inhibitor efficacy, are shown in **Table 2**.

4. Notes

1. It is essential that the time of Ag pulsing is kept to a minimum. Prolonged pulsing with Ag may add variables to the results, including serum protease action on the Ag or peptide regurgitation, and prevents an accurate time-course from being established. In addition, the use of peptide-pulsed or infected controls must be performed with similar APC, because the effects of different trafficking of MHC-I and Ag in different cells types has not been thoroughly examined, and may provide an additional variable to the results seen.
2. Cells other than primary cells, such as RMA-S (in the murine system) or T2 (in the human system), are commonly used to address the role of TAP in presentation of exogenous Ag. It is important to use the appropriate parental cell line

(RMA or .174, respectively) for comparison, rather than an unrelated cell type. Both RMA and .174 express the TAP molecules that RMA-S and T2 lack, but they also express additional proteins. To definitively state that the lack of TAP accounts for a lack of presentation of exogenous Ag, TAP must be transfected, or otherwise expressed, in cells originally lacking it, and a restoration of presentation demonstrated.

3. For contamination to be ruled out, it is essential that the peptide representing the minimal T-cell determinant is known, to allow direct comparison between intact and peptide Ag. When the readout is cytotoxicity, a half maximal killing would be expected to be achieved by a maximum of 10^{-10} M peptide, and often as low as 10^{-12} M.

4. When using peripheral blood lymphocytes from human donors the repertoire of Ags that can be examined is limited to Ags that the donors may have been exposed to naturally, such as influenza, or to which the donors have been immunized, e.g., hepatitis B surface Ag. In all cases, it must be established that the cells from the donors respond to peptide-pulsed APC in every experiment.

5. In all Ag-presenting assays of this type, controls must be included for the sensitivity of the CTLs. This can be achieved most readily by including a dose response of the cytotoxic cells to titrated peptide doses pulsed onto the surface of APC. In the absence of such sensitivity tests negative results or changes in results, because of inhibitor usage, may be interpreted incorrectly.

6. It is unlikely that a hybridoma will be as sensitive as a CTL line or clone. Results comparing the two different readouts must take account of this observation. Hybridomas are notorious for undergoing a decrease in sensitivity following overgrowth in tissue culture. Thus, the sensitivity of the hybridoma to a titered peptide, pulsed onto the surface of an APC population, must be established regularly, if not in every experiment.

7. Cells fixed at different time-points should be in separate tubes or dishes. Fixation of cells in a single well of a multi-well plate will allow diffusion of paraformaldehyde fumes from one well to the next during the 10-min time period, allowing fixation of cells in neighboring wells, and giving false results.

8. Failure to wash away the 0.2 M glycine will result in the death of responding T-cells during extended culture.

9. Any dramatic change in the pH of the medium will indicate that either the fixative or the quenching agent may not have been washed away thoroughly.

10. Washing in serum free medium should be performed in medium known not to contain β_2m, or any protein that may contain β_2m. For instance, crude BSA may contain relatively large amounts of β_2m, and so should not be included in these washes, or during the 48-h incubation in serum free medium.

11. Incubation of cells for 48 h in serum-free medium is required to deplete the cells of the majority of exogenous β_2m. Shorter term incubations are possible if the cells are very sensitive to the lack of fetal bovine serum.

12. The inclusion of a peptide titration is to ensure that β_2m has been depleted from the medium. Under such conditions, the efficiency of peptide pulsing should be

greatly reduced, or even ablated. The efficiency of β_2m depletion can be determined by the effects on the efficiency of peptide pulsing.

13. This protocol assumes that the Ag is soluble, and can be passed through a Biogel P-10 column. If the Ag is particulate, it can be washed extensively to rid the Ag preparation of free, unconjugated FITC.

14. If the Ag used is particulate, but will still pass through a 0.2-μm filter (e.g., an inactivated virus), the preparation should be filtered to ensure that no large aggregates are present. Otherwise, the filtering step should be excluded.

15. When possible, this protocol should be performed with cells in an adherent state. Using cells adhered to glass cover slips allows washing in large volumes at regular intervals to be accomplished more easily than using cells in suspension. It is possible to adhere cells that are normally nonadherent to glass or plastic, by first coating the cover slips with concanavalin A or poly-L-lysine.

16. Important information may also be obtained by examining cells, using a fluorescence microscope, after uptake of FITC-labeled Ag. The morphology of intracellular vesicles containing Ag may provide evidence of distinct endocytic pathways involved in Ag uptake. In addition, co-localization with other fluorescently labeled endocytosed proteins, or endocytic markers, may provide clues about intracellular Ag trafficking.

17. The method used to ascertain the time-course of cell surface presentation following Ag pulsing will depend exclusively on the determinant being studied. The presentation of some determinants will be affected less by fixation than others, and quenching of an Ag signal by fixation will necessitate the use of alternative methods. The method described is the minimal fixation possible, and allows the use of fixed cells as targets in a CTL assay *(21)*. Other possible methods of fixation include a 30-s period in 0.05% glutaraldehyde, or 30 min in 1% paraformaldehyde, both of which allow effective recognition of the Ova 257–264 determinant in complex with H2-K^b by a T-cell hybridoma, following fixation *(13)*. If the determinant used is fixation-sensitive, the sensitivity of presentation to BFA may be utilized, with BFA added at various time points after addition of Ag to produce a time course.

18. When cytochalasin is used, its efficacy must be established in the absence of any effect on Ag presentation. This can be accomplished by staining APCs with Rhodamine phalloidin (Rh-Ph), which will bind to the filamentous form of actin, stabilizing it, and preventing its disassembly. To stain actin filaments within cells (filaments should not be visible after cytochalasin treatment) stain APC in serum-free medium with 5 µg/mL Rh-Ph (diluted from stock solution of 50 µg/mL in EtOH) for 30 min, wash extensively and examine by fluorescence microscopy. In some studies, cytochalasin B, which is less specific in its effect on actin polymerization, has been used, instead of the preferable cytochalasin D.

19. Inhibitors of fluid-phase endocytosis can inhibit receptor-mediated endocytosis at higher concentrations, and are toxic at some concentrations. All presentation experiments must be conducted with these facts in mind. Extended time periods of incubation with amiloride or DMA *(8)* can dramatically affect cell viability. In

addition, only a correlation between an inhibitor-mediated effect on Ag uptake, and an effect on Ag presentation, can be taken as evidence that fluid phase endocytosis is a requirement for Ag presentation.

20. Chloroquine *(22)* is a lysosomotropic amine that prevents acidification of endosomal compartments, decreasing the efficiency of endosomal proteolysis. However, in addition to these effects, chloroquine also dramatically affects intracellular trafficking, and may deleteriously alter MHC-I processing and presentation. Chloroquine is often used as a reagent in transfection protocols, suggesting that it may increase the efficiency of transport from endosomal compartments into the cytosol. Thus, instead of an inhibitory affect, chloroquine may enhance Ag presentation *(8)*. In the absence of any effect on class I presentation, the efficacy of the chloroquine preparation must be established in parallel, by demonstrating inhibition of MHC-II processing.

21. Leupeptin *(23)* is a specific inhibitor of some endosomal proteases, particularly cathepsin S, which has a prominent role in MHC-II processing and presentation. In the absence of any effect, the efficacy of the leupeptin preparation can be established by demonstrating a leupeptin-mediated effect on the formation of sodium dodecyl sulfate stable MHC-II dimers in treated cells, or by demonstrating inhibition of MHC-II processing.

22. NH_4Cl *(22)* is a lysosomotropic amine similar in action to chloroquine. However, the nonspecific effects associated with chloroquine have not been described when NH_4Cl is used. In the absence of any effect on class I presentation, the efficacy of the NH_4Cl preparation must be established in parallel, by demonstrating inhibition of MHC-II processing.

23. When using both proteasome inhibitors, it should be noted that the generation of some peptide determinants for presentation on MHC-I has been demonstrated to be proteasome-independent *(24)*, and may be decreased by proteasome activity. Because of this observation, the role of the proteasome in the generation of the determinant from endogenous Ag should be ascertained before examining the role of the proteasome in presentation of exogenous Ag.

24. zLLL (also known as MG132) is an inhibitor of the majority of the proteasome functions *(25)*. However, it is also an inhibitor of other cellular proteases such as calpain, and effects on protein synthesis have been described at higher concentrations. As such, it is important to compare the effects of zLLL with N-acetyl-leucyl-leucyl-methioninal (calpain inhibitor II) which has similar activity against other cellular proteases, but minimal activity against the proteasome. It is important to ascertain the lowest effective inhibitor concentration before use of this reagent. In addition, the effects of zLLL on the proteasome are reversible, so a control, in which zLLL is removed and proteasome activity allowed to recover, should be included.

25. Lactacystin is the most specific inhibitor of the proteasome yet described, and inhibits the chymotryptic activity irreversibly *(26)*. It is important to ascertain the lowest effective inhibitor concentration to use in the cell type being studied; undesirable effects of proteasome inhibitors have been demonstrated, such as

reduction of free ubiquitin pools, induction of stress response, interference with the cell cycle, and even, following extended incubation periods, induction of apoptosis.

26. BFA is a fungal metabolite that prevents intracellular trafficking of membrane and secreted proteins out of the secretory pathway. It prevents trafficking of newly synthesized MHC-I molecules from reaching the cell surface *(27)*. However, BFA may also reduce the number of peptide-receptive class I molecules on the cell surface, and it has been demonstrated to inhibit transport of peptide-loaded MHC-II complexes from endosomes to the cell surface *(28)*. Thus, it is important to use BFA for the minimal time period necessary, and even then BFA sensitivity should not necessarily be equated with a requirement for peptide loading in the ER. In the absence of any BFA sensitivity, the efficacy of the BFA preparation should be demonstrated in parallel, by showing an effect on presentation of cytosolic Ag, either virally derived or loaded by electroporation or osmotic lysis of pinosomes. Because BFA effects are reversible, a control, in which BFA is removed and intracellular transport allowed to recover, should be included.

Acknowledgments

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4

Functional Analysis of Antigen Processing and Major Histocompatibility Complex Class II-Restricted Presentation

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1. Introduction

Class II major histocompatibility complex (MHC-II) antigens (Ags) are integral membrane glycoproteins that have the unique capacity to bind preprocessed peptides, and present them to the immune system. These peptides are derived through a complex series of intracellular processing events that diverge among the plentitude of Ags presented in the context of MHC-II. The trimeric complex, consisting of peptide and class II α- and β-chains, is recognized by epitope specific CD4^{+} T-cells. This fine specificity exhibited by the T-cell receptor is fundamental to the induction of tolerance and pathogen clearance. Therefore, delineating the mechanisms by which certain epitopes are generated and presented to CD4^{+} T-cells may be useful in the development of immunotherapies for solid tumors and autoimmune diseases, and more efficacious vaccines.

1.1. Ag-Presenting Cells

Although all nucleated cells present MHC-I surface Ags, only select cell types express surface MHC-II under physiologically normal conditions *(1)*. These specialized cells, referred to as professional antigen-presenting cells (pAPCs), include B-cells, macrophages, dendritic cells, and thymic epithelia. Within pAPCs, the MHC-II encoded α- and β-polypeptides are assembled in the lumen of the endoplasmic reticulum, in association with the non-MHC-encoded chaperone molecule invariant chain, Ii. The Ii serves two functions, in that it shields the peptide binding groove formed by the nascent $\alpha\beta$ heterodimer, and targets the complex through the Golgi to the endocytic system

(1,2). Predominantly, these αβIi complexes transport directly to endosomes, although a subset of complexes may first transit to the plasma membrane prior to delivery into endosomes. Ii is cleaved by acid proteases found within late endosomes and lysosomes, and once the Ii is cleaved and removed, the αβ heterodimer is liberated to bind peptide. In designing experiments to study Ag processing and MHC-II restricted presentation, the role of the Ii should be considered. This is especially true when using non-pAPC transfected to express class II *(3)*.

Ags, taken up by APC through endocytosis or phagocytosis, traverse the increasingly acidic vesicles of the endocytic system, where they are denatured and cleaved by resident proteases *(4–6)*. These acidic proteases, termed cathepsins include, but are not limited to the cysteine proteases, cathepsins B, H, L, and S, and the aspartyl proteases, cathepsins D and E. An MHC-II-rich vesicular structure, termed MIIC, has been identified as a peptide-loading compartment in pAPCs *(7)*. Whether this compartment is unique to class II-expressing APCs is not clear. Nonetheless, the MIIC has lysosome-like morphology, and shares endosomal/lysosomal markers, such as lyosomal associated membrane protein (LAMP) and proteases. Moreover, the MIIC is rich in the MHC-encoded molecule, human leukocyte antigen DM (HLA-DM), which has been shown to catalyze the removal of the Ii fragment, class II-associated invariant chain peptide (CLIP), and to facilitate the loading of Ag peptide *(8)*. In fact, in the absence of DM, the repertoire of peptides presented is biased toward αβ:CLIP. The majority of peptides are bound by class II in the MIIC, but peptide loading has also been shown to occur within clathrin-coated recycling endosomes *(7)*. The precise compartment for peptide loading seems to be epitope-dependent.

It is important to realize that the expression levels of MHC-II and endosomal proteases will vary with the type and activation state of the APC. Thus, it is rational to assume that the repertoire of peptides, and overall ability of an APC to stimulate CD4$^+$ T-cells, will vary as well. The cytokine interferon γ, for example, can induce the expression level and activity of cathepsin B in murine macrophages and B-lymphoblastoid cell lines *(9)*. However, under the same conditions, MHC-II will only be upregulated in the macrophages *(10)*. Furthermore, immature dendritic cells exhibit high levels of phagocytosis and Ag processing, with little surface expression of MHC-II. However, upon maturation, they display dramatically reduced levels of phagocytosis and Ag processing, and become extremely potent APCs, expressing high levels of surface class II *(5)*.

1.2. T-Cells

CD4$^+$ T-lymphocytes, often referred to as T-helper cells, express receptors that recognize specific peptide–αβ complexes presented on the surface of APC. This specific recognition provides the first step in developing tolerance or elic-

iting a protective immune response. In primary T-cells, this recognition is not sufficient for activation, because they require co-stimulation through additional surface molecules *(5)*. In fact, in the absence of this secondary signal, the responding T-cell may be induced to enter a state of nonreactive anergy or apoptosis. Therefore, in addition to peptide–$\alpha\beta$ complexes, an APC must also express co-stimulatory molecules, such as B-7 and CD40, which bind the T-cell surface Ags CD28 and CD40L, respectively, to induce activation of the T-cell. The necessity of co-stimulation must be considered in the study of class II-restricted Ag presentation to primary T-cells. Often, co-stimulation can be compensated for by the use of monoclonal antibodies to crosslink and activate CD28 or CD40L. Once activated, CD4$^+$ T-cells secrete cytokines that drive the immune response. Chief among these cytokines is interleukin 2 (IL-2) which can act in an autocrine fashion, to stimulate the CD4$^+$ T-cell, and in an endocrine fashion, to stimulate, for example, cytotoxic CD8$^+$ T-cells. In vitro, IL-2 provides an excellent measure of T-cell activation.

T-hybridoma cell lines have proven to be an invaluable tool for the study of class II-restricted presentation of specific Ag epitopes. These cells are constructed by fusing an Ag-specific CD4$^+$ murine T-cell with an immortalized cell, such as that derived from a thymoma. Following subcloning and screening, the resulting immortalized cells express a single T-cell receptor specific for a known peptide-$\alpha\beta$ complex *(11)*. A key advantage in the development of T-hybridoma cell lines is their expression of a T-cell receptor with known specificity and measurable production of cytokines, upon recognition of peptide–MHC-II complexes. In addition, but equally as important, the necessity for co-stimulation is often greatly reduced or abolished with these immortalized cells.

The methods discussed here are directed toward analysis of exogenous Ag processing and MHC-II-restricted presentation, using an in vitro T-cell stimulation assay. These methods employ the suspended APC and T-cell hybridoma lines. However, these protocols can easily be modified to incorporate the study of primary T-cells, as well as primary and/or adherent APCs.

2. Materials

1. Tissue culture media suitable for APC. T-hybridoma cell lines are generally grown in RPMI supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 μ/mL streptomycin, and 50 μM β-mercaptoethanol (T-cell media). Tissue culture flasks.
2. IL-2 dependent T-cell line, HT-2, maintained in T-cell media supplemented with 20% T-Stim (Collaborative Biomedical Products, Bedford, MA).
3. Concentrated stock solutions of Ag, synthetic peptide, and pharmacological reagents. Store in small aliquots at –20°C or –80°C.
4. 96-well u-bottom tissue culture plates and 24-well tissue culture plates.
5. 1% paraformaldehyde, pH 7.4, made by adding 1 g paraformaldehyde to 100 mL

phosphate-buffered saline (PBS) and warming to 56°C in a fume hood. It may take up to 60 min to completely dissolve, and should be filter sterilized (*see* **Note 1**).

6. Sterile Hank's balanced salt solution (HBSS) or PBS.
7. Sterile microcentrifuge tubes.
8. ^{3}[H]-Thymidine (Thy) diluted in T-cell media to 40 µCi/mL (or 1 µCi/25 µL).
9. Equipment to harvest the cells and quantitate incorporated ^{3}[H]-Thy, preferably a cell harvester with glass-fiber filters.

3. Methods

3.1. Employing Pharmacological Agents to Disrupt Processing of Exogenous Proteins in APCs

The following procedure describes a method of treating suspended APCs with proteolytic inhibitors, to analyze their effect on Ag processing and MHC-II-restricted presentation, (*see* **Notes 2** and **3**). Controls using synthetic peptide should be performed concurrently (*see* **Subheading 3.2.**).

CAUTION: All solutions and consumables that come in contact with cells must be sterile.

1. Wash the APCs by centrifugation at 500*g* followed by aspiration of spent media. Resuspend in fresh tissue culture media, and repeat wash procedure.
2. Resuspend the APC pellet in fresh tissue culture media at 1×10^6 cells/mL.
3. Aliquot 1 mL resuspended APC into each well of a labeled 24-well tissue culture plate.
4. Add protease inhibitor from concentrated stock solutions, directly to the appropriate wells (*see* **Note 4**). Mix gently but thoroughly with a pipet.
5. Incubate 30 min at 37°C in a tissue culture incubator to allow inhibitor to enter cells.
6. Transfer 100 µL of the APC from each well into a sterile microcetrifuge tube (*see* **Note 5**). Identical microcentrifuge tubes should be prepared, each with: no Ag, plus Ag, or pulsed with peptide control (*see* **Subheading 3.2.**).
7. Add the Ag directly to the appropriate microcentrifuge tubes containing the APC (*see* **Note 6**).
8. Incubate at 37°C. The length of incubation will vary, depending on the APCs and Ag. A time-course assay, using increasing incubation times, can be used to find optimal incubation periods.
9. Wash the APC by adding 500 µL cold HBSS or PBS to each microcentrifuge tube and centrifuging at 500g for 2 min. Aspirate the supernatant, and repeat the wash procedure.
10. Gently resuspend the pellet in 250 µL cold 1% paraformaldehyde.
11. Wash the APCs twice in HBSS or PBS, then twice in T-cell media, as in **step 1**.
12. Resuspend the pellet in 500 µL T-cell media.

3.2. Assessment of MHC Class II Ag Function Via Synthetic Peptide Presentation

The mechanisms of Ag processing are complex and seem to vary for different Ags *(6)*. Often, the cellular factor(s) being analyzed not only act on Ag

processing, but have a role in MHC-II maturation as well. This method describes the use of a synthetic peptide that mimics the naturally processed epitope presented by MHC-II for recognition by the responding T-cell hybridoma. This synthetic peptide, added to the APC, binds surface class II, and provides a measure of surface MHC-II expression and function. With this method, perturbations in MHC-II protein function by pharmacological agents and inhibitors can be distinguished from effects on Ag processing.

1. With 1.5–3 h remaining in the Ag processing assay described above, remove the microcentrifuge tubes containing APC designated to receive the synthetic peptide control.
2. Add the synthetic peptide directly to the APCs from the concentrated stock (*see* **Note 7**).
3. Gently mix by finger-flicking each tube, and return the cells to the incubator for the remainder of the Ag processing incubation described above.
4. Gently mix, every 30 min, until the end of the incubation period.
5. Fix the cells as described in **Subheading 3.1.**, **steps 9–13**.

3.3. Detection of Ag Presentation, Using an Ag-Specific T-Hybridoma Cell Line

Once the APCs have been fixed with 1% paraformaldehyde, the cells are ready to be co-cultured with an Ag-specific T-hybridoma cell line (*see* **Note 8**). This is performed in a 96-well u-bottom microtiter plate for suspended APCs.

1. Wash the T-cells in fresh T-cell media by centrifugation at 500g for 5 min, followed by aspiration of the supernatant.
2. Resuspend the T-cell pellet in fresh T-cell media at 1×10^5 cells/mL.
3. Using a multichannel pipet, aliquot 100 μL T-cells (1×10^4 cells) into each well of the 96-well microtiter plate.
4. Aliquot 100 μL of fixed APCs from each microcentrifuge tube, in triplicate, into the appropriate wells containing the T-cells.
5. Incubate at 37°C, 5% CO_2, for 24 h.
6. Freeze plate at –80°C for >4 h (*see* **Note 9**).

3.4. Bioassay to Detect Ag-Specific T-Cell Hybridoma Activation

Upon recognition of a specific peptide–MHC-II complex, T-hybridoma cells will secrete cytokines, such as IL-2 and IL-4. Therefore, the measure of cytokines, such as IL-2, can be used to quantitate the level of class II-restricted presentation. This is accomplished by utilizing a cytokine-dependent cell line, such as HT-2, which is strictly dependent on IL-2 for proliferation (*see* **Notes 10** and **11**).

1. Thaw the T-cell–APC co-culture plate by placing it at 37°C for approx 45 min.
2. 6×10^5 HT-2 cells are required for each 96-well plate in the assay. With 10 mL T-cell

Table 1
Common Inhibitors[a] Used in Analyzing Ag Processing

Inhibitor	Target	Comments
Leupeptin[b]	Cysteine and serine proteases	Reversible inhibitor, aqueous soluble
Pepstatin A	Aspartyl proteases	Reversible inhibitor, concentrated solutions soluble in DMSO or EtOH
E-64	Cysteine proteases	Irreversible inhibitor, aqueous soluble
Chloroquine	Acidic organelles	Reversible inhibitor, aqueous soluble, indirectly inhibits acidic proteases
Brefeldin A	Endoplasmic Reticulum to Golgi transport	Reversible inhibitor, soluble in DMSO or EtOH

[a] Concentrated stocks of 100–1000X should be made, to minimize the volume added to cells.
[b] Leupeptin can be stored 4–6 wk at –20°C.

media in a 15 mL conical tube, wash the HT-2 cells by centrifugation at 500*g,* followed by aspiration of the supernatant. Repeat this wash procedure two additional times.

3. Resuspend the HT-2 cells in fresh T-cell media (6×10^5 HT-2 cells should be resuspended in 3 mL fresh T-cell media for each plate in the assay).
4. Using a multichannel pipet, aliquot 25 μL into each well of a fresh 96-well microtiter plate.
5. Centrifuge the thawed plate at 1000*g*, to pellet cell debris.
6. Using a multichannel pipet, transfer 80 μL supernatant from the T-cell–APC plate to the corresponding wells in the HT-2 plate.
7. Incubate 8–16 h.
8. Add 25 μL ^{3}[H]-Thy (1 μCi) to each well.
9. Continue incubation an additional 8–16 h.
10. Harvest the cells, and measure incorporated ^{3}[H]-Thy.

4. Notes

1. When making 1% paraformaldehyde, it may be necessary to add concentrated NaOH dropwise, until the paraformaldehyde goes into solution. The pH must then be adjusted to 7.4, and the solution filter-sterilized using a 22-μm filter.
2. Before using pharmacological agents designed to inhibit cellular processes, it is important to know which concentrations, if any, are toxic to the cells. Furthermore, different cells may display varying levels of sensitivity to certain drugs. Critical concentrations can be determined by performing a toxicity curve, using increasing amounts of the pharmacological agent in question. **Table 1** lists sev-

eral inhibitors that have been used to analyze acidic protease activity and/or vesicular trafficking in Ag processing and MHC-II-restricted presentation.

3. APC function is dependent on adequate expression of surface MHC-II proteins. Therefore, it may be necessary to cytokine pretreat some APCs, such as macrophage and dendritic cells. The macrophage cell line THP-1, for instance, must be stimulated with IFN-γ 24–48 h prior to the addition of T-cells, to optimize MHC-II expression.

4. Some proteolytic inhibitors can pass easily through the plasma membrane, others must enter cells through endocytosis. Pretreatment of APCs with inhibitors is suggested, while minimizing any toxicity caused by these drugs.

5. When using adherent cells as APCs, the cells can be plated directly into a 96-well flat-bottom microtiter plate. Ag and peptide can be subsequently added, and washings carried out by drawing off fluids, using a multichannel pipet. Care should be taken after fixation, because any residual paraformaldehyde will be toxic to the hybridoma T-cells.

6. After adding Ag to the APC, it may be useful to place the microcentrifuge tubes on a rotator in the 37°C incubator. This will reduce the settling of the cells, and help mix the solution.

7. The optimal final concentration of synthetic peptide added to the APC should be determined by titrating the peptide, while keeping the effector:target ratio constant.

8. The optimal T-cell:APC ratio during co-culture will vary, depending on the type of APCs, the relative efficiency of processing and presentation of the Ag, and the particular T-hybridoma cell line. This can be determined by increasing the number of APC while keeping the T-cell number constant (e.g., 1×10^4 cells/well). Such cell titrations are also useful in comparing Ag-specific MHC-II restricted presentation among different APCs.

9. Following co-culture of APCs and T-cells, it is important that the 96-well plate be allowed to completely freeze at –80°C, to obtain complete cell lysis after thawing. Accordingly, care should be taken when stacking multiple 96-well microtiter plates, because this will result in unequal freezing. Therefore, overnight freezing is recommended; however, 3–4 h is usually sufficient. Unused supernatant containing the thawed APC–T-cells can be returned to –80°C and reassayed.

10. Subculture HT-2 cells at $5 \times 10^3 - 2 \times 10^4$ cells/mL every 3 d in fresh T-cell media supplemented with 20% T-Stim reagent. T-Stim reagent is a cytokine cocktail produced by concanavalin A treatment of rodent T-cells. After this culture period, the cells can be used immediately or cultured at room temperature for up to 36 h. These cells are cytokine starved, and will be most receptive to cytokines produced by the T-cell hybridomas. Just prior to use, wash the HT-2 cells at least 3× in fresh T-cell media, to remove residual growth factors. An aliquot of HT-2 cells is diluted into fresh T-cell media with T-Stim reagent, for propagation in a tissue culture incubator.

11. In addition to the use of IL-2-dependent cell lines, commercial cytokine ELISA kits are available. The sensitivity of these assays is somewhat lower than the bioassay, but the production of many different cytokines can be monitored.

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5

Quantitation of Major Histocompatibility Complex Antigen Endocytosis Using Capture ELISA

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1. Introduction

Major histocompatibility class I and II (MHC-I and MHC-II) antigens (Ags), as well as a variety of cell surface receptors, have been shown to enter the endocytic pathway of antigen-presenting cells (APCs). MHC Ags endocytose into clathrin-coated pits, which pinch off from the plasma membrane and form clathrin-coated vesicles *(1–4)*. These vesicles rapidly uncoat and fuse together or with pre-existing vesicular structures called "early endosomes." Selected molecules within early endosomes return to the plasma membrane, possibly via carrier vesicles *(5)*. This pathway has been best described for endocytosed transferrin receptors (TfR) *(6)*.

MHC-I Ags are expressed on the cell surface of all nucleated cells, yet the process of endocytosis and recycling for class I Ags has been observed only in a few cell types. MHC-I Ags have been shown to rapidly internalize from the surface of mitogen activated T-lymphocytes, T-lymphomas, and macrophage/monocyte cells, but not B lymphocytes or B lymphomas *(1,7–9)*. Transgenic mice expressing a nonrecycling glysosylphosphatidylinositol (GPI-linked) MHC-I were found to be deficient in generating cytolytic T-lymphocyte responses *(10)*. It has therefore been suggested *(7–10)* that this recycling pathway plays a pivotal role in the function of these Ag presenting molecules.

MHC-II molecules are distributed throughout endosomal compartments, where they may intersect and bind Ag peptides. Studies have shown that newly synthesized class II bind processed peptides as they traffic through late endosomal compartments to the plasma membrane. This pathway can be blocked by inhibitors of protein synthesis and is typically dependent on invariant chain targeting of class II proteins to these endosomal vesicles *(11,12)*.

Passage of mature cell surface MHC II through acidic early endosomes may facilitate peptide loading or the exchange of weakly bound peptides for those of higher affinity, thereby enhancing Ag presentation *(13,14)*. The recycling of MHC-II Ags may also allow access to unique pools of peptides found within these organelles.

Previous assays to measure endocytosis and recycling of MHC Ags were cumbersome, involving iodinated antibodies (Abs), cleavable radiolabeling reagents *(15)* or biotinylated F(ab) fragments detected with radiolabeled avidin *(16)*. Biotin-labeling of cell surface Ags is an established methodology for monitoring protein transport to the cell surface *(17)*, polarized sorting *(18)*, and endocytosis *(19)*, without perturbing surface expression or endocytic transport of proteins. Sulfosuccinimidyl-2-(biotinamido) ethyl-1, 3-dithiopropionate (sulfo-NHS-S-S-biotin) is used to chemically label cell surface proteins on APC. NHS is a highly reactive ester group, which introduces a biotin tag covalently into any polypeptide that contains either an unblocked NH_2-terminal amino acid or an exposed amino group of a reactive lysine residue *(20)*. Biotinylation of surface proteins provides a rapid and safe alternative to radioactive labeling that is independent of ligand binding.

Direct quantitation of cell surface and endocytosed biotin-tagged proteins has been time-consuming, and relied on polyacrylamide gel electrophoresis (PAGE) followed by densitometry. Protein biotinylation of viable cells allows direct, accurate quantitation of surface expression, endocytosis, and recycling for a wide variety of plasma membrane proteins, including MHC Ags. The basal levels of TfR or MHC-II Ag endocytosis, measured using this technique, were comparable to results obtained with more complex radioactive assays. Confirmation that the biotin label tags only cell-surface proteins was obtained via studies of an endosomal/lysosomal protease, cathepsin D. This intracellular protease was biotinylated in detergent-lysed cells, but could not be tagged by cell surface biotinylation. Biotin-labeling has also been shown not to promote the endocytosis of proteins lacking internalization signals such as the B-lymphocyte Fc receptor *(21)*. Biotinylation, followed by a capture enzyme-linked immunosorbent assay (ELISA), provides a noninvasive technique for chemically tagging cell-surface proteins and allowing monitoring of endocytosis, recycling, and turnover of cell surface proteins.

2. Materials

1. APC, culture media, and tissue culture flasks.
2. Sulfo-NHS-S-S-biotin (Pierce, Rockford, IL). Resuspend in Hank's balanced salt solution (HBSS) at 10 mg/mL. Biotin should be stored desiccated at –20ºC, and the solution made fresh daily.

3. HBSS, and HBSS with 5 mM Tris-HCl, pH 7.4.

4. Glutathione stripping solution: 0.05 M glutathione (reduced-form), 0.075 M NaCl, and 0.001 M EDTA. Dissolve these reagents in water, and just prior to use, add 0.075M NaOH, followed by fetal calf serum (FCS) to a final concentration of 10% (pH ~8.6; do not adjust). Keep the solution on ice, and use cold.

5. Primaquine: 0.3 mM primaquine in RPMI-HEPES.

6. Sucrose solution: 0.45 M sucrose in HBSS.

7. Lysis buffer: HBSS with 1% Triton X-100 and the protease inhibitors phenyl-methylsulfonyl fluoride (PMSF) (0.02 mM) and tosyl lysine chloromethyl ketone (TLCK) (0.01 mM).

8. 96 well EIA/RIA high protein binding plates.

9. Blocking solution: 5% FCS in phosphate-buffered saline (PBS).

10. Purified Abs for capture ELISA: Dilute in PBS to 1–10 µg/mL (to be determined for each antibody).

11. Avidin-peroxidase: 2.5 µg/mL in PBS with 10% calf serum.

12. 2,2'-azino-di[3-ethylbenzthiazoline sulfonate] (ABTS) (Bio FX, Randells-town, MD).

13. ELISA wash buffer: PBS plus 0.05% Tween-20 (PBS-T).

14. Anti-MHC-I or -II specific Abs for coating Protein A-Sepharose beads for immunoprecipitation.

15. Tris Saline: 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100.

16. 2X reducing sample buffer: 20 mL glycerol, 2 mL 2-mercaptoethanol, 4 g sodium dodecyl sulfate (SDS), 1.52 g (0.13 M) Tris, 1 mg bromophenol blue, pH to 6.8 with HCl, 78 mL water.

17. Standard SDS-PAGE reagents *(22)*.

18. 1.0% bovine serum albumin (BSA) in PBS-T.

19. Streptavidin-horseradish peroxidase (HRP) at 0.5 µg/mL in PBS.

20. Enhanced chemiluminescence (ECL) reagents for Western blotting.

3. Methods

Biotinylation is ideal for labeling cell surface proteins on living cells (*see* **Note 1**). Sulfo-NHS-S-S-Biotin is advantageous, because of its water solubil-ity and membrane impermeability, thus allowing only cell-surface proteins to be labeled. Endocytosis of the biotin-tagged proteins can be detected following incubation of cells at 37°C. Treatment of these cells with a reducing reagent releases the biotin label from cell-surface proteins; endocytosed molecules are protected within the cell, and retain their biotin label. The amount of biotin bound to a specific receptor or Ag can be quantitated using an antibody capture technique and avidin HRP. As an additional means of detecting class I or II Ags tagged with biotin, Western blots can be performed, followed by quan-titation using densitometry.

3.1. Biotin Labeling of Cell Surface Ags

1. Dissolve sulfo-NHS-S-S-biotin in HBSS at 10 mg/mL and store on ice (*see* **Note 2**).
2. For each sample, wash 3×10^7 cells $3 \times$ with cold HBSS (*see* **Note 3**).
3. For each sample, resuspend cells in 1 mL cold HBSS and add 50 µl biotin (final concentration of 0.5 mg/mL).
4. Rotate cells at 4°C for 15 min to promote biotin-labeling of proteins.
5. Wash cells $3 \times$ with cold HBSS plus 5 mM Tris-HCl, pH 7.4, to quench any excess NHS.
6. To determine the total amount of a protein labeled with biotin on the cell surface, proceed as described in **Subheadings 3.4.** and **3.5.**
7. To determine the total level of each protein both on the surface and within cells, lyse the cells (as described in **Subheading 3.4.**) prior to biotinylation, then quench excess biotin with 5 mM Tris-HCl, pH 7.4 (*see* **Note 4**).

3.2. Endocytosis of Biotin-Labeled Surface Ags

1. Resuspend biotinylated cells in 2 mL prewarmed HBSS.
2. Incubate in a 37°C water bath for varying lengths of time, to allow endocytosis (5–60 min). As a control, one sample of biotinylated cells should remain on ice, to block endocytosis prior to glutathione stripping. This sample permits determination of gluthathione (GSH) stripping efficiency (*see* **Note 5**).
3. Primaquine blocks the cycling of proteins back to the plasma membrane, but allows normal endocytosis. Treat cells with 0.3 mM primaquine in RPMI-HEPES during the incubation at 37°C, to allow endocytosis without recycling back to the cell surface (*see* **Notes 6** and **7**).
4. Endocytosis through clathrin-coated pits can be blocked by treating cells with 0.45 M sucrose in HBSS for 10 min at 37°C prior to biotinylation, with an additional sucrose treatment during the incubation at 37°C following biotinylation (*see* **Notes 7** and **8**).

3.3. Glutathione Stripping of Cell Surface Biotin Label from Proteins

1. Resuspend biotinylated samples in 5 mL cold glutathione solution, to release biotin label from proteins at the cell surface.
2. Rotate cells at 4°C for 25 min.
3. Pellet the cells, and resuspend in 5 mL fresh cold glutathione solution.
4. Rotate for 30 min at 4°C.
5. Wash cells $3 \times$ with HBSS, prior to lysing or additional warming.
6. To demonstrate the continuous cycling of endocytosed proteins back to the cell surface, glutathione-stripped cells were rewarmed to 37°C, as described in **Subheading 3.2., steps 1** and **2**, for varying lengths of time (5–60 min) to permit endocytosis and recycling.
7. A second glutathione treatment performed as described above (**Subheading 3.3., steps 1–4**), will remove any labeled proteins that recycled back to the plasma membrane during the second warming.

3.4. Preparation of Cell Lysates
for Quantitation of Biotin Labeling and Ag Endocytosis

1. Following biotin labeling, lyse cells at 4°C in HBSS with 1% Triton X-100 and the protease inhibitors PMSF (0.02 mM) and TLCK (0.01 mM). The final concentration of cells must be at least 10^7 cells/mL, to permit detection of labeled proteins.
2. Incubate the lysed cells on ice for 10 min.
3. Centrifuge the lysates at 200g for 5 min at 4°C, to remove the intact nuclei.
4. To label both surface and intracellular proteins, lyse cells with detergent prior to biotinylation.

3.5. ELISA for Quantitation of Biotin-Labeled Antigens

1. Purified Abs specific for receptors or Ags of interest should be diluted to 1–10 µg/mL in PBS (*see* **Notes 9** and **10**).
2. For each sample to be tested, add 100 µL appropriate diluted Ab per well (in triplicate), and incubate overnight at 4°C.
3. Coat additional wells with 100 µL of 5% FCS or an irrelevant isotype-matched Ab as controls.
4. Wash the Ab-coated wells 3 × with 300 µL/well PBS-T.
5. Add 300 µL/well PBS plus 5% FCS, and incubate at room temperature for 10 min. Repeat 2 ×, to block the wells.
6. Wash the wells 3 × with PBS-T.
7. Add 100 µL/well cell lysates (in triplicate), and incubate for 2 h at 4°C.
8. As controls, add 100 µL/well cell lysis buffer to Ab-coated wells, and 100 µL/well cell lysates to wells coated with control Ab or FCS.
9. Wash 5 × with PBS-T.
10. Add 100 µL/well avidin-peroxidase at 2.5 µg/mL in PBS plus 10% heat-inactivated calf serum.
11. Incubate at room temp for 30 min.
12. Wash 5–8 × with PBS-T.
13. Add 100 µL/well ABTS.
14. Read the color reaction at 405 nm.

3.6. Statistical Analysis

1. To calculate biotin labeling of an Ag at the cell surface or following endocytosis, subtract the average value absorbance at 405 nm for control or FCS sample wells from the average absorbance of the test sample. These corrected sample values are then divided by either the corrected average for total Ag labeled in cells or by the corrected average cell surface label for each protein. Multiply by 100 to yield the adjusted percentage of protein biotinylation for each Ag.
2. Triplicate data is used to calculate standard deviations in each measurement.
3. Absorbance values can be compared with way analysis of variance (ANOVA). The Tukey HSD procedure can be used to define differences when the ANOVA is significant.

3.7. Western Blot

The techniques of immunoprecipitation and Western blotting can be used following the steps in **Subheadings 3.1–3.4.** in order to confirm the specificity of Abs used in the capture ELISA. A rough quantitation of endocytosis and glutathione stripping efficiency can also be determined by immunoprecipitation and Western blotting, using densitometry.

1. Immunoprecipitate biotin-labeled cell lysates with anti-MHC-I or -II Abs at 5 µg/mL by rotating samples at 4°C for 2 h.
2. Add 50 µl Protein A-Sepharose beads precoated with rabbit antimouse immunoglobulin G.
3. Rotate at 4°C for 30 min.
4. Wash 3–5 × with Tris-saline buffer.
5. Elute the Ag–Ab complexes with 50 µl reducing SDS-PAGE sample buffer, by boiling for 5 min.
6. Run samples on 10% SDS-PAGE, according to a standard protocol *(22)*.
7. Transfer to nitrocellulose.
8. Block the nitrocellulose in PBS-T plus 1% BSA overnight at 4°C.
9. Probe the blot with streptavidin conjugated to HRP at 1 µg/mL in PBS for 30 min at 4°C.
10. Develop blot with ECL reagents.
11. Expose to X-ray film.

3.8. Quantitating Surface Half-Life of Proteins, Using Biotin Labeling

A modification of the method described above to measure endocytosis can be used to determine the surface half-life of a variety of cell-surface receptors and Ags.

1. Biotinylate cells, as described in **Subheading 3.1.** using biotin solution that has been filter-sterilized.
2. Wash the biotinylated cells 3 × with sterile HBSS plus 5 m*M* Tris-HCl, pH 7.4.
3. Resuspend the cells in media to an appropriate concentration $(0.5–1 \times 10^6$ cells/mL).
4. Remove equal volume aliquots at various time intervals, starting with a zero timepoint.
5. Wash the aliquots 3 × with HBSS, and freeze the cell pellets at –80°C.
6. Thaw the pellets, and lyse them in lysis buffer with a final concentration of 1×10^7 cells/mL.
7. Use the lysates in the ELISA, as described in **Subheading 3.5.**
8. Calculate the percentage of each protein remaining in the cell at each time point, by dividing relative to the absorbance reading at zero time. As before, the absorbance readings should be corrected by subtracting the absorbance of controls.

4. Notes

1. NHS-SS-biotin has an extended spacer arm, which reduces steric hindrances and allows the biotin groups access to avidin. NHS-SS-biotin is also water-soluble, allowing the biotinylation to be carried out in the absence of organic solvents. Buffers containing amines (such as Tris or glycine) should be avoided, because these compete with protein biotinylation reactions.

2. Carry out all steps, other than the warming for endocytosis, at 4°C or on ice. All centrifuge spins should also be done at 4°C. This is essential, because even slight warming of cells or buffers permits protein endocytosis.

3. The protocol described here is for suspended cells, and must be slightly modified for adherent cells. Plate 1×10^7 cells on a 150×25 mm plate (one plate/ sample), and allow cells to attach overnight. The assay is then performed directly on the plate with all washes, biotinylation (5 mL at 0.5 mg/mL), and glutathione stripping being done with the cells still attached to the plate. For the warming step, place 5 mL of prewarmed HBSS on the cells, and incubate at 37°C in a CO_2 incubator. Add at least 2 mL lysis buffer to each plate, and incubate on ice for 10 min. Remove the cells using a cell scraper, and transfer the lysates to a 15-mL tube. Allow the lysis to continue for 5 min on ice before spinning out the intact nuclei. Perform the ELISA of cell lysates, as described in **Subheading 3.5.** using 200 μL lysate per well, rather than 100 μL.

4. We recommend staggering the start of each sample, so that each reaches the lysis step at approximately the same time. Alternatively, each sample can be pelleted and frozen at –80°C before the lysis step. The ELISA can then be performed the following day.

5. The efficiency of the glutathione stripping varies between experiments, and for different receptors within an experiment. In order to determine the glutathione-stripping efficiency, one sample of biotinylated cells should remain on ice prior to glutathione stripping, to block any endocytosis. Typically, 95–100% surface biotin can be stripped back off the surface using the glutathione solution.

6. Primaquine treatment, which nonspecifically disrupts endosomal/lysosomal function, can be used as an additional probe to establish recycling of biotin-labeled proteins. Primaquine has been shown to slow the rate of recycling of TfR *(23,24)* and MHC-II *(2)*, and thereby increases the amount of biotin-labeled proteins detected within endosomes, using our assay.

7. Primaquine and sucrose treatments may be toxic to cells, especially if used for extended periods of time. The toxicity of each treatment should be tested for each cell line prior to use.

8. Exposure of cells to hypertonic media blocks receptor mediated endocytosis by preventing the formation of clathrin-coated pits *(25)*. The addition of hypertonic sucrose solution reduces the endocytosis of MHC-II proteins to background level, confirming that endocytosis of MHC II is mediated by clathrin-coated pits.

9. Purified Abs are more efficient at capturing Ags than crude preparations of tissue culture supernatant containing antibodies. We suggest using only purified Abs. Not all Abs will work well in a capture ELISA, and the optimal concentration for

each Ab varies. It is important to test each Ab at a range of concentrations. Surface biotinylate a sample of cells, and perform the ELISA to determine the ability of each Ab to capture its Ag and to find the optimal concentration.

10. The TfR is an excellent control. It is known to rapidly recycle and the pathway it follows has been extensively studied *(6)*. Because this technique allows for simultaneous monitoring of multiple receptors, we recommend that the TfR be followed as a control in each experiment.

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6

In Vitro Generation of Functional Human and Murine Dendritic Cells

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1. Introduction

Dendritic cells (DC) that reside in tissues such as the epidermis, lung, or spleen, and those that circulate in the blood, are functionally and phenotypically immature *(1,2)*. In this immature or precursor state, DCs can process protein antigens (Ags), and phagocytose and process particulate matter efficiently; however, they are poor stimulators of T-lymphocytes. As DCs migrate from the epidermis to the lymphoid organs, several phenotypic and functional changes occur: they acquire several co-stimulatory molecules; upregulate major histocompatibility complex class II (MHC-II), DEC-205 Ag receptor, and granule Ag M342; become less phagocytic; lose their protein-processing capabilities; and eventually become potent stimulators of T-lymphocytes. Similar to their in vivo properties, DCs freshly isolated from the blood, skin, or spleen are functionally and phenotypically immature, but after 1–2 d in culture, they mature into DCs with strong T-cell-stimulatory capacity, with a concomitant loss in protein/Ag processing capabilities. The stimuli for maturation in vivo could be exposure to proinflammatory stimuli, such as lipopolysaccharide (LPS), interleukin 1 (IL-1), IL-6, or tumor necrosis factor α (TNF-α) (e.g., encountered during infection, transplant rejection, or hypersensitivity reactions). In vitro, this maturation process can be mimicked by the cytokine differentiation of proliferating mouse or human progenitor cells, or of nonproliferating precursors of human or murine monocytes/macrophages (*see* **Note 1**).

1.1. Limitations of Direct Isolation of DCs

The frequency of human or murine DCs circulating in the peripheral blood is very low (0.1–0.2%), making direct isolation a difficult procedure that first involves depletion of T-cells and adherent cells, followed by density gradient centrifugation to isolate low-buoyant-density cells *(3)*. The cell yield from one leukapheresis (50 mL) is approx 5×10^6 cells, and, of these, <50% stain for DC markers (MHC-II and CD83). Higher purity can be obtained by cell-sorting or positive selection using magnetic beads. Thus, the direct isolation of DCs is labor-intensive, and the low yield has limited its widespread use *(4)*.

1.2. Generating DCs by Cytokine Differentiation of Monocytes or CD34+ Cells

An alternative method to generate a relatively homogenous population of functionally mature human DCs is by cytokine differentiation of monocytes (CD14+) or peripheral blood hematopoietic progenitor (PBHP) CD34+ cells. Generation of DCs from monocytes is relatively simple, and generally results in consistently high yields that range from $100–200 \times 10^6$ DCs per leukapheresis (50 mL). Using similar techniques, approx 5×10^6 DCs can be generated from 50 mL whole blood. Thus, isolation of DCs by the cytokine differentiation of monocytes is the method of choice in studies in which large amounts of Ag-presenting DCs are required. However, there are two main drawbacks to this procedure. First, because monocytes do not survive freezing well, experiments must be performed on fresh cells, and this imposes a limitation on the number of experiments that can be performed on the cells of single donor. Second, because the DCs that have been generated by this method have no proliferative capacity, they are less amenable to retroviral gene transfer.

These limitations of monocyte-derived DCs can be overcome by using DCs derived by the cytokine differentiation of PBHP CD34+ cells. First, following storage under appropriate conditions, aliquots of CD34+ cells from a single donor can be used for the generation of DCs at any given time, increasing the number of experiments that can be performed from the cells of a single donor. Second, the yield of DCs by this method is much higher (~20–100 DCs/CD34+ cell). Third, PBHP CD34+-differentiated DCs can be engineered to express Ags or cytokines more easily than monocyte-derived DCs. The major limitation of this technique is the significant expense in purifying CD34+ cells. This chapter focuses on generation of large numbers of human DCs from monocytes and progenitor cells. Included is a protocol for generating DCs from proliferating mouse bone marrow (BM) progenitors.

2. Materials
2.1. Generation of Human DCs from Monocytes

1. Leukapheresis pack (50–100 mL) or buffy coat obtained from the blood bank, or 50 mL heparinized whole blood. Use of frozen mononuclear cells (MNCs) is routinely unsuccessful.

2. RPMI 1640 (Life Technologies, Gaithersburg, MD).
3. Antibiotics: penicillin, gentamycin, streptomycin (Life Technologies). Antibiotics are omitted if the DCs will be used for live bacterial or mycobacterial infection studies.
4. HEPES (Life Technologies).
5. Autologous plasma or 5% heat-inactivated human serum (fetal bovine serum [FBS] results in high background proliferation in mixed lymphocyte reactions (MLRs); human serum can be prepared from multiple normal volunteers, and pooled).
6. Complete media: RPMI 1640, 5% heat-inactivated serum, antibiotics, and 2 m*M* HEPES.
7. Recombinant human cytokines: r(h) Granulocyte macrophage-colony stimulating factor (GM-CSF), r(h) IL-4, (R and D Systems, Minneapolis, MN). Store 1000X stocks of cytokines in 50-µL aliquots at –70°C for 6 mo. Once thawed, use within 4 wk, and do not refreeze.
8. Phosphate-buffered saline (PBS) without calcium and magnesium, containing 0.2 m*M* EDTA (Life Technologies).
9. Tissue culture flasks, 15- and 50-mL conical centrifuge tubes with caps, and sterile pipets.
10. Ficoll-Hypaque solution (1.077 g/L Nycomed).
11. Metrizamide (14.5%, Sigma, St. Louis, MO).
12. Antibodies (Abs) specific for cell surface markers (Pharmingen, San Diego, Ca), fluorescein isothiocyanate (FITC) and/or PE-labeled, with isotype controls.
13. Access to flow cytometric analysis (e.g., FACS Calibur with Simultest software from Becton Dickinson, San Jose, CA).

DC culture and incubations are at 37°C in a humidified 5% CO_2 incubator, and all manipulations are conducted in the tissue culture hood, unless stated otherwise.

2.2. Techniques to Generate Human DCs from Progenitor Cells

1. Access to blood bank apheresis unit.
2. Access to bone marrow transplant (BMT) unit for large scale purification of CD34^{+} cells.
3. Ceprate SC stem cell concentrating system (Cell-Pro, Bothell, WA) or Isolex immunomagnetic bead technology (Baxter Healthcare, Mundelein, IL).
4. Recombinant human cytokine stem cell factor (SCF), GM-CSF, TNF-α and IL-4 (all from R&D Systems).
5. Complete media containing Iscove's modified Dulbecco's medium, serum (10% FBS or 5% autologous human serum, or heat-inactivated human serum prepared from normal donors), penicillin/streptomycin, sodium pyruvate, nonessential amino acids and 4 m*M* glutamine.
6. Freezing media. If DCs will be propagated in fetal calf serum (FCS) or bovine serum, then progenitor cells can be frozen in 90% FBS and 10% dimethyl sulfoxide (DMSO). If DCs will be propagated in serum free conditions, then CD34^{+} cells

can be frozen in 10% DMSO (Research Industry), 1% human serum albumin (HSA) (Immuno-US), 90% X-vivo media (BioWhittaker). This method of freezing CD34$^+$ cells is effective, and cells remain viable, even after 18 mo.

7. Tissue culture supplies, such as 6 well plates, flasks, and cryogenic vials.
8. Abs (Pharmingen), FITC- and or PE-labeled, with isotype controls.
9. Access to flow cytometric analysis (e.g., FACS Calibur with Simultest software from Becton Dickinson).

Tissue culture manipulations are performed in tissue culture hood, and all incubations at 37°C in a humidified 5% CO_2 incubator, unless stated otherwise.

2.3. Generation of DC from Mouse BM Progenitor Cells

1. Mice 4–8 wk weighing >18 g (male mice have higher yields).
2. Cytokines: recombinant murine GM-CSF and IL-4 (R and D Systems) (*see* **Note 2**).
3. RPMI-1640 (Life Technologies) ice-cold (alternatively PBS containing 0.1% bovine serum albumin).
4. RPMI-1640 containing 5% FCS, penicillin 100 U/mL, streptomycin 100 µg/mL, 2m*M* HEPES.
5. Sterile gauze pads.
6. 3-mL syringe with 25-G needle.
7. Dissecting tools, such as forceps, scissors.
8. Tissue culture grade 15- and 50-mL conical polypropylene tubes, 6-well plates (Corning).
9. Pasteur pipets.
10. Petri dish.
11. Cell strainer (70 µm, Falcon)
12. Red blood cell lysing buffer (Sigma).
13. Monoclonal Abs for FACS analysis: CD86, I-A^d, CD40, CD11c, CD11b, CD54, CD3, B220, and isotype controls (Pharmingen); NLDC 145 Ab for DEC205 (Caltag).
14. Access to flow cytometric analysis (e.g., FACS Calibur with Simultest software from Becton Dickinson).

Tissue culture manipulations are carried out in the hood, and all incubations at 37°C in a humidified 5% CO_2 incubator, unless stated otherwise.

3. Methods

3.1. Generation of Human DCs from Monocytes

3.1.1. Isolation of Peripheral Blood MNCs

1. Dilute the leukapheresis or buffy coat with PBS (1:4 ratio) or whole blood with PBS (1:2 ratio) and place 35 mL diluted leukocyte/blood suspension in a 50-mL conical tube.

2. Using a sterile 10-mL cotton-plugged Pasteur pipet, aspirate 14 mL Ficoll-Hypaque solution, then place the tip of the pipet at the bottom of the 50-mL conical, and slowly release approx 12 mL Ficoll-Hypaque solution, taking care to avoid air bubbles.

3. Centrifuge the conical tube at $800g \times 30$ min at RT. The cells separate as follows (from bottom to top): red cells and neutrophils pellet to the bottom (~5 mL); a clear layer of ficoll (~12 mL); peripheral blood mononuclear cells (PBMNCs) (monocytes, macrophages, and lymphocytes) form a thin layer above the Ficoll; and, finally, the topmost layer consists of PBS and plasma containing platelets (~30 mL).

4. Aspirate and discard the top 20 mL. Using a 10-mL Pasteur pipet, carefully aspirate the remaining 10 mL, which will include the thin MNC layer at the interface, and transfer to another 50-mL conical tube. Wash twice with 20 mL PBS, and centrifuge at $400g$) to pellet the MNCs.

3.1.2. Isolation of Monocytes/Macrophages from PBMNCs

1. Resuspend the MNC pellet in complete RPMI, at a concentration of $2–3 \times 10^6$/mL and transfer to a tissue culture flask (T-75).

2. Place the tissue culture flask horizontally in a 5% CO_2 humidified incubator at 37°C *(4)* for 2 h. At the end of 2 h, the monocytes/macrophages will have adhered to the plastic.

3. Hold the flask vertically, and aspirate all the media. This will remove all the nonadherent lymphocytes. Add 10 mL prewarmed (at 37°C) RPMI-1640 to the bottom of the flask, and gently tilt the flask horizontally a few times (this step is required to wash the adherent cells).

4. Hold the flask vertically, and aspirate all the medium that may contain any residual nonadherent lymphocytes. This washing step can be repeated if small, round lymphocytes are still observed under a phase-contrast microscope.

5. Add 15 mL ice-cold PBS, containing 0.2 m*M* EDTA, to the flask, and incubate it horizontally in the tissue culture hood for 10 min. Detach the adherent cells by vigorous pipeting with a 10-mL cotton-plugged Pasteur pipet.

6. Transfer the cells to a 50-mL conical, and count the cells. Pellet the harvested monocytes by centrifuging at $400g \times 10$ min, and resuspend to a cell density of 5×10^6/mL in PBS.

7. An optional step, to remove any additional contaminating lymphocytes, is to overlay the cell suspension with a 14.5% metrizamide solution (volume of cell suspension:metrizamide is 2:1) and centrifuge at $600g \times 10$ min (optional step).

8. Carefully harvest the thin layer of monocytes at the interface, wash with 0.2 m*M* EDTA in PBS, and resuspend in complete RPMI (containing 5% human serum, 20 µg/mL gentamycin, and 2 m*M* HEPES) at a final concentration of 1×10^6/mL.

3.1.3. Culture of Monocytes with Cytokines

1. Add 50 ng/mL r(h)GM-CSF and 10 ng/mL r(h)IL-4 to the monocyte culture and incubate at 37°C in a humidified incubator. The concentrations of the cytokines given reflect the final concentration.

2. On d 3 and d 5 of culture, remove 80% of the media, and replace it with fresh media containing GM-CSF (50 ng/mL) and IL-4 (10 ng/mL).

3. On d 7, remove 80% of the media, and replace it with fresh media containing r(h) GM-CSF (50 ng/mL), IL-4 (10 ng/mL) and TNFα (10 ng/mL) (*see* **Note 3**).

4. Within 2–3 d, large clusters of cells, with characteristic DC features, are seen. The nonadherent/semiadherent cells, which appear as clumps or isolated floating cells, are DCs (50–75% of the cells). To remove the DCs, transfer the media from the flask to a 50-mL conical. To remove the remainder of loosely adherent cells, incubate the flask with 15 mL PBS/2 m*M* EDTA solution for 10 min. Repeated pipeting at this stage will break up the clumps and semiadherent cells. Transfer to the 50-mL conical, and count the cells. Pellet ($400g \times 5$ min), and resuspend at desired concentration.

5. The DCs generated are morphologically homogeneous and express high levels of MHC-I and -II, CD1a, ICAM-1, CD11b, CD11c, CD40, CD83, CD86, and CD33, and are negative for CD3 (T-lymphocyte marker), CD19 (B-lymphocyte marker), CD14 (monocyte marker), and CD56 (natural killer cell marker). Until the culture conditions are well established in the laboratory, staining every DC preparation for MHC-II, CD83, or CD1a is advisable, to ensure that the technique is reproducible.

3.2. Techniques to Generate Human DCs from Progenitor Cells

3.2.1. Apheresis of Normal Volunteers

After obtaining informed consent, the light-density cells from the peripheral blood of normal donors pretreated with granulocyte-colony stimulating factor (G-CSF, Amgen, Thousand Oaks, CA) are collected by apheresis (*see* **Notes 4** and **5**).

1. Normal volunteers are treated with 10 µg/kg/d G-CSF subcutaneously for 5–6 d, to increase the percentage of CD34$^+$ PBHP cells in the peripheral circulation *(7)*.

2. Draw blood every other day in a purple-top tube (that contains EDTA) for determination of leukocyte counts and FACS staining for CD34$^+$ cells. The total leukocyte count increases to 40,000–80,000/mm^3 by d 5, and the absolute number of CD34$^+$ cells increases by ~50-fold. CD34$^+$ cell counts usually peak on d 5 or 6 of G-CSF treatment, and the apheresis is usually timed to coincide with this peak.

3. The apheresis procedure requires the expertise and close cooperation of the blood bank and/or the BMT unit, where this procedure is performed on a routine basis for the isolation and purification of progenitor cells for BMT. The goal is to apherese at least 10 L blood via two large bore 14 G peripheral access lines in a period of 3–4 h. The entire procedure may take 4–5 h, depending on the blood flow rate via the access lines and the time required to obtain peripheral access.

3.2.2. Purification of CD34$^+$ PBHP Cells

1. PBHP cells are further enriched for CD34$^+$ cells by positive selection, using either the Ceprate SC stem cell concentrating system (Cell-Pro) or the Isolex immunomagnetic bead technology (Baxter Healthcare), according to instructions of the manufacturer. This procedure usually takes 3 h, and enriches PBHP cells

to greater than 70% CD34$^+$ cells. The average yield from each donor ranges from 50 to 300 $\times$ 10^6 CD34$^+$ cells.

2. The CD34$^+$ cells are frozen in 5–10 $\times$ 10^6 cells/mL aliquots in 10% DMSO, 1% HSA, 90% X-vivo media (BioWhittaker) in cryogenic vials (Nunc) (for DC generation in serum-free conditions) or in FBS (for DC generation in FBS).

3. CD34$^+$ cells are allowed to freeze slowly by storing at –70°C in styrofoam containers overnight, then are transferred the next day to liquid nitrogen vapor phase.

3.2.3. Cytokine Differentiation of CD34$^+$ PBHP Cells into Mature DCs

1. Prewarm the complete media, and aliquot 9 mL into a 15-mL conical tube.
2. Rapidly thaw the frozen vial containing CD34$^+$ cells by gently shaking the vial in a 37°C water bath (*see* **Note 6**).
3. In a tissue culture hood, transfer the contents of the vial into the 15-mL conical tube containing 9 mL media. To remove the DMSO, centrifuge this tube at 400*g* $\times$ 10 min, and transfer the pelleted cells to 5 mL complete media.
4. Culture the CD34$^+$ PBHP cells in 6-well plates at a concentration of 0.5 $\times$ 10^6/mL (2–4 mL/6-well plate). The media used is complete media supplemented with the following recombinant growth factors: 20 ng/mL SCF; 50 ng/mL GM-CSF (*see* **Note 7**).
5. Approximately 20–25% of the cells are nonviable, and this may be the contaminating monocytes, which do not survive freezing well. By the third day, the cells start dividing.
6. Change the medium every 2–3 d, and maintain the cell concentration at 0.3 $\times$ 10^6/mL. Do not allow the concentration to increase above 1 $\times$ 10^6/mL.
7. On d 6 of culture, add IL-4 and TNF-α at 10 ng/mL to the culture. The DCs derived from the cytokine-differentiated CD34$^+$ PBHP cells can be kept in culture for a total of 12–14 d. In each experiment, the differentiating cells should be stained for cell-surface markers for DCs, monocytes, and lymphocytes. There is at least a 20-fold expansion from each CD34$^+$ cell. However, there are some donors who give a higher yield of ~100-fold expansion. The yield may vary from laboratory to laboratory, and may depend on the viability and purity of the frozen CD34$^+$ cells at the initiation of culture.
8. By d 14 of culture, more than 99% of cells are CD33$^+$, suggesting that the predominant cell population is of the myeloid series. The portion of cells that stain for T-lymphocytes (CD3) or B-lymphocytes (CD19) is consistently less than 1–3%. There is a donor-to-donor variability (~20–30%) in the levels of some cell surface markers. Nevertheless, in all instances, the cytokine-differentiated PBHP cells are predominantly of myeloid origin, as evident by the high expression levels of CD33 (range: 90–98% cells), and low expression levels of CD3 (range: 0–3%) and CD19 (range: 0–3%). These cells have phenotypic characteristics of DCs, such as expression of CD1a (range: 20–81%), CD80 (range: 15–74%), CD86 (range: 18–81%), HLA-DR (40–99%), and adhesion molecules, such as CD11a (30–85%), CD11b (26–69%), CD11c (33–84%), and CD54 (17–76%).

3.3. Generation of DC from Mouse Bone Marrow Progenitor Cells

This procedure was described first by Inaba *(13,14)*, and has been used extensively by many investigators successfully. BM progenitor cells, cultured in the presence of GM-CSF and IL-4, differentiate into DCs. As the progenitors differentiate, they proliferate and are easily amenable to transduction by retrovirus-based gene-transfer techniques. The typical yield from the BM progenitor cells derived from a single 6-wk BALB/c mouse is $5–12 \times 10^6$ DCs.

1. Mice are euthanized according to the protocols established at the local institution and acceptable to the Institutional Animal Care and Use Committee. After euthanization, carefully dissect both the femurs and tibias from each mouse, and place in ice-cold RPMI-1640 (can substitute ice-cold PBS containing 0.1% bovine serum albumin).
2. Transfer each long bone onto sterile gauze, and remove the muscles by blunt dissection.
3. All steps henceforth should be done in the tissue culture hood. Immerse the bone stripped of all muscle in a Petri dish containing 70% EtOH for 45 s, and wash twice with ice-cold RPMI-1640.
4. Transfer to a clean Petri dish, and cut the end of the long bones with scissors. Flush the marrow with 2 mL RPMI using a 3-mL syringe attached to a 25-G needle. Transfer the marrow suspension from all the bones in a 50-mL conical tube kept on ice.
5. Place a cell strainer on top of a 50-mL conical tube. Remove the particulate matter from the marrow suspension by filtering it through the cell strainer into the 50-mL conical.
6. To lyse the red blood cells, add 3–10 mL red blood cell lysing buffer for 2–3 min, and pellet the cells at 400*g* for 10 min. Some investigators routinely deplete lymphocytes by using Abs against B- and T-cells in the presence of complement (*see* **Note 8**). However, we find that, if the nonadherent cells are removed on d 2 and 4, there is minimal contamination with lymphocytes.
7. Resuspend the pelleted cells in 6-well plates at a concentration of $1–2 \times 10^6$/mL in RPMI and 5% FCS containing 50 ng/mL GM-CSF and 1 ng/mL IL-4, and incubate at 37°C and 5% CO_2.
8. On d 2 and d 4 of culture, remove the nonadherent cells by gently suctioning off the media and replacing with fresh media containing growth factors.
9. At d 6–7 of culture, classic DC clusters with veiled morphology can be seen. The proliferating aggregates of DCs can be dislodged by gentle pipeting with RPMI 5% FCS. Pellet the cells at 400*g* for 5 min, and resuspend to 1×10^6/mL in fresh medium containing growth factors in the appropriate 96- or 24-well plates for Ag pulsing and/or Ag-presenting experiments.
10. By FACS staining, between 50 and 85% of these cells stain for classical DC markers. In a representative experiment, we found the cells to have the following surface staining: CD86 (82%), MHC-II (80%), CD40 (77%), CD11b (95%), CD11c (30%), NLDC 145 (50%), CD54 (92%), and CD3 and B220 (<5%).

4. Notes

1. Human and murine DC lines have been described that express CD1a, and take up, process, and present soluble Ags and induce MLR *(5)*. These cell lines are not available commercially.

2. Use the same source of cytokines throughout the project, once the culture techniques are established in the laboratory. Varying doses of GM-CSF, ranging from 10 to 50 ng/mL, have been used to differentiate BM cells toward the DC lineage. These varying dosages could result from the differences in the source from which the cytokine was purchased, with resulting differences in biological activity. Alternatively, they could be the result of individual investigator preferences. In this laboratory, 50 vs 20 ng/mL GM-CSF (R and D Systems) resulted in DCs with a higher percentage (~15–30%) of cells positive for co-stimulatory molecules.

3. Instead of adding recombinant cytokines, at d 7 of cell culture approx 50% of the media may be replaced with monocyte conditioned media *(5)* and cultured for an additional 4 d, and the nonadherent cells (DCs) harvested, as described in **Subheading 3.1.3.** To generate monocyte-conditioned media, culture PBMNCs ($3–5 \times 10^6$/mL, approx 8–10 mL cells/100-mm dish) on a human immunoglobu-lin G (Organon Teknika)-coated Petri dish for 30 min at 37°C. Remove the nonadherent cells, and culture the adherent cells for 24 h at 37°C in 10 mL complete medium. Harvest the supernatant 24 h later, and freeze in 5-mL aliquots at –20°C. These supernatants can be used as a source for monocyte-conditioned media (source of TNF-α, IL-1, IL-6, and interferon-α).

4. Many reports describe various culture conditions for generation of DCs from progenitors from many sources, such as BM, peripheral blood leukocytes of normal volunteers, as well as cancer patients and cord blood of newborns (6–12). The advantages of the protocol described are its reproducibility, large yields, and ability to freeze the progenitor cells for use at a later date to generate DCs. Several independent experiments can be performed with DCs generated from the CD34$^+$ cells of a single donor, thus reducing donor-to-donor variation. This option is not feasible when using progenitor cells from cord blood samples, because yields of CD34$^+$ progenitor cells from cord blood or from a leukapheresis pack, are lower than those from the procedure described. The yield of CD34$^+$ cells from patients with inherited diseases, cancer, or recent chemotherapy may be lower, and the biological functions of these cells may be different from those of normal adult volunteers *(6)*. Based on our calculations, to obtain 5×10^7 CD34$^+$ progenitor cells, it was as expensive but more time consuming to purify 10–15 cord blood samples (which may not be available at the same time) than to apherese one normal adult donor after G-CSF mobilization.

5. G-CSF administration to normal volunteers has now been accomplished in several studies *(6,7),* and at the dosage used can be associated with bone pain, malaise, headache, and flu-like symptoms. Typically, these side effects of G-CSF can be relieved by an analgesic, such as ibuprofen, and usually resolve completely within 48 h of stopping the treatment *(6,7)*. The apheresis procedure involves the use of a standard anticoagulant, acid citrate dextrose, which may cause

cramping and tetany, because of hypocalcemia. This is usually relieved by calcium supplementation or stopping the apheresis.

6. On thawing the frozen CD34$^+$ cells, one may often see clumps that contain cells, platelets, and fibrin. Investigators often remove them, thinking the cells are dead, but if they are left in culture, after 48–72 h, the cells separate and proliferate, thus increasing the yield.

7. Alternative protocols for growth of progenitor-derived DCs in serum-free conditions have been described using transforming growth factor-β1 *(10)*. CD-40L and IL-3 have also been used to support DC growth from progenitors independent of GM-CSF *(11)*. Addition of SCF or Flt-3 ligand to in vitro cultures increases the yield of CD1a$^+$ DCs by 3–6-fold *(12)*.

8. Some investigators routinely deplete lymphocytes and MHC-II cells by using a cocktail of Abs from hybridomas to B220 (clone RA303A1), CD4 (clone G.K.1.5), CD8 (clone 3.155), and MHC-II (clone M5/114). All the hybridoma clones are available from American Type Culture Collection (location). After red cell lysis, the cells are resuspended at 10×10^6/mL with hybridoma supernatants (1:20) and rabbit complement (1:20), and incubated for 1 h at 37°C in a shaking water bath, to induce complement mediated lysis. If a more pure population of DCs is required, the lymphocytes can be depleted further by magnetic bead selection. Other contaminants include immature DCs that have low levels of MHC-II and lack CD86. For functional proliferation assays, the ratio of DCs to T-lymphocytes at 1:20 gave maximum thymidine incorporation.

Acknowledgment

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7

Genetic Engineering of Dendritic Cells Using Retrovirus-Based Gene Transfer Techniques

Seema S. Ahuja

1. Introduction

Dendritic cells (DC) can be genetically engineered to constitutively express a gene of interest that could be either an immune-modulating cytokine, or an antigen (Ag) derived from a tumor/pathogen. There are numerous strategies for ex vivo transfer of genes or Ags into DCs. These include nonviral techniques (such as electroporation and liposomes) or recombinant viral-based vectors (e.g., retrovirus, adenovirus, herpes simplex virus, and avian and vaccinia viruses) *(1–4)*. The major advantages of retrovirus vectors are that they are effective in achieving stable and highly efficient transduction of genes into primary cells, such as DCs/monocytes/macrophages (*see* **Note 1**). The major disadvantages are that the cells to be transduced must be in mitosis (i.e., actively dividing for reverse transcription and stable viral integration), and small size genes are more efficiently transduced than larger ones.

A retrovirus genome is made of RNA, but, in the presence of reverse transcriptase produces a linear double-stranded DNA copy in host cells. The retrovirus enters a host cell using a specific viral receptor on the host cell surface. The viral DNA integrates into the host genome at random locations, where it is called the provirus. The provirus then replicates with the host DNA and is passed on to all the progeny. After integration, the viral long-terminal repeat (LTR) promoter at the 5' end of the genome becomes active and directs the synthesis of a full-length copy of the viral genome, which terminates in the 3' LTR.

The replication incompetent retrovirus vector is generated from cloned proviral sequences by removing all the sequences required to generate an infectious virus (e.g., the viral proteins Gag, Pol, and Env), and retaining the

sequences required for transmission (e.g., packaging sequence, reverse transcription, and integration signal). In place of the sequences encoding viral proteins, DNA sequences (cytokine genes or tumor or pathogen Ags) of interest can be cloned into the retroviral vector and expressed using the transcriptional regulatory sequences of the 5' LTR (*8–14*; *see* **Note 2**). To generate the replication-incompetent retrovirus, the vector DNA is introduced into a packaging cell line where it integrates into the host genome and generates vector RNA. The packaging cell line is a murine or avian cell line that encodes the viral structural proteins (Gag, Pol and Env). The structural proteins recognize the packaging sequences in the vector RNA, and preferentially package it to produce the retrovirus. The retrovirus buds from the surface of the packaging cell line into the culture supernatant that is used as a source of virus stock. The virus stock contains the replication-incompetent retrovirus that can efficiently infect target cells once, and transmit the gene of interest by integrating into the host genome, but cannot replicate and infect other cells, thus providing a level of safety (*see* **Note 3**).

This chapter initially describes the method to generate the retrovirus vector and viral supernatant (*see* **Fig. 1**). This is followed by a description of the protocol to transduce primary DCs differentiating from progenitor cells.

2. Materials
2.1. Generation of Retrovirus Vector

1. Plasmid encoding the retrovirus genome derived from moloney murine leukemia virus containing a multiple cloning site. The vector that we use is designated as "MFGS" *(5–7)* and it lacks an antibiotic selection gene (*see* **Note 3**). However, similar retroviral vectors are now commercially available (e.g., from Clontech, Palo Alto, CA) which also contain an antibiotic selection marker such as puromycin or neomycin.
2. cDNA of the gene of interest (e.g., interferon γ and interleukin 12).
3. Primers.
4. Restriction endonucleases.
5. DNA polymerase, such as *Taq* or *Pfu* (Stratagene, LaJolla, CA).
6. Molecular biology reagents for DNA cloning (e.g., mini/maxi prep kits), Topo TA cloning kits (Invitrogen, Carlsbad, CA).

2.2. Generation of Retrovirus

1. Retroviral plasmid DNA, containing the cDNA of interest, cloned into the multiple cloning site. If this vector does not contain an antibiotic resistance gene, then one will need a eukaryotic vector that encodes an antibiotic resistance gene.
2. Packaging cell line appropriate for the retroviral vector and the host cell to be transfected (some cell lines are commercially available from Clontech). For example, the Ψ crip packaging cell line produces an envelope which can infect
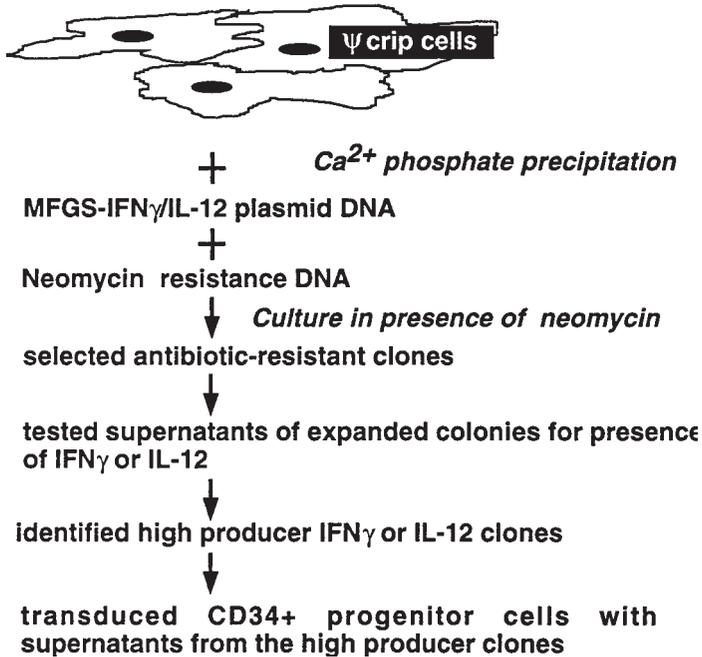
Fig. 1. Generation of retrovirus and transduction of DCs.

both human and murine cells. In contrast, the Ψ cre cell line produces a retrovirus that can infect murine cells very efficiently *(1)*.

3. Media for growing the packaging cell line (Dulbecco's modified Eagle's medium [DMEM] can be used for Ψ crip or Ψ cre packaging cells).

4. Calf or fetal bovine serum (select the appropriate serum, because many of the packaging cell lines require calf serum. instead of fetal bovine serum).

5. Complete media would be media (DMEM) containing 10% serum (check the source of the packaging cell line for serum requirements).

6. Antibiotics that correspond to the antibiotic resistance gene present in the retroviral vector for prokaryotic cells.

7. 2 *M* CaCl$_2$ (prepared fresh).

8. 2X Hydroxyethylpiperazine-N'-ethanesulfonic acid (HeBS) HEPES-buffered saline is prepared fresh and used within 2 wk (8 g NaCl, 0.32 g KCl, 0.125 g Na$_2$PO4$_2$·H$_2$O, 2 g dextrose, 10 g HEPES, H$_2$O to 500 mL and pH adjusted to 7.0).

9. 10-cm tissue culture dishes and 6-well plates, cloning cylinders, cell scrapers (Falcon/Costar).

10. 0.05% Trypsin, 0.05 EDTA m*M* (Life Technologies, Gaithersburg, MD).

11. Polybrene (Sigma, St. Louis, MO).

12. NIH3T3 cell line.

All incubations involving tissue culture are carried out at 37°C in a humidified 5% CO_2 incubator, and manipulations are carried out in a tissue culture hood.

2.3. Harvesting Viral Supernatant

1. Tissue culture reagents.
2. Freezing medium: 90% fetal calf serum and 10% DMSO.

2.4. Transduction of DCs

1. DCs.
2. Viral supernatant.
3. Polybrene (Sigma).
4. Tape (Bel Art cat. no. F13463-005).
5. Media for DCs (*see* Chapter 6).

3. Methods

3.1. Generation of the Retrovirus Vector

It takes about 2–3 mo to identify a high producer clone. This process essentially involves introduction of the retroviral vector DNA into the packaging cell line via the calcium phosphate precipitation method, placing the cell line under antibiotic selection, and screening for the appropriate high titer clone.

1. Design specific primers flanking the 5' and 3' end of the cDNA with two specific features. First, to ensure that the DNA cassette is inserted only in 5'- to 3'- orientation, add additional sequences to make the polymerase chain reaction (PCR) amplicon compatible with the restriction endonuclease site in the multiple cloning site in the retrovirus vector. Second, design the 5'- and 3'- primer as close as possible to the translation initiation codon (methionine) and the stop codon, respectively. We have found that incorporating into the 5'-PCR primer nucleotide sequences for the type II restriction endonuclease (distant cutter) can be used to maintain the fidelity of the first methylation/ATG site. For example, the recognition sequence for BsmBI is 5'-CGTCTC(N1)-3'. This sequence can be incorporated into the 5'-PCR primer as follows: CG TCT CAC *ATG,* followed by the appropriate coding sequences. Following digestion with the restriction endonuclease *Bsm*BI, the PCR amplicon will have a 5' overhang of *CATG* that is compatible with the *Nco*I restriction endonuclease site present in the multiple cloning site of the vector that we have the most experience with (MFGS). Third, maintain an intact Kozak sequence surrounding the translation initiation site.
2. Using these primers, the DNA fragment of choice is PCR-amplified with *Pfu* polymerase (Stratagene) or any other DNA polymerase with proofreading capacity, then cloned into a prokaryotic vector, such as pBluescript. The disadvantage of *Pfu* is that the PCR amplicon has flush ends, thus requiring blunt-end cloning strategies. An alternative strategy that we use is to mix the polymerases *Taq* and *Pfu* at a 15:1 ratio. This polymerase mix allows for the proofreading properties of

Pfu and the amplicon has a T overhang, facilitating cloning into very efficient TA cloning vectors that use the properties of Topo polymerases for ligation (Topo TA cloning kits, Invitrogen).

3. The PCR amplicon is sequenced (double-stranded), to ensure sequence fidelity. This extra step has ensured a high success rate in producing biologically active cytokines. We have found that even minor conservative changes in the coding regions can significantly influence expression levels.

4. To isolate the DNA insert, the vector is digested with appropriate enzymes that flank the insert, electrophoresed, gel-purified, and ligated into the retroviral vector, that has also been digested with identical restriction enzymes.

3.2. Generation of Retrovirus

1. One day before transfection, plate the packaging cells in 10 cm tissue culture dishes at 10–20% density (1/5 split of confluent cells). For a fibroblast cell line (most packaging cell lines are adherent cell lines) this roughly translates to approx 500,000 cells/10-cm dish.

2. Replace the media next morning (day of transfection) with 10 mL fresh media.

3. Prepare the DNA mixture containing calcium chloride in a total volume of 0.5 mL. To a microcentrifuge tube, add 62 μL 2 *M* $CaCl_2$ and 40 μg retroviral vector DNA, and bring the volume up to 0.5 mL with water. In a 15-mL sterile polystyrene, clear conical tube, add 0.5 mL of 2X HeBS. Start aerating the HeBS solution by bubbling air (use a thin 1–2 mL pipet). At the same time, gradually add the DNA mixture dropwise to the HeBS solution. Let the DNA–HeBS solution mixture stand in the hood for 30–45 min, until a fine, hazy white precipitate is formed. If one is using a retroviral vector without any selection marker, co-transfect 36 μg retroviral DNA + 4 mg SV2Neo vector *(5–7)*.

4. Add this mixture to the 10-cm dish containing the packaging cells, and incubate overnight (14–16 h) at 37°C in a humidified 5% CO_2 incubator.

5. The next day, tilt the dish, and aspirate all the media. Wash twice with complete media, add 20 mL fresh complete media, and return to the incubator.

6. 48 h later, split the cells (use either 0.05 m*M* trypsin, 0.05% EDTA for 3–5 min or gentle unidirectional scraping with a cell scraper) from this plate into 10 plates (10 cm tissue culture dishes) and add the selection antibiotic to the media, and incubate for at least 14 d.

7. Depending on the antibiotic selection, the nontransfected cells die (usually by d 4), and the transfected cells grow slowly, over a period of 2–3 wk, into colonies. Single surviving cells can be identified after prolonged search under the phase-contrast microscope, but this is not recommended. Replace the media with fresh media after 5–7 d (optional).

8. After 2 wk, hold the plate above one's head, against the overhead light, and identify white, opaque colonies. Make a circle around the colonies, and mark them at the bottom of the dish, using a laboratory marking pen. Approximately 2–4 clones per plate can usually be identified.

9. Pick well-isolated colonies with a cloning cylinder and transfer to a 24-well plate. As the cells grow, split them frequently, until a duplicate set of each clone has been grown in 6-well plates.

10. Freeze the cells from one set of clones growing in 6-well plate in 90% FBS and 10% DMSO, and maintain the other set until 20–30 colonies have been picked.

11. Harvest the viral supernatant for each clone. When the cells growing for each clone are at 80% confluence, replace the media with 1.5–2 mL fresh media (just enough to coat the surface of cells). This media, which is harvested 12 h later, contains the replication-incompetent virus, as well as the protein product of the gene encoded in the retroviral vector.

12. The protein product, in the case of cytokine genes, can easily be measured using ELISA, and the remainder of the supernatant is frozen at –70°C, until all the clones are isolated and their supernatants frozen. The clone producing the highest level of cytokine usually also has the highest titer of virus, but some of the slower-growing clones can have high viral titers with modest levels of cytokine production.

13. To identify the clone with high viral titer, supernatants from each clone are screened for ability to confer cytokine production to NIH3T3 cells. Plate NIH3T3 cells at 40% confluence in 6-well plates.

14. The following day, gradually thaw the viral supernatants from all the clones to room temperature, in the hood. Dilute them 1:1 with fresh complete media, and add polybrene to 6 µg/mL (transduction medium).

15. Aspirate all the media from the NIH3T3 plates, and add 2mL transduction medium from each clone, and incubate the plate for 6 h.

16. After 6 h, aspirate the transduction mixture, wash once, and replace with fresh medium. This transduction can be repeated once daily (2–4 ×) if the sensitivity of the assay that will be used to measure the protein product is low, or a very high transduction efficiency is desired. Prolonging the duration of transduction (>6 h/d), in our experience, was not useful in enhancing transduction efficiency. Polycation polybrene could be toxic to primary cells on prolonged incubation of >12 h.

17. For most cytokine retrovirus transductions, we found high titers detectable as early as 24 h after the first transduction. The high viral titer clones were identified as the clones whose supernatant conferred the highest cytokine production to NIH3T3 cells. Usually, we could identify 4–6 high-producer clones from 30 clones picked.

3.3. Harvesting Viral Supernatant

1. Once a high-titer clone is identified, the original vial of the clone should be thawed and expanded in 4–6 175 mL tissue culture flasks, until they are 70–80% confluent.

2. Add enough fresh media to barely coat the surface of the flask and incubate for 12 h.

3. Harvest the medium from each flask after 12 h; centrifuge at 300g for 5 min to remove particulate matter and freeze the supernatants in 3-mL aliquots at –70°C.

The viral supernatant is stable for at least 6 mo, and it has been used even a year later with no loss in transduction efficiency. Once thawed, the viral supernatant should not be refrozen.

4. After the viral supernatant is harvested, the cells of the high-producer clone should be frozen (12–18 vials) per high producer clone. One can regrow the high-titer clone and harvest fresh supernatant at a later date, when needed.

3.4. Transduction of DCs

The protocol for transducing DCs is very simple, and similar for mouse and human progenitor cells. The protocol for murine DCs is described in the following subheading. For successful transductions, the DCs differentiating in vitro from bone marrow cells/progenitor cells are cultured in 6-well plates, as described in Chapter 6 of this volume.

1. On d 3, 4, and 5 of murine bone marrow culture, the media is gently aspirated and replaced with 2 mL transduction mixture (viral supernatant:media, 1:1, containing 6 µg/mL polybrene, 50 ng/mL granulocyte-macrophage colony-stimulating factor, and 1 ng/mL interleukin 4).
2. Seal the edges of the 6-well plate with 0.5-in thick tape, and centrifuge the plate in a bucket with a horizontal platform (Sorvall tabletop centrifuge) at 800*g* for 60 min at RT *(5–7,10)*. Sealing the plate prevents the risk of contamination during the centrifugation step.
3. Transfer the plate to the tissue culture hood. Clean hands with soap and water and 70% EtOH then gradually remove the tape with ungloved hands. While removing the tape, hold the top of the 6 well plate steady with the other hand, because sudden jerky movements can spill the media from one well to the other. After incubating the plate for 5 h at 37°C, the transduction medium is aspirated, and replaced with fresh media containing growth factors. Repeat the transduction step on 2 d subsequently.
4. As early as 24 h after the first transduction, the protein product of the cloned gene in the retrovirus can be detected, and it peaks at 48 h after the third transduction. If it is a cytokine, it can be measured in the supernatant by commercially available ELISA reagents. The nature of the transduced DCs can be analyzed by fluorescence-activated cell sorting analysis for a cell surface marker for DC and intracellular staining for the cytokine. If the reagents for protein or cytokine detection are not available, their presence can be confirmed by Western blotting, PCR, or Northern blots. However, an assay to analyze the proteins' biological activity is important to ensure that the protein is being produced and expressed in its biologically active form.

4. Notes

1. The transduction efficiency can be as high as 80% (percentage of cells transduced can be in the range of 20–80%). Very high levels of a particular cytokine can have direct effects on the differentiating DCs. These changes may be subtle and need to be moni-

tored with functional assays for Ag presentation and DC surface markers. For example, transduction of human-progenitor-derived DCs with a retrovirus encoding human interferon-γ resulted in a consistent decrease in colony forming units and lower proliferation of the progenitor cells and increase in major histocompatibility complex class II and class I expression *(5–7)*. In our laboratory, the level of murine IL-12 p *(70)* produced by DCs 24 h after the first transduction is as high as 20 ng/mL *(6)*.

2. In many instances, the virus-derived vectors or the ampicillin resistance genes in the plasmid vector can contribute to Ags presented by the transduced cells and induce vector/viral-related immune response *(8)* . A simple alternative is to use retroviral vector without a selection marker or to use a retrovirus vector without any cloned products as a control *(5–7)*. We have found that, the smaller the cloned fragment in the retroviral vector, the higher the expression of the protein product. For protein products whose biologically active form is a heterodimer, one can construct bicistronic fusion constructs in which one product is driven off the 5'-LTR and the second gene is driven of an internal ribosomal entry site element. Alternatively, one can fuse two genes with a linker sequence *(9)*.

3. Biological safety issues must be considered when working with retroviruses, and the biosafety instructions regarding disposal and handling and helper virus assay must be obtained from the commercial source or investigator who supplies the retrovirus vector and packaging cell line *(1,2)*.

Acknowledgment

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8

Use of Vaccinia Virus Expression Vectors to Investigate Antigen Processing and Presentation

E. John Wherry, Deepa Rajagopal, and Laurence C. Eisenlohr

1. Introduction

Vaccinia virus (VV) has been used extensively to study the generation of antigenic (Ag) peptides by the major histocompatibility complex (MHC) class I and class II (MHC-I and MHC-II) Ag processing pathways *(1–5)*. The ease with which recombinants can be constructed makes VV ideally suited to assess the impact of alterations in Ag structure, intracellular location, and expression levels on processing and presentation by MHC-I and MHC-II molecules. In addition, the wide tropism of VV allows for Ag expression in a variety of cell lines and in vivo applications.

VV is a large double-stranded DNA virus that can easily accommodate insertions of up to 25 kb. Extensive characterization of the mechanisms of transcription and the viral life cycle allows the choice of numerous well-described promoters active during different phases of viral replication *(6–9)*. Further, VV can be stored at –80°C almost indefinitely, and remains relatively stable at 4°C. The lytic nature of VV infection may be undesirable for some applications. In these cases, antigenically similar nonlytic viruses, such as modified vaccinia virus Ankara (MVA) *(10)* may prove useful. Alternatively, many cytopathic effects of the late phase of VV replication may be avoided by blocking DNA replication, and thus the late (lytic) phase of the viral life cycle, with cytosine β-D-arabinofuranoside (ara-C).

There are many variations of the methods described in this chapter, and many optimizations can be determined experimentally. The following protocols describe basic methods for the generation and plaque purification of a recombinant VV (rVV), a simple standard method of titering the virus, and methods to confirm the integrity of the recombinant, with emphasis on techniques neces-

sary for T-cell assays. In addition, protocols for standard assays used to assess in vitro and in vivo MHC-I and MHC-II restricted T-cell responses using VV as an expression system are described.

1.1. General Considerations

Whenever possible, polypropylene 15- and 50-mL conical tubes should be used to minimize the chance of virus leakage caused by cracking of polystyrene tubes. In addition, virus particles tend to aggregate when frozen. Thawed virus must be sonicated to disrupt viral aggregates.

1.2. Safety Considerations

Institutional guidelines should be considered before working with VV. Some institutions require vaccination prior to laboratory handling of VV. In all cases, proper precautions should be taken. Any waste containing VV should be autoclaved, treated with a solution of 10% bleach, or both.

2. Materials

2.1. General Needs

All chemicals not specifically noted can be obtained from Sigma (St. Louis, MO).

1. Tissue culture needs (plates, flasks, media, and so on).
2. BSS-BSA: 0. 15 M NaCl, 5 mM KCl, 1.22 mM MgSO$_4$, 1.3 mM K$_2$HPO$_4$, 0.74 mM KH$_2$PO$_4$ (mix K$_2$HPO$_4$ and KH$_2$PO$_4$ and adjust to pH 7.2), 10 mM HEPES, pH 7.2, 2.5 mM CaCl$_2$, 0.1% w/v BSA (*see* **Note 1**).
3. Fetal calf serum (FCS).
4. Dulbecco's modified Eagle's medium (DMEM) + 5% FCS for tissue culture.
5. Sonicator (e.g., Branson Sonifier 450)
6. ara-C.

2.2. Recombination and Plaque Purification

1. Wild type (WT) VV (WR strain, American Type Culture Collection [ATCC] WR-1354). The WR-1354 strain is the most commonly used strain for generating recombinants, but other strains, such as Wyeth or Lister, have also been used.
2. VV recombination plasmid (e.g., pSC 11 *see* **ref.** *11*).
3. CV-1 cells (ATCC CCL 70; African green monkey kidney cells).
4. 143B TK⁻ cells (ATCC CRL 8303; Human Osteosarcoma thymidine kinase [TK⁻] cells).
5. 5-Bromo-2'-deoxyuridine (BrdU, Boehringer Mannheim, Indianapolis, IN).
6. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; solutions in dimethyl sulfoxide are stored as aliquots in –20°C in amber vials).
7. Low melting point agarose (LMP) (Gibco-BRL, Grand Island, NY). Make up a 1.8% solution in sterile water, melt in a microwave and autoclave.

8. Eagle Minimum Essential Medium (EMEM), 2X (with sodium bicarbonate and L-glutamine, without phenol red; Gibco-BRL).
9. CellPhect Transfection Kit (Pharmacia, Piscataway, NJ).
10. Crystal violet: 0.1% solution in 20% EtOH and water.

2.3. VV Sequencing

1. Igepal CA-630 (Noted by Sigma to be indistinguishable from NP-40).
2. Polyoxyethylenesorbitan monolaurate (Tween-20).
3. Proteinase K.
4. Oligonucleotide primers designed to anneal to VV recombination plasmid, 2 pairs.

2.4. Cytoxic T-Lymphocyte Assay, Priming Assays, and Limiting Dilution Analysis

1. Inbred mouse strains.
2. 0.5-mL insulin syringes.
3. MHC-matched target cells.
4. Triton X-100 (Fisher Scientific, Pittsburgh, PA).
5. 2-Mercaptoethanol (2-ME) (Gibco-BRL).
6. Gentamicin sulfate solution.
7. Assay medium: RPMI-1640 + 10% FCS, 5.5 x 10^{-5} M 2-ME, 10 µg/mL gentamicin for spleen T-cell cultures.
8. An alternative method of restimulation (virus, transfectants, peptide).
9. Source of interleukin 2 (IL-2) (supernatant from G310 cell line, concanavalin A supernatant, rIL-2).
10. V-bottom 96-well plates (limiting dilution analysis [LDA] only, Costar, Corning, NY).
11. MVA *(10)*.
12. ^{51}Cr as Na chromate in NaCl solution (Amersham, Arlington Heights, IL).
13. γ counter.

2.5. Enzyme-Linked Immunospot

1. Enzyme-linked Immunospot (ELISPOT) plates, multiscreen 96-well filtration plate, 0.45 µm surfactant-free mixed cellulose, ester membrane (Millipore, France).
2. Anticytokine antibodies(Abs) (capture and biotinylated detection Abs, Pharmingen, San Diego, CA).
3. Horseradish peroxidase avidin D (Vector, Burlingame, CA).
4. Sigma Fast 3,3'-diaminobenzidine tablet set (DAB peroxidase substrate).
5. Coating buffer: 0.015 M Na borate, 0.15 M NaCl, pH 8.5.
6. Dilution buffer: PBS + 1% BSA.
7. Wash solution: PBS + 0.25% Tween 20.
8. Blocking solution: PBS + 5% BSA.
9. Sealing film, seal plate for 96-well plates.

2.6. Class II Assays/LacZ Hybridomas

1. Potassium (K) ferrocyanide.
2. K ferricyanide (light-sensitive).
3. $MgCl_2$
4. 4-methyl umbelliferyl β-D galactoside (4MUG).
5. Glycine.
6. EDTA.
7. Formaldehyde.
8. Glutaraldehyde.

3. Methods

3.1. Generation of Recombinants

Recombinant VV can be generated easily by simultaneous infection of CV-1 cells with WT VV and transfection with a rVV plasmid containing the gene of interest. Disruption of the TK locus of WT VV in successful recombinants allows selection using the thymidine analog BrdU. Additionally, the plasmid may contain a second marker (often the *lacZ* gene) for selection of recombinant plaques, using a chromogenic substrate. After infection/transfection of CV-1 cells, three rounds of plaque purification on 143B TK⁻ cells are performed to isolate a pure recombinant virus (blue plaques if using *lac Z)* from the background spontaneous TK⁻ mutant VVs (*see* **Note 2**). In total, the generation of a recombinant virus from recombination to expansion and titering requires approx 3 wk.

1. Plate CV-1 cells at a density of 4.2×10^4 cells/cm^2 of surface area (approx 4×10^5 in a well of a 6-well plate), and allow to adhere overnight.
2. Remove the media from the well and add enough sterile BSS–BSA to cover the well (approx 0.4 mL for one well of a 6-well plate) and approx 2.5×10^4 PFU (0.05 PFU/cell) wild type VV. Place in incubator, and rock plate every 15–20 min.
3. After 90 min, begin DNA precipitation, using the CellPhect Transfection Kit. Plasmid DNA should be purified using Qiagen or other techniques. Add 3.4 µg DNA in 40.7 µL sterile water to 40.7 µL CellPhect buffer A. Mix by flicking, and allow to sit for 10 min. Add 81.4 µL CellPhect buffer B, vortex at midspeed for 3 s and allow to sit for 15 min. Suction virus from well. Add 1.35 mL DMEM with 5% FCS and 10 µg/mL gentamicin to well. Add all of the calcium phosphate–DNA precipitate to well, and place in the incubator.
4. After 2–3 d, when there is complete destruction of the cell monolayer, harvest the CV-1 well. Use a TB syringe plunger to gently scrape the cells from the well (*see* **Note 3**). Transfer the cells and media to a sterile 1.5-mL Eppendorf tube, and spin at 3000*g* for 1–2 min. Remove the supernatant, add 0.2 mL sterile BSS–BSA and subject the resuspended virus–cell mixture to three rounds of freezing on dry ice, thawing in a 37°C water bath, and vortexing for 1 min, sonicating for

1 min at 50% pulse in a Branson Sonifier 450 cup sonicator (or equivalent) before the last vortex.

5. Plaque purification. Plate 143B TK⁻ cells at a density of 4.2×10^4 cells/cm² in a 6-well plate, and allow to adhere overnight (for this initial plaquing, plating of 143B TK⁻cells at 8.4×10^4 cells/cm² may result in better separation of, but smaller, plaques). On day 0 infect 143B TK⁻ cells with dilutions of the CV-1 extract (although not essential, a brief spin at 3000*g* will pellet cell debris, leaving virus in suspension). Remove media from 143B TK⁻ cells, and add 450 µL sterile BSS–BSA. Infect one well with a 1:10 dilution of the CV-1 lysate by adding 50 µL CV-1 extract. Mix well, and transfer 50 µL to the next well, for a 1:100 dilution of the lysate (usually 2–3 dilutions are enough). Place in the incubator for 2 h, rocking every 15–20 min. Carefully remove BSS–BSA and add mixture of 2X EMEM (supplemented with 10% FCS and 10 µg/mL gentamicin), sterile 1.8% LMP, and 25 µg/mL BrdU (from a sterile 5 mg/mL stock in H_2O). For one well, this will be 0.75 mL 2X EMEM (prewarmed in a 45ºC water bath) + 0.75 µL LMP (melted and equilibrated to 45ºC in a water bath) + 7.5 µL BrdU (*see* **Note 4**). Add 1.5 mL/well, and allow to cool completely before returning to the incubator (if the overlay is not completely solid before returning to the incubator, it will remain semisolid and prevent proper plaquing).

6. On the morning of d 3, overlay wells with X-gal. Melt LMP, and equilibrate LMP and 2X EMEM as above. Wells will be overlaid with 1 mL total of a mix of 0.5 mL 2X EMEM + 0.5 mL LMP + 5 µL X-gal (5% w/v in DMSO). Add overlay, allow to cool and return to incubator. Blue plaques should appear within a few hours (but occasionally take longer).

7. Picking and labeling plaques. Spontaneous TK⁻ mutant viruses will yield background nonrecombinant (clear) plaques in the presence of BrdU. It is important to identify recombinant plaques that are as isolated as possible for further purification. Additionally, occasional erroneous recombinations may occur. Pick isolated blue plaques as soon as they can be identified (*see* **Note 5**). Identify and circle three isolated blue plaques, labeling the most isolated or most promising "A", the next "B", and third "C." The largest plaques do not always result in the most efficient plaquing during the next round. Pick the plaques, using a sterile 200–1000 µL pipet tip attached to a bulb. Be sure to pick around the edges of the plaque, and carefully scrape the bottom of the plate with the tip. VV grows as a cell-associated virus spreading from cell to cell via membrane contact. Therefore, the majority of the virus in a plaque will be around the edges of the plaque, where there are cells actively producing virus, not at the center of the plaque where there are few cells remaining. Transfer plaques to sterile 1.5 mL Eppendorf tubes with 0.5 mL sterile BSS–BSA. Freeze–thaw–vortex, and sonicate as above, and repeat the plaquing procedure, using two dilutions for each plaque, (usually 50 and 5 µL are sufficient [A, B, and C]). Again, pick three plaques, and label similarly (thus second-round plaques will be named AA, AB, AC, BA, and so on, according to which wells yield the best plaques). Repeat for a third round. Usually,

picking three plaques/round is sufficient, and the labeling system indicates which plaques are most desirable. Thus, plaque ABA would be more promising than BBA. The virus can then be expanded and sequenced, or tested to confirm the integrity of the inserted gene.

8. Viral expansion. Once a pure recombinant plaque is identified, approx 200 µL of the disrupted plaque can be added directly to a 6-well plate of 143B TK$^-$ cells seeded at 4.2×10^4 cells/cm^2 (*see* **Note 6**). After 2 d the well is scraped and harvested, as was done for the CV-1s above. After the three rounds of freeze/thaw/vortex and a brief spin, add most of the extract to more 143B TK$^-$ wells, to amplify the virus (saving a small amount as a backup). This extract can be used to infect a T-75 flask seeded 1d previously with 143B TK$^-$ cells at 8.5×10^4 cells/cm^2 (approx 6.6×10^6 cells/flask). Harvest after 2 d of infection (*see* **Note 7**) using a cell scraper, transfer to a sterile 50 mL polypropylene conical and centrifuge for 5 min at 1500g. Remove the supernatant and resuspend in 1 mL BSS–BSA, freeze–thaw–vortex three rounds, sonicate, and spin at 1500g. Transfer the supernatant to a new tube, vortex gently for 20 s and aliquot in sterile microcentrifuge tubes and store at –80°C (*see* **Note 8**). During virus expansion spontaneous TK$^-$ mutants (nonrecombinants) may outgrow the desired recombinant *(12)*, particularly if the recombinant protein expressed at high levels has the potential to interfere with cell growth (*see* **Note 9**).

3.2. Titer

Viral titers of 10^7 to 5×10^8/mL are achievable following expansion in 143B TK$^-$ cells. Titers above 7.5×10^7 are preferred, minimizing the volume of virus used in assays (decreasing the potential adverse effects of the cellular extract). To this end, high-titer virus usually begets higher-titer stock (*see* **Note 10**). HeLa cells adapted to grow in roller bottles can also generate large amounts of high- titer virus, as an alternative method; however, it will often be inappropriate to compare viruses that have been expanded in different cell types. In some cases the addition of trypsin to the cell pellet before the three rounds of freeze-thaw-vortex may yield higher titers (presumably by releasing cell associated virions). However, the trypsin remains in the virus stock, and the effects on subsequent experiments may be detrimental. We avoid this practice.

Titering VV is performed on 143B TK$^-$ cells allowed to adhere overnight in 6-well plates. There can be some variability associated with titering. For this reason new 143B TK$^-$ cells should be thawed approximately every 6–8 wk. To further ensure accurate titers, all viruses to be used in a particular experiment should be titered together or against a standard and their titers adjusted.

1. Plate 143B TK$^-$ cells at 4.2×10^4 cell/cm^2 in 6 well plates, one plate/virus to be titered. Allow to adhere overnight.
2. Remove an aliquot of virus to be titered from –80°C, thaw, and sonicate as above.

3. Each virus should be titered in true duplicate (two separate dilution series). For each dilution series, we use three wells of a 24-well plate (6 wells/virus) to dilute virus in DMEM with 5% FCS 10^2-,10^4- and 10^6-fold (additional dilutions may be needed for purified or very-high-titer virus). A general procedure is as follows: Add 10 µL of virus to 990 µL media in one well. Mix thoroughly with a P1000 set at ∪800 µL by pipeting up and down 10×. Transfer 10 µL to a second well containing 990 µL media. Mix. Transfer 15 µL to a third well containing 1485 µL media. Mix, and add exactly 500 µL from the third well to a well of 143B TK⁻ plate (no need to remove the media from the plate first). Repeat the titration for accuracy (*see* **Note 11**). A single replicate for each dilution is probably sufficient, but two wells for each may be used if necessary. These wells will be used to assess the titer by overlaying with crystal violet. Transfer the remainder to a third well to be overlaid with X-gal. The volume added to this well is not as critical, because it will only be used to check for spontaneous TK⁻ mutants, not to calculate a titer. Incubate for exactly 2 d at 37°C. Although VV grows mostly as a cell-associated virus, movement of plates during this incubation should be avoided. Cells or virus particles may become dislodged and seed secondary plaques. Waiting until d 3 to overlay also results in an increase in secondary plaques.

4. Remove media, and overlay crystal violet wells with 0.1% crystal violet, 20% EtOH, incubate 5 min, and remove. Overlay the X-gal wells as above. Incubate until blue appears. Count plaques in crystal violet wells (marking counted plaques with a pen), and calculate the titer (average number of plaques/well multiplied by the dilution factor [10^6 in this case]/amount transferred to the well [titer = {number of plaques} {10^6} /{0.5 mL}]).

3.3. Verification of Recombinant

Numerous methods are available to confirm insertion into the VV genome and/or expression of the desired protein. If the recombinant gene product is easily detected using Abs, then immunofluorescence, western blot, and immunoprecipitation may offer rapid and convenient methods to screen for expression of the desired protein. If Abs are not available, or if confirmation of the nucleotide sequence is necessary, polymerase chain reaction (PCR) amplification of VV DNA, followed by sequencing, offers a straightforward method of assuring that the gene of interest is inserted. This method is similar to that used by Roper et al. *(13)* and is described below. However, alternative methods, including Southern, Northern, and dot blot hybridization, may be used to assess recombination and gene expression *(14,15)*.

The method below uses two pairs of primers annealing to regions of the recombination plasmid outside the gene of interest. The first set is used to PCR amplify the appropriate region of DNA, and the second set (annealing inside the first set) for forward and reverse sequencing reactions.

1. Inoculate a 143B TK⁻ well with a small amount of stock virus (1–10 μL). Allow infection to proceed (2–3 d) until near 100% cytopathic effect. Scrape well as above, and transfer 10 μL to 1.5-mL microcentrifuge tube.
2. Add 10 μL of a mixture of 0.9% Igepal CA-630 (NP40 equivalent), 0.9% Tween 20 and 1.2 μL 5 mg/mL proteinase K. Incubate 30–60 min at 37°C or 30 min at 45°C.
3. Heat inactivate the proteinase K at 94°C for 10 min.
4. Spin at 14,000*g* for 10 s and use 10 μL supernatant in a 100-μL PCR reaction, using the first set of primers, which should anneal far enough from the start and stop sites of the inserted gene to allow the nested set of sequencing primers to bind optimally.
5. Gel-purify the PCR product, and purify the DNA. Sequence the purified product using the second set of primers. These primers should anneal more than 20 bp from the start and stop sites, but still inside the first set of primers. The primers used for PCR can also be used for sequencing, however, primers annealing to the end of a PCR product may result in inconsistent sequences. Designing the 5' primer to allow assessment of the integrity of the promoter also is helpful.

This procedure can be performed directly on crude virus preps, although PCR seems to work more consistently on freshly grown virus. Additionally, this procedure can be used to sequence a virus scraped from a single plaque of a plate overlaid with agar.

3.4. In Vitro Class I-Restricted Chromium (^{51}Cr)-Release Assay

A standard ^{51}Cr-release assay offers a simple method for determining presentation of correctly processed and presented MHC-I-bound peptides, using cytotoxic T lymphocytes (CTLs). Expression of recombinant proteins by VV allows assessment of the impact of intracellular location of, mutations in, and the expression levels of the Ag-processing starting substrate on the generation of CTL-recognized epitopes. Epitope-specific CTL populations can be generated by priming mice and restimulating in vitro, or by maintaining CTL clones. If performed efficiently, this assay requires approx 10–11 h. A longer period of time from the start of infection can result in substantially elevated spontaneous lysis and variable results, because of the lytic nature of the late phase of VV replication.

1. For an assay using a single effector population, approx 5×10^5 MHC matched target cells (this number accounts for some loss caused by the infection and washes) are infected with 10 PFU/cell of the recombinant VVs to be tested (*see* **Note 12**). The appropriate volume of virus is added to 15-mL conicals, and is adjusted to 200 μL total with BSS–BSA. Target cells are set at 2.5×10^6/mL and 200 μL is added to the 200 μL of virus + BSS–BSA. Cells are allowed to infect at 37°C on a rotator for 1 h. The assay can easily be scaled-up for more than one effector population. If MHC-matched target cells are unavailable, targets may be co-infected with separate VVs expressing the appropriate restricting element and

the Ag of interest (usually 5 PFU/cell of each virus; a total infection of more than 15 PFU/cell may result in elevated nonspecific lysis).

2. After 1 h, 2–10 mL complete medium (the same medium normally used for the target cells) is added to target cells, and they are rotated at 37°C for an additional 3 h (*see* **Note 13**).

3. During the 3 h incubation, 96-well plates containing the effector populations are set up. Three replicates for each of four dilutions of effector: target (E:T) ratios are plated, by adding 150 μL effectors in assay medium to the top row of the plate, and diluting 50 μL into 100 μL of assay medium in the next three rows, to generate 1:3 dilutions of effectors. 50 μL is discarded from the fourth row. For each target, also set up (on a separate plate) 5 wells with 100 μL assay medium and 5 wells with 100 μL 1% Triton X-100 in PBS for measurement of spontaneous and maximal ^{51}Cr release. Place plates in 6% CO_2 incubator until addition of targets.

4. After the 3 h incubation, spin the infected targets for 5 min at 1000*g*. Aspirate as much of the media as possible (*see* **Note 14**). Label cells in 100 μCi ^{51}Cr/l0^6 cells in 50 μL assay medium/10^6 cells. Resuspend the pellet by flicking or scraping the tubes before adding ^{51}Cr. Add ^{51}Cr and return to rotator at 37°C for 1 h (longer if necessary).

5. Centrifuge targets for 5 min at 1000*g*. For disposal purposes, it may be helpful to separate high-activity and low-activity waste. Remove high-activity ^{51}Cr to an appropriate container by pipeting. Resuspend pellet by flicking or scraping and add 5–10 mL cold PBS, and centrifuge again. Decant PBS wash (low-activity waste) in appropriate container (*see* **Note 15**). Repeat wash.

6. Resuspend cells in appropriate volume of assay medium (*see* **Note 16**). Plate out 100 μL targets into wells containing effectors and mediums and totals, using multidosing pipet (*see* **Note 17**). Wash multidoser with H_2O between targets. Incubate plates at 37°C, 6% CO_2 for 4 h.

7. Remove plates from incubator carefully to avoid disturbing the cells that have settled to the bottom of each well. Although usually unnecessary, plates can be spun briefly to ensure no cells are harvested with the supernatant. Transfer 100 μL supernatant from each well to γ counter tubes, and chase with 100 μL 10% bleach, using a multichannel pipet. To avoid differences in the total time of co-incubation, plates should be harvested in the same order as they were plated out. Measure ^{51}Cr release using a γ counter.

8. Calculate percent lysis by:

Percent Lysis = [(Experimental – Spontaneous)/(Maximal – Spontaneous)] × 100

Several alternatives to this method are noteworthy. First, infection/transfection can sometimes be used to first screen plasmids, before making rVVs *(16)*. In this method, target cells are infected with WT VV for 90 min then transfected with the VV recombination plasmid using Lipofectin Reagent (Gibco-BRL). However, this method is challenging as high transfection efficiency, not possible with many cell types, must be achieved. L929 murine fibroblasts can be transfected

efficiently using this technique. Second, MHC-I-restricted T-cell hybridomas (T_{Hyb}) offer an alternative readout to ^{51}Cr release. The *lacZ*-transfected T_{Hyb} pioneered in the Shastri laboratory (Berkeley, CA) offer another method, and are described in more detail in **Subheading 3.6.2.** below *(17)* and in Chapter 22. Assays using these T_{Hyb} for class I-restricted responses are identical to the class II assays described below.

3.5. In Vivo Class I-Restricted Assays

In many cases it is desirable to assess the generation of a CD8^{+} T-cell response in vivo. The general capacity to prime for an epitope-specific response can be assessed relatively easily, using VV. In addition, the number of epitope-specific CD8^{+} T-cells activated in vivo can be directly quantitated, using LDA *(18,19)* and interferon (IFN)-γ ELISPOT *(20)*. Intracellular staining for IFN-γ *(21),* and staining with MHC-I/peptide tetramers *(21,22)* offer alternative techniques for measuring CTL population sizes, but are not discussed here.

3.5.1. Priming Assay

1. Dilute all viruses to appropriate PFU in 250 µL BSS–BSA. Generally, 10^7 PFU/ mouse is an appropriate starting dose, but, to see relevant differences in priming capacity, dilutions of virus will probably need to be used.
2. Prime mice intraperitoneally, using insulin syringes (*see* **Note 18**). After 2–3 wk spleens can be removed. Primary ex vivo CTL activity can be detected in some cases, but usually an in vitro restimulation is necessary. For restimulation, a second method of epitope presentation is necessary. The use of synthetic peptide is a common method of restimulating for an epitope-specific CTL response. Peptide can be added directly to cultures in which it will bind empty cell-surface class I molecules (*see* **Note 19**). Alternatively, cells transfected with Ag can be irradiated and added to the culture, or a second virus expressing the Ag of interest can be used to infect a percentage of the spleen cells as stimulators. As an example, the following steps focus on the use of influenza virus-infected spleen cells for restimulation.
3. Homogenize, wash, and count spleen cells. If comparing different primings, spleen populations must be adjusted to the same cell density and total cell number/flask during restimulation.
4. For each priming, two-thirds of the cells will be used to generate epitope-specific CTL, and one-third for VV-specific CTL, which will be used to confirm equal priming. Naive spleen cells are used as stimulators, and added to the responding spleen populations at a 1:3 ratio of stimulator to responders. Pellet the fraction to be used as stimulators, and infect those to be used for VV stimulators with WT VV (approx 1 PFU/cell) + an equivalent volume of BSS–BSA, and those to be used for influenza epitope stimulators with 0.5 mL influenza (allantoic fluid, approx 2000 hemagglutinating units (HAU) for $0.3\text{-}1 \times 10^7$ cells) + 0.5 mL BSS–

BSA. Incubate at 37°C (or room temperature for flu) for 1 h on rotator or shaking every 15 min.

5. Restimulations are performed at 6–7 d in T-75 flasks (40 mL total volume assay medium) for epitope-specific, and T-25 flasks (12 mL total volume) for VV-specific, populations. At the end of the 1 h infection add 10 mL BSS–BSA and spin the infected antigen-presenting cells (APC). Resuspend in assay medium, and add to the responder populations in flasks in the appropriate total volume.

6. Assess CTL activity using ^{51}Cr-release assay, as in **Subheading 3.4.**

3.5.2. Limiting Dilution Analysis

A more quantitative estimate of Ag-specific cytotoxic T lymphocyte precursors (CTLp) elicited in vivo **c**an be obtained using LDA. LDA underestimates the true frequency of Ag specific precursors by 5–100-fold *(21)*, but the data regarding kinetics, duration, and relationship between responses is equivalent to data from systems using MHC tetramers or intracellular cytokine staining *(23)*. In addition, LDA allows assessment of the cytolytic capacity of a CTLp population. In some cases, Ag-specific CTLp frequencies may be at the lower limit of detection for flow cytometric techniques. Therefore, LDA is described briefly below.

1. Prime mice as for a priming assay.
2. Remove spleens, homogenize, wash, and count, as above. Naïve cells from an unprimed spleen will also be used as APC.
3. Irradiate the cells to be used as APC, and infect or pulse with peptide (*see* **Note 19**). The lytic nature of VV may adversely affect the restimulation of low numbers of CTLp in microliter wells. To avoid these problems, the antigenically related, but nonlytic, MVA *(10)* may be used.
4. Make 4–8 dilutions (24 or more replicates each) of the responder populations and plate out in 96-well V-bottom plates (100 µL/well) (*see* **Note 20**).
5. Spin and wash infected (or peptide pulsed) APC and resuspend in assay medium at a concentration of 2.5–5 × 10^6/mL with 2X IL-2 (usually 10–40 U/mL, but it should be determined experimentally) and add to plates with responders for a total of 200 µL/well.
6. After 6–7 d wells can be tested for cytotoxic activity vs peptide-pulsed or infected targets. Each well will be split two ways, to assay for epitope-specific (or VV-specific) lysis and for background lysis, against unpulsed or uninfected targets. Enough MHC-matched targets for 10^4 targets/well are labeled with ^{51}Cr at a concentration of 100 µCi/10^6 cells in a volume of 50 µL/10^6 cells in the presence of 10^{-6} peptide or 5–10 PFU/cell MVA for 1 h.
7. Split wells two ways. Because some volume may have evaporated, 80 µL from each well is added to 20 µL fresh media in 96-well round-bottomed plates.
8. Labeled cells are washed as for a normal ^{51}Cr release assay and plated out into wells containing the appropriate populations of effectors. Mediums and totals are plated as for the ^{51}Cr release assay above. Plates are incubated at 37°C for 7–8 h to allow detectable lysis even by low numbers of effectors from the restimulation.

9. Harvest the assay as above, and calculate percent lysis for each well.
10. Positive wells are identified as those that cause lysis of the appropriate targets (infected or peptide pulsed) 3 standard deviations above the mean lysis of the wells of effectors incubated with control targets (no peptide or uninfected) for that group. Wells that cause significant lysis of control wells are eliminated from consideration. Precursor frequencies can be calculated by linear regression analysis using the cutoff of 37% of negative wells to give an estimate of frequencies. However, the X^2 method described by Taswell *(24)* will give a more accurate estimate of frequency.

3.5.3. ELISPOT

Because LDA underestimates the true frequency of CTL precursors, alternative approaches are becoming popular. MHC-I tetramers and intracellular cytokine staining (mentioned above) offer the advantage of allowing analysis of Ag-specific populations by flow cytometry, but may require high precursor frequencies to allow detection (roughly 1:1000–5000). ELISPOT analysis allows quantitation of smaller populations of CTLp with accuracy equivalent to fluorescence-activated cell sorting techniques *(21)*.

1. Prime mice as above.
2. One d before removing spleens, coat the wells of a 96-well nitrocellulose-backed plate with an optimal concentration of anticytokine Ab (10–40 µg/mL in freshly made coating buffer).
3. The following day discard the coating Ab, and wash the plate 3 X with wash buffer (PBS + 0.25% Tween 20). Dry plate (*see* **Note 21**), and coat each well with blocking solution (PBS + 5% BSA). Incubate 30 min at 37°C.
4. Discard blocking solution. Wash 3 X with wash solution, and add complete medium, to rinse away any remaining Tween-20. Incubate 10 min at 37°C.
5. Remove spleens, homogenize, lyse red cells using standard procedures, wash, and count remaining spleen cells.
6. Harvest MHC-matched stimulator cells (spleen cells from naïve mice may also be used as stimulators). Stimulators are pulsed with peptide, assuring that only the response of MHC-I restricted cells will be measured. The VV CTLp can be assessed by infecting stimulator cells with WT VV. This may detect responses of CD4+ cells, but will still allow confirmation of equal priming for the overall anti-VV immune response. Alternatively, infection of cells with a second virus expressing the same Ag or use of cells transfected with the Ag of interest may be useful. Pulse cells with peptide or infect with virus for 1 h.
7. Wash and resuspend target cells at 5×10^5–5×10^6/mL (10^4–10^5/50 µL) in complete media. The optimal density of target cells/well should be determined experimentally. Discard media from plate and dry. Add 50 µL target cells to appropriate wells.
8. Wash spleen populations several times, and set dilutions to appropriate concentration, usually 5×10^6/mL (2.5×10^5/50 µL) is a good starting concentration for

epitope-specific responses and $5 \times 10^4/50$ μL for VV-specific responses. Add 50 μL splenic populations to appropriate wells and incubate at 37°C 18–24 h.

9. Wash plate extensively (9 X) with wash solution and dry.
10. Add secondary biotinylated anticytokine Ab (4 μg/mL), and incubate for 2 h at room temperature.
11. Wash plate 6 X and dry.
12. Add optimal concentration of avidin-peroxidase (or other developing reagent), and incubate for 2 h at room temperature.
13. Wash plate 5 X with wash solution, and once with water.
14. Add developing substrate. Once spots have developed, wash plate with water, and allow to dry (*see* **Note 22**).
15. Count spots, using a dissecting microscope, and calculate CTLp frequencies (*see* **Note 23**).

3.6. In Vitro Class II-Restricted Responses

Studying MHC-II-restricted Ag processing and presentation using VV provides challenges that are not encountered in MHC-I-restricted assays. The traditional methods of measuring class II-restricted responses in vitro are proliferation of, and/or IL-2 production by, T-cells from a primed mouse or T-cell hybridomas. In our hands, VVs are not suited for such assays, presumably because of the lengthy culture periods (2–3 d) with a lytic virus. This problem might be alleviated by making the virus replication incompetent. This can be achieved by psoralen-UV treatment *(25,26)* or by use of the nonlytic MVA *(10),* but we have not tested these possibilities. Instead, we prefer to use assays of shorter duration which use either a cytolytic assay similar to the ^{51}Cr-release assay described in **Subheading 3.4.** utilizing class II-restricted T-cell clones, or the assay devised by Shastri et.al. employing T_{Hyb} that produce β-galactosidase (β-gal) upon activation *(17; see* Chapter 22*)*.

An additional caveat for using VV in class II-restricted T-cell assays is that purified virus must be used. Crude virus preparations can contain exogenous Ag and may complicate the interpretation of results. Purification of virus may be performed by centrifugation on a sucrose gradient, as is well described elsewhere *(27)*.

3.6.1. The Cytotoxic T-Cell Assay

The lower cytolytic activity of primary CD4$^+$ cultures, compared to CD8$^+$ cultures, has been attributed to lower perforin expression, as well as lower precursor frequency of Fas ligand-expressing cells *(28)*. The cytotoxic assay using class II-restricted clones, has, therefore, been shown to require Fas-expressing target cells. The longer assay time usually used for class II-restricted assays may allow upregulation of Fas ligand, however, the lytic nature of the VV would then be problematic (*see* **Note 24**). Therefore, assays

are done in a fashion similar to those for class I-restricted responses, except the co-incubation of effectors and targets is much shorter than traditional class II assays, to avoid nonspecific lysis caused by the VV infection, but somewhat longer than the class I-restricted assays, to allow Fas–Fas ligand-mediated killing. Such an assay is described in brief below:

1. Target cells (2×10^7/mL) are coincubated with various dilutions of the virus to be tested for 1 h at 37°C in BSS–BSA in conical tubes.
2. Make up the volume to 5 mL with assay medium, and incubate for an additional 4 h at 37°C.
3. Label the cells as described for class I assays.
4. Add 1×10^4 radiolabeled targets to round bottom well plates, then add effector cells to obtain the desired E:T ratios.
5. Incubate for 12–14 h at 37°C, and harvest half of the supernatant. The percentage specific lysis is calculated as described earlier.

3.6.2. Lac Z Inducible T_{Hyb}

The *lac-Z* inducible Ag–MHC complex specific T_{Hyb} provide a convenient system of assay for the study of both class I- and class II-restricted Ag presentation. The assay is primarily based on the development of T_{Hyb} which express β-gal upon recognition of the cognate MHC–peptide complex. This has been made possible by the development of a fusion partner, BWZ.36, transfected with the nuclear factor of activated T-cells (NFAT)–1acZ construct *(17)*. For optimal use in the VV system, however, the VV recombination plasmid should contain an alternate selectable marker such as β-glucuronidase *(10)*. Alternatively, ara-C can be added, to block expression of β-gal expressed from a late VV promoter, however, we get highly variable results using this approach, and the former method is preferred.

For generating class II-restricted *lacZ* inducible T_{Hyb}, CD4$^+$ T-cell clones provide a readily usable, and perhaps more effective, alternative to splenocytes from primed mice for the fusion procedure. The clones are routinely maintained by biweekly restimulations in the presence of irradiated virus-infected or peptide-pulsed, syngeneic spleen cells. The cells can be used for fusion 3 d following a restimulation. Several modifications of standard T-cell fusion procedures have proven useful, and are described in **Note 25**.

Maintenance of the T_{Hyb} is often challenging because of instability. The cause is unknown. Some T_{Hyb} are more stable than others, perhaps reflecting differences in the particular T-cell clone used. Repeated subcloning (in some cases more than 10 X) by limiting dilution is advisable, and can eventually result in a stable cell line. Once the T_{Hyb} are established they are routinely maintained in assay medium.

The assay procedure using *lacZ* inducible T_{Hyb} is described below. Several methods are available for detection of T-cell activation by measurement of β-gal activity. For additional information, readers should see **ref. *17*** and Chapter 22.

1. Infect APCs, as above, with 5 PFU/cell of the rVV and/or pulse with dilutions of the peptide to be tested.
2. Wash APCs once with BSS–BSA or PBS, and plate at 5×10^4 per well in 100 μL assay medium in a flat bottom 96-well plate.
3. Add specific T_{Hyb} at 1×10^5 cells per well in 100 μL assay medium and incubate plates overnight at 37°C, in 6% CO_2.
4. Spin plates at $200 \times g$ for 5 min. The medium is suctioned off gently, and 100 μL PBS added to each well. Plates are washed a second time in a similar manner. The cells are processed further, depending on the substrate used for the assay. Two methods for detecting activation of *lacZ* T_{Hyb} are given below.

3.6.2.1. DETECTION OF LACZ T_{Hyb} ACTIVATION: VISUALIZATION OF BLUE CELLS

1. Activated cells can be visualized using an insoluble substrate that is cleaved by β-galactosidase resulting in activated cells turning blue. Fix cells with 50 μL fixative (2% formaldehyde and 0.2% glutaraldehyde in water) for 5 min at 4°C. Wash once with PBS and add 50 μL/well of the substrate solution prepared as follows: To 10 mL PBS add 100 μL of 0.5 *M* K ferrocyanide, 100 μL of 0.5 *M* K ferricyanide (light sensitive), 1 mL of X-gal (10 mg/mL stock in DMSO), and 100 μL of 0.2 *M* $MgCl_2$.
2. Incubate plates overnight with substrate, and count blue cells microscopically.

3.6.2.2. QUANTITATION OF LACZ T_{Hyb} ACTIVATION, USING A FLUORESCENT SUBSTRATE

Counting blue cells is a tedious (and potentially error-prone) process. Quantitation of T-cell activation can also be assessed using the fluorescent substrate 4MUG.

1. The initial assay procedure remains the same except cells are not fixed. Instead, following overnight incubation, 50 μL of the following substrate is added directly to the cells. To 10 mL PBS, add 6.96 μL 5.5×10^{-2} *M* 2-ME, 100 μL 0.9 *M* $MgC1_2$, 1.25 mL Igepal CA-630 (NP40 equivalent) and 10 μL 4MUG (33 mg/mL stock).
2. Incubate plates for 4 h at 37°C, then add 50 μL/well of stop buffer (300 m*M* glycine and 10 m*M* EDTA).
3. Transfer 50 μL to a new plate. Read the fluorescence in a cytofluorescence plate reader at 360 nm (excitation) and 460 nm (emission) (*see* **Note 26**).

Class II-restricted in vivo experiments have not worked well in our hands. However, other groups *(29,30)* have used modified Ag or immunization proto-

cols to generate in vivo CD_4^+ T-cell responses using VV. Routing of the Ag to the endosomal/lysosomal compartment, using a modified Ag containing the endosomal sorting signal from the lysosomal associated membrane protein (LAMP) has been shown to enhance class II–peptide expression from recombinants *(29)*. In addition, expression of hen egg lysozyme linked to the invariant chain may result in more efficient class II-restricted Ag presentation in vivo *(30)*. Physical manipulation of the route of entry by subcutaneous or footpad immunization in Freund's adjuvant has also been reported to bring about efficient class II priming using VV *(29)*. Therefore, generation of a system to examine in vivo class II Ag-processing and presentation using VV may require Ag and or priming protocol modifications.

4. Notes

1. PBS–BSA (PBS with 0.1% w/v BSA) may be substituted for BSS–BSA.
2. Nonrecombinant spontaneous TK^- mutant VVs arise in the presence of BrdU. It is critical that plaque-purified recombinants be free of TK^- mutants. These mutants have a growth advantage compared to true recombinants, in many cases altering the effective dose of Ag expressed from a given titer of virus.
3. When harvesting virus from 6-well plates, especially early in recombinant selection (CV-1 harvest), it is critical to guard against accidental splashes from one well to another. Transfer of even a minute amount of virus may allow selection of the wrong recombinant in subsequent rounds of plaque purification. It may be helpful to cover other wells with the lid of the plate when harvesting one well.
4. Ensure that the melted LMP is not too hot before adding to the cells. Proper cooling in a 45ºC water bath ensures no cell damage occurs.
5. Extended incubation with X-gal may be somewhat toxic to cells or virus, as reflected by the subsequent plaquing. Therefore, when isolated blue plaques (as determined by microscopic examination) appear, they should be picked as early as possible. In addition, overlaying plaques at day 2 after infection can yield plaques for purification. However, plaques may take longer to become apparent (turn blue), and may not contain as much virus.
6. Expanding virus (and plaquing) on standardized numbers of 143B TK^- cells will yield more consistent and reproducible results. Plaquing can be performed in 6-well plates seeded 1 day previously, at a density of either 4.2×10^4 or 8.4×10^4 cells/cm^2 (this corresponds to 4×10^5 or 8×10^5 cells/each well of a 6-well plate; the latter results in smaller plaques, and may only be advantageous when plaquing from CV-1 extracts). The condition of the 143B TK^- cells can affect the expansion and titering of virus; therefore, overgrowth should be avoided, and fresh cells thawed every 6–8 wk. In addition, subcloning TK^- may help optimize viral expansion and/or titering.
7. Despite the fact that the cytopathic effect of infection often appears greater 3 d postinfection, in our hands, a higher-titer stock is recovered after 2 d.

8. After centrifugation of the cell debris from the virus stock, the virus-containing supernatant is transferred and vortexed in a new tube, before aliquoting. This step is taken because of the stratification of virus particles that may occur during high-speed centrifugation.

9. Expression of proteins with cytotoxic effects is possible using the VV system, but an alternative means of expression is necessary, since expression of the protein during generation and selection of recombinants would preclude viral replication. One such inducible system drives recombinant Ag expression from a T7 promoter, rather than a VV promoter. After plaque purification and expansion, target cells are co-infected with the recombinant and with T7 polymerase-expressing VV to assess the effect of expression of the desired protein.

10. In general, low-titer virus begets low-titer virus. Better results are usually obtained by successive smaller expansions, rather than one initial try at a large-scale expansion. The following procedure usually yields a good titer. First, expand most of the selected plaque-purified virus in 1–2 wells of a 6-well plate. Harvest as above and re-infect 3–6 wells of a 6-well plate with most of the extract. The extract from this harvest can be used to infect a T-75 flask. If good cytopathic effect is not seen, the T-75 extract can be used to re-infect a second T-75. If a larger stock of virus is needed, infecting a T-175 with most of the virus from a T-75 expansion will usually yield a reasonable titer. Viruses expressing proteins that are slightly toxic may be selected against with numerous rounds of expansion. In some cases it will be necessary to re-make the recombinant from the original plasmid, and re-expand the virus.

11. To ensure as much reproducibility as possible, specific pipets should be designated for titering purposes. Note, however, that most day-to-day variation in titering of the same stock will result from other factors (e.g., state of the cells used for titering).

12. The condition of the target cells used for CTL assays can have a dramatic impact on subsequent results. Cells should be growing at log phase at the time they are harvested for the assay. Extended periods of culture should also be avoided. Furthermore, some cells may require exceedingly high multiplicities of infection for productive infection.

13. At any point during the VV infection for a CTL assay, Brefeldin A (BFA) may be added to prevent further egress of MHC-I/peptide complexes to the cell surface *(31)*. In this way, the efficiency of presentation can be gauged. In addition, inclusion of various agents (e.g., protease inhibitors) may adversely affect T-cell function. Addition of BFA following ^{51}Cr labeling and washing can obviate the need to have the inhibitor present during the co-incubation with T-cells. Another area in which BFA is useful is the confirmation of equal target cell infection. The use of VV-specific CTL to confirm equal infection is complicated by the magnitude of the anti-VV response, often resulting, in similar killing, even at different E:T ratios. Addition of BFA at various times following infection may limit presentation such that a titration of specific lysis is observed with different E:T ratios.

14. The total volume in which target cells are resuspended for ^{51}Cr labeling can significantly impact the efficiency of labeling. Therefore, when removing media before labeling, it is important, not only to remove as much as possible, but also to be as consistent as possible from tube to tube. It is helpful, using vacuum suction, to go back to each tube a second time, after it has stood for 1–2 min, to remove any additional media that has drained from the walls of the tube.

15. In many cases, simply pouring off the PBS used to wash targets is sufficient. However, a single motion should be used, because any reflux may dislodge the cell pellet. In some cases, target cells do not form a tight pellet, and manually pipeting off the PBS is necessary to avoid loss of target cells.

16. It is possible to count target cells before plating out, to adjust to the desired cell density. However, the variability in counting may be as great as that from cell loss during the washes. If all tubes have been handled similarly, the total amount of ^{51}Cr released (maximal lysis with 1% Triton) will indicate whether the number of cells/well for each target was equivalent. In addition, the notion of an E:T ratio is somewhat arbitrary, using bulk population of CTL, because the number of true epitope-specific effectors is not known. One exception is comparing infected and uninfected targets. In this case, infected and uninfected cells may be lost to different degrees during washes, and counting before plating out may be of use.

17. When plating, targets should be added to the Triton-containing wells last. Imperceptible splashes from the detergent wells may cause very high lysis in the 1–2 wells plated out subsequently, as a result of detergent on the tip of the pipet. Washing the multidoser with water between targets is sufficient to remove any detergent, as well as to prevent carryover of cells from one target to the next.

18. The smaller the total volume injected, the better. Likewise, small-gauge needles, such as insulin syringes, work best. Both help reduce any reflux of inoculum at the injection site. Further, primed mice should always be caged separately from unprimed, to prevent any cross-contamination caused by virus leakage (which may be a problem with a large inoculum volume or large-gauge needle) or shedding (though unlikely with VV).

19. Many protocols can be used to restimulate in vitro effector CTL populations. The use of peptide or Ag-transfected cells will require a titration, to determine the optimal concentration of either in a restimulation culture. In addition, a source of exogenous IL-2 may be necessary. A common starting concentration for peptide restimulation is 10^{-6} *M* with the addition of 10–50 U/mL of IL-2. Transfected cells may be added (after irradiation) at a ratio of 1:25–1:50 (transfectants:spleen cells).

20. V-bottom 96-well plates allow for more efficient restimulation of CTLp than round bottom plates, perhaps because better cell contact is achieved.

21. After each wash of the ELISPOT plates, wells should be dried completely. Nitrocellulose wells should be removed from the plastic backing and allowed to air-dry, before reassembling the plate and continuing the procedure.

22. Available peroxidase substrate tablet sets are a convenient method for developing these assays. However, these substrates do not dissolve completely and must

be filtered (0.22 μm syringe filter) to reduce background specks. These may be further reduced by preparing the substrate mixture from the appropriate powdered reagents (also filtered before use).

23. To ease counting and storage of ELISPOT results, the nitrocellulose backing of the wells containing spots can be removed using adhesive film for microplates (*See* **Subheading 2.5., step 9**). Place the adhesive cover on the back of the dried nitrocellulose membranes, making sure to press firmly around the edges of each well. Carefully peel back the adhesive film, taking with it the membrane from each well. This can be challenging if the nitrocellulose is not firmly attached to the film.

24. Because Fas expressing target cells are necessary for assays using class II-restricted T-cell clones, selection of target cells for high Fas expression by flow cytometry can significantly improve assay results *(28)*.

25. The plating of cells following fusion should be done at a higher density than described. Irradiated splenocytes can be used as feeders, to increase the cell density/well. However, as an alternative, a larger number of T-cell clones and fusion partners may be used and plated out at a higher density/well. Also subsequent testing of the T_{Hyb} should be done only when the wells reach confluence (the growth can be seen visually).

26. Fluorescence of the substrate is light-sensitive, and therefore plates should be protected from light when necessary. Presence of air bubbles can also give rise to abnormal fluorescence values which may be corrected by brief centrifugation.

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9

Sindbis Virus-Based Vectors for the Study of Class I Antigen Presentation In Vitro and In Vivo

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1. Introduction
1.1. Alphaviruses

Alphavirus-based vectors have received considerable attention in the fields of antigen (Ag) presentation, epitope mapping, vaccinology, and gene therapy *(1–12)*, because of their relative ease of manipulation, purification to high titers, and ability to express large amounts of protein *(13)*. Several members of the Alphavirus genus, including Sindbis virus (SIN), Semliki Forest virus, and Venezuelan equine encephalitis virus, have been modified genetically for use as viral expression vectors for foreign Ags *(1,3,8,13–17)*. SIN virions are comprised of an icosahedral nucleocapsid surrounded by a plasma-membrane-derived envelope containing two viral glycoproteins, E1 and E2 *(18)*. The nucleocapsid contains a single stranded, positive (+) sense RNA gene of approx 11,700 nucleotides, which is capped and polyadenylated, and associated with 240 copies of the viral capsid protein.

Following viral entry into the cell, RNA replication occurs exclusively in the cytoplasm, and progeny virions mature by budding from the plasma membrane. Replication is mediated by four viral replicase proteins encoded within the 5' two-thirds of the genome, and proceeds through synthesis of a full-length complementary (–) strand RNA. The (–) RNA serves as a template for synthesis of two discrete RNA species: genome-length (+) strands for packaging into progeny virus and the more abundant subgenomic mRNA encoding the virion structural proteins. Transcription of subgenomic (SG) mRNA occurs from a highly active internal promoter, known as the SG or junction region (JR) promoter.
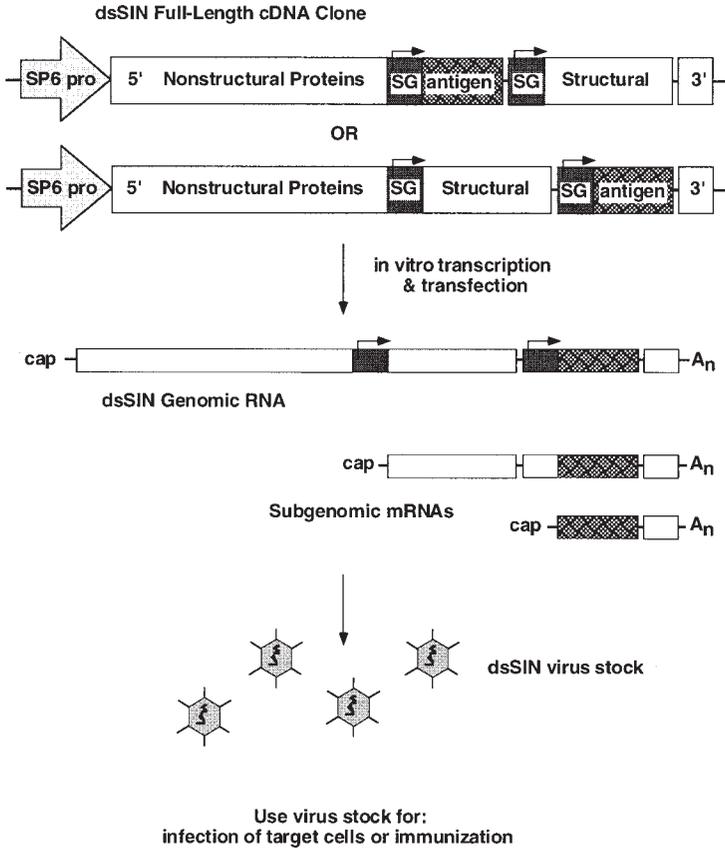
Fig. 1. Schematic for the generation of recombinant dsSIN vectors.

A common theme for all alphavirus-derived expression vectors is the use of this SG promoter for expression of heterologous genes.

1.2. SIN vectors

The construction of plasmids containing a full-length cDNA copy of the alphaviral genome, downstream of promoters for either prokaryotic or eukaryotic DNA-dependent RNA polymerases *(10,19)*, provided a molecular approach for modifying the viral genome to incorporate foreign genes. Infectious virus may thus be obtained by transfection of cells with either plasmids containing viral cDNA or with RNA transcribed in vitro. Three basic types of expression systems have been derived from such cDNA clones: replication-competent double SG promoter SIN vectors (dsSIN; *1*), replication-incompetent SIN replicons *(13)*, and layered plasmid DNA vectors harboring SIN

A

Vector Replicon

Genomic Defective Helper **+**

Split Defective Helpers **OR**

B

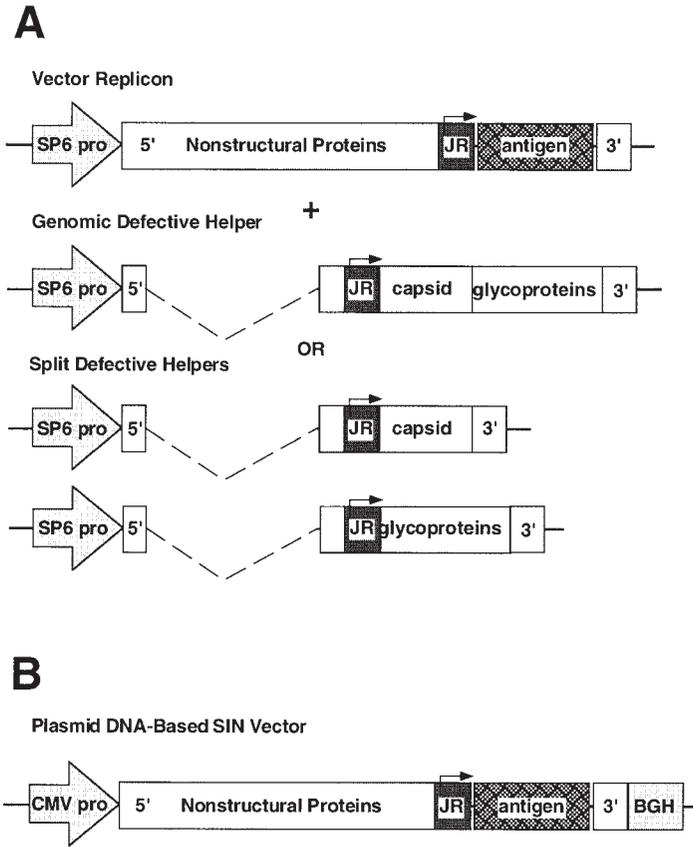**Plasmid DNA-Based SIN Vector**

Fig. 2. Schematic of SIN replicons and layered plasmid vectors.

replicons *(10)*. Generation of dsSIN viruses and SIN replicons require transfection of in vitro transcribed RNA while plasmid DNA vectors are directly transfected into susceptible cells. A brief description facilitating differential choice of these vectors for expression of Ags for major histocompatibility complex (MHC) presentation is provided below.

1.2.1. dsSIN Vectors

Recombinant dsSIN vectors that are both replication- and packaging-competent were constructed by insertion of an additional SG promoter into the viral 3'-end noncoding region, to drive expression of foreign genes (*1,16,18*; **Fig. 1**). In a subsequent generation of vectors, the second SG promoter was placed upstream of its natural location, preceding the SIN structural protein reading frame *(7,16,18)*. Heterologous sequences encoding a desired Ag are

inserted immediately downstream, and are therefore under transcriptional control of the second SG promoter. Because of the relatively large size of dsSIN vectors, limited cloning strategies are available for insertion of foreign DNA. Therefore, shuttle plasmids, with multiple cloning sites flanked by SIN sequences, are typically employed as cloning intermediates *(1)*.

In vitro transcription of dsSIN vectors using bacteriophage SP6 polymerase yields infectious RNA, which is transfected into susceptible cells to produce a high-titered stock of infectious virus. Replication results in transcription of two SG mRNAs, one from each promoter. Translation of the foreign Ag occurs directly from its specific SG mRNA at levels comparable to the structural SIN proteins, which can be as high as 10^8 molecules/cell *(13)*. The recombinant virus stock may be stored long-term for future infection of cells and Ag expression. For both dsSIN recombinant virus configurations, multiple passages in cultured cells often lead to deletion of the insert, particularly for those recombinants harboring inserts >2 kb in length. This should be considered in experimental design (*see* **Note 1**).

1.2.2. Replicon Vectors

Unlike the dsSIN vector, SIN replicon RNAs are replication (or propagation)-incompetent, because of the complete replacement of the virus structural protein genes with the heterologous sequence (e.g., Ag) to be expressed (*13*; **Fig. 2**). Following in vitro transcription of SIN replicons, the RNA may be transfected directly into the desired cell type for transient expression, or first packaged into virion-like vector particles by providing the deleted SIN structural proteins *in trans*. Once produced, stocks of packaged vector particles may also be stored long-term for future infection of cells and class I Ag presentation studies. Typically, vector packaging has been performed by co-transfection of cells with replicon RNA and one or more additional in vitro transcribed SIN defective helper (DH) RNAs, which lack the replicase genes and packaging signal, but maintain the *cis* sequences necessary for amplification and the coding sequences for the structural proteins *(14,16,17,20–22)*. Packaging cell lines for the production of alphavirus vector particles have been developed recently, eliminating the need for co-transfection of in vitro transcribed vector and DH RNAs *(22)*.

1.2.3. Plasmid DNA-Based SIN Vectors

As an alternative to in vitro transcription approaches, layered plasmid DNA vectors harboring SIN replicons have been developed (*10*; **Fig. 2**). Without a prior need for in vitro transcription or packaging into vector particles, these vectors are fully functional after direct transfection into susceptible cells in vitro and in vivo. The first layer consists of a eukaryotic RNA polymerase promoter (e.g., CMV or RSV), which transcribes in vivo a SIN replicon RNA. Upon transport to

the cytoplasm, the SIN replicon catalyzes it own amplification, using the viral replicase, and expresses the foreign gene via the highly active SG promoter. Again, similar to in vitro transcribed vector replicons, these DNA-based vectors are replication (or propagation)-incompetent, because of the complete replacement of the viral structural protein genes by the heterologous sequence. They can also be used to generate vector particles by cotransfection with DNA-based detective helpers or by transfection into packaging cell lines.

1.3. SIN Vectors to Study Ag Processing and Presentation In Vitro and In Vivo

The direct incorporation of Ag-encoding foreign genes into SIN vector cDNA constructs, followed by transcription into functional RNA in vitro or in vivo, obviates requirements for selection and purification of recombinants from wild-type (WT) virus. The ease and rapidity of generating pure recombinant virus or vector particle stocks, as well as DNA-based vectors, makes this expression system a useful tool for studies at all levels, including Ag presentation to effector T-cells *(1,23,24)*, in vitro restimulation of memory T-cells *(25)*, and induction of cell-mediated immunity by vaccination *(1,3,4,7–9,11)*.

1.3.1. Ag Presentation by Target Cells

Recombinant dsSIN provides an efficient and convenient means for in vitro mapping and fine-structure analysis of T-cell epitopes expressed via the endogenous Ag presentation pathway *(23–25)*. Although synthetic peptides have been useful in identifying novel epitopes, the constraints on peptide presentation by the cellular processing machinery can be more severe than anticipated. The effects of naturally emerging mutations, both flanking and within epitopes, the influence of linker residues in multiple-epitope polypeptides, and other parameters affecting proteolysis, peptide transport, and class I assembly *(26)*, can only be assessed by *de novo* expression of the respective Ag. SIN vectors provide an excellent source for such *de novo* Ag expression *(23–25)*.

1.3.2. Stimulator Cells

The generation of epitope-specific effector cytotoxic T-lymphocytes (CTL) from splenocytes of immunized mice generally requires in vitro expansion and differentiation on feeder cells presenting the corresponding epitope. This is commonly achieved by addition of synthetic peptide to splenocyte cultures, provided the CTL epitope has been identified. However, in situations in which the epitope has not been precisely defined, naïve syngeneic splenocytes or cell lines, infected with either the homologous or heterologous source of Ag compared to the immunizing agent, also are effective for in vitro stimulation. The use of a heterologous vector has the advantage of avoiding the stimulation of T-cells specific for epitopes in the vector backbone,

thereby enhancing expansion of Ag-specific CTL, and reducing nonspecific killing of target cells in CTL assays. Cell lines or splenocytes infected with recombinant SIN expressing CTL epitopes thus provide alternatives to recombinant vaccinia virus infected feeders or peptides for in vitro stimulation purposes *(25)*.

1.3.3. Immunogens

Each of the three vector configurations are immunogenic when administered directly into animals, capable of priming Ag-specific CTL and antibody responses *(1–4,7–9,11,12,21,22)*. These observations have generated considerable interest in the application of alphavirus-derived vectors for human and veterinary vaccines, in addition to their use as research tools. However, with the exception of alphavirus plasmid DNA-based vaccines, the technologies necessary for scale-up beyond the research laboratory have only recently become available *(22)*.

2. Materials

2.1. Plasmid Vectors

Various plasmids have been developed from SIN cDNA clones, and only representative examples are listed below. The viral strain backbone, including regulatory elements and restriction sites, is found in the respective references. The most commonly used dsSIN vector, designated "pTE3'2J", is derived from the original full-length SIN cDNA clone named Toto 1101 *(1,19)*.

1. dsSIN vector: pTE3'2J *(1)*.
2. dsSIN shuttle vector: pH3'2J1 *(1)*.
3. SIN replicon vector: SINrep5 *(17)*.
4. SIN defective helper: DH-BB *(17)*.
5. Plasmid DNA-based SIN vector: pSIN2.5 *(11)*.

2.2. Cloning Reagents

(*See* **ref. 27** for general procedures.)

1. Restriction endonucleases and buffers.
2. T4 DNA ligase.
3. 3 *M* NaOAc, pH 5.2.
4. Tris-EDTA (TE) buffer: 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 7.4.
5. EtOH.
6. A mixture of Tris–saturated phenol (Roche Molecular Biochemicals, Indianapolis, IN) and chloroform (1:1).
7. Ether.
8. Competent *Escherichia coli* (e.g., DH5α or JM109).
9. RNase free H_2O (*see* **Note 2**).

2.3. Transcription Reagents (see *Note 2*)

1. 5X SP6 polymerase buffer (usually supplied by manufacturer with enzyme; Promega, Madison, WI, or Gibco-BRL, Grand Island, NY): 200 m*M* Tris-HCl, pH 7.6, 30 m*M* MgCl$_2$, 10 m*M* spermidine
2. 2.5 m*M* ribonucleoside triphosphate (rNTP) mix, containing 2.5 m*M* of adenoside-5-triphosphate, guanosine-5-triphosphate, uridine-5-triphosphate, and cytidine-5-triphosphate (10 m*M* stocks from Promega, Madison, WI or Roche Molecular Biochemicals)
3. 200 m*M* dithiothreitol
4. 10 mg/mL bovine serum albumin (New England Biolabs, Beverly, MA)
5. Capping reagent: 20 m*M* 7 mG(5')ppp(5')G (N. E. Biolabs).
6. SP6 Polymerase (Promega or Gibco-BRL).
7. RNasin (Promega).

2.4. Transfection Reagents (see *Note 2*)

1. DOTAP (Roche Molecular Biochemicals) for lipid-mediated transfection.
2. 20 m*M* HEPES, pH 7.4.
3. Snap cap 5-mL polystyrene round bottom tubes.
4. RNase-free phosphate-buffered saline (PBS) (e.g., BioWhittaker, Walkersville, MD) for electroporation using Gene Pulser II unit from Bio-Rad (Hercules, CA).

2.5. Agarose Gel Electrophoresis Reagents and Equipment

1. Tris-borate-EDTA (TBE) running buffer.
2. TE buffer: 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 7.4.
3. 6X loading dye.
4. 10% (w/v) SDS.
5. 0.5 *M* EDTA, pH 8.0 (*see* **ref. *27*** for general procedures, and *see* **Note 2**).

2.6. Tissue Culture Reagents (All Sterile)

1. Fetal bovine serum (FBS).
2. 2.5% trypsin solution (e.g., Irvine Scientific, Santa Ana, CA).
3. Penicillin-streptomycin solution or gentamicin sulfate solution (e.g., Irvine Scientific).
4. Dulbecco's modified Eagle's medium (DMEM) (supplemented with 8% FBS and 1 mL penicillin-streptomycin solution/100 mL or 1 mg Gentamicin/100 mL).
5. MEM.
6. PBS.
7. SeaKem ME agarose for plaque assay overlay (FMC Bioproducts, Rockland, ME).
8. Neutral red solution (Gibco-BRL).

2.7. Cell Lines

1. Baby Hamster Kidney 21 (BHK-21) cells (American Type Culture Collection clone 13).

2. P815 (H-2^d) nonadherent mastocytoma cells.

Both cell lines are propagated in DMEM supplemented with 8–10% FBS and antibiotics. BHK cells are trypinized in 0.25% trypsin solution (dilute 2.5% stock in versene). For short-term cultures, such as during viral infections, BHK cells can be maintained in MEM + 5% FBS.

3. Methods

3.1. Cloning of Foreign DNA into Full-Length dsSIN cDNA Clone Using Shuttle Vector

The choice of expression system, as discussed above, dictates which vector is most suitable for desired gene expression. For purposes of simplicity, the following subheading focuses primarily on the frequently used dsSIN system for Ag presentation studies *(1)*. Foreign sequences may be cloned directly into full-length dsSIN vectors or first subcloned into shuttle plasmids. For example, dsSIN vectors, such as pTE3'2J, containing the entire SIN genome under control of the SP6 promoter, have limited cloning strategies for inserting foreign DNA, because of their length (>13 kb). A shuttle plasmid, designated pH3'2J1 (~3 kb), containing a multiple cloning site with strategically placed restriction sites flanking the SG promoter at the 5'-end and the poly (A) site at the 3'-end, respectively, facilitates cloning *(1)*. Preferably, foreign inserts should be restricted to less than 2 kb in length (*see* **Note 1**). Although the originally described vectors contain a *XhoI* site for linearization prior to transcription, other unique sites have been engineered into these vectors (*see* **Note 3**).

3.2. Propagation of Virus from Clone

3.2.1. In Vitro Transcription of Full-Length dsSIN Genomic RNA

1. Linearize full-length plasmid dsSIN cDNA encoding Ag (for pTE3'2J, use *XhoI*; *see* **Notes 3** and **4**)
2. Check for complete digestion on 0.8% agarose gel.
3. Purify linearized DNA by extracting twice with a mixture of Tris–saturated phenol and chloroform (1:1), followed by two ether extractions (ether is upper phase) or $CHCl_3$ extraction. When using ether, blow off residual ether under N_2 stream, or evaporate with open lid for approx 10 min. Add one-tenth vol 3 *M* NaOAc and 2.5 vol cold 95% EtOH to precipitate DNA. Centrifuge precipitated DNA, wash pellet with 70% EtOH, dry pellet, and resuspend in RNase-free dH_2O at a concentration of approx 300 ng/μL (*see* **Note 5**).
4. In vitro transcription reactions are set up to the appropriate scale (for example, 10 μL are sufficient for 10 mL virus stock, with titers of approx 10^9 PFU/mL) at room temperature (RT), by adding the following reagents in order (keep individual components on ice): 1.0 μL RNase-free dH_2O; 4.0 μL 2.5 m*M* nucleoside

triphosphate mix; 2 μL 5X SP6 buffer; 0.5 μL 20 m*M* 7mG(5')ppp(5')G; 0.25 μL 200 m*M* dithiothreitol; 0.25 μL RNasin (40 U/μL); 0.5 μL bovine serum albumin (10 mg/mL); 0.25 μL SP6 polymerase (20 U/μL). Last, add 1.5 μL linear template DNA (approx, 400–500 ng), and immediately place in 37°C bath (*see* **Note 5**). For multiple reactions, a cocktail of reagents may be prepared, and appropriate volumes added to individual DNA templates. Larger-scale transcription reactions can be carried out, provided relative concentrations of reagents are maintained (e.g., in volumes up to 500 μL).

5. Incubate for 60–90 min at 37°C.
6. Check quality of RNA transcripts on a 0.7% TBE–agarose gel containing 0.1% SDS without ethidium bromide (EtBr). The gel is made by dissolving agarose in RNase-free TBE buffer, and adding 10% SDS immediately before casting to make a final concentration of 0.1%. Prepare samples as follows, and include a template-only control: 8 μL TE buffer, 1 μL 10% SDS, 0.25 μL 0.5 *M* EDTA, 2 μL transcription reaction (or DNA control). Heat sample to 70ºC for 2 min, quick-cool on ice, add loading dye, and load entire sample onto gel. Use RNase-free TBE buffer containing 0.1% SDS without EtBr as running buffer. When the dye is near the bottom, remove gel, and stain in TBE buffer containing EtBr (30–60 min) on a shaker platform. Destain in dH$_2$O for a short time, if desired. Visualize RNA transcripts using UV light, and compare transcription reactions to a lane containing the DNA template control. Typically, the RNA runs at a lower apparent mol wt compared to the DNA template; RNA products should furthermore run as a distinct band, with very little smearing indicative of incomplete transcripts (**Fig. 3**).
7. Store remaining transcription reaction at –20°C or –80°C until used for transfections.

3.2.2. RNA Transfection

RNA may be transfected into a variety of cell types for virus production, although BHK-21 and chicken embryo fibroblast monolayers are the most common. BHK-21 cells are primarily used for convenience. These cells are propagated in DMEM supplemented with 8% FCS, and passaged by treatment with 0.25% trypsin. If overgrown or overtrypsinized, these cells tend to form clumps, resulting in poor transfection efficiencies. Both liposome-mediated transfection and electroporation yields high-titer recombinant dsSIN virus. For propagation-incompetent replicon stocks, electroporation is the preferred method, because of significantly higher transfection efficiencies necessary for multiple RNAs. Each procedure is described below.

3.2.2.1. RNA Transfection Using DOTAP

1. Plate BHK-21 cells 1–2 d prior to transfection in 60-mm dishes, so that cells are no more than 80–90% confluent at the time of transfection. Confluent or clumpy cells result in poor transfection efficiency. Generally, 2 × 60 mm dishes for each dsSIN vector are sufficient to produce an ample stock from 10 μL transcription
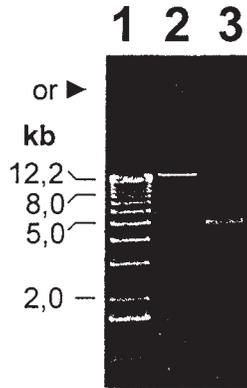
Fig. 3. Infectious dsSIN RNA derived by in vitro transcription of cDNA. Using a linearized pTE3'2J plasmid as template, infectious RNA was transcribed and analyzed on an 0.7 % agarose gel, as described in **Subheading 3.2.1**. Lane 1: 1 kb ladder DNA marker (BRL); lane 2: linearized DNA; lane 3: 2 µl RNA transcription reaction.

    reaction. If the assay is simply to check for viability of a construct, one 60 mm dish is sufficient. Always include a positive (known RNA transcript) and negative (DNA template) control.

2. Dilute transcription reaction (8 µL) to 200 µL with HEPES (volumes are for 2×60 mm dishes, so cut recipe in half for one transfection).
3. Set up transfection mixtures in snap-cap polystyrene tubes as follows per 60-mm dish: 70 µL HEPES plus 30 µL DOTAP, then add 100 µL HEPES already containing RNA or DNA control.
4. Incubate the transfection mixture 10 min at RT.
5. Wash plates 2× with prewarmed (37°C) serum free MEM.
6. Add 2 mL MEM with no or minimal serum (<2%); then add 200 µL transfection mixture with gentle swirling to obtain an even distribution.
7. Incubate 30 min at 37°C, then add 2 mL medium + 5% FCS.
8. Continue incubation at 37°C. Cytopathic effect (cells are rounded and begin floating) is first seen approx 24–30 h later.
9. Harvest supernatants after 30–36 h into 15-mL conical tube, wash cells with 1–2 mL PBS, and combine wash with supernatant. To check for Ag expression, lyse cells directly in dish by adding 200 µL Laemmli sample buffer, transferring to microcentrifuge tube, and boiling at least 5 min. Load 10 µL on mini-protein gel for Western analysis, if polypeptide contains an antibody reactive domain, or is tagged with an immunogenic peptide.
10. Clarify culture supernatants containing recombinant dsSIN virus by centrifugation for 20–30 min at 4°C (approx 1300*g* in tabletop centrifuge).
11. Transfer supernatant to fresh tube, dilute to 10 mL/60 mm dish, aliquot at 0.2–0.5 mL, and store aliquots at –80°C.

3.2.2.2. RNA Transfection by Electroporation

1. Expand BHK-21 cells by passage on two successive days. Cells from overgrown cultures should be avoided for optimal electroporation efficiency. Prepare sufficient T125 or T225 flasks to have 10^7 cells available per sample to be electroporated, plus extra for losses during manipulation.
2. Wash monolayers with RNase-free PBS, and trypsinize cells to detach, being careful not to overtrypsinize. Gently resuspend cells in medium plus 10% FCS, and transfer to 50-mL conical tube.
3. Pellet the cells by centrifugation at 4°C for 5 min (approx 200g in tabletop centrifuge).
4. Keep cells on ice from this point, and be very gentle at all resuspension steps.
5. Pour off media, disperse cell pellet by tapping side of tube, and gently resuspend cells in 30 mL ice-cold PBS (use only RNase-free PBS) by repetitive pipeting.
6. Pellet cells again as above. Resuspend each cell pellet (e.g., from one T225 flask) in 30 mL ice-cold PBS. Remove a small sample and count cells. At least 10^7 cells should be available for each RNA sample to be electroporated.
7. Centrifuge again as above and repeat wash for third time.
8. During the centrifugation, place RNA (use a minimum of 5 µL transcription reaction per 10^7 cells) into chilled, sterile tube (e.g., screw-cap, or snap-cap). If cotransfecting vector replicon and DH RNAs, add equimolar amounts of each RNA.
9. Pour off PBS, and gently resuspend cells in PBS at a concentration of 10^7/mL.
10. Add 1 mL cells to RNA, mix, and transfer to a prechilled 0.4-cm gap Gene Pulser cuvet.
11. Electroporate in a Bio-Rad Gene Pulser II unit, set with the following parameters: resistance, infinity; capacitance, 25 µF; voltage, 1700 V. Pulse twice, and allow the cells to recover at RT for approx 10 min.
12. Transfer cuvette contents into 10 mL media containing 10% FCS, and plate as desired (typically into 100 mm dish).
13. Incubate at 37°C for approx 24 h, at which time culture supernatants containing recombinant dsSIN virus or packaged vector replicons may be harvested and clarified as outlined in **Subheading 3.2.2.1.**
14. Electroporation efficiency for RNAs may be monitored by using SIN vectors expressing reporters, such as GFP or β-galactosidase, in parallel with the foreign insert.

3.2.3. SIN Virus Plaque Assay

1. Plate BHK-21 cells in DMEM + 8% FCS into 60 mm dishes 1–2 d prior to plaque assay, to obtain cells at approx 80–90% confluency on day of titration. Confluent monolayers from $3 \times$ T75 flasks are sufficient to seed 24×60-mm plates. Infections can be carried out in serum-free MEM or DMEM.
2. Serially dilute recombinant dsSIN virus (e.g., 200 µL into 1.8 mL MEM) down to 10^{-8}, using a fresh pipet or tip for every dilution (carryover is large).

3. Aspirate medium from cells; washing is not necessary.
4. In duplicate or triplicate, add 200 µL/plate of diluted virus, starting at 10^{-4}. Use two plates for mock infection with 200 µL medium for uninfected control plates.
5. Incubate at 37°C, rocking every 15 min, for 60 min.
6. Prepare 0.6% agarose for overlay during incubation. The following protocol for 100 mL is sufficient for 20 plates. Prewarm serum-free medium at 42°C in a water bath. Weigh out 0.6 g agarose into 10 mL H_2O and autoclave 15 min. Let agarose cool under hood for a few minutes (<10 min), slowly add 50 mL preheated medium, and mix well with agarose, let cool at 42°C in water bath for at least 10 min, and add 40 mL medium + 5% FCS (alternatively, separately add serum to 2–2.5% just before plating).
7. Overlay cells with 5 mL agarose, and let solidify at RT.
8. Incubate at 37°C approx 42–48 h.
9. Add 3 mL neutral red solution in serum-free medium per 60-mm plate (4 mL neutral red /100 mL medium).
10. Incubate at 37°C, counting plaques 4–6 h later. Plaques are colorless and round, compared to the neighboring uninfected cell monolayer. At lower dilutions (e.g., 10^{-5}), the entire monolayer should be lysed, compared to mock-infected controls. Count plaques at the dilution that yields approx 20–200 plaques/plate, and calculate PFU/mL. SIN titers in supernatants typically range from 10^8 to 10^{10} PFU/mL (*see* **Note 6**).

3.3. Infection of Cells for CTL Assays or Stimulation

Alpha viruses generally have a broad range of susceptible host cells, including those of insect, avian, and mammalian origin *(1,13,18)*. However, some clonal virus strains may have more limited tropism as a result of adaptation to laboratory cell lines. Furthermore, dsSIN replication can be severely modulated in cell lines expressing murine MHC-I molecules in a haplotype- and allele-dependent manner *(28)*. Whereas class I molecules of the H-2^k haplotype show minimal inhibition, the H-2^d haplotype exhibit strong, and H-2^b haplotype intermediate inhibitory effects. Nevertheless, the inhibitory effects are most dramatic at low multiplicities of infection (MOI), and several cell lines support sufficient replication at high MOI (20–50 PFU/cell) for class I presentation. Murine cell lines that can be used to study class I Ag presentation, following infection with recombinant dsSIN virus, include P815 mastocytoma cells (H-2^d), L929 fibroblasts (H-2^k), EL4 T-lymphoma cells (H-2^b), and MC57 fibroblasts (H-2^k) *(1,25;* *see* **Note 7**). An example is provided in **Fig. 4**, which demonstrates sensitization of both D^d- and L^d-restricted CTL clones by dsSIN-infected P815 targets.

3.3.1. Preparation of Infected Target Cells

1. Prepare suspension of susceptible cells; if target cells require trypsinization, wash trypsinized cells with medium prior to infection.
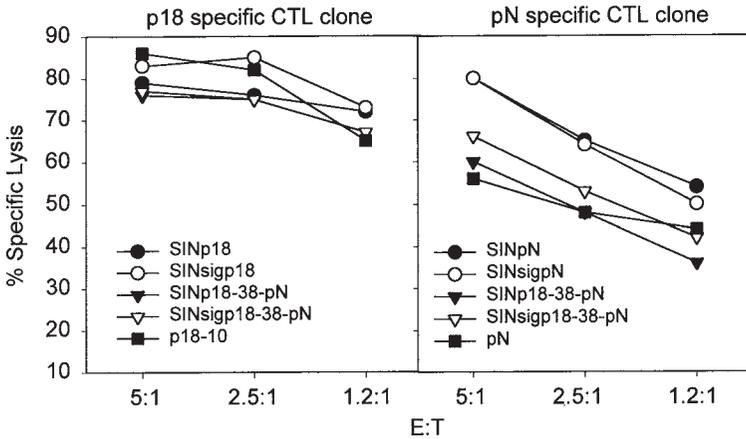
Fig. 4. Sensitization of CTL by dsSIN-infected target cells. Various recombinant dsSIN constructs expressing the D^d-restricted p18 epitope derived from HIV-1$_{IIIB}$ gp160 (RGPGRAFVTI), the L^d-restricted pN epitope derived from the mouse hepatitis virus nucleocapsid protein (APTAGAFFF), or both epitopes in tandem array *(29)*, were tested for recognition by either p18- or pN-specific CTL clones at the indicated effector to target (E:T) ratios. Recombinants were generated by cloning minigenes encoding the single epitopes preceded by a methionine (SINp18, SINpN), the single epitopes preceded by a heterologous signal sequence for insertion into the endoplasmic reticulum (SINsigp18, SINsigpN), and chimeric polypeptides containing both epitopes flanked by native amino acids and linked by a 38-amino acid spacer derived from gp160 *(29)*, with and without the signal sequence (SINpN-38-p18 and SINsigpN-38-p18, respectively). P815 cells were infected at an MOI of 25 PFU/cell, and incubated for 4 h prior to ^{51}Cr labeling. The data show efficient presentation of either epitope synthesized *de novo* in infected cells, independent of processing requirements for the various Ag precursors. Lysis of infected cells was comparable to peptide-coated targets (p18–10 and pN, respectively).

2. Resuspend 10^6 cells in 0.2–0.5 mL MEM.
3. Infect cells with recombinant dsSIN virus at 20–50 PFU/cell for 1 h at 37°C. Include a negative control, by infecting cells with a heterologous dsSIN not expressing the epitope.
4. Gently rock tubes every 15 min.
5. Refeed cells with 1 mL medium + 5% FCS, and incubate for 4 h at 37°C (*see* **Note 8**).
6. Collect cells by centrifugation and label with 80–100 µCi ^{51}Cr for 1 h at 37°C, swirling every 15 min. Wash cells 3× with MEM + 2% FCS, and resuspend in RPMI + 2% FCS at 10^5 cells/mL for CTL assays.
7. CTL assays use 10^4 target cells/well and various effector ratios. Harvest 100 µL supernatant after 4 h incubation at 37°C for determination of released radioactivity from lysed cells.

3.3.2. Infection of Feeder Cells

1. Susceptible feeder cells are essentially infected as described in **Subheading 3.3.1.** and irradiated prior to or following infection. The cell numbers required for infection are dependent on the number of responder cells from immunized mice to be stimulated. Cultures using infected cell lines for stimulation are set up at a responder: stimulator ratio of 40:1 *(25)*.
2. Infect stimulator cell lines at 50 PFU/cell for 1 h at 37°C, shaking every 15 min.
3. Irradiate cells with the appropriate dose, either prior to or after the infection.
4. Set up cultures with responder cells in RPMI complete medium containing 10% prescreened FCS.
5. Incubate for 5–6 d at 37°C, and test for Ag-specific CTL activity.

3.4. Immunization of Mice to Induce CD8 T-Cells

Adult mice (>5 wk of age) can be immunized with recombinant dsSIN vectors by intramuscular, subcutaneous, or intraperitoneal injection to prime Ag specific CD8 T-cells *(9)*. Even high virus doses (up to 10^9 PFU) are tolerated in mice, without showing obvious signs of symptoms or disease (e.g., ruffled fur). CD8 T-cell priming can be monitored by measuring the frequency of interferon γ secreting responder cells in enzyme-linked immunospot assays, as described *(9,30)*. The frequency of responding cells may vary at least fivefold, depending on the epitope itself. Although ex vivo cytolytic activity specific for foreign Ag is not observed in splenocytes, in vitro restimulation of memory cells results in efficient Ag-specific cytolytic activity (*1,30*; *see* **Note 9**). An advantage of dsSIN as an immunogen, compared to other viral vectors, is the fact that WT SIN appears to only induce CD8 responses in a limited number of mouse strains *(31,32)*. Skewing of CD8 T-cell expansion caused by endogenous viral epitopes is thereby minimized.

For packaged SIN vector replicons, immunization is best performed by the intramuscular, intradermal, intranasal or subcutaneous routes, with titers of vector particles between 10^3–10^4IU *(22)*. Plasmid DNA-based SIN vectors are administered only by the intramuscular route (in PBS), with amounts typically ranging from 10 ng to 10 µg *(11)*. If desired, boosting with plasmid DNA- or replicon-based SIN vectors may be performed at 2–3 wk.

4. Notes

1. The stability of dsSIN viral progeny is limited by both the size and position of the foreign insert *(2,7,16,18)*. Although the 3' noncoding region can accommodate inserts up to 4 kb without compromising packaging of the resulting viral genome, large inserts are typically unstable. Loss of expression with large inserts can be observed after a single passage at low MOI, thereby restricting the use of recombinants to the stock derived from the original transfection. The dsSIN system is therefore most suitable for

expression of smaller inserts (<2 kb), preferentially truncated genes or minigenes encoding CTL epitopes *(1,2,16,18,30)*. However, if expression of longer inserts is required, 5' recombinants appear to be more stable than the corresponding 3' recombinants *(7)*. The level of Ag expression appears to be independent of the 5' or 3' position, relative to the SIN structural open reading frame *(7)*. Similar insert size constraints have not been observed for packaged SIN vector replicons and plasmid DNA-based vectors.

2. RNase-free solutions are available commercially, or can be made by using diethyl pyrocarbonate-treated, autoclaved H_2O for buffer stocks *(27)*.

3. Vectors containing unique *Not*I or *Mlu*I sites for linearization have been constructed *(2,24)*, and further modifications may be made by insertion of a linker sequence into the current linearization site.

4. Plasmid DNA does not have to be purified extensively, e.g., CsCl banding, or Qiagen columns (Valencia, CA) are not required. Miniprepped plasmid DNA prepared by alkaline lysis protocols *(27)* is of sufficient quality.

5. It is critical not to use too much DNA template, to reduce the risk of generation of intermediate-length transcripts at the expense of full-length transcripts. For efficient transcription reactions, it is also important to fully remove residual phenol after DNA extractions, either by $CHCl_3$ or ether.

6. Titers are inversely correlated with the length of insert: higher-titered virus stock are obtained with smaller-sized inserts (e.g., a minigene expressing a CTL epitope) (*2*; Bergmann, unpublished observations). Although virus titers are stable at –80°C, virus and packaged vector preparations are sensitive to repetitive freeze–thawing (more than 2–3 times).

7. The murine cell lines J774.1 and BC10ME (both H-2[d]), and human B lymphoblastoid cell line CIR (CIR/D[d], CIR/L[d]), and its Ag-processing defective T2 derivatives (T2/L[d], T2/D[d]), used to assess transporter associated with Ag presentation-independent processing, do not present dsSIN encoded endoplasmic reticulum-targeted, preprocessed CTL epitopes, in the authors' experience. This may result from limited tropism or inhibitory effects of MHC on replication *(28)*.

8. Incubation times after 1 h adsorption, can be reduced to 2 h, including the time of ^{51}Cr labeling, if higher MOI are used *(1,23)*. For P815 cells infected at an MOI of 20 PFU/cell, no cell loss was observed after 4 h infection time. Presentation of dsSIN-encoded preprocessed epitopes is also not inhibited by the proteasome inhibitors, N–acetyl–L–leucina–L–norleucinal (LLnL) or lactacystin (Bergmann, unpublished observations).

9. CD8 T-cell priming to K[d] restricted epitopes in vivo has been shown to be efficient *(1,9)*. However, although D[d] restricted responses are also readily primed *(30)*, induction of CD8 T-cells by dsSIN viruses expressing L[d]-restricted CTL epitopes appears impaired despite efficient presentation by dsSIN-infected P815 cells in vitro (M. Tsuji and Bergmann, unpublished observations).

Acknowledgments

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10

Precision Genotyping of Human Leukocyte Antigen-A, -B and -C Loci Via Direct DNA Sequencing

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1. Introduction

Class I human leukocyte antigen (HLA-I) alleles have been described as the most polymorphic human antigens *(1–4)*. To date, there are more than 500 HLA-I alleles which have been officially assigned by the World Health Organization Nomenclature Committee for Factors of the HLA System *(5)*. This number continues to grow, with approximately one new HLA-I allele reported weekly *(6)*.

Since the 1960s, serology has been the traditional method for HLA-I typing *(7–9)*. Serologic typing with alloantibodies provided the realization that HLA-I molecules differed from individual to individual, and the initial system for classifying or grouping the many different HLA-I molecules in the human population was based on patterns of serologic crossreactivity *(10–13)*. Indeed, nomenclature for new and existing HLA molecules is either directly or indirectly based on an HLA molecule pattern of serologic crossreactivity.

Early in the 1990s, it became clear that particular major histocompatibility complex class I (MHC-I) polymorphisms, although capable of triggering alloreactive T cells, cannot be detected by antibodies *(14–18)*. DNA-sequence-based research studies of MHC-I evolution found that serology could not detect particular polymorphisms, and sometimes grouped distant class I molecules together, based on small stretches of homology at dominant serologic epitopes, and sometimes grouped closely related class I molecules apart, because they differed at a dominant serologic epitope *(14–18)*. Because a sound understanding of alloreactivity, disease association, and autoimmunity requires allele-level, or high-resolution, HLA typing, precision alternatives to serologic typing have been in development for several years.

Overview of High-Resolution Class I Sequence-Based Typing of HLA-A, -B, and -C Loci.

Whole blood/cryopreserved cells/tissue etc.

⇩   Extract genomic DNA

Locus specific amplification of HLA - A, B or C (1°)

⇩   1/100 dilution of 1° PCR - A & B locus
     1/50 dilution of 1° PCR – C locus

Nested amplification of exons 2 & 3; (four 2° PCR reactions/sample)

⇩   Bind amplicons to combs

Directly sequence PCR products on streptavidin-coated combs with T7 sequenase

⇩   Input heterozygous sequence data into
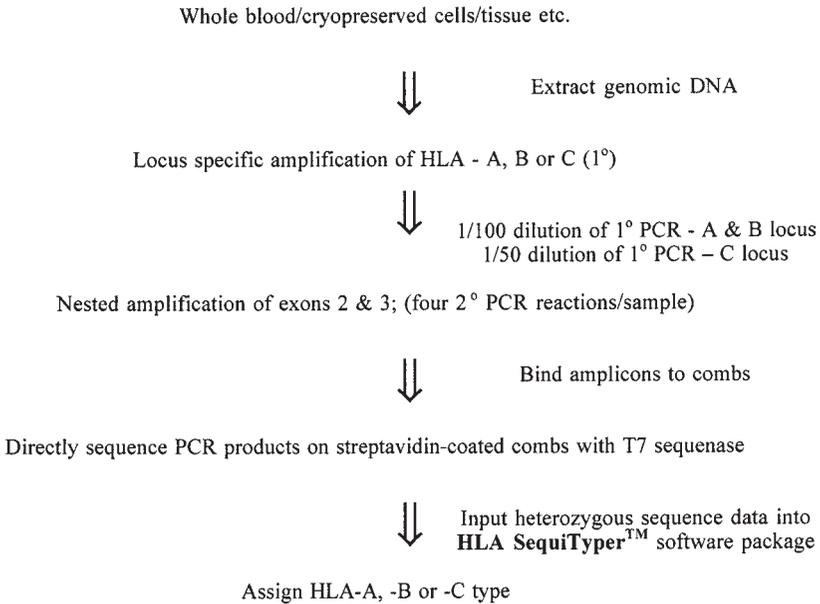   **HLA SequiTyper**™ software package

Assign HLA-A, -B or -C type

Fig. 1. Overall strategy for determining a class I HLA sequence-based type with direct DNA sequence analysis. Genomic DNA is extracted from samples and an HLA-A, -B, or -C locus-specific PCR is performed. This locus-specific PCR is then used as a template for a second (nested) PCR where exons 2 and 3 are separately amplified with biotinylated and M13-tailed PCR primers. The biotinylated product is then bound to a streptavidin-coated comb, and the sequencing reactions are performed on the comb. Heterozygous sequence data is then analyzed using the HLA SequiTyper software and a class I HLA-A, -B, or -C type is assigned.

One of the goals within this laboratory has been the development of a DNA sequence-based method for typing HLA-I molecules (**Fig. 1**). HLA-I molecules differ from immunoglobulins, the receptors of T-lymphocytes, and, to some extent, HLA-II molecules, in that class I molecules have no real hypervariable region. The polymorphisms that distinguish class I molecules from one another are evenly distributed over the 546 nucleotides (nt) that encode exons 2 and 3 *(19)*. An even distribution of polymorphism makes it difficult to predict where two class I alleles may differ, and the design of oligo-nt primers and probes for typing by polymerase chain reaction (PCR) and/or hybridization thus becomes cumbersome, because a large expanse of DNA must be screened for potential mutations. Beginning from genomic DNA, we have therefore designed a direct (i.e., no cloning) approach, by which exons 2 and 3
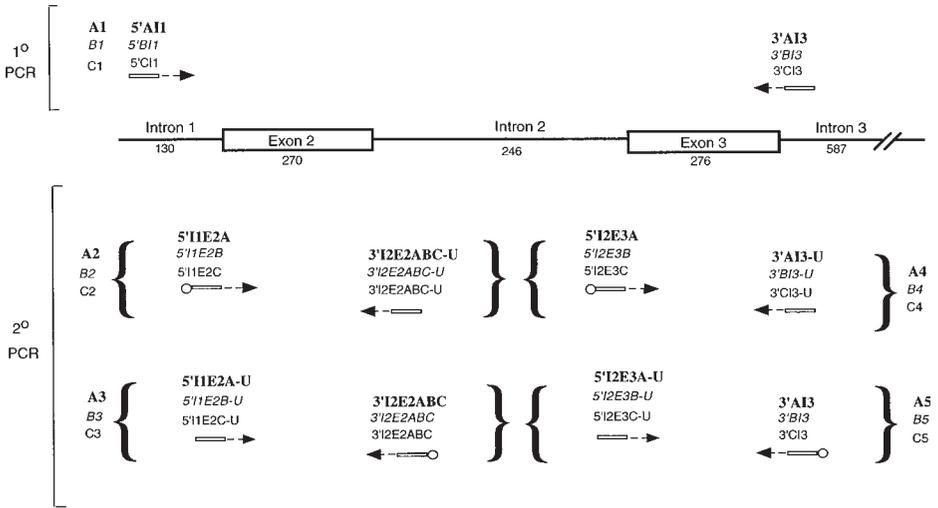
Fig. 2. Sequence-based typing strategy for direct heterozygous sequencing of HLA-A, -B, -C exons 2 and 3. Genomic DNA, represented here as a linear diagram of exons 2 and 3 flanked by introns 1, 2, and 3, acts as template for the primary (first-round) amplicon of approx 914 bp in length. This primary locus-specific PCR product then serves as template for four nested and heminested secondary (second-round) PCR reactions, shown below the linear diagram of exons 2 and 3. Oligonucleotide primer mixes A2, A3, A4, and A5 generate separate exon 2 and 3 HLA-A amplicons of approx 340 bp in length, with an M13 universal primer site on one end and biotin on the other. The HLA-A, - B, or -C exon 2 and 3 biotin PCR products are then bound to a streptavidin-coated support, upon which bidirectional DNA sequencing reations are performed. 5'-biotin moiety; ○, 5'-M13 universal primer tail; U.

of HLA-A, -B, or -C can be completely DNA sequenced (**Fig. 2**). The resulting class I sequence is then interpreted with software designed for resolving heterozygous DNA sequences.

Validation of this approach comes from 2292 HLA-A, 895 -B, and 4271 -C typings performed over the course of the past 2 yr. A majority of the samples (>95%) were from the National Marrow Donor Program's (NMDP) research repository. All of the NMDP samples typed were from frozen aliquots of blood, monocytes, or lymphocytes. All NMDP typings were performed with no previous typing information (blindly), and in duplicate, with another laboratory using a different technology, to assure accuracy. In 1998–1999, the discrepancy rate between laboratories was below 5%. Another quality control measure is our typing of samples for the International Cell Exchange, where select (often obscure) samples are typed by hundreds of laboratories, and results compared.

Class I sequence-based typing (SBT) has proven 98% accurate with Cell Exchange samples. Perhaps the best validation of our approach is that it has detected 47 new alleles in the past 2 yr (*20*; and unpublished data). Thus, the class I SBT method described herein is validated and robust for detecting known and unrealized HLA-I molecules.

To facilitate amplification of exons 2 and 3 from HLA-A, -B or -C, a nested amplification protocol is currently employed (**Fig. 2**). An allele-specific amplification of HLA-A, -B or -C is initially performed followed by a nested amplification of exons 2 and 3 (one primary + four nested PCR reactions per sample facilitate bidirectional sequencing of exons 2 and 3). The second PCR affords a locus- and exon-specific biotinylated PCR product of approx 320 bp, tailed with an M13 sequencing primer site. The bidirectional solid-phase DNA sequencing of exons 2 and 3 is then accomplished by Cy5-dye-labeled primer chemistry (M13 universal primer) and T7 DNA polymerase, employing the Amersham Pharmacia Biotech (APB) AutoLoad Solid Phase Sequencing Kit. The resulting fragments are separated by electrophoresis, and detected on a APB ALFExpress DNA sequencer. HLA types are assigned by APB HLA SequiTyper software, using published HLA-I A, -B or -C sequences as a database.

2. Materials

A Perkin-Elmer (Norwalk, CT) model 9700 thermocycler is used in the laboratory to perform all PCR. Other models of thermocyclers may also be suitable for this purpose (*see* **Note 1**). An APB (Piscataway, NJ) AlfExpress automated DNA sequencer is used for all sequencing. The software package HLA SequiTyper version 2.00 is used to analyze sequence data and determine an HLA-A, -B, or –C type for each sample (*see* **Note 2**).

2.1. Genomic DNA Extraction

1. 200 μL whole blood (in citrate/EDTA), granulocytes, peripheral blood, or Epstein-Barr virus-transformed cells (*see* **Note 3**).
2. DNA Extraction Kit: Genomic DNA is extracted using the Qiagen QIAmp Blood Tissue kit (Qiagen, Valencia, CA).

2.2. Primary PCR

1. H_2O: Deionized ultrafiltered water (DIUF) (Fisher Scientific, Pittsburgh, PA, 4L), 4l.
2. 2-mercaptoethanol (Sigma, St. Louis, MO, 100 mL).
3. PCR nt mix (10 m*M* each deoxyribonucleoside triphosphate [dNTP]), (Roche Molecular Biochemicals, IN).
4. 10X PCR buffer: 500 m*M* Tris-HCl, pH 8.8, 150 m*M* $(NH_4)_2SO_4$, 0.5 m*M* EDTA, 1 mg/mL gelatin (dissolve at 50–55ºC) (0.22 μm sterile-filtered).

Table 1
First-Round PCR: HLA-I SBT Primers

Primer	Amplicon	Localization (intron)	Orientation	Bases from exon	Primer sequence 5' To 3'	Primer length	Amplicon length
5'AI1	A1	1 (34–46)	sense	105	**S**GC CTC TG**Y** GGG GAG AAG CAA	21	938
3'AI3	A1	3 (26–57)	anti-sense	46	GGG AGA **Y**CT A**Y**A GGC GAT CAG G	22	
5'BI1	B1	1 (38–57)	sense	92	GAG GAG **MR**A GGG GAC CGC AG	20	940
3'BI3	B1	3 (37–68)	anti-sense	56	AG**S** CCA TCC CCG **S**CG ACC TAT	21	
5'CI1	C1	1 (42–62)	sense	87	CGA GG**K** GCC CGC CCG GCG A	19	911
3'BCI3	C1	3 (12–33)	anti-sense	32	AGA TGG GGA AGG CTC CCC ACT	21	

5. Amplification primers (in pmol/µL) (*see* **Table 1** and **Note 4**):
   a. A Locus: sense, 5'AI1 (10); anti-sense, 3'AI3 (10).
   b. B Locus: sense, 5'BI1 (10); anti-sense, 3'BI3 (10).
   c. C Locus: sense, 5'CI1 (10); anti-sense, 3'CI3 (10).
6. *Taq* polymerase (5U/µL), (Fisher Scientific, 500 U/vial) (*see* **Note 5**).
7. DNA template (25–125 ng).

2.3. Secondary PCR

1. DIUF water (Fisher Scientific, 4L).
2. PCR nt mix (10 m*M* each dNTP), (Roche Molecular Biochemicals).
3. 10X PCR buffer: Fisher Scientific buffer A, contains 15 m*M* MgCl$_2$ (*see* **Note 6**).
4. *Taq* polymerase (5U/µL), (Fisher Scientific, 500 U/vial) (*see* **Note 5**).
5. Amplification Primers (in pmol/µl) (*see* **Table 2** and **Note 4**):
   a. A2/B2/C2 mixes: sense (10), anti-sense (10).
   b. A3 mix: sense(15), anti-sense (15).
   c. B3/C3 mixes: sense (10), anti-sense (10).
   d. A4 mix: sense (15), anti-sense (15).
   e. B4/C4 mixes: sense (10), anti-sense (10).
   f. A5 mix: sense (15), anti-sense (15).
   g. B5/C5 mixes: sense (10), anti-sense (10).
6. Template (first-round) PCR product is diluted 1:100 for the HLA–A and -B loci and 1:50 for the -C locus.

2.4. Sequencing

1. AutoLoad Solid-Phase Sequencing Kit (*see* **Note 7**).

   Kit Reagents:

   a. T7 DNA polymerase (Sequenase): 8 U/µL in 25 m*M* Tris-HCl (pH 7.5), 0.25 *M* NaCl, 5 m*M* dithiotheitol (DTT) and 50% glycerol.
   b. Enzyme dilution buffer: 20 m*M* Tris-HCl (pH 7.5), 5 m*M* DTT, 100 ug/mL BSA, and 5% glycerol.
   c. Annealing buffer: 1 *M* Tris-HCl (pH 7.6), and 100 m*M* MgCl$_2$.
   d. Extension buffer:304 m*M* citric acid Tris-HCl (pH 7.5), 324 m*M* DTT and 40 m*M* MnCl$_2$ (pH 7.5).
   e. nt (dNTP/ddNTP) mixes.
   f. Dimethyl sulfoxide (DMSO).
   g. Stop solution: 100% deionized formamide and dextran blue 2000 (5 mg/mL).
   h. Sequencing combs: 8-tooth streptavidin-coated sequencing combs (50 pieces).
   i. 10-Well plates: 10-well plates for PCR product capture, denaturation, and annealing steps (30 pieces).
   40-Well plates: 40-well plates for sequencing reactions (10 pieces).
2. 0.1 *M* NaOH: 1.000+/-0.005 *M* volumetric solution of NaOH diluted to 0.1 *M* (*see* **Note 8**).

3. Binding and Washing Solution: 2 *M* NaCl, 10 m*M* Tris-HCl (pH 7.5), 1 m*M* EDTA (*see* **Note 8**).
4. Tris-EDTA (TE) buffer: 10 m*M* Tris-HCl, 1 m*M* EDTA (pH 8.0) (*see* **Note 8**).
5. Cy5-labeled M13 universal primer (forward [-20]): 5'Cy5-GTA-AAA-CGA-CGG-CCA-GT (*see* **Note 4**).
6. DIUF water, (Fisher Scientific, 4 L).

2.5. Gel Casting

1. PagePlus 40% concentrate (Amresco, 500 mL).
2. Urea, (APB, 500g).
3. N'N'N'N'-tetramethylethylenediamine (TEMED), (APB, 25 mL).
4. Ammonium persulfate (10% solution) (APB, 25g).
5. Boric acid (APB, 500g).
6. EDTA-disodium salt, (APB, 100*g*).
7. Tris (APB, 500*g*).
8. Bind-silane (APB, 25 mL).
9. Isopropanol (Sigma, 500 mL).
10. Glacial acetic acid (Fisher Biotech, 500 mL.
11. DIUF water (Fisher Scientific, 4 L).
12. EtOH 200-proof.

3. Methods
3.1. Genomic DNA Extraction

Genomic DNA (gDNA) is routinely prepared from 200 μL frozen whole blood (citrate–EDTA), using the commercially available Qiagen QIAamp blood kit according to the manufacturer's protocol, which is compatible with subsequent amplification and sequencing protocols, and facilitates high-throughput batch processing of samples. Alternative extraction procedures yielding similar quantities/ purity of DNA suitable for PCR may be employed, although particular HLA-A, -B, and -C alleles have been known to drop-out or not amplify, with alternative extraction methods.

3.2. Primary PCR

1. First-round PCR master mix (25 μL reaction): 2.5 μL of 10X PCR buffer (preincubate at 65°C for 10 min to ensure $MgCl_2$ is dissolved), 2.5 μL of DMSO, 0.25 μL of 1 *M* 2-mercaptoethanol, 0.5 μL of PCR nucleotide mix, 1.2 μL primer mix (*see* **Note 9**), 0.125 μL *Taq* polymerase (5 U/μL), 5 μL of template gDNA (25–125 ng), 12.925 μL H_2O.
2. Cycling conditions for the Perkin-Elmer 9700 thermocycler (*see* **Note 1**): 96°C for 2 min then cycle at 94°C for 30 s, x°C for 50 s + 1 s/cycle (-A locus $x = 58$; -B locus $x = 56$; -C locus $x = 54$), and 72°C for 30 s + 2 s/cycle. Repeat for a total of 39 cycles, then one cycle of 5 min at 72°C. Hold at 4°C.

Table 2
Second Round (2 °) PCR: HLA-I SBT Primers

Primer amplicon length	Amplicon	Localization	Orientation (intron)	Bases from exon	Primer sequence 5' to 3'	Primer length	Amplicon length
*5'I1E2A	A2	1 (84–100)	sense	47	BIOTIN-G CGC CKG GAS GAG GGT C	17	351
3'I2E2ABC-U	A2	2 (18–34)	anti-sense	34	*GTA AAA CGA CGG CCA GT*G TCS TGA CCT SCG CCC C	34	
5'I1E2A-U	A3	1 (84–100)	sense	47	*GTA AAA CGA CGG CCA GT*G CGC CKG GAS GAG GGT C	34	351
*3'I2E2ABC	A3	2 (84–34)	anti-sense	34	BIOTIN-G TCS TGA CCT SCG CCC C17		
*5'I2E3A	A4	2 (234–251)	sense	33	BIOTIN-G GGG GAC YGG GCT GAC C	17	355
3'AI3-U	A4	3 (26–57)	anti-sense	46	*GTA AAA CGA CGG CCA GT*G GGA GAY CTA YAG GCG ATC AGG	39	
5'I2E3A-U	A5	2 (234–251)	sense	33	*GTA AAA CGA CGG CCA GT*G GGG GAC YGG GCT GAC C	34	355
*3'AI3	A5	3 (26–57)	anti-sense	46	BIOTIN-G GGA GAY CTA YAG GCG ATC AGG	22	
*5'I1E2B	B2	1 (84–100)	sense	47	BIOTIN-G CGC CGG GAG GAG GGT C	17	351
3'I2E2ABC-U	B2	2 (18–34)	anti-sense	34	*GTA AAA CGA CGG CCA GT*G TCS TGA CCT SCG CCC C	34	
5'I1E2B-U	B3	1 (84–100)	sense	47	*GTA AAA CGA CGG CCA GT*G CGC CGG GAG GAG GGT C	34	351
*3'I2E2ABC	B3	2 (18–34)	anti-sense	34	BIOTIN-G TCS TGA CCT SCG CCC C17		
*5'I2E3B	B4	2 (240–256)	sense	28	BIOTIN-A CKG KGC TGW CCG CGG G	17	360
3'BI3-U	B4	3 (37–68)	anti-sense	56	*GTA AAA CGA CGG CCA GT*A GGC CAT CCC CGG CGA CCT AT	38	
5'I2E3B-U	B5	2(240–256)	sense	28	*GTA AAA CGA CGG CCA GT*A CKG KGC TGW CCG CGG G	34	360
*3'BI3	B5	3 (37–68)	anti-sense	56	BIOTIN-A GGC CAT CCC CGG CGA CCT AT	21	
*5'I1E2C	C2	1 (99–116)	sense	32	BIOTIN-T CGG GCG GGT CTC AGC C	17	336
3'I2E2ABC-U	C2	2 (18–34)	anti-sense	34	*GTA AAA CGA CGG CCA GT*G TCG TGA CCT GCG CCC C	34	
5'I1E2C-U	C3	1 (99–116)	sense	32	*GTA AAA CGA CGG CCA GT*T CGG GCG GGT CTC AGC C	34	336
*3'I2E2ABC	C3	2 (18–34)	anti-sense	34	BIOTIN-G TCG TGA CCT GCG CCC C	17	
*5'I2E3C	C4	2 (237–263)	sense	21	BIOTIN-T GAC CRC GGG GGC GGG G	17	329
3'BCI3-U	C4	3 (12–33)	anti-sense	32	*GTA AAA CGA CGG CCA GT*A GAT GGG GAA GGC TCC CCA CT	38	
5'I2E3C-U	C5	2 (237–263)	sense	21	*GTA AAA CGA CGG CCA GT*T GAC CRC GGG GGC GGG G	34	329
*3'BCI3	C5	3 (12–33)	anti-sense	32	BIOTIN-A GAT GGG GAA GGC TCC CCA CT	21	

BIOTIN: denotes 5' TEG-biotinylation; italics denote complementary M13 tail; and wobbled positions are bold and denoted by standard IUPAC nomenclature: K = G/T, R = A/G, S = C/G, Y = C/T, W= A/T, M= A/C.

3. Confirm amplification of the PCR product by running 5 µL PCR product on a 2% agarose gel (*see* **Note 10**).

3.3. Secondary (Nested) PCR

1. Second-round PCR Master Mix (60 µL reaction): 6.0 µL of 10X buffer A (preincubate at 65°C for 10 min to ensure $MgCl_2$ dissolved), 1.2 µL of PCR nt mix, 1.2 µL exon-specific primer mix (*see* **Note 11**), 0.3 µL of *Taq* polymerase, 45.3 µL of H_2O, and 6.0 µL diluted first-round PCR product.
2. Cycling conditions for the Perkin-Elmer 9700 (*see* **Note 1**): 95°C for 1 min, x°C for 1 min (-A locus $x = 54$; -B locus $x = 56$; -C locus $x = 56$) 72°C for 1 min. Repeat for a total of 30 cycles, then one cycle of 72°C for 5 min. Hold at 4°C.
3. Confirm PCR reaction fidelity/amplification product size/yield by agarose gel electrophoresis (2.0% gel), with suitable DNA ladder (*see* **Note 12**).

3.4. Sequencing

Routinely, solid-phase sequencing is performed using the AutoLoad Kit (APB). The AutoLoad Solid Phase Sequencing Kit is designed to facilitate the direct sequencing of PCR products on the ALF family of automated DNA sequencers. PCR is performed using one M13 universal tailed primer and one biotinylated primer. The PCR product is captured on a specially designed sequencing comb containing immobilized streptavidin, and the nonbiotinylated strand of the PCR product is removed by alkaline denaturation. The immobilized single-stranded product, which remains bound to the sequencing comb, is then used as a template in dideoxy sequencing reactions, using a fluorescently (Cy5) labeled M13 universal primer. The products of the sequencing reaction remain bound to the immobilized template strand until the comb is loaded onto the sequencing gel. The wells of the sequencing gel are filled with stop solution, which releases the fluorescently labeled sequencing fragments from the combs. The combs are removed from the sequencing gel, and the electrophoresis/detection is started through a linked computer, using ALFwin software.

The AutoLoad Solid Phase Sequencing Kit may be used to sequence 10 PCR products simultaneously. The kits provide sufficient combs, reaction plates, and reagents to sequence 100 PCR products with T7 polymerase. One eight-tooth sequencing comb is capable of capturing two templates, because four teeth are required per template (A,C,G,T). The combs efficiently capture the products (~320 bp) generated by PCR amplification of HLA-A, -B and -C exons 2 and 3. We have observed poor capture of PCR products, and hence a weak and insufficient sequencing signal, with combs that have less than 4 mm streptavidin. We suggest that a ruler be used to ensure that teeth on the sequencing combs are coated to a depth ≥4 mm.

3.4.1. Immobilization of the Biotinylated PCR Product

These instructions describe the simultaneous sequencing of 10 PCR products using the AutoLoad Solid Phase Sequencing Kit. A total of five eight-tooth sequencing combs are required for 10 PCR templates. The five sequencing combs can be attached together via the plastic connectors located on each comb, and processed as a single unit. The reagent volumes stated in the following instructions are the volumes required to sequence 10 templates. The number of sequencing combs and some reagent volumes must be adjusted if sequencing fewer than 10 templates.

Before starting, confirm that the PCR product is of the correct size and that the quality and quantity are sufficient, by agarose gel electrophoresis (i.e., a single sharp band of the expected length and quantity is visible). Typically, 40–50 μL PCR reaction contains at least 2 pmol product. This can also be expressed as 1.4 μg/kb PCR product, i.e., to sequence a 500 bp PCR fragment, ~700 ng of the product is needed.

1. Add 80 μL 0.5X binding wash buffer to each well of a 10-well plate.
2. Add 40–50 μL of PCR products to each of the 10 wells (*see* **Note 13**).
3. Place the five eight-tooth sequencing combs (attached together) in the plate wells, and mix gently by moving the combs up and down 2–3x.
4. Incubate the 10-well plate at 65°C for 30 min.
5. Remove the comb set and 10-well plate from the heat block, and incubate for 5 min at RT. Then replace evaporated water in each of the 10 wells with 80 μL Fisher DIUF.
6. Incubate the 10-well plate at 65°C for an additional 30 min, to ensure complete immobilization of the PCR products on the combs.

3.4.2. Template Denaturation

1. Remove the combs from the 65°C heat block and place in a petri dish containing TE, making sure that the streptavidin coating on the comb is completely immersed in the TE buffer for 30 s.
2. Repeat **step 1** twice, using fresh TE each time.
3. Blot the combs on a Kimwipe, to remove excess solution, then place them in a new 10-well plate containing 120 μL 0.1 *M* NaOH in each well. Incubate for 10 min at RT.
4. Prepare the annealing mix (for one comb set): 1040 μL Fisher DIUF, 120 μL annealing buffer, and 40 μL Cy5 M13 universal primer (2 pmol/μL).
5. Vortex well, and add 120 μL to each well of a new 10-well plate. Place the plate on ice until ready to proceed to **step 6**.
6. Place the 10-well plate containing the annealing mix on the 65°C heat block, to prewarm during the next three wash steps.
7. After the 10-min denaturation step, remove the combs, and place in a Petri dish containing NaOH. Wash for 30 s.

8. Blot the combs on a Kimwipe, and place in a Petri dish containing TE buffer. Wash for another 30 s.
9. Blot the combs on a Kimwipe and place in a petri dish containing Fisher DIUF water. Wash for a final 30 s.

3.4.3. Primer Annealing

1. Blot the combs on a Kimwipe, to remove all excess solution, then place them in the prewarmed 10-well annealing plate containing 120 µL annealing mix in each well. Incubate for 10 min at 65ºC. During this 10 min incubation proceed to **steps 2** and **3**.
2. Dilute the T7 Sequenase with cold enzyme dilution buffer, to a concentration of 6 U/µL. Mix well, and place on ice.
3. Prepare the sequencing master mixes (one for each A, C, G, and T):

<div align="center">(1 comb set/10 reactions)</div>

dNTP/ddNTP mix (A,C,G or T)	30 µL
Annealing buffer	20 µL
Extension buffer	10 µL
DMSO	20[a]/30 µL[b]
Fisher DIUF water	110[a]/100 µL[b]
T7 Sequenase [6 U/µL]	10 µL

Note: DMSO concentration: [a]A and B locus 10% (v/v), [b]C locus 15% (v/v).
4. Remove the combs from the 65ºC heat block and incubate an additional 10 min at RT followed by two washes in TE buffer.

3.4.4. Sequencing Reactions

1. During the 10 min incubation at RT, vortex each master mix and dispense 19 µL of the A master mix, into each A well of a fresh 40-well plate. Repeat for each of the other 3-nt master mixes, placing 19 µL into the appropriate wells (order: A, C, G, T) (*see* **Note 14**). Place the plate on ice, until needed.
2. Prewarm the 40-well plate for 1 min on the 37ºC heat block.
3. Add the combs to the 40-well plate, and mix gently by moving the combs up and down 2–3 ×. Incubate for exactly 5 min, then terminate the sequencing reactions by placing the comb set in a Petri dish containing fresh TE (*see* **Note 15**).

3.5. Gel Casting

1. Prepare a 10X TBE stock solution for the sequencing gel mix (500 mL): 60.5 g Tris, 1.85 g EDTA, 25.5 g boric acid, and 440 mL Fisher DIUF H_2O. Filter using a 0.22-µm or 0.45-µm filter, and store at 4ºC until required.
2. Prepare a 10X TBE stock solution for the running buffer (1 L): 121.0g Tris, 3.7g EDTA, 51.0 g boric acid, and 880 mL Ultrapure H_2O.
3. Prepare the sequencing gel mix (one gel, 6% PagePlus) (*see* **Note 16**): 19.8 g Urea, 7.95 mL PagePlus 40% conc., 5.5 mL 10X Tris-borate-EDTA, and 25.3 mL Fisher DIUF H_2O. Filter using a 0.22-µm or 0.45-µm filter.

4. Initiate polymerization of the sequencing gel by adding 330 µL freshly made 10% APS solution and 33 µL TEMED.

5. Cast a 0.5 mm sequencing gel, and allow it to polymerize for 5 hours (*see* **Note 17**).

3.6. Loading Sequencing Reactions on the ALFExpress

1. Remove all dust and gel debris from the gel cassette using isopropanol.

2. Set the electrophoresis conditions: run time, 360 min; voltage, 1900V; current, 60mA; power, 25W; temperature, 55°C; and sampling interval, 2 s.

3. Fill both the upper and lower buffer tanks with 1 L 0.6X TBE running buffer.

4. Remove the comb carefully, and rinse each gel well, to remove residual urea and gel debris.

5. When the temperature of the gel has reached 55°C, load 10 µL of stop solution into each gel well, including each of the blank end wells, to minimize edge effects.

6. Place the sequencing combs in the wells, being careful not to displace the stop solution (*see* **Note 18**).

7. Push the combs down to within approx 1 mm of the bottom of the wells.

8. Allow to stand 15 min, then carefully remove the combs, close the lid, and begin the run.

9. Analyze data, using the APB HLA SequiTyper version 2.00 software.

4. Notes

1. If using a thermocycler other than the Perkin Elmer 9700, it may be necessary to adjust the annealing temperatures accordingly.

2. System requirements for this software package are as follows:
   Hardware: CPU, recommended Pentium processor of 133 MHz or higher; graphics adapter, VGA or higher; memory, recommended 32 Mb; free disk space, 10Mb. Software: HLA SequiTyper is developed for use in the Microsoft Windows 95 operating system environments.

3. If using whole blood as the sample, use blood stored in citrate–EDTA, because heparin has been shown to negatively affect the DNA extraction.

4. Use only high-performance liquid chromatography purified primers in the PCR and sequencing. Make sure to request triethylene-glycol linker for the biotinylated primers.

5. Other brands of *Taq* polymerase may be shown to work; however, our experience is with the Fisher brand *Taq*.

6. This 10X PCR buffer usually comes with the *Taq* polymerase.

7. Never remove T7 polymerase stock from storage at –20°C, except momentarily to remove an aliquot. During use, keep all reagents on ice until required.

8. For best results, make the TE buffer fresh each week, and the NaOH fresh daily.

9. Use the appropriate primer mix: A1 for the -A locus amplification, B1 for the -B locus, and C1 for the -C locus.

10. Sometimes, a PCR band may not be visible; however, proceed with the secondary PCR.

11. Use the appropriate primer mix for the desired amplification, e.g., if amplifying the -A locus, perform four separate secondary PCR amplifications: one using the

A2 primer mix, another using the A3 mix, another with A4, and another with A5. This allows one to sequence both exons 2 and 3 bidirectionally, assuring accuracy in typing.

12. Proceed with sequencing if you have nice, tight, strong PCR bands for each of the four secondary PCR products.

13. To achieve proper comb orientation when loading sequencing gels, the pegs on the sequencing combs should point toward the user when placed on the plate wells.

14. Make sure not to introduce any bubbles while dispensing the master mixes. If bubbles should be introduced, remove them from the well, because they will deleteriously affect the sequencing.

15. The combs containing the sequencing reaction products may be stored in TE for up to 24 h.

16. Alternatively, the APB Reprogel may be used.

17. You may run a gel up to 24 h after polymerization. Keep the gel moist and out of the light until use.

18. The pegs on the combs should point toward the user.

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11

Photolabeling the Transporter Associated with Antigen Processing

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1. Introduction

Crosslinking reagents are valuable tools used to determine the quarternary and subunit structures of proteins *(1,2)*. Other applications for crosslinkers include the determination of protein–protein interactions, analysis of proteins in cellular membranes, and the identification of novel peptide-binding proteins *(3–5)*. Crosslinkers function by coupling reactive groups to amino acid side chains of peptides, and are divided into two groups; homobifunctional or heterobifunctional, depending on whether the available functional groups are similar or different, respectively. They can be further subdivided into groups that react either chemically or photochemically, i.e., requiring activation by ultraviolet light (UV) *(6,7)*.

The transporter associated with antigen processing (TAP) is a heterodimer of TAP-1 and TAP-2 subunits. It is primarily localized to membranes of the endoplasmic reticulum (ER). TAP binds to and facilitates the transport of cytoplasmic peptides, generated by the proteasome, into the endoplasmic reticulum, for association with major histocompatibility complex I (MHC-I) molecules *(8–11)*. TAP provides a critical peptide transport function in MHC-I antigen processing.

Crosslinkers, both photoreactive and chemical, have been exploited to study the structure and function of TAP. In these studies, the crosslinker is conjugated to peptides that are known to be efficiently transported by TAP. The conjugation of the photoreactive, heterobifunctional crosslinker, N-hydroxysuccinimidyl-4-azidobenzoate (HSAB; *see* **Fig. 1**) to a natural epitope, Nef7B,
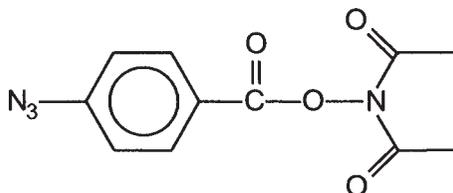
Fig. 1. Structure of HSAB.

was used to determine the ability of TAP to bind peptides that differ in length and sequence, and also to demonstrate that the peptide-binding site of TAP is composed of both TAP-1 and TAP-2 subunits *(12,13)*. In addition, other studies employing HSAB conjugated to a peptide derivative of Nef7B, KB11 (KB11-HSAB), demonstrated that TAP reconstituted in proteoliposomes can maintain its ability to bind peptide *(14)*. A more recent study *(15)* involving both KB11-HSAB and the chemical crosslinker, ethylene glycol *bis*(succinimidyl succinate), showed that the herpes simplex virus inhibitor, ICP47, destabilizes the TAP heterodimer. Finally, another photoreactive crosslinker, 4'-(trifluoromethyl-diazirinyl)-phenylalanine, has been used to identify regions of TAP-1 that bind peptides *(16)*.

In an effort to develop new photoreactive peptides for labeling TAP, we conjugated HSAB to three naturally occurring MHC-I epitopes: the histone-derived epitope B27#3 *(17)*, and two tumor-associated epitopes, MAGE-1.a and MAGE-1.b *(18)*. By creating a panel of photoreactive peptides for TAP, we hope to gain further insight into the relationship between the binding and transport of peptides by TAP. This chapter focuses on the methods used to conjugate the photocrosslinker, HSAB, to the B27#3, MAGE-1.a, and MAGE-1.b peptides, and to characterize the conjugates in TAP photoaffinity labeling experiments. In addition to giving insight into the structure and function of TAP, photoreactive peptides may be used to identify novel peptide-binding proteins, and to elucidate the mechanism of peptide-binding of proteins such as the ER-resident heat shock protein, gp96, and heat-shock protein 70 *(19–21)*.

2. Materials
2.1 Conjugation of HSAB to Peptides

1. 100 m*M* sodium (Na) borate, pH 8.5 (store at room temperature[RT]) (*see* **Note 1**).
2. 1 m*M* each, of the following synthesized peptides (*see* **Note 2**) in 100 m*M* Na borate, pH 8.5 (stable at –20°C): KB11 (AKVPLRPMTYKA), B27#3 (RRYQKSTEL), MAGE-1.a (SAYGEPRKL), MAGE-1.b (EADPTGHSY).
3. 30 m*M* HSAB in dimethyl sulfoxide (DMSO) (make fresh, as required).

2.2. Purification and Quantitation of Peptide-HSAB Conjugates

1. C-18 reverse-phase, high-performance liquid chromatography (HPLC) column (Vydac, Hesperia, CA).
2. HPLC-grade water.
3. Solution A: 0.1 % trifluoroacetic acid–/HPLC grade water.
4. Solution B: 70 % HPLC-grade UV transparent acetonitrile (CH_3CN), 0.085 % trifluoroacetic acid–HPLC-grade water.
5. Series of twofold dilutions (in water) of 30 mM HSAB (made in DMSO) in the range of 2–32 μM.
6. Centrifugal vacuum concentrator (SC110A SpeedVac Plus Concentrator, Savant Instruments, Holbrook, NY).

2.3. Iodination of Peptide–HSAB Conjugates

1. 20 nmol HPLC-purified peptide-HSAB conjugate.
2. 200 mM sodium phosphate (NaH_2PO_4), pH 7.4.
3. 2 mg/mL chloramine-T (make fresh in 200 mM NaH_2PO_4, pH 7.4).
4. 2 mg/mL sodium metabisulfite (make fresh in 200 mM NaH_2PO_4, pH 7.4).
5. 50 mM NaH_2PO_4, pH 7.0.
6. Glass wool.
7. 5.2 mL column of Sephadex G-10 in a 5 mL pipet.
8. 1 mCi sodium iodide (^{125}INa) (CAUTION: volatile, radiation hazard).
9. Automatic γ counter (1470 Wizard Automatic, Wallac, Gaithersburg, MD).

2.4. Preparation of Crude Membranes

1. B-lymphoblastoid cell line, Raji.
2. RPMI-1640, 10 % bovine calf serum, 2 mM glutamine, 1 mM Na pyruvate, and 100 U/mL penicillin and streptomycin.
3. Tris-buffered saline (TBS): 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.02% Na azide; store at 4°C.
4. 50 mM phenylmethylsulfonyl fluoride (PMSF) (store at –20°C).
5. 10 mM Tris-HCl, pH 7.4 (store at 4°C).
6. Intracellular transport buffer (ICT): 50 mM HEPES, pH 7.0, 78 mM KCl, 4 mM $MgCl_2$, 8.4 mM $CaCl_2$, 10 mM EGTA (store at 4°C).
7. 0.4 M dithiothreitol (store at –20°C).
8. 100 mg/mL bovine serum albumin (store at –20°C).

2.5. Photolabeling of TAP in Membranes

1. 96-well plates.
2. UV light (254 nm, Spectroline model XX-15F; Spectronics, Westbury, NY).
3. Solubilization buffer: TBS containing 1% Triton X-100 (store at 4°C).
4. 0.5 M iodoacetamide (IAA) (store at –20°C).
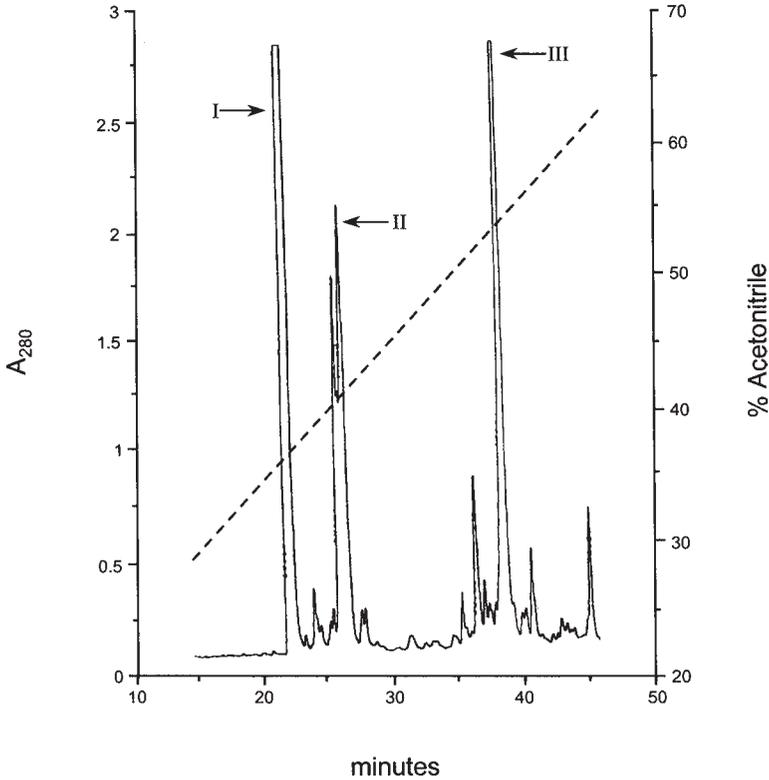5. Anti-TAP-2 monoclonal antibody 435.3.

Fig. 2. Chromatogram of HPLC-purified KB11-HSAB. 0.5 mg of KB11-HSAB was purified by HPLC, as described in **Subheading 3.2. step 1.** Peak I represents unconjugated HSAB, peak II represents the majority of the photoreactive peptide, KB11-HSAB, and peak III contains residual KB11-HSAB. The peaks were eluted after 22, 26, and 38 min, respectively. The dotted line represents the CH_3CN gradient.

6. 50% suspension of protein G-Sepharose.
7. Wash buffer: TBS containing 0.5% Triton X-100 (store at 4°C).
8. 30% (w/v) acrylamide, 0.8% (w/v) *bis*-acrylamide.
9. Upper gel buffer: 0.5 *M* Tris-HCl, 0.4% sodium dodecyl sulfate (SDS), pH 6.8.
10. Lower gel buffer: 1.5 *M* Tris-HCl, 0.4% SDS, pH 8.8.
11. N, N, N', N'-tetramethylethylenediamine.
12. 10% ammonium persulfate (store at –20°C).
13. Vertical SDS polyacrylamide gel electrophoresis (SDS-PAGE) apparatus (V16 model, Life Technologies, Grand Island, NY).
14. 10X SDS sample buffer: 0.5 *M* Tris-HCl, pH 6.8, 50% glycerol, 10% SDS, 0.05% bromophenol blue, 1% β-mercaptoethanol.
15. 10X Running buffer: 0.25 *M* Tris-base, 1.92 *M* glycine, 1% SDS.

16. X-ray film.
17. Gel dryer (Speed Gel SG210D, Savant).

3. Methods

3.1. Conjugation of HSAB to Peptides

1. Add 100 µL of 30 m*M* HSAB (in DMSO) to every 1 mL 1 m*M* peptide (in Na borate buffer; *see* **Subheading 2**), in a dark area (*see* **Note 3**).
2. Incubate reaction for 1 h at RT (*see* **Note 4**).

3.2. Purification and Quantitation of Peptide–HSAB Conjugates

1. 1 mL (approx 1–1.5 mg) of peptide–HSAB material is purified by HPLC, using a C18 reverse-phase column and a 45-min gradient of 0–70 % CH_3CN (solution B) (*see* **Note 5**). Load 0.5 mL of peptide–HSAB material at a time onto the column. Column flow rate is set at 1 mL/min. Monitor the absorbance at 280 (A_{280}) to determine the elution of major peaks. Collect fractions that represent all major peaks (*see* **Fig. 2**).
2. Lyophilize the collected fractions overnight in the dark. Resuspend each lyophilized fraction in 50 µL water. Measure the A_{280} of a 1:50 dilution of each fraction. Determine the concentration of each fraction by extrapolating the value from a standard curve of serial dilutions of HSAB (ranging from 2–32 µ*M*) versus the A_{280}. Aliquot 20 nmoles of peptide from each fraction and store at –80°C.

3.3. Iodination of Peptide–HSAB Conjugates

1. Plug a 5-mL pipet with glass wool, and pour a 5.2-mL column of Sephadex G-10. Equilibrate the column with two column volumes of 50 m*M* NaH_2PO_4, pH 7.0.
2. Combine 50 µL 200 m*M* NaH_2PO_4, pH 7.4, 20 nmol peptide–HSAB conjugate, 20 µL 2 mg/mL chloramine-T, and 1 mCi ^{125}INa. Incubate reaction, covered, at RT, for 2.5 min (*see* **Note 6**).
3. Add 20 µL 2 mg/mL Na metabisulfite, then apply the reaction mixture to the Sephadex G-10 column. Allow reaction mixture to enter the column bed. Collect the first fraction by eluting with 3 vol of 170 mL 50 m*M* NaH_2PO_4, pH 7.0 then collect an additional five fractions of 500 µL each. Store fractions at 4ºC.
4. Make a 1:100 dilution of each collected fraction in water. Measure the counts per minute (cpm) of 10 µL of each fraction dilution using an automated γ counter (*see* **Note 7**).

3.4. Preparation of Crude Membranes

1. Resuspend 1×10^8 Raji cells in 2 mL cold TBS containing 0.5 m*M* PMSF (*see* **Note 8**). Spin the suspension at 2000*g* at 4°C for 5 min. Save the supernatant, and resuspend the pellet in 1 mL cold 10 m*M* Tris-HCl, pH 7.4. Spin again at 2,000*g* at 4°C for 5 min. Pool the two supernatants, and spin at 100,000*g* for 45 min at 4°C, to pellet the membranes.
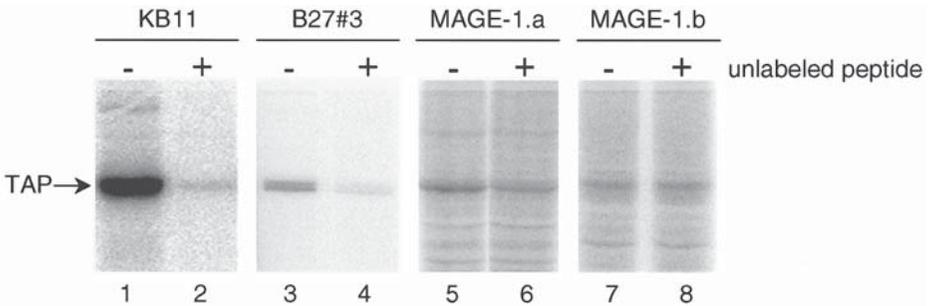
Fig. 3. Photolabeling of TAP with various photoreactive peptides. Raji membranes were left untreated, or were treated with 50 µ*M* each of unlabeled KB11 (lane 2), B27#3 (lane 4), MAGE-1.a (lane 6), and MAGE-1.b (lane 8). Membranes were then photolabeled with ^{125}I-KB11-HSAB (lanes 1 and 2), ^{125}I-B27#3-HSAB (lanes 3 and 4), ^{125}I-MAGE-1.a-HSAB (lanes 5 and 6), or ^{125}I-MAGE-1.b-HSAB (lanes 7 and 8), as described in **Subheading 3.5.** Crosslinked membranes were solubilized in SDS sample buffer, and analyzed directly by SDS-PAGE (10%) and autoradiography (lanes 1, 2, 5–8). Alternatively, crosslinked membranes were solubilized and immunoprecipitated as described in **Subheading 3.5.**, and analyzed by SDS-PAGE (10%) and autoradiography (lanes 3 and 4).

2. Resuspend the membrane pellet in 1 mL of cold ICT buffer containing 1 m*M* dithiothreitol and 4 mg/mL bovine serum albumin.

3.5. Photolabeling of TAP in Membranes

1. Aliquot 100 µL cell membrane-ICT suspension (from 1×10^7 cells each, 0.4 mg total protein) into wells of a 96-well plate on ice. In the dark, add 4 µ*M* (*see* **Note 9**) radiolabeled photoreactive peptide, and incubate on ice for 30 min. For competition experiments, add 50 µ*M* unlabeled peptide to membranes, and incubate for 10 min on ice, prior to the addition of the radiolabeled photoreactive peptide. Expose samples to UV light for 6 min on ice. Transfer samples to microcentrifuge tubes, and wash twice with 1 mL cold ICT buffer, by centrifuging at 15,000*g* at 4°C for 5 min. Resuspend washed photolabeled membrane pellets in 100 µL 1X SDS sample buffer, boil for 5 min, and spin at 15,000*g* at RT for 5 min, to clarify the supernatant. Analyze samples directly by SDS-PAGE (10%) and autoradiography.

2. Alternatively, photolabeled TAP can be immunoprecipitated by solubilizing each washed photolabeled membrane pellet in 1 mL solubilization buffer containing 0.5 m*M* PMSF and 5 m*M* IAA, for 1 h on ice. Spin extracts at 15,000*g* for 5 min at 4°C, to clarify. Transfer the supernatants to fresh tubes, and immunoprecipi-

tate each with 5 µL purified anti-TAP-2 monoclonal antibody, 435.3 *(22)* and 25 µL protein G-Sepharose, by rotating at 4ºC for 1 h. Wash the antibody–protein G complexes 3 × with 1 mL wash buffer, boil in 100 µL 1X SDS sample buffer for 5 min, and analyze by SDS-PAGE (10%) and autoradiography.

As an example, photoreactive peptides were made from a histone-derived peptide, B27#3, and two melanoma-associated epitopes, MAGE-1.a and MAGE-1.b (*see* **Subheading 2.1.**). These peptides were chosen based on their inhibitory concentration of 50% (IC_{50}) values, i.e., the concentration of competitor peptide necessary to produce 50% transport inhibition of a reporter peptide *(18)*. In addition, the photoreactive peptide, KB11-HSAB, was used as a positive control, to demonstrate efficient photolabeling of TAP. As expected, peptides with low IC_{50} values, indicating that they are good substrates for TAP (B27#3, MAGE-1.a), photolabeled TAP more efficiently than the peptide with a higher IC_{50} value (MAGE-1.b) (*see* **Fig. 3**). These results confirm previous peptide binding and transport studies of TAP.

4. Notes

1. 100 m*M* stock solution of Na borate, pH 8.5, should be prepared by making a 50 m*M* solution according to the formula weight, to give a final stock concentration of 100 m*M*.
2. Peptides chosen for these experiments must include a tyrosine residue in the primary sequence, for iodination. HSAB conjugation to the peptide may occur at either the amino-terminal end of the peptide, or to the ε-amino group of lysine residues, and this could affect the interpretation of the results.
3. The conjugation reaction should be performed in the dark, since HSAB is light-sensitive.
4. Peptides conjugated to HSAB can be stored at –20°C until HPLC is performed.
5. Perform an HPLC test run of a 50-µL sample of each peptide–HSAB conjugate, to determine the elution times of the major peaks. Save all major peaks from the HPLC purification step at –80°C until the peak that contains the peptide–HSAB conjugate is determined.
6. The iodination procedure should be performed, in dim light, in a chemical fume hood. Iodinate 20 nmol of each HPLC peak that does not represent free HSAB, and test the radiolabeled peptides in photolabeling experiments (this is the only conclusive way to determine which peaks contain the photoreactive peptide).
7. The cpm of the 10 µL of each fraction dilution actually represents cpm/0.1 µL, and should, therefore, be multiplied by 10 to determine the cpm/µL. The specific activity of iodinated photoconjugates ranges from 15 to 40 cpm/fmol.
8. Large quantities of Raji cells for preparation of crude membranes may be obtained by growing the cells in spinner flasks. Raji cells can reach concentrations of 1.8×10^6 cells/mL and should be split to $3–5 \times 10^5$ every 2–3 d. Also, 1×10^8 Raji cell pellets may be stored at –80°C for preparation of crude membranes. Crude membranes, resuspended in ICT buffer, can be snap-frozen in liquid nitro-

gen and stored at –80ºC for photolabeling experiments.

9. Because there is some variability in photolabeling TAP, it is necessary to titrate the photoreactive peptide to optimize the photolabeling of TAP. For example, 4 μM radiolabeled photopeptide (as mentioned in **Subheading 3.3.5.**) represents the optimal amount of KB11-HSAB used in these experiments. However, the amounts of the photoconjugates of B27#3, MAGE-1.a, and MAGE-1.b necessary to photolabel TAP may be greater than 4 $\mu M,$ and, therefore, various amounts of each should be tested.

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12

Application of One-Dimensional Isoelectric Focusing to Separate Different Major Histocompatibility Complex Class I Alleles and Determine Their Allelic Interactions with Transporter Associated with Antigen Processing (TAP)

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1. Introduction

Major histocompatibility complex class I (MHC-I) molecules are highly polymorphic cell surface expressed molecules that bind antigenic (Ag) peptides in the endoplasmic reticulum (ER) and transport them to the cell surface for presentation to cytotoxic T-cells *(1)*. The MHC-I complex is composed of a 45 kDa polymorphic heavy chain (HC) noncovalently associated with the 12 kDa light chain, β_2-microglobulin (β_2m), together forming a peptide-receptive heterodimer *(1)*. The third subunit is the Ag peptide, which is derived from cytosolic proteins and delivered into the ER by the peptide transporter associated with Ag processing, TAP *(2)*. Correct assembly of the heterotrimeric MHC-I complexes in the ER is essential for their stable expression at the cell surface.

In 1994 it was established *(3,4)* that assembly of MHC-I complexes involves the transient association of the peptide-receptive MHC-I HC/β_2m heterodimers with TAP. Upon peptide binding, the MHC-I molecules dissociate from TAP and leave the ER, which indicates that TAP might be directly involved in the assembly and peptide loading of MHC-I molecules. Since those studies, three additional ER resident proteins, calreticulin, tapasin, and Erp57, have been found to associate with the TAP–MHC-I complex *(5–10)*, thereby forming a large MHC-I peptide loading complex, in which tapasin is thought to bridge the class I molecule to TAP *(5,10)*.

The close proximity of MHC-I molecules to TAP is suggested to facilitate their loading of TAP-transported peptides in the ER lumen. However, we recently showed that not all MHC-I molecules interact equally well with TAP *(11)*. In fact, a considerable number of the human class I alleles studied associated very poorly or not at all with TAP. These class I alleles are still efficiently loaded with peptide and present Ags at the cell surface, which indicate that the interaction with TAP is not an absolute requirement for proper loading of every MHC-I allele. This is supported by recent studies using the tapasin-negative human cell line .220, in which MHC-I molecules do not associate with TAP, and fail to present Ags at the cell surface *(5,12,13)*. In these cells, expression of a truncated, soluble tapasin protein restored Ag presentation by the transfected human leukocyte antigen (HLA)-B8 allele, although TAP–MHC-I interaction was not detectable *(14)*. This indicates that, in the HLA-B8 transfected .220 cells, it is the interaction of MHC-I molecules with tapasin, rather than with TAP, that is important for efficient MHC-I peptide loading and surface expression *(14)*. Whether this applies to other HLA class I (HLA-I) alleles as well, remains to be established. The allele-specificity of MHC-I–TAP interactions has recently been extended to include the interaction of MHC-I alleles with calreticulin (5). Based on the observed allele-specificities of MHC-I molecules, it is of interest to study the different class I alleles separately, and to determine their individual interactions with TAP, as well as with other components of the MHC-I peptide loading complex.

In humans, MHC-I molecules are encoded by three loci, HLA-A, -B, and -C, in which each locus encodes many different alleles *(15)*. An individual can express up to six different HLA class I alleles. Because class I alleles have very similar molecular weights, it is not possible to separate them by standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis. However, HLA-I alleles differ in their amino acid composition, and thus have different isoelectric points. It is therefore possible to separate the different class I alleles expressed in a cell by one-dimensional isoelectric focusing (1D-IEF), in which proteins are separated in a gel according to their isoelectric point *(16)*.

Described here is a method for separating different HLA-I alleles, and studying their individual interactions with TAP, using the 1D-IEF technique. The protocol described is a modified version of a procedure published previously *(16)*. The method involves three major steps: metabolic labeling of cells to radioactively label HLA-I molecules; immunoprecipitation of HLA class I molecules from cell lysates with specific antibodies (Ab), and separation and analysis of the HLA class I molecules by 1D-IEF.

The expression of HLA-I alleles can be visualized by labeling living cells metabolically with [^{35}S]methionine/cysteine (Met/Cys) or, alternatively, by

Western blotting. However, metabolic labeling of cells is required for visualization of early stages of MHC-I assembly. The cells are subsequently lysed in a mild detergent, such as digitonin, which preserves the interaction between TAP and HLA-I molecules *(3,4)*. It is thus possible to immunoprecipitate the radiolabeled class I molecules directly, or to determine their interaction with TAP by studying the co-immunoprecipitation of HLA-I molecules with TAP *(3–5,11)*. The isolated HLA-I alleles are analyzed by 1D-IEF, in which the alleles will be separated according to their isoelectric points. We have employed this method to electrophoretically separate the different MHC-I alleles expressed in a cell, study the kinetics of assembly, maturation and intracellular transport of the different MHC-I alleles, and identify their individual interactions with other proteins, such as TAP *(11,16–18)*.

2. Materials

2.1. Metabolic Labeling of Cells

1. Human cells expressing HLA-I alleles of interest (*see* **Note 1**).
2. Complete media: RPMI-1640, supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine.
3. Starvation media: Met/Cys-free RPMI-1640 media supplemented with 10% FCS.
4. Chase media: complete media supplemented with 1 mM Met and Cys.
5. [^{35}S]-Met/Cys mixture (usually 80% / 20%).
6. 2% (w/v) digitonin stock solution (Calbiochem, La Jolla, CA) (*see* **Note 2**).
7. Lysis buffer: 1% (w/v) digitonin, 50 mM Tris-HCl (pH 7.5), 5 mM MgCl$_2$, 150 mM NaCl.

2.2. Immunoprecipitation of Free and TAP-associated MHC-I Alleles

1. Nonspecific Ab: Normal rabbit serum (NRS) or normal mouse serum.
2. MHC-I-specific Abs: *W6/32*, mAb recognizing β$_2$m-associated HLA-A, -B, and -C alleles; *αHC*, polyclonal rabbit serum against free HLA-A, -B, and -C HCs.
3. TAP-specific Abs: *αTAP-1 and αTAP-2*, polyclonal rabbit sera recognizing the ATP-binding domain of human TAP-1 and TAP-2, respectively (*see* **Note 3**). Both anti-TAP sera recognize the assembled TAP-1–TAP-2 complex, as well.
4. Protein A-Sepharose CL-4B (in 20% EtOH, Pharmacia Biotech, Uppsala, Sweden).
5. Washing buffer: 0.5% digitonin (w/v), 50 mM Tris-HCl (pH 7.5), 5 mM MgCl$_2$, 150 mM NaCl.

2.3. Analysis of MHC-I Molecules by 1D-IEF

1. Two glass plates; one plate 30 (horizontal) × 19 cm (vertical), and one plate 30 (horizontal) × 21 cm (vertical) (*see* **Note 4**).
2. Three spacers (1 cm wide, 1 mm thick).

3. A Teflon comb (1 mm thick) accommodating 20–25 samples.
4. 1% (w/v) agarose at 65°C (Gibco-BRL, Life Technologies, Paisley, Scotland).
5. Clamps.
6. Gel chamber.
7. Urea (Merck, Darmstadt, Germany).
8. 10% (v/v) NP-40 solution (BDH Laboratory Supplies, Dorset, UK).
9. Acrylamide/*bis*-acrylamide (30 / 1.6%) solution (Serva, Heidelberg, Germany).
10. Ampholines, pH 5.0–7.0, pH 7.0–9.0 and pH 3.5–10.0 (Pharmacia Biotech).
11. 10% (w/v) ammonium peroxodisulfate (APS) (Merck).
12. Tetramethylethylendiamine (TEMED) (Sigma, St. Louis, MO).
13. Lower electrode buffer: 20 mM H_3PO_4 (1.3 mL of a 89% H_3PO_4 solution/L) (Merck).
14. Upper electrode buffer: 50 mM NaOH (2 g NaOH pellets/L) (Merck).
15. IEF sample buffer: 9.5 M urea, 2% (v/v) NP-40, 2% (v/v) ampholine pH 3.5–10.0, 5% β-mercaptoethanol.
16. IEF overlay buffer: IEF sample buffer diluted 4 × with water, add some bromophenol blue to verify correct migration during electrophoresis.
17. Hamilton syringe.
18. Power supply with voltage capacity of 1000 V (e.g., ECPS 3000/150, Amersham Pharmacia, Buckinghamshire, UK).
19. Dimethylsulfoxide (DMSO) (Riedel-de Haen, Seelze, Germany).
20. 2,5-Diphenyloxazol, scintillation grade (PPO) (Merck).
21. Whatman 3MM paper or gel-blotting paper (Danel, Germany).
22. Kodak X-omat AR film (Rochester, NY).

3. Methods

3.1. Metabolic Labeling of Cells

3.1.1 Expression of MHC-I Alleles and Their Interaction with TAP (see **Note 5**)

1. Harvest 1×10^7 cells (5×10^6 cells/immunoprecipitation) by centrifugation for 5 min at 400g in a benchtop centrifuge (*see* **Note 6**).
2. Wash the cells by resuspending in prewarmed phosphate-buffered saline (PBS) (37°C), centrifuge 5 min at 400g to pellet the cells, and aspirate the PBS.
3. Resuspend cells in 0.5 mL starvation media, and incubate for 45 min in incubator (37°C, 5% CO_2).
4. Spin down cells (5 min, 400g), resuspend cell pellet in 100 µL fresh starvation media, and add 100 µCi [^{35}S]-Met/Cys (*see* **Note 6**). Incubate for 15–30 min at 37°C (5% CO_2), to label sufficient amounts of newly synthesized proteins.
5. Wash cells once in cold PBS, and spin them down (5 min, 400g).
6. Lyse cells in 1 mL ice-cold lysis buffer containing 1% digitonin and leave lysis on ice for at least 1 h.

7. Spin down nuclei (10 min, 1000g), and transfer postnuclear lysate to new microcentrifuge tube.

3.1.2. Pulse-Chase Analysis to Follow Kinetics of MHC-I Assembly, Maturation, and TAP Interaction

1. 7×10^7 cells (1×10^7 cells are used for every chase time) are harvested, and washed once in PBS, as described in **Subheading 3.1.1.** (*see* **Note 6**).
2. Resuspend cells in 1 mL starvation media, and incubate for 45 min at 37°C (5% CO_2).
3. Following starvation, cells are pelleted (5 min, 400g) and resuspended in 300 µL fresh starvation media.
4. Label cells metabolically with 300–500 µCi [^{35}S]-Met/Cys for 15 min at 37°C and 5% CO_2 (*see* **Note 6**).
5. Stop incorporation of label by adding 7 mL complete media supplemented with 1 mM Met/Cys (chase media, 37°C).
6. Continue incubation at 37°C. At time-points 0, 10, 30, 60, 120, 240, and 480 min, take out 1 mL suspension (1×10^7 cells) into 10 mL ice-cold PBS.
7. Pellet cells by centrifuging at 400g and aspirate PBS.
8. Lyse each cell aliquot in 1 mL lysis buffer containing 1% digitonin for 1 h on ice.
9. Remove nuclei by centrifuging 10 min at 1000g. Transfer each lysate to new microcentrifuge tube.

3.2. Immunoprecipitation of Free and TAP-Associated MHC-I Alleles

Radioactively labeled MHC-I alleles are immunoprecipitated from the cell lysates produced as described in **Subheadings 3.1.1.** or **3.1.2.** All steps described below should be performed at 4°C.

1. Preclear each cell lysate by a nonspecific precipitation step with NRS at 4°C for 2 h (rotating) (*see* **Note 7**). Recover immunoprecipitates with protein A Sepharose (25 µL 50% solution), by incubation for 30 min.
2. Spin down protein A-Sepharose beads (2 min, 10,000g, 4°C) and transfer lysate to new microcentrifuge tube.
3. Repeat the nonspecific preclear step for 1 h, and recover immunoprecipitates with protein A-Sepharose beads.
4. Specific immunoprecipitation: Split each lysate into two portions (0.5 mL each, representing a lysate of 5×10^6 cells), and incubate one portion with mAb W6/32 to recover β$_2$m-associated HLA-A, -B and -C alleles (*see* **Note 8**). The other portion is incubated with anti-TAP-1 or anti-TAP-2 serum (αTAP-1 or αTAP-2, respectively) to recover TAP/MHC-I complexes (*see* **Note 9**).
5. Incubate for 1–2 h at 4°C (rotating), and recover immunoprecipitates with protein A-Sepharose for 30 min.
6. Spin down protein A-Sepharose beads (2 min, 10,000g, 4°C) and wash 3 × in 0.5 mL washing buffer containing 0.5% digitonin.
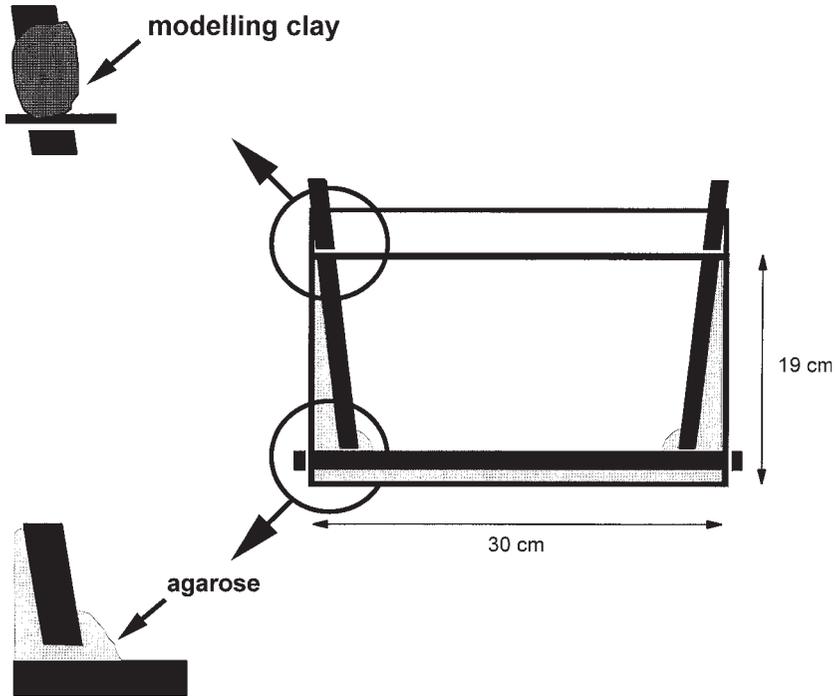
Fig. 1. Preparation of the 1D-IEF gel. The side spacers between the two glass plates are tilted toward the bottom spacer, to prevent the gel from slipping out during electrophoresis. Agarose is applied along all three spacers and in lower corners, to seal glass plates before pouring the gel. The polymerized gel is built in the gel apparatus with modeling clay.

3.3. Separation and Analysis of MHC-I Molecules by 1D-IEF

3.3.1. Preparation of the 1D-IEF Gel

1. Two glass plates, three spacers, and a Teflon comb. Clean the glass plates and spacers thoroughly, and finish off with alcohol. Clean the Teflon comb with hot water (no alcohol).
2. Put the two side spacers, slightly tilted (1–2 cm) toward the bottom spacer, between the glass plates, to prevent the gel from slipping out during electro-phoresis (*see* **Fig. 1**).
3. Leave a space (2–3 mm) between the bottom spacer and the side spacers, and put the clamps all around the plates on the spacers.
4. Put agarose along all spacers, to seal the gel. Take care that the lower corners, where the side and bottom spacers almost meet, are filled with agarose (*see* **Fig. 1**).
5. Prepare gel solution for a 4.5% polyacrylamide 1D–IEF gel (total volume: 55 mL): 30.25g urea, 11.0 mL 10% (v/v) NP-40, 8.25 mL acrylamide/*bis*-acrylamide (30 / 1.6%), 12.1 mL water (Millipore).

6. Warm up the gel solution in hot water bath, while swirling, to dissolve the urea.
7. When solution is at room temperature (RT) again, add the following ampholines:
   2.2 μL   ampholine pH 5.0–7.0
   220 μL   ampholine pH 7.0–9.0
   550 μL   ampholine pH 3.5–10.0
8. Add 200 μL 10% (w/v) APS and 100 μL TEMED to the gel solution, mix quickly, and pour the gel immediately between the glass plates, completely to the top.
9. Immediately, insert the Teflon comb. Avoid air bubbles.
10. Polymerization of the gel takes 30–60 min. (*see* **Note 10**).

3.3.2. Loading of Samples and Electrophoresis of the 1D-IEF Gel

1. Remove the comb and the bottom spacer. The comb should be removed very carefully, in little steps, to keep wells intact. Remove urea crystals from the wells.
2. Fill lower gel chamber with the lower electrode buffer (20 m*M* H_3PO_4).
3. Build the gel in the gel apparatus, with modeling clay between the upper corners of the largest plate and the gel apparatus (*see* **Fig. 1**). Alternatively, use a glass plate with "ears" as the small glass plate.
4. Remove air bubbles under the gel.
5. **Important:** avoid leakage of the buffer during the electrophoresis. Therefore, seal the glass plates to the gel apparatus with 1% agarose. Also, add agarose along the clay, to avoid leakage between the clay and the glass plates/gel apparatus.
6. Prepare the immunoprecipitates (from **Subheading 3.2.**) for loading, by adding 30 μL IEF sample buffer to each tube of washed protein A Sepharose beads.
7. Incubate samples for 15 min at RT, and spin down the beads (2 min, 10,000*g*)
8. Apply the supernatant to the 1D-IEF gel with a Hamilton syringe. Load empty lanes with a similar amount of IEF sample buffer (*see* **Notes 11** and **12**).
9. Apply 30 μL IEF overlay buffer on top of each loaded sample. This avoids deamination of glutamine and asparagine residues.
10. Carefully, fill up the wells with the upper electrode buffer (50 m*M* NaOH). **Important:** Apply buffer with a plastic Pasteur pipet along the glass plate over the wells, to avoid mixing buffer with the samples.
11. Fill up the upper gel chamber with 50 m*M* NaOH, and connect the gel apparatus to the power supply.
12. Electrophoresis: Set voltage limit at 1000 V, and adjust the power so that the initial voltage is 350 V, which will prevent excessive heat formation during electrophoresis. Within 2–3 h, voltage rises to 1000 V.
13. Continue electrophoresis for 13–16 h (*see* **Note 13**).

3.3.3. Enhancement, Drying and Autoradiography of the 1D-IEF Gel

1. Remove the gel from the glass plates carefully. The gel is very fragile (4.5%) and should not be touched by hand.
2. Soak the gel, directly after electrophoresis, in DMSO (*see* **Notes 14** and **15**). The gel should be completely covered by DMSO.
3. Shake the gel for 30 min RT, and pour off the DMSO, which can be reused once or twice.

4. Add fresh DMSO, and shake again for 30 min. Remove the DMSO.
5. Soak the gel in a DMSO–PPO solution (220 g PPO–800 mL fresh DMSO), and leave it shaking for 1 h (*see* **Note 16**).
6. Remove the DMSO–PPO solution, which can be reused about 8–10×.
7. Soak the gel in water. The gel will become white.
8. Wash the gel 3–4× in water. Leave the gel shaking in the last wash for 15–20 min.
9. Carefully transfer the gel to wet Vitawrap/plastic folio (*see* **Note 17**). Put wet Whatman 3MM paper on top of the gel.
10. Dry the gel under vacuum at 60°C for 1.5 h.
11. Expose the dried gel to Kodak X-omat AR film.

4. Notes

1. An example is given using human cells in suspension to study the MHC-I molecules, but other cell types (murine cell lines, peripheral blood leukocytes, and so on) may be used as well.
2. Make a 2% stock solution of digitonin by adding 2 g digitonin into 100 mL boiling water (add digitonin powder carefully, since solution will boil up quickly). Boil solution for 5 min while stirring, and let it cool off to RT. Filter off any precipitate and store solution at RT, or at 4°C. This 2% digitonin solution is stable for 1–2 mo. **CAUTION**: Digitonin powder is toxic, so always wear gloves, and avoid contact with skin or eyes.
3. The TAP-1 and TAP-2 antisera used here were made in the laboratory as follows: A glutathione S-transferase (GST) fusion protein was constructed containing the adenosine triphosphate-binding domain of either TAP-1 (amino acids 507–748) or TAP-2 (amino acids 434–703). The GST fusion proteins were overexpressed in *Escherichia coli*, isolated as inclusion bodies by differential centrifugation, and injected into rabbits. Obviously, other Abs against the TAP-1 and TAP-2 proteins can be used as well.
4. The size of the 1D-IEF gel described in this protocol is 30 (horizontal) × 19 cm (vertical), which gives a very good resolution after electrophoresis under the described conditions. Gels of other sizes can also be used. However, very broad gels tend to slip from under the glass plates during electrophoresis. For a very narrow gel, the electrical field is not homogeneous, and will not give an optimal resolution.
5. It should be noted that the interaction between MHC-I molecules and TAP may not be direct, but mediated by the tapasin molecule *(5,10)*.
6. The amount of cells and radioactive label described in this protocol is what we normally use for efficient labeling and immunoprecipitation. However, this is just an example, and the amount of cells and radioactive label can be changed as desired. A labeling period of 15-30 min is too short to label TAP molecules and, consequently, TAP cannot be visualized under the described conditions. TAP has a long half-life, and cells should be metabolically labeled overnight to visualize the TAP proteins. For studying the early events of assembly of MHC-I HC-β_2m-TAP complexes, a labeling period of 60–90 s is recommended.
7. Preclearing of cell lysate with NRS can be performed overnight as well.

8. The mAb W6/32 recognizes free or peptide-loaded MHC-I HC/β_2m–complexes, but not TAP-associated MHC-I molecules *(11)*.
9. Optional: To verify that the TAP-associated molecules (migrating in the 1D-IEF gel), indeed represent MHC-I alleles, half of the αTAP-1 or αTAP-2 immunoprecipitates are denatured in 50 μL 1% SDS (95°C, 5 min). Add 1 mL lysis buffer, and reprecipitate with a polyclonal Ab recognizing free HLA-I HCs (αHC). Load the reprecipitated samples next to the αTAP immunoprecipitates on the 1D-IEF gel to compare migration patterns.
10. We recommend making the gel at least 3–4 h before use, to allow better polymerization of the gel. This will result in generation of a more regular gradient during electrophoresis.
11. Do not load samples in the outer most wells, because the gel may be "smiling" here during electrophoresis.
12. If several different cell lines are analyzed, and some of the expressed MHC-I alleles are not known, it is recommended to load samples with the largest number of shared class I alleles next to each other on the 1D-IEF gel. This will facilitate identification of the unknown alleles in the gel.
13. For an optimal gradient, electrophoresis should be performed for at least 13 h and no longer than 20 h.
14. CAUTION: DMSO is toxic and gloves should always be used. Cover the tray, containing gel and DMSO, with a lid, and place it, shaking, in a hood or a properly ventilated room.
15. Fluorography of the gel can be facilitated by using either DMSO–PPO or Enhance (New England Nuclear, Boston, MA). We prefer the DMSO–PPO treatment, because it better stabilizes the fragile 4.5% IEF gel. An additional advantage of using DMSO–PPO is that some of the ampholine bands become visible (should be straight bands) after 15 min incubation in DMSO, visualizing the regularity of the pH gradient obtained during electrophoresis.
16. DMSO–PPO solution is made 1 d in advance, to let the PPO dissolve completely. CAUTION: PPO is toxic, so always use gloves and weigh out the PPO in a chemical hood. The DMSO–PPO solution is stored at RT, and can be reused at least 10 × (or until the gels are no longer stabilized by the DMSO–PPO treatment). Keep the solution away from water, since PPO will precipitate in the presence of water.
17. After DMSO–PPO treatment, the IEF gel is less fragile, and can be handled carefully, but always use wet gloves when touching the gel.

Acknowledgments

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Analysis of Major Histocompatibility Complex Class I Interactions with Endoplasmic Reticulum Proteins

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1. Introduction

Major histocompatibility complex class I (MHC-I) molecules alert cytotoxic T-lymphocytes to the presence of intracellular pathogens and tumors by presenting short peptides derived from pathogen- or tumor-specific proteins. To present a peptide, an MHC-I heavy chain (HC) binds the peptide and a small accessory protein called β_2-microglobulin (β_2m) in the endoplasmic reticulum (ER), and then migrates to the cell surface. The outer domains of the MHC-I HC and the antigenic peptide are then available to bind to T cell receptors.

During the process of assembling with β_2m and peptide, the MHC-I HC interacts with several ER proteins, including the transporter associated with antigen processing (TAP), calreticulin, and tapasin *(1–3)*. Each of these three proteins binds preferentially to the open form of the MHC-I HC *(1–5)*, and is responsive to peptide-induced folding *(4–5)*. Furthermore, the binding of all of these three ER proteins is abrogated by mutations in three disparate locations on the class I HC, positions 86, 134, and 227, which suggests possible cooperativity in their binding *(5,6)*.

The conformational- and site-specificity of this complex has been shown most clearly through a technique employing a combination of immunoprecipitations and Western blots *(4–7)*. A critical aspect of this technique is the use of thoroughly characterized antibodies (Ab) for the immunoprecipitation step, which can specifically recognize the open, peptide-free forms of murine and human MHC-I HC/β_2m heterodimers *(8–14)*. After immunoprecipitation of the wild-type or mutant forms of open class I from a cell lysate, the presence or absence of association between class I and co-precipitating ER proteins is
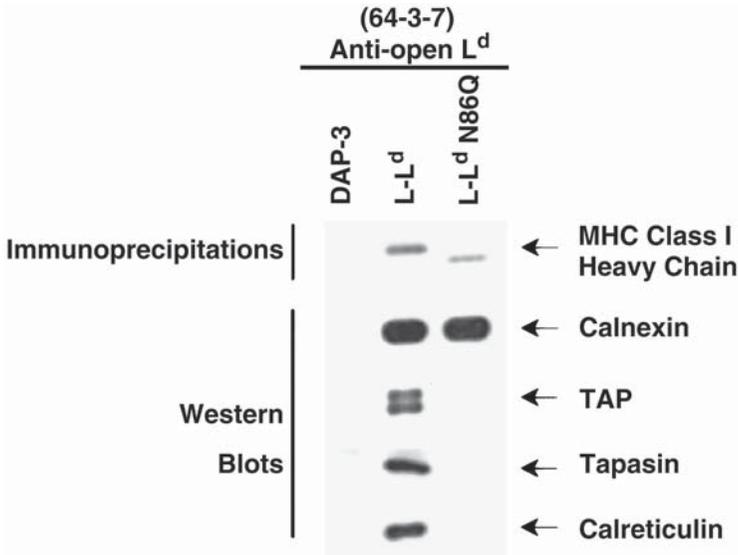
Fig. 1. Detection of the loss of association between class I and TAP, tapasin, and calreticulin, resulting from a mutation at position 86 of the class I HC through immunoprecipitation of the HC (shown in the autoradiograph) and identification of co-precipitated assembly complex proteins by Western blotting.

assayed by a sensitive Western blotting technique, as described in the following subheadings. Comparison of wild-type and mutant forms of class I HCs has identified class I sites important for binding to assembly complex proteins, and, accordingly, may lead to advances in the ability to manipulate immune regulation at this level.

An example of data derived using this immunoprecipitation and Western blotting procedure is shown in Fig. 1. Association of ER proteins with the murine MHC-I H2-L^d HC is contrasted with the loss of association of such proteins with an L^d mutant (asparagine→glutamine at position 86), which lacks the normal MHC-I α1 domain glycosylation site.

2. Materials

2.1. Immunoprecipitation

1. Sterile phosphate-buffered saline (PBS), pH 7.4: 140 mM NaCl, 8 mM Na$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$.
2. Cells expressing MHC-I molecules, e.g., murine Ltk$^-$ DAP-3 (H-2^k) fibroblast cells transfected with *Ld* gene (referred to as L-L^d) *(14)* cultured at 37°C, 5% CO$_2$ in RPMI-1640 medium with 10% fetal calf serum (v/v), 2 mM L-glutamine, 1 mM Na pyruvate, 0.1 mM per amino acid of non-essential amino acids, 25 mM

HEPES, penicillin (100 U/mL), and streptomycin (0.1 mg/mL). Expression of the L^d molecules is maintained by selection with Geneticin selective antibiotic (Gibco-BRL, Gaithersburg, MD), at 0.4 mg/mL (active concentration).

3. 5 mM EDTA in PBS.

4. Labeling media: RPMI-1640 Medium without L-methionine (Gibco-1640), plus 5% fetal calf serum, 2 mM L-glutamine, and 25 mM HEPES.

5. EasyTag Express [^{35}S]-Protein Labeling Mix (New England Nuclear [NEN], Boston, MA).

6. Charcoal traps (NEN) and 18 gauge hypodermic needles.

7. Activated carbon.

8. 60- and 100-mm tissue culture dishes.

9. Tris-buffered saline (TBS), pH 7.4: 10 mM Tris-HCl, 151 mM NaCl, adjust to correct pH with HCl. Store at 4°C.

10. Cell wash buffer: 20 mM iodoacetamide (IAA) in PBS. Make wash buffer fresh on the day of cell harvest, and use chilled on ice. IAA is light-sensitive, therefore cover the solution in aluminum foil. Caution should be used when weighing IAA and handling the solution, because it is toxic and a severe irritant.

11. 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate (CHAPS) stock solution: 1% CHAPS (w/v) in TBS. CHAPS is light-sensitive, store covered at 4ºC.

12. CHAPS lysis buffer: 1% CHAPS, with 20 mM IAA and 0.2 mM phenyl-methylsulfonyl fluoride (PMSF) from a stock solution (100 mM in 100% EtOH). Lysis buffer is made fresh, and used chilled. Immediately before use, precipitating Ab is added to the lysis buffer at a concentration of approx 10 μg/mL.

13. CHAPS wash buffer: 20 mM IAA in 0.1% CHAPS, prepared from 1% CHAPS stock solution and TBS. Make fresh, cover in foil, and use chilled on ice.

14. Protein A-Sepharose beads (PAS) (Amersham Pharmacia Biotech, Piscataway, NJ): Prepare a PAS stock by suspending 1.5 g dry PAS in 50 mL 0.1% CHAPS in TBS, and divide into 25-mL aliquots in 50-mL centrifuge tubes. Incubate overnight at 4°C, and spin at 140g, 4°C, for 5 min. Carefully remove supernatant by suction, and bring each tube to a final volume of 50 mL with 0.1% CHAPS. Store at 4°C. Immediately before use, prepare a PAS pellet by spinning down 0.5 mL PAS stock per sample at 140g and remove supernatant. Resuspend the PAS in the original volume of CHAPS wash buffer, and aliquot 0.5 mL suspension into individual tubes. Centrifuge, remove supernatant, and chill on ice until needed.

15. Protein elution buffer: 125 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS) (w/v), 12% glycerol (v/v), and 2% (w/v) bromophenol blue. Store at room temperature.

16. The Ab used for immunoprecipitation of murine L^d is 64-3-7. Antibody 64-3-7 is an IgG2 Ab that recognizes the α1 domain of open, peptide-free L^d *(8–14)*. L^d forms with the 64-3-7 serologic epitope do not have the epitopes surrounding the L^d peptide-binding cleft that are recognized by several conformation-sensitive monoclonal antibodies (e.g., 30-5-7), which suggests that the L^d 64-3-7$^+$ form has an open/unfolded groove *(12)*. Addition of peptide to 64-3-7$^+$ L^d causes fold-

ing, as demonstrated by titration of radioiodinated peptide ligand into ^{35}S-methionine-labeled cell lysates and immunoprecipitation of the L^d molecules. With addition of peptide ligand, the amount of 64-3-7$^+$ L^d decreased, and the amount of 30-5-7+ L^d increased *(11)*. Furthermore, the iodinated peptide was co-precipitated in a dose-dependent fashion with the folded (30-5-7$^+$) L^d form, and could be visualized, along with L^d, on the autoradiograph *(11)*. In vivo, 64-3-7$^+$ and folded, peptide-occupied L^d have a precursor–product relationship, as exhibited by pulse-chase analysis. As chase time increases, 30-5-7$^+$ L^d levels rise and 64-3-7$^+$ L^d levels drop *(11)*. Similarly, the antibody used for human class I HC immunoprecipitation is HC10. HC10 is an IgG2a that reacts preferentially with human leukocyte antigen (HLA)-B and -C HCs *(13)*. A reciprocal relationship exists between HC10$^+$ HLA forms and folded HLA forms (forms recognized by the W6/32 Ab). For example, L-B27/hβ_2m cells cultured with peptides have a decreased population of cell-surface, HC10-reactive molecules, and increased numbers of W6/32$^+$ molecules *(14)*. Furthermore, when exogenous peptide ligands are provided, the half-life of W6/32$^+$ molecules on L-B27/hβ_2*m* is lengthened by about 3 h, and the half-life of HC10$^+$ molecules is reduced by the same amount of time *(14)*. These antibodies are all used at 5 µL ascites/immunoprecipitation (10 µg/mL).

2.2. Electrophoresis and Autoradiography

1. SDS-polyacrylamide gel electrophoresis (PAGE) running buffer: 3.5 m*M* SDS, 25 m*M* Tris-base, 192 m*M* glycine, pH 8.4. A 5X stock solution is made by dissolving 5.0 g SDS, 15 g Tris-base, and 72 g glycine in deionized water. The stock is diluted to 1X as needed.
2. 8% and 4–20% Tris-glycine precast gels (Novex, San Diego, CA).
3. XCell II mini-cell electrophoresis unit (Novex).
4. MultiMark Multi-Colored Standard (Novex).
5. Gel fixative: 10% acetic acid (v/v), 30% methanol (v/v) in deionized (DI) water.
6. Methanol (Fisher, Pittsburgh, PA).
7. Amplify fluorographic reagent (Amersham Pharmacia Biotech).
8. Glycerol.
9. 3MM Chromatography paper (Whatman, Fisher Scientific, Pittsburgh, PA).
10. Kodax Biomax MR film.

2.3. Western Blotting

1. Half strength Towbin's transfer buffer: 12 m*M* Tris base, 96 m*M* glycine, pH 8.1–8.5. If the pH is not in this range, remake the buffer: Do not adjust. Store at 4ºC, and use chilled.
2. Xcell II Blot Module (Novex).
3. Blotting pads (Novex).
4. Transfer membrane: Immobilon-P polyvinylidene flouride (PVDF) (Millipore).
5. Membrane-blocking buffer: 10% (w/v) dry milk dissolved in 0.05% Tween 20 in PBS.
6. 0.05 and 0.3% Tween 20 (v/v) in PBS.
7. Primary Abs diluted in blocking buffer: 64-3-7 or HC10 at a dilution of 1:1000,

to detect class I HCs *(8–14)*, anti-TAP serum diluted to 1:500 (503, specific for mouse TAP *(4)*, or 1478, specific for human TAP *(5)* anticalreticulin serum (Stressgen, Victoria, BC, Canada) at a dilution of 1:750, antihuman tapasin serum at a dilution of 1:1000 *(3)* antimouse tapasin serum, diluted to 1:150 *(6)* and rabbit anticalnexin serum at a dilution of 1:500 *(14)*.

8. Secondary Abs: Goat antimouse IgG (H + L) biotin-conjugated (Caltag, Burlingham, CA) and goat antirabbit IgG (H + L) biotin-conjugated (Caltag). Both goat antimouse and antirabbit are used at a dilution of 1:2000 in 0.05% Tween-20.
9. Horseradish peroxidase(HRP)-conjugated streptavidin (Zymed, San Francisco, CA). HRP-conjugated streptavidin is used diluted to 1:20000 in 0.05% Tween-20.
10. Enhanced chemoluminescence (ECL) Western blotting reagents (Amersham Pharmacia Biotech).
11. Kodak BioxMax MR or BioMax Light Film.

3. Methods

3.1. Immunoprecipitation

1. Remove cells to be metabolically labeled from flasks with EDTA/PBS and wash $3 \times$ in 10 mL chilled PBS. Resuspend washed cells in labeling media at 5×10^6 cells/mL and plate 2×10^7 cells in a 60-mm tissue culture dish. Preincubate cells for 30 min at 37°C, 5% CO_2, then add [^{35}S]-protein labeling mix to each plate at 100 µCi/mL. Label the cells for an incubation period of 30 min at 37°C, 5% CO_2 (*see* **notes 1** and **2**). Following incubation, remove the cells from each plate with a cell scraper, and wash $3 \times$ with 10 mL cell wash buffer. At this point, the cells can be frozen at –70°C, and the immunoprecipitation finished later.
2. Resuspend the cell pellets in 0.5 mL CHAPS lysis buffer with precipitating Ab, then transfer to 1.5-mL microcentrifuge tubes. Incubate for 1 h on ice, then centrifuge at 18,000g, 4°C, for 15 min, removing nuclei and cellular debris.
3. Transfer the cell lysate (supernatant) to PAS pellets, and incubate for 45 min on ice. Each sample should be gently bubbled every 10 min, using a Pasteur pipet and rubber bulb.
4. After the incubation, wash the PAS-bound Ab and antigen $4 \times$ with 5 mL CHAPS wash buffer. After addition of the wash buffer to the samples for the first and second wash, centrifuge immediately (140g /4°C/5 min). For the third and fourth wash, observe a wait period of 10 min before centrifugation.
5. Solubilize sample proteins by adding 50 µL protein elution buffer and boiling for a period of 5 min (*see* **Note 3**). Samples may be loaded for electrophoresis immediately, or frozen at –70°C.

3.2. Electrophoresis and Autoradiography

1. Each precast gel should be rinsed in DI water before use, and all frozen immunoprecipitation samples should be boiled for 2 min, then spun at 18,000g for 2 min before loading (*see* **Notes 4** and **5**). Assemble the gel apparatus, and fill the buffer

chambers with 1X SDS-PAGE running buffer. Any bubbles trapped along the foot of the gel can be removed by tipping the gel box and gently tapping it on the corner. Load 10 µL/well of each sample, or the mol wt marker, with a Hamilton glass syringe. Any empty wells should be filled with an equal amount (10 µL) of elution buffer, to prevent band spreading and uneven running of adjacent lanes. Separate proteins by electrophoresis at a constant 125 V.

2. For an autoradiograph of the immunoprecipitated protein, remove the gel from the cassette, and soak in gel fixative for 30 min. Next, treat the gel in Amplify with 2% (v/v) glycerol for 20 min then dry gel on a gel drier at 60°C for 1.5 h, sandwiched between a piece of 3MM paper and plastic wrap. To produce the autoradiographs, place the gel in a X-ray cassette, and expose it to Kodak BioMax film for a time period that gives the desired band intensity.

3.3. Western Blotting

1. For a Western blot of class I HC or associated proteins, the gel is not fixed, but instead equilibrated in transfer buffer for 15 min. One transfer membrane per gel should be prepared by immersion in 100% methanol for 30 s, DI water for 1.5 min, and, finally, transfer buffer for a minimum of 15 min. 3MM paper, two per gel, and blotting pads, four per gel, should be saturated in transfer buffer before use. Take care to press all bubbles from pads, because bubbles block protein transfer.

2. Create a sandwich with the above items, by first placing two sponges in a container filled with transfer buffer to a level slightly above the sponges. Next, place one sheet of chromatography paper on top of the transfer pads. Gently float the gel on the excess buffer, until it is properly aligned with the paper and sponges. Once the gel is in the correct orientation, lift the entire assembly out of the tray and place into the cathode core of the blot module. Place the membrane onto the gel, and any bubbles should be pressed out with a smooth object. To complete the sandwich, the membrane is covered with the second 3MM paper and the remaining two pads. The sandwich should fit securely between the cathode and anode, with the membrane on the anode side. Fill the blot module with enough half-strength Towbin's buffer to cover the top of the sandwich. Transference of the immunoprecipitated proteins to the membrane is carried out for 2 h at a constant 30 V (*see* **Note 6**).

3. Following the transfer, notch the top right corner of the protein side of the membrane, and incubate the membrane, protein-side-up, overnight, at 4°C, in blocking buffer.

4. Incubate the membrane in the primary Ab, diluted in the appropriate amount of blocking buffer for 2–4 h at RT on a platform rocker. Following the proper incubation period, rinse the membrane quickly 3 × in 0.05% Tween-20 in PBS, and then wash the membrane 3 × for periods of 10 min in fresh 0.05% Tween-20.

5. Apply the biotin-conjugated secondary Ab to the membrane at a dilution of 1:2000 in 0.05% Tween 20. Incubate for 1 h at RT, with rocking. Wash as in **step 4**.

6. Add HRP-strepavidin at a dilution of 1:20,000 in 0.05% Tween-20 and rock for 1 h. Wash as above, except increase the percentage of Tween-20 to 0.3%.

7. Visualize the protein by soaking the membrane in equal amounts of ECL reagents 1 and 2, mixed together immediately before use, for 1 min. Remove the membrane, and let the excess detection reagents drip off. Before the membrane dries completely, seal it between two layers of clear plastic wrap, and protect from light.

8. Expose the membrane to Kodak Biomax Light or MR film for various exposure times, ranging from a few seconds to hours, depending on the intensity of the signal (*see* **Note 7**).

4. Notes

1. It is important to note that ^{35}S-labeled amino acids, especially ^{35}S-methionine, release volatile radioactive decomposition material, both during the thawing process, and during incubation at 37°C *(16)*. Accordingly, several steps must be taken to limit personnel exposure and laboratory contamination. While washing cells to be metabolically labeled, vent [^{35}S]-protein labeling mix using a charcoal trap and needle. This releases any pressure, and removes any decomposition products that have built up during storage and thawing. Also, plates of cells being labeled should be placed in a larger plate containing activated charcoal, which is a very efficient absorber of volatile radioactive gases. A tray of charcoal, placed inside of the cell culture incubator used for labeling, is also a wise precaution to avoid incubator contamination. Additionally, the water inside the incubator should be changed often, since the volatile products are very water-soluble, and quickly contaminate the incubator water. Finally, radioactivity surveillance swipes should be conducted after each labeling, not only of the work area, but also of all equipment used, paying special attention to the rubber gaskets, another high-affinity area for the radioactive material released during labeling.

2. If autoradiographs are not required, the immunoprecipitations should be performed without first radiolabeling the cells. In this case, after harvesting, the cells are washed 3 × in cell wash buffer, and then lysed. As an alternative to an autoradiograph to examine levels of the immunoprecipitated protein, a Western blot can be conducted, using a primary Ab specific for the precipitated protein.

3. It is important at all times to keep the immunoprecipitations on ice, but especially after adding the elution buffer, because endogenous proteases are highly active in SDS–sample buffer. It is best to add the elution buffer to the samples and boil immediately.

4. When boiling frozen immunoprecipitation samples, do not boil the mol wt marker.

5. Novex offers their protein separation gels in several different buffer systems, each with its benefits and shortcomings. The Tris-glycine gel systems seem to be the most flexible, and yield the most consistent separation over a wide range of protein sizes; however, the shelf life is very short (1–2 mo). The Tris-acetate gels have proven to give the best protein transfer, and have a longer shelf life. They do, however, require a Tris-acetate running and transfer buffer, and have a limited selection of gel percentages to chose from. Finally, the Bis-Tris gels provide

good separation and resolution, especially of larger proteins, as well as a long shelf life, but, again, force a choice from a limited selection of gel percentages, and will not work with the standard protein elution buffer.

6. Half-strength Towbin's transfer buffer is used vs full-strength Towbin's because it grants enough ionic strength for proper transfer, but avoids generating excessive heat. Therefore, it is unnecessary to cool the blot apparatus with ice during transfer.

7. Between exposures to film, developed membranes should be stored sealed in plastic wrap at 4°C. This will prolong emission of light by ECL. Nonetheless, dry areas will occasionally develop while attempting to get later exposures. In this case, redevelop the blot by incubating the membrane in equal amounts of ECL reagents 1 and 2. It is possible to reprobe a membrane, so crucial membranes should be stored wet, and covered in plastic wrap at a temperature of 4°C after initial immunodetection.

Acknowledgments

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Methods of Purification of Heat Shock Protein-Peptide Complexes for Use as Vaccines Against Cancers and Infectious Diseases

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1. Introduction

Immunization of mice, with preparations of heat shock proteins (HSPs) isolated from tumors or virus-infected cells, has been shown to elicit specific protective immunity against the tumor or the virus-infected cells used as the source of the HSP. This phenomenon has been shown to be general, in that specific immunogenicity of tumor-derived HSP preparations has been demonstrated in the cases of hepatoma *(1)*, fibrosarcoma *(2–8)*, lung carcinoma *(9)*, prostate cancer *(10)*, spindle cell carcinoma *(9)*, colon carcinoma *(9)*, and melanoma *(9)*, in mice and rats of different haplotypes. These tumors include chemically induced *(1–8,10)*, UV-induced *(11)* and spontaneous tumors *(9)*, and efficacy has been demonstrated in prophylactic *(1–8,10,11)* as well as in therapeutic models *(9)*. In the case of viral models, HSP preparations from cognate cells have been shown to elicit a virus-specific cellular immune response against influenza virus *(12)*, SV40 *(13)*, vesicular stomatitis virus *(14)* and lymphocytic choriomeningitis virus *(15)*. HSP preparations from cells transfected with model antigens (Ags) such as ovalbumin and β-galactosidase have been shown to elicit Ag-specific cytotoxic T-lymphocytes (CTL)s against ovalbumin *(16)* or β-galactosidase, respectively *(17)*.

The immunogenicity of HSP-peptide complexes has obvious applications in vaccination against cancers and infectious diseases *(18)*. With respect to cancers, HSP-peptide complexes isolated from a patient's cancer can serve as customized, patient-specific therapeutic vaccines *(19)*. Despite the current enthusiasm for shared Ags of human tumors *(20, 21)*, the need for patient-

specific vaccines is highlighted by the classical observation that tumors of individual mice and rats are individually distinct with respect to their antigenicity (*see* **ref.** *22* for review and *see* **ref.** *23* for a fuller discussion of the merits of shared vs unique Ag vaccines of human cancers). With respect to infectious diseases, the immunogenicity of HSP-peptide vaccines provides an opportunity to complex known Ag peptides with HSPs and use such complexes to elicit peptide-specific CTL response, despite their exogenous presentation *(16,24)*.

This chapter describes the methods used in purification of a number of HSPs *(2,25–27)*, along with their peptide cargo, and also the use of such HSP-peptide complexes in prophylactic and therapeutic vaccination against cancer and infectious diseases.

2. Materials

2.1 Purification of HSPs

Chromatographic separations can be carried out manually or through automated equipment, such as the fast protein liquid chromatography (FPLC) system (Pharmacia, Uppsala, Sweden) or the Biocad system (Perkin Elmer), at 4°C. The systems consist of two reciprocating pumps, a solvent controller to program the gradients, and an injection valve. Fractions are collected in a Frac-100 or other fraction collector. The Mono Q HR 5/5 column can be purchased from Pharmacia. Several equivalent alternatives exist.

2.1.1. Purification of gp96

2.1.1.1. BUFFERS (*SEE* **NOTE 1**)

1. Phosphate-buffered saline (PBS): 10 m*M* phosphate buffer, pH 7.4, 2.7 m*M* KCl, 150 m*M* NaCl.
2. Concanavalin A (ConA) PBS (use fresh): 5 m*M* phosphate buffer, pH 7.4, 180 m*M* NaCl, 2 m*M* CaCl$_2$ (add dropwise, with stirring, from 1 *M* stock in water), 2 m*M* MgCl$_2$ (add dropwise, with stirring, from 1 *M* stock in water), 1 m*M* phenyl-methylsulfonylfluoride (PMSF), from 100X stock in EtOH, (add just before use, and extremely slowly, dropwise).
3. 10% Methyl-∝-D-Mannose Pyranoside: 45 mL ConA PBS, 5g Methyl-∝-D-Mannose Pyranoside.
4. FPLC buffer: 5 m*M* phosphate buffer, pH 7.0.
5. FPLC buffer A: FPLC buffer with 0.3 *M* NaCl.
6. FPLC buffer B: FPLC buffer with 0.7 *M* NaCl.
7. Lysis buffer (hypotonic buffer): Make fresh buffer and use 4 × the volume (in mL) the size of the tissue (in g), 30 m*M* NaHCO$_3$ pH 7.0 containing 1 m*M* PMSF.
8. 0.2 *M* PMSF: Add 1.74g PMSF to 30 mL ethanol, make up to 50 mL with EtOH (*see* **Note 2**).

1. Primary: Grp94 Ab-1 (Neomarkers; anti-gp96).
2. Secondary: Rabbit antirat immunoglobulin G-horseradish peroxidase (HRP) (Sigma).

2.1.2. Purification of HSP70

2.1.2.1. BUFFERS

1. Lysis buffer: as in purification of gp96.
2. Buffer D: 20 mM Trizma base, 20 mM NaCl, 15 mM β-mercaptoethanol (*see* **Note 3**), 3 mM MgCl$_2$, 0.5 mM PMSF.
3. Stir, and adjust pH to 7.5 with glacial acetic acid.
4. Variations of buffer D: buffer D + 0.5 M NaCl, buffer D + 3 mM adenosine triphosphate (ATP) (Mg salt), freshly made, buffer D + 3 mM adenosine diphosphate (ADP) (Na salt), freshly made.
5. FPLC buffer A: 20 mM sodium phosphate (NaH$_2$PO$_4$), 20 mM NaCl, pH 7.0.
6. FPLC buffer B: FPLC buffer A + 1 M NaCl.

2.1.2.2. ANTIBODIES FOR IMMUNOBLOTTING

1. Primary: Anti-72/73 kDa HSP monoclonal antibody (Ab) (Stressgen).
2. Secondary: Goat antimouse–HRP (Sigma).

2.1.3. Purification of HSP90

2.1.3.1. BUFFERS

1. EP$_{200}$ buffer: 20 mM NaH$_2$PO$_4$, l mM EDTA, 200 mM NaCl, pH 7.4.
2. EP$_{600}$ buffer: 20 mM NaH$_2$PO$_4$, l mM EDTA, 600 mM NaCl, pH 7.4.

2.1.3.2. ABS FOR IMMUNOBLOTTING

1. Primary: 1:1 mixture of HSP84 Ab1 and HSP86 Ab1 (Neomarkers).
2. Secondary – Goat antirabbit – HRP (Sigma).

2.1.4. Purification of Calreticulin

2.1.4.1. BUFFERS

1. Carboxymethyl (CM)-Sephadex cation exchange chromatography buffer: 25 mM Na-citrate, pH 5.1.
2. diethylaminoethyl (DEAE) anion exchange chromatography buffer: 20 mM NaH$_2$PO$_4$, pH 6.0 (Buffer A) and Buffer A with 1 M NaCl (buffer B).

2.1.4.2. ABS FOR IMMUNOBLOTTING

1. Primary: Anticalreticulin (CRT) polyclonal antibody (rabbit antirecombinant human CRT), recognizes rabbit, rat, *Xenopus*, and mouse CRT. (Affinity Bioreagents).
2. Secondary: Goat antirabbit–HRP (Sigma).

3. Methods

3.1. Purification of HSPs

3.1.1. Purification of gp96

3.1.1.1. Column Preparations

1. Con A-Sepharose (Pharmacia) column: column volume should be approx one half the volume of starting tissue. Wash column with 10× column volume of Con A PBS.
2. PD-10 column (Pharmacia): Wash 3× with appropriate buffer immediately before use. Follow the manufacturer's instructions for exchange of buffer. After use, wash column twice with 0.1% Na azide, cap, and store.
3. DEAE-Sepharose column: column volume should be equal to that of the Con A-Sepharose column. Wash repeatedly with 0.3 M FPLC A, until the pH of buffer eluting from the column is the same as the buffer applied to the column.

3.1.1.2. Purification

1. Cell lines: harvest cells. Wash cell pellet 3× with PBS. Resuspend cell pellet in lysis buffer. Place cells on ice (4°C) for 30 min. Lyse cells in a dounce homogenizer until approx >80% of cells are lysed, as determined microscopically. Go to purification procedure.
2. Tissue: remove organ, put in PBS on ice. Add PMSF to 2 mM final concentration. Homogenize with blender.
3. Spin cell or tissue lysate for 90 min at 100,000g (27,000 rpm in a Beckman SW28 rotor).
4. Add ammonium sulfate ([NH$_4$]$_2$SO$_4$ to the supernatant to 50% saturation (314 g/L × Volume of supernatant (L) = Amount added) (*see* **Note 4**).
5. Stir slowly at 4°C for 1 h.
6. Spin for 30 min (4°C) at 40,500g.
7. Save supernatant; measure volume.
8. Add (NH$_4$)$_2$SO$_4$ to 80% saturation (210g/L × Vol supernatant [L] = Amount added) (*see* **Note 4**).
9. Stir at 4°C for 1 h.
10. Spin for 30 min (4°C) at 40,500g.
11. Save, and solubilize pellet in Con A PBS (10 mL/mL pellet).
12. Apply to prewashed Con A-Sepharose column, collect unbound material, and reapply. Wash column with Con A PBS until OD$_{280}$ drops to baseline.
13. Apply one-third × column vol of 10% methyl-∝-D-mannose pyranoside in Con A PBS.
14. Incubate the column for 10 min at 37°C.
15. Elute column with 2 column vol 10% methyl-∝-D-mannose pyranoside in Con A PBS. Collect fractions of one-third column vol each, and check OD$_{280}$. Pool all protein-containing fractions.
16. Apply pooled material onto pre-equilibrated PD-10 columns according to the manufacturer's instructions, and change buffer to FPLC buffer A, according to the manufacturer's instructions.

17. Apply protein-containing sample on pre-equilibrated DEAE-Sepharose or other equivalent ion-exchange column.
18. Wash column with 10 bed vol of FPLC buffer A, or more, until OD_{280} drops to baseline.
19. Elute column with 2 column vol of FPLC buffer B. Collect fractions of one-third column vol each, on ice.
20. Analyze fractions by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by silver-staining and immunoblotting with anti-gp96 Ab.
21. Pool, and store gp96-containing fractions at 4°C for immediate use, or at –20°C for long-term storage (*see* **Notes 5** and **6**).

3.1.2. Purification of HSP70

3.1.2.1. Column Preparations

1. ADP/ATP agarose (Sigma): Swell column according to manufacturer's instructions, and equilibrate with buffer D ($10 \times$ column vol). For column preparation, use 1 mL swollen ADP/ATP agarose gel for every g/mL starting tissue. ADP-agarose is to be used if immunogenic HSP70-peptide complexes are required. If HSP70 preparations that are free of peptides are needed, ATP-agarose is to be used.
2. Blue Sepharose (Pharmacia): Swell Blue Sepharose beads per the manufacturer's instructions, and equilibrate with buffer D. For column preparation, use 1 mL of swollen Blue Sepharose gel for every g/mL of starting tissue.

3.1.2.2. Purification

1. Prepare cell/tissue supernatant as previously described for gp96 (*see* **Subheading 3.1.1.2, steps 1–3**) by spinning at 100,000*g*.
2. Keep supernatant; measure volume.
3. Change buffer, using PD10 or other gel filtration column to buffer D (*see* **Note 7**).
4. Apply samples to ADP/ATP-agarose columns pre-equilibrated with buffer D.
5. Wash column with buffer D + 0.5 *M* NaCl (~15X column volume).
6. Wash with buffer D alone, until the eluate is free of protein by Bradford assay (~5–10 $\times$ column vol).
7. Add one-third column vol of 3 m*M* ADP (or ATP) in buffer D. Add a few drops more of buffer on top of column to keep it moist. Cap column, and incubate 30 min at room temperature.
8. Elute with 5 column vol of buffer D containing 3 m*M* ADP/ATP at the end of the 30 min incubation.
9. Change buffer using PD10 or equivalent columns to FPLC buffer A.
10. Filter samples with 0.45-μm filters. Apply sample to FPLC system (Mono Q, Pharmacia) pre-equilibrated with FPLC buffer A.
11. Wash with FPLC buffer A, and elute using a 20–600 m*M* NaCl gradient.
12. Collect 0.25-mL fractions and run on a 10% SDS-PAGE. Verify presence and homogeneity of the HSP70-containing fractions by silver-staining and immunoblotting.

13. Pool HSP70-containing, fractions and store at –20°C or 4°C, depending on the planned time of usage (*see* **Note 8**).

3.1.3. Purification of HSP90

3.1.3.1. COLUMN PREPARATIONS

Mono Q HR 5/5 and Superose 12HR 10/30 columns were obtained from Pharmacia Biotech and used as per the per manufacturer's instructions.

3.1.3.2. PURIFICATION (MODIFIED FROM REF. *26*)

1. Prepare cell/tissue supernatant as previously described for gp96 (**Subheading 3.1.1.2., steps 1–3**) by centrifuging at 100,000*g* for 90 min.
2. Change buffer of supernatant to EP_{200} buffer, by gel filtration through PD10 or equivalent columns.
3. Inject samples (8 mL) via a 50-mL Superloop (335×30 mm) (Pharmacia) onto a Mono Q HR 5/5 column (50×5 mm) equilibrated with EP_{200} buffer.
4. Wash the column for 10 min with EP_{200} buffer at a flow rate of 1 mL/min.
5. Carry out elution with a linear gradient from EP_{200} buffer to EP_{600} buffer, in 30 min.
6. Collect fractions (1 mL), and assay for protein content.
7. Inject samples (500 μL) eluted from the Mono Q column onto a Superose 12 HR 10/30 column (300×10 mm) equilibrated with EP_{200} buffer.
8. Perform chromatography at a flow rate of 0.5 mL/min (operating pressure 2 Mpa).
9. Collect 1-min fractions, assay for protein content, and analyze by SDS-PAGE and immunoblotting.
10. Pool HSP90-containing fractions, and store at –20°C until further use (*see* **Note 9**).

3.1.4. Purification of calreticulin

3.1.4.1. COLUMN PREPARATION

1. CM-Sephadex cation-exchange column: Equilibrate column with the cation-exchange buffer (2 mL bed vol is recommended for use for each g/mL of starting cells or tissue).
2. DEAE anion exchange column: Equilibrate column with the DEAE anion exchange buffer A (0.5 mL bed vol for each mL of starting cells or tissue).

3.1.4.2. PURIFICATION

1. The initial steps of purification of calreticulin are the same as for gp96.
2. Exchange the buffer of the unbound material from Con A column to 25 m*M* Na-citrate buffer, pH 5.1, using a PD10 or equivalent column.
3. Apply the solution onto a CM-Sephadex column pre-equilibrated with 25 m*M* Na-citrate buffer, pH 5.1. Collect unbound material.
4. Exchange the buffer of the unbound material with DEAE buffer A, using PD10 or other columns as before (**step 2**).

5. Apply the solution to DEAE-Sephacel column pre-equilibrated with DEAE buffer A.
6. Wash column using DEAE buffer A with 0.1 M NaCl.
7. Elute column with a linear gradient of 0.15–1 M NaCl in the NaH_2PO_4 buffer.
8. Collect fractions (one-third × bed vol each), and analyze by SDS-PAGE, silver staining, and immunoblotting, using the polyclonal Ab to CRT.
9. Pool CRT-containing fractions and store at –20°C or 4°C, depending on the planned time of usage (*see* **Note 10**).

3.2. Detection of Immunogenicity of HSP Preparations

3.2.1. Tumor Rejection

The gp96, HSP90, HSP70, and CRT preparations obtained from tumor cells or tissues can be used to elicit tumor immunity *(1–5,7,27)*. Typically, HSPs are injected subcutaneously under the nape of the neck, in 100–500 µL vol PBS, twice at weekly intervals(*see* **ref. 28** for a detailed analysis of effects of other routes of immunization and doses upon HSP immunogenicity). The animals are challenged with live cancer cells injected intradermally, 1 wk after the second immunization, and the kinetics of tumor growth is monitored. Although the preceding account seems relatively straightforward, a number of parameters should be observed carefully, in order to do a satisfactory tumor rejection assay. The following precautions are recommended.

1. Mice or rats should be immunized with different doses of HSPs. As an example, groups of five mice each should be immunized with 1, 5, 10, or 20 µg gp96/mouse/injection. The immunogenicity of HSPs can be significantly dose-dependent, and inclusion of a number of doses assures that one is operating within a suitably broad range *(2,4)*.
2. The number of live cancer cells used for challenge should be determined carefully, by prior titration of different doses of cancer cells. Unless one is very comfortable with a given challenge dose, animals vaccinated with HSPs should be challenged with three different doses bracketing the estimated appropriate dose. Thus, for each of the four doses of HSPs described earlier, one may consider three groups of three different challenges *(4)*.
3. A control, unimmunized group is included. However, in order to establish a higher degree of confidence in the control group, two groups of control animals may be used, one group to be challenged before the immunized groups have been challenged, and the other, after. This is recommended, because occasionally challenging a large number of mice may take up to 2 h, during which period the cells used for challenge may lose viability. In this case, mice immunized later will show an artificially high tumor rejection response.
4. In the same context, the viability of cells used for challenge should be between 98 and 100%. The nonviable cells serve as effective vaccines, and, thus, a given number of viable cells will be more tumorogenic by itself than if mixed with nonviable cells. Cells should be placed at 4°C in an appropriate medium, without calf serum, and not in PBS while waiting to be used in challenge.

5. The tumor challenges should be given intradermally, rather than subcutaneously, except if specifically intended to be subcutaneous. Monitoring the kinetics of tumor growth is done with greater accuracy with intradermal tumors.

6. The biochemical integrity of the HSP preparation must be determined by SDS-PAGE on the day it is used for immunization or challenge. This is significant, because some HSPs are prone to autodegradation *(2,29–30)* and others to loss caused by aggregation.

7. Animals should also be immunized with HSPs purified from normal tissues and/or other tumors as negative controls. Similarly, animals should be immunized with the intact irradiated cancer cells from which the HSPs were derived, and with other cancer cells.

8. The tumors should be measured every other day, along two perpendicular axes. If feasible, tumor thickness should also be measured. The data should be plotted in terms of average tumor diameter and also tumor volume, in order to get a complete picture of tumor growth. Tumors should also be examined attentively for texture, signs of inflammation, and necrosis.

9. It may be obvious that the health of animals used in vaccination is of seminal significance, but it is worthwhile to state this explicitly. Animals carrying bacterial or viral infections can be artificially permissive or resistant to tumor growth, and may easily mask a vaccination effect. Similarly, the genotype of animals used for vaccination must be identical (and not merely close) to that of the tumors used in the study. Observation of this sacred dictum may sometimes require an extensive survey of previous literature and several unsatisfactory telephone calls, in order to determine the source of the animals that were used for induction of tumors. However, attention to this detail during planning of the experiments will vaccinate the investigator against future shock (*see* **ref. *31***).

10. The observances recommended in **Subheading 3.2.1.** lead to large and perhaps cumbersome tumor rejection assays, easily involving over 100 mice in several groups. However, precisely because these assays are so expensive and time-consuming, it is essential that they be done well, if they are to be done at all. Although only prophylactic vaccination in tumor systems has been discussed above, prophylactic vaccination in viral models *(16)* and therapeutic administration of HSP preparations, requires similar precautions, and has also resulted in significant life prolongation, tumor remissions, and complete regressions in tumor-bearing mice *(9)*.

3.2.2. Generation of Ag-Specific, MHC-I-Restricted CTLs

The gp96, HSP70, and CRT preparations obtained from tumor cells or virus-infected or transformed cells can be used to elicit cognate Ag-specific, MHC-I-restricted, CTLs *(11,13,14,16,27,32)*. Such experiments have not been attempted so far with HSP90 preparations. Typically, mice are immunized twice by weekly subcutaneous injection of HSP preparations derived from cognate cells. The HSP preparation is delivered in PBS, without any adjuvants. Indeed, the use of a number of adjuvants, such as Freund's complete and incom-

plete adjuvants, the Gerbu adjuvant, and colchicine, has led not only to lack of an immunoenhancing effect, but to an abrogation of the response observed with HSP in PBS alone (S. Janetzki, M. Daou and P. K. Srivastava, unpublished observations). Typically, HSP preparations between 5 and 20 µg are used per injection and the precise optimal quantity varies somewhat between cell lines. Spleens are harvested between 4–7 d after the final immunization, and splenocytes are co-cultured with the cognate stimulator cells for 5–7 d, as appropriate. At the end of this period, the T-cells are tested for cytotoxicity, against a panel of target cells, by a chromium[51] release assay. The CTL activity may also be tested by cytokine release by activated T-cells. One may observe better detection of CTL response by one assay than another, depending upon the particular system used. The CTL responses generated in this manner are Ag-specific and MHC-I- and CD8-restricted. Our results also suggest that the CTLs generated by vaccination with HSP–peptide complexes are qualitatively different than the CTLs elicited by vaccination with intact cells bearing the appropriate Ag, which would indicate a hitherto unsuspected diversity among CTLs.

An interesting aspect of vaccination with HSP–peptide complexes is their ability to cross-prime, i.e., to elicit an MHC-restricted, Ag-specific CTL response in mice of any haplotype, as long as the HSPs are derived from cells that express the cognate Ag *(14,32)*. This is possible because the association of peptides with HSPs occurs before their association with the MHC molecules, and, thus, in contrast to the MHC-associated peptides, the HSP-associated peptides are not preselected with respect to any particular haplotype *(32)*. This phenomenon has obvious applications in generation of vaccines against diseases in which the protective Ags have been identified.

4. Notes

1. Buffers and samples applied to FPLC columns are filtered through a 0.45-µm sterile filter (Nalgene, Rochester, NY). All chemicals are analytical grade products from Sigma, unless otherwise stated.
2. This stock solution is stable for 1 mo.
3. The molarity of undiluted β-mercaptoethanol solution is 14.3 *M*.
4. Add slowly, while stirring at 4°C, in order to avoid high local concentrations of $(NH_4)_2SO_4$.
5. Approximately 30–100 µg of apparently homogenous preparations of gp96 are obtained from each g/mL starting cells or tissue. The variability results mostly from the choice of cells or tissues, and from the degree of homogenization.
6. One may encounter the problem of instability in purified gp96 preparations. A preparation that appears homogeneous immediately after purification may develop a number of lower, and, occasionally, even higher mol wt bands after a week of storage. The higher-mol wt bands appear to arise from an SDS-resistant

association of the intact gp96 with some of the degradation products. All these bands will still be detected by an Ab to gp96. The degradation is seen more reproducibly in more concentrated preparations, and therefore we do not recommend storing gp96 at concentrations higher than 0.1 mg/mL. The basis of this instability is presently unclear. Recent evidence suggests that gp96 molecules contain protease activity *(29–30)*. Further, the purified preparation may occasionally appear to be a set of 2–5 closely spaced bands, between 94 and 110 kDa in size. This heterogeneity, whose structural basis is presently unclear, is not the result of differences in glycosylation and does not appear to be a consequence of degradation *(6)*.

7. For purification of HSP70 from liver, apply the 100,000*g* supernatant first to a Blue Sepharose column (Pharmacia), to remove albumin. Take flowthrough, and proceed.

8. Approximately 50–200 µg of apparently homogenous preparations of HSP70 is obtained from each g/mL starting cells or tissue. The yield is dependent on the size of the cytosolic compartment in the starting tissue.

9. Approximately 200–400 µg apparently homogenous preparations of HSP90 is obtained from each g/mL of starting cells or tissue. The yield is dependent on the size of the cystolic compartment in the starting tissue.

10. Approximately 10–20 µg of apparently homogenous preparations of CRT is obtained from each g/mL starting cells or tissue. It is our estimate that this represents a poor recovery. More efficient methods of purification are under development.

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15

Random Peptide Libraries

A Tool for Analyzing Peptide Specificity
of Major Histocompatibility Complex Class I Molecules

James Stevens and Geoffrey W. Butcher

1. Introduction

Major histocompatibility complex I (MHC-I) molecules bind short peptides, usually 8–11 amino acids in length, for presentation to CD8+ T-cells. Polar residues at either end of the peptide binding groove act to lock the peptide in, via its amino and carboxyl termini. Additional stability is provided along the groove by restrictive pockets, which can accommodate only certain amino acid side chains. Consequently, each type of MHC-I molecule can only bind peptides with particular residues at certain positions, and these have been termed anchor residues.

One result of the recognition of the peptide N-terminus is that all peptides are bound at the same point, although this is not the case for MHC-II molecules, which permit the peptide to overhang the ends of the peptide binding groove. As a consequence, the most common method used to determine the nature of peptides binding to any MHC-I molecule is the pool-sequencing method of Falk et al. *(1)*, which usually consists of an initial immunoprecipitation step using a specific antibody (Ab) coupled to a solid support. The resulting MHC-Ab complex is then acidified, thus releasing the bound peptides, which can then be separated and purified from the heavy and light chains (HC and LC) by reverse-phase high-performance liquid chromatography. Pooling the predetermined peptide-containing fractions, and subjecting the pool to automated sequence analysis, enables the yield and relative importance of each amino acid at each sequencing cycle to be determined and a binding motif to be produced. This method has proven useful for obtaining peptide-binding data for MHC-I alleles from different species, and less so for MHC-II molecules *(2)*.
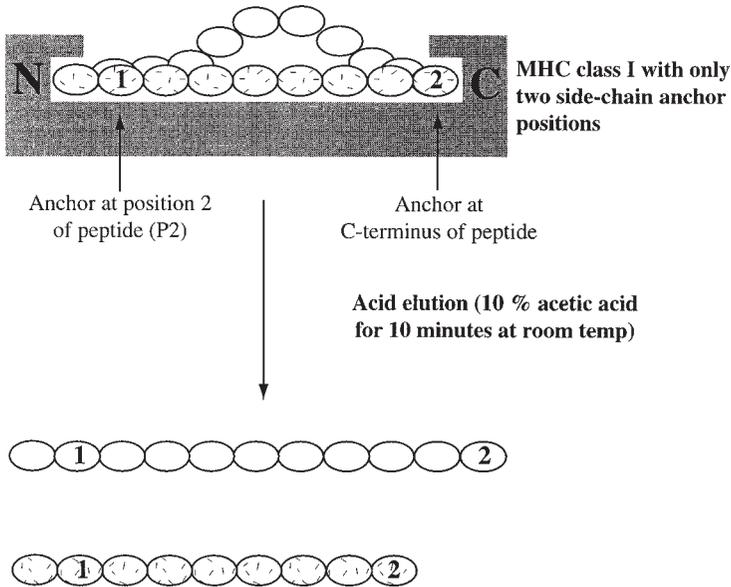
Fig. 1. Peptides of different lengths can bind to the same class I molecule. The two peptides illustrated both satisfy the anchor requirements (at P2 and C-terminus) for this molecule. However, when eluted, pool sequencing from the N-terminus will easily distinguish the first anchor (labeled 1), but the second anchor (labeled 2) will be diluted by the signals from longer peptides. Use of fixed-length random peptide libraries circumvents this problem.

However, there are a number of drawbacks that limit the universal applicability of this method. For its success, the method relies on a specific Ab for the purification of the MHC-I under study, but this is not always available. Abs can often have crossreactivities with other known or unknown class Ia or class Ib molecules *(3)*, and these molecules might be co-expressed with the molecule under study, thus, a binding motif may not be unique to a single class I molecule. If expression of the molecule under study is low, or if it has a restricted tissue distribution, such as the nonclassical MHC-I molecules *(4,5)*, then cloning and expression in a suitable cell line may be required, but this is time-consuming, can be expensive for maintenance of the derived cell lines, and good expression is not guaranteed. When eluting natural peptides, the C-terminal anchor residue is not always clearly defined by the pool-sequencing method, because class I alleles can bind peptides of varying lengths *(6–8)*, thus spreading the C-terminal signal over several sequencing cycles (*see* **Fig. 1**).

A recently described alternative strategy circumvents all the above potential problems *(9,10)*, thus enabling the study of MHC-I molecules for

which expression is low. Described here is an in vitro protocol to determine the peptide-binding motif of any MHC-I molecule. A bacterial expression system is used to produce recombinant protein of truncated HCs of MHC-I molecules and the LC, β_2-microglobulin (β_2m). This obviates the need for a specific Ab or a rich cellular source of class I molecules. The cloned HC is truncated just after the $\alpha 3$ domain, but before the transmembrane region (usually at residue 276). A histidine (His) tag is also cloned at the C-terminus of the HC, thus providing a simple method for rapid purification, based on the selective affinity of proteins with polyhistidines for a nickel-metal chelate adsorbent. Ni-charged nitrilotriacetic acid agarose (Ni-NTA) is used as the chelating adsorbent, because it binds metal ions more stably than other available resins, and can therefore retain ions under a wide variety of stringent wash conditions.

Truncated HCs are expressed as inclusion bodies, solubilized using 8 *M* urea, and purified as enriched monomers on the Ni-NTA adsorbent. The LC, β_2m, is expressed and solubilized as inclusion bodies in a way similar to the HC. Purification is achieved by using dialysis to refold the protein, then subjecting the dialyzed solution to ion exchange chromatography. Subsequent assembly of the denatured HC and refolded LC molecules is then performed in the presence of a fixed-length random peptide library. After purification by gel filtration, the monomeric complexes are acidified to elute the bound peptides, which are pooled and sequenced to produce a binding motif for the molecule under study.

2. Materials

All equipment used, including pipets, glassware, and plasticware, should be clean and autoclaved. Stock solutions for all reagents are prepared exactly as described in **ref. *11***, unless otherwise indicated.

2.1. Cloning and Bacterial Transformation of MHC-I cDNAs

1. (LB)-Medium: Dissolve 10 g Bacto-Tryptone, 5 g Bacto-yeast extract, and 10 g NaCl per liter water. Adjust pH to 7.5 with NaOH, and autoclave to sterilize.
2. An appropriate protein expression vector (*see* **Note 1**).
3. A suitable antibiotic, depending on the selected expression vector.
4. Bacterial strains: Any bacterial strain that is capable of maintaining plasmids, and gives high transformation efficiencies and good plasmid yields, is suitable for cloning purposes. Suitable bacterial hosts include the *Escherichia coli* K12 strains JM109, DH5α, HB101, XL-1 Blue (Stratagene) or NovaBlue (Novagen). For protein expression, the general purpose expression host, BL21 (DE3), is used.

2.2. Expression and Purification of Insoluble MHC-I Molecules on Ni-NTA Agarose

1. Prepare a 0.5 *M* stock by dissolving 2.979 g of isopropyl-β-D-thiogalacto-pyranoside (IPTG) into 25 mL water and store aliquots at –20°C.

2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer: Prepare a 3X stock solution by dissolving 0.9 g SDS (6% w/v) and 0.9 mg bromophenol blue (0.006% w/v) in 4.5 mL glycerol (30% v/v), 2.813 mL 1.0 M Tris-HCl, pH 6.8 (0.1875 M v/v), 2.25 mL β-mercaptoethanol (15% v/v), and make up the solution to 15 mL with Milli-Q water. A 1X solution is made by the addition of an appropriate volume of water. Alternatively, a sample buffer of similar composition can be purchased from various commercial sources, such as Bio-Rad (which only requires the addition of β-mercaptoethanol).

3. Lysis buffer: 10 mM Tris-HCl, 1 mM EDTA, 100 μg/mL phenylmethylsulfonyl fluoride (PMSF), 0.1 % Triton X-100. Adjust pH to 7.5 with NaOH.

4. Denaturing buffer A: 8.0 M urea, 0.1 M NaH$_2$PO$_4$, 0.01 M Tris-HCl. Adjust pH to 8.0 with NaOH immediately prior to use.

5. Denaturing buffer B: 8.0 M urea, 0.1 M NaH$_2$PO$_4$, 0.01 M Tris-HCl. Adjust pH to 6.3 with hydrochloric acid (HCl) immediately prior to use.

6. Denaturing buffer C: 8.0 M urea, 0.1 M NaH$_2$PO$_4$, 0.01 M Tris-HCl. Adjust pH to 5.9 with hydrochloric acid, immediately prior to use.

7. Denaturing buffer D: 8.0 M urea, 0.1 M NaH$_2$PO$_4$, 0.01 M Tris-HCl. Adjust pH to 4.5 with hydrochloric acid, immediately prior to use.

8. Ni-NTA agarose.

2.3. Expression and Purification of Recombinant β$_2$-Microglobulin

1. Lysis buffer: 10 mM Tris-HCl, 1 mM EDTA, 100 μg/mL PMSF, 0.1 % Triton X-100. Adjust pH to 7.5 with NaOH.

2. Denaturing buffer: 10.0 mM Tris-HCl, 8.0 M urea. Adjust pH to 7.5 with NaOH immediately prior to use.

3. Dialysis tubing: It is essential to choose a membrane with a cutoff of 12 kDa or less, otherwise, unwanted loss of β$_2$m will occur during dialysis.

4. Dialysis buffer: 10 mM Tris-HCl. Adjust pH to 7.5 with NaOH.

5. Fast protein liquid chromatography (FPLC) buffer A: 10 mM Tris-HCl. Adjust pH to 7.5 with NaOH and filter through a 0.2 μm filtration device, prior to use.

6. FPLC buffer B: 10 mM Tris-HCl, 500 mM NaCl. Adjust pH to 7.5 with NaOH and filter through a 0.2 μm filtration device prior to use.

2.4. In Vitro Refolding of MHC-I Molecules Around a Random Peptide Library

1. A nonamer random peptide library is used (*see* **Note 2**). The peptide, usually supplied as a solid, is dissolved in dimethy sulfoxide at a concentration of 10 mg/mL. Concentration can be checked by protein determination, using a suitable kit, such as the Micro BCA assay from Pierce.

2. PMSF: A 200 mM (200X) stock solution is made by dissolving 100 mg in 2.87 mL anhydrous methanol or EtOH. The solution can be stored at 4°C for up to 9 mo.

3. Refold buffer: 50 mM Tris-HCl, 400 mM arginine, 0.1 mM EDTA, 0.1 mM PMSF. The pH of the solution is adjusted to pH 8.0 with hydrochloric acid, and the solu-

tion is filtered through a 0.2-μm filtration device, and stored at 4°C. PMSF is added to give 0.1 m*M* final concentration, once all other components have been added to the refold mixture, including the HC/LC and peptide (*see* **Note 3**).

4. A suitable device to allow concentration of the refold mixture, such as the range of centrifugal filtration devices offered by Amicon. Methods (*see* **Subheading 3.4.**) describes the use of Centricon-10 for small-scale studies and Centriprep-10 devices for the large-scale preparations. The mol wt cutoff of these devices (10 kDa) is sufficient to retain proteins greater than 12 kDa.

5. Gel filtration buffer: 10 m*M* Tris-HCl, 100 m*M* NaCl. Adjust the pH to 8.0 with NaOH, and filter through a 0.2-μm filtration device, prior to use.

6. Gel filtration column, such as a Superdex 75 column (Amersham Pharmacia Biotech) which allows the separation of refolded complexes (~40 kDa) from β_2m (~12 kDa) and misfolded high-mol-wt aggregates.

2.5. Elution and Purification of Bound Peptides

1. Acetic acid (AA): 100 and 10% stocks are required. A 10% stock solution is made by adding 1 mL glacial AA to 9 mL Milli-Q water. Use only AA with high purity, and store the solution at room temperature (RT).

2. A suitable device to allow the separation of peptides from the HC and LC components of the MHC-I complex, such as the Centricon-3 unit from Amicon, which has a mol wt cutoff of 3 kDa, and allows small peptides through, while holding up the larger components of the class I complex.

3. Reverse-phase column: Although any C_{18} column should be suitable, each column and system should be calibrated to determine the range of acetonitrile (CH_3CN) concentrations required for the elution of peptide pools. This laboratory uses an Applied Biosystems Aquapore (250×1 mm) Brownlee C_{18} column.

4. Reverse-phase buffer A. 0.025% trifluoroacetic acid (TFA) in Milli-Q water. The solution is filtered through a 0.2-μm filtration device, prior to use.

5. Reverse-phase buffer B. 0.025% TFA, 90% CH_3CN. The CH_3CN should be HPLC-grade, and the solution should be filtered through a filter suitable for organic solutions, such as the Millex-LCR units from Millipore.

2.6. Pooled Peptide Sequencing

1. Polybrene-coated glass-fiber filters, specific for protein sequencing. Other materials for solid-phase adsorption of the eluted and pooled nonamer peptides may be available. Advice should be sought from the sequencing facility to be used.

2. A suitable protein sequencer, or access to a protein sequencing facility.

3. Methods

3.1. Cloning and Bacterial Transformation of MHC-I cDNAs

The regions coding for the full-length β_2m, and for amino acids 1–276 of the HC of the class I molecule under study, are amplified by the polymerase chain reaction (PCR), using suitable oligonucleotide primers. The truncated sequence

is ligated into the desired plasmid (*see* **Note 1**) in the correct reading frame, using conventional cloning methods *(11)*. The ligation mix is transformed into a suitable host for construct amplification and DNA sequencing. Once the sequence for the construct has been confirmed, 1–20 ng generated plasmid is transformed into the bacterial strain, BL21(DE3). At the same time, plasmid without insert should be transformed into the bacteria, as a positive control for transformation and as a negative control for expression studies in the next subheading. Various amounts of the transformed bacteria are plated on LB agar plates containing a suitable antibiotic. The next day, single colonies are picked for further analysis.

3.2. Expression and Purification
of Insoluble MHC-I HC Molecules on Ni-NTA-Agarose

3.2.1. Initial Test for Expression

To test for expression, two discrete colonies from the plate of potentially correct clones and a colony from the plate with plasmid only (negative control), are picked and inoculated into 5 mL fresh LB + antibiotic. These are grown in a shaker incubator (200–300 rpm; 37°C) to stationary phase.

1. Next day, 1 mL of each culture is added to 9 mL fresh LB + antibiotic, prewarmed to 37°C. The fresh cultures are grown in a shaker (200–300 rpm at 37°C), until growth is mid-log phase (OD_{600} is 0.6).
2. Samples (1 mL) of the cultures are removed, and, in the remaining mixtures, protein expression is induced by the addition of IPTG, to a final concentration of 1 m*M*. The tubes are returned to the shaker for a further 3 h. After removal, the 1 mL samples are transferred to microcentrifuge tubes, and centrifuged (approx 3,000g) for 5 min. The pellets are each resuspended into 450 µL 1X SDS-PAGE sample buffer, placed in an 80°C heating block (or water bath) for 5 min, then frozen at –20°C, until further analysis can be performed (*see* **step 5**).
3. After 3 h induction with IPTG, 2× 1 mL samples are removed from each tube, and centrifuged as before. The pellets from one set of 3 h incubation samples are each resuspended into 450 µL 1X SDS-PAGE sample buffer, heated at 80°C for 5 min, then frozen at –20°C, until further analysis can be performed (*see* **step 5**). These will be compared to the uninduced samples for protein expression.
4. In order to determine if protein is expressed in a soluble form or as inclusion bodies, the pellets from the remaining set of the 3 h induction are resuspended in 300 µL PBS, and subjected to sonication to lyse the bacteria. The lysed suspensions are then centrifuged at maximum speed in a microcentrifuge, and the supernatants carefully removed to a fresh microcentrifuge tube containing 150 µL 3X SDS-PAGE sample buffer. These are also heated at 80°C for 5 min and then frozen at –20°C. The lysed pellets are subjected to two cycles of PBS washing and centrifugation, then resuspended in 450 µL 1X SDS-PAGE sample buffer, heated at 80°C for 5 min, and frozen, as before.

5. For analysis, total protein lysates from the uninduced samples (produced in **step 2**) and IPTG-induced samples (produced in **step 3**), as well as soluble and insoluble fractions (produced in **step 4**) are analyzed by SDS-PAGE. All samples should be reheated to 80°C for 5 min and centrifuged at maximum speed for 3 min in a microcentrifuge prior to loading the gel. HCs should run at 30–35 kDa.

Successful induction should produce truncated HCs as inclusion bodies. In the literature this is the case for all studies to date *(8–10,12)*.

3.2.2. Full Scale Preparation

The following protocol describes induction and purification of HC protein from inclusion bodies in a 1-L bacterial culture. An appropriate scale-up should be performed for larger preps.

1. A 20-mL bacterial culture is grown overnight in LB + antibiotic. The next day, this is used to inoculate two 1-L flasks, each containing 500 mL LB and the appropriate antibiotic. The inoculated flasks are placed in a shaker incubator (300 rpm at 37°C) and when the cultures reach mid-log phase ($OD_{600} = 0.6$), protein expression is induced for 3–4 h by the addition of IPTG, to a final concentration of 1 mM.

2. Bacteria are harvested by centrifugation at 4000g for 15 min. At this stage, the bacterial pellet can be stored at –70°C.

3. If frozen, the bacterial pellet is allowed to warm up to RT slowly, by placing the frozen tube in a beaker of cold tap water for 10–15 min. The pellet (approx 3 g wet wt) is resuspended in 20 mL lysis buffer (20% w/v), disrupted by sonication, and centrifuged (25,000g). The supernatant is removed, and the cell pellet is washed twice more with lysis buffer (20 mL each).

4. The washed pellet is solubilized by resuspension and mixing in 30 mL denaturing buffer A for 1 h at RT, after which the solution is centrifuged (25,000g), to remove any insoluble material.

5. Urea-solubilized protein is mixed with 4 mL Ni-charged Ni-NTA matrix for 1 h. The matrix is then loaded into a suitable column, and washed with 30 mL denaturing buffer A. The column is then washed with 15 mL denaturing buffer B, which washes out most of the *E. coli* proteins, and pure HC protein is eluted by washing with 15 mL denaturing buffer C and 15 mL denaturing buffer D. Fractions (1 mL) are collected for buffers B, C, and D elutions. Each of these buffer washes is sufficient so that the A_{280} of the eluant is zero before the next buffer wash is started. When scaling-up the protocol for a larger bacterial culture, the volumes of Ni-NTA matrix and buffer washes should be increased accordingly, to account for the higher yields.

6. Protein concentration for each fraction is determined by the bicinchoninic acid (BCA) assay (Pierce) or another suitable assay, and protein purity is assessed by SDS-PAGE, which in the presence and absence of a reducing agent should confirm that the mixture eluting in the pH 5.9 buffer wash is mostly composed of monomers, whilst protein eluting in the pH 4.5 buffer comprises large-mol-wt

multimers, resulting from crosslinking by disulfide bonds. Only the monomer-containing fractions are used for subsequent refolding work with random libraries (*see* **Note 4**). The fractions are pooled and frozen at –70°C.

7. A sample of the protein to be used should be submitted for protein sequencing, to confirm that the N-terminus is as expected.

3.3 Expression, Refolding, and Purification of Recombinant β_2m

The following protocol describes induction and purification of β_2m from inclusion bodies from a 1-L bacterial culture.

1. A 20-mL bacterial culture is grown overnight in LB + antibiotic. The next day, this is used to inoculate two 1-L flasks, each containing 500 mL LB and the appropriate antibiotic. The inoculated flasks are placed in a shaker incubator (300 rpm at 37°C), and, when the cultures reach mid-log phase ($OD_{600} = 0.6$), protein expression is induced for 3–4 h by the addition of IPTG to a final concentration of 1 m*M*.

2. Bacteria are harvested by centrifugation at 4000*g* for 15 min. At this stage, the bacterial pellet can be stored at –70°C.

3. If frozen, the bacterial pellet is allowed to slowly warm up to RT, by placing the frozen tube in a beaker of cold tap water for 10–15 min. The pellet (approx 3g wet wt) is resuspended in 20 mL lysis buffer (20% w/v), disrupted by sonication, and centrifuged (25,000*g*). The supernatant is removed, and the cell pellet is washed a further 2× with lysis buffer (20 mL each).

4. The washed pellet is solubilized by resuspension and mixing in 30 mL denaturing buffer for 1 h at RT, after which the solution is centrifuged (25,000*g*), to remove any insoluble material.

5. Urea-solubilized β_2m is next refolded by dialysis. The sample is placed in dialysis tubing that has a mol wt cutoff of <12 kDa, and is dialyzed against 4 L dialysis buffer at RT, with a minimum of three changes of fresh dialysis buffer. The dialyzed sample is then centrifuged to remove any precipitate and applied to a Q-Sepharose column (Amersham Pharmacia Biotech) pre-equilibrated with dialysis buffer. If the β_2m to be purified is from a species other than human, mouse, or rat, initial experiments should be performed with a salt gradient (0–500 m*M* NaCl), to determine the correct concentration to elute and separate monomeric β_2m from multimeric forms. If multiple species of β_2m are eluted at different concentrations, fractions should be subjected to SDS-PAGE, in the presence and absence of reducing agents, to assess the monomeric nature of the samples. If the preparation of solubilized inclusion bodies is of sufficient purity, a step elution can be performed (*see* **Note 5**).

3.4. In Vitro Refolding of MHC-I Molecules Around a Random Peptide Library

Refolding is performed by the dilution method *(12,13)*. Small-scale refolds are first performed to determine the amount of peptide required to achieve a
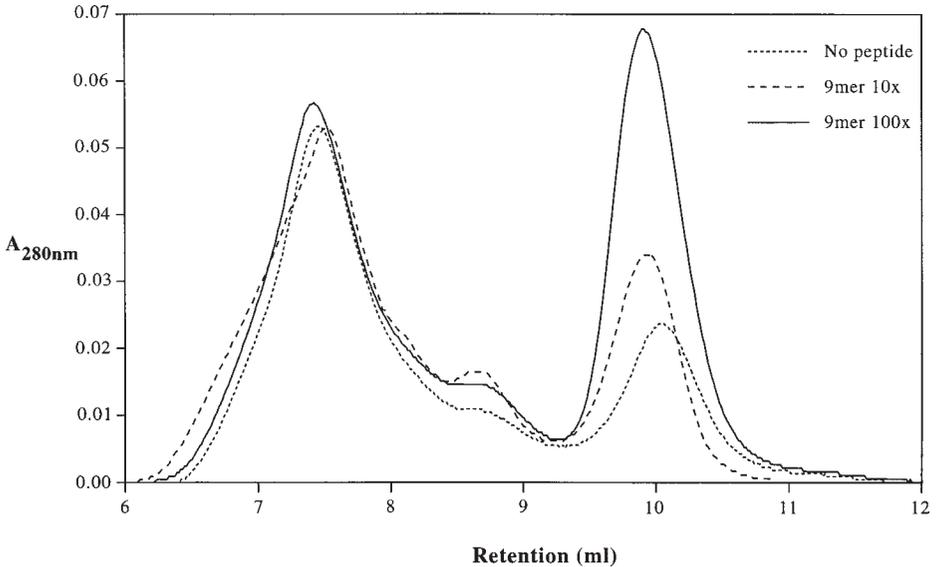
Fig. 2. Effect of peptide concentration on the successful refolding of the rat class I molecule, RT1-A^u. At least two concentrations should be tested in the initial refolding experiments, because different class I alleles have been shown to require different peptide concentrations for effective refolding *(9,13,17)*. Reproduced with permission from **Ref. *9***.

measurable peptide-specific increase in complex formation, which is performed to avoid wasting valuable peptide, and because quantitative differences in requirement for peptide were observed between different rat class I alleles *(9)*. Such refold experiments are performed in a total volume of 2 mL. β_2m (48 µg, 4 nmol) and peptide (20 µg, 20 nmol; or 200 µg, 200 nmol) is added to 1.5 mL refold buffer. As a control, a tube with no peptide is also prepared. Denatured HC (62 µg; 2 nmol) is added, and refold buffer added, to give a final volume of 2 mL in each tube, and final molar ratios of 1:2:0, 1:2:10, and 1:2:100 (HC: β_2m:peptide). The solutions are left for 24–48 h at RT, after which the refold mixtures are concentrated to 100 µL, using a Centricon-10 (Amicon). Refolded class I complex is purified by gel filtration on an FPLC Superdex 75 column (Amersham Pharmacia Biotech) pre-equilibrated in gel filtration buffer. A typical elution profile is shown in **Fig. 2**, and illustrates the effect of concentration of the nonamer random peptide library on the extent of refolding for the rat MHC-I molecule, RT1-A^u. The peak eluting at 7.5 mL corresponds to material of mol wt >100 kDa, and is composed of improperly refolded LC and HC, whereas the peak at 10 mL contains properly refolded soluble class I complexes of mol wt ~40 kDa. The LC (β_2m) is not shown in this figure, but it elutes with a retention of 15 mL.
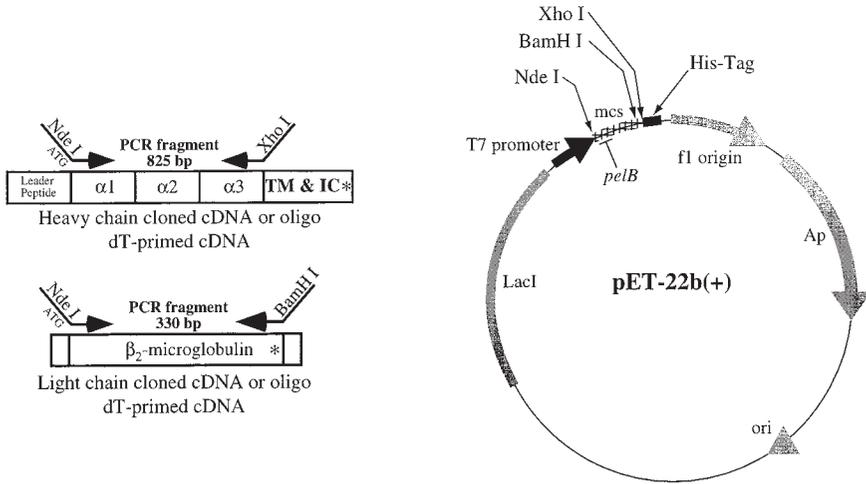
Fig.3. Cloning scheme used for the production of rat β_2-microglobulin and truncated HC (* represents the position of the natural stop codon), using the ampicillin-resistant pET22b(+) expression vector. The PCR primers are designed to introduce unique restriction sites into the PCR product. *Nde*I is introduced into both proteins at the upstream site. These are subsequently used to ligate the genes into the plasmid, in correct frame. Truncation of the HC to remove the transmembrane (TM) and intracellular domain (IC), and ligation with *Xho*I allows transcription to proceed through the Histag coding region, used for purification of the protein. β_2-microglobulin has its own stop codon, so it can be cloned downstream at any site within the multiple cloning site (mcs).

Once the required peptide concentration is determined, the refold conditions are scaled-up to 30–40 mL. After 24–48 h at 20ºC, the mixture is concentrated to 0.5–1 mL with a Centriprep-10 (Amicon), and this is also applied, in a single purification run, to a larger gel filtration column, or as multiple runs to the smaller one used for the test study. Fractions containing the refolded complex are pooled, and the peptides bound to the refolded complex are released and purified as described in the next subheading.

3.5. Elution and Purification of Bound Peptides

Peptides are eluted by first concentrating the purified complex to 1350 µL then acidifying the complex with 150 µL 100% AA for 5 min. This is then centrifuged through a Centricon-3 unit (Amicon). A further 500 µL 10% AA is added to the unit and this is also centrifuged. The released peptides in the flowthrough are then separated by reversed-phase chromatography, using an Applied Biosystems Aquapore (250 × 1 mm) Brownlee C_{18} column with an

CH$_3$CN gradient (0–90%) in 0.025% TFA. In this system, peptides elute between 6.75% and 40.5% CH$_3$CN. Fractions collected in this range are pooled, concentrated, and submitted for sequencing. Any large discrete peaks in the elution profile should be pooled and submitted separately for sequencing.

3.6. Pooled Peptide Sequencing

Purified peptides are applied to polybrene-coated glass-fiber filters, and sequenced by Edman degradation, using a suitable protein sequencer, such as an ABI model 492 sequencer, with 10A data analysis software. Results should be provided as yield of each amino acid per cycle. As described *(1,9,14,15)*, a pmol yield >150% of the previous cycle is usually scored as significant; for cycles 7–10, this cutoff value can be reduced to 120% to allow for cycle-to-cycle sample loss. When using a nonamer library, one should see a marked drop in the amino acid yield between cycles 9 and 10.

4. Notes

1. Each laboratory will have different preferences for suitable vectors. Any vector is suitable for β$_2$m production, but for the truncated HC, consideration must be given to the incorporation of the His tag at the C-terminus. We routinely use the pET system, because of its high expression levels and tight control over basal expression. In particular, the ampicillin resistant pET-22b(+) vector is used (*see* **Fig. 3**), because it carries both an optional N-terminal *pelB* signal sequence for potential periplasmic localization of proteins and an optional C-terminal His tag sequence. This vector allows cloning of the same gene into one vector for expression and targeting to different parts of the bacterium, if soluble proteins are required. However, for this particular type of study, solubility is not an issue, so, for both the rat truncated HC and the LC, the *pelB* signal sequence is excised (using the *Nde*I restriction site), and the genes for both β$_2$m and the truncated HC are ligated with their own initiation codon. Only for the truncated HC is the optional C-terminal His-Tag coding sequence used. Because of the need for truncation, the gene is ligated without its own stop codon, in frame with the plasmid-encoded His-Tag sequence (using the *Xho*I restriction site).
2. A number of companies now offer combinatorial peptides. Care should be taken before ordering to check how the library is made and what quality controls the company performs to ensure randomness at each position. For a nonamer library, complete random couplings should yield 19^9 possible peptide combinations (cysteine is omitted from the library, thus preventing unwanted disulfide bond formation). In all recent work *(9,16)*, peptide libraries were obtained from ECHAZ Microcollections (Tübingen, Germany).
3. PMSF is water-labile, with an effective half-life of about 1 h at pH 7.5, and should therefore be added last when refold experiments are set up.
4. By purifying the HC component this way, monomer chains are enriched from multimers that are produced by the formation of interdisulfide bonds, created

during bacterial translation. The use of reducing agents during solubilization will break apart these multimers, but they can also complicate ensuing purification and refolding steps, e.g., refolding buffers would require the presence of an optimum redox potential (obtained by the use of glutathione in both oxidized and reduced forms), to allow correct formation of disulfide bonds. Success using this method is not guaranteed; initial experiments in this laboratory, to denature aggregates of RT1-A[a] in the presence of reducing agents, and to refold them with a specific peptide, were unsuccessful. For all three rat molecules produced in **ref. 9**, the fraction of monomer produced was 50% of the total HC production, but, because induction was high (10–20 mg/L), a loss of 50% at this stage was deemed to be acceptable to maintain the simplicity of the method, and thus allow its general use.

5. Recombinant forms of rat, human, and mouse β_2m have been produced and purified *(9,13,17)*. All forms were expressed as inclusion bodies, and both the human and mouse forms were used for refolding and crystallization studies, without further purification. Only the monomeric form of the rat molecule was purified prior to refolding studies *(9,13,17)*. The method described here should also be applicable to the other forms. Pure monomeric rat β_2m (>95% purity) is achieved by washing the loaded column with 50 m*M* NaCl *(9)* and, when using the bacterial construct provided by Professor Don Wiley *(13)*, monomeric human β_2m (>95% purity) is eluted with 100 m*M* NaCl, using this purification scheme (personal observation).

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16

Neural Network Method for Predicting Peptides that Bind Major Histocompatibility Complex Molecules

Kamalakar Gulukota and Charles DeLisi

1. Introduction

Peptides that bind major histocompatibility complex (MHC) products exhibit allele-specific sequence motifs. The motifs, however, are neither necessary nor sufficient for binding. In particular, about 66% of the peptides that exhibit motifs do not bind the corresponding MHC molecule. This chapter describes a neural network based method for identifying MHC-binding peptides. The neural net is superior to simple motif searches in eliminating false positives; its behavior can be coarsely tuned to the strength of binding desired, and its applicability is easily extendable to any desired MHC allele.

MHC molecules are promiscuous, in that they bind many different peptides. Identifying peptides that bind MHC molecules plays a critical part in understanding the immune response in autoimmune disorders *(1,2)* and viral infections *(3–5)* and is also important for designing peptide vaccines *(6–8)*. Because of the fact that experiments to measure the binding affinities of peptides to MHC are time consuming and expensive, considerable effort has gone into computational methods for identifying MHC-binding peptides *(9–11)*.

One of the earliest methods is based on sequence motifs. Peptides that bind MHC tend to have allele-specific sequence motifs. For example, peptides that bind human leukocyte antigen (HLA)-A0201 tend to be 8–10 residues long, and have a leucine or isoleucine at position 2, and a leucine or valine at the C-terminus. These observations are treated as rules, to facilitate the identification of HLA-A0201-binding peptides. These simple rules may be extended to allow or disallow other residues at other positions *(13)* but, however modified, they suffer from two fundamental limitations: basing the binding decision on only a few positions, and treating preferences for certain amino acids at these

positions as absolute requirements. The result of these shortcomings is a substantial number of false positives: almost two thirds of the peptides so chosen will not bind.

More detailed methods have been designed, which treat preferences more appropriately, but still make the assumption that individual side chains of the peptide independently bind to the MHC: the so-called independent binding of sidechains or IBS assumption (*11,14–16*; Weng, Z., and Weng, C. D., unpublished results). The IBS assumption appears plausible in the context of the structures of peptides when bound to MHC *(17)*; their relatively extended structure implies that direct interactions between residues that are far from each other in sequence are probably weak. However, even if direct interactions are ignored, at least two other contributions to the free energy of binding can violate IBS.

First, the interaction with MHC of a given residue can be strongly dependent on its neighboring residues. For example, the constraints on the conformations of a residue are stricter, if its adjacent residue has a large side chain, than if the neighbor has a small side chain. Thus, the structure and contribution to overall binding free energy of a given residue at a given position may be different in peptides of different sequences. Second, although the IBS assumption may be plausibly satisfied by the bound form of the peptide, there is no reason to assume even approximate adherence to the IBS rule by the peptide in its free state. Since the binding free energy of a peptide is the difference between its free energies in the free and the bound states, this too can cause a significant variation from IBS. Thus, although patterns exist in the sequences of MHC-binding peptides, these patterns are not readily discernible, and certainly are more complicated than simple sequence motifs. Therefore, this is a classic problem of pattern discrimination; an artificial neural network (ANN) *(18,19)* can be used to solve it *(20)*.

2. Materials

The following are required for building a neural network to predict peptides that can bind MHC molecules.

1. Software that can simulate a neural network. Specifically, the system should have the capacity to simulate neurons with sigmoidal output functions, and back-propagation *(18)* should be available as a training algorithm. Both are available in most commercial and freeware neural network simulators.
2. A database of peptide sequences, along with measurements of their binding affinities to the MHC allele of interest. The larger this database, the more robust the learning by the neural network. As far as possible, the database should reflect the test set of peptides.
3. A test set of peptide sequences, for which we are interested in predicting the binding affinity. If a separate test set is not available, the original database can be split into a training set and a test set, or a jackknife method may be used.

3. Methods

The main steps in building neural nets to solve the problem of MHC–peptide are as follows: gather a training set, design and train the neural network, test it on a testing set, and, finally, tweak the design until optimal performance is achieved.

1. Generate a data set. Binding measurements for approx 500 peptides will be required for reliable training and testing. If doing that many measurements in-house is not feasible, and if the interest is in a common MHC allele, such as HLA-A2, relatively large amounts of binding data have been published, and these may be used as the data set (*see* **Note 1**). Data sets with systematic biases should be avoided. For example, there should be a good distribution between peptides that have the appropriate motif and those that do not. Also, there should be a good distribution of binding affinities within the data set.

2. Decide the desired fate for each peptide. The desired output of the neural net for each peptide should be set. This is usually 1, if the peptide binds, and 0, if it does not. Binding affinity measurements are not binary in nature, and so a cutoff value for binding needs to be chosen (*see* **Note 2**).

3. Procure or develop software for simulating an ANN. Many packages (commercial as well as freeware) are available for simulating a neural net. Most packages have the minimal features needed for this application: ability to simulate sigmoidal neurons and to implement the back-propagation learning algorithm.

4. Amino acid representation. Each amino acid (aa) must be represented as a vector of bits (0 or 1). Other representations, such as using numbers from 1 to 20, are possible, but not advisable (*see* **Note 3**). The most straightforward representation is to use a vector of 20 bits for each aa. For example, glycine (Gly) could be a 1 followed by 19 0s. Some classification of aa, based on hydrophobicity, charge, or size, can be used to reduce the number of bits (*see* **Note 4**), but different classifications will probably result in different predictions. In any case, each aa would be represented by an array of L bits, so that a peptide of 9 aa would be represented by an array of 9 L bits, where bits 1–L would represent position 1, L + 1 to 2 L would represent position 2, and so on.

5. Design the architecture of the ANN. The architecture that the authors recommend is a fully connected feed-forward neural net, with one hidden layer. Such a net has three layers of neurons: an input layer, a hidden layer, and an output layer. One hidden layer is probably sufficient to capture the complexity of patterns for most MHC alleles, but, if the pattern is very subtle, increasing the number of hidden layers to two should be considered (*see* **Note 5**). Each neuron in a layer is connected to every neuron in the next layer and there are no feedback loops. The number of neurons in the input layer is equal to the number of bits in the input vector. Thus, if the size of the peptide changes, the design of the ANN needs to change. The recommended number of neurons in the output layer is one, i.e., the ANN performs optimally for binary predictions (*see* **Note 6**). Thus, the most significant design decision to make is the size of the hidden layer. Good

results can be obtained using a single hidden layer of 50 neurons. This initial design of the ANN can be tuned to the specific problem in the testing phases.

6. Split the data set into a training set and a testing set. The temptation to place most of the data into the training set should be avoided. Although this does improve the training of the neural net, it leaves little room for validation, which is an integral part of designing neural nets (*see* **Note 7**). It is best to allocate approx one-half, chosen randomly, to each data set. However, care must be taken that a good sampling of binding and nonbinding peptides goes into both the training set and the testing set.

7. Train the ANN. Each peptide in the training set must then be encoded as described in **step 4** and fed to the ANN, to determine its predicted output. The training set is fed repeatedly, using back-propagation to set the weights of the net, i.e., to train it. Most neural network packages allow this learning to continue until some measure of the error drops below a threshold or the number of training cycles (called epochs) crosses a predetermined number. Two measures of error are most commonly used: maximum error or mean squared error. Either can be used, but maximum error is preferable (*see* **Note 8**).

8. Test the ANN. Extensive validation is critically important (*see* **Note 9**). The weights of the ANN should be frozen (all learning should be stopped); peptides in the testing set should be encoded according to the rules in **step 4** and fed to the ANN. Both the sensitivity and the specificity should be determined (**Note 10**) for the testing set.

9. Tune the ANN. Given the sensitivity and specificity of a given realization of the neural net, its architecture can be altered to improve performance. The chief adjustable parameters are the number of neurons in the hidden layer and the number of hidden layers. Increasing either increases the number of connections or weights in the ANN, and increasing the number of weights usually requires larger amounts of data for reliable learning. As a general rule, the ANN of the least complexity, which gives the desired performance, should be used (*see* **Notes 11–16**).

4. Notes

1. It is important to ensure a uniformly high quality for the binding-affinity measurements, but, when collating data from different publications and laboratories, this can be difficult. Different laboratories may measure binding differently, and the different techniques may not correlate well with each other. All measurements should be converted into a single unit. This sounds like sophomoric advice, but many binding measurements require careful thought to convert into equilibrium binding units, such as inverse n*M*. It is especially important not to mix measurements of kinetic quantities, such as half-life of MHC-peptide complexes *(21)* and inhibitory concentration of 50% (IC50) measurements *(13)* because they will not generally be linearly related. Even the simple inverse relation between IC50 and affinity is valid only under a restricted range of conditions, and more general relation *(11)* needs to be used, if measurements fall outside that range.

2. When equilibrium binding affinities are known for the peptides in the data set, a cutoff value should be chosen, e.g., all peptides whose IC50 is greater than 500 n*M* may be considered nonbinding. The chosen cutoff value should be relatively close to the chemically relevant value for binding, because the implicit assumption underlying the use of ANN is that binding and nonbinding peptides have distinguishable amino acid sequence patterns. For example, assume that only peptides with IC50 <500 n*M* have relevant binding. For the ANN, this means that peptides that bind with IC50 <500 n*M* have a sequence pattern that is absent from those with IC50 >500 n*M*. The ANN can learn this distinguishing pattern only if the cutoff value for IC50 is set at 500 n*M*. If it is set at a significantly different level, the classes of binding and nonbinding peptides will not be pure. Their patterns will be clouded by patterns from the other class, violating the implicit assumption that there exists a distinguishing pattern.

3. In principle there is no bar against using incremental numbers, e.g., 1 for Gly, 2 for alanine (Ala) and so on. Then only one neuron will be needed to encode each amino acid. However, such a scheme inherently implies order relationships between amino acid to the neural net. For instance, amino acid 15 will be considered closer to amino acid 14 than to 8. Such relationships may not exist, or may not be relevant for binding properties. It is much better to allow any such relationships to emerge naturally, than to impose them with an arbitrarily selected numbering scheme. It is also possible to come up with a more economical scheme than using 20 bits to represent each amino acid. In principle, five bits are sufficient to uniquely represent all 20 amino acid. Although such schemes have less error than using incremental numbers, they also are arbitrary, and convey relationships between aa that may not be relevant. For example, consider what happens if Gly is encoded as (00000), tryptophan (Trp) as (00001), and Ala as (11111). Inherent in these input patterns is a closer relation between Gly and Trp than that between Gly and Ala. Such confounding input patterns may unnecessarily complicate the separation between binding and nonbinding peptides.

4. Arbitrarily chosen numbering schemes should be avoided, but carefully designed amino acid classifications *(22)* will significantly reduce the number of neurons, which, in turn, reduces the number of connections in the ANN, and the amount of data needed to train it. Conversely, training with the same amount of data is more robust when the number of connections is lower. When amino acid classifications are used, it is important to relate the classification to the effect of the amino acid on the binding properties of the peptide. This may be subtly different from a classification of amino acids based on their own properties. Further, it is important to ensure that the performance of the ANN is not too sensitive to minor changes in the classification scheme.

5. When the number of hidden layers is increased, the complexity of the ANN, the number of connections in it, and its need for data, all increase. Although the ability to identify subtle patterns is better, because of increased complexity, it only works if a large amount of input data is available for training.

6. A single neuron in the output layer means that only a binary prediction is expected, i.e., if the output level of the neuron crosses a threshold (usually 0.5), the peptide is predicted to bind, otherwise, it is predicted not to bind. Specifically, no prediction is made regarding the strength of binding. It may be tempting to relate the actual output level of the neuron to the binding affinity. However, any observed correlation in this respect should be considered serendipitous, unless proven otherwise. An alternative method for predicting binding affinities is to increase the number of neurons in the output layer. For example, the strength of binding can be correlated with the number of neurons in the output layer whose output level is 1. This approach needs many positive and negative examples at each affinity level. Even more significantly, the implicit assumption in this approach is that there exists a pattern that distinguishes strongly binding peptides from weakly binding peptides. Usually, no support exists for such an assumption. Using ANN to predict the binding affinity of peptides that bind HLA-A2 has been discouraging, probably for these reasons.

7. ANNs are inherently statistical methods. Individual predictions of an ANN mean less for its validation than its overall performance on a data set. Thus, it is important to keep the training and testing sets sufficiently large. Exhaustive training (by making the training set as large as possible) and comprehensive testing (which requires a large test set) are therefore mutually contradictory goals. The best division can be found by using training sets of different sizes, and choosing the minimum size that gives acceptable performance.

8. Let the expected output (0 or 1) on the Ith peptide be $X(I)$ and let the ANN prediction be $P(I)$; then, the error of prediction on this peptide is $E(I) = $ abs $(X[I] - P[I])$ where abs signifies the absolute value. Maximum error is defined as the maximum of all $E(I)$ for the data set, and mean squared error is the arithmetic mean of all $(E[I])^2$. If training is continued until the maximum error drops below a threshold, overtraining of the neural net may occur. In this scenario, the ANN will perform perfectly on the training set (i.e., there will be zero mispredictions), even though it may make mistakes on the testing set. Even so, maximum error is a good measure to use for the initial attempts. If the ANN performs acceptably under this regimen, the implication is that a relatively strong pattern has been discovered. The ANN may fail under the maximum error criterion, for two reasons: it may fail to converge, or its performance on the testing set may be unacceptable. If the ANN fails to converge (i.e., even prolonged training does not lead to error below threshold), it may mean that there are specific examples in the training set that do not conform to an overall pattern strongly apparent in the rest of the data set. In this case, it is better to train the ANN to minimize mean-squared error, which is less sensitive to specific examples of outliers. For HLA-A2, acceptable performance was found using a maximum error of 0.1 as the threshold.

9. The fact that an ANN can be trained only implies that the data are internally consistent. More specifically, it means that a distinguishing pattern exists in the training set, and that this pattern has been learned by the ANN. Whether this pattern is general enough to be applicable outside the training set is an open ques-

Table 1
Performance of a Neural Net

	Real binders	Real non-binders
Predicted Binders	True Positives (TP)	False Positives (FP)
Predicted Non-binders	False Negatives (FN)	True Negatives (TN)

tion. Without proof of this outside applicability, the ANN is useless. Therefore, the need to test the ANN on a testing set of statistically significant size cannot be overemphasized.

10. The overall performance on the test set can be evaluated using the 2×2 matrix in **Table 1**.

11. Sensitivity, or the ability to identify real binders, is defined as TP/(TP + FN), and specificity, or the ability to identify nonbinders, is defined as TN/(TN + FP). Other measures of performance include positive predictive value (defined as PPV = TP/[TP + FP]), negative predictive value (defined as NPV = TN/[TN + FN]) and Accuracy (defined as the proportion of all predictions that are correct, Acc = ([TP + TN]/[TP + FP + TN + FN]). Of all these measures, sensitivity and specificity are raw measures of performance, in that they do not depend on the prevalence of binding peptides in the data set. PPV, NPV, and Acc on the other hand, are strongly dependent on this prevalence, and should be used cautiously.

12. It is important to use both sensitivity and specificity to evaluate the overall performance. Using only one measure can be misleading, since some methods will do very well in that one measure, even though their overall performance is poor. As an extreme example, a method that predicts all peptides to be binders will have a sensitivity of 1, but its specificity will be 0.

13. It is important to use the smallest possible ANN that gives acceptable results. Thus, once the evaluation on a testing set is completed, the design of the ANN should be re-engineered, in order to reduce the number of connections in it. This can be done by reducing the number of hidden layers, and by reducing the number of neurons in the hidden layer. There are formal rules *(19)* for the approximate size of the data set required for training an ANN of a given complexity, but these rules are often too general to be applicable. A rough rule is to use only one hidden layer, with about 30% as many neurons as there are in the input layer.

14. The distribution of peptides in an actual test set may look dramatically different from that in the training set. For example, a typical application is to scan an immunogenic protein for peptides that may bind the MHC of interest. The peptides from such a scan will typically have relatively random sequences. Only a small proportion of these peptides will be real binders. Therefore tuning the ANN should not be based on performance measures such PPV, NPV, or Acc, which have a heavy dependence on the prevalence of binding peptides in the data set. It is much safer to rely on sensitivity and specificity.

15. The reason to look for a balance between sensitivity and specificity, rather than rely on any one measure, is that no method is perfect. That is no method has a

sensitivity and specificity of 100%. It is unlikely that such a method could be devised using only peptide sequence, even if the training set is relatively large. A method for perfect prediction would probably include more detail, such as predicting the structure of the given peptide *(23)* and evaluating its free energy of binding *(24)*. At present such procedures *(25)*, although feasible for a handful of peptides, are too complicated and time-consuming to apply routinely to a large number of peptides. The greatest advantage of the imperfect but simple tools, based on ANN, is that they can be applied rapidly to a large number of peptides.

16. The sensitivity of the neural net is strongly dependent on the number of binding peptides in the training set. If this number is very low, the ANN has too few examples, and the pattern that it learns will not be broadly applicable. The ideal training set will have sequence qualities similar to the target test set. Because this is almost impossible to achieve, an alternative is to make the training set as large as possible. As more of ANN predictions are experimentally tested, these new measurements can be added to the training set. Thus, it is possible and desirable to retrain the ANN periodically, as more data becomes available.

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17

Analysis of T-Cell Response
Using Altered Peptide Ligands

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1. Introduction

T lymphocytes interact with protein antigens (Ags) in the form of peptides bound to self-major histocompatibility complex (MHC) molecules displayed at the surface of antigen-presenting cells (APC) *(1,2)*. Dissection of the different T-cell receptor (TCR)-mediated functions elicited by peptide Ag stimulation is essential for understanding the mechanisms that govern T-cell immunity. Historically, it was believed that each individual T-cell clone recognized a single peptide, and T-cell activation was thought to be an all-or-nothing phenomenon. The current interpretation is that the exquisite specificity of T-cell recognition of a given peptide is not absolute, because of crossreactive T-cell responses with other sequentially or structurally related peptides *(3–7)*. Additionally, recent studies *(8,10)* using altered peptide ligands (APLs) or analog peptides displaying amino acid subsitutions at key TCR contact positions of the Ag peptide have revealed that TCR can interpret subtle modifications in its ligand, resulting in differential activation of T-cell functions.

Much direct evidence has been amassed *(11–14)* showing that the TCR can deliver selective transmembrane signals, depending on the fine specificity of the Ag determinant recognized. Based on their functional properties, APLs can be divided into three nonmutually exclusive categories: the full-agonist, the partial-agonist, and the antagonist peptides. Full-agonist peptides activate all the T-cell functions mediated by the wild-type (WT) peptide. Alternatively, differential signaling through the TCR–CD3 complex, by partial-agonist peptides, results in selective activation of some, but not all, WT peptide-induced

T-cell functions. TCR signaling by partial agonists can lead to T-cell activation or anergy *(15,16)*, selective induction of T-helper 1 (Th1) or Th2 CD4^{+} T-cell subsets, or partial activation of T-cell functions (cytolytic activity/proliferation/lymphokine release) *(17–20)*. Additionally, co-incubation studies have demonstrated that APLs can also behave as antagonists of the WT peptide ligand and thereby inhibit certain signals delivered by the specific Ag to T-cells *(21–23)*.

Natural APLs have been implicated in thymic development *(10,24)*. Using in vitro culture models, recent studies have shown that APLs can influence both CD4^{+} and CD8^{+} T-cell thymic development *(25–27)*. When included in fetal thymic organ cultures from MHC I-deficient TCR transgenic mice, peptide variants which were antagonist APLs for mature T-cells expressing the same TCR could positively select thymocytes *(26)*. In contrast, naturally occurring partial- or full-agonist self-peptides, although supporting positive selection at low concentration, induced negative selection at high concentrations *(27)*. These observations have demonstrated the role of natural APLs as a major component of the immune system. In other studies, synthetic peptide analogs have been engineered in order to manipulate in vivo T-cell responses in an Ag-specific fashion. Immunomodulation using analogs of pathogenetic peptides has proven effective in the treatment of some autoimmune diseases in rodents *(28–30)*.

Collectively, these studies emphasize the contribution of naturally occurring analog peptides to the selection of T-cell repertoire in the developing thymus, and to the induction and regulation of in vivo adult peripheral T-cell responses *(31)*. Furthermore, based on these accumulated studies, it has become clear that synthetic peptide analogs represent an invaluable tool for the study of fine mechanisms underlying T-cell immunity and for manipulating T-cell responses in health and disease. The following describes working methodologies designed to identify APLs with agonistic and antagonistic properties and to study their ability to modulate T-cell responses to WT Ag peptides.

2. Materials
2.1. Immunization

1. Complete Freund's adjuvant (Difco, Detroit, MI).
2. Phosphate buffered saline (PBS, pH 7.2).
3. AIM-V containing L-glutamine (L-Gln) and antibiotics (Gibco-BRL, Grand Island, NY, no. 12055-091).
4. Sterile syringes (1 mL) + needles (26.5 gauge).

2.2. Proliferation Assay

1. 96-well culture microplates (flat-bottom) (Corning, Corning, NY).
2. AIM-V containing L-Gln and antibiotics (Gibco-BRL).

3. ^{3}H-thymidine (1 mCi for 10 plates) (ICN Pharmaceuticals, Costa Mesa, CA).
4. Cell harvester (Skatron or Wallac, Waltham, MA).
5. Scintillation liquid.
6. Radioactive β-counter.

2.3. Murine ELISA Spot Procedure

1. Specific filter 96-well plates (Unifilter 350 Polyfiltronics, Rockland MA).
2. Incubation medium : AIM-V containing L-Gln and antibiotics (Gibco-BRL, no. 12055-091), 1% fetal calf serum (FCS).
3. Washing solutions: sterile PBS; PBS; PBS containing 0.025% Tween-20 (PBS-T).
4. Blocking solution: sterile PBS containing 1% bovine serum albumin.
5. Incubation solution: PBS containing 1% bovine serum albumin and 0.025% Tween-20.
6. Primary antibodies (Abs) (i.e., purified rat antimouse cytokines, Pharmingen, San Diego, CA) and secondary Abs (i.e., biotin rat antimouse cytokines, Pharmingen).
7. Conjugate: avidin-horseradish peroxidase.
8. Substrate solution: 800 μL 3-amino,9-ethyl carbazole (AEC) (Pierce, Rockford, IL) (10 mg/mL) plus AEC buffer: 24 mL 0.1 M Na acetate buffer, pH 5.0. Filter this solution.
9. H_2O_2 (30%).

3. Methods

Here are two techniques that can be utilized to determine whether a peptide behaves as an agonist, a partial agonist, and/or an antagonist, compared to a known immunogenic (WT) reference peptide (*see* **Note 1**). Briefly, mice are immunized with the WT peptide, then, after 10 d, lymph node and spleen cell suspensions are prepared. These cells are placed in culture, along with various concentrations of the WT immunizing peptide (positive control) or with the analog peptide at different doses (to test for agonist properties), or in the presence of both WT and analog peptides at different ratios (to test for antagonist properties). T-cell functions are then determined using the proliferation assay and the ELISA spot procedure, as described below.

3.1. Immunization and Preparation of Lymphoid Cells

1. Prepare 50–200 μg peptide (either WT or analog peptide) solubilized in 50 μL PBS and emulsified in complete Freund's adjuvant (CFA) (Difco).
2. Using a 1 mL sterile syringe and a 26.5-gauge needle, inject the Ag–CFA emulsion in hind footpad of a mouse (50 μL).
3. 9–10 d after injection, sacrifice the mouse, and collect the popliteal lymph nodes and the spleen.
4. In a sterile Petri dish containing 5 mL serum-free AIM-V medium, a lymphoid cell suspension was prepared by laceration, then maceration of the lymphoid organs, using the head of a 5 mL sterile syringe (*see* **Note 2**).

3.2. Proliferation Assay

Following immunization, Ag peptides that are immunogenic induce clonal expansion of specific T-cells in draining lymph nodes, and, to a lesser extent, in spleen. The response peaks after 9–10 d in the lymph nodes, then decreases rapidly. Alternatively, because of the homing of memory T-cells, Ag-specific primed T-cells can be detected more than 1 yr after immunization in the spleens of mice. Clonal expansion is mostly driven by the secretion of IL-2 although other lymphokines, including IL-4, contribute to this process. Clonal expansion is a crucial event following Ag recognition and T-cell signaling, and it is a crucial event in T-cell-mediated immunity.

1. Prepare suspension of 2×10^6 lymph node cells/mL and 10^7 spleen cells/mL in Dulbecco minimum Eagle's medium (DMEM), supplemented with 2 mM L-Gln, 50 µM 2-mercaptoethanol, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% FCS.
2. Wash the cells twice in AIM-V serum-free medium.
3. Place in culture 2×10^6 lymph node cells or 10^7 spleen cells in 1 mL AIM-V medium alone, containing 2 mM Gln, or with 1–50 µM (final concentration) of control WT peptide or analog peptide, in 96-well culture dishes for 4 d (100 µL cell suspension well) in a CO_2 incubator (5% CO_2 at 37°C).
4. Add 1 mCi [^{3}H]-thymidine for the last 18 h of culture.
5. Harvest the cells on filters (using automatic cell harvester), then dry the filters in a microwave oven.
6. Add scintillation liquid, and count radioactivity.

3.3. ELISA Spot Procedure

The technique of ELISA spot allows the measurement of cytokine secretion at a single-cell level. This sensitive method can detect cytokine-secreting cells in the order of one cell per million in a polyspecific/polyclonal T-cell population). Using this technique, one can establish the frequency of Ag-reactive T-cells, as well as their phenotype (type 1/type 2), as determined by their lymphokine patterns (type 1: IL-2, interferon γ and type 2: IL-4, IL-5)

1. Coat the 96-well plate with 100 µL/well primary Abs previously diluted in sterile PBS (concentration range for anticytokine Abs: 2–5 µg/mL) (*see* **Note 3**). Wrap the plate, and keep it overnight at 4°C (or up to 90 h).
2. On the day of the experiment, empty the wells by shaking the plate over a basin in the hood. Wash the plate twice with sterile PBS (200 µL/well). Block free sites by incubating the plate for 90 min with 150 µL/well blocking solution.
3. Remove the blocking solution and wash the plate 3 X with sterile PBS (200 µL/well). Keep the last wash on the plate until needed.
4. Remove the last wash, and add the prepared Ags, stimulator, and responder cells in AIM-V medium supplemented with 1% FCS, in order to have 200 µL/well at the end (*see* **Note 4**).

5. Gently tap the side of the plate to redistribute the cells. Put the plate at 37°C and in 5% CO_2 for 24h (IL-2, IL-4) or 48 h (IFN-γ, IL-5).

6. When the incubation time is finished, discard medium, and wash 3 X with PBS and 4 X with PBS-T. Leave the last wash on the plate for 5 min at room temperature, then discard wash. After each wash, the plate should be inverted and gently tapped onto paper towels, to remove all the liquid.

7. Add 100 µL/well of the second Ab (2 µg/mL in PBS-BSA-Tween). Wrap the plate, and refrigerate overnight.

8. The next day, prepare a dilution (1/2000) of the horseradish peroxidase-avidin conjugate in incubation solution. Empty the contents of the plate, and wash it 3 X with PBS-T. Then add 100 µL/well of the conjugate. Cover with foil, and incubate for 90 min at room temperature.

9. Before the end of the incubation, prepare the solution containing the substrate. Filter this solution.

10. Wash the plate 4 X with PBS, then add 12 µL H_2O_2 to the substrate solution, and keep this solution under foil. Empty the plate, and add 100 µL/well of this substrate. Keep the plate under foil, and follow the development for 2–20 min.

11. When the plate is developed (when no new spots appear in the absence of red background), empty it, and rinse 5–10 X with dH_2O to stop the reaction. Dry the plate in a fume hood covered with foil.

12. Count the spots (ImmunoSpot Analyzer, C.T.L, Cleveland, OH).

4. Notes

1. There is no universal practice in the prediction of the comparative functional properties of a peptide analog and its WT counterpart. In some studies, systematic amino acid substitutions, with each of the 20 common amino acids, have been applied at each position of the immunogenic peptide. Alternatively, other studies have focused on conservative or nonconservative substitutions at putative key residues in the WT peptide, based on its physicochemical features. No clear-cut methology has emerged from these studies on how to design analog peptides with defined activities. However, it is clear that in order to bind to the same MHC restriction element as the WT peptide, the analog peptide should contain a recognized MHC-binding motif. Also, in the case of peptides whose TCR contact residues have been described, it is clear that these positions represent preferential sites for amino acid substitutions.

2. In selected experiments, T-cells can be purified using commercial T-cell purification columns (R & D, Minneapolis MN), and separated into CD4+ and CD8+ T-cell subsets, then incubated in vitro with irradiated or mitomycin C-treated splenocytes (as APCs) and peptide Ags.

3. **Steps 1–5** should be performed in sterile conditions, and **steps 9–11** require the use of a chemical hood.

4. In ELISA spot assays utilizing purified T-cells, the ratio between T-cells and irradiated or mitomycin C-treated splenocytes (used as APCs) is crucial. This laboratory found that a ratio of 1:3 between T-cells and APCs is optimal.

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18

Assays of Thymic Selection

Fetal Thymus Organ Culture and In Vitro Thymocyte Dulling Assay

Kristin A. Hogquist

1. Introduction

Many of the important properties of T-cells are not imprinted by the genetic program of the cell. Clonotypic expression of receptors, major histocompatibility complex (MHC) restriction, and self tolerance are all properties that are determined somatically during the development of an individual T-cell precursor in the thymus. Three selection checkpoints operate during development to imprint these properties: β selection, positive selection and negative selection. β selection occurs during an early stage of thymic development *(1)*. This checkpoint ensures clonotypic expression of the T-cell receptor (TCR) β-chain, by signaling the termination of further rearrangement at that locus, in cells that have successfully produced a β-chain to pair with the invariant pre-Tα-chain. Positive and negative selection act at a later stage of development *(2)*. Here, intact α/β-TCRs are checked for their ability to interact with MHC–self ligands. The prevailing affinity model suggests that receptors with a low affinity for MHC–self ligands trigger survival, subsequent maturation, and lineage commitment *(3)*. This positive selection step assures that the T-cell repertoire of an individual is MHC restricted to the alleles that individual expresses. Receptors having a high affinity for MHC–self ligands trigger activation-induced apoptosis. It is thought that the threshold for activation of peripheral T-cells is at least as high as that of apoptosis in the thymus. Thus, negative selection contributes to a self-tolerant T-cell repertoire.

Although it is well established that antigenic peptides induce negative selection if present during development in the thymus, the precise nature of the ligand(s) for positive selection has been more controversial *(4)*. This has been a difficult subject to study for several reasons, one of which is that the process of positive selection is not reproduced with fidelity in vitro. This is in part because thymic stromal cells, which are obligatory for positive selection *(5)*, rarely maintain their normal in vivo characteristics upon isolation. In addition, thymocytes, removed from the intact lobe, undergo rapid changes in the TCR complex *(6)*. However, for many years, immunologists have been using an organ culture (OC) approach to study thymic development (*see* **Notes 1** and **2**). Here, the two intact thymic lobes of a fetal mouse (*see* **Notes 1** and **2**) are placed at the liquid–air interface, and cultured under high humidity for up to 3 wk *(7)*. Thymocytes proliferate and undergo appropriate developmental changes during this time. The ex vivo nature of the culture allows addition of drugs *(8,9)*, antibodies (Abs) *(10,11)*, cytokines *(12,13)* retrovirus *(14,15)*, and peptides *(16,17)*. In such a manner, this technique has proved useful for elucidating issues of β selection *(18)*, positive selection *(16)*, and negative selection *(19)*, as well as general proliferative *(12,20)* and differentiative *(5)* properties of thymocytes. This review presents detailed technical information on performing fetal thymic organ cultures (FTOC).

In some cases, the technique of FTOC can be burdensome. For example, we recently wanted to screen hundreds of chromatography fractions for the presence of self peptides that would induce positive selection. OCs, which typically require 10 mL media for a seven day period, were impractical, given the small yield of self peptide present in the fractions. Thus, we sought to utilize an in vitro assay to detect the ligands for positive selection. Two published assays, which showed promising preliminary results, were a thymocyte–stromal cell reaggregate assay *(5)*, and a thymocyte–stromal cell co-culture assay *(21)*. Both of these techniques require the time-consuming and expensive isolation of stromal cells from large numbers of animals. However, they do allow the greater experimental flexibility of being able to vary the stromal cell content. In many cases, such flexibility is not required. Thus, we designed an inexpensive, short-term, in vitro assay to detect peptide ligands of relevance to thymic selection. This assay is based on the observation that CD4/CD8 double positive (DP) thymocytes (the cells upon which selection acts) undergo rapid downregulation of both co-receptors (DP dulling) upon engagement of ligands for either positive or negative selection *(22,23)*. In the initial report of this assay, we used CD8 overexpression to enhance the sensitivity for class I bound peptides *(24)*. Such a modification was subsequently shown to be unnecessary *(25)*, and makes the approach more broadly applicable. This chapter reports

details of the DP dulling assay methodology, and discusses limitations to its use and interpretation.

2. Materials

2.1. Fetal Thymus OC

1. Timed pregnant mice: Timed pregnant mice can be ordered from several vendors, such as Jackson Labs (Bar Harbor, ME), Harlan (Indianapolis, IN), Charles River (Wilmington, MA), or Simonsen (Gilroy, CA). Alternatively, one can set up timed matings, if particular transgenic or knockout strains are needed that are not commercially available. To do timed matings in a colony, add three breeding age females per cage with one male, overnight. Females maintained on a light—dark cycle tend to ovulate about every 4–5 d. Thus, one can expect two pregnant females from three timed mating cages (nine females), assuming all the animals are of optimal breeding age and in good health. It has been the author's experience that males can be used up to 10 mo of age, and females between 4 wk and 4 mo of age. To increase the efficiency of timed matings one can theoretically do two things. First, use only females in estrus (i.e., ovulating females). The stage of the estrous cycle of a female can be determined by checking the appearance of the vagina *(26)*. Secondly, use females that have been superovulated by injection of pregnant mare's serum, followed by human chorionic gonadotropin *(26)*. The author has found that it is easier and more efficient simply to increase the number of breeding cages, using randomly cycling females. Be sure to house males individually, because they will fight. Also, once a male has been with females overnight, do not use him again for 1 wk, because the sperm count drops for several days after a male has mated.

   The next morning, remove the females, and check for the presence of a copulation plug. This consists of coagulated proteins from the sperm, and can be easily seen. Those females with a detectable plug are likely to be pregnant, and can be set aside for the indicated period of time. It is not critical to check for a plug. However, since the remaining females can be cycled back in with a male for additional timed matings, this can make your matings more efficient.

   Since most timed matings are done overnight, the precise gestational age can vary up to 16 h. For this reason, one should pay close attention to the timed mating terminology. For example, in these experiments we use mice at a gestational age of 16 d (g.d. 16). Here, the morning when the mice are taken apart is considered day 1. Theoretically then, the mice are somewhere between 15 and 15.7 d of age when the thymus is excised. Others consider the morning the mice are taken apart to be day 0. In this case, the mice are somewhere between 16 and 16.7 d of age at g.d. 16. If using timed pregnant mice from a vendor, be sure to inquire what terminology they adhere to.

2. RP10 media: RPMI (Gibco-BRL, Gaithersburg, MD) containing 10% fetal calf serum, 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, 50 U/mL penicillin, 50 mg/mL streptomycin, 50 mg/mL gentamycin sulfate, and 5 mM HEPES.

3. Humidified incubator delivering 5% CO_2.
4. Ice-cold phosphate-buffered saline (PBS) prepared according to standard lab protocol.
5. 100-mm plastic Petri dishes.
6. 6-well tissue-culture-treated plates.
7. Dissecting microscope.
8. Goose-neck lamp (if a lumination source is not included with the microscope).
9. Laminar horizontal flow-hood.
10. Two pairs of dissecting scissors.
11. One pair of fine dissecting scissors (sterile).
12. Four pairs of forceps: two regular, one fine curved (such as Roboz # RS-5137, Rockville, MD), and one ultra-fine (such as Roboz, # RS-4905), all sterilized.
13. Package of sterile 4×4 gauze.
14. 0.5 in. 25-G needles.
15. 1.5 in. 25-G needles.
16. 13-mm cellulose ester filters with grids (0.45 µm pore size) (Millipore, Bedford, MA).
17. 7–12 gel-foam sponges (UpJohn, Kalamazoo, MI).
18. Plasticware dish with lid.

2.2. DP Dulling Assay

1. Mice which accumulate pre-selection thymocytes: The DP dulling assay typically is used to study development of cells bearing a single rearranged TCR (*see* **Notes 3** and **4**). Although it has been used to study mice with a normal repertoire *(27)*, such an application is unusual. Most TCR transgenics are used when expressed on a background that supports positive selection of that TCR. However, this is inappropriate for the DP dulling assay, because in vivo encounter of positive selection ligands alters the thymocytes' ability to sense those same ligands in a DP dulling assay. Thus, the TCR transgenic needs to be backcrossed to a strain that does not support positive or negative selection of that receptor. In such animals, preselection thymocytes accumulate in vivo. Thymocytes from these mice should be harvested from animals between 3 and 8 wk of age.
2. RP10 media: RPMI (Gibco-BRL) containing 10% fetal calf serum, 5×10^{-5} *M* 2-mercatoethanol, 2 m*M* L-glutamine, 50 U/mL of penicillin, 50 mg/mL streptomycin, 50 mg/mL gentamycin sulfate, and 5 m*M* HEPES.
3. Humidified incubator delivering 5% CO_2.
4. Hank's balanced salt solution (HBSS) (Gibco-BRL).
5. 96-well-flat bottom tissue-culture-treated plates.

2.3. Flow Cytometry

1. CX-80 stainless steel mesh (Small Parts, Miami Lakes, FL).
2. 60×15 mm Petri dishes.
3. Several 3-cc syringe plungers (Becton Dickinson, Franklin Lakes, NJ).

4. Round-bottom 96-well polycarbonate plates.
5. Fluorescence-activated cell sorting (FACS) buffer: PBS + 1% FCS + 0.02% Na azide.
6. 12×75 mm polystyrene tubes (Falcon no. 2052, Lincoln Park, NJ).
7. Flow cytometer capable of reading three fluorescent parameters.
8. CellTracker Green 5-chloromethyl flourescence diacetate (CMFDA) (Molecular Probes, Eugene, OR).
9. 5 μm latex beads (Interfacial Dynamics, Portland, OR).
10. Abs: listed below are Abs typically used to assess mouse thymic development. All are available conjugated to various fluorochromes, from vendors such as Pharmingen (San Diego, CA):
    a. Anti-CD8α (53–6.7).
    b. Anti-CD4 PE (RM4–5).
    c. Anti-CD3ε (2C11).
    d. Anti-TCRβ (H57–597).
    e. Anti-TCRγδ (GL3).
    f. Antiheat stable antigen (HSA) (J11d).

3. Methods

3.1. Fetal Thymus OC

3.1.1. Preparation of the Culture Plate

1. Boil the Millipore filters $3\times$ in dH$_2$O, to remove contaminants, and to sterilize. The final time, cover with foil, and, when cool, transfer into the hood. Place the filters in media.
2. Place 2 mL RP10 media in each well of a 6-well plate. Add drug, peptide, Ab, and so on, at this point, if required. Remove a strip of gel foam sponge, and cut it into three squares, placing each square in one well of the plate. Using one straight and one curved sterile forceps, soak the gel foam and tease the air bubbles out. Place one Millipore filter on each gel foam square. Place the plate in a plasticware container that has a dH$_2$O soaked paper towel in the bottom. Prewarm the container in the incubator.

3.1.2. Excision of Fetal Thymus

1. The thymus of a fetal mouse can be excised easily after g.d. 14. The later the lobe is removed from the animal, the more efficient development occurs in OCs. However, lobes from animals older than g.d. 17 should not be used because the size of the thymic lobe at g.d. 17 is large enough to preclude the efficient exchange of gases and nutrients to the inside of the lobe, and significant death of thymocytes will occur. Some investigators *(27)* have modified the FTOC protocol to use chunks of either older fetal lobes or newborn lobes. In the latter case, the newborn thymus was cut into several pieces, prior to culture. However, this technique is inappropriate for any experiment in which the total number of thymocytes needs to be determined, since one can control neither the numbers of cells that

start out in each chunk nor the integrity of the chunk during the week of culture. We use g.d. 16 thymi. Most commonly, we use a strain has both a transgene and a targeted knock-out gene, and is on a C57BL/6 × 129 background. At g.d. 16, a small number of the cells in the thymus have begun to express CD4 and CD8. This is appropriate for these experiments, which examine positive and negative selection events. However, for experiments in which β selection is being tested, one should use g.d. 14 thymic lobes.

2. Remove the uterine sacs from each side of the euthanized pregnant female mouse and place them in a Petri dish with 10 mL sterile, cold PBS. From this point, everything should be performed in the horizontal laminar flow hood. Remove the individual embryos from the sac, using the fine-curved forceps and a fine scissors. Sever the placenta from each embryo, and remove the sac tissue. Place the exposed fetus in a fresh dish of cold PBS. Prepare all tissue like this prior to doing the thymectomy.

    To remove the thymus, place the fetus on top of a stack of four gauze pieces under the objective of the microscope. Using a 1.5 in. 25-G needle, pin it through the mouth, pulling the head back to do so. Use a 0.5 in. 25-G needle to pin it near the base of the tail. Using another 0.5 in. 25-G needle, tease away the tissue above the thymus. The thymi are most easily identified when kept in correct anatomic position (above the heart, with one lobe on each side of the esophagus), so be careful not to disrupt the position of the lobes while teasing the chest cavity tissue. Pluck the lobes out using the ultrafine forceps, and place each one on a square of the prepared filters. Be careful not to rupture the thymic capsule. If the fetal litter has mice of different genotypes, record the position of each lobe on the filter. In this case, it is helpful to have premarked the round filter with an ink pen, for orientation, (do this prior to boiling the filters). If the individuals of the litter must be genotyped, take a small sample of tail tissue at this point from which to prepare DNA. Return the 6-well plate to the incubator, in its plasticware box. Leave the cover loose to allow CO_2 diffusion, but to minimize humidity loss.

3. Replace the media in the cultures every other day. Carefully suction off the existing media with a sterile Pasteur pipet. Add 1.2 mL fresh warm media. Replacement more often may be required, depending on the stability in media of any drugs or peptides being used. Seven days of culture are sufficient for good thymic development from a g.d. 16 lobe.

3.1.3. Harvest and Analysis of Thymocytes by Flow Cytometry

After the indicated period of culture time, the thymocytes can be harvested from the lobes, using any technique that would apply to collecting adult thymocytes. Because fetal thymic lobes contain only ~2×10^5 cells after 1 wk of culture, attention should be paid to minimize cell loss. This lab uses sterile stainless steel screens. A 5-cm square piece of screen is cut and nicked at each corner. The sides are folded down, the piece flamed to sterilize, and this "box"

is placed, open side down, in a 60–15 mm Petri dish, with 0.75 mL HBSS. The fetal thymic lobe is plucked from the filter, using ultrafine forceps, and placed on the steel screen. In cases in which thymic development was severely impaired by presence of a drug or compound, the residual lobe is difficult to pluck, and must be scraped off. Under conditions of moderate to good development, the lobe is easy to pick up. Then, using the black tip of the plunger from a sterile 3-cc syringe, the thymic lobe(s) are pressed through the screen. The 0.75 mL HBSS containing the thymocytes is transferred to a tube, and the screen is rinsed with another 0.75 mL HBSS, which is added to the first tube. Using this method, we routinely harvest the cells from a single thymic lobe. If the experimental design tolerates pooling lobes prior to harvest, that can be done; but often single-lobe analysis is useful, because statistics can be applied to the data set.

Most applications of this technique involve analysis of T-cell development in the lobes by flow cytometry, using Abs to thymic differentiation antigens (typically CD4, CD8, and TCR). Standard protocols for doing flow cytometry are used. FTOC cells can also be sorted and subjected to polymerase chain reaction analysis of DNA *(28)* or reverse transcription-polymerase chain reaction analysis of RNA, or tested for a number of functional activities, including proliferation *(29)* and cytolysis *(30)*.

3.2. DP Dulling Assay

3.2.1. Isolation of Thymocytes and Presenting Cells

The DP dulling assay involves an 18-h incubation of preselection thymocytes with antigen presenting cells (APCs). Any APC can be used including macrophages, whole spleen cells, fibroblasts, and thymic stromal cells. The primary consideration is how to exclude the APCs from the flow cytometry analysis of thymocytes, after the incubation period. Even though APCs do not typically express CD4 and CD8, they must be excluded because their autofluorescence is often in the DP dull gate. We have used several different methods: adherent APCs, thymocytes from congenic marked mice, and labeling of APC with CellTracker dye. The latter is the most widely applicable technique, and is presented here. Monolayers or suspension cultures of APCs are labeled with 500 nM CellTracker Green CMFDA in RPMI without serum, for 15 min. The cells are washed twice and incubated at 37°C for an additional 30 min in RP10, then washed 2 X more. Thymocytes are harvested from young preselection TCRtg mice, according to the protocol used for harvesting fetal thymocytes (except that 5 mL HBSS is used, instead of 0.75 mL). The cells are washed 2 X and counted. The thymocytes are co-cultured with labeled APCs in 96-well flat-bottom tissue-culture-treated dishes. The ratio of 5×10^5 thy-

mocytes to 3×10^5 APCs/well is optimal for seeing DP dulling in response to endogenous ligands *(25)*. Incubate at 37°C for 18 h.

3.2.2. Analysis of Thymocytes by Flow Cytometry

1. Again, standard protocols for staining lymphoid cells can be applied. However, because the assay may often be used for large numbers of samples, it is convenient to do the antibody staining in 96-well plates. Therefore, after the co-culture incubation period, transfer the entire contents of each well to a 96 well round bottom assay plate. Centrifuge the plate for 5 min. Carefully flick off the media, using a single, smooth motion. Resuspend the cells by gently tapping the plate, and add 200 µL of FACS buffer to each well. Centrifuge again, this time resuspending the cell pellets in FACS buffer containing Abs to CD4 phycoerythrin (PE) and CD8 (Tricolor). After 30 min on ice, wash the samples 2 X and analyze using a flow cytometer. The APCs, which are labeled with the green dye, can be gated out of the analysis.
2. Typically, the percentage loss of cells from a DP bright gate is quantitated, after gating out dead cells and APCs. In cases of strong TCR stimuli, however, many of the DP dull cells die during the culture. Thus, in some circumstances, it may be preferable to analyze live and dead thymocytes. Note that, when this happens, the background level of DP dulling will be high, because 15–30% of thymocytes die without any stimuli, most of which are DP dull.
3. In the DP dulling assay, the use of phagocytic APCs may be problematic. Some of the thymocytes in 18-h cultures will die and be engulfed by phagocytic APCs. A direct comparison between phagocytic and nonphagocytic APC then requires additional analysis. The best method is a comparison of the absolute numbers of cells remaining, which can then be multiplied by the percentage of cells which are DP bright. This gives one a value of absolute numbers of cells in the DP bright gate. Instead of counting the cells remaining in each well, which would be very time consuming, one can use the flow cytometer to count the cells. To do this, resuspend each well in precisely 200 µL FACS buffer. Add 40,000 latex beads to each sample, prior to collection, and set the cytometer to collect 10,000 beads. Therefore, one quarter of each sample is collected, and the amount of specific death is calculated by comparing the number of thymocytes remaining in the DP bright gate in experimental vs control samples.

3.3. Summary

The in vitro methods of FTOC and DP dulling assay have been useful for defining the structural ligand requirements for positive selection of thymocytes. Both techniques, but especially the latter, have restrictions on their interpretation, which are outlined in the notes. The ability to study a cellular process in vitro has been, and will continue to be, of critical importance for immunologists. However, one should always be careful to confirm a hypothesis using in vivo techniques, whenever possible.

4. Notes

1. In OCs, the whole thymus, or a piece of it, is placed at the air–liquid interface, to promote the optimal exchange of nutrients and gases. The use of an intact fetal thymus, taken between GD 14 and 16, offers several advantages. First, the size of the lobe at this stage is ideal for promoting growth and differentiation without significant cell loss. Second, at this early stage, the organ has been seeded by thymic precursors in vivo. Thus, one can study the wave of development resulting from this initial population, essentially without further recruitment of cells (APC or precursors) from the blood or exodus of cells from the lobe. Third, intact FTOCs have been shown to retain a normal phenotype and three-dimensional architecture *(31)*. Thus, the precursors present give rise to both the γδ- and αβ-T-cells, in a fashion similar to what occurs in a fetal mouse in vivo *(7)*.

2. For the most part, development in the fetal thymus follows the same rules as in the adult thymus. A large number of TCR transgenics and MHC-altered or -deficient mice, which were used to discover the details of thymic selection in adult animals, show a similar phenotype in FTOCs. However, some differences between fetal and adult thymic development have been noted. For example, the fetal thymus produces unique populations of γδ-T-cells, compared to the adult thymus *(32)*. These cells seed specific peripheral sites, and are thought to serve a unique role in the immune system. FTOCs mimic the appropriate fetal pattern of γδ differentiation *(33,34)*. Fetal thymocytes also do not express the enzyme terminal deoxynucleotide transferase. As a consequence, those T-cells which develop early in the mouse have fewer nontemplated nucleotide additions to their TCR rearrangements *(35)*. This enzyme does seem to be expressed in fetal thymic tissues kept in OCs *(36)*, and, as a consequence, the receptors developing in FTOC have nontemplated nucleotide additions. Another interesting example of differences between fetal and adult mice has come about by assessment of mice deficient for bcl-2 *(37,38)*. Such animals have normal thymic development in the fetal period. However, within a few wk after birth, the thymus is profoundly lacking in its capacity to produce αβ-T-cells. This could point to a difference between fetal and adult development requirements, or it could be the result of an impairment of precursor homing in adult mice, and not have to do with T-cell development *per se*. The possibility of differences between fetal and adult development should be kept in mind when interpreting data from FTOC.

3. TCR transgenics have had an explosive impact on understanding of T-cell development *(39)*. Their main utility is that they allow the tracking of cells with a single receptor specificity. That same receptor can be studied in vitro and in vivo, allowing the direct comparison of structural/kinetic information about the receptor to its developmental properties in vivo. Nonetheless, there are caveats to their use. First, the expression of the TCR transgene is not identical to that of an endogenous TCR. Most TCR-αβ transgenic receptors are expressed earlier in development than endogenous TCRs *(40)*. This can lead to the inappropriate expression of αβ receptors in cells of the γδ lineage *(41)* and can give rise to

certain abnormalities in FTOCs *(28)*. TCR transgenes are also expressed at a slightly higher level on the surface of immature thymocytes, compared to endogenous receptors *(42)*, and this may lead to artifacts. For example, the commonly accepted property of thymocytes of undergoing deletion at the DP stage has been mostly determined using TCR transgenics. Thus, if and how often this happens in a normal mouse will await the production of mice expressing a rearranged TCR in its appropriate chromosomal location (a knock-in mouse). Second, the fact that nearly all of the thymocytes and T-cells in a TCR transgenic express a single receptor, although the attractive aspect of this technology, can also lead to problems. One example is the use of peptide injection to study thymic deletion in TCR transgenics. In such mice, not only can the peptide theoretically act directly on thymocytes when presented by thymic APCs, but it is also presented to nearly all of the peripheral T-cells of that same specificity. This overwhelming response in the periphery can produce cytokines that feed back and affect thymic development in a manner that would not be expected to occur in normal mice, in which the percentage of cells responding is somewhere around $1/10^6$ in the periphery. This has been shown to cause peptide-independent deletion of thymocytes *(43)*. Alternatively, in TCR transgenics, the fate of the majority of the thymocytes is dependent on the MHC molecules present in the animal. Thus in nonselecting TCR transgenic mice (particularly if the animals are deficient in the recombinase activating genes [RAG^o] as well), no thymocytes mature. Theoretically, the absence of this population could affect the homeostatic mechanisms that normally operate in the thymus *(44)* (and in the periphery *[45]*). Since homeostatic feedback mechanisms in the thymus are mostly unexplored to date, the potential to overlook these types of issues is great. Concerns about the monoclonal nature of the TCR transgenic repertoire can be overcome by use of adoptive transfer of T-cells *(46)* if studying peripheral T-cell biology, or of mixed bone marrow chimeras, if studying thymic development. However, to date, no technique to address this issue in FTOCs has been proposed.

4. The use of the DP dulling assay to draw conclusions about thymic selection is very restricted. Foremost should be the consideration that a positive selection ligand, even when present on thymic stromal cells, rarely leads to the full process of positive selection in in vitro co-cultures. Thus, this assay cannot be used to study the process of immature thymocyte survival or differentiation. We have used it exclusively to identify candidate peptides that could be ligands for positive selection, which was subsequently confirmed using FTOC *(24)*. It is possible that the assay could prove useful for studying the subtle differences in very early TCR signaling events between positive- and negative-selection ligands *(42)*. However, any conclusions would have to be confirmed using FTOC or in vivo approaches. Another important point to stress, regarding the use of the DP dulling assay, is that it does not distinguish between positive- and negative-selection ligands. Both types of ligands cause DP dulling activity. We have found a correlation between the strength of response in the DP

dulling assay and the thymic selection outcome *(24)*, but a quantitative threshold is difficult to assign, especially because the outcome in vivo is also dose-dependent *(29,47,48)*.

Acknowledgments

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19

Protocol for Diversion of a CD4⁺ Response to the T-Helper 2 Cell Pathway

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1. Introduction

Differentiated CD4⁺ T-cells produce a restricted set of cytokines, allowing their subdivision into two discrete populations: T-helper 1 (Th1), characterized by secretion of interleukin 2 (IL-2) and interferon γ (IFN-γ); and Th2, selectively producing IL-4, IL-5, and IL-10 *(1)*. Polarized subsets of antigen(Ag)-specific CD4⁺ Th1 and Th2 cells can be induced in vivo by Ag priming, and their development is primarily influenced by the cytokine milieu during the initial phase of the immune response. Among cytokines, decisive roles are played by IL-12 and IL-4, driving T-cell responses toward the Th1 or Th2 phenotype, respectively *(2,3)*. The polarization in Th1 and Th2 cells is also influenced by several other factors, including non-major histocompatibility complex (MHC) genetic polymorphism *(4–6; see* **Note 1***)*, ligand-T-cell receptor (TCR) interaction *(7,8)*, Ag dose *(9,10)*, and mode of Ag administration *(11*; *see* **Note 2***)*.

The functional significance of CD4⁺ T-cell subsets was first clearly demonstrated in vivo by studying the immune response to infectious agents *(12)*. In these situations, the ability of pathogens to preferentially stimulate either Th1 or Th2 responses depends on several factors, including non-MHC-linked genetic predisposition of the host, type of infecting organism, stage of infection, and microbial load. For example, high and low microbial loads have been shown to polarize the immune response towards either a Th2 or a Th1 phenotype *(6)*, which can be protective or deleterious, depending on the pathogen. As a possible explanation for the predominance of the Th2 response in overwhelming infections, it has been hypothesized that continuous release of low

amounts of protein Ags from pathogenic microorganisms may polarize the immune response toward a Th2 phenotype in susceptible mouse strains. Consistent with this hypothesis, continuous administration of any soluble protein Ag induces, in normal and β_2-microglobulin-deficient BALB/c mice, the selective development of Ag-specific Th2 cells, which depends on the production of endogenous IL-4 *(13)*.

We have used this model system to examine the role of route of administration, soluble protein Ag dose (*see* **Note 3**), and non-MHC-linked genetic polymorphism in the polarization towards a Th2 response *(11)*. Polarization of the T-cell response to a Th2 phenotype can be obtained when as low as 0.3–1 µg protein Ag are administered by miniosmotic pump over a 10 d period (30–100 ng/d). Although inhibition of Th1 cell development is observed in all mouse strains tested, strong Th2 development depends on a non MHC-linked genetic polymorphism and is only observed in mice on the BALB background and partially in DBA/2 mice *(11)*. This pattern of Th2 responses is predictive of disease outcome following *Leishmania major* infection *(14)*.

As described above, systemic administration of protein Ag to BALB/c mice results in the selective development of Ag-specific CD4+ Th2 cells *(13; see* **Note 4***)*. Having identified a protocol able to induce vigorous unipolar Ag-specific Th2 responses in BALB/c mice, three different modes of Ag administration have been compared, and it has been found that Th2 cells could be induced by protein administered continuously sc, or by single bolus ip, but not by iv injection (*see* **Notes 2** and **3**). This may reflect different pharmacokinetic effects and/or type of antigen-presenting cell (APC) involved. Conversely, Th1 responses were inhibited independently of the mode of soluble Ag administration at any Ag dose tested (*see* **Note 5**). This further indicates, as previously suggested *(13,15)*, that inhibition of Th1 responses and Th2 cell expansion may be unrelated (*see* **Note 6**). Unlinked regulation of Th1 and Th2 responses is clearly shown by the differential effect of non-MHC genetic polymorphism which selectively controls Th2, but not Th1, development following soluble Ag administration. The importance of the route and dose of soluble Ag administration, as well as the demonstration that strong Th2 responses could be obtained only in mice on the BALB background, explains the previous conflicting reports analyzing the effect of soluble Ag administration on Th2 cell development (*16–18; see* **Note 7**).

Th2 cell induction following soluble Ag administration allows three phenotypes to be distinguished: Th2-prone (BALB/c, BALB/b, BALB/k), Th2-averse (B10.D2, C3H, C57BL/6), and Th2-intermediate (DBA/2) *(11)*. These three phenotypes predict disease outcome following *L. major* infection *(14)*. However, not all protocols described that selectively induce Th2 cell responses in vivo are predictive of disease outcome following *L. major* infection (*see* **Note 8**).
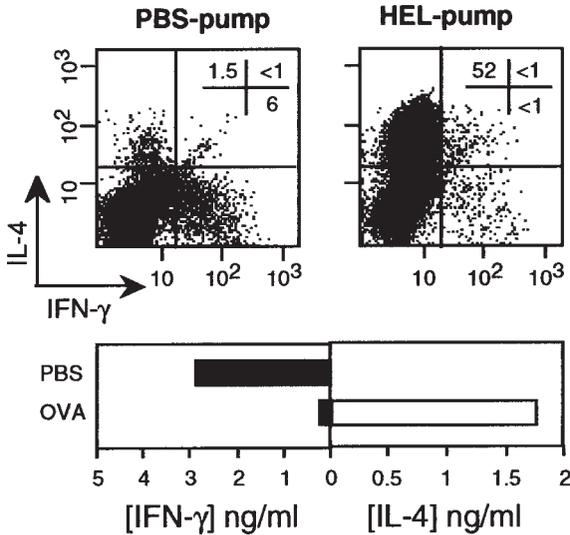
Fig. 1. Priming for Th2 cell development by soluble Ag administration to BALB/c mice. BALB/c mice (three mice/group) were implanted with mini-osmotic pumps containing PBS or 3 nmole HEL/mouse, 12 d before immunization with HEL in CFA (1 nmol/mouse). Immune LNCs were pooled and cultured at 6×10^5 cells/well, in the presence of 10 m*M* HEL. IFN-γ and IL-4 secretion were measured by ELISA in culture supernatants at 72 h. Immune LNCs from the same cultures were then washed and cultured for an additional 72 h in complete medium without Ag. After Ficoll separation, T-cell blasts were collected, activated with PMA and ionomycin for 4 h in the presence of BFA for the last 2 h, and fixed. Staining for IFN-γ and IL-4 synthesis by CD4+ T-cells were performed as described.

For instance, immunization of adult or neonate mice with protein in incomplete Freund's adjuvant (IFA) has been reported to selectively induce Th2 cell development, but no differences could be observed between BALB/c and the Th1-biased B10.PL mice *(19)*.

Results in **Fig. 1** exemplify a prototypic Th2-prone (BALB/c) phenotype. Continuous administration of hen egg-white lysozyme (HEL) to BALB/c mice, prior to immunization with HEL–complete Freund's adjuvant (CFA) resulted in decreased IFN-γ and strongly induced IL-4 secretion by HEL-restimulated LNC. HEL-stimulated lymph node cells (LNC) were then cultured for another 3 d, and analyzed at the single-cell level for the intracellular production of IFN-γ and IL-4. IL-4-producing CD4+ T-cells increased by about 35-fold and IFN-γ-producing cells decreased to nearly undetectable levels in BALB/c mice implanted with HEL-pump, compared to phosphate-buffered saline (PBS)-treated control mice.

2. Materials

1. Mini-osmotic pumps: Alzet 2001 (Alza, Palo Alto, CA).
2. Metal stitches (Michel BN 507, Aesculap, Germany).
3. PBS (without calcium, magnesium, and Na bicarbonate).
4. IFA (Difco, Detroit, MI).
5. CFA: containing H37a mycobacteria (Difco).
6. Antigens: HEL or ovalbumin (OVA) (Sigma, St. Louis, MO).
7. 96-well flat-bottom culture plates, low-evaporation lid (Costar, Cambridge, MA).
8. 96-well U-bottom culture plates (Costar).
9. HL-1 medium (BioWhittaker, Walkersville, MD), supplemented with 2 m*M* L-glutamine (Sigma) and 50 mg/mL gentamicin (Sigma).
10. Complete medium: RPMI-1640 (Sigma) supplemented with 2 m*M* L-glutamine, 50 µg gentamicin, 10% fetal calf serum (FCS) (Serono), and 50 m*M* β-mercaptoethanol.
11. 1 µg/mL Ionomycin (Sigma,) and 50 ng/mL phorbol–myristate acetate (PMA) (Sigma).
12. Brefeldin A (BFA) (Novartis, Basel, Switzerland).
13. 4% paraformaldehyde (Fluka).
14. Fluorescence-activated cell sorting (FACS) medium: PBS containing 5% FCS and 0.1% NaN_3.
15. Saponin buffer: PBS containing 5% FCS, 5% saponin (Sigma), 0.1% NaN_3.
16. Antibodies (Abs) for FACS staining: phycoerythrin (PE)-conjugated rat antimouse IL-4 (11B11-PE PharMingen); Fluorescein isothiocyanate (FITC)-conjugated rat antimouse IFN-γ (XMG1.2-FITC PharMingen); Cy-Chrome-labeled anti-CD4 (L3T4, PharMingen, San Diego, CA).
17. 96-well polyvinyl microtiter plates (Falcon).
18. Carbonate buffer: 1.91 g Na_2CO_3, 3.52 g $NaHCO_3$, 0.2 g NaN_3 dissolved in 1 l H_2O, pH 9.6.
19. Test solution for ELISA: PBS containing 5% FCS and 1g/L phenol (Sigma).
20. IL-4 ELISA: for capture anti-IL-4 monoclonal antibody (mAb) 11B11 (PharMingen), for detection anti-IL-4 biotinylated mAb BVD6-24G2 (PharMingen); alkaline phosphatase-conjugated streptavidin (Jackson Immunoresearch, Avondale, PA).
21. IFN-γ ELISA: for capture anti-IFN-γ mAb AN-18.17.24 *(20)*, for revelation peroxidase-conjugated anti-IFN-γ XMG1.2 mAb *(21)*.
22. Developing substrate for IL-4 ELISA: *p*-nitrophenylphosphate disodium (40 mg tablets, Sigma) in diethanolamine buffer, pH 9.6 (87 mL diethanolamine, Sigma; 0.2 g NaN_3; 0.1 g MgCl; adjust the pH to 9.6 by adding HCl. The volume is made up to 1 L by addition of deionized (DI) water).
23. Developing substrate for IFN-γ ELISA: 1 mL tetramethylbenzidine-H_2O_2 solution (TMB) 120 mg 3,3'–,5,5'-tetramethylbenzidine (Fluka), in 5 mL acetone (Fluka), and 0.5 mL 30% H_2O_2 solution (Merck), diluted in 20 mL substrate buffer (6.3 g citric acid monohydrate (Fluka), in about 800 mL DI water. Adjust the pH value to 4.1 by addition of 4 *N* KOH. Add DI water, to bring the volume up to 1 L.

3. Method

3.1 Soluble Ag Administration

Soluble protein is delivered by mini-osmotic pumps or single bolus ip injection.

3.1.1. Mini-Osmotic Pump Preparation and Implantation

1. Fill Alzet 2001 pumps with 50–100 µg soluble protein (e.g., OVA or HEL) diluted in PBS, before implantation. The mean fill-volume of the mini-osmotic pumps is approx 220 µL and the pumping rate around 1 µL/h for 10 d.
2. Anesthetize a mouse (BALB background) with ether.
3. Place the mouse on its abdomen, so that its back faces the operator.
4. Swab the back of the mouse with 70% EtOH.
5. Using scissors, make 5-mm-long incision in the skin of the lumbar region, perpendicular to the mouse axis.
6. Gently introduce forceps to make a 3-cm-long pocket, and insert the Alzet 2001 pump subcutaneously, with the pump regulator pointing cranially.
7. Clip the wound with metal surgical stitches, covering the surgical wound (2–3 metal stitches are sufficient).
8. 10–12 d after pump implantation, immunize mice into the hind footpads (*see* **Subheading 3.2.**).

3.1.2. Soluble Ag Administration by Single Bolus IP

1. Inject 50–100 µg protein, diluted in 200 µL PBS, intraperitoneally into mice, using an insulin syringe.
2. 10–12 d after protein administration, immunize mice in the hind footpads (*see* **Subheading 3.2.**).

3.2. Immunization

1. Prepare a glass syringe fitted with a needle into a rubber stopper, to prevent leaking of the protein solution.
2. Fill the syringe with the desired volume of protein solution (protein is diluted in PBS). Add an equal volume of IFA or CFA.
3. Emulsify for 1 min, using an homogenizer.
4. Remove the needle from the rubber stopper, and slowly insert the piston into the syringe.
5. Using a new needle, drop 50 µL emulsion into water. If prepared correctly, the drop of emulsion should not disperse.
6. Inject 50 µL emulsion/foot pad sc (1 nmol/mouse should be injected, thus, the final protein concentration in the emulsion should be 1 nmol/100 µL).

3.3. Preparation of Lymph Node Cell Suspension

1. Kill the mouse by cervical dislocation.
2. Dip the mouse in EtOH 70%.
3. Place the mouse on its abdomen, and pin it down on a board in a laminar flow cabinet.

4. Pull the skin at the heel to the thigh, so that the popliteal area is well exposed.
5. Remove the lymph nodes with sterile forceps.
6. Transfer them into a tube containing 2 mL RPMI.
7. Prepare a LNC suspension, using a loosely fitting potter homogenizer.
8. Fill up the tube to 10 mL and transfer the contents, filtering it through sterile gauze, into another tube. The gauze should remove cell debris and clumps.
9. Mix 10 µL cell suspension with 10 µL counting solution (trypan blue 1:10 in PBS) and fill the counting chamber with 10 µL. Count living cells (the dead cells are dark-blue stained). Calculate the total cell number and the final volume required (6×10^5 cells/well).
10. Centrifuge the tubes 10 min at 290g.
11. Remove the medium, and fill up to the final volume, using HL-1 medium.
12. LNC are cultured (6×10^5/well) in synthetic HL-1 medium with different Ag concentrations (three-fold dilutions, starting from 100 µg/mL OVA or HEL). In a typical assay Ag is distributed in 96-well flat-bottom plates (100 µL/well), before adding LNC (100 µL/well from a suspension of 6×10^6 LNC/mL).
13. Incubate the plates at 37°C in an humidified atmosphere of 5% CO_2 in air for 72 h.
14. For IFN-γ and IL-4 determination, collect culture supernatants at 72 h (*see* **Subheadings 3.4.1.** and **3.4.2.**).

3.4. Th Phenotype Characterization

3.4.1. IFN-γ Secretion Assessed by Two-Site Sandwich ELISA

1. Coat polyvinyl microtiter plates with 100 µL/well AN-18 anti-IFN-γ capture Ab (2.5 µg/mL), in carbonate buffer overnight at room temperature (RT).
2. Wash with DI water 6×, then gently tap the plates on absorbent paper.
3. Incubate samples (50 µL/well), diluted in test solution, together with 50 µL peroxidase-conjugated XMG1.2 (IFN-γ detection Ab) overnight at RT. A standard curve is generated in each plate, using serial twofold dilutions of rIFN-γ from 1000 to 15 pg/mL.
4. After overnight incubation, wash 5× with DI water containing 0.1% Tween 20. After washing, tap plates on absorbent paper.
5. Add 100 µL/well developing substrate for IFN-γ.
6. Stop the reaction at desired time, which is determined by the intensity of the color in the standard curve, by adding 50 µL 1 mM H_2SO_4.
7. Read at 450 nm with an automated microplate ELISA reader.

3.4.2. IL-4 Secretion Assessed by Two-Site Sandwich ELISA

1. Coat polyvinyl microtiter plates with 100 µL of anti-IL-4 Ab (1 µg/mL) in PBS overnight at 4°C.
2. Wash with PBS 6×, then gently tap the plates on adsorbent paper.
3. Incubate samples (100 µL/well), diluted in test solution overnight at 4°C. A standard curve is generated in each plate, using a serial dilution of rIL-4 from 1000 to 15 pg/mL.
4. After overnight incubation, wash 5× with PBS containing 0.1% Tween-20. After washing, tap plates on absorbent paper.

5. Add 100 µL anti-IL-4-biotinylated mAb (final dilution 0.1–0.25 µg/mL in test solution) for 1 h at 37°C.
6. Wash 5× with PBS containing 0.1% Tween-20. After washing, tap plates on absorbent paper.
7. Add 100 µL alkaline phosphatase-conjugated streptavidin (diluted 1:5000 in test solution) for 30 min at 37°C.
8. Wash 5× with PBS containing 0.1% Tween-20. After washing, tap plates on absorbent paper.
9. Add 100 µL/well of developing substrate for IL-4.
10. Stop the reaction at desired time, which is determined by the intensity of the color in the standard curve, by adding 50 µL 3 *N* NaOH.
11. Read at 405 nm with an automated microplate ELISA reader.

3.4.3. Ag-Induced Intracellular Synthesis of IFN-γ and IL-4

1. Culture LNC (6×10^5/well) in 96-well flat-bottom plate in HL-1 medium with 100 µg/mL protein.
2. Harvest cells after 72 h of culture. Wash, and restimulate them in 96-well U-bottom plates ($0.5–1 \times 10^6$ cells/well), in complete medium, with 1 µg/mL PMA and 50 ng/mL ionomycin, for 4 h at 37°C, with 10 µg/mL BFA added for the last 2 h. Alternatively, reculture LNC for an additional 72 h in complete medium, before PMA/ionomycin restimulation.
3. Spin down (200*g* for 5 min), remove the medium, and wash once with 200 µL PBS containing 10 µg/mL BFA.
4. After washing, resuspend LNC in 50 µL/well PBS containing 10 µg/mL BFA, before adding an equal volume of 4% paraformaldehyde for cell fixation.
5. After fixing for 20 min at RT, add 100 µL complete medium, and spin down cells (200*g* for 5 min). Discard the supernatant.
6. Store LNC in complete medium at 4°C for up to 2 d, or stain them immediately for intracytoplasmic cytokines, as described below (**steps 7–12**).
7. Wash LNC with 200 µL saponin medium. Spin down LNC (200*g* for 5 min at RT). Discard the supernatant.
8. Add 100 µL saponin medium for 10 min, to allow membrane opening.
9. Add an additional 100 µL saponin medium, and spin down cells. Discard the supernatant.
10. Incubate with FITC-conjugated rat antimouse IFN-γ and PE-conjugated rat antimouse IL-4 (mix the two Abs in saponin medium, to get a final concentration of 1 µg/mL for each Ab). Add 50 µL diluted Ab,s and incubate for 25 min at RT.
11. Wash by adding 200 µL/well saponin medium, and spin down cells at 200*g* for 5 min. Discard the supernatants (repeat twice).
12. Wash with 200 µL FACS medium twice.
13. Stain the cell membrane for CD4. Add 50 µL of Cy-Chrome-labeled anti-CD4 (diluted 0.4 µg/mL in FACS medium).
14. Wash 2× with 200 µL FACS medium.
15. Add 50 µL FACS medium, and transfer LNC to FACS tube containing 500 µL PBS.

4. Notes

1. Our analysis of Th subset plasticity in BALB/c mice shows that the development toward Th1 can be fully diverted to Th2 by pretreatment with soluble protein Ag. Note that this diversion is observed, among the mouse strains we tested, only in mice on BALB background (BALB/c, BALB/k, and BALB/b), and partially in DBA/2 mice *(11)*.

2. The diversion is induced by pretreatment with soluble protein Ag administered by mini-osmotic pump or ip, but not iv, injection.

3. Pretreatment with proteins, but not peptides, induces diversion to Th2 *(15)*.

4. The deviation to Th2 is abrogated by co-administration of IL-12, which redirects the response to Th1. Conversely, the inhibition of Th1 cell development, once established, cannot be broken by IL-12 administration *(22)*. However, primed Th2 cells are still partially sensitive to exogenous IL-12, consistent with residual IL-12 signaling in early Th2 cells *(23)*. Unlike early Th2, fully polarized mouse Th2 cells have been found in-vitro-resistant to phenotype reversal *(23,24)*, probably because of loss of IL-12 responsiveness in Th2 cells *(23)*. In contrast, human Th2 cells could be transiently reverted by IL-12 to Th0 or Th1 *(25,26)*. These different results appear to reflect a different regulation of interleukin 12 receptor (IL-12R) β_2-chain expression between mouse and human Th subsets *(27,28)*. In both mouse and human cells, the IL-12R β_2-subunit, the signal-transducing component of the IL-12R *(29)*, is expressed on Th1, but not Th2 clones, and it is induced during differentiation of naïve CD4$^+$ T-cells along the Th1, but not the Th2, pathway *(27,28)*. However, mouse Th2 cells lose expression of the IL-12R β_2-subunit, as the result of absence of IFN-γ in culture; human Th2 cells express low but functional levels of the IL-12R β_2-subunit. In addition, IFN-γ upregulates in vitro the IL-12R β_2-subunit in mouse cells *(27)*; this is enhanced by type I IFNs in human cells *(28)*.

5. Pretreatment of BALB/c mice with soluble protein Ag leads to selective inhibition of IL-12R β_2 transcripts in Ag-induced CD4$^+$ cells, thus providing an explanation for the inhibition of Th1 cell development. However, at present, the possibility cannot be excluded that other molecular changes, such as variations in the signal-transducing capacity of Stat4, may also play a role. The inhibition of IL-12R β_2 transcripts is also consistent with the priming of Th2 cells induced by soluble Ag. The IL-12R β_1 transcripts are not overtly affected by soluble Ag administration, in agreement with their similar expression in Th1 and Th2 cells *(27,28)*. Administration of IL-12 enhances expression of IL-12R β_1 and β_2 mRNA, implying that CD4$^+$ cells from mice injected with soluble protein express the receptor for IL-12. Thus, IL-12 administration leads to enhancement of its own receptor, and can thereby provide a co-stimulatory signal necessary for Th1 cell development.

6. The inhibition of the Th1 response by pretreatment with soluble Ag is not linked to the development of a strong Th2 response, because soluble Ag administration inhibits Th1 development in any mouse strain tested, irrespective of Th2 cell induction *(11,13,16,17)*. Therefore, inhibition of Th1 does not depend on Th2 cell induction, and soluble Ag presented in vivo to uncommitted precursor T-cells

switches off their potential to become Th1, while simultaneously driving, in the appropriate genetic background, Th2 cell development. This implies that the decreased IL-12R β_2 mRNA expression, following soluble Ag administration, accounts for inhibition of Th1 cell development, and is necessary but not sufficient for Th2 cell development. Soluble protein Ag appears to be selectively presented in vivo by B cells *(30,31)*, which may favor Th2 development *(32)*, because they fail to secrete IL-12 *(33)*. IL-12 administration could functionally convert B-cells into professional APC-like dendritic cells, which present Ag and secrete IL-12 in vitro *(34,35)* and in vivo *(33)*, thereby driving primarily Th1 responses.

7. The decision of naïve CD4^{+} T-cells to develop into Th1 or Th2 effector cells is not simply related to whether the priming conditions include high levels of inflammatory cytokines, such as IL-12. In the *L. major* model, it has been suggested that the T-cell response, which occurs in the absence of strong pathogen-driven signals, may reflect a strain-specific default development of Th cells *(5)*. Genetic differences have been demonstrated between T-cells of BALB/c vs B10.D2 mice in their Th phenotype acquisition under neutral conditions in vitro, although this model was not completely predictive of disease outcome, since the Th2 phenotype was more pronounced in DBA/2 than in BALB/c T-cells. More recently, it has been hypothesized that the susceptibility of BALB/c mice to *L. major* infection may reside in their inability to sustain IL-12-dependent development to the Th1 pathway, rather than an intrinsic capacity of BALB/c T-cells to differentiate toward the Th2 phenotype *(23)*. The locus controling this genetic effect has been mapped to a region of chromosome 11, containing a cluster of genes important for T-cell differentiation, including IL-4, IL-5, IL-3, and IFN regulatory factor-1 *(36)*.

8. Polarized Th1 populations, obtained from mice infected with *L. major (37)*, secrete Th2 cytokines, upon reculture in vitro with Ag and IL-4. This Th1 to Th2 switch may result from heterogeneity at the population level, because Th1 clones could not be converted to Th2 *(38)*, but Th2 cells could be obtained from undifferentiated precursors present in highly polarized Th1 cell populations *(39)*. Regarding the switch from Th2 to Th1 cells, ongoing Th2 responses to *L. major* could be converted to Th1 by combined treatment with Pentostam to reduce parasite load and IL-12 *(40)*.

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Quantitating Presentation of MHC Class I-Restricted Antigens

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1. Introduction

CD8^{+} cytotoxic T lymphocytes (T_{CD8+}) play major roles in the acute response to intracellular pathogens and in maintaining the latent state in chronic virus infections. There is great hope that T_{CD8+} responses can be manipulated to treat neoplasms. Major histocompatibility complex (MHC) class I molecules display endogenously processed antigenic (Ag) peptides to T_{CD8+}. This shapes the developing T_{CD8+} repertoire in the thymus and enables the delivery of immune effector molecules to antigen-presenting cells (APCs) in the periphery.

It has been widely observed that T_{CD8+} responses toward pathogens are usually focused on only a fraction of the potential determinants encoded by the pathogen. This hierarchical response pattern of the immune system is described as immunodominance *(1)*. The mechanisms that underlie this phenomenon are just now being dissected. The most critical factor is that the determinant bind to class I molecules above a threshold affinity (~500 n*M*). Next, the determinant must be efficiently liberated and delivered to class I molecules in the endoplasmic reticulum. Lastly, there must be T_{CD8+} cells capable of responding to the class I peptide complex.

To understand the molecular mechanisms of the T_{CD8+} response to pathogens, it is necessary to have a quantitative understanding of the contribution of each step in the generation of peptide–class I complexes. This chapter describes a number of methods for quantitating the number of class I–peptide complexes expressed on the cell surface by APCs in vitro. This information is vital, both for understanding the immunodominance hierarchy, and for producing vaccines with enhanced immunogenicity because Ag presentation is often a limiting factor in immunogenicity.

The most precise method for quantitating class I–peptide complexes is the use of monoclonal antibodies (mAbs) that mimic the specificity of the T-cell receptor (TCR), termed T-AG, for T-cell antigen mAbs. These have been produced from phage libraries *(2)*, or by standard hybridoma methodology *(3)*. Alternatively, multivalent TCRs obtained from specific T_{CD8+} have been used to similar effect (*4*; *see* **Note 1**). These reagents enable the rapid and precise cytofluorographic quantitation of complex number on the surface of individual APCs. However, there are several major drawbacks, the foremost being that they are difficult to produce, and that they are far less sensitive than the most sensitive T_{CD8+} (hundreds to thousands of complexes for T-AGs vs 1–10 complexes for such T_{CD8+}).

A more universal approach is the biochemical method pioneered by Rötzschke et al. *(5)*, in which peptides present in high performance liquid chromatography (HPLC) fractions from acid extracts, are titrated in functional assays of T_{CD8+} recognition, compared to a known amount of peptide *(6)*. This method requires large numbers of cells, is always arduous, and often expensive. Another liability is that intracellular complexes (which may not make it to the cell surface) are included with cell surface complexes in the pool of peptides recovered from cells. Additionally, estimations of complex number depend on the assumption that the naturally processed peptide is identical to the synthetic peptide used as a standard of comparison, and that the efficiency of recovery is similar to that of a synthetic peptide doped into control extracts. Despite these limitations, this is the only method that may be routinely used to quantitate peptide–class I complexes. In some instances it may be possible to measure peptides in unfractionated acid extracts. Because of the presence of large amounts of competing peptides (also present in HPLC fractions, but at lower concentrations relative to the target peptide), fractionation can only be avoided for abundant or high-affinity peptides. The presence of competing peptides is controlled (as in the case of HPLC-purified peptides) by the efficiency of recovery of the doped synthetic peptide.

It is possible to gain an idea of the relative amounts of peptide-class I complexes, using a simpler assay based on the recognition of APCs by T_{CD8+}. This approach is most accurate when comparing presentation of the same determinant expressed in different contexts, but it may also be used to compare different determinants, if the sensitivity of the T_{CD8+} is accounted for. The most commonly used assay is the lysis of target cells (usually assessed by release of ^{51}Cr) or the activation of T_{CD8+}. This assay is convenient, economical, and highly sensitive, but it is basically a quantal assay: cells expressing a threshold number of complexes (or 1000-fold more) are lysed, and those expressing subthreshold amounts are spared. It is capable, therefore, of distinguishing only between the grossest differences in Ag presentation. In this chapter is described

a modification of this assay that greatly enhances its ability to distinguish between differences in the efficiency of Ag presentation. Although this assay can be very useful, and may be sufficient to answer some questions, it is important to recognize that ultimately the true quantitation of peptide-class I complexes requires the use of T-AGs, or quantitation of peptides isolated from cells.

The method is based on the kinetic appearance of peptide-class I complexes. Transport of new complexes to the cell surface is blocked either by formaldehyde fixation of cells or by exposing cells to brefeldin A (BFA) *(7–8)*, a fungal metabolite that blocks the egress of proteins from the early secretory pathway. BFA is active in most cells, but there are a number of cell lines that are resistant to its action. Since loading of peptides onto class I molecules in most circumstances occurs in the endoplasmic reticulum, BFA can be used to block the generation of cell surface peptide-class I complexes, and thereby provides a measure of kinetics of complex generation. Because generation of Ag peptides is usually the limiting step in Ag presentation *(9)*, the kinetics of presentation reflect the kinetics of peptide generation.

1.1. The System

To exemplify how peptide quantitation is accomplished directly and indirectly, we use the mouse influenza virus system. The T_{CD8+} response in BALB/c mice to influenza A virus Puerto Rico/8/34 (PR8) involves at least five naturally presented determinants presented by H-2K^d *(10)*, plus other determinants restricted by MHC molecules other than K^d *(11)*. To simplify matters, we refer only to two determinants, both derived from nucleoprotein (NP), NP39–47 and NP147–155. In this system, NP147–155 is an immunodominant determinant with a moderate binding affinity for K^d ($K_d \sim 100$ nM) and NP39–47 is a subdominant determinant with a high binding affinity for K^d (K$^d \sim 10$ nM). Following infection with PR8, the number of T_{CD8+} responding to $NP_{147–155}$ is ~20-fold higher than the response to $NP_{39–47}$. To understand this phenomenon, it is necessary to quantitate the amounts of peptide–class I complexes produced by APCs.

2. Material

2.1. BFA Kinetics Assay

1. Virus (nominal Ag). Generation of nominal Ag stocks will vary with the system. PR8 is most conveniently grown in the allantoic cavity of 10-d embryonated chicken eggs. PR8 and embryonated chicken eggs can be purchased inexpensively (Spafas, Charles River Spafas Laboratory, North Franklin, CT). Virus should be present at more than 1000 hemagglutinating units (HAU/mL).
2. APCs: These again will vary with the nominal Ag system employed. For PR8, many cell lines may be used; none actually produce infectious virus, but all viral gene products are synthesized, and can potentially be presented to T_{CD8+}, given

the proper class I molecule. For what follows, we use P815 mastocytoma cells. Many subclones of P815 cells are highly resistant to PR8 infection, yet, given the right subclone and the proper infection conditions, the cells can produce great amounts of viral proteins. Importantly, PR8 is also able to infect a subpopulation of splenocytes, which serve as convenient APCs for in vitro stimulation of T_{CD8+}.

3. Cell culture, media, and buffers: All media were purchased from Life Technologies (Gaithersburg, MD) unless otherwise indicated. P815 cells (H-2^d) were maintained in Dulbecco's modified Eagle's (DME)-10: DMEM containing 10% fetal bovine serum (FBS), $5 \times 10^{-5}M$ β-mercaptoethanol, antibiotics, and 2 mM glutamine. T_{CD8} were stimulated and maintained in RPMI-1640 medium containing the above supplements (RP-10) and 10 U/mL recombinant human IL-2 (SAIC Frederick, Frederick, MD). ^{51}Cr-release assays were performed with Iscove's modified DMEM supplemented with 10% FBS (I-10). P815 cells are infected with PR8 in Autopow MEM (Life Technologies), supplemented with 0.1% bovine serum albumin (BSA) and 20 mM HEPES, and adjusted to pH 6.6 (AIM 6.6) and all washing used a balanced salt solution (BSS) supplemented with 0.1% BSA (w/v) (BSS–BSA).

4. Red blood cell lysis buffer: 0.83% ammonium chloride in 17 mM Tris-HCl, pH 7.2.

5. Peptides: All peptides were synthesized, HPLC-purified and analyzed by mass spectrometry by the Biologic Resource Branch, National Institute of Allergy and Infectious Diseases (Rockville, MD). Peptides were dissolved in DMSO at 1 mM as stock solution and stored at –20°C.

6. Paraformaldehyde solution (16%) (EM sciences, Ft.Washington, PA, Cat. no. 15710), kept frozen and thawed at 60°C to solubilize.

7. Sodium chromate (Na^{51}CrO$_4$, Amersham, Piscataway, NJ).

8. BFA can be obtained least expensively from Sigma. A 10 mg/mL stock solution in methanol is stored at –20°C.

2.2. Ag Peptide Extraction

1. Roller bottles (Corning, Corning, NY).

2. Flow A:0.1% Trifluoroacetic Acid (TFA) (Pierce, Rockford, IL) in H$_2$O; and Flow B**:** 0.1% TFA in acetonitrile (CH$_3$CN, Burdick & Jackson, Muskegon, MI).

3. Ten Broeck homogenizer (PGC Scientific, Frederick, MD) with 0.003" clearance.

4. Beckman (Palo Alto, CA) 15-mL ultracentrifuge tubes.

5. 3K cutoff filter (Macrosep, filtron 3K, Pall Filtron, Northborough, MA).

6. Peptide retention standard (Pierce), dissolved in 500 μL H$_2$O, 0.1% TFA, 5% CH$_3$CN.

7. C18 column (Delta pack, 5μm, 100Å, 3.9×300 mm, Waters, Milford, MA).

3. Methods
3.1. T_{CD8+} Immunization and Restimulation

1. 8–10 wk-old female BALB/c mice are injected i.p. with 1 mL of a 1/10 dilution of PR8 in allantoic fluid.

2. At least 2 wk after virus immunization, splenocytes are dissociated from the spleen by disrupting gently on a metal mesh in BSS–BSA, then passed through a 21-gauge needle 2–3× to achieve single cell suspension (splenocytes may also be prepared by teasing or using a Ten Broeck homogenizer). RBC are then lysed with lysis buffer (2 mL per spleen) at room temperature for 3–5 min.

3. Spleenocytes are stimulated in RP-10 with 10 U/mL interleukin 2 (IL-2) in 6-well plates, by incubating 3×10^7 splenocytes with 6×10^5 peptide-pulsed P815 cells exposed to 1 nM synthetic peptide for 1 h at 26°C, then washed. To prevent their replication in culture, P815 cells are irradiated with 200 Gy prior to peptide pulsing.

4. After a 4 d incubation at 37°C in air–CO$_2$ (95–5%), live cells are recovered by centrifugation in Ficoll-Hypaque gradient, and recultured at $5–10 \times 10^6$ cells per well in RP-10–IL2.

5. After 3 d more of culture, T_{CD8+} can be used in standard ^{51}Cr-release assays. At this point it is also possible to carry the T_{CD8+} as short term lines, by restimulating the cells weekly as described above (restimulation at d 4 is not necessary).

3.2. BFA Kinetics Assay and ^{51}Cr-Release Assay

1. Log-phase P815 cells are harvested, and washed twice in BSS–BSA (*see* **Note 2**).

2. Cells are suspended at 10^7 cells/mL in AIM in a 15-mL centrifuge tube, and an equal volume of PR8 containing allantoic fluid is added (*see* **Notes 3** and **4**).

3. Cells are incubated for 1 h at 37°C in a water bath, resuspended by flicking every 20 min, before adding 10 vol of prewarmed I-10.

4. Cells are then centrifuged at 500g for 5 min. Discard most of the supernatant, and resuspend the pellet in the minimum volume still remaining (~20 µL for each 10^6 cells), and label with ^{51}Cr (100 µCi for each 10^6 cells) at 37°C for 50 min.

5. Cells are then washed twice with 15 mL BSS–BSA, with all of the supernatant removed by aspiration. Cells are resupended at 10^5/mL and 100 µL aliquots are added to the wells of round-bottom 96-well plates that already contain four three-fold dilutions of T_{CD8+} in 100 µL I-10 (starting at an effector:target [E:T] cell ratio of 10–20:1). Assays are usually performed in triplicate.

6. Plates are incubated for 8 h at 37°C in air–CO$_2$ (91–9%). 20 µL BFA at 100 µg/mL in I-10 are added to sets of wells at the following times after incubation at 37°C to a final concentration of 10 µg/mL: 0, 0.5, 1, 2, 3, 5, 7 h (*see* **Note 5**).

7. In parallel, T_{CD8+} are tested for lysis of ^{51}Cr-labeled P815 cells incubated with 10-fold dilutions of synthetic peptide to establish the sensitivity of the T_{CD8+}. This is essential for comparing presentation of different determinants. Untreated ^{51}Cr-labeled target cells are included as a negative control. Also, labeled targets are incubated without effector cells, with and without detergent, to establish maximal and control release values (*see* **Note 6**).

8. 100 µL of supernatant is collected from each well, using a multichannel pipetor, and the radioactivity in supernatants is determined using a γ-counter. It is not necessary to change tips if tips are rinsed with water between rows. The water is delivered to the sample tube to maximize the accuracy of harvesting. The percent specific lysis is then determined as:

$$\% \text{ Specific release} = \frac{(\text{T}_{CD8+} \text{ induced release} - \text{Spontaneous release})}{(\text{Release by detergent} - \text{Spontaneous release})} \times 100$$

3.3. Kinetics Assay with Fixed APCs and Hybridoma Read-Out

1. Cell culture and infection are performed as described in **Subheading 3.2.**
2. After 1-h infection at 37°C in a water bath, infected cells are aliquoted in prewarmed I-10, and cultured at 37°C. At each sampling time-point, an aliquot of the infected cells is washed and fixed with 1% paraformaldehyde in PBS, at room temperature for 20 min (*see* **Note 7**), then washed 2× with cold BSS–BSA, and maintained on ice.
3. After the final time-point, cells are resuspended in I-10, and serial three-fold dilutions are distributed into round-bottom 96-well plates, starting with 3×10^5 APCs/well. Approximately 10^5 hybridoma cells in 100 μL I-10 are added to each well.
4. Cells are incubated at 37°C for 24 h in air–CO_2 (91–9%). If hybridoma cells expressing β-galactosidase (β-gal) under the control of the IL-2 promoter are used, the amount of β-gal produced, or the number of cells expressing β-gal is determined as described *(12)*. Alternatively, most hybridoma cells secrete IL-2 and/or interferon γ. These may be assayed by ELISA, intracellular staining, or, as described below, using a cytotoxic lymphoid line (CTLL) proliferation assay.

3.4. CTLL Proliferation Assay

1. CTLL cells are cultured in RP-10 with 50 U/mL IL-2 (*see* **Note 8**).
2. Log-phase CTLL cells are harvested and washed 3× in FBS-free RPMI-1640, and 5000 CTLL cells are then aliquoted in 150 μL RP-10 into assay wells already containing 50 μL supernatant collected from the hybridoma assay.
3. After 18 h of culture at 37°C, 1 μCi of ^{3}H-thymidine is added to each well.
4. 6 h later, the cells are harvested onto glass-fiber filter membranes, and the incorporated ^{3}H in the proliferating CTLL is then measured, using a liquid β-scintillation counter.

3.5. HPLC Extraction of Ag Peptides from Whole Cells

1. To obtain large numbers of cells, P815 cells are cultured in 1 L DME-10 in roller bottles. From each roller bottle, ~2×10^9 cells can be harvested.
2. Infect 10^9 P815 cells, as described in **Subheading 3.2.** for 6–8 h, while having enough cells to include the following controls (*see* **Note 9**): uninfected cells or cells infected with a control virus, and uninfected cells spiked with the defined determinant of interest at a concentration estimated to reflect the amount of natural peptide present (*see* **Note 10**).
3. Wash the cells twice with PBS, and either freeze them at –70°C for future processing, or proceed as follows.
4. Resuspend the cell pellet in 10 mL 0.1% TFA/H_2O, then add 5 mL 1% TFA/H_2O.
5. For the spike control group, add peptide at this point.

6. Disrupt the cells by 10 strokes, using a Ten Broeck homogenizer.
7. Collect homogenate into a 50 mL polypropylene centrifuge tube.
8. Sonicate in the tube for 30 s at 4°C, setting the pulse duty cycle at 50% on an Ultrsonics sonicator (Heat Systems, Farmingdale, NY).
9. Further mix for 30 min at 4°C on rotating wheel at ~2 rpm.
10. Centrifuge at 1000g for 20 min, and collect supernatants into 15-mL Beckman tubes.
11. Centrifuge the supernatant at 10,000g for 30 min.
12. Collect the supernatants, and filter through a 0.45 μM filter (Millipore, Bedford, MA).
13. Pass the filtrate through a 3K cutoff filter. Make certain the filter is tightly assembled, by pressing it in very hard and ascertaining the filter is properly aligned. Centrifuge at 4300g for 6–12 h, dilute the leftover material (usually about 1.5 mL, because of the dead space of the filter) with 5 mL of 0.1% TFA/H_2O, then further filter it through the same filter.
14. Collect all the filtrate, which can be safely stored at 4°C for at least a few days.
15. Concentrate the samples on a SpeedVac (Savant, Holbrook, NY) to a volume <500 µL.
16. Centrifuge the samples for 2 min on a microcentrifuge, then pass them through a 0.22 μM filter (Millipore).
17. Samples can be safely stored at 4°C until fractionated by HPLC.

3.6. HPLC Running Conditions

1. Please see related books and manufacturer's operating manuals for detailed HPLC operating conditions, including initiation and washing. The conditions used for the two NP determinants are suitable for many peptides, but can be adjusted to achieve greater purification (*see* **Note 11**).
2. All extracted materials are fractionated on a C18 column.
3. The starting condition is 5% B–95% A, for 5 min, then the gradient is increased linearly over the next 25min to 50% B–50% A. Switch to 95% B–5% A in the next 10 min, hold for 5 min and switch back to the initial 5%–B 95% A in the next 5 min. Hold for the next 10 min.
4. Collect fractions of 0.5 mL (or less); most peptides elute between 20 and 30 min (*see* **Note 12**).

3.7. T$_{CD8+}$ Detection of Ag Activities in HPLC Fractions

1. Culture RMA/S-K^d cells at 26°C overnight (12–14 h), to increase the number of peptide receptive molecules (do not incubate at 37°C until molecules have been stabilized by incubation with peptides) (*see* **Note 13**). Human β_2-microglobulin (Sigma, St. Louis, MO) may be added to the media at 5 µg/mL, to maximize the effect.
2. ^{51}Cr-label RMA/S-K^d cells at 26°C for 60 min, then wash twice with BSS–BSA.
3. Resuspend the cells in FBS-free DME medium (*see* **Note 14**) at 2×10^5/mL and aliquot 10^4 (50 µL) cells into a well containing 5 µL of the HPLC fraction to be tested.

4. Incubate for 30 min at 26°C.
5. Effector T_{CD8+} cells are then added into each well in 150 μL I-10. The specificity and activity of effector cells should be established prior to the assay, because of the precious nature of the HPLC fractions.
6. Centrifuge the plates at 220g to enhance E:T cell contact, and incubate for 4–5 h at 37°C.
7. Harvest supernatants, count, and calculate the released ^{51}Cr activities, as described in **Subheading 3.2.** (*see* **Note 15**).

3.8. T_{CD8}+ Quantitating Ag Activities in Fractions

1. Make three-fold serial dilutions of the fractions with Ag activity (*see* **Note 16**), and also make three-fold dilutions of the standard peptide, over a final concentration range of 10^{-10}–10^{-14} M (some class I allomorphs require 1000-fold higher peptide concentrations).
2. Pulse the ^{51}Cr-labeled RMA/S-K^d targets for 30 min in a 26°C CO$_2$ incubator, and add effector T_{CD8+} cells, as described above.

3.9. Calculating Copy Number of Ag Peptide/APC

The number of peptides recovered from APCs is derived from the following calculations:

$$X = (C_{std})(D)(V)$$

where X = unknown peptide concentration in an active fraction;
C_{std} = concentration of standard peptide that causes 50% of the specific release obtained with a saturating amount of peptide; D = dilution fold of the fraction that results in 50% of the specific release obtained with a saturating amount of peptide; and V = volume of the active fraction in mL.

Total molecule number in the fraction $(N) = (X)(6.02 \times 10^{23})/1000$

$$\text{Recover efficiency } (E) = \frac{(D_{spiked} \times C_{std} \times \text{Fraction volume})}{\text{Spiked peptide amount}} \times 100\%$$

$$\text{Copy no./APC} = \frac{(E)(N)}{\text{APC no.}}$$

4. Notes

1. The affinity of the TCR is too low for cytofluorographic detection, so TCRs must be used in multimeric form, to boost the functional avidity of the interaction. T-AGs are selected on the basis of having sufficient avidity for detection.
2. Everything should be well organized to achieve accurate timing of the kinetics study. Prewarmed solutions should be used to keep the cell growth continuous.
3. It is most reliable to use viral infections for Ag-delivery, although electroporation or liposomal delivery *(12)* of soluble Ags can also be used. Because electro-

poration is associated with cell damage that results in poor retention of ^{51}Cr, T_{CD8+} triggering should be measured by other means.

4. The infection times and MOI should be adjusted as needed. For example, the kinetics of presentation may be so rapid as to result in saturation of lysis even 2 h postinfection. In this event, decrease the infection time or MOI. It is also possible to ^{51}Cr-label the target during the infection to reduce the period prior to addition of effector cells (but the effect on infection should be examined). Time resolved europium assays can be used instead of ^{51}Cr to minimise assay times. Similarly, if the kinetics of presentation is slow, the period prior to ^{51}Cr labeling may be extended.

5. To enhance kinetic accuracy, BFA should be diluted in I-10 to make a 10X solution in a 96-well plate and distributed using a multichannel pipetor. The 10X solution can be diluted all at once for the entire assay. Do not exceed the 10X concentration; at higher concentrations, BFA precipitates will form.

6. The initial round of T_{CD8+} stimulation, especially if involving $T_{CD8}+$ specific for subdominant determinant(s), is generally more efficient in our hands if a 50:1 ratio of responder:stimulator (peptide-pulsed APCs) is used. Also, peptides should be pulsed at no more than 1000-fold the concentration needed to obtain 50% of the maximal lysis in CTL assays.

7. Use freshly diluted paraformaldehyde. Most class I-restricted determinants survive the fixation, and fixation can actually enhance the stability of weak-binding peptides.

8. Upon withdrawing IL-2, CTLL cells should undergo apoptosis within 18 h or so. This property is dependent on culturing cells with appropriate FBS. It may be necessary to screen different lots of FBS for their ability to support IL-2-dependent CTLL proliferation. It is strongly recommended to maintain CTLL in log-phase growth, to keep their strict IL-2-dependence.

9. As an additional control, it is important to run a MHC mismatched infection group to demonstrate the MHC-dependence of peptide recovery.

10. The amount of peptide used for spiking should be as close as possible to the amount of naturally processed peptide, to accurately assess the efficiency of recovery. The use of large amounts of synthetic peptides can result in contamination of the HPLC system, with disastrous consequences.

11. CAUTION: Care should be exercised to avoid peptide contamination in sample loading. It is strongly recommended to wash the sample loading path thoroughly, first with CH_3CN, then by 0.1% TFA/H_2O. Denatured and trypsinized hen egg albumin could be used after each sample to remove residual sample-derived activity; and retention standards should be run just before each sample. Separate sampling syringes are also strongly recommended.

12. Fraction size should be minimized, to decrease competition for binding to class I molecules, and to increase resolution.

13. Low temperature incubation enhances the sensitivity of RMA/S target cells by 10-fold.

14. FBS contains proteases that destroy Ag peptides; hence, pulsing should be performed in FBS-free media.

15. If peptide detection is at threshold values even after using low temperature-induced RMA/S targets, it may be possible to enhance sensitivity using freshly

stimulated T_{CD8+} instead of long-term lines or clones. In our hands, combining fractions, followed by concentration, has never helped to enhance the detection.

16. To make the standard dose-response curves, dilution of the fractions from both peptide-spiked control and samples to be tested must be performed in the same assay to ensure accuracy.

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21

Identification of CD8+ T-Cell-Stimulating Antigen Genes in cDNA Libraries

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1. Introduction

Molecular identification of the major histocompatibility complex (MHC)-bound antigenic (Ag) peptides recognized by CD8+ T-cells has remained elusive until recently, because of the lack of a simple and efficient method to isolate these Ags *(1)*. Each MHC-I molecule presents thousands of distinct peptides, only one of which is usually recognized by a given T-cell *(2,3)*. This enormous complexity of the peptide pool presents a formidable challenge to the identification of the Ag peptide in complex Ags such as transplanted tissues and tumors. Here is described the expression-cloning method developed in this laboratory for the identification of the donor Ag gene that has been applied to the identification of histocompatibility Ags. This and similar methods have also been successful in the identification of CD8+ T-cell Ags in tumors, allogeneic cells, and in autoimmunity *(4–7)*.

The Ag peptides presented by the MHC-I molecules on the cell surface are derived from endogenously synthesized proteins. Thus, in principle, the Ag peptides can be identified by one of three distinct approaches. First, the Ag peptide can be defined by biochemical purification and mass spectrometry among the mixture of peptides eluted from the relevant MHC molecule *(8,9)*. Second, if prior knowledge of the chromosomal location of the Ag gene is available, it may lead to the identification of the Ag gene by positional cloning *(10–13)*. Finally, the Ag-encoding gene can be identified by expression cloning in a cDNA library *(6,14,15)*. The expression-cloning strategy, like the biochemical purification strategy, does not require any prior knowledge of the Ag gene or the Ag peptide. However, the expression cloning strategy has proved to be simpler, and has led to the identification of far more Ag genes, because it does not require sophisticated instrumentation or training.

Prior to embarking on an expression-cloning approach to identify the CD8+ T-cell Ag gene of interest, a specific and sensitive T-cell clone should be in hand for use as a probe with which to screen the library. In addition, the MHC molecule required for presenting the Ag peptide by the antigen presenting cell (APC) must be known. This is readily accomplished by determining which anti-MHC-specific antibody will inhibit the Ag-specific response of the T-cell clone. Once these requirements are satisfied, the expression-cloning strategy requires that small pools of a cDNA library from the Ag bearing cells be transfected into recipient cells lacking the Ag. The relevant MHC molecule may be constitutively expressed in the recipient APC or, if absent, its cDNA can be co-transfected, together with the cDNA pools. Because the MHC-I Ag processing pathway is conserved, expression of the transfected cDNAs in the recipient APC allows the generation of the appropriate peptide–MHC complexes that can be detected by the response of the CD8$^+$ T-cell clone. The Ag gene is then isolated from the cDNA pool by repeating the screen with individual cDNA clones. We have used the *lacZ-* inducible, CD8$^+$ T-cell hybridomas described in Chapter 22 of this volume, as sensitive probes for screening cDNA libraries and have isolated several CD8$^+$ T-cell-stimulating Ag genes. The following subheadings describe the three essential elements of the expression-cloning strategy: first, the identification of an appropriate recipient cell for Ag presentation: second, the construction of the cDNA library in pools; and, third, the screening and isolation of the CD8$^+$ T-cell stimulating Ag gene from the cDNA library.

2. Materials

1. Superscript Choice System II cDNA Library Kit (Gibco-BRL, Rockville, MD).
2. RNeasy mRNA Isolation Kit (Qiagen, Valencia, CA).
3. pcDNA1 vector and MC1061/P3 ultracompetent bacteria (Invitrogen, Carlsbad, CA).
4. RPMI 1640 medium supplemented with 2 m*M* glutamine, 1 m*M* pyruvate, 50 µ*M* 2-mercaptoethanol, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% fetal bovine serum (FBS) (Hyclone, Logan, UT).
5. U-bottom and flat-bottom 96-well microtiter plates.
6. 2X transfection medium (2X TF) consists of RPMI 1640 medium, supplemented, as described in **Subheading 2.4.** above, with 10% Nuserum (Collaborative Bio-medical, Bedford, MA) and diethylaminoethyl (DEAE) dextran (0.2 mg/mL), chloroquine (0.2 m*M*), and the restricting MHC-I cDNA (20 ng/mL) and B7-2 cDNA (10 ng/mL). DEAE dextran stocks should be made at a concentration of 10 mg/mL, and chloroquine stocks at 10 m*M* in dH$_2$O, filtered through a 0.2-µm filter and stored in aliquots at –20°C.
7. dimethyl sulfoxide (DMSO), molecular biology grade.
8. 1X phosphate-buffered saline (PBS), is diluted 1:20 from a 20X stock. 20X PBS is made by dissolving 10.25 g NaH$_2$PO$_4$•H$_2$O and 90 g Na$_2$HPO$_4$•7H$_2$O in about 2 L ddH$_2$O. Adjust to the required pH (7.4). Add 350.7 g NaCl, and make up to a total volume of 4L.

9. Chlorophenol red β-D-galactopyranoside (CPRG) reagent: PBS with 9 m*M* MgCl$_2$, 0.125 % NP-40 and 0.15 m*M* CPRG, (Boehringer Mannheim).
10. Stop buffer: 300 m*M* glycine and 15 m*M* Na$_2$ EDTA, pH 12.0.
11. Microplate reader at a wavelength of 595 nm and a reference wavelength at 655 nm (Bio-Rad, Richmond, CA).
12. Luria-Bertani (LB) broth : 10 g Bacto-Tryptone, 5 g Bacto-yeast, and 10 g NaCl are added to 1 L dH$_2$O, and the pH is adjusted to 7.4. The broth should be auto-claved and cooled below 55°C, before adding antibiotics. For LB agar, add 15 g bacto-agar to broth before autoclaving.
13. Ampicillin is made as a 100 mg/mL stock in dH$_2$O, and sterilized by filtration through a 0.2-μm filter, and tetracycline is made as a 25 mg/mL stock in 95% EtOH. Both are stored at –20°C.
14. Solution I: 50 m*M* glucose (dextrose), 25 m*M* Tris-HCl, pH 8.0, and 10 m*M* EDTA (GTE). Autoclave and store at 25°C.
15. Solution II: 0.2 N NaOH, 1% (w/v) sodium dodecyl sulfate (SDS). It is not nec-essary to autoclave, store at 25°C.
16. Solution III: 5 *M* potassium acetate solution, pH 4.8, prepared by adding 60 mL 5 *M* potassium acetate (K⁺OAc⁻), 11.5 mL glacial acetic acid, and water to 100 mL. Autoclave and store at 25°C.
17. Isopropanol.
18. 3M Scotch pad tape pads, UPC part no. 021200-61618.
19. 1X Tris-EDTA (TE): 10 m*M* Tris-HCl, pH 8.0 and 1 m*M* EDTA. Autoclave and store at 25°C.

3. Method
3.1. Choice of Recipient Cells for Screening the cDNA Library

The most important criteria to consider when choosing a recipient cell are whether the recipient APC will stimulate the T-cell probe without transfection of the Ag cDNA and result in an unacceptably high background; whether the recipient cell is an efficient APC that expresses the costimulatory and adhesion molecules; and whether the recipient APC is readily transfectable. Several cell lines used for transient expression of cDNAs are available from American Type Culture Collection (ATCC). For Ag presentation, we prefer LMtk⁻ fibroblasts *(14)*, because they are readily transfectable and are excellent APCs. We have also had success with COS7 cells in expression screens, and, although they express higher levels of the transfected cDNA, they are not as efficient at Ag presentation as are LMtk⁻ cells in stimulating murine T-cells (unpublished observations). To determine the best choice for the recipient APC, test (by the method described below) either LMtk⁻ or any available cell line that satisfies the above mentioned criteria.

1. Test LMtk⁻, COS7, or another cell line for its ability to stimulate the T-hybrid in the absence of Ag-encoding cDNA by titrating cDNA for the restricting MHC-I mol-

ecule or pcDNA1 vector DNA in a flat-bottom 96-well plate. Dilute MHC-I cDNA into 2X TF to 2 µg/mL.

2. Set up a DNA titration in triplicate, by pipeting 50 µL/well 2X TF medium with a multichannel pipetor, leaving one well empty, to start the titration as a no-DNA negative control.

3. Add 75 µL cDNA in 2X TF (**step 1**) to the empty well, and titrate cDNA (1:3) by serial dilution (25 µL per step).

4. Add 50 µL/well recipient APC at a concentration of 6×10^5 cells/mL (3×10^4 cells/well), spin plates for 2 min at 850*g*. Incubate at 37°C for 90 min.

5. Shock transfected cells by flicking off the supernatant, and adding 100 µL/well 10% DMSO in PBS. Spin plates as above, flick off supernatant, and add 100 µL/well medium with 10% FBS. Culture transfected APCs in 96-well plates for 48 h at 37°C.

6. Co-culture transfected APCs with the T-hybrid for 16–20 h at 37°C, after adding 100 µL/well of T hybrid at a concentration of 1×10^6 cells/mL (1×10^5 cells/well).

7. Spin the 96-well plates at 850*g* for 2 min, and flick off the supernatant. Add 100 µL/ well PBS, repeat spin as before, and flick off supernatant. Add 100 µL/well CPRG in Z buffer, to assay for T-cell activation. Incubate plates at 37°C for up to 4 h, and measure conversion of CPRG to chlorophenol red on a microplate reader at 595 nm, with a reference wavelength of 655 nm. To inactivate the β-galactosidase and stop the generation of the CPRG product, add 50 µL/well of the Stop buffer.

8. Compare the response by the T hybrid to the MHC-I titration vs the vector titration and the Ag-bearing cells as a positive control, to determine whether the presence of endogenous genes expressed in the recipient cells will affect the cDNA screening assay. Choose cells with the lowest possible background (*see* **Notes 1** and **2**).

3.2. Ag Expressing Donor Cells and cDNA Library Construction

Reliable commercial cDNA library kits, which contain all reagents necessary for library construction and detailed instructions, are available. We routinely use the Superscript System II (Gibco-BRL) for constructing the cDNA libraries, according to manufacturer's instructions, and find the mammalian expression vector pcDNA I (Invitrogen) is optimal for library construction, in part because of the high levels of expression of inserted genes.

1. Test available target cell lines and cell suspensions from tissues for their ability to stimulate the Ag-specific T-cell hybrid (*see* **Note 3**).

2. Isolate poly(A)+ mRNA from cells that are most efficient at stimulating the T-hybrid.

3. Construct a cDNA library in the mammalian expression vector pcDNA1, according to the manufacturer's instructions. The cDNAs can be generated using oligo-dT primers with a unique restriction site to allow unidirectional cloning into the BstXI or the BstXI/NotI sites of the pcDNA I vector. Carry out test transformations to determine the titer of the library, i.e., how many bacterial colonies are obtained/µL ligation.

3.3. Generation of cDNA Pools in 96-Well Plates

After obtaining the cDNA ligated to the vector, it is necessary to transform bacteria, to plate them in small pools of ~50–100 colonies/well, and to prepare the plasmid DNA in 96-well plates. We usually prepare a batch of cDNA library pools in 20 96-well plates, with each plate consisting of a total of ~$5–10 \times 10^3$ colonies. More batches can be prepared and screened if the Ag gene is not found in the first screen. The bacterial pools are grown in the 96-well plates, and the plasmid DNA is prepared in the 96-well plates, to facilitate the subsequent screening protocol.

1. Transform the equivalent of 1×10^5 cfu of the primary cDNA library into electrocompetent MC1061/P3 cells, and allow cells to recover in 3 mL LB broth, while shaking at 37°C without any antibiotics, for 20 min (*see* **Note 4**).
2. Dilute transformation into 400 mL LB broth with 50 μg/mL ampicillin and 25 μg/mL tetracycline.
3. Plate 200 μL/well of the transformation into 20 U-bottom 96-well plates. Culture bacteria on a rotary shaker at 190 rpm and 37°C for 48 h.
4. Plate 1 mL of the transformation on a 150-mm LB agar plate with antibiotics, and culture overnight (16 h) at 37°C. Count CFU on LB agar plate to confirm CFU/well in 96-well plates (*see* **Note 5**).
5. After 48 h, pellet bacteria by spinning the 96-well plates at 480*g* for 10 min. Flick off supernatant, and loosen pellets by vortexing plates for 10–20 s (*see* **Note 6**).
6. Add 50 μL solution I (GTE) to each well. Gently agitate plate by hand, taking care not to spill from one well to another. This step increases the yield, but is not crucial. Alternatively, allow plates to sit for 3 min.
7. Add 100 μL solution II (NaOH–SDS) to each well. Wait 3–5 min for cells to completely lyse. The cell suspension should turn from cloudy to clear, and will be viscous.
8. Add 50 μL solution III potassium acetate (KOAc) to each well. Cover each plate with a sheet of 3M adhesive paper, and ensure that a seal is made around each well. Shake plates vigorously by hand, or vortex to precipitate the *Escheria coli* genomic DNA and protein (*see* **Note 7**). Spin plates at 4°C for 15 min at 1320*g,* to pellet the precipitate.
9. Insert pipet tips, using a multichannel pipetor above the edge of the U in the bottom of the well, take care not to disturb the flocculent white pellet at the bottom. Draw up 125 μL supernatant, and transfer to a clean, labeled, U-bottom 96-well plate. The same set of 12 pipet tips can be used for an entire 96-well plate. Change tips for each new plate. It is useful, when processing numerous plates, to be consistent when removing the supernatant, by starting in row A and moving toward row H. Do this for every plate. Do not try to recover all of the fluid in the wells. Quality is more important than quantity. Extra care should be taken to avoid transferring any of the white pellet.
10. Add 125 μL isopropanol to each well of the new plate. Cover the plates with another sheet of 3M adhesive paper, and ensure that each well has a seal around it. Shake vigorously to mix, and precipitate the plasmid DNA and RNA. Chill at

–20°C for at least 2 h (preferably overnight), to increase the efficiency of the precipitation.

11. Spin plates for 20 min at 1320*g* at 4°C, flick off the supernatant, dry pellets by inverting plates and blotting onto bench paper, then leave them inverted in a laminar flow hood for 10 min. Resuspend pellets in 100 μL 1X TE buffer. Store plates at 4°C for short-term, and at –20°C for long-term storage.

3.4. Isolation of CD8+ T-Cell-Stimulating Ag Gene in cDNA Library

The cDNAs in each well of the 96-well plate are transfected into appropriate recipient APCs selected above. After allowing 48 h for expression of the protein products, and Ag processing, the presence of the peptide–MHC-I complexes is detected using the *lacZ* response induced in the T-cell hybrid.

1. Label 96-well flat-bottom plates to correspond to each cDNA library plate to be screened. Make up 5 mL 2X TF medium for each plate to be screened, and include the restricting MHC-I-encoding cDNA to a concentration of 20 ng/mL, if required (*see* **Note 8**).
2. Transfer 50 μL/well 2X TF medium to each 96-well plate. Add 3 μL cDNA to each well, using a multichannel pipet. Use one set of tips for each plate to be screened, and transfer cDNA from row A to row H (*see* **Note 9**).
3. Add recipient APC in a final volume of 50 μL/well (3–5 × 10^4 cells/well). Spin plates at 850*g* for 2 min to pellet cells, and culture at 37°C for 90 min.
4. Flick off the supernatant, and shock cells by adding 100 μL/well 10% DMSO in PBS. Spin plates for 2 min at 850*g*, flick off supernatant, and add to the transfected APCs 100 μL/well RPMI medium with 10% FBS. Culture plates at 37°C for 48 h.
5. Co-culture transfected APCs with the T-cells for 16–20 h at 37°C, after adding 100 μL/well T hybridoma, at a concentration of 1 × 10^6 cells/mL (1 × 10^5 cells/well).
6. Repeat **step 7** in **Subheading 3.1.** above, to measure T-cell activation (*see* **Note 10**).
7. Compare T-cell responses to cDNA pools to the background response with vector only transfection.
8. After a positive cDNA pool has been identified in the expression screen with the T hybrid, immediately confirm the validity of the positive by testing for the requirement of the restricting MHC-I molecule in obtaining the T-cell response. Titrate 5 μL positive cDNA pool in a transfection, in which 2X TF medium contains either the restricting MHC-I cDNA or an irrelevant MHC-I cDNA. Repeat **steps 3–7** of **Subheading 3.4.** (*see* **Note 2**).

3.5. Cloning the T-Cell Ag cDNA

1. From the corresponding well containing the putative positive cDNA pool in the bacterial DNA plate, dilute 1 μL DNA into 9 μL of 1X TE. Transform 1 μL 1:10 dilution into the electrocompetent MC1061/P3 bacteria, and add 2 mL LB broth without any antibiotics.
2. Shake the transformation at 37°C for 20 min (*see* **Note 4**). Plate 50, 150, and 350 μL of the transformation onto three LB agar plates with antibiotics, and culture at 37°C for 16–20 h.

3. Pick 300 individual colonies (about $5 \times$ the estimated pool size) that are well seperated, and culture the bacteria at 37°C in three 96-well U-bottom plates (200 μL/well LB broth with antibiotics), for a cDNA pool size of one CFU/well.

4. Isolate plasmid DNA from the individual bacterial colonies by repeating **steps 5–11** of **Subheading 3.3.**

5. Rescreen the individual cDNA clones by transfecting the clonal cDNAs into recipient APCs, as described in **steps 1–7** of **Subheading 3.4.**

6. Positives in this secondary screen should be present at the same complexity as the original cDNA pool. Confirm positive cDNA clones by co-transfection with the restricting MHC-I molecule and an irrelevant MHC-I molecule as a negative control. Repeat **step 8, Subheading 3.4.**

7. After confirming the Ag reactivity of the cDNA clone, it can then be sequenced. The sequence can be compared with those in the databases, to determine whether the cDNA represents a known or a new gene. The Ag peptide can also be identified by either testing candidate synthetic peptides predicted by computer algorithms or by testing deletion constructs, to narrow down the Ag activity.

4. Notes

1. The recipient APC should not express the Ag, however, screening for T-cell Ags has been successful, even when the recipient APC expressed low levels of the Ag. In this situation, as soon as possible, confirm the putative positive pools by titrating the cDNA with either the restricting MHC-I cDNA or an irrelevant MHC-I cDNA. This avoids the tedium of chasing false positives.

2. To identify a putative positive pool, compare the response of the T-cell, as measured by the absorbance at 595 nm, with the absorbance for the background in the vector-only control and other cDNA pools tested in the same experiment. We have found genuine Ag positive cDNA pools that were as low as 1.2-fold above background. This emphasizes the importance of selecting recipient APCs with as low a background as possible.

3. T-cell response is not only a measure of the presence of the Ag peptide, but also the ability of the cell expressing the Ag to act as an APC. Therefore, if the cell expresses co-stimulatory or adhesion molecules, it will stimulate the T-cell more efficiently. Strong T-cell response to a particular APC does not necessarily reflect the overall abundance of the Ag-encoding mRNA in that cell. Consider using mRNA from a different Ag-expressing cell if the initial screens fail to yield the Ag cDNA.

4. When culturing the bacteria transformed with the cDNA library in liquid medium, it is important to remember that bacteria with smaller cDNAs have a growth advantage, compared to those with larger cDNAs. To avoid any possible discrepancies in the representation of small vs large cDNAs in the library, culture the bacteria for 20 min to recover after transformation. Longer culture may allow the bacteria to divide, before plating the cDNA into pools, and will increase the complexity of the cDNA library.

5. The measure of complexity of the cDNA library in 96-well plate cultures is determined by plating the transformation onto an agar plate. The accuracy of this estimate can be off by up to twofold. Approximately 20 random bacterial colonies should be picked, and minipreps analyzed to confirm the presence of inserts and to determine average insert size.

6. Care should be taken to avoid spilling any residual supernatant from well to well during vortexing. If too much supernatant remains, lower-speed settings on the vortex may be required.

7. It is important to completely precipitate all proteinaceous material, particularly any DNase activity that could degrade the plasmid during storage.

8. MHC-I-encoding cDNA is required when the recipient APC does not express the restricting class I molecule. No advantage has been found to making stable transfectants of the recipient cells expressing the restricting MHC-I molecule.

9. To facilitate screening, we recommend using the same set of tips per plate. Consistency in transferring the cDNA to the plates from row A to row H simplifies analysis of the screen, because a strong positive may be seen titering down in adjacent wells.

10. Positive wells may be scored more easily as above-background activity, if the plates are allowed to develop over a longer period of time. This is done by developing them for 20 min at 37°C, followed by 12–16 h at 23°C.

Acknowledgments

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Generation of Antigen-Specific, *LacZ*-Inducible T-Cell Hybrids

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1. Introduction

The recognition of a peptide-bound major histocompatibility complex (MHC) molecule on the surface of antigen-presenting cells (APCs) is the central event in T-cell activation. The enormous complexity of the peptide pool displayed by MHC molecules makes it virtually impossible to detect physically the presence of a given peptide–MHC complex on the APC surface. Yet, T-cells have evolved to carry out precisely this function with an extraordinary efficiency, i.e., the detection of rare peptide–MHC complexes among the multitude of other peptides presented by the MHC molecules on the APC surface. Therefore, all practical assays for detecting the expression of a particular peptide–MHC complex depend on the measurement of some aspect of the T-cell response that is specifically initiated when the T-cell receptor (TCR) and its CD4 or CD8 coreceptor is engaged by the appropriate peptide–MHC complex.

Conventional assays measure a variety of T-cell functions, such as lytic activity of CD8+ T-cells or cytokine expression by CD4+ T-cells. Depending on the frequency of antigen(Ag)-specific T-cells in the sample, such assays can be carried out ex vivo, but, more typically, short- or long-term T-cell lines, or clones derived from these bulk lines, are used. Maintenance and assay of these T-cell lines and clones requires periodic restimulation with APCs and cytokines, making the procedures tedious and expensive.

Here are described methods for the generation of β-galactosidase (*lacZ*)-inducible murine CD4+ and CD8+ T-cell hybrids. In contrast to normal T-cells, these hybrids offer several advantages. The hybrids are relatively easy to generate and maintain in culture, and the *lacZ* assay provides a sensitive, rapid, inexpensive,

nonradioactive method for measuring T-cell activation. With chromogenic substrates, the *lacZ* assay also provides almost instant results. The *lacZ*-inducible hybrids have been successfully used as sensitive probes for the identification of unknown T-cell-stimulating Ags (*see* Chapter 21 of this volume), as well as for the study of Ag-processing pathways *(1–4)*.

The *lacZ* T-cell activation assay is based on the principle that engagement of the TCR with its peptide–MHC ligand results in expression of nuclear factor of activated T-cells (NFAT) activity, which regulates the transcription of the endogenous interleukin 2 gene, but can also direct transcription of heterologous reporter genes *(5)*. We took advantage of this tightly regulated NFAT transcriptional activity to measure Ag-specific T-cell activation by assaying *lacZ* expression in T-cells transfected with the reporter NFAT–*lacZ* construct *(6)*. To allow generation of T-cells with inducible *lacZ* expression, and with any desired Ag specificity, the BWZ.36 fusion partner *(7)* was generated from the original TCRα⁻β⁻ BW5147 cell line *(8)*. By fusing normal T-cells with this fusion partner, numerous Ag-specific, *lacZ*-inducible T-cell hybrids have been generated. For reasons that are not clear, the expression of endogenous CD8, but not CD4, is extinguished in T-cell hybrids. Thus, fusion with the normal BW5147 fusion partner, as well as with its derivative, BWZ.36, allows only the generation of CD4⁺ T-cell hybrids. To overcome this problem for generating CD8⁺ T-cell hybrids, CD8α was also transfected into the BWZ.36 cells. The resulting BWZ.36/CD8α cells constitutively express CD8α and have been successfully used to generate αβ-TCR⁺ and CD4⁺ or CD8⁺ T-cell hybrids. Experience in generating γδ-TCR⁺ hybrids is currently limited.

TCR mediated induction of *lacZ* activity in the BWZ36/CD8α-derived T-cell hybrids can be measured using a variety of *lacZ* substrates *(7)*. The most common *lacZ* assay in this laboratory uses the chromogenic substrate, chlorophenol red-β-D-galactopyranoside (CPRG). Cleavage of CPRG by *lacZ* results in production of chlorophenol red, which is conveniently quantitated by measuring its absorbance at 575–595 nm using a standard 96-well-plate reader. Other alternatives include the less sensitive, but cheaper, chromogenic substrate *o*-nitrophenyl β-D-galactopyranoside (ONPG) or the slightly more sensitive, but also more expensive, fluorogenic substrates, 4-methylumbelliferyl β-D-galactoside (MUG) or fluorescein di-β-D-galactopyranoside (FDG), which may be used if fluorescence plate readers are available. The FDG substrate can also be used for measurement of *lacZ* activity by flow cytometry in viable cells *(6,9)*. Yet another alternative is to stain fixed *lacZ*⁺ cells with the 5 bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) substrate *(7)*, which allows a microscopic visualization of the activated T-cells because of their intense blue color. Although the number of blue cells is tedious to quantitate, it can provide a sensitive readout when the number of APCs is limiting, as in expression cloning

strategies for isolating T-cell-stimulating Ag genes, or among tumor-infiltrating cells *(10–13)*.

2. Materials

1. Complete culture medium: RPMI 1640 (Gibco-BRL, cat. no. 11875-085) supplemented with glutamine, Na pyruvate (Gibco-BRL, cat. no. 11360-070), 2-mercaptoethanol (Sigma, cat. no. M-7522), penicillin + streptomycin (Gibco-BRL, cat. no. 10378-016), geneticin (G418), and 10% fetal bovine serum (FBS) (Hyclone, Logan UT, cat. no. SH30071.03).
2. Fusion medium: complete medium without 10% FBS. Incubate ~250 mL of this medium overnight in the CO_2 incubator, to prewarm and maintain proper pH.
3. Polyethylene glycol (PEG): 50% PEG1500 (Boehringer Mannheim, cat. no. 783641).
4. Drug selection medium: To complete medium, add the following to obtain the indicated final concentration: 2X Aminopterin (Sigma A5159), 2X hypoxanthine and thymidine (HT) (Sigma H-0137) (HAT) and 2 mg/mL hygromycin (Gibco-BRL, cat. no. 10687-010).
5. Drug withdrawal medium: To complete medium, add the following to obtain the indicated final concentration: $1 \times$ HT (Sigma H0137) + 1 mg/mL hygromycin.
6. CPRG reagent (pH 7.2–7.4). Dissolve 45.5 mg CPRG (Boehringer Mannheim, cat. no. 884 308) in 500 mL 10 m*M* phosphate buffer (pH 7.2–7.4), containing 1 m*M* $MgCl_2$ and 0.125% NP-40 (Calbiochem, cat. no. 492015). The CPRG reagent can be stored in dark brown bottles at 4°C for up to several weeks.
7. Stop buffer (pH ~12.0). Dissolve 22.52 g glycine (300 m*M)* and 5.58 g Na_2-EDTA (15 m*M)* in water, adjust pH with 10 *M* NaOH, and make up to 1L.

3. Methods

The efficiency of obtaining drug resistant hybrid cells in optimal experiments is ~1 in $0.6–1 \times 10^5$ normal cells fused. Of these, up to 50% can be Ag-specific for strong Ags, but may be only 1% for weaker Ags. To increase the probability of obtaining hybrids with desired specificity, use at least $10–20 \times 10^6$ activated T-cells for the fusion.

3.1. Preparation of Normal T-Cells and Fusion Partner

1. Normal T-cells: Use CD8^{+} or CD4^{+} T-cell lines, or clones from immunized mice. The cells must have been stimulated with the Ag 2–3 d earlier (*see* **Note 1**). Harvest, count, and resuspend the cells in 10 mL prewarmed fusion medium. Ideally use $~10 \times 10^6$ cells.
2. The fusion partner BWZ.36/CD8α cells are grown in complete culture medium. Split 1:2 the day before the fusion. On the day of fusion, harvest, count, and resuspend to 1×10^6/mL in prewarmed fusion medium.
3. Mix an equal number of normal T-cells to be fused with the BWZ36/CD8α fusion partner cells in a 50 mL conical tube.

4. Spin down the cells at 850g for 2 min, and, by tilting the tube at a 75-degree angle, aspirate off as much medium as possible, taking care not to aspirate the cell pellet itself.

3.2. Fusion with PEG

1. Gently tap the bottom of the conical tube to loosen the cell pellet.
2. Place tube in a 250 mL beaker containing 37°C water from a water bath (*see* **Note 2**).
3. Slowly, over 1–2 min, dribble 0.5 mL warm 50% PEG1500 along the side of the tube, while swirling the cell pellet (*see* **Note 3**).
4. Add 10 mL prewarmed fusion medium along the side of the tube, while swirling the contents. This step dilutes out the PEG. Continue to swirl the tube gently, to mix the PEG uniformly with the medium.
5. Leave the cells in a 37°C water bath for 8 min.
6. Spin down the cells at 300g for 2 min. Aspirate off the supernatant, and break the pellet by tapping the tube.
7. Resuspend the pellet in complete medium to 3×10^5 normal T-cells/mL.
8. Using a multichannel pipeter, plate out 0.1 mL/well of the cell suspension in 96-well, flat bottom plates. This results in 3×10^4 normal T-cells/well.
9. Leave plates overnight at 37°C in a humidified CO_2 incubator.

3.3. Drug Selection of HAT^r and Hygromycin^r (Hyg^r) Hybridomas

1. Add 0.1 mL drug-selection medium, consisting of complete medium containing 2× aminopterin + 2× HT + 2 mg/mL hygromycin, the day after the fusion (*see* **Note 4**).
2. Incubate at 37°C in a humidified incubator for 7–14 d. Most-cells should die within 2–3 d. Colonies of growing hybrid cells should be visible under the microscope around 8 d after the fusion.

3.4. Ag-Specificity Screen

The T-cell hybrids can be screened for their Ag specificity after the cells have grown to cover about one-half to two-thirds of the well, which is usually around 10–14 d after the fusion. In the best experiments, each 96-well plate yields about 40–50 wells containing growing hybridoma cells. If this is the case, cells in all the wells in the plate (including those without a colony) can be screened, to make the manipulations easier. Alternatively, if the number of hybrids per 96-well plate is <10–20, the wells with growing hybrids can be transferred to another 96-well plate. Total volume of medium in each well should be 0.2 mL. These are the master plates. Do not let the hybrids die because of overgrowth, until it is clear whether they should be expanded or discarded. The cells can be split 1:2 in the 96-well plate, if there are delays in setting up the screen.

1. Prepare appropriate APCs in a 96-well plate. These can be 2×10^5 spleen cells or $30–50 \times 10^3$ cell line (e.g., fibroblast-cell line) cells ± Ag peptide (100 nM for CD8$^+$ T-cells, 10 µM for CD4$^+$ T-cells), if available. This is the test plate. Keep

total volume/well to 150 µL. Transfer 50 µL hybrid cells from each well of the master plates (or from those wells selected for screening) to each of the test and control wells in the test plate. Control wells are without the Ag. These can be APC+medium alone or APC which do not express the Ag. This is to ensure that the *lacZ* response observed is Ag-specific.

2. Add fresh medium to the master plate to make the total volume 0.2 mL/well. Return the plate to the incubator.

3. After overnight incubation, test for *lacZ* activity induced in the T-cells, as described below (*see* **Subheading 3.7.** *see* **Note 5**).

4. Transfer the hybridoma cells selected for further experiments to 24 well plates in drug withdrawal medium (1× HT + 1 mg/mL hygromycin). After 10 d, resume growth in complete medium (*see* **Note 6**). Hygromycin is no longer necessary.

3.5. Subcloning Hybrids

Hybrids should be subcloned by limiting dilution as soon as possible, to avoid being overtaken by nonresponsive cells that may have been present in the original well, or may have arisen because of chromosomal instability in the hybrids. The procedure should be repeated whenever the performance of cells appears to be suboptimal.

1. Count cells accurately with a hemocytometer. Dilute cells in complete medium, to yield 0.5 or 1 cell/0.2 mL. Plate out 0.2 mL/well of one 96-well plate for each dilution, and leave in a humidified incubator at 37°C. Dividing cells should be visible after 2–3 d, and colonies in about 1 wk.

2. Clones should be tested for their Ag-specificity as above for the initial screen.

3.6. Frozen Stocks and Maintenance

Once hybrids of interest have been identified, expand incrementally from 24-well to six well plates or T-25 flasks, and, finally, to T-75 flasks. Freeze down at least three different batches of $2–4 \times 10^6$ cells/vial in 10% dimethyl sulfoxide (DMSO)/FBS as the freezing medium.

1. Pellet cells at 850*g* for 2 min; resuspend in appropriate volume of freezing medium. Add to labeled, chilled Nunc vials. Place the vials in a styrofoam tube rack, and leave at –70°C for up to a few days, then transfer the vials to liquid nitrogen. Make appropriate entry in the inventory book (*see* **Note 7**).

2. Maintain cells in complete medium. HAT or hygromycin is no longer required (*see* **Note 8**).

3.7. CPRG Assay for Measuring lacZ Expression in Activated T-Cells

Before assaying for *lacZ* activity, the cells are washed with PBS to remove the culture medium, which contains phenol red as the pH indicator dye, and is the same color as the enzymatic reaction product, chlorophenol red.

1. To remove the medium, spin the 96-well plate in a centrifuge at $850g$ for 2 min, and deftly flick off the supernatant into the sink.
2. With a multichannel pipeter, and a disposable reagent reservoir, add 0.1 mL PBS to each well of the plate. Tap side of plate gently to mix.
3. Spin plate again at $850g$ for 2 min. Flick off the PBS wash.
4. Lysis and enzyme assay. Using a multi-channel pipeter and a reagent reservoir, add 0.1 mL CPRG reagent to each well. Cells are lysed by the NP-40 detergent, and release the intracellular *lacZ,* which then acts on the CPRG substrate.
5. Incubate plate at 37°C for 4 h. Occasionally inspect plates visually during the incubation for the red reaction product, to ensure that they do not overdevelop when the T-cell response is strong. Alternatively, if the T-cell response is weak, the plates can be left to develop overnight at room temperature.
6. Stopping the enzyme reaction. Add 50 µL Stop buffer to each well with the multichannel pipeter.
7. Measurement of enzymatic product. Remove plastic covers from the plates, and read absorbance at 575–595 nm and at 655 nm as the reference wavelength, using a 96-well plate reader (*see* **Note 9**).
8. Ag-specific T-cell responses are usually severalfold over background. In secondary screens, confirm the specificity of T-cell reactivity by carrying out dose titrations of APC and/or the Ag (*see* **Note 10**).

4. Notes

1. Normal T-cells are obtained from immunized animals. In a typical experiment to generate C57Bl/6 (B6) anti-BALB.B specific CD8$^+$ T-cells, the B6 animals were injected intraperitoneally thrice at weekly intervals with 20×10^6 BALB.B spleen cells. Four d after the last injection, the spleen was removed from the immunized animal. A single cell-suspension of the spleen cells was co-cultured with irradiated BALB.B spleen cells. Four d later, the actively dividing cells (mostly T-cells) were harvested, and further enriched by centrifugation through a Ficoll-Hypaque cushion. Viable cells at the interphase were collected, washed, counted, and used for the fusion *(14)*. These T-cells can also be further propagated by weekly restimulation cycles in presence of IL-2, to generate a long-term line that can be fused, with a concomitant increase in the frequency of Ag-specific T-cell hybrids. Numerous protocols are available for generating Ag-specific CD4$^+$ or CD8$^+$ T-cell responses *(7,15)*.
2. During the fusion reaction, maintaining the temperature at 37°C is very important for the membrane events that occur during and immediately after the PEG mediated fusion event. Because of the small volume, the cells can rapidly come down to room temperature. Therefore, it is strongly advised to use a 250 mL beaker filled with 37°C water from a water bath, and to keep the tube inside the beaker during the fusion reaction.
3. The incubation time with PEG is critical, and, in our experience, deviations of more than 30 s dramatically lower the fusion efficiency.
4. The BWZ36/CD8α fusion partner has the NFAT-*lacZ* and the CD8α constructs respectively under hygromycin and G418 antibiotic selection. The hybrids are normally selected for growth in HAT + hygromycin-containing medium. Hygromycin is included to maintain selection pressure on the NFAT–*lacZ* construct. For rea-

sons not clear, addition of G418, which should theoretically help maintain expression of the CD8α construct in the hybrids, is detrimental to the overall fusion efficiency, and should not be included in the fusion or selection media.

5. Wells containing growing hybrids should be tested for their Ag-specificity and subcloned as soon as possible. Subcloning should be repeated several times with unstable hybrids, to improve their stability and Ag sensitivity.

6. HAT selects against growth of the hypoxanthine-guanine phosphoribosyl transferase-sensitive (HGPRT) fusion partner but allows growth of the hybrid cells. The cells grow slower in HAT, because they are forced to use their less efficient nucleotide salvage pathway. Under no circumstances should cells growing in HAT medium be directly transferred to normal medium. The cells will immediately die, because the A in HAT (aminopterin, an irreversible dihydrofolate reductase [DHFR] inhibitor) remains within the cells, and can only be diluted out over several generations. Meanwhile, HT is necessary to allow cells to keep using the salvage pathway until they can use their DHFR dependent *de novo* nucleotide synthesis pathway.

7. Freeze aliquots, and store in liquid nitrogen, as soon as possible. The hybrids are maintained by culturing in normal growth medium. Split cells 1:10 or 1:20 every 2–3 d. Some hybrids are more stable than others in their Ag-specific responses, and can be used for months, before they gradually lose reactivity. It is advised to freeze $2–4 \times 10^6$ cells per freezing vial. We use 10% DMSO in FBS as the freezing medium.

8. Do not allow hybrids to overgrow during weekends. Even if the cells survive, they may rapidly lose their functional responsiveness. Always try to grow cells at a semiconfluent density of $<10^6$/mL.

9. Assay plates can be stored up to 1 wk after stopping the reaction with the Stop buffer at room temperature. To prevent changes in product concentration caused by evaporation, it is advised that the plates be read as soon as possible.

10. Standard T-cell responses are measured using $30–100 \times 10^3$ T-cell hybrids/well of a 96-well plate. The APCs are titrated down from 1×10^6/well for spleen cells or 1×10^5/well for cell lines (e.g., fibroblast cell lines). In initial experiments, controls with concanavalin A (2–4 µg/mL) or phorbol–myristate acetate (10 ng/mL) plus ionomycin (1 µ*M)*, or crosslinked anti-CD3 or anti-TCR, may be used as strong positive controls for T-cell responsiveness without APCs. Ag-specific T-cell responses rarely reach this level.

Acknowledgments

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