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Michael J.P. Lawman
Patricia D. Lawman *Editors*

Cancer Vaccines

Methods and Protocols

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Edited by

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Preface

Cancer immunotherapies, which include cancer vaccines, are novel therapeutic modalities being added to the armamentarium for cancer management/treatments that are finally becoming available to cancer patients around the world. In contrast to chemo- and radiotherapies, cancer vaccines are not normally associated with severe side effects, and unlike these therapies which directly kill the tumor cells and normal rapidly dividing cells in the body, cancer vaccines and other immunotherapies exert their effect by stimulating the body's immune system to focus on the cancer cells alone, remove them, and consequently reduce the severity of the disease, generally without toxicity. Given these characteristics, cancer vaccines offer cancer patients a more focused and gentler means of cancer treatment that is far less detrimental to their bodies and is cognizant of the patient's wish for a better quality of life.

The status of the patient's immune system is the vital biological element affecting the outcome of cancer immunotherapy. However, each individual's immune status is in turn affected by factors including age of the person, stage of the disease, prior treatment (chemotherapy or radiation therapy), tumor-induced immunosuppression, and the overall well-being of that person. As the term "immunotherapy" implies, the cells of the immune system perform the primary role in mediating the outcome of an immunotherapeutic regimen.

Most cancer vaccines to date have been designed to treat cancers that have already developed and therefore are termed "therapeutic." The purpose of these cancer vaccines is to stop cancer cell growth and eventually reduce the tumor burden. Some experts in the field believe that cancer vaccines may be best suited to prevent cancer from returning or to eliminate cancer cells that were not killed by other, more conventional treatments.

Whether used as adjunctive or stand-alone therapies, the development of effective cancer vaccines requires a thorough understanding of the innate and adaptive immune system, immune effector cells, and cancer cells. However, despite the plethora of clinical and basic knowledge of cancer and the immune system, the issue boils down to the simple fact that the immune system, in most cases, does not see cancer cells as being "nonself" and thus dangerous. Even when the immune system does recognize some element of danger, it does not usually mount a clinically significant response against well-established tumors. This is mainly due to the fact that cancer cells have developed mechanisms that make it challenging for the immune system to target them for removal. The most significant issue is that cancer cells express normal "self"-antigens on the cell surface in addition to specific cancer-associated antigens, giving the abnormal cells an advantage against immune surveillance. Furthermore, during their rapid proliferation, these cancer cells frequently undergo further genetic mutations that may consequently lead to the loss or down-regulation of the cancer-associated antigens. Finally, cancer cells generate soluble factors that function to suppress an anticancer immune response.

Producing an effective therapeutic cancer vaccine has proven to be challenging. To be effective, cancer vaccines must achieve two objectives. First, cancer vaccines must stimulate

a robust tumor-specific immune responses against the correct target. Second, the immune responses must be potent enough to overcome the means by which cancer cells evade the adaptive immune response.

Therapeutic cancer vaccines can be divided into two broad categories, namely, (1) whole-cell vaccines, which encompass autologous, allogeneic, and dendritic cell vaccines, and (2) peptide or protein antigen vaccines. Dendritic cell vaccines fall into both "camps," since this category can include the use of peptide and/or protein antigens as well as whole-cell lysates in the production of these vaccines.

The whole-cell vaccine approach encompasses the use of inactivated whole-tumor cells and/or whole-cell lysate as the vaccine. As such, these whole-cell vaccines present an array of tumor cell-associated antigens to the patient's immune system. The approach of using whole-tumor cell as a vaccine eliminates the significant problem of having to identify the crucial antigen(s) for that cancer, most of which remain unknown, but almost always requires some type of immune adjuvant.

Peptide or protein antigen vaccines can be comprised of synthetic or purified native moieties that are representative of the tumor cell antigens displayed by the target tumors. These antigens can be used to immunize patients and have been shown to generate an immune response capable of destroying cells in the body that display these antigens. These types of cancer vaccines are dependent upon knowing the major tumor cell markers/antigens, their structure, and, if peptides are generated, the important epitope(s) required to generate a tumor-specific immune response. Dendritic cells, which orchestrate the function of immune cells, are often used as the "delivery vehicles" for these synthetic peptides and proteins to the immune system.

Researchers continue to acquire the elements and knowledge required in order to design cancer vaccines that can potentially accomplish both goals, i.e., to evoke a tumor-specific response and overcome the immuno-evasive mechanisms employed by the tumor cells. The purpose of this current volume is to gather many of the methods that have been developed to manufacture these cancer vaccines under one cover. The chapters are grouped according to the purpose or the aim of the cancer vaccine, namely, the manipulation and modification of immune cells; the manipulation and modification of tumor cells; and the manipulation of immune/tumor interactions and various delivery mechanisms. The volume also covers the subject of cancer vaccines in a more global sense with its section on the advances, challenges, and future of cancer vaccines.

In bringing this volume together, we have attempted to gather experts in the various subspecialty fields of cancer vaccines to share their expertise with current and future cancer vaccinologists, researchers, and clinicians. To this end, the authors have shared their experiences and given helpful "tips" through the Notes section in each chapter to aid in the development of future cancer vaccine design. It is hoped that the methods and protocols that have already been developed will lead to the further generation of cancer vaccines that are both safe and efficacious and that cancer vaccines will be the standard of care in the very near future.

The coeditors, Dr. Michael Lawman and Dr. Patricia Lawman, are grateful to the many authors who took time from their busy schedules to contribute to this volume. Without

their efforts, this book would never have materialized. In addition, the coeditors offer special thanks to Dr. Venkata Narasimhulu Kuppala. Speaking for all the contributing authors, we also are very grateful for the advice, encouragement, and support given to us by Dr. John and Jan Walker, editors in chief for the series *Methods in Molecular Biology*, and to the publishers Humana Press and Springer Science + Business Media for the opportunity to attempt this project.

Tampa, FL, USA

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Part I

Manipulation and Modification of Immune Cells: Dendritic Cells

Chapter 1

Single-Step Antigen Loading and Maturation of Dendritic Cells Through mRNA Electroporation of a Tumor-Associated Antigen and a TriMix of Costimulatory Molecules

Daphné Benteyn, An M.T. Van Nuffel, Sofie Wilgenhof, and Aude Bonehill

Abstract

Dendritic cells (DC) are key players in several types of cancer vaccines. Large numbers of DC can easily be generated in closed systems from the monocyte fraction of the peripheral blood. They are the professional antigen-presenting cells, and electroporation of mRNA-encoding tumor antigens is a very efficient and a relatively simple way to load the DC with antigen. The co-electroporation of a tumor antigen of choice and the combination of 3 costimulatory molecules, including CD70, caTLR4, and CD40L (TriMix-DC), leads to fully potent antigen-presenting DC able to generate a broad immune response.

Here we describe the in vitro transcription of the mRNA and the subsequent generation and electroporation of autologous DC used for the treatment of melanoma patients.

Key words Leukapheresis, Dendritic cells, mRNA, Electroporation, Immunomonitoring, Tumor antigen, TriMix

1 Introduction

Dendritic cell (DC)-based cancer vaccines are hot topics in the antitumor immunology area. DC are the most professional antigen-presenting cells and are attractive candidates for therapeutic manipulation of the immune system to induce novel or enhance insufficient antitumor immune responses present in cancer patients. The types of tumor-associated antigens (TAA) for DC loading, DC culture, and maturation steps are key variables in the development of DC-based products. Different approaches have been used for antigen loading, and both defined (peptides, protein, mRNA) [1–7] and undefined (tumor mRNA, tumor lysates) antigens are used [8–10]. Also, for the maturation of immature DC, different clinical grade maturation protocols are used, among which are classical cytokine cocktails [11], Toll-like receptor ligands [12], and TriMix maturation [6, 13].

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Our expertise lies in the development of antigen-encoding mRNA-electroporated DC-based cancer vaccines. mRNA is a non-integrating molecule with a short half-life leading to a transient antigen expression mimicking an infection. When treating patients with mRNA-electroporated DC, there is no need for prior knowledge of the patient's HLA type as mRNA encoding the full-length TAA ensures presentation of the full antigenic spectrum of epitopes [5]. Both $CD8^+$ and $CD4^+$ T cells are necessary to coordinate an antitumor response leading to tumor regression [14–17]. mRNA coding for the TAA can be genetically modified to present peptides in both MHC class I and class II molecules by linking the TAA with a MHC class II targeting signal [18]. In addition, several parts of the mRNA can be optimized to enhance the transcription rate and stability. In general, mRNA is rapidly degraded because of its short half-life and several other characteristics that cause mRNA instability, including rare codon usage, low GC-content, or presence of negatively cis-acting motifs, hampering protein translation. Recently, it has been shown that a rational gene design, based on modern bioinformatics, followed by the de novo generation of a synthetic gene may help to circumvent this problem. Several studies have proven the positive impact of in silico cDNA optimization [19, 20].

To enable a DC-based vaccine to be fully potent, costimulatory signals are necessary. TriMix is the combination of three molecules, comprising CD40L, constitutive active TLR4 (caTLR4), and CD70-encoding mRNA, which in combination with the TAA are capable of generating functional mature antigen-presenting DC, further referred to as TriMix-DC, which are able to generate specific immune responses [21, 22]. All these molecules can be efficiently loaded into DC in a single step by co-electroporation. A major advantage of this approach is that there is no need to pre-incubate the DC for up to 48 h with soluble maturation signals like pro-inflammatory cytokines or TLR ligands to achieve maturation, which can render the cells "exhausted" and inferior for vaccination purposes. As a result, TriMix-DC can be injected into the patient within a few hours after electroporation and will mature and secrete most of their immunostimulatory cytokines and chemokines in situ.

Different routes of immunization can be combined to broaden the tissue distribution of antigen-specific T cells induced by the treatment. Investigators showed that immunization by different routes induces specific T cells situated at different tissue sites resulting in the eradication of tumors located at different body sites [23]. As reported by our group, the combination of intradermal (ID) and intravenous (IV) vaccination results in a broad T cell response induced by the DC treatment [13] and leads to enhanced clinical responses [24].

We here describe in detail the production and administration procedure of TriMix-DC.

2 Materials

2.1 Production of Capped mRNA

1. pST1/CD40L, pST1/CD70, pST1/sig-caTLR4, and pST1/sig-TAA-DC-Lamp plasmids (Fig. 1).
2. GenElute HP Endotoxin-Free Plasmid Maxiprep Kit (Sigma-Aldrich, Diegem, Belgium).
3. *Sap*I restriction enzyme (Fisher Scientific, Erembodegem, Belgium).
4. 3 M sodium acetate, pH 5.2 (nuclease-free).
5. 100 % and 70 % ethanol (EtOH).
6. Sterile RNase, DNase, and endotoxin-free water.
7. mMESSAGE mMACHINE® T7 Ultra Kit (Life Technologies, Merelbeke, Belgium).

2.2 Generation of Immature DC (iDC)

1. Peripheral blood mononuclear cells (PBMC) collected through leukapheresis (*see* **Note 1**).
2. 4-tray Cell Factories (*see* **Notes 2** and **3**) (VWR Nunc, Leuven, Belgium).

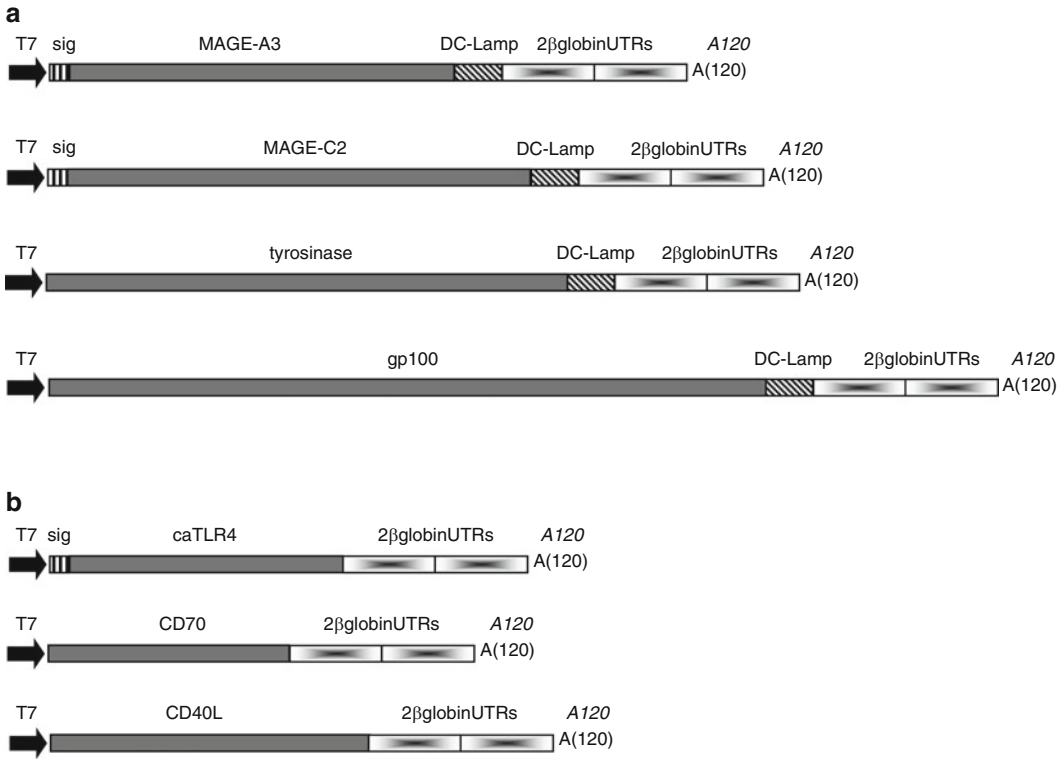

Fig. 1 Schematic representation of the different mRNAs. (**a**) Schematic representation of the mRNA encoding the 4 TAA comprising the vaccine. The T7 promoter, β-globin 3′ untranslated regions (UTRs), poly(A) tail (A120), signal sequence (sig) of Lamp-1, and the HLA class II targeting sequence (DC-Lamp) are shown. (**b**) Schematic representation of mRNAs coding for the TriMix molecules

3. X-VIVO™-15 medium.
4. Phosphate buffered saline (PBS).
5. Roswell Park Memorial Institute (RPMI)-1640 medium.
6. Granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) (*see* **Note 4**).
7. Heat-inactivated autologous plasma (AP) (*see* **Note 5**).
8. CELL-DYN Sapphire hematology analyzer (Abbott, Waver, Belgium).

2.3 Electroporation of DC for Vaccination

1. Serum-free RPMI-1640 medium.
2. Opti-MEM reduced serum medium without phenol red (Life Technologies).
3. 4-mm electroporation cuvettes (Immunosource Cell Projects, Schilde, Belgium).
4. Gene Pulser XCell electroporator (Bio-Rad, Nazareth, Belgium).
5. DC culture medium consisting of RPMI-1640 medium supplemented with 1 % AP, 1,000 U/mL of GM-CSF, and 500 U/mL of IL-4.
6. 15- and 50-mL tubes.
7. Ultra-Low Attachment T-75 flask (Elscolab Corning, Kruibeke, Belgium).
8. Cryopreservation medium consisting of AP + 10 % DMSO (Acros Organics, Geel, Belgium) and 2 % glucose.
9. 1-mL cryopreservation tubes.
10. Cryofreezing container (Cryo 1 °C freezing container, rate of cooling −1 °C/min) (VWR Nalgene, Leuven, Belgium).
11. PBS.
12. Human serum albumin (HSA).
13. 1-mL and 20-mL syringes.

3 Methods

The methods below describe (1) the production of capped mRNA, (2) the generation of autologous immature DC, and (3) subsequent electroporation of the DC with TriMix and TAA for combined ID and IV administration to the patient.

3.1 Production of Capped mRNA

For the production of capped mRNA suitable for electroporation, the following procedures must be performed: (1) cloning of the gene(s) of interest into a suitable vector and production of plasmid DNA, (2) linearization of the plasmid DNA, and (3) in vitro transcription of capped mRNA.

For preparation of TriMix-mRNA, the DNA sequences encoding the CD40L, CD70, and caTLR4 proteins are cloned into the pST1 vector [25] (provided by Dr. U. Sahin, Johannes Gutenberg University, Mainz, Germany) by using standard molecular cloning techniques. The extracellular part of the TLR4 is deleted resulting in the caTLR4 with the intracellular and transmembrane fragments [26] flanked by a signal sequence. For preparation of TAA mRNA, the DNA sequence encoding full-length TAA, flanked by the signal sequence of the Lamp-1 and the HLA class II targeting sequence of DC-Lamp [18], is cloned into the pST1 vector by using standard molecular cloning techniques. The signal sequence will translocate newly synthesized proteins to the endoplasmic reticulum while the DC-Lamp targeting sequence will provide transport to the HLA class II compartments (*see* **Note 6**).

The pST1 vector contains a bacteriophage T7 promoter, which controls in vitro transcription. Downstream from the insert, a poly(A) tail of 120 adenines is present. Between the coding region and the poly(A) tail, 2 consecutive human β-globin 3′ untranslated regions (UTRs) are present. Before in vitro transcription of the mRNA, the plasmids are linearized (*see* **Note 7**) with the *Sap*I restriction enzyme, resulting in an unmasked poly(A) tail with a free 3′ end (Fig. 1). After cloning, sufficient amounts of plasmid DNA are prepared with the GenElute HP Endotoxin-free Plasmid Maxiprep Kit according to the manufacturer's instructions. Optionally, DNA sequences can be optimized in silico to maximize translational efficiency (*see* **Note 8**).

3.2 Production of In Vitro Transcribed Capped mRNA

Prior to the in vitro transcription, linearization of 100 μg plasmid with 100 U *Sap*I restriction enzyme in a total volume of 500 μL is performed, followed by ethanol precipitation. In vitro transcription of capped mRNA is performed with T7 RNA polymerase by using the mMESSAGE mMACHINE® T7 Ultra Kit according to the manufacturer's instructions. This kit is designed for the in vitro synthesis of large amounts of efficiently and correctly capped mRNA with a poly(A) tail suitable for cancer vaccines (*see* **Note 9**). After transcription, the remaining plasmid DNA is removed by DNase treatment to reduce the risk of introducing foreign DNA into the cells. Size and integrity of the mRNA are checked by gel electrophoresis and quantity and purity are determined by spectrophotometry. Good quality mRNA is then stored at −20 °C in small aliquots (*see* **Note 10**).

3.3 Generation of Immature DC

This method describes the generation of clinical grade DC in vitro from plastic adherent monocytes in GM-CSF and IL-4 containing medium.

1. Adjust the concentration of the washed peripheral blood mononuclear cells (PBMC) to 10×10^6 cells/mL X-VIVO™-15 medium supplemented with 2 % AP.

2. Bring 800 mL of the cell suspension into 1× 4-tray Cell Factory.
3. Allow plastic adherence of the DC precursors ($CD14^+$ monocytes) for 1.5–2 h at 37 °C (*see* **Note 11**).
4. Remove the nonadherent cells for cryopreservation and wash the Cell Factory once with 250 mL of X-VIVO™-15.
5. Bring 800 mL of RPMI-1640 medium supplemented with 1 % AP, 1,000 U/mL of GM-CSF, and 500 U/mL of IL-4 into the Cell Factory.
6. Incubate the cells at 37 °C and 5 % CO_2 in a humidified incubator.
7. On days 2 and 4 of DC culture, add the same amount of GM-CSF and IL-4 to the cells as on day 0 in 20 mL of RPMI-1640 medium supplemented with 1 % AP.
8. On day 6 of DC culture, the cells are harvested for subsequent vaccine preparation.
9. An in-process quality control is performed on day 6 including viability, sterility, and mycoplasma detection.

3.4 Electroporation of DC for Vaccination

1. Prepare a 50-mL tube with 30 mL of RPMI-1640 medium supplemented with 1 % AP, 1,000 U/mL of GM-CSF, and 500 U/mL of IL-4 (at 37 °C).
2. Adjust the physical parameters of the Gene Pulser XCell electroporator as follows: voltage 300 V, capacitance 450 μF, and resistance ∞Ω.
3. Wash 50×10^6 DC with 10 mL of Opti-MEM.
4. While performing the washing step, prepare the following mRNA electroporation mix in a final volume of 600 μL of Opti-MEM:

   20 μg of CD40L mRNA

   20 μg of CD70 mRNA

   20 μg of caTLR4 mRNA

   60 μg of sig-TAA-DC-Lamp mRNA
5. Resuspend the washed DC in the mRNA electroporation mix and transfer into a 4-mm electroporation cuvette.
6. Insert the cuvette into the electroporation chamber and trigger the pulse.
7. Immediately after the electroporation, transfer the DC to the 50-mL Falcon tube with 30 mL of RPMI-1640 medium supplemented with 1 % AP, 1,000 U/mL of GM-CSF, and 500 U/mL of IL-4 and rinse the electroporation cuvette twice with DC culture medium.

8. If you have performed different electroporations (with different TAA), pool the different DC into 1 Ultra-Low Attachment T-75 flask and incubate the electroporated DC for 3.5–4 h in a humidified incubator at 37 °C and 5 % CO_2 (*see* **Note 12**).
9. Harvest the electroporated DC and freeze them at $\pm 12.5 \times 10^6$ per cryotube (*see* **Note 13**).
10. Perform quality control (*see* **Note 14**).
11. For vaccination, thaw four cryotubes of electroporated DC (*see* **Note 13**) and let them rest for 1.2 h in X-VIVO™-15 supplemented with IL-4 and GM-CSF.
12. For intravenous administration, resuspend 20×10^6 electroporated DC in 15 mL of 0.9 % NaCl/1 % HSA and transfer the DC to a sterile 20-mL syringe.
13. For intradermal administration, resuspend 4×10^6 electroporated DC in 250 μL PBS supplemented with 1 % HSA and transfer the DC to a sterile 1-mL syringe.
14. The DC can now be used for vaccination.

3.5 Patients, Treatment Schedule, Clinical Evaluation, and Immunomonitoring

We treat patients with recurrent stage III or stage IV melanoma in academic, single-center clinical trials. These patients are incorporated in the study after written informed consent and with approval of the study protocol by the institutional ethical committee and national competent authorities.

DC therapy is administered by 4 biweekly intradermal (ID) and intravenous (IV) injections, and a 5th administration is scheduled 8 weeks after the 4th immunization in the absence of disease progression. TriMix-DC are administered IV during a 15-min infusion by constant flow rate in a peripheral vein. At the same time, TriMix-DC are injected ID at two different anatomical sites (axilla and inguinal region). Each patient is closely monitored for at least 1 h after the end of the IV administration (*see* **Note 15**).

Patients undergo two leukaphereses, one before treatment and one after the 4th administration (*see* **Note 16**). DC therapy is prepared from the first leukapheresis and the nonadherent fraction of both aphereses is frozen for immunomonitoring.

Tumor response assessments (by RECIST) are performed by [(18)F]-fluorodeoxyglucose positron emission tomography/computed tomography (FDG-PET/CT) at baseline and in weeks 8, 16, and 24.

Immunomonitoring is performed both on delayed type IV hypersensitivity (DTH) skin infiltrating lymphocytes (SKILs) [27] and on peripheral blood $CD8^+$ T cells [13]. One week after the fourth DC administration, a small amount of TriMix-DC (5×10^5) is injected ID to induce a DTH from which skin biopsies are taken 48 or 72 h later. After 2.5 weeks of in vitro culture in the presence of 100 U/mL IL-2, SKILs are harvested.

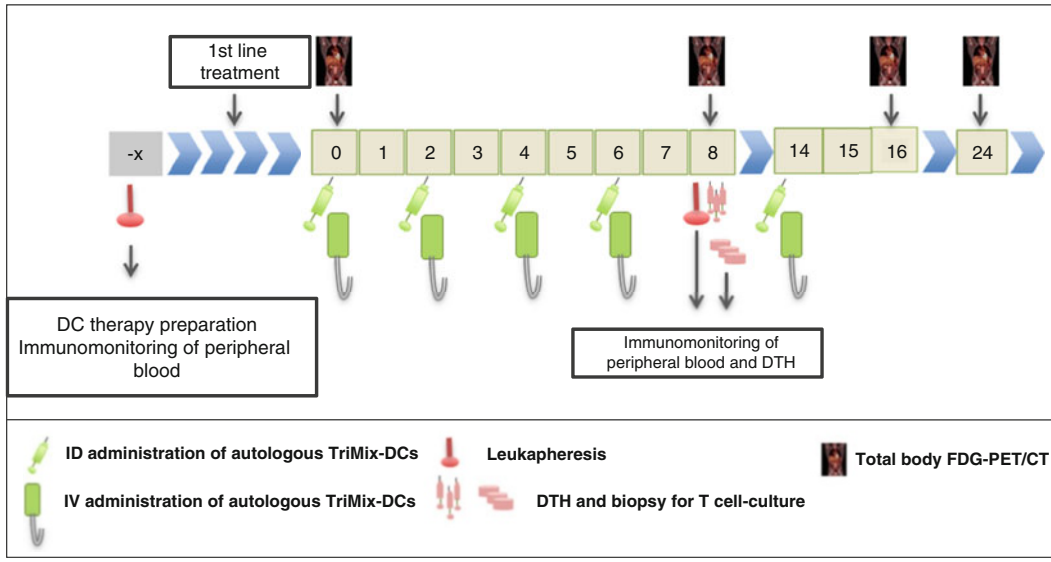

Fig. 2 Treatment schedule of melanoma patients receiving ID and IV administration of autologous TriMix-DC. Clinical evaluation and immunomonitoring time points are indicated

Their antigen-specific activation (CD137 upregulation [28]), cytolytic capacity (CD107a upregulation [29]), and cytokine release (IFN-γ and TNF-α) in response to autologous EBV-B cells expressing the antigens present in the TriMix-DC vaccine are assessed. For immunomonitoring of the peripheral blood of the patients, $CD8^+$ T cells are stimulated weekly with autologous TriMix-DC co-electroporated with one of four different tumor antigens at a 10:1 ratio for 2 or 3 weeks. Their antigen specificity is then determined as for the SKILs.

A schedule of the TriMix-DC administrations, leukaphereses, clinical evaluation, and immunomonitoring assays is given in Fig. 2.

4 Notes

1. Leukapheresis is performed with a COBE Spectra Apheresis System (CaridianBCT, Zaventem, Belgium) under continuous supervision of a trained physician, and approximately 12 L of blood is processed. The leukapheresed PBMC are then washed with a COBE 2991 Cell Processor (CaridianBCT) to remove contaminating platelets. Samples from the washed cell suspension are tested for hematocrit, total white blood cell count, and platelet count.
2. DC for vaccination must be produced in a clean room following the current guidelines of Good Manufacturing Practice. We have designed a system to produce large amounts of DC in

a closed system using Cell Factories suitable for clinical use [30]. Tubing sets with sterile connections and septa for injections to transfer the PBMC to the culture vessel and to perform the necessary washing steps, the addition of cytokines and AP, and the harvesting were designed. Typically, four to five Cell Factories can be filled with the PBMC of one single leukapheresis.

3. When granulocyte contamination of the leukapheresis product is less than 5 %, monocyte enrichment is performed by counterflow elutriation instead of by plastic adherence. Before elutriation, monocyte and granulocyte content of the PBMC are measured using the CELL-DYN Sapphire hematology analyzer. Continuous counterflow elutriation of leukapheresed PBMC is performed with the Elutra Cell Separation System (CaridianBCT) with single-use, functionally sealed disposable Elutra sets (CaridianBCT). After priming, the leukapheresis product is loaded via the inlet pump into the constantly rotating (2,400 rpm) elutriation chamber. The automation mode produces five elutriation fractions (F1–F5), each specified by a centrifuge speed, elutriation buffer flow rate, and a process volume. All fractions are collected in RPMI-1640 medium supplemented with 1 % AP. The final monocyte-enriched fraction (F5) is collected into the final collection bag when the centrifuge is stopped, i.e., the collection of the chamber content with the rotor off. All procedures are conducted according to the manufacturer's recommendations. After elutriation, DC culture is started by seeding $400–600 \times 10^6$ monocytes per 4-tray Cell Factory in 800 mL of RPMI-1640 medium supplemented with 1 % AP, 1,000 U/mL of GM-CSF, and 500 U/mL of IL-4.
4. GM-CSF and IL-4 are prepared in-house but are also commercially available. The cytokines prepared in-house are animal-protein- and endotoxin-free. Their biological activity is titrated against standards obtained from the National Institute for Biological Standards and Controls (NIBSC, South Hills, UK).
5. AP is collected from each patient and decomplemented at 56 °C for 50 min. Then, plasma is centrifuged at $23,000 \times g$ for 20 min at 4 °C. Human AB serum can be used as an alternative.
6. The primary aim of this approach is to obtain HLA class II-mediated presentation of antigen-derived $CD4^+$ helper T cells in addition to HLA class I-mediated $CD8^+$ cytotoxic T cells. Both are pivotal for the induction of an effective and long-lasting antitumor immunity [17]. Tumor-specific antigens MAGE-A3 and MAGE-C2 and differentiation antigens gp100 and tyrosinase were chosen. Almost every TAA-encoding sequence can be used for cloning.

7. Plasmid DNA must be linearized with a restriction enzyme downstream of the insert to be transcribed. Circular plasmid templates will generate extremely long, heterogenous RNA transcripts. It is important to examine the linearized template DNA on a gel to confirm that cleavage is complete.
8. Our observations report increased expression of CD40L and caTLR4 after in silico optimization (GENEART). Nevertheless, for some genes, no major impact on gene expression is observed, including CD70 expression.
9. A modified cap, Anti-Reverse Cap Analog (ARCA), is used which allows T7 RNA polymerase to synthesize RNAs capped exclusively in the correct orientation. Substitution of traditional cap analog with ARCA allows for synthesis of capped RNAs that are 100 % functional, in contrast to transcription reactions using traditional cap analog where only half of the cap analog is incorporated in the correct orientation. As a result, ARCA Cap mRNA molecules are more efficiently translated and much higher protein expression levels can be achieved than from mRNA made with the standard cap.
10. Gel electrophoresis of the transcribed mRNA should confer one single, sharp band. If not, mRNA might be degraded or improperly digested. mRNA quantity is measured at 260 nm and 280 nm. Pure RNA has an A260/A280 ratio of 1.9–2.1. If not, RNA might be contaminated with protein or DNA. After aliquoting and freezing, avoid freeze-thawing the mRNA.
11. Plastic adherence of the PBMC in Cell Factories is not feasible in RPMI-1640 medium. Therefore, we use X-VIVO™-15 medium.
12. We typically perform eight electroporations, 2× with 50×10^6 DC with TriMix-mRNA plus TAA (either gp100, MAGE-A3, MAGE-C2, or tyrosinase). This yields enough electroporated DC for one treatment cycle.
13. DC are frozen in cryotubes at $\pm 12.5 \times 10^6$ DC per tube in 1 mL of AP with 10 % DMSO and 2 % glucose. The DC are slowly frozen to −80 °C using a cryofreezing container and subsequently stored in liquid nitrogen until use. For thawing, DC are quickly thawed in a 37 °C water bath, transferred to DC culture medium, and pelleted at room temperature. The thawed DC are then resuspended in 5 mL of pre-warmed culture medium. Cell number and viability are determined with trypan blue. Typically, cells are >80 % viable and >90 % of the frozen cells are recuperated after thawing.
14. The final product must be monitored and reported prior to its release for clinical use. The endpoints for the quality control are the number of DC, purity (determined by flow cytometric light scatter properties) and viability (determined by trypan

blue exclusion), electroporation efficiency (measured by the % of cells expressing CD70), the immuno-phenotype (including CD40, CD80, CD83, CCR7, CD14 expression), and functional characterization by IL-12p70 secretion between 0–24 h and 24–48 h. DC-therapy samples are analyzed for sterility by long-term microbiological culture and tested for mycoplasma infection by PCR.

15. Post-infusion grade 2 chills are observed in some patients receiving IV infusion. Chills typically start about 15 min after the end of the IV infusion of TriMix-DC and resolve spontaneously within 30 min.
16. In an ongoing phase II clinical trial, patients are treated with a combined ID/IV [ID (4×10^6 DC) and IV (20×10^6 DC)] TriMix-DC therapy in combination with an antibody directed against the cytotoxic T-lymphocyte antigen 4 (anti-CTLA-4; ipilimumab). In this study, patients undergo a leukapheresis before the start of the treatment and a buffy coat at the end of the treatment.

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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