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Improved Method of *In vitro* Dendritic Cells Expansion for Immunotherapy

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Abstract

Developing efficient human dendritic cell-based immunotherapeutic vaccines is one of primary goals of cancer immunotherapy. This is hurdled by necessity to obtain extensive amounts of patient's dendritic cells, which consist a relatively small population in peripheral blood. This requirement entails the need to take large volume of patient's blood, which may be a limiting factor, since patient's condition is already undermined by developing tumor. In this paper we present an optimized method for obtaining large amounts of functional dendritic cells derived from proliferating monocytes to minimize necessary volume of blood taken from the patient. We have confirmed that overexpression of c-Myc and BMI1 genes leads to monocyte proliferation and optimized the method of transgene delivery to monocytes by novel bicistronic lentivirus vector containing sequences of both genes.

Key words: c-Myc, Bmi1, dendritic cells, immunotherapy, cancer vaccines

INTRODUCTION

Currently, multiple clinical trials of different immunotherapeutic approaches to cancer treatment are carried out. [1-4] Results of these trials are quite encouraging, which leads to the fact that in the nearest future immunotherapy will be widely used in clinics along with the radiotherapy and chemotherapy. One of the most widespread types of immunotherapy, both in experimental and clinical medicine, is immunotherapeutic vaccination with modified dendritic cells.

Dendritic cells are the main antigen-presenting cell population of the human immune system. The ability of dendritic cells to absorb foreign agents, digest and effectively present foreign antigens to T-lymphocytes on their surface, makes them highly promising in the immunotherapy of different diseases including cancer.

Different ways exist for production of immunotherapeutic vaccines containing dendritic cells activated against tumor cells. Two most common methods are loading dendritic cells with tumor antigens by cultivation with lysates of tumor cells; and genetic modification by loading dendritic cells with antigens to a specific type of tumor by genetic engineering [5, 6]. Vaccines created by later method were shown to be efficient against melanoma [7], malignant glioma [8], B-cell lymphoma [9], myeloma [10], myeloid leukemia [11] and many others.

One of the main drawback in cancer vaccine production is the need to withdraw sufficiently large amounts of dendritic cells from the patient (approximately 10^8 cells), while in human blood they are present in a relatively small amount. [12-14] Most popular strategy to obtain necessary amount of dendritic cells, which is widely used in the experimental science and medicine, is *in vitro* differentiation from non-proliferating progenitors, $CD14^+$ monocytes. Differentiation of monocytes into dendritic cells occurs by cultivation with granulocyte-macrophage colony-stimulating growth factor (GM-CSF) and IL-4 interleukin. [15] Despite the fact that monocytes are much more frequent in peripheral blood than dendritic cells, there remains a need to take a sufficiently large amount of blood from the patient to make an effective cancer vaccine. Such technique of obtaining dendritic cells significantly increases the risk of invasiveness of cancer.

This problem can be solved by obtaining proliferating population of CD14+ monocytes or dendritic cells. In the work [16] Haruto et al. present a method of generation of a large number of dendritic cells from the proliferating monocytes expanded by forced expression of c-Myc and BMI1 genes. In this work we improved this method, making it more efficient and accessible.

MATERIALS AND METHODS.

Cell culture

HEK-293T (human embryonic kidney) cells were cultured in DMEM/F12 (Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12) with 2 mM L-glutamine, 10% FBS (fetal bovine serum) and antibiotics penicillin and streptomycin. Peripheral blood was taken from a healthy donor. Peripheral blood mononuclear cells (PBMC) were obtained from blood by Ficoll density centrifugation. Fraction of $CD14^+$ monocytes were isolated from PBMC by EasySep human CD14 positive selection kit (StemCell Technologies, USA). Human monocytes and monocyte-derived dendritic cells were cultured in AIM-V serum free media with 50 ng/ml rhGM-CSF (Peprotech, UK) and 50 ng/ml rhM-CSF (Peprotech, UK). To obtain monocyte-derived dendritic cells, monocytes were cultured for 4 days in medium supplemented with 50 ng/ml rhGM-CSF (Peprotech, UK) and 50 ng/ml rhIL-4 (Peprotech, UK). The medium was replaced every 2 days with fresh cytokines.

Plasmid construction

pLenti-Ef1alpha-Myc-puro (pL-Myc), pLenti-Ef1alpha-BMI-puro (pL-BMI)

BMI1 and c-Myc genes were amplified from PBMC cDNA with by Tersus HS DNA Polymerase (Evrogen, Russia) and cloned by sites XbaI and BamHI in pLenti-EF1aplha-MCS lentiviral vector. Amplification of

the c-Myc fragment was conducted with 5'-AGAGAGTCTAGAACCATGGATTTTTTTCGGGTAGTGGAAAACCAGCAG-3' forward and 5'-AGAGAGGGATCCGGCGCACAAGAGTTCCGTAGCTGTTC-3' reverse primers. Amplification of the BMI-1 fragment was conducted with 5'-AGAGAGTCTAGAACCATGCATCGAACAACGAGAATCAAGATC-3' forward and 5'-AGAGAGGGATCCGGACCAGAAGAAGTTGCTGATGACCC-3' reverse primers.

pLenti-Ef1aplha-BMI-T2A-Myc-puro (pL-BMI-T2A-Myc)

BMI and c-Myc puro were amplified from pLenti-Ef1alpha-Myc-puro, pLenti-Ef1alpha-BMI-puro vectors. Direct primer of c-Myc gene and reverse primer of BMI1 contained the sequence of T2A. The fragment BMI-T2A-Myc was obtained by overlap PCR and cloned in the lentiviral vector pLenti-EF1aplha-MCS by sites XbaI and SpeI. Amplification of the c-Myc fragment was conducted with 5'-AGAGAGTCTAGAACCATGGATTTTTTTCGGGTAGTGGAAAACCAGCAG-3' forward and 5'-CACGTCACCGCATGTTAGAAGACTTCCTCTGCCCTCTCCGCTTCCCGCACAAGAGTTCCGTAGCTG-3' reverse primers. Amplification of the BMI-1 fragment was conducted with 5'-GAAGTCTTCTAACATGCGGTGACGTGGAGGAGAATCCCGGCCCTATGCATCGAACAACGAGAATC-3' forward and 5'-AGAGAGACTAGTTCAACCAGAAGAAGAAGTTGCTGATGACCC-3' reverse primers.

Virus preparation

For virus packaging were used 2nd generation lentiviral packaging plasmids: psPAX2 and VSV-G envelope expressing plasmid pMD2.G. psPAX2 and pMD2.G were gifts from Didier Trono (Addgene plasmid # 12260 and # 12259). Also we used helper plasmid pRSV-Rev-T2A-Vpx described in our previous work [17] for more efficient transduction of monocytes. HEK-293T cell line was transfected with psPAX2, pMD2.G and pRSV-Rev-T2A-Vpx and one of the pLenti-Ef1alpha-Myc-puro, pLenti-Ef1alpha-BMI-puro, pLenti-Ef1aplha-BMI-T2A-Myc-puro plasmids, respectively with the help of transfection reagent Turbofect (Thermo Scientific, USA). Cell supernatant was collected after 24, 48 and 72 hours of incubation, filtered through 0.45 μm filter, concentrated with PEG 8000 and pelleted at 6000 g for 30 min. Viruses were titrated on HEK-293T cells and the titer was determined by the use of flow cytometry. The virus stocks were frozen in RPMI 1640 medium supplemented with 10% FBS at -80 °C.

Transduction of monocytes

Monocytes were transduced by cultivation with viruses containing BMI1, c-Myc, BMI1-T2A-c-Myc genes for 8 hours in presence of polybrene (8μg/ml). After 8 hours, medium was changed to fresh AIM-V.

SDS-PAGE electrophoresis and Western blotting

Protein samples for SDS-PAGE electrophoresis were prepared by cell lysing in ice-cold RIPA lysis buffer (150 mM NaCl, 5 mM EDTA pH 8.0, 50 mM Tris pH 8.0, 1%, NP-40, 0.1% sodium deoxycholate, 0.1% SDS) with protease inhibitors (Amresco, USA) for 10 minutes and centrifugation at 13400 rpm for 10 minutes at 4 °C. Supernatant was mixed with 4x sample buffer (277.8 mM Tris-HCl, pH 6.8, 44.4% (v/v) glycerol, 4.4% LDS, 0.02% bromophenol blue, 10% 2-mercaptoethanol) and incubated at 95°C for 5 minutes. Samples were run on 10% SDS-PAGE gel under reducing conditions. Separated proteins were blotted onto PVDF (Polyvinylidene fluoride) membrane (Hybond GE Life Sciences, USA). Membrane was blocked in 5% solution of dry milk in PBS-T and incubated with primary rabbit polyclonal antibodies specific to c-Myc (Santa Cruz, USA) overnight at +4°C. After washing membrane with PBS-T (PBS, 0.05% Tween 20) PVDF was incubated with peroxidase-labeled goat anti-rabbit antibodies (Santa Cruz, USA) for 1 hour at room temperature. Peroxidase activity was measured by phemtoLUCENT Luminol Solution (GBioscience, USA) and visualized by GeneGnome (Syngene, USA). β-Actin was detected with mouse monoclonal β-Actin antibodies (C4, Santa Cruz, USA).

CellTiter-Glo 2.0 luminescent cell viability test

The number of monocytes during their proliferation period was measured by CellTiter-Glo 2.0 Luminescent Cell Viability Assay. Cells were seeded in the 96-well plate in 100 μl at 30000 per well. For preparation of CellTiter-Glo reagent the CellTiter-Glo buffer was mixed with the CellTiter-Glo substrate in ratio 1:1. 100 μl of the CellTiter-Glo reagent was added in each well and the condense of the plate was mixed for 2 minutes on an orbital shaker. Cells with the reagent were incubated at room temperature for 10 minutes to stabilize luminescent signal. The luminescence was detected on the microplate reader (Dynex Triad LT, USA)

RESULTS

Production of proliferating monocyte culture

In order to confirm the possibility of obtaining proliferating population of $CD14^+$ monocytes by ectopic expression of BMI1 and c-Myc genes we built the lentivirus vectors for separate expression of BMI1 and c-Myc. Viral particles were packaged in HEK-293T cells with the use of packaging plasmids psPAX2, PMD2 and helper plasmid pRSV-Rev-T2A-Vpx, which was designed earlier in our laboratory. In our previous work, it was shown that presence of Vpx protein in the virus envelope significantly increases efficiency of transduction of dendritic cells due to its ability to suppress of SAMHD1 protein responsible for anti-viral defense in dendritic cells. Monocytes were inoculated in 6 cm plates, $5x10^5$ cells per plate, and co-transduced with viruses containing BMI1 and c-Myc genes in 2 repeats. Non-infected monocytes were used as negative control, which were also inoculated in 6cm plate at a density of $5x10^5$ cells per plate. Cells were counted weekly during 4 weeks after infection.

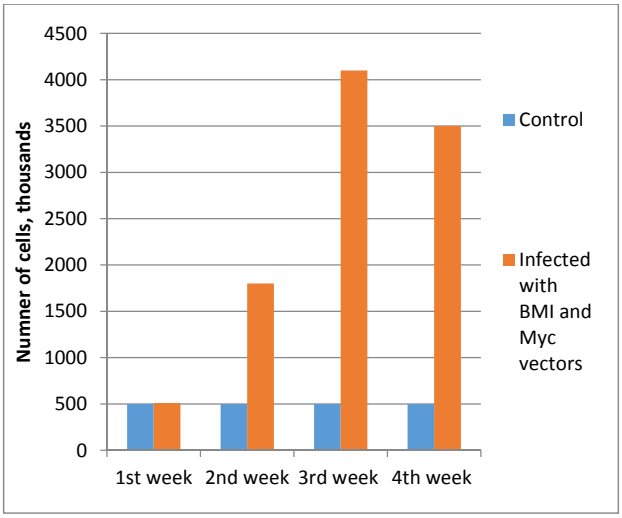

Figure 1. Dynamics of monocyte proliferation during 4 weeks after transduction with pL-BMI and pL-Myc viruses.

Within two weeks after co-infection, the population of monocytes tripled and reached its peak after three weeks with an increase in cell number of over 8-fold (Figure 1).

The fact that BMI1 and c-Myc overexpression in monocytes could influence their proliferative abilities was also confirmed by CellTiter-Glo 2.0 luminescent cell viability test. The monocytes derived from health donor were seeded in the 96-well plate (30 0000 cells per well) and transduced with the same titer of viruses containing BMI1 and c-Myc genes as in an experiment described above. CellTiter-Glo 2.0 test was conducted in 3 repeats every week during 4 weeks after the infection. Increase in the luminescence activity for more than three weeks confirmed the proliferative activity of monocytes (Table 1).

Differentiation of monocytes into dendritic cells

To show that population of proliferating monocytes can be differentiated into dendritic cells and used in different immunotherapeutic methods we conducted *in vitro* differentiation by cultivating monocytes with with 50 ng/ml rhGM-CSF (Peprotech, UK) and 50 ng/ml rhIL-4 (Peprotech, UK) for 4 days (Figure 2).

Optimization of method

We optimized the method of obtaining a proliferating monocyte culture described above to reduce the number of transductions of monocytes. We constructed a vector containing c-Myc and BMI1 genes simultaneously, following each other under one promoter spitted by T2A cleavage sequence (Figure 3). By infecting monocytes with viral particles containing this vector, we increase the probability of getting both required genes in each cell as compared to a separate infection with BMI1 and c-Myc viruses. Thus, we increase the number of proliferating monocytes in the infected population. Also, reducing the number of infections leads to the increase in cell survival.

Table 1: CellTiter-Glo 2.0 luminescent cell viability analyses of proliferating monocyte culture during 4 weeks after transduction of pL-BMI and pL-Myc viruses.

	1st week	2nd week	3rd week	4th week
Average of 3 repeats (Luminescence intense)	143	429	1 287	988
CV %	15	5	4	12

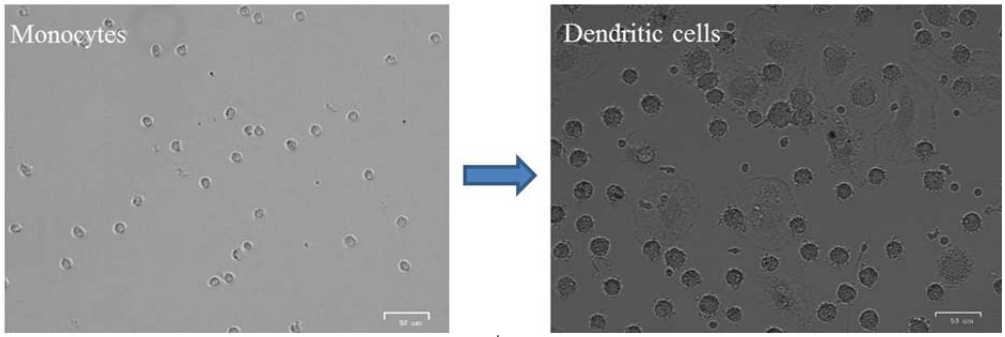

Figure 2: Maturation of CD14$^+$ monocytes into dendritic cells

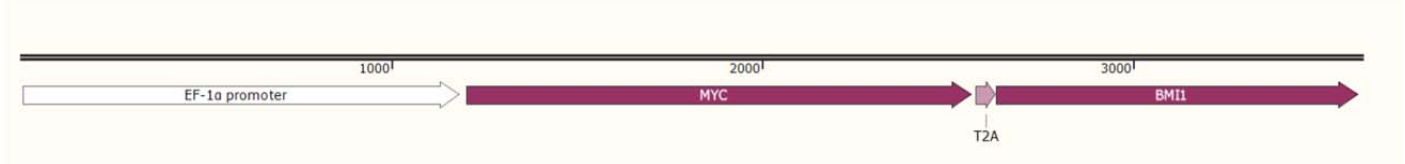

Figure 3: Construction of vector containing BMI1 and c-Myc sequences

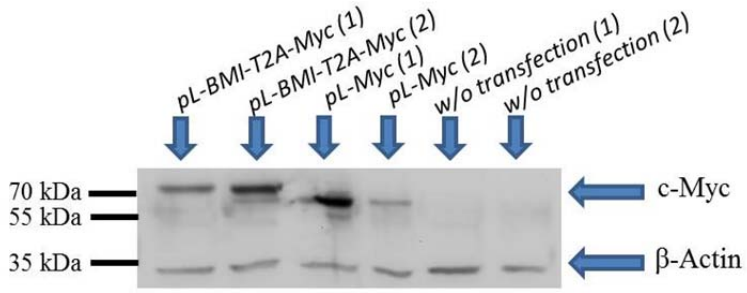

Figure 4: Expression of c-Myc in HEK-293T transiently transfected with vectors pL-BMI-T2A-Myc in 2 repeats, pL-Myc in two repeats, and HEK-293T without transfection.

We confirmed expression of c-Myc from vectors pLenti-Ef1alpha-Myc-puro and pLenti-Ef1aplha-BMI-T2A-Myc-puro in equal amounts, by transient transfection of HEK-293T cell with these vectors and western blotting of cell lysates (Figure 4).

DISCUSSION

In this paper we confirmed that ectopic expression of BMI1 and c-Myc can result in proliferation of $CD14^+$ monocytes acquired from peripheral blood. Proliferating culture of $CD14^+$ monocytes was produced by transduction of monocytes derived from a healthy donor with BMI1 and c-Myc-expressing lentivectors. Efficiency of transduction was increased by introduction of HIV-2 Vpx protein into viral particles. The number of cells in transduced population increased more than 8-fold during 4 weeks of observation. Proliferating $CD14^+$ monocytes were characterized by normal viability and active metabolism, confirmed by the CellTiter-Glo 2.0 test. Ability of this culture to be used for generation of large amounts of viable dendritic cells was proved by successful differentiating of proliferating monocytes.

CONCLUSION

To improve the efficiency of the method described above we constructed vector pLenti-Ef1aplha-BMI-T2A-Myc-puro, which contains both BMI1 and c-Myc sequences divided self-splitting peptide T2A. This vector ensures the same level of c-Myc and BMI1 expression as vectors pLenti-Ef1aplha-Myc-puro and pLenti-Ef1aplha-BMI1-puro. This optimization increases the probability of overexpression of BMI1 and c-Myc genes simultaneously in a single cell, which leads to producing of larger number of proliferating monocytes capable of differentiation into dendritic cells.

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