Optimizing Dendritic Cell Preparation for Fusion with Melanoma Cells

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ABSTRACT

Background: Fusion of dendritic cells (DCs) with melanoma cells could reinforce the antigenicity of tumors as a strategy for the treatment of malignant melanoma. However, the insufficient quantity of DCs and the low fusion efficiency limits the development of such approach. **Objective:** To define the dosage of the stimulating factors as well as the induction condition for the optimal DCs preparation and cell fusion. **Methods**: DCs were generated from murine bone marrow cells, and cultured with four different concentrations of the granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4). DCs were confirmed to be mature by detecting the expression of MHC-II, CD11c, CD80, and CD83 by flowcytometry. DCs-melanoma fusion cells were generated using polyethylene glycols (PEG) with different molecular weights and the fusion efficiency was detected by fluorescence-activated cell sorter (FACS). **Results:** The largest quantity of DCs was found when cells were cultured with 1000 U/ ml of GM-CSF and 500 U/ml of IL-4 ($1.69 \pm 0.04 \times 10^6$ ml^{-1}, $p<0.001$ when compared with the other three groups). The expression levels of MHC-II and CD83 on day 7 after incubation were significantly lower than those on day 3 (MHC-II: $p<0.001$; CD83: $p<0.001$). The efficiency of cell fusion under induction of PEG-3000 was significantly higher than that of PEG-4000 ($15.4 \pm 0.56\%$ vs. $11.1 \pm 0.45\%$, $p<0.001$). **Conclusions:** The largest quantity for mature DCs was stimulated with 1000 U/ml of GM-CSF and 500 U/ml of IL-4 and the highest fusion efficiency was under induction of PEG-3000.

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INTRODUCTION

Malignant melanoma is considered as a significant and growing public health burden worldwide due to its high incidence and poor prognosis (1). Melanoma tumors are highly resistant to chemotherapy, but more responsive to immunological treatments (2) and could be subject to T cell-mediated immunity (3). Therefore, therapeutic vaccination has been considered as a promising approach for the treatment of melanoma tumors.

Most human tumors, including metastatic melanoma, are poorly immunogenic (4) and are often aggressive to the host immune system (5). The immunotherapy with antigen-presenting cells (APC)-based tumor vaccine could improve the immunogenicity and the antigen presenting capability of tumor cells and has been demonstrated as an effective strategy to augment the host immune response to tumor (6). Dendritic cells (DCs), as the most powerful APCs, can capture antigens, process and present antigenic fragments, elicit T cell-mediated antitumor immunity and induce effective tumor-specific cytotoxic T lymphocytes (CTL) (7,8). Previous studies reported that DCs-melanoma fusion could circumvent cancer growth as well as prevent the development and metastasis of melanoma through reinforcing the antigenicity and cytolytic T cell-mediated immunity (9,10). In addition, DCs-melanoma fusion cell could induce rejection of established metastases in animal studies (11). Furthermore, treatment with vaccines using fused DC-melanoma cells would induce strong and extensive immunological responses as well as antitumor activity and remarkably prolong survival time of patients with malignant melanoma in clinical studies (7,12).

Though there was strong preclinical evidence supporting the use of DCs-melanoma fusions for cancer vaccination, conflicting effects of the treatments have been observed in clinical trials (13,14). These contradictory results may have been caused by paucity of mature DCs and low efficiency of cell fusion (13,14). Generation of large numbers of DCs from mouse bone marrow can be influenced by several factors among which the granulocyte-macrophage colony stimulating factor (GM-CSF) and Interleukin-4 (IL-4) have been reported to be of great importance (15). In addition, among numerous means to create hybrid cells, polyethylene glycol (PEG) mediated fusion has been reported to be an effective approach to increase the fusion efficiency, since PEG fused cells could induce more efficient CTL responses (11). In the current study, we intended to explore the optimized condition for DCs preparation through calculating the amount of mature DCs from bone marrow stimulated by combinations with different concentration of GM-CSF and IL-4 as well as for DCs-melanoma fusion through detecting the fusion efficiency induced with different molecular weights of PEG.

MATERIALS AND METHODS

Cell lines and Mice. B16, a mouse melanoma cell line, was obtained from the American Type Culture Collection (ATCC, Manassas, USA). Female specific-pathogen-free (SPF) C57BL/6 mice, 6-8 weeks old weighing 18-20 g on average were provided by the Animal Experimental Center of Dalian Medical University, China [license No. SCXK (Liao) 2008 - 0002].

Generation of DCs. DCs were separated from mouse bone marrow according to the previous description with some modification (15). Briefly, the bone marrow was flushed

from femurs and tibias of mice using a syringe with complete medium containing RPMI 1640 (Gibco, NY, USA), 10% heat-inactivated fetal calf serum (FCS) (Gibco, NY, USA), 2 mmol/L glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies, Tokyo, Japan). After centrifugation for 5 min (500 g, 4 °C), less fluids of marrow supernatant were resuspended with erythrocyte lysis buffer (Sigma, St. Louis, MO, USA) to lyse the red blood cells. After 3 hours incubation at 37 °C with 5% CO_2 in complete medium, the adherent bone marrow cells were plated on 6-well culture plates at 1×10^6 cells per well in a complete medium with the combination of different doses of GM-CSF and IL-4. The medium containing the same amount of cytokines was changed every 2 days. On the 5th day, the culture supernatant was replaced by complete medium enriched with TNF-α (1000 U/ml; Sigma, St. Louis, MO, USA). The morphology of mature DCs was analyzed by inverted microscope (Leitz Labovert FS, Foster City, CA) and the cell number was quantified by counting chamber on the 7th day.

Detecting the Dose Effects of GM-CSF and IL-4. In order to investigate the effect of different doses of stimulating factors on the quantity of generated DCs, the bone marrow cells were divided into four groups under the same cell concentration and stimulated with different doses of GM-CSF and IL-4 (Sigma, St. Louis, MO, USA). The divided groups were shown in Table 1.

Table 1. Groups with combination of different concentration of GM-CSF and IL-4.

Cytokines	IL-4 (500 U ml^{-1})	IL-4 (1000 U ml^{-1})
GM-CSF (500 U ml^{-1})	Group A	Group D
GM-CSF (1000 U ml^{-1})	Group B	Group C

GM-CSF: granulocyte-macrophage colony stimulating factor ; IL-4: interleukin-4.

Flowcytometry Analysis. The phenotypes of mature and immature DCs were identified by FACS analysis following staining with FITC-conjugated mAbs against MHC-II, CD-83 (PeproTech, Rocky Hill, NJ, USA) or PE-conjugated mAbs against CD-80 (PeproTech, Rocky Hill, NJ, USA), CD-11c (Biolegend, CA, USA). DCs were collected on day 3 and day 7, respectively, and resuspended with PBS containing 1% bovine serum albumin (BSA) (Sigma, St. Louis, MO, USA). The cells were incubated with corresponding antibody for 30 min at 4°C after adjusting cell density to 1×10^6 ml^{-1}, and then analyzed with a FAC Scan Flowcytometer (Becton Dickinson, NJ, USA).

Immunofluorescence Analysis. Mature DCs were washed with PBS and fixed with 4% paraformaldehyde for 20 min. The permeabilized cells were obtained by using 0.1% Triton X-100 (Sigma, St. Louis, MO, USA) for 10 min and incubated in 3% BSA for 30 min at room temperature. Then mature DCs were incubated with rat anti-mouse CD83 (AbD Serotec, Oxford, United Kingdom) at dilution of 1:200 overnight at 4°C. In addition, cells were washed with PBS and incubated with 1:200 goat anti-rat FITC-IgG (Santa Cruz, CA, USA) for 1 hour at 37°C. Cell nuclei were dyed by 4', 6'-diamidino-2-phenylindole (DAPI) diluted in 1:5000 for 15 min. The stained mature DCs were viewed under fluorescence microscope.

Cell Fusion. DCs and melanoma cells were fused using PEG. In order to determine the difference of fusion efficiency between PEG-3000 (MW= 3000) and PEG-4000 (MW=4000; Sigma, St. Louis, MO, USA), the DCs and B16 cells were labeled with PKH67 and PKH26 (Sigma-Aldrich, St. Louis, MO, USA), respectively, and B16 cells were irradiated with 5000 cGy after staining. The cell fusion was processed at a ratio of 2:1 between melanoma cells and DCs in a solution of 50% PEG-300 or PEG-400 in DMSO. After induction with PEG, the DCs/melanoma cell pellets were then slowly joined with pre-warmed RPMI 1640 medium by gentle stirring and then incubated overnight. The fused cells were observed using an inverted fluorescence microscope the next day and the fusion efficiency of PEG-3000 and PEG-4000 group was detected by a FACS Calibur.

Statistical Analysis. The significant differences between groups were explored using t-test and one-way analysis of variance (one-way ANOVA) followed by the least significant difference (LSD) test by SPSS 16.0 software SPSS Inc, Chicago, IL, USA). P values less than 0.05 was considered statistically significant.

RESULTS

Optimal Concentrations of GM-CSF and IL-4 for DCs Preparation. According to the results, the cell counts of Group A, B, C and D were 0.45 ± 0.03, 1.69 ± 0.04, 1.48 ± 0.04 and 0.94 ± 0.02 × 10^6 mL^{-1}, respectively. There were significant differences between the four groups ($p<0.001$). The pairwise multiple comparison suggested that the maximum cell density (1.69 ± 0.04 ×10^6 mL^{-1}) was observed in Group B which contained 1000 U/ml of GM-CSF and 500 U/ml of IL-4 (Figure 1B; $p< 0.001$ compared with the other three groups). In Group B, numerous DCs showed the typical dendritic cell morphology with obvious burr-like protrusion under the inverted microscope (Figure 1B).

Maturation of Dendritic Cells. The expressions of MHC-II, CD80 and CD83 on day 3 and day 7 were analyzed using FACS, respectively (Fig. 2A). The expression levels of MHC-II and CD83 on day 7 were higher than those on day 3 (MHC-II: 86.5 ± 6.2 % vs. 45.7 ± 3.2 %, $p<0.001$; CD83: 79.2 ± 3.8 % vs. 14.52 ± 1.4 %, $p<0.001$; Figure 2B). However, the expression levels of CD80 on day 7 were lower than those on day 3 (CD80: 22.2 ± 1.8 % vs. 30.2 ± 1.1 %, $p=0.003$). Furthermore, CD11c showed a decreased expression from day 3 to day 7 (CD11c: 32.1 ± 1.1 % vs. 28.5 ± 1.6 %, $p=0.03$).

The morphology of mature DCs on day 7 stained by CD83-FITC was observed under inverted fluorescence microscopy. The high expression of CD83 on the DCs membrane presented to be green and stained cells showed a typical dendritic morphology (Figure 2C).

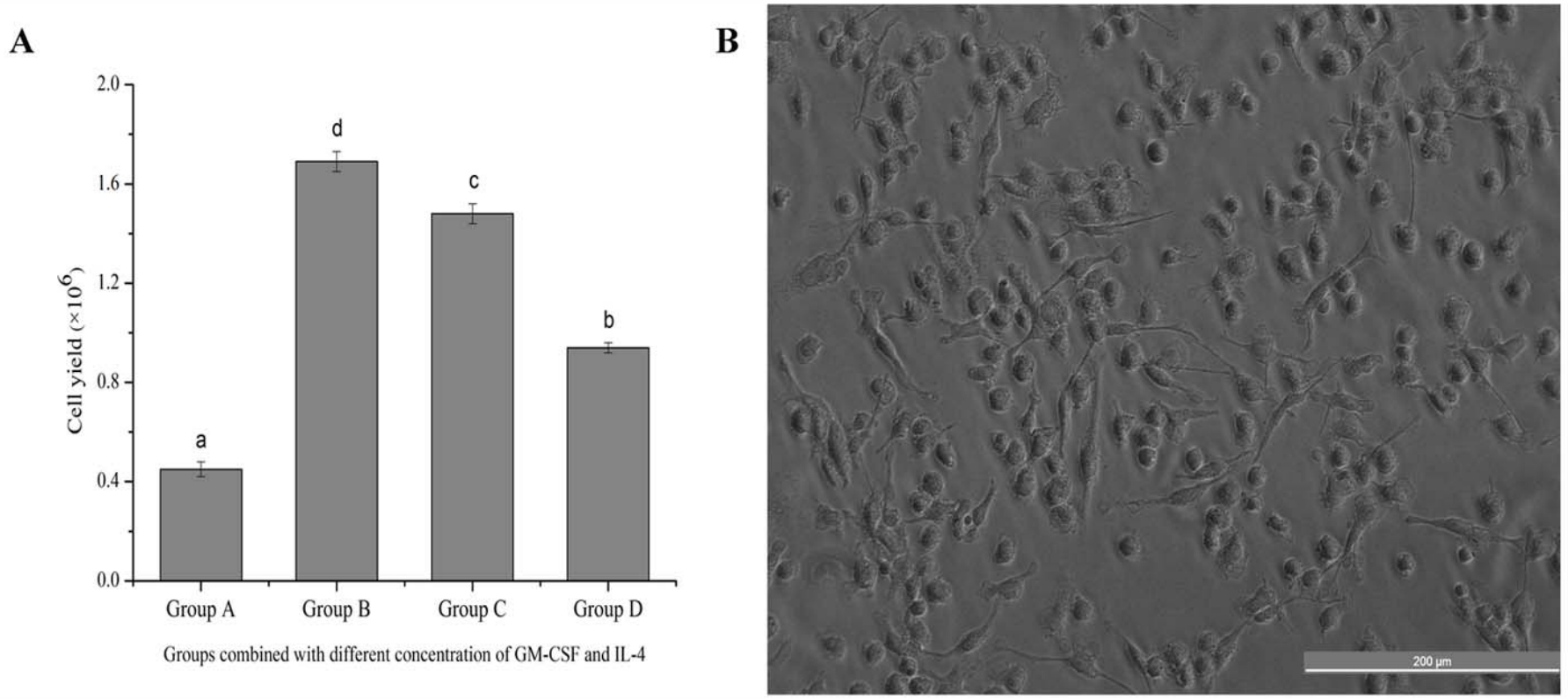

Figure 1. Detection the mature DCs quantity induced by different concentration of granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin-4 (IL-4). (A) Cell yield was determined by the DCs quantity generated form bone marrow under stimulation of different concentration of GM-CSF and IL-4. Group A, 500 U ml^{-1} of GM-CSF and 500 U ml^{-1} of IL-4; Group B, 1000 U ml^{-1} of GM-CSF and 500 U ml^{-1} of IL-4; Group C, 1000 U ml^{-1} of GM-CSF and 1000 U ml^{-1} of IL-4; Group D, 500 U ml^{-1} of GM-CSF and 1000 U ml^{-1} of IL-4. Data were presented as mean ± standard deviation (*N*=3) of cell yield. The significant differences among groups were subjected to one-way ANOVA followed by the LSD test. The values with different letters were significantly different. (B) The morphology of dendritic cells in Group B under the inverted microscope.

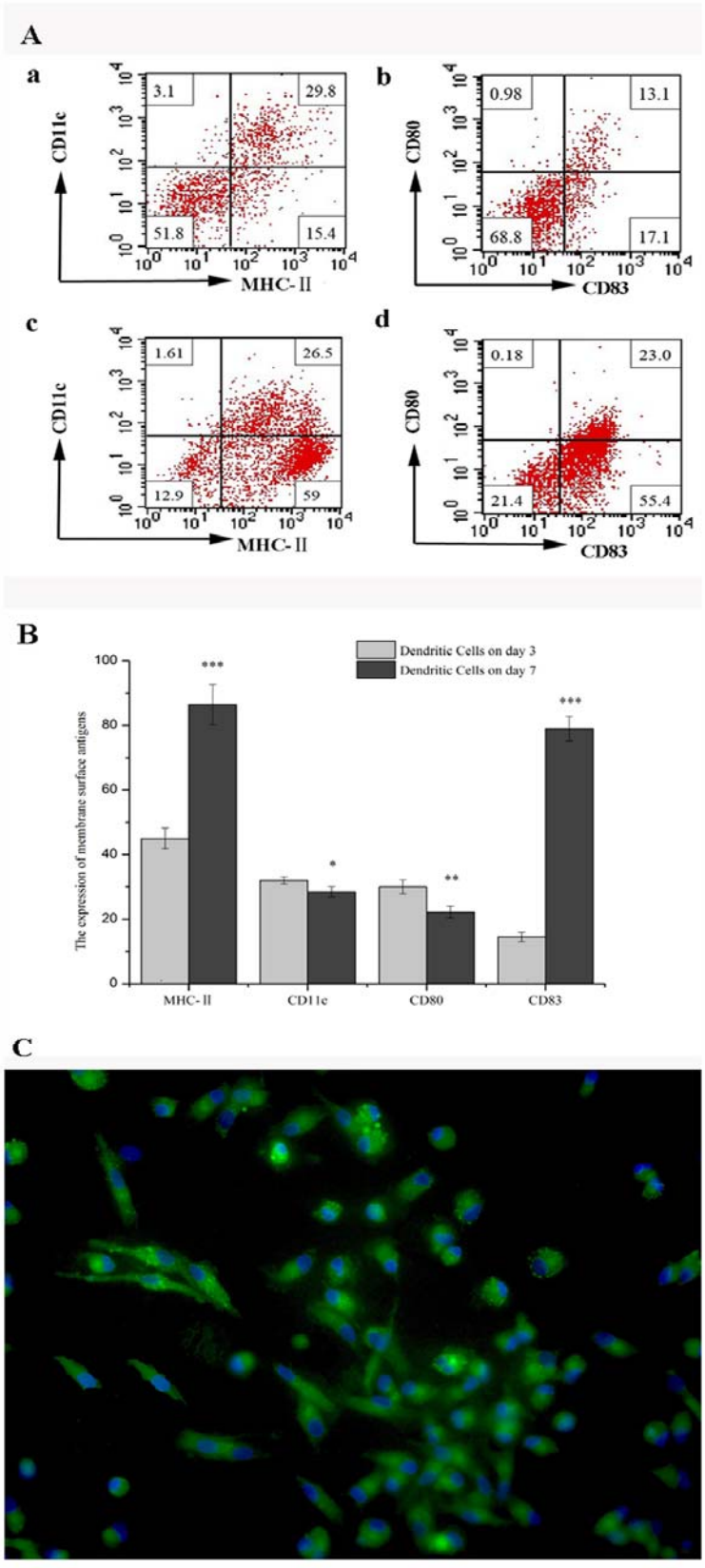

Figure 2. Detection the maturation of dendritic cell (DCs) by flowcytometry analysis and immunofluorescence. (A) The expression of membrane surface molecules including MHC-II, CD11c , CD80 and CD83 was detected by FACS. a and b displayed the expression of MHC-II, CD11c, CD80 and CD83 on d ay 3; c and d displayed the expression of MHC-II, CD11c, CD80 and CD83 on on day 7. (B) The differential expression of MHC-II,CD11c, CD80 and CD83 on day 3 and day 7 was displayed after th ree independent experiments. Data were presented as mean ± standard deviation (*N* = 3). T he significant differences among groups were subjected to t-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (C) The morphology of mature DCs on day 7 stained by CD83-FITC was displayed under inverted fluorescence microscopy.

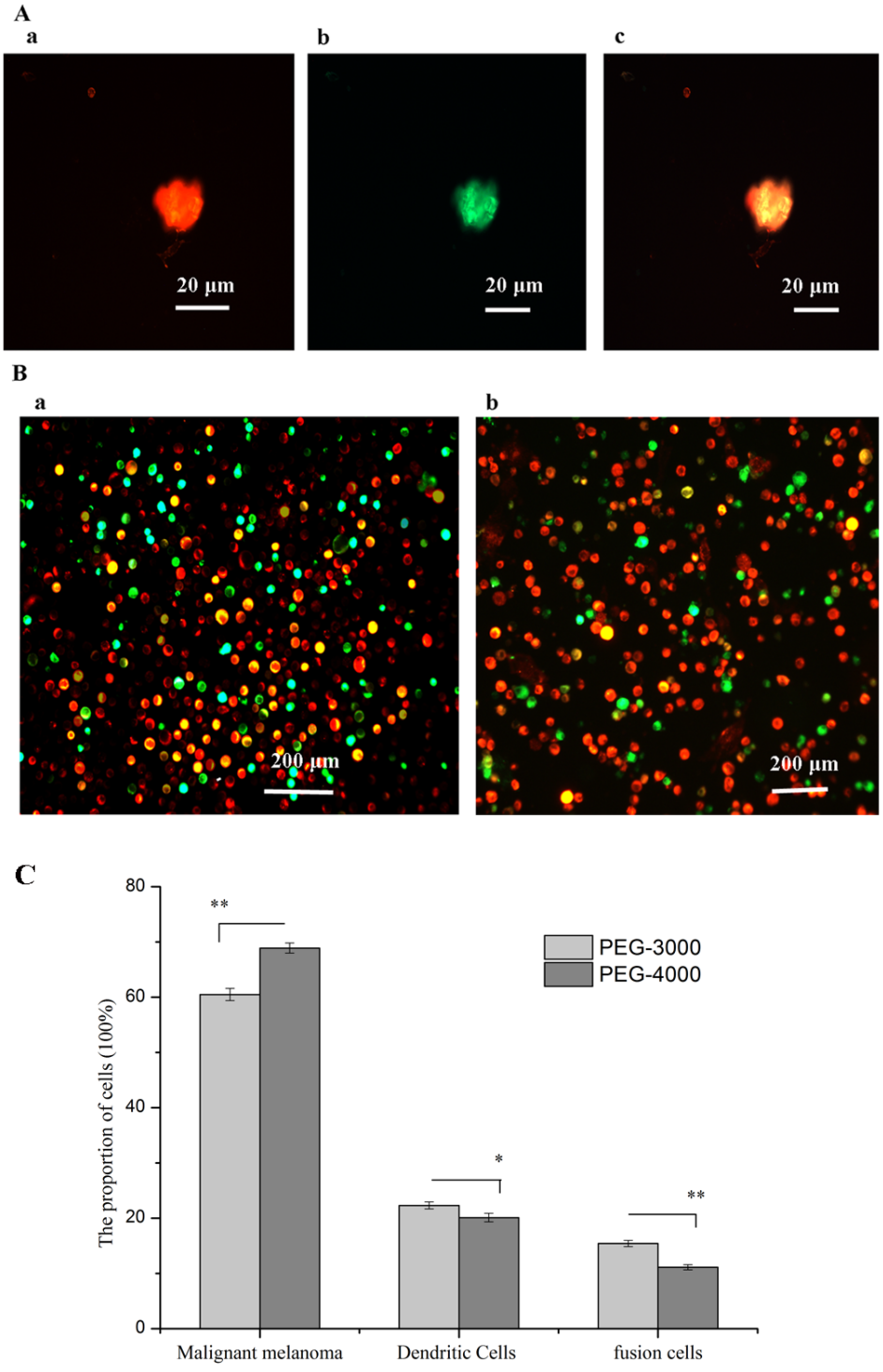

Figure 3. Detection the fusion efficiency induced by different molecular weight of polyethylene glycol (PEG). (A) Display of single cell hybrid both labeled with PKH67 (green) and PKH26 (red) under inverted fluorescence microscope at high magnification. (B) The quantity of fusion cells under induction of different molecular weight of PEG. a. Cell fusion induced by PEG-3000; b. Cell fusion induced by PEG-4000. (C) Analysis of the proportion of cells after induction with different molecular weight of PEG. Data were presented as mean ± standard deviation (N = 3). The significant differences among groups were subjected to t-test. * $P < 0.05$.

Optimal Molecular Weight of PEG for DCs-Melanoma Fusion. The fusion of DCs and melanoma cells was induced by PEG-3000 and PEG-4000, respectively. DCs labeled with PKH67 emitted green, and B16 cells labeled with PKH26 emitted red. The fusion cell could be seen under the emission of the red light alone and also be seen under the green light alone. While under simultaneous emitting of both red and green light, fusion cells presented to be yellow due to overlapping of these two lights. The typical morphology of mature dendritic cell with small burr-like protrusions could also be seen (Figure 3A).

Under inverted fluorescence microscope, there were more yellow cells after the induction of PEG-3000 compared with PEG-4000 (Figure 3B). The proportions of B16 cells, DCs and DCs-melanoma fusion cells were also calculated using FACS Calibur. According to the result, the fusion efficiency after induction of PEG-3000 was significantly higher compared with PEG-4000 (15.4 ± 0.56 % vs. 11.1 ± 0.45 %, $p<0.001$). The portion of B16 cells after induction of PEG-3000 was lower than that of PEG-4000 (60.5 ± 1.12 % vs. 68.2 ± 0.98 %, $p<0.001$). However, the portion of DCs after induction of PEG-3000 was higher than that of PEG-4000 (22.3 ± 0.62% vs. 19.9 ± 0.96 %, $p=0.04$; Figure 3C).

DISCUSSION

DC-melanoma fused cells have been used as a tumor vaccine for malignant melanoma due to their ability to efficiently present tumor-derived antigens by DC-derived antigen presentation machinery and increase an effective cell-mediated anti-tumor response (16). The augmented clinical application of DCs-melanoma vaccine largely depends on the effecient cell fusion of DCs and melanoma cells. However, the deficiency in the frequency (16) and maturation state of DCs (13) and the low fusion efficiency resulting from improper choice of induction condition (17) may limit the development of the treatment with DCs-melanoma vaccine.

Mature DCs have strong antigen presenting function due to higher expression of MHC and costimulatory molecules and are significantly better at CTL induction (18,19). Hence, abundant mature DCs are essential for the successful construction of DCs-melanoma vaccine. DCs derived from bone marrow are immature and the stimulating factors including GM-CSF and IL-4 could induce the production of large numbers of mature DCs from mouse bone marrow (15,20). Studies showed that GM-CSF could stimulate the proliferation and differentiation of DCs in vitro (20,21). While several reports have compared the effects of GM-CSF in the presence or absence of IL-4 on generation of mature dendritic cells and the results showed that DCs cultured in the presence of GM-CSF and IL-4 would be more mature than GM-CSF alone (18,26). But the dosage of GM-CSF and IL-4, which makes a significant impact on the quantity of DCs, is not well defined. According to the previous reports, the commonly used concentration of GM-CSF and IL-4 are 500 U/ml and 1000 U/ml (15,22-25). In this study, different doses of GM-CSF and IL-4 have been combined and then divided into four groups. Among the four groups, Group B consisting of 1000 U/ml of GM-CSF and 500 U/ml of IL-4 achieved the highest frequency of DCs. Also the cells in this group were non-adherent and showed typical morphology of mature dendritic cell under the inverted microscope (Figure 1). All these results indicated that the combination of 1000 U/ml of GM-CSF and 500 U/ml of IL-4 was the optimal dose for the generation of

mature DCs and the dose combination of these two stimulating factors would provide a meaningful reference of culture condition for cell preparation of DCs based vaccine.
We also explored the phenotype changes during the maturation of DCs through detecting the expression of surface molecules, such as MHC-II, CD83, CD80 and CD11c. The maturation of DCs has always been coupled with the high expression of MHC-II and co-stimulatory molecules (26). In our study, the significantly increased expression of MHC-II and CD83 were observed, which was consistent with the previous studies (27-29). In addition, the morphology of mature DCs stained by CD83-FITC under inverted fluorescence microscopy verified the successful preparations of mature DCs (30). However, there were decreased expressions of CD80 and CD11c in our study. The abnormal expression of these molecules may be caused by the addition of different concentration of cytokines including GM-CSF and IL-4 and having changed more DCs to macrophages during the process of dendritic cell differentiation.
The PEG polymer of has provided an ideal choice for cell fusion with low toxicity, water solubility and ease of handling. The molecular weights used for cell fusion ranges from 1000 to 10000 D (31). Early studies showed that molecular weight of PEG could affect the efficiency of cell fusion (31-33). PEG-3000 and PEG-4000 in the concentration of 50% were chosen in our study due to their closest osmotic pressure to DCs and low probability in causing cell rupture. Although PEG is commonly used for DCs-tumor cell fusion, a comparison of the fusion efficiency of PEGs with different molecular weights has not been reported. According to our results, there was significantly higher fusion efficiency after induction with PEG-3000 than PEG-4000. A previous study showed that PEG with larger molecular weight could be more effective in causing the aggregation of cells (32) but are more toxic to cells. PEGs with larger molecular weights were reported to lead to cell dehydration and disruption (34). This can be the reason why the portion of DCs under the treatment of PEG-4000 was lower.
The dosage of stimulating factors including GM-CSF and IL-4 and the molecular weight of PEG were considered in this study to explore the most appropriate conditions for DCs-melanoma fusion. The optimal concentrations of GM-CSF and IL-4 for DCs preparation were 1000 and 500 U/ml, respectively, and the optimal PEG type for DC-melanoma cell fusion was PEG-3000. This research provides a reference for mature DCs preparation and cell fusion for future studies and clinical applications.

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