

Production of functional dendritic cells from mouse bone marrow

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Abstract— Currently, immune cell-based therapies, particularly those that utilize dendritic cells (DCs), are a promising therapy approach for cancer treatment. Therefore, DC therapy is the focus of many studies in many laboratories worldwide that are developing novel cancer therapies. This study aimed to develop a reproducible procedure to produce functional DCs from mouse bone marrow for DC therapy research. Bone marrow was collected from mouse femur bones by flushing with buffered saline. These cells were used to isolate mononuclear cells (MNCs) by Ficoll gradient centrifugation. MNCs were cultured in RPMI 1640 medium supplemented with 20 ng/mL of IL-4 and 20 ng/mL of GM-CSF to induce maturation of immature DCs. The results showed that this procedure induced cells exhibiting the DC phenotypes, such as the expression of CD40, CD80, and CD86, high phagocytic capacity, strong production of IL-12, and efficient stimulation of T-CD4 lymphocytes. These results suggest that this procedure can be used to produce functional DCs in future studies that use DCs for immune therapy.

Keywords— Breast cancer stem cells, Dendritic cells, IL-12 mRNA, Immune therapy, Tumor lysates primed DCs.

INTRODUCTION

Dendritic cells (DCs) are professional presenting cells that can process and present antigens to both T and B cells (Steinman, 1991). *In vivo*, DCs will trigger and initiate immune response. These cells can be differentiated from myeloid progenitors and lymphoid progenitors. Therefore, stimulation of DCs *in vivo* has also been considered as a potential therapy for cancer treatment. In almost all studies, there were two cytokines used to stimulate DCs, including the flt3 ligand (FL) and granulocyte macrophage colony-stimulating factor (GM-CSF) (Hanada et al., 1996; Maraskovsky et al., 1996; Pulendran et al., 1999).

However, DCs will present suitable antigens when they are induced with antigens *in vitro* before injection into the body. In fact, in cancer patients, DCs interact with tumor cells, and these DCs could become tolerant

and also display non-immunologic effects against tumors and the tumor microenvironment (Dhodapkar et al., 2008). Therefore, DCs must be matured by antigens in an *in vitro* culture system prior to use.

In an early study, Inaba et al. (1992) used GM-CSF to induce maturation of hematopoietic precursors into DCs after 6–8 days (Inaba et al., 1992). Subsequently, other studies optimized the procedure to produce mature DCs when combined with GM-CSF and IL-4 (Labeur et al., 1999; Saunders et al., 1996). Thereafter, almost all studies used both GM-CSF and IL-4 to produce DCs for DC therapy. These mature DCs must express CD80, CD86, and CD40 as well as MHC class II molecules. Moreover, DCs can also produce some interleukins (ILs), especially IL-12, or efficiently stimulate T lymphocytes *in vitro* and *in vivo*.

Here, we describe a simple method to produce large numbers of DCs *in vitro* from mouse bone marrow cells using GM-CSF and IL-4. Using this method, DCs completely exhibited the DC phenotypes, including both morphological and other characteristics of DCs. This method provides a model to produce and study DCs *in vitro* and DC therapies in mouse models.

MATERIAL AND METHODS

Cell culture and differentiation of monocytes into dendritic cells

The mice were euthanized by cervical dislocation, in which the neck of each mouse was grasped firmly in one hand and the other hand was used to sharply pull at the base of the tail. Prior to its placement in the glass cabinet for manipulation, the mouse model was doused with 70% alcohol. A small cut was made in the abdominal area with a pair of sterile scissors, just above the mid-line. This was then followed by the removal of the membrane layer to reveal the organs in the mid-section of the body. The femurs of the mice were dislodged and submerged in PBS (phosphate buffer saline) supplemented with antibiotic-mycotic (Sigma-Aldrich, St Louis, MO).

Bone marrow cells were used to isolate mononuclear cells by centrifugation in a Ficoll gradient. All obtained mononuclear cells were maintained at 37°C with 5% CO₂. We added IL-4 to the culture medium, which contains GM-CSF for culturing DCs, because a combination of GM-CSF and IL-4 is better able to stimulate mixed leukocyte reactions. The culture medium that was used for all of the experiments was RPMI-1640 (Sigma-Aldrich, St Louis, MO) supplemented with 2 mM/L glutamine, 100 µg/mL penicillin, 100 µg/mL streptomycin, and 10% heat-inactivated fetal bovine serum (FBS). To produce immature DCs (iDCs), adherent cells were cultured for 6 days in medium containing recombinant GM-CSF and IL-4 at concentrations of 20 ng/mL each. At days 7–12, these cells matured with TNF-α (20 ng/mL), a different cytokine that was added to the complete media. At day 12, mature DCs (mDCs) were confirmed to have DC phenotypes through flow cytometry detection of CD14 (for monocytes), CD40, D80, and CD86 (for DCs).

Dextran-FITC uptake assay

The phagocytic capacities of iDCs and mDCs were analyzed according to a previously published protocol (Phuc et al., 2011). Briefly, 5×10⁴ cells were incubated for 1 h with dextran-FITC (1 mg/mL; Sigma-Aldrich, St Louis, MO) in 100 µL of culture medium at 37°C and 4°C as negative control. Cells were then washed with cold PBS supplemented with 1% bovine serum albumin (BSA) four times before flow cytometry analysis. Phagocytic ability of DCs was confirmed by the appearance of cell populations that expressed FITC fluorescent signals.

Stimulation of CD4⁺ T lymphocyte proliferation

Stimulation of CD4⁺ T lymphocyte proliferation was evaluated following the previously published protocol (Phuc et al., 2011). The assessment was performed in different groups with the ratio of DC/lymphocytes as follows: 0.25:100, 0.5:100, 1:100, 2:100, and 8:100. The control groups included DC+PHA (phytohemagglutinin, Sigma-Aldrich, St Louis, MO), PHA, and PHA+lymphocytes. PHA concentration was 50 mg/L, and the experiment was conducted on 96-well plates (Nunc, Roskilde, Denmark) and repeated four times. CD4⁺ T lymphocyte proliferation was measured by MTT assay using the Cell Growth Determination Kit, MTT based (Sigma-Aldrich, St Louis, MO). Twenty microliters of MTT was added into each well of the 96-well plates, followed by incubation for 4 h and addition of 150 µL of DMSO. Plates were then thoroughly mixed for 10 min until the crystals completely dissolved. Absorption values (A-values) for each well were measured at a wavelength of 490 nm using micro-plate reader DTX 880 (Beckman Coulter, Brea, CA). An offset value of A and absorption value of a control group (DC+PHA) reflect lymphocyte proliferation. An offset value of absorption in the lymphocyte+PHA and PHA groups showed proliferation of lymphocytes in the control group. All results were analyzed by Staraphic 7.0 software.

Quantity of cytokine/chemokine production

To detect the secretion of cytokines, monocytes were further induced with TNF-α after induction with GM-CSF and IL-4, as described above, in culture medium in 24-well plates for 24 h. Supernatant was collected and frozen at -80°C until analysis. The quantity of cytokine IL-12 in the supernatant was determined by

ELISA (Abcam, Cambridge, UK) and read on a DTX 880 (Beckman Coulter, Brea, CA).

RESULTS

Induced monocytes express DC phenotypes

We observed that monocytes formed small groups similar to the culture of monocytes from human bone marrow (Bai et al., 2002), peripheral blood (Ratta et al., 1998) or umbilical cord blood (Liu et al., 2002; Shi et al., 2005) as well as to those observed in some previous studies addressing bone marrow-derived dendritic cells (Lutz et al., 1999; Lutz and Rossner, 2007; Lutz et al., 2000; Scheicher et al., 1992). Most of the cells had expanded cytoplasm and small dendrite-like structures. Morphologically, the differentiated cells had some characteristics of DCs. These cells had

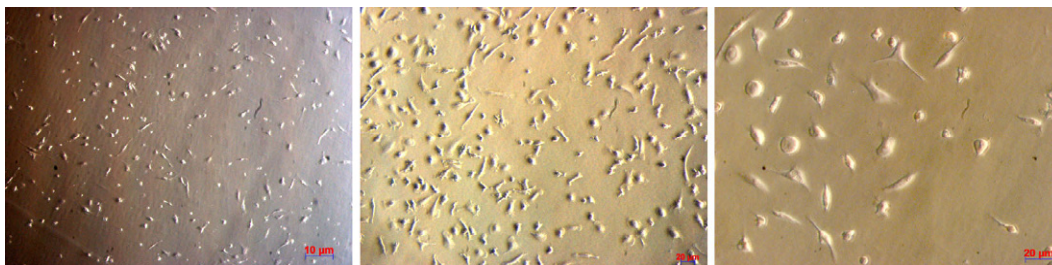


Figure 1. Dendritic cells were induced from mouse bone marrow cells. Bone marrow cells were obtained from murine bone marrow (A) and induced in medium with IL-4 and GM-CSF for 6 days (B) and 12 days (C).

relatively uniform shapes with large heterogeneity of nuclei, many mitochondria and vacuoles, and relatively few particles in the cytoplasm. Cell shape is comparable to DCs (Fig. 1).

The results of DC marker analysis are presented in Fig. 2. It was observed that monocytes expressed CD14 but did not express CD40, CD80, and CD86 before induction; they expressed CD86 and CD40 but did not express CD14 and CD80 after induction of iDCs; and they expressed CD40, CD80, and CD86 but did not express CD14 after induction of mDCs.

Differentiated DCs from monocytes were functional *in vitro*

Antigen phagocytosis

To evaluate the function of DCs, we measured the phagocytic ability of DCs *in vitro*. Phagocytic activity was assessed by measuring the ability of cells to consume dextran-FITC. Our data showed that monocytes after induction increased their phagocytic ability to $92.28 \pm 9.25\%$ in iDCs and $78.54 \pm 11.33\%$ in mDCs ($p < 0.05$). Meanwhile, phagocytic ability was only $8.11 \pm 3.13\%$ ($n = 3$) in the group of cells that were cultured in the medium without GM-CSF, IL-4, and TNF- α . In addition, cells that were cultured at 4°C could not engulf the dextran-FITC (Fig. 3).

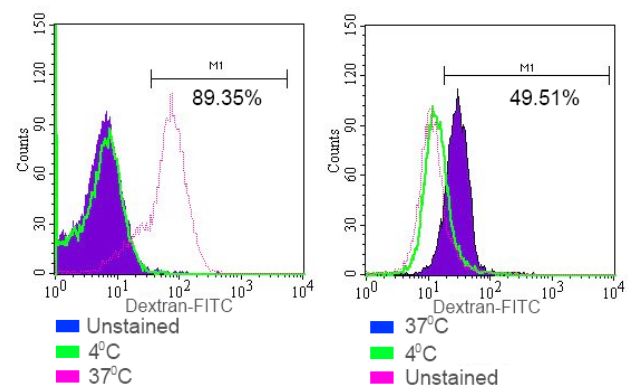


Figure 3. Percentage of induced monocytes that consumed dextran-FITC. (A) iDCs strongly consumed dextran-FITC, while mDCs more weakly consumed dextran-FITC (B). 4°C : DCs that were incubated at 4°C ; Unstained: DCs that were not incubated with dextran-FITC; 37°C : DCs that were incubated at 37°C .

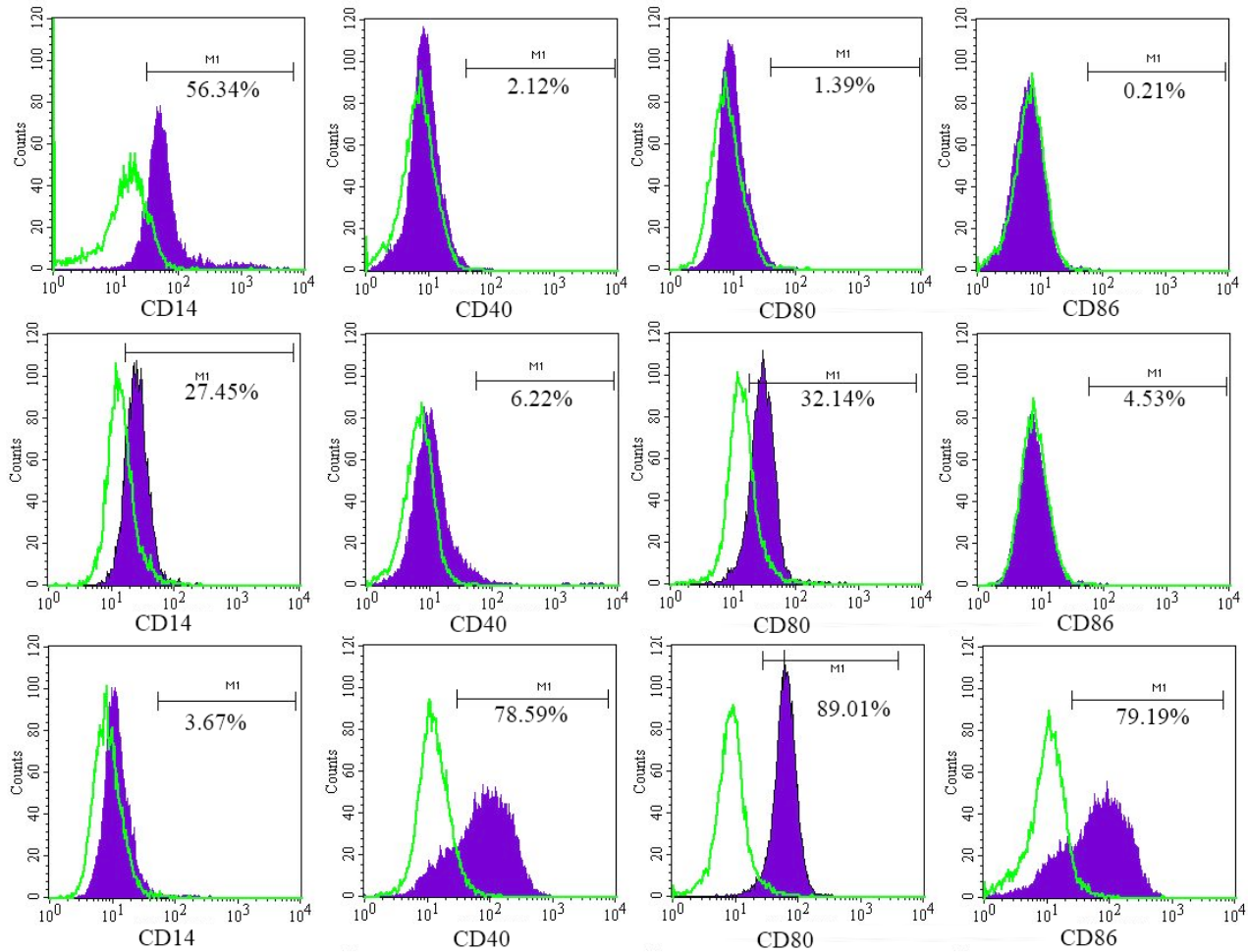


Figure 2. DC markers analysed by flow cytometry. Monocytes weakly expressed CD14, but did not express CD40, CD80, and CD86 (A–D). Monocytes induced with GM-CSF and IL-4 expressed CD86, weakly expressed CD80, but did not express CD14 and CD40 (E–H). Mature DCs expressed CD40, CD80, and CD86 but did not express CD14 (I–M).

Induced monocyte-stimulated T lymphocytes

The expression of CD80 and CD86 on the surface of the DCs was associated with the activity of activated T lymphocytes. The results showed that induced DCs by monocytes with the cytokines GM-CSF, IL-4, and TNF- α triggered T cell proliferation. **Fig. 4** reveals that the activity of DCs was significantly different compared with that of the control group ($p < 0.05$) and was augmented when DC concentrations increased ($p < 0.05$).

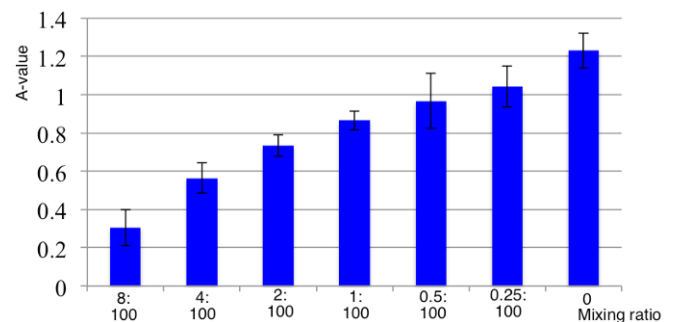


Figure 4. Stimulation of lymphocyte proliferation by DCs. Lymphocytes were increasingly stimulated as the proportion of DCs:lymphocytes increased.

A-values (**Fig. 4**) are offset of OD values that were measured in the lymphocyte+PHA control group and experimental groups. Because PHA is the strongest stimulant of lymphocytes, the OD value of the control sample was the highest. Therefore, the A-value was smaller when the growth capacity of experimental cells was higher. In a similar manner, the A-value was larger if the stimulation by DC was lower. The experimental results also revealed that DCs differentiated from bone marrow monocytes had the ability to stimulate lymphocyte cells *in vitro*. This ability depended on the ratio of mixing between DCs and lymphocyte cells; the more DCs added, more lymphocytes were stimulated. Activity of stimulating lymphocyte proliferation is a critical characteristic of DCs *in vivo*. This feature helps enhance immunity in cancer treatment, which is a key point for success of this approach.

Induced monocytes produced IL-12

To determine the mechanism of activated T cell proliferation, the secretion of IL-12 was evaluated after induction because IL-12 is the most important interleukin in the activation of T lymphocytes. In fact, DCs always interact with other cells in the body. This interaction can occur directly by cell-cell communication via the interaction of surface proteins (as described above) through B7 (CD80 or CD86) with CD28 on lymphocytes; alternatively, this interaction can occur indirectly over a greater distance through cytokines. Typically, DCs produce IL-12 after induction with antigens. IL-12 is a signal that induces CD4⁺ T cells into the Th1 phenotype. Then, it activates the immune system to attack antigens that are presented by DCs.

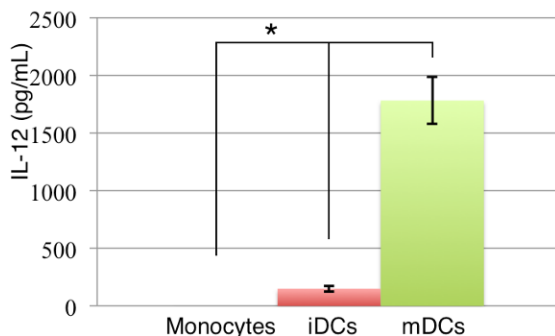


Figure 5. IL-12 concentration of groups. Monocytes: the samples (n=3) of monocytes before induction with cytokines. iDCs: the samples of monocytes after induction with GM-CSF and IL-4; mDCs: the samples of monocytes after induction with GM-CSF, IL-4, and TNF- α . IL-12

concentration of induced groups significantly increased compared with the control group (monocytes).

IL-12 quantity was analyzed by ELISA (**Fig. 5**) and it was shown that there was a statistically significant difference ($p < 0.5$) between monocytes with and without cytokine treatment.

DISCUSSION

DCs are the most professional antigen-presenting cells in our bodies. Unlike macrophages, they have the ability to present antigens not only to T cells but also to B and NK cells. Within our biological system, DCs can be differentiated by hematopoietic stem cells (HSCs). When there is any stimulation by risk factors through toll-like receptors (TLRs), DCs phagocytose small portions of the membrane of the inducers through a process called nibbling. Subsequently, small fragments are processed and sent to their cell surface using MHC molecules. In the mature stage, mDCs express CD80 (B7.1), CD86 (B7.2), and CD40. These are essential co-receptors that stimulate T cell activity. By the expression of mentioned co-receptors, DCs have the power to provoke memory T and naïve T cells, as well as other forms of T cells. Hence, appearance of these surface proteins is a prerequisite for DCs to activate other cells of the immune system. Numerous studies that created DCs from monocytes or HSCs from umbilical cord blood (UCB), bone marrow (BM), or peripheral blood succeeded in enhancing the expressivity of these markers (Ferlazzo et al., 2000; Goriely et al., 2001; Liu et al., 2002; Reddy et al., 1997; Reis e Sousa et al., 1997). In this report, we showed that the DCs in our study also expressed similar proteins, including, for example, CD80 and CD86. This evidence reveals that induced cells have the capacity to excite other cells in the immune system, especially naïve T and memory T cells.

This point is demonstrated by the results of our investigation of the ability of DCs to activate T cells. In the experiment to stimulate CD4⁺ T cells by induced monocytes, we clearly saw remarkable T cell proliferation when DCs were co-cultured with CD4⁺ T cells extracted from allogenic bone marrow samples. Furthermore, the increase in T cells was dosage-dependent when the same amount of T cells was mixed with variable amounts of DCs. This trend was facilitated by direct interaction between DCs and CD4⁺ T cells through CD80 and CD86 co-receptors. It

is known that the stimulatory effect of DCs on T cells is not only caused by direct interaction but also by the indirect action of cytokine signals, which are released by DCs. In the human body, IL-12, an important interleukin, is produced by either DCs or macrophages and B cells. IL-12 initiates differentiation of naïve T cells into Th0 and then into Th1 and Th2 cells. IL-12 also plays a pivotal role in NK and T cell activities; it intensifies the toxicity of NK cells and CD8+ T cells. Fortunately, DCs from bone marrow can release IL-12. Data from this study indicated that induced DCs with a suitable cocktail of cytokines had significantly elevated IL-12 secretion. This evidence again supports our conclusion that authentic DCs were derived from mouse bone marrow.

Not only did induced DCs from bone marrow have a DC-like shape and ability to activate T cells, they also had high phagocytic capacity and antigen presentation, which are indispensable for DCs to perform their functions in immune therapy. In this report, we showed that phagocytosis is feasible by DCs through dextran-FITC uptake assay. After incubation with dextran, $92.28 \pm 9.25\%$ of iDCs and $78.54 \pm 11.33\%$ of mDCs participated in dextran uptake and expressed strong fluorescent signals. Hence, we created functional DCs with all of the typical characteristics of normal DCs, and these results are similar to those of some previously published publications (Burdek et al., 2010; Morelli et al., 2001). These cells have DC-like shapes, phagocytic capacities, and process and present antigens to stimulate other cells in the immune system, especially T cells, through direct cell-cell interaction or indirectly by IL-12 secretion.

CONCLUSION

DCs are professional antigen-presenting cells. The roles of DCs are different from those of macrophages in that they can present antigens to both lymphocytes (T and B cells) as well as NK cells. Mouse DCs can be easily produced from bone marrow cells by induction in RPMI-1640 medium supplemented with 2 mM/L glutamine, 100 µg/mL penicillin, 100 µg/mL streptomycin, 10% heat-inactivated FBS, 20 ng/mL IL-4, and 20 ng/mL GM-CSF for 7 days. These DCs entirely exhibited the DC phenotypes such as specific markers of DCs and strong phagocytic abilities, IL-12 production, and T cell stimulation. This procedure can be applied to produce functional DCs in future studies.

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ABBREVIATIONS

BM: Bone marrow; BSA: Bovine serum albumin; CD: Cluster of differentiation; DC: Dendritic cell; ELISA: enzyme-linked immunosorbent assay; FL: Flt3 ligand; GM-CSF: Granulocyte macrophage colony-stimulating factor; HSC: Hematopoietic stem cell; iDC: Immature DC; IL: Interleukin; MHC: Major Histocompatibility Complex; mDC: mature DC; MNC: Mononuclear cell; PBS: Phosphate buffer saline; PHA: Phytohemagglutinin; TLR: Toll like receptor; TNF- α : Tumor necrosis factor- α ; UCB: Umbilical cord blood

Competing interests

The authors declare that they have no competing interests.

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