Chapter 2

Generation of Multiple Peptide Cocktail-Pulsed Dendritic Cells as a Cancer Vaccine

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Abstract

Cancer immunotherapy based on dendritic cell (DC) vaccination has promising alternatives for the treatment of cancer. A central tenet of DC-based cancer immunotherapy is the generation of antigen-specific cytotoxic T lymphocyte (CTL) response. Tumor-associated antigens (TAA) and DC play pivotal roles in this process. DCs are well known to be the most potent antigen-presenting cells and have the most powerful antigen-presenting capacity. DCs pulsed with various TAA have been shown to be effective in producing specific antitumor effects both in vitro and in vivo. Several types of tumor antigens have been applied in cancer treatment including tumor RNA, lysates, apoptotic bodies, heat shock protein, peptides from TAA, and allogeneic tumor cells. Among them, the use of immunogenic HLA-A*0201-specific epitopes from multiple TAA enhances induction of antigen-specific CTL and associated therapeutic efficacy in HLA-A*0201$^+$ cancer patients. The current chapter provides a detailed protocol of generating multiple peptide cocktail-pulsed DC to elicit CTL with a broad spectrum of immune responses against the related tumor antigens.

Key words Cancer immunotherapy, Dendritic cells, Multiple peptide, Tumor-associated antigens, Cytotoxic T lymphocytes

1 Introduction

Dendritic cells (DCs) are the most attractive and potent antigen-presenting cells for targeted immunotherapy in cancer. First, several physiological aspects of DC including DC type and maturation status can be easily manipulated during ex vivo generation. Second, tumor-associated antigens (TAA) can be loaded in a controlled and feasible manner using peptides, proteins, or RNA. Third, autologous tumor cells such as dying tumor cells or whole tumor RNA can be used as tumor antigens to target patient-specific DC vaccination for successful cancer immunotherapy [1–7]. Animal models demonstrated that TAA-loaded DCs are capable of eliciting protective and

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therapeutic antitumor responses [8, 9]. Clinical trials also showed immunologically and clinically promising effects of antigen-loaded DC administered as a cancer vaccine [10, 11].

Although DC-based immunotherapy is a promising approach to augment tumor antigen-specific cytotoxic T lymphocyte (CTL) responses in cancer patients, tumor immune escape mechanism via downregulation or complete loss of TAA and MHC class I molecules, escaping death receptor signaling, impaired antigen processing may limit the susceptibility of tumor cells to the immune attack [12]. Therefore, targeting of multiple TAA and concomitant generation of CTL responses may represent one strategy to circumvent this potential drawback. Recently, several studies demonstrated that cancer immunotherapy using DC pulsed with multiple peptide cocktail derived from multiple (4 or 5) TAA with repeated boosting generates feasible and efficient cellular antitumor responses in patients with hormone-refractory prostate cancer and multiple myeloma [13–15].

We describe here a universal protocol to generate DC pulsed with multiple peptide cocktail based on our and other groups. It is necessary that more suitable, immunogenic TAA and powerful DC should be chosen for a strong and efficient antitumor immune responses using multiple peptide cocktail-pulsed DC.

2 Materials

2.1 Isolation of $CD14^+$ Monocytes and $CD3^+$ Lymphocytes from Peripheral Blood

1. Vacutainer blood collection tubes with sodium heparin (Becton Dickinson, Franklin Lakes, NJ, USA).
2. 15-mL and 50-mL polypropylene tubes.
3. Lymphoprep $d = 1.077$ (Axis-Shield Po CAS, Oslo, Norway).
4. 1× phosphate-buffered saline (PBS).
5. Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen, Gibco® by Life Technologies™, Grand Island, NY, USA) with 10 % fetal bovine serum (FBS).
6. MACS buffer: 0.5 % bovine serum albumin (BSA) and 2 mM EDTA in PBS and pH 7.2 (Miltenyi Biotec, Auburn, CA, USA).
7. Medium for human $CD14^+$ monocytes: IMDM with 10 % FBS.
8. Medium for human $CD3^+$ T cells: Roswell Park Memorial Institute (RPMI)-1640 (Invitrogen) with 10 % FBS.
9. Isolation columns for human $CD14^+$ monocytes and $CD3^+$ T cells (Miltenyi Biotec).
10. MACS separation kit (Miltenyi Biotec).
11. CD14 microbeads, human (Miltenyi Biotec).

12. CD3 microbeads, human (Miltenyi Biotec).
13. MACS columns and MACS separators (MS, LS) (Miltenyi Biotec).
14. Allegra X-12 centrifuge (Beckman Coulter, Brea, CA, USA).

2.2 Generation of Immature and Mature DC

1. Medium for DC culture: IMDM with 10 % FBS.
2. Medium for washing the cells: 1× PBS.
3. 6-well or 24-well culture plates.
4. Cytokines for DC differentiation:
   (a) 50 ng/mL recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) (Peprotech, Rocky Hill, NJ, USA) or 1,000 U/mL GM-CSF (Immunex, Seattle, WA, USA).
   (b) 20 ng/mL recombinant human interleukin-4 (IL-4) (Peprotech) or 1,000 U/mL IL-4 (R&D Systems, Minneapolis, MN, USA).
5. Cytokines for DC maturation 1 [13, 14]:
   (a) 20 ng/mL recombinant human tumor necrosis factor-α (TNF-α) and GMP grade (CellGenix, Freiburg, Germany).
   (b) 10 ng/mL recombinant human interleukin-1β (IL-1β) and GMP grade (CellGenix).
   (c) 1,000 U/mL recombinant human interleukin-6 (IL-6) and GMP grade (CellGenix).
   (d) 1 μg/mL prostaglandin 2 (PGE_2) (Pharmacia & Upjohn, Dubendorf, Switzerland).
6. Cytokines for DC maturation 2 [15]:
   (a) 1,000 U/mL recombinant human interferon-α (IFN-α) (R&D Systems, Minneapolis, MN, USA).
   (b) 10 ng/mL recombinant human TNF-α (TNF-α) (R&D Systems).
7. Cytokines for DC maturation 3 (with simple modification: αDC1-polarizing cocktails) [16]:
   (a) 50 ng/mL recombinant human TNF-α (Peprotech).
   (b) 25 ng/mL recombinant human IL-lβ (Peprotech).
   (c) 3×10^3 IU/mL recombinant human IFN-α; (Intron A-IFN-α-2b) (LG Life Science, Chonbuk, Korea).
   (d) 100 ng/mL recombinant human interferon-γ (IFN-γ) (Peprotech).
   (e) Poly-I:C 20 μg/mL (Sigma-Aldrich, St. Louis, MO, USA).

2.3 Synthesis of Multiple Peptide Cocktail Derived from Tumor Antigens

1. Two popular databases for MHC ligands and peptide motifs are available.
   (a) Peptide-binding database 1: Bioinformatics & Molecular Analysis Section (BIMAS), http://www-bimas.cit.nih.gov/molbio/hla_bind/
   (b) Peptide-binding database 2: SYFPEITHI, http://www.syfpeithi.de/.

2.4 Binding Affinity and Stability

1. T2 cell line (ATCC, Manassas, VA, USA).
2. Human β2-microglobulin (working concentration of 3 μg/mL) (Sigma-Aldrich).
3. PBS with BSA (PBA) [20]: 0.9 % sodium chloride (NaCl), 0.5 % BSA, 0.02 % sodium azide (NaN_3), or FACS buffer, 1× PBS and 1 % FBS.
4. The Brefeldin A (BFA) solution: 1,000×, working concentration of 3 μg/mL (eBiosciences Inc., San Diego, CA, USA).
5. Fluorescein isothiocyanate (FITC)-labeled anti-HLA-A*0201 monoclonal antibody (mAb) BB7.2 (Becton Dickinson Pharmingen).
6. FACS calibur cell sorter (Becton Dickinson Pharmingen).
7. Win MDI version 2.9 (Bio-Soft Net, developed by *John Trotter*, Salk Institute, San Diego, CA, USA).

2.5 DC Pulsing by Multiple Peptide Cocktail

1. Multiple peptides derived from tumor-associated antigen may be obtained from various sources.
2. 6-well culture plates (Becton Dickinson Pharmingen).
3. 15-mL polypropylene tubes.
4. IMDM medium with 10 % FBS.

2.6 DC Harvest and Storage

1. Cryotubes.
2. 2× freezing medium A: RPMI-1640 and 40 % FBS.
3. 2× freezing medium B: RPMI-1640 and 20 % dimethyl sulfoxide (DMSO).
4. Cryo 1 °C Nalgene™, freezing container (Thermo Fisher Scientific Inc, Rochester, NY, USA) with isopropanol.

2.7 Phenotypic Analysis of Multiple Peptide Cocktail-Pulsed DC

1. Mouse antihuman CD80 mAb conjugated with PE (Becton Dickinson Pharmingen).
2. Mouse antihuman CD83 mAb conjugated with FITC (Becton Dickinson Pharmingen).
3. Mouse antihuman CD86 mAb conjugated with PE (Becton Dickinson Pharmingen).
4. Mouse antihuman CCR7 mAb conjugated with FITC (R&D Systems).

5. Mouse IgG1, k, isotype control (Becton Dickinson Pharmingen).
6. Mouse IgG1, k, isotype control (Becton Dickinson Pharmingen).
7. Mouse IgG_{2A}, isotype control (R&D Systems).

2.8 In Vitro Induction of Multiple Peptide-Specific CTL

1. IL-2, 25 ng/mL (Peprotech).
2. Interleukin-7 (IL-7) 10 ng/mL (Peprotech).
3. 50 mL CTL medium: 22.5 mL RPMI-1640, 22.5 mL, AIM-V (Invitrogen), 5 mL FBS, and 0.5 mL penicillin-streptomycin. The AIM-V medium is a mixture of HEPES-buffered Dulbecco's Modified Eagle Medium and Ham's Nutrient Mixture F12 that had been supplemented with purified human albumin, transferrin, insulin, and a proprietary mixture of purified factors.

3 Methods

3.1 Isolation of Peripheral Blood Mononuclear Cells (PBMC)

1. Collect blood in heparinized tubes and dilute 1:2 with 1× PBS
2. Overlay 30 mL of diluted blood over 15 mL of Lymphoprep in each 50-mL tube.
3. Centrifuge at 1,000 × *g* for 25 min, at room temperature or 21 °C (acceleration, 5; deceleration, 0).
4. Harvest the buffy coat layer (PBMC fraction) after centrifugation.
5. Wash the cells twice with 1× PBS at room temperature.

3.2 Isolation of $CD14^+$ Monocytes and $CD3^+$ Lymphocytes from PBMC

1. Suspend PBMC in cold (4–8 °C) MACS buffer: PBS pH 7.2, 0.5 % BSA, and 2 mM EDTA.
2. Isolate $CD14^+$ monocytes and $CD3^+$ lymphocytes by the positive selection systems, respectively, according to the manufacturer's instructions (*see* **Note 1**).
3. Store the isolated $CD3^+$ lymphocytes in vapor phase of liquid nitrogen until needed.

3.3 DC Generation (See Note 2)

1. After the last wash of the monocytes, add fresh culture medium (IMDM with 10 % FBS), containing at least 50 ng/mL GM-CSF and 20 ng/mL IL-4 at a seeding density of 5×10^5 cells/mL/24-well plate or 2×10^6/2 mL/6-well plate.
2. On day 2 of the culture, discard half of the medium and add the same amount of fresh medium, pre-warmed to room temperature, with the 2× concentrated growth factors (100 ng/mL of GM-CSF and 40 ng/mL of IL-4).
3. On day 4 of the culture, repeat **step 2**.
4. On day 6, take out the half of the spent medium and add new medium containing GM-CSF (optional) and 2× concentrated

DC maturation cytokines, e.g., 1× αDC1-polarizing cocktail: 20 μg/mL of Poly-I:C, 50 ng/mL of TNF-α, 25 ng/mL of IL-1β, 3,000 IU/mL of IFN-α, and 100 ng/mL of IFN-γ.

5. Incubate for 42–48 h.

3.4 Analysis of Cytokine Production and Phenotypes in DC

1. On day 8, collect the supernatants from the cultures of mature DC. These supernatants represent "during maturation."
2. Harvest DC to 15-mL conical tubes and wash thoroughly to remove all the cytokines.
3. Plate the cells at 2×10^4 cells/100 μL/well in flat-bottomed 96-well plates.
4. Add the cytokine-inducing stimulus: 1 of 2 CD40 ligand (CD40L)-based stimuli, such as 5×10^4 cells/100 μL/well CD40L-transfected J558 cells [17] or 1 μg/mL of soluble CD40L [18, 19]. The induction of cytokine production is routinely performed in a final volume of 200 μL/flat-bottomed 96-well plates.
5. Following either of two methods of stimulation, the 24 h supernatants are harvested. These supernatants represent "after maturation and CD40L stimulation."
6. Perform the ELISA to measure human IL-12p70 and IL-10 secretion, for Th1 cytokine and Th2 cytokine, respectively, using the supernatants (during maturation and after maturation and CD40L stimulation) from cultures according to the manufacturer's instructions.

3.5 Synthesis of Multiple Peptide Cocktail

1. To predict T cell epitope candidates, select MHC type, e.g., HLA-A*0201. Choose a "mer," e.g., nonamers-9 amino acids; paste your amino acid sequence from source such as "MedLine"; and choose "run" to start analysis (*see* **Notes 3** and **4**).
2. Confirm the purity of commercially synthesized peptides (use >98 % purity by HPLC and Mass Spectrophotometry).
3. Dissolve the synthetic peptides in DMSO or distilled water according to the manufacturer's recommendations.
4. Store at −20 °C until needed.
5. Irrelevant peptides are used as controls.

3.6 Binding Affinity of Multiple Peptide Cocktail

1. Peptides binding to HLA-A*0201 molecules are measured using the T2 cell line according to a protocol described previously [20].
2. Wash T2 cells 2× in serum-free RPMI-1640 medium.
3. Place 8×10^4 cells T2 cells in serum-free RPMI medium into a V-bottom 96-well plate at total peptide concentrations ranging from 0 μg/mL to 50 μg/mL with 3 μg/mL of β2-microglobulin.

4. Incubate overnight at 37 °C in 5 % CO_2 in humidified air.
5. Wash T2 cells once with cold (4 °C) PBA: 0.9 % NaCl, 0.5 % BSA, 0.02 % NaN_3, or FACS buffer.
6. Stain T2 cells with the first antibody BB7.2 (HLA-A*0201-specific mAb).
7. Incubate for 30 min at 4 °C.
8. Wash 2× with cold PBA.
9. Stain T2 cells with FITC-labeled $F(ab')_2$ fragments of goat anti-mouse IgG as the second antibody.
10. Incubate for another 30 min at 4 °C.
11. Wash the cells 1×.
12. Measure the fluorescence at 488 nm on a FACScan flow cytometer.
13. Quantify the HLA-A*0201 expression according to the following formula:

$$\frac{\textit{Mean fluorescence with peptide} - \textit{Mean fluorescence without peptide}}{\textit{Mean fluorescence without peptide}} \times 100.$$

3.7 Stability of Multiple Peptide Cocktail

1. Wash the multiple peptide-pulsed T2 cells.
2. Incubate with BFA at 3 μg/mL to block the protein transport of newly synthesized HLA-A*0201 molecules.
3. Stain the cells with BB7.2 HLA-A*0201 mAb and FITC-labeled $F(ab')_2$ fragments of goat anti-mouse IgG sequentially at 0, 2, 4, 6, and 14 h post-BFA treatment.
4. Measure the peptide/HLA-A*0201 complex stability by flow cytometry.

3.8 DC Pulsing by Multiple Peptide Cocktail

1. Prepare 2×10^5 cells/mL in IMDM with 10 % FBS in each 15-mL tube.
2. Pulse the cells with multiple peptides at a concentration of 25–40 μg/mL (6.25–10 μg/peptide/mL) (*see* **Note 5**).
3. Incubate the cells for 2–4 h at 37 °C with multiple peptides and swing slightly every 30 min.
4. Harvest cells into a 15-mL conical tube and wash 2× using IMDM with 10 % FBS.
5. Count the cells.

3.9 DC Harvest and Storage

1. Spin the cells at 335 × *g* for 5 min at room temperature or 21 °C (acceleration, 5; deceleration, 0).
2. Prepare labeled cryotubes and store at 2–8 °C.
3. Resuspend the cells with 0.5 mL 2× cold freezing medium A.

4. Pipette 0.5 mL of DC in 2× cold freezing medium A into each cryotube and add 0.5 mL of 2× cold freezing medium B for a final concentration of 10^6 DC/mL.
5. Keep cryo 1 °C freezer container at −80 °C for at least 2 h or overnight.
6. Store DC in the vapor phase of liquid nitrogen.

3.10 Phenotypic Analysis of Multiple Peptide-Pulsed DC

Perform flow cytometric analysis of mDC. Stain the cells separately with mouse antihuman CD80 mAb conjugated with PE, mouse antihuman CD83 mAb conjugated with FITC, mouse antihuman CD86 mAb conjugated with PE, and mouse antihuman CCR7 mAb conjugated with FITC.

3.11 In Vitro Induction of Multiple Peptide-Specific CTL

1. Multiple peptide-specific CTL are generated ex vivo by repeated multiple peptide stimulation of $CD3^+$ T lymphocytes from HLA-A*0201 donors.
2. Harvest 2×10^5 cells/mL mDC to polypropylene tubes.
3. Wash thoroughly to remove all the cytokines.
4. Add multiple peptide at a concentration of 25–40 μg/mL (6.25–10 μg/peptide/mL) to mDC (*see* **Note 6**).
5. Incubate at 37 °C for 2–3 h.
6. Wash thoroughly to remove unbound peptides.
7. Resuspend the mDC in 1 mL of CTL medium.
8. Prepare 2×10^6 cells/mL $CD3^+$ T cells from the same (autologous) donor in 1 mL of CTL medium.
9. Plate mDC at 2×10^5 cells/mL/well in 24-well plates.
10. Add 2×10^6 cells/mL/well $CD3^+$ T cells in 24-well plates.
11. Co-culture mDC and $CD3^+$ T cells for 3 days.
12. After 3 days, add 25 ng/mL of rhIL-2 and 10 ng/mL of rhIL-7. The cells usually need to be fed with 50 % of fresh 25 ng/mL of rhIL-2-containing and 10 ng/mL of rhIL-7-containing medium every 2 days and transferred to new wells. The cultures reach quiescence about day 10–14 and need to be restimulated.
13. At 10–12 days after the first stimulation, restimulate the cells with irradiated (20 Gy), multiple peptide-pulsed autologous PBMC (at 1:1 ratio), with multiple peptide-pulsed T2 cells (at 2:1 ratio) or DC (at 10:1 ratio) (*see* **Note 7**).
14. The cells should be expanded for another 10–14 days in the presence of 25 ng/mL rhIL-2. This restimulation step allows the demonstration of the CTL activity induced by mDC and facilitates ELISPOT analysis of antigen-specific responses by reducing the nonspecific background. At days 20–24 (10–12 days after the second stimulation), the frequency of antigen-specific T cells is analyzed by IFN-γ ELISPOT according to the manufacturer's instructions (*see* **Note 8**).

4 Notes

1. Standard isotonic Percoll (SIP) solution and 10× concentrated "acidic" PBS can be used to isolate PBMC (the fraction of monocytes and lymphocytes) from the fresh blood [21].
2. It is important to select a batch of FBS and the source of medium. There can be significant differences between several different batches of FBS in their ability to support the DC1 maturation. DC1s are DC which can effectively elicit type 1 cytotoxic T lymphocytes (Tc1) production of high IFN-γ and low IL-4. The source of medium can make a difference as well.
3. Usually, naïve (unmodified) and/or heteroclitic (enhanced) nonapeptide can be used in multiple peptide cocktails. Heteroclitic peptides can be made by replacing one amino acid with the other different amino acid [22].
4. The sequence of multiple peptides from several tumor-associated antigens, which are associated with tumor pathogenesis and are highly expressed on the tumor cells, can be reviewed for peptides that could potentially bind to HLA-A*0201 using a peptide-binding database. After comparing the predictive binding scores, several peptide candidates are selected that could potentially bind with HLA-A*0201 molecules [5, 22].
5. If multiple peptides are applied, use one culture dish or one polypropylene tube for each peptide.
6. To avoid potential competition in HLA-A*0201 affinity among the specific peptides, do not avoid excess concentration of multiple peptide, e.g., 24–40 μg/mL total, 6.25–10 μg/peptide, to pulse the DC during CTL generation.
7. Cultures can be restimulated every 7–10 days with irradiated multiple peptide-loaded T2 cells, PBMC, or DC for a total of 2–4 cycles to generate multiple peptide-specific CTL.
8. In order to further characterize the function of antigen-specific CTL, MHC class I peptide multimer (tetramer) staining and cytolytic assays are available.

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