

Murine Dendritic Cells for Immunotherapy and Vaccine Development: Generation, Optimization and Transduction

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ABSTRACT

Introduction: Dendritic cells (DCs) play crucial roles in cellular immunity as the most powerful antigen presenting cells. They have been widely used for antigen delivery in vivo and in vitro. There are different ways to generate DCs and also gene transduction. In this study we introduce some optimization in order to produce high amount of well differentiated murine DCs for potential immunotherapy and vaccine development applications. **Methods:** Murine bone marrow cells were isolated from male BALB/c mice and the cells were cultured with complete RPMI in presence of the same ratio of IL-4 and GM-CSF. Some changes were made in the medium and the lysis buffer applications to increase the differentiation rate of the cells. Lentiviral virions were applied to transfer the genes of interest to DCs with no pre-maturation steps. CD11c, MHC-II, CD80 and CD86 were assessed by flow cytometry. **Results:** The optimized steps led to significant increase in number of the isolated cells. IL-4 usage in a similar dose to GM-CSF led to macrophage formation inhibition. Lentiviral virions resulted in successful gene delivery along with well-maturated DCs. **Conclusion:** The introduced optimized steps could be followed in different DC applications by using lentiviral virions to transduce DCs, independent of the pre-maturation steps.

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INTRODUCTION

Dendritic Cells (DC) are capable to stimulate a variety of immune cells including naïve and memory CD8⁺ T-cells, CD4⁺ helper T-cells as well as B-cells which characterize them as most powerful antigen presenting cells [1]. At their immature stage, DCs process foreign antigens to the immune system in blood and tissues. Presentable antigen uptake stimulates DC maturation and promotes their migration to the lymph nodes, where the interaction with the immune effector cells can directly occur [2, 3]. While antigen presentation is being processed, the biosynthesis of co-stimulatory receptor molecules, such as CD80, CD86 and CD40 on DC plasma membrane is upregulated. The DC receptors bind to CD28 receptors on the Th₀ cell membrane, which results in IL-12 or IL-10 cytokines secretion and subsequently T cell differentiation [4-8]. The antigen could be from different

sources including whole protein, selected peptides or mRNAs [3, 9, 10].

Several experimental methods have been investigated to optimize DC use in immunotherapy and vaccine development. DC vaccines are generally autologous bone marrow derived cells which are then matured *in vitro* and uploaded with the antigen of interest before application. This process consists of DCs generation in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) plus interleukin 4 (IL-4) for one week, followed by stimulation. As a growth factor for the stem cells, GM-CSF increases the expression of class II major histocompatibility complex (MHC-II) antigens and enhances the DCs antigen presenting capacity. IL-4 inhibits the development of macrophages and granulocytes and can keep

the DCs in immature stage which makes them more capable of exogenous antigens processing.

Maturation is an essential step for DC functions which enhances their antigen presenting ability. There have been many studies on DC maturation *ex vivo* by stimuli including microbial products, lipopolysaccharide (LPS), prostaglandin E (PGE), CD40 ligand and other combinations of molecules [11-14]. In this study, we present an optimized murine DC generation in different steps by applying lentiviral virions to transfer the genes of interest and to mature the DCs at the same time.

MATERIALS AND METHODS

Ethics Approval

This study was approved by the ethics committee of Pasteur Institute of Iran, Tehran (No# IR.PII.REC.1398.2).

Reagents

RPMI 1640 culture medium and FBS (fetal bovine serum, Gibco, Germany), Penicillin-Streptomycin and L-Glutamine (Biosera, France) were applied to culture the cells. Murine GM-CSF (mGM-CSF) and murine IL-4 (mIL-4; PeproTech, Rocky Hill, USA) were used to stimulate DC formation. CD11c and MHC-II (FITC eBioscience™), CD80, CD86 (PE eBioscience™) murine monoclonal antibodies were applied to evaluate DC's formation and maturation.

Murine DC Generation

We applied 2 male BALB/c mice which were 8-10 weeks old. The mice were kept according to the standard conditions provided by Pasteur Institute of Iran. They were anesthetized and euthanized by cervical dislocation. Back legs above the hip

joint were cut in order to access the femur and tibia, leaving knee and ankle joints in place. The muscle and tissue were rubbed by a sharp Scalpel. The isolated bones were transferred to a petri dish containing cold PBS containing 2X penicillin/streptomycin (20 µl/ml). The both ends of the bone were cut with scissors as close to the joints as possible. A 2 ml syringe was filled with ice-cold RPMI complete media and was inserted into the bone and flushed out the bone marrow into a centrifuge tube on ice until the bones were completely white. The femurs and tibias were removed and the muscle and tissue were removed. The cells were flushed into a falcon containing complete RMPI containing FBS 20% and penicillin/streptomycin 2X using a 2 ml sterilized syringe (Fig. 1).

The cell suspension was washed twice in RPMI medium by 5 min centrifugation in a 15 ml centrifuge tube at 1200 RPM in a refrigerated centrifuge (4°C). The cells were re-suspended in 2 ml cold Ammonium-Chloride-Potassium (ACK) lysis buffer and incubated for 5 min at room temperature in order to eliminate red blood cells. The ACK was removed following RPMI addition and centrifugation. The isolated cells were adjusted to 2×10^6 cells/ml in a non-treated 6-well plate with complete RPMI 1640 culture medium at the presence of 20 ng/ml mGS-CSF and 20 ng/ml mIL-4. The plate was placed in a CO₂ incubator (37° C, 5% CO₂). IL-4 was applied at the same ratio as GM-CSF. Fresh complete RPMI 1640 culture medium containing mGM-CSF and mIL-4 was added every other day. No cell type was removed during the whole process. The morphology of the cells was followed by an inverted microscope every day. On the 6th day, the non-adherent cells were harvested and collected to evaluate DC's CD markers by flow cytometry using CD11c monoclonal antibody.



Fig. 1. Cell isolation from murine bone marrow.

DCs Transduction

On day 6 when the majority of the cells achieved DC phenotype, recombinant lenti virions were applied to transduce the DCs, as described previously [15]. The virions were added at Transduction Unit (TU) of 20 without any maturation stimuli. The recombinant virions were tittered by HIV-1 p24 ELISA according to the provided protocol (XpressBio, USA). The cells were cultured at 37°C. Green fluorescent protein (GFP) expression, as a part of pCDH vector genome, was investigated by a fluorescent microscope, 24 h after the transduction. Three days after the transduction, suspended and loosely adherent cells were collected and CD11c, CD80, CD86

and MHC-II expressions were evaluated by flow cytometry. No cell type was removed during the process.

RESULTS

DC Morphology Evaluation

Murine bone marrow cells were isolated from two femurs and tibias in total number of 33 million. A selection of the cell differentiation images by inverted microscopy from day 0 till day 6 are shown in Fig. 2.

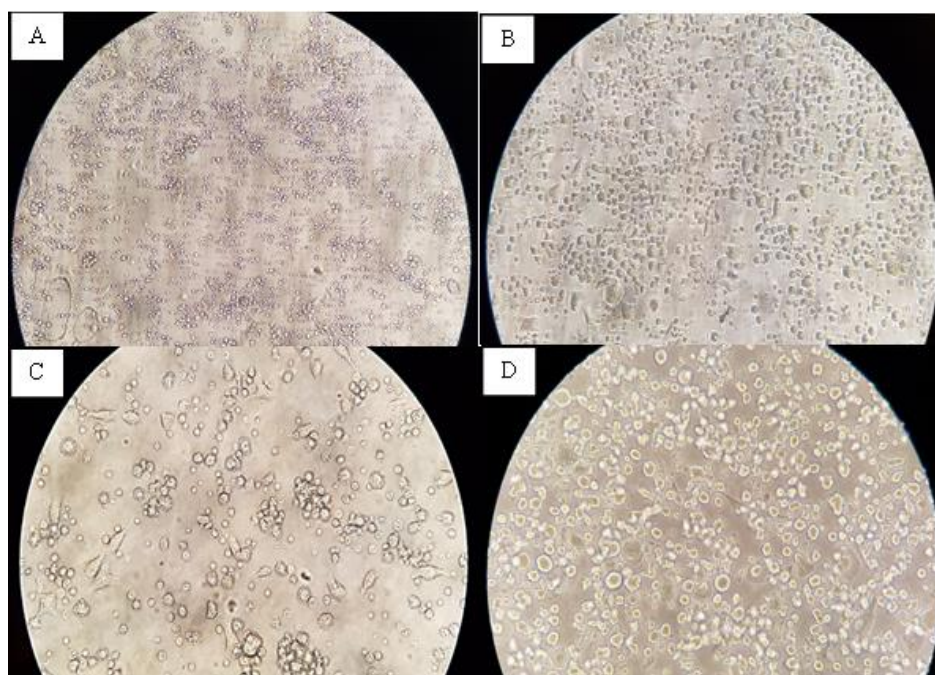


Fig. 2. Bone marrow cell differentiation images on different days. **A)** The isolated cells on day 0. **B)** Initiation of differentiation, 2 days after the cell culture. **C and D)** The well differentiated DCs on days 4 and 6, respectively.

The differences in IL-4 concentration in cell formation are shown in Fig. 3. It was clear that IL-4 could prevent macrophage formation when it was applied at the same concentration as mGM-CSF (Fig. 3. A-C). The left panel depicts the treatment of the isolated cells with 20 ng of IL-4 whereas

the right panel presents the cells treated with 10 ng of IL-4. It was clear that IL-4 could prevent macrophage formation when it was applied at the same concentration as mGM-CSF (Fig. 3 A-C).

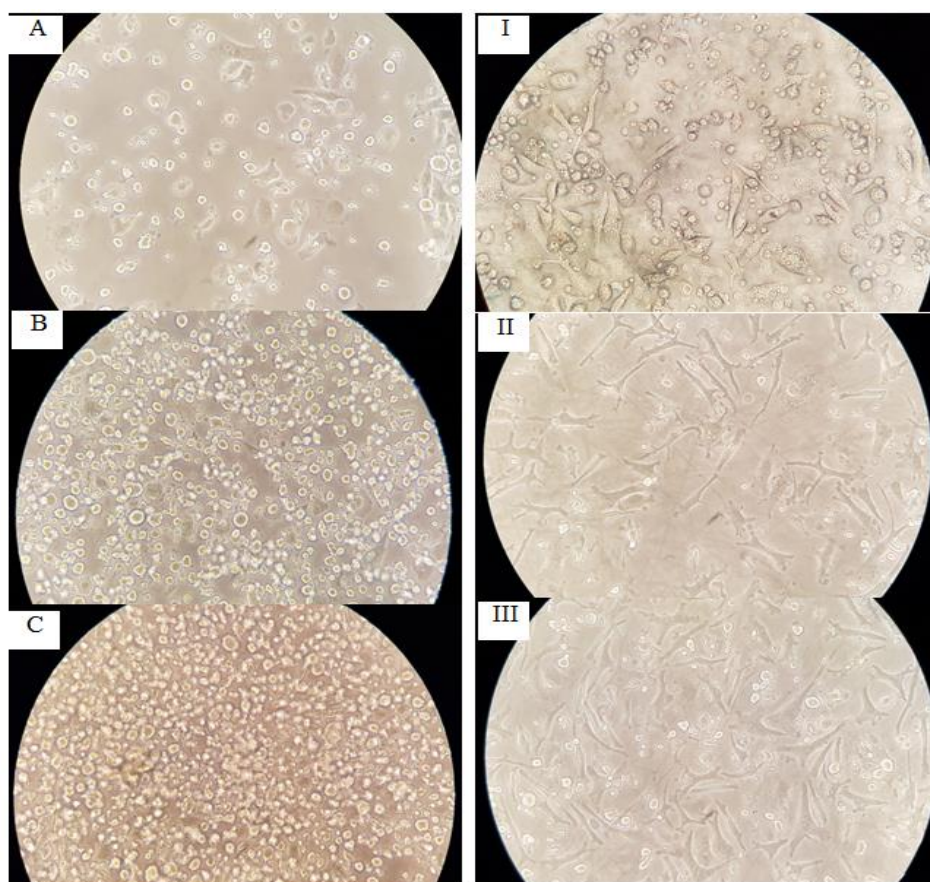


Fig. 3. Differences in cell differentiation by applying different concentrations of mIL-4. The left side panel (**A, B and C**) were cultured at the presence of 20 ng/ml of IL-4 on days 3, 5 and 6. The images on the right side panel (**I, II, III**) depict cells which were cultured at the presence of 10 ng/ml of IL-4 on the same days.

CD Markers Assessment

In order to assess DCs generation, flow cytometry was applied to evaluate expression of CD11c as the main murine CD marker on day 7. As shown in Fig. 4, two different

antibodies, conjugated with FITC or PE were applied. Nearly 88% of the treated cells expressed CD11c whereas approximately 16% of the control cells were CD11c positive.

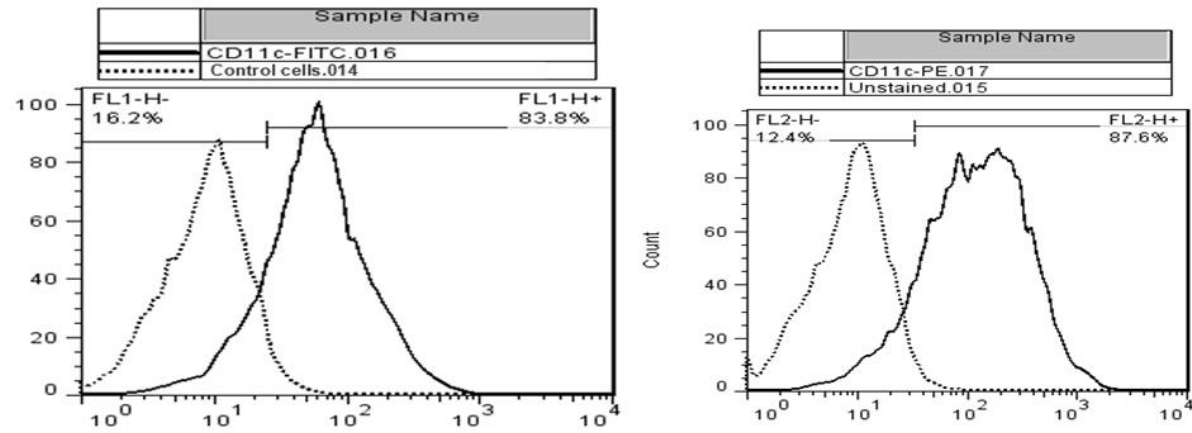
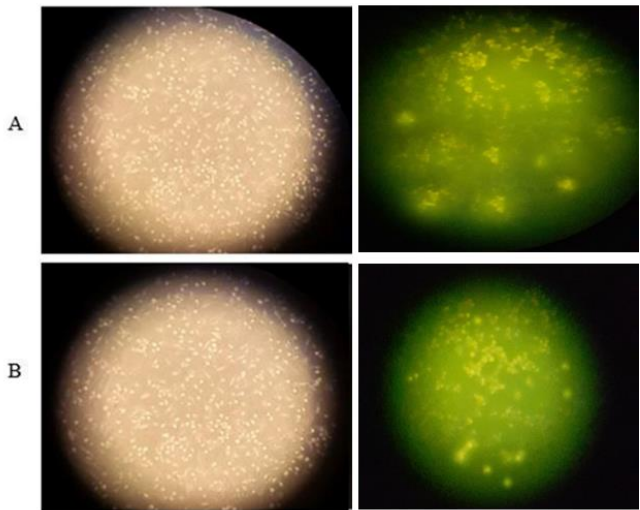


Fig. 4. CD11c evaluation by flow cytometry using anti CD11c (conjugated FITC/PE).



The generated virions were applied at TU of 20 on day 6 to transduce the confirmed DCs. The GFP expression was evaluated 24-48 h after the transduction (Fig. 5).

On day 9, the cells were collected to be assessed for MHC-II, CD80 and CD86 maturation markers as shown in Fig. 6.

As shown in Fig. 4, CD11c as the main marker of murine DC was well-expressed on both un-transduced and transduced cells. The expression of this marker indicated the successful differentiation of the DCs. The maturation markers CD80, CD86 and MHC-II then were investigated. As shown in Fig. 6, there was a significant difference between the groups of DCs in term of maturation which showed that the DCs were successfully transduced resulting in expression of CD80 and CD86 as maturation markers on the transduced DCs. MHC-II was also highly expressed on transduced DCs in comparison with the isotype control.

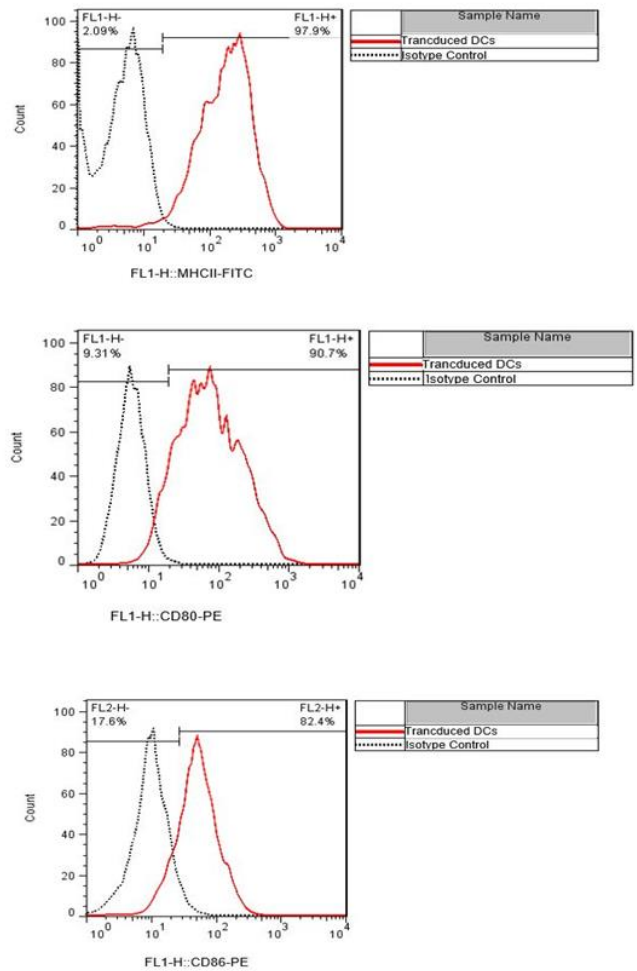


Fig. 6. CD marker expression on transduced CS. A) MHC-II, B) CD80 and C) CD86 maturation markers in comparison with the isotype control.

DISCUSSION

DCs represent a heterogeneous population although they originate from the bone marrow. DC subsets are divided into two main sorts, myeloid and plasma cytoid. Peripheral blood mononuclear cells (PBMCs) could differentiate to DCs under certain conditions, when exposed to pathogen-associated molecular patterns (PAMPS), including bacterial LPS, viruses, flagellin, or tumor-associated antigen (TAA), leading to a cytokine-mediated inflammatory response. DC vaccines are based on antigen-loaded autologous DCs as an active immunotherapy which could be applied to induce immune responses against a specific target [16-18]. There are several protocols to generate DCs from the bone marrow cells and also DC transduction. In this study, we aimed at optimization of the pertaining cell isolation as well as the cell transduction protocols.

The first optimized step was the age of the mice. We conducted two series of cell isolation in which firstly, male BALB/c mice 3-6 months were investigated according to their more effective young cell types, evaluated in previous studies [19]. The total number of cells from 4 femurs and tibias from two mice were nearly 5 million. In the next experiment, we applied 8-10 months mice which resulted in isolation of 33 million cells. Moreover, the procedure of bone marrow cell isolation includes applying 70% ethanol in many protocols [20, 21]. In two series of the procedures we performed, once a protocol with ethanol and the other time with PBS containing penicillin/streptomycin 2X, the result indicated that ethanol might reduce the number of cells. Therefore, in the optimized method, ethanol was replaced with PBS.

ACK is necessary to remove the red blood cells. Notably, the time of incubation is considerable. According to our experiences, the optimum time for incubation is 5 minutes. The longer time could negatively affect the obtained cell number. In some protocols, the suspended cells are removed on day 3 [22]. In this optimized procedure, no cell sorts were removed and fresh RPMI media containing FBS 20% and mGM-CSF (20 ng) with mIL-4 (20 ng) were added every other day. Combination of GM-CSF with IL-4 is known to drive monocytes to DCs (mDCs) *in vitro* [23]. Moreover, IL-4 DCs have been reported to be more efficient at inducing allogeneic T cell proliferation [24]. In the conducted procedures, we firstly applied 10 ng/ml of mIL-4 according to the previous protocols [25, 26] although some applied protocols did not use this cytokine [27]. As it is clearly shown in Fig. 3, the application of the same concentrations of both cytokines (*i.e.* 20 ng/ml), resulted in much more DC generation rather than the lower concentration. In fact, the lower concentration of mIL-4 resulted in macrophage formation (Fig. 3 I, II and III). Therefore, proper concentration of mIL-4 was evaluated as an important factor which could strongly inhibit macrophage formation.

In order to mature the generated DCs, LPS, exposure to microorganisms and simple chemicals such as polybrene have been widely used prior to the transduction [28, 22, 29, 30]. In a study by Dai *et al.*, DCs were generated in a similar way with no cell type removing. However, they applied polybrene to mature DCs before the transduction [31]. However, it has been shown that polybrene could negatively affect cell proliferation [32]. In another study, NiCl₂ and CoCl₂ have been shown to induce apoptosis in DCs [33]. In this study, we applied recombinant lentiviral virions to transduce the generated DCs without a need for prior maturation. The CD marker evaluation resulted in highly expressed CD80, CD86 and MHC-II in

comparison with the cell controls which means that virions are powerful tools to transfer the target gene and to induce DC maturation simultaneously. In conclusion, this study has provided an optimized murine DCs generation protocol which leads to high DC population with potential applications in immunotherapy and vaccine development. Moreover, DCs transduction and maturation were simply achievable by lentiviral virions without any pre-maturation steps.

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CONFLICT OF INTEREST

The authors declare they have no conflict of interests.

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