# Generation and cryopreservation of clinical grade Wilms' tumor 1 mRNA-loaded dendritic cell vaccines for cancer immunotherapy

**Chapter 3**

# Generation and cryopreservation of clinical grade *Wilms' tumor 1* mRNA-loaded dendritic cell vaccines for cancer immunotherapy

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**Running Head:** *WT1* mRNA-electroporated dendritic cell vaccines

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**Abstract**

First described in the seventies, dendritic cells (DC) are currently the subject of intense investigation to exploit their unique antigen-presenting and immunoregulatory capacities. In cancer, DC show promise to elicit or amplify immune responses directed against cancer cells by activating natural killer (NK) cells and tumor antigen-specific T cells. Wilms' tumor 1 (WT1) protein is a tumor-associated antigen that is expressed in a majority of cancer types and has been designated as an antigen of major interest to be targeted in clinical cancer immunotherapy trials. In this chapter, we describe the generation, cryopreservation and thawing of clinical grade autologous monocyte-derived DC vaccines that are loaded with WT1 by messenger RNA (mRNA) electroporation. This in-house developed transfection method gives rise to presentation of multiple antigen epitopes and can be used for all patients without restriction of human leukocyte antigen (HLA) type.

**Key words:** Dendritic cells, electroporation, RNA, clinical grade, Wilms' tumor 1, WT1

## 1. Introduction

Dendritic cells (DC) are currently being investigated in clinical trials to treat cancer patients, due to their unique capacity to direct immune responses toward tumor cells by activating natural killer (NK) cells and tumor-specific T cells [1-3]. Presentation of tumor-associated antigens by DC in an active state can license T cells to attack tumor cells.

Because of their low numbers *in vivo*, most DC used in clinical vaccination trials are obtained by *ex vivo* differentiation of monocytes in the presence of interleukin (IL)-4 and granulocyte macrophage colony-stimulating factor (GM-CSF) [2,4]. Alternative protocols to generate immunostimulatory monocyte-derived DC include differentiation of monocytes in the presence of IL-15 [5-8] or interferon-α [9].

In our laboratory, a safe and efficient method was developed to load DC *in vitro* with tumor-associated antigens by means of messenger RNA (mRNA) electroporation [10,11]. This non-viral gene transfer technique results in high, transient expression of the encoded protein(s) without the risk of insertional mutagenesis, in contrast to DNA electroporation, and is now widely implemented in immunotherapy to load DC with antigens and/or immunomodulatory molecules [12,13,7,14-17].

Our main tumor-associated antigen of interest is the Wilms' tumor protein 1 (WT1). WT1 has been evaluated as having top priority as target antigen in tumor immunotherapy trials because it fulfills several criteria, including expression in a variety of cancer types, high immunogenicity and involvement in oncogenesis [18-20]. Loading of DC with the WT1 protein by mRNA electroporation leads to presentation of multiple epitopes and can be performed for all patients, without the need to take the human leukocyte antigen (HLA) type into consideration [12,14,21].

For logistical reasons, for each patient we generate several DC vaccines out of one leukapheresis product [22]. DC vaccines are then stored in liquid nitrogen and thawed two hours before injection. Here we will describe the generation, cryopreservation and thawing of clinical-grade autologous monocyte-derived DC vaccines loaded with WT1 by mRNA electroporation. These DC have been successfully used to prevent or postpone relapse in patients with acute myeloid leukemia (AML) [16], are currently tested in a multicenter randomized controlled phase II trial in AML patients, and are also being investigated in patients with different types of solid tumors [22].

## 2. Materials

### *2.1. Generation of human monocyte-derived DC*

1. Monocytes, obtained from leukapheresis products after CD14 magnetic bead labeling and isolation with the CliniMACS system (Miltenyi Biotec), according to the manufacturer's protocol
2. Cell culture medium consisting of CellGro DC Medium (CellGenix) supplemented with 1% human AB serum (PAA), 250 U/mL recombinant human (rh)IL-4 (Miltenyi) and 800 U/mL rhGM-CSF (Gentaur)
3. Opti-MEM Reduced Serum Medium, phenol red-free (Gibco)
4. Prostaglandin E2 ($PGE_2$; Prostin E2, Pfizer)
5. Rh tumor necrosis factor (TNF)-α (Miltenyi)
6. Keyhole limpet hemocyanin (KLH, Immucothel)
7. T175 flasks (BD Falcon)
8. Centrifuge
9. 50-mL tubes

10. 5-mL, 10-mL, 25-mL, 50-mL pipettes

### *2.2. mRNA electroporation of mature DC*

1. Gene Pulser XCell Electroporation System for eukaryotic cells, including main unit, CE unit and ShockPod cuvette chamber (BioRad)
2. 4-mm electroporation cuvettes
3. Cell culture medium at room temperature consisting of CellGro DC Medium (CellGenix) supplemented with 1% human AB serum (PAA), 250 U/mL rhIL-4 (Miltenyi) and 800 U/mL rhGM-CSF (Gentaur)
4. Opti-MEM Reduced Serum Medium (Gibco)
5. 50-mL tubes
6. Transfer pipettes
7. Micropipettes and filter tips for volumes from 5 to 1000 µL
8. 6-well low adherence plates (Corning)
9. 0.9% NaCl
10. 20 µg of 1 µg/µL mRNA encoding WT1 (Curevac) per electroporation
11. 5-mL, 10-mL, 25-mL, 50-mL pipettes

### *2.3. Cryopreservation of electroporated mature DC*

1. Cold cryopreservation medium consisting of human AB serum (PAA) supplemented with 10% dimethyl sulfoxide (DMSO; Cryosure) and 2% glucose (Glucosteril, S.A. Laboratories)
2. 1.5 mL-cryopreservation tubes
3. Cryofreezing container (Nalgene Cryo 1°C Freezing Container, with a rate of cooling of -1°C/minute)

### *2.4. Thawing of electroporated mature DC and preparation of the vaccine*

1. 37°C water bath
2. Small zip lock bags
3. 6-well low adherence plate (Corning)
4. Cold thawing medium consisting of CellGro DC Medium (CellGenix) and 1% human AB serum (PAA)
5. Warm incubation medium consisting of CellGro DC Medium (CellGenix), supplemented with 1% human AB serum (PAA), 250 U/mL rhIL-4, 800 U/mL rhGM-CSF, 2.5 µg/mL $PGE_2$ and 600 U/mL TNFα
6. 50-mL tubes
7. 0.9% NaCl
8. 1-mL syringe
9. 23G x 1'' (0.6 x 25 mm) needle
10. Transport box

## 3. Methods

Carry out all procedures at room temperature unless otherwise specified. In order to generate clinical grade DC vaccines, all procedures should be carried out in a clean room, separately patient per patient **(see Note 1**).

### *3.1 Generation of human monocyte-derived DC*

1. Transfer the monocytes from the CliniMACS cell collection bag to 50-mL tubes and determine the cell volume, concentration and viability.
2. Centrifuge at 480 g for 6 minutes.

3. Culture the monocytes in T175 flasks at a minimum of 50 x $10^6$ and a maximum of 70 x $10^6$ total cells per flask in 50 mL culture medium.
4. Resuspend and pool the cell pellet(s) in culture medium (50 mL x number of T175 flasks required) and distribute the cell suspension into the T175 flasks using a 50-mL pipette.
5. Keep the flasks in a horizontal position at 37°C and 5% $CO_2$ in a humidified incubator for 8 days (start of the culture is day 0).
6. At day 3 or 4, add 250 U/mL rhIL-4 and 800U/mL rhGM-CSF to the culture medium.
7. At day 6, add 2.5 µg/mL $PGE_2$ and 600 U/mL TNFα to the culture medium for DC maturation and 10 µg/mL KLH as an adjuvant.
8. At day 8, harvest the mature DC from the T175 flasks:
    8.1. Tap the flask on the bottom and the sides to loosen the cells.
    8.2. Resuspend the cells with a 25-mL pipette and pipette the cell suspension up and down to detach as many cells as possible.
    8.3. Transfer the cell suspension to a 50-mL tube.
    8.4. Rinse the bottom of the flask with 25-mL Opti-MEM by pipetting up and down with a 25-mL pipette.
    8.5. Transfer the cell suspension to a 50-mL tube.
    8.6. Harvest, collect and pool the cells from all flasks into 50-mL tubes.
    8.7. Centrifuge at 480 g for 6 minutes.
    8.8. Remove the supernatant using a pipette.

### *3.2. mRNA electroporation of mature DC (see Note 2)*

1. Resuspend the cell pellets in Opti-MEM and pool the cell pellets into 100 mL in two 50-mL tubes **(See Note 3)**.
2. Centrifuge at 480 g for 6 minutes.

3. Repeat the washing step with Opti-MEM**.** Resuspend the cell pellets in one 50-mL tube in a volume of Opti-MEM suitable for accurate cell counting **(See Note 4)**.
4. Determine cell number and viability.
5. Calculate the number of electroporations required. Each electroporation is performed in a minimum of 200 µL and a maximum of 500 µL Opti-MEM, with a maximum total cell concentration of 50 x $10^6$/500 µL.
6. Centrifuge the cell suspension at 480 g for 6 minutes.
7. During centrifugation, prepare 50-mL tubes with 10 mL culture medium per electroporation, as well as empty 50-mL tubes.
8. After centrifugation, remove the majority of the supernatant with a pipette.
9. Remove the remaining supernatant using a filter tip.
10. Resuspend the cell pellet in Opti-MEM (500 µL x number of electroporations required), taking the cell pellet volume into account.
11. Distribute the cell suspension into the cuvettes using a filter tip.
12. Adjust the electroporation settings on the gene pulser: time constant protocol, time 7 millisec; voltage 300 V; cuvette 4 mm.
13. Add 20 µg of mRNA to the cell suspension, tap the cuvette to disperse the mRNA throughout the suspension **(See Note 5).**
14. Insert the cuvette into the ShockPod cuvette chamber inside the laminar flow, close the chamber and trigger the pulse.
15. Immediately after electroporation, add culture medium to the cell suspension with a transfer pipette and transfer this mixture of medium and cell suspension to a second empty 50-mL tube.

16. Rinse the cuvette twice with the remaining fresh culture medium and transfer the rinse suspension and the rest of the remaining medium to the second 50-mL tube, to obtain a cell concentration of about 5 x $10^6$/mL.
17. Repeat this for each electroporation.
18. Distribute the cell suspension into 6-well low adherence plates at 3-4mL/well.
19. Incubate for 2 hours at 37°C and 5% CO2 in a humidified incubator.
20. Harvest the electroporated mature DC with a transfer pipette and transfer cell suspension to 50-mL tubes.
21. Rinse the wells with 0.9% NaCl, and add to the rest of the cell suspension to the 50-mL tubes.
22. Centrifuge at 480 g for 6 minutes.
23. Remove the supernatant with a pipette.
24. Resuspend the cell pellets in 0.9% NaCl and pool them in one 50-mL tube in a volume suitable for accurate cell counting **(See Note 4)**.
25. Determine cell number and viability.

***3.3 Cryopreservation of electroporated mature DC (see Note 6)***

1. Centrifuge the cell suspension at 480 g for 6 minutes.
2. Remove the supernatant with a pipette.
3. Resuspend the cell pellet in cold cryopreservation medium at a viable cell concentration of 10—20 x $10^6$/mL **(See Note 7).**
4. Distribute the cell suspension into labeled cryovials **(See Note 8).**
5. Transfer the vials to a freezing container and place it immediately in a -80°C freezer.
6. After 48 hours, transfer the cryovials to the gas phase of liquid nitrogen.

### *3.4. Thawing of electroporated mature DC and preparation of the vaccine*

1. Thaw a vial of electroporated mature DC in a 37°C water bath with constant, moderate agitation until there is still a small clump of frozen suspension left in the vial.
2. Dilute the thawed cells in 30 mL cold thawing medium in a 50-mL tube as soon as possible.
3. Rinse the vial with the cold thawing medium and transfer the rinsing suspension back to the 50-mL tube.
4. Centrifuge at 480 g for 6 minutes.
5. Remove the supernatant with a pipette and resuspend the cell pellet in 6 mL warm incubation medium.
6. Distribute the cell suspension at 3 mL per well in a low adherence 6-well plate.
7. Incubate for 2 hours at 37°C and 5% $CO_2$ in a humidified incubator.
8. Harvest the cells with a transfer pipette and transfer the cell suspension to a 50-mL tube.
9. Rinse the wells with 0.9% NaCl, and add to the rest of the cell suspension in the 50-mL tube. Fill up to 30 mL with 0.9% NaCl.
10. Centrifuge at 480 g for 6 minutes, and remove supernatant with a pipette.
11. Resuspend the cell pellet in 30 mL 0.9% NaCl in order to wash the cells.
12. Centrifuge at 480 g for 6 minutes, and remove supernatant with a pipette.
13. Repeat washing step.
14. Resuspend the cell pellet in 10 mL 0.9% NaCl.
15. Determine cell number and viability.
16. Fill up to 30mL with 0.9% NaCl, centrifuge at 480 g for 6 minutes, remove supernatant with a pipette.
17. Resuspend the cell pellet with 0.9% NaCl to obtain the appropriate concentration required for vaccination (i.e. 10 x $10^6$/500 µL), taking the cell pellet volume into account.

18. Aspirate 600-700 µL of the cell suspension with a labeled 1-mL syringe and 23 G needle. This will allow to administer 500 µl of the DC suspension to the patient, following the loss of volume during purging of the air from the dead volume of the syringe and needle.
19. The vaccine is now ready for administration. Transport the vaccine in a labeled zip lock bag inside a sealed transport box.

**4. Notes**

1. The entire procedure for the manufacturing of DC vaccines, from isolation of the monocytes to preparation of the vaccines, must be performed in a clean room facility grade A in B, following the guidelines of Good Manufacturing Practice (GMP). All reagents used must be GMP-grade and the materials must be certified for use in a GMP-grade facility.

   Traceability of all used products and of the DC vaccines produced must be ensured. Quality control of the working environment must be ensured by daily sterility measurements and continuous monitoring of the air quality. Batch quality control of the cell products must be ensured by performing sterility controls at different time points during production, as well as at the end point, which is the thawed DC vaccine before administration. Next to sterility controls, extra quality controls must be performed on the cell products, including cell recovery, concentration, viability, phenotypic markers, protein expression (quality control of the electroporation efficiency) and migratory capacity.
2. Working with RNA requires an RNAse-free environment. It is important to wear gloves and use filter tips throughout the procedure.
3. The three washing steps of the mature DC with Opti-MEM prior to electroporation ensures serum-free and thus RNAse-free conditions required for mRNA electroporation.

4. In order to determine a correct volume for cell counting, the detection limits of the counting device must be taken into account. Using a HORIBA ABX MICROS 60 OT Hematology Analyzer (Horiba) with the detection limit maximum at 20 x $10^6$ cells per mL, we resuspend the cells in 50 mL for counting.
5. When electroporating 20 x $10^6$ cells or less, 10 µg of mRNA per electroporation is sufficient. It is important to work quickly during the electroporation procedure, in order to avoid mRNA degradation after it has been added to the cell suspension and to reduce the exposure time of cells to serum-free conditions. After pulsing, the cells must be resuspended as quickly as possible in serum-containing medium to speed up the resealing process of the membrane pores.
6. When working with DMSO, it is important to perform freezing of the cells as quickly as possible and in cold DMSO solution, since the cryoprotectant DMSO is toxic for cells at room temperature.
7. The concentration and the total number of cells to be cryopreserved per vial depends on the number of viable cells required for vaccine administration as specified in the trial protocol. After thawing, the possibility of a loss of 50% of the cells should be taken into account. Thus for the preparation of 1 vaccine dose of e.g. 10 x $10^6$ DC, ideally 20 x $10^6$ DC should be cryopreserved in 1 cryopreservation vial.
8. In order to ensure traceability of the vaccines produced, labeling of all cell products is very important. Labels should mention product number, patient ID, production date, expiry date and storage conditions.

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