Video Article

Overlapping Peptide Library to Map Qa-1 Epitopes in a Protein

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Abstract

Qa-1 (HLA-E in human) belongs to a group of non-classical major histocompatibility complex 1b (MHC-Ib) molecules. Recent data suggest that Qa-1 molecules play important roles in surveying cells for structural and functional integrity, inducing immune regulation, and limiting immune responses to viral infections. Additionally, functional augmentation of Qa-1-restricted $CD8^+$ T cells through epitope immunization has shown therapeutic effects in several autoimmune disease animal models, *e.g.* experimental allergic encephalomyelitis, collagen-induced arthritis, and non-obese diabetes. Therefore, there is an urgent need for a method that can efficiently and quickly identify functional Qa-1 epitopes in a protein. Here, we describe a protocol that utilizes Qa-1-restricted $CD8^+$ T cell lines specific for an overlapping peptide (OLP) library for determining Qa-1 epitopes in a protein. This OLP library contains 15-mer overlapping peptides that cover the whole length of a protein, and adjacent peptides overlap by 11 amino acids. Using this protocol, we recently identified a 9-mer Qa-1 epitope in myelin oligodendrocyte glycoprotein (MOG). This newly mapped MOG Qa-1 epitope was shown to induce epitope-specific, Qa-1-restricted $CD8^+$ T cells that enhanced myelin-specific immune regulation. Therefore, this protocol is useful for future investigation of novel targets and functions of Qa-1-restricted $CD8^+$ T cells.

Video Link

The video component of this article can be found at https://www.jove.com/video/56401/

Introduction

Qa-1 belongs to a group of non-classical major histocompatibility complex 1b (MHC-Ib) molecules in mice. Its human homolog is HLA-E. Previous evidence has demonstrated that Qa-1 molecules have important biological functions. Firstly, Qa-1 molecules play an important role in surveying cells for structural and functional integrity. In this regard, Qa-1 molecules have evolved several strategies to monitor the normal function of a cell. One such strategy enables Qa-1 molecules to form complexes with a processed leader peptide (epitope), *i.e.* the Qa-1 determinant modifier (Qdm) that is processed from classical MHC-Ia molecules in the endoplasmic reticulum[1]. These Qa-1/Qdm complexes later display on the surface of a cell and bind to inhibitory NKG2A receptors on NK cells to inhibit NK killing activity[2]. If the expression of MHC-Ia molecules is lost, a cell (e.g. a malignant cell) becomes sensitive to NK killing[2]. The other strategy enables Qa-1 molecules to form new Qa-1/epitope complexes on the surface of a cell that is deficient in TAP (transporter associated with antigen processing)[3] and/or ERAAP (endoplasmic reticulum aminopeptidase associated with antigen processing)[4] (both deficiencies often occur in malignant cells). The cell that expresses these new Qa-1/epitope complexes can then be recognized and eliminated by the epitope-specific Qa-1-restricted $CD8^+$ T cells. Secondly, Qa-1 molecules induce immune regulation[5]. In this regard, Qa-1/epitope complexes have been shown to stimulate $CD8^+$ regulatory T (Treg) cells that are important for the prevention of immune-mediated damage of self-tissues[6,7,8,9,10]. Thirdly, Qa-1-restricted $CD8^+$ Treg cells have been shown to limit immune responses against viral infection[11].

Therefore, specific augmentation of epitope-specific Qa-1-restrictred $CD8^+$ T cells is a potentially promising strategy for the elimination of abnormal cells, for the enhancement of immune regulation, and for the control of the magnitude of virus-induced immune responses. While it has not been determined whether augmentation of epitope-specific Qa-1-restricted $CD8^+$ T cells can enhance immune surveillance and limit virus-induced immune responses, our laboratories and others have clearly demonstrated that immunization with Qa-1 epitopes can augment the function of Qa-1-restricted $CD8^+$ Treg cells specific for pathogenic autoimmune $CD4^+$ T cells, leading to efficient control of $CD4^+$ T cell-mediated autoimmune diseases in a variety of animal models such as experimental allergic encephalomyelitis (an animal model of human multiple sclerosis)[6,10], collagen-induced arthritis (an animal model of human rheumatoid arthritis)[7], and non-obese diabetes (an animal model of human type 1 diabetes)[8]. Additionally, we have discovered that immunization with a tissue-specific Qa-1 epitope leads to specific control of immune-mediated inflammation in that tissue through augmentation of $CD8^+$ Treg cells[12]. The above successes of preclinical studies indicate a need for a full evaluation of Qa-1 epitope immunization for the treatment of tissue-specific immune-mediated diseases and potentially for the therapy of other diseases associated with deficiencies in TAP and ERAAP.

Accordingly, there is a demand for a technology that can reliably and quickly analyze Qa-1 epitopes in a protein. In this regard, a limited number of biologically important Qa-1 epitopes has been described. Most of these Qa-1 epitopes were identified serendipitously during the

study of $CD8^+$ T cell responses to bacteria[13], cells deficient in TAP[3], cells deficient in ERAAP[4], and cells that cause EAE[6,9]. Therefore, a high throughput technique is desirable for the identification of biologically important Qa-1 epitopes in a defined protein. In the following, we describe an overlapping peptide (OLP) library strategy that maps functional Qa-1 epitopes in a protein using Qa-1-resrticted $CD8^+$ T cell lines specific for the OLP pool (OLP_pool) of a protein.

Protocol

All experiments were done in compliance with an Institutional Animal Care and Use Protocol approved by Animal Care and Use Committee at the University of Texas at El Paso and Loma Linda University.

1. Generation of an OLP Library Covering the Whole Length of a Protein

1. Design an OLP library in which all peptides are 15-mer in length, and adjacent peptides overlap by 11 amino acids.
   NOTE: In the MOG OLP study, sequence of the MOG precursor [Mus musculus] was retrieved from NCBI protein database by following this link: https://www.ncbi.nlm.nih.gov/protein/NP_034944. The MOG precursor (247 amino acids), which contained both signal and mature peptides, was used because most of reported Qa-1 (HLA-E) epitopes were located in signal peptides (e.g. Qdm[1]). Beginning at its N-terminus, the sequences of 15-mer peptides were identified such that two adjacent peptides overlapped by 11 amino acids (**Figure 1**). Hence, exactly 59 OLPs were identified in the 247 amino acid MOG precursor. However, the last OLP can range from 12 to 15-mer depending on the length of the protein. Additionally, we choose 15-mer library because we and others have shown that 15-mer peptides, when added into dendritic cells (DCs) or macrophages, can be efficiently processed into epitopes for recognition by $CD8^+$ T cells[14,15].
2. Purchase each individual peptide commercially. 5 mg per peptide should be enough for the screening. OLPs and truncated peptides (Step 6.1) can be desalted peptides (purity is approximately 50 - 70%). The purity of optimal peptide (Step 6.2) that will be used for the generation of tetramer and for future biological analyses should be >90%. Reconstitute the peptides under sterile condition.
3. Make 50 mg/mL individual peptide stocks in 100% DMSO. For 5 mg of each peptide, add 100 µL of DMSO into each tube, mix, and store the peptides at -20 °C. These stocks will be used to make an OLP_pool stock for the generation of $CD8^+$ T cell lines reactive to the OLP_pool. In addition, these stocks will also be used to make 10 mg/mL individual peptide stocks for determining the ability of each individual peptide to stimulate a Qa-1-restricted response in an OLP_pool-reactive $CD8^+$ T cell line.
4. Make OLP_pool stock by adding an equal volume of each peptide into a fresh tube. This OLP_pool stock contains 100% DMSO. In the MOG OLP study, there were 59 OLPs[12]. Hence, concentration of each peptide in the OLP_pool was 847.46 µg/mL (50 mg/mL ÷ 59).
5. Make 10 mg/mL individual peptide stocks by diluting (5x) the 50 mg/mL stock in sterile H_2O in a V-bottom 96-well plate (this stock contains 20% DMSO). Make these stocks in a 96-well plate because the determination of the Qa-1-restricted response of each individual peptide will be performed in a 96-well plate using an 8- or 12-channel multichannel pipette.
   NOTE: All peptides, including OLPs and truncated peptides, should be diluted in either a V-bottom 96-well plate or a 96-well sample rack filled with 1 mL tubes since the peptide library screening is performed in 96-well plates using a multichannel pipette. If it is difficult to dilute a peptide, add 1 N NaOH drop wise to help dissolve the peptide.

2. Priming of $K^{b-/-}$ $D^{b-/-}$ $CD8^+$ T Cells with the OLP_pool-pulsed $K^{b-/-}$ $D^{b-/-}$ Dendritic cells (DCs).

NOTE: There are two major Qa-1 alleles: one is Qa-1^a, and the other is Qa-1^b. Since the animals commonly used for academic research, *e.g.* C57BL/6 and Balb/c mice, carry Qa-1^b, this protocol describes the procedure for mapping Qa-1^b epitopes in a protein. $CD8^+$ T cells used in this protocol are purified from $K^{b-/-}D^{b-/-}$ mice (C57BL/6 background) in which $CD8^+$ T cells are restricted mostly by non-classical MHC-Ib molecules including Qa-1.

1. **Produce bone marrow-derived DCs as previously described[12].**
   1. Briefly, culture bone marrow single cell suspensions (1 x 10^6 cells/mL) in RPMI-10 medium (RPMI 1640 supplemented with 10% fetal bovine serum, 5.5 x 10^{-5} M 2-mercaptoethanol, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acid) containing 10 U/mL IL-4 and 100 U/mL GM-CSF in a 6-well plate (4 mL/well) at 37 °C, 5% CO_2.
   2. Two days later, remove non-adherent cells carefully and add fresh media and cytokines.
   3. After culturing the cells for another two days, transfer non-adherent cells containing fresh media and cytokines into a new 6-well plate.
   4. Culture the cells for another two days and replenished with fresh media and cytokines containing LPS (0.1 µg/mL) to activate the DCs.
   5. 24 h later, collect the DCs for experiments.

2. **Irradiate the DCs with 3000 Rads.**
   1. Alternatively, treat the DCs (5 x 10^7 cell/mL) with mitomycin C (50 µg/mL) in PBS at 37 °C for 20 min. Add RPMI-0 (RPMI 1640 without serum) to fill the tube (~12 mL) and spin the cells for 10 min in a table-top centrifuge at 300 x g. Discard supernatants and repeat the washing procedure two more times. These three washes are critical because any trace amount of mitomycin C may inhibit the response of $CD8^+$ T cells during the DC-T cell co-culture.

3. Adjust the DC concentration to 5 x 10^6 cells/mL in a serum-free medium (AIM-V serum-free medium supplemented with 5.5 x 10^{-5} M 2-mercaptoethanol, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acid).
4. Add the OLP_pool stock solution, which contains 100% DMSO, to the DCs such that final DMSO concentration will be 0.5% (200x). In the MOG OLP study, the concentration of each OLP in the OLP_pool was 847.46 µg/mL; hence the final concentration of each OLP in the DC culture was 4.2 µg/mL (847.46 µg/mL ÷ 200). However, this concentration can be scaled up to 100 µg/mL as long as the DMSO concentration in the cell culture remains less than 1%.
5. Incubate the DCs at room temperature for 3 h and shake the cells gently every 15 min.

6. During this incubation period, purify $CD8^+$ T cells from the harvested spleen or lymph nodes of $K^{b-/-}D^{b-/-}$ mice using a commercial $CD8^+$ T cell purification kit, adjust the cell concentration to 10 x 10^6 cells/mL in the serum-free medium containing 50 U/mL interleukin 2 (IL-2) and 100 U/mL of IL-7.
7. Now, add the $CD8^+$ T cells into a 48-well plate as 0.5 mL/well (after the addition of 0.5 mL/well of the OLP_pool-pulsed DCs in Step 2.8, the final concentration of the $CD8^+$ T cells will be 5 x 10^6 cells/mL).
   NOTE: $CD8^+$ T cells from mouse spleen and lymph nodes can be purified using CD8 Positive Isolation Kit by following the instruction provided by the manufacturer. $CD8^+$ T cells obtained are free of beads.
8. Spin down the OLP_pool-pulsed DCs (300 x g, 10 min) at RT. Reconstitute the OLP_pool-pulsed DCs to 2 x 10^6 cells/mL in the serum-free medium and add 0.5 mL/well into the 48-well plate that contains the $CD8^+$ T cells (final concentration of the OLP_pool-pulsed DCs is 1 x 10^6 cells/mL, final concentration of $CD8^+$ T cells is 5 x 10^6 cells/mL, final concentration of IL-2 is 25 U/mL, and final concentration of IL-7 is 50 U/mL). Culture the cells at 37 °C and 5% CO_2.
9. On day 4, remove and discard about 400 µL of the culture medium and add 500 µL of the fresh serum-free medium containing 100 U/mL IL-2 and 100U/mL of IL-7 to each well. Incubate the cells at 37 °C and 5% CO_2.
10. On day 7 or 8, re-stimulate the OLP_pool-primed $CD8^+$ T cells with the OLP_pool.

3. Restimulation of the Primed $CD8^+$ T Cells with Macrophages Pulsed with the OLP_pool

1. Four days before re-stimulation of the OLP_pool-primed $CD8^+$ T cells, prepare 2% (v/v) polyacrylamide bead solution: wash 2 g of polyacrylamide beads twice in 20 mL endotoxin-free H_2O or PBS. Pellet the polyacrylamide beads by centrifugation (400 x g) for 5 min and resuspend in 100 mL of PBS. Autoclave at 15 lb/m for 20 min. Store at room temperature.
2. Inject mice intraperitoneally with 1 mL/mouse of the sterile 2% polyacrylamide bead solution to attract the migration of monocytes/macrophages into the peritoneal cavity[16].
3. **Four days later, sacrifice the animals by CO_2 overdose.**
   1. Under sterile conditions, cut a small opening at the center of the abdomen such that the opening is just enough for passing a 5 mL transfer pipette. Fill the transfer pipette with RPMI-0 and insert the pipette into the abdomen cavity through the opening.
   2. Rinse the abdomen cavity by pipetting. Pipette out as much liquid as possible from the abdomen cavity into a sterile tube (these are peritoneal macrophages). Repeat the rinse step 4 - 5 times.
4. **Irradiate the peritoneal macrophages with 3000 Rads.**
   1. Alternatively, treat the peritoneal macrophages with mitomycin C (follow the procedure described in Step 2.2).
5. Adjust the concentration of the peritoneal macrophages to 5 x 10^6 cells/mL in the serum-free medium. Add OLP_pool stock (the final DMSO concentration is less than 1%) and M-CSF (final concentration=100 U/mL).
   NOTE: In this MOG OLP study, we used 0.5% DMSO. Thus, MOG OLP_pool stock was diluted 200x (*e.g.* 1 µL of MOG OLP_Pool stock was added into 199 µL of peritoneal macrophages). Hence, the final concentration of each OLP was 4.2 µg/mL).
6. Add 200 µL/well (1 x 10^6 cells/well) in a 48-well tissue culture plate and incubate the plate at 37 °C for 4 h.
7. Remove the non-adherent cells and the polyacrylamide beads by gentle washing with 200 µL/well of the pre-warmed RPMI-0.
8. Collect and pool the OLP_pool primed $CD8^+$ T cells from Step 2.10. Adjust the OLP_pool primed $CD8^+$ T cell concentration to 1 x 10^6 cells/mL in the serum-free medium containing 25 U/mL IL-2 and 50 U/mL of IL-7. Add 1 mL/well to the 48-well plate that contains the OLP_pool-pulsed peritoneal macrophages.
9. Four days later, replenish the medium in the 48-well plate. Remove and discard about 400 µL of the culture medium in the 48-well culture plates. Add 500 µL of fresh serum-free medium containing 100 U/mL IL-2 and 100 U/mL of IL-7 into each well. Culture the cells at 37 °C and 5% CO_2.
10. Three or four days later, examine the OLP_pool-restimulated $CD8^+$ T cells for OLP_pool-specific, Qa-1-restricted response by enzyme-linked immunospot assay (ELISPOT). After this point, re-stimulate the $CD8^+$ T cells every 7 - 10 days.
    NOTE: Macrophages are preferred for the re-stimulation of the OLP_pool-primed $CD8^+$ T cells. We noticed that $CD8^+$ T cells re-stimulated by macrophage grew better than DCs *in vitro*.

4. Determination of OLP_pool-specific, Qa-1-restricted Response in an OLP_pool-restimulated $CD8^+$ T Cell Line

NOTE: OLP_pool-specific, Qa-1-restricted response in an OLP_pool-restimulated $CD8^+$ T cell line is determined by IFNγ secretion following stimulation by the OLP_pool in the presence of C1R or C1R.Qa-1^b cells using an IFNγ ELISPOT assay. C1R cells can be obtained commercially. C1R.Qa-1^b cells can be generated by transducing the C1R cells with the Qa-1 lentiviral vector.

1. Add 100 µL/well of a capture anti-IFN-γ antibody diluted in coating buffer (PBS) into the wells of an ELISPOT plate. Seal and incubate the plate at 4 °C overnight.
2. On the second day, discard the coating buffer containing the capture anti-IFN-γ antibody and add 200 µL/well-blocking solution (serum-free medium) and incubate the plate for 2 h at room temperature.
3. **Irradiate the C1R and C1R.Qa-1^b cells with 9,600 Rads.**
   1. Alternatively, treat the C1R and C1R.Qa-1^b cells with mitomycin C as described in the Step 2.2 except that the treatment time will be 30 min.
4. Adjust the C1R and C1R.Qa-1^b cells in the serum-free medium to 4 x 10^6 cells/mL.
5. Discard the blocking solution in the plate and add the C1R and C1R.Qa-1^b cells at 50 µL/well (200,000 cells/well) and mix.
6. Add 50 µL/well of 100x diluted OLP_pool stock (in the serum-free medium) and mix properly. Incubate the plate at room temperature for 2 - 3 h.
7. Adjust the OLP_pool-restimulated $CD8^+$ T cells to 1 - 2 x 10^6 cells/mL in the serum-free medium containing 150 U/mL of IL-7 and add 50 µL/well (50,000 - 100,000 cells/well) to the plate. Centrifuge the plate (58 x g) for 5 min.

8. Incubate the plate at 37 °C and 5% CO_2 overnight.
9. Aspirate cell suspension. Wash wells 2 times with deionized (DI) water (250 µL/well). Allow wells to soak for 5 min at each wash step.
10. Wash wells 3 times with Wash Buffer I (PBS containing 0.05% Tween20). Allow wells to soak for 1 min at each wash step. Discard wash buffer.
11. Add 100 µL of detection antibody diluted in a dilution buffer (PBS containing 10% fetal bovine serum (FBS)).
12. Incubate the plates at room temperature for 2 h.
13. Discard detection antibody solution. Wash wells 3 times with 250 µL/well Wash Buffer I. Allow wells to soak for 1 min at each wash step.
14. Add 100 µL/well of enzyme conjugate (Streptavidin-HRP) diluted in the dilution buffer at 1:100 dilution.
15. Incubate the plates for 1 h at room temperature.
16. Discard enzyme conjugate solution. Wash wells 4 times with 250 µL/well Wash Buffer I. Allow wells to soak for 1 min at each wash step.
17. Wash wells 2 times with 250 µL/well Wash Buffer II (PBS).
18. Add 100 µL of Substrate Solution (mix 1 drop=20 µL of AEC Chromogen with 1 mL of AEC substrate) to each well. Monitor spot development for 5 to 60 min.
19. When desired results start to appear, stop the substrate reaction by washing wells with distilled water.
20. Air-dry the plates at room temperature for 2 h or overnight until it is completely dry. Removal of the plastic tray under the plates will facilitate drying. Store plates in a sealed plastic bag in the dark until it is analyzed.
21. Enumerate spots manually by inspecting under a dissecting microscope or using an ELISPOT plate reader. See the representative result in **Figure 2**.

5. Determination of Individual Peptides in the OLP_pool which Stimulate to the Qa-1 Restricted IFN-γ Secretion in an OLP_pool-specific $CD8^+$ T Cell Line

1. Determine the individual peptides that stimulate the OLP_pool-specific, Qa-1-restrictred IFN-γ secretion in an OLP_pool-specific $CD8^+$ T cell line using the above described IFN-γ ELISPOT assay (final concentration of each individual peptide used for the ELISOPT assay is 10 µg/mL). See representative results in **Figure 3**.
   NOTE: An OLP-specific, Qa-1-restircted response is defined as a response that is at least three times of the response in the presence of C1R.Qa-1^b cells but without the OLP. In the MOG OLP study, OLP68, OLP96, and OLP105 all met this criterion. Therefore, all the three OLPs potentially contain Qa-1 epitopes[12] (**Figure 3**). However, the OLP105 consistently gave the strongest response; we hence performed a detailed analysis of the OLP105 (**Figure 4** and **Figure 5**)[12].

6. Identification of the Optimal Qa-1 Epitope in a 15-mer OLP which Stimulates the Epitope-specific, Qa-1-restricted Response in an OLP_pool-specific $CD8^+$ T Cell Line

1. Synthesize C- and N-terminally truncated peptides of a 15-mer OLP as shown in **Figure 4**.
2. Examine IFN-γ secretion in an OLP-specific $CD8^+$ T cell line by stimulating the $CD8^+$ T cells with each of the truncated peptides as well as the parental 15-mer OLP in the presence of C1R or C1R.Qa-1^b cells using the above described IFN-γ ELISPOT assay. The final concentration of each individual peptide used for the ELISPOT assay is 10 µg/mL. A peptide is defined as the optimal Qa-1 epitope if the peptide: 1) consistently gives similar or stronger IFN-γ response, as compared to the original 15-mer peptide, in the presence of C1R.Qa-1^b cells; 2) is between 8 - 10-mer (9-mer peptide preferred) which are the peptide lengths bound to MHC-I molecules[15,17]. See **Representative Results** in **Figure 5**.

Representative Results

Design of an OLP library covering the whole length of a protein

Beginning at the N-terminus of a protein, each peptide is 15 amino acids (15-mer). Hence, the first peptide spans position 1 to position 15. The N-terminus of the second peptide overlaps with the C-terminus of the first peptide by 11 amino acid. Hence, the second peptide spans the position 5 to position 19. Design the rest of the peptides to the end of C-terminus of the protein (**Figure 1**). We chose the 15-mer library because we and others have shown that 15-mer peptides, when added into dendritic cells (DCs) or macrophages, can be efficiently processed into epitopes for recognition by $CD8^+$ T cells[14,15]. The number of OLPs in a library depends on the length of a protein. Additionally, the last OLP can range from 12 to 15-mer depending on the length of the protein. For example, myelin oligodendrocyte glycoprotein (MOG) has a length of 247 amino acids. The MOG OLP library contains 59 OLPs[12]. Representative results presented in this manuscript are from the analysis of the MOG OLP library.

Determination of OLP_pool-specific, Qa-1-restricted response in an OLP_pool-stimulated $CD8^+$ T cell line

We generated a $CD8^+$ T cell line that was primed by $K^{b-/-}D^{b-/-}$ DCs pulsed with MOG OLP_pool (MOG_pool) and restimulated by MOG_pool-pulsed $K^{b-/-}D^{b-/-}$ peritoneal macrophages as described in the aforementioned protocol (Protocol 1, 2, and 3). To determine potential MOG_pool-specific, Qa-1-restricted response in this $CD8^+$ T cell line, we examined the response of this $CD8^+$ T cell line to the MOG_pool in the presence of either C1R or C1R.Qa-1^b cells using the IFN-γ ELISPOT assay (**Figure 2**) (Protocol step 4). Our data showed that the MOG_pool-stimulated $CD8^+$ T cell line secreted a low level of IFN-γ in the presence of C1R.Qa-1^b cells (32 Spot forming cells or SFCs) but not C1R cells (2 SFCs), suggesting the presence of $CD8^+$ T cells which responded to Qa-1-binding epitopes derived from C1R cells (C1R cells are human B lymphoblastoid cells). SFCs were increased when the MOG_pool was added into the well that contained C1R cells (78 SFCs), suggesting the presence of $CD8^+$ T cells which responded to non-Qa-1 epitopes. SFCs were dramatically increased when the MOG_pool was added into the well that contained C1R.Qa-1^b cells (320 SFCs), suggesting the presence of $CD8^+$ T cells which responded to Qa-1-binding peptides. The data also demonstrate that the MOG_pool contains Qa-1-binding epitope(s).

Determination of individual peptides in the OLP_pool which contribute to the Qa-1-restricted IFN-γ secretion in an OLP_pool-reactive CD8$^+$ T cell line

To determine the peptides in the OLP_pool which stimulated the Qa-1-restricted IFN-γ secretion in a MOG_pool-reactive CD8$^+$ T cell line, we stimulated the MOG_pool-reactive CD8$^+$ T cells with individual 59 OLPs in the presence of either C1R or C1R.Qa-1^b cells (**Figure 3**) (Protocol step 5). Our data showed that few SFCs were observed in the wells that contained C1R cells and the OLPs. In contrast, SFCs increased in all wells that contained C1R.Qa-1^b cells and the OLPs. From **Figure 2**, we learned that CD8$^+$ T cells in the presence of C1R.Qa-1^b alone also formed SFCs. Therefore, the increased SFCs in most wells could be due to non-specific stimulation as a result of the presentation of Qa-1 epitopes derived from intracellular proteins in the C1R cells by the Qa-1 molecules. However, the OLPs in the 3 framed wells in **Figure 3C** (B8, D12, and E9), which matched the 3 highlighted OLPs in the **Figure 3A** (OLP68, OLP96, and OLP105), met the criterion for defining an OLP-specific, Qa-1-restricted IFN-γ response as described in the Protocol step 5.1[12]. The data suggest that the 3 OLPs contain Qa-1 epitope(s). Since the OLP105 consistently produced the highest response, we performed detailed analysis of the OLP105[12].

Design of a truncated peptide library for a 15-mer OLP

A 15-mer OLP, which has been determined to stimulate Qa-1-restricted IFN-γ secretion in the OLP_pool-reactive CD8$^+$ T cell line, needs to be analyzed for the optimal epitope using a truncated peptide library. Such truncated peptide library is designed by progressively truncating the 15-mer OLP by 1 amino acid at its N- and C-termini (**Figure 4**). Similar to other MHC class I molecules, Qa-1 molecules mainly bind to 8 - 10-mer peptides. Therefore, we recommend that the shortest truncated peptide is 6-mer in length.

Identification of the optimal Qa-1 epitope in a 15-mer OLP which stimulates Qa-1-restricted IFN-γ secretion in an OLP_pool-reactive CD8$^+$ T cell line

The above N- and C-terminally truncated peptides are tested for the strength in stimulating IFN-γ secretion in a CD8$^+$ T cell line specific for the OLP_pool or the 15-mer OLP using the IFN-γ ELISPOT described above. In the study of Qa-1 epitopes in MOG[12], we generated a CD8$^+$ T cell line that was specific for the MOG OLP105 (**Figure 5A**). Further analysis demonstrated that the N-terminally truncated 9-mer peptide met the two criteria for an optimal epitope as described in the Protocol step 6.2 (**Figure 5B**). Therefore, we concluded that the N-terminally truncated 9-mer peptide was the optimal Qa-1 epitope.

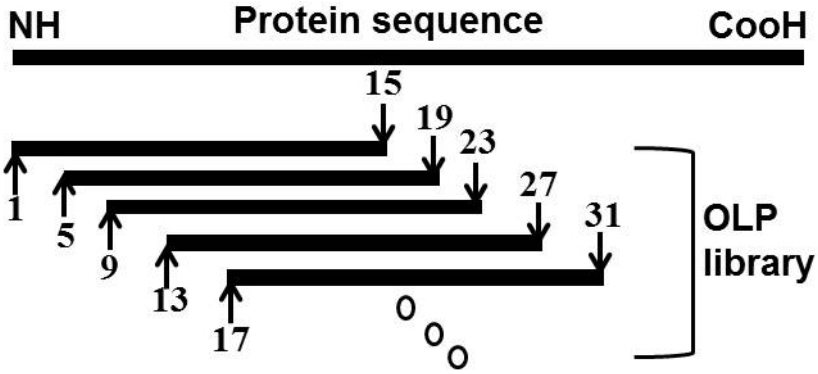

Figure 1: Design of an OLP library covering the whole length of a protein. Beginning at the N-terminus, peptides of 15 amino acid (15-mer) length were identified. The N-terminus of each peptide, except for the first peptide, overlapped with the C-terminus of the previous peptide by 11 amino acids.

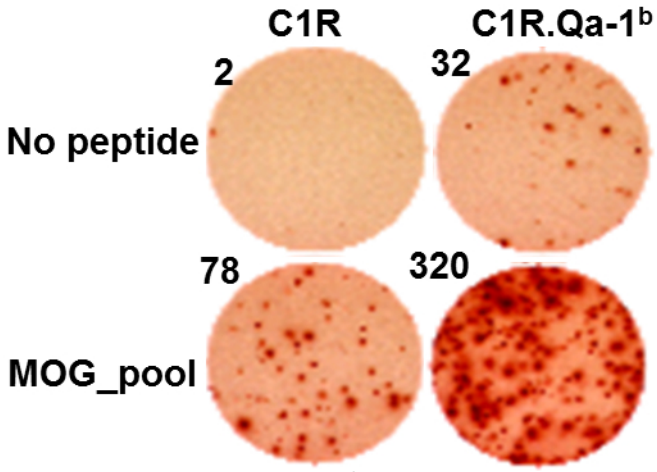

Figure 2: MOG_pool-stimulated CD8$^+$ T cells were partially MOG_pool specific and Qa-1-restricted[12]. *In vitro* CD8$^+$ T cells stimulated by the MOG OLP_pool (MOG_pool) were examined for response to the MOG_pool in the presence of either C1R or C1R.Qa-1^b cells as antigen presenting cells using an IFN-γ ELISPOT assay. Representative ELISPOT images are shown. Numbers of spot-forming cells (SFCs) are shown at the upper left corners of each well. CD8$^+$ T cells: 50,000 cells/well. C1R or C1R.Qa-1^b cells: 200,000 cells/well.

A. Layout of MOG OLP IDs

	1	2	3	4	5	6	7	8	9	10	11	12
A	49	50	51	52	53	54	55	56	57	58	59	60
B	61	62	63	64	65	66	67	68	69	70	71	72
C	73	74	75	76	77	78	79	80	81	82	83	84
D	85	86	87	88	89	90	91	92	93	94	95	96
E	97	98	99	100	101	102	103	104	105	106	107	Med

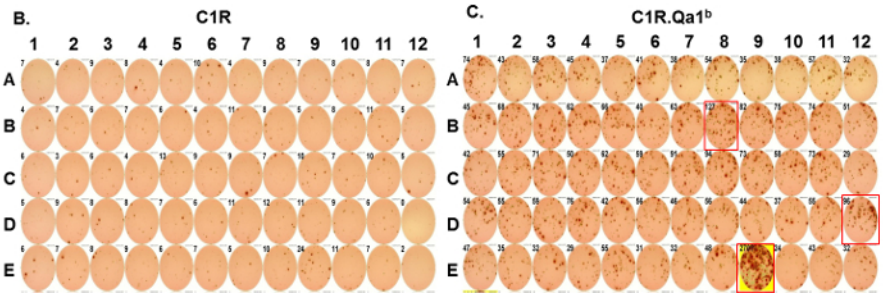

Figure 3: Analysis of the OLPs in the MOG_pool which were responsible for the Qa-1 restricted response. (A) Layout of the V-bottom 96-well plate that contained the 10 mg/mL individual MOG OLPs. Numbers in the wells represented OLP IDs. The highlighted 3 wells contained the OLPs that met the criterion for an OLP-specific, Qa-1-restricted IFN-γ response as described in the Protocol step 5.1[12]. **(B)** Representative SFCs in each well that contained an OLP (final concentration = 4.2 µg/mL, OLP ID matched that in "A"), the MOG_pool-reactive CD8$^+$ T cells (50,000 cells/well), and C1R cells (200,000 cells/well). **(C)** Representative SFCs in each well that contained an OLP (final concentration = 4.2 µg/mL, OLP ID matched that in "A"), the MOG_pool-reactive CD8$^+$ T cells (50,000 cells/well), and C1R.Qa-1^b cells (200,000 cells/well). The 3 framed wells contained the OLPs that met the criterion for an OLP-specific, Qa-1-restricted IFN-γ response[12]. The figure has been adapted from reference[12]. Please click here to view a larger version of this figure.

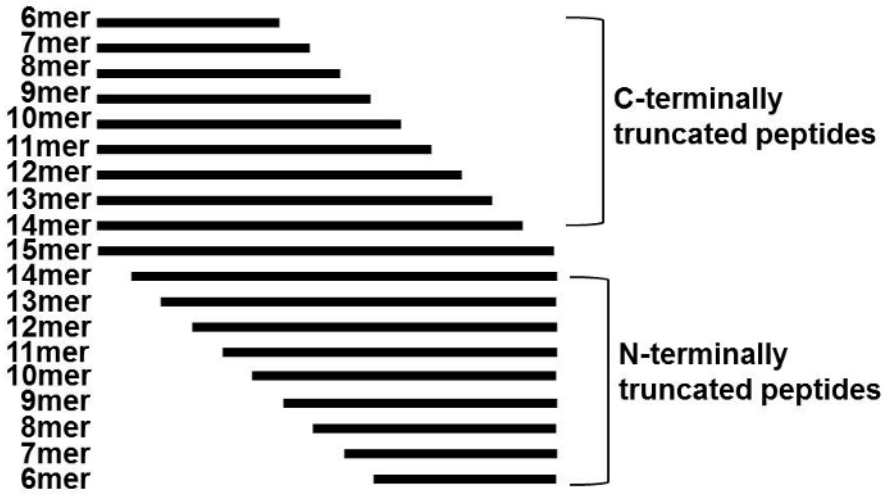

Figure 4: Design of truncated peptides of a 15-mer peptide. A 15-mer peptide was progressively truncated at its N- and C-termini by 1 amino acid to 6-mer. The 15-mer and its truncated peptides were then synthesized. Please click here to view a larger version of this figure.

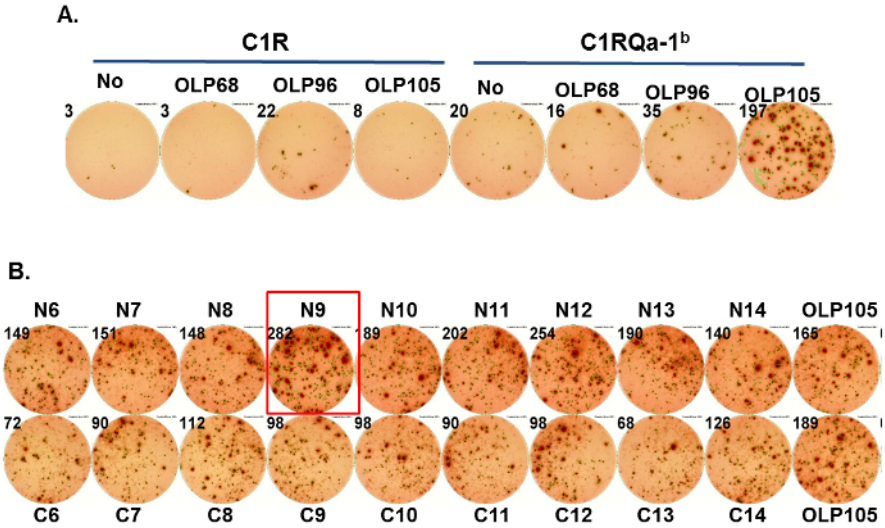

Figure 5: Analysis of the optimal Qa-1 epitope in the OLP105. (A) An OLP105-stimulated $CD8^+$ T cell line was examined for response to the OLP68, OLP96, and OLP105 in the presence of C1R or C1R.Qa-1^b cells using an IFN-γ ELISPOT assay. Representative IFN-γ SFCs (numbers at the upper left corners) are shown. **(B)** The OLP105-specific $CD8^+$ T cell line, as shown in (A), was examined for response to the individual N- and C-terminally truncated peptides, as shown in **Figure 4**, in the presence of C1R.Qa-1^b cells using the IFN-γ ELISPOT assay. OLP105: a 15-mer OLP. N6-14: N-terminally truncated OLP105 peptides. C6-14: C-terminally truncated OLP105 peptides. $CD8^+$ T cells: 50,000 cells/well. C1R.Qa-1^b cells: 200,000 cells/well. Numbers at the upper left corners were SFCs per 50,000 $CD8^+$ T cells. Red rectangle marked the well that met the criteria for defining the optimal Qa-1 epitope in an OLP as described in the Protocol step 6.2. Please click here to view a larger version of this figure.

Discussion

Here, we have described a protocol for analyzing Qa-1 epitopes in a protein. In relation to this protocol, several other strategies were also reported previously. First, allogeneic $CD8^+$ T cell lines and clones were used for the identification of the Qdm[1]. Second, a putative Qa-1-binding motif from the analysis of Qdm was used for the identification of the HSP60p216-224 and a TCRBV8.1 epitope[9,18]. Third, individual overlapping peptides from a protein were used to immunize animals. Subsequently, $CD8^+$ T cells isolated from the immunized animals were used to identify the TCRBV8.2 peptide p42-50 which was subsequently confirmed for binding to Qa-1[6,10,19]. Fourth, functional $CD8^+$ T cell lines in combination with cDNA display library were used for the identification of the FL9 Qa-1 epitope[4].

In reference to the previous techniques, the use of allogeneic $CD8^+$ T cell lines and clones may not be suitable for analyzing Qa-1 epitopes that are presented during physiological immune responses. Additionally, accumulating data suggest that the previously described binding motif may only partially represent the peptide-binding capacity of Qa-1 molecules. Therefore, the exact Qa-1-binding motif is still not known[4]. Furthermore, immunization with individual peptide for the identification of Qa-1 epitopes is laborious. Finally, cDNA display library strategy involves the construction of many vectors that carry different peptide lengths for cell transfection. In contrast, the OLP library strategy described here is simple, and the mapped peptides are known to stimulate Qa-1-restricted $CD8^+$ T cells. Therefore, our strategy has a unique advantage.

The strategy described here uses OLP_pool-specific $CD8^+$ T cells which are generated by *in vitro* priming and restimulation. An alternative source of the $CD8^+$ T cells can be from draining lymph nodes of a $k^{b-/-}D^{b-/-}$ mouse that is immunized with the source protein[20]. Such immune $CD8^+$ T cells can then be examined for IFN-γ responses to OLPs and truncated peptides for the identification of Qa-1 epitopes. Theoretically, the *in vivo* immune $CD8^+$ T cells are closer to the physiological condition than the *in vitro* generated $CD8^+$ T cells. However, we found that the response of immune $CD8^+$ T cells directly purified from immunized mice was weak and often required *in vitro* restimulations for a satisfactory peptide screening result. Additionally, this protocol is designed for future translation to human study of HLA-E epitopes wherein *in vivo* immunization is not practical.

Technically, the critical part of this protocol is the generation of $CD8^+$ T cell lines that are specific for the OLP_pool or an individual OLP. Since Qa-1/peptide complexes are unstable compared to classical MHC-I/peptide complexes[21], after antigen presenting cells are pulsed with the OLPs or a peptide, the pulsed cells should not be washed extensively. We have found that peptide-pulsed cells, if washed extensively, lose or significantly reduce the ability to stimulate $CD8^+$ T cells. Therefore, we recommend washing the peptide-pulsed antigen presenting cells once immediately before they are co-cultured with $CD8^+$ T cells.

Also, because of the instability of Qa-1/peptide complexes, we recommend the use of a serum-free medium for the peptide pulsing and the stimulation of $CD8^+$ T cells. Additionally, we routinely add 50 U/mL of IL-7 during $CD8^+$ T cell culture as well as during the IFN-γ ELISPOT assay. The purpose of the IL-7 is to maintain the viability and function of $CD8^+$ T cells. IL-7 by itself does not stimulate $CD8^+$ T cells to produce IFN-γ.

Similar to all strategies, Qa-1 epitopes mapped by this strategy also require further investigation for biological significance. For example, one important question is whether the mapped epitope can be physiologically presented. To address this question, DCs can be transduced with a lentiviral vector that expresses the epitope source protein (e.g. MOG In the MOG OLP study). Subsequently, response of the epitope-specific $CD8^+$ T cells to the transduced DCs is examined. A positive response suggests that the epitope can be physiologically processed and presented by DCs. In addition, functional consequences as a result of the epitope recognition by $CD8^+$ T cells should be further investigated. In this regard, the analyses of Qdm and FL9 Qa-1 epitopes have led to the conclusion that Qa-1 molecules are important for immune surveillance[1,4]. Additionally, the studies of HSP60p216, TCRBV8.1 peptide, and the p42-50 have led to the conclusion that Qa-1-restricted $CD8^+$ T cells regulate immune responses through targeting pathogenic $CD4^+$ T cells [6,7,9,19]. Furthermore, the study of MOG_{196} has for the first time revealed that Qa-1-restricted $CD8^+$ T cells could regulate immune responses by directly targeting a tissue to prevent it from damage by potentially pathogenic autoimmune cells[12]. Finally, functional analysis of an epitope-specific, Qa-1-restricted $CD8^+$ T cell can be assisted by tetramer. Such tetramer can be produced in NIH tetramer core facility (http://tetramer.yerkes.emory.edu/) or a company. To request the generation of a tetramer, one may send the functional data that support binding of the newly mapped optimal Qa-1 epitope to Qa-1 protein for their approval for the generation of a Qa-1 tetramer.

In conclusion, previous studies have demonstrated that Qa-1-restricted $CD8^+$ T cells play an important role in the regulation of local immune responses[12]. In this regard, tissue damages can be caused by an immune response that directly targets the tissue. Additionally, collateral damages can be caused by an immune response that targets an invading pathogen. Therefore, understanding the role of Qa-1-restricted $CD8^+$ T cells in these two different tissue damages is important for their clinical applications. To achieve this goal, a thorough analysis of Qa-1 epitopes in a self-tissue or an invading pathogen is necessary. This protocol will be suitable for these analyses.

Disclosures

Authors declare no conflict of interest.

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